Poliovirus and rotavirus detection in water:

evaluating and applying environmental surveillance methods

Jill Christin Falman

A thesis submitted in partial fulfillment of the requirements for the degree of:

Master of Science

University of Washington

2017

Committee:

John Scott Meschke

Joseph Cook

Christine S Fagnant

Program Authorized to Offer Degree:

Department of Environmental and Occupational Health Sciences

School of Public Health

University of Washington

Abstract

Poliovirus and rotavirus detection in water:

evaluating and applying environmental surveillance methods

Jill C Falman

Chair of Supervisory Committee:

Professor John Scott Meschke

Environmental and Occupational Health Sciences

Human enteric viruses are responsible for the majority of acute waterborne diseases. Most people affected are children under five years old. Symptoms of disease can include diarrhea, gastroenteritis, and paralysis. However, human infections can also be asymptomatic, which allows viruses to go undetected and circulate within the environment. Environmental surveillance is a tool that can supplement disease-based surveillance by sampling, concentrating, and analyzing sewage and other environmental surface waters for pathogens. Polioviruses and group A rotaviruses are enteric viruses of major global public health concern and water has been implicated in their transmission. Environmental surveillance for the detection of poliovirus has expanded in recent years to supplement clinical detection of wild polioviruses and vaccine-derived polioviruses in support of polio eradication efforts. Surveillance of other enteric viruses

typically relies on disease-based surveillance and any environmental surveillance employed is for a limited sampling period. Recently, the introduction of new live, attenuated rotavirus vaccines has created a need for environmental monitoring to characterize rotavirus strains in wastewater. The epidemiology of these strains is useful to inform vaccine efficacy. This research focused on addressing environmental sampling needs. First, the evaluation and optimization of a secondary concentration step for improved detection of poliovirus in wastewater is presented. Next, environmental surveillance sampling is applied to enable molecular characterization of group A rotavirus strains in circulation in three communities in Nairobi, following the introduction of the Rotarix® vaccine in Kenya. A skimmed-milk flocculation (5%) method is identified as an economically feasible, time efficient, and high recovery secondary concentration method for poliovirus detection. Genetic characterization in selected Nairobi communities reveals a diversity of rotavirus strains in post-vaccine Kenya, with the emergence of serotype G3. Environmental surveillance for non-polio enteric viruses is often ad hoc to support outbreak investigations or inform vaccine efficacy. Though, it is expected to expand in coming years as better detection methods are developed and the global community pursues eradication of more vaccine preventable diseases.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Equations

Acknowledgements

This thesis would not have been possible without the support and patience of colleagues, friends, and family. I would like to thank Scott Meschke for giving me the opportunity to work in his lab as part of the polio project and travel to South Africa for additional training. Christa Fagnant and Alex Kossik provided me with a solid foundation for viral lab work and were unwavering in their emotional and technical support along the way. Both Christa and Alex were instrumental in my journey to become a scientist and I am very grateful for their time, expertise, and friendship. I would also like to acknowledge the additional Meschke lab and support staff – Nicky Beck, Jeff Shirai, Nicolette Zhou, and Christine Tran – for additional knowledge and encouragement. Additional graduate and undergraduate students provided humor, lab assistance, and comradery: Erika Keim, Elena Jaffer, Nicole Van Abel, Tori Balta, Joanna Harrison, Liliana Sánchez, Alero Agberen, Yarrow Linden, and Daysha Gunther. Thank you to Joe Cook for being an excellent teacher and his willingness to serve on my committee. I would further like to recognize Maureen Taylor and her lab staff at the University of Pretoria, Department of Medical Virology and Nicci Page and her team at NICD. A special thanks to Walda van Zyl for being amazing and treating me like family. The many trips to the West Point Treatment Plant were made possible thanks to Phuong Truong, Eric Eifrig, and technicians. Finally, thank you to Adam, my parents, and friends for continued love and support.

Funding

Research was supported by funding from the Paul G. Allen Family Foundation (Grant Number NPT.1938-603689) and the Global Opportunities in Health Fellowship through the University of Washington, Department of Global Health.

1. Introduction

Human enteric viruses are responsible for the majority of acute waterborne diseases worldwide (Fong and Lipp, 2005; Wyn-Jones and Sellwood, 2001). Human infections are often asymptomatic, but can induce various symptoms, including diarrhea, gastroenteritis, and even paralysis. Morbidity and mortality are most common and severe among the immunocompromised, pregnant women, children, and the elderly (Gerba et al., 1996). Enteric viruses often multiply within the gastrointestinal tract of their hosts and are then shed in the feces in high numbers (typically between 10^5 to 10^{11} viruses/g stool) for up to several months (Salim, et al., 1990; Blacklow & Greenberg 1991). Consequently, enteric viruses have been found to persist in water after treatment at wastewater treatment plants and are frequently detected in surface waters in developing countries (Petrinca et al., 2009).

Surveillance of disease typically relies on clinical case reporting. Infected, symptomatic individuals with access to and who seek health care are accounted for in clinical surveillance systems. However, those who are infected and asymptomatic or do not access health care can shed virus into the waste stream and are not captured in disease-based surveillance (Manor et al., 2014; Asghar et al., 2014; Mangal et al., 2013). Environmental surveillance complements clinical surveillance by testing wastewater and wastewater-impacted surface waters for the presence of viruses (Hovi et al. 2012; GPEI, 2015).

1.1 Environmental surveillance

Environmental surveillance of enteric viruses consists of sampling, concentrating, and analyzing sewage and other environmental surface waters for pathogen(s) of interest. Monitoring the environment is useful to detect the presence of viral circulation in a region and subsequently

inform population risk. Monitoring individuals occurs through evaluation of clinical specimens, while monitoring a population occurs through the evaluation of sewage samples representative of the population. Furthermore, understanding the epidemiology and circulation of an enteric virus can prompt health officials to implement supplementary activities (*i.e.* immunization campaigns) before clinical cases emerge. Strain-specific information can also provide insights into vaccination coverage and virus mutations after the introduction of a vaccine. Environmental surveillance of enteric viruses, including poliovirus, rotavirus, Hepatitis A virus, Hepatitis E virus, norovirus, and nonpolio enteroviruses have been conducted in sewage, river, and estuarine samples for detection and characterization (Asghar et al., 2014; Pellegrinelli et al., 2013; Yanez et al., 2014; Hellmer et al., 2014; Zheng et al., 2013; Lu et al., 2015; Hazam et al., 2010). Viral environmental sampling is labor intensive, costly, and requires routine sampling. Consequently, most enteric virus surveillance in water is short-term (*e.g.* one year) because environmental surveillance is used in response to a suspected outbreak or to monitor for a limited time following an outbreak. The exception is environmental surveillance of poliovirus. In the global effort to eradicate poliovirus, routine surveillance in environmental wastewater and wastewater-impacted surface waters is used to monitor transmission in communities as a supplement to diseased-based clinical surveillance. Environmental surveillance continues to expand to help identify remaining poliovirus in endemic areas, as a warning of new importations, and elimination of vaccine viruses (WHO, 2013). Similarly, there is an expectation environmental surveillance activities will expand for other vaccine preventable diseases, like gastrointestinal disease from rotavirus.

Poliovirus and rotavirus are two enteric viruses of major global public health concern and water has been implicated in their transmission (Leclerc et al., 2002; Lodder et al., 2012).

Poliovirus is the only human enteric virus close to eradication. In 2016, there were only 37 wild poliovirus (WPV) and 5 vaccine-derived poliovirus (VDPV) cases globally (GPEI, 2017) compared to 215,000 estimated rotavirus cases (WHO, 2016d). However, both viruses are transmitted via the fecal-oral route and the majority of those infected are asymptomatic (Hovi et al., 1986; Minor, 2016; Estes and Kapikian, 2007). Accordingly, environmental surveillance can provide valuable data of viral circulation in the absence of clinical cases.

1.2 Specific Aims

The research that will be presented is focused on development of environmental surveillance sampling methods for enteric viruses, specifically poliovirus and applications for rotavirus characterization. The specific aims are

1. To adapt and identify secondary concentration methods suitable to improve detection of poliovirus type 1 (PV1) in wastewater samples initially concentrated using positively-charged ViroCap™ filters.
2. To adapt and optimize the skimmed-milk flocculation method and compare PV1 recovery, cost feasibility, and time efficiency with the polyethylene glycol (PEG)/sodium chloride (NaCl) precipitation method for secondary concentration of wastewater samples, initially concentrated using ViroCap filters.
3. To characterize group A rotavirus (RVA) strains in circulation in the environment in three urban communities in Nairobi following the introduction of the Rotarix® vaccine in Kenya.

Chapter 2: Poliovirus

2.1 Introduction

In 1988, the Global Polio Eradication Initiative (GPEI) launched as a public-private partnership to eradicate poliomyelitis (polio) worldwide (WHO, 2016b). Polio is caused by poliovirus infection and mainly affects children under five years old, elderly, and immunocompromised individuals (Gerba et al., 1996). Poliovirus is a single-stranded, positive-sense RNA virus with about 7500 nucleotides and is classified within the Human Enterovirus C species of the picornaviridae (Minor, 2016). The non-enveloped virus is generally shed for three to six weeks and often longer for immunocompromised individuals compared to healthy infected individuals (Melnick, 1996; Tebbens et al., 2013; Burns et al., 2014). There are three serotypes (1, 2, and 3) for which an inactivated polio vaccine (IPV) and a live attenuated oral polio vaccine (OPV) were developed by Jonas Salk and Albert Sabin, respectively (Salk et al., 1954; Sabin et al., 1985). Both vaccines have significant roles in the polio eradication plan. The OPV is widely used in endemic and at-risk areas for poliovirus transmission because the attenuated vaccine-virus can provide passive immunization (Minor, 2016). The IPV is used in areas where polio has been eliminated and so there is low risk for transmission due to better mucosal immunity provided by the IPV (Onorato et al., 1991; WHO, 2013).

Although the OPV can serve an important protective function, there is a potential for excreted vaccine-virus to survive, be transmitted by the fecal-oral route, and circulate within community surface waters. The more it circulates among human hosts, the more it replicates and exchanges genetic material. This increases the likelihood of a mutation into a VDPV that has the potential to cause paralysis (Shaghaghi et al., 2016; Jorba et al., 2016; Tebbens et al., 2006). There are three types of VDPV: circulating vaccine-derived poliovirus (cVDPV),

immunodeficiency-related vaccine-derived poliovirus (iVDPV), and ambiguous vaccine-derived poliovirus (aVDPV) (GPEI, 2017). In under-immunized populations with continued person-to-person transmission of VDPVs, the circulating vaccine-virus can mutate and reacquire neurovirulence. This can ultimately lead to an outbreak of paralytic cases and is called cVDPV. People with severe immunodeficiencies are unable to clear an intestinal OPV infection (typically cleared in healthy people within six to eight weeks) and consequently can have prolonged intestinal replication of viruses. Therefore, they excrete iVDPVs for long periods of time. Finally, aVDPVs represent VDPVs isolated from people with no known immunodeficiency or from sewage of an unknown source.

Until April 2016, a trivalent OPV was used to protect against all three poliovirus serotypes (WHO, 2016c). However, the type 2 vaccine strain (Sabin virus) mutates more rapidly than the type 1 and 3 vaccine strains (WHO, 2016a). Therefore, it is more common for the type 2 vaccine strain to become virulent. This, combined with the last known case of wild PV type 2 occurring in 1999, contributed to its removal from OPV (Hagan et al., 2015). There is a planned cessation of the bivalent OPV in 2019 so the OPV will be entirely replaced with IPV (WHO, 2013).

The number of poliovirus cases has fallen by over 99% since the founding of GPEI, with endemic cases remaining in Afghanistan, Pakistan, and Nigeria (WHO, 2017). An estimated one in 200 people infected with poliovirus exhibit the hallmark symptom of infection – acute flaccid paralysis (AFP) (WHO, 2017). Disease-based clinical surveillance through AFP case identification is the gold standard for polio surveillance (WHO, 2013). The global effort to eradicate polio relies on the strength of environmental poliovirus surveillance data to confirm the presence of WPV and VDPV in the absence of clinical cases and offers insights into the

international spread of poliovirus. Typical environmental viral monitoring involves primary concentration and sometimes secondary concentration methods for detection.

Early concentration methods of enteric viruses in water were developed in the 1960s and 1970s. These initial methods were limited because they generally processed only small sample volumes (~ 1 L) (Ikner et al., 2012). Aqueous polymer two-phase separation, hydroextraction, soluble ultrafiltration, and ultracentrifugation are among the small volume concentration methods that have been shown to recover enteric viruses, including poliovirus, but they are not considered feasible for large volume processing (Shuval et al., 1967, 1969; Hill et al., 1971; Gartner, 1967; Nupen, 1970; Cliver & Yeatman, 1965; Grabow, 1968; Rao and Labzoffsky, 1969). More recently, these methods are often used following primary concentration as a reconcentration step for viral detection, which will further be referred to as secondary concentration. The primary concentration methods used today typically use electropositive or electronegative filtration media to process large volumes (up to 1,000 L) of water (Ikner et al., 2012; Abbaszadegan et al., 1993). As eradication nears, poliovirus is expected to circulate in low numbers. The overall sensitivity of detection is affected by the sample volume collected and concentration methods employed.

The WHO Guidelines for Environmental Surveillance of Poliovirus provide recommendations for concentration of poliovirus from sewage. Grab sample collection was an early approach for viral evaluation and is the featured sampling method for environmental surveillance (WHO, 2003). This method processes 500 mL from a 1-L grab sample. The widespread use of this method is attributed to the reasoning that wastewater contains high viral loads because of the large quantities of enteric viruses shed in feces and so a 500-mL sample is adequate for positive detection. However, as poliovirus nears eradication, there are fewer

infectious viral particles present in many waste streams. The bag-mediated filtration system (BMFS) was developed to concentrate larger volumes in the field and in low-resource settings (Fagnant et al., 2014). The BMFS uses the VIRus ADsorption and ELution (VIRADEL) method based on electrostatic interactions between electronegative poliovirus with the positively-charged ViroCap filter for primary concentration of poliovirus. The method has been field-validated in Kenya and Pakistan for environmental surveillance of poliovirus in selected source waters. The method was compared to the recommended WHO grab sample which was concentrated by aqueous polymer two-phase separation by which a 500 mL sample is mixed with dextran and polyethylene glycol, let to stand overnight, and the lower concentrated phase is collected (7-12 mL) (WHO, 2003). The BMFS has used PEG/NaCl precipitation, as described in methods 2.4.5, different from two-phase separation, for secondary concentration.

In addition to PEG/NaCl precipitation, other viral secondary concentration methods have demonstrated a range of recoveries. Methods based on organic flocculation, aluminum hydroxide precipitation-hydroextraction, adsorption-elution, ultrafiltration, and ultracentrifugation have been used as secondary concentration techniques for poliovirus (Ikner et al., 2012). Many of the early studies looked at secondary concentration of poliovirus from tap water. Since the water matrix for wastewater, where poliovirus is most prevalent, is characterized by high organic matter, applicable concentration methods should be optimized to maximize the effective volume assayed while taking into account organic inhibitors that could affect viral recovery efficiency.

This study sought to compare PEG/NaCl precipitation to four secondary concentration methods with potential feasible, simple, and inexpensive applications for environmental surveillance of poliovirus. Beef extract-Celite (Dahling and Wright, 1986b; Rhodes et al., 2011; Melnick, 1996), flat disc filtration, skimmed-milk flocculation (Calgua et al., 2008), and

concentration by InnovaPrep® Concentrating Pipette were adapted as secondary concentration methods following primary concentration using ViroCap filters.

2.2 Materials and Methods

2.2.1 Virus preparation and cell culture

PV1 was used in this study. Stocks of PV1 vaccine strains were prepared by confluent lysis of buffalo green monkey kidney (BGMK) cell monolayers (Sobsey et al., 1978). PV1 was provided by Mark Sobsey (University of North Carolina) while BGMK cells were initially provided by Dan Dahling (United States Environmental Protection Agency). Viruses were extracted with Vertrel XF (E. I. du Pont de Nemours and Company, Wilmington, DE, USA) and purified stocks stored at -80ºC. BGMK cells were grown in 75-cm^2 flasks or 9.5-cm^2 wells containing Eagle's minimum essential media (Corning, 10-010-CV) supplemented with 10% fetal bovine serum (ATCC 30-2020).

2.2.2 Water samples

Ten-liter samples of influent wastewater were collected from a wastewater treatment plant in Seattle, WA. The water matrix was stored at 4°C, used within 24 hours, and mixed well before use.

2.2.3 Primary concentration by BMFS

For each experiment, 10-L water samples were processed through positively charged ViroCap filters using a peristaltic pump and eluted in a 1.5% beef extract/glycine solution. A peristaltic pump was used rather than the BMFS to conserve resources and laboratory space. The

pump essentially mimics the BMFS gravity filtration process deployed in the field. The 2" ViroCap filters have an average pore size of 2-3 µm, and contain glass microfibers coated with alumina nanofibers (Fagnant et al., 2014; Karim et al., 2009). After filtration, filters were stored at 4ºC for up to 4 hours and then eluted by adding sterile 1.5% beef extract (BBL™ Beef Extract Powder, Bection, Dickinson, and Company, Sparks, MD), 0.05 M glycine buffer (TCI G0099, Tokyo, Japan), pH 9.5 with a 30-minute filter contact time. Eluates were immediately pH-adjusted to 7.0-7.5 using 1 M HCl and 1 M NaOH. Eluates were stored at -20ºC until secondary concentration. As PV was eliminated from the United States and PV vaccination is a standard protocol for children under the age of five, it was assumed the eluates contained no poliovirus and therefore used them as the sample matrix for subsequent experiments. The sample matrix will herein be referred to as eluate stocks.

2.2.4 Secondary concentration preliminary investigations

Preliminary investigations involved adaptation of each method for use with primary concentrate from ViroCap filters. The following secondary concentration methods were tested: 1) beef extract-Celite; 2) flat disc filter; 3) InnovaPrep Concentrating Pipette; 4) skimmed-milk flocculation; and 5) PEG/NaCl precipitation in preliminary investigations. The secondary concentration step was evaluated independent of the primary concentration process. During this evaluation, 100-mL eluate stocks were spiked with approximately 10^3 plaque forming units (PFU) PV1. These were mixed thoroughly by shaking (10-15 minutes, 200 RPM, room temperature [20 - 25°C]).

2.2.4.1 Beef extract-Celite

To concentrate the spiked eluate stock by the beef extract-Celite method, 0.1 g celite (Sigma Aldrich Celite® 577, fine, St. Louis, MO) was added per 100 mL of sample and the samples were shaken (room temperature, 10 minutes, 200 RPM). The samples with Celite added were filtered by vacuum filtration using an AP20 filter (2.0 µm pore size, glass fiber filter with binder resin, Millipore Corp, Bedford, MA). The viruses were eluted from the Celite by passing 10 - 20 mL 1 X phosphate-buffered saline (PBS) (pH 9.0) through the filter. This final PBS eluate was collected in a fresh, sterile flask, adjusted to a pH of 7.0 - 7.5 using 1 M HCl and 1 M NaOH, and assayed.

2.2.4.2 Flat disc filter

For secondary concentration using a flat disc filter, the spiked eluate stock was diluted with an additional 500 mL of autoclaved DI water and adjusted to pH 7.0 - 7.5 using 1 M HCl and 1 M NaOH. The sample was filtered by vacuum filtration through a flat disc filter (90-mm diameter ViroCap filter, Scientific Methods). Next, a 1.5% beef extract, 0.05 M glycine, pH 9.5 solution was added to the filter inlet. Only 10 mL of beef extract/glycine solution were needed for full filter contact. After a 30-minute contact time, the secondary eluent was filtered. The filtrate was collected in a glass flask, pH adjusted to 7.0 - 7.5 using 1 M HCl and 1 M NaOH, and assayed. Due to flaws in the filter housing, including leaking because the device could not be fully sealed, the filtration process was simulated using a vacuum-filtration set-up with the same positively charged filter adsorption – elution process. In the simulation, the 90-mm filter was cut to fit in a 60-mm diameter magnetic flat disc filter holder. The spiked eluate stock was filtered by vacuum-filtration. The filter paper was then removed using sterile forceps and placed in 10 mL

1.5% beef extract, 0.05 M glycine, pH 9.5 solution for 30 minutes. Elution was performed by placing the filter paper back on the funnel, pouring the beef extract/glycine solution onto the filter, and collecting by vacuum-filtration.

2.2.4.3 InnovaPrep Concentrating Pipette

Concentration by the InnovaPrep Concentrating Pipette was performed using ultrafiltration hollow fiber polysulfone pipette tips (InnovaPrep CC08022, 0.2 µm, 82 cm^2, Drexel, MO). The spiked eluate stock from influent wastewater was used for four experiments, while spiked 1.5% beef extract, 0.05 M glycine, pH 7 solution and 1 X PBS (pH 7.4) were used as proxy matrices for the remaining preliminary experiments. For all experiments, 100 mL of the spiked matrix (eluate stock, beef extract/glycine solution, or tap water) was divided into two 50-mL aliquots for processing. Each 50-mL stock was concentrated by a hollow fiber polysulfone pipette using the InnovaPrep Concentrating Pipette. The ultrafiltration hollow fiber polysulfone pipette tips were unable to process more than 50 mL of primary concentrate eluate stock per pipette, even when instrument settings were optimized to increase the flow buffer. The filters also clogged after processing 50 mL of the beef extract solution and PBS. The manufacturer's factory settings were used for the four experiments that processed the spiked eluate stocks and PBS. The spiked beef extract solution samples were processed with the following custom instrument settings. Valve open: 35; valve closed: 100; pulse count: 2; flow buffer: 12; and extraction delay: 6 (note: the manufacturer did not assign units to these settings and therefore no units are provided). Following concentration, one to six extractions were performed using one of four elution fluids prepared by InnovaPrep: 0.075% Tween 20 PBS (HC08000), 0.075% Tween 20 Tris (HC08001), 0.075% Tween 20 PBS, pH 9.5 (custom order), and Dulbecco's Modified

Eagle's Medium (DMEM) (custom order). Extraction fluid was collected, diluted in up to 12.3 mL 1 X PBS, pH 7.4, and assayed.

2.2.4.4 Skimmed-milk flocculation (1%)

For concentration by skimmed milk flocculation, first 1 mL of 1% w/v skimmed-milk solution (Oxoid, Ltd., Hants, UK) was added per 100 mL of the spiked eluate stock (Calgua et al., 2008). Next, the sample was pH adjusted to 3.0 – 4.0 using 1 M HCl. This acidification step created a flocculation process by which the skimmed-milk, other eluate stock particulates, and PV1 clumped together into a floc. Samples were shaken (room temperature or 4ºC, 200 RPM) and shaking times were evaluated (4 hours, 8 hours, and overnight [18 hours]). Samples were centrifuged (4500 x G, 30 minutes, 4ºC) and the supernatant was carefully removed. In some cases, the supernatant was evaluated in the viral plaque assay. The pellets were resuspended in 1 X PBS (pH 7.4, 10 to 20 mL), vortexed (5 - 10 minutes, maximum speed) until resuspended, and assayed.

2.2.4.5 PEG/ NaCl precipitation

To the spiked eluate stock, 14 g PEG 8000 (Acros Organics, Geel, Belgium) and 0.2 M NaCl per 100 mL sample were added and shaken vigorously (~5 minutes) at room temperature until dissolved. The samples were then shaken (4ºC, 200 RPM, overnight [18 hours]). Samples were centrifuged (4500 x G, 30 minutes, 4ºC), and the supernatant was carefully removed. The pellets were resuspended in 1 X PBS (pH 7.4, 10 to 20 mL), vortexed (5 - 10 minutes, maximum speed) until resuspended, and assayed.

2.2.5 Secondary concentration comparisons

Two competitive secondary concentration methods were compared for recovering PV1 from wastewater concentrates. These experiments evaluated the secondary concentration step only. Following primary concentration, eluates were pooled, mixed by hand, and divided into aliquots of 100-mL eluate stocks. Each eluate stock was seeded with 100-µl PV1 for approximately 10^3 PFU PV1 in the eluate. The samples were thoroughly mixed by shaking (10-15 minutes, 200 RPM, at room temperature). Secondary concentration methods evaluated were PEG/NaCl precipitation and skimmed-milk flocculation (5% w/v). The skimmed-milk flocculation method was also optimized to reduce sample processing time while maintaining high virus recovery.

2.2.5.1 PEG/NaCl precipitation

PEG/NaCl precipitation samples were processed as in Section 2.2.4.5.

2.2.5.2 Skimmed milk flocculation (5%)

For concentration by skimmed milk flocculation, some samples were first amended with 3.33g artificial sea salts (Sigma-Aldrich, Co., S9883, St. Louis, MO) to 100-mL eluate stock. Then 1-mL of a 5% w/v skimmed-milk solution (Oxoid, Ltd., Hands, UK) was added to the 100-mL spiked eluate stock. Next, the sample was pH adjusted to 3.0 – 4.0 using 1 M HCl to produce flocs, as described in Section 2.2.4.4. Samples were shaken (room temperature, 2 hours, 200 RPM). Samples were centrifuged (4500 x G, 30 minutes, 4ºC) and the supernatant was carefully removed. The pellets were resuspended in 1 X PBS (pH 7.4, 10 to 20 mL), vortexed (5 - 10 minutes, maximum speed) until resuspended, and assayed.

2.2.6 Skimmed-milk flocculation (5%) centrifuge speed evaluation

The same method for skimmed-milk flocculation (5%) was used as described previously in Section 2.2.5.2 with the exception of the centrifuge speed. Experiments evaluated centrifuge speeds at 3500, 4000, and 4500 x G for any effect on the PV1 recovery outcome for the skimmed-milk flocculation method.

2.2.7 Purification

Secondary concentration comparison samples in Section 2.2.5 and 2.2.6 (skimmed-milk flocculation 5% and PEG/NaCl precipitation) were purified by Vertrel XF extraction. A 1:10 ratio of Vertrel XF was added to samples. Samples were vortexed for five minutes, placed on ice for three minutes, and then vortexed for five minutes before centrifugation (15 minutes, 4ºC, 3000 x G). Directly following centrifugation, the supernatant was removed and centrifuged (15 minutes, 4ºC, 3000 x G). Finally, the supernatant was removed and assayed.

2.2.8 Viral plaque assay

Viruses were enumerated by plaque assay on 95% confluent BGMK cell monolayers using Avicel (FMC Health and Nutrition, Philadelphia, PA, USA) as overlay media (Matrosovich et al., 2006). All assays were performed in duplicate using 200 µl of relevant dilutions in 1 x PBS (pH 7.4) onto 9.5 cm^2 wells. Infected cells were incubated (37°C, 5% CO_2, 40-48 hours) and stained (2% crystal violet in 20% methanol). Plaques were counted for infectious virus enumeration.

2.2.9 Recovery Calculation

The cell culture assay involves counting plaques for infectious virus enumeration. PV1 concentration in the sample and titer was calculated using a weighted average (Equation 1). The total PV1 in the sample and titer were calculated by multiplying the final concentration and volume (Equations 2, 3). PV1 percent recovery was calculated by dividing the recovered viral PFUs from the total PV1 dosed by the seeded viral PFUs (Equation 4).

Equation 1

$$Viral_{titer} = \frac{Total\ PFU}{Total\ volume\ plated}\ x\ dilution\ factor\ to\ first\ plated\ dilution$$

$Viral_{titer}$ (PFU/mL) =PV1 concentration in final sample volume; total PFU (PFU) = plaque counts among seeded or recovered sample dilutions; total volume plated (mL) = volume plated onto BGMK cells.

Equation 2

$$PFU_{seeded} = PV1\ stock\ titer\ x\ volume\ seeded\ (x\ dilution\ factor\ as\ necessary)$$

PFU_{seeded} (PFUs) = PV1 seeded into sample; PV1 stock titer (PFU/mL); volume seeded (mL)

Equation 3

$$PFU_{recovered} = viral\ titer\ of\ sample\ x\ sample\ volume$$

$PFU_{recovered}$ (PFU) = PV1 recovered from final sample; viral titer of sample (PFU/mL); sample volume (mL)

Equation 4

$$Percent\ Recovery = \frac{PFU_{recovered}}{PFU_{seeded}} x\ 100$$

2.2.10 Statistical Analysis

Unpaired Student's t-tests were used to compare recoveries between different methods.

2.2.11 Controls

Negative controls of 1 X PBS, beef extract/glycine solution, and unseeded control volumes of eluate stocks were plated (200 µL) onto BGMK cells to ensure absence of culturable enteric viruses. A dilution series of frozen PV1 stock was assayed as a positive control and to compare seeded values with recovered values.

2.3 Results

2.3.1 Preliminary Experiment Results

Analysis of PV1 recovery from the preliminary evaluation of secondary concentration methods showed highest recovery values with PEG/NaCl precipitation and skimmed-milk flocculation (Figure 1). Average PV1 percent recoveries and 95% confidence intervals are reported for the five evaluated methods: beef extract-Celite (41.77 ± 20.66); flat disc filter (17.24 ± 9.95); InnovaPrep Concentrating Pipette for all samples (24.65 ± 24.03); skimmed-milk flocculation (1%) (78.92 ± 21.03); and PEG/NaCl precipitation (68.97 ± 33.42) (Table 1). The four InnovaPrep Concentrating Pipette experiments that processed primary concentrate eluate from influent wastewater had the lowest recoveries with an average recovery of 0.32% using Tween/Tris elution to extract PV1 from the ultrafiltration pipette material. The spiked PBS samples concentrated by the InnovaPrep Concentrating Pipette (n=4) had a 2.97% average PV1 recovery. PV1 recovery using the InnovaPrep Concentrating Pipette to process the beef extract/glycine solution samples (n=6) was higher (average 55.32%) and used 1 X PBS, pH 9.5

(n=5) and DMEM (n=1) elution for viral extraction from the pipette. The beef extract solution processed samples required up to six extractions. Each extraction uses approximately 1 – 1.5 mL of elution fluid (fluid is stored in pressurized CO_2 cans provided by InnovaPrep).

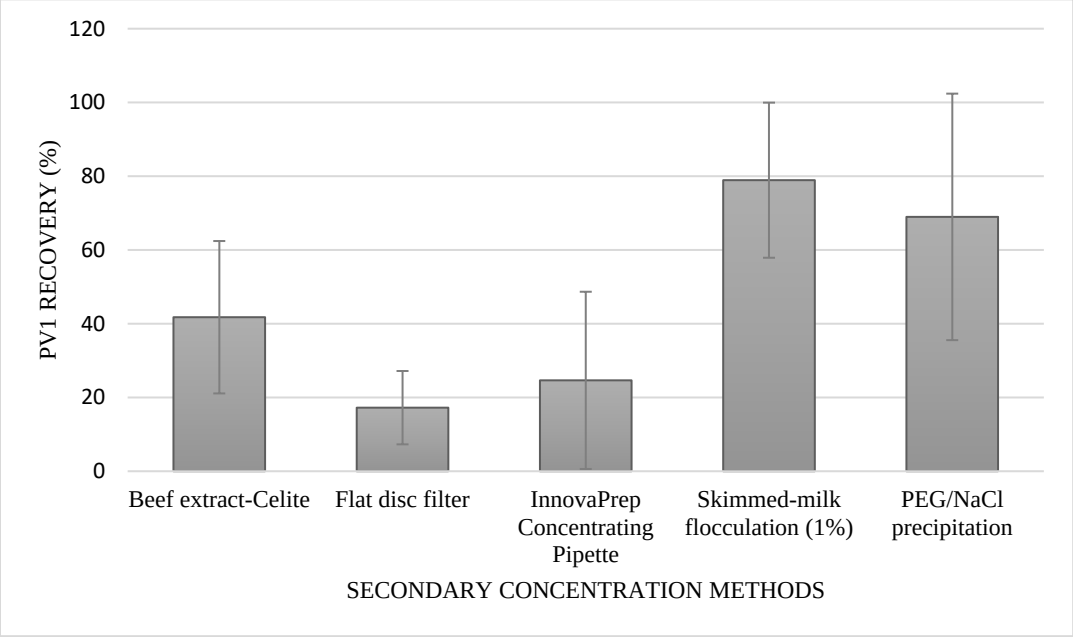

Figure 1 Average PV1 recovery for preliminary secondary concentration methods
Error bars represent 95% confidence intervals.

Table 1 PV1 recovery for preliminary secondary concentration methods

Method	**PV1 recovery**			
	n	**Mean (%)**	**95% LCL (%)**	**95% UCL (%)**
Beef extract-Celite	10	41.77	21.10	62.43
Flat disc filter	3	17.24	7.29	27.18
InnovaPrep Concentrating Pipette	14	24.65	0.62	48.68
Skimmed-milk flocculation (1%)	16	78.92	57.89	99.96
PEG/NACL precipitation	4	68.97	35.55	102.39

LCL = lower confidence limit; UCL = upper confidence limit.

In preliminary experiments, the 1% skimmed-milk flocculation method (n=16) had an average PV1 recovery of 78.92% for all shaking incubation times and temperatures. This method was performed using 4-hour and 18-hour (overnight) shaking incubation times. All 4-hour experiments were shaken at room temperature (n=6) and the 18-hour experiments were evaluated at room temperature (n=7) and 4°C (n=3). The average four hour, overnight room temperature, and overnight 4°C PV1 recoveries (%) were 115.88 ± 40.20, 51.98 ± 8.41, and 67.89 ± 19.69, respectively (Figure 2).

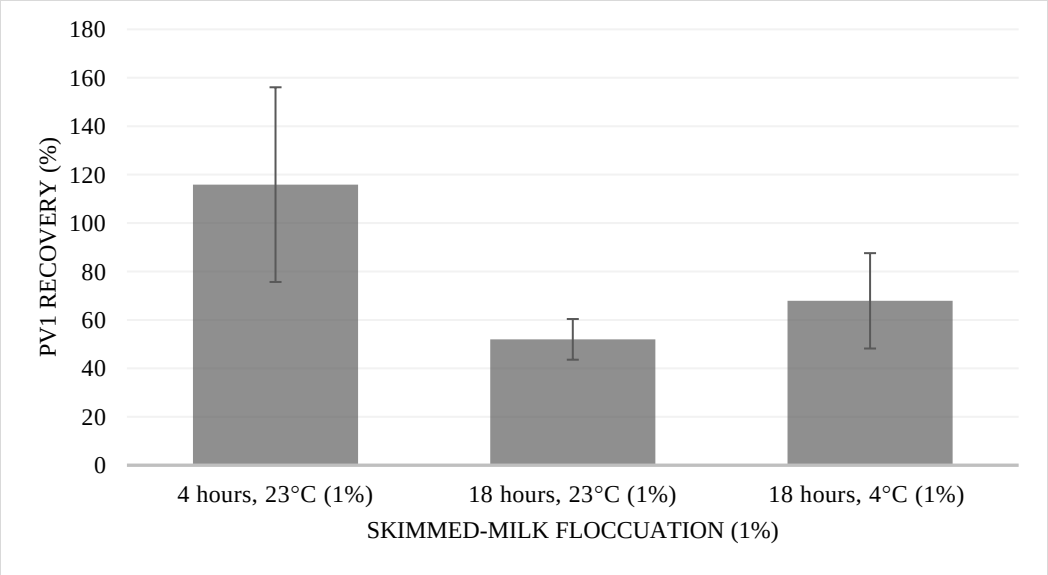

Figure 2 PV1 recovery from skimmed-milk flocculation (1%) by shake time and temperature
Error bars represent 95% confidence intervals.

There was no purification step performed for the preliminary experiments. As a result, cytotoxicity was observed in the BGMK cells for some experiments. Experiments with cytotoxicity observed in more than half of all the wells were not included in the analysis. Cytotoxicity was found present in 2 out of 10 beef extract-Celite samples (20%), 3 out of 16 skimmed-milk flocculation samples (18.75%), and three out of five PEG/NaCl precipitation samples (60%). Less than half of all the wells in the assay were cytotoxic so these results were not excluded from analysis. Two additional PEG/NaCl precipitation experiments were removed from analysis due to high cytotoxicity in the assays. The flat disc filter and InnovaPrep Concentrating Pipette samples had no observed cytotoxicity.

The preliminary results determined the beef extract-Celite, flat disc filter, and InnovaPrep Concentrating Pipette methods non-competitive. Of the five methods evaluated, they had the lowest average PV1 percent recoveries. The beef extract-Celite method required basic laboratory materials, such as a vacuum, graduated cylinder, magnetic flat disc filter holder, and filter paper

and had higher PV1 recoveries than the other two non-competitive methods. However, compared to the PEG/NaCl precipitation and skimmed-milk flocculation (1%), the recovery values were lower. Although there were only three samples processed for the flat disc filter method, the initial recovery values were the lowest of the five methods and simulating the flat disc method using vacuum filtration was challenging. Since the flat disc filter leaked, the simulation was necessary. This required manually lifting the filter paper off of the filter column and soaking it in beef extract solution. It is possible this lifting and soaking process did not accurately simulate the actual filter and may have affected recovery in the transport process. The InnovaPrep Concentrating Pipette demonstrated improved recoveries with use of PBS, pH 9.5 elution fluid, although recovery values were inconsistent and these recovery values were from beef extract solution samples, not primary concentrate eluate from filtered influent wastewater. In addition, the instrument's inability to filter more than 50 mL of sample required an added ultrafiltration pipette tip and more elution fluid per sample. Consequently, the InnovaPrep Concentrating Pipette incurs higher costs and is unable to effectively and consistently reconcentrate PV1 in samples.

2.3.2 Competitive Method Comparison Results

Since PEG/NaCl precipitation and skimmed-milk flocculation methods were deemed competitive, continued investigations compared the two methods. In a comparison of the two competitive methods, mean PV1 recovery (%) in PEG/NaCl precipitation (n=8) yielded 59.53 ± 16.04 and skimmed-milk flocculation (5%) including samples amended with sea salts and without (n=16) yielded 88.50 ± 15.18 (Figure 3). Skimmed-milk flocculation (5%) had significantly higher PV1 recoveries than PEG/NaCl precipitation (p=0.030).

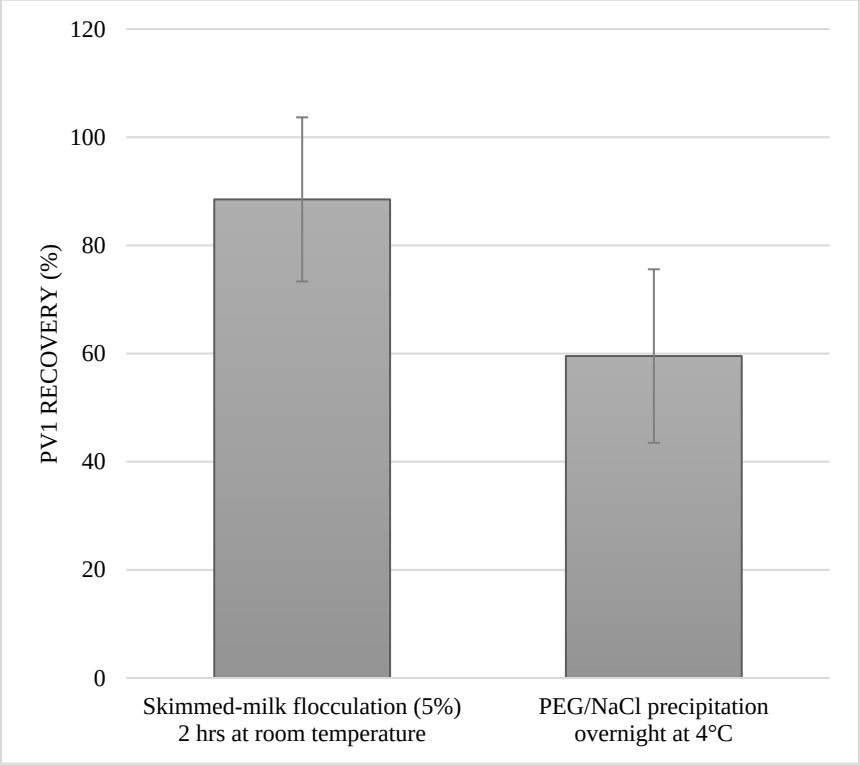

Figure 3 Comparison of competitive methods: skimmed-milk flocculation (5%) and PEG/NaCl precipitation Skimmed-milk flocculation values include experiments with and without sea salt amendments. Error bars represent 95% confidence intervals.

Further evaluating the skimmed-milk flocculation method by distinguishing between added sea salts and without showed a statistically significant (p=0.017) higher average PV1 (%)

recovery for 5% skimmed milk flocculation without the sea salt addition (n=8; 106.11 ± 20.58) than the same method with the sea salts (n=8; 70.9 ± 14.97) (Figure 4). Comparing each of these individually to PEG/NaCl precipitation, only the non-sea salt amended skimmed-milk flocculation (5%) samples were significantly higher (p=0.004).

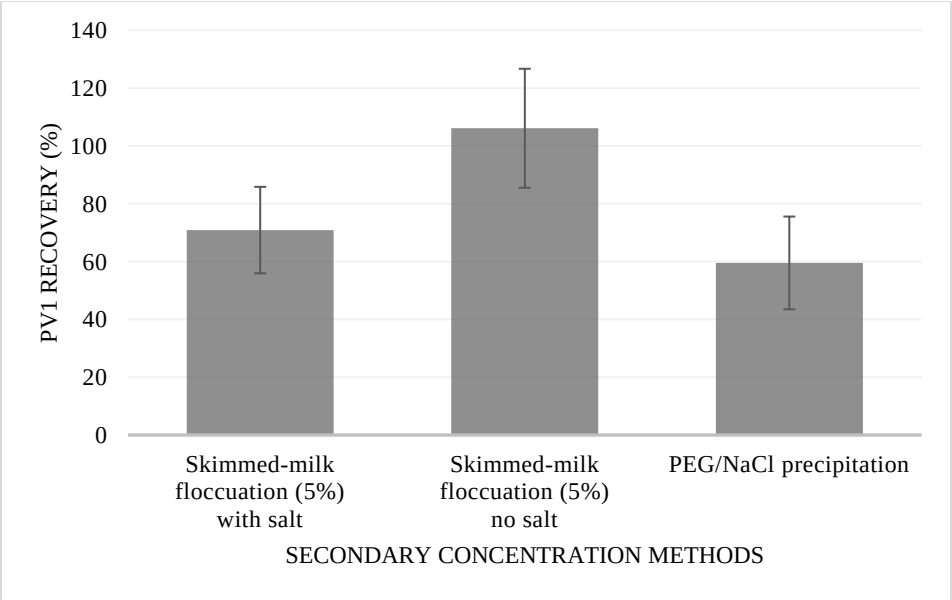

Figure 4 Comparison of competitive secondary concentration methods by sea salt amendments
Error bars represent 95% confidence intervals.

2.3.3 Skimmed-milk flocculation (5%) centrifuge speed

The 5% skimmed-milk flocculation process was further investigated by centrifuge speed to demonstrate usability in settings where centrifuges speeds are limited. Speeds were evaluated at 3500 x G, 4000 x G, and 4500 x G. Only two samples were processed for each rate of centrifugation so results, however, these experiments showed the lower speeds yielded higher PV1 recoveries. The 3500, 4000, and 4500 x G experiments showed an average PV1 (%) recovery of 120.83, 98.74, and 90.61, respectively (Figure 5). No rate of centrifugation was found to be significantly different from another ($p > 0.05$).

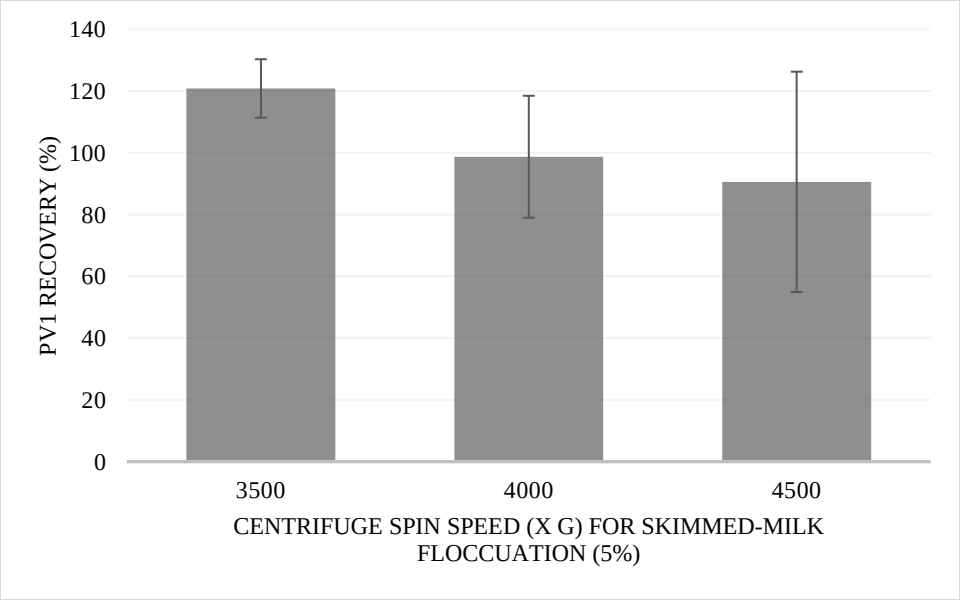

Figure 5 Skimmed-milk flocculation (5%) centrifuge speed analysis
Error bars represent 95% confidence intervals.

2.4 Discussion

Evaluation of the secondary concentration methods enabled us to identify methods with the potential to improve detection of poliovirus in wastewater samples in the field. In the preliminary laboratory investigations, we explored feasibility and viral recovery efficiency. Although the beef extract-Celite method is simple to perform and does not require refrigeration or centrifugation, the results were variable. Previous studies demonstrated Celite (diatomaceous silica) can be used as a binding agent to poliovirus (Dahling and Wright 1986a, 1988; Rhodes et al., 2011). However, Dahling and Wright (1986a) reported differences in virus recovery among different Celite and beef extract lots. We followed the Celite concentration method described by Rhodes et al. (2011) using 1.5% BBL beef extract powder (212303), Celite 577, and PBS, pH 9.0 eluent, but our water matrix differed. Rhodes et al. (2011) spiked poliovirus into tap water and we used influent wastewater as a sample matrix. Organic inhibitors in the wastewater could have

affected the poliovirus binding to the Celite. This may explain why our mean PV1 recovery was lower (41.77%) than the Rhodes et al. reported recovery from tap water (89.5%).

The flat disc filter method was developed to further concentrate PV1 using an additional adsorption-elution step. Simulating the flat disc method using vacuum filtration was challenging. Since the flat disc filter housing leaked, the simulation was necessary. This required manually lifting the filter paper off the filter column and soaking it in beef extract solution. It is possible this lifting and soaking process did not accurately simulate filter adsorption and may have affected recovery in the transport process. In addition, suspended organic particles from the influent wastewater samples could have been recovered with PV1 in the primary concentration process. Since the elution volume used in secondary concentration was only 10 mL, it is possible the presence of these particles caused preferential adsorption to the organic particles so they desorbed rather than go through the positively charged filter. Neither the flat disc filter nor InnovaPrep Concentrating Pipette methods showed cytotoxicity in the viral plaque assay. Organic inhibitors may have been trapped on the filters in each method due to low elution efficiency since neither method had high PV1 recoveries.

We did not expect the InnovaPrep Concentrating Pipette to be a cost-efficient method for small-scale sample processing because the initial investment in the instrument was $10,000. Still, we weighed the costs with the anticipated benefits of automation and rapid processing. There were limitations inherent in the instrument's automation step. After adjusting flow buffer settings for concentration, the ultrafiltration pipette tip was only able to concentrate 50 mL of sample. After 50 mL, the sample could be eluted by extraction, and another ultrafiltration pipette tip could be inserted to concentrate an additional 50 mL of sample. At the time of these experiments, ultrafiltration pipette tips cost $25 each. In addition, using two pipette tips required use of

additional elution fluid for extraction, and were available only from the manufacturer at $23 each (15 – 20 extractions per can). This also increased the processing time and reduced the likelihood of PV1 recovery because recovery depended on eluting the virus off two pipette tips for a 100 mL sample. Thus, there was greater likelihood of losing virus to the pipette tips because there is a greater surface area for the viruses to contact and there was a larger dilution factor in using two pipette tips as opposed to one. In considering field study applications, it is possible the sample volume used for secondary concentration may be greater than 100 mL and would affect cost, method efficiency, and viral recovery.

The skimmed-milk flocculation method simplified the procedure described by Calgua et al. (2008). Our adapted preliminary (1%) method reduced the total processing time to four hours and used a lower centrifuge speed for easier adoption by laboratories with lower speed centrifuges. The optimized method increased the skimmed-milk powder solution to 5% (w/v) to investigate if a two-hour shaking time would be feasible. Increasing the skimmed-milk solution was intended to increase the number of available flocs to which PV1 could bind. By comparing the results from the preliminary (1% for four hours) and optimized (5% for two hours) methods, the preliminary method appears to demonstrate a higher mean PV1 recovery (115.88%) ($p=6$) than the optimized method (88.50%) ($n=16$). The two were not significant from another ($p=0.135$). However, cytotoxicity observed in the viral plaque assay from the preliminary method may have skewed the recovery values in its favor as these samples required assaying a higher dilution factor, thereby reducing the effective volume assayed and sensitivity. The optimized method incorporated a purification step to reduce the potential for cytotoxicity in the viral plaque assay. Therefore, the results from two methods are not directly comparable. A future study may consider comparing the two concentrations at the two-hour processing time. The

addition of artificial sea salts to some samples demonstrated lower recovery for PV1, but we did not measure conductivity of the initial matrix to observe differences among salt concentrations. Since wastewater composition can vary by time and location, laboratories would have to measure conductivity regularly if salt composition were to be determined as a necessary component in future studies. This may be useful in considering improved viral detection, but we did not explore it in this study due to laboratory efficiency considerations. Future investigations in the use of sea salt in for skimmed-milk flocculation may consider evaluating the effects based on the type of sea salts used and the conductivity of each sample matrix. All of the skimmed-milk flocculation experiments used pH strips with a range 0 – 14. Thus, there was inherent pH variability in the organic flocculation step when we acidified the sample. We decided to use disposable pH strips instead of a pH meter for biosafety and feasibility purposes. Using a pH meter for this step would require a regular decontamination step and access to a pH instrument. The centrifuge speed evaluation for this method suggests reducing the centrifuge speed to 3500 x G will not lower PV1 recovery. These initial speed investigations indicate a lower speed may improve recovery. Future experiments could consider lowering the speed to evaluate viral recovery.

The PEG 8000 used in PEG/NaCl precipitation is more expensive and difficult to acquire in many countries outside of the United States. This study used PEG 8000 because it was available in the laboratory, but there may be differences observed in PEG 6000, the commonly used molecular weight elsewhere. This presents an opportunity evaluate PEG/NaCl precipitation using PEG 6000 and comparing to skimmed-milk flocculation (5%) in the future.

There were general limitations across all methods evaluated. The influent wastewater was collected from the same wastewater treatment plant, but at varying times of day across different

seasons from 2015 – 2016. Changes in sewage composition may affect the method recoveries. Though, we tried to control for this by using the same composite eluate samples for the skimmed-milk flocculation (5%) and PEG/NaCl precipitation comparison experiments. Viral aggregation and the assay methodology may explain the greater than 100% PV1 recovery values. Aggregation is common in laboratory settings when investigators spike virus to wastewater samples. We did not include a pre-filtration step of the spiked PV1 to reduce aggregation effects. There are inherent limitations to the viral plaque assay for poliovirus detection in wastewater (WHO, 2003). Dahling and Wright (1986b) optimized the BGMK cell line to provide a more effective enterovirus assay of waterborne viruses. This study used BGMK cells to enumerate poliovirus. Since the cell line is not selective for poliovirus, it is possible non-polio enteroviruses were present in the influent wastewater and were detected in the plaque assay. We assayed the unseeded eluate stocks, but since the entire sample is not used in the assay, non-polio enteroviruses could have been responsible for plaque formation in some experiments. The well plate size affects plaque-forming growth and counting can be subjective, leading to reporting errors. As noted earlier, cytotoxicity skewed the recovery values, especially when compared to plaque assay results without cytotoxicity. Despite these imperfections, compared to polymerase chain reaction (PCR), the plaque assay shows virus viability, enables a larger assay volume, and is important for understanding viral circulation. Finally, if we had assayed samples in triplicate instead of duplicate, we would have strengthened our statistical findings.

Given the high PV1 recovery achieved from the skimmed-milk flocculation (5%) method, in addition to the low cost of materials, general accessibility of laboratory equipment required, and short processing time, we recommend skimmed-milk flocculation (5%) be validated in the field for PV1 detection. When compared to PEG/NaCl precipitation, there is a

clear cost benefit in moving forward with skimmed-milk flocculation (Table 2). Lower costs and access to materials is important in any laboratory, but they are even more important in low resource settings with limited funds and supplies. Furthermore, in tropical settings, it can be difficult to keep samples cold. Since the skimmed-milk flocculation method eliminates the need for samples to be shaken at 4°C, the method is likely more feasible in these settings than PEG/NaCl precipitation. Nevertheless, it is important to recognize room temperature can be higher than 20 - 25°C in warmer climates. Therefore, laboratories in warmer climates may need to set-up a temperature chamber to maintain 20 - 25°C during the shaking process. Achieving temperature control within this range is still more feasible than at 4°C.

Table 2 Cost benefit of PEG/NaCl precipitation vs. skimmed-milk flocculation methods

Method	***Cost***	***Time***	***Standardization***	***Temperature***
PEG/NaCl precipitation	PEG is reportedly more expensive in other countries	Standard shaking time is overnight (18-hours)	Variations in molecular weight and PEG manufacturers	4°C overnight
Skimmed-milk flocculation	Skim milk powder is < 1% the cost of PEG needed per sample analyzed	Shortened shaking time to 2-hours	Variation in skimmed-milk manufacturers	Room temperature (20 - 25°C)

This study was limited in the evaluation of viral recovery using only PV1, because poliovirus type 2 and poliovirus type 3 are still circulating. A secondary concentration method for poliovirus detection should be able to detect the three serotypes. Therefore, future studies should evaluate poliovirus recovery of all three. Nevertheless, field testing is recommended in the near-term to compare with PEG/NaCl precipitation and two-phase separation.

3. Rotavirus

3.1 Introduction

Group A rotaviruses (RVs) can cause severe and fatal diarrhea in children under five years of age worldwide (Tate, 2016). The RV genome consists of 11 segments of double-stranded RNA and each encodes one protein, except segment 11 which can code for two proteins in some RV strains (Estes and Desselberger, 2012). Of these proteins, six are structural and the others are non-structural. VP4 and VP7 are two outer capsid, structural proteins that constitute a dual classification system for RV types. The VP4-specific types are termed P-types and the VP7-specific types are termed G-types (Estes and Kapikian, 2007) . Globally, human infections have been mainly caused by six rotavirus genotypes, G1P[8], G2P[4], G3P[8], G4P[8], G9P[8], and G12P[8] (Dóró et al., 2014). Circulation of rotavirus types can vary across geography, income, and season (Bowen et al., 2016; Bányai et al., 2012).

In July 2014, Kenya introduced the GlaxoSmithKline Biologicals' monovalent, live, attenuated human rotavirus vaccine, Rotarix® (G1 serotype and P[8] genotype) into the Expanded Programme of Immunization (PATH, 2016; Ruiz-Palacios, 2006). Prior to the introduction of the vaccine, G1, G8, G9, and P[8] were the dominant strains circulating in eastern Kenya (Kiulia et al., 2014). Between 2009 and 2011, G12 was detected in the country for the first time. Between 2007 and 2008 types G1, G9, G10, G11, and G12 were detected in an urban wastewater stream in Kibera, with G12 (100%) and G1 (90%) predominating (Kiulia et al., 2010). Kibera, in the Nairobi area, is recognized as the largest informal settlement in Kenya with informal structures, poor sanitation, and roaming animals (i.e. pigs, chickens, and dogs). Kibera is predominantly a refugee community, as is the Eastleigh community nearby.

The United Nations High Commissioner for Refugees (UNHCR) estimates there are 553,912 refugees in Nairobi (UNHCR, 2017). Urban refugees are often highly mobile and reluctant to seek healthcare services out of fear of deportation (Pavanello et al., 2010). This presents a challenge for surveillance of disease. In 2008, the World Health Organization (WHO) coordinated a Global Rotavirus Surveillance Network by bringing together existing surveillance networks to establish a standardized global sentinel hospital surveillance network for RV disease (Agocs et al., 2014; WHO, 2008). The data collected from the global network help document and describe rotavirus infections from children who are symptomatic and seek care. However, clinical surveillance is not able to capture RV infected individuals who are asymptomatic and others who do not utilize health services. Environmental surveillance (ES) complements clinical surveillance by RV detection in a community's wastewater. Understanding the circulating RVA strains can impact understanding of the efficacy of current RV vaccines.

Rotarix vaccination in Kenya grew from 277,675 children in 2014 to 1,017,042 in 2015 (WHO, 2016d). As a result of increasing vaccination coverage, genotyping circulating rotavirus is useful to evaluate vaccine-associated changes in genotype distribution within communities. This study sought to characterize the RVA strains in circulation in the environment in three urban communities in Nairobi following the introduction of the Rotarix vaccine in Kenya.

3.2. Methods

3.2.1 Water Samples

From April 2015 to September 2015, water samples (n=55) were collected, at approximately two week intervals, from four sampling locations in Nairobi, Kenya. The sites included two sewer sample sites (Eastleigh A and Eastleigh B) from a neighborhood that

predominantly houses a refugee community, a fecally contaminated stream running through the large informal settlement, Kibera, and a latrine waste stream located in the informal settlement of Mathare (Starehe).

3.2.2 Concentration

Samples were processed and viruses were recovered using the newly developed bag-mediation filtration system (BMFS). Briefly, using the BMFS, 2.9 ± 0.4 L of water samples were filtered through positively-charged ViroCap filters (Scientific Methods, Inc., Granger, IN). Viruses were eluted from the filters using a 1.5% beef extract (BBL^{TM} Beef Extract powder; Becton, Dickinson and Company, Sparks, MD) 0.05 M glycine (Merck KGaA,, Darmstadt, Germany), buffer pH 9.5 with a 30 minute filter contact time before being eluted using a peristaltic pump (Fagnant et al., 2014). Viruses were further concentrated by PEG/NaCl precipitation (14% (w/v) PEG 6000 [Merck] and 0.2 M NaCl [Merck]) to a final volume of 10mL in 1 X PBS (pH 7.4) (Sigma-Aldrich Co., St. Louis, MO) (Fagnant et al., 2014).

3.2.3 Genetic detection of RVAs

3.2.3.1 Nucleic acid extraction

Nucleic acid was extracted from 1-mL virus concentrate using the semi-automated Nucli-SENS® EasyMAG® instrument (BioMérieux, Marcy l'Etoile, France) as per manufacturer's protocol. Prior to nucleic acid extraction, 10 µL of mengovirus ((1 x 10^5 genome copies [gc])/10 µL) was added to each sample as an extraction control (Le Guyader et al., 2009). The extraction efficiency was determined by comparison of the cycle threshold (Ct) of the initial input versus that of the sample.

3.2.3.2 Detection

Mengovirus (5-µL extracted nucleic acid) was detected qualitatively by real-time reverse transcription-polymerase chain reaction (RT-PCR) using a commercial kit (mengo@ceeramTools™ Kit, Ceeram s.a.s, La Chappelle-Sur-Erdre, France). Rotavirus (5-µL extracted nucleic acid) was detected qualitatively using a commercial kit with an internal amplification control (rotavirus@ceeramTools™ Kit, Ceeram s.a.s, La Chappell-Sur-Erdre, France) as per manufacturer's instructions.

3.2.3.3 Genotyping

Molecular methods developed for typing RVs from clinical specimens were applied for the characterization of the VP7 and VP4 genes from the environmental RVA strains. Rotavirus strains were typed by semi-nested RT-PCR methods with primers specific for regions of the genes encoding the VP7 (G-type) and VP4 (P-type) proteins (Appendix A) (WHO, 2009).

3.2.3.3.1 First Step Amplification

Unless stated to the contrary, all reagents were supplied by Promega Corp. Madison, WI. In brief, the first round reaction (primary amplification): Enzyme reverse transcriptase and gene-specific primers (VP7 primers: sBeg9 and End9; VP4 primers: con2 and con3) were used in the first step amplification to convert viral RNA to cDNA. Each 10 pmol primer was mixed with 10 µl of extracted double-stranded RNA, heated for 2 minutes at 95°C, transferred to ice, and then 8 µl of reverse transcriptase (RT) mix was added and incubated for 60 minutes at 42°C. The following RT mix was used: 10 mM deoxynucleotide triphosphates, Protoscript II RT Reaction Buffer 5X, 0.1 M 10X DTT (New England Biolabs Inc., Ipswich, MA), Protector RNase

inhibitor – cDNA Synthesis Transcriptor 1st strand (Roche Diagnostics GmbH, Mannheim, Germany), and Protoscript II RT (New England Biolabs Inc.). The cDNA was amplified by *Taq* polymerase by adding 10 µl of the cDNA products to 40 µl of the following PCR mix: 10 mM deoxynucletide triphosphates, 5X Go *Taq* Flexi buffer, 25 mM $MgCl_2$, and Go *Taq* G2 Flexi DNA polymerase. Initial denaturation for 1 minute at 95°C was followed by 35 cycles of 1 minute at 95°C, 1 minute at 42°C, and 1 minute at 72°C, with a final extension step of 7 minutes at 72°C.

3.2.3.3.2 Nested and Genotyping

The second nested PCR typing step utilized G- and P-specific primers to generate amplicons to be analyzed based on size. The RT-PCR products were used as a template for a seminested PCR using VP7 primers 9con1 and EndA. An additional seminested multiplex PCR was subsequently applied by using primers specific for genotype regions of the VP7 (Gouvea et al., 1994; Iturriza-Gomara, et al., 2004) and VP4 (Gentsch et al., 1992) genes. Seminested and typing multiplex reactions were done using the following PCR mix: 10 mM deoxynuclelotide triphosphates, 5 x Go *Taq* Flexi Buffer (Promega), 25 mM $MgCl_2$ (Promega), 1 µl of each 10pmol primer, and Go *Taq* G2 Flexi DNA polymerase (Promega). Initial denaturation for 1 minute at 95°C was followed by 35 cycles of 1 minute at 95°C, 1 minute at 42°C, and 1 minute at 72°C, with a final extension step of 7 minutes at 72°C. Finally, 10 µl of the nested RT-PCR products were separated on a 1.5% agarose gel using a 100-bp DNA marker and visualized by ethidium bromide staining.

3.3 Results

Analysis by real-time RT-PCR showed 89% (49/55) of the samples positive for RVA. Nested RT-PCR detected prevalent RVA genotypes G1 (73%), G3 (63%), G8 (16%), G9 (51%), G10 (6%), G12 (8%), P[4] (6%), P[6] (4%), P[8] (90%), and untypeable (3%) (Table 3). G1, G3, G9, and P[8] predominated. The fecally-contaminated stream and sewer sites shared the detected G-types and P[8]. The P[4] and P[6] types were only detected in the stream sites (Kibera and Starehe).

Table 3 Rotavirus genotype prevalence detected from stream or sewer samples in Nairobi, Kenya

	Sampling Site				***Site Type***		***Total***
	Eastleigh A	**Eastleigh B**	**Kibera**	**Starehe**	**Stream**	**Sewer**	**All Sites**
G types	n=10	n=11	n=15	n=13	n=28	n=21	n=49
G1	8 (80%)	7 (64%)	11 (73%)	10 (77%)	21 (75%)	15 (71%)	36 (73%)
G3	8 (80%)	7 (64%)	6 (40%)	10 (77%)	16 (57%)	15 (71%)	31 (63%)
G8	2 (20%)	2 (18%)	2 (13%)	2 (15%)	4 (14%)	4 (19%)	8 (16%)
G9	6 (60%)	4 (36%)	9 (60%)	6 (46%)	15 (54%)	10 (48%)	25 (51%)
G10	1 (10%)	0 (0%)	1 (7%)	1 (8%)	2 (7%)	1 (5%)	3 (6%)
G12	1 (10%)	1 (9%)	1 (7%)	1 (8%)	2 (7%)	2 (10%)	4 (8%)
Untypeable	0 (0%)	0 (0%)	2 (13%)	0 (0%)	2 (7%)	0 (0%)	2 (4%)
P types	n=10	n=11	n=15	n=13	n=28	n=21	n=49
P[4]	0 (0%)	0 (0%)	2 (13%)	1 (8%)	3 (11%)	0 (0%)	3 (6%)
P[6]	0 (0%)	0 (0%)	2 (13%)	0 (0%)	2 (8%)	0 (0%)	2 (4%)
P[8]	10 (100%)	11 (100%)	11 (73%)	12 (92%)	23 (82%)	21 (100%)	44 (90%)
Untypeable	0 (0%)	0 (0%)	1 (7%)	0 (0%)	1 (4%)	0 (0%)	1 (2%)

Also analyzed for G2, G4, P[9], P[10], P[11], and P[14] types

3.4 Discussion

We were able to characterize RVA strains from wastewater samples in selected Nairobi sites and provide an epidemiological baseline of RVA strain circulation in the environment. The results inform circulation for the representative population. Since the study's sampling sites are not representative of Nairobi's entire population, it is possible there are correlating changes in strain diversity in communities with high Rotarix coverage.

There was high detection of RVA in these communities. Vaccination coverage in these specific communities is unknown. In general, immigrant populations at sites contribute to low

vaccination rates. We suspect rotavirus vaccination coverage at these four sample sites was low in 2015, because total coverage across Kenya was only 19% the first year following the vaccine introduction (WHO, 2016d). As vaccination coverage in Kenya increases over time, it is useful to compare site specific coverage and compare to the circulating strains. Since these sites represent predominantly refugee communities with poor sanitation, population dynamics likely plays a role in continued circulation, in addition to vaccination status.

Prior to Rotarix introduction in Kenya, dominant strains were G1, G8, and G9 (Kilulia et al., 2014; Agutu et al., 2017; Page et al., 2010). Prevalence of these serotypes in the sample communities is consistent with pre-vaccine reports. The emergence of G12 in Kenya was first detected in Kenya during a 2009-2010 surveillance period and corresponds to detection of G12 at all four sites (Kiulia et al., 2014). G3 was briefly predominant in Kenya from 1999-2000, but reported prevalence prior to Rotarix introduction had declined (Kiulia et al., 2008). This study observed G3 consistently detected in samples and supports G3 emergence detected in a Nairobi hospital study in 2012 (Agutu et al., 2017).

In sub-Saharan Africa, G2 is routinely detected with G1, although in Kenya G2 has only been detected at low levels and infrequently (Mwenda et al., 2010). Our findings are consistent with the previous findings in Kenya, as we did not detect G2 in any samples (Agutu et al., 2017; Nyangao et al., 2010; Kiulia et al., 2014). Surveillance in communities after the introduction of the Rotarix vaccine has detected G2P[4] genotypes at a higher prevalence compared to previous strain diversity (Kirkwood et al., 2011). Since we suspect low vaccination coverage in the communities represented by our samples, it makes sense these strains were not prevalent.

Predominance of the Rotarix vaccine strains, G1 and P[8], in our study may be indicative of the Rotarix vaccine, but results are limited by non-specific vaccine genotyping methods. Given

the pre-vaccine observations of dominant G1 and P[8] genotypes and low vaccination coverage, we do not expect these strains are vaccine-related.

The diversity of RVA genotypes, including animal type G10, may be explained by frequent mobility of people in these communities, poor sanitation, and close contact with domesticated animals. Therefore, continuous surveillance of mammalian and human RVA strains is important to understand strain shifts and the risk for direct interspecies transmission events.

Rotarix has demonstrated efficacy against severe rotavirus gastroenteritis caused by unrelated serotypes (Ward et al., 2009, Correia et al., 2010; Steele et al., 2012; Linhares et al. 2008). If vaccination coverage is low across the study populations, we expect increased strain diversity with increased immunization coverage. Still, there are programmatic and social challenges to increase coverage in these communities (Cherian et al., 2012). In order to better understand vaccination coverage and effects among refugee predominant populations, environmental surveillance will be a useful tool to monitor for strain changes and vaccine efficacy.

3.5 Conclusion

The prevalence of non-vaccine strains may be explained by site population dynamics and poor environmental conditions. Pre-vaccine data from wastewater and clinical specimens highlight the circulation of non-vaccine RV strains within these areas in Kenya. Serotype G3 had not previously been detected in Nairobi, but these results demonstrate it has emerged as a dominant circulating strain. Compared to clinical specimens, wastewater provides a more accurate representation of the RVA strains circulating in a community. Environmental samples can also indicate emerging strains before the presentation of disease. Therefore, ongoing

environmental strain surveillance is needed to assess the effect of RV vaccination, especially in impoverished areas with a large migrant population.

References

1. Abbaszadegan, M., Huber, M.S., Gerba, C.P., and Pepper, I.L. (1993) Detection of enteroviruses in groundwater with the polymerase chain reaction. *Applied and Environmental Microbiology* **59**: 1318.
2. Agocs, M.M., Serhan, F., Yen, C., Mwenda, J.M., de Oliveira, L.H., Teleb, N. et al. (2014) WHO Global Rotavirus Surveillance Network: a strategic review of the first 5 years, 2008-2012. In, p. 634.
3. Agutu, M.-T., Ongus, J., Kombich, J., Kamenwa, R., Nyangao, J., Kagira, J. et al. (2017) Prevalence and genetic diversity of rotavirus infection in children with acute gastroenteritis in a hospital setting, Nairobi Kenya in post vaccination era: a cross- sectional study. *The Pan African Medical Journal* **26**.
4. Asghar, H., Diop, O.M., Weldegebriel, G., Malik, F., Shetty, S., El Bassioni, L. et al. (2014) Environmental surveillance for polioviruses in the Global Polio Eradication Initiative. *The Journal of infectious diseases* **210 Suppl 1**: S294.
5. Blacklow, N.R., and Greenberg, H.B. (1991) Viral Gastroenteritis. *The New England Journal of Medicine* **325**: 252-264.
6. Bowen, M.D., Mijatovic-Rustempasic, S., Esona, M.D., Teel, E.N., Gautam, R., Sturgeon, M. et al. (2016) Rotavirus Strain Trends During the Postlicensure Vaccine Era: United States, 2008– 2013. *The Journal of Infectious Diseases* **214**: 732-738.
7. Burns, C.C., Diop, O.M., Sutter, R.W., and Kew, O.M. (2014) Vaccine- derived polioviruses. *The Journal of infectious diseases* **210 Suppl 1**: S283.
8. Bányai, K., László, B., Duque, J., Steele, A.D., Nelson, E.A.S., Gentsch, J.R., and Parashar, U.D. (2012) Systematic review of regional and temporal trends in global rotavirus strain diversity in the pre rotavirus vaccine era: Insights for understanding the impact of rotavirus vaccination programs. *Vaccine* **30**: A122-A130.
9. Calgua, B., Mengewein, A., Grunert, A., Bofill-Mas, S., Clemente-Casares, P., Hundesa, A. et al. (2008) Development and application of a one-step low cost procedure to concentrate viruses from seawater samples. *Journal of Virological Methods* **153**: 79-83.
10. Cherian, T., Wang, S., and Mantel, C. (2012) Rotavirus vaccines in developing countries: The potential impact, implementation challenges, and remaining questions. *Vaccine* **30**: A3-A6.
11. Cliver, D.O., and Yeatman, J. (1965) ULTRACENTRIFUGATION IN THE CONCENTRATION AND DETECTION OF ENTEROVIRUSES. *Applied microbiology* **13**: 387.
12. Correia, J.B., Patel, M.M., Nakagomi, O., Montenegro, F.M.U., Germano, E.M., Correia, N.B. et al. (2010) Effectiveness of monovalent rotavirus vaccine (Rotarix) against severe diarrhea caused by serotypically unrelated G2P4 strains in Brazil. *The Journal of infectious diseases* **201**: 363.

13. Dahling, D.R., and Wright, B.A. (1986a) Optimization of the BGM cell line culture and viral assay procedures for monitoring viruses in the environment. *Applied and Environmental Microbiology* **51**: 790.

14. Dahling, D.R., and Wright, B.A. (1986b) Recovery of viruses from water by a modified flocculation procedure for second-step concentration. *Applied and Environmental Microbiology* **51**: 1326.

15. Dunn, W.N. (2008) *Public policy analysis : an introduction*. Upper Saddle River, N.J.: Upper Saddle River, N.J. : Pearson Prentice Hall.

16. Dóró, R., László, B., Martella, V., Leshem, E., Gentsch, J., Parashar, U., and Bányai, K. (2014) Review of global rotavirus strain prevalence data from six years post vaccine licensure surveillance: Is there evidence of strain selection from vaccine pressure? *Infection, Genetics and Evolution* **28**: 446-461.

17. Estes, M., Kapikian, A.Z. (2007). Rotaviruses. In: Fields Virology, fifth ed. Lippincott Williams & Wilkins, Philadelphia.

18. Estes, M.K., and Desselberger, U. (2012) Rotaviruses: cause of vaccine- preventable disease yet many fundamental questions remain to be explored. In, pp. 369-372.

19. Fagnant, C., Beck, N., Yang, M.-F., Barnes, K., Boyle, D., and Meschke, J. (2014) Development of a novel bag-mediated filtration system for environmental recovery of poliovirus. *Journal of Water and Health* **12**: 747-754.

20. Fields, B.N., Knipe, D.M., and Howley, P.M. (2007) *Fields' virology*. Philadelphia: Philadelphia : Wolters Kluwer Health/Lippincott Williams & Wilkins.

21. Fong, T.-T., and Lipp, E.K. (2005) Enteric Viruses of Humans and Animals in Aquatic Environments: Health Risks, Detection, and Potential Water Quality Assessment Tools. *Microbiology and Molecular Biology Reviews* **69**: 357.

22. Gartner, H. (1967). Retention and recovery of polioviruses on a soluble ultrafilter. In G. Berg (Ed.), *Transmission of viruses by the water route.* (pp. 121-127). London. Interscience.

23. GPEI. (2015). Guidelines on environmental surveillance for detection of polioviruses. Global Polio Eradication Initiative. World Health Organization, Geneva, Switzerland. Available at: http://polioeradication.org/wp-content/uploads/2016/07/GPLN_GuidelinesES_April2015.pdf.

24. GPEI. (2017). Vaccine-derived poliovirus. Accessed 4 June 2017. http://polioeradication.org/polio-today/polio-prevention/the-virus/vaccine-derived-polio-viruses/.

25. Gentsch, J.R., Glass, R.I., Woods, P., Gouvea, V., Gorziglia, M., Flores, J. et al. (1992) Identification of group A rotavirus gene 4 types by polymerase chain reaction. *Journal of Clinical Microbiology* **30**: 1365.

26. Gerba, C.P., Rose, J.B., and Haas, C.N. (1996) Sensitive populations: who is at the greatest risk? *International Journal of Food Microbiology* **30**: 113-123.

27. Gouvea, V., Santos, N., and Timenetsky, M.d.C. (1994) Identification of bovine and porcine rotavirus G types by PCR. *Journal of Clinical Microbiology* **32**: 1338.

28. Grabow, W. (1968) The virology of waste- water treatment. *Water Research* **2**: 675-701.

29. Hagan, J.E., Wassilak, S.G.F., Craig, A.S., Tangermann, R.H., Diop, O.M., Burns, C.C., and Quddus, A. (2015) Progress toward polio eradication-- worldwide, 2014- 2015. *Morbidity and Mortality Weekly Report* **64**: 527.

30. Hazam, R., Singla, R., Kishore, J., Singh, S., Gupta, R., and Kar, P. (2010) Surveillance of hepatitis E virus in sewage and drinking water in a resettlement colony of Delhi: what has been the experience? *Arch Virol* **155**: 1227-1233.

31. Hellmer, M., Paxeus, N., Magnius, L., Enache, L., Arnholm, B., Johansson, A. et al. (2014) Detection of Pathogenic Viruses in Sewage Provided Early Warnings of Hepatitis A Virus and Norovirus Outbreaks. *Applied And Environmental Microbiology* **80**: 6771-6781.

32. Hill, W., Akin, E., and Benton, W. (1971) Detection of viruses in water: a review of methods and application. *Water Research* **5**: 967-995.

33. Hovi, T., Shulman, L.M., Van Der Avoort, H., Deshpande, J., Roivainen, M., and De Gourville, E.M. (2012) Role of environmental poliovirus surveillance in global polio eradication and beyond. *Epidemiology and Infection* **140**: 1-13.

34. Hovi, T., Huovilainen, A., Kuronen, T., Pöyry, T., Salama, N., Cantell, K. et al. (1986) OUTBREAK OF PARALYTIC POLIOMYELITIS IN FINLAND: WIDESPREAD CIRCULATION OF ANTIGENICALLY ALTERED POLIOVIRUS TYPE 3 IN A VACCINATED POPULATION. *The Lancet* **327**: 1427-1432.

35. Ikner, L., Gerba, C., and Bright, K. (2012) Concentration and Recovery of Viruses from Water: A Comprehensive Review. *Food Environ Virol* **4**: 41-67.

36. Iturriza-Gómara, M., Kang, G., and Gray, J. (2004) Rotavirus genotyping: keeping up with an evolving population of human rotaviruses. *Journal of Clinical Virology* **31**: 259-265.

37. Jorba, J., Diop, O.M., Iber, J., Sutter, R.W., Wassilak, S.G., and Burns, C.C. (2016) Update on vaccine- derived polioviruses- worldwide, January 2015- May 2016. *Morbidity and Mortality Weekly Report* **65**: 763-769.

38. Karim, M.R., Rhodes, E.R., Brinkman, N., Wymer, L., and Fout, G.S. (2009) New Electropositive Filter for Concentrating Enteroviruses and Noroviruses from Large Volumes of Water. *Applied and Environmental Microbiology* **75**: 2393.

39. Kirkwood, C.D., Boniface, K., Barnes, G., and Bishop, R. (2011) Distribution of Rotavirus Genotypes After Introduction of Rotavirus Vaccines, Rotarix (R) and RotaTeq (R), into the National Immunization Program of Australia. *Pediatric Infectious Disease Journal* **30**: S48-S53.

40. Kiulia, N.M., Kamenwa, R., Irimu, G., Nyangao, J.O., Gatheru, Z., Nyachieo, A. et al. (2008) The Epidemiology of Human Rotavirus Associated with Diarrhoea in Kenyan Children: A Review. *Journal of Tropical Pediatrics* **54**: 401-405.

41. Kiulia, N.M., Netshikweta, R., Page, N.A., Van Zyl, W.B., Kiraithe, M.M., Nyachieo, A. et al. (2010) The detection of enteric viruses in selected urban and rural river water and sewage in Kenya, with special reference to rotaviruses. *Journal of Applied Microbiology* **109**: 818-828.

42. Kiulia, N.M., Nyachieo, A., Nyaga, M.M., Seheri, M.L., Wolfaardt, M., Van Zyl, W.B. et al. (2014) Rotavirus G and P types circulating in the eastern region of Kenya: Predominance of G9 and emergence of G12 genotypes. *Pediatric Infectious Disease Journal* **33**: S85-S88.

43. Le Guyader, F.S., Parnaudeau, S., Schaeffer, J., Bosch, A., Loisy, F., Pommepuy, M., and Atmar, R.L. (2009) Detection and Quantification of Noroviruses in Shellfish. *Applied and Environmental Microbiology* **75**: 618.

44. Leclerc, H., Schwartzbrod, L., and Dei-Cas, E. (2002) Microbial agents associated with waterborne diseases. In *Crit Rev Microbiol*, pp. 371-409.

45. Linhares, A.C., Velázquez, F.R., Pérez-Schael, I., Sáez-Llorens, X., Abate, H., Espinoza, F. et al. (2008) Efficacy and safety of an oral live attenuated human rotavirus vaccine against rotavirus gastroenteritis during the first 2 years of life in Latin American infants: a randomised, double- blind, placebo- controlled phase III study. *The Lancet* **371**: 1181-1189.

46. Lodder, W.J., Buisman, A.M., Rutjes, S.A., Heijne, J.C., Teunis, P.F., and de Roda Husman, A.M. (2012) Feasibility of Quantitative Environmental Surveillance in Poliovirus Eradication Strategies. *Applied and Environmental Microbiology* **78**: 3800.

47. Lu, J., Zheng, H., Guo, X., Zhang, Y., Li, H., Liu, L. et al. (2015) Elucidation of Echovirus 30's Origin and Transmission during the 2012 Aseptic Meningitis Outbreak in Guangdong, China, through Continuing Environmental Surveillance. *Applied And Environmental Microbiology* **81**: 2311-2319.

48. Mangal, T.D., Aylward, R.B., and Grassly, N.C. (2013) The Potential Impact of Routine Immunization with Inactivated Poliovirus Vaccine on Wild-type or Vaccine-derived Poliovirus Outbreaks in a Posteradication Setting. *American Journal of Epidemiology* **178**: 1579-1587.

49. Manor, Y., Shulman, L., Kaliner, E., Hindiyeh, M., Ram, D., Sofer, D. et al. (2014) INTENSIFIED ENVIRONMENTAL SURVEILLANCE SUPPORTING THE RESPONSE TO WILD POLIOVIRUS TYPE 1 SILENT CIRCULATION IN ISRAEL, 2013. *Eurosurveillance* **19**: 1.

50. Melnick, J.L. (1996) Current status of poliovirus infections. *Clinical Microbiology Reviews* **9**: 293.

51. Minor, Philip D. (2016). "Chapter 1. An Introduction to Poliovirus: Pathogenesis, Vaccination, and the Endgame for Global Eradication." *Poliovirus: Methods and Protocols*. Ed. Javier Martin. New York: Methods in Molecular Biology. vol. 1387, DOI 10.1007/978-1-4939-3292-4_1.

52. Mwenda, J.M., Ntoto, K.M., Abebe, A., Enweronu-Laryea, C., Amina, I., McHomvu, J. et al. (2010) Burden and epidemiology of rotavirus diarrhea in selected African countries: preliminary results from the African Rotavirus Surveillance Network. *The Journal of infectious diseases* **202 Suppl**: S5.

53. Nupen, E.M. (1970) Virus studies on the windhoek waste- water reclamation plant (South-West Africa). *Water Research* **4**: 661-672.

54. Nyangao, J., Page, N., Esona, M., Peenze, I., Gatheru, Z., Tukei, P., and Steele, A.D. (2010) Characterization of human rotavirus strains from children with diarrhea in Nairobi and

Kisumu, Kenya, between 2000 and 2002. *The Journal of infectious diseases* **202 Suppl**: S187.

55. Onorato, I.M., Modlin, J.F., McBean, A.M., Thoms, M.L., Losonsky, G.A., and Bernier, R.H. (1991) Mucosal immunity induced by enhance- potency inactivated and oral polio vaccines. *The Journal of infectious diseases* **163**: 1.

56. Page, N., Esona, M., Armah, G., Nyangao, J., Mwenda, J., Sebunya, T. et al. (2010) Emergence and characterization of serotype G9 rotavirus strains from Africa. *The Journal of infectious diseases* **202 Suppl**: S55.

57. PATH. (2016). Rotavirus vaccine country introductions: maps and list. Seattle, WA. Accessed 2 March 2017. http://www.path.org/vaccineresources/details.php?i=2235.

58. Pavanello, S., Elhawary, S., and Pantuliano, S. (2010). "Hidden and Exposed: Urban Refugees in Nairobi, Kenya." Working Paper. Overseas Development Institute. Available at: https://www.odi.org/publications/4786-hidden-and-exposedurban-refugees-nairobi-kenya.

59. Pellegrinelli, L., Bubba, L., Primache, V., Pariani, E., Battistone, A., Delogu, R. et al. (2016) Surveillance of poliomyelitis in Northern Italy: results of Acute Flaccid Paralysis Surveillance and Environmental Surveillance, 2012-2015. *Human vaccines & immunotherapeutics*: 0.

60. Petrinca, A.R., Donia, D., Pierangeli, A., Gabrieli, R., Degener, A.M., Bonanni, E. et al. (2009) Presence and environmental circulation of enteric viruses in three different wastewater treatment plants. *Journal of Applied Microbiology* **106**: 1608-1617.

61. Rao, N.U., and Labzoffsky, N.A. (1969) A simple method for the detection of low concentration of viruses in large volumes of water by the membrane filter technique. *Canadian Journal of Microbiology* **15**: 399-403.

62. Rhodes, E.R., Hamilton, D.W., See, M.J., and Wymer, L. (2011) Evaluation of hollow-fiber ultrafiltration primary concentration of pathogens and secondary concentration of viruses from water. *Journal of Virological Methods* **176**: 38-45.

63. Ruiz-Palacios, G.M., Pérez-Schael, I., Velázquez, F.R., Abate, H., Breuer, T., Clemens, S.C. et al. (2006) Safety and Efficacy of an Attenuated Vaccine against Severe Rotavirus Gastroenteritis. *The New England Journal of Medicine* **354**: 11-22.

64. Sabin, A.B. (1985) Oral Poliovirus Vaccine: History of Its Development and Use and Current Challenge to Eliminate Poliomyelitis from the World. *The Journal of Infectious Diseases* **151**: 420-436.

65. Salim, A.F.M., Phillips, A.D., and Farthing, M.J.G. (1990) Pathogenesis of gut virus infection. *Bailliere's Clinical Gastroenterology* **4**: 593-607.

66. Salk, J.E., Krech, U., Youngner, J.S., Bennett, B.L., Lewis, L.J., and Bazeley, P.L. (1954) Formaldehyde treatment and safety testing of experimental poliomyelitis vaccines. *American journal of public health and the nation's health* **44**: 563.

67. Shaghaghi, M., Shahmahmoodi, S., Abolhassani, H., Soleyman-Jahi, S., Parvaneh, L., Mahmoudi, S. et al. (2016) Vaccine- Derived Polioviruses and Children with Primary Immunodeficiency, Iran, 1995-2014. *Emerging Infectious Diseases* **22**: 1712-1719.

68. Shuval, H.J., Cymbalista, S., Fatal, B., & Goldblum, N. (1967). Concentration of enteric viruses in water by hydro-extraction and two phase separation. In G. Berg (Ed.), *Transmission of viruses by the water route* (pp. 45-55). London. Interscience.

69. Shuval, H.J., Fattal, B., Cymbalista, S., & Goldblum, N. (1969). The phase-separation method for the concentration and detection of viruses in water. *Water Research*, 3, 225-240.

70. Sobsey, M.D., Carrick, R.J., and Jensen, H.R. (1978) Improved methods for detecting enteric viruses in oysters. *Applied and Environmental Microbiology* **36**: 121.

71. Steele, A., Neuzil, K., Cunliffe, N., Madhi, S., Bos, P., Ngwira, B. et al. (2012) Human rotavirus vaccine Rotarix (TM) provides protection against diverse circulating rotavirus strains in African infants: a randomized controlled trial. *BMC Infectious Diseases* **12**: 213.

72. Tate, J.E., Burton, A.H., Boschi-Pinto, C., Parashar, U.D., Agocs, M., Serhan, F. et al. (2016) Global, Regional, and National Estimates of Rotavirus Mortality in Children <5 Years of Age, 2000–2013. *Clinical Infectious Diseases* **62**: S96-S105.

73. Tebbens, R.J.D., Pallansch, M.A., Kew, O.M., Cáceres, V.M., Jafari, H., Cochi, S.L. et al. (2006) Risks of Paralytic Disease Due to Wild or Vaccine- Derived Poliovirus After Eradication. *Risk Analysis* **26**: 1471-1505.

74. Tebbens, R.J.D., Pallansch, M.A., Chumakov, K.M., Halsey, N.A., Hovi, T., Minor, P.D. et al. (2013) Expert Review on Poliovirus Immunity and Transmission. *Risk Analysis* **33**: 544-605.

75. UNHCR. (2017). Population Statistics. Available at: http://popstats.unhcr.org/en/overview#_ga=1.265253199.1776679616.1486168248.

76. Wallis, C., Henderson, M., and Melnick, J.L. (1972) Enterovirus concentration on cellulose membranes. *Applied microbiology* **23**: 476.

77. Ward, R.L., and Bernstein, D.I. (2009) Rotarix: a rotavirus vaccine for the world. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **48**: 222.

78. WHO. (2003). Guidelines for environmental surveillance of poliovirus circulation. Geneva. 2003. Available at: http://apps.who.int/iris/bitstream/10665/67854/1/WHO_V-B_03.03_eng.pdf.

79. WHO. (2008). Generic protocol for monitoring impact of rotavirus vaccination on gastroenteritis disease burden and viral strains. Geneva. Available at: http://apps.who.int/iris/handle/10665/69913.

80. WHO. (2009). *Manual of Rotavirus Detection and Characterization Methods*. Department of Immunization, Biologicals, Vaccines. 156 Pp, 2009. Available at: http://www.who.int/immunization/monitoring_surveillance/burden/vpd/surveillance_type/sentinel/WHO_IVB_08.17_eng.pdf?ua=1.

81. WHO. (2013). Polio eradication & endgame strategic plan 2013-2018. Available at: http://polioeradication.org/wp-content/uploads/2016/07/PEESP_EN_A4.pdf.

82. WHO. (2016a). GPEI Annual Report 2015. Available at: http://polioeradication.org/tools-and-library/policy-reports/annual-reports/.

83. WHO. (2016b). Governance. *GPEI.* Accessed 16 Nov. 2016. http://polioeradication.org/who-we-are/governance/.

84. WHO. (2016c). Semi-annual status report: January to June 2016. Available at: http://polioeradication.org/wp-content/uploads/2016/10/Status-Report_Jan-Jun2016.pdf.

85. WHO. (2016d). Vaccines and Biologicals. WHO/UNICEF coverage estimates for 1980-2015. 15 July 2016. http://apps.who.int/immunization_monitoring/globalsummary/timeseries/tswucoveragepol3.html.

86. WHO. (2017). Poliomyelitis fact sheet. Available at: http://www.who.int/mediacentre/factsheets/fs114/en/.

87. Wyn-Jones, A.P., and Sellwood, J. (2001) Enteric viruses in the aquatic environment. In. Oxford UK, pp. 945-962.

88. Yanez, L.A., Lucero, N.S., Barril, P.A., Díaz, M.D.P., Tenaglia, M.M., Spinsanti, L.I. et al. (2014a) Evidence of Hepatitis A virus circulation in central Argentina: Seroprevalence and environmental surveillance. *Journal of Clinical Virology* **59**: 38-43.

89. Yanez, L.A., Lucero, N.S., Barril, P.A., Díaz, M.D.P., Tenaglia, M.M., Spinsanti, L.I. et al. (2014b) Evidence of Hepatitis A virus circulation in central Argentina: Seroprevalence and environmental surveillance. *Journal of Clinical Virology* **59**: 38-43.

90. Zheng, H., Lu, J., Zhang, Y., Yoshida, H., Guo, X., Liu, L. et al. (2013) Prevalence of Nonpolio Enteroviruses in the Sewage of Guangzhou City, China, from 2009 to 2012. *Applied And Environmental Microbiology* **79**: 7679-7683.

APPENDIX A

VP7 Primers

Primer		Sequence 5' – 3'	Position (nt)	Amplicon Length
First Round				
sBeg9		ggC TTT AAA AgA gAg AAT TTC	1 – 21	1062bp
End9		GGTCACATCATACAATTCTAATCTAAG	1062 - 1036	27 mer
Second Round PCR (nested)				
9con1		TAGCTCCTTTTAATGTATGG	37 – 56	20 mer, 904bp
EndA		ATAGTATAAAATACTTGCCACCA	922 – 944	23 mer
Genotyping Round (Gouvea and Iturriza-Gomara)				
G8	aAT8v	GTCACACCATTTGTAAAYTCAC	178 – 199	767 bp
G1	aBT1	CAAGTACTCAAATCAATGATGG	314 – 335	631 bp
G2	aCT2	CAATGATATTAACACATTTTCTGTG	411 – 435	534 bp
G3	mG3	ACGAACTCAACACGAGAGG	250 – 269	692 bp
G4	aDT4	CGTTTCTGGTGAGGAGTTG	480 – 498	465 bp
G9	mG9	CTTGATGTGACTAYAAATAC	757 – 776	188 bp
G10	mG10	ATGTCAGACTACARATACTGG	666 – 687	276 bp
G12	G12b	GGTTATGTAATCCGATGGCG	504 – 524	438 bp
	EndA	ATAGTATAAAATACTTGCCACCA	922 – 944	

VP4 Primers

Primer		Sequence 5' – 3'	Position (nt)	(Amplicon) Length
First Round				
con3		TGGCTTCGCTCATTTATAGACA	11 - 32	22mer, 876bp
con2		ATTTCGGACCATTTATAACC	868 - 887	20mer
Second Round PCR (nested)				
VP4F		TATGCTCCAGTNAATTGG	132 – 149	18mer, 663bp
VP4R		ATTGCATTTCTTTCCATAATG	775 – 795	21mer
Genotyping Round (Gentsch)				
P[8]	1T-1V	TCTACTGGATCGACGTGC	339 – 356	213 bp
P[4]	2T-1	CTATTGTTAGAGGTTAGAGTC	474 – 494	351 bp
P[6]	3T-1	TGTTGATTAGTTGGATTCAA	259 – 278	135 bp
P[9]	4T-1	TGAGACATGCAATTGGAC	385 – 402	259 bp
P[10]	5T-1	ATCATAGTTAGTAGTCGG	575 – 594	462 bp
P[11]	mP11	GTAAACATCCAGAATGTG	305 – 323	180 bp
P[14]	p4943	GGTGTAGTTCCTGCGTA	538 – 554	414 bp
	VP4F	TATGCTCCAGTNAATTGG	132 – 149	