SPATIAL AND TEMPORAL DISTRIBUTION OF ENTERIC VIRUSES IN WASTEWATER AND SURFACE WATER

by

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A Dissertation Submitted to the College of Science in Partial Fulfilment of the Requirements for the Award of the Degree of Master of Science in Biology and Biotechnology of BIUST

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January, 2018

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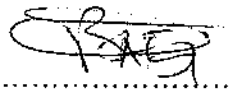
Signature  .....

CERTIFICATION

The undersigned certifies that she has read and hereby recommends for acceptance by the College of Science, a dissertation/thesis titled: "Spatial and temporal distribution of enteric viruses in wastewater and surface water", in fulfilment of the requirements for the degree of Master of Science in (Biology and Biotechnological Sciences) of the BIUST.

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Dr Lemme P. Kebaabetswe

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Date: 09/05/2018

ACKNOWLEDGEMENTS

I wish to convey my sincere gratitude and appreciation to my supervisor Dr Lemme P. Kebaabetswe for her professional guidance, valuable constructive comments and patience. I could not wish for a better and friendlier supervisor.

I am thankful to the Botswana International University of Science and Technology (BIUST) for sponsoring this project, the African Network of Earth Science Institutions (ANESI) for offering me a Mobility Grant to do my preliminary sample analysis at the University of Pretoria, African German Network of Excellence in Science (AGNES) Mobility Grants for Junior Researchers for funding me to complete my sample analysis at the University of Pretoria. Without their financial support this project would never have been successful. I am particularly grateful to Dr Janet Mans, Professor Maureen Taylor, and staff at the Medical Virology Laboratory (University of Pretoria) for their warm hospitality during my research visits. I am grateful for all your efforts, assistance and advices.

I thank the entire staff and postgraduate students of the Department of Biological Sciences and Biotechnology for their various contributions to this study.

Lastly but not least, I would like to express my very great appreciation to my family; their amazing love and support enabled me to endure.

DEDICATION

I dedicate this piece of work to my husband, Redman, my son Masa and daughter Asele. Thank you for always being there for me; your support kept me going.

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List of Abbreviations

bp – base pairs

cDNA – complementary deoxyribo nucleic acid

dsRNA – double stranded RNA

HBGA - histo-blood group antigens

IRF – Interferon regulatory transcription factor

MBR – Membrane bioreactor

ML - Mega Litres

NoV – Norovirus

NSP – Non-structural proteins

nt – terminal nucleotide

ORF – Open reading frame

PABP – polyA binding protein

PBS – Phosphate buffer solution

PEG – Polyethylene glycol

RoV – Rotavirus

rt RT PCR – real time reverse transcriptase polymerase chain reaction

VP – viral proteins

WTP - Wastewater treatment ponds

Abstract

Waterborne diseases, especially infectious diarrhoea, remain a public health concern particularly in developing countries where many lack access to safe clean water. The quality of water is assessed using bacterial indicators. However, they may not fully imitate the threat from other non-bacterial pathogens like enteric viruses. The aim of the study was to ascertain and establish the viral load, the seasonal and spatial distribution of rotavirus and norovirus (GI and GII) in sewage and river water samples. A total of 59 samples of raw and treated sewage as well as surface water, were collected from a sludge activated wastewater treatment plant in Gaborone, and Notwane River. Viruses were recovered from water samples and concentrated using the Polyethylene glycol/NaCl precipitation. The detection of enteric viruses was performed using molecular analysis (real-time RT-PCR) in concentrated water samples collected over a period of 12 months from November 2015 to October 2016. The enteric viruses were detected throughout the study period except for norovirus GI, which was not detected in June. Rotavirus was the most prevalent and was detected throughout the study period with the highest number of positive samples (76.3%), followed by norovirus GII (42.4%) and norovirus GI (38.9%). Norovirus GI had the highest number of positive samples in May (where all the samples collected were positive), followed by December and February.

The enteric viruses were detected in all the study sites. Norovirus GI was mostly detected in S1 (inlet), followed by S5 (Notwane river in Matebele). Detection rate in S2 (secondary settling tank), S3 (maturation ponds) and S4 (Notwane river in Oodi) was almost the same. Similarly norovirus GII was mostly detected in S1 followed by S4. Sites S2, S3 and S5 had same detection rates. Rotavirus was the most prevalent with the highest detection in all the sites. S1 and S2 recorded the highest number of positive samples, whereas S3, S4 and S5 had the same numbers. The viral loads were still high after the treatment process. There was no significant association between physicochemical parameters and viral loads, except for pH which had a significant relationship with rotavirus and norovirus GII ($p = 0.05$). This study enhances an understanding of the occurrence and quantification of the enteric viruses in wastewater before and after treatment, which is important to guide policy makers in devising relevant interventions that could determine disinfectant dose and ensure sanitary safety levels of treated water.

CHAPTER 1
INTRODUCTION

1.1 Introduction

Waterborne diseases, especially diarrhoea remains a public health concern worldwide, particularly in developing countries where many lack access to safe clean water (Njume and Goduka, 2012). Diarrhoeal diseases remain the second most common cause of under 5's mortality, and rotavirus and norovirus being the most common causing agents in both developed and developing countries (Tamura *et al.* 2010). The most common form of diarrhoea is the infectious diarrhoea, which is largely associated with bacterial pathogens, viruses and protozoa (Palombo, 2006; Hodges and Gill, 2010). Even though diarrhoea caused by bacterial pathogens and protozoa is common, enteric viruses cause significant health issues due to low infectivity (Wyn-Jones and Sellwood, 2001). Given similar exposures, infection risk from viruses is reported to go upto 10 000 fold greater than from pathogenic bacteria (Fong and Lipp, 2005).

Enteric viruses can occur naturally in environmental waters, but mostly get introduced through anthropogenic activities such as sewage leakages, urban runoff, and agricultural runoff (Haramoto *et al.* 2004; Lodder and Husman, 2005). Sewage discharge is known to be the main pollutant of water sources and therefore it needs an adequate treatment before disposal in the environment (Payment *et al.* 2001). These viruses are conveyed via the fecal–oral route, where they have been implicated in diarrhoeal outbreaks resulting from contaminated drinking water, recreational and urban waters (Fong and Lipp, 2005). Sapoviruses, astroviruses, rotaviruses, noroviruses and adenoviruses are the main enteric viruses known to cause diarrhoea in humans (Clark and McKendrick, 2004; Lopman *et al.* 2004; Ren *et al.* 2013).

In Botswana, little is known about the incidence and persistence of enteric viruses in water sources despite their importance in causing diarrhoea, a significant public health concern in the country. Enteric viruses have been detected from clinical and food samples (Basu *et al.* 2003; Kasule *et al.* 2003; Mattison *et al.* 2010; Kebaabetswe *et al.* 2005). This study therefore intends to detect enteric viruses with focus on rotavirus and norovirus from different water sources in urban and rural Botswana, using molecular techniques.

1.2 Statement of problem

Diarrhea is caused by pathogens such as parasites, viruses, or bacteria and characterized by frequent and watery bowel movements. Like other waterborne diseases, diarrhea is primarily spread through contaminated water. In Botswana, diarrhea is one of the leading causes of morbidity (8.8% of all causes) and mortality (9.9% of all deaths) (Central Statistics office, 2015).

The presence of enteric viruses in the environment poses a health hazard to communities, particularly where wastewater effluent is discharged into water sources which run through human settlements and the water is used for agricultural and domestic purposes. Even though over 97% of Batswana are reported to have access to safe water (IBP Inc, 2008), inequities in access to safe water still exist. Many rural communities still depend on untreated water from open water bodies, exposing them to enteric viruses. Moreover, even with communities with access to safe water, enteric viruses are known to be resistant to disinfectants such as chlorine (Fong and Lipp, 2005; Dubois *et al.* 1997), and therefore can possibly present a health hazard even after treatment.

Discharge of wastewater effluent onto the environment is a primary anthropogenic activity that leads to a decline in water quality in many surface water bodies. Although wastewater is known to harbor pathogens, (Obi and Okocha, 2007), its role in transmitting the disease causing pathogens particularly enteric viruses has been overlooked (Peterson, 2001). Currently there is limited information about the environmental occurrence of enteric viruses in Botswana. The limitation retards efforts to devise relevant and effective interventions to curb the health problems associated with enteric viruses such as recurring annual diarrhoea outbreaks, and leads to the underestimation of their health burden. The few studies conducted in Botswana on enteric viruses (Basu *et al.* 2003; Mattison *et al.* 2010; Rowe *et al.* 2010; Kebaabetswe *et al.* 2005) were performed on clinical samples. Furthermore, the studies conducted in Botswana to investigate water quality mainly used bacterial and physicochemical indicators to assess water quality (Masamba and Mazvimavi 2008; Mfundisi *et al.* 2009; Mmualefe and Torto 2011; Tubatsi *et al.* 2014). Hence this study intends to shed light on the occurrence and distribution of enteric viruses in Gaborone wastewater treatment plant and downstream of the plant in Notwane River. The Gaborone wastewater treatment plant discharges the effluent in to the Notwane River, which flows across several settlements. The communities near the settlements use the water from the

river for domestic, recreational and agricultural purposes. The results of this study will therefore contribute to the knowledge base for management and control of virus-induced diarrhea and other related diseases. Moreover, it will inform policy in developing guidelines pertaining to reuse and treatment of wastewater with particular reference to enteric viruses. The study will also establish the environmental and public health risks posed by discharge of wastewater effluent into the river.

1.3 Objectives

1.3.1 General objective

This study sought to determine the seasonal and spatial occurrence of enteric viruses with a focus on rotavirus and norovirus in wastewater and surface water in Gaborone.

1.3.2 Specific objectives

To determine the variation of the enteric viruses' concentrations between the Gaborone wastewater treatment plant (influent and effluent) and surface water (Notwane River).

To examine whether the concentrations of enteric viruses differs across months.

To identify whether a relationship exists between physicochemical water quality and enteric viral loads.

To assess the efficiency of the treatment plant in removing enteric viruses from wastewater.

1.4 Significance of study

Enteric viruses are considered to be the leading etiological agents for gastroenteritis worldwide and implicated in about 30 – 90% of gastroenteritis cases (Bon *et al.* 1999; Clark and McKendrick, 2004; Haramoto, *et al.* 2004). The viruses get introduced into the environment, most importantly in water bodies, through anthropogenic activities such as sewage discharge, urban and agricultural runoff.

Obtaining information on viruses in the aquatic environment will consequently contribute to better public health as it will facilitate the provision of appropriate advice to the public and authorities on water management and treatment. Currently, drinking water safety is solely

assessed through bacterial indicators such as fecal coliforms which are often ineffectual proxies for pathogenic viruses. Diarrhoea outbreaks associated with drinking water that meets criteria for safe water have occurred and these may further prove that viral and protozoan pathogens maybe the cause. Moreover, this confirms shortcomings in water quality surveillance where only bacterial indicators are used to determine the quality of drinking water. The potential for using human enteric viruses as significant indicators of water quality is gaining more scientific interest (Lin and Ganesh, 2013); hence it is crucial that more research is done.

Different natural and anthropogenic activities interact to influence the distribution of enteric viruses, thus making their occurrence in the environment highly variable, and warranting thorough investigations. To curb the problem of enteric viruses and their associated health risks, it is important to take into consideration and attempt to eliminate factors that contribute to their high prevalence. Water quality monitoring and viral epidemiology are essential steps in identifying polluted and potentially unsafe water sources. There is often an underestimation of the public health burden of viruses and therefore doing environmental viral surveillance can shed more light on the possible health problems that could be associated with enteric viruses.

The proposed study therefore, intends to gather information on the occurrence and prevalence of norovirus and rotavirus, in a waste treatment plant and open water sources downstream of the plant. The knowledge and data generated from this study, will therefore help in safe guarding the health of communities that use water from these sources in their day to day activities, and guide policy makers in making evidence based decisions. The government of Botswana is considering recycling wastewater for domestic use, a step which requires research based evidence on the prevalence of enteric viruses in wastewater. Identification and quantification of enteric viruses in water sources is critical for the development of effective preventive measures against viral infections and diseases. Moreover, other researchers will find the information generated from this study useful as it will allow for comparative analysis between environmental isolates and clinical isolates.

The study will employ molecular techniques to detect and quantify enteric viruses of interest. To determine the public health risk caused by human enteric viruses in water, a reliable, sensitive, and practical method for detecting small concentrations of viruses is needed. In the past, culture methods with limited sensitivity were used and these made detection of viruses a

challenge. Molecular methods of virus detection such as polymerase chain reaction (PCR) assays have improved environmental virology research since they are more sensitive, specific and able to detect a wide group of viruses in environmental samples (Girones *et al*, 2010). PCR is one of the most-sensitive methods available for viral monitoring.

CHAPTER 2
LITERATURE REVIEW

2.1 Overview of enteric viruses

The term 'enteric viruses' refers to all viruses which may be present in the gastrointestinal tract and may cause disease or the infection may be asymptomatic (Fong and Lipp, 2005; Okoh *et al.* 2010; Hamza *et al.* 2011). Viruses belonging to *Adenoviridae* (adenoviruses), *Picornaviridae* (e.g enteroviruses), *Caliciviridae* (e.g noroviruses, caliciviruses), and *Reoviridae* (e.g rotaviruses) groups are the most commonly studied. Enteric viruses have been implicated in non-bacterial gastroenteritis caused by drinking contaminated water. They are found in the gastrointestinal tract of the host and hence their route of transmission is the fecal-oral route. High numbers of upto 10^{12} virus particles per gram of stool are usually detected in viral diarrhea patients (Gerba, 2000; Cook *et al.* 2004; Bosch, 2010). Transmission of enteric viruses can be through human contact, fomites, food, and water. Less than 10 viral particles can cause an infection and the probability of infection when one is exposed to one rotavirus is approximately 31%, with 1 Plaque-forming unit (PFU) required for causing diarrhoea in one in a hundred adults who had no previous exposure to the virus (Teunis *et al.* 2008; Bosch *et al.* 2006; Dennehy, 2015).

Enteric viruses are a threat to both human and animals. They are host specific and cause several diseases and symptoms in humans and animals. Viral infections mostly lead to diarrhea, but they do also lead to other complications such hepatitis, and conjunctivitis. Other diseases such as encephalitis and meningitis have been reported in immune-compromised humans (Lee and Kim, 2002; Okoh *et al.* 2010; Hamza *et al.* 2011).

Enteric viruses occur naturally in water bodies, however their presence is primarily due to anthropogenic activities which includes leaking septic systems, sewage, urban and agricultural runoff (Fong and Lipp, 2005; Bosch, 2010). The viruses can be conveyed through rivers, groundwater, inadequately treated water, as well as aerosols from sewage treatment plants (Lee and Kim, 2002). Pathogenic viruses from sewage and other fecal waste sources are the main pollutants of rivers (Lodder and Husman, 2005). The survival of viruses outside the host is

affected by various environmental conditions and factors such as heat, moisture and pH. These and other factors will vary in presence and extent among different environments (Rzeżutka and Cook, 2004). Sewage contaminated water is known to harbor over 100 viruses pathogenic to humans (Bosch *et al.* 2006).

Wastewater treatment plants have been very useful in improving the quality of wastewater effluent before being discharged into receiving waters. But on the other hand, they have been implicated for contaminating the same water bodies and introducing pathogens such as enteric viruses (Okoh *et al.* 2010). The treatment technique used in a particular wastewater plant determines the concentration of the viruses and other pathogens, and the relative risk of disposal thereof. Raw sewage contains high numbers of viruses and present treatment techniques fail to completely eliminate the viral pathogens, thereby making them environmental pollutants when treated sewage gets disposed into the environment. Wastewater treatment processes ranging from activated sludge treatment to chlorination techniques have been shown to eliminate approximately 50 - 90% of viruses from influent (Cloette *et al.* 1998; Okoh *et al.* 2010). Despite being used internationally as standards for monitoring water quality, bacterial indicators such as coliforms and *Escherichia coli* fail to give a reliable clue of the virological quality of water (Bosch *et al.* 2006).

The enteric viruses that are of primary health importance are the *Caliciviridae* particularly noroviruses, and *Reoviridae* (especially rotaviruses), which are considered the principal causing agents of gastroenteritis worldwide. This study will therefore focus on rotavirus and norovirus (GI and GII).

2.1.1 Structural organization of rotavirus

Rotavirus (RoV) belongs to the family *Reoviridae* which consists of 11 genera (Pesavento *et al.* 2003). About seven rotavirus serogroups, A to G, are known and only A-C is known to cause diseases in humans (Rahman *et al.* 2007) and serogroup A being the most common human rotavirus (Wyn-Jones and Sellwood, 2001).

The non-enveloped virus particles are icosahedral and measure about 70-75 nm in diameter. The virus genome is 18.5 kb genome with 10-12 segments encoding six structural proteins (viral proteins (VP) 1-4, 6 and 7) and five or six non-structural proteins (NSP1-NSP5/6) (Figure 2.1). The genome is double stranded RNA, and the complete virus particles have a triple layered protein capsid. The six structural proteins in the multilayered capsid organization integrate the bases for cell entry, host specificity, and the enzymatic functions needed for endogenous transcription.

Each genome segment codes for one viral protein, except for gene 11 which encodes for two viral proteins (NSP5 and NSP6). The innermost core layer has the VP2 the genomic RNA, enzymatic complexes, VP1 (the RNA-dependent RNA polymerase) and the VP3 (a guanylyl transferase and methylase). The intermediate layer is made up of VP6 whereas the outer layer consists of VP7 (glycoprotein) and VP4 (hemagglutinin and cell attachment protein); VP7 form the smooth external surface, and VP4 forms spikes that extends and protrudes from the viral surface (Figure 2.1B). The VP4 is the main attachment protein responsible for determining host range, virulence and induction of protective immunity while the VP3 serves as a capping enzyme for viral RNA (Ciarlet and Estes, 2001; Arias *et al.* 1996; Dennehy, 2007; Okoh *et al.* 2010). NSP4 is a virulent gene which functions as an enterotoxin. The NSP1 is an RNA binding protein which interacts with the interferon regulatory transcription factor 3 (IRF3). The NSP3 is mainly essential for viral protein translation (Groft and Burley, 2002). All the other non-structural proteins are essential for virus replication and they have been shown to affect replication (Estes *et al.* 2001).

The VP4 and VP7 are the two outer capsid proteins involved in the neutralization of the rotavirus (Steele *et al.* 2002; Kiulia *et al.* 2014). The VP7 stimulates production of neutralizing antibodies in the host, whereas the VP4 elicits production of neutralizing antibodies important

for rotavirus virulence (Steele *et al.* 2002). This study focused on detecting group A rotavirus, the major cause of morbidity (Kiulia *et al.* 2014). The real-time RT-PCR detection of rotavirus for the present study used primers and probe which targets the NSP3 in the rotavirus genome.

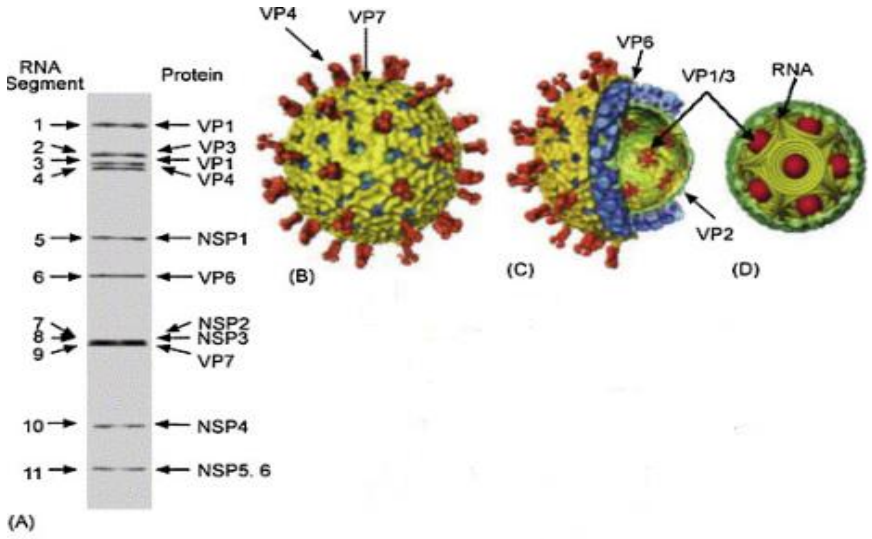


Figure 2.1: Structure of Rotavirus. The 11 segments and the proteins encoded by each segment (A), Outer viral proteins, VP4 and 7 (B), other structural proteins (C) and viral RNA (D). Modified from Desselberger (2014).

The rotavirus genome contains three ORFs (Open reading frames). The first ORF expresses non-structural proteins; ORF2 encodes capsid protein while the third ORF codes for a 212 amino acid protein whose function is not known (Mattion *et al.* 1991, Glass *et al.* 2000). The third ORF potentially plays a role in replication or assembly since its presence in the genome is conserved. The 11 segments of rotavirus have completely conserved 5'and 3'ends with a partially converted complementarity (Li *et al.* 2010; Desselberger 2014).

2.1.2 Rotavirus pathogenicity

The virus interactions with the host intestinal enterocytes are the initial steps of rotavirus infection. The virus attaches to the enterocytes through sialic acid containing receptors, with integrins acting as post-attachment receptors, all localized on lipid rafts of the enterocytes (Baker and Prasad, 2010) (Figure 2.2). The attachment is mediated by the VP7 subunit of VP4. The virus then enters the host cells through receptor-mediated endocytosis or direct membrane penetration, with solubilisation of the outer capsid proteins. The outer protein layer, formed by the spike protein (VP4) and the glycoprotein (VP7) is lost from the virion during entry, producing a double layered particle. The virus penetrates into the cytoplasm through the cytoplasmic or the endosome membranes. The VP4 will be cleaved by trypsin to produce VP8 and VP5 and consequently enhance rotavirus infectivity (Figure 2.2). This cleavage is required for the virus to enter the cell (Estes and Greenberg, 2013). Once within the host's small intestines, rotavirus will infect the enterocytes of the upper epithelium. The plus strand ssRNA is synthesized through mediation of the VP1, VP2 and VP3. Finally, the viroplasm forms, viral RNA packaging starts, while the minus strand RNA is synthesized, before the virus particles are released (Patton *et al.* 1999; Desselberger, 2014). The viroplasm is a large cytoplasmic inclusion body which is present in rotavirus infected cells and acts as a site in which viral (+) RNAs are packaged into cores and simultaneously replicated into dsRNAs. The NSP2 and NSP5 are the two proteins responsible for formation of viroplasm (Patton *et al.* 2006). Translation and transcription of the viral proteins occurs within the host cell. The VP7 and NSP4 are involved during the maturation of the viral particles until cell lysis (Gil *et al.* 2000; Estes and Greenberg, 2013; Okoh *et al.* 2010**)**. The NSP4 is also responsible for stimulating intestinal secretions and alters epithelial cell integrity.

Since rotavirus infection occurs in the small intestines which are responsible for digestion and absorption, the viral infection often results in loss of nutrients and water absorption, dehydration, malnutrition and ultimately leading to death (Pesavento *et al.* 2003). Rotavirus-induced diarrhoea manifests itself though several mechanisms including malabsorption secondary to enterocyte destruction, stimulation of the nervous system as well as villus ischemia. The severity of infection is affected by both viral and host properties and often associated with mild inflammation of the intestines, villus blunting and enterocyte vacuolization.

(Lundgren and Svensson (2003) mentioned that rotavirus generally causes diarrhoea by altering the function of the small intestinal epithelium.

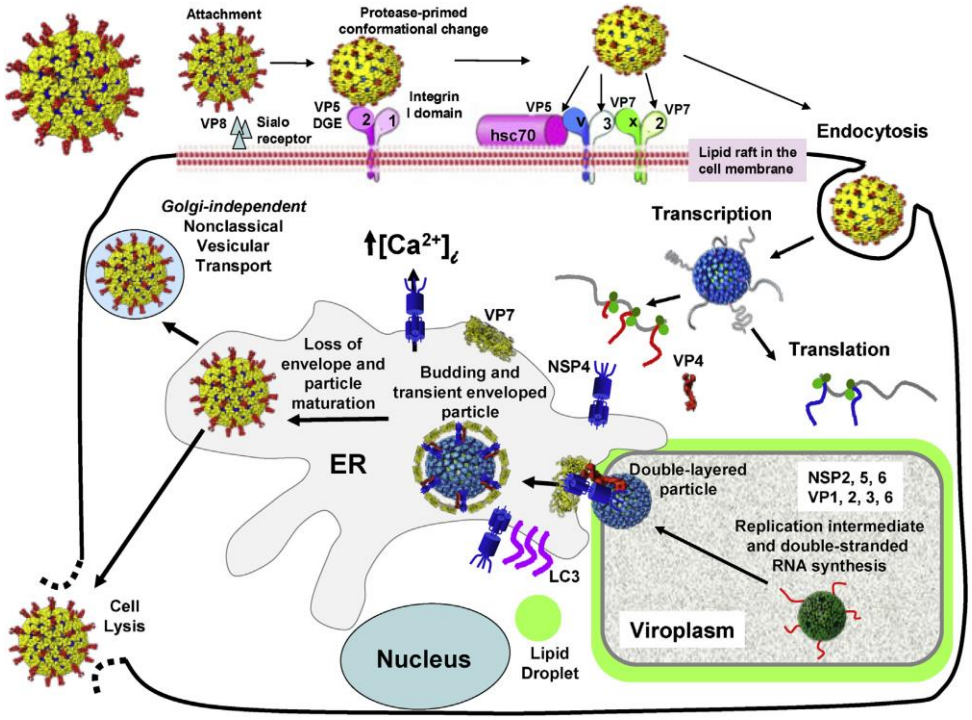


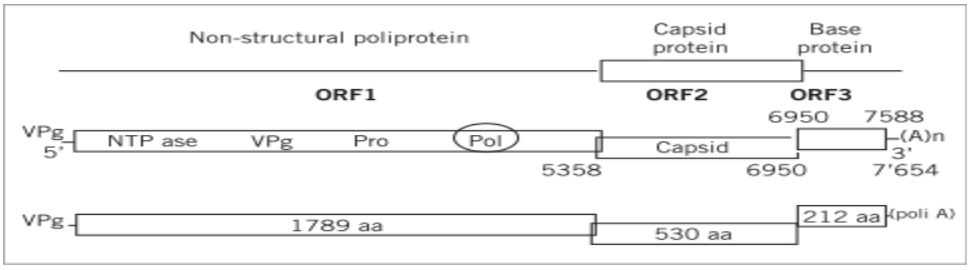
Figure 2.2: The rotavirus replication cycle from the attachment stage to virion release. From Estes and Greenberg (2013).

During cell infection, the rotavirus overcomes most of the antiviral mechanisms initiated by host cells. Being an obligate parasite, rotavirus takes over host machinery causing a shut-off of protein synthesis in host cell protein synthesis. The mechanism and translation initiation factors involved in this shut-off are not yet well understood, but it has been speculated that the process is mediated by the inhibition of the activity of translation factor eIF2 (Mitzel *et al.* 2003; Lopez and Arias, 2012). It is however possible that upon viral infection, the host cell continues to synthesize certain proteins and prevent the translation of viral mRNAS, while rotavirus on the other hand will respond by allowing the efficient translation of its proteins. Moreover, it is during the early infection stage that NSP1 and VP6 are expressed but more VP6 is expressed than NSP1 (Mitzel *et al.* 2003). The non-structural protein (NSP) 3 is responsible for inhibiting the translation of cellular mRNAs and modifies the intracellular localization of the polyA-binding protein (PABP) (Lopez and Arias, 2012).

2.1.3 Structural organization of norovirus

Norovirus (NoV, formerly known as Norwalk-like caliciviruses or small round-structured viruses), belong to the family *Caliciviridae* and consist of a non-enveloped, single stranded and positive-sense RNA genome (Gallimore *et al.* 2007), of approximately 7.5 - 7.7 kb (Okoh *et al.* 2010; Karst *et al.* 2015) and a diameter of approximately 38 nm (Donaldson *et al.* 2008). A triple layered protein capsid encloses the genome. The capsid consists of the outer capsid proteins VP4 (protease-sensitive) and VP7 (glycoprotein), inner capsid protein VP6, and the core proteins VP1, VP2 and VP3 (Hardy, 2005; Tayeb *et al.* 2010). Norovirus consists of a single major protein VP1 on its exterior surface that forms the capsid (Okoh *et al.* 2010). The VP1 has a shell (S) and protruding (P) domains. The S domain is responsible for controlling the shell structure whereas the P domain is for antigenicity and cellular receptor binding of the virus (Okoh *et al.* 2010). The viral RNA is covalently linked to VPg, a viral protein which provides a cap at the 5'end and implicated for transporting the genome to sites of negative strand synthesis (Figure 2.3) (Donaldson *et al.* 2008).

(A)



(B)

Figure 2.3: The human norovirus genome showing the nonstructural proteins in ORF1 and the structural proteins in ORF 2 and 3 (A). The ORFs 1, 2 and 3 have 1789, 530 and 212 amino acids respectively (B). From Scipioni *et al.* 2008.

Figure 2.3 shows that noroviruses have three ORFs. The first, ORF1, encodes the 200 kDa non-structural polyprotein cleaved by three viral 3C-like protease into 6 proteins, while ORF2 and ORF3 encodes the major 60 kDa VP1 and minor VP2 capsid proteins respectively, (Yan *et al.* 2012; Bull *et al.* 2005; Scipioni *et al.* 2008). The polyprotein in ORF1 is essential for viral

replication and VP2 for packaging the genome into virions. The norovirus Pol in ORF1 has been shown to be essential for *in vitro* viral replication (Donaldson *et al.* 2008, Thorne and Goodfellow, 2014; Mans *et al.* 2013).

The noroviruses consist of 5 genogroups (GGI, GGII, GGIII, GGIV, and GV) classified based on the major capsid protein sequence (Karst *et al.* 2015). Only genogroups GI, GII and GIV have been reported to cause human diseases, with GII being the most common causing majority of epidemics (Gallimore *et al.* 2007). This study therefore focused on norovirus GI and GII, and real time RT-PCR amplification of the polymerase/capsid region spanning the ORF1/2 junction was employed (Mans *et al.* 2014).

2.1.4 Norovirus pathogenicity

For norovirus to cause an infection, they start by attaching to host gut cells through association of attachment factors with the host protein receptors (Figure 2.4). Noroviruses have carbohydrate receptor binding properties which enable them to bind to the host's gut-expressed carbohydrates thereby enhancing its pathogenicity. These carbohydrates act as host cell receptors. The virus will recognize the terminal sialic acid residues of carbohydrates on the host surface for attachment and bind host cells via sialic acid moieties, glycolipids and glycoproteins (Hutson *et al.* 2004; Thorne and Goodfellow, 2014). Infection by noroviruses also depends on the identification of the human histo-blood group antigens (HBGAs) as receptors. This infection stage controls host exposure and resistance against noroviruses (Tan and Jiang, 2014). Upon entry into the host cell, the cellular translation mechanisms and the VPg at the 5'end of the genome will mediate viral genome translation. The viral protease NS6 will cleave the ORF 1 polyprotein before a replication complex is formed through NS1/2 and NS4 interactions. The NS7 will generate the genomic RNA from a negative strand intermediate using VPg dependent mechanisms. Finally, the viral replicated genome is then packaged and released (Thorne and Goodfellow, 2014). During norovirus infection, the VPg is essential for priming transcription and initiation of translation (Hodges and Gill, 2010).

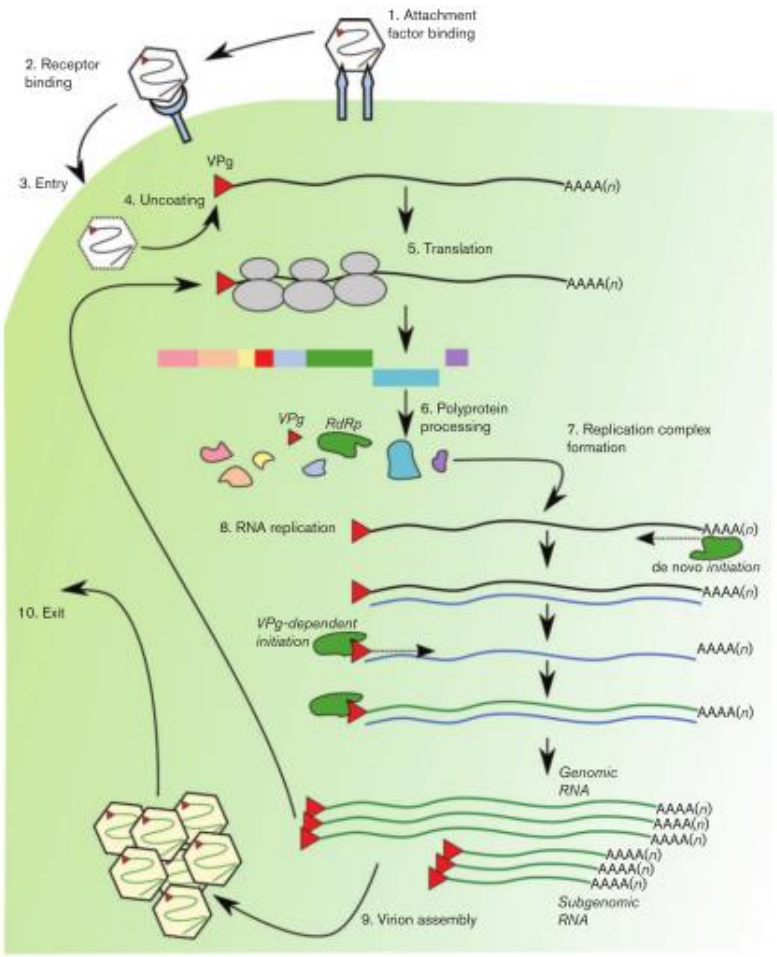


Figure 2.4: Norovirus reproductive cycle showing the virus particles starts by attaching and finally packaging and release. From Thorne and Goodfellow (2014).

Pathologically, infection of norovirus is accompanied by the villi broadening and blunting, limited absorption of substances such as D-xylose, fat and lactose as well as intestinal inflammation (Al-Sailawi, 2015; Karst *et al.* 2015). In addition to gastroenteritis, symptoms of norovirus infection include nausea, abdominal cramps, low grade fever and malaise (Dennehy, 2011; Lee and Pang, 2013). Even though norovirus-induced diarrhoea does not result is structural damage to the intestines; it does alter the secretory and/or absorptive processes (Troeger *et al.* 2009; Karst *et al.* 2015). The activity of the brush boarder enzyme is decreased during acute infection as well as other structural and functional changes of the duodenum have been reported to occur during infection (Troeger *et al.* 2009).

2.2 Seasonal and spatial distribution of rotavirus and norovirus

Enteric viruses are shed in large numbers by infected persons and have the ability to persists in the environment and survive wastewater treatment processes (Kiulia *et al.* 2010). Viruses persist longer in the environment than the enteric bacteria (Bosch, 2010). Their circulation in the environment has been shown to be seasonal (Arraj *et al.* 2008). Several studies have observed and detected viruses more frequently during winter and spring than summer (Nordgren *et al.* 2009; Tamura *et al.* 2010; Maunula *et al.* 2012; Lenaker *et al.* 2017), but seasonality varies between countries. Enteric viruses survive and tolerate fluctuating environmental conditions hence they are often able to survive for longer periods of up to months (Lin and Ganesh, 2013). Environmental factors such as land cover, hydrological conditions, pH, temperature and turbidity affect the fate of enteric viruses, which vary from one geographic location to the other (Lenaker *et al.* 2017; Yang *et al.* 2012; Elmahdy *et al.* 2016; Embrey *et al.* 2004).

Rotaviruses are spread through the fecal-oral route, water and contaminated surfaces (Lopman *et al.* 2012). They can survive on surfaces and water bodies for weeks. Even though rotavirus is detected in the environment all year round, literature has reported display of distinct seasonality, with the viruses being most prevalent during the cold dry winters (Parashar *et al.* 2006; Fischer *et al.* 2007). Basu *et al.* (2003) also noted seasonality, with peaks during autumn -winter, of rotavirus shedding in children with gastroenteritis in Botswana. However the seasonal peaks tend to vary from one geographic location to the other depending on local climatologically and anthropogenic factors (Sato *et al.* 2010). Furthermore, the occurrence of rotavirus species has been reported to vary spatially and temporally (Okoh *et al.* 2010).

Gastroenteritis caused by norovirus is highly seasonal. The infectious viruses tend to prefer cool, dry environments for optimum survival. Like rotavirus, transmission of norovirus occurs all year round, but cases and outbreaks have shown a cold weather peak with fewer summer peaks (Lopman *et al.* 2004). A study by Ayukekbong *et al.* (2014), detected norovirus in fecal samples all year round but noted a peak at the beginning of the rainy summer season. Human norovirus is quite stable outside its host and can remain infective for upto months outside its host (Karst *et al.* 2011; Kotwal and Cannon, 2014). The viruses are resistant to disinfectants and therefore can survive wastewater treatment processes and thus get discharged into the environment with the final effluent. The viral particles are reported to have the ability to survive for up to two weeks in

environmental surfaces and up to two months in water (Lopman *et al.* 2004), which enhances their stability in the environment.

2.3 Virus detection using real time reverse transcriptase – polymerase chain reaction (PCR)

The health concerns which arises from use and contact with wastewater warrants the need for reliable detection systems for pollutants, including pathogens. Enteric viruses have low infectious doses and occur in low levels in water bodies; therefore sensitive detection assays are vital. There is no tissue culture system for propagating viruses and electron microscopy had been routinely used in the latter years even despite having low sensitivity (Kageyame *et al.* 2003). However, since the 1980s, the use of molecular techniques has gained popularity over the conventional methods due to their immense versatility and sensitivity (Straub *et al.* 1995; Fong and Lipp, 2005). The conventional methods which were used before were laborious, time consuming and inefficient.

Real-time PCR has thus become the state-of-the-art technology for the detection and quantification of nucleic acids and it is reportedly 100 – 1000 fold more sensitive than conventional methods (Nadin-Davis *et al.* 2009). Real time RT-PCR is now an invaluable resource for environmental virologists (Connell *et al,* 2012). There has been some developments of and rapid growth of commercially available real time RT-PCR reagents and kits for detection which greatly increased the options available to laboratories and decreasing the risk of sample contamination. Real time PCR and commercial kits have been used to successfully detect enteric viral pathogens in the environment and wastewater (Rodriguez *et al.* 2009; Kiulia *et al.* 2010; Mans *et al.* 2013; Calgua *et al.* 2013). This technique has enabled detection of viruses which do not grow in cell culture. These commercial kits often use primer sets which exhibits the lowest detection limits under optimized conditions (Nadin-Davis *et al.* 2009; Rodriguez *et al.* 2009).

CHAPTER 3
METHODOLOGY

3.1 Description of study site

The study was conducted at the Gaborone wastewater treatment plant and along the Notwane River in Botswana. Gaborone is the capital city of Botswana and has a population of 231 592 (Central Statistics Office, 2011). The treatment plant has a capacity of 65 Mega Liters (ML) per day, with a current inflow average of 57 ML/day and outflow of approximately 50 ML/day. The plant services Gaborone city, Phakalane, Tlokweng, Mogoditshane and Metsimotlhabe. The latter are settlements located directly adjacent to the capital city Gaborone. Phakalane is 12.5km away from Gaborone; Tlokweng, 22km; Mogoditshane 8km; and Metsimotlhabe, 19.6km. The treatment plant receives mostly domestic wastewater, industrial wastewater, storm water and the septage from tankers. The water from the treatment plant is used by the City Council to water public gardens, some surrounding hotels also use it to water gardens, and by the government for the Glen Valley irrigation scheme. The rest of the final effluent is discharged into the Notwane River and several small scale farmers along the river use it for irrigating their crops. Some individuals also use the effluent for watering their private gardens.

The Gaborone wastewater treatment plant uses an activated sludge process which involves aeration of the wastewater with the help of microbial suspension. Following the aeration, the solid is separated from the liquid and the clarified effluent is discharged while the excess biomass discarded as waste or excess sludge. The rest of the remaining biomass is sent to the aeration tank. The plant comprises of the following: (i) Inlet works: where debris is passed through mechanically raked screens; flow measurement is regulated and degritting occurs, (ii) Primary settlement: where water is allowed to settle and sludge will be collected at the bottom, (iii) Activated sludge reactor with surface aerators and secondary settling tanks; and (iv) Maturation ponds in Broadhurst: The final step in treatment of wastewater is the polishing and disinfection of the effluent at the maturation ponds through exposure to sunlight before discharge into the Notwane River.

Notwane is a perennial river in south eastern Botswana traversing through Gaborone, Phakalane, Oodi, Matebele and other downstream settlements in Botswana. The river is

recharged by mainly rainfall and it is dependent on effluent from the Gaborone wastewater treatment plant. The sampling sites were selected across a transect from the wastewater treatment plant (S1 – S2) and along the Notwane river (S3 – S5), (Figure 3.1 and 3.2). The distance between the sampling sites is shown in Figure 3.2

3.2 Experimental design

The study area consisted of five (5) sampling points, S1-S5 (Figure 3.1).

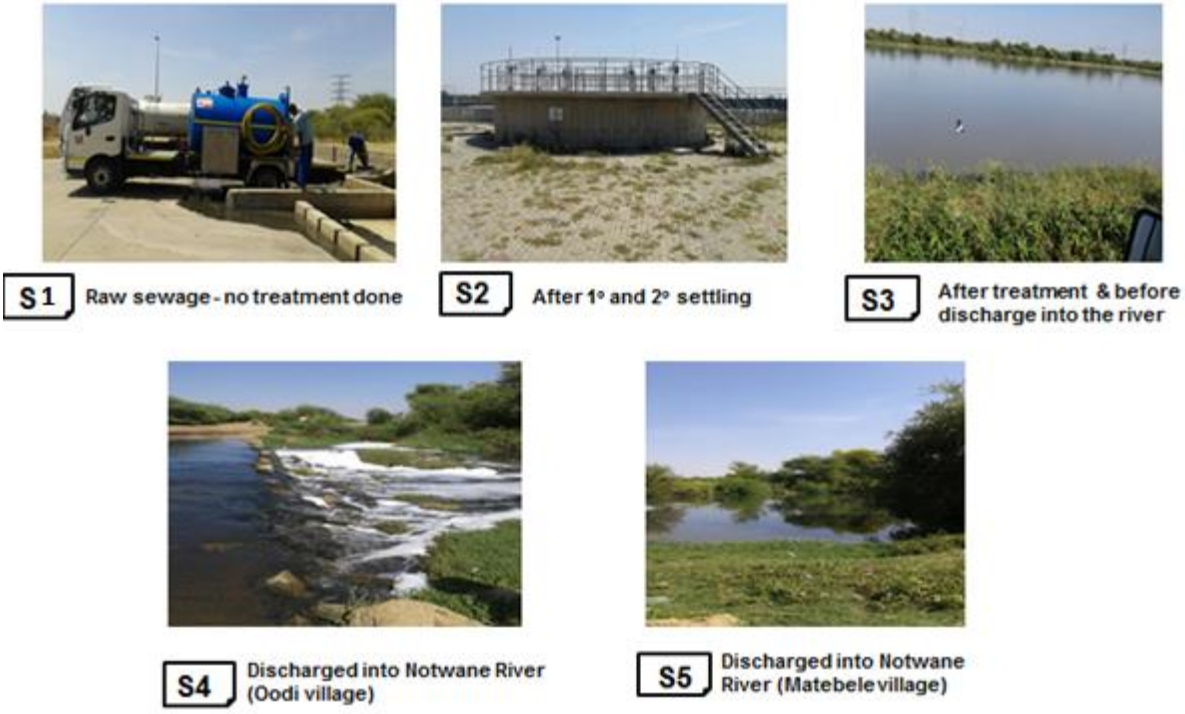


Figure 3.1: The five sampling sites identified in the study area, S1 – S5. The wastewater is received at S1, undergoes treatment at S2 and S3, before it is discharged into the river at S4 and S5.

Site 1 is the initial site where wastewater comes in from households and industries. The raw sewage water is separated from the debris/litter by going through the sieves. The samples collected here are raw sewage (influent).

Site 2 is the primary settling tank, after the influent has undergone the primary and secondary treatment involving the activated sludge treatment processes. The tank is circular with a collection hopper for collecting sludge, and fitted with a baffle for removing matter. The scum

forming on the surface is removed periodically while the sludge moves to hoppers by gravity where it is removed.

Site 3 is the converging point of the maturation ponds. There are 10 maturation ponds converging at this point and discharging effluent into the Notwane river. At this site, there are pipes that channel the water to different places for irrigation purposes. The maturation ponds are where tertiary treatment occurs which includes removal of nutrients and algae, and are shallow to allow penetration of light. At this stage the effluent quality is enhanced through the effect of sunlight to ensure the effluent quality is suitable for downstream reuse. Wastewater treatment ponds (WTPs), which consist of shallow engineered basins, stabilize and treat wastewater via natural processes driven by sunlight and the proliferation and predation of biological flora. The configuration consists of a facultative pond followed by one or more maturation ponds. Maturation ponds are designed to remove pathogens. Previous investigations have demonstrated that WTP systems consisting of a facultative pond followed by two maturation ponds can provide > 4-log10 removal of bacterial indicators (von Sperling and Mascarenhas, 2005); however, the extent of enteric virus removal in WTPs is not as well understood.

Site 4 is where Notwane River passes through Oodi village. The village has a population of 5,874 (Central Statistics Office, 2011). Residents use the water for agricultural purposes such as vegetable farming and domestic animals (cattle, goats) drink from the river.

The last sampling site is just after Oodi village, where the river goes on and passes through Matebele village (Site 5). The village has a population of 2,586 (Central Statistics Office, 2011). Similar to the community in Oodi, the residents in this village also use the water for agricultural purposes.

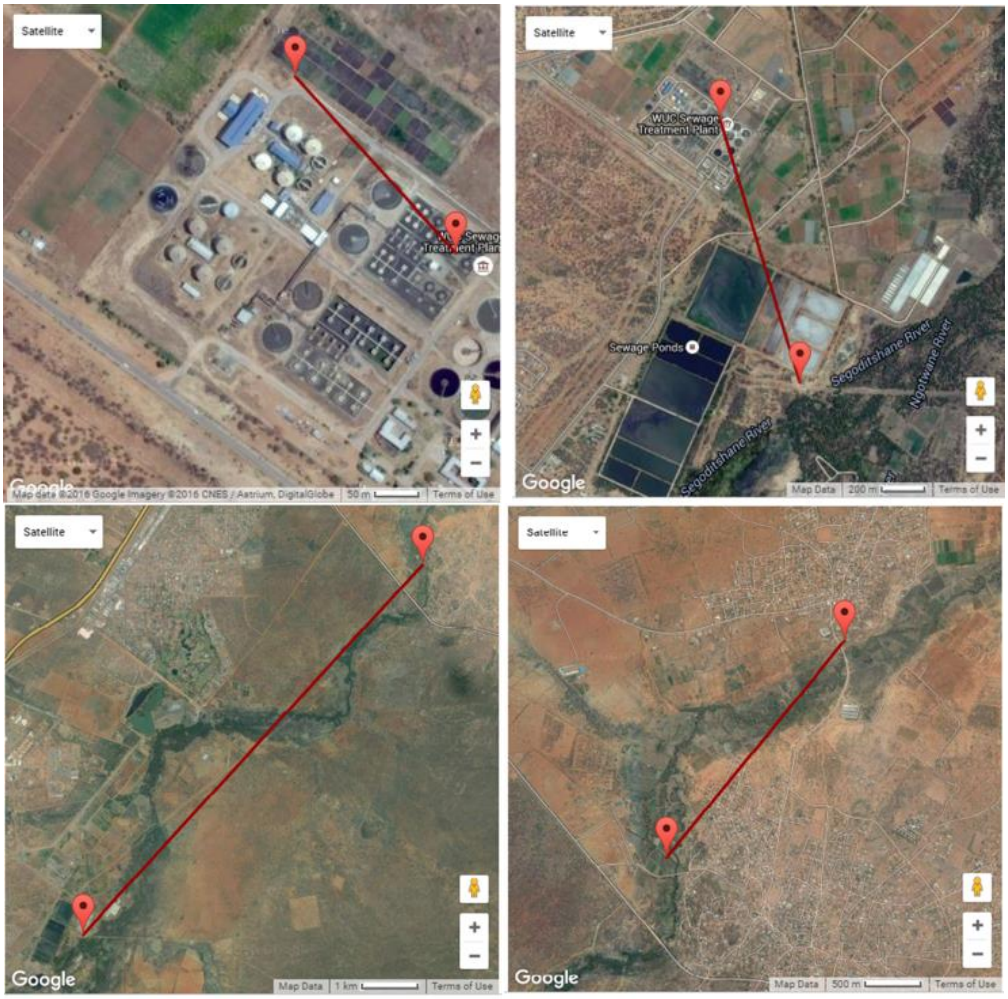


Figure 3.2: Satellite image showing distance between each sampling sites (S1-S5)**.** S1-S2 = 0.251 km, S2-S3 = 1.162 km, S3-S4 = 8.516 km, S4-S5 = 2.384 km.

3.3 Materials and methods

3.3.1 Wastewater samples collection

Samples were collected from a wastewater treatment plant and Notwane river on a monthly basis over a period of 12 months, and comprised of untreated and treated wastewater. Each month one sample was collected from each of the five sampling sites, giving a total of 59 samples after 12 months. (No sample was collected during the first month of sampling at site S4,).

All samples were collected between 0800 hours and 1000 hours, and 25 cm below the water surface. This was done to ensure consistency. The occurrence of viruses in water bodies varies across depth and with temperature. An assessment of a cross-sectionally integrated technique across different depth by Martin *et al.* (1992) showed that microbial water quality tends to improve in deeper waters (>25 cm below surface) and on the surface. Water samples (500 ml) were collected in autoclaved plastic bottles and kept in cooler boxes packed with ice until processing. The sample temperature, turbidity, dissolved oxygen and pH were recorded on site.

3.3.2 Virus concentration using polyethylene glycol (PEG)/sodium chloride (NaCl) precipitation

To prepare the PEG/NaCl solution, 500 g of PEG and 87 g of NaCl was dissolved in about 800 ml of distilled water. The PEG solution was then heated in a 50 ^{o}C incubator with constant stirring until it dissolved. The final volume was adjusted to 1000 ml and sterilised by autoclaving.

To concentrate the virus, 500 ml of water samples collected was divided into 250 ml portions and centrifuged for 10 minutes at 1000 x *g*. The supernatants were pooled and the pellets kept at 4 ^{o}C until further processing. The pH of the pooled supernatant mixture was adjusted to neutral (pH 7-7.2) using 1 M sodium hydroxide (NaOH) or 1 M hydrochloric acid (HCl). The final volume of the supernatant was measured and 0.25 volumes of the PEG/NaCl solution added to the supernatant in 1 L bottles. The supernatant was then incubated on ice for 1 hour in a shaker incubator followed by centrifugation for 30 minutes at 10 000 x *g*. The upper phase was discarded and the remaining pellet dissolved in 10 ml phosphate buffer solution (PBS). The same solution was used to dissolve pellets from the first step for the same sample.

Approximately 0.2 volumes of chloroform were added to the suspension and shaken vigorously for 1 minute. The suspension was centrifuged for five minutes at 10 000 x *g*. The upper phase was collected and transferred into sterile 15 ml tubes for storage at -20 ^{o}C. Part of the upper phase was aliquoted into 2 ml epperndoff tubes and used for RNA extraction.

3.3.3 RNA extraction

Commercial QIAamp UltraSens Virus extraction kits (Qiagen, Hilden, Germany), used by van Zyl *et al.* (2006), were used following the manufacturer's instructions. One (1) ml sample from the concentrated supernatant was thawed and equilibrated to room temperature (15–25 °C) and transferred into a 2 ml microcentrifuge tube. Buffer AC (0.8 ml) and carrier RNA solution (5.6 µl) were added to the sample. Samples were then spiked with 10 µl of mengovirus to assess recovery efficiency. Contents were mixed thoroughly by first inverting the microcentrifuge tube three times followed by vortexing for 10 seconds. The mixture was then incubated at room temperature for 10 minutes before centrifuging at 1200 x *g* for three minutes. The supernatant was discarded and 300 µl buffer AR warmed to 60°C and 20 µl proteinase K were added. The mixture was vortexed until the pellet was completely resuspended and incubated for 10 minutes at 40 °C and buffer AB added. The 700 µl lysate was carefully transferred to a QIAamp spin column and centrifuged at 5000 x *g* for one minute. The QIAamp spin column was placed into a new 2 ml collection tube while the tube containing the filtrate was discarded. Then 500 µl buffer AW1 was added to the QIAamp spin column and sample centrifuged at 6000 x *g* for 1 minute. After centrifuging, the QIAamp spin column was again placed into a new 2 ml collection tube before buffer AW2 (500 µl) was added to the QIAamp spin column and the sample centrifuged at full speed (16,000 x *g*) for 3 minutes. The filtrate RNA was stored at -80 ^{o}C until use.

3.3.4 Virus detection using real time RT-PCR

Real time RT-PCR was done with Roche LightCycler^{TM} instrument for screening and quantification of rotavirus and norovirus (GI and GII).

3.3.4.1 Detection of Rotavirus

The Rotavirus@CeeramTools^{TM} kit (Ceeram S.A.S., LAChappelle-Sur-Erdre, France), also used previously by Perez-Mendez *et al.* (2014), were used. Immediately before each test rotavirus nucleic acid extracts were heated at 95 ^{o}C for one minute to denature the double stranded RNA, and placed on ice until use. Five (5) µl of the extracted RNA was mixed with PC master mix, RT-PCR Enzyme mix and DNAse free water into capillary tubes. The master mix comprised of specific nucleotides sets with FAM^{TM} and VIC –TAMRA probes, ROX fluorescence reference as well as specific primers (Table B1). The RT reaction mixture was incubated in a LightCycler^{TM} instrument (Roche Molecular Biochemicals, Lewes, Sussex, UK) to estimate the relative concentration of the RNA recovered. The thermal cycling and amplicon detection incubations used were as outlined below- initial denaturation at 95 ^{o}C for five seconds, and 33 cycles of incubation at 96 ^{o}C. Measurement of the specific PCR amplicon concentration at the end of the incubation was done by measuring the fluorescent signal at 530 nm, the wavelength at which colour compensation was also done. Primers and probe used for real-time RT-PCR (Table B1) were targeting the conserved non-structural protein region, NSP3 (Jothikumar *et al.* 2009; Zeng *et al.* 2008). The negative and positive amplification controls were included.

3.3.4.2 Detection of Norovirus

Norovirus GI and GII were detected quantitatively with commercial kits, NorovirusGI@CeeramTools^{TM} and NorovirusGII@CeeramTools^{TM} kits, (Ceeram S.A.S., LAChappelle-Sur-Erdre, France), also used by Baert *et al.* (2011). Five (5) µl of the extracted RNA was mixed with PC master mix, RT-PCR Enzyme mix and DNAse free water into capillary tubes. The master mix comprised of probes as well as specific primers (QNIF4 and NVILCR as forward and backward GI primers respectively; QNIF2D and COG2R as forward and backward GII primers respectively) (Table B2). The reaction mixture was incubated in a LightCycler^{TM} instrument (Roche Molecular Biochemicals, Lewes, Sussex, UK) to estimate the relative concentration of the RNA recovered. The thermal cycling and amplicon detection incubations used were as outlined below- 30 seconds at 30 ^{o}C, denaturation for 5 seconds at 95 ^{o}C, and 45 cycles of amplification. Measurement of the specific PCR amplicon concentration at the end of the incubation was done by measuring the fluorescent signal at 530 nm, the wavelength at which

colour compensation was also done. The detected virus was characterized by RT-PCR amplification of the polymerase/capsid region spanning the ORF1/2 junction (van Abel *et al.* 2017).

3.3.5 Statistical analysis

Statistical analysis was performed using Graphpad prism Version 7.03 (Graphpad Software Inc., San Diego, USA) software. Graphs were made using SIGMA PLOT 12.0 Systat Software, Inc. (Addilink Software Scientific, S.L. Barcelona, Spain). Two-way analysis of variance (ANOVA) was performed to test for significant difference in viral loads across sites and months. The critical p-value for all the tests was set at 95% confidence level ($p < 0.05$). Tukey's multiple comparison test was performed where ANOVA showed some significant difference. Linear regression was done to test for association between viral loads and physicochemical parameters.

CHAPTER 4

RESULTS

4.1 Occurrence of the enteric viruses

Over the study period, a total of 59 samples were collected and analysed. All the studied enteric viruses were detected in all the study sites over the study period with varying detection rates (Figure 4.1, Appendix A). Norovirus GI was mostly detected in S1, followed by S5. Detection rates, presented herein as number of positive samples attained, was almost the same for Norovirus GI in S2, S3 and S4 (Figure 4.1). Similarly, Norovirus GII was mostly detected in S1, followed by S4. Sites S2, S3 and S5 had almost same detection rates for Norovirus GII (Figure 4.1). Rotavirus was the most prevalent with the highest detection in all the sites (Figure 4.1). S1 and S2 recorded significantly highest number of positive samples ($p = 0.0003$).

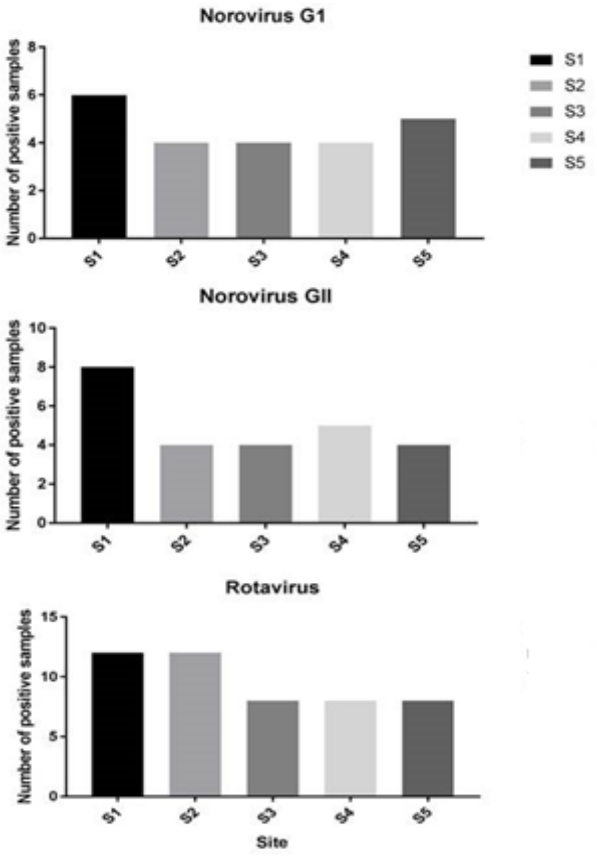


Figure 4.1: Number of positive samples per site. S1 (inlet), S2 (primary settling tank), S3 (maturation ponds), S4 (Notwane river at Oodi), S5 (Notwane river at Matebele).

Enteric viruses were detected throughout the study period except for Norovirus GI which was not detected in June (Figure 3.2). Norovirus GI had the highest number of positive samples in May, followed by December and February. Slightly above a third (37.3%) of all samples collected tested positive for norovirus GI. Norovirus GII was detected throughout the study period and had the highest number of positive samples in December where it was detected in all the sites. For the entire study period, 42.4% of all samples tested positive for norovirus GII. From July to November, same number of positive samples was recorded. Rotavirus was detected throughout the study period and had the highest number of positive samples (81.3%).

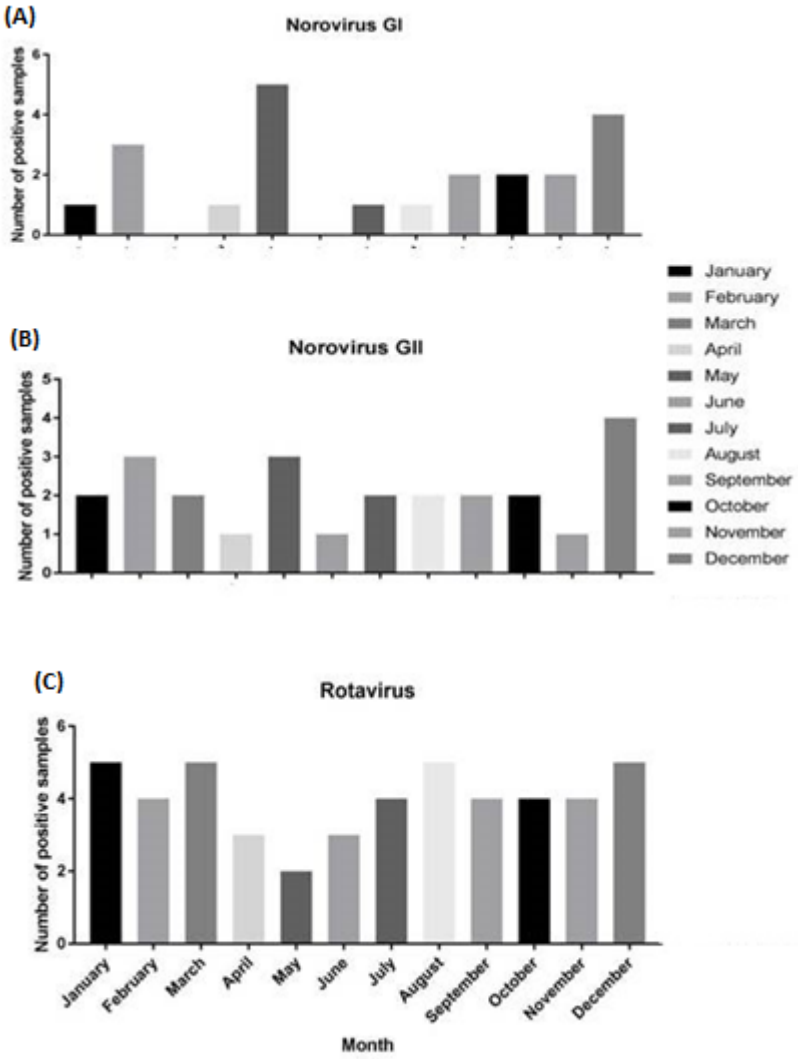


Figure 4.2: Number of positive samples per month (A) Norovirus GI (B) Norovirus GII and (C) Rotavirus.

Even though the viral loads of norovirus GI seemed to vary across sites (Figure 4.2a), ANOVA did not reveal any statistically significant difference ($F_{(11,44)}$ = 1.21, p = 0.309). Similarly, no statistically significant difference ($F_{(4,44)}$ =1.894, p = 0.128) was revealed across different months throughout the study period, even though the viral loads seemed to be different (Figure 4.3).

For norovirus GII, viral loads seemed to be lower in S1 than in other sites but the difference was not statistically significant when tested with ANOVA ($F_{(4,\ 44)}$ = 1.698, p = 0.168). However, when the viral loads for different months were compared, they varied significantly ($F_{(11,\ 44)}$=2.334, p = 0.0231). Further pairwise comparisons using Tukey's multiple comparison test revealed that the significant difference was between September and all the other months (P<0.005). During the month of September the viral load of norovirus GII was significantly higher than all the other months (Figure 4.3 b).

Rotavirus viral loads were higher in the months of February, April, May and June (Figure 4.2c). ANOVA did not reveal any statistically significant difference in rotavirus viral loads across the different months sampled ($F_{(4,44)}$ = 1.833, p = 0.144). Rotavirus viral loads displayed some variation across sites but the differences were not statistically significant ($F_{(11,44)}$ = 1.597, p = 0.1334).

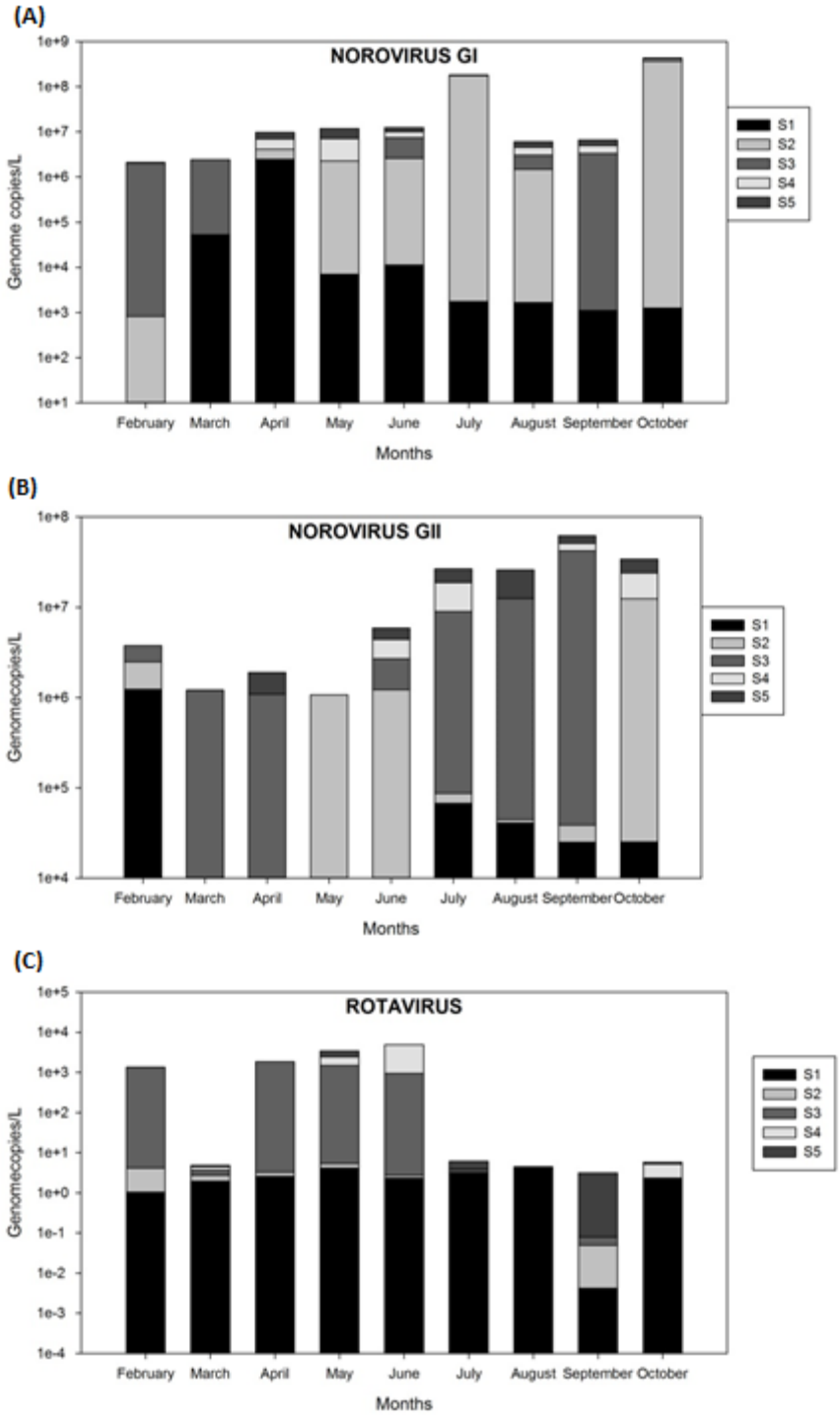


Figure 4.3: Variation of viral loads across the study sites for different months. (A) Norovirus GI (B) Norovirus GII (C) Rotavirus.

4.2 Association between physicochemical water quality and viral loads

pH was a significant predictor of rotavirus and norovirus GII viral loads, whereas there was no significant relationship between the other physicochemical parameters with viral loads (Table 4.1).

Table 4-1 Regression coefficients where viral loads were the dependent variable.

	Independent Variables	Coefficient	Standard Error	t - value	p–value
ROTAVIRUS	pH	7.46	0.068	2.201	0.05**
	DO	0.0009	0.0066	1.37	0.179
	Turbidity	0.0177	0.025	0.703	0.486
	Temperature	0.001	0.009	1.0052	0.321
NOROVIRUS GI	pH	7.53	0.0648	1.474	0.149
	DO	3.67	0.479	7.66	0.75
	Turbidity	94.23	20.11	4.686	0.513
	Temperature	21.29	0.771	0.764	0.449
NOROVRUS GII	pH	7.456	0.068	2.021	0.05**
	DO	3.438	0.505	6.782	0.318
	Turbidity	98.36	21.4	4.61	0.396
	Temperature	20.99	0.827	0.506	0.615

**** Statistically significant**

4.3 The efficiency of the waste treatment plant in removing enteric viruses from wastewater

To check for the efficiency of wastewater treatment plant, viral concentration of the influent (S1) and effluent (S2) were compared. The enteric viruses in this study were detected in both the influent (before treatment) and the effluent (after treatment). When comparing the viral concentrations between the influent and effluent, there was no distinct trend (Figure 4.4a and b). However, with respect to rotavirus, the viral load for influent was greater than at the effluent (Figure 4.4c).

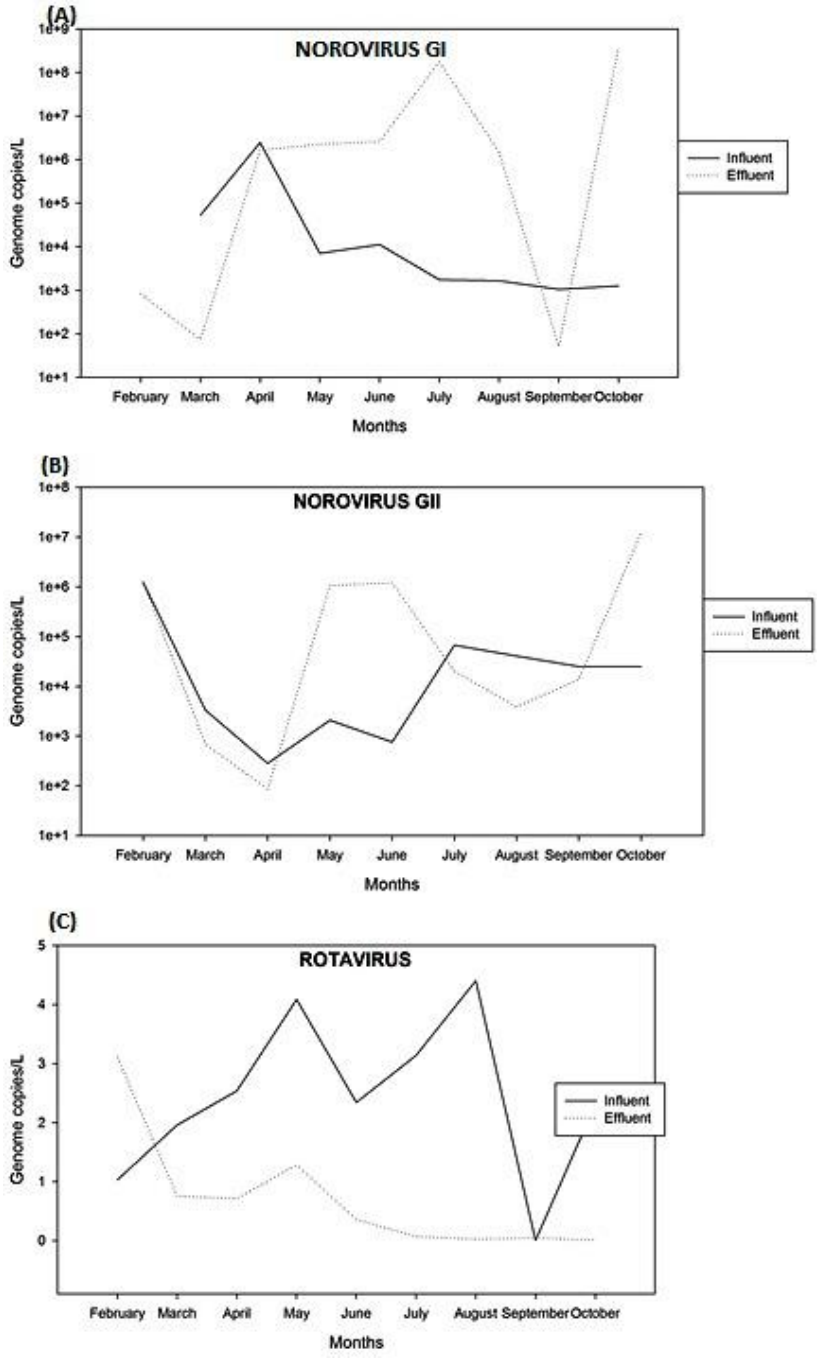


Figure 4.4: Virus concentration of the influent (S1) versus effluent (S2) at Gaborone waste treatment plant for different months for February to November (A) Norovirus GI (B) Norovirus GII (C) Rotavirus. (No viral quantification was done for November, December and January)

CHAPTER 5
DISCUSSIONS

Surface water remains an important source of water for many communities. It is used for irrigation, agriculture as well as domestic purposes. However, most wastewater treatment plants often discharge their final effluent into surface water bodies, ultimately leading to contamination. Contaminated surface water harbors pathogens which cause a significant public health risk warranting thorough investigations. The work presented here was undertaken to determine the spatial and temporal occurrence and distribution of enteric viruses (rotavirus, norovirus GI and norovirus GII) from the Gaborone wastewater treatment plant (influent and effluent) and surface water (Notwane River). The results of this study depict rotavirus as the most prevalent enteric virus (76.3% positive samples) compared to norovirus (GI, 38.9% and GII, 42.4%). Similarly, He *et al.* (2012) observed to be the most prevalent (48.1%) in urban surface waters of Beijing, China when compared to astrovirus and norovirus, with viral concentrations ranging from 0 to 18.27 genome copies/L. Rotaviruses are known to be stable in the environment and resistant to the processes used during wastewater treatment (Xagoraraki *et al.* 2014), and this could possibly explain their widespread prevalence. Norovirus has been reported as the second most common cause of viral gastroenteritis after rotavirus (Aragao *et al.* 2010; Tamura *et al.* 2010). Even though less frequently detected than rotavirus in the study area, norovirus was fairly present, with norovirus GII (25 positive samples) being more prevalent than GI (22 positive samples). A similar trend where norovirus GII outnumbered GI was also observed by Perez-Sautu *et al.* (2012). It is not clear why norovirus GII is mostly detected, but GI is reportedly scarce and relatively an uncommon group than GII (Tao *et al.* 2015).

In this study, all the viruses were detected all year round at varying viral loads across months. Rotavirus was more prevalent during the months of February, April, May and June (the difference between months was not statistically significant). The months mentioned are generally cooler winter months in Botswana and these results agree with other studies elsewhere which have noted that is generally more prevalent in winter and spring than in summer and autumn (Hejkal *et al.* 1984; Maunula *et al.* 2012; Lenaker *et al.* 2017). Elmahdy *et al.* (2016), recorded rotavirus prevalence of 20.8% in summer compared to 45.8% in winter in Brazil. In the present

study, norovirus GI was detected for all the months except February. Viral loads varied across the study sites with no distinct trend on which conclusions can be drawn on month(s) with highest prevalence. However, in general Notwane river sites (S4 and S5) recorded highest viral loads in May, just at the beginning of the winter season in Botswana. Similar to these study observations, Perez-Sautu *et al.* (2012) noted higher genome copies of norovirus GI and GII in colder months than warmer months. In Botswana, Basu *et al.* (2003) reported higher prevalence of rotavirus in stools of children with gastroenteritis.

The observed prevalence trend could be attributed to exposure to less sunlight in winter than in summer. High temperatures and more sunlight are able to inactivate viruses by denaturing their proteins which could lead to higher viral loads during cold winters (Desselberger, 2014). A report by Romero *et al*. (2011), states that while high temperatures inactivates the virus, lower temperatures tend to promote lower die-off rate of these viruses (Geldenhuys and Pretorius, 1989). For the same reason, viral particles survive longer if they are settled in soil underwater where it is cooler (Embrey *et al.* 2004*)*. Nordgren *et al.* (2009) reported norovirus GI to be more prevalent with high concentrations in winter months whereas GII was more during summer. They also noted that GI is more stable in wastewater, while GII on the other hand displayed larger seasonal peaks. Even though they can occur throughout the year, enteric viruses usually follow seasonal patterns (Sastry and Burgard, 2005) and the pattern tend to vary with respect to geographic location and season (Lipp *et al.* 2001). Changes in the concentration and distribution of enteric viruses for example are reported throughout the year (Kitajima *et al.* 2014). In water, virus survival mainly depends on temperature, exposure to ultraviolet (UV), and presence of microbiological flora (Bosch *et al.* 2006). Exposure to sunlight does have an effect on virus survival by inactivating the virus through direct mechanism involving the absorption of photons by the virus, resulting in damage to the genome or capsid proteins (Verbyla and Mihelcic, 2015).

Both viruses were found in raw sewage, treated sewage as well as Notwane river water. These results confirm the notion that virus contamination can still persist after wastewater treatment (Rzeżutka and Cook, 2004; Dubois *et al.* 1997). Virus detection occurs in both influent and effluent because apparently wastewater treatment processes can only reduce viral loads but do not completely eliminate the viruses (Rusinol *et al.* 2015). With respect to viral loads across the three types of samples, there were instances where raw sewage seemed to have lower viral concentrations than the effluent. This is contrary to what was expected because the treatment

processes should reduce the viral loads hence less concentration of viruses expected in the effluent than raw sewage. It is possible that the viral loads could have been underestimated due to the presence of inhibitors in raw samples thereby reducing PCR efficiency. Inhibitors can originate from the sample or be introduced in the laboratory during sample processing and they include mostly organic substances (bile salts, phenol, ethanol, polysaccharides, humic acids etc.) and few inorganic substances (e.g calcium and magnesium ions) (Bessetti, 2007; Schrader *et al*, 2012). Inhibitors tend to reduce after water treatment in effluent. Wastewater contain a substantial amount of clinical waste that can be inhibitory to the enzymes used in nucleic acid amplification techniques, such as the Taq polymerase thus resulting in underestimation of viral nucleic acids. The higher viral loads after the treatment process could also reflect the inefficiency of the plant in removing the virus. The molecular structures of the enteric viruses play a critical role in making them to be resistant to most wastewater treatment processes (Fong and Lipp, 2005; da Silva *et al.* 2007); hence they can be detected even after treatment. Nonetheless, some researches such as Nordgren *et al.* (2009) have documented a reduction of norovirus GI and GII concentration at the primary settling tanks.

The enteric viruses detected in the study persisted along the river at varying concentrations. These viruses are known to have the ability to survive in freshwater sources and sewage and remain infective for up to 120 days at temperatures ranging between 20 and 30 ^{o}C (Fong and Lipp, 2005). Survival and persistence of viral particles is dependent on several environmental factors including the interaction of viruses with particulates, microorganisms, and macroinvertebrates. Virus internalization by higher trophic-level organisms can have both protective and a detrimental effect on virus viability. The impact of virus-particle associations on sunlight disinfection in the pond also influences the persistence of viruses in the environment. The current results reveal no association between viral load with physicochemical water quality parameters (turbidity, temperature, and dissolved oxygen) except for pH which showed a significant relationship with rotavirus and norovirus concentrations. Rotavirus particles are very resistant to environmental conditions such as temperature hence their stability in the environment (Desselberger, 2014).

After pathogens from wastewater have been released into the environment, their fate is also determined by the sorption-desorpton processes. Suspended viral particles tend to adhere to

colloidal particles thereby enhancing their survival by reducing exposure to environmental stress factors such as sunlight (Yang *et al.* 2012), hence the concentrating effect of more turbid water on viral load (Embrey *et al.* 2004*)*. Although in some instances, physical parameters can correlate positively with viral loads (López-Gálvez *et al.* 2016), it has also been demonstrated that at times the relationship is not predictable due to the complex and dynamic interactions in the natural environment (Abad *et al.* 1994; Cromeans *et al*. 2010). Similar observations where there was no correlation between viral loads and physicochemical indicators have been documented by Espinosa *et al*. (2008). These study outcomes are therefore not un-reasonable given that the relationship between viruses and other parameters is relatively complex and it is difficult to separate various ecological factors affecting their survival in the natural environment (Faust *et al*. 1975; Rzeżutka and Cook, 2004).

As already mentioned, sunlight can have inactivation effect on viruses through its ultraviolet rays and maturation ponds mainly take advantage of this mechanism. The effectiveness of this mechanism however depends on the strength of radiation, the optical and physicochemical characteristics of pond water, as well as the properties of the particular virus. One of the most common virus genome deformations that results from the absorbance of photons is the formation of pyrimidine dimers (especially thymine dimers), which can prevent replication. Since RNA does not contain thymine, RNA viruses are typically more resistant to direct light-mediated inactivation than DNA viruses (Lytle and Sagripanti, 2005). With no significant relationship for most parameters, the results of this study can draw support from some research observations that viral inactivation do vary according to intrinsic characteristics of each type of virus and among different environmental samples (Espinosa *et al*. 2008; Cromeans *et al*. 2010).

It is key to acknowledge that a few physicochemical parameters (pH, dissolved oxygen, turbidity and temperature) were selected for this research, but there are other factors that can influence survival of viruses at different temporal and spatial scales. Factors such as vegetation composition could have had an effect on viral concentration and survival other than physicochemical parameters. Viral circulation pattern in the environment is further known to vary across geographic regions and even influenced by the epidemiological community profile (Embrey *et al.* 2004*)*, hence the varying viral loads recorded in the present study.

CHAPTER 6
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

Freshwater resources are limited and a growing number of water bodies may be affected by freshwater scarcity due to changes in climate, urbanization, population, and land use. It is therefore of uttermost importance that research on the quality and level of pollution on current water resources be prioritized, particularly enteric viruses, which have not been fully studied and there is little understanding about them and yet they are of public health concern. This study has detected and quantified enteric viruses in the environmental matrices, hence it has contributed to the scientific body of knowledge by giving information about the occurrence and persistence of enteric viruses in the environment after discharge from the wastewater treatment plant. The existence and fate of enteric viral particles is an important component of water quality to consider amidst the increasing demand for water. Available freshwater sources are threatened by pollution from domestic, agricultural and industrial waste, where gallons of liters of wastewater are discharged into the environment yearly.

The government of Botswana is now considering re-using wastewater to curb the escalating problem of water shortage. Not only will this approach reduce water shortage, it will also reduce pollution of the available fresh water sources as wastewater is the major pollutant of open water sources. Hence, this study has shed crucial information on the efficiency of wastewater treatment process that even when treated, the wastewater treatment processes do not completely eliminate pollutants such as pathogens. The detection and high viral loads of norovirus and after treatment may suggest that the wastewater treatment processes in the plant do not have good performance to eliminate pathogens. This information is important to consider when wastewater is to be recycled for domestic use. The frequent detection of the enteric viruses and their RNA, whether infectious or not, in the effluent from the wastewater treatment plant, as observed in this study, indicates wide dispersion of these enteric viruses in the environment. Consequently, this implies that viral contamination resulting from the use of these treated waters can possibly pollute open and ground water sources and therefore needs to be addressed.

With reference to waterborne diseases management, the information on the distribution of enteric viruses by countries and regions plays an essential role in the development of immunization strategies which are crucial in reducing the morbidity and mortality associated with viruses such as rotavirus and norovirus.

6.2 Study limitations

A few limitations from this study include minimal number of samples that were collected and analyzed. The use of molecular techniques for virus isolation is costly, and due to the high cost, only a few samples could be done. The study could have generated more data if the number of samples per month and site was increased together with increased sampling period. Wastewater, especially raw sewage, contains various inhibitors which may have led to underestimation of viral loads. Even though PCR is a generally rapid and sensitive method, these inhibiting substances can affect its sensitivity because it is an enzymatic reaction. Partial inhibition results in decreased sensitivity whereas total inhibition gives false negative results (Schrader *et al.* 2012). Real-time PCR uses fluorescent probes for detecting PCR products and therefore does not distinguish between viable and non-viable viruses; hence it gives no indication of virus infectivity. This therefore warrants further investigations which employ infectivity assays and microscopy techniques to fully understand the health risks posed by these enteric viruses.

6.3 Future studies

Studies on identification of enteric virus recombinants that may be more virulent and pathogenic than the already circulating strains need to be conducted. A previous study showed that the exposure to multiple virus strains in sewage contaminated water can potentially lead to new strains (van der Berg *et al.* 2005). Hence, it is also important to couple detection and quantification of viral loads with genotyping and molecular sequencing in order to know the different viral strains that are circulating in the environment.

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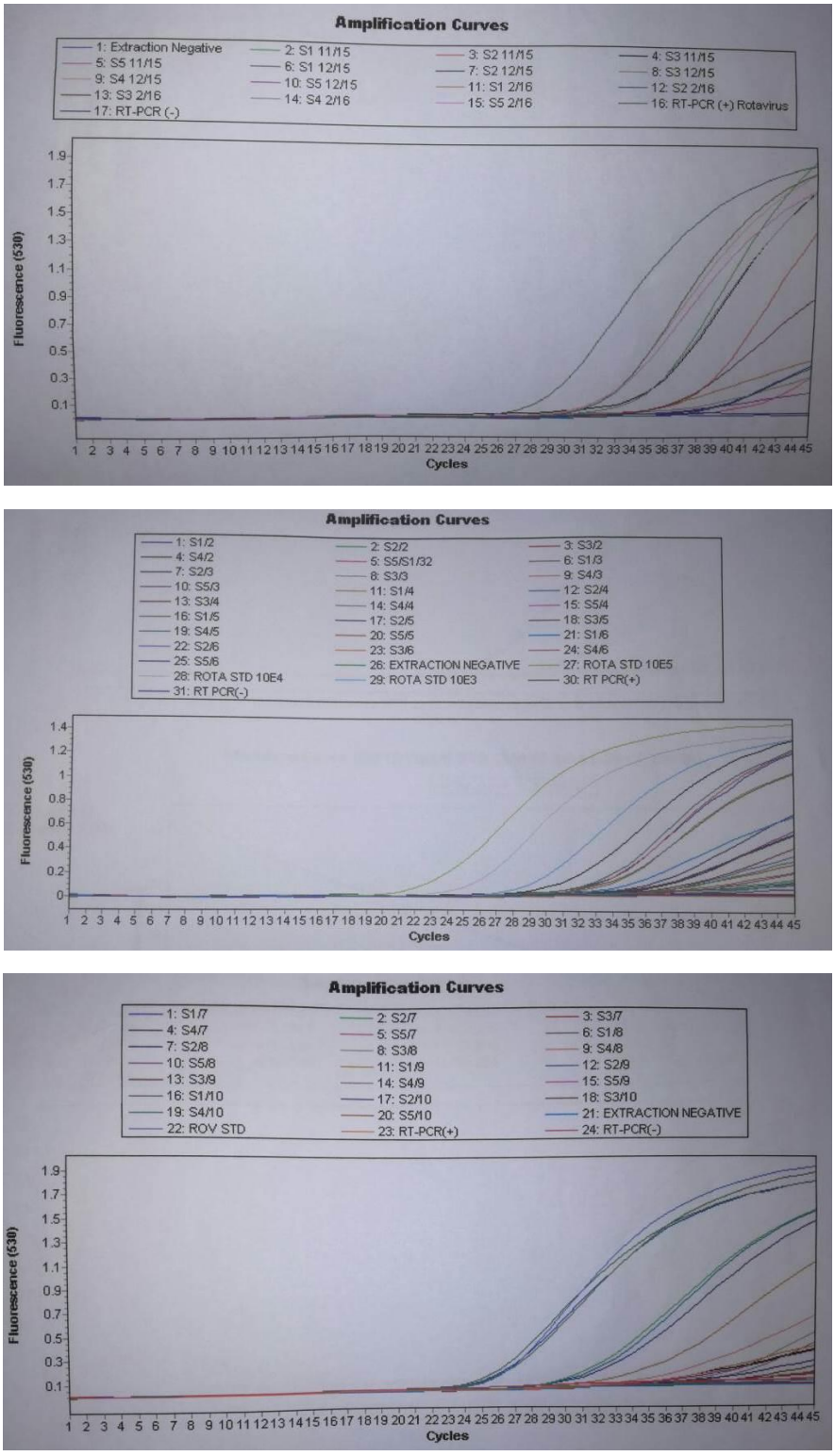
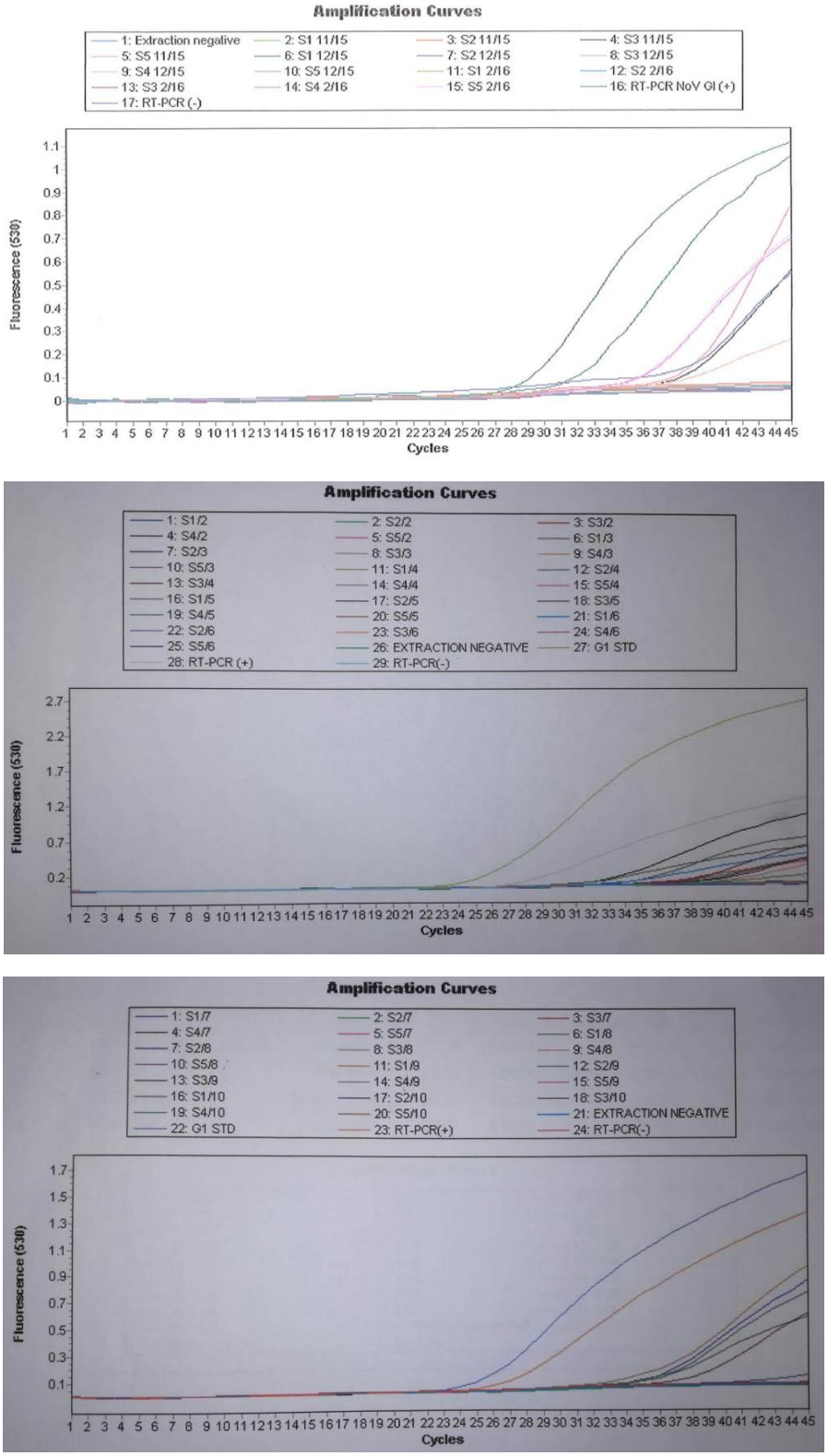
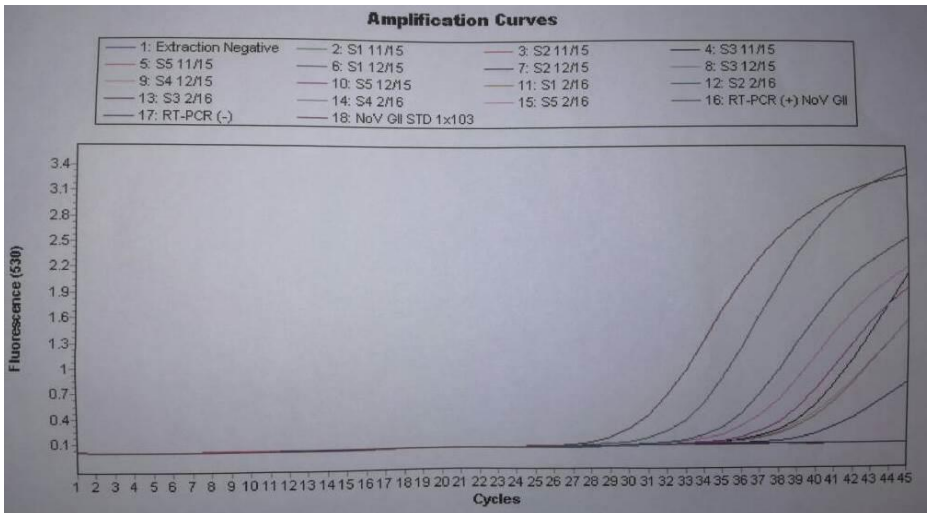
APPENDIX A

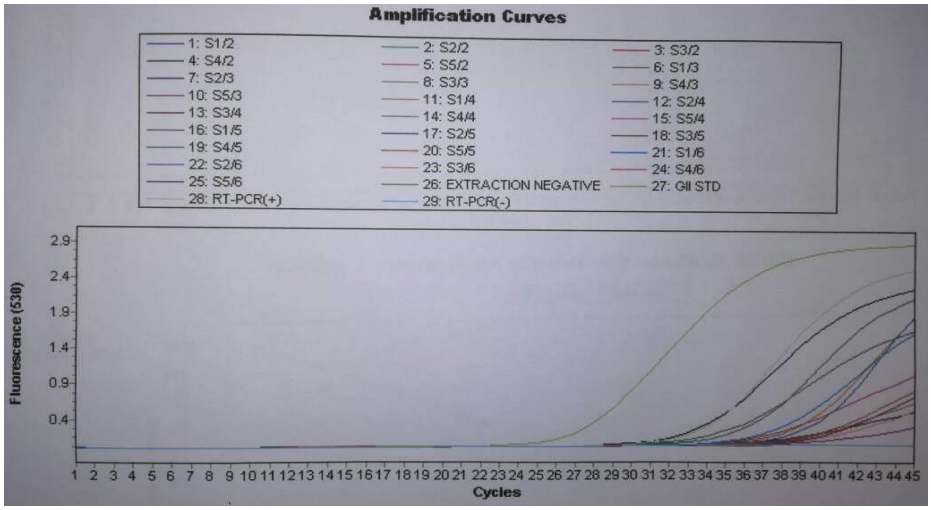


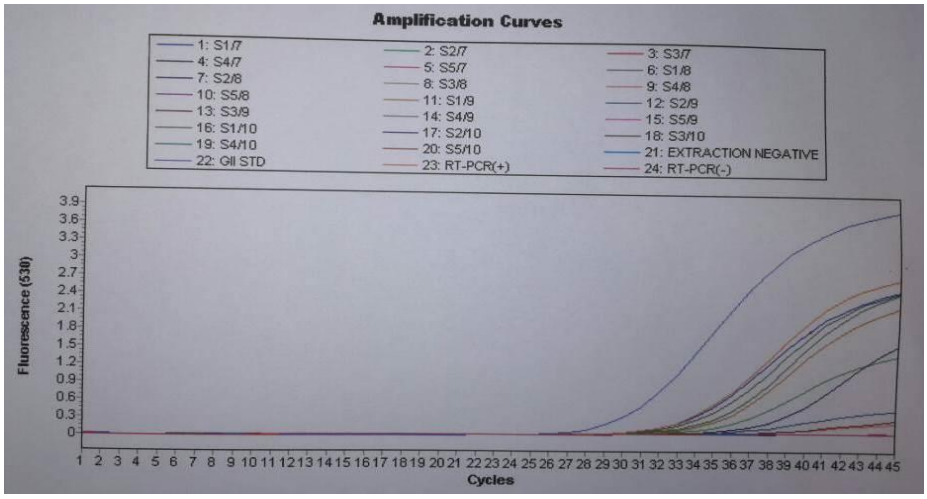
A1: Rotavirus amplification curve. S1 – S5 samples.



A2: Norovirus GI amplification curve. S1 – S5 samples.







A3: Norovirus GII amplification curve. S1 – S5 samples.

APPENDIX B

Table B1: Rotavirus primers used in the study

Primer	Sequence 5'- 3'	Position
NSP3 Forward primer	ACCATCTACACATGACCCTC	963–982
Reverse primer	GGTCACATAACGCCCC	1034–1049
TaqMan probe	FAM-ATGAGCACAATAGTTAAAAGCTAACACTGTCA A-TAMRA	984–1016

Table B2: Norovirus GI and GII primers used in the study

Virus detected	Primer	Sequence 5'- 3'	Position
Norovirus GI	QNIF4 (Forward)	CGCTGGATGCGNTTCCAT	5291–5308
	NV1LCR (Reverse)	CCTTAGACGCCATCATCATTTAC	5354–5376
	NVGG1 probe	FAM-TGGACAGGAGAYCGCRATCT-TAMRA	5321–5340
Norovirus GII	QNIF2 (Forward)	ATGTTCAGRTGGATGAGRTTCTCW GA	5012–5037
	COG2R(Reverse)	TCGACGCCATCTTCATTCACA	5080–5100
	QNIFS probe	FAM-AGCACGTGGGAGGGCGATCG-TAMRA	5042–5061