Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Jing Shi Date

GI and GII Norovirus Shedding After Experimental Human Infection

By

Jing Shi
Master of Public Health

Department of Epidemiology

_____

Dr. Christine L. Moe
Thesis Advisor

_____

Dr. Pengbo Liu
Field Advisor

GI and GII Norovirus Shedding After Experimental Human Infection

By

Jing Shi

Bachelor of Science
Fudan University, China
2009

An abstract of
A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Public Health
in Department of Epidemiology
2012

Abstract

GI and GII Norovirus Shedding After Experimental Human Infection

By Jing Shi

Norovirus (NoV) infection is the main cause of epidemic, acute non-bacterial gastroenteritis in United States and worldwide. However, little is yet known about the duration and magnitude of NoV excretion. In addition, the factors that determine NoV shedding patterns in stools remain unknown. In this study we investigated 51 subjects who had been experimentally challenged with Norwalk virus (NV) and 15 subjects who had been challenged with Snow Mountain virus (SMV). Stool specimens were examined for NoV by quantitative real-time reverse transcription-PCR. We also examined the relationship between covariates (age, gender, blood type, symptom severity, and pre-challenge anti-NoV serum IgG) and outcome variables (median viral loads, peak shedding titer, duration of shedding, cumulative virus shedding, and rate of increase and decrease in fecal virus titer). A total of 15 (29%) subjects were infected with NV, and nine (60%) subjects were infected with SMV. Among infected subjects, the median duration of viral shedding was 20 and 6 days for subjects infected with NV and SMV, respectively. NV had a similar median peak virus titer compared to SMV (3.03×107 genomic copies/g of stool vs. 1.06×107 genomic copies/g of stool). Peak virus titers were most commonly found in stools collected after resolution of symptoms. Prolonged duration of NoV shedding and the high virus titer in stools likely facilitate the transmission of NoV from infected persons to susceptible hosts through the fecal-oral route. Additionally, we found that subjects with asymptomatic infections had mean virus titers in stool similar to those with symptomatic infections, which indicates that individuals with asymptomatic NoV infections may play a significant role in virus transmission. No statistically significant correlation was found between any of the outcome variables and the co-variates. Further studies are needed to identify the factors that determine the magnitude and duration of NoV shedding.

GI and GII Norovirus Shedding After Experimental Human Infection

By

Jing Shi

Bachelor of Science
Fudan University, China
2009

A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Public Health
in Department of Epidemiology
2012

Acknowledgements

I would like to thank Pengbo Liu, whom I worked with in the lab for over a year perfecting all the laboratory methods and techniques, and who offered much needed insight into the numerous challenges faced at every stage of this project. I would also like to thank the other members of the group whose work contributed to the successful completion of this thesis Julia Sobolik, Amy Kirby, Meredith Lichtenstein. Lastly, I would like to thank Christine Moe for providing me with the opportunity to work on this project and for her thoughtful criticisms of my writing.

TABLE OF CONTENTS

Chapter I

Background/Literature Review

1. Introduction

Noroviruses (NoVs) are the most common cause of acute gastroenteritis in the United States and are a major cause of viral gastroenteritis in humans worldwide (1). Although gastroenteritis is a mild, self-limiting disease in the U.S. and other industrialized countries, high morbidity and high incidence of hospitalization are associated with it. Globally, the mortality caused by acute gastroenteritis is estimated at 3 – 5 million annually, including over 1.8 million deaths in children under 5 years of age worldwide (2, 3).

As molecular diagnostic techniques for NoV infection have improved in performance and become more widely available, substantial advances have been made in NoV epidemiology, immunology, diagnostic methods, and infection control. In addition, the periodic emergence of epidemic strains and outbreaks in specific populations (e.g., the elderly in nursing homes) have been characterized. This article reviews these recent advances as well as the biologic, clinical and epidemiologic features of NoVs.

2. Viral particle structure of NoVs

NoV virions consist of a capsid and a nucleic acid but lack a lipid envelope. The virus genome consists of a characteristic single-stranded, polyadenylated RNA with a positive polarity. The genome contains approximately 7.7 kilobases that is protected from the environment by a protein capsid (1). The capsid is composed of the major capsid protein, known as viral protein 1 (VP1), and a few copies of a second small, basic,

structural protein known as VP2 (4). The genome contains three open read frames (ORF). The second and third ORFs encode VP1 and VP2, respectively.

3. Classification

Because of lack of precise detection methods, the virus classification was not accurately defined until the development of genome-specific assays, such as reverse transcription-polymerase chain reaction (RT-PCR). These sensitive assays allow many strains of NoVs to be cloned and sequenced from stool and emesis samples. Based on the similarity across highly conserved regions of the genome, NoVs can be classified into at least five genogroups (GI, GII, GIII, GIV, GV), consisting of at least 31 genetic clusters or genotypes (5). Of these five genetic groups, GI, GII and GIV are found in humans, with GI and GII NoVs containing the majority of the human NoVs and presenting the largest genetic diversity. By phylogenetic analysis, other caliciviruses that infect cattle, pigs and mice also fall within the NoV genus. The bovine caliciviruses form a proposed GIII, and the murine NoVs cluster into a proposed GV. GIII NoVs are most closely related to GI NoVs, and the members of GV NoVs are closer to GII NoVs than those of GI by sequence comparisons (6, 7). NoVs in GI can be further subdivided into seven genetic clusters, designated I.1 to I.7, and GII viruses can be further divided into at least 19 genotypes, of which GII.4 is responsible for > 85% of outbreaks in United States (8). Strains within a genetic cluster typically show ≥ 80% VP1 amino acid identity in the major capsid protein sequence. Strains within the same genogroup show ≥ 60% identity, and strains in different genogroups show ≤ 50% identity (4). The relationship between the

high degree of genetic variability in NoV and its infectivity is not fully understood. However, recent studies indicated that an increase in the number of outbreaks in an immunologically naïve population is often associated with the emergence of a new variant (8).

4. Transmission

The most important mode of NoV transmission is the fecal-oral route. Indirect evidence in epidemiological studies suggests the virus transmission can also be airborne via explosive vomiting (9, 10). Transmission through infectious vomit has been suggested as an explanation for the rapid and extensive spread of disease outbreaks in closed settings, such as hospitals, hotels, cruise ships, and day-care centers. Inapparent contamination of shared restrooms may be a source of infection in many settings (11). In many outbreaks, primary infection results from the ingestion of fecal-contaminated food or water, whereas person-to-person contact, aerosolized vomitus, fomites, and infected food handlers may lead to secondary infection or further propagate the epidemic. Transmission is very efficient as there are certain characteristics of NoVs that enhance their ability to spread during outbreaks. The virus is highly infectious, with an median infectious dose of 18 viruses (12). Infected persons remain contagious even after symptoms resolve because asymptomatic viral shedding continues for up to three weeks (13). In addition, the virus is relatively stable on hard surface, in contaminated environment or on hands. It survives for a long period at a wide range of temperatures

from freezing to 60 ^{o}C and is relatively resistant to common disinfectants such as chlorine (14).

5. Clinical features and treatment

NoVs infect persons of all ages. The average incubation period of the virus is 12-48 hours. Clinical symptoms are characterized by abdominal cramps, nausea, vomiting, and diarrhea (2). Accompanying symptoms include headache, fever, chills, and myalgia occurring in 25%-50% of infected persons (15). About half of infected individuals develop a low-grade fever (101-102^{o} F) that typically resolves within 24h (16). Symptom development can be either gradual or abrupt. Children <1 year generally demonstrate diarrhea as the predominant feature of the illness, whereas persons >1 year of age experience nausea and vomiting more than diarrhea (3). Diarrheal stool is non-bloody, lacks mucus, and may be loose and watery. The duration of symptoms is approximately 2-3 days. However, recent studies have shown that the median duration of illness can be longer (i.e., 4-6 days) in patients affected during hospital outbreaks and in children < 11 years of age (16). Around 30% of infections from NoVs are asymptomatic; however, all infected individuals can be contagious because NoV can be detected in stools for up to 3 weeks by sensitive diagnostic assays in persons with either symptomatic or asymptomatic infections (17).

Clinical features are usually self-limiting, and most patients recover uneventfully without sequalae. Those who are unable to maintain hydration, typically the very young and the elderly, are at risk for dehydration, resulting in electrolyte disturbances that may

require hospitalization. Approximately 10% of people with NoV require a medical visit, including hospitalization (18). Deaths can occur in those unable to maintain hydration, especially elderly people and children under 5 years of age (19).

No antiviral treatments are available to treat NoV. Treatment focuses on preventing and treating dehydration secondary to the disease. Hydration is usually maintained using oral rehydration solutions that provide essential electrolyte replacement plus glucose or sucrose. Symptomatic treatment for headache, nausea, and myalgia can be provided using analgesics and antiemetic. Bismuth subsalicylate has been found to decrease abdominal cramping, but has no effect on viral shedding (16). There is no role for antibiotic therapy for the treatment of uncomplicated viral gastroenteritis.

6. Epidemiology

NoV are considered the most common viral etiologic agents of epidemic food-borne and waterborne viral gastroenteritis. Data from the United States and European countries have demonstrated that NoV is responsible for approximately 50% of all reported gastroenteritis outbreaks (2). According to surveillance data from CaliciNet, the outbreak surveillance network for NoV that is maintained by the CDC, GII viruses were responsible for > 85% of the outbreaks reported from March 2009 to January 2011 (8). Additionally, periodic increases in NoV outbreaks tend to occur in association with the emergence of new GII.4 strains that evade population immunity (8).

Although diarrhea outbreaks can occur all over the year, some seasonality patterns have been observed. These patterns are different in Northern and Southern hemispheres.

In Northern hemisphere, gastroenteritis caused by NoV is more commonly seen in winter and early spring. In the Southern hemisphere, outbreaks are more frequent over the spring and summer (20). In an outbreak, primary cases often result from exposure to contaminated food or water, whereas person-to-person spread among contacts of primary cases further propagates the epidemic.

A lack of sensitive diagnostic methods has historically limited the ability to study the etiology and epidemiology of gastroenteritis caused by NoV. Increased availability of PCR testing and the development and simplification of detection methods have the potential to revolutionize the ability to detect and trace this common human disease to sources of contamination such as food and water.

7. Diagnostic and detection methods

Over the last three decades, advances in the diagnosis and detection of NoV have progressed from the tests designed to identify the virus in stools using electron microscopy to the current ability to identify the presence of scant amount of virus in stool collected 1-2 weeks after the onset of illness using nucleic acid detection by reverse transcriptase polymerase chain reaction (RT-PCR).

7.1 Electron Microscopy

Before molecular diagnostic assays were developed, electron microscopy (EM) was the major diagnostic approach for viral gastroenteritis by observing viral particles in fecal samples. This technique is based on size and morphological characteristics. Viral

particles under the electron microscope are round, measuring about 27 to 30 nm in diameter, and exhibits an icosahedral symmetry.

One advantage of EM detection is that it provides a direct way to identify the virus by visualization, allowing performance of an examination without a preconceived concept of the etiological agent. However, a viral load of > 10^6/ml of stool is required in order to be detected, and, thus this technique can only be successfully used in the very early part of the illness (21). In addition, EM requires expensive equipment and trained personnel. Therefore, with the development of molecular detection assays, the utilization of EM is becoming less frequent.

7.2 Immuno Electron Microscopy

Immuno electron microscopy was one of the first generation diagnostic techniques for NoV. It was designed to enhance the detection of the virus by the addition of immune serum. Immune sera can help aggregate and highlight viral particles in stool suspensions. The virus clumping that occurs in the presence of specific antibody enables its detection and has improved the diagnostic capability . However, this technique is only useful for specimens collected during the first 24-48 hours after the onset of the disease.

7.3 Reverse Transcriptase Polymerase Chain Reaction

The cloning of NV led to the development of RT-PCR assays to detect NoVs in clinical and environmental specimens, such as water and food. Because amplification via RT-PCR provides a highly sensitive technique in which a very low copy number of RNA molecules can be detected, it is used widely in commercial and research laboratories

allowing detection of virus in specimens collected late in illness, when the quantity of virus is low.

The major advantage of the RT-PCR assay is that it can detect NoV RNA from fecal samples with as low as 100 particles/ml even after the acute clinical infection is resolved. NoV can be detected in fecal samples stored at 4^{o} C for several months and at -70^{o} C for many years. Additionally, RT-PCR has been useful in molecular epidemiology studies to identify the source of infection and detect many strains of NoVs. Although RT-PCR is used around the world as a standard tool for routine diagnosis of NoV infection, some strains may escape detection. Another disadvantage is that these assays require exquisite care to prevent contamination in the laboratory.

7.4 Real-time RT- PCR

The quantification of NoVs in human clinical specimens and environmental sample relies heavily on the use of reverse transcription (RT)-PCR. Early conventional RT-PCR focused on endpoint dilution titration to estimate virus titers; recent development of real-time quantitative RT-PCR (qRT-PCR) provides a faster and more sensitive approach than conventional RT-PCR for detection and quantification of NoVs (22).

The procedure follows the general principle of polymerase chain reaction. Tow common methods for detection of products in real-time PCR are: (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA, and (2) sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target. Real-time PCR is combined with reverse transcription to

quantify mRNA. The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes. The limit of detection for qRT-PCR assay is ≈ 40×10^6 copies/g feces (23).

To date, real-time RT-PCR is the most sensitive method of NoV quantification (22). However, results can be compromised by contamination in the laboratory. Also, few laboratories are equipped to perform this analyses.

7.5 Enzyme-Linked ImmunoSorbent Assays (ELISA)

ELISA was developed in 1970 and is defined as a biochemical technique mainly used to detect the presence of an antibody or an antigen in a sample. It is also known as an Enzyme ImmunoAssay (EIA).

ELISA for NoV detection provides a rapid, technically simple assay system that can be used to inform the surveillance of gastroenteritis outbreaks, especially in Public Health Laboratories with high sample throughput (24). In 2011, the FDA approved RIDASCREEN® Norovirus 3rd Generation Elisa as the first commercial assay at all in the USA for detection of NoVs of genogroup I and II in human stool samples (25). This assay is useful for the detection of NoV outbreaks where there are multiple stool samples available for testing; however, due to its sensitivity, RT-PCR is still required for routine NoV detection in sporadic cases investigation.

8. Human Challenge Studies

Because there is no small animal model of human NoV infection, and NoVs are not cultivable, there have been a number of NoV human challenge studies since the 1970s. The first NoV human challenge study was first reported in 1971 by Dolin et al (26), where transmission of acute infectious nonbacterial gastroenteritis to volunteers were studied by oral administration of virus inoculum. Since that time, multiple NoV human challenge studies have contributed greatly to our knowledge of host susceptibility, immune response to infection, the dose-response relationship, clinical course of infection, and evaluation of norovius vaccine candidates. These studies have also provided valuable reagents (virus in stool, sera with NoV-specific antibodies) to study NoV infections. These studies are one of very few human-challenge experimental models with infectious agents and are only conducted after careful ethical review, and rigorous safety precautions and medical supervision to protect the human subjects.

NoV human challenge studies have been very useful in investigation of NoV shedding. The uniqueness of these challenge studies lies in their ability to collect serial samples from carefully-monitored NoV infections where the exact time of exposure was known. Atmar et al. demonstrated a prolonged virus shedding of 4 weeks with a median peak virus titer as high as 10^{10} genomic copies/g feces among subjects experimentally challenged with NV (23). The severity of NV-associated viral gastroenteritis was reflected by a mean modified Vesikari score of 5.5 (27).

However, data on NoV shedding patterns is still limited. Because human challenge studies are expensive, and few laboratories have developed the expertise to conduct

human challenge study. Additionally, the majority of the challenge studies were based on Norwalk virus (GI.1). There is limited information about infections with other NoV strains, including the predominant GII/4 strain. Finally, findings from human challenge studies may not be generalized to other populations, since only healthy adults are eligible for challenge studies.

9. Host Susceptibility

On the basis of data from experimentally-infected human volunteers, genetic resistance to Norwalk virus (NV) was suggested more than 30 years ago when a subset of individuals was repeatedly susceptible to NV infection, whereas a second subset was repeatedly resistant to infection (28). It was hypothesized that a virus receptor on the host cell could possibly be the factor affecting a person's susceptibility to NV infection. Subsequent studies of Norwalk virus-like particles (VLPs) demonstrated that human ABH histo-blood group antigens (HBGAs) are regarded as putative receptors and may influence susceptibility to NV (29). Studies also found that the secretor phenotype is associated with host susceptibility: Norwalk VLPs bound to gastroduodenal epithelial cells from individuals who were secretors (Se+), but not to cells from non-secretors (Se-) (30).

Several enzymes are important in the synthesis of HBGAs, including an α(1,2)fucosyltransferase that is encoded by the fucosyl transferase 2 (FUT 2) gene. In secretor-positive (Se^+) individuals, the FUT2 gene is expressed, and its product produces the carbohydrate H type-1 found on the surface of epithelial cells and in mucosal

secretions. Approximately 80% of Northern Europeans and Caucasian Americans are Se+ (4). Because carbohydrate binding is essential for NV infection, individuals who are non-secretors (Se-), who do not express the FUT 2 fucosyltransferase and consequently do not make H type 1 oligosaccharide ligand required for NV binding, are resistant to NV infection. Secretors of blood types O and A are predicted to be at greatest risk of NV infection and disease (4). Persons in whom the blood group B antigens are expressed are less likely to become infected with NV, as Norwalk VLPs bind less to B histo-blood group antigens than to A or H histo-blood group antigens (4).

However, genetic differences in susceptibility to NV infection did not explain the fact that of the Se^+ populations that encoded a functional FUT2 gene, a portion was resistant to infection (30). This suggested that resistance to NV infection is multifactorial. A memory immune response or some other unidentified factor, may also afford protection from NV infection.

10. Immunity and Vaccine

Because of lack of an animal model and ability to grow NoVs in cell culture, data on immunity development after NoV infection is obtained from human challenge studies. Studies indicated that approximately 50% of people exposed to the virus acquired homologous immunity, which is correlated with the serum antibody level (31). However, people with pre-existing high antibody levels to NoV may become ill if exposed to the virus. Immunity is usually short-term (6-14 weeks), and subjects who were symptomatic could be re-infected when challenged 2-3 years later with the same NoV inoculum (28).

Currently, there is no vaccine to prevent human NoV infection, however, the development of VLP-based vaccines are in progress. Norwalk VLPs are produced by the expression and spontaneous self-assembly of the major capsid protein VP1 in recombinant systems. They can induce both systemic and mucosal immune responses in mice and humans when delivered intranasally or orally (32). El Kamary et al. first reported clinical data from two phase I clinical trials evaluating the immune responses to a monovalent adjuvanted Norwalk VLP vaccine administered intranasally (33). Although the Norwalk VLP vaccine was demonstrated to be safe and immunogenic, it is unknown whether the elicited immune responses are strong enough to prevent NoV illness. In 2011, Atmar et al. conducted a phase I/phase II clinical trial assessing the safety, immunogenicity, and efficacy of an investigational, inranasally delivered VLP vaccine, and demonstrated that NoV VLP vaccine provides protection against acute viral gastroenteritis after challenge with a homologous virus (27). This study provided the first evidence that a NoV vaccine could provide protection against NoV.

Although these are important steps in NoV vaccine development, there are several major challenges. First, the frequency of NoV infections in the community suggests that natural infection does not provide long-term immunity, which raises concerns that vaccines will also not provide long-term immunity. Second, the apparent rapid evolution of NoV suggests that the NoV vaccine may only provide protection against infection with a homologous virus, but lacks cross-protection among NoV strains from different genogroups. NoV vaccines may encounter challenges similar to those for influenza, in which comprehensive strain surveillance is needed to identify and evaluate the most prevalent strains that need to be included in a vaccine. Third, the efficacy rate in the

initial trial was about 50% or less, which is not high enough to make it available to the public because most routine childhood vaccines aim for 80% to 90% efficacy. Fourth, clinical data are entirely from human challenge studies with healthy adult volunteers. Ultimately, the researchers will need to test the vaccine in the field and examine efficacy in the most vulnerable populations – children and the elderly.

11. Prevention and Control

NoV outbreaks are currently interrupted by the control and maintenance of hand hygiene, exclusion and isolation of infected persons, and environmental disinfection. Strict personal hygiene and the proper disinfection of environmental surfaces are also critical for prevention of food-handler-associated transmission.

Appropriate hand hygiene is likely the single most important method to prevent NoV transmission and infection. Studies suggest that proper hand washing with soap and running water for at least 20 seconds is the most effective way to reduce NoV contamination on the hands. Hand sanitizers may serve as an effective adjunct in between proper handwashings but should not be considered a substitute for soap and water handwashing (34). As an additional preventive strategy, no bare-hand contact with ready-to-eat foods (foods edible without washing, cooking, or additional preparation to achieve food safety) is recommended (14).

Considering the highly infectious nature of NoV, exclusion and isolation of infected persons are often the most practical means of interrupting transmission of virus and limiting contamination of the environment. This is particularly important in institutional

settings where people reside or congregate, such as long-term--care facilities, acute-care hospitals, and food service facilities. For example, it is recommended that food handlers be excluded from work during, and for 48-72 hours after recovery from NoV gastroenteritis to prevent transmission of virus (14).

The use of chemical disinfectants is one of the key approaches to interrupt NoV spread from contaminated environmental surfaces. Attention should be given to the likely areas of greatest environmental contamination such as bathrooms and high-contact surfaces (e.g., door knobs and hand rails). Currently, the most effective disinfectant for NoV is chlorine bleach (35). For items that cannot be subjected to chlorine, heat disinfection (i.e., pasteurization to 140°F [60°C]) has been suggested (36). Other disinfection approaches such as ozone, hydrogen peroxide, or coating surfaces with antimicrobial materials (e.g., titanium dioxide [TiO2] film) also have been proposed for routine environmental control of NoV (35, 37).

Chapter II

Manuscript

ABSTRACT

Norovirus (NoV) infection is the main cause of epidemic, acute non-bacterial gastroenteritis in United States and worldwide. However, little is yet known about the duration and magnitude of NoV excretion. In addition, the factors that determine NoV shedding patterns in stools remain unknown. In this study we investigated 51 subjects who had been experimentally challenged with Norwalk virus (NV) and 15 subjects who had been challenged with Snow Mountain virus (SMV). Stool specimens were examined for NoV by quantitative real-time reverse transcription-PCR. We also examined the relationship between covariates (age, gender, blood type, symptom severity, and pre-challenge anti-NoV serum IgG) and outcome variables (median viral loads, peak shedding titer, duration of shedding, cumulative virus shedding, and rate of increase and decrease in fecal virus titer). A total of 15 (29%) subjects were infected with NV, and nine (60%) subjects were infected with SMV. Among infected subjects, the median duration of viral shedding was 20 and 6 days for subjects infected with NV and SMV, respectively. NV had a similar median peak virus titer compared to SMV (3.03×107 genomic copies/g of stool vs. 1.06×107 genomic copies/g of stool). Peak virus titers were most commonly found in stools collected after resolution of symptoms. Prolonged duration of NoV shedding and the high virus titer in stools likely facilitate the transmission of NoV from infected persons to susceptible hosts through the fecal-oral route. Additionally, we found that subjects with asymptomatic infections had mean virus titers in stool similar to those with symptomatic infections, which indicates that individuals with asymptomatic NoV infections may play a significant role in virus transmission. No statistically significant correlation was found between any of the outcome variables and the co-variates. Further studies are needed to identify the factors that determine the magnitude and duration of NoV shedding.

INTRODUCTION

Noroviruses (NoVs) are the leading cause of acute non-bacterial human gastroenteritis worldwide (38-40). Outbreaks caused by NoV involve people of all ages and occur in a variety of settings, including hospitals, nursing homes, cruise ships, catered events, and on military bases (13, 41-44). Highly contagious, NoVs are commonly transmitted through fecal-oral spread, direct person-to-person contact, environmental contamination, and ingestion of contaminated food or water (2).

NoVs are members of the *Caliciviridae* family, and most of the strains responsible for human gastroenteritis belong to genetic clusters within genogroups I (GI) and II (GII), with GII.4 viruses and variants reported as the predominant outbreak strains in the United States since late 2005/early 2006 (8, 45). The prototype Snow Mountain virus (SMV), designated as GII.2, was first recognized in a waterborne outbreak in 1976 in Colorado (46) and this genotype is the causative agent of an estimated 8% of NoV outbreaks from 1996 to 1997 (47). Less commonly identified than GII viruses, the prototype Norwalk virus (NV), designated as GI.1, was associated with only an estimated 5% of NoV outbreaks from 1996 to 1997 (47). However, it is unclear why GII viruses have become pandemic whereas other NoVs are not.

The duration and magnitude of NoV shedding in stool influences NoV dissemination and transmission to other individuals and has implications for outbreak control measures. Therefore, it is important to understand the course of NoV shedding. To date, much of our knowledge about NoV shedding has been derived from human challenge studies,

hospital studies, and community-based epidemiology studies. Rockx et al. found in a community-based cohort study in Netherlands that symptoms lasted for a median of five days for Norwalk-like viruses (NLV) infection, and that NLV shedding lasted for up to three weeks after onset of illness (17). High prevalence of prolonged norovirus shedding and illness was also observed among hospitalized patients (48). Long-term shedding after the disappearance of clinical symptoms was also observed in infants, children, and adults, with a continuation of viral excretion for an average of 2-3 weeks (13, 41).

However, quantification of NoVl load in stool was very accurate until the recent development of the quantitative real-time reverse transcript PCR assay (qRT-PCR). Quantitative RT-PCR provides a rapid and accurate method for detection, titration, and typing of NoV in environmental and clinical specimens (22, 49). A few studies have used qRT-PCR to examine fecal viral load and reported that NoV shedding peaked in the acute stage of illness and continued for more than 2 weeks after onset, with median viral loads ranging from 10^7 to 10^9 genomic copies/g feces (13, 23, 50, 51). Interestingly, a few studies also observed that asymptomatic individuals had mean viral loads similar to those of symptomatic individuals (23, 50). Although it is becoming clear that prolonged NoV shedding can occur in both symptomatic and asymptomatic shedders, data on the magnitude of NoV shedding is still limited. In addition, the factors that determine the duration and magnitude of NoV excretion remain unknown.

To address many of these needs, the goal of this study was to describe the quantitative course of NoV shedding after experimental inoculation and determine what factors affect the duration and magnitude of NoV shedding in stools. We compared the length and titer of GI and GII virus excretion in fifty-one volunteers challenged with NV and in fifteen

volunteers challenged with SMV, using qRT-PCR. We examined the relationship between median viral loads, peak shedding titer, duration of shedding, cumulative virus shedding, and rate of increase and decrease in fecal virus titer with age, gender, ethnicity, blood type, symptom severity, and the titer of pre-challenge NV/SMV serum IgG. The results from this study will contribute to our understanding of NoV shedding patterns and the factors that affect the virus shedding. This information can guide the development of strategies to reduce the risk of NoV transmission and infection.

MATERIAL AND METHODS

Study participants and sample collection

Fifteen eligible adult volunteers were enrolled in a human challenge study with SMV conducted at the University of North Carolina General Clinical Research Center (GCRC) from October 2000 through March 2002 (TABLE 1). Participants received different dosages of inoculum ranging from 30 to 3×10^5 genomic equivalent copies. Stool, sera, and saliva samples were collected and clinical symptoms and signs were recorded for the first five days post-challenge and at follow-up visits on days 8, 14, 21 post-challenge, as described elsewhere (52).

A NV human challenge study was conducted from February 2008 through September 2009. Fifty-one healthy adults were admitted to the Emory University Clinical Interaction Network and randomized into control and intervention groups (TABLE 1). Volunteers received NV inoculum (8FIIb, 10^4 genomic equivalent copies) in artificially-seeded oysters with or without high hydrostatic pressure processing (HPP) treatment (400 MPa at 25^{o}C, 600 MPa at 6 ^{o}C, or 400 MPa at 6 ^{o}C) for 5 min. Volunteers remained in the Emory University Clinical Interaction Network for the first five days post-challenge and returned for follow-up visits on days 8, 14, 21, 28, and 35 post-challenge for blood, saliva, and stool sample collection and recording of gastrointestinal symptoms, as described in (53). All samples collected from both human challenge studies were archived at -80 ^{o}C if they were not immediately processed.

Laboratory Studies

All pre-challenge serum samples were tested for anti-NoV IgG by ELISA using recombinant NV-like, or SMV-like particles (VLPs) as the antigen and alkaline phosphatase-labeled rabbit anti-human IgG (Sigma-Aldrich Co., St. Louis, MO) as the detector antibody, as described by Lindesmith et al. (52).

For viral RNA extraction, a 10% (wt/vol) stool suspension was prepared with water (20% [vol/vol]) and an equal volume of Vertrel XF (DuPont, Wilmington, DE), and centrifuged at 13,000 × g for 10 minutes. Viral RNA was extracted from 140 ul of the supernatant using the QIAamp Viral RNA Mini kit vacuum protocol (Qiagen, Valencia, CA). RNA extracts were stored at -20^{o}C until tested.

To detect and quantify NV RNA in fecal specimens, quantitative real-time reverse transcription – polymerase chain reaction (qRT-PCR) using Qiagen one-step RT-PCR kit (Qiagen, Valencia, CA) was performed on a Stratagene Mx3000P real-time PCR instrument (Stratagene, La Jolla, CA) using Norwalk-specific primers (NVKS1 and NVKS2) and probe (NVKS3) as described by Liu et al.(22). The quantification of RNA was calculated by MxPro software based on the C_T value and known copy numbers using the NV RNA standard curves.

The SMV-specific qRT-PCR assay was performed using methods described above, with the following modification. The primers and probe for the SMV- specific qRT-PCR assay were designed to correspond to the SMV RNA polymerase region (sense primer SMV-F: 5'- CAG GAA CCC ATG TTC AGG TGG ATG AG-3', which is complementary to SMV nt 5003-5028; antisense primer SMV-R: 5'- TCG ACG CCA

TCT TCA TTC ACA -3', which is complementary to SMV nt 5080- 5100; and probe SMV-P: 5' –FAM/ TGG GAG GGC GAT CGC AAT CT/BHQ1-3', which is complementary to nt 5048- 5067). PCR modifications included a completion of 45 amplification cycles at 95^{o}C for 15 sec and at 54^{o}C for 60 sec. The viral load was calculated by MxPro software based on the C_T value and known copy numbers using the SMV RNA standard curves.

All samples were tested in duplicate wells, and the average copy number was calculated. For determination of the virus titer, both wells had to show amplification. Specimens with ambiguous results were retested with the same extract or with a new extract of the sample. Stool specimens from NoV-infected subjects were included as positive controls in each extraction and qRT-PCR, and water was included as a negative control.

Scoring System for Severity of Gastrointestinal Symptoms

During the first five days at the Emory University Clinical Interaction Network, we maintained records of self-reported symptoms (nausea, abdominal cramping, headache, chills, myalgia, and fatigue) and objective conditions (fever, vomiting, and diarrhea). The severity of diarrhea and vomiting was also measured as duration and number of episodes per 24-hour period. For the purposes of this study, the severity of gastrointestinal symptoms was assessed using a 17-point numerical scoring system that was modified from Ruuska and Vesikari's description (27, 54) . All NoV-infected volunteers were

given a numeric score according to this modified Vesikari scoring system as a measurement of symptom severity (TABLE 2).

Definitions

Infection was defined as qRT-PCR detection of NoV RNA in any post-challenge stool sample as described by Liu et al. (22). "Symptomatic" was defined as a subject with at least one of the following: nausea, abdominal cramps, headache, chills, myalgia, fatigue, vomiting, and diarrhea (≥ 3 unformed stools in any continuous 24-hour period). To be included as a symptom, fever (oral temperature ≥37.6 ^{o}C) had to be associated with one or more other symptoms. Volunteers were classified as asymptomatic or symptomatic. Viral gastroenteritis was defined as illness with diarrhea (alone) for any continuous 24-hour period or one vomiting episode plus one of the following: abdominal cramps, nausea, fever, myalgia, chills, fatigue, or headache (23). First and last day of shedding were defined as the first and last day that an assayed stool sample was positive for NoV RNA by qRT-PCR as described in (30).

Data Collection and Statistical Analysis

Data quality was monitored through established sample-tracking sheets and standardized data entry protocols. Standardized error checking was completed through double data entry by separate operators. Databases were cross-checked, and any discrepancies were resolved by reviewing the hard-copy files.

The raw data obtained by qRT-PCR were log-transformed. For each infected volunteer, the “cumulative virus shedding” was calculated by summing the virus titer from each positive stool sample. Normality was assessed for all variables using the Shapiro-Will test. For variables that were found to be not normally distributed, non-parametric statistical tests and measures of central tendency (i.e. medians) were calculated. A Spearman’s correlation coefficient was calculated to assess the relationship between covariates (age, gender, blood type, severity of symptoms, and baseline anti-Norwalk/anti-SMV IgG) and outcome variables. Due to the small sample size, the Wilcoxon rank sum test was used, where appropriate, to compare median values. Fisher's exact two-tailed test was performed to analyze categorical variables with small sample size. All data analyses were performed using the statistical software package suite SAS 9.2 (SAS Institute Inc., Cary, NC). A p-value < 0.05 was considered significant.

RESULTS

In the NV human challenge study, the population was 57% male, 39% white, and 20% other races, with an average age of 26 years (range 18 to 51, Table 1). All participants were positive secretors (Se^+). In the SMV human challenge study, the study population was 53% male, 73% white, and no other races, with an average age of 31 years (range 21 to 54, Table 1). Four volunteers were seronegative pre-challenge, the rest were seropositive. The secretor status of three participants was negative (Se^-), the remaining 12 were Se^+. One Se^- volunteer was infected with SMV and also became ill.

A total of 15 (29.4%) persons inoculated with NV met the definition for NV infection. Of these, ten (66.7%) experienced one or more symptoms, and five subjects (33.3%) had asymptomatic NV infection during the five-day post-challenge period in the hospital. Among the symptomatic subjects, eight met the definition for viral gastroenteritis. The two who did not meet this definition had ≥3 symptoms but that did not include vomiting or diarrhea. Of the eight subjects who had viral gastroenteritis, seven had diarrhea and six had vomiting (Table 3). Other signs and symptoms were abdominal cramps (n=3), headache (n=2), chills (n=3), myalgia (n=3), fatigue (n=5), nausea (n=7) and fever (n=6). The two subjects who did not fulfill the criteria for gastroenteritis had nausea (n=2), abdominal cramps (n=1), headache (n=1), myalgia (n=1), and fatigue (n=1). Two subjects only had fever and were therefore classified as asymptomatic. The median duration of clinical symptoms was one day (TABLE 4). The median incubation period for subjects with symptomatic infection is one day (TABLE 4).

Fifteen volunteers were challenged with SMV, and nine (60%) had SMV infection. All nine (100%) SMV-infected subjects were classified as symptomatic (TABLE 4). The median incubation period is one day. Of these nine persons with symptomatic SMV infection, eight fulfilled the criteria for gastroenteritis. All eight participants with viral gastroenteritis had fever and headache. Five of them had diarrhea and six had vomiting (TABLE 3.). Other clinical symptoms included nausea (n=7), abdominal cramps (n=7), chills (n=4), myalgia (n=5), and fatigue (n=6). One subject, who did not meet the definition of gastroenteritis, had fever and abdominal cramps. The median number of symptoms for SMV-infected subjects was eight, whereas NV-infected subjects had a median of five symptoms (Figure 1). The clinical symptoms for SMV-infected subjects lasted a median of two days.

The severity of clinical signs and symptoms for NV and SMV infections was compared by the numeric score generated according to the 17-point scale modified Vesikari scoring system. The score of participants infected with NV ranged from 1-8, with an average score of 3.53 (Std. D=2.56, Figure 2). The score of participants infected with SMV ranged from 0-6, with an average score of 3.22 (Std. D=1.86, Figure 2). Comparisons of these average scores showed no statistically significant difference (p=0.3676).

NV shedding, as measured by qRT-PCR, was first detected at a median of two days (range 1-4 days) after inoculation and lasted a median of 20 days post-inoculation (range 5-35 days). On average, the duration of NV shedding was similar for the participants who were symptomatic compared to those persons who were asymptomatic (21 days vs. 19 days, p=0.4632, Table 3). However, two symptomatic subjects, who did not meet the

criteria of clinical gastroenteritis, shed NV for 26 days and 33 days, respectively. No pre-symptomatic shedding was found in NV-challenged volunteers. SMV shedding was first detected at a median of two days (range 1 to 3 days) after inoculation and lasted a median of six days (range 3 to 25 days) after inoculation. One subject, who did not meet the definition of gastroenteritis, only shed SMV for three days post-challenge (Table 3). The median duration of SMV shedding was significantly shorter than the median duration of NV shedding (6 days vs. 20 days, p=0.0347, Wilcoxon rank sum).

NV concentration in feces, as measured by qRT-PCR, peaked at a median of 3.5 days (range 2-14 days) after inoculation; NV titer for symptomatic subjects peaked at a median of three days, whereas the median time of peak shedding for asymptomatic subjects was four days after inoculation. The median peak titer of NV shedding was 3.03×10^7 (range 1.35×10^5 – 3.69×10^8, Figure 3) genomic copies/g feces, and all NV-infected participants shed $\geq 10^4$ copies/g until at least day 15 (Figure 4). Persons who were symptomatic had a higher median peak NV titer than those who were asymptomatic (3.75×10^7 vs. 3.74×10^6 genomic copies/g feces, p=0.0650, Wilcoxon rank sum). Study subjects who met the clinical definition of gastroenteritis had similar median peak NV titer as those who did not have gastroenteritis (3.75×10^7 vs. 1.35×10^8, p=0.4632, Wilcoxon rank sum). On average, NV-infected subjects shed a total of 7.59×10^9 genomic copies over the course of their infection based on the titers of the NV-positive stool specimens. No significant difference in cumulative virus shedding was found between subjects who were and were not classified as symptomatic (8.56×10^9 vs. 5.81×10^9 genomic copies, p=0.1700, student *t* test).

The SMV viral titer in feces from the nine infected subjects peaked at a median of three days after inoculation (Figure 4). The highest fecal concentrations of virus were detected in two (22.2%) participants after their clinical signs had resolved. The median peak fecal virus titer was 1.06×10^{7} (range 3.83×10^{4} – 6.96×10^{8}) genomic copies/g feces. A comparison between the median peak titer of NV and SMV in infected subjects indicated that NV-infected symptomatic individuals had a slightly higher median peak titer than SMV-infected symptomatic individuals (3.75×10^{7} vs. 1.06×10^{7} copies/g of stool, p=0.3168, Wilcoxon rank sum). On average, SMV-infected subjects shed a total of 6.78×10^{9} genomic copies/g of stool during the course of their infection. Our results also showed that SMV-infected individuals and NV-infected individuals had similar mean cumulative virus shedding (6.78×10^{9} vs. 7.59×10^{9}, genomic copies/g of stool, p=0.3168, Wilcoxon rank sum).

To elucidate the factors that influence the course of NoV excretion, we examined the relationship between median viral titer, peak shedding titer, duration of shedding, cumulative virus shedding, rate of increase and decrease in fecal virus titer with age, gender, ethnicity, blood type, symptom severity, and the titer of pre-challenged NV/SMV serum IgG, using correlation analysis. No statistically significant correlation was found between any of the outcome variables and the co-variates (data not shown).

DISCUSSION

The past decade has witnessed vast improvements in NoV detection methods, surveillance, and awareness. Methods such as real-time RT-PCR have enabled rapid, broadly reactive, and highly sensitive NoV diagnoses. Numerous molecular epidemiological studies have increased our understanding of these important viruses. However, estimated NoV incidence remains high and relatively few data describe the quantity and duration of the NoV shedding in feces as determined by modern assays. The goal of this study was to describe the quantitative course of NoV shedding after experimental inoculation.

The duration and timing of NoV shedding is problematic in terms of preventing transmission. NV was detected in stool samples for a median of three weeks and for up to five weeks after virus inoculation, whereas SMV was detected in stool samples for a median of one week and for up to three weeks after virus inoculation. Despite the fact that GII NoVs have been the dominant genotype in human NoV outbreaks, our findings indicating that the duration of SMV shedding was significantly shorter than that of NV shedding.

The median peak virus titers for NV- and SMV-infected subjects were 3.03×10^7 genomic copies/g feces and 1.06×10^7 genomic copies/g feces, respectively. The peak virus titers were most commonly found in stool samples collected after resolution of symptoms. These observations help explain the epidemiologic observations of NoV outbreaks linked to food handlers who had recovered from symptomatic infection.

It is recommended by US Food and Drug Administration (FDA) that food handlers be excluded from work during, and for 48-72 h after gastrointestinal illness symptoms subside to prevent transmission of virus. However, the results from our study suggest that NoVs are shed for a longer duration even after resolution of symptoms, indicating the need to exclude these individuals from food contact for longer periods.

Interestingly, subjects with asymptomatic Norwalk infections also shed virus for a median of three weeks, suggesting that asymptomatic carriers may be infectious for a prolonged period of time. Similar peak titer and cumulative virus shedding were observed for infected symptomatic and asymptomatic individuals, which may account for the increased number of infections and the predominance of an asymptomatic transmission route. Asymptomatic NoV excretion has been recognized in food service facilities with outbreaks (55) and without outbreaks (56). Although there was not sufficient evidence to prove NoV transmission by asymptomatic infected individuals, asymptomatic food handlers were postulated to be the source of infection (55). In addition, NoVs are considered highly contagious, with an estimated median infectious dose of 18 genomic copies (12). Taken as a whole, these results suggested the importance of transmission by people who are infected, but not ill, and the potential hazard from these highly contagious viruses.

Recently, several studies have also used quantitative RT-PCR to examine NoV fecal load. Chan et al. reported a median viral load of 8.4×10^5 genomic copies/g of stool for NoV GI in adult patients with NoV-associated gastroenteritis in Hong Kong (57), whereas the mean viral loads of GI observed by Ozama et al. (50) were 2.79×10^7 genomic copies/g of stool in symptomatic and asymptomatic food handlers in Japan. Data for both

of these studies were derived from outbreaks and sporadic cases. Few studies have used human inoculation to examine fecal viral load. Atmar et al. reported in a human experimental NV challenge study that the median peak NV viral load was 10^{11} genomic copies/g of stool in infected subjects with and without viral gastroenteritis (23). The median peak NV titer observed in our study (3.03×10^{7} genomic copies/g of stool) was similar to the mean reported by Ozawa et al (2.79×10^{7} genomic copies/g of stool). We also found that the median peak virus titer and the average cumulative virus shedding for symptomatic NV-infected subjects was approximately 10-fold higher than those of asymptomatic infected subjects, although the difference was not statistically significant most likely owing to the relatively small number of participants in each subgroup. Higher viral loads in persons who had symptomatic gastroenteritis was also reported by Amar et al. , compared to those who had asymptomatic NV infections (58). Therefore, our findings suggest that symptomatic NV infection was associated with higher peak virus shedding and higher total virus shedding after inoculation. We did not see an association of peak virus titer with symptom duration.

Potential reasons for the different results across studies may include difference in specimen storage, RNA extraction efficiency, the stringency and sensitivity of the real-time assays used (generic assays designed to be broadly reactive vs. assay designed specifically for NV detection), virulence of the infecting strains, differences in the populations studied (e.g., age, ethnicity), differences in sample collection (single samples from outbreaks and sporadic cases vs. serial samples from human challenge studies), and the small number of asymptomatic infected persons in our study.

At present, GII NoVs are the most prevalent cause of NoV outbreaks. Several studies have speculated that the pre-dominance of GII strains over GI strains may be attributed to the higher viral load of GII NoV in feces. Chan et al. reported the median cDNA viral load of NoV genogroup II of 3.0×10^8 genomic copies/g, which is $\geq$ 100-fold higher than that of genogroup I in the fecal specimens of clinic patients with NoV associated gastroenteritis (57). Additionally, Ozawa et al. reported a mean viral load of 3.81×10^8 genomic copies/g of stool for GII NoV, which is a log unit higher than that of genogroup I in stool samples (50). However, the median peak SMV titer observed in our study (1.06×10^7 genomic copies/g of stool) is 10-fold lower than the 10^8 median/mean GII NoV titer reported in the prior studies (50, 57). One potential reason could be the differences in the infecting strains. In the two abovementioned studies, over 50% were tested positive for GII/4 strain of all the positive stool specimens examined for GII NoV. Our study, though, focused entirely on subjects challenged with GII/2 strains. It is possible that GII/2 strains may have lower shedding and transmissibility compared to GII/4 strains. Additionally, our findings found that both NV-infected subjects and SMV-infected subjects had similar median peak virus titer and cumulative NoV shedding, suggesting that NoV titers in stool may not correlate well with the epidemiology of NoV infections.

This is the first study to describe the magnitude and duration of SMV shedding and examine the association between various host factors and NoV shedding patterns. Another strength of this research is that we collected and analyzed serial stool samples from carefully-monitored NoV infections where the time of exposure was known and all the post-challenge stools were collected for seven days. However, our study had several

limitations. First, although the majority of the infected subjects had NoVs-negative stools by the last follow-up visit, one subject with NV infection and two subjects with SMV infection still had virus in the last stool samples collected at the end of the follow-up period; we cannot exclude the possibility that these persons shed NoV for a longer period. Second, because of the timing of stool collection during the follow-up period, we are not able to determine the exact day on which NoV shedding ended. Third, the doses of NV inoculum that the participants received in the NV challenge trial were different from the doses of SMV inoculum in the SMV challenge trial, and therefore we could not examine the relationship between NoV dose, NoV virulence and NoV shedding.

In conclusion, we found that NoV-infected subjects experienced symptoms early in the course of infection, and that NoV shedding can extend to a median of 2-3 weeks after the resolution of symptoms, with a median peak virus titer of 10^7 genomic copies/g of stool. We speculate that the prolonged duration of NoV shedding and the high virus titer in stools facilitate the transmission of NoV from infected persons to susceptible hosts through the fecal-oral route. Additional studies are needed to: (i) determine the courses of shedding among different strains of NoV, (ii) model the association between NoV dose, NoV virulence and NoV shedding, (iii) examine implication of NoV shedding patterns for prevention and control of NoV transmission.

TABLES

TABLE 1. Baseline characteristics of study subjects challenged with Norwalk virus (NV) and Snow Mountain virus (SMV)

	No. (%) of subjects challenged with:	
Characteristic	**NV (N=51)**	**SMV (N=15)**
Ethnicity		
African American	21 (41.2)	4 (26.7)
Caucasian	20 (39.2)	11 (73.3)
Other	10 (19.6)	0 (0)
Gender		
Male	29 (56.9)	8 (53.3)
Female	22 (43.1)	7 (46.7)
Secretor Status		
Positive	51 (100)	12 (80)
Negative	0 (0)	3 (20)
Blood Type		
A	15 (29.4)	4 (26.7)
B	5 (9.8)	1 (6.7)
O	30 (58.8)	8 (53.3)
AB	1 (2.0)	2 (13.3)
	Mean (S.D.)	Mean (S.D.)
Age, year	26.1 (8.0)	30.7 (9.3)
Serum IgG, µg/ml *	38.9 (28.7)	23.9 (19.2)

*Serum IgG was measured pre-challenge.

TABLE 2. Modified Vesikari Score (MVS) Components

	0 Points	**1 Point**	**2 Points**	**3 Points**
Diarrhea duration, days	0	1-4	5	≥6
Maximum no. of diarrheal stools/24-h	0	1-3	4-5	≥6
Vomiting duration, days	0	1	2	≥3
Maximum no. of vomiting episodes/24-h	0	1	2-4	≥5
Maximum recorded fever, oral, ^{o}C	≤37.0	37.1-38.4	38.5-38.9	≥39.0
Dehydration	None		IV Treatment	

TABLE 3. Clinical symptoms among study subjects infected with Norwalk virus (NV) and Snow Mountain virus (SMV)

Symptom(s)	No. (%) of infected subjects with symptoms	
	NV (N=15)	SMV (N=9)
Chills	3 (20.0)	4 (44.4)
Abdominal Cramps	4 (26.7)	8 (88.9)
Diarrhea [a]	7 (46.7)	5 (55.6)
Fatigue	6 (40.0)	6 (66.7)
Fever [b]	8 (53.3)	9 (100.0)
Headache	3 (20.0)	8 (88.9)
Myalgia	4 (26.7)	5 (55.6)
Nausea	9 (60.0)	7 (77.8)
Emesis	6 (40.0)	6 (66.7)
No Symptom [c]	3 (20.0)	0 (0.0)

[a]Diarrhea is defined as ≥ 3 unformed stools for any continuous 24-hour period.

[b]Fever is defined as oral temperature ≥37.6 ^{o}C.

[c]No symptom here is defined as without any listed clinical symptoms including fever. However, fever alone is not considered a symptom in the definition of "symptomatic".

TABLE 4. Virus shedding among study participants challenged with Norwalk virus (NV) and Snow Mountain virus (SMV)

Subject No.	First-last study days[a] postchallenge when symptoms[b] present	First-last study days stool qRT-PCR positive	Day peak virus titer	Peak qRT-PCR virus titer (log_{10}/g)	Cumulative qRT-PCR virus titer (log_{10}/g)[c]
Symptomatic (SMV) [d]					
Met clinical definition of gastroenteritis [e]					
SMV-1	2-4	2-27	3	8.84	10.63
SMV-2	2-3	1-25	6	7.02	9.17
SMV-3	1-5	2-9	2	8.20	9.87
SMV-10	2-4	2-7	2	4.58	6.99
SMV-11	3-4	3-8	4	7.50	9.70
SMV-12	2-4	3-9	4	6.81	8.26
SMV-13	2-4	3-9	3	3.60	6.07
SMV-15	1-2	2-10	2	7.05	9.55
Did not meet clinical definition of gastroenteritis					
SMV-4	3-4	2-5	3	6.32	8.68
Median (SMV)	2-4[g]	2-8[g]	3	7.02	8.77
Symptomatic (NV)					
Met clinical definition of gastroenteritis [e]					
NV-4	1-2	2-20	2	7.61	9.26
NV-15	2-3	3-23	7	6.50	8.82
NV-34	2-3	2-37	3	8.29	9.93
NV-36	2-3	3-14	4	8.11	10.43
NV-37	2-3	3-28	4	7.36	10.18
NV-40	2-3	2-17	3	7.54	8.95
NV-46	2-3	3-8	3	7.42	9.82
NV-54	2-3	3-22	3	8.16	10.05
Did not meet clinical definition of gastroenteritis					
NV-14	1-5	2-35	5	6.69	8.80
NV-17	2-3	3-29	4	8.42	10.12
Median	2-3[g]	2-21[g]	3	7.57	9.64
Asymptomatic (NV) [f]					
NV-3	N/A	2-10	3	5.48	7.81
NV-9	N/A	3-29	14	6.66	8.64
NV-12	N/A	2-27	4	6.57	8.84
NV-16	N/A	4-9	4	5.13	7.65
NV-29	N/A	2-23	3	8.57	10.44
Median	N/A	2-23	4	6.57	8.68
Overall Median	2-3[g]	3-23[g]	4	7.42	9.28

[a]Study days are reported as calendar days; Day 1 is defined as the first day post-challenge.
[b]Symptoms include abdominal cramps, nausea, fever (≥37.6 ºC), myalgia, chills, fatigue, diarrhea, vomiting, and headache.
[c]The "cumulative virus shedding" was calculated by summing up the virus titer shedded from each positive stool sample for each infected subject.
[d]"Symptomatic" was defined as a subject with at least one symptom, not including fever. To be classified as a symptom, fever had to be associated with at least one other symptoms.
[e]Viral gastroenteritis was defined as illness with diarrhea (alone) for any continuous 24 hour period or one vomiting episode plus one of the following: abdominal cramps, nausea, fever, myalgia, chills, fatigue, or headache.
[f]A subject with only fever was considered as asymptomatic.
[g]The first number represents the median of the first day and the second number represents the median of the last day.

FIGURES

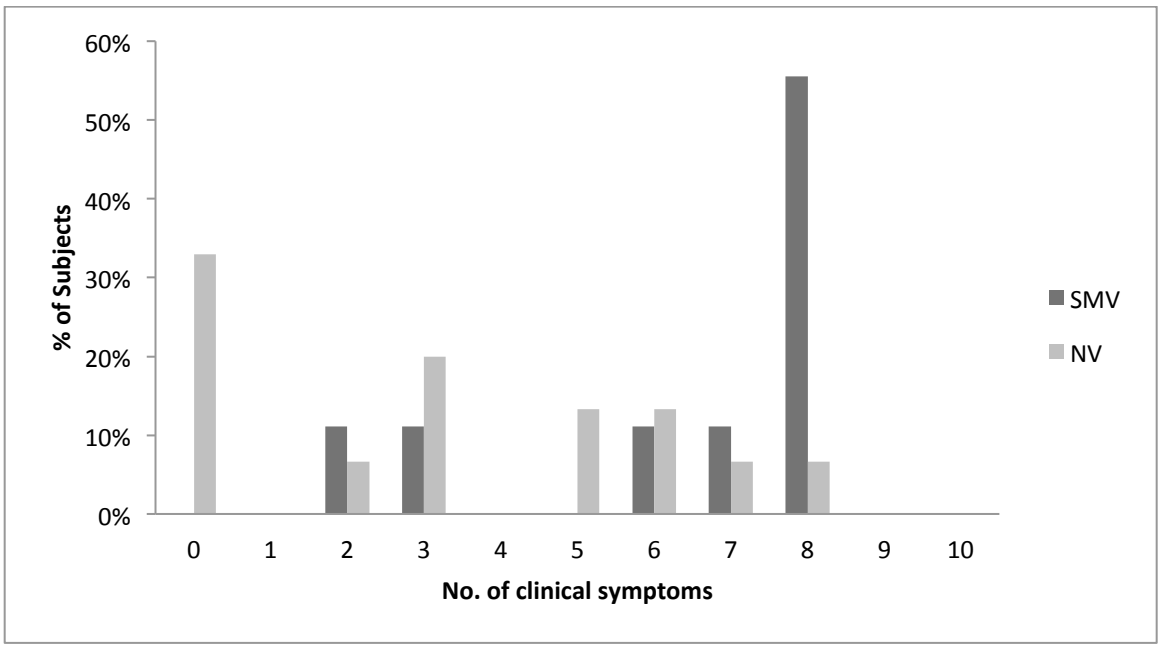

Figure 1. Distribution of clinical symptoms among NV infected (N=15) and SMV infected subjects (N=9)

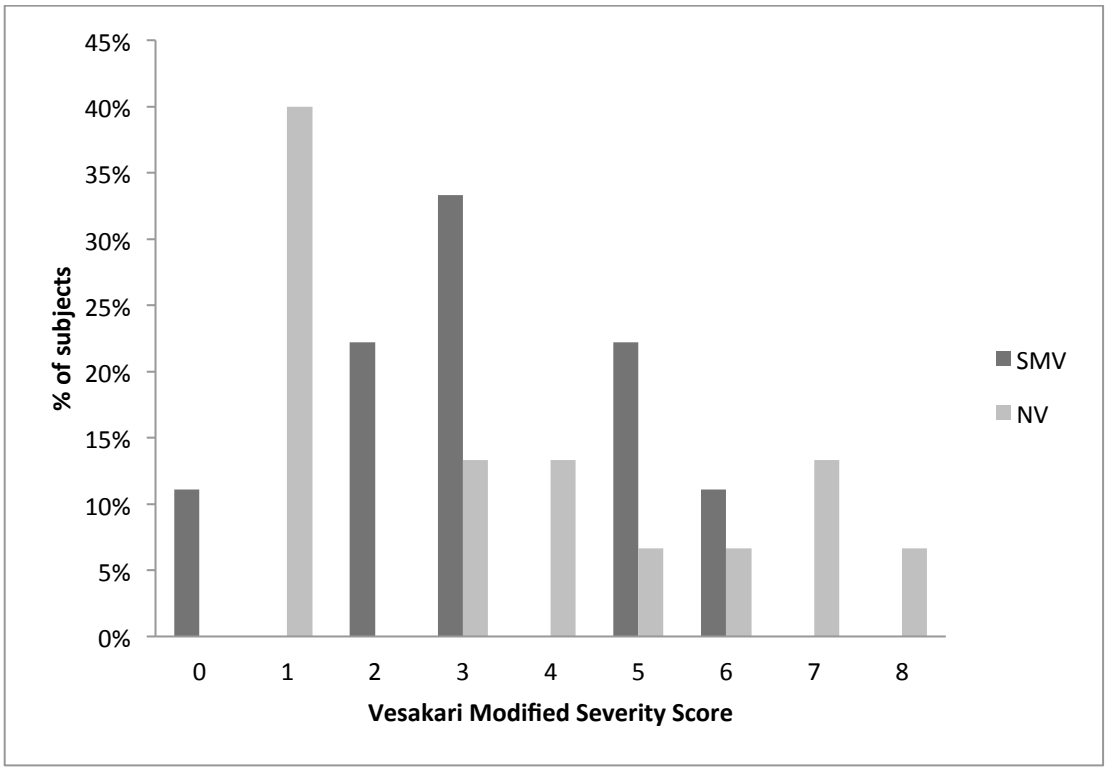

Figure 2. Distribution of the modified Vesakari Score among symptomatic subjects infected with Norwalk virus (N=15) and Snow Mountain virus (N=9)

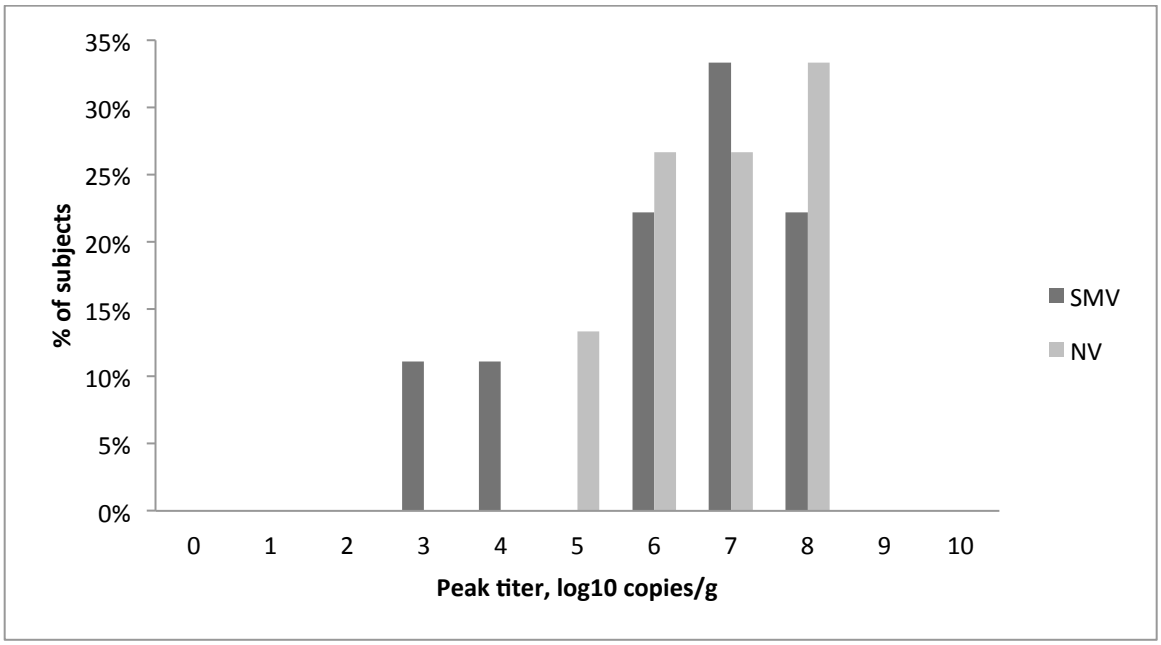

Figure 3. Distribution of virus peak qRT-PCR titer (log10/g) among infected subjects

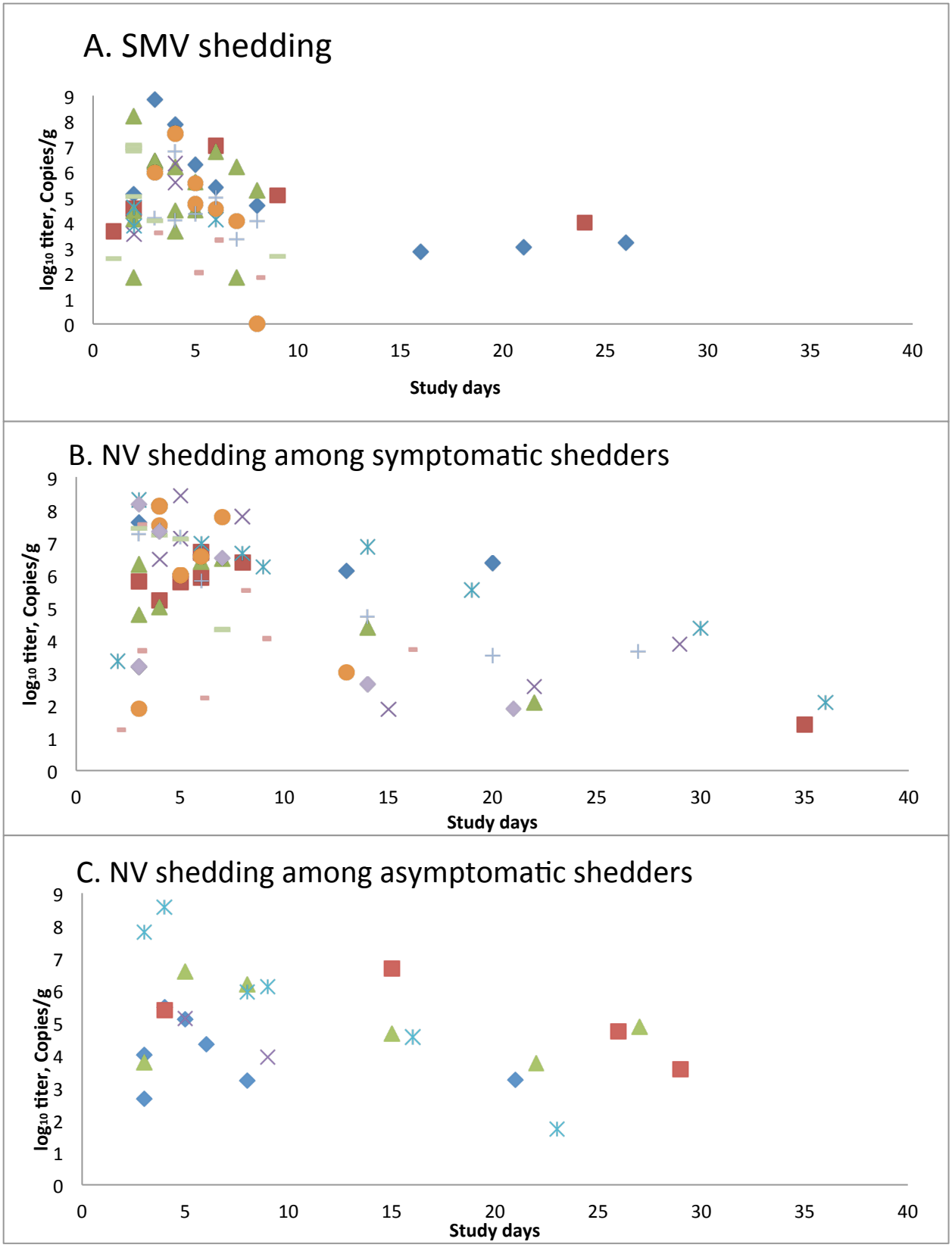

Figure 4. Shedding of Norovirus in stools. Panel A, B, C show the virus titers as measured by qRT-PCR in stool samples collected from participants who were challenged with SMV (Panel A), symptomatic shedders who were challenged with Norwalk virus (Panel B), and asymptomatic shedders who were challenged with Norwalk virus (Panel C).

REFERENCES

1. Morillo, S.G. and C. Timenetsky Mdo, *Norovirus: an overview.* Revista da Associacao Medica Brasileira, 2011. **57**(4): p. 453-8.

2. Patel, M.M., et al., *Noroviruses: a comprehensive review.* Journal of Clinical Virology, 2009. **44**(1): p. 1-8.

3. Thornton, A.C., K.S. Jennings-Conklin, and M.I. McCormick, *Noroviruses: agents in outbreaks of acute gastroenteritis.* Disaster Manag Response, 2004. **2**(1): p. 4-9.

4. Hutson, A.M., R.L. Atmar, and M.K. Estes, *Norovirus disease: changing epidemiology and host susceptibility factors.* Trends in Microbiology, 2004. **12**(6): p. 279-87.

5. Zheng, D.P., et al., *Norovirus classification and proposed strain nomenclature.* Virology, 2006. **346**(2): p. 312-23.

6. Oliver, S.L., et al., *Molecular characterization of bovine enteric caliciviruses: a distinct third genogroup of noroviruses (Norwalk-like viruses) unlikely to be of risk to humans.* Journal of Virology, 2003. **77**(4): p. 2789-98.

7. Karst, S.M., et al., *STAT1-dependent innate immunity to a Norwalk-like virus.* Science, 2003. **299**(5612): p. 1575-8.

8. Vega, E., et al., *Novel surveillance network for norovirus gastroenteritis outbreaks, United States.* Emerging Infectious Diseases, 2011. **17**(8): p. 1389-95.

9. Kirkwood, C.D. and R.F. Bishop, *Molecular detection of human calicivirus in young children hospitalized with acute gastroenteritis in Melbourne, Australia, during 1999.* Journal of Clinical Microbiology, 2001. **39**(7): p. 2722-4.

10. Caul, E.O., *Small round structured viruses: airborne transmission and hospital control.* Lancet, 1994. **343**(8908): p. 1240-2.

11. Ho, M.S., et al., *Viral gastroenteritis aboard a cruise ship.* Lancet, 1989. **2**(8669): p. 961-5.

12. Teunis, P.F., et al., *Norwalk virus: how infectious is it?* Journal of Medical Virology, 2008. **80**(8): p. 1468-76.

13. Aoki, Y., et al., *Duration of norovirus excretion and the longitudinal course of viral load in norovirus-infected elderly patients.* Journal of Hospital Infection, 2010. **75**(1): p. 42-6.

14. Parashar, U., et al., *"Norwalk-like viruses". Public health consequences and outbreak management.* MMWR Recomm Rep, 2001. **50**(RR-9): p. 1-17.

15. LeBaron, C.W., et al., *Viral agents of gastroenteritis. Public health importance and outbreak management.* MMWR Recomm Rep, 1990. **39**(RR-5): p. 1-24.

16. Treanor, J. and R. Dolin, eds. *Norwalk Virus and Other Caliciviruses*. 5 ed. Vol. 2. 2000. 1949-1956.

17. Rockx, B., et al., *Natural history of human calicivirus infection: a prospective cohort study.* Clinical Infectious Diseases, 2002. **35**(3): p. 246-53.

18. Shirato, H., et al., *Norovirus and histo-blood group antigens.* Japanese journal of infectious diseases, 2011. **64**(2): p. 95-103.

19. De Rougemont, A., et al., *[Norovirus infections: an overview].* Med Sci (Paris), 2010. **26**(1): p. 73-8.

20. Marshall, J.A., et al., *Incidence and characteristics of endemic Norwalk-like virus-associated gastroenteritis.* Journal of Medical Virology, 2003. **69**(4): p. 568-78.

21. Atmar, R.L. and M.K. Estes, *Diagnosis of noncultivatable gastroenteritis viruses, the human caliciviruses.* Clinical Microbiology Reviews, 2001. **14**(1): p. 15-37.

22. Liu, P., et al., *Quantification of Norwalk virus inocula: Comparison of endpoint titration and real-time reverse transcription-PCR methods.* Journal of Medical Virology, 2010. **82**(9): p. 1612-6.

23. Atmar, R.L., et al., *Norwalk virus shedding after experimental human infection.* Emerging Infectious Diseases, 2008. **14**(10): p. 1553-7.

24. Burton-MacLeod, J.A., et al., *Evaluation and comparison of two commercial enzyme-linked immunosorbent assay kits for detection of antigenically diverse human noroviruses in stool samples.* Journal of Clinical Microbiology, 2004. **42**(6): p. 2587-95.

25. Morillo, S.G., et al., *Norovirus 3rd Generation kit: an improvement for rapid diagnosis of sporadic gastroenteritis cases and valuable for outbreak detection.* Journal of Virological Methods, 2011. **173**(1): p. 13-6.

26. Atmar, R.L., et al., *Norovirus vaccine against experimental human Norwalk Virus illness.* New England Journal of Medicine, 2011. **365**(23): p. 2178-87.

27. Parrino, T.A., et al., *Clinical immunity in acute gastroenteritis caused by Norwalk agent.* New England Journal of Medicine, 1977. **297**(2): p. 86-9.

28. Hennessy, E.P., et al., *Norwalk virus infection and disease is associated with ABO histo-blood group type.* Journal of Infectious Diseases, 2003. **188**(1): p. 176-7.

29. Lindesmith, L., et al., *Human susceptibility and resistance to Norwalk virus infection.* Nature Medicine, 2003. **9**(5): p. 548-53.

30. Johnson, P.C., et al., *Multiple-challenge study of host susceptibility to Norwalk gastroenteritis in US adults.* Journal of Infectious Diseases, 1990. **161**(1): p. 18-21.

31. Vinje, J., *A norovirus vaccine on the horizon?* Journal of Infectious Diseases, 2010. **202**(11): p. 1623-5.

32. El-Kamary, S.S., et al., *Adjuvanted intranasal Norwalk virus-like particle vaccine elicits antibodies and antibody-secreting cells that express homing receptors for mucosal and peripheral lymphoid tissues.* Journal of Infectious Diseases, 2010. **202**(11): p. 1649-58.

33. Liu, P., et al., *Effectiveness of liquid soap and hand sanitizer against Norwalk virus on contaminated hands.* Applied and Environmental Microbiology, 2010. **76**(2): p. 394-9.

34. *Updated norovirus outbreak management and disease prevention guidelines.* MMWR Recomm Rep, 2011. **60**(RR-3): p. 1-18.

35. Baert, L., et al., *The reduction of murine norovirus 1, B. fragilis HSP40 infecting phage B40-8 and E. coli after a mild thermal pasteurization process of raspberry puree.* Food Microbiol, 2008. **25**(7): p. 871-4.

36. Hudson, J.B., M. Sharma, and M. Petric, *Inactivation of Norovirus by ozone gas in conditions relevant to healthcare.* Journal of Hospital Infection, 2007. **66**(1): p. 40-5.

37. Atmar, R.L. and M.K. Estes, *The epidemiologic and clinical importance of norovirus infection.* Gastroenterology Clinics of North America, 2006. **35**(2): p. 275-90, viii.

38. Schmid, D., et al., *An outbreak of Norovirus infection affecting an Austrian nursing home and a hospital.* Wien Klin Wochenschr, 2005. **117**(23-24): p. 802-8.

39. Sulik, A., et al., *[Norovirus infection in children hospitalized with acute gastroenteritis in northeastern Poland].* Przeglad Epidemiologiczny, 2007. **61**(3): p. 477-82.

40. Murata, T., et al., *Prolonged norovirus shedding in infants <or=6 months of age with gastroenteritis.* Pediatric Infectious Disease Journal, 2007. **26**(1): p. 46-9.

41. Wikswo, M.E., et al., *Disease transmission and passenger behaviors during a high morbidity Norovirus outbreak on a cruise ship, January 2009.* Clinical Infectious Diseases, 2011. **52**(9): p. 1116-22.

42. Mayet, A., et al., *Food-borne outbreak of norovirus infection in a French military parachuting unit, April 2011.* Euro Surveill, 2011. **16**(30).

43. Schmid, D., et al., *Foodborne gastroenteritis outbreak in an Austrian healthcare facility caused by asymptomatic, norovirus-excreting kitchen staff.* Journal of Hospital Infection, 2011. **77**(3): p. 237-41.

44. Zheng, D.P., et al., *Molecular epidemiology of genogroup II-genotype 4 noroviruses in the United States between 1994 and 2006.* Journal of Clinical Microbiology, 2010. **48**(1): p. 168-77.

45. Morens, D.M., et al., *A waterborne outbreak of gastroenteritis with secondary person-to-person spread. Association with a viral agent.* Lancet, 1979. **1**(8123): p. 964-6.

46. Fankhauser, R.L., et al., *Molecular epidemiology of "Norwalk-like viruses" in outbreaks of gastroenteritis in the United States.* Journal of Infectious Diseases, 1998. **178**(6): p. 1571-8.

47. Siebenga, J.J., et al., *High prevalence of prolonged norovirus shedding and illness among hospitalized patients: a model for in vivo molecular evolution.* Journal of Infectious Diseases, 2008. **198**(7): p. 994-1001.

48. Trujillo, A.A., et al., *Use of TaqMan real-time reverse transcription-PCR for rapid detection, quantification, and typing of norovirus.* Journal of Clinical Microbiology, 2006. **44**(4): p. 1405-12.

49. Ozawa, K., et al., *Norovirus infections in symptomatic and asymptomatic food handlers in Japan.* Journal of Clinical Microbiology, 2007. **45**(12): p. 3996-4005.

50. Takanashi, S., et al., *Detection, genetic characterization, and quantification of norovirus RNA from sera of children with gastroenteritis.* Journal of Clinical Virology, 2009. **44**(2): p. 161-3.

51. Lindesmith, L., et al., *Cellular and humoral immunity following Snow Mountain virus challenge.* Journal of Virology, 2005. **79**(5): p. 2900-9.

52. Leon, J.S., et al., *Randomized, double-blinded clinical trial for human norovirus inactivation in oysters by high hydrostatic pressure processing.* Applied and Environmental Microbiology, 2011. **77**(15): p. 5476-82.

53. Ruuska, T. and T. Vesikari, *Rotavirus disease in Finnish children: use of numerical scores for clinical severity of diarrhoeal episodes.* Scandinavian Journal of Infectious Diseases, 1990. **22**(3): p. 259-67.

54. Barrabeig, I., et al., *Foodborne norovirus outbreak: the role of an asymptomatic food handler.* BMC Infect Dis, 2010. **10**: p. 269.

55. Gallimore, C.I., et al., *Asymptomatic and symptomatic excretion of noroviruses during a hospital outbreak of gastroenteritis.* Journal of Clinical Microbiology, 2004. **42**(5): p. 2271-4.

56. Okabayashi, T., et al., *Occurrence of norovirus infections unrelated to norovirus outbreaks in an asymptomatic food handler population.* Journal of Clinical Microbiology, 2008. **46**(6): p. 1985-8.

57. Chan, M.C., et al., *Fecal viral load and norovirus-associated gastroenteritis.* Emerging Infectious Diseases, 2006. **12**(8): p. 1278-80.

58. Amar, C.F., et al., *Detection by PCR of eight groups of enteric pathogens in 4,627 faecal samples: re-examination of the English case-control Infectious Intestinal Disease Study (1993-1996).* European Journal of Clinical Microbiology and Infectious Diseases, 2007. **26**(5): p. 311-23.

Chapter III

Conclusions/Public Health Implications/Future Directions

Conclusions:

- NoV-infected subjects experienced symptoms early in the course of infection; symptoms resolved rapidly (2-3 days)
- Prolonged duration of NoV shedding was prevalent among NoV-infected symptomatic and asymptomatic subjects. NoV shedding can be extended to a median of 2-3 weeks after the resolution of clinical symptoms.
- GI and GII NoV-infected subjects shed high amounts of virus during their course of infection. Both GI and GII NoV shedders had similar median peak virus titer (3.75×10^7 vs. 1.06×10^7 copies/g of stool) and cumulative virus shedding (7.59×10^9 vs. 6.78×10^9, genomic copies/g of stool).
- Symptomatic subjects had median virus titers (3.75×10^7 vs. 3.74×10^6 genomic copies/g of stool) and cumulative virus shedding similar to those of asymptomatic subjects (8.56×10^9 vs. 5.81×10^9 genomic copies of stool).
- None of the host factors we examined (age, gender, blood type, symptom severity, and pre-challenge anti-NoV serum IgG) were significant predictors of the duration or magnitude of NoV shedding.

Public Health Implications:

- The prolonged duration of NoV shedding and the high virus titer in stools may facilitate the transmission of NoV from infected persons to susceptible hosts through the fecal-oral route.

- Data from this study suggest that symptomatic food handlers need to be excluded from the workplace for a period longer than 48-72 hours. From a more economically-practical perspective, we suggest that symptomatic food handler be temporarily reassigned to a job that does not involve handling food for an additional week after recovery from illness.
- How to reduce NoV transmission from food handlers with asymptomatic infection is especially challenging because asymptomatic food handlers may continue to work without recognition of their infection and potential to transmit virus to others.

Future directions:

Additional studies are needed to:

- Determine the course of infection and viral shedding among subjects with different strains of NoV.
- Model the association between NoV dose, NoV virulence and NoV shedding and examine the implications of NoV shedding patterns for prevention of virus transmission.