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

Veronica Costantini, Esther K. Morantz, Hannah Browne, Khalil Ettayebi, Xi-Lei Zeng, Robert L. Atmar, Mary K. Estes, Jan Vinjé

Human noroviruses are a 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. We grew jejunal HIE cultures (J2 or J3 lines) and Wnt3a-producing cells (CRL-2647 cells) as undifferentiated 3D cultures in the presence of CMGF and without growth factors (CMGF−), differentiation media, and 3 conditioned media (Wnt3a, R-spondin, and Noggin) were prepared as reported previously (13,16,17).

We grew jejunal HIE cultures (J2 or J3 lines) as undifferentiated 3D cultures in the presence of CMGF and without growth factors (CMGF−), differentiation media, and 3 conditioned media (Wnt3a, R-spondin, and Noggin) were prepared as reported previously (13,16,17).

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. For each set of infections, we immediately froze 1 plate at −70°C and incubated a duplicate plate at 37°C in 5% CO2 for 72 h and froze it at −70°C. 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 %L 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
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/ no. tested %</th>
</tr>
</thead>
<tbody>
<tr>
<td>GI.1</td>
<td>GI.P1</td>
<td></td>
<td>4</td>
<td>0.5–19.3 × 10^3</td>
<td>&gt;18</td>
<td>2000</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td>GI.3</td>
<td>GI.P3</td>
<td></td>
<td>2</td>
<td>0.3–59.4 × 10^4</td>
<td>0–12</td>
<td>2013</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td>GI.Pd</td>
<td></td>
<td></td>
<td>2</td>
<td>1.2–8.7 × 10^4</td>
<td>&gt;18</td>
<td>2005</td>
<td>−70°C</td>
<td>Outbreak</td>
<td></td>
</tr>
<tr>
<td>GI.4</td>
<td>GI.P4</td>
<td></td>
<td>1</td>
<td>2.2 × 10^2</td>
<td>&gt;18</td>
<td>2000</td>
<td>−70°C</td>
<td>Outbreak</td>
<td>0/1 (0)</td>
</tr>
<tr>
<td>GI.7</td>
<td>GI.P7</td>
<td></td>
<td>3</td>
<td>2.6–16.4 × 10^3</td>
<td>0–12</td>
<td>2010</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>GI.1</td>
<td>GI.Pg</td>
<td></td>
<td>1</td>
<td>1.4 × 10^6</td>
<td>0–12</td>
<td>2017</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>0/1 (0)</td>
</tr>
<tr>
<td>GI.2</td>
<td>GI.P16</td>
<td></td>
<td>3</td>
<td>0.2–52.6 × 10^3</td>
<td>0–12</td>
<td>2017</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>1/2 (50)</td>
</tr>
<tr>
<td>GI.3</td>
<td>GI.P21</td>
<td></td>
<td>2</td>
<td>1.0–6.4 × 10^4</td>
<td>0–12</td>
<td>2012</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>0/1 (0)</td>
</tr>
<tr>
<td>GI.P12</td>
<td></td>
<td></td>
<td>2</td>
<td>1.6–4.4 × 10^4</td>
<td>0–12</td>
<td>2012</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>1/2 (50)</td>
</tr>
<tr>
<td>GI.P16</td>
<td></td>
<td></td>
<td>4</td>
<td>0.2–14.1 × 10^4</td>
<td>0–12</td>
<td>2012</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td>GI.4 Den Haag</td>
<td>GI.P4 Den Haag</td>
<td>3</td>
<td>1.3–161.6 × 10^4</td>
<td>&gt;18</td>
<td>2010</td>
<td>4°C</td>
<td>0/1 (0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GI.4 New Orleans</td>
<td>GI.P4 New Orleans</td>
<td>1</td>
<td>4.1 × 10^6</td>
<td>0–12</td>
<td>2013</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>0/1 (0)</td>
<td></td>
</tr>
<tr>
<td>GI.4 Sydney</td>
<td>GI.Pe</td>
<td>22</td>
<td>3.5 × 10^3–2.1 × 10^3</td>
<td>0–12</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></td>
<td>1</td>
<td>1.1 × 10^4</td>
<td>0–12</td>
<td>2010</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>0/1 (0)</td>
</tr>
<tr>
<td>GI.6B</td>
<td>GI.P7</td>
<td></td>
<td>1</td>
<td>7.6 × 10^4</td>
<td>0–12</td>
<td>2015</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>0/1 (0)</td>
</tr>
<tr>
<td>GI.6</td>
<td>GI.P7</td>
<td></td>
<td>4</td>
<td>0.1–8.4 × 10^4</td>
<td>0–12</td>
<td>2012</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td>GI.7</td>
<td>GI.P7</td>
<td></td>
<td>2</td>
<td>0.3–9.1 × 10^4</td>
<td>&gt;18</td>
<td>2010</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>0/2 (0)</td>
</tr>
<tr>
<td>GI.14</td>
<td>GI.P7</td>
<td></td>
<td>1</td>
<td>6.1 × 10^4</td>
<td>0–12</td>
<td>2016</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>1/1 (100)</td>
</tr>
<tr>
<td>GI.17</td>
<td>GI.Pe</td>
<td></td>
<td>1</td>
<td>3.4 × 10^6</td>
<td>0–12</td>
<td>2010</td>
<td>−70°C</td>
<td>Sporadic</td>
<td>1/1 (100)</td>
</tr>
<tr>
<td>GIV</td>
<td>GIV.P1</td>
<td></td>
<td>3</td>
<td>0.3–13.6 × 10^3</td>
<td>&gt;18</td>
<td>2016</td>
<td>4°C</td>
<td>Outbreak</td>
<td>0/3 (0)</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 GI 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 GI 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...
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).

**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...
Emerging Infectious Diseases • www.cdc.gov/eid • Vol. 24, No. 8, August 2018

Our data also demonstrate that, after initial confirmation of variant strains, demonstrating that the HIE model is robust. Od showed consistent increase in viral titers of these 3 GII.4 variants. Repeated infections conducted over a 1-year period showed successful replication of 6 GII norovirus genotypes (GII.1, 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.

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.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.

Table 2. Norovirus fecal filtrates successfully cultivated in study of human norovirus replication in human intestinal enteroids

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Genotype, RdRp-Capsid*</th>
<th>Mean norovirus RNA copies/µL</th>
<th>Participant age, mo</th>
<th>Collection date†</th>
<th>Storage condition‡</th>
<th>Fold virus RNA increase, log10§</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP01015</td>
<td>GII.Pg-GII.1</td>
<td>1.4 × 10⁵</td>
<td>4</td>
<td>2017 Mar</td>
<td>−70°C</td>
<td>696 (2.8)</td>
</tr>
<tr>
<td>NP36980</td>
<td>GII.P16-GII.2</td>
<td>1.9 × 10³</td>
<td>132</td>
<td>2017 Feb</td>
<td>−70°C</td>
<td>56 (1.8)</td>
</tr>
<tr>
<td>N741656</td>
<td>GII.P12-GII.3</td>
<td>1.4 × 10⁵</td>
<td>9</td>
<td>2012 Aug</td>
<td>−70°C</td>
<td>181 (2.3)</td>
</tr>
<tr>
<td>G3868</td>
<td>GII.P4 Den Haag-GII.4 Den Haag</td>
<td>2.0 × 10⁵</td>
<td>12</td>
<td>2015 May</td>
<td>−70°C</td>
<td>155 (2.2)</td>
</tr>
<tr>
<td>G3829</td>
<td>GII.P4 New Orleans-GII.4 New Orleans</td>
<td>4.1 × 10⁵</td>
<td>5</td>
<td>2013 Apr</td>
<td>−70°C</td>
<td>227 (2.4)</td>
</tr>
<tr>
<td>A5413</td>
<td>GII.Pe-GII.4</td>
<td>1.6 × 10⁶</td>
<td>12</td>
<td>2012</td>
<td>−70°C</td>
<td>487 (2.7)</td>
</tr>
<tr>
<td>R3702</td>
<td>GII.Pe-GII.4 Sydney</td>
<td>7.7 × 10⁶</td>
<td>46</td>
<td>2015 Jan</td>
<td>−70°C</td>
<td>1236 (3.0)</td>
</tr>
<tr>
<td>V0882</td>
<td>GII.Pe-GII.4 Sydney</td>
<td>4.0 × 10⁶</td>
<td>20</td>
<td>2015 Feb</td>
<td>−70°C</td>
<td>998 (3.0)</td>
</tr>
<tr>
<td>V0900</td>
<td>GII.Pe-GII.4 Sydney</td>
<td>5.1 × 10⁵</td>
<td>17</td>
<td>2015 Feb</td>
<td>−70°C</td>
<td>1102 (3.0)</td>
</tr>
<tr>
<td>H4423</td>
<td>GII.Pe-GII.4 Sydney</td>
<td>5.9 × 10⁵</td>
<td>12</td>
<td>2015 Feb</td>
<td>−70°C</td>
<td>125 (2.1)</td>
</tr>
<tr>
<td>H4587</td>
<td>GII.Pe-GII.4 Sydney</td>
<td>4.4 × 10⁵</td>
<td>21</td>
<td>2015 Apr</td>
<td>−70°C</td>
<td>448 (2.7)</td>
</tr>
<tr>
<td>C8993</td>
<td>GII.Pe-GII.4 Sydney</td>
<td>1.7 × 10⁷</td>
<td>19</td>
<td>2015 Mar</td>
<td>−70°C</td>
<td>223 (2.3)</td>
</tr>
<tr>
<td>CDC830‡</td>
<td>GII.Pe-GII.4 Sydney</td>
<td>2.9 × 10⁶</td>
<td>996</td>
<td>2016 Apr</td>
<td>4°C</td>
<td>121 (2.1)</td>
</tr>
<tr>
<td>NP36994</td>
<td>GII.P16-GII.4 Sydney</td>
<td>2.1 × 10⁵</td>
<td>14</td>
<td>2016 Dec</td>
<td>−70°C</td>
<td>340 (2.5)</td>
</tr>
<tr>
<td>NP36994</td>
<td>GII.P7-GII.14</td>
<td>6.1 × 10⁴</td>
<td>22</td>
<td>2016 Dec</td>
<td>−70°C</td>
<td>47 (1.6)</td>
</tr>
<tr>
<td>G3734</td>
<td>GII.Pe-GII.17</td>
<td>3.4 × 10⁵</td>
<td>11</td>
<td>2010 Oct</td>
<td>−70°C</td>
<td>96 (1.9)</td>
</tr>
</tbody>
</table>

*Dual genotyping based on sequencing partial RdRp and capsid regions (28,27). RdRp, RNA-dependent RNA polymerase.
†When month was not available, only year of collection is reported.
‡Samples were kept from collection time until testing at the indicated temperature.
§Virus RNA increase after 3 days postinfection expressed as fold increase and log10 fold increase. For each filtrate, data represent mean viral RNA increase from 3 experiments with 3 technical replicates each.
¶CDC830 was stored at 4°C for 2 mo before cultivation.

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 × 10⁵ RNA copies/well), B) GII. P12-GII.3 (5.3 × 10⁵ RNA copies/well), C) GII.P16-GII.2 (3.2 × 10⁵ RNA copies/well), or D) GII.Pg-GII.1 (9.3 × 10⁵ RNA copies/well). After 1 h at 37°C in 5% CO₂, 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. 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; 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. 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. 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.1 human noroviruses are not affected by alcohol. In a comprehensive study comparing different cultivable surrogate viruses for human norovirus, Cromans 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. 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 GII 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

Figure 5. Confirmatory testing for human norovirus replication in human intestinal enteroids (HIEs). We inoculated jejunal HIE monolayers (J3 line) with A) GI.4 Pe-GI.4 Sydney (3.3 × 10⁵ RNA copies/well), B) GI.12-GI.3 (5.3 × 10⁵ RNA copies/well), C) GI. P16-GI.2 (3.2 × 10⁵ RNA copies/well), or D) GI.Pg-GI.1 (9.3 × 10⁵ RNA copies/well). After 1 h at 37°C in 5% CO₂, monolayers were washed, and media was replaced with differentiation media and incubated at 37°C and 5% CO₂. At 24, 48, and 72 hours postinfection, we removed the medium and added fresh differentiation media. At 96 hours postinfection, we removed the medium. We extracted RNA and quantified it by quantitative reverse transcription PCR from the supernatant at each time point. Data represent mean ± SD of 2 experiments with 3 wells for each time point. Dotted lines represent RT-qPCR limit of detection.

Emerging Infectious Diseases • www.cdc.gov/eid • Vol. 24, No. 8, August 2018
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 (16) can be successfully implemented in another laboratory. 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

---

**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.

---

**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) N741656_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.

This study was supported by the intramural food safety program at the Centers for Disease Control and Prevention and in part by Agriculture and Food Research Initiative Competitive Grant 2011-68003-30395 from the US Department of Agriculture, National Institute of Food and Agriculture and PHS grants P01AI 057788 and P30 DK 56338. Names of specific vendors, manufacturers, or products are included for public health and informational purposes; inclusion does not imply endorsement of the vendors, manufacturers, or products by the Centers for Disease Control and Prevention or the US Department of Health and Human Services.

### About the Author

Dr. Costantini is an associate service fellow at the National Calicivirus Laboratory, Division of Viral Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention in Atlanta, Georgia, USA. Her research interests include all aspects of viral gastroenteritis with an emphasis on norovirus immunology and vaccines.


Address for correspondence: Veronica Costantini, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop G04, Atlanta, GA 30329-4027, USA; email: vcostantini@cdc.gov

April 2017: Emerging Viruses

• Biologic Evidence Required for Zika Disease Enhancements by Dengue Antibodies

• Neurologic Complications of Influenza B Virus Infection in Adults, Romania

• Implementation and Initial Analysis of a Laboratory-Based Weekly Biosurveillance System, Provence-Alpes-Côte d’Azur, France

• Transmission of Hepatitis A Virus through Combined Liver–Small Intestine–Pancreas Transplantation

• Influence of Referral Pathway on Ebola Virus Disease Case-Fatality Rate and Effect of Survival Selection Bias

• Plasmodium malariae Prevalence and csp Gene Diversity, Kenya, 2014 and 2015

• Presence and Persistence of Zika Virus RNA in Semen, United Kingdom, 2016

• Three Divergent Subpopulations of the Malaria Parasite Plasmodium knowlesi

• Variation in Aedes aegypti Mosquito Competence for Zika Virus Transmission

• Outbreaks among Wild Birds and Domestic Poultry Caused by Reassorted Influenza A(H5N8) Clade 2.3.4.4 Viruses, Germany, 2016

• Highly Pathogenic Avian Influenza A(H5N8) Virus in Wild Migratory Birds, Qinghai Lake, China

• Design Strategies for Efficient Arbovirus Surveillance

• Typhus Group Rickettsiosis, Texas, 2003–2013

• Detection and Molecular Characterization of Zoonotic Poxviruses Circulating in the Amazon Region of Colombia, 2014

• Reassortment of Influenza A Viruses in Wild Birds in Alaska before H5 Clade 2.3.4.4 Outbreaks

• Incidence and Characteristics of Scarlet Fever, South Korea, 2008–2015

• Markers of Disease Severity in Patients with Spanish Influenza in the Japanese Armed Forces, 1919–1920

• Molecular Identification of Spirometra erinaceieuropaei in Cases of Human Sparganosis, Hong Kong

• Zika Virus Seroprevalence, French Polynesia, 2014–2015

• Persistent Arthralgia Associated with Chikungunya Virus Outbreak, US Virgin Islands, December 2014–February 2016

• Assessing Sensitivity and Specificity of Surveillance Case Definitions for Zika Virus Disease

• Detection of Zika Virus in Desiccated Mosquitoes by Real-Time Reverse Transcription PCR and Plaque Assay

• Surveillance and Testing for Middle East Respiratory Syndrome Coronavirus, Saudi Arabia, April 2015–February 2016

• Antiviral Drug-Resistant Influenza B Viruses Carrying H134N Substitution in Neuraminidase, Laos, February 2016

• West Nile Virus Seroprevalence, Connecticut, USA, 2000–2014