Variation in Severity of Symptoms Associated with Two Snow Mountain Virus Inocula

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Abstract

Background: Norovirus (NoV) is a leading cause of epidemic non-bacterial acute gastroenteritis in young children and adults globally. Snow Mountain Virus (SMV), the prototype of genogroup II and genotype II NoV, has been used in three human challenge studies to examine the infectivity, pathogenicity, and immune response to NoV. Methods: This is a secondary data analysis. Clinical and laboratory data from two previously completed SMV human challenge trials using two different inocula (Inoculum 1 and Inoculum 2) were analyzed to compare infectivity, illness (including modified Vesikari severity scores of gastroenteritis in those subjects with clinical symptoms), viral shedding, and serum IgG conversion. SMV Inoculum 2 is a second-generation inoculum prepared from a stool sample collected from a study subject who was infected with SMV Inoculum 1. Results: Of 15 subjects orally challenged with SMV Inoculum 1 between 2000 and 2002, nine were infected, and seven presented with acute gastroenteritis. Of 33 subjects orally challenged with SMV Inoculum 2 between 2016 and 2018, 25 were infected, and nine presented with acute gastroenteritis. There were no statistically significant differences in overall infection and illness rates between subjects challenged with Inoculum 1 vs. Inoculum 2. However, subjects infected with Inoculum 1 experienced more severe clinical symptoms of acute gastroenteritis and had higher severity scores (6.00 vs. 2.94, P = 0.003) compared with those infected with Inoculum 2. We also observed that infection with Inoculum 2 resulted in longer viral shedding compared with Inoculum 1. This analysis also indicated that secretor-positive subjects had more severe gastroenteritis than secretor-negative subjects. Among ill subjects, no association was observed between challenge dose and severity of acute gastroenteritis. Conclusions: Understanding the differences between these two SMV inocula is critical for NoV vaccine evaluation because illness and viral shedding are two important outcomes in NoV challenge studies to determine vaccine efficacy. Using a less pathogenic inoculum for a vaccine trial will require more participants to meet the target reduction in illness when evaluating the efficacy of candidate vaccines.

INTRODUCTION

Norovirus (NoV) is a leading cause of epidemic non-bacterial acute gastroenteritis in children under five years and adults worldwide [1, 2]. In the United States, NoV infection is associated with an estimated 71,000 hospitalizations and 21 million total illnesses per year [3]. NoV infection is characterized by the acute onset of vomiting, diarrhea, nausea, abdominal cramps, and/or fever, which generally last for 48-72 hours. NoV transmission occurs through contaminated water, food, hands, and environmental surfaces, and person to person by the fecal-oral route [4].

The NoV genome is a linear, positive-sense, single-stranded RNA, and these viruses are classified into at least ten genogroups (GI-GX) and 49 genotypes based on the major structural protein (VP1) amino acid sequence diversity [5]. Only genogroups I (GI), GII, and GIV have been associated with human gastroenteritis. NoV

GII is further divided into 22 genotypes [6]. Snow Mountain Virus (SMV) is the prototype strain of genogroup II genotype II NoV.

Human challenge studies with Norwalk virus (NV, the prototype of NoV genogroup I and genotype I) demonstrated a strong association between NV infection and secretor status as determined by the *FUT2* gene [7, 8]. *FUT* 2 encodes an α(1,2) fucosyltransferase that is responsible for the synthesis of H antigen, and individuals with H antigen expression are considered secretor positive. Previous human challenge studies indicated that secretor-negative individuals do not become infected with NV regardless of the dose [7], but the relationship between secretor status and genogroup II NoV infection is less clear. We observed SMV infection and illness in both secretor-positive and secretor-negative subjects challenged with SMV [9]. Secretor-negative subjects are not completely protected from GII.4 infections [10].

The human challenge model has been used to study the pathogenesis and immunology of NoV infection, and the efficacy of NoV vaccine candidates. We have conducted two SMV human challenge studies, one between 2000 to 2002 with a first generation SMV inoculum [11] and the second between 2016 to 2018 with a second generation SMV inoculum [9].

The objectives of this analysis were to compare infection (defined by serum IgG conversion and/or detection of SMV RNA in stool by RT-PCR or RT-qPCR) and illness (defined by diarrhea, vomiting, and other clinical symptoms) among subjects challenged with the first SMV inoculum [11] prepared by Dolin et al. around 1980 [12] and the second SMV inoculum prepared in 2009 by Dr. Baric [9]. This analysis also examined the severity of illness and the duration of the viral shedding among human volunteers challenged with the two SMV inocula. The results from this study will contribute to our understanding of SMV infectivity and pathogenesis.

MATERIALS AND METHODS

SMV human challenge study 1 [11]: 15 healthy human subjects were challenged with one dose of SMV inoculum, ranging from 31.7 to 3.17×10^5 genome copies quantitated by RT-qPCR [13]. Subjects remained in the hospital clinical research unit for five days after challenge. Stool, serum, vomitus, saliva samples and clinical information were collected daily. Subjects were also followed up on days 7, 15, 30, and 45 post-challenge for stool and serum sample collection [11].

SMV human challenge study 2 [9]: The study consisted of four sequential cohorts and a placebo group. In cohorts 1 through 3 (all subjects were secretor positive), individuals per cohort were challenged with 1.2 x 10^4 genome equivalent copies (GEC) (cohort 1), 1.2 x 10^6 GEC (cohort 2) and 1.2 x 10^7 GEC (cohort 3) of SMV. Cohort 4 only included secretor-negative subjects, and they were challenged with a dose of 1.2 x 10^7 GEC. Subjects remained in the hospital clinical research unit for five days after challenge. Stool, serum, vomitus, saliva samples and clinical information were collected. Subjects were also followed up on days 7, 15, 30, and 45 post-challenge for stool and serum sample collection. For this secondary data analysis, a total of 33 subjects who received the SMV inoculum and completed the study [9] were included in this study.

All the laboratory assays, including detection of anti-SMV IgG in serum, detection of secretor status, and quantification of SMV RNA in stool have been described previously [9, 11]. SMV infection was defined as RNA detection in any post-challenge stool sample by RT-PCR or specific RT-qPCR with CT values < 40 in duplicate reactions and/or anti-SMV serum IgG conversion by ELISA in any post-challenge serum sample vs. pre-challenge serum sample. Illness was defined as those infected with SMV who presented with diarrhea [?]3 loose or liquid stools or [?]300 g of loose or liquid stools in any continuous 24-h period, or one or more vomiting episodes during the inpatient period and with one other clinical sign or symptom such as fever, abdominal cramps, nausea, headache, chills, fatigue, or myalgia.

STATISTICAL ANALYSIS

The databases from the two SMV human challenge studies were merged into a single database for analyses. Data were analyzed using SAS 9.4 (SAS, Cary, NC) for Windows. Categorical data were analyzed using a chi-square test or Fisher's 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 scores of illness were calculated using an 18-point numerical scoring system, which was modified from Ruuska and Vesikari's score system [14]. The scoring system used information on the duration of diarrhea and vomiting, the maximum number of episodes of diarrhea and vomiting within a 24- hour period, and whether the subject experienced certain clinical signs or symptoms, including headache, fever, chill, fatigue, nausea, abdominal cramp, and myalgia ("yes", "more than one" or "no"). Fever was further graded at four levels ([?]37.0degC, 37.1degC-38.4degC, 38.5degC-38.9degC, [?]39degC) by the clinical staff. Table 1 shows the severity score criteria of the modified Vesikari score system [14].

RESULTS

Analysis of the demographic characteristics of the subjects in the two SMV trials indicated no significant differences in sex, age, and secretor status between subjects challenged with SMV Inoculum 1 and Inoculum 2 (Table 2).

We compared infection and illness rates between the subjects who received SMV Inoculum 1 (n=15) to those who received SMV Inoculum 2 (n=33). Overall, SMV infection occurred in 9 of 15 (60%) subjects challenged with Inoculum 1 and in 25 of 33 (75.7%) subjects challenged with Inoculum 2. Illness occurred in 7 of 15 (46.7%) subjects challenged with Inoculum 1 and in 9 of 33 (27.3%) subjects challenged with Inoculum 2. There were no statistically significant differences between the infection (60.0% vs. 75.7%) and illness (46.7% vs. 27.3%) rates in the two trials (Table 3).

In addition to examining the infection and illness rates following SMV challenge, we explored possible differences in severity of illness in the 16 infected subjects who met our definition of illness (Table 4). Subjects infected with SMV Inoculum 1 had a mean severity score of 6.00 (95% CI: 4.97, 7.03], whereas the mean severity score for subjects infected with SMV inoculum 2 was significantly lower 2.94 (95% CI: 1.74, 4.14, P = 0.003) (Table 4, Figure 1). Furthermore, our analysis indicates that Inoculum 1 was associated with more severe acute gastroenteritis even at doses more than 100-fold lower than the doses used for the Inoculum 2 challenge. In addition, we found secretor-positive subjects had significantly higher mean modified Vesikari severity scores compared to secretor-negative subjects ($P < 0.001$) at the dose of $1.2x10^7$ genome copies (Figure 1). When we combined all data from both inocula and different challenge doses, we did not find a significant association between $\log_{10}$ inoculum dose and modified Vesikari scores for either inoculum (Inoculum 1 and 2, P = 0.951 and P = 0.905, respectively).

The RT-PCR results (Table 5) indicated that the NV positive rate was not significantly different in subjects infected with Inoculum 1 vs. inoculum 2 in stool samples collected during the first three days post challenge, but subjects infected with Inoculum 1 had significantly more ($P < 0.001$) PCR-positive stools during days 4-6 post challenge (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, SMV RNA was detected in three stool samples (9.1%) from days 15-45 post-challenge from subjects challenged with Inoculum 2, but no subject challenged with Inoculum 1 shed virus at 15 and 45 days post-challenge.

Finally, we compared anti-SMV serum IgG conversion (>4-fold vs. pre-challenge) in subjects challenged with Inoculum 1 and Inoculum 2 (Table 5). During days 1 - 3 post challenge, none of subjects in both groups showed anti-SMV serum IgG conversion. During days 4 - 6 post-challenge, eight subjects (57.1%) infected with Inoculum 1 showed anti-SMV serum IgG conversion compared to none of the subjects infected with Inoculum 2 ($P < 0.001$) during that period. Most serum IgG conversion occurred between 15 and 30 days post challenge, but there was no significant difference between the two trials in the overall proportion of subjects with seroconversion.

DISCUSSION

This analysis compared the infectivity and virulence of two SMV inocula used in two different human challenge trials by examining infection rates, illness rates, severity of illness, viral shedding, and IgG seroconversion among subjects challenged with a single dose of one of these inocula. Inoculum 1 was prepared

at NIH sometime between 1977 and 1979 from the stool filtrate of a subject infected in the original Snow Mountain Virus outbreak in Colorado in December 1976. This safety-tested inoculum was first used in a human challenge study with 12 subjects conducted at NIH by Dolin et al. [12] and then again in a human challenge study with 15 subjects conducted between 2000-2002 at the University of North Carolina-Chapel Hill [11]. Inoculum 2 was prepared from the stool filtrate of an infected subject in the 2000-2002 human challenge trial and was then used in a human challenge study with 44 subjects at Emory University from 2016-2018 [9]. The goal of this analysis is to examine whether the infectivity, pathogenicity, and virulence of the SMV changed due to passage in the human host by assessing differences in the outcome measures of the two more recent challenge trials.

Both inocula were associated with similar overall infection rates as measured by the proportion of challenged subjects who developed infection and illness after challenge in each trial. However, the median infectious dose (ID_{50}) of Inoculum 2 was about 100-fold higher than that estimated for Inoculum 1 [13]. Comparing the severity scores of infected subjects with clinical symptoms indicated that subjects infected with SMV Inoculum 2 had less severe acute gastroenteritis and more delayed viral shedding even though these subjects were challenged with a much higher dose than the subjects who were infected with Inoculum 1. These results are consistent with what we recently reported [15] that subjects challenged with a first-generation NV inoculum (8FIIa, 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 (8FIIb, prepared in 1997 from the stool filtrate of a subject infected with the first NV inoculum).

Viral shedding and clinical illness are two important outcomes evaluated in NoV challenge trials. Based on outbreak investigations and human challenge studies, most infected subjects experience clinical symptoms along with viral shedding in their feces during the first several days of infection [16]. Symptoms usually resolve after 48-72 hours, however viral shedding can continue for up to three weeks [16] and even longer in immunocompromised subjects [17, 18]. In this study, all subjects infected with SMV Inoculum 1 shed virus for up to six days, but 3% of subjects infected with Inoculum 2 had viral shedding between days 15 to 45 days post challenge. We hypothesize that the less severe clinical illness, but possibly longer SMV shedding, observed in subjects infected with Inoculum 2 may be associated with viral mutations that reduced the virulence of the inoculum, rather than host factors or laboratory assessment methods. However, it is possible that the RT-qPCR assay used to measure viral shedding in the more recent challenge trial with Inoculum 2 is more sensitive and better able to detect prolonged shedding compared to the conventional RT-PCR assay used in the trial with Inoculum 1 [19].

As the prototype of NoV GII.2, SMV is associated with a small percentage of NoV outbreaks, and little is known about the mutation of this virus. Swanstrom et al., [20] reported that the sequence of the P2 domain of SMV strains collected between 1976 and 2010 has evolved over time but less extensively than has been reported for GII.4 NoV strains. Mutations in the surface protein could significantly alter the antigenicity of representative strains, which could also change viral function, pathogenesis, transmission, and infectivity. Studies of other RNA viruses indicate that viral mutations can be deleterious or favorable to the pathogens in terms of infectivity and virulence. Both host and viral factors may explain the change in infectivity and virulence that we observed between the two SMV inocula. Some host factors, including acquired and innate immunity prior to challenge and secretor status, can affect the likelihood of NoV infection and illness. Other host factors, including age, sex, and race/ethnicity may or may not impact the risk of NoV infection and illness. Rouphael et al [9] reported that prechallenge anti-SMV serum IgG concentration, carbohydrate-binding blockade antibody, and salivary IgA were not associated with infection with Inoculum 2. Given the evidence from viral evolution of other RNA viruses such as influenza and SARS-CoV-2 [21-23], we hypothesize that the less severe symptoms associated with the second generation SMV inoculum in this study may be due to intra-host SMV mutations during the course of infection in the subject who was the original source of Inoculum 2 and possibly further inter-host mutations in the subjects who were infected with Inoculum 2 after challenge.

This analysis is the first to compare the clinical outcomes associated with two different SMV inocula used

in two human challenge trials [9,11]. We examined detailed clinical and laboratory data collected from well-controlled human challenge studies with nearly identical protocols that included follow-up sample collection and analyses to carefully characterize infection, illness, and immune response. The limitations of this analysis include small sample size in both studies that may not provide enough power to detect a significant difference in some outcomes, retrospective analysis of two studies that span over 20 years and had different study populations, and slightly different laboratory methods for measuring viral shedding and serum antibodies. Finally, we were not able to assess how differences between inocula preparation in the late 1970's (Inoculum 1) vs. 1997 (Inoculum 2) and storage may have affected the infectivity, pathogenicity, and virulence of these two inocula. The time between inoculum preparation and the challenge trials that provided the data we analyzed was approximately 20 years for both inocula: Inoculum 1 (1979-2000) and Inoculum 2 (1997-2016).

Understanding the difference in the severity of illness associated with these two SMV inocula is critical for those who plan to use second generation NoV inoculum to evaluate new NoV vaccine candidates in vaccination-challenge trials. As we observed for the NV inocula, the second generation SMV Inoculum 2 was associated with a higher ID_{50} and less severe clinical illness when compared with the first generation SMV inoculum. If second generation NoV inoculum does not elicit expected symptomatic illness rates in challenged subjects, it will become more complex and costly to conduct vaccination-challenge trials because they may require larger numbers of study subjects and challenges with higher doses of inoculum in order to assess the efficacy of a candidate vaccine to reduce illness.

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Table 1: Modified Vesikari score (18-point scale) [14] for SMV-infected subjects

with clinical symptoms

Clinical symptoms	Clinical symptoms	Clinical sympto
Duration of diarrhea days		
0	0	0
1	1	1
2-3	2	2
4	3	3
Maximum number of diarrhea stools/24h	Maximum number of diarrhea stools/24h	Maximum numb
0	0	0
1-3	1	1
4-5	2	2
6	3	3
Duration of vomiting days	Duration of vomiting days	Duration of vom
0	0	0
1	1	1
2	2	2
3	3	3
Maximum number of vomiting episodes /24h	Maximum number of vomiting episodes /24h	Maximum numb
0	0	0
1-3	1	1
4-5	2	2
6	3	3
Chills	Chills	Chills
No	0	0

Clinical symptoms	Clinical symptoms	Clinical sympto
Yes	2	2
Headache, Nausea, Abdominal cramp, and Myalgia	Headache, Nausea, Abdominal cramp, and Myalgia	Headache, Naus
Yes[?]1	Yes[?]1	1
No<1	No<1	0
Fever	Fever	Fever
37.0C°	0	0
37.1-38.4C°	1	1
38.5-38.9C°	2	2
39C°	3	3

Table 2: Characteristics of subjects challenged with SMV Inoculum 1 and Inoculum 2

	No. (%) of subjects challenged with	No. (%) of subjects challenged with	
Characteristics	Inoculum 1 (N=15)	Inoculum 2 (N=33)	P
Sex			0.48[a]
Male	7(46.7)	20(60.6)	
Female	8(53.3)	13(39.4)	
Race/Ethnicity			0.019[b]
African American	4(26.7)	22(66.6)	
White	11(73.3)	9(27.3)	
Other	0(0)	2(6.1)	
Secretor Status			0.81[b]
Positive	11(73.3)	25(75.7)	
Negative	4(26.7)	8(24.3)	
Age (years)	30.7(9.3)[c]	32.9(9.3)[c]	0.35[d]

[a]Chi-square P-value

[b]Fisher's exact test P- value

[c]Mean (Standard deviation)

[d]Two-sample *t*- test P-value

Table 3: Comparison of infection and illness in subjects challenged with SMV Inoculum 1 and Inoculum 2

	Inoculum 1 (N=15)	Inoculum 2 (N=33)		
Characteristics	No. (%)	No. (%)	Total	P[a]
Infection[b]	9 (60.0)	25 (75.7)	34(70.8)	0.54
Illness[c]	7 (46.7)	9 (27.3)	16(33.3)	0.41

SMV: Snow Mountain Virus

[a]Chi-square test P-value

[b]Infection was defined as SMV RNA detection in any post-challenge stool sample by specific RT-PCR or RT-qPCR and/or anti-SMV serum IgG conversion by ELISA in any post-challenge serum 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 or more vomiting episodes during the inpatient period and with one additional clinical sign or symptom such as fever, abdominal cramps, nausea, headache, chills, fatigue, or myalgia

Table 4: Comparison of mean illness severity score between subjects infected with Inoculum 1 and Inoculum 2

			Modified Vesikari Score 95% CI	Modified Vesikari Score 95% CI	Modified Vesikari Score 9
Inoculum	N	No. Illness	Mean	SD	95% CI
1	15	7	6.00	1.85	(4.97, 7.03)
2	33	9	2.94	3.43	(1.74, 4.14)

CI: Confidence interval

[a]Mann-Whitney P value indicating the probability of a statistically significant difference in mean severity scores between subjects with illness challenged with Inoculum 1 and Inoculum 2

Table 5. PCR detection of SMV RNA in post-challenge stool samples and anti-SMV IgG seroconversion in post-challenge serum samples from human subjects challenged with SMV Inoculum 1 and Inoculum 2

	Inoculum 1 (N=15)	Inoculum 1 (N=15)	Inoculum 2 (N=33)	Inoculum 2 (N=33)	
	No	No. Positive[a] (%)	No	No. Positive[b] (%)	P
RT-PCR results					
Day 1-3	15	9(60.0)	33	23(69.7)	0.60[c]
Day 4-6	15	7(46.6)	32	0(0)	<0.001[d]
Day 15	15	0(0)	32	1(3.1)	-
Day 30	13	0(0)	31	1(3.2)	-
Day 45	2	0(0)	28	1(3.6)	-
Serum IgG conversion[e]					
Day 1-3	15	0(0)	33	0(0)	-
Day 4-6	14	8(57.1)	32	0(0)	<0.001[c]
Day 15	15	8(53.3)	32	13(40.6)	0.41[c]
Day 30	14	7(50.0)	31	15(48.4)	0.92[c]
Day 45	1	1(100)	29	13(48.3)	0.96[d]

[a]RT-PCR positive was defined as visible SMV-specific amplified PCR product on agarose gel in the study with Inoculum 1

[b]RT-qPCR positive was defined as CT values< 40 in duplicate reactions in the study with Inoculum 2

[c]Chi-square test

[d]Fisher's exact test

"-" Statistical analysis was not performed due to small sample size

[e]Serum conversion was defined as anti-SMV serum IgG conversion (>4 fold) by ELISA in any post-challenge serum sample vs. pre-challenge serum sample

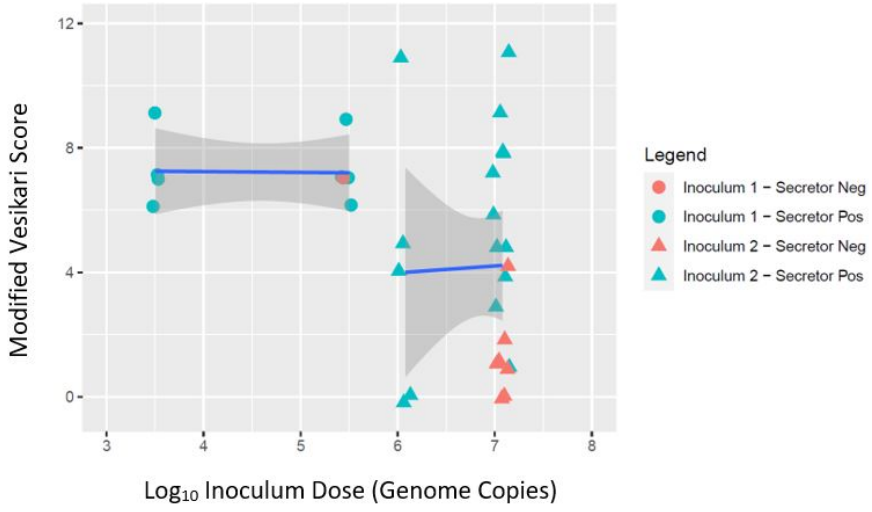

Figure 1. Association between the modified Vesikari severity score of gastroenteritis and $\log_{10}$ challenge dose of SMV inoculum (genome copies). Simple linear regressions were performed for subjects in two challenge studies and fitted line (in blue) and confidence interval (in gray) are presented.

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