Video Article

A Small Volume Procedure for Viral Concentration from Water

Brian R. McMinn[1], Asja Korajkic[1]

[1]U.S. EPA

Correspondence to: Brian R. McMinn at McMinn.Brian@epa.gov

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Abstract

Small-scale concentration of viruses (sample volumes 1-10 L, here simulated with spiked 100 ml water samples) is an efficient, cost-effective way to identify optimal parameters for virus concentration. Viruses can be concentrated from water using filtration (electropositive, electronegative, glass wool or size exclusion), followed by secondary concentration with beef extract to release viruses from filter surfaces, and finally tertiary concentration resulting in a 5-30 ml volume virus concentrate. In order to identify optimal concentration procedures, two different electropositive filters were evaluated (a glass/cellulose filter [1MDS] and a nano-alumina/glass filter [NanoCeram]), as well as different secondary concentration techniques; the celite technique where three different celite particle sizes were evaluated (fine, medium and large) followed by comparing this technique with that of the established organic flocculation method. Various elution additives were also evaluated for their ability to enhance the release of adenovirus (AdV) particles from filter surfaces. Fine particle celite recovered similar levels of AdV40 and 41 to that of the established organic flocculation method when viral spikes were added during secondary concentration. The glass/cellulose filter recovered higher levels of both, AdV40 and 41, compared to that of a nano-alumina/glass fiber filter. Although not statistically significant, the addition of 0.1% sodium polyphosphate amended beef extract eluant recovered 10% more AdV particles compared to unamended beef extract.

Video Link

The video component of this article can be found at https://www.jove.com/video/51744/

Introduction

Human enteric viruses are important causative agents of waterborne diseases [1-3], but are generally present in low numbers in contaminated environmental waters, making their detection difficult without concentration. Procedures used to concentrate viruses typically include a filtration step, followed by filter elution, and secondary concentration of the filter eluate. A common filtration procedure relies on use of charged membranes such as electropositive filters (recently reviewed in [4,5]). These filters rely on capturing viruses suspended in water using electrostatic interactions between the filter surface (positively charged) and targeted virus particles (negatively charged). Two electropositive filters that are commercially available rely on this technology, the glass/cellulose and nano-alumina/glass fiber filters. The glass/cellulose filter costs are up to 10 times that of the nano-alumina/glass fiber, which limit the use of the glass/cellulose filters for routine virus monitoring. Recent studies have concluded differences are nominal between these two filters in recovery of enteroviruses from ambient water [6,7], justifying the use of a cheaper filter alternative. Other filter options such as electronegative and glass-wool filters have been studied, however, they either require the pretreatment of source water (electronegative filters) or are not commercially available (glass-wool filters). The development of virus concentration procedures has mostly focused on optimizing primary concentration techniques (filters) in order to improve virus recoveries from water. However, secondary concentration procedures, which reduce the volume of eluant typically from 1 L to milliliter volumes, can also have a significant impact on virus recoveries [8].

Secondary concentration of enteric viruses typically relies on a flocculating agent such as some types of beef extract (organic flocculation) or skimmed milk flocculation [9-12] to remove virus particles from filter surfaces. Recently, another secondary concentration procedure using beef extract coupled with the addition of celite (fine particle) has shown promise for recovering adenovirus, enterovirus, and norovirus [8,13,14]. Celite concentration works under similar principles to that of the organic flocculation method in that virus particles attach to and are released from particles (floc or celite) by altering the pH of the suspension solution. Comparisons between these two secondary concentration techniques have been evaluated in recovery of spiked adenovirus (AdV) types 40 and 41 [8]. This study concluded that the two secondary concentration techniques were statistically similar in recovery of adenoviruses. However, the organic flocculation method requires a 30 min. incubation at pH 3.5, while the celite technique requires a shorter incubation (10 min) at pH 4.0. The organic flocculation also requires the use of expensive laboratory equipment (centrifuges) to collect floc particles during tertiary concentration, the celite technique in contrast uses only basic laboratory equipment (vacuum filtration) to separate celite particles from suspension.

Certain combinations of filters and secondary elution techniques can also affect virus recoveries. One study concluded that certain combinations of primary (electropositive filters) and secondary concentration techniques (celite or organic flocculation) had a significant impact of recovery of adenovirus [13]. These findings suggest that optimization is required in order to optimally recover target virus from a given water matrix when

using these techniques. Optimization is a time consuming, arduous process many researchers actively avoid since numerous variables will be evaluated (filter type/brand, pH elution solution, celite/organic flocculation).

For this study, a procedure was developed to identify optimal conditions for virus concentration from water using spiked human adenovirus strains 40 and 41. Presumably, since each virus type displays a unique capsid morphology and specific capsid charge, concentration protocols may need to be optimized for every virus target in order to attain optimal viral recovery. This study provides an approach for AdV 40 and 41 concentration by: 1) evaluating virus recoveries in tap water using electropositive filter discs followed by 2) evaluation of an established organic flocculation method versus the celite technique as a secondary concentration, and 3) evaluation of elution buffers for tertiary concentration.

Protocol

1. Preparation of Glassware and Filter Housings

1. Unless otherwise noted, sterilize all glassware, filter housings and solutions at 121 °C for 15 min. To ensure sterility, cover all openings or exposed surfaces with either aluminum foil or tape-secured paper prior to sterilization.
2. Assemble filtration apparatus by attaching filter housing (47 mm diameter) to 1 L side arm Erlenmeyer flask. Collect filters required: 47 mm diameter electropositive/electronegative disc filters.
3. Prepare 1 L of 1.5% beef extract (flocculating; produces floc particles when pH of solution is lowered to 3.5, or non-flocculating; which does not aggregate at pH 3.5), with 0.05 M glycine, by dissolving 15 g of beef extract in 1 L of deionized water and adding 3.75 g glycine.
   NOTE: Non-flocculating beef extracts will require the addition of celite prior to tertiary concentration.
4. Add elution additive sodium polyphosphate to beef extract at a 0.1% concentration.
5. Add 1 M hydrochloric acid solution, drop wise, to mixing beef extract, pH solution to desired pH. Autoclave beef extract for 15-30 min at 121 °C.
6. Measure 0.1 g of fine, medium, or large particle celite (for use with non-flocculating beef extract only).
7. Prepare vacuum/suction by attaching compatible tubing to Erlenmeyer side-arm flask and to the vacuum outlet.

2. Preparation of Solutions and Virus Stock

1. Prepare 1 L of 1 M HCl.
2. Prepare 1 L of 1 M NaOH.
3. Prepare 1x PBS solution (137 mM NaCl, 2.7 mM KCl, 1.47 mM K_2PO_4, and 4.3 mM NaH_2PO_4).
   1. Adjust pH of 1x PBS to 9.0 using 1 M NaOH.
4. Prepare and dilute stock virus to 10^4-10^5 most probable number (MPN) ml^{-1} by mixing 1 ml of stock virus with 9 ml of PBS (pH 7), as previously described [8,13]. Store at -80 °C in 1 ml aliquots.
   1. Sterilize virus stock by syringe filtering (0.22 µm pore size) to remove potential contaminants.
5. Dechlorinated Tap Water
   1. Measure 1 L of sterile tap water using a sterile graduated cylinder and pour into a 2 L beaker containing magnetic stir bar.
   2. Add 0.7 g of sodium thiosulfate and mix until granules dissolve. Adjust pH of tap water to 7-7.5 if needed using 1 M HCl or 1 M NaOH solution.
   3. Thaw virus spike (1 ml) and add entire volume of spike to 1 L of dechlorinated sterile tap water. Mix for 10 min.
   4. Prepare a method blank (dechlorinated tap water without adenovirus) with each experiment and process in the same manner as the spiked sample.

3. Preparation of Millipore Filter Apparatus

1. Remove sterile covering over filter housing. Ensure proper fit between filter housing upper bowl and lower filter screen to avoid sample leakage during filtration.
2. Remove sterile covering from 1 L Erlenmeyer flask. Ensure that the cork of the filter unit fits snugly in top opening of Erlenmeyer flask, creating a good seal.
3. Remove top bowl of filter unit from lower filter screen, and place 47 mm electropositive filter disk squarely over screen with sterile forceps. Attach filter housing bowl to bottom screen and lock into place by twisting counterclockwise.

4. Tap Water Filtration (Primary Concentration)

1. Measure 100 ml of virus spiked tap water using sterile graduated cylinder.
2. Pour 100 ml of virus spiked tap water into filter housing containing either a 47 mm pleated glass/cellulose filter or a nano-alumina/glass fiber disk filter.
3. Allow water to slowly pass through filter. Apply gentle vacuum to remove remaining residuals of tap water from filter surface.
4. If using trypsin treatment, add 5 ml of 0.25% trypsin to filter surface and incubate for 5 min.
5. Wash trypsin from filter surface using 50 ml of sterile deionized water.

5. Virus Elution from Filter

1. Remove filter housing from 2 L Erlenmeyer flask, and place onto 250 ml side-arm flask.
2. Measure 100 ml of beef extract using graduated cylinder. Slowly pour 100 ml of beef extract into filter housing.
3. Allow beef extract to pour through filter, capturing beef extract in a small (250 ml) Erlenmeyer flask. Apply vacuum to pull through residual beef extract from filter.

6. Celite Secondary Concentration

1. Transfer flasks containing the eluted viruses to a stir plate. Add sterile magnetic stir bar and place on stir plate, mixing to create slight vortex.
2. Add 0.1 g of celite powder to 100 ml of beef extract and allow celite to disperse. Place sterile pH probe into beef extract.
3. Add drop wise 1 M HCl to beef extract to achieve pH 4.0. Allow to slowly mix for 10 min.
4. Place 47 mm pre-filter onto filter housing (as previously described) using a 2 L Erlenmeyer flask.
5. Pour 100 ml beef extract/celite mixture over pre-filter. Allow beef extract to pour through. Apply slight vacuum to pull through residual beef extract from filter.
6. Place metal tube clip on end of filter housing spout. Attach sterile 15 ml polypropylene collection tube to metal tube clip. Place filter housing back into Erlenmeyer flask.
7. Add 5 ml of 1x PBS buffer, pH 9.0 over celite collected on pre-filter.
8. Allow PBS to drip through pre-filter. Apply slight vacuum to pull through residual PBS into 15 ml collection tube.

7. Organic Flocculation Secondary Concentration

1. Transfer the beakers containing the eluted virus to a stir plate. Add sterile magnetic stir bar to 100 ml of beef extract and mix to create slight vortex.
2. Place sterile pH probe into beef extract.
3. Add 1 M HCl drop wise to beef extract and adjust pH to 3.5. Mix for 30 min.
4. Pour the eluate into 250 ml centrifuge conical tubes and centrifuge at 2,500 x g for 15 min at 4 ºC.
5. Pour off supernatant taking care not to not to disrupt the pellet. Resuspend the pellet in 5 ml of 1x PBS, pH 9.
6. Centrifuge the suspension at 4,000 x g for 10 min at 4 ºC. Pour off the supernatant and discard the pellet.
7. Adjust the pH to 7-7.5, filter sterilize through 0.22 µm syringe filter and freeze at -80 °C.

8. Viral Nucleic Acid Extraction

1. Extract viral nucleic acids using an appropriate commercial kit according to manufacturer's instructions.

9. AdV 40/41 qPCR

1. Use primers and probes, as well as reaction mix and thermal cycling profiles as published [8,13].
   NOTE: Estimated limit of detection for the assay is approximately 5 qPCR units per reaction.
2. Add components of PCR amplification mixtures at concentrations as follows: 10 mM Tris (pH 8.3), 50 mM KCl, 4.5 mM $MgCl_2$, 10 mM dNTPs, 10 µM primers and 1 µM probe and 0.5 µl of polymerase so that final volumes of 45 µl can be added to appropriate number of wells within a 96-well PCR reaction plate.
3. Load 45 µl of PCR amplification mixture to each appropriate well of a 96-well, fast reaction PCR plate.
4. Add 5 µl of DNA extracted sample to appropriate wells of the PCR plate.
5. Cover plate with heat resistant cover seal, spin the plate to move any droplets to bottom of PCR plate wells.
6. Set PCR amplification cycles as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min.

10. Data Analyses

1. Analyze triplicate measurements of the serial 5-fold dilutions of the samples spanning 1:5 to 1:625 dilution range in order to determine the most probable number (MPN) of the viral particles, as previously described [8,13].
2. Analyze serial 5-fold dilutions of adenovirus experimental spikes spanning 1:5 to 1:15,625 dilution range in order to determine the most probable number (MPN) of the viral particles.
3. Using the EPA MPN calculator (http://www.epa.gov/nerlcwww/online.html), adjust the calculator to account for type of dilution (1:5 or 1:10), number of dilutions performed, and number of replicate samples in dilution series.
4. Perform $\log_{10}$ transformation of the data, followed by normalizing to a unit of volume (*e.g.*, 1 ml, 100 ml, *etc.*). Calculate recoveries for given experiment by dividing number of viral particles present in sample concentrates with number of viral particles present in experimental spikes.
5. Evaluate the effect of the different experimental variables (*e.g.,* different celite types, pH ranges, different filter types and different secondary concentration techniques) on AdV recoveries by performing parametric or non-parametric statistical tests (*e.g.*, paired t-test, one or two-way ANOVA) depending on the distribution of the data and the experimental design.

Representative Results

Celite selection

Three different types of celite were tested prior to the selection of the best performing variant. Celites with fine to medium sized particles produced the highest adenovirus recoveries. Use of larger celites resulted in lower recoveries for both AdV40 and 41 (range 32%-100%) (**Figure 1**). Average recoveries of adenovirus 40 were 144% ± 52% (fine), 115% ± 28% (medium) and 82% ± 53% (large) particle celites and for AdV41, 132% ± 39% (fine), 83% ± 25% (medium) and 50% ± 11% (large). One-way ANOVA indicated the optimized celite technique recovered statistically similar levels of AdV40 (*P* = 0.7920) and 41 (*P* = 0.1439) to that of the organic flocculation method, which recovered 75% ± 19% and 109% ± 16%, respectively. **Figure 1** illustrates the differences among secondary concentration techniques and between AdV types.

Filter type evaluation

One hundred milliliter volumes of virus-spiked, tap water were concentrated using either a pleated glass/cellulose or nano-alumina/glass fiber disc filter and were processed using either the celite or organic flocculation method. Filters were eluted with beef extracts at pH 7.5, 9.0, 9.5, 10, 11 and 12 to determine which would be most effective in eluting AdV particles from filters. The glass/cellulose filter coupled with beef extract at pH 10 and celite, produced the highest recoveries (by qPCR/MPN) for AdV40 (52% ± 22%) and AdV41 (64% ± 4%) which are shown in **Figures 2** and **3**. Beef extract pH 10 recovered significantly higher AdV41 than all other pH values (*P* value range: 0.0045-0.041), while AdV40 followed a similar, although somewhat weaker trend (*P* value range: 0.025-0.042). A glass/cellulose filter was also evaluated in conjunction with the organic flocculation method. For AdV40, significantly higher recoveries were gained using beef extract pH 10 (40% ± 10%) (*P* value range: 0.010-0.027), and for AdV41, both pH 9.5 and 10 outperformed all other beef extract pH tested (*P* value range: 0.023-0.027) (**Figures 2** and **3**). Overall, the celite technique with beef extract pH 10 was found to be superior to the organic flocculation method in direct comparisons for recovery of both AdV strains.

Optimizations of the beef extract additives

The optimized method (glass/cellulose filter using beef extract pH 10 coupled with celite secondary concentration) was compared to two other treatments: 1) addition of 0.25% trypsin and 2) supplementing the beef extract with 0.1% sodium polyphosphate. The addition of trypsin did not appear to improve recovery of AdV as only 16% ± 6% and 24% ± 14% of AdV40 and 41 inputs, respectively were recovered (**Figure 4**). Beef extract supplemented with 0.1% sodium polyphosphate yielded the highest recoveries of AdV40 and 41 compared to that of the previous optimized method. AdV40 recovery appeared to increase by 13%, and AdV41 recovery increased 7% over the previous method, but neither was statistically significant (*P* value: 0.146). Use of higher percentages of sodium polyphosphate 1%, 3% and 5% (data not shown) was found to be ineffective, requiring large volumes of acid to adjust pH of these solutions to 4.0 during secondary concentration.

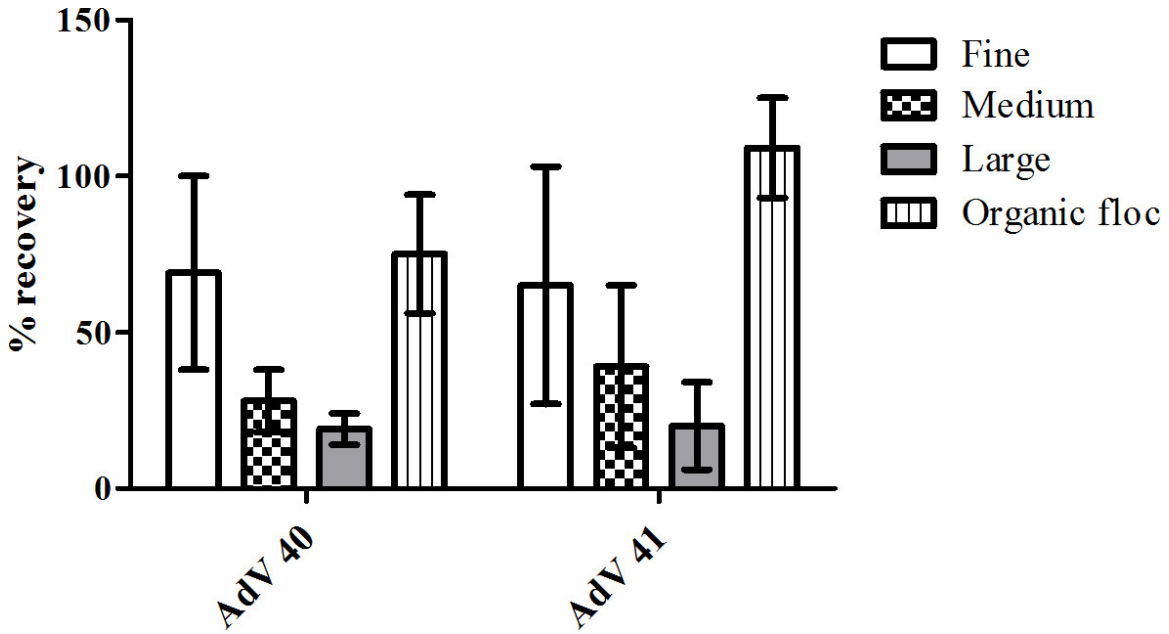

Figure 1: Percent recovery of AdV-40 and AdV-41 based on secondary concentration technique (Celite versus Organic floc) and the type of Celite used. Error bars represent standard deviation (n = 3)[8].

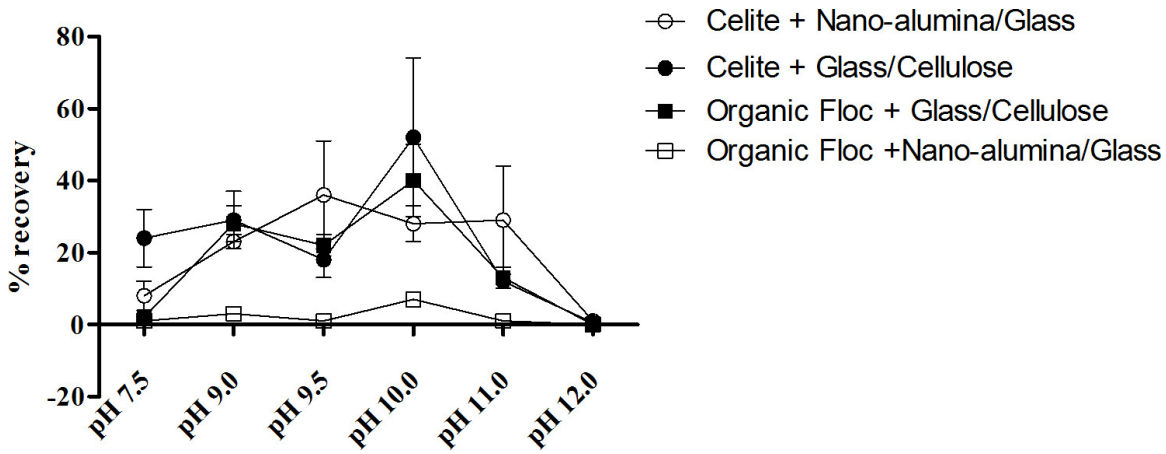

Figure 2: Percent recovery of AdV-40 using two types of secondary concentration techniques (Celite and organic floc) and two types of filters (Glass/Cellulose and Nano-alumina/Glass) at varying pH levels of beef extract. Error bars represent standard deviation (n = 3)[13].

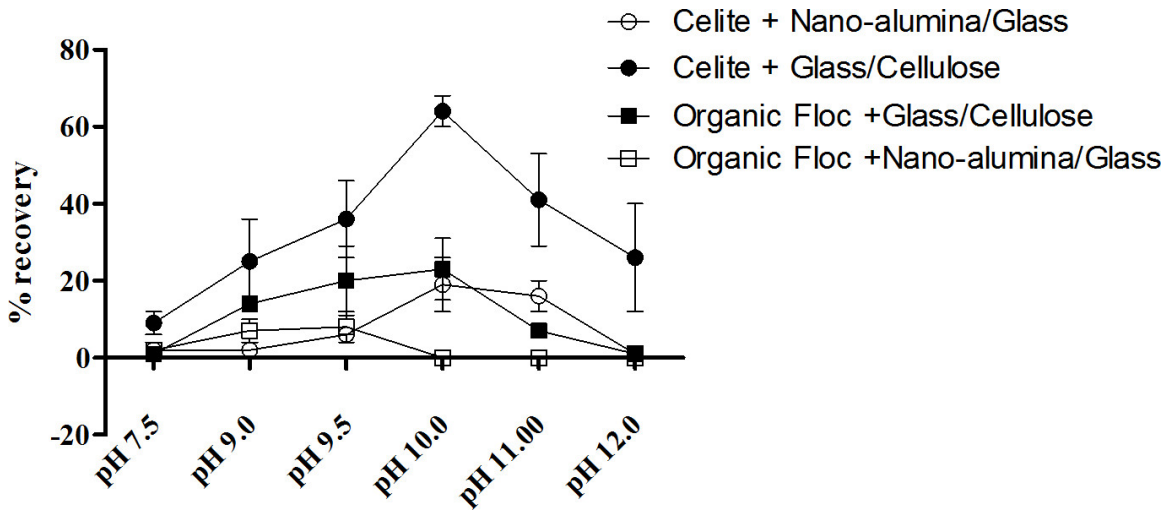

Figure 3: Percent recovery of AdV-41 using two types of secondary concentration techniques (Celite and organic floc) and two types of filters (Glass/Cellulose and Nano-alumina/Glass) at varying pH levels of beef extract. Error bars represent standard deviation (n = 3)[13].

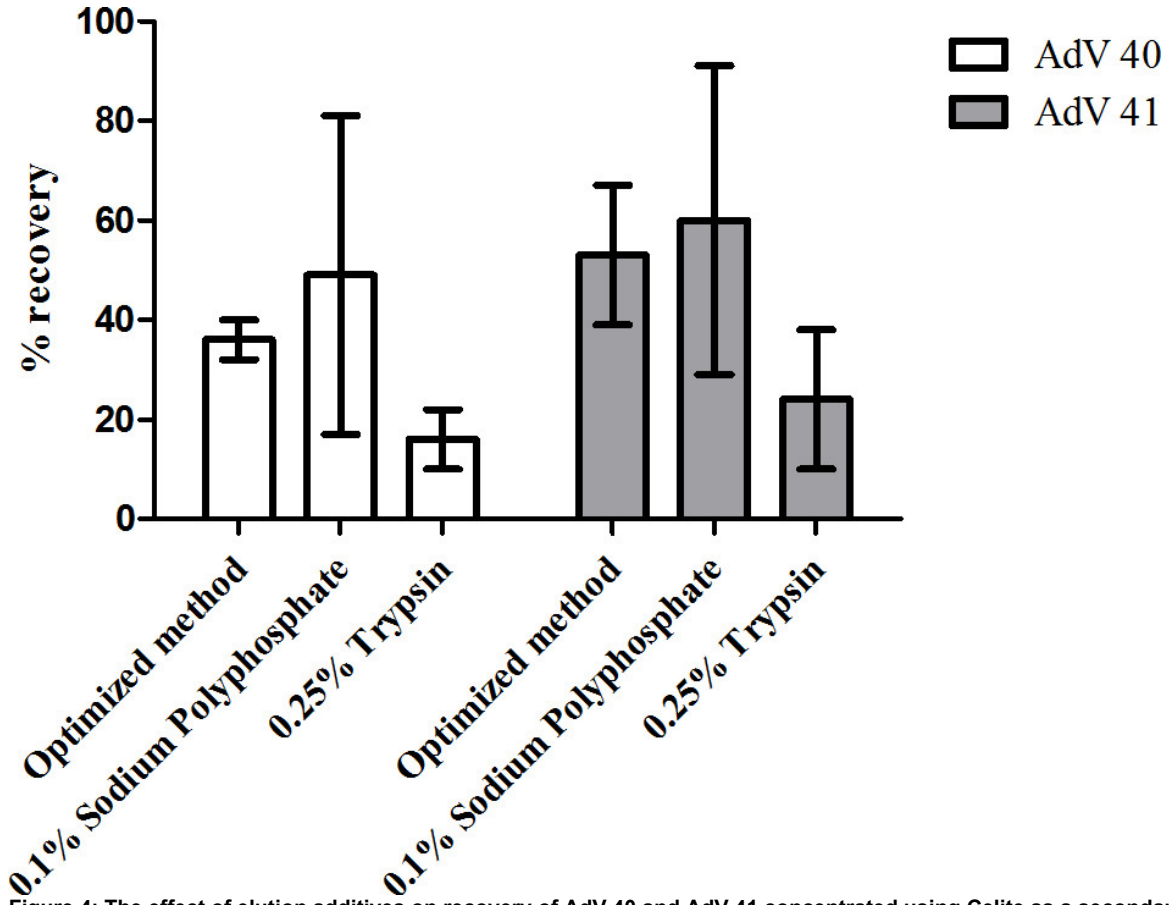

Figure 4: The effect of elution additives on recovery of AdV 40 and AdV 41 concentrated using Celite as a secondary concentration technique and Glass/Cellulose. Error bars represent standard deviation (n = 3)[13].

Discussion

Electropositive filters are useful in concentrating viruses from water; however these filters can differ in their structure and composition which could in turn alter their effectiveness. Compounding this problem, capsid structures and charges vary between virus strains requiring concentration techniques be tailored to ensure optimal recovery [15]. Through simple modifications of the existing concentration techniques (*e.g.,* electropositive filters, beef extract elution), more effective concentration of target viruses can be achieved [16,17].

Research to date has typically focused on recovery of virus by modifying primary concentration (filters) steps, but little attention has been given to subsequent concentration procedures which could also influence virus recovery [18]. It has been demonstrated that different secondary concentration techniques can affect virus recoveries [6,13,19,20]. Celite concentration has been shown to recover similar levels of virus to that of other secondary concentration methods [8,14] from a variety of matrices, while using quicker and simpler sample processing procedures.

Due to varying outer capsid structures, certain viruses can become physically trapped within the filter matrix [21], necessitating the evaluation of different additives to filter elution protocols. For example, addition of surfactant, sodium polyphosphate improved both bacterial and viral recovery during primary concentration [6,13,22-24]. On the other hand, low recoveries observed following addition of trypsin indicate that proteases do not aid in cleavage of the protein fibers on the outer viral capsid. Furthermore, while not uncommon [7,25,26], it is noteworthy that some of our recoveries were in excess of 100%. As previously shown [7,25,26], this is likely due to the clumping of laboratory prepared virus used as spike.

The small volume (100 ml virus spiked water) concentration approach presented can be a practical way to develop and optimize virus concentration methods, due to its simplicity and low consumption of resources along with quick sample processing time. Using such small volumes of water, the concentration of PCR inhibitors within the sample is also minimized, however it is recommended that each source water be evaluated for the presence of PCR inhibitors prior to experimentation. Since numerous choices of both filters and secondary concentration techniques exist, small volume concentration techniques allow for quick evaluations of numerous variables. It is hoped that these techniques could be deployed prior to large scale filtration experimentation to aid in identifying which conditions are optimal for target virus concentration per given water matrices.

Disclosures

No competing financial interests.

The views expressed in this article are those of the authors and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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