Clam (Corbicula fluminea) as a Potential Sentinel of Human Norovirus Contamination in Freshwater

Xunyan Ye
University of Southern Mississippi

Follow this and additional works at: https://aquila.usm.edu/dissertations

Part of the Aquaculture and Fisheries Commons, Biology Commons, Immunology of Infectious Disease Commons, and the Other Immunology and Infectious Disease Commons

Recommended Citation
Ye, Xunyan, "Clam (Corbicula fluminea) as a Potential Sentinel of Human Norovirus Contamination in Freshwater" (2012). Dissertations. 761.
https://aquila.usm.edu/dissertations/761

This Dissertation is brought to you for free and open access by The Aquila Digital Community. It has been accepted for inclusion in Dissertations by an authorized administrator of The Aquila Digital Community. For more information, please contact aquilastaff@usm.edu.
The University of Southern Mississippi

CLAM (CORBICULA FLUMINEA) AS A POTENTIAL SENTINEL OF
HUMAN NOROVIRUS CONTAMINATION IN FRESHWATER

by

Xunyan Ye

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

May 2012
ABSTRACT

CLAM (CORBICULA FLUMINEA) AS A POTENTIAL SENTINEL OF HUMAN NOROVIRUS CONTAMINATION IN FRESHWATER

by Xunyan Ye

May 2012

The purpose of this study was to evaluate and validate the use of the clam *Corbicula fluminea* as a sentinel of human noroviruses (HuNoV) contamination in freshwater. The first specific aim was to develop a new method to extract HuNoV RNA from contaminated bivalves (e.g. oysters, clams) that would be much faster than existing methods. The procedure developed includes an initial total RNA extraction using TRI Reagent, followed by HuNoV RNA concentration and purification using biotinylated probe-capture technology. HuNoV RNA is finally detected by real-time RT-PCR. Using bivalve homogenates spiked with HuNoV, 100 PCR detection units of the virus was detectable. Compared to published methods that require an initial virus purification step, the new method is much faster to complete. Approximately 3 h are needed to purify NoV RNA using the new method compared to at least 8 h using conventional methods. Coupled with real-time RT-PCR, the new method can detect HuNoV in contaminated bivalves within 8 h. The detection limit of the method was 10 -100 PDU of HuNoV. In addition, the method was successfully applied for HuNoV detection in live artificially-contaminated oysters, wild oysters, and also for murine norovirus (MNV-1) and HuNoV detection in clams.
The second specific aim was to evaluate the ability of *C. fluminea* to bioaccumulate and depurate HuNoV using MNV-1 as a surrogate of HuNoV. Clams were exposed to MNV-1 in 10 L artificial pond water for 6 h, 1, 2, and 3 d in an environmental chamber. Depuration experiments were carried out in 80 L artificial pond water for 0, 1, 4, 7, 10, and 15 d at 10°C and 20°C. MNV-1 was detectable after 6 h and 1 d exposure in clams exposed to virus concentrations of $10^6$ PFU·L$^{-1}$ and $10^4$ PFU·L$^{-1}$, respectively. The amount of bioaccumulated MNV-1 increased as the exposure period increased from 6 h to 3 d. The lowest virus concentration at which exposed clams were PCR-positive was $10^2$ PFU·L$^{-1}$ after 2 d exposure at 20°C. Clams bioaccumulated MNV-1 more quickly at 20°C than at 10°C ($p < 0.05$). The virus was persistently detected in contaminated clams during depuration at both 10°C and 20°C. Depuration occurred significantly more quickly at 20°C than at 10°C ($p < 0.05$). The results indicate that the clam is likely to be useful as a sentinel for detecting NoV contamination in freshwater.

The third specific aim was to determine whether *C. fluminea* is effective as a sentinel of HuNoV contamination in natural freshwater. Clams were collected from Lake Serene in Hattiesburg, Mississippi where HuNoV has never been detected from Oct 2010 to Jul 2011, and translocated to 9 sites at 4 freshwater creeks in Gulfport and Long Beach where the creek water flowed into Mississippi Sound. HuNoV RNA was isolated from clams ($n = 588$) using the biotinylated probe hybridization method mentioned above and detected by qRT-PCR. Correct identity of the virus was accomplished by sequencing some of the amplified RT-PCR products (HuNoV capsid N-terminal/shell domain). qRT-PCR results showed that HuNoV GI and GII were detectable in the translocated *C.
*fluminea* mainly during the warmer months (Apr to Jul and Oct), but not during the colder months (Dec to Mar). Based on sequence comparisons, the HuNoV detected in translocated clams were classified into GI/17 and GII/4, respectively. Statistical analysis using binary logistic regression showed that water temperature and turbidity (p = 0.026 and p = 0.038, respectively), but not pH, salinity, or current velocity (p = 0.476, p = 0.425, and p = 0.174, respectively), were significant factors affecting HuNoV presence/absence in clams in freshwater creeks.

In conclusion, it was found that the freshwater clam *C. fluminea* can be translocated and serve as an effective sentinel of HuNoV contamination in freshwater of low turbidity during warm months.
CLAM (*CORBICULA FLUMINEA*) AS A POTENTIAL SENTINEL OF
HUMAN NOROVIRUS CONTAMINATION IN FRESHWATER

by

Xunyan Ye

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

Approved:

______________________________
Shiao Y. Wang
Director

______________________________
R. D. Ellender

______________________________
Glen Shearer

______________________________
Bobby Middlebrooks

______________________________
Susan A. Siltanen
Dean of the Graduate School

May 2012
ACKNOWLEDGMENTS

I would like to thank the dissertation director and my mentor, Dr. Shiao Wang, for his financial support, guidance, patience, and wisdom through this project over the years, and also for his help in my daily life. I would also like to thank the committee members, Dr. R. D. Ellender, Dr. Bobby Middlebrooks, and Dr. Glen Shearer for their valuable advice about this project.

Additional thanks to Dr. Jacquanila Woods (U. S. Food and Drug Administration, Dauphin Island, AL, USA) for the human norovirus clinical samples. Thank you also to Dr. Herbert W. Virgin, IV (Washington University) for the murine norovirus stock samples.

I also would like to thank all the previous and current lab mates in JST 707 for their camaraderie. A special thank goes to my husband, Yun Zhang, for his spiritual support all these years. This dissertation is dedicated to my parents.

This project was funded by the U. S. Environmental Protection Agency, Gulf of Mexico Program Office through grants MX-96401204 and MX-96429505-0, and the Mississippi Coastal Impact Assistance Program/U. S. Dept of the Interior.
# TABLE OF CONTENTS

ABSTRACT .........................................................................................................................ii

ACKNOWLEDGMENTS........................................................................................................v

LIST OF TABLES..................................................................................................................viii

LIST OF ILLUSTRATIONS..................................................................................................ix

LIST OF ABBREVIATIONS................................................................................................xii

CHAPTER

I.  INTRODUCTION.............................................................................................................1

The Problem and the Proposed Solution
Contribution of This Study

II.  BACKGROUND..........................................................................................................4

Environmental Water Contamination
Norovirus (NoV)
*Corbicula fluminea (C. fluminea)*
References

III.  DEVELOPMENT OF A NOROVIRUS RNA EXTRACTION METHOD IN OYSTERS USING BIOTINYLATED PROBE HYBRIDIZATION TO TARGET VIRAL RNA: A PRELUDE TO RNA EXTRACTION IN CLAMS.................................................................29

Abstract
Introduction
Materials and Methods
Results
Discussion
Acknowledgements
References

IV.  EVALUATION OF *CORBICULA FLUMINEA* AS A SENTINEL OF HUMAN NOROVIRUS CONTAMINATION IN FRESHWATER USING MURINE NOROVIRUS AS A SURROGATE OF HUMAN NOROVIRUS IN THE LABORATORY STUDY........................................53
Abstract
Introduction
Materials and Methods
Results
Discussion
Acknowledgements
References

V. VALIDATION OF THE CLAM CORBICULA FLUMINEA AS A SENTINEL OF HUMAN NOROVIRUS CONTAMINATION IN FRESHWATER CREEKS

Abstract
Introduction
Materials and Methods
Results
Discussion
Acknowledgements
References

VI. CONCLUSIONS

APPENDIXES
# LIST OF TABLES

Table

1. Some clinically significant enteric viruses......................................................5
2. Primer and probe sequences for HuNoV detection........................................38
3. The proportion of samples in which HuNoV was detected by real-time RT-PCR using RNA isolated by the described hybrid capture method. Oyster homogenates were spiked with different amounts of HuNoV indicated..................43
4. The proportion of oysters in which HuNoV was detected by real-time RT-PCR using RNA isolated by the described hybrid capture method. Oysters were exposed to HuNoV in 10 L seawater in the laboratory......................................................43
5. Detection of HuNoV in wild oysters using RNA isolated by the described hybrid capture method.................................................................44
6. Typical high and low temperatures (°C) for various Mississippi cities............61
7. MNV-1 bioaccumulation rates of *C. fluminea*..............................................62
8. Temperature effects on MNV-1 bioaccumulation rates of *C. fluminea*...........63
9. MNV-1 depuration by *C. fluminea* ..............................................................64
10. Oligonucleotide primer and probe sequences for MNV-1 detection...............67
11. GPS coordinates of clam translocation sites along Mississippi Sound..........83
12. Oligonucleotide primer and probe sequences for HuNoV detection and genotyping.................................................................87
13. HuNoV GI and GII detection in 294 extracts in 9 creek sites using TaqMan RT-PCR.................................................................................90
14. Real time RT-PCR results of HuNoV GII detection in spiked clam homogenates.................................................................93
LIST OF ILLUSTRATIONS

Figure

1. Unrooted phylogenetic trees based on amino acid sequence alignments of Calicivirus VP1 ................................................................. 6
2. The NoV genomic structure ................................................................................................................................. 7
3. Phylogenetic tree representing NoV genogroup classification .............................................................. 9
4. Closed C. fluminea ................................................................................................................................................. 17
5. Opened C. fluminea ................................................................................................................................................. 17
6. Biotin-based probe hybridization technology ................................................................................................. 36
7. Flow diagram showing the procedures of the three sample treatment protocols assessed for their ability to recover HuNoV RNA. RT-PCR assay was used for HuNoV detection ................................................................................................................................. 37
8. The effect of different probe hybridization and capture times on C_T values during HuNoV detection by real-time RT-PCR. A) Initial study using probe hybridization times longer than 1 h and probe capture times at least 30 min. B) Subsequent study using probe hybridization times shorter than 1 h and probe capture times less than 30 min ................................................................................................................................. 40
10. The effect of hybridization volumes on HuNoV RNA isolation. Error bars: standard deviation ................................................................. 42
11. MNV-1 bioaccumulation rates of clams. Error bars: standard deviation ................................................................. 68
12. Temperature effect on MNV-1 bioaccumulation by clams. Error bars: standard deviation ................................................................. 69
13. Bioaccumulation limit of clams. Error bars: standard deviation. ND: not detectable ................................................................. 70
14. MNV-1 depuration rates of clams. Error bars: standard deviation ................................................................. 70

Clam translocation sites along Mississippi Sound. Google 2011. Round dots in red represent creek sites from left to right: CC0, CC1, CC2, Condo, and AOC ..........82

Conventional RT-PCR amplification of HuNoV GI in field translocated clams. Expected PCR amplicon is 330 bp. Lane M: 100 bp DNA ladder; Lane 1: clam sample from CC2 in Apr 2011; Lane 2: clam sample from CC2 in May 2011; Lane 3: RNase-free water substituted for clam extract (negative control).............89

Conventional RT-PCR amplification of HuNoV GII in field translocated clams collected in Oct 2010. Expected PCR amplicon is 344 bp. Lane M: 100 bp DNA ladder; Lanes 1-2: clam samples from Trautman Ave. creek; Lanes 3-4: clam samples from AOC; Lanes 5-7: clam samples from Condo creek; Lane 8: RNase-free water substituted for clam extract (negative control)......................89

Phylogenetic tree constructed on the basis of the partial sequences of the HuNoV capsid gene. The distance was calculated using P-distance method, and the tree was plotted using the neighbor-joining (N-J) method. The numbers at each branch indicate the bootstrap values for the clusters supported by that branch. An outgroup virus (sapovirus strain Manchester) was used. GI: genogroup I; GII: genogroup II. The GenBank accession numbers of the reference strains are in the brackets followed by each strain name in the figure ........................................89

Conventional RT-PCR amplification of HuNoV GII in 100 µL of clean clam homogenates spiked with 10 µL of each 10-fold serial dilution of HuNoV GII positive stool extract. Expected PCR product was 344 bp. Lane M: 100 bp DNA ladder; Lanes 1-2: Clam homogenates spiked with 100 dilution of HuNoV GII positive stool extract, replicates A and B, respectively; Lanes 3-4: Clam homogenates spiked with 10^4 dilution of HuNoV GII positive stool extract, replicates A and B, respectively; Lanes 5-6: Clam homogenates spiked with 10^2 dilution of HuNoV GII positive stool extract, replicates A and B, respectively; Lanes 7-8: Clam homogenates spiked with 10^3 dilution of HuNoV GII positive stool extract, replicates A and B, respectively; Lane 9: Nuclease free water (negative control of viral RNA isolation); Lane 10: HuNoV GII positive stool extract (positive control of viral RNA isolation); Lane 11: Nuclease free water (negative control of conventional RT-PCR); Lane 12: RNA directly extracted from the stool extract (positive control of conventional RT-PCR)..................................93

Relationship between temperature and the HuNoV presence/absence in clams ....95

Relationship between turbidity and the HuNoV presence/absence in clams ......95
23. Relationship between pH and the HuNoV presence/absence in clams ..........96
24. Relationship between salinity and the HuNoV presence/absence in clams .......96
25. Relationship between water velocity and the HuNoV presence/absence in clams ..............................................................................................................97
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>BLR</td>
<td>Binary logistic regression</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effects</td>
</tr>
<tr>
<td>C&lt;sub&gt;T&lt;/sub&gt;</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees centigrade</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DG</td>
<td>Digestive gland</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle’s minimum essential medium</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>HuNoV GI</td>
<td>Human norovirus genogroup I</td>
</tr>
<tr>
<td>HuNoV GII</td>
<td>Human norovirus genogroup II</td>
</tr>
<tr>
<td>µL</td>
<td>Microliter</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MNV-1</td>
<td>Murine norovirus 1</td>
</tr>
<tr>
<td>MS</td>
<td>Mississippi</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>m·S⁻¹</td>
<td>Meters per second</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>nG</td>
<td>Nanogram</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>ntu</td>
<td>Nephelometric turbidity unit</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDU</td>
<td>PCR detection units</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque forming units</td>
</tr>
<tr>
<td>ppt</td>
<td>Parts per thousand</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>USDA</td>
<td>U. S. Department of Agriculture</td>
</tr>
<tr>
<td>USFDA</td>
<td>U. S. Food and Drug Administration</td>
</tr>
</tbody>
</table>
CHAPTER I

INTRODUCTION

The Problem and the Proposed Solution

A large number of water bodies in the U. S. are considered to be impaired on the basis of their microbiological qualities. Microbial contamination in coastal water results in broad economic losses due to beach closures and closures of fisheries. To date, we use indicator organisms to monitor the microbiological quality of water. Indicator organisms are defined as microbes that indicate the potential presence of pathogens in the environment (Griffin et al., 2001). Microbiological indicators include total coliforms, fecal coliforms, E. coli, and enterococci. These indicators have been used to analyze the degree of water pollution for over a century. A good indicator should be applicable to all types of water, be present when pathogens are present, survive longer than the hardiest pathogen, and not reproduce outside the animal host. In addition, the density of the indicator in contaminated water should have a direct correlation with the degree of pollution (Griffin et al., 2001). However, E. coli and Enterococci survive, grow and establish populations in natural environments such as freshwater lakes and streams, as well as sediments (Byappanahalli et al., 2003; Flood et al., 2010; Hardina and Fujioka 1991; Power et al., 2005; Whitman and Nevers 2003; Yamahara et al., 2007). Indicator bacteria are inactivated more readily than some waterborne pathogens during wastewater disinfection (Blatchley et al., 2007), and during sunlight exposure (Nasser et al., 2007). Numerous studies have also shown a lack of correlation between these indicator bacteria and pathogens, such as Salmonella spp. (Lemarchand and Lebaron, 2003; Carr et al., 2010) and Campylobacter spp. (Horman et al., 2004). The lack of correlation raises
concern about the reliability of using the traditional indicator method to accurately predict health risks (Boehm et al., 2009).

Due to the shortcomings of using indicator organisms to monitor water quality, direct pathogen detection in water has been considered. The direct monitoring of human pathogens in waters involves two steps: the concentration of the pathogen from water, and the detection and identification of the recovered pathogen. With regards to viral pathogens, a major challenge is the critical first step where the virus must be concentrated from a relatively large volume of water because it becomes diluted once shed in water. Even when dilute, many viruses pose health risks because they are highly infectious even at low doses (such as norovirus and rotavirus). Other difficulties with the initial concentration step include the need for specialized training and equipments, the extensive labor required to process the multiple steps and thus the high associated expense.

The filter-feeding clam *Corbicula fluminea* can be used as a relatively simple and inexpensive first step for concentrating pathogens from large bodies of water, and thus serve as a sentinel of pathogens contamination in water. Clams can bioaccumulate different solid particles and microorganisms, mainly in the digestive gland. Virus contaminated clams indicate virus contamination of the surrounding water. *C. fluminea* is common, widespread, resistant to environmental toxicants, and easily collected and transported for translocation as sentinels.

**Contribution of This Study**

The freshwater clam (*Corbicula fluminea*) was evaluated as a sentinel of HuNoV contamination in artificial freshwater in the laboratory study using a cultivable murine
norovirus as a surrogate of human norovirus and then validated to be an effective sentinel of HuNoV contamination in natural freshwater creeks in Mississippi.
CHAPTER II

BACKGROUND

Environmental Water Contamination

Beach water could be contaminated from various sources. The most frequent cause is polluted creeks and sewage outfalls feeding into beach water. Because current water treatment practices are unable to provide virus-free wastewater effluent, pathogenic viruses can be routinely introduced into beach water (Rao and Melnick, 1986). Environmental waters can become contaminated with both animal and human feces. Human feces are more likely to contain human-specific enteric viruses, such as rotavirus, norovirus, and enterovirus, thus, human fecal contamination of recreational waters is generally regarded as a greater risk to human health than fecal contamination from other animal sources. Enteric viruses refer to a group of viruses found in the intestinal tract of humans and animals. There are more than 100 viral entities associated with human feces. The health significance of these agents in humans ranges from hepatitis, poliomyelitis (polio), and gastroenteritis to innocuous infections. Some of the most important are listed in Table 1. Contamination of the marine environment can exact high risks to human health as well as result in significant economic losses due to closures of beaches and shellfish harvesting areas.

Direct monitoring for human pathogens can determine their presence or absence in waters, and thus circumvents the need to assay for often-ambiguous indicator organisms, such as *E. coli*, *Enterococcus spp*, and *Clostridium perfringens*. However, a direct monitoring approach is difficult to perform using current pathogen concentration methods, as human pathogens become greatly diluted once shed in a large body of water.
With regards to viruses, dilution does not solve the health risk problem as many viruses have a low infectious dose. For example, the infectious dose of norovirus (NoV), the most common viral agent of acute gastroenteritis in humans, is as low as 10 to 100 virus particles (Parashar et al., 2001).

Table 1

Some clinically significant enteric viruses

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astroviridae</td>
<td>Mamastrovirus</td>
<td>human astroviruses</td>
</tr>
<tr>
<td>Adenoviridae</td>
<td>Mastadenovirus</td>
<td>human adenoviruses</td>
</tr>
<tr>
<td>Caliciviridae</td>
<td>Norovirus</td>
<td>noroviruses (Norwalk-like viruses)</td>
</tr>
<tr>
<td></td>
<td>Sapovirus</td>
<td>human sapoviruses</td>
</tr>
<tr>
<td>Parvoviridae</td>
<td>Parvovirus</td>
<td>human parvoviruses</td>
</tr>
<tr>
<td></td>
<td></td>
<td>non-polio enteroviruses: coxsackievirus A &amp; B,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>echoviruses,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and human enteroviruses (types 68 to 71)</td>
</tr>
<tr>
<td>Picornaviridae</td>
<td>Enterovirus</td>
<td>echoviruses,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and human enteroviruses (types 68 to 71)</td>
</tr>
<tr>
<td>Reoviridae</td>
<td>Rotavirus</td>
<td>human rotaviruses</td>
</tr>
</tbody>
</table>

Norovirus (NoV)

Name Origin and Classification

Norovirus (NoV) was originally named *Norwalk virus* after Norwalk, Ohio, USA, where an outbreak of acute gastroenteritis occurred among children at Bronson Elementary School in November 1968. The name NoV (*Norovirus* for the genus) was approved by the International Committee on Taxonomy of Viruses in 2002. Several other names have been used for NoV including: Norwalk-like viruses (NLVs), caliciviruses
(because they belong to the virus family *Caliciviridae*), and small round structured viruses (SRSV).

The *Caliciviridae* family is composed of small (27 to 40 nm), nonenveloped, icosahedral viruses that possess a linear, positive-sense, single-stranded RNA (ssRNA) genome. The *Caliciviridae* family includes six viral genera (Fig. 1) (Farkas, 2008): *Lagovirus, Vesivirus, Sapovirus, Norovirus, Becovirus and Recovirus*. Noroviruses (NoV) and Sapoviruses (SV) are called human caliciviruses and infect predominantly humans causing epidemic gastroenteritis. *Vesivirus* and *Lagovirus* contain only animal strains and are characterized by unique disease states. For example, San Miguel sea lion virus, a vesivirus, causes vesicular disease in sea lions and other pinniped species; rabbit hemorrhagic disease virus (RHDV), a lagovirus, which causes an often fatal hemorrhagic disease in rabbits (Green et al., 2000). *Becovirus* and *Recovirus* represent two tentative genera not yet accepted. NoV is the major cause of nonbacterial epidemic gastroenteritis, a disease that usually occurs in family or community-wide outbreaks.

![Unrooted phylogenetic trees based on amino acid sequence alignments of Calicivirus VP1 (Farkas, 2008).](image)
**Genomic Organization and Protein**

NoV encodes a 7.6 kb positive-sense, single-stranded RNA genome with three open reading frames (ORFs) (Fig. 2). ORF1 is over 5 kb and encodes a 200 kDa polyprotein, which is autoprocessed by a virally encoded 3CL protease to yield the non-structural viral replicase proteins essential for viral replication. These include p48, NTPase (important in NTP binding and hydrolysis of NTP), p22, VPg (which covalently links to the RNA genome), the 3CL proteinase (Pro), and an RNA dependent RNA polymeras (Pol). ORF2 is 1.8 kb and encodes VP1, which forms the viral capsid. ORF3 is 0.6 kb and encodes VP2, a 22 kDa minor basic structural protein that has been hypothesized to function in packaging the genome into virions (Glass et al., 2009).

![Genomic Structure Diagram](asyanka.png)

**Figure 2.** The NoV genomic structure (Asanaka et al., 2005)

**Phylogeny and Nomenclature**

Based on the sequence of the capsid gene, noroviruses have been classified into five genogroups (GI - GV) (Fig. 3) (Fankhauser et al., 1998; Koopmans et al., 2002; Zheng et al., 2006). GI, GII and GIV infect human and are called human norovirus (HuNoV), with the exception of three porcine-specific viruses within GII (GII-11, 18 and 19). GIII infects bovine species (Liu et al., 1999; Oliver et al., 2003) and GV infects mice (Hsu et al., 2007; Karst et al., 2003; Müller et al., 2007; Thackray et al., 2007; Kim et al., 2010; Ramirez et al., 2008). The genogroups can be further divided into different genetic clusters or genotypes. Each HuNoV genogroup (GI, GII and GIV) is divided into
different clusters (Fig. 3). Genetic clusters are designated numerically following identification of the genogroup to which they belong (e.g., GII.4). Distinct strains are further subdivided within a genetic cluster (Fig. 3). The name of NoV individual isolates routinely use *strain/year/country* nomenclature (e.g., Stepping Hill/2001/UK) and NoV strains are commonly named after the places or regions where the strains were first isolated, e.g., Montgomery County, Snow Mountain, Mexico, Hawaii, Parmatta, Taunton, and Toronto viruses. The name of the country is shown as a two-letter code (except where the name is obvious) along with the strain name. These codes are as follows: AU, Australia; CA, Canada; DE, Germany; Fr, France; JP, Japan; NL, Netherlands; NZ, New Zealand; Sau, Saudi Arabia; UK, United Kingdom; US, United States (http://intl-journals.asm.org).

Analyses of the full-length genomic sequence of several NoVs indicated that viral strains within a genogroup share 69-97% similarity, while strains in different genogroups are only 51-56% similar. The ORF1/2 junction is the most highly conserved sequence in the NoV genome, maintaining 86-100% identity within a genogroup in the subsets of strains tested (Kageyama et al., 2003).
Figure 3. Phylogenetic tree representing NoV genogroup classification (Koopmans et al., 2002; Zheng et al., 2006).
Clinical Features and Transmission

HuNoV infects persons of all ages (Rockx et al., 2002). Young or old people and pregnant women may be more vulnerable. Clinical HuNoV infection generally has an incubation period of 24 – 48 h and is characterized by acute onset of nausea, vomiting, abdominal cramps, myalgias, and non-bloody diarrhea. The symptoms are usually resolved in 2-3 days. However, the median duration of illness can be longer in patients affected during hospital outbreaks and in children less than 11 years of age (Rockx et al., 2002; Lopman et al., 2004). Further, patients continue to shed the virus long after symptoms have resolved. Deaths have been reported during outbreaks in nursing homes (Dedman et al., 1998; Chadwick et al., 2000).

The fecal-oral route is generally the most important mode of NoV transmission. Transmission through infectious vomit, contaminated food or water further propagates the epidemic (Becker et al., 2000). Several characteristics of HuNoV that facilitate their spread in epidemics include: (1) the low infectious dose of HuNoV which may be as low as 10 particles to infect people (Teunis et al., 2008); (2) the prolonged duration of viral shedding, even after symptoms resolve, increases the risk of secondary spread; (3) the stability of the virus at a temperatures from freezing to 60°C (Patel et al., 2009) and in relatively high concentrations of chlorine (Duizer et al., 2004); and (4) repeated infections can occur throughout life with re-exposure, likely because of lack of complete cross-protection against the diverse HuNoV strains and inadequate long-term immunity.

Epidemiology

HuNoV is the major cause of nonbacterial epidemic gastroenteritis in humans worldwide. In the U.S., CDC estimates that nationally 21 million cases of acute
gastroenteritis a year are due to HuNoV infection. Among the 232 outbreaks of HuNoV illness reported to CDC from July 1997 to June 2000 in the US, 57% were foodborne, 16% were due to person-to-person spread, and 3% were waterborne. In 23% of outbreaks, the cause of transmission was not determined. Norovirus contamination in water can be found throughout the year (Haramoto et al., 2005; Sano et al., 2006). Waterborne outbreaks of HuNoV disease in community settings have often been caused by recreational water and sewage contamination of wells. GI and GII account for the majority of HuNoV cases. However, outbreaks of the GII.4 genocluster occur much more frequently than any other genocluster with the GII genogroup, and GI outbreaks occur even less frequently (Fankhauser et al., 2002; Ike et al., 2006).

**Host Susceptibility, Immunology, and Pathogenesis**

Information on host susceptibility and immunology is obtained mainly from human volunteer studies and HuNoV outbreaks, since HuNoV does not easily grow in cell culture. In volunteer studies, villus atrophy in duodenal biopsies and presence of malabsorptive diarrhea were described (Dolin et al., 1972; Agus et al., 1973; Dolin et al., 1975). Previous volunteer studies also showed that (1) while infected volunteers develop immunity after a HuNoV challenge (Wyatt et al., 1974; Parrino et al., 1977), immunity appeared short-lived (several weeks to months); (2) this immunity did not necessarily extend to heterologous virus challenge (Wyatt et al., 1974); (3) previous existing antibodies against HuNoV were not correlated with protection against the same HuNoV inocula when the same subjects were challenged 2-3 years later (Parrino et al., 1977). Some individuals with a high level of antibodies against HuNoV were even more susceptible to HuNoV challenge than those with no or lower levels of antibodies (Parrino
et al., 1977). Individuals with blood type O are more often infected, while blood types B and AB can confer partial protection against symptomatic infection (Hutson et al., 2002).

Despite the lack of suitable tissue culture or animal models, significant advances in HuNoV attachment and pathogenesis research have been achieved. HuNoV binds to the highly polymorphic histo-blood group antigens (HBGAs) (Harrington et al., 2002; Marionneau et al., 2002; Hutson et al., 2003), which act as the putative cellular receptors, and strains from different genoclusters bind various HBGAs. Human HBGAs are complex glycans present on the surface of red blood cells, on the epithelia of the gastrointestinal and respiratory tracts, or as free antigens in biologic fluids such as saliva, milk, and intestinal contents (Marionneau et al., 2001). Three major human HBGA families, namely, the Lewis, secretor, and ABO families, are involved in HuNoV recognition. Up to now, eight distinct receptor binding patterns of HuNoV have been described (Huang et al., 2003; Tan and Jiang, 2005). HuNoV may recognize HBGAs on gastrointestinal cells of clams, mussels, and oysters, leading to a possible mechanism of bioaccumulation. Recently, serial passage of HuNoV in gnotobiotic pigs, with occurrence of mild diarrhea and shedding, and immunofluorescent detection of the HuNoV structural and nonstructural proteins in enterocytes confirmed HuNoV replication in gnotobiotic pigs (Cheetham et al., 2006). The gnotobiotic pig model may be useful to study the pathogenesis of human NoV infections. Recent research also showed that HuNoV can infect and replicate in a physiologically relevant 3 dimensional (3-D), organoid model of human small intestinal epithelium (Straub et al., 2007). The results demonstrate that the highly differentiated 3-D cell culture model can support the natural growth of HuNoV and facilities the study of HuNoV pathogenesis.
Detection Methods

Methods used to detect NoV rely on the detection of viral particles or viral RNA. Detection of viral particles by electron microscopy is labor intensive and relatively insensitive because a concentration of at least $10^6$ virions mL$^{-1}$ is required (Griffin et al., 2003; Kageyama et al., 2003). Many molecular detection assays that detect a wide variety of NoV strains have been developed. Examples include conventional reverse transcription polymerase chain reaction (RT-PCR) (Ando et al., 1995; Green et al., 1995a; Vinjé and Koopmans, 1996; Anderson et al., 2003), nucleic acid sequence-based amplification (NASBA) (Greene et al., 2003; Moore et al., 2004a), enzyme linked immunosorbent assays (ILESA) (Michael and Rainer, 2004) and real time RT-PCR assays (Kageyama et al., 2003; Hohne and Schreier, 2004; Richards et al., 2004; Jothikumar et al., 2005). Although conventional RT-PCR is currently the primary NoV detection method, post-amplification steps, such as gel electrophoresis combined with probe hybridization or sequencing, are still necessary to confirm the identity of the amplified products and to prevent the misinterpretation of false positive results due to non-specific amplification (Alain and Danielle, 2006). NASBA has less consistent signals than TaqMan real-time PCR for HuNoV GII detection with the Kageyama system (Alain and Danielle, 2006).

Real-time PCR has become more popular due to the following characteristics. Real-time PCR assays are more sensitive and specific than ELISA (enzyme-linked immunosorbent assay) for detecting NoV in stool specimens (Michael and Rainer, 2004). The assay does not require post-PCR processing and is more appropriate for quantitative detection of NoV RNA than conventional RT-PCR. It also has more consistent signals
than NASBA for HuNoV GII detection with Kageyama system (Alain and Danielle, 2006). Finally, caps of reaction tubes used in TaqMan PCR do not need to be opened for electrophoresis after PCR thereby reducing the chance of PCR contamination. Despite the availability of multiple assays to detect NoV, detection of NoV in bivalves can be still be problematic because the sample processing and RNA extraction protocols used for the initial virus isolation step are still time consuming to complete. In addition, because multiple steps are required, for example, procedures used by Mullendore et al. (2001), Myrmel et al. (2004), Jothikumar et al. (2005), Gentry et al. (2009) and Le Guyader et al. (2009), loss of viral RNA during each of the steps remains problematic. Diagnostic procedures published by scientists at the U. S. Food and Drug Administration (USFDA) as well as U. S. Department of Agriculture (USDA) appear to require 1-2 days (DePaola, et. al., 2010; Kingsley, 2007).

**Surrogate of Human Noroviruses – Murine Norovirus**

Murine norovirus (MNV-1) was identified in 2002 as a new mouse virus, and is the first NoV to be grown in cell culture (Karst et al., 2003; Wobus et al., 2004). Although MNV-1 was initially isolated from severely immunocompromised mice, subsequent studies demonstrated that this virus also infects wild-type mice. MNV-1 causes a disseminated infection that include necrosis of the spleen, liver, lung, brain and intestines in mice with deficient innate immune responses (Karst et al., 2003). In contrast, MNV-1 causes a subclinical infection without remarkable tissue pathology in immunocompetent mice, and infection is limited to the intestines, liver, spleen, lymph nodes, and lungs (Hsu et al., 2005; Karst et al., 2003; Mumphrey et al., 2007). During the last few years, more than 60 additional isolates of MNV have been identified and
sequenced, and they comprise the single genogroup V (Thackray et al., 2007). None of these additional MNV strains have been reported to cause clinical disease in immunocompetent mice (Hsu et al., 2006; Müller et al., 2007; Thackray et al., 2007).
Corbicula fluminea (C. fluminea)

History and Characteristics

Corbicula fluminea (C. fluminea) (Fig. 4 and 5), the freshwater Asian clam is in the order Veneroida and the family Corbiculidae. It is harvested by humans throughout the world for consumption and when removed from its shell, used as fish bait.

The clam is an invasive non-native filter-feeder found in abundance throughout most aquatic systems in the United States. Original, they were found in temperate to tropical southern Asia, west to the eastern Mediterranean, and in Africa, except in the Sahara desert, as well as the Southeast Asian islands south into central and eastern Australia (Morton 1986). The earliest verifiable record of this species in North America was at Nanaimo, Vancouver Island, British Columbia in 1924. Asian clams are believed to have established a viable population on the west coast of the United States sometime prior to 1938 (Cherry et al. 1980). While they are typically considered a freshwater species, they are salt-tolerant to 13 ppt for short periods, and higher if allowed to acclimate. Estuarine populations have been documented in the San Francisco and Chesapeake Bays.

The clam is hermaphroditic, with single genopores on each side of the body. Reproduction and larval release occur biannually in the spring and in the late summer. The clam is believed to practice self-fertilization, enabling rapid colony regeneration when colony populations are low. Normally, their lifespan ranges from 3-5 years but can reach a maximum of 7 years. The size is usually smaller than 50 mm in diameter. Though the clams can be found in any habitat, they prefer flat areas with combinations of fine
clean sand, coarse sand and clay. The clam has limited mobility and may be a good indicator of site-specific potential for pathogen bioaccumulation.

*Figure 4. Closed C. fluminea*  
*Figure 5. Opened C. fluminea*

**Pathogen Bioaccumulation by Corbicula fluminea**

*Corbicula fluminea* can filter up to 2.5 L water per h per clam to obtain food and in the process bioaccumulate viruses. They are common, widespread, and resistant to environmental toxicants, so it is recommended for freshwater contaminant bioaccumulation studies by the National Water Quality Assessment Program (Crawford and Luoma, 1993). *C. fluminea* can be left at selected sites for various lengths of time where they can filter large volumes of water to obtain food and bioaccumulate viruses. Both humans and animals harvest them as a food source, suggesting that they might expose consumers to pathogens when eaten raw. Chemicals such as organochlorines and pesticides as well as pathogens have been detected in *C. fluminea*. The pathogens include human enteric protozoans (*Cyclospora cayetanensis*, *Cryptosporidium parvum* and *Giardia lamblia*) and NoV (Saitoh et al., 2007). Factors influencing virus bioaccumulation by shellfish include hydraulic characteristics of water flow, virus type, temperature, virus concentrations in water, salinity and pH (ionic changes), and suspended solids (SS) or turbidity (Sobsey et al., 1991; Le Guyader et al., 2006; Tian et al., 2006; Tian et al., 2007).
References


Boehm AB, Ashbolt NJ, Colford JM, Dunbar LE Jr., Fleming LE, Gold MA, Hansel JA,


Gentry J, Vinje J, Lipp EK. 2009. A rapid and efficient method for quantitation of genogroups I and II norovirus from oysters and application in other complex


CHAPTER III
DEVELOPMENT OF A NOROVIRUS RNA EXTRACTION METHOD IN OYSTERS USING BIOTINYLATED PROBE HYBRIDIZATION TO TARGET VIRAL RNA: A PRELUDE TO RNA EXTRACTION IN CLAMS

For submission to the Journal of Virological Methods

Abstract

Human Noroviruses (HuNoV) are the most frequent cause of acute gastroenteritis following the ingestion of pathogen-contaminated raw or improperly cooked oysters. Although highly sensitive methods to detect HuNoV in oysters using RT-PCR are already available, isolation of either HuNoV RNA or virions from oyster remains a cumbersome process. I developed a new method to extract HuNoV RNA from contaminated oysters that is much faster compared to existing methods. The procedure includes an initial extraction of total RNA using TRI Reagent followed by HuNoV RNA concentration and purification using a biotinylated probe capture technique. The purified HuNoV RNA is subsequently detected by real-time RT-PCR. The virus was detectable in oyster homogenates spiked with as little as 100 PCR detection units (PDU) of HuNoV. Compared to published methods that require an initial virus purification step, the new method is much faster to complete. Approximately 3 h are needed to purify NoV RNA using the new method compared to at least 8 h using conventional methods. Coupled with real-time RT-PCR, the new method can detect HuNoV in contaminated oysters within 8 h. The detection limit of the developed method was 10 -100 PDU of HuNoV. In addition, the method was used successfully to detect HuNoV in live artificially-contaminated oysters and wild oysters.
Introduction

Noroviruses (NoVs) are non-enveloped, icosahedral viruses with a positive-sense RNA genome and constitute a genus in the family Caliciviridae (Green et al., 2000). Based on genome sequence data, Koopmans et al. (2002) initially separated NoVs into five genogroups (GI - V), but Mesquita et al. (2010) proposed the addition of a new genogroup GVI. Among them, GI, GII and GIV NoVs infect humans (Patel et al., 2009) and are called human noroviruses (HuNoV). NoVs are the most common cause of gastroenteritis outbreaks worldwide, accounting for ~50% of all-cause gastroenteritis outbreaks worldwide (Patel et al., 2009). Because oysters can concentrate NoVs, outbreaks of NoV gastroenteritis are often associated with the consumption of raw or improperly cooked oysters (Koopmans et al., 2004; Lees et al., 2000; Schwab et al., 2000).

Methods used to detect NoV in oysters rely on the detection of viral particles or viral RNA and both present difficulties. Detection of viral particles by electron microscopy is labor intensive and relatively insensitive because at least $10^6$ virions ml$^{-1}$ is required (Griffin et al., 2003; Kageyama et al., 2003). Detection of NoV RNA by reverse transcription polymerase chain reaction (RT-PCR) is highly sensitive but current NoV RNA extraction protocols (Gentry et al., 2009; Jothikumar et al., 2005; Kingsley, 2007; Le Guyader et al., 2009; Mullendore et al., 2001; Myrmel et al., 2004) are time consuming to perform and use multiple steps during which viral RNA could be lost. For examples, the glycine, polyethylene glycol, Tri–reagent, poly dT viral RNA extraction protocol of Kingsley and Richards (2001) has separate steps for virus elution, virus precipitation, total RNA isolation and viral RNA purification. In a study comparing
methods, Schultz et al. (2007) found that each of the three published methods included in the study (Beuret et al., 2003; Le Guyader et al., 2000; Mullendore et al., 2001) required at least one day to process six samples from tissue homogenization to viral RNA isolation.

In the present study, I describe a rapid and efficient viral RNA extraction method that greatly reduces the amount of time needed to detect HuNoV in oysters using real-time RT-PCR. The new method was developed and optimized using oyster homogenates spiked with HuNoV GII-positive stool extract and then tested using live artificially-contaminated oysters and wild oysters.

Materials and Methods

Virus stock and oyster samples

The HuNoV GII-positive clinical sample, chloroform extracted from stool specimens of patients with gastroenteritis, was kindly provided by Dr. Jacquelina Woods (U.S. Food and Drug Administration, Dauphin Island, AL, USA). This HuNoV sample was stored at 4°C and used to spike oyster homogenates during method development and to artificially contaminate live oysters. To determine the titer of the sample, HuNoV GII detection by real-time RT-PCR (described below) was performed using 10 µL of each 10-fold serial dilutions from $10^0$ to $10^5$ in 1X PBS (Phosphate Buffered Saline). The most dilute sample that remained RT-PCR-positive was $10^{-3}$ and the titer of the clinical sample was determined to be $10^5$ RT-PCR detection units (PDU)·ml$^{-1}$.

Live oysters (*Crassostrea virginica*) (n = 20) used for tissue spiking experiments were purchased from a seafood market (Crystal Seas Seafood, Pass Christian, MS). Oyster digestive glands were pooled, homogenized using a manual Potter-Elvehjem
tissue grinder and stored at -80°C until use. To determine the detection limit of the HuNoV assay method, 100 µL subsamples of the homogenate were spiked with the HuNoV GII-positive stool extract and serially diluted in PBS. The amount of HuNoV spiked corresponded to 100, 10 and 1 PDU. Each sample was prepared in duplicates and the experiment was repeated three times. Digestive gland homogenates without spiked stool extract served as negative control. Total RNA was extracted and tested for the presence of HuNoV as described below.

To compare the efficacy of the HuNoV assay method developed in the present study to other published methods, samples of oyster homogenate containing the same amount of HuNoV were prepared and assayed. For each preparation, one gram of thawed oyster digestive gland homogenate was spiked with HuNoV GII-positive fecal extract containing 1,000 PDU HuNoV. After thorough mixing, six 100 µL subsamples were transferred to new tubes. Two subsamples were processed using each of three RNA extraction methods. This comparison was performed 3 times.

Oysters (n = 30) used in laboratory HuNoV exposure experiments were collected from a salt marsh at East Beach, Ocean Springs, MS, USA and maintained in 150 L of artificial seawater in the laboratory for three days before use. These oysters were fed 500 µL of Shellfish Diet 1800 (Reed Mariculture, Inc., Campbell, CA) daily. To expose oysters, HuNoV-positive stool extract diluted in PBS was first spiked into tanks holding 10 L of natural seawater (24 ppt) to reach a final virus concentration of 100 and 2,000 PDU·L⁻¹. Ten oysters were added to each tank after one hour during which water was continuously circulated using a small submersible pump. Oysters in a tank not spiked with HuNoV served as negative control. Three oysters were sampled after one and three
days. The digestive glands were dissected, viral RNA extracted and tested for the presence of HuNoV as described below.

Different batches of oysters (n = 51) from a marina in Ocean Springs, and salt marshes in Ocean Springs and Pass Christian, MS, USA were also collected and tested to determine whether HuNoV could be detected in wild populations using the present method. Oyster digestive glands were stored individually at -80°C for subsequent RNA extraction and HuNoV detection.

**NoV RNA isolation**

The HuNoV RNA extraction method described herein is based on sequence hybridization technology using a biotinylated probe (Fig. 6). Total RNA was first extracted from HuNoV-positive stool extracts or oyster tissue using TRI Reagent (Molecular Research Center, Inc., Cincinnati, Ohio) according to the manufacture’s protocol. To isolate HuNoV RNA, the resulting total RNA pellet was first dissolved in 60 µL of DEPC-treated water, heated at 94°C for 5 min, and quick chilled on ice for 5 min. Afterwards, 2 µL of 1 µM biotinylated COG2R hybridization probe (Table 2), 1 µL of RNasin® Plus RNase Inhibitor (Promega Corp, Madison, WI) and 7 µL of 10X hybridization buffer (0.4 M NaCl, 40 mM HEPES, 1 mM EDTA, pH 6.6) were added to each 60 µL RNA sample. To facilitate hybridization of HuNoV RNA to the biotinylated COG2R probe, the samples were incubated at 45°C for 1 h with continuous agitation using a Thermomixer 5436 (Eppendorf North America, Inc., Westbury, NY) at 1,000 rpm·min⁻¹.

To capture HuNoV RNA, 10 µL of washed streptavidin-coated magnetic bead (Dynabeads MyOne™ Streptavidin C1, Life Technologies, Grand Island, NY) and 5.9 µL
of DEPC-treated 5 M NaCl were added and the samples mixed continuously for 30 min at 25°C. To isolate HuNoV RNA, the magnetic beads were captured using a Magnetic Separation Stand (Promega Corp, Madison, WI), washed three times with 1X washing buffer containing 5 mM Tris-HCl (pH 7.5), 0.5 mM EDTA and 1 M NaCl. To elute the captured HuNoV RNA, 10 µL of DEPC-treated water were added to each sample and heated at 94°C for 5 min. After brief centrifugation, the magnetic beads were captured again using the Magnetic Separation Stand, and the supernatant containing HuNoV RNA transferred to a new tube for subsequent analysis by real-time RT-PCR. DEPC-treated water and HuNoV RNA extracted directly from the HuNoV were used as negative and positive controls, respectively.

Variables tested during method optimization included the amount of time for probe-target hybridization and probe capture, PEG (polyethylene glycol) effects on isolation efficiency, reaction volumes. PEG binds water, and thus is commonly used as precipitant for plasmid DNA isolation, protein crystallization, and virus concentration. Since HuNoV is dilute in environmental samples, I expected that adding of PEG to the hybridization reaction (biotinylated probe and target RNA) in the RNA extraction method might help the probe hybridize with the viral RNA genome. To determine if PEG can improve the RNA extraction efficiency, 12.6 µL of PEG (50%, autoclaved) or DEPC-treated water were added to the 70 µL of RNA extraction reaction to make a final concentration of PEG at 7.5% or 0%, respectively. Experiments were performed in duplicates. The resulting purified HuNoV was detected by real time RT-PCR.

The efficacy of HuNoV detection using the present RNA isolation method was compared to those achieved using two published methods with slight modifications (Fig.
7). With the method of Beuret et al. (2003), TRI Reagent was used to extract RNA instead of QIAamp Viral RNA Mini Kit (GIAGEN Inc., Valencia, CA). With the method of Baert et al. (2007), QIAamp Viral RNA Mini Kit was used in place of the RNeasy Mini Kit (GIAGEN Inc., Valencia, CA). Oyster homogenates were spiked with the virus so that each subsample contained 100 PDU HuNoV. Two subsamples were extracted using each of the three methods and the experiment was performed 3 times. The amount of HuNoV detected in RNA isolated using each of the methods was compared by real-time RT-PCR as described below.
Figure 6. Biotin-based probe hybridization technology
Figure 7. Flow diagram listing the three sample treatment protocols assessed for their ability to recover HuNoV RNA. RT-PCR assay was used for HuNoV detection.

Detection of HuNoV using real-time RT-PCR

Primers used for reverse transcription and PCR of HuNoV cDNA are listed in Table 2. ImPron-II Reverse Transcriptase (Promega Corp, Madison, WI) was used according to the manufacturer’s protocol for cDNA synthesis, and EconoTaq DNA Polymerase (Lucigen Corporation, Middleton, WI) was used for real-time PCR assays. Water was included as a negative control at both the reverse transcription and PCR steps. Plasmid DNA containing cloned HuNoV cDNA was used as the standard during real-time PCR. Amplification reactions contained 2 µL cDNA, 400 nM each primer (JJV2F and
COG2R), 200 nM probe (RING2-TP), 0.5 U EconoTaq DNA polymerase, 200 µM dNTPs, and 1X EconoTaq Buffer in a total volume of 10 µL. Real-time PCR was performed using a Rotor-Gene 6000 thermal cycler (Corbett Research, New South Wales, Australia). cDNA amplification was carried out using a melting step at 95°C for 10 min followed by 45 cycles of 95°C for 15 sec to melt DNA and 60°C for 60 sec to anneal and extend primers. The size of the expected amplicon was 98 base pairs.

Table 2

*Primer and probe sequences for HuNoV detection*

<table>
<thead>
<tr>
<th>Primer/probe</th>
<th>Sequence (5′–3′)</th>
<th>Positiona</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotinylated COG2R</td>
<td>BIO**-TCGACGCCATCTTCATTCCACA</td>
<td>5100-5080</td>
<td>This study</td>
</tr>
<tr>
<td>JJV2F</td>
<td>CAAGAGTCAATGTTTAGGTGGATGAG</td>
<td>5003-5028</td>
<td>Jothikumar et al., 2005</td>
</tr>
<tr>
<td>COG2R</td>
<td>TCGACGCCATCTTCATTCCACA</td>
<td>5100-5080</td>
<td>Kageyama et al., 2003</td>
</tr>
<tr>
<td>RING2-TP</td>
<td>FAM**-TGGGAGGGCGATCGCAATCT-BHQd</td>
<td>5048-5067</td>
<td>Kageyama et al., 2003</td>
</tr>
</tbody>
</table>

Note. a Nucleotide position based on Lordsdale virus (Genbank accession no. X86557); **BIO, biotin; †FAM, fluorescein; ‡BHQ, Black Hole Quencher.

*Statistical analysis*

The statistical significance (P < 0.05) of differences in the amount of HuNoV detected using the three RNA isolation methods and in the different hybridization conditions was evaluated using one-way ANOVA (SPSS version 13.0). Tukey’s HSD test was used to test the significance of differences among means.
Results

Method development

Real-time RT-PCR results showed that there was no significant difference (p < 0.05) in cycle threshold (Cₜ) values using probe-target hybridization and probe capture times that ranged from 5 h to 1 h and 2 h to 30 min, respectively (Fig. 8A). Further experimentation showed that additional savings in time could be achieved by shortening the hybridization and capture times to 1 min and 10 min, respectively (Fig. 8B). However, reducing the probe capture time to 1 min reduced the assay sensitivity significantly (p < 0.05) resulting in Cₜ values that increased by approximately four cycles (Fig. 8B). The combination of 1 min for probe-target hybridization and 10 min for probe capture provided the fastest HuNoV RNA extraction protocol without sacrificing assay sensitivity.
Figure 8. The effect of different probe hybridization and capture times on C<sub>T</sub> values during HuNoV detection by real-time RT-PCR. A) Initial study using probe hybridization times longer than 1 h and probe capture times at least 30 min. B) Subsequent study using probe hybridization times shorter than 1 h and probe capture times less than 30 min. Error bars: standard deviation.
Adding PEG significantly decreased the viral RNA extraction efficiency ($p < 0.05$). The $C_T$ values were significantly higher when 7.5% of PEG was added to the hybridization reactions compared to reactions without PEG (Fig. 9).

![PEG (7.5%) Effects on the Efficiency of Viral RNA Isolation](image)

**Figure 9.** The effect of PEG on HuNoV RNA isolation. Error bars: standard deviation.

There was no significant difference between two different reaction volumes 70 µL and 35 µL on viral RNA isolation efficiency (Fig. 10) ($p < 0.05$). As a result, 70 µL reaction volume was used in experiments for the development of RNA extraction method and 35 µL was used for experiments using clams as a sentinel of NoV contamination.
Comparison of Viral RNA Purification Volume (uL)

Figure 10. The effect of hybridization volumes on HuNoV RNA isolation. Error bars: standard deviation.

Detection of HuNoV in oysters

The target capture method can be used to successfully isolate HuNoV from oysters within 3 h. Coupled with RT-PCR, HuNoV in contaminated oysters can be detected within 8 h. Using oyster tissue homogenates, HuNoV was detected consistently in samples spiked with 100 PDU and 10 PDU of HuNoV, but not at the more dilute level of 1 PDU (Table 3). Using live artificially-contaminated oysters in the laboratory, HuNoV was detected in oysters exposed to the virus at a concentration of $2 \times 10^4$ PDU·L$^{-1}$ for one and three days but not in oysters exposed to $1 \times 10^2$ PDU·L$^{-1}$ (Table 4). Oysters not exposed to HuNoV were all RT-PCR negative. Three of the 51 wild oysters tested were contaminated with HuNoV (Table 5). All three contaminated oysters were collected from Davis Bayou near the Gulf Coast Research Laboratory (GCRL) in Ocean Springs,
MS. Oysters collected from Pass Christian and the Ocean Springs Harbor were RT-PCR negative for HuNoV (Table 5).

Table 3

The proportion of samples in which HuNoV was detected by real-time RT-PCR using RNA isolated by the described hybrid capture method. Oyster homogenates were spiked with different amounts of HuNoV as indicated

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Proportion of samples in which HuNoV was detected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 PDU</td>
</tr>
<tr>
<td>1</td>
<td>4/4</td>
</tr>
<tr>
<td>2</td>
<td>4/4</td>
</tr>
<tr>
<td>3</td>
<td>4/4</td>
</tr>
<tr>
<td>Total</td>
<td>12/12</td>
</tr>
</tbody>
</table>

Table 4

The proportion of oysters in which HuNoV was detected by real-time RT-PCR using RNA isolated by the hybrid capture method. Oysters were exposed to HuNoV in 10 L seawater in the laboratory

<table>
<thead>
<tr>
<th>HuNoV concentration (PDU/L)</th>
<th>Proportion of oysters in which HuNoV was detected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 day exposure</td>
</tr>
<tr>
<td>0 PDU (control)</td>
<td>0/5</td>
</tr>
<tr>
<td>100</td>
<td>0/3</td>
</tr>
<tr>
<td>2,000</td>
<td>3/3</td>
</tr>
</tbody>
</table>
Table 5

Detection of HuNoV in wild oysters using RNA isolated by the described hybrid capture method

<table>
<thead>
<tr>
<th>Sampling sites</th>
<th>Sampling dates</th>
<th>Number of oysters tested</th>
<th>Number of positive oysters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Davis Bayou, Ocean Springs, MS</td>
<td>7/13/09; 10/28/09; 3/17/10; 12/19/11</td>
<td>44</td>
<td>3</td>
</tr>
<tr>
<td>Ocean Springs Harbor, MS</td>
<td>3/17/10</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Pass Christian, MS</td>
<td>7/13/09</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

Comparison to two other RNA extraction methods

Our results showed that the described target-capture method enhances the sensitivity of HuNoV detection by real-time RT-PCR when compared to two common RNA extraction methods. We were not able to detect HuNoV in RNA extracted from oyster tissue homogenates spiked with 100 PDU HuNoV using the modified Baert method (Baert et al., 2007). Compared to RNA extracted using the modified Beuret method (Beuret et al., 2003), the present method detected HuNoV sooner during real-time PCR. The mean C_T value obtained with RNA isolated using the modified Beuret method (31.4 ± 0.2) was significantly (p < 0.05) larger than the mean C_T value obtained with RNA isolated using the target-capture method (25.6 ± 0.4).
Discussion

Consumption of shellfish contaminated with NoV has long been recognized as a problem worldwide. To better monitor contamination, several highly sensitive molecular assays to detect HuNoV are available (e.g., Jothikumar et al., 2005; Kageyama et al., 2003; Kou et al., 2006). However, an effective method to first isolate NoV RNA for subsequent detection by one of the molecular assays is still urgently needed. One problem is the multiple steps and thus large amount of time required to process samples to maximize detection sensitivity. For example, the GPTT viral RNA extraction protocol first reported by Kingsley and Richards (2001) uses a glycine buffer to elute virus from homogenized shellfish tissue, polyethylene glycol to precipitate virus, TRI reagent to isolate total RNA and then poly-dT beads to purify viral RNA. Kingsley (2007) reports that the extraction method can be performed in less than 8 hr. Similar approaches used by other researchers to extract viral RNA from oysters are likely to take longer because of additional steps. For example, the methods used by DePaola, et. al. (2010) and Mullendore et al. (2001) included a second elution process and an extra chloroform extraction step. Schultz et al. (2007) found that each of the three published methods included in the study (Le Guyader et al., 2000; Mullendore et al., 2001; Beuret et al., 2003) required at least one day to isolate viral RNA from oysters.

I developed a faster method to detect norovirus in oysters using a DNA probe hybridization technique to first enrich target viral RNA before RT-PCR. The method differs from previously published methods in the approach used to enrich viral RNA. Among the various methods used heretofore, a common approach was to first isolate the virus from oyster tissues before RNA extraction. Methods that used such an approach
were time consuming because of the various adsorption and elution, and precipitation and centrifugation steps. My approach is faster because it obviates the initial virus isolation step. Total RNA is first purified from oyster tissue and then HuNoV RNA is selectively captured using a biotinylated DNA probe that hybridizes to HuNoV RNA. The biotinylated DNA:RNA duplex is selectively isolated by using streptavidin coated magnetic beadsto immobilize the duplex while oyster RNA is selectively removed. The HuNoV RNA is released by heating the DNA:RNA bead complex and then detected by real-time RT-PCR. The entire procedure can be completed in 3 hr. With an estimated additional 5 h to perform RT-PCR, results can be obtained in a total of 8 h.

During development of the method, I noticed that the source of polypropylene microcentrifuge tubes used makes a profound difference in the sensitivity of the assay. Those that work well can be easily distinguished. After the streptavidin magnetic beads have been added to capture the biotinylated DNA:RNA duplex, the uniformly brown solution should become clear quickly when the tube is placed against a magnet. With tubes that work well, the vast majority of beads congregate on the side of the tube next to the magnet with little elsewhere. These, paradoxically, are frequently the less expensive generic, private label microcentrifuge tubes. With tubes that do not work well, often labeled “low adhesion” and more expensive, a noticeable amount of the brown magnetic beads adhere elsewhere inside the tube. We surmise that is some type of weak attractive force between either biotin or streptavidin and plastic treated to prevent adhesion of DNA.

My results show that the new method is more sensitive than the modified Baert method (2007) and Beuret method (2003). In addition, the detection limit of 10 - 100
PDU can compete with those reported by other researchers, although direct comparisons of HuNoV detection limit are not easy because of different RT-PCR procedures, polymerases and quantification methods. For example, Häfliger et al. (1997) reported the detection of 3,300 PDU of NoV from oysters. Dix and Jaykus in 1998 reported the detection of 450 PDU of NoV from 50 g of clams. Kingsley (2007) reported 14.5 RT-PCR$_{50}$ for murine norovirus (MNV-1), a close genetic surrogate for HuNoV genogroups I and II, by using the GPTT method. One RT-PCR$_{50}$ unit defined as the amount of virus that yields a positive RT-PCR amplification 50% of the times.

As described earlier, the method was developed using HuNoV GII, the most prevalent human genogroup worldwide (Bull et al., 2006). To determine whether the method could be used for both HuNoV GI and GII RNA isolation, viral RNA was isolated from a mixture of HuNoV GI and GII positive clinical samples using the new method and tested by RT-PCR. RNA from both viruses were isolated simultaneously without sacrificing assay sensitivity compared to sensitivity obtained by isolating RNA from each genogroup separately (results not shown).

In summary, I developed a protocol that provides a convenient, fast and sensitive way to isolate HuNoV RNA from oyster tissues. It is likely that the method described can be used with appropriate biotinylated probes to isolate RNA from shellfish contaminated with other RNA viruses such as rotavirus, astrovirus, and hepatitis A virus.
Acknowledgements

I thank Dr. Jacquelina W. Woods at USFDA for HuNoV positive clinical samples. This study was financially supported by the EPA Gulf of Mexico Program (MX96429505-0) and the Mississippi Coastal Impact Assistance Program/U.S. Dept of the Interior (M09AF16192).
References


Estes K, Matson DO, Nakata S, Neill JD, Studdert MJ and Thiel HJ. 2000. Taxonomy of


Kingsley DH, Richards GP. 2001. Rapid and efficient extraction method for reverse
transcription-PCR detection of hepatitis A and Norwalk-like viruses in shellfish.


CHAPTER IV
EVALUATION OF CORBICULA FLUMINEA AS A SENTINEL OF HUMAN NORO VIRUS CONTAMINATION IN FRESHWATER USING MURINE NORO VIRUS AS A SURROGATE OF HUMAN NORO VIRUS IN THE LABORATORY STUDY

Human viruses generally occur at low concentrations in environment waters. A major challenge with existing virus monitoring methods is the critical first step when dilute virus must be concentrated from large bodies of water. The process is labor intensive and some procedures require specialized training and equipment. Filter-feeding bivalves, on the other hand, may provide a simple and inexpensive method to concentrate virus from natural waters. The objective of the study was to determine whether the freshwater clam Corbicula fluminea can be used as a sentinel to detect human norovirus (HuNoV) in freshwater. Clams were exposed to murine NoV (MNV-1, a surrogate for human NoV) in 10 L artificial pond water for 0.5, 6, 24, 48, and 72 h in an environmental chamber. Depuration experiments were carried out in 80 L artificial pond water for 0, 1, 4, 7, 10, and 15 d at 10°C and 20°C. NoV RNA was isolated using a biotinylated probe hybridization method developed in our lab and detected by reverse transcription TaqMan qPCR. MNV-1 was detectable after 6 h and 1 d exposure in clams exposed to virus concentrations of $10^6$ PFU·L$^{-1}$ and $10^4$ PFU·L$^{-1}$, respectively. The amount of bioaccumulated MNV-1 increased as the exposure period increased from 6 h to 27 h. The lowest virus concentration at which exposed clams were PCR-positive was $10^2$ PFU·L$^{-1}$ after 48 h exposure at 20°C. Clams bioaccumulated MNV-1 more quickly at 20°C than at 10°C (p < 0.05). The virus was persistently detected in contaminated clams during
depuration at both 10°C and 20°C. Depuration occurred significantly more quickly at 20°C than at 10°C (p < 0.05). In summary, the freshwater clam *C. fluminea* bioaccumulates NoV quickly and depurates slowly. The clam is likely to be useful as a sentinel for detecting NoV contamination in freshwater.

**Introduction**

The human noroviruses (HuNoV) within the genus *Norovirus* cause at least 95% of nonbacterial gastroenteritis outbreaks, and 50% of all gastroenteritis outbreaks in all age groups throughout the world. HuNoVs have been reported to be second only to rotaviruses in causing severe childhood gastroenteritis (Sakai et al., 2001; Glass et al., 2009), considering the recent success in employing universal rotavirus vaccination in infants and young children, NoV will likely become the main viral agent of childhood gastroenteritis in the near future.

HuNoV cannot be easily cultured in the lab, and several different surrogates have been used for the HuNoV study. Attenuated vaccine strains of polioviruses and the male-specific bacteriophage MS2 have frequently been used as surrogates for HuNoV. Feline calicivirus (FCV), from the genus *Vesivirus*, can be propagated in cell culture, it also has been extensively studied as a surrogate for HuNoVs in environmental survival and inactivation studies (D’Souza et al., 2006; Duizer et al., 2004). Recently, the first NoV to be propagated in cell culture was reported (Wobus et al., 2004). This virus, designated mouse norovirus1 (MNV-1), MNV is morphologically and genetically similar to HuNoVs, thus shows considerable promise as a HuNoV surrogate (Wobus et al., 2006). In the present study, MNV-1 was used as a surrogate of HuNoV.
Freshwater clams *Corbicula fluminea* (*C. fluminea*) is a suspension feeder able to filter detrital particles of 1.5 to 10 μm at a rate of up to 2.50 liters/h (McMahon et al., 1991). *C. fluminea* is long lived and present all year but has high filtration and assimilation rates at the warmer water temperature characteristic of late spring and early summer. However, one study showed that incubating *C. fluminea* at 10°C compared to 20°C water temperatures did not affect the number of *Cryptosporidium parvum* oocysts detected in the exposed clams (Miller et al., 2005). *C. fluminea* is able to bioaccumulate organic pollutants and heavy metals (Doherty et al., 1990; Basack et al., 1997; Inza et al., 1997; Narbonne et al., 1999). Moreover, documents also showed that bivalves can harbor environmental-derived human pathogenic bacteria and viruses as a result of concentrating pathogens from the surrounding water, and therefore may be useful for monitoring water pollution (Ayres et al., 1978; Trollope, 1984). With regard to NoV and clam the specifically, however, there is only one study about detection of NoV in clams (*Corbicula fluminea*) (Saitoh et al., 2007) and little information exists regarding the properties of these processes by bivalves. In addition, NoV contamination in the water environment is found throughout the year (Haramoto et al., 2005; Sano et al., 2006), so the fate of NoV in clams under different conditions of water temperature is worth elucidating.

To evaluate the clam *C. fluminea* as a sentinel of HuNoV contamination in freshwater, experiments were conducted under simulated environmental conditions in environmental chambers. The specific objectives were to determine: (1) if clam *C. fluminea* can bioaccumulate MNV-1; (2) MNV-1 bioaccumulation rates of *C. fluminea*; (3) temperature effect on MNV-1 bioaccumulation rates of *C. fluminea*; (4) MNV-1
bioaccumulation limit of *C. fluminea* (the lowest virus concentration in water at which viruses are PCR-positive for exposed clams); (5) temperature effect on MNV-1 depuration by *C. fluminea*.

**Materials and Methods**

*Preparation of murine norovirus stocks.* RAW 264.7 cells (Cat. #: TIB-71), a mouse leukaemic monocyte macrophage cell line, were purchased from American Type Culture Collection. Cells were grown in complete Dulbecco's modified eagle's medium (DMEM). Complete DMEM was DMEM supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Inc., Logan, Utah, USA), penicillin (100 U·mL\(^{-1}\)) and streptomycin (100 μg·mL\(^{-1}\)) in a TC-75 cm\(^2\) flask (Corning Incorporated, Corning, NY, USA). Cells were grown and maintained according to standard animal cell culture protocols and kept at 37°C with 5% CO\(_2\). In brief, cells were taken out of the liquid nitrogen tank and placed into a 37°C water bath immediately. A 1:3 split was performed with a medium change every 3 days.

To make frozen stocks of cells, subcultured cells (70% confluence, 4 d old) were lifted from flasks with cell scrapers and the resulting cell suspension was poured into 50 mL centrifuge tubes. The tubes were centrifuged at 200 g at 25°C for 5 min using a clinical centrifuge (International Equipment Company, TN, USA). Without touching cell pellets, almost all supernatant (about 500 μL left) in the tubes was removed and discarded. The bottoms of tubes with the cell pellets in them were flicked with an index finger to gently suspend the cell pellets. Ten mL freezing medium (complete DMEM with 5% dimethyl sulfoxide or DMSO) was added to the cell suspension. The cell suspension was mixed gently with a pipette tip and then transferred to 2 mL frozen vials. Vials each
containing 1.2 mL of cell suspension (10^7 CFU·mL^-1 as determined by Hemacytometer) were placed into Nalgene™ Cryo 1°C Freezing Container (USA). The container was stored at -80°C freezer (less than 1 week) and then transferred to liquid nitrogen for permanent storage.

Murine norovirus (MNV-1) was a gift from Dr. Virgin at Washington University. MNV-1 was propagated in RAW 264.7 cells. Cells were seeded into a TC-75 cm² flask so that an approximately 80-90% cell monolayer formed within 24 h. Immediately prior to infection, the growth medium was removed and 250 μL of viral stock in 2 mL of serum-free DMEM was added to the flask. The cell monolayer was incubated for 1 h at 37°C with 5.0% CO₂, and then washed twice with the serum-free DMEM. Following the two washes, 10 mL of medium supplemented with 5% FBS was added into the flask. The flask was then incubated for 48 hours, until approximately 90% viral-induced cytopathic effects (CPE) (rounding of cells, loss of contact inhibition and cell death) was observed. The flask was then stored at -80°C. After 24 h storage, the flask was then allowed to thaw at room temperature. After three cycles of freeze and thaw, the content of the flask was completely removed and centrifuged at 3,000 rpm for 5 minutes to remove all cellular debris. The supernatant was then removed and aliquoted into 5 mL microcentrifuge tubes containing 2.0 mL of the virus and 2.0 mL of FBS. The viral aliquots were stored at -80°C for permanent storage.

The titer of MNV-1 in plaque-forming units (PFU) was determined using plaque assay. In brief, RAW 264.7 cells were seeded into 6-well plates (Corning Incorporated, Corning, NY, USA) at a rate of 2 × 10^6 cells/well. The density allowed the formation of a confluent monolayer within 24 hours. Five hundred μL of each 10-fold dilution of MNV-
1 in complete DMEM were inoculated onto the murine cell line grown in the 6-well plates, following aspiration of the medium and two cell washes with DMEM without FBS. Plates were incubated for one hour at 37°C in a humidified 5% CO₂ incubator, with gentle rocking every 15 min to allow an even distribution of the viral inoculums. All liquid was removed from the plates and cells were covered with 2 mL/well of a 1.5% low melting point agarose overlay (USB, Cleveland, Ohio, USA). To prepare the 1.5% low melting point agarose overlay, the total amount of agarose overlay needed (2 mL/well x number of wells) was calculated, and half of the total amount of 3% low melting point agarose was prepared in sterile water in microwave. Half of the total amount of 2x eagle’s minimum essential medium (EMEM) (Lonza, Walkersville, MD, USA) supplemented with 10% FBS, penicillin (200 IU·mL⁻¹), streptomycin (200 μg·mL⁻¹), L-Glutamin (2%), and HEPES (10 mM) was prepared in a separate bottle. The 3% agarose and the complete medium were equilibrated to 37°C and 42°C in water bathes, respectively, and mixed together in one bottle to make the 1.5% low melting point agarose overlay. At the end of the incubation, the virus inocula were aspirated. Two mL of the agarose overlay were added to each well (touched the side of the well, did not pipette directly on the cells). The agarose was allowed to solidify (about 5-10 min). The plates were incubated at 37°C for 48 h.

To visualize the plaques, the second 1.5% SeaKem GTG agarose (FMC Corporation, Rockland, ME, USA) overlay was prepared. The total amount of agarose overlay needed (2 mL/well x number of wells) was calculated, and half of total amount of the 3% SeaKem GTG agarose was prepared in sterile water in microwave. Half of the total amount of 2x EMEM supplemented with 10% FBS, penicillin (200 IU·mL⁻¹),
streptomycin (200 μg·mL\(^{-1}\)), L-Glutamin (2%), HEPES (10 mM), and 2.0% neutral red solution (These were the final concentration of each component) was prepared in a separate bottle. Both solutions were equibibrated to 56°C in a water bath, mixed together in one bottle, and left the resulting 1.5% SeaKem GTG agarose overlay in the water bath until ready to use. Two mL of the 1.5% overlay were added to each well. The cells were allowed to take up the neutral red for 4 h before visualizing the plaques.

**Clams (Corbicula fluminea).** Freshwater clams *C. fluminea* (30 ± 5 mm length) were collected from a clean site (Lake Serene) in Hattiesburg, Mississippi, USA. Clams were transported in the natural freshwater to the WetLab at the University of Southern Mississippi and maintained in artificial pond water (Lenore, 1998). Clams were placed in an environmental chamber set at 20°C with the photoperiod of 12 h every day, allowing clams to acclimate to the laboratory condition for 3 d before any experiments. Throughout the studies, clams were monitored daily for mortality based on movement.

**MNV-1 bioaccumulation by clams.** To determine if *C. fluminea* can bioaccumulate MNV-1, 2.2 \(\times\) 10\(^5\) PFU of MNV-1 and 500 µL of Shellfish Diet 1800 (Reed Mariculture Inc., Campbell, CA, USA) were spiked into 10 L of artificial pond water in a bucket in an environmental chamber at 20°C. It is well known that clams are active in late spring and early summer, when the average temperature in Mississippi is approximately 20°C (Table 6). A temperature recorder from HoBo was used to monitor the temperature and photoperiod in the environmental chambers. The spiked water was recirculated with a pump for 0.5 h, then 5 clams acclimated to 20°C were placed into the bucket with spiked water. The bucket exposure then commenced at 20°C with 12 h of photoperiod per day. The spiked water was changed every day to keep the virus concentration in the water as
consistent as possible. Calcium hypochlorite (bleaching powder) was used to disinfect the viruses. Calcium hypochlorite is widely used for water treatment and as a bleaching agent. This chemical is considered to be relatively stable and has greater available chlorine than sodium hypochlorite (liquid bleach). One pound of calcium hypochlorite in granular form can treat up to 10,000 gallons of drinking water according to the manufacturer, and the concentration used in the study was 0.1G·L⁻¹water. After 72 h exposure, 3 active clams were sampled. DG was obtained manually from each clam. Clam dissection tools were rinsed after each clam dissection. Following dissection, one DG was considered as one sample. DG samples were homogenized with blades, and 100 µL of the homogenate was used for RNA extraction immediately as described later or stored at -80°C for RNA extraction later. MNV-1 in the clams was detected by RT-PCR as described later.

A virus positive control and a virus negative control were included during the exposure. The positive control was to test the percentage of the spiked viruses which was available in the water but did not stick to the bucket during exposure. To do this, 2.2 x 10⁵ PFU of MNV-1 was spiked into aerated 10 L of artificial pond water in the bucket. The virus titer of spiked water was 10³ PFU·mL⁻¹ and no clams were placed in the bucket. After 24 h exposure, 500 µL of the spiked water was collected in replicates and filtered with 0.2 µM HT Tuffryn membrane (Pall Corporation, Ann Arbor, MI, USA). The virus concentration in the water was determined by plaque assay (Lee et al., 2004). The negative control was to test if the artificial pond water, bucket, and clams used for bucket exposure had MNV-1 contamination before spiking. To do this, everything was the same as positive control except that 5 clams were placed in the bucket instead of viruses. After
24 h, clams from the positive control were collected and were subjected to MNV-1 detection by TaqMan assay. A total of 3 independent trials were performed.

Table 6

Typical high and low temperatures (°C) for various Mississippi cities

<table>
<thead>
<tr>
<th>City</th>
<th>Jan</th>
<th>Feb</th>
<th>Mar</th>
<th>Apr</th>
<th>May</th>
<th>Jun</th>
<th>Jul</th>
<th>Aug</th>
<th>Sep</th>
<th>Oct</th>
<th>Nov</th>
<th>Dec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gulfport</td>
<td>16/6</td>
<td>18/8</td>
<td>21/11</td>
<td>25/15</td>
<td>29/19</td>
<td>32/22</td>
<td>33/23</td>
<td>33/23</td>
<td>31/21</td>
<td>26/16</td>
<td>21/11</td>
<td>17/7</td>
</tr>
<tr>
<td>Jackson</td>
<td>13/2</td>
<td>16/3</td>
<td>20/7</td>
<td>24/11</td>
<td>28/16</td>
<td>32/20</td>
<td>33/22</td>
<td>33/21</td>
<td>30/18</td>
<td>25/11</td>
<td>19/6</td>
<td>14/3</td>
</tr>
<tr>
<td>Meridian</td>
<td>15/2</td>
<td>17/3</td>
<td>21/7</td>
<td>25/10</td>
<td>29/16</td>
<td>32/20</td>
<td>34/21</td>
<td>34/21</td>
<td>31/18</td>
<td>26/11</td>
<td>20/6</td>
<td>16/3</td>
</tr>
</tbody>
</table>

Notes. The above information was from US travel weather; The average temperature in Mississippi in Dec and Jan is 9.4°C; The average temperature in Mississippi in Apr and May is 20.5°C

MNV-1 bioaccumulation rates of clams. As shown in Table 7, MNV-1 at concentrations of $10^4$ PFU·L$^{-1}$ and $10^6$ PFU·L$^{-1}$ was spiked into each aerated 10 L of artificial freshwater maintained at 20°C, respectively. After 0.5 h of circulation of the spiked water, clams (n = 5) was added to each spiked water sample and bioaccumulation commenced. Three exposed clams were sampled at different time periods: 0.5, 6, 24, 48, and 72 h. To perform a virus positive control during the exposure, 2.2 x $10^5$ PFU of MNV-1 was spiked into aerated 10 L of artificial freshwater. No clams were placed in the water. After 1 24 h exposure, 500 µL of the spiked water was collected in replicates and filtered with 0.2 µM HT Tuffryn membrane (Pall Corporation, Ann Arbor, MI, USA). The sample was subjected to plaque assay (Lee et al., 2004). To perform a virus negative control during the exposure, everything was the same as positive control except that 5 clams were placed in the bucket instead of viruses. After 24 h exposure, clams were collected and subjected to MNV-1 detection by quantitative RT-PCR (qRT-PCR) assay. A total of 3 independent trials were performed.
Table 7

*MNV-1* bioaccumulation rates of *C. fluminea*

<table>
<thead>
<tr>
<th>Bucket</th>
<th>Sample ID</th>
<th>Content</th>
<th>Exposure period (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td># 1</td>
<td>5 clams + $10^5$ PFU MNV-1</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td># 2</td>
<td>5 clams + $10^5$ PFU MNV-1</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td># 3</td>
<td>5 clams + $10^5$ PFU MNV-1</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td># 4</td>
<td>5 clams + $10^3$ PFU MNV-1</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td># 5</td>
<td>5 clams + $10^5$ PFU MNV-1</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td># 6</td>
<td>Negative control</td>
<td>5 clams, w/o MNV-1</td>
<td>72</td>
</tr>
<tr>
<td># 7</td>
<td>Positive control</td>
<td>$10^3$ PFU MNV-1 w/o clams</td>
<td>24</td>
</tr>
</tbody>
</table>

*Temperature effect on MNV-1 bioaccumulation by clams.* The experimental design is showed in Table 8. Clams were acclimated to 20°C and 10°C, respectively. Ten clams were exposed to $10^7$ PFU of MNV-1 in 10 L of artificial freshwater at 20°C and 10°C, respectively. Triplets were done at each temperature. After 24 h exposure, 6 active clams were sampled from each bucket at each temperature. The clams were subjected to MNV-1 RNA extraction and detection. A total of 3 independent trials were performed.
Table 8

*Temperature effect on MNV-1 bioaccumulation by C. fluminea*

<table>
<thead>
<tr>
<th>Bucket</th>
<th>Exposure temperature (°C)</th>
<th>Sample ID</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td># 1</td>
<td></td>
<td>Experimental group</td>
<td>10 clams + 10⁷ PFU MNV-1</td>
</tr>
<tr>
<td># 2</td>
<td></td>
<td>Experimental group</td>
<td>10 clams + 10⁷ PFU MNV-1</td>
</tr>
<tr>
<td># 3</td>
<td>10</td>
<td>Negative control</td>
<td>10⁷ PFU MNV-1, w/o clams</td>
</tr>
<tr>
<td># 4</td>
<td>10</td>
<td>Positive control</td>
<td>5 clams, w/o MNV-1</td>
</tr>
<tr>
<td># 5</td>
<td>10</td>
<td>Positive control</td>
<td>10⁷ PFU MNV-1, w/o clams</td>
</tr>
<tr>
<td># 6</td>
<td></td>
<td>Experimental group</td>
<td>10 clams + 10⁷ PFU MNV-1</td>
</tr>
<tr>
<td># 7</td>
<td>20</td>
<td>Negative control</td>
<td>10 clams + 10⁷ PFU MNV-1</td>
</tr>
<tr>
<td># 8</td>
<td>20</td>
<td>Positive control</td>
<td>10 clams + 10⁷ PFU MNV-1</td>
</tr>
<tr>
<td># 9</td>
<td></td>
<td>Negative control</td>
<td>10 clams, w/o MNV-1</td>
</tr>
<tr>
<td># 10</td>
<td></td>
<td>Positive control</td>
<td>10⁷ PFU MNV-1, w/o clams</td>
</tr>
</tbody>
</table>

*MNV-1 bioaccumulation limit of clams.* Bioaccumulation limit is defined as the lowest virus concentration at which exposed clams are PCR-positive for virus detection. MNV-1 was spiked into 10 L of artificial freshwater to get a final viral concentration of 10¹, 10², 10³, and 10⁴ PFU·L⁻¹, respectively. Then the bioaccumulation commenced. The spiked water in each bucket was changed after 24 h exposure. Clams were collected from each bucket after 48 h exposure and subjected to MNV-1 RNA extraction and detection. A total of 3 independent trials were performed.

*MNV-1 depuration by clams.* The experimental design is showed in Table 9. Eighty clams were exposed to 1.6 x 10⁸ PFU of MNV-1 in 10 L of artificial freshwater at 20°C. After 1 d exposure, 40 exposed clams were collected and placed into 80 L of clean artificial freshwater for depuration at 20°C. Another 40 exposed clams were placed into
another 80 L of clean artificial freshwater at 15 °C for 8 h, and then transferred to a clean 80 L of artificial freshwater for depuration at 10 °C. The water in the depuration tanks at both 20 °C and 10 °C was changed every other day. Six clams at each temperature were collected after 0, 1, 4, 7, 10, and 15 d depuration, respectively. The collected clams were subjected to MNV-1 RNA extraction and detection. A total of 3 independent trials were performed.

Table 9

*MNV-1 depuration by C. fluminea*

<table>
<thead>
<tr>
<th>Content (in 10 L water)</th>
<th>Bioaccumulation temperature (°C)</th>
<th>Depuration temperature (°C)</th>
<th>Depuration volume (L)</th>
<th>Depuration period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 clams + 10^7 PFU MNV-1</td>
<td>20</td>
<td>20</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>80</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>80</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>80</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>80</td>
<td>15</td>
</tr>
</tbody>
</table>

0
**MNV-1 RNA isolation.** Clams from the laboratory study were rinsed with deionized water and digestive gland (DG) was obtained. Homogenization was done using a Precellys 24 homogenizer (Bertin Technologies, France). Zirconia beads (BioSpec Products, Inc., Bartlesville, OK, USA) at 1.0 mm and 2.0 mm in diameter were added to each sample, respectively. Homogenization was done at 6,000 rpm for 30 sec for a total of 3 times. One hundred µL of homogenate from each DG sample was subjected to total RNA isolation. An extra 100 µL of homogenate spiked with 1,000 PFU of MNV-1 stock was used as an RNA isolation positive control. Clams directly collected from Lake Serene were used for a negative control.

The viral RNA isolation method described herein is based on sequence hybridization technology using a biotinylated probe. Total RNA was first extracted from DG samples using TRI Reagent (Molecular Research Center, Inc., Cincinnati, Ohio) according to the manufacture’s protocol. The resulting total RNA pellet was first dissolved in 30 µL of DEPC-treated water, heated at 94°C for 5 min and then quick chilled on ice for 5 min. Afterwards, 1 µL of 1 µM biotinylated MNVKS2 probe (Table 10), 0.5 µL of RNasin Plus RNase Inhibitor (Promega Corp, Madison, WI), and 3.5 µL of 10X hybridization buffer (0.4 M NaCl, 40 mM HEPES, 1 mM EDTA, pH 6.6) were added to the RNA samples. To facilitate hybridization of MNV-1 RNA to the biotinylated probe, the samples were incubated at 45°C for 10 min with continuous agitation using a Thermomixer 5436 (Eppendorf North America, Inc., Westbury, NY) at 1,000 rpm·min⁻¹. To capture viral RNA, 5 µL of washed streptavidin-coated magnetic beads (Dynabeads MyOne™ Streptavidin C1, Life Technologies, Grand Island, NY) and 2.96 µL of DEPC-treated 5 M NaCl were added and the samples mixed continuously for
30 min at 25°C with agitation for one time in the 30 min. The magnetic beads were captured using a Magnetic Separation Stand (Promega Corp, Madison, WI), washed three times with 1x washing buffer containing 5 mM Tris-HCl (pH 7.5), 0.5 mM EDTA and 1 M NaCl. To elute the captured viral RNA, 10 µL of DEPC-treated water were added to each sample and heated at 94°C for 5 min. After brief centrifugation, the magnetic beads were captured again using the Magnetic Separation Stand, and then the supernatant containing purified viral RNA was transferred to a new tube for subsequent analysis by real-time RT-PCR.

*TaqMan* real time RT-PCR assays for MNV-1 detection. MNV-1 RNA was reverse transcribed using gene specific primer MNVKS2 and the ImProm-II Reverse Transcriptase according to the manufacturer’s procedures. Briefly, 5.75 µL of purified MNV-1 RNA and 0.5 µL of primer MNVKS2 at concentration of 20 µM were used in a total reaction volume of 10 µL. Reverse transcription positive control and negative control were performed in each run of reverse transcription. The PCR reaction mixture contained 2 µL of the reverse transcription products, 500 nM of each of the primers (MNVKS1 and MNVKS2) and 250 nM of the TaqMan probe (MNVKS3), 0.5 U of EconoTaq DNA Polymerase (Lucigen Corporation, Middleton, WI, USA), 200 µM of each of the deoxynucleoside triphosphates (dNTP), and nuclease-free water for a total reaction mixture of 10 µL. cDNA from 10-fold serial dilutions of MNV-1 stock was used to generate the standard curve. The reaction mixture was subjected to a real time PCR TaqMan assay on the Rotor-Gene 6000 real time DNA amplification system (Corbett Research, New South Wales, Australia). The following conditions were used for MNV-1 amplification: a single cDNA melting step at 95°C for 15 min followed by 40 cycles of
95°C for 15 sec to melt DNA, 55°C for 60 sec to anneal primers, and 72°C for 30 sec to extend primers. All primers and probes are listed in Table 10.

Table 10

*Oligonucleotide primer and probe sequences for MNV-1 detection*

<table>
<thead>
<tr>
<th>Primer/probe</th>
<th>Sequence (5′–3′)</th>
<th>PCR amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotinylated</td>
<td>BIO&lt;sup&gt;a&lt;/sup&gt;-CCAAGCTCTCACAAGCCTTC</td>
<td></td>
<td>The study</td>
</tr>
<tr>
<td>MNVKS2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MNVKS1</td>
<td>AGGTCATGCGAGATCAGCTT</td>
<td>159</td>
<td>Bae et al. 2008</td>
</tr>
<tr>
<td>MNVKS2</td>
<td>CCAAGCTCTCACAAGCCTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MNVKS3</td>
<td>FAM&lt;sup&gt;b&lt;/sup&gt;-CAGTCTGCGACGCATTGAGAA-BHQ&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note.<sup>a</sup> BIO, biotin;<sup>b</sup> FAM, fluorescein;<sup>c</sup> BHQ, Black Hole Quencher.

*Statistical analysis.* Differences in the temperature effects on bioaccumulation rates and depuration rates of *C. fluminea* were evaluated by one-way ANOVA (SPSS version 13.0). Post hoc comparisons were done by using the Tukey HSD test. Significance was declared at p < 0.05.

**Results**

*MNV-1 bioaccumulation by Corbicula fluminea.* The TaqMan assay results showed that the spiked MNV-1 could be detected in all exposed clams, which indicates that *C. fluminea* can bioaccumulate MNV-1 when the MNV-1 concentration is 10<sup>4</sup> PFU·L<sup>−1</sup> (10<sup>5</sup> PFU per 10 L) at 20°C. In addition, based on plaque assay results, 80% of spiked MNV-1 was still available in the positive control bucket (which contained MNV-1 and water, but no clams) after 24 h, which means that 80% of spiked viruses were available in the water for MNV-1 bioaccumulation by clams. No viruses were detectable for negative control
bucket (contained clams and water, but no viruses), which means that no MNV-1 existed in the artificial pond water, bucket, and clams collected from Lake Serene.

*MNV-1 bioaccumulation rates of clams.* To determine MNV-1 bioaccumulation rates by clams, clams were exposed for different time periods (0.5, 6, 24, 48, and 72 h) at two different MNV-1 concentrations in 10 L of water. The TaqMan assay results showed that MNV-1 was detectable after 6 h and 24 h exposure in clams exposed to virus concentrations of $10^6$ PFU·L$^{-1}$ and $10^4$ PFU·L$^{-1}$, respectively. The amount of bioaccumulated MNV-1 increased as the exposure period increased from 6 h to 72 h (Fig. 11).

![Figure 11. MNV-1 bioaccumulation rates of clams. Error bars: standard deviation.](image)

*Temperature effect on MNV-1 bioaccumulation by clams.* To compare the temperature effects on MNV-1 bioaccumulation rates by *C. fluminea*, two different temperatures ($10^\circ$C and $20^\circ$C) for MNV-1 bioaccumulation by *C. fluminea* were tested. Clams
bioaccumulated MNV-1 significantly more quickly at 20°C than at 10°C (Fig. 12, p < 0.05).

Figure 12. Temperature effect on MNV-1 bioaccumulation by clams. Error bars: standard deviation.

Bioaccumulation limit of clams. To determine the lowest virus concentration at which exposed clams were PCR-positive, clams were placed at different concentrations of MNV-1 in water (10<sup>1</sup>, 10<sup>2</sup>, 10<sup>3</sup>, 10<sup>4</sup> PFU·L<sup>-1</sup>). The TaqMan assay showed that bioaccumulated MNV-1 was detected when MNV-1 was spiked at the concentration of 10<sup>2</sup>, 10<sup>3</sup>, and 10<sup>4</sup> PFU·L<sup>-1</sup>, but not at the lowest concentration (10<sup>1</sup> PFU·L<sup>-1</sup>) (Fig. 13). In conclusion, the lowest virus concentration at which exposed clams were PCR-positive was 10<sup>2</sup> PFU·L<sup>-1</sup> after 48 h exposure at 20°C.
Figure 13. Bioaccumulation limit of clams. Error bars: standard deviation. ND: not detectable.

*MNV-1 depuration by clams.* To determine MNV-1 depuration rate and the temperature effects on the depuration rate of clams, depuration experiments were performed in environmental chambers. The virus was persistently detected in contaminated clams during 0, 1, 4, 7, 10, and 15 d depuration at 20°C. The same results were observed for depuration at 10°C. However, depuration occurred significantly more quickly at 20°C than at 10°C (p < 0.05) (Fig. 14).

*Figure 14.* MNV-1 depuration by clams. Error bars: standard deviation.
Discussion

This study was the first to evaluate freshwater clam (*C. fluminea*) as a sentinel of HuNoV contamination in freshwater by using MNV-1 as a surrogate of HuNoV. I compared the rates at which clam bioaccumulated MNV-1 at two viral concentrations; and temperature effects on the rates of MNV-1 bioaccumulation and depuration by clams. These two temperatures, 10°C and 20°C, represent the winter as well as early summer conditions of Mississippi, respectively.

Although bivalves like clams can harbor environmental-derived human pathogenic bacteria and viruses as a result of concentrating pathogens from the surrounding water (Ayres et al., 1978; Trollope, 1984), and HuNoV was detected in freshwater *C. fluminea* (Saitoh et al., 2007), the question of whether clams can actually accumulate HuNoV has not been answered. The present study demonstrated that freshwater *C. fluminea* bioaccumulated MNV-1 and the bioaccumulated MNV-1 in the exposed clams can be detectable as soon as after 0.5 h exposure. Further experiments showed that the higher concentration of virus in the water, the shorter exposure period for the bioaccumulated MNV-1 to become detectable in clams, and that clams bioaccumulated MNV-1 at both 10°C and 20°C. These results indicate that clams could be used as a sentinel of MNV-1 contamination in freshwater at both warm and cold temperatures. However, the bioaccumulation rate was significant slower at 10°C than at 20°C, indicating that clams could be a better sentinel at 20°C than at 10°C. The reason for the high bioaccumulation rate at warm temperature may be that the optimal temperature for *C. fluminea* to be active is 14-22°C (Fraysse et al., 2000).
The experiments of bioaccumulation limit showed that the lowest MNV-1 concentration at which exposed clams were PCR-positive was as low as $10^2 \text{ PFU}\cdot\text{L}^{-1}$ after 48 h exposure. Because the 10 L of spiked water was changed 24 h exposure, so the total water volume that clams filtered was 20 L after 48 h exposure. Considering of unlimited natural water in natural creeks, the bioaccumulation limit of clams in creeks should be lower than the limit obtained from the lab exposure study, which makes the clams an even more sensitive sentinel of virus contamination in freshwater creeks than that in the laboratory exposure study.

Bioaccumulation is a process allowing clams to pick up particle matters and "self-purge" in a controlled aquatic environment. The machnism of virus bioaccumulation by clams has been studied. Tian et al., (2007) showed that multiple histo-blood group antigens (HBGA) are expressed on gastrointerstinal tissues of clams, mussels, and oysters. NoVs can bind to the HBGA, which could be the major mechanism of bioaccumulation of NoVs by these bivalves, and which could also indicate that conventional depuration cannot eliminate NoV from clam tissues. Le Guyader et al., (2006) also showed that viral particles bind specifically to oyster digestive ducts (midgut, main and secondary ducts, and tubules) by carbohydrate structures with a terminal N-acetylgalactosamine residue in an $\alpha$ linkage (same binding site used for recognition of human HBGA).

Depuration is part of the normal filter-feeding activities of clams. Depuration is a clam sanitation process that can occur in controlled land-based tanks, and usually contains recirculating or flow-through water (Richards, 1988). Studies have showed that depuration is not an effective method to completely eliminate viruses from shellfish (Hay et al., 1986; Hernroth et al., 2006; Le Guyader et al., 2006; Schwab et al., 1998; Ueki et
al., 2007). The depuration experiments showed that the bioaccumulated MNV-1 was still
detectable after 15 d depuration at both temperatures (10°C than at 20°C), although
depuration rate is significant faster at 20°C than at 10°C. The data suggested that if clams
are used to determine virus contamination in natural creek waters, then contaminated
clam field transplantation might be a useful strategy. Clam translocation means clams
from a site free of virus contamination can be transplanted to freshwater creeks for
determining of potential virus contamination in the water. One advantage of field
transplantation is that the virus contamination can be determined for freshwater creeks
where no clam population exists initially.

Acknowledgments

I thank Dr. Herbert W. Virgin IV at Washington University for MNV-1 stock.
The research was financially supported by EPA Gulf of Mexico Program - Project
MX96429505-0 and the Mississippi Coastal Impact Assistance Program/U.S. Dept of the
Interior.
References


CHAPTER V

VALIDATION OF THE CLAM CORBICULA FLUMINEA AS A SENTINEL OF
HUMAN NOROVIRUS CONTAMINATION IN FRESHWATER CREEKS

Human viruses generally occur at low concentrations in environmental waters. A major challenge with current virus-monitoring methods is the critical first step of concentrating viruses from water. The process is time consuming, labor intensive, expensive, and often results in poor recovery of viruses. Earlier studies showed that the filter-feeding clam C. fluminea was able to concentrate murine norovirus from artificially contaminated water in the laboratory and thus may provide a simple and inexpensive method to concentrate norovirus in the field. The purpose of the present study was to validate whether C. fluminea can be used as a sentinel of Human Norovirus (HuNoV) contamination in natural freshwater. Clams were collected from a lake (Lake Serene) in which HuNoV has never been detected in Hattiesburg, Mississippi from Oct 2010 to Jul 2011, and translocated to 9 sites at 4 freshwater creeks flowing into the Mississippi Sound. HuNoV RNA was isolated from clams (n = 588) using a biotinylated probe hybridization method and detected by qRT-PCR. Amplified products (HuNoV capsid N-terminal/shell domain) were sequenced bi-directionally. qRT-PCR results showed that HuNoV GI and GII were detectable in the translocated C. fluminea mainly during the warmer months (Apr to Jul and Oct), but not during the colder months (Dec to Mar). Based on our phylogenetic analysis, the HuNoV genome detected in translocated clams was classified into GI/17 and GII/4, respectively. Statistical analysis using binary logistic regression showed that water temperature and turbidity (p = 0.026 and p = 0.038, respectively), but not the pH, salinity, or current velocity, were significant factors
affecting HuNoV presence/absence in clams. In conclusion, the freshwater clam *C. fluminea* can serve as an effective sentinel of HuNoV contamination in freshwater of low turbidity during warm months with clean *Corbicula* translocation strategy.

Introduction

Beach water could be contaminated from various sources. The most frequent source is polluted creeks and sewage outfalls feeding into beach water, because current water treatment practices are unable to provide virus-free wastewater effluents, and thus pathogenic viruses are routinely introduced into beach water. Others sources of coastal water contamination include dumping of wastes at sea; and the exploration and exploitation of the sea bed and ocean floor, etc. Among the numerous noxious pollutants that are discharged into beach water, human enteric viruses, such as rotavirus, norovirus, and enterovirus, may represent a major source of public health and economic problems (Sorber, 1983).

Human noroviruses (HuNoV) are a group of viruses that cause the “stomach flu” or gastroenteritis in people of all age groups. They are the most common cause of viral gastroenteritis worldwide and are routinely implicated in waterborne outbreaks (WHO 2003; Kageyama et al. 2004; Nygard et al. 2004; Yoder et al. 2008). They are especially virulent in the elderly, as evidenced by recent reports of 19 deaths associated with HuNoV acute gastroenteritis in 2006 in long-term care facilities in the United States (Estes et al., 2006; Koopmans et al., 2004; Hutson et al., 2000; MMWR, 2007). These viruses are members of the Caliciviridae family. They are non-enveloped viruses, 27 to 35 nm in diameter with icosahedral symmetry, and possess a single-stranded, positive-sense RNA genome of 7.5 to 7.7 kb (Atmar and Estes, 2001).
Detection of HuNoV present in creek waters flowing into beach water can predict the HuNoV contamination of the beach water, and provide valuable information about safety of beach water. However, direct monitoring of HuNoV in beach waters can be difficult. Direct pathogen detection in water involves two steps: the concentration of the virus from waters, and the detection and identification of the recovered virus. A major challenge with all current virus-monitoring methods is the critical first step of concentration of the viruses from large bodies of waters (with poor recoveries), because viruses are always shed in low numbers in waters.

On the other hand, however, freshwater clams *Corbicula fluminea* are filter-feeders, and was proved to be a useful sentinel of HuNoV contamination in artificial freshwater in the previous laboratory study using MNV-1 as a surrogate of HuNoV. The objective of this study was to validate clam as a sentinel of HuNoV contamination in freshwater creeks. The aim was twofold: 1) to detect and genotype HuNoV in freshwater creeks flowing into Mississippi Sound using *Corbicula fluminea* as a sentinel; 2) to determine the relationship of environmental factors and the HuNoV presence/absence in clams in the freshwater creeks.

**Materials and Methods**

_Creek sites._ Four freshwater creeks (Fig. 15 and 16; Table 11), each contains 2 sampling sites except for the Coffee Creek which has 3 sites, were chosen as clam translocation sites based on a contamination history of Human Polyomaviruses (a study in our lab from May 2009 to Aug 2010), and human specific fecal markers Methanobrevibacter smithii and Bacteroidales (Flood et al., 2011), and the fact that these creeks are freshwater tributary that feed into the Mississippi gulf coast, have some sewer and some septic tank
areas, and have high human and animal population. Nine creek sites (Trautman Ave., 7A.CC, Nicholson Ave. S, Nicholson Ave. N, CC0, CC1, CC2, Condo, and AOC) (Fig. 15 and 16; Table 11) at these 4 creeks were tested for HuNoV contamination by using clams as a sentinel.

Figure 15. Clam translocation sites along Mississippi Sound. Google 2011. Round dots represent creek sites from left to right: Trautman Ave. creek, 7A.CC, Nicholson Ave. S, and Nicholson Ave. N.
Figure 16. Clam translocation sites along Mississippi Sound. Google 2011. Round dots represent creek sites from left to right: CC0, CC1, CC2, Condo, and AOC.
Table 11

*GPS coordinates of clam translocation sites along Mississippi Sound*

<table>
<thead>
<tr>
<th>Creek location</th>
<th>Creek name</th>
<th>Creek site</th>
<th>GPS Coordinate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long Beach, MS</td>
<td>Trautman</td>
<td>7A.CC</td>
<td>N30°20.530'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trautman Ave.</td>
<td>W089°09.633'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N30°20.830'</td>
</tr>
<tr>
<td>Nicholson Ave.</td>
<td></td>
<td>NCS (Nicholson Ave. S)</td>
<td>W089°09.442'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCN (Nicholson Ave. N)</td>
<td>N30°20.936'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N30°21.254'</td>
</tr>
<tr>
<td>Gulfport, MS</td>
<td>Coffee</td>
<td>CC0 (Coffee Creek 0)</td>
<td>30.384815</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-89.060787</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CC2 (Coffee Creek 2)</td>
<td>N30°22.682'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W089°03.308'</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CC1 (Coffee Creek 1)</td>
<td>N30°22.776'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W089°03.379'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anniston Oak</td>
<td>AOC (Anniston Oak Ave. Creek)</td>
<td>N30°23.246'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Condo</td>
<td>W089°01.138'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N30°22.963'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>W089°01.539'</td>
</tr>
</tbody>
</table>
Translocation of Corbicula fluminea. Clams (n = 588) were translocated to these 9 creek sites at 4 creeks. In each field trial, clams (n = 10) were placed in a mesh grid at each creek site and left undisturbed to filter the water. Metal sticks (about 1 meter) were used to anchor mesh grids and marked locations of the clams in creeks. A minimum of 6 exposed clams were collected at each sampling site after 2 d or 5 d exposure and transported to the lab on ice for HuNoV detection.

**HuNoV GI and GII RNA isolation.** For each sampling site, 100 µL of homogenate from each DG sample was used for total RNA extraction for determining the presence of HuNoV GI and GII. A second 100 µL of homogenate from the combination of three DG samples from the creek site was spiked with 1,000 PDU of a HuNoV GII positive clinical sample. The spiked sample was used as a positive control of RNA extraction and RT-PCR. A third 100 µL of homogenate from clean clams collected from Lake Serene was used as a negative control. RNA was extracted from both unknown and control samples using biotinylated probe hybridization method as described above in the development of HuNoV RNA extraction method in chapter III. However, HuNoV RNA of both GI and GII were purified from total RNA at the same time in a single extraction. Briefly, 29 µL instead of 30 µL of total RNA was used, and 1 µL of biotinylated COGIR (Table 12) at concentration of 1µM was also added to the previously described HuNoV GII RNA purification reaction in chapter III. The resulting purified HuNoV RNA was released from bead complex by adding 20 µL of DEPC treated water followed by heating at 94°C for 5 min. The resulting purified HuNoV RNA was stored at -20°C (< 3 weeks) or -80°C for long term storage.
TaqMan real time RT-PCR assays for HuNoV GI and GII detection. Viral RNA was reverse transcribed using gene specific primers and the ImProm-II Reverse Transcriptase according to the manufacturer’s procedures. Briefly, 5.75 µL of purified HuNoV RNA and 0.5 µL of primer mixture (COG1R and COG2R at concentration of 20 µM each) were added in a total reaction volume of 10 µL. The PCR reaction mixture contained 2 µL of the reverse transcription products, 400 nM of each of the HuNoV primer set (COG1F and COG1R for GI or JJV2F and COG2R for GII), 300 nM RING1a and 100 nM RING1b probes for HuNoV GI or 200 nM RING2-TP probe for HuNoV GII, 0.5 U of EconoTaq DNA Polymerase (Lucigen Corporation, Middleton, WI, USA), 200 µM of each of the deoxynucleoside triphosphates (dNTP), and nuclease-free water for a total reaction mixture of 10 µL. Plasmid DNA containing cloned HuNoV GI cDNA or GII cDNA was used as standards for HuNoV amplification. The reaction mixture was subjected to a PCR assay on the Rotor-Gene 6000 real time DNA amplification system (Corbett Research, New South Wales, Australia). The following conditions were used for amplification of HuNoV GI and GII: a single cDNA melting step at 95°C for 10 min followed by 45 cycles of 95°C for 15 sec to melt DNA and 60°C for 60 sec to anneal and extend primers. All primers and probes were listed in Table 12. PCR were done in replicates for each DG homogenate sample. So, a total of 6 PCR reactions were performed for 3 DG homogenate samples in each creek site in each month.

Genotyping of HuNoV. To further determine the genotypes of HuNoV in contaminated clams from these creeks, conventional RT-PCR was performed for all HuNoV GI or GII real time PCR positive samples. In brief, purified viral RNA was amplified by conventional RT-PCR assay using an OneStep RT-PCR kit (QIAGEN, CA, USA). Ten
µL of viral RNA was amplified with 0.4 µM of each oligonucleotide primer (G1SKF and G1SKR for GI, G2SKF and G2SKR for GII) in a final reaction volume of 25 µL. After 30 min of RT at 42°C, followed by heat activation of Taq polymerase for 15 min at 95°C, PCR consisting of 40 cycles at 94°C for 30 sec, 50°C for 30 sec, and 72°C for 60 sec followed by a final extension for 5 min at 72°C was performed. The PCR product was part of the capsid N-terminal/shell (N/S) domain of HuNoV. All conventional RT-PCR products of the expected size (330 bp for GI, 344 bp for GII) were purified using a Zymoclean™ Gel DNA Recovery kit (Zymo Research, CA, USA) according to the manufacturers’ protocols, and bi-directionally sequenced by Operon.com. Primers and PCR products were in separate tubes. Briefly, 10 µL of primer (G1SKF, G1SKR, G2SKF or G2SKR) at concentration of 2 µM and 10 µL of purified PCR products at concentration of 30 ng·µL⁻¹ in replicates (for bi-directional sequencing) were made. All samples were shipped overnight to the sequencing company at room temperature. Sequences of all primers and probes were listed in Table 12.
Table 12

Oligonucleotide primer and probe sequences for HuNoV detection and genotyping

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Primer/probe</th>
<th>Sequence (5′–3′)</th>
<th>Position</th>
<th>PCR amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HuNoV GI detection</td>
<td>Biotinylated COGIR</td>
<td>BIO(^d)CTTAGACGCCATCATCATTYAC</td>
<td>5375-5358(^b)</td>
<td></td>
<td>The study</td>
</tr>
<tr>
<td></td>
<td>COGIF</td>
<td>CGYTGATGCGNTTYCATGA</td>
<td>5291-5310(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>COGIR</td>
<td>CTTAGACGCCATCATCATTYAC</td>
<td>5375-5358(^b)</td>
<td>85</td>
<td>Kageyama et al. 2003</td>
</tr>
<tr>
<td></td>
<td>RING1a</td>
<td>FAM(^d)-AGATGCGATCYCCTGTCCA-BHQ(^f)</td>
<td>5340-5359(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RING1b</td>
<td>FAM(^d)-AGATGCGGTCTCCTGTCCA-BHQ(^f)</td>
<td>5340-5321(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Biotinylated COG2R</td>
<td>BIO(^d)TCGACGCCATCTTCATTCA</td>
<td>5100-5080(^b)</td>
<td></td>
<td>The study</td>
</tr>
<tr>
<td></td>
<td>JLV2F</td>
<td>CAAGAGTCAATGTAGTTAGTGATGAG</td>
<td>5003-5028(^b)</td>
<td>98</td>
<td>Jothikumar et al. 2005</td>
</tr>
<tr>
<td></td>
<td>COG2R</td>
<td>TCGACGCCATCTTCATTCA</td>
<td>5100-5080(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RING2-TP</td>
<td>FAM(^d)-TGGGAGGGCGATCGCAATCT-BHQ(^f)</td>
<td>5048-5067(^f)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G1SKF</td>
<td>CTGCCCGAATYTGAATGTA</td>
<td>5342-5361(^b)</td>
<td>330</td>
<td>Kojima et al. 2002</td>
</tr>
<tr>
<td></td>
<td>G1SKR</td>
<td>CCAACCCARCCATTTRTACA</td>
<td>5671-5653(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G2SKF</td>
<td>CNTGGGAGGGCGATCGCAA</td>
<td>5058-5076(^b)</td>
<td>344</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G2SKR</td>
<td>CCRCCNGCATHCCRTTRTACAT</td>
<td>5401-5389(^b)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note. \(^a\) N = A/T/G/C; \(^b\) R = A/G; \(^c\) Y = C/T; \(^d\) H = A/C/T; \(^e\) Nucleotide positions based on Norwalk virus (Genbank accession no. M87661); \(^f\) Nucleotide positions based on Lordsdale virus (Genbank accession no. X86557); \(^g\) BIO, biotin; \(^h\) FAM, fluorescein; \(^i\) BHQ, Black Hole Quencher.

*Phylogenetic analysis.* The HuNoV capsid sequences (237 to 340 nt) of the unknown strains from the field translocated clams were aligned with genomes of the reference strains from GenBank. The names and the accession numbers of these stains are provided.
in the legend for Fig. 19. All sequences were converted to FASTA format in NCBI and saved as TEXT files. The sequencing alignment was done by using ClustalW of MEGA 5.05 software. A phylogenetic tree was constructed using the neighbor-joining (N-J) technique and P-distance method using MEGA 5.05 software, and the bootstrap cycles were set at 1,000.

*Comparison of sensitivity between real time RT-PCR and conventional RT-PCR for HuNoV detection.* One hundred µL of clam homogenate without HuNoV contamination was spiked with 10 µL of each 10-fold serial dilution of HuNoV GII positive stool extract (10^0 to 10^3). Viral RNA was extracted from the spiked samples using biotinylated probe hybridization method as described previously in the study. The resulting purified viral RNA was eluted in 20 µL of DEPC-treated water. The same RNA template was used for both real time RT-PCR and conventional RT-PCR as described previously in the study. The resulting products from conventional RT-PCR were subjected to agarose gel electrophoresis.

*Relationship between environmental factors and the HuNoV presence/absence in clams.* Five environmental factors including temperature (°C), pH, salinity (ppt, parts per thousand), turbidity (ntu, nephelometric turbidity unit), and current velocity (m·S⁻¹, meters per second) in creek waters were measured in the translocation experiments. Each factor was measured twice in each field trial. The first measurement of each factor was recorded when clams were placed in the creeks, and the second measurement of each factor was recorded when exposed clams were collected from the creeks. Average numbers of each factor were calculated and used for statistical analysis. A binary logistic regression (BLR) (SPSS version 20) analysis was performed to determine if the
environmental factors had effects on the HuNoV presence/absence in clams. BLR is the technique most commonly used to model such a binary (i.e., presence/absence) response. The presence/absence of HuNoV in clams was treated as the dependent variable (i.e., a binary variable). When HuNoV was present, it was assigned the value 1. When HuNoV was absent, it was assigned the value 0. Relationships were considered significant when the p value for the model chi square was < 0.05.

Results

_HuNoV detection in translocated clams._ The TaqMan RT-PCR results about HuNoV detection in creek waters by using clams as a sentinel of HuNoV contamination is shown in Table 13. No HuNoV was detected in the clean clams from Lake Serene (Data not shown). HuNoV were detectable in the translocated clams mainly during the warmer months (Apr to Jul and Oct) in almost every creek site, but not during the colder months (Dec to Mar). HuNoV were mainly detectable in the CC1 (14 out of 59) and CC2 (13 out of 59). And creek sites CC1 and CC2 were contaminated with both GI and GII simultaneously in Apr 2011. GII were detectable more frequently than GI. Of the 294 extracts, 24 (8.0%) and 35 (11.7%) were positive for GI and GII, respectively, in the 10 month study (Oct 2010 to Jul 2011).
Table 13

*HuNoV GI and GII detection in 294 extracts in 9 creek sites using TaqMan RT-PCR*

<table>
<thead>
<tr>
<th>Exposure periods</th>
<th>No. of positive samples (GI, GII)</th>
<th>Total No. of positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7A.CC</td>
<td>Trautman</td>
</tr>
<tr>
<td>Oct 21 - 26, 2010</td>
<td>0, 2</td>
<td>0, 3</td>
</tr>
<tr>
<td>Oct 26 - 28, 2010</td>
<td>0, 1</td>
<td>0, 1</td>
</tr>
<tr>
<td>Nov 9 - 11, 2010</td>
<td>0, 0</td>
<td>0, 1</td>
</tr>
<tr>
<td>Nov 11 - 16, 2010</td>
<td>ND, 0</td>
<td>ND, 0</td>
</tr>
<tr>
<td>Dec 9 - 12, 2010</td>
<td>0, 0</td>
<td>0, 0</td>
</tr>
<tr>
<td>Jan 11 - 13, 2011</td>
<td>0, 0</td>
<td>0, 0</td>
</tr>
<tr>
<td>Feb 16 - 18, 2011</td>
<td>0, 0</td>
<td>0, 0</td>
</tr>
<tr>
<td>Mar 28 - 30, 2011</td>
<td>0, 0</td>
<td>0, 1</td>
</tr>
<tr>
<td>Apr 19 - 21, 2011</td>
<td>0, 0</td>
<td>0, 0</td>
</tr>
<tr>
<td>May 17 - 19, 2011</td>
<td>0, 0</td>
<td>0, 0</td>
</tr>
<tr>
<td>Jun 14 - 16, 2011</td>
<td>0, 0</td>
<td>0, 1</td>
</tr>
<tr>
<td>Jul 12 - 14, 2011</td>
<td>1, 0</td>
<td>0, 0</td>
</tr>
<tr>
<td>Total No. of positive samples</td>
<td>GI, GII</td>
<td>1, 3</td>
</tr>
<tr>
<td></td>
<td>GI + GII</td>
<td>4</td>
</tr>
</tbody>
</table>

Note. “ND”: Not determined

*Genotyping of HuNoV in translocated clams.* Results from agarose gel electrophoresis showed that 8 out of 59 qRT-PCR positive samples produced bands: 330 bp for HuNoV GI and 344 bp for HuNoV GII (Fig. 17 and 18, respectively). Of the 8 samples, 2 and 6 were positive for HuNoV GI (Fig. 17) and HuNoV GII (Fig. 18), respectively. These 2 GI-positive samples were collected in CC2 in Apr and May 2011, respectively. These 6 GII-positive samples were collected in Trautman Ave. creek, AOC, and Condo creek in Oct 2010.
Figure 17. Conventional RT-PCR amplification of HuNoV GI in field translocated clams. Expected PCR amplicon is 330 bp. Lane M: 100 bp DNA ladder; Lane 1: clam sample from CC2 in Apr 2011; Lane 2: clam sample from CC2 in May 2011; Lane 3: RNase-free water substituted for clam extract (negative control).

Figure 18. Conventional RT-PCR amplification of HuNoV GII in field translocated clams collected in Oct 2010. Expected PCR amplicon is 344 bp. Lane M: 100 bp DNA ladder; Lanes 1-2: clam samples from Trautman Ave. creek; Lanes 3-4: clam samples from AOC; Lanes 5-7: clam samples from Condo creek; Lane 8: RNase-free water substituted for clam extract (negative control).

Based on the phylogenetic analysis, HuNoV detected in the clam samples was classified into genogroup I genotype 17 (GI/17) and genogroup II genotype 4 (GII/4). Specifically, the one from CC2 in Apr 2011 was classified as GI/17, and the one from CC2 in May 2011 failed to sequencing reactions because of not enough PCR product. All the 6 amplicons from Oct 2010 were classified as GII/4 (Fig. 19).
Figure 19. Phylogenetic tree constructed on the basis of the partial sequences of the HuNoV capsid gene. The distance was calculated using P-distance method, and the tree was plotted using the neighbor-joining (N-J) method. The numbers at each branch indicate the bootstrap values for the clusters supported by that branch. An outgroup virus (sapovirus strain Manchester) was used. GI: genogroup I; GII: genogroup II. The GenBank accession numbers of the reference strains are in the brackets followed by each strain name in the figure.

Comparison of sensitivity between real time RT-PCR and conventional RT-PCR. Previous results showed that 59 out of 294 RNA extracts were contaminated with either HuNoV GI or GII (Table 13) based on real time RT-PCR; however, only 8 out of the 59 positive samples (Fig. 17 and 18) were positive based on conventional RT-PCR. Thus, the proposed reason was that real time RT-PCR was more sensitive than conventional RT-PCR for HuNoV detection in clams. The hypothesis was tested by clam tissue spiking experiments. The results showed that spiked HuNoV GII can be detected at all dilution
levels (10^0 to 10^{-3}) of HuNoV GII stool extract when real time RT-PCR was used for viral detection (Table 14). However, spiked HuNoV GII can only be detected at the dilution levels of 10^0 and 10^{-1} (Lanes 1, 2, 3, and 4 in Fig. 20), but not the dilutions of 10^{-2} and 10^{-3} (Lanes 5, 6, 7, and 8 in Fig. 20) when conventional RT-PCR was performed. In summary, real time RT-PCR was 100 fold more sensitive than conventional RT-PCR for HuNoV detection in clam samples.

Table 14

*Real time RT-PCR results of HuNoV GII detection in spiked clam homogenates*

<table>
<thead>
<tr>
<th>No.</th>
<th>HuNoV amounts</th>
<th>C_T values (mean)^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Stock of HuNoV GII stool extract (1,000 PDU)</td>
<td>25.3 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>1:10 dilution of HuNoV GII stool extract (100 PDU)</td>
<td>28.7 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>1:100 dilution of HuNoV GII stool extract (10 PDU)</td>
<td>31.6 ± 0.3</td>
</tr>
<tr>
<td>4</td>
<td>1:1,000 dilution of HuNoV GII stool extract (1 PDU)</td>
<td>34.6 ± 0.3</td>
</tr>
<tr>
<td>5</td>
<td>Negative control of RNA isolation</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>Positive control of RNA isolation</td>
<td>23.5 ± 0.1</td>
</tr>
<tr>
<td>7</td>
<td>Negative control of reverse transcription</td>
<td>None</td>
</tr>
<tr>
<td>8</td>
<td>Positive control of reverse transcription</td>
<td>19.5 ± 0.3</td>
</tr>
</tbody>
</table>

Note: ^a N=4; C_T values were based on 100 µL of clean clam homogenates were spiked with 10 µL of each dilution of HuNoV GII stool extract (10^0 - 10^{-3}).
**Figure 20.** Conventional RT-PCR amplification of HuNoV GII in 100 µL of clean clam homogenates spiked with 10 µL of each 10-fold serial dilution of HuNoV GII positive stool extract. Expected PCR product was 344 bp. Lane M: 100 bp DNA ladder; Lanes 1-2: Clam homogenates spiked with 10^0 dilution of HuNoV GII positive stool extract, replicates A and B, respectively; Lanes 3-4: Clam homogenates spiked with 10^1 dilution of HuNoV GII positive stool extract, replicates A and B, respectively; Lanes 5-6: Clam homogenates spiked with 10^2 dilution of HuNoV GII positive stool extract, replicates A and B, respectively; Lanes 7-8: Clam homogenates spiked with 10^3 dilution of HuNoV GII positive stool extract, replicates A and B, respectively; Lane 9: Nuclease free water (negative control of viral RNA isolation); Lane 10: HuNoV GII positive stool extract (positive control of viral RNA isolation); Lane 11: Nuclease free water (negative control of conventional RT-PCR); Lane 12: RNA directly extracted from the stool extract (positive control of conventional RT-PCR).

**Relationship between environmental factors and the HuNoV presence/absence in clams.**

Statistical analysis using binary logistic regression showed that water temperature and turbidity (p = 0.026 and p = 0.038 in Fig. 21 and 22, respectively), but not the pH, salinity, or current velocity (p = 0.476, p = 0.425, p = 0.174 in Fig. 23, 24, and 25, respectively), were significant factors affecting HuNoV presence/absence in clams. In Fig. 21 to 25, "0 and 1" in the Y axes represented HuNoV was not detected and detected in clams using real time RT-PCR, respectively. The logistic curve in Fig. 21 showed that the likelihood of HuNoV presence in clams increased as the water temperature increased, indicating that clam sentinel was more sensitive during warm months than cold months for HuNoV detection in freshwater. The logistic curve in Fig. 22 showed that the likelihood of HuNoV presence in clams decreased as the turbidity increased, indicating that clam sentinel was more sensitive in low turbidity than high turbidity for HuNoV detection in freshwater creeks. The statistical results also showed that interaction among
each environmental factors had no significant effect on the HuNoV bioaccumulation by clams (p = 0.087).

**Figure 21.** Relationship between temperature and the HuNoV presence/absence in clams.

**Figure 22.** Relationship between turbidity and the HuNoV presence/absence in clams.
Figure 23. Relationship between pH and the HuNoV presence/absence in clams.

Figure 24. Relationship between salinity and the HuNoV presence/absence in clams.
Figure 25. Relationship between water velocity and the HuNoV presence/absence in clams.

Discussion

Maintenance of beach water safety is important, as contamination of the water can
effect high risks to human health as well as resulted in significant economic losses due to
beach closures and shellfish harvesting areas. Beaches such as Myrtle Beach, S.C., and
Doheny State Beach in Orange County, Calif., two of the more commonly contaminated
beaches, were closed 54 d and 312 d, respectively, in 2004. Mississippi Gulf Coast
Beaches including Hancock County, Harrison County, and Jackson County were closed

Freshwater clams *C. fluminea* were validated to be able to bioaccumulate HuNoV
from freshwater creeks. Clams are preferential filter feeders rather than detritus feeders
(McMahon et al., 1991). The presence of HuNoV in the clam tissue would be indicative
of water contamination rather than sediment contamination. The present study showed
that HuNoV GI and GII were detectable in the translocated C. fluminea mainly during the warmer months (Apr to Jul and Oct), but not during the colder months (Dec to Mar). The data indicates HuNoV contamination of the creeks where clams were translocated, and it was mainly the CC1 (14 out of 59) and CC2 (13 out of 59) but not the clams from the rest 7 creek sites in Mississippi. Sequencing data showed that two HuNoV genotypes in seven clam samples from creeks were detected. One sample from CC2 was demonstrated to be contaminated with GI.17, and the majority of the samples (6 out of 7) were contaminated with GII.4. The data suggest that GII.4 was the prevalent genotype in creeks in Mississippi in US just as it is in many other countries in the world. Documents have shown that GII.4 accounts for the majority of norovirus outbreaks all over the word (Bull et al., 2006; Donaldson et al., 2008) and causes a more severe gastroenteritis than other noroviruses in young children (Huhti et al., 2011). Between 1995 and 2006, four major GII.4 strain pandemics have been identified. The first one was recognized in the mid-1990s (Noel et al., 1999). During that time, strain US95/96 was responsible for about 55% and 85% of the norovirus outbreaks in the US and Netherlands, respectively (Vinje et al., 1997). The second one was recognized between 2000 and 2004. During that time, US95/96 was replaced by two new GII.4 variants, Farmington Hills and GII.4b. Farmington Hills (Fankhauser et al., 2002) ultimately accounted for 80% of norovirus acute gastroenteritis outbreaks in the US (Widdowson et al., 2004), and GII.4b simultaneously emerged and caused outbreaks in Europe (Lopman et al., 2004; Medici et al., 2006; Phan et al., 2006). In 2004, the third pandemic caused by the Hunter GII.4 variant detected in Australia, Europe, and Asia (Bull et al., 2006; Phan et al., 2006; Kroneman et al., 2006). This strain was subsequently replaced in early 2006 by two new
cocirculating GII.4 variants in the United States and Europe, Laurens (2006a) and Minerva (2006b) (CDC, 2007; Kroneman et al., 2006; Siebenga et al., 2008). Although different clam specimens were proved to have the same genotype GII.4 in the present study, the possibility of the coexistence of 2 or more HuNoV genotypes in the same genogroup in a single clam specimen cannot be completely excluded. A cloning step before sequencing might be necessary for a detailed analysis of HuNoV genotype in clams. The clam sample from CC2 in May 2011 failed to sequencing reactions because DNA template was not enough for sequencing. Fifty nine clam samples were HuNoV positive based on real time RT-PCR, however, only 8 out of the 59 samples were positive based on conventional RT-PCR. The reason was that real time RT-PCR (Table 14) was 100 fold more sensitive than conventional RT-PCR (Fig. 20) for HuNoV detection in clam samples.

The negative results of HuNoV contamination in clams based on real time PCR need to be further confirmed as true negative. To do this, one strategy is to detect Enterococci (EN) in translocated clams used for HuNoV detection. EN is an indicator of fecal pollution in environmental waters and is relatively abundant compared to human viral pathogens like HuNoV. If statistically significant higher C_T values of EN for clean clams from Lake Serene than these for exposed clams from creek sites are observed, which would suggest that clams were active (not “sleeping” or dead) during the exposure periods. Based on this rationale, PCR negative results for HuNoV, but significantly lower C_T values for EN in these exposed clams compared to clean clams are observed, which can confirm that these PCR negative results are true negative and thus indicates no viruses are present in these creek sites.
Environmental factors such as variations in water temperature, pH, current velocity, turbidity and salinity were monitored because these factors may all play a critical role in the pathogen bioaccumulation by clams in freshwater systems. The results showed that water temperature had a significant effect on the HuNoV bioaccumulation by clams, and the likelihood of HuNoV presence increased as the water temperature increased. This data further confirmed the results from temperature effects on bioaccumulation rates of *C. fluminea* using MNV-1 as a surrogate of HuNoV in the laboratory study, which showed that *C. fluminea* bioaccumulated MNV-1 more quickly at higher water temperature (20°C) than at lower temperature (10°C). The results also showed the likelihood of HuNoV presence in clams decreased as the turbidity increased, indicating that virus bioaccumulation by clams becomes slowly or stop bioaccumulation after turbidity increased to a certain value.

In summary, the field study validated that the clam *C. fluminea* can serve as an effective sentinel of HuNoV contamination in freshwater of low turbidity during warm months with clean *Corbicula* translocation strategy.

**Acknowledgments**

I thank Dr. Jacquelina W. Woods at USFDA for HuNoV positive clinical samples. The research was financially supported by EPA Gulf of Mexico Program - Project MX96429505-0 and the Mississippi Coastal Impact Assistance Program/U. S. Dept of the Interior.
References


Hutson AM, Atmar RL, Estes MK. 2004. Norovirus disease: changing epidemiology and


CHAPTER VI
CONCLUSIONS

The freshwater clam *C. fluminea* was evaluated as a sentinel of HuNoV contamination by using culturable MNV-1 as a surrogate of HuNoV in the lab. The evaluation results showed that *C. fluminea* bioaccumulated NoV quickly and depurated slowly, and that *C. fluminea* bioaccumulated and depurated MNV-1 more quickly at 20°C than at 10°C. The data indicates that the clam is likely to be useful as a sentinel for detecting NoV contamination in freshwater. To validate the sentinel, clams were translocated to natural creeks for detecting HuNoV contamination in freshwater creeks flowing into Mississippi Sound. The study showed that HuNoV was detected in creeks using *C. fluminea* as a sentinel and GII.4 was the main genotype in the creeks. In addition, the MNV-1 and HuNoV RNA isolations involved in the laboratory and field studies were done by using the biotinylated DNA probe hybridization method developed in the project. In conclusion, the clam *C. fluminea* can serve as an effective sentinel of HuNoV contamination in freshwater of low turbidity during warm months with clean *Corbicula* translocation strategy.
APPENDIXES

SEQUENCES OF AMPLICONS FROM CONVENTIONAL RT-PCR FOR HUNOV DETECTION

> CC2, Apr 2011, 331 bases
TCTGCCGAATTTGTAAATGATGGCCTCTAAGGACGCCCAACAAACATGGA<REMOVED>CTGA GCCCTATACATGAGCCCGCCTCTGGCTGGGCCAGAAGGCTGGCAACTGCT GCCTGGTGGCCACCTGGCATATGGTGACCCCTTTCTTATCTCAATTTTGGCCAATG

> Trautman, sample #1, Oct 2010, 236 bases
ACGCCAACCCATCTGATGGGTCCGCAGCCAGCCTCGTCCAGAGGTCAACAAAGGTCATGGCTTTGGAGCCCGTTGCCGGTGCCGCTATTGCGGCGCCTGTAGCGGGCCAACAAAATGTAATTGACCCCTGGATTAGAAATAATTTTGTACAAGCCCTGGTGGAGAGTTCACAGTATCCCTAGAAACGTCCAGGTGAAAT

> Trautman, sample #2, Oct 2010, 277 bases
TCTTGGGAGGGCGATCGCAATCTGGCTCCAGCTCCAGTGTGAATGAAGATGGCGTCGAGTGACGCCAACCCATCTGATGGGTCCGCAGCCAGCCTCGTCCAGAGGTCAACAAAGGTTATGGCTTTGGAGCCCGTTGCCGGTGCCGCTATTGCGGCGCCTGTAGCGGGCCAACAAAATGTAATTGACCCCTGGATTAGAAATAATTTTGTACAAGCCCTGGTGGAGAGTTCACAGTATCCCTAGAAACGTCCAGGTGAAAT

> AOC, sample #1, Oct 2010, 279 bases
GGAGGGTGATCGCAATCTGGCTCCCAATTTGTGTAATGAAGATGGCGTGGAGTACGCGCAACCCTTCTGATGGCGTCCGCAAGCCAGCCTCGTCTCCAGAGGTCAACAAAGGTCATGGCTTTGGAGCCCGTTGCCGGTGCCGCTATTGCGGCGCCTGTACGAGGGCCAACAAAATGTAATTGACCCCTGGATTAGAAATAATTTTGTACAAGCCCTGGTGGAGAGTTCACAGTATCCCTAGAAACGTCCAGGTGAAAT

> AOC, sample #2, Oct 2010, 342 bases
TGGGAGGGCGATCGCAATCTGGCTCCCAATTTGTGTAATGAAGATGGCGTGGAGTACGCGCAACCCTTCTGATGGCGTCCGCAAGCCAGCCTCGTCTCCAGAGGTCAACAAAGGTCATGGCTTTGGAGCCCGTTGCCGGTGCCGCTATTGCGGCGCCTGTACGAGGGCCAACAAAATGTAATTGACCCCTGGATTAGAAATAATTTTGTACAAGCCCTGGTGGAGAGTTCACAGTATCCCTAGAAACGTCCAGGTGAAAT
ACTATGGAGCGCGCCCTTAGGCCCCTGATCTGAATCCCTACCTATCTCATTTGG
CCAGAATGTACAACGGACATGCGCGG

> Condo, sample #1, Oct 2010, 340 bases
CTTGGGAGGGCGATCGCAATCTGGCTCCCATGGTTTGTGAATGAGATGGCGT
CGAATGACGCAAACATCTGATGGGTCGCAGGCCAGCTCCTCAGGAAGGT
CAACAATGAGTTATGGCTTTGGGAGCCCGTTGCGGCTGCGCTATTGCAGCG
CCTGTCGCGGGGCAAACAAAAATGTAATTGACCCCTGAGTTAGAAATATTTTG
TACAAGCCCATGCTGGGAGAGTTCACAGTTATCCCTAGAAACGCTCCAGGTGA
AATACTATGGAGCGCGCCCTTAGCCTGATCTGAATCCCTACCTATTACCTTCTCATTT
GGCCAGAATGTAACGCGACATGCGG

> Condo, sample #2, Oct 2010, 233 bases
GAGANGGCGTCGATGACGCGCAAACCATCAGTATGGGTCGCAGCCAGCCTGG
CCCAGAGGTCAACAATGAGTTGTATGGCTTTTGGAGCCCGTTGCGGCTGCGCT
ATTGCGGCCCGTGTACCGGCTGCAACAAAATTGTAAATGACCCCTGAGTTAGAA
ACAATTTGTACAGCCCTGTTGGAGAGTTTCACAGTTATCCCTAGAAACGCT
GCCAGGTTGAAATCCTATGGAGCGCGCCCTTAGGCCCCTGATT