QUANTITATIVE DETECTION OF ROTAVIRUS AND ENTEROVIRUS IN RAW SEWAGE USING REVERSE TRANSCRIPTION DROPLET DIGITAL PCR

By

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ABSTRACT

QUANTITATIVE DETECTION OF ROTAVIRUS AND ENTEROVIRUS IN RAW SEWAGE USING REVERSE TRANSCRIPTION DROPLET DIGITAL PCR

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The goal of this study was to survey the concentrations of rotaviruses (RVs) and enteroviruses (EVs) in raw sewage using reverse transcription droplet digital PCR (RT-ddPCR) and to compare the use of ViroCap filters, polyethylene glycol/sodium chloride (PEG/NaCl) precipitation and mixed cellulose ester (HA) filter methods for virus recovery. Sewage samples were collected from lagoons in Kenya (KE) (5 L, n=10) and Michigan (MI), United States (USA) (10 L, n=10). From four wastewater treatment plants (WWTPs) in Virginia (VA), USA (0.05 L, n=42) and one WWTP in California (CA) (2 L, n=18). Samples were concentrated either using ViroCap, PEG/NaCl or HA filter method. Nucleic acid was extracted using either the QIAamp Viral RNA Mini Kit or the bioMerieux NucliSens easyMag. Detection and quantification of RV and EV was done using RT-ddPCR. Rotavirus was detected at a geometric mean concentration of 1.31E+05 genome copies/L (gc/L) (CA), 2.71E+04 gc/L (KE) and 1.48E+05 gc/L (VA). Enterovirus at 1.39E+06 gc/L (CA), 3.72E+06 gc/L (KE) and 6.18E+03 gc/L (VA). The mean RV concentrations using PEG and ViroCap methods in MI lagoon was statistically significant ($p<0.01$). The bag-mediated filtration system (BMFS) using ViroCap filters was an inexpensive method when concentrating large volumes. Therefore, we recommend the use of BMFS with ViroCap for routine monitoring of viruses in polluted water sources in low resource countries while PEG and HA filters can be used across all environmental virology laboratories where resources are available.

This thesis is dedicated to my parents Peninah Mukami Kiulia and Eliud Kiulia Mukuongo, for their love and support, and for teaching me the two most important things in life - compassion and humility. To my beloved uncle Dr. Jason Mwenda for instilling in me the virtues of hard work, determination and focus. To my brother, sisters and their children (my nieces and nephews) for their continued prayers, support and encouragement and for believing in me. In memory of my late grandfather M'Mukuongo Thanyuki and M'Laikuru M' Imathiu (Ntakuo) for always encouraging me to work hard. To my beloved friends, thank you all for your prayers throughout this journey. Finally, to the almighty God for seeing me through the treacherous journey of education and for always blessing and holding my hand when I was feeling low and almost giving up, I uplift and adore you.

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KEY TO ABBREVATIONS

AFP	Acute flaccid paralysis
ATCC	American Type Culture Collection
BGM	Buffalo green monkey kidney
bp	Base pair
CDC	Centers for Disease Control and Prevention
CI	Confidence interval
CNS	Central nervous system
Ct	Cycle threshold
CPE	Cytopathic effects
°C	Degrees celcius
ddPCR	Droplet digital polymerase chain reaction
DNA	Deoxyribonucleic acid
ds	Double stranded
E	Echovirus
EM	Electron microscopy
EPA	Environmental Protection Agency
EPI	Expanded program of immunization
EV	Enterovirus
GAVI	Global Alliance for Vaccines and Immunization
GPLN	Global Polio Laboratory Network
G	Genogroup
g	Gram
h	Hour

IPV	Inactivated polio vaccine
EV	Enterovirus
HAV	Hepatitis A virus
HCL	Hydrochloric acid
IPR	Institute of Primate Research
kb	Kilobase
L	Litre
µL	Microliter
µM	Micromolar
mg	Milligram
mL	Millilitre
min	Minute
mm	Millimeter
NaCl	Sodium chloride
NoV	Norovirus
NPEVs	Non-poliovirus
NR	Not reported
NSP	Non-structural protein
nt	Nucleotide
NTR	Non-translated region
OPV	Oral polio vaccine
ORF	Open reading frame
PBS	Phosphate-buffered saline
PV	Poliovirus
PCR	Polymerase chain reaction

PEG	Polyethylene glycol
RNA	Ribonucleic acid
RT	Reverse transcription
rt	Real time
RV	Rotavirus
ss	Single-stranded
UNICEF	United Nations Children's Fund
USA	United States of America
VP	Viral protein
WHO	World Health Organization
WPV	Wild-type poliovirus

CHAPTER 1: INTRODUCTION

1.1 Waterborne pathogens and implication for public health

Human enteric viruses are among the waterborne pathogens that cause severe cases of diarrhea diseases globally. Contamination of water sources used for drinking and recreation by pathogenic human enteric viruses is the major cause of non-bacterial gastroenteritis outbreaks worldwide (Fong and Lipp, 2005). It is estimated that as of 2016 approximately 4.7 billion people use piped water while 2.1 billion people use non-piped water (WHO/UNICEF, 2017). The recent report by the Joint Monitoring Programme (JMP) for Water Supply and Sanitation by WHO and UNICEF estimate that about 2.3 billion people lack basic sanitation with the majority (892 million) either practicing open defecation (WHO/UNICEF, 2017). The other portion (856 million) uses facilities such as pit latrines that are not well-built e.g having no concreate slab, while other use hanging latrines or bucket latrines. As of 2016, 844 million people lack basic drinking water service (WHO/UNICEF, 2017) and about 780 million do not have access to safe and clean drinking water. In Africa, unsafe drinking water, poor hygiene and inadequate sanitation are the main cause of diarrheal diseases due to exposure to waterborne pathogens such as *vibrio cholerae*, *Salmonella typhimurium* that causes cholera and typhoid fever respectively. Other cases occur due to exposure to non-bacterial agents, which includes enteric viruses such as rotaviruses (RV), noroviruses (NoV) and enteroviruses (EVs) just to name a few. Protozoa e.g *Cryptosporidium, Entamoeba* and *Giardia.* Children are the most affected globally where 1 out of every 5 deaths under the age of 5 is associated with a water-related disease which account for great burden of diarrheal disease in children < 5 years of age. Under the United Nations (UN), safe water supply and adequate sanitation to protect public health are among the basic human rights (UN, 2010), thus, safe and clean

drinking water is one of the deliverables under the UN's Sustainable Development Goals (SDGs). In low-income countries, hygiene and sanitation impact heavily on population health and the burden of waterborne disease. Studies have shown that contaminated water contains mainly enteric viruses in addition to other pathogens (Braeye et al., 2015; Koroglu et al., 2011; Mellou et al., 2014).

1.2 Methods for recovery of enteric viruses in raw sewage

To recover viruses from raw sewage, the best methods used should be simple, rapid, inexpensive, and consistent (Ikner et al., 2012). Published studies have reported on several techniques used to recover enteric viruses from raw sewage and they include ultrafiltration, ultracentrifugation, adsorption elution using positively or negatively charged membranes or filters, flocculation and two-phase separation with polymers (Arraj et al., 2008; Grassi et al., 2010; Kargar et al., 2013; Kitajima et al., 2014; Kiulia et al., 2010). All these methods have their pros and cons. For instance, using ultracentrifugation the advantage is that the processing of the samples can be done without adjusting the pH and that no elution step is needed. The major drawback is the cost of buying the high-speed ultracentrifuge that can cost up to $35,000, thus, cannot be affordable in low resource countries (Fumian et al., 2010; Prata et al., 2012).

The detection and quantification of viruses in water samples is a multistage process consisting of sample preparation, concentration, detection, quantification and viral typing (Mattison and Bidawid, 2009; Wyn-Jones, 2007). To recover viruses from raw sewage, attributes of a good method include high efficiency, ability to recover many types of viruses, precision and accuracy are needed. Viral cell culture methods are often more expensive and take many weeks to obtain the results compared to molecular approaches but do provide information on infectivity (Ikner et al., 2012).

When choosing a method for efficient viral recovery, several factors should be considered for instance; the physicochemical quality of water such as pH, conductivity, turbidity, and organic matter (Ikner et al., 2012). In most studies, several techniques have been used to recover enteric viruses from sewage and they include ultrafiltration, Polyethylene Glycol/Sodium Chloride precipitation, ultracentrifugation, adsorption elution using positively or negatively charged membranes or filters, flocculation and two-phase separation with polymers (Arraj et al., 2008; Kargar et al., 2013; Kitajima et al., 2014). The detection, identification and quantification of RV in environmental samples is cumbersome and its recovery requires collection and concentration of a large volume sample (Ikner et al., 2012). New methods developed for the concentration of poliovirus (PV) in sewage as part of the environmental surveillance of PV (Fagnant et al., 2014) have yet to be evaluated for RV which will be needed as the vaccine for RV is further distributed.

1.3 Methods for detection and quantification of enteric viruses in environmental samples

The detection of pathogenic viruses in water sources is an important aspect of water quality monitoring and management; however, tracking of enteric viruses in environmental waters is a challenge, labor intensive and expensive. However, advancement in the development and application of molecular tools has made it possible to quantify enteric viruses including emerging and newly recognized enteric viruses in environmental samples (Alhamlan et al., 2013; Ng et al., 2012; Pu et al., 2016).

Molecular biology tools for rapid detection and characterization enteric viruses are important in environmental samples analysis (Bibby and Peccia, 2013; Ng et al., 2012). To examine the infectivity of the isolated viruses, cell culture system has always been the gold standard. Most enteric viruses like NoV lack reliable cell lines to propagate and

grow viruses to aid in their detection and identification. This is because they are normally in low levels in environmental samples (Atmar and Estes, 2001). Though the cell culture of NoV has been a challenge. Recently Ettayebi and coworkers (Ettayebi et al., 2016) reported great success in cultivating multiple human NoVs strains in cell culture using enterocytes in stem cell–derived, non-transformed human intestinal enteroid monolayer cultures. In this technique, they noted that the use of Bile was a critical factor for propagation to succeed (Ettayebi et al., 2016).

The ability to detect and quantify enteric viruses in environmental water sources is also cumbersome due to the presence of many assay inhibitors (Bosch et al., 2008). The recent development of droplet digital PCR (ddPCR) has revolutionized and made the detection and quantification of viruses easy and less cumbersome. This novel technique is able to quantify RNA genome copies without the need to develop a standard curve (Rački et al. 2014). Droplet digital PCR is not dependent on amplification efficiency and therefore may provide more accurate measurements than qPCR. The ddPCR technique is used widely in clinical research (Hall Sedlak and Jerome, 2014; Kiselinova et al., 2014; Palmer, 2013; Sedlak and Jerome, 2013; Strain et al., 2013) but ddPCR has been applied in very few limited studies to detect and quantify enteric viruses in wastewater (Coudray-Meunier et al., 2015; Ishii et al., 2014; Kishida et al., 2014; Racki et al., 2014b).

There are many research gaps in the literature on quantitative data on RV concentration in raw sewage. Over the last 2 decades, very few peer-reviewed publications (3 in Africa, 3 in Asia, 4 in Europe, 6 in Americas and 1 in Middle East) that have reported the occurrence of RV in raw sewage with a mean prevalence of 60% (range 8.3-100%) (see chapter 2, Table 2.2). In Africa countries, namely; Kenya, Tunisia and Egypt the rate of RV detection ranges from 8.3-69.2% (Kamel et al., 2010; Kiulia et al., 2010; Sdiri-Loulizi et al., 2010). In Asia occurrence of RV in raw sewage was reported in China (He et al.,

2011) at 44.4% prevalence and in India a detection rate of 77% was found (Vivek et al., 2013) In Europe the rate of RV detection in untreated water ranged between 37.5-100% (see Table 2.2 in Chapter 2); (Arraj et al., 2008; Grassi et al., 2010; Hellmer et al., 2014; Ruggeri et al., 2014). In the Americas, the occurrence of RV in raw sewage was reported in studies from Brazil at 58% (Ferreira et al., 2009b; Fumian et al., 2013; Vecchia, 2012) Venezuela at 66.7% (Rodriguez-Diaz et al., 2009) Argentina at 100% (Barril et al., 2010), and the USA at 58.3% (Kitajima et al., 2014). Just like RV there is plausible quantitative data on the concentration of EVs in raw sewage. Most studies report on the presence and absence of limited studies reporting on the quantitative data (as shown in Table 2.3 of chapter 2). Looking at all these studies (Table 2.2) there is inadequate RV concentration data in raw sewage. Therefore, the quantitative data on RV and EV generated from my research will contribute knowledge in the literature thus trying to bridge the knowledge gap. These data are very important in risk assessment studies since the data on pathogen concentration are input as key parameters and variables for quantitative microbial risk assessment (QMRA) (Haas et al., 2014; Mena, 2007; Symonds et al., 2014)

1.4 Scientific questions, research hypotheses and objectives

1.4.1 Scientific questions

a) What are the concentrations of rotavirus and enteroviruses in raw sewage using droplet digital PCR?

b) Is rotavirus concentration in Kenya lagoons higher that the rotavirus concentration in a US lagoon?

c) Are the rotavirus and enterovirus concentrations recovered using Virocap recovery methods higher than that recovered using the PEG virus recovery method?

d) Is there any difference between rotavirus concentration and enterovirus concentration in raw sewage from various US wastewater treatment plants?

1.4.2 Research hypotheses

a) The occurrence of rotavirus and enterovirus in raw sewage is the same in both developing (Kenya) and developed countries (USA).

b) Virocap, PEG or HA membrane filters method used for virus concentrate and recovery in raw sewage samples yields higher RV genome copies.

1.4.3 Objectives

a) To determine the occurrence of rotaviruses (RVs) and enteroviruses (EVs) in raw sewage

b) To assess and compare the use of ViroCap filters and the polyethylene glycol/sodium chloride precipitation (PEG/NaCl) technique for the recovery of enteric viruses from raw sewage samples.

c) To evaluate RT-ddPCR as a tool to detect and quantify RV and EV in raw sewage and compare the concentrations of RV and EV from different geographical settings (Kenya and the United States.

d) To optimize the sampling preparation method to enable quantification of RNA viruses in raw sewage.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction to enteric viruses

2.1.1 Rotavirus

Rotavirus was discovered in 1973 by Ruth Bishop and coworkers via electron microscopy in duodenal mucosa from children, who were hospitalized with acute gastroenteritis (Bishop et al., 1973). Since then it has been recognized as a major cause of severe dehydrating diarrhea in human and animals (Estes and Greenberg, 2013).

2.1.1.1 Virology and classification

Rotavirus (RV) is a double stranded (ds) ribonucleic acid (RNA), non-enveloped virus with a triple-layered icosahedral capsid measuring 55 to 75 nm in diameter and an inner layer that encloses the viral genome (Estes and Greenberg, 2013). They are grouped in the genus *Rotavirus* in the Reoviridae family (Attoui et al., 2012; Estes and Greenberg, 2013). The RV genome has eleven dsRNA segments which encode for 6 viral structural proteins (VP1-VP4, VP6 and VP7) and 6 nonstructural proteins (NSP1-NSP6) (Desselberger, 2014; Estes and Greenberg, 2013). Rotaviruses are classified into at least seven distinct serogroups (A- G), based on distinctive antigenic and genetic properties (Estes and Greenberg, 2013). Among these groups, group A, B and C rotaviruses are known to cause disease in humans and animals, whereas other groups (E-G) have only been associated with animal infection (Estes and Greenberg, 2013).

Among these viral proteins, VP7 and VP4 are the two outer capsid proteins that elicit neutralizing antibodies and are the major proteins incorporated in the RV vaccine (Estes and Greenberg, 2013; Vesikari et al., 2006). The VP7 and VP4 have both been used for the classification of the RV, where the VP7 defines the G genotype (Glycoprotein) and

VP4 which is a Protease-sensitive protein defines the P genotype (Estes and Greenberg, 2013).

These viral proteins also play a key role in virus entry and infection of the host cells (Estes and Greenberg, 2013). The NSP4 is the major protein that plays a role as an enterotoxin that induces diarrhea (Estes and Greenberg, 2013). The VP6, the inner shell protein (middle capsid layer), determines the group and subgroup specific antigen and is the most abundant capsid protein in the RV virion (Estes and Greenberg, 2013; Trask et al., 2012b). This protein plays an important role as an adaptor for the outer capsid proteins, which are important for attachment and entry into a host cell and virions assembly (Trask et al., 2012a; Trask et al., 2012b). The VP6 protein has also been a target for vaccine development (Jalilvand et al., 2015; Ward and McNeal, 2010).

2.1.1.2 Transmission of rotavirus

Rotavirus transmission of primarily through fecal–oral route either by person-to-person contact or by ingestion of fecally contaminated water and food (Bosch, 2010; Rodriguez-Lazaro et al., 2012). The RV incubation period takes about 5-7 days but it may be as short as 48 hours (Estes and Greenberg, 2013; Lee et al., 2013). In young children, at onset the symptoms are sudden and are presented by watery diarrhea, vomiting, fever, abdominal discomfort and dehydration (Estes and Greenberg, 2013; Mwenda et al., 2010). The virus can also affect older children and adults but the severe disease is seen more in children below two years of age (Anandan et al., 2014; Gastanaduy et al., 2013; Parashar et al., 2009; Tate et al., 2016; Tate et al., 2012).

Like other enteric viruses such as noroviruses, RV has been associated with waterborne related outbreaks (Altzibar et al., 2015; Braeye et al., 2015; Koroglu et al., 2011). Fecal contaminated water sources used to supply drinking water to a community is a major

environmental contributor to the burden of waterborne related infection worldwide. Therefore, frequent monitoring of water sources for the presence of waterborne pathogens is an important approach to ensure the safety of drinking water. Detection and quantification of enteric viruses in raw sewage is necessary to understand community health risk, especially for fecally contaminated drinking water sources, shellfish harvesting waters, and recreational waters.

2.1.1.3 Occurrence of rotavirus in raw sewage

The presence of rotavirus in water sources is of public health concern due to its potency, persistence and impact on young children (Estes and Greenberg, 2013; Fong and Lipp, 2005; O'Ryan et al., 2012; Ward et al., 1986). Monitoring of enteric pathogens in sewage systems is one of the best methods to assess the presence of pathogens that are circulating in a specific community. Despite having a vaccination available, RV continues to cause severe gastroenteritis worldwide. Although the occurrence of RV in raw sewage have been documented, the number of published literature reporting on the density and/or concentration of this virus in sewage is very few (da Silva et al., 2017). Globally, very few studies have reported the concentration of RV in sewage (He et al., 2011; Kitajima et al., 2014; Kiulia et al., 2015).

2.1.1.4 Global burden of rotavirus disease

Globally RV causes severe diarrhea in young children and animals (Estes and Greenberg, 2013). Among the groups of RV, group A RVs causes the most severe acute gastroenteritis in young infants and children worldwide (Estes and Greenberg, 2013).

This virus is of serious public health concern due to morbidity and mortality associated with it (Parashar et al., 2009; Tate et al., 2016; Tate et al., 2012; WHO, 2013a).

Globally, the prevalence rate of RV disease varies from country to countries and geographical region (Table 2.1). Several studies that have documented the burden of RV disease reporting prevalence rates that ranges from 6-56.3%. The number of cases enrolled range between 128 to 6057 young children under the age of 5, and these studies were conducted between 1 year to 3 years (Table 2.1).

In the last decade, there has been an increase in the need to assess the burden of RV gastroenteritis in children less than five years of age, due to the increase in childhood morbidity and mortality across the globe (Tate et al., 2016). The global RV surveillance network spearheaded by World Health Organization (WHO) estimated an annual global mortality of approximately 453,000 (range 420,000 to 494,000) as of 2008 (Tate et al., 2012). The fatalities rate accounted for about 5% of all child deaths and a cause-specific mortality rate of 86 deaths per 100 000 population aged below 5 of years (Tate et al., 2012; WHO, 2013b). Global mortality estimates as of 2013 had reduced significantly to approximately 215,000 from an estimated 453,000 in 2008 and this is attributed to the introduction of an RV vaccine in many countries around the world (Tate et al., 2016; Tate et al., 2012).

Table 2.1: Summary of the prevalence rates of rotavirus infection in different countries 2002-2014.

Continent	Country	Duration of study	# of Samples recruited	(%) Prevalence	Reference
Africa	Kenya	2009-2011	500	38	Kiulia et al., 2014b
	Libya	2007-2008	1090	31.5	Abugalia et al., 2011
	Morocco	2006-2009	1388	41.7	El Qazoui et al., 2014
	Morocco	2011	335	26.6	El Qazoui et al., 2014
	Tunisia	2007-2010	435	27.6	Hassine-Zaafrane et al., 2011
	Sierra Leone	2005	128	37.5	Jere et al., 2011
	South Africa	2003-2006	3191	22.8	Seheri et al., 2010
Asia	Cambodia	2005-2007	2281	56	Nyambat et al., 2009
	China	2008-2009	766	27.94	Ouyang et al., 2012
	China	2011-2012	767	34.3	Sai et al., 2013
	India	2009-2012	1191	39	Babji et al., 2014
	India	2007-2012	756	38.4	Tiku et al., 2014
	India	2009-2011	1807	35.9	Mathew et al., 2014
	India	2004-2008	412	19.2	Mishra et al., 2010
	Lao PDR	2005-2007	1158	54	Aloun et al., 2009
	Myanmar	2004-2005	2179	56.3	Moe et al., 2009
	South Korea	2005-2007	6057	22	Lee et al., 2009
	South Korea	2007-2008	702	25.2	Shim et al., 2012
	Taiwan	2005-2007	3435	25	Wu et al., 2009
Europe	Albania	2007-2010	1066	21	Kota et al., 2014
	France	2001-2004	457	48.8	Lorrot et al., 2011
	Spain	2006-2008	2048	40.1	Sanchez-Fauquier et al., 2011
Middle East	Iran	2009-2010	163	46.02	Kargar and Akbarizadeh, 2012
	Israel	2007-2008	472	39.1	Muhsen et al., 2009
	Saudi Arabia	2002-2003	1000	6	Tayeb et al., 2008
South America	Argentina	2004-2007	710	19.7	Esteban et al., 2010
	Venezuela	2013	480	21	(Gonzalez et al., 2011)

Table 2.2: Summary of the occurrence of rotavirus in raw and untreated sewage from selected countries in Africa, Asia, Europe, Middle East and Americas

Continent	Country	Year of study	# samples collected (n)	# Positive (RV%)	Sample Volume	Method of viral recovery	Mean concentration (GC/L)	Reference
Africa	Egypt	2006-2007	72	6 (8.3)	2L	Adsorption–elution	NR	Kamel et al., 2010
	Kenya	2007-2008	13	9(69.2)	1L	Ultracentrifugation	NR	Kiulia et al., 2010
	Tunisia	2003-2007	125	53 (42.4)	100mL	Ultracentrifugation	NR	Sdiri-Loulizi et al., 2010
Americas	Argentina	2009	52	52 (100.0)	1.5L	Ultracentrifugation	NR	Barril et al., 2010
	Bolivia	2012	10	10 (100.0)	60 mL	Filtration with 0.45um after acidification	3.1E+07 GC/L	Symonds et al., 2014
	Brazil	2004-2005	24	11 (45.8)	2L	Electronegative filter	NR	Ferreira et al., 2009a
	Brazil	2009-2010	24	24 (100.0)	42mL	Ultracentrifugation	2.40E+05 GC/L	Fumian et al, 2011
	Brazil	2009	7	2 (28.6)	500mL	Electronegative filter	NR	Vecchia et al., 2012
	USA	2011-2012	12	8 (67.0)	100mL	Electronegative filter	2.8E+06 GC/L	Kitajima et al., 2014
	Venezuela	2007-2008	12	8 (66.7)	35mL	Ultracentrifugation	NR	Rodríguez-Díaz et al, 2009
Asia	China	2006-2007	10	10 (100.0)	1mL	Ultracentrifugation	NR	He et al, 2008
	China	2006-2007	36	16 (44.4)	1mL	Ultracentrifugation	3.12E+03 GC/L	He et al, 2011
	India	2009-2010	144	111 (77.0)	30mL	Ultracentrifugation	NR	Vivek et al, 2013
Europe	France	2003-2004	29	11 (37.9)	1L	Adsorption	NR	Arraj et al, 2008
	Italy	2006-2007	16	37.5	1L	Ultrafiltration	NR	Grassi et al, 2010
	Italy	2010-2011	285	60.4	65mL	Ultracentrifugation	NR	Ruggeri et al, 2014
	Sweden	2013	7	100.0	1L	Adsorption to milk proteins	NR	Hellmér et al, 2014
Middle East	Iran	2010-2011	15	33.3	1L	Two phase sedimentation	NR	Karger et al 2013

2.1.2 Enteroviruses

Enteroviruses (EVs), one of the enteric virus groups, have been associated with diverse syndromes from febrile illness to neurological diseases (aseptic meningitis, acute flaccid paralysis, poliomyelitis) respiratory (myocarditis), gastrointestinal and skin diseases. (Caro et al., 2001; Pallansch M and Roos R, 2013). Poliovirus is among the EVs that are well studied and there are two vaccines approved for use to address disease prevention.

2.1.2.1 Virology and classification

Enteroviruses are classified within the family *Picornaviridae* and assigned in the genus *Enterovirus* (Knowles et al., 2012). Enteroviruses are small non-enveloped, positive-strand RNA viruses (Knowles et al., 2012) and are about 27 to 30 nm in diameter with an icosahedral symmetry (Knowles et al., 2012; Pallansch M and Roos R, 2013). Enteroviruses like other picornaviruses have a similar genome structure of approximately 7,500 to 8,000 nucleotides (nt) long, with single large open reading frame (ORF) prior to a long 5'-untranslated region (5' UTR), They also have a small virus-encoded protein VPg (or 3B) that is attached to the 5' non-translated region (NTR) end of the viral genome, a 3' end poly(A) tail with a variable length that ranges between 65 to 100 nt. As of 2016, the genus *Enterovirus* is currently classified into twelve (12) distinct species namely; *Enterovirus A-H, J,* with another species *Enterovirus I* being proposed to be included in the list, the *Rhinovirus A-C*. EV species A comprises of the Coxsackievirus A2–16, and Enterovirus A71, A76, A89, A90, A91, A114, A119, A120, A121. The HEV species B includes the following; Coxsackie B1–6, A9, echovirus 1–7, 9,11–21, 24–27, 29–33 and the Enterovirus B69, 73–75, 77–88, 93, 97, 98, 100, 101, 106, 107, and 111. The HEV species C (Coxsackievirus A1, 11, 13, 17, 19, 20, 21, 22, 24, Enterovirus C95, 96, 99, 102, 104, 105, 109, 113, 116–118 and PV 1–3 while HEV species D includes EV-D68,

D70, D94, D111 (Lugo and Krogstad, 2016) as approved by the ICTV in 2013. As of 2015, the three PV serotypes that have been used for many years are assigned to the species *Enterovirus C* and the species *Poliovirus* no longer exists (ICTV, 2015). This classification has been possible thorough the use of advanced molecular technology for characterizing viruses (Betancourt and Shulman, 2016; Knowles et al., 2012).

2.1.2.2 Transmission of enteroviruses

Enteroviruses infect both gastrointestinal (GIT) and respiratory tract (RT) (Pallansch M and Roos R, 2013) and just like other enteric viruses that infect the GIT their spread and transmission is through ingestion of fecal contaminated sources such as water or food.

2.1.2.3 Occurrence of enteroviruses in raw sewage

The abundance of EV in sewage is due to their universal distribution in a population, which is widespread globally (Betancourt and Shulman, 2016). The occurrence of EVs in raw sewage is shown in figure 1.3 which has been quantifying using either qPCR or cell culture. There is a variation in occurrence of EV due to the limitation and differences in recovery and detection and because the abundance of viruses in raw sewage in a different location around the world do vary because also the shedding rates within a community are different. The detection and quantification of EV in raw sewage is of public health importance because pathogenic virus may be circulating silently in a population. Several studies on Environmental surveillance (ES) of PVs in raw sewage have been ongoing for several decades in different countries around the world, for instance, The ES for PV in Israel has been ongoing since 1989 (Berchenko et al., 2017; Manor et al., 2014), with composite sewage samples collected monthly at the mouth of sewage treatment facilities. During the ES period, wild-type poliovirus (WPV-1) was detected even without any clinical

case being detected and this strain was related to a strain identified in Egypt towards the end of 2012. The detection of this strain revealed that there was a silent epidemic going on even without any case being isolated in clinical samples and this could have been as a result of asymptomatic transmission. Finland has been monitoring sewage regularly since the outbreak PV in early the 1980s (1984-85) (Hovi et al., 2001) but no PV has been detected in sewage since then (Hovi et al., 2001). The ES of PV in sewage is a sensitive approach to monitor silent PV circulation in populations that are being served by that sewerage system (Hovi et al., 2001). The ES approach is important since it can help in decision making on when to launch a supplementary immunization program.

Table 2.3: Summary of the occurrence of enteroviruses in raw sewage

Continent	Country	Year of study	samples collected (n)	# Positive (EV%)	Sample Volume	Method of viral recovery	Concentration	Reference
Africa	Ivory Coast	2008-2009	68	30 (44.1)	1 L	***Dextran T40 & PEG 600)	NR	Momou et al., 2014
	Kenya	2007-2008	13	9 (69.2)	1L	PEG/NaCl precipitation	NR	Kiulia et al, 2010
	Tunisia	2009-2010	172	52 (30.2)	2 L	PEG/NaCl precipitation	NR	Ibrahim et al., 2014
	South Africa	2002-2005	100	43 (42.5)	10 L	Glass-wool	NR	Ehlers et al., 2005
	South Africa	2001-2003	213	176 (86.63)	50 mL	PEG/NaCl precipitation	NR	Pavlov et al., 2006
Americas	Bolivia	2012	10	10 (100)	60 mL	#Filtration	**4.2E+02-6.2E+02 IU/L	Symonds et al, 2013
	USA	2008-2019	10	10 (100.0)	20 L	*#1MDS	*2.10E+05 GC/L	Simmon & Xagoraraki et al., 2011
	USA	2011-2012	12	12 (100.0)	100 mL	Electronegative membrane filter	1.0E+06 GC/L	Kitajima et al., 2014
	Venezuela	2007-2008	12	9 (75)	35 mL	Ultracentrifugation	NR	Rodriguez-Diaz et al 2009
Asia	China	2009-2012	240	216 (90)	1 L	Electronegative membrane filter	NR	Zheng et al, 2013
	Japan	2003-2004	72	71 (100.0)	100 mL	Electronegative membrane filter	*3.2E+02 GC/L	Katayama et al, 2008
	Japan	2011-2012	14	10 (71.0)	100 mL	Electronegative membrane filter	**4.47E+04-2.8E+06 GC/L	Haramoto and Otagiri, 2014

*Mean virus concentration; **range of virus concentration; GC/L genome copies per liter; iU/mL infectious units per milliliter; NR not reported; PFU/L plaque forming units per liter; L liter; ***Two Phase separation using Dextran T40 and PEG 600
#Filtration with 0.45um after acidification; *#1MDS cartridge filters

Table 2.3 (Cont'd)

	Singapore	2007	18	17 (94)	1 L	PEG/NaCl precipitation	NR	Aw and Gin, 2011
Europe	Italy	2005-2008	1392	702 (50.4)	500 mL	***Dextran T40 & PEG 600)	NR	Battistone et al., 2014
	Netherland	1998-1999	72	72 (100.0)	10 L	Electronegative membrane filter	*1.0E+02 PFU/L	Lodder and de Road Husman 2015
	Spain	2001-2006	74	62 (84.0)	20 mL	Direct Filtration using 0.22 um pore size filters	*5.4E+03-3.74+04 PFU/L	Costa-Longares et al, 2010
	UK	2009-2010	40	37 (92.5)	100 mL	Centrifugation and Filtration	NR	Harvala et al, 2014
Oceania	New Zealand	2003-2004	30	18 (60)	1 L	Beef extract flocculation	4.68E+06 GC/L	Hewitt et al, 2011

2.2 Environmental surveillance of rotavirus; lesson learnt from the Global Polio Eradication Initiative.

Clinical surveillance programs for human infectious diseases is aimed at monitoring disease patterns and pathogens strains in the human populations. Therefore, most of the clinical surveillance data on the circulating pathogens come from samples clinical studies especially from patients seeking medical assistance in health centers and hospitals. However, ES of enteric viruses could serve as an additional tool to monitor the transmission of pathogens in a population. This has been a success in the agenda of the Global Polio Eradication Initiative (GPEI) in their effort to eradicate polio globally (Hovi et al., 2012). Several active surveillance programs on ES of PV are ongoing in many developing countries where WPV polio has been occurring and where Oral Polio Vaccine (OPV) is being used (Etsano et al., 2016). These countries include Kenya, Angola, Nigeria, India and Pakistan (Asghar et al., 2014). Rotavirus surveillance system for monitoring the circulating genotypes/strains can learn from this initiative so as to supplement the clinical data.

In respect to RV, the WHO has been spearheading the hospital-based surveillance of RV associated disease globally. This has led to a well-documented and a rich database on the burden of the RV disease, the RV strains in circulation pre-and post vaccine introduction. The data collected as a result of hospital based surveillance in Africa and Asia played a very crucial role in helping the WHO inform policy and decisions regarding the introduction of the RV vaccines as soon as possible in the Africa and Asian countries where the burden of the RV disease was high (WHO, 2013b).

The already licensed introduced RV vaccines are live attenuated vaccines that are administered orally like the OPV. The OPV has been linked to the emergence of vaccine derived poliomyelitis where most of the vaccine strains have been showed to revert to virulence form (Etsano et al., 2016; Jorba et al., 2016). Just like the ES program with

PV (Etsano et al., 2016; Hovi et al., 2012), there is much interest in monitoring sewage for both vaccine strains and wild types to understand the efficacy of the RV vaccine and to monitor the circulation RV strains in correlation with the data generated from clinical samples (Fumian et al., 2011). The identification of RVs occurring in the environment will provide an additional source of information regarding the genotypes circulating in the community which provides an evidence base for policy makers with regard to decisions in the introduction the RV vaccines.

Several studies have demonstrated the benefit of ES as an additional tool to determine the epidemiology of RV genotypes circulating in a surrounding community (da Silva et al., 2017; Fumian et al., 2011; Kiulia et al., 2010). Studies on molecular characterization of RV genotypes in clinical, wastewater and sewage are important to understand the impact of the vaccine on the RV genotypes circulating in a community. The segmented genome of rotavirus readily reassorts during co-infection and it plays a key role in virus evolution (Estes and Greenberg, 2013). The reassortment may lead to the emergence of novel RV variants. Monitoring clinical samples alone using hospital based surveillance systems cannot provide all the data needed to understand the diversity of rotavirus genotypes. Therefore, ES can play a key role in supplementing the clinical data and provide the spatial and temporal distribution and hotspots associated with rotavirus discharges.

Several studies have reported the use of an ES of rotavirus approach to monitoring the circulation rotavirus strain after RV vaccine introduction (Fumian et al., 2011; Hassine-Zaafrane et al., 2015; Kiulia et al., 2010). For instance, a study in Brazil Fumian and coworkers carried a one year ES of RV genotypes in circulation after the introduction of the Rotarix vaccine (Fumian et al., 2011). This study, evaluated the spread of group A RV in the environment after the RV1 was introduced in Brazil by monitoring wastewater

treatment plants in Rio for a full calendar year. In the study the most prevalent RVA genotypes detected were the G2, P[4], P[6] based on VP4 and VP7 classification. Though the study did not detect any RV vaccine-like strains from the sewage samples using nucleotide sequencing technique, it highlighted the importance of ES as a tool to study RV epidemiology in the surrounding human population. It also stressed its usefulness in supplementing vaccine monitoring programs. A study in Tunisia by Hassine-Zaafrane and colleagues (Hassine-Zaafrane et al., 2015) that looked at the distribution of RV strains in sewage samples and compared them with RV strains in clinical data. This study demonstrated great diversity of RV genotypes in circulation including detecting animal rotavirus strains namely; P[1], P[5] and P[11] (Hassine-Zaafrane et al., 2015). While the study in Kenya also documented the emergence of animal strains G5 and G10 that has not yet been detected in the clinical samples (Kiulia et al., 2010).

All these studies have demonstrated the importance of environmental surveillance of RV and stress the need to re-think the strategy in carried out simultaneous studies both using hospital based and environmental surveillance systems. Therefore, monitoring of rotavirus in sewage polluted water and raw sewage systems will be the best methods to assess the presence of RV strains that are circulating in a specific community and if there are any novel or emerging strains.

CHAPTER 3: MATERIAL AND METHODS

3.1 Site descriptions

3.1.1 Kenya sampling locations

The three sampling sites in Kenya (Kibera, Karen and Maua) were selected because these sites were near health facilitates where clinical surveillance and epidemiological data on enteric viruses was available (Kiulia et al., 2008; Kiulia et al., 2007; Kiulia et al., 2010; Kiulia et al., 2014b; Kiulia et al., 2006; Mwenda et al., 2010).

The Kibera site, a densely populated informal settlement located at Latitude -1.318889 and Longitude 36.794167 (see Figure 3.1) drains its human wastewater receptacles (latrines) and other black water into the Mutoine river. The river is highly polluted with human fecal matter as well as a multitude of urban runoff from the informal settlement. The river passes through the Nairobi dam and joins the Nairobi river downstream, that joins the Tana river and ultimately ends up into the Indian Ocean. The Mutoine river is used by many slum dwellers for washing clothes and irrigating their fresh produce (tomatoes, Kales) farms and this end up in the Nairobi vegetable market. Downstream about 3 KM away the street family that lives alongside this river uses it for domestic purposes like bathing and children in these areas usually swim and play in this polluted river.

The Karen Lagoon located at Latitude -1.330247 and Longitude 36.713083) (Figure 3.1) is in the upper Nairobi (near Karen shopping center). Although most houses in the area are connected to a sewerage system or have septic tanks. The raw sewage is drains directly to a lagoon. In the surrounding area are many flower vendors that use the water draining on the outlet to water their flowers and some water edible vegetables. The Karen lagoon also have open roadside drainage channels that carries water downstream that ends up into the Mbagathi river.

The Institute of Primate Research (IPR) Lagoon (GPS coordinates; Latitude -1.365412, Longitude 36.71328) (Figure 3.1) is located within the Ololua forest in the upper Nairobi area. The IPR is a large biomedical research center that houses various non-human primates used for research in infectious diseases and reproductive sciences. The IPR has its own sewerage system from the non-human primate housing, the laboratory facilities and the staff residential housing. The water going into the lagoon includes both black and grey water and is drained directly to the Lagoon for biological treatment and after which it end up to the Mbagathi river. Downstream of this river many people use the river to water their farms where they grow some fresh produce like lettuce, kale, tomatoes, and cabbages but mostly for personal or household usage and a few for small commercial purposes.

The Maua Methodist Hospital (MMH) Lagoon (GPS coordinates; Latitude 0.222887, Longitude 37.941794), is located about 270 KM from Nairobi capital center, In Maua town, Meru county. In this town, there is neither a sewerage system nor any wastewater and/or sewerage treatment plant. Due to lack of a proper sewerage system in the town, the MMH management built its own sewerage system within the hospital and connected it to a Lagoon about 2 KM away. This lagoon is also treated through the biological treatment process. All the treated sewage is then directed to the Mboone river. The river is highly polluted with raw sewage coming from the MMH lagoon, the surrounding overflowing latrines. This river has been previously investigated for the presence of enteric viruses (Kiulia et al., 2014a; Kiulia et al., 2010). The MMH lagoon was chosen since there are well described studies with detailed data on the molecular epidemiology of enteric viruses both in clinical and environmental samples, though the data are not quantitative.

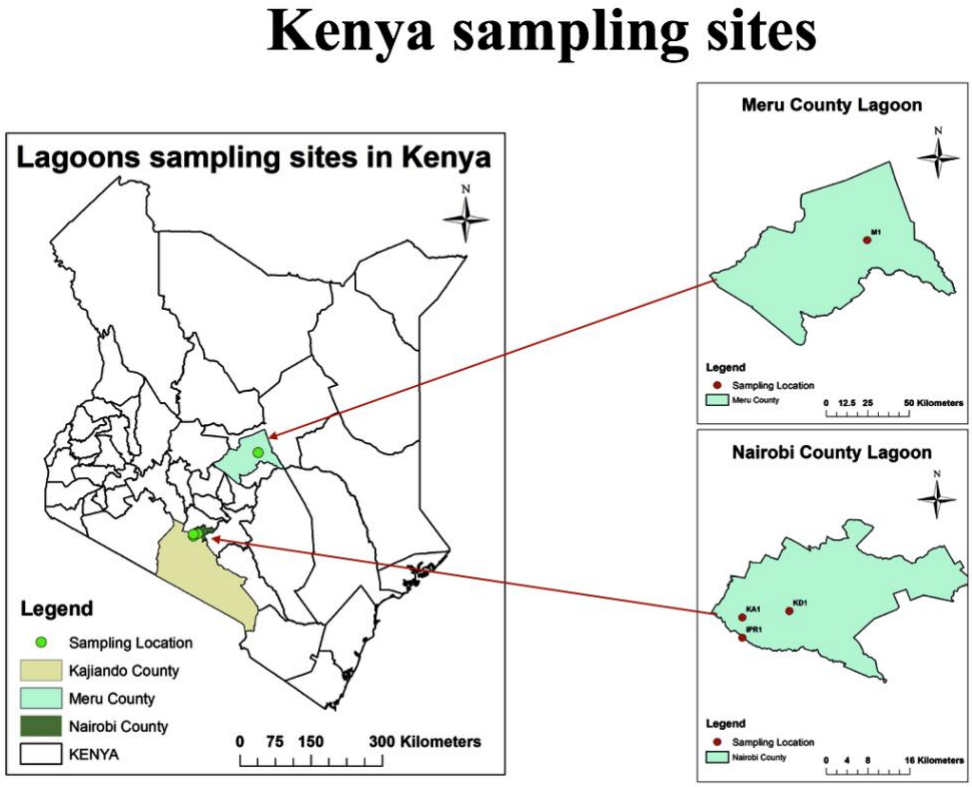


Figure. 3.1: A Kenya map showing all the sampling sites

Figure legend: The sampling sites in Nairobi County (KAI – Karen Lagoon, KD1 – Kibera slum wastewater and IPR – Institute of Primate Research Lagoon. The Meru County sampling site, M1 – Maua Methodist Hospital Lagoon.

3.1.2 The United States (USA) sampling location

The USA sampling sites were at 3 locations, California, Michigan and Virginia.

The lagoon in Michigan is located in the city of Belding (Latitude 43.077465 and Longitude -85.243205) and it covers approximately 50 acres. It designed to have 5 Pond (cell), where Pond 1 which is the receiver of raw sewage covers 1.3 acres and is 12 feet deep, Pond 2 covers 22 acres and is 6 feet deep, Pond 3 is 15 acres of 6 feet in depth while Pond 4 & 5 covers 7.5 acres and measures 6 feet in depth. The lagoon accommodates a total volume of approximately 100 million gallons and it receives approximately 0.6 million gallons per day.

In California, we sampled at the San Luis Rey Wastewater Treatment Plant/Water Reclamation Facility operated by the City of Oceanside (SLRWWTP) located in Oceanside, California. The SLRWWTP is a conventional wastewater treatment plant with a rated secondary treatment capacity of 13.5 million gallons per day (MGD). After headworks and flow equalization, the flow is split between two separate treatment trains designated as Plant 1 and Plant 2. The SLRWWTP's original configuration consisted solely of Plant 1 designed for an average annual flow of 9.4 MGD. Plant 2 was later implemented as a two-phase 4 MGD/8 MGD expansion but only operates with the 4 MGD capacity during wet weather months. Over the course of the study, average treated flows for Plant 1 and Plant 2 were 7.40 MGD and 3.13 MGD, respectively. Plant 2, while normally used in wet weather months only, remained in operation in all but three sampling events due to summertime construction on portions of the Plant 1 process. Both plants are designed with conventional activated sludge treatment consisting of primary clarifiers, aeration basins, and secondary clarifiers. While the majority of flow from both plants is discharged via ocean outfall, only about 3% of the total secondary effluent flow is filtered and chlorinated to meet Title 22 recycled water demands. The SLRWWTP process flow diagram is shown in Figure 2.2**.**

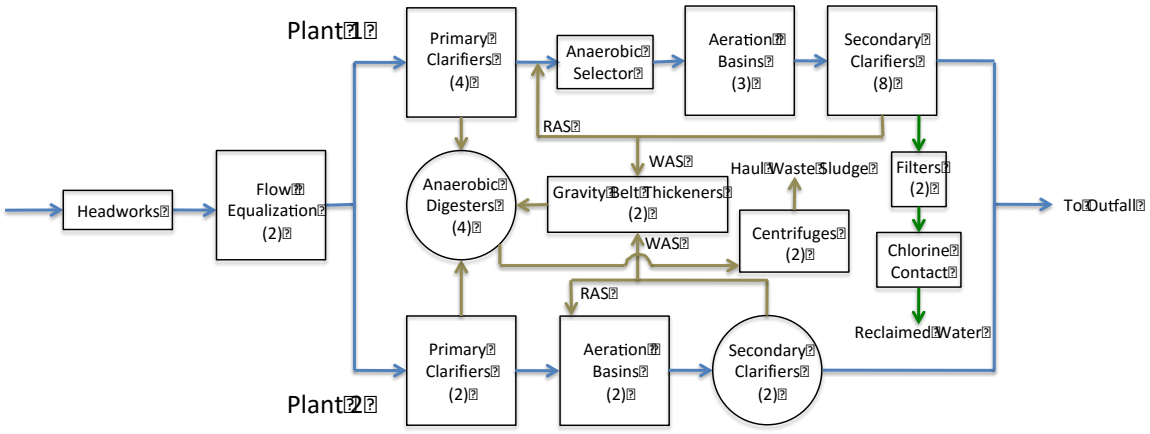


Figure 3.2: The San Luis Rey Wastewater Treatment Plant Process Flow Diagram

3.2 Raw sewage samples collection

3.2.1 Kenya lagoon sampling

In June 2015, 10 raw sewage (influent) grab samples were collected (5 L) from three lagoons and/or ponds in Kenya as described in section 3.1.1 (Karen Lagoon n=2), IPR lagoon (n=2), MMH lagoon (n=4) and from a wastewater canal in Kibera slum, Nairobi, Kenya (n=2). All the samples were transported in a cooler box from the sites to the Enteric Pathogens and Water Research Laboratory, IPR, Nairobi where the samples were processed and viruses concentrated within 6 hours using the Virocap method as described in section 3.3.1, and later the 0.22um filtered viral concentrates shipped to the Water Quality, Environmental and Molecular Microbiology (WQEMM) Lab, Department of Fisheries and Wildlife, Michigan State University, USA, for nucleic acid extraction and quantification using advanced molecular techniques as described in section 3.5.

3.2.2 USA lagoon sampling

Raw sewage samples (10 L, n=10) were collected from a lagoon in Michigan, US in July 2016. These 10 influent grab samples were collected from two sampling sites namely; Pond A (n=5) and Pond B (n=5). The samples were transported in cooler boxes to the WQEMM laboratory for viral recovery.

3.2.3 USA Wastewater Treatment Plant sampling

From August 2015 to July 2016 (12 months) raw sewage (influent) samples of 2 L (n=18) were collected from a Wastewater Treatment Plant (WWTP) in the California (CA), United States (US). A second set of samples were collected in 4 different WWTPs in Virginia (VA), US WWTP; plant A (n=12), plant B (n=12), plant C (n=12) for a period of 12 months while plant D (n=6) was collection for a period of 6 months. California samples

were collected as composite samples and transported with coolants to the Water Quality, Environmental and Molecular Microbiology (WQEMM) Lab, Department of Fisheries and Wildlife, Michigan State University (WQEMM) laboratory for RV recovery and downstream analysis. The Virginia grab samples were transported on ice < 6°C to Hampton Roads Sanitation District's Central Environmental Laboratory (HRSD CEL) for viral recovery and downstream analysis.

3.3 Sample processing and virus recovery

3.3.1 Samples processing flow chart

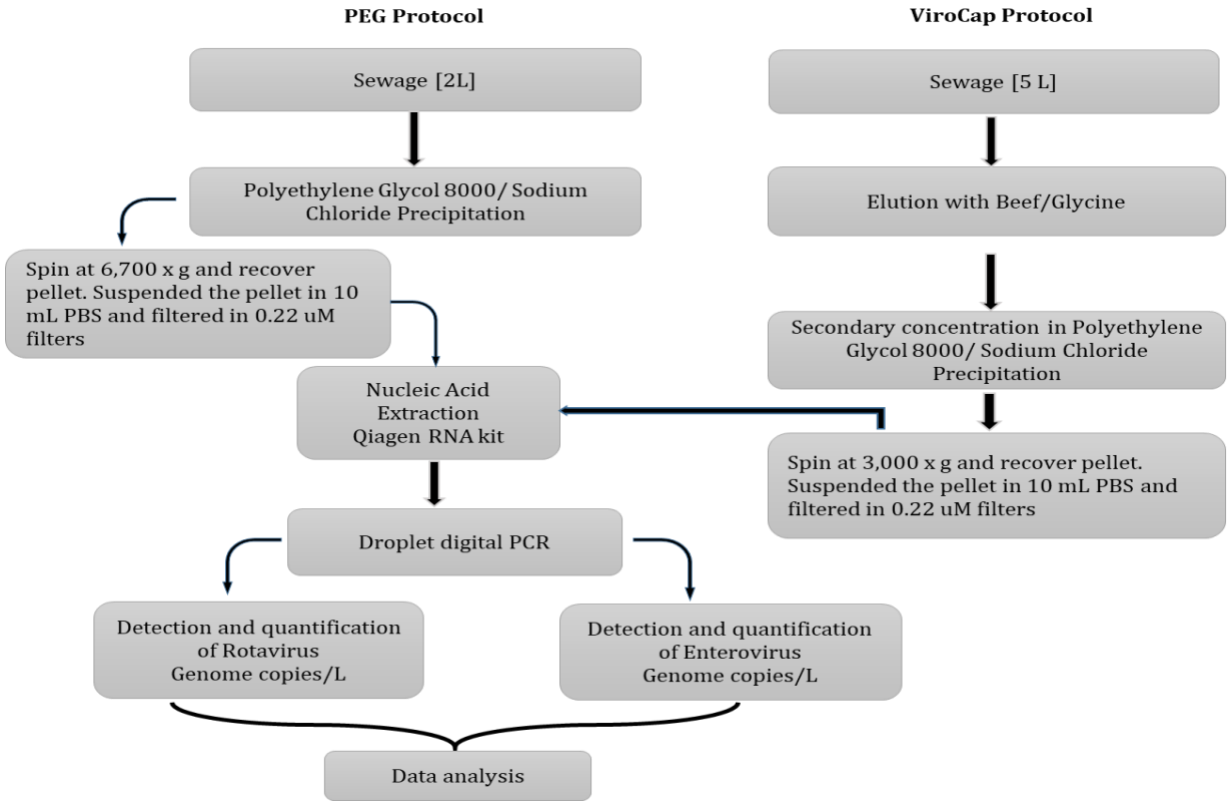


Figure 3.3: Samples processing flow chart.

3.3.2 Viral concentration using ViroCap filters

Enteric viruses were recovered from the Kenyan lagoons samples and a set of samples from the US lagoon using the ViroCap™ filters (Scientific Methods, Granger, Indiana, USA) through an adsorption-elution procedure (Fagnant et al., 2014). Briefly, in Kenya samples (5 L) and US (7.5 L) were filtered through the ViroCap filters at a flow rate of 0.25 L/ min. The negatively charged viruses adsorb to the ViroCap filters during filtration taking approximately 30 minutes. Filters were then eluted with 1.5% Beef extract, with 0.05 M Glycine buffer, pH 9.5 (GBEB; 0.05M glycine [Sigma-Aldrich Inc, St Louis, MO, USA]; 0.5% beef extract [BBLTM Becton Dickinson and Co., Sparks, MD]). Immediately after elution the pH of the eluate was neutralized to pH 7.0 using 1 M HCl (JT Baker, PA, USA). The 125 mL eluate was subjected to secondary concentration using the polyethylene glycol/sodium chloride precipitation (PEG) method. The resultant pellet was then re-suspended in 5 mL phosphate buffered saline pH 7.2 (PBS; Sigma-Aldrich Inc, St Louis, MO) and then filtered through a 0.22 µm filter. The 0.22 µm filtered viral concentrates from Kenya were shipped in dry ice to the WQEMM Lab, USA, for nucleic acid extraction and quantification via appropriate permits and approvals with the Center for Disease Control and Prevention (CDC).

3.3.3 Viral concentration using polyethylene glycol

Recovery of RV from the raw sewage (2 L) from the US (CA – WWTP) and from a set of the US lagoon samples was done using the PEG/NaCl precipitation method described by Shieh et al. (Shieh et al., 1995). Briefly, 2 L of raw sewage was precipitated by mixing it with 8% PEG 800 and 0.3M NaCl and stirred to mix well and left to stand overnight (18 hours) at 4°C. This was then centrifuged at 6,700 x g for 30 min and the pellet re-suspended in 20 mL phosphate-buffered saline (PBS) (pH 7.2). Recovered RV

suspensions were clarified by the addition of 20 mL of chloroform (Merck) to 20 mL of the recovered viral suspension followed by rigorous vortexing for 30 seconds. The mixture was then centrifuged at 1,700 × g (Eppendorf 5402D Microcentrifuge, Hamburg, Germany) for 30 min. The sediments and the sample was further centrifuged at 1,700 x g for 30 min. The supernatant was filtered through the 0.22-µm filter and stored at -80°C for further analysis.

3.3.4 Viral concentration using HA filters

Fifty mL of VA wastewater samples were concentrated using mixed cellulose ester HA filters (HAWP04700; Millipore, Billerica, MA, USA). Prior to filtration, samples were acidified with 20% HCl to a pH of 3.5 then amended with MgCl to a final concentration of 25nM. Filters were immediately stored at 80°C then processed within one week.

3.4 Nucleic acid extraction

3.4.1 Extraction of RNA using QIAamp Viral RNA Mini Kit at WQEMM laboratory Michigan

Genomic viral nucleic acid was extracted from the 0.22 µM filtered viral concentrates using the Qiagen RNA extraction kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions, with slight modification where a starting volume was 200 µL. A negative extraction control (NEC) (nuclease-free water) was included for quality control. Extracted nucleic acid was eluted in 60 µL of the elution buffer and stored in 10 µL aliquots at -80°C until further analysis.

3.4.2 Extraction of RNA using NucliSENS easyMag platform at HRSD CEL Laboratory Virginia

Prior to extraction using bioMerieux NucliSENS easyMag, 10 µL of 1 x 10^6 genome copies/µL Hep G Armored RNA (Asuragen, Austin, TX, USA) was spiked in the lysis buffer with the HA filter. All extractions were performed according to the manufacturer's protocol specific B 2.0.1 with modification. The protocol is modified with a 30 minute off board lysis using 2 mL of lysis buffer, and 100 µL of magnetic silica beads. The samples, standards, and negative extraction control (NEC) were extracted according to this protocol and then eluted to a final volume of 100 µL. Positive genomic RNA standards were from American Type Culture Collection (ATCC, Manassas, VA, USA) for group A human RV (ATCC number VR-2018DQ).

3.5 Detection of rotavirus using droplet digital RT-PCR.

Rotavirus was detected and quantified using a one-step RT-ddPCR assay using published primers and probe targeting the non-structural protein 3' (NSP3) region of the RV genome (Zeng et al., 2008), using a One-Step RT-ddPCR advanced kit (Bio-Rad, CA, USA) following the manufacturer's instructions. In short, the reaction mixtures in a final of volume 20 µL consisting of 5 µL of the One-Step RT-ddPCR Supermix (Bio-Rad, CA, USA), 2 µL of RT enzyme, 1 µL of 300 mM dithiothreitol (DTT) solution (Bio-Rad, CA, USA), 1 µL each of the forward and reverse primers (900nM), 1 of 250nM probe (Eurofins Genomics Co., AL, USA), 5 µL molecular grade RNAse free water and 4 µL of RNA was used. The RNA was denatured at 95°C for 5 min and kept on ice for 5 min prior addition to the reaction. Four µL of RNA from each sample or molecular grade RNAse free water as a negative template control (NTC) was transferred into individual wells using multichannel pipette on a disposable eight-channel droplet generator cartridge (Bio-Rad, CA, USA). Each oil well was then filled with 70 µL of droplet generation oil (Bio-Rad, CA,

USA) and the prepared cartridge then loaded into the QX 200 droplet generator (Bio-Rad, CA, USA).

After droplet generation, the suspension of droplets from each was transferred using a multichannel pipette into a 96-well polypropylene plate (Bio-Rad, CA, USA), heat sealed with foil, and amplified in a conventional calibrated Bio-Rad thermal cycler (Bio-Rad, CA, USA). The PCR cycling conditions consist of 30 min reverse transcription at 60°C, 5 min initial denaturation at 95°C, followed by 45 cycles of a two-step thermal profile of 30 s denaturation at 94°C and 60 s annealing-elongation at 60°C, and a final 10 min denaturation step at 98°C. After thermal cycling, the plate was transferred to the QX 200 droplet reader (Bio-Rad, CA, USA). The positive droplets, containing amplification products, was then analyzed by discriminating positive from the negative droplets by applying a fluorescence amplitude threshold in QuantaSoft software version 7.21 (Bio-Rad, CA, USA). Samples inhibition was gauged by comparing sample internal control concentrations to NEC internal control concentrations. Inhibition was rarely an issue; however inhibited samples were diluted until there was less than a 0.5 $\log_{10}$ difference between sample and NEC internal control concentrations. All NEC and no template controls (NTC) had 0 positive droplets.

The sampling location, number of samples collected and the method of virus concentration and nucleic acid extraction are summarized in Table 3.1 while the primers and probes used in this study are shown in Table 3.2

Table 3.1: Summary of sampling locations, number of samples and volume collected, method of virus concentration and nucleic acid extraction.

Location	Facility	# Samples (n)	Volume (L)	Method of Viral recovery	Nucleic acid extraction method
Kenya	Lagoon	10	5	ViroCap	QIAamp Viral RNA Mini Kit
Michigan	Lagoon	10	7	ViroCap	QIAamp Viral RNA Mini Kit
Michigan	Lagoon	10	2	PEG	QIAamp Viral RNA Mini Kit
California	WWTP	18	2	PEG	QIAamp Viral RNA Mini Kit
Virginia	WWTP Plant A	12	0.05	HA	bioMerieux NucliSENS easyMag
	WWTP Plant B	12	0.05	HA	bioMerieux NucliSENS easyMag
	WWTP Plant C	12	0.05	HA	bioMerieux NucliSENS easyMag
	WWTP Plant D	6	0.05	HA	bioMerieux NucliSENS easyMag

Table 3.2: The primers and Taqman probes used for detection of rotaviruses and enteroviruses.

Virus	Primer & Probe	Sequence (5'-3') *	Reference
Rotavirus	VP6F	GAC GGV GCR ACT ACA TGG T	Zeng et al. 2008
	VP6R	GTC CAA TTC ATN CCT GGT G	
	VP6P	[FAM] CCA CCR AAY ATG ACR CCA GCN GTA [BHQ1]	
Enterovirus	EntF	CCTCCGGCCCCTGAATG	US EPA Method 1615
	EntR	ACCGGATGGCCAATCCAA	
	EntP	FAM-CGGAACCGACTACTTTGGGTGTCCGT-TAMRA	

*International Union of Pure and Applied Chemistry (IUPAC) codes used to indicate degenerate positions; R= A / G, Y= C / T, M= A / C, K= G / T, S= C / G, W= A / T, B= C / G / T, D= A / G / T, H= A / C / T, V= A / C / G, N= A / C / G / T.
The degenerate base symbols are an IUPAC representation for a position on a DNA sequence that can have multiple possible alternatives. For instance, in the sequence VP6F, the R in the DNA sequence can be either a A or G.

3.6 Thermal gradient optimization

To optimize the primer and probe annealing temperature 10 fold serial dilutions (10^{-1} to 10^{-10}) viral copies per reaction of purified viral RNA from ATTC WA strain of virus was made in PCR RNAse free water. This was used as a RNA template to assess the optimal annealing temperature of the primer and probes before use. The standard RT-ddPCR cycling condition was modified at the annealing step by replacing the annealing temperature with a thermal gradient between 50°C and 65 °C for 1 min extension time.

All PCR reactions were carried out using a CFX96 Bio-Rad thermal cycler (Bio-Rad, ÇA, USA).

3.7 Statistical analyses

The data in this study was analyzed as one factor experiment and fitted using the statistical model as outlined in equation 1.

Rotavirus Concentration $_{ij}$ = μ + *method* $_{i}$ + *site (Method)* +ε_{ij} = Where i = 1, 2,3 and j=1,2,3,4,5,6,7 (equation 1)

The degrees of freedom for the model components was calculated as follows; 1 for the grand mean (μ); Method (3-1) = 2, The site (method) = 5, and the residual (ε_{ij}) was 80. The sites (locations) of samples collection were nested within the type of method used at that site and the degree of freedom for this was 5. The (ε_{ij}) used was the error term in the individual samples collected in each site/location. To check for normality of the residuals, visualization of the normal probability plot and histogram of the residuals were assessed (see Appendix A.5, Figure A.5.1A & 1B). To check for Homogeneity of variances visualisation of the residual v. predicted plots values and of the side-by side box and Whisker plots was done (see Appendix A.5, Figure A.5.2A & 2B), in addition, a Levene's test was conducted (see Appendix A.5, Figure A.5.3A & 3B). Most of the data from environmental samples tend to follow a poisson distribution. Therefore, for analysis the data are usually transformed either using logarithm$_{10}$ ($\log_{10}$) or natural log (LN). In this study, the data was transformed using natural log before analysis.

Descriptive statistics were conducted with Microsoft Excel 2016 (Microsoft Corp., Redmond, WA). The EV and RV concentration data were expressed as mean, standard deviation (SD), median, geometric mean, minimum and maximum. One-way ANOVA was done to compare the mean difference of RV concentration between the methods of virus

recovery (HA, PEG and ViroCap) and the site (sampling location). A Tukey's Honestly Significantly Different test (Tukey HSD) (A.5.4, Table 4.3.) for pairwise comparison was used to test differences among virus recovery methods and sampling sites and/or location. A two independent samples t-test was conducted to compare the mean difference of RV concentration quantified using ViroCap and PEG method in the US lagoon. Box plots were constructed using BoxPlotR, a convenient online tool available at http://boxplot.tyerslab.com (Spitzer et al., 2014). One-way analysis of variance (ANOVA) and A Tukey's Honestly Significantly Different test (Tukey HSD) was performed with SAS version 9.4 of the SAS System for Windows (SAS Institute Inc. Cary, NC, USA) and differences between means of selected enteric virus concentration were considered to be statistically significant at $\alpha < 0.05$ (95% confidence interval).

CHAPTER 4: RESULTS

4.1 Statistical evaluation of data

The data were analyzed to test for normality and the distribution of the residuals. Looking at the histogram the data were skewed to the right thus deviating from normality. Levene's test also showed that there was no difference among the variances on the methods used for rotavirus concentration and recovery (Appendix 1 Figure 3a and b). However, the plot of the residuals vs predicted values indicated that larger variances tend to be associated with larger predicted values. To use the dataset for analysis the data were log transformed and the final analysis were based on the log transformed data (See Appendix A.6, supplementary material for the dataset and the SAS code used).

4.2 Concentration of rotavirus in raw sewage

All the samples collected (Kenya lagoon n=10), Michigan, US lagoon (n=10), and US-CA WWTP (n=18) were analyzed and quantified for RV. Minimum, maximum, mean ± SD and geometric mean RV concentrations (genome copies/L [gc/L]) are presented in Table 4.1. Rotavirus was detected in all the samples analyzed, accounting for 100% in the Kenya (10/10) and US lagoons (10/10), and (18/18) in the US (CA) WWTPs. In Virginia (VA) 100% of the samples were positive for rotavirus in the four plants evaluated: plant A (12/12), plant B (12/12), plant C (12/12) and plant D (6/6) (Table 4.1).

Rotavirus was detected in the Kenyan lagoons at a mean concentration of 1.09E+05 ± 1.90E+05 genome copies (gc/L) (range 3.24E+03 to 5.84E+05 gc/L) and a geometric mean of 2.71E+04 gc/L. In the US lagoon, the samples concentrated using ViroCap had a mean of 7.68E+02 ± 9.41E+02 gc/L (range 7.20E+01 to 2.83E+03 gc/L), while the samples concentrated using PEG had a mean of 1.48E+04 ± 8.97E+03 gc/L (range 2.76E+03 to 2.81E+04 gc/L) (Figure 4.1, Table 4.1,).

The US (CA) WWTP had a mean concentration of 4.92E+05 ± 8.19E+05 gc/L (range 5.04E+03 to 2.74E+06 gc/L and a geometric mean of 1.31E+05 gc/L. The Virginia WWTPs (A, B, C and D) mean RV concentrations were 4.77E+05 ± 1.05E+06, 1.10E+06 ± 1.52E+06, 9.04E+05 ± 1.44E+06 and 1.83E+06 ± 2.19E+06 gc/L, respectively (Table 4.1).

Table 4.1: Summary of rotavirus concentrations in raw sewage in Kenya and the USA.

			Rotavirus concentration (genome copies/L)				
Country	**Source (Conc method)**	**n (%) RV +**	**Mean ± SD**	**Median**	**Geo mean**	**Min**	**Max**
Kenya	Lagoon (Virocap)	10 (100)	1.09E+05 ± 1.90E+05	2.27E+04	2.71E+04	3.24E+03	5.84E+05
USA	Lagoon (Virocap)	10 (100)	7.68E+02 ± 9.41E+02	2.50E+02	3.99E+02	7.20E+01	2.83E+03
USA	Lagoon (PEG)	10 (100)	1.48E+04 ± 8.97E+03	1.34E+04	1.16E+04	2.76E+03	2.81E+04
USA	WWTP (PEG)	18 (100)	4.92E+05 ± 8.19E+05	3.59E+04	1.31E+05	5.04E+03	2.74E+06
USA	WWTP Plant A (HA)	12 (100)	4.77E+05 ± 1.05E+06	3.67E+04	4.71E+04	6.63E+02	3.70E+06
USA	WWTP Plant B (HA)	12 (100)	1.10E+06 ± 1.52E+06	1.97E+05	2.53E+05	1.56E+04	4.24E+06
USA	WWTP Plant C (HA)	12 (100)	9.04E+05 ± 1.44E+06	1.52E+05	1.33E+05	6.63E+02	3.74E+06
USA	WWTP Plant D (HA)	6 (100)	1.83E+06 ± 2.19E+06	7.90E+05	6.03E+05	3.40E+04	4.93E+06

Conc – Concentration; Geo mean – geometric mean; US – United State; PEG-Polyethylene Glycol; WWTP – Wastewater treatment plant; HA- HA filters (nitrocellulose membranes) and ViroCap – ViroCap filters

4.3 Variation of rotavirus concentration in Kenya and the USA raw sewage

Figure 4.1 shows the comparison of RV concentrations (Log_{10} RNA genome copies/L) in different sampling locations (lagoons and wastewater treatment plants in Kenya and the US) along with the methods used for virus concentration and recovery. There was high concentration of RV detected in both US WWTPs as compared to the 2 lagoons in Kenya and US ($p<0.01$, T-test). There was significant mean difference of RV concentration when comparing the ViroCap concentration vs PEG method in the US lagoon ($p<0.01$, Figure 4.1). However, there was no mean difference of RV concentration in both CA and VA WWTPs $p>0.05$ (Figure 4.1). The RV concentrations in both US wastewater treatment plants (CA and VA) varied, although the mean difference was not significant ($p>0.05$), while the RV concentration in the Kenyan lagoon was higher than the US lagoon with about 1-2 log_{10} RNA gc/L but the difference was not significant ($p>0.05$) despite data that shows the method used produced lower concentrations (Figure 4.1).

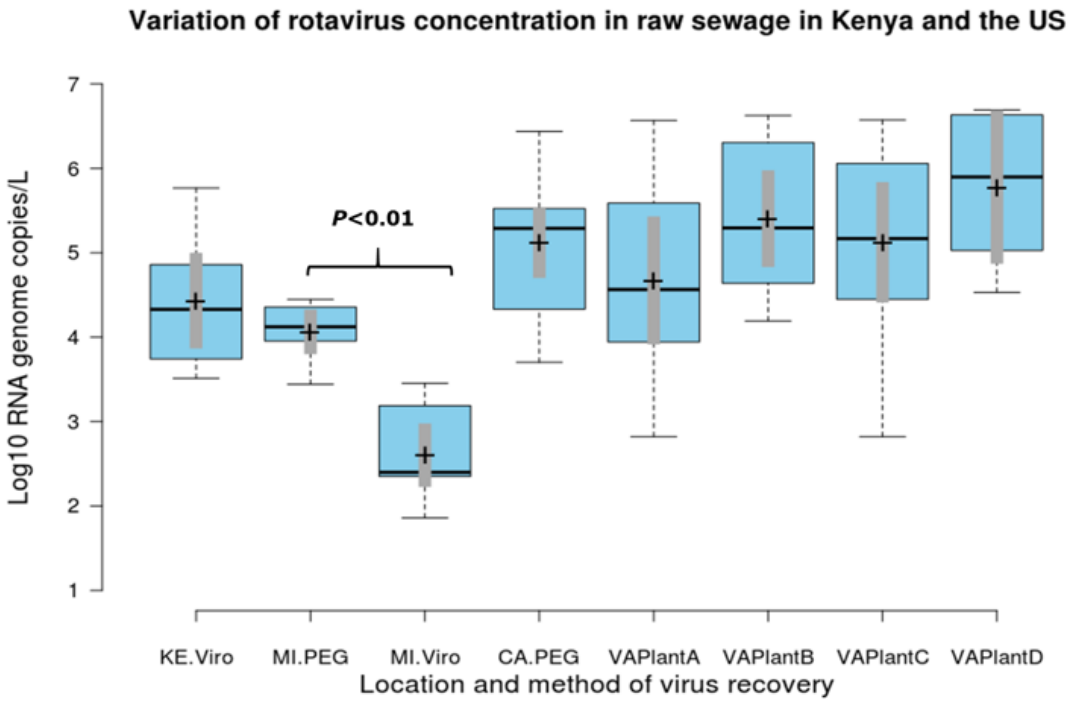


Figure 4.1: Boxplot showing variation in RV concentrations in raw sewage in Kenya and the US sampling locations.

Figure 4.1 shows variation of RV concentrations (Log_{10} RNA genome copies/L) in different sampling locations in Kenya and the US. Sample means are indicated by crosses and 95% confidence intervals of the means are indicated by shaded bars superimposed on the box plots. Sampling location abbreviations are as follows: KE.Viro – Kenya lagoons virus concentrated using ViroCap; MI.PEG- Michigan lagoon virus concentrated using PEG; MI.Viro; Michigan lagoon virus concentrated using ViroCap; CA.PEG- California wastewater treatment plant virus concentrated using PEG; VAPlant A-D- Virginia wastewater treatment plant A-D virus concentrated using HA membrane filters.

4.4 Occurrence and concentrations of enterovirus in raw sewage in Kenya and the USA

Enteroviruses were detected in all the samples analyzed (100%) (Table 4.2). Enterovirus was detected a concentration of 1.18E+04 in KE, 3.72E+06 in CA, while in Virginia it was

5.73E+03 gc/L (Plant A), 5.73E+03 gc/L (Plant B), 7.60E+03 gc/L (Plant C) and 5.65E+03 gc/L (Plant D) respectively (Table 4.2).

Table 4.2: Summary of enterovirus concentrations in sewage in Kenya and the USA.

				**EV (gc/L)
Country	**Source**	**Samples (n)**	**# (%) EV +**	**Geometric Mean**
Kenya	Lagoon	10	10 (100)	1.18E+04
USA	Lagoon	20	20 (100)	ND
USA	WWTP-CA	18	18 (100)	3.72E+06
USA	*WWTP Plant A	12	12 (100)	5.67E+03
USA	*WWTP Plant B	12	12 (100)	5.73E+03
USA	*WWTP Plant C	12	12 (100)	7.60E+03
USA	*WWTP Plant D	6	6 (100)	5.65E+03

gc/L – genome copies/L; ND – Not done; US – United State; CA- California, WWTP – wastewater treatment plant; *samples collected from Virginia; ** quantified with RT-ddPCR.

When one way ANOVA was done, the overall F statistics (F = 1.7, p = 0.2739, Table 4.3) showed that there was no mean difference in rotavirus concentrations across the methods of virus recovery used in this study (i.e PEG, ViroCap and HA filters), while based on Tukey-Kramer pairwise comparisons, the mean difference in rotavirus concentrations was not statistically significant ($p>0.05$, Table 4.5). Although when a two independent samples t-test was conducted to compare the mean difference of RV concentration quantified using ViroCap and PEG method in the US lagoon this was statistically different ($p<0.01$, t-test, Table 4.4).

Table 4.3: One-Way ANOVA to compare the mean rotavirus concentrations among the three virus recovery methods.

Type 3 Tests of Fixed Effects				
Effect	**NumDF**	**Den DF**	**F value**	**Pr > F**
Methods (PEG, ViroCap & HA)	2	5	1.7	0.2739

Table 4.4: One sample t-test to compare the mean rotavirus concentrations in Michigan lagoon.

Method	Variance	DF	t value	Pr >|t|
Pooled	Equal	18	7.34	<0.0001
Satterthwaite	Unequal	16.095	7.34	<0.0001

Table 4.5: Tukey-Kramer pairwise comparisons for rotavirus concentrations means among the methods of virus recovery.

Differences of Least Squares Means								
	Method	_Method	Estimate	Standard error	DF	t value	Pr>|t|	Adj P
Method	HA	PEG	0.9729	1.5617	5	0.62	0.5606	0.8146
Method	HA	ViroCap	3.4724	1.5735	5	2.21	0.0784	0.1628
Method	PEG	ViroCap	2.4995	1.8006	5	1.39	0.2238	0.4138

4.5 Temporal trends of rotavirus raw sewage in California and Virginia, US

Temporal changes in concentrations of RV in wastewater did not vary over time in CA samples (Figure 4.2). This was in contrast to VA where the weather varies more in temperature and rainfall where there were some slight differences observed (Figure 4.3). In VA, WWTP A had lower RV concentration levels compared to the other plants, although the difference was not significant $p>0.05$ (Figure 4.3). Wastewater treatment plants B and C had varied RV concentrations levels throughout the sampling period while plant D had higher levels (range $\log_{10}$ 4.53 to 6.69) than all the other plants from February to June 2017 though, the variation was not significant $p>0.05$ (Figure 4.3).

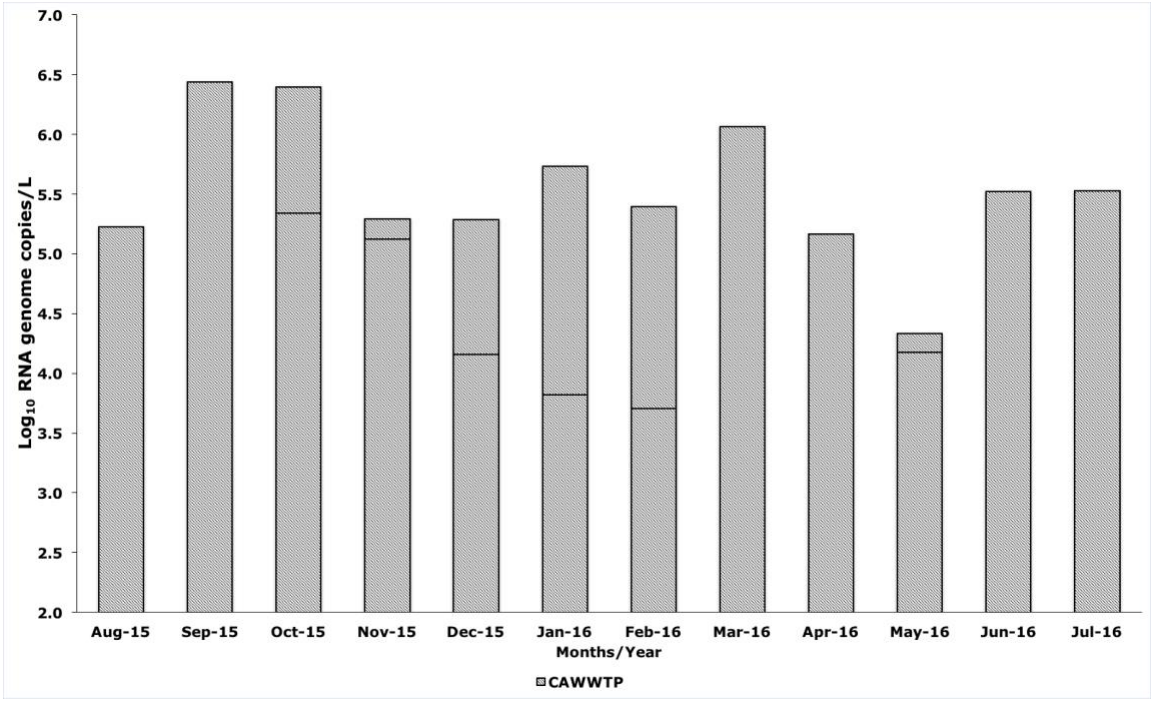


Figure 4.2: Temporal trends of RV concentrations in a wastewater treatment plant in CA - Aug 2015-July 2016.

In the bar graph above (Figure 4.2) the line in the bars indicate where 2 samples were collected during the same month.

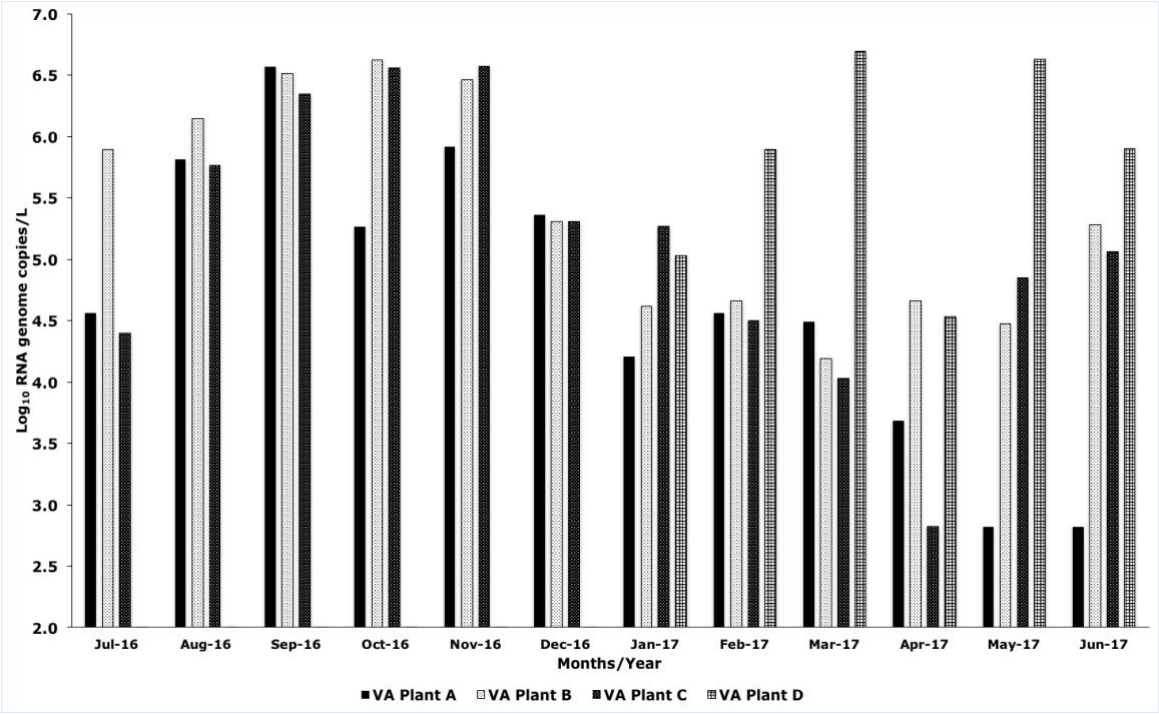


Figure 4.3: Temporal trends of RV concentrations in four wastewater treatment plants in VA - July 2016-June 2017.

4.6 Temporal variation of rotavirus concentrations and enterovirus in raw sewage in California US

Figure 4.4 shows the temporal variation of RV compared to EV (Log_{10} RNA gc/L) in raw sewage in CA during the sampling period August 2015 to July 2016. Enterovirus was detected at higher levels than RV in CA WWTP, with a difference of about 1 log_{10} (range 0.5 – 2 log_{10}) (Figure 4.4). During the months of September and October 2015, the concentration of RV was higher (10^6 log_{10} RNA gc/L) compared to the months of August, November and December 2015 (< 10^6 log_{10} RNA gc/L) (Figure 4.3). The EV concentration during the same months was slightly higher, i.e August (6.53 log_{10} scale RNA gc/L), September (6.59), October (6.43; 6.82), November (6.33; 7.72), December 2015 (5.61) log_{10} RNA gc/L respectively, whereas for RV it was August 2015 (5.23), September 2015 (6.44), October 2015 (5.34; 6.40), November 2015 (5.12) and December 2015 (4.15). Apart from May 2016 where the RV concentration was below 10^5 for all other months January-March 2016 and April, June and July 2016 the RV concentration was above 10^5 (Figure 4.4).

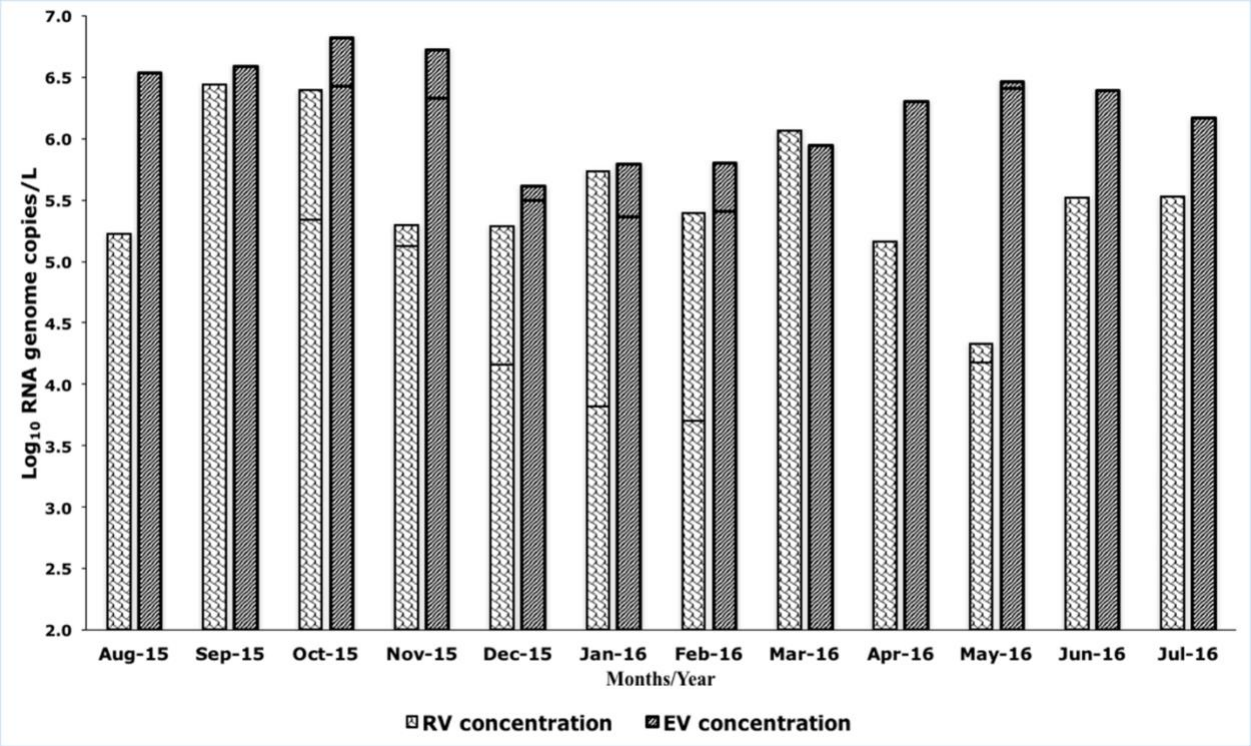


Figure 4.4: Temporal trends of EV and RV concentrations and in a wastewater treatment plant in CA -Aug 2015-July 2016.

CHAPTER 5: DISCUSSION

The goal of the present study was to survey the concentrations of RVs and EVs in raw sewage using reverse transcription droplet digital PCR (RT-ddPCR). Additionally, to compare the use of adsorption-elution (ViroCap filters), polyethylene glycol/sodium chloride (PEG/NaCl) precipitation and HA filter methods for virus recovery and concentration. In this study, RV and EV were selected due to their epidemiological importance and public health impact (Betancourt and Shulman, 2016; da Silva et al., 2017; La Rosa et al., 2012). In Kenya, quantitative data on RV and EV was lacking and therefore, these two viruses were quantified so as to determine their concentrations in raw sewage. While in the United States, data on the occurrence of RV and EV in raw sewage and their concentrates was also very limited.

Despite only a 2-4 log reduction of viruses is achieve, the removal and inactivation of viruses in lagoons or wastewater treatment facilities is an important public health goal (Verbyla et al., 2017; Verbyla and Mihelcic, 2015). Contamination of surface waters with pathogenic enteric viruses can occur either directly or indirectly through discharge of raw/untreated or inadequately treated sewage, leaking sewer system and defective wastewater treatment (Lodder and de Roda Husman, 2005; Sowah et al., 2017).

Lack of adequate wastewater treatment facilities to treat and disinfect raw sewage prior to discharge directly to surface water in some low resource countries is of great public health concern. Most of these countries uses the lagoon systems to treat their raw sewage. Several factors play a key role in determining the level of pathogen concentrations in any water matrix and these includes inactivation via solar ultraviolet radiation (UV), dilutions factors, die-off rates and attenuation (Alexander et al. 1986; Ferguson et al. 2003; Pedley et al. 2006; Dowd et al. 2000). Unintentional ingestion of

fecally polluted water containing pathogenic enteric viruses can cause severe acute gastroenteritis and other illness in the population using this water for domestic, recreation or irrigation. Major waterborne outbreaks associated with recreational water and consumption of edible fresh produce irrigated with contaminate water have been reported and this is of public health concern.

In this study, the RV and EV concentration in raw sewage from Kenyan lagoons, Michigan lagoon and raw sewage from wastewater treatment plants in the US was evaluated and quantified. Rotaviruses and EVs were detected in is all raw sewage samples analyzed in Kenya and the USA suggesting that the presence of these pathogens in the environment is of public health concern.

Comparing to a similar study that was carried in Kenya in the same sampling location a decade ago, the rates of RV detection in raw sewage was comparable (Kiulia et al. 2010). The RV concentrations rates in the Kenya lagoons was also similar to a study in Brazil lagoon system. In the Brazil study the detection of RV was of 2.16E+06 gc/L and 6.10E+06 gc/L (Rigotto et al. 2010), 5.6E+04 gc/L (Vieira et al. 2012) and 2.40E+05 gc/L respectively (Fumian et al. 2011). When RV concentrations in Kenya lagoon was compared to a similar lagoon in Uganda, the levels of RV were lower in Uganda (Katukiza et al. 2014).

Sampling period in the US WWTPs covered the whole calendar year (12 months) and there was no noticeable temporal variation noted even though RV is known to occur with different frequency in temperate climate (Figure 3.2-3.4). This outcome is in agreement with other studies carried out in the Americas (Brazil and Argentina) where RV is reported to circulate all year round (Barril et al., 2015; Prez et al., 2015). The difference in RV concentrations in Kenya and US could have been as a result of the use of two different viral concentrating method, ViroCap filters vs polyethylene glycol/sodium chloride

precipitation technique. The low levels of virus concentrations noticed in samples where ViroCap was used could have been as a result of the use of glycine-beef extract. The ViroCap method uses beef-glycine extract for elution of viruses and this could have resulted in viral inhibition that may have affected the virus yield.

In this study, the concentrations of RV detected in both US and Kenya raw sewage samples were higher than those reported in other studies in China by almost 2 logs (Table 2.2) while it was on the same range with other studies in Americas (Table 2.2). These studies used very little volume of raw sewage samples for analysis (1 mL- 100 mL) while in the present study 1 L – 7.5 L of raw sewage was used. Therefore, the amount of volume used for virus recovery should be evaluated further as to the optimal collection to enhance detection but deter poor recoveries.

In the present study EV was detected in 100% of samples analyzed which was higher than that reported in Italy of 62 % (Iaconelli et al., 2017) while it was similar to studies in the same country (Cesari et al., 2010; Pellegrinelli et al., 2013).

In terms of temporal variation of RV and EV there was slight trend on virus concentrations seen during the sampling period (Figure 3.4) and this should be further explored by carrying out studies lasting for a duration of more than one year so as to determine the actual seasonality of these viruses in raw sewage.

Rotavirus is a major cause of acute gastroenteritis in young children globally and the shedding of the virus is high in an ill patient leading to presence of this virus in the sewer systems (da Silva et al., 2017). Fecally contaminated water sources used to supply drinking water to a community are a major environmental contributor to the burden of waterborne related infection worldwide. Therefore, monitoring of pathogenic enteric

viruses such as enteric RV and EV in wastewater and fecally polluted water sources is essential to supplement the clinical monitoring of these viruses and determine the concentration and the diversity of genotypes in circulation. In a study done in Kenya pathogenic enteric viruses were detected in raw sewage with a higher diversity of RV genotypes in circulation (Kiulia et al., 2010). In the present study which was done in same sampling site but in difference sampling period, RV was quantified although characterization and genotyping of the strains in circulation was not done and therefore, the diversity of RV genotype in circulation was not reported.

Over the last few years, studies quantitative microbial risk assessment (QMRA) have estimate the health risk of RV infection in recreation and domestic water (Chigor et al., 2014; He et al., 2011; Katukiza et al., 2014; Machdar et al., 2013; Mara et al., 2007; Prez et al., 2015; Seidu et al., 2008; Verbyla et al., 2016; Westrell et al., 2004). These studies used water collected from rivers (Chigor et al., 2014; Prez et al., 2015), open drainage channels, unprotected springs (Katukiza et al., 2014) and secondary effluent (He et al., 2011) to estimate the health risk of RV infection. To our knowledge no studies have used RV concentration data from raw sewage for risk estimation. The RV data generated from this study since it is from raw sewage can be used to estimate the health risk associated with RV since the surrounding population in Kenya uses effluent from the lagoons systems to irrigate both edible and non-edible fresh produce and this is of public health importance. It is suggested that irrigation of sewage impacted water be evaluated using a risk assessment approach in the future using rotavirus data.

Molecular detection methods for viruses such as qPCR and conventional rt-PCR have been used for decade to detected, identify and characterize RV in both clinical and

environmental samples (Bosch et al., 2008). Newly developed methods like ddPCR which has been shown to have better reproducibility, precision and accuracy in detection and quantifying viruses and its advantage compared to qPCR is direct quantification without relying on a standard curve (Hindson et al., 2013; Racki et al., 2014b). This technique is yet to be used to detect and quantify RV in water sources including sewage. New methods also developed for to concentrate and recover PV during PV environmental surveillance (Fagnant et al., 2014) in sewage has yet to be evaluated for RV and other enteric viruses which will be needed as the vaccine for RV is further distributed. Therefore, in this study, ddPCR was evaluated as a tool to detect and quantify RV in raw sewage, to compare the RV concentrations from different geographical settings and to optimize the best sampling preparation procedure to use that capable of quantifying RNA viruses in raw sewage and the volume of sample that can yield higher RV concentration three methods of virus concentration was used.

One of the findings of this study is that the use of methods for virus recovery that are less expensive for instance, the use of bag mediated filtration system (BMFS) using ViroCap filters, a method that does not require expensive equipment because filtration is done via gravity (see appendix A.1) and can be used in any laboratory in low resource country and still yield sufficient RV genome copies as evident by the range of RV concentration quantified in Kenyan samples (3.24E+03 gc/L to 5.84E+05 gc/L). When the two methods (ViroCap Vs PEG) were compared using samples from the same US lagoon the mean difference of RV concentration (7.67E+02 Vs 1.48E+04) was statistically significant $p<0.05$. The difference of this variation could have been due to the use of glycine-beef extract during the elution of viruses, which could have added some inhibitors

in the samples, although the use of ddPCR has been showed to minimize any inhabitation during PCR amplification (Racki et al., 2014a).

This study also provides useful data and gives insight on RV concentration in raw sewage in Kenya. This is important because no other study has documented or reported on the RV concentration in Kenyan wastewaters, although other data for instance, on the prevalence and occurrence of RV in Kenya water sources is available (Kiulia et al., 2015; Kiulia et al., 2010). The present study has also provided additional data on occurrence and RV concentration in the US and using droplet digital PCR. There was high concentration of RV detected in US WWTP than the lagoons as showed in Figure 3.1. The reason for these low levels in lagoon could have be due to photo-inactivation of the virus through sunlight, dilution effect from the grey water that could reduce the viral loads being discharged to the lagoon system. In Kenya, the lagoons are designed in a way where all residential and commercial building sewer lines have a single pipe that combines both the grey water and sewage from residential and business premises and the wastewater and sewage is directly discharged to the lagoon.

Globally, very few studies have reported the data on RV concentration. Quantitative and quality data are needed is needed to estimate the health risk of RV infection to help policy decision making on the control of diarrhea caused by RV. Despite recent advances in molecular biology of infectious pathogens and the use of other advanced molecular tools like such as qPCR many challenges to quantify dsRNA virus are expected due to the step needed such as denaturing of the ds RNA, reverse transcription and amplification and other factors like presence of inhibitors in the sample which limits accurate quantifying the viruses (Racki et al., 2014a; Racki et al., 2014b). The ddPCR, has revolutionized this process where absolute quantification of viral RNA is achieved without the need of

developing a standard curve and that virus can be quantified with precision and with more accuracy (Hall Sedlak and Jerome, 2014; Lui and Tan, 2014; Racki et al., 2014b; Sedlak and Jerome, 2013).

To determine if ddPCR can be used to detect and quantify RNA viruses in sewage, primers and probes that have been used to detect RV in clinical and environmental samples were used and known RV standards of RV from ATTC as a positive control. In the present study, we found that the RT-ddPCR assay was easier to use with RV and EV RNA because the time and step required to develop the standard curve for absolute quantification in qPCR assay was not applicable for this assay. Therefore, the RT-ddPCR can be applied when quantifying other RNA viruses in environmental samples. As the ddPCR assay prices comes down and as more studies to quantifying enteric viruses in clinical and environmental samples, this assay can in future replace qPCR.

The results from this study highlights how raw sewage can harbor most viruses that could cause infection to the naïve population. For instance, rotavirus one of the major causes of severe diarrhea in infants and young children (Tate et al., 2016) was detected at higher concentration on all the samples analyzed. This virus has a vaccine that is already in use in most countries globally, which is a live attenuated vaccine.

This study had some limitations; the molecular characterization of strains circulating of the positive samples could have been done to determine the genotype diversity of RV and EV in raw sewage. The other limitation was that we did not use both virus concentration method in all sampling sites. For instance, in Kenya only the ViroCap method was used while in California virus it was the PEG method. Thus, to make a fair comparison we could have concentrated the virus in all the sampling sites using the three virus concentration methods.

The quantification of viruses is critical in the assessment of the efficiency of virus removal during wastewater treatment and also in QMRA. The data generated from this study has added more quantification data on the occurrence and quantification of RV in sewage since very limited data have been reported.

CHAPTER 6: CONCLUSION AND FUTURE RESEARCH

This study examined two methods used for virus recovery and concentration methods. The bag mediated filtration system (BMFS) using ViroCap filters was an inexpensive method when concentrating larger volume samples. Therefore, we recommend the use of BMFS with ViroCap for routine monitoring of viruses in polluted water in low resource countries. While PEG and HA membrane filters can be used across all environmental virology laboratories where resources are available. Although in our study the ViroCap did not perform compared to PEG to concentrate viruses in raw sewage. This should be further examined.

This thesis also highlights the benefit of environmental surveillance for EV and RV in raw sewage and untreated water sources as a tool to supplementary clinical data that are being generated in the ongoing hospital and laboratory based surveillance for clinical enteric viruses. Rotavirus infection and thus the occurrence of the virus in sewage is a global phenomenon and if the population is not vaccinated and the sewage is not adequately treated there is a significant risk of waterborne disease. Studies focusing on RV removal during wastewater treatment processes should be carried out since only a few studies have reported on treatment particularly as the push for water reuse is implemented in areas where water is scarce. More research is needed that focuses on quantification of RV concentrations in raw and untreated sewage, since partial or limited data are available.

Research on the environmental surveillance of RV and other enteric viruses of public health importance should be encouraged. In this study, we did not characterize if the RV strains identified were wild-type or vaccine type. Therefore, future research should focus

on carrying out molecular characterization and typing of the enteric viruses detected in raw sewage to ascertain the diversity of the genotypes that may be circulating in the community and also to monitor the emergence of new genotypes and serotypes that might emerge as a result of vaccine selective pressure due to the use of the monovalent or pentavalent RV vaccine globally. These studies will be helpful in monitoring the impact of the RV vaccination in the population since the typing of the detected virus isolates in raw sewage by differentiating between the wild type and vaccine strains will give an indication if the vaccine coverage is optimal in the community. Other studies should be carried out to develop droplet digital PCR assays and protocols for genotyping of RV and/or see if the ddPCR platform can be used as a tool for typing of RV strains. If this is achieved it will be a breakthrough since the current typing methods relies on the use of conventional PCR with partial or full genome sequencing to assign the RV genotypes.

This thesis has contributed quantitative data for rotavirus and enterovirus in the global database. It has also for the first time provided new quantitative data on RV and EV in raw sewage in Kenya.

APPENDICES

Appendix A.1: Demonstrating the use of ViroCap filters during field sampling

Figure A.1: Illustration for the use of the bag mediated filtration system using ViroCap in the field. The bottom image shows the ViroCap filter.

Appendix A.2: Abstract presented at local and international conferences

A.2A: Kiulia NM, Rose JB. New Tools for Quantification and Detection of Rotavirus In Raw Sewage. [Oral Presentation]. IWA World Water Congress &Exhibition, Brisbane, Australia, 9th – 13th 2nd October 2016.

A.2B: Kiulia NM, Rose JB. Quantification of rotavirus in raw sewage to address global emissions to surface waters using digital droplet PCR. [Oral Presentation]. 1st Africa Graduate Student Association (AGSA) – Michigan State University, Michigan, USA, 2nd April 2016.

A.2C: Kiulia NM, Rose JB. Quantitative detection of rotavirus and enterovirus in raw sewage using reverse transcription droplet digital PCR. [Poster Presentation]. ASTMH 66th Annual Meeting, The Baltimore Convention Center Baltimore, Maryland USA, 5th – 9th November 2017.

Appendix A.3: Article published in peer review journal

A.3A: Kiulia NM, Hofstra N, Vermeulen L, Obara MA, Medema GJ, Rose JB. Global occurrence and emission of rotaviruses to surface waters. Pathogens 2015; 4(2), 229-255.

Appendix A.4: Visual representation of droplet digital PCR results

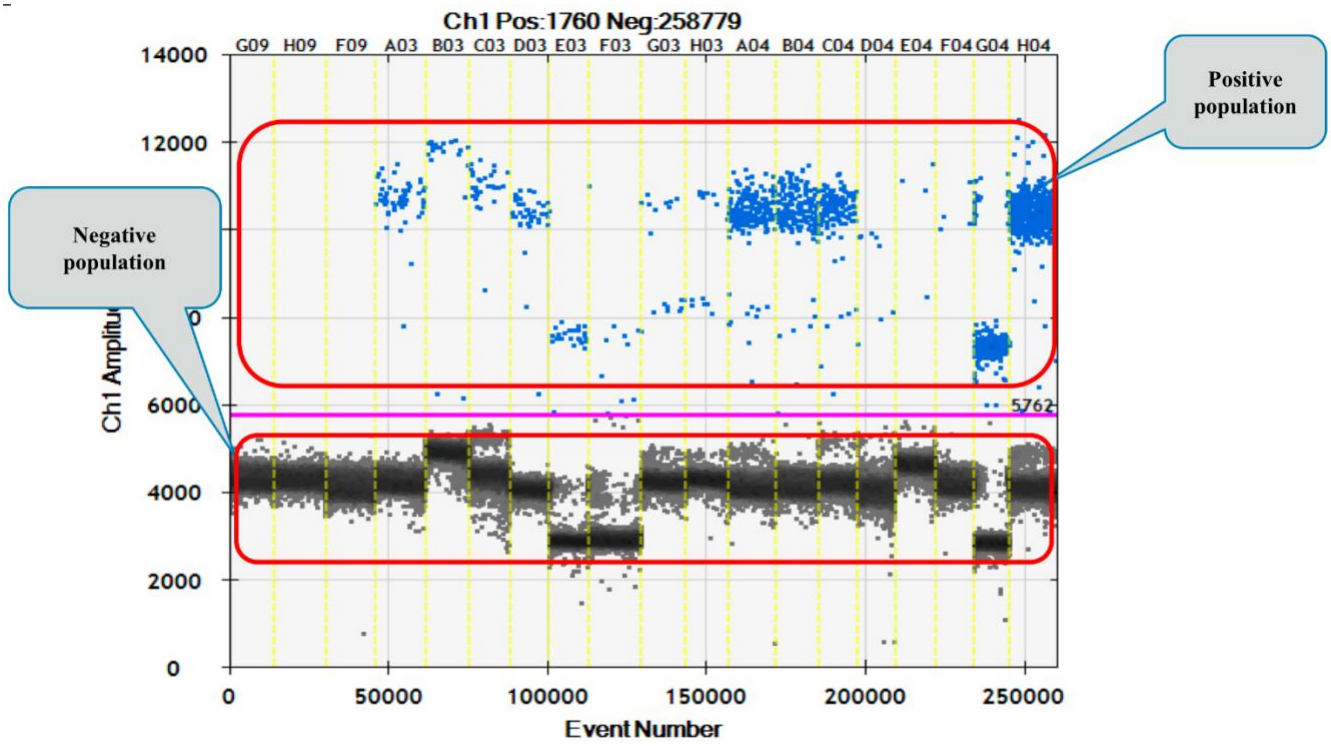
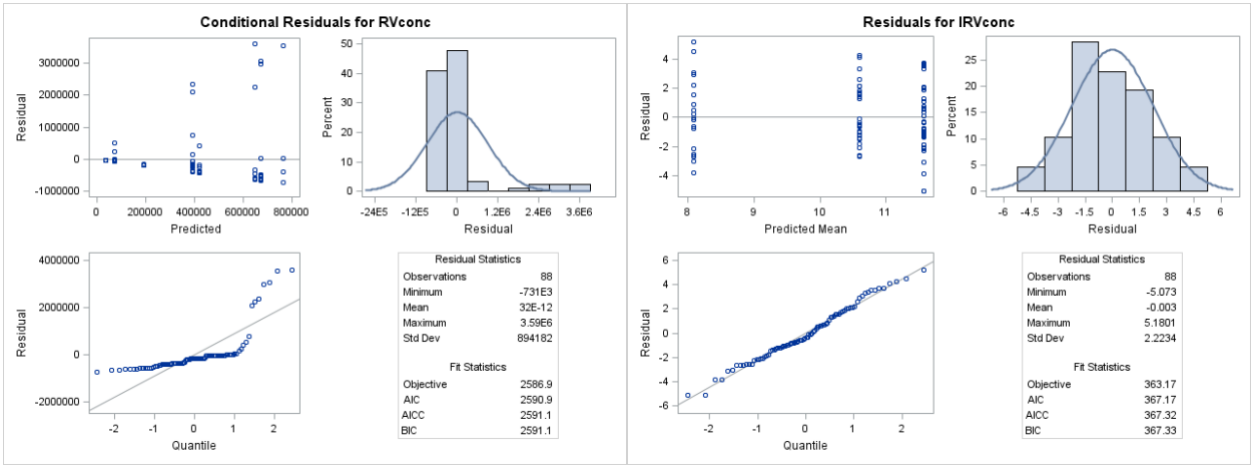


Figure A.2: Visual image showing the ddPCR results for RV after flow enumeration.

The upper cluster shows the positive droplets while the lower cluster shows the negative droplets. The y axis represents the channel 1 amplitude (fluorescent intensity) while and the x axis represents the total number of event in each sample.

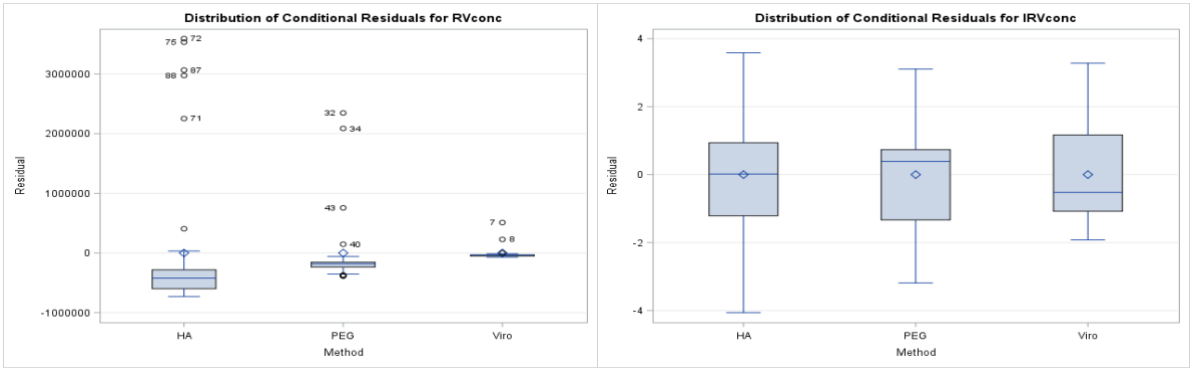
Appendix A.5: SAS output



a **b**

Figure A.3: Normal probability plots and histograms of the residuals.

(a) original/untransformed data and (b) log transformed data.



a **b**

Figure A.4: Plots of the residuals vs. predicted values and side-by-side box plots of the residuals.

(a) Data Not transformed, (b) after log transforming the data.

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Method	2	5	2.41	0.1849

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Method	2	5	1.43	0.3226

Figure A.5: Levene's test output of untransformed and log transformation data.

Differences of Least Squares Means									
Effect	Method	_Method	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
Method	HA	PEG	0.9729	1.5617	5	0.62	0.5606	Tukey-Kramer	0.8146
Method	HA	Viro	3.4724	1.5735	5	2.21	0.0784	Tukey-Kramer	0.1628
Method	PEG	Viro	2.4995	1.8006	5	1.39	0.2238	Tukey-Kramer	0.4138
Method	HA	PEG	0.9729	1.5617	5	0.62	0.5606		.
Method	HA	Viro	3.4724	1.5735	5	2.21	0.0784		.
Method	PEG	Viro	2.4995	1.8006	5	1.39	0.2238		.

Figure A.6: Tukey-Kramer for pairwise comparison between the methods (HA, PEG and ViroCap).

Appendix A.6: SAS code used for data analysis

```
data RV;
input Site$ Method$ RVconc;
lRVconc=log(RVconc);
datalines;
KE      Viro    3.24E+03
KE      Viro    4.20E+03
KE      Viro    5.52E+03
KE      Viro    1.50E+04
KE      Viro    3.04E+04
KE      Viro    7.92E+03
KE      Viro    5.84E+05
KE      Viro    3.01E+05
KE      Viro    6.12E+04
KE      Viro    7.24E+04
MIBA    PEG     2.76E+03
MIBA    PEG     9.72E+03
MIBA    PEG     2.23E+04
MIBA    PEG     3.00E+03
MIBA    PEG     1.16E+04
MIBA    PEG     2.35E+04
MIBA    PEG     1.51E+04
MIBA    PEG     2.27E+04
MIBA    PEG     2.81E+04
MIBA    PEG     9.00E+03
MIBB    Viro    1.54E+03
MIBB    Viro    2.49E+02
MIBB    Viro    7.20E+01
MIBB    Viro    2.24E+02
MIBB    Viro    3.71E+02
MIBB    Viro    2.83E+03
MIBB    Viro    2.52E+02
MIBB    Viro    1.75E+03
MIBB    Viro    1.58E+02
MIBB    Viro    2.26E+02
CA      PEG     1.68E+05
CA      PEG     2.74E+06
CA      PEG     2.18E+05
CA      PEG     2.48E+06
CA      PEG     1.96E+05
CA      PEG     3.72E+04
CA      PEG     1.43E+04
CA      PEG     1.93E+05
CA      PEG     6.60E+03
CA      PEG     5.38E+05
CA      PEG     5.04E+03
CA      PEG     2.47E+05
CA      PEG     1.15E+06
CA      PEG     1.45E+05
```

```
CA      PEG    1.50E+04
CA      PEG    2.15E+04
CA      PEG    3.32E+05
CA      PEG    3.35E+05
VBH     HA     6.63E+02
VBH     HA     6.63E+02
VBH     HA     1.24E+04
VBH     HA     4.80E+03
VBH     HA     3.11E+04
VBH     HA     3.67E+04
VBH     HA     1.33E+04
VBH     HA     1.60E+04
VBH     HA     4.89E+04
VBH     HA     2.30E+05
VBH     HA     8.27E+05
VBH     HA     1.83E+05
VJR     HA     1.91E+05
VJR     HA     2.98E+04
VJR     HA     2.71E+04
VJR     HA     4.58E+04
VJR     HA     1.56E+04
VJR     HA     4.58E+04
VJR     HA     4.80E+04
VJR     HA     4.13E+04
VJR     HA     3.20E+05
VJR     HA     2.03E+05
VJR     HA     2.90E+06
VJR     HA     4.24E+06
VNA     HA     3.82E+05
VNA     HA     7.97E+05
VNA     HA     4.30E+06
VNA     HA     3.40E+04
VVI     HA     1.16E+05
VVI     HA     7.11E+04
VVI     HA     1.51E+05
VVI     HA     2.22E+03
VVI     HA     1.07E+04
VVI     HA     3.16E+04
VVI     HA     7.69E+04
VVI     HA     1.87E+05
VVI     HA     6.89E+05
VVI     HA     2.03E+05
VVI     HA     3.74E+06
VVI     HA     3.65E+06
;

* Write and check the model
RVconc = mu + method + Site(Method)+ e1
88        1    2        5          80
Where e1= is error term in the individual samples;
```

```
TITLE "This is for initial analysis -checking the model";
PROC MIXED data=RV method=type3;
CLASS Method Site;
model RVconc= Method;
random Site(Method);
run;

ods graphics on;
TITLE "Assumption checking and getting the residuals";

proc mixed data=RV plots=all;
class Method Site;
model RVconc= Method/outp=myres;
random Site(Method);
/* outpred= your_new_data_set_name -
this creates the new data set where the output results will be*/
run;
proc print data=myres;
run;
TITLE "Levene's test and getting squared values of the residuals";
data myres;set myres;
sqres=resid*resid;
*testing;
proc mixed data=myres;
class Method Site;
model sqres= Method;
random site(Method);
run;

ods graphics off;

ods graphics on;
TITLE "Assumption checking after log-transformation";

proc mixed data=RV plots=all;
class Method Site;
model lRVconc=Method/outp=myres;
random Site(Method);
run;

data myres;set myres;
sqres=resid*resid;
proc mixed data=myres;
class Method Site;
model sqres=Method;
random Site(Method);
run;
quit;
ods graphics off;
```

```
ods graphics on;

TITLE " Main analysis using method as factor, Note the method is nested within the
sites -Sasha";

proc mixed data=RV;
class Method Site;
model lRVconc=Method;
random Site(Method);
run;

TITLE "Analysis with accounting for equal and unequal variances";

proc mixed data=RV;
class Method Site;
model lRVconc=Method;
random Site(Method);
run;

proc mixed data=RV;
class Method Site;
model lRVconc= Method/ddfm=SATTERTH;
repeated /group= Method;
random Site(Method);
run;

TITLE "treatment means (methods) and their standard errors";
proc mixed data=RV;
class Site Method;
model lRVconc=method/ddfm=SATTERTH;
repeated /group=method;
random Site(Method);
lsmeans method/pdiff alpha=0.05;
run;
quit;
ods graphics off;
ods graphics on;

TITLE " Getting the treatment means (methods) and their standard errors
in the analysis with equal variances and Post Hoc Pairwise comparison";

proc mixed data=RV;
class Method Site;
model lRVconc=method;
random Site(Method);
lsmeans method/pdiff adjust=tukey;
lsmeans method/pdiff;
run;
quit;
```

```
ods graphics off;

TITLE " TTEST for the to compare the mean rotavirus concentration in the Michigan
lagoon";

data RV;
input Rvconc Site;
lRVconc=log(RVconc);
cards;
2.76E+03     1
9.72E+03     1
2.23E+04     1
3.00E+03     1
1.16E+04     1
2.35E+04     1
1.51E+04     1
2.27E+04     1
2.81E+04     1
9.00E+03     1
1.54E+03     2
2.49E+02     2
7.20E+01     2
2.24E+02     2
3.71E+02     2
2.83E+03     2
2.52E+02     2
1.75E+03     2
1.58E+02     2
2.26E+02     2
;
proc print;
run;

TITLE "PROC TTEST MIBA vs MIBB";
Proc ttest data= RV;
class Site;
var Rvconc;
RUN; /* MIBA =1  MIBB =2/*
/*nnnnnn*/

TITLE "PROC TTEST MIBA vs MIBB";
Proc ttest data= RV;
class Site;
var lRVconc;
RUN; /* MIBA =1 MIBB =2/*
```

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