PREVALENCE AND GENETIC CHARACTERIZATION OF ROTAVIRUS INFECTIONS AMONG CHILDREN UNDER FIVE YEARS IN MUTAHO HEALTH DISTRICT, GITEGA PROVINCE AND BUJUMBURA MUNICIPALITY, BURUNDI

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Prevalence and Genetic Characterization of Rotavirus Infections among Children under Five Years in Mutaho Health District, Gitega Province and Bujumbura Municipality, Burundi.

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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This thesis has been submitted for examination with our approval as University Supervisors

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DEDICATION

I dedicate this thesis to my beloved parents, the late Julien Baranzira and Anésie Simbare. I cannot forget, my lovely father, the first day you took me to school and your passion to help me revise my lessons every day. My dear loving mother, you never ceased to encourage me to go forth in my studies, though you did not go to school. You did whatever was possible in the limit of your power and in accordance with Christian principles to keep me at school after the death of my father when I was still 6. Thank you infinitely for your great deeds. May the Lord God bless you all and preserve you.

I also dedicate this thesis to my wife Scholastique Ntihabose who showed unconditional love, courage, patience and support throughout the period of my absence in my home country due to my studies in this foreign country.

Further, I dedicate this thesis to my lovely children Bénie Fransiska Anthia Akimana, Louis Careb Ishimwe and Zélie Dolissa Iteka. Follow my footprints and go beyond what your father has achieved.

Finally, I dedicate this thesis to my brother Marc Iragarurirakure, my sisters Consolate Banyankimbona and Sylvie Hakizimana, their spouses and their children. You supported me in my studies in many different ways.

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ABBREVIATIONS AND ACRONYMS

Ag	Antigen
AFRSN	African Rotavirus Surveillance Network
AIDS	Acquired Immune Deficiency Syndrome
CDC	Center for Disease Control and Prevention
cDNA	Complementary DNA
CHWs	Community Health Workers
DLP	Double-layered particle
dsRNA	Double stranded Ribo-Nucleic Acid
EIA	Enzyme Immuno-Assay
ELISA	Enzyme Linked Immuno-Sorbent Assay
EM	Electron Microscopy
ER	Endoplasmic Reticulum
GAVI	Global Alliance for Vaccines Initiative
GoB	Government of Burundi
INSP	National Institute of Public Health
ISTEEBU	Burundi Institute of Statistics and Economic Studies
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute
LC3	Light chain-3
Mabs	Monoclonal antibodies
MoH	Ministry of Health
MoI	Ministry of interior
mRNA	Messenger Ribo-Nucleic Acid
MSc	Master of Science
NERC	National Ethical Review Committee
NSPs	Non-Structural Proteins
OD	Optical Density
OPD	Out-Patient Department

OR	Odds Ratio
ORS	Oral Rehydration Solution
PAGE	Polyacrylamide Gel Electrophoresis
PATH	Program for Appropriate Technology in Health
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Pcs	Pieces
PIN	Personal Identification Number
PPAT	Passive Particle Agglutination Test
SPSS	Statistical Package for the Social Sciences
TLP	Triple Layered Particle
UNDP	United Nations Development Program
UNICEF	United Nations Children's Fund
US	United States
VAD	Vaccine Accessibility and Delivery
VP	Viral Protein
WBG	World Bank Group
WHO	World Health Organization

ABSTRACT

Rotavirus is the leading cause of severe diarrhea in children under five years worldwide. In Burundi, rotavirus took the lives of more than 3,500 children under 5 years, each year, in the pre-rotavirus vaccine era. In 2013, the rotavirus vaccine was implemented in Burundi. Nonetheless, persistent diarrhea in Mutaho health district raised questions of whether uncommon rotavirus strains were circulating and challenging the efficacy of rotavirus vaccine in that part of Burundi. In Burundi, no study has been carried out to document the genetic diversity of circulating rotavirus strains. This cross-sectional study aimed at determining the prevalence and genetic characteristics of rotavirus infections among children under five years of age in Mutaho Health District and the Municipality of Bujumbura, in Burundi. Stool specimens were collected from 646 children presenting with acute diarrhea. In addition, data on the socio-demographic characteristics and the vaccination status of the study subjects were collected using a questionnaire. These specimens were screened for rotavirus antigen using Diagnostar® rapid test kit and confirmed by ELISA. Samples positive by ELISA underwent Polyacrylamide Gel Electrophoresis (PAGE), RT-PCR, G and P genotyping by multiplex semi-nested PCR using a cocktail of type specific primers or by sequencing. The difference between different proportions or the significance of the association between rotavirus prevalence, G and P type prevalence and exposure variables including socio-demographic characteristics and vaccination status of the study participants as well as the season were tested using Chi-square test or estimated as Odds-ratio. Furthermore, the VP4 and VP7 sequences from this study were subjected to molecular evolutionary analysis and phylogenetic analysis using p-distance nucleotide substitution model and maximum likelihood method respectively. The overall prevalence of rotavirus was 6.2% (40/646). Rotavirus detection rate increased as the amount of rainfall went down, showing a significant negative association between the two variables ($r = -0.875$; $P = 0.0001$). The prevalence of the genotype G1 was significantly higher in Bujumbura Municipality than Mutaho health district while G12 predominated in Mutaho health district (OR = 7.33; $P = 0.026$). Three different P types were identified; P [8] the most common, followed by P [6] and P [4]. The most common G/P combination genotype was G1P [8] (45.5%), followed by G12 P [8] (41.0%), G1P [6] (4.5%), G12 P [6] (4.5%) and G12 P [4] (4.5%). The emergence of G12 rotavirus strains with potential to challenge the efficacy of rotavirus vaccines was highlighted. Furthermore, the high degree of divergence between the VP7 and VP4 amino acid sequences of G1P [8] rotavirus strains from this study and the rotavirus vaccine stain, Rotarix, showed their potential to escape from the protection conferred by vaccination. It is recommended that strain surveillance for rotavirus in Burundi should be continuous to monitor trends in the occurrence of these prevailing and potentially emerging new rotavirus strains.

CHAPTER ONE

INTRODUCTION

1.1Background information

Diarrheal illness remains a major threat to child health worldwide (Abugalia *et al.*, 2011). It is one of the principal causes of morbidity and mortality among children in developing countries where numerous cases occur without identification of the specific etiologic agents (Kapoor *et al*., 2009). In Burundi, diarrheal diseases are the third leading cause of death among children under five years, after malaria and acute respiratory infections (GoB/MoH, 2016).

Several different groups of viruses have been shown to be responsible for the high incidence of acute viral diarrhea among children during their first few years of life (Moyo *et al*., 2007). Among them, rotavirus is the most important etiological agent in severe dehydrating diarrhea (Wilhelmi, Roman, & Sanchez-Fauquier, 2003). According to the WHO, rotavirus is the most common cause of severe diarrheal disease hospitalizations in young children in Africa (Raini *et al*., 2015; UNICEF & WHO, 2009).

Globally, rotavirus infections result in more than 450,000 deaths each year in children under five years and are responsible for millions of hospitalizations (PATH, 2013; UNICEF & WHO, 2009). In Africa, rotavirus takes the lives of 232,000 African children under five each year, accounting for more than 50 percent of the global total of rotavirus associated deaths (PATH, 2014). In Burundi, prior to rotavirus vaccine introduction, approximately one third of all under-five diarrheal disease hospitalizations and more than 3,500 deaths among children under five years were attributable to rotavirus, each year (PATH, 2013).

Rotavirus-induced gastroenteritis is more severe than gastroenteritis from other causes and more often results in dehydration, hospitalization and if not treated, shock, electrolyte imbalance and death (Bernstein, 2009).

The Global Alliance for Immunizations ranked rotavirus as a priority for vaccine. In Burundi, the vaccination against rotavirus was implemented in 2013 (GAVI, 2013). Nevertheless, to ensure success of vaccination against rotavirus, it is important to document locally circulating strain(s) against which to provide protection (Raini *et al*., 2015).

Rotavirus is characterized by substantial genetic diversity, as evidenced by the presence of multiple serotypes. The most common rotavirus strains associated with gastroenteritis worldwide include the following serotypes: G1P [8], G2P [4], G3P [8], G4P [8], G9P [8], G9P [6], P [6] G1P [6], G2P [6], G3P [6], G4P [6], G1P [9] and G3P [9]. These strains are responsible for 95% of pediatric rotavirus diarrhea worldwide (Bernstein, 2009). G1P [8] was particularly prevalent in North America, Australia, and Europe (>70% of infections) but less common in South America, Asia, and Africa (20%–30%). Furthermore, G9 has emerged in recent years as an important strain, with the highest rates in South America and Australia. Other serotypes continue to emerge including G5, G8, and G12 rotavirus strains (Santos & Hoshino, 2005).

The segmented nature of rotavirus genome favors re-assortment between different rotavirus strains during co-infections, which may result in a progeny with high genomic diversity. Virtually all the eleven rotavirus genes are involved in re-assortment events (Maunula & Bonsdorff, 2002). In addition, accumulation of point mutations, due to the high error rate of the viral RNA dependent RNA polymerase, causes further genetic diversity amongst human rotavirus strains (Ianiro, Delogu, Fiore, & Ruggeri, 2015). Identification of rotavirus strains with novel P/G combinations in Malawi and elsewhere emphasized the ability of rotavirus to

undergo re-assortment at high frequency. These re-assortments may result in the formation of potentially new rotavirus strains (Ramachandran *et al*., 1998). When these mutations occur at genotype-specific regions of the VP7 and/or VP4 encoding genes, they can lead to the loss of complementarity with the typing primers, and result in either a failure to genotype or mistyping results (Iturriza-Gómara, Kang, & Gray, 2004).

According to the World Health Organization (WHO), in countries considering a rotavirus vaccination program, strain surveillance is needed to determine the most important genotypes against which to provide protection (WHO, 2009; Aly, Khairy, Johani, & Balkhy, 2015). To date, no study has been conducted to determine molecular characteristics of rotavirus strains circulating in Burundi.

1.2. Statement of the problem

In Burundi, before the introduction of rotavirus vaccine, under five mortality rate due to diarrheal diseases was estimated at 18% (WHO, 2014), making them the third leading cause of child mortality after malaria and acute respiratory infections. An estimated 5,605 Burundian children under five years died in 2010 due to dehydrating diarrhea. Rotavirus alone took the lives of more than 3,500 Burundian children under five years, each year, in the pre-rotavirus vaccine era. Furthermore, approximately one third of all under-five diarrheal diseases hospitalizations were attributable to rotavirus. Therefore, the vaccination against rotavirus was implemented in Burundi in 2013 (PATH, 2013).

However, the incidence of dehydrating diarrhea did not reduce significantly in Mutaho health district. Compared with other health districts of Burundi, Mutaho had the highest incidence rates of dehydrating diarrhea with 16, 538; 14,845 and 14,925 new cases in 2013, 2014 and 2015 respectively. Children under five years were the most affected by dehydrating diarrhea in that health district. The number of new

cases of diarrhea in the rest of health districts, considered independently, ranged from 261 to 7,417 in 2013 and 298 to 8,447 in 2014. In Bujumbura municipality, the number of new cases of dehydrating diarrhea was 8,130 in 2013 but was reduced considerably to 4,121 (49.3% reduction) in 2015 (GoB/MoH, 2014; GoB/MoH, 2015; GoB/MoH, 2016).

That situation raised questions of whether uncommon rotavirus strains were circulating in Mutaho health district, leading to a reduction in the efficacy of rotavirus vaccine currently used in Burundi. These questions were unanswered since there has been no study conducted to shed light on the prevalence of rotavirus infections and the genetic diversity of rotavirus strains circulating in Burundi.

1.3 Justification of the study

In Burundi, data on the prevalence of rotavirus infections and the genetic diversity of locally circulating rotavirus strains are scarce. The rotavirus vaccine, Rotarix, was introduced in Burundi in 2013 (GAVI, 2013), to tackle the high rates of morbidity and deaths associated with rotavirus in that country. This vaccine has been shown to be safe and effective in clinical trials in Africa, Asia, Europe, Latin America, and the US (PATH, 2013).

The WHO recommended countries considering a rotavirus vaccination program to conduct a rotavirus strain surveillance in order to determine the most important genotypes against which to provide protection (Gatheru *et al*., 1993) (WHO, 2009; Aly *et al*., 2015). Actually, common rotavirus strains causing severe disease change from year to year and from country to country (Kirkwood, 2010). Furthermore, uncommon rotavirus strains, with novel G and P specificities are increasingly being identified in different parts of the world, with potential to challenge the efficacy of currently used rotavirus vaccines (Donato *et al*., 2014).

Burundi has not implemented a laboratory based rotavirus surveillance system yet. Actually, no study has been conducted to determine the genotypes of rotavirus strains circulating in that country so far. However, besides the WHO recommendation (WHO, 2009), previous studies highlighted that following rotavirus vaccines introduction, antigenically distinct novel or rare strains could be selected and spread, decreasing vaccine efficacy; due to increased immune pressure against wild-type rotavirus strains circulating in the community (Kirkwood, 2010).

Therefore, it is difficult to rule out the possibility of the presence of uncommon rotavirus strains capable to escape from the protection conferred by the monovalent rotavirus vaccine, Rotarix, in Burundi; particularly in Mutaho health district where persistency of dehydrating diarrhea was observed in a post-vaccination era (from 2013 to 2015).

Determining the prevalence of rotavirus infections in Mutaho heath district and Bujumbura municipality could shed light on the current proportions of diarrhea cases caused by rotavirus. Furthermore, investigating the molecular characteristics of rotavirus strains circulating in Mutaho health district and Bujumbura municipality could allow for the detection of uncommon rotavirus strains in Burundi.

Moreover, the findings from this study would provide an opportunity for initiation of rotavirus strain surveillance in Burundi. Identifying strains over time could affect future vaccine strategies and detect any regional differences of genotype prevalence (Khoshdel, Parvin, Doosti, & Eshraghi, 2014).

1.4 Research questions

1.4.1 What is the proportion of rotavirus infections among children under five years presenting with acute diarrhea in Mutaho Health District, Gitega Province and Bujumbura municipality, Burundi?

1.4.2 Which rotavirus electropherotypes are circulating among children under 5 years presenting with acute diarrhea in Mutaho health district and Bujumbura municipality, Burundi?

1.4.3 Which G and P genotypes of rotavirus are circulating among children under 5 years presenting with acute diarrhea in Mutaho health district and Bujumbura municipality, Burundi?

1.4.4 To which extent are these genotypes related to each other, to rotavirus vaccine strains and to comparable genotypes of rotavirus circulating in other countries of the world?

1.4.5 What are the socio-demographic characteristics and vaccination status of the children under five years infected with rotavirus in Mutaho health district and Bujumbura municipality, Burundi?

1.5 Study objectives

1.5.1 General objective

To determine the prevalence and molecular characteristics of different rotavirus strains circulating among children under five years of age presenting with diarrhea in Mutaho Health District, Gitega Province and the municipality of Bujumbura, Burundi, in 2018.

1.5.2 Specific objectives

1. To establish the prevalence of rotavirus among children under five years presenting with acute diarrhea in Mutaho health district and Bujumbura municipality, in 2018;

2. To determine the electropherotypes of rotavirus strains circulating among children presenting acute diarrhea in Mutaho health district and Bujumbura municipality, in 2018;
3. To determine the genotypes of rotavirus strains circulating among children under five years presenting with acute diarrhea in Mutaho health district and Bujumbura municipality in 2018;
4. To determine the degree of similarity within VP7 and VP4 nucleotide sequences of rotavirus strains identified in Mutaho health district and Bujumbura municipality, the degree of similarity between them and comparable nucleotide sequences of rotavirus vaccine strains and reference rotavirus strains available in GenBank database in 2018;
5. To describe the socio-demographic characteristics and vaccination status of children under five years infected with rotavirus, as well as the seasonal patterns of rotavirus infections, in Mutaho health district and Bujumbura municipality, in 2018.

CHAPTER TWO
LITERATURE REVIEW

2.1 Biology of rotavirus

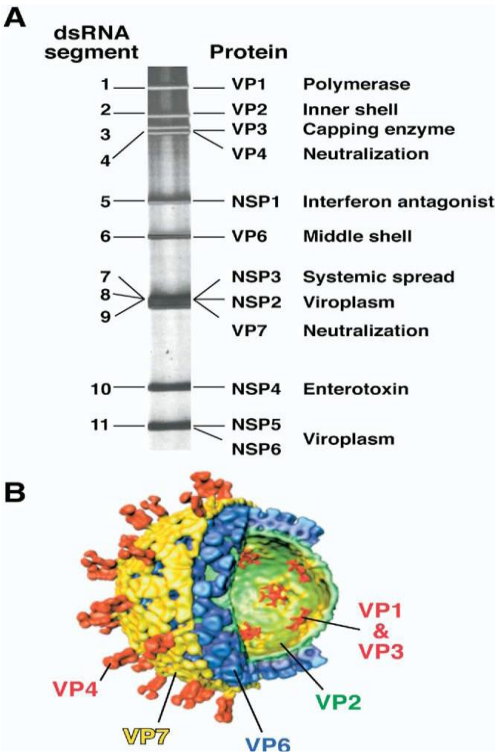
Rotaviruses are members of the *Rotavirus* genus classified into the *Reoviridae* family (Payne, Wikswo, & Parashar, 2011; WHO, 2009). The virus has a characteristic wheel-like shape (rota is Latin for wheel) (Fischer & Gentsch, 2004; Desselberger *et al*., 2009) (Figure 2.1).

It's a triple-layered icosahedral virus consisting of an inner core containing viral proteins VP1, VP2 and VP3 encoded by RNA segments 1–3; a middle capsid made up of protein VP6 encoded by the segment 6; an outer capsid made up of a VP7 shell encoded by segment 9 (or 7 or 8, depending on strain) and a VP4 spike protein encoded by RNA segment 4 (Fischer & Gentsch, 2004; Gouvea *et al.*, 1990).

The capsid proteins are responsible for many of the serologic properties of group A rotaviruses. Host antibodies to the VP6 protein define the rotavirus group antigen, while antibodies to VP7 and VP4 define G and P serotypes, respectively. Because antibodies to VP7 and VP4 also elicit protective immunity, vaccines have been targeted to these two proteins (WHO, 2009).

Rotaviruses genome consists of 11 segments of double-stranded RNA encoding six structural proteins that make up virus particles (viral proteins [VPs]) and 6 nonstructural proteins (NSPs) (Greenberg & Estes, 2009) (Figure 2.1). Each helix or segment is a gene numbered 1 to 11 by decreasing size. The segmented nature of the rotavirus genome facilitates re-assortment during multiple infections. As the gene segments encoding VP7 and VP4 can segregate independently during re-assortment, different G- and P-type combinations can be found in co-circulating rotaviruses (Dennehy, 2008; Iturriza-gómara *et al*., 2004). The emergence of antibody escape-

mutants can result from accumulation of point mutations which occur at high rate in rotaviruses. When genotype-specific regions of the VP7 and/or VP4 encoding genes are affected by these mutations they can result in the loss of complementarity with the typing primers leading to a failure to genotype or mistyping results (Adah, Rohwedder, Olaleyle, & Werchau, 1997).



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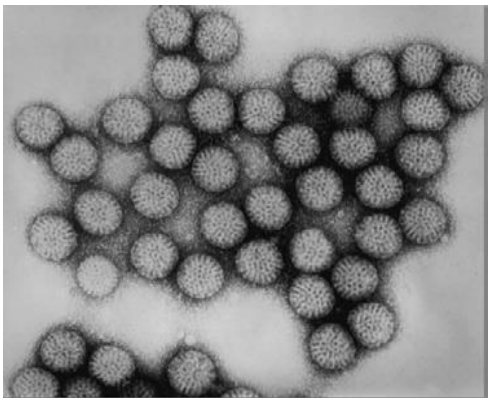

Source: Epidemiology and prevention of vaccine-preventable diseases (CDC, 2015)

Figure 2.1: Architectural features of rotavirus

These figures show the structure, morphologic features and different proteins produced by rotaviruses. (A) PAGE gel showing 11 dsRNA segments comprising the rotavirus genome. The gene segments are numbered on the left and the corresponding proteins are indicated on the right. (B) A cut-away view of the rotavirus TLP showing the inner VP6 (blue) and VP2 (green) layers and the transcriptional enzymes (shown in red) anchored to the VP2 layer. The spike proteins VP4 is colored in orange and the outermost VP7 layer in yellow.

Source: Rotaviruses: From Pathogenesis to Vaccination. *Gastroenterology* 2009; 136:1939–1951

2.2 Classification of rotaviruses

The *Rotavirus* genus includes at least seven serogroups (A to G) that may be distinguished on basis of the antigenic properties and the genomic characteristics of the inner capsid protein, VP6. Of the seven serogroups, three (A – C groups) are known to be human pathogens (Desselberger *et al*., 2009). Most of rotavirus infections in humans are associated with group A rotaviruses (Burke & Desselberger, 1996). Furthermore, the VP6 bears different epitopes which allow to differentiate different subgroup (SG) specificities of group A rotaviruses i.e. SG I and SG II (Fischer & Gentsch, 2004).

The VP7 (a glycoprotein or G-type antigen) and VP4 (a protease-sensitive protein or P-type antigen) are the basis of a binary classification system for group A rotaviruses (Ianiro *et al*., 2015). That classification system is based on the immunological reactivities and gene structures of the two outer capsid proteins (VP4 and VP7) that independently elicit neutralizing antibodies (Matthijnssens *et al*., 2008). VP7 types are classified as serotypes by neutralization assays or as genotypes by sequencing; results from the 2 assays are concordant (Gouvea *et al*., 1990), so rotaviruses are referred to by their G serotype alone (e.g. G1, G2, G3, and so forth). VP4 serotypes also are classified by neutralization and sequencing assays, but results from the two assays are sometimes discordant, so a dual nomenclature has been adopted for the VP4 serotype and genotype classification (Greenberg & Estes, 2009). The P serotype, when known, is denoted by a Arabic number (e.g., P1A), and the P genotype is denoted by an Arabic number within squared brackets (e.g. P [8]). If both are known, the P serotype precedes the P genotype (e.g. P1A [8]) (Kirkwood, 2010).

2.3 Replication of rotaviruses

Besides their significant pathogenicity, rotaviruses show unusual aspects of structural complexity with particular replication features (Greenberg & Estes, 2009). Rotavirus entry, activation of transcription, morphogenesis, cell lysis, particle release, and the distant action of viral proteins are Ca^{2+} dependent processes (Ruiz, Cohen, & Michelangeli, 2000).

Rotavirus cell attachment is a complex, multistep process which requires several specific interactions between different domains of the two viral proteins, VP7 and VP4, and surface molecules of epithelial cell (Crawford *et al*., 2018). Rotavirus attachment involves cell receptors found at mature enterocytes at the tips of villi but the host cell range may be extended to other types of cells (Greenberg & Estes, 2009). The marked cell tropism observed in vivo for the mature enterocytes of the small intestine has suggested that these cells bear specific receptors for the virus. In this respect, different cell surface molecules act as attachment and entry receptors and include integrins (α2β1, αvβ3, and αxβ2) and a heat shock protein (hsc70), and have been found to be associated with cell membrane lipid microdomains (Lopez & Arias, 2006). In vitro, VP4 is cleaved by the exogenous protease, trypsin (or trypsin-like proteases such as furin), into subunits VP5 and VP8 which is responsible for the attachment of the triple-layered particle onto the cell membrane and the subsequent penetration (figure 2.2) of the TLP to the endosome through endocytosis (Crawford *et al.*, 2001)

The dissociation of the VP7 layer and the subsequent release of the DLP into the cytoplasm are triggered by reduction in the concentration of intracellular calcium (Ruiz *et al*., 2000). The polymerase complex (comprised of VP1 and VP3) is stimulated by the uncoating event. Therefore, the DLPs are transcriptionally active. These particles function as molecular machines, producing capped viral (+) ssRNAs that are extruded from transcribing particles into the cytoplasm. Rotavirus

transcription proceeds via a conservative mechanism in which the parent strands of the genome segments are retained within the DLP while the daughter strands are extruded as (+) ssRNA products through channels located at the vertices of the particle (Patton, Carpio, & Spencer, 2004). These plus-ssRNAs have two distinct functions; as mRNAs for the viral proteins synthesis and as template for (-) ssRNA synthesis to yield the dsRNA genome segments (Arnoldi & Burrone, 2009).

In the cytoplasm, (+) ssRNAs are translated into proteins and replicated to produce new genomic RNA. The proteins in the core of the incoming particles (DLPs) possess all the enzymatic activities required to produce the viral transcripts from the viral genome double-stranded RNA (dsRNA) because eukaryotic cells lack RNA polymerases that transcribe mRNA from dsRNA templates (Greenberg & Estes, 2009).

Once critical amounts of viral proteins are synthesized, they accumulate in cytoplasmic inclusion bodies called viroplasms which are formed early (2 to 3 hours) after infection. The viral proteins identified in the viroplasms include the structural proteins VP1, VP2, VP3 and VP6 and the non-structural ones NSP2 and NSP5 (Arnoldi & Burrone, 2009).

The site for assembly and replication is thought to be the viroplasms. The dsRNA is synthesized by VP1 within the inner VP2 core. VP6 then assembles onto the VP2 core to stabilize it and to form the DLP (Patton *et al*., 2004). After the first steps of virus assembly, DLP made in viroplasms bud through the membrane of the endoplasmic reticulum acquiring a transient envelope (Arnoldi & Burrone, 2009). Budding into the endoplasmic reticulum is mediated by the NSP4 (Greenberg & Estes, 2009). The transient envelope acquired upon internalization into the endoplasmic reticulum lumen is then lost and replaced by VP7 and VP4 outer layer with particles becoming mature TLPs. Upon maturation, endoplasmic reticulum-accumulated TLPs are released from cells either by cell lysis or by delivery of

particles to the apical plasma membrane of polarized cells by a non-classic trafficking pathway that bypasses the Golgi apparatus and lysosomes and does not result in apparent cell membrane disruption (Arnoldi & Burrone, 2009) (Figure 2.2).

In the absence of Ca^{2+}, virus morphogenesis is stopped at the double-layered particle step and VP7 is excluded from hetero-oligomeric complexes (NSP4 and VP4) that participate in the budding of double-layered particles into the endoplasmic reticulum (Poruchynsky, Maass, & Atkinson, 1991)**.**

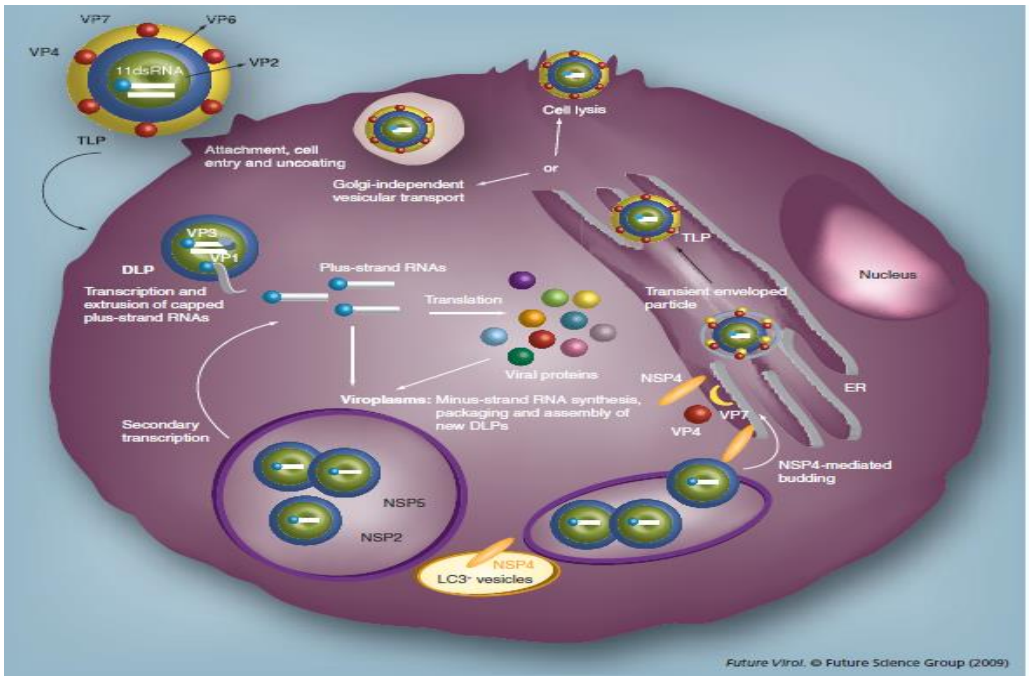

Figure 2.1: Schematic illustration of the replication cycle of rotavirus.

This diagram shows the replication cycle of rotaviruses from entry to exit of new virus progenies (Arnoldi & Burrone, 2009).

2.4 Rotavirus transmission and epidemiology

Human rotaviruses were discovered in 1973 by Ruth Bishop and her co-workers (Bishop, 2009) a decade after the first animal rotaviruses were visualized (Greenberg & Estes, 2009). Within 10 years of discovery, it was well known that rotaviruses occurred worldwide and were associated with approximately 20%– 30% of severe diarrheal diseases that required hospitalization in children under five years of age. Globally, rotavirus causes approximately 2 million hospitalizations and takes the lives of more than 450,000 children under 5 years of age, each year (Parashar, Hummelman, Bresee, Miller, & Glass, 2003) (Figure 2.3).

Studies from western Europe found that 50% of cases of gastroenteritis in children under 5 years of age who were treated in emergency departments were associated with rotavirus and that the infection caused 230 deaths per year (Soriano-Gabarro, 2006). In Africa, rotavirus takes approximately the lives of 232,600 children under five years, more than 600 each day (PATH, 2014). Vaccination is the best way to prevent rotavirus disease and the severe diarrhea that it causes (PATH, 2013).

Currently, two orally administered rotavirus vaccines i.e. Rotarix and RotaTeq are available. Large-scale trials in Africa, Asia, Europe and America demonstrated the safety and effectiveness of both rotavirus vaccines (Madhi *et al.*, 2010). In Africa, these trials showed that rotavirus vaccines reduced severe rotavirus disease by more than 60% during the first year of life, when children are at greatest risk for severe rotavirus diarrhea. Further, researchers have found herd immunity following the use of rotavirus vaccines (Patel, Glass, Desai, Tate, & Parashar, 2012).

In Burundi, before the introduction of rotavirus vaccination, rotavirus took the lives of more than 3,500 children under five years, each year. Furthermore, it was estimated that one third of all under five diarrheal disease hospitalizations were attributable to rotavirus (PATH, 2013). Therefore, rotavirus vaccination was

introduced in Burundi in 2013 (GAVI, 2013). However, to date, there has been no study conducted to assess the efficacy of the currently used rotavirus vaccine i.e. Rotarix in Burundi, over six years after its introduction in the national immunization program.

Concerning transmission of rotavirus, unlike many bacterial enteric pathogens, rotaviruses subsist in temperate and tropical climates, as well as in developed and less-developed social settings (Payne *et al*., 2011). Rotaviruses are very stable and may remain viable in the environment for weeks or months if not disinfected (CDC, 2015), which facilitates fecal– oral transmission both through close person-to-person contact (Payne *et al*., 2011) and contact with contaminated environmental surfaces (Greenberg & Estes, 2009; Bernstein, 2009). Respiratory transmission might also have a role in dissemination (Bernstein, 2009).

Rotaviruses are shed in the feces in large amounts up to 10^{10} particles per gram of stool of infected child. Limited infectivity studies indicated that 10 or fewer particles were likely infectious. The large amount of virus shed is probably the reason why improvements in hygiene and sanitation in developed countries did not greatly reduced the incidence of rotavirus disease (Greenberg & Estes, 2009).

In temperate climates, rotavirus disease shows seasonality, occurring in the cooler, dryer months of the year. Seasonality is less marked closer to the equator but the disease is more pronounced during drier and cooler months (Dennehy, 2008). The mechanism responsible for this seasonality is not clear (Greenberg & Estes, 2009).

The factors associated with rotavirus diarrhea in sub-Saharan Africa included nutritional status, poor food hygiene, low birth weight, the dry season and age under 2 years. Exclusive breastfeeding, particularly in infants, has been shown to offer protection against rotavirus diarrhea (Clemens *et al*., 1993). In addition, transplacentally acquired immunoglobulin G and immunoglobulin A in breastmilk

protect children aged <3 months against rotavirus infection (Salim, Karyana, Sanjaya-putra, Budiarsa, & Soenarto, 2014).

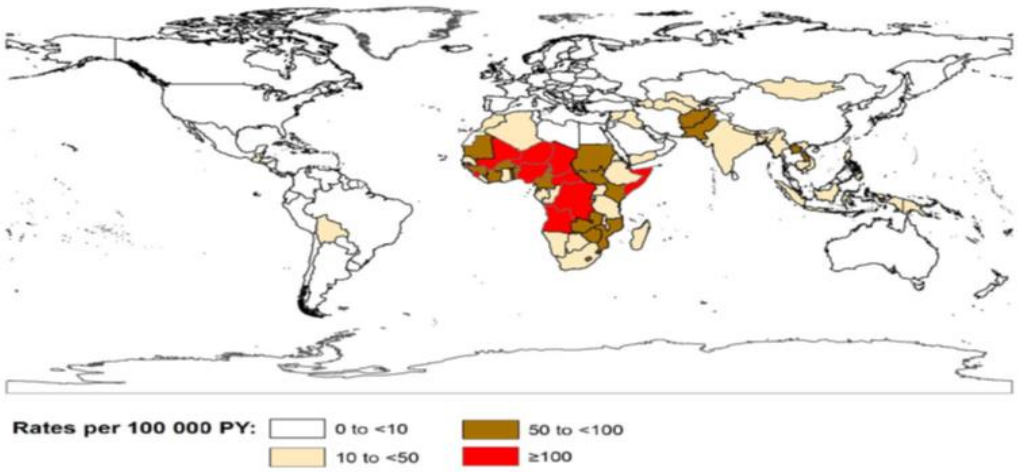

Figure 2.2: Rates of rotavirus mortality among children <5 years of age, by country, 2013.

Abbreviation: PY, person-years. This figure depicts the distribution of under five deaths associated with rotavirus by country, 2013, with the highest child death rates occuring in sub-saharan Africa where Burundi is geographically situated.

Source: Global, regional and national estimates of rotavirus (Tate *et al*., 2016).

2.5 Pathogenesis

Rotavirus infection can be symptomatic or asymptomatic. The outcome of infection depends on both viral and host factors (Burke & Desselberger, 1996) The age is the forestanding host factor that affects clinical outcome of rotavirus infection (Greenberg & Estes, 2009). Repeated infections occur from birth to old age, but

natural immunity renders the majority of infections asymptomatic after the first years of life (Payne *et al.*, 2011). Therefore, rotavirus can infect adults, but severe symptomatic disease is relatively uncommon and can result from infections with an unusual rotavirus strain (Greenberg & Estes, 2009).

Actually, common rotavirus strains causing severe disease change from year to year and from country to country through accumulation of point mutations that can lead to antigenic changes; re-assortment that leads to viruses with novel genetic and antigenic characteristics; and direct transmission of animal strains into a human host (Iturriza-Gómara et al., 2004; Kirkwood, 2010). All these changes often result in the formation of rotavirus strains with the capabilities to escape from natural or vaccine-induced host immunity.

In 2010, a large outbreak of severe rotavirus gastroenteritis occurred in the Alice Springs region of the Northern territory, Australia and was found to be associated with a G1P [8] rotavirus strain. Surprisingly, the outbreak occurred 43 months after the introduction of the G1P [8] rotavirus vaccine Rotarix. Whole genome sequencing of the 2010 outbreak rotavirus strain revealed numerous amino acid differences compared to the Rotarix vaccine strain (Donato *et al*., 2014).

Host factors influencing the clinical outcome of rotavirus infection include also malnutrition which is known to aggravate rotavirus diarrhea by delaying small intestinal recovery and inducing modification of intestinal inflammatory responses (Ramig, 2004).

Virus virulence is related to properties of the proteins encoded by a subset of the 11 viral genes. Virus virulence is multigenic and has been associated with genes 3, 4, 5, 9, and 10. Gene 3 encodes the capping enzyme (VP3) that affects the levels of viral RNA replication; genes 4 and 9 produce the outer capsid proteins (VP4 and VP7 respectively) required to initiate infection; the gene 5 codes for a protein (NSP1) that

functions as an interferon antagonist; the gene 10 codes for the nonstructural protein NSP4 which functions to regulate calcium homeostasis, virus replication, and as an enterotoxin (Greenberg & Estes, 2009).

The virus infects the mature enterocytes of the villus tip of the small intestine (Lorrot & Vasseur, 2007) suggesting that differentiated enterocytes express factors required for efficient infection and replication (Ramig, 2004). Infection often leads to fever, vomiting, and diarrhea in children (Dennehy, 2008). Several different mechanisms are involved in rotavirus diarrhea including malabsorption resulting from enterocyte destruction, a virus-encoded toxin, stimulation of the enteric nervous system (ENS), villus ischemia (Ramig, 2004) , virus-induced down-regulation of the expression of absorptive enzymes, and functional changes in tight junctions between enterocytes that lead to paracellular leakage (Greenberg & Estes, 2009). There is a secretory component of rotavirus diarrhea that is thought to be mediated by activation of the enteric nervous system and the effects of NSP4—the first described virus-encoded enterotoxin (Tafazoli, Zeng, Estes, & Magnusson, 2001).

Studies of the virus and the effects of NSP4 alone, in cultured cells and animal models, indicated that rotavirus-induced diarrhea results in part from activation of cellular Cl^- channels, which increases secretion of Cl^- and consequently water (Morris *et al*., 1999). In addition, absorption of Na^+, water, and mucosal disaccharidases are decreased (Ramig, 2004). Malabsorption results in the transit of undigested mono and disaccharides, carbohydrates, fats, and proteins into the colon (Lorrot & Vasseur, 2007). The undigested bolus is osmotically active, and the colon is unable to absorb sufficient water, leading to an osmotic diarrhea (Ramig, 2004).

The role of the innate immune response in rotavirus infection, and specifically in IFN-induced antiviral effects, has been examined both in vivo and in vitro. Levels of type I and II IFNs increase in rotavirus-infected children and animals. Type I and II IFNs are able to limit rotavirus infection in vitro (Greenberg & Estes, 2009). Studies

in vivo have shown that the systemic virulence of selected rotavirus strains was increased and a lethal biliary and pancreatic disease was induced when IFN signaling was blocked during rotavirus infection (Feng *et al*., 2008).

Concerning the role of acquired immunity, studies in mice showed that B cells were the primary determinant of protection against reinfection after natural infection while CD8+ T cells were responsible for shortening the course of primary infection (Cunliffe, Bresee, & Hart, 2002).

Rotavirus infection is not limited to the intestine (Nakano *et al*., 2011). It is clear that all infected individuals and animals undergo at least a short period of viremia and the virus can be detected in the several other tissues of immunocompetent hosts (Greenberg & Estes, 2009). Rarely, involvement of extra-intestinal sites, including the respiratory tract, liver, kidney, lymph nodes, and central nervous system, has been reported (Dennehy, 2008).

2.6 Rotavirus detection and characterization methods

The clinical symptoms associated with rotavirus (RV) gastroenteritis are not sufficiently characteristic to distinguish between rotavirus infection and other causes of gastroenteritis. Therefore, laboratory procedures, including electron microscopy (EM), enzyme-linked immunosorbent assays (ELISA), passive particle agglutination tests (PPAT), polyacrylamide gel electrophoresis (PAGE) or reverse transcription-polymerase chain reaction (RT-PCR) are necessary to confirm a clinical diagnosis of rotavirus gastroenteritis (WHO, 2009; Iturriza-Gómara *et al*., 2004).

2.6.1 Electron microscopy (EM)

Electron microscopy is highly specific for detection of rotavirus (Fischer & Gentsch, 2004) and is as sensitive as some EIAs. However, the method is too labor intensive for routine detection of rotavirus in large numbers of stool specimens. In addition,

EM requires an expensive instrument and highly trained personnel and cannot distinguish between rotaviruses of different groups (WHO, 2009).

2.6.2 Rotavirus detection by cell culture

Isolation and cultivation of human rotaviruses from clinical fecal specimens is difficult and adaptation to growth in vitro requires multiple rounds of passage in primary cells. Cultivation of animal and human rotaviruses in cells requires proteolytic activation of the viral attachment protein using trypsin (Arnold, Patton, & Mcdonald, 2012).

The propagation and quantification of rotavirus is generally performed in rhesus monkey kidney cells (MA104) (Estes, Graham, Gerba, & Smith, 1979; WHO, 2009). However, it is important to note that different rotavirus strains vary in their capacity to grow in culture (Arnold *et al*., 2012). When growing rotavirus from clinical samples, cytopathic effect and cell lysis can take several days (up to 1 week) and might be difficult to distinguish from the cell death that will also appear in the control vials/tubes (WHO, 2009).

2.6.3 Serological methods

2.6.3.1 Rotavirus detection by ELISA

Rotaviruses can be detected in fecal specimens by ELISA (Iturriza-Gómara *et al*., 2004) that uses rotavirus specific antibodies to capture antigen onto wells of plastic plates. The antigen is then detected in a colorimetric reaction using a second rotavirus-specific antibody coupled to a detector enzyme. The ELISA format is highly sensitive and specific and is adaptable to large sample volumes in the 96-well plate format. The optical density (OD) results can be easily recorded with a standard plate reader, permitting analysis of results with standard computer programs (WHO, 2009).

2.6.3.2 Rotavirus serotyping

Historically, the most common strain characterization method has been to start with an enzyme-linked immunosorbent assay (ELISA), using serotype-specific monoclonal antibodies (Mabs) to define the VP7 serotype directly in stool specimens (Fischer & Gentsch, 2004). The five most common rotavirus G serotypes (G1, G2, G3, G4 and G9) can be assigned a serotype directly from fecal material using several ELISA formats incorporating monoclonal antibodies (Mabs) that bind in a serotype-specific manner to the VP7 protein. Similarly, VP6 subgroupings I and II can be assigned using binding specificity of VP6 Mabs (Iturriza-Gómara *et al*., 2004; WHO, 2009). Other serotype-specific Mabs have proven valuable for detection of less common serotypes such as G5, G6, G8 and G10. Several Mabs to serotype G9 have been important in the detection of this serotype subsequently determined to be common worldwide (Kirkwood *et al*., 2003). Serotyping assays based on Mabs specific for the three most common human rotavirus P (VP4) serotypes/subtypes, P1A[8], P1B[4], and P2A[6], have also been developed (Coulson, 1993). However, the many cross-reactive epitopes observed between different P serotypes/subtypes in rotavirus field isolates precludes the use of this assay for routine P typing studies (WHO, 2009). In addition, Mabs used for serotyping are not readily available. Moreover, this method often fails to identify uncommon and newly appearing rotavirus strains (Fischer & Gentsch, 2004).

2.6.3.3 Detection of rotavirus by Passive Particles Agglutination Test (PPAT)

Latex particles or red blood cells coated with rotavirus antibodies (Abs) will agglutinate in the presence of rotavirus antigens (Ag) to produce macroscopically visible aggregates. Although agglutination tests may be performed more rapidly than EM or ELISAs, they are less sensitive and prone to nonspecific reactivity (Iturriza-Gómara *et al*., 2004).

2.6.3.4 Immunochromatography

Immunochromatography tests are easy, rapid and useful assays for detection of rotavirus (Nguyen *et al*., 2007). Enzyme-linked immunosorbent assays (ELISA) was the method of choice in many laboratories because it has high sensitivity, and a built-in control for non-specific reaction. Recently, polymerase chain reaction (PCR) has been used broadly in epidemiological surveillances because of its ability to genotype samples that could not be typed by ELISA (Khamrin, Tran, Chan-it, Thongprachum, & Okitsu, 2011). Those methods, however, need special equipment and are time consuming to perform.

The development of rapid immunochromatography (IC) based detection kits facilitates work in third world countries where there is lack of human and material resources for conducting laboratory tests in hospital as well as in epidemiological researches (Nguyen *et al*., 2007). A study carried out in Japan on the comparison of the rapid methods for screening of group A rotavirus in stool samples demonstrated that the immunochromatography kits evaluated in that study could be used as an alternative method for the rapid screening of group A rotavirus in fecal specimens, especially during acute gastroenteritis outbreak season (Khamrin et al., 2011).

2.6.4 Molecular characterization of Rotavirus

2.6.4.1 Polyacrylamide Gel Electrophoresis (PAGE)

Rotavirus dsRNA can be detected in clinical specimens by extraction of the viral RNA and analysis by electrophoresis on a polyacrylamide gel followed by silver staining (Fischer & Gentsch, 2004; Iturriza-Gómara *et al.*, 2004; Halloran *et al*., 2000).

Rotavirus genome consists of 11 segments of dsRNA. During electrophoresis through the gel, these negatively charged macromolecules separate according to their

size. The patterns of dsRNA can be visualized in the gel by staining with silver nitrate. Silver staining is a sensitive procedure to detect small amounts of nucleic acid in polyacrylamide gels. Silver ions form a stable complex with nucleic acids (WHO, 2009).

The dsRNA extracted from group A rotaviruses can be split into four size classes: four large segments, two medium-sized segments, three small segments, and the two smallest segments. Group A human and animal rotaviruses also display two electropherotypes: “long” and “short” (Iturriza-Gómara *et al*., 2004).

Human rotavirus groups A, B and C have distinct patterns of gene distribution. The electropherotypes correlate with the presence of viruses of specific groups A, B and C (Iturriza-Gómara *et al*., 2004). For example for group A rotavirus, they will have characteristic patterns like in figure 2.4 (WHO, 2009).

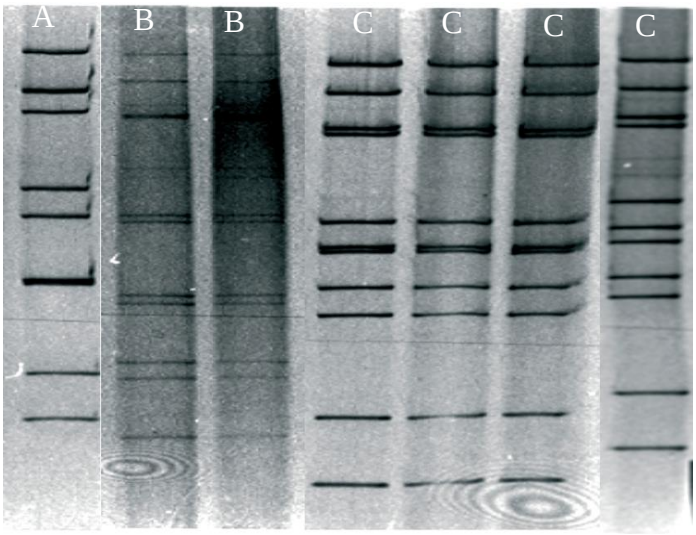

Figure 2.4: PAGE gel showing differences in segments migration configuration of group A, B and C rotaviruses.

Source: Manual of rotavirus detection and characterization methods (WHO, 2009).

2.6.4.1.1 Classification of different rotavirus electropherotypes

In an attempt to distinguish and to classify different rotavirus electropherotypes, the following procedure was adopted: The 11 RNA bands as determined by PAGE were divided into 4 groups including the large bands 1, 2, 3 and 4 (Group I); medium-size bands 5 and 6 (Group II), small bands 7, 8 and 9 (Group III) and smallest bands 10 and 11 (Group IV) (Iturriza-Gómara *et al*., 2004). This method allowed to define several distinct RNA patterns within each group, each pattern being referred to by a, b, c, d, e, f, g, etc (Lourenco, Nicolas, Cohen, Scherrer, & Bricout, 1981) (Figure 2.5).

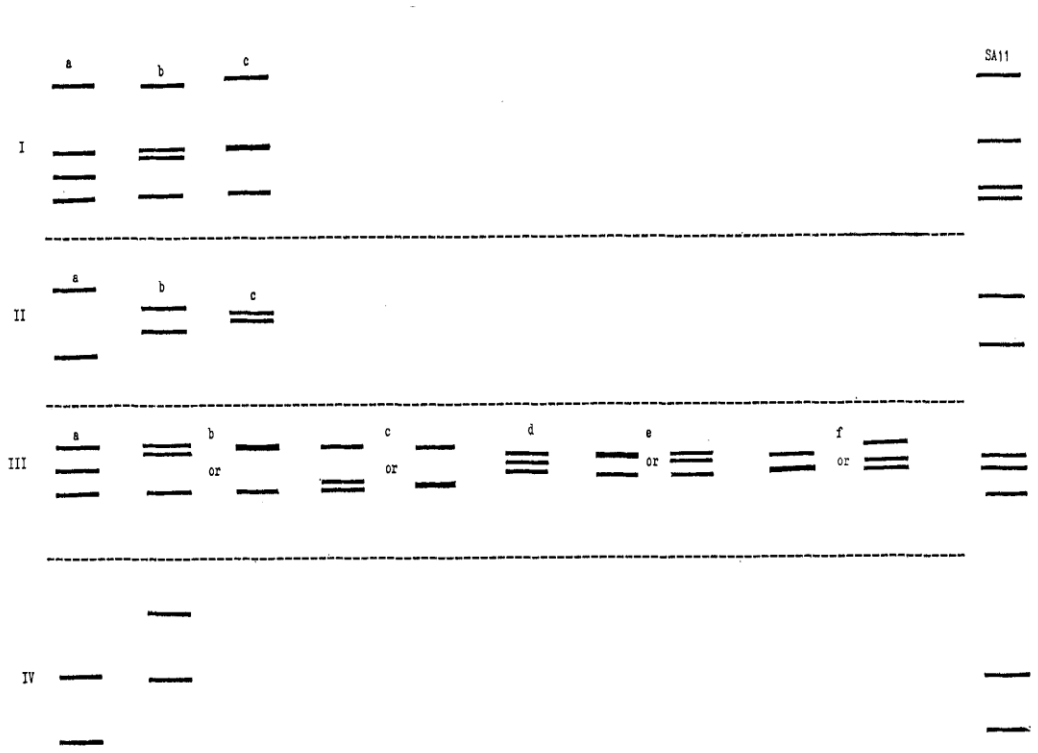

Figure 2.3: Subdivision of the RNA bands into 4 size-group

Source: Rotavirus gastroenteritis in Italian Children. (Lourenco *et al*., 1981).

Overall, short electrophoretic patterns exhibit a larger segment 11 (encoding NSP5) that migrates more slowly and is located between gene segments 9 and 10 whereas long RNA patterns are recognised by faster migration of the gene segment 11 (Matsui, Mackow, Matsuno, Paul, & Greenberg, 1990).

The electropherotype of each isolate could therefore be expressed as the combination of its specific group band patterns. For example, the electropherotype of the isolate shown in slot 6 can be written Ib IIb IIIg IVa or bbga. The RNA band patterns were classified based on the distance between different RNA segments (Cascio, Vizzi, Alaimo, & Arista, 2001) (Figure 2.6).

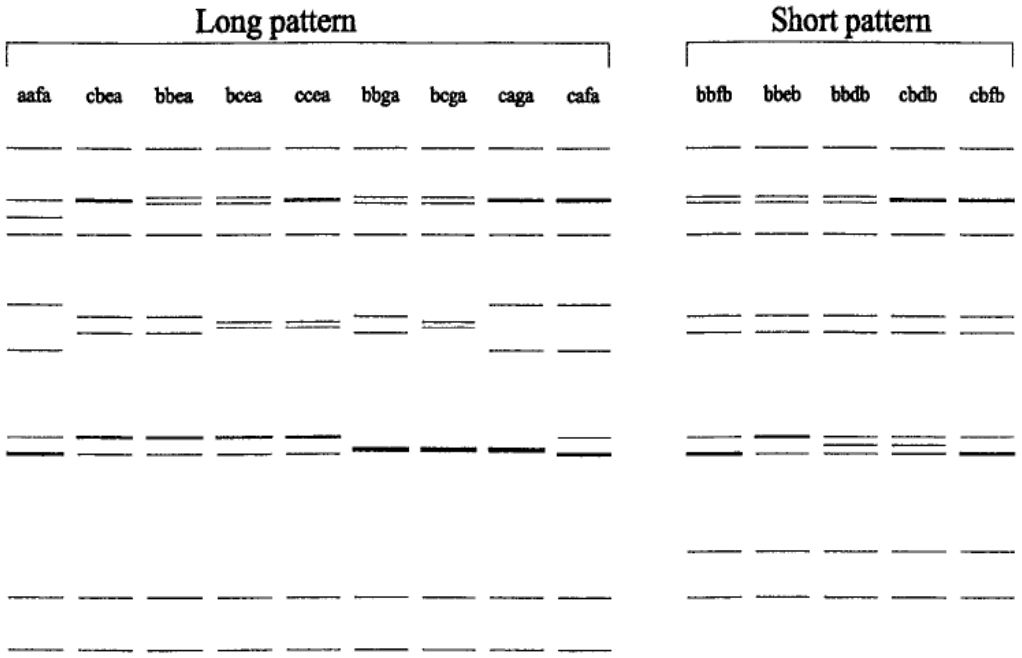

Figure 2.4: Schematic illustration of the electrophoretic migration of different e-types

Source: Rotavirus gastroenteritis in Italian Children. (Cascio *et al*., 2001).

The PAGE method has sometimes been used in the diagnosis of group A rotavirus infections for surveillance studies. However, this method is very labor intensive and time consuming (WHO, 2009).

2.6.4.2 Amplification (RT-PCR)

Rotaviruses in clinical specimens can be detected and G and P types determined by extraction of the viral RNA from fecal specimens and analysis by semi-nested RT-PCR with primers specific for regions of the genes encoding the VP7 or VP4 (Fischer & Gentsch, 2004). The objective is to obtain genotype-specific PCR products for analysis on an agarose gel or sequencing gel. RT-PCR of rotavirus dsRNA has three steps: 1) denaturation of dsRNA, 2) reverse transcription of dsRNA, and 3) amplification of cDNA. PCR consists of these steps: 1) heating the DNA to be amplified to separate the two template strands, 2) adding two primers that are complementary to the region to be amplified, 3) adding a heat-stable DNA polymerase enzyme that catalyzes the extension of the primers using the DNA strand as template, and 4) repeating the cycle, with the newly synthesized cDNA heat-denatured and the enzymes extending the primers attached to the liberated single DNA strands. The genotypes are then determined based on the size of the product after analysis by agarose gel electrophoresis (WHO, 2009).

2.7 Prevention and control

Diarrhea is more prevalent in the developing world due, in large part, to the lack of safe drinking water, sanitation and hygiene, as well as poorer overall health and nutritional status (UNICEF & WHO, 2009). Improvements in access to safe water and adequate sanitation, along with the promotion of good hygiene practices (particularly hand washing with soap), can help prevent childhood diarrhea. In fact, an estimated 88 per cent of diarrheal deaths worldwide are attributable to unsafe water, inadequate sanitation and poor hygiene (Black, Morris, & Bryce, 2003).

Nonetheless, public health interventions aimed at improving water, food and sanitation are unlikely adequately to control rotavirus related disease (Fischer & Gentsch, 2004; WHO, 2009). Due to the limited effect of sanitation-based strategies for preventing the spread of the virus, several rotavirus vaccines have been developed, out of which two oral vaccines (RotaTeq and Rotarix) have been licensed (Moyo *et al*., 2014). RotaTeq, approved in February 2006 by the US Food and Drug Administration, is a live, oral vaccine that contains a combination of 5 human/bovine reassortant rotaviruses that replicate poorly in the gut (Payne *et al*., 2011). In contrast, Rotarix is a live-attenuated human rotavirus vaccine prepared from a single human strain (P1A [8] G1) that replicates well in the gut (Bernstein, 2009).

Vaccine program objectives include the prevention of moderate to severe disease but not necessarily of mild disease associated with rotavirus (Dennehy, 2008). The two rotavirus vaccines i.e. Rotarix and RotaTeq are currently saving lives and improving the health of children in different countries where they are being used (PATH, 2013). The introduction of rotavirus vaccines in developed countries has significantly reduced diarrheal mortality and hospitalizations (Palma *et al*., 2010). So far, every ecological study has shown a decline ranging between 49% and 89% in hospital admissions for rotavirus-confirmed in children younger than five years within two years of vaccine introduction, in eight countries including El Salvador and the U.S (Patel *et al*., 2012). Further, clinical trials in African countries found that rotavirus reduced severe rotavirus disease by more than 60% during the first year of life (PATH, 2014).

In Burundi, one of the Sub-Saharan countries with the highest child death rates from rotavirus disease (> 300 deaths per 100,000), the rotavirus vaccine Rotarix was introduced in 2013 (GAVI, 2013). It was expected not only to save lives of Burundian children but also to reduce the tremendous economic and health burden of rotavirus disease, thereby contributing to poverty reduction.

The introduction of the rotavirus vaccine represented the latest step of Burundi in its implementation of comprehensive diarrhea control strategies (PATH, 2013). Between 2004 and 2010, Population Services International (PSI) distributed and marketed oral rehydration solution (ORS) in Burundi under the brand Orasel® as part of a USAID-funded project. This project significantly increased knowledge of the signs of dehydration due to diarrhea and the approaches of treatment including use of ORS (Kassegne, Kays, & Nzohabonayo, 2011).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study design

This was a health facility-based cross-sectional study analyzing stool samples from children under five years attending selected health facilities in Mutaho health district, Gitega Province and the municipality of Bujumbura, Burundi. Further, the study involved the collection of socio-demographic data, data on date of onset of diarrhea and vaccination status of the study participants. The study was conducted from November 2017 to July 2018.

3.2 Study site

The study was conducted in six health facilities within Mutaho health district and seven health facilities in Bujumbura Municipality. In Mutaho health district, the study sites included Mutaho district hospital, Mutoyi Hospital, Mutoyi, Rwisabi, Bugendana and Mutaho health Centers. In Bujumbura Municipality, the study was conducted at the Prince Regent Charles Hospital (HPRC), Buyenzi community health center (CMC Buyenzi), Bwiza-Jabe, Kamenge, Mirango, Kinama and Buterere health centers.

Mutaho health district is situated in Gitega Province, in the eco-climatic region of Kirimiro, in central plateaus of Burundi. This region is characterized by a high altitude between 1350-2000m (Nzigidahera, 2012). This health district covers two communes namely; Mutaho and Bugendana.

On the other hand, the Municipality of Bujumbura is one of the 18 Provinces of Burundi. It consists entirely of the city of Bujumbura, the economic capital of Burundi. It consists of three communes namely; Ntahangwa, Mukaza and Muha corresponding to three health districts namely; the Northern health district, the

central and Southern health district respectively. It covers an area of 86.54 km^2. It is situated in Western Burundi, in the Imbo floodplain, a low altitude land (774-1000 m of altitude) on the shores of the Lake Tanganyika (IADH, 2017).

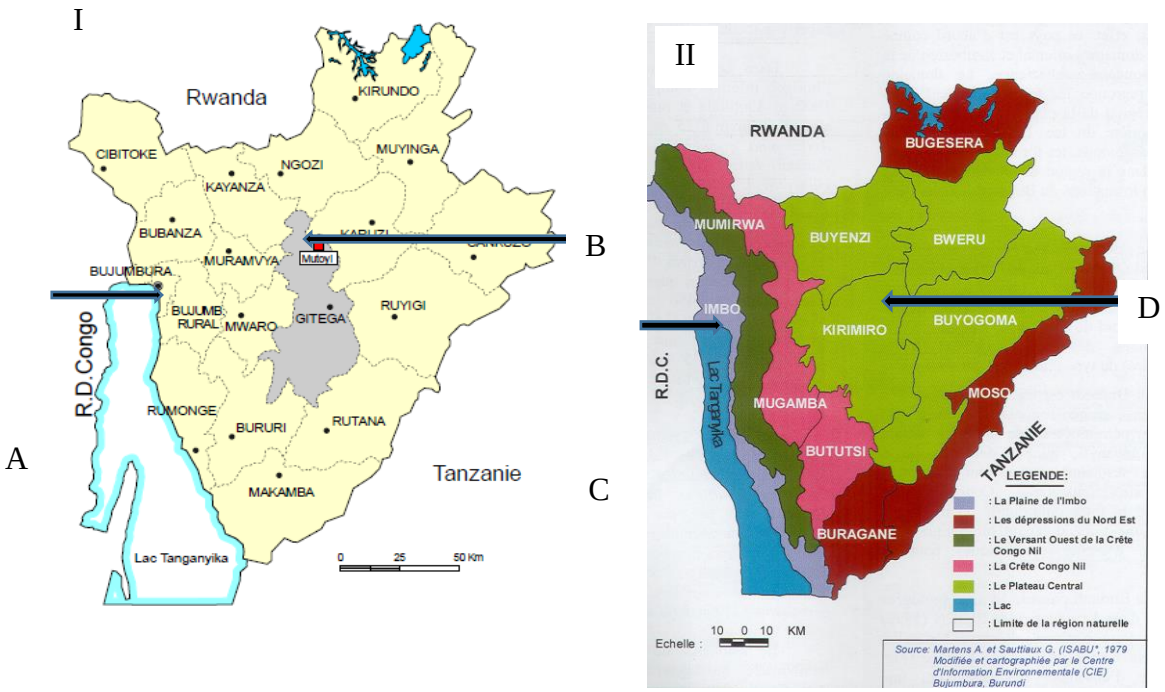

Figure 3.1: Geographic localization of Gitega Province, Mutaho health district and Bujumbura municipality and different eco-climatic regions lodging the sites of the study.

A: Bujumbura municipality, B: Mutaho health district, C: Imbo floodplain, D: Kirimiro eco-climatic region

Sources:

Figure 3.1.I Mutoyi hospital report of activities, 2015 (Mutoyi Hospital, 2016).

Figure 3.1.II Government of Burundi/Ministry of water, environment, land management and urban planning (Nzigidahera, 2012).

The data from a census conducted in 2008 across the entire country showed that the population of the two communes covered by Mutaho health district was 65,354 for Mutaho and 108,387 for Bugendana (GoB/MoI, 2008). The population served by this health district was estimated at 205,000 in 2015 (Hôpital Mutoyi, 2015). On the other hand, the population of the municipality of Bujumbura was estimated at 497,166 with 274,976 male and 222,190 female (GoB/MoI, 2008).

Mutaho Health district has a total of 14 health facilities of which two are primary level referral hospitals (Mutaho District Hospital and the Mutoyi Hospital) and twelve health centers (GoB/MoH, 2015). Annual reports of the Burundian MoH revealed that Mutaho health district had the highest incidence rates of diarrheal illnesses compared to the rest of health districts of the country (GoB/MoH, 2014; GoB/MoH, 2015; GoB/MoH, 2016). Furthermore, over 60% of households living in Gitega Province where Mutaho health district is located had less access to a source of clean drinking water. Moreover, 56.2% of children living in that province were suffering from malnutrition (ISTEEBU, 2015). In terms of health, the municipality of Bujumbura is subdivided into three health districts (the North Health District, the Central Health District and the Southern Health District). It has 5 national hospitals and 89 health centers (IADH, 2017).

Mutaho health district is essentially a rural area where most of people depend on agriculture and livestock for their livelihood (Mutoyi Hospital, 2016). A survey conducted between 2013 and 2014 revealed that 79% of the households living in Gitega Province was affected by poverty. In contrast, the level of poverty in households living in the municipality of Bujumbura was relatively less pronounced (ISTEEBU, 2015).

Mutaho health district and the Municipality of Bujumbura, situated in Kirimiro and Imbo eco-climatic regions respectively, have a tropical wet climate characterized by an alternation of the rainy season which runs generally from October to May and the

dry season which runs from June to September. Kirimiro region has an annual average precipitation ranging from 1,200 mm to 1,600 mm per year. The Imbo floodplain lodging the municipality of Bujumbura has a mean annual precipitation ranging from 800 mm to 1,100 mm (Nzigidahera, 2012; WBG, 2019).

3.3 Study population

The study population comprised children under five years attending selected health facilities with acute diarrhea, in Mutaho health district and the municipality of Bujumbura, during our study time period.

3.3.1 Inclusion criteria

- Children under five years (0-59 months) with diarrhea not exceeding seven days. Diarrhea is defined, according to WHO guideline, as the occurrence of three or more, loose, liquid, or watery stools within 24 hour period (WHO, 2014).
- Children admitted to the inpatient department (IPD) or under care in the outpatient department (OPD) of selected health facilities within the period of specimen collection;
- Written informed consent from the child's parent/guardian.

3.3.2 Exclusion criteria

- Children presenting with bloody diarrhea,
- Children presenting with other symptoms of gastroenteritis than diarrhea,
- Children failing to provide stool specimen.

3.4 Sample size determination

The minimum number of study subjects was determined by using a previously described formula (Pourhoseingholi, Vahedi, & Rahimzadeh, 2013):

$$n = \mathrm{g} * \left(\frac{Z^2 * P * (1 - P)}{(\mathrm{d})^2}\right)$$

Where:

n = estimated minimum sample size

P = expected proportion of under five children with acute diarrhea in Mutaho health district and the municipality of Bujumbura.

$z_{(1-\alpha/2)}$ = 1.96 is the Z-score corresponding to a 95% confidence level.

d= desired precision
g = design effect

In this study, we assumed a prevalence of 18.1%, the proportion of children with rotavirus diarrhea in Dar es Salaam, Tanzania; an East African country where Burundi is also geographically situated (Moyo *et al*., 2007). Therefore, the minimum sample size required to estimate the true proportion of children with diarrhea caused by rotavirus in Mutaho health district and the municipality of Bujumbura, with a desired 95% confidence interval and a precision of 0.05 was the following:

p= 0.181 thus, 1-p= 1-0.181
1-p = 0.819

$$n = 2 * \left(\frac{(1.96)^2 * .181 * (1 - .181)}{(.05)^2}\right)$$

n= 2*228, thus n= 456

Table 3.1: Distribution of study participants by health facility (n =646)

	Health facility	New cases of acute diarrhea as of 2017	Expected number of participants to be enrolled (%)	Number of participants enrolled (%)
Mutaho health district	Mutoyi hospital	265	7 (3)	19 (4.8)
	Mutaho district hospital	190	5 (2.2)	13 (3.2)
	Bugendana health center	20	2 (0.9)	35 (8.8)
	Mutoyi health center	7251	199 (87.3)	206 (51.5)
	Mutaho health center	478	13 (5.7)	50 (12.5)
	Rwisabi health center	84	2 (0.9)	77 (19.2)
Subtotal 1		8,288	228 (100)	**400 (100)**
Bujumbura municipality	HPRC	168	33 (14.5)	12 (4.9)
	Bwiza-Jabe health center	4	1 (0.5)	10 (4.1)
	Mirango health center	513	99 (43.4)	47 (19.1)
	Kamenge health center	7	1 (0.4)	136 (55.2)
	Kinama health center	272	53 (23.2)	27 (11.0)
	Buterere health center	207	40 (17.5)	4 (1.6)
	Buyenzi health center	2	1 (0.5)	10 (4.1)
Subtotal 2		1173	228 (100)	**246 (100)**
Grand Total		9,461	456	6**46**

3.5 Sampling design

Study participants were consecutively enrolled until the minimum sample size was achieved. The recruitment of study participants was integrated within the day-to-day activities of each health facility involved in this study. The clinicians explained to parents/guardians about the study in Kirundi and then a consent was obtained from those who agreed to participate.

3.6 Data and fecal specimens collection

A structured questionnaire administered by a clinician was used to collect both clinical and demographic information from the consenting parents/guardians (Appendix III). This activity involved attending clinicians. For stool specimen collection, the parents/guardians were given wide mouthed plastic sterile containers (polypots). The specimen was collected the same day by the parent/guardian and immediately brought to the laboratory for analysis. Attending clinicians provided necessary information while seeking informed consent from children's parents/guardians. Sample collection was carried out concurrently in all selected health facilities but separately in Mutaho health district and Bujumbura Municipality, until achievement of the required sample size. The consent form was translated in Kirundi, the national language of Burundi.

3.7 Laboratory procedures.

3.7.1 Specimen handling and processing

Upon samples receipt at the facility, they were screened for rotavirus antigen. After rotavirus antigen detection using rapid diagnostic test kit (Diagnostar® Rotavirus/Adenovirus Combo rapid test; Healgen Scientific LLC, 5213 Maple Street, Bellaire, TX 77401, USA), negative specimens were discarded. Positive specimens from Mutaho health district were placed in a cool box with ice bags (temperature of $+4^0$C) and transported to the district hospital laboratory where aliquots were prepared by transferring substantial amounts of stools into cryovials and stored in refrigerators at -20^0C. From there, at the end of the week, they were transported to the NIPH laboratory where they were stored at -80^0C until shipment to Kenya.

Positive samples from the municipality of Bujumbura were directly transported to the NIPH laboratory. From there, they were transported to the processing laboratory at

KEMRI, Center for Virus Research (CVR) in the rotavirus laboratory for further analyses. Transportation of stool specimens complied with national and international (IATA) regulations for the transport of infectious substances. In this regard, a triple packaging system with appropriate label (UN 3373 for BIOLOGICAL SUBSTANCES CATEGORY B) on the outer packaging was used. Further, a declaration of infectious substances was addressed to the transport company (FEDEX BURUNDI). A cold chain was maintained by use of dry ice in order to preserve the quality of the specimens during transport.

3.7.2 Rotavirus antigen detection by ELISA

Enzyme Linked Immuno-Sorbent Assay (ELISA) was performed as previously described (AFRSN, 2017; WHO, 2009). Briefly, one milliliter of 10% fecal suspension was prepared by adding a pea-sized portion (approximately 0.1g or 100µL for liquid specimens) of stool sample into 2 mL cryotubes containing 1mL of Prospect^{TM} Rotavirus sample diluent, vortexed and clarified by centrifugation at 2000 rpm for 5 minutes. Thereafter, the clarified supernatant was used for group A rotavirus antigen detection using a solid phase sandwich type Enzyme Linked Immunosorbent Assay (ELISA) method according to the manufacturer's instructions (Prospect^{TM} Rotavirus Microplate Assay, Oxoid Ltd, United Kingdom). The antigens captured onto wells of a plastic plate were detected in a colorimetric reaction using a second rotavirus-specific antibody coupled to a detector enzyme. The optical density (OD) results were recorded with a standard plate reader set at 450nm. Samples with optical density (OD) values above the cut-off value (OD of the negative control + 0.20) were considered as positive for rotavirus antigen. A positive and a negative control supplied with the kit were used per plate for quality control purpose and for precision in the calculation of the cut-off value. The procedures for detection were undertaken according to the manufacturer's instructions.

3.7.3 Phenol-chloroform method for the extraction of RNA from stool specimens.

Extraction of rotavirus ds RNA by a phenol-chloroform method was performed as described in previous studies (AFRSN, 2017; WHO, 2009). A pea sized amount of fecal material (0.1g for solid stool or 100 µL for liquid stool) was added to 900 µL of distilled water in a screw cap test tube to make a 10% stool suspension. The suspension was well mixed by vortexing. Then 450 µL of the stool suspension was placed in a clean Eppendorf tube whereby 50 µL of pre-warmed 1M Sodium acetate (1M NaAc) containing 1% Sodium Dodecyl Sulfate (SDS), PH, 5.0 was added. The mixture was vortexed for 10 seconds and incubated in a 37^{o} C water bath for 15 minutes. After incubation, 500 µl of phenol/chloroform (1:1) was added to the Eppendorf tube, vortexed for 1 minute and incubated for a further 15 minutes in a 56^{o}C water bath. After opening and immediately resealing the tubes to reduce air pressure within the tubes and prevent popping open during mixing, the tubes were vortexed for 1 minute and centrifuged for 5 minutes at 12,000 rpm. Then the upper aqueous phase containing the dsRNA was carefully removed and transferred into new clean Eppendorf tubes, taking care to avoid any interface material as this contains protein and DNA that would contaminate the extraction and potentially degrade the RNA. After addition of 40 µL (1/10 volume) of 3M NaAc, the tubes were filled with ice-cold absolute ethanol and gently mixed by turning them over and over for 6 times. The tubes were then incubated at -20^{o}C for 2 hours after which they were centrifuged at 4 ^{o} C for 20 minutes to pellet the ds RNA. After, the supernatant was poured off and the tubes containing the RNA allowed to air-dry. Finally, the pellet was re-suspended in 30 µL PAGE sample dye before loading on a PAGE gel.

3.7.4 Gel Casting

Gel casting was performed as previously described (AFRSN, 2017; WHO, 2009). Glass plates were thoroughly cleaned with absolute ethanol (96%) and assembled for gel casting.

A 10% resolving gel was prepared by adding 15.8 mL of distilled water, 10 mL of acrylamide stock, 3.75mL of resolving gel buffer, 30 µL of Tetramethylethylenediamine (TEMED) and 1000 µL of ammonium persulfate into a falcon tube. The acrylamide solution was immediately poured between the glass plates and allowed to polymerize for 60 minutes. A 3% spacer gel was then made by adding 6.8 mL of distilled water, 1.6 mL of acrylamide stock, 1.25 mL of spacer gel buffer, 10 µL of TEMED and 500 µL of ammonium persulfate into a new falcon tube. This spacer gel was loaded on top of the resolving gel and a comb was immediately inserted to form wells. This spacer gel was allowed to polymerize for 45 minutes after which the comb was carefully removed. The glass plates were assembled onto the electrophoresis apparatus and then inserted into the electrophoresis tank which was subsequently filled with 1x Tris-Glycine running buffer. The ds RNA samples suspended in PAGE sample dye were loaded into the gel according to labelling on worksheet.

3.7.5 Polyacrylamide Gel Electrophoresis (PAGE)

The rotavirus dsRNA was run on a 10% polyacrylamide resolving gel and a 3% spacer gel was used to enhance resolution of the ds RNA segments. The electrophoresis was run at 15mA for 15 hours, in a Tris-glycine buffer at room temperature. Two known samples containing short and long electropherotype (e-type) rotavirus strains respectively were used as positive controls (on different gels) along with a sample known to be negative for rotavirus which was used as a negative control. The gels were stained using silver nitrate staining solution as described in

the Laboratory Manual developed by the African Rotavirus Workshop in South Africa (WHO, 2009).

3.7.6 Silver nitrate staining

Silver nitrate staining of rotavirus RNA segments was performed as described in previous studies (AFRSN, 2017; WHO, 2009). Briefly, the gel was first removed from the glass plates, the bottom right hand corner cut for gel orientation and the spacing gel cut and discarded. Then 200mL of fixing solution 1 was added to the gel in a container and incubated for 30 minutes on an orbital shaker. After, the fixing solution 1 was drained off and replaced with 200 mL of fixing solution 2 and incubated for 30 minutes on the orbital shaker. The fixing solution 2 was then drained off and silver nitrate staining solution added and incubated for 30 minutes on the orbital shaker. The silver nitrate staining solution was then drained off and the gel washed twice with distilled water for 2 minutes each time. After gel washing, 50 mL of developing solution was added to the gel which was gently agitated by hand for 30 seconds to remove any black precipitate. The 50 mL of developing solution was drained off and another 200 mL developing solution added to the gel and incubated until the 11 rotavirus ds RNA segments could be seen (approximately 5 minutes). After, the developing solution was drained off and stop solution added, a10 minute-incubation followed. Finally, the gel was rinsed in distilled water and the migration patterns visualized on an illuminator. Electropherotypes were classified by dividing the 11 ds RNA segments into four groups, as previously described (Cascio *et al*., 2001).

3.7.7 RT-PCR amplification

3.7.7.1 Extraction of ds RNA by QIAgen method

The ds RNA of rotavirus was extracted according to the manufacturer's instructions using the QIamp viral RNA mini kit (Qiagen/Westburg, Leusden, Netherlands) as previously described (AFRSN, 2017). Briefly, 10% stool suspensions were prepared by adding a pea-sized portion of stool sample (0.1g or 100µL for liquid sample) into cryotubes containing 1mL of distilled water. The suspensions were mixed by vortexing and clarified by centrifugation. Thereafter, 140 µL of the supernatant was added into a well labeled 1.5mL Eppendorf tube containing 560 µL of prepared Buffer AVL containing carrier RNA and mixed by pulse-vortexing for 15 seconds. After 10 minutes of incubation at room temperature followed by a brief centrifugation, 560 µL of absolute ethanol (96-100%) was added to the sample, mixed by pulse-vortexing for 15 seconds and briefly centrifuged. Subsequently, 630 µL of the solution was carefully applied to the QIamp Mini Spin columns placed into 2 mL collection tubes and then centrifuged at 8,000 rpm for 1 min. This step was repeated for the remaining 630 µL of the solution and then, the collection tubes containing the filtrate were discarded. After this step, 500 µL of Buffer AW1 was added to the content of the QIAamp Mini Spin column placed in a clean 2 mL collection tube and centrifuged at 8,000 rpm for 1 min. The Mini Spin Column was placed in a new 2 mL Collection tube and 500 µL of Buffer AW2 was added to its content and centrifuged at full speed (14,000 rpm) for 3 minutes. The old collection tubes containing the filtrate were discarded and then, 60 µL of Buffer AVE equilibrated at room temperature was added to the Mini Spin columns placed in sterile labelled 1.5 mL Eppendorf tubes followed by incubation at room temperature for 5 minutes. Finally, the QIAamp Mini spin columns were centrifuged at 8,000 rpm for 1 minute, the columns were discarded remaining with the 1.5mL Eppendorf tubes containing the viral RNA which were kept at -20^{o}C.

3.7.7.2 RT-PCR amplification of rotavirus ds RNA

Amplification was performed as described in the laboratory manual developed by the African Rotavirus Surveillance Workshop In South Africa (AFRSN, 2017; WHO, 2009). RT-PCR of rotavirus ds RNA involved three steps:

- The ds RNA denaturation,
- Reverse transcription of ds RNA
- PCR amplification of the newly synthesized cDNA.

For VP7-RT-PCR, a full-length 1062 bp gene segment 9 encoding the VP7 glycoprotein was reverse transcribed and amplified by using the primers sBeg9 [nucleotide (nt) 1→21, 5'-GGCTTTAAAAGAGAGAATTTC-3'] and End 9 (nt 1062←1036, 5' GGTCACATCATACAATTCTAATCTTAAG-3'). For VP4-RT-PCR, a full length 876 bp gene segment 4, encoding the VP4 protein was reverse transcribed and amplified by using outer primers Con3 and Con2 (Gouvea *et al*., 1990; Fischer & Gentsch, 2004).

Briefly, separately for VP7 and VP4 encoding gene segments, 1 µL of each random primer (forward and reverse primers) was added into a PCR tube containing 8 µL of extracted ds RNA. After a brief centrifugation, the ds RNA was incubated for 5 minutes at $+94^0$ C for denaturation and then immediately chilled in an ice bath for 5 minutes. Working on ice, a total volume of 3.2 µL of RT master-mix per reaction (1 µL dNTPs, 0.2 µL AMV RTase and 2.0 µL of AMV buffer) was added into each labelled PCR tube containing the extracted ds RNA, followed by incubation at 42^0 C for 30 minutes for reverse-transcription. Subsequently, the newly synthesized cDNA was subjected to PCR amplification under conditions described in table 3.2. For this first round PCR, the total volume of master-mix prepared per reaction was 40µl in these proportions: 4 µL of dNTPs, 4 µL of Taq Buffer, 2.4 µL of $MgCl_2$ (50mM), 30

µL of ddH2O and 0.3 µL of Taq Polymerase. The volume of master-mix required per reaction was multiplied by the number of reactions to be performed.

Table 3.1: Thermocycler program for the amplification of cDNA

Step	Temperature (^{0}C)	No. of cycles	Time (min)
Denaturation	94	1	2
Denaturation	94	30	1
Annealing	42		1
Extension	72		1
Final extension	72	1	7

3.7.7.3 G and P typing of group A rotavirus strains.

Rotavirus G and P genotyping was performed using semi-nested type specific multiplex PCR. For G (VP7) typing, the first round PCR product was subjected to genotyping with a cocktail of primers specific to eight human rotavirus genotypes G1- G4, G8, G9, G10 and G12 (aBT1, aCT2, aET3, aDT4, aAT8, AFT9, mG10 and bG12) and the consensus primer End 9 (Table 3.3).

Table 3.3: Oligonucleotide primers for G-typing

Primer	**Sequence (5'-3')**	**Position**	**Genotype**
aBT1	CAAGTACTCAAATCAATGATGG	314-335	G1
aCT2	CAATGATATTAACACATTTTCTGTG	411-435	G2
aET3	CGTTTGAAGAAGTTGCAACAG	689-709	G3
aDT4	CGTTTCTGGTGAGGAGTTG	480-498	G4
aAT8	GTCACACCATTTGTAAATTGG	178-198	G8
aFT9	CTAGATGTAACTACAACTAC	756-776	G9
mG10	ATGTCAGACTACAGATACTGG	666-687	G10
bG12	CCGATGGACGTAACGTTGTA	548-567	G12

P (VP4) typing involved a cocktail of primers specific to the five human rotavirus P genotypes P[4], P[6], P[8], P[9], and P[10] (2T-1, 3T-1, 1T-1, 4T-1, 5T-1) and the Con3 primer (Fischer & Gentsch, 2004; Gouvea *et al*., 1990) (Table 3.4). Briefly, working on ice, 40µL of master-mix comprising 4µL of 10x Taq buffer, 2.4 µL of 25mM $MgCl_2$, 0.3 µL of Taq Polymerase (5u/ µL), 4 µL of dNTPs (10mM) was added into a PCR tube containing 1µL of first PCR product, 1 µL of reverse primer (End 9 for VP7 and Con 3 for VP4) and 1 µL of each type specific primer. The PCR was run after a brief centrifugation. The second PCR products (7 µL per sample) were then resolved by electrophoresis on a 1% TAE-agarose gel at 94V for 30 minutes followed by determination of the genotypes based on their specific size observed under UV light. Positive and negative controls (Two known samples, one positive and another negative for rotavirus) as well as a 100 bp ladder (molecular weight marker) were used for quality control purpose and for precision in the measure of amplicon size.

Table 3.4: Oligonucleotide primers for P-typing

Primer	Sequence (5'-3')	Position	Genotype
2T-1	CTATTGTTAGAGGTTAGAGTC	474-494	P[4]
3T-1	TGTTGATTAGTTGGATTCAA	259-278	P[6]
1T-1	TCTACTTGGATAACGTGC	339-356	P[8]
4T-1	TGAGACATGCAATTGGAC	385-402	P[9]
5T-1	ATCATAGTTAGTAGTCGG	575-594	P[10]

3.7.7.4 Sequencing of VP7 and VP4 genes of non-typeable rotavirus strains by RT-PCR

Non-typeable rotavirus strains by RT-PCR may result from genetic variations in genotype-specific regions of the VP7 and /or VP4 encoding genes of globally

common rotavirus strains. Samples failing to genotype by RT-PCR underwent DNA sequencing using first round PCR products.

Sequencing reactions were performed in the Master cycler pro 384 using the ABI BigDye Terminator V3.1 Cycle sequencing Kit (Applied Biosystems) following the manufacturer's instructions. The same outer primers for PCR (sBeg9 and End 9 for VP7 and Con 2 and Con 3 for VP4) were used for DNA sequencing. The fluorescent-labeled fragments were purified from the unincorporated terminators with the BigDye terminator purification kit (Applied Biosystems). The samples were then injected to electrophoresis in an ABI 3730xl DNA Analyzer (Applied Biosystems).

3.7.7.5 Phylogenetic analysis of the VP7 and VP4 genes nucleotide sequences

Nucleotide sequences were analyzed by the nucleotide BLAST services (NCBI) for sequence homology search. The MEGA 6.0 software was used for phylogenetic and molecular evolutionary analyses (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). The evolutionary distances between rotavirus strains from Burundi, vaccine strains and reference strains from GenBank database were computed using the p-distance nucleotide substitution model. The maximum likelihood method was used for phylogenetic analysis (Tamura & Nei, 1993). The phylogenetic tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The reliability of the tree topology was tested using the bootstrap resampling analysis (1000 replicates).

3.8 Data management and analysis

3.8.1 Data entry and storage

Participants in the study were identified with their admission numbers for hospitalized children, the OPD numbers for outpatient children, re-coding done to identify samples with the unique code known to only the investigator as well as the

date of specimen collection. All the data obtained from each patient in the study were entered into a spread sheet created in MS Excel where the data was stored and protected using passwords known only to the principal investigator.

3.8.2 Data processing and analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS for IBM software version 21.0). The Chi-square test was used to test the statistical significance of differences observed between different proportions. The association between rotavirus positivity, G and P genotypes prevalence and socio-demographic characteristics was estimated as odds ratio (OR). A P value <0.05 was considered as an indicator of statistical significance. Descriptive summaries were presented in tables and figures.

3.9 Ethical consideration

Authorization to carry out this study was sought from the Burundian Ministry of Public Health and the Fight against AIDS (MoH) and ethical clearance was obtained from the National Ethics Review Committee in Burundi (BERC 14/10/2017) (Appendix IV). It was voluntary and written informed consent was obtained from children's parents or guardians to allow children participating in the present study. The consent form was translated in Kirundi. This study did not involve any invasive procedure. In fact, the parents/guardians collected the stool specimen in sterile containers in the normal defecation process. This study therefore did not subject the participants to any pain. Anonymity and confidentiality of the subjects was strictly upheld at all stages of the study.

CHAPTER FOUR

RESULTS

4.1 Socio-demographic characteristic of study participants

A total of 646 participants were enrolled in this study. More than a half (55%, 355/646) of children who participated in this study were male. The mean age for these children was 21.58 months (SD: 14.29). Over 60% of them were under 2 years of age. The study participants residing in Mutaho health district accounted for 61.9% (400/646) of all the study participants. According to their parents/guardians or care takers, 584 (90.4%) were vaccinated against rotavirus, 36 (5.6%) were not vaccinated and the vaccination status of 26 (4.0%) of them was not known (Table 4.1).

4.2 Prevalence of rotavirus infections among children under 5 years

Group A rotavirus antigens were detected in 40 out of 646 (6.2%) specimens tested by ELISA. The highest prevalence of rotavirus infections was observed in the 54-59-month-old age-group (12.0%, 3/25) followed by the 12-17-month-old age-group (11%, 11/115). Children younger than 1 month of age, those aged 30-35 months, 42-47 months and 48-53 months had the lowest prevalence of rotavirus infections (0/3; 0/44; 0/19 and 0/30 respectively).

Concerning the vaccination status of children under five years presenting with acute diarrhea in Mutaho health district and Bujumbura municipality, according to their parents/guardians, 585 out of 646 (90.6%) children who participated in this study were vaccinated against rotavirus (including those who received one dose of Rotarix). The highest percentage of children reported as vaccinated was observed in children aged 30-35 months (100%, 44/44) and the lowest percentage was observed in children aged 53 to 59 months. Interestingly, the highest prevalence (12.0%, 3/25) of rotavirus infections was observed in this latter age-group (53-59 months).

Furthermore, children less than 1 month of age had the lowest prevalence of rotavirus infections (0%, 0/3) though they were not vaccinated against rotavirus (Table 4.1).

Table 4.1: Prevalence of rotavirus infections and vaccination status of children under five years presenting with acute diarrhea by age-group (n=646)

	Vaccination status				ELISA results	
Age-group (months)	Non-vaccinated (%)	Vaccinated (%)	Not ascertained (%)	Total (%)	Negative (%)	Positive (%)
< 1	2 (66.7)	**1 (33.3)***	0	3 (0.5)	3 (100)	0
1 to 5	3 (8.8)	28 (82.4)	3 (8.8)	34 (5.3)	32 (94.1)	2 (5.9)
6 to 11	5 (3.0)	152 (91.6)	9 (5.4)	166 (25.7)	157 (94.6)	9 (5.4)
12 to 17	4 (3.5)	108 (93.9)	3 (2.6)	115 (17.8)	104 (90.4)	**11 (9.6)**
18 to 23	3 (3.9)	72 (93.5)	2 (2.6)	77 (11.9)	70 (90.9)	**7 (9.1)**
24 to 29	4 (5.3)	70 (93.3)	1 (1.3)	75 (11.6)	69 (92.0)	6 (8.0)
30 to 35	0	44 (100)	0	44 (6.8)	44 (100)	0
36 to 41	1 (1.7)	54 (93.1)	3 (5.2)	58 (9.0)	56 (96.6)	2 (3.4)
42 to 47	1 (5.3)	17 (89.5)	1 (5.3)	19 (2.9)	19 (100)	0
48 to 53	5 (16.7)	22 (73.3)	3 (10.0)	30 (4.6)	30 (100)	0
54 to 59	7 (28.0)	17 (68.0)	1 (4.0)	25 (3.9)	22 (88.0)	**3 (12.0)**
Total	35 (5.4)	585 (90.6)	26 (4.0)	646 (100)	606 (93.8)	**40 (6.2)**

*Children younger than 1 month of age wrongly reported by parents/guardians as vaccinated against rotavirus (Social desirability bias, since the first dose of Rotarix is started at 6 weeks of age).

The prevalence of rotavirus was significantly higher in the municipality of Bujumbura than Mutaho health district (OR = 2.6, P = 0.005). However, there was no significant association between the prevalence of rotavirus infections and the gender of study participants (OR = 0.9, P = 0.874) or the age of participants (OR = 1.73, P = 0.086). In addition, no significant difference of rotavirus detection rate was found between children reported by parents/guardians as vaccinated and non-vaccinated children (OR = 0.399, P = 0.565) (Table 4.2).

Table 4.2: Association between socio-demographic characteristics, vaccination status of participants and rotavirus detection rate (n = 646)

Variable	No. tested (%)	Rotavirus positive by ELISA	OR	95% CI	P. value
Age					
0-23 month	395 (61.1)	29 (7.3)	1.728	0.81-3.91	0.086
24-59 moths	251 (38.9)	11 (4.4)	Ref		
Gender					
Male	355 (55.0)	21 (5.9)	0.9	0.47-1.71	0.874
Female	291 (45.0)	19 (6.5)	Ref		
Residence					
Mutaho Health District	400 (61.9)	16 (4.0)	Ref	1.35-4.99	**0.005**
Municipality of Bujumbura	246 (38.1)	24 (9.7)	2.6		
Vaccination status					
Vaccinated	585(90.6)	39 (6.7)	Ref		
Non vaccinated	35 (5.4)	1 (2.9)	0.412	0.01-2.60	0.322
Status not known	26 (4.0)	0			
Seasonality					
Rainy season	385 (59.6)	4 (1.0)	Ref		
Dry season	261 (40.4)	36 (13.8)	**15.2**	**5.35-43.4**	**0.0001**

The prevalence of rotavirus was significantly higher in dry than rainy season with a peak of rotavirus detection rate during the month of July 2018 (15.8%) which accounted for 82.5% of all rotavirus infection cases recorded during the study (r = -875; P = 0.0001) (Table 4.3).

Table 4.3: Association between the prevalence of rotavirus infections and monthly mean precipitation

Month	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul
Average rainfall (mm)	146	154	133	134	149	176	85	7	3
Rotavirus prevalence (%)	0	0	0	1.9	0.6	0	6.1	6	15.75
Pearson's coefficient of correlation	**-0.875**								

In Burundi, the rainy season occurs during the months of October-May and the dry season from June to September. The month of July is the driest and the coolest month of the year in Burundi with an average precipitation of 3 mm and a mean temperature of 17.6^{o}C (Figure 4.1).

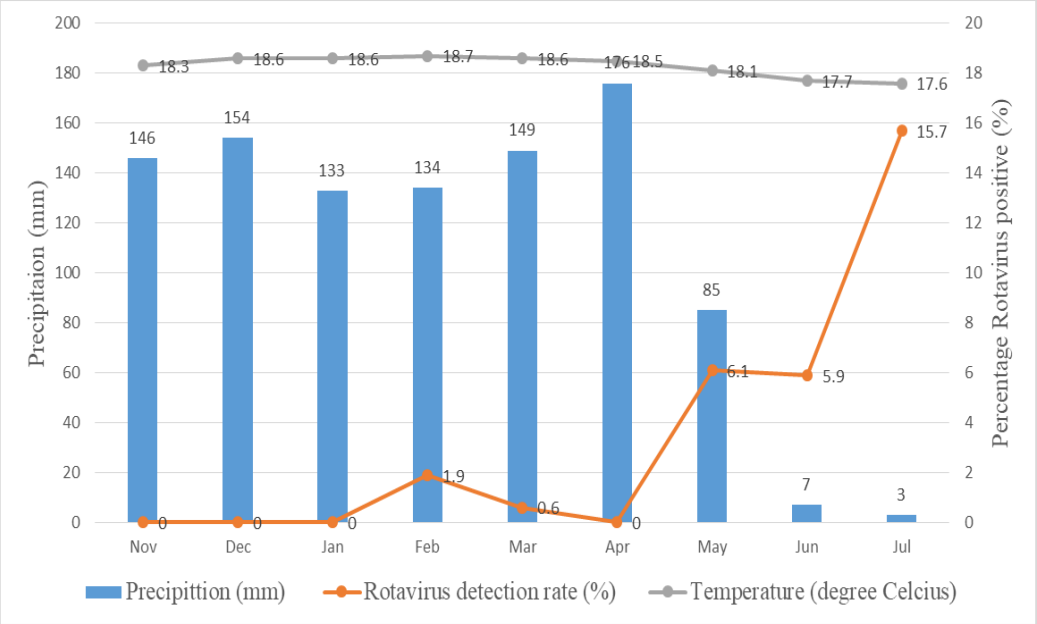

Figure 4.1: Trend of rotavirus detection rate over different months of the year and its association with the level of precipitation in Mutaho health district and Bujumbura municipality, Burundi

4.3 Distribution of rotavirus electropherotypes

Polyacrylamide Gel Electrophoresis was performed for 32 samples out of which 24 (75%) showed identifiable electropherotypes (e-types). Analysis of rotavirus electropherotypes revealed that 100% (24/24) of rotavirus strains isolated in Mutaho health district and the municipality of Bujumbura were long electropherotypes of group A rotavirus, with the typical 4-2-3-2 grouping of ds RNA segments. However, if we consider the group III of RNA segments, we noticed two distinct patterns of RNA segments migration. The first one is recognized by co-migration of gene-segments 7 and 8 and was designated as "Long electropherotype bbea". The second long RNA pattern showed a co-migration of gene segments 7, 8 and 9 whereby they seem to form a unique band. It was designated as "Long electropherotype bbga" (Figure 4.2).

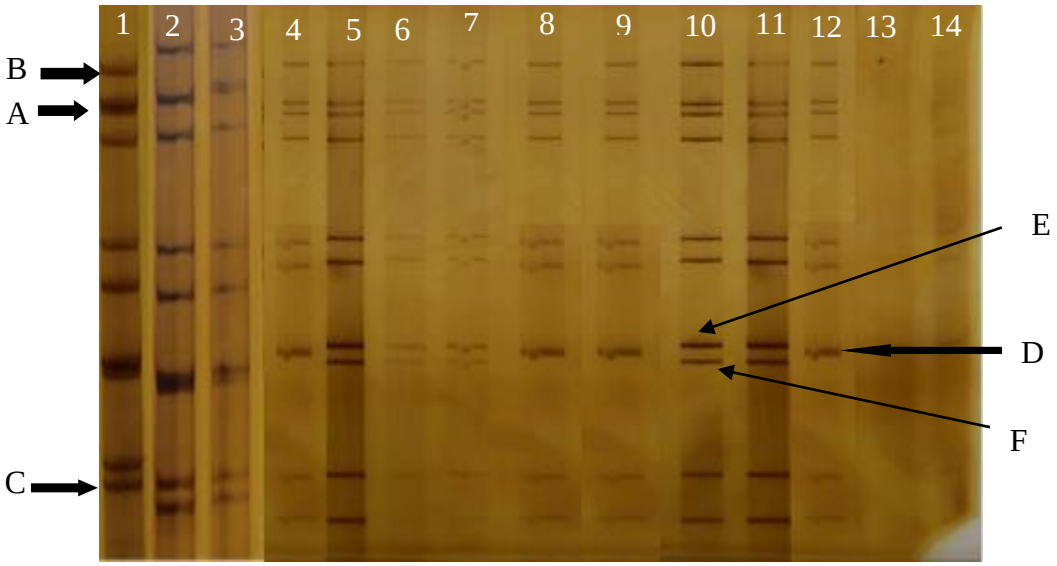

Figure 4.2: Polyacrylamide Gel Electrophoresis (PAGE) and silver staining of the ds RNA of rotavirus strains isolated in Mutaho health district and the municipality of Bujumbura.

Lane 1, short electropherotype, positive control; lanes 5, 6, 7, 10 and 11, long electropherotype bbea; lanes 2, 3, 4, 8, 9 and 12, long electropherotype bbga. The lanes 13 and 14 did not show any identifiable electropherotype. A: ds RNA-segment 1, B: ds RNA segment 2, C: ds RNA segment 11, D: co-migration of ds RNA segments 7, 8 and 9, E: Co-migration of ds RNA segments 7 and 8, F: ds RNA segment 9

The predominant long e-type was bbea which accounted for 54.2% (13/24) of all long e-types identified in this study against 45.8% (11/24) for the long pattern bbga. The long electropherotypes bbea and bbga were found to be significantly associated with G1 and G12 genotypes of rotavirus respectively ($p = 0.0001$). Over 90% (22/24) of all e-types of this study were isolated in dry season. Nonetheless, the distribution of different e-types was not significantly associated with season ($p = 1$).

Furthermore, there was no significant difference of e-types distribution between vaccinated and non-vaccinated children (p =1) (Table 4.4).

Table 4.4: Distribution of different long electropherotypes isolated among children with diarrhea in Mutaho health district and the Municipality of Bujumbura

	Electropherotypes (n = 24)		
	bbea	bbga	
Variable	n (%)	n (%)	P. value
G-types			
G12	0	11 (100)	
G1	13 (100)	0	**0.0001**
Period			
February – May	1(50)	1(50)	
June – July	12 (54.5)	10 (45.5)	1
Vaccination status			
Vaccinated	12 (52.2)	11 (47.8)	
Non vaccinated	1 (100)	0	1

4.4 Distribution of identified G and P types.

4.4.1 G (VP7) types

Of the 40 stool specimens confirmed to be positive for rotavirus antigens by ELISA, 32 samples were available for RT-PCR and genotyping and/or PCR products sequencing. For G- typing, 6 samples were PCR negative, 26 samples were successfully genotyped. Two G-genotypes, G1 and G12 were identified out of 7 searched for. The genotypes G2, G3, G4, G8 and G9 were not found (Figure 4.3).

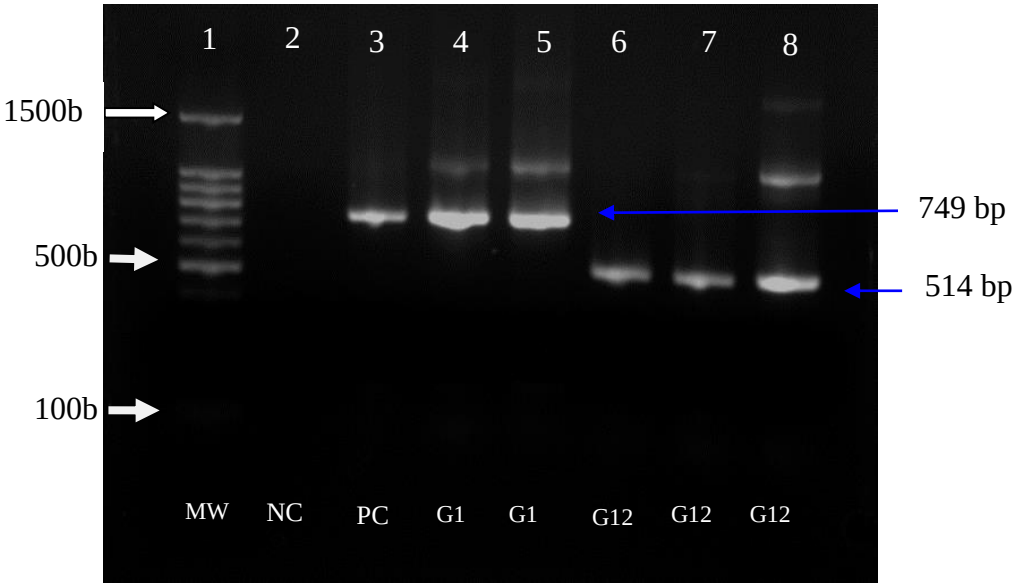

Figure 4.3: Agarose gel showing different G-types identified in Mutaho health district and the municipality of Bujumbura.

Lane 1(100 bp molecular weight marker), Lane 2 (Negative control), Lane 3 represents the positive control rotavirus strain, 749bp), Lanes 4 and 5 represent G1 rotavirus positive samples (749 bp), Lanes 6-8 represent G12 rotavirus positive samples (514 bp).

The genotype G1 was found predominant (53.8%, 14/26), followed by G12 (46.2%, 12/26). The distribution of G-types was not found to be associated with the age, gender and the vaccination status of children who participated in this study, nor was it significantly associated with season. However, the prevalence of G1 genotype of group A rotavirus was significantly higher in Bujumbura Municipality than in Mutaho health district while the G12 genotype predominated in Mutaho Health district, a rural area where most of the population depend on agriculture and livestock for their livelihood (OR= 7.333, P = 0.026) (Table 4.5).

Table 4.5: Prevalence of G genotypes of group A rotavirus strains detected in Mutaho health district and Bujumbura municipality

Variable	G1 n (%)	G12 n (%)	OR	95% CI	P. value
Age					
0 - 23 months	9 (56.3)	7 (43.7)	1.286	0.479-3.452	0.618
24 - 59 months	5 (50.0)	5 (50.0)	Ref		
Gender					
Male	9 (69.2)	4 (30.8)	Ref		
Female	5 (38.5)	8 (61.5)	0.278	0.040-1.798	0.119
Residence					
Mutaho health district	3 (27.3)	8 (72.7)	**7.333**	1.272-42.294	**0.026**
Bujumbura Municipality	11 (73.3)	4 (26.7)	Ref		
Vaccination status					
Vaccinated	13 (52.0)	12 (48.0)			
Non vaccinated	1 (100)	0			
Status not known	0	0	-	-	0.345
Seasonality					
Rainy season	1 (7.1)	1 (8.3)	Ref		
Dry season	13 (92.9)	11 (91.7)	1.182	0.066-21.175	0.72

4.4.2 P (VP4) types

P-typing was successfully performed for 25 samples while 7 samples were PCR negative. P-typing allowed the identification of three different P-types with the most common P-type being P[8] accounting for 84.0% (21/25) of all rotavirus P-types circulating in Mutaho Health District and in the municipality of Bujumbura, followed by P [6] and P [4] accounting for 8.0 % (2/25) each. The genotypes P [9] and P [10] were not found (Figure 4.4).

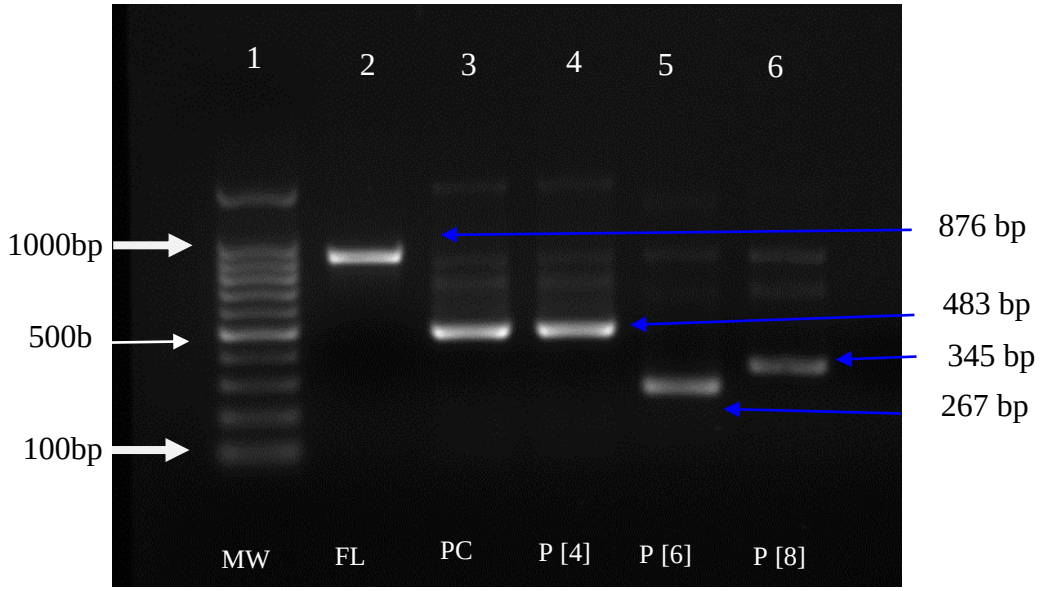

Figure 4.4: Agarose gel showing different P-types identified in Mutaho health district and Bujumbura municipality.

Lane 1 (100bp molecular weight marker), Lane 2 (876bp full length [FL] VP4 PCR product), Lane 3 represents the positive control (P [4] rotavirus strain), Lane 4 represents the P [4] (483bp) positive sample, Lane 5 represents P [6] (267bp) positive sample and Lane 6 represents P [8] (345bp) positive sample.

The distribution of P-types was not significantly associated with season (P = 0.072), the age of study subjects (P = 0.618), the gender of participants (P = 0.119). Further, there was no significant difference of P-types distribution between vaccinated and non-vaccinated children who participated in this study (P= 0. 906) (Tale 4.6).

Table 4.6: Prevalence of P genotypes of group A rotavirus detected in Mutaho health district and the Municipality of Bujumbura

Variable	P[4] n (%)	P[8] n (%)	P[6] n (%)	OR	95% CI	P. value
Age						
0 - 23 months	1 (6.3)	14 (87.5)	1 (6.3)	1.509	0.944-2.413	0.086
24 - 59 months	1 (11.1)	6 (66.7)	2 (22.2)	Ref		
Gender						
Male	1 (10.0)	7 (70.0)	2 (20.0)	Ref		
Female	1 (6.7)	13 (86.6)	1 (6.7)	1.352	0.860-2.125	0.192
Residence						
Mutaho health district	1 (9.1)	9 (81.1)	1 (9.1)	1.15	0.750-1.763	0.966
Bujumbura Municipality	1 (7.1)	12 (85.7)	1 (7.1)	Ref		
Vaccination status						
Vaccinated	2 (8.3)	20 (83.3)	2 (8.3)			
Non vaccinated	0	1 (4.8)	0			
Status not known	2 (8.0)	21 (84.0)	0	-	-	0.906
Seasonality						
Rainy season	0	1 (50)	1(50)	Ref		
Dry season	2 (8.7)	20 (87.0)	1 (4.3)	0.954	0.625-1.457	0.072

4.4.3. G/P combination genotypes

For each rotavirus isolate, G typing and P typing results were combined to get the corresponding G/P combination genotype. G1P [8] was found to be the most common combination (45.5%, 10/25), followed by G12 P [8] (41.0%), G1 P [6] (4.5%), G12 P [6] (4.5%) and G12 P [4] (4.5%) (Figure 4.5).

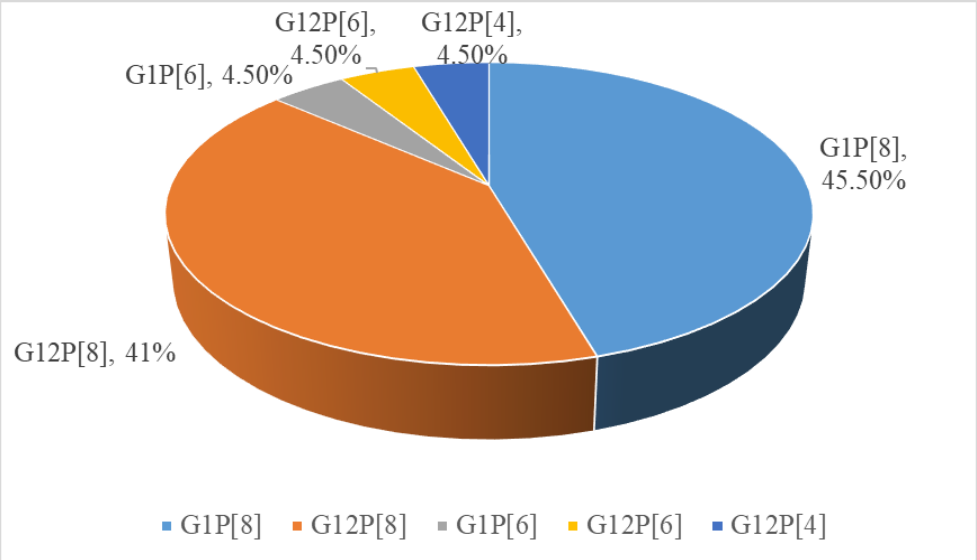

Figure 4.5: G/P combination genotypes identified in Mutaho health district and Bujumbura municipality

4.4.4 DNA sequencing of VP7 and VP4 first PCR amplicons

DNA sequencing was performed for samples that failed to genotype by RT-PCR and for some samples successfully genotyped by RT-PCR and randomly selected for sequencing in order to confirm PCR typing results. In total, 24 samples were subjected to DNA sequencing for P typing including 12 samples for confirmation of PCR results and 12 samples non typeable by RT-PCR. The latter were subsequently identified as P [8] rotavirus strains by DNA sequencing. For G-typing, 12 samples were subjected to sequencing for PCR results confirmation purpose. In total, DNA sequencing was successfully performed for 21 samples for P-typing and 10 samples for G-typing. (Appendix VI for VP7 and VP4 nucleotide sequences).

4.5 Analysis of the VP7 and VP4 nucleotide sequences

4.5.1 Analysis of the VP7 nucleotide sequences.

Phylogenetic analysis based on deduced amino acid sequences of the VP7 protein showed that amino acid similarity ranged from 93.9 to 100% within the Burundian G1 rotavirus strains. Rotavirus G1 strains from this study showed 93.6 to 99% amino acid similarity to reference G1 strains from India, Mali, Togo and USA (GenBank accession numbers for reference G1 strains are KT387247, KP882967, KP752653 and MF168292). Interestingly, only 88.8 to 92.2% amino acid similarity was found between Burundian G1 rotavirus strains and the rotavirus vaccine-strain Rotarix (GenBank accession number JN849114), despite their shared G1 specificity.

The VP7 nucleotide sequences of Burundian G12 rotavirus strains showed 95.4 to 99% similarity to each other at deduced amino acid level. Compared with the VP7 nucleotide sequences of reference rotavirus strains from Mozambique and USA, they showed a similarity ranging from 94 to 99% at deduced amino acid level (GenBank accession numbers: KY426082 and MF 168292) (Figure 4.6)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1. GU565057.1_RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P75																							
2. JN849114.1_RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P1A8	0.061																						
3. JN849152.1_RVA/Human-wt/BEL/BE1520/2009/G1P8	0.056	0.005																					
4. KP222840.1_RVA/Human-wt/MOZ/21168/2011/G12P6	0.245	0.255	0.250																				
5. KP752653.1_RVA/Human-wt/TGO/MRC-DPRU2209/2009/G1P8	0.082	0.071	0.066	0.255																			
6. KP882967.1_RVA/Human-wt/MLI/Mali-043/2008/G1P8	0.066	0.056	0.051	0.250	0.015																		
7. KT387246.1_RVA/Hu-wt/DIB/RMRC-11-03-106/2013/G1P8	0.077	0.066	0.061	0.255	0.015	0.010																	
8. KT387247.1_RVA/Hu-wt/DIB/RMRC/2013/G1P8	0.102	0.092	0.087	0.281	0.041	0.036	0.026																
9. KY426082.1_RVA/Human-wt/MOZ/0211/2012/G12P6	0.245	0.255	0.250	0.000	0.255	0.250	0.255	0.281															
10. LC374192.1_RVA/Human-wt/NPL/10N4155/2010/G12P6	0.240	0.260	0.255	0.005	0.260	0.255	0.260	0.286	0.005														
11. MF168292.1_RVA/Human-wt/USA/VU12-13-50/2013/G12PX	0.240	0.260	0.255	0.010	0.260	0.255	0.260	0.286	0.010	0.005													
12. MF184823.1_RVA/Human-wt/IND/N259/2004/G1G10P8	0.066	0.056	0.051	0.250	0.015	0.010	0.010	0.036	0.250	0.255	0.255												
13. MK043944.1_COVAS/Osmanbad/Hu155	0.082	0.071	0.066	0.260	0.020	0.015	0.005	0.031	0.260	0.265	0.265	0.015											
14. RVA/BDI/CN01/G1P8	0.097	0.077	0.071	0.270	0.026	0.031	0.020	0.041	0.270	0.276	0.276	0.031	0.026										
15. RVA/BDI/CN02/G1P8	0.092	0.071	0.066	0.265	0.020	0.026	0.015	0.041	0.265	0.270	0.270	0.026	0.020	0.005									
16. RVA/BDI/CN03/G12P8	0.245	0.265	0.260	0.015	0.260	0.255	0.260	0.286	0.015	0.020	0.026	0.255	0.265	0.276	0.270								
17. RVA/BDI/CN04/G1P6	0.087	0.077	0.071	0.265	0.015	0.020	0.010	0.036	0.265	0.270	0.270	0.020	0.015	0.020	0.015	0.270							
18. RVA/BDI/CN05/G1P8	0.092	0.071	0.066	0.265	0.020	0.026	0.015	0.041	0.265	0.270	0.270	0.026	0.020	0.005	0.000	0.270	0.015						
19. RVA/BDI/CN06/G1PX	0.092	0.082	0.077	0.270	0.031	0.026	0.015	0.031	0.270	0.276	0.276	0.026	0.020	0.036	0.031	0.276	0.026	0.031					
20. RVA/BDI/CN07G1P8	0.092	0.071	0.066	0.265	0.020	0.026	0.015	0.041	0.265	0.270	0.270	0.026	0.020	0.005	0.000	0.270	0.015	0.000	0.031				
21. RVA/BDI/CN10/G1P8	0.133	0.112	0.107	0.306	0.066	0.066	0.061	0.041	0.306	0.311	0.311	0.071	0.066	0.046	0.046	0.311	0.061	0.046	0.061	0.046			
22. RVA/BDI/CN11/G12P6	0.250	0.260	0.255	0.005	0.260	0.255	0.260	0.286	0.005	0.010	0.015	0.255	0.265	0.276	0.270	0.010	0.270	0.270	0.276	0.270	0.311		
23. RVA/BDI/CN12/G1PX	0.107	0.087	0.082	0.281	0.036	0.041	0.031	0.056	0.281	0.286	0.286	0.041	0.036	0.020	0.015	0.286	0.031	0.015	0.046	0.015	0.061	0.286	
24. RVA/BDI/CN9/G12P8	0.250	0.281	0.276	0.051	0.281	0.276	0.281	0.306	0.051	0.056	0.061	0.276	0.286	0.296	0.291	0.036	0.291	0.291	0.296	0.291	0.332	0.046	0.306

Figure 4.6: Degree of divergence between amino acid sequences of the VP7 protein of Burundian rotavirus strains and reference rotavirus strains from GenBank database

Burundian G1 rotavirus strains clustered with the Indian G1P [8] rotavirus strain RVA/Hu-wt/DIB/RMRC-11-03-107 with similarity ranging from 96.9 to 99.5% at deduced amino acid level, despite their different P genotypes (P[8] and P[6]).

On the other hand, G12 rotavirus strains from this study were very closely related and clustered with reference rotavirus strains from Mozambique, South Africa and USA. These Burundian G12 rotavirus strains formed a monophyletic cluster, despite their difference in P specificities (P [6] and P [8]) (Figure 4.7).

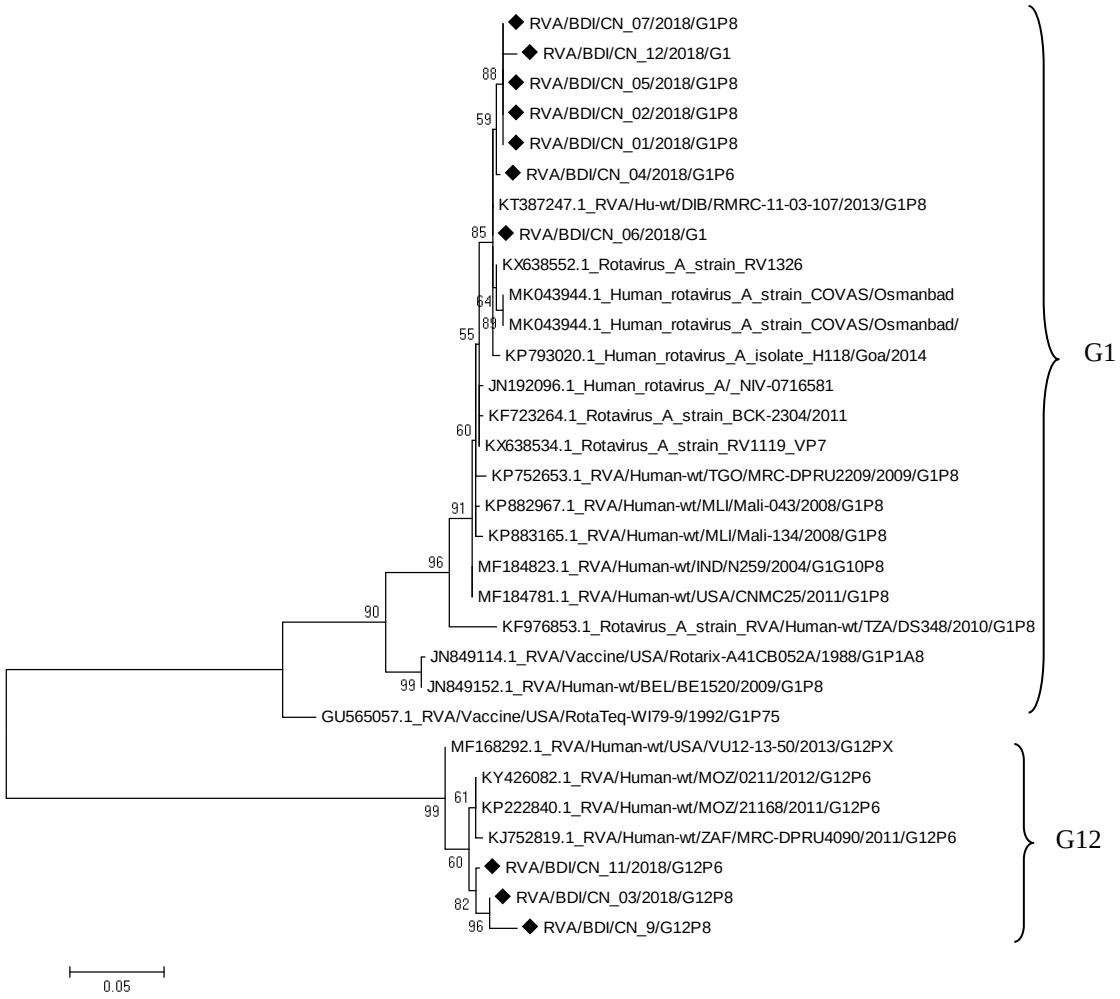

Figure 4.7: Phylogenetic tree based on the nucleotide sequences of the VP7 encoding gene of rotavirus strains identified in Mutaho health district and Bujumbura Municipality, Burundi, 2018

Reference rotavirus strains are indicated by accession numbers while rotavirus strains from this study are indicated by a diamond symbol. The numbers in the branches indicate the bootstrap values. The scale indicates nucleotide substitution per site. The VP7 gene nucleotide sequences of our study were submitted directly to GenBank and were assigned accession numbers from MK 685890 to MK 685896.

4.5.2 Analysis of the VP4 nucleotide sequences

Phylogenetic analysis based on deduced amino acid sequences of the VP4 gene showed that sequences varied by only 0 to 2.2% within the Burundian P [8] rotavirus strains. Rotavirus P[8] strains from this study and reference rotavirus strains from India, the Democratic Republic of Congo, South Africa and Mozambique exhibited similarities ranging from 98 to 100% at deduced amino acid level (GenBank accession numbers MF373733, KJ870781, KJ752342, and KP222858) (Figure 4.8).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1. RVA/BDI/CD02/G12P8																							
2. RVA/BDI/CD04/G1P8	0.022																						
3. RVA/BDI/CD05/G1P8	0.000	0.022																					
4. RVA/BDI/CD06/G12P8	0.000	0.022	0.000																				
5. RVA/BDI/CD07/G12P8	0.000	0.022	0.000	0.000																			
6. RVA/BDI/CD09/G12P8	0.000	0.022	0.000	0.000	0.000																		
7. RVA/BDI/CD10/G12P8	0.000	0.022	0.000	0.000	0.000	0.000																	
8. RVA/BDI/CD11/G12P8	0.000	0.022	0.000	0.000	0.000	0.000	0.000																
9. RVA/BDI/CD12/G1P8	0.000	0.022	0.000	0.000	0.000	0.000	0.000	0.000															
10. RVA/BDI/CD01/GXP8	0.000	0.022	0.000	0.000	0.000	0.000	0.000	0.000	0.000														
11. RVA/BDI/CN14/G1P8	0.018	0.004	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018													
12. RVA/BDI/CN15/G12P8	0.000	0.022	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.018												
13. RVA/BDI/CN17/G1P8	0.018	0.004	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.000	0.018											
14. RVA/BDI/CN19/G1P8	0.018	0.004	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.000	0.018	0.000										
15. RVA/BDI/CN20/GXP8	0.018	0.004	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.000	0.018	0.000	0.000									
16. RVA/BDI/CN21/G12P8	0.000	0.022	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.018	0.000	0.018	0.018	0.018								
17. RVA/BDI/CN22/G1P8	0.018	0.004	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.000	0.018	0.000	0.000	0.000	0.018							
18. KJ752342.1_RVA/Human-wt/ZAF/MRC-DPRU1191/2009/G12P8	0.000	0.022	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.018	0.000	0.018	0.018	0.018	0.000	0.018						
19. KP222858.1_RVA/Human-wt/MOZ/21140/2011/G12P8	0.004	0.027	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.022	0.004	0.022	0.022	0.022	0.004	0.022	0.004					
20. KJ870781.1_RVA/Human-wt/COD/KisB614/2008/G1P8	0.018	0.004	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.000	0.018	0.000	0.000	0.000	0.018	0.000	0.018	0.022				
21. MF373733.1_RVA/Hu-wt/DIB/RMRC-11-04-0586/2016/G1P8	0.000	0.022	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.018	0.000	0.018	0.018	0.018	0.000	0.018	0.000	0.004	0.018			
22. KJ870783.1_RVA/Human-wt/COD/KisB616/2008/G1P8	0.022	0.009	0.022	0.022	0.022	0.022	0.022	0.022	0.022	0.022	0.004	0.022	0.004	0.004	0.004	0.022	0.004	0.022	0.027	0.004	0.022		
23. JN849151.1_RVA/Human-wt/BEL/BE1520/2009/G1P8	0.080	0.075	0.080	0.080	0.080	0.080	0.080	0.080	0.080	0.080	0.071	0.080	0.071	0.071	0.071	0.080	0.071	0.080	0.084	0.071	0.080	0.075	
24. HG917355.1_RVA/Vaccine/USA/Rotarix-AROLA490AB/1988/G1P1A8	0.084	0.080	0.084	0.084	0.084	0.084	0.084	0.084	0.084	0.084	0.075	0.084	0.075	0.075	0.075	0.084	0.075	0.084	0.088	0.075	0.084	0.080	0.004

Figure 4.8: Degree of divergence between amino acid sequences of the VP4 protein of Burundian P [8] rotavirus strains, rotavirus vaccine-strain Rotarix and reference rotavirus strains from GenBank database.

A comparison between rotavirus P[6] strains from this study and reference rotavirus P[6] strains from the Democratic Republic of Congo, Mozambique, Zimbabwe, South Africa, and India showed VP4 amino acid similarity ranging from 95.6 to 99.6% (GenBank accession numbers KJ870892, KP222867, KJ753832, KJ752342 and JX878655) (Figure 4.9).

	1	2	3	4	5	6	7	8	9	10
1. RVA/BDI/CN16/2018/G1P6										
2. RVA/BDI/CN23/2018/G12P6	0.049									
3. KP222867.1_RVA/Human-wt/MOZ/21178/2011/G2P6	0.044	0.004								
4. KJ753719.1_RVA/Human-wt/ZAF/MRC-DPRU2344/2008/G2P6	0.044	0.004	0.000							
5. KJ752342.1_RVA/Human-wt/ZAF/MRC-DPRU1191/2009/G12P8	0.341	0.341	0.341	0.341						
6. KJ870892.1_RVA/Human-wt/COD/KisB554/2010/G8P6	0.044	0.004	0.000	0.000	0.341					
7. JX878655.2_RVA/Hu-wt/RUS/Omsk/O1299/2012/G2P6	0.009	0.040	0.035	0.035	0.341	0.035				
8. KJ870740.1_RVA/Human-wt/COD/KisB537/2009/G1P6	0.044	0.004	0.000	0.000	0.341	0.000	0.035			
9. KJ753832.1_RVA/Human-wt/ZWE/MRC-DPRU1158/G2G9P6	0.044	0.004	0.000	0.000	0.341	0.000	0.035	0.000		
10. HG917355.1_RVA/Vaccine/USA/Rotarix-AROLA490AB/1988/G1P1A8	0.323	0.319	0.319	0.319	0.084	0.319	0.323	0.319	0.319	

Figure 4.9: Degree of divergence between deduced amino acid sequences of the VP4 protein of Burundian P [6] rotavirus strains and reference rotavirus strains from GenBank database.

The Burundian P [8] rotavirus strains BDI/CD03, BDI/CN22, BDI/CD04, BDI/CN14, BDI/CN17, BDI/CN19 and BDI/CN20 were more closely related and exhibited very high amino acid similarity ranging from 99.6% to 100% to each other (Figure 4.10). They clustered very closely with the Congolese P [8] rotavirus strains COD/Kis602, COD/Kis614, COD/Kis616 and the South African P [8] rotavirus strain ZAF/MRC-DPRU1808 and exhibited amino acid similarities ranging from 99% to 100% to them.

The Burundian P [8] rotavirus strains formed another separate cluster including BDI/CN15, BDI/CN21, BDI/CD01, BDI/CD11, BDI/CD10, BDI/CD07, BDI/CD06,

BDI/CD05, BDI/CD02, BDI/CD08, BDI/CD09, the Indian P [8] rotavirus strain RVA/Hu-wt/DIB/RMRC-11-04-0586 and the rotavirus strain H125/Goa/2014. With a G12 specificity in almost all of them (Except the isolate BDI/CD05 which was G1), they showed a very close genetic relationship among them and the similarity within this cluster ranged from 99 to 100% at deduced amino acid level (Figure 4.10).

On the other hand, Burundian P [6] rotavirus strains formed two distinct clusters. The Burundian BDI/CN16/G1P [6] isolate clustered with the Russian RVA/Hu-wt/RUS/Omsk/01299/2012/G2P [6] and the BDI/CN23/G12P [6] isolate formed another monophyletic cluster with P [6] rotavirus strains from the Democratic Republic of Congo, Zimbabwe and South Africa. The two Burundian P [6] rotavirus strains, with different G-genotypes (G1 and G12) were distantly related with 95.1% similarity at deduced amino acid level (Figure 4.10).

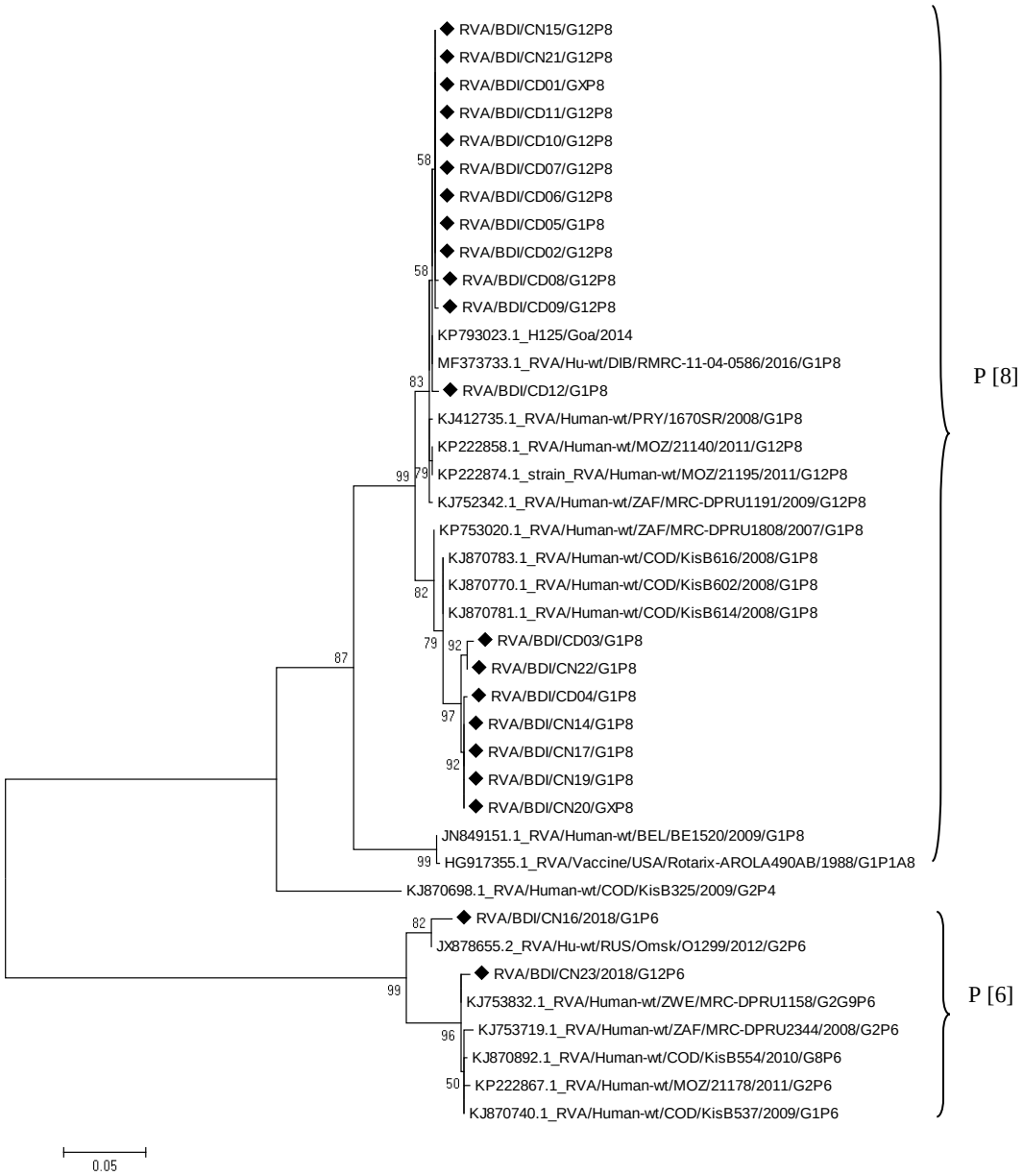

Figure 4.10: Phylogenetic tree based on the nucleotide sequences of the VP4 gene of rotavirus strains identified in Mutaho health district and Bujumbura municipality, Burundi, 2018.

Reference rotavirus strains are indicated by accession numbers while rotavirus strains from this study are indicated by a diamond symbol. The numbers in the branches indicate the bootstrap values. The scale indicates nucleotide substitution per site. The nucleotide sequences for VP4 gene of our study were submitted directly to GenBank and were assigned accession numbers from MK 685872 to MK 685896.

CHAPTER FIVE

DISCUSSION

5.1 Prevalence and seasonality of rotavirus infections.

This study showed that the overall prevalence of rotavirus infections was 6.2%. However, monthly detection rates varied significantly and showed a peak reaching 15.75% in July, the driest month of the year in Burundi (WBG, 2019). Rotavirus detection rate increased significantly as the level of precipitation went down.

This seasonal pattern of rotavirus infections could be attributed to the shortage in safe drinking water experienced in dry season (taps dry out). This situation expose people to drawing water from unsafe sources including rivers, with the risk to draw virus-laden debris since the level of water is dramatically reduced at that period. In fact, it is believed that rotavirus is spread predominantly through fecal-oral transmission, through water, person-to-person contact or contaminated environmental surfaces (Greenberg & Estes, 2009; Payne *et al*., 2011).

In addition, a number of authors suggested that rotavirus may also spread through the air (Levy, Hubbard, & Eisenberg, 2009). Dry conditions would tend to encourage the formation of virus-laden dust from fecally contaminated diapers, bedding and clothing. Also, small particles would tend to stay suspended in the air and then reach a susceptible individual (Brandt *et al*., 1982). However, this mode of transmission is still in theory (CDC, 2015). A similar seasonal pattern of rotavirus infections was described in Kenya, Zambia and Bangladesh (Levy *et al*., 2009).

The burden of rotavirus gastroenteritis among children under 5 years in post vaccination era highlighted by this study was also reported in other African countries (Agutu *et al*., 2017; McHaile *et al*., 2017). However, the overall prevalence of rotavirus infections observed in this study was lower than those reported in other

East African countries including Kenya which reported an overall prevalence ranging from 14.5% to 31.5% (Muendo et al., 2018; Raini et al., 2015; Agutu et al., 2017) and Tanzania with an overall prevalence ranging from 21.5% to 24.6% (McHaile *et al.*, 2017; Moyo *et al*., 2014).

Randomized trials demonstrated that Rotarix was effective in preventing severe rotavirus disease in African infants at 61.2% (Muendo *et al.*, 2018; Steele *et al*., 2012). Therefore, the efficacy of Rotarix coupled with a high vaccine coverage (greater than 90% in 2018) (WHO/UNICEF, 2019) might have resulted in a significant reduction in morbidity and mortality associated with rotavirus disease among Burundian infants and young children. A similar substantial reduction in rotavirus disease following the introduction of Rotarix in the national program of immunization was reported in 2015 South Africa (Magagula *et al*., 2015).

5.2 Association between the prevalence of rotavirus infections and socio-demographic characteristics of study participants.

The highest prevalence of rotavirus infections was observed in children aged 54 to 59 months and the lowest prevalence of rotavirus infections was observed in children younger than one month of age, children aged 30-35 months and those aged 48-53 months.

The highest prevalence of rotavirus infections was observed in children aged 54 to 59 months suggesting a high vulnerability of this age-group to rotavirus infections. Some factors including the malnutrition might have played a role in this increased vulnerability to rotavirus diarrhea. Evidence has shown that children with poor health and nutritional status are more vulnerable to serious infections like acute diarrhea and suffer multiple episodes every year (UNICEF & WHO, 2009). Actually, 48.8% of children aged 6 to 59 months were affected by chronic malnutrition, according to the Burundian MoH statistics (GoB/MoH, 2016). In addition, unlike the age-groups

30-35 months and 48-53 months, the highest proportion of children non-vaccinated against rotavirus was observed in this age-group (53 to 59 months). In fact, this was an age-group of children born in early 2014, a few months after Rotarix vaccine was launched in Burundi, which could explain the low rotavirus vaccine uptake in that age-group. Concerning children younger than one month of age, it is believed that children aged up to 3 months are generally protected by maternal antibodies acquired by trans-placental transfer (Greenberg & Estes, 2009). These findings are not consistent with those from studies conducted in Kenya, Uganda and Tanzania where the highest prevalence of rotavirus infections was observed in children under two years of age (Raini *et al.*, 2015; Nakawesi *et al.*, 2010; McHaile *et al.*, 2017).

Further, it emerged from this study that rotavirus was significantly more prevalent in Bujumbura Municipality than Mutaho health district. This could be attributed to difference in periods and duration of sample collection in these two areas. In Bujumbura Municipality, sample collection started later when dry season was imminent.

The findings from this study did not show a significant difference of rotavirus infections prevalence between vaccinated and non-vaccinated children. This could be attributed to a misclassification of the study participants into vaccinated and non-vaccinated groups. For instance, some parents/guardians to children younger than 1 month wrongly reported that their children were vaccinated against rotavirus while the first dose of Rotarix is generally given at 6 weeks of age. As a vaccination card that could evidence the parents/guardians answers with respect to the vaccination status of their children was not a requirement for children to be offered health care services, some parents did not carry it.

5.3 Rotavirus electropherotypes identified in Mutaho health district and Bujumbura municipality

The Polyacrylamide Gel Electrophoresis (PAGE) results revealed that only rotavirus long electropherotypes were circulating among children under five years presenting with acute diarrhea in Mutaho health district and the Municipality of Bujumbura. Contrary to findings from previous studies in Kenya, Zimbabwe and Ireland (Agutu *et al*., 2017; Halloran *et al*., 2000; Maruta *et al*., 2014), there were no short electropherotypes identified in this study.

Further, a relatively low diversity of long electropherotypes was observed in this study, in comparison with other countries like Italy, Nigeria, Ireland and Slovakia where greater numbers of long electropherotypes variations were reported (Cascio *et al*., 2001; Ayolabi, Ojo, & Armah, 2013; Halloran *et al*., 2000; Tietzova & Petrovicov, 2000). This little variation of electropherotypes could be attributed to the relatively low genetic diversity of rotavirus strains identified in this study whereby only two G genotypes i.e. G1 and G12 rotavirus strains were identified. However, the long electropherotypes identified in this study showed two distinct RNA patterns i.e. bbea and bbga which were found to be significantly associated with G1 and G12 genotypes respectively. Similar electrophoretic migration patterns for G1 rotavirus strains were described in previous studies conducted elsewhere (Cunliffe *et al*., 2009; Ndung'u *et al*., 2017).

Nevertheless, a number of studies revealed the absence of absolute correlation between electropherotypes and G-types as the long electrophoretic pattern bbea was found to be common in G1, G2, G3 and G4 rotavirus strains (Halloran *et al*., 2000; Tietzova & Petrovicov, 2000). In addition, the long e-type bbga associated with G12 rotavirus strains in our study was previously reported to be in association with G3 rotavirus strains in other studies (Cascio *et al*., 2001).

5.4 Rotavirus G and P types identified in Mutaho health district and Bujumbura municipality.

The G and P genotypes identified in our study sites were previously reported in the Democratic Republic of Congo, Tanzania, Kenya and South Africa (Heylen *et al*., 2014; Moyo *et al*., 2014; Ndung'u *et al*., 2017; Magagula *et al*., 2015).

In addition, the G/P genotype combination G1P[8] predominant in Bujumbura Municipality is recognized to be predominant worldwide and is globally responsible for 50-60% of group A rotavirus gastroenteritis among children (Halloran *et al.*, 2000; Moyo *et al*., 2014). This also suggested that rotavirus strains spread across different countries of the globe as a result of human and animal mobility. These genotypes include the emerging G12P [6], G12P [4], G12P [8] rotavirus strains and globally common rotavirus strains G1P [8] and G1P [6].

The genotype G12, identified first in 1987 in the Philippines (Taniguchi, Urasawa, Kobayashi, Gorziglia, & Urasawa, 1990) was detected in Burundi in this study, with considerable proportions (46.2%, 12/26). In this study, it was predominantly in association with P [8] but also P [6] and P [4] at lesser frequencies. Since 1998, a decade after their first detection in the Philippines, G12 strains have been identified in Asia, Europe, South America, and North America, suggesting their possible emergence worldwide (Rahman *et al*., 2007). In Africa, G12 rotavirus strains were detected in Ghana, Nigeria, Ivory Coast and the Democratic Republic of Congo (Heylen *et al*., 2014; Ndze *et al*., 2013). They were also reported in East African countries including Kenya, Tanzania and Uganda (Ndze *et al*., 2013). Of note, none of these countries reported a high prevalence of G12 rotavirus strains comparable to the one observed in this study. The high prevalence with which G12 rotavirus strains were detected and the great variability of P specificities found in its association in this study may be an indicator of the ongoing evolution of rotavirus strains especially through genetic re-assortment among different host specific rotavirus strains. In

addition, these findings suggest the constant spread of these rotavirus strains across the globe due to increasing mobility of humans and animals.

The G12 rotavirus strains were found to be significantly predominant in Mutaho health district than Bujumbura municipality. Mutaho health district is a rural area where people depend mainly on agriculture and livestock including pig farming for their livelihood. These activities and less organized pig farming in particular might have contributed to the high prevalence of G12 rotavirus strains in this area as pigs are believed to be a source of new rotavirus genotypes through a mechanism of genetic re-assortment.

It is believed that human G12 rotavirus strains result from evolution of porcine rotaviruses, especially through the exchange of genomic segments among the different host specific rotaviruses (Malik *et al*., 2014). This evolution may lead to the emergence of novel porcine rotaviruses having the capability to infect humans. This is consistent with findings from a study conducted in India which suggested that this emerging genotype might have a porcine origin in view of the degree of relatedness between human and porcine G12 rotavirus (Rahman *et al*., 2007). Porcine rotavirus A strains with G12 genotype for which comparative sequence analysis clustered with human G12 strains with maximum identities of 93.6% to 94.5% at deduced amino acid level were first reported in India in 2006.

These emerging rotavirus strains have the potential to challenge the efficacy of currently used rotavirus vaccines i.e. Rotarix (GSK Biologicals, Rixensart, Belgium) and RotaTeq (Merck & Co., Whitehouse Station, NJ, USA) as they share neither G nor P-type with these vaccines. The monovalent Rotarix vaccine currently used in Burundi comprises a G1P[8] human rotavirus strain while the pentavalent vaccine RotaTeq is a human-bovine reassortant vaccine comprising the genotypes G1, G2, G3, G4, G9 and P[8] on a bovine strain background (Cunliffe *et al*., 2009). The

efficacy of Rotarix against severe gastroenteritis caused by G12 rotavirus strains was estimated at only 51.5% (Steele *et al*., 2012).

5.5 Evolutionary relationships between rotavirus strains identified in Mutaho health district and Bujumbura municipality and reference rotavirus strains

Phylogenetic analyses showed that G1 rotavirus strains from this study were closely related to each other and to reference rotavirus strains from other countries including India, Togo, Mali and USA. These findings supports the idea of global spread of rotavirus strains as a result of human and animal mobility.

Interestingly, based on the VP7 encoding gene, G1P [8] rotavirus strains were distantly related to the rotavirus vaccine-strain Rotarix with only 88.8 to 92% similarity at deduced amino acid level. This degree of similarity is much lower than that reported recently in Tanzania (Moyo *et al*., 2014). This substantial and increasing degree of divergence suggests that G1P [8] rotavirus strains circulating in Burundi may have changed and keep changing over time through accumulated mutations making them different from original vaccine strains isolated a long time ago (Afrad *et al*., 2013). These results supported the idea that G1P [8] rotavirus strains circulating in Burundi may acquire the capability to escape from the protection provided by the vaccine Rotarix.

VP7 and VP4 nucleotide sequences analysis showed that Burundian P [8] rotavirus strains were combined with different G-types (G1 and G12) suggesting a recent genetic re-assortment involving the VP7 gene segment. This diversification of P [8] rotavirus strains has potential implications on the efficacy of rotavirus vaccines in use. Rotavirus P [8] strains associated with G1 and G12 genotypes were previously reported in the Democratic Republic of Congo (DRC), but in a pre-rotavirus vaccine era (Heylen *et al*., 2014).

They clustered very closely with the Congolese P [8] rotavirus strains COD/Kis602, COD/Kis614, COD/Kis616 and the South African P [8] rotavirus strain ZAF/MRC-DPRU1808 and exhibited amino acid similarities ranging from 99% to 100% to them. The clustering pattern (monophyletic cluster) with a very high degree of relatedness between Burundian and Congolese rotavirus strains based on their VP4 gene sequences indicated that rotavirus spread across the two countries, as a result of human and animal mobility.

Actually, human movements between Burundi and DRC were increased due to conflicts and insecurity hitting the eastern part of DRC. At the same time, the number of Burundians crossing the border towards DRC for business purposes is large. The DRC, a neighbor country of Burundi, launched a rotavirus vaccine very recently, in 2018, in some provinces of the country (GAVI, 2019).

5.6 Limitations of the study

The stool specimens analyzed in this study were collected and stored in Burundi from November 2017 and transported to Kenya in August 2018. This implies possible degradation of the specimens during transport and storage since screening results, on field, had shown higher rates of rotavirus positivity (12.5%, 81/646). Furthermore, during collection of socio-demographic data and data on the vaccination status, the vaccination card was not required to evidence the vaccination status reported by parents/guardians. This may have resulted in a social desirability bias. Moreover, different factors likely to influence the susceptibility of vaccinated children to rotavirus infections (nutritional status, completion of the doses of the vaccine, immunization status, and low weight at birth, etc) were not taken into consideration in the measure of the association between vaccination status and rotavirus positivity. Finally, the present study did not cover a 12-month period to give a full picture of seasonal variations of rotavirus infections.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This cross sectional study showed that the overall prevalence of rotavirus infections among children under five years presenting with acute diarrhea in Mutaho health district and the municipality of Bujumbura was lower than those previously reported in other countries of the East African region, which suggested a possible substantial reduction in rotavirus disease following the introduction of rotavirus vaccination in Burundi.

From this study, it emerged that only long electropherotypes were circulating in different sites of this study. However, two different long e-types i.e. bbea and bbga associated with G1 and G12 genotypes of rotavirus respectively were identified in this study.

Further molecular characterization revealed that G1P [8] and G12P [8] were the most common genotypes of rotavirus in different sites of this study. In addition, other emerging G12 rotavirus strains including G12P [6] and G12P [4] were detected albeit at relatively lower frequencies. Emerging G12 human rotavirus strains were significantly predominant in Mutaho health district, a rural area where most people depend on agriculture and livestock for their livelihood. These emerging rotavirus strains raise public health concerns as they share neither G nor P genotypes with currently used rotavirus vaccines and hence have the potential to challenge the efficacy of these vaccines.

Phylogenetic analysis of the VP7 and VP4 nucleotide sequences of rotavirus strains from this study revealed an increasing degree of divergence between G1P [8] rotavirus strains and the rotavirus vaccine-strain Rotarix in Burundi. This has

important implications for the efficacy of rotavirus vaccine i.e. Rotarix, currently used in Burundi.

Children aged 53 to 59 months were the most affected by rotavirus diarrhea, which may be associated with malnutrition affecting over 50% of Burundian children and the low rotavirus vaccine uptake observed in this age-group. Furthermore, a seasonal pattern was found in the occurrence of rotavirus infections, with a sharp increase in dry season, particularly in July which is the driest month of the year in Burundi. The shortage in safe drinking water experienced in Burundi during dry season (GoB/UNDP, 2007) may have a role in the increased rotavirus transmission.

6.2 Recommendations

In light of the above conclusions from this study, the following recommendations were made:

1. To initiate and maintain a continuous rotavirus strain surveillance in Burundi to monitor trends in the occurrence of these prevailing and potentially emerging new rotavirus strains. In this respect, the Ministry of Public Health and the Fight against AIDS should establish a laboratory-based surveillance of rotavirus strains circulating in that country.

2. To use PAGE as an alternative method for molecular characterization of rotavirus strains circulating in Burundi, since it could allow to predict the G-genotypes of rotavirus circulating in that country.

3. The genotype G12 should be incorporated into the rotavirus vaccine-strains in order to tackle the rotavirus disease associated with this emerging genotype of growing importance worldwide.

4. Further research studies should be conducted to determine the genetic relationships between human and animal G12 rotavirus strains and the factors underlying the seasonal pattern of rotavirus infections in Burundi.

5. To investigate the factors predisposing children under five years, in particular those aged 53 to 59 months, to rotavirus infections and take action accordingly.

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APPENDICES

Appendix I: Consent form (Parent/Guardian)

CONSENT FORM (English version)

Identification No. …… **Interviewer: _____**

Project title: Prevalence and genetic characterization of rotavirus infections among children under five years in Mutaho health district, Gitega Province and the municipality of Bujumbura, Burundi

PRINCIPAL INVESTIGATOR

Cassien NDUWIMANA, MSc (Med Virology)-JKUAT, ITROMID

RESEARCH SUPERVISORS

1. Dr. Raphael Lihana, Ph.D.- KEMRI
2. Dr. Eddy Okoth Odari, Ph.D.- JKUAT

INTRODUCTION (for principal investigator)

My name is Cassien NDUWIMANA, a Masters student at Jomo Kenyatta University of Agriculture and Technology's Institute of Tropical Medicine and Infectious Diseases- KEMRI in Kenya. I'm undertaking a research study on rotavirus in children under five years in Mutaho health district. Therefore, I am kindly requesting you to take part in this research study. Your participation in the study will help us to identify rotavirus strains causing diarrhea in children under five years in Mutaho health district and the municipality of Bujumbura. The purpose of this form is to make sure that you have all information about the study before you decide to participate in it. Remember your participation is voluntary**.** The information you

provide will be held in strict confidence and will be used for the purpose of this study.

INTRODUCTION (for other personnel other than the principal investigator)

My name is _____. I wish to introduce you to this study undertaken by Cassien NDUWIMANA, a Masters student at Jomo Kenyatta University of Agriculture and Technology, Institute of Tropical Medicine and Infectious Diseases. He is undertaking a research study on rotavirus in under five children, in Mutaho health district and the municipality of Bujumbura. Your participation in the study will help us to identify rotavirus strains circulating in Mutaho health district. The purpose of this form is to make sure that you have all information about the study before you decide to join the study. Remember your participation is voluntary**.** The information you provide will be held in strict confidence and will be used for the purpose of this study.

Why is the study being done?

The MoH reports have suggested high incidence rates of dehydrating diarrhea in this Health District, particularly in 2013, 2014 and 2015. This research aims at characterizing the strains of rotavirus causing diarrhea in children under 5 years of age in Mutaho health district, Gitega Province and in the municipality of Bujumbura. Results of this study will help to improve services and programs aimed at preventing rotavirus infections. The results of this study will be given to the MoH authorities who will take the necessary action depending on the outcomes. You will benefit from the free test and the results will be given back to your physician (or nurse) for management. In addition, the study will have a long term benefit to the general population as the results will be instrumental in policy formulation with respect to rotavirus vaccine introduction and use within the health sector of the government of Burundi.

What is involved in the study?

The study involves collecting stool samples from children attending Mutaho district hospital, Mutoyi hospital, Bugendana, Rwisabi and Mutaho and Mutoyi health centers in Mutaho health district; Mirango, Kamenge, Kinama, Bwiza-Jabe and Buterere health centers and the Prince Regent Charles hospital in the municipality of Bujumbura, presenting with diarrhea. You will be provided with a sterile container within which to collect the stool during the clinic visit. Ward patients will be given containers in which stools will be collected in the morning. Positive samples will be identified using rapid diagnostic test and thereafter transported to KEMRI in Kenya for further analyses.

CONSENT FOR STORAGE AND TRANSPORTATION OF SAMPLES

YES ☐ **NO** ☐

Please check one box only

How long will the study take?

The study is projected to take eight months.

What are the risks of the study?

This study does not involve any invasive procedure. You will collect the stools in the containers in the normal defecation process. This will therefore not subject you to any pain.

Is there any benefit to taking part in the study?

Apart from the free test for rotavirus (upon consent from subjects), you will not have any further direct benefits. However, the results of the study will be shared with the MoH authorities and other relevant authorities who will take the necessary course of

action. As mentioned in the introduction, the findings from this study will be instrumental in policy formulation with respect to rotavirus vaccine introduction and use within the health sector of the government of Burundi.

What about confidentiality?

All the information obtained will be strictly confidential and data will be password protected. Only the Principal investigator, the research supervisors and if need be, appointed authority from the KEMRI or the National Ethical Review Committee will be able to access the information. Your children will remain anonymous during and after the study, being identified only by the Personal Identification Numbers (PIN) assigned to him. The results obtained will be made available to the health care providers only with your consent.

How many people participate in the study?

A total of 456 children are expected to participate in the study.

What are the costs?

You do not pay anything for your children to participate in this study.

Summary of your rights as a participant in a research study

This study is absolutely voluntary, you will be free to withdraw from the study at any point and will not be penalized in any way and you are not also waiving any of your legal rights by signing this informed consent document.

Contact information

The following persons will be available for contact in the event of any research related questions, comments or complaints.

Principal Investigator: Cassien NDUWIMANA, JKUAT.
Telephone: (+257) 76 349 611(Burundi)
(+257) 79 017 974 (Burundi)
(+254) 789 581 685 (Kenya)
Email: cassimbare@gmail.com

And in case of complaints regarding rights of participation, one can always contact:

BURUNDI/NERC, THE SECRETARY: Telephone: …….
Mobile:

PERMISSION

I have read the Consent Form and conditions of this project. All my questions have been answered and I hereby give my voluntary consent.

Consent for stool collection: **Yes** ☐ **No** ☐
Please check one box only
Signature of parent/guardian …….. Date ……
Printed name of parent/guardian ……...
Name and signature of the witness _____sign _____
* Only in case the subject is not literate and a thumb is used. *Must not be the person collecting specimen
Signature of person obtaining consent_____ Date_____
Printed name of person obtaining consent _____
(Must be the principal investigator or individual who has been designated in the checklist to obtain consent)
Signature of principal investigator _____DATE_____
(Affirming subject's eligibility for the study and that informed consent has been obtained).

Appendix II: Consent Form explanation in Kirundi

URUPAPURO RWO KUGARAGAZA UBUSHAKE (Umuvyeyi/Umurezi)

Inomero_____ Uwugirisha ikiganiro_____

Umutwe w'icigwa: Urugero rw'indwara zo gucibwamwo n'ubushakashatsi kuri kamere mpererekanwa y'imigera ya Rotavirisi itera izo ndwara mubana b'imyaka iri munsi y'itanu mukarere k'ubuvuzi ka Mutaho, Intara ya Gitega, no mugisagara ca Bujumbura, mu Burundi.

Umushakashatsi mukuru

Kasiyano NDUWIMANA, Umunyeshure kuri Kaminuza y'uburimyi n'ubuhinga yitiriwe Jomo Kenyatta (JKUAT)

Abamuyoboye m'ubushakashatsi

Muganga Raphael Lihana, PhD-KEMRI
Muganga Eddy Okoth Odari, PhD-JKUAT

Intangamarara (Mugihe ishikirijwe na nyene kugira ubushakashatsi)

Nitwa Kasiyano NDUWIMANA, nkaba nd'umunyeshure ariko akurikirana ibijanye n'imigera y'indwara yitwa amavirisi, kuri Kaminuza y'Uburimyi n'Ubuhinga yitiriwe Jomo Kenyatta, Igisata ITROMID, i Nairobi muri Kenya. Ndiko nkora ubushakashatsi ku migera yitwa Rotavirisi mubana bari munsi y'imyaka itanu, mu karere k'ubuvuzi ka Mutaho. Ni kubw'iyo mpamvu nagomba kubasaba ngo muterere muri ubu bushakashatsi. Ukugira uruhara kwanyu muri ubu bushakashatsi bizodufasha kumenya amoko y'umugera Rotavirisi atera indwara zo gucibwamwo mu bana b'imyaka iri munsi y'itanu muri aka Karere k'Ubuvuzi ka Mutaho no

mugisagara ca Bujumbura. Intumbero y'uru rupapuro n'ukugira ntihagire ico twokwibagira kubamenyesha mu bijanye n'iki cigwa imbere y'uko mugiramwo uruhara.

Kugira uruhara muri ikicigwa nt'agahato kajamwo. Ivyo muza kudushikiriza bizoguma mw'ibanga ntangere kandi bizokoreshwa muri iki cigwa gusa.

Intangamarara (Mugihe ishikirijwe n'uwundi atari nyene kugira ubushakashatsi)

Nitwa…….. Nifuza kubinjiza muri ubu bushakashatsi bwateguwe na Kasiyano NDUWIMANA, umunyeshure ariko akurikirana ibijanye n'imigera y'indwara. yitwa amavirisi kuri Kaminuza y'Uburimyi n'Ubuhinga yitiriwe Jomo Kenyatta, Igisata ITROMID, i Nairobi muri Kenya. Ariko akora ubushakashatsi ku migera yitwa Rotavirisi mubana bari munsi y'imyaka itanu, mu karere k'ubuvuzi ka Mutaho. Ni kubw'iyo mpamvu nagomba kubasaba ngo muterere muri ubu bushakashatsi. Ukugira uruhara kwanyu muri ubu bushakashatsi bizodufasha kumenya amoko y'umugera Rotavirisi atera indwara zo gucibwamwo mu bana b'imyaka iri munsi y'itanu muri aka Karere k'Ubuvuzi ka Mutaho. Intumbero y'uru rupapuro n'ukugira ntihagire ico twokwibagira kubamenyesha mu bijanye n'iki cigwa imbere y'uko mugiramwo uruhara. Kugira uruhara muri ikicigwa nt'agahato kajamwo. Ivyo muza kudushikiriza bizoguma mw'ibanga ntangere kandi bizokoreshwa muri iki cigwa gusa.

Kubera iki cigwa categuwe gukorwa?

Ama raporo y'Ubushikiranganji bw'Amagara y'abantu n'Ukugwanya Ikiza ruhonyanganda SIDA yaragaragaje urugero runini rw'indwara zo gucibwamwo zunyurwa n'umwumira muri aka karere k'ubuvuzi, na cane mu myaka ya 2013, 2014 na 2015. Ubu bushakashatsi bufise intumbero yo kumenya amoko y'imigera yo

m’umurwi wa Rotavirisi zitera izo ndwara mu bana b’imyaka iri munsi y’itanu muri aka Karere k’ubuvuzi ka Mutaho, Intara ya Gitega no mugisagara ca Bujumbura. Ibizova muri ubu bushakashatsi bizofasha guteza imbere ibikorwa vy’ibisata bijejwe kugwanya ingwara zo gucibwamwo zitewe na Rotavirisi. Bizoshikirizwa abarongoye Ubushikirangaji bw’amagara y’abantu no kurwanya ikiza rohanyanganda SIDA bo bazofata ingingo zibereye.

Umwana wanyu araza kugirirwa igipimo c’umusarani k’ubuntu. Inyishu z’igipimo ziraza gushikirizwa umuganga wanyu kugira zimufashe kuvura neza umwana wanyu. Vyongeye, ibizova muri iki cigwa bizogira ikimazi muri kazoza ku benegihugu kuko bizomurikira abajejwe gutunganya amategeko ajanye no kwinjiza no gukoresha incanco zo gukinga Rotavirisi mu gisata c’amagara y’abantu mu Burundi.

N’igiki gikenewe muri ikicigwa?

Muri iki cigwa, tuzokwegeranya imisarani itanzwe n’abana baje kwivuza canke bagwariye mu Bitaro vy’akarere ka Mutaho, ibitaro vy’i Mutoyi, amavuriro ya Bugendana, Rwisabi na Mutaho na Mutoyi mukarere k’ubuvuzi ka Mutaho; amavuriro ya Mirango, Kamenge, Kinama, Bwiza-Jabe na Buterere hamwe n’ibitaro vyitiriwe umuganwa Karori mu gisagara ca Bujumbura, bagwaye ingwara yo gucibwamwo. Turabaha agacupa mushiramwo umusarani w’umwana wanyu uyu munsi nyene. Abari mu bitaro nabo nyene turakabaha maze bazoshiremwo umusarani w’umwana bagwaje mu gitondo. Turapima dukoresheje ubuhinga bubangutse maze abo tuza gusanga bafise uyo mugera, tuzoca tubitwara mu Kigo c’Ubushakashatsi KEMRI muri Kenya kugira tubandanye ibipimo.

Kutwemerera kubika no gutwara umusarani w’igipimo

EGO ☐ OYA ☐

Musabwe kwuzuza agakiramende kamwe rudende

Iki cigwa kizotwara igihe kingana gute?

Bitegekanijwe ko iki cigwa gitwara amezi umunani.

N'ibihe vyoshobora kugushikira muri iki cigwa utari uvyiteze?

Muri iki cigwa, nta buhinga busaba kwinjiza ivyuma m'umubiri w'umurwayi burimwo. Musabwa gutora umusarani w'umwana wanyu mugihe kama co kwituma kwiwe. Nta kibabaza na kimwe rero kirimwo.

Hoba harimwo akunguko k'uwagiye muri ikicigwa?

Uretse igipimo kitarihwa c'umugera Rotavirisi, nt'akandi kunguko bwite uwugiye muri iki cigwa aronka. Ariko, nk'uko twabivuze, ibizova muri iki cigwa bizomenyeshwa abarongoye ubushikiranganji bw'amagara y'abantu n'ukurwanya ikiza ruhonyanganda SIDA kugira bibafashe gufata ingingo zibereye. Ibizovamwo bizokwunganira mugutunganya ibijanye no kwinjiza no gukoresha incanco zo gukinga umugera wa Rotavirisi mu gisata c'amagara y'abantu mu Burundi.

Mbega ibijanye no kuzigama amabanga y'umurwayi muri iki cirwa bimeze gute?

Ivyo mutubwira vyose bijanye n'iki cigwa bizozigamwa mw'ibanga ntangere kandi n'ivyo tuzotora mu bushakashatsi bizokingirwa hakoreshejwe "igitigiri kabanga". Umushakashatsi mukuru wenyene canke bibaye ngombwa, abamuyoboye mu bushakashatsi, abategetsi b'ikigo kijejwe ubushakashatsi KEMRI canke abo m'Umurwi w'Igihugu ujejwe iyubahirizwa ry'amategeko m'Ubushakashatsi nibo bazoshobora kumenya ayo makuru. Umwana wanyu nta zina na rimwe tuzomwandikako kiretse inomero imuranga aza kuba yahawe. Inyishu z'igipimo ziza guhabwa umuganga wanyu k'uruhusha rwanyu.

Iki cigwa kizojamwo abana bangahe?

Abana bangana amajana abiri na mirongo ibiri n'umunani (456) nibo bitezwe kugira uruhara muri ikicigwa.

Bisaba kuriha amafaranga angahe?

Kuja muri iki cigwa ni k'ubuntu.

Incamake y'uburenganzira bw'uwugize uruhara muri ikicigwa

Kugira uruhara muri iki cigwa ni k'ubushake bw'umwe wese, n'uburenganzira bwanyu kubivamwo umwanya uwariwo wose mubishatse kandi nkurikizi n'imwe. Vyongeye, ntaburenganzira na bumwe muhabwa n'amategeko mutakaza mugusinya uru rupapuro rwo kugaragaza ubushake bwo kugira uruhara mu cigwa.

Imirongo mwodutorako

Aba bantu bakurikira bazokwakira ibibazo vyose bijanye n'ubu bushakashatsi, abitwara, n'abafise ivyo baterera.

Umushakashatsi mukuru: Kasiyano NDUWIMANA, JKUAT.

Terefone: (+257) 76 349 611(in Burundi)

(+257) 79 017 974 (in Burundi)

(+254) 789 581 685 (in Kenya)

Email: cassimbare@gmail.com

K'uwufise ivyo yarenganijwe bijanye n'uburenganzira bwiwe bw'uwagize uruhara mu cigwa, ashobora guterefona kuri:

BURUNDI/NERC: UMUNYAMABANGA

Telephone: …….

Mobile: ……

UBURENGANZIRA

Nasomye uru rupapuro rwo kugaragaza ubushake n'ibisabwa muri uyu mugambi. Naronse inyishu ku bibazo vyose nabajije none ndiyemeje kugira uruhara muri iki cigwa.

Kwiyemeza kuzana umusarani w'umwana: ego ☐ oya ☐

Musabwe kwuzuza mugakiramende kamwe

Igikumu c'umuvyeyi/umurezi_____itariki _____

Amazina y'umuvyeyi/umurezi _____

Amazina n'igikumu vy'icabona*_____Igikumu (signature)_____

* Mugihe umuvyeyi canke umurezi ataciye kuntebe y'ishure. * Nt'ategerezwa kuba uwuhawe urupapuro rwo kugaragaza ubushake bwo kugira uruhara mucigwa.

Igikumu vy'uwakira abaja mucigwa _____ itariki _____

Amazina y'uwakiriye abitavye icigwa_____

Ategerezwa kuba umushakashatsi mukuru canke uwundi yagenywe nawe akaba ari no k'urutonde rw'abakira abitavye icigwa.

Igikumu c'umushakashatsi mukuru _____ itariki _____

(Vyemeza ko uwitavye icigwa abikwiriye kandi yagaragaje ubushake bwo kucitaba).

Appendix III: Questionnaire for demographic and clinical information collection

Questionnaire for demographic and clinical information collection
1. Identification No. …….. The patient code will be supplemented with the first three letters of the first and the last name of the patient and the first four letters of the health facility in this order: Initials of the health facility/Patient code/initial of the patient's name **e.g. Muta/C12-321/NA-J**
2. Age : ……..months Date of birth:……/……/……..
3. Gender: Male ☐ Female: ☐
4. Department: Inpatient ☐ Outpatient ☐
5. Rotavirus vaccination status: Vaccinated ☐ Not vaccinated ☐ Unascertained ☐
6. Frequency of diarrhea: ……/day
7. Duration of the diarrhea:……..days Diarrhea is defined, according to WHO guideline, as the occurrence of three or more, loose, liquid, or watery stools within 24 hour period
8. Consistency of the stool: Watery ☐ Mucoid ☐ Bloody ☐ Other ☐
9. Name of the person collecting the information and the stool specimen: **…….**
10. Date:……/……../…….. Time:……..

Appendix IV: Ethical clearance letter

Comité National d'Ethique pour la protection des êtres humains participants à la recherche biomédicale et comportementale

Bujumbura, le 14 octobre 2017

A Monsieur Cassien NDUWIMANA

Investigateur Principal

Objet : Décisions du Comité National d'Ethique

Monsieur,

Le Comité National d'Ethique a analysé votre projet de recherche « Prévalence et caractérisation génétique des infections à Rotavirus chez les enfants de moins de cinq ans dans le district sanitaire de Mutaho, province de Gitega, Burundi ».

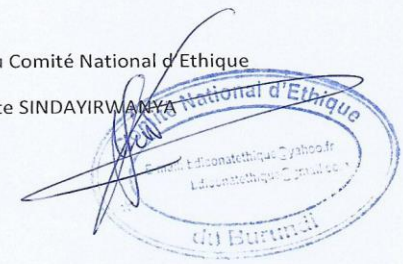
Après avoir analysé les aspects éthiques du projet conformément aux réglementations internationales dans ce domaine, le Comité National d'Ethique du Burundi a approuvé le projet. Cette approbation est valable pour une année renouvelable du 14 octobre 2017 au 13 octobre 2018.

Toutefois, si des modifications du protocole devraient y être apportées, une demande préalable devra être adressée au Comité National d'Ethique avant application.

Veuillez agréer, Monsieur, l'expression de ma considération distinguée.

Le Président du Comité National d'Ethique

Pr. Jean Baptiste SINDAYIRWANYA



Appendix V: Abstract of the published data

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Prevalence and Genetic Characterization of Rotavirus Infections Among Children Under Five Years in Mutaho Health District, Gitega Province and Bujumbura Municipality, Burundi

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Abstract

Rotavirus is the leading cause of severe diarrhea in children under five years worldwide. It is ranked as a priority for vaccine. In Burundi, vaccine against rotavirus was implemented in 2013. The impact of recent rotavirus vaccination on morbidities in Burundi is not well established. Moreover, no study has been carried out to document the genetic diversity of rotavirus strains circulating in Burundi. This cross-sectional health facility-based study aimed at determining the prevalence and molecular characteristics of rotavirus infections among children under five years of age in Mutaho Health District and the Municipality of Bujumbura, in Burundi. Stool specimens were collected from children presenting with acute diarrhea. These specimens were tested for rotavirus antigen using Diagnostar® rapid test kit. Positive stool samples were confirmed at the Kenya Medical Research Institute (KEMRI) by ELISA. Positive confirmed samples underwent RT-PCR, G and P genotyping by multiplex semi-nested PCR using a cocktail of type specific primers or by sequencing. A total of 646 participants were enrolled in this study. The overall prevalence of rotavirus was 6.2% (40/646) with 4.0% (16/400) in Mutaho health district and 9.7% (24/246) in the Municipality of Bujumbura. Rotavirus detection rate tended to increase as the level of precipitation went down, showing a significant negative association between the two variables. (OR = 15.2; P = 0.0001). In addition, rotavirus detection rate was higher in Bujumbura Municipality than in Mutaho health district (OR = 2.6; P = 0.005). Two G genotypes were identified, G1 the predominating G genotype accounted for 53.8% (14/26) followed by G12 (46.2%, 12/26). The prevalence of the genotype G1 of Group A rotavirus was significantly higher in Bujumbura Municipality than in Mutaho health district while G12 predominated in Mutaho health district (OR = 7.33; P = 0.026). Rotavirus strains from pigs might have contributed to the high prevalence of human G12 rotavirus in that area. Three different P types were identified P[8] the most common, followed by P[6] and P[4]. The most common G/P combination genotype was G1P[8] which accounted for 45.5% of all rotavirus genotypes identified, followed by G12 P [8] (41.0%), G1P [6] (4.5%), G12 P [6] (4.5%) and G12 P [4] (4.5%). The emergence of G12 rotavirus strains which share neither G nor P genotypes with currently used rotavirus vaccines raises public health concerns as they have the potential to challenge their efficacy. Therefore, we recommend to initiate and maintain a continuous rotavirus strain surveillance in Burundi so as to monitor trends in the occurrence of these prevailing and potentially emerging new strains.

Keywords: Rotavirus, diarrhea, genetic diversity, prevalence, Mutaho, Bujumbura, children
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Appendix VI: List of 7 and VP4 nucleotide sequences of Burundian group A rotavirus strains and their GenBank accession numbers

>MK 685871/RVA/CD02_Bdi/G12P [8]/2018/VP4

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