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Comparison of infection and illness between two Norwalk virus inocula (8FIIa and 8FIIb)

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Abstract

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By Mumu Rahman

Noroviruses (NoVs) are a leading cause of epidemic gastroenteritis. Human challenge studies have been used to examine the infectivity, pathogenicity, and host immune response to NoV. Several human challenge studies have been performed using Norwalk virus (a prototype Genogroup I NoV) inocula purified from the stools of infected individuals. Surprisingly little is known about differences in infection and illness in human volunteers challenged with different preparations of Norwalk virus (NV) inocula. The goal of this study was to compare the response among study subjects who received a secondary NV inoculum (8FIIb), compared to subjects who received its precursor, the 8FIIa. We investigated a total of 160 subjects: 76 subjects were experimentally challenged with NV inoculum 8FIIa, and 84 subjects were challenged with 8FIIb. We compared the difference in infection, illness, mean severity score, blood types, and duration of viral shedding between two groups of subjects. We also examined the demographic characteristics and secretor status of 8FIIa- and 8FIIb-inoculated subjects. There were no statistically significant differences in overall infection and illness rates between subjects inoculated with 8FIIa and 8FIIb. However, subjects challenged with 8FIIA dose above the ID50 had significantly more illness (94.1%) compared to subjects who were challenged with high doses of 8FIIb (47.1%), *P*=0.0. Comparison of mean severity score between the two groups of inoculated subjects did not show any significant difference. We observed that infection with 8FIIb was significantly associated with longer duration of viral shedding (P=0.02), and there was a significant difference in duration of viral shedding by dose for both inocula (P=0.00). The results from this study contribute to our understanding and knowledge about NoV infections, pathogenesis, and viral shedding. This information can guide future NoV human challenge studies to test candidate NoV vaccines and treatment efficacy.

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TABLE OF CONTENTS

Chapter III: Conclusions/Public Health Implications/Future Directions

Comparison of infection and illness between two Norwalk virus inocula (8FIIa and 8FIIb)

Chapter I: Background/ Literature Review

Introduction

Acute gastroenteritis is defined as inflammation and/or infection of the digestive tract, causing nausea, vomiting, diarrhea and/or abdominal pain that lasts less than 14 days (94). In the United States, acute gastroenteritis caused more than 1.5 million outpatient visits, 200,000 hospitalizations and approximately 300 deaths per year among children younger than five years of age, according to the Center for Disease Control and Prevention (CDC) (1). Acute gastroenteritis causes an estimated 2 million deaths per year among children less than five years old in low-income countries (1). Viral gastroenteritis accounts for about 50% to 70% of acute gastroenteritis cases in the United States, with NoVs being the leading cause (2, 3).

Noroviruses (NoVs), are the major cause of viral gastroenteritis, worldwide. They cause diarrhea and profuse vomiting which is self-limited. Although, in those who are immunocompromised or have weak immune systems, such as the elderly and young children, NoV infection can develop into a more severe and long-term illness (4-7). NoVs are highly transmissible, resistant to conventional cleaning agents, and have a low infectious dose. They are considered as Category B potential bioterrorism agents, according to the National Institute of Allergy and Infectious Diseases classification of pathogens important for biodefense (NIAID Biodefense Research), since they have ideal properties as enteric outbreak pathogens (8).

There is currently no vaccine available to prevent human NoV infection and no specific therapy to treat it, due to a lack of cell culture systems and small animal models of human NoV infection. For NoV vaccine development, human challenge studies have been necessary to understand characteristics of virus infectivity, pathogenicity, and host immune response.

Structure of NoVs and Classification

NoV are RNA viruses in the family *Caliciviridae*. The caliciviruses are positive-sense, single-stranded RNA viruses. Human caliciviruses have been difficult to study because of their inability to grow in conventional cell culture and the lack of an animal model. The human NoV genome is linear, positive-sense RNA, which is ~7.6 kb in length (9). The genome is capped on the 5' end by the virally encoded protein, VPg, and polyadenylated at the 3' end (10). The NoV genome contains three to four open reading frames (ORFs). ORF1 codes for non-structural proteins (including VPg), ORF2 codes for the NoV capsid protein (VP1), and ORF3 codes for a minor structural protein (VP2) (Figure 1).

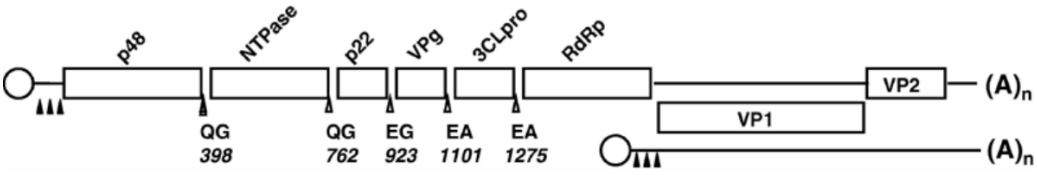

Figure 1: Norovirus protein structure and function.

(*FEMS Microbiol Lett. 2005;253(1):1-8. doi: 10.1016/j.femsle.2005.08.031, FEMS Microbiol Lett | © 2005 Federation of European Microbiological Societies)*

NoVs can be described by their diversity and are currently classified into seven genogroups [GI] to [GVII], and >40 genotypes (12-13) (Figure 2). Genetic strains show ≥80% VP1 amino acid identity in the major capsid protein sequence within a cluster, and strains in the same genogroup show ≥60% identity. However, strains in different genogroups show ≤ 50% identity (17).

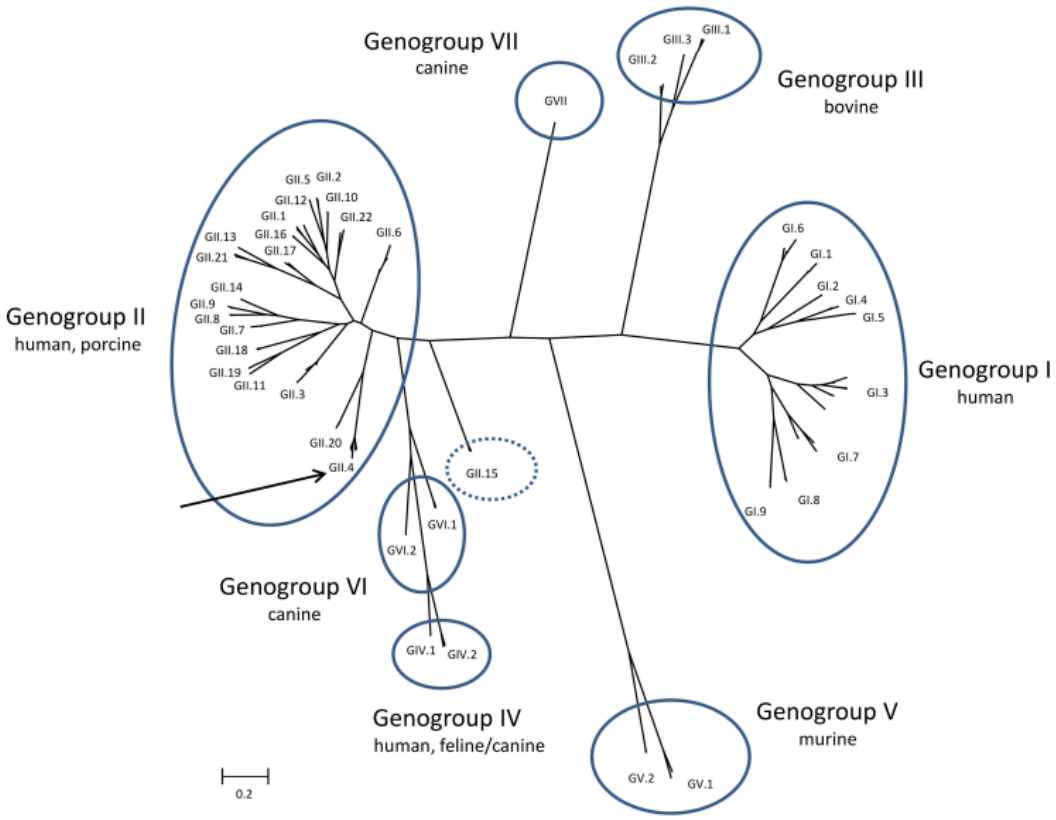

Figure 2: Classification of NoVs into 7 genogroups (GI to GVII) based on amino acid sequence diversity in the complete VP1 capsid protein. The scale bar here represents the number of amino acid substitutions per site. Adapted from Vinjé (53).

NoV infection in humans is caused by, from highest to lowest frequency, genogroup GII (predominantly GII.4), GI, and GIV (some genotypes of which

infect pigs) (14-15). GII viruses can be further divided into at least 19 genotypes, among them GII.15, which is only detected in humans (53). GII.4 is responsible for >85% of outbreaks in the United States (16).

It is critical to understand the genetic diversity and infectivity of NoVs, as recent studies indicate that the emergence of a new variant is frequently associated with an increase in the number of outbreaks in an immunologically naïve population (18).

Modes of Transmission and Risk factors

Transmission of NoVs primarily occurs via four general routes: direct person-to-person, foodborne, waterborne, or environmental fomite (19). Airborne transmission of NoV infection can occur by virus-containing aerosol droplets produced by vomiting (20) and toilet flushing (21). The genetic diversity of NoV has public health relevance. Certain genotypes are associated with different modes of transmission and severity of disease outcomes. Genogroup I viruses (for example GI.6) are more often associated with food and waterborne outbreaks (69), whereas GII.4 viruses are strongly associated with person-to-person transmission and transmission in healthcare settings (70). GII.4 infections are associated with greater disease severity, hospitalization, and deaths than those caused by other GII and GI viruses (71).

NoVs are immensely contagious, with an estimated median infectious dose (ID_{50}) of 18 genome equivalent copies for Norwalk virus (22). Asymptomatic viral shedding can occur for up to 21 to 24 days for normal host, but it can be longer in

immunocompromised hosts, which makes the infected persons remain contagious, even after symptoms resolve (23). NoVs withstand freezing and heating to 60^{o}C. They are relatively stable and resistant to vinegar, alcohol, and high sugar concentration, which allows them to survive on fruits and prepared foods for long periods of time (24).

Contact with an infected person is the strongest risk factor for community-acquired disease. Usually, young children acquire infection from outside of the house, such as school or playground, whereas older children and adults acquire their infection from within the household (25). Exposure to a different spectrum of NoV strains could be a risk factor for NoV infection in foreign travelers. An estimated 14% of NoV outbreaks globally are due to food borne transmission (25). Younger age distribution, asymptomatic infection, and a higher overall disease incidence are reported in low-income countries (25).

Clinical features of Norovirus infection and treatment

Symptomatic infection

People of all ages can be infected by NoV. The median incubation period for NoV infection is 1.2 days, according to a recent systematic review of the literature (30). Recent studies have shown that the median incubation period could be longer (4-6 days) in patients who get infected in hospital outbreaks (34). The predominant symptoms of NoV infection include nausea, vomiting, diarrhea, and abdominal

cramps (31) (Table 1). Typically, 25%-50% of infected persons also develop headache, low-grade fever, chills, and myalgia (32). Diarrheal stool is non-bloody, lacks mucus, and may be loose and watery.

Table 1 Clinical Features Associated with Norovirus Infection

Symptoms	***cases***	***Percentage (%)***
Diarrhea	1186	78.8
Vomiting	964	64.9
Abdominal pain	945	67.0
Nausea	728	51.6
Fever (≥38°C)	453	31.2
Headache	426	31.0
Myalgia	313	24.1
Chills	184	15.4
General malaise	117	7.6

Source: C. Arias et al. 2010.

Total of 1544 cases. According to Arias et al., 2010, NoV gastroenteritis is defined as two or more loose stools in 24 h and/or vomiting twice or more in 24 h, with additional symptoms including nausea, fever, abdominal pain, headache, myalgia, general malaise and chills.

NoV symptoms are usually self-limited; however, viral shedding can continue to occur 22 days or more after symptoms resolved (33). Although most of the NoV infections do not have long term consequences, a recent study showed that one of the important long-term sequelae associated with NoV infection is irritable bowel syndrome (IBS), with 3-36% of all NoV infections resulting in post-infectious IBS (40). No antiviral treatments are currently available to treat NoV infection. Preventing and treating dehydration secondary to the disease is the focus of treatment. Oral rehydration solutions, which provide essential electrolytes and

glucose or sucrose replacement, are usually used to maintain hydration. Approximately 10% of cases require hospitalization (35), and death may occur in those who are unable to maintain hydration, especially in very young children and the elderly (36). Immunocompromised individuals may develop prolonged and severe disease with NoV infection and may have prolonged viral excretion (39).

Asymptomatic infection

Asymptomatic NoV infections are a very important source of transmission of the virus. According to the observational study by Qi and colleagues, the estimated prevalence of asymptomatic NoV infection is 7% globally, with higher prevalence in Africa (15%), Mesoamerica (14%) and South America (11%) (26). among other factors, the high genetic variability of NoV could be the most likely contributing factor to its prevalence. Studies have shown that NoV genogroup II is probably responsible for 80% of asymptomatic infection, with children younger than 6 months more susceptible to asymptomatic infection (27). Host genetic factors, such as secretor status, plays an important role in the development of symptomatic and asymptomatic infection. Secretor-positive individuals are more susceptible to the worldwide dominant NoV GII.4 strain, whereas those who are secretor-negative develop asymptomatic infection mostly from the non-GII.4 NoV strains (28-29).

Pathogenesis and host susceptibility

Human volunteer studies give us more insight into NoV pathogenesis, development of immunity, and host susceptibility after NoV infection (37). Observations from human challenge studies indicate that NoV infection first attacks the proximal small bowel, which causes expansion of the villi and shortening of the microvilli and development of patchy lesions in the mucosa that ultimately lead to diarrhea (33). Transient gastroparesis occurs with viral infection and may cause nausea and vomiting, which mostly resolves with resolution of the illness (33).

Experimentally-infected human volunteer studies indicate that some people might be genetically resistant to NoV infection, while others are genetically susceptible to NoV infection (38). A virus receptor on the host cell may be the primary factor affecting a person's susceptibility to NoV infection. Studies of NoV virus-like particles (VLPs) show that Histo-Blood Group Antigens (HBGAs), may play an important role as a possible receptor binding site for NoV (41). Studies also indicate that host secretor phenotype status is related to host susceptibility. Gastroduodenal epithelial cells were infected by Norwalk VLPs in secretor-positive (Se+) individuals whereas gastroduodenal epithelial cells in secretor negative (Se-) individuals did not get infected by Norwalk VLPs (42). People who express the fucosyltransferase 2 (FUT2) gene are susceptible to Norwalk virus infection, while those who have a non-functional FUT2 gene are genetically resistant to the Norwalk virus. The FUT2 gene is responsible for expression of the H type 1 HBGA on the surface epithelium. A decreased risk of Norwalk virus

infection and illness is associated with those who have blood group B, as Norwalk VLPs bind less to B HBGA than to A or H HBGAs(41).

Many studies have also shown that in the Se+ population, among those who have a functional FUT2 gene, a portion is resistant to NoV infection (42). This suggests that there are multiple determinants of NoV infection.

Individuals resistant to one NoV strain could be susceptible to other NoV strains, as other NoVs display different ABH and Lewis carbohydrate-binding profiles (56-57). Until now, carbohydrate binding of one GI and seven GII NoV VLPs have been reported. Among them, the most extensively studied interaction was the single GI VLPs binding to carbohydrates (55). The carbohydrate-binding properties of GII VLPs are varied in different ways, and their binding specificities are different. Previous human challenge studies have shown that VLPs from one GII.4 strain (VA387), which is clustered with the current predominant NoV strains in circulation, bind to saliva and synthetic carbohydrates of all secretors, regardless of their ABO type. In contrast, VLPs from GII.2 (Snow Mountain virus) bind the saliva of secretors of blood type B and AB, but not of type O (56-57). This indicates that carbohydrate-binding properties are strain-specific.

Identifying residues that are important for binding and the carbohydrate-binding sites on NoVs would help predict the carbohydrate-binding characteristics of a NoV on the basis of its amino acid sequence (55). Further research needs to be conducted to explore unidentified factors or memory immune responses in order to identify why some individuals are protected against, or resistant to, NoV infection.

Immunity and vaccine

People develop short-term immunity (6-14 weeks) after NoV infection (37). Human challenge studies have shown that subjects who were symptomatic were re-infected two to three years later when challenged with the same NoV inoculum (37). A recent study showed that, among people who are exposed to the virus, 50% of them developed homologous immunity, which was correlated with serum antibody levels (43). However, people who have pre-existing high antibody levels (serum IgG or IgA) to NoV infection may also develop disease if exposed to the virus. A study by Ajami, Kavanagh and Ramani et al. followed about 75 US students who travelled to Mexico (44). Serum samples were collected from each traveler, before and during travel, to determine the antibody titers against GII.4 NoV VLPs. Among the 75 travelers, 62 had IgA and all 75 of the travelers had IgG serum antibodies against the GII.4 VLPs (44). Seroprevalence of both IgA and IgG antibodies before travelling had no effect on the likelihood of contracting GII.4 NoV infection (44). These study results indicate that, regardless of whether antibodies are present, prior NoV infection does not necessarily protect an individual from reinfection. NoV specific antibodies and their exact role and efficacy in preventing infection are yet to be determined through further research.

Currently, no vaccine is available to prevent human NoV infection. A recombinant system is used in the expression and spontaneous self-assembly of the major capsid protein, VP1, to produce and develop the Norwalk VLP-based vaccine against Norwalk virus. A study by El Kamary et al. evaluated the immune

response to a monovalent adjuvant Norwalk virus VLP vaccine, administered intranasally. This was the first study to demonstrate that the Norwalk virus VLP-based vaccine, administered intranasally, was safe and immunogenic (58). However, it is not known whether the elicited immune responses are strong enough to prevent NoV infection. Atmar et al. conducted a clinical trial using the intranasally-delivered VLP vaccine and demonstrated that the Norwalk VLP vaccine could provide protection against acute gastroenteritis after challenge with a homologous virus (45). VLPs are highly immunogenic, and Norwalk virus VLPs induce both systemic and mucosal immune responses in mice and humans. However, the NoV VLP vaccine may prevent illness but not prevent infection (59). Mucosal vaccines have the ability to skew the adaptive immune response toward a $CD4^+$ helper type 1, which is associated with effective protection against viruses (60). A modified cholera toxin, used as mucosal adjuvant with Norwalk virus VLPs, has been shown to enhance systemic immunoglobulin G (IgG) immune response in mice when administered orally or intranasally. However, as the cholera toxin may lead to potential adverse effects, it cannot be used in the human NoV vaccine (61). Toll-like receptors (TLRs), including TLR7 agonists consisting of monophosphoryl lipid A (MPL), are currently being developed as adjuvants and have been shown to induce higher antibody titers than other commonly used adjuvants in vaccines (58).

There are some major challenges which play an important role in NoV vaccine development: i) NoVs require a very low infectious dose, utilize a human reservoir, are stable in the environment (withstanding conditions ranging from

freezing to 60^{o}C), may not provide long-term immunity, rapidly evolve new variant strains, and have multiple routes of transmission – these are all factors that contribute to the persistence of this virus in the population (63); ii) it is not possible to routinely culture these viruses and, therefore, it is difficult to evaluate the level of protection afforded by a candidate vaccine (63); and iii) limited cross-protection to NoV genotypes within the same genogroup is observed, however, there is very little cross-protection between NoV genogroups, suggesting that a multivalent vaccine may be necessary to protect susceptible individuals. All of these challenges have hindered the development of robust, safe and efficacious NoV vaccines (63).

8FIIa and 8FIIb inoculum

NoVs are very contagious, requiring a low inoculum in the host to cause infection. Numerous human challenge studies have been conducted since 1971 to examine the histopathology, pattern of illness, clinical treatment, and immune response associated with NoV infections (81). The first inoculum was 8FIIa, which was produced by a second passage filtrate derived from a volunteer who had received first passage stool inoculum from a previous volunteer who had ingested diarrheal stool from an acute case in the original Norwalk virus 1968 outbreak in Ohio (81). The new (second) inoculum was produced sometime between 1990 and 1996, by Atmar and Estes (83), from a subject infected with 8fIIa in their challenge studies conducted from 1985 to 1990 (82).

Before the human challenge study conducted with the 8FIIa inoculum, volunteers were screened by medical evaluation for other common enteric pathogens and human immunodeficiency virus (HIV) and underwent liver function tests. Another inoculum, designated as second-generation inoculum 8fIIb, originated from a single stool sample from a NV-infected volunteer with a high titer of viral RNA confirmed by NV-specific primer (Moe et al., 1994) (49). In further purification of 8FIIb inoculum, the stool was diluted 1:5 with 1× phosphate-buffered saline (PBS) and extracted twice with an equal volume of 1,1,2-trichloro-1,2,2-trifluoroethane (Freon) (Sigma, St. Louis, MO). Both Freon extracts (the aqueous phase) were pooled and filtered through a 0.2 μm Nylon® filter (Millipore, Billerica, MA) prior to analysis for viral titer using dilution series PCR (endpoint titration) (49). The same protocol was also used to titrate the 8fIIa inoculum.

Human Challenge studies

Because of the lack of routine cell culture systems for NoV and animal models of human NoV infection, human challenge studies have contributed enormously to our knowledge of host susceptibility, the dose-response relationship, immune response to infection, clinical course of infection, and the development of NoV vaccine. A number of human challenge studies have been conducted since 1970 (Table 2). The first NV human challenge study was conducted in 1971 by Dolin et al. (47).

Table 2. Summary of Norovirus Human Challenge Studies

		All cases			Secretor (+)			Secretor (-)	
Human Challenge studies	NoV strain	N	No. (%) Infected	No. (%) Ill	N	No. (%) infected	No (%) Ill	N	No. (%) infected
Frenck 2012	GII.4	40	17 (42)	12 (30)	23	16 (70)	12 (52.1)	17	1 (5.9)
Seitz 2011	NV	13	10 (77)	10 (77)	13	10 (77)	10 (77)		
Atmar 2011	NV	41	34 (83)	29 (71)	41	34 (83)	29 (71)		
Leon 2011	NV	15	7 (47)	5 (33)	15	7 (47)	5 (33)		
Atmar 2008	NV	21	16 (76)	11 (52)	21	16 (76)	11 (52)		
Lindesmith 2005	SM	15	9 (60)	7 (47)	12	8 (67)		3	1 (33)
Lindesmith 2003	NV	77	34 (44)	21 (27)	55	35 (64)	21(38)	21	0
Graham 1994	NV	50	41 (82)	34 (68)					
Johnson 1990	NV	42	31 (74)	25 (60)					
Parrino 1977	NV	12	6 (50)						
Wyatt 1974	NV, MC, HI	23		16 (70)					
Dolin 1971	SM	12		9 (75)					

* SM, Snow Mountain virus; NV, Norwalk virus; MC, Montgomery County virus; HI, Hawaii virus; GII.4, genogroup 2, genotype 4. Source: Simmons, K., Gambhir, M., Leon, J., & Lopman, B. (2013). Duration of immunity to NoV gastroenteritis. *Emerging infectious diseases*, *19*(8), 1260–1267. doi:10.3201/eid1908.130472

These human challenge studies are conducted after careful ethical review, rigorous safety precautions, and high levels of medical supervision, to ensure that

there should be no harm to the human subjects. Investigation of NoV shedding in human challenge studies has provided critically important knowledge. Atmar et al. reported that the median peak virus titer could be as high as 10^{10} genomic copies/g feces among subjects experimentally challenged with NV (46).

There are some limitations of these human challenge studies; for example, the majority of the challenge studies used Norwalk virus GI.1 strain, with limited information about infections with other strains, such as GII.4. Generalization of these study results to other populations is not possible, since only healthy adult volunteers were eligible to participate in these studies.

Diagnostic and detection methods

The burden of NoV gastroenteritis has been examined using molecular methods for the detection, identification and characterization of NoVs. Electron microscopy (EM), immuno-electron microscopy (IEM) and radioimmunoassay were used to detect NoVs in the 1970's and 1980's (19). In 1990, a new molecular diagnostic test was used to detect and sequence the virus genome directly from a fecal specimen; this was called reverse transcription-polymerase chain reaction (RT-PCR) (19).

1.1 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR allows the detection and amplification of DNA by using RNA as a template. The RNA is reverse transcribed into complementary DNA (cDNA),

using reverse transcriptase. Amplification via RT-PCR provides a highly sensitive technique, by which a very low copy number of RNA molecules can be detected. It is now widely used in commercial and research laboratories and can detect the virus in specimens collected at various stages of infection even when the quantity of virus is low. The major advantage of this technique is that RT-PCR can detect NoV RNA from fecal samples with as low as 100 particles/ml, even after the acute stage of clinical illness is resolved. RT-PCR has been useful in the detection of many strains of NoVs and in molecular epidemiological studies to identify the source of infection. NoV can be detected in fecal samples stored at 4^{o}C for several months and at -70^{o}C for many years. Although it is the diagnostic tool widely used to identify NoV infection, some disadvantages include its reduced clinical specificity and the fact that this assay requires exquisite care to prevent contamination in the laboratory.

1.2 Real-time RT- PCR

The recent development of real-time quantitative RT-PCR (qRT-PCR) provides a quicker and more sensitive approach than conventional RT-PCR for detection and quantification of NoVs. Quantitative PCR is used to detect, characterize and quantify NoV RNA in clinical and environmental samples. In qRT-PCR, RNA transcripts are quantified by reverse transcription into cDNA and then qPCR is subsequently carried out. Fluorescent labeling enables the collection of data, as the PCR progresses. Two common methods for detection of products in real-time PCR are: i) non-specific fluorescent dyes that intercalate with double-stranded DNA, and ii) 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.

To date, qRT-PCR assay is highly specific, sensitive and offers multi-throughput potential, with the possibility to multiplex for amplification of multiple targets. However, the results can be compromised by contamination in the laboratory. Also, few clinical laboratories are well equipped to perform this analysis as a routine diagnostic method.

Prevention and control

Identification of the mode of transmission of NoV and its interruption by the control of contamination of food and water, maintenance of strict hygiene by food-handlers and reduction of secondary propagation of outbreaks through person-to-person spread are the crucial steps in the prevention and control of NoV outbreaks.

Current guidelines issued by public health agencies for managing NoV outbreaks include: implementing policies concerning hand hygiene, patient isolation and grouping of patients based on symptoms, exclusion of ill workers, visitor

restrictions, enhanced environmental cleaning and disinfection, and ward closures (72).

Food contamination with NoV can occur at various stages: during production, processing, and preparation of food and service. Commonly consumed raw foods, such as shellfish, fruits and leafy vegetables, are the most commonly reported foods associated with foodborne NoV outbreaks (74). Contaminated foods should be removed and appropriately disinfected, and food-handlers should remain off work for at least 48 hours after symptom resolution (75). Staff members involved in food preparation, storage and serving food should adhere to the US FDA food code, to prevent foodborne NoV outbreaks (76). Eliminating bare hand contact with ready-to-eat foods is one of the key infection control measures for preventing foodborne NoV outbreaks. Oyster-associated NoV outbreaks should be controlled by surveillance of shorelines to identify possible sites of contamination of water and enforce the prohibition of overboard dumping of fecal wastes from boats (77).

Maintenance of hand hygiene is one of the most effective ways to control the spread of NoV gastroenteritis during an outbreak. Proper handwashing with soap and running water, for at least 2 minutes, is the most effective way to reduce NoV infection (50). Alcohol-based hand sanitizers should be used as an adjunct to hand-washing, as there is inconclusive evidence for their effectiveness (73). Gloves and masks should be used during the clean-up of vomitus, as they are effective ways to limit the further spread of aerosolized NoVs (71).

Environmental hygiene maintenance plays an important role in NoV infection control. The use of chemical disinfectants is one of the most effective methods used to interrupt NoV spread from contaminated environmental surfaces. Close attention should be given to highly exposed areas and frequently touched surfaces, such as door handles, telephones, mobile phones, and bathroom units. Several studies have demonstrated that chlorine bleach is the most effective disinfectant for NoVs (51). Sodium hypochlorite solution (≥1000 p.p.m) is reliable for achieving a greater than 3 $\log_{10}$ reduction of human NoV on surfaces. Other effective disinfection approaches that can be adopted when chlorine is unavailable, or when surfaces cannot be subjected to it, include pasteurization to 140^{0}F (52), ozone, hydrogen peroxide, and coating surface with antimicrobial materials (e.g., titanium dioxide [TiO2] film) (78). Steam cleaning is effective for soft furnishings, such as rugs, carpets, chairs, and other fabrics (79). Cleaning and disinfection processes should start from unaffected areas and move to affected areas, with special care given to high-contamination areas (79).

Chapter II: Manuscript

Abstract

Noroviruses (NoVs) are a leading cause of epidemic gastroenteritis. Human challenge studies have been used to examine the infectivity, pathogenicity, and host immune response to NoV. Several human challenge studies have been performed using Norwalk virus (a prototype Genogroup I NoV) inocula purified from the stools of infected individuals. Surprisingly little is known about differences in infection and illness in human volunteers challenged with different preparations of Norwalk virus (NV) inocula. The goal of this study was to compare the response among study subjects who received a secondary NV inoculum (8FIIb), compared to subjects who received its precursor, the 8FIIa. We investigated a total of 160 subjects: 76 subjects were experimentally challenged with NV inoculum 8FIIa, and 84 subjects were challenged with 8FIIb. We compared the difference in infection, illness, mean severity score, blood types, and duration of viral shedding between two groups of subjects. We also examined the demographic characteristics and secretor status of 8FIIa- and 8FIIb-inoculated subjects. There were no statistically significant differences in overall infection and illness rates between subjects inoculated with 8FIIa and 8FIIb. However, subjects challenged with 8FIIA dose above the ID50 had significantly more illness (94.1%) compared to subjects who were challenged with high doses of 8FIIb (47.1%), *P*=0.0. Comparison of mean severity score between the two groups of inoculated subjects did not show any significant difference. We observed that infection with 8FIIb was significantly associated with longer duration of viral shedding (P=0.02), and there was a significant difference in duration of viral shedding by dose for both inocula (P=0.00). The results from this study contribute to our understanding and knowledge about NoV infections, pathogenesis, and viral shedding. This information can guide future NoV human challenge studies to test candidate NoV vaccines and treatment efficacy.

Introduction

Noroviruses (NoVs) are a leading cause of epidemic gastroenteritis. They are also an important cause of sporadic acute gastroenteritis worldwide. NoV infection occurs in a variety of settings, including hospitals, nursing homes, military bases, cruise ships, and catered events, and involves people of all ages, from children to elderly persons (80). NoVs are highly contagious and are transmitted through the fecal-oral route via direct person-to-person contact, environmental contamination, or ingestion of fecal-contaminated food or water (19).

Noroviruses are RNA viruses in the family *Caliciviridae*. The human NoV genome is a linear, positive-sense, single-stranded RNA (9). NoV infection in humans is caused by, from highest to lowest frequency, GII (predominantly GII.4), GI, and GIV (14-15). GII.4 is responsible for > 85% of outbreaks in the United States (16). It is critical to understand the genetic diversity and infectivity of NoVs as recent studies show that the emergence of a new variant is frequently associated with an increase in the number of outbreaks in an immunologically naïve population (18).

We acquire most of our knowledge about the NoVs from the study of outbreaks and experimental human challenge studies, since there are difficulties with growing the NoV in cell culture and there is lack of an animal model. Norwalk virus (NV) is a prototype Genogroup I NoV. Several studies have shown that individuals with blood group O are at increased risk of Norwalk virus infection, and Norwalk virus-like particles (VLPs) bound to gastroduodenal epithelial cells,

from individuals who are secretor positive (se+), but not to cells who are secretor negative, Se (-). People who express fucosyltransferase 2 (FUT2) gene are susceptible to Norwalk virus infection, while those who have a nonfunctional FUT2 gene are genetically resistant to Norwalk virus (41-42). The FUT2 gene is responsible for the expression of the histo-blood group antigen H type 1 on the surface epithelium. A decreased risk of Norwalk virus infection and illness is associated with those who are blood group B, and Norwalk virus VLPs bind less to B histo-blood group antigen than to A or H histo-blood group antigens.

NoV infections can be symptomatic and asymptomatic. The predominant symptoms of NoV infection include nausea, vomiting, diarrhea, and abdominal cramps (31). Between 25% to 50% infected persons also develop a headache, low grade fever, chills, and myalgia (32). Diarrheal stool is non-bloody, lacks mucus, and may be loose and watery. About 30% of Norwalk virus infections in human challenge studies are asymptomatic (97), although symptomatic infection is of greater concern, both for individual and public health reasons.

There is no vaccine available to prevent human NoV infection and no specific therapy to treat it. For NoV vaccine development, human challenge studies are necessary, and these require safe, well-characterized inocula. It is important to understand characteristic features of NoV inocula, such as persistence, infectious dose, illness-producing dose, and precise quantification of virus concentration in the inocula. Numerous human challenge studies have been conducted since 1971 to examine the histopathology, pattern of illness, clinical treatment, and immune response associated with NoV infections (81). NV 8FIIa is the original NV

inoculum that was obtained from the feces of individual experimentally challenged in the 1970s. Second generation NV inoculum, designated as 8fIIb, was produced by a single stool sample that originated from a volunteer infected with 8FIIa, and was confirmed to have a high titer of viral RNA using RT-PCR and NV-specific primers (49).

The objectives of this study were to compare illness (defined by illness with diarrhea (alone) in any continuous 24- hour period or one or more vomiting episodes plus one of the following symptoms: abdominal cramps, fever, nausea, chills, myalgia, fatigue or headache (46) and infection (defined by seroconversion and detection of NV RNA in stool by RT-PCR) status in human volunteers inoculated with NV 8FIIa (early inoculum) and 8FIIb (recent inoculum) by the research team at Emory University. This study will also examine the effect of inoculum dose on probability of infection and duration of viral shedding among human volunteers challenged with 8FIIa and 8FIIb. The results from this study will contribute to our understanding and knowledge about NoV clinical infections, pathogenesis and immune response.

MATERIAL AND METHODS

Study participants and sample collection

Human challenge studies

Clinical and laboratory data were obtained from four previously completed NV human challenge studies: NV Dose-range (July 1994-June 1997), NV low Dose

(Jan 1997- Dec 1998), NV Ondansetron Trial (Oct 1997- May 2002) and NV Inactivation in Oysters (Feb 2008- Sep 2009), conducted by the Moe Research Team.

Table 3 summarizes key features of these studies, including study period, inoculum used, and the number of subjects participated in the studies.

Table 3. Norwalk Virus Human Challenge Studies Conducted by Moe Research Team

Trial	**Study Period**	**Inoculum (Dose)**	**No. of subjects[a]**
Dose Range (VT)	July 1994 -June 1997	8FIIa $10 – 10^7$ PDU[b]	45
Low Dose (LD)	Jan 1997 – Dec 1998	8FIIa 0.1 – 10 PDU	31
Ondansetron Trial (GW)	Oct 1997 – May 2002	8FIIb $10^7 – 10^8$ PDU	33
NV Inactivation in oysters (OY)	Feb 2008 – Sep 2009	8FIIb 10^4 genomic copies.	51

aTotal subjects=160
[b]PDU = PCR-detectable units.

Inocula preparation

Inocula for these human challenge studies were prepared by dilution of a stock suspension of Norwalk virus. “8FIIa” is the primary inoculum prepared from the original Norwalk virus isolate in 1971 [Dolin et al., 1971]. Secondary inoculum, which is known as “8FIIb”, was prepared in 1997 from a stool sample from one of the infected subjects in a challenge study in 1995 and was used in a three challenge studies. The inoculum was prepared from a 20% stool suspension,

extracted with Freon and then with 1 x phosphate buffered saline (PBS). The first two extractions were titered, combined and filtered through 0.2 μm Nylon filter. The filtrate was titered and safety tested for a range of pathogens, using standard methods and protocol (22).

Three of the human challenge studies, including “VT”, “LD”, and “GW” Trial participants, received different doses of inoculum, ranging from 10 to 3 X 10^8 PCR-detectable units (PDU), determined by endpoint titration RT-PCR (41). The PDU dose was converted to genome equivalent copies (GEC) by calculation of change factor (from endpoint titration RT-PCR and RT-qPCR titer estimation) for each inoculum and multiplying that change factor with each PDU unit for each inoculum and dose. Mean endpoint titration (PDU/ml) for 8FIIa 7.6x10^7, which is equivalent to mean quantitative RT-PCR (GEC/ml) for 8FIIa 2.9x10^9 and mean endpoint titration (PDU/ml) for 8FIIb 5.9x10^7 which is equivalent to mean quantitative RT-PCR (GEC/ml) for 8FIIb 7.2x10^8. Conversion factor for 8FIIa was 38.16 and 8FIIb was 12.20.

Before challenge and at days 1-5, 8, 14 and 21 days after the challenge.

In the ‘OY’ trial, fifty-one healthy, secretor-positive adults were admitted to the Emory University clinical trial unit in the hospital and randomized into control and intervention groups. Participants received NV 8FIIb inoculum 1.0x10^4 GEC/ml in artificially-seeded oysters, 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. Participants remained in the Emory University clinical trial unit 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 collections and recording of gastrointestinal symptoms, as described in (84).

Laboratory Tests

Stool specimens from all the studies were tested by RT-PCR for NV RNA. A 10% (wt/vol) stool suspension was prepared with water (20%) and an equal volume of Vertrel XF (Dupont, Wilmington, DE), and centrifuged at 13,000 x g for 10 minutes, for viral RNA extraction. Viral RNA was extracted from 140 µl of the supernatant using the QIAamp viral RNA Mini kit vacuum protocol (Qiagen, Valencia, CA). RNA extracts were stored at -20°C, until tested. NV RNA in fecal specimens was detected by conventional or quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) using Qiagen one-step RT-PCR kit (Qiagen, Valencia, CA), using Norwalk-specific primers (NVKS1 and NVKS2) and probe (NVKS3), as described by Liu et al (49). MxPro software, based on the CT value and known copy numbers using the NV RNA standard curves, was used to calculate the quantification of RNA. A stool specimen from NV-infected subjects was included as a positive control in each extraction and qRT-PCR, and water was included as a negative control. All samples were tested in duplicate wells, and the average copy number was calculated. Both wells had to show amplification for determination of the virus titer. Subjects with NV RNA detected in their stool by RT-PCR after Day 1 post-challenge were classified as infected.

All serum samples were tested for Norwalk virus-specific serum IgG by EIA, using Norwalk virus VLP 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. (84). Seroconversion was defined as a greater than or equal to 4-fold increase above Day 0 (pre-challenge serum) anti-NV serum IgG titer in any post-challenge serum sample. Subjects who seroconverted were classified as NV infected.

Saliva samples were secretor phenotype, as described in (85). DNA was extracted from saliva cell pellets using the QIAamp DNA mini-kit (Qiagen, Valencia, California). A fragment of the FUT2 gene was PCR -amplified with GAGGAATACCGCCACATCCGGGGGAGTAC (forward) and ATGGACCCCTACAAAGGTGCCCGGCCGGCT (reverse) primers and digested with Avall (84). The G428A mutation abrogated this restriction site (84). Norwalk VLP-binding to saliva was determined, as previously reported (85).

All study protocols and data collection tools were reviewed and approved by the University of North Carolina at Chapel Hill School of Medicine Institutional Review Board (VT, LD, and GW studies) or the Emory University Institutional Review Board (OY study).

Definitions

Infection was defined as NV RNA in any post-challenge stool sample detected by conventional RT-PCR or qRT-PCR (as described by Liu et al (49)) and/or

seroconversion, (as described by Lindesmith et al. (84)). Viral gastroenteritis or symptomatic infection was defined as illness with diarrhea (alone) in any continuous 24- hour period or one or more vomiting episodes plus one of the following symptoms: abdominal cramps, fever, nausea, chills, myalgia, fatigue or headache (46). "Diarrhea" was defined as ≥3 unformed stools in any continuous 24- hour period and "fever" was defined as oral temperature ≥37.6 ^{o}C. NV seroconversion was defined as a ≥4-fold increase in NV-specific serum IgG units based on an assigned concentration of total NoV IgG units in the reference serum (95). The severity of clinical signs and symptoms for NV infection associated with the 8FIIa and 8FIIb inocula was compared according to a modified Vesikari scoring system, which is the numeric score generated according to the 17-point scale. (98)

Statistical Analysis

All the databases were merged together into a single database for analyses. Data were analyzed with STATA/SE 13.0 software for Windows. Categorical data were analyzed using χ^2 test or Fisher exact test. Continuous variables were analyzed using the t-test. Multivariate logistic regression analysis was performed using multiple independent variables for identifying factors associated with NV infection. Data quality was monitored by establishing sample-tracking sheets and standardized data entry protocol. Any discrepancies between the data sets were resolved by reviewing the hard-copy files and cross-checking the databases. A P-value of <0.05 was considered significant.

Results

As human passage may result in adaptations that can change the infectivity or transmission properties of a pathogen (22), we wanted to examine the infectivity of the secondary 8FIIb inoculum and compare it to its precursor, the 8FIIa inoculum. First, we analyzed information on the demographics, secretor status, and blood group of 160 subjects who were inoculated with either NV 8FIIa or 8FIIb (Table 4). The subjects who received the two inocula differ with respect to secretor status (P=0.00) and ethnicity (P=0.01) (Table 4). More secretor-positive subjects received the 8FIIb inocula because the importance of secretor status for susceptibility to NV infection was known at the time that these 8FIIb studies were conducted. More (34.1%) African-American subjects were inoculated with 8FIIb compared to 25% who received 8FIIa, whereas 51.2% of the subjects who received 8FIIb were Caucasian compared to 71.1% who received the 8FIIa inoculum. A greater proportion of females received the 8FIIb inoculum (59.5%) compared to the 8FIIa inoculum (44.7%), but this difference was borderline significant (P=0.06) (Table 4). There were no significant differences in ABO blood group and age between the subjects challenged with 8FIIa and 8FIIb inocula.

Overall infection rates and illness rates among infected subjects were not significantly different between subjects who received the 8FIIa inoculum compared to subjects who received the 8FIib inoculum (Table 5). Among 76 subjects inoculated with 8FIIa, 28.9% (22/76) developed infection and 23.7% (18/76) of infected subjects developed illness. Among the 84 subjects who were

inoculated with 8FIIb, 38.1% (32/84) developed infection and 21.4% (18/84) of infected subjects developed illness.

Because NV infection is associated with secretor status (42), we examined infection rates and illness for the 156 subjects where secretor status was clearly determined. Table 6 shows NV infection classification based on RT-PCR detection of NV RNA in stool and seroconversion and illness rates for subjects who received either 8FIIa or 8FIIb. This data clearly showed a strong association between secretor status and markers of NV infection (P=0.00). Among 128 Se (+) subjects, 53 (41.4%) had PCR (+) stool, while none of the 28 Se (-) subjects had PCR (+) stool. Among the 128 Se (+) subjects, 48 (37.5%) seroconverted, while no seroconversion occurred in those who were Se (-). Out of 54 infected Se+ subjects, 36 developed symptomatic infections (67%, P=0.00) (Table 6).

Previous human challenge studies have shown that ABO blood types are associated with susceptibility to symptomatic NV infection (41). In our study, we analyzed different blood types and their association with NV infection and illness among infected participants, in both 8FIIa- and 8FIIb-inoculated subjects (Table 7). We found that subjects with blood group O were more likely to become infected compared to blood groups A or B, but this difference was not quite statistically significant (P=0.07). No statistically significant difference was found when comparing illness rates among infected persons with different blood types, but the number of infected subjects with blood group B was very small. Because study subjects were challenged with different doses of 8FIIa and 8FIIb, in order to compare the infectivity and pathogenicity of these two inocula, we

stratified the dose response by greater than and less than or equal to the median infectious dose (ID_{50}). The ID50 was estimated using the data for all four studies and the Spearman-Kaerber method. Subjects in the OY study who ingested HPP-treated oysters with NV were not included in this ID50 estimation because the exact NV dose they ingested could not be determined ie. HPP treatment resulted in a dose equal to or at some level below 10^4 GEC. Table 8 summarizes the in0cula titer, volume ingested, dose in GEC, log10 dose, number of challenged subjects and number of infected subjects for the four studies. The estimated ID_{50} was $2.63x10^4$ GEC.

We then examined the proportion of subjects who had RT-PCR positive stool samples, seroconverted, and had illness for each inoculum, stratified by ID50 (Table 9). There were no significant differences in infection (RT-PCR positive stools and seroconversion) between subjects who received either 8FIIa or 8FIIb inocula. However, subjects challenged with 8FIIa doses above the ID50 had significantly more illness (94.1%) compared to subjects who were challenged with high doses of 8FIIb (47.1%), P=0.01

The severities of clinical signs and symptoms for subjects infected with either 8FIIa and 8FIIb NV inocula were compared by the numeric score generated according to the 17-point scale modified Vesikari scoring system (Schnadower, D. et al., 2013) (Table 10). The severity score for subjects infected with 8FIIa ranged from 1-9, with an average score of 2.26 (95% CI: 1.76, 2.77), and the severity score of subjects infected with 8FIIb inoculum ranged from 0-5, with an average score of 1.94 (95% CI: 1.39, 2.49) (Table 10). Comparison of these average scores showed no statistically significant difference (P=0.45).

Several factors may be associated with risk of NV infection. Identification of those factors can help us to know about susceptibility to NV infection. To identify factors associated with NV infection in human volunteers challenged with 8FIIa and 8FIIb, multivariate logistic regression analysis was performed (Table 12), which included inoculum, gender, ethnicity, blood type, pre-challenge IgG (titer at Day 0), $\log_{10}$ dose in genome equivalent copies (GEC) and age. This analysis was performed on the 128 subjects who were known to be potentially susceptible to infection because of their positive secretor status. The only significant risk factor identified for infection was GEC dose at the highest quartile (p=0.0). As seen in the analyses stratified by inoculum and ID50 (Table 9-NEW), there was no significant difference between risk of infection for subjects challenged with 8FIIa vs. 8FIIb (p=0.13). Gender, ethnicity, pre-challenge IgG titer, blood type, and age did not show any statistically significant association with NV infection in secretor-positive human volunteers challenged with 8FIIa and 8FIIb (Table 12).

Prolonged viral shedding can cause a high number of secondary infections and illness and a high level of endemic exposure. We defined "long duration of viral shedding" as when a subject had a RT-PCR (+) stool after day 5 post-challenge. There was a total of 29 study subjects who had RT-PCR (+) stool after Day 5 post-challenge. We evaluated the association between 8FIIa and 8FIIb challenge and RT-PCR (+) stool greater than 5 days and less than or equal to 5 days post-challenge (Table 13-NEW). We observed that infection with 8FIIb was significantly associated with longer shedding: 67.7% of subjects infected with

8FIIb had RT-PCR positive stools after Day 5 compared to 36.4% of subjects infected with 8FIIa (p=0.02).

We also evaluated if there was an association between long duration of viral shedding and inocula dose (GEC) (Table 14) and observed a significant difference in duration of viral shedding by dose (p=0.0). The majority of subjects with RT-PCR (+) stools after Day 5 post-challenge received a dose between 10^4-10^6 GEC (69.0%) compared to 62.5% of subjects who had RT-PCR (+) stools for less than 5 days post-challenge who were challenged with >10^6 GEC.

Discussion

Norwalk virus is highly contagious. Comparison of infection and illness in subjects who received the 8FIIa inoculum vs. the 8FIIb inoculum provides information about any differences in infectivity and pathogenicity between the two inocula. Human volunteer studies are the main source of understanding about the pathogenesis, infection, and illness associated with NV. Comparison of two inocula from our study results indicate that the two inocula had the same infectivity as measured by the proportion of challenged subjects who developed infection (defined as NV RNA in any post-challenge stool sample detected by conventional RT-PCR or qRT-PCR and/or seroconverted to positive as described by Liu et al. after ingesting a single inoculum). They showed the same infectivity when stratified by dose and considering multivariate logistic regression model (Table 9 &12). This result suggest that adaptation during a single passage through a human subject did not change the infectivity of the 8FIIb inoculum. Previous analysis of the data from these human challenge study (Tenuis, et al., 2008) also reached the same conclusion.

Interestingly, we saw a difference in illness rate between 8FIIa and 8FIIb challenged participants when the dose was higher than ID50 (Table 9). Subjects who were infected from a higher dose of the 8FIIb inoculum were significantly less likely to develop symptoms of acute gastroenteritis (47.06%) compared to those subjects who became infected from a high dose of 8FIIa (94.12%). This result suggest that the 8FIIb may have adapted to be less pathogenic after serial passage through hosts or cell culture. Difference in host immune response may

have played an important role here. However, we did not see any difference in mean severity score between 8FIIa and 8FIIb challenged participants.

It is crucial to know about the duration of NV shedding and association with NV inocula and dose to prevent its transmission. We compared subjects challenged with NV inocula 8FIIa and 8FIIb and duration of viral shedding and observed that a higher proportion of subjects infected with 8FIIb developed long duration of viral shedding (after day 5), but less illness compared to subjects infected with 8FIIa (Table 13). There are several possible reason for this difference, including virulence of the infecting inocula, differences in the populations studied (e.g., age, immune status), host immune response to different inocula to development of illness that is associated with the longer duration of vial shedding.

We also saw a difference in duration of viral shedding by infected participants when stratified by inocula dose (Table 14). Our analyses indicated that a higher percentage of subjects with RT-PCR (+) stool after Day 5 post-challenge received a dose between 10^4 – 10^6 GEC compared to subjects who had RT-PCR (+) stools for less than 5 days post-challenge. Our results indicated that a dose 10^4 – 10^6 GEC can cause longer duration of viral shedding regardless of illness status. Previous NV human challenge studies have shown that viral shedding in stool occurs simultaneously with the onset of illness, and serologic response to infection was associated with virus dose and longer duration of viral shedding (90, 96). This result suggests that a subject's immune status may be associated with longer duration of viral shedding. Participants who had previous

NV infections and had pre-challenge anti-NV antibodies, may shed virus for a short duration after challenge and infection with a high dose, whereas, participants with no previous NV infection or pre-challenge anti-NV antibodies may develop illness after challenge with a low dose and shed virus for long duration.

This is the first study to document the comparison of infection and illness status in 8FIIa- and 8FIIb -inoculated subjects. One strength of our study is that the clinical symptoms data and serial stool samples were collected and analyzed from carefully-monitored NV infections, where the time of exposure and dose were known. Further, all the post-challenge clinical symptoms and stool sample data were collected for seven days post-challenge. There were some limitations of our study. First, in the modified Vesikari scores (Table 10), the assessment of symptom severity was limited as dehydration and treatment were not considered. Second, we were unable to determine the exact day, on which NV shedding ended because of the timing of stool collection during the follow-up period.

TABLES

TABLE 4. Baseline Characteristics of Subjects Challenged with Norwalk Virus Inocula 8FIIa and 8FIIb

	No. (%) of subjects challenged with		
Characteristic	**8FIIa** (N=76)	**8FIIb** (N=84)	***P***
Gender			
Male	42 (55.26)	34 (40.48)	0.06[a]
Female	34 (44.74)	50 (59.52)	
Ethnicity			
African American	19 (25.0)	28 (34.15)	
Caucasian	54 (71.05)	42 (51.22)	
Asian	0 (0)	7 (8.54)	0.01[b]
Multiracial	3 (3.95)	5 (6.10)	
ND[#]	0 (0)	2 (1.25)	
Secretor status			
Positive	50 (65.79)	78 (92.86)	0.00[a]
Negative	22 (28.95)	6 (18.18)	
Weak	3 (3.95)	0 (0)	
ND[c]	1 (1.32)	0 (0)	
Blood Type			
A	27 (36.0)	29 (37.66)	
B	4 (5.33)	10 (12.99)	0.27[b]
O	41 (54.67)	37 (48.05)	
AB	3 (4.0)	1 (1.30)	
ND[d]	1 (1.32)	7 (8.33)	
Age (year)	27. 67 (7.99)[d]	28.70 (9.17)[b]	0.45[e]

[a]Pearson chi *P*-value, P-value indicating probability of statistically significant baseline characteristics difference between 8FIIa and 8FIIb.
[b]Fisher's exact *P*-value
[c]ND: Not determined
[d]Mean (S.D)
[e]two-sample t-test *P*-value,
Total subjects=160

TABLE 5. Infection and Illness in Subjects Challenged with Norwalk virus 8FIIa and 8FIIb

Characteristics	8FIIa	8FIIb	*P*[a]
Infection[b]	No. (%)	No. (%)	
Yes	22 (28.95)	32 (38.10)	0.22[c]
No	54 (71.05)	52 (61.90)	
Illness[d]			
Yes	18 (81.81)	18 (56.25)	0.08[e]
No	4 (18.18)	14 (43.75)	

[a]*P*-value: [c]Pearson chi-square and [e]Fisher exact *P*-value indicating probability of statistically significant difference in infection vs. no infection/illness among infected participants vs. no illness between 8FIIa and 8FIIb.
[b]Infection was defined as NV RNA in any post-challenge stool sample detected by conventional RT-PCR or qRT-PCR and/or seroconverted to positive as described by Liu et al.
[d]Illness was defined as with diarrhea (alone) in any continuous 24- hour period or one vomiting episode plus one of the following symptoms: abdominal cramps, fever, nausea, chills, myalgia, fatigue or headache (46)
Total subjects 8FIIa (N=76), 8FIIb (N=84).

Table 6. PCR Detection, Seroconversion and Illness in Secretor Positive and Negative Subjects Inoculated with 8FIIa and 8FIIb.

		RT-PCR[a]		Seroconversion[b]		Illness[c]	
Secretor status	**N**	**Positive No (%)**	***P*[d]**	**Yes (%)**	***P*[d]**	**N**	**Yes (%)**
Positive	128	53 (41.41)		48 (37.50)		54	36 (66.67)
Negative	28	0 (0)	0.00	0 (0)	0.00	-	-
Total	156					54	

[a]RT-PCR (Reverse transcription PCR): detection of virus RNA in stool
[b]Seroconversion was defined as a ≥4-fold increase in NV-specific serum IgG units (ng/ml) based on an assigned concentration of total NoV IgG units in the reference serum (95)
[c]Illness" was defined as with diarrhea (alone) in any continuous 24- hour period or one vomiting episode plus one of the following symptoms: abdominal cramps, fever, nausea, chills, myalgia, fatigue or headache (46).
[d]Fisher's exact *P*-value indicating probability of statistically significant difference in RT PCR (+) stool/ seroconversion/ Illness among infected participants between secretor (+) and secretor (-) subjects

Table 7 Blood Type and Norwalk Virus Infection and Illness among 8FIIa and 8FIIb Inoculated Subjects

		Infection[a]		Illness[b]		
Blood type	**N**	**Yes (%)**	***P*[c]**	**N**	**Yes (%)**	***P*[d]**
A	56	13 (23.21)		13	8 (61.54)	
B	14	5 (35.71)	0.07	5	2 (40.0)	0.35
O	78	33 (42.31)		33	24(72.73)	

[a]Infection was defined as NV RNA in any post-challenge stool sample detected by conventional RT-PCR or qRT-PCR and/or seroconverted as described by (Liu et al).
[b]illness was defined as with diarrhea (alone) in any continuous 24- hour period or one vomiting episode plus one of the following symptoms: abdominal cramps, fever, nausea, chills, myalgia, fatigue or headache (46)
[c]Pearson chi-square, [d]Fisher's exact *P*-value indicating probability of statistically significant difference in Infection /Illness among infected participants in ABO blood groups Subjects.
Total subjects: 148. (12 participants were not included because of their blood type was not determined)

Table 8. The Dose-response Data for Infection and The ID50 Calculation

INOCULUM	TITER GEC/ul	VOL OF INOCULUM (ul)	DOSE (GEC)	LOG10 DOSE	# EXPOSED	# INFECTED	NOTES
8FIIa	$2.9x10^6$	10^{-6}	2.9	0.46	8	0	LD
8FIIa	$2.9x10^6$	10^{-5}	$2.9x10^1$	1.46	9	0	LD
8FIIa	$2.9x10^6$	10^{-4}	$2.9x10^2$	2.46	8	3	VT & LD
8FIIa	$2.9x10^6$	10^{-3}	$2.9x10^3$	3.46	3	2	VT
8FIIb	$7.2x10^5$		$1x10^4$	4.00	20	9	OY
8FIIa	$2.9x10^6$	10^{-1}	$2.9x10^5$	5.46	8	7	VT
8FIIb	$7.2x10^5$	1	$7.2x10^5$	5.86	8	2	GW
8FIIa	$2.9x10^6$	1	$2.9x10^6$	6.46	6	3	VT
8FIIb	$7.2x10^5$	10	$7.2x10^6$	6.86	18	14	GW
8FIIb	$7.2x10^5$	30	$2.16x10^7$	7.33	1	1	GW
8FIIa	$2.9x10^6$	10	$2.9x10^7$	7.46	3	2	VT
8FIIa	$2.9x10^6$	100	$2.9x10^8$	8.46	5	5	VT
TOTAL					97	48	
DOSE	LOG_{10} (ID_{50})	ID50[a]					
Mean	4.4	$2.63x10^4$					
St dev	0.4						

[a]The ID50 was estimated using the data for all four studies and the Spearman-Kaerber method.

Table 9. PCR Detection, Seroconversion and Illness in Secretor-Positive Individuals, Stratified by Inoculum Dosage

	Dose		RT-PCR[a]	Infection[b]		Illness[c]
Inoculum	GEC[d]	N	Positive (%)	Yes (%)	N	Yes (%)
8FIIa	>ID_{50}[e]	22	17 (77.27)	17 (77.27)	17	16 (94.12)
8FIIb	> ID_{50}	27	14 (51.85)	17 (62.96)	17	8 (47.06)
	P[f]		0.08	0.36		0.01
8FIIa	≤ ID_{50}	28	5 (17.86)	5 (17.86)	5	2 (40.0)
8FIIb	≤ ID_{50}	51	17 (33.33)	15 (29.41)	15	10 (66.67)
	P		0.19	0.29		0.35

[a]RT-PCR (Reverse transcription PCR): detection of virus RNA in stool.
[b]Infection was defined as NV RNA in any post-challenge stool sample detected by conventional RT-PCR or qRT-PCR and/or seroconverted to positive as described by (Liu et al).
[c]Illness was defined as with diarrhea (alone) in any continuous 24- hour period or one vomiting episode plus one of the following symptoms: abdominal cramps, fever, nausea, chills, myalgia, fatigue or headache (46).
[d]GEC=Genome equivalent copies.
[e]ID_{50}(median dose 50) =$2.63X10^4$, [a]RT-PCR
[f]Fisher's exact P-value indicating probability of statistically significant difference in RT-PCR (+), infection, illness among infected participant between 8FIIa and 8FIIb inoculated subjects by inoculum dosage.
Total subjects: 128 (secretor positive)

Table 10. Modified Vesikari Score (MVS) Components

Parameter	1 Point	2 Points	3 Points
Diarrhea			
Maximum number of stools per day	1-3	4-5	≥6
[a]Diarrhea duration (day)	1	2	≥3
Vomiting			
Maximum number of vomiting per day	1	2-4	≥5
Vomiting duration (day)	1	2	≥3
Maximum body temperature, oral (°C)	37.1-38.4	38.5-38.9	≥39.0

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

Adapted from: Schnadower, D., Tarr, P. I., Gorelick, M. H., O'Connell, K., Roskind, C. G., Powell, E. C., … Freedman, S. B. (2013). Validation of the modified Vesikari score in children with gastroenteritis in 5 US emergency departments. *Journal of pediatric gastroenterology and nutrition*, *57*(4), 514–519. doi:10.1097/MPG.0b013e31829ae5a3

Table 11. Comparison of Mean Severity Score of Ill subjects stratified by Norwalk virus Inocula.

Inoculum	N	Mean	95% CI		*P
			Low	high	
8FIIa	76	2.26	1.76	2.77	0.45
8FIIb	32	1.94	1.39	2.49	

*P= t-test *P*-value indicating probability of statistically significant difference in mean severity score between inoculum 8FIIa and 8FIIb.
Total subjects: 108 (Subjects where symptoms information was available). One person was excluded because illness status was not determined.

Table 12. Multivariate Logistic Regression Analysis of Norwalk Virus Infection in Human Volunteers.

Characteristics	OR[a]	95% CI[b]		*P*[c]
		Lower	Upper	
Inoculum				
8FIIa	1			
8FIIb	0.36	0.09	1.36	0.13
Gender				
Female	1			
Male	0.56	0.22	1.41	0.22
Ethnicity				
Asian	1			
African American	0.98	0.08	12.0	0.99
Caucasian	0.76	0.06	8.81	0.82
Multiracial	0.64	0.03	13.17	0.77
Blood group				
A	1			
B	0.65	0.13	3.32	0.61
O	2.35	0.81	6.62	0.12
Pre IgG[d]				
<8	1			
8 - 10	1.17	0.30	4.50	0.82
>10	1.19	0.24	5.77	0.83
Log_{10} Dose (GEC)[e]				
<4 (lower quartile)	1			
4 - 6	4.98	0.92	26.88	0.06
>6 (upper quartile)	26.93	5.4	134.26	0.00

Table 12 Cont'd. Multivariate Logistic Regression Analysis of Norwalk Virus Infection in Human Volunteers.

Characteristics	OR[a]	95% CI[b]		*P*[c]
		Lower	Upper	
Age (years)[f]				
18 - 22	1			
23 - 30	0.77	0.25	2.38	0.66
31 - 53	1.41	0.41	4.84	0.59

[a]OR: odds ratio; [b]CI: confident interval; [c]*P*: p-value; [d]Pre-IgG: Pre-challenge IgG (ng/ml), [e]GEC=Genome equivalent copies, Pre-IgG and Dose GEC (Genome equivalent copies) value transformed into $\log_{10}$, [f]Age: (18-53) years; Total subjects =160,

Table 13. Long Duration of Viral shedding in Infected Subjects Challenged with Norwalk Virus Inocula 8FIIa and 8FIIb

[a]RT-PCR in stool	Inoculum		[b]*P*
	8FIIa No (%)	8FIIb No (%)	
>5 days	8 (36.36)	21 (67.74)	0.02
≤5 days	14 (63.64)	10 (32.26)	
Total	22	31	

[a]RT-PCR (Reverse transcription PCR): detection of virus RNA in stool.
[b]*P*-Fisher-exact *P*- value indicating probability of statistically significant difference in long duration of viral shedding in Subjects Challenged with Norwalk virus 8FIIa and 8FIIb.
Total subjects: 53.

Table 14. Duration of Viral Shedding in Infected Subjects Stratified by Inoculum dose

Log_{10} Dose (GEC[b])	RT-PCR in stool[a]		*P*[c]
	>5 days No (%)	≤5 days No (%)	
<4 (lower quartile)	2 (6.90)	3 (12.50)	
4 - 6	20 (68.97)	6 (25.0)	
>6 (upper quartile)	7 (24.14)	15 (62.50)	0.00
Total	24	29	

[a]RT-PCR (Reverse transcription polymerase chain reaction): detection of virus RNA in stool.
[b]GEC=Genome equivalent copies, GEC value transformed into log_{10}
[c]*P*-Fisher-exact *P*-value indicating probability of statistically significant difference in duration of viral shedding and inoculum dosages.
Total subjects: 53

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Chapter III

Conclusions/ Public Health Implications/Future Directions.

Conclusions/ Public Health Implications/Future Directions

In conclusion, we found that NV inoculum 8FIIa and 8FIIb have the same infectivity, and non-secretor status provides total resistance to NV infection as well as symptomatic infection among 8FIIa- and 8FIIb-inoculated subjects. The lower illness rate among subjects infected with high doses of 8FIIb may be due to changes in the virulence of the inoculum or difference in host immune response. Serologic response to different inocula may modify the effect of dose on illness and duration of viral shedding among 8FIIa- and 8FIIb-challenged participants.

Future studies of the efficacy of candidate NoV vaccines or NoV treatments may require human challenge studies with well-characterized NoV inocula. This study provides valuable information on two NV inocula in terms of infectivity, pathogenesis, and virulence and presents evidence of their stability in terms of infectivity and possible attenuation of virulence. Further study is needed to examine possible differences in immune response after challenge with 8FIIa and 8FIIb NV inocula.