Development of a novel bag-mediated filtration system for environmental recovery of poliovirus

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ABSTRACT

Poliovirus (PV) is on the verge of global eradication. Due to asymptomatic shedding, eradication certification requires environmental and clinical surveillance. Current environmental surveillance methods involve collection and processing of 400-mL to 1-L grab samples by a two-phase separation method, where sample volume limits detection sensitivity. Filtration of larger sample volumes facilitates increased detection sensitivity. This study describes development of a pumpless in-field filtration system for poliovirus recovery from environmental waters. Recovery of PV types 1, 2, and 3 were compared for glass wool, ViroCap, and NanoCeram (PV1 only) filters. Seeded experiments were performed using 10^5 plaque forming units of PV inoculated into 10-L volumes of secondary effluent, surface water, or a 50:50 mixture of each at pH 7.0. Filter eluates were plated onto buffalo green monkey kidney cells for virus enumeration by plaque assay. Across all water types, recovery from glass wool filters for PV1, PV2, and PV3 averaged 17%, 28%, and 6%, respectively. Recovery from ViroCaps for PV1, PV2, and PV3 averaged 44%, 70%, and 81%, respectively. 10-L samples of moderate turbidity water were processed through ViroCap filters in less than 30 minutes using a pumpless, bag-mediated filtration system. Bag-mediated filtration offers a simple, compact, and efficient method for enhanced environmental PV surveillance.

Key words | environmental sampling, filtration, poliovirus, wastewater

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INTRODUCTION

Polioviruses (PVs) are a group of three distinct serotypes (PV1, PV2, and PV3) genetically classified as *Enterovirus C* species within the genus *Enterovirus* in the family *Picornaviridae*. They have a single-stranded positive sense RNA genome, and are extremely robust, remaining stable for days to weeks at room temperature (World Health Organization (WHO) 2004). PV is most commonly transmitted through the fecal-oral route and can be shed up to 4 weeks by infected individuals (WHO 2004). PV causes poliomyelitis, which primarily impacts children under the age of five, and can result in irreversible acute flaccid paralysis (AFP) (Nathanson & Kew 2010).

Efforts to eradicate poliovirus began in 1988 by the WHO. Poliomyelitis cases have been reduced over 99% since 1988; in 2012 cases declined 66% from the previous year, with only 222 reported cases globally (WHO 2013b). While significant gains have been made, PV is still endemic in Nigeria, Afghanistan, and Pakistan (WHO 2013b). Additionally, in 2013 the WHO reported polio outbreaks in Ethiopia, Cameroon, Somalia, Kenya, and Syria (WHO 2013a).

The WHO's 'gold standard' approach to PV surveillance entails monitoring clinical cases for AFP (WHO 2003, 2004). However, within populations of high vaccine coverage, AFP surveillance may not be sufficient to detect low PV levels because few cases present with severe clinical signs (Poyry *et al.* 1988; Birmingham *et al.* 1997). Amongst unvaccinated individuals, 0.1–1% of those infected will experience AFP (Hovi *et al.* 1986).

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As vaccine coverage increases and overall cases are reduced, environmental surveillance of PV becomes increasingly important. Symptomatic, asymptomatic, and vaccinated individuals spread the virus in their fecal matter; on average, infected individuals excrete 10^7 virions/day (WHO 2003; Lodder *et al.* 2012). This can result in PV's circulation within a population with no clinical cases arising for months to years (Eichner & Dietz 1996). Environmental sampling examines viral presence in centralized wastewater flows and wastewater impacted rivers and streams. Thus, environmental surveillance examines large populations for PV presence amongst asymptomatic individuals.

Currently, environmental surveillance is applied as supplementary to AFP surveillance (Hovi 2006). Sampling at key points along a sewer line can pinpoint a PV outbreak to a specific location (Hovi 2006). Through filtration of 10 L raw sewage and ultrafiltration secondary concentration to 20 mL, as few as 25 virions can be detected per liter from environmental samples (Lodder *et al.* 2012). In a study where $3–6 \times 10^{10}$ $CCID_{50}$ (50% cell culture infective dose) of PV1 was flushed down a toilet, PV was detectable 20 km downstream the sewer line for 4 days (Hovi *et al.* 2001). This indicates that PV could be detected from a single grab sample if 1 in 10,000 persons were infected (Hovi *et al.* 2001). However, this method assumes that each step of the method is 100% efficient and that wastewater flows are ~100 liter per person per day – assumptions that are unlikely to occur in practice in areas currently endemic or subject to outbreaks (WHO 2003).

Current methods for environmental sampling include collection of single or composite 400-mL to 1-L grab samples, or use of a trap sampling device (e.g., a bag of macroporous glass) (Hovi *et al.* 2001; Deshpande *et al.* 2003; El Bassioni *et al.* 2003; WHO 2003; Chowdhary & Dhole 2008), and concentration of samples by a two-phase separation method (Hovi *et al.* 2001, 2005; Deshpande *et al.* 2003; El Bassioni *et al.* 2003; WHO 2003; Manor *et al.* 2007; Chowdhary & Dhole 2008). These methods are advantageous because they are easy to perform, but they require transport of liquid samples back to a laboratory for concentration. Further, the overall sensitivity of the method is limited by the small volume collected and concentration method employed.

If recovery efficiencies are maintained or improved, increasing sampling volume proportionally raises sensitivity of detection. However, PV sampling sites can occur in remote locations where access is limited and transport of large water volumes is difficult. In-field filtration facilitates collection of considerably larger sample volumes. However for filtration to serve as a viable option, sample collection methods must accommodate collection site limitations, including ease of access and lack of power supply. Additionally, filtration methods should be easy to use, offer high capture and recovery efficiencies, be inexpensive, and easy to decontaminate. The objective of this study was to develop a simple in-field filtration sampling system for PV recovery from environmental waters. Accordingly, a pumpless bag-mediated filtration system (BMFS) was developed and three filter types (glass wool, NanoCeram, and ViroCap) were evaluated for recovery of PV from seeded water samples of varying quality.

METHODS

Study organisms

Stocks of the vaccine strains of Poliovirus type 1 (PV1), poliovirus type 2 (PV2), and poliovirus type 3 (PV3) were prepared by confluent lysis of buffalo green monkey kidney (BGMK) cells monolayers (Sobsey *et al.* 1978). Poliovirus strains were kindly provided by Steve Oberste (United States Centers for Disease Control and Prevention), while BGMK cells were initially provided by Dan Dahling (United States Environmental Protection Agency). Viruses were extracted with Vertrel XF (Dupont) and purified stocks stored at −80 °C. Viruses were enumerated with a previously described plaque assay on 95% confluent BGMK cells, except modified to include an Avicel (FMC Biopolymer) overlay rather than agarose (Sobsey *et al.* 1978; Matrosovich *et al.* 2006). All assays were performed in triplicate using 200 μL aliquots of relevant dilutions. Infected cells were incubated at 37 °C and 5% CO_2 for 48 hours, then stained with 2% crystal violet in 20% methanol. Plaques were counted for infectious virus enumeration.

Filter media

Two filtration media were evaluated in this study, glass wool and NanoCeram® filter media (Argonide, Sanford, FL, USA). Glass wool (Johns Manville R-11) was washed as

described by Vilagines *et al.* (1993). Briefly, glass wool was soaked in deionized water for 15 minutes, then soaked for 15 minutes in 1 M HCl and rinsed with deionized water until circumneutral pH was reached. The glass wool was then soaked in 1 M NaOH for 15 minutes, and rinsed with deionized water. After reaching neutral pH, it was stored in phosphate buffered saline (PBS) at pH 7.0. Glass wool filters were packed to a density of 0.73 g/cm^3 in reusable, autoclavable housings (4.5 cm diameter by 10 cm long) manufactured from polypropylene. Cartridges were well packed to avoid short-circuiting. Filter cartridges were then stored for up to 1 month prior to use, at 4 °C with the media saturated in sterile PBS at pH 7.0. Filters were flushed prior to filtration with 200 mL PBS, pH 7.0 and the flushed filtrate tested with pH strips to ensure it remained at pH 7.0.

ViroCap™ and NanoCeram are commercially available pleated cartridge filters containing the same positively charged filter media. The filtration media has an average pore size of 2–3 μm, and contains glass microfibers coated with alumina nanofibers (Karim *et al.* 2009). The ViroCap contains a 5 cm long by 7 cm diameter pleated filter cartridge, in a disposable polypropylene housing (Scientific Methods, Granger, Indiana, USA). NanoCeram filters contained 12.5 cm long by 7 cm diameter disposable filter cartridges in a reusable housing.

Water

Ten-liter samples of tap water, surface water, and secondary effluent were collected for filter recovery experiments. Tap water was drawn from a tap in Seattle, WA, USA and autoclaved for 30 minutes to dechlorinate. Secondary effluent (pre-chlorination) was collected from a wastewater treatment plant in Seattle, WA. Surface water was collected from Lake Union in Seattle, WA. Water samples were pH-adjusted to 7.0 with 1 M HCl and 1 M NaOH, using a Denver Instruments UltraBasic pH meter. After pH adjustment, a 10-L volume of a 50:50 mixture of secondary effluent and surface water was prepared. Water was stored at room temperature until use.

Filtration

A total of 10^5 plaque forming units (PFU) of PV were seeded to 10 mL PBS, vortexed for 30 seconds, and then the entire volume added to a 10-L water sample. After thorough mixing, the sample was passed through one of the filter configurations at a rate of 2 L/min. Duplicate runs for each condition were performed at a given experimental trial. Each condition was performed on three separate experimental trials. Filters were stored at 4 °C until elution. Filters were eluted no more than 6 hours after filtration.

Elution

Glass wool filters were eluted as described by Lambertini *et al.* (2008). Briefly, a sterile 80 mL solution of 3% beef extract, 0.05 M glycine buffer, pH 9.50 was injected into the filter outlet, saturating the filter media. After 15 minutes, another 80 mL was injected. Air was then injected until all eluate was collected. After elution, glass wool media was disposed of and filter cartridges were washed and autoclaved for 30 minutes prior to re-use.

ViroCap filters were eluted by pumping 175 mL sterile 1.5% beef extract, 0.05 M glycine buffer, pH 9.50, into the filter and letting it stand. After 30 minutes, this was pumped out and collected, and then pumped back into the filter and let to stand for another 30 minutes. This process was repeated once more, for a total filter contact time of 90 minutes.

NanoCeram virus filters were eluted as described by the United States Environmental Protection Agency (Fout 2001). Briefly, 500 mL sterile 1.5% beef extract, 0.05 M glycine buffer, pH 9.50, was pumped into the filter. After 1 minute, this was pumped out and collected. It was pumped back into the filter, let to stand for 15 minutes, then pumped out and collected.

All eluates were immediately pH-adjusted to 7.0–7.5 using 1 M HCl and 1 M NaOH. Eluates were stored at 4 °C until analysis. Eluates were serially diluted into PBS prior to analysis. All samples were assayed by viral infectivity assay within 18 hours of elution. Viral recovery was calculated by dividing the recovered viral count by the seeded viral count.

Controls

Unseeded control volumes of each water type (lake water, mixed water, and secondary effluent), PBS, and beef

extract/glycine eluant, were plated onto BGMK cells to ensure absence of culturable enteric viruses. Additionally, known concentrations of PV were plated in each water type to evaluate impact on viral enumeration. Similarly, assay inhibition controls were run by filtering 10 L unseeded water, eluting the filters, and subsequently adding ~10^5 PFU PV1 to the eluate. Neither assay inhibition controls nor various water types substantially impacted viral enumeration.

Bag-mediated filtration system

A 10-L polyurethane-coated nylon sock bag was designed and fabricated. A detachable brass ring was inserted into holes on the bag's top. A rope was attached to the ring; the bag lowered into water to fill, and then hauled up by the rope for sample recovery. The bag was hung onto a customized, collapsable tripod stand by reinforced holes on the bag's side. A filter was attached to a port at the bottom of the bag. Samples were allowed to filter under gravity until head was inadequate to move the remaining sample through the filter. The bag was then sealed with a clamp, and the remainder of the sample filtered by rolling the top of the bag to generate adequate pressure to drive the sample (Figure 1).

Sampling system apparatus disinfection

Disinfection trials were performed to evaluate field based decontamination procedures for the BMFS. A total of 10^5 PFU PV1 was pipetted onto 2-cm^2 areas of the sampling bag and the PVC tubing. The areas were allowed to dry and then treated by immersion in 1% bleach for either 10 seconds or 5 minutes. Dried controls were immersed in deionized (DI) water for 10 seconds or 5 minutes. After an appropriate contact time, sites were swabbed with a PBS/2% sodium thiosulfate solution. Swabs were eluted by vortexing for 60 seconds. Triplicate trials were run for each surface type and each contact time. Infectious viruses in swab eluates were quantified by three replicate plaque assays on BGMK cells, as described previously. Log10 reductions were calculated by the difference between the log10 concentrations of the swab eluates for the DI controls and the log10 concentrations of the swab eluates for bleach treated samples.

RESULTS AND DISCUSSION

In preliminary studies using dechlorinated tap water, the two commercially available filters out-performed the glass wool filters for PV1 recovery. ViroCap recoveries were highest averaging 64%, with NanoCeram recoveries averaging near 50%. The difference in recovery between the two commercial filters was likely due to elution volume, since each filter type contains the same filter media. In the current study, recoveries of PV1 from dechlorinated tap water by glass wool filters averaged only 32%, substantially less than previously reported. Earlier studies reported recoveries from 77–98% (Vilagines *et al.* 1993, 1997; Gantzer *et al.* 1998; Ehlers *et al.* 2005; Lambertini *et al.* 2008; Deboosere *et al.* 2011). These previous studies used sodocalcic glass wool (Rantigny 725 from Saint Gobain, Isover-Orgel, France) for their filter media, which is not readily available in the United States. As a result the present study used Johns Manville R-11 glass wool based on unpublished reports of similar

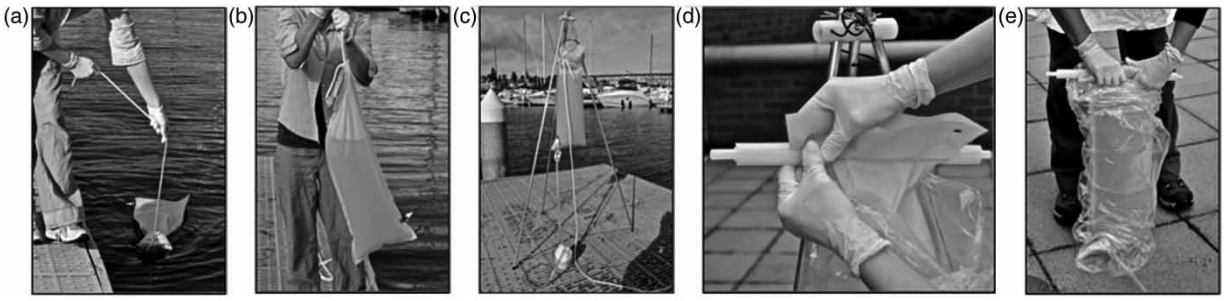

Figure 1 | Photographs of sample collection and filtration. (a) Sample collection; (b) raise sample out of water; (c) hook sample on tripod stand and attach filter to port; (d) attach clamp to seal bag; (e) roll bag to force water through filter.

performance (Mark Borchardt, personal communication). The difference in filter media may have contributed to the disparity in recovery.

PV1 recoveries from surface water, secondary effluent and a 50:50 mix are summarized in Figure 2 for each filter type. The ViroCap filter had a PV1 recovery efficiency ranging from 38–48% in the three water matrices. These results are consistent with previously reported recovery efficiencies (37–44%) of PV1 in 20 L deionized water and artificial seawater by the ViroCap filter (Bennett *et al.* 2010). The ViroCap filters performed better than or similarly to the NanoCeram filters for PV1 recovery. However, for 10 L filtered, the NanoCeram's elution volume results in only a 20-fold concentration, verses a 57-fold for the ViroCap. Additionally, NanoCeram filters are more difficult to use from an operational standpoint and require additional connection parts. For these reasons, the NanoCeram was not pursued for further investigations. PV1 recovery on glass wool filters ranged from 15–18%. The ViroCap filter obtained recoveries about 2 times more efficiently than the glass wool filters, similar to the variation in dechlorinated tap water.

Recoveries of PV2 using ViroCap and glass wool filters are summarized in Figure 3. ViroCap filter recovery exceeded the recovery with glass wool filters for each water type. Virocap recoveries averaged greater than 70% across all water types. Recoveries from glass wool filters were most similar in surface water, but recoveries from secondary effluent and the 50:50 mixture of effluent and surface water were substantially lower than observed for the ViroCap filters.

Figure 4 summarizes the observed PV3 recovery results for ViroCap and glass wool filters from the three matrices.

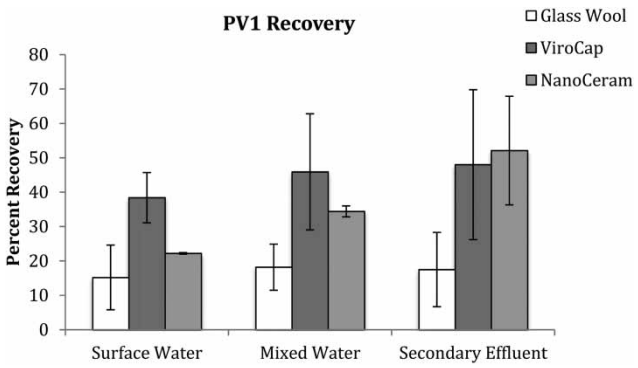

Figure 2 | PV1 recovery from water matrices. Error bars represent ± standard deviation.

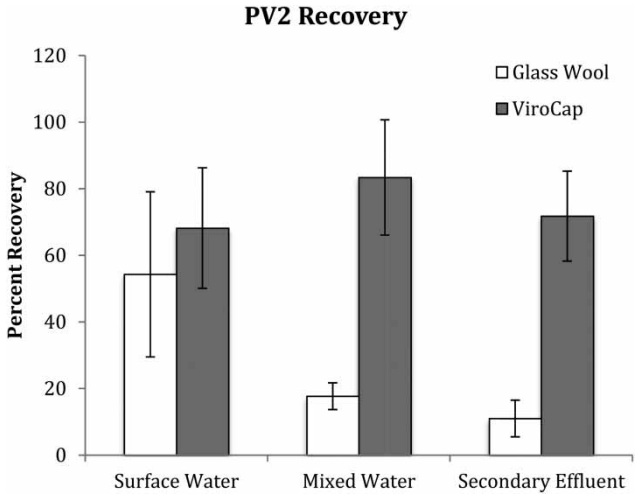

Figure 3 | PV2 recovery from water matrices. Error bars represent ± standard deviation.

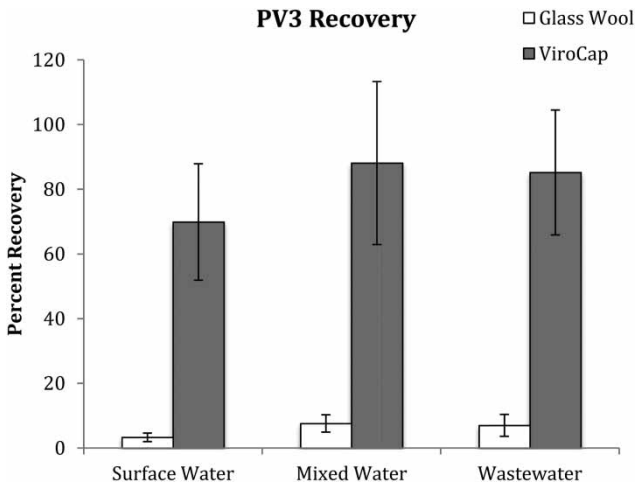

Figure 4 | PV3 recovery from water matrices. Error bars represent ± standard deviation.

In each comparison, PV3 recovery was lower for glass wool than for the ViroCap filters. The difference in recoveries observed from the two filter types was most disparate for PV3. While ViroCap recoveries remained high (70–>80%), glass wool recoveries were nominal (3–8%).

Recovery of PV1, PV2, and PV3 was higher in secondary effluent and mixed water than in lake water, with the exception of PV2 on glass wool filters. This is likely due to adsorption of PV onto organic matter or soil in the wastewater. Because the filters remove turbidity from water, PV adsorbed to this organic matter is likely to be captured more efficiently than the PV in water with less organic matter.

The dramatic difference in recovery observed for PV1 and PV2, as compared to PV3 for glass wool filters may be related to differences in the virus surface charge between the serotypes. However, losses in recovery efficiency coincided with the purchase of a new glass wool lot. Follow-up experiments with PV1 and the new lot of glass wool suggest that variances in glass wool lots substantially impact viral recovery (data not shown). This raises concern for interlaboratory consistency of this method.

Additionally, Lambertini *et al.* (2008) discovered viral recovery on glass wool filters is significantly impacted by water pH (Lambertini *et al.* 2008). Optimal pH for poliovirus recovery is estimated at 6.5, with decreasing recovery as pH increased, down to nearly 0% recovery at a pH of 9.0 (Lambertini *et al.* 2008). Therefore, if environmental samples have a naturally high pH then pH adjustment by acidification would be necessary for effective recovery. This added step complicates sampling and reliability, and may present a barrier to field sampling in developing countries.

In comparison, ViroCap filter recovery efficiency was high for both PV2 (56–89%) and PV3 (70–88%). These positively charged filters are not as sensitive to fluctuations in pH, due to the strong positive charge imparted by the alumina nanofibers below pH 9.2. Additionally, as a ready-to-use commercially available product, lot variations are less likely than for glass wool, which requires substantial washing and conditioning steps.

ViroCap filter eluate is appropriate for PV detection by tissue culture or reverse transcription polymerase chain reaction (RT-PCR). While tissue culture is the gold standard for viral detection, molecular methods offer the ability to distinguish between wild type PV and vaccine strains (which are commonly found in environmental waters where the oral polio vaccine is used).

For field sampling, the BMFS coupled with the ViroCap filters was demonstrated to be an effective alternative to the use of electric pumps for filtration, and is easy to operate by a single operator. The ring's structural stability prevented the bag from collapsing upon entry to the water source. A petite operator could fill the bag with a 10 L sample volume, raise it out of the water, and hook it onto a tripod stand for filtration. In the laboratory, 10-liter volumes of surface water, secondary effluent, and primary effluent could be passed easily through the ViroCap filters with the BMFS within 30 minutes or less (Table 1). Application of the BMFS and ViroCap filters to raw wastewater in Nairobi resulted in clogging of the filters after passage of 2–8 liters; however, filters could still be eluted and viral recoveries were high (data not shown). This was consistent with the filterable volumes of raw sewage observed in laboratory studies (Table 1). Debris loading in raw sewage samples is a real concern and investigations are being conducted to address this, including design of a pre-filtration/screening apparatus, redesign of the filter housing to enlarge choke points, and enlarging the bag valve. Incorporation of these aspects to the design should increase the raw sewage filtration capacity of the ViroCap filter.

The two-phase separation method currently used by the WHO provides 50–100-fold concentration of samples (WHO 2003). Even with filtration of only a 2 L sample (the lowest volume observed for raw sewage), after polyethylene glycol concentration of the eluate the current method results in a concentrate volume of 4 mL, a 500-fold concentration. With 10 L sampled, the BMFS sample is concentrated 2500-fold. This results in 5–500 times increased sensitivity over the currently preferred WHO method assuming similar methods of analysis. Assuming recoveries observed in this study for PV1 (~40%), and analysis of the full concentrate volume, the current method has a theoretical detection limit of range <0.5 to 2 viruses per liter. For PV2 and PV3, which had greater observed recoveries, the theoretical detection limit of the method would be <1 virus per 5 liters.

Disinfection of the major reusable components of the BMFS (sampling bags and tubing) is summarized in Figure 5.

Table 1 | Filterable volumes with BMFS system using ViroCap filters

Water type	Minimum volume filtered (L)	Maximum volume filtered (L)	Average volume filtered (L)	Standard deviation	*n*
Lake	> 10	> 10	> 10	0	5
Secondary effluent	> 10	> 10	> 10	0	3
Primary effluent	> 10	> 10	> 10	0	2
Raw sewage	2.9	7.0	5.0	1.5	5

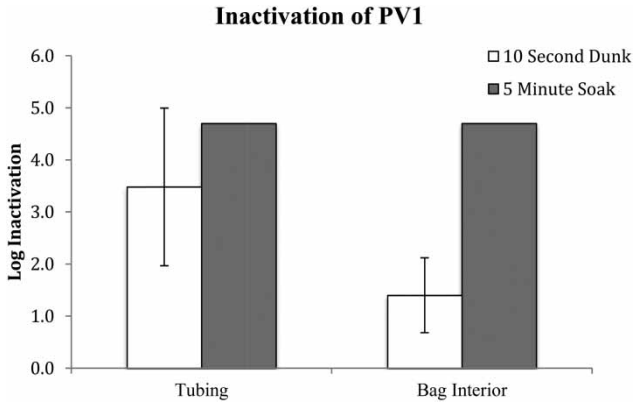

Figure 5 | Log inactivation of PV1 by submersion in 0.0584% free available chlorine. Error bars represent ± standard deviation.

Submersion of the sampling bags and tubing in 1% (v/v) bleach solution (0.0584% free available chlorine) for a contact time of 5 minutes achieved a greater than 4.7 log inactivation of PV1. The bag clamp and tubing clamp are plastic and can also be disinfected by soaking in 1% bleach solution for 5 minutes. The metal ring and towrope are autoclavable or can be disinfected in boiling water.

For ease of transport, all components of the BMFS fit into a backpack. The loaded backpack weighs 4.5 kilograms. The backpack contains a separated cooler section, which accommodates two icepacks and up to four filters. Icepacks refrigerate the sample for transport to the laboratory, which increases viral survival prior to analysis.

CONCLUSIONS

As PV vaccine coverage increases and PV environmental surveillance becomes increasingly relevant, improvements over current methodology are necessary. Collection and processing of large sample volumes in the field offer a means to reduce or streamline sample processing bottlenecks in the laboratory. The BMFS is an effective option for power-free sampling in remote locations. ViroCap filters offer reliable and effective concentration of viruses from environmental waters. The sampling method developed in this study could be used to improve environmental surveillance for PV. A field validation study is needed to compare the efficacy of the newly developed method in parallel with current WHO recommended methods for environmental surveillance.

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