MOLECULAR DETECTION OF WATERBORNE HUMAN AND BOVINE ENTERIC VIRUSES IN A MULTI-USE COASTAL WATERSHED IN SOUTHEAST GEORGIA, USA.

by

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(Under the Direction of Erin K. Lipp)

ABSTRACT

The stringent host specificity of enteric viruses makes them good library-independent markers for identification of water pollution sources. Here we developed molecular assays targeting human enteroviruses (HEV), bovine enteroviruses (BEV) and human adenoviruses (HAdV) to examine the microbiological water quality in the lower Altamaha River, Georgia. Water samples were collected monthly from five tidally influenced stations, and analyzed by (RT)-nested PCR and dot-blot hybridization. Human adenoviruses, HEV and BEV were detected in 36.67%, 56.67% and 36.67% of surface water samples. Two-thirds of the samples tested positive for either HEV or HAdV and simultaneous recovery occurred in 25.71 % of samples. Recovery of these viruses was directly related to dissolved oxygen and streamflow, and inversely related to water temperature, rainfall and chlorophyll-*a* concentrations but not significantly related to coliform indicator levels. Viral detection by PCR is an easy-to-use tool for rapid assessment of fecal contamination sources.

INDEX WORDS: enteric viruses, fecal contamination, water quality, nested-PCR

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B.S., Boise State University, 2000

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2004

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DEDICATION

To my beloved grandfathers:

Fong Eng Leong
(9/10/1927-6/21/2001)

Tan Keng Sian
(10/17/1917-6/21/2004)

ACKNOWLEDGEMENTS

First of all, I would like to thank my major advisor, Dr. Erin Lipp, for her continuous support of my work and for all the hours spent on reviewing this thesis. I also would like to thank my committee members, Dr. Marsha Black and Dr. Dana Cole, for their input and guidance; everyone in our lab: Carrie, Ethell, Joy, Katie and Tricia, for encouragement and help in the completion of this project; and my family and friends, for always being there for me, your understanding and efforts to keep in touch ☺ Last but not least, I would like to thank the the staff at the Department of Environmental Health Science, Ms. Heather McEachern, Ms. Ella Willingham, and Ms. Regina Davis, for always being friendly and helping to keep my documents and records on track, you have made my life so much easier!

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CHAPTER 1

INTRODUCTION

Over 100 types of pathogenic viruses are excreted in human and animal wastes. These viruses, collectively known as enteric viruses, are frequently detected in large numbers in sewage and other fecally contaminated waters. The stringent host specificity of these viruses makes them potential markers for identifying major sources of fecal contamination in environmental waters. In the literature review (Chapter 2), the characteristics, pathogenicity, occurrences and detection in aquatic environments of two of the most studied groups of enteric viruses as potential water quality indicators (enteroviruses and adenoviruses) are discussed in detail.

Rapid population growth and urban development along waterways and coastal areas has led to decreasing water quality in the lower Altahama River, Georgia. In Chapter 3, research is presented on the examination of the effects of upstream anthropogenic activities (e.g., urban runoff, leaking septic systems, sewage overflow etc.) and agricultural operations on the microbiological water quality of the river. We developed molecular assays targeting human enteroviruses (HEV), bovine enteroviruses (BEV) and human adenoviruses (HAdV) to identify major sources of fecal contamination in the river. Water samples were collected monthly from five tidally influenced stations between July and December 2002. Samples were analyzed by (RT)-nested PCR and dot-blot hybridization. Viral data were correlated to bacterial indicators and environmental and water quality variables such as water temperature, streamflow, salinity, pH, dissolved oxygen (DO) and rainfall using ANOVA and binary logistic regression. Eleven and 17 of the 30 surface water samples tested positive for HAdV and HEV, respectively.

Two-thirds of the samples tested positive for either HEV or HAdV and the viruses occurred simultaneously in 25.71 % of samples. BEV were detected in 11 of 30 surface water samples. Binary logistic regression analysis showed that the presence of both human and bovine enteric viruses were not significantly related to either fecal coliform or total coliform levels. The presence of these viruses was directly related to dissolved oxygen and streamflow, but inversely related to water temperature, rainfall in the 30 days preceding sampling and chlorophyll-*a* concentrations.

In chapter 4, conclusions are made suggesting that viral pathogen detection by PCR is a highly sensitive and easy-to-use tool for rapid assessment of water quality and fecal contamination when information regarding potential sources of contamination is desired. Human adenoviruses and human enteroviruses may be used to identify human fecal contamination in water based on extensive research that has been done on host-specificity, survival, transport, and detection of the viruses in water. The application of animal-specific viruses, e.g. bovine enteroviruses to trace the source of fecal contamination in water, however, will need additional analyses of prevalence and specificity of the viruses in different geographical areas.

CHAPTER 2

LITERATURE REVIEW

Introduction

Enteric viruses may be present naturally in aquatic environments, or more commonly are introduced through human activities such as leaking sewage and septic systems, urban runoff, agricultural runoff and in the case of estuarine and marine waters, sewage outfall and boat dumping. Over 100 types of pathogenic viruses are excreted in human and animal wastes (101). These viruses can be transported in the environment through ground water, estuarine water, seawater, rivers, aerosols emitted from sewage treatment plants, insufficiently treated water, drinking water and private wells that receive treated or untreated wastewater either directly or indirectly (9, 88, 92, 123, 138, 160). These viruses, collectively known as enteric viruses, usually are transmitted via the fecal-oral route, and primarily infect and replicate in the gastrointestinal tract of the hosts. Enteric viruses are shed in extremely high numbers in the feces of infected individuals, typically between 10^5 and 10^{11} virus particles per gram of stool (38).

Commonly studied groups of enteric viruses belong to the families *Picornaviridae* (polioviruses, enteroviruses, coxsakieviruses, hepatitis A virus and echoviruses), *Adenoviridae* (adenoviruses), *Caliciviridae* (noroviruses, caliciviruses, astroviruses and small round-structured viruses) and *Reoviridae* (reoviruses and rotaviruses). Although enteric virus infections are primarily associated with diarrhea and self-limiting gastroenteritis in humans, they may also cause respiratory infections, conjunctivitis, hepatitis, and diseases that have high mortality rates such as aseptic meningitis, encephalitis and paralysis in immunocompromised individuals (83).

In addition, some enteric viruses have been linked to chronic diseases such as myocarditis and insulin-dependent diabetes (53, 83). Enteric virus infections in animals such as cattle and swine are normally asymptomatic, but can lead to abortion, neurological disorder and mortality (73, 76, 90, 98).

Enteric viruses can be transmitted by food, water, fomites and person-to-person. In addition to causing acute diseases, they are of public health concern due to their low infectious dose (58). For example, the probability of infection from exposure to one rotavirus is 31 % and no more than one plaque forming unit (PFU) is required to cause infection in one percent of healthy adults with no antibody to the virus (127). Haas et al. (58) concluded that the risk of infection when consuming viruses in drinking water is 10 to 10,000 fold greater than that of pathogenic bacteria at similar exposures (14, 58). Because of the potential for contamination from a variety of sources, enteric viruses in water are of particular concern. Since the 1980s, with significant advancements in the area of environmental virology, enteric viruses have been recognized as the causative agents in many non-bacterial gastroenteritis cases and outbreaks (14). Enteric viruses have been isolated from and linked to outbreaks originating from contaminated drinking water sources, recreational waters (e.g., swimming, canoeing, surfing, etc.), urban rivers and shellfish harvested from contaminated waters (22, 32, 72, 88, 95, 108, 118). Between 1975 and 1979, water, followed by shellfish, were reported to be the main vehicles in outbreaks of vehicle-associated viral disease in the U. S. (25). Non-potable water, such as seawater, is also important; enteric viruses are able to persist for extended periods in the marine environment, which increases their probability for exposure risk by recreational contact and accumulation in shellfish (95). Because shellfish are filter feeders, the concentration of viruses accumulated in their edible tissues may be much higher than in the surrounding water (1).

Consumption of shellfish harvested from enteric virus-contaminated waters often has led to human outbreaks (15, 16, 95).

In many countries, including the U. S., regulators are still relying solely on bacterial indicators such as fecal coliform and total coliform bacteria to assess the microbiological quality of water; however, bacterial indicators do not always reflect the risk from many important pathogens, such as viruses, stressed pathogenic bacteria (viable but non-culturable), and protozoa (47, 72, 111). Infectious enteric viruses have been isolated from aquatic environments that are in compliance with bacterial indicator standards and there have been several viral-related outbreaks linked to ingestion of waters that met fecal coliform standards (28, 99). One of the major drawbacks in using fecal coliform bacteria and other traditional indicators (e.g., enterococci) is that these indicators may be found in both human and animal feces, and naturally in soils. Furthermore, they may regrow in the environment after being excreted from their host (140). The ability to identify the dominant sources of fecal pollutants in aquatic environments has become increasingly important in water quality management and remediation; however, tracking the host source of bacterial indicators in environmental waters is impossible without laborious and extensive assays such as multiple antibiotic resistance profiling and ribotyping (33, 113). Complicating matters, studies have shown that in coastal and marine waters traditional bacterial indicators generally die off quickly when compared to viruses and protozoa (13, 139).

Viral pathogens, because of their host specificity, have been suggested as one of the most promising tools to determine the sources of fecal contaminants in aquatic environments and may be used in conjunction with bacterial indicators to assess water quality and improve public health surveillance (100). Pathogenic viruses are generally more resistant than bacterial indicators during conventional wastewater treatment such as chlorination and filtration, and are able to

withstand lipid solvents (44, 72, 145). In the environment, enteric viruses can survive under a wide pH range (pH 3 -10) and for extended periods at low temperatures (83). Viruses have been reported to survive and remain infective for up to 130 d in seawater, up to 120 d in freshwater and sewage, and for up to 100 d in soil at 20-30 ºC (72, 150). These survival periods surpass those reported for fecal coliform and other indicator bacteria in similar environments (102). Finally, because viruses have an obligate host requirement, there is no potential for regrowth in the environment. In general, enteric viruses show great potential to be used as water quality indicators to assess the risks associated with infectious virus transmission as well as to identify the dominant source of fecal contamination in waters.

Enteric Viruses as Pathogens

Enteric viruses represent a diverse group. Most of the mammalian viruses, such as picornaviruses, rotavirus, and noroviruses, are non-enveloped RNA viruses while adenoviruses are the only group with double-stranded DNA. The impracticability in monitoring for the presence of all viral pathogens has lead to the concept of an indicator organism. Two of the most studied groups of enteric viruses as potential water quality indicators are the enteroviruses and adenoviruses.

Enteroviruses. Enteroviruses consist of poliovirus, coxsackieviruses, echoviruses, and the numbered enteroviruses. As of 2003, 89 serotypes of enteroviruses have been identified and ratified by the Executive Committee of the International Committee on Taxonomy of Viruses (ICTV) (19). Enteroviruses are single-stranded RNA viruses with an icosahedral capsid ranging from 20 to 30 nm in diameter. About 70 % (62 serotypes) of non-polio enteroviruses have been associated with human infections and 30 % with animal infections (73). Enteroviral infections in

humans are reported to peak in summer and early fall, which also coincides with increased water recreational activities and water contact (83).

Enteroviruses as human pathogens. Enteroviruses can cause a wide spectrum of diseases in humans. All enteroviruses are transmitted by the fecal-oral route but clinical outcomes may go beyond gastroenteritis, as some viruses travel from the intestinal tract to other organs. Polioviruses usually infect their host by attacking the central nervous system and cause paralysis in victims (poliomyelitis). Coxsackieviruses have not only been associated with respiratory system infections and gastroenteritis, but also insulin-dependent diabetes and heart diseases, such as myocarditis and pericarditis (53, 83, 154). Echoviruses are generally less infectious than other enteroviruses and are usually associated with the common cold and respiratory diseases. The numbered enteroviruses have not been studied extensively but have been isolated from patients with bronchiolitis, conjunctivitis, meningitis and paralysis resembling poliomyelitis (83).

Enteroviruses as animal pathogens. Animal-specific enterovirus infections in hosts such as cattle and pigs are often asymptomatic but may cause diseases ranging from diarrhea to reproductive failure and neurological disorders (85, 90). Two bovine enteroviruses (BEV), three porcine enteroviruses (PEV), one ovine enterovirus (OEV) and 11 porcine teschoviruses (PTV) (ten were formerly classified as porcine enteroviruses) have been identified (80).

Based on a study in Maryland, U.S.A., bovine enteroviruses (BEV) have a prevalence of 76 % in farmed cattle. While they are usually nonpathogenic, BEV have been linked to diarrhea and abortions in some infected cattle (90). Porcine enteroviruses (PEV) have a prevalence of 65 % in pigs and wild hogs (20). PEV and PTV have been identified as the etiologic agents of the neurological disorder known as Teschen-Talfan disease, polioencephalomyelitis, vesicular

diseases, myocarditis, pneumonia, diarrhea, fertility disorders and dermal lesions in swine (36, 39, 63, 81).

Swine vesicular disease virus (SVDV), a porcine variant of human coxsackievirus B5 (CVB5) causes lesions in pigs that are indistinguishable from those caused by foot-and-mouth disease virus, an aphthovirus (43). Because swine vesicular disease is highly contagious, difficult to eradicate, and there is no effective vaccine, control measures often necessitate the slaughter of infected and contacted animals, which lead to severe economic losses (43). Transmissions of this disease include direct contact among infected animals and environmental contamination (31).

Adenoviruses. Adenoviruses were first isolated from humans and identified as the causative agent of epidemic febrile respiratory disease among military recruits in the 1950s (68, 124). Adenoviruses are non-enveloped, range from 90 to 100 nm in diameter and consist of double-stranded DNA (77).

In 1998, adenoviruses were included in the "Candidate Contaminant List" (CCL) as part of the Safe Drinking Water Act by the Environmental Protection Agency, USA, and are one of only four viruses on the list (the three others are caliciviruses, coxsackieviruses and echoviruses) (148). Adenoviruses are included because of their public health implications and their frequent occurrence in many aquatic environments. In addition, adenoviruses have been shown to be up to 60 times more resistant to UV irradiation than RNA viruses, such as enteroviruses and hepatitis A virus (49, 103). All adenovirus with human or mammalian hosts are classified under genus *Mastadenovirus* (71).

Adenoviruses as human pathogens. Fifty-one serotypes of human adenoviruses (HAdV) have been identified (57). Human adenoviruses are the second most important viral pathogen of childhood gastroenteritis after rotavirus (27). They have been cited to cause

symptomatic infections in several organ systems, including the respiratory system (pharyngitis, acute respiratory disease and pneumonia), eye (conjunctivitis), gastrointestinal tract (gastroenteritis), central nervous system (meningoencephalitis) and genitalia (urethritis and cervicitis) (27, 77). Human adenovirus types 40 and 41 have been associated with gastroenteritis in children, while human adenovirus type 4 is linked to persistent epidemics of acute respiratory disease in the United States (29, 100). Transmission includes the fecal-oral route and inhalation of aerosols (72). The viruses are shed for extended periods in feces, urine and respiratory secretions of infected persons (27).

In contrast to the view that only adenoviruses that infect the intestinal tract of hosts will be excreted in feces, adenoviruses type 5, the non-enteric adenovirus strain that accounts for 11 % of clinical adenovirus cases reported to World Health Organization, is also frequently detected in aquatic environments (88, 144).

Adenoviruses as animal pathogens. Humans are not the only host for adenoviruses; animal-specific adenoviruses infect a wide range of hosts, including other mammals, birds, reptiles, amphibians and fish (125). Five porcine adenoviruses (PAdV), five bovine adenoviruses and six ovine adenoviruses (OAdV) have been classified under the genus *Mastadenovirus* (5). Most adenoviruses infecting fowl (FAdV) have been classified as *Aviadenovirus* (5).

Infection with PAdV is usually nonpathogenic although cases of mild diarrhea or mild respiratory signs in swine have been noted (61). PAdV also has been isolated from pigs with encephalitis and pneumoenteritis (71, 78). Some BAdV, such as BAdV-3, have been shown to replicate in cattle, and produce mild or no clinical signs, but several other serotypes have been linked to keratoconjunctivitis, acute febrile disease, pneumonenteritis, and acute and fatal enteric diseases in calves (71, 89, 98).

Among avian adenoviruses, chicken embryo lethal orphan adenovirus (CELO), classified as the type 1 fowl adenovirus, is wide spread among chicken populations but has never been associated with serious disease and does not induce clinical signs when experimentally inoculated in chickens (42). Avian adenoviruses that often induce clinical signs or cause fatalities in avian species include infectious bursal disease virus (causes immunosuppressive disease that may lead to death or impaired growth in young chickens), hemorrhagic enteritis virus (infecting turkeys and causes intestinal haemorrhages accompanied with immunosuppression), egg drop syndrome virus (only infects hens in the laying period, causing loss of color and thin-shelled eggs, and reduction in egg production) (42, 66, 67, 152).

Animals infected by adenoviruses have been shown to excrete the infectious viruses through their feces and can potentially become infected through the ingestion of fecal-contaminated water or food (71, 151).

Detection of Enteroviruses and Adenoviruses in Aquatic Environments

Monitoring for the presence of human enteric viruses in environmental waters began in the 1940s (55). In early studies of the occurrence of human enteric viruses in aquatic environments, cell culture was the most widely used technique for detection and isolation of infectious enteric viruses. Other viral detection methods typically used for clinical samples, such as radioimmunoassay, immunofluorescence, complement fixation and enzyme-linked immunosorbent assay, were either too costly or lacked the sensitivity to detect viruses in environmental samples (53). The basic steps of virological analysis from environmental waters are sampling, virus concentration (and purification), and detection with cell culture assays or, more recently, molecular methods such as polymerase chain reaction (PCR) and hybridization.

Virus concentration methods. Because the levels of enteric viruses in natural environments often are low, large volumes of water (up to thousands of liters) are frequently concentrated before analysis by inoculation on cultured host cells or by molecular methods (54, 93, 153). Different types of filters and filtration methods, such as cartridge filters (electropositive or electronegative), glass fiber filters, glasswool filters, vortex flow filtration, tangential flow filtration and acid flocculation traditionally have been used to collect and concentrate viral particles from water samples (46, 53, 72, 94, 114). Because of the small size of viral particles, mechanical filtration is often not possible; therefore adsorption-elution methods are employed. These involve manipulation of charges on the virus surface, using pH changes to maximize their adsorption to charged filters (94, 114). Adsorption-elution of viruses with an electropositive filter (i.e., 1MDS Zetapor Virosorb; CUNO, Meriden, CN) is one of the most commonly used and is EPA's Information Collection Rule (ICR) designated method for recovery of enteric viruses from drinking water (149). These filters require no manipulation of pH because most enteric viruses are negatively charged at ambient pH conditions (94). However, electropositive filters are easily clogged and have low recovery rate for viruses in marine water; the presence of salt and alkalinity of seawater cause low absorption of viruses to the filters (97).

Electronegative filters show higher virus recoveries than electropositive filters from marine water and waters of high turbidity (37, 79, 94, 97). Under ambient conditions enteric viruses are negatively charged and will adsorb to a negatively charged membrane only in the presence of Mg^{2+} (i.e., salt), other multivalent cations, or more commonly under acidic conditions (when their net charge becomes positive) (137, 156). Katayama et al. (79) developed a modified virus concentration method with a high virus recovery rate in seawater (up to 73% of poliovirus recovered from 1 L) and minimal inhibitory effects. This method uses adsorption with

a type-HA, negatively charged membrane (Millipore, Billerica, MA), rather than a cartridge used in traditional methods (94). After the sample is filtered and viruses adsorbed to the membrane, an acid rinse step is used to remove cations, i.e., salt, and other inhibitors while keeping viruses attached to the membrane. In addition, an inorganic eluting medium (NaOH) that has less inhibitory effects in polymerase chain reaction (PCR) assays than the commonly used organic eluting medium, beef extract, is used (62, 79); however, a high pH beef extract solution is the most widely used eluting medium to elute absorbed viruses from cartridge filters and has worked well with cell culture assays (4, 128, 132). In PCR, the use of NaOH as an eluent provides a good alternative to other methods that attempt to remove PCR inhibitors from beef extract solution such as resin treatments, polyethylene glycol precipitation-resuspension techniques, immunomagnetic capture and glass purification, which can be expensive and complicated (79).

For improved recovery of viruses from freshwater, such as groundwater, river and tap water, Haramoto et al. (62) modified the virus concentration method of Katayama et al. (79) by precoating a type-HA, negatively charged membrane (Millipore, Billerica, MA) with $AlCl_3$ prior to filtering samples, yielding a mean poliovirus recovery of 109 % from 10 liters of seeded MilliQ water.

Ultrafiltration methods such as vortex flow filtration (VFF) and tangential flow filtration (TFF) are alternatives to adsorption-elution techniques and have been shown to be efficient in recovering viruses from marine water (a recovery rate of 72 % for T2 bacteriophage in seeded samples with VFF) (53, 119). Both filtration devices utilize a flow pattern that forces water through a cylindrical filter with pressure while keeping and retaining particles from filters to avoid clogging (119). These methods require minimal manipulation of water; samples can be processed under natural pH and an elution step is not needed (72). The typical volume of water

processed is 20 L, which is concentrated to ~ 50 ml (53).TFF requires prefiltration of water samples to remove plankton and suspended solids. VFF has been shown to be more time efficient because prefiltration of samples is not required and has a higher viral recovery rate than TFF but tends to concentrate more PCR inhibitors with the viruses (72). However, both VFF and TFF are less cost- and time effective than adsorption-elution because of the high cost of equipment and limitations on volume of sample that can be concentrated at one time.

Concentrated or eluted water samples usually are further concentrated and purified to reduce the final volume of samples to one or two ml for processing (62, 72, 79, 94, 111). Commonly used secondary concentration methods include organic flocculation (recommended by USEPA's Information Collection Rule (ICR) protocol), polyethylene glycol (PEG) precipitation and centrifugal ultrafiltration (ultraconcentration based on a molecular weight cut-off, such as Centriprep YM-30 or YM-50 concentrator columns (Millipore, Billerica, MA))(72, 79, 94, 149).

In organic flocculation, buffered beef extract is used to precipitate viruses from concentrated samples by reducing the pH to 3.5. The precipitate is then centrifuged to form a pellet before being dissolved in sodium phosphate (149). The PEG precipitation procedure consists of precipitating viral particles by adding of 0.5 M NaCl and 7 % PEG to beef extract with constant stirring for 2 h at 4 ºC followed by centrifugation. The virus pellet is then resuspended in tris-buffered saline (TBS) (37). Again, the use of beef extract in these procedures has been reported to cause inhibitory effects in PCR assays (4, 128). Ultrafiltration concentration methods do not require manipulation of samples and has shown a high virus recovery; seeded MilliQ water samples concentrated by Centriprep YM-50 filter units, give a mean polioviruses recovery of 74 % (62).

Cell culture assay. Concentrated samples can either be extracted for viral nucleic acid analysis (PCR amplification) or inoculated onto common cell lines such as the Buffalo Green Monkey kidney (BGM) cells, MA104 cells, RD cells, A549 cells, FRhK-4 cells, CaCo-2 cells, specific to each virus type for quantification and isolation of infectious viruses (94, 120). The cell culture technique was the most widely used technique to determine the occurrence of infectious enteroviruses in environmental samples before the development of molecular based methods such as PCR in the late 1980s and early 1990s and is still the best method to isolate and determine infectivity of viruses from environmental samples (Table 2.1). After inoculating a chosen cell line, flasks are evaluated for the presence of damaged cells or rounding of cells and sloughing of the monolayer (cytopathogenic effects (CPE)) as evidence for viral infection.

The major drawback to the cell culture assay is that it is very laborious and time-consuming; it requires days to weeks of incubation and several passages to confirm both positive and negative results. In addition, some samples may be cytotoxic but appear as CPE on cells. A universal cell line that can be used for culturing all enteric viruses has not been established and there are many viruses that cannot be detected through cell-culture assay either because they do not produce CPE, are extremely slow-growing or do not grow on established cell lines (23, 94, 121). For example, adenoviruses, one of the most important human pathogens that are often detected in greater numbers than enteroviruses in wastewater, are slow growing, often do not produce CPE and are consistently underestimated when fast-growing enteroviruses are present (70, 144). Likewise, noroviruses, one of the major causative agents for viral gastroenteritis and foodborne outbreaks, cannot be propagated in cell culture (62).

Viral nucleic acid extraction. Both concentrated samples and infected cultured cells can be extracted for viral nucleic acids and purified to remove cell debris and inhibitors before being

amplified and detected by PCR (54, 94). One of the most widely used methods for viral nucleic acid extraction and purification was developed by Boom et al. (11) based on guanidium thiocyanate (GuSCN) extraction and silica columns to bind and wash nucleic acids. This method is rapid, easy to use, and efficient in removing inhibitors (72, 121). Casas et al. (21) developed an extraction method with the use of GuSCN and an inorganic solvent to purify both viral RNA and DNA in a single extraction step. Extraction kits based on modifications of these methods are available commercially (54, 72, 94). Other methods for viral nucleic acids extraction and purification include proteinase K treatment followed by phenol-chloroform extraction and ethanol precipitation, sonication and heat treatment (2, 17, 23, 51, 86, 106).

PCR detection of viral pathogens. Molecular techniques have been used extensively to detect enteric viruses from environmental samples since the early 1990s. Molecular viral detection assays, such as PCR and hybridization, usually are based on the detection of a part of the viral genome that is highly conserved with broad homology within a specific group of viruses (3, 30). PCR-based assays offer several advantages over cell-culture assays in detecting viral pathogens from environmental samples. PCR is rapid, highly sensitive and specific if a well-designed assay is developed. PCR viral detection is less laborious and time-consuming, and also more specific and sensitive than cell culture (Table 2.1) (24, 59, 72). Results from PCR assays can be obtained within 24 h of sampling compared to days or weeks of incubation for cell-culture assay (55, 113). PCR is capable of differentiating specific viruses (72, 79, 120). For example, PCR primers can be designed to target whole virus orders (e.g., enteroviruses or adenoviruses), or specific to a single type of virus (e.g. poliovirus) or tailored for virus serotypes within a host group (e.g. human, cattle and pigs) (57, 159).

In addition, PCR is highly sensitive and capable of detecting viruses that are present in low numbers in environmental samples and are either difficult to grow in cultured cells or replicate without producing CPE (23, 94, 121). The high level of sensitivity in PCR assays has indicated that cell culture detection alone may underestimate the true level of contamination in environmental sources. Pina et al. (120) suggested that PCR has led to higher detection rate of adenoviruses in environmental samples. Borchardt et al. (12) detected enteric viruses (enteroviruses, rotavirus, Norwalk-like virus and hepatitis A virus) from four (8%) of fifty household wells by PCR, while no virus was detected by cell culture. Unlike cell culture, however, the infectivity of viruses detected by molecular methods is often unknown.

While PCR detection methods offer a high level of sensitivity, this property may also increase the risk for false-positive results due to low levels of contamination. In order to reduce false positive rates, stringent quality control measures such as using aerosol-resistant pipette tips or positive displacement pipettors, decontamination of instruments between experiments and physical separation of pre- and post-PCR products, are required in processing the samples to prevent cross-contamination and ensure the quality of PCR products. Likewise, false-negative may also be a problem when inhibitors in environmental samples are present. Humic and fulvic acids, heavy metals, and phenolic compounds may inhibit the activity of polymerase enzyme (142, 158, 161). Additional manipulations including resin treatments, polyethylene glycol precipitation-resuspension, immunomagnetic capture and glass purification are sometimes required to remove inhibitors (14). Additives may also be used in PCR, directly, to reduce the effects of inhibitory compounds.

Among the problems with traditional PCR has been the inability to enumerate viruses. Recently, conventional PCR has been modified to improve specificity, sensitivity and efficiency,

but also to quantify the number of viruses detected (Table 2.1). Some variations of conventional PCR include nested-PCR, multiplex PCR and real-time PCR (for quantification). Seminested-PCR and nested-PCR assays increase the sensitivity and specificity of PCR with the use of an internal primer or primer set and are sometimes used as a confirmation step. Nested-PCR assays for adenoviruses by Allard et al. (3) and Van Heerden et al. (153) were shown to have increased sensitivities when compared to conventional PCR, with detection limits of one adenovirus particle and 10^{-2} PFU, respectively. However, nested-PCR has been shown to have a high probability of carryover contamination when PCR products from the first round of PCR are transferred to the reaction-mixture for the nested PCR reaction (72, 79).

The application of multiplex PCR (where several sets of primers against several targets are included in a single PCR reaction) may save time and costs because several types of viruses can be detected in a single PCR assay (41). The development of a multiplex PCR assay, however, is not easy and requires careful optimization of reaction mixtures and PCR conditions (41, 50, 146). The original effort of Fout et al. (41) to develop a multiplex PCR assay that would detect five enteric viruses (i.e., enteroviruses, reovirus, rotavirus, hepatitis A virus and Norwalk virus) was not successful and instead, two multiplex PCR assays had to be developed to detect the five targeted virus groups. Fout et al. (41) also noted that even with optimal conditions, enteroviruses and Norwalk virus were not amplified as efficiently as other virus groups. A multiplex PCR developed by Green and Lewis (50) to detect enterovirus, rotavirus and hepatitis A worked well in analyzing seeded samples but a large number of non-specific PCR products were formed when environmental samples were analyzed; secondary PCR was required to confirm positive samples.

Real-time PCR provides quantitative data for the presence of enteric viral genomes in environmental samples with the use of a fluorescent dye, such as SYBR Green (Molecular Probes, Eugene, OR) that will bind to amplified cDNA, or with fluorochrome-tagged probes that fluoresce when bound to complementary sequences in the amplified region. The procedure is less time-consuming because a confirmation step such as agarose gel elelctrophoresis and additional hybridization are generally not required. The entire analysis can be done in a closed system, which may reduce potential for contamination. Real-time PCR assays have shown comparable or increased detection sensitivity to conventional PCR in several studies (7, 34). Beuret (7) reported that real-time RT-PCR detection of norovirus and enteroviruses in seeded samples shows an increased sensitivity of a factor of 10 and 10^2, respectively, when compared to the conventional RT-PCR protocol. The real-time RT-PCR assay developed by Donaldson et al. (34) for detection of enteroviruses showed a detection limit of 9.3 viral particles ml^{-1} for seawater and 155 viral particles g^{-1} for sponge. However, the cost of a real-time PCR machine is still substantially more expensive than a conventional PCR machine and in some cases, real-time PCR has been shown to be less sensitive than conventional RT-PCR and nested-PCR (113). Noble et al. (113) reported that human adenovirus 40 was detected by real-time PCR in only two of the four samples positive for adenoviruses by conventional nested-PCR; none of the samples that were positive for enteroviruses by conventional RT-PCR was detected by real-time RT-PCR.

While PCR-based methods offer many advantages in sensitivity, specificity and efficiency over cell culture, they still cannot provide information on the infectivity of viruses detected with the reliability of cell culture. Recently, however, several studies have combined cell culture and PCR, and reported that this method improves the specific detection of infectious enteric virus from environmental samples. The hypothesis behind this method is that after

inoculating a cell line, only infectious viruses, if present, will propagate; the cells can then be extracted and tested for viruses by PCR before CPE is noted. This is also appropriate for viruses that do not produce CPE but still infect and grow in a cell line. Chapron et al. (23) noted that an integrated cell culture-RT-nested-PCR (ICC-RT-PCR) procedure provided increased sensitivity compared to the conventional cell culture method (CPE only) (149). By ICC-RT-PCR, 68.9% of samples were positive for an infectious virus, compared to 17.2% determined by traditional cell culture (23). Detection of infectious adenoviruses also showed significant improvement with this method; the percentage of positive environmental samples (including sewage, sludge, river and shellfish samples) increased from 28.6 % by conventional cell culture to 50 % by ICC-PCR (52). ICC-(RT)-PCR also increases the frequency at which viruses are detected from environmental samples that normally have very low levels of infectious enteric viruses, including potable water (88). In Korea, 65.2 % (15 of 23) of tap water samples were positive for infectious enteric viruses by integrated cell culture and (RT)-multiplex nested PCR compared to a detection rate of below 10 % from similar studies in the 1980s and early 1990s (48, 88). With this method, infectious adenoviruses which do not usually produce CPE were detected in 39.1 % (9 of 23) of tap water samples; enteroviruses and adenoviruses were detected simultaneously in 21.7 % (five) samples (88). ICC-PCR can also produce results in a shorter period than traditional cell culture (i.e., $\leq$ 3 d) (52). However, recent work by Ko et al. (82) suggests that carryover of nucleic acids of inactivated viruses inoculated onto cultured cell might result in a false-positive result from samples containing no infectious viruses. To address this, Ko et al. (82) developed an ICC-RT-PCR based assay to detect viral mRNA rather than DNA in the case of adnoviruses; mRNA is only transcribed by infectious adenoviruses during replication. After exposure to

different doses of UV radiation, adenovirus DNA was detected consistently in inoculated cell culture lysate by PCR even when adenovirus mRNA could no longer be detected (82).

Recently several studies that examined the relationship between the presence of viral genomes and the infectivity of those viruses in environmental samples have found that viruses (particularly RNA-based viruses) detected by PCR usually are infectious and there actually may be little difference between detection of viruses from the environmental samples by PCR and by cell culture (35, 147, 157). Wetz et al. (157) showed that the detection rate for polioviruses varied little between cell culture and RT-PCR in unfiltered seawater. Because RNA degrades relatively rapidly in the environment (in a few minutes) compared to DNA, viruses that are no longer infectious because of damage to the capsid also experience damage to the RNA on the same time scale, thus becoming undetectable by both cell culture and RT-PCR (91). Tsai et al. (147) showed that naked enteroviral RNA could not be detected by RT-PCR and dot-blot hybridization after 2 days of incubation at both 4 ºC and 23 ºC in unfiltered seawater. Skraber et al. (136) observed that though the poliovirus genome has a higher persistence than an infectious poliovirus, the loss in detection of the viral genome is directly correlated to the disappearance of infectious virus, suggesting that viral nucleic acids may indeed serve as an efficient indicator for infectious viruses in aquatic environments.

Occurrence of Enteroviruses and Adenoviruses in Aquatic Environments

Infectious enteric viruses, especially enteroviruses and adenoviruses, have been isolated from various types of water including groundwater, treated sewage, marine water, rivers, streams and drinking water under various environmental conditions (72, 84, 87, 88, 92, 131). Factors controlling the occurrence, survival and distribution of enteric viruses in the environment include

host excretion, water temperature, susceptibility to sunlight inactivation, virus attachment to suspended solids, and other environmental variables such as water composition (including the presence of predators), rainfall and streamflow (93, 144).

Host excretion. Many researchers have observed peaks in both human enteric virus infections and excretion in summer and early fall, which also coincides with increased water recreational activities and human-water contact (83, 109, 130, 153). In a year-long survey of the occurrence of adenoviruses in drinking water in South Africa, adenovirus detection peaked in July, when up to 30 % and 60 % of treated and raw water samples were positive for adenoviruses, respectively (153). Furthermore, there appears to be a connection between environmental and clinical isolates in a given year within specific geographic areas. A study that compared clinical and sewage isolates of enteroviruses from Milwaukee, Wisconsin collected between 1994 and 2002, found that the predominant clinical serotype was most often also the predominant sewage serotype for that year (130). For example, in 1998, echovirus 30 accounted for 50.0 % of sewage isolates and 46.1 % of clinical cases, and in 1990, 79.7 % of sewage isolates and 60.3 % of clinical cases were echovirus 11 (130). In some cases, early spring sewage enterovirus isolates could help to predict serotypes that would predominate clinically during the following summer (130). One of the explanations for this phenomenon is that a "new" serotype may cause asymptomatic or mild infections that do not require clinical attention in the earlier season and as that serotype becomes predominant during peak infection season, clinical cases of that serotype are identified and diagnosed (130) .

Water temperature. While peaks in clinical cases and sewage isolates for enteric viruses were often observed in late summer and early fall, Green and David (50) isolated a higher number of infectious enteroviruses from raw sewage and final effluent during the winter months.

In several studies, enteric viruses have been reported to survive longer and occur more frequently at lower temperatures in natural environments (i.e., seawater, river, groundwater) (50, 92, 93, 157). High temperatures can damage the virus capsid or nucleic acids, which might prevent adsorption of the virus to its host, and may inactivate enzymes required for replication (8). Lipp et al. (93) detected enteroviruses from an estuary in southwest Florida only when water temperature was below 23 ºC. In an *in vitro* study, enhanced poliovirus survival and detection was observed at 22 ºC as compared to 30 ºC in seawater (157). In artificial seawater, viruses were detected by RT-PCR for at least 60 days at 22 ºC, but only for only 30 days at 30 ºC (157). Similarly, Gantzer et al. (45) showed that in seawater, it took 671 days to inactivate 90 % of poliovirus and hepatitis A at 4 °C and only 25 days at 25 °C.

Sunlight inactivation. Next to temperature, ultraviolet (UV) radiation is the most common factor leading to virus inactivation. Sinton et al. (134) found that bacteriophage inactivation rates in sunlight are ten times higher than their inactivation rates in the dark. This is consistent with the findings by Johnson et al. (74), who observed 90% and 99.9% inactivation of polioviruses in marine water after 24 h incubation in dark and exposure to sunlight, respectively. Despite their susceptibility to UV radiation, viruses are more resilient than many other pathogens and indicator bacteria to that effect (44, 74). Furthermore, dsDNA adenoviruses are extremely stable when exposed to UV because their undamaged DNA strand may serve as a template for repair by host enzymes (49, 145).

Adsorption to suspended solids and sediment. Association of viruses with solids is believed to increase their persistence in natural environments by offering protection from enzymes, other degrading factors and UV inactivation (45, 50, 86, 104).

In a comparative virus-soil sorption study, poliovirus 1 (i.e., enterovirus) was shown to be the most sorptive to all soil types tested, followed by Norwalk virus and the F+ RNA coliphage, MS2 (104). Green and Lewis (50) reported that enteroviruses and hepatitis A could be detected throughout the year in sediment in the immediate vicinity of a sewage outfall even though enterovirus concentrations peaked in the wastewater during winter months. Likewise, during wet weather when contaminants were loaded into an estuary, Ferguson et al. (40) could isolate enteric viruses in both water and sediment samples, but during the dry season, only viruses persisted in the sediment. Furthermore, during the same dry season, Ferguson et al. (40) isolated enteroviruses from sediment samples collected as far as 23 km from the sewage overflow point. Because of frequent detections of pathogenic microorganisms in solids, Brookes et al. (18) suggested taking into account the survival and accumulation of microbes in sediments as well as the likelihood of their resuspension and re-distribution by natural and anthropogenic disturbance when assessing water quality issues related to public health risk.

Composition of water. In certain cases, composition of water (i.e., nutrient concentrations, predators, and dissolved oxygen) might have a significant effect in addition to water temperature and other factors on virus survival. In a study comparing *in vitro* and *in situ* survival in seawater, viruses survived significantly longer at lower temperatures in laboratory conditions but despite a similar temperature range in field studies, there was no significant difference in poliovirus survival between seasons in natural water (155). Likewise, Wetz et al. (157) showed that virus survival at both 22 °C and 30 °C in unfiltered natural seawater was much shorter than survival in filtered seawater or artificial seawater at either temperature. Finally, sampling season (summer *versus* winter) was shown to have a greater effect than incubation temperature on survival of both infectious poliovirus and poliovirus RNA (136). Survival of

infectious Poliovirus-1 seeded in river water collected during winter was greater than that of fecal coliforms regardless of incubation temperatures whereas the opposite is observed in summer water (136). Therefore, in addition to temperature, UV effects and adsorption to solids, viral persistence in natural waters may be strongly related to predation by flagellates, extracellular proteases, nucleases and other enzymes (110, 112, 143). Salinity has not been shown to have a direct effect on virus survival, although accelerated inactivation of fecal indicators has been reported in higher salinities (13, 45, 86, 139).

Overall, factors that influence the occurrence and survival of enteric viruses in waters, such as water temperature, suspended solids, turbulence, sunlight intensity, host excretion, nutrient content of water and predation have been extensively studied, and these parameters should be included when attempting to predict the presence of viral pathogens in the environment.

Adenoviruses and Enteroviruses as Water Quality Indicators and Microbial Source-Tracking Tools

The enteroviruses were the first enteric virus group studied in fecal-contaminated waters in the 1940s and continued to be among the most well-studied (55). Enteroviruses are included by the European Union regulations governing water quality as a parameter for evaluating viral pollution of a water body because they can easily be isolated and quantified as plaque-forming units (PFU) in cell culture and vaccine-related poliovirus is prevalent in contaminated waters (96, 122). Studies conducted in Europe and other parts of the world recently have suggested including adenoviruses as an index of pollution of human origin in waters because they have been shown to be more persistent and present in greater numbers than enteroviruses in sewage

and contaminated aquatic environments (70, 72, 84, 120, 145). Muniain-Mujika et al. (107) studied the prevalence of viral pathogens in shellfish from three sites in Spain with different levels of fecal contamination; 47 %, 19 % and 24 % of their samples were positive for human adenoviruses (HAdV), human enteroviruses (HEV) and hepatitis A virus (HAV), respectively. They proposed using HAdV as a molecular index for viral contamination in shellfish because HAdV was detected in all samples positive for HEV and HAV (107). In addition, Pina et al. (120) reported that the presence of human adenoviruses in sewage samples is highly correlated to the presence of hepatitis A viruses and human-specific bacteriophages, such as those infecting *Bacteriodes fragilis* HSP40.

In many watersheds experiencing water quality deterioration, pollutants originating from non-point sources such as urban runoff, forests, wildlife, and agricultural runoff (including contamination by manure application and unrestricted access of livestock and wildlife to rivers and streams) are difficult to identify for proper management and remediation planning. Enteroviruses and adenoviruses, just as other types of viruses, have a narrow host range. For example, human enteroviruses only infect humans, and cannot cause infection in cattle or fowl and vice versa. Because human and animal enteric viruses are excreted in large number in infected hosts, a fecal pollution tracking method based on the host specificity of viruses has been hypothesized as a useful indicator system for the presence of contaminants originating from specific sources (e.g., human sewage, cattle, or swine farms) (73, 90, 98, 113). In 1995, Metcalf et al. (105) were among the first researchers to hypothesize that molecular detection techniques based on host specificity of viral pathogens in environmental samples would allow the determination of the sources of contaminants and improve surveillance for public health;

however, this system has not been widely used and most methods for tracking pollution sources rely on microbial indicators (i.e., *E. coli* or enterococci).

Microbial source tracking (MST) methods currently being used can be categorized into four basic groups (Table 2.2): 1) genotypic library-based methods, i.e., ribotyping, repetitive extragenic palindromic-PCR (rep-PCR), and pulsed field gel electrophoresis (PFGE), which differentiate sources of pollutants by matching the genetic patterns of isolated bacteria to a library with bacterial isolates from known sources, 2) phenotypic library-based methods, i.e., antibiotic resistance analysis (ARA), and carbon source utilization (CSU), which differentiate sources of pollutants by matching the growth pattern of a bacterium, such as *E. coli* or enterococci, on a suite of antibiotics or carbon sources with those of isolates from a library of sources, 3) library-independent bacterial host-specific markers, i.e. identifying host-specific genetic markers of bacteria such as host-specific *Bacteroides-Prevotella* 16S rDNA markers with terminal restriction fragment length polymorphism (t-RFLP) or length heterogeneity PCR (LH-PCR), and 4) direct measurement of viral pathogens and bacteriophages with different host groups, i.e., HEV, HAdV, BEV and the F + RNA coliphages (56, 129, 133).

Both genotypic and phenotypic library-based methods have the advantages of being quantitative, highly sensitive, reproducible, and may be used to classify isolates from multiple sources (60, 117). Parveen et al. (117) reported that ribotyping of *E. coli* correctly classified an average of 82 % of human and non-human isolates. Antibiotic resistance patterns in fecal streptococci have been used successfully in a rural Virginia watershed to determine that cattle were the predominant source of fecal contamination and fecal coliform levels were reduced by an average of 94 % after appropriate remediation actions (60). The major drawback of library-based methods is the requirement of a large isolate database, which can be extremely

labor intensive, time-consuming and maybe geographically specific (56, 64, 129, 133). In addition, these methods have shown a high false positive rate when tested with spiked samples and in the case of ARA, bacterial isolates have to show antibiotic resistance to be typed (56, 129, 133).

The host-specific *Bacteroides-Prevotella* 16S rDNA markers have been used to differentiate between human and non-human sources of fecal contamination in a multi-use estuary in Oregon and a recreational beach in California (6, 10). In contrast to library-based methods, bacterial host-specific markers do not require a reference library or a cultivation step, testing is rapid and easy to perform with PCR (though non-quantitative), and has been reported to have a very low false positive and false negative rate when differentiating human and non-human sources of contaminants from spiked samples (56, 129, 133). However, the survival and distribution of bacterial host-specific markers in aquatic systems have not been extensively studied and this method is currently applicable only to a limited number of host groups (129).

The F + RNA coliphages are the most extensively studied and well characterized phages for use in source tracking (129, 135). The F + RNA coliphages are divided into four subgroups that are highly associated with different host categories. Analysis typically involves enumeration of the phages on host cells (*E. coli*) followed by serotyping or genotyping to determine the subgroup of the bacteriophage (69). Detection, enumeration and subtyping of the F + RNA coliphage is easy to perform and straightforward; however, its low occurrence in human feces and other aquatic environments despite its frequent detection in wastewater indicates that the phages might be able to proliferate in sewage (65). In addition, its survival rate in marine and tropical waters is varied and exceptions to the associations between coliphage subgroup and

particular host group have been reported, e.g., subgroup II and III coliphages (human-specific) have been isolated from pigs (26, 126).

Detection of host-specific viral pathogens with molecular assays, such as PCR and its variants, are less laborious and time-consuming than library-based microbial source tracking techniques because a reference database is not required and results generally can be obtained in a relatively short period (hours to a day) (113). Through different primer sets that target enteric viruses within a specific host group, PCR detection of viral pathogens can be used to directly identify the major source of contamination in environmental samples. In addition, primers are usually developed from conserved regions of the viral genome that show high stability and do not change appreciably with time or environmental conditions (113). Though enteric viruses occur in low numbers in some aquatic environments, recent modifications and improvements in viral concentration, extraction and detection techniques have allowed detection from waters that generally have very low number of viruses, such as potable water (88). Furthermore, the occurrence, survival and transport of theses viruses under different environmental conditions have been well characterized (14, 53, 120).

Recent studies reveal that tracking of human-specific enteroviruses (HEV) and adenoviruses (HAdV) show promising and reliable results in indicating the presence of human sewage and discriminating between human and non-human pollution sources in environmental waters (113, 120). In one study, PCR detection of human enteric viruses from mixed fecal samples of human- and nonhuman-origin has shown that HAdV is more specific than HEV in that it picked up most samples with human feces but none of the solely nonhuman-origin samples (120).

Animal-specific enteroviruses and adenoviruses have also showed great potential as indicators for fecal contamination of animal origin (73, 90, 98). In a study that examined the prevalence of bovine enteroviruses (BEV) in a closed herd of cattle, other animals on the premises and environmental samples in the area, Ley et al. (90) found BEV in feces of 76 % of cattle, 38% of white-tailed deer and in one of three geese. BEV were also isolated from streams and rivers that received runoff from the farm and in oysters collected in the rivers. Ley et al. (90) concluded that with additional analyses of BEV in animals from other areas, BEV might serve as marker for bovine fecal contamination. Jiménez-Clavero et al. (73) detected porcine teschoviruses (PTV) RNA in water and fecal samples from five pig farms located in different parts of Spain, but not in fecal samples from other animals. In addition, Jiménez-Clavero et al. (73) also suggested that PCR-based identification of virus species was a more reliable and sensitive marker than conventional chemical water quality indicators such as nitrate and nitrite readings (73). PTV RNA was detected as far as 3 km downstream from the discharge, where the impacts on nitrates and nitrites were no longer observed (73). Maluquer de Motes et al. (98) reported a high prevalence of bovine adenoviruses (BAdV) and porcine adenoviruses (PAdV) in animal feces but all of the human sewage-contaminated samples tested negative. All three studies above suggest that while animal-specific viruses have been shown to have a high prevalence in aquatic environments directly influenced by the particular animal source, the analysis of a large number of samples from different geographical areas is necessary to validate the application of animal-specific viruses for identifying the source of fecal contamination (73, 90, 98).

Conclusions

Enteric viruses are important pathogens that are frequently isolated from waters directly or indirectly influenced by fecal contamination and have been associated with many waterborne outbreaks (25, 53, 72, 93, 120). Given that traditional bacterial indicators have been shown to be inappropriate for viruses and other pathogens, and direct detection methods now exist for easy analysis of viral pathogens, surveillance for pathogens directly may be warranted to better protect public health (93, 102). Factors that have been shown to affect the occurrence and survival of viruses can be incorporated into models that predict the levels of viral contamination in specific types of water and can contribute to efforts to control contamination. In addition, the stringent host specificity of enteric viruses suggests that they can be good library-independent indicators for identifying sources of water pollution. Molecular detection (e.g., PCR and hybridization) of viral pathogens is rapid, highly specific and sensitive, and with the use of quantitative (real-time) PCR, concentration of viral pathogens in environmental samples can be determined. PCR assays can be developed based on genotypic differences between viruses with different host groups and be used to better characterize sources of contamination in aquatic environment so that an appropriate and cost-effective water quality remediation plan can be developed. However, additional research to study the prevalence and distribution of animal-specific viruses in environmental waters is required to validate the use of these viruses for source-tracking purposes.

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Table 2.1. Comparison of common methods for the detection of enteric viruses from environmental sources.

Method	Advantage(s)	Disadvantage(s)	Ref.
Cell culture	Infectivity can be determined Provides quantitative data	Long processing time; takes days to weeks Relatively more expensive than conventional PCR Not all viruses can grow on cultured cells	(94, 141)
PCR (RT-PCR)	Rapid Increased sensitivity and specificity compared to cell culture	Presence or absence only (non-quantitative) Inhibitors present in environmental samples may interfere with PCR amplification Infectivity cannot be determined	(54, 92)
Nested-PCR (semi-/heminested)	Increased sensitivity compared to conventional PCR Can replace PCR confirmation steps, such as hybridization	Potential risk of carryover contamination when transferring PCR products	(72, 120, 153)
Multiplex PCR	Several types, groups or species of viruses can be detected in a single reaction Saves time and cost	Difficult to achieve equal sensitivity for all targeted virus species/group/type May produce non-specific amplification in environmental samples	(41, 50)
Real-time PCR	Provides quantitative data Confirmation of PCR products is not required, saves time Can be done in a closed system, which reduces risk of contamination compared to nested-PCR	Expensive equipment Occasionally less sensitive than conventional PCR and nested-PCR	(7, 34, 113)
ICC-PCR	Improves detection of infectious viral pathogens when compared to conventional cell culture Detects viruses that do not produce CPE in cell culture Provides results in half the time for conventional cell culture	Less time-efficient and more costly than direct PCR detection Carryover detection of DNA of inactivated viruses inoculated onto cultured cells is possible	(23, 52, 82)

Table 2.2. Comparison of methods that may be used for microbial-source tracking in aquatic environments.

Method	Advantage(s)	Disadvantage(s)	Ref.
Genotypic, library-based			
Ribotyping	Quantitative	Large isolate database required; geographically specific	(64, 117)
	Highly sensitive & reproducible	Labor intensive & time consuming	
	Classifies isolates from multiple sources	High percentage of inconclusive results	
PFGE	Sensitive, discriminative and reproducible	Labor intensive & time consuming	(75, 115)
	Quantitative	May be too sensitive for discriminating multiple sources	
Phenotypic, library-based			
ARA	Rapid	Large isolate database required; geographically specific	(60, 116)
	Classifies isolates from multiple animals sources	Isolates have to show antibiotic resistance to be typed	
		Antibiotic resistance traits are not stable	
		No consensus on combination & dose of antibiotic used	
Library-and culture-independent (bacterial host-specific markers)			
t-RFLP, LH-PCR	Rapid & easy to perform	Survival & distribution of molecular markers in aquatic environments are not well-studied	(6, 10)
	No database or cultivation required	Expensive equipment	
	High accuracy in differentiating human & non-human sources	Currently applicable only to a limited number of host groups	
Direct measurement of host-specific viruses			
PCR	Library independent & directly relates to health risk	Non-quantitative in conventional PCR	(34,93,113)
(viral pathogens)	Rapid & straightforward	Requires more sensitive detection methods	
	Detects conserved regions of a viral genome; may not have geographical limits	Limited knowledge of prevalence of animal-specific viruses in aquatic environments	
PCR & phage typing	Subgroups are well-correlated to sources	Serotyping is expensive and time-consuming	(69, 126)
(F + RNA coliphage)	Straightforward	Low survival in marine & tropical waters	
		May proliferate in sewage	
		Exceptions between association of coliphage subgroup & host group have been noted	

CHAPTER 3

MOLECULAR DETECTION OF WATERBORNE HUMAN AND BOVINE ENTERIC VIRUSES IN A MULTI-USE COASTAL WATERSHED[1]

[1] Theng-Theng Fong, Dale Griffin and Erin K. Lipp. To be submitted to *Applied and Environmental Microbiology*.

ABSTRACT

Rapid population growth and urban development along waterways and coastal areas has led to decreasing water quality. To examine the effects of upstream anthropogenic activities on microbiological water quality, methods for source specific testing are required. In this study, molecular assays targeting human enteroviruses (HEV), bovine enteroviruses (BEV) and human adenoviruses (HAdV) were developed and used to identify major sources of fecal contamination in the lower Altamaha River, Georgia, USA. Two-liter grab samples were collected monthly from five tidally influenced stations between July and December 2002. Samples were analyzed by RT- and nested PCR. PCR results were confirmed by dot-blot hybridization. Eleven and 17 of the 30 surface water samples tested positive for HAdV and HEV, respectively. Two-thirds of the samples tested positive for either HEV or HAdV and the viruses occurred simultaneously in 25.71 % of samples. BEV were detected in 11 of 30 surface water samples. Binary logistic regression analysis showed that the presence of both human and bovine enteric viruses was not significantly related to either fecal coliform or total coliform levels. The presence of these viruses was directly related to dissolved oxygen and streamflow, but inversely related to water temperature, rainfall in the 30 days preceding sampling and chlorophyll-*a* concentrations. The stringent host specificity of enteric viruses makes them good library-independent indicators for identification of water pollution sources. Viral pathogen detection by PCR is a highly sensitive and easy-to-use tool for rapid assessment of water quality and fecal contamination when public health risk characterization is not necessary.

Keywords: source-tracking, enterovirus, fecal contamination, bovine, adenovirus, water quality

INTRODUCTION

Fecal coliform bacteria and other bacterial indicators have been used by most water quality regulators in the United States for over a century as standard tools to measure fecal contamination and determine if a body of water is suitable for its designated use (e.g. fishing, potable use, recreational, industrial, wildlife preserve, etc.). These standards have helped to improve water sanitation and protect public health (34); however, there are several drawbacks to these indicators that make them unreliable for predicting the occurrence of many waterborne pathogens and identifying fecal contamination sources. Fecal coliform bacteria may be found in both human and animal feces; therefore, tracking and monitoring the source of contamination is impossible without sophisticated microbial source tracking techniques such as multiple antibiotic resistance profiling, ribotyping and pulsed field gel electrophoresis, which require an extensive strain database and can be laborious and costly (15, 68). Coliform standards often fail to predict the occurrence of many waterborne human pathogens such as pathogenic bacteria, the protozoan parasites *Cryptosporidium* and *Giardia* as well as enteric viruses, which are most often the cause of disease from recreational exposure (29, 34, 76). Furthermore, traditional bacterial indicators generally die off quickly in marine water when compared to viruses and protozoa (40, 50, 51, 57, 60, 72, 87). Studies have shown that human pathogenic viruses have been isolated from sites with no violation of coliform standards (34, 50, 51) and outbreaks of gastroenteritis have been associated with water supplies with acceptable fecal coliform counts (e.g.,10, 13, 36, 37).

Over 100 types of pathogenic viruses have been found in sewage-contaminated aquatic environments, such as ground water, coastal marine water, coastal river water, aerosols emitted from sewage treatment plants, insufficiently treated water drinking water and private wells that received treated or untreated wastewater either directly or indirectly (e.g., 6, 30, 47, 66, 71, 89).

These viruses, collectively known as enteric viruses, are transmitted via the fecal-oral route, and primarily infect and replicate in the gastrointestinal tract of the hosts. Enteric viruses are excreted in high concentrations in human and animal feces and, in certain cases, urine (53, 62). Infected individuals suffering from viral gastroenteritis and hepatitis may excrete from 10^5 to 10^{11} viral particles per gram of stool, with average levels between 10^6 and 10^8 viral particles per gram of stool for enteroviruses and hepatitis A virus, respectively (20, 21, 26, 88). Enteric virus concentrations in raw sewage and polluted surface water have been estimated at around 10^2 viral particles 100 ml $^{-1}$ and 1 to 10 viral particles 100 ml $^{-1}$, respectively (28, 73, 80). Enteric viruses also are more resistant than many other sewage-associated pathogens and bacterial indicators to extreme environmental conditions and conventional wastewater treatment such as chlorination, UV radiation, and filtration (40, 77, 78). These viruses can also remain infective for long periods in the environment; they have been reported to survive for up to 130 d in seawater, up to 120 d in freshwater and sewage, and for up to 100 d in soil at 20 - 30 ºC (1, 9, 81, 85). These survival periods surpass those reported for fecal coliform and other indicator bacteria in similar environments (40, 54). Therefore, the traditional bacterial indicators are poor proxies to monitor the presence of pathogenic viruses.

The host specificity of enteric viruses and their prevalence in sewage and fecal-contaminated waters suggest that they can be promising library-independent microbial source tracking tools for polluted environmental waters (41, 48, 52, 58). Human enteroviruses, which consist of poliovirus, coxsakieviruses A and B, echoviruses and the numbered-enteroviruses, have been included by the European Union regulations governing water quality as a parameter for evaluating viral pollution of a water body because they can easily be isolated and quantified as plaque-forming units (PFU) in cell culture (62, 65). Recent studies

conducted in Europe have also suggested using adenoviruses as an index of pollution of human origin in waters given their high numbers in sewage and contaminated aquatic environments (38, 40, 44, 45, 62). Human adenoviruses (HAdV) are the only human enteric viruses that contain double-stranded DNA instead of RNA, potentially are more stable in various environments, and are more resistant to UV irradiation and other water purification treatments than other human enteric viruses because they are able to use the host cell DNA repair mechanism to repair damages in their DNA caused by UV irradiation (27, 38, 55). Adenoviruses have been found to survive three to five times longer in seawater, wastewater and tap water than poliovirus (18). Both human enteroviruses (HEV) and HAdV are readily detected in surface water as well as coastal waters that have received anthropogenic inputs (2, 8, 11, 40, 50, 62, 64). Recently, animal-specific enteroviruses and adenoviruses have been identified and may be used as indicators to identify and monitor fecal contamination originating from cattle farms, swine farms, or other animal sources (41, 48, 52).

The use of PCR-based viral pathogen detection assays to identify the source categories (human and animals) of fecal pollution in coastal rivers has not been previously evaluated. In this study, we examined the extent and the relative importance of fecal contamination from agricultural (cattle), anthropogenic activities and development upstream in coastal reaches of the lower Altamaha River, Georgia, USA, by detecting three groups of host-specific enteric viruses: human enteroviruses (HEV), human adenoviruses (HAdV) and bovine enteroviruses (BEV). We also compared the findings from PCR-based assays to concurrently collected bacterial indicator data and other environmental variables such as rainfall, streamflow, and water temperature to evaluate the use of this assay in defining estuarine water quality in a mixed-use watershed.

MATERIALS AND METHODS

Sampling sites. The Altahama is the largest river of the Georgia coast and the second largest basin in the eastern United States (24). It drains more than one-fourth of the state, with a drainage area of approximately 7,107 km ^{2} (24). The Altamaha supports more than 30 % of Georgia's $80 million commercial fishery and about one-third of Georgia's $350 million recreational fishery according to the Altamaha Riverkeeper (4). The lower Altamaha River acts as a conduit for discharging the combined flow from two major rivers in Georgia: the Ocmulgee River and the Oconee River. Urban development, population growth and a growing ecotourism industry in the coastal areas as well as upstream urban, agricultural and industrial discharges have degraded the quality and productivity of the lower Altamaha River markedly, and there is evidence of increasing coastal salinity, harmful algal populations, and declining fishery stocks in the river (24).

Samples were collected from five stations along a 15-km stretch of the lower Altamaha River, located between Glynn and McIntosh counties, in conjunction with the Georgia Marine Extension Service, Brunswick, GA (Fig. 3.1). Sampling stations were located approximately 11 km and 23 km from regulated commercial shellfish harvesting areas in McIntosh and Glynn counties, respectively. Although not regulated, all sampling sites were within areas considered to be shellfish supporting by the State (23). The sampling stations are surrounded by marsh islands and wetlands that are mainly inhabited by hogs, cattle ("Cow Island") and waterfowl, and are within the waterfowl management area. Samples were collected monthly from July to December 2002 to observe and track the changes in enteric virus loading in the river between wet and dry seasons. All samples were collected on an outgoing tide, starting with station 1, which is located at the Altamaha Sound (mouth of the river) and ending with station 5, which is located farther

inland, west of the Broughton Island and downstream from a commercial fish camp (Fig. 3.1). At each station, a grab sample was collected from just below water surface. Water samples were collected from the bottom of the water column in July, but because of processing difficulties due to high turbidty no additional samples were collected. Two liters from each sample was kept on ice (~ 4°C) and processed within 24 hours of collection. Salinity, water temperature, pH, chlorophyll *a*, turbidity, dissolved oxygen (DO) and weather conditions (i.e., ambient temperature, rainfall, and wind etc.) were noted for each sample at collection. Streamflow data for the area was obtained from the USGS stream gauge at Doctortown, GA (USGS station 02226000). Rainfall data for Glynn County, GA, was obtained from the Georgia Automated Environmental Monitoring Network (www.georgiaweather.net). The Georgia Marine Extension Service provided fecal coliform and total coliform counts for each sample.

Concentration of viruses. Water samples were concentrated according to a method described by Katayama et al. (43) with the following modifications. Samples were acidified to a pH of 3.5 to 4.0 by adding 10% acetic acid prior to filtration. Acidified water samples were filtered through a type-HA, negatively charged membrane (Millipore, Billerica, MA) with 47-mm diameter and a 0.45-µm pore size. Because of the high turbidity, the volumes of water filtered ranged between 0.5 and 2.0 L. A volume of 100 ml 0.5 mM H_2SO_4 was then passed through the membrane, and viral particles were finally eluted with 10 ml of 1 mM NaOH. Eluate was recovered in a tube containing 0.1 ml of 50 mM H_2SO_4 and 0.1 ml of 100 x TE buffer for neutralization. All 10-ml eluates were stored at -20 °C. Eluates were further purified, concentrated and desalted with Centriprep YM-50 concentrator columns (Millipore, Billerica, MA). The final volume of concentrated eluate recovered was about two milliliters. Concentrates were split in half and stored at – 80 °C.

Extraction of viral RNA and DNA. Concentrated samples were extracted for viral RNA and purified through commercial spin columns based on the method of Boom et al. (7), using an RNeasy Mini Kit (Qiagen, Valencia, CA). Briefly, 200 µl of concentrated sample were lysed with guanidine-isothiocyanate and homogenized before ethanol was added to the lysate to provide ideal binding conditions. The lysate was then loaded onto the silica-based spin column provided by the kit and viral RNA was adsorbed to the spin column according to the manufacturer's protocol. This allows the lysate to be efficiently washed to remove the debris and PCR inhibitors. Purified viral RNA was then eluted and resuspended in 50 µl of RNase-free water. Each concentrated and purified sample was serially diluted to a concentration of 10^{-3}.

Concentrated water samples were extracted and purified for viral DNA using a DNeasy Tissue Kit (Qiagen, Valencia, CA). The DNeasy Tissue Kit works under similar principles as the RNeasy Kit except that viral DNA, not viral RNA was extracted from the samples. In brief, proteinase K provided by the kit was added to 200 µl of samples to lyse the viral particles. DNA was then adsorbed to the silica-based spin column provided by the kit and samples were washed twice to remove the debris and enzyme inhibitors. Extracted DNA was eluted and resuspended in 50µl of AE buffer (elution buffer) provided by the kit. Each concentrated and purified sample was serially diluted to a concentration of 10^{-3}.

Oligonucleotides. Primers and probes were selected from highly conserved regions of the HEV, HAdV and BEV genomes, which allowed for detection of multiple members from each group of viruses. For HEV detection, the pan-enterovirus primer set (ENT-up-2 and ENT-down-1) developed by De Leon et al. (14) was used in conjunction with another HEV primer set (ENT-up-1 and ENT-down-2; developed by J.H. Paul at the University of South Florida) to develop a RT-nested-PCR assay for the pan enterovirus group (Table 3.1). The

nested-HEV primers were selected from the 5' untranslated region (UTR) of HEV genomes by aligning with previously published sequences available on the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). HEV primer sets were able to pick up at least 25 different HEV; Echovirus 22 was not detected (14, 16). The BEV primer set was identified by aligning and evaluating the 5' UTR sequences of BEV genomes available on the NCBI database (Table 3.1). The sequences amplified by the BEV primer set were BLAST searched in Genbank and showed exact match with seven different strains of bovine enteroviruses (strains PS87, RM2, SL305, K2577, BOT/209/67, BEV261, and VG527) and one sheep enterovirus isolate (strain 82Sh2R). The primers were tested on Poliovirus 1 (strain Lsc) and HAdV 2, and showed no cross-reaction with either of the virus groups. The nested-primer sets for HAdV designed by Allard et al. (3) were selected from the DNA sequence of the open reading frame of hexon genes of HAdV (Table 3.1). The HAdV primers sets are able to identify 47 HAdV serotypes, including the more common HAdV types 2, 40, and 41 (3, 64). The internal probe used for HEV dot blot hybridization was described by DeLeon et al. (14) and is able to pick up 25 different enteroviruses (33). Internal probes for BEV and HAdV were developed in this study (Table 3.1).

Reverse Transcription-Nested PCR for HEV. HEV were amplified with reverse transcription-nested-polymerase chain reaction (RT-nested-PCR). RT-nested-PCR was performed with the RNA-PCR core kit by Applied Biosystems (Foster City, CA). Because enteroviruses are single-stranded RNA viruses, enteroviral RNA was reverse transcribed to create complementary DNA (cDNA) before amplification by nested-PCR. The reaction mixture for RT consisted of 2.5 µl of concentrated and purified sample RNA, and 7.5 µl of reaction mixture. The RT reaction mixture contained 5.0 mM $MgCl_2$, 1X PCR buffer, 0.75 mM

deoxynucleosides (dNTPs), 2.5 μM random hexamer (as provided in the kit), 2.5 U μl^{-1} reverse transcriptase enzyme, and 1.5 U μl^{-1} RNase inhibitor. The temperature cycle for RT was 22 °C for 10 min, 42 °C for 15 min and 99 °C for 5 min. Samples were cooled to 4 °C before the addition of master-mix for the first round of PCR (PCR I). All reactions were performed in a DNA Engine® PTC-0200 thermal cycler (MJ Research, Inc. Waltham, MA).

In order to optimize the detection sensitivity by PCR, we tested several concentrations of primers, $MgCl_2$, and dNTPs as well as annealing temperatures and numbers of cycles. During PCR 1, the 5' UTR of the viral cDNA was amplified with primers ENT-up-1 and ENT-down-1, yielding amplicons of 333 basepairs (bp) in size. PCR I was carried out by adding 20 μl of PCR-master-mix to the reaction mixture from RT. The PCR I reaction mixture had a final concentration of 2.9 mM $MgCl_2$, 1X PCR buffer, 0.4 mM dNTPs, 0.2 μM of each primer, 2.1 U μl^{-1} *Taq* polymerase enzyme and 1X Eppendorf TaqMaster® (Brinkmann Instruments, Inc. Westbury, NY). PCR I consisted of 40 cycles of denaturing at 95 °C for 30 s, annealing at 57.7 °C for 30 s, and extension at 72 °C for 45 s. During the last cycle of amplification, an extra five minutes for extension were included.

One microliter of amplified PCR product from PCR I was transferred into the master-mix for the second round of PCR (PCR II). The reaction mixture for PCR II had a final volume of 50 μl containing 2.5 mM $MgCl_2$, 1X PCR buffer, 0.2 mM dNTPs, 0.2 μM of each primer (ENT-up-2 and ENT-down-2), and 2.5 U μl^{-1} *Taq* polymerase enzyme. PCR II consisted of 40 cycles of denaturing at 95 °C for 30 s, annealing at 56.5 °C for 30 s, and extension at 72 °C for 30 s. As with PCR I, a final extension of five minutes was included during the last cycle. Amplicons were 154 bp. Poliovirus 1 (vaccine strain Lsc, courteously provided by Dr. C. P. Gerba, University of Arizona) was used as a positive control and molecular grade nuclease-free

water was used as a no-template negative control. The equivalent original volume of water analyzed by this HEV RT-nested PCR reaction for each sample ranged between 2.5 ml and 10 ml, depending on volume filtered for adsorption-elution.

RT-PCR for BEV. RT for BEV was performed under the same conditions as RT for HEV. Total volume and final concentrations in the PCR reaction mixture for BEV were also the same as for the reaction mixture in PCR I for HEV. The cDNA was amplified for 40 cycles, which consisted of denaturing at 95 °C for 30 s, annealing at 56 °C for 30 s, and an extension at 72 °C for 1 min followed by a final five-minute extension. The resulting amplicons were about 270 bp. BEV type 1 (ATCC VR-248) was used as the positive control; human poliovirus 1 (strain LSc), HAdV type 2 and molecular grade nuclease-free water were used as negative controls to test for cross-reaction and contamination, respectively. As with HEV, the equivalent original volume of water analyzed for each sample ranged between 2.5 ml and 10 ml.

Nested-PCR for HAdV. Nested-PCR for HAdV was performed by amplifying the open reading frame of the hexon gene of adenoviruses following the protocol of Pina et al. (62) with modifications in the concentration of primers, dNTPs, annealing temperature and the addition of Eppendorf TaqMaster® (Brinkmann Instruments, Inc. Westbury, NY) into PCR reaction mixtures. TaqMaster® was added to stabilize the *Taq* DNA Polymerase and make it less sensitive to exogenous PCR inhibitors. Two rounds of PCR consisting of 40 cycles each were performed. In the first round of PCR (PCR I), 1.5 µl of sample DNA were added to a 23.5 µl-reaction mixture. The reaction mixture contained 1.5 mM $MgCl_2$, 1X PCR buffer, 0.5 mM deoxynucleoside (dNTPs), 0.8 µM of each primer, 1X TaqMaster® and 2.5 U μl^{-1} *Taq* polymerase enzyme. Three µl of PCR I product were used as template for PCR II. Primers AV-A1 and AV-A2 were used in PCR I; primers AV-B1 and AV-B2 were used in PCR II. Both

rounds of PCR were performed under the same conditions: initial DNA denaturation at 94 °C for 4 min followed by 40 cycles of denaturation at 92 °C for 30 s, annealing at 60 °C for 30 s, and an extension at 72 °C for 1 min. A final extension at 72 °C for five min was added during the last cycle of amplification. Amplicons of 300 bp and 142 bp were produced from PCR I and PCR II, respectively. HAdV type 2 was used as the positive control (courteously provided by Dr. C.P. Gerba), and molecular grade nuclease-free water was used as a no-template negative control. The equivalent original volume of water analyzed for each sample ranged between 1.5 ml and 6 ml.

Visualization and confirmation of PCR products. Twelve and one half microliters of PCR products were analyzed by gel electrophoresis on a 2.2% average strength Omnipur® agarose gel (EM Science, Darmstadt, Germany). The gel was stained in ethidium bromide and viewed under UV light. PCR products were then confirmed by dot-blot hybridization with biotin-labeled probes (Table 3.2) internal to the amplified viral regions and detected by chemiluminescence following the protocol for Southern-Light™ chemiluminescent detection system for biotin-labeled DNA from Applied Biosystems (Bedford, MA) (14, 33, 50). All blots were hybridized overnight at 37 °C. Stringency wash temperatures for HEV, HAdV and BEV probes were 47 °C, 65 °C and 51 °C, respectively.

Detection efficiency. The sensitivities of all PCR assays were determined by limiting dilution experiments of pure virus stocks in cell culture lysates (~10^8 viral particles ml $^{-1}$ for HEV and HAdV and ~10^7 ml $^{-1}$ for BEV). Viral particles were counted using the method described by Noble and Fuhrman (59), with the exception that viruses were stained with SYBR Gold rather than SYBR green I nucleic acid gel stain (Molecular Probes, Eugene, OR). Viral RNA (enteroviruses) and DNA (adenoviruses) extracts were serially diluted to 10^{-8}, reversed transcribed (HEV and BEV), and amplified by PCR methods described previously.

The efficiency of viral detection after concentration by adsorption-elution was also evaluated using a seeded study. MilliQ water was used as a control. A fresh estuarine water sample (with salinity 5 $^{0}/_{00}$ and pH 7.9) was filtered through 0.45 μm membrane filters to remove particles. Prior to inoculation, the filtered estuarine water sample was exposed to UV light for at least 24 hours to reduce or eliminate any background viruses. For quality control, two samples were taken from each type of water before inoculation as pre-seed controls. One liter each of the two water types were spiked with known amounts of HEV, BEV and HAdV, and serially diluted to one virus particle ml $^{-1}$. Viruses were added to the different water types and mixed at room temperature for at least an hour before processing began. Seeded samples were concentrated and extracted following the protocols as described above and detected by (RT-)PCR. All PCR products were then confirmed by dot-blot hybridization following the protocol described previously.

Statistical analysis. The Pearson correlation test was used to evaluate relationships among water quality and environmental variables. Binary logistic regression was used to analyze the relationship between the occurrence of viruses and levels of bacterial indicators and other environmental variables collected in this study. Minitab® Release 12.2 (Minitab, Inc., State College, PA) was used for logistic regression and correlation analyses. The analysis of variance (ANOVA) and subsequent comparisons to determine differences in mean levels of bacterial indicators and other environmental variables were performed using Graph Pad Prism (GraphPad Software, Inc., San Diego, CA). In all cases, significance was determined at the 95% confidence level.

RESULTS

Physical and chemical parameters related to water quality. A total of 35 samples were collected (including five samples that were collected from the bottom of the water column in July). Water temperature ranged between 10.52 ˚C (December) and 31.02 ˚C (July). Average water temperatures were similar for July, August, September, and October (means range between 27.7 ˚C and 29.5 ˚C) and dropped significantly as winter approached (November mean 20.24 ˚C; December mean 11.03 ˚C; $p < 0.001$). Increases in dissolved oxygen levels corresponded to the decrease in water temperature ($r = -0.791$, $p < 0.001$). Dissolved oxygen levels were the lowest in August with an average of 2.86 mg L^{-1}, and the highest in December with an average 9.35 mg L^{-1}. Salinity varied between sites and sampling dates and ranged between 0.09 $^{0}/_{00}$ (station 5 in December) and 23.13 $^{0}/_{00}$ (station 1 in September). Station 5 (located farthest inland) consistently had the lowest salinity (mean 0.99 $^{0}/_{00}$). Salinity levels were significantly lower at all stations in November (mean 2.91) and December (mean 2.85) ($p = 0.01$) and were moderately influenced by increased streamflow ($r = -0.5446$, $p = 0.0019$) (Table 3.4).

Total monthly precipitation ranged from a seasonal high of 16.51 cm in August to a winter-time low of 5.59 cm in December, which was significantly lower than precipitation levels in any other month (Fig. 3.2). However, streamflow was the lowest in August, 50.82 m^3 s^{-1} and the highest in December, 321.84 m^3 s^{-1} (Fig. 3.2).

Biological water quality indicators. Chlorophyll-*a* measurements did not vary significantly among stations and the averages ranged between 7.52 µg L^{-1} (station 5) to 8.54 µg L^{-1} (station 3). Monthly averages were more variable and ranged from 10.69 µg L^{-1} in August to 4.86 µg L^{-1} in December, which was significantly lower than in other months ($p < 0.001$).

Over the course of the study, fecal coliform levels ranged between 2 MPN 100 ml $^{-1}$ (station 1, July) and 170 MPN 100 ml $^{-1}$ (station 5, September) (Table 3.2). Increases in fecal coliform levels corresponded moderately to decreasing salinity ($r = -0.648$, $p < 0.001$). Fecal coliform levels were the lowest at station 1 (located at the mouth of the river), with a geometric mean of 7.1 MPN 100 ml $^{-1}$, and increased up the river where station 5 consistently had the highest levels and a geometric mean of 74 MPN 100 ml $^{-1}$ (Table 3.2). Total coliform levels ranged between 11 MPN 100 ml $^{-1}$ (station 1, July) and 2400 MPN 100 ml^{-1} (station 5, December), following the same trend as fecal coliform levels.

Viral detection efficiency. Pure cultures of HEV and HAdV could each be detected to a 10^{-8} dilution (~ 4 viral particles ml $^{-1}$) with RT-nested-PCR and nested-PCR, respectively. The BEV was detected by RT-PCR to a 10^{-3} dilution (~ 40,000 viral particles ml $^{-1}$).

The efficiency of viral detection after concentration by adsorption-elution was evaluated in a seeded study. In MilliQ water, the equivalent of 4 HAdV particles ml $^{-1}$ were detected by nested-PCR. HEV were detected to a concentration of 40 particles ml $^{-1}$ with RT-nested-PCR. Both HAdV and HEV were detected to a level of 40 particles ml $^{-1}$ in filtered estuarine water. BEV were detected to a concentration of 4×10^{5} viral particles ml $^{-1}$ both in MiliQ and filtered estuarine water with RT-PCR.

Dot blot hybridization showed no significant improvement in detection efficiency for seeded HAdV (detection limits remain 4 viral particles ml $^{-1}$ in MilliQ water and 40 viral particles ml $^{-1}$ in filtered estuarine water). However, the additional hybridization step did improve the detection of HEV by an order of magnitude. HEV was detected by hybridization to a concentration of 4 particles ml $^{-1}$ in both MilliQ water and filtered estuarine water. Detection efficiency was relatively low for BEV but also improved by 10 - 100 fold after dot-blot

hybridization in which they were detected to a concentration of 4×10^4 viral particles ml^{-1} and 4×10^3 viral particles ml^{-1} in filtered estuarine water and MilliQ water, respectively.

Detection of human enteric viruses from environmental samples. HEV were detected in 17 out of 30 (56.67 %) surface water samples and two out of five (40 %) bottom water samples (collected only in July). HAdV were detected in 11 (36.67 %) surface samples and one (20 %) bottom sample. 66.67 % of surface water samples and 40 % of bottom water samples were positive for either HEV or HAdV. The viruses were detected simultaneously in nine (25.71 %) samples (including one of the bottom water samples); this consisted of 40.9 % of those samples in which either HEV or HAdV were detected.

Human enteric viruses were most frequently detected at stations 1 and 4, followed by station 5, station 2 and station 3 (Table 3.3). Six out of seven (85.7 %) samples taken from station 1 were positive for either HEV or HAdV. HEV were detected from four (66.67 %) surface samples, and from the bottom sample collected in July, and HAdV was detected from three (50 %) surface samples at station 1. At station 4, HEV and HAdV were each detected from four out of six (66.67 %) surface samples; HEV and HAdV were detected concurrently from three (50 %) surface samples. For station 5, HEV and HAdV were each detected from three out of six (50 %) surface samples, and HEV and HAdV were detected simultaneously from the bottom sample collected in July. Four HEV positive and only one HAdV positive samples were detected from station 2. Human enteric viruses were only detected at Station 2 from July to October. Station 3 seemed to be the least contaminated station throughout the study period with only two HEV positive (October and November) samples. No HAdV was detected from station 3. Overall, two out of five (40 %) bottom samples (collected in July) tested positive for one or more viruses: station 1 was positive for HEV and station 5 was positive for both HAdV and

HEV. The percentage of positive HEV and HAdV samples at each sampling station is shown in Fig. 3.3.

The occurrence of human enteric viruses demonstrated a clear seasonal trend in which the frequency of detection increased at lower water temperatures and increased streamflow (Fig. 3.2). Both of these conditions reached their greatest extent in December at which time HEV and HAdV were detected simultaneously in 80% of the samples. Conversely, only three samples (stations 1, 2 and 5) were positive for any human virus in July (average temperature 31.02 ˚C) and two of these samples (stations 1 and 5) were collected from the bottom of the water column (Table 3.3).

The occurrence of human enteric viruses was significantly related to streamflow level on the day samples were collected, mean daily rainfall for the 30 days preceding the sample collection and other water quality variables (i.e., water temperature, DO levels and chrolophyll-*a*) but not related to rainfall on the sample collection day and mean daily rainfall for up to seven days preceding the sample collection, salinity, pH, and nutrient levels. Binary logistic regression models using streamflow (on sampling days), water temperature, 30-d mean rainfall, DO and chlorophyll-*a* concentrations as independent variables were able predict the presence or absence of HEV and HAdV together in a sample ($p < 0.05$) (Table 3.4). Generally, the presence or absence of human enteric viruses was directly (positively) related to streamflow and DO, and inversely related to temperature, rainfall and chrolophyll-*a* concentrations. Fecal coliform and total coliform levels in sampling area were generally low and did not show any correlation to viral detection (Table 3.4).

Detection of bovine enteric viruses from environmental samples. BEV were detected from 11 out of 30 (36.67 %) surface water samples and none of the bottom water samples. Over

the course of study, station 4 was the most contaminated site for BEV; BEV was detected from four out of six (66.67 %) surface samples. Station 1, station 3 and station 5 each had two BEV positive samples while only one BEV positive sample was discovered at station 2. The frequency of BEV positive samples at each sampling station is shown in Fig. 3.3.

The occurrence of BEV demonstrated a seasonal trend similar to those of human enteric viruses with frequency of detection increasing at lower water temperatures and increased streamflow (Fig. 3.2). BEV was detected from three out of five (60 %) stations in November and all stations in December. Neither July nor August had any positive BEV samples. Binary logistic regression analysis for the occurrence of BEV using streamflow on sampling days, 30-d mean rainfall, water temperature and other water variables (i.e., DO levels and chrolophyll-*a*) as independent variables showed similar relationships for BEV as those for human enteric viruses (Table 3.4). There was nosignificant relationship between the presence of BEV and total or fecal coliform counts (Table 3.4).

DISCUSSION

Freshwater demand in the 24 counties of Georgia's coast has increased tremendously from 1980 to 1997 because of population growth and increased water use by industry and agriculture (19). Furthermore, exponential population growth and rapid development along coastal rivers in general has generated many concerns about decreasing water quality (24). Population growth and urbanization have resulted in increased pumpage of groundwater and freshwater, conversion of open lands into nonpermeable surfaces, changes in hydrologic conduits, and increased wastewater discharge (19). These changes have affected water quality

through saltwater intrusion and the addition of sediment, toxic chemicals, pathogenic microorganisms, and nutrients into the coastal rivers and estuaries.

Water quality issues in the Altamaha River. In 1999, water quality data collected by the United States Geological Survey (USGS) in the Altamaha River Basin indicated DO and fecal coliform impairments in many segments of the Altamaha tributaries (22, 24). The State of Georgia has conducted several studies, in accordance with Section 303(d) of the Clean Water Act and the U. S. Environmental Protection Agency (EPA) Water Quality Planning and Management Regulations (40 CFR Part 130), to develop fecal coliform bacteria and DO total maximum daily loads (TMDLs) for waterbodies in the Altamaha River basin that are listed as either not supporting or partially supporting designated use classifications, due to exceedence of water quality standards (22, 24). One of the main purposes of the TMDL analysis is to identify the source categories or individual sources of fecal pollution in a watershed and the amount of loading contributed by each of these sources so that the long-term effects of anthropogenic activities and development upstream on microbial water quality can be better monitored (24). Currently, there are more than 100 permits allowing treated sewage, discharge from paper and pulp operations, and other pollutants to be discharged into the Altahama River (5). Typical nonpoint sources of fecal pollution in the Altahama River include urban development (storm-water runoff and leaking sewer collection lines), leaking septic systems, land application of agricultural manure, livestock grazing, and wildlife (24). Based on an EPA survey in 2001, five percent of the septic systems in the watershed leak (24). In the Altamaha River basin, animal waste might be one of the main contributors to non-point source contamination via poultry litter, hog and cow manure application, feces deposition and runoff from confined agricultural livestock and unconfined animals (i.e., deer, hogs and other wildlife) (24). Marsh islands

surrounded our sampling stations are inhabited primarily by cattle, hogs (for hog hunting) and waterfowl (Katy Austin, GA Marine Extension Services, personal communication).

Fecal indicator bacteria. Several studies have suggested that fecal coliform levels can not be used to predict the occurrence of human viruses and this finding is consistent with the results of our study (33, 62, 82, 87). Though human and bovine enteric viruses were frequently detected at our sampling stations, fecal coliform readings at sampling stations were generally low and never exceeded the Georgia's recreational water quality control one-time sampling limit of 500 most probable number (MPN) 100 ml $^{-1}$ during our study period (23) (Fig. 3.4). Fecal coliform levels ranged between 13 and 130 MPN 100 ml $^{-1}$ at station 4, which was ranked the highest in human and bovine enteric virus contamination, combined. Fecal coliform bacteria, therefore, may not be a reliable indicator to assess risks associated with enteric viruses in the coastal rivers of Georgia because a symptomatic infection in hosts can be caused by less than 1 PFU of enteric virus (67).

Enteric viruses as water quality assessment tools. The levels of enteric viruses in natural environments (e.g., seawater, rivers, and streams) usually are low and detection methods with high sensitivity and specificity are needed to study the occurrence of these viruses. The molecular detection (i.e., PCR and dot blot hybridization) of viruses offers several advantages over traditional viral assays, such as cell culture. PCR viral detection is less laborious and time-consuming, but more specific and sensitive than cell culture (12, 35, 40). PCR is capable of detecting viruses that are either difficult to grow in cultured cells or replicate without producing cytopathogenic effects in cells (11, 51, 63). For example, adenoviruses, one of the most important human pathogens present in polluted water, are slow growing, often do not produce cytopathogenic effects in cells and therefore are consistently underestimated when fast-growing

enteroviruses are present (38, 75). Pina et al. (62) suggested that the highly sensitive PCR detection method had led to higher detection rate of adenoviruses in environmental samples. While PCR detection methods offer high sensitivity, the risk for contamination and false-positive results exists. In this study, stringent quality control measures were practiced in processing the samples, especially during PCR to prevent cross-contamination and ensure the quality of our PCR products.

In addition to its high sensitivity, PCR is also highly specific and capable of differentiating specific groups of viruses (40, 43, 62). Through different primer sets that target specific virus groups, the major sources of contamination in samples can be identified. PCR is less time-consuming and requires less effort than bacterial strain typing because a database of strains is not required. Samples can be tested and assigned a source directly. Virus isolation, extraction and detection generally can be done in one day whereas cell culture and bacterial strain typing would take at least a few days or months.

One of the problems that is commonly associated with PCR detection of enteric viruses is the presence of PCR inhibitors such as humic and fulvic acids, heavy metals, and phenolic compounds in environmental samples (74, 86, 90). In this study, we followed the improved virus concentration protocol developed by Katayama et al. (43). Katayama et al. (43) found that adding an acid rinse step between the adsorption and elution steps substantially improved viral elution and detection efficiency. Katayama et al. (43) also suggested replacing beef extract solution traditionally used for viral concentration (e.g., 61, 70, 84) with sodium hydroxide (NaOH) as the elution medium because NaOH does not contain PCR inhibitors commonly found in beef extract, while offering similar virus recovery. Current methods to remove PCR inhibitors from beef extract, such as resin treatments, polyethylene glycol precipitation-resuspension

techniques, immunomagnetic capture and glass purification are expensive and complicated (43). Dilution of concentrated and purified samples before PCR amplification in this study has also helped to improve detection efficiency; some of the samples were positive for viruses only in diluted extracts (data not shown). In our seeded experiment, we demonstrated comparable detection sensitivity for all virus types between MilliQ water (control) and pre-filtered estuarine water, indicating high recovery rate and minimal inhibitory effects of PCR inhibitors in estuarine water. The use of dot-blot hybridization also decreased chances of false negatives where samples were negative by gel electrophoresis but positive by hybridization.

The application of (RT)-nested-PCR protocols to detect HEV and HAdV in our study increased the detection limit when compare to conventional PCR protocols (14). Nested-PCR is generally more specific and has shown a higher level of sensitivity in detecting enteric viruses from environmental samples (64). The detection limit for HAdV by nested-PCR as used in this study was ~ 4 viral particles ml $^{-1}$, which is consistent with previously reported sensitivities as high as one purified viral particle (3). This is about 100 - 1,000 times more sensitive than cell culture assays, given that about 10 - 100 viral particles are required to produce one PFU in cell culture (64). The RT-nested-PCR protocol for HEV detection developed in this study was also shown to have a sensitivity of ~ 4 viral particles ml $^{-1}$. Previous un-nested PCR assays had a reported sensitivity of 1 x 10^3 viral particles ml $^{-1}$ (33).

The application of dot-blot hybridization after PCR further increased detection sensitivity, especially in natural water samples, and prevented false-negative results as well as confirming PCR positives by gel electrophoresis. In our seeded experiment, dot-blot hybridization improved detection sensitivity of HEV by about one order of magnitude in filtered estuarine water and one to two orders of magnitude for BEV in MilliQ water and filtered

estuarine water. There was no significant improvement for HAdV in MilliQ water or filtered estuarine water; however, in environmental samples, our rate of detection was higher after dot-blot hybridization in all cases. This finding is consistent with the results from previous studies in which dot-blot hybridization increases detection sensitivity by at least an order of magnitude (14, 33, 79). For our environmental samples, dot-blot hybridization increased the number of HAdV, HEV and BEV positive samples compared to gel electrophoresis by 71 %, 111 % and 100 %, respectively.

Human and animal fecal loading in the lower Altamaha. The prevalence of positive human enteric virus samples detected in this study suggests that the water quality at the lower Altamaha River is affected by contaminants of human-origin such as wastewater (including leaking septic systems) and urban runoff. The Altamaha River drains one-fourth of the state of Georgia and therefore contamination may reflect both upstream as well as local pollution. The most human viral loading was noted at station 1, which is located near the Altamaha Sound. Contamination at this station may be higher because in addition to the lower Altamaha River, it also receives flow from the Atlantic Intracoastal Waterway (which flows through Savannah River, GA, the Altamaha Sound and ends in Florida). Station 4, which ranked the highest in both bovine and human enteric virus contamination, is also influenced by the Atlantic Intracoastal Waterway (which passes a marsh island near station 3 inhabited primarily by cattle, locally known as "Cow Island") and the South Altamaha tributary (Fig. 3.1). Sampling station 5, our most upstream site and located on the South Altamaha tributary, ranked second in human enteric virus load. A commercial fish camp with restaurant and lodging, upstream from the station might have contributed to human fecal contamination. All of our sampling stations, except station 5, are designated as waters generally supporting shellfish (23). Because shellfish are filter

feeders, they tend to accumulate viruses in their edible tissues and concentration of viruses in shellfish may be much higher than in the surrounding water (1). Thus, the detection of human enteric viruses around the area suggests a potential public health risk if contaminated shellfish are consumed.

BEV also were frequently detected in the study area, despite a reduced detection efficiency compared to the human enteric viruses. This suggests that bovine species also contribute to fecal loading in this watershed and indicates potential for transmission of zoonotic pathogens.

In our study, all viruses were detected at a higher frequency in December, reflecting the importance of low temperature and high streamflow in viral survival and loading, respectively. This finding is consistent with previous reports that the viability and the stability of viral particles are highly influenced by water temperature (18, 42, 50, 57, 83, 85). The important effect of streamflow in the loading of viruses in coastal waters has also been demonstrated by Goyal et al. (31) and Lipp et al. (50). Increase in streamflow may have caused more remote influx of viral pollutants as well as more widespread viral loading. Seasonal cycles in viral infection and excretion in the population also might have played a role in the elevated detection of enteric viruses in winter months (39). Although some researchers have related the detection of enteric viruses to rainfall events (e.g., 25, 32), our results showed no immediate response of viral detection to rainfall events and an inverse response with rainfall 30 days preceding sampling, which may reflect a complex hydrology in this watershed.

As previously mentioned, a high frequency of viral detection was observed in December, when the average water temperature was significantly lower than in other sampling months. However, HEV and HAdV were found in two (40 %) and one (20 %) of samples, respectively,

collected in July from the bottom of the water column (mean water temperature, 29.07 °C). The detection of these viruses from samples collected at greater depth suggests that sediment might serve as a reservoir for enhanced survival of viruses (8). Resuspension of sediments and tidal movement (samples were collected during out-going tide) during sampling may cause the release of viruses into water column. In a virus-soil sorption study, poliovirus 1 (i.e. enterovirus) demonstrated strong sorption to clay-loam, the predominant soil type found at our sampling areas (56). Also, light attenuation could have played a role in viral stability and survival; viruses at the bottom of water columns are able to survive for a longer period because they are protected from direct UV irradiation and photodamage (42, 50, 69). Unfortunately, only one round of samples were collected from these depths and therefore, we are unable to compare frequency of detection to the surface water.

Although direct-PCR and dot-blot hybridization offer quick, highly specific and sensitive ways to detect enteric viruses from environmental samples, the detection of viruses through this method does not necessary represent public health risk because little is known about the infectivity of the viruses. Because PCR can detect nucleic acids from both infectious and damaged (non-infectious) viruses, data derived from direct PCR is most useful when the infectivity of these viruses is not an important issue or not of public health concern (i.e., solely to track the source of water pollution). Several studies show that viral RNA and DNA are significantly more persistent than infectious viruses (up to 3.5 times) (46, 85); however, those studies were conducted under artificial or sterile environments, such as artificial seawater, prefiltered seawater or DI water. Studies that examined the stability and survival of viruses in natural environments have shown that PCR detection of RNA viruses, such as enteroviruses, may indicate that they were recently infectious because liberated RNA will disappear in as soon

as a few minutes in wastewater (40, 49). Likewise, in natural marine water, the detection rate is similar for cell culture and RT-PCR (17, 85). In addition, a study of human enteric viruses in contaminated coastal waters in Florida showed that a high percentage of samples positive by RT-PCR were also positive by cell culture (50).

Animals also contribute to fecal contamination in aquatic environments and can contribute to contamination with zoonotic pathogens; therefore, the ability to identify the major source categories of fecal contamination is important in managing both water quality and human health risk. Several studies have evaluated the prevalence of animal-specific enteric viruses (i.e., bovine enteroviruses, porcine teschoviruses, bovine and porcine adenoviruses) in animal feces and the environment (41, 48, 52, 58). Findings of this study along with previous works suggest that molecular detection of animal-specific viruses can be highly sensitive and specific markers for tracing sources of fecal contamination; however, more testing will need to be done to study the geographical and seasonal distribution of these viruses to make them universal markers.

In conclusion, the stringent host specificity of enteric viruses makes them good library-independent indicators for identifying sources of water pollution. PCR can be used to detect and differentiate specific groups of viruses when infectivity data are unnecessary in decision-making. Detection assays specific for other groups of animal enteric viruses, such as avian and porcine enteric viruses could be developed to better characterize sources of contamination. More importantly, given the low infectious dose of enteric viruses (67), the isolation of these viruses in the coastal water, even at low levels could indicate a public health risk for recreational activities and shellfish harvesting. However, additional research is needed to properly correlate levels of contamination and public health risks.

ACKNOWLEDGMENTS

This study was funded by the Georgia Sea Grant College Program. Special thanks to Ms. Patricia Smith and Mr. Ethell Vereen Jr. for help with sample processing. Ms. Katy L. Austin and the sampling team from the Marine Extension Service of the University of Georgia in Brunswick provided valuable support in sample collection and transportation, and provided water quality data and coliform readings. Dr. Charles P. Gerba (Univ. of Arizona) provided strains of poliovirus and adenovirus for use as positive controls; Dr. George Lukasik provided cultures of bovine enteroviruses; and Dr. John H. Paul assisted with primer development for human enteroviruses.

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TABLES

Table 3.1. Nucleotide sequences for primers and probes used for PCR amplification and dot-blot hybridization of human enteroviruses (HEV), bovine enteroviruses (BEV) and human adenoviruses (HAdV).

Virus	Primers & Probes	Sequence (5' to 3')	Target	Amplicon (bp)	Reference
HEV	ENT-up-1	GTAGATCAGGTCGATGAGTC			This study
	ENT-down-1	ACYGGRTGGCCAATC[*]	5'-UTR	330	(14)
	ENT-up-2[b]	CCTCCGGCCCCTGAATG			(14)
	ENT-down-2[b]	ATTGTCACCATAAGCAGCC	5'-UTR	154	This study
	EV-probe	[a]TACTTTGGGTGTCCGTGTTTC			(14)
BEV	BEV-up	GAGTAGTCCGACTCCGCWCC[*]			This study
	BEV-down	CAGAGCTACCACTGGGGT	5'-UTR	270	This study
	BEV-probe	[a]AAYCGACCAATAKVNGC[*]			This study
HAdV	AV-A1	GCCGCAGTGGTCTTACATGCACATC			(3)
	AV-A2	CAGCACGCCGCGGATGTCAAAGT	Hexon gene	300	(3)
	AV-B1[b]	GCCACCGAGACGTACTTCAGCCTG			(3)
	AV-B2[b]	TTGTACGAGTACGCGGTATCCTCGCGGTC	Hexon gene	143	(3)
	AV-probe	[a]ACGCACGACGTAACCACAGAC			This study

[a] 5'end is biotin-labeled

[b] internal primer set

[*] Y= G+T, R= A+G, W= A+T, K= G+T, V= A+C+G, N= A+T+C+G

Table 3.2. The range and geometric mean MPN fecal and total coliform bacteria counts at each sampling station in the lower Altamaha River between July and December 2002. Station 5 ranked the highest in both fecal coliform and total coliform counts while station 1 ranked the lowest in both counts.

Station	Fecal coliform bacteria (MPN 100 ml $^{-1}$)	Total coliform bacteria (MPN 100 ml $^{-1}$)
1	2-23	11-170
	7	51
2	8-34	30-700
	22	91
3	13-70	50-1600
	34	238
4	13-130	30-900
	36	162
5	30-170	110-2400
	74	786

Table 3.3. Occurrence of human enteroviruses (HEV), human adenoviruses (HAdV) and bovine enteroviruses (BEV) at each sampling station in the lower Altamaha River between July and December 2002. Sites are ranked by occurrence of viruses with one (1) being the most contaminated. Station 4 ranked the highest in both human and bovine fecal contamination.

	Human Impact			Bovine Impact	
Station	HEV positive samples	HAdV positive samples	Rank	BEV positive samples	Rank
1	July[a], Aug, Oct, Nov, Dec	Sept, Oct, Dec	1	Nov, Dec	2
2	July, Aug, Sept, Oct	Sept	3	Dec	3
3	Oct, Nov		4	Sept, Dec	2
4	Aug, Oct, Nov, Dec	Sept, Oct, Nov, Dec	1	Sept, Oct, Nov, Dec	1
5	July[a], Aug, Dec	July[a], Sept, Dec	2	Nov, Dec	2

[a] bottom water sample

Table 3.4. Binary logistic regression analyses for the occurrence of human enteroviruses (HEV), human adenoviruses (HAdV) and bovine enteroviruses (BEV) using water quality or environmental variables. Overall, the presence of viruses (human and bovine) was directly (positively) related to streamflow and dissolved oxygen (DO), and inversely related to water temperature, rainfall and chlorophyll-*a* concentration. The presence of viruses was not significantly related to either fecal or total coliform levels.

	HAdV		HEV		Both HEV & HAdV[a]		BEV	
Parameters	Concordance (%)	P-value	Concordance (%)	P-value	Concordance (%)	P-value	Concordance (%)	P-value
DO	75.6	0.031*	59.3	0.625	85.8	0.012*	88.5	0.018*
Water Temp	75.6	0.062	63.8	0.183	81.8	0.012*	86.6	0.006*
T. coliform	51.2	0.431	44.3	0.429	48.9	0.233	42.6	0.567
F. coliform	50.7	0.545	52.9	0.643	57.4	0.377	44	0.811
Streamflow[b]	70.8	0.040*	54.3	0.246	79	0.009*	85.6	0.005*
Rainfall[c]	70.8	0.08	54.3	0.204	79	0.012*	85.6	0.003*
Chlorophyll-*a*	74.2	0.034*	42.5	0.956	80.1	0.019*	80.9	0.009*

[a] HEV and HAdV were detected in the same sample simultaneously

[b] streamflow value on sampling days was used

[c] mean rainfall 30d preceding sampling was used

* significant ($P < 0.05$)

FIGURE LEGEND

FIG. 3.1. Sampling stations (1 through 5) in the lower Altahama River, Georgia, USA. The Altamaha River and the South Altamaha are the main sources of flow toward the coast. The Intracoastal Waterway provides flow from coastal areas primarily to stations 1, 2 and 4.

FIG. 3.2. Percentage of samples positive for human enteroviruses (HEV) and human adenoviruses (HAdV) (detected simultaneously), and bovine enteroviruses (BEV) by month *versus* mean monthly water temperature (ºC) and streamflow (10 m^3 s^{-1}), along the lower Altamaha River between July and December 2002. (N = 5).

FIG. 3.3. Percentage of human enterovirus (HEV), human adenovirus (HAdV) and bovine enterovirus (BEV) positive samples at the five stations along the lower Altahama River between July and December 2002. (N = 7).

FIG. 3.4. Occurrence of human enteroviruses (HEV), human adenoviruses (HAdV) and bovine enteroviruses (BEV), versus MPN fecal coliform counts (100 ml^{-1}). All viruses were detected when fecal coliform counts were below the one-time sampling maximum threshold of 500 MPN 100 ml^{-1} (23).

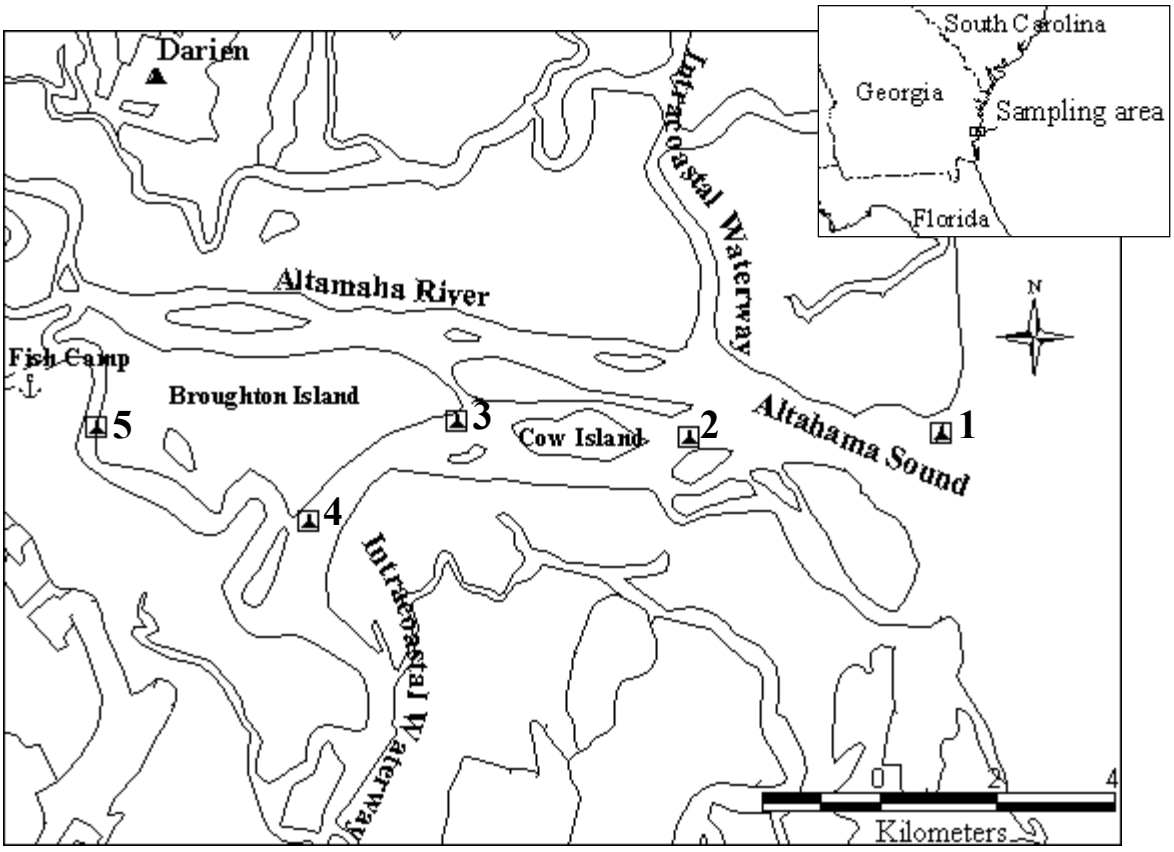
Darien
Altamaha River
Fish Camp
Broughton Island
Cow Island
Intracoastal Waterway
Altahama Sound
Intracoastal Waterway
1
2
3
4
5
South Carolina
Georgia
Florida
Sampling area
N
0
2
4
Kilometers

FIG. 3.1

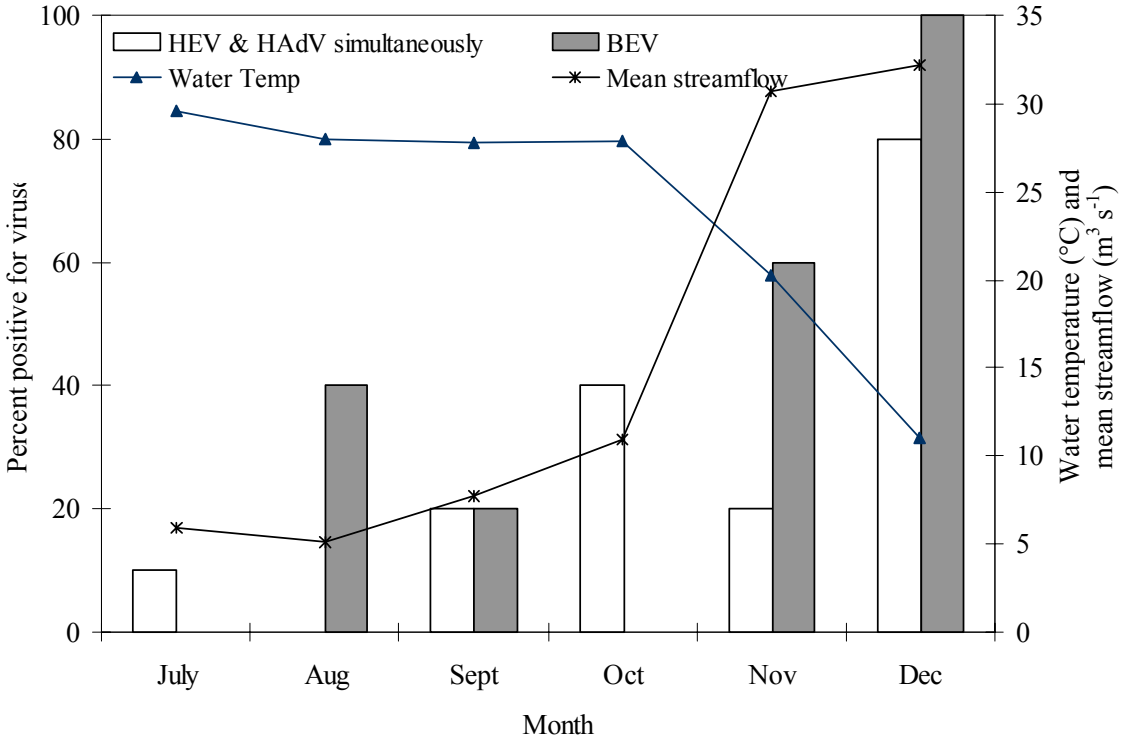
HEV & HAdV simultaneously
BEV
Water Temp
Mean streamflow
Percent positive for virus
Water temperature (°C) and mean streamflow (m3 s-1)
100
80
60
40
20
0
35
30
25
20
15
10
5
0
July
Aug
Sept
Oct
Nov
Dec
Month

FIG. 3.2

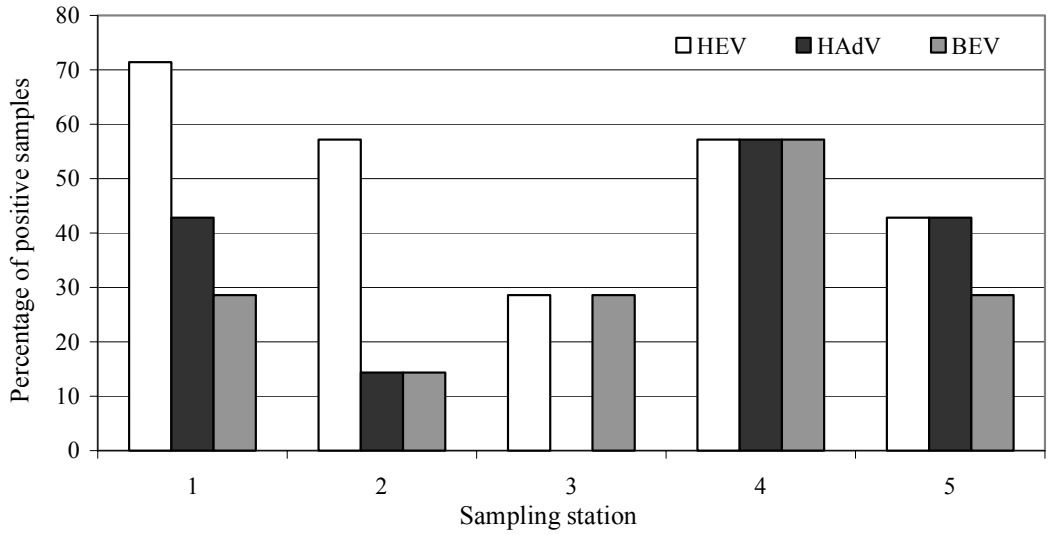
80
70
60
50
40
30
20
10
0
Percentage of positive samples
HEV
HAdV
BEV
1
2
3
4
5
Sampling station

FIG. 3.3.

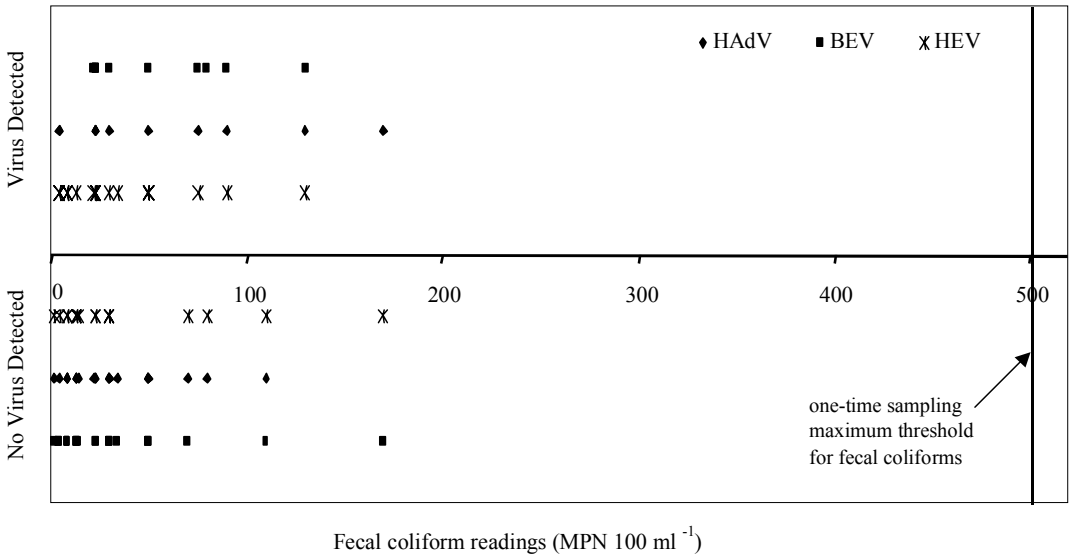
HAdV
BEV
HEV
Virus Detected
No Virus Detected
0
100
200
300
400
500
one-time sampling
maximum threshold
for fecal coliforms
Fecal coliform readings (MPN 100 ml -1)

FIG. 3.4.

CHAPTER 4

CONCLUSIONS

The enteric viruses are frequently detected in large numbers in sewage and other fecally-contaminated waters. They can cause a wide spectrum of diseases in human and animals. The presence of these viruses in aquatic environments poses a public health risk because they are generally transmitted via the fecal-oral route. Unfortunately, bacterial indicators, such as fecal coliforms, total coliforms and enterococci, are not good proxies for enteric viruses because enteric viruses generally survive longer than bacterial indicators under various environmental conditions. The characteristics, pathogenicity, occurrence and detection of waterborne enteric viruses, especially enteroviruses and adenoviruses have been studied extensively. Both the enteroviruses and adenoviruses show great potential to serve as indicators for the presence of other viral pathogens and fecal contamination in aquatic environments. The enteroviruses have been included by the European Union regulations governing water quality as a parameter for evaluating viral pollution of a water body. Recently, several researchers hypothesized that molecular pathogen detection techniques based on virus host specificity in environmental samples would allow for determination of the sources of contaminants and improve surveillance of public health.

We used polymerase chain reaction-based viral pathogen detection assays to examine the extent and relative importance of fecal contamination from upstream agricultural and anthropogenic andactivities, and urban development in coastal reaches of the lower Altamaha River, Georgia, USA, by detecting three groups of host-specific enteric viruses: human

enteroviruses (HEV), human adenoviruses (HAdV) and bovine enteroviruses (BEV). To evaluate the use of this assay in defining estuarine water quality in the mixed-use watershed, we then compared the findings from PCR-based assays to concurrently collected bacterial indicator data and other environmental variables such as rainfall, stream-flow, and water temperature.

The prevalence of positive human enteric virus samples detected in this study suggests that the water quality at the lower Altamaha River is heavily affected by contaminants of human-origin such as wastewater (including leaking septic systems) and urban runoff. Eleven (36.67%) and 17 (56.67%) of the 30 surface water samples tested positive for HAdV and HEV, respectively. Two-thirds of the samples either tested positive for HEV or HAdV and the viruses occurred simultaneously in 25.71 % of samples. BEV were detected in 11 of 30 surface water samples. The detection of BEV in this area suggests that bovine species also contribute to fecal loading in this watershed and indicates potential for transmission of zoonotic pathogens.

Fecal coliform readings at sampling stations were generally low and never exceeded the Georgia recreational water quality control one-time sampling limit of 500 MPN 100 ml^{-1} during our study period. Moreover, binary logistic regression analysis showed that the presence of both human and bovine enteric viruses was not significantly related to either fecal coliform or total coliform levels. Fecal coliform bacteria, therefore, may not be a reliable indicator to assess risks associated with enteric viruses in the coastal rivers of Georgia.

The presence of human and bovine viruses was directly (positively) related to dissolved oxygen and streamflow, but inversely (negatively) related to water temperature, rainfall in the 30 days preceding sampling and chlorophyll-*a* concentrations. Increase in streamflow may have caused more remote influx of viral pollutants as well as more widespread viral loading. Although a higher frequency of viral detection was observed in winter months and viruses have been

reported to survive longer under lower temperatures, the detection of HEV and HAdV from two (40 %) and one (20 %) of samples collected in July from the bottom of the water column, respectively, suggests that sediment might serve as a reservoir for enhanced survival of viruses. Resuspension of sediments and tidal movement (samples were collected during out-going tide) during sampling may cause the release of viruses into water column. Overall, factors that influence the occurrence and survival of enteric viruses in waters, such as water temperature, suspended solids, turbulence, sunlight intensity, host excretion, nutrient content of water and predation have been extensively studied, and these parameters should be included when predicting the presence of viral pathogens in the environment.

Although conventional molecular viral pathogen detection methods (direct-PCR and dot-blot hybridization) are not quantitative and do not provide information about the infectivity of viruses, they are rapid, highly specific and sensitive ways to detect enteric viruses from environmental samples when infectivity data are unnecessary in decision-making. The stringent host specificity of enteric viruses makes them good library-independent indicators for identifying sources of water pollution. In this case, PCR is an efficient method to detect and differentiate specific groups of viruses for fecal contamination source identification. Detection assays specific for other groups of animal enteric viruses, such as avian and porcine enteric viruses could be developed to better characterize sources of contamination.