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Short communication

A simple method to recover Norovirus from fresh produce with large sample size by using histo-blood group antigen-conjugated to magnetic beads in a recirculating affinity magnetic separation system (RCAMS)

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ABSTRACT

Human norovirus (NoV) outbreaks are major food safety concerns. The virus has to be concentrated from food samples in order to be detected. PEG precipitation is the most common method to recover the virus. Recently, histo-blood group antigens (HBGA) have been recognized as receptors for human NoV, and have been utilized as an alternative method to concentrate human NoV for samples up to 40 mL in volume. However, to wash off the virus from contaminated fresh food samples, at least 250 mL of wash volume is required. Recirculating affinity magnetic separation system (RCAMS) has been tried by others to concentrate human NoV from large-volume samples and failed to yield consistent results with the standard procedure of 30 min of recirculation at the default flow rate. Our work here demonstrates that proper recirculation time and flow rate are key factors for success in using the RCAMS. The bead recovery rate was increased from 28% to 47%, 67% and 90% when recirculation times were extended from 30 min to 60 min, 120 min and 180 min, respectively. The kinetics study suggests that at least 120 min recirculation is required to obtain a good recovery of NoV. In addition, different binding and elution conditions were compared for releasing NoV from inoculated lettuce. Phosphate-buffered saline (PBS) and water results in similar efficacy for virus release, but the released virus does not bind to RCAMS effectively unless pH was adjusted to acidic. Either citrate-buffered saline (CBS) wash, or water wash followed by CBS adjustment, resulted in an enhanced recovery of virus. We also demonstrated that the standard curve generated from viral RNA extracted from serially-diluted virus samples is more accurate for quantitative analysis than standard curves generated from serially-diluted plasmid DNA or transcribed-RNA templates, both of which tend to overestimate the concentration power. The efficacy of recovery of NoV from produce using RCAMS was directly compared with that of the PEG method in NoV inoculated lettuce. 40, 4, 0.4, and 0.04 RTU can be detected by both methods. At 0.004 RTU, NoV was detectable in all three samples concentrated by the RCAMS method, while none could be detected by the PEG precipitation method. RCAMS is a simple and rapid method that is more sensitive than conventional methods for recovery of NoV from food samples with a large sample size. In addition, the RTU value detected through RCAMS-processed samples is more biologically relevant.

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1. Introduction

Noroviruses (NoV) cause an estimated 23 million annual cases of sporadic and epidemic gastrointestinal (GI) disease in the United States annually, accounting for half to two-thirds of all food-borne illnesses (Daniels et al., 2000; Glass et al., 2000). The most common and best characterized route of infection is through the consumption of oysters, which are contaminated directly at the source (Daniels et al., 2000). Recent outbreaks of food-borne illness have shed light and raised concerns about the growing problem of contamination of fresh produce such as salads (Seymour and Appleton, 2001). As this RNA virus cannot be grown in cell culture, reverse-transcription polymerase chain reaction (RT-PCR) is the primary tool for detection of the virus (Jiang et al., 1992). Given the low infectious doses required to cause illness, and the complexity of food samples, it has been challenging to detect NoV in food samples directly. Methods used previously to concentrate NoV include ultracentrifugation, polyethylene glycol (PEG) precipitation, adsorption/elution, and immunomagnetic separation (Atmar and Estes, 2001; Gentry et al., 2009). Drawbacks are that these methods can be time-consuming (PEG precipitation and adsorption/elution) or strain specific (immunomagnetic separation). Other methods have been developed to detect NoV using histo-blood group antigen (HBGA)-coupled magnetic beads (Cannon and Vinje, 2008; Tian et al., 2008). Synthetic HBGA oligosaccharides have been applied to recover NoV from water samples and porcine gastric mucin-conjugated to magnetic beads has

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been used to capture NoV from virus spiked small samples (up to 40 mL). Recently, Morton et al. reported detection of NoV from ready to eat food by using iCropTheBug and Pathatrix methods for food samples with a large volume (Morton et al., 2009). However, inconsistent results were found by using the Pathatrix system. In addition, it remains unclear if HBGA-based methods are superior to the commonly used PEG method as no direct comparison was performed. The work presented here is to determine the optimal conditions for using HBGA-conjugated beads to concentrate NoV from produce wash with sample size of 250 mL with a RCAMS system and directly compare the efficacy to the tradition PEG precipitation method.

2. Materials and methods

2.1. Preparation of porcine stomach mucin-conjugated magnetic beads (PGM-MB)

Type III Porcine Gastric Mucin (PGM) purified from porcine stomach mucosa was sourced from Sigma (St. Louis, MI). PGM was conjugated to MagnaBind™ carboxyl-derivatized beads (Pierce Biotechnology, Rockford, IL) to produce PGM-MB suspension using a scaled-up procedure derived from the manufacturer's protocol. The beads were resuspended with 10 mL of PBS and aliquotted for use and storage at 4 °C for up to 2 months.

2.2. Preparation of NoV samples

A GII.4 NoV was used in the study (Tian et al., 2010). The virus sample was diluted (1:10) into buffer (PBS pH 7.2). The stool suspension was clarified of gross solids by low-speed centrifugation (2000 RCF for 20 min), and the supernatant was then filtered through a 0.45 μm autovial syringeless filter (Whatman, Kent, UK). The virus was serially diluted and the extracted viral RNA was quantitated as real-time RT unit (RTU) by quantitative real-time RT-PCR.

2.3. Modification of Pathatrix Ultra consumable

We modified the Pathatrix Ultra consumables to both enhance PGM-MB collection and reduce suspension solution retention. Magnetic beads were added into the samples and mixed, rather than applied to the collection chambers as described by the manufacturer. To reduce turbulent swirls at the collection chamber, and suspension solution retention when draining back into the sample bag, both valves were removed to resemble the original Pathatrix consumable. To enhance PGM-MB circulation (by reducing fluid flow "dead spots") within the sample bag and to reduce retention of both PGM-MB and suspension solution by the sponge, the sponge at the submerged end was also removed.

2.4. Analysis of RCAMS performance

10 μL of NoV sample was diluted into 250 mL of buffer (CBS, pH 3.6) in a 500 mL sample bag (Whirl-Pak, Stomacher) and mixed thoroughly. From the dilute NoV solution, 140 μL was transferred into a microcentrifuge tube and set aside at 4 °C ("input"). 50 μL of PGM-MB was added to the remaining dilute NoV solution and mixed thoroughly. A modified Pathatrix Ultra consumable was placed into the sample bag containing the PGM-MB-NoV suspension, and the assembly was installed into a Pathatrix unit. The apparatus was run on a manual setting ("Wash" speed) for 30, 60, 120 or 180 min at 25 °C. After the run, the collection chamber ("Capture Phase") containing captured PGM-MB-NoV was disconnected from the apparatus while remaining in its magnetic holder. As this was done, the post-suspension medium from the collection chamber and tubing was carefully drained back into the sample bag. The collection chamber was removed from the magnetic holder and the captured PGM-MB-NoV was rinsed from the collection chamber with 560 μL of viral lysis buffer (Qiagen), into a microcentrifuge tube and set aside at 4 °C ("concentrated"). The post-suspension medium from the sample bag was distributed into four 50 mL screw-cap centrifuge tubes and centrifuged at 2819 RCF for 106 min at 4 °C. After centrifugation, 140 uL of the supernatant was transferred into a microcentrifuge tube and set aside at 4 °C ("Remaining"). The remaining supernatants from all centrifuge tubes were carefully decanted without disturbing the pellets, and discarded. The pellets were sequentially resuspended and combined using 560 μL of viral lysis buffer ("uncollected free beads"). Both "concentrated" and "uncollected beads" samples were allowed to lyse for 10 min, placed in a magnetic separation rack for 5 min, and the separated suspension media were carefully aspirated into new microcentrifuge tubes. 560 μL of viral lysis buffer was added to both "input" and "remaining" and allowed to lyse for 10 min. All four fractions were extracted for viral RNA using the Qiagen QIAamp Viral RNA Mini spin column kit in accordance with the manufacturer's protocol.

2.5. Assessment of PEG precipitation of NoV

From the diluted NoV solution, 140 μL was transferred into a microcentrifuge tube and stored at 4 °C for use later in the procedure ("input"). PEG (10% final conc.) and NaCl (0.3 M final conc.) was added to the ~250 mL diluted NoV solution, mixed thoroughly and stored at 4 °C overnight to precipitate NoV. The precipitated suspension was centrifuged at 2819 RCF for 106 min at 4 °C. The resulting supernatants were carefully decanted from each centrifuge tube and combined into a single sterile 250 mL flask. From this post-centrifugation supernatant, 140 uL was transferred into a microcentrifuge tube and stored at 4 °C for use later in the procedure ("Remaining"). 560 μL of viral lysis buffer was used to sequentially re-suspend and recombine every pellet. The combined, resuspended pellet solution ("concentrated") was allowed to further lyse the precipitated viruses for an additional 5 min. 560 μL of viral lysis buffer was added to each microcentrifuge tube containing "input"; and "remaining" and allowed to lyse for 10 min. Together with the resuspended pellet solution ("concentrated") already in viral lysis buffer, all three fractions were extracted for viral RNA using the Qiagen QIAamp Viral RNA Mini spin column kit. Extracted viral RNA was quantitated by quantitative real-time RT-PCR.

2.6. Quantitation of extracted viral RNA via real-time RT-PCR and standard curve

We quantitated NoV samples by quantitative real-time RT-PCR on a MX3000P QPCR system (Stratagene, La Jolla, CA) using an OneStep RT-PCR kit (Qiagen USA, Valencia, CA) in accordance with the manufacturer's protocol. Primers and probe sequences from Kageyama et al. were synthesized with modified fluorophores and quenchers (Kageyama et al., 2003; Tian et al., 2008). Cycling times and temperatures were 50 °C for 50 min and 95 °C for 15 min, followed by 45 cycles of 95 °C for 10 s, 53 °C for 25 s, and 62 °C for 70 (Burkhardt et al., 2006). A standard virus titer curve was generated by extracting viral RNA from serially-diluted (10^{-2} to 10^{-7}) virus stock. Quantitative real-time RT-PCR was performed and resulting Ct values were plotted against their respective dilution. The highest measurable Ct was assigned the value of one RT-PCR unit (RTU), and a logarithmic trendline was plotted ($R^2 = 0.9942$).

2.7. Statistical analysis

We analyzed data by either Student t-test or one-way ANOVA. All data analyzed represented duplicates for each experimental condition

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