Optimizing methods for the detection and quantification of infectious human norovirus from fresh berries using human intestinal enteroids

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Summary
Human norovirus (HuNoV) and hepatitis A virus (HAV) are responsible for most foodborne outbreaks associated with berries. The current FDA standard method for detection of these viruses in berries relies on identification of viral RNA. A recent breakthrough in HuNoV cell culture allowed the detection of infectious HuNoV using human stem cells that are grown as 3D human intestinal enteroids (HIE). We will optimize the FDA standard method for recovery of infectious HuNoV and HAV from berries using HIE and FRhK-4 cells, respectively. The optimized method's percent recovery, detection limit, as well as the relationship between viral RNA and infectivity will be determined. The optimized method will be used to investigate the persistence of infectious viruses on berries.

Objectives
1. Optimize the FDA-based method for the detection of infectious HuNoV from various berries using HIE, establish the method's detection limit for infectious HuNoV from various berries, and determine the relationship between infectious HuNoV and viral RNA copies recovered from various berries. Tulane virus (TV; a norovirus surrogate) and HAV will be initially used to optimize the method.
2. Determine the persistence of infectious HuNoV and HAV on various fresh berries under postharvest conditions.

Methods
First, TV and HAV will be used on strawberries to determine the baseline recovery of infectious viruses using the FDA method. Then, the method will be optimized as shown in **Figure 1**. The percent recovery of infectious viruses will be determined to guide optimization efforts. General bacteria on berries will be quantified to determine their effect on viral recovery. Once the method is optimized it will be validated with HuNoV on HIE. The method's detection limit will be determined using serially diluted infectious viruses. Viral RNA detection by RT-qPCR will be compared to presence/absence of infectious viruses to determine the relationship between infectious viruses and Ct values. Second, various berries will be spot-inoculated with the viruses and sampled over time to determine viruses' persistence under post-harvest conditions.

Results to Date
Strawberries were inoculated with TV at ~5 log TCID50/50 g and processed according to the FDA method to determined baseline recovery of infectious viruses (**Figure 2**). Tulane virus was recovered at ~3 log when dried on strawberries, with no significant difference in titers to viruses recovered from process control or from 6% tris-glycine beef extract (TGB) (**Figure 3A**). The recovery of infectious TV from strawberries was 1.4% (**Table 1**). Also, 6% TGB was cytotoxic, leading to an assay detection limit of ~1000 particle/ml. In contrast, TV in elution buffer alone was recovered at significantly higher titer in 1% than 6% TGB (**Figure 3B**). Comparing two methods for concentrating viruses in 1% TGB alone, revealed that ultracentrifugation allowed higher recovery of viruses at 11% versus 1% with ultrafiltration (**Table 2**).

Benefits to the Industry
The current FDA standard method for HuNoV and HAV detection in berries relies on detection of viral RNA by molecular assays, which may or may not indicate the presence of infectious viruses. Having an optimized method for the detection of infectious viruses would allow the berry industry a better understanding of the risks associated with the detection of viral RNA. In addition, the knowledge about the persistence of infectious HuNoV and HAV on various berries under post-harvest conditions is important for devising better control strategies of these viruses on berries. Overall, the berry industry would benefit from reducing foodborne illnesses through consumption of safe food, reducing product recalls and the associated damage to brands.

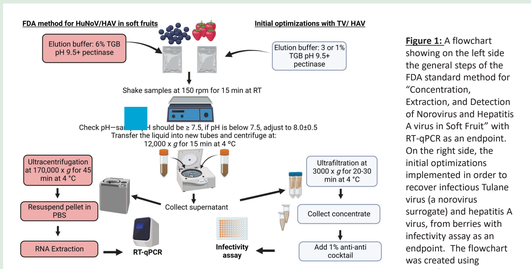

Figure 1: A flowchart showing on the left side the general steps of the FDA standard method for "Concentration, Extraction, and Detection of Norovirus and Hepatitis A virus in Soft Fruit" with RT-qPCR as an endpoint. On the right side, the initial optimizations implemented in order to recover infectious Tulane virus (a norovirus surrogate) and hepatitis A virus, from berries with infectivity assay as an endpoint. The flowchart was created using BioRender.com

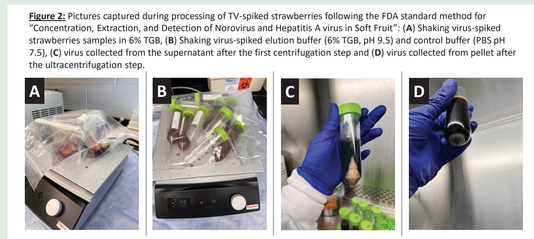

Figure 2: Pictures captured during processing of TV-spiked strawberries following the FDA standard method for "Concentration, Extraction, and Detection of Norovirus and Hepatitis A virus in Soft Fruit": (**A**) Shaking virus-spiked strawberries samples in 6% TGB, (**B**) Shaking virus-spiked elution buffer (6% TGB, pH 9.5) and control buffer (PBS pH 7.5), (**C**) virus collected from the supernatant after the first centrifugation step and (**D**) virus collected from pellet after the ultracentrifugation step.

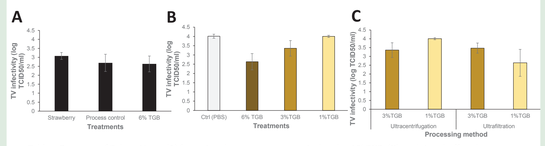

Figure 3: Recovery of Tulane Virus: (**A**) from strawberries, process control and in 6% TGB, (**B**) in elution buffer alone with 6, 3 or 1% beef extracts, (**C**) in elution buffer alone processed by either ultracentrifugation or ultrafiltration

Tables: Recovery of Tulane Virus: (**1**) from strawberries, process control and in 6% TGB, (**2**) in elution buffer alone processed by either ultracentrifugation or ultrafiltration

Table 1: Treatment	Recovery (%)
Control (TV+PBS)	11.2 ± 2.9
TV+ 6% TGB	0.6 ± 0.4
(TV+ Strawberry), virus drying, + 6% TGB	1.4 ± 0.6
Process control (TV +strawberry+6% TGB)	0.7 ± 0.5

Table 2	Recovery (%)	
Treatment	Ultracentrifugation	Ultrafiltration
TV+ 3% TGB	3.3 ± 3	3.7 ± 2.6
TV +1% TGB	11 ± 1.5	1 ± 1