MAJOR ARTICLE

Determination of the 50% Human Infectious Dose for Norwalk Virus

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Background. Noroviruses are the most common cause of gastroenteritis in the United States. An understanding of the infectious dose of these viruses is important for risk assessment studies.

Methods. Healthy adults were enrolled in a randomized, double-blind, placebo-controlled evaluation of different dosages of Norwalk virus. Eligible subjects were monitored for clinical gastroenteritis, and infection status was determined. The presence of virus in vomitus was also assessed.

Results. Fifty-seven persons were enrolled; 8 received placebo and an additional 8 persons were considered to be nonsusceptible on the basis of being secretor negative. Twenty-one persons were infected (all blood group O or A), and 67% of those infected developed viral gastroenteritis. The 50% human infectious dose was calculated to be 3.3 reverse transcription polymerase chain reaction units (approximately 1320 genomic equivalents [gEq]) for secretor-positive blood group O or A persons and 7.0 (approximately 2800 gEq) for all secretor-positive persons. The time of illness onset was inversely correlated with inoculum dose. The maximal concentration of virus shedding was higher for persons with gastroenteritis. Norwalk virus was identified in 15 of 27 (56%) vomitus samples at a median concentration of 41 000 gEq/mL.

Conclusions. The 50% human infectious dose measured is higher than previous estimates and similar to that of other RNA viruses.

Clinical Trials Registration. NCT00138476.

Keywords. norovirus; infectious dose; virus shedding; serology; secretor.

Human noroviruses are the most common cause of gastroenteritis in the United States, where the infection causes a significant health burden [1, 2]. The inability to propagate human noroviruses in vitro and the lack of a readily available small animal model has led to the use of experimental human infection models to study viral pathogenesis and immunity [3–8]. Many of these studies have used the prototypical human norovirus, Norwalk virus, as the challenge virus.

Histoblood group antigens (HBGAs) are glycans present on epithelial cells and in body secretions. Their expression is determined genetically by a number of different enzymes [9], including fucosyl transferase 2 (FUT2). Persons who do not express a functional FUT2 enzyme are naturally resistant to infection with Norwalk virus, even at high dosages [10, 11]. We sought to determine the 50% human infectious dose (HID_{50}) of Norwalk virus in susceptible healthy adults (ie, those with a functional FUT2 enzyme) and to evaluate the effect of dose on disease expression.

MATERIALS AND METHODS

Study Design

The study was a randomized, double-blind, placebo-controlled evaluation of different dosages of Norwalk virus. Subjects were enrolled into 1 of 5 groups between September 2004 and October 2011. Each group was

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enrolled sequentially and contained up to 12 subjects. The first 4 groups were randomized 5:1 to receive virus or placebo (sterile water). The virus inoculum dosages used for groups 1 to 4 were 4800, 48, 4.8, and 0.48 reverse transcription polymerase chain reaction (RT-PCR) units, respectively. The last group (group 5) was randomized 1:2 to receive 4.8 or 0.48 RT-PCR units of Norwalk virus; placebo was not used for this group because of the low infection frequency observed for group 4 (ie, 0.48 RT-PCR units). The clinical protocol was reviewed and approved by the institutional review boards of the Baylor College of Medicine and The Methodist Hospital, and written informed consent was obtained from each study participant. The study was registered at ClinicalTrials.gov (NCT00138476). Parts of this study have been described in previous publications [12–16].

Virus Inoculum

The derivation of the Norwalk virus challenge pool (lot 42 399) has been described previously [13, 17]. Following its preparation, the challenge pool was stored at <−70°C in individual 1-mL aliquots until the day of challenge. The titer of undiluted virus inoculum was initially determined to be 120 000 RT-PCR units by most probable number (MPN) RT-PCR [18]. Subsequent studies using the real-time quantitative RT-PCR assay described below demonstrated that 1 RT-PCR unit of Norwalk virus as determined by MPN RT-PCR contained approximately 400 genomic equivalents (gEq). On the day of virus challenge, a 1-mL vial of the pool containing 120 000 RT-PCR units of virus/mL was thawed and serially diluted in sterile water to obtain the final inoculum dosage for administration. The inoculum was stored on wet ice until administered within several hours of preparation. The dosage range was 0.48–4800 RT-PCR units of Norwalk virus and was administered in a 2-mL volume.

Study Participants

Healthy adults (18–50 years of age) who provided informed consent were screened for eligibility within 4 weeks of virus inoculation. Eligible subjects were secretor positive, had no serious chronic diseases, passed screening laboratory studies (liver and renal function, blood counts, hepatitis and HIV serology), had no history of nonbacterial gastroenteritis within 3 months of inoculation and had negative fecal screening studies for bacterial and protozoal enteric pathogens within 28 days of inoculation, were not exposed to persons at high risk of more severe norovirus infection (including immunocompromised persons, children, and elderly), and did not have jobs identified with a significant risk of transmission to others at risk (eg, food handlers, healthcare workers, airline workers). Persons of blood group B or AB were excluded from enrollment for group 5 only. Study participants were required to pass a test showing an understanding of the protocol.

Challenge Study

Study participants were admitted in groups of 2–6 persons to the General Clinical Research Center (GCRC) at Baylor College of Medicine at The Methodist Hospital on the day of inoculation (day 0). Each subject drank 500 mg sodium bicarbonate in 120 mL water, followed 2 minutes later by 2 mL of the virus inoculum or placebo and then 5 minutes later by 2 sodium bicarbonate (650 mg each) tablets with 120 mL of water. Symptoms were evaluated every 4 hours (while the subjects were awake) for the duration of the GCRC stay, and all fecal samples were collected as described previously [13]. In addition, vomitus samples were collected while the subjects were in the GCRC. The subjects remained in the challenge unit for a minimum of 96 hours. Criteria for discharge included no vomiting or watery stools for at least 18 hours. After discharge, all fecal samples were collected through day 21 and then weekly thereafter for up to 8 weeks postinoculation. Serum samples were collected before inoculation and at approximately days 2, 7, 14, 28, and 180 postinoculation.

Definitions

Norwalk virus infection was defined as fecal virus excretion (by antigen enzyme immunoassay [EIA] or RT-PCR) or a ≥4-fold increase in serum antibody titer measured by antibody EIA (from preinoculation to 28 days postinoculation). Viral gastroenteritis was defined as moderate diarrhea alone (>200 g watery [immediately takes the shape of the container] feces) or as 1 episode of vomiting with at least 1 other symptom (abdominal cramps/pain, nausea, bloating, loose feces, fever ≥37.6°C, myalgia, or headache) for any continuous 24-hour period.

Laboratory Studies

Norwalk virus-specific antigen and antibody EIAs were performed as described previously [5, 13, 19]. Norwalk-specific serum immunoglobulin G (IgG) and immunoglobulin A (IgA) assays were determined using a dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA) [15], and HBGA blocking antibody responses were measured as previously described [16]. H type 1 glycan (Glycotech) was used for virus-like particle capture for the first 35 enrollees and H type 3 glycan (Glycotech) for the last 22 participants. Quantitative real-time and immunomagnetic capture RT-PCR assays were performed as described previously for measuring Norwalk virus excretion in fecal samples [13]. Quantitative real-time RT-PCR was also used to measure virus in vomitus and to quantify virus in the challenge pool. Secretor status was determined by measuring the presence of histoblood group antigens in a salivary sample, with persons having A, B, or Lewis b (Le^b) being scored as secretor positive [16]. Some secretor-negative subjects were enrolled in each of the first 4 groups before it was discovered that the monoclonal antibody (anti-Le^b, Immucor) used to identify Le^b cross-reacted with Le^a (present in nonsecretors). For group 5, UEA-1 lectin (Sigma-Aldrich) and a second

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Table 1. Characteristics of Study Participants

Characteristic	Virus Group (n = 49)	Placebo (n = 8)	All Subjects (N = 57)
Age, y, median (range)	26 (20–50)	33.5 (23–49)	27 (20–50)
Male sex, No.	27	4	31 (54%)
Race/ethnicity, No.			
White	28	2	30
African American	5	1	6
Hispanic	4	3	7
Asian	9	2	11
Multiracial	3	0	3
Blood type[a]			
O	25	5	30
A	18	1	19
B	4	1	5
AB	2	1	3
Secretor positive	41	7	48

[a] Three secretor-negative persons in the virus group were blood group A (n = 2) or B (n = 1).

monoclonal anti-Le^b (Covance) that does not cross-react with Le^a were used to identify secretor-positive subjects [16].

Statistical Analysis

Parametric data were compared with Student *t* test and nonparametric data with Wilcoxon rank-sum or Kruskal–Wallis tests. Categorical variables were analyzed using χ^2 or Fisher exact tests. Linear regression was used to assess the effect of dosage on time to the development of gastroenteritis, and logistic regression was used to determine the HID_{50}. The 95% confidence intervals (CIs) for the HID_{50} were calculated based on the Fieller theorem.

RESULTS

Demographics

Fifty-seven persons were enrolled in the study (Table 1). The median age was 27 years, and 54% of the participants were male. Eight persons who received a virus-containing inoculum were subsequently found to be secretor negative. Three persons dropped out of the study early, 1 after the day 7 follow-up and 2 after the day 30 follow-up; all 3 had sufficient data available to be included in the determination of the HID_{50}.

Infection Status

Twenty-one persons were infected with Norwalk virus (Table 2); all were secretor positive and either blood group O or A. None of the 8 placebo recipients, none of the 8 nonsecretors, and none of the 6 persons who were blood group B or AB and received virus became infected. The HID_{50} for blood group O and A persons is estimated to be 3.3 RT-PCR units (95% CI, 1.1–9.4); the estimate is higher (7.0 RT-PCR units [95% CI, 1.4–62.5]) if all secretor-positive individuals challenged with Norwalk virus are included in the analysis (Figure 1). Because 1 RT-PCR unit is approximately 400 gEq, the HID_{50} for the blood group O and A secretor-positive individuals and all secretor-positive individuals is estimated to be approximately 1320 (95% CI, 440–3760) and 2800 (95% CI, 290–25 000) gEq, respectively (Figure 1).

Clinical Illness and Safety

All but 1 infected person had symptoms during the 4 days after inoculation, with nausea, abdominal cramps, and malaise being the most common (Table 3). All 14 of the persons who fulfilled the definition of gastroenteritis vomited at least once and 7 had watery diarrhea. Symptoms were present in 6 of the 7 subjects who did not fulfill the definition of gastroenteritis, 2 of whom had 1 episode of watery fecal sample that weighed <100 g.

The time from inoculation to onset of vomiting or watery diarrhea (Table 4) increased with decreasing inoculum dosage ($P = .001$, linear regression). Thus, the median onset in the lowest-dosage groups (4.8 RT-PCR units or less) was 11 hours later than that in the highest-dosage group (4800 RT-PCR units). Increasing inoculum dosage also was associated with increasing duration of illness ($P = .04$, linear regression).

The symptoms and signs associated with participation in the study were largely those of viral gastroenteritis. Severe symptoms included 3 episodes of severe nausea, 2 episodes of severe diarrhea, and 1 episode of severe headache in 5 persons, all of

Table 2. Infection and Illness Status by Dosage Group

Challenge Dosage (RT-PCR Units)	No. per Group	No. Secretor Positive	No. (%) Infected of Secretor Positive	No. Blood Groups O or A	No. (%) Infected of Blood Groups O or A	No. (% of Infected) With Viral Gastroenteritis
4800	9	7	6 (86%)	6	6 (100%)	4 (67%)
48	10	8	7 (88%)	7	7 (100%)	4 (57%)
4.8	14	13	7 (54%)	12	7 (56%)	5 (71%)
0.48	16	13	1 (8%)	11	1 (9%)	1 (100%)
0	8	7	0 (0%)	6	0 (0%)	NA

Abbreviations: NA, not applicable; RT-PCR, reverse transcription polymerase chain reaction.

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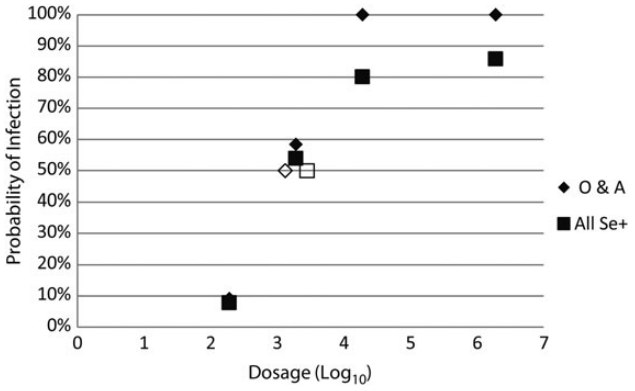

Figure 1. Probability of infection based upon inoculum dosage. The infection frequency observed for secretor-positive (Se+) persons who were blood group O or A or any blood group (All Se+) is shown for each dosage group expressed as $\log_{10}$ genomic equivalents, where 1 reverse transcription polymerase chain reaction unit is approximately 400 genomic equivalents. The open boxes represent the calculated 50% human infectious dose estimates, and the closed boxes represent observed infection frequencies.

whom had viral gastroenteritis. The largest 24-hour volume loss was 3.6 L (1.8 L watery diarrhea and 1.7 kg vomitus), but no other subject had >2 L output in 24 hours and none required intravenous volume repletion. All but 1 of the persons who vomited subsequently received between 1 and 3 doses of ondansetron to control nausea. There was 1 serious adverse event during the study and it was assessed as unrelated. A placebo recipient developed a partial retinal detachment 74 days after receipt of study product.

Virology

All but 1 of the persons who were infected with Norwalk virus had virus present by RT-PCR in at least 5 different fecal samples; 1 person did not return fecal samples after study day 3

Table 3. Clinical Symptoms Among Infected Persons

Symptom	No. With Symptom and Gastroenteritis (n = 14)	No. With Symptom and No Gastroenteritis (n = 7)
Nausea	14	5
Vomiting	14	0
Abdominal cramps	13	4
Malaise	12	4
Anorexia	9	5
Headache	9	3
Myalgia	7	3
Watery diarrhea (No. with >200 g)	7 (6)	2 (0)
Fever (>37.5°C)	4	2
Chills	4	2

Table 4. Viral Gastroenteritis Onset and Duration

Dosage Group (RT-PCR Units)	Median Hours to Onset of Vomiting or Watery Diarrhea (Range)*	Median Duration in Hours of Gastroenteritis Illness (Range)**
4800 (n = 4)	29 (20–31)	24 (18–60)
48 (n = 4)	37 (32–37)	11 (8–34)
≤4.8 (n = 6)	40 (35–50)	15 (8–24)

Abbreviation:RT-PCR, reverse transcription polymerase chain reaction.

*P = .001, linear regression.

**P = .04, linear regression.

and the last 2 fecal samples collected were positive. One subject (number 731) had a solid stool sample approximately 20 hours after illness onset without virus being identified in the sample; the first virus-positive fecal sample was identified approximately 40 hours after resolution of symptoms. For all other ill subjects, virus was present in all tested samples collected during illness.

The duration of shedding for persons from whom stool samples were collected for at least 4 weeks ranged from 6 days to 55 days. Excluding the subject who did not provide stool samples beyond the first 3 days after inoculation, the median duration was 29 days in persons who fulfilled the definition of gastroenteritis compared to 19 days for those without gastroenteritis (P = .14, Wilcoxon rank-sum test). Similarly, virus shedding duration for persons who received a dose of 48 or 4800 RT-PCR units was 29 days compared with 20 days for those who received a lower inoculum dosage (P = .12, Wilcoxon rank-sum test). The maximal fecal virus concentration was greater in persons with viral gastroenteritis compared to those without (geometric mean titer [GMT], 160 billion vs 10 billion gEq/mL, respectively, P = .005, Student t test), although no differences were observed in GMT maximal fecal virus concentration based upon inoculum dosage received.

Virus antigen was identified in fecal samples of 18 of 21 infected persons; 1 person with gastroenteritis and 2 persons without viral gastroenteritis had no antigen-positive stool samples. All antigen-positive stool samples were also positive by RT-PCR. The median duration of antigen-positive stools was 5 days (range, 1–9 days). The duration of antigen-positive fecal samples was similar among ill and nonill persons (5 vs 6 days, respectively, P = .29, Wilcoxon rank-sum test).

Twenty-seven specimens of emesis were collected from 12 ill subjects; specimens from 2 ill participants were not obtained. Norwalk virus was identified in 15 (56%) of the specimens, with a range in titer from <2200 to 12 million gEq/mL. The median concentration of virus-positive samples was 41 thousand (interquartile range, 3800–240 000) gEq/mL.

Serology

All infected persons had at least a 16-fold increase in serum pan–enzyme-linked immunosorbent assay antibody titer at

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Table 5. Results of Total Serum Antibody, Immunoglobulin G, Immunoglobulin A, and Histoblood Group Antigen-Blocking Antibody Assays

	Infected					Not Infected				
Assay	Day 0 (n = 21)	Day 7 (n = 21)	Day 14 (n = 20)	Day 30 (n = 20)	Day 180 (n = 20)	Day 0 (n = 36)	Day 7 (n = 36)	Day 14 (n = 35)	Day 30 (n = 36)	Day 180 (n = 34)
Total serum antibody (panELISA)										
GMT (95% CI)	3800 (1360, 10 700)	19 800 (9960, 39 400)	551 000 (319 000, 951 000)	702 000 (411 000, 1 200 000)	97 400 (61 700, 154 000)	1920 (881, 4170)	1680 (767, 3660)	1620 (713, 3700)	1610 (742, 3510)	1600 (718, 3570)
Seroresponse frequency (95% CI)	NA	57% (36%, 78%)	100% (86%, 100%)	100% (86%, 100%)	90% (77%, 100%)	NA	0% (0%, 8%)	0% (0%, 8%)	0% (0%, 8%)	0% (0%, 8%)
GMFR (95% CI)	NA	5.2 (3.1, 8.9)	111 (55, 225)	142 (74, 272)	20 (10, 39)	NA	0.9 (.8, 1.0)	0.8 (.7, .9)	0.8 (.7, 1.0)	0.8 (.7, 1.0)
	Day 0 (n = 21)	Day 7 (n = 21)	Day 14 (n = 20)	Day 30 (n = 20)	Day 180 (n = 20)	Day 0 (n = 35)	Day 7 (n = 35)	Day 14 (n = 34)	Day 30 (n = 35)	Day 180 (n = 33)
IgG										
GMT (95% CI)	8.9 (4.7, 17)	23 (15, 37)	424 (280, 642)	577 (382, 873)	109 (80, 149)	5.6 (3.4, 9.1)	5.4 (3.4, 8.7)	5.3 (3.2, 8.8)	5.4 (3.3, 8.9)	5.8 (3.3, 10)
Seroresponse frequency (95% CI)	NA	33% (13%, 53%)	100% (86%, 100%)	100% (86%, 100%)	75% (56%, 94%)	NA	0% (0%, 8%)	0% (0%, 8%)	0% (0%, 8%)	0% (0%, 9%)
GMFR (95% CI)	NA	2.6 (1.6, 4.2)	47 (27, 83)	64 (36, 113)	12 (6.7, 22)	NA	1.0 (.9, 1.1)	0.9 (.8, 1.1)	1.0 (.8, 1.1)	1.0 (.9, 1.2)
	Day 0 (n = 21)	Day 7 (n = 21)	Day 14 (n = 20)	Day 30 (n = 20)	Day 180 (n = 20)	Day 0 (n = 35)	Day 7 (n = 35)	Day 14 (n = 34)	Day 30 (n = 35)	Day 180 (n = 33)
IgA										
GMT (95% CI)	2.7 (1.5, 5.2)	22 (13, 40)	379 (225, 638)	153 (93, 252)	14 (8.2, 23)	2.4 (1.7, 3.3)	2.3 (1.7, 3.2)	2.3 (1.7, 3.2)	2.3 (1.6, 3.2)	2.2 (1.6, 3.2)
Seroresponse frequency (95% CI)	NA	67% (47%, 87%)	100% (86%, 100%)	100% (86%, 100%)	60% (39%, 81%)	NA	0% (0%, 8%)	0% (0%, 8%)	0% (0%, 8%)	0% (0%, 9%)
GMFR (95% CI)	NA	8.2 (4.2, 15.7)	139 (77, 252)	56 (32, 99)	5.1 (3.3, 7.8)	NA	1.0 (.9, 1.1)	1.0 (1.0, 1.0)	1.0 (.9, 1.0)	1.0 (.9, 1.0)
	Day 0 (n = 21)	Day 7 (n = 21)	Day 14 (n = 20)	Day 30 (n = 20)	Day 180 (n = 20)	Day 0 (n = 35)	Day 7 (n = 35)	Day 14 (n = 34)	Day 30 (n = 35)	Day 180 (n = 23)
HBGA blocking assay										
GMT (95% CI)	36	ND	918	1075	472	25	ND	21	20	24
Seroresponse frequency (95% CI)	NA	ND	95% (85%, 100%)	100% (86%, 100%)	80 (62%, 98%)	NA	ND	0% (0%, 8%)	0% (0%, 8%)	0% (0%, 12%)
GMFR (95% CI)	NA	ND	24 (14, 41)	28 (17, 48)	13 (7.5, 21)	NA	ND	0.8 (.7, 1.0)	0.8 (.6, 1.0)	0.9 (.7, 1.2)

Abbreviations: CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; GMFR, geometric mean fold rise; GMT, geometric mean titer; HBGA, histoblood group antigen; IgA, immunoglobulin A; IgG, immunoglobulin G; NA, not applicable; ND, not done.

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some timepoint following infection (Table 5). None had a 4-fold rise by day 2 after inoculation, but 12 (57%) had a rise by day 7 postinoculation. All infected persons had a rise on days 14 and 28 after inoculation (see Supplementary Table 1; 1 subject was lost to follow-up after day 7 but had a 32-fold rise on this day). The peak geometric mean fold rise occurred at day 28, and antibody levels at 6 months were still higher than at baseline. Similar results were seen for other serological assays (Table 5), although serum IgA antibody levels rose sooner and peaked earlier (at 14 days). The prechallenge serum HBGA blocking antibody level was significantly lower among those who developed gastroenteritis (Supplementary Figure 1, $P = .006$, Wilcoxon rank-sum test); the same was not observed for prechallenge serum antibody levels measured using the other serological assays.

DISCUSSION

Dose-response data are an important component in the generation of quantitative risk assessments for infectious agents [20]. There are limited data examining the quantitative estimates of norovirus exposure to infection risk [18, 21, 22] and only 1 previous report evaluated the HID_{50} [8]. In the current study, we found that the HID_{50} was approximately 1320 gEq for secretor-positive persons who were blood type O or A. We previously have observed that persons who express blood group B are less likely to develop disease following Norwalk virus exposure [23]. In this study, none of the persons who expressed blood group B became infected. However, if the data from these persons are included in the calculation of the HID_{50}, the estimate increases to 2800 gEq.

Teunis and colleagues determined the HID_{50} of Norwalk virus in an experimental human infection model while making assumptions and adjustments for virus aggregation; they reported an HID_{50} estimate that ranged from 18 to 1015 gEq depending on their modeling assumptions [8]. Interestingly, no person in their study administered a dosage of 324 gEq or less was infected, and only 3 of 9 administered a dosage of 3240 gEq became infected. In addition to the potential impact of aggregation described by Teunis et al [8], another potential reason for the number of gEq to be higher than the estimated infective dose is the presence of noninfectious genomes that result from the error-prone viral RNA-dependent RNA polymerase. The particle to plaque-forming unit (PFU) for murine norovirus has been reported to range from 100 to 10 000 gEq [24, 25], which is similar to that reported for picornaviruses (up to 1000 gEq/PFU) [26]. Another factor that could affect virus infectivity is the host's preexisting immunity [17] such that a larger amount of virus is required to infect persons with some immunity. We were not able to observe an impact of preexisting immunity on susceptibility to infection, although we were able to confirm our prior observation of an effect of serum levels of HBGA-blocking antibody levels on the development of gastroenteritis [16].

The inoculum dose did not have an effect on the frequency of the development of gastroenteritis among those who were infected (ie, illness was as likely to develop in those administered lower dosages as in those who received higher dosages). However, higher inoculum dosages were associated with shorter incubation times and higher peak virus shedding. Similar inverse relationships between inoculum dosage and incubation period have been noted for other enteric pathogens (*Salmonella typhosa*, *Vibrio cholerae*) in human experimental infection models [27, 28].

Norovirus transmission has been associated with vomiting events [29, 30], and norovirus has been observed in vomitus samples and oral mouthwash samples [31, 32]. Our study provides an estimate of the frequency and the first quantitative determination of levels of virus contamination of vomitus. Viral RNA was observed less frequently in vomitus than in the feces, and viral levels in vomitus samples were much lower than observed in stool. Nevertheless, 1 mL of vomitus was found to contain as much as 9000 HID_{50} of virus. These levels of virus emphasize the importance of disinfecting sites contaminated by vomitus.

Human experimental infection studies that enhance the likelihood that an exposed person will become infected include host genetic background (eg, secretor status), virus strain (eg, genotype), host immunity (eg, as measured by serum HBGA-blocking antibody), and level of exposure. These factors can also influence disease manifestations (eg, preexisting immunity) and incubation period, as well as the level and duration of virus shedding. The quantitative assessments made in this and similar studies should be useful for future hazard analyses.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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