Infectious Virus Confirmation in Foods Using Human Intestinal Enteroids

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Abstract

One of the major goals of CFSAN's research program is the development of an assay to confirm the presence of infectious norovirus in foods associated with outbreaks. Current detection methods of norovirus from food samples can only identify the presence of a viral genome, which does not provide any information on virus infectivity. The recent breakthrough of the successful cultivation of human norovirus from clinical samples in human intestinal enteroids (HIEs) has provided a promising tool to look into growth conditions of different norovirus strains and titration of infectious virus from food matrixes.

Introduction

Purpose: We determined whether the human intestinal enteroids system can be used to identify infectious norovirus particles that have been extracted from spiked lettuce or frozen berries, two commodities that are often associated with viral foodborne outbreaks.

FDA Mission Relevance Statement: The development of a reliable cell culture model for human norovirus is vital to confirming the presence of infectious virus in a potentially contaminated source (food or environmental sample).

1. Weigh 50 g of food and inoculate with 100ul of 10% stool suspension

2. Let air dry for 1h at RT

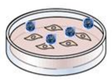
3. Elute virus with 0.1M Tris-HCl-0.05-M glycine-1% beef extract buffer, pH 9.2, with Polyvinylpyrrolidone and pectinase on a shaking platform (30 min at RT)

4. Clarify eluate with centrifugation at 10,000xg at 4°C

5. Concentrate the virus by ultracentrifugation for 170,000xg for 1h at 4°C

6. Resuspend pellet in media, sonicate and clarify particles at 4500 rpm/5min

7. Infect enteroids (1h adsorption, 3 washes) and collect samples at times 0 and 3 days post infection (dpi)



8. Extract RNA from cells and supernatants with Trizol

9. Quantify virus recovery from foods and replication by qRT-PCR with in-house full length transcripts

Fig. 1. Virus isolation protocol for raspberries, lettuce, and strawberries. Method adapted from FDA BAM (Chapter 26B).

Materials and Methods

We first established that two norovirus strains, GII.4[P16] and GII.6[P7] can be successfully grown in the HIE's. Briefly, 50g of either lettuce or frozen berries were artificially inoculated with 10% stool suspensions, and subsequently the virus was eluted with a 0.1 M Tris-HCl-0.05 M Glycine-1% Beef extract buffer and finally concentrated by ultracentrifugation. The resulting virus particles were inoculated onto differentiated HIE's, and after 1h of adsorption at 37°C, virus was washed off and the cells were incubated for 3 days at 37°C. Cells/virus were collected and RNA was isolated and quantified by RT-qPCR with in-house whole genome transcripts.

Positive (5+ fold increase)	Negative (<5-fold increase)
GII.4 Sydney (BCM strain)	GII.1 (2016)*
GII.4[P16] (011617)*	GII.4 (2014)*
GII.4[P16] (1036 NVRL)	GII.7 V*
GII.4[P16] (1041 NVRL)	GII.17 (118-2)^
GII.4[P16] (1046 NVRL)	GI.x*
GII.4 Sydney (13-39)+	GII.4[P16] Sydney (1043 NVRL)
GII.6 (14-55)+	GII.6 (15-65)+
GII.4 Sydney (15-59)+	GII.4 New Orleans/Sydney (16-78)+
GII.6[P7] (2014 stool)*	GII.6[P7] EP3*
	GII.6[P7] EP4*
	GII.6[P7] EP5*
	GII.6[P7] EP6*
	GI.6[P11]^
	GI.3[P3] (#2)^
	GI.3[P3] (#3)^
	GI.3[P3] (461-1)^
	GI.3[P3] (461-2)^
	GI.3[P3] (461-3)^
	GII.6[P7] (2014 vomit)*

Figure 2. Summary of human Norovirus strains tested in J2 HIE's.
* in-house samples
NVRL samples provided by Dr. Suzie Coughlan, NVRL, UCD, Dublin, Ireland
+ samples provided by Dr. Sabah Bidawid, Health Canada, Ottowa, CN
^samples provided by Julia Wolfe, Orange County Public Health Laboratory, Santa Ana, CA

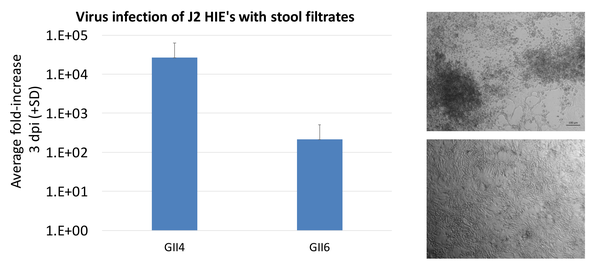


Figure 3. Human noroviruses GII.4 and GII.6 replicate in J2 HIE's. Average fold-increases of GII.4 or GII.6 at 3 dpi compared to 1 hpi. Results are averaged from 7 independent experiments. GII.4 range: 1.5e3-8.5e4 fold-increase. GII.6 range: 18-858-fold increase. Upper panel: GII.4 sometimes causes cytopathic effect (cpe) in J2 HIE's at 3 dpi. Lower panel: GII.6 does not cause cpe at 3 dpi. Both images are 5x magnification.

Results and Discussion

Results: Preliminary results indicate that although there was variability in log-fold increase of each virus in each experiment, successful recovery from foods and replication of both strains was possible, as measured by RT-qPCR, that could reach up to a 5 log increase in genome copies.

Sample	Input	1 hpi	72 hpi
GII.6	3.4e2	1.2e3	1.4e7
GII.6	Udt	5.9e2	1.9e7
GII.6	Udt	3.9e2	4.2e6
GII.4	1.6e5	6.4e3	6.0e7
GII.4	Udt	4.4e3	1.3e8
GII.4	Udt	6.1e2	1.0e8

Table 1. Representative samples of replication of 2 norovirus strains isolated from artificially spiked frozen raspberries. The numbers represent average total genome copies per well. **5/6 GII.6 and 8/8 GII.4 isolates (13/14 total) replicated in J2 HIE cells.** Only 3/14 inputs were detectable by qRT-PCR, indicating high levels of PCR inhibition in the extracts (Udt is below the detectable limit in the qRT-PCR reaction).

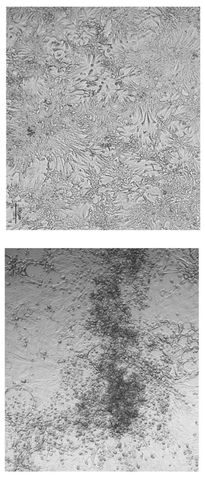


Figure 4. Cytopathic effect in J2 enteroids infected with GII.4, but not GII.6, norovirus isolated from frozen raspberries. Upper panel, GII.6-spiked berry extract at 3 dpi, 5x magnification. Lower panel, GII.4-spiked berry extract at 3 dpi, 5x magnification.

Sample	Input	1 hpi	72 hpi
GII.6	2.6e4	1.9e2	1.5e2
GII.6	5.6e4	3.1e2	Udt
GII.6	5.1e5	3.0e2	6.4e3
GII.4	3.4e5	4.0e3	1.1e6
GII.4	1.2e6	9.0e2	1.8e7
GII.4	3.1e5	3.6e2	Udt

Table 2. Representative samples of replication of 2 norovirus strains isolated from artificially spiked fresh lettuce. The numbers represent average total genome copies per well. **1/11 GII.6 and 9/12 GII.4 isolates (10/23 total) replicated in J2 HIE cells.** All 23/23 inputs were detectable by qRT-PCR, indicating low levels of PCR inhibition in the lettuce extracts.

Sample	Input	1 hpi	72 hpi
GII.6	Udt	4.0e2	2.6e7
GII.6	7.1e2	Udt	Udt
GII.6	Udt	2.5e2	5.1e2
GII.4	7.4e2	Udt	5.9e7
GII.4	4.0e3	2.8e2	1.6e7
GII.4	3.5e2	Udt	Udt

Table 3. Representative samples of replication of 2 norovirus strains isolated from artificially spiked frozen strawberries. The numbers represent average total genome copies per well. **3/11 GII.6 and 9/12 GII.4 isolates (12/23 total) replicated in J2 HIE cells.** 17/23 inputs were detectable by qRT-PCR.

Summary:

- The cultivation of J2 HIE's has been successfully established in our laboratory
  - We have 2 in-house strains of human norovirus that robustly replicate in the J2's
    - GII.P16-GII.4 (2017) and GII.P7-GII.6 (2014)
    - GII.4 strain sometimes shows cytopathic effect, but GII.6 does not
- We have shown proof-of concept that human noroviruses extracted from foods CAN be cultivated in the J2 HIE's
  - Robust replication can be achieved from berry isolates
  - GII.4 replicated more consistently and with higher titers regardless of food matrix
  - GII.6 replicated better when isolated from berries
    - Berry extracts have much more inhibitory compounds than lettuce extracts for PCR detection

Conclusion

Conclusion: These studies begin to address the challenges faced with the development of a cell culture model aimed at confirming the presence of low, infectious virus load in naturally contaminated food matrices. Future studies will need to determine the lower limit of viral load that can lead to successful replication in the HIE system.

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