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Virus recovering from strawberries: Evaluation of a skimmed milk organic flocculation method for assessment of microbiological contamination

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ABSTRACT

Skimmed milk organic flocculation method was adapted, optimized and compared with polyethylene glycol (PEG) precipitation and filtration methods for recovering viruses from a strawberry matrix. Spiking experiments with norovirus genogroup II genotype 4 (NoV GII.4) and murine norovirus 1 (MNV-1) demonstrated that the organic flocculation method associated with a glycine elution buffer, filter bag and cetyltrimethylammonium bromide (CTAB) showed a recovery percentage of 2.5 and 32 times higher than PEG precipitation and filtration methodologies for NoV recovering. Furthermore, this method was used for investigating NoV and human adenoviruses (HAdVs) in 90 samples of fresh strawberries commercialized in Rio de Janeiro markets. NoV GI and GII were not detected in those samples and MNV-1, used as internal process control (IPC), was recovered in 95.5% (86) of them. HAdVs were detected in 18 (20.0%) samples and characterized by nucleotide sequencing as Human *Mastadenovirus* specie F and as type specie HAdV-2. Bacterial analysis did not detect *Salmonella* spp. and *Listeria monocytogenes*, however, 3.3% of fecal coliforms were detected in those samples. These results indicate the organic flocculation method as an alternative for recovering enteric viruses from strawberries, emphasizing a need for virus surveillance in food matrices.

1. Introduction

Foodborne illnesses cause a large number of hospitalizations worldwide and it is estimated that more than 67% of them are associated with viral infections due to contamination of fresh produce or processed foods by virus-containing fecal material (Atreya, 2004; Painter et al., 2013). Pre-harvest irrigation or failure in hygiene procedures during harvesting is suggested as possible sources of virus contamination (Richards, 2001). Enteric viruses such as noroviruses (NoVs) are related to acute gastroenteritis (AG) outbreaks worldwide by consumption of fresh foods such as vegetables, shellfish and fruits, especially red fruits, strawberries and raspberries. Other viruses such as human adenoviruses (HAdVs) are also implicated (Maunula et al., 2009; Mäde et al., 2013; Rodriguez-Manzano et al., 2013).

NoVs are RNA viruses belonging to genus *Norovirus*, Caliciviridae family. They are classified in seven different genogroups (G) and more than 35 genotypes are described (Vinje, 2015; Zheng et al., 2006). NoV GI, GII and GIV are related to infect humans, being NoV GII the most prevalent in foodborne infections (Koopmans et al., 2002).

HAdVs are DNA viruses belonging to the Adenoviridae family and genus *Mastadenovirus*, presenting 57 types, being the enteric type species HAdV-40 and HAdV-41 mostly associated with foodborne diseases (Greening, 2006). HAdVs are found at consistently high levels in sewage all year round (Wyn-Jones et al., 2011) and are used as indicator viruses for human fecal contamination in water (Hewitt et al., 2013).

Virus detection in food requires appropriate elution and concentration techniques which need to be adapted for different food matrices as well as for the use of internal process controls (IPC), mainly to monitor the presence of enzymatic reaction inhibitors in those matrices (Stals et al., 2012). In the last few years, many methods and IPCs for

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recovering viruses using distinct elution buffers associated to polyethylene glycol precipitation (PEG), ultracentrifugation or positively charged filters for virus concentration have been evaluated (Baert et al., 2008a; Brassard et al., 2012; Comelli et al., 2008; Mäde et al., 2013; Morales-Rayas et al., 2009; Park et al., 2008; Rzezutka et al., 2005; Stals et al., 2011; Wobus et al., 2006).

This study aimed to adapt, optimize and compare a skimmed milk organic flocculation method for virus recovering with other methods described previously such as PEG and filtration using negatively charged membranes. The organic flocculation method was first described for virus detection in seawater (Calgua et al., 2008) and some modifications were made for virus detection in fruits in order to be applied in laboratories of food microbiology. Investigation of NoV GI, GII, HAdV and bacterial analyses were carried out to assess microbiological contamination of fresh strawberries commercialized in Rio de Janeiro, Brazil. *Salmonella* spp., *Listeria monocytogenes* and fecal coliforms, previously associated with strawberries consumption and cultivation were also investigated (Laidler et al., 2013; Szymczak et al., 2014).

2. Material and methods

2.1. Viruses and food samples

A NoV GII.4 stool sample (GenBank accession number JX975591) was obtained from the Regional Reference Gastroenteritis Laboratory collection, at the Oswaldo Cruz Institute, Rio de Janeiro—RJ, Brazil. Murine norovirus-1 (MNV-1) kindly provided by Dr. Herbert W. Virgin from Washington University School of Medicine, was propagated in RAW 264.7 cells (a macrophage-like Abelson leukemia virus-transformed cell line derived from BALB/c mice), according to de Abreu Corrêa and Miagostovich (2013). Real time PCR TaqMan system was used to quantify the absolute number of genome copies (gc)/mL (Yin et al., 2001) used for the spiking experiments.

Samples of fresh strawberries (*Fragaria vesca* var.) were obtained from local commercial sources in three different municipalities (Petrópolis, Niterói and Rio de Janeiro), all located in the state of Rio de Janeiro, Brazil.

2.2. Quantitative amplification method (qPCR) detection limit

Viral stocks and their 10-fold dilutions (10^0–10^6) were used in triplicate to evaluate the detection limit of each virus. The amount of virus inoculated in strawberry samples in spiking experiments was determined according to qPCR detection limit.

In the artificial contamination experiments the possible loss of viral load during steps of concentration and recovery was evaluated. NoV GII and MNV-1 were seeded onto the food surface by direct application of ten-fold dilutions (10^6, 10^5 and 10^4 cg/mL) of 250 μL and 100 μL, respectively.

2.3. Experimental design of the study

Primarily, a skimmed milk organic flocculation method was adapted and optimized for a food matrix by experimental evaluation using different variables such as elution buffers, containers and final treatment with cetyltrimethylammonium bromide (CTAB). After standardization, this method (1) was compared with other two viral concentration methods as PEG precipitation (method 2), and filtration using negatively charged membranes combined with ultra-filtration (method 3). The final concentrate sample (2 mL) obtained from each method was used for RNA extraction and quantified by quantitative polymerase chain reaction (qPCR) using TaqMan® assays. Afterwards, the most efficient recovering method was applied to assess the viral contamination of fresh strawberries commercialized in Rio de Janeiro. NoV GI, NoV GII and HAdV were investigated in a total of 90 samples randomly obtained during June–September 2013 (six samples/week) from distinct markets. For these experiments, MNV-1 was used as IPC. Additionally, the presence of *Salmonella* spp., *L. monocytogenes* and fecal coliform was also investigated.

2.4. Spiking experiments

2.4.1. Method 1

In order to optimize the flocculation method (Calgua et al., 2008), artificial contamination experiments were performed. Briefly, fresh strawberry samples (25 g) were spiked by direct application of 250 μL of NoV GII.4 and 100 μL of MNV-1 onto food surfaces for 2 h at room temperature. For each variable: a) containers (beaker and filter bag), b) elution buffers (PBS and Glycine 0.05 M/Tris–HCl 0.1 M) and, c) final treatment (CTAB), the samples were assayed in triplicate, using one sample as negative control (seeded with 350 μL of PBS 1×) and processed at the same time as the others (Fig. 1). When a beaker was used as a container, the elution step was performed for 1 h, using 400 mL of each buffer (PBS and glycine 0.05 M/Tris–HCl 0.1 M). For the filter bag (Nasco®, Fort Atkinson, Wisconsin, USA), the elution was performed for 30 min, using 225 mL of a buffer.

Before the flocculation step, the eluates were transferred to sterile beakers; the pH was adjusted to 3.5 using HCl 6 N and then skimmed milk at a final concentration of 1% was added. The organic flocculation process was performed for 3 h with constant stirring. After this step, the flocculated samples were centrifuged (8000 ×*g* for 30 min) and the pellet was re-suspended in 1 mL of a phosphate buffer (pH 7.5; Na_2HPO_4 0.2 M/NaH_2PO_4 0.2 M; 1:2 *v*/*v*). Before the RNA extraction step, the final concentrated samples were incubated (or not) at 56 °C with a final concentration of 1.25% CTAB and 0.45 M NaCl for 30 min (Baert et al., 2008b).

2.4.2. Methods 2 and 3

Spiking experiment procedures for both viruses were also performed in triplicate for assessing a virus recovery rate using the PEG precipitation method (Stals et al., 2011), and filtration using negatively charged membranes combined with ultra-filtration, first described by Fumian et al. (2009) and adapted by de Abreu Corrêa and Miagostovich (2013). Fig. 1 presents an outline of the methods used for the experiments.

2.5. Virus detection and molecular characterization

Viral RNA/DNA was extracted from 140 μL of the concentrated samples, using the QIAamp viral RNA mini kit® (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Synthesis of complementary DNA (cDNA) was performed using random primers (Invitrogen®, USA) for RNA virus detection.

MNV-1, NoV (GI and GII) and HAdV qPCR using a TaqMan® system (ABI PRISM 7500, Applied Biosystems, Foster City, CA, USA), were performed using a set of specific primers and probes as presented in Table 1. For all genomic quantification a standard curve was performed with eight points of serial plasmid dilutions (10^7–10^0 genomic copies (gc)/reaction) that yields a slope of −3.59 and a reaction efficiency of 0.90.

qPCR reactions consisted of 5 μL of cDNA/DNA sample, 12.5 μL universal master mix (Applied Biosystems, Foster City, CA, USA), corresponding primers and hydrolysis probe. The following conditions were used for the amplification step: activation of the uracil N-glycosylase for 2 min at 50 °C, and activation of AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) for 10 min at 95 °C followed by 45 cycles of amplification (15 s at 95 °C and 1 min at 60 °C). An ABI PRISM 7500™ real time PCR system (Applied Biosystems, Foster City, CA, USA) was used in all assays. All samples were tested in duplicate using both undiluted and 1:10 diluted RNA/DNA, totalizing four qPCR reactions per sample. Samples were considered positive when at least one replica was detected at the cycle threshold (Ct) 38 or lower.