Determining the Relationship of Human Enteric Viruses in Clinical, Wastewater, and Environmental Samples Utilizing Molecular and Cell Culture Techniques

Jacquelina Susann Williams Woods

University of Southern Mississippi

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DETERMINING THE RELATIONSHIP OF HUMAN ENTERIC VIRUSES IN CLINICAL, WASTEWATER, AND ENVIRONMENTAL SAMPLES UTILIZING MOLECULAR AND CELL CULTURE TECHNIQUES

by

Jacqueline Susann Woods

Abstract of a Dissertation Submitted to the Graduate School of The University of Southern Mississippi in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

May 2010
ABSTRACT

DETERMINING THE RELATIONSHIP OF HUMAN ENTERIC VIRUSES IN CLINICAL, WASTEWATER, AND ENVIRONMENTAL SAMPLES UTILIZING MOLECULAR AND CELL CULTURE TECHNIQUES

by Jacquelina Susann Woods

May 2010

This study was the first to examine five significant enteric viruses in human fecal material, sewage, and oysters to show a genetic relationship between human enteric viruses and different sample matrices. Fecal samples were collected from an area hospital and examined for norovirus genotype I (NoV GI), norovirus genotype II (NoV GII), hepatitis A virus (HAV), adenovirus (ADV), and enteroviruses. During this study, sewage samples were collected from a Waster Water Treatment Plant (WWTP) in Mobile, AL and oyster sentinels were placed at 0.1 nautical miles (nm) (station 1), 0.2nm (station 2), 1.5nm (station 3), and 4nm (station 4) downstream from the WWTP. Samples were examined by molecular methods for the five virus groups; HuAdv, HAV, and enteroviruses were examined by cell culture methods. Samples positive by molecular methods were further examined by sequencing PCR products of NoV, HuAdv, and enteroviruses. Of the 401 fecal samples analyzed, human NoV, HuAdv, and enterovirus was detected in 4.7%, 13.8%, and 2.5% of samples respectively. HAV was not detected in any fecal, oyster, sewage, or tissues culture samples. HuAdv was detected in the sewage influent and effluent and station 1 in all samples tested during the study.

Enterovirus was detected in 5 out of 7 of influent sample sets and 1 out of 7 of oyster
concentrates. The detection rate for viruses in oysters placed at stations 1 and 2 were similar for all viruses tested including male-specific bacteriophage (MSB). Sequence analysis of NoV GII for the September and December sample set revealed ≥99% sequence homology for stool isolates and oyster isolates at station 2. Sequence analysis for HuAdv for the December samples revealed ≥99% sequence homology between the influent, oyster isolates, and tissue culture isolates. NoV GII and HuAdv were detected at all stations during December sampling utilizing real-time PCR and RT-PCR, respectively. NoV GII was detected at all stations by conventional RT-PCR for the February samples at all stations. This study showed that there is significant genetic relatedness between clinical and environmental isolates.
ACKNOWLEDGEMENTS

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I would like to give a special thanks to my husband, Dr. Kelly Woods, and my children Kristen, Jaden, and Kelli, for their love and support which enabled me to complete this work.
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CHAPTER I

INTRODUCTION

Human Enteric Viruses in the Environment

Human enteric viruses pose a significant health treat in the aquatic environment since they are transmitted via the fecal oral route. Human activities such as faulty septic systems, agricultural runoff, urban runoff, sewage outfall and wastewater discharge from vessels are ways enteric viruses are introduced into the environment. There are approximately one hundred forty enteric viruses found in humans and approximately one billion per gram of feces where at least ten percent of the population can shed these viruses at any give time (Griffin, 2003). Enteric viruses can be transferred throughout the environment by attaching to particulates in groundwater, estuarine water, seawater and rivers, estuaries, shellfish grown in contaminated waters, and by aerosols emitted form sewage treatment plants (Bosch, 1998). The fate of these enteric viruses can take many routes (Figure 1). Humans can be exposed to enteric viruses through various routes; crops grown in land irrigated with wastewater or fertilized with sewage, shellfish grown in contaminated water, sewage polluted recreational waters and contaminated drinking water. In a waterborne disease outbreak study between 1946 and 1980, water system deficiencies that contributed to these outbreaks were categorized under five major headings: (i) use of contaminated untreated surface water, (ii) use of contaminated untreated groundwater, (iii) inadequate or interrupted treatment (iv) distribution network problems, and (v) miscellaneous (Lippy and Waltrip, 1984). Deficiencies in treatment and distribution of water contributed to more than 80% of the outbreaks.
Figure 1. Routes of enteric virus transmission (Bosch, 1998).

The most commonly studied enteric viruses belong to the families of single stranded RNA viruses (ssRNA) \{Picornaviridae (enteroviruses, polioviruses, coxsakieviruses, hepatitis A virus, and echoviruses), Caliciviridae (noroviruses, caliciviruses, and astroviruses)\} double stranded DNA (dsDNA) Adenoviridae (adenoviruses), and double-stranded RNA (dsRNA) Reoviridae (reoviruses and rotaviruses). These enteric viruses have cellular and molecular structures that make them resistant to current water treatment
processes. Emerging enteric viral pathogens like Aichi virus (ssRNA), sapovirus (ssRNA) and picobirnaviridae (bi-segmented dsDNA) have properties similar to currently studied enteric viral pathogens in that they are non-enveloped, resistant to heat inactivation, stable at low pH, resistant to chlorination or resistant to UV light inactivation (Bosch, 1998). Paroviruses (the smallest known enteric viruses with ssRNA and high heat resistance) and polyomaviruses (includes JC virus, BK virus and simian virus 40 are non-enveloped dsDNA viruses) can also be considered emerging viruses but do not cause acute gastroenteritis as the most commonly studied enteric viruses (Bofill-Mas et al., 2000; Brauniger et al., 2000; Engelbrecht et al., 1980)

Although enteric virus infections are associated primarily with self limiting gastroenterititis in humans, they may also cause respiratory infections, conjunctivitis, hepatitis, and disease that have high mortality rates, such as aseptic meningitis, encephalitis, and paralysis in immunocompromised individuals (Kocwa-Haluch, 2001) (Table 1). In addition, some enteric viruses have been linked to chronic diseases such as myocarditis and insulin-dependent diabetes (Griffin et al., 2003).

Human enteric viruses can be transmitted by water, food, fomites, and by human contact. They typically have a low infectious dose which makes them an immediate public health concern. In some instances, such as norovirus infections, the infectious dose can be a little as one to ten virions with a secondary attack rate of 50% (Koopmans and Duizer, 2004). The risk for infection when consuming viral contaminated water is at least ten fold greater than that for pathogenic bacteria with similar exposures (Haas et al., 1993).
Table 1
Pathogenic Human Enteric Viruses

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<td>Mamastrovirus</td>
<td>Astrovirus</td>
<td>Gastroenteritis</td>
</tr>
<tr>
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<td>Coxsackievirus A,B</td>
<td>Gastroenteritis, Herpangina, Rash, Myocarditis, Pericarditis, Diabetes, Pancreatitis, Meningitis</td>
</tr>
<tr>
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<td>Hand-Foot-Mouth, Neurological Disease</td>
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<tr>
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<tr>
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<td>Hepatitis A</td>
<td>Gastroenteritis, Hepatitis</td>
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<tr>
<td>Rotavirus</td>
<td>Rotavirus A</td>
<td>Gastroenteritis</td>
</tr>
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<td>Human Adenovirus</td>
<td>Gastroenteritis, Respiratory Illness, Conjunctivitis</td>
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<td>Norwalk virus</td>
<td>Gastroenteritis</td>
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<td>Human Parvovirus</td>
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<td>Sappovirus</td>
<td>Gastroenteritis</td>
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</tbody>
</table>
Enteric viruses in water are of particular concern because of the potential for contamination from a variety of sources. Because significant advances have been made in the area of environmental virology, enteric viruses have now been recognized as the causative agents in many nonbacterial gastroenteritis cases and outbreaks identified in the past as unknown etiological origin (Bosch, 1998). Enteric viruses have been detected and linked to many outbreaks from contaminated waters and foodstuff (Beuret et al., 2002; Daniels et al., 2000; Munnoch et al., 2004).

Enteric viruses encompass a diverse group of organism that can be transferred by the fecal oral route. Since it is not practical to monitor all pathogenic viruses and bacterial indicators have not been shown to be effective viral surrogates; therefore an indicator of viral contamination should be a human enteric virus or bacteriophage.

Enteric Viruses in Sewage and Shellfish

Molluscan bivalves are shellfish that have two shell halves which hinge together. Commercial types commonly harvested and sold in the United States are the Pacific oyster (*Crassostrea gigas*), Eastern oyster (*Crassostrea virginica*), Quahog clam (*Mercenaria mercenaria*), and blue mussel (*Mytilus edulis*) (NOAA, 2007). These animals attach a substrate or bury themselves in the water floor. Molluscan bivalves vary in their characteristics and habitat. When out of the water, most animals close their shell tight to retain a marine environment around their internal parts (Lees, 2000). Most shellfish can survive weeks out of the water under refrigeration, but their taste typically reflects the age of the animal.

Individuals infected with enteric viruses transmitted by the fecal oral route can shed billions of viral particles in their feces. Subsequently, there are several different
enteric viruses occurring in large numbers in sewage. Sewage treatment plants remove the majority of viruses and other microorganisms but removal efficiency, which can range between 87-99%, varies between groups of organisms and the type of treatment (Burkhardt et al., 2005). Although enteric viruses may be present in low concentrations after treatment, it only takes one virion to cause disease. Once in the environment, enteric viruses can survive for weeks to months either in the water column or by attaching to particulate matter and accumulating in sediments (Bosch, 1998). In the process of filter feeding, bivalve shellfish concentrate and retain human pathogens from their surrounding water, thus making microbial contamination levels in shellfish tissue significantly greater than those in overlying water (Cabelli, 1988). The risks posed by bioaccumulation of pathogenic microorganisms are exacerbated by the traditional consumption of shellfish raw or lightly cooked. This circumstance is unique to bivalved shellfish and it represents a special case among microbial hazards associated with food that dates back to the 1800’s (Rippey, 1984). Recent epidemiological evidence suggests that human enteric viruses are the most common etiological agent implicated in the transmission of infectious disease due to the consumption of contaminated shellfish (CDC, 2009). As these viruses are retained in the shellfish, they do not increase in number because they are obligate intracellular parasites and require human cells in which to replicate. Current microbiological indicators serve as a predictor of fecal contamination in shellfish growing areas and have been somewhat successful in preventing bacterial gastrointestinal infections but this practice is believed to have limited predictive value for viral enteric pathogen contamination in shellfish (Pina et al, 1998; Goyal, 2006).
Human diseases other than gastroenteritis caused by enteric viruses are meningitis, respiratory disease, jaundice, eye infections, and heart anomalies. Currently, human norovirus (HuNoV) is the most common etiological agent identified in viral gastroenteritis (Lynch et al., 2006). In recent years the incidence of gastroenteritis caused by enteric viruses have not significantly increased while advances in research and technology have allowed for better detection methods and understanding of these viruses. While most enteric viruses are found more commonly in the winter or during colder temperatures, the ability of the shellfish to accumulate viruses coupled with increased community illnesses in colder climates increases the risk of gastrointestinal illnesses associated with shellfish consumption (Mounts et al., 2000). This phenomenon has been documented in several outbreaks, which occurred during cold times of the year (Woods et al., 2007). In addition to preferring colder climates, cold storage or immediate freezing of shellfish after harvest can be ideal conditions for maintaining enteric viruses in shellfish. In a recent outbreak, shellfish that had been imported were flash frozen immediately upon harvesting. These shellfish were later consumed and subsequently implicated in a shellfish outbreak of gastroenteritis (Woods et al., 2007).

Survival of Enteric Viruses in the Environment

Dissemination of enteric viruses is not only dependent on its interaction with a host, but on its interaction with the environment outside the host. Viruses possess no inherent metabolism outside the host and may be thought of as inert particles that do not require nutrients to persist outside the host. They somehow possess a level of toughness which allows them to remain infectious during various conditions in the environment as they are transferred from one host to another. The shear number of enteric viral diseases
transmitted by fecal oral route in the environment demonstrates their robustness (Rippey, 1994; Mead et al., 1999).

Enteric viruses increase their chance of transmission the longer they can survive outside the host. Various environmental conditions and other factors such as heat, moisture and pH will affect their chances of survival (Bosch, 1998). These conditions will vary in their presence and extent among different environments. To fully comprehend the risks that enteric pathogens pose, it would be fortunate to have a complete knowledge of enteric virus survival in the environment and the factors which influence their survival.

Most studies used to determine the potential for survival of enteric viruses have been conducted using basic principles. A known number of infectious viruses have been artificially introduced into a sample of water, food, soil etc., and the sample stored under conditions relevant to those in the environment. After a specified time, the viruses are extracted and enumerated. There are varying methods for extraction of viruses from the environment and from foods. Typical methods of detection involve molecular detection or cell culture. Using cell culture plaque assays for culturable viruses along with molecular methods for detection allows comparison of infectious particles remaining in the sample with the amount of virus that was introduced into the sample. Statistical analysis can be used to determine the significance of the results.

In a 1994 study, Abad et al reported the enteric viruses persisted for extended periods on several types of materials commonly found in institutions and domestic environments. The stability of the virus was affected by the type of surface contaminated and relative humidity. Overall HAV was found to be more resistant to inactivation than
enteric adenovirus and poliovirus. Adenovirus and poliovirus exhibited a significant
decrease in titer when exposed to desiccation whereas HAV showed lower decrease in
titer. This study showed that poliovirus is probably not a good model for the behavior of
enteric virus survival in the environment and HAV appears to by quite stable when
exposed to different environmental conditions. In a 2007 study, Gerba and Kennedy
looked at the survival of enteric viruses on swatches of clothes during laundering using
detergent with and without bleach (sodium hypochlorite). Enteric viruses (adenovirus,
rotavirus, and hepatitis A virus) were inoculated onto sterile 58-cm² swatches and
launched with 3.2 kg of cotton T-shirts and underwear, and a soiled pillowcase. It was
found that washing with detergent alone was not sufficient to remove or inactivate the
virus and the viruses easily contaminate the other non-inoculated clothes. Using bleach in
conjunction with detergent eliminated at least 99.99% of the infectious viruses.

Survival of enteric viruses in environmental water has been investigated
considerably. Utilizing simulated natural conditions, Loh et al. (1979) inoculated
poliovirus into samples of coastal water from the plume of a sewage outlet and samples
of water from 6.4 km away from the plume. Samples were mixed continuously and
incubated at 24°C for 4 days. The virus titer had dropped slightly after 1 day with a
complete inactivation observed after 72 to 120 h. In this study there was data to suggest
that a virus-inactivating component of a biological nature was present in the sewage
polluted water and the water retrieved miles away from the plume. In 1980, Fujioka et al.
substantiated this evidence in a study where the antiviral activity of the seawater samples
was lost when it were challenged with filtration, boiling, or autoclaving. A study
conducted by Hurst et al. in 1989, analyzed the long term survival of species of
enteroviruses in surface freshwater. Over a period of 12 weeks, temperatures of -20, 1, and 22 °C was shown to have virus inactivation levels of 0.4-0.8, 4-5, and 6.5-7.0 log reduction, respectively. Many physical and chemical parameters appeared crucial to virus survival, including turbidity and suspended solids.

Throughout the years, there have been many recorded cases of viral gastroenteritis attributed to contaminated foods. Whether the food was contaminated by irrigation or washing in contaminated waters, infected food handlers, or accumulation of viruses in foodstuff, the information available demonstrated the potential of enteric viruses to persist in a foodstuff. In a study conducted by Grigor’eva et al. (1965), tomatoes, white cabbage, and sweet peppers were planted in pots outdoors and irrigated with water inoculated with coxsackievirus A5, A7 and A14. The fruit and leaves were analyzed up to 20 days later and virus infectivity as assessed through the infection of newborn mice. Coxsackievirus continued to be infectious between 3-4 days on the cabbage leaves, 7 to 10 days on the sweet pepper leaves, and 15 to 18 days on the tomato leaves. Another irrigation study conducted by Tierney et al. (1977), a 99% loss of detectable virus was noted 4 to 5 days after irrigation with water contaminated with poliovirus. Drying and sunlight was found to have considerable negative influence on the persistence of infectious viruses on irrigated foods and this was demonstrated by Kott et al. (1974) when comparing survival of poliovirus seeded on parsley indoors and outdoors. In 2002, Croci et al. studied the survival of HAV on fresh produce. In this study samples of lettuce, fennel, and carrots were immersed in sterile distilled water supplemented with a HAV suspension of 5 log tissue culture infectious dose (TCID$_{50}$/ml. The samples were stored at 4 °C after contamination and analyzed at 0, 2, 4, 7, and 9 days. The HAV
remained viable after 9 days of storage with only a 2 log reduction of TCID$_{50}$ after washing. The HAV was viable before washing until day 4 on the fennel and carrots. After washing at day 7, HAV was not detected on the fennel and carrots at day seven. Lettuce, like green onions, is multilayered can retain particles that can harbor contaminants during harvesting and packing which has the potential to cause outbreaks like the one that occurred in 2003 (Wheeler et al., 2005).

Although the majority of foodborne viral outbreaks can be traced to food contaminated by infected food handlers (Koopmans and Duizer, 2004), survival and persistence of enteric viruses in shellfish represents a unique challenge because of the nature of these filter feeding animals. Molluscan shellfish accumulate the virus from contaminated harvest water and when contaminated shellfish are consumed raw or slightly cooked, there is a potential for infection to occur. There are several recorded outbreaks of gastroenteritis where shellfish contaminated with enteric viruses was implicated as the vehicle of transmission (Berg et al., 2000; Butt et al., 2004; Gallimore et al., 2005, Woods et al., 2007). There has also been several studies demonstrating the survival and persistence of enteric viruses in shellfish. A 1970 study conducted by DiGirolamo et al. (1970) examined the survival of poliovirus in chilled, frozen, and processed Pacific (Crassostrea gigas) and Olympia (Ostrea luridia) oysters. After 15 days of storage at 5°C, infectious virus in the Olympia oyster was reduced by 60% and 13% remained infectious after 30 days. In the frozen Pacific oyster, infectious virus was reduced by less than 10% in 4 weeks and by 12 weeks, only 10% of the infectious virus remained. In 2004, Hewitt and Greening studied the survival and persistence of norovirus, HAV, and feline calicivirus (FCV) in marinated mussels. NoV, HAV, and
FCV were inoculated into marinated green-lipped mussels (*Perna canaliculus*) and marinade liquid and held at 4°C for up to 4 weeks. Survival of HAV and FCV was determined by TCID$_{50}$ and persistence of the non-culturable NoV was determined by RT-PCR assay. Over 4 weeks, HAV survived exposure to the marinade at a low pH (3.75). There was a 1.7 log reduction in HAV TCID$_{50}$ titer but no reduction in the NoV or HAV RT-PCR titer after 4 week.

Persistence and survival of enteric viruses on or in foods provides a challenge. Further work is required to gain a better understanding of enteric viruses causing illnesses. Development of robust and reliable detection methods for recovery of these viruses will provide additional information necessary to recovery clinically significant enteric viruses in the environment and foods.

Detection of Enteric Viruses

In the past, detection of enteric viruses in environmental and food samples largely depended on whether the agent grew in cell culture. For those types that do, such as enteroviruses and adenoviruses, detection by virus replication in cell culture demonstrated infectivity as well as their presence. The ability to detect viruses by cell culture is a clear advantage when assessing whether environmental samples or foods are microbiologically hazardous. The capacity to do quantitative assays is also a bonus of cell culture. Cytopathic effect or virus-specific killing or lysing of cells is visible by ordinary light microscopy. Cell culture assays were the most widely used protocol for detection of enteric viruses until the 1990’s (Farrah 1977; Rao et al., 1984; Goyal 2006). While cell culture offers quantitative analysis and infectivity, the high cost, long turnaround time, and labor intensive efforts are drawbacks. There are many cell lines suitable for growing
enteric viruses (Table 2). Buffalo Green Monkey (BGM) cell line has been shown to give higher plaques forming units per milliliter (PFU/ml) and faster CPE for coxsackieviruses and polioviruses (Chonaitree et al., 1985).

Table 2

*Cell Lines Used for Isolation of Enteric Viruses*

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Origin</th>
<th>Tissue</th>
<th>Virus Isolated</th>
</tr>
</thead>
<tbody>
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<td>Enterovirus</td>
</tr>
<tr>
<td>A549</td>
<td>Human</td>
<td>Lung Carcinoma</td>
<td>Adenoviruses</td>
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<tr>
<td>MRC-5</td>
<td>Human</td>
<td>Human Diploid Fibroblast</td>
<td>Enteroviruses</td>
</tr>
<tr>
<td>PMK</td>
<td>Rhesus Monkey</td>
<td>Primary Rhesus Monkey Kidney</td>
<td>Enteroviruses</td>
</tr>
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<td>RD</td>
<td>Human</td>
<td>Rhabdomysarcoma</td>
<td>Enterovirus</td>
</tr>
<tr>
<td>FRhK</td>
<td>Rhesus monkey</td>
<td>Kidney</td>
<td>Hepatitis A</td>
</tr>
<tr>
<td>Vero</td>
<td>African Green Monkey</td>
<td>Kidney</td>
<td>Poliovirus</td>
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<td>BGM</td>
<td>Buffalo Green Monkey</td>
<td>Kidney</td>
<td>Enterovirus</td>
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<td>Human</td>
<td>Cervix adenocarcinoma</td>
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<td>Enterovirus</td>
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<tr>
<td>PLC/PRF/5</td>
<td>Human</td>
<td>Primary Liver Carcinoma</td>
<td>Enterovirus</td>
</tr>
</tbody>
</table>

Cell lines obtained from ATCC

There are a host of enteric viruses that are capable of growth utilizing cell culture, but two significant enteric viruses, human noroviruses and wild-type HAV, are difficult or unable to propagate in cell culture. Cell culture would apparently not be a proactive
monitoring protocol given the length of time required to complete most assays and difficulties of propagation. However, cell culture used in conjunction with detection emerging assays can be used to address the issue of viability while comparing the sensitivity of each representative assay.

Detection of enteric viral pathogens with molecular based PCR assays has successfully been utilized for a number of years (Ando et al., 1995; Caro et al., 2001; Croci et al., 1999; Chapron et al., 2000). Conventional PCR methodology utilizes a pair of oligonucleotides or primers, each hybridizing to one strand of double stranded DNA (dsDNA) target. The types of primers used can be (i) random primers—short single stranded DNA fragments with all possible combinations of bases, (ii) polythymine primers—sixteen base long thymine primers that will hybridize with the polyadenine end of the mRNA and (iii) specific primers—only the targeted region specific to the primers will be amplified. The primers act as a substrate for DNA polymerase which creates a complementary strand by the way of sequential addition of deoxynucleotides. The process of PCR can be summarized into three steps: (i) denaturation—dsDNA is separated by an increase in temperature, (ii) annealing—the temperature is decreased to allow the primers to anneal to the separated DNA, (iii) extension—the extension of the DNA fragment with the primers attached by addition of deoxynucleotides. For RNA viruses such as enteroviruses, NoV, and HAV, RT-PCR, or the conversion of RNA to cDNA, is necessary. During reverse transcription, a primer is required for the reverse transcriptase (RNA-dependent DNA polymerase) to initiate the synthesis of cDNA from the viral RNA. For DNA viruses reverse transcription is not a necessary step. The final PCR product is analyzed by electrophoresis in the presence of ethidium bromide in which
the correct size of the product can be examined visually by ultraviolet light.

Hybridization with digoxigenin-labeled probes or genetic sequencing can be used to further identify PCR products. If the products examined are from environmental samples, cloning of the PCR products may also be required, as there can be multiple strains of individual viruses present.

Real-time quantitative PCR or qPCR is used to quantitatively determine the amount of original target present in the sample (Gibson et al., 1996; Mackay et al., 2002). During a qPCR assay, the amplicon produced during each cycle can be quantified using SYBR Green (nonspecific attachment to dsDNA), or by using a fluorescent internal probe (specific hybridization) (Mackay et al., 2002). For SYBR green assays, analysis of the melting curves of the amplicons as the amplicons have different Tm (melting temperature). For fluorescent internal probes, fluorescence is measured during each cycle, and when the amount of fluorescence exceeds the background level (threshold level), the sample is scored as positive. The number of cycles required to reach the threshold level, commonly referred to as the cycle threshold value (Ct), correlates with the amount of target in the sample prior to amplification (Gibson et al., 1996). Real-time PCR is an excellent tool for detection of enteric viruses in environmental samples and has been used successfully to determine the concentrations of viral genomes (Donaldson et al., 2002; Heim et al., 2003).

Multiplex PCR, which utilizes multiple primer sets within a single PCR reaction, can be used to simultaneously detect different groups of viruses. However, this multiple viral detection can be difficult to optimize because of the different annealing temperature requirements of dissimilar primer sets and because of the properties of the viral nucleic
acids found between viral groups (Fong and Lipp, 2005). Real-time PCR (qPCR) has been quite successful for detection of multiple enteric viruses, because it can analyze each target independently in the same assay by using specific internal probes binding to different fluorochromes that the real-time PCR equipment can analyze independently (Kageyama et al., 2003; Logan et al., 2006). Furthermore, the PCR products can be of a similar size, providing better amplification efficiency. For viruses that grow poorly in cell culture, the detection by PCR integrated with cell culture (i.e., ICC-PCR) drastically reduces the time needed for detection (Reynolds et al., 2001). The detection of enteroviruses in water can be between 3 and 14 days using cell culture, 5 days using integrated cell culture, and less than a day using direct real-time PCR. Because viruses are normally present in very low concentrations in environmental samples, the level of sensitivity of most PCR is advantageous for detection of low copy number. While the sensitivity of PCR is beneficial, the presence of inhibitory substances (i.e. humic acid or heavy metals) in concentrated environmental samples is of concern. Internal controls for real-time PCR have been developed to determine the presence of inhibitors in a sample and ensure that reaction conditions are optimal (Burkhardt et al., 2005).

While there has been tremendous progress in molecular detection assays, complications remain. Even though most molecular based assays are specific, sensitive, rapid and cost efficient there has been no development of a universal method or standardization. Perhaps future development of molecular based assays that can establish infectivity will combine best of cell culture and PCR when it comes to the detection of enteric viruses in the environment.
Contribution of Present Study

Since HuNoV and wild-type hepatitis A (HAV) are difficult or impossible to propagate, assessing the levels of culturable viruses, such as enteroviruses and adenoviruses, in clinical and environmental matrices may provide the opportunity to indirectly determine the viability of viruses detected by RT-PCR and cell culture. HuNoV GI and GII, HAV, enteroviruses, and adenoviruses and were examined in human fecal samples, sewage treatment plants’ (STP) influent and effluent, and shellfish as sentinels downstream of the STP. Conventional RT-PCR, real-time RT-PCR, and real-time PCR were utilized as detection methods. Cloning and sequencing was used to further characterize strains of the enteric viruses. Current indicator organisms and male specific bacteriophage levels were examined in the influent, effluent, and shellfish samples. Tissue culture was utilized for propagation of enteroviruses and adenoviruses. The goal of this study was to demonstrate: a) a link between enteric viruses found in different environmental matrices and clinical specimens and; b) assess a sewage treatment plants’ ability to reduce their viral load.
CHAPTER II
LITERATURE REVIEW

History of Norovirus and Hepatitis A Virus and Its Role in Disease

Norovirus

An outbreak of acute gastroenteritis occurred among students and teachers in a school in Norwalk, Ohio in 1968 (Adler and Zickl, 1969). The initial attack rate had a morbidity of 50% and a secondary attack produced a higher rate of 82% (Atmar and Estes, 2001). Nausea and vomiting occurred in >90% of those affected, while diarrhea occurred in 38% of those affected. The clinical onset of the illness was typically 12 to 24 hours with duration of 12 to 60 hours. Upon subsequent transmission of stool filtrates to human volunteers in 1972, a small round-structured virus (SRSV’s) ~ 27-nm was identified by electron microscopy (Kapikian et al., 1972). Later studies revealed that other SRSV’s morphologically similar to NoV caused gastroenteritis, but Norwalk virus remained the prototype of these fecal viruses (Atmar and Estes, 2001). Before the discovery of NoV, most cases of gastroenteritis not attributed to bacteria were thought to be cause by nonbacterial gastroenteritis (Rippey, 1994). In 1981, Greenberg et al. published data proposing that NoV might be a calicivirus. In 1993, Jiang et al., provided molecular evidence that NoV was a calicivirus with a 7.6 kilo-base(kb) viral genome consisting of a positive sense, single-stranded, polyadenylated RNA. The genome consists of three open reading frames (ORFs), which code for the nonstructural proteins including the RNA polymerase (ORF1), capsid protein (ORF2), and a minor structural protein (ORF3). Noroviruses can be separated into five genogroups (GI, GII, GII, GIV, and GV) based on the sequence comparison of the RNA polymerase and capsid regions.
(Ando et al., 1995; Zheng et al., 2006). Genogroup I infects humans only, genogroup II infects humans and swine, and genogroup IV infects humans and canine. Genogroup III infects bovine animals and genogroups V infects mice. Of the five genogroups, the classification scheme for the different clusters and strains identifies NoV GI containing 8 clusters, GII containing 17 clusters, and GIV containing 1 cluster (Table 3). Noroviruses have an assigned nomenclature where strains are named after the geographic location of the outbreak from which they are first described. The genogroups and genotypes were characterized and classified based on the RNA polymerase region and the complete capsid gene sequences (Ando et al., 1995; Zheng et al., 2006). To date, the most common genogroups implicated in gastrointestinal infections is genogroups II.
Table 3

*Genogroups, Clusters, and Strains of Human Norovirus* (Zheng et al., 2006)

<table>
<thead>
<tr>
<th>Cluster</th>
<th>GI</th>
<th>Strain</th>
<th>Cluster</th>
<th>GII</th>
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Noroviruses are transmitted primarily through the fecal oral route, consumption of fecally contaminated food or water, or by person to person contact. There is a high rate of secondary infection that can occur by airborne transmission. Outbreaks commonly occur in schools, nursing homes, hospitals, camps, daycare centers, and any other close community situation. Because norovirus is not listed as a reportable disease to health officials, estimates of the level of infection may not be accurate. To date, noroviruses are estimated to be responsible for two-thirds of the non-bacterial food-borne illness and nearly all (96%) of the non-bacterial gastrointestinal illnesses each year in the United States (Lynch et al., 2006). Virtually any food may be implicated in norovirus transmission, but bivalved molluscan shellfish present a relatively high risk because of their ability to concentrate viruses from contaminated waters.

To date, the ability to cultivate norovirus has been futile, although several attempts have been made. A Duzier et al., study (2004) utilized 27 different cell lines in an unsuccessful attempt to cultivate norovirus. Straub et al., (2007) utilized human embryonic intestinal epithelial cells (INT-407) with 3-D tissue culture. The use of INT-407 cells with 3-D tissue culture appeared to be promising as this method provided the closest attempt at mimicking the structure of in vivo cells. The difficulty with cultivation of norovirus may be explained by its specific requirements or receptors needed for attachment to cells in order for replication to occur. Experiments with recombinant norovirus particles and human gastrointestinal biopsies showed preferential binding to epithelial cells of the pyloric region of the stomach and to enterocytes on duodenal villi (Duzier et al., 2004). Human specific blood groups antigens H1 type expression were
shown to be necessary for norovirus attachment to the cells and most routine cell cultures lack the characteristics of these specialized human intestinal epithelial cells.

The majority of background information on the biological properties of norovirus has been obtained through humans who volunteer for human feeding studies (Dolin et al., 1971; Teunis et al., 2008). Infectivity can only be assessed in human dose response experiments and the infectious dose had been determined to be around 10 virus genomes (Teunis et al., 2008). This is very critical when considering norovirus survival. Norovirus remains infectious under refrigeration and freezing conditions, it survives well in the environment and it is resistant to heat and drying conditions. This can be demonstrated in an outbreak at a long term care facility where norovirus survived on fomites and continued to cause infection two weeks after the initial peak of illnesses (Wu et al., 2005). Norovirus will continue to be a significant health threat worldwide as this virus continues to evolve. The lack of a tissue culture cell line for effective propagation of norovirus will hinder complete understanding of how this virus causes infection in addition to decreased progression on the development of a productive vaccine.

*Hepatitis A Virus*

Hepatitis A is a non-enveloped RNA virus 27 to 32 nm in diameter. It has an icosahedral symmetry and belongs to the genus *Hepatovirus* of the *Picornaviridae* family. HAV has a positive-polarity single-stranded 7.5 kb genome with a single ORF with three distinct regions (P1, P2, and P3) (Cohen et al., 1987). Region P1 consists of four capsid proteins VP1-VP4. Region P2 consists of non-structural protein 2A-2C and region P3 consists of non-structural proteins and virus-specific proteins (VPg) (Cohen et al., 1987). HAV demonstrates a high degree of antigenic (amino acid) and genetic
(nucleotide) conservation throughout the genome (Cohen et al., 1987; Lemon et al., 1992). Although this high propensity for conservation exists, there is still enough diversity to define HAV genotypes and sub-genogroups (Robertson et al., 1992). The genomic regions commonly used to define HAV genogroups include (i) the C terminus of the VP3 region, (ii) the N terminus of the VP1 region, (iii) the 168-bp junction of the VP1/P2A regions, (iv) the 390-bp region of the VP1-P2B regions, and (v) the entire VP1 region (Robertson et al., 1992; Hutin et al., 1999). A total of six genotypes have been identified: genotype 1A, 1B, II, III, IV, V, and VI. Genotypes I, II, and III are of human origin, and IV, V, and VI genotypes are of non-human primate origin. Genotype I and III are the most prevalent genotypes isolated from humans (Nainan et al., 2006). Because there is only one serotype of HAV, individuals infected by HAV in one part of the world are protected from re-infection by HAV in another part of the world.

Infections with HAV can produce effects that range in severity from asymptomatic to death from fulminant hepatitis. Infections with HAV are typically self-limiting and do not result in chronic liver disease. The virus in shed in the feces and peak fecal excretion, hence infectivity, occurs prior to the onset of symptoms (Lednar et al., 1985). Clinical manifestations can increase with age and with older children and adults, symptoms are typically present with jaundice occurring in 70% of those infected (Lednar et al., 1985). An average incubation period is 28 days with a range of 15 to 50 days. Symptoms include gastroenteritis, fever, malaise, anorexia, nausea, abdominal discomfort, dark urine, and jaundice, all which may last up to 2 months. Chronic liver disease has not been shown to persist, although 15 to 20% of those infected may have prolonged or relapsing disease lasting up to 6 months (Glikson et al., 1992). Fulminant
hepatitis is a rare complication in HAV infections, only occurring in <1% of those infected, with the highest rates occurring in young children and elderly adults who may have underlying liver conditions (Nainan et al, 2006). Among those with fulminant disease, reported findings demonstrate nucleotide and/or amino acid substitutions in the 5’ untranslated region, P2 region and the P3 region of the HAV genome (Fujiwara et al., 2001; Nainan et al., 2006).

Cell culture propagation of human and non-human HAV has occurred in African green monkey kidney and fetal rhesus monkey kidney cells (Flehmig, 1980; Daemer et al., 1981). Propagation of HAV of human origin is quite different than propagation of other picornaviruses of human origin. HAV of human origin requires an extensive adaptation period or serial passages before it grows in cell culture, and once it is adapted, HAV becomes attenuated as demonstrated by not producing disease in experimentally inoculated nonhuman primates (Feinstone et al., 1983). Mutations causing attenuation in viral nucleic acid could play a significant role in the adaptation of HAV in cell culture (Daemer et al., 1981; Cohen et al., 1987). The attenuated strain HM-175 was adapted in cell culture and this strain is currently used a vaccination agent for HAV (Cohen et al., 1987).

HAV is a major cause of acute hepatitis in developed countries, while in developing countries it can be considered endemic (Jothikumar et al., 2005). HAV is transmitted primarily by the fecal-oral route, via person-to-person contact, contaminated food, or water, while the other common hepatitis viruses (hepatitis B and hepatitis C) are typically transmitted by blood or body fluids. HAV is stable in the environment when associated with organic material and it is resistant to low pH and heating (Hollinger et al.,
The name ‘hepatitis’ is derived from the fact that HAV replicates in the liver and affects the liver. The source of the infectious agent, however, cannot be identified in approximately 50% of reported hepatitis A cases in the U.S., partly because of the long incubation before the appearance of symptoms (Nainan et al., 2006). Only 2-5% of reported hepatitis A cases each year are attributed to contaminated food (Lynch et al., 2006). Since 1961, the U.S. major outbreaks (> 30 cases per outbreak) of hepatitis A associated with the consumption of bivalve molluscan shellfish have decreased. Specifically, from 1989 through 2004, there were no major shellfish associated HAV outbreaks reported. This reduction in cases could be due to the availability of the hepatitis A vaccine, which became available in 1995, or because routine surveillance may not detect cases related to foodborne transmission and cases may accrue gradually or be dispersed among a number of public health jurisdictions (Amon et al., 2003). Also, in developed countries, sewage treatment and hygiene practices have improved. However, in 2005, a multi-state outbreak of hepatitis A was reported among restaurant patrons who consumed raw and undercooked oysters (Shieh et al., 2007). Because foodborne outbreaks of HAV can cause considerable morbidity and even mortality, it is imperative that HAV be isolated and identified as the implicated pathogen.

History of Enterovirus and Adenovirus and Its Role in Disease

Enterovirus

Human enteroviruses are members of Picornaviridae family and Enterovirus genus. They are icosahedral, non-enveloped with a diameter of 27-30 nm. The genome is 7.5 kb to 8.5 kb and is composed of single stranded positive polarity RNA. Enteroviruses are further divided into the subgenera/species of poliovirus, coxsackieviruses,
echoviruses, and enteroviruses and they are marked according to their serotypes. The poliovirus group consists of 3 different serotypes. Type 1 and 3 are recognized as epidemic while type 2 as endemic (Rajtar et al., 2008). Coxsackieviruses consist of groups A and B where the A group contains 24 serotypes and the B group contains 6 serotypes. In 2003, the International Committee on Taxonomy of Viruses classified Enteroviruses into 5 groups of species based on their molecular properties (Khetsuriani et al., 2006) (Table 4).

Table 4

**Classification of Human Enteroviruses (Khetsuriani, 2006)**

<table>
<thead>
<tr>
<th>Groups of enteroviruses</th>
<th>Species of enteroviruses</th>
</tr>
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<tbody>
<tr>
<td>Enterovirus-A</td>
<td>Coxsackievirus A2-8, 10, 12, 14, 16</td>
</tr>
<tr>
<td></td>
<td>Enterovirus 71, 76, 89, 90, 91</td>
</tr>
<tr>
<td>Enterovirus-B</td>
<td>Coxsackievirus A9</td>
</tr>
<tr>
<td></td>
<td>Coxsackievirus B1-6</td>
</tr>
<tr>
<td></td>
<td>Echovirus 1-7, 9, 11-21, 24-27, 29-33</td>
</tr>
<tr>
<td></td>
<td>Enterovirus 69, 73-75, 77-78, 79-88, 100-101</td>
</tr>
<tr>
<td>Enterovirus-C</td>
<td>Coxsackievirus A1, 11, 13, 17-22, 24</td>
</tr>
<tr>
<td></td>
<td>Poliovirus 1-3</td>
</tr>
<tr>
<td>Enterovirus-D</td>
<td>Enterovirus 68, 70</td>
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</tbody>
</table>

Infections due to enteroviruses are common, causing a range of disease including pharyngitis and poliomyelitis. In the United States, it is estimated that 30-50 million enterovirus infections occur a year, of which only 5-15 million are symptomatic (Rajtar et
The virus is spread by the fecal oral route and person to person through direct contact with secretions of an infected individual. The incubation period is usually 3 to 7 days with virus transmission lasting 3 to 10 days after symptoms appear. Replication occurs in the gastrointestinal track, but can occur in other tissues such as nerve and muscle (Colbere-Garapin et al., 1989; Rajtar et al., 2008). Polioviruses typically infect their host by attacking the central nervous system causing paralysis (poliomyelitis) in infected individuals. The spread of poliovirus has been limited by the development and use of vaccines. The Sabin trivalent oral live attenuated vaccine (OPV) consists of three live attenuated strains of 1, 2, and 3 serotypes grown in cell culture. The Salk vaccine is trivalent inactivated polio vaccine (IPV) given by injection. The Sabin vaccine has an advantage over the Salk vaccine in that it elicits secretory IgA antibody production in addition to IgA, IgM and IgG serum antibody production (Howard, 2005).

Coxsackieviruses have been associated with respiratory infections, gastroenteritis, insulin-dependent diabetes, myocarditis, and pericarditis (Griffin et al., 2003). Echoviruses are typically less infectious and are usually associated with the common cold and other respiratory diseases. The numbered enteroviruses have not been widely studied, but they are generally associated with bronchiolitis, conjunctivitis, meningitis, and paralysis (Kocwa-Haluch, 2001; Fong and Lipp, 2005). Also, enteroviruses are one of few enteric viruses which produce viremia in infected patients.

Enteroviruses are resistant to most concentrations of chlorine used in sewage treatment and they are tolerant to cold and warm temperatures. This makes them ideally suited for survival in the environment. Stability of enteroviruses in the environment is dependent on temperature, humidity, and UV radiation. In order to inactivate 90% of
poliovirus in salt water environment, 671 days at 4°C is required; on the other hand, an increase the temperature to 25°C reduces the inactivation time by 25 days (Rajtar et al., 2008). Symonds et al. 2009 collected sewage influent and effluent from several waste water treatment plants (WWTP) across the United States and revealed that 75% of the sewage influent and 8.3% of the effluent contained enteroviruses. In 2003, the largest European outbreak of enterovirus related infection occurred in Belarus (Amvrosieva et al., 2006). Over 1300 people became ill and water contaminated with echovirus and coxsackievirus was identified as the source of the infection. Aside from water and sewage samples, enteroviruses have been detected in food samples. In 1914, the first described food borne outbreak was linked to milk contaminated with poliovirus (Jubb, 1915). After pasteurization of milk was adopted, transmission of enterovirus by contaminated milk decreased dramatically. Despite its demonstrated presence in the environment and sewage, there have been very few foodborne related outbreaks due to enterovirus.

**Adenovirus**

Adenoviruses are members of the *Adenoviridae* family and the *Mastadenovirus* genus, which comprises five genera and infects hosts across the extended spectrum of vertebrates (Wigand and Adrian, 1986; Jiang, 2006). Human adenoviruses is a double stranded DNA virus containing a non-enveloped icosahedral shell with five live projections form each of the 12 vertices (Stewart et al., 1993). Its DNA is linear with about 35 kb and encoded for more than 30 structural and non structural proteins (Friefeld et al., 1984). In 1953, the first adenovirus was isolated from human adenoid tissue (Rowe et al., 1953). There are 51 serotypes of adenovirus and they are divided into six species
based on their hemeagglutination properties, their oncogenic potential in rodents and DNA homology or GC content (Jiang, 2006) (Table 5).

Table 5

**Serotype Classification of Human Adenovirus (Jiang, 2006)**

<table>
<thead>
<tr>
<th>Subgroup/Species</th>
<th>Serotype</th>
<th>Site of Infection</th>
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<tbody>
<tr>
<td>A</td>
<td>12, 18, 31</td>
<td>gastrointestinal tract</td>
</tr>
<tr>
<td>B</td>
<td>3, 7, 16, 21, 50 (B1)</td>
<td>lung, urinary tract</td>
</tr>
<tr>
<td></td>
<td>11, 14, 34, 35, (B2)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1, 2, 5, 6</td>
<td>upper respiratory tract</td>
</tr>
<tr>
<td>D</td>
<td>8, 9, 13, 5, 17, 19, 22, 23, 24, 26, 27, 30, 32, 33, 36, 37, 38, 39, 42, 43, 44, 45, 46, 47, 48, 48, 51</td>
<td>eye, gastrointestinal tract</td>
</tr>
<tr>
<td>E</td>
<td>4</td>
<td>respiratory tract</td>
</tr>
<tr>
<td>F</td>
<td>40, 41</td>
<td>gastrointestinal tract</td>
</tr>
</tbody>
</table>

Infections with adenoviruses can result in a wide range of clinical symptoms. Subgroup A, D and F are sites for gastrointestinal infections. Subgroup B is responsible for lung and urinary tract infections. Subgroup C and E are responsible for respiratory tract infections. Serotypes 40 and 41 are the cause of most adenovirus associated gastroenteritis and serotypes 4 and 7 are associated with most cases of ARD (acute respiratory disease) in the Unites States (CDC, 2005). Most adenovirus are self-limiting except cases where the infected individual is immunocompromised. However, in 2007
there were cases of a new emerging strain of adenovirus 14 that caused fatal respiratory
disease in healthy individuals (MMRW, 2007a). After the primary infection, immunity is
conferred to the causative adenovirus serotype.

Human adenoviruses are specific to humans even though adenoviruses infect a
range of animals. In 2005, Cox et al reported no viable human adenovirus detection in
feces of cattle, valve, pig, sheep, horse, dog, poultry, wombat, cat, kangaroo, possum,
wood duck, rat, wild pig, fox, rabbit, ferry cat, goat, carp, and deer. In domestic sewage
worldwide, human adenoviruses have been detected in high concentrations and their
detection in sewage seems to have little seasonal variability (Bosch, 1998; Carter, 2005;
Jiang, 2006). As with most enteric viruses, adenovirus survives better in the environment
and sewage treatment than the current indicator bacteria. Adenoviruses have increased
resistance to UV light and this increased resistant could be due to the DNA repair
mechanism of the host cell. Because of the environmental stability of adenovirus, they
have been suggested as an indicator of viral pollution (Pina et al., 1998). In current
literature, adenovirus has been associated with waterborne outbreaks and foodborne
outbreaks has suspected, but not confirmed (Goyal, 2005; CDC, 2005)

Enteric Viruses and Indicator Bacteria: Why Indicators Do Not Predict
Viral Contamination

Human enteric pathogens of main concern from sewage contamination are NoV
and HAV. Conventional sewage treatment plants utilizing primary and secondary
treatment typically reduce enteric viruses by 2 logs (Burkhardt et al., 2005). Many of the
viruses present in the effluent remain infectious and chemical disinfection processes vary
in their ability to inactivate enteric viruses (NRC, 2004). These viruses are more resistant
to heat, disinfection and pH changes than are most enteric bacteria (NRC, 2004). HAV may retain their infectivity after exposure to low pH while NoV can remain infective after exposure to low pH, refrigeration, and freezing. NoV and HAV survive well on inanimate surfaces and NoV is considered to be resistant to inactivation in the presence of 3.75-6.25 mg chlorine/L, which is the concentration used to treat a water supply after a contamination incident (NRC, 2004). Sewage effluent often contains relatively high concentrations of viruses due ineffectiveness of chlorine disinfection. Furthermore, sewage treatment plants occasionally bypass untreated sewage during wet weather by design, and many urban sewage systems discharge combined sewer overflows directly to receiving waters (NRC, 2004). Considering these factors, it is plausible to indicate that a constant and predictable relationship does not exist among indicator bacteria and viruses in estuarine waters and shellfish (Pina et al., 1998; Lee et al., 2004; Kingsley, 2006).

The public health risk associated with fecal material from animal sources versus human sources is in question. Terrestrial mammals carry bacterial species pathogenic to humans; however, these have generally not been associated with shellfish-borne illnesses. Rather, sewage-associated human illnesses appear most frequently to have a viral etiology, and viruses tend to be species specific. Recently, a NoV GII.4 like sequence was detected in fresh manure from animal pig pens. Also, partial GII.4 genomic sequence was detected in cattle feces (Mattison et al., 2007). These findings demonstrate a plausible route for indirect zoonotic transmission of noroviruses through the food chain considering that most productive shellfish growing estuaries are often those most subject to rainfall runoff from animal non-point sources (Mattison et al., 2007). Extensive closures due to high fecal coliform indicator counts from non-point animal sources have
been identified as one of the major concerns of state regulatory agencies and industry members from coastal areas; a great deal of research is required to assess human health risks from wild and domestic animal runoff (Elliot and Colwell, 1985; Kilgen, 1989; Calci et al., 1989). Therefore, an indicator of human enteric viruses in water and in seafood is needed. Some indicators that have been proposed include poliovirus type 1, enterococci, *E. coli*, coliphages, fecal streptococci, *Clostridium perfringens*, *Pseudomonas aeruginosa*, *Bifidobacterium* species, *Rhodococcus* species, *Streptococcus bovis*, *Bacteroides* phages, F+ phages, and adenoviruses (Elliot and Colwell, 1985; Kilgen, 1989; Richards, 1985, Pina et al., 1998). None of the suggested indicators appears to be an adequate indicator of human health risk from enteric virus pathogens in seafood of water, and none of them would predict the presence of naturally occurring bacterial pathogens such as members of the *Vibrionaceae*. Molecular detection of enteric viruses from clinical isolates and environmental samples along with cell culture may offer some insight into virus viability and possible viral indicators.
CHAPTER III

MATERIALS AND METHODS

Collection and Analysis of Stool Samples

A total of 401 stool samples were collected between September 2008 and March 2009. The samples were collected at local clinics in standard bacterial transport media (Meridian Bioscience, Inc., Cincinnati, OH) and delivered to the hospital by currier. Once bacterial analyses at the hospital were complete, samples were stored at 4°C until retrieval. Samples were identified based on patients’ zip code, collection day, and month. The zip code was used to determine the catchments area of the patients and subsequent sewage treated by Mobile’s WWTP (waste water treatment plant). Once the samples were retrieved, all samples were analyzed for ADV, enteroviruses, HAV, human NoV GI and GII using real-time PCR or RT-PCR. Each stool sample was diluted 1 to 10 utilizing 50 µl of patient stool and 450 ml tissue culture grade phosphate buffered saline (t.c. PBS) (8.0 g NaCl, 0.2 g KCl, 0.12 g KH₂PO₄, 0.91 g Na₂PO₄ per liter) pH 7.5. Five hundred microliters of chloroform was added to the stool suspension and briefly vortexed. The sample was centrifuge at 5000 x g for 5 min. Fifty microliters of the top aqueous layer was removed and added to a clean 0.5 ml thin walled PCR tube. The 50 µl of extract was placed in a heat block. The samples were heat-liberated at 95°C for 5 min (Schwab, 1997). The liberated RNA or DNA was tested for NoV GI, NoV GII, HAV, ADV, and enteroviruses. The remaining aqueous layer was stored in a sterile DNase/RNAse free microcentrifuge tube at 20°C for further use.
Collection and Analysis of Sewage Samples

Mobile WWTP has a capacity of over 20 MGD (million gallons per day) and services approximately 200,000 people in the Mobile County area. Two 500 ml samples of primary influent and final effluent were collected in polypropylene co-polymer bottles from Mobile WWTP each month 3 days before the removal of the oyster sentinels (protocol discussed below). Sodium thiosulfate tablets (Whirl-Pak® Nasco, Fort Atkinson, WI) were added to the effluent bottles prior to collection to bind chlorine and prevent additional inactivation of indicators microbes. All sewage samples were analyzed for adenoviruses, enteroviruses, hepatitis A, HuNoV genotype I and II, fecal coliforms, E. coli, and male specific bacteriophage.

Concentration of Enteric Viruses in Sewage

An ultracentrifugation (Cruz, 2005) protocol was used concentrate aliquots of influent and effluent for enteric viruses. Forty ml of influent and effluent was added to polycarbonate ultracentrifuge tubes, weighted and balance. Samples were spun at 107,100 x g for 1 hr at 4 °C. Supernant was discarded and 4 ml of 0.25 N glycine (3.75 g per liter pH 9.5) was added to the pellet. Samples were vortexed and placed on ice for 30 min. Four ml of cold 2X t.c. PBS (16.0 g NaCl, 0.4 g KCl, 0.24 g KH2PO4, 1.82 g Na2PO4 per liter pH 7.2) were added and samples were spun at 1584 x g for 20 at 4 °C. Supernant was removed and added to clean polycarbonate centrifuge tube. Thirty ml of t.c. PBS was added to each tube for balancing and the samples were spun at 107, 100 x g for 1 hr at 4 °C. Supernant was discarded and the pellet was resuspended in 400 µl t.c. PBS. Samples were divided into four 100ul aliquots and stored at -80 °C until analysis.
Extraction of viral RNA and viral DNA

Pellets were extracted for RNA utilizing 6M guanidium isothiocyanate as a lysis solution and the RNeasy Mini kit (Qiagen, Valencia, CA). For extraction of DNA viruses, Qiagen DNA Blood and Tissue kit (Qiagen, Valencia, CA) was used. Extracted RNA and DNA were tested by real-time RT-PCR and PCR, respectively.

Indicator and Bacteriophage Analysis of Sewage

Male-specific bacteriophage (MSB) densities was determined by using a modified double-agar-overlay method with *E. coli* HS (pFamp) RR (ATCC #700891) as the host strain (Cabelli, 1988; Debartolomeis, 1991). Fecal coliforms densities, as described by Dufour 1975, were determined utilizing a HC membrane (Millipore Corp. Bedford, MA) filtration method along with mTEC agar (Becton, Dickinson, and Company, Franklin, Lakes, NJ) protocol to enumerate fecal coliforms and *Escherichia coli*.

Collection and Analysis of Shellfish

Shellfish (*Crassostrea virginica*) were collected monthly from a shellfish harvest area of Mobile Bay, AL. Oysters were depurated for 2-3 weeks and relocated 0.1 (station 1 or S1), 0.3 (station 2 or S2), 1.75 (station 3 or S3), and 3.75(station 4 or S4) nmi (nautical miles) down stream from the Williams sewage outfall during the months of September 2009 thru March 2009 (Figure 2).
Figure 2. Oyster Sentinel Locations in Mobile Bay (USFDA, 2009). In the large graph, the dark grey area represents land and the light grey area represents the bay.
Oysters were relocated as sentinels for 12 to 21 days, depending on weather conditions which could hamper the ability to retrieve the sentinels. Concentration and extraction of shellfish was performed utilizing a slightly modified method developed at FDA’s Gulf Coast Research Lab, Dauphin Island, AL (Mullendore et al., 2001).

**Virus Concentration and RNA Extraction.**

The oyster extraction method utilized (Mullendore et al, 2001, Shieh et al., 2003) was as follows: fifteen whole oysters were washed, shucked and the digestive diverticula from the oysters were removed to obtain a total of 25 g sample. The digestive diverticula was homogenized with 7X volume of H₂O. A total of 175 g of the homogenate was added into a tared 500-ml centrifuge bottle. Conductivity was measured using a 4-ml aliquot of the homogenate (Myron L Company, Model ARH1, Carlsbad, CA) and the homogenate was adjusted to less than 2000 µS (micro siemens). Viruses were absorbed onto the particulate by adjusting the pH to 4.8±0.3 and the mixture centrifuged for 20 min at 2,000 x g at 4°C. Following centrifugation, the supernatant was discarded. The pellet was eluted with 175 ml of 0.75M glycine-0.15M NaCl and pH adjusted to 7.5 ± 0.2, followed by an additional elution with 87.5ml of 0.5M threonine-0.15M NaCl. The eluates were combined and precipitated with 8% PEG-0.3M NaCl and incubated for 3 h or overnight at 4°C. Precipitates were spun and the pellet was resuspended in 12-ml of t. c. PBS. Samples were extracted first with 12-ml of chloroform, vortexed for 1 min and then centrifuged at 1,700 x g for 30 min at 4°C. The upper aqueous phase was transferred to a clean, 50ml conical tube. The remaining portion was extracted with 6-ml of 0.5 M-threonine-0.15 M NaCl and centrifuged as previously described. Both aqueous phases were combined and precipitated with 8% PGE-0.3 M NaCl for 3 h or overnight at
4°C. Following precipitation, samples were centrifuged at 20800 x g for 15 min at 4°C and pellets were extracted for RNA utilizing 6M guanidium isothiocyanate as a lysis solution and the RNeasy Mini kit (Qiagen, Valencia, CA). DNA was extracted using the DNA Blood and Tissue kit (Qiagen, Valencia, CA). Extracted RNA and DNA were tested by real-time RT-PCR and qPCR for NoV GI and GII, enteroviruses, ADV, and HAV.

*Indicator Bacteria and Bacteriophage Analysis of Shellfish*

Fecal coliform and *E. coli* densities were determined using a conventional five-tube, three-dilution MPN procedure with minimal modifications to the FDA Bacteriological Analytical Manual (BAM) and American Public Health Association (APHA) recommended procedures for the examination of shellfish APHA, 1970. Modifications to this procedure include blending of the shellfish meats and liquors without dilution buffer; this was necessary due to the multiple microbial analyses performed on each shellfish sample. Following homogenization, a 1:10 dilution of homogenate (10 g) was prepared with PBS (7.65 g NaCl, 0.21 g KH$_2$PO$_4$, 0.724 g Na$_2$PO$_4$ per liter pH 7.4). Ten ml of this dilution, a 1-g equivalent, was transferred to five tubes of 10-ml of double strength lauryl typtose broth (LST; Difco, Sparks, MD). One ml aliquots (0.1-g equivalent) were transferred to five tubes of single strength LST, while five 1-ml aliquots of a 1:100 dilution were also to single strength LST. Presumptive positive tubes were confirmed for fecal coliforms and *E. coli* using EC-MUG (Difco Laboratories, Sparks, MD) medium (Rippey et al., 1987). Male-specific bacteriophage (MSB) densities were determined using a modified double-agar-overlay method with *E. coli* HS (pFamp) RR as the host strain (Cabelli, 1988).
Cell Culture of Shellfish and Sewage Extracts

Five different cell lines: BGM (US EPA, Cincinnati, OH), A549 (ATCC #A45334 Manassas, VA), RD (GCSL, Dauphin Island, AL), Caco-2 (ATCC #HTB-37), and FRhk-4 (GCSL) were used for culture of enteric viruses from waste water and shellfish extracts. Minimal Essential Media (MEM, Invitrogen, Carlsbad, CA) with 10% Fetal Bovine Serum (FBS, Invitrogen, Carlsbad, CA) was used for BGM, FRhk-4, and RD cell lines and Dulbecco’s Modified Eagle’s Media (DMEM, Invitrogen, Carlsbad, CA) with 15% FBS was used for A549 and Caco-2 cell lines. All cell line media contained the added components purchased from Invitrogen, Carlsbad, CA: sodium bicarbonate, hepes, GlutaMax, kanamycin, gentamycin, and non-Essential Amino Acids. Cell lines prepared for infection also contained fungizone (Invitrogen, Carlsbad, CA). Each cell line was grown, incubated at 37 °C in 5% CO₂, and maintained in 75 cm² flasks (Costar, Corning, CA). Once 90% confluent, 24-well plates (Costar, Corning, CA) were made for each cell line. Separate incubators were used for passage cell and infected cells. The shellfish and sewage concentrates (influent and effluent) were extracted with chloroform to eliminate possible cell toxicity. Adenovirus type 40 Dugan strain (ATCC VR-931), the poliovirus type 3 (Sabin strain), and HAV (HM175/18f GCSL) were used as positive controls for demonstrable cytopathic effect (CPE). PBS was used as the negative control. A 1 to 5 dilution was made of the influent, effluent, and shellfish extracts and 40µl, equivalent to ~0.05g of shellfish tissue was inoculated into 2 wells of the 24 well plate for each cell line. An additional 1 to 10 dilution of the extracts were made and inoculated into 2 wells of a 24 well plate for the corresponding cell lines (Figure 3). For virus adsorption, the plates were incubated for 1.5 hrs at 37°C with rocking and rotating every 15 min. After
incubation, 1 ml of 2% MEM or DMEM-FBS was added to each well. Plates were incubated for 3 weeks at 37°C and were read with inverted microscope (Olympus, Germany) at day 1, 3, 7, 10, 14, and 21 for observance of CPE. An additional 1 ml of corresponding media was added to each well over the 3 week incubation (Figure 3). Following the 3 week incubation, all plates were freeze thawed and cell lysate was chloroform extracted and tested by real-time PCR or RT-PCR (Figure 4). The TCID50 and PFU conversion was determined using the Reed and Muench calculation (Figure 4) (Reed, 1938).

![Figure 3. Diagram of Cell Line Inoculum](image)

Figure 3. Diagram of Cell Line Inoculum
**Reed & Muench Calculator**

1. Enter the starting dilution: initial dilution: 0.02
2. Enter the dilution factor: dilution factor: 10
3. Enter the volume tested per well: ml: 0.03

<table>
<thead>
<tr>
<th>This is your calculated dilution series:</th>
<th>2.00E-02</th>
<th>2.00E-03</th>
<th>2.00E-04</th>
<th>2.00E-05</th>
<th>2.00E-06</th>
<th>2.00E-07</th>
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</thead>
<tbody>
<tr>
<td>total wells:</td>
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<td>2</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>total wells:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>positive wells:</td>
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<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>These values are calculated automat...</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>negative wells:</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<td>2</td>
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<td>cum pos:</td>
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<tr>
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<td>3</td>
<td>4</td>
<td>8</td>
<td>10</td>
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<tr>
<td>% infected:</td>
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<td>0.00</td>
<td>0.00</td>
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<td>0.00</td>
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<tr>
<td>prop dist:</td>
<td>4.5</td>
<td>-0.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

6. This is your TCID50
TCID50: 6.32E-03

7. This is your TCID50/ml
TCID50/ml: 5.27E+03

*Figure 4.* Reed and Muench calculator used to enumerate TCID\(_{50}\) in cell culture
Figure 5. Detection of enteric viruses in oysters utilizing ICC-PCR, cell culture and direct PCR (Choo and Kim, 2006)
Identification of Extracts by Molecular Techniques

Quantitative real-time PCR and RT-PCR for viral RNA detection has allowed rapid, sensitive detection and enumeration of pathogenic viruses present in wastewater and shellfish. For this reason, viral extracts were identified utilizing real time PCR and qRT-PCR with previously published primers and probes (Table 6).

Table 6.

*Primers and Probes used in this Study.*

<table>
<thead>
<tr>
<th>Norovirus</th>
<th>GI</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>CGYTGGATGCGNTTYCATGA (Kageyama, 2003)</td>
<td>84bp</td>
</tr>
<tr>
<td>Reverse</td>
<td>CTTAGACGCCATCATATTYAC (Kageyama, 2003)</td>
<td></td>
</tr>
<tr>
<td>Probe 1</td>
<td>Cy5&lt;sup&gt;1&lt;/sup&gt;-AGATYGCGATCYCCTGTCCA-IBRQ&lt;sup&gt;2&lt;/sup&gt; (Kageyama, 2003)</td>
<td></td>
</tr>
<tr>
<td>Probe 2</td>
<td>Cy5-AGATCGCGGTCTCCTGTCCA-IBRQ (Kageyama, 2003)</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>TGGACICGYYGGICCYAAYCA (Beuret, 2002)</td>
<td>212bp</td>
</tr>
<tr>
<td>Reverse</td>
<td>GAAACGCATCCARCGGAACAT (Beuret, 2002)</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>CNTGGGAGGGCGATCGCAA (Kojima, 2002)</td>
<td>324bp</td>
</tr>
<tr>
<td>Reverse</td>
<td>CCRCCNGCATRHCCRTTRTACAT (Kojima, 2002)</td>
<td></td>
</tr>
</tbody>
</table>

Norovirus GII

| Forward          | CARGARBCNATGTYYAGRTGGATGAG (Kageyama, 2003) | 97bp         |
| Reverse          | TCGACGCATTTCTCATTCA (Kageyama, 2003) |              |
| Probe            | Cy3<sup>3</sup>-TGGGAGGGCGATCGCAATCT-IBRQ (Kageyama, 2003) |              |
| Forward          | TGGACICGYYGGIICCAYAAYCA (Beuret, 2002) | 212bp        |
| Reverse          | GAAYCTCATCCAYCTGAACAT (Beuret, 2002) |              |
| Forward          | CNTGGGAGGGCGATCGCAA (Kojima, 2002) | 324bp        |
| Reverse          | CCRCCNGCATRHCCRTTRTACAT (Kojima, 2002) |              |
Table 6 (continued).

<table>
<thead>
<tr>
<th>HAV</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward</strong> ATAGGGTAACAGCGCCGATAT (Gardner, 2003)</td>
<td>89bp</td>
</tr>
<tr>
<td><strong>Reverse</strong> AATGCATCCACTGGATGAG (Gardner, 2003)</td>
<td></td>
</tr>
<tr>
<td><strong>Probe</strong> Cy5-AGACAAAAACCATTCAACGCCGAGG-IBRQ (Gardner, 2003)</td>
<td></td>
</tr>
</tbody>
</table>

**Adenovirus**

| **Forward** GCC CCAGTGGTCTTACATGCACATC (Hein, 2005) | 127bp |
| **Reverse** GCACGGTGCTGGTTCTAAACTT (Hein, 2005)  |      |
| **Probe** FAM4-TGCACCAGACCCCGGCTACGTACTCCGA-BHQ15 (Hein, 2005) | |

**Internal Control**

| **Forward** GACATCGATATGGGTGCGC (Depaola, 2010) | 146bp |
| **Reverse** AATATTCGCGAGAGCAGATGCAG (Depaola, 2010)  |    |
| **Probe** TxRed6-TTCATGCGTCTCCCTGGAATGTG-IBRQ (Depaola, 2010) | |

**Enterovirus**

| **Forward** CCTCCGGCCCCTGAATG (Donaldson, 2002) | 196bp |
| **Reverse** CACCGGATGGCCAATCCAA (Donaldson, 2002)  |     |
| **Probe** Cy5-CGGACACCCAAAGTAGTGCCTCGG-IBRQ (Donaldson, 2002) | |

Primers and probes for this study were purchased from Integrated DNA Technologies (Coralville, IA).

1Cy5™, Cyanine 5, 2IBRQ, Iowa Black® RQ, 3Cy3™, Cyanine 3, 4FAM™, 6-Carboxyfluorescein, 5BHQ1, Black Hole Quencher®-1, 6TxRed, Texas Red®-X NHS Ester
Real-time Reverse Transcription--PCR. Norovirus and Enterovirus.

This assay was originally designed as a 4-plex assay with detection of NoV GI, NoV GII, enterovirus, and an internal amplification control (IAC). During this study, enterovirus detection was assayed in a separate reaction. Positive controls used for NoV GI and GII were in vitro RNA transcripts of sequences cloned from positive clinical samples previously identified as NoV (Burkhardt et al, 2006). Positive controls used for enterovirus were in vitro RNA transcripts of sequences cloned from poliovirus type 3 Sabin. Primers and probes for NoV GI and GII targeted the most conserved region of the ORF1-ORF2 junction (Table 6) (Kageyama et al., 2003). Primers and probes for enterovirus targeted the 5’ UTR of the enterovirus genome (Table 6) (Donaldson et al., 2002). Real-time RT-PCR for detection of NoV GI, NoV GII and enterovirus with an RNA IAC was performed in a 25-µl reaction using a One-Step RT-PCR Kit (Qiagen, Valencia, CA). The primer concentrations for the NoV targets were 300 nM each and the concentrations for the IC primers (IC 46 F and 194R) were 75 nM each. The primer concentrations for the enterovirus target were 400 nM each with IC primers concentration previously described. The 5’ nuclease probe concentration for NoV, enterovirus, IC targets were 100, 300, and 150 nM each, respectively. The final concentration of MgCl₂ in each RT-PCR reaction was 4 mM. Thermal cycling was run using a SmartCyclerII system® with the following conditions: 50°C for 3000 s, 95°C for 900 s followed by 50 cycles of 95°C for 10 s, 53°C for 25 s, 62°C for 70 s. Fluorescence was read at the end of the 62°C elongation step SmartCycler II®. Default analysis parameters were used, except for the manual threshold fluorescence units that were set to 10. Samples positive with the initial primer and probe sets for NoV GI and/or NoV GII (Kageyama et al., 2003) were
amplified with primers from the B region or C region by conventional RT-PCR as previously described (Table, 6) (Beuret et al., 2002; Kojima et al., 2002). The RT-PCR products amplified from the region B primers (MON 431, 432, 433, and 434), region C primers (G1GSKF, G1GSKR, G2GSKF, and G2GSKR), and enterovirus primers were run on a 2% agarose and visualized using ethidium bromide (0.5µg/ml). Fragment sizes were compared with commercially available size standards (Invitrogen, Carlsbad, CA).

The positive gel bands corresponding to the correct product size were excised and extracted using Qiagen gel extraction kit (Qiagen, Valencia, CA). Extracted products were cloned with TOPA-TA according to manufactures instructions (Invitrogen) or amplified using M13 labeled primers (Woods et al., 2007). Products were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Clones and M13 labeled products were sequenced utilizing Big Dye Terminator (Beckman Coulter, Fullerton, CA).

Real-time Reverse Transcription PCR for HAV

The positive control used for HAV was the vaccine strain (HM175/18f; subgenotype 1B) propagated in house utilizing FRhK cell line. Real-time RT-PCR for detection of HAV with an RNA IC was in a 25-µl reaction using a One-Step RT-PCR Kit (Qiagen). The primer concentrations for HAV and the IC were 300 nM and 75 nM, respectively, the 5’ nuclease probe concentrations for HAV and the IC targets were 200 and 150 mM, respectively. The final concentration of MgCl₂ in the RT-PCR reaction was 4 mM. Thermal cycling was performed using a SmartCyclerII system® with the following conditions: 50°C for 3000 s, 95°C for 900 s followed by 50 cycles of 95°C for 10 s, 53°C for 25 s, 64°C for 70 s. Fluorescence was read at the end of 70°C elongation.
step SmartCycler II®. Default analysis parameters were used, with the exception being the manual threshold fluorescence units which were set to 10.

**Real-time PCR of Adenovirus**

Positive controls for adenovirus were extracted DNA obtained from cell lysate of adenovirus type 40 Dugan strain (ATCC VR-931). The real-time PCR cycling protocol and reaction component concentrations were optimized for detection of the hexon gene of all 51 serotypes of adenovirus (Heim et al., 2003). The 25-μl reaction contained the following: 1X PCR Buffer (Invitrogen, Carlsbad, CA), 5 mM MgCl₂, 0.4-mM each dNTP (Qiagen, Valencia, CA), 1.25 U Platinum Taq polymerase (Invitrogen), 400 nM each forward and reverse adenovirus primer, and 200-nM of a 5’nuclease probe for adenovirus. Real-time PCR cycling was run using the SmartCycler II® system utilizing at the following cycling parameters: 95°C for 120 s, followed by 50 cycles of 95°C for 3 s, 55°C for 10 s, and 65°C for 60 s. Default analysis parameters were used except the manual threshold fluorescence units setting was adjusted to 10. This real-time PCR assay has been previously shown to have a limit of detection of 10 targets per reaction (Heim et al., 2003) Products were run on a 2% agarose and visualized using ethidium bromide (0.5μg/ml). Fragment sizes were compared with commercially available size standards (Invitrogen, Carlsbad, CA).

The positive gel bands corresponding to the correct product size were cut and extracted using Qiagen gel extraction kit (Qiagen, Valencia, CA). Products were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Extracted products were cloned using TOPO TA cloning kit according to manufactures instructions or amplified using M13 labeled primers (Woods et al.,
Clones and M13 labeled products were sequenced utilizing Big Dye Terminator (Beckman Coulter, Fullerton, CA).

**Quantification of Noroviruses, Enteroviruses and Adenoviruses using RT-PCR Units**

Quantification of NoV, enterovirus, and adenovirus levels were based on standard curve \( r^2 > 0.99 \) using real-time RT-PCR assay. Standard curves were based upon the end-point dilutions and the endpoint would be established where only 2 of 3 positive reactions were assigned a value of 1 RT-PCR unit. Negative samples were described as a RT-PCR unit with a value of 0.

**Sequencing of Viral Isolates from Stool, Sewage, Oyster Extracts and Cell Lysate**

RT-PCR or PCR products of corresponding were purified with Qiagen Gel Extraction kit (Qiagen, Valencia, CA). Products were sequenced directly utilizing M13 primers (Woods et al., 2007) or cloned with the TopoTA (Invitrogen, Carlsbad, CA) cloning kit with *E.coli* as the host according to manufactures instructions. Sequencing was conducted with the Big Dye Terminator sequencing kit (Beckman Coulter, Fullerton, CA) and the CEQ 8000 (Beckman Coulter, Fullerton, CA) sequence analyzer. Sequence alignments were obtained using BioEdit (Hall, 1999). Sequence alignment and comparison was performed using the NCBI bl2seq and MEGA4 (Tamura et al., 2007) alignment program.
CHAPTER IV

RESULTS

Virus Positive Stool Samples

Stool samples were collected from a local hospital and analyzed for NoV GI, GII, human enteroviruses, ADV, and HAV. The zip codes of each positive sample are listed in Table 7. Based on a map of the MAWWS (Mobile Area Water and Sewage System) and the zip codes obtained from the patient stool samples, between 38 and 80% of the positive samples were in the MAWWS catchment. Other zip codes were from local treatment plants within 40 square miles of MAWWS.
Table 7

*Zip Codes of Virus Positive Stool Samples*

<table>
<thead>
<tr>
<th>September</th>
<th>October</th>
<th>November</th>
<th>December</th>
<th>January</th>
<th>February</th>
<th>March</th>
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<tbody>
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<td>36605</td>
<td>36695</td>
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</tr>
</tbody>
</table>
Of the 401 samples collected, 4.7%, 2.5% and 13.8% were positive for NoV GII, enterovirus, and HuADV, respectively. NoV G1 and HAV were not detected in any of the stool samples. December had the greatest number of NoV positive samples and there were no NoV positive samples for the month of November. The number of enteroviruses positive samples accounted for 2.5% of total positive samples. During the months of September and March, no enteroviruses were detected and the greatest number of positives occurred during the month of October (Table 8).

Table 8

*Number of Samples Collected Each Month and Number Virus Positive*

<table>
<thead>
<tr>
<th>Month</th>
<th>Sept 08</th>
<th>Oct 08</th>
<th>Nov 08</th>
<th>Dec 09</th>
<th>Jan 09</th>
<th>Feb 09</th>
<th>Mar 09</th>
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</thead>
<tbody>
<tr>
<td># samples</td>
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<td>36</td>
<td>31</td>
<td>96</td>
<td>51</td>
<td>49</td>
<td>55</td>
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<td>0</td>
<td>6</td>
<td>1</td>
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<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Adv pos</td>
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<td>4</td>
<td>9</td>
<td>16</td>
<td>5</td>
<td>4</td>
<td>8</td>
</tr>
</tbody>
</table>

Adenoviruses were detected in stool samples for each month samples that were collected. Gel analysis of the 127 bp product from October samples are shown in figure 6. The highest number of positive HuADV and NoV stool samples were collected in the month of December. December was also the month that had the most stool samples collected. During the months of September and March there were no enteroviruses detected. The highest percentage positives of NoV occurred during the month of March and the highest percentage of adenovirus positives occurred during the month of
November (Table 9). The highest percentage of enterovirus positives occurred during the month of October.

![Figure 6](image)

*Figure 6.* Gel photo of adenovirus 127 bp product from October stool sample set. Lanes: 1) stool 16; 2) stool 22; 3) stool 24; 4) stool 31; 5) POS adenovirus positive control; 6) NEG negative control; L: (100 bp ladder)

Table 9

*Percentage Positive of Samples Collected*

<table>
<thead>
<tr>
<th>Month</th>
<th>Sept 08</th>
<th>Oct 08</th>
<th>Nov 08</th>
<th>Dec 09</th>
<th>Jan 09</th>
<th>Feb 09</th>
<th>Mar 09</th>
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</thead>
<tbody>
<tr>
<td>#samples</td>
<td>83</td>
<td>36</td>
<td>31</td>
<td>96</td>
<td>51</td>
<td>49</td>
<td>55</td>
</tr>
<tr>
<td>% coll</td>
<td>20.7</td>
<td>9.0</td>
<td>7.7</td>
<td>23.9</td>
<td>13.7</td>
<td>12.2</td>
<td>13.7</td>
</tr>
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<td>NoV pos</td>
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<td>2.0</td>
<td>6.1</td>
<td>7.3</td>
</tr>
<tr>
<td>Ent pos</td>
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<td>6.4</td>
<td>2.1</td>
<td>3.9</td>
<td>2.0</td>
<td>0</td>
</tr>
<tr>
<td>Adv pos</td>
<td>10.8</td>
<td>11.1</td>
<td>29.0</td>
<td>16.7</td>
<td>9.8</td>
<td>8.2</td>
<td>14.5</td>
</tr>
</tbody>
</table>
Nucleotide sequences from positive stool samples were analyzed utilizing BLAST (www.ncbi.nlm.nih.gov/BLAST). Multiple strains, subgroups and serotypes were identified during each month (Table 10).

Table 10.
Classification of Positive Stool Sample Isolated During Each Month

<table>
<thead>
<tr>
<th>September</th>
<th>October</th>
<th>November</th>
<th>December</th>
<th>January</th>
<th>February</th>
<th>March</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADV 31</td>
<td>ADV 41</td>
<td>ADV 12</td>
<td>NoV GII.4</td>
<td>NoV GII.4</td>
<td>NoV GII</td>
<td>NoVGII.4</td>
</tr>
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<td>NoV GII.4</td>
<td>ADV 5</td>
<td>ADV 41</td>
<td>NoV GII.7</td>
<td>Poliovirus3</td>
<td>NoV GII.7</td>
<td>ADV 12</td>
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<td>ADV 5</td>
<td>ADV 2</td>
<td>ADV 2</td>
<td>NoV GII.4b</td>
<td>ADV 2</td>
<td>ADV 41</td>
<td>ADV 6</td>
</tr>
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<td>Echovirus30</td>
<td>ADV 41</td>
<td>ADV 41</td>
<td>Cosack A4</td>
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<td>Enterovirus90</td>
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<td>Enterovirus71</td>
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<td>ADV C</td>
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</tr>
</tbody>
</table>

Enterovirus was detected the least with only 10 positive samples. Figure seven represents an example of gel analysis of the 196 bp product of an enterovirus positive stool sample for the month of October. For the ten positive enterovirus stool samples, only seven were able to be sequenced. Nucleotide sequence analysis using nucleotide BLAST yielded 97-100% identities for all enteroviruses analyzed. Analysis of the sequence of the enterovirus positive stool samples were from groups A-C. There were no positive stool samples from group D (Figure 8). There were no identical sequences for enteroviruses isolated from stool samples. Sequence identities for Echovirus 30 were 100% identity with BLAST analysis. During the months of January and February
poliovirus 3 and coxsackievirus A4 were 100% identity with their corresponding nucleotide BLAST.

*Figure 7.* Gel of enterovirus positive stool sample. Lanes; L; (100 bp ladder); 1) 5ul October stool sample 11; 2) negative control; 3) enterovirus positive control.
Figure 8. Phylogenetic tree showing relationship between sequences of positive enterovirus stool samples. Alignment was done with ClustalW using the 196 bp of the 5’ untranslated region. Month, sample type and virus are represented as; Oct= October, Feb= February, Dec= December, inf= influent sample, EV 90= enterovirus 90, Echo30= Echovirus 30, CoxA4= coxsackievirus A4, CoxA24= coxsackievirus A24, and CoxB4= coxsackievirus B4. The tree was constructed using neighbor-joining method (MEGA 4.0). Scale bar represented 1.0 substitutions per base position. GenBank reference strains are included. See table 4 for representative of enterovirus groups.
For NoV positive stools, 12 of the 19 positive stool samples were able to be identified with the 212 or 342 bp product (Figure 9). Most samples sequenced were identified as HuNoV genotype II and no genogroup I was identified during qRT-PCR or sequence analysis. There were only 3 different strains identified; NoV GII.4, GII.4b, and GII.7. No strains showed 100% identity in nucleotide blasts but there were several strains with ≥97% identities with nucleotide blast. When comparing NoV sequences from September and October samples, the sequences were 99% identical. The September NoV positive samples stool 44 and 45 were from the same zip code but the October positive sample stool 29 was from a different zip code in the greater Mobile area. All three of those isolated were 100% identical when analyzed by BLAST. Bootstrap values based on 500 replicated ranged from 30 to 97%. The sequence from the original norovirus outbreak in Norwalk, OH was used as the outgroup phylogenetic tree constructs. Sequences from NoV genogroups II and IIb were used as reference strains (Figure 10).
Figure 9. Gel analysis of stool sample 44 and 45 for NoV of 342 and 212 bp products. Lanes 1-7 represent 342 bp product and lanes 8-13 represent 212 bp product. September stool isolates 44 and 45 were analyzed by RT-PCR with 3µl and 2µl volumes. Lanes: 1) 3µl stool 44; 2) 2ul stool 44; 3) 3µl stool 45; 4) 2µl stool 45; 5) negative control; 6) positive control; 7) positive control; L; (100 bp ladder); 8)3µl stool 44; 9) 2ul stool 44; 10) 3µl stool 45; 11) 2µl stool 45; 12) negative control; 13) positive control
Figure 10. Phylogenetic tree showing relationship between sequences of positive human norovirus stool samples. Alignment was done with ClustalW using the 212 bp of RdRp. Month, sample type and virus are represented as; Sept= September, Oct= October, Feb= February, Dec= December, Jan= January, NoV= norovirus, GI= norovirus genotype I, and GII= norovirus genotype II. Tree was constructed using neighbor-joining method (MEGA 4.0). Scale bar represented 0.1 substitutions per base position. GenBank reference strains are included. Bootstrap value are indicated as % of 500 replicates and HuNoV GI is used as the outgroup.
For the ADV positive stool, 38 of 55 isolates were sequenced. Isolates unable to be sequenced Subgroups A, B, C, D, and F were identified by using the 127 nt sequence utilizing BLAST. All sequences showed ≥95% identity. ADV type 41 was found in the majority of stool samples tested, although type 41 was not detected during the month of September. ADV type 2, which affects the respiratory tract, appeared to be the second most common isolate identified. With the phylogenetic tree construct, all of the ADV positives were correctly classified according to the different subgroups A-F (Figure 11). Stool 3 and stool 14 showed ≥95 % homology with adenovirus 41 during the month of October and they were 100% identical. October stool samples 4, 16, and 22 were 100% identical and showed ≥97% identity with adenovirus serotype 2. November stool isolates 7, 22, and 23 were 100% identical and showed ≥95 identities with adenovirus serotype 41. During the month of December stool 15, 16 and 17 were 100% identical and showed ≥95 identities with adenovirus serotype 41. During the month of December stool 15, 16 and 17 were 100% identical and showed ≥95 identities with adenovirus serotype 41. There were no adenovirus positive stools in January that were 100% identical. During the months of February, stool 16 and 17 were 100% identical; March stools 40, 42, and 49 were 100% identical and all showed ≥95 identity with adenovirus serotype 41.
Figure 11. Phylogenetic tree showing relationship between sequences of positive ADV stool samples. Alignment was done with ClustalW using the 127 bp of the adenovirus hexon gene. Month, sample type and virus are represented as; Sept= September, Oct= October, Nov= November, Feb= February, Dec= December, Jan= January, Mar= March, and ADV= adenovirus. The tree was constructed using neighbor-joining method (MEGA 4.1). Scale bar represented 0.1 substitutions per base position. GenBank reference strains are included. Bootstrap value are indicated as % of 500 replicates.
Bacterial Indicators in Sewage and Shellfish

Fecal Coliform Levels in Sewage

Sewage samples were collected from Mobile’s WWTP 1 to 3 days before retrieving the oyster sentinels. Indicator levels in influent was reduced 6 to 7 logs which yielded 99.99% reduction in bacterial load for all months of the study and for each indicator species. During the colder months of December, January, and February, indicator bacteria were reduced by 7 logs. Fecal coliform and *E. coli* levels in the influent and effluent were similar for all collection months. The highest levels of fecal coliforms in the effluent occurred during the month of October (Figure 12). The highest levels effluent of *E. coli* occurred during the month March and the lowest occurred during the month of September (Figure 13).
Figure 12. Fecal coliform densities in sewage. Levels were determined using a HC membrane. Results are expressed as log10 fecal coliforms per 100 ml. The blue bar represents influent and the pink bar represents effluent for each collecting months.
Figure 13. *E. coli* densities in sewage. Levels were determined using HC membrane. Results are expressed as $\log_{10} E. coli$ per 100 ml. The purple bar represents influent and the yellow bar represents effluent for each collecting month.

**Fecal Coliform Levels in Shellfish**

Fecal indicators in shellfish varied from month to month depending on water temperature and salinity. The temperature and salinity levels were recorded for each station when the oyster sentinels were placed at each station and when the sentinels were removed from each station. September saw the highest water temperature with an average of 26.9°C. February had the lowest average water temperature 9.4°C (Figure 14). The highest average salinity of 17.6 ppt (parts per thousand) occurred in the month of November and the lowest levels of salinity occurred during the month of January and March with average salinity levels of 3.2 and 2.3 ppt, respectively.
Figure 14. Temperature and salinity for each sampling month. The four points for each month are temperature and salinity for station 1, 2, 3, and 4. Wtemp = water temperature measured in °C and salinity was measured as ppt.

Fecal coliform levels in shellfish ranged from 20/100g to ≥16,000/100g (Figure 15). Fecal coliform levels were highest during the warmer month of September and lowest during the month of January. During most months, fecal indicators decreased in concentration as distance from MAWWS outfall to station 4 increased. In one instance the fecal coliform levels and *E. coli* levels were higher for station 2 than for station 1. A p value <0.05 indicated that there was a relationship between the presence of fecal coliforms and the presence of *E. coli* in shellfish (Figure 16).
Figure 15. Fecal coliform levels in Shellfish for Each Month of Sample Collection. Data is displayed as log pfu/100g. Each set of bars for each corresponding month represent stations 1-4. Fecal indicator levels were determined using MPN 5-tube 3 dilution.
Figure 16. Linear regression of fecal coliforms and *E. coli*. A graph of fecal coliform densities and *E. coli* showing the relationship between the level of each indicator. Densities are presented as log/100g. Indicators were determined using 5-tube 3-dilution with minimal modification to BAM and APHA protocols.

**Bacteriophage in Sewage and Shellfish**

Male-specific bacteriophage (MSB) levels were determined in sewage and shellfish using the double agar overlay method. All sewage influent samples required at least one dilution to ensure that the plate count results were not too numerous to count. Bacteriophage levels in sewage influent were greatest during the month of September at $2.4 \times 10^5 /100$ ml (Figure 17). The second highest levels in the influent occurred during the month of March. December and January had similar levels in the influent samples.
Levels of bacteriophage in the influent averaged $10^5$ and the average bacteriophage levels in the effluent were $10^2$. The percent reduction in the sewage effluent for bacteriophage were during the months of September, October, November, January, February, and March were 99.99, 97.10, 99.98, 99.84, 99.91, 99.86, and 99.90, respectively. Although the highest levels for bacteriophage influent occurred during the month of September, the reduction levels were also greatest in September.

![Bacteriophage Levels in Sewage Influent and Effluent](image)

*Figure 17.* Bacteriophage levels in sewage influent and effluent. The levels are expressed as log phage/100ml. Levels are listed for each month that sampling occurred.

Bacteriophage levels in shellfish appeared to vary based on water temperature.

The correlation between fecal coliforms and bacteriophage was determined using a linear regression model (Figure 18). The p value was greater than 0.05 which indicates no significant correlation between the presence of fecal coliforms and the presence of
bacteriophage in shellfish. A p value <0.05 using Fisher exact test with multiple regressions indicates that there was a statistical relationship between the presence of NoV GII and bacteriophage (Table 11).

Table 11

Correlation Between Presence of NoV GII and Indicators

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</table>
Figure 18. Linear regression plot of fecal coliforms and bacteriophage in shellfish. The fecal coliforms and bacteriophage are expressed as log per 100 g.

Bacteriophage was consistently detected at station 1 and 2 for each sampling month (Figure 19). Levels were highest at station 1 and 2 during the months of February and December and lowest for those stations during the months of January and March. During the months of September, December, and February, bacteriophage were detected at all four stations with levels ranging from 5 to 3921 PFU/100ml. The recorded average water temperature for the sentinel stations was lowest for the month for February. Bacteriophages were present at station 3 for five out of seven collection months. Station 1 was positioned closest to the WWTP outfall but in 4 out of 7 collection months, the bacteriophage levels in shellfish were higher for station 2.
Figure 19. MSB levels in shellfish and water temperature for each sampling month at each station. Station 1=burgundy, Station 2= green, Station 3=pink, station 4=blue. Bacteriophage levels are expressed as log PFU per 100g.
Virus Positive Sewage and Shellfish Samples

*Virus Positive Sewage Samples*

Direct analysis for enteric viruses was done on sewage concentrates. The concentrate were extracted for RNA or DNA and analyzed by PCR/RT-PCR. Norovirus GII was detected in the influent for all sampling months and detection of NoV GII in the effluent only occurred in the month of October (Figure 20). Norovirus GI was only detected in the influent samples for the months of September and October. The highest levels of GII occurred during the month of March and the lowest levels occurred during the month of January. Analysis of sequences 212 nt sequence of NoV RdRp from the influent and effluent yielded products of GI and GII. Influent sequences for the months of September, October, November, December and January, February and March showed 99, 98, 99, 99, 98, 99 and 98 percent identities, respectively, when analyzed with nucleotide BLAST. Phylogenetic analysis of influent from September, October, December, and February was completed (Figure 21). Partial sequences for sewage influents were obtained from the samples collected during the months of November, January, and March and were not included in the phylogenetic analysis.
Figure 20. Detection of NoV in sewage influent and effluent. Influent and effluent levels were determined for each sampling month. Values are expressed as log RT-PCR units per 100ml. The blue bar represents G1 influent and the burgundy bar represents G2 influent. The red bar represents GII effluent. NoV GI was not detected in the effluent.
Figure 21. Phylogenetic tree showing relationship between sequences of positive NoV sewage samples. Alignment was done with ClustalW using the 212 bp of RdRp. Month, sample type and virus are represented as; Sept= September, Oct= October, Feb= February, Dec= December, inf= influent sample, GII= NoV GII. Tree was constructed using neighbor-joining method (MEGA 4.0). Scale bar represented 1.0 substitutions per base position. GenBank reference strains are included. Bootstrap values are indicated as % of 500 replicates.

Enteroviruses were detected in sewage influent for all sampling months but were not detected in any effluent samples. Levels were highest for the month of September and lowest for the month of February (Figure 22). Sequence analysis of the 196 bp product
showed ≥96% identities with nucleotides BLAST. Echovirus 30, coxsackievirus B4, enterovirus 90 and enterovirus 71 were identified in sewage isolates. Phylogenetic analysis of the 196 nt sequences from influent were constructed based on neighbor-joining method using MEGA 4.1 (Figure 23).

Figure 22. Enterovirus levels in sewage influent for each sampling month. Levels are expressed as log_{10} RT-PCR units per 100 ml. The pink bar represents influent levels for the corresponding month. Enteroviruses were not detected in any effluent samples during this study.
Figure 23. Phylogenetic tree showing relationship between sequences of positive enterovirus sewage isolates. Alignment was done with ClustalW using the 196 bp of the 5' untranslated region. Tree was constructed using neighbor-joining method (MEGA 4.0). Month, sample type and virus are represented as; Sept= September, Oct= October, Feb= February, Dec= December, Lys= cell lysate, inf= influent sample, EV= enterovirus, Echo= Echovirus, CoxA4= coxsackievirus A4, and CoxB4= coxsackievirus B4. Scale bar represented 2 substitutions per base position. GenBank reference strains are included. Bovine enterovirus and simian enterovirus were used as outgroups. Bootstrap values are indicated as % of 500 replicates.

Adenoviruses were present in the sewage influent and effluent for all sampling months (Figure 24). The highest levels for influent and effluent occurred during the month of March. Adenovirus viral loads were reduced by 93.2, 99.0, 94.8, 87.4, 92.1, 87.5, and 87.2% for September, October, November, December, January, February, and March, respectively. Of the seven collecting months, adenovirus viral loads were reduced
by 1.5 logs on average. Sequence analysis of the 127 bp product for positive influent and effluent showed ≥96% identities with nucleotide blast. Phylogenetic trees of the 127 nt sequences were constructed based on neighbor-joining methods by using MEGA 4.1 (Figure 25). Adenovirus 12, 52, and 41 were identified in sewage influent and effluent samples. October and March sequence analysis of influent and effluent showed 99% identities when aligned.

**Figure 24.** Detection of Adenoviruses in sewage influent and effluent. Influent and effluent levels were determined for each sampling month. Values are expressed as log RT-PCR units per 100ml. The blue bar represents adenovirus influent and the burgundy bar represents adenovirus effluent.
Figure 25. Phylogenetic tree showing relationship between sequences of positive adenovirus sewage isolates. Alignment was done with ClustalW using the 127 bp of the hexon gene. Tree was constructed using neighbor-joining method (MEGA 4.1). Month, sample type and virus are represented as; Sept= September, Oct= October, Feb= February, Dec= December, Jan= January, Mar= March, inf= influent sample, eff= effluent sample, and ADV= adenovirus. Scale bar represented 0.1 substitutions per base position. GenBank reference strains are included. Murine adenovirus was used as outgroups. Bootstrap values are indicated as % of 500 replicates.
**Virus Positive Shellfish Samples**

*Norovirus.*

Detection of enteric viruses in shellfish was done utilizing a modified adsorption-elution protocol. HuNoV GI, GII, human adenoviruses, and human enteroviruses were extracted and detected by qPCR or qRT-PCR. NoV GII was detected by qRT-PCR yielding a 97 bp amplicon and norovirus GI was detected by qRT-PCR yielding a 84 bp amplicon. In addition to qRT-PCR analysis, samples positive for NoV were amplified with conventional RT-PCR with another set of primers yielding a 212 for GI and GII, a 324 bp product for GI, and a 342 bp product for GII. Products of adenovirus and enterovirus extraction yielded amplicon sizes of 127 and 196 bp, respectively. All shellfish extracts amplified by conventional RT-PCR were sequenced and analyzed using nucleotide BLAST, BioEdit, and MEGA 4.0.

Norovirus GII were consistently detected by qRT-PCR at stations 1 and 2 for each sampling month (Figure 26). Norovirus GI was detected at station 2 during the month of September and at station 1 during the month of October by qRT-PCR. During the month of December, norovirus GII was detected at all 4 stations. The 212 bp fragment for GII positive samples was amplified for the December, February, and March sample set. The 342 bp fragment for GII was amplified for the September samples only. No GI samples were able to be amplified by conventional RT-PCR. Water temperature was highest during the month of September and lowest during February. NoV GII levels were highest during the month of February. There was no NoV detected during the month of January although the average water temperature was below 13°C and the average salinity was
around 3 ppt. Although the average salinity in March was 3 ppt, NoV GII was detected at station 1 and station 2.

The 342 bp amplicon from station 2 of the September sample set was sequenced and analyzed with nucleotide BLAST. The sequence showed ≥99% identities with nucleotides BLAST and ≥99% identities with September stool sample 44 and 45. The 213 bp amplicon from station 1 and station 2 of the December sample set was sequenced and analyzed with nucleotide BLAST. The sequences from station 2 showed ≥99% identities with nucleotide BLAST and ≥99% with December stool 52. Phylogenetic trees of the 342 nt sequence was constructed based on neighbor-joining methods by using MEGA 4.1 software (Figure 27). Bootstrapping (500 replicates) gave reliable values of >75 on the node for September stool and oyster samples. Phylogenetic trees of 212 nt sequence was constructed based on neighbor-joining methods by using MEGA 4.1 software (Figure 28). Bootstrapping (500 replicates) gave reliable values of >75 on the node for December stool 52 and station 2 oyster samples.
Figure 26. Detection of HuNoV in shellfish. NoV GI and GII levels were determined for each positive sample set from qRT-PCR assay. Values are expressed as log RT-PCR units per 100ml. The burgundy bar represents G1 levels at each station and the green bar represents G2 levels for the corresponding station. The blue line represents salinity and the yellow line represents water temperature. GI was only detected during the months of September and October.
Figure 27. Phylogenetic tree showing relationship between sequences NoV sequences from September stool and oyster. Alignment was done with ClustalW using the 342 bp of capsid region. Tree was constructed using neighbor-joining method (MEGA 4.1). Scale bar represented 0.001 substitutions per base position. GenBank reference strains are included. Bootstrap value are indicated as % of 500 replicates.
Figure 28. Phylogenetic tree showing relationship between sequences NoV GII sequences from December stool and oyster. Alignment was done with ClustalW using the 212 bp of RdRp region. Tree was constructed using neighbor-joining method (MEGA 4.1). Scale bar represented 1 substitution per base position. GenBank reference strains are included. Bootstrap values are indicated as % of 500 replicates.
Adenovirus.

Adenovirus was detected at station 1 in shellfish extracts for all sampling months and station 2 for six out of seven sampling months (Figure 29). During the month of December, adenoviruses were detected at stations 1-4. Adenovirus and bacteriophage detection were comparable for December, January, February, and March. Although the salinity levels for January and March were \( \leq 3 \) ppt, adenovirus and bacteriophage were detected at station 1 and 2. Sequence analysis of the 127 bp amplicon showed \( \geq 94\% \) identities with nucleotide BLAST sequences. All adenovirus positive stool, sewage, and oyster sequence alignments with \( \geq 99\% \) identities with were identified as adenovirus 41 with nucleotide BLAST. Adenovirus isolated from September stations 1 and 2 and effluent showed 99\% identity when aligned and was identified as adenovirus type 41. October station 1, influent and effluent showed \( \geq 99\% \) identity when aligned and was identified as adenovirus type 41. Adenovirus isolated during December from stations 1, 2, 3, 4 and effluent showed 100\% identity when aligned and was identified as serotype 41. Isolates from March stool 42 and stool 49, station 1 and station 2 showed 99\% identity when aligned and were identified as adenovirus type 41. Phylogenetic trees of 127 nt sequence from stool, sewage, and oyster was constructed based on neighbor-joining methods by using MEGA 4.1 software for September, October, November, December, January, February, and March sample set (Figures 30, 31, 32, 33, 34 and 35).
Figure 29. Detection of Adenoviruses and bacteriophage in shellfish. Adenovirus levels were determined for each positive sample set from qPCR assay. Values are expressed as log PCR units per 100ml. Bacteriophage level are expressed as log PU/100ml. The pink bar represents bacteriophage levels as each station and the green bar represents adenovirus levels for the corresponding station. The blue line represents salinity and the yellow line represents water temperature.
Figure 30. Phylogenetic tree showing relationship between sequences from September positive adenovirus shellfish isolates and effluent samples. Alignment was done with ClustalW using the 127 bp of the hexon gene. Tree was constructed using neighbor-joining method (MEGA 4.1). Scale bar represented 0.05 substitutions per base position. GenBank reference strains are included. Bootstrap values are indicated as % of 500 replicates.
Figure 31. Phylogenetic tree showing relationship between sequences from October positive adenovirus shellfish isolates and sewage samples. Alignment was done with ClustalW using the 127 bp of the hexon gene. Sample type and virus are represented as; inf= influent, eff= effluent sample, Lys= cell lysate, and ADV= adenovirus. Tree was constructed using neighbor-joining method (MEGA 4.1). Scale bar represented 5 substitutions per base position. GenBank reference strains are included. Bootstrap values are indicated as % of 500 replicates.
Figure 32. Phylogenetic tree showing relationship between sequences from November positive adenovirus shellfish isolates and stool samples. Alignment was done with ClustalW using the 127 bp of the hexon gene. Tree was constructed using neighbor-joining method (MEGA 4.1). Scale bar represented 0.5 substitutions per base position. GenBank reference strains are included. Bootstrap values are indicated as % of 500 replicates. Murine adenovirus was used as the outgroup.
Figure 33. Phylogenetic tree showing relationship between sequences from December positive adenovirus shellfish isolates and stool samples. Alignment was done with ClustalW using the 127 bp of the hexon gene. Tree was constructed using neighbor-joining method (MEGA 4.1). Scale bar represented 0.5 substitutions per base position. GenBank reference strains are included. Bootstrap values are indicated as % of 500 replicates. Murine adenovirus was used as the outgroup.
Figure 34. Phylogenetic tree showing relationship between sequences from January positive adenovirus shellfish isolates and stool samples. Alignment was done with ClustalW using the 127 bp of the hexon gene. Tree was constructed using neighbor-joining method (MEGA 4.1). Scale bar represented 0.1 substitutions per base position. GenBank reference strains are included. Bootstrap values are indicated as % of 500 replicates. Murine adenovirus was used as the outgroup.
Figure 35. Phylogenetic tree showing relationship between sequences from February and March positive adenovirus shellfish isolates and stool samples. Alignment was done with ClustalW using the 127 bp of the hexon gene. Tree was constructed using neighbor-joining method (MEGA 4.1). Scale bar represented 0.2 substitutions per base position. GenBank reference strains are included. Bootstrap values are indicated as % of 500 replicates. Murine adenovirus was used as the outgroup.
Cell Culture Analysis of Sewage and Shellfish Samples

A total of 28 oyster samples from and 14 sewage samples were analyzed by cell culture method using A549, BGMK, CaCo-2, and RD and by ICC-PCR (Table 12). Station 2 had the highest number of samples exhibiting CPE in the A549 cell line followed by the influent sample. Only 1 out of seven effluent samples exhibited CPE in the A549 cell line representing 7% of the samples exhibiting CPE. When assaying for adenovirus with the ICC-PCR method for A549 lysates 50% of the samples were positive. CPE in the BGMK cell line occurred in 19% of the samples assayed. ICC-PCR had a detection rate of 30% when assaying for enteroviruses. Less than 10% of the samples exhibited CPE in the CaCo-2 cell line. Only 9.5% of the samples tested positive for ICC-PCR method when assaying for enteroviruses. CPE was exhibited in 14.2% in the RD cell line and 19% of the samples were positive for enteroviruses using ICC-PCR. Overall BGMK cell line exhibited the highest level of CPE compared to CaCo-2 and RD. Altogether; infectious viruses were detected in 20.0% of the oyster samples and 17.8% of the sewage samples.
Table 12

*Adenovirus and Enterovirus Detection Frequency in Cell Culture and ICC-PCR Assays with A549, BGMK, CaCo-2, and RD Cell Line. CC = Cell Culture, ICC-PCR = Integrated Cell Culture-PCR, S1-S4 = Oyster Stations 1-4, INF = Influent. EFF = Effluent.*

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When comparing the detection of adenoviruses by direct detection and cell culture, adenoviruses were detected 57.1% by direct method and 42.9% by cell culture (Table 13). Enteroviruses were detected 10.7% by direct method and 10.7% by cell culture. During the month of December, adenoviruses were detected at station 1-4 by direct method and by cell culture. Virion levels by direct detection are expressed as PCR or RT-PCR units/ml and cell culture levels are expressed as PFU/ml. When comparing PCR units/ml to PFU/ml the ratio ranges from 1 to 100 to 1 to 10000. The ratio was the highest when there was no detection by the direct method. Sequence analysis of the A549 lysate showed that only adenovirus type 41 was identified. Sequence analysis of BGMK, CaCo-2, and RD lysate identified as enterovirus 90, echovirus 30, and coxsackievirus B4, respectively.
Table 13

Detection of Adenovirus and Enteroviruses at Each Station for Each Month. Calculations by Direct Methods Were Done Using Standard Curves and Numbers Represent RT-PCR units/100g. Detection of Adenovirus and Enterovirus by Cell Culture Methods Were Done by Observing CPE and Determining PFU/100ml Which are Represented in Figure. S1, S2, S3, S4 are Stations 1, 2, 3, and 4, CC = Cell Culture, –CPE = No Cytopathic Effect Observed, and ND = Not Detected

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<tr>
<td>December</td>
<td>Direct</td>
<td>9x10²</td>
<td>ND</td>
<td>2x10⁵</td>
<td>ND</td>
<td>4x10²</td>
<td>ND</td>
<td>7</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>2x10⁶</td>
<td>-CPE</td>
<td>3x10⁶</td>
<td>-CPE</td>
<td>3x10⁶</td>
<td>-CPE</td>
<td>3x10⁶</td>
<td>-CPE</td>
</tr>
<tr>
<td>January</td>
<td>Direct</td>
<td>3x10²</td>
<td>1</td>
<td>86</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td></td>
<td>CC</td>
<td>-CPE</td>
<td>-CPE</td>
<td>1x10⁵</td>
<td>-CPE</td>
<td>-CPE</td>
<td>-CPE</td>
<td>-CPE</td>
<td>-CPE</td>
</tr>
<tr>
<td>February</td>
<td>Direct</td>
<td>2x10²</td>
<td>ND</td>
<td>6x10²</td>
<td>ND</td>
<td>9x10²</td>
<td>ND</td>
<td>ND</td>
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</tr>
<tr>
<td></td>
<td>CC</td>
<td>-CPE</td>
<td>-CPE</td>
<td>-CPE</td>
<td>-CPE</td>
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<td>-CPE</td>
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<tr>
<td>March</td>
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<td>600</td>
<td>ND</td>
<td>605</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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</tr>
<tr>
<td></td>
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<td>1x10⁵</td>
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CHAPTER V
DISCUSSION

Importance of Present Study

The goal of this present study was to demonstrate a relationship between viral isolates from the community, local sewage treatment plant, and oysters placed as sentinels. The contributions of this study are 1) community survey of enteric viruses isolated from stool samples 2) demonstrating WWTP infectiveness in removal of enteric viruses 3) demonstrating a dilution effect of enteric virus levels in oysters downstream from WWTP 4) establishing a molecular relationship between community and oyster enteric virus isolates 5) isolation of culturable viruses in oysters where molecular identification showed significant relationship between community and oyster isolates 6) demonstration of bacterial indicators inability to index for enteric virus contamination of shellfish and 7) demonstrate molecular relationship between enteric viruses isolated from community, sewage, and oysters.

Virus Detection in Stool

Enteric viruses are the most common causes of infections in human population. Advancements in technology have allowed for better detection and isolation of theses microorganisms. Different strains or groups can circulate in the community and environment at any given time. During this study, 20% of the stool samples contained enteric viruses. Based on a map of the MAWWS and the zip codes obtained from the patient stool samples, two out of the seven months that samples were collected had less than fifty percent of stool samples that were not in the MAWWS catchment. Overall there was about 38% of the positive stool samples were from people not in the MAWWS
catchment based on the zip codes; majority of these positive samples were in neighboring towns. When this fact is considered, it may not be that significant considering that most of the positive samples showed 100% identity when analyzed by nucleotide BLAST and virus transmission does not know boundaries. Also, ill persons from out of town would visit doctors who would send samples to the hospitals.

There were eight different ADV serogroups detected from the community isolates. The most common isolates, 4 and 7, which causes Acute Respiratory Disease (ARD) in young military recruits was not detected (CDC, 2005). In addition, virulent strain of adenovirus 14, which was circulating in 2006 and 2007 and caused the death of 10 individuals, was not isolated (MMWR, 2007a). However, the common childhood serogroups 1, 2, and 5 were isolated. The detection of serogroups 2 and 5 appeared to dominate during the warmer months, while detection of enteric HuADV 40 and 41 predominated during the cooler months. Adenovirus type 41 was first detected in stool samples during the month of November although adenovirus 41 was detected in sewage and oyster samples collected during September and October. During the month of December, only enteric HuADV was detected.

During the months of December and March, enteroviruses were not detected in any stool samples. Different strains of enterovirus infections are found during varying months of the year but primarily during the spring and fall months. In addition, reported peak occurrence for enterovirus infection occurs during the summer month and tapers off during the fall months (MMRW, 2006). The annual incidence of enterovirus disease is estimated at 10-15 million cases in the United States (CDC, 2005). The approximate rate could be 1 in 27 or 3.63%. Non-polio enteroviruses are second only rhinoviruses or the
“common cold” as the most common viral infectious agent in humans. Considering these facts you would expect the detection rate of enteroviruses in the stool samples collected to be greater than 2%. Perhaps if respiratory specimens had been collected with stool samples, the detection rate would have been closer to the estimated annual incidence for the United States. There were five different serogroups of enterovirus detected during this study. The lowest reported incidence of enterovirus infections in the United States during the winter months (CDC, 2005). This is consistent with the monthly occurrence of the enterovirus detection stool samples collected during this study. Echovirus 30 was isolated during the months of October, November, and December echovirus 30 ranked number two in MMWR surveillance of enteroviruses in 2005. Coxsackievirus A4 and enterovirus 71 were isolated during the month of February and they ranked 14 and 15, respectively. During 2008, an enterovirus 71 outbreak occurred in China causing 78 deaths. Later it was found that this particular strain of enterovirus 71 was not circulating the United States or Europe at the time (Swiss Federal Department of Foreign Affairs, 2009).

According to the BLAST search, enterovirus 71 detected during this study was 93% related to the recent Chinese strains causing infections. Most of the enterovirus positive stool samples were from group A and group B as these groups causes most of the cases of enterovirus infections (MMRW, 2005). During the month of January, poliovirus 3 was isolated from a single stool sample. The current vaccine series in the United States for poliovirus consists of an IPV (inactivated poliovirus vaccine) which typically is not shed in the stool as with the OPV (oral poliovirus vaccine) which offers gastrointestinal immunity. Although the OPV is not included in the poliovirus vaccine series in the
United States, some physicians may continue to give OPV or perhaps there are visitors from other countries which may still receive OPV.

Most NoV positive samples occurred during the cooler months. For epidemiological purposes, identification of sequences with ≥97% homology with noroviruses can be considered highly significant and within the same cluster or strain. This was the case with positive HuNoV stool isolates and when comparing positive stool and shellfish isolates. There were only two strains isolated from stool samples, GII.4 and GII.7. Most of the GII.4 strains identified showed ≥99% homology to the Minerva strain, which was circulating during 2006 and 2007 (MMWRb, 2007). There were no NoV positive samples detected during the month of November, which is unusual considering there were 5 positive samples the previous month. NoV GI was not detected in any stool samples. This is not uncommon since GII infections predominate. The number of positive samples peaked during the month of December. During this month, there were more NoV and adenovirus positives, but the highest percentage of positives of adenovirus per samples collected occurred during the month of November. HAV was not detected in any of the stool samples analyzed. After further investigation, it was discovered that the number of reported cases of HAV for the state of Alabama was 6 from July 2008 to July 2009 (MMRW, 2009). The availability of the HAV vaccine has perhaps reduced the number of reported cases of HAV across the United States.

Bacterial Indicators, Bacteriophage, and Viruses in Sewage and Shellfish

Current guidance for controlling public health risks associated with shellfish consumption rely sanitary surveys and closures of harvest areas based on river stages. (Wilt, 1974). The inability of fecal coliforms to assess the risk of enteric viral
contamination in shellfish has been well documented (Sobsey et al., 1987; Goyal, 2006; Flannery et al., 2009). During this study, the inability of indicator bacteria to accurately predict the presence of enteric virus contamination was confirmed. The reduction of bacterial indicators in WWTP effluent was \( \geq 99\% \) for all sampling months. Indicator levels in oyster samples only exceeded the recommended guidance level of 230 MPN/100g in 25% of oysters analyzed between November and March, while the detection of NoV for the same period was 35% when the indicator levels were within the acceptable guidance levels. During the same time period, ADV was detected in shellfish at a rate of 50%. Although the oyster sentinels were placed in areas considered restricted classification of growing area, this clearly demonstrates the inability of fecal coliforms to index for enteric virus contamination and the sewage treatments plant failure to effectively remove viruses during treatment.

With the advancement of technology and the development of improved extraction techniques, detection of enteric viruses in sewage and shellfish have become less of a daunting task. During this study, NoV, adenoviruses and enteroviruses was detected in sewage and shellfish samples. NoV GII, enterovirus, and adenoviruses were detected in all effluent samples. NoV GI in influent was only detected during two sampling months. There was no NoV GI detected in the effluent during the sampling period. Adenoviruses were detected in all influent samples and effluent samples. Detecting NoV GII in all sewage influent while finding NoV GI in only two sample sets is consistent with finding associated with outbreaks; norovirus GII is implicated in more gastroenteritis infections that norovirus GI (Patel, 2009). The fact that enterovirus were not detected in any effluent samples was a little surprising. But given the fact that poliovirus vaccine series are given
as IPV and not OPV has decreased the presence of enteroviruses in sewage as most enteroviruses do not replicate in the gastrointestinal tract as poliovirus does.

During the months of January and March the salinity levels were \( \leq 3.2 \text{ppt} \) at stations 1-4. Oyster pumping efficiency decreases significantly at low salinities. This is reflected in the January data set as there were very low enteric virus pathogens detected in the oysters. There was low detection of fecal coliforms, bacteriophage and enteric viruses. However, this did not occur during the month of March due to the fact that the salinity levels were low only at the time the oyster baskets were retrieved. Fecal coliform levels were greatest during the month where the highest average water temperature occurred, September. The month with the lowest fecal coliform levels were January, but lowest average water temperature occurred during the month of February. The range of indicator levels, 20 to \( \geq 16,000/100\text{g} \) were typical levels seen in shellfish, depending on water temperature and salinity. During the month of October, station 2 indicator levels were higher than station 1 indicator levels and this occurred again during the month of December. For bacteriophage and viruses, station 2 had higher levels than station 1 over 50% of the time the samples were collected. Because station 1 was closest to the outfall, virus accumulation could have been slowed because of exposure chlorine residual associated effluent discharge.

Bacteriophage has been proposed as an alternative indicator organism for enteric viruses in the shellfish and the environment (Dore et al., 2000; Leclerc et al., 2000; Goyal, 2006). In this study, bacteriophage levels were consistent with levels from previous studies (Shieh et al., 2003; Daskin et al., 2007). As oyster sentinels were place further from the sewage treatment plant, the levels of bacteriophage detected in shellfish
decreased. Bacteriophage was present at station 1 and 2 for each month samples were collected. Station 1 is closest to the outfall but in 4 out of 7 collection months, the bacteriophage levels were higher for station 2. Station 1 was closest to the outfall and was exposed to more fresh water as this is reflected by lower salinities. Bacteriophage provided a better indicator of the presence enteric viruses than the current bacterial indicators. During 2000 study, Dore et al findings indicated that when bacteriophage levels were below 50 per 100g, NoV was not detected in any oysters. Interpreting the data from the Dore study, shellfish with levels 50 PFU per 100 g of shellfish should be safe from enteric viruses. During this study, there was only one instance were bacteriophage levels were less than 50 PFU per 100 g and NoV GII was present. This occurred during the month of March and at station 2. Station 2 is 0.3 nmi from the sewage treatment plant and this distance would be a restricted area in accordance with NSSP model ordinance guidelines (Frost, 1925). Bacteriophage as an indicator may be useful for shellfish but not for sewage because it is consistently present in sewage at significant levels.

There was no detection of enterovirus in the oysters past station 1. There have been several studies showing the rapid inactivation and reduction of enteroviruses once they are in the environment (Bosch, 1998; Fong et al., 2005) Also, there was no instances were enteroviruses were detected in the effluent. As with the stool isolates, enterovirus isolated from the sewage and oyster samples were common circulating serogroups. Adenovirus was detected from stool isolated each month that samples were collected and adenovirus was detected in all influent and effluent samples collected. Adenovirus type 41 was the most common subgroup isolated from the stool, sewage and oyster samples.
In a study conducted by Choo and Kim (2006), six different subgroups of ADV were detected in oysters. In this current study, only 2 different subgroups were detected in oysters, 12 and 41. During the month of December, adenoviruses were detected at all stations. Sequence analysis showed $\geq 99$ identity between stool, sewage and oyster isolates for adenovirus 41. Since adenovirus 41 is an enteric virus, this could easily explain its increased presence in stool, sewage and oyster isolates as enteric viruses survive more readily under strenuous environmental conditions (Bosch, 1998).

NoV GII was consistently detected at stations 1 and 2 for each sampling month, with the exception of January. As stated earlier, the salinity levels in shellfish was significantly low during the month of January, which decreases oyster pumping efficiency. NoV GII was detected at station 3 and 4 in during the months of December and February. The average water temperature for these months was 11°C. This was the lowest average water temperature for each sampling month except for January. Detection of enteric viruses at station 4 is significant in that area is under consideration for harvesting of shellfish for relaying. Since NoV is not culturable, the presence of NoV at any detectable level is considered potentially harmful. The level of virions detected at station 4 during the month of December was 2 per 100 g. Feeding studies have been done to determine the levels for NoV required to induced infection and it was found that 1 to 10 virion are capable of causing disease (CDC, 2006; Tennis, 2008).

Culturable Adenoviruses and Enteroviruses

Detection of enteric viruses by direct, ICC-PCR and cell culture provides definitive information about the presence of infectious adenoviruses and enteroviruses in sewage and shellfish isolates. Cell lines A549, BGMK, RD and CaCo-2 were used for
propagation of adenoviruses and enteroviruses in sewage and oyster isolates. Although adenoviruses and enteroviruses are susceptible to A549 and BGMK, adenoviruses are isolated at much higher frequencies with A549 cell lines and enteroviruses are isolated at much higher frequencies with BGMK cell lines (Hashimoto et al., 1991; Greening et al., 2002; Lee et al., 2002). Based on previous studies, some adenovirus and enteroviruses share similar target cell receptors and enteroviruses typically exhibit CPE faster than adenoviruses in BGMK cells; therefore, only A549 cells were analyzed by ICC-PCR for detection of adenovirus.

The positives samples by direct method for October did not correspond with the CPE positives in the BGMK cell line. Although there was CPE and ICC-PCR positives in the BGM cell line for the 1:50 dilution for station 3 during the month of October, there was no CPE detected for S4 but there was ICC-PCR detection for station 4. It is not unusual to have ICC-PCR detection without CPE as there are many enteroviruses that do not demonstrate CPE. The RT-PCR units for station 2 were 0.2 RT-PCR units/ml and the calculated PFU was $3.63 \times 10^3$/ml.

The detection of adenovirus and enterovirus by ICC-PCR was higher than just culture alone indicating and increased sensitivity for the detection of infectious virion by ICC-PCR. Although ICC-PCR detected infectious adenoviruses and enteroviruses, viral contamination levels could still be underestimated because individual cells propagate certain types of adenoviruses and enteroviruses. The detection rate for adenoviruses by direct, ICC-PCR and cell culture was higher than enteroviruses. This is consistent with levels found in stool, sewage and oyster isolates. Adenovirus levels were higher than all other enteric viruses analyzed in all aspects as adenovirus is a DNA viruses while NoV,
and enteroviruses are RNA viruses. Adenovirus, whose genome consists of DNA, can survive and persist longer in the environment than other RNA enteric viruses (Sobsey, 1989; Gerba et al., 2002; Ko et al., 2005). This can possibly be explained by the proofreading capabilities of DNA viruses or by the attachment of adenoviruses to particulates. The BGMK cell line was most effective at production of CPE for enteroviruses and Caco-2 produced the least CPE. ICC-PCR was most effective for A549 and BGMK cell lines. Only 3 samples were detected by ICC-PCR that did not exhibit CPE, two were at station 4 and effluent sample. There were 4 samples that demonstrated CPE but no adenovirus or enteroviruses were detected. This can be explained by noting that other enteric viruses not amplified by PCR during this study such as reoviruses can exhibit CPE in BGMK cell lines (Irving et al., 1981). Detection of enteroviruses and adenovirus by direct and cell culture methods were in agreement in 25% and 55% of positive samples, while ICC-PCR and cell culture were in agreement with 63% and 80% of positive samples respectively. Overall it was determined that the detection of adenoviruses and enteroviruses by direct method is less sensitive compared to ICC-PCR.

Summary

In summary, this study showed the genetic relationship between enteric viruses in the community and those isolated from sewage and shellfish. This study also demonstrated culturable enteric viruses in shellfish samples where non-culturable are detected by real-time RT-PCR. Two of the seven sampling months showed detection of NoV GII at the stations furthest from the WWTP discharge. Genetic analyses of stool and oyster samples collected during the month of December had a ≥ 99% identity for NoV GII when analyzed by nucleotide BLAST. Also, the GII.4 strains isolated showed ≥99%
identity with the 2007-2008 circulating Minerva strain. Adenovirus isolated from stool, sewage, and oysters demonstrated ≥99% identities when analyzed by Genebank. In addition to sequence identities being highly similar for stool sewage, and oyster isolates, culturable adenoviruses were detected at station 3 and station 4 during the month of December.

During this study it was observed that enteric adenoviruses 41 was not detected in stool isolates until the month of November while they were present in sewage samples during the warmer month of September. Adenoviruses were also present in the influent and effluent during all collection months and present at station 1 and 2 during 6 of the 7 collection months. The highest levels of adenoviruses were detected in oysters during the month of November which corresponded to the highest percentage positive of adenoviruses during the month of November.

The detection of enteroviruses in sewage and shellfish was surprisingly low, considering that higher levels were consistently detected at the same WWTP during a similar study five years ago. Since ICC-PCR and cell culture was utilized for enteroviruses and there was not a significant increase in detection of infectious enteroviruses, this could indicate that the levels of enteroviruses present in influent and effluent has decreased over the years.

With the simultaneous detection of NoV GII and culturable viruses at the station 4, which is furthest from the treatment plant, an extrapolation could be made that the detection of non-culturable enteric viruses along with culturable enteric viruses may strengthen the question of viability of NoV.
Through this study, I was able to demonstrate a molecular relationship between community and oyster isolates of enteric viruses. Future studies which would include more sampling months could provide additional insight into the seasonality and changes in circulation strains of enteric viruses.
APPENDIX

SEQUENCE DATA

September Sequence Data Set

Adenovirus 31 stool 39
CCCCTTTTTTGTGTTCTTGTTGTGCGCGGGCGAATTGCACCAGACCGGGACTC
AGTACTCCAGAGGCGTCTCTGACCAGGCAGGCGATGTGCATGTAAGACCAACTGGGGA
CAAAAGCG

Norovirus GII stool 44
TGGATGAGATTCTCAGATCTGAGCACGTGGGAGGGCGATCGCAATCTGGCTCCCAGTTTTGTGAATGAAGATGGCGTCGAATGACGCCAACCCATCTGAGTTGTCCGCAGCCAGCCTCGTCCCAGAGGTCAACAATGAGGTTATGGCTTTGGAGCC
CGTTGTCGTTGCGCTATTGCCGGCGCTGATGCGGCGCCTGTAGCGGGCCAACAAAATGTAATTGACCCCTGGATTAGAAACAATTTTGTACAAGCCCCTGGTGGAGAGTTCACAGTATCCCCTAGaAACGCTCCAGGGTAAATAACTATGGAGCGCGCCCTTAGGCCCTGATCTGAATTCCTACTATCTCATTTGGCC

Norovirus GII stool 45
TGGATGAGATTCTCAGATCTGAGCACGTGGGAGGGCGATCGCAATCTGGCTCCCAGTTTTGTGAATGAAGATGGCGTCGAATGACGCCAACCCATCTGAGTTGTCCGCAGCCAGCCTCGTCCCAGAGGTCAACAATGAGGTTATGGCTTTGGAGCC
CGTTGTCGTTGCGCTATTGCCGGCGCTGATGCGGCGCCTGTAGCGGGCCAACAAAATGTAATTGACCCCTGGATTAGAAACAATTTTGTACAAGCCCCTGGTGGAGAGTTCACAGTATCCCCTAGaAACGCTCCAGGGTAAATAACTATGGAGCGCGCCCTTAGGCCCTGATCTGAATTCCTACTATCTCATTTGGCC

Norovirus GII Station 2
TGGGAGGGCGATCGCAATCTGGCTCCCAGTTTTGTGAATGAAGATGGCGTCGAATGACGCCAACCCATCTGAGTTGTCCGCAGCCAGCCTCGTCCCAGAGGTCAACAATGAGGTTATGGCTTTGGAGCC
CGTTGTCGTTGCGCTATTGCCGGCGCTGATGCGGCGCCTGTAGCGGGCCAACAAAATGTAATTGACCCCTGGATTAGAAACAATTTTGTACAAGCCCCTGGTGGAGAGTTCACAGTATCCCCTAGaAACGCTCCAGGGTAAATAACTATGGAGCGCGCCCTTAGGCCCTGATCTGAATTCCTACTATCTCATTTGGCC

Adenovirus 5 Stool 1
TGGGGTTTCATAAACTTGTTATTCAGGCTGAAGTACGTCTCGGTGGCGCGGGCA
AAACTGCACCAGCCGGGCTCGTACTGCTCGGCGGCAGGCGTCCTCTGCTCCGGGAGATG
TGCAATGACCGGACTTCGGCGGCGGCGG
Adenovirus 50 stool 5
CATCGCCGGACAGGATGCTTCCGGAGTACCTGAGTCCGGGTCTGGTGCAGTTC
GCCCGCGCCACAGACACCTAATCTCAATCTGGGGAACAAGTTTAGGAACCCCA
CCGTGGCGCCACACCCTATGATGT

Adenovirus 12 stool 39
GGGTTTCTAAACTTGTCCACGGGTGGAAGTTAGTGTCGGGTGGCCGGGCGGAA
ATTGCAACCAGACCGGGACTCAGGTACTCCAGGGGCTCTGAGCCGCGATGTG
CATGAAGACCTGAGGGCGGTC

Adenovirus 12 stool 41
GCCACGCTGGGTTTCTAAACTTGTCCACGGGTGGAAGTTAGTGTCGGGTGGCC
GGGCGGCAACTGCAACCCAGCCGGGACTCAGGTACTCCAGGGCTCTGAGCCG
CGATGTGCACTGTAAGACACCCT

Adenovirus 6 stool 51
CCACGCTGGGTTTCTAAACTTGTCCACGGGTGGAAGTTAGTGTCGGGTGGCC
GGGCGGCAACTGCAACCCAGCCGGGACTCAGGTACTCCAGGGCTCTGAGCCG
CGATGTGCACTGTAAGACACCCT

Adenovirus 41 Station 2
AATACTATCGGTGGCGCGGGCAAACTGCACCAGGCCGGACTCAGGTCTCCAGG
CCGGGCTAGGCCCGGCGATGTGCACTGTAAGACCCG

Adenovirus 41 Effluent
GCCACGCTGGGTTTCTAAACTTGTCCACGGGTGGAAGTTAGTGTCGGGTGGCC
GGGCGGCAACTGCAACCCAGCCGGGACTCAGGTACTCCAGGGCTCTGAGCCG
CGATGTGCACTGTAAGACCCG

Adenovirus 41 Effluent Lysate
GCCACGCTGGGTTTCTAAACTTGTCCACGGGTGGAAGTTAGTGTCGGGTGGCC
GGGCGGCAACTGCAACCCAGCCGGGACTCAGGTACTCCAGGGCTCTGAGCCG
CGATGTGCACTGTAAGACCCG

Adenovirus 41 Station 1
GGCCAGTGCCACCGGTGGGTTTCTAAACTTGTCCACGGGTGGAAGTTAGTGTC
GGGCGGCAACTGCAACCCAGCCGGGACTCAGGTACTCCAGGGCTCTGAGCCG
CGATGTGCACTGTAAGACCCG

Adenovirus 5 Effluent Lysate (partial sequence)
TCTTACATCGCACTTCTCCGGCCAGGACCGCCCTCCGGGACTCAGGTACTCCAG
GGGCGGCAACTGCAACCCAGCCGGGACTCAGGTACTCCAGGGCTCTGAGCCG
CGATGTGCACTGTAAGACCCG
Norovirus GII stool 44
GCCAGTTGGAGCAGGGGCTAATCATGAAGATCCATCTGAATCAATGATTC
CACACTCTCAAAGACCCATACAATTGATGTCCTTACTGGAGGGAGGCCGCACT
CCACGGGCAACATTCTACAATGAAAATCTAGAAATAGTCATTGCAGAGCTA
AAAGAAGGTGGCAGGGATTTTTTACGTGCCCCAGGAGAGCCCAATGTCAGGT
GGATGA

Norovirus GII stool 45
GCCAGTTGGAGCAGGGGCTAATCATGAAGATCCATCTGAATCAATGATTC
CACACTCTCAAAGACCCATACAATTGATGTCCTTACTGGAGGGAGGCCGCACT
CCACGGGCAACATTCTACAATGAAAATCTAGAAATAGTCATTGCAGAGCTA
AAAGAAGGTGGCAGGGATTTTTTACGTGCCCCAGGAGAGCCCAATGTCAGGT
GGATGA

Norovirus GII Influent
TAGACTAGGGGTTCCAACCATGAAGACCCATCTGAAACAATGATTCCACT
CCCAGAGCCATACAATTGATGTCCTTACTGGGGAGGCCGCTCTCCACGG
CCCAGCATTTCTACAGCAAAATCAGCAATTAGTCTATTGCAAGAGCTGAAAGA
GGTGGTAGATGGATTTTTTACGTGCCCCAGACAAGAGCCCAATGTCAGGT
GGATGA

October Sequence Data Set

Adenovirus 41 Influent
TCATTGTAAAACATACGGCCAGTGCCGCGGGTTTCTAAACTTGTTCCCCCA
GGCTGAAAGTGATACGCTACGCTGCGGCAGGCAAACACTGCACAGGCGCGGACTCAG
ATACTCGGAAGGCTCCTGCCCCAG

Adenovirus 41 Station 1
CCTATGTTCCCCAGGGTGAAGTACGTATCGGTGGCGGGCGGGCAAACACTGCACCA
GGCCCGGACTCAGATACCCAGGCGCCTGCTCGCCCCGCCGACTGATGTGATGTAAGA
CCACTCGGGCGGGTCATACGGGCAG

Adenovirus 41 Effluent
GCCACCGTGGGTTTCTAAACTTGTTCCCCAGGGCTAAGTACGATACCGTCGCGC
GCAGGGCTACGCTACGCTGCGGCAGGCGCCTGCTCGCCCGGCGATGTGCATGTAAGAA
CCACTCGGGCGGGTCATACGGGCAG

Adenovirus 41 Stool 3
GCTAGAAGTACGTCTCCTCGGTTGGCCGCGGGCAACACTGCACAGCCCGGCGGCCTCAG
GTACTCGGAGGCGTCCCTGCCCCAGGATGTGATGTAGAAGACACACTGCGGGCCTG
CATAGACTAGTATTCTCGAA
Adenovirus 2 Stool 4
CATCTCGGCGCCAGGACGCTCGGAGTACCTGAAGCCCCCCGGCTGAGTTTG
GCCCGGCACACCGAGACGTACTTTCACGCTGGAATAAACAGTTTAGAAACAC
CCGTGGCAGCTTGCCG

Adenovirus 5 Stool 14
GCCCGGCAGGCTGGAAGTACGTTCTCGGTCGG
CGCGGGCAACTGCACTACGGCTGAGTACGTTCTCGGTCGG

Adenovirus 41 Station 2 Cell Lysate
CCTATGCTCCAGGCTGGAAGTACGTTCTCGGTCGG
CGCGGGCAACTGCACTACGGCTGAGTACGTTCTCGGTCGG
CGAGATGTCATGTAAGACCA

Adenovirus 41 Station 4 Cell Lysate
GCCACGGTGGGGTTTCTAAACTTGTTATTCAGGCTGGAAGTACGTTCTCGG
CGCGGGCAACTGCACTACGGCTGAGTACGTTCTCGGTCGG
CGAGATGTCATGTAAGACCA

Adenovirus 5 Stool 24
CCACGGTGGGGTTTCTAAACTTGTTATTCAGGCTGGAAGTACGTTCTCGG
CGCGGGCAACTGCACTACGGCTGAGTACGTTCTCGGTCGG
CGAGATGTCATGTAAGACCA

Adenovirus 2 Stool 22stool 22
GTGCCACGGTGGGGTTTCTAAACTTGTTATTCAGGCTGGAAGTACGTTCTCGG
CGCGGGCAACTGCACTACGGCTGAGTACGTTCTCGGTCGG
CGAGATGTCATGTAAGACCA

Echovirus 30 BGMK Cell Lysate Station 2
CTACTTGTTAAACGACGTGCAGTCCTCCGGCCCTGGAATGGCGCTGTAATCTCA
ACTGCGGAGCAGATACCCACACACCGAGTGGCGAGTCTGTAACAGGCAAC
TCCGCAAGGAAACGGACTACTTGGGTTGTCGTCCTCTTTTTTTTTATAGCT

Echovirus 30 Stool 12
AGTCTCCGGCCCTGGAATGGCGCTAATCTCACTGCAGGAGCAGATACCCAC
ACGGCCAGTGGGCAGTCTGTAACAGGCAACTCCGCAAGGAAACGGACTAC
TTGGGGTTCGTCGTCCTTCTTTTTTTTTTTATACGTGGCTGTTATGGGACATT
GAGAGATTGTTGCGCATATAGCTATTGGATTGGCCATCCGGTGTCTCATA
Norovirus GI Influent
TCAGACCCTTCAGAGACATTGTTGCCACACACCCAAAGAAAAGTACAATTGATGCTCACTCTTGGGAGAGGCCTCACTCCATGGTGAAAAATTCTACAGAAAGATCTCCAGCAAAGTCATACATGAAATCAAGACTGGTGGGTTGGAGATGTACGTC
CCAGGATGGCAGGCCATGTGCTGGCTGCGC

Norovirus GII Influent
CTNCCCAAAGACCCATACAATTGATGCTCCCTACTGGGAGAGGCCGCACTCCACGGCCCAACATTCTACAGACACCAGCGGACTCAGGTACTCCGAGGCATCCTGACCGGCCATGTGCATGTAAGACAGCATGACTGGCAGAGCTAAAAGAAGGTGGTATGGATTTTTACGTGCCCAGACAAGGCGCAATGTTCAGAGTG
GATGAGAGTTCCGTCATAGCTAGTAAATCACATAGATAAG

November Sequence Data Set

Adenovirus 12 Station 2
GCCACGGGTGGGTTTCCTAAACCTGTTTTCCAGGGTGGAAGTATAGTAGGTGCCTCGTGCCGCGGGCGAATGACCAGACCGGGACTGCAGGTACTCCGAGGCATCCTGACCGGCCATGTGCATGTAAGACAGCATGACTGGCAGAGCTAAAAGAAGGTGGTATGGATTTTTACGTGCCCAGACAAGGCGCAATGTTCAGAGTG
GATGAGAGTTCCGTCATAGCTAGTAAATCACATAGATAAG

Adenovirus 12 Effluent
GCCACGGGTGGGTTTCCTAAACCTGTTTTCCAGGGTGGAAGTATAGTAGGTGCCTCGTGCCGCGGGCGAATGACCAGACCGGGACTGCAGGTACTCCGAGGCATCCTGACCGGCCATGTGCATGTAAGACAGCATGACTGGCAGAGCTAAAAGAAGGTGGTATGGATTTTTACGTGCCCAGACAAGGCGCAATGTTCAGAGTG
GATGAGAGTTCCGTCATAGCTAGTAAATCACATAGATAAG

Adenovirus 41 Station 2 Cell Lysate
GCCACGGGTGGGTTTCCTAAACCTGTTTTCCAGGGTGGAAGTATAGTAGGTGCCTCGTGCCGCGGGCAAACTGCACCAGCCAGCCGGACTGCAGGTACTCCGAGGCATCCTGACCGGCCATGTGCATGTAAGACAGCATGACTGGCAGAGCTAAAAGAAGGTGGTATGGATTTTTACGTGCCCAGACAAGGCGCAATGTTCAGAGTG
GATGAGAGTTCCGTCATAGCTAGTAAATCACATAGATAAG

Adenovirus 41 Station 4 Cell Lysate
GCCACGGGTGGGTTTCCTAAACCTGTTTTCCAGGGTGGAAGTATAGTAGGTGCCTCGTGCCGCGGGCAAACTGCACCAGCCAGCCGGACTGCAGGTACTCCGAGGCATCCTGACCGGCCATGTGCATGTAAGACAGCATGACTGGCAGAGCTAAAAGAAGGTGGTATGGATTTTTACGTGCCCAGACAAGGCGCAATGTTCAGAGTG
GATGAGAGTTCCGTCATAGCTAGTAAATCACATAGATAAG

Adenovirus 41 Stool 30
GCCACGGGTGGGTTTCCTAAACCTGTTTTCCAGGGTGGAAGTATAGTAGGTGCCTCGTGCCGCGGGCAAACTGCACCAGCCAGCCGGACTGCAGGTACTCCGAGGCATCCTGACCGGCCATGTGCATGTAAGACAGCATGACTGGCAGAGCTAAAAGAAGGTGGTATGGATTTTTACGTGCCCAGACAAGGCGCAATGTTCAGAGTG
GATGAGAGTTCCGTCATAGCTAGTAAATCACATAGATAAG

Adenovirus 41 Stool 20
GCCACGGGTGGGTTTCCTAAACCTGTTTTCCAGGGTGGAAGTATAGTAGGTGCCTCGTGCCGCGGGCAAACTGCACCAGCCAGCCGGACTGCAGGTACTCCGAGGCATCCTGACCGGCCATGTGCATGTAAGACAGCATGACTGGCAGAGCTAAAAGAAGGTGGTATGGATTTTTACGTGCCCAGACAAGGCGCAATGTTCAGAGTG
GATGAGAGTTCCGTCATAGCTAGTAAATCACATAGATAAG

GCGATGTGCATGTAAGACAGCATGACTGGCAGAGCTAAAAGAAGGTGGTATGGATTTTTACGTGCCCAGACAAGGCGCAATGTTCAGAGTG
GATGAGAGTTCCGTCATAGCTAGTAAATCACATAGATAAG
Adenovirus 41 Stool 19
GCCACGGGTGAGGTGTTTTCTAAACTTGTTCCCAAGGGTGCAAGTACGTATCGGTGCGT
GCCGGCAACTGCACACAGGCCCGGACTTCAGATACTGCCGAGGGGTTCCTGGCCCG
GCCATGTGCATGTAAGACCAC

Adenovirus 41 Stool 7
GCCACGGGTGAGGTGTTTTCTAAACTTGTTCCCAAGGGTGCAAGTACGTATCGGTGCGT
GCCGGCAACTGCACACAGGCCCGGACTTCAGATACTGCCGAGGGGTTCCTGGCCCG
GCCATGTGCATGTAAGACCAC

Adenovirus 41 Stool 22
GCCACGGGTGAGGTGTTTTCTAAACTTGTTCCCAAGGGTGCAAGTACGTATCGGTGCGT
GCCGGCAACTGCACACAGGCCCGGACTTCAGATACTGCCGAGGGGTTCCTGGCCCG
GCCATGTGCATGTAAGACCAC

Adenovirus 41 Stool 23
GCCACGGGTGAGGTGTTTTCTAAACTTGTTCCCAAGGGTGCAAGTACGTATCGGTGCGT
GCCGGCAACTGCACACAGGCCCGGACTTCAGATACTGCCGAGGGGTTCCTGGCCCG
GCCATGTGCATGTAAGACCAC

Adenovirus 41 Stool 17
GCCACGGGTGAGGTGTTTTCTAAACTTGTTCCCAAGGGTGCAAGTACGTATCGGTGCGT
GCCGGCAACTGCACACAGGCCCGGACTTCAGATACTGCCGAGGGGTTCCTGGCCCG
GCCATGTGCATGTAAGACCAC

Adenovirus 41 Stool 3
GCCACGGGTGAGGTGTTTTCTAAACTTGTTCCCAAGGGTGCAAGTACGTATCGGTGCGT
GCCGGCAACTGCACACAGGCCCGGACTTCAGATACTGCCGAGGGGTTCCTGGCCCG
GCCATGTGCATGTAAGACCAC

Adenovirus 41 Stool 21
GCCACGGGTGAGGTGTTTTCTAAACTTGTTCCCAAGGGTGCAAGTACGTATCGGTGCGT
GCCGGCAACTGCACACAGGCCCGGACTTCAGATACTGCCGAGGGGTTCCTGGCCCG
GCCATGTGCATGTAAGACCAC

Adenovirus 41 Stool 2
GTGCCACGGGTGAGGTGTTTTCTAAACTTGTTATTCAGGCTGAAGTACGTCTCGGTGCG
GCCGGCAACTGCACACAGGCCCGGACTTCAGATACTGCCGAGGGGTTCCTGGCCCG
GCCATGTGCATGTAAGACCAC

Adenovirus 41 Stool 8
GTGCCACGGGTGAGGTGTTTTCTAAACTTGTTATTCAGGCTGAAGTACGTCTCGGTGCG
GCCGGCAACTGCACACAGGCCCGGACTTCAGATACTGCCGAGGGGTTCCTGGCCCG
GCCATGTGCATGTAAGACCAC

Adenovirus 41 Stool 16
AACTTGTTCCCAAGGGTGCAAGTACGTATCGGTGCGTGGCGCCGGCAACTGCAACCA
GCCGGCAACTGCACACAGGCCCGGACTTCAGATACTGCCGAGGGGTTCCTGGCCCG
GCCATGTGCATGTAAGACCAC

Adenovirus 2 Stool 8
GTGCCACGGGTGAGGTGTTTTCTAAACTTGTTATTCAGGCTGAAGTACGTCTCGGTGCG
GCCGGCAACTGCACACAGGCCCGGACTTCAGATACTGCCGAGGGGTTCCTGGCCCG
GCCATGTGCATGTAAGACCAC

Adenovirus 41 Stool 16
AACTTGTTCCCAAGGGTGCAAGTACGTATCGGTGCGTGGCGCCGGCAACTGCAACCA
GCCGGCAACTGCACACAGGCCCGGACTTCAGATACTGCCGAGGGGTTCCTGGCCCG
GCCATGTGCATGTAAGACCAC
Echovirus 30 BGMK Station 3 Cell Lysate
CTACTTGTAAAAACGACGTGCCAGTCTCCGGGCCTGAATTGGCTAATCTCTA
ACTGCACAGACGATACCCACACACAGCGAGCGTAGCTGTGTAACGGGCAAC
TCCGCAGCGACCCGAACCGACTACTTTTGGGTGTCCTGTTTCCCTTTTATATAC
GGCTGCTTA

Enterovirus 90 Stool 5 (repeat blast)
AGTCCTCCGGCCCCTGAATGCAGCTAAATCCCAACCACCGAGCAAGTGCCCAC
ATACCAGTARAGTCTTGTGTAACGGGCAACGGGCTACTACGTGATGGCTGCTTAACG
TTGGGTGTCCTGTTTTATTTTTATATATGCTGCTTTATAGTGACAAATC
TAAGATTTGTTATCATATAGCTTTTGGATTGGCCATCCGGTGGGTCATAGCTG

December Sequence Data Set

Norovirus GII Stool 49
GGGGGCCCAACCATAGAAGACCCATCTGAAAACATGATACCCACACTCCCAAGA
GGCCCATACAAATGATGTTTTACTGGTGAGGGCAGTCATCCACAGGCCCACAC
ATTCTACAGCAAAAATACGTAAACTGGTCATTTGACAGGAGGTGCG
ATGGATTTTTACGTGCAAGAAAAGGACGCACTGTCAGAGATGGAGATGAGTTC
GGTC

Norovirus GII Stool 52
CCAGTGGACCGAGGGGCCTAATCATGAGAAACCAGTACGAGAGCATGGTCC
TCATTCTCAGCGGCCACACAGCTCATGCCCCTTTCTTGAGGCTCTCACTGCA
ATGGTTCCAGTTTTCAAGAAAATGATGCTCATAATGAATTAA
GAGTGGTGGCTCTGAGTTTTTACGTGCCCAGACAGAGGCGCATGCAGGTGGATGAG

Norovirus GII Station 2
CCAGTGGACCGAGGGGCCTAATCATGAGAAACCAGTACGAGAGCATGGTCC
TCATTCTCAGGGGCCACACAGCTCATGCCCCTTTCTTGAGGCTCTCACTGCA
ATGGTTCCAGTTTTCAAGAAAATGATGCTCATAATGAATTAA
GAGTGGTGGCTCTGAGTTTTTACGTGCCCAGACAGAGGCGCATGCAGGTGGATGAG

Norovirus GII Stool 53
ACGTAAAAATCCCATGCCCCACCTTTTATAGCCTGCAATGACTAATTTGCTGAT
TTTACTGTAAGATGTTGGGCCTGGAGTGCTGCCCTCCAGTAAGGACATC
AATTGTATGGGCTTTTGGAGAGTGTTGGGATCATGCATGTCAGATGGGTCTTCATG
ATTAGGGCCCCCCTCGTCAACTGGGCGTCATATTACAA
Norovirus GII Station 3
GCTCTTTGTCGAGGCACATAAAACTCCATACCACCCTCTTTAATCTCATTGAT
GACCACCTCTGGCTAATTTTTCTTGTAHAATCGGACCAGCAGCTGAGGGAAGGCCTCAC
CAAGAAGGGGCATGAGTGCATGTTGGCCGCTGAGAATGAGGAACCATGCTCTCATC
GACGGGTTCATCATGATTAGGCCCCCTGGTCCAACTGGCCGTCAGTTATTACAA

Norovirus GII Influent
AGAACCCGTATGAGAGCATGGTTCCTCATTCCCAGCGGGCCATCAACTCATG
GCCCCTTCTTTGGTGAGGCCCTCATGCTAATCTGTTTTTCAGAGAAAGGTA
GCAAAATGGTGCTCAATGAATTAAAGAGTGCTGGTGCTGGAGGTTTTTACGTGCC
CAGACAAAGGGCCATGTTGAGGATGAGATGTCAGTCTCAATGCTGTTATCC
TGA

Norovirus GII Stool 4
CGGCCAGTGTCGAGGGGGGCCCTAATCATGAAGATCCATCTGAATCAATGGAT
TCCACACTCTCAAAAGACCCATCAACTCATGATGTCTTTAATCTGGAAGAGGCCGCA
CTCCACGGCCCAACTTACAGTAAAATACAGAAATTAGCTATTGCGAGGC
TTAAAGAAGGGTGCCATGGAGTTTTTACGTCGCCCAGCAAGAGCAGCAATGTTCAG
GTGG

Norovirus GII Stool 38
CGGCCAGTGTCGAGGGGGGCCCTAATCATGAAGATCCATCTGAATCAATGGAT
TCCACACTCTCAAAAGACCCATCAACTCATGATGTCTTTAATCTGGAAGAGGCCGCA
CTCCACGGCCCAACTTACAGTAAAATACAGAAATTAGCTATTGCGAGGC
TTAAAGAAGGGTGCCATGGAGTTTTTACGTCGCCCAGCAAGAGCAGCAATGTTCAG
GTGG

Adenovirus 41 Station 2
GCCACGCTGAGGGGGTTTCTAAACTTCCAGGCTGAAGTACGTATCGGTGGC
GCCGCAAAGCAGCCGCCGAACGGTGGACATCAGTACGTCGCTGGCC
GCGGCAAATTACAGGCCGCCGAGCTCACGATACCGCGGTCCGCTGCCC
GCCATGTGCATGTAAGACCAC

Adenovirus 41 Station 3
GCCACGCTGAGGGGGTTTCTAAACTTCCAGGCTGAAGTACGTATCGGTGGC
GCCGCAAAGCAGCCGCCGAACGGTGGACATCAGTACGTCGCTGGCC
GCGGCAAATTACAGGCCGCCGAGCTCACGATACCGCGGTCCGCTGCCC
GCCATGTGCATGTAAGACCAC

Adenovirus 41 Station 4
GCCCCAGTGGTCATACATGCACATCAGCCGCAGGACGCCTCGAGGATATCTG
AGTCCGCGGCTTGCTGAGTTTGGCCGCAGCAGAGAATACGTTACGCTGCTG
GGAACAAAGTTCAGAAAACCAC
Adenovirus 41 Influent
GCCCCAGTGGTCTTACATGCACATCGCCGCGAGCCAGCACGCTCAGTGATATCTG
AGTCCGGGCTCCTGGAATTTGCCCAGCCGACCAGATACATACCTACCCCTGG
GGGAACAGTGGTAAACCCCA

Adenovirus 41 Effluent
GCCACGGGTGGGTTTCTAAAACTTGTCCCAGGCTGAAGTACGTATCGGTGGC
GCGGGGCAAACGCGCCGGACTCAGATACCTCCGAGGCGTCTGCCCG
GCCGTGTGCACTGTAAGACACC

Adenovirus 41 Stool 16
GCCACGGGTGGGTTTCTAAAACTTGTCCCAGGCTGAAGTACGTATCGGTGGC
GCGGGGCAAACGCGCCGGACTCAGATACCTCCGAGGCGTCTGCCCG
GCCGTGTGCACTGTAAGACACC

Enterovirus 71 Influent
ATCCTAATCGCCGAGCACATGCTCACAAACCAGTAGGGGTGTTGTCAGTAACG
GCCAACCTCTGCAGCGGGAACCGACTCCTTACTTGGGTGCTCAGTCTTTTTATTTAATTC
TTATATTGGTCTCCTATGAGCAATCAAAGAATTGTGCAATAGCTATTG
GATTGGCCATCCGGTAGGTCATAGCTGTTTCATGAAA

Adenovirus 41 Stool 17A127
GCCACGGGTGGGTTTCTAAAACTTGTCCCAGGCTGAAGTACGTATCGGTGGC
GCGGGGCAAACGCGCCGGACTCAGATACCTCCGAGGCGTCTGCCCG
GCCGTGTGCACTGTAAGACACC

Adenovirus 41 Stool 15
GCCACGGGTGGGTTTCTAAAACTTGTCCCAGGCTGAAGTACGTATCGGTGGC
GCGGGGCAAACGCGCCGGACTCAGATACCTCCGAGGCGTCTGCCCG
GCCGTGTGCACTGTAAGACACC

Enterovirus 90 Influent
AGTCCTCGCCGCCCCTGAATGGGCTAATCCCAACCACGGAAGCAGTTGCACTC
AAAACATGGGTGATCGTCATACACGCTAAGCTTACGTGCTGAGCAGCGAACCAGACTAC
TTTGGGTGTCAGTCCCCCTTTTCATTATATTATGGATTGAGTGTCAATTC
TAAGATTGTATCTATATAGCATTGTTTGATGATGCAATCCGAGGGGTGGTCTAGCTG

Echovirus 30 Influent BGMK Cell Lysate
AGTCCTCGCCGCCCCTGAATGGGCTAATCCCAACCACGGAAGCAGTTGCACTC
AAAACATGGGTGATCGTCATACACGCTAAGCTTACGTGCTGAGCAGCGAACCAGACTAC
TTTGGGTGTCAGTCCCCCTTTTCATTATATTATGGATTGAGTGTCAATTC
TAAGATTGTATCTATATAGCATTGTTTGATGATGCAATCCGAGGGGTGGTCTAGCTG

Echovirus 30 Influent CaCo-2 Cell Lysate (partial sequence)
CCTGAAATGCGGCTAAATCTCTACGGAGCAGATACCACACGCGGAGTGGG
AGTCTGCTGTAACGGGGAACCTCCGAGGCGACAC
January Sequence Data Set

Norovirus GII Stool 4  
CCATGAAGATCCATCTGAATCAATGATTCCACACTCTCAAGACCCATACAA  
TTGATGTCTTACTGGGAGAGGCCGCACTCCAGGCCCAACATTCTACAGTA  
AAATCAGTAAATTAGTCATTGCAAGCTAAAGAAAGGGTGACATGGATTTTTA  
CGTGCCCAAGCAAGGCTAATTGCAGTGGATGAGATTTCTCAGATCTGAGC  
ACGT

Poliovirus 3 Stool 17  
GTGCAACGCTTTGCAACCCCAGCAAGCCAGCTGTAACGCGCAGTCGTGCGC  
GGAACCGACTACTTTGCTGTCCGTGTTCCTCTTTTATTCTTTGAATGGCTGCTTATGGTGACAATCATAGATTGTTATCATAGCTGCTTTATGCTGTATACGCCTAC  
TTGCGAGTTACCTGCTTATGCTTATGCTGCTTATGCTGTATACGCCTAC  
TTAAGATTGTATCATATAGCTGTGATTGGATGGCCTCGATCCAGCT

Enterovirus 90 Influent  
AGTCCTCCGGCCCTGAATGCGGCTAATCCCAACCACGAGGAGAAGGTGTCAC  
AAACCAGCAAGTGGGCTTGTGTAACGCGTAAGTCTGTGCGGGAACGACTAC  
TTGCGAGTTACCTGCTTATGCTTATGCTGCTTATGCTGTATACGCCTAC  
TTAAGATTGTATCATATAGCTGTGATTGGATGGCCTCGATCCAGCT

Adenovirus 41 Station 1  
GCCACGGTGGGGTTTCTAAACTTGTTCCCCAGGCTGAAGTACGTATCGGTGGC  
GCGGGCAAACTGCAAGCCCGGAACACTCAAGGCGTCTCGGCGCTCCCGGCGATGTGCATGTAAGACCT  
ACTGCGGCGGCTCATAAGACAC

Adenovirus 52 Influent  
CTTGTTCATCCCAAGCTGAAGTACTGCACTCAGGTACTCCGATGTTGTGGCCGCGGCTCATAAGACAC

Adenovirus 2 Stool 22  
GTGCCACGGTGCGGGTTTCTAAACTTGTTTATCCAGGCTGAAGTACGTATCGGTGGC  
GCGGGCAAACTGCAAGCCCGGAACACTCAAGGCGTCTCGGCGCTCCCGGCGATGTGCATGTAAGACCT  
ACTGCGGCGGCTCATAAGACAC

Adenovirus 41 Stool 24  
GCCACGGTGCGGGTTTCTAAACTTGTTTATCCAGGCTGAAGTACGTATCGGTGGC  
GCGGGCAAACTGCAAGCCCGGAACACTCAAGGCGTCTCGGCGCTCCCGGCGATGTGCATGTAAGACCT  
ACTGCGGCGGCTCATAAGACAC

Adenovirus 31 Stool 37  
TAGAGTTTGCTCCTGCTGACCGCGGCGATGTTGTGACGTTAAGACCAGACTGCGGCTCATAAGACAC
Adenovirus Group C Stool 40 (partial sequence)
GCCCGGGCGCACAGGTACCTCCGAGGCGTCTCTGGCCCGAGATGTGCATGTAAGAACACTGGGCGGCTAGCTGAATTACTAGAAA

Adenovirus 41 Stool 50 (partial sequence)
GCCCGGGCGCACAGGTACTCCGAGGCGTCTCTGGCCCGAGATGTGCATGTAAGAACACTGGGCGGCTAGCTGATTTACTAGAAA

Adenovirus Group C Stool 49 (partial sequence)
GGCGCGGGCGACAGGTACCTCCGAGGCGTCTCTGGCCCGAGATGTGCATGTAAGAACACTGGGCGGCTAGCTGATTTACTAGAAA

Adenovirus 41 Stool 42
GCTGAAGTACGTATCGGTGGCGCGGGCAAGCTCACGCACTGGGCGGAGGCGTCTCTGGCCCGAGATGTGCATGTAAGAACACTGGGCGGCTAGCTGATTTACTAGAAA

Adenovirus 41 Stool 51
GCCACGGGCTGAGTACTCCGAGGCGTCTCTGGCCCGAGATGTGCATGTAAGAACACTGGGCGGCTAGCTGATTTACTAGAAA

Norovirus GII Station 1
ACGGCCAGTTGGACGAGGGGGCCTAATCATGAGAACCCGTACGAGAGCATGGGCTCCCTCATTCTCAACGGGCCACACCACTCATGCGGCCCCCTCTCTGGGATTTTTACGTGCCCAGGCAAGAGCCAATGTTCAGGTGGATGAGAGTTC

Norovirus GII Stool 33
ACGAGGGGGCCTAATCATGAGAACCCATCTGAATCAATGATCCCACACTCTGAACAGCACTCATATGCGGCTCAGGTGGATGAGAGTTC

Norovirus GII Influent
GCCTCTTGTCTGGGCACGTAAAACTCCATACCACACTCTTAATCTCATGATGACCATTTTGCTAACTTTCTTGTAAAACTGGGGACCATGCATTGAGGCCTCACAAAGAAAGGCTCATGAGCTGTGTGGCTCGCTGGGAATGAGGAACCATGCCTCAAAGGGTGCTCATGATTAGGCCCCTCGTCCAACTGGCCGTAAGTTATATAAACATGAAAG

February Sequence Data Set
Norovirus GII Stool 49
GTGGGACGAGGGGGCCTAAATCATGAGAAACCATATGAGAGCATGGTCCCTCA
TTCCACGGCGGACCACACGCTCATGGGCCCCTTTCGGGAGGCTTTCACTGCAT
GGCCCTCAGTTTTTAAAGAAGGTTAGCAAGATGCTCATCAATGAAATCAAAAA
GTGGTGCTGCTGAAATTCTATGTGCCCCAGACAAGAGGCGCATGTTTCA

Norovirus GII Stool 16
GCCACGGTGTTGTTTTCTAAAATCTGTTCCTCAGGGCTGGAAGTACGTATCGGTGCC
GCGGGCAACTGCACCAGGCCGCCAAGCTCAGATACTCCGAGGCGTCCTGCCC
GCGATGTGCATGTAAGACACT

Adenovirus 41 Stool 17
GCCACGGTGGGGTTTCTAAACTTGTTCCCCAGGGCTGGAAGTACGTATCGGTGCC
GCGGGCAACTGCACCAGGCCGCCAAGCTCAGATACTCCGAGGCGTCCTGCCC
GCGATGTGCATGTAAGACACT

Coxsackievirus B4 Influent
AGTCCCTCCGGCCCTGAATGCGGCTAACTCCTAATCTGCGGAGCAGACACCCAC
AAAGCGATGGGCGACTCTGGTAAACGGGCAACTCTGCAGCGGGAACGACTAC
TTGGGTGCTCGGTCTCTTCTATATTCTTACACTGCGTTATATGGTGCAAAAT
GAAAGATTGTTAATTGCTATATTGGGATTGGGCCCCATTCCGGTG

Coxsackievirus A4 Stool 25
TCTTGTAAAACGACGGCCAGTCCTCCGGCCCCTGAATGCGGCTAATCCTAAC
GCGGAGCACAACACCCCTCAACCCAGGGGCGAGTGTGTCGCTGTAACGGGCAACTCT
GCAGCGGAAACCGACTACTTGGGGGTGTC

Enterovirus 71 Stool 25 (partial sequence)
CGGAACCGACTACTTTGGGTGCTCGGTCTTCTTCTTCTTTTATTTTTATCTTCTG
GTATGGTGACAATTTAAAGAATTTGTTACCATAATAGCTATTTGGGATTGGCCATCCGG
TGGGTGCTAGCTGGTTTCCATAGAA

March Sequence Data set

Norovirus GII Stool 33
GAATCAATGATCCTCACTCTCAAAAGACCCATATAAATATGCTCTTATACGG
AGAGGGCGGCACTCCAGCGCCACATTCTACATGAAAAATCAGGAAATTTAGTC
ATTGCAGAGCTTAAAAGAAGGGTGCATGGGATTTTTACGTGCACCCAGCGAGGC
GAATGTTCAGATGGTAGAGTTCGGT

Adenovirus 41 Station 1
GCCACGGTGTTGTTTTCTAAAATCTGTTCCTCAGGGCTGGAAGTACGTATCGGTGCC
GCGGGCAACTGCACCAGGCCGCCAAGCTCAGATACTCCGAGGCGTCCTGCCC
GCGATGTGCATGTAAGACACT
Adenovirus 41 Station 2
GCCACGGTGTTTCTAAACTTGTTCCTCCGCCAGCTGAAGTACGTATCGGTGGC
GCGGGCGACACTGCACCAGGCCCGGACTCAGATACTCCGAGGCGTCCTGCCCG
GCGATGTTGCAATGTAAGACCACTG

Adenovirus 41 Influent
GCCACGGTGTTTCTAAACTTGTTCCTCCGCCAGCTGAAGTACGTATCGGTGGC
GCGGGCGACACTGCACCAGGCCCGGACTCAGATACTCCGAGGCGTCCTGCCCG
GCGATGTTGCAATGTAAGACCACTG

Adenovirus 41 Effluent
GCCACGGTGTTTCTAAACTTGTTCCTCCGCCAGCTGAAGTACGTATCGGTGGC
GCGGGCGACACTGCACCAGGCCCGGACTCAGATACTCCGAGGCGTCCTGCCCG
GCGATGTTGCAATGTAAGACCACTG

Adenovirus 12 Stool 18
GCCACGGTGTTTCTAAACTTGTTCCTCCGCCAGCTGAAGTACGTATCGGTGGC
GCGGGCGACACTGCACCAGGCCCGGACTCAGATACTCCGAGGCGTCCTGCCCG
GAGATGTTGCAATGTAAGACC

Adenovirus 6 Stool 23
GCCACGGTGTTTCTAAACTTGTTCCTCCGCCAGCTGAAGTACGTATCGGTGGC
GCGGGCGACACTGCACCAGGCCCGGACTCAGATACTCCGAGGCGTCCTGCCCG
AGATGTTGCAATGTAAGACC

Adenovirus 2 Stool 31
GTGCCACGGTGTTTCTAAACTTGTTCCTCCGCCAGCTGAAGTACGTATCGGTGGC
GCGGGCGACACTGCACCAGGCCCGGACTCAGATACTCCGAGGCGTCCTGCCCG
CCGAGATGTTGCAATGTAAGACC

Adenovirus 6 Stool 33
GCCACGGTGTTTCTAAACTTGTTCCTCCGCCAGCTGAAGTACGTATCGGTGGC
GCGGGCGACACTGCACCAGGCCCGGACTCAGATACTCCGAGGCGTCCTGCCCG
GAGATGTTGCAATGTAAGACC

Adenovirus 41 Stool 40
GCCACGGTGTTTCTAAACTTGTTCCTCCGCCAGCTGAAGTACGTATCGGTGGC
GCGGGCGACACTGCACCAGGCCCGGACTCAGATACTCCGAGGCGTCCTGCCCG
GCGATGTTGCAATGTAAGACC

Adenovirus 41 Stool 42
GCCACGGTGTTTCTAAACTTGTTCCTCCGCCAGCTGAAGTACGTATCGGTGGC
GCGGGCGACACTGCACCAGGCCCGGACTCAGATACTCCGAGGCGTCCTGCCCG
GCGATGTTGCAATGTAAGACC
Adenovirus 12 Stool 35
GCCACGGTGGGGTTTCTAAAACCTTGTTCCTCCAGGGTGAAGTAGGTGTCCGTGGC
GCGGGCGAATTGCACCAGACCGGGACTCAGGTACTCCAGGAGGCGCTCTGACCG
GCGATGTGCATGTAAGACCG

Adenovirus 2 Stool 38
GTGCCACGGTGTTTCTAAAACCTTGTTCCTCCAGGTGAAGTAGGTGTCCGTGGC
GCGCGGGCAAACTGCACCAGGCCGGGCTCAAGGTACTCCAGGAGGCACGTCCCTGACCG
CCGAGATGTGCATGTAAGACCG

Adenovirus 41 Stool 49
GCCACGGTGTTTCTAAAACCTTGTTCCTCCAGGGTGAAGTAGGTGTCCGTGGC
GCGGGCAAACTGCACCAGGCCGGGCTCAAGGTACTCCAGGAGGCGCTCTGACCG
GCGATGTGCATGTAAGACCG

Adenovirus 6 Stool 39
GCCACGGTGTTTCTAAAACCTTGTTCCTCCAGGGTGAAGTAGGTGTCCGTGGC
GCGGGCAACTGCACCAGGCCGGGCTCAAGGTACTCCAGGAGGCGCTCTGACCG
GAGATGTGCATGTAAGACCG
REFERENCES


efficiently and produce plaques on a human cell line A549, derived from lung carcinoma. J. Virol. 65: 2429-2435.


138. NOAA Fisheries of the US. 2007. US Commercial Landings


176. **USFDA report.** 2009. Minimum dilution of wastewater treatment plant effluent discharge required to ensure the safety of molluscan shellfish.


185. http://www.water-research.net/watertreatment/chlorination.htm
188. www.med.yale.edu/micropath/pdf/Infectivity%20calculator.xls