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Human Norovirus in Artificial and Environmental Marine Water: Development of Antibody Based Rapid Methods

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HUMAN NOROVIRUS IN ARTIFICIAL AND ENVIRONMENTAL MARINE WATER: DEVELOPMENT OF ANTIBODY BASED RAPID METHODS

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This work is dedicated to Rosalind Glover. You have always believed in me and for that I am eternally grateful.

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"Rejoice always, pray without ceasing, in everything give thanks; for this is the will of God in Christ Jesus for you" 1 Thessalonians 5:16-18.

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Abstract

Norovirus (NoV) is the principal cause of viral gastroenteritis in the United States. It has been linked to filter-feeding molluscan shellfish, that bioaccumulate the virus from contaminated surrounding waters. The consumption of raw or undercooked contaminated oysters may result in acute gastroenteritis. We investigated the occurrence of NoV GI and GII and microbial indicators of fecal contamination in oysters and harvesting water from areas along the Louisiana Gulf Coast. We developed a filtration and concentration method for the detection of NoV from oyster harvesting waters. Lastly, this body of work compares commonly used molecular techniques (RT-PCR) and a commercial enzyme immunoassay for the detection of NoV. One oyster sample was positive for norovirus GII at 3.5 ± 0.2 $\log_{10}$ genomic equivalent copies/g digestive tissues, however the surrounding water tested negative for NoV. Zeolite granules were used for the filtration of norovirus-seeded waters. Beef Extract (10%) in McIlvaine's buffer was the optimal elution buffer resulting in an average percent recovery of 41.76 $\pm$ 0.07 ($p<0.05$). Artificial and environmental waters with 20ppt salt had an observed average percent recovery of 40.79 $\pm$ 0.19 and 18.95 $\pm$ 0.24, respectively which was significantly higher than 0, 5, 10, 15, and 25ppt ($p<0.05$). The observed percent recoveries for artificial and environmental waters were 44.03 $\pm$ 0.20 and 34.36 $\pm$ 0.02, respectively. The percent recovery for artificial and environmental water using TaqMan® Fast Virus 1-Step RT-qPCR was 38.85% $\pm$ 0.27 and 19.77% $\pm$ 0.07, respectively. In comparison, SuperScript® III Platinum One-Step qRT-PCR exhibited an average percent recovery of 11.12% $\pm$ 0.183 and 15.55% $\pm$ 0.225 for artificial and environmental waters. The EIA assay assay was not sensitive enough to detect NoV in the elution samples despite RT-qPCR methods quantifying the virus concentration between 10^4 and 10^5 genomic copies/ml. As such, it is not an effective method for the detection of NoV from environmental water matrices without RT-qPCR as a secondary

validation method. This body of work provides an effective method to detect norovirus in oyster harvesting waters. Our results emphasize the need for regular monitoring of pathogenic viruses in oyster harvesting areas to reduce viral gastroenteritis incidences.

Chapter 1 Review of Literature

1.1 NoV taxonomy, nomenclature, and genetic diversity: Gastroenteritis, often referred to as the 'stomach flu', is the inflammation of the stomach and intestines due to a bacterial, parasitic, or viral etiologic agent. Bacterial agents often responsible for gastroenteritis include: *Campylobacter jejuni, Escherichia coli, Salmonella* spps., *Shigella, Yersina,* and *Staphlyococcus* spps. Viral contamination in food and water matrices is significantly more prevalent than bacterial contamination. Subsequently, viral contamination is the predominate cause of gastroenteritis worldwide. Viruses often known to be etiological agents for gastroenteritis include: Rotaviruses, Astroviruses, Adenoviruses, and most common, Caliciviruses (1-6). Because it is caused by bacterial or viral agents, gastroenteritis results in varying degrees of diarrhea, nausea, and vomiting. Fevers are not typically associated with the illness. However, a low-grade fever is possible if the causative agent is a bacterial pathogen. Although death is not typically associated with gastroenteritis, it can occur when dehydration is induced due to untreated profuse diarrhea and vomiting. Diarrhea diseases are the cause of approximately 2.5 million deaths per year, with a disproportionately high occurrence in developing countries (7-9). Deaths due to diarrhea diseases, such as gastroenteritis, most often affect children and infants. In 1982, a published review calculated an annual mortality rate of 14 per thousand in children under 5 years of age and 23 per thousand in infants under 1 year of age (9). Although a staggering statistic, the same review determined that 4.5 million deaths per year were due to diarrhea-specific illnesses. However, a more recent study found that value to be significantly lower; at 2.5 million per year (8). Undoubtedly, there is a decline in the mortality rate caused by diarrhea-specific illnesses but an increase has been observed in the associated morbidity rates, especially among children in developing countries (8).

As aforementioned, there are several viruses known as causative agents of gastroenteritis. Viral gastroenteritis occurs sporadically and exhibits an acute onset. Viruses in the Caliciviridae family, specifically Norovirus, are most often associated with epidemic occurrences of viral gastroenteritis. However, endemic spreads have been known to occur. Caliciviridae is a family of small round structured positive viruses that are class IV members of the Baltimore Classification System. The prefix "*calici"* is derived from the Latin word "*calyx*" which translates to cup or chalice. Appropriately named, *Caliciviridae* viruses have characteristic cup-shaped depressions on their capsid surfaces (10). Vesicular exanthema, caused by *vesicular exanthema of swine virus* (VESV) was the first illness associated with *Caliciviridae* viruses (11). Due to its shape and size, VESV was originally classified in the picornaviridae family of viruses. However, it was later determined that the replication mechanism and structure of VESV differed from that of the typical genera of picornaviridae(12, 13). Three distinguishable characteristics separate viruses classified as picornaviridae from those classified as caliciviridae. First, viruses in the caliciviridae family have segmented open reading frames (ORF) while picornaviruses have one large ORF. This difference supports the theory that viruses in these two families have different replication mechanisms (14). Caliciviridae viruses do not have a methylated cap on the 5' end of their RNA strand. There is, instead, a small viral protein covalently linked to the RNA (15). This small protein is not included in picornaviruses nor is it required for their infectivity. Due to the key differences between newly discovered viruses such as VESV and those classified in the picornaviridae family, the International Committee on Taxonomy of Viruses published a new family of positive viruses named *Caliciviridae* (16). Viruses classified in the *Caliciviridae* family are comprised of

a single-stranded, polyadenylated, positive-sense RNA genome (17). Furthermore, this family of viruses has one major structural protein that encodes the icosahedral viral capsid.

In viral taxonomy, viral families are further arranged into genera. Initially, *caliciviridae* was subdivided based on the hosts different viruses infected (18). However, as more studies were published, it became increasingly evident that similar viruses infected a wide range of hosts resulting in the need for a more specific classification method. As a result of the need for a new classification method, the definition of genus in the *caliciviridae* was redefined as 'genetically distinct clades of viruses' (18). Two phylogenic methods determined that there were four major genera within *caliciviridae*: *Lagoviruses, Noroviruses, Sapoviruses, and Vesiviruses* (Figure 1) *(19, 20).* In 2006, Oliver et al., determined that *Neboviruses* have two ORFs but are genetically distinct and should be listed as a new clade within the *Caliciviridae* family (21). In 2009, *Neboviruses* were classified as a new genera within *Caliciviridae* (22).

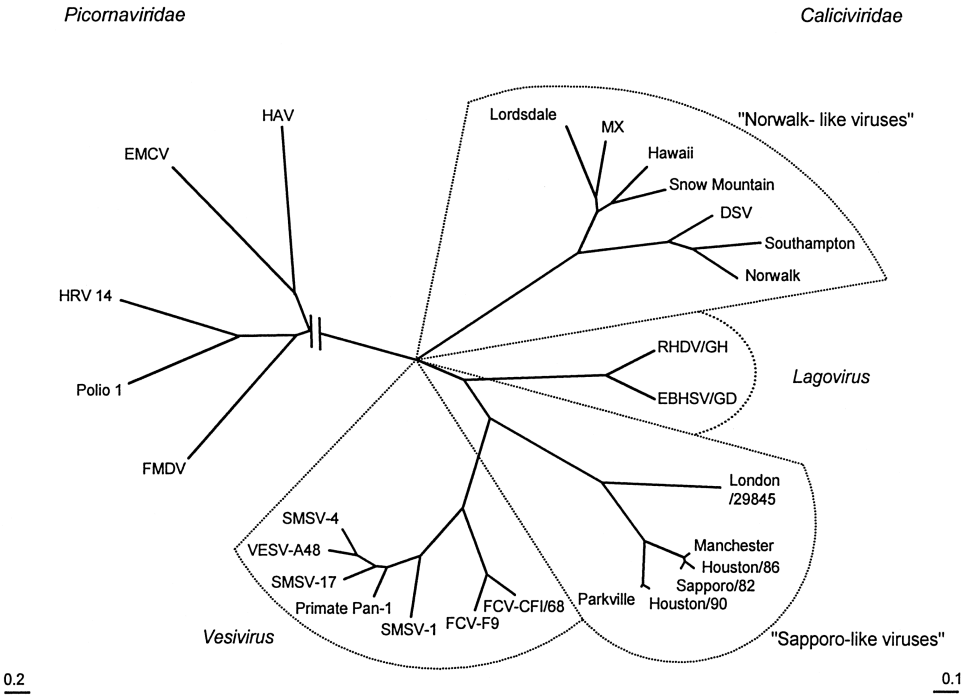

Figure 1 Phylogeny of Caliciviridae. Source: Green et al. (18)

Each of the five genera within *Caliciviridae* infects different hosts. However, only *Noroviruses* and *Sapoviruses* infect humans and are etiological agents for viral gastroenteritis (5, 23).

Until 1972, etiological agents responsible for nonbacterial gastroenteritis had been elusive. Viruses were widely thought to be responsible for the widespread illness as bacteria was not always present in stool filtrates during outbreaks. In addition, stool filtrates free of bacteria still resulted in gastroenteritis (24). In 1972, Norovirus (NoV), formerly referred to as 'Norwalk-like virus,' was first visualized using immune electron microscopy and determined to be the causative agent of the 1968 acute gastroenteritis outbreak in Norwalk, Ohio (25-27). Often referred to by common names such as 'Snow Mountain virus', NoV nomenclature is detailed. NoV strains are named based on the species infected, genus, virus name, strain, year of isolation, and country of isolation (6). Similarly, to the use of common names, NoV is often written with its associated common name and the genogroup and cluster as an associated suffix. For example, Snow Mountain virus is a common name that is used to refer to Hu/NLV/184-01388/1990/US (6). NoV is the principal cause of viral gastroenteritis and is responsible for the majority of foodborne illness in the United States (28). NoV is also the leading cause of all deaths due to gastroenteritis at a rate of approximately 797 deaths per year (29). NoV is comprised of 5 genogroups and 32 genotypes (also referred to as clusters) of which only genogroups I, II, and IV infect humans (Figure 2) (30, 31). Genogroup I, II, and IV contain over half of the 32 clusters with each having 8, 19, and 1 respectively (30, 32).

New strains of NoV are categorized into genogroups based on their capsid protein characteristics and the sequence of genes encoding viral RdRp (30). Strains with similar genome sequences and capsid properties are classified together. The large amount of

genotypes associated with NoV can be attributed to constant mutation within the hypervariable region of the protruding domain (P2) on the major structural protein VP1.

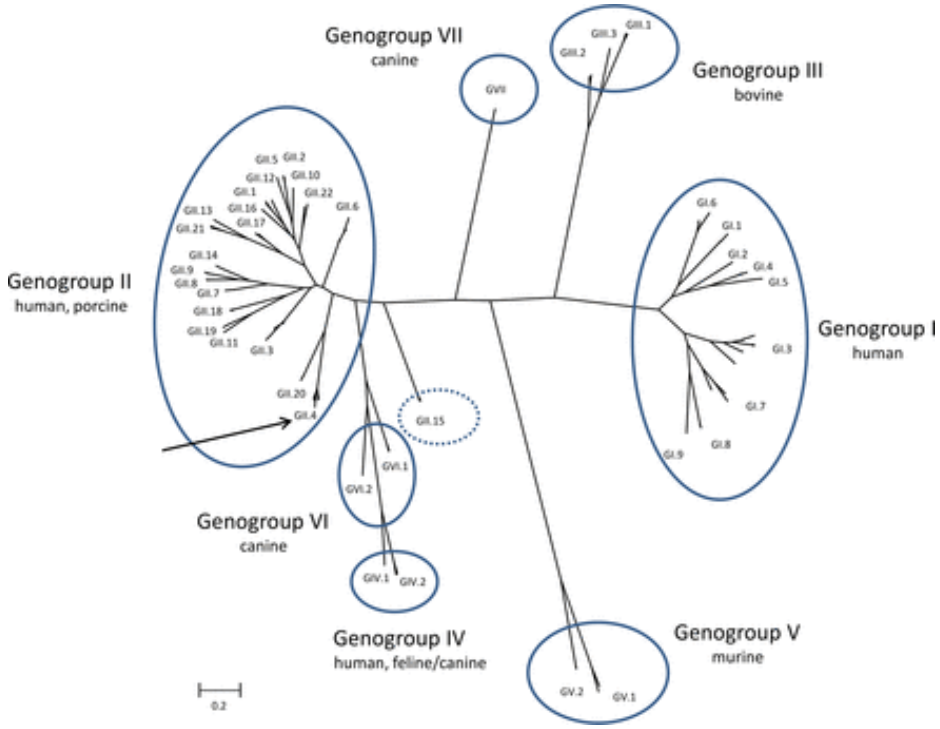

Figure 2. NoV genogroups based on VP1 amino acid sequence diversity. Source: Vinje (33)

Noroviruses within genogroups exhibit 45-61% difference between their associated VP1 genes. Similarly, within genotypes there is approximately 14-44% differences and 0-14% differences within strains (30). New genotypes are developed when there are significant differences in the VP1 sequence of a new NoV strain compared to those within established genotypes (34). The difference between the new strain and existing viruses in established clusters should exceed 44%. If the new viral strain exceeds the difference range for every cluster, then a new one is formed. NoV GII.4 is the predominate cause of gastroenteritis pandemics with a new strain of GII.4 emerging every 2 to 3 years (35, 36). Studies have shown that NoV GII.4 evolves at a rate 1.7 times faster than all other genogroups, thus

resulting in a more rapid rate of antigenic drift (36). In addition, there is approximately 5% difference in the VP1 sequences between different GII.4 variants (37).

1.2 NoV genome, virion structure, and proteins: NoV has a compact, positive-sense, single stranded, non-segmented RNA genome. It is 7.5kbp in length and is poyladenylated at the 3' terminal (38, 39). NoV genome is organized into three open reading frames (ORF) with the exception of murine NoV genogroup V, which has a fourth ORF that overlaps ORF 2 (Figure 3) (30, 40). NoV ORFs encode the nonstructural and structural viral proteins. ORF 1 encodes a polyprotein that is cleaved by viral 3C protease (NS6) into 6 nonstructural proteins including RNA-dependent RNA-polymerase (RdRp) and NS6 (41, 42). ORF 2 and 3 encode the major and minor structural proteins VP1 and VP2, respectively.

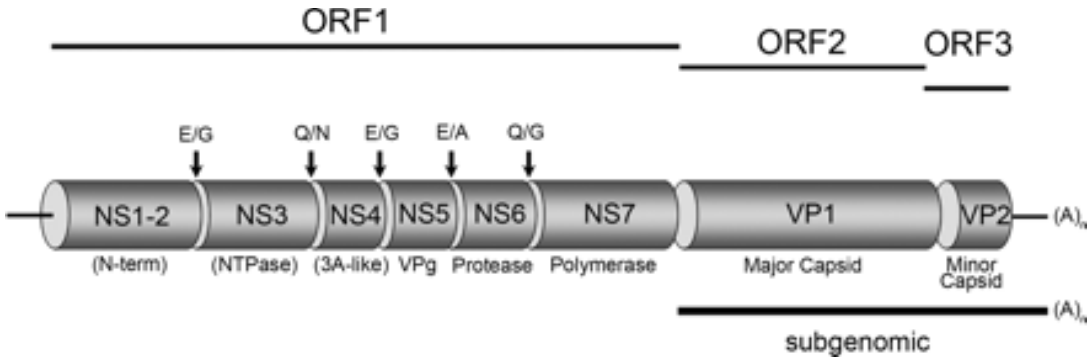

Figure 3. NoV ORFs and associated proteins. Source: Hyde et al., (43)

Caliciviridae viruses have a unique feature in that their nonstructural proteins are located at the 5' end of RNA before the structural proteins which are found at the 3' terminus (17, 44, 45). In addition, NoV does not have methylated caps at the 5' end of its RNA genome rather it is covalently linked to viral protein VPg which is thought to play a role in translation initiation (15, 39, 46, 47). Furthermore, studies have shown that caliciviruses void of VPg at the 5' terminus are noninfectious (15).

Stem-loops and hairpin secondary structures have been located respectively at the 5' and 3' ends of NoV RNA genomes (48, 49). The function of NoV secondary structures is poorly understood, however theories as to their mechanism and role have been published. The 3' terminal hairpin structure has characteristics similar to cis-acting replication elements (cre) in picornaviruses (48). NoV 5' terminal stem loops are thought to play a significant role in viral translation due to the shortcomings of the untranslated regions (UTR) of the NoV genome. Short UTRs are found at the 5' and 3' terminals of the NoV RNA genome (50, 51). UTRs play a significant role in viral translation, replication, pathogenesis by interacting with cell translation machinery and viral replicase (48, 52, 53). UTRs are typically long, enabling them to function. However, NoV UTRs are short. The secondary stem loop structures are believed to function similarly to UTRs and initiate translation (48).

Each of NoV ORF encodes one or more proteins. Subgenomic RNA containing ORF 2 encodes major structural protein (SP) VP1. VP1 is approximately 58 to 60kDA and 530 to 555 amino acids in length (54). Due to the lack of cell line propagation for NoV, the structure and function of VP1 has often been characterized using virus-like particles (VLPs); particles that are similar in structure to native virus particles but lack RNA (55, 56). VP1 provides the icosahedral capsid surrounding NoV. In 1994, Prasad et al., first viewed the NoV capsid using x-ray crystallography (57). The capsid was determined to be 38 nm in diameter, exhibit a T=3 icosahedral symmetry, and be comprised of 90 dimers of VP1 (56). In addition, cup-like depressions characteristic of the *Calicivirdae* family were present at the 3-fold and 5-fold axis of symmetry (56, 57). The VP1 structural protein can be divided into two domains: the N-terminal shell (S) and C-terminal protruding (P), each playing a significant role in the viral capsid formation (Figure 4).

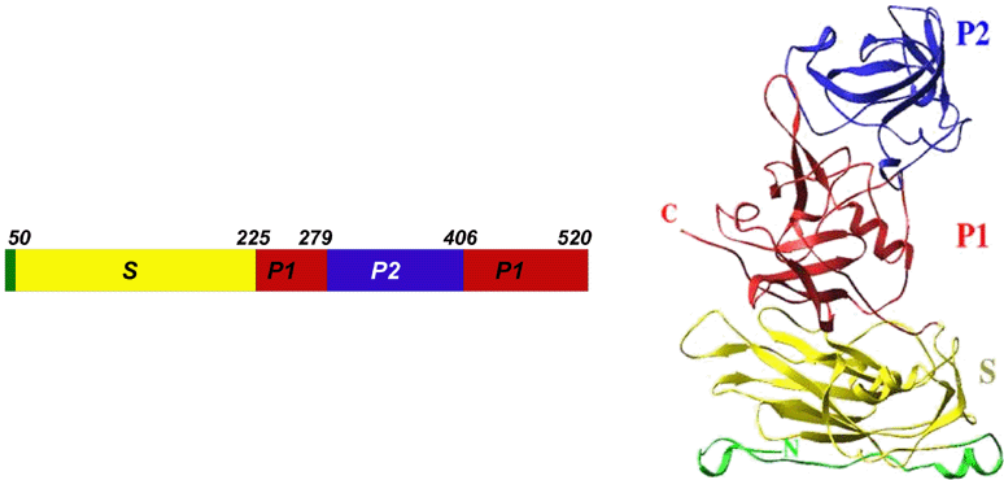

Figure 4. NoV major structural protein VP1. Source: Hardy et al. and Prasad et al. (54, 57)

The S domain is a highly conserved region that contains all of the material necessary for the initiation of capsid assembly (57). The P domain has a conserved and hypervariable region respectively within its subdomains P1 and P2 (57). Characteristics of the P domain make it likely that its function is to provide stability to the viral capsid (57). The S and P domain interact to form dimeric VP1 protrusions that can be observed using an electron microscope. The structure of VP1 is such that two well-conserved regions (S and P1) border the hypervariable P2 region with P2 being a 127 amino acid insertion in the P1 subdomain (Figure 4). Although an insertion in P1, the surface of the P2 subdomain is exposed as it forms the outer tip of the viral protrusions (Figure 5) (56). As such P2 may play a significant role in carbohydrate antigen interactions and receptor binding, however a true receptor cannot be determined until NoV is cultured with a cell line (58).

NoV ORF 3 encodes minor structural protein VP2 which is 208 to 268 amino acids in length. Although believed to be extremely basic in function and structure, much of the information regarding VP2 is elusive. Only one to two dimers of VP2 are associated with each NoV virion and there is a high amount of variability in the VP2 sequence between different virus strains (59). Research has shown that VP2 is not necessary for VLP assembly thus

further supporting theories that it is a minor structural protein that merely stabilizes VP1 in the NoV capsid (60, 61). However, in the absence of VP2, feline calicivirus (FCV) was no longer infectious; potentially indicating a more significant role for VP2 in NoV (61).

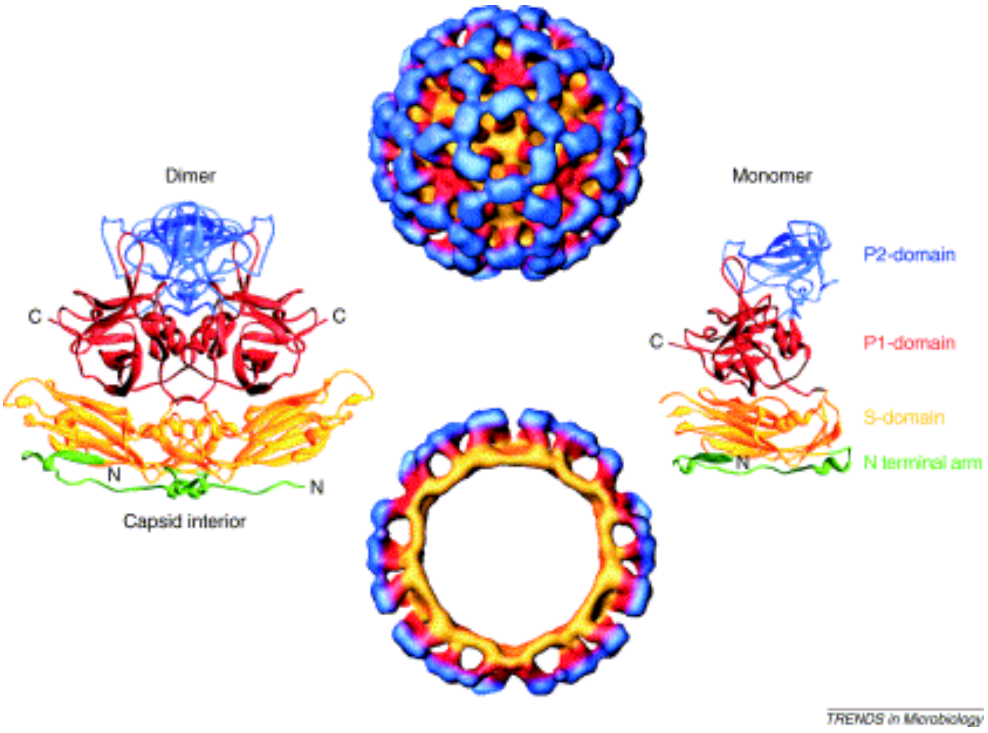

Figure 5. NoV capsid structure. Source: Huston et al., (62)

Currently, the exact location of VP2 in the NoV structure is unknown. However, studies have shown that VP1 and VP2 interact in the S domain of the N-terminal, inside the NoV capsid. This indicates that VP2 may function in viral capsid assembly (63).

Human Norovirus cannot be propagated in immortal cells. Information regarding gene expression and protein function is derived from culturable NoVs such as Murine Norovirus (MNV) (50). In addition, valuable information regarding protein expression is gained using immortalized cell lines (64-66). As previously mentioned, ORF1 is a polyprotein that is cleaved by PRO, commonly called 3C-like protease (referred to as NS6 in MNV) producing six nonstructural proteins (NP) (Figure 6) (5, 43, 67).

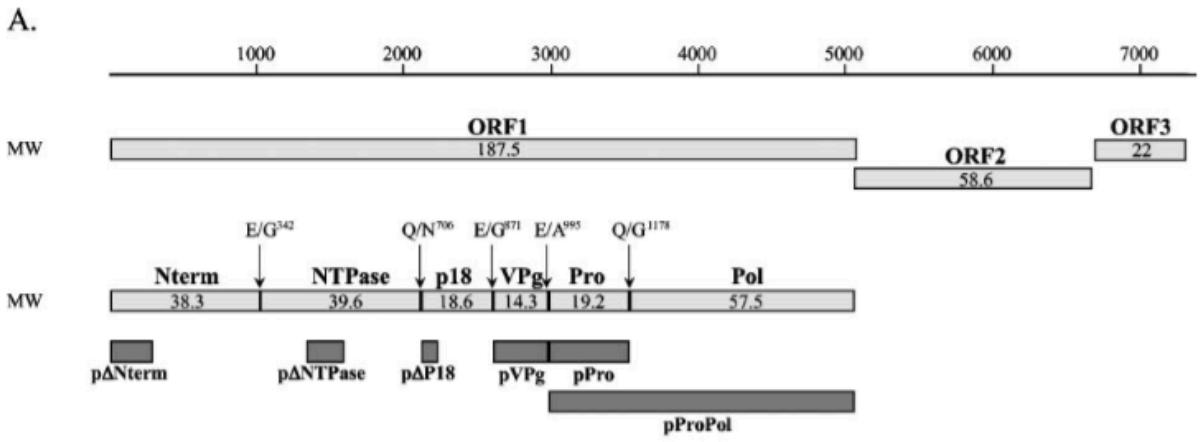

Figure 6 MNV ORF cleavage sites and nonstructural proteins. Source: Sosnovtsev et al., (42)

Sosnovtsev et al., determined there are five dipeptide cleavage sites in MNV ORF1: ^{341}E/G^{342}, ^{705}Q/N^{706}, ^{870}E/G^{871}, ^{994}E/A^{995}, and ^{1177}Q/G^{1178} (42). ORF1 is coded from the N to the C terminus as sequence conservation is greater toward the latter (54). Similarly the N terminus amino acid sequence and length is considerably different between NoV genotypes I and II (54). The six NP of NoV are encoded as follows in ORF1: p48, NTPase, p22, VPg, Pro, and Pol/3Dpol (42, 54). The latter encoded proteins found in the C terminus of ORF1 are the most conserved and characterized of the NP (42). NoV nonstructural and structural proteins differ greatly in function. Structural proteins, both minor and major encompass all of the information necessary to initiation capsid formation and provide structure stability. In comparison, nonstructural proteins function in the genetic mechanisms of NoV occurring both within the capsid and host cell. Each of the nonstructural proteins has individual functions and some work in conjunction with one another.

NoV p48 also referred to a N-term (NS1/2 in MNV) does not exhibit similar characteristics with any other viral protein except parachoviruses (54). Parachoviruses viral proteins have a Hbox/NC sequence that functions in cell proliferation. There is an observed

Hbox/NC sequence in p48 as such it is believed to have similar function to that of the parachovirus protein with the analogous sequence feature (54, 68). In addition to having a role in cell proliferation, p48 is believed to function in the formation of membrane replication complexes and Golgi disassembly (5, 54, 69, 70).

NTPase, commonly referred to as p41 (NS3 in MNV) is believed to be an RNA helicase homologous to poliovirus 2C (referred to at ATPase) and HCV NS3 (54, 71, 72). There are 3 identified motifs (A, B, and C) on NTPase categorizing it in the Superfamily III of RNA helicases. It is believed that NTPase hydrolyzes ATP and unwinds viral nucleic acids (72, 73). Studies have shown that despite its ability to hydrolyze ATP, NTPase lacks the ability to unwind synthetic RNA:DNA complexes (54). Although similar to ATPase and HCV NS3, proteins with confirmed unwinding abilities, NTPase may potentially lack the functionality of other RNA helicase. A significant amount of information is needed to fully characterize NTPase in NoV, however studies have reported that when NTPase is inhibited viral RNA synthesis does not occur (71).

Several of NoV nonstructural proteins' functions are vague including p22 often referred to as 3A-like (NS4 in MNV). The common name 3A-like used interchangeably with p22 is derived from the similar location p22 in NoV shares with 3A in picornaviruses. Much of the information regarding NoV replication complex has been elusive. It was not until recently that researchers observed the potential role p22 plays in NoV replication complex formation (74). Prior to this newly discovered role, p22 was simply one of several protein precursors in a proteolytic pathway (41). Researchers theorized that p22 could function similarly to 3A in picornaviruses in the localization of replication complexes and inhibition of protein secretion (75). Studies published in 2010 and 2012 determined p22 functions in the membrane

localization of the replication complex and inhibits protein secretion (74, 76). NS4 in MNV, a p22 homologue was observed to play a role in the formation of the replication complex, Golgi disassembly, and inhibition of cellular protein secretion (74). Similarly, p22 serves the same roles as NS4, however it uses different mechanisms. Nonstructural protein p22 contains an Endoplasmic Reticulum export signal (ERES). There is a theorized interaction between p22 ERES and COPII protein that results in the direct uptake of p22 by COPII vesicles. This potential interaction results in COPII vesicles bypassing the Golgi during localization thus causing Golgi disassembly and inhibition of protein secretion (76). Unlike p22, NS4 lacks an ERES. As such, although similar in function p22 and NS4 fulfill their viral roles using different mechanisms.

VPg is a viral nonstructural protein covalently linked to the 5' end of caliciviruses (39). Little information is known regarding the function of VPg, however the use of MNV has proven beneficial in gaining knowledge of the protein structure and potential interactions. In 2013, Leen et al., observed the structure of VPG in FCV and MNV using nuclear magnetic resonance spectroscopy (77). VPg was found to have a compact helical core comprised of hydrophobic and salt-bridge interactions that is bordered with flexible N and C terminal regions. Due to a Tyr residue within the helical core of VPg seemingly renders it unable to bind viral polymerase; however studies have proven the ability of VPg to unwind exposing its tyrosine residue. Despite the valuable structural information gained about VPg, no relationship was found between the viral protein function and structure (77). Several studies have provided evidence that suggest VPg functions in the recruitment of translational machinery. These studies have shown VPg interacts with viral eIF4F a translation initiation factor and eIF3 (78, 79). The characterization of VPg is still poorly developed. However, it is clear that the viral protein's

interaction with translation initiation complexes is not limited to those required for translation to occur. Chaundry et al., determined eIF3 is not required for the initiation of translation in caliciviruses (79). VPg is required for caliciviruses to be infectious; however it is not required for translation to occur. Suitable substitutes such as m^7G have been observed to function in the same capacity as VPg (80). Similarly, when VPg was removed from caliciviruses translation was still initiatied, however there was a significant reduction in viral proteins (39).

Pro (NS6 in MNV) is a well characterized nonstructural protein that similar to 3C proteases in picornaviruses (5). Pro functions in the proteolysis of ORF1 and cleaves poly(A) binding proteins that inhibit cellular translation (59, 66). The structure of Pro was reported by Nakamura et al., and determined to have chymotrypsin folds and an active site stabilized by Hydrogen bonds (81). Similar to chymotrypsin-like proteases, Proactive site consists of a catalytic triad required for proteolysis activity. Cys139, His30, and Glu54 form the catalytic triad in Pro, however studies have shown Glu54 is not required for Pro to function rather it functions in increasing proteolysis activity (81, 82). Pro has two enzyme substrate binding sites (S1 and S2) that bind substrate P1 and P2 in NoV ORF1. S1 contains His157, a specificity site that senses and cleaves P1 at the glutamine or glutamic acid residue. Similarly, the hydrophobic S2 interacts with the sidechain of the amino acid residue on P2. Interestingly, studies have shown that that mutation of His157 does not eliminate Pro proteolytic activity, however a drastic reduction is observed.

Second to PRO, Pol/3DPol's (NS7 in MNV) structure and function is well-characterized. Pol/3DPol is the viral RNA-dependent RNA polymerase (RdRp) which synthesizes the negative sense RNA intermediate used as the template strand during genome replication (48). Pol/3DPol structure and function is similar to that of the viral RdRp found in rabbit hemorrhagic

disease virus. Both viral RdRp have structural features commonly found among RdRp such as fingers, palm, and thumb domains (83, 84). Norovirus Pol/3DPol contains a C terminal located in the active site which is near an aspartic acid residue (38, 54). As reviewed by Hardy et al., this unique characteristic of Norovirus RdRp may result in structural similarities to the thumb insertion of Hepatitis C which functions in the stabilization of primers during the initiation of RNA synthesis (54).

1.3 NoV translation, replication, and pathogenesis: Several viruses in the calicivirdae family bind to carbohydrate structures. For example, RHBV and MNV have been shown to bind to H-antigens and sialic acid, respectively (85). Similarly, human noroviruses (HuNoV) and Sapoviruses are observed to bind human histo-blood group antigens (HBGA) and Lewis antigens (Le), carbohydrate antigens commonly found on red blood cells, saliva, and tissue such as the intestines (86). Due to the numerous caliciviruses which contain carbohydrate receptors, its has been proposed that the ancestor of caliciviruses also formed a binding-complex with carbohydrates (85). NoV recognition and binding to HBGAs is strain-specific. Different strains of NoV bind either A and/or B, and H antigens or Le, and H antigens. Only one NoV strain: Farmington Hills 2002 has been found to bind both A and Le antigens (87). Both GI and GII contain strains of NoV that bind either AB strains or Le strains with GII.4 binding the most groups of HBGAs (88). Mutational studies have shown that altering genes such as FUT2 (fucosyltransferase) knocks out the expression of H type 1 or Lewis B antigens in the small intestine resulting in resistance to NoV GI.1 infection (89). In addition, Shirato et al., reported that NoV VLPs tend to bind more tightly to type 1 carbohydrates which are commonly found on the surface of the small intestine, indicating the possibility for NoV tissue specificity (88). Although studies have provided an understanding of what binds to NoV, the binding interaction

is widely theorized as no culture method for NoV exists. NoV is believed to bind to HBGAs on the surface of epithelial cells in the gastroduodenal junction (90). The highly variable and surface-exposed P2 domain of the capsid protein contains the binding-site for HBGAs. The binding of HBGAs within the P2 domain results in specific and nonspecific interactions. Within the P2 domain site 1 and site 2 amino acids form a binding pocket and stabilize the interaction (58). Site 1 within P2 interacts with the fucose of HBGAs via hydrogen bonds. Site 2 forms additional interaction bonds, however they too are weak. Due to the weak nature of the bonds researchers have theorized that long-distance non-specific binding may occur to aid in stabilizing the HBGA and P2 interaction (91, 92). The internalization of NoV by host cells remains a mystery, however binding to an unidentified receptor is required. Studies have shown that MNV entry into murine macrophages does not occur via clathrin and caveolin-mediated endocytosis. Furthermore, MNV uptake by host cells requires host cholesterol and dynamin II, a protein involved in endocytosis (93, 94). Despite the elusive nature of norovirus propagation in an immortalized cell line information regarding entry into host cells can be gleaned from MNV entry mechanisms.

The major and minor structural proteins of NoV play a significant role in attachment and entry into host permissive cells. Upon release into the host cell cytoplasm, the viral genome is uncoated and initial translation proceeds from the 5’ to 3’ terminus (ORF1 to ORF3). NoV is a positive-sense single-stranded RNA and thusly serves as the mRNA template for initial translation round. As previously described, RNA-binding protein VPg is attached at the 5’ terminus of all calicivirdae viruses and is required for infectivity, but not translation. Although translation will proceed in the absence of VPg, it does function in recruiting host cell translation machinery. The host cell translation initiation factor recognizes and interacts with VPg. Direct

interactions between VPg and components of the translation initiation factor complex, specifically eIF4E and eIF3 have been observed. During and after the translation of ORF1 it is processed by Pro, a viral protease which cleaves ORF1 into six nonstructural proteins which play a role in the formation of the replication complex. Translation proceeds from ORF1 to ORF3. Unlike the nonstructural proteins, VP1 and VP2 are translated from polycistronic subgenomic RNA and do not function in viral replication (95).

Post-translation of parental RNA, NoV replication proceeds resulting in the proliferation of positive-sense genomic and subgenomic RNA. NoV replication is not fully understood as no suitable cell culture method has been proposed for NoV propagation in an immortal cell line. As such, a sizeable amount of information regarding NoV replication has been gleaned from MNV studies. NoV is a Class IV member of the Baltimore Classification meaning its viral genome can serve as mRNA and encode viral proteins. Prior to replication occurring, the replication complex forms in the perinuclear region of the host cell. The replication complex is composed of several host membranes (ER, endosomes, and trans-golgi complex) and is formed via initiation and recruitment by nonstructural proteins p48 and p22. Formation of the replication complex has yet to be studies in cells infected with HuNoV RNA. NoV replication occurs via a (-) sense intermediate synthesize using viral RdRp. The NoV parental genome strand is (+) sense and mechanism for initiation by the viral RdRp to synthesize (-) RNA is not fully understood. Thorne et al., provides a well-rounded overview of the two proposed mechanisms for viral RdRp initiation (5). Viral RdRp initiates the synthesis of the (-) sense intermediate by two proposed methods: de novo and VPg (65). As previously discussed, VPg plays a role in the initiation of translation, however a link between VPg and (-) sense NoV RNA has yet to be proven. In comparison, loop sequences found in the S domain of VP1 interact

with the viral RdRp supporting the theory that initiation occurs via de novo mechanisms (64). Positive-sense single stranded genomic and subgenomic RNA is synthesized from the double-stranded RNA and is VPg-dependent. Nucleotidylation, also referred to as guanylation is the formation of a phosphodiester bond between the guaninine of RdRp and tyrosine of VPg. This interaction is required for NoV infectivity; as such VPg is required for NoV infectivity. Although nucleotidylation is required for the synthesis of both genomic and subgenomic RNA, several downstream mechanisms have been proposed for subgenomic RNA synthesis. As described in Thorne et al., early termination during (-) RNA synthesis may result in (-) RNA serving as the template for (+) sense subgenomic RNA synthesis (5). Studies have also theorized that secondary structures such as stem-loops found upstream from ORF2 in (-) sense RNA may promote the synthesis of subgenomic (+) sense RNA (48). Post-replication genomic RNA localizes to the assembly site due to the localization signal in ORF1. The exact location and mechanism for assembly, encapsulation, and exit for NoV virions is unknown, however members of caliciviridae induce host cell apoptosis as an exit strategy.

According to the Center for Disease Control and Prevention (CDC), annually, Human Norovirus (HuNoV) is the cause of approximately 20 million cases of nonbacterial acute gastroenteritis, 70,000 hospitalizations, and nearly 800 deaths among young children and elderly patients. In general, waterborne human enteric viruses pose a greater health risk than enteric bacteria due to the low infectious dose; which may be as little as one virion (96). NoV is easily transmitted and often found in closed, small communities such as schools, cruise ships, nursing homes, and hospitals. NoV is transmitted through direct contact with a contaminated source such as faeces and vomit. Any exposed individual can become infected with NoV but severe and prolonged symptoms are most often associated with infants, young children,

immunocompromised individuals, and the elderly. Symptoms of NoV occur 12 to 48 hours after exposure with the infection, typically lasting 12 to 72 hours. Infected individuals experience symptoms common for gastroenteritis such as nausea, vomiting, diarrhea, and at times dehydration; some of which have been associated with the pathophysiological effects caused by NoV infection. Although NoV exhibits a rapid onset and resolution, infected individuals are capable of shedding the virus for an extended period of time; even after symptoms disappear which further contributes to the high transmission rate (97, 98).

NoV infections result in pathophysiological changes within the intestines of infected individuals. In addition, specific short-lived immune responses have been observed. Intestinal biopsies from infected human volunteers show significant changes such as: broadening villi, enlarged and pale mitochondria, intercellular edema, abnormal epithelial cells, and lesions (99-102). Although abnormal, the intestinal epithelial cells remain intact post-infection and the lesions resolve within two weeks. NoV infection is believed to occur within the epithelial cells of the intestine. However, research has shown that apoptosis of enterocytes occurs in infected individuals (103). It remains unclear whether the observed apoptosis is due to direct or distant interactions with NoV virions. However, it has been theorized that an increase in $CD8^+$ lymphocytes results in the release of perforin, thus inducing apoptosis. The pathophysiological changes associated with NoV infection contribute to the associated symptoms. The shortening microvilli and slow gastric emptying are responsible for malabsorption and vomiting, respectively.

In addition to pathophysiological changes, short-lived immune responses occur in the presence of NoV infections. Adaptive immunity has been proven to play a significant role in the immune systems response to NoV. In the absence of B and T-cells high levels of MNV-1 was

observed in mice (31). Cytotoxic T-cells in the duodenum have been observed 0-6 days post-onset of NoV symptoms (103). Immune responses in infected individuals are short-lived and due to the high mutation rate within the variable region of the P2 domain, little immunological memory exists in patients when exposed to NoV on a repeated basis.

1.4 NoV in oyster harvesting waters: Seawater surrounding the oysters can become contaminated with HuNoV through various sources such as direct discharge of human or animal waste into the body of water (104). In addition, bivalve molluscan shellfish are known to actively concentrate microorganisms and viruses. Therefore, seafood can concentrate HuNoV and cause foodborne viral illness if consumed raw. Infected individuals may shed as many as 10^6 to 10^{10} infectious virons per gram of faeces, and raw sewage can contain anywhere from 10^3 to 10^5 infectious virons per liter. While there are over 100 different enteric viruses that have been observed in human faeces, Hepatitis A virus (HAV) and HuNoV are the ones most commonly implicated in seafood-borne outbreaks. Due to its low infectious dose and high transmission rate, HuNov is a public health concern.

There are numerous filtration and concentration techniques to detect the presence of HuNoV, however none of the current techniques are optimal or available for use with a range of contaminate sources. Several methods often used for concentrating enteric viruses include adsorption/elution, electronegative and electropositive membranes, and ultrafiltration. The adsorption elution method, commonly referred to as VIRADEL (105) involves the adsorption of viral particles to a filter by charge interaction. The viral particles are then eluted from the membrane by a pH- adjusted solution. The most common elution buffer is beef extract, however in recent years beef extract is no longer used as it has inhibitory effects on PCR (106, 107). Two different types of filters can be used for the viradel method: an electronegative or

electropositive filter. Several studies have shown that the recovery of viruses from seawater using a positively charged membrane is quite poor (108), however the presence of multivalent salts help facilitate the binding of the virus to a negatively charged membrane (107). Viruses in water typically have a negative surface charge, thus the water sample pH must be conditioned to change the charge on the viral particles (109). An electronegative filter in conjunction with aluminum or magnesium proved successful in recovering human norovirus from various water matrixes, not including seawater (110). Results from several studies (111-113) conclude that the presence of salt is necessary for optimum viral adsorption to a membrane filter (108). Typically there is a wide range in percent recovery rates, some studies reporting percent recovery rates as high as 16 to 84% in mineral and river water and 3 to 14% in seawater for HuNoV using electronegative membranes (114). These vast differences in virus recovery may be due to virus type rather than filter type, water matrix, or sample volume (109). Granular zeolites are known as molecular filters, and are widely used in industry for water purification due to low cost, strong ion-exchange property and large adsorption capacity (115). Zeolites are hydrated crystalline tectoaluminosilicate that have the ability to organize molecules similar to their uniform pore size (116). Some zeolites contain microporous hydrated aluminosilicates crystals with well-defined structures containing AlO_4 and SiO_4 tetrahedral linked through the common oxygen atoms and have a strong affinity for ammonia (117). Various studies suggested the ability of zeolite to adsorb virus and remove contaminants from water. In several studies zeolite was documented to remove 99% of viruses and 100% of *E. coli* from the water, and adsorb up to 5 logs of viruses in less than 1 minute (118-121). The adsorption capacity of zeolite rendered it suitability in concentration of viruses from seawater. In order to increase the recovery rate of viruses from water a secondary concentration step is needed. Beyond primary

concentration several studies conduct a secondary concentration step using a Centriprep YM-50 centrifugation unit to reduce the final volume of the concentrate (107, 122, 123). Although effective the use of a Centriprep YM-50 device is an unnecessary expense. Currently, policies set in place by governing authorities' mandates regular monitoring of oyster harvesting waters for microbial contamination, they do not regulate the method of detection. Furthermore, policies only require harvesting site closures when microbial loads are above a certain threshold. Policies do not require harvesting sites to be closed during occurrences of suspected NoV outbreaks. In addition to the lack of regulations regarding detection methods, no policies require harvesting water testing for viral contamination. As such, harvesting waters in the LA area are not directly tested for the presence of viral contaminates during suspected outbreaks. Due to the lack of regular testing and the difficulty associated with analyzing environmental water samples there is little data on virus occurrences in marine water, especially in Louisiana. In order to gain valuable information regarding the prevalence of viral contaminates in marine waters researchers must conduct a survey over an extended period of time or regularly monitor viral contamination as is done with bacterial loads.

1.5 Justification: Louisiana is located in the southern part of the United States in an area commonly known as the "Gulf Region." The Gulf Region is comprised of southern states that share a boarder with the Gulf of Mexico. Gulf States, including Louisiana play a major role in the commercial fishing industry in the United States. According to the "Fisheries of the United States 2012" report released by the National Oceanic and Atmospheric Organization, commercial fishery accounted for 11.6 billion pounds of seafood in the United States with Louisiana contributing 1.2 billion pounds. In 2012, approximately 33.1 million pounds of oysters were harvested in the United States accounting for 155.1 million dollars. The Gulf Coast region

accounted for 62% (20.4 million pounds) of the 33.1 million pounds of oysters harvested (124). Louisiana is widely known for its diverse aquaculture, however it continuously leads the nation in oyster harvesting providing 8 to 12 million pounds per year accounting for nearly 1/3 of the oyster supply within the United States (125).

In 1998, the Food and Drug Administration (FDA) released a risk assessment in which 33.6% of all Louisiana residents who consumed oysters did so raw. This figure was drastically smaller in other Gulf States such as Texas (16.5%), Florida (11.4%), and southern California (11.2%). A survey of 4,860 participants released in 2003 by the Interstate Shellfish Sanitation Conference (ISSC) found that of the population within LA, TX, FLA, and CA those who consume oysters raw are typically 40 years old, Caucasian, and males. In addition, the results of the survey showed that 4% of those who consume raw oysters are at risk for bacterial or viral illnesses due to weakened immune systems (126).

Bivalve molluscan shellfish are known to actively concentrate microorganisms and viruses. As such, oysters can concentrate norovirus and cause foodborne viral illness if consumed raw or undercooked. Due to a high percentage of consumers, who prefer to eat raw oysters, safety measures must be in place to reduce the public health risk. Throughout the world, numerous norovirus outbreaks have been linked to contaminated shellfish (127-132). Over the past decade, several norovirus outbreaks (predominately genotypes I and II) in the Louisiana area have been linked to the consumption of raw oysters from local harvesting sites (Table 1). On average the number of ill individuals is relatively low indicating the rapid investigation and recall of food products by Louisiana officials.

Table 1: NoV Outbreaks in Louisiana Linked to Louisiana Oyster Harvesting Beds from 2005-2015

Date	Location	Number Ill	Harvesting Area
March 2010	Restaurant	14	1
March 2010	Restaurant	19	7
March 2010	Restaurant	9	7
April 2012	Restaurant	14	23
December 2012	Other	9	30

Data based on Norovirus Annual Report 2010 by Louisiana Office of Public Health- Infectious Disease Epidemiology Section

Current policy set forth by the Environmental Protection Agency (EPA) recommends quantifying fecal coliforms and *Escherichia coli* in waters as microbial measures indicating the presence of human enteric viruses. In accordance, the Louisiana Department of Health and Hospitals (LDHH) Oysters Division uses microbial indicator levels as the determining factor in closing molluscan shellfish harvesting areas. The use of microbial indicators as a means for closing harvesting sites has several gaps that result in an increased likelihood for NoV outbreaks to occur. This method is not reliable, as bacterial indicators do not efficiently reflect the occurrence of enteric viruses (133). The closure of Louisiana oyster harvesting sites due to possible NoV contamination is economically inefficient. Harvesting sites may be closed for prolonged periods of time for suspected NoV potentially costing LA 13.5 to 23 million dollars a year in revenue loss (125). Although only a small percentage of the total revenue generated by LA oysters (approximately 11 to 20 percent), the financial loss accumulates with each closure resulting in a significant profit loss. *As such, there were several goals for this research project. The first objective for this dissertation was to d*etermine the utility of direct detection of HuNoV contamination in molluscan shellfish harvesting waters relative to traditional and novel fecal

indicators, as a means by which to predict human virus contamination in this important food commodity. This was successfully achieved through the performance of monthly surveys of selected shellfish harvesting waters for the presence and levels of: traditional fecal indicators (i.e., aerobic plate count, generic *E. coli*, and enterococci), novel indicators (male-specific and somatic coliphages), and NoV contamination (genotypes I and II). The second objective for this body of work was to develop and optimize a primary filtration and concentration method for the rapid and efficient detection of NoV GII in molluscan shellfish harvesting waters. In addition, immunomagnetic separation (IMS) was developed and employed for the secondary concentration of NoV GII to further removed potential inhibitors. The final objective for this body of work was to compare the newly developed filtration and concentration method to several established methods often used when detecting NoV in environmental water samples. The results of this project provide a new rapid and sensitive primary and secondary concentration method for the detection of NoV GII contamination in molluscan shellfish harvesting waters. The findings in this study suggest that testing directly for viruses in harvestings waters should be regulated closely like bacterial indicators. In doing so, there will be a reduction in the risk associated with NoV outbreaks caused by oysters and an increase in the protection of public health. The methods described in this research project are easily adaptable, cost effective, and when employed have the potential to reduce the profit loss bared by Louisiana due to oyster harvesting site closures caused by suspected NoV outbreaks.

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Chapter 2 Surveillance of Enteric Viruses and Microbial Indicators in Eastern Oysters and Harvest Waters Along the Louisiana Gulf Coast

2.1 Introduction: Noroviruses (NoV) are the leading cause of acute and epidemic gastroenteritis in humans. The Centers for Disease Control and Prevention (CDC) estimated that approximately 16.1% of the norovirus outbreaks in the U.S. with known transmission routes are foodborne (1). NoV belong to *Caliciviridae* family, and consist of a single stranded, positive sense RNA genome. To date, six genogroups of NoV (GI to GVI) have been identified, and are comprised of more than 38 genotypes. A tentative GVII has been recently proposed (2, 3). Genogroups I, II, and IV infect humans, and the rest are isolated from other species (4, 5). Despite the extensive genetically divergent nature of noroviruses, the GII.4 strains remain the predominant cause of the NoV outbreaks worldwide (6).

Pathogenic enteric virus particles are shed in large numbers into the faeces or vomit of infected individuals and enter the environmental waters by direct discharge or the release of wastewater. The viruses are either suspended or precipitated, and can survive for weeks to months while retaining their infectivity (7-9). As a result, filter-feeding mollusks inhabiting contaminated waters bioaccumulate naturally occurring or anthropogenic microbial pathogens, and if consumed either raw or inadequately cooked, transmit them to humans (7, 10).

Cases and outbreak incidences of NoV infections due to the consumption of contaminated raw or partially cooked shellfish are frequently reported worldwide (11-14). According to the CDC, mollusks accounted for 19% of foodborne NoV outbreaks in the U.S. from 2009-2012 (15). Bacteriological standards have been developed by the U.S. FDA and Interstate Shellfish Sanitation Conference (ISSC), namely National Shellfish Sanitation Program (NSSP), on using total or fecal coliforms densities for the regular monitoring and classification of harvest waters to assure sanitary quality of shellfish (16), where as in the E.U.,

the regulations have focused on fecal coliforms in oyster tissues (10). These measures effectively enhanced the health of the shellfish consumers against diseases of bacterial origin (11); however, pathogenic viruses in oysters have been detected even when levels of microbial indicators in oyster or harvest waters remained low (17, 18).

Coliphages are viruses that infect *E. coli* and are naturally present in the intestinal tract of animals. Male-specific coliphages (FRNA bacteriophages), a subset of coliphages, resembles size and genome characteristics of many enteric viruses and have been proposed as a suitable viral indicator of fecal contamination and human enteric virus (including NoV) in oysters and water but their effectiveness has not been studied intensively in Louisiana oysters (17, 19-22).

This study is the first report on the surveillance of NoV GI and GII and microbial indicators of fecal contamination both in oysters and harvest waters along the Louisiana Gulf Coast. In addition, we assessed the effectiveness of fecal indicators as determining factors for the viral safety of Louisiana oysters with regard to noroviruses.

2.2 Materials and Methods:

2.2.1 Sample collection: Biweekly samples of Eastern oysters (*Crassostrea virginica*) and harvest waters were collected from five commercially open shellfish harvesting areas along Louisiana Gulf Coast within a period of January to November 2013. For each sampling, harvest waters were grab sampled above the oyster beds followed by dredging oysters within approximately 65 m^2 of each sampling location. Data obtained from the analyses of the samples from the sampling areas 9 to 11 (Plaquemines Parish) and areas 12 to 13 (Jefferson, Plaquemines and Lafourche Parishes) were clustered as sites A and B, respectively (Figure 7). Oysters were double bagged in polyethylene bags, along with the water samples were kept on ice, and processed within 24 h of collection.

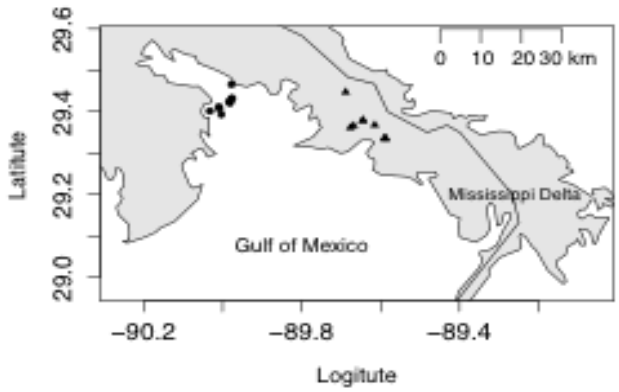

Figure 7: Sampling locations. Triangles denote sampling sites “A” and circles denote sampling area “B”

2.2.2 Oyster processing: Upon sample arrival, oysters were washed using cold tap water, and shucked under sterile conditions. For enumeration of microbial indicators, 10-12 whole viable oysters (without abductor muscles) including liquor were homogenized and analyzed immediately. For the virological analyses, digestive tissues (digestive diverticula and stomach) of 10-12 viable oysters were dissected, homogenized, and undergone virus extraction protocol as follows.

2.2.3 Microbial indicators: Standard membrane filtration technique using 47-mm mixed cellulose ester membrane filters (EMD Millipore, Billerica, MA) was utilized to quantify viable bacterial indicators of fecal contamination in the water samples. Fecal coliforms were enumerated using m-FC agar (Difco, Sparks, MD) according to EPA Method 9222. *E. coli* colonies were enumerated on modified membrane-thermotolerant *Escherichia coli* agar (Modified mTEC, Difco) following EPA Method 1603 (23). Enterococci were quantified using enterococcus indoxyl-β-D-glucoside agar (mEI, Difco) based on US EPA Method 1600 (24). Coliphages were quantified using a single agar layer method according to the U.S. EPA Method 1602 (25) which *E. coli* HS(pFamp)R (ATCC 700891) and *E. coli* CN-13 (ATCC 700609) were utilized as host strains for male-specific and somatic coliphages, respectively.

The plaque forming units (PFU) were enumerated, and reported as $\log_{10}$ PFU/100mL of water sample.

For the bacterial enumeration of oyster samples, a 1:2 suspension (w/v) of oyster homogenate and subsequent decimal dilutions in phosphate-buffered saline (PBS, 0.02 m*M* NaH_2PO_4, 0.02 *M* Na_2HPO_4, 0.15 *M* NaCl, pH 7.0) were prepared. Aerobic plate counts (APC) were counted using pour-plating technique on standard plate count agar (Neogen, Lansing, MI) following incubation for 48 h at 35 °C. To enumerate fecal coliforms and *E. coli*, multiple tube fermentation technique (5 tube-3 dilutions) was used as described by American Public Health Association for the examination of shellfish (26).The data were reported as $\log_{10}$ most probable numbers (MPN)/100 g oyster. Male-specific coliphages (MSC) and somatic coliphages (SC) were enumerated from 15 mL (eq. 15 g) whole oysters using a modified double-agar-overlay method developed for the analysis of oysters. The data were reported as $\log_{10}$ PFU/100g oyster.

2.2.4 Virus concentration and RNA extraction: Viruses were concentrated from 1 L of the duplicate water samples using the adsorption-elution method as described by Katayama and others (27) and modified by Fong and others (28). RNA was extracted from 200 μl of the viral concentrate using the RNeasy Mini Kit (Qiagen, Germantown, MD). For the oyster samples, an adsorption-elution method by incorporating ultracentrifuge was utilized for extraction of enteric viruses from 4 g of digestive tissues following the U.S. FDA Gulf Coast Seafood Laboratory protocol (Woods and Burkhardt III 2011). In this method, virus concentrates (200 μL) were extracted for RNA, utilizing 6 *M* guanidine thiocyanate (Fisher Scientific, Fair Lawn, NJ) for the virus lysis, and RNeasy Mini Kit (Qiagen, Germantown, MD) following the manufacturer's

instruction with minor modifications, in which 15 min hold time was given after adding the washing buffers. Extracted RNA were immediately analyzed, or stored at -80 °C until required.

2.2.5 Detection and quantification of enteric viruses: TaqMan quantitative real-time Reverse Transcription-Polymerase Chain Reaction (RT-qPCR) was used for the detection and quantification of NoV GI and GII by targeting the most conserved, sensitive and broadly reactive ORF1-ORF2 junctions in NoV, as described by Kageyama and others (29) and Jothikumar and others (30). For the oysters, pathogenic enteroviruses (EV) that is, *Poliovirus*, *Echovirus*, Human *Coxackievirus*, Human *Rhinovirus*, and Human *Enterovirus* were also analyzed by coamplifying the 5' untranslated region of the enteroviral genome with a panenterovirus primer set utilizing primer and probes developed by Donaldson and others (2002). Cepheid SmartCycler® II system (Sunnyvale, CA) was used for all the RT-qPCR analyses.

Detection and quantification of NoV GI and GII in the harvest water were followed according to Gentry and others (31). The reaction mixture used a SuperScript® III Platinum One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA) in a 25 µL reaction mixture and 2.5 µL RNA template. A homogenous internal amplification control (IAC) was incorporated in all reactions to evaluate PCR inhibition (Jennifer Gentry-Shields, North Carolina State University, personal communication). Reverse-transcription was carried out at 50°C for 15 min, followed by enzyme activation for 2 min at 95 °C, and 45 cycles of 15 s at 94 °C, 15 s at 55 °C and 20 s at 72 °C (threshold = 30).

Analyzing enteric viruses in oysters digestive tissues followed a multiplex RT-qPCR assay for simultaneous detection and quantification of NoV GI, GII, and EV along with a heterogeneous IAC as optimized by Burkhardt and others (32) and Nordstrom and others (33).

Reaction mixture used Qiagen® OneStep RT-PCR kit (Valencia, CA) for a total volume of 25 µL per reaction and 3.0 µL of RNA template. The templates were reverse-transcribed at 50 °C for 50 min, and then the HotStarTaq DNA polymerase was activated at 95 °C for 15 min, followed by thermal cycling for 10 s at 95 °C, 25 s at 53 °C, and 70 s at 62 °C for a total of 50 cycles, and a final extension at 72 °C for 10 min (threshold = 10). Reactions considered positive when the emission intensities exceeded the threshold during the first 46 cycles. All the reactions were carried out in duplicate. NoV GI and GII RNA standards (10^9 Genomic Equivalent Copies (GEC)/µL) were kindly provided by Dr. Christian Moe's laboratory at Emory University (Atlanta, GA), and Human Poliovirus 3 stock (attenuated Sabin strain) kindly provided by Dr. William Burkhardt at the U.S. FDA Gulf Coast Seafood Laboratory (Dauphin Island, AL), and were utilized as positive controls and for RNA quantification.

2.2.6 Outbreak sample: The Molluscan Shellfish Program - Louisiana Department of Health and Hospitals in January 4, 2013, reported a norovirus outbreak in Cameron Parish due to possible consumption of contaminated oysters. Oyster and water samples were collected from the suspected area (located in Cameron Parish, basin 3, area 30: 29.85139, -93.37995) on January 17, 2013, and analyzed along with a stool specimen, which was sent to LSU/AgCenter Food Microbiology Laboratory approximately 14 days after the onset of the acute gastroenteritis symptoms from one of the affected individuals known to have consumed raw oysters from the suspected area and exhibited gastrointestinal symptoms typical of norovirus. A 20% suspension of stool specimen was clarified by centrifugation at 12,400 ×g for 5 min. The RNA was extracted from 150 µL of the suspension and analyzed accordingly.

2.2.7 Sequencing and genotyping: Sequencing was performed either by direct sequencing of M13-tailed RT-qPCR products (34) or sequencing the amplified junction region between

ORF1 and ORF2 (Region C) of the viral genome (35, 36) cloned into a pCR2.1-TOPO TA vector using TOPO® TA Cloning® Kit, with TOP10 *E. coli* (Life Technologies). Sequences were read on an ABI Prism 3130 Genetic Analyzer (Life Technologies), and processed on 4Peaks (version 1.7.2, Nucleobytes Inc., Amsterdam, Netherlands) and CLC Sequence Viewer (version 7.5, CLC Bio, Aarhus, Denmark). The query sequences were aligned against the nucleotide database representing different taxonomic groups available at The National Center for Biotechnology Information (NCBI) by utilizing The Basic Local Alignment Search Tool (BLAST) (37). Phylogenic analysis of the sequences was inferred by using the Maximum Likelihood method based on Tamura-Nei model (38) by employing MEGA (version 6.0), a molecular evolutionary genetics analysis tool developed by Tamura and others (2013). The sequences of the reference strains were retrieved from the GenBank sequence database deposited at NCBI (2, 39).

2.2.8 Statistical analysis: All the analyses were carried out in duplicates and reported as mean ± standard error. Significant differences among mean ranks and multiple comparisons were evaluated using Kruskal Wallis test at $\alpha = 0.05$. Pearson product-moment correlation coefficients (r) were used to assess dependency and correlation among variables, respectively. Software RStudio (version 0.98.1028, RStudio Inc., Boston, MA) was used for the statistical analyses and visualization. Data of water surface temperature used in this study was obtained from the Giovanni online data system, developed and maintained by NASA Goddard Earth Sciences Data and Information Services Center (GES DISC); a threshold of 24 °C was considered to categorize the data to warm months (May through October) against cold months (November through April).

2.3 Results and Discussion: All the sampling locations (areas 9 through 13) were among the most active commercial oyster harvesting along the Louisiana Gulf Coast and remained open during the sampling period; however, due to adverse weather conditions (heavy rain, fogginess, storm, or water level) sample collection from some specific areas or times was not possible. Figure 8 shows monthly sea surface temperature of the sampling areas along Louisiana Gulf Coast in 2013. Due to the lack of a reliable cell culture system, RT-qPCR has been the most widely utilized method for the detection and quantification of NoV in complex food and environmental samples where the level of virus contamination is usually low. In this study, protocols previously optimized for the analysis of oysters and harvest waters were used (17, 18, 40, 41).

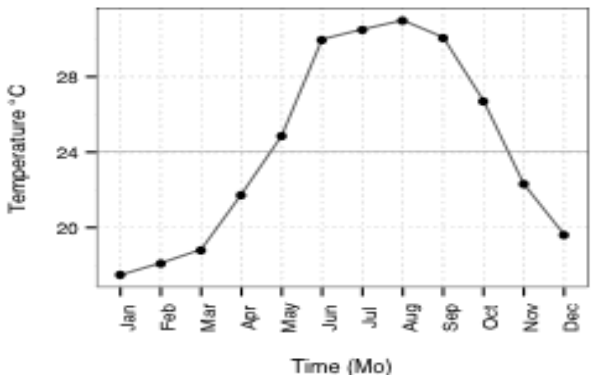

Figure 8. Monthly temperature (°C) of sea surface in Louisiana Gulf Coast in 2013. The horizontal line denotes the 24 °C threshold to distinguish cold and warm months.

2.3.1 Microbial indicators: Microbial indicators in both oysters and harvest waters were relatively low with no significant difference across sampling sites ($p > 0.05$, Tables 2-3). Mean density of APC in oysters was 5.47 ± 0.13 $\log_{10}$ CFU/100g, and lower than 6.5 log/100g as previously reported in Gulf Coast oysters (17). Enterococci along with other fecal indicators can be used to evaluate sanitary condition of shellfish harvest water (42). This group of bacteria is accumulated in oyster tissues

(43); however they have not yet been considered as a sanitary monitoring criterion in oysters (16). In our study, enterococci were analyzed only in harvest water, and present in all the samples at 0.50 ± 0.08 log_{10} CFU/100mL.

Shellfish harvest areas in the U.S. are classified based on the sanitary survey of water and monitoring the concentration of fecal or total coliforms in the surface water (16). Both fecal coliforms and *E. coli* were detected in all the water samples with average concentrations of 0.69 ± 0.07 and 0.38 ± 0.05 log_{10} CFU/100mL, respectively. According to the NSSP, the mean concentration of fecal coliforms in "approved" classification of shellfish growing water should not exceed 1.15 log_{10} CFU/100mL for mTEC test, with 10% of the samples not exceeding 1.49 log_{10} CFU/100mL (16). In our study, fecal coliforms in the water samples were present within the acceptable limits.

Table 2: Microbial indicators in oysters harvested from site A and B, mean ± SE

		Month				
Microbial indicators	Site	March	April	July	September	October
APC		4.70 ± 0.03	4.76 ± 0.16	6.19 ± 0.13	6.11 ± 0.17	5.87 ± 0.01
Fecal coliforms		0.69 ± 0.00	1.97 ± 0.23	0.69 ± 0.00	0.92 ± 0.23	0.69 ± 0.00
E. coli	A	0.69 ± 0.00	0.92 ± 0.23	0.69 ± 0.00	0.69 ± 0.00	0.69 ± 0.00
MSC		1.04 ± 0.00	1.04 ± 0.00	1.04 ± 0.00	1.04 ± 0.00	1.04 ± 0.00
SC		1.04 ± 0.00	1.11 ± 0.07	1.04 ± 0.00	1.04 ± 0.00	1.04 ± 0.00
		January	February	June	August	November
APC		4.56 ± 0.28	5.55 ± 0.09	6.06 ± 0.32	5.78 ± 0.16	5.37 ± 0.09
Fecal coliforms		2.24 ± 0.09	1.15 ± 0.46	0.69 ± 0.00	0.69 ± 0.00	0.87 ± 0.17
E. coli	B	0.92 ± 0.23	0.69 ± 0.00	0.69 ± 0.00	0.69 ± 0.00	0.87 ± 0.17
MSC		1.04 ± 0.00	1.04 ± 0.00	1.15 ± 0.11	1.04 ± 0.00	1.04 ± 0.00
SC		1.30 ± 0.15	1.04 ± 0.00	1.04 ± 0.00	1.04 ± 0.00	1.12 ± 0.08

Acronyms: APC: aerobic plate count, MSC: male-specific coliphages, SC: somatic coliphages.
Units: APC: log_{10} CFU/100g; fecal coliforms and *E. coli*: log_{10} MPN/100g, MSCand SC: log_{10} PFU/100

Table 3: Microbial indicators in harvest waters from site A and B, mean ± SE

Microbial indicators	Site	Month					
		April	February	July	March	October	September
Enterococci	A	0.39 ± 0.16	1.06 ± 0.23	0.16 ± 0.15	0.52 ± 0.10	0.15 ± 0.03	0.31 ± 0.24
Fecal coliforms	A	0.68 ± 0.13	N.A.	0.33 ± 0.21	0.54 ± 0.12	N.A.	1.28 ± 0.20
E. coli	A	0.46 ± 0.19	0.83 ± 0.49	0.21 ± 0.15	0.56 ± 0.03	0.55 ± 0.10	0.31 ± 0.24
MSC	A	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.54 ± 0.09	0.00 ± 0.00	0.35 ± 0.40
SC	A	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.54 ± 0.09	0.00 ± 0.00	0.00 ± 0.00
		February		June	August		November
Enterococci	B	0.89 ± 0.16		0.08 ± 0.09	0.34 ± 0.31		0.93 ± 0.20
Fecal coliforms	B	N.A.		0.45 ± 0.05	0.69 ± 0.47		1.01 ± 0.21
E. coli	B	0.89 ± 0.27		0.10 ± 0.19	0.14 ± 0.18		0.51 ± 0.28
MSC	B	0.15 ± 0.21		0.00 ± 0.00	0.00 ± 0.00		0.08 ± 0.15
SC	B	0.30 ± 0.43		0.08 ± 0.15	0.00 ± 0.00		0.00 ± 0.00

N.A.: missing data due to some technical issues. Acronyms: MSC: male-specific coliphages, SC: somatic coliphages. Units: Enterococci, fecal coliforms and *E. coli*: $\log_{10}$ CFU/100mL, MSC and SC: $\log_{10}$ PFU/100mL.

Eastern oysters growing in estuarine waters of the Gulf Coast bioaccumulate fecal coliforms to a concentration of approximately 4.4 times greater than their surrounding water (44). In total, 33.3% and 77.8% of oyster samples of sites A and B, respectively, were positive for fecal coliforms. Similarly, lower prevalence of *E. coli* was observed in the site A with 22.2% positive samples against 66.7% in site B. The overall means of fecal coliforms and *E. coli* in oysters (sites A and B combined) were 1.08 ± 0.10 and 0.76 ± 0.04 $\log_{10}$ MPN/100g, respectively. Both were lower than the safety levels of 2.52 or 2.36 $\log_{10}$ MPN/100g respectively in ≥1 or ≥2 out of 5 sub-samples.

In our study, the prevalence of MSC and SC were remarkably low. Out of 17 water samples, only 29.4% (0.09 ± 0.04 $\log_{10}$ PFU/100mL) and 23.5% (0.06 ± 0.03 $\log_{10}$ PFU/100mL) were positive for MSC and SC, respectively. In oysters, no MSC was detected in site A where as approximately 33.3% of the oyster samples from site B were positive for MSC averaging 1.05 ± 0.01 $\log_{10}$ PFU/100g. Somatic coliphages were detected in 16.7% of the oysters (site A and B) at 1.08 ± 0.02 $\log_{10}$ PFU/100g. Similar concentrations of MSC in U.S.

market oysters have been reported by DePaola and others (17). However, higher levels of MSC (>3 $\log_{10}$ PFU/100g) have been found in oysters from the U.K. commercial harvesting areas (45). In general, even though there were no significant difference between site A and B in terms of the bacterial indicators ($p > 0.05$), it was apparent that site B showed higher prevalence of fecal contamination.

2.3.2 Trends and correlations: In previous studies, Eastern oysters (*C. virginica*) from the Gulf Coast showed seasonality for the accumulation of MSC (increasing from late November through January) but not in the case of fecal coliforms and *E. coli* (44). We did not observe any distinctive temporal effect on microbial indicators in oysters except for the APC, which was significantly ($p < 0.05$) higher during warm months in agreement with Shieh and others (46). It could be due to a increased rate of digestion in oysters at elevated temperatures (47). These observations, however, differ from the report of DePaola and others (17) where the concentrations of MSC, fecal coliforms, and *E. coli* in oysters reached their highest levels in the summer at 0.9 $\log_{10}$ PFU/100 g, >3.3 $\log_{10}$ PFU/100 g and 2.3 $\log_{10}$ PFU/100 g, respectively with no observed seasonal trend for APC (averaged 6.5 $\log_{10}$ CFU/100 g).

Overall, no strong positive correlation ($r < 0.45$) was observed between microbial indicators in oysters (data not shown). In the case of water samples, enterococci remarkably correlated with fecal coliforms ($r = 0.63$, $p = 0.000$) and *E. coli* ($r = 0.64$, $p = 0.000$). In the case of the coliphages, most of the obtained data from oysters and water samples fell below or around the detection limit, therefore, no strong evidence of correlations with bacterial indicators were observed ($r \leq 0.45$). Campos and others (7) obtained strong correlation between fecal coliforms and *E. coli*, MSC, and APC in shellfish. During our study, the highest correlation among microbial indicators between oysters and water samples were observed between fecal

coliforms in water and *E. coli* in oysters (r = 0.36, p = 0.059). Our results are is agreement with Wu and others (2011) that the data linking microbial indicators to virus and bacterial pathogen contamination in water is equivocal requiring further examination.

2.3.3 Norovirus detection: Despite low levels of fecal contamination in the open areas for oyster and harvesting water collection, NoV GII was detected in oysters collected from area 12 (site B) in June 2013. NoV GI or GII was not detected in any of the eighteen water samples collected. The RT-qPCR cycle threshold (Ct) values of the positive samples were 42.3 ± 0.2 in which was corresponded to 3.53 ± 0.20 $\log_{10}$ GEC/g oyster digestive tissues (r^2 = 0.99, and RT-qPCR efficiency = 96.25%, Figure 9). Secondary extraction of NoV from the oyster samples generated a positive signal as well (data not shown).

Oyster-associated NoV outbreaks often contain multiple genotypes, and comprise total of 2-3 $\log_{10}$ GEC/g of digestive tissues (8, 12, 13, 48). However, the association of RNA quantity with the risks to human health may depend on the methodology employed for the downstream analysis of the viral genome (13). To date, no NoV outbreak has been linked to the oysters harvested in June 2013 from the area 12 (site B). The 98-nucleotide NoV GII sequence obtained from RT-qPCR analysis of the oyster sample (Figure 10) showed 90% query coverage and 98% identity (expected value of $1:10^{40}$) with the NoV GII sequences deposited at NCBI nucleotide database (data not shown). Even though, this clearly indicates that the reaction was true positive for NoV GII, the RT-qPCR targeted sequence (ORF1-ORF2 junction) is a highly conserved region in NoV GII, and not suitable for genotyping NoV strains (29). So far, no alternative conserved PCR primers have been recognized for the confirmation of NoV positive RT-qPCR assays (Knight and others 2013). Positive NoV RNA and negative reactions all with the internal controls were also incorporated in all the samples analyzed. Due

to the low concentration of NoV in the samples (high Ct value), genotyping through the amplification and sequence analysis of regions B, C or D of the viral genome was not possible (data not shown) as previously reported (17, 35, 41).

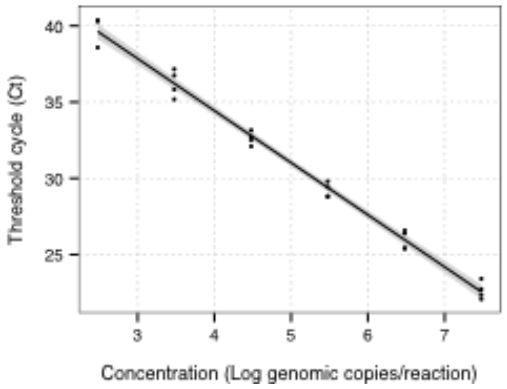

Figure 9. NoV Calibration curve (the cycle threshold (Ct) from the multiplex RT-qPCR assay as a function of NoV GII RNA concentration per reaction. The gray shaded area denotes the 95% confidence interval.

```
                    20                  40
CARGARBCNATGTTYAGRTGGATGAGGTTTTCTGACTTGAGCACGTGGG

          60                  80
AGGGCGATCGCAATCTGGCTCCCAGTTTTGTGAATGAAGATGGCGTCGA
```

Figure 10. RT-qPCR amplicons sequence from the NoV GII positive oyster sample (5' to 3'). Note: IUPAC codes used to indicate the degenerate positions resulted from the degeneracy of the forward primer, COG2F (Kageyama and others 2003): Y, C or T; R, A or G; B, not A; N, any.

2.3.4 Outbreak samples: Table 4 summarizes densities of the microbial indicators in the suspected oysters and harvest water from Cameron Parish (area 30). The concentrations of fecal coliforms and *E. coli* in oysters were slightly higher than the majority of the samples analyzed from sites A and B. Higher levels of indicators in oysters and water could be indicative of the presence of noroviruses and other pathogenic enteric viruses (18, 20). RT-qPCR analysis of the suspected oysters harvested from the area 30 (Calcasieu Lake, LA) and the overlaying water did not indicate any NoV contamination. However, the stool specimen

obtained from the individual who consumed raw oysters from area 30 was positive for GII (8.55 ± 0.00 $\log_{10}$ GEC/g).

Table 4: Microbial indicators in oysters and harvest waters from Cameron Parish (area 30), mean ± SE.

Microbial indicators	Oysters	Harvest waters
Enterococci	-	1.02 ± 0.03
APC	4.63 ± 0.01	-
Fecal coliforms	1.77 ± 0.17	0.57 ± 0.10
E. coli	1.15 ± 0.46	0.97 ± 0.06
MSC	1.04 ± 0.00	0.65 ± 0.30
SC	1.04 ± 0.00	0.83 ± 0.00

Acronyms: APC: aerobic plate count, MSC: male-specific coliphages, SC: somatic coliphages. Units (harvest waters): Enterococci, fecal coliforms and *E. coli*: $\log_{10}$ CFU/100mL, MSC and SC: $\log_{10}$ PFU/100mL. Units (oysters): APC: $\log_{10}$ CFU/100g, fecal coliforms and *E. coli*: $\log_{10}$ MPN/100g, MSC and SC: $\log_{10}$ PFU/100g

Phylogenic analysis of the NoV viral genome revealed that the strain belonged to the GII.4 Sydney, which has been the dominant NoV outbreaks strain in the U.S. during 2013 and 2014 (2). Other strains (GI.2, GI.3, GI.4, GII.b, GII.e, GII.2, GII.6, GII.12 and GII.13) have been identified in the shellfish or clinical specimens obtained from shellfish-associated NoV outbreaks, however the NoV genotypes identified in oysters could rarely be linked to the outbreak cases (8, 12, 14, 48). The nucleotide sequence of the stool NoV GII determined in this study is deposited in GenBank under the accession number KP455650.

Our assumption for not detecting NoV in the outbreak-suspected oysters would be the lack or low concentration of the NoV in the oysters (< 1.7 $\log_{10}$ GEC/g digestive tissues) were available in the area on the sampling date, or secondary transmission which masks the connection between sources and outbreaks (12). According to the CDC, food handlers have been considered as primary source of NoV transmission contributing to about 70% of the NoV outbreaks with known sources in the U.S. (15).

2.4 Conclusion: This study surveyed noroviruses and microbial indicators of fecal contamination in oysters and harvest waters taken from commercial harvesting areas along Louisiana Gulf Coasts, and to evaluate the effectiveness of microbial indicators for assessing viral safety of oysters. Microbial indicators (fecal coliforms, *E. coli* and coliphages) detected in oysters and harvest waters were used as an indication of fecal contamination. Based on the current standards of fecal coliforms in shellfish harvest water in the U.S., all the samples were within the acceptable ranges defined by the NSSP, and could not reliably predict the occurrence of NoV in the oysters. Whether detecting NoV in the tested oysters can be considered a health hazard is rather complicated because no robust tissue culture technique has been developed for laboratory growth of norovirus to distinguish between infectious and noninfectious noroviruses (40), and current RT-PCR methods are not able correlate molecular detection results with the NoV infectivity (35, 40).

Due to the difference in the rate of depuration among enteric viruses and fecal indicators in oysters, incorporating more efficient microbial sanitary indicators of depurated shellfish has been suggested (43). Also, postharvest multiplication of fecal coliforms and *E. coli* can occur in oysters that make applying sanitary measures for oysters at the harvest time ambiguous (17). Male-specific coliphages have been recently proposed as a better indicator of fecal contamination of U.K. market-ready oysters as they showed a seasonality consistent with the trend of shellfish-associated gastroenteritis outbreaks (45). In addition, the NSSP set a MSC density of 1.70 $\log_{10}$ PFU (or 50 PFU) per 100 gram of oysters as the threshold for the closure of sewage contaminated shellfish growing area (16). In our study, the concentrations of coliphages in oysters and harvest waters could indicate fecal contamination; however their concentrations were very low and barely detectable. As a result, it is unlikely that they can

reliably indicate a potential health risk (17, 20). Therefore, monitoring of harvesting waters and oysters for pathogenic enteric viruses is crucial (49). Recently, dual criterion of NoV detection and elevated MSC (>1.70 $\log_{10}$ PFU/100g) in oysters has been suggested to flag for potential public health issues (17).

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Chapter 3 Novel Method For Rapid Detection Of Human Norovirus GII In Oyster Harvesting Waters Using Zeolite Granules Coupled With Immunomagnetic Separation

3.1 Introduction: Norovirus (NoV), member of the family Caliciviridae is a single strand RNA enteric virus that causes acute gastroenteritis in those infected. NoV has seven identified genogroups, three of which pose a public health concern: GI, GII, and GIV, and approximately 40 different genotypes (1). Throughout the years NoV GII.4 has remained the predominate cause of NoV outbreaks worldwide (2). According to the Center for Disease Control and Prevention (CDC), annually NoV is the cause of approximately 58 percent of all cases of nonbacterial acute gastroenteritis (3). The cost associated with NoV is exorbitant averaging approximately 2 billion dollars a year, of which 184 million dollars is associated with seafood contamination (4).

NoV is easily transmitted through direct contact with a contaminated source such as faeces and vomit. Furthermore, infected individuals can shed the virus for an extended period of time in their faeces even after symptoms have disappeared (5-7). Infected individuals may shed as many as 10^9 infectious virions per gram of faeces (5). Similarly, raw sewage can contain anywhere from 10^3 to 10^5 infectious virons per liter. NoV potentially enters environmental waters in several ways. Sewage contamination and boat discharge have been extensively studied and are common sources for NoV contamination in shellfish harvesting waters (8-12). Once in the environment, NoV is stable to degradation due to environmental factors. In surface waters, NoV can survive for weeks to months possibly due to adsorption by organic matter and binding to inorganic matter (13-15). In addition to resisting degradation, NoV can remain infective while suspended in environmental waters, thus posing a public health risk as most waterborne human enteric viruses exhibit a low infectious dose (16). Bivalve molluscan shellfish are known to actively concentrate microorganisms and viruses. As

such, seafood can concentrate NoV and cause foodborne viral illness if consumed raw or undercooked. Several studies have examined the link between NoV contamination in shellfish and fecal pollution in harvesting waters (11, 17, 18).

In order to reduce the amount of illness due to contact with a NoV contaminated source sensitive, rapid, and reliable detection methods are essential. Several methods often used for concentrating enteric viruses include adsorption/elution, electronegative and electropositive membranes, and ultrafiltration. The adsorption elution method involves the adsorption of viral particles to a filter by charge interaction (19). The viral particles are then eluted from the membrane by a pH- adjusted solution. The most common elution buffer for NoV recovery from filtration membranes is beef extract; however in recent years beef extract is no longer used as it has inhibitory effects on several molecular techniques including real-time polymerase chain reaction (RT-PCR) (20-22).

Concentrating human enteric viruses in environmental water samples has proven to be a difficult task. There is a need for an improved primary and secondary concentration method that makes possible rapid detection while providing a high recovery rate to aid in addressing the public health concern. In effort to increase the percent recovery of NoV from environmental water matrices and provide rapid detection of NoV in oyster harvesting waters, we employed the use of zeolite granules for the primary filtration and concentration of NoV contaminated waters. Additionally, we employed immunomagnetic separation(IMS) as a secondary concentration step to further reduce the presence of inhibitors in the elution prior to RT-PCR.

3.2 Materials and Methods:

3.2.1 Virus stock: NoV positive fecal (GII.4 Sydney) was obtained from the stool of an infected individual associated with a NoV outbreak in January 2013 (Cameron Parish, La., area 30) (11). Positive fecal samples were confirmed using RT-PCR and sequencing during

previous studies (11). NoV fecal stock solutions were prepared as previously described in Haramoto et al., 2009. One milliliter of NoV GII positive fecal sample was diluted in 9ml of phosphate buffer saline. The diluted mixture was centrifuged for 10 minutes at 9500 rpm. Following centrifugation the supernatant was collected and stored at -80^{o}C and used as positive NoV stocks (2x10^{8}gec/ml) (23).

3.2.2 Zeolite: Zeobrite®Xtreme granular zeolite (Zeotech Co., Fortworth, TX) was used for the primary filtration and concentration of NoV from various water matrices. The zeolite used during this study had an internal negative charge and a positive surface charge enabling it to bind both positive and negative ions. The zeolite granules had a size range of 0.3-1.4 mm in diameter and a total surface area of 45-60 m^{2}/g. The porous granules have the ability to capture particles ranging in size from 2 to 20 microns.

3.2.3 Artificial and Environmental Water Samples: Varying volumes of NoV stock were inoculated into artificial and environmental oyster harvesting waters. Artificial marine water was prepared by adding marine salt to distilled water resulting in a final salinity of 20 parts per thousand (ppt). Environmental water samples were collected at Hackberry Bay in Lafourche Parish, Louisiana (29.4088324, -90.0303508). Environmental water samples were collected via grab-samples below the surface of the water and stored at -20^{o}C until analysis. Salinity, temperature, and turbidity were measured at the time of sampling using a YSI 30 Salinity, Conductivity, and Temperature reader (YSI Incorporation., Yellow Springs, OH). Prior to filtration, environmental waters were thawed at 25^{o}C for 24 hours. Water samples were adjusted to the optimal viral binding salinity (20ppt) prior to zeolite filtration. None of the waters were pre-filtered or chemically treated.

3.2.4 Optimization of elution buffer: Six different groups of elution buffers were tested for their ability to elute NoV from the zeolite granules (Table 5). Each buffer varied in chemical and physical properties (molarity and pH). The optimal elution buffer was chosen based on the highest percent recovery of NoV calculated based on the RT-PCR results. Each buffer was tested in 3 replicates and each replicate was duplicated (n=6). We used SAS software to determine the difference between the means using Tukey's HSD (SAS Institute Inc., Cary, NC). The optimal buffer was then used to test for the optimal salinity and temperature for the elution of NoV off of zeolite granules.

Table 5: Average percent recovery of NoV from zeolite granules using preliminary buffers tested based on chemical and physical properties[1]

Molarity		
Buffer	Molarity	Average Percent Recovery
EDTA	0.1-1M	4.67± 0.03
NaCl	1-6M	N.D[2]
pH		
Buffer	pH	Average Percent Recovery
Phosphate	1-10	N.D
Glycine	1-10	N.D
SDS	1-10	N.D
Percent weight/volume (%w/v)		
Buffer	(%w/v)	Average Percent Recovery
Beef Extract	3	53.44± 0.15
Beef Extract in FBS	3	7.54 ± 0.02
Beef Extract in MI	3	14.76 ± 0.13

[1]No. of trials, 3.
[2]N.D – Not Detected

3.2.5 Virus concentration and elution using zeolite: Artificial and environmental marine water (100ml) was seeded with NoV GII.4 Sydney positive virus stock. The seeded water (140 µl) was aliquot into an eppendorf tube labeled "pre" and stored at 2-8^{o}C until viral RNA

extraction. In duplicate, 50ml of water was filtered through 2g of pre-soaked zeolite. As described by the manufacturer instructions, prior to filtration the zeolite granules were soaked in NoV-negative distilled H_2O to activate the surface charges. The zeolite was thoroughly dried and combined with 1.5ml of 10% beef extract in McIlvaine's buffer. The mixture was rigorously shaken for 15 minutes at 25^{o}C. Finally, 140 µl of the supernatant was aliquot into a tube labeled "elu" and used for viral RNA extraction.

3.2.6 Immunomagnetic beads: Tosylactivated magnetic beads (Dynabeads M-280, ThermoFischer Scientific, Waltham, MA) were coupled to Anti-Norovirus GII.4 mouse monoclonal antibodies, produced using VLP GII.4 Minerva 2006 as an immunogen (Abcam, Cambridge, MA). As instructed on the manufacturer's website, 5ug of pre-washed M-280 beads were coupled with 100ug of monoclonal antibodies (1.0mg/ml) in 0.1M borate buffer pH 9.5. Ammonium sulphate (3M) in borate buffer was added and the mixture was incubated at 4^{o}C for 48 hours. The antibody-coupled beads were placed on a magnet and the supernatant was removed. The beads were incubated at 37^{o}C in phosphate-buffered saline pH 7.4 with 0.5% (w/v) bovine serum albumin (BSA) for 1 hour. The beads were again placed on the magnet and the supernatant was discarded. Phosphate-buffered saline pH 7.4 with 0.1% (w/v) BSA was added resulting in a final antibody-coated bead concentration of 20mg/ml. Epoxy magnetic beads were coupled to Anti-Norovirus GII.4 mouse monoclonal antibodies (Abcam, Cambridge, MA) using the Dynabeads Antibody Coupling Kit (ThermoFischer Scientific, Waltham, MA).

3.2.7 Virus concentration and elution using zeolite coupled with immunomagnetic separation: Artificial and environmental marine water (100ml) was seeded with NoV virus stock. A sample of the seeded water (140 µl) was aliquot into an eppendorf tube labeled "pre"

and stored at 2-8^{o}C until viral RNA extraction. In duplicate, 50ml of the seeded water was filtered through 2g of pre-soaked zeolite. Prior to filtration, the zeolite granules were soaked in NoV-negative distilled water. The zeolite was thoroughly dried and combined with 1.5ml of 10% beef extract in McIlvaine's buffer. The mixture was rigorously shaken for 15 minutes at 25^{o}C. The eluent was centrifuged twice at 8rpm for 1 minute to remove any excess zeolite. After centrifugation, 33uL of M-280 antibody-coated beads were added to the supernatant and incubated at 37^{o}C for 1 hour on a rotator. The solution was placed on a magnet and the supernatant was removed. The beads were washed twice in PBS pH 7.4. Finally, 140uL of PBS was added to the beads and labeled "elu" for viral RNA extraction.

3.2.8 Viral RNA extraction: All viral RNA extractions were completed using a Qiamp Viral RNA Minikit (Qiagen Sciences Inc., Germantown, MD) immediately after NoV elution from the zeolite granules. Briefly, 140 µl of the "pre" and "elu" samples were mixed with 560µl of AVL, vortexed, and allowed to sit for 10 minutes at room temperature. After viral lysis, 560µl of 99-100% ethanol was added to the mixture and vortexed for 15 seconds. The sample mixtures were added to minispin columns and centrifuged at 8.0rpm for 30 seconds. The columns were washed with AW1 and AW2 wash buffers and transferred into new collection tubes. Viral RNA was eluted from the minispin columns using 35µl of AVE. Sample eluents were immediately placed on ice and analyzed via RT-PCR.

3.2.9 RT-PCR: RT-PCR was used for the detection and quantification of NoV GII in the various water matrices. RT-PCR was performed in duplicate using a Cepheid SmartCycler II (Cepheid, Sunnyvale, CA). Each RT-PCR template tube contained 11.1µl of RNA and 8.9 µl of master mix. The master mix included 5 µl of TaqMan® Fast Virus 1-Step Master Mix (Life Technologies Co. Carlsbad, CA), 900 µM of each primer (JJV2F and COG2R), and 150 µM of

probe (Ring2P). RT-PCR amplification targeted the ORF1-ORF2 junction as described in Kageyama et al., 2003 (24). The thermal cycling conditions were as follows: reverse transcription was at 50°C for 5 min, initial denaturation at 95°C for 100 s, and 40 cycles of amplification (95°C for 15 s, 55°C for 15 s, 72°C for 30 s).

3.2.10 Standard curve Standard curves were used for the extrapolation of data for unknown RNA samples. Ten-fold dilutions were carried out in duplicate starting from known GII RNA standards (10^9 genomic equivalent copies/µl). RT-PCR was conducted on each dilution in duplicate and a standard curve was developed. The amplification efficiency (E) was calculated using the following equation: $E = (10^{-slope}) - 1$.

3.3 Results and Discussion:

3.3.1 Elution Buffer Optimization and Efficiency: Buffer Optimization: Four of the 60 preliminary buffers tested were capable of eluting NoV off of zeolite granules (Table 5). The four buffers included: 3% Beef Extract, 3% Beef Extract in Fetal Bovine Serum, 3% Beef Extract in McIlvaines Buffer, and 0.5M EDTA. However, upon further validation 0.5M EDTA did not result in the recovery of NoV from the granules. Each of the five beef extract buffers were able to elute NoV from zeolite, however there was a significant difference between the percent recoveries of each buffer ($p<0.05$). The 10% beef extract in McIlvaines buffer (% w/v) proved most efficient in eluting NoV with a recovery range of 34.95 to 54.24% and an average recovery of 41.76 $\pm$ 0.07% (Table 6). Each of the other beef extract elution buffers including: 3% beef extract, 3 and 10% beef extract in fetal bovine serum, and 3% beef extract in McIlvaine's buffer resulted in an average percent recovery less than 25% in artificial marine water.

Table 6: Percent recovery of NoV from zeolite granules using beef extract buffers[1]

Buffers	Genomic Copies (GC/ml) Inoculated[2]	Recovered[2]	Percent Recovery[3]
3% BE	$3.28x10^6$ ($2.64x10^6$-$3.92x10^6$)	$8.58x10^5$ ($1.86x10^5$-$3.53x10^6$)	10.54 (5.79-16.47)[ab]
3% BE/FBS	$4.87x10^6$ ($2.99x10^6$-$6.99x10^6$)	$4.81x10^5$ ($3.43x10^5$-$5.80x10^5$)	10.58 (6.52-13.78)[ab]
10% BE/FBS	$2.33x10^6$ ($9.54x10^5$-$4.42x10^6$)	$4.21x10^6$ ($1.94x10^5$-$1.17x10^6$)	19.92 (7.50-32.88)[ab]
3% BE/MI	$6.10x10^6$ ($2.99x10^6$-$1.08x10^7$)	$7.35x10^5$ ($6.15x10^5$-$8.39x10^5$)	15.25 (7.04-23.84)[ab]
10% BE/MI	$6.35x10^6$ ($4.87x10^6$-$8.31x10^6$)	$2.68x10^6$ ($1.72x10^6$-$4.51x10^6$)	41.76 (34.95-54.24)[a]

[1] No. of trials, 6.
[2] Average RNA genomic copies and range. Large variability in range is due to inhibitors in solid fecal material
[3] Percent recovery and standard deviation. Superscripts represent mean comparison report based on Tukey's HSD method. Conditions with different connecting letters are significantly different ($p<0.05$).

3.3.2 Salinity, Temperature, and Time: The salinity of the seeded water had a significant impact on the percent recovery of NoV (Table 7). There was a statistical significant difference between the percent recoveries of NoV at the various salinities with 10 and 20ppt proving optimal for NoV binding to zeolite granules. 20ppt was statistically different from the other salinities tested except 10ppt.

Table 7: Average percent recovery of NoV from artificial water based on salinity using zeolite granules[1]

Salinity	Genomic Copies (GC/ml) Inoculated[2]	Recovered[2]	Percent Recovery[3]
10	$8.33x10^6$ ($2.53x10^6$-$6.46x10^6$)	$8.55x10^5$ ($4.36x10^5$-$1.87x10^6$)	30.00 (9.86-73.84)[ab]
15	$1.04x10^7$ ($3.03x10^6$-$6.85x10^6$)	$5.85x10^5$ ($3.55x10^5$-$9.76x10^5$)	13.35 (11.01-15.92)[b]
20	$6.35x10^6$ ($2.26x10^6$-$3.75x10^6$)	$1.07x10^6$ ($5.59x10^5$-$1.88x10^6$)	40.79 (17.74-75.80)[a]
25	$2.45x10^7$ ($8.89x10^6$-$1.61x10^7$)	$7.43x10^5$ ($3.32x10^5$-$1.41x10^6$)	7.56 (2.53-15.83)[b]

[1]No. of trials, 6.
[2]Average RNA genomic copies and range. Large variability in range is due to inhibitors in solid fecal material
[3] Percent recovery and standard deviation. Superscripts represent mean comparison report based on Tukey's HSD method. Conditions with different connecting letters are significantly different ($p<0.05$).

Although statistically similar to 10ppt, 20ppt resulted in the highest recovery of NoV with an average percent recovery of 40.79 ± 0.19. An incubation temperature and time curve for the inoculation of NoV with zeolite granules in the elution buffer was developed, however no significant difference was observed (data not shown). As such, this study employed 25°C for 15 minutes as the optimal incubation parameters as NoV recovery was the highest at 35.21%.

3.3.3 Recovery of NoV GII in artificial and environmental waters: Zeolite filtration: NoV seeded in artificial water was recovered off of zeolite granules using the optimal conditions previously described. The recovery range for artificial water was 17.74-75.80% with an average recovery of 40.79 ± 0.19% (Table 8). In comparison, the percent recovery range for NoV in environmental waters was 5.63-60.90 with an average recovery of 18.95 ± 0.24% (Table 8).

Table 8: Average percent recovery of NoV from artificial and environmental oyster harvesting waters using zeolite granules and optimal elution conditions[1,2]

		Genomic Copies (GC/ml)		
	Water Matrices	Inoculated	Recovered	Percent Recovery
Zeolite[3]	Artificial	$2.93x10^6(2.26x10^6\text{-}3.75x10^6)$	$1.19x10^6$ $(5.59x10^5\text{-}1.88x10^6)$	40.79 ± 0.19
	Environmental	$1.40x10^6(6.01x10^5\text{-}1.85x10^6)$	$1.83x10^5$ $(9.24x10^4\text{-}4.02x10^5)$	18.95 ± 0.24
Zeolite with IMS[4]	Artificial	$2.84x10^6(2.62x10^6\text{-}3.29x10^6)$	$1.24x10^6$ $(6.24x10^5\text{-}1.90x10^6)$	44.03 ± 0.20
	Environmental	$4.49x10^5(4.40x10^5\text{-}4.56x10^5)$	$1.54x10^5$ $(1.41x10^5\text{-}1.61x10^5)$	34.36 ± 0.02

[1] Samples from Hackberry Bay, LA
[2] No. trials = 6
[3] Conditions: BE (10%) in McIlvaines Buffer, 20ppt, 25°C
[4] Conditions: BE (10%) in McIlvaines Buffer, 20ppt, 25°C, IMS using Tosylactivated IMBs

3.3.4 Zeolite filtration coupled with Immunomagnetic Separation: Although both epoxy (M-270) and tosylactivated (M-280) immunomagnetic antibody-coupled beads were able to recover NoV from artificial water, the percent recovery of M-280 was significantly higher with an average recovery of 35.53% ($p<0.05$). As such, the zeolite protocol was coupled with immunomagnetic separation using M-280 tosylactivated monoclonal antibody-bound beads. Removal of NoV RNA from the M-280 beads was more efficient using an AVL lysis buffer compared to release via 99^{o}C water bath or incubation at 99^{o}C for 5 minutes in a thermal cycler (data not shown). The percent recovery range for artificial water was 23.24-72.23 with an average percent recovery of 44.03 $\pm$ 0.20 (Table 8). In comparison, the percent recovery range for environmental water was 30.88-35.62 with an average percent recovery of 34.36 $\pm$ 0.02 (Table 8).

3.3.5 Comparison of NoV Recovery between zeolite without and with IMS: NoV was recovered from artificial and environmental oyster harvesting waters using zeolite granules in the absence of immunomagnetic separation (IMS) at 40.79 $\pm$ 0.19 and 18.94 $\pm$ 0.24%, respectively. Similarly, NoV was recovered from artificial and environmental waters using zeolite coupled with IMS as a secondary concentration step. Coupled with IMS, zeolite filtration resulted in an increased recovery in both water matrices. Zeolite with IMS resulted in a percent recovery of 44.03 $\pm$ 0.20. In comparison, zeolite coupled with IMS applied to environmental waters had a percent recovery of 34.36 $\pm$ 0.02. The observed percent recovery of NoV in both artificial and environmental waters was higher when zeolite was coupled with IMS compared to the percent recoveries using zeolite alone (Table 8). In addition, the percent recovery range within the 6 trials for both artificial and environmental water was significantly smaller when zeolite was coupled with IMS ($p<0.05$). Although the percent recoveries were higher in the

presence of IMS, the average percent recovery was reduced in environmental waters compared to artificial waters regardless of the use of IMS. There was approximately a 21% reduction between the percent recovery of NoV in artificial waters versus environmental waters when zeolite was used. Similarly, a 9% reduction was observed between the two waters when zeolite was coupled with IMS. However, the observed reduction in the percent recovery between artificial and environmental waters was substantially reduced when zeolite was coupled with IMS.

The percent recovery of NoV from seeded artificial and environmental water was used to determine the efficiency of the detection parameters including: salinity, incubation time and temperature, elution buffer, and use of IMS. NoV was detected using RT-PCR and quantified via extrapolation from a standard curve of known NoV concentration. However, although able to detect NoV results from RT-PCR cannot determine whether the NoV is infective as a tissue culture method has yet to be developed (25). Granular zeolites have been employed in previous studies and are documented to remove 99% of viruses and 100% of *E. coli* from water, and adsorb up to 5 logs of viruses in less than 1 minute (26-29). In addition to its antiviral and antimicrobial properties, zeolite has been shown to be effective in the reduction of both enveloped and non-enveloped viruses (27). The use of dual-charged zeolite as a filter to bind NoV proved effective. Our study found NoV in artificial water had an average recovery of 40.79 $\pm$ 0.19 while NoV seeded in environmental oyster harvesting waters had an average recovery of 18.95 $\pm$ 0.23. This difference is largely in part due to the presence of inhibitors in environmental waters. In the environment, most enteric viruses, including NoV have a negative charged (30-32). The zeolite used in this study had a dual surface charge, as such it is suspected that NoV bound to the zeolite via electrostatic interaction. However, due to a higher

recovery rate in salt water versus fresh water matrices electrostatic interaction cannot be the only interaction binding NoV to the zeolite granules. EDTA (ethylenediaminetetraacetic acid) is a chelating agent that has been documented to remove aluminum from zeolite (33). The removal of aluminum from the zeolite granules by EDTA did not result in the cessation of the NoV-zeolite interaction further proving the existence of interactions beyond electrostatic interaction.

Salt has the ability to increase electrostatic interactions thereby increasing virus adsorption to membranes. River and seawater have an abundance of cations and anions including: Na^{+}, Mg^{2+}, Ca^{2+}, K^{+}, Sr^{2+}, and Cl^{-}. The presence of trivalent and divalent cations in water has the ability to increase electrostatic interaction between virus particles and adsorption membranes (34-38). However, monovalent cations have the ability to increase adsorption of NoV to membranes as well. Previous studies have shown that salt is needed for increased adsorption of viral particles to filter membranes (31, 34, 35). Although salt has proven to increase adsorption, some studies have indicated that too much salt can decrease electrostatic interaction resulting in a decrease in viral adsorption (31). In our study, the optimal salinity concentration was 20ppt. The environmental waters collected for this study have a natural salt content that is predominately NaCl. NaCl is an anti-chaotropic salt thereby having the ability to strengthen hydrophobic bonds between NoV and zeolite granules (36). The protein capsid of virus typically contains amino acid residues such as glutamic acid, aspartic acid, histidine, and tyrosine (39). Salt bridge interaction has been shown to occur between viruses and membranes and could increase virus adsorption (35). NoV contains a glutamic acid residue on its viral capsid that could potentially form a salt bridge interaction with the zeolite granules increasing viral adsorption.

Beef extract has long been used as an alkaline protenacious buffer for the elution of NoV from various membranes. Typically, beef extract is used at concentrations between 1 to 3%, however several studies have indicated its ability to inhibit RT-PCR and other molecular protocols used to detect NoV (20). Several studies have attempted to reduce the presence of inhibitors in beef extract (22, 40, 41). This study used a higher concentration of beef extract compared to others (10 % w/v) as such, we employed immunomagnetic separation to further purify the eluent and reduce the presence of inhibitors.

Tosylactivated paramagnetic beads are often used when conducting immunomagnetic separation to capture NoV(42-45). However, Epoxy beads have the ability to bind to antibodies and could potentially be used for IMS. Our study found that Epoxy beads are not as efficient in recovering NoV, however they can be used as an alternative to M-280 beads. This difference may be due to the random orientation binding exhibited by Epoxy beads. In comparison, tosylactivated beads facilitate increased interaction in the Fab region resulting in optimal orientation for binding. In addition to an increased recovery, we observed more non-specific binding with tosylactivated beads than epoxy.

We observed an overall higher recovery of NoV from artificial and environmental water using zeolite filtration coupled with immunomagnetic separation indicating that IMS did successfully reduce the presence of inhibitors prior to RT-PCR. Zeolite filtration and concentration coupled with IMS was capable of detecting NoV in environmental water. The percent recovery of NoV in our study was higher than most published adsorption-elution methods (23, 37, 46-48).

3.4 Conclusion: Our study provides a rapid sensitive concentration method for the detection of NoV from water matrices particularly oyster harvesting waters. The zeolite filtration method

detected NoV in seeded waters with salinities ranging from 10ppt to 25ppt. The newly developed zeolite filtration method is easily adaptable to field studies and does not require the use of complex machinery. Furthermore, zeolite filtration combined with IMS and RT-PCR was rapid taking approximately 2.5 hours and results in relatively high percent recoveries of NoV from oyster harvesting waters. Due to its ability to effectively concentrate NoV from marine water without the need for pretreatment modification and its rapid analysis, zeolite filtration could potentially be used for rapid virus concentration from shellfish growing waters at low cost, and be conveniently transported to the lab for analysis.

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Chapter 4 Comparison of Taqman Quantitative Real Time RT-PCR Methods and A Commercial Enzyme Immunoassay for the Detection of Norovirus GII in Artificial and Environmental Water Matrices

4.1 Introduction: In the United States, gastroenteritis caused by foodborne illness is responsible for approximately 38.4 million illnesses, 71,878 hospitalizations, and 1,686 deaths (1). Norovirus (NoV), the predominate etiological agent of gastroenteritis is commonly associated with foodborne outbreaks caused by the consumption of raw or undercooked shellfish (2, 3). The cost associated with NoV is exorbitant averaging approximately 2 billion dollars a year, of which 184 million dollars is associated with seafood contamination (4). In addition to treatment expenses exceeding 273 million dollars per year, suspected NoV contamination results in oyster harvesting site closures for prolonged periods of time costing states such as Louisiana 13.5 to 23 million dollars annually in revenue loss (5, 6). Norovirus is easily transmitted through direct contact with contaminated sources such as faeces and vomit. Oyster harvesting areas located in fresh water such as lakes and rivers are vulnerable to fecal contamination by sewage discharges from boats, failing septic systems, runoff from urban storm water, and sewer overflows. NoV has yet to be propagated in an immortal cell line, thus delaying the development of vaccinations and significantly limiting quantifying detection methods (7).

NoV is comprised of 5 genogroups and 32 genotypes (also referred to as clusters) of which only genogroups I, II, and IV infect humans (8, 9). Genogroup I, II, and IV contain over half of the 32 clusters with each having 8, 19, and 1 respectively (8, 10). NoV has a compact, positive-sense, single stranded, non-segmented RNA genome which is organized into three open reading frames (ORFs) (8, 11). Due to the lack of a cell culture or animal model for NoV, molecular methods such conventional, multiplex, and Taqman quantitative real-time Reverse-Transcription polymerase chain reaction (RT-qPCR) are used to detect and quantify NoV from

various matrices (12-17). RT-qPCR is considered to be highly sensitive, specific, and cost effective (14, 18). Most taqman RT-qPCR methods employed for the detection and quantification of NoV use similar primers which most often target the ORF1 and ORF2 junction as it is the most reactive and highly conserved region within the NoV genome (19). However, RT-qPCR methods may vary in the type of reverse transcriptase and polymerase used thus potentially impacting the synthesis of cDNA and the assay specificity, respectively. In effort to rapidly and effectively detect NoV in water matrices, we employed the use of zeolite granules and immunomagnetic separation (previously discussed in chapter 3) coupled with a commercial enzyme immunoassay (EIA), or one of two different RT-qPCR methods in effort to determine which method was most sensitive and applicable to environmental matrices.

4.2 Materials and Methods:

4.2.1 Virus stock: NoV positive fecal (GII.4) was obtained from the stool of an infected individual associated with a NoV outbreak in January 2013 (Cameron Parish, La., area 30) (15). Positive fecal samples were confirmed using RT-PCR and sequencing during previous studies (15). NoV fecal stock solutions were prepared as previously described in Haramoto et al. (20). One milliliter of NoV GII positive fecal sample was diluted in 9ml of phosphate buffer saline. The diluted mixture was centrifuged for 10 minutes at 9500 rpm. Following centrifugation the supernatant was collected and stored at -80^{o}C and used as positive NoV stocks ($2x10^{8}$gec/ml).

4.2.2 Artificial and Environmental Water Samples: Artificial water samples were prepared as previously described in Chapter 3. Environmental water samples were collected at Hackberry Bay in Lafourche Parish, Louisiana (29.4088324, -90.0303508). Primary and secondary NoV concentration was conducted using the optimal conditions validated in Chapter 3 (publication pending). As previously discussed, zeolite granules were used for the primary

filtration of NoV seeded water. NoV was eluted from the granules at 25^{o}C using beef extract (10%) in McIlvaines' buffer. Secondary concentration was conducted using tosylactivated immunomagnetic beads coated in NoV monoclonal antibodies. Each downstream assay within a trial was based on the same water sample.

4.2.3 Viral RNA extraction: All viral RNA extractions were completed using a Qiamp Viral RNA Minikit (Qiagen Sciences Inc., Germantown, MD) immediately after NoV elution from the zeolite granules. Briefly, 140 µl of the "pre" and "elu" samples were mixed with 560µl of AVL, vortexed, and allowed to sit for 10 minutes at room temperature. After viral lysis, 560µl of 99-100% ethanol was added to the mixture and vortexed for 15 seconds. The sample mixtures were added to minispin columns and centrifuged at 8.0rpm for 30 seconds. The columns were washed with AW1 and AW2 wash buffers and transferred into new collection tubes. Viral RNA was eluted from the minispin columns using 35µl of AVE. Sample eluents were immediately placed on ice and analyzed via RT-qPCR.

4.2.4 RT-qPCR:

4.2.4.1 SuperScript® III Platinum One-Step qRT-PCR: Detection and quantification of NoV GII using TaqMan quantitative real-time Reverse Transcription-Polymerase Chain Reaction (RT-qPCR) was completed according to Gentry and others (13). The reaction mixture used a SuperScript® III Platinum One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA) in a 25 µL reaction mixture and 2.5 µL RNA template. A homogenous internal amplification control (IAC) was incorporated in all reactions to evaluate PCR inhibition. The primers used targeted the most conserved, sensitive and broadly reactive ORF1-ORF2 junctions in NoV (19, 21). Reverse-transcription was carried out at 50°C for 15 min, followed by denaturation for 2 min at

95 °C, and 45 cycles of 15 s at 94 °C, 15 s at 55 °C and 20 s at 72 °C (threshold = 30) using a Cepheid SmartCycler® II system (Sunnyvale, CA).

4.2.4.2 TaqMan® Fast Virus 1-Step RT-qPCR: RT-qPCR was performed in triplicate using a Cepheid SmartCycler II (Cepheid, Sunnyvale, CA). Each RT-qPCR template tube contained 11.1µl of RNA and 8.9 µl of master mix. The master mix included 5 µl of TaqMan® Fast Virus 1-Step Master Mix (Life Technologies Co. Carlsbad, CA), 900 µM of each primer (JJV2F and COG2R), and 150 µM of probe (Ring2P). RT-qPCR amplification targeted the ORF1-ORF2 junction as described in Kageyama et al., 2003 (19). The thermal cycling conditions were as follows: reverse transcription was at 50°C for 5 min, initial denaturation at 95°C for 100 s, and 40 cycles of amplification (95°C for 15 s, 55°C for 15 s, 72°C for 30 s).

4.2.4.3 Standard curve Standard curves were used for the extrapolation of data for unknown RNA samples. Ten-fold dilutions were carried out in duplicate starting from known GII RNA standards (10^9 genomic equivalent copies/µl). RT-PCR was conducted on each dilution in duplicate and a standard curve was developed. The amplification efficiency (E) was calculated using the following equation: $E = (10^{-slope}) - 1$.

4.2.5 Enzyme Immunoassay (EIA): EIA was conducted using the RIDASCREEN® Norovirus 3rd generation EIA kit which employs the use of NoV monoclonal antibodies (mAbs). NoV seeded water samples were (100µl) was suspended in 500µl of a protein-buffered NaCl solution with 0.1% Kathon CG. The suspension was vortexed well and allowed to settle for 10 minutes. After removing the supernatant from the stool suspension, 100µl was added to separate pre-coated microwells. Next, 100µl of anti-NoV biotin-conjugated mAbs were added to each well and the plate was incubated at 25°C for 1hour. The liquid from the microwell plate was discarded and each well was washed 5 times using a phosphate-buffered NaCl solution

containing 0.1% thimerosal. After the wash step, 100µl of streptavidin peroxidase in a protein solution was added to each well. The plate was covered and incubated at 25^{o}C for 30 minutes. Following incubation, the wash step was repeated and 100µl of a substrate solution with hydrogen peroxide in TMB was added. The microwell plate was incubated for 15 minutes at 25^{o}C in the dark. Lastly, 50µl of 1N sulphuric acid was added to each plate to stop the color change reaction. Each OD value was obtained with 10 minutes of adding the 1N sulphuric acid using a spectrophotometer at 450nm wavelength. A NoV positive and negative control (diluent 1) was ran with each assay.

4.2.5.1 Standard curve: A standard curve was developed for the EIA assay. Briefly, six ten-fold dilutions were carried out in duplicate starting from known GII solid fecal standards (10^{8} copies/g). In addition to EIA, RT-PCR was conducted on each dilution in duplicate and a standard curve was developed.

4.3 Results and Discussion: The TaqMan® Fast Virus 1-Step RT-qPCR was successful in detecting NoV in artificial and environmental marine water. As previously discussed in chapter 3, the method used has an expected average percent recovery of 44.08% for artificial water and 34.36% for environmental matrices (Table 10). The percent recovery for artificial and environmental water using TaqMan® Fast Virus 1-Step RT-qPCR was 38.85% $\pm$ 0.27 and 19.77% $\pm$ 0.07, respectively. In comparison, SuperScript® III Platinum One-Step qRT-PCR exhibited an average percent recovery of 11.12% $\pm$ 0.183 and 15.55% $\pm$ 0.225 for artificial and environmental waters (Table 10). SuperScript® III Platinum One-Step qRT-PCR exhibited a lower sensitivity than TaqMan® Fast Virus 1-Step RT-qPCR in the detection of NoV ($p<0.05$).

Throughout this study, SuperScript® III Platinum One-Step qRT-PCR was observed to be approximately one log lower in its detection of NoV compared to the TaqMan® Fast Virus method (data not shown).

The EIA assay was not an effective method for the detection of NoV from environmental water matrices (Table 9). When the OD value calculation were completed as outlined by the kit instructions, none of the samples provided a positive result. When the OD correction value of 0.150 was removed from calculations the EIA assay did prove successful in detecting NoV in the pre-filtration samples. However, this assay was not sensitive enough to detect NoV in the elution samples despite RT-qPCR methods quantifying the virus concentration between 10^4 and 10^5 genomic copies/mL.

Table 9 RIDASCREEN® Norovirus 3rd generation EIA Qualitative Results for the Detection of Norovirus GII from Artificial and Environmental Waters[1,2]

Water Matrices	Pre-Filtration Qualitative Analysis and Mean OD Value	Elution Qualitative Analysis and Mean OD Value
Artificial	Positive 0.1186 ± 0.02	Negative 0.033 ± 0.01
Environmental	Positive 0.0746 ± 0.01	Negative 0.013 ± 0.09

[1]No. of trials, 6.
[2] OD Values do not reflect the RIDASCREEN® Norovirus 3rd generation EIA correction factor of 0.150

As previously stated an OD correction factor of 0.150 required in the leaflet for the EIA assay resulted in a cut off that was too high for our sample analysis. As such, we recalculated the cut off value by averaging the negative controls OD values and creating the cut off ten percent below the value. The average OD value for the negative controls was 0.036. As such, samples were identified as negative if the corresponding OD value was 0.033 or less. If the

sample OD value was 0.0407 or above, it was identified as positive. Lastly, if the sample OD value was between 0.033 and 0.0407, it was labeled undetermined which would require it to be assayed again. Based on the EIA results using our methodology (Table 9), qualitative enzyme-linked immunoassays are can be used as a preliminary screen for the detection of NoV in environmental matrices, however secondary molecular testing should be used for validation. Several studies have examined the use of EIA and found them suitable for the detection of NoV from faeces (22-25). Kirby et al., reported a 63% sensitivity for RIDASCREEN EIA when used for detecting NoV in the stool of infected individuals (25). Our study neither supports nor refutes this claim, however it does prove that the scope of RIDASCREEN EIA may be limited to stool samples. Furthermore, several studies have reported that EIA assays do work in detecting NoV, however due to their low sensitivity and narrow scope RT-qPCR should be used to validate both EIA positive and negative samples (26-29). Due to the high accuracy and sensitivity of RT-qPCR compared to EIA assays it is the superior method when detecting NoV in environmental matrices.

SuperScript® III Platinum One-Step qRT-PCR is most commonly employed for the detection of NoV in various sample matrices, however the use of TaqMan® Fast Virus 1-Step RT-qPCR is rapidly increasingly especially in environmental studies (30-32). The two RT-qPCR methods used in this study differ in their master mix components, reverse transcriptase, DNA polymerase, and cycling times. Unlike SuperScript® III Platinum One-Step, TaqMan® Fast Virus 1-Step is a 4X formulation which allows for more target sample and less master mix. This is a critical difference as an increase in formula concentration increases assay time while decreasing the necessary volume of supplies.

In addition to faster results, TaqMan® Fast Virus 1-Step uses a Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV) which is most effective in the synthesis of cDNA from mRNA greater than 5kb in length. SuperScript® III Platinum One-Step, uses SuperScript® III Reverse Transcriptase which allows for cDNA synthesis at higher temperatures and for difficult secondary structures. Norovirus has been shown to have stem/loops and hairpin secondary structures at the 5' and 3' end of its genome (33, 34). The increased sensitivity exhibited by TaqMan® Fast Virus 1-Step may support the theory that the role of the secondary structures of NoV is limited to viral protein translation and not viral replication. The manufacturer of TaqMan® Fast Virus 1-Step, Life Technologies Co. has indicated that their trademark inhibitor (included in their mastermix) is more effective than traditional inhibitors such as RNAse Inhibitor. The increased sensitivity of TaqMan® Fast Virus 1-Step mixture in this study further potentially supports this claim.

4.4 Conclusion: This study proves that the use of EIA is not as effective as RT-qPCR for the detection of NoV from environmental matrices. It further validates previous studies claims that the RIDASCREEN EIA is limited in its scope of application and should not be employed without the use of RT-qPCR for confirmatory tests. Our work successfully shows that TaqMan® Fast Virus 1-Step can be applied for the detection of NoV in environmental matrices and may have increased sensitivity compared to the commonly employed SuperScript® III Platinum One-Step method. Lastly, this study, as a continuation of the previous shows that zeolite filtration combined with IMS and TaqMan® Fast Virus RT-qPCR effectively concentrates and detects NoV from marine water, thus proving its beneficial potential for application in shellfish growing waters.

Table 10 Comparison of TaqMan® Fast Virus 1-Step and SuperScript® III PlatinumOne-Step For The Detection Of Norovirus GII from Artificial and Environmental Water Matrices[1]

		Genomic Copies (GC/ml)		
	Water Matrices	Inoculated[4]	Recovered[4]	Percent Recovery
TaqMan® Fast Virus 1-Step RT-qPCR[2]	Artificial	$2.93x10^6(2.26x10^6\text{-}3.75x10^6)$	$4.24x10^5$ $(2.23x10^5\text{-}5.85x10^5)$	38.85 ± 0.27
	Environmental[3]	$1.40x10^6(6.01x10^5\text{-}1.85x10^6)$	$5.48x10^5$ $(4.37x10^5\text{-}6.24x10^5)$	19.77 ± 0.07
SuperScript® III Platinum One-Step RT-qPCR	Artificial	$1.77x10^6(1.20x10^5\text{-}2.96x10^6)$	$2.25x10^4$ $(9.75x10^3\text{-}3.88x10^4)$	11.12 ± 0.183
	Environmental	$1.16x10^6(1.75x10^5\text{-}2.14x10^6)$	$5.02x10^4$ $(3.94x10^4\text{-}7.25x10^4)$	15.55 ± 0.225

[1] No. trials 6
[2] RT-qPCR method employed in chapter 3
[3] Samples from Hackberry Bay, LAConditions: BE (10%) in McIlvaines Buffer, 20ppt, 25°C
[4] Mean genomic copies/ml and range

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Conclusions

This body of work takes a well-rounded approach to detecting NoV in Louisiana oyster harvesting waters. This study surveyed noroviruses and microbial indicators of fecal contamination in oysters and harvest waters taken from commercial harvesting areas along Louisiana Gulf Coasts, and to evaluate the effectiveness of microbial indicators for assessing viral safety of oysters. Observed levels of microbial indicators detected in harvesting waters were within the acceptable ranges defined by the NSSP, however they could not reliably predict the occurrence of NoV in the oysters. As such, a rapid and reliable detection method was needed. To meet this need, our study provides a rapid sensitive concentration method for the detection of NoV from water matrices particularly oyster harvesting waters. Lastly, this study compared commonly employed EIA and RT-qPCR detection methods. It proves that the use of EIA are not as effective as RT-qPCR for the detection of NoV from environmental matrices. In addition, it proves that TaqMan® Fast Virus 1-Step can be applied for the detection of NoV in environmental matrices thus providing rapid results. Overall our study supports the need for frequent and continued monitoring of harvesting waters for NoV in order to reduce the potential public health risk.

Vita

Morgan A.C Maite, a native of Grove City, Ohio received her bachelor's of science at Spelman College in 2012. During her undergraduate career, she worked with several companies including Coca Cola Enterprises and Sherwin Williams further fueling her passion for product and workplace safety. In the summer of 2011, she attended the Pre-Doctoral Program offered by Louisiana State University studying under the guidance of Dr. Marlene Janes and Dr. Evelyn Gutierrez. Upon graduation from Spelman College, she chose to continue on to higher education and entered the graduate school in the School of Nutrition and Food Science at Louisiana State University. She hopes to receive her Doctorate in May 2016 and plans to continue working in food safety and quality assurance.