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Pathogenic Enteric Viruses in Louisiana Oysters and Environmental Waters

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PATHOGENIC ENTERIC VIRUSES IN LOUISIANA OYSTERS AND ENVIRONMENTAL WATERS

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To my beautiful wife, Parichehr

&

My beloved parents, Ashraf and Ali

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ABSTRACT

Norovirus (NoV) and pathogenic enteroviruses are the major causes of gastroenteritis in humans worldwide, and are usually transmitted through direct or indirect exposure to raw or partially treated sewage. Filter-feeding shellfish concentrate virus particles from the water and transmit them to humans. The occurrence of norovirus GI and GII and fecal indicators in Louisiana eastern oysters (*Crassostrea virginica*) and harvest water were investigated on a biweekly basis for almost one year. Only one oyster sample was positive for NoV GII at 3.5 $\log_{10}$ genomic copies/g digestive tissues. A stool specimen obtained from an infected individual associated with a norovirus outbreak and the suspected oysters were also analyzed. The norovirus in the stool belonged to GII.4 Sydney; however, the oyster and water were negative and could not be linked. Densities of microbial indicators (fecal coliforms, *Escherichia coli*, and coliphages) were low, and weakly correlated. A municipal secondary wastewater treatment plant (New Orleans, Louisiana), as a potential source of environmental contamination, was further investigated on a monthly basis for one year. Enteric viruses were detected in the influent and effluent wasters year-round. NoV GII was more abundant than NoV GI, and along with EV increased in influent during fall and spring. NoV GI concentration was higher in influent in fall. Densities of indicator bacteria (enterococci, fecal coliforms and *E. coli*) did not show strong monthly or seasonal patterns. Averaged monthly removal for enteric viruses and male-specific coliphage ranged between 0.95 and 1.63 $\log_{10}$, and were lower than the indicator bacteria (4.36 $\log_{10}$), indicating higher resistance of viruses to the treatment. Male-specific coliphage was the only indicator that correlated with NoV GII densities in both influent and effluent (r = 0.48 and 0.76, respectively) and monthly

removal (r = 0.72), indicating that it can be more reliable than indicator bacteria to monitor NoV GII load and microbial removal. Various norovirus genotypes were identified (GI/1, 3 and 4; GII/3, 4, 13 and 21), dominated by GI.1 and GII.4 strains. These observations emphasize the need for direct monitoring of pathogenic enteric viruses in oysters and waters to reduce the risks of viral gastroenteritis incidence.

CHAPTER 1. INTRODUCTION TO NOROVIRUS

1.1. Nomenclature, virion and genome structure

Norovirus (formerly known as "Norwalk-like virus") was first visualized in 1972 using immune electron microscopy on infectious stool filtrate derived from an outbreak of gastroenteritis in an elementary school in Norwalk, Ohio (Kapikian 2000). Norovirus belongs to the family *Caliciviridae* (*calici* originated from the Latin word *calyx* means cup) (Green 2007), which are categorized into five genera, namely *Norovirus*, *Nebovirus*, *Sapovirus*, *Vesivirus* and *Lagovirus*. Among these, *Norovirus* and *Sapovirus* can infect humans and cause gastroenteritis (Thorne and Goodfellow 2014).

So far, more than 32 norovirus (NoV) genotypes composing numerous strains have been identified and, according to their genome sequence homologies, have been categorized into five genogroups (GI to GV, Figure 1) in which GI, GII and GIV are prevalent in humans; the rest (GIII to GV) are found in animals and are comprised of bovine NoV, Murine NoV (MNV) and canine NoV (Green 2007, Hall et al. 2014, Karst et al. 2014). Recently NoV GVI was identified in domestic dogs (Mesquita et al. 2010), and a tentative GVII has been proposed (Tse et al. 2012, Vinjé 2015). Genogroups GI and GII possess only 53% nucleotide and 65% amino acid similarities (Wang et al. 1994). Despite their extensive genetically divergent nature, the NoV GII.4 strains remain the predominant cause of the NoV outbreaks worldwide (Siebenga et al. 2009).

Norovirus is a non-enveloped virus, and composed of a virion protein, genome-linked protein (VPg), and a positive-sense single-stranded RNA (Green 2007). The buoyant density of Norwalk virus in cesium chloride (CsCl) ranges from 1.33 to 1.41 g cm^{-1} (Kapikian et al. 1973).

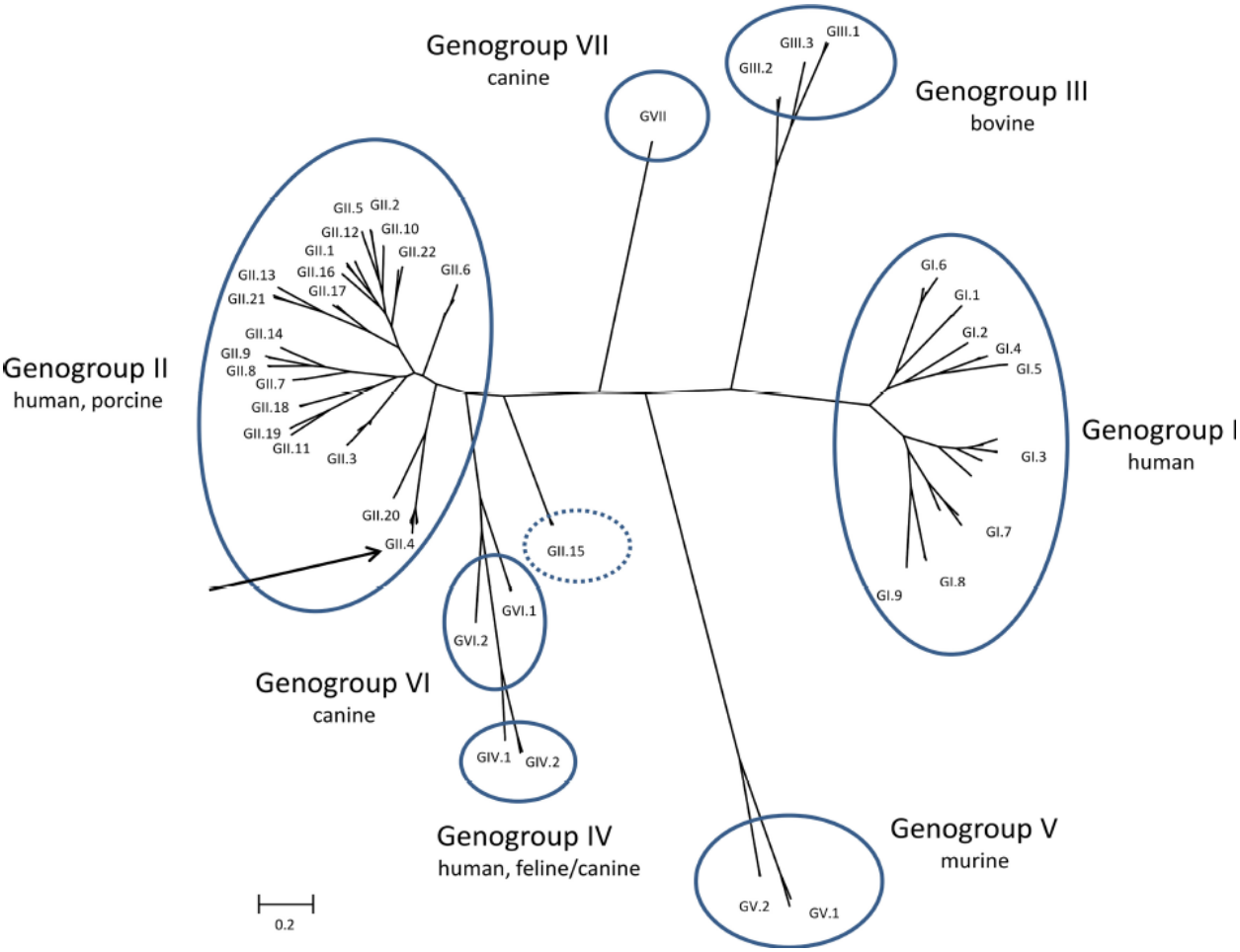

Figure 1. Classification of noroviruses into seven genogroups (GI to GVII) based on amino acid sequence diversity in the complete VP1 capsid protein (the scale bar refers to the number of amino acid substitutions per site). Source: Vinjé (2015)

The structural protein of each virion is composed of 180 copies of 59 kDa major protein (VP1) with 530 amino acids, and one or two copies of a 30 kDa minor protein (VP2) (Greenberg et al. 1981, Prasad et al. 1996). Figure 2 represents a typical structure of norovirus. The capsid protein of noroviruses is composed of 90 dimers that form a shell from which 90 arch-like capsomers protrude at the local and strict two-fold axis. The arches form 32 large hollows at the icosahedral five- and three- fold positions with a cup-like structure on the surface (Green 2007). The capsid protein (VP1) can self-assemble into a virus-like particle (VLPs) without incorporation of

genomic RNA or the minor capsid protein. The virus-like particles retain their antigenicity similar to intact virion strains, and are able to induce immunological responses to the infected individuals. This can help the development of vaccines, cell culture techniques and diagnostic tests (Jiang et al. 1992).

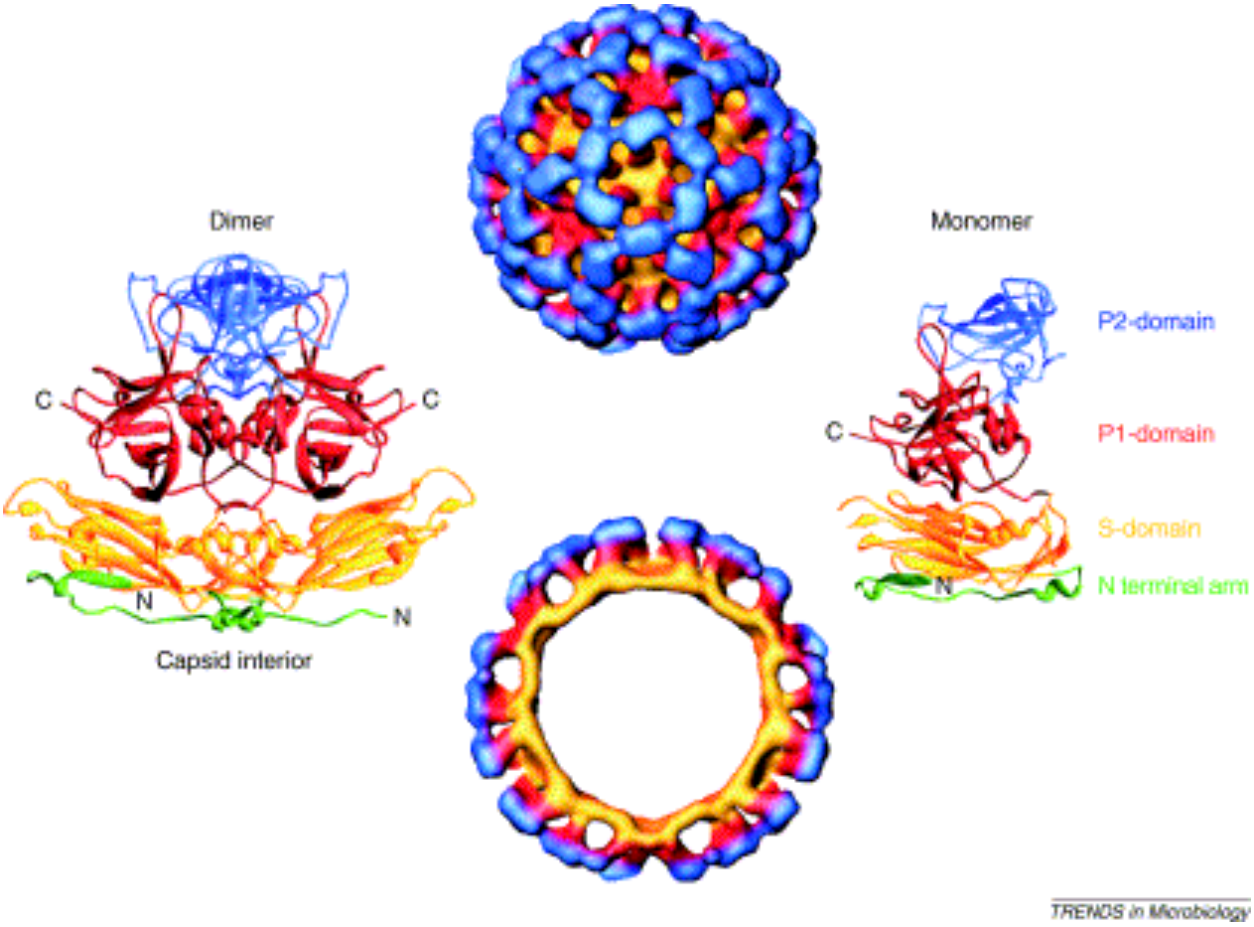

Figure 2. The Norwalk virus-like particle. Source: Huston et al. (2004)

The viral genome (7.4-7.7 kb) has a polyadenylated tail at the 3' end, and is coated by a non-enveloped icosahedral capsid with 27-40 nm in diameter (Green 2007). The distinguishing characteristic of the *Calicivirus* genome is a 5' non-structural polyprotein preceding the single viral structural capsid protein (Clarke and Lambden 1997). Depending on the genus, the viral genome is composed of two or three major open reading frames (ORFs). Three ORFs are

recognized in the NoV viral genome (Figure 3). ORF1 encodes the nonstructural proteins that are processed co- and post-translationally by the viral 3C-like protease (3CL Pro). The coding order in ORF1 proceeds N to C terminus, p48, NTPase, p22, VPg, 3CL Pro and RdRp (Hardy 2005). Despite heterogeneity in the amino acid sequence of the ORF1 polyprotein, the location of the cleavage sites and sequence preference for glutamic acid or glutamine residues in the P1 position are well conserved among noroviruses. The last two proteins, 3CL Pro and RdRp are the most genetically conserved and well-characterized calicivirus nonstructural proteins (Sosnovtsev et al. 2006).

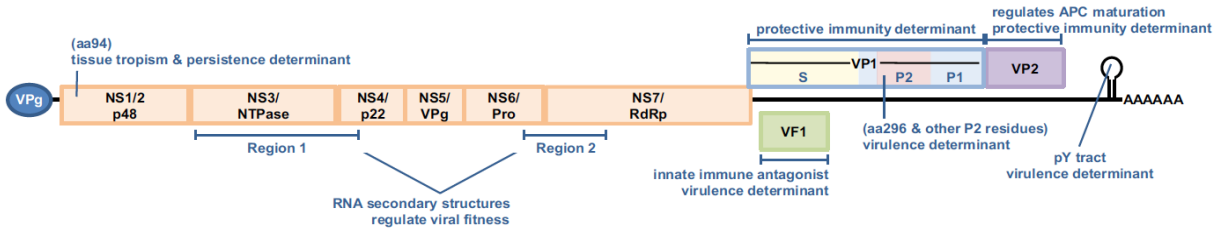

Figure 3. Norovirus virulence determinants. Source: Karst et al. (2014)

The ORF2 and ORF3 are translated from a subgenomic RNA, and encode the major and minor capsid proteins, VP1 and VP2, respectively (Thorne and Goodfellow 2014). The VP1, encoded by ORF2, is divided into two major domains, the well conserved N-terminal or internal shell (S) and the C-terminal protruding (P) domains connected by a flexible short hinge forming dimeric VP1 arches. The P domain is further divided into P1 and P2 subdomains, composing the leg and the head of the protruding P dimers, respectively (Prasad et al. 1999). The minor structural protein (VP2), encoded by ORF3, is believed to be located on the interior of the virion; even though it is not essential in virus-like particles (VLP) formation but it may function in RNA viral encapsidation. Similar to many plant viruses, RNA packaging occurs through an interaction of the viral RNA with the basic N-terminal domain of the capsid protein. The capsid protein of

caliciviruses is acidic (pI = 5.64), and lacks a basic N-terminal arm (Glass et al. 2000). Nonstructural proteins are responsible for applications such as protease activity, viral genome replication and virion localization (Hardy 2005).

A fourth ORF is identified in murine norovirus (MNV) genome that overlaps the ORF2 coding region, and is translated during virus infection. The encoded protein (virulence factor 1, VF1) antagonizes the innate immune response to infection (McFadden et al. 2011). NoV genomic and subgenomic RNAs are covalently linked to a small virus-encoded protein (VPg), and the 3' ends are polyadenylated (Karst et al. 2014). Calicivirus coding genome is flanked by short stretches of UTRs. In other (+)ssRNA viruses that have larger UTRs, it is believed that these UTRs interact with the cellular translation machinery and/or the viral replicase components to regulate translation and/or replication. The stem-loops present within the coding region in caliciviruses genome may play a similar role (Simmonds et al. 2008).

1.2. Attachment and replication strategies

Inability of a reliable cell culture system to grow NoV has limited the understanding of NoV life cycle, and the development of a vaccine and antiviral drug (Karst et al. 2014). Similar to other viruses that replicate on mucosal surfaces, and particularly in the gastrointestinal tract, proteolytic cleavage of outer capsid protein in NoV is an important step in the replication cycle of the virus, and probably not important for the infectivity of the virus. The cleavage product is the source of the soluble protein occurs in stools of infected individuals and perhaps has a role in immune response to the pathogen (Hardy et al. 1995).

The small intestine is believed to be the primary site of human NoV infection and replication (Karst et al. 2015). NoV recognizes human histo-blood group antigens (HBGAs) as receptors. The A/B-binding group and the Lewis-binding group are the major epitopes involved

in this interaction. Strains in the A/B binding group recognize the A and/or B and H antigens, but not the Lewis antigens, while strains in the Lewis-binding group react only to the Lewis and/or H antigens. Strains in both binding groups can be found in both NoV GI and GII. High polymorphism in human HBGAs and involvement of multiple epitopes could be a reason for the diversity in virus-ligand binding indicating important role of human HBGA system in NoV evolution (Huang et al. 2005). Different strains of NoV specifically recognize different human HBGAs on intestinal epithelial cells as receptors (Huang et al. 2003). Following attachment, the virion releases the genome into the host's cytoplasm, and then the genome-linked protein initiates translation. After translation of ORF1, necessary protein are produced to induce (-)ssRNA which later are used as a template for transcription to full-length genomic and sub-genomic RNA. VP2 may function in RNA genome packaging (Hardy 2005).

Once the VPg-linked RNA genome is released into the cytoplasm of a permissive cell, it is likely that the VPs forms multiple contacts with components of the translation initiation factor complex, then the genome is used as an mRNA template for viral genome translation. There are evolutionary conserved RNA structures at both ends of calicivirus genomes that interact with host cell factors to promote viral replication and translation. Viral proteins VP1 and VP2 are translated primarily from the subgenomic RNA, as the subgenomic RNA is present at high levels in infected cells. Once the translation of ORF2 (VP1) is accomplished, ribosomes are probably still bound to RNA to reinitiate the translation of ORF3 (VP2) (Thorne and Goodfellow 2014). Norovirus replication resembles other (+)ssRNA viruses, and occurs in association with intracellular membranes (Wobus et al. 2004).

Similar to all positive-sense RNA viruses, genome replication takes place via a negative-sense RNA intermediate by the viral RNA-dependent RNA polymerase (RdRp, NS7). This

process occurs following the translation of incoming parental viral RNA. In this regard the mRNA template functions as the template for the formation double-stranded replicative form. Next is the synthesis of positive-sense genomic and subgenomic RNA. During the replication process, an extensive modification of cellular transcriptional and translational apparatus occurs with infection that benefits virus replication (Thorne and Goodfellow 2014). The minus strand is synthesized by RNA-dependent RNA polymerase (RdRp), and acts as a template for the synthesis of the full-length genomic RNA from which non-structural proteins are translated, including RdRp, helicase and protease (Simmonds et al. 2008). Protein VP1 is able to self-assemble into virus-like particles (VLPs). While the S domain has all properties to assemble a stable icosahedron, it is suggested that the interaction between the P and S domains stabilizes the assembled particles and perhaps attributes to maintain the appropriate size on the particle (Bertolotti-Ciarlet et al. 2002). Viral assembly, encapsidation and the exit of NoV are unknown (Thorne and Goodfellow 2014). Induction of apoptosis by murine norovirus through the mitochondrial is observed as an exit strategy in caliciviruses (Bok et al. 2009).

1.3. Detection and genotyping

Norwalk virus was first discovered by Kapikian et al. (1972) using immune electron microscopy to study stool filtrate obtained from a gastroenteritis outbreak in Norwalk, Ohio. Inactivated convalescent serum from experimentally infected volunteers was utilized as a specific antibody to aggregate viruses and coated them by specific antibodies that facilitated direct visualization and detection. Cloning of Norwalk virus in 1990 advanced molecular characterization of noroviruses significantly (Xi et al. 1990). Underestimation of recombination rate, error-prone RNA replication, and the lack of a suitable *in vitro* culture system, has limited studies on NoV (Xue et al. 2013). Murine norovirus has been the only norovirus that grows well

in cell culture by infecting macrophages and dendritic cells. The similarities of MNV with other members of *Caliciviridae* have recognized it as a suitable surrogate for virology purposes (Wobus et al. 2006). Efforts have been made to cultivate norovirus in a 3-dimentional tissue culture in an organoid model of human small (Straub et al. 2007) and large (Straub et al. 2007) intestine epithelium. Recently, promising results were obtained by Jones et al. (2014) who found that human NoV infects B cells at the presence of HBGA-expressing enteric bacteria as a stimulatory factor for the infection. In this regard, NoV must find its way into the intestinal epithelium to access target B cells.

Due to the lack of a reliable cell culture system, various molecular detection and genome sequencing techniques have been developed for the identification and classification of NoV in clinical specimens or environmental samples. PCR-based assays have been the gold standard for the rapid and sensitive detection of NoV in clinical (stool, serum) as well as food or environmental samples where the level of virus contamination is usually low.

A breakthrough was achieved by Kageyama et al. (2003) who improved detection of GI and GII using a real-time reverse-transcription PCR (RT-qPCR) by targeting the junction of ORF1-ORF2 junction (from C-terminal RdRp to the N-terminal capsid), which is the most conserved region in both GI and GII genome allowing sensitive detection of the viral genome (Figure 4). Other methods have been designed for the detection of noroviruses, such as loop-mediated isothermal amplification, LAMP (Fukuda et al. 2007), microarray (Martínez et al. 2015), nucleic acid sequence-based amplification, NASBA (Lamhoujeb et al. 2009), enzyme immune assays, EIA (Shigemoto et al. 2014), enzyme-linked immunosorbent assay, ELISA (Geginat et al. 2012) and surface plasmon resonance biosensor (Yakes et al. 2013).

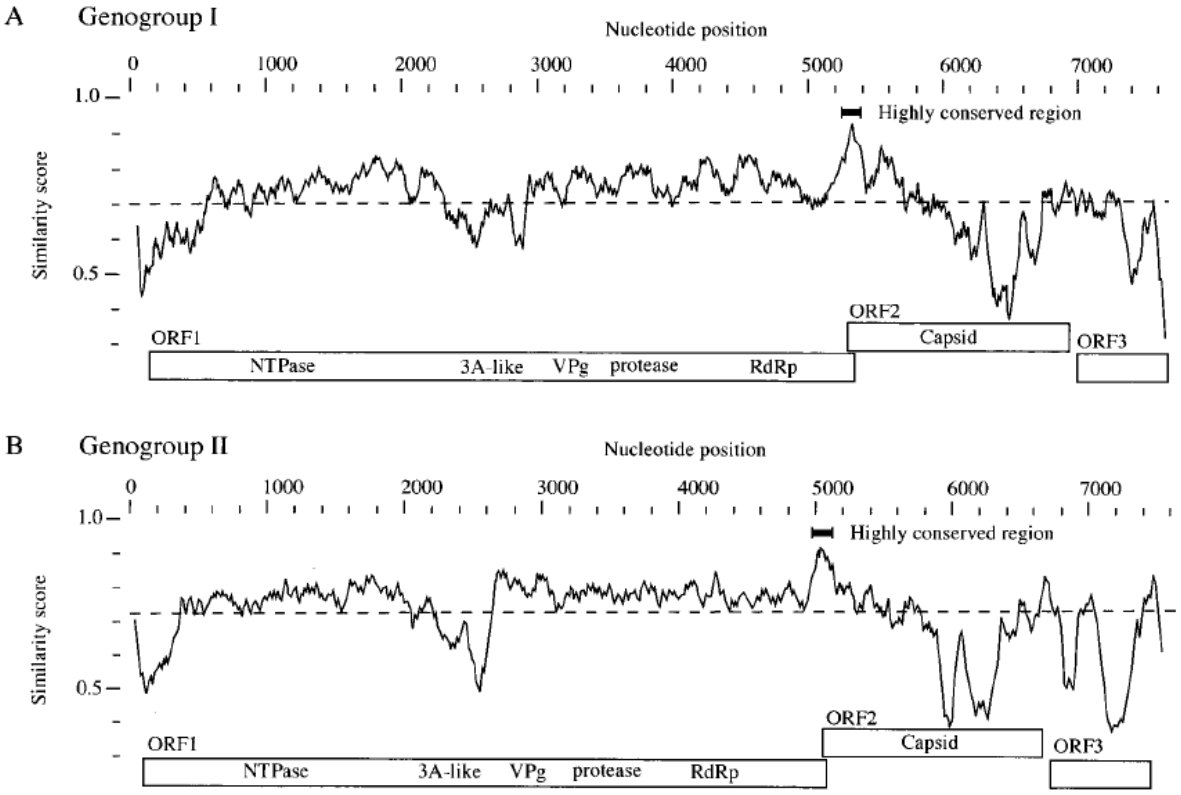

Figure 4. Norovirus genome conserved sequences identified by PlotSimilarity (A: genogroup I, B: genogroup II). Source: Kageyama et al. (2003)

Noroviruses are genetically diverse even within each genogroup (Wang et al. 1994). The RT-qPCR assay target the ORF1-ORF2 junction is designed for a highly conserved region in both NoV GI and GII, and is not suitable for genotyping different strains (Kageyama et al. 2003). So far, no alternative conserved PCR primers have been recognized for the confirmation of NoV positive RT-qPCR assays (Knight et al. 2013). However, various regions (A to E) of NoV viral genome are amplified using end-point RT-PCR assays for genotyping purposes (Figure 5, Mattison et al. 2009).

Recent advances in genotyping are achieved by sequencing the region C or D of genome sequences, which target the ORF2 regions, which partially encodes capsid region, VP1 (Mattison et al. 2009). The C-terminus of VP1 protein demonstrates separation of uncorrected distances of

strains within clusters, between clusters and between genogroups (Vinjé et al. 2004). This region includes the protruding (P1/P2) domains of the capsid; the epitopes that take part in binding to histo-blood group antigens (Hutson et al. 2003). Two short stretches of conserved amino acids, DPDT and YQLKPVGTA for GI strains, and POE and LAPM motifs for GII strains were found within the designated region D. The new primer sets targeting this stretches of amino acids showed amplification in 95% of the samples (Vinjé et al. 2004). Due to the lack of a standardized approach, typing of NoV variants is sometimes questioned by what defines a genotype and what is the best genomic region for classification. Overall, the maximum pairwise difference of VP1 amino acid sequences within NoV genotypes is 0.21 for GI and 0.15 for GII, and the minimum difference between NoV genotypes is 0.20 for GI and 0.13 for GII (Kroneman et al. 2013).

A pairwise distance cutoff of 20% divergence in the complete VP1 amino acid sequence (ORF2) had been used since mid 1990s to assign a new NoV genotype (Vinjé et al. 2000). Later the standard for classification of a new cluster was reduced to at least 15% pairwise distance difference in complete VP1 amino acid sequence (Zheng et al. 2006). However, some NoV variants such as GII.4 undergone a high level of nucleotide substitution over the past three decades in which only 5% of amino acid variation in VP1 could classify GII.4 subclusters (Kroneman et al. 2013, Zheng et al. 2010).

Emerging the recombinants and high diversity of NoV strains, make it challenging to link norovirus cases when multiple strains are involved in an outbreak (Le Guyader et al. 2006b). Recombination hotspots in GII.4 are near the open reading frame 1/2 (ORF1/2) and ORF2/3 overlaps, as well as within ORF2, which encodes the viral capsid, at the junction of the shell and

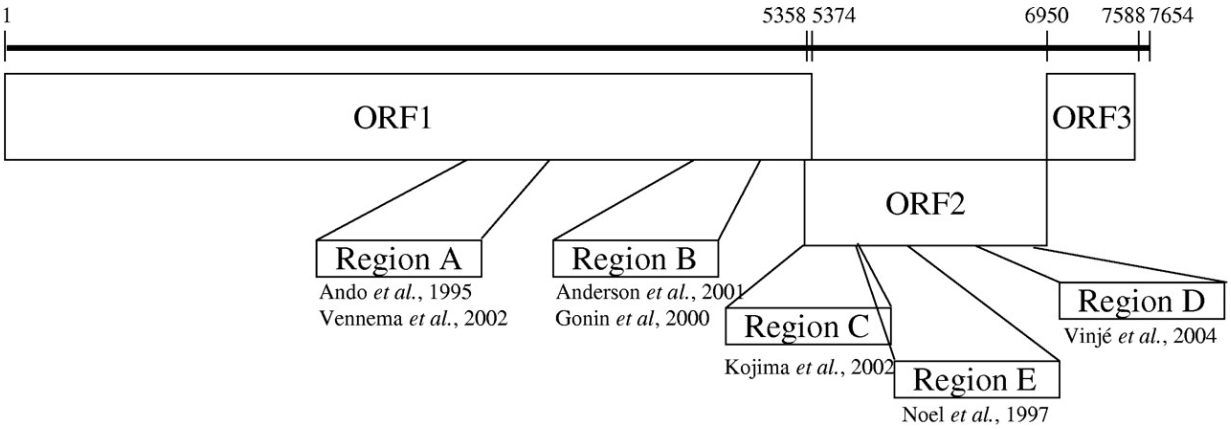

Figure 5. Schematic representation of the NoV genome and the partial sequence regions (A to E) are been used for genotyping. Numbers refer to the nucleotide position in the GI.1 Norwalk virus genome (GenBank accession number M87661). Source: Mattison et al (2009)

protruding domains (Eden et al. 2013). Norovirus GII.b is another recombinant that contains ORF1 from GII.b and ORF2 from other genotypes. The ORF2 genotype of GII.b recombinants in Australia comprised GII.1 (47.6%), GII.3 (47.6%), and GII.13 (4.8%) for 2002-2005 to GII.1 (2.0%), GII.3 (94.1%), GII.13 (2.0%), and GII.21 (2.0%) for 2006-2011 (Bruggink and Marshall 2013). Fumian et al. (2012) detected a novel inter-genotype recombinant GII.7_GII.20 in stool samples in an Amazon community of Brazil. GII.20 was first observed in Germany, Sweden, and China, and has a low prevalence worldwide. Emergence of novel NoV recombinants contributes to rapid evolution and antigenic variation in response to the community and the emergence of pandemic outbreaks (Eden et al. 2013). Novel GII.P16_GII.3 and GII.P16_GII.13 recombinants are recently reported from a recent norovirus outbreak in Italy that was the first recorded occurrence of GII.P16_GII.13 in Europe (Medici et al. 2014). Presence of recombinants and the continuing emergence of new NoV lineages, led to a proposed dual nomenclature using both ORF1 and VP1 sequences (Kroneman et al. 2013). Later, in a study of a panel of fecal specimens, it was observed that region C typing offered a higher success rate than region D (78%

over 52%); however, region D provides more information to distinguish the sequence diversity exists within GII.4 strains (Mattison et al. 2009).

The data of NoV outbreaks in the U.S. are deposited and curated by the U.S. Centers for Disease Control and Prevention (CDC) since 2009 in a comprehensive electronic surveillance network, called *CaliciNet* (Vega et al. 2011). NoV sequences and epidemiologic information in *CaliciNet* are contributed by certified participants who performed molecular typing of NoV strains by using standardized laboratory protocols (RT-PCR methods target the region C or D) followed by DNA sequence analysis of the amplicons (preferably region D). Analyzes of the P2 region (VP1) along with analysis region C or region D is required to differentiate genetically closely related variants, such as GII.4 New Orleans vs. GII.4 Minerva. Another network is *ViroNet* established at the National Microbiology Laboratory in Winnipeg (Canada) for the identification and comparison of NoV outbreak strains (Anonymous 2011). A global network called *NoroNet* expanded by Food-borne Viruses in Europe network (FBVE) is established to share virological, epidemiological and molecular data on NoV (Duizer et al. 2008).

1.4. Clinical features and contamination routes

Norovirus is a major causative agent of viral gastroenteritis in humans, and has caused the most gastroenteritis outbreaks in humans worldwide at least during the last decade (Vega et al. 2014). The strain diversity exists among NoV affect immune evasion and viral adaptation to interaction with a variety of human histo-blood group antigens, and results in the persistence of the pathogen in a community (Iturriza-Gómara and Lopman 2014). After *Clostridium difficile*, NoV has been the second contributor to gastroenteritis-associated deaths in the U.S. from 1999-2007 (Hall et al. 2012). Norovirus ranks fourth among foodborne pathogens in the U.S. in terms of economic burden, which is estimated at $2.3 billion in a typical year (USDA/ERS 2014).

The CDC has estimated that 48% of all reported foodborne outbreaks in the U.S. (2009-2012) with a single known cause are associated with noroviruses. Most NoV contaminations have been occurred during food preparation, in which restaurants were the most common setting (64%) and food workers were implicated as the source in 70% of the outbreaks with the reported contributing factor. Only 67 outbreaks were associated with specific food categories dominated by raw crops (e.g. leafy vegetables) (30%), fruits (21%), and mollusks (19%). NoV outbreaks are more common in winter (55%) during December-February. Approximately 48% of the NoV outbreaks were linked to food in the U.S. (2009-2012) and 0.26% of the reported outbreaks were waterborne (Hall et al. 2014).

There is not any age restriction regarding the illness from human NoV (Thackray et al. 2007). About one third of infected individuals may shed viruses without eliciting symptoms (Graham et al. 1994). NoV commonly causes a mild and severe acute gastroenteritis in humans (Ahmed et al. 2014, Iturriza-Gómara and Lopman 2014). The illness is usually occurs within 15-48 hours. Symptoms include severe vomiting, watery diarrhea, nausea, abdominal cramps, fever and general malaise (Dolin et al. 1972); however, it can be more severe, more complicated and even fatal in immunocompromised and physically stressed individuals such as in healthcare-associated outbreaks (Hutson et al. 2004, Iturriza-Gómara and Lopman 2014).

Although the illness symptom may disappear within a few days, an infected individual may shed viruses 18 hours to 28 days after inoculation. The peak of shedding is observed in fecal samples after resolution of the symptoms with level of about 10^{11} genomic copies per gram of feces (Atmar et al. 2008). In this regard, an accidental release, one per 1000 people, in the water can have a remarkable impact on the loads of pathogens in the water. Children (less than 18 years old) have higher incidence of viral enteric infections than adults and shed more viruses in

their feces (Gerba 2000). A recent study estimated that the 50% human infectious dose (HID50) of NoV is similar to other RNA viruses and ranges between 3.01 and 3.45 $\log_{10}$ virions (Atmar et al. 2014). The presence of NoV RNA in the serum and cerebral fluids of some patients is reported, as reviewed by Karst et al. (2015).

Human NoV are usually transmitted through oral-fecal routes (Thorne and Goodfellow 2014). Food commodities can be contaminated with pathogenic enteric viruses, and transmit the pathogens to human. Various NoV outbreaks have been associated with the consumption of raw or partially cooked bivalve filter-feeder mollusks grown in contaminated water or the crops irrigated with contaminated water, and the exposure to sewage-polluted drinking or recreational waters. Other transmission routes are through direct contact with infected people or contaminated surfaces that usually result from poor personal hygiene, and the incidental ingestion of aerosolized vomitus particles (Green 2007, Hall et al. 2014, Jaykus 2000). Domestic animals (calves and pigs) or wild animals (rats in sewage systems) have been found to host human NoV indicating the possibility of pathogen transmission to humans (Prato et al. 2013, Smiley et al. 2002, Wang et al. 2005).

NoV infections among the people in healthcare facilities, hospitals and nursing homes are commonly high reaching to a median attack rate of 50% among the patients and customers, according to a systemic review by Harris et al. (2010). Spread of NoV in confined environments such as cruise ships occurs more rapidly among individuals due to increased contacts, shared sanitation facilities, and commonality of food supplies. Occurrence of NoV outbreak among cruise ships passengers has become a burden for truism industry worldwide (Kak 2009). In a survey study of houseboats, NoV was detected on interior of boat surfaces including 83% of bathrooms, 40% of kitchens and 100% of doorknobs (Jones et al. 2007). Cooks and food

handlers who have vomited in sinks, in wastebaskets, and on the floors of their workplace can infect other peoples, contaminate the kitchen environment, and eventually spread the virus (Moe 2009).

1.5. Occurrence in environmental water and wastewater systems

Waters bodies (river, estuarine, well and wastewater) may harbor pathogenic enteric viruses primarily through contamination with human contaminated body fluids and sewage (Anderson et al. 2003, Gentry et al. 2009, Maalouf et al. 2010a, Ueki et al. 2005). The discharge of human wastes into the marine water is subjected to dilution, bio-sedimentation, and degradation of viruses due to sunlight, salinity, pH and temperature. Therefore, NoV is usually found in lower concentrations in surface water than wastewater (Maalouf et al. 2010a). Viruses can be adsorbed onto the suspended solids present in the sewage and discharged to the aquatic environment. The particulate matter either become suspended in the water column, or absorbed onto larger particle and precipitate in the sediment and survive for weeks to months (Bosch et al. 2006, Lees 2000). Clayey to sandy sediments in both freshwater and marine sediments can reside infectious viruses for a couple of months (Bosch et al. 2006, Gerba et al. 1977, Maalouf et al. 2010a, Skraber et al. 2009).

Disturbance of water sediments through currents, storms, or human activities (boating) can suspend the sediments and increase the exposure rate of viruses (Gerba et al. 1977, Le Guyader et al. 2008, Maalouf et al. 2010a). In this regard, biological factors, climate conditions, hydrological and meteorological events (such as rainfall, river flows, storms, sediment erosion, water depth, tidal cycle) and the pattern of impacting pollution sources influence the abundance and temporal distribution of microbial indicators in water and shellfish, reviewed by Campos et

al. (2013). For example, Le Guyader et al. (2006b) found that heavy rain was responsible for a severe contamination of seafood with noroviruses.

Raw sewage and partially treated wastewater have been considered as major sources of environmental contamination with noroviruses and other enteric pathogens (Flannery et al. 2013, Kitajima et al. 2012, Ueki et al. 2005). The presence of NoV in the wastewater treatment plant (WWTP) effluents shows the inefficiency of the complete elimination of NoV by the conventional secondary treatment of wastewater. This indicates that the discharge of both raw and treated sewage introduce enteric pathogens into the environmental waters (Kitajima et al. 2012, Lodder and de Roda Husman 2005). The public health risk associated with the discharge of contaminated wastewater increases during a NoV epidemic case, or the aftermath of a heavy rain that cause overflow of raw or partially treated sewage into the environment (Astrom et al. 2009, Lowther 2011, Westrell et al. 2006).

Secondary treatment of wastewater is a common practice to treat nontoxic sewage from domestic or industrial wastes. The process involves a preliminary removal of the debris and coarse materials, followed by a primary treatment by the physical processes including and screening and sedimentation, followed by biological (e.g. activated sludge, trickling filter and oxidation ponds) and chemical (e.g. disinfection) unit processes and nutrient removal. Chlorination and UV irradiation are two conventionally used methods for the disinfection of wastewater before discharge into the water (Bitton 2011). In general, viruses have shown to be more resistant than bacteria to the UV irradiation or chlorination (Bitton 2011, Francy et al. 2012, Tree et al. 2005). Studies showed that RNA viruses are more resistant than DNA viruses to UV inactivation, as was observed in NoV surrogate, male-specific coliphage, MSC (Rodriguez et al. 2014). Inactivation kinetics of human NoV under UV irradiation resembles other

nonenveloped (+)ssRNA viruses (Park et al. 2011). NoV GI and GII outbreaks due to the contamination of well water have been reported in the U.S., China and Sweden (Anderson et al. 2003, Riera-Montes et al. 2011, Zhou et al. 2012). It has been speculated that sandy, porous soil has low ability for adsorption and subsequently removal of viruses from the well water (Anderson et al. 2003).

Norovirus is more resistant to chlorine treatment of water than many bacteria and viruses (Keswick et al. 1985). Usually ≤1 mg/mL chlorine for 30 min reduces bacterial numbers effectively; however, enteric viruses are more resistant to chlorine (Bitton 2011). Some microorganisms such as feline calicivirus (surrogate for NoV) and *E. coli* are more sensitive to chlorine in which exposure to 30 mg/mL chlorine for 5 min reduced the count for 4 $\log_{10}$; however, MSC showed more resistance to chlorine (Tree et al. 2005). A 4-$\log_{10}$ reduction of NoV GII.4 in drinking water is observed when the viruses were exposed to 0.1 and 0.5 mg/mL free chlorine for 120 and 0.5 min, respectively (Kitajima et al. 2010). Similarly, Shin and Sobsey (2008) reported that NoV GI strain 8FIIa is not highly resistant to free chlorine disinfection of drinking water. However, the aggregation of enteric viruses in water or other wastewater solids reduces the efficacy of chlorine and UV disinfectants, and help viruses maintain their infectivity when discharged in to the environmental waters (Bitton 2011, Keswick et al. 1985, Sobsey et al. 1991, Templeton et al. 2005).

Non-disinfected effluent may have similar prevalence of NoV as the raw sewage (Hewitt et al. 2011). Prevalence and intensity of NoV and enteric viruses in large WWTP (i.e. larger population size) are usually more consistent due to the more frequent occurrence of infection and comparative homogeneity of wastewater (Hewitt et al. 2011). Noroviruses have been found in wastewater influents in Japan at 3.2-5.2 $\log_{10}$ genomic copy (GC)/100mL for NoV GI, and 3.7-

6.0 $\log_{10}$ GC/100mL for NoV GII (Kitajima et al. 2012). Hewitt et al. (2011) observed 1.1-3.6 $\log_{10}$ GC/100mL for GI, and 1.2-4.5 $\log_{10}$ GC/100mL for GII in influent, and 1.2-4.1 $\log_{10}$ GC/100mL for GI and 1.9-4.5 $\log_{10}$ GC/100mL for GII in effluent. Enteric viruses can occur at 1.8-5.7 $\log_{10}$ GC/100mL in the influents and reduce to 0.5-4.3 $\log_{10}$ GC/100mL in effluent, as observed in the study of a WWTP in New Zealand (Hewitt et al. 2011). Symonds et al. (2009) analyzed sewages from 12 WWTP from the coastal U.S. and found adenovirus and picobirnaviruses in 100% of the raw sewages followed by enteroviruses (75%) and NoV (58%). The authors observed fluctuations in the virus prevalence in the effluent across different plants.

1.6. Occurrence in oysters

1.6.1. Oyster biology as relates to virus uptake

Eastern oyster (*Crassostrea virginica*, family *Ostreidae*), also known as the American oyster or Virginia oyster, is a commercial molluscan bivalve well adapted in inhabiting estuarine waters in various climates worldwide (Kennedy et al. 1996). Adult eastern oysters can grow and reproduce well under temperature of 24-26 ºC, salinity of 7.5-27 ppt, depth of 0.3-12 m. Louisiana vast coastal wetland area has provided suitable habitats for oysters to thrive, and made the state a leader in the U.S. in terms of the harvest volume and quality accounting for an average of 34% of the nation's harvested oysters. Louisiana Department of Wildlife and Fisheries (LDWF) is estimated 10.9 million pounds of the oysters harvested in 2012 with more than $35 million annual worth of dockside sales of oysters in Louisiana. The oyster industry utilizes the public oyster grounds both to seed oysters for transplantation to private leases and to harvest sack-sized oysters (≥3 inches). Oyster reefs are considered as a key element in estuarine ecosystems by maintaining shorelines, providing hard substrate for other aquatic animals such as invertebrates, and shelter and forage habitat for many species such as crabs, fish, etc.

Additionally, oysters have important role in water quality by filter-feeding from the surrounding water (LDWF 2013).

Oysters store energy mostly as the form of glycogen. Concentration of glycogen affects spawning and gonad maturation (Kennedy et al. 1996). Glycogen concentration in the eastern oysters of Louisiana varies from 467 to 2,960 mg (average of 1,326 mg) per 100g of raw oyster, and depends on the time of the year and the geographical location. The concentration of the glycogen in Louisiana oysters increases from December to May and again reduces from June to November (Sidwell et al. 1979). The spawning peak in Louisiana oyster is in late May, early June and September (Pollard 1973). Growth of oysters depends on environmental conditions, position and density in the reef (Stanely and Sellers 1986). Filter-feeding behavior allows an individual adult oyster to pump about 13 L of water per hour through the gills, and obtain food and necessary oxygen for the metabolism. Three mechanisms are explained for the selection of suspended particles that are 1) preferential clearance on the ctenidia, 2) preingestive clearance on the labial palps, and 3) differential absorption in the gut, also known as post-digestive (Shumway 1985).

Oysters can entrap the viruses from the surrounding water and act as intermediate vector (Wang et al. 2008). Filtration occurs both through physical and ionic entrapment (Maalouf et al. 2010b). A bioaccumulation experiment showed that eastern oysters filter MSC, a surrogate for enteric viruses, with a concentration factor up to 99-fold (Burkhardt and Calci 2000). Surface properties of virus particles, rather than size, influence the bioaccumulation of viruses by filter-feeding marine bivalves. The bioaccumulation capacity of viruses in oysters is high but finite, and depends on the numbers of the sites for virus adsorption and the concentration of virus in the water. Studies showed that the saturation level of reovirus type III and Semliki Forest virus in

New Zealand rock oyster (*C. glomerata*) is 4×10^{10} particles per oyster (Bedford et al. 1978). Various enteric viruses (such as NoV, enterovirus, rotavirus and astrovirus) have been detected in the harvested shellfish in France (Le Guyader et al. 2000).

Similar to NoV, enteroviruses contain a large group of pathogenic enteric viruses from genus Enterovirus (family *Picornaviridae*) and are classified into poliovirus, coxsackievirus, echovirus and the numbered enteroviruses (Fong and Lipp 2005). The genomic size and genomic and capsid structures of enteroviruses is similar to NoV, transmitted by the fecal-oral routes, and responsible for a wide range of rather serious infections in humans with diverse clinical syndromes such as gastroenteritis and meningitis (Fong and Lipp 2005, Khetsuriani et al. 2006).

A bioaccumulation study showed that virus uptake in shellfish is influenced by mucosal acidity. At pH 7.0, poliovirus has the highest affinity to shellfish mucus, but diminishes when the pH is dropped to 3.0. This also can explain the passage of viruses into the stomach and gut in the acidic environment of the digestive tract (Di Girolamo et al. 1977). Studies showed that the gills and digestive tissues of oysters are main tissues for the adsorption of NoV followed by the mantle cilia (Wang et al. 2008). Digestive tissues comprise approximately 10% of the body mass of an adult oyster, and are known as the site where majority of the NoV bind (Schwab et al. 1998). Virus particles bind specifically to the receptors of digestive ducts in midgut, main and secondary ducts and tubules using a carbohydrate structure having a terminal N-acetylgalactosamine residue in an α linkage. This binding site is the same site used for the recognition of human histo-blood group antigens, HBGA (Le Guyader et al. 2006a).

Maalouf et al. (2011) observed that NoV bioaccumulation is highly strain dependent. The GI.1 bioaccumulates in the digestive tissues of oysters on a dose-dependent manner (Maalouf et al. 2011), and has the highest level of ligand expression during the cold months when most of the

outbreaks occur (Maalouf et al. 2010b). However, GII strains bioaccumulate less efficiently in oysters with no seasonal influence. In this regard, GII.4 has lower bioaccumulation efficiency and seasonal effect than GII.3. Furthermore, GII.3 temporarily concentrates in gills and mantle before accumulate in digestive tissues. The authors postulated that binding to the sialic acid-containing ligand that are present almost in all tissues may contribute to the retaining of virus particles in the gills or mantle and result in to rapid destruction (Maalouf et al. 2011). Recently, phagocytic blood cells (hemocytes) of eastern oysters have been found to play role in retention of virus particles including enteric viruses (Provost et al. 2011).

The binding in viruses is so strong that depuration does not eliminate NoV from the contaminated tissues (Le Guyader et al. 2006a). According to the National Shellfish Sanitation Program (NSSP), depuration is the process of reducing the pathogens that may be present in oysters by holding them in a controlled aquatic environment as the treatment process. The current required depuration time for oysters is 44 hours (NSSP 2013). A depuration process of 48 h removes 95% of *E. coli* in oysters, whereas only 7% removal has been observed for NoV GI.1 (Schwab et al. 1998). In another study, a depuration for up to 10 days was not effective to eliminate NoV GII.6 from oysters (Ueki et al. 2007). Inefficient depuration of NoV along with their long-term persistence in shellfish and transmission variations among the strains have led to the conclusion for the presence of more specific mechanisms for NoV bioaccumulation in oysters. If non-specific binding mechanism present, it would be less efficient than specific ligand-mediated binding (Maalouf et al. 2010b). Therefore, the absence of bacterial indicators such as *E. coli* do not guarantee virus-free shellfish, and the health risk to consumers still exists even if the shellfish are considered safe from bacteriological standpoint (Doré et al. 2000).

1.6.2. Outbreaks and health risks

Norovirus outbreaks due to the consumption of raw or partially cooked oysters or other shellfish have been a global issue. Discharge of partially treated wastewater from WWTP or direct sewage discharge from boaters has been associated with the contamination of oysters and the subsequent incidence of gastroenteritis outbreaks (Berg et al. 2000, Ueki et al. 2005).

The occurrence of NoV GI and GII in 3.9% of U.S. market raw oysters has been reported where their levels were 10-100 times lower than the levels detected in outbreak investigations (Woods and Burkhardt III 2010). A two-year survey showed NoV contamination in the 76.2% of oysters sampled from 39 commercial harvesting areas in the U.K. The maximum concentrations of NoV GI and GII was 6.21 and 6.26 $\log_{10}$ genomic copies/100g digestive tissues, respectively, and dominated by GI strains (Lowther et al. 2012b). Norovirus infected 49.4% of the mussels in NW Spain at 5.9×10^3 to 1.6×10^9 RNA copies/g digestive tissue for NoV GI and 6.1×10^3 to 5.4×10^6 RNA copies/g digestive tissue for NoV GII. In this study, a higher contamination rate was observed during cold months i.e. from March to October (Manso and Romalde 2013). Results of a survey in detecting NoV in seven coastal cities in China markets showed that approximately the 13% of shellfish are contaminated with NoV GI.3 and GII.12 dominating by GII.12 (Ma et al. 2013). Similarly, Ma et al. (2013) observed higher densities of NoV in the retail oysters in China in spring and winter. Even though it is not possible to correlate the concentration of NoV viral genome with the virion infectivity, comparison of NoV RNA presented in non-outbreak samples with those were associated with outbreaks indicates that oysters containing less than 4 $\log_{10}$ NoV RNA copies per 100 gram are less likely to pose health risks to humans (Lowther et al. 2012a).

Other enteric viruses such as hepatitis A virus, enterovirus, rotavirus, astrovirus and adenovirus have been also occasionally detected in oysters worldwide particularly in human sewage impacted areas (DePaola et al. 2010, Le Guyader et al. 2000, Muniain-Mujika et al. 2003). Louisiana oysters harvested from approved areas were linked to hepatitis outbreaks in 1973. It is believed that flooding Mississippi River transferred the fecal pollution into the oyster growing areas on the east side of the river and contaminated the oysters (Mackowiak et al. 1976). In another event, several enteric virus outbreaks in France were associated with the consumption of oysters, which had been harvested following a flood (Le Guyader et al. 2008). Oyster-associated enteric virus outbreaks especially those were linked with sewage contamination commonly caused by multiple NoV strains, or multiple viruses. It has not been confirmed whether infection with multiple viruses contribute to more severe symptoms (Gallimore et al. 2005, Le Guyader et al. 2008).

1.6.3. Intervention techniques in food

Noroviruses are resistant to many environmental stressors, such as multiple freeze-thaw cycles (Richards et al. 2012), short time exposure to high temperatures (60 ºC) or acidic condition at pH 2.7 (Green 2007). NoV remains infectious under the natural intrinsic (pH, Aw) and extrinsic (temperature, time, redox potential) conditions associated with food (Jaykus 2000). Non-thermal intervention methods have shown promising results in reducing NoV; however, a few have focused on infectivity of human NoV after treatment. Even though NoV virus-like particles have shown resistance to high-pressure processing (Lou et al. 2012), a clinical trial reported complete inactivation of infectious NoV GII.8IIb in oysters at 600 MPa at 6 ºC or 25 ºC (Leon et al. 2011). In a similar study, by incorporating porcine gastric mucin (PGM)-conjugated magnetic beads (PGM-MBs) to distinguish infectious and noninfectious NoV, Ye et al. (2014)

reduced >4 $\log_{10}$ of NoV GI and GII in oyster and clams. Cold atmospheric pressure plasma has been successful in reducing NoV load on the contact surfaces; however the application of such techniques on food has yet to be investigated (Ahlfeld et al. 2015). Evidences on the application of compounds such as salt (Park and Ha 2014), mulberry (*Morus alba*) juice (Lee et al. 2014) and persimmon extract (Kamimoto et al. 2014) on reducing NoV and its surrogates in foods have been reported.

1.7. Microbial indicators in shellfish and water

Microbial indicators (such as fecal coliforms and *E. coli*) have been primarily utilized by regulatory agencies to monitor the sanitary conditions of drinking water, wastewater, recreational waters and oysters in terms of fecal pollution assessment and management practices such as wastewater treatment process control and shellfish harvest water classification (Ashbolt et al. 2001, Louisiana Department of Environmental Quality (LDEQ) 2011, National Shellfish Sanitation Program (NSSP) 2013). According to the Louisiana Water Quality Regulations, the discharges of sanitary publically owned WWTP in the Mississippi River have the fecal coliforms limitation of 200 CFU/100 mL (2.30 $\log_{10}$ CFU/100 mL) on a weekly average and 400 CFU/100 mL (2.60 $\log_{10}$ CFU/100 mL) on a monthly average (LDEQ 2011). Most Probable Number (MPN) criteria of total and fecal coliforms have been established by NSSP to assess the sanitary condition of depurated shellfish and the harvest water. For example, "in using a single dilution, the geometric mean value for the classification standard was required to remain at the level of both the 3 and 5-tube tests (70 MPN/100 mL for total coliforms and 14 MPN/100 mL for fecal coliform organisms in the Approved classification)". Then the measure of variability is adjusted using the upper two-sided 95% confidence limits (NSSP 2013).

Fecal coliforms and *E. coli* are indigenous members of the intestinal flora of healthy humans and warm-blooded animals (Leclerc et al. 2001, Tallon et al. 2005). Other microbial indicators such as bacteroides, enterococci, sulphite-reducing clostridia, *Clostridium perfringens* and bacteriophages have been suggested as alternative fecal indicators (Ashbolt et al. 2001). Coliphages are viruses that infect *E. coli* and are naturally present in the intestinal tract of animals. They have been intensively used for source tracking purposes. Male-specific coliphages (MSC or 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 (Brion et al. 2002, Chung et al. 1998, DePaola et al. 2010, Doré et al. 2000, Rodriguez et al. 2014). The concentration of microbial indicators (*E. coli*, total coliforms and MSC) in raw sewage is usually almost constant, and does not reflect the occurrence of human NoV in the final effluent but may indicate fecal population carriage rate (Haramoto et al. 2006, Wu et al. 2011). Contribution of human sewage water to viral contamination of shellfish growing areas and the occurrence of outbreak has led to the development of coliphages criteria for monitoring swage impacts on shellfish and shellfish growing waters (Maalouf et al. 2010a). Sewage-contaminated water has a high potential of harboring pathogenic enteric viruses such as NoV, hepatitis A virus, enteroviruses and rotaviruses (Wyer et al. 2012).

The risk associated with the consumption of raw shellfish contaminated with pathogenic enteric viruses still exits even when the oysters may be considered bacteriologically safe (DePaola et al. 2010, Myrmel et al. 2004). There is evidence of shellfish-associated NoV outbreaks where harvest water was negative for NoV (McIntyre et al. 2012). Monitoring the microbial indicators in shellfish showed that changes in MSC correlates with NoV suggesting its

potential as a useful indicator of virus contamination and depuration process efficiency (Doré et al. 2000). A 50 PFU limit of MSC in 100g of shellfish has been proposed by NSSP to assess the occurrence of raw untreated sewage contamination and emergency closure of the growing area (NSSP 2013). A drawback of using MSC is that not all of these coliphages are human specific (Havelaar et al. 1986) and only a subgroup of MSC (Type III) has found to be closely related to the release of human fecal wastes in water (Brion et al. 2002).

However, direct screening of pathogenic human enteric viruses in shellfish has been found as the most promising human-specific viral marker in environmental waters and shellfish (DePaola et al. 2010, Muniain-Mujika et al. 2003, Myrmel et al. 2004, Wong et al. 2012). Enteroviruses and adenoviruses, just as other types of viruses, have a narrow host range. For example, human enteroviruses infect only humans and cannot cause infection in cattle or fowl and vice versa (Fong and Lipp 2005). The health risks associated with pathogens originated from nonhuman animal wastes are less well investigated (Bosch et al. 2006).

1.8. Justification

Several viral gastroenteritis outbreaks worldwide have been associated with the direct or indirect human exposure to raw or partially treated sewage, which usually contain high concentrations of various types of pathogenic enteric viruses, in particular noroviruses. Filter-feeding molluscan shellfish such as oysters and clams have the ability to concentrate small particles including virus particles from the surrounding waters, and transmit to humans. In the recent years, the U.S. FDA has reported several norovirus outbreaks in which they were traced back to Louisiana oysters following the analysis of fecal samples from the infected individuals and tracing back the cases to the potential food sources were consumed. To date, no report was

found in the literature to directly link a NoV outbreak with the oysters were contaminated in the growing waters in Louisiana.

Regulatory agencies in Louisiana and several states have utilized bacterial indicators of fecal contamination (total coliforms, fecal coliforms and *Escherichia coli*) as the microbial criteria to monitor sanitary conditions of water and shellfish. Recently, the US National Shellfish Sanitation Program proposed a microbial limit for male-specific coliphage in oysters for reopening areas were closed due to high impact sewage pollutions. There is limited information available on the current sanitary status of Louisiana eastern oysters harvested from commercial areas. This will help regulatory agencies to have hands-on information on the safety of Louisiana oysters, and to establish more effective sanitary measures to reduce public health risks and the burden of foodborne diseases associated with enteric viruses. The objectives of the study was to identify shellfish harvest areas that will reflect varying levels of human fecal contamination, perform bi-weekly survey of selected shellfish harvest waters[1] and shellfish for noroviruses, pathogenic enteric viruses and microbial indicators (aerobic plate count, fecal coliforms, and *E. coli* and coliphages), evaluate the correlation between pathogens and the microbial indicators, and identify the dominant NoV genotypes found in the water and/or oysters.

The second part of this study aimed to investigate the prevalence and densities of noroviruses and pathogenic enteroviruses in a secondary wastewater treatment plant in Louisiana. Human sewage is a major source of environmental contaminations with enteric

[1] The data from the harvest water analyses are shared by my research collaborator, Morgan Maite, from a portion of her Ph.D. research under Dr. Marlene Janes's (2012-current).

pathogens. Discharge of raw or partially treated sewage into the recreational, irrigation or oyster growing waters, or any cross contamination with drinking water can have serious health risk consequences. The purpose of this research was to investigate the pathogenic enteric viruses load in the influent and effluent wastewater treatment plant in Louisiana on a monthly basis within a one-year period to determine the viral and microbial indicators entering the plant and discharging into the Mississippi River. The objectives were to determine microbial removal, the correlation of microbial indicators with the enteric pathogens, and identify the dominant norovirus genotypes the wastewater system. The outcomes of this research will not only help regulatory agencies and plant managers to enhance the treatment efficiency, and implement more reliable microbial criteria, it can help health professionals to better understand the prevalence and intensity of the pathogenic enteric viruses circulate within a community, and help the development of more efficient vaccines and cell culture systems.

1.9. References

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CHAPTER 2. ENTERIC VIRUSES IN LOUISIANA OYSTERS AND HARVEST WATER[2]

2.1. Introduction

Noroviruses (NoV) are the leading cause of acute and epidemic gastroenteritis in humans. 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 32 genotypes. A tentative GVII has been recently proposed (Green 2007; Tse et al. 2012; Vinjé 2015). Genogroups I, II, and IV infect humans, and the rest are isolated from other species (Green 2007; Hall et al. 2014). Despite the extensive genetically divergent nature of noroviruses, the GII.4 strains remain the predominant cause of the NoV outbreaks worldwide (Siebenga et al. 2009).

Pathogenic enteric virus particles are shed in large numbers into the feces or vomit of infected individuals, and enter the environmental waters by direct discharge or the release of sewage. The viruses are either suspended or precipitated, and can survive for weeks to months while retaining their infectivity (Bosch et al. 2006; Campos and Lees 2014; McIntyre et al. 2012). As a result, filter-feeding mollusks inhabiting contaminated waters bioaccumulate the microbial pathogens, and if consumed raw or inadequately cooked, transmit them to humans (Campos and Lees 2014; Oliveira et al. 2011).

[2] This chapter has been accepted for publication at the *Journal of Food Science*. Naim Montazeri, Morgan Maite, Da Liu, Jiemin Cormier, Matthew Landry, John Shackleford, Lucina E. Lampila, Eric C. Achberger and Marlene E. Janes. Surveillance of enteric viruses and microbial indicators in the eastern oysters (*Crassostrea virginica*) and harvest waters along Louisiana gulf coast.

Norovirus infections associated with shellfish consumption are frequently reported worldwide (Alfano-Sobsey et al. 2012; Lowther et al. 2012b; Rajko-Nenow et al. 2014; Rippey 1994). The Centers for Disease Control and Prevention (CDC) estimated that approximately 16.1% (465/2,895) of the norovirus outbreaks in the U.S. (2009-2013) with known transmission routes are foodborne (Vega et al. 2014). According to a similar study, 19% (13/67) of foodborne NoV outbreaks in the U.S. (2009-2012) with a specific food category are attributed to the mollusks (Hall et al. 2014). 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 (NSSP 2013), whereas in the E.U., the regulations have focused on fecal coliforms in oyster tissues (Oliveira et al. 2011). These measures effectively enhanced the health of the shellfish consumers against diseases of bacterial origin (Rippey 1994); however, pathogenic viruses in oysters have been detected even when densities of microbial indicators in oyster or harvest waters remained low (DePaola et al. 2010; Lowther et al. 2012b).

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 the 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 (Brion et al. 2002; Chung et al. 1998; DePaola et al. 2010; Doré et al. 2000; Rodriguez et al. 2014).

This study is the first report on the surveillance of NoV GI and GII and microbial indicators of fecal contamination in both 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. Material and methods

2.2.1. Sample collection

Biweekly samples of eastern oysters (*Crassostrea virginica*) and 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 analysis 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 (Table 1., Figure 6). Oysters were double bagged in polyethylene bags, along with the water samples were kept on ice, and processed within 24 h of collection.

2.2.2. Oyster processing

Upon sample arrival, oysters were washed using cold tap water, and shucked under sterile conditions. For the 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.

Table 1. Dates and locations of sample collection from commercial oyster harvesting areas along the Louisiana Gulf Coast

Sampling date	Area	Site	Latitude	Longitude
January 14, 2013	13	b	29.40147	-90.03231
January 28, 2013	12	b	29.42425	-89.98367
February 25, 2013	13	b	29.40818	-90.00840
March 18, 2013	9	a	29.33497	-89.58835
April 8, 2013	10	a	29.37898	-89.64500
April 22, 2013	11	a	29.36612	-89.66843
June 3, 2013	12	b	29.42071	-89.98174
June 24, 2013	13	b	29.41158	-90.00987
July 1, 2013	9	a	29.33588	-89.59035
July 15, 2013	10	a	29.36766	-89.61490
July 29, 2013	11	a	29.36139	-89.67645
August 5, 2013	12	b	29.46582	-89.97770
August 26, 2013	13	b	29.43233	-89.97580
September 9, 2013	9	a	29.33515	-89.58707
September 16, 2013	10	a	29.37881	-89.64475
October 21, 2013	11	a	29.44718	-89.68850
November 18, 2013	12	b	29.43075	-89.97806
November 24, 2013	13	b	29.39345	-90.00330

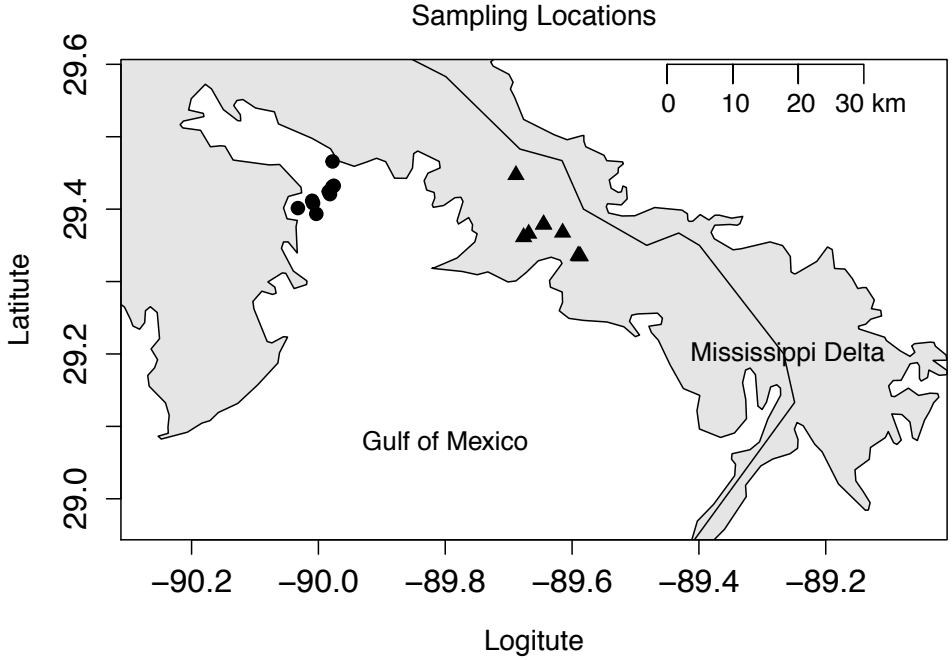

Figure 6. Oyster and harvest water sampling locations along the Louisiana Gulf Coasts. Triangles denote sampling sites “A”, and circles denote sampling area “B”.

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 9222D (U.S. EPA 2003). *E. coli* colonies were enumerated on modified membrane-thermotolerant *Escherichia coli* agar (Modified mTEC, Difco) following EPA Method 1603 (U.S. EPA 2009). Enterococci were quantified using membrane-enterococcus indoxyl-β-D-glucoside agar (mEI, Difco) based on US EPA Method 1600 (U.S. EPA 2002). Coliphages were quantified using a single agar layer method according to the U.S. EPA Method 1602 (U.S. EPA 2001) in 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 and 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 (Greenberg and Hunt 1984). The data were reported as $\log_{10}$ most probable numbers (MPN)/100g oyster. Male-specific coliphages (MSC) and somatic coliphages (SC) were enumerated from 15 mL (eq. 15 g) of the oyster

homogenate using a modified double-agar-overlay method developed for the analysis of oysters by Howell (2009). 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 et al. (2002) and modified by Fong et al. (2005). 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 the 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. The extracted RNA was eluted in 40 μL THE RNA Storage Solution (1 m*M* sodium citrate, pH 6.4, Ambion), and immediately analyzed or stored frozen at -80 ºC 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 et al. (2003) and Jothikumar et al. (2005). For the oysters, pathogenic enterovirus serotypes (EV), including poliovirus, coxsackievirus, echovirus and enteroviruses, 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 et al. (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 samples were followed according to Gentry et al. (2009b). 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 oyster digestive tissues followed a multiplex RT-qPCR assay for the simultaneous detection and quantification of NoV GI, GII and EV along with a heterogeneous IAC as optimized by Burkhardt et al. (2006) and Nordstrom et al. (2007). 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. Christine Moe's laboratory at Emory University (Atlanta, GA), and human poliovirus 3 stock (attenuated Sabin strain) was 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

A norovirus outbreak likely due to the consumption of contaminated oysters from Cameron Parish, LA was reported by the Molluscan Shellfish Program - Louisiana Department of Health & Hospitals in January 4, 2013. Oyster and water samples were collected from the suspected area in Calcasieu Lake (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 NoV gastroenteritis symptoms from one of the affected individuals known to have consumed raw oysters from the suspected area. 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 (Woods et al. 2011) or sequencing the amplified junction region between ORF1 and ORF2 (region C) of the viral genome (Kojima et al. 2002; Mattison et al. 2009) 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) (Altschul et al. 1997). A phylogenic tree of the partial capsid gene sequences was inferred by using the Maximum Likelihood method based on Tamura-Nei model (Tamura and Nei 1993) by employing MEGA (version 6.0), a molecular evolutionary genetics analysis tool developed by Tamura et al. (2013). The sequences of the reference strains were retrieved from the GenBank sequence database deposited at the NCBI (Vinjé 2015; Zheng et al. 2006).

2.3. 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 the correlation among variables. Software RStudio (version 0.98.1028, RStudio Inc., Boston, MA) was used for the statistical analyses and data visualization. Values of water surface temperature used in this study were obtained from the Giovanni online data system, developed and maintained by NASA Goddard Earth Sciences Data and Information Services Center (GES DISC). Temperatures are measured from approximately 10 μm below the surface (infrared bands) to 1 mm (microwave bands) depths using radiometers. A threshold of 24 °C was considered to categorize the data to warm months (May to October) and cold months (November to April).

2.4. Results and Discussion

All the sampling locations (areas 9 through 13) were among the most active commercial oyster harvesting areas along the Louisiana Gulf Coast and remained open during the sampling period; however, due to adverse weather conditions (heavy rain, fog, storm, or water level)

sample collection from some specific areas or times was not possible. Figure 7 shows monthly sea surface temperature of the sample collection areas along the 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, the protocols previously optimized for the analysis of oysters and harvest waters were used (Figures 8-10, Table 2, DePaola et al. 2010; Gentry et al. 2009a; Knight et al. 2013; Lowther et al. 2012b; Woods et al. 2011).

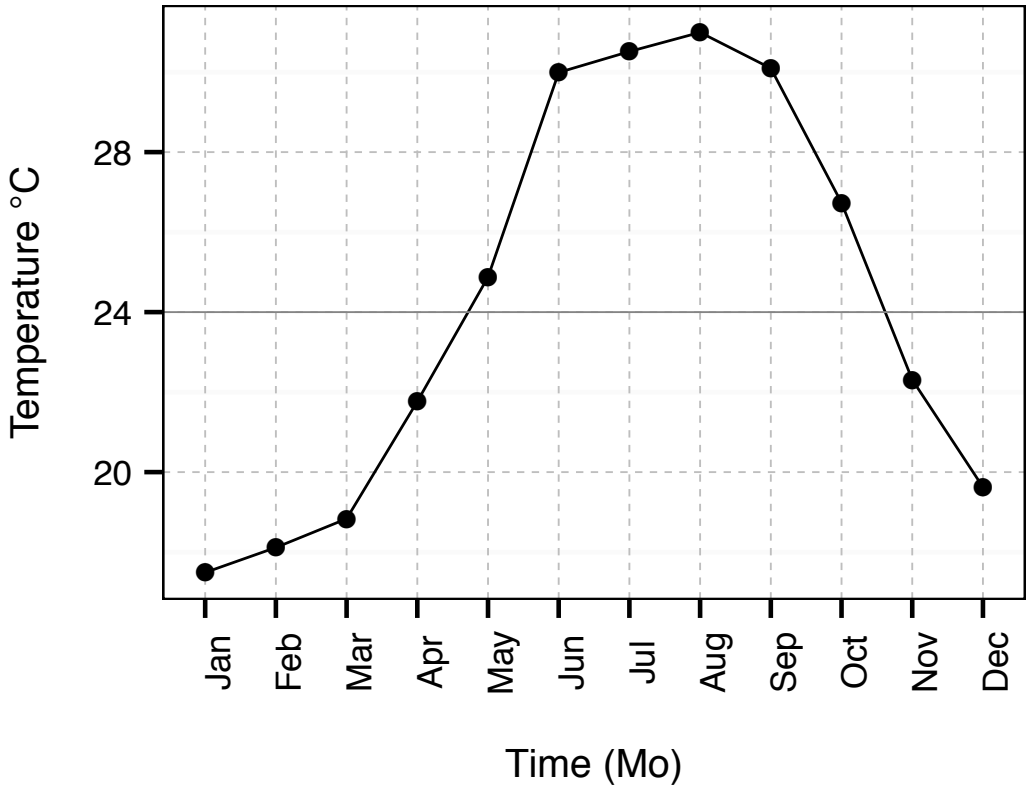

Figure 7. Monthly temperature (°C) of sea surface in Louisiana Gulf Coast in 2013. The horizontal line denotes the 24 °C threshold to distinguish between cold and warm months.

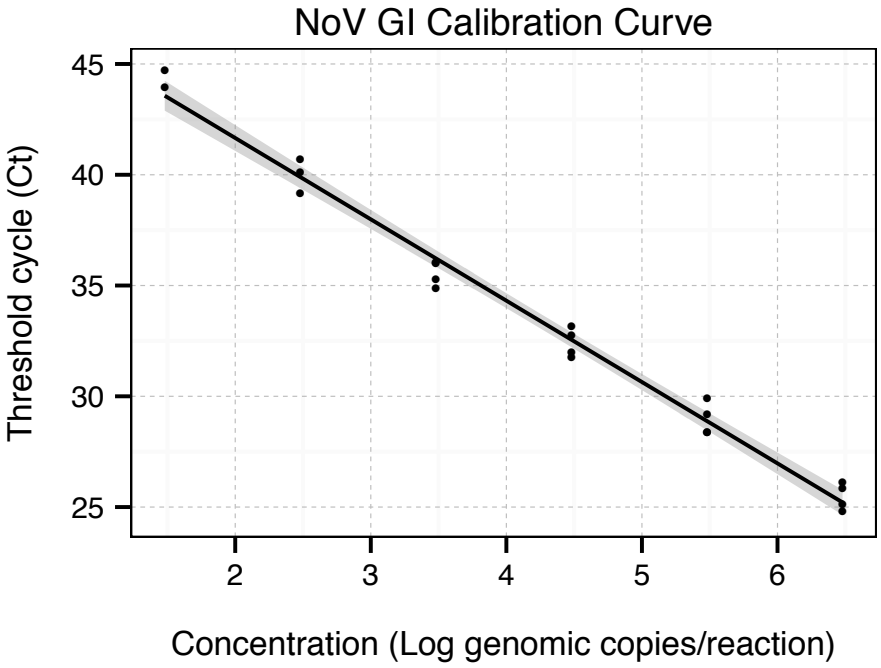

Figure 8. NoV GI calibration curve

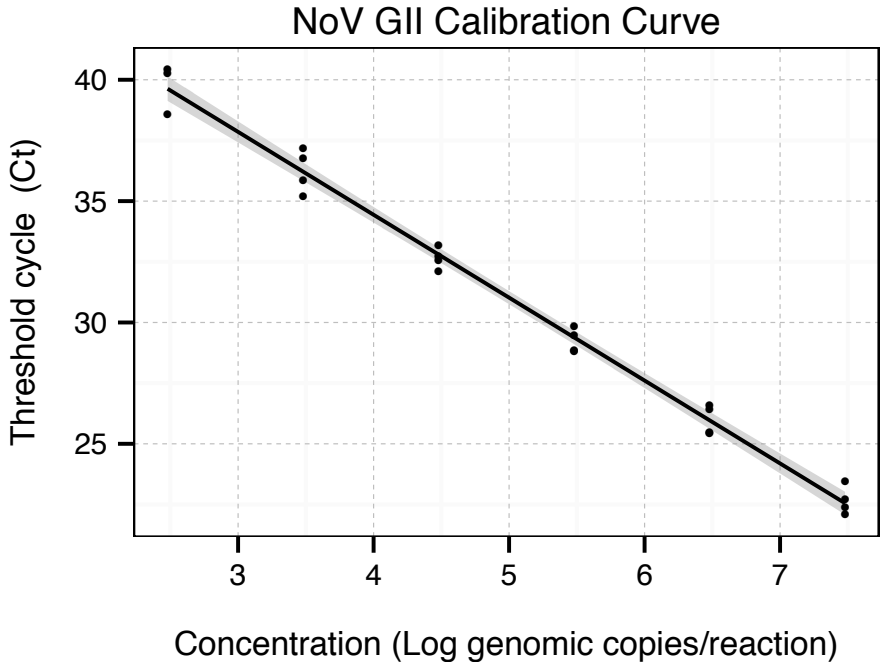

Figure 9. NoV GII calibration curve

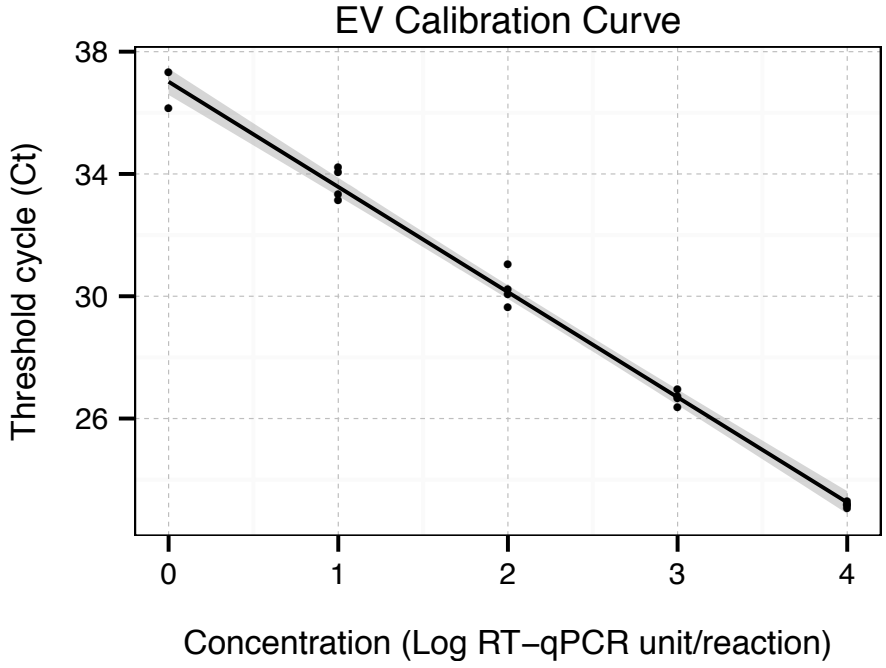

Figure 10. EV calibration curve

Table 2. RT-qPCR performance for the detection and quantification of NoV and enteroviruses in oysters

	Linear range	R^2	Slope	Y-intercept	RT-qPCR efficiency (%)	Amplification factor
NoV GI[1]	1.48 to 6.48	0.986	-3.672	48.99	87.22	1.87
NoV GII[1]	2.48 to 7.48	0.989	-3.415	48.09	96.25	1.96
EV[2]	0.00 to 4.00	0.992	-3.440	37.02	95.29	1.95

[1] $\log_{10}$ GC/reaction; [2] $\log_{10}$ RT-qPCR unit/reaction

2.4.1. Microbial indicators

Microbial indicators in both oysters and harvest waters were relatively low with no significant difference across the sampling sites ($p > 0.05$, Tables 3-4). 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 (DePaola et al. 2010). Enterococci along with other fecal indicators can be used to evaluate sanitary condition of shellfish harvest water (U.S. EPA 2002). This group of bacteria is accumulated in oyster tissues (Love et al. 2010); however, they have not yet been

considered as a sanitary monitoring criterion in oysters (NSSP 2013). In our study, enterococci were analyzed only in harvest water, and present in all the samples at an average value of 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 (NSSP 2013). 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 (NSSP 2013). In our study, fecal coliforms in the water samples were present within the acceptable limits.

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 (Burkhardt and Calci 2000). 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 of 5 sub-samples (U.S. FDA 2011).

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, whereas

Table 3. Microbial indicators in oysters from site A and B, mean ± SE.

		Month				
Microbial indicators	Site	March	April	July	September	October
APC	A	4.70 ± 0.03	4.76 ± 0.16	6.19 ± 0.13	6.11 ± 0.17	5.87 ± 0.01
Fecal coliforms	A	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	A	1.04 ± 0.00	1.04 ± 0.00	1.04 ± 0.00	1.04 ± 0.00	1.04 ± 0.00
SC	A	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	B	4.56 ± 0.28	5.55 ± 0.09	6.06 ± 0.32	5.78 ± 0.16	5.37 ± 0.09
Fecal coliforms	B	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	B	1.04 ± 0.00	1.04 ± 0.00	1.15 ± 0.11	1.04 ± 0.00	1.04 ± 0.00
SC	B	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, MSC and SC: $\log_{10}$ PFU/100g.

Table 4. Microbial indicators in harvest waters from site A and B, mean ± SE.

		Month					
Microbial indicators	Site	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. (not available): 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.

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 et al. (2010). However, higher levels of MSC (>3 $\log_{10}$ PFU/100g) have been found in oysters from the U.K. commercial harvesting areas (Doré et al. 2000). In general, even though there were no significant difference between site A and B in terms of the bacterial indicators ($p > 0.05$), site B showed a higher level of fecal pollution.

2.4.2. 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* (Burkhardt and Calci 2000). 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 et al. (2003). It could be due to an increased rate of digestion in oysters at elevated temperatures (Loosanoff 1958). These observations, however, differ from the report of DePaola et al. (2010) 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/100g, >3.3 $\log_{10}$ PFU/100g and 2.3 $\log_{10}$ PFU/100g, respectively with no observed seasonal trend for APC (averaged 6.5 $\log_{10}$ CFU/100g).

Overall, no strong positive correlation ($r < 0.45$) was observed between microbial indicators in oysters (data not shown). Densities of enterococci in the water samples were correlated with fecal coliforms ($r = 0.63$, $p < 0.05$) and *E. coli* ($r = 0.64$, $p < 0.05$). 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 was

observed ($r \leq 0.45$). Campos et al. (2013) obtained strong correlation between fecal coliforms and *E. coli*, MSC, and APC in shellfish (Campos et al. 2013). 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 in agreement with Wu et al. (2011) that the data linking microbial indicators to virus and bacterial pathogen contamination in water is equivocal requiring further examination.

2.4.3. 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 and GII were not detected in any of the eighteen water samples collected. The mean threshold cycle (Ct) values of the positive samples was 42.3 ± 0.2, and corresponded to 3.53 ± 0.20 $\log_{10}$ GEC/g oyster digestive tissues. Secondary extraction of NoV from the oyster samples generated a similar 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 (Alfano-Sobsey et al. 2012; Lowther et al. 2012a; McIntyre et al. 2012; Rajko-Nenow et al. 2014). 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 (Lowther et al. 2012a). 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 11, Appendix 1) 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 is a highly conserved region in

NoV GII, and not suitable for genotyping (Kageyama et al. 2003). So far, no alternative conserved PCR primers have been recognized for the confirmation of NoV positive RT-qPCR assays (Knight et al. 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 (Boxman et al. 2011; DePaola et al. 2010; Mattison et al. 2009; Woods et al. 2011).

IUPAC codes used to indicate the degenerate positions resulted from the forward primer (COG2F): Y, C or T; R, A or G; B, not A; N, any. Kageyama et al. (2003).

Figure 11. RT-qPCR amplicons sequence from the NoV GII positive oyster sample (5' to 3').

2.4.4. Outbreak samples

Table 5 summarizes densities of the microbial indicators in the suspected oysters and harvest water from the area 30 at the Calcasieu Lake (Cameron Parish, LA). 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 (Chung et al. 1998; Lowther et al. 2012b). RT-qPCR analysis of the suspected oysters 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). Phylogenic analysis of the NoV viral genome revealed that the strain belonged to the GII.4 Sydney (Figure

12), which has been the dominant NoV outbreaks strain in the U.S. during 2013 to 2014 (Vinjé 2015). 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 (Alfano-Sobsey et al. 2012; McIntyre et al. 2012; Rajko-Nenow et al. 2012; Rajko-Nenow et al. 2014; Verhoef et al. 2010). The nucleotide sequence of the stool NoV GII determined in this study is deposited in GenBank under the accession number KP455650.

Table 5. 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.

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 (Alfano-Sobsey et al. 2012; Boxman et al. 2011). 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., 2009-2012 (Hall et al. 2014).

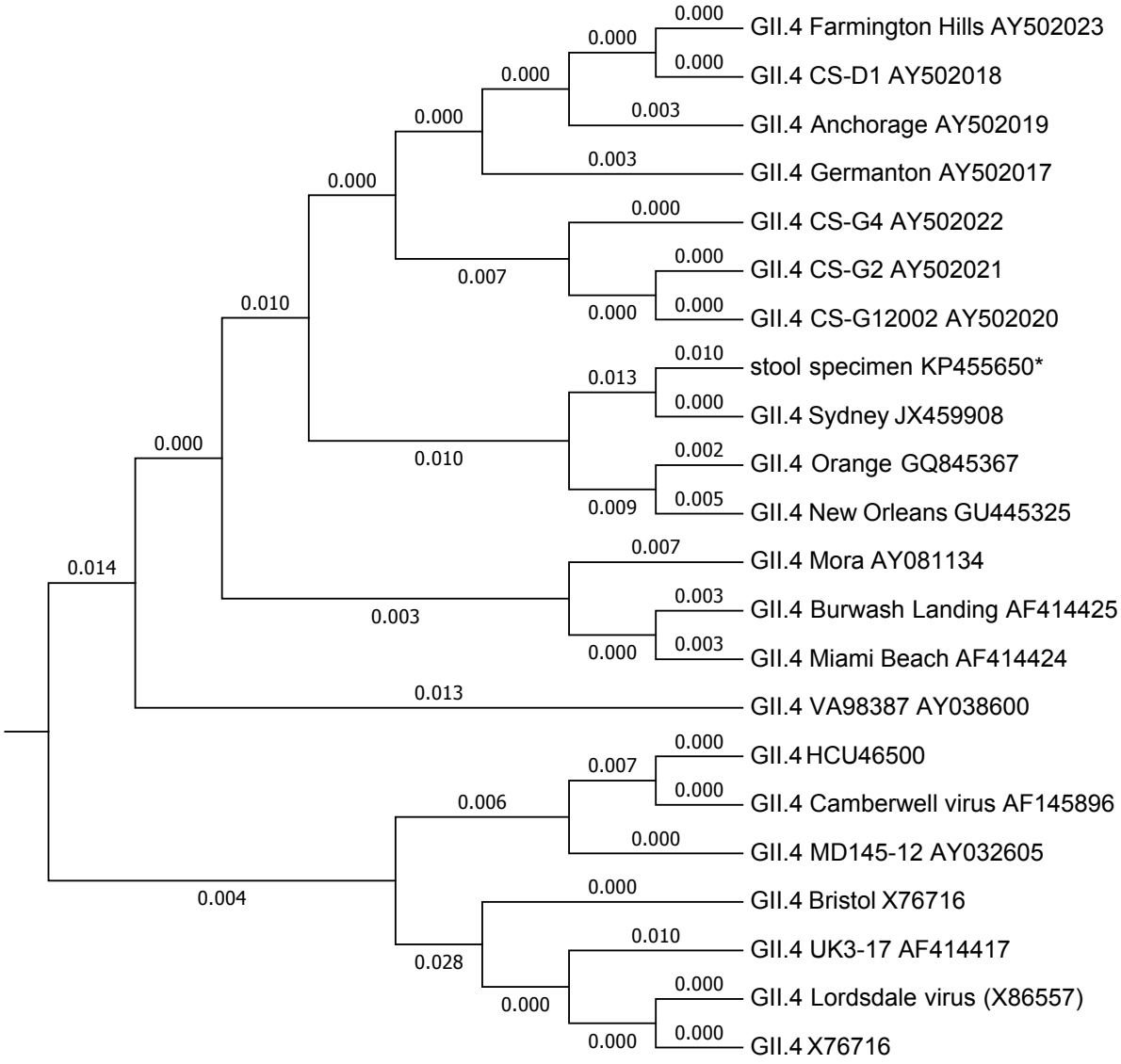

Figure 12. Selected region of the phylogenic tree (NoV GII.4 strains) constructed for genotyping the NoV GII detected in the outbreak stool specimen. The numbers on the branches indicate the branch lengths. Each strains name is followed by the reference sequences obtained from NCBI (Vinjé 2015; Zheng et al. 2006).

2.5. Conclusions

This study surveyed noroviruses and microbial indicators of fecal contamination in the oysters and harvest waters taken from commercial harvesting areas along Louisiana Gulf Coasts, and to evaluate the effectiveness of microbial indicators for assessing the 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 (Knight et al. 2013), and current RT-PCR methods are not able to correlate molecular detection results with the NoV infectivity (Knight et al. 2013; Mattison et al. 2009).

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 (Love et al. 2010). 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 (DePaola et al. 2010). Male-specific coliphages have been recently proposed as a better indicator of fecal contamination of U.K. market-ready oysters as they showed seasonality consistent with the trend of shellfish-associated gastroenteritis outbreaks (Doré et al. 2000). 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 (NSSP, 2013). 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 (Chung et al. 1998; DePaola et al. 2010). Therefore, monitoring of harvesting waters and oysters for pathogenic enteric viruses is crucial (Hernandez-Morga et al. 2009). Our study supports the dual criterion of NoV detection

and elevated MSC (>1.70 $\log_{10}$ PFU/100g) in oysters to flag for potential public health issues (DePaola et al. 2010).

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CHAPTER 3. ENTERIC VIRUSES IN THE MUNICIPAL WASTEWATER

3.1. Introduction

Enteric viruses are responsible for a wide range of infections in humans with diverse symptoms. Infected individuals shed millions of virus particles in their feces or body fluids, which eventually enter sewage systems. Enteric viruses may naturally occur in aquatic environments as well, but human activities, in particular sewage discharge is the primary source of environmental contaminants (Fong and Lipp 2005, Lees 2000, Skraber et al. 2011). Among the pathogenic enteric viruses, norovirus, enterovirus, adenovirus, astrovirus and rotavirus have been frequently found in municipal wastewaters worldwide (Fong and Lipp 2005, Kitajima et al. 2012, Myrmel et al. 2006, Symonds et al. 2009). Several gastroenteritis outbreaks have been linked directly or indirectly to human exposure of raw or partially treated sewage-contaminated water or foods (Lees 2000).

Municipal wastewaters usually undergo a secondary treatment before being discharged into the environment. The process involves mechanical treatment for removing solids followed by biological and chemical treatments, nutrient removal and discharge (Bitton 2011). Primary treated (physically processed) wastewater or the effluent water that does not undergo a disinfection process may still harbor infectious enteric viruses similar to the raw sewage (Hewitt et al. 2011, Myrmel et al. 2006). To protect water quality and public safety, fecal coliforms or *E. coli* are used to monitor fecal pollution in wastewater discharge or environmental waters. In general, viruses are more resistant than bacteria to UV treatment or chlorination, two common disinfection strategies in secondary wastewater treatment (Bitton 2011, Francy et al. 2012). The aggregation of viruses in water or other wastewater solids reduces the efficacy of disinfectants, and helps viruses maintain their infectivity when discharged into the environmental waters

(Bitton 2011, Keswick et al. 1985). As a result, the bacterial indicators may not be able to reflect the occurrence of enteric viruses in water efficiently. In this regard, monitoring bacteriophages such as male-specific coliphages (MSC) or even direct measurement of enteric viruses such as enteroviruses and adenovirus have been suggested as more reliable criteria to assess sewage pollutions of human origins in water (Fong and Lipp 2005, Rodriguez et al. 2014).

Norovirus (genus Norovirus, family *Caliciviridae*) causes the most cases of human gastroenteritis worldwide at least during the last decade. Norovirus (NoV) is a positive sense single stranded (+ssRNA) virus comprising six genogroups (GI to GVI) and more than 32 genotypes. Genogroups I, II and IV are responsible for disease in humans, whereas the rest have been found in animals (Green 2007, Vega et al. 2014, Vinjé 2015). Despite the extensive genetically diverse nature, the GII.4 strains remain the predominant cause of the NoV outbreaks in humans globally (Pringle et al. 2015). Enteroviruses (genus Enterovirus, family *Picornaviridae*) are composed of poliovirus, coxsackievirus, echovirus and the numbered enteroviruses (Fong and Lipp 2005). The genomic size, and genomic and capsid structures of enteroviruses is similar to noroviruses. Enterovirus serotypes (EV) are usually transmitted through the fecal-oral routes, and are responsible for a wide range of infections in humans with diverse clinical syndromes such as gastroenteritis and meningitis (Fong and Lipp 2005, Khetsuriani et al. 2006).

In this study, we monitored the loads of pathogenic enteric viruses and microbial indicators in a municipal secondary wastewater treatment plant (WWTP) in the U.S. (New Orleans, Louisiana). The molecular techniques used to analyze viral genomes did not permit absolute quantification of infectious viruses. This is primarily due to the negative effect of inhibitory compounds on optimum amplification of viral RNA and the inability of PCR in

distinguishing intact over damaged virus particles. According to the available literature, this is the first report on the year-round monitoring of pathogenic enteric viruses, microbial indicators and NoV diversity in a municipal WWTP influent and effluent in the U.S.

3.2. Material and methods

3.2.1 Wastewater treatment plant and sampling

A municipal WWTP in New Orleans (Louisiana) with an annual average flow of 98 million gallons per day (MGD) for 2013 was studied. The wastewater is generated from New Orleans on the east bank of the Mississippi River with an estimated population of 378,715 (U.S. Census Bureau 2013). The sanitary sewage system collects wastewater using a gravity collection system, and transfers it to the treatment plant through a series of pumping stations. A 24 h a day secondary treatment process employs a high-purity oxygen modification of the activated sludge system, including clarification through sedimentation, chlorination (0.5 mg/L) for disinfection and finally discharge of effluent directly into the Mississippi River (Louisiana). Solids are either returned to the process or wasted and disposed through incineration and then landfill. Monthly samples from influent and effluent waters were obtained from July 2013 to June 2014. Each month, 2 L of 24 h composite influent and effluent samples were collected (4 ºC). Even though the samples were collected on the same day, the influent and effluent samples were temporally separate due to the retention time for the influent water to go through the treatment. Effluent samples were collected prior to release into the Mississippi River and were dechlorinated with 0.63 *M* sodium thiosulfate at 0.5 mL/L of sample. The samples were stored on ice, transported to the Food Microbiology laboratory at Louisiana State University AgCenter, and analyzed within 24 h. Biochemical oxygen demand (BOD), total suspended solids (TSS) and volatile suspended solids (VSS) data were provided by the WWTP laboratory, and were measured according to the

Standard Methods for the Examination of Water and Wastewater protocols No. 5210, 2540D and 2540E, respectively (Rice et al. 2012).

3.2.2. Microbial indicators

Microbial indicators of fecal contamination (fecal coliforms, thermotolerant *E. coli*, enterococci and male-specific bacteriophages) were quantified in influent and effluent waters using the U.S. Environmental Protection Agency (U.S. EPA) standard membrane filter techniques. Fecal coliforms were enumerated on m-FC medium (Difco, Sparks, MD) after incubation at 45 ºC for 24 h (U.S. EPA 2003). Thermotolerant *E. coli* were counted on modified membrane-thermotolerant *E. coli* agar (m-TEC, Difco) in which the plates were initially incubated at 35 ºC for 2 h to resuscitate injured or stressed bacteria, and then incubated at 45 ºC for 24 h (U.S. EPA 2009). Enterococci (*Enterococcus faecalis*, *E. faecium*, *E. avium* and variants) were quantified on enterococcus membrane-indoxyl-β-d-glucoside agar (mEI, Difco) following incubation for 24 h at 41 ºC (U.S. EPA 2002). Quantities of bacteria were reported as logarithm of colony forming units (CFU) per 100 mL of wastewater samples ($\log_{10}$ CFU/100 mL). Single agar layer (SAL) plaque assay on 2× tryptic soy agar (Difco) containing 3.0 mg/mL of each ampicillin sodium salt and streptomycin sulfate salt (Sigma-Aldrich, Steinheim, Germany) was used for the male-specific coliphages plaque assay using *E. coli* HS(pFamp)R (ATCC 700891) as the host strain. The plaque forming units (PFU) were counted after incubating the plates for 16-24 h at 37 ºC, and reported as $\log_{10}$ PFU per 100 mL wastewater sample. Male-specific (F+) coliphage MS2 (ATCC 15597-B1) was used as positive control along with the samples (U.S. EPA 2001).

3.2.3. Enteric viruses

3.2.3.1. Extraction and concentration of enteric viruses

An ultracentrifuge method was used for the extraction and concentration of enteric viruses from 60 mL of the wastewater samples as developed by the U.S. FDA Gulf Coast Seafood Laboratory at Dauphin Island, AL (U.S. FDA 2010). The RNA was extracted using RNeasy Mini Kit (Qiagen, Germantown, MD) following the manufacturer's instruction with minor modifications (U.S. FDA 2010), in which 15 min hold time was given for each washing steps. The extracted RNA was eluted in 40 µL THE RNA Storage Solution (1 m*M* sodium citrate, pH 6.4, Ambion), and immediately analyzed or stored frozen at -80 ºC until required.

3.2.3.2. Determination of pathogenic enteric viruses

Detection and quantification method followed a multiplex real-time TaqMan-based RT-PCR (RT-qPCR) for simultaneous detection of NoV GI, GII and enterovirus serotypes (EV) along with a heterogeneous internal amplification control (IAC) optimized by Burkhardt et al. (2006) and Nordstrom et al. (2007). The sequence of the primers and probes for NoV GI and GII target sensitive and broadly reactive ORF1-ORF2 junctions, as designed by Kageyama et al. (2003). The primers for EV amplified the 5' untranslated region (UTR) of the enteroviral genome with a panenterovirus primer set (Donaldson et al. 2002). 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. A Cepheid SmartCycler® II system (Sunnyvale, CA) was used for all the RT-qPCR analyses. The templates were reverse-transcribed at 50 °C for 50 min, and then the 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 were considered positive when the emission intensities exceeded the

threshold during the first 46 cycles. All reactions were carried out in duplicate. NoV GI and GII RNA standards (10^9 Genomic Copies (GC)/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's laboratory (U.S. FDA Gulf Coast Seafood Laboratory, Dauphin Island, AL), and were utilized as positive controls and for RNA quantification by analyzing serial decimal dilutions of extracted RNA, and assigning the value of one RT-qPCR unit per reaction to the highest dilution showing a positive threshold cycle (Ct) value (Richards et al. 2004). No template control was included in all the analyses to overcome any uncertainty of false-positive responses due to cross-contamination or reaction failure.

3.2.3.3. Sequencing and genotyping noroviruses

The noroviruses samples were genotyped on a bimonthly basis starting from July 2013 until May 2014 to identify the predominant strains present in the influent and effluent waters. The ORF1-ORF2 junction (region C) of the NoV viral genome was amplified by a hemi-nested PCR on a C1000 thermal cycler (Bio-Rad, Hercules, CA) by utilizing a OneStep RT-PCR kit (Qiagen), 0.4 µ*M* of each oligonucleotide primer, and 5 units of SUPERase.In RNase Inhibitor (Ambion, Foster City, CA) in a final reaction volume of 25 µL. Primer sequences were obtained from the previous studies (Kageyama et al. 2003, Kojima et al. 2002). For the first PCR, 5 µL of extracted RNA was amplified by incorporating COG1F/G1SKR and COG2F/G2SKR primers for NoV GI and GII, respectively. RT-PCR condition was as following: RT for 30 min at 42 ºC, heat activation of *Taq* DNA polymerase for 15 min at 95 °C, PCR consisting of 40 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 60 s and a final extension for 7 min at 72 °C. The PCR amplicons were purified by electrophoresis using 2% agarose gel (4.83 volts/cm) containing 0.5 µg/mL ethidium bromide, and extracted by utilizing QIAquick Gel Extraction kit per the

manufacturer's instruction (Qiagen, Valencia, CA). Extracted DNA (1 μL) was subjected to a second PCR with G1SKF/G1SKR primers for GI and G2SKF/G2SKR primers for GII under the same condition excluding the RT step (Kojima et al. 2002, Mattison et al. 2009). PCR amplicons (330 bp for GI and 344 bp for GII) were gel-purified and 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 BigDye terminator cycle sequencing reaction kit (Applied Biosystems) on an ABI Prism 3130 automated sequencer Genetic Analyzer (Life Technologies), and processed on CLC Sequence Viewer (version 7.5, CLC Bio, Aarhus, Denmark). Phylogenic trees of the partial capsid gene sequences (ORF2 region, 264 nucleotide for GI and 252 nucleotide for GII) were inferred by the maximum likelihood analysis based on the Tamura-Nei model (Tamura and Nei 1993) using the MEGA 6 software (Tamura et al. 2013). Reference strains were retrieved from the GenBank sequence database deposited at NCBI (Vinjé 2015, Zheng et al. 2006).

3.3. Data analysis

The logarithmic removal was calculated by subtracting logarithmic concentrations of variables in effluent from influent according to Francy et al. (2012). Significant differences among mean ranks and multiple comparisons were evaluated using Kruskal Wallis test at $\alpha = 0.05$. Univariate logistic regression model was used to evaluate the contribution of each microbial group to the occurrence of others. Pearson product-moment correlation coefficient (r) was used to assess the correlation among variables. Software RStudio (version 0.98.1091, RStudio Inc., Boston, MA) was used for the statistical analyses and visualization. Local minimum and maximum weather temperature data were obtained from the National Oceanic and Atmospheric Administration's National Climatic Data Center (NOAA-NCDC), Carrollton

station (latitude: 29.934º, longitude -90.136º), and reported as midrange values. A threshold of 21 °C was considered to categorize the data into warm months (June-May) and cold months (November-April). Seasons were classified as spring (March-May), summer (June-August), fall (September-November) and winter (December-February).

3.4. Results

3.4.1. PCR performance

Analysis of serially diluted standard RNA showed RT-qPCR efficiencies of 87.22%, 96.25% and 95.29% for NoV GI, GII and EV, respectively; the coefficients of determination (R^2) ranged from 0.986 to 0.992 (Figures 8-10, Table 2). Promising results were obtained from the end-point hemi-nested PCR. Even though, in some cases, the RT-PCR did not yield bright or visible bands, the agarose gel purification of the amplicons yielded 100% positive reactions in the second PCR (Appendix 2-3).

3.4.2. Pathogenic enteric viruses and microbial indicators

Monthly composite influent and effluent waters were obtained when no flooding or heavy rain had occurred within 24 h prior to each sample collections. Figures 13 and 14 represents the weather temperature, and BOD, TSS and VSS content of wastewater samples, respectively. Enteric viruses were observed nearly all year round (Figure 15). Overall, 83.3% of the influent samples (10/12) and 33.3% of the effluent samples (4/12) were positive for NoV GI, whereas NoV GII and EV were more prevalent and detected similarly in 100% (12/12) of the influent and 83.3% (10/12) of the effluent samples. The concentration of NoV GII was greater than NoV GI and EV in both influent and effluent waters ($p < 0.05$). Fecal coliforms and *E. coli* and MSC were detected in all the influent and effluent samples while enterococci was observed in 100%

(12/12) of the influent and 66.7% (8/12) of the effluent waters, all with a slight fluctuation over time (Table 6, Figure 16).

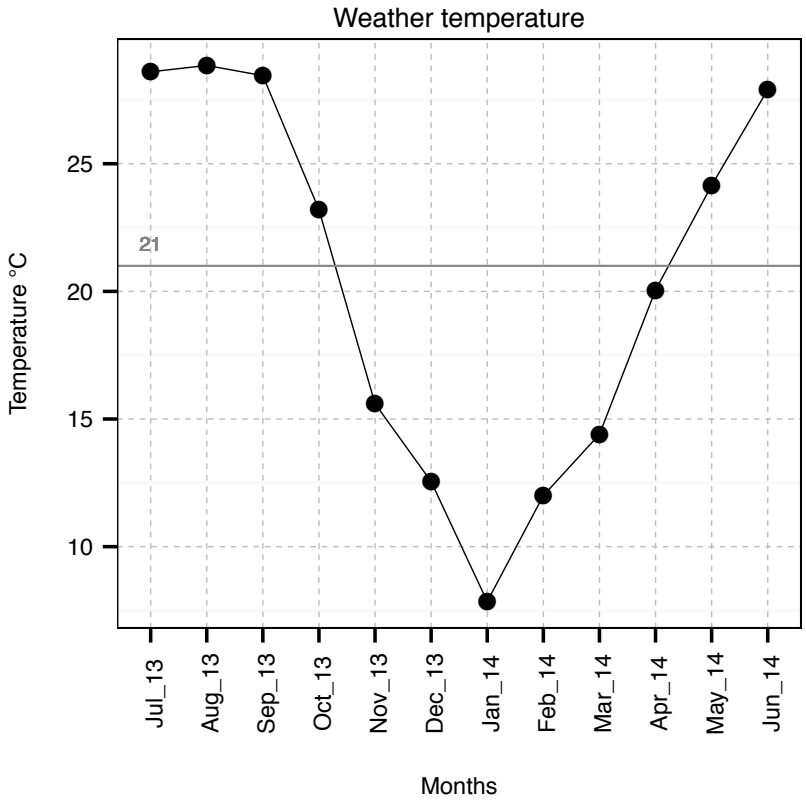

Figure 13. Mid-range weather temperature (ºC) in New Orleans (LA) for each sample collection day

3.4.3. Trends and seasonality

Among the indicator bacteria, only *E. coli* densities in influent was affected by seasonality ($p = 0.016$), reaching the highest concentrations in summer and fall (averaged 6.2 ± 0.0 $\log_{10}$ CFU/100mL), with higher loads in warmer months (averaged 6.2 ± 0.0 $\log_{10}$ CFU/100mL) than cold months (averaged 5.9 ± 0.1 $\log_{10}$ CFU/100mL, $p = 0.03$). Stronger fluctuations were observed in the densities of pathogenic enteric viruses (NoV GI, NoV GII and EV) with distinct trend over time in NoV GII and EV (Figure 15). Similar to the MSC, the concentration of NoV GII and EV in the influent increased up to April (cold months), which

resulted a sharp increase in seasonal concentrations during spring (March-May), with the means of 4.7 ± 0.1 $\log_{10}$ PFU/100mL, 5.9 ± 0.2 $\log_{10}$ GC/100mL and 1.7 ± 0.2 $\log_{10}$ RT-qPCR unit/100mL for MSC, NoV GII and EV, respectively. Similarly, EV showed another increase in fall with an average value of 1.8 ± 0.1 $\log_{10}$ RT-qPCR unit/100mL.

Only in the case of NoV GI and GII, the monthly changes in the influent could reflect their concentrations in influent (r = 0.41 and 045, respectively, $p < 0.05$). Among the pathogenic enteric viruses, NoV GI and EV densities in the influent and GII in the effluent were correlated with the weather temperature (r = -0.56, 0.42 and -0.59, respectively, $p < 0.05$). The strongest correlations ($r > 0.45$) between enteric pathogens and microbial indicators were found between NoV GII and MSC both in the influent and effluent (r = 0.48 and 0.76, respectively, $p < 0.05$). In addition, univariate logistic regression analyses indicated the occurrence of NoV GII on the level of MSC in the effluent (odds ratio (OR) = 1.45, 97.5% confidence interval (CI) = 1.06 to 1.98, p = 0.030). EV was correlated with NoV GII in the influent (r = 0.45); however the presence or absence of EV was unrelated to the NoV GII densities ($p > 0.05$). Among the indicator bacteria, enterococci showed the strongest correlation with the fecal coliforms ($r >$ 0.63) and *E. coli* ($r > 0.77$), followed by fecal coliforms and *E. coli* ($r > 0.55$) in both influent and effluent waters. In this regard, the presence or absence of enterococci in the effluent was in accordance with the concentration of *E. coli* (OR = 1.48, CI = 1.22 to 1.79, p = 0.0007), and coliforms (OR = 1.41, CI = 1.19 to 1.67, p = 0.0006).

3.4.4. Microbial removal

Secondary treatment of wastewater resulted in significant removal in all microbial groups. The removal was more pronounced in indicator bacteria (4.36 $\log_{10}$, $p > 0.05$), followed by NoV GII, EV and MSC (1.48 $\log_{10}$, $p > 0.05$) and then NoV GI (0.95 $\log_{10}$). In general, log

removals in indicator bacteria and NoV GI were similar during warm versus cold months; however, for the rest of the microbial groups higher removal was observed in warm months. Close similarity in monthly log removal was found between MSC and NoV GII (r = 0.72), and between MSC and EV (r = 0.73). Among all the microbial groups, microbial removal in NoV GII and EV were correlated with weather temperature (r = 0.70 and 0.57, respectively). In general, log removal in the indicator bacteria did not have strong seasonality ($p > 0.05$), whereas it was higher in MSC during fall and summer (1.65 $\log_{10}$), NoV GI during fall and winter (1.17 $\log_{10}$), and in NoV GII in summer and fall (2.32 $\log_{10}$), and EV in summer (1.74 $\log_{10}$).

3.4.5. Genetic diversity of noroviruses

Seven norovirus genotypes were identified from a total of 72 clones (Figures 17 and 18, Table 7). NoV GI.1 Norwalk was present in all the samples except for the influent water in January, comprising 72.2% (13/18) and 83.3 % (15/18) of the influent and effluent clones, respectively; followed by GI.3 and GI.4 which each comprised 11.1% (4/36) of the GI clones. NoV GII.4 Sydney dominated the NoV GII genotypes, and was identified in 22.2% (4/18) of the influent and 50.0% (9/18) of the effluent clones. The rest of the GII.4 genotypes (30.6%, 11/18) could not be distinguished at a strain level. Other genotypes were GII.3 (19.4%, 7/36), GII.13 (13.9%, 5/36) and GII.21 (2.78%, 1/36).

3.5. Discussion

Due to the inability to grow human NoV in cell culture, RT-qPCR has been used extensively for the detection and quantification of NoV in environmental matrices and clinical specimens, particularly where the virus densities are usually low (Fong and Lipp 2005, Knight et

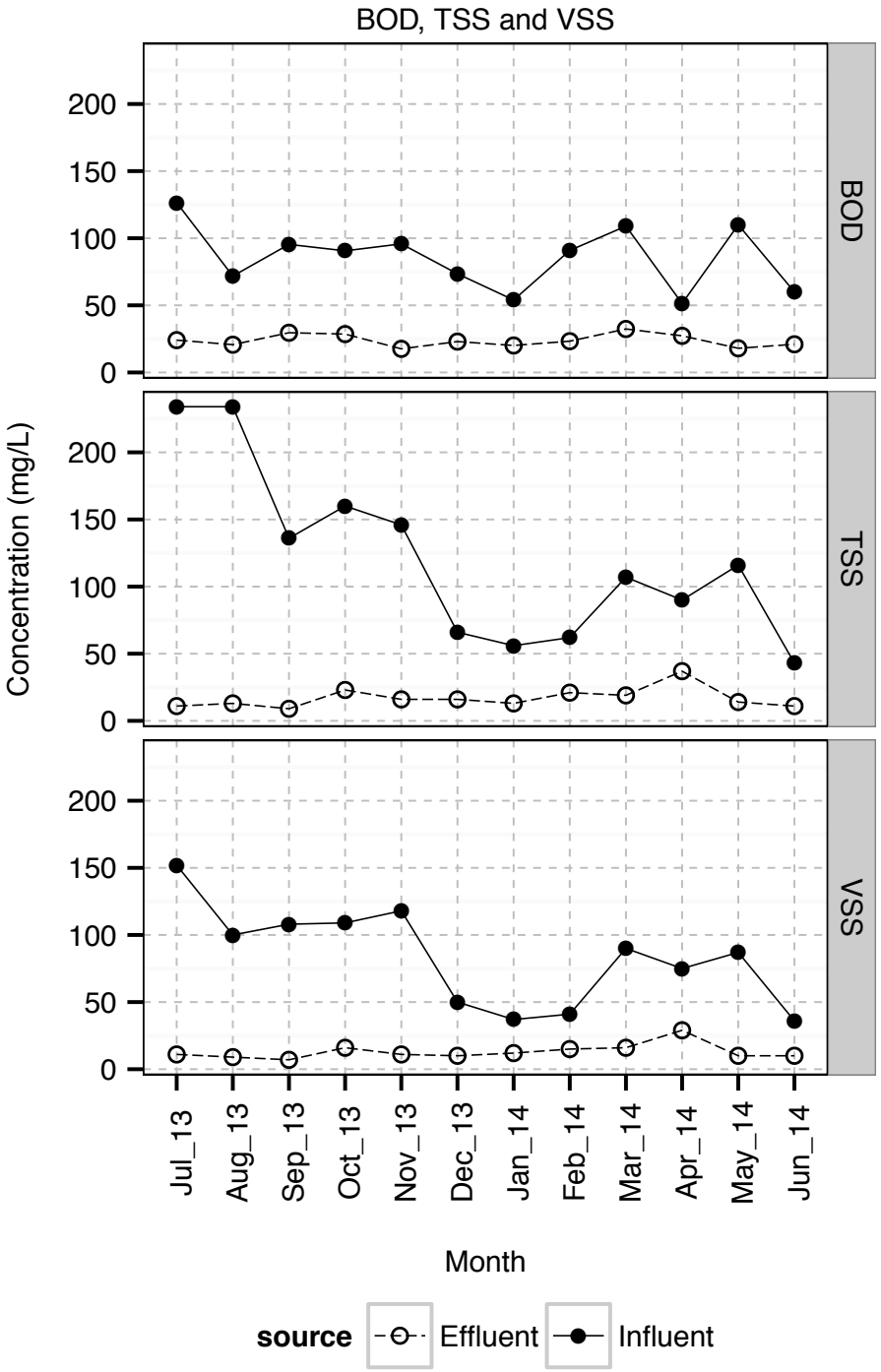

Figure 14. Concentrations of biochemical oxygen demand (BOD), total suspended solids (TSS) and volatile suspended solids (VSS) in the wastewater influent and effluent waters.

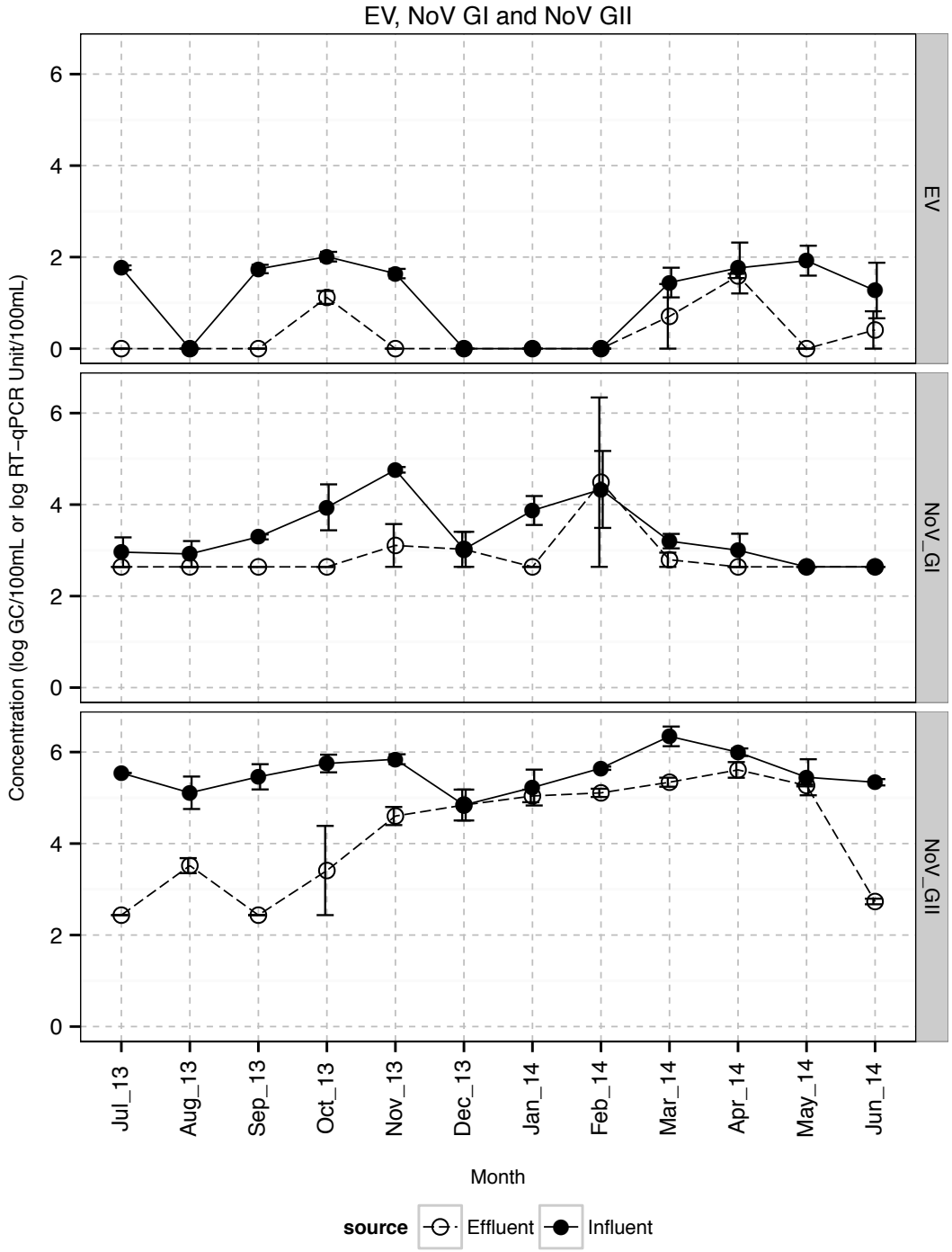

Figure 15. Concentrations of NoV GI, GII and EV in the wastewater influent and effluent (mean ± SE).

Table 6. Concentrations of indicator bacteria in the influent and effluent of the wastewater treatment plant over the sampling month*

	Fecal coliforms		*E. coli*		Enterococci	
Month, year	Influent	Effluent	Influent	Effluent	Influent	Effluent
July, 2013	5.58 ± 0.13^{e}	2.62 ± 0.38^{ab}	6.22 ± 0.08^{a}	2.50 ± 0.00^{b}	4.99 ± 0.18^{abc}	0.17 ± 0.12^{a}
August, 2013	6.04 ± 0.07^{abcd}	1.81 ± 0.34^{cdf}	6.17 ± 0.05^{ab}	1.67 ± 0.07^{d}	5.22 ± 0.19^{a}	0.60 ± 0.30^{b}
September, 2013	6.25 ± 0.22^{abc}	1.38 ± 0.35^{defg}	6.22 ± 0.01^{a}	1.52 ± 0.02^{e}	5.08 ± 0.13^{abc}	0.13 ± 0.00^{c}
October, 2013	6.14 ± 0.32^{abcd}	1.90 ± 0.03^{bcd}	6.24 ± 0.06^{a}	2.17 ± 0.09^{c}	5.08 ± 0.01^{ab}	0.83 ± 0.02^{ab}
November, 2013	6.27 ± 0.27^{ab}	1.11 ± 0.08^{efg}	6.22 ± 0.02^{a}	1.44 ± 0.13^{e}	5.09 ± 0.16^{abc}	0.13 ± 0.00^{c}
December, 2013	6.06 ± 0.21^{abcde}	3.99 ± 0.08^{a}	6.07 ± 0.26^{ab}	3.47 ± 0.09^{e}	5.37 ± 0.12^{a}	0.92 ± 0.09^{ab}
January, 2014	5.86 ± 0.03^{bcde}	0.54 ± 0.15^{g}	5.78 ± 0.02^{bc}	0.57 ± 0.03^{h}	4.59 ± 0.21^{c}	0.13 ± 0.00^{c}
February, 2014	5.70 ± 0.06^{de}	0.81 ± 0.25^{fg}	5.43 ± 0.17^{c}	1.03 ± 0.03^{gh}	4.59 ± 0.24^{bc}	0.13 ± 0.00^{c}
March, 2014	6.20 ± 0.209^{abc}	2.43 ± 0.14^{ab}	6.13 ± 0.09^{abc}	2.79 ± 0.12^{ab}	5.50 ± 0.50^{a}	0.43 ± 0.03^{b}
April, 2014	5.82 ± 0.06^{cde}	2.19 ± 0.19^{abc}	5.49 ± 0.10^{c}	2.09 ± 0.01^{c}	4.77 ± 0.05^{bc}	0.17 ± 0.04^{c}
May, 2014	6.34 ± 0.15^{ab}	1.55 ± 0.02^{def}	5.94 ± 0.04^{abc}	1.15 ± 0.01^{fg}	4.98 ± 0.03^{abc}	0.13 ± 0.00^{c}
June, 2014	6.41 ± 0.17^{a}	1.83 ± 0.26^{bcd}	6.22 ± 0.03^{a}	1.23 ± 0.06^{f}	5.35 ± 0.04^{a}	0.13 ± 0.00^{c}

* Units: $\log_{10}$ CFU/100mL. Note: Within each microbial group and source (influent or effluent), means with the same letter are not significantly different. All values are reported as mean ± SE.

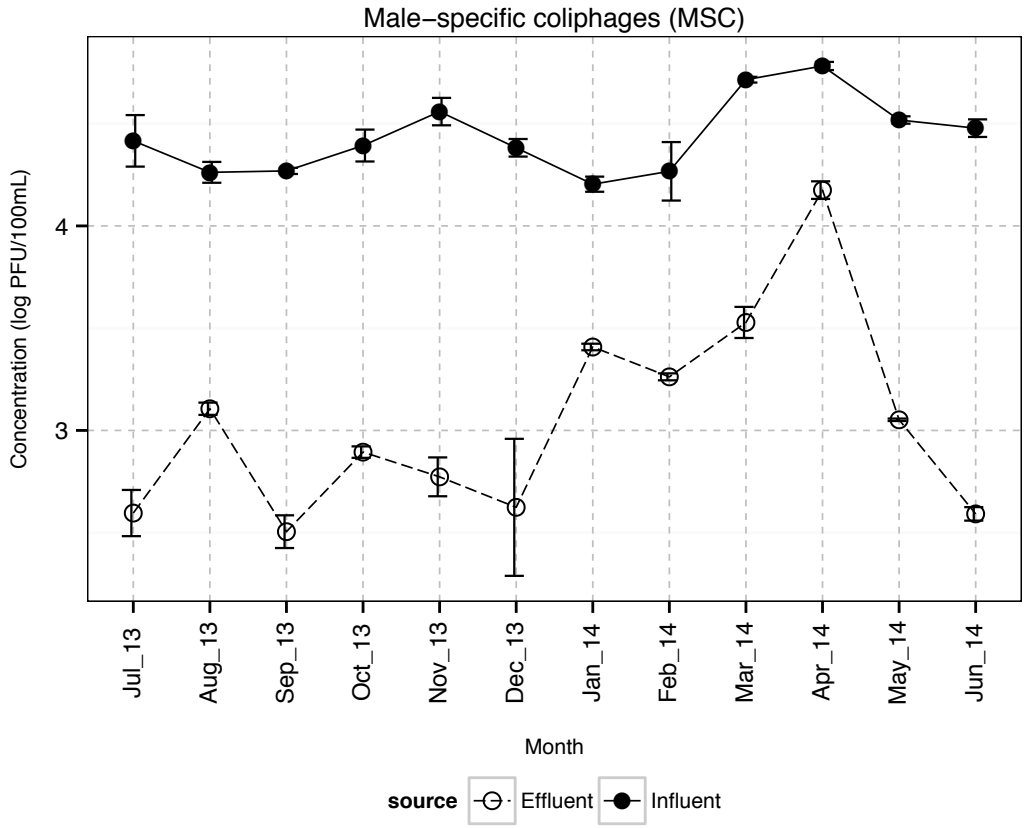

Figure 16. Concentrations of male-specific coliphages (MSC) in the wastewater influent and effluent (mean ± SE).

al. 2013, Pringle et al. 2015, Vinjé 2015). Wastewater contains humic compounds, divalent cation and salts, which may occur in the PCR template, negatively inhibit the amplification efficiency and underestimate quantification or cause false-negative results (Hewitt et al. 2011, Toze 1999). The internal amplification control used in the RT- qPCR reactions was able to monitor any inhibitory effect. In this regard, the virus and RNA extraction protocols were efficient in removing the majority of the inhibitory compounds. The potential effects of the remaining inhibitory substances were mitigated by serial dilution of the RNA template prior to analysis.

The concentration of NoV GII was higher than GI, which is in accordance with previous reports and epidemiological trends in the U.S. and globally (Hall et al. 2014, Hewitt

et al. 2011, Skraber et al. 2011). Epidemiological data in the U.S. and worldwide have shown that NoV outbreaks peak in the colder winter months globally, with 55% of the cases in the U.S. occurring in December-February (Ahmed et al. 2013, Hall et al. 2014), which is in agreement with our observation of high NoV GII densities (5.6 $\log_{10}$ GC/100mL) in the influent in November-March, and previous reports (Katayama et al. 2008, Skraber et al. 2011, Victoria et al. 2010).

The dominant GI genotypes with the order of prevalence were GI.1 Norwalk, GI.3c and GI.4. We did not identify any GI.6; however, it was the dominated GI genotype isolated from forty nine WTTP in Netherland (van den Berg et al. 2005). The GI.1 was the dominated NoV strain in the influent; however, this genotype has been associated with only less than 1% of the outbreaks in the U.S. during 2009-2013, as opposed to GI.6 (49.7%), GI.3 (24.6%) and GI.4 (9.9%) (Vega et al. 2014). It suggests that non-epidemic or sub-clinical gastroenteritis incidences in the community might be contributed to the high prevalence of GI.1 strains that were reflected in the influent samples (Myrmel et al. 2006). Other NoV genotypes that have been reported in WWTP are GI/2, 5-9, 11, 12 & 14 and GII/1, 2, 5-12, 15-17 (Kitajima et al. 2012, Rajko-Nenow et al. 2013).

NoV GII.4 has been largely associated with the gastroenteritis outbreaks in the U.S. and worldwide, and the most frequently identified genotype in foodborne NoV outbreaks (Pringle et al. 2015) and wastewater (Kitajima et al. 2012, Rajko-Nenow et al. 2013, Skraber et al. 2011). According to epidemiological studies, about 94% of all outbreaks in the U.S. during 2009-2013 have been typed as NoV GII.4 New Orleans or GII.4 Sydney. These two strains have been known as emerging recombinants and implicated severe gastroenteritis outbreaks worldwide (Pringle et al. 2015, Vega et al. 2014). The GII.13 and GII.21 that were detected

in our study have been rarely reported, and have been responsible for less than 1% of the NoV outbreaks in the U.S., 2009 to 2013 (Vega et al. 2014). The presence of multiple genotypes in wastewaters may contribute to the emergence of recombinant strains with potentially more severe public health risks (van den Berg et al. 2005, Vega et al. 2014).

In general, the concentrations of EV in the wastewater samples were lower than what Hewitt et al. (2011) reported in multiple WWTP, in which EV exceeded NoV GI and GII concentrations, and reached 1.8-5.7 and 0.5-4.3 $\log_{10}$ GC/100mL in the influent and effluent, respectively. Katayama et al. (2008) found that adenoviruses concentration in the influent of a WWTP in Japan was higher than NoV and EV. The concentration of EV in the influent was within the range as reported by others (Katayama et al. 2008). Epidemiological studies of enterovirus infection cases in the U.S. have shown remarkable seasonality, increasing sharply during summer and fall with a peak in August (Khetsuriani et al. 2006). We observed that the highest concentrations of EV occurred in fall and spring and the lowest concentrations in spring and winter (Figure 15).

High prevalence of enteric pathogens in wastewater throughout the year reflects the circulation of the pathogens in a population. Therefore, even when the rates of epidemiological and clinical cases are low the potential health risks still exist (Cheng et al., 2012, da Silva et al. 2007, Haramoto et al. 2006, Hewitt et al. 2011, Katayama et al. 2008, Myrmel et al. 2006, van den Berg et al. 2005, Victoria et al. 2010). Secondary treatment has shown to be effective in reducing the microbial load but inefficient to completely remove them. In accordance with previous reports, log removal was lower in pathogenic enteric viruses than indicator bacteria (Victoria et al. 2010). Microbial removal in EV was 1.4 $\log_{10}$,

Figure 17. Phylogenetic tree for norovirus GI strains using 264 nucleotides of the partial capsid gene sequences. Reference strains start with GI followed by the GenBank accession numbers (Vinjé 2015, Zheng et al. 2006). Samples start with the months, followed by the source (In: influent/Ef: effluent), replicates (A-C) and the GenBank accession numbers.

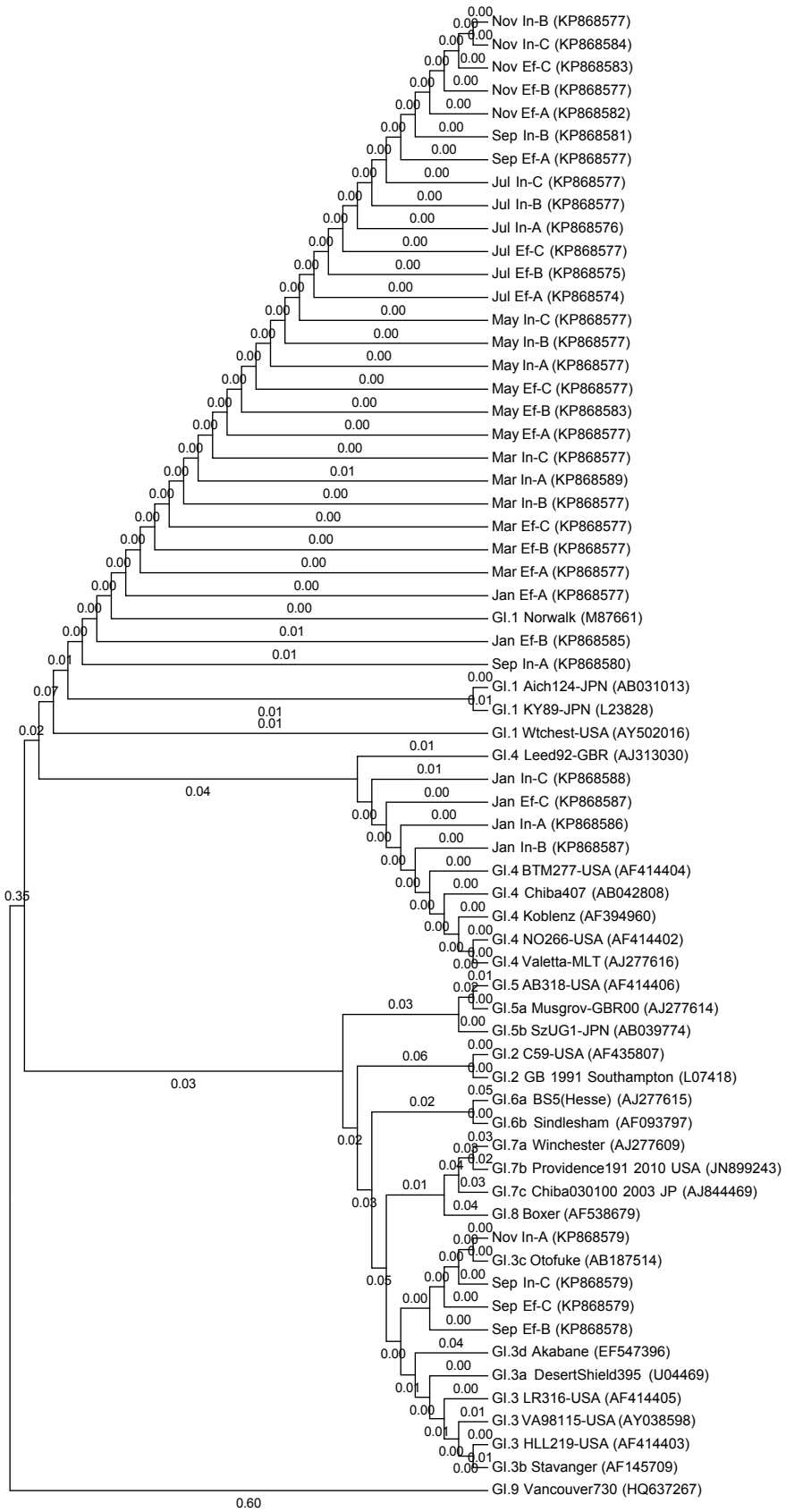
Nov In-B (KP868577)
Nov In-C (KP868584)
Nov Ef-C (KP868583)
Nov Ef-B (KP868577)
Nov Ef-A (KP868582)
Sep In-B (KP868581)
Sep Ef-A (KP868577)
Jul In-C (KP868577)
Jul In-B (KP868577)
Jul In-A (KP868576)
Jul Ef-C (KP868577)
Jul Ef-B (KP868575)
Jul Ef-A (KP868574)
May In-C (KP868577)
May In-B (KP868577)
May In-A (KP868577)
May Ef-C (KP868577)
May Ef-B (KP868583)
May Ef-A (KP868577)
Mar In-C (KP868577)
Mar In-A (KP868589)
Mar In-B (KP868577)
Mar Ef-C (KP868577)
Mar Ef-B (KP868577)
Mar Ef-A (KP868577)
Jan Ef-A (KP868577)
GI.1 Norwalk (M87661)
Jan Ef-B (KP868585)
Sep In-A (KP868580)
GI.1 Aich124-JPN (AB031013)
GI.1 KY89-JPN (L23828)
GI.1 Wtchest-USA (AY502016)
GI.4 Leed92-GBR (AJ313030)
Jan In-C (KP868588)
Jan Ef-C (KP868587)
Jan In-A (KP868586)
Jan In-B (KP868587)
GI.4 BTM277-USA (AF414404)
GI.4 Chiba407 (AB042808)
GI.4 Koblenz (AF394960)
GI.4 NO266-USA (AF414402)
GI.4 Valetta-MLT (AJ277616)
GI.5 AB318-USA (AF414406)
GI.5a Musgrov-GBR00 (AJ277614)
GI.5b SzUG1-JPN (AB039774)
GI.2 C59-USA (AF435807)
GI.2 GB 1991 Southampton (L07418)
GI.6a BS5(Hesse) (AJ277615)
GI.6b Sindlesham (AF093797)
GI.7a Winchester (AJ277609)
GI.7b Providence191 2010 USA (JN899243)
GI.7c Chiba030100 2003 JP (AJ844469)
GI.8 Boxer (AF538679)
Nov In-A (KP868579)
GI.3c Otofuke (AB187514)
Sep In-C (KP868579)
Sep Ef-C (KP868579)
Sep Ef-B (KP868578)
GI.3d Akabane (EF547396)
GI.3a DesertShield395 (U04469)
GI.3 LR316-USA (AF414405)
GI.3 VA98115-USA (AY038598)
GI.3 HLL219-USA (AF414403)
GI.3b Stavanger (AF145709)
GI.9 Vancouver730 (HQ637267)

Figure 18. Phylogenetic tree for norovirus GII strains using 252 nucleotides of the partial capsid gene sequences. Reference strains start with GII followed by the GenBank accession numbers (Vinjé 2015, Zheng et al. 2006). Samples start with the months, followed by the source (In: influent/Ef: effluent), replicates (A-C) and the GenBank accession numbers.

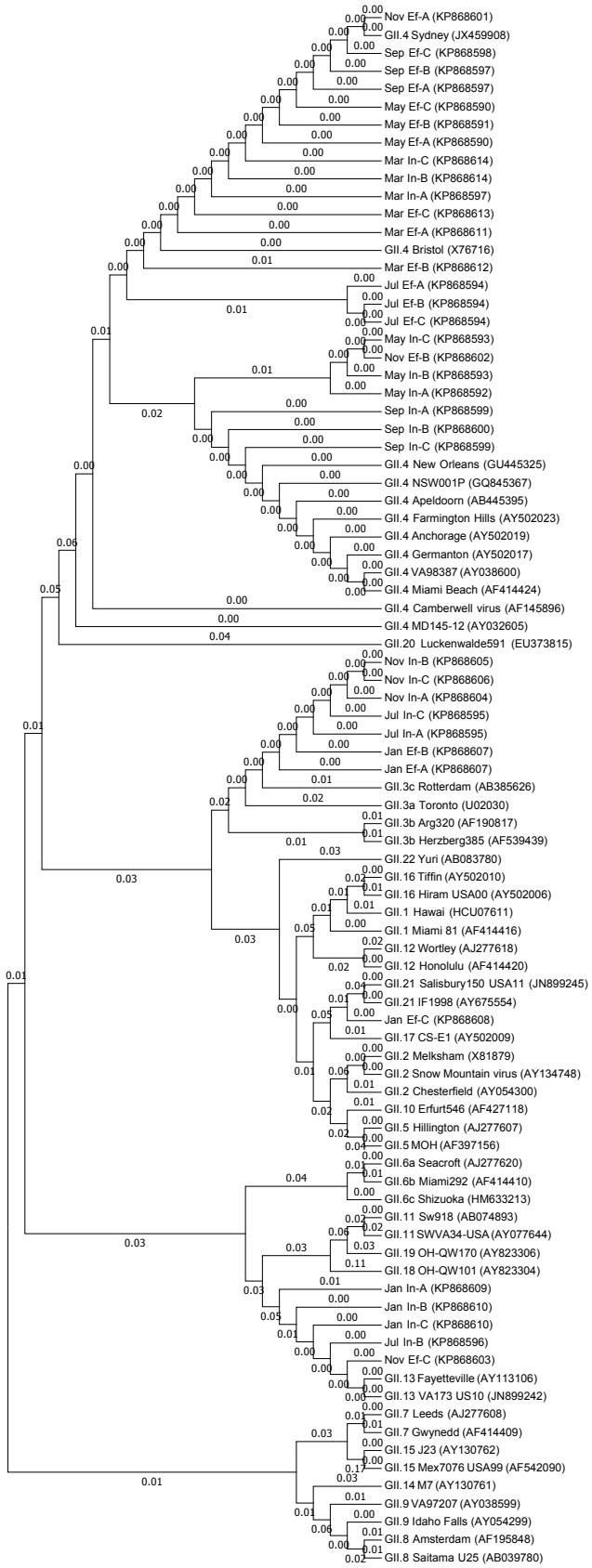
Nov Ef-A (KP868601)
GII.4 Sydney (JX459908)
Sep Ef-C (KP868598)
Sep Ef-B (KP868597)
Sep Ef-A (KP868597)
May Ef-C (KP868590)
May Ef-B (KP868591)
May Ef-A (KP868590)
Mar In-C (KP868614)
Mar In-B (KP868614)
Mar In-A (KP868597)
Mar Ef-C (KP868613)
Mar Ef-A (KP868611)
GII.4 Bristol (X76716)
Mar Ef-B (KP868612)
Jul Ef-A (KP868594)
Jul Ef-B (KP868594)
Jul Ef-C (KP868594)
May In-C (KP868593)
Nov Ef-B (KP868602)
May In-B (KP868593)
May In-A (KP868592)
Sep In-A (KP868599)
Sep In-B (KP868600)
Sep In-C (KP868599)
GII.4 New Orleans (GU445325)
GII.4 NSW001P (GQ845367)
GII.4 Apeldoorn (AB445395)
GII.4 Farmington Hills (AY502023)
GII.4 Anchorage (AY502019)
GII.4 Germanton (AY502017)
GII.4 VA98387 (AY038600)
GII.4 Miami Beach (AF414424)
GII.4 Camberwell virus (AF145896)
GII.4 MD145-12 (AY032605)
GII.20 Luckenwalde591 (EU373815)
Nov In-B (KP868605)
Nov In-C (KP868606)
Nov In-A (KP868604)
Jul In-C (KP868595)
Jul In-A (KP868595)
Jan Ef-B (KP868607)
Jan Ef-A (KP868607)
GII.3c Rotterdam (AB385626)
GII.3a Toronto (U02030)
GII.3b Arg320 (AF190817)
GII.3b Herzberg385 (AF539439)
GII.22 Yuri (AB083780)
GII.16 Tiffin (AY502010)
GII.16 Hiram USA00 (AY502006)
GII.1 Hawai (HCU07611)
GII.1 Miami 81 (AF414416)
GII.12 Wortley (AJ277618)
GII.12 Honolulu (AF414420)
GII.21 Salisbury150 USA11 (JN899245)
GII.21 IF1998 (AY675554)
Jan Ef-C (KP868608)
GII.17 CS-E1 (AY502009)
GII.2 Melksham (X81879)
GII.2 Snow Mountain virus (AY134748)
GII.2 Chesterfield (AY054300)
GII.10 Erfurt546 (AF427118)
GII.5 Hillington (AJ277607)
GII.5 MOH (AF397156)
GII.6a Seacroft (AJ277620)
GII.6b Miami292 (AF414410)
GII.6c Shizuoka (HM633213)
GII.11 Sw918 (AB074893)
GII.11 SWVA34-USA (AY077644)
GII.19 OH-QW170 (AY823306)
GII.18 OH-QW101 (AY823304)
Jan In-A (KP868609)
Jan In-B (KP868610)
Jan In-C (KP868610)
Jul In-B (KP868596)
Nov Ef-C (KP868603)
GII.13 Fayetteville (AY113106)
GII.13 VA173 US10 (JN899242)
GII.7 Leeds (AJ277608)
GII.7 Gwynedd (AF414409)
GII.15 J23 (AY130762)
GII.15 Mex7076 USA99 (AF542090)
GII.14 M7 (AY130761)
GII.9 VA97207 (AY038599)
GII.9 Idaho Falls (AY054299)
GII.8 Amsterdam (AF195848)
GII.8 Saitama U25 (AB039780)

Table 7. Summary of the dominant Norovirus GI and GII strains identified in the influent and effluent of wastewater.

Month	Source	NoV GI	NoV GII
July, 2013	Influent	GI.1 Norwalk (3/3)	GII.3c Rotterdam (2/3), GII.13
	Effluent	GI.1 Norwalk (3/3)	GII.4 (3/3)
September, 2013	Influent	GI.1 Norwalk (2/3), GI.3c	GII.4 (3/3)
	Effluent	GI.1 Norwalk (1/3), GI.3c	GII.4 Sydney (3/3)
November, 2013	Influent	GI.1 Norwalk (2/3), GI.3c	GII.3c Rotterdam (3/3)
	Effluent	GI.1 Norwalk (3/3)	GII.4 Sydney (1/3), GII.4 (1/3), GII.13 (1/3)
January, 2014	Influent	GI.4 (3/3)	GII.13 (3/3)
	Effluent	GI.1 Norwalk (2/3), GI.4 (1/3)	GII.3c Rotterdam (2/3), GII.21 (1/3)
March, 2014	Influent	GI.1 Norwalk (3/3)	GII.4 Sydney (3/3)
	Effluent	GI.1 Norwalk (3/3)	GII.4 Sydney (2/3), GII.4 (1/3)
May, 2014	Influent	GI.1 Norwalk (3/3)	GII.4 (3/3)
	Effluent	GI.1 Norwalk (3/3)	GII.4 Sydney (3/3)

The numbers in the parentheses denote the number of clones were identified.

which is similar to the previous reports (Hewitt et al. 2011). The NoV removal through the treatment was 1.0-1.6 $\log_{10}$ and was lower than 2.7 $\log_{10}$ observed by van den Berg et al. (2005).

In general, higher concentrations of NoV GI and GII viral genome in the influent resulted in a higher concentration in the effluent; however, the amount of virus removal was varied over time and not related to their initial densities except for NoV GI. Fluctuation in treatment efficiency is expected in a WWTP and can affect microbial removal (Cheng et al. 2012, Symonds et al. 2009). Our results (Figure 15) indicate that NoV GI can be approximately 4.8 times more resistant to the secondary treatment and disinfection than NoV GII, which agrees with the previous reports (Cheng et al. 2012, da Silva et al. 2007). Furthermore, MSC removal may indicate the efficiency of secondary treatment on removing NoV GII in WWTP. Male-specific coliphages (along with *E. coli*) has been suggested as a good indicator of virus removal across conventional secondary disinfection process (Francy

et al. 2012). Assuming a similar abundance and survival of NoV and MSC, we could conclude that at least a portion of NoV GII detected in the effluent maintained their infectivity due to simultaneous detection of viable MSC plaques and the concentrations of NoV GII viral genomes, as suggested by Hewitt et al. (2011) who reported the parallel occurrence of cultivable enteric viruses and PCR concentrations. However, similar to fecal coliforms and *E. coli*, not all the coliphage types are human specific. Non-human fecal matters may pollute water but not necessarily pose a health risk due to enteric viruses. Among four genogroups of MSC, human sewage usually contains groups II and III, in which group II has correlated well with human fecal pollution in water (Brion et al. 2002, Havelaar et al. 1986).

A recommended way to monitor viral contamination is the direct detection of pathogens without using indicators (Fong and Lipp 2005). Regarding the high prevalence of some enteric viruses in the influent, direct monitoring of enteric viruses such as adenoviruses, enteroviruses, NoV and preferably picobirnaviruses has been suggested as viral indicators of fecal contamination (Symonds et al. 2009). Whether the detected levels of NoV or EV in the WWTP samples are considered a health hazard is rather complicated because current RT-PCR assays may not reflect virus infectivity (Knight et al. 2013, Mattison et al. 2009). Additionally, a strong correlation between the removal of viral genome (particularly RNA) and loss of infectivity has been reported that could be due to the rapid degradation of RNA in the environment (reviewed by Fong and Lipp 2005). Assuming similar survival and abundance of NoV and enteroviruses, Hewitt et al. (2011) suggested that at least a proportion of NoV detected in the effluents could be infectious due to the simultaneous occurrence of cultivable enteric viruses and PCR concentrations. Therefore, the secondary treated

wastewater can contribute to release of infectious virus particles in the environment and increase the harmful health effects of human exposure to enteric pathogens.

3.6. Nucleotide sequence accession numbers

The NoV sequences identified in our study can be found in GenBank under the following accession numbers: KP868574, KP868575, KP868576, KP868577, KP868578, KP868579, KP868580, KP868581, KP868582, KP868583, KP868584, KP868585, KP868586, KP868587, KP868588, KP868589, KP868590, KP868591, KP868592, KP868593, KP868594, KP868595, KP868596, KP868597, KP868598, KP868599, KP868600, KP868601, KP868602, KP868603, KP868604, KP868605, KP868606, KP868607, KP868608, KP868609, KP868610, KP868611, KP868612, KP868613 and KP868614.

3.7. Conclusions

According to our monthly surveillance of a municipal WWTP (July 2013-June 2014, New Orleans, Louisiana) we found that the occurrences of pathogenic enteric viruses in wastewater can reflect the circulation of the pathogens in a population but may not follow the epidemiological and clinical trends. Viruses were more resistant than bacteria to the secondary treatment of wastewater; however, the health risks associated with survived infectious virions could not be confirmed. In general, NoV GII were more abundant than NoV GI and EV. Male-specific coliphages may be more reliable than bacterial indicators for monitoring NoV GII load in the wastewater and treatment removal success. This relationship indicates the likelihood of infectious NoV GII occurrence in the effluent water. Therefore,

direct monitoring of infections pathogens are still preferred over bacterial indicators particularly when the effluent water to be further utilized for human activities.

3.8. Acknowledgments

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FINAL CONCLUSIONS

This study provides important information regarding the viral safety of Louisiana oysters and harvest waters, and the viral load in a secondary wastewater treatment plant influent and effluent with regard to noroviruses (NoV) and pathogenic enteroviruses (EV). This information can help regulatory agencies to establish strategies to prevent and control the risks associated with enteric pathogens in sewage-contaminated environments. Identification of multiple NoV genotypes in wastewater influent indicated the presence of underreported strains circulating among the population that can be considered for the development of more effective vaccines and cell culture systems.

There is always a high potential for the presence of infectious enteric viruses in the raw and secondary treated municipal wastewater. Virus removal during the secondary treatment and chlorine disinfection is low, and can vary over time. Therefore, raw and secondary treated human sewage must be handled carefully before discharge. It is crucial to monitor the effluent water for the infectious enteric pathogens if to be utilized for human activities. Discharge of human sewage into the water can cause contamination of molluscan shellfish with enteric viruses. NoV can also occur in the oysters where the fecal pollution both in oysters and harvest waters are low from the microbial fecal indicators standpoints. These observations provide further evidence to emphasize the necessity of direct monitoring of NoV in oysters to reduce the burden of foodborne diseases.

APPENDIX 1. NUCLEOTIDE BLAST OF RT-QPCR NOV POSITIVE SAMPLES (EXCERPTED)

BLAST ®

Basic Local Alignment Search Tool

NCBI/ BLAST/ blastn suite/ **Formatting Results - 746K1FX1015**

- Formatting options
- Download

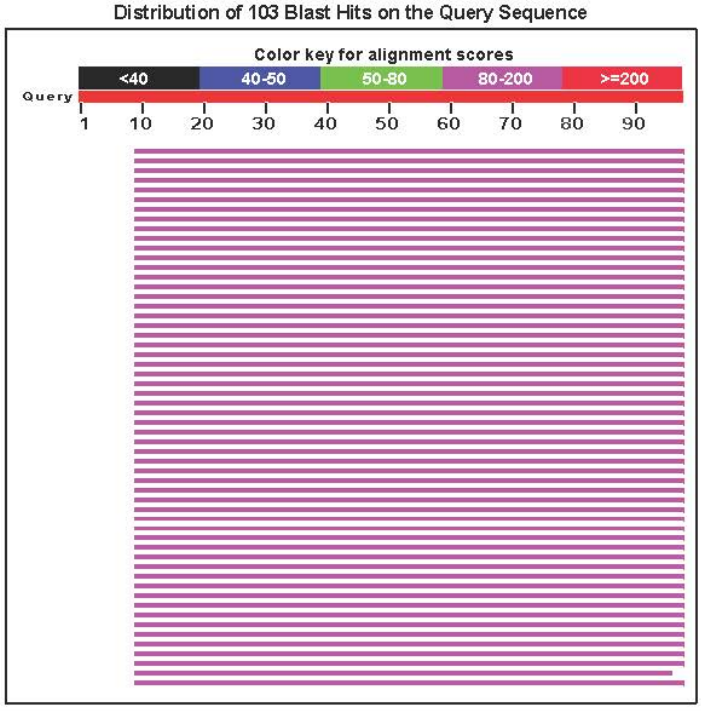
Blast report description

Nucleotide Sequence (98 letters)

RID	746K1FX1015 (Expires on 11-25 00:33 am)		
Query ID	lcl|45293	**Database Name**	nr
Description	None	**Description**	Nucleotide collection (nt)
Molecule type	nucleic acid	**Program**	BLASTN 2.2.30+
Query Length	98		

Graphic Summary

Distribution of 103 Blast Hits on the Query Sequence



Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Norovirus GII isolate 33 RNA-dependent RNA polymerase and capsid protein genes, partial cds	175	175	90%	1e-40	98%	KM044166.1
Norovirus GII isolate 13 RNA-dependent RNA polymerase and capsid protein genes, partial cds	175	175	90%	1e-40	98%	KM044122.1
Norovirus Hu/G2.2/SW2011039-1 /Nanning/2011/CHN RdRp and capsid genes, partial cds	175	175	90%	1e-40	98%	KM246928.1
Norovirus Hu/G2.2/SW2011006-3 /Nanning/2011/CHN RdRp and capsid genes, partial cds	175	175	90%	1e-40	98%	KM246918.1
Norovirus GII/Hu/JP/2002/GII.P16_GII.17 /Saitama/T87, complete genome	175	212	90%	1e-40	98%	KJ196286.1
Norovirus Hu/GII.2/CGMH47/2011/TW ORF1 gene, partial cds; and VP1 and VP2 genes, complete cds	175	175	90%	1e-40	98%	KC464505.1
Norovirus Hu/GII/IDH3744/2010/IND genes for RNA dependent RNA polymerase, capsid protein, partial cds	175	175	90%	1e-40	98%	AB757793.1
Norovirus Hu/GII/IDH3682/2010/IND genes for RNA dependent RNA polymerase, capsid protein, partial cds	175	175	90%	1e-40	98%	AB757792.1
Norovirus Hu/4CAU38/Seoul/South Korea/2012 RNA-dependent RNA polymerase and capsid protein genes, partial cds	175	175	90%	1e-40	98%	KC110857.1
Norovirus Hu/GILL70/Seoul/South Korea/2012 RNA-dependent RNA polymerase and capsid protein genes, partial cds	175	175	90%	1e-40	98%	KC110856.1
Norovirus Hu/GII.16-GII.2/SG4017-02 /2011/SG RNA-dependent RNA polymerase and major capsid protein genes, partial cds	175	175	90%	1e-40	98%	JX480623.1
Norovirus Hu/Wuhan/DHGX-2/CHN/2010 RNA-dependent RNA polymerase and capsid protein genes, partial cds	175	175	90%	1e-40	98%	JQ751041.1
Norovirus Hu/Wuhan/E2120/CHN/2010 RNA-dependent RNA polymerase and capsid protein genes, partial cds	175	175	90%	1e-40	98%	JQ751040.1
Norovirus Hu/Wuhan/E2116/CHN/2010 RNA-dependent RNA polymerase and capsid protein genes, partial cds	175	175	90%	1e-40	98%	JQ751039.1
Norovirus Hu/GII/L53/Beijing/2011 RNA-dependent RNA polymerase and capsid protein genes, partial cds	175	175	90%	1e-40	98%	JQ889817.1
Norovirus Hu/GII.2/OH10026/2010/JP genes for RNA polymerase, VP1, capsid protein, complete and partial cds	175	175	90%	1e-40	98%	AB662900.1
Norovirus Hu/GII.2/OH10025/2010/JP genes for RNA polymerase, VP1, capsid protein, complete and partial cds	175	175	90%	1e-40	98%	AB662899.1

Description	Max score	Total score	Query cover	E value	Ident	Accession
Norovirus Hu/GII.2/OH10021/2010/JP genes for RNA polymerase, VP1, capsid protein, complete and partial cds	175	175	90%	1e-40	98%	AB662897.1
Norovirus Hu/GII.2/OH10020/2010/JP genes for RNA polymerase, VP1, capsid protein, complete and partial cds	175	175	90%	1e-40	98%	AB662896.1
Norovirus Hu/GII.2/OH10015-2/2010/JP genes for RNA polymerase, VP1, capsid protein, complete and partial cds	175	175	90%	1e-40	98%	AB662895.1
Norovirus Hu/GII.2/OH10013/2010/JP genes for RNA polymerase, VP1, capsid protein, complete and partial cds	175	175	90%	1e-40	98%	AB662894.1
Norovirus Hu/GII.2/OH10012/2010/JP genes for RNA polymerase, VP1, capsid protein, complete and partial cds	175	175	90%	1e-40	98%	AB662893.1
Norovirus Hu/GII.2/OH10006/2010/JP genes for RNA polymerase, VP1, capsid protein, complete and partial cds	175	175	90%	1e-40	98%	AB662889.1
Norovirus Hu/GII.2/OH09032/2009/JP genes for RNA polymerase, VP1, capsid protein, complete and partial cds	175	175	90%	1e-40	98%	AB662884.1
Norovirus Hu/GII.2/OH09028/2009/JP genes for RNA polymerase, VP1, capsid protein, complete and partial cds	175	175	90%	1e-40	98%	AB662881.1
Norovirus Hu/GII.2/OC10058/2010/JP genes for RNA polymerase, VP1, capsid protein, complete and partial cds	175	175	90%	1e-40	98%	AB662880.1
Norovirus Hu/GII.2/OC10012-2/2010/JP genes for RNA polymerase, VP1, capsid protein, complete and partial cds	175	175	90%	1e-40	98%	AB662876.1
Norovirus Hu/GII.2/OC09072/2009/JP genes for RNA polymerase, VP1, capsid protein, complete and partial cds	175	175	90%	1e-40	98%	AB662870.1
Norovirus Hu/GII.2/OH08020/2008/JP genes for RNA polymerase, VP1, capsid protein, complete and partial cds	175	175	90%	1e-40	98%	AB662868.1
Norovirus Hu/GII.2/OC08154/2008/JP genes for RNA polymerase, VP1, capsid protein, complete and partial cds	175	175	90%	1e-40	98%	AB662861.1
Norovirus Hu/GII.4/Beijing/55171 /2008/CHN RNA-dependent RNA polymerase gene, partial cds; and capsid and minor structural protein genes, complete cds	175	175	90%	1e-40	98%	GQ856476.1
Norovirus Hu/GII/2005/8162/St. Petersburg/RUS RNA-dependent RNA polymerase and capsid genes, partial cds	175	175	90%	1e-40	98%	FJ383877.1
Norovirus Hu/GII/2005/7821/Odessa/RUS RNA-dependent RNA polymerase and capsid genes, partial cds	175	175	90%	1e-40	98%	FJ383876.1
Norovirus Hu/NLV/VannesL23 /1999/France RNA-dependent RNA polymerase and capsid protein genes, partial cds	175	175	90%	1e-40	98%	AY682551.1
Norovirus Hu/NLV/GII/Neustrelitz260 /2000/DE from Germany, complete genome	175	212	90%	1e-40	98%	AY772730.1

Description	Max score	Total score	Query cover	E value	Ident	Accession
Norovirus Hu/GII/IDH1801/2009/IND genes for RNA-dependent RNA polymerase, capsid protein, partial cds	166	166	90%	4e-38	97%	AB592964.1
Norovirus Hu/GII/IDH1521/2009/IND genes for RNA-dependent RNA polymerase, capsid protein, partial cds	166	166	90%	4e-38	97%	AB592962.1

Alignments

Norovirus GII isolate 33 RNA-dependent RNA polymerase and capsid protein genes, partial cds
Sequence ID: **gb|KM044166.1|** Length: 988 Number of Matches: 1
Range 1: 675 to 763

Score	Expect	Identities	Gaps	Strand	Frame
175 bits(85)	1e-40()	87/89(98%)	0/89(0%)	Plus/Plus	

Features:

```
Query  10   ATGTTYAGRTGGATGAGGTTTTCTGACTTGAGCACGTGGGAGGGCGATCGCAATCTGGCT  69
            ||||| || ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  675  ATGTTCAGATGGATGAGGTTTTCTGACTTGAGCACGTGGGAGGGCGATCGCAATCTGGCT  734

Query  70   CCCAGTTTTGTGAATGAAGATGGCGTCGA  98
            ||||||||||||||||||||||||||||
Sbjct  735  CCCAGTTTTGTGAATGAAGATGGCGTCGA  763
```

Norovirus GII isolate 13 RNA-dependent RNA polymerase and capsid protein genes, partial cds
Sequence ID: **gb|KM044122.1|** Length: 988 Number of Matches: 1
Range 1: 675 to 763

Score	Expect	Identities	Gaps	Strand	Frame
175 bits(85)	1e-40()	87/89(98%)	0/89(0%)	Plus/Plus	

Features:

```
Query  10   ATGTTYAGRTGGATGAGGTTTTCTGACTTGAGCACGTGGGAGGGCGATCGCAATCTGGCT  69
            ||||| || ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  675  ATGTTCAGATGGATGAGGTTTTCTGACTTGAGCACGTGGGAGGGCGATCGCAATCTGGCT  734

Query  70   CCCAGTTTTGTGAATGAAGATGGCGTCGA  98
            ||||||||||||||||||||||||||||
Sbjct  735  CCCAGTTTTGTGAATGAAGATGGCGTCGA  763
```

Norovirus Hu/G2.2/SW2011039-1/Nanning/2011/CHN RdRp and capsid genes, partial cds
Sequence ID: **gb|KM246928.1|** Length: 386 Number of Matches: 1
Range 1: 10 to 98

Score	Expect	Identities	Gaps	Strand	Frame
175 bits(85)	1e-40()	87/89(98%)	0/89(0%)	Plus/Plus	

Features:

```
Query  10   ATGTTYAGRTGGATGAGGTTTTCTGACTTGAGCACGTGGGAGGGCGATCGCAATCTGGCT  69
            ||||| || ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10   ATGTTTAGGTGGATGAGGTTTTCTGACTTGAGCACGTGGGAGGGCGATCGCAATCTGGCT  69

Query  70   CCCAGTTTTGTGAATGAAGATGGCGTCGA  98
            ||||||||||||||||||||||||||||
Sbjct  70   CCCAGTTTTGTGAATGAAGATGGCGTCGA  98
```

Norovirus Hu/G2.2/SW2011006-3/Nanning/2011/CHN RdRp and capsid genes, partial cds
Sequence ID: **gb|KM246918.1|** Length: 388 Number of Matches: 1

Range 1: 10 to 98

Score	Expect	Identities	Gaps	Strand	Frame
175 bits(85)	1e-40()	87/89(98%)	0/89(0%)	Plus/Plus	

Features:

```
Query  10  ATGTTYAGRTGGATGAGGTTTTCTGACTTGAGCACGTGGGAGGGCGATCGCAATCTGGCT  69
           ||||| || |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10  ATGTTTAGGTGGATGAGGTTTTCTGACTTGAGCACGTGGGAGGGCGATCGCAATCTGGCT  69

Query  70  CCCAGTTTTGTGAATGAAGATGGCGTCGA  98
           ||||||||||||||||||||||||||||||
Sbjct  70  CCCAGTTTTGTGAATGAAGATGGCGTCGA  98
```

Norovirus GII/Hu/JP/2002/GII.P16_GII.17/Saitama/T87, complete genome
Sequence ID: **gb|KJ196286.1|** Length: 7535 Number of Matches: 2
Range 1: 5009 to 5097

Score	Expect	Identities	Gaps	Strand	Frame
175 bits(85)	1e-40()	87/89(98%)	0/89(0%)	Plus/Plus	

Features:

```
Query  10    ATGTTYAGRTGGATGAGGTTTTCTGACTTGAGCACGTGGGAGGGCGATCGCAATCTGGCT  69
             ||||| || |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5009  ATGTTCAGGTGGATGAGGTTTTCTGACTTGAGCACGTGGGAGGGCGATCGCAATCTGGCT  5068

Query  70    CCCAGTTTTGTGAATGAAGATGGCGTCGA  98
             ||||||||||||||||||||||||||||||
Sbjct  5069  CCCAGTTTTGTGAATGAAGATGGCGTCGA  5097
```

Range 2: 1 to 18

Score	Expect	Identities	Gaps	Strand	Frame
37.4 bits(18)	35()	18/18(100%)	0/18(0%)	Plus/Plus	

Features:

```
Query  79  GTGAATGAAGATGGCGTC  96
           ||||||||||||||||||
Sbjct  1   GTGAATGAAGATGGCGTC  18
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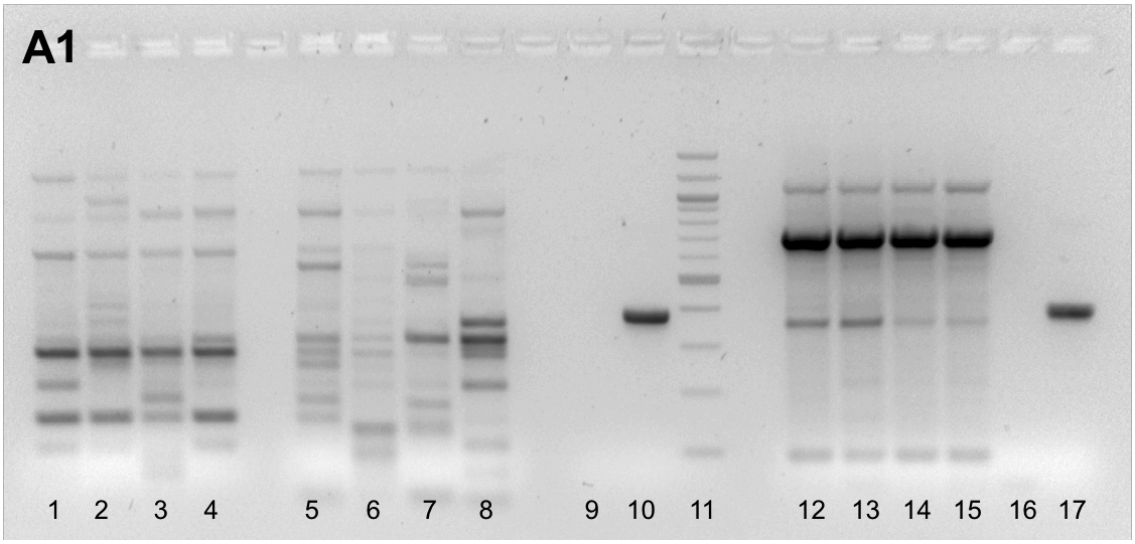
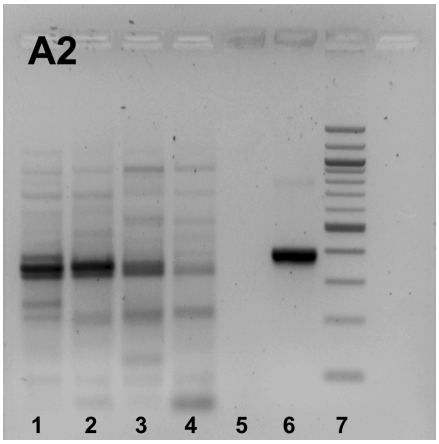
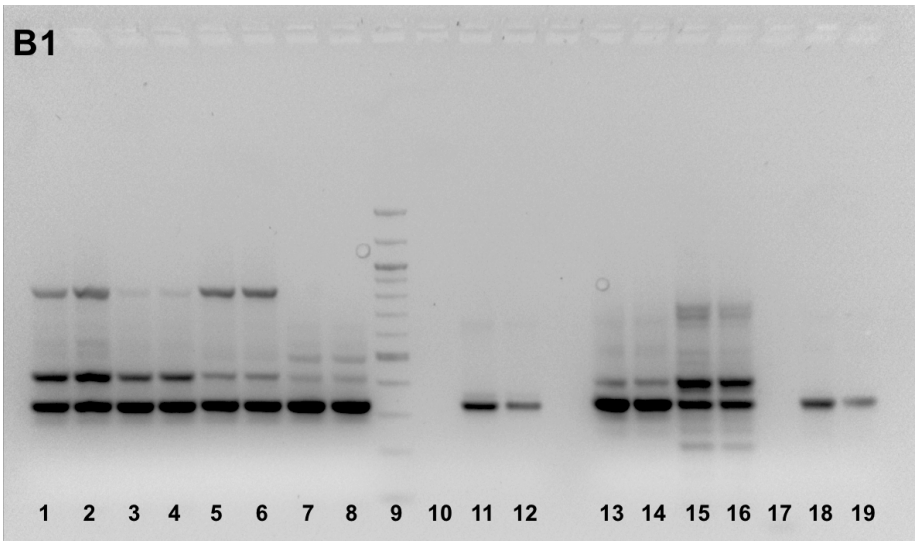
APPENDIX 2. GEL ELECTROPHORESIS OF THE AMPLICONS FROM THE FIRST PCR REACTION

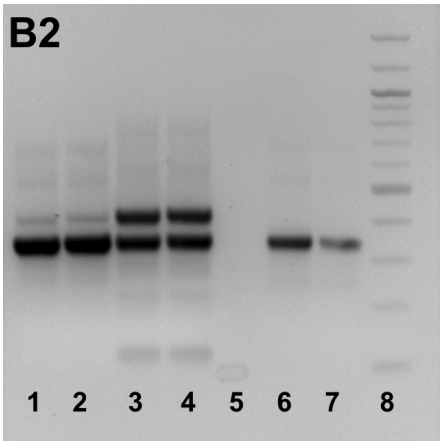




A1: L1-4, NoV GI effluent; L5-8, NoV GI influent; L9, NoV GI negative control, L10, NoV GI positive control, L11, 100 bp DNA ladder; L12-15 NoV GII effluent; L16 NoV GII negative control; L17, NoV GII positive control. **A2**: L1-4, NoV GII influent; L5, NoV GII negative control; L6, NoV GII positive control, L7, 100 bp DNA ladder.

APPENDIX 3. GEL ELECTROPHORESIS OF THE AMPLICONS FROM THE SECOND PCR REACTION





B1: L1-4, NoV GI effluent; L5-8, NoV GI influent; L9, 100 bp DNA ladder, L10, NoV GI negative control; L11-12, NoV GI positive control; L13-16, NoV GII effluent; L17, NoV GII negative control; L18-19, NoV GII positive control. **B2**: L1-4, NoV GII influent; L5, NoV GII negative control; L6-7, NoV GII positive control, L8, 100 bp DNA ladder.

VITA

Naim Montazeri Djouybari, born and raised in Iran, received his bachelor's and masters' degrees in Fisheries at Azad University. In 2009 he moved to the U.S. and obtained a second masters in Seafood Science from the University of Alaska Fairbanks and conducted his research on the safety of cold-smoked salmon. To pursue his passion in food safety, Naim enrolled the Ph.D. program of Food Science at Louisiana State University in 2012, and conducted his research on norovirus in oysters and environmental waters. He is a candidate to receive his doctorate of philosophy in May 2015, and plans to begin his professional career as a research scientist.