Human noroviruses are the leading cause of epidemic and endemic acute gastroenteritis worldwide and a leading cause of foodborne illness in the United States. Recently, human intestinal enteroids (HIEs) derived from human small intestinal tissue have been shown to support human norovirus replication. We implemented the HIE system in our laboratory and tested the effect of chlorine and alcohols on human norovirus infectivity. Successful replication was observed for 6 norovirus GII genotypes and was dependent on viral load and genotype of the inoculum. GII.4 viruses had higher replication levels than other genotypes. Regardless of concentration or exposure time, alcohols slightly reduced, but did not completely inactivate, human norovirus. In contrast, complete inactivation of the 3 GII.4 viruses occurred at concentrations as low as 50 ppm of chlorine. Taken together, our data confirm the successful replication of human noroviruses in HIEs and their utility as tools to study norovirus inactivation strategies.
with inactivation of infectious human norovirus, no consensus has been reached on the best surrogate for human norovirus (24,25). In this study, we demonstrate the successful implementation of HIE cultures, show successful replication of several human norovirus genotypes, and demonstrate the applicability of HIEs to evaluate the efficacy of chlorine and alcohols on reducing virus infectivity.

Materials and Methods
Detailed methods and description of HIE cultures, gene expression analysis, viral infections, norovirus detection, and statistical analyses are provided in the online Technical Appendix (https://wwwnc.cdc.gov/EID/article/24/8/18-0126-Techapp1.pdf). This investigation was determined by the Centers for Disease Control and Prevention (CDC) to be public health nonresearch and therefore not subject to institutional review board review.

Fecal Samples
We included 80 human norovirus-positive fecal samples (12 genogroup [(G)] I, 65 GII, and 3 GIV) collected during 2000–2017 in the study (Table 1). Samples were stored at 4°C or –70°C from collection time until the time of testing. All samples were tested during April 2016–December 2017.

Human Intestinal Enteroid Culture
Baylor College of Medicine (Houston, TX, USA) provided secretor-positive jejunal HIE cultures (J2 and J3 lines) and Wnt3a-producing cells (CRL-2647 cells). Calvin Kuo (Palo Alto, CA, USA) kindly provided R-spondin-producing cells. Gijs van den Brink (University of Amsterdam, Amsterdam, the Netherlands) kindly provided Noggin-producing cells. Calvin Kuo (Palo Alto, CA, USA) kindly provided R-spondin-producing cells. Gijs van den Brink (University of Amsterdam, Amsterdam, the Netherlands) kindly provided Noggin-producing cells. We grew jejunal HIE cultures (J2 or J3 lines) as undifferentiated 3D cultures in the presence of CMGF⁺ and without growth factors (CMGF⁻). After 7 days, highly dense 3D cultures were either split 1:2, frozen in LN₂, or dissociated into a single cell suspension and plated as undifferentiated monolayers. After culture for 24 h at 37°C in 5% CO₂, we replaced CMGF⁻ and added 100 µL of differentiation medium containing 1% sow bile, 500 µmol/L GCDCA, or 500 µM GCDCA plus 50 µmol/L ceramide to each well. We recovered jejunal HIE cultures from 2 donors (J2 and J3) frozen at passage 7 (P7) from LN₂ and grew them as 3D cultures in Matrigel (BD Biosciences, San Jose, CA, USA). Within 24 hours, cells formed small cystic or multilobular HIEs and continued to grow in the presence of CMGF⁺ medium (Figure 1). With each passage, the number of 3D HIEs doubled, reaching a maximum Matrigel capacity of 100/plug. We were able to culture HIEs for ≥4 months (16–17 consecutive passages) (Figure 2, panel A). We were able to culture HIEs for ≥4 months (16–17 consecutive passages) (Figure 2, panel A).

Infection Experiments and Viral Replication
We performed all infections in triplicate on 100% confluent 4-day-old differentiated HIE (J3 line) monolayers, except when specified that the J2 line was used. In some experiments, we pretreated monolayers with 1% sow bile included in the differentiation medium 48 h before infection. In other experiments, we differentiated HIE monolayers without pretreatment, and infected them in the presence of 500 µmol/L of glycochenodeoxycholic acid (GCDCA; Sigma, St. Louis, MO, USA) or with 500 µM GCDCA plus 50 µM ceramide.

To determine viral infectivity, we inoculated duplicate 96-well plates with 100 µL of fecal filtrate (online Technical Appendix) at 1:10, 1:100, and 1:1000 dilution. After 1 h incubation at 37°C in 5% CO₂, we washed the monolayers twice with CMGF⁻ and added 100 µL of differentiation medium containing 1% sow bile, 500 µmol/L GCDCA, or 500 µM GCDCA plus 50 µmol/L ceramide to each well. We used quantitative reverse transcription real-time PCR to determine the amount of norovirus RNA from input virus and from HIE monolayers at 1 hour postinfection (hpi) and at 72 hpi. Standard curve based on quantified RNA transcripts was included.

Inactivation Treatments
Alcohol Treatment
We diluted 10% fecal filtrates 1:10 in 70% ethanol or isopropanol solutions and incubated them for 1 min or 5 min. We then neutralized the alcohols in the samples by adding 9 volumes of CMGF supplemented with 10% fetal bovine serum (FBS). We included a nontreatment control and an alcohol neutralization control in each experiment.

Chlorine Suspension Assays
We prepared fresh chlorine stock solutions at 1,000 ppm and 10,000 ppm by diluting commercial bleach (6% sodium hypochlorite) in cell culture–grade water. We diluted 20 µL of 10% fecal filtrates in an appropriate volume of chlorine stock solutions to achieve a series of total chlorine concentrations of 5–5,000 ppm. After incubating the solutions for 1 min at room temperature, we added sodium thiosulfate (final concentration 50 mg/L) to neutralize free chlorine. We included a nontreatment control and a chlorine neutralization control in each experiment.

Results
HIE Model and Small Intestine Complexity
We recovered jejunal HIE cultures from 2 donors (J2 and J3) frozen at passage 7 (P7) from LN, and grew them as 3D cultures in Matrigel (BD Biosciences, San Jose, CA, USA). Within 24 hours, cells formed small cystic or multilobular HIEs and continued to grow in the presence of CMGF⁺ medium (Figure 1). With each passage, the number of 3D HIEs doubled, reaching a maximum Matrigel capacity of 100/plug. We were able to culture HIEs for ≥4 months (16–17 consecutive passages) (Figure 2, panel A).
A). We confirmed that the number of highly proliferative stem cells increased over time, as shown by enhanced transcriptional levels of LGR5+ (Figure 2, panel B), whereas differentiated monolayers’ LGR5+ expression levels were greatly reduced and lineage-specific markers (SI, ALPI, TFF3, MUC2, FFA4R) were increased (Figure 2, panel C).

### Table 1. Human norovirus–positive fecal samples tested on 80 jejunal HIEs in study of human norovirus replication in HIEs

<table>
<thead>
<tr>
<th>Genotype*</th>
<th>Capsid</th>
<th>RdRp</th>
<th>No. samples</th>
<th>Mean norovirus RNA copies/μL</th>
<th>Patient age group</th>
<th>Collection date†</th>
<th>Storage condition‡</th>
<th>Outbreak or sporadic</th>
<th>No. inactivated/ sporadic %</th>
<th>No. inactivated/ sporadic samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>GI.1</td>
<td>GI.P1</td>
<td>4</td>
<td>0.5–19.3 × 10³</td>
<td>&gt;18</td>
<td>4</td>
<td>2000</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>0/4 (0)</td>
<td></td>
</tr>
<tr>
<td>GI.3</td>
<td>GI.P3</td>
<td>2</td>
<td>0.3–59.4 × 10⁴</td>
<td>0–12</td>
<td>1</td>
<td>2013 Jul</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>0/4 (0)</td>
<td></td>
</tr>
<tr>
<td>GI.4</td>
<td>GI.Pd</td>
<td>2</td>
<td>1.2–8.7 × 10⁴</td>
<td>&gt;18</td>
<td>1</td>
<td>2015 Nov</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>1/2 (50)</td>
<td></td>
</tr>
<tr>
<td>GI.7</td>
<td>GI.P7</td>
<td>3</td>
<td>2.6–16.4 × 10³</td>
<td>0–12</td>
<td>1</td>
<td>2010 Nov</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>0/3 (0)</td>
<td></td>
</tr>
<tr>
<td>GI.1</td>
<td>GI.Pg</td>
<td>1</td>
<td>1.4 × 10⁶</td>
<td>0–12</td>
<td>1</td>
<td>2017 May</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>1/1 (100)</td>
<td></td>
</tr>
<tr>
<td>GI.2</td>
<td>GI.P16</td>
<td>3</td>
<td>0.2–52.6 × 10³</td>
<td>0–12</td>
<td>2</td>
<td>2017 Feb</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>1/2 (50)</td>
<td></td>
</tr>
<tr>
<td>GI.3</td>
<td>GI.P21</td>
<td>2</td>
<td>1.0–6.4 × 10⁴</td>
<td>0–12</td>
<td>1</td>
<td>2012 Mar</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>0/1 (0)</td>
<td></td>
</tr>
<tr>
<td>GI.4</td>
<td>Haag</td>
<td>3</td>
<td>1.3–161.6 × 10⁴</td>
<td>&gt;18</td>
<td>1</td>
<td>2010 May</td>
<td>4°C</td>
<td>Outbreak</td>
<td>0/1 (0)</td>
<td></td>
</tr>
<tr>
<td>GI.4</td>
<td>New</td>
<td>1</td>
<td>4.1 × 10⁵</td>
<td>0–12</td>
<td>1</td>
<td>2013 Apr</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>0/1 (0)</td>
<td></td>
</tr>
<tr>
<td>GI.4</td>
<td>Sydney</td>
<td>22</td>
<td>3.5 × 10⁵–2.1 × 10⁷</td>
<td>0–12</td>
<td>3</td>
<td>2012</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>1/3 (30)</td>
<td></td>
</tr>
<tr>
<td>GI.5</td>
<td>GI.P22</td>
<td>1</td>
<td>1.1 × 10⁴</td>
<td>0–12</td>
<td>1</td>
<td>2010 Nov</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>0/1 (0)</td>
<td></td>
</tr>
<tr>
<td>GI.6</td>
<td>GI.P7</td>
<td>4</td>
<td>0.1–8.4 × 10⁴</td>
<td>0–12</td>
<td>1</td>
<td>2015 Jan</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>0/1 (0)</td>
<td></td>
</tr>
<tr>
<td>GI.7</td>
<td>GI.P7</td>
<td>2</td>
<td>0.3–9.1 × 10⁴</td>
<td>&gt;18</td>
<td>1</td>
<td>2012 Feb</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>0/2 (0)</td>
<td></td>
</tr>
<tr>
<td>GI.14</td>
<td>GI.P7</td>
<td>1</td>
<td>6.1 × 10⁴</td>
<td>0–12</td>
<td>1</td>
<td>2016 Dec</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>1/1 (100)</td>
<td></td>
</tr>
<tr>
<td>GI.17</td>
<td>GI.P6</td>
<td>1</td>
<td>3.4 × 10⁴</td>
<td>0–12</td>
<td>1</td>
<td>2010 Oct</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>1/1 (100)</td>
<td></td>
</tr>
<tr>
<td>GIV</td>
<td>GIV.P1</td>
<td>3</td>
<td>0.3–13.6 × 10³</td>
<td>&gt;18</td>
<td>3</td>
<td>2016 May</td>
<td>4°C</td>
<td>Outbreak</td>
<td>0/3 (0)</td>
<td></td>
</tr>
</tbody>
</table>

*Dual genotyping based on sequencing partial RdRp and capsid regions (26,27). HIE, human intestinal enteroids; RdRp, RNA-dependent RNA polymerase.
†When month was not available, only year of collection is reported.
‡Samples were stored at the indicated temperature.
Human Norovirus Infection of Jejunal HIEs

A previous study demonstrated the replication of GII.4 norovirus in HIEs (16). To evaluate whether those results could be reproduced, we infected jejunal HIEs (line J3) with GII.4 fecal filtrates (GII.P4 Den Haag-GII.4 Den Haag, GII.P4 New Orleans-GII.4 New Orleans, GII.Pe-GII.4 Sydney, and GII.P16-GII.4 Sydney). At 72 hpi, we detected 100- to 1,100-fold increases in viral RNA copies per well for all GII.4 fecal filtrates compared with viral RNA levels detected at 1 hpi (Figure 3, panel A; Table 2).

To further evaluate the reproducibility of the system, we included GII.4 fecal filtrates (GII.4 Den Haag, GII.4 New Orleans, and GII.4 Sydney) in each infection experiment conducted during August 2016–2017. We observed consistent replication of the 3 strains without significant differences in viral titers (Figure 3, panel B). The mean log_{10} increase was 2.7 (95% CI 2.68–2.82; n = 35) for GII.4 Sydney, 2.4 (95% CI 2.29–2.45; n = 33) for GII.4 Den Haag, and 2.3 (95% CI 2.17–2.46; n = 18) for GII.4 New Orleans. We observed significantly higher-fold increases on viral RNA titers for GII.4 Sydney compared with GII.4 Den Haag and GII.4 New Orleans (p<0.0001).

To evaluate whether HIEs support replication of non-GII.4 strains, we inoculated monolayers with different GI-, GII-, and GIV-positive fecal filtrates (Table 1). We observed viral replication for GII.Pg-GII.1 (2.6 log_{10}), GII.P16-GII.2 (2.8 log_{10}), GII.P12-GII.3 (2.3 log_{10}), GII.P7-GII.14 (1.7 log_{10}), and GII.Pe-GII.17 (1.9 log_{10}) strains (Table 2; Figure 3, panel C). We did not observe replication of GI, GIV, and other GII genotypes. We also confirmed that both J2 and J3 HIE lines support human norovirus replication without significant differences in -fold change between the 2 cell lines (Figure 3, panel D).

Most (15/16; 94%) samples that successfully replicated had been stored at –70°C (Table 2) and were collected from children <2 years of age (13/16; 81%) (Figure 3, panel E). CDC830 was stored at 4°C for 2 months before cultivation; this sample was collected from an adult 83 years of age.

We next evaluated replication of human norovirus in HIEs by assessing the kinetics of infection for 4 GII genotypes (GII.1, GII.2, GII.3, and GII.4 Sydney). Consistent with a successful infection, norovirus RNA levels increased at 12 hpi, reaching a plateau at 24 hpi; no significant further increase at 72 hpi was observed for any of the genotypes (Figure 4). Despite a similar viral input level (3.3–9.3 × 10^5 copies/well), GII.4 Sydney infected HIEs with higher efficiency than did the other 3 genotypes (Figure 4), as shown by higher levels of viral RNA in cells and supernatant.

To further confirm the production and release of norovirus from cells, we quantified viral titers in supernatants collected from cell cultures and replaced the differentiation media every 24 hours after infection (Figure 5). We detected viral RNA in supernatants collected at 24 hpi and 48 hpi for all infections. Higher and more consistent levels of norovirus RNA were detected in HIE infected with GII.4 Sydney up to 96 hpi, whereas the initial RNA levels detected for GII.1, GII.2, and GII.3 declined and became undetectable after 48 hpi for GII.1 and GII.2. These data clearly demonstrate that, although HIEs are permissive to infection with different norovirus genotypes, GII.3 and GII.4 replicated with higher efficiency.

We also compared the amount of input viral RNA with the success of replication. Samples that replicated successfully had a significantly higher input titer compared with strains that did not replicate (p<0.0001) (Figure 6, panel A). Stratified by genotype, the effect of the initial input amount of virus was observed for infections performed with GII.1, GII.2, GII.4 Den Haag, GII.4 New Orleans, and GII.4 Sydney viruses (Figure 6, panels B, C). To further confirm the role of the amount of virus...
Human Intestinal Enteroids and Virus Inactivation

inoculum on the success of replication, we infected HIE monolayers with 10-fold serial dilutions of GII.3 and GII.4 fecal filtrates. The dose required to produce infection in 50% of the inoculated wells (ID$_{50}$) was $2.1 \times 10^3$ genome copies/well for GII.4 Den Haag, $4.4 \times 10^2$ genome copies/well for GII.4 Sydney, and $4.0 \times 10^3$ genome copies/well for GII.3, based on the Reed-Muench method (Figure 7) (28).

Figure 2. Characterization of differentiated and undifferentiated HIE in a study of human norovirus replication in HIEs. A) Quantification of undifferentiated HIE generated on each passage. Undifferentiated HIEs derived from 2 donors (J2P7 and J3P7) frozen at passage 7 (P7) were recovered from LN, and embedded in Matrigel (BD Biosciences, San Jose, CA, USA) (4 plugs per HIE). Cell count was performed at day 7. On that day, undifferentiated HIEs were split 1:2 or 1:3, depending on density, and seeded again in Matrigel. All available wells (n > 4) per passage were counted. Error bars indicate SD. B) Analysis of stem cell proliferation marker gene LGR5 expression by quantitative reverse transcription PCR in undifferentiated HIEs. HIEs were embedded into Matrigel, seeded in individual wells, and cultured in the presence of complete media with growth factors. RNA was isolated from 2 wells at 1 hour postseed (day 0) and each day during days 3–9. LGR5 expression was normalized to GAPDH and expressed as fold change relative to day 0 (n = 2 wells/bar). Two different passages were assayed (P12 and P20). Error bars indicate SDs; asterisks indicate significant difference from day 0: *p<0.05; **p<0.001. C) Heat map based on log (2–ΔΔCt) comparing gene expression levels for markers of differentiated small intestinal epithelial cells between undifferentiated and 4-day differentiated HIE monolayers. Experiments were performed with 3 independent cell passages (P12, P16, and P20). Transcripts were normalized to GAPDH levels. Shown are markers for enterocytes (EC), Paneth cells (PC), enteroendocrine cells (EE), goblet cells (GC), and stem cells (SC). Gene symbols: ALPI, intestinal-type alkaline phosphatase; CHGA, chromogranin A; DEFA5, defensin α 5; FFAR4, free fatty acid receptor 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LGR5, leucin-rich repeat-containing G-protein-couple receptor 5; LYZ, lysozyme; MKI67, marker of proliferation Ki-67; MUC2, mucin 2; SI, sucrose isomaltase; SYP, synaptophysin; TFF3, trefoil factor 3. 3D, 3-dimensional; HIE, human intestinal enteroid.

Inactivation of Human Norovirus by Alcohols

We next evaluated the efficacy of alcohols to inactivate infectious human norovirus by using 3 successfully replicating GII.4 viruses (GII.4 Den Haag, GII.4 New Orleans, and GII.4 Sydney). Although replication levels of fecal filtrates exposed to 70% ethanol for 1 and 5 minutes were significantly lower compared with nontreated fecal filtrates (p<0.05), none of the GII.4 viruses was completely inactivated by ethanol.
In addition, we treated 2 GII.4 Sydney fecal filtrates (R3702 and CDC830) with 70% ethanol or 70% isopropanol for 5 minutes to rule out the possibility that the observed inactivation patterns were sample specific (Figure 8, panel B). We observed no complete inactivation for any of the tested samples, although the replication levels after treatment with 70% ethanol were up to 0.7 log_{10} lower, and we found no reduction after exposure to isopropanol (Figure 8, panel B). Following treatment of the fecal filtrates with 70% isopropanol for 5 minutes, norovirus input RNA was still detectable, whereas input titers after 70% alcohol treatment were reduced (1.3–2.9 log_{10}).

Inactivation of Human Norovirus by Chlorine

To evaluate the ability of chlorine to effectively inactivate infectious human norovirus, we treated fecal filtrates of 3 GII.4 viruses with increasing concentrations of chlorine (5–5,000 ppm) for 1 minute. Compared with nontreated controls, all chlorine concentrations ≥50 ppm completely inactivated GII.4 Den Haag, GII.4 Sydney (Figure 8, panels C, D), and GII.4 New Orleans (data not shown). Norovirus input RNA was detectable in all samples that were treated with ≤600 ppm of chlorine.

Discussion

Since the discovery of Norwalk virus, many attempts have been made to culture human noroviruses; most efforts were unsuccessful, or the results were not reproducible in other laboratories (2–6,9,10). The successful long-term expansion of intestinal epithelial organoids has been a major breakthrough in the field of in vitro culture of intestinal epithelium (12,14,15). Recent studies show that HIEs support...
replication of human norovirus and other enteric viruses (13,16,29) and enable analysis of the innate immune response against these viruses (30). In this study, we showed successful replication of 6 GII norovirus genotypes (GII.1, GII.2, GII.3, GII.4, GII.14, and GII.17), including 3 GII.4 variants. Repeated infections conducted over a 1-year period showed consistent increase in viral titers of these 3 GII.4 variant strains, demonstrating that the HIE model is robust. Our data also demonstrate that, after initial confirmation of infectivity, storage of fecal samples at –70°C will preserve virus infectivity for at least 1 year.

We showed successful replication for 6 of the 14 genotypes tested in this study, although the success rate varied. Strain-specific differences have been reported for other viruses grown in HIEs (13,29). For example, compared with echovirus 11 and coxsackievirus B, enterovirus 71 replicates to significantly lower levels in HIEs (29). Enteroids also support robust replication of human rotavirus

**Figure 4.** Evaluation of human norovirus replication in human intestinal enteroids (HIEs) by assessment of kinetics of infection for 4 GII genotypes. We inoculated jejunal HIE monolayers (J3 line) with A) GII.4 Pe-GII.4 Sydney (3.3 × 10^5 RNA copies/well), B) GII. P12-GII.3 (5.3 × 10^5 RNA copies/well), C) GII.P16-GII.2 (3.2 × 10^5 RNA copies/well), or D) GII.Pg-GII.1 (9.3 × 10^5 RNA copies/well). After 1 h at 37°C in 5% CO_2, monolayers were washed, and medium was replaced with differentiation media and incubated for 3 d. For the growth curve, we extracted RNA from frozen lysates (cells and supernatant) at the indicated time points. For the cells vs. supernatant experiment, we removed supernatants by centrifugation before harvesting the cells. Data represent mean ± SD of 2 experiments with 3 wells for each time point. Dotted lines represent quantitative RT-PCR limit of detection.
strains Ito (G3P[8]) and Wa (G1P[8]) but not the attenuated G1P[8] human rotavirus vaccine strain (13). Ettayebi et al. also demonstrated that GII.4 Sydney strains infect enteroids with higher efficiency than do GI.1, GI.3, and GI.17 viruses (16); in our study, GII.4 norovirus strains replicated at higher efficiency than did GI.1, GI.2, and GI.3 viruses. In addition, the ID50 values for GI.4 and GI.3 viruses were slightly lower (2–5×) than reported previously (16). Taken together, these results indicate that high viral RNA titers are not a guarantee for successful replication, perhaps suggesting that fecal specimens that do show norovirus replication may contain large numbers of noninfectious particles.

Until now, evaluation of control measures for human norovirus, including disinfection measures, has relied primarily on the use of cultivable surrogate viruses (24). Although these viruses are similar in size and genome organization, none completely mimics the inactivation patterns of human norovirus based on reduction of viral RNA titers. We demonstrated that the HIE model can be used to evaluate the effectiveness of alcohols and chlorine against human norovirus. Although 5 minutes of exposure to 70% ethanol and isopropanol slightly reduced viral RNA levels, overall, the alcohols did not inactivate GI.4 viruses. These results are in agreement with a previous study that, based on lack of reduction of viral RNA titers, suggested that GI human noroviruses are not affected by alcohol (24). In a comprehensive study comparing different cultivable surrogate viruses for human norovirus, Cromeans et al. (24) showed that Tulane virus, but not feline calcivirus or MNV, was resistant to alcohols. Using HIEs, we now demonstrate that human norovirus closely resembles Tulane virus when measuring inactivation by alcohols.

For chlorine, our data showed that complete inactivation of 3 different GI.4 strains could be achieved with concentrations as low as 50 ppm. These results are consistent with a recent report indicating that treatment with chlorine concentrations <50 ppm were not sufficient to inactivate human norovirus in secondary effluents from water treatment plants (31). In conclusion, our inactivation data demonstrate that chlorine, but not alcohol, completely inactivates human norovirus and that evaluation of inactivation strategies based only on detection of viral RNA does not always reflect the effectiveness of the treatment.

Our study has several limitations. First, the success rate of samples with a moderate viral RNA titer was relatively low, and thus far we have had no success with GI and GIV samples. However, because we were also not able to replicate several high viral load GI.4 samples, other, currently unknown, factors also contribute to successful replication. Second, although we demonstrated that infectious particles are produced and we were able to measure complete inactivation by chlorine treatment, we analyzed only viral RNA levels, not protein levels. Further work is needed on the amount of chlorine required to inactivate human norovirus because we used fecal samples, which inherently have
a high chlorine burden, and we measured total chlorine, whereas the level of free chlorine is what actually determines inactivation. Finally, the HIE model is costly and labor intensive; additional improvements are required to make it more affordable and widely available.

In conclusion, we confirmed that the HIE system to culture human norovirus科学技术 works. The culture system supported identical levels of replication of a panel of human norovirus strains consistently for >1 year. The success of replication depends on genotype and initial virus titer but also on other unknown factors. Additional HIE cell lines need to be tested or cultures need to be enriched for enterocytes (32) because replication of some noroviruses is restricted by cell line characteristics (16). In addition, whether infectivity is limited by the presence of virus-specific fecal antibodies and the possibility that cellular host factors may prevent or limit replication of certain

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**Figure 6.** Comparison of amount of input viral RNA with success of human norovirus replication in human intestinal enteroids (HIEs). A) We infected HIE monolayers with undiluted or prediluted (1:10; 1:100; 1:1000) 10% fecal filtrates. Each dot represents the input norovirus RNA per well of a single experiment (n = 168) that resulted in successful (n = 78) or unsuccessful (n = 90) virus replication. Boxes represent 25th percentile, median, and 75th percentile, and whiskers show the minimum and maximum values. Circles indicate GII.4 genotypes and triangles non-GII.4 genotypes. ***p<0.001 by Mann-Whitney test. B, C) Role of initial norovirus RNA input in successful (+) and unsuccessful (−) human norovirus infections. We infected HIE monolayers with undiluted or prediluted (1:10; 1:100; 1:1000) 10% fecal filtrates and incubated them at 37°C in 5% CO₂ for 3 d. We extracted RNA and quantified it by quantitative reverse transcription PCR from frozen lysates (cells and supernatant) at 1 hour postinfection and 3 days postinfection. Data points represent individual experiments. Bars represent mean ± SD. Dotted lines represent RT-qPCR limit of detection. Samples that successfully replicate at high, but not low, concentration colored are colored and listed in Table 2.

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**Figure 7.** Determination of ID₅₀ required for human norovirus replication in human intestinal enteroids (HIEs). We inoculated HIE monolayers in triplicate with 10-fold serial diluted fecal filtrates A) A5413_GII.4 Sydney, B) G3868_GII.4 Den Haag, or C) NT741656_GII.3 RNA copies and incubated them for 1 h at 37°C. We washed the monolayers 3 times and cultured them in differentiation media for 3 d. We extracted RNA and quantified it by quantitative reverse transcription PCR from frozen lysates (cells and supernatant) at 1 hour postinfection and 3 days postinfection. We calculated ID₅₀ using the Reed-Muench method (28). ID₅₀, 50% infectious dose.
genotypes all indicate that more research is needed to further optimize this cultivation system.

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References


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Human Norovirus Replication in Human Intestinal Enteroids as Model to Evaluate Virus Inactivation

Technical Appendix

Materials and Methods

Fecal Samples

This investigation was determined by CDC to be public health non-research, and therefore was not subject to institutional review board review. We included 80 fecal samples in the study (Technical Appendix Table). We collected the samples during 2000–2017 from children ≤12 years old (n = 62) and adults (n = 18). We collected 67 samples from sporadic cases and 13 samples from norovirus outbreaks. This collection included 12 genogroup (G) I, 65 GII, and 3 GIV human norovirus positive fecal samples. We stored the samples at the indicated temperature from collection time until the time of testing. We prepared 10% fecal suspensions by adding 0.5 g of whole fecal sample or 500 µL of liquid fecal sample to 4.5 mL of PBS. We vortexed the fecal suspensions for 30 seconds, kept them at room temperature for 5 minutes, and vortexed them again. We sonicated the samples 3 times for 1 minute at the highest setting, with 1-minute periods on ice after each sonication step. We removed the solids by centrifugation for 10 minutes at 10,000 × g and serially filtered the supernatants through 5 µm, 1 µm, 0.45 µm, and 0.22 µm filters. Aliquots of the resulting 10% clarified fecal filtrate were stored at −70°C.

Ten-fold serial dilutions of the 10% fecal filtrate (until viral RNA input was undetectable by real-time RT-PCR) were tested in triplicate. All samples were tested during April 2016–December 2017.

Human Intestinal Enteroid Culture and Media

Secretor-positive jejunal HIE cultures (J2 and J3 lines) and Wnt3a-producing cells (CRL-2647 cells) were provided by Baylor College of Medicine. R-spondin-producing cells were
kindly provided by Dr. Calvin Kuo, Palo Alto, CA. Noggin-producing cells were kindly provided by Dr. Gijs van den Brink, University of Amsterdam, Netherlands. Complete media with and without growth factors (CMGF+ and CMGF−, respectively), differentiation media, and Wnt3a- R-spondin- and Noggin -conditioned media were prepared as reported previously (1,2).

Jejunal HIE cultures (J2 or J3 lines) were grown as undifferentiated 3-dimensional (3D) cultures, as described previously (2) with minor modifications. Briefly, HIEs were recovered from liquid nitrogen (LN2), suspended in 20 μL of Matrigel® (≈40 crypts), plated in a single well of a 24-well plate, and grown as 3D cultures in CMGF+ medium supplemented with 10 μM Y-27632 (Sigma). We replaced the medium every 48 hours. After 7 days, highly dense 3D cultures were either split 1:2 and embedded in Matrigel, frozen in liquid nitrogen for further use, or dissociated into single cell suspension and plated as undifferentiated monolayers, as described previously (2) (Technical Appendix Figure 1).

For all infections, we washed undifferentiated HIEs with 0.5 mM EDTA in ice-cold PBS (without calcium chloride–magnesium chloride) and dissociated into single cell suspension with 0.05% trypsin/0.5 mM EDTA. We seeded 96 well plates with 1–2 × 10^5 cells/well to form monolayers, as described previously (2). After 24 hours, CMGF+ supplemented with 10 μM Y-27632 was replaced with differentiation medium, which was refreshed every 48 hours during 4 days.

**Total RNA Isolation and Gene Expression Analysis**

We isolated total RNA from differentiated and undifferentiated HIE monolayers using MagMAX-96 Total RNA Isolation Kit according to the manufacturer’s instructions. For each sample, 1 μg of RNA was reverse transcribed using the High Capacity RNA to cDNA kit (Applied Biosystems, Foster City, CA, USA). We performed real-time PCR amplification using TaqMan Fast Advance master mix and TaqMan Gene Expression assays (Life Technologies, Grand Island, NY, USA) or IDT PrimeTime qPCR Assays (Technical Appendix Table 1) in an Applied Biosystems 7500 platform. Expression levels were normalized to GAPDH and fold-change of expression level was calculated using the comparative Ct method (2^−ΔΔCt), as described previously (3). Heat maps were created using GraphPad Prism7.0 (GraphPad Software, La Jolla, CA, USA).
Infection Experiments and Viral Replication

We performed all infections in triplicate on 100% confluent 4-day-old differentiated HIE (J3 line) monolayers in 96-well plates, except when specified that J2 line was used. In some experiments, monolayers were pretreated with 1% sow bile included in the differentiation medium 48 hours before infection. In other experiments, HIE monolayers were differentiated 4 days before infection without pretreatment, and infected in the presence of 500 μM of glycochenodeoxycholic acid (GCDCA; Sigma) or 500 μM GCDCA plus 50 μM of a ceramide. Ten percent fecal filtrates were pre-diluted 1:10, 1:100, 1:1000, and 1:10000 in PBS. Each dilution was further diluted 1:20 in CMGF− with 1% sow bile, 500 μM GCDCA, or 500 μM GCDCA plus 50 μM of a ceramide. In all experiments, monolayers were washed twice with CMGF− and inoculated with 100 μL of each fecal filtrate (Technical Appendix Figure 2). Bile was collected from sows and piglets under a study protocol approved by the Animal Care and Use Committee of Baylor College of Medicine and was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (4).

To determine viral infectivity, we inoculated duplicate plates. After 1 hour of incubation at 37°C and 5% CO₂, we washed the monolayers twice with CMGF− and added 100 μL of differentiation medium containing 1% sow bile, 500 μM GCDCA, or 500 μM GCDCA plus 50 μM ceramide to each well. For each set of infections, 1 plate was immediately frozen at −70°C and a duplicate plate was incubated at 37°C, 5% CO₂ for 72 hours and frozen at −70°C. Viral RNA was extracted from input virus, and HIE monolayers at 1 hour postinfection (hpi) and 3 days postinfection (dpi) were determined by RT-qPCR as described below (Technical Appendix Figure 2).

Norovirus Detection, Quantification, and Genotyping

We extracted viral RNA from input virus and HIE monolayers at 1 hpi and 3 dpi using the KingFisher instrument and MagMAX–96 Viral RNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer’s instructions. For infected cells, we performed RNA extraction with minor modifications. Briefly, we added 250 μL of lysis buffer directly to each well and incubated them for 10 minutes at room temperature. We then transferred the cell lysates to a processing plate for RNA extraction according to the manufacturer’s instructions. Norovirus RNA was detected by GI/GII TaqMan real-time RT-PCR (RT-qPCR) (5). We generated standard
curves using 10-fold serial dilutions of GI.4, GII.4, and GIV RNA transcripts. The real-time RT-PCR limit of detection was 200 RNA copies per 5 μL of RNA (or 4.0 × 10^3 RNA copies/well). Samples with titer below the limit of detection were arbitrarily assigned to half of the limit of detection (100 RNA copies per 5 μL of RNA or 2.0 × 10^3 RNA copies/well). Positive samples were genotyped by a dual typing RT-PCR using oligonucleotide primer sets specific for GI and GII viruses (5). PCR products were visualized on a 2% agarose gel and purified by ExoSAP-IT (Affymetrix, Cleveland, OH, USA). Genotypes were assigned by phylogenetic analysis using the unweighted-pair group method with reference sequences used by CaliciNet (6) for capsid typing, or the Norovirus Typing Tool, version 2.0 (7,8).

**Virus Growth Kinetics**

For growth curves, cells versus supernatant, and replace-media experiments, growth kinetics were performed by inoculating HIEs with human norovirus at 3.3–9.3 × 10^5 copies/well. Differentiated HIE monolayers (J3 line) grown in triplicate wells of a 96-well plate were inoculated with GII.1, GII.2, GII.3, or GII.4 Sydney strains. After a 1-hour incubation at 37°C, 5% CO₂, plates were washed twice with CMGF⁻ and 100 μL of differentiation medium was added. For each set of infections, 1 plate was immediately frozen at −70°C and the remaining plates were incubated at 37°C, 5% CO₂ for the length of the experiment. Plates were frozen at 12, 24, 48, and 72 hpi. An extra plate at 96 hpi was included for the replace-media experiment. For growth curves, we extracted RNA from frozen lysates (cells and supernatant). For cells versus supernatant experiments, we removed supernatants before cells were harvested at each time point. For the replace-media experiment, we collected cell culture supernatants and replaced them with an equal volume of fresh differentiation media at each time point. Viral RNA levels in frozen lysates, cells, or supernatants were determined by RT-qPCR. The input virus used for each experiment is described in each figure legend.

**Infectious Dose 50% (ID₅₀)**

Determination of ID₅₀ was performed by inoculating HIEs with 10-fold serial dilutions of human norovirus. Differentiated HIEs monolayers (J3 line) grown in 6 wells of a 96-well plate were inoculated with 10-fold serial dilutions of GII.3, GII.4 Den Haag, or GII.4 Sydney. After a 1-hour incubation at 37°C, 5% CO₂, plates were washed twice with CMGF⁻ and 100 μL of differentiation medium was added. For each set of infections, 1 plate was immediately frozen at
−70°C and the remained plates were incubated at 37°C, 5% CO2 for 72 hours. We determined viral RNA levels in frozen lysates, cells, or supernatants by RT-qPCR. Wells that showed virus replication as an increase in genomic copies per well at 72 hours versus 1 hpi were scored as positive. ID50 was calculated by the Reed-Muench method (9).

Inactivation Treatments

Alcohol Treatment

We diluted 10% fecal filtrates 1:10 in 70% ethanol or isopropanol solutions. After incubation for 1 minute or 5 minutes, we neutralized the samples by 1:10 dilution in CMGF− supplemented with 10% FBS. A nontreatment control (10% fecal filtrate diluted 1:100 in PBS) and a neutralization control (10% fecal filtrate diluted 1:10 in PBS and 1:10 in CMGF− supplemented with 10% FBS) were also included. All alcohol solutions were made fresh by addition of the appropriate volume of cell culture grade water (Life Technologies) to molecular biology grade absolute ethanol or isopropanol (Fisher Scientific, Fairlawn, NJ, USA).

Chlorine Suspension Assays

We prepared fresh chlorine stock solutions at 1,000 ppm and 10,000 ppm by dilution of commercial bleach (Clorox, 6% sodium hypochlorite) in cell culture grade water (Life Technologies). For chlorine inactivation experiments, we diluted 20 μL of 10% fecal filtrate in an appropriate volume of 10,000 ppm or 1,000 ppm stock solutions to achieve final chlorine concentrations of 5000, 1000, 800, 600, 400, 200, 100, 50, and 5 ppm. After exposure for 1 minute, we added sodium thiosulfate (final concentration: 50 mg/L) to neutralize free chlorine. A nontreatment control (10% fecal filtrate diluted in PBS) and a neutralization control (10% fecal filtrate diluted in PBS and sodium thiosulfate) were also included. All incubations were performed at room temperature. All inactivation experiments and infections were done in triplicate.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA). Experiments were performed at least 3 times (3 technical replicates each time) from independent enteroid preparations, as indicated in the figure legends. Data are presented as mean ± SD. Except when specified, a Student’s t test was used to determine statistical significance. Specific p values are detailed in the figure legends.
References


### Technical Appendix Table. RT-qPCR primers and probes to analyze gene expression levels for markers of differentiated and undifferentiated small intestinal epithelial cell types

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### Technical Appendix Figure 1. Human intestinal enteroid culture (propagation and differentiation).

Jejunal HIE cultures (J2 or J3 lines) were grown as undifferentiated 3-dimensional (3D) cultures. HIEs were recovered from liquid nitrogen, suspended in 20 μL of Matrigel (~40 crypts), plated in a single well of a 24-well plate, and grown as 3D cultures in CMGF⁺ medium supplemented with 10 μM Y-27632 (Sigma). The medium was replaced every 48 hours. After 7 days, highly dense 3D cultures were either split 1:2 and embedded in Matrigel, frozen in liquid nitrogen for further use, or dissociated into single cell suspensions and plated as undifferentiated monolayers, as described previously (2).
Technical Appendix Figure 2. To determine viral infectivity, duplicate plates were inoculated. After 1 hour of incubation at 37°C and 5% CO₂, monolayers were washed twice with CMGF⁻ and 100 μL of differentiation medium containing 1% sow bile, 500 μM GCDCA, or 500 μM GCDCA plus 50 μM ceramide was added to each well. For each set of infections, 1 plate was immediately frozen at −70°C and a duplicate plate was incubated at 37°C, 5% CO₂ for 72 hours and frozen at −70°C. Viral RNA was extracted from input virus and HIE monolayers at 1 hour postinfection and 3 days postinfection were determined by RT-qPCR.