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Faculty of Science
Department of Biological Sciences

**PREVALENCE OF *CRYPTOSPORIDIUM PARVUM, GIARDIA INTESTINALIS* AND MOLECULAR CHARACTERIZATION OF ROTAVIRUS ASSOCIATED WITH DIARRHEA IN CHILDREN BELOW FIVE YEARS OLD IN GABORONE, BOTSWANA.**

KURENZVI LINEAGE
ID NUMBER: 201505902

Supervisor: DR T.K SEBUNYA
Co-supervisor 1: MS T. COETZEE
Co-supervisor 2: DR G. PAGANNOTI

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# DECLARATION

The work contained in this dissertation was completed by me at the University of Botswana between 2015 and 2019. It is an original work except where due reference is made and neither has been nor will be submitted for an award at any other University. This dissertation has been submitted with approval of University Supervisors.

Signature_____ Date_____.
Supervisor: Dr. T. K Sebunya

Signature_____ Date_____.
Co-supervisor: Ms. T Coetzee

Signature_____ Date _____.
Co-supervisor: Dr. G Paganotti

# DEDICATION

I dedicate this dissertation to my husband Courage Gwenikweni and my children Blessed and Bernice for their understanding and support over this few years that I left them for my studies. I also dedicate this work to my mother, Enecy Mwanyadza Tuso for her unconditional faith and support in all my endeavors.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| 5HT | 5- hydroxytryptamine |
| AIDS | Acquired Immune Deficiency Syndrome |
| cDNA | Complementary Deoxyribonucleic acid |
| DLP | Double Layered Particle |
| DNA | Deoxyribonucleic acid |
| dNTPs | deoxyribonucleoside triphosphates |
| dsRNA | double-stranded Ribonucleic Acid |
| ELISA | Enzyme Linked Immunosorbent Assay |
| ER | Endoplasmic Reticulum |
| EtBr | Ethidium Bromide |
| HCl | Hydrochloric acid |
| IHC | Immunohistochemistry |
| $MgCl_2$ | Magnesium Chloride |
| NaAcetate | Sodium Acetate |
| NAAT | Nucleic Acid Amplification Test |
| PAGE | Polyacrylamide Gel Electrophoresis |
| PCR | Polymerase chain reaction |
| RNA | Ribonucleic acid |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| *spp* | Species |
| SLP | Single Layered Particle |
| TAE | (Tris-acetate-EDTA) |
| TLP | Triple Layered Particle |

| | |
|---|---|
| UV | Ultraviolet |
| ORT | Oral Rehydration Therapy |
| RV | Rotavirus Vaccine |
| RVA | Group A Rotavirus |
| SDS | Sodium Dodecyl Solution |
| ICA | Immunochromatographic Assay |
| WHO | World Health Organization |

# ABSTRACT

***Background*:** Diarrhea remains a major cause of mortality and morbidity in children under the age of five years across the globe. In Botswana, the control of diarrheal diseases aims at reducing the burden caused by diarrhea as well as improving the quality of lives of children. Despite all these efforts, gastroenteritis is still one of the major causes of death and illnesses in young children in Botswana. To ensure the acceleration towards the reduction of diarrhea in children, recent information on pathogens causing the disease should be documented. The aim of this study was to determine the occurrence of *Cryptosporidium parvum*, *Giardia intestinalis* and molecular characteristics of rotaviruses in Botswana.

***Methods***: A case study was carried out on 200 stool specimens from symptomatic pediatric patients and 100 asymptomatic children under the age of five years from selected hospitals and clinics in Gaborone. The Ziehl Neelsen staining technique was used for detection of *Cryptosporidium parvum* and wet mount procedure for detection of *Giardia intestinalis*. Confirmation of samples that tested negative for the parasites was done using immunochromatographic assay. The enzyme-linked immunosorbent assay was used to screen for rotavirus. Rotavirus electrophoretypes from ELISA positive specimens were detected by polyacrylamide gel electrophoresis. Molecular characterization of rotavirus was conducted by reverse transcriptase-polymerase chain reaction using genotype specific primers that target VP4 and VP7. Selected PCR amplicons were sequenced and analyzed by Clustal W. Distance matrices were constructed by using the Kimura 2 parameter nucleotide substitution model in MEGA 6.06. Phylogenetic analysis was carried out by utilizing the neighbor joining model with 1000 bootstrap replicates and sequences were compared with reference strains from GenBank using the Basic Local Alignment Search Tool.

***Results***: Prevalence rates of 20.5% (41/200), 16.5% (33/200) and 11.0% (22/200) in diarrhea cases were observed for *Cryptosporidium parvum*, *Giardia intestinalis* and rotavirus, respectively. Four

percent (*n=8*) of diarrheic specimens had multiple infections. Peak infections of *G. intestinalis* and *C. parvum* were frequently observed during hot and rainy season, while peak prevalence for rotavirus occurred in April and July. For rotavirus, long electrophoretypes occurred more frequently (56.25%) than short electrophoretypes (25%). The most G/P combination observed was GIP[8] (7/15, 46.7%) followed by G2P[4] (2/15, 13%) and G3P[8] (1/15, 7%). One mixed strain, G1+G2P[4,8], was found in 13% (2/15) of case samples. Twenty percent of the specimens were non-typeable. Phylogenetic analysis of VP4 and VP7 sequences clustered rotavirus strains from Botswana within G1 lineages 1 and 2, G3-lineage 1, P[8] lineage 3 and P[4] lineage 5 together with Southern African strains, but distantly related to Rotarix.

***Conclusion***: This study suggests that *Cryptosporidium parvum*, *Giardia intestinalis* and rotavirus are important pathogens in the burden of diarrhea in children below the age of five years in Botswana. Rotavirus strain diversity was similar to that previously observed worldwide. Findings in this study suggests that rotavirus surveillance is an important tool to assess the impact of the ongoing vaccination program. Monitoring of circulating rotavirus strains is essential for assessment of effectiveness of current vaccines in Botswana.

# CHAPTER 1

# INTRODUCTION

## 1.1 Background

Diarrhea is one of the leading causes of deaths and illnesses in children less than five years worldwide (Guerrera, 2015). It is a common disease in the developing countries, especially in areas with poor hygiene, poor sanitation and limited access to safe water (Levine *et al*., 2017). Diarrheal diseases are rarely fatal in developed countries, except at extremes of age and in immunocompromised individuals (You *et al*., 2015).

Children under the age of five years suffer three or four diarrheal episodes annually and one in five cases results into death (Walker *et al*., 2013). Prolonged and recurrent diarrhea in children leads to malnutrition, which results in negative effects on growth and development of children (Pinkerton *et al*., 2016). In recent years, the introduction of vaccination, water and sanitation hygiene (WASH) programs, Oral Rehydration Therapy (ORT) and adequate nutrition measures reduced mortality and morbidity caused by diarrhea in children (Darvesh *et al*., 2017). However, there have been rising concerns about potential increase in diarrheal associated deaths in most countries due to climate change (Alexander *et al*., 2013; Falkenberg & Saxena, 2018).

The WHO Child Health Epidemiology Reference Group estimated that about 16% of deaths in African children under the age of five are as a result of diarrheal diseases (Alkema *et al*., 2014). By the year 2016, diarrhea was the fifth leading cause of death in children below the age of five, causing approximately 446,000 deaths annually (Troeger *et al*., 2018a). Enteric illnesses are one of the major causes of hospitalizations, mortalities and outpatient visits in young children in Botswana (Creek *et al.*, 2010; Mokomane *et al*., 2016). High case fatality ratios of upto 2.8% had been previously reported in diarrheal children in Botswana (Pernica *et al*., 2016; Welch *et al*., 2013). Past studies in

Botswana reveal that acute diarrhea shows a bimodal annual pattern amongst young children, with peaks around March and October (Alexander *et al*., 2013; Mach *et al*., 2009).

The most common causes of viral diarrhea are noroviruses, rotaviruses, adenoviruses, sapoviruses and astroviruses. Ingestion of fecal contaminated water or food is the major route of transmission of viral diarrhea (Bányai *et al*., 2018). Viral gastroenteritis affects all age groups worldwide, and people who are more susceptible to severe symptoms are children below the age of two years, immunocompromised individuals and premature infants (Dennehy, 2011; Lamberti *et al*., 2016). In 2016, rotavirus was the most identified viral pathogen in children hospitalized with diarrhea, accounting about one third of all diarrhea cases and approximately 128,515 deaths (Troeger *et al*., 2018b). Two live attenuated oral rotavirus vaccines, RotaTeq™ and Rotarix® were recommended by the World Health Organization in national immunization programmes to fight against rotavirus in young children (Tate *et al*., 2010). The introduction of the 2 dose monovalent rotavirus vaccine (RV1, Rotarix®), which includes one human attenuated strain G1P[8], led to a great reduction of rotavirus associated diarrhea in Botswana but changing diversity of circulating strains had been seen in most studies conducted in the post vaccination era (Gastañaduy *et al*., 2016; Goldfarb *et al*., 2014).

The most prevalent parasites that cause diarrheal diseases are *Entamoeba histolyca, Giardia intestinalis, Cryptosporidium parvum, Microsporidia* and *Cyclospora cayetanis*. Transmission of parasitic infections is mainly through consumption of water and food contaminated with cysts (Kotloff *et al*., 2017). This study will be focused on *Cryptosporidium* and *Giardia* as protozoa that cause diarrhea in under-fives of Gaborone. The occurrence of both *Cryptosporidium* and *Giardia* has two peaks in Botswana (Alexander *et al*., 2012), although seasonal distribution of these two pathogens is not quite understood in other studies. *Cryptosporidium* and *Giardia* are ubiquitous and

under certain conditions, cysts can remain viable for several months in the environment. The small size of both *Cryptospodium* and *Giardia* cysts can allow them to pass through conventional water plant filters and they can be highly resistant to some disinfectants at low concentrations. Due to these characteristics and their association with drinking and recreational water, *Cryptosporidium* and *Giardia* pose a significant threat to humans (Carmena, 2010). In many countries where diarrheal diseases are common, efforts had been made to determine prevalence of *Cryptosporidium* and *Giardia* and possible sources of contamination that can lead to human infections (Squire & Ryan, 2017; Yoder and Beach, 2010).

*Cryptosporidium* was the fifth leading cause of diarrheal mortality in children below the age of five years, in 2016, causing approximately 44.8 million episodes of diarrhea and 48,300 deaths annually (Khalil *et al*., 2018). It has been estimated that over 2.9 million of *Cryptosporidium* infections occur annually in children below two years of age in Sub-Saharan Africa (Squire & Ryan, 2017). *Cryptosporidium* prevalence range from as low as 2% to 60% in Botswana (Creek *et al*., 2010; Goldfarb *et al*., 2014; Rowe *et al*., 2010) and affects children ≤ 24 months of age more than any other age group (Alexander *et al*., 2012). This indicates the need to revisit and evaluate the burden of cryptosporidiosis in Botswana.

*Giardia* outbreaks were reported in 37% of global waterborne transmission of protozoan parasites between 2011 and 2016 (Efstratiou et *al*., 2017). In 2010, WHO estimated that *Giardia* caused approximately 28.2 million cases of diarrhea annually as a result of contaminated food (World Health Organization, 2015). Prevalence rates that range from < 1% to > 72% in children have been documented for *Giardia* in most African countries (Squire & Ryan, 2017). The occurrence of *Giardia* ranges from 1 to 10% in Botswana (Alexander *et al*., 2012; Rowe *et al*., 2010). Although prevalence rates are low for *Giardia* in Botswana, some studies observed that chronic giardiasis

modulates some symptoms of rotavirus infection (Bhavnani *et al*., 2012). Co-infection in giardiasis patients also intensifies severity of diarrhea especially in young children (Cotton *et al*., 2015). This highlights the global significance of *Giardia* in public health. Association between *Giardia* and other highly prevalent diarrhea pathogens has not been well investigated in Botswana, a country that once reported high case fatality ratios in diarrheal children (Welch *et al*., 2013). Determining prevailing etiological causes of diarrhea is crucial for surveillance studies and it helps in designing more specific prevention measures and vaccination strategies.

## 1.2 Statement of problem

*Cryptosporidium, Giardia* and rotavirus have been detected in diarrhoea suffering children throughout the world. In major outbreaks of diarrhea in Botswana, *Cryptosporidium, Giardia* and rotavirus were among the isolated microorganisms in young children (Creek *et al*., 2010). Morbidity and mortality from childhood diarrhea had been falling in Botswana, but climate change is expected to increase the risk of diarrheal diseases (Alexander *et al*., 2013; Carlton *et al*., 2016). Several studies have found an association between temperature and diarrheal episodes (Musengimana *et al*., 2016; Zhou *et al*., 2013). Up- to date estimates of the extent to which *Cryptosporidium, Giardia* and rotavirus are prevalent in the under-five population create awareness within the health care system and communities.

Co- infections among diarrhea causing pathogens had gained more attention worldwide. This has not be well investigated in Botswana, where diarrhea continues to be one of the leading cause of childhood illnesses. The monovalent rotavirus vaccine Rotarix® had been incorporated in the national immunization programme of Botswana since 2012, but there are variations in the dominating strains from time to time (Gastañaduy *et al*., 2016; Goldfarb *et al*., 2014). To gain further

insight into genetic variability of rotavirus strains circulating in Botswana, identified rotavirus strains were sequenced and analyzed in this study.

## 1.3 Research questions

- What is the epidemiology of *Cryptosporidium parvum*, *Giardia intestinalis* and rotavirus in children with diarrhea in Gaborone in 2017?
- What are the genetic characteristics of rotavirus strains circulating in Gaborone after 5 years of rotavirus vaccine introduction into the national immunization programme of Botswana?
- What is the impact of vaccination on epidemiology and molecular characteristics of the rotavirus strains circulating in children around Gaborone after Rotarix® introduction in the national immunization programme?

## 1.4 Research Hypothesis

- *Cryptosporidium parvum*, *Giardia intestinalis* and rotavirus are prevalent in children suffering from diarrhea in Gaborone.
- There is a significance relationship between age, seasonal patterns and diarrhea causing pathogens of this study (*Cryptosporidium parvum*, *Giardia intestinalis* and rotavirus).
- There is diversity of rotavirus electrophoretypes, genotypes and serotypes among rotavirus strains isolated in children below five years of age attending healthcare facilities in Gaborone.

## 1.5 Study objectives

The purpose of this study is to detect, identify and determine the prevalence of *Cryptosporidium parvum, Giardia intestinalis* and rotavirus in children below the age of five years in Gaborone, Botswana.

**The specific objectives of this study are:**

- To detect and identify *Cryptosporidium parvum, Giardia intestinalis* associated with diarrhea in the under-five population of Gaborone using microscopy and immunochromatographic assay techniques.
- To detect circulating strains of rotavirus in children below the age of five years in Gaborone using enzyme linked immunosorbent assay.
- To assess genetic diversity of VP4 and VP7 of rotavirus by RT-PCR and sequencing.
- To compare nucleotide sequences found in this study with reference strains.
- To establish genetic lineages between different VP4 and VP7 subgroups.
- To find potential association between *Cryptosporidium parvum, Giardia intestinalis* and rotavirus in children suffering from diarrhea.

## 1.6 Significance of the study

The findings of this study will provide crucial information on the importance of *Cryptosporidium parvum*, *Giardia intestinalis* and rotaviruses in the overall burden of diarrhea amongst the under-five population of Gaborone. The information attained can be beneficial to the Ministry of Health and Child Welfare as well as Non-Governmental Organizations that are interested in improving children welfare status. This study will provide a comprehensive picture on the genotypic distribution of rotavirus strains which is required in improving rotavirus vaccination and evaluating rotavirus vaccine efficacy. Findings of this study will also help in coming up with more specific intervention measures that can reduce the burden of diarrhea in under-fives. The results of the study may contribute to knowledge on seasonal variations of *Cryptosporidium parvum*, *Giardia intestinalis* and rotaviruses in Gaborone, which can be used as a basis in future studies.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 *Cryptosporidium spp.* and *Giardia spp.*

### 2.1.1 Classification of *Cryptosporidium parvum* and *Giardia intestinalis*

*Cryptosporidium* was discovered to be a cause of infection in humans in 1976 and during the early 1980s it was found as the major cause of chronic diarrhea in people with AIDS (Sallon *et al*., 1988). *Cryptosporidium* was associated with childhood nutrition and early childhood death in third world countries in the mid 1990s (Collins *et al*., 2006). *Cryptosporidium parvum* is one of the species that cause a disease called cryptosporidiosis. *C. parvum* is classified under Phylum Apicomplexa, Class Sporozoasida, Subclass Coccodiasina, Order Eucoccidiorida, Suborder Eimeriorina, family *Cryptospordiidae* and genus *Cryptosporidium* (Ghazy *et al*., 2015). *Cryptosporidium parvum* occur in three developmental stages: meronts, gamonts (sporozoites) and oocysts. Around 27 species of *Cryptosporidium* are known and they are named on the basis of the host they occur in, parasite morphology and site of infection (Ryan *et al*., 2014). The other main cause of cryptosporidiosis in humans is *C. hominis* while other species like *C. canis*, *C. felis*, *C. meleagridis*, and *C. muris* are found in multiple host species (Šlapeta, 2013).

*Giardia intestinalis* is a non-invasive, flagellated protozoan parasite that can cause acute, watery diarrhea called giardiasis by infecting the upper small intestine (Einarsson *et al*., 2016). It affects all mammals and the parasite can exist at higher levels in water (Ryan & Cacciò, 2013). *Giardia intestinalis* is classified under Phylum Protozoa, Subphylum Sarcomastigophora, Superclass Mastigophora, Class Zoomastigophora, Order Diplomonadida, and Family Hexamitidae. Some other species of *Giardia* that are more important in humans and animals are G. *agilis*, *G. muris*, *G. ardeae*, *G. psittaci* and *G. microti*, *G. cricetidarum* and *G. peramelis* (Brynildsrud *et al*., 2018).

### 2.1.2 Morphology of *Cryptosporidium parvum and Giardia intestinalis*

The oocysts of *Cryptosporidium parvum* (Figure 2.1) appear as round to oval in shape and approximately 4-6µm in diameter. Oocysts usually contain four sporozoites and an eccentric residual body. Sporozoites possess an apical complex at the anterior part. The apical complex consists of micronemes, a single rhoptry, and dense granules (Thompson & Ash, 2016). Every sporozoite can develop into a spherical shape and undergo merogony to form type 1 meront which contains 8 merozoites. Merozoites are released and attach again to the surface of the epithelial cell to undergo another merogony forming either type 1 or type 2 meront (Ryan *et al*., 2014).

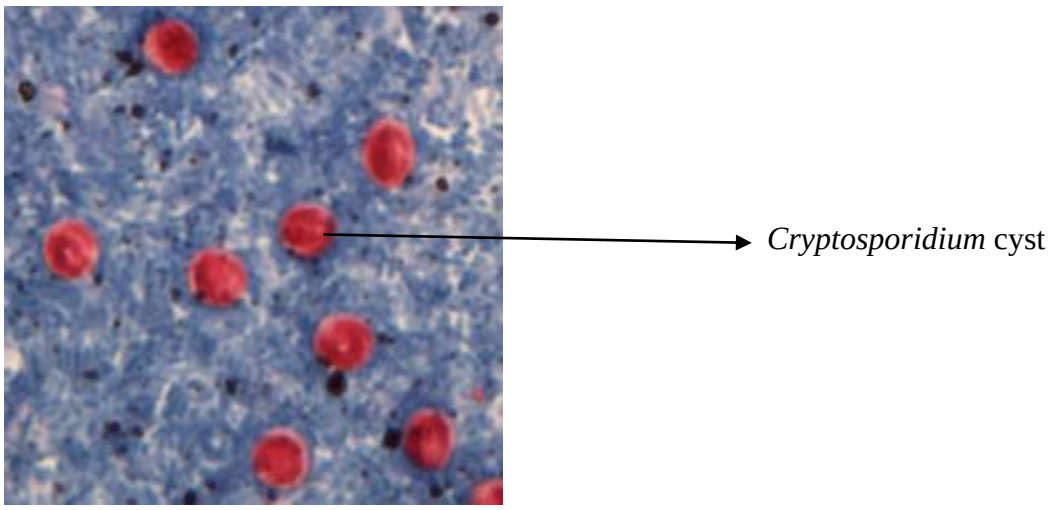


**Figure 2.1** *Cryptosporidium parvum* cysts after stained by Modified Ziehl Neelsen (Centers for Disease Control and Prevention, 2019).

*Giardia intestinalis* cysts (Figure 2.2) are pear shaped, has a broad anterior and a much attenuated posterior. They are approximately 8-20µm long and 5-12µm wide. Trophozoites of *Giardia intestinalis* have 8 flagella that arise on a ventral surface near the parallel nuclei which give them the falling leaf type of motility (Einarsson *et al*., 2016). Trophozoites of *Giardia intestinalis* have two nuclei and two median bodies. It is relatively flattened, comprising of a large sucking disk on the anterior ventral which plays an important role in attaching to the mucosa of the host (Plutzer *et*

*al*., 2010). Upon ingestion of contaminated foods, *Giardia* cysts excysts in the gastrointestinal tract and excystation is facilitated by the acidity of the stomach and proteases found within the stomach. The cysts will then release trophozoites that will attach to the surface of the intestinal epithelium by its ventral suckling disk. Trophozoites reproduce by binary fission forming new sporozoites which will be excreted in feces (Ryan & Cacciò, 2013).

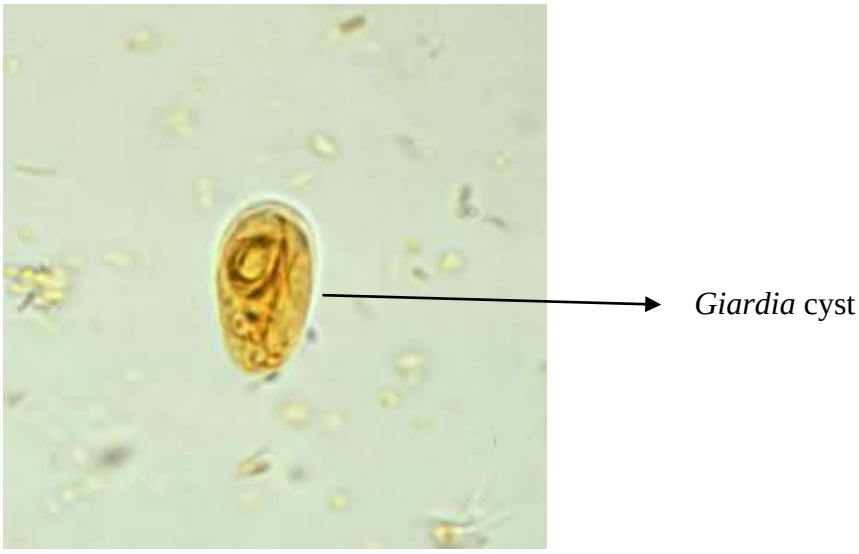


**Figure 2.2** Iodine stained cyst of *Giardia intestinalis* (Centers for Disease Control and Prevention, 2017).

### 2.1.3 Epidemiology of *Cryptosporidium* and *Giardia* infections

#### 2.1.3.1 Prevalence

*Cryptosporidium* and *Giardia* infections are more common in countries and communities that are overcrowded with poor sanitation conditions. Giardiasis is usually associated with cryptosporidiosis. *Giardia* infections associated with *C. hominis* had been observed in America, Australia and Africa (Painter *et al.*, 2015). *Giardia* and *Cryptosporidium* had been found to be major causes of water borne diseases in the United States and Europe, whilst they are major causes of

parasitic foodborne infections in Africa (Checkley *et al*., 2015). The relationship between gender, geographic location, seasonal variation and the occurrence of giardiasis and cryptosporidiosis is not quite understood since several conflicting reports have been observed in most studies. Although cryptosporidiosis and giardiasis affects all age groups, they are generally common in children aged two years and below in all continents (Chalmers *et al*., 2018).

**Table 1** Prevalence of *Cryptosporidium* and *Giardia* in Botswana.

| Year | Prevalence rate (%), Age group (years) | |
|---|---|---|
| | *Cryptosporidium* | *Giardia* |
| 2006 | 60, ≤ 5(Creek *et al*., 2006) | |
| 2003-2008 | 2.5, all age groups (Rowe *et al*., 2010) | 0.8, all age groups (Rowe *et al*., 2010) |
| 2012 | 10 , ≤ 5 (Alexander *et al*., 2012) | 7, all age groups (Alexander *et al*., 2012) |
| 2011-2013 | 8.3, ≤ 5 (Pernica *et al*., 2016) | 5.3, ≤ 5 (Pernica *et al*., 2016) |
| 2012-2013 | 16.8, ≤ 13 years (Goldfarb *et al*., 2014) | 8.6, ≤ 13 years (Goldfarb *et al*., 2014) |

In most countries, *Cryptosporidium* is second to rotavirus as a cause of moderate to severe diarrhea in the under-five population (Kotloff *et al*., 2013). Cryptosporidiosis is a notifiable disease in the European Union and outbreaks had been reported from Denmark, England, Scotland and Nordic countries (Guzman-Herrador *et al*., 2015). In Botswana, prevalence rates that range from 2-60% have been observed in past studies (Table 1).

Approximately 300 million people are affected by giardiasis annually. In humans, the prevalence of giardiasis is between 2% and 3% in industrialized countries whilst it can reach upto 30% in developing countries (Zylberbeg *et al*., 2017). There is limited information on the prevalence of *Giardia* in Botswana, but previous studies reveal that 1 to 10% of diarrhea hospitalizations are caused by *Giardia* (Table 1). Consistent seasonal patterns and regional variations had been observed for giardiasis in some countries like New Zealand, showing a relatively small increase of infections in summer (Lal & Hales, 2015). Some studies in African nations like Zambia reported a remarkably higher prevalence of *G. intestinalis* in summer and wet season as compared to the dry season (Siwila *et al*., 2011).

#### 2.1.3.2 Transmission of *Cryptosporidium* and *Giardia* infections

Transmission of infectious *Cryptosporidium parvum* and *Giardia intestinalis* cysts can be waterborne, foodborne, or fecal-oral transmission. Parasites can be introduced to the human body through direct and several indirect routes of transmission. Direct transmission is mainly through the fecal- oral route from infected hosts and this can be animal to animal, animal to human, human to animal or human to human (Squire & Ryan, 2017). Ways of direct transmission include drinking water contaminated with the parasites and eating contaminated uncooked foods. Indirect transmission include environmental contamination, contact with contaminated surfaces and close contact with other infected people or infected animals (Efstratiou *et al*., 2017; Thompson & Ash, 2016). Post treatment contamination is also one of the leading cause of waterborne giardiasis and cryptosporidiosis in regions where water treatment is available. Water storage tanks in households, reservoirs, and accumulation of biofilms in distribution pipes can lead to contamination of water after its treatment (Plutzer *et al*., 2010).

Inhalation of oocysts was reported to be another mode of transmission of *Cryptosporidium* in immunocompromised patients and children (Sponseller *et al*., 2014). *Cryptosporidium parvum* is capable of completing its life cycle in 2 days within a single host and form cysts that can be released from the body through excretions or formites before transmission to new host (Clode *et al.*, 2015). On the other side, *Giardia* infections can also be transmitted via heterosexual or homosexual anal-oral sexual contact and the cysts are highly infectious, about 10 cysts can lead to infection in humans (Certad *et al*., 2017).

#### 2.1.3.3 Symptoms of cryptosporidiosis and giardiasis

Signs and symptoms of infection caused by *Cryptosporidium parvum* include watery diarrhea, dehydration, lack of appetite, weight loss, stomach pains, stomach cramps, fever, nausea and vomiting. Cryptosporidiosis can start 2 to 10 days after infection (Ryan *et al*., 2018). Reinfections can lead to recurrence of the disease in immunocompromised hosts and young children causing prolonged and persistent diarrhea. This makes acute diarrhea caused by *C. parvum* to be severe or life threatening in patients with compromised immunity (Checkley *et al.*, 2015). Symptoms of giardiasis caused by *Giardia intestinalis* include flatulence, floaty greasy stool, abdominal cramps, nausea, dehydration and weight loss. They usually manifest 1 to 3 weeks after exposure to *G. intestinalis* and can last for a period of 2 to 6 weeks (Mmbaga & Houpt, 2017).

### 2.1.4 Prevention and control of *C. parvum and G. intestinalis* infections

Parasitic infections can be prevented by good hygiene practices like carefully washing hands, being careful when handling animals and avoid foods that are not well cooked. Avoiding fecal exposure, swallowing recreational water and drinking untreated water can also reduce chances of infection by *C. parvum* and *G. intestinalis* (Cacci & Chalmers, 2016). Surfaces, including children's toys should

be cleaned thoroughly using cleaning solutions like detergents. Raw foods must be washed thoroughly with clean uncontaminated water before consumption. Other pathogen killing methods such as blanching can be used after washing raw foods to reduce chances of infection. All foods should be wholly cooked since partial cooking may fail to destroy all *Giardia* and *Cryptosporidium* cysts (Macpherson & Bidaisee, 2015; Ryan *et al*., 2018).

### 2.1.5 Detection and characterization of *Cryptosporidium parvum* and *Giardia intestinalis*

Cryptosporidiosis can be diagnosed by examination of stool samples. The stools are usually examined in multiples before a final result is decided on a given sample. The most frequently used laboratory diagnostic testing in hospitals is light microscopy, mainly because of its high affordability and its simplicity (Van den Bossche *et al.*, 2015). Morphological features are clearly observed by using staining techniques like acid-fast staining and Ziehl Neelsen staining. However, microscopic detection limit is only 50,000 to 500,000 cysts per gram of stool (Weber *et al*., 1991) and the process is tiresome, time consuming and requires experienced technicians. *Giardia intestinalis* cysts can be observed directly on fecal smears stained with common stains like iodine, methylene blue glycerol and trichrome. Cysts of *G. intestinalis* can also be concentrated before staining using sucrose, zinc sulphate or sodium nitrate (Baig *et al*., 2012; Koehler *et al*., 2014).

Immunological methods such as Enzyme immunoassays (EIA) and rapid immunochromatographic assays (ICA) for commercially available kits can be used in detecting the antigens for both *Giardia intestinalis* and *Cryptosporidium parvum*. The use of ICA in laboratory testing of parasites is increasingly becoming popular since it is easy to perform, results are interpreted over a short period of time and more than one parasite can be detected in a single test format (Garcia *et al*., 2003). Most studies have shown that ICA has high specificity although sensitivity can vary from one test kit to another (Chalmers *et al*., 2011). Identification of *Cryptosporidium* and *Giardia* up to species level

can be done using molecular methods like PCR and sequencing. These molecular methods have extremely high specificity and sensitivity and they can be carried out over a short period of time since many reactions can be done in batches (Kabir *et al*., 2018). Despite of all the advantages of molecular methods, many laboratories in developing countries still rely on microscopy during routine tests to avoid high costs that can be incurred by methods like PCR.

## 2.2 Rotavirus

### 2.2.1 Structure, morphology and molecular characteristics of Rotavirus

Rotavirus (Figure 2.3) is a non-enveloped, double stranded RNA virus that belongs to the family *Reoviridae*. A mature and infectious rotavirus particle is approximately 100nm in diameter and composed of a three layered icosahedral protein capsid that surround the genome (Esona & Gautam, 2015). The three concentric shells surrounding it are the outer layer, intermediate layer and an inner core layer (Fig 2.3C). The shells enclose 11 gene segments (Figure 2.3A) that encodes 6 structural proteins (VP1, VP2, VP3, VP4, VP6, VP7) and 6 nonstructural proteins (NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6). Each segment codes for at least one protein except for gene 9 and 11 which codes for 2 proteins (Beards, 2017). The outermost shell of the virus contains VP7 and VP4. Classification of rotaviruses is mainly based on these two proteins found on the outer layer since they are responsible for neutralization and induction of antibodies that are used in immune protection. The intermediate layer contains VP6, and the inner core layer consists of VP2 which enfolds VP1 and VP3 as shown in Figure 2.3D (Desselberger, 2014).

Three distinct types of rotavirus particles namely TLP's (triple layered particles), DLP's (double layered particles) and SLP's (single layered particles) can be observed by an electron microscope. TLP's are complete infectious particles and they are approximately 100nm in diameter (Figure

2.3B). DLP's appear as rough particles and they lack the outer shell (Figure 2.3E, Figure 2.3F). SLP's are aggregated and can be described as cores. SLP's lack the outer shell, intermediate shell and sometimes genomic RNA (Dormitzer, 2015; Esona & Gautam, 2015).

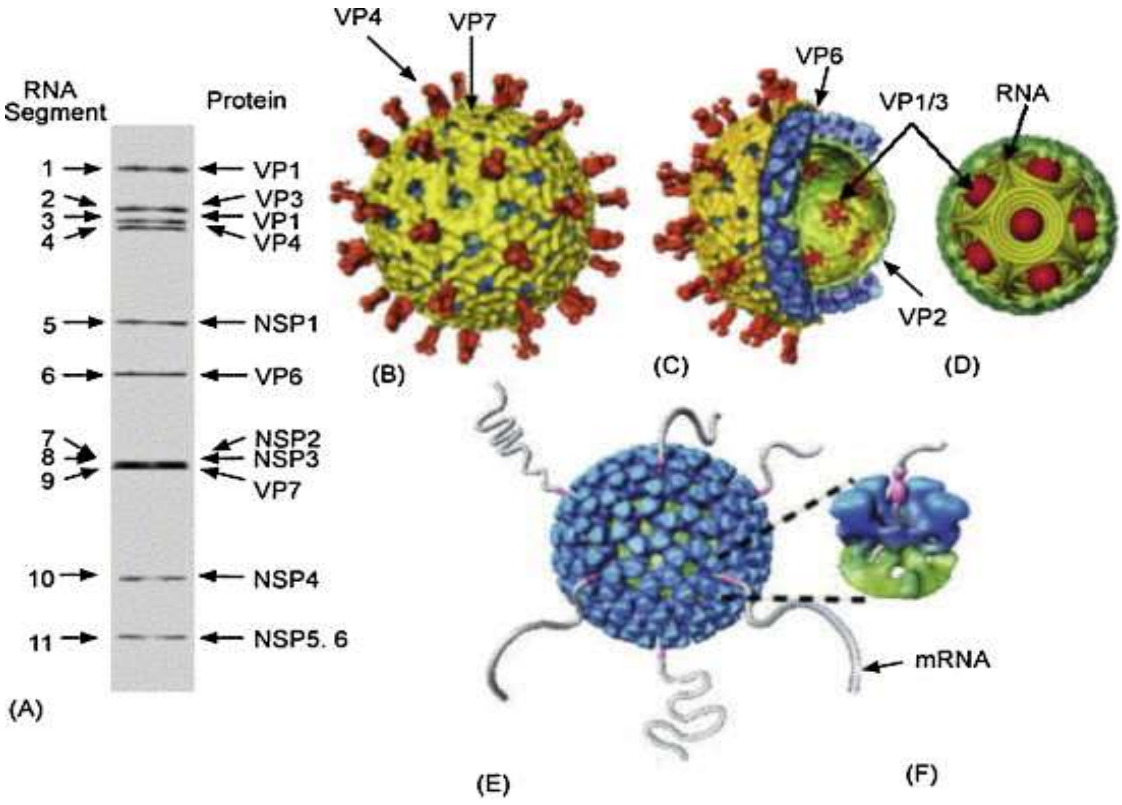


**Figure 2.3** Rotavirus structure. (A) PAGE gel showing 11 dsRNA segments comprising of the human rotavirus genome. (B) Cryo-EM reconstruction of the rotavirus triple-layered particle. (C) A cutaway view of the rotavirus TLP. (D) Schematic depiction of genome organization in rotavirus. (E and F) Model from Cryo-EM reconstruction of transcribing DLPs (Desselberger, 2014).

### 2.2.2 Genomic structure and organization of Rotavirus

Rotaviruses consists of segmented, non- enveloped, double stranded ribonucleic acid (dsRNA) genome (Figure 2.4) with 11 segments. The genome comprises of approximately 18,555 nucleotides in total that ranges from 0.6 to 3.3 kilo base pairs in size (Beards, 2017). The gene segments of the

dsRNA are found in the virus core capsid which are numbered from the largest to the smallest and can produce four electrophoretype patterns when separated by PAGE (polyacrylamide gel electrophoresis). The segments are designated by 4 large segments, 2 medium-sized segments, 3 smaller segments and 2 smallest segments (Norkin, 2010). When this basic pattern is not seen, this means that the virus being investigated may be Group A avian rotavirus, non-Group A rotavirus, a Group A rotavirus with rearrangements within the gene segments or a new and unique Group A rotavirus (Matthijnssens *et al*., 2008b; Papp *et al*.,2013).

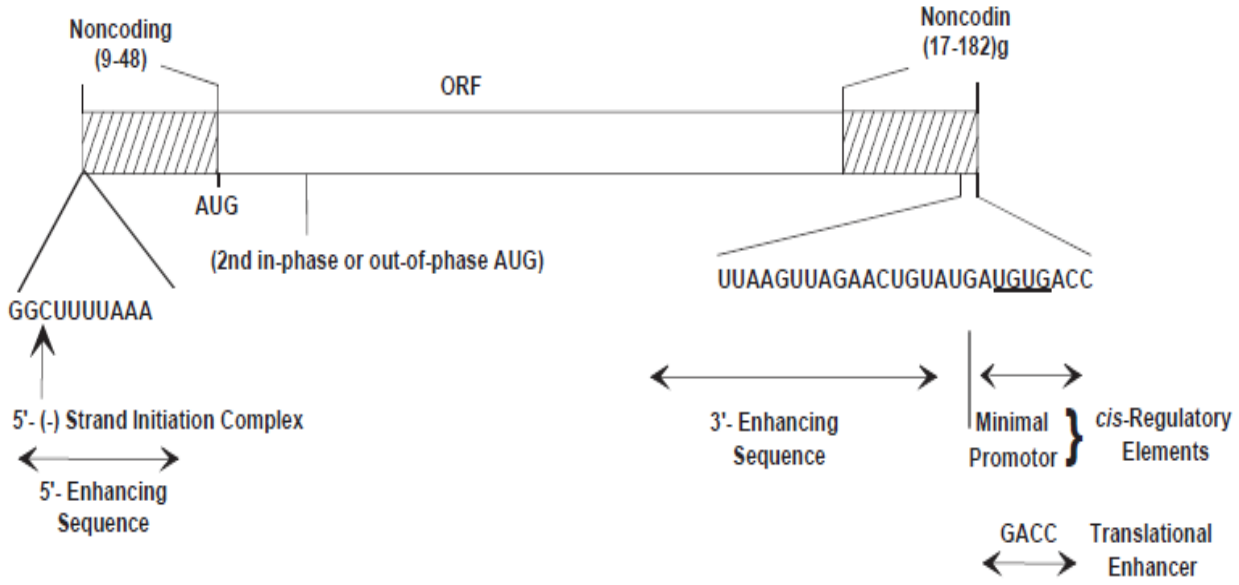


**Figure 2.4** The major features of rotavirus genes structure. The arrows at the bottom show cis-regulatory elements of rotavirus messenger RNA [mRNA] (Estes & Greenberg, 2013).

The weight of rotavirus gene segments ranges from $0.2\times10^6$ daltons to $2.2\times10^6$ daltons with a total molecular weight of approximately $11\times10^6$ daltons to $14\times10^6$ daltons (Desselberger, 2014). The viral core contain its own RNA-dependent RNA-polymerase that it uses to transcribe the RNA segments into active mRNA. Each viral RNA segment contain 5'-methylated cap structures but without polyA

tail. Positive-sense RNA segments have short completely conserved 5' and 3' terminal nucleotide sequences 5'-GGC…ACC-3' of about 7 to 9 nucleotides. The 5'- terminal consensus sequence 5'-GGC(A/U)(A/U)U(A/U)A(A/U)(A/U) appears only once in the fourth gene segment. The 3' end consensus sequence (UGACC) is conserved in all 11 viral genes [the last four nucleotides of the mRNAs are translation enhancers] (Greenberg & Estes, 2009). All 11 RNA segments begins with a 5′-guanidine which is followed by a set of conserved sequences. These conserved sequences are part of the 5′ noncoding sequences and are followed by an open reading frame that codes for the protein product. A set of noncoding sequences (containing a different subset of conserved terminal 3'-sequences) that ends with 3' terminal cytidines is found after the stop codon. The lengths of the 3'- and -5' noncoding sequences vary for different genes (Franco *et al*., 2017; Li *et al*., 2010).

The conserved terminal sequences of rotavirus genome segments contain cis-acting signals important for transcription, RNA translation, RNA transport, replication, assembly or encapsidation of the viral gene segments. All the mRNAs of the virus share common cis-acting signals since they are replicated by the same polymerase, but each segment contain a specific signal unique to it alone because segments must be distinguished from one another during packaging (McDonald & Patton, 2011). The highly conserved noncoding regions may contain gene specific packaging signals. The untranslated regions (UTR) of the RNA segments (+ sense) are 9 to 48 nucleotides long at the 5' end and 17 to 182 nucleotides at the 3' end. All sequenced genes for rotavirus possess at least one long ORF after the first initiation codon. Gene sequences are A+U rich (ranging from 58% to 67%) (Estes & Greenberg, 2013).

### 2.2.3 Gene coding assignments of rotaviruses

The structural proteins of the rotavirus genome build up the viral particle, and the nonstructural proteins (NSPs) function either in the viral replication cycle (Figure 2.5) or interaction with host proteins to influence the pathogenesis or immune response (Sadiq *et al.*, 2018).

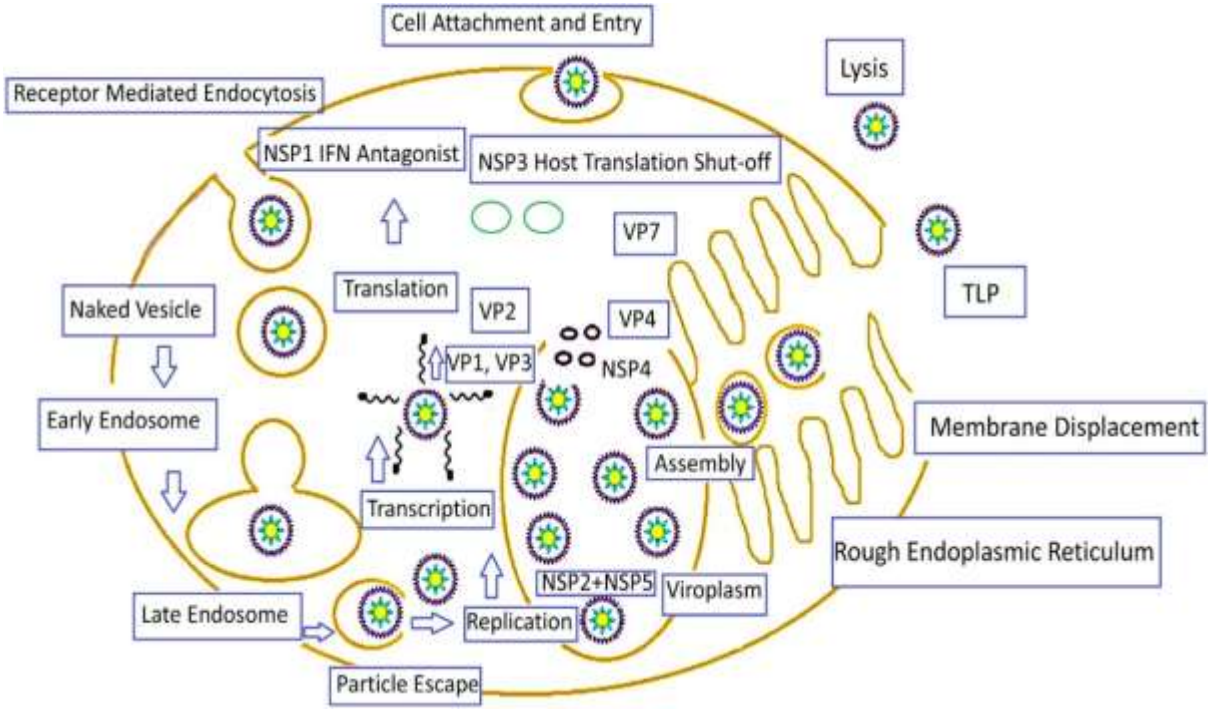


**Figure 2.5** Schematic diagram of the life cycle of rotavirus and roles of all the gene coding assignments (Sadiq *et al*., 2018).

#### 2.2.3.1 Non-structural proteins

The non-structural proteins, NSP1 to NSP6, are essential for rotavirus replication because they modify the cell functions to enable the release of new virions from the infected cells (Holloway & Coulson, 2013). NSP1 binds IRF3 and is capable of inhibiting interferon response during rotavirus infection. NSP2, together with NSP5 is involved in the synthesis and packaging of viral RNA during viroplasm synthesis (Hu *et al*., 2012). NSP3 binds to viral mRNA at the end of the 3' and promotes viral protein synthesis (Gratia *et al*., 2015). NSP4 is essential for rotavirus replication, transcription and morphogenesis. It is required for the outer capsid assembly. NSP4 is a transmembrane glycoprotein that accumulates in the ER and acts as an enterotoxin that cause diarrhea when someone is infected by rotavirus (Trask *et al.,* 2012a). NSP5 acts as a replication intermediate and is a phosphoprotein that works together with NSP2 during RNA synthesis and packaging. NSP6 is not coded for by most rotavirus strains, but when present it interacts with NSP5 in viroplasms and its function is not clear (Martin *et al*., 2013).

#### 2.2.3.2 Inner and core proteins

VP1 is the viral RNA-dependent RNA polymerase enzyme for rotavirus. It is a minor component of the virus central core and it comprises 2% of the total viral mass. VP1 has a molecular weight of approximately 125,000 daltons in Group A and C rotaviruses while in group B is approximately 136000 daltons (Pesavento, 2006). VP1 is associated with RNA transcription and replicase. VP1, together with VP3 forms part of the RNA-dependent RNA polymerase activity associated with double shelled particles (Desselberger, 2014). The innermost layer of the virus is made up of VP2 protein, to which the VP1 and VP3 proteins are attached from the inferior side. VP2 forms the major protein component of rotavirus central core and it is the third most abundant protein in DLPs. Several studies have shown that VP2 has nucleic acid binding activity. VP2 plays an important role

in the structure and function of the viral central core (Hu *et al*., 2012). VP3 is a minor sub-core protein with a molecular weight of approximately 98,000 daltons. The protein consists of amino acid sequence that has multiple repeats of amino acids. VP3 acts as a replication intermediate and has guanilytransferase and methyltransferase activity (Estes & Greenberg, 2013).

The inner capsid layer of the virus is comprised of VP6 which is a product of gene segment 6. It is the major structural protein of the inner viral capsid, weighing approximately 44,816 daltons and constitutes about 51% of the virion (Desselberger, 2014). Because of this large composition and stability of VP6 on the virion, its group epitopes can be detected easily during diagnostic assays. The protein plays important structural, immunological and morphogenic functions during infection. VP6 protein induces heterotypic cross-protective immunity by eliciting T cell (CD4+) responses and circulating IgA antibodies which neutralize the virus by intracellular action (Badillo-Godinez *et al*., 2015). It is also associated with viral RNA transcriptase activity although it does not possess any polymerase activity (Desselberger & Huppertz, 2011).

#### 2.2.3.3 Outer capsid proteins

##### 2.2.3.3.1. VP7

Serotype specificity in human rotaviruses is determined by outer capsid two major proteins, VP7 and VP4. Up to date, 37 different G- and 51 P- types have been characterized for rotavirus in both animals and humans worldwide (RCWG, 2018; Bányai *et al*., 2017).

VP7 is a 34kDa outer capsid glycoprotein encoded by genome segment 7, 8 or 9 depending on the strain (Franco *et al*., 2017). VP7 is highly immunogenic and can induce neutralizing antibodies specific to the G serotypes of group A rotavirus. G1, G2, G3, G4, G9 and G12 are mainly identified in humans. G3-G5, G9 and G11 typically infects pigs. G3, G4, G9 are common in both humans and swine (Dormitzer, 2015). The protein is synthesized with 2 different amino termini and an amino

acid terminal hydrophobic domain that anchors it in the membrane of the ER during its synthesis (Aoki *et al*., 2009). With other copies of 780 molecules, VP7 forms the major component of the outer capsid of rotavirus. It appears to be involved in binding of the virus particle to cell surface during replication (Desselberger, 2014).

Sequence analysis of VP7 gene segment in past studies revealed an Open Reading Frame (ORF) of 326 amino acids that begins with an initiation codon with a weak consensus sequence followed by an in-frame initiation codon with strong consensus sequences 30 codons downstream. These two initiation codons precedes a hydrophobic amino acid region that acts as a signal sequence which directs VP7 to the endoplasmic reticulum. In some strains, a third initiation codon is found 42 nucleotides downstream from the hydrophobic region (Aoki *et al*., 2009; Estes & Greenberg, 2013). In most VP7 molecules, a glycosylation site at amino acid 69 is present and other glycosylation sites are located at amino acid 146, 238 and 318. Conserved cysteine residues in VP7 are found in amino acids 82, 135, 165, 191, 207, 244 and 249. Conserved proline residues are found at amino acids 58, 86, 112, 131, 167, 197, 254, 275 and 279 (Estes & Cohen 1989; Green *et al*., 1987).

**2.2.3.3.2 VP4**

VP4 is a product of gene segment 4. It is an 88,000 daltons, non -glycosylated component of the outer capsid of the rotavirus particle that appears as a homodimer and defines the P serotypes of different rotavirus isolates (Estes & Greenberg, 2013). VP4 protein form projections that can be found on the two-fold symmetry axes of the icosahedron. The protein plays a role in binding to cellular receptors on the surface of the cell, penetration of the virus, infectivity and haemagluttination (Mc Donald & Patton, 2011).

VP4 is cleaved by trypsin into an amino-terminal part, VP8* (molecular weight 28000 daltons), and a carboxy-terminal moiety, VP5* (molecular weight is 60000 daltons) so as to prime the virus for

infection. Both cleavage products are components of the virions and cleavage reactions facilitates penetration of the virus into the cell, enhancing virus infectivity (Trask *et al*., 2012b). Past studies had revealed that the two trypsin cleavage sites are at conserved regions of arginine residues 241 and 247 and are conserved in every VP4 sequence (Pesavento *et al*., 2006). Other conserved cysteine residues are found at positions 216, 318, 380 and 774 in all rotavirus strains. In most rotavirus strains, an additional potential trypsin cleavage site exists at either lysine or arginine before the 3' arginine site at amino acid 246 (Estes & Cohen, 1989). Variable sites were found between amino acid 140 to 160 and amino acid 180 to 210 close to the amino terminus in the region of VP8*. Another variable site had been found between amino acid 580 and 610 in the VP5* product (Arias *et al*., 1996; Kulkarni *et al*., 2014). Sequence analysis of previous studies show that VP4 contains 775 amino acids in humans, whilst animal rotaviruses contains a long ORF with 776 amino acids due to an additional cysteine residue at position 203 (Estes & Cohen, 1989; Pesavento *et al*., 2006).

### 2.2.4 Classification of rotaviruses

Rotavirus classification into a separate genus within the Reoviridae family was based on similarities between capsid structure, genome organization and replication (Esona & Gautum, 2015). Rotaviruses can be classified according to the migration patterns after polyacrylamide gel electrophoresis (Eiden *et al*., 1984). The virus can be classified into long, short, supershort or atypical electrophoretypes. Classification of rotavirus into groups is based on the immunologic structure of the major core capsid protein VP6, which accounts about half of the total core proteins. VP6 allows classification of rotaviruses into ten distinct groups [Group A to J] (Bányai *et al*., 2017; Matthijnssens *et al*., 2012b). Most human rotavirus infections are associated with Group A as compared to all other groups. Different subgroups of Group A rotaviruses are differentiated by

epitopes of VP6 into SG I, SG II, SG I + II, or SG non-I, non-II viruses. Most human rotaviruses fall under class I and II (Desselbeger, 2014).

Serotype and genotype specificities are determined by VP4 (or P serotypes, where P is for protein-sensitive) and VP7 (or G serotypes, G is for glycosylated protein). Rotaviruses with more than 89% amino acid similarity are considered to belong to the same G or P genotype, but some studies revealed a few discordances between the P serotypes and P genotypes. This had been as a result of more genotypes than serotypes that have been identified, due to lack of monospecific P antisera. Therefore, P types are identified as serotypes by Arabic numbers and as genotypes by Arabic numbers in square brackets (Matthijnssens *et al*., 2011).

Classification of rotaviruses was further expanded by Matthijnssens and colleagues, basing classification on nucleotide similarities. In this genotyping system, constellations were designated in the following order: VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5 followed by an Arabic number for the genotype like Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, where x is an integer and represents the genotype of a particular segment (Matthijnssens *et al*., 2008b). This classification system is more valuable in rotavirus analysis since it is widely used and is frequently updated. Rotavirus strains can be Wa-like strains, DS-1 like strains or AU-strains. The Wa-like strains are non-G/P genotypes (I1-R1-C1-M1-A1-N1-T1- E1-H1) and consists of G/P genotypes like G1P[8], G3P[8], G4P[8], or G9P[8]. The DS-1 like strains are characterized by non- G/P genotypes (I2-R2-C2-M2- A2-N2-T2-E2-H2) and usually comprises of G/P genotype G2P[4]. AU-I like strains are minor and are characterized by non-G/P genotypes (I3-R3-C3-M3-A3-N3-T3-E3-H3) consisting of G/P genotype G3P[9] (Matthijnssens *et al*., 2008a; Nakagomi T. & Nakagomi O., 1989).

### 2.2.5 Genetic diversity of rotaviruses

There are three ways that leads rotaviruses to be very diverse. These are gene reassortments, point mutations and gene rearrangements. Point mutations in rotaviruses are related to the proneness of its RNA-dependent RNA-polymerase (since the enzyme do not have proofreading activity) encoded by the VP1 gene segment. Accumulation of point mutations caused by insertions, deletions and duplications during replication leads to antigenic drift of rotaviruses (Kirkwood, 2010; Martella *et al*., 2010). Gene rearrangements contributes a low percentage in genetic diversity of rotaviruses. Rearrangements occurs when the characteristic 4-2-3-2 electrophoretic pattern of the RNA segments is altered and replaced by a different migrating pattern of the bands. This can occur when two rotavirus strains infect a single cell at the same time, resulting in the formation of a chimera gene segment (Navarro *et al*., 2013; Troupin *et al*., 2010). Gene reassortments are the largest contributor to rotavirus genetic diversity and usually occurs when one cell is infected by two rotaviruses at the same time. In most instances, gene reassortments led to numerous exchanges of gene segments between human and animal rotaviruses or between rotaviruses from two different animal species leading to a high number of genotypes known today (McDonalod & Patton, 2011).

### 2.2.6 Diagnosis of rotaviruses

Rotavirus antigen can be detected by fluorescent antibody (FA) staining, immunoperoxidase staining and enzyme immunoassay. Other techniques such as electron microscopy, reverse transcription-polymerase chain reaction, nucleic acid hybridization, sequence analysis, and culture are used primarily in research settings (Soltan *et al*., 2016). Rotavirus antigen can be identified in the serum of patients 3 to 7 days after disease onset, but at present, routine diagnostic testing is based primarily on testing of fecal specimens (Zhang *et al*., 2013).

#### 2.2.6.1 Enzyme linked Immunosorbent Assay

The most widely used and available method for confirmation of rotavirus infection is by detection of rotavirus antigen in stools by enzyme-linked immunoassay (EIA) using commercially available test kits. These kits are simple to use, inexpensive, and very sensitive. Most of commercially available ELISA kits utilizes broadly reactive monoclonal antibodies against epitopes of VP6 glycoprotein (Zhang *et al*., 2014). The method is reliable even in cases where specimen transportation is prolonged. Rotavirus antigen detection with ELISA is practical, easy to perform, does not need several specialized laboratory equipment and allow rapid diagnosis. There are two types of ELISA, direct and indirect immunoassay. Amongst these two types of ELISA, the double antibody sandwich is the most common procedure. In this assay, the antigen is immobilized to a solid surface, and then a specific antibody linked to an enzyme will be applied to the solid surface so that it can bind to the antigen. A substrate will be added to the plates at the end, followed by incubations, so that the enzyme will be detected (Lequin *et al*., 2005). Subgroup-specific monoclonal antibodies in an ELISA can be detected by subgroups defined by epitopes on the VP6 protein. Four subgroups had been described in literature: subgroup I, subgroup II, subgroup I + II and subgroup non-I non-II (Matthijnssens *et al*., 2012b).

#### 2.2.6.2 Reverse transcriptase polymerase chain reaction

Detection of specific DNA or RNA sequences can be applied on any virus. These assays can be specific for a single virus or for a group of related viruses. Polymerase Chain Reaction (PCR) is one of the nucleic acid detection method that can be used on rotavirus specimens (Estes & Greenberg, 2013). PCR is more sensitive than enzyme immunoassay, and it is one of the widely used Nucleic Acid Amplification Tests (NAAT) technique because of its simplicity and applicability (Reddington *et al*., 2014). RT-PCR can be used to analyze all the eleven segments of the viral genome for

rotavirus but most studies focus on only VP7 and VP4 proteins since they are used for major classification of human Group A rotaviruses (Desselberger, 2014). RT-PCR use RNA as a template, where the RNA is reverse transcribed into cDNA followed by standard PCR procedure which amplify the cDNA. Most RT-PCR assays employ one-step or two-step of reverse transcription followed by amplification approach so as to increase sensitivity for rotavirus detection (World Health Organization, 2016).

Like any classical PCR, RT-PCR for rotavirus comprises of three major steps, namely denaturation, annealing and extension. During denaturation, high temperatures of up to $90^0$C to $97^0$C are used to denature the DNA. The second step involves the use of primers that will anneal with the DNA template strands to prime extension. The final step in PCR involves extension that occurs at the end for the annealed primers to create a complimentary copy of a strand of DNA and double the DNA quantity. VP7 is usually amplified by primer set sBeg9/Beg9 and End9/EndA (Figure 2.6) which are selected from the highly conserved regions of rotavirus genome. For VP4, genotyping primers con3 and con2 (Figure 2.7) are used (Das *et al*., 1994; Gentsch *et al*., 1992; Gouvea *et al*., 1990). Detection by RT-PCR method is based on the fluorogenic 5'nuclease assay whereby DNA polymerase cleaves the probe at the 5' end and separates the reporter dye from the quencher dye only when the probe hybridizes to the target DNA. This cleavage results in the fluorescent signal generated by the cleaved reporter dye. The process generates thousands or millions of copies of a particular DNA sequence (Modrow *et al*., 2013).

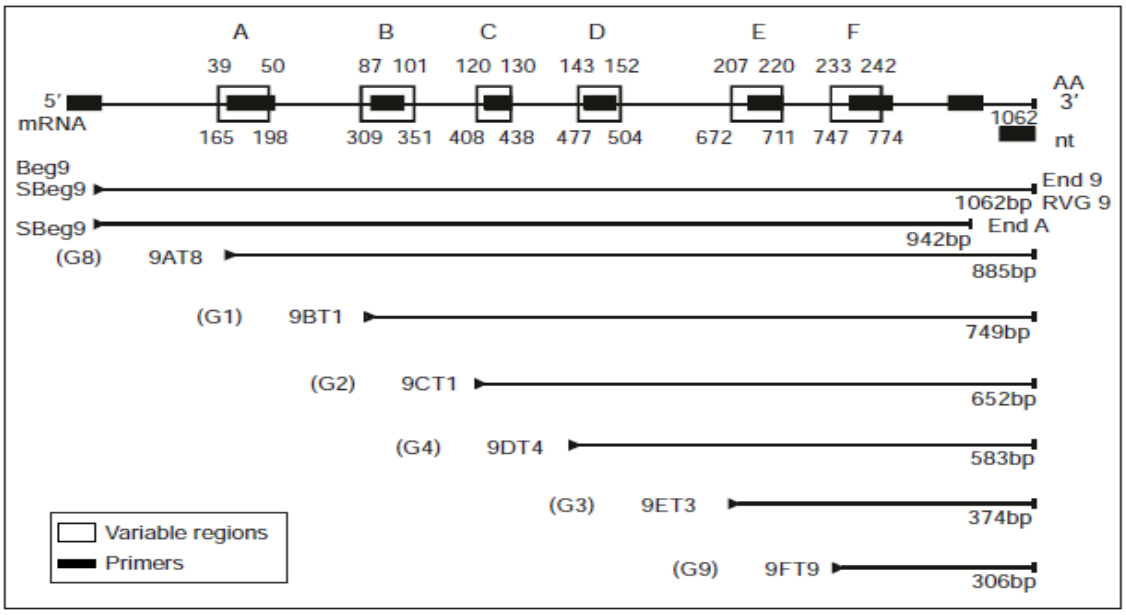


**Figure 2.6** Schematic illustration of VP7 genotyping by PCR using sBeg9/Beg9, End9/EndA/RVG9, and a primer cocktail (World Health Organization, 2016).

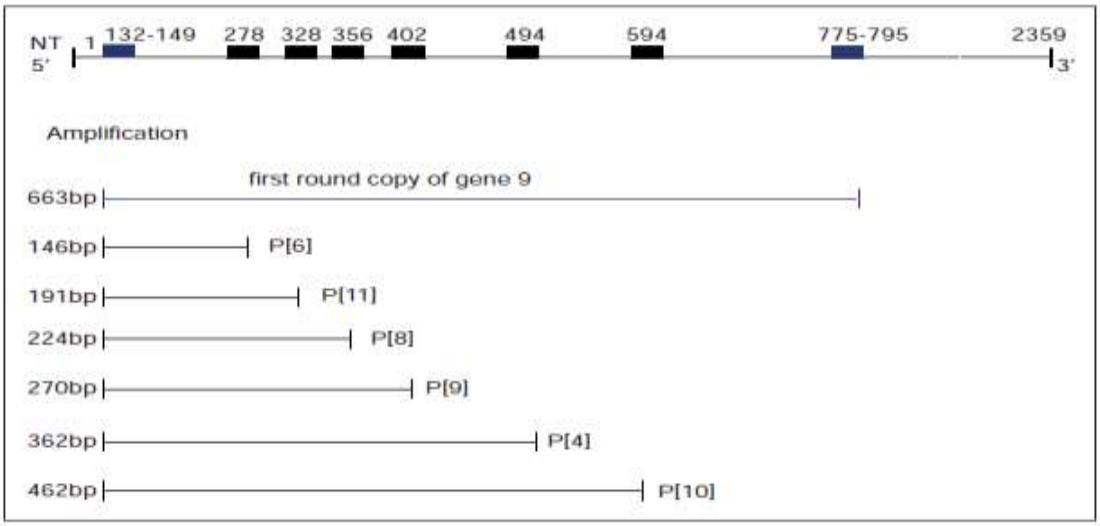


**Figure 2.7** Schematic illustration of VP4 genotyping using con2/con3 and a primer cocktail (Gentsch *et al*., 1992).

#### 2.2.6.3 Nucleotide sequencing

Sequence analysis is the standard procedure for confirmation and identification of rotavirus strains previously genotyped by RT-PCR. PCR amplification products can be sequenced out using cycle sequencing reaction or polysequencing (Metzker, 2010). A variety of consensus primer-pairs have been described for the VP7 gene for rotaviruses and this includes beg9/end9 and VP7-F/VP7-R, 9con1/9con2 and 9con1-L/VP7-R deg. Consensus primers for VP4 gene fragments include con2/con3, HumCom5/HumCom3 and VP4-F/VP4-R. After sequencing, the genotype of the strain can be determined by comparing the genes of strains with known VP4 or VP7 types from the GenBank database (Reddington *et al*., 2014; World Health Organization, 2016). Information that is generated from sequencing can be used in precise identification, genotyping, detection of presence or absence of mutations related to antiviral drug resistance or unusual clinical manifestations. Next generation sequencing (NGS) plays an important role in diagnostic virology where it is applied on antiviral susceptibility testing as well as discovery of known and novel viruses (Goodwin *et al*., 2016).

#### 2.2.6.4 Polyacrylamide gel electrophoresis

The dsRNA of Rotavirus can be extracted by several methods and analyzed by polyacrylamide gel electrophoresis (PAGE) followed by silver staining. PAGE is an analytical method used to separate components of a protein mixture according to their sizes (Kabir *et al*., 2016). The method is based on a principle that charged molecules migrate in an electric field towards an electrode with an opposite charge. Negatively charged macromolecules of the 11 segments of Rotavirus dsRNA separate according to size during electrophoresis and migration patterns can be visualized after silver staining. Silver staining allow silver ions to form a stable complex with the nucleic acids, making them to be visible as bands (Herring *et al*., 1982).

The dsRNA of Group A rotaviruses can be classified into 2 major electrophoretypes; long and short electrophoretypes. Super short electrophoretypes have been identified in some studies (Matsui *et al*., 1990). The short electrophoretypes phenotype results from a partial duplication in gene 11, which causes it to migrate more slowly than gene segment 10. The $11^{th}$ gene segment of long electrophoretypes migrates faster than the $10^{th}$ gene segment (Santos & Hoshino, 2005). Viral dsRNA can also be grouped in 4 different classes that is, 4 large segments, 2 medium-sized segments, 3 small segments and 2 smallest segments. Results for PAGE analysis are easier to interpret, but the whole procedure is time consuming and requires trained and experienced technologists (World Health Organization, 2016).

### 2.2.7 Prevalence and seasonal variation of rotavirus infections

Rotavirus is widely spread in both developing and developed countries. It is one of the leading causes of diarrhea in children under the age of five years. By the age of three, almost every child would have been infected once or more by the virus (Troeger *et al*., 2017). Children that are between the age of six months and two years, premature infants, immuno-compromised individuals and the elderly are more vulnerable to the virus (Walker *et al*., 2013). There has been an over 90% reduction of rotavirus hospitalizations in industrialized and low income countries since the implementation of rotavirus vaccines in immunization programs (Shah *et al*., 2017).

The prevalence of specific rotavirus strains varies with geographic area and seasons (Al-Thani *et al*., 2013). In regions with temperate climate, five serotypes of Group A rotavirus, G1P-, G2P-, G3P-, G4P- and G9P- accounts for 90% of clinical rotavirus globally. Among P types found with these G types, P [4], P [6] and P [8] are the most prevalent (Shah *et al*., 2017). Several surveillance studies done in Africa show that most cases of rotavirus infections are related to G1 co-circulating with other strains even in vaccinated children. However, circulating strains have been found out to vary

from year to year (Mukaratirwa *et al*., 2018; Ouermi *et al*., 2017; Page *et al*., 2018). Some strains like G2 have been reported worldwide to occur every 3 to 4 years and in seasons of high prevalence followed by low prevalence. In recent studies, G9 seems to emerge in Africa whereas G4 and G8 strains occur in sporadic isolation. Most rotavirus strains isolated in African countries are highly associated to P [8], P [6] and P [4] (Seheri *et al*., 2018). In Botswana, G2P[4] and G1P[8] have been reported to be the most predominant rotavirus genotypes in the post vaccination era (Table 1), while G1 and G3 associated with P[8] and P [6] were predominant before vaccine introduction.

**Table 2** Prevalence of rotavirus in children with diarrhea in Botswana

| Year | Prevalence (%) | Prevalence in children ≤2 years (%) | Dominant strain |
|---|---|---|---|
| 1999-2001 | 17 (Kasule *et al*., 2003) | 86 (Kasule *et al*., 2003) | G1P[8] (Kasule *et al*., 2003) |
| 2001-2002 | 9.2 (Basu *et al*., 2003) | 91 (Basu *et al*., 2003) | Not genotyped |
| 2003-2004 | 13 (Kebaabetswe *et al*., 2005) | 94 (Kebaabetswe *et al*., 2005) | G3P[6] (Kebaabetswe *et al*., 2005) |
| 2011-2013 | 36 (Pernica *et al*., 2016) | 74 (Pernica *et al*., 2016) | Not genotyped |
| 2013-2015 | 40 (Gastañaduy *et al*., 2016) | 99 (Gastañaduy *et al*., 2016) | G2P[4] (Gastañaduy *et al*., 2016) |

The occurrence of rotavirus infections tends to follow a seasonal pattern in most countries globally. In temperate climates, rotavirus gastroenteritis is most prevalent in winter season as compared to other seasons. In tropical and subtropical climates, the distribution of rotavirus infections is not clear, but it occurs all year round, reaching its peaks in the drier and cooler months (Dormitzer, 2015).

### 2.2.8 Transmission of rotavirus infections

Rotavirus is very stable and may remain viable in the environment for weeks or months if not disinfected and this makes the virus to be of much public concern. The virus is shed in very high concentrations in stools of infected people (more than $10^9$ virus particles/g) and only a few particles (10 to 100 particles) of the virus are capable of causing infection (Dóró *et al.,* 2015). Rotavirus spreads by fecal-oral route or through respiratory droplets. Vehicles of transmission can be through person to person contact, contact with vomitus, respiratory secretions as well as contact with fecal contaminated surfaces. Ingestion of contaminated food and water can transfer the virus into the body (Estes & Greenberg, 2013).

### 2.2.9 Symptoms and pathogenesis of rotavirus induced diarrhea

Rotavirus infection induces several symptoms that range from mild to severe and in some cases asymptomatic. Clinical features, stool appearance and characteristics of rotavirus gastroenteritis are not specific and stools can only be confirmed for rotavirus diarrhea by laboratory testing. Symptoms of rotavirus-acquired diarrhea usually appear within two days of infection (incubation period) and this include forceful vomiting, fever, loss of appetite and watery diarrhea that last for a period of three to eight days (Hagbom *et al*., 2012; Lee *et al*., 2013).

Watery diarrhea in rotavirus infections is triggered by damage to the epithelial cell line and changes in intracellular $Ca^{2+}$ ion concentration when mature enterocytes are infected. After ingestion of rotavirus particles, they enter the small intestines then goes to mature enterocytes by calcium-dependent endocytosis or direct entry (Greenberg & Estes, 2009). Increased $Ca^{2+}$ ion concentration by NSP4 inhibits $Na^+$ co-transporters and reduces the capacity of absorption of the epithelium of intestines and some organic molecules remain unabsorbed. This increases the osmolarity in

intestines hence absorption of water from the epithelium, leading to osmotic diarrhea (Desselberger, 2014).

Vomiting in rotavirus-acquired gastroenteritis is mainly caused by the release of 5HT (5-hydroxytryptamine) by enterochromaffin cells of the intestines. After its release, 5-HT interacts with $5\text{-HT}_3$ leading to the stimulation of the vagal efferent nerve projecting to the vomiting center of the brain. Usually, $5\text{-HT}_3$ receptor antagonists in anti-emetic drugs are used to stop vomiting in children suffering from rotavirus related diarrhea (Hagbom *et al*., 2012). Fever in people infected by rotavirus is stimulated by the release of pyrogens from viral infected cells. Pyrogens like prostaglandins have an effect on temperature and are capable of stimulating the secretion of water that is secreted as sweat in patients with fever (Rivero-Calle *et al*., 2016; Scher & Pillinger, 2009).

**2.2.1.0 Prevention of rotavirus infections**

There are several ways that can help in preventing rotavirus illnesses. Frequent washing of hands highly reduces the risks of infection. Contaminated materials can be treated in temperatures higher than $50^0$C. Treatment of objects and surfaces in 95% ethanol can remove the outermost layer of the virus, which contains VP7 and VP4 that plays a major role in infectivity of rotavirus (Dennehy, 2013). In cases of fecal accidents, surrounding areas should be properly cleaned and disinfected. Infected people can be excluded from places like childcare institutes, schools and work until vomiting and diarrhea ceases. Food handlers should avoid handling of food until there is no vomiting and diarrhea for 48 hours (Cortese & Parashar, 2009).

#### 2.2.1.1 Past and current rotavirus vaccines

##### 2.2.1.1.1 Past discontinued rotavirus vaccines

Since the early 1980s, efforts to find rotavirus vaccine were underway. The first two rotavirus vaccine candidates were live bovine vaccines RIT4237 and WC3. RIT4237 was derived from Lincoln isolate bovine virus NCDV with serotype G6P[6] and was given as a single dose before the rotavirus season. The vaccine was not affected by breastfeeding, it was safe and immunogenic in young infants (Vesikari *et al*., 1985) but its failure to demonstrate consistent protective efficacy in Africa led to it being discontinued from trials. The bovine vaccine strain WC3 (Winstar Cow) was developed by cell culture and was derived from VP6 serotype 6 that was isolated at Winstar Institute in Philadelphia (Clark *et al*., 1996). The vaccine was safe, nonreactogenic and immunogenic in young infants but demonstration of poor protection in Ohio and Central African Republic led to its development being discontinued (Albert, 1987).

Bovine rotavirus vaccines were replaced by RRV-1, which was a rhesus rotavirus vaccine isolated from diarrhea stools of a rhesus monkey and characterized as VP7 serotype 3 unrelated to human strains. RRV-1 was safe and immunogenic in children (Perez-Schael *et al*., 1997; Vesikari & Joensuu, 1996). However, one third of infants that were vaccinated with RRV developed low grade fever for 3 to 4 days after administration of the vaccine. Inconsistent protective in trials led to its discontinuation (Flores *et al*., 1987). RRV-1 was later named Rotashield after being reasserted with human rotavirus so as to express the human rotavirus antigen G1 to G4 and Rhesus G3. Although fever occurred more frequently after vaccination in the third trial (Vesikari & Joensuu, 1996), the vaccine was found to be safe and immunogenic. This vaccine was discontinued after one year of its introduction due to increased risks of intussusceptions within the first 10 days after its administration (Centers for Disease Control and Prevention, 1999).

#### 2.2.1.1.2 Current rotavirus vaccines

From the year 2006, two effective vaccines namely RotaTeq™ and Rotarix® were prequalified by WHO and recommended for routine immunization of children. Eight-one countries have managed to introduce Rotarix® or RotaTeq™ in their routine national immunization programs by the year 2016 (Madhi *et al*., 2016; Shah *et al*., 2017). Rotarix® (GlaxoSmith Kline Biologicals, Rixensart, Belgium) is one of the widely used vaccines against rotavirus. It is a live vaccine that contains the attenuated monovalent G1 and P[8] human rotavirus strain. Rotarix® is recommended to be orally administered in 2 doses from 6 to 12 weeks of age followed by intervals of at least 4 weeks between first and second dose up until 24 weeks of age (McCormack & Keam, 2009). Rotarix® vaccine demonstrated 49-77% efficacy in clinical trials in some African countries like Botswana (Gastañaduy *et al*., 2016), Malawi (Cunliffe *et al*., 2012) and South Africa (Steele *et al*., 2012).

RotaTeq™ (Merck and Co, PA, USA) is a live, oral attenuated, bovine-human reassorted rotavirus vaccine containing five reassortant viruses with G1P[7], G2P[7], G3P[7], G4P[7] and G6P[8]. It can be orally administered in 3 doses between 6 to 12 weeks, followed by subsequent doses at 4-week to 10-week intervals but the last dose should be administered before 32 weeks of age (Ciarlet & Schödel, 2009). In trials in USA and other developing countries, RotaTeq™ have shown that it can give upto 74% protection against rotavirus induced diarrhoea and 100% protection against severe rotavirus diarrhoea (Burnett *et al*., 2018). Over the past years, Rotarix® and RotaTeq™ had been safely and effectively implemented in countries they were introduced. Intussusception risks (1.9 and 1.5/100 000 children) for the first 21 days after the first dose of Rotarix or RotaTeq had been documented (Stowe *et al*., 2016) but the benefits of these vaccines outweighs the risks.

There are some rotavirus vaccines currently used in some specific countries. The lamb rotavirus strain vaccine, Lanzhou Lamb Rotavirus (LLR) is a monovalent lamb vaccine strain G10P[2] licensed in China in 2000. LLR was observed to have 13 to 52% protection against rotavirus diarrhea

and 15-75% against moderate to severe rotavirus diarrhea (Zhen *et al*., 2015). Rotavin-M1™ (Polyvac, Viet Nam) is another oral vaccine licensed in Vietnam that was derived from an attenuated G1P[8] strain isolated from a child in Vietnam. The vaccine is safe and immunogenic in young children although effectiveness studies are still ongoing (Tu *et al*., 2012). High incidences of intussusceptions had been observed in children vaccinated by Rotavin-M1™ (Van Trang *et al*., 2014) but phase II clinical trial in comparison with Rotarix® conducted on infants revealed no differences in terms of mild adverse effects after dosage (Anh *et al*., 2012).

ROTAVAC (Bharat Biotech International Limited) and ROTASIIL (Serum Institute of India) are currently licensed in India but not prequalified by World Health Organization. ROTAVAC consists of a naturally occurring G9P[11] reassortant strain combined with one bovine rotavirus gene and 10 human rotavirus genes. ROTASIIL is a live attenuated human bovine reassortant pentavalent vaccine consisting of rotavirus serotypes G1, G2, G3, G4 and G9 (Deen *et al*., 2018; Naik *et al*., 2017). Both ROTAVAC and ROTASIIL demonstrated safety in several clinical trials but used sample sizes were small to detect any slight risk caused by the presently prequalified vaccines (Ella *et al*., 2018; Kulkarni *et al*., 2017).

# CHAPTER 3

# MATERIALS AND METHODS

## 3:1 Experimental design

### 3.1.1 Study design, study site and study period

This study was conducted in Gaborone, the largest and capital city of Botswana. A case study was conducted on diarrheic children aged 0 to 60 months between March and November 2017. Diarrheal participants were children below the age of five years whose stool samples were submitted to Princess Marina Hospital, Bokamoso Private Hospital, and Diagnofirm medical microbiological laboratories. The control population was made up of asymptomatic non-diarrheic children below the age of five from the Pediatric Ward of Princess Marina Hospital and Child Welfare Clinics in Gaborone. Enrolment of non-diarrheic children was subjected to their parental or guardian consent.

### 3.1.2 Study population and sample size

Diarrheal fecal specimens were collected from 200 children below 5 years of age seeking medical attention at Princess Marina Hospital, Bokamoso Private Hospital and Diagnofirm medical microbiological laboratory between March and November 2017. The sample size was calculated using the Cochran's formula:

$n_0 = [z^2pq]/e^2$

where $n_0$= required sample size,

z= standard normal deviate

p= the proportion in a particular target population estimated to have a particular characteristic under study,

q= 1-p, and

e= desired level of precision (Kotrlik & Higgins, 2001).

Since previous reports in Botswana with similar research settings for *Cryptosporidium*, *Giardia* (Alexander *et al*., 2012) and rotavirus (Kebaabetswe *et al*., 2005) observed prevalence rates that range from 10 to 13%, a p-value of 0.15 was thought to be adequate for the study. Using the above formula, and 5% margin of error, the estimated sample size was 197, therefore 200 samples were found to be enough for the study.

A ratio of 2:1 was chosen for proportion between cases and controls since it was difficult to get samples from non-diarrheic children due to cultural beliefs and other unclear reasons from parents or guardians. The control group population consisted of 100 non-diarrheic children seeking help from Princess Marina Pediatric Ward and asymptomatic children from several Child Welfare Clinics in Gaborone. Samples were collected from collection points daily on weekdays. General information on the study participants like age and gender of the children enrolled in the study was provided by clinics and microbiological laboratories during collection of samples. All samples that were received by the microbiology laboratories enrolled in the study were obtained until required sample size was reached. Non-diarrheic participants were selected at random.

### 3.1.3 Inclusion and exclusion criteria

All samples from diarrhea patients within the age of 0-59 months were included in the study. All children without diarrhea, with the appropriate age from Child Welfare Clinics and Princess Marina Pediatric Ward who were willing to participate in the study and have consent to participate were included in the control group. Children whose age was above 60 months at the time of sample collection were excluded. All children who were not consented by their parents or guardians were also excluded from the study.

### 3.1.4 Sample collection

Samples from children who met all the inclusion criteria were requested from the microbiological laboratories, wards and clinics enrolled in the study. Samples were collected in sterile containers and labeled according to the date of collection and sample number. Transportation of samples to the Virology laboratory at the University of Botswana was done on an ice box. All samples were divided into two parts before storage. Stool samples that were intended for the study of parasites were analyzed microscopically and stored at -20$^0$C without any preservative. A 10% stool suspension was made by adding 9 parts of injection water to each part of specimens intended to be analyzed for rotavirus. The mixture was vortexed for a few seconds for the sample to be completely suspended in water before storage at 4$^0$C until time of analysis.

## 3.2 Laboratory methods

### 3.2.1 Detection of *Giardia* cysts

A drop of iodine and saline drops were added to a match-head sized stool sample on a glass slide. A smear was prepared by mixing gently. Smears were covered by a glass slide and examined under the light microscope, initially at 10× objective then at 40× objective (Baig *et al*., 2012).

### 3.2.2 Detection of *Cryptosporidium* cysts

All stool samples were detected for the presence of *Cryptosporidium parvum* by means of the Modified Ziehl Neelsen method (Fayer *et al*., 2000). Fecal smears were prepared directly from the stool sample and allowed to air dry. Smears were fixed in methanol for 3 minutes first then by passing briefly through a flame. After fixing, smears were stained with carbol fuchsin (0.34 % fuchsin and 4%w/v phenol) for 20 minutes, and then rinsed thoroughly under running tap water. Stained slides were decolorized using acid alcohol for 20 seconds (acid alcohol contained 1% HCl

in methanol). After 20 seconds, slides were rinsed thoroughly under running tap water before counterstained with 0.4% malachite green for 60 seconds. Slides were rinsed thoroughly under running tap water, air dried, then blotted on toweling paper to remove excess water and examined under the light microscope using 40× objective before 100× objective.

### 3.2.3 Confirmation of *Cryptosporidium parvum* and *Giardia intestinalis* in samples that test negative after microscopy

The presence or absence of *Cryptosporidium parvum* and *Giardia intestinalis* in stool samples that tested negative was confirmed by means of a One-step *Crypto+Giardia* combo test card (CerTest Biotec S.L., Zaragoza, Spain). Manufacturer's instructions were followed. The principle of this test is based on qualitative immunochromatographic assay for the determination of *Cryptosporidium parvum* and *Giardia intestinalis* in stool samples.

### 3.2.4 Rotavirus detection

#### 3.2.4.1 Rotavirus Antigen Detection

Rotavirus from the stool samples was screened using ELISA kits for Group A Human Rotavirus (IVD Research Inc., Carlsbad, CA, USA). Instructions from the manufacturer's manual were followed. The results were read visually first, then spectrophotometrically at 450nm using a microplate reader. Samples were regarded positive for the presence of rotavirus when the absorbance reading was 0.15 and above. All samples recording an absorbance reading of less than 0.15 OD were regarded as negative.

### 3.2.4.2 Extraction of the dsRNA for PAGE

Double stranded RNA (dsRNA) was extracted from 16 samples that were strongly positive for group A rotavirus antigen using the phenol-chloroform (reagents listed in Table i of appendix 1) method (World Health Organization, 2016). To a sterile eppendorf tube, 450µl stool suspension was added then 50µl of pre warmed solution of 1M NaAcetate (sodium acetate) with 1% SDS (sodium dodecyl sulphate). The mixture was vortexed for 10 seconds and incubated at $37^0$C for 15 minutes. An equal amount of phenol chloroform was added and then vortexed for 1 minute before incubated for 15 minutes at $56^0$C. Tubes were opened and immediately resealed and further vortexed for 1 minute before centrifuged at 12000rpm for 3 minutes. The upper aqueous phase was transferred to another tube. Phenol-chloroform (250µl) was added to the solution, then phenol-chloroform extraction was repeated again. Approximately 40µl of 3M NaAcetate (pH 5.0) and 700 µl of ice cold absolute ethanol was added to the dsRNA solution and mixed gently by inversion 4 to 6 times. The solution was incubated at $-20^0$C for 2 hours, then at $70^0$C for 30 minutes. RNA was pelleted by centrifugation at 12000rpm for 15 minutes at a temperature of $4^0$C and ethanol was decanted immediately. Tubes were inverted on a paper towel to dry. The remaining pellet was resuspended in 30µl of loading buffer.

### 3.2.4.3 Polyacrylamide gel electrophoresis of double-stranded RNA for rotavirus

Polyacrylamide gel electrophoresis (PAGE) was carried out so as to determine the migration patterns of the rotavirus segmented genome (recipes of reagents are in Table iii, Appendix 1). Gel electrophoresis was carried out in 10% acrylamide slab gels with a 4% stacking gel [Appendix 1, Table ii] (World Health Organization, 2016). A discontinuous buffer system without sodium dodecyl sulphate was used. Aliquots for each sample were loaded on the gel and electrophoresis was done at 150V for 2 hours at room temperature (Laemmli, 1970).

The dsRNA segments were visualized after staining gels with silver nitrate using the technique of Herring *et al*., (1982). After electrophoresis gels were fixed in 200ml fixing solution 1 for 30 minutes on a rotary shaker at room temperature then fixed for the second time in 200ml fixing solution 2. Gels were the stained by adding 200ml of silver nitrate solution. Silver nitrate solution was aspirated and gels were washed in distilled water three times for 15 seconds each. To remove any black precipitate, 50ml of developing solution was added to the gels and agitated by hand for 30 seconds before it was aspirated. The remaining 200ml developing solution was added (all recipes for silver staining solutions are listed in Appendix 1, Table iv). Gels were rotated at room temperature for 5 minutes. Developing solution was drained, and 200ml of stopping solution was used to stop the reaction. The gels were dried and photographed on a light box.

### 3.2.4.4 RNA extraction and reverse transcription

Double stranded RNA to be used for RT-PCR was extracted by means of the ZR Viral RNA kit (Zymo Research, Orange, CA, USA), following instructions from the manufacturer's manual. The eluted dsRNA was immediately stored at -70$^0$C until further analysis. Reverse transcription was done (for 15 samples that were showing good nanogram readings) using the Protoscript II cDNA Synthesis Kit (New England Biolabs). The RNA that was extracted by ZR Viral RNA kit was used as a template for reverse transcription. For each sample, the following reagents were added to a reverse transcriptase tube on ice: 4µl total dsRNA, 2µl of Oligo-dT primer [d(T)$_{23}$ VN], 10µl Protoscript II Reaction Mix (consisting of deoxynucleoside triphosphates [dNTPs] and an optimized buffer), 2µl Protoscript II Enzyme Mix (made up of reverse transcriptase and murine RNase inhibitor). A volume of 2µl nuclease-free $H_2O$ was added to each reaction to make up a total of 20µl. Tubes were then incubated at 42$^0$C for 1 hour and the enzyme was inactivated by placing the tubes at 80$^0$C for 5 minutes. For the control reaction without reverse transcriptase, 4µl total dsRNA

was mixed with 2 µl d (T) $_{23}$ VN, 10 µl Protoscript II Reaction Mix and 4µl nuclease-free $H_2O$ in a reverse transcriptase tube on ice. Tubes were then incubated at $42^0$C for 1 hour. All the cDNA products were stored at $-20^0$C until further analysis.

### 3.2.4.5 RT-PCR for dsRNA of rotavirus

The cDNA generated from reverse transcription was used as a template for PCR. Amplification was mainly done in 2 rounds: consensus (first round) and a multiplex (second round) PCR. The method of Gouvea and colleagues was used for G-typing (Gouvea *et al*., 1990) and P-typing was carried out using the method of Gentsch and colleagues (Gentsch *et al*., 1992). For first round amplification, specific primer pair con2/con3 were used for VP4 and sBeg9/End9 for VP7 (sequences of the consensus primers are in Table v of Appendix 2).

### 3.2.4.6 G-typing (VP7) by PCR

For VP7 genotype amplification, PCR was done in a total volume of 50µl. PCR reactions contained 4.5µl buffer (Bioline), 2.0µl of 50µM $MgCl_2$ (magnesium chloride), 1.0µl of 10µM dNTPs (deoxynucleoside triphosphates), 1.0µl of 10µM sBeg9, 1.0µl of 10µM End9, 0.2µl Taq polymerase (Bioline), 35.3µl nuclease free water and 5µl cDNA. The reaction was gently mixed by pulsing in a microcentrifuge for 5 seconds. PCR tubes were then placed in a preheated $94^0$C thermal cycler and cycled under the following conditions: 2 minutes at $94^0$C; 35 cycles of 1 minute at $94^0$ C, 1 minute at $52^0$C, 1 minute at $72^0$C; a final step of 7 minutes at $72^0$C, then held at $15^0$C.

A multiplex PCR system was used to amplify serotype G of VP7. PCR reactions were prepared by mixing 4.8µl buffer (Bioline), 2.5µl of 50µM $MgCl_2$, 1.0µl of 10µM dNTPs, 1.0µl of 10µM End9, 1.0µl of 10 µM of each of the six serotype specific primers, 0.2µl Taq polymerase (Bioline), 32.5µl nuclease free water and 2 µl of cDNA product from first round amplification. Serotype specific

primers used for G-typing are aBT-1, aCT-2, aET-3, aDT-4, aAT-8, and aFT-9 (details on the primer sequences are in Table vi of Appendix 2). PCR reaction mixtures were pulsed for 5 seconds then placed in a preheated thermal cycler and reaction was run using the following conditions: 2 minutes at $94^0$C; 35 cycles of 1 minute at $94^0$C, 1 minute at $50^0$C, 1 minute at $72^0$C; then 7 minutes at $72^0$C. Tubes were held at $15^0$C. After amplification, 10µl of each PCR product was diluted in 6 times gel loading buffer and examined by gel electrophoresis. The gels used were 1.5% agarose gels containing ethidium bromide (20µl ethidium bromide solution in 50µl agarose gel). Samples were run alongside a 100 base pair molecular ladder in Tris-borate-EDTA (TBE) buffer at 100V for 30 to 45 minutes at room temperature. At the end of electrophoresis, gels were photographed under ultraviolet (UV) light.

A semi- nested PCR was performed on several samples that failed to be typed by Multiplex PCR. The PCR amplification was done in a final volume of 25µl. PCR reactions were prepared by mixing 2.4µl buffer (Bioline), 1.3µl of 50µM $MgCl_2$, 0.5µl of 10µM dNTPs, 0.5µl of 10µM End9, 0.2µl Taq polymerase (Bioline), 0.5µl of 10 µM of any of the six of the serotype specific primer, 17.6µl nuclease free water and 2µl of the cDNA product from first round amplification. Cycle conditions used were the same as in Multiplex PCR. The PCR products were examined as above.

#### 3.2.4.7 P-typing (VP4) PCR

Consensus primer mixtures were prepared by adding 4.5µl buffer (Bioline), 2.5µl of 50 µM $MgCl_2$, 1.0µl of 10µM dNTPs, 1.0µl of 10µM con3, 1.0µl of 10µM con2, 0.2µl Taq polymerase (Bioline), 34.8µl nuclease free water and 5µl of cDNA. The mixture was centrifuged for 5 seconds and PCR reactions were immediately placed in a preheated $94^0$C thermal cycler and cycled under the following conditions: 2 minutes at $94^0$C; 35 cycles of 1 minute at $94^0$ C, 1 minute at $50^0$C, 1 minute

at $72^0$C; extended for 7 minutes at $72^0$C, then held at $15^0$C. PCR products were also subjected to 1.5% agarose gel electrophoresis at 100V for 30 to 45 minutes before photographed under UV light.

Second round amplification for serotype P of VP4 was done using con3 and five P-type specific primers P[4], P[6], P[8], P[9] and P[10] (primer sequences are shown in Table vii of Appendix 2). PCR mixtures were prepared by adding 4.8µl buffer (Bioline), 2.5µl of 50µM $MgCl_2$, 1.0µl of 10µM dNTPs, 1.0µl of 10µM con3, 1.0µl of 10µM of five of the serotype specific primer, 0.2µl Taq polymerase (Bioline), 33.5µl nuclease free water and 2µl of the cDNA from first round amplification. The solution was denatured at $94^0$C for 4 minutes followed by 30 cycles of PCR at $94^0$C for 1 minute, $45^0$C for 2 minutes and $72^0$C for 1 minute. This was followed by a final elongation at $72^0$C for 7 minutes. Reactions were held at $15^0$C. PCR products were examined by gel electrophoresis method used for first round amplicons.

### 3.2.4.8 Sequencing

Out of the 15 samples that were tested by PCR, 10 specimens were selected for sequencing at Inqaba Biotechnical Industry (Pretoria, South Africa). The Nimagen BrilliantDye™ v3.1 Terminator Cycle Sequencing Kit (NimaGen BV, Nijmenden, The Netherlands) was used for sequencing, following manufacturer's instructions. Amplicons were representatives of both VP4 and VP7 strains for selected specimens. The primers that were used for RT-PCR were also used as sequencing primers.

Prior to sequencing, amplicons were subjected to PCR using the NEB OneTaq 2X Master Mix with Standard Buffer (M0482) kit (New England Biolabs, Inc) according to manufacturer's procedure. After PCR, amplicons were visualized on a 1% agarose gel (Cleaver Scientific Ltd) stained with EZ-vision® Bluelight DNA Dye (Amresco, USA) to see the integrity of the PCR. PCR products were purified by the Exonuclease 1 (NEB M0293) and Shrimp Alkaline Phosphatase (NEB M0293) following the manufacturer's protocol (New England Biolabs, Inc). Fragments were then sequenced

using the Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1. Sequencing products were purified using the ZR-96 DNA Sequencing Clean-up Kit™ (Zymo Research, Orange, CA, USA) and later injected on the ABI PRISM™ 3500XL Genetic Analyser with a 50cm array (Applied Biosystems, Thermo Fisher Scientific, Carlsbad, USA).

## 3.3 Data analysis and data presentation

### 3.3.1 Microscopic and Serological data

All raw data generated from serological tests and electrophoresis were analyzed using Microsoft Excel software Version 2013 (Microsoft Corporation, USA). To summarize data, frequencies and percentages were calculated for all categorical variable and ranges were generated for continuous variables such as age groups. Assuming that the data follows a normal distribution, the Chi-square test was used to test for statistical significance. A p-value of less than 0.05 was considered statistically significant for independent variables.

### 3.3.2 Sequence assembly, genotyping and phylogenetic analysis

The chromatograms of the sequences were assembled and visually analyzed using BioEdit software version 7.2.5 (Hall, 1999). Genotype identification of the sequences was achieved by comparing sequences from this study with reference sequences in the NCBI GenBank database, using BLAST [Basic Local Alignment Search Tool] (Altschul *et al*., 1990). Sequences of Botswana VP7 and VP4 strains from this study were compared with those of the vaccine strains Rotarix® (JX943614) G1P[8] and RotaTeq (GU565079) G3P[7]. Clustal W (Thompson *et al*., 1994), incorporated in BioEdit software version 7.2.5 (Hall, 1999) was used for multiple sequence alignments. The aligned VP7 and VP4 sequences were exported to MEGA software version 6.06 (Tamura *et al*., 2013) where they were used to estimate distance matrices using the Kimura 2-parameter nucleotide substitution

model (Kimura, 1980) as well as constructing a mid-point rooted neighbor-joining tree (Saitou & Nei, 1987). The robustness of each tree branch was estimated by performing 1,000 bootstrap replicates.

### 3.4 Ethical considerations

Permission to conduct this study was obtained from the Institutional Review Board of University of Botswana (Ref. No: UBR/RES/IRB/GRAD/297), the Botswana Ministry of Health in Gaborone (Ref. No: HPDME 13/18/1) and Princess Marina Hospital Research and Ethics Committee (Ref. No: PMH5/79 [302-1-2017]). Permission was also obtained from all the authorities of respective hospitals and clinics where recruitment of participants took place. Written consent forms were signed by parents or guardians of the participants where applicable.

# CHAPTER 4

# RESULTS

## 4.1 Prevalence

At least one of the three microorganisms were detected in 43.5% (87/200) of diarrheic specimens from the under-five population of Gaborone. Approximately 56.5% (113/200) of the diarrheic samples did not reveal any of the three agents of diarrhea involved in this study. Most of the diarrheic samples were collected from children who were below 24 months of age and 40.4% of the samples in this age group contained detectable rotavirus, *Giardia intestinalis* or *Cryptosporidium parvum.* Only 8 samples (4%) had more than one microorganism detected. Among the control samples (from children without diarrhea), at least one microorganism was detected in 2% of the samples tested.

Microscopically, *Cryptosporidium* cysts were found in only 3% of the case samples and detected in 20.5% of the samples by qualitative immunochromatographic assay. One percent (1%) of the control samples had detectable *C. parvum* by ICA (images of representative positive samples for both microscopy and immunochromatographic assay are presented in Appendix 3, Figure i and ii respectively). The association of C. *parvum* with the total samples from children with diarrhea was 1:4.9. This indicates that from all the cases of children who were seeking treatment for diarrhea in Gaborone between March and November 2017, approximately 1 in 5 children had *C. parvum* related diarrhea.

*Giardia intestinalis* oocysts were detected in only 1% of the total diarrheic samples by microscopy but confirmation by ICA revealed that 16.5% of the samples had detectable cysts (images in Appendix 3, Figure iii and ii, respectively). None of the control samples had detectable *G. intestinalis* when tested by both methods. Six samples (3%) from symptomatic children observed

with *G. intestinalis* were also positive for *C. parvum*. The association between *G. intestinalis* and diarrheic samples from children in Gaborone was approximately 1:6.

Of all the 200 diarrheic stool specimens that were screened for Group A rotavirus using ELISA, 11.0% had detectable Group A rotavirus antigens. Only 1 sample (1%) of the control samples had detectable rotavirus antigens. One percent (1%) of the diarrheic samples were found to be infected by both *G. intestinalis* and rotavirus. One sample from the controls had detectable confections between *C. parvum* and rotavirus. Figure 4.1 below summarizes prevalence of microorganisms investigated in this study.

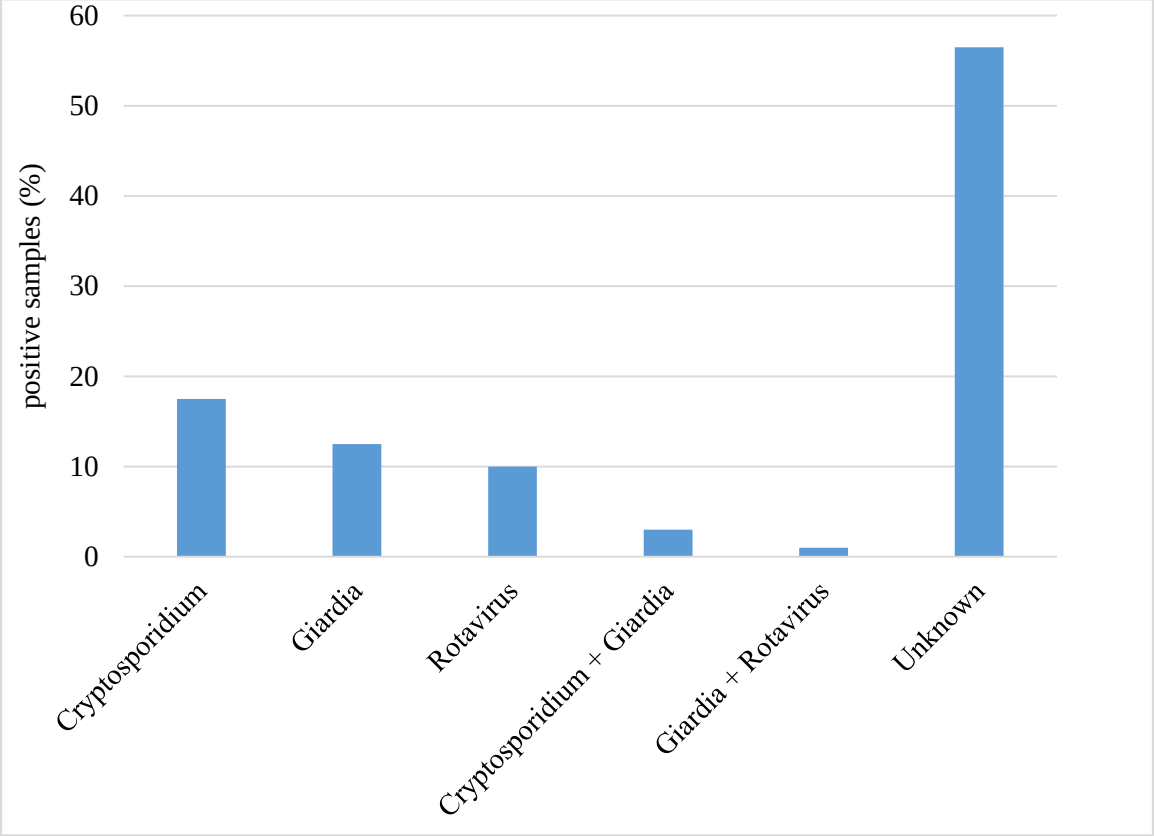


**Figure 4.1** Prevalence of *Cryptosporidium parvum* (detected by ICA), *Giardia intestinalis* (detected by ICA) and rotavirus (detected by ELISA) in children below the age of five years in Gaborone.

### 4.1.1 Age distribution of *Cryptosporidium parvum*, *Giardia intestinalis* and rotavirus infections

There was a great variation in prevalence of rotavirus, *G. intestinalis* and *C. parvum* in all age groups (Figure 4.2). Out of the 22 (11%) rotavirus positive samples that were collected from diarrheic children ≤5 years, 15 samples (68.2%, p= 0.93) were from children aged ≤2 years and only 7/22 (31.8%) were for children over 2 years of age. Most positive cases of *C. parvum* and *G. intestinalis* were found in children who were ≤36 months of age with frequencies of 32/41 (78%, p= 0.100) and 27/33 (81.8%, p=0.449) respectively.

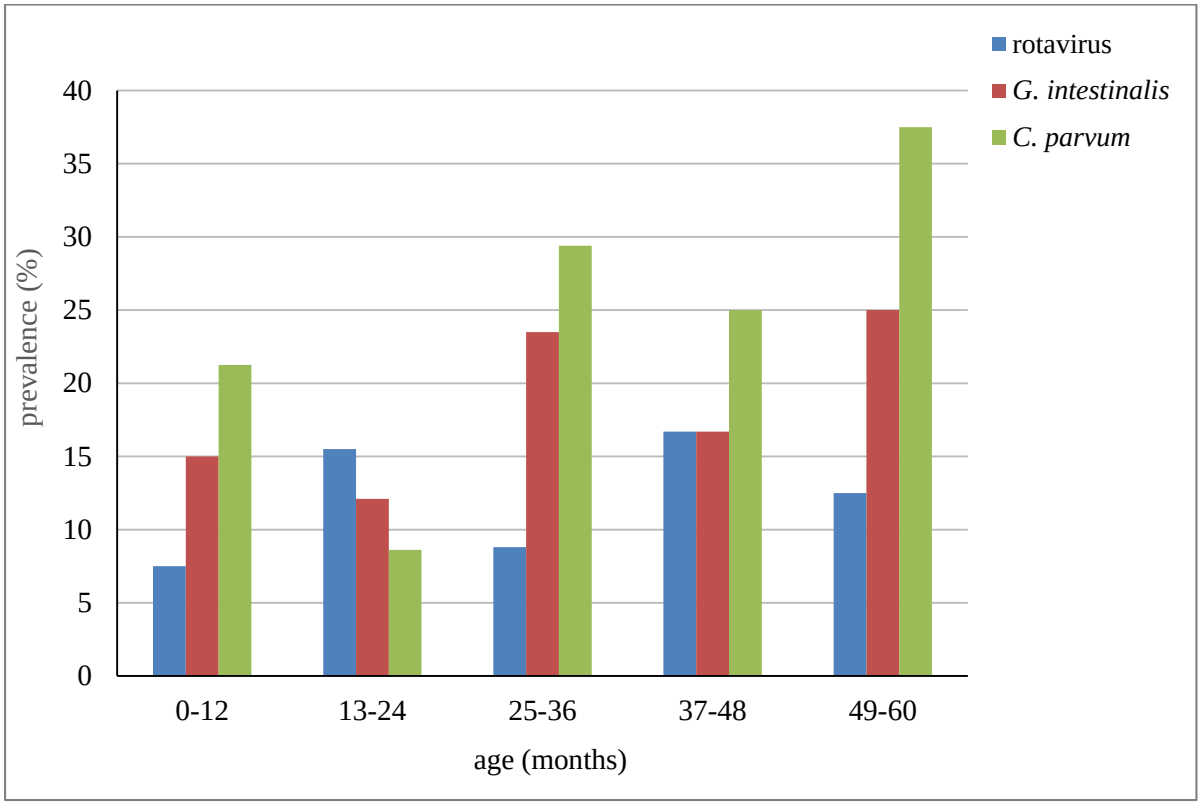


**Figure 4.2** Variation by age of *C. parvum*, *G. intestinalis* and rotavirus in stool samples of children suffering from diarrhea in Gaborone, 2017.

### 4.1.2 Gender distribution of *Cryptosporidium parvum, Giardia intestinalis* and rotavirus infections

Most cryptosporidiosis patients were male children and cases recorded prevalence of approximately 60% (p=0.037). There was no significant relationship between gender and *G. intestinalis* infections (54.5%, p=0.895) although it affected more female children (Figure 4.3) than male children (Figure 4.4). An equal number of females and males were affected by rotavirus (Figure 4.3, Figure 4.4) and there was no statistical difference (p= 0.727) between gender and infection by rotavirus.

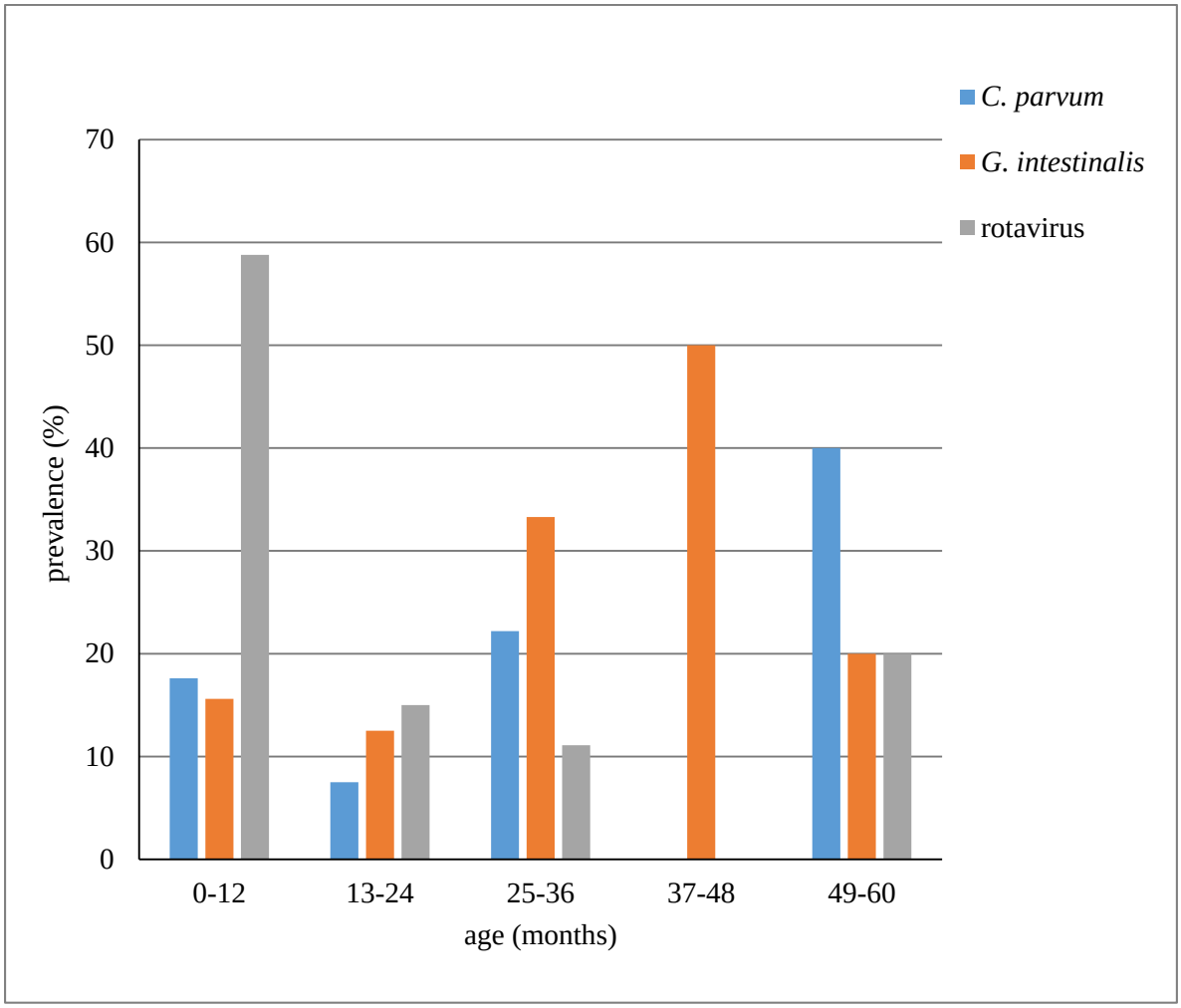


**Figure 4.3** Prevalence of *Cryptosporidium. parvum*, *Giardia intestinalis*, and rotavirus in stool samples of female children with diarrhea in Gaborone, 2017.

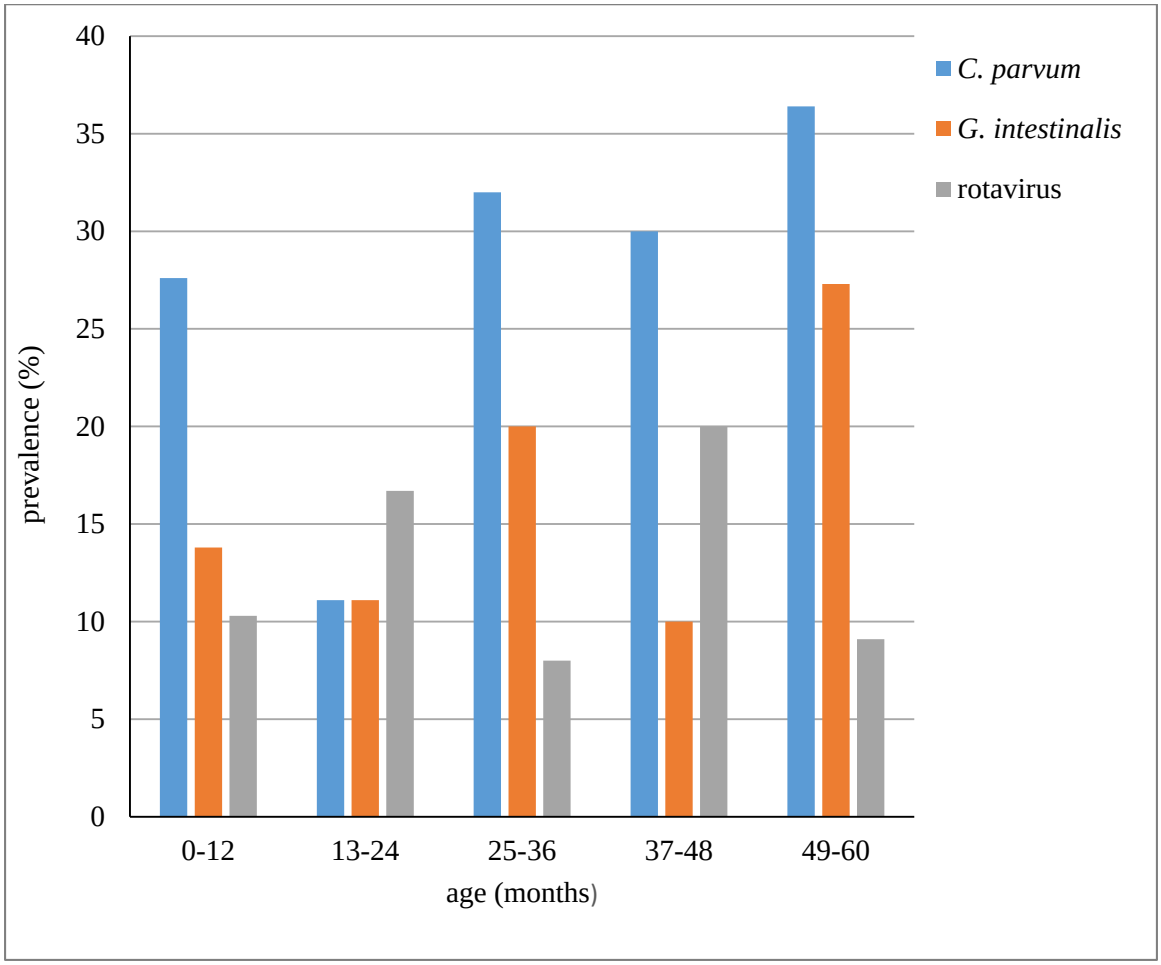


**Figure 4.4** Age specific prevalence of *C. parvum*, *G. intestinalis*, and rotavirus in stool samples of male children suffering from diarrhea in 2017, Gaborone.

### 4.1.3 Seasonal distribution of *C. parvum*, *G. intestinalis* and rotavirus infections

*Cryptosporidium parvum*, *Giardia intestinalis* and rotavirus infections occurred throughout the entire study period, which was March to November 2017 (Figure 4.5). Incidences of rotavirus were at peak during the onset of winter season in April and in July (which is part of winter in Botswana). Cryptosporidiosis reached its peak in October. Slight fluctuations in *G. intestinalis* infections were seen frequently throughout the entire study period, but peak infections were in August.

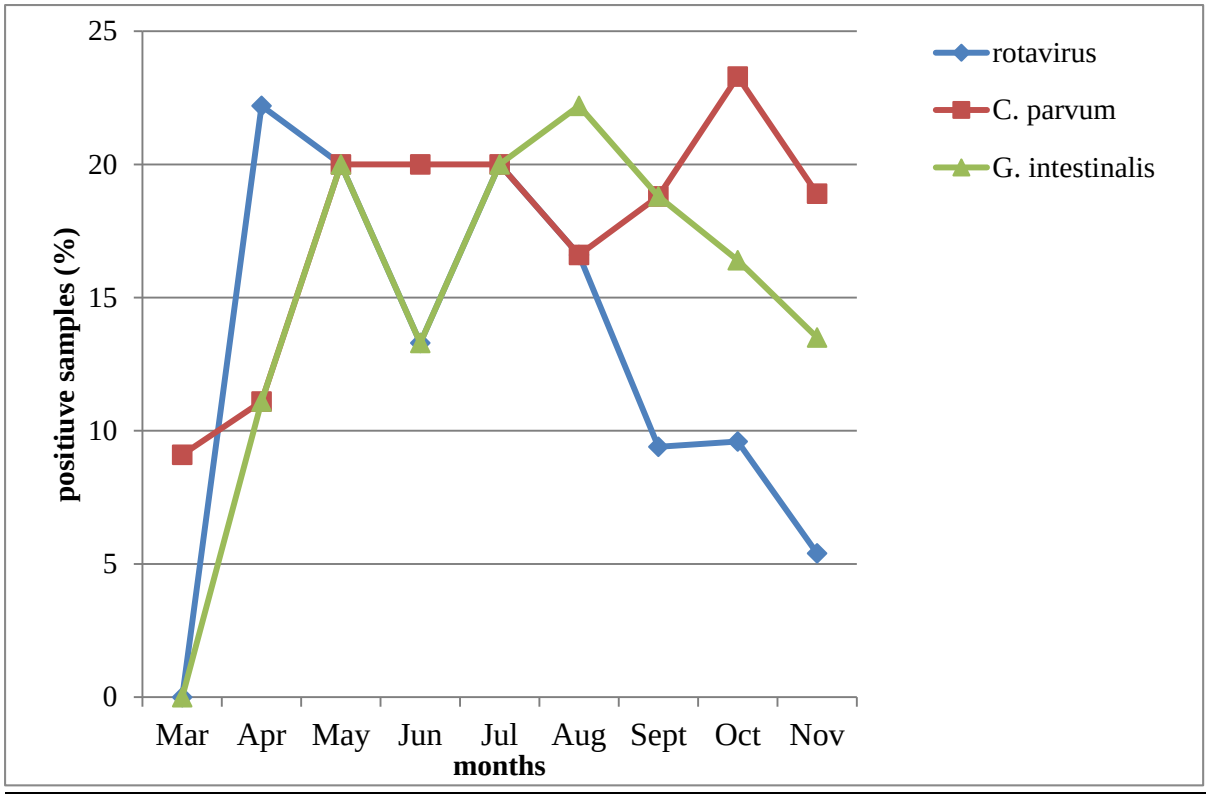


**Figure 4.5** Seasonal distribution of *Cryptosporidium parvum*, *Giardia intestinalis* and rotavirus in children affected by diarrhea in Gaborone between March and November 2017.

## 4.2 Molecular epidemiology of rotavirus strains

### 4.2.1 Rotavirus electrophoretypes

Out of the 22 samples that tested positive for rotavirus, dsRNA was extracted from sixteen samples that were strongly positive for ELISA. The double-stranded RNA isolates were used to perform PAGE. Most of the isolates revealed the 4-2-3-2 gene migration pattern of Human Group A Rotavirus (Appendix 3, Figure iv). A higher percentage of the electrophoretypes from the diarrheic samples were long (56.25%), and only 25% were short electrophoretypes. Two samples (12.5%) had more than 11 gene segments, suggesting infection by more than one strain of rotaviruses. No clear migration was found in one (6.25%) of the diarrheic sample. Prevalence of electrophoretypes from this study is summarized in Figure 4.6 below.

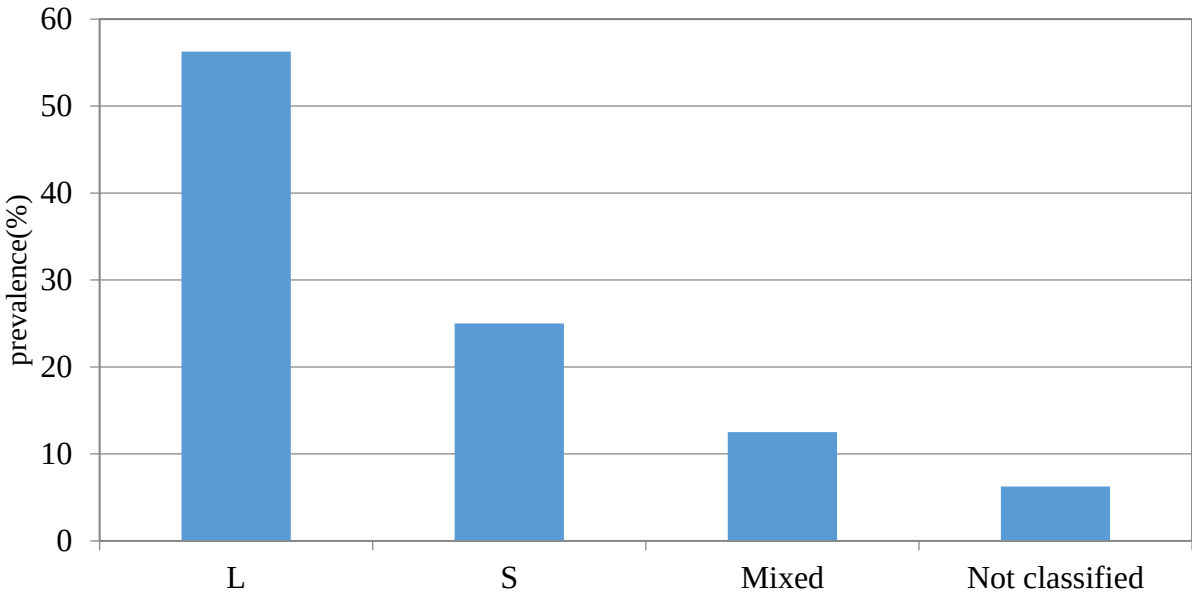


**Figure 4.6** Distribution of electrophoretic patterns on a 10% PAGE for rotavirus strains extracted from samples of diarrhea-suffering children under the age of five years in Gaborone (March to November 2017). L indicates long profiles and S is for short profiles.

### 4.2.2 Genetic diversity of rotavirus by Multiplex PCR

All of the15 samples from diarrheic children that were tested by PAGE were confirmed by RT-PCR. Only 3 G genotypes, G1, G2 and G3 were observed (Figure 4.7). G1 predominated (Appendix 3, Figure vi) accounting for approximately 60% of the strains detected followed by G2 (26%). G3 (7%) was the least detected genotype. Most of the detected G types were associated with either P[4] or P[8] (Appendix 3, Figure v). The uncommon rotavirus G/P combination G1P[6] was detected in approximately 7% (1/15) of the samples by RT-PCR. Two cases (13.3%) were found to have an uncommon combination G1+G2P[4,8]. Twenty percent (20%) of the specimens tested by PCR were untypeable. There was no apparent variations in the G-P genotype distribution amongst different age groups but all unusual G-P combinations and mixed strains were detected in younger age groups as compared to older age groups.

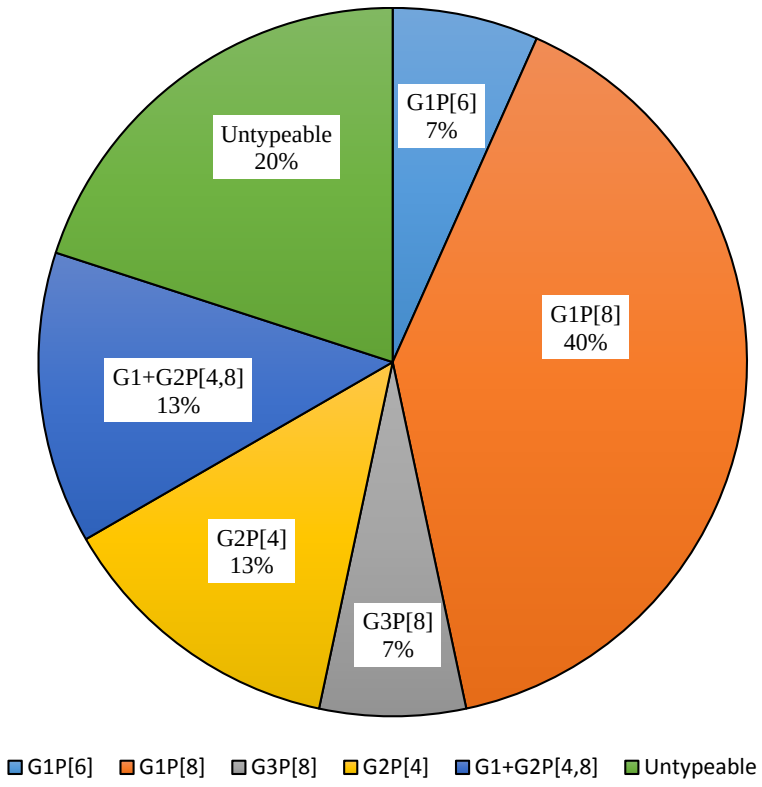


**Figure 4.7** P and G type combinations of rotavirus strains isolated from children below the age of five years in Gaborone, March to November 2017.

### 4.2.3 Sequencing results

Sequencing results from sequence alignment confirmed the genotyping results obtained from RT-PCR. Sequence alignment and phylogenetic analysis of the VP7 genes of the 10 rotavirus strains detected in Gaborone showed 96-99% nucleotide sequence identity after being compared to corresponding G1 and G3 strains internationally. One sample that was thought to be G1P[6] by RT-PCR was confirmed to be G1P[8] after sequencing. Sequencing of the G2 strains detected by RT-PCR was not successful and hence no sequence data could be obtained for phylogenetic analysis. VP4 gene sequences of rotavirus strains from this current study were 96-97% identical to some regional and international P[4] and P[8] strains.

### 4.2.4 Phylogenetic analysis of G-types of rotaviruses

Phylogenetic analysis of the G1 strains of Botswana clustered G1 sequences into 2 lineages (Figure 4.8). Five of the G1 strains RVA/Human-wt/BWA/O14/2017/G1, RVA/Human-wt/BWA /063/2017/G1, RVA/Human-wt/BWA/141/2017/G1, RVA/Human-wt/BWA/145/2017/G1 and RVA/Human-wt/BWA/161/2017/G1 shared more than 99% nucleotide sequence identity. These sequences clustered in lineage 1 and were 98-100% identical to some South African (KJ753805, KJ752278, KJ753123 and KP753022), Malawian (MG181474, MG181331 and MG181507) and Mozambican (KP222814, KP222809) G1 strains. These G1 strains also clustered with some international strains previously reported in Thailand (DQ512974, DQ512981), India (JN192064) and Belgium (JN849122). A distant relationship was found between these five VP7 strains and Rotarix (the vaccine currently used in the national vaccination programme of Botswana).

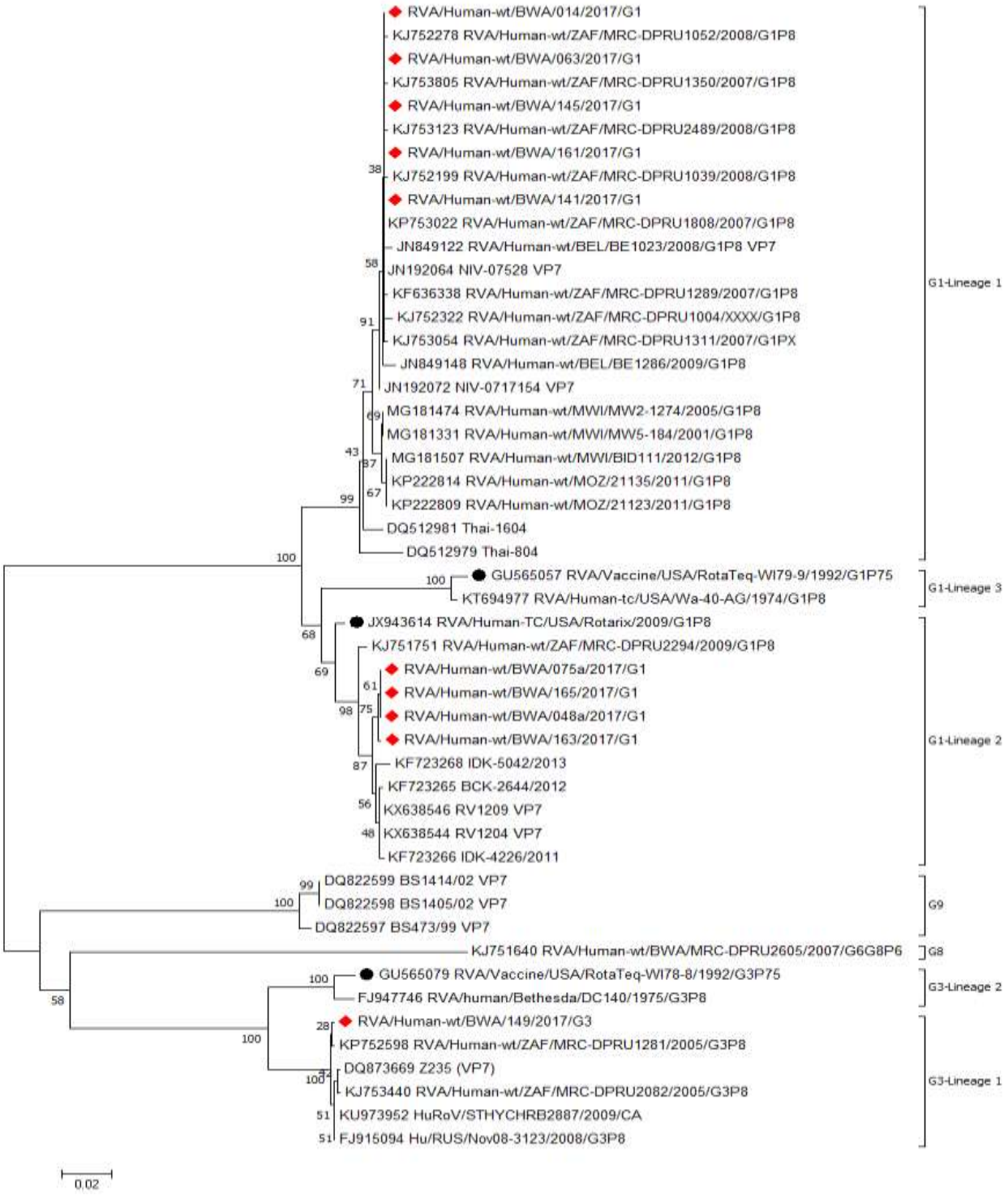


**Figure 4.8** Phylogenetic analysis of VP7 nucleotide sequences of representative G genotypes of rotaviruses isolated from children with diarrhea in Gaborone in 2017. The number at each branch indicates percentage bootstrap support. Representative sequences of strains of this study are indicated with a red filled diamond shape and vaccines with a dark filled circle. Variation scale is indicated by the bar at the bottom of the phylogenetic tree.

The other four G1 sequences from this current study: RVA/Human-wt/BWA/048a/2107/G1, RVA/Human-wt/BWA075a/2017/G1, RVA/Human-wt/BWA/163/2017/G1 and RVA/Human-wt/BWA/165/2017/G1 fell into lineage 2 and exhibited 97-99% identity among themselves. They were more than 99% identical to strains discovered earlier in India (KX638546), South Africa (KJ751751), China (DQ873669) and also distantly related to Rotarix. One G3 sequence observed in this study (RVA/Human-wt/BWA/149/2017/G3) belonged to lineage 1 and showed that it was closely related to G3 strains previously identified in South Africa (KP752598,KJ753186 and KJ753440) and China (DQ873669). All the VP7 sequences of strains in this study were distantly related to previously published sequences from Botswana (DQ822597, DQ822598 DQ822599 and KJ751640) deposited in GenBank.

### 4.2.5 Phylogenetic analysis of rotavirus P-types

All VP4 serotype P[8] sequences observed in this study shared 100% nucleotide sequence identity with each other (Figure 4.9). The P[8] strains detected clustered in lineage 3 and were closely related to sequences of rotavirus P[8] previously identified in South Africa (KJ753803 and KJ753121), Malawi (MG181483), Gambia (KJ752287), Senegal (KJ751560) and Belgium (HQ392119, JN849147). P[8]. Nucleotide sequences from this study did not cluster with Rotarix (found in lineage 1) and RotaTeq (found in lineage 2). P[4] strains (RVA/Human-wt/BWA/048b/2017/P4 and RVA/Human-wt/BWA/075b/2017/P4) of this current study shared 99% nucleotide identity with each other and were similar to other strains reported in Malawi (MG181912 and MG181835), Belgium (KR705171), and Mauritius (KP752663). However, sequence analysis of the G2 types (confirmed by RT-PCR) related to these strains failed to be determined.

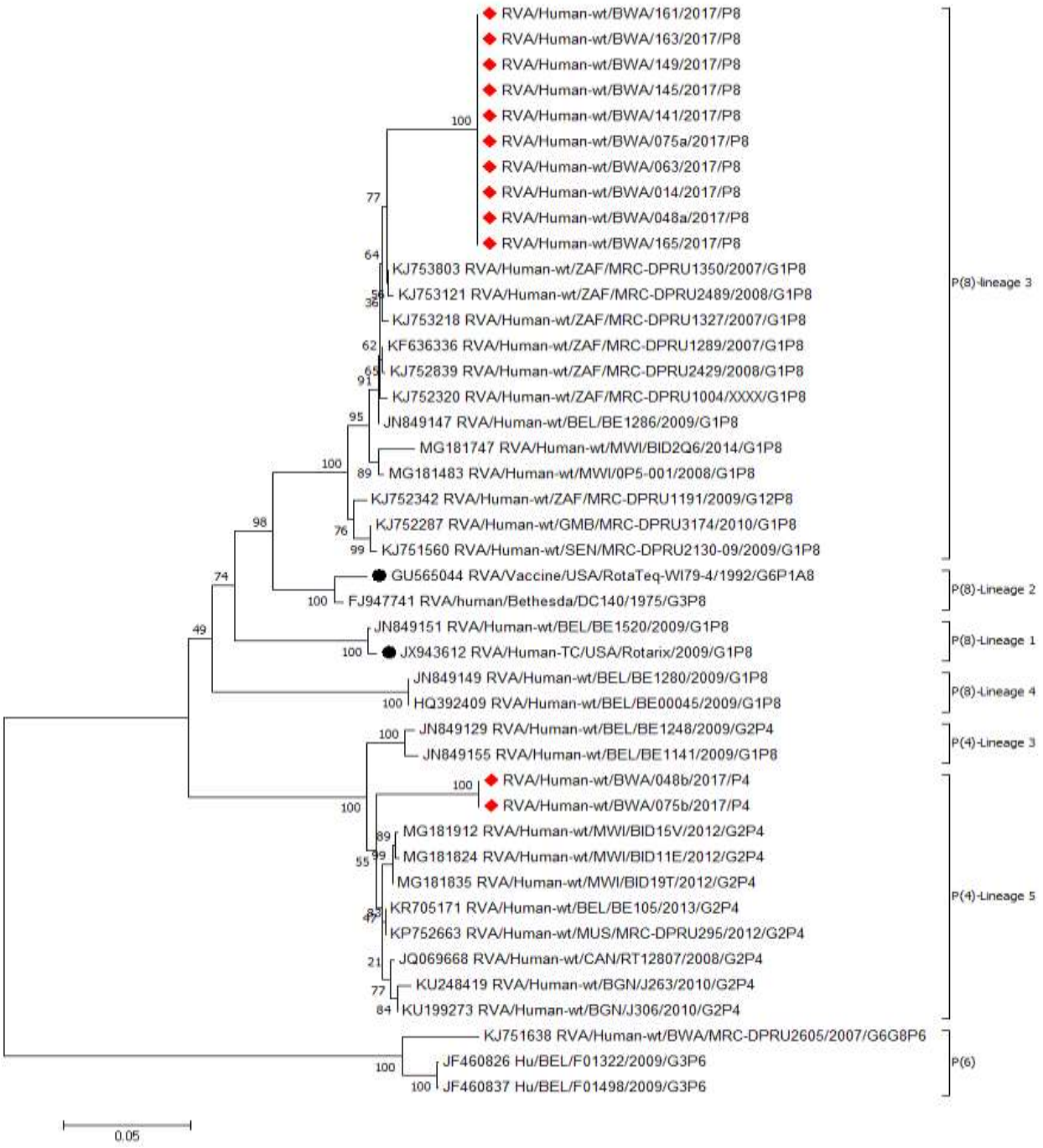


**Figure 4.9** Phylogenetic analysis of representative VP4 nucleotide sequences of P[4] and P[8] genotypes of rotaviruses detected in the study. The phylogenetic tree was constructed based on the neighbor-joining method with 1000 replicates using the MEGA 6.06 program. Percent bootstrap support is indicated by numbers on each branch node. Representative strains from this study are indicated with a red diamond shape and rotavirus vaccine strains by a filled dark circle. Reference strain sequences were obtained from GenBank. The bar at the bottom of the trees indicates variation scale.

# CHAPTER 5

# DISCUSSION

## 5.1 Prevalence

Diarrheal diseases remain a major concern in causing morbidity and mortality of children. In many developing countries including Botswana. *Cryptosporidium parvum, Giardia intestinalis* and rotaviruses are medically important microorganisms associated with diarrhea especially in set-ups where there is no proper hygiene and sanitation (Kotloff, 2017; Riahi *et al*., 2018). For all the diarrheic specimens in this study 43.5% had detectable rotavirus, *C. parvum* or *G. intestinalis*. The remaining 56.5% of the diarrhea cases might have been related to other diarrhea-causing pathogens that were not part of this investigation. These might be bacteria (for example, *Escherichia coli, Campylobacter jejuni, Vibrio cholera, Shigella spp.* and *Salmonella spp.*), viruses (for example, norovirus and enteric adenoviruses) or helminthes [examples are *Ascaris lumbricoides* and *Strongyloides stecoralis*] (American Academy of Pediatrics, 2016).

Most of the diarrhea cases involved in this current study were from children who were below 24 months of age (40.4% of the samples were positive for rotavirus, *C. parvum* or *G. intestinalis*). Several past studies in Botswana also revealed high prevalence of diarrhea in children ≤24 months of age (Alexander *et al*., 2012; Arvelo *et al*., 2010). This finding is also consistent with results from other African countries and the United States (Aldeyarbi *et al*., 2016; Painter *et al*., 2015). The high occurrence of diarrhea in this age group may be caused by more exposure to pathogens when children develop more physical movements and explore around their environment. Most children in this age group also have a tendency of picking objects from the ground to the mouth, increasing the risk to foodborne infections. Exclusive breastfeeding is also an uncommon practice to most working mothers in Gaborone (Ogwu *et al*., 2016), yet breastfeeding is the best way to reduce diarrhea in

children below 24 months old (Kumar *et al*., 2016; Richard *et al*., 2018). The sources of other milk or foods increase exposure to diarrheal infections in young children.

Among the investigated three diarrhea causing microorganisms in this study, *C. parvum* was the most prevalent pathogen. These results confirmed previous reports that identified *Cryptosporidium* as one of the leading cause of diarrhea in children as compared to *Giardia* and rotavirus in Botswana (Goldfarb *et al*., 2014). Similar findings have been reported in Libya, Tanzania and India (Ghenghesh *et al*., 2016; Moyo *et al*., 2011; Sarkar *et al*., 2014). However, rotavirus had been reported as the most prevalent pathogen of diarrhea in some studies where both *Cryptosporidium* and *rotavirus* had been investigated in Botswana (Pernica *et al*., 2016). Lower prevalence of rotavirus than *C. parvum* observed in this study can be as a result of increased efficiency of the monovalent rotavirus vaccine Rotarix® currently used in Botswana (Gastañaduy *et al*., 2016).

Co-infections among *C. parvum* and *G. intestinalis* observed in this study are similar to findings reported in earlier studies in Libya and China (Ghenghesh *et al*., 2016). Simultaneous transmission of enteric pathogens in young children may be facilitated by lack of proper sanitation and hygiene practices after frequent contact with contaminated surfaces in schools and homes (Dóró *et al*., 2015; Pengpid & Peltzer, 2012). Some specimens in this study were positive for *G. intestinalis* and rotavirus. Similar findings were previously observed in Ecuador and Côte d'Ivoire (Bhavnani *et al*., 2012; Koffi *et al*., 2014). Pathogenicity of *Giardia* in the presence of rotavirus may be caused by successful attachment of the *Giardia* trophozoites central disk to the viral infected epithelium (Müller & Von Allmen, 2005).

### 5.1.1 *Cryptosporidium parvum*

*Cryptosporidium parvum* was detected in 20.5% of stool specimens of children who were suffering from diarrhea in Gaborone. This finding differs from the 2.2% (Rowe *et al*., 2010), 16% (Goldfarb *et al*; 2014) and 10% (Alexander *et al*., 2012) prevalence that were previously observed in some other studies in Botswana. This high prevalence is probably justified by increased rates of consumption of water from possibly contaminated household water storage containers caused by recent water rationing in Botswana (Kadibadiba *et al*., 2018). The poor storm water drainage system of Gaborone also increases fecal contamination rates of water bodies in the rainy season, therefore increasing chances of the occurrence of *C. parvum* cysts in water and irrigated plants (Sakijege *et al*., 2012). High variations in prevalence can also be attributed to different research set ups, differences in sample population, differences in sample size and previous methods used for detection of cryptosporidiosis.

Cryptosporidiosis was detected across all age groups, but most frequently in children aged 36 months and below. This agree with past studies in Botswana, which reports higher percentages of *Cryptosporidium* cases for children in this age group as compared to older children (Alexander *et al*., 2012). Outcomes of several previous studies globally also revealed that cryptosporidiosis is age specific and affects children in lower age groups more frequently than any other age group (Khalil *et al*., 2018; Kotloff *et al*., 2017). High prevalence in this age group is probably due to lack of understanding or awareness of basic hygiene rules. Exposure to contaminated food and water as they pass through weaning period also increases chances of *C. parvum* infections (Adler *et al*., 2017; Kotloff *et al*., 2013).

In this study, most cryptosporidiosis infections significantly affected males (60%) than females. Although gender has not been considered as a risk factor in some past reports in Botswana (Pernica *et al*., 2016), Ethiopia (Wegayehu *et al*., 2013) and Cuba (Bello *et al*., 2011), higher prevalence of

*Cryptosporidium*-related diarrhea had been reported in male children than females in countries like Yemen and Australia (Al-Shamiri *et al*., 2010; Lal *et al*., 2015). This association could be explained by behavioral activities of male children that increase exposure to *C. parvum*.

*Cryptosporidium parvum* was detected in all months within the period of study. Most cases of *C. parvum* were observed in October (23.3%), May (20%), June (20%) and September (18.8%). The peak month of cryptosporidiosis transmission (October), coincided with the hot and rainy season. *Cryptosporidium*-related acute diarrhea was also found to be highly prevalent in October in Chobe district (Alexander *et al*., 2013). In most countries, cryptosporidiosis also displays spring and summer peaks (Jagai *et al*., 2009; Lal *et al.*, 2015; Xiao, 2010). High prevalence of cryptosporidiosis in hot and rainy months can be caused by high water saturation of the land surface. This leads to efficient transportation of cysts into water bodies by runoff, followed by incomplete purification before water is pumped into domestic supplies (Carlton *et al*., 2016). In cities, recreational activities like swimming also increases in summer hence increasing the risk of ingesting *C. parvum* cysts by preschool children. Generally, water consumption in humans increase as we approach summer, hence an increase in chances of getting infected by *C. parvum*. However, a clear seasonal distribution of *C. parvum* related diarrhea failed to be established in this study due to a short period for sample collection.

### 5.1.2 *Giardia intestinalis*

*Giardia* plays an important role in childhood gastroenteritis in most African countries, but the actual epidemiological situation of *Giardia* infections is unknown in Botswana. Giardiasis is usually linked to socio-economic level of a country, with prevalence ranging between 2-7% in most industrialized regions and reaching up to 40% in developing countries (Ahmed *et al*., 2018; Painter *et al*., 2015; Squire & Ryan, 2017).

This study revealed that there was a prevalence of 16.5% of giardiasis by ICA technique. This prevalence falls in the same range with rates that had been observed earlier in Estonia (16.8%), Sweden (13.1%) and Bulgaria (23%) (European Centre for Disease Prevention and Control, 2016). However, earlier studies in Botswana revealed prevalence of *Giardia* is less than 10% in most places (Alexander *et al*., 2012; Goldfarb *et al*., 2014; Pernica *et al*., 2016; Rowe *et al*., 2010). The higher prevalence of giardiasis observed in this study can be explained by effects of urbanization and water scarcity on cities like Gaborone. Wastewater irrigation has recently increased in Gaborone, and high pathogen densities of wastewater increase chances of contamination of plants (to include vegetables) and pose disease risk on urban populations (Falkenberg & Saxena., 2010; Spanakos *et al*., 2015; Qadir *et al*., 2010). Discrepancy of results could also have been caused by differences in research settings, differences in sample population and methods used in this current study. The low frequency of *G. intestinalis* infections in asymptomatic children observed in this study agrees with earlier reports that the rate of detection of *Giardia* oocysts in non-diarrheic children is very low than in symptomatic children (Al-Mohammed, 2011; Ramírez *et al*., 2015).

*Giardia intestinalis* was observed to have moderate prevalence in the age group 0-12 months while highest prevalence occurred in children between 25 to 36 months and 49 to 60 months of age. Its presence in early childhood is supported by most studies worldwide including Botswana (Alexander *et al*., 2012; Einarsson *et al*., 2016) and may be as a result of underdeveloped immune system. High incidences of *G. intestinalis* infections in intermediate childhood age of this study can be as a result of children playing in very close contact with other potentially infected children in child-care settings (Painter *et al*., 2015). Lack of previous exposure to *G. intestinalis* could also render children to be more susceptible to infection and illness even at a later age. Similar findings were reported in Bangladesh, where infections caused by *Giardia* were high in children more than 2 years of age (Suman *et al*., 2011). In this study, no significant relationship was found between gender and

giardiasis. This can be explained by the fact that most samples with detectable *G. intestinalis* came from pre-school aged children where they engage in same activities, hence the lack of association between gender and *G. intestinalis* infection in this study. However, most researchers associates *Giardia* distribution to socio-cultural and behavioral differences between male and female children (Al Saqur *et al*., 2017).

*G. intestinalis* also occurred throughout the entire study period, except the month of March. Its absence in March may be attributed to low diarrhea cases in that month. Most giardiasis cases were observed in August (22.2%), May (20%), July (20%) and September (18.8%). Although the study was conducted on a short period of time for seasonal variation evaluation, a small spring peak on giardiasis in this study was similar to previous observations in Botswana (Alexander *et al*., 2013) and New Zealand (Lal & Hales, 2015). An increase in the shedding of *G. intestinalis* oocysts as the winter season approached (from April to July) that was observed was also evident in another study in Iraq (Muhsin *et al*., 2016). Currently, Gaborone is wetter in early winter and late spring rainfalls have been observed, increasing chances of cysts to be deposited into water reservoirs (Moalafhi *et al*., 2012). This might explain higher prevalence rates in May and August observed in this current study.

### 5.1.3 Rotavirus

Worldwide, rotavirus has been consistently identified as the common pathogen associated with severe diarrhea in children that are below the age of five years (Troeger *et al*., 2018a). For the 200 stool samples collected from under-fives suffering from diarrhea in Gaborone between March and November 2017, 11.0% were found to be positive for rotavirus. This is slightly lower than the 13% reported by Kebaabetswe *et al*., (2005), in a similar study for children below five years in Northern Botswana. The slight decrease may be due to the introduction of rotavirus vaccine in immunization

programs in Botswana (Enane *et al*., 2016; Goldfarb *et al*., 2014; Mokomane *et al*, 2018). Similar trends had also been observed in South Africa and Rwanda (Madhi *et al.*, 2016; Tate *et al*., 2016). However, information on whether participants of this study and their siblings were vaccinated or not was not collected.

Only one sample (1%) from the control group population had detectable rotavirus. Rotavirus may be present in the body just before the child have symptoms of diarrhea within the incubation period. The time from initial infection to symptoms (incubation period) for rotavirus disease varies from one to three days. The virus can also be detectable up to 8 days after children develop diarrhea (Bennett *et al*., 2015). Moreover, some research findings points out that several asymptomatic infections can occur in children in the first five years of life (Lewnard *et al*., 2017).

Rotavirus infections were found across all age groups but affected approximately 68.2% of children ≤24 months. Frequency of infections decreased with increasing age. A high prevalence of infections in children ≤24 months is in accordance with some previous studies in Botswana, where upto 99% of all rotavirus cases came from children less than 24 months old (Gastañaduy *et al*., 2016; Pernica *et al*., 2016). Same findings were also reported in Bangladesh (Rahman *et al*., 2017) and South Rajasthan (Ameta *et al*., 2015). Some studies in China observed that 50% of children are affected by rotavirus before the age of one year and 90% before the age of two (Wu *et al.*, 2016). High prevalence of rotavirus in this age group is probably caused by lack of immunity and increased exposure to viral contaminated foods as they pass through weaning (Plenge-Bönig *et al*., 2010). After the introduction of solid foods to children, hygiene also play an important role in the reduction of rotavirus infection (Fawzy *et al*., 2011). Mobility in toddlers leads them to touch infected surfaces as well as interacting with other children, hence increasing exposure to the virus. Reduction in occurrence of rotavirus after the age of 24 months could be attributed to acquired immunity caused

by subsequent infections as children grow. Advanced studies on rotavirus show that prior symptomatic infection with rotavirus reduces severity and risk of subsequent rotaviral diarrhea (Bányai *et al*., 2018; Burnett *et al*., 2018).

There was no significant difference in the rate of infection between male and female children that were affected by rotavirus. This finding differ from observations made in several studies that suggest that male children are more affected by rotavirus than female children (Ansari *et al*., 2013; Seheri *et al*., 2014). Lack of gender prevalence difference in this study can be as a result of few samples that had been used in this study.

Rotavirus infections recorded peaks in April and July and these months coincides with the cooler and colder season of Botswana. Fewer cases were observed in September to November, which are part of the hot and wet season. This pattern was similar to findings in previous studies in Botswana where rotavirus infections were at peak in winter as compared to some other seasons (Basu *et al*., 2001; Kebaabetswe *et al*., 2005; Welch *et al*., 2013). Same findings had been documented in studies in other nations like Kenya and China (Agutu *et al.,* 2017; Dian *et al*., 2017). Previous studies observed that low temperatures reduce humidity and encourages people to stay in closed indoors where contaminated air can be easily breathed and contaminated surfaces easily touched (Patel *et al*., 2013), leading to an increase in rotavirus infections. However, some researchers also deduced that there is no unifying explanation for the global varying seasonality of rotavirus (Baker & Alonso, 2018). Further future evaluations on the impact of rotavirus vaccination on seasonal patterns of rotavirus-related diarrhea may help to understand the relationship of prevalence and seasonality of rotaviruses.

## 5.2 Molecular diversity and phylogenetic analysis of rotavirus strains

Continuous monitoring of the common prevailing strains of rotavirus is a necessity so that the effectiveness of all interventions involved towards its occurrence is ensured. Previous studies in Botswana revealed that prevalence of rotavirus genotypes have changed from 2002 up to date. The three G genotypes (G1, G2 and G3) and 2 P genotypes (P[4] and P[8]) detected in this study had been observed in several past studies in Botswana (Gastañaduy *et al*., 2016; Kasule *et al*., 2003; Kebaabetswe *et al*., 2005), other African countries like Kenya and Mozambique (Agutu *et al*., 2017; João *et al*., 2018) as well as developed countries like Finland (Hemming *et al.*, 2013). Results have shown that all the G1 strains observed were associated with the long electrophoretic pattern whilst G2 and G3 were associated with the short electrophoretic pattern. Similar associations were reported in previous studies in Botswana (Kasule *et al*., 2003) and abroad (Seheri *et al*., 2018).

It is of worthy to note that G4, G8 and G9, which are some of the common G types worldwide were not detected in this study. Absence of G4 and G8 in this study agrees with findings in a recent surveillance study in Botswana where the two were not among detected genotypes (Gastañaduy *et al*., 2016) but were detected in several studies before Rotarix® introduction (Kasule *et al*., 2003; Kebaabetswe *et al*., 2005). Moreover, G4 have not been observed in neighboring countries like South Africa and the rest of Africa for many past years (Seheri *et al*., 2018). Absence of G4, G8 and G9 after vaccine introduction suggests that Rotarix® may have high efficacy against the five major G genotypes circulating worldwide as well as cross-protection against serotypes not included in the vaccine's composition.

Serotype G1 had been found to be the most predominant circulating rotavirus G-serotype worldwide (Luchs &Timenetsky, 2016). In this study, G1 was the most predominant G-serotype and was found mostly alone or in association with G2. In similar previous studies done in Botswana, G1 contributed 59% of the total circulating G serotypes in children between 1999 and 2001 (Kasule *et al*., 2005),

followed by its subsequent decrease while G3 serotype increased in prevalence (Kebaabetswe *et al*., 2005). G3 was replaced by G2 and G1 in the post vaccination era in Botswana (Gastañaduy *et al*., 2016). Globally, epidemiological studies also observed G1 as one of the most prevalent strain even after the introduction of the vaccine (Abebe *et al*., 2018; Mukaratirwa *et al*., 2018). All of the G1s from this study were associated to P[8] of the VP4 protein. Analysis of theVP7 gene sequences of Botswana G1 strains from this study revealed 2 lineages of G1 strains (lineage 1 and 2). Five of the G1 sequences clustered in lineage 1 and closely related to some G1P[8] strains discovered in some Southern African countries, specifically South Africa, Malawi and Mozambique. This observation indicates that the serotype G1 rotaviruses observed might be direct descendants of previous strains from neighboring countries rather than emergence from point mutations. South Africa share borders with both Botswana and Mozambique and cross migration is high between the 3 countries therefore circulation of similar strains is not unusual. Further analysis will be essential to prove this hypothesis. The other four G1 sequences of this study formed a separate cluster in G1 lineage 2, and were more similar to sequences of G1 strains observed in India than other African strains. This observation suggests that these G1 rotaviruses might have been introduced in Botswana by continental migration or genetic reassortment. Monitoring of strains from other continents into Botswana would be essential in finding out the relationship between movement of people and other reservoirs with local rotavirus epidemiology.

An increase of occurrence of G2 had been noted in several countries, including developed countries, where the monovalent G1P[8] had been introduced suggesting selective pressure of Rotarix on the genotype (Matthijnssens *et al*., 2012a). In this study, G2 was the second leading cause of rotavirus associated diarrhea and this agrees with some local previous studies (Kebaabetswe *et al*., 2005) although it was the most predominant strain in rotavirus acquired illnesses in one post vaccination study in Botswana (Gastañaduy *et al*., 2016). However, circulating rotavirus genotypes may vary

on a yearly basis and their occurrence may also be affected by cyclic patterns of rotavirus strain as well as seasonal variations (Mwenda *et al*., 2014). All the G2 strains were observed exclusively in combination with P[4] and P[8] and similar associations were observed in most Southern African countries (Seheri *et al*., 2018) and Finland (Hemming *et al*., 2013). Due to poor DNA quality and low viral titers, phylogenetic analysis of G2 sequences from this study was impossible. However, phylogenetic analysis of the P[4] sequences clustered the G2P[4] strains of this current study in P[4] lineage 2 with other strains from Malawi, Mauritius and Belgium, suggesting that they might have been derived from a common origin.

Genotype G3 for human rotavirus was first detected in Botswana in the early 2000s and was one of the most predominant genotype although sequences of observed strains were not determined in those studies (Kebaabetswe *et al*., 2005). In this present study, G3 was the least detected G-type and this observation is supported by several studies that show changes in strain dominance in the post-vaccination era (Mukaratirwa *et al*., 2018; Mwenda *et al*., 2014). This finding also correlates with reports from Eastern and Southern African countries after Rotarix vaccine introduction (Seheri *et al*., 2018). The single G3 observed in this study was associated with P[8] and phylogenetic analysis revealed that the strain belonged to G3 lineage 2. This G3 strain (from this current study) demonstrated more than 99% homology and identity with strains previously identified in South Africa and China indicating that they might have descended from a common ancestor.

A substantial amount of mixed infections were detected in this study. In other African countries, rotavirus strains of G types G1, G2, G8, G9 and P types P[4], P[6], P[8], P[9] had been isolated from drinking water, which is one of the environmental factors that promote mixed infections in developing countries (Chigor & Okoh, 2012; Aminu *et al*., 2010; Verheyen *et al*., 2009). Phylogenetic analysis of the specimens with mixed infections revealed that G1+G2P[4,8] was caused by infection by both G1P[8] and G2P[4] strains closely related to strains from South Africa

and Malawi respectively. However, complete genome sequencing of mixed strains would be a necessity to determine any degree of natural reassortment.

## 5.3 Conclusion

Cryptosporidiosis was much more common than giardiasis and rotavirus infections. Most *Cryptosporidium parvum* and *Giardia intestinalis* infections were observed in children ≤36 months of age while rotavirus was more prevalent in children ≤24 months. Cryptosporidiosis and giardiasis transmissions were at peak during the hot and rainy months of Botswana, while rotavirus had the highest prevalence in winter. Further analysis to understand seasonal peaks of *C. parvum*, *G. intestinalis* and rotavirus infections is required so as to establish more intervention measures that can disrupt transmission in children. Information on circulating genotypes, serotypes and viral lineages of the rotavirus strains was presented. This study demonstrated that rotavirus strains circulating in Botswana are similar to those frequently occurring worldwide. Phylogenetic analysis of the isolated rotavirus strains of Botswana indicate that there is transmission between Botswana and some southern African countries, especially South Africa and Malawi. All the VP7 and VP4 sequences of rotavirus strains of Gaborone differed from both Rotarix and RotaTeq vaccine strains, which may indicate the presence of wild strains, although vaccination information of participants was not inquired. To obtain more understanding of the epidemiology of rotavirus strains in the region, more and continuous surveillance studies of circulating rotavirus genomes need to be done. Epidemiological surveillance of rotavirus is also crucial so as to detect effectiveness of rotavirus vaccines used in the country.

## 5.4 Limitations of the study

This study faced several limitations. The study would have been more effective if the risk factors were assessed in this study. This had been effected by collection of diarrheic samples from microbiology laboratories instead of collecting directly from participants hence lack of more information from patients. The study was also a hospital based study and therefore results obtained are unlikely to be a true reflection of the burden of rotavirus, *Cryptosporidium parvum* and *Giardia intestinalis* in the community.

Although the study was a case control study, participants from the two populations were not matched by age and sex. This limited the ability to generalize prevalence of diarrhea cases related to *C. parvum*, *G. intestinalis* and rotavirus in Gaborone.

For the case population, samples used were from tertiary hospitals which is a specialized study population. In this case, only severe cases admitted in these hospitals were used and does not include less severe cases of diarrhea. The control group was also made up of children from Child Healthcare facilities and children admitted because of other diseases rather than diarrhea. The study was going to be a better population based study if the controls were obtained from the same places where the cases were sourced from.

Sample collection from the control group relied more on parents or guardians than the investigator, since it was done at home. There was a high probability that some samples were not collected properly or submitted outside the correct submission time range, and this had a major impact on microscopic analysis of specimens for *C. parvum* and *G. intestinalis*. Single stool collection might have led to under detection of parasites. Lastly, due to resource constraints, samples were only tested for two parasites, and other parasites that had been previously detected together with *G. intestinalis* and *C. parvum* in similar research settings were not investigated. Because of the same reason, all

positive samples for both *C. parvum* and *G. intestinalis* were not analyzed by gel electrophoresis and PCR amplification.

## 5.5 Recommendations

- Further studies are required to ascertain the association of all the diarrhea causing pathogens from this study and seasonal variation so as to increase understanding of transmission and epidemiology of *Cryptosporidium parvum*, *Giardia intestinalis* and rotavirus diseases in Botswana.
- There is need to investigate associated risk factors in children affected by *C. parvum, G. intestinalis* and rotavirus in Gaborone. Genotyping of *G. intestinalis* and *C. parvum* would also help in understanding transmission and epidemiology of these microorganisms in Botswana.
- More investigations on co-infections of *G. intestinalis* and rotavirus is needed in Botswana since *G. intestinalis* is an opportunistic pathogen in rotavirus affected patients.
- Continuous monitoring of rotavirus vaccines and circulating strains is necessary so as to assess if changes are sustained over a long period of time. More vaccine effectiveness studies should be contacted in Botswana. This will support evaluation of the impact of rotavirus vaccine distribution that is caused by vaccine-driven selective pressure.

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# APPENDICES

## Appendix 1: Buffers

**Table i** Preparation of reagents used in RNA extraction by the phenol/chloroform method

| **Buffer** | **method** |
|---|---|
| 10% SDS stock | Add 1 g of SDS to 10 ml of distilled water. Dissolve in a 65°C water bath. |
| 1 M sodium acetate (NaAc) containing 1% SDS | Dissolve 8.2 g of sodium acetate in 60 ml of distilled water. Add 10 ml of 10% SDS stock and mix. Adjust the pH to 5.0 with glacial acetic acid, and make up to 100 ml with sterile distilled water. Heat the solution to 42°C if a precipitate is present prior to use. |
| Phenol-chloroform (1:1) | Mix equal volumes of citrate-saturated phenol, pH 4.3, and chloroform. Place in a dark or foil-covered bottle. Store at 4°C. |
| 3 M NaAc, pH 5.0 | Dissolve 4.92 g of sodium acetate in 10 ml of distilled water. Make up to 20 ml with distilled water. |

**Table ii** spacer and resolving gel recipe

| **Reagent** | **4% spacer gel** | **10% resolving gel** |
|---|---|---|
| Distilled water | 5.1ml | 9.9ml |
| 30% acrylamide stock | 1.2ml | 6.3ml |
| resolving buffer pH 8.8 | 0.9ml (pH 6.8) | 2.4ml (pH 8.8) |
| TEMED | 4 µl | 10 µl |
| 10% APS | 112 µl | 282 µl |

**Table iii** Preparation of reagents for PAGE

| **Reagent** | **Method of preparation** |
|---|---|
| 30% acrylamide stock | Dissolve 30 g of acrylamide and 0.8 g of N,N'methylene bis-acrylamide in 100 ml of distilled water. Filter before use. Place the solution in a dark or foil-covered bottle, and store at 4°C. |
| 1N hydrochloric acid (HCl) | Add 8.6 ml of concentrated HCl to 91.4 ml of sterile distilled water. |
| Resolving gel buffer (1.5 M, pH 8.8) | Dissolve 18.15 g of Tris base in 40 ml of distilled water. Adjust the pH to 8.8 with 1N HCl. Make up to 100 ml with distilled water. |
| Stacking gel buffer (0.5 M, pH 6.8) | Dissolve 5.98 g of Tris base in 50 ml of distilled water. Adjust the pH to 6.8 with 1N HCl. Make up to 100 ml with distilled water. |
| 10% (w/v) ammonium persulphate (APS) | Dissolve 0.1 g of APS in 1 ml of distilled water just prior to use. Store at 4°C for a maximum of 3 days. |
| 5 x Tris-glycine running buffer | Dissolve 15.1 g of Tris base and 94 g of glycine in distilled water, and make up to 1,000 ml with distilled water. |
| 1 x Tris-glycine running buffer | Dilute 200 ml 5 x Tris-glycine buffer with 800 ml of distilled water. |
| PAGE sample running dye | Dissolve 10 mg of bromophenol blue and 1 ml of glycerol in 5 ml of stacking gel buffer. Make up to 10 ml with distilled water. |

**Table iv** Preparation of buffers used in silver staining

| Buffer/ solution | Method of preparation |
|---|---|
| Fixing solution 1 | Add 80 ml of ethanol and 10ml acetic acid to 110 ml of dH20. |
| Fixing solution 2 | Add 20 ml of ethanol and 1ml acetic acid to 180 ml of dH20 |
| Silver nitrate solution | Dissolve 0.37 g of AgN03 in 200 ml of dH20. |
| Developing solution | Add 2 ml of 36% formaldehyde to 250 ml of distilled water. Just before use, dissolve 7.5g of NaOH in this solution. |
| Stopping solution | Add 10 ml of acetic acid to 200 ml of dH20. |

## Appendix 2: Oligonucleotide primer sequences

**Table v** Consensus primer sequences used for P-typing and G-typing

| Primer | Sequence( 5'-3') | Position | Genotype |
|---|---|---|---|
| Con2 | ATTTCGGACCATTTATAACC | nt 868-887 | Group A |
| Con3 | TGGCTTCGCCATTTTATAGACA | nt 11-32 | Group A |
| sBeg9 | GGCTTTAAAAGAGAGAATTTC | nt 1-21 | Group A |
| End9 | GGTCACATCATACAATTCTAATCTAAG | nt 1062-1036 | Group A |

**Table vi** G-type specific oligonucleotide primer sequences

| Primer | Sequence( 5'-3') | Position | Genotype |
|---|---|---|---|
| G1 | CAAGTACTCAAATCAATGATGG | nt 314-335 | aBT-1 |
| G2 | CAATGATATTAACACATTTTCTGTG | nt 411-435 | aCT-2 |
| G3 | CGTTTGAAGAAGTTGCAACAG | nt 689-709 | aET-3 |
| G4 | CGTTTCTGGTGAGGAGTTG | nt 480-498 | aDT-4 |
| G8 | GTCACACCATTTGTAAATTCG | nt 178-198 | aAT-8 |
| G9 | CTAGATGTAACTACAACTAC | nt 757-776 | aFT-9 |

**Table vii** P-type specific oligonucleotide primers

| Primer | Sequence( 5'-3') | Position | Genotype |
|---|---|---|---|
| P[4] | CTATTGTTAGAGGTTAGCGTC | nt 474- 494 | 2T-1 |
| P6 | TGTTGATTAGTTGGATTCAA | nt 259-278 | 3T-1 |
| P8 | ACTTGGATAACGTGC | nt 339-356 | 1T-1 |
| P9 | TGAGACATGCAATTGGAC | nt 385-402 | 4T-1 |
| P10 | ATCATAGTTAGTAGTCGG | nt 575-594 | 5T-1 |

## Appendix 3: Images of results from this study

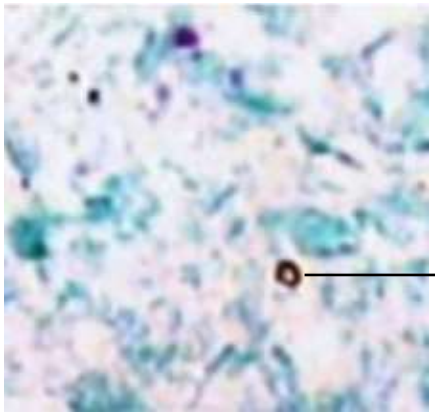


**Figure i** Ziehl Neelsen stained *Cryptosporidium parvum* cysts from one sample of a child suffering from diarrhea in Gaborone between March and November 2017.

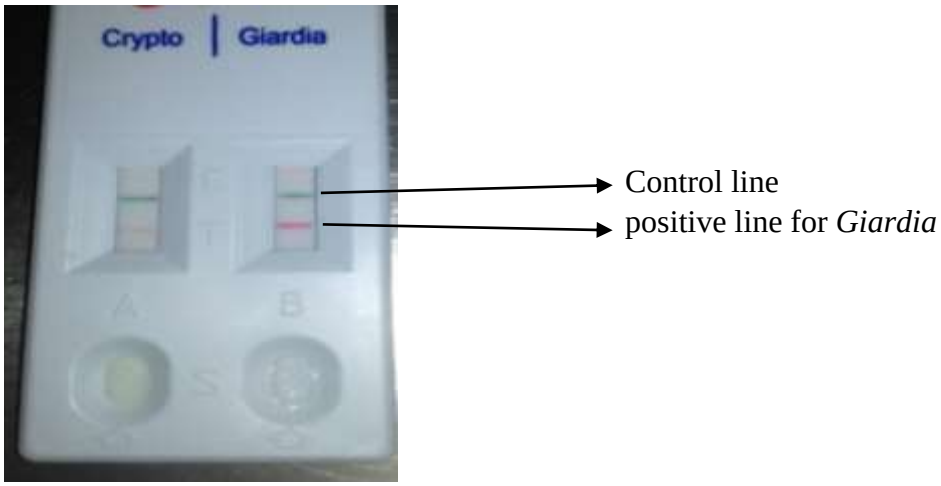


**Figure ii** ICA disc showing a sample positive for both *Cryptosporidium parvum* and *Giardia intestinalis*. The upper line opposite C label is for the control and appearance of a line across the label T indicates the presence of the corresponding microorganism in the sample.

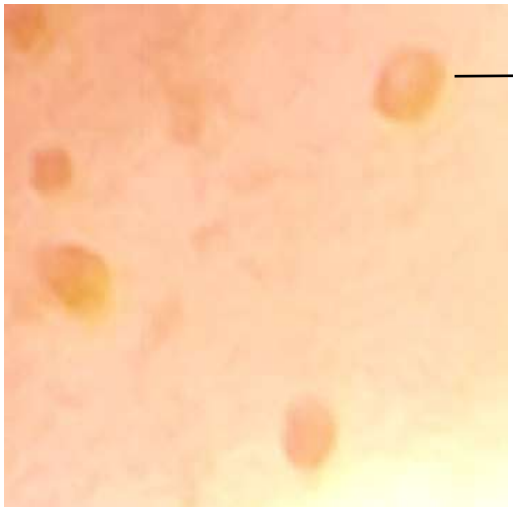


**Figure iii** Iodine stained *Giardia intestinalis* cysts detected in one of the stool samples.

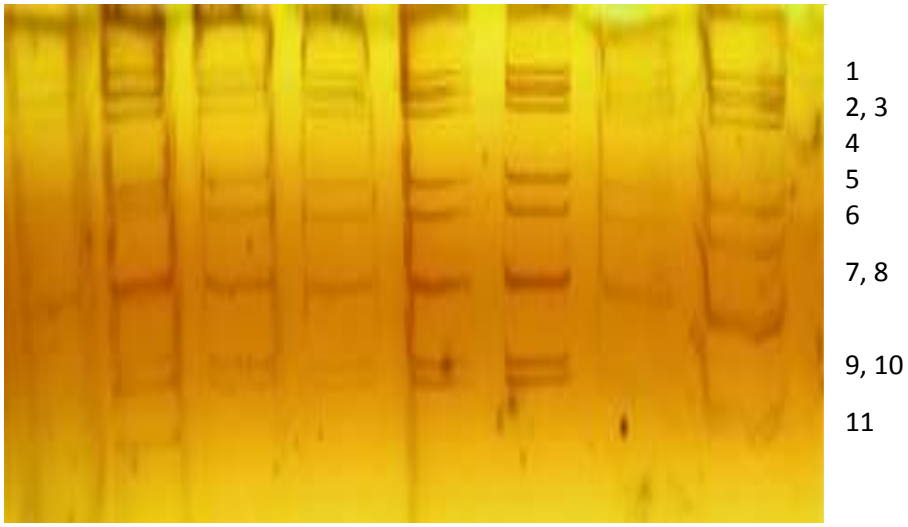


**Figure iv** A 10% polyacrylamide gel that show pattern of rotavirus electrophoretypes from ELISA positive samples of children below the age of five years in Gaborone, 2017. Lanes, 2, 3, 4, show short electrophoretypes while lane 1, 5 and 6 show long electrophoretypes.

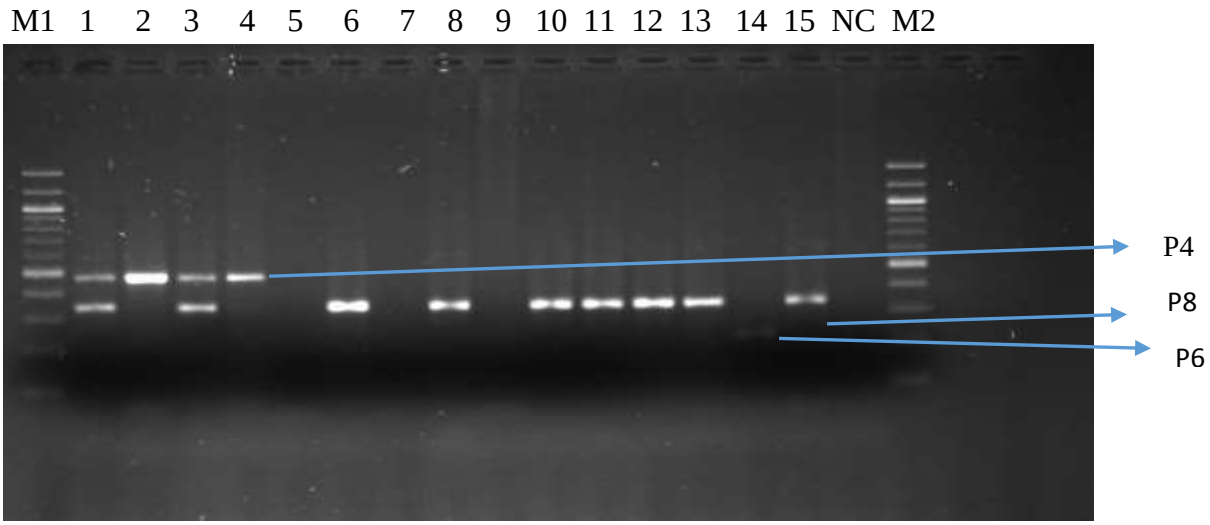


**Figure v** Human rotavirus VP4 genotypes detected on 1.5% agarose gel after a multiplex RT-PCR (using con3 and Gentsch primers) on samples from diarrhea-suffering children attending selected healthcare facilities in Gaborone in 2016. Lanes 1 and 17: 100 bp molecular DNA marker; lane 16, negative control; lanes 7, 9, 11, 12, 13, 14 and 16: P[8] (expected amplicon band size 345bp), lanes 3 and 5: P[4] (expected amplicon band size 483bp); lane 15: P[6] (expected band size 274bp). Lane 2 and 4 are mixed strains P [4.8] strains.

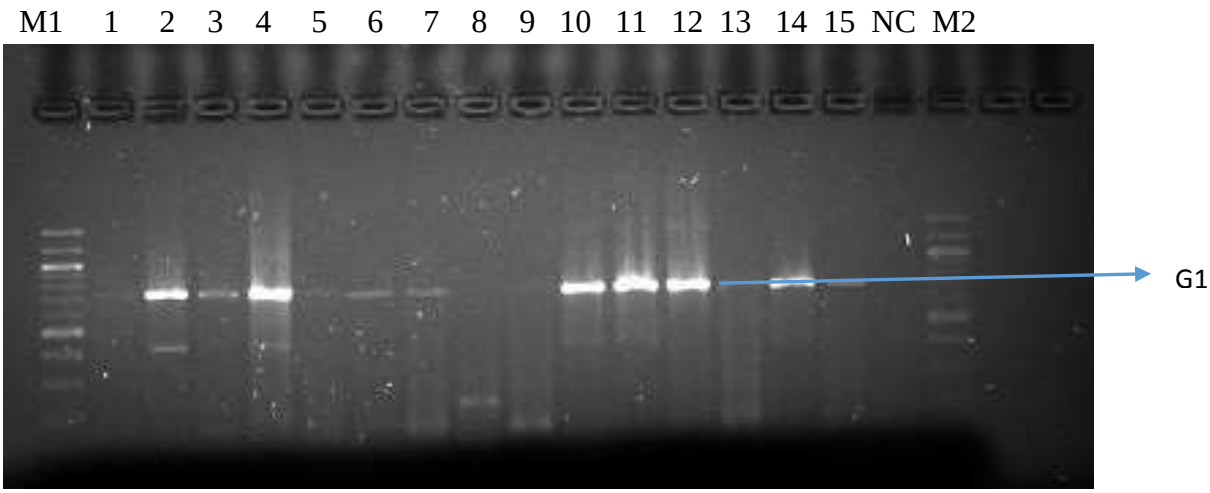


**Figure vi** Representatives of rotavirus G genotypes detected on 1.5% agarose gel after a multiplex RT-PCR of 15 ELISA positive samples from under five population suffering from diarrhea from selected healthcare facilities in Gaborone in 2017. Lane 1: 100 bp molecular DNA marker; lane 14 is a negative control; lanes 3, 6, 7, 10, 11 and 12: G1 (expected band size 749bp). Lane 2 and 4 are G1+G2 strains.