Food Microbiology

Evaluation of skimmed milk flocculation method for virus recovery from tomatoes

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\textbf{A B S T R A C T}

This study aimed to evaluate the elution-concentration methodology based on skimmed milk flocculation from three varieties of tomatoes (\textit{Solanum lycopersicum} L. \{globe\}, \textit{Solanum lycopersicum} var. \textit{cerasiforme} \{cherry\} and hybrid cocktail \{grape tomato\}) for further monitoring of field samples. Spiking experiments were performed to determine the success rate and efficiency recovery of human norovirus (NoV) genogroup II, norovirus murine-1 (MNV-1) used as sample process control virus and human adenovirus (HAdV). Mean values of 18.8\%, 2.8\% and 44.0\% were observed for NoV GII, MNV-1 and HAdV, respectively with differences according to the types of tomatoes, with lower efficiency for cherry tomatoes. Analysis of 90 samples, obtained at commercial establishments in the metropolitan region of Rio de Janeiro State, revealed 4.5\% positivity for HAdV. Bacterial analysis was also performed with no detection of \textit{Salmonella} spp., \textit{L. monocytogenes} and fecal coliforms. Data demonstrated that the skimmed milk flocculation method is suitable for recovering HAdV from tomatoes and highlights the need for considering investigation in order to improve food safety.

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Introduction

Enteric viruses are described as important contaminants of fresh foods as vegetables and fruits, considering the inad- equate system of water irrigation or inappropriate food handling as possible routes of contamination. Among those, noroviruses (NoV) are the main agent causing acute gastroenteritis (AG) outbreaks associated with consumption of fresh products worldwide. Additionally, NoV Gi, GII, and GIV can infect human, NoV GI.4 is the most prevalent genotype related to foodborne infection.

Introduction

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Additionally, other viruses such as human adenoviruses (HAdV) have been also investigated in water and food samples. Even though they are rarely associated with foodborne illnesses some of them are associated with cases of gastroenteritis. Currently, they have been investigated as indicators of human fecal contamination mainly due to their resistance to adverse environmental conditions, absence of seasonality and its high concentration detected in wastewater samples. HAdVs are DNA viruses belonging to the Adenoviridae family and genus Mastadenovirus with 67 types reported.

The increasing number of viruses foodborne outbreaks have resulted in a growing number of studies that evaluate elution and concentration methods from different food matrices, as well as the use of sample processes control viruses (SPCVs) as murine norovirus-1 (MNV-1), bacteriophage Ff7 and others. In 2013, the International Organization for Standardization (ISO), together with the European Committee for Standardization (CEN), standardized methodologies for recovering NoV and hepatitis A virus from matrix foods that were validated recently.

This study aims to expand previous studies that adapted successfully skimmed milk flocculation method to recover viruses from strawberries. Here, we assess NoV, MNV-1 and HAdV success rate and efficiency recoveries from three varieties of tomatoes as well as assess their microbiological quality by investigating NoV Gi and GII, HAdV, Salmonella spp., Listeria monocytogenes and fecal coliforms from samples obtained at market places at the Great Metropolitan Region of Rio de Janeiro State.

Materials and methods

Viruses and food samples

A NoV GII.4 stool sample (GenBank accession number JX975591) was obtained from the Regional Reference Gastroenteritis Laboratory collection, at Oswaldo Cruz Institute, Rio de Janeiro-RJ, Brazil. Murine norovirus-1 (MNV-1) was kindly provided by Dr. Herbert W. Virgin (Washington University School of Medicine) and propagated in RAW 264.7 cells (a macrophage-like A549 cell line derived from BALB/c mice; ATCC® TIB-71™), according to de Abreu Corrêa and Miagostovich. HAdVs type 2 was propagated in HEK 293 cells (human embryonic kidney cells; ATCC® CRL1573™) obtained from the Regional Reference Gastroenteritis Laboratory collection, at Oswaldo Cruz Institute, Rio de Janeiro, RJ, Brazil.

Three species of tomatoes as Solanum lycopersicum L. (globe), Solanum lycopersicum var. cerasiforme (cherry) and hybrid cocktail (grape) were obtained from distinct markets in Rio de Janeiro.

For field analysis 90 tomatoes samples (45 globe and 45 grape tomatoes) were randomly obtained from March to September, 2014 (three–five samples per week). All samples were inoculated with MNV-1, used as SPCV.

Spiking experiments for assessing efficiency of virus recovering using skimmed flocculation method

Artificial contamination was carried out in duplicate in three independent experiments totaling six assays for each virus. The quantitative PCR (qPCR) TaqMan™ system was used to quantify the absolute number of genome copies (gc)/reaction used for those experiments.

Twenty-five grams of tomato samples were spiked by direct application of 250 μL of NoV GII.4 (1 × 10⁶ gc/reaction), 100 μL of MNV-1 (5 × 10⁵ gc/reaction) and 200 μL of HAdV (1 × 10⁵ gc/reaction) onto food surfaces for 2 h at room temperature. The values of gc/reaction for NoV GII.4 and MNV-1 spikes were obtained according to the formula shown in Eq. (1), where N is the average number of amplified copies, based on the standard curve; D is the dilution of extracted nucleic acid; V (μL) represent the volumes of cDNA produced (Vc); of the eluted nucleic acid (Vc); the suspension of virus particles inoculated in the sample (Vd); of cDNA was added to the TaqMan (Vr) reaction; of the template used for cDNA synthesis (Vt); and nucleic acid extracted from the viral particle (obtained by cell culture or stool suspension) (Vs). For HAdV, the same calculation, excluding Vc and Vd. One negative control (seeded with 350 μL of phosphate saline buffer (PBS) 1x) was included and processed at the same time together with the other samples.

\[
N = \frac{nDV_c \times V_c + V_d \times V_d + V_h}{V_a}
\]  

(1)

Skimmed milk flocculation method was performed as described by Melgaço et al using the inclusion of cetyltrimethylammonium bromide (CTAB) (Fig. 1).

RNA/DNA extraction and viral detection

Viral RNA/DNA was extracted from 140 μL of concentrated samples, using QIAamp viral RNA mini kit (Qiagen, Valencia, CA, USA), according to manufacturer’s instructions. Synthesis of complementary DNA (cDNA) was performed for NoV and MNV-1 detection using random primers, pd(N)6 (Amersham Biosciences, UK) for RNA virus detection.

QPCR using TaqMan™ assays were carried out using a set of specific primers and probes described previously. Reactions were performed using TaqMan Universal PCR Master Mix® (Applied Biosystems, California, USA) according to the manufacturer in ABI 7500® (Applied Biosystems).

For all genomic quantification, a standard curve was performed with eight points of serial plasmid dilutions (10⁻³–10⁻⁶ gc/reaction). All the standard curves yield a slope of -3.59 and a R² (reaction efficiency) of 0.90. An ABI PRISM
Skimmed milk flocculation
Elution in glycine Tris-HCl (pH 9.5)

pH of filtrated adjusted to 3.5
Skimmed milk flocculation for 3 hours
Centrifugation at 8 000 xg for 30 min
Resuspension of pellet in 1 mL phosphate buffer (pH 7.5)
Treatment with CTAB No treatment with CTAB

Final concentrate for RNA extraction

**Fig. 1 – Flow-chart of the viral elution-concentration method.**

7500™ real-time PCR system (Applied Biosystems, Foster City, CA, USA) was used. All samples were tested in duplicate using both undiluted and 1:10 diluted RNA, totaling four qPCR reactions per sample. Samples were considered positive when at least one replica was detected at the cycle threshold (Ct) 40 or lower.

**Bacterial analysis**

Salmonella spp. analysis was performed using a semi-automated VIDAS® system (BioMérieux, France) kit using VIDAS® Salmonella (SLM) according to manufacturer’s instructions. For L. monocytogenes, the culture method by selective enrichment technique was carried out according to standard methodology (Food and Drug Administration’s Bacteriological Analytical Manual online (BAM-FDA)). Fecal coliform was investigated using a Petrifilm™ Coliform Count Plate (3M, USA) according to the manufacturer’s instructions.

**Data analysis**

Recovery of NoV GII, MNV-1 and HAdV from tomatoes samples was qualitatively and quantitatively analyzed according. Qualitative analysis of viral recovery was performed to determine recovery success rate, calculated as the number of qPCR reactions with successful NoV GII.4, MNV-1 or HAdV recovery per number of qPCR reactions performed. Quantitative recovery analyses from samples yielded recovery efficiency (%), calculated per individual sample as mean number of recovered viral genomic copies per inoculated number of NoV GII.4, MNV-1 or HAdV, genomic copies.

Statistical analysis of NoV GII.4 and MNV-1 recovery rates was performed using the nonparametric Mann–Whitney (MW-test), and Wilcoxon (t-test) tests followed by a Kolmogorov–Smirnov (KS-test) test. All statistical analyses were performed using GraphPad Prism 5.01 (GraphPad Software, San Diego, CA, USA). Significance levels were set at 0.05.

**Results**

**Efficiency of virus recovering**

Table 1 shows success rate and recovery efficiency obtained from spiking experiments. No viruses were detected in PBS negative controls. For NoV GII.4, the recovery success rate was of 100% in all specimens, except for cherry and recovery efficiency that ranged from 5.2% to 33.4% with better results for globe and grape tomatoes (Table 1). CTAB treatment did not show significant increase in recovery success rate for all specimens. However, when comparing globe with cherry tomatoes CTAB revealed an increase in recovery efficiency for the first one (p = 0.0043).

For MNV-1 recovery success rate ranged from 45.8% to 87.5%, with lowest values results for cherry tomatoes. Recovery efficiency ranged from 0.6 to 4.2, also with lower results for cherry tomatoes.

For HAdV recovery success rate reach 100% for globe and grape tomatoes with efficiency or recovery of 60.7 and 27.4%, respectively.

**Field study**

HAdV was detected in four samples, three globe and one grape (4.5%) of the 90 samples tested, with concentrations ranging from 10³ to 10⁶ gc/g in 25 g of tomatoes. All samples were negative for NoV G1 and NoV GII. MNV-1 used as SPCV was detected in all samples evaluated. No samples showed contamination by Salmonella spp. or L. monocytogenes (absence in 25 g). Fecal coliform levels were <10 CFU/g in all samples tested.

**Discussion**

The use of organic flocculation method for virus recovery from tomatoes showed variable results among viruses and species studied, both for success rate and efficiency recoveries. In general, the method showed higher efficiency recoveries for NoV GII.4 and HAdV from tomato globe, with percentage of 33.4% and 60.7%, respectively. Considering the varieties analyzed the low recovery percentages obtained for cherry tomatoes was remarkable. Due to unsatisfactory results obtained for virus recovery from this variety, cherry tomatoes were not included in the field study. Previously, low virus recovery efficiency of cherry tomatoes was reported by Pan et al. suggesting problems of adsorption of virus on the food surface.
Table 1 — Recovery success rate (%) and recovery efficiency (%) of skimmed flocculation analyzed in 24 qPCR reactions for norovirus genogroup II (NoV GII), murine norovirus 1 (MNV-1) and human adenovirus type 2 (HAdV-2).

| Method          | Types of tomatoes       | Treatment | NoV GII.4 |         | MNV-1 |         | HAdV-2 |         |
|-----------------|-------------------------|-----------|-----------|---------|-------|---------|--------|---------|
|                  |                         |           | Positive samples (%) recovery success | Recovery efficiency (% mean range) | Positive samples (%) recovery success | Recovery efficiency (% mean range) | Positive samples (%) recovery success | Recovery efficiency (% mean range) |
| Skimmed milk floculation | Globe Tomato           | CTAB      | 24 (100.0) | 33.4    | 7.9–66.3 | 18 (75.0) | 4.1    | 1.7–9.5 | 24 (100.0) | 60.7 | 9.8–92.7 |
|                  |                        | No CTAB   | 22 (91.6)  | 18.1    | 5.8–31.6 | 21 (87.5) | 4.1    | 1.6–5.2 |               | –   | –         |
|                  | Cherry tomato          | CTAB      | 20 (83.3)  | 5.2     | 0.4–12.9 | 11 (45.8) | 0.6    | 0.0–1.2 |               | –   | –         |
|                  |                        | No CTAB   | 21 (87.5)  | 9.8     | 0.5–27.0 | 11 (45.8) | 1.9    | 0.0–5.7 |               | –   | –         |
|                  | Grape tomato           | CTAB      | 24 (100.0) | 16.9    | 0.3–52.7 | 18 (75.0) | 2.3    | 0.05–5.2| 24 (100.0) | 27.4 | 2.8–53.2 |
|                  |                        | No CTAB   | 24 (100.0) | 29.6    | 2.5–110.9 | 19 (79.2) | 4.2    | 0.2–11.1|               | –   | –         |

(-) Not done.
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Conflicts of interest

The authors declare no conflicts of interest.

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