CPS 2022 RFP – FINAL PROJECT REPORT

Project Title:

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

Project Period:

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Objectives:

1. Optimize the FDA-based method to enable detection of infectious HuNoV from various berries using HIE cell culture system, establish the optimized 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.
2. Determine the persistence of infectious HuNoV on various fresh berries under postharvest conditions.

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FINAL REPORT

Summary of Findings and Recommendations

Human norovirus (HuNoV) and hepatitis A virus (HAV) are responsible for a number of foodborne illness outbreaks associated with berries. The FDA/BAM chapter 26 and ISO 15216 standardized methods consist of three main steps that basically allow the elution, separation and concentration of viruses from berries. The final detection of viruses is achieved by RT-qPCR which quantifies viral RNA through cycle threshold (Ct) values. These Ct values are inversely proportional to the amount of viral RNA; however, their relationship to virus infectivity is not well delineated. A recent breakthrough allowed the detection of infectious HuNoV by using 3D human intestinal enteroids (HIE). In this project, it was found that the first step of the FDA/BAM method, i.e. virus elution, is the most critical step that affected recovery of infectious virus from strawberries. By lowering the elution buffer beef percentage and pH, higher recovery for infectious Tulane virus, a surrogate for human norovirus, was achieved (~75%). The RT-qPCR Ct limit of detection of the FDA/BAM method was found to be similar to the ISO 15216 method for both hepatitis A virus and Tulane virus, ranging from 35-36 and 37-38, respectively. Whether using the BAM or ISO methods, infectious HAV and Tulane virus are less likely to be directly recovered from strawberries when the RT-qPCR Ct >36. Furthermore, Ct >40-44 is less likely to predict the presence of infectious viruses per 50 g of strawberries. On day 7 of strawberries stored at 4°C, infectious HAV was significantly reduced by ~0.6 log, while HuNoV was not detected by HIE. Overall, results from this study provide better insights into the interpretation of virus Ct values obtained from strawberries whether using the BAM or ISO methods and enhance our knowledge into infectious virus persistence on strawberries stored under refrigeration.

Abstract

Human norovirus (HuNoV) and hepatitis A virus (HAV) are responsible for several foodborne illness outbreaks associated with berries. The current FDA/BAM and ISO standardized methods for virus detection from berries rely on the detection of viral RNA by RT-qPCR, which does not necessarily indicate the presence of infectious viruses. Historically, HuNoV lacked permissive cells to estimate its infectivity. A recent breakthrough allowed the detection of infectious HuNoV by using 3D human intestinal enteroids (HIE). The objectives of this study were: to optimize the steps of the BAM method for recovery of infectious Tulane virus (TV), a HuNoV surrogate, from strawberries, to determine the detection limit of BAM and ISO methods and the relationship between RT-qPCR Ct values and infectivity of viruses on strawberries, and finally to investigate the persistence of infectious HuNoV and HAV on strawberries stored under refrigeration. Viruses were spiked on strawberries, and then recovered using either the BAM or ISO methods, followed by quantification using RT-qPCR and $TCID_{50}$ infectivity assays. The results showed that optimizing percent beef extract and pH of the BAM elution buffer significantly increased infectious TV recovery to ~75%. The RT-qPCR Ct limit of detection for TV and HAV using BAM and ISO methods were not significantly different and ranged between 35-36 and 37-38, respectively. Logistic model indicated that there is a low probability of directly recovering infectious TV and HAV from strawberries at Ct >36. Furthermore, linear regression analyses showed that Ct >40-44 is less likely to predict the presence of infectious viruses on strawberries. On day 7 of strawberries stored at 4°C, infectious HAV was significantly reduced by ~0.6 log, while HuNoV was not detected by HIE. Overall, this study provides better insights on Ct values interpretation and on the persistence of infectious viruses on strawberries stored under refrigeration.

Background

Berries can be consumed fresh without cooking, and, if they get contaminated anywhere from farm to table, they may pose a health risk to consumers. Human norovirus (HuNoV) and hepatitis A viruses (HAV) are the two most common viruses that lead to illness outbreaks associated with berry consumption. Viruses do not multiply on food or in the environment and this necessitates the use of highly sensitive molecular methods to detect them. The FDA/BAM chapter 26/Section A2 standardized method for "Concentration and Extraction of Enteric Viruses from Soft Fruit: Fresh and Frozen" is divided into three main steps. The first step is intended to elute the viruses from the berries by shaking them in a specific elution buffer made of Tris Base, Glycine and 6% beef extract (6% TGBE, pH 9.5). The second step is designed to separate the viruses from the berry debris by centrifugation at a specific speed and period. Finally, the purpose of the third step is to concentrate the viruses into smaller volumes by ultracentrifugation at a specific speed and period. The international Organization for Standardization (ISO) established a method for the detection of HuNoV and HAV in food matrices (ISO 15216). The ISO method relies on the elution of viruses from berries using a similar elution buffer to BAM, but with a lower percentage of beef extract (1% TGBE, pH 9.5). Then, the virus concentration is achieved by using polyethylene glycol (PEG) mixed with sodium chloride (NaCl). For both methods, the concentrated viruses are then subjected to extraction of genetic material using commercial RNA extraction kits, followed by virus-specific molecular testing by RT-qPCR to determine the presence and to quantify viral genetic material. The RT-qPCR, which generates cycle threshold (Ct) values that are inversely proportional to the amount of viral RNA. However, a major drawback to the BAM and ISO methods, is that both methods rely on the detection of a small piece of viral RNA genome amplified by RT-qPCR. It is widely known that the presence of viral RNA does not indicate, and most likely overestimates, the presence of infectious viruses.

A cell culture model is needed to check whether a specific virus is infectious. Virus infectivity is confirmed if the virus can replicate inside cells, cause cytopathic effect or increase its RNA levels within the cells. HuNoV was recalcitrant to growth in cell culture until recently, when the virus was reported to successfully replicate in human intestinal enteroid (HIE) cells derived from human intestinal stem cells. This cell culture model is costly, labor-intensive and is not commercially available. In addition, infectious HuNoV from fecal samples of sick humans is not commercially available and cannot be generated using the HIE model. Because obtaining infectious HuNoV that replicates in HIE in high titers is difficult, Tulane virus (TV) can initially be used as a surrogate to understand experimental factors that may affect HuNoV. This is because TV (1) is easily culturable and hence can be generated in high titers, (2) belongs to the same family as HuNoV, and shares similar structure, morphology, genome size and binding characteristics to histo-blood group antigens, as HuNoV and (3) is transmitted through the fecal-oral route like HuNoV. Therefore, the first objective of this study was to examine and optimize the various steps of the BAM method for recovery of infectious TV, to determine the method's detection limit, and to define the relationship between RT-qPCR Ct values and virus infectivity using infectious TV and HAV. The second objective was to investigate the persistence of infectious HuNoV and HAV on strawberries stored under refrigeration.

Research Methods and Results

<u>Objective 1:</u>
Task#1: Optimize the FDA-based method to enable detection of infectious HuNoV from various berries using HIE cell culture system
Task #2: Establish the optimized method's detection limit for infectious HuNoV from various berries
Task #3: Determine the relationship between infectious HuNoV and viral RNA copies recovered from various berries

Task #1: In the first task of objective 1 of this project, all three steps of the BAM method were examined and optimized to determine which step affects the recovery of infectious viruses.

i- Optimization of first step, the elution of viruses from strawberries:

The first step in the BAM method is intended to elute the viruses from the surface of 50 g berry samples by shaking at 150 rpm for 15 min at room temperature in 30 ml of elution buffer made of 6% beef extract, Glycine and Tris Base, with a final pH adjusted to 9.5 (6% TGBE, pH 9.5). It is thought that an alkaline buffer allows greater removal of adsorbed viruses to acidic food such as berries.

To optimize the first step, various buffers and ways of virus elution were tested and compared to the elution buffer of the BAM method (6% TGBE, pH 9.5). These elution buffers included: DMEM/2% FBS (pH 7), carbonated water (pH 4) and variation on the percent beef extract and the pH of the standardized elution buffer (6% TGBE, pH 8 and 1% TGBE pH 8). In addition, two other ways for eluting viruses were tested: (1) homogenization of the contaminated strawberries in DMEM/2% FBS (pH 7) followed by the step of the BAM method and (2) one cycle of freeze-thawing the contaminated strawberries at -20 °C/20 °C for 10 h/1 h, and collecting the oozing liquid which then gets directly tested for infectious viruses by the $TCID_{50}$ assay. The TV inocula used to contaminate strawberries ranged between $5x10^4$ to $9x10^4$ $TCID_{50}$/50 g of strawberries. Each experiment included triplicates of 50 g of strawberries and then each experiment was independently repeated a total of three times. One-way or two-way ANOVA were used to determine significant differences among different means of one or two factor treatments, respectively.

The results showed that the average baseline recovery of infectious TV by the BAM elution buffer (6% TGBE, pH 9.5) was ~10%. Changing the pH of the 6% TGBE buffer to pH 8, gave a significantly higher recovery of infectious TV at ~60%. However, when the percentage of beef extract was lowered from 6 to 1% in TGBE (pH 8), the recovery was further improved ~75%. Other tested buffers or ways of elution such as DMEM/2%FBS (pH 7), carbonated water (pH 4), homogenization and freeze/thaw gave similar or worse recovery of infectious TV (~10, 5, 23 and 0.5 %, respectively) as compared to the standardized elution buffer. Therefore, the first step of the BAM method is very critical for recovery of infectious viruses and the elution buffer 1% TGBE (pH 8) is the best among the tested elution buffers and ways of elution.

ii- Optimization of second step, the clarification of viruses from berry debris:

The second step in the BAM method is intended to separate the eluted viruses from the strawberry debris recovered in the first step. The latter is achieved by centrifugation at 12,000 x g for 15 min at 4 °C. By the end of this centrifugation step, the viruses are expected to stay in the supernatants while the pellet is usually discarded. To test the effect of centrifugation speed on recovery of infectious viruses, TV was suspended directly in the standard elution buffer (6% TGBE, pH 9.5) in 1 ml aliquots and then subjected to various centrifugation speeds (2,000 to 10,000 x g for 15 min at 4 °C). After centrifugation, the supernatants and the pellets were collected, and infectious viruses were tested in both fractions.

The results showed that the recovery of infectious TV in the supernatant increased from ~45% to 85% as the centrifugation speed increased from 2000 to 10,000 x g, respectively. However, the increase in speed also increased the percent recovery of infectious TV in the pellet from 5 to 12%. A significant positive linear relationship was found between centrifugation speed and the percentage recovery of infectious viruses present in both the supernatants and the pellets. Using this linear relationship, it can be predicted that at the speed of 12,000 x g used in the BAM method, nearly 98.9% of the viruses can be recovered. Therefore, the centrifugation parameters in this step are optimal for virus recovery in the supernatant while minimizing infectious viruses' loss in the pellet.

iii- Optimization of third step, the concentration of viruses into small volumes:

Because the BAM method elutes viruses from 50 g samples of strawberries in 30 ml of elution buffer, the third step is to concentrate this volume into smaller volumes of ~ 1 ml that are suitable for molecular analyses by RT-qPCR. For this reason, ultracentrifugation at very high speeds is used (170,000 x g for 45 min at 4 °C) in the BAM method. Following ultracentrifugation, it is expected that the viruses will be concentrated in the pellet, while the supernatant is usually discarded. To determine the effect of speed and time on the concentration of the viruses in the pellets, another speed at a longer period was tested (125,000 x g for 120 min) and compared to the BAM's combination of speed and time. Following ultracentrifugation, both the supernatant and the pellet fractions were collected and tested for recovery of infectious viruses.

The results showed that using the BAM speed and time combination, a baseline fold increase of infectious TV in the pellet was at 1.8-fold, while the supernatant still contained infectious viruses at 0.3-fold of what was initially present before ultracentrifugation. The lower speed for longer period of ultracentrifugation (125,000 x g for 120 min) improved the viruses in pellets to ~2.5-fold increase; however, this was not significantly different than the speed/time used by BAM. Furthermore, at 125,000 x g for 120 min, infectious TV were still detectable in the supernatant at a similar level to the BAM speed and time. Overall, both tested speeds and time combinations resulted in about 25% of infectious TV to be lost in the supernatants.

The ISO 15216 method adopted a simpler way of pelleting viruses through the addition of commercially available chemicals (PEG+NaCl), then performing regular centrifugation (i.e. without the need for expensive equipment). Therefore, this virus precipitation method was evaluated for recovery of infectious TV in the pellet. The results showed that infectious TV was concentrated at 2.6-fold of what was initially present prior to addition of PEG+NaCl. Retesting this precipitation method without the addition of NaCl, showed that a similar fold increase of infectious TV in the pellet (~2.1 fold). Comparing the ISO and BAM ways of concentrating viruses in the pellets revealed no significant difference in fold-increase of infectious viruses in the pellets.

Overall, results from task#1 indicate that the first step is the most critical step for improving recovery of infectious viruses from strawberries. Both the pH and percent beef extract significantly affected the recovery of infectious viruses. Using 1% TGBE (pH 8), improved the recovery of infectious viruses from baseline 10% to 75%. The second step did not affect much the recovery of infectious viruses. However, in the third step, there is still a need for further improving the concentration step to capture the infectious viruses lost in the supernatant.

Task#2: Determining the limit of detection of the BAM and ISO methods

In this task, both HAV and TV were used to determine the RT-qPCR and infectivity limit of detection (LOD) of the BAM and ISO methods. Ten-fold serial dilutions of each virus were made in water. Strawberries were contaminated using each of the ten-fold serially diluted TV or HAV. For each virus, one subset of the contaminated strawberries was subjected to the BAM method and the second subset was subjected to the ISO method. All viruses recovered from strawberries were quantified using RT-qPCR assays specific to TV and HAV. The infectivity titers for TV and HAV were also quantified using the TCID50 infectivity assays. Each experiment was independently repeated a total of three times.

The results showed that using the BAM method, the LOD for infectious TV on strawberries was 1.8 ± 0 log $TCID_{50}$/50 g of strawberries, corresponding to Ct 33.2 ± 2.2, while the LOD for infectious HAV was 1.6 $TCID_{50}$/50 g of strawberries, corresponding to Ct 34.1 ± 0.4. Using the ISO method, the LOD for infectious TV on strawberries was 1.7 ± 0.1 log $TCID_{50}$/50 g of strawberries, corresponding to Ct 36.2 ± 0.5, while the LOD for infectious HAV was 1.8 $TCID_{50}$/50g, corresponding to Ct of 35.6 ± 4.5. Comparing all

recovered HAV and TV from strawberries by both ISO and FDA/BAM revealed no statistically significant difference between the processing methods for RT-qPCR Ct values. Next, the RT-qPCR LODs for viral RNA of TV and HAV were determined: the LOD for TV was at Ct 36-38 and for HAV at Ct 35-37, for which no infectious viruses can be directly detected from strawberries by $TCID_{50}$ assay.

Task# 3: Determining the relationship between virus infectivity and RNA copies

In this task, the relationship of virus infectivity titers to Ct values obtained from strawberries was determined. Multiple linear regression and multiple logistic regression were used to determine relationships between infectivity and Ct values or RNA titers.

Results showed that multiple logistic regression model fitted to the overall TV and HAV infectivity titers recovered from strawberries revealed that there is a higher probability (>0.5) of directly detecting infectious viruses from strawberries when the Ct value is <36. This logistic model had positive and negative predictive powers at 93.7 and 75%, respectively.

Because the initial infectivity titers of the viruses that were spiked on strawberries are known, regression analyses between these viruses' infectivity titers and Ct values obtained from strawberries were performed. Results from regression analyses showed a significant linear relationship for both BAM and ISO methods for TV (R^2= 0.98 and 0.97, $p \leq 0.001$) and for HAV (R^2 =0.95 and 0.93, $p \leq 0.01$). Using the linear regression equations for both TV and HAV for both the BAM and ISO methods, it can be predicted that at Ct between 40-44, the strawberries initially had one-unit infectious virus (1 $TCID_{50}$) per 50 g. Therefore, it is unlikely that the detection of viral RNA on strawberries at Ct >40-44 can predict the presence of infectious viruses using current infectivity assays.

<u>Objective 2:</u> Determine the persistence of infectious HuNoV on various fresh berries under postharvest conditions.

A limited amount of a fecal sample containing infectious HuNoV (GII.4) that was shown previously to replicate in the HIE model was obtained from our CDC collaborator. The 3D enteroids were maintained in culture for 1 month before they were differentiated into monolayers that were used in HuNoV infections. Strawberries were spiked with HuNoV and HAV and then, following a brief period of air-drying the viral droplets, the strawberries were incubated inside a refrigerator at 4 °C for 7 days (longer incubation periods resulted in mold formation). On days 0, 1, 3 and 7, viruses were eluted from strawberries using 1% TGBE (pH 8) as an elution buffer, while the rest of the steps were followed as indicated by the BAM method. On each day, HuNoV and HAV total RNA were quantified by virus-specific RT-qPCR. The $TCID_{50}$ infectivity assay was used to quantify HAV infectivity titers, while the HIE system was used to determine presence/absence of infectious HuNoV. Furthermore, on each sampling day, the total heterotrophic bacteria on the surface of the strawberries were quantified using R2A media.

Results showed that HAV total RNA copies remained unchanged on strawberries under refrigeration throughout the 7 days storage period, while infectious HAV showed a significant 0.6 log reduction starting on day 7. Similarly, HuNoV total RNA copies remained unchanged on strawberries under refrigeration throughout the 7 days storage period, while no infectious HuNoV was detected by HIE on day 7. Total heterotrophic bacteria count significantly increased on day 1 by ~0.8 log and this increase was maintained on day 7 (~0.7 log increase). However, there were no significant correlations between total virus RNA for HuNoV or HAV and bacteria counts. Therefore, in case of accidental fecal contamination of fresh strawberries stored under refrigeration, infectious HuNoV and HAV seem to start declining significantly by day 7.

Outcomes and Accomplishments

The FDA/BAM method consists of three main steps that basically allow the elution, separation and concentration of viruses from berries. The final detection of viruses is achieved by RT-qPCR which quantifies viral RNA through cycle threshold (Ct) values. These Ct values are inversely proportional to the amount of viral RNA; however, their relationship to virus infectivity is not well delineated. Results from this project allowed the identification of the critical step in the FDA/BAM method that affected recovery of infectious virus from strawberries. The elution buffer beef percentage and pH were optimized to allow higher recovery for infectious Tulane virus, a surrogate for human norovirus. The RT-qPCR limit of detection of the FDA/BAM method was found to be similar to the ISO 15216 method for both hepatitis A virus and Tulane virus. Whether using the BAM or ISO methods, infectious hepatitis A virus and Tulane virus are less likely to be directly recovered from strawberries when the RT-qPCR Ct >36. Furthermore, linear regression analyses showed that Ct >40-44, obtained by BAM or ISO methods, is less likely to predict the presence of infectious viruses per 50 g of strawberries. Infectious human norovirus and hepatitis A virus were significantly inactivated on strawberries stored at 4 °C by day 7. Overall, results from this study provide better insights into the interpretation of virus Ct values obtained from strawberries whether using the BAM or ISO methods and enhance our knowledge into infectious virus persistence on strawberries stored under refrigeration.

APPENDICES

Publications and Presentations

Two peer-reviewed manuscripts are expected to be published from this project.
Final results from this project will be shared at the 2025 CPS Research Symposium and at other food protection conferences.

Budget Summary

This project received a total of $196,951 in research funds, and the majority of funds were spent.