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Human Norovirus Infection of Caco-2 Cells Grown as a 3-Dimensional Tissue Structure

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Abstract

Human norovirus (hNoV) infectivity was studied using a 3-dimensional model of large intestinal epithelium. Large intestine Caco-2 cells were grown in rotating wall vessel bioreactors for 18-21 days at 37°C and then transferred to 24-well tissue culture plates where they were infected with GI.1 and GII.4 human noroviruses collected from human challenge trials and various outbreak settings, respectively. Compared to uninfected cells, transmission micrographs of norovirus infected cells displayed evidence of shortening or total loss of apical microvilli, and vacuolization. Quantitative reverse transcription real-time PCR (qRT-PCR) indicated an approximate 2-3 Log_{10} increase in viral RNA copies for the infected cells. A passage experiment examined both the ability for continued viral RNA and viral antigen detection. In the passaged samples 1.01×10^6 copies/mL were detected by qRT-PCR. Immune electron microscopy using primary antibody to hNoV GI.1 capsids in conjunction with 6 nm gold-labeled secondary antibodies was performed on crude cellular lysates. Localization of antibody was observed in infected but not for uninfected cells. Our present findings, coupled with earlier work with the 3-dimensional small intestinal INT407 model, demonstrate the utility of 3-D cell culture methods to develop infectivity assays for enteric viruses that do not readily infect mammalian cell cultures.

Keywords

Noroviruses; Infectivity; Cell Culture; *In vitro* organoids; Quantitative PCR

INTRODUCTION

Human Noroviruses (hNoV) are the leading cause of non-bacterial gastroenteritis worldwide. In the United States alone, it is estimated that 23 million people per year become infected (Centers for Disease Control and Prevention (U.S.). 2007). Typically, exposed individuals exhibit severe gastrointestinal symptoms within 12-24 hrs of exposure, and symptoms last from 24-72 hrs after onset of symptoms. Peak viral shedding in feces averages 11 days post infection, and infected individuals may continue to excrete virus for

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greater than 30 days (Atmar *et al*. 2008). Normally, there are no chronic complications due to infection by noroviruses. Nevertheless, for some compromised individuals, persistent gastrointestinal disease, isolation of the virus in cerebrospinal fluid in an encephalitis patient, and even death can occur (Karst 2010). With the significant number of publically reported norovirus outbreaks in 2006 (Centers for Disease Control and Prevention (U.S.). 2007), questions regarding their evolution (Frankhauser *et al*. 2002), and what these changes may bring in terms of increased virulence and possible mortality are raised. It is apparent that proactive study is required to determine what physical control measures are needed to mitigate or prevent subsequent outbreaks.

The difficulties with studying the human noroviruses *in vitro* have been well documented. Consistent infection and replication of these viruses *in vitro*, has not been demonstrated to any great extent (Duizer *et al*. 2004; Lay *et al*. 2010; Straub *et al*. 2007). Duizer *et al*., (2004) investigated a number of human and animal cell lines, methods for differentiation, and various treatments to find a cell line and conditions that would support replication, but was unsuccessful. Straub *et al*. (2007) had initial success with a 3 – dimensional culture of small intestinal epithelial cells (3-D INT 407). However, poster presentations at national and international conferences by other investigators show that using these same methods have not been successful. This may be due, in part, to contamination of ATCC INT 407 stocks with HeLa cells (ATCC written communication to recipients of the INT407 cell line). Synthetic biology approaches have shown that full-length cDNA or full-length viral RNA can be transfected into cells, but only one round of viral replication can occur (Asanaka *et al*. 2005; Guix *et al*. 2007). Furthermore, this approach requires approximately 10^8 viral copies to transfect 10^5 cells. Of the transfected cells, approximately 100 to 150 cells are infected. Lay *et al*. (2010), recently published findings that human noroviruses do not infect human macrophages or dendritic cells. Their work was motivated by the observation that murine noroviruses efficiently infect and replicate in murine macrophages and dendritic cells (Wobus *et al*. 2006; Wobus *et al*. 2004).

Several studies have demonstrated the utility of growing cells as 3-dimensional tissues for studying pathogenesis of other infectious diseases including enteric pathogens (Carterson *et al*. 2005; Carvalho *et al*. 2005; Honer zu Bentrup *et al*. 2006; Lamarca *et al*. 2005; Long and Hughes 2001; Long *et al*. 1999; Nickerson and Ott 2004; Nickerson *et al*. 2005; Nickerson *et al*. 2001). In the case of the 3-D small intestinal (INT 407) epithelial model for human norovirus infectivity, well-formed apical microvilli are expressed and these may serve as the initial site of hNoV attachment (Straub *et al*. 2007). Microvilli are not well expressed in conventional 2-dimensional (2-D) monolayers for either small or large intestinal epithelial models (Honer zu Bentrup *et al*. 2006; Nickerson *et al*. 2001) and this key factor of cellular differentiation could be a potential factor that explains why 2-D cultures have been largely unsuccessful for developing an infectivity assay for human noroviruses.

We sought to improve the reproducibility of the human norovirus infectivity assay that we had previously developed using the 3-D INT 407 cell line (Straub *et al*. 2007) by investigating an alternate cell line grown as a dynamic 3-dimensional tissue culture. Despite being a heterogeneous cell line with multiple normal and cancer cellular phenotypes, we chose Caco-2 cells based on the following observations. Grown longer term (> 2 weeks) as 3-dimensional trans-well membrane cultures, some cellular phenotypes differentiate to produce apical brush borders characteristic of normal small and large intestines (Peterson and Mooseker 1992). Histoblood group antigens on epithelial cell surfaces and mucosal secretions, produced by a functioning α (1,2) fucosyltransferase (*FUT2*) enzyme (e.g. positive secretor status), have been hypothesized as receptors for hNoV, and Caco-2 has been demonstrated to have these receptors (Harrington *et al*. 2004; Harrington *et al*. 2002; Hutson *et al*. 2003; Marionneau *et al*. 2002; White *et al*. 1996). In addition, White *et al*.,

(1996) demonstrated that norovirus-like particles were internalized in Caco-2 cells grown as traditional 2-D cell cultures. Thus, the aim of our study was to determine if Caco-2, grown as a dynamic 3-dimensional cell culture, could differentiate into cells expressing brush borders, and if these differentiated cells could support infection by Genogroup I and Genogroup II noroviruses.

METHODS

Generation of the 3-D Caco-2 Large Intestinal Cell Model

The colorectal adenocarcinoma (Caco-2) cell line was obtained from the American Type Culture Collection (ATCC HTB-37™, ATCC, Manassas, VA). Cell monolayers were propagated in 75 cm^2 tissue culture flasks at 37°C and 5% CO_2 until they were approximately 90% confluent. Tissue culture media consisted of MEM alpha (containing L-glutamine, vitamins, ribonucleosides, and deoxyribonucleosides, but not sodium bicarbonate; Life Technologies, Carlsbad, CA), 2.2 g/L $NaHCO_3$ (Sigma Chemical Company, St. Louis, MO), 20% heat inactivated fetal bovine serum (Sigma), 0.5 μg/mL Fungizone® (Invitrogen, Carlsbad, CA), and 100 U each of penicillin and streptomycin (Invitrogen). Cells were trypsinized and resuspended in 10 mL MEM alpha with 20% heat inactivated FBS and enumerated using a Coulter Counter (Beckman Coulter, Fullerton, CA). A minimum of 2×10^6 cells (total cell count) was used to seed the bioreactor.

Approximately 250 mg of Cytodex-3 porous collagen-I coated microcarrier beads (Sigma) were washed 3X in 1X Hanks Balanced Salt Solution without calcium and magnesium (HBSS, Sigma). On the final wash the HBSS was aspirated to approximately 1 mL above the bead suspension, and then transferred to a 55 mL reusable slow turning lateral vessel bioreactor (Synthecon, Houston, TX) fitted with 5 and 10 mL leur-lock type syringes. The hydrated bead suspension volume was approximately 2.5 mL. Cells were then transferred to the bioreactor and incubated in a 37°C, 5% CO_2 incubator without rotation for 1 hour to allow cells to attach to the microcarrier beads. Media was then added to the bioreactor through the 5 and 10 mL syringes until the bioreactor was full, and the 5 mL syringe was nearly filled with media. Air bubbles in the bioreactor were expelled through the 5 and 10 mL syringes. When free of bubbles, the stopcocks between the syringes and bioreactors were closed and the bioreactor was attached to the base and the rotation speed was set at 18 RPM. The bioreactor was inspected daily for air bubbles, and if detected, the bioreactor was detached from the base, the stopcocks opened, and bubbles expelled. After inspection, the bioreactor was attached to the base and returned to its normal rotational speed. Approximately 90% of the media was exchanged after 5 days, and then exchanged daily for 13-16 more days. After 18-21 days in the bioreactor, the mature aggregates were transferred to a 50 mL conical centrifuge tube and washed 3 times with 1X HBSS. Aggregates were then transferred to 24-well tissue culture plates (50 μL per well, approximately 1×10^5 cells/mL) using a micropipettor fitted with a wide bore pipette tip. Some aggregates were transferred to 1 mL microcentrifuge tubes and characterized for markers of cellular differentiation as previously described (Honer zu Bentrup *et al*. 2006; Nickerson *et al*. 2001; Straub *et al*. 2007). Trypan Blue dye exclusion was performed to assess the viability of the cells prior to infection. Greater than 95% of the cells were viable after culture for 18-21 days. In addition, some aggregates were further characterized by examination of ultra-thin sections using transmission electron microscopy. Depending on the cell yield, three to four 24-well plates were produced from one bioreactor.

Norovirus Stocks

Human norovirus stocks were obtained from several sources. Genogroup I.1 (GenBank Accession Number M87661) viruses were kindly provided by C.L. Moe at Emory

University from a previous human challenge trial. Stool samples from two anonymous patients (denoted as Patient 3 and Patient 4), collected pre and post challenge with GI.1, were identified as hNoV negative (3-1, and 4-1) and positive stool samples (3-9 and 4-3), respectively. Genogroup II samples, identified as sample 386 and 1G, were kindly provided by C.P. Gerba at the University of Arizona and were obtained from various outbreak settings. Sequencing data of the RNA dependent RNA polymerase gene, provided by the University of Arizona, indicated that strain 386 is most closely related to GenBank EF126966 (Norovirus Hu/GII.4/Nijmegen115/2006/NL) and 1G is most closely related to GenBank AB240184 (Norovirus Hu/NV/Hokkaido/284/2004/JP).

All hNoV samples were resuspended in 1X PBS as 10% (w/v) slurry. The samples were vigorously vortexed, and then the solids were pelleted by centrifugation at 3,000 x g for 10 min. The supernatant was filtered through a 0.2 μm pore size filter. From the filtrate, 50 μL of virus was processed for quantitative reverse transcription real-time PCR (herein abbreviated as qRT-PCR) using a Qiagen RNEasy Plus kit (Qiagen, Valencia, CA) per manufacturer's instructions. Details for viral enumeration by qRT-PCR follow.

Infectivity Assays

For infectivity assays a 1:1,000 dilution of the viral stocks (and hNoV negative stool samples), prepared in sera free MEM alpha, was used to infect the aggregates in the 24-well tissue culture plates. The predicted initial quantity of virions in these dilutions, determined by qRT-PCR, were as follows. For the two different genogroup I samples, the initial predicted titer added to each well was 802 ± 58 and 6,390 ± 681 copies for samples 3-9 and 4-3, respectively. For the Genogroup II viruses, 386 had an initial predicted titer of 41 ± 7 copies and 1G had a predicted titer of 529 ± 59 copies. The total volume in each well was approximately 150 μL (100 μL of sample and 50 μL of cells). This volume was sufficient to just cover the cells and allow for maximal viral contact with the cell suspension. Plates were incubated for 1 hr at 37°C in a 5% CO_2 incubator. Plates were gently rocked every 15 minutes.

For the 1 hr and all subsequent time points, wells were completely sampled, without replacement, at each time point. For any given time point, some wells would be processed to assess viral RNA amplification and others for microscopic analysis. At 1 hr post infection, cells in all wells were overlaid with 1 mL of sterile sera free MEM alpha. After overlaying the cells with media, wells designated as the 1 hr post infection time point were harvested using a micropipettor fitted with a wide bore pipette tip to maintain tissue integrity, and transferred to 1.5 mL microcentrifuge tubes. Cells were centrifuged at 1,500 x g for 3 min and the supernatant was aspirated and discarded. A portion of the tissue aggregates was flash frozen in liquid nitrogen and stored at -80°C for qRT-PCR. The qRT-PCR copies at 1 hr post infection in the tissue aggregates was the observed titer that had attached to the cells and this was used as a baseline when calculating viral RNA amplification. Another portion of the tissue aggregates was fixed with 2.5% glutaraldehyde in 1X PBS and stored at 4°C for further analysis by transmission electron microscopy. This same harvesting process was repeated for subsequent time points up to 1-week post infection.

Microscopic Analysis of Infection

Initial observation of the uninfected and infected tissue aggregates for cytopathic effect (CPE) was made using a Micromaster® inverted microscope with phase contrast infinity corrected optics (Westover Scientific, Mill Creek, WA). Images were captured and saved as jpeg images using a Micron USB2 Camera and Software (Westover Scientific). The tissue aggregates were examined for potential cytopathic effect (CPE) before harvesting the cells for further examination by TEM or qRT-PCR.

Transmission Electron Microscopy of Tissue Thin Sections

Sub-cellular CPE was assessed by transmission electron microscopy using the methods described by Straub *et al*. (Straub *et al*. 2007). Ultra-thin sections (70 nm) were affixed on 100 mesh copper grids coated with forvar and carbon (Electron Microscopy Sciences (EMS), Hatfield, PA) and were post-stained with 1% aqueous uranyl acetate (EMS) and 1% Reynolds lead citrate (Reynolds 1963) before viewing on a Tecnai T-12 transmission electron microscope (FEI Co., Hillsboro, OR) operating at 120 kV with LaB filament. Images were collected digitally using a 2x2K UltraScan 1000 charge-coupled device (Gatan Inc., Pleasanton, CA).

Immune Electron Microscopy

Immune electron microscopy (IEM) was performed on GI.1 infected (4-3) and uninfected tissues (4-1). For these experiments, IEM was performed on viruses that had been passaged through the 3-D Caco-2 cell cultures two times. After freeze thaw lysis and extraction with decafluropentane, the titer measured by qRT-PCR for the infected tissues after passage was 1.01×10^6 copies/mL. The primary antibody was a mouse monoclonal antibody to a peptide in the GI.1 capsid (P2B2; Abcam; Cambridge, MA), and the secondary antibody consisted of 6 nm gold particles conjugated with goat anti-mouse IgG (EMS). Controls for this procedure consisted of 1) uninfected cells and 2) infected cells with the primary antibody omitted.

To ensure that viruses were cell-associated, harvested tissue aggregates were washed 3X in sterile 1X PBS. Briefly, tissue aggregates, free of media, were resuspended in PBS, gently pelleted by centrifugation (1,500 x g for 3 min.), and the supernatant aspirated. This process was repeated two additional times. After the 3rd wash, the supernatant was aspirated and the pellets were resuspended in 500 μL of 1X PBS. Viruses were released from the cells by freezing at -80°C and thawing at room temperature. The crude lysate was extracted with an equal volume of Vertrel (decafluropentane; Sigma, St. Louis, MO), and the aqueous phase was recovered. Uninfected cells were processed in the same manner. The aqueous phase was then processed for IEM.

Processing of the EM grids consisted of a series of different buffer and antibody solutions arrayed on a hydrophobic piece of Parafilm (VWR), and all steps were performed at room temperature. After the TEM grids were exposed face down to drops of hNoV extracts for 10 min, they were processed by transfer to 100 μL drops of respective solutions by the following protocol: fixation in 2% paraformaldehyde in 1X PBS (EMS) for 15 min, 3 washes in 1X PBS, (2 min each), 1% bovine serum albumin (BSA) in 1X PBS (3 min), blocking in 2% BSA (20 min), and a wash with 1% BSA (3 min). For grids receiving primary antibody, the optimal dilution was determined to be 1:100 in 1% BSA. For controls where primary antibody was omitted, grids were exposed to 1% BSA only. Primary antibody exposure (or mock) proceeded for 60 min. The grids were then washed 3 times in 1% BSA, exposed to secondary antibody, (1:20 in 1% BSA, 60 min), washed 3x in 1% BSA and then 3x in 1X PBS. The grids were post-fixed in 2% glutaraldehyde in 1X PBS (EMS) for 5 min, finally washed 3x in 1X PBS and 3x in distilled water. Grids were counterstained with Nano-W (Methylamine Tungstate, Nanoprobes, Yaphank, NY) for 30 s, blotted dry, and then imaged.

Nucleic Acid Extraction From Stools and Cells

To prepare stool samples and tissue aggregates for qRT-PCR analysis, materials were processed using Qiagen's RNEasy Plus kit (Qiagen, Valencia, CA). The protocol was modified to include the use of a QiaShredder column (Qiagen) and a genomic DNA removal column allowing total RNA to be analyzed for virus or host RNA. Tissue aggregates (50 μL

with some residual liquid) were suspended in 350 μL of buffer RLT containing 0.146% beta mercaptoethanol (ß ME, 10 μL of a 14.6% solution added to each mL of buffer RLT) and vortexed until the microcarrier beads dissolved. The suspension was homogenized by passing it through a QiaShredder column (Qiagen). The filtrate was then added to the genomic DNA removal column. The filtrate was recovered, and the remainder of the procedure per manufacturer's instructions, was followed exercising the following options. After the last wash with buffer RPE, the columns were transferred to clean collection tubes and centrifuged at 16,100 x g for 1 min to ensure ethanol removal. The column was eluted with nuclease-free distilled water (2 × 30 μL) yielding a final volume of approximately 60 μL (approximately the same volume starting the procedure). This allowed direct comparison of viral copy number in either stool or cellular pellets without mathematical conversion (e.g. raw copy number at 1 hr post infection could be directly compared to copy number at 1 week post infection). RNA concentration was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Human GAPDH and STAT-1 were also measured by qRT-PCR as a secondary assessment of host RNA concentration for other studies. For human GAPDH and STAT-1, validated qPCR kits were purchased from Applied Biosystems (Applied Biosystems, Foster City, CA), and after cDNA synthesis, qPCR was performed on the ABI 7500 Fast Real-time PCR System (Applied Biosystems). For uninfected cells, total RNA recovery was roughly consistent regardless of time point. For infected cells, total RNA concentration increased as a function of increased viral RNA copies in the cells over time (Table 1).

Quantitative PCR Standards

Amplification of the PCR products to produce qRT-PCR standards, cloning the amplicons into plasmids, generation of RNA transcripts, and purification of the transcripts employed standard protocols provided by kits from various suppliers. The Genogroup I standard transcript is based on sample 155 (GI.1) from Straub *et al*. (Straub *et al*. 2007), while the Genogroup II was based on sample VS122 (provided by C.P Gerba from a previous study). PCR primers (Table 2) were designed to amplify a fragment that was slightly longer than the segment amplified by real-time PCR. Degenerate primers were used to generate the standards due to the high amount of sequence diversity in hNoV strains.

Quantitative Reverse Transcription PCR

Quantitative RT-PCR for Genogroup I and Genogroup II followed the protocol of Kageyama *et al*. (Kageyama *et al*. 2003), with the following modifications. Real-time PCR was performed on an ABI 7500 Fast Real-Time PCR System, but using a modified thermal cycling protocol (2 min at 50°C for Uracil DNA Glycosylase digestion, 10 min at 95°C, and then 45 cycles of denaturation for 15 sec at 95°C and primer annealing and extension for 1 min at 56°C), and standards for Genogroup I and Genogroup II were generated from *in vitro* RNA transcripts as described above.

For standards, mock infected, negative stool infected, hNoV infected, and no template controls, reverse transcription was performed using SuperScript III reverse transcriptase following the manufacturer's recommended protocol (Invitrogen, Carlsbad, CA). From the RT reaction, 2 μL of cDNA (or 2 μL of nuclease free water for no template controls) was used in each real-time PCR reaction. Primer and probe concentrations and thermal cycling protocols were identical to Kageyama *et al*. (Kageyama *et al*. 2003), with the exceptions noted above. The Kageyama protocol was ultimately chosen because, for environmental sampling, flexibility is required to detect any potential GI and/or GII strains. Their protocol targets the most conserved region in all known norovirus genomes (e.g the ORF1 ORF2 junction). To quantitatively measure and compare viral RNA amplification within an infection trial, all time points were run on the same 96-well plate as the standards and no-

template controls. This was because the Kageyama protocol typically had an efficiency of 85 to 100% (slope = -3.7 to -3.3 for 85 and 100% efficiency, respectively). By running an entire experiment on one plate, the variability in PCR amplification efficiency could be mitigated. Thus, even if the amplification efficiency was low, log increases in viral amplification could still be assessed because they were compared against the same standard curve. In addition, PCR inhibition from the samples could also be assessed by comparing the slopes of the samples with the slope of the slopes of the standards (Applied Biosystems Technical Communication). During the exponential phase of amplification, equivalent slopes of the samples and standards indicated that there is no PCR inhibition in the processed samples (Figure 1). All time points, standards and no template controls were performed in triplicate.

RESULTS

Cytopathic Effects from Human Norovirus Infection

Our first experiment was conducted using the 3-D Caco-2 cells along with hNoV negative (4-1) and hNoV positive stool samples (Sample 4-3, GI.1) from the Emory human challenge trial to determine 1) if there was a pathological difference that could be readily observed between non-infectious vs. infectious stools, and 2) if stool samples, even diluted in sera-free media, had toxic effects for these cells. Figure 2 shows that there were little, if any, pathological differences between the cells regardless of the time point when these tissue aggregates were examined using an inverted microscope. Figure 2H is from sample 4-3 after 72 hrs post infection and showed some suspect cytopathic effect (CPE). This was, however, an exception rather than the rule for the infected samples, and similar sample characteristics were also occasionally observed in uninfected controls or negative stool sample challenge at any time point. In subsequent challenges with the additional GI.1 patient sample (3-9), and GII.4 samples, no detectable CPE was observed when these aggregates were examined using the inverted microscope at 100 – 200 X magnification. These initial results show that toxicity due to components in the stool, regardless of whether it is free from virus or contains virus, is not a factor for infectivity studies using 3-D Caco-2 cells and the stool samples selected for this study.

These same samples were analyzed by TEM and the results were dramatically different. Figure 3 compares the 3-D Caco-2 cells challenged with hNoV negative stool (Sample 4-1, Panels A, C, E, and G) and hNoV positive stool (Sample 4-3, Panels B, D, F, and H). Grown as 3-D tissue aggregates, and similar to the results of other studies (Nickerson and Ott 2004; Nickerson *et al*. 2001; Straub *et al*. 2007), 3-D Caco-2 cells are further differentiated from cells grown using conventional cell culture methods. Notably, numerous well-formed apical microvilli are clearly seen in the uninfected cells (Figure 3, left column of photos). Figure 3, Panel C best illustrates both the presence of the apical microvilli, and the presence of tight junctions between two cells. Infected cells, however, generally lose their microvilli, and become thoroughly vacuolated. Figure 3, Panel H (72 hrs post infection) illustrates a relatively healthy cell (apical microvilli present) adjacent to a presumably infected cell (apical microvilli absent and vacuolated). Observational changes in the structure of the brush border upon infection with norovirus in this study are consistent with observations for both early and very recent human biopsy studies (Dolin *et al*. 1975; Schreiber *et al*. 1973; Troeger *et al*. 2009).

Immune Electron Microscopy Reveals Antibody Localization

IEM was performed on hNoV infected (GI.1) and uninfected tissue culture. In this study, the viruses had been passaged through cell culture two times. When qRT-PCR was performed after the lysed cells had been extracted with decafluoropentane, the measured titer of hNoV

was 1.01×10^6 copies/mL. Even with organic extraction, there was a fair amount of cellular debris that had partitioned into the aqueous phase, and this was the likely cause of being unable to visualize virions. Nevertheless, despite not being able to clearly discern viral structure, Figure 4 shows localization of the 6 nm gold-labeled secondary antibodies for norovirus infected cells. This micrograph also shows that they are concentrated in aggregates, suggesting that the viruses are in aggregates as well. Controls with infected cells in which the primary antibody was omitted (Figure 4 A), and controls with uninfected cells but with both primary and secondary antibodies added, did not reveal any localization of the gold secondary antibodies. The inability to visualize viral particles in positive specimens is not without precedence. In the human biopsy studies pathology of the cells was observed but viral particles were not (Dolin *et al*. 1975; Schreiber *et al*. 1973; Troeger *et al*. 2009). For gnotobiotic piglets, tissue biopsies showed the presence of viral antigen on the apical surfaces of duodenal epithelial cells of the sacrificed animals, but no true viral particles were observed in these cells (Cheetham *et al*. 2006; Cheetham *et al*. 2007). Immune localization taken together with increases in viral copies measured by qRT-PCR provides evidence consistent with norovirus infectivity in cell culture.

Viral Copy Number Increases Significantly in Infected 3-D Caco-2 Cells

The realization that cells within the tissue aggregates were possibly infected with hNoV, and that these same cells remained attached to the tissue structure on the collagen microspheres led us to focus our investigation on virus interaction with these cells. Table 3 shows the qRT-PCR results for cells that had been analyzed both by phase-contrast microscopy on the inverted microscope (Figure 2) and TEM (Figure 3). What is evident from this table is that there was nearly a 3 Log_{10} increase in viral titer for the 4-3 samples from 1 hr through 72 hrs post infection. The 48 hr 4-1 sample was presumably contaminated through an aerosolization event during the infectivity assay because both the infectious (4-3) and non-infectious samples (4-1) were adjacent to each other on the 24-well plate. Evidence for a true viral contamination event vs. a PCR contamination event was reflected in the total RNA concentration for these samples. The 1 and 24 hr samples for 4-1 were 0.9 and 1.6 ng/μL, respectively. These concentrations are very similar for cells that were not exposed to fecal material over 72 hrs (Table 1). However, the total RNA concentration for the 4-1 48 hr sample was 108 ng/μL. This observation was consistent with increases in total RNA concentration for cells infected with norovirus over 72 hrs (Table 1). Elevated total RNA concentration was also observed in the 4-1 72 hr pi sample (20 ng/uL). From the qRT PCR plots, the possible beginning of a true amplification event was observed in 1 of 6 replicates. Nevertheless, because threshold was not crossed at the end of 45 cycles, these samples were judged to be negative by PCR. This experimental design error with norovirus negative stool samples was avoided in later trials by keeping non-infectious samples on different 24-well plates and in different incubators. From that point forward, all other negative stool samples used to challenge cells were negative by qRT-PCR. No template controls and cells not exposed to fecal material (mock infected with sterile tissue culture media) were always negative by qRT-PCR throughout this study.

Tables 4 and 5 illustrate the results for additional Caco-2 infectivity trials for an additional GI.1 challenge and GII.4 viruses, respectively. Because we did not see a plateau at 72 hrs post infection for the previous trial, we chose to incubate the samples for up to 1-week. For the GI.1 samples, titers of the original stool samples revealed that sample 3-9 had a qRT PCR titer that was approximately 1 Log_{10} less than sample 4-3 (802 ± 58 copies and 6,390 ± 681 copies for 3-9 and 4-3, respectively). In this case (sample 3-9), qRT-PCR was negative at 1 hr, and 48 hrs post infection, indicating that the viral titer was below the limit of detection of our assay. There was, however, a significant increase in viral copy number as the infection proceeded to 72 hrs and 1-week post infection. Norovirus was detected in

sample 4-3 at all time points. A decrease in Ct of approximately 3.3 – 3.7 cycles (depending on amplification efficiency) represents a 1 Log_{10} increase in viral copy number. Thus for samples 3-9 and 4-3, we observed an approximate 2 – 3 Log_{10} increase in viral titer for these infectivity trials (Tables 3 and 4).

From the original titers in our GII.4 stool samples we estimated that the viral copy numbers delivered to the cells for samples 386 and 1G were approximately 40 copies and 500 copies per well, respectively. From the data for sample 1G in Table 5, we estimate that approximately 10% of our expected viral dose attached to, and infected the 3-D Caco-2 cells. If this is the case, then for sample 386, we estimate that approximately 4 of the 40 copies that were applied to each well subsequently attached to the cells with subsequent infection. Virus was not detected at 1 and 48 hrs post infection, was easily detected at 72 hrs, but was not detected at 1-week post infection. Because each time point represents the contents of an independently sampled well, it is possible that the first replicate of the 1-week post infection sample did not result in attached viruses, and thus, we would not detect virus in that sample. For a second biological replicate we did not detect viruses until observing 68 ± 42 copies at 1-week post infection, and this observation supports our hypothesis that the proportion of viruses that can attach to and infect the Caco-2 cells was likely very low. Sample 1G, which has a viral titer in stool that is approximately 1 Log_{10} greater than the measured stool titer in sample 386, was detected at all time points, but was very close to the limit of detection at 1 and 48 hrs post infection. Nevertheless, significant amplification occurred at 72 hrs (approximate 2 Log_{10} increase in viral count), but no further replication occurred between 72 hrs, and 1-week post infection.

DISCUSSION

An Infectivity Assay vs. a Stable Replication System

There has been a long-standing need to be able to assess potential infectivity of human noroviruses recovered from water, food, and environmental surfaces that have been contaminated in various outbreak settings. Our results for infection of the 3-D Caco-2 cells with human norovirus indicate that 1) cellular pathology (namely, the loss or shortening of apical microvilli) is consistent with observations in previous *in vivo* studies (Cheetham *et al*. 2006; Dolin *et al*. 1975; Schreiber *et al*. 1973; Souza *et al*. 2008; Troeger *et al*. 2009) and 2) viral RNA copy number is significantly increasing (> 2 Log_{10}) as the course of the infection proceeds. Because noroviruses are positive-sense RNA viruses with no DNA stage, the only way that viral RNA copies can increase is if the virus infected host cells, the viral RNA was translated to produce a functioning RNA-dependent RNA polymerase, and that this viral enzyme directed synthesis of new viral RNA.

For the results presented here, we are confident that we have developed an infectivity assay that could be used by laboratories to assess norovirus infectivity when these viruses are recovered from environmental samples. It is important to point out that this infectivity assay may not be a true replication system. Viral RNA could be amplifying, but not necessarily producing infectious progeny virions. This may provide a partial explanation of why we were unable to visualize new virions despite antibody localization (Figure 4). However given that the antibody localization studies were based on passaged viruses, there is a possibility that we have both an infectivity assay and replication system. For virion visualization, it is likely that more sample cleanup (e.g. purification by ultracentrifugation of the crude lysate through a $CsCl_2$ or sucrose gradients) will be required.

If the norovirus assay is only an infectivity assay, it would be analogous to the cell-culture infectivity assay for the protozoan *Cryptosporidium parvum* (Rochelle *et al*. 2002). Allowed to infect HCT-8 cells, the environmentally resistant oocysts undergo excystation and

sporozooites (and other life-cycle stages) are detected inside the cells. Nevertheless, this infectivity assay does not produce significant numbers of environmentally resistant hard-walled oocysts that are detected in water samples. For additional infectivity studies, investigators must still rely on oocysts raised in appropriate animal models. For noroviruses, the analogy would be if RNA amplification occurs without producing infectious virions. Nonetheless, the assay described here would still be appropriate to answer the problem of determining infectivity of noroviruses isolated from environmental settings.

The Importance of Variability In Norovirus Isolates

By testing both Genogroup I and Genogroup II isolates, we are confident that this infectivity assay will be applicable to any environmental isolate of human norovirus. The Genogroup I samples from Emory were important for several reasons including 1) highly screened, and well defined virus stocks that have a low probability of contamination with other pathogens, 2) stool samples harvested from different patients on different days to account for human variability and also showing that shed human noroviruses remain infectious days after symptoms have passed, and 3) with negative stool samples from the same patients, we were better able to assess true CPE (assessed by TEM) vs. a potential cytotoxicity event. The University of Arizona samples were equally important because they represent likely scenarios for environmental and public health monitoring. These scenarios include, but are not limited to, 1) stool samples from infected patients may or may not be available, 2) mixed Genogroups of hNoV could be recovered, 3) samples could be potentially contaminated with other microorganisms including co-infecting viruses, bacteria, or protozoan pathogens (especially if they are recovered from wastewater), and 4) percent recovery of highly dilute viruses is low. The combination of infecting 3-D cell cultures and combining this with a broadly reactive quantitative PCR assay(s) will be the most useful method for environmental monitoring. Alternate assays such as viral antigen detection in infected cell cultures (Guix *et al*. 2007) could also be useful provided that 1) the Genogroup and strain are known (e.g. GI. 1) and 2) specific antibodies exist.

Hypotheses Regarding the Reproducibility of this Assay

Of all publications regarding assay development for human noroviruses that have used differentiated Caco-2 cells, none have clearly specified what differentiated means. Especially given the heterogeneity of this cell line (Peterson and Mooseker 1992), and if these cells were grown as 2-dimensional (standard tissue culture flasks), not as 3-dimensional cell cultures on trans-well membranes (Peterson and Mooseker 1992) or dynamic cultures (Nickerson and Ott 2004), what these cells differentiate into could be dramatically different across laboratories. While this may not be problematic for many CPE producing viruses recovered from environmental samples, for human noroviruses, the state of differentiation may be critical.

Positive secretor status appears to be the critical factor governing human susceptibility to norovirus infection (Thorven *et al*. 2005). If secretor status alone is important, there are a number of human cell lines that express the correct histoblood group antigens on their cell surfaces, including Huh-7 and Caco-2 (Guix *et al*. 2007), and even INT 407 (Straub *et al*. 2007). However, even with over-expression of the *FUT2* gene, which governs secretor status, this factor alone led to greater attachment, but not entry of progeny noroviruses into the cells to complete a subsequent round of infection (Guix *et al*. 2007).

In our continued research, it is our hypothesis that the common denominator that leads to successful norovirus infection of the 3-D Caco-2 cells is clonal populations of cells that consistently express brush borders. This is the population we are working with and report in this paper. If this hypothesis is correct, reproducibility of the norovirus assay both within a

single laboratory and across independent laboratories could be problematic, given the heterogeneity of this cell line (Peterson and Mooseker 1992).

In our continued work to develop a reproducible norovirus assay, we have experienced this problem of heterogeneity of the Caco-2 cell line firsthand. We have two different populations of Caco-2 cells. One population is from the Washington State University (WSU) Archive, and the other population is from the same low-passage stock we had received from ATCC and report in this paper. Grown both as 2-D and 3-D cell cultures, the two populations are dramatically different (Figure 5). The WSU archived cells appear to be mostly non-polarized. Furthermore, when these 3-D cells are infected, no viral RNA amplification occurs, whereas we can reproduce our results with brush-border producing Caco-2 cells. Furthermore, we obtained and grew the $C2_{bbe1}$ cell line (ATCC CRL-2102) as a 3-D culture. This clone was developed by Peterson and Mooseker (1992) in their work to develop a cell line from Caco-2 that consistently produced brush-borders. $C2_{bbe1}$ also supports infection by human noroviruses. Finally, we have also tested HuTu-80 cells grown as 3-D cell cultures. These cells are positive secretors and polarized when grown as 3-D cells, but they do not produce apical brush borders. These cells cannot be infected by human noroviruses. For a reproducible norovirus infectivity assay, our hypothesis is that in addition to positive secretor status, cell lines must also produce apical brush borders

CONCLUSIONS

This is the second report where 3-dimensional cell cultures have shown promise in developing an *in vitro* assay for human noroviruses. In this second report, the 3-dimensional Caco-2 cells appear to be more consistent both in terms of cellular response to norovirus infection (namely, loss or significant shortening of apical microvilli) and significant viral RNA amplification (> 2 Log_{10}). In our continued work, we are finding that, in addition to the positive secretor status of the human cell lines, consistent expression of apical microvilli is likely key to reproducibility of this assay. It is yet to be determined whether the methods of generating 3-dimensional tissues: transwell membranes (Peterson and Mooseker 1992) or dynamic, physiological fluid shear cultures (Nickerson and Ott 2004) also plays a role not only in they way cells differentiate, but also in their susceptibility to norovirus challenge. Whichever method is used to produce 3-dimensional tissue cultures, we believe it is important that investigators characterize their differentiated cells prior to attempting the norovirus assay. Namely, we believe it is important to have a significant population of cells with apical expressed brush borders. This can be accomplished by relatively simple histopathology techniques and/or electron microscopy.

Acknowledgments

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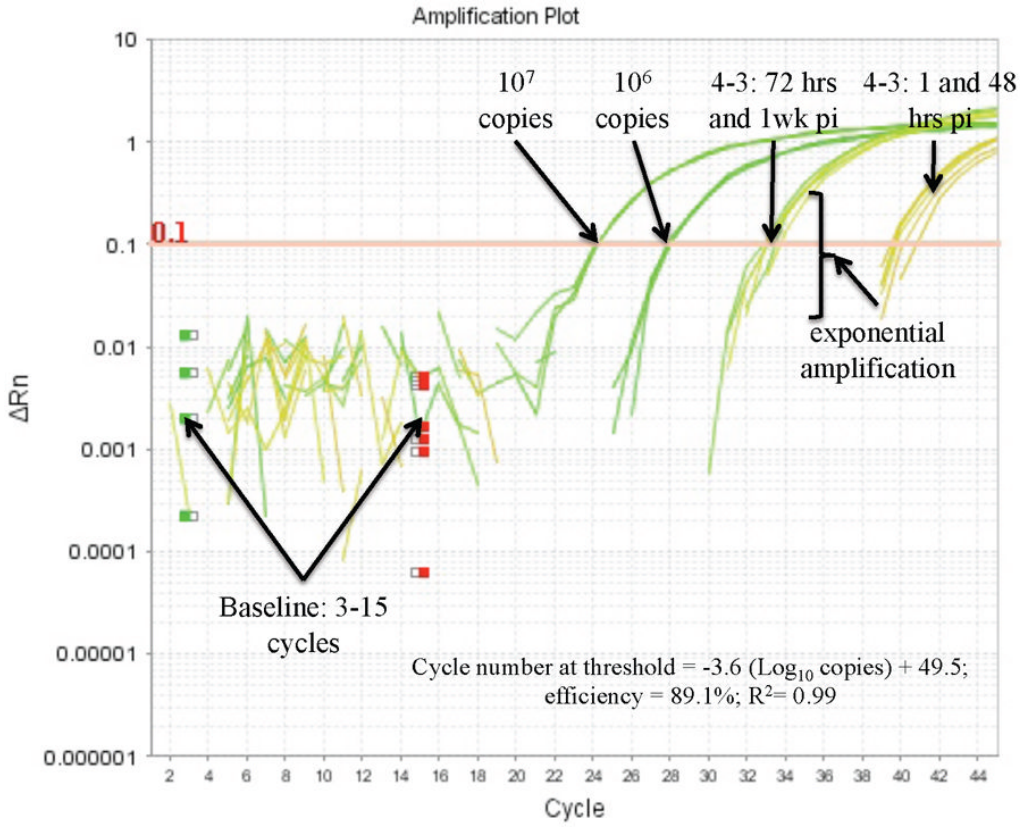

Figure 1.

Logarithmic amplification plot of a quantitative reverse transcription real-time PCR experiment comparing hNoV infected Caco-2 cells with standards. For clarity only the 10^7 and 10^6 copy standards are displayed. The data for the infected cells is presented in Table 4. Note that the slopes of the samples vs. standards are similar in the exponential phase of amplification. This indicates that the samples had little to no PCR inhibition throughout the time course of the experiment. For all experiments, threshold for positive amplification was ΔRN = 0.1, and baseline was set between 3 and 15 PCR cycles.

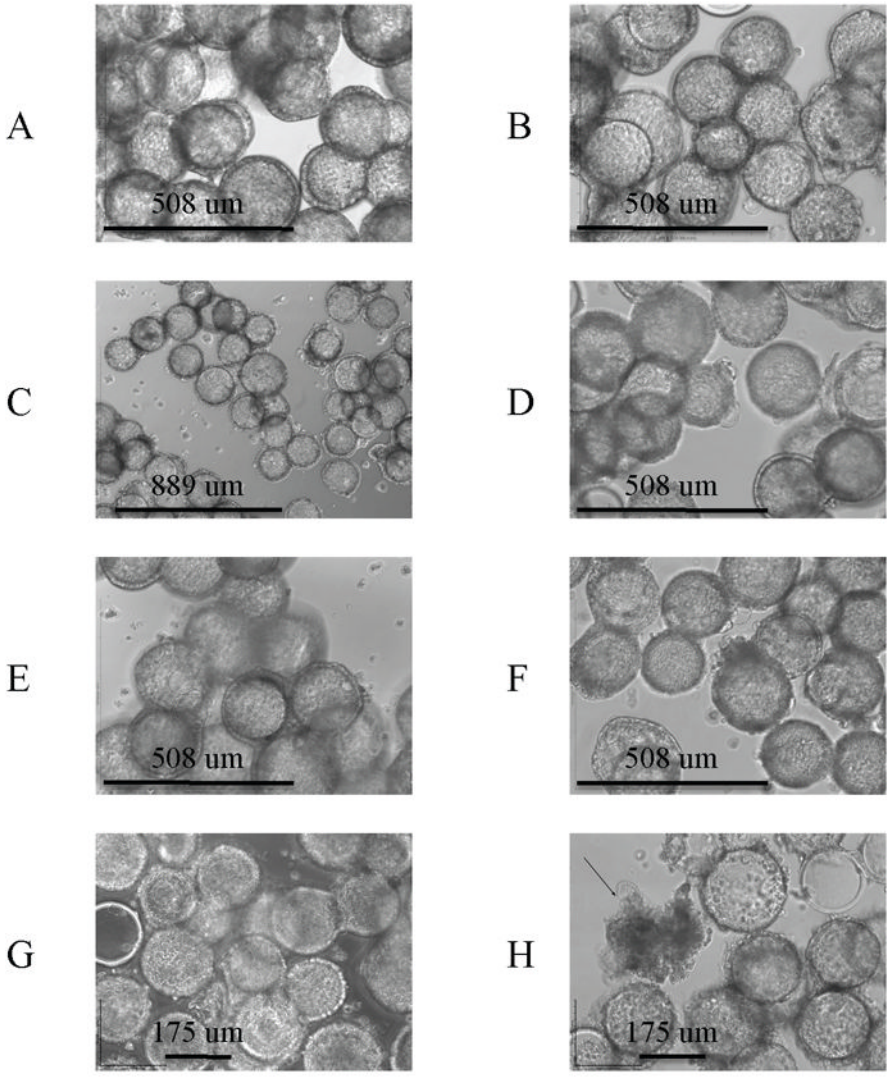

Figure 2.

Lack of gross pathology observed in NoV infected 3-D Caco-2 cells. Observations of the 3-D tissue aggregates under an inverted microscope showed little if any, indication of infection by hNoV. Samples 4-1 and 4-3 were stool samples from the same patient harvested pre (4-1) and post (4-3) infection with GI.1 hNoV from a previous human challenge trial. The left column of photos (A, C, E, G at 1, 24, 48, and 72 hrs post-challenge, respectively) show the course of challenge using 4-1), and the right column of photos (B, D, F, and H at1, 24, 48, and 72-hrs post-challenge, respectively) show the course of a challenge using 4-3. While (H, arrow) showed preliminary signs of infection, it was the exception rather than the rule and similar examples could be observed in uninfected controls at 72 hrs post challenge.

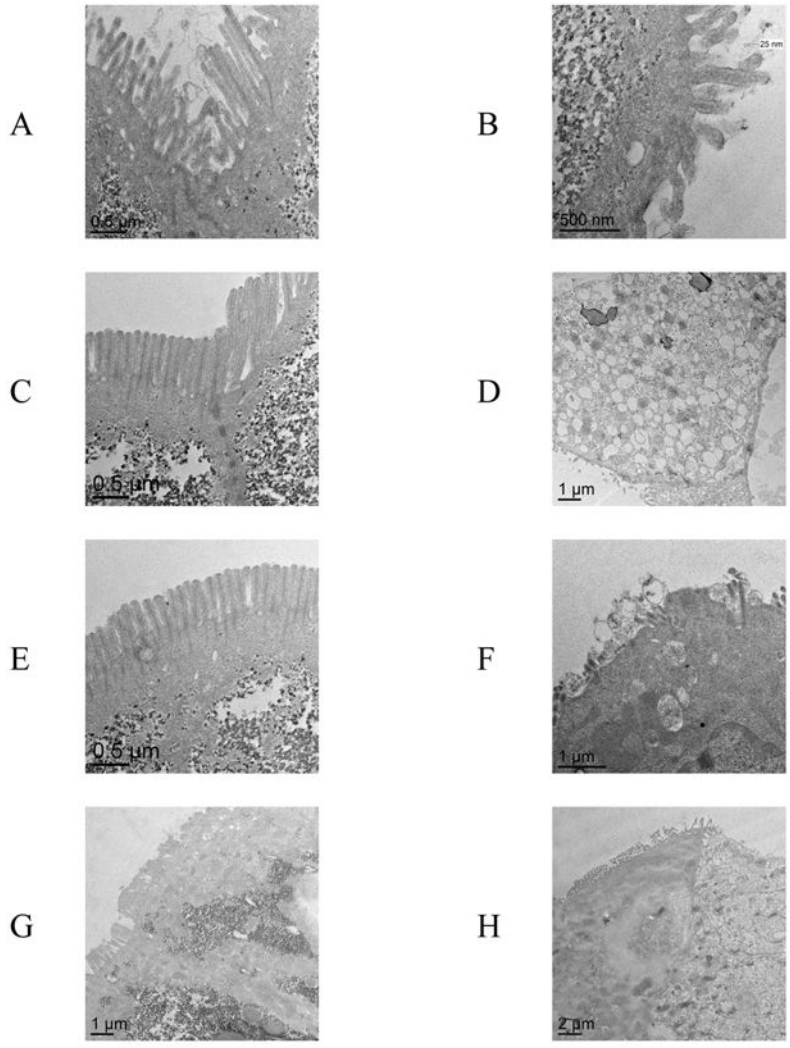

Figure 3.

Transmission electron micrographs comparing stool sample challenges of the 3-D Caco-2 cells using 4-1 (negative) and 4-3 (hNoV positive) stool samples. The left column of photos (A, C, E, and G at1-hr, 24-hrs, 48 hrs, and 72-hrs post-challenge, respectively) show the course of a challenge using 4-1, and the right column of photos (B, D, F, and H at1-hr, 24-hrs, 48 hrs, and 72-hrs post-challenge, respectively) show the course of a challenge using 4-3. Note the presence of microvilli on all cells challenged with hNoV negative stool sample (left column of photos) and the presence of tight junctions (C). In (H), a relatively healthy cell with microvilli is adjacent to cells that are presumably infected with hNoV.

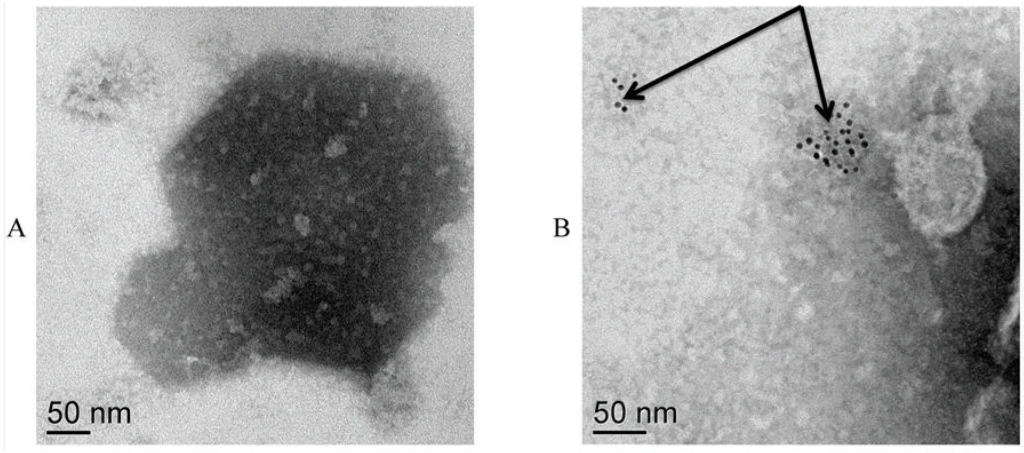

Figure 4.
Immune electron microscopy of cell lysates from GI.1 hNoV infected 3-D Caco-2 cells (A) Failure of localization of secondary antibody when primary antibodies were omitted. (B) Localization of 6 nm gold secondary antibodies (arrows) when infectious samples received both primary and secondary antibody.

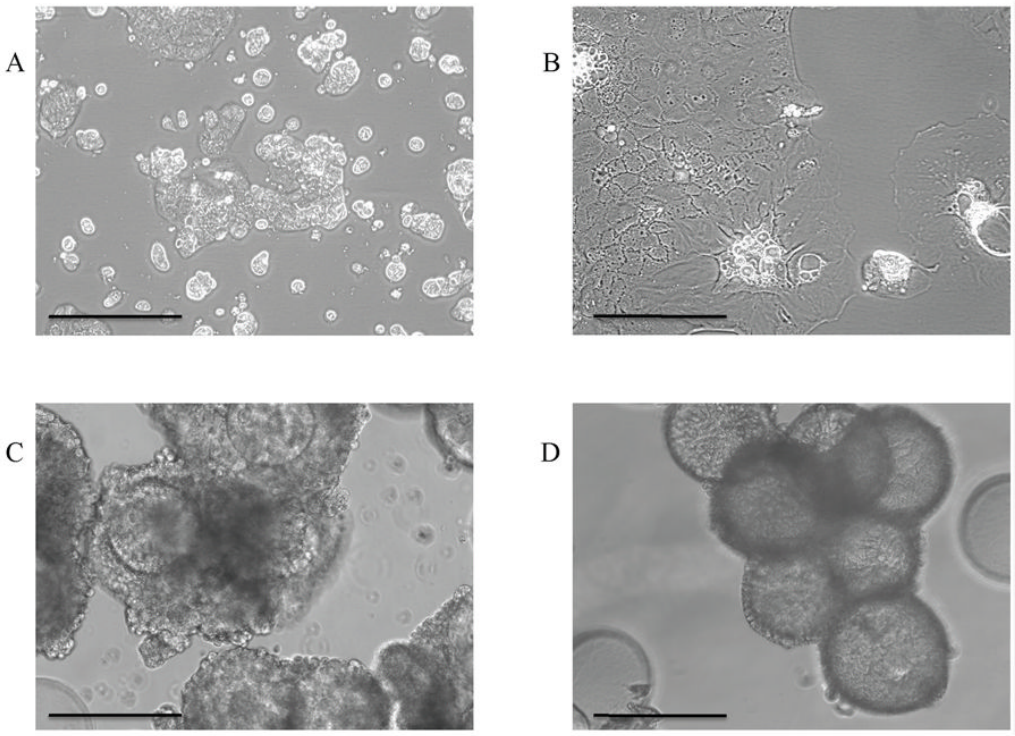

Figure 5.
Observational phenotypes of Caco-2 cells grown in 75 cm^2 flasks (A and B) and as dynamic 3 – dimensional cell cultures (C and D). Cells shown in A and C are from Washington State University, and cells shown in B and D were purchased directly from ATCC. Bar = 250 microns.

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Table 1

Measured RNA concentrations in uninfected and infected cells.

Treatment	Time (hrs pi)	Total RNA (ng/μL)	Human GAPDH Ct	Human STAT-1 Ct	Norovirus Ct[1]
Uninfected	1 hr	1.4	21.7 ± 0.12	29.1 ± 0.14	No amplicon
	24 hrs	1.3	21.7 ± 0.10	30.2 ± 0.07	No amplicon
	48 hrs	1.7	21.2 ± 0.11	29.5 ± 0.01	No amplicon
	72 hrs	1.7	22.1 ± 0.15	30.3 ± 0.24	No amplicon
Infected[2]	1 hr	2.2	21.6 ± 0.03	28.8 ± 0.17	43.7 ± 0.9
	24 hrs	2.5	19.8 ± 0.05	28.5 ± 0.06	38.5 ± 0.1
	48 hrs	77.0	14.4 ± 0.17	23.4 ± 0.03	34.9 ± 0.4
	72 hrs	130.7	13.8 ± 0.01	22.4 ± 0.17	33.3 ± 0.2

[1] Data for norovrius Ct is shown again in Table 3. Decreasing Ct indicates increasing RNA concentration.

[2] Infected with Genogroup I.1, sample 4-3.

Table 2

PCR primers to generate real-time PCR standards for human norovirus genogroups GI and GII.

Genogroup	PCR Primers	Primer Sequence (5' - 3')[1]	Primer Position[2]
GI	NGI15280	aggccatgttccgctggat	5280-5298
	NGI15280A	aRgcHatRttYcgYtggat	5280-5298
	NGI15357	gcgtccttagacgccatcatcatt	5357-5380
	NGI15357A	gcgYccttagacgccatcatcatt	5357-5380
GII	NGII24995	tgcccagacaagagccaatgtt	4995-5016
	NGII24995A	tRccMagRcaRgaRBcNatgtt	4995-5016
	NGII25083	gtcactcgacgccatcttcatt	5083-5105
	NGII25083A	gYSaYtcgacgccatcttcatt	5083-5105

[1] R = G or A; H = T, C, or A; Y = T or C; M = C or A; B = G, C, or T; S = c or G; N = A, C, G, or T.

[2] Based GenBank Accession number M87661 (GI) and AF145896 (GII).

Table 3

RT real-time PCR results for the genogroup GI.1 viral challenge corresponding to Figures 2 and 3. Sample 4-1 is norovirus-free stool and sample 4-3 is norovirus positive stool.

Sample	Mean Quantity (qRT PCR copies)	Std dev	Mean Ct	Std dev
Mock infection 1 hr pi[a]	None detected		>45 cycles	
Mock infection 24 hr pi	None detected		>45 cycles	
Mock infection 48 hr pi	None detected		>45 cycles	
Mock infection 72 hr pi	None detected		>45 cycles	
4-1 1 hr pi	None detected		>45 cycles	
4-1 24 hr pi	None detected		>45 cycles	
4-1 48 hr pi[b]	1,250	± 348	39.3	± 0.3
4-1 72 hr pi	None detected		>45 cycles	
4-3 1 hr pi	171	± 85	43.7	± 0.9
4-3 24 hr pi	2,200	± 676	38.5	± 0.1
4-3 48 hr pi	36,206	± 6,244	34.9	± 0.4
4-3 72 hr pi	132,919	± 37,863	33.3	± 0.2

[a] pi = post infection.

[b] Cross contamination event at the time of infection. Measured total RNA concentrations for 4-1 were 0.9 ng/μL, 1.6 ng/μL, 108 ng/μL, and 20 ng/μL for 1, 24, 48, and 72 hrs pi, respectively.

Table 4

Second independent trial infecting 3-D Caco-2 cells with non-infectious and hNoV containing stool samples.

Sample	Mean quantity (RT real-time PCR copies)	Std dev	Mean Ct	Std dev
No template	None detected		> 45 cycles	
3-1[a] 1 hr pi	None detected		> 45 cycles	
3-1 48 hr pi	None detected		> 45 cycles	
3-1 72 hr pi	None detected		> 45 cycles	
3-1 1 week pi	None detected		> 45 cycles	
4-1 1 hr pi	None detected		> 45 cycles	
4-1 48 hr pi	None detected		> 45 cycles	
4-1 72 hr pi	None detected		> 45 cycles	
4-1 1 week pi	None detected		> 45 cycles	
3-9[a] 1 hr pi	None detected		> 45 cycles	
3-9 48 hr pi	None detected		> 45 cycles	
3-9 72 hr pi	8,880	± 2,070	35.7	± 0.4
3-9 1 week pi	17,200	± 3,560	34.6	± 0.3
4-3[b] 1 hr pi	635	± 243	40.0	± 0.6
4-3 48 hours pi	779	± 57	39.6	± 0.1
4-3 72 hours pi	41,100	± 9,280	33.2	± 0.4
4-3 1 week pi	47,800	± 6,840	32.9	± 0.2

[a]This is a GI.1 sample from a second patient in the Emory human challenge trial. This patient's stools were harvested prior to infection (3-1) and 6 days post infection (3-9), and the copy number was approximately 1 Log_{10} lower than patient 4-3.

Table 5

Norovirus GII.4 copies from infected 3-D Caco-2 cells measured from the cell pellet. Both samples 386 and 1G were norovirus positive stools.

Sample	Mean quantity (qRT PCR copies)	St dev	Mean Ct	Std dev
Mock infection 1 hr pi[a]	None detected		>45 cycles	
Mock infection 48 hr pi	None detected		>45 cycles	
Mock infection 72 hr pi	None detected		>45 cycles	
Mock infection 1 week pi	None detected		>45 cycles	
386 1 hr pi	None detected		>45 cycles	
386 48 hrs pi	None detected		>45 cycles	
386 72 hrs pi	9,375	± 1,048	33.4	± 0.18
386 1 week pi	None detected [b]			
386 1 hr pi	None detected [c]		>45 cycles	
386 48 hrs pi	None detected		>45 cycles	
386 72 hrs pi	None detected		> 45 cycles	
386 1 week pi	68	± 42	38.4	± 1.0
1G 1 hr pi	29[d]	± 17	42.6	0.99
1G 48 hrs pi	11[e]		44.0	
1G 72 hrs pi	2324	± 180	35.6	0.18
1G 1 week pi	1563	± 329	36.2	0.32

[a] pi = post infection.

[b] With low titer, and an independently measured well, it is possible that there was no detectable virus-infected cells in this well.

[c] Biological replicate; different cells were infected with the same stock as the first replicate.

[d] Expected number of copies was approximately 10% of the expected titer

[e] Only 1 of 3 replicates was positive so there was no average or standard deviation.