

Defining cell culture conditions to improve human norovirus infectivity assays

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ABSTRACT

Significant difficulties remain for determining whether human noroviruses (hNoV) recovered from water, food, and environmental samples are infectious. Three-dimensional (3-D) tissue culture of human intestinal cells has shown promise in developing an infectivity assay, but reproducibility, even within a single laboratory, remains problematic. From the literature and our observations, we hypothesized that the common factors that lead to more reproducible hNoV infectivity *in vitro* requires that the cell line be (1) of human gastrointestinal origin, (2) expresses apical microvilli, and (3) be a positive secretor cell line. The C2BBE1 cell line, which is a brush-border producing clone of Caco-2, meets these three criteria. When challenged with Genogroup II viruses, we observed a 2 Log₁₀ increase in viral RNA titer. A passage experiment with GII viruses showed evidence of the ability to propagate hNoV by both quantitative reverse transcription polymerase chain reaction (qRT-PCR) and microscopy. In our hands, using 3-D C2BBE1 cells improves reproducibility of the infectivity assay for hNoV, but the assay can still be variable. Two sources of variability include the cells themselves (mixed phenotypes of small and large intestine) and initial titer measurements using qRT-PCR that measures all RNA vs. plaque assays that measure infectious virus.

Key words | C2BBE1 cells, infectivity, noroviruses, qRT-PCR, three-dimensional cell culture

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INTRODUCTION

The human noroviruses (hNoV) continue to be one of the most difficult viruses to study due to the challenge of developing a relevant and robust *in vitro* model for their study (Duizer *et al.* 2004; Asanaka *et al.* 2005; Guix *et al.* 2007; Straub *et al.* 2007, 2011). There are several conflicting reports regarding the actual cell type in the gastrointestinal tract that hNoV infect. For murine noroviruses, Wobus *et al.* (2004) reported that macrophages are the primary cell type. In contrast, Lay *et al.* (2010) found that hNoV does not infect human macrophages. Most recently, two studies have reported localization of noroviruses in the cells of the lamina propria and Brunner's glands (Bok *et al.* 2011; Chan *et al.* 2011). Thus, questions regarding what cell types should comprise a relevant *in vitro* cell culture system for the hNoV remains in question.

With the exception of the Bok *et al.* (2011) and Chan *et al.* (2011) studies, all other research suggests that hNoV likely infects the epithelial cells of the small intestine, and that the chemistry of the histoblood group antigens (HBGA)

expressed on the apical surfaces of gastrointestinal epithelial cells (e.g. secretor status) is critical for symptomatic hNoV infection (Thorven *et al.* 2005). This hypothesis is based on several lines of evidence. First, biopsies of human volunteers showed significant pathology of the proximal small intestine after hNoV infection (Schreiber *et al.* 1973; Dolin *et al.* 1975). Studies with gnotobiotic animals further confirmed viral interaction with the epithelial cells of the small intestine, and antibodies detected viral capsid proteins on the apical microvilli in these animals (Cheetham *et al.* 2006; Souza *et al.* 2008). *In vitro* studies, mostly employing Caco-2 cells, showed that hNoV and hNoV virus-like particles bind to HBGA that are expressed on the cell membrane (White *et al.* 1996; Harrington *et al.* 2002, 2004; Marionneau *et al.* 2002; Hutson *et al.* 2003). Finally, Thorven *et al.* (2005) demonstrated that a point mutation in the *FUT2* gene, which encodes for positive secretor status, changes the chemistry of the cell surface receptor (non-secretors), and these individuals do not become symptomatic when infected with hNoV.

Our hypothesis is that the reproducibility of *in vitro* hNoV infectivity assays is based on several key factors. First, since the primary target of hNoV appears to be the epithelial cells of the gastrointestinal tract, gastrointestinal cell lines grown in a manner that better recapitulates the architecture of their *in vivo* constructs from which they were derived is likely critical (Barrila *et al.* 2010). Second, we had observed that growth of the gastrointestinal cells as three-dimensional (3-D) organotypic tissue constructs alone was insufficient for success (Straub *et al.* 2007, 2011). Our best success came when these tissue constructs expressed apical microvilli. Finally, based on the literature and our observations, secretor status is important. Combining these factors of 3-D organotypic gastrointestinal cell cultures, apical expression of microvilli, and positive secretor status, we present new data, not discussed in Straub *et al.* (2011), on improvements in reproducibility for the *in vitro* infectivity assay for hNoV.

METHODS

Characterization of the C2BBE1 cell line

The C2BBE1 cell line (ATCC CRL-2102™; ATCC, Manassas, VA) is a clone of Caco-2 and was chosen because this clone consistently produces apical microvilli when grown as a 3-D trans-well culture (Peterson & Mooseker 1992). Polymerase chain reaction (PCR) and restriction enzyme digest based on Thorven *et al.* (2005) was performed to verify positive secretor status of this cell line. In this study, C2BBE1 cells were grown and differentiated as dynamic 3-D organotypic cultures on collagen I coated microspheres in rotating wall vessel bioreactors for 18–21 days as previously described (Straub *et al.* 2011) with the following exceptions. MEM alpha was replaced with Dulbecco's Modified Eagles Media with L-glutamine (DMEM; ATCC), heat inactivated fetal bovine serum was reduced from 20 to 10%, and Insulin Transferrin Selenium (ITS; Life Technologies, Carlsbad, CA) was added to a final concentration of 1X.

Differentiated C2BBE1 tissue structure was assessed using immunofluorescent staining for villin to assess brush border formation and scanning electron microscopy (SEM) (Figure 1). Cells were fixed and stained following manufacturer recommendations (Cell Signaling, Danvers, MA). Working dilutions for all antibodies was 1:100 in 1% normal goat serum (Santa Cruz Biotechnologies, Santa Cruz). Villin-1 antibody (Cell Signaling) was developed

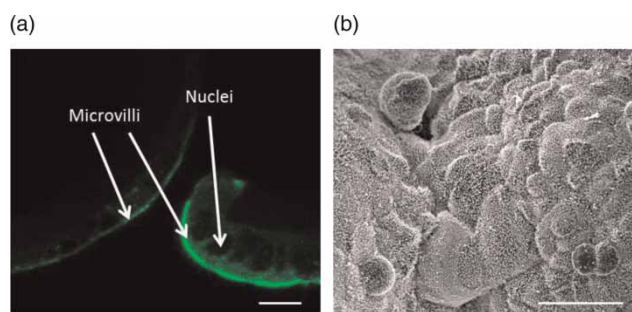


Figure 1 | Immunofluorescent staining of 3-D organotypic cultures of C2BBE1 with villin-1 (a) and visualization of apical microvilli by scanning electron microscopy (b). Scale bars are 20 µm.

with 1:100 Alexa Fluor® 488-conjugated with goat anti-rabbit IgG (Life Technologies).

SEM was used to characterize uninfected and infected C2BBE1 cells in the hNoV passage experiment described in this study. Cells were washed three times in phosphate-buffered saline (Life Technologies) after fixing in glutaraldehyde. Lipids were stained with 1% osmium tetroxide (Electron Microscopy Science, Hatfield, PA) for 1.5 h, washed three times in water, and gradually dehydrated in an ethanol series for 15 min (25, 33, 50, 75, and 100%). After dehydration, the samples were then transferred to a 0.2 µm pore polycarbonate track-etched membrane filter in a vial for critical point drying (CPD). The CPD instrument (Pelco CPD2; Ted Pella, Inc., Redding, CA) was pre-cooled and processed according to an automated CPD scheme, reaching a temperature of 35 °C at 1,200 lb/in², with CO₂ as a transitional fluid. Samples were mounted on aluminum SEM stubs covered with double-sided carbon adhesive tape prior to sputter coating with carbon. Samples were evaluated at room temperature in the scanning electron microscope at 2–3 kV.

Infectivity assays

For the data reported in this study, NoV GGII outbreak isolate 8G was kindly provided by C.P. Gerba, was used to infect cells. This isolate is similar to 1G used in our previous study (Straub *et al.* 2011) except that the initial stool titer (ca. 5.6×10^6 copies per PCR reaction) is approximately 10 times greater than 1G. The results reported for this study represent three independent trials. Each of these trials used independently grown batches of cells and different lots of cell culture stocks from ATCC. Where possible, we never exceed 10 passages from the original ATCC stock to minimize overgrowth of the abnormal phenotypes described by

Peterson & Mooseker (1992). As before, viruses were diluted 1:1,000 or 1:10,000 in sera free DMEM and allowed to infect cells for 72 h for up to 5 days. For the viral passage experiment, tissue aggregates were transferred to a conical centrifuge tube and allowed to settle at 1X gravity for 5 min. Supernatant culture media was aspirated, and the tissue aggregates were resuspended in 5 mL of fresh sera free DMEM. This suspension was subjected to a 2X freeze (−20 °C) thaw (room temperature) lysis and then extracted with an equal volume of Vertrel (decafluoropentane, Sigma, St Louis, MO). Naïve tissue aggregates in 24-well tissue culture dishes were infected with 0.5 mL/well of the extracted virus and allowed to infect for 1 h. Infected cells were washed, overlaid with 1 mL of media, and infection proceeded for an additional 72 h. Time matched controls for all experiments consisted of ‘mock infected’ cells. The mock-infected cells were processed in the same manner as infected cells. At selected time points, cells were harvested and viral nucleic acid was extracted as previously described (Straub *et al.* 2011). Quantitative reverse transcription PCR (qRT-PCR) was performed as a two-step PCR (reverse transcription using random primers, followed by qPCR). GII qPCR was performed using the protocol described by Kageyama *et al.* (2003).

RESULTS AND DISCUSSION

We present the results of three independent trials where NoV GGII outbreak isolate 8G was used to infect the cells. Two of these studies are passage 0 (P0) trials: Diluted stool sample was applied to the cells and allowed to infect the cells (Table 1). In the second independent trial we compared a low passage stock of Caco-2 that was used in our previous study that was shown to produce apical microvilli (Straub *et al.* 2011) with C2BBE1. The third trial was a P1 trial where viruses from a P0 challenge were harvested, and then these viruses were used to infect the cells (Table 2 and Figure 2). Additional passage experiments were not performed in the work presented here.

In general, when 3-D organotypic gastrointestinal Caco-2 cell cultures produce apical microvilli, we observed better evidence of human norovirus RNA replication (Straub *et al.* 2011). The C2BBE1 clone of the Caco-2 cell line does produce apical microvilli more consistently than its parent Caco-2 as previously reported by Peterson & Mooseker (1992). Phenotypes representing both large (shorter microvilli length, approximately 0.6 µm) and small intestine (longer microvilli

Table 1 | Viral RNA amplification observed in two independent P0 trials

	Time post infection	Ct	Ct SD	Copies	Copy SD
Trial 1 (C2BBE1)	1 h	34.9	0.1	2,728	224
	48 h	33.7	0.3	5,911	1,321
	5 days	26.5	0.2	559,129	55,671
Trial 2a (Caco-2) ^a	1 h	>45	NA	NA	NA
	24 h	33.18	0.2	15,299	1,774
	72 h	32.15	0.3	29,634	4,979
Trial 2b (C2BBE1)	1 h	>45	NA	NA	NA
	24 h	33.26	0.3	14,519	2,338
	72 h	35.89	0.2	2,685	351

^aIn this trial, we compared a low passage Caco-2 stock with C2BBE1. This Caco-2 stock was a different batch of cells and obtained from ATCC. These data were not reported in Straub *et al.* (2011).

Table 2 | Viral RNA amplification observed in the passage experiment

	Time post infection	Ct	Ct SD	Copies	Copy SD
P0 (C2BBE1)	1 h	>45	NA	NA	NA
	5 days	39.0 ^a	NA	363	NA
P1 (C2BBE1)	1 h	>45	NA	NA	NA
	72 h	37.2	0.6	1181	425

^aOnly 1/3 replicates was positive by PCR.

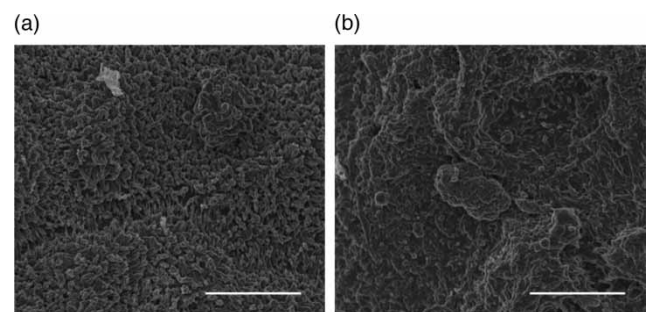


Figure 2 | Comparison of uninfected (a) and infected (b) cells in the P1 experiment at 48 h post infection. Scale bar is 4 µm.

length, up to 1.6 µm) were observed by Peterson & Mooseker (1992) and in our observations as well. If the hNoV do have a tropism for the small intestine, and cannot infect the large intestine, then despite our best efforts, this source of variability cannot be controlled in this cell line. Moreover, being a cancer cell line, we may also be limited by the number of times the cells are passaged before the cell line is overrun by the aberrant cancer cell phenotypes (Straub *et al.* 2011).

For the data presented in [Table 1](#), the first trial showed over a 2 Log_{10} increase in viral RNA copies. The second trial results were more ambiguous. In this second trial, viruses were diluted to 1:10,000, and we were unable to determine an initial titer by PCR. For the Caco-2 cells we again were able to observe a 2 Log_{10} increase in viral titer at 24 h post infection followed by a slight increase at 72 h for the Caco-2 cells, but a decrease in viral titer from 24 to 72 h in the C2BBE1 cells. We were unable to determine the reasons for this decrease in viral titer, but we hypothesize that the nature of how the assays are conducted (at each time point the 3-D tissue aggregates in the 24-well tissue culture dishes are sampled independently and without replacement) may provide a partial explanation. With a lower dose infection (based on the standard curve our limit of detection is approximately 100 copies per PCR reaction), possible viral clumping, and independent sampling, it is possible that the true number of infectious virions was lower. Despite the variability in the results we still observed over a 2 Log_{10} increase in viral titer, by the end of the infection trial for all treatments. Uninfected controls showed no evidence of viral RNA as expected.

The passage experiment provided more evidence of the ability for the viruses to be propagated in cell culture. Here, viruses were diluted to the point where we were unable to measure titer by PCR. After 5 days of infection, only one of three replicates showed replication of viral RNA. After processing the viruses for passage, cells were infected, and after 72 h, all three replicates were positive by PCR ([Table 2](#)). Similar to trends observed in the TEM images from [Straub *et al.* \(2011\)](#), in the SEM images shown in [Figure 2](#), microvilli in the infected cells appeared to be blunted and the apical surfaces appeared more damaged, in comparison with their matched uninfected controls. Taken together with the PCR data for this passage experiment, our observations suggest that the viruses can be passaged at least one time. This observation is supported by our previous work where we showed specific localization of antibody to GI.1 viruses in a P1 sample ([Straub *et al.* 2011](#)).

CONCLUSIONS

In vitro infectivity assays for hNoV are still challenging even within our laboratory. Based on three studies, it is apparent that human gastrointestinal epithelial cells, grown as organotypic 3-D tissues, are necessary for success. Furthermore, positive secretor status also appears to be a key factor in success based on our results with HT-29 cells

which are negative secretors ([Straub *et al.* 2007](#)). The factors affecting reproducibility could lie among the cell lines used and how initial titer is measured. Caco-2 and its clone C2BBE1 are representative of enterocytes found in both the small and large intestine. However, if noroviruses have a tropism for the small intestine, this could become problematic. INT407 provides variable results, presumably due to HeLa contamination (ATCC). The small intestinal cell line CCL 241 (ATCC) is a secondary cell line that is not immortalized and is negative for periodic acid-Schiff (PAS) staining. This indicates that this cell line may not differentiate into multiple cell types like INT407 ([Nickerson *et al.* 2001](#)), and multicellular constructs may be necessary for replication as well.

A second source of variability is understanding the proportion of virions that are infectious at the beginning of the experiment. For cytopathic effect (CPE) producing viruses such as the murine noroviruses, initial infectious titer can be measured by plaque assays. For hNoV quantitative RT-PCR must be used, and this measures RNA from infectious, intact but non-infectious virions, and any free RNA that has not already been hydrolyzed by natural RNases in food, environmental, or clinical matrices. Thus it is possible that when replication is not observed, it could be due to a stock that either has non-infectious virions or free, intact RNA. Treatment by RNaseA would be expected to remove the intact RNA, but depending on the nature of the non-infectious virions, RNaseA and even treatment with Proteinase K may not remove detectable RNA. It is important to note that even after RNaseA treatment, disinfection studies where quantitative RT-PCR is compared to plaque assays, the time required for 99% viral inactivation is longer when measured by PCR vs. infectivity ([de Abreu Correa *et al.* 2012](#)). Thus, better methods are needed to assess infectious virus titer when relying on molecular methods.

In our system, a 'run away' infection leading to the production of a high titer virus stock does not occur as it does for other CPE producing enteric viruses. We observe at best a $2\text{--}3 \text{ Log}_{10}$ increase in viral RNA titer, and then infection plateaus. In a recently published study, [Finkbeiner *et al.* \(2012\)](#) found a similar phenomenon in regards to clinical isolates of human rotavirus infecting human gastrointestinal organoids. These organoids had been generated using induced pluripotent stem cells. In contrast, laboratory adapted rotavirus could easily replicate in the human intestinal organoids similar to their established cell culture system. In addition, the role of innate immunity in the 3-D organotypic cultures requires further characterization to determine if innate mechanisms could also limit replication.

Results from Guix *et al.* (2007) using infectious RNA started a preliminary investigation into the role of innate immunity in the failure of the progeny virions to infect adjacent cells, but no conclusions could be drawn from their studies regarding the potential cellular pathways that may be involved. Regardless of the mechanisms limiting viral replication, this assay is showing that viruses recovered from clinical stool samples are infectious.

In conclusion, our studies are providing more promise for developing a robust, *in vitro*, infectivity assay for the hNoV. With the 3-D organotypic cultures, we are observing repeated evidence of epithelial cell stress (blunted microvilli and vacuolized cells) due to viral infection that mimics results of the human challenge trials. In addition, we usually observe a concomitant increase in viral RNA titer that would indicate viral replication. The next steps are to test the system with samples recovered from food and environmental samples. We demonstrated the ability to amplify virus from lower titer stocks (<100 viral RNA copies), which would be important for samples recovered from the environment. Stool samples not containing virus do not appear to be toxic to the 3-D organotypic cultures (data not shown), and this would indicate that the system could handle potential toxins that may co-concentrate with food and environmental sample processing.

ACKNOWLEDGEMENTS

The authors wish to thank William Chrisler for his assistance with confocal microscopy (Figure 1(a)). This research was funded in part by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under contract no. NO1-AI-30055 and the United States Environmental Protection Agency STAR Grant Program (Grant R833831010). Confocal and scanning electron microscopy was funded in part by the United States Department of Energy Laboratory Directed Research and Development (LDRD) grant for J. R. Hutchison as part of a human microbiome community preliminary study. A portion of this research was performed using EMSL, a national scientific user facility sponsored by the Department of Energy's Office of Biological and Environmental Research located at Pacific Northwest National Laboratory. PNNL is operated by Battelle Memorial Institute for the United States Department of Energy under contract DE-AC06-76RLO 1830.

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First received 28 May 2012; accepted in revised form 2 October 2012