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Infection and Illness in Human Subjects Challenged with Two Snow Mountain Virus (SMV) Inocula

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Infection and Illness in Human Subjects Challenged with Two Snow Mountain Virus (SMV) Inocula

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An abstract of
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Abstract

Infection and Illness in Human Subjects Challenged with Two Snow Mountain Virus (SMV) Inocula

By Hongyan Qu

Background
Snow Mountain Virus is the prototype strain of genogroup II and genotype 2 NoV. Since the 1970s, a number of NoV (including SMV) human challenge studies have been performed, mainly in the United States, to study NoV immune response, pathogenesis, and vaccine efficacy.

Objectives
The aim of this study is to evaluate infection and illness in subjects challenged with two different SMV inocula and identify factors associated with viral shedding and clinical symptoms.

Methods
We analyzed data sets from two SMV human challenge studies previously conducted in 2000-2002 and 2015-2018, respectively. Clinical and laboratory data were analyzed for infection and illness rates, severity scores of acute gastroenteritis in subjects with clinical symptoms, viral shedding, and serum IgG/IgA conversion. Logistic regression analysis was used to examine what factors were associated with post-challenge infection and illness.

Results
The two clinical datasets had a total of 49 subjects. 15 subjects were orally challenged with a first generation SMV inoculum 1 between 2000-2002, and 34 subjects were orally challenged with a second generation SMV inoculum 2 between 2015-2018. There were no statistically significant differences in overall infection and illness rates between subjects challenged with inoculum 1 and inoculum 2. However, individuals challenged with inoculum 1 experienced more severe clinical symptoms of acute gastroenteritis, demonstrating significantly higher severity scores (6.00 vs. 2.94, P=0.003) compared with those challenged with inoculum 2. We also observed that pre-challenge serum blockade antibody titers 50% (BT_{50}) were associated with protection from SMV infection (P=0.046) but not with illness (P=0.146) after controlling for covariates. In addition, the data showed that subjects infected with inoculum 2 tended to have longer viral shedding compared with those infected with inoculum 1.

Conclusions
Understanding the difference between the two SMV inocula is critical for NoV vaccine evaluation because illness and viral shedding are two important outcomes.

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Infection and Illness in Human Subjects Challenged with Two Snow Mountain Virus (SMV) Inocula

Chapter I: Introduction and Literature Review

Introduction

NoV is a leading cause of foodborne illness and acute gastroenteritis in young children globally (1). Over 21 million cases are reported annually in the United States (2). These viruses cause outbreaks in a variety of settings including schools, healthcare facilities, nursing homes, and military bases, etc. (3-6). NoV can infect a population at any age; however, higher mortality and morbidity occur in children under 5 years old, in senior populations, and especially among those with immunocompromised conditions. (7). NoV infection generally causes clinical symptoms of acute gastroenteritis that include vomiting, diarrhea, nausea, headache, fever, chills, and these symptoms usually last a short period of time. Vomiting and diarrhea are the most common symptoms that are present in approximately 50% of the patients in different settings (8). Vomiting and diarrhea are also the main clinical manifestations among children under 5 years old with NoV infection (9).

NoV are a member of the *Caliciviridae* family that comprises a genetically and antigenically diverse genus. NoV are single-stranded RNA viruses of about 7.5kb in length that contain three open reading frames (ORFs) (10). NoV are classified into five genogroups (GI-GV), and each genogroup is further divided into genotypes based on the ORF2 region. Genogroups GI, GII, and GIV only infect humans. Among the human genotypes, genogroup II genotype 4 strains cause about 70-80% of NoV outbreaks in the world for the past decades (11). Snow Mountain Virus is the prototype strain of genogroup II genotype 2 NoV. Since the 1970s, a number of NoV human challenge studies have been performed, mainly in the United States, to better understand NoV immune response, pathogenesis, and vaccine efficacy.

Since 1994, Dr. Christine Moe's research team at the Rollins School of Public Health has conducted nine NoV challenge studies with different objectives. Among these studies, two human challenge studies, one between 2000 and 2002 with the first generation of SMV inoculum (12) and another between 2015 and 2018 with the second generation of SMV inoculum (13), were conducted. Clinical samples (stool, serum, saliva, and emesis) from the study subjects were tested for NoV and antibodies during the study period.

Epidemiology of NoV infection

NoV is a leading cause of acute gastroenteritis outbreaks in communities and a major etiological agent of acute diarrhea in children under 5 years old and in adults in the world (1, 14). Glass et al (14) showed that 60% of acute gastroenteritis cases in the U.S were caused by NoV. With over 21 million annual NoV cases in the United States, the CDC estimated that 71,000 of those cases ended up with hospitalization (2). Boga et al (15) indicated that in Asturias and Spain, 50% of sporadic NoV-associated diarrheal cases in younger children were reported in summer. NoV can infect a population of any age; However, higher mortality and morbidity occur in children under 5 years old and in the senior population, especially among those with immunocompromised conditions.

Many studies reported that NoV transmission occurs in various settings such as airplanes, cruise ships, health care facilities, elementary schools, colleges, and military (3-6, 16, 17). In these settings, NoV transmission is mainly transmitted through fecal-oral route via vomitus and fecal materials. Other transmission routes were also reported including airborne, waterborne, and foodborne (18-29). A systematic review analyzed PCR-confirmed human NoV outbreaks from 1993 to 2011 and indicated that 54% (363/666) of outbreaks were transmitted through foodborne

route; 35% (294/830) of outbreaks were associated with food service settings (30). Genotypes of NoV may also affect modes of transmission. A study investigated 3,960 outbreaks from 2009 to 2013 and found that 72% (2,853/3,960) of outbreaks were caused by the GII.4 genotype, and among those 2,853 outbreaks, 1,838 outbreaks were transmitted through person-to-person transmission route but non-GII.4 genotypes such as GI. 3, GI.7, GII,12 were also transmitted via person-to-person transmission route (31).

NoV persists well in the environment, and disinfectants are difficult to kill the virus on environmental surfaces, which results in repeated NoV outbreaks (32). NoV are highly contagious, but infectivity varies among virus genogroups, and human vulnerability is caused by their genetic characteristics such as secretor status. Genogroups GII and GI are attributed to the majority of human outbreaks, and the GII.4 genotype remained the predominant genotype in the last two decades (33). A study analyzing five foodborne outbreaks found that infection was associated with exposure to NoV-contaminated oysters (34).Several studies have reported that the median infectious dose (ID_{50}) for secretor-positive subjects for GI and for GII was approximately 0.29 (95% Cl:0.015-0.61) and 0.4 (95% Cl:0.04-0.61), respectively. In addition, the median illness dose for secretor-positive subjects for GI and GII was 0.13 (95% Cl:0.007-0.39) and 0.18 (95% CI: 0.017-0.42), respectively. In contrast to secretor-positive subjects, secretor-negative subjects challenged with GII NoV had a significant lower rate of infection (33-35). The mechanism of NoV infectivity for subjects with different secretor status may involve different affinity of NoV VLPs (virus-like particles) binding to carbohydrates on the epithelial cell surfaces of the gastrointestinal tract or in saliva of those subjects with different carbohydrate phenotypes (35, 36).

Researchers have tested candidate NoV vaccines for prevention and control of NoV infections. The tested animal models included mouse, chimpanzees, gnotobiotic pigs, and rats, etc. (37-42). Although these animal models do not develop infections with the majority of the human NoV genotypes, GI.1 and GII.4 NoV have been shown to infect chimpanzees and gnotobiotic pigs (38, 39). When cell culture systems were tested, a few studies reported that NoV can replicate in the human Huh-7 cells and BHK21 cells, and a murine NoV can only grow in the murine macrophage (43-45). These findings advanced our understanding of interactions between virus and host and can provide insight for vaccine research for NoV.

NoV as a cause of diarrheal disease and gastroenteritis in young children

NoV is recognized as an important cause of acute diarrheal disease and sporadic gastroenteritis in young children globally. In developing countries, pediatric gastroenteritis may result in death among infected children. A systematic review reported 200,000 diarrheal deaths per year of children under age of 5 years old in developing countries (46). In Mongolia, it was reported that 37.3 infant deaths per 1000 live births in 1999 were associated with respiratory tract infections, parasitic infections and infectious diarrhea (47). Between July to August 2003, 36 stool specimens were collected from 25 different households from two areas in Mongolia, and 9 of 36 samples (25%) were positive for NoV (48). NoV genotypes varied with locations in the world, and asymptomatic infections are common in children. Many studies have reported that GII.4 and GII.3 were frequently detected in fecal specimens of children under five years old (49, 50).]. For example, a seroprevalence study found that GII.4 infections were significantly higher than GII.3 infections in children under 2 years of age (49-53) in Xi'an, China. In Vellore south India, 53 of 350 (15.1%) children who were hospitalized with acute gastroenteritis were positive for NoV,

and 55 of 500 (11%) children who had diarrhea in the community tested positive for NoV, and 13 of 173 (7.5%) children who didn't have diarrhea were tested positive for NoV (54). In Xi'an, China, 41 of 201(20.4%) hospitalized children with diarrhea were positive for NoV, and 19 of 53 (35.8%) hospitalized asymptomatic children were positive for NoV (53). In Malawi, 220 of (11.3%) 1,941 children five years old or younger with/without acute diarrhea in the hospital were positive for NoV, and GII.4 genotypes were the predominant strains (52). Because the GII.4 genotype has a longer virus shedding period (5 days to 6 months), infected children are likely become NoV reservoirs for person-to-person transmission, especially through fecal matter. In the Netherlands, a prospective cohort study reported that 26% of patients' fecal specimens contained NoV for up to 21 days (9). In Japan (55), 23 (32.4%) of 71 infants 6 months or younger had clinical NoV infections, with a median duration of NoV shedding of 16 days.

After infection with NoV, patients can shed viruses for a long time. For example, NoV could still be detected in fecal specimens collected 5 to 47 days in the follow-up feces specimens after initial infections (55). Several studies reported NoV infection in immunocompromised immunocompetent children, such as children with cartilage hair hypoplasia, experienced an even longer excretion for up to 180 days (50, 51).

NoV outbreaks in different settings

NoV outbreaks occur in a variety of settings that have dense living conditions and shared use of dining equipment. In such conditions, people have close contact, and it is difficult to follow a good personal hygiene. In schools and childcare settings, acute gastroenteritis outbreaks frequently occurred in the last 20 years with different modes of transmission (4, 5, 18, 56) In 1999, a large foodborne NoV outbreak in a daycare center in Sweden was reported, and a

secondary transmission was associated with cases in the daycare and their households (56). In 2001, an outbreak of acute gastroenteritis in elementary school students illustrated that aerosolized viral particles from students' vomit were probably associated with NoV transmission (18). In 2007, an epidemiologic investigation of NoV outbreak in an elementary school (Colombia) showed the virus was spread through the sharing of computer mice and keyboards contaminated by the fingers of cases. In this outbreak, the contaminated surface (environment) was the main route of transmission (4). In 2008, a study reported that NoV was transmitted through vomitus in three universities in different states because of close dormitory quarters (5).

In military settings, NoV outbreaks occurred among military personnel, cadets, support personnel, and crowded US ground troops causing more than 1,000 hospitalizations in the French military parachuting unit, in the U.S. Air Force Academy, and in the military forces (57-59). The transmission modes of these outbreaks were mainly via foodborne, food services, and person-to-person, and caused by densely populated troops and persistent virus excretion.

In cruise ship settings, outbreaks of acute gastroenteritis have been documented for decades, and the etiology of many of these outbreaks is known to be NoV (6, 60, 61). The main mode of transmission among these outbreaks was person-to-person contact and by sharing rooms with infected persons or exposure to infectious virus in vomitus in public areas. In 1977, 521 of 814 cruise ship passengers were infected with NoV in an explosive outbreak of acute gastroenteritis that lasted for four consecutive cruises (62). In 2002, the VSP (CDC's Vessel Sanitation Program) recorded cases of acute gastroenteritis on cruise ships entering the US. Among the 21 reported outbreaks, five were caused by NoV from July 1st to December 2nd (63).

In nursing home settings, many studies have reported outbreaks of gastroenteritis caused by NoV, with transmission route mainly through person-to-person contact between residents and staff in long-term care facilities and caregivers. In nursing homes, NoV is likely to spread between person-to-person due to frequent contact opportunities. However, new research argued that NoV spreads in nursing homes mainly through virus in aerosols (64). From 2009 to 2012, CDC reported that 80 percent of outbreaks in long-term care facilities in the U.S. were recorded as non-foodborne (64, 65). Other studies suggested that in nursing homes, residents may have mental conditions and their personal hygiene might be compromised which facilitates widespread transmission of NoV (66).

NoV genome organization and phylogenetic tree

In 1972, Albert Kapikian first identified Norwalk virus under the immune electron microscope by examining stool samples from diarrhea cases in a school outbreak (Figure 1) (67). Since then, many studies have detected NoV RNA in contaminated food, water, and other environmental samples, stool, and vomitus specimens using molecular techniques such as RT-PCR, real-time RT-PCR, and DNA sequencing. These studies indicated that NoV is an important cause of acute gastroenteritis outbreaks and sporadic gastroenteritis cases in different geographic regions. NoV belong to the *Caliciviridae* family with a genetically and antigenically diverse genus. NoV contains a single-stranded RNA genome of approximately 7.5kb that encodes three open reading frames (ORFs). The ORF1 encodes a large polyprotein that is cleaved into seven nonstructural proteins. The ORF2 encodes the major capsid structural protein (VP1) with a molecular weight of 58 - 60kDa. It contains the S and P major domains. The P2 section of the P domain is on the surface of the virus capsid and is part of the virus that binds to Histo-Blood Group Antigens

(HBGAs) on the surface of the host cells that are believed to serve as the cellular binding receptor for human NoV. The ORF3 encodes a 22-29 kDa molecular weight minor protein (VP2), which is responsible for a natural regulator in virus packaging (10) and VP2 only presents one or two copies in one viral virion (10).

NoV viruses are classified into five genogroups (GI-GV) and each genogroup is further divided into genotypes based on the diversity of the ORF2 region. Genogroups GI, GII, and GIV only infect humans (11, 68). Among these genotypes, genotype 4 in GII causes about 70-80% of NoV outbreaks in the world over the past decades. Snow Mountain Virus (SMV) is the prototype strain of the GII NoV, and data show that it causes about 8% NoV outbreaks (69). Some studies reported that GII.4 strains have a higher percentage of amino acid diversity compared to other GII.2 strains. Based on the molecular and epidemiological data of NoV outbreaks in multiple countries, a phylogenetic tree of NoV was constructed that revealed the diversity of this virus family (Figure 2) (70). This phylogenetic approach also allows scientists to identify novel variants worldwide and plays an important role in the prevention and control of NoV.

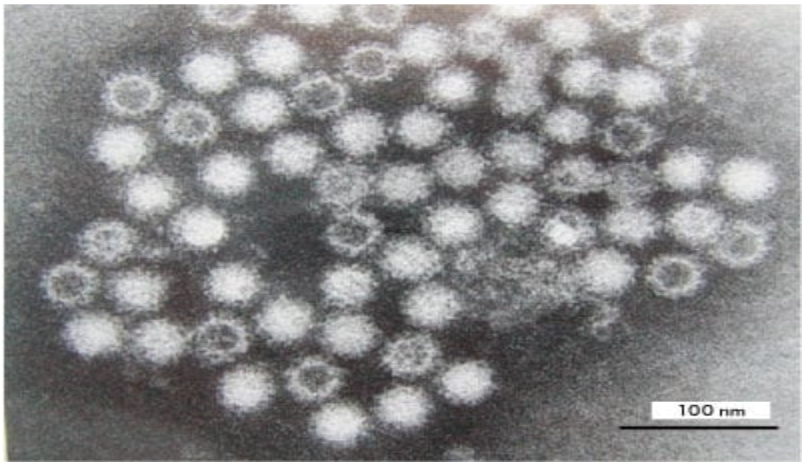

Figure 1: Transmission electron microscope photograph of human NoV particles with Uranyl acetate staining. Bar=100nm (67)

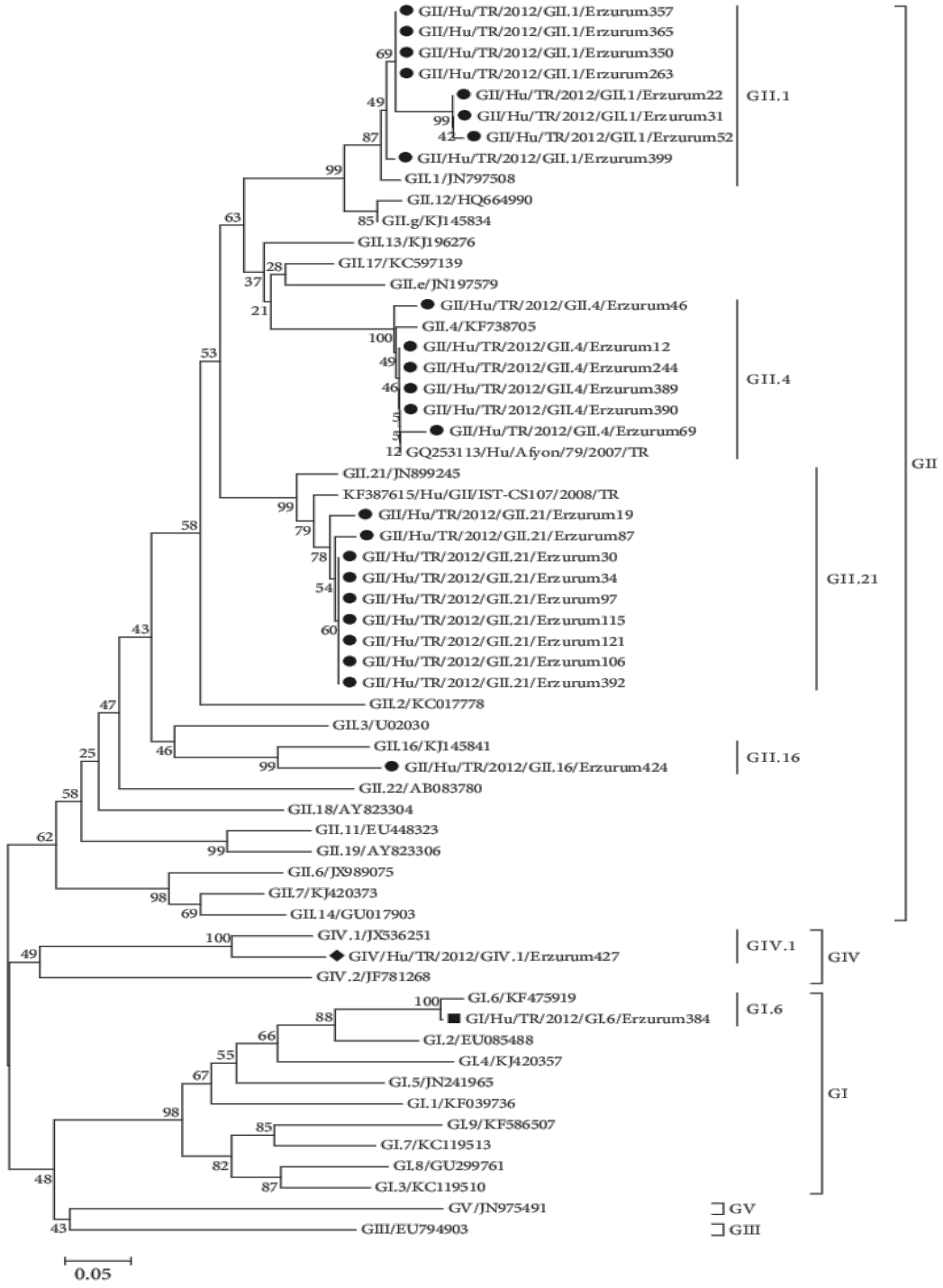

Figure 2: Phylogenetic tree of NoV strains (70).

Clinical Symptoms of NoV Infection

Symptoms of adult infection

NoV infections generally present clinical symptoms of acute gastroenteritis, including vomiting, diarrhea, nausea, headache, fever, chills, and a short course of illness. Among these symptoms, vomiting and diarrhea are the most common manifestations that present in approximately 50% of patients (Table 1). Lopman et. al. analyzed 4 outbreaks in England (71) and found that 1,225 of 1,551 cases (79%) reported diarrhea and 1,039 of 1,551 cases (67%) reported vomiting. The average duration of illness was 2-3 days, but it can last longer in some patients. In an outbreak of 137 NoV-infected staff at child centers in Sweden (56), 71.5% of patients experienced diarrhea, 64.1% vomiting, 96.8% nausea, 87.7% stomach pain, 63.6% headaches, and 44.7% fevers over 38.5 °C. From a human challenge study in the United States (72) , 21 infected individuals exhibited clinical symptoms similar to natural NoV infections. Among the 21 infected subjects, 67% experienced vomiting and 33% had watery diarrhea. Abdominal cramps were reported in 62% of patients, nausea in 67%, and headache in 43% (72).

Table 1. Symptoms of Adult Infection in Selected NoV Outbreaks and Human Challenge Studies

Study	Location	Setting	No. of Patient	Symptom (%)	Reference
Outbreak	Miaoli, Taiwan	Total patients	184	Diarrhea (87.5%) Vomiting (25.5%)	Tseng et al. (2011)
		Hospitalized patients	172	Abdominal pain (4.9%)	
		Healthcare workers	7	Fever (2.2%)	
		Nursing-home residents	5		
Outbreak	Avon, England	Total patients	1,500	Diarrhea (79%) Vomiting (67%)	Lopman et al. (2004)
		Hospital patients	691	Duration 2-3days	
		Hospital staff	482		
		Nursing home residents	266		
		Nursing home staff	112		
Outbreak	Sweden	Child centers	137	Diarrhea (71.5%) Vomiting (64.1%) Nausea (96.8%) Stomach pain (87.7%) Headache (63.6%) Chills (44.3%) Fever (44.7%) Myalgia (48.2%)	GÖtz et al. (2001)
Outbreaks	US	Military troops	90	Diarrhea (67%) Vomiting (80%) Nausea (88%) Abdominal pain (76%) Headache (22%) Fever/Chills (41%) Photophobia pain (3%)	Arness et al. (2000)
Outbreak	England	Bakery plant employees	135	Diarrhea (80%) Vomiting (70%) Abdominal pain (80%) Fever (43%) Headache (43%) Duration 2 days	Brugha et al. (1999)

Human challenge study	US	21	Water diarrhea (33%) Abdominal cramps (62%) Vomiting (67%) Nausea (67%) Anorexia (43%) Headache (43%) Myalgia (33%) Fever (19%) Chills (19%) Malaise (57%)	Atmar et al. (2014)
Human challenge study	US	40	Nausea (40%) Abdominal gurgling (65%) Malaise (43%) Abdominal pain (81%) Headache (43%0 Anorexia (23%) Chills (18%) Muscle aches (13%) Fever (0%)	Frenck et al. (2012)

NoV Symptoms in young children (<5 years)

Vomiting and diarrhea are the main clinical manifestations of NoV infection among children under 5 years old (Table 2). In a NoV outbreak in Ohio, United States (73), 167 of 199 cases (84%) had vomiting, 169 of 199 (85%) occurred with nausea, 123 of 199 (62%) occurred with abdominal cramps, and 87 of 199 (44%) occurred with diarrhea. These symptoms disappeared 12-24 hours post-onset in most patients; however, a small portion of patients had symptoms that lasted more than 48 hours. In Sweden (56), nausea was observed in 93.1% of NoV patients, stomach pain in 88.7%, and vomiting in 80.6%, and diarrhea in 52%. Vomiting lasted a median duration of 6 hours, and diarrhea lasted a median duration of 15 hours. Studies conducted in the Netherlands, Japan, and the United States agreed that diarrhea and vomiting occurred in over 90% and 70% of NoV patients,

Table 2. Clinical Symptoms in Children Under 5 Years with NoV Infection

Study	Location	Setting	No. Patients	Symptoms (%)	Reference
Outbreak	Ohio, US	Elementary school	199	Nausea (85%) Vomiting (84%) Abdominal cramps (62%) Lethargy (53%) Diarrhea (44%) Fever (32%) Chills (5%)	Adler et al. (1968)
Outbreak	Sweden	Childcare centers	136	Diarrhea 66/127 (52%) Vomiting 108/134 (80.6%) Nausea 94/101 (93.1%) Stomach pain 94/106 (88.7%) Headache 30/69 (43.5%) Chills 16/77 (20.8%) Fever 31/88 (35.2%) Myalgia 11/63 (17.5%)	GÖtz et al. (2001)
Outbreak	Netherlands	Community	69	Diarrhea (90%) Vomiting (67%) Fever (32%) Nausea (41%) Abdominal pain[a] (35%) Abdominal cramp[a] (38%)	Rockx et al. (2002)
Outbreak	China	Hospital patients	40	Diarrhea (100%) Vomiting (77.5%) Fever (57.5%) Dehydration (25%)	Wang et al. (2012)
Cross-sectional	Southern India	Hospital patients	12	Diarrhea (100%) Vomiting (42%) Nausea (50%) Dehydration (8%)	Kang et al. (2000)
Cross-sectional	Japan	Hospital patients	59	Diarrhea (94.9%) Vomiting (94.9%) Fever (20.3%) Dehydration (32.2%) Duration 5 days	Murata et al. (2007)

Outbreak	US	Hospital/Com	15[a]	Diarrhea (90%) Emesis (30%)	Munir et al. (2013)

[a] Immunocompromised children

respectively (55). These results are consistent with those of studies conducted in China and Southern India (9, 51), where 100% of NoV patients had diarrhea and 77.5% experienced vomiting. A community-based study by Rockx et al (74) found that the median duration of illness was 6 days in infants younger than 6 months old, and 4 days in children between 1 and 4 years of age. Diarrhea was the primary symptom for the first 5 days and lasted up to 28 days from the onset of clinical symptoms. The large variation in observed symptom duration might be explained by host biological differences and virus types as multiple genotypes often co-circulate in a community.

Asymptomatic NoV Infection

Studies have shown that NoV are associated with both symptomatic and asymptomatic infections among adults and children worldwide (75, 76). Asymptomatic infections of NoV were identified in volunteer studies and outbreaks (77). Table 3 summarizes asymptomatic infection of NoV in young children in multiple countries.

Table 3. Summary of NoV Asymptomatic Pediatric Infections in Multiple Countries

Country	Population	% (positive samples/total samples)	References
Cameron	5-15y	29.6 (16/54)	Ayukekbong et al., 2011
Botswana	children	31.0 (8/16)	Mattison et al., 2010
Ghana	<11y	7.0 (27/367)	Silva et al., 2008
Nicaraguan	≤ 5y	11.7 (19/163)	Bucardo et al., 2010
Mexican	≤2y	29.8 (48/161)	GarcÍa et al., 2006
Brazil	0.5-5y	37.5 (21/56)	Oliveira et al., 2014
England	<18y	26.2 (246/938)	Phillips et al., 2010
	adults	9.1 (115/1267)	

NoV asymptomatic infections were detected in 16 of 54 (29.6%) children in Cameroon (78) and in 21 of 56 (37.5%) children 5 years old or younger in Brazil (79). A study in England reported 9.1% asymptomatic NoV infection in adults, compared with 26.2% asymptomatic NoV infection in children (80), suggesting that asymptomatic infections are very common. Asymptomatic individuals can act as significant virus reservoirs that shed the virus into their surroundings which may result in NoV outbreaks. The occurrence of these NoV outbreaks was determined using the four Kaplan criteria (81): 1) check stool for the absence of bacterial pathogens, 2) mean (or median) duration of symptoms for 12 to 60 hours, 3) the presence of vomiting in more than half of subjects, and 4) the mean (or median) incubation period between 24 to 48 hours.

NoV Human Challenge Studies

To study the pathogenesis and immune response to NoV infection, human challenge studies were conducted (Table 4) because it is difficult to grow human NoV *in vitro* or replicate NoV in small animal models. In 1947, 1953, and 1971, three human challenge studies were conducted (82-84), respectively. These studies showed that clinical symptoms observed in volunteer studies were similar to that of the natural disease that occurred in outbreaks. In the 1972 human challenge study (85), it investigated the biological properties of Norwalk virus and found that NV particle was 36 nm in size, insensitive to acid, lacked a lipid envelope, and was tolerant to heat of 60°C for 30 minutes. In 1974, Wyatt et al (86) conducted a NoV cross-challenge study in volunteers aiming to determine whether antigenically-related NoV agents confer immunity to subsequent challenge with another NoV agent. This study showed that the immunity caused by the Norwalk and Hawaii agents did not protect from disease by subsequent challenge with the other virus, suggesting that Norwalk and Hawaii are antigenically dissimilar agents. However, the immunity

caused by Montgomery virus showed protection against Honolulu virus challenge, suggesting these two agents are antigenically similar. In the 1990s, several researchers (87-90) studied the duration of immunity and antibody titer in volunteers challenged with Norwalk virus. Their results showed that immunity to NV lasted at least 6 months and pre-existing antibodies did not provide protection against Norwalk virus infection. In addition, another human challenge study demonstrated that the virus could be detected in feces within 25 to 72 hours post-challenge, with the highest virus titer found in feces at 15 hours. The investigators also observed that anti-NoV IgG titers increased by more than 4-fold in those who had vomiting using a new ELISA with recombinant virus particles as the antigen source (89). In these studies, asymptomatic infections were first reported where the virus was detected in stools of some subjects who had no clinical symptoms. In the 2000s, several studies (91-95), (12, 36) used human challenge experiments to understand humoral and cellular immune responses, challenge dose-response, and host genetic factors that influenced susceptibility to infection with different NoV genotypes. One study found that Norwalk virus, SMV, and HV viral-like particles (VLPs) had different abilities to bind to histo-blood group antigens, and serum antibodies produced in challenged human volunteers could inhibit this binding (91). This result showed that potential antibody-mediated neutralization of NoV could be an important approach for evaluating NoV vaccine response. Another challenge study (12) showed that infection with SMV strain was not associated with secretor status. In addition, one early challenge study performed in 2002 (96) with Norwalk virus showed that host secretor type influences Norwalk VLP binding and infection and only secretor positive individuals are susceptible to Norwalk virus infection. One recent study showed that among secretor-positive individuals, pre-existing blocking antibodies protect individuals against clinical gastroenteritis after NV challenge (72).

TABLE 4. Summary of Norovirus Human Challenge Studies

				All cases			Secretor (+)			Secretor (-)	
Journal	Year	Author	NoV Strain	N	No (%) Infected	No (%) Illness	N	No (%) infected	No (%) Illness	N	No (%) infected
J Exp Med	1947	Gordan et al		34		10 (29)					
J Exp Med	1953	Jordan et al		91		15(16)					
J Infect Dis	1971	Dolin et al	SMV	16		10 (62)					
Proc Soc Exp Biol Med	1972	Dolin et al	NV	29		16 (55)					
				23		7 (30)					
J Infect Dis	1974	Wyatt et al	NV, MC, HI	23		16(70)					
J Infect Di	1975	Thornhill et al	NV	23		11 (47)					
N Engl J Med	1977	Parrino et al	NV	12		6 (50)					
J Infect Dis	1990	Johnson et al	NV	42	31 (74)	25 (60)					
J Infect Dis	1994	Graham et al	NV	50	41 (82)	34 (68)					
J Infect Dis	1995	Okhuysen et al	NV	38	26 (83)	16 (94)					
Nat Med	2003	Lindesmith et al	NV	77	34 (44)	21 (27)	55	36 (64)	21 (38)	21	0
J Virol	2005	Lindesmith et al	SMV	15	9 (60)	7 (47)	12	8 (67)		3	1 (33)
J Med Virol	2008	Teunis et al	NV	21	16 (76)	11 (52)	21	16 (76)	11 (52)		
J Infect Dis	2012	Frenck et al	GII.4	40	17 (42)	12 (30)	23	16 (70)	12 (52)	17	1 (6)
J Infect Dis	2014	Atmar et al	NV	49	21 (42)	14 (66)	41	21 (51)	14 (34)	8	0 (0)

*SMV, Snow Mountain Virus; NV, Norwalk Virus; MC, Montgomery County Virus; HI, Hawaii Virus; GII.4, genogroup 2, genotype 4. GI.1, genogroup 1, genotype 1.

Laboratory Diagnosis of NoV Infection

Electrical Microscope (EM)

In 1972, Albert Kapikian first identified the prototype Norwalk virus with the size of a 27-nm under an electron microscope in filtered human stool samples collected from an outbreak of acute gastroenteritis in an elementary school in Norwalk, Ohio, USA (97). This report indicated that traditional EM methods could detect NV antigens when viral shedding levels are high. The advantage of EM is that sample preparation time is short and the specific virus morphotype can be detected if the virus is present. The disadvantage of this method is that the specificity to differentiate structurally similar viruses such as NoV and sapoviruses is low. In addition, the sensitivity of EM method is relatively low. Richards et al (98) showed that the sensitivity of EM was 23.9% for detecting NV antigens in individuals with confirmed with NV infections. Pang et al (99) reported 403 false-negative results by EM in 2,486 PCR-positive stool samples from a one-year prospective study. Disadvantages of the EM method are that EM instruments are expensive to maintain and the operation needs specialized technical training. Therefore, only specialized laboratories can use EM for NoV diagnosis.

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

The conventional NoV RT-PCR assays use reverse transcriptase to synthesize complementary DNA from an RNA template extracted from patient stool samples, and the DNA is then amplified with NoV-specific primers and Taq DNA polymerase in a thermocycler. The procedure ends up with an amplified DNA product that can be examined by polyacrylamide or agarose gel electrophoresis. Jiang et al (100) showed for the first time that conventional RT-PCR detected Norwalk virus in stool samples from volunteers in a human challenge study and its

sensitivity was 100 times higher compared to a dot blot hybridization method. The positive detection rate in 55 stool samples was 67.2% detected by conventional RT-PCR. Although the RT-PCR assay has become the gold standard for the detection of NoV in gastroenteritis cases in epidemiological studies, it also has limitations that led to its limited use in clinical diagnostic laboratories. This is because of the high variability associated with multi-step procedures, low yield of amplified products, long run time, labor-intensive, and significant risk of contamination when conducting PCR in an open lab environment. Over time, most of these challenges have been overcome by following well developed protocols with good quality control/quality assurance practices, development of faster thermocyclers, and automation of some parts of the procedure. Another limiting factor is the genetic variation of NoV. The first-generation RT-PCR NoV assay using various primers did not detect NoV RNA in all clinical samples well. Ando et al (101) used second-generation assays targeting conserved regions of the virus genome to examine stool samples from patients with gastroenteritis who were insensitive to various primers among unknown 22 small round-structured virus strains due to the diversity of NoV genomes. Ando et al (101) designed two specific RT-PCR primers, one GI related, and the other GII related. Six unknown strains that belonged to GI, and 16 unknown strains that belonged to GII were detected by the specific RT-PCR primers in 22 small round-structured viruses previously classified by immune election microscopy. Clearly, first- and second-generation RT-PCR assays further increased the sensitivity of NoV detection. Vinjé et al (102) illustrated the sensitivity of five RT-PCR assays including conventional RT-PCR, a panel of second-generation RT-PCR assays, and nested RT-PCR for detection of NoV in a panel of 91 stool specimens. The overall sensitivity rate of the five RT-PCR assays ranged from 52 to 73%. The sensitivity for genotype GI NoV detection was from 54 to 100%, while GII detection was from 58 to 85%.

Real-time quantitative RT-PCR (RT-qPCR) detection of NoV RNA

Currently, real-time RT-qPCR is commonly used for the detection of NoV in stool samples with SYBR green or TaqMan platforms. It addresses some limitations of conventional RT-PCR and EM methods. The advantages of real-time RT-qPCR are manifested in many aspects, such as pre-optimized reagents and amplification conditions, simplified detection and design of primers and probes by software, higher throughputs, ease of reproducibility, and development of multiplex testing for various NoV genotypes in a single reaction, reduced risk of contamination, higher specificity and sensitivity, and software-driven automation of operations compared to conventional RT-PCR. Kageyama et al (103) showed that real-time qPCR has a higher NoV detection rate than conventional RT-PCR and nested RT-PCR, and it was able to detect NoV RNA in 80 of 81 fecal specimens that were positive by EM. The conserved region used to design primers for RT-qPCR not only achieved high sensitivity but also detected a wide range of NoV genotypes including GII.17. Pang et.al (104) developed a multiplex real-time RT-qPCR detection method and detected NoV genogroup GI and GII in stool samples from 97 outbreaks and 726 stool samples from sporadic diarrhea cases, respectively. For this method to be widely used in clinical laboratories globally, there is a need to accurately characterize these NoV detections and to assess the relative performance characteristics of genotyping assays to improve assay sensitivity and specificity. Two similar well-controlled comparative studies were performed in 2003 (102, 105) and a one-step duplex GI/GII TaqMan RT-PCR assay was designed using a modified primer-probe set first reported by Kageyama et al (103). This assay can be finished within 90 minutes with a good reliability and has a clinical sensitivity of 91% for NoV detection. In addition, its sensitivity is higher than the SYBR green real-time RT-PCR method performed by Richards et al (106), but it is slightly lower than the TaqMan assay

developed by Hohne and Schreier (107). The advantage of this assay is that it can detect GI and GII in a single reaction.

Currently, commercial NoV RT-qPCR kits have become available, but the sensitivity of these kits varies based on the different manufacturers. Butot et al (108) showed that AnDiaTec (Germany) and Generon (Italy) NoV real-time RT-PCR kits were unable to detect 59 GI NoV genotypes, while the Generon kit had a lower rate of detection for GII NoV genotypes. Dunbar et al (109) evaluated the efficacy of the RIDAGENE (R-Biopharm, Darmstadt, Germany) NoV real-time RT-PCR in a routine diagnostic laboratory. The overall sensitivity and specificity of the RIDAGENE kit were 98%, with higher sensitivity for detecting GII compared to GI. This kit can detect multiple GI and GII genotypes. Real-time RT-qPCR can be used not only to detect NoV RNA in clinical specimens, but also to detect NoV RNA in environmental samples such as water and food. Currently, CEERAM (France) NoV GI and GII kits and foolproof NoV Detection Kit Biotecon Diagnostics (Germany) are used to detect NoV in environmental samples such as food, surface, seawater, wastewater, and water.

ELISA

ELISA (Enzyme-Linked Immunosorbent Assay) can be used as a complementary method to RT-qPCR diagnostics to screen for NoV antigen in stool samples or anti-NoV antibodies in saliva, and serum. Antigen-detection ELISAs need polyclonal antibodies or MAbs (monoclonal antibodies) that are usually generated using VLP (virus-like particles) as antigen to immunize various animal species. The advantage of ELISA is simple and fast and does not require complex equipment for detection. This approach has the potential to be widely disseminated due

to the use of standard techniques. Vipond et al (110) suggested that the shortcomings of ELISA limited its use to in-house applications rather than a commercial assay due to the lack of sufficient quantity and quality of NoV antigens. With advancement of molecular cloning techniques, it became more feasible to produce highly purified antigens that could led to the availability of commercial kits. ELISA techniques are now frequently used for rapid NoV diagnosis. Denka Seiken Co. Ltd in Tokyo, Japan, and DakoCytomation Ltd at Ely, UK provide two commercial kits (SRSV(II)-AD and IDEIA NLV ELISA), respectively, for detection of human NoV antigens in stool samples. MAbs of the GI and GII, respectively, are coated on the surface of microwells to capture GI and GII antigens from stool samples, and the captured GI and GII antigens are detected by conjugated polyclonal or MAb antibody. When the normalized ratio of sample to negative control is greater than or equal to 2, it indicates the presence of virus antigen in the sample. Bruin et al (111) showed that the sensitivity and specificity was 38% and 96%, respectively, for the Dako kit, and 36% and 88%, respectively, for the Ridascreen kit, using 158 fecal samples from 23 outbreaks. The Dako kit failed to detect two GI strains and one GII strain, while the Ridascreen kit failed to detect five GI and GII strains. Using the same two ELISA kits Burton et al (112) detected antigenically diverse human NoV in stool samples and showed that two ELISA kits have low sensitivity and detection limit for four subgroups of human NoV. These results suggested that both ELISA kits were not sufficient to be a substitute for RT-qPCR methods for the detection of human NoV in clinical diagnosis or outbreaks. Haruko Shirato (113) described a saliva-VLP binding assay, based on an ELISA-based binding assay, to detect and quantify the binding of NoV VLP to HBGAs. This study (113) showed that the total reaction time for HBGA binding to NoV takes about 8 hours.

ELISA can be used to detect IgM, IgA and IgG serum antibodies. The principle of this method is to use VLP of NoV to capture serum antibodies that react with the VLP, followed with a reaction using a goat anti-human immunoglobulin conjugated with an enzyme, such as horseradish peroxidase or alkaline phosphatase. A number of human challenge studies have used ELISA to examine the antibody response, such as IgM, IgA, and IgG, after NoV challenges. Gray et al (114) demonstrated that NoV-specific IgM, IgA, and IgG responses were significantly different among symptomatic and asymptomatic volunteers using indirect enzyme-linked immunosorbent assays. Jiang et al (44) used NoV capsid protein as antigen to detect NoV IgM, IgA, and IgG antibodies in serum. Treanor et al (115) used an ELISA method to detect subclass-specific serum antibody response in adults challenged separately with NoV, Snow Mountain, or Hawaii virus. This method detected fourfold or greater serum IgG antibody response in 15 of 20 volunteers challenged with NoV. Serum antibody response to IgA and IgM were also detected in 6 of 15 volunteers challenged with Snow Mountain Virus and in 2 of 12 volunteers challenged with the Hawaii virus.

Blocking antibodies

Since it is still difficult to culture human NoV and there is no small animal model for *in vivo* human NoV replication, scientists developed a blockade antibody assay that measures the antibodies that block the ability of carbohydrate ligands to bind to NoV VLPs. This blocking assay is highly sensitive and may serve as a measure of protective immunity. However, this method cannot distinguish blocking antibodies generated from different NoV genotypes. Reeck et. al. (116) showed that blocking antibody titers continued to rise after the NoV challenge in human volunteers and peaked at 28 days. More importantly, the blocking antibody titers

remained high and were still detectable on day 180 after challenge. Atmar et al., (117) indicated that the geometric mean titer of blocking antibodies peaked on day 42 post-challenge. Bok et al., (38) used chimpanzees as animal models and demonstrated the persistence of NV-specific blocking antibody titers, ranging from 1:800 to 1:25,600 between 3- and 6-weeks post infection. HBGA blocking assay can be a surrogate method for measuring virus-neutralizing serum antibodies due to its ability to inhibit NoV VLP binding to HBGAs. Reeck et al (116) demonstrated that 6 of 6 subjects with measurable blockade titers prior to virus challenge were 100% free of gastroenteritis after being challenged. However, only 2 of 12 subjects who developed gastroenteritis after challenged had pre-existing blockade antibodies ($P<0.02$). This study also found that subjects without pre-existing serum blocking antibodies had longer viral shedding compared to those with pre-existing blocking antibodies (116). Although the HBGA-blocking assay was used to detect serum virus-neutralizing antibodies in human challenge studies, scientists have also performed experiments to test the ability of serum antibodies to block the binding of recombinant NoV VLPs to H1 or H3 synthetic glycans. The objective of these studies is to use this artificial system to screen serum blocking antibodies in specimens from outbreaks or sporadic clinical gastroenteritis cases.

In addition, some studies have reported that the presence of high levels of heterotypic pre-existing blockade antibodies failed to protect against NoV infection and illness, indicating that there is no consistent, stable immunity over long periods of time (118). Despite the complexity of the host immune response to NoV infection, researchers have found a relationship between pre-challenge NoV-blocking antibody levels and lack of clinical symptoms following experimental challenges, which led to the identification of the potential protective relevance of

blocking antibodies. This discovery helps guide research strategies for developing new vaccines against NoV and further explains the natural host immune response to the virus

NoV Vaccine Development

A major challenge in NoV vaccine development is that there is no easy cell culture system that can grow human NoV, making it difficult to measure neutralizing antibodies. Although HBGA-blocking antibodies can be used as surrogate for neutralizing antibodies, it involves the complex issues of host genetic variability. In addition, our understanding of heterotypic immunity is limited, and the evolution of NoV genogroups and genotypes is rapid, especially the evolution of genogroups GII.4, GII.7, and GII 17 in special immunocompromised populations such as transplant patients. Understanding the immune profile of NoV variants plays a key role in the success of vaccine development.

Despite all the potential obstacles to the development of an effective vaccine, scientists remain committed to the research and have developed VLP-based NoV vaccines. These VLP vaccines can be monovalent GI.1 or bivalent GI.1/GII.4. Animal model studies have demonstrated that the inoculation of mice with GII.4 VLPs and chimpanzees with GI.1 VLPs resulted in homologous immunity rather than heterologous protection (91). In a clinical trial of the NoV GI.1 VLP vaccine by both oral and intranasal administration, Tacket et al (119) indicated that in volunteers who received 250 μg of VLP vaccine, the number of IgA antibody-secreting cells peaked on day 7 after the first vaccination and on day 28 day after the second vaccination. Serum anti-VLP IgG titers were detected in 90% (9 of 10) volunteers after two doses of 250 μg on days 0 and 21 days, respectively. Ball et al (120) showed that serum IgG anti-VLP titers increased >fourfold in 83% (15 of 18) of volunteers after a single dose of 259 μg. Atmar et al., (121) conducted vaccination with a GI.1 VLP vaccine in healthy secretor-positive adults and

then they were challenged with NoV genotype GI.I. The results showed that GI.1 VLP vaccine significantly reduced illness in the vaccination group compared to the placebo group (37% vs 69%, *P*=0.006) and reduced infection after challenge with GI.I strain (61% vs 82%, *P*=0.05). A bivalent GI.I/GII.4 VLP vaccine has also been evaluated in healthy adults (122). This GII.4 VLP antigen is similar to the GII.4 strains of NoV virus that caused widespread NoV outbreaks globally in the past 30 years. Bernstein et al (123) randomized healthy adults to 2 doses of GI.1/GII.4 VLP vaccine followed by challenge with GII.4 virus. The results showed that the vaccine group did not have a significant reduction in illness and infection compared to the placebo group (27 of 50 vs 30 of 48). But the bivalent VLP vaccine significantly reduced the modified Vesikari score from 7.3 to 4.5 (*P*=0.002).

In summary, based on the current evidence, vaccination has been shown to provide homotypic protection against illness and infection in clinical studies. The next steps in developing an effective NoV vaccine will be addressing many challenges such as immunogenicity, vaccine efficacy, and heterologous immunity.

Chapter II: Manuscript

Abstract

Background
Snow Mountain Virus is the prototype strain of genogroup II and genotype 2 NoV. Since the 1970s, a number of NoV (including SMV) human challenge studies have been performed, mainly in the United States, to study NoV immune response, pathogenesis, and vaccine efficacy.

Objectives
The aim of this study is to evaluate infection and illness in subjects challenged with two different SMV inocula and identify factors associated with viral shedding and clinical symptoms.

Methods
We analyzed data sets from two SMV human challenge studies previously conducted in 2000-2002 and 2015-2018, respectively. Clinical and laboratory data were analyzed for infection and illness rates, severity scores of acute gastroenteritis in subjects with clinical symptoms, viral shedding, and serum IgG/IgA conversion. Logistic regression analysis was used to examine what factors were associated with post-challenge infection and illness.

Results
The two clinical datasets had a total of 49 subjects. 15 subjects were orally challenged with a first generation SMV inoculum 1 between 2000-2002, and 34 subjects were orally challenged with a second generation SMV inoculum 2 between 2015-2018. There were no statistically significant differences in overall infection and illness rates between subjects challenged with inoculum 1 and inoculum 2. However, individuals challenged with inoculum 1 experienced more severe clinical symptoms of acute gastroenteritis, demonstrating significantly higher severity scores (6.00 vs. 2.94, P=0.003) compared with those challenged with inoculum 2. We also observed that pre-challenge serum blockade antibody titers 50% (BT_{50}) were associated with protection from SMV infection (P=0.046) but not with illness (P=0.146) after controlling for covariates. In addition, the data showed that subjects infected with inoculum 2 tended to have longer viral shedding compared with those infected with inoculum 1.

Conclusions
Understanding the difference between the two SMV inocula is critical for NoV vaccine evaluation because illness and viral shedding are two important outcomes.

Introduction

NoV is a leading cause of acute epidemic non-bacterial gastroenteritis in children <5 years (124) and adults of all ages (125) worldwide. In the United States, NoV infection is associated with an estimated 71,000 hospitalizations and 21 million illnesses per year (126). NoV infection is characterized by the acute onset of vomiting, diarrhea, nausea, abdominal cramps, and fever, which generally last for 2-3 days. Transmission occurs primarily through fecal-contaminated water, food, hands, and environmental surfaces, and person-to-person by the fecal-oral route (127).

NoV genome is a linear, positive-sense, and single-stranded RNA, and the virus is grouped into at least ten genogroups (GI-GX) and 49 genotypes based on the major structural protein (VP1) sequence diversity (68). Among those genogroups, only genogroup I (GI), GII, and GIV are associated with human gastroenteritis. NoV GI is further divided into 9 genotypes, and GII is further divided into 22 genotypes (68). Snow Mountain virus (SMV) is the prototype strain of genogroup II genotype II NoV. Genogroup II genotype 4 (GII.4) are the most prevalent strains detected in outbreaks around the world for the past two decades (128).

The human challenge model has been used as an important tool for studying the pathogenesis and immunology of NoV infection, and the efficacy of NoV vaccine candidates. Since 1994, Dr. Christine Moe and her research team have conducted 9 NoV challenge studies with different objectives. Among these studies, two human challenge studies, one between 2000 and 2002 with the first generation of SMV inoculum (12) and another between 2015 and 2018 with the second generation of SMV inoculum (13), were conducted.

The objectives of this analysis were to compare infection (defined by seroconversion and detection of SMV RNA in stool by RT-qPCR) and illness (defined by clinical symptoms) status

among subjects challenged with the early SMV inoculum (inoculum 1) prepared by Dolin et al around 1980 (12, 129) or a SMV inoculum prepared around 2009 (13) (inoculum 2) in previous human challenge trials conducted by Dr. Moe. The severity of illness, duration of the viral shedding, and serum IgG and IgA conversion among human volunteers challenged with the two SMV inocula were compared. The relationship between pre-challenge serum blockade antibody and post-challenge infection using logistic regression analysis was analyzed. The results from this analysis will contribute to our understanding of SMV infectivity and pathogenesis.

MATERIAL AND METHODS

Human challenge studies

SMV human challenge study 1: This study was conducted between 2000-2002 to examine the infectivity of the first generation of SMV inoculum (inoculum 1) at the University of North Carolina (12). Fifteen healthy human subjects were admitted and completed the challenge study. Each subject was challenged with one dose of SMV inoculum, ranging from 10 to 10^5 genome copies. Subjects remained in the hospital clinical research unit for 5 days after the challenge. Stool, serum, vomitus, saliva samples, and clinical information were collected. Subjects were also followed up on days 8, 14, and 21 post-challenge for sample collection (12).

SMV human challenge study 2: This study was performed between October 2015 and November 2018 at Emory University's Hope Clinic, and the results were reported by Rouphael et al (13). The primary objectives of this study were to evaluate the dose-response relationship between infection and illness and immune responses after a double-blind, placebo-controlled human challenge study with a new second generation SMV inoculum (inoculum 2) that was prepared from a stool specimen from an infected subject in Study 1. The study consisted of 4 sequential cohorts. In cohorts 1 through 3, nine individuals per cohort were challenged with SMV and the

remaining two subjects received the placebo. Cohorts of subjects were sequentially challenged with one of three doses of the SMV challenge stock beginning with 1.2×10^4 genome equivalent copies (GEC) with the objective to determine the optimal challenge dose. Once illness and safety data were available for a cohort, real-time review of the data was undertaken by the protocol team to determine the appropriate dose for the subsequent cohort. For example, if 75% or fewer subjects in Cohort 1 (challenged with a dose of 1.2×10^4 GEC of SMV) became ill, Cohort 2 received a dose that was two logs higher (1.2×10^6 GEC). If greater than 75% of subjects in Cohort 1 became ill, Cohort 2 would have received a dose that was two logs lower (1.2×10^2 GEC). Once the optimal challenge dose was determined through Cohorts 1 through 3, an additional cohort (Cohort 4), composed of eight secretor-negative subjects and three secretor-positive subjects, was enrolled in the study to determine the safety, illness, and infection with the SMV challenge stock in secretor-negative individuals. All individuals in this cohort were challenged with a dose of 1.2×10^7 GEC of SMV.

Definition of SMV infection

Volunteers were determined to be infected if the virus in feces was detected by SMV-specific RT-PCR (Human Challenge Study 1) or RT-qPCR (Human Challenge Study 2) on any post-challenge day or at least a 4-fold increase in SMV-specific serum IgG from pre-challenge to day 45 after challenge by ELISA (Table 5).

Measurement of serum antibodies

SMV-specific serum immunoglobulin A (IgA) and IgG were measured by SMV VLPs (virus-like particles), (12) for serum samples collected at day 1 (pre-challenge) and days 2-6, 15, 30, and 45 of the post-challenge. Antibody concentrations were reported in μg/L.

SMV-specific carbohydrate blockade antibody was measured using an adaptation of a previous protocol developed for Norwalk virus (Atmar et al (130)) for samples collected at day 1 and days 6, 15, 30, and 45 after SMV challenge with inoculum 2, not in early study with inoculum 1.

All the assays, including the detection of anti-SMV IgG and IgA antibody in serum, detection and quantification of SMV RNA in stool, and detection of SMV blockade antibody have been described previously (12, 13).

Statistical Analysis

The databases from the two SMV human challenge studies were merged into a single database for analyses. Any discrepancies between the data sets were resolved by reviewing the hard-copy files and cross-checking the databases. Data were analyzed using SAS 9.4 (SAS, Cary, NC) for Windows. Categorical data were analyzed using χ^2 test or Fisher exact test. Continuous variables were analyzed using the *t* test or Mann-Whitney *U* test. A *P*-value of <0.05 was considered significant. Severity score criteria for subjects with clinical symptoms were calculated using an 18-point numerical scoring system modified from Ruuska and Vesikari's score system (131) based on the duration of diarrhea and vomiting, the maximum number of episodes of diarrhea and vomiting within 24-hours period, headache, fever, chill, fatigue, nausea, abdominal cramps, and myalgia that was based on whether the subject experienced these symptoms "yes, more than one" or "no". Fever was graded at four levels ($\leq$37.0°C, 37.1°C-38.4°C, 38.5°C-38.9°C, $\geq$39°C) by clinical diagnostic criteria in the hospital. Table 6 shows the severity score criteria determined by NoV clinical symptoms and the previous Vesikari score system (131).

Results

First, we analyzed the demographics of subjects who were inoculated with SMV inoculum 1 and inoculum 2. There was no significant difference in gender, age, ethnicity, and secretor status between subjects inoculated with the SMV inoculum 1 and inoculum 2 (Table 7). However, more African American subjects participated in the study with inoculum 2 than the study with inoculum 1 (64.7% vs. 26.7%), while more white subjects participated in the study with inoculum 1 (73.3%) compared to the study with inoculum 2 (29.4%).

We examined infection and illness rates among the subjects and compared the subjects who received the SMV inoculum 1 (n=15) to those who received the SMV inoculum 2 (n=34). Overall, SMV infection occurred in 9 of (60%) 15 subjects infected with inoculum 1 and in 25 of (73.5%) 34 subjects infected with inoculum 2. Illness occurred in 7 of (46.7%) 15 subjects infected with inoculum 1 and 11 of (32.4%) 34 subjects infected with inoculum 2. There were no statistically significant differences in overall infection (60.0% vs. 73.5%) and illness (46.7% vs. 32.4%) rates (Table 8).

In addition to overall infection and illness following SMV challenge, we were interested in exploring possible differences in the severity of illness in the 18 infected subjects who met our definition of illness (Table 9). The severity of illness was assessed using the 18-point numerical scoring system that was modified according to Ruuska and Vesikari's description (130). The severity score for subjects infected with SMV inoculum 1 showed an average score of 6.00 [95% CI (confidence interval): 4.97, 7.03], and the severity score of subjects infected with SMV inoculum 2 showed an average score of 2.94 (95% CI: 1.74, 4.14) (Table 9). Comparison of these average scores showed a statistically significant difference ($P = 0.003$).

We also examined virus shedding rates in subjects challenged with inoculum 1 and inoculum 2 post-challenge (Table 10). The RT-PCR results indicated that viral shedding was not significantly different in subjects infected with inoculum 1 and inoculum 2 in stool samples collected during days 1 and 3 post challenge, but subjects infected with inoculum 1 had significantly more ($p<0.0001$) PCR-positive stools during days 4-6 (46.6%) compared to none of the subjects infected with inoculum 2 having PCR-positive stool samples during that period of time. When shedding duration was compared between these two groups, three stool samples from Days 15-45 post-challenge from subjects infected with inoculum 2 had SMV RNA (8.8%), but no subject infected with inoculum 1 shed virus between 15 and 45 days post-challenge.

We also observed serum IgG conversion in subjects infected with inoculum 1 and inoculum 2 post-challenge (Table 10). During days 1-3 post-challenge, none of the subjects in both groups showed SMV serum IgG conversion. During day 4-6 post-challenge, eight subjects infected with inoculum 1 showed significantly more ($P<0.0001$) serum IgG conversion compared to none of the subjects infected with inoculum 2 showed serum IgG conversion during that period. Most serum IgG conversion occurred between 15 and 30 days after the challenge, but there was not a significant difference between the two groups. At day 45 post-challenge, 13 subjects infected with inoculum 2 still showed serum conversion, and only 1 subject infected with inoculum 1 showed serum conversion but this was not a significant difference ($P=0.28$) (Table 10).

We used logistic regression models to examine what host factors were associated with SMV infection and illness (Table 11 and 12). Serum IgA pre-challenge concentration (log_{10}), serum IgG pre-challenge concentration (log_{10}), pre-existing serum blockade titer (BT_{50}) using 12.5 as the reference, secretor status (negative as reference), race (white as reference), and age (age≤30 as reference) were included in the models. This analysis was only performed for the study with

inoculum 2 because the study with inoculum 1 did not have serum blockade antibody data. Logistic regression analysis indicated that pre-challenge serum blockade antibodies levels (serum blockade titer BT_{50}=50) were associated (*P*=0.046) with post-challenge infection but not with illness (*P*=0.295) after controlling for covariates. Another interesting result was that serum IgG baseline concentration was likely positively associated (*P*=0.071, borderline significance) with post-challenge infection. This association may reach significance with a larger sample size.

Discussion

NoV are a leading cause of foodborne diseases and acute gastroenteritis in young children globally. Over 23 million of NoV cases were reported annually in the United States (1, 2). To study the pathogenesis and immunity of NoV, human challenge studies are conducted because growing human NoV *in vitro* is difficult, and no small animal model is available. In this study, comparison of infection and illness in subjects challenged with SMV inoculum 1 and inoculum 2 demonstrates whether there is a difference in infectivity and illness among subjects inoculated with the first and second generation of SMV inocula. This study indicates that the two SMV inocula had similar infectivity as measured by the proportion of challenged subjects who developed infection and illness after challenge with a single dose of SMV inoculum. Interestingly, analysis of the severity scores of those infected subjects with clinical symptoms showed that those subjects challenged with SMV inoculum 2 had less clinical symptoms of acute gastroenteritis, and more delayed viral shedding was observed in some subjects infected with inoculum 2 compared to those challenged with inoculum 1. These results are consistent with what we recently reported (132) that subjects challenged with a first generation Norwalk virus inoculum (prepared in 1971) had significantly higher severity scores of acute gastroenteritis, but

shorter duration of viral shedding compared with those challenged with a second generation NV inoculum (prepared in 1997). Another reason for subjects challenged with SMV inoculum 2 having less clinical symptoms of acute gastroenteritis was possibly because of higher pre-challenge serum blockade antibody titer in some subjects challenged with inoculum 2 than in subjects challenged with inoculum 1 since higher blockade antibody levels protect subjects from subsequent infection and reduce the severity of clinical symptoms of acute gastroenteritis [114]. Our logistic regression analysis indicated that pre-challenge serum blockade antibody levels (serum blockade titer BT_{50}=50) were negatively associated (P=0.046) with post-challenge infection but not with illness (P=0.295) after controlling for covariates (Table 11 and 12). This result is consistent with Reeck et al (116) who observed that those with higher titers of pre-challenge blockade antibody against Norwalk virus had good protection against NV challenge.

Most subjects demonstrated clinical symptoms, along with viral shedding in their feces, for the first several days of infection. A previous Norwalk virus human challenge study indicated those without pre-existing serum blockade antibodies had a significant higher viral titers than those with pre-existing serum blockade antibodies and more severe clinical symptoms of acute gastroenteritis compared to those with preexisting blockade antibodies (116). In our study, all infected subjects challenged with SMV inoculum 1 shed virus for six days post-challenge, but three episodes (8.8%) of viral shedding occurred between days 15 and 45 in subjects challenged with inoculum 2. We hypothesize that this long viral shedding may be associated with reduced virulence of inoculum 2 and accumulation of NoV amino acid/nucleotide mutations in the genome (133, 134). Another reason for this long viral shedding may be related to pre-challenge blockade antibody levels since our logistic regression model (Tables 11 and 12) showed that

blockade antibody protects subjects from post-challenge infection. In addition, long viral shedding may be associated with virus detection methods. In the recent study with SMV inoculum 2, viral shedding was assessed by a more sensitive RT-qPCR, but in the early study with inoculum 1, a less sensitive conventional RT-PCR was used to test the stool samples for SMV RNA. Clearly, these different PCR methods may have sensitivity difference but are not sufficient to cause a significant disparity in the duration of viral shedding between the two studies since our previous publication indicated that NV-qPCR was only 1.1-1.6 times more sensitive than endpoint titration RT-PCR (135). We hypothesize that the less severe clinical symptoms of acute gastroenteritis, but possibly longer SMV shedding, observed in those subjects inoculated with inoculum 2 are associated with viral factors, such as viral mutations, rather than host factors and/or laboratory assessment methods.

Both host and viral factors are likely associated with more or less severe clinical symptoms of acute gastroenteritis in subjects challenged with NoV inoculum. Host factors that may affect SMV illness include acquired and innate immunity prior to challenge, age, gender, ethnicity, and individual genetic characteristics, such as secretor status, but these factors may or may not affect probability of SMV infection. Virus inoculum factors include virus source, inoculum titer, how the inoculum was prepared and stored, and possible virus evolution, etc. Among these factors, we hypothesize that viral mutations may have occurred in the second generation SMV inoculum (inoculum 2) and may have played a role in the change of the severity of acute gastroenteritis of those subjects infected with inoculum 2. As the prototype of NoV genogroup II, SMV causes a small percentage of NoV outbreaks, and little is known about the mutation of this virus. Swanstrom et al., reported that the sequence of the P2 domain of SMV strains has evolved over

time but less extensively than has been reported for GII.4 NoV strains. As a RNA virus, mutations in the SMV surface protein can significantly alter its antigenicity in the strains between 1976 and 2010 (136), which could also change viral function, pathogenesis, transmission, and infectivity, etc. In addition, studies of other RNA viruses indicate that viral mutations can be deleterious or favorable to the pathogens in terms of infectivity. For example, highly pathogenic avian influence viruses (H5N1 and H7N7) demonstrated enhanced virulence in mice after a few *in vivo* passages (137), whereas the H9N2 avian influenza virus showed gradual loss in lethality for chicken embryos after serial passages (138). Further evidence indicated that amino acid substitutions or deletions of influenza genome were associated with changes in influenza infectivity and pathogenesis (137-139). This phenomenon has been observed in recent years with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic in the world. Since COVID-19 was first recognized in 1999, SARS-CoV-2 has continued to mutate in the gene encoding the spike (S) protein, and at least five variants of concern (VOC) have been reported, Alpha, Beta, Gamma, Delta, and Omicron. The emergence of each VOC has resulted in new waves of infections and adaptive mutations in the SARS-CoV-2 genome that alter its transmission, hospitalization, and mortality rates in human hosts. Omicron variants, compared to its ancestral variants, had significantly higher transmission rates but lower hospitalization and mortality rates (140, 141). Given the evidence from influenza and SARS-CoV-2, we hypothesize that the less severe symptoms associated with second generation SMV inoculum 2 infection in this study is likely because of intra-host SMV mutations during the course of infection in the infected subject who was the original source of the inoculum 2 and further inter-host mutations in the subjects who were infected with inoculum 2 after challenge.

Interestingly, the results of less severe symptoms and longer viral shedding associated with SMV inoculum 2 in this study are consistent with the recent publications (13, 142) indicating that the infectivity of inoculum 2 stock was significantly lower than inoculum 1 based on ID_{50} estimation of both inocula using β Poisson methodology (142) and SMV human challenge model (13). If inoculum 2 does not elicit more severe symptomatic illness, this will have serious implications for the use of this inoculum in future human challenge studies to test candidate NoV vaccines since it will be more complex and expensive to do those trials because a larger number of study subjects will be required to assess the efficacy of the vaccine to prevent illness.

TABLES

Table 5. Serum SMV IgA and IgG Antibody GMC[a](µg/L) and GMFR[b] with 95% Confidence Intervals by Time Points

		Inoculum 1 (N=15)			Inoculum 2 (N=34)		
Time Point	N	GMC (95% CI)	GMFR (95% CI)	N	GMC (95% CI)	GMFR (95% CI)	*P*
Day 1							
IgA(log_{10})	0	-	-	34	3.90 (3.66-4.17)	-	
IgG(log_{10})	15	4.33 (4.10-4.58)	1.00	33	5.13 (4.99-5.28)	1.00	<0.00001[c]
Day 5							
IgA(log_{10})	0	-	-	32	4.08 (3.90-4.27)	1.02 (1.00-1.04)	
IgG(log_{10})	15	4.44 (4.23-4.66)	1.02 (0.99-1.06)	31	5.18 (5.04-5.33)	1.00 (0.99-1.01)	<0.0001[c] 0.2128[d]
Day 6							
IgA(log_{10})	0	-	-	32	4.13 (3.84-4.45)	1.04 (0.97-1.11)	
IgG(log_{10})	13	5.03 (4.67-5.42)	1.18 (1.08-1.30)	29	5.26 (5.11-5.41)	1.02 (1.01-1.03)	0.2482[c] 0.0045[d]
Day 15							
IgA(log_{10})	0	-	-	32	4.58 (4.29-4.90)	1.15 (1.06-1.25)	
IgG(log_{10})	14	5.14 (4.81-5.50)	1.19 (1.09-1.31)	29	5.73 (5.50-5.97)	1.11 (1.06-1.16)	0.0051[c] 0.1231[d]
Day 30							
IgA(log_{10})	0	-	-	31	4.47 (4.13-4.83)	1.12 (1.03-1.23)	
IgG(log_{10})	13	5.05 (4.68-5.45)	1.15 (1.07-1.25)	28	5.78 (5.56-6.01)	1.12 (1.07-1.17)	0.0015[c] 0.4741[d]
Day 45							
IgA(log_{10})	0	-	-	27	4.61 (4.44-4.78)	1.13 (1.07-1.17)	
IgG(log_{10})	1	5.43	1.15	27	5.78 (5.56-6.01)	1.12 (1.07-1.17)	0.5165[c] 0.8857[d]

[a]GMC: geometrics mean concentration

[b]GMFR: geometrics mean fold rise compared to pre-challenge

[c]Two sample t-test p-value for GMC

[d]Two sample t-test p-value for GMFR

"-" missing data

Table 6. Modified Vesikari Score (131)(18-point scale) for SMV Infected Volunteers with Clinical Symptoms

Clinical symptoms	Points
Duration of diarrhea days	
0	0
1	1
2-3	2
≥4	3
Maximum number of diarrhea stools/24h	
0	0
1-3	1
4-5	2
≥6	3
Duration of vomiting days	
0	0
1	1
2	2
≥3	3
Maximum number of vomiting episodes /24h	
0	0
1-3	1
4-5	2
≥6	3
Chills	
No	0
Yes	2
Headache, Nausea, Abdominal cramp, and Myalgia	
Yes≥1	1
No<1	0
Fever	
≤37.0C°	0
37.1-38.4C°	1
38.5-38.9C°	2
≥39C°	3

Table 7. Baseline Characteristics of Subjects Challenged with SMV Inoculum 1 and Inoculum 2

Characteristic	No. (%) of subjects challenged with Inoculum 1 (N=15)	Inoculum 2 (N=34)	*P*
Gender			0.43[a]
Male	7(46.7)	20(58.8)	
Female	8(53.3)	14(41.2)	
Ethnicity			0.014[b]
African American	4(26.7)	22(64.7)	
White	11(73.3)	10(29.4)	
Other	0(0)	2(5.9)	
Secretor Status			0.81[b]
Positive	11(73.3)	26(76.5)	
Negative	4(26.7)	8(23.5)	
Age (years)	30.7(9.3)[c]	33.5(9.5)[c]	0.33[d]

[a]Pearson χ^2 *p* value.
[b]Fisher's exact *p* value.
[c]Mean (Standard deviation).
[d]Two-sample *t* test *p* value.

Table 8. Comparison of Infectious and Illness Challenged with SMV Inoculum 1 or Inoculum 2

Characteristics	Inoculum 1 (N=15) No. (%)	Inoculum 2 (N=34) No. (%)	Total	*P*[a]
Infection[b]	9(60)	25(73.5)	34(69.3)	0.5
Illness[c]	7(46.7)	11(32.4)	18(36.7)	0.36[d]

SMV: Snow Mountain Virus
[a]Pearson χ^2 *P* value.
[b]Infection was defined as Snow Mountain Virus RNA by specific RT-qPCR and anti-GII.2 SMV serum IgG conversion by ELISA in any post-challenge stool sample
[c]Illness was defined as with diarrhea ≥3 loose or liquid stools or ≥300 g of loose or liquid stools in any continuous 24-h period or one vomiting during the inpatient period and with one of the clinical signs such as fever, abdominal cramps, nausea, headache, chills, fatigue, or myalgia.
[d]*t* test for illness only among infected subjects between SMV Inoculum 1 and Inoculum 2 showed no significant difference (*P*=0.36).

Table 9. Comparison of Mean Illness Severity Score between Subjects Infected with Inoculum 1 and inoculum 2

				95% CI		
Inoculum	N	Mean	SD	Low	High	*P*[a]
1	15	6	1.85	4.97	7.03	0.003
2	34	2.94	3.43	1.74	4.14	

CI: Confidence interval

[a]Mann-Whitney *P* value indicating the probability of statistically significant difference in mean severity scores of those infected with inoculum 1 and inoculum 2

Table 10. PCR Positive Stool and IgG Seroconversion in Post-Challenge Clinical Specimens from Human Subjects

	Inoculum 1 (N=15)		Inoculum 2 (N=34)		
	N[c]	Positive (%)	N[c]	Positive (%)	*P*
PCR-positive stool					
Day 1-3	15	9(60)	34	23(67.65)	0.6043[a]
Day 4-6	15	7(46.67)	32	0(0)	<0.0001[b]
Day 15	15	0(0)	32	1(3.13)	-
Day 30	13	0(0)	31	1(3.23)	-
Day 45	2	0(0)	28	1(3.57)	-
Anti-SMV IgG seroconversion					
Day 1-3	15	0(0)	34	0(0)	-
Day 4-6	14	8(57.14)	32	0(0)	<0.0001[b]
Day 15	15	8(53.33)	32	13(40.63)	0.4140[a]
Day 30	14	7(50)	31	15(48.39)	0.9202[a]
Day 45	1	1(100)	29	13(44.83)	0.2769[a]

[a]Chi-Square

[b]Fisher's Exact Test

[c]Number of stool samples tested from infected subjects

Table 11. Logistic Regression Model to Examine the Association Between Various Host Factors and Post-Challenge Infection Adjusted for Covariates

Model Parameter	Parameter Estimate	SE[a]	Odds Ratio	95% CI[b]	*P*
Intercept	-14.7	9.95			0.131
Anti-SMV Serum IgA pre-challenge concentration (Log_{10})	1.07	1.08	2.92	0.36-24.00	0.318
Anti-SMV Serum IgG pre-challenge concentration (Log_{10})	3.3	1.83	27.04	0.76-967.4	0.071
Pre-existing serum blockade titer					
Serum blockade titer=12.5(reference)	-	-	-	-	-
Serum blockade titer=25	-3.5	1.92	0.03	0.001-1.30	0.068
Serum blockade titer=50	-5.24	2.62	0.01	0.001-0.92	0.046
Secretor Status					
Negative (reference)	-	-	-	-	-
Positive	-1.84	1.51	0.16	0.01-3.03	0.222
Race					
White (reference)	-	-	-	-	-
Black or African American	-1.8	1.42	0.17	0.01-2.69	0.206
Other	12.22	355.4			0.973
Age (years)					
≤30 (reference)	-	-	-	-	-
30-40	-0.591	1.50	0.25	0.03-10.53	0.694
>40	-0.59	1.47	0.55	0.09-27.65	0.76

[a]SE: standard error
[b]CI: Confidence interval

Table 12. Logistic Regression Model to Examine the Association Between Various Host Factors and Post-Challenge Illness Adjusted for Covariates

Model Parameter	Parameter Estimate	SE	Odds Ratio	95% CI	*P*
Intercept	-27.66	161.1			0.864
Anti-SMV Serum IgA pre-challenge titer (log_{10})	0.5	1.02	1.65	0.23-12.04	0.622
Anti-SMV Serum IgG pre-challenge titer (log_{10})	3.02	2.08	20.41	0.34-999.9	0.148
Pre-existing serum blockade titer					
Serum blockade titer=12.5(reference)	-	-	-	-	-
Serum blockade titer=25	0.303	1.61	1.35	0.06-31.70	0.851
Serum blockade titer=50	-2.2	2.1	0.11	0.002-6.75	0.295
Secretor Status					
Negative (reference)	-	-	-	-	-
Positive	13.31	160.8			0.934
Race					
White (reference)	-	-	-	-	-
Black or African American	-3.32	1.69	0.04	0.001-0.99	0.049
Other	-14.193	322.2	0.001		0.965
Age (years)					
≤30 (reference)	-	-	-	-	-
30-40	-0.8	1.48	0.45	0.002-8.24	0.589
>40	-0.97	1.75	0.38	0.01-11.63	0.58

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Chapter III: Conclusions / Limitations / Public Health Significance

Conclusions / Limitations / Public Health Significance

Individuals challenged with SMV inoculum 1 experienced more severe clinical symptoms of acute gastroenteritis compared with those challenged with SMV inoculum 2. The results of less severe symptoms and may be longer viral shedding associated with SMV inoculum 2 in this study suggest that the infectivity of inoculum 2 stock was significantly lower than inoculum 1. Understanding the difference between the two SMV inocula is critical for NoV vaccine evaluation because illness and viral shedding are two important outcomes. The limitations of this study include small sample size in both studies for data analysis, retrospective analysis from two studies that span over 10 years, different study objectives and study population, and slightly different laboratory methods for testing viral shedding and serum antibodies.

If the second generation of SMV inoculum is less infectious than the first generation of SMV inoculum, it may need to recruit a large number of study subjects for future human challenge studies either studying SMV immune response/pathogenesis or measuring SMV vaccine candidates with clinical trials. This will make future human challenge studies more complicated and expensive.