WHOLE-GENOME ANALYSES OF ROTAVIRUS STRAINS CIRCULATING PRE- AND POST- ROTATEQ™ VACCINE INTRODUCTION IN RWANDA

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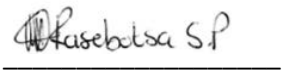
DECLARATION

"I, Sebotsana Paula Rasebotsa, declare that the Master's Degree research dissertation or interrelated, publishable manuscripts/published articles, or coursework Master's Degree mini-dissertation that I herewith submit for the Master's Degree qualification Masters of Medical Sciences with specialisation in Virology at the University of the Free State is my independent work and that I have not previously submitted it for a qualification at another institution of higher education."



Sebotsana Paula Rasebotsa

This dissertation is dedicated to my family, especially my mother, Mrs. Moshupologo Hilda Rasebotsa for being my pillar of strength

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LIST OF ABBREVIATIONS

A-type	Interferon Antagonist
aa	Amino Acid
AICc	Akaike Information Criterion
ATM	Amplicon Tagment Mix
BLASTn	Nucleotide Basic Local Alignment Search Tool
C-type	Core Shell Protein
cDNA	Complementary Deoxyribonucleic Acid
CTL	Cytotoxic T-lymphocytes
ddH_20	Double-distilled Water
DLP	Double-layered Particle
dNTPs	Deoxyribonucleotide Triphosphates
DPRU	Diarrheal Pathogens Research Unit
dsRNA	Double-stranded Ribonucleic acid
E-type	Enterotoxin
EB	Elution Buffer
EIA	Enzyme Immuno-Assay
ELISA	Enzyme-Linked immunosorbent Assay
EM	Electron Microscopy
ER	Endoplasmic Reticulum
G-type	Glycoprotein
GAVI	Global Alliance for Vaccines and Immunizations
H-type	Phosphoprotein
HSREC	Health Sciences Research Ethics Committee
HT1	Hybridization Buffer 1
I-type	Intermediate Protein
IFIH1	Interferon-induced Helicase C Domain-containing Protein 1
ISGF3	Interferon-stimulated Gene Factor 3
JAK/STAT	Janus Kinase/signal Transducer and Activators of Transcription
$LiCl_2$	Lithium Chloride
M-type	Methyltransferase
MEGA 6	Molecular Evolutionary Genetics Analysis 6
mRNA	Messenger RNA
N-type	NTPase
NaOH	Sodium Hydroxide
NF-kB	Nuclear Factor Kappa-light-chain-enhancer of Activated B cells
NGS	Next-generation Sequencing
NPM	Nextera PCR Master Mix

NSP	Non-structural Proteins
NT	Neutralize Tagment Buffer
nt	Nucleotide
OPV	Oral Poliovirus Vaccines
ORF	Open Reading Frame
ORS	Oral Rehydration Solutions
ORT	Oral Rehydration Therapy
P-type	Protease Sensitive
PCR	Polymerase Chain Reaction
PGM	Ion Torrent Personal Genome Machine
R-type	Viral RNA-dependent RNA Polymerase
RdRp	RNA-dependent RNA Polymerase
RRL-SA	Rotavirus Reference Laboratory in South Africa
RSB	Resuspension Buffer
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RVA	Rotavirus Group A
RVB	Rotavirus Group B
RVC	Rotavirus Group C
RVD	Rotavirus Group D
RVE	Rotavirus Group E
RVF	Rotavirus Group F
RVG	Rotavirus Group G
RVH	Rotavirus Group H
RVI	Rotavirus Group I
RVJ	Rotavirus Group J
ssRNA	Single-stranded RNA
T-type	Translation Enhancer
TD	Tagment DNA Buffer
ToRs	Terms of References
TSA	Technical Service Agreement
UFS	University of the Free State
UFS-NGS Unit	University of the Free State-Next Generation Sequencing Unit
vdG1P[8]	Vaccine-derived G1P[8]
VP	Viral Proteins
WHO	World Health Organization
WHO/AFRO	World Health Organization Regional Office for Africa

ABSTRACT

Children living in developing countries are constantly faced with the burden of diarrheal infections that account for over 1.6 million death cases globally. Rotavirus group A (RVA) has been identified as one of the viruses implicated in most viral-induced diarrhoeal infections in children less than five years worldwide. In Rwanda, over 3500 RVA related mortality cases were reported yearly prior to the implementation of the RotaTeq® vaccine in 2012 to overcome this burden, which led to a significant decrease in rotavirus infections. Africa has a huge diversity of rotavirus strains compared to other developed continents especially Europe and North America, thus requiring a deeper understanding of this phenomenon. This study aimed at characterizing all the 11-segments of RVA strains circulating in Rwanda pre- and post-vaccine introduction as part of the World Health Organization (WHO) supported African rotavirus pilot surveillance program. The study was based on 158 rotavirus positive samples that were collected from children presenting symptoms associated with rotavirus infection between 2011 and 2016. The rotavirus double-stranded ribonucleic acid (dsRNA) was extracted from the viral particles and converted into complementary deoxyribonucleic acid (cDNA) prior to library preparation for whole-genome sequencing with an Illumina MiSeq platform. Several bioinformatics tools were utilized to construct phylogenetic trees and the proteins structures. From the sequenced samples, 36 samples were identified as G1P[8] strains, and five samples were reassortant strains. Ten G1P[8] strains were identified pre-vaccine introduction while 26 were identified post-vaccine introduction. Thirty-five of the G1P[8] strains expressed pure Wa-like genome constellations, while one of the strains that was identified in 2012 exhibited a genome constellation typical of a RotaTeq® vaccine strain. On the other hand, the five reassortant strains were identified post-vaccine introduction between 2013-2015. Whole-genome analysis revealed that the G4P[4], G9P[4] and one G12P[8] reassortant strains exhibited both the Wa-like and the DS-1-like genome constellations while two G12P[8] strains had all the three genogroup constellations. Furthermore, the phylogenetic analysis of most of the G1P[8] strains revealed that they segregated according to their vaccination status; strains identified pre-vaccine introduction clustered together while post-vaccine strains also formed a separate cluster. The five reassortant strains were closely related to human RVA strains in all the gene segments and RotaTeq® vaccine strains in the VP1, VP2, NSP2, NSP4, and NSP5 gene segments. Analysis of the neutralization epitopes and cytotoxic T-lymphocytes (CTL) of the G1P[8] strains revealed multiple amino acid substitutions, with some changes influencing the change in polarity thus deemed to be radical in nature. A similar trend was also observed in the reassortant strains, with 27 amino acid substitutions in the VP7 epitope region and only three substitutions in the VP4 epitope region. Changes observed in these epitope regions have the potential of generating vaccine-escape mutants that may undermine the effectiveness of the rotavirus vaccine with time. Whole-genome

sequencing has proven to provide information that could have been missed when looking only at the outer capsid proteins. It is thus important to continue conducting rotavirus whole-genome studies to unpack the hidden information behind the huge diversity of rotavirus strains in African countries such as Rwanda.

Keywords: rotavirus, Rwanda, whole-genome characterization, reassortment, epitope region, RotaTeq®, Rotarix®, diarrhoea, vaccine-derived strain, genome constellation

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CHAPTER ONE: INTRODUCTION

1.1. Background

Rotaviruses are among the leading cause of virus-induced mortality in children less than 5 years globally, with more than half of these deaths occurring in sub-Saharan Africa (Tate et al., 2016; Troeger et al., 2018a). In 2016, more than 1.6 million global mortality was attributed to diarrhoeal diseases (Troeger et al., 2018a). Rotavirus is commonly implicated in gastroenteritis, a medical condition characterized by inflammation of the stomach and intestines (Leung et al., 2005). The virus can easily be transmitted through the faecal-oral route, causing symptoms such as diarrhoea, malaise, vomiting, and fever (Leung and Robson, 2007). The symptoms usually start two days after infection, with an acute onset of fever and vomiting, followed by frequent watery stool, and the infection may last for three to eight days (Leung and Robson, 2007; Parashar et al., 2013). It is also associated with significant dehydration that could lead to death in children due to their high vulnerability to electrolyte imbalance resulting from diarrhoea and vomiting (Leung and Robson, 2007).

Rotavirus is regarded as "the democratic virus" due to its ability to infect populations across the social-economic status (Parashar et al., 2013). Although rotavirus infection has been implicated in high mortality in children, vaccination against the virus has proven to be the most reliable prevention measure accompanied by improved sanitation (Fischer et al., 2007; Tate et al., 2016). Furthermore, zinc supplements, oral rehydration therapy (ORT), and intravenous fluids have also been recommended as effective treatments for diarrhoea by the WHO (Santosham et al., 2010). Despite this recommendation, such treatments are not holistically accessible by several low-income countries including sub-Saharan African countries. For instance, in 2013 alone, four countries (Democratic Republic of the Congo, India, Nigeria, and Pakistan) accounted for half of all rotavirus deaths in the world (Tate et al., 2016). The lack of timely access to quality health care, frequent concurrent infections, malnutrition, and climatic factors contributes to the high rate of rotavirus mortality in third world countries (Elliott, 2007; Parashar et al., 2009; Wazny et al., 2013).

Four rotavirus vaccines (Rotarix®, RotaTeq®, Rotavac® and Rotasil®) are currently pre-qualified by WHO for prevention of rotavirus infections (WHO, 2018). The efficacy of rotavirus vaccines differs between countries with different socio-economic statuses. High-income countries that introduced Rotarix® in their national immunization program reported 84% vaccine effectiveness while low- and middle-income countries reported efficacy of 57-75% (Jonesteller et al., 2017). Furthermore, high-income countries that

introduced RotaTeq® vaccine in their national immunization program reported 90% vaccine effectiveness while low-income countries documented efficacy of 45% (Jonesteller et al., 2017). Low-income countries have been suspected to experience lower efficacy of the vaccines due to factors such as maternal antibodies, malnutrition, and co-administration of rotavirus vaccines and oral poliovirus vaccines (OPV) (Moon et al., 2010). Rotavirus vaccines are more effective when administered before natural immunity is acquired through natural infections; therefore, administering the vaccine after natural rotavirus infection may also affect the effectiveness of the rotavirus vaccines (Patel et al., 2009). The most prevalent rotavirus genotype combinations detected in Africa between 2006 and 2015 were G1P[8], G2P[4], G9P[8], G2P[6], G12P[8], and G3P[6] (Mwenda et al., 2010; Seheri et al., 2018, 2014).

The establishment of the Global Rotavirus Surveillance Network in 2008 has created a platform to constantly monitor changes that may have been influenced by the introduction of rotavirus vaccines and to understand better the dynamics of rotavirus strain diversity in different settings all over the world (Aliabadi et al., 2019). The mandate of the network is to perform exceptional diagnostic tests (Reverse transcription-polymerase chain reaction, antigen detection assays, polyacrylamide gel electrophoresis, and electron microscopy) for diarrhoea induced by rotavirus and report on the strains circulating worldwide (Parashar et al., 2013; WHO, 2017). This study forms part of the WHO pilot program to undertake rotavirus surveillance at the whole-genome level in Africa, in collaboration with the University of the Free State-Next Generation Sequencing (UFS-NGS) Unit, with a particular focus on rotavirus strains circulating in Rwanda.

1.2. Problem Statement

Over the years, rotavirus surveillance studies in Africa were conducted through conventional genotyping of the two outer capsid proteins (VP4 and VP7), thus providing an information gap with regards to the whole-genome constellation of the virus (Nyaga et al., 2020). The first African rotavirus surveillance study was conducted in June 2006 through December 2008, established in 12 sites in 10 African countries (Mwenda et al., 2010). Ghana, Kenya, Uganda, and Zambia were the initial countries included in the surveillance study in 2006, while Cameroon, Tanzania, Zimbabwe, and Ethiopia constituted the four additional countries included in the study in 2007. Togo and Mauritius were subsequently included in the study in 2008. The study revealed a considerable diversity of strains and a higher burden of rotavirus disease in African countries. Untypable strains were also documented in the study, and most of them were

suspected to originate from animals (Mwenda et al., 2010). Similarly, Seheri and co-workers reported on a huge diversity of rotavirus strains circulating in African countries with a high percentage of untypable strains and mixed genotypes between 2007 and 2011 (Seheri et al., 2014).

In a six-year rotavirus surveillance study conducted by Seheri *et al*. (2018), G1P[8], G2P[4] and G9P[8] rotavirus strains were detected through conventional genotyping as the prevalent cause of rotavirus induced acute gastroenteritis in young children less than five years in 15 Eastern and Southern African countries pre- and post-vaccine introduction. Furthermore, Botswana, Brazil, Malawi, South Africa, and Thailand have reported on the changes in strain circulation pre- and post- rotavirus vaccine introduction (Carvalho-Costa et al., 2019; Gómez et al., 2014; Jere et al., 2018; Luchs et al., 2019; Mokomane et al., 2019; Page et al., 2018; Tacharoenmuang et al., 2016). Interestingly, temporal strain variation was also reported in Rwanda after RotaTeq® introduction with G8P[4] circulating at a high proportion in 2013, followed by G4P[8] and G12P[8] in 2014, which were replaced by G1P[8] strains in 2015 (Seheri et al., 2018). With the dynamic changes in circulating strains in most countries globally, including Rwanda, it is imperative to study the overall genotype and evolution of the circulating strains before and after vaccine rollout.

1.3. Aim

The aim of this study was to determine the vaccine impact on the circulating rotavirus strains on a whole-genome level pre- and post- RotaTeq® vaccine introduction in Rwanda.

1.4. Hypothesis

The introduction of RotaTeq® vaccine in Rwanda influenced the change in circulating strains post-vaccine introduction.

1.5. Objectives

The aim of the study was achieved through these specific objectives:

- Synthesis of cDNA for whole-genome sequencing of rotavirus A positive specimens from Rwanda.
- Determination of distinctive phylogenetic features pre- and post-vaccination from the whole-genome sequence data from Rwanda.
- Exploration of the probable evidence of rotavirus vaccine pressure from Rwanda between 2011 and 2016.

1.6. Dissertation organization

The dissertation consists of contributions in the form of reprints of a published article and an article under internal review for publication. Specifically, chapter one is the introductory chapter which outlines the general background of the project based on the research proposal, as well as the aim and objective of the study. Chapter two provides information on the literature review based on the comprehensive summary of published research on rotavirus. Chapter three outlines the comprehensive methodology utilized to address the study objectives that could not be placed under publication chapters. The subsequent chapters (Chapter four and five) are presented to address the study objectives using two peer-reviewed manuscripts with an introduction, literature review, methodology, general discussion, and concluding remarks. Chapter four has been published in *Scientific* Reports journal, while chapter five is under internal review for publication in *Frontiers* in Microbiology journal. The final chapter of the dissertation (Chapter six) highlights the general discussion, conclusion of the project as well as limitations and probable recommendations. A reference list is provided at the end of the dissertation, followed by the appendices.

CHAPTER TWO: LITERATURE REVIEW

2.1. Background

Rotaviruses were first identified in humans in 1973 by Ruth Bishop and colleagues in the duodenal mucosa of children under five years (Bishop et al., 1973). Their viral particles have a wheel-like structure and an icosahedral shape with rotational symmetry as seen under an electron microscope, hence the name rotavirus originates from a Latin word "rota," meaning wheel (Flewett, 1983). Rotavirus is a genus within the family *Reoviridae* associated with acute gastroenteritis in both animals and humans, grouped into ten distinct antigenic Groups A-J, based on the distinct antigenic and genetic differences on the VP6 protein (Bányai et al., 2017; Estes and Kapikian, 2007; Matthijnssens et al., 2011; Mihalov-Kovács et al., 2015). Rotavirus Groups A, B (RVB), C (RVC), and H (RVH) are implicated in infecting humans (Bányai et al., 2012; Parashar et al., 2006). Rotavirus Groups D-G and I are only found in animals. Rotavirus Group B can infect humans, cattle, sheep, pigs, deer, and rats, while RVC has been detected in pigs, humans, cattle, dogs, and ferrets (Estes and Kapikian, 2007). Groups D (RVD) , F (RVF) and G (RVG) rotaviruses exclusively affect birds, while Group E (RVE) was detected only in pigs, albeit with no sequence available (Estes and Kapikian, 2007; Johne et al., 2011; Martella et al., 2010; Stucker et al., 2015). Group H rotavirus affects humans and pigs (Molinari et al., 2014; Nagashima et al., 2008; Nyaga et al., 2016), and Group I (RVI) rotavirus affects dogs (Mihalov-Kovács et al., 2015). Group J (RVJ) has also been recently identified in bats and currently undergoing ratification (Bányai et al., 2017). Of the rotavirus groups, RVA is of most medical importance and the most prevalent rotavirus group in humans, especially in children less than five years of age (Estes and Kapikian, 2007). RVA has also been sporadically detected in numerous species, including monkeys, giraffes, racoons, camels, and mice (Evans, 1984; Jere et al., 2014; Matthijnssens et al., 2011; Mulherin et al., 2008).

In 2016, diarrhoea was identified as the eighth leading cause of mortality responsible for over 1.6 million death globally (Naghavi et al., 2017; Troeger et al., 2018a). Diarrhoeal diseases can be attributed to over 20 different viral, bacterial, and parasitic pathogens (Glass et al., 2014). A considerable proportion of diarrheal induced death cases in children under five years are attributable to rotavirus, norovirus, calicivirus, and *Escherichia coli* infections (Lanata et al., 2013). In sub-Saharan Africa, approximately 104 733 of those death cases were attributed to RVA in children under five years (Troeger et al., 2018b). Infections due to RVA are primarily established in the small intestine, where surface tissues are destroyed and nutrient absorption is prevented thus resulting in diarrhoea (Leung et al., 2005; Leung and Robson,

2007). Rotaviruses have an incubation period of one to three days with an estimated infectious dose of 100–1000 viral particles (Bernstein, 2009; Ward et al., 1986). Symptoms associated with infected infants are fever, vomiting, abdominal pain, and watery diarrhoea that may last three to seven days (Crawford et al., 2017; Glass et al., 2014; Leung and Robson, 2007). Immunocompromised children often present a prolonged virus shedding period for a year or more, which may constitute a potent reservoir for infection (Glass et al., 2014). The most common cause of rotavirus mortality is dehydration and electrolyte imbalance resulting in cardiovascular failure (Glass et al., 2014). In addition to gastroenteritis, RVA infection has been associated with hepatic abscess, respiratory infections, seizures and pneumatosis intestinalis (Capitanio and Greenberg, 1991; Grunow et al., 1985; Lynch et al., 2001; Zheng et al., 1991). The route of transmission for RVA is primarily through the faecal-oral route influenced by poor hygiene practises and can survive at ambient temperatures for long periods (Abad et al., 1994; Estes and Kapikian, 2007; Parashar et al., 2013; Ansari et al., 1991).

The seasonality of rotavirus infections varies in numerous countries, reflecting the difference in climate conditions (Mwenda et al., 2010). In Ghana, increased rotavirus infections are usually observed in dry, cool months (January – February), while Uganda reports less distinct seasonal peaks due to the equatorial weather all year round (Bwogi et al., 2016; Enweronu-Laryea et al., 2014; Mwenda et al., 2010). Furthermore, studies report that in Morocco, rotavirus infections peak in cold months (October - December), while South Africa usually reports the rotavirus seasonal peak in winter, between April and August (Benhafid et al., 2013, 2012; Mwenda et al., 2010; Page, 2006). In Rwanda, the RVA infections are most prevalent in the dry season (July) due to limited water supply and poor hygiene practices (Uwimana et al., 2015). Although rotaviruses are generally species-specific, evidence of zoonotic transmission has, however, been documented (Cook et al., 2004; Maringa et al., 2020). The segmented nature of RVA makes them prone to reassortment events during coinfection with two or more strains, thus influencing the diversity of the virus (Nyaga et al., 2015).

2.2. Rotavirus structure

Rotaviruses are non-enveloped dsRNA viruses with a segmented genome classified by size, ranging from 667 bp to 3302 bp (Figure 2.1) (Estes and Kapikian, 2007; Estes and Cohen, 1989). The segments encode for six structural viral proteins (VP); VP1-VP4, VP6, and VP7, and five sometimes six non-structural proteins (NSP) (NSP1-NSP5/6) forming 11 segments (Table 2.1). The mature viral particles, of approximately 100 nm in diameter, are made up of three-layered icosahedral capsids; outer, middle, and inner capsid layers (Estes and Greenberg, 2013).

The outer capsid layer incorporates the VP7 (Glycoprotein [G-type]) and spike-like projections of VP4 (Protease Sensitive [P-type]) (Estes and Kapikian, 2007; Estes and Cohen, 1989). The VP7 is highly immunogenic, induces the formation of neutralizing antibodies, and essential in viral attachment to the host cell (Estes and Cohen, 1989). The VP4 is also immunogenic and plays a major role in attachment and cellular penetration (Jayaram et al., 2004; Svensson et al., 1987). In the presence of trypsin, VP4 splits into VP5* and VP8*, which increases the viral infectivity and penetration of the virus into the cell (Estes and Cohen, 1989). The middle capsid layer, which is entirely made up of VP6 (Intermediate Protein [I-type]), is highly antigenic and often the target of the serological diagnosis when determining the rotavirus sero/genogroups A-J (Bányai et al., 2017; Estes and Kapikian, 2007; Estes and Cohen, 1989; Matthijnssens et al., 2011; Mihalov-Kovács et al., 2015). The VP2 (Core Shell Protein [C-type]) encases the VP1 (Viral RNA-dependent RNA Polymerase [R-type]) and the VP3 (Methyltransferase [M-type]) forming the inner capsid layer which encloses the nucleic acid material (Estes and Kapikian, 2007; Estes and Cohen, 1989; Gentsch et al., 2005; Greenberg and Estes, 2009). The NSP is generally essential for replication and morphogenesis. The NSP1 (Interferon Antagonist [A-type]), is an RNA-binding protein that blocks interferon response, and NSP2 (NTPase [N-type]) is involved in RNA packaging. The NSP3 (Translation Enhancer [T-type]) is responsible for shutting down the cellular protein synthesis while NSP4 (Enterotoxin [E-type]) induces diarrhoea. The NSP5/NSP6 (Phosphoprotein [H-type]) are single-stranded RNA (ssRNA) and dsRNA binding modulators of NSP2 (Estes and Cohen, 1989).

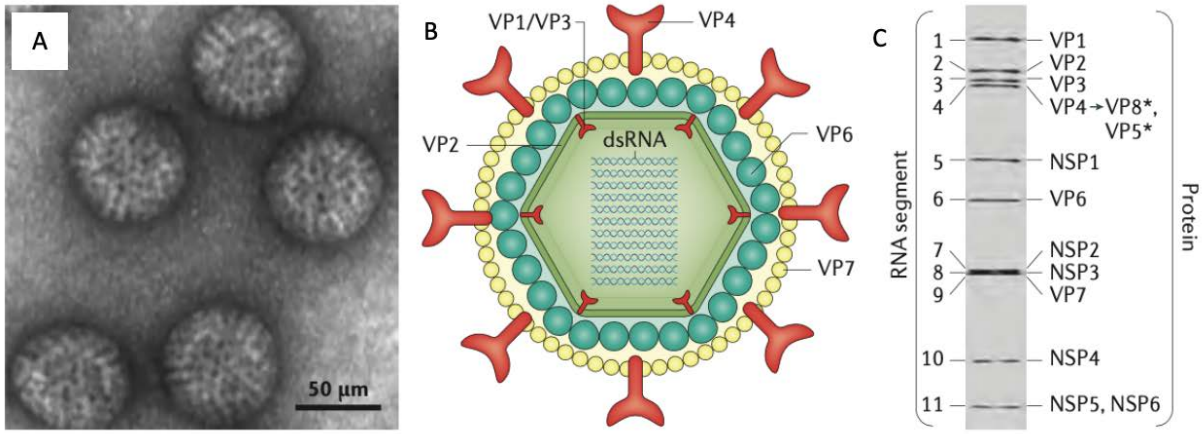


Figure 2.13: Rotavirus structure and the 11 gene segments. **A)** Rotavirus viral particles visualized by electron microscopy. **B)** The triple-layered structure of rotavirus representing the dsRNA viral genome, the outer capsid proteins (VP7 and VP4), the middle capsid layer (VP6), and the inner capsid layer (VP2 enclosing the VP1 and VP3). **C)** The electrophoretic separation of the 11 RNA segment along with their gene-protein assignments. Adapted with permission (Appendix B) (Crawford et al., 2017).

Table 2.4 Properties of the rotavirus proteins along with their descriptions and location in the three-layered icosahedral capsids.

Protein	Gene Segment	Size (bp)	Location	Description
VP1	1	3302	Inner capsid layer	Viral RNA-dependent RNA Polymerase [R-type]
VP2	2	2684, 2717	Inner capsid layer	Core shell Protein [C-type]
VP3	3	2591	Inner capsid layer	Methyltransferase [M-type]
VP4	4	2359	Outer capsid layer	Protease Sensitive [P-type]
NSP1	5	1563, 1567	Non-structural	Interferon Antagonist [A-type]
VP6	6	1356	Middle capsid layer	Intermediate Protein [I-type]
NSP3	7	1064, 1074	Non-structural	Translation enhancer [T-type]
NSP2	8	1059	Non-structural	NTPase [N-type]
VP7	9	1062	Outer capsid layer	Glycoprotein [G-type]
NSP4	10	750, 751	Non-structural	Enterotoxin [E-type]
NSP5/NSP6	11	664, 821	Non-structural	Phosphoprotein [H-type]

The 11-genome segments grouped into structural (orange) and non-structural (green) proteins. VP: Viral Protein, NSP: Non-structural Proteins, bp: base pairs (Adapted from Estes and Greenberg, 2013).

2.3. Diagnosis and genome classification

Over the years, the outer capsid proteins (VP7 and VP4) have been used for binary classification of rotavirus strains into G (Glycoprotein) and P (Protease-sensitive) genotypes, respectively (Estes and Kapikian, 2007). Initially, RVA was diagnosed using electron microscopy (EM), which proved to be tedious, expensive, time consuming, and required a high level of expertise (Anderson and Weber, 2004). Improved diagnostic methods introduced over the years to detect and characterize RVA strains were antigenic-based immunoassays (Enzyme-linked immunosorbent Assay [ELISA] and Enzyme Immuno-Assay [EIA]), RNA-RNA hybridization, Reverse Transcription Polymerase Chain Reaction (RT-PCR), real-time or quantitative qPCR, Sanger sequencing and next-generation sequencing (NGS) (Anderson and Weber, 2004; Gentsch et al., 1992; Pang et al., 2004; Wilde et al., 1991). An extended schematic nomenclature for classification of rotavirus, the genome is Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, which encodes for VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 gene segments (x indicates the genotype number) was endorsed for the whole-genome classification of rotaviruses in 2008 (Matthijnssens et al., 2008; Matthijnssens et al., 2011).

Three genogroup constellations have been established for RVA strains. The Wa-like (G1-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1) and DS-1- like (G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2) are the major constellations, while the AU-1-like (G3-P[9]-I3-R3-C3-M3-A3-N3-T3-E3-H3) is a minor genogroup. Most strains that possess the Wa-like constellation are from porcine origin, while DS-1-like strains are from the bovine origin and the AU-1-like strains are mostly of canine and feline origin (Matthijnssens et al., 2008). Presently, at least 36 G, 51 P, 26 I, 22 R, 20 C, 20 M, 31 A, 22 N, 22 T, 27 E, and 22 H genotypes of humans and various animal rotavirus species have been assigned by the Rotavirus Classification Working Group (RCWG, 2020). Some genotypes are endemic and spread rapidly across human populations like the G1P[8] and the G2P[4], while others are sporadically detected in humans, mostly those that have evolved as a result of several mechanisms of genetic diversity, other factors such as atypical genotypes, mixed or coinfections and animal strains with zoonotic potential (Bányai et al., 2012; Maringa et al., 2020; Mwangi et al., 2020; Nyaga et al., 2015, 2014; Strydom et al., 2019a, 2019b). Genotype G3 has been reported to have the widest host-range in contrast to other genotypes such as G13-G27 and P[16]-P[37], which have been observed in cattle, pigs, and avian species with limited detection (Abe et al., 2010; Matthijnssens et al., 2011).

2.4. Replication cycle

Viruses interact with the host cell at all stages of replication (cell entry to cell exit) and these interactions are crucial for the production of new viruses (Randall and Goodbourn, 2008). Although hosts have evolved a defence mechanism against pathogens, viruses have also evolved strategies to avoid host immune responses. Rotavirus infection is established through the attachment of different glycan receptors on the host epithelial cells via the VP4's VP8* domain (Figure 2.2) (Crawford et al., 2017). Viral entry is mediated after the initial binding, the VP7 and the VP4's VP5* domain thus interacting with numerous co-receptors at the lipid raft. Depending on the rotavirus strain, different pathways (clathrin-independent, clathrin-dependent, and caveolin-independent endocrytic pathways) have been proposed as models that internalize the virus.

The intracellular calcium concentration is essential for regulating replication, morphogenesis, and pathogenesis (Bugarčić and Taylor, 2006). Reduced calcium concentration in the endosome prompts the dissociation of the outer capsid layer, resulting in the release of the VP2 and VP6 proteins which are transcriptionally active double-layered particle (DLP) into the cytoplasm where transcription and translation takes place (Crawford et al., 2017). The NSP2 and NSP5 interact to form viroplasm, where RNA synthesis and translation occurs using viral messenger RNA (mRNA) (Ramani et al., 2016; Saxena et al., 2016). Furthermore, RNA is packaged into the new DLPs. The newly formed DLP binds to NSP4, which serves as an intracellular receptor, and the DLP buds into the endoplasmic reticulum (ER) (Crawford et al., 2017). The NSP4 is essential in increasing the cytoplasmic calcium level required for virus replication. Viral particle maturation occurs after the VP4 and VP7 proteins assemble on the DLP to form a triple layered particle in the ER. The progeny are eventually released from the cell through cell lysis or Golgi-independent non-classical vesicular transport into the intestinal lumen (Trask et al., 2012).

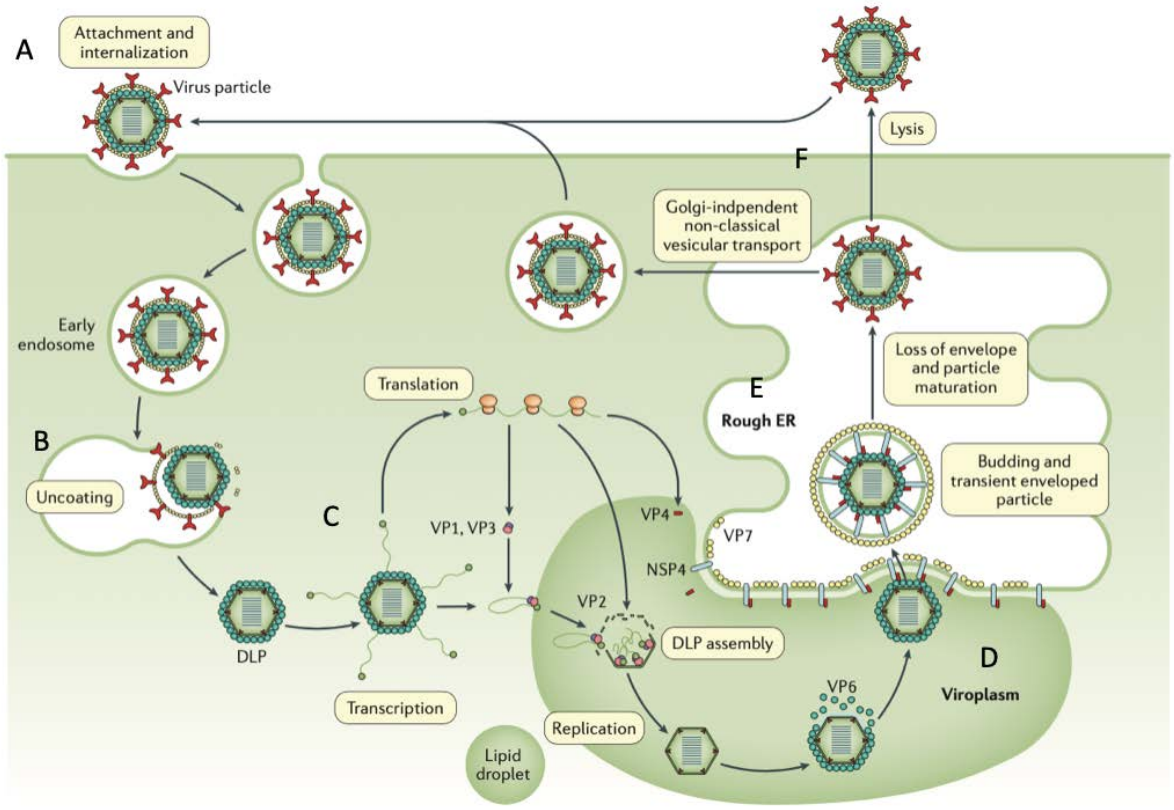


Figure 2.14: The rotavirus replication cycle. **A)** Rotavirus particle attaches to the cell and establish infection. It is internalized and the endosome is formed. **B)** Uncoating of the virus is initiated by low calcium levels thus releasing a transcriptionally active double-layered particle. **C)** Transcription and translation take place to produce the 11 proteins. **D)** Rotavirus replication occurs in the viroplasm followed by the assembly of the double-layered particle, which simultaneously packages the dsRNA. **E)** The double-layered particle buds into the endoplasmic reticulum where the viral particle matures after the addition of VP7 and VP4 protein. **F)** After maturation, the progeny are released through cell lysis or Golgi-independent non-classical vascular transport. Adapted with permission (Appendix B) (Crawford et al., 2017).

2.5. Immune response

Rotavirus-induced immune response in humans is not entirely understood; however, data from animal models are available (Desselberger and Huppertz, 2011; Franco et al., 2006). Cellular and humoral immune responses are reported to be involved in acquiring immunity against rotavirus infection (Offit, 1996). Rotavirus antigens are recognized by pattern recognition receptors (Adenosine triphosphate-dependent RNA helicase DDX58 and interferon-induced helicase C domain-containing protein 1 [IFIH1]) in enterocytes or cells of the immune system (microphages, dendritic cells or adaptive B cells and T cells) (Broquet et al., 2011; Offit, 1996). The rotavirus-specific B cells and CTL are stimulated and directed to the VP6 protein inhibiting the viral transcription process (Aiyegbo et al., 2013). Pattern recognition receptors, interferons and proinflammatory cytokines are responsible for initiating the type I and type III interferon response mediating the clearance of rotaviruses by the innate immune response.

The recognition of rotavirus antigens by the pattern recognition receptors influences the assembly of signalling complexes by ligand-activated sensors (López et al., 2016). This process ultimately promotes the activation of the antiviral program. The signalling complexes triggers the activation of host transcription factors (interferon 3, interferon 7, and Nuclear Factor kappa-light-chain-enhancer of activated B cells [NF-kB]) and their translocation to the nucleus where type I and type III interferon, proinflammatory molecules, and cytokines responses are initiated (Lin et al., 2016; López et al., 2016). Type I and type III interferons are thus recognized by interferon receptors present on the surface of neighbouring cells, ultimately activating the Janus kinase/signal transducer and activators of transcription (JAK/STAT) signalling pathway (López et al., 2016). The STAT1 and STAT2 proteins are phosphorylated and interact with the interferon regulatory factor 9 protein thus forming a heterotrimeric complex interferon-stimulated gene factor 3 (ISGF3). The ISGF3 complex is translocated to the nucleus leading to downstream transcription and expression of interferon-stimulated genes. The products from the interferon-stimulated genes subsequently inhibit the viral infection and alters other cellular functions. Rotaviruses are also reported to have several features that influence a poor innate immune response, such as the inhibition of the signalling pathways by NSP1 or other rotavirus proteins when activated by recognition receptors (Crawford et al., 2017; Holloway et al., 2014; López et al., 2016).

2.6. Rotavirus evolutionary mechanisms

Rotaviruses are constantly evolving through numerous evolutionary mechanisms (Taniguchi and Urasawa, 1995). The error prone RNA-dependent RNA polymerase (RdRp) is implicated in influencing genetic drifts (generating spontaneous sequential point mutations) within a viral population, especially for dsRNA viruses like rotaviruses. It has been reported that a progeny rotavirus genome will contain at least one mutation in the genome that differentiates it from the parent strain (Blackhall et al., 1996). The same RdRp replicates each gene. However, different rates of mutations are established due to different immune or host-selection pressures. The outer capsid proteins are suspected of evolving more rapidly than those of internal structural proteins because they are targets for host-neutralization antibody response (Taniguchi and Urasawa, 1995). Genomic rearrangement is commonly observed in rotavirus strains excreted by chronically infected immunocompromised children and animals (Pedley et al., 1984). It can also be generated in cell culture through serial passages of rotavirus strains at a high multiplicity of infections (Alam et al., 2008; Patton et al., 2001). The rearranged genes are usually involved in head-to-tail duplication downstream of the open reading frame (ORF) and rarely occur within the ORF (Patton et al., 2006; Taniguchi et al., 1996). This has been observed in RNA segments coding for NSP1, NSP3-NSP5, and VP6 (Desselberger, 1996; Taniguchi et al., 1996).

Genetic recombination in viruses occurs when there is coinfection in the same host cell by two different parent strains, resulting in a progeny containing genes from both parent strains. A limited number of recombination events have been reported within the RVA (Maringa et al., 2020; Parra et al., 2004; Phan et al., 2007). Genomic reassortment in rotaviruses occurs when a single cell is infected with several distinct but compatible rotavirus strains, also known as genetic shift (Mwangi et al., 2020; Nyaga et al., 2013; Ramig and Ward, 1991). Over the years, natural reassortment between human and animal strains has been frequently reported, also known as zoonotic transmission. The zoonotic transmission has been observed at a higher frequency in developing countries because of humans and domestic animals living in close proximity (Cook et al., 2004; Glass et al., 2006; Maringa et al., 2020; Moon et al., 2016; Naylor et al., 2015). Reassortment between rotavirus strains of different groups has not been detected (Ramig and Ward, 1991; Taniguchi and Urasawa, 1995). However, the NSP1 gene of avian group A strain clusters together with strains in the NSP1 of group D, thus suggesting a possible reassortment may have occurred between the two groups (Taniguchi and Urasawa, 1995; Trojnar et al., 2010).

2.7. Prevention and treatment

Children under five years were prone to severe RVA disease presentation with exposure to multiple rotavirus infections in their first two to three years of life before widespread vaccine introduction in 2006 (Velázquez et al., 1996). Primary rotavirus infections are generally more severe than subsequent infections that are usually mild or asymptomatic (Bishop et al., 1983; Ward, 2008). Children presenting signs of dehydration due to rotavirus induced diarrhoea are usually treated with zinc supplements, ORT, and intravenous fluids (Crawford et al., 2017). However, the best prevention method is the use of rotavirus vaccines. Several rotavirus vaccines have thus been developed over the years to ease the rotavirus disease burden (Table 2.2).

Table 5.2 Basic features and administration recommendations of currently licensed rotavirus vaccines.

Product	Manufacturer	Composition	Doses	Formulation/storage
Globally licensed				
RotaTeq®	Merck & Co. Inc., USA	G1, G2, G3, G4, P[8] and G6P[5]	3 doses (6, 10 and 32 weeks)	Liquid 2-8 °C or below -20 °C for 24 months
Rotarix®	GlaxoSmithKline (GSK) Biologicals, Belgium	G1P[8]	2 doses (6 and 24 weeks)	Liquid 2-8 °C or below -20 °C for 36 months
Rotavac®	Bharat Biotech International Limited India, India	G9P[11]	3 doses (6, 10 and 14 weeks)	Liquid frozen 2-8 for 7 months or °C -20 °C long term
Rotasil®	Serum Institute of India, India	G1, G2, G3, G4, G9 and G6P[5]	3 doses (6, 10 and 14 weeks)	Lyophilized < 25 °C for 30 months or < 40 °C for 18 months
Nationally licensed				
Rotavin-M1	POPLYVAC, Centre for Research and Production of Vaccines and Biologicals, Vietnam	G1P[8]	2 doses (6 and 12 weeks)	Liquid frozen 2-8 °C for 2 moths or -20 °C for 24 months
Lanzhou lamb rotavirus vaccine	Lanzhou Institute of Biological Products Co., Ltd., China	G10P[15]	1 dose (6 weeks to 3 years)	Liquid

(Adapted from Burke et al., 2019)

2.7.1. WHO prequalified vaccines

The first rotavirus vaccine introduced to the national immunization program of the United States in 1998 was RotaShield® (Wyeth Laboratories, U.S), a rhesus-human reassortant vaccine (CDC, 2011). However, the vaccine was discontinued from the market because it was suspected to cause intussusception and bloody stools in children (CDC, 1999; Glass et al., 2005). In 2006, the WHO pre-qualified the use of two live attenuated oral vaccines, Rotarix® (RV1, GlaxoSmithKline Biologicals, Belgium) and RotaTeq® (RV5, Merck & Co. Inc., USA). Rotarix® and RotaTeq® were introduced in the immunization program of many countries including African countries like Botswana, Ghana, Malawi, Rwanda, South Africa and Zambia by the end of 2013 (ROTA Council, 2016). In addition to these two vaccines, India has recently introduced two indigenously developed vaccines, Rotavac® (nHRV, Bharat Biotech International Limited India, India) and Rotasil® (BRV-PV, Serum Institute of India, India) (WHO, 2018). The estimated efficacies of both the indigenous Indian vaccines are comparable to that of Rotarix® and RotaTeq® when administered in low-income countries (Armah et al., 2010; Zaman et al., 2010). For several low-income nationals, the implementation of the rotavirus vaccine is supported by the Global Alliance for Vaccines and Immunizations (GAVI), the Vaccine alliance. In 2019, 46 countries received GAVI support for rotavirus vaccine introduction out of 73 countries that are GAVI eligible (IVAC, 2019).

RotaTeq® is a pentavalent human-bovine reassortant vaccine administered as three oral doses at 6, 10, and 32 weeks (Heaton and Ciarlet, 2007; Matthijnssens et al., 2010). This vaccine consists of four human reassortant strains expressed on the VP7 protein (G1, G2, G3, or G4) and a bovine rotavirus strain on the VP4 protein (P7[5]). The fifth reassortment was derived from the VP7 protein (G6) of a bovine rotavirus strain and the VP4 protein (P1A[8]) from a human rotavirus strain. Rotarix® is a monovalent live attenuated human G1P[8] rotavirus strain (RIX4414) given as two oral doses, administered at 6 and 24 weeks (GSK, 2019; Ward and Bernstein, 2009). Post vaccine introduction, rotavirus related hospitalization declined among children less than five years and demonstrated herd immunity among adults above the age bracket to receive the vaccine (Patel et al., 2009). Specifically, since the global introduction of these two oral vaccines globally, rotavirus hospitalization of children less than five years has drastically declined from about 527 000 mortality rate in 2000 to approximately 215 000 in 2013 and it further decreased to 128 500 deaths in 2016 (Armah et al., 2010; Begue and Perrin, 2010; Troeger et al., 2018b). Both Rotarix®

and RotaTeq® vaccines have been reported to confer homotypic and heterotypic protection against multiple RVA strains (Glass et al., 2006; Ward and Bernstein, 2009).

Similarly, the WHO also prequalified the use of Rotavac® and Rotasil® in the national immunization program of India in 2018. Rotavac® is a monovalent neonatal human attenuated G9P[11] rotavirus vaccine administered at 6, 10, and 14 weeks (Bharat Biotech, 2019). It has been reported to have a higher efficacy as compared to other rotavirus vaccines in high mortality settings and the infectivity/immunogenicity of Rotavac® is enhanced when it interacts with breast milk (Bharat Biotech, 2019). In contrast, Rotasil® is a bovine-human reassortant live attenuated lyophilized rotavirus vaccine consisting of human G1, G2, G3, G4, G9, and 10 genes from the UK bovine rotavirus G6P[5], administered at 6, 10, and 14 weeks (Kapikian et al., 2005; Zade et al., 2014). It is the first thermostable (-20 °C to 42 °C) rotavirus vaccine, unlike RotaTeq®, Rotarix® and Rotasil® which require an uninterrupted cold chain (2-8 °C or below -20 °C) during storage and transportation (GSK, 2019; Merck, 2008; Naik et al., 2017). Maintaining a cold chain in developing countries is a considerable challenge; thus Rotasil® offers a promising avenue to simplify vaccine distribution, storage, and transportation (Naik et al., 2017; WHO, 2018).

2.7.2. Nationally licenced vaccine

Other vaccines such as Rotavin-M1 (POPLYVAC, Centre for Research and Production of Vaccines and Biologicals, Vietnam) and Lanzhou lamb rotavirus vaccine (LLR, Lanzhou Institute of Biological Products Co., Ltd., China) have been licensed locally in Vietnam and China, respectively (Table 2.2) (Kirkwood and Steele, 2018). Rotavin-M1 is a live attenuated G1P[8] oral vaccine-derived from a strain (KH0118-2003) isolated from a child in Vietnam (Anh et al., 2012; Burke et al., 2019). It was licensed in 2012 and administered in two doses scheduled at 6 and 12 weeks of age. Rotavin-M1 has been reported to show safety and immunogenicity profile in children similar to that of Rotarix®. However, one interesting distinction between the two vaccines is that 65% vaccine shedding was observed for Rotarix® after the first dose, while Rotavin-M1 exhibited only 44-48% vaccine shedding (Anh et al., 2012). Lanzhou lamb rotavirus vaccine is a live attenuated lamb G10P[15] group A strain administered as a single oral dose to infants scheduled at 6 weeks to 3 years of age (Kirkwood and Steele, 2018). It was licensed in China in 2000 (Li et al., 2018). The Lanzhou lamb rotavirus vaccine is 35% effective against gastroenteritis and 53% against moderate to severe diarrhoea (Zhen et al., 2015). Zhang and co-workers reported that the Lanzhou

lamb rotavirus vaccine provides cross-protection against gastroenteritis caused by both G9 and G3 strains (Li et al., 2019).

2.7.3. Treatment of rotavirus infection

Children presenting with the rotavirus disease may develop symptoms for about five days with zero to two vomiting episodes within 12 hours; a few lose watery stools per day with a low-grade fever (Crawford et al., 2017). Medical intervention will be required if the symptoms last longer than a week with increased vomiting episodes and frequent episodes of watery diarrhoea (Crawford et al., 2017). The key treatment concept includes ORT or intravenous rehydration. Oral rehydration therapy is recommended in response to mild and moderate dehydration in children with acute diarrhoea which includes rehydration and maintenance of fluids with oral rehydration solutions (ORS) combined with the adequate dietary intake (CDC, 2003; Hartling et al., 2006). Intravenous rehydration is mostly recommended when dehydration becomes severe due to excessive vomiting, reduced consciousness, and intestinal ileus (CDC, 2003). The use of ORT and intravenous rehydration is highly advantageous for economically disadvantaged populations as they are less expensive but equally effective oral solutions. However, several low-income countries are still phased with challenges accessing such treatments.

Other treatment methods include zinc supplements, probiotics, and antiviral therapy. Zinc supplements administered for 10 – 14 days can also significantly decrease the prevalence and duration of diarrhoeal episodes by improved absorption of water and electrolytes in the intestine, promoting faster regeneration of the gut epithelium and enhancing immune response (Bettger and O'Dell, 1981; Gebhard et al., 1983; Shankar and Prasad, 1998). Lactic acid-producing bacteria such as *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Bifidobacteria,* and *Enterococcus faecium,* as well as the yeast *Saccharomyces boulardii* are also commonly used as probiotics. These probiotics have been reported to limit the duration of gastroenteritis successfully. However, they are not included in the global standard of treatment for children with rotavirus (Crawford et al., 2017). Studies have also reported that an antiviral drug such as nitazoxanide can reduce the duration of diarrhoeal episodes by interfering with the viral morphology thus inhibiting the replication of rotaviruses (La Frazia et al., 2013; Mahapatro et al., 2017; Rossignol, 2014).

2.8. Rotavirus vaccine introduction in African countries

Rotavirus vaccines have enormously improved child health morbidity and reduced diarrhoea-associated mortality (Jonesteller et al., 2017). As of April 2020, 107 countries had introduced rotavirus vaccines (ROTA Council, 2020). These includes 103 national introductions and 4 sub-national introductions. Although more than 70% of sub-Saharan African countries have introduced rotavirus vaccines into their national immunization programs, the rotavirus disease burden still remains high compared to high-income countries (Tate et al., 2016, 2012). Five sub-Saharan African and South Asian countries including Angola, the Democratic Republic of Congo, India, Nigeria, and Pakistan, account for half of the rotavirus deaths worldwide. Of the 35 African countries that have embraced and introduced the rotavirus vaccines, RotaTeq® was introduced only in five countries (Burkina Faso, Gambia, Libya, Morocco, and Rwanda) (IVAC, 2019; Seheri et al., 2018). The first African country to introduce Rotarix® was South Africa in 2009, while Rwanda was the first low-income country in the world to introduce RotaTeq® vaccine in May 2012 but switched to Rotarix® in April 2017 (Madhi et al., 2012; Ngabo et al., 2016b; Seheri et al., 2018). Countries such as Ethiopia, Kenya, Tanzania, Uganda, Zambia, and Zimbabwe introduced Rotarix® vaccine from 2012 to 2015 (Weldegebriel et al., 2018). In Rwanda, RotaTeq® vaccine coverage of 99% was reached by 2013, administered at 6, 10, and 14 weeks of age (Gatera et al., 2016; Ngabo et al., 2016a; Sibomana et al., 2018; WHO, 2020). In 2019, the RotaTeq® vaccine coverage of 98% was reported in Rwanda (WHO, 2020). Studies suggest that if all GAVI-eligible countries introduce rotavirus vaccines, an estimate of 180 000 deaths would be averted (Atherly et al., 2012).

2.9. Surveillance data of African countries

Although, rotavirus vaccines were intended to provide protection against multiple rotavirus strains (Buttery et al., 2011; Tsolenyanu et al., 2016). There are constant gene reassortment, point mutation, and gene rearrangement of the virus that are probably responsible for lower efficacy in regions that have reported excellent strain diversity, thus necessitated continuous surveillance (Bányai et al., 2011). The outer capsid proteins are primary targets for vaccine development due to their ability to induce neutralizing antibodies and thus the antigenic changes in their structure may also impact vaccine effectiveness (Maranhão et al., 2012; Zeller et al., 2012). Generally, while the impact of rotavirus vaccines has been significant post introduction, studies have reported poor performance of live oral vaccines for rotavirus in developing countries (Velasquez et al., 2017). The documented feasible justification for the

low efficacy of the vaccines in Africa and Asia may be interference by maternal antibodies, co-infection with multiple enteric pathogens, genetic diversity, malnutrition, and limited access to treatment (Boschi-Pinto et al., 2008; Dagan et al., 1990; Glass et al., 2006; Moon et al., 2016; Naylor et al., 2015). Rotavirus associated mortality in sub-Saharan Africa accounts for > 80% of the disease burden with only 8 countries; Burkina Faso, Chad, Cote d'Ivoire, Democratic Republic of Congo, Ethiopia, Niger, Nigeria, and Uganda (Troeger et al., 2018b).

The analysis of Wa-like and DS-1-like genome constellations has revealed that both constellations are successful in causing and rapidly spreading rotavirus infection in humans (Matthijnssens and Van Ranst, 2012). The most prevalent rotavirus genotypes worldwide in the last 20 years are G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8] which accounted for 74.7% of all strains circulating globally (Bányai et al., 2012; Leshem et al., 2014; Mwenda et al., 2010; Nyaga et al., 2020; Santos and Hoshino, 2005; Seheri et al., 2014; Todd et al., 2010). These six strain combinations cause over 90% of rotavirus disease in Australia, Europe, and North America. In South America and Africa, they account for 83% and 55%, respectively, with frequent detection of unusual genotypes (Santos and Hoshino, 2005).

Unusual strains have been detected at high frequencies in countries such as Malawi (G8P[6] and G8P[4]), Brazil (G5P8]), Hungary (G6P[8]), and India (G10P[11]). In the years between 2010 and 2015, G1P[8] (23.8%), G2P[4] (11.8%) and G9P[8] (10.4%) were reported as the predominant cause of acute gastroenteritis in Eastern and Southern African countries among children less than five years (Seheri et al., 2018). The yearly fluctuation of G1P[8] strain was observed in Rwanda (2011 and 2015), Zambia (2012 and 2014), Tanzania (2010-2011 and 2014- 2015) and Zimbabwe (2014-2015). An increase in the prevalence of G2P[4] and G2P[6] genotypes in Ethiopia, Tanzania, Zambia, Zimbabwe, and Madagascar was also reported (Seheri et al., 2018). Moreover, uncommon genotypes such as G9P[4], G9P[6], G3P[4] and G3P[6] are generally detected in low frequencies globally (Collins et al., 2015; Cunliffe et al., 2010; Giri et al., 2019; João et al., 2020; Lartey et al., 2018; Lennon et al., 2008; Page et al., 2018; Rasebotsa et al., 2020; Seheri et al., 2018; Yamamoto et al., 2015).

In Rwanda, a strong seasonal pattern was observed with more diarrhoeal hospital admissions from May to September and a peak in July (Ngabo et al., 2016b; Uwimana et al., 2015). The pattern coincided with the rotavirus season (dry season), where there are less rainfall and insufficient water supply coupled with poor hygiene and sanitation (Uwimana et al., 2015). Studies have also associated infections with climate change response, with the highest number of infections observed during colder and drier times of the year (Levy et al., 2009). After the introduction of RotaTeq® in Rwanda, the proportion of rotavirus induced diarrhoeal hospitalization declined by 25 - 44% among children less than five years during the first three years post-vaccine introduction (Sibomana et al., 2018). Immediately after rotavirus vaccine implementation, an increase in rotavirus hospital admissions to children over five years of age was observed (Ngabo et al., 2016b). However, a decrease in rotavirus positive diarrhoeal admissions was observed in all age groups, suggesting that children not eligible for vaccination are also protected through the decrease of RVA transmission in the population (Clarke et al., 2011; Lambert et al., 2009; Ngabo et al., 2016b). Temporal strain variation was reported in Rwanda post-vaccine introduction with G8P[4] circulating at high proportion in 2013, G4P[8] and G12P[8] in 2014, which were replaced by G1P[8] strains in 2015 (Seheri et al., 2018). The global use of the vaccines has been suspected to be the potential cause of evolutionary changes in the genome (Hoshino et al., 2004; Kirkwood, 2010). As numerous mechanisms influence diversity of the rotaviruses, long-term rotavirus surveillance is essential in determining the vaccine impact along with changes in the circulating strains in the African region (Kirkwood, 2010; Seheri et al., 2018).

2.10. Next-generation Sequencing

In previous years, detecting viruses in a clinical or environmental sample was very challenging and time-consuming. In 1977, Frederick Sanger and colleagues developed a ground breaking DNA sequencing method, Sanger sequencing, that enabled the detection of various organisms, including viruses (Sanger et al., 1977). Sanger sequencing has been used for many years and is considered as the gold standard in elucidating genetic information. This triggered the development of new technologies such as NGS over the years that substantially reduced DNA sequencing time and costs while remarkably producing massive sequencing data (Grada and Weinbrecht, 2013). Moreover, NGS produces more accurate, less biased, and reliable results enabling comprehensive analysis of genomes, epigenome, transcriptomes that make a significant contribution to biological and biomedical research (Metzker, 2010; Shendure and Ji, 2008). Next-generation sequencing platforms such as LifeTechnology Ion Torrent Personal Genome Machine

(PGM) and the Illumina Miseq are frequently applied in health, agricultural, and environmental fields (Grada and Weinbrecht, 2013).

Next-generation sequencing technologies require library preparation, amplification, and sequencing steps. The PGM uses emulsion PCR to amplify single library fragments onto microbes, while the Illumina Miseq platform uses bridge amplification to form template clusters on a flow cell (Berglund et al., 2011; Quail et al., 2012). Moreover, both platforms rely on sequencing by synthesis. The DNA libraries are used as templates, from which new DNA fragments are synthesized. Sequencing is carried out by cycles of washing and flooding the DNA library fragments with known nucleotides in a sequential order to significantly reduce raw error rates, while simultaneously recording the DNA sequence digitally. The PGM platform detects and records a new nucleotide when the pH changes each time a hydrogen ion is released upon incorporation in a growing DNA strand (Quail et al., 2012). In contrast, the Illumina Miseq platform detects and records the sequence each time a new fluorescently labelled reversible-terminator nucleotide is incorporated in a growing DNA strand (Bentley et al., 2008). The sequencing data generally undergoes several analysis steps such as removal of adaptor sequence and low quality read as well as mapping the data to a reference genome or conducting *de novo* assembly and finally the analysis of the compiled sequences through various bioinformatics platforms (Grada and Weinbrecht, 2013). Both NGS platforms are reported to be viable options for sequencing in terms of utility and ease of workflow (Marine et al., 2020; Quail et al., 2012).

The Illumina MiSeq platform was utilized in this project. The key application of an Illumina MiSeq platform offers capabilities for small whole-genome sequencing of microbes and viruses, targeted gene sequencing and 16S metagenomic (Illumina, 2020). Using a high throughput kit, the platform can produce up to 15Gb of output with 25 million sequencing reads and 2 x 300 bp reads lengths in a single run with a run time of 4-55 hours. The use of NGS platforms has been efficient in characterizing and deciphering the whole-genome sequence of circulating rotavirus strains globally (Bwogi et al., 2017; Esona et al., 2017; Ianiro et al., 2013; Jere et al., 2018, 2011; Komoto et al., 2014; Maringa et al., 2020; Mukherjee et al., 2011; Mwangi et al., 2020; Nyaga et al., 2018, 2015, 2014, 2013, 2020; Rasebotsa et al., 2020; Saikruang et al., 2016). Whole-genome studies have a significant potential in providing comprehensive insight into the impact of vaccines on both wild type and atypical rotavirus strains (Nyaga et al., 2020). Such studies can also aid in the rational design of the next-generation subunit vaccines. Moreover, the frequency of reassortment

events under natural conditions and their epidemiological fitness against wild type strains can be determined.

2.11. Rotavirus whole-genome studies

The advancements in sequencing methods, such as NGS, have made it easier to determine the overall genetic makeup and evolutionary pattern of RVA strains (Nyaga et al., 2020). Over the years, evolutionary mechanisms such as gene reassortment, rearrangement, and zoonotic transmissions have led to the emergence of novel RVA strains (Kirkwood, 2010). Moreover, some instances of zoonotic transmissions of RVA strains can only be confirmed through whole-genome analysis (Martella et al., 2010; Santos and Hoshino, 2005). The pure Wa-like and DS-1-like rotavirus genome constellations generally have an evolutionary fitness advantage (Heiman et al., 2008; McDonald et al., 2009). However, the emergence of novel strains has been documented worldwide (Agbemabiese et al., 2017; Arana et al., 2016; Donato et al., 2012; Jere et al., 2018; Mwangi et al., 2020; Strydom et al., 2019a). The 11 rotavirus genome segments exhibits different levels of diversity, with NSP1 and NSP4 genome segment being more diverse than the other segments (Dunn et al., 1994; Nyaga et al., 2015). The VP1 and VP2 genome segments were reported to be more conserved (Matthijnssens et al., 2008).

The DS-1-like constellation is generally linked to G2P[4] strains (Matthijnssens et al., 2008). Although, non G2P[4] DS-1-like strains have been identified in Africa (G1P[8], G3P[6] and G8P[4]), Asia (G1P[4] and G12P[6]) and Europe (G3P[8]) (Agbemabiese et al., 2017; Arana et al., 2016; Bwogi et al., 2017; Heylen et al., 2013; Mwangi et al., 2020; Nyaga et al., 2015; Saikruang et al., 2016; Strydom et al., 2019a). The G1P[8] strains commonly exhibits a typical Wa-like genotype constellation (Magagula et al., 2015). However, several whole-genome studies have reported intergenogroup reassortment of G1P[8] strains in Brazil, Japan, Philippines, Thailand, Vietnam (Fujii et al., 2014; Komoto et al., 2015; Luchs et al., 2019; Nakagomi et al., 2017). In Africa, the first atypical G1P[8] strains with a DS-1-like backbone were detected in Blantyre, Malawi, predominantly during the post-vaccine introduction period (Jere et al., 2018). Recently, South Africa also reported the first DS-1-like G1P[8] strain. This particular reassortment occurred pre-vaccine introduction, suggesting that the event might have been influenced by natural evolutionary events (Mwangi et al., 2020).

In a study conducted in Kenya, a G2P[4] strain with a Wa-like NSP2 genotype on the DS-1-like backbone was observed (Ghosh et al., 2011). The phylogenetic analysis of the strain also revealed that while the other segments were closely related to the prototype and other human G2P[4] strains, the NSP2 genome appeared to be closely related to rotavirus genes of artiodactyl origin highlighting the complex evolutionary dynamics of African G2P[4] rotavirus strains. Moreover, a G9P[6] strain from South Africa with a predominantly DS-1-like backbone also exhibited a Wa-like NSP2 genotype which was suggested to have emerged through intergenogroup reassortment (Nyaga et al., 2013). A similar trend was observed on G12 rotavirus strains isolated in the Philippines and Bangladesh (Rahman et al., 2007). Most G4P[6] strains have the Wa-like genome constellation and have been frequently detected in Korea, they are considered to be rare in other countries (Lee et al., 2019). However, a complete genome sequence of a G4P[6] strain isolated from Zambia was submitted on GenBank as RVA/Human-wt/ZMB/MRC-DPRU1752/XXXX/G4P[6] exhibiting a DS-1-like genome constellation.

Despite the benefit of rotavirus vaccines, cases of vaccine shedding, horizontal transmission of vaccine strains and vaccine-derived strains have been documented globally (Boom et al., 2012; Gower et al., 2020; Hemming and Vesikari, 2014; Hsieh et al., 2014; Than et al., 2015). Whole-genome analysis has made it easier to differentiate wild-type rotavirus strains from vaccine-derived strains (Magagula et al., 2015; Sakon et al., 2017). A study conducted in Nicaragua reported two G1P[8] strains that exhibited an NSP2 gene with a DS-1-like genotype identical to that of the RotaTeq® vaccine. In Brazil, where Rotarix® was introduced, a G1P[8] strain with an AU-1-like NSP3 was reported. The strain was evident of a vaccine-derived reassortment because it also clustered along with the Rotarix® vaccine during phylogenetic analysis (Rose et al., 2013a). The detection of vaccine-derived strains may have led to reduced rotavirus vaccine effectiveness over time. Such findings emphasize the importance of conducting rotavirus whole-genome studies in order to obtain conclusive data on the complex evolutionary dynamics of RVA strains, especially in Africa (Nyaga et al., 2020; Seheri et al., 2018). Although studies have been conducted on the outer capsid proteins of rotavirus strains circulating in Rwanda (Seheri et al., 2018; Uwimana et al., 2015), there still a dearth of information regarding the entire backbone of those strains.

CHAPTER THREE: METHODOLOGY

3.1. Ethical statement and study design

The ethical clearance for this study was granted by the UFS, Health Sciences Research Ethics Committee (HSREC) under the reference UFS-HSD2019/1601/2810. The research was conducted at the UFS-NGS Unit, Division of Virology, Faculty of Health Sciences, UFS. Archived genotyped rotavirus stool specimen (n=158) from children younger than five years from Rwanda were retrieved from the Diarrheal Pathogens Research Unit (DPRU), which is a WHO rotavirus Regional Reference Laboratory in South Africa (RRL-SA). The targeted stool samples were from the pre- (2011) and post- (2012-2016) vaccine introduction era sampled from children in all five provinces (Figure 3.1). The viral dsRNA was isolated from the stool samples and purified in preparation for cDNA synthesis. The cDNA was synthesized and fragmented to prepare DNA libraries for sequencing on an Illumina MiSeq platform, followed by data analysis using various bioinformatics software. This study forms part of a bigger project aimed at addressing the Terms of References (ToRs) for Technical Service Agreement (TSA) between the UFS-NGS Unit and the WHO, to perform whole-genome sequencing of rotavirus strains from the World Health Organization Regional Office for Africa (WHO/AFRO) rotavirus surveillance network, under the reference HSREC130/2016(UFS-HSD2016/1082).

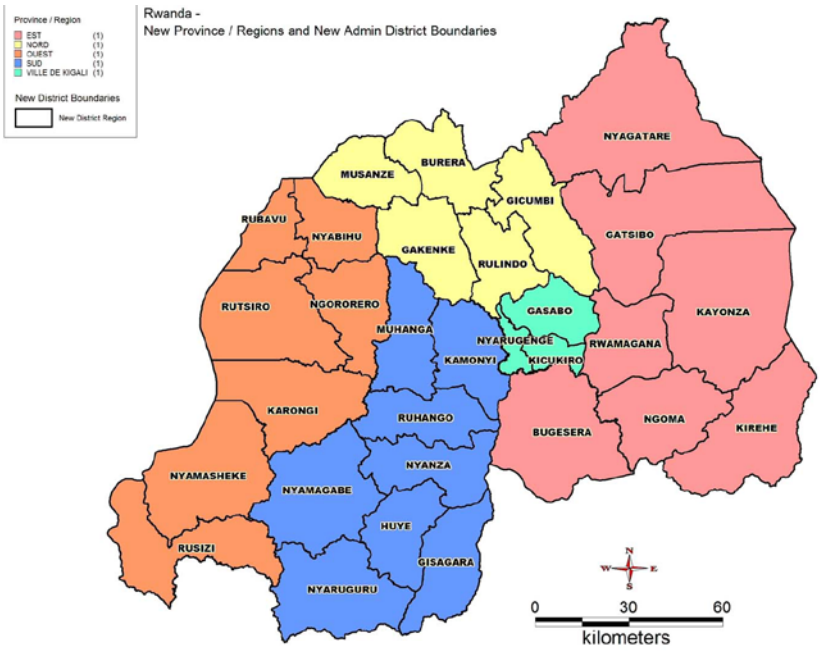


Figure 3.1: The map of Rwanda indicating all the five provinces. Each province is indicated with a different colour; orange (Western province), yellow (Northern province), blue (Southern province), green (Kigali province) and red (Eastern province). Adapted (Kalisa et. al., 2016)

3.2. Extraction of rotavirus dsRNA from faecal samples

Rotavirus dsRNA was extracted following a protocol described previously by Nyaga et al. (2018). Briefly, in a Biosafety cabinet (Esco Technologies Pty Ltd, Gauteng, South Africa), a 10% dilution was formulated using approximately 100 mg of the stool sample added to a sterile 2 ml microcentrifuge tube (Thermo Scientific, QSP, New Hampshire, United States) containing 200 µl of sterile phosphate buffer solution, pH 7.2 (PBS, Sigma-Aldrich, St. Louis, USA). To extract the dsRNA in the aqueous phase, 1 ml of TRI-reagent (Molecular Research Center, Inc, Cincinnati, OH, USA) was added to the tube and thoroughly mixed by vortexing (Labnet International,Inc., New Jersey, United States), followed by an incubation period of five minutes at room temperature. Subsequently, 270 µl of chloroform (Sigma-Aldrich, St. Louis, USA) was added to the tube, vortexed (Labnet International,Inc., New Jersey, United States) again, and incubated for three minutes at room temperature. The blend was later centrifuged (Eppendorf, Hamburg, Germany) at 16 000 x *g* for 20 minutes at 4°C. Thereafter, the supernatant was added to a new 2 ml microcentrifuge tube (Thermo Scientific, QSP, New Hampshire, United States) containing 1 ml ice-cold isopropanol (Sigma-Aldrich, St. Louis, USA) to dissolve any salts that might be chelated to the dsRNA sample while precipitating the dsRNA for five minutes at room temperature. The blend was centrifuged (Eppendorf, Hamburg, Germany) at 16 000 x *g* for 30 minutes at 20°C and the supernatant was discarded, leaving behind a pellet at the bottom of the tube, which was air-dried for ten minutes. The dsRNA was recovered from the bottom of the tube by adding 95 µl of the Qiagen Elution Buffer, EB (Qiagen, Hilden, Germany). The tube was sporadically vortexed (Labnet International,Inc., New Jersey, United States) and each sample (2 µl) was visualized on a 1% agarose gel (ThermoFisher Scientific, Waltham MA, USA) stained with Pronasafe (Condalab, Madrid, Spain).

Lithium chloride, 8 M $LiCl_2$, (Sigma-Aldrich, St. Louis, USA) was used to enrich the extracted total RNA by removing ssRNA because rotavirus is a dsRNA virus. $LiCl_2$ (Sigma-Aldrich, St. Louis, USA) was prepared by dissolving 1.69 g $LiCl_2$ (Sigma-Aldrich, St. Louis, USA) into 5 ml nuclease-free water. A volume of 30 µl of the 8 M $LiCl_2$ (Sigma-Aldrich, St. Louis, USA), while still hot, was transferred to a 2 ml microcentrifuge tube (Thermo Scientific, QSP, New Hampshire, United States) containing the extracted nucleic material and incubated for 20 minutes at room temperature with a further incubation period of 16 hours in a 4°C water bath. The mixture was centrifuged (Eppendorf, Hamburg, Germany) at 16 000 x *g* for 30 minutes at 4°C to precipitate the $LiCl_2$ (Sigma-Aldrich, St. Louis, USA) and the supernatant was transferred into a new 2 ml microcentrifuge tube (Thermo Scientific, QSP, New Hampshire, United States).

The Qiagen MinElute gel extraction kit (Qiagen, Hilden, Germany) was used to purify the dsRNA. Briefly, a Prism Mini centrifuge (Labnet International, Inc., New Jersey, United States) was used to spin down the sample and 330 µl QG buffer (Qiagen, Hilden, Germany) was added to the 2 ml microcentrifuge tube (Thermo Scientific, QSP, New Hampshire, United States) containing the dsRNA sample. The sample was gently mixed by vortexing (Labnet International,Inc., New Jersey, United States), centrifuged (Prism Mini centrifuge, Labnet International, Inc., New Jersey, United States) and subsequently transferred into a new 1.5 ml tube containing a MinElute spin column (Qiagen, Hilden, Germany) to allow adsorption of the dsRNA on the silica-gel membrane of the column while washing away impurities introduced during the RNA extraction. The tube was centrifuged (Eppendorf, Hamburg, Germany) at 16 000 x *g* for one minute at 4°C. The column was transferred into a new 1.5 ml microcentrifuge tube (Qiagen, Hilden, Germany) and 35 µl of the EB buffer (Qiagen, Hilden, Germany) was added at the centre of the membrane to elute the bound dsRNA. The tube was incubated for one minute then centrifuged (Prism Microcentrifuge, Labnet International,Inc., New Jersey, United States) at 16 000 x *g* for another minute. The column was discarded leaving behind the purified dsRNA visualized on a 1% agarose gel (ThermoFisher Scientific, Waltham MA, USA) and quantified using BioDrop-µLITE spectrophotometer (Biodrop, Cambridge, United Kingdom), which measures the concentration of low volume nucleic acid samples.

3.3. Complementary DNA synthesis

A maxima H Minus ds cDNA synthesis kit (ThermoFisher Scientific, Waltham MA, USA) was used to synthesize cDNA from total RNA. The conversion of RNA to cDNA was brought by the introduction of reverse transcriptase to the rotavirus sample, which catalyses the formation of DNA from utilizing RNA as a template. Minor modifications to the maxima H Minus ds cDNA synthesis protocol were made as per UFS-NGS Unit cDNA synthesis standard operating procedure, to optimize it specifically for rotavirus by adding 13 µl of the dsRNA into a PCR (Polymerase Chain Reaction) tube (Eppendorf, Hamburg, Germany) and incubating it in a pre-heated (105°C) thermocycler (Merck, Darmstadt, Germany) at 95°C for five minutes. The incubation step allowed the dsRNA to unwind and separate into an elongated ssRNA. The tube was centrifuged (Prism Mini centrifuge, Labnet International, Inc., New Jersey, United States) followed by the addition of 1 µl of the random hexamer primer (ThermoFisher Scientific, Waltham MA, USA). The mixture was briefly centrifuged on a Prism Mini Centrifuge (Labnet International,Inc., New Jersey, United States). Subsequently, the tube was incubated at 65°C for five minutes in a thermocycler (Merck, Darmstadt, Germany) to allow the random primer to anneal randomly to the RNA template

strand. The tube was briefly pulse centrifuged (Prism Mini Centrifuge, Labnet International,Inc., New Jersey, United States) and placed on ice. A volume of 5 µl of the 4X first strand reaction mix (ThermoFisher Scientific, Waltham MA, USA) along with 1 µl of the first strand enzyme mix (ThermoFisher Scientific, Waltham MA, USA) were added to the mixture in the PCR tube (Eppendorf, Hamburg, Germany) followed by ten minutes of incubation at 25°C and elongation of the strand for 2 hours at 50°C in a Thermocycler (Merck, Darmstadt, Germany). Final denaturation was achieved by heating the blend for five minutes at 85°C. The final product of this reaction was the first strand.

The first strand was used as a template to generate the second strand by introducing an *E.coli* RNase H which inserts nicks into the RNA, allowing the DNA polymerase I to replace the RNA strand with deoxyribonucleotides. Finally, the *E.coli* DNA ligase closed the gaps to complete the ds cDNA strand. This was all achieved by adding 55 µl of the second strand nuclease-free water (ThermoFisher Scientific, Waltham MA, USA), 20 µl 5X second strand reaction mix (ThermoFisher Scientific, Waltham MA, USA) and 5 µl second strand enzyme mix (ThermoFisher Scientific, Waltham MA, USA) to a PCR tube (Eppendorf, Hamburg, Germany) containing 12 µl of the reaction mixture from the first strand synthesis. The tube was incubated for 60 minutes at 16°C in a Thermocycler (Merck, Darmstadt, Germany). Thereafter, the reaction was halted by adding 6 µl of 0.5 M EDTA, pH 8.0 (ThermoFisher Scientific, Waltham MA, USA). Residual RNA was removed by adding 10 µl RNase I (ThermoFisher Scientific, Waltham MA, USA) to the second strand reaction tube leaving behind the cDNA.

3.4. cDNA purification

An MSB PCRAPACE purification kit (Invitek Molecular, Berlin, Germany) was used to purify the synthesized rotavirus cDNA samples. Briefly, 250 µl of the Binding Buffer (Invitek Molecular, Berlin, Germany) was transferred to a 2 ml microcentrifuge tube (Thermo Scientific, QSP, New Hampshire, United States) containing 50 µl of the cDNA sample and vortexed (Labnet International,Inc., New Jersey, United States). The blend was transferred into a 2 ml receiver tube (Invitek Molecular, Berlin, Germany) containing a spin filter (Invitek Molecular, Berlin, Germany), incubated for one minute at room temperature, and centrifuged (Prism Microcentrifuge, Labnet International,Inc., New Jersey, United States) for four minutes at 21 000 x *g*. This step allows the separation of the PCR product from impurities such as deoxyribonucleotide triphosphates (dNTPs), primers, and enzymes introduced during the synthesis of the cDNA. Furthermore, the spin filter (Invitek Molecular, Berlin, Germany) was inserted into a sterile 1.5 ml

receiver tube (Invitek Molecular, Berlin, Germany) and 10 µl of the EB (Invitek Molecular, Berlin, Germany) was added directly at the centre of the spin filter (Invitek Molecular, Berlin, Germany) to elute the cDNA. The tube was incubated for one minute at room temperature and centrifuged (Prism Microcentrifuge, Labnet International,Inc., New Jersey, United States) for one minute at 11 000 x *g* in readiness for validation using a fluorometric assay.

3.5. Quality control using Qubit™ 4 Fluorometer

Qubit™ 4 Fluorometer (Life Technologies, California, United States), a benchtop fluorometer, was used to quantify the purified rotavirus cDNA in preparation for DNA library preparation. A Qubit™ working solution was prepared by making a 1:200 dilution of the Qubit™ reagent (Life Technologies, California, United States) and Qubit™ buffer (Life Technologies, California, United States) (Figure 3.2). The assay standards (Life Technologies, California, United States) were prepared by adding 10 µl of standard 1 and 2 in separate tubes containing 190 µl of the working solution. The standards (Life Technologies, California, United States) were used to calibrate the instrument prior to quantifying the samples. Furthermore, 1 µl of the rotavirus sample was added to a Qubit™ tube (Life Technologies, California, United States) containing 199 µl of the working solution. All the Qubit™ tubes (Life Technologies, California, United States) were vortexed (Labnet International,Inc., New Jersey, United States) and incubated at room temperature for two minutes. The Qubit™ tubes (Life Technologies, California, United States) were inserted in the Qubit™ 4 Fluorometer (Life Technologies, California, United States) and the readings were recorded. Samples with high concentration (concentrations higher than 0.2 ng/µl) were normalized by adding EB (Qiagen, Hilden, Germany) while samples that had low concentration (concentration lower than 0.2 ng/µl) were excluded from further processing.

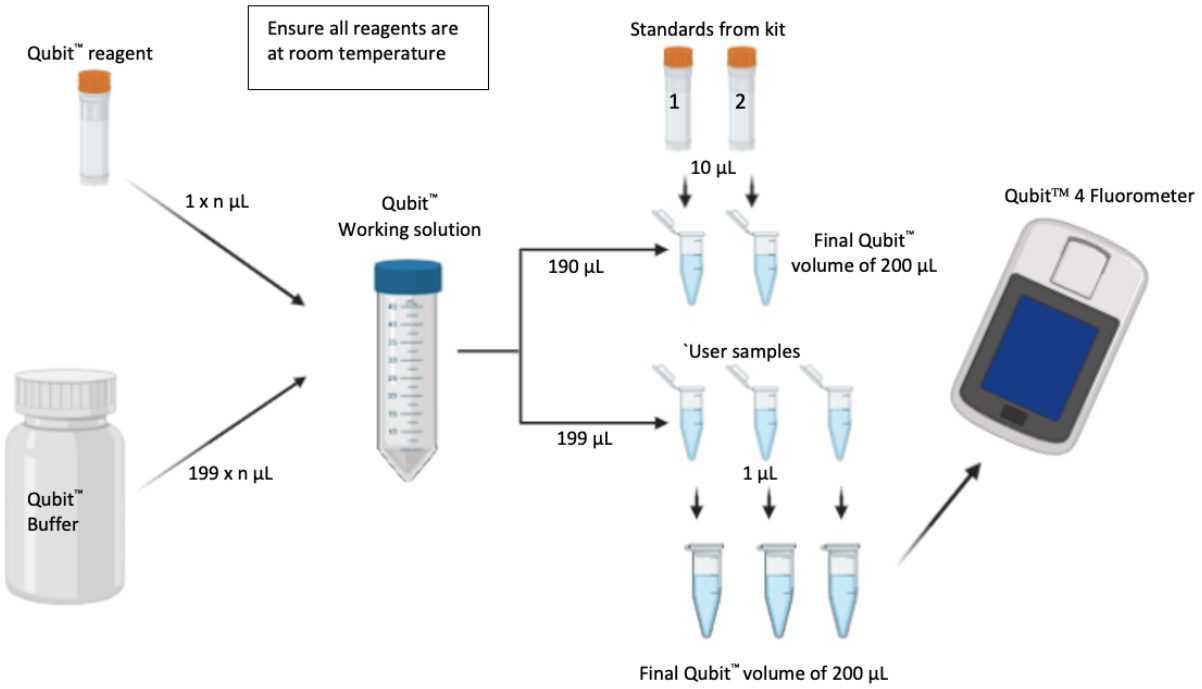


Figure 3.2: Qubit™ 4 Fluorometer workflow. The Qubit™ working solution was prepared by adding 199 x n µl of the Qubit™ buffer and 1 x n µl of the Qubit™ reagent into a single tube. A volume of 190 µl of the solution was transferred into a tube containing 1 µl of the sample, while 190 µl of the working solution was transferred to two tubes containing standard 1 and standard 2, respectively. The standards were used to calibrate the Qubit™ 4 Fluorometer in preparation for quantifying the samples (Created with BioRender.com, 26 August 2020)

3.6. DNA Library preparation

The Nextera® XT DNA library preparation kit (Illumina, Inc., California, United States) was used for the library preparation for sequencing on an Illumina MiSeq (Illumina, Inc., California, United States) platform. The rotavirus cDNA samples with concentrations ranging from 0.2–0.3 ng/µl were used for library preparation as the starting cDNA concentration for the enzymatic fragmentation and adapter ligation to the templates. In brief, the genomic DNA was enzymatically fragmented into smaller templates (~ 300 bp) using Nextera transposomes. The ends of the templates were ligated by amplification using a thermocycler (Merck, Darmstadt, Germany), which simultaneously added the sequencing oligos, the Illumina instrument-specific P5 and P7 adaptors and unique indexes for de-multiplexing into individual entities. Prior to that, the amplified DNA was size-selected and purified by removing PCR inhibitors to approximately 300 bp templates using AMPure XP beads (Beckman Coulter, Indiana, United States). This allowed removal of low and high molecular weight while library fragments for an optimal library free from over or under-clustering during sequencing. The DNA libraries were normalized to a uniform concentration of 4 nM after fragment length validation on the Agilent Bioanalyzer (Agilent Technologies, California, United States) and ultimately normalized to 20 pM and into a final concentration of 8 pM. The libraries were pooled together and denatured in readiness for sequencing using an Illumina MiSeq (Illumina, Inc., California, United States) platform.

3.6.1. Detailed genomic DNA tagmentation and library amplification

The tagmentation step introduced transposome enzymes into the template DNA, which fragmented the template into small fragments. Illumina chemistry specific adapter sequences (P5 and P7) were then added to the fragmented genomic DNA (Figure 3.3). In a 96-well PCR plate (Soreson Bioscience Inc., Utah, United States), 10 µl of the Tagment DNA Buffer, TD, (Illumina, Inc., California, United States) was added followed by 5 µl of the normalized DNA (0.2 – 0.3 ng/µl). A volume of 5 µl of the Amplicon Tagment Mix, ATM, (Illumina, Inc., California, United States) was added to the blend and centrifuged (Hermle, Labnet International, Inc., New Jersey, United States) at 280 x *g* for one minute at 20°C. Furthermore, the 96-well PCR plate (Soreson Bioscience Inc., Utah, United States) was placed on a thermal cycler (Multigene Optimax, Labnet International, Inc., New Jersey, United States) with a pre-heated lid (105°C) at 55°C for five minutes. Subsequently, 5 µl of the Neutralize Tagment Buffer, NT, (Illumina, Inc., California, United States) was added to the plate and centrifuged (Hermle, Labnet International, Inc., New Jersey, United States) at 20°C for one minute at 280 x *g* and incubated at room temperature for five minutes.

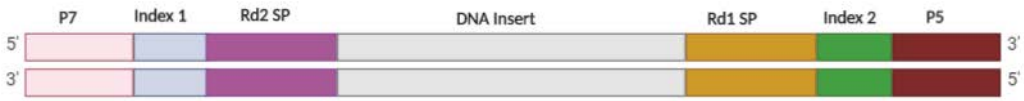


Figure 3.3: DNA fragment ligated with indexes and Illumina chemistry specific adapter sequences (Created with BioRender.com, 26 August 2020)

An Illumina sample manager software (Experiment Manager Software version 1.15, Illumina, Inc., California, United States) was utilized to assign each sample with a specific index to uniquely label and differentiate between each specific sample after de-multiplexing in Illumina control software. The libraries were uniquely labelled by adding 5 µl of the prescribed index (1 and 2) (Illumina, Inc., California, United States) (Illumina, Inc., California, United States) to each specific sample. A Nextera PCR Master Mix, NPM, (Illumina, Inc., California, United States) of 15 µl was added to the blend and centrifuged (Hermle, Labnet International, Inc., New Jersey, United States) at 280 x *g* for one minute at 20 °C. The 96-well PCR plate (Soreson Bioscience Inc., Utah, United State) containing the blend was placed on a pre-heated (105°C) thermal cycler (Multigene Optimax, Labnet International, Inc., New Jersey, United States). The programme run on the thermal cycler (Multigene Optimax, Labnet International, Inc., New Jersey, United States) with the following conditions: 72°C for three minutes, 95°C for 30 seconds and 12 cycles of 95°C for 10 seconds, 55°C for 30 seconds and 72°C for 30 seconds followed by 72°C for five minutes and the reaction was put on hold at 4°C.

3.6.2. Library clean-up and quantification

Firstly, the libraries were centrifuged (Hermle, Labnet International, Inc., New Jersey, United States) at 280 x *g* for one minute at 20°C to prevent sample loss by concentrate the liquid at the bottom of the well. The PCR product (50 µl) was transferred to a new 96-well PCR plate (Soreson Bioscience Inc., Utah, United States) followed by the addition of 30 µl AMPure XP beads (Beckman Coulter, Indiana, United States). The beads selectively bind to the 300 bp DNA fragments. The blend was thoroughly mixed using a Micro-Multiple Genie (Scientific industries, Inc., New York, United States) at 280 x *g* for two minutes and placed on a magnetic stand (PerkinElmer, Massachusetts, United States) for two minutes allowing the beads to concentrate at the bottom of the plate. The supernatant was discarded and the beads were washed twice with freshly prepared 80% ethanol (Merck, Darmstadt, Germany) (200 µl), followed by an incubation

period of 30 seconds on the magnetic stand (PerkinElmer, Massachusetts, United States) to remove impurities (excess ATM, NPM, and indexes) introduced during the tagmentation and library amplification step. The supernatant was carefully removed to prevent the carry-over of ethanol that might disrupt downstream processes. The beads were air-dried for five minutes at room temperature and 52.5 µl of the Resuspension Buffer, RSB, (Illumina, Inc., California, United States) was added before the beads started showing cracks. The plate was centrifuged (Hermle, Labnet International, Inc., New Jersey, United States) at 280 x *g* for two minutes and incubated at room temperature for an additional two minutes. Furthermore, the plate was placed on a magnetic stand (PerkinElmer, Massachusetts, United States) allowing the beads to form a pellet at the bottom. A volume of 50 µl of the supernatant was transferred to a new 96-well PCR plate (Soreson Bioscience Inc., Utah, United States).

Library quantification was conducted using Agilent Technology 2100 Bioanalyzer (Agilent Technologies, California, United States) and Qubit™ 4 Fluorometer (Life Technologies, California, United States) (Figure 3.2). Briefly, 1 µl of the library was added to the high sensitivity chip and ran on an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, California, United States) which allowed for easy viewing of the fragment size of the libraries. In preparation for library pooling and to ensure equal library representation in the pooled samples, the Bioanalyzer (Agilent Technologies, California, United States) and the Qubit™ 4 Fluorometer (Life Technologies, California, United States) results were normalized to 4 nM following unit conversion from ng/µl into nM using the expression below:

$$\frac{concentration\ in\ ng/\mu l}{(660\ gmol/x\ Average\ library\ size)} \times 10^6 = concentration\ in\ nM$$

3.6.3. Quality control using an Agilent Technology 2100 Bioanalyzer

An Agilent Technology 2100 Bioanalyzer (Agilent Technologies, California, United States), a chip-based capillary electrophoresis machine, was used to assess the size and integrity of the amplified library as a quality control measure. This quality control step was done before pooling the libraries to ensure the quality of the prepared libraries were good to avoid generating a poor quality run. Briefly, the Gel-Dye mix was prepared by adding 15 µl of the high sensitivity DNA dye concentrate (Agilent Technologies, California, United States) into the high sensitivity DNA gel matrix vial (Agilent Technologies, California, United States). The mixture was vortexed (Labnet International,Inc., New Jersey, United States) for ten seconds and pulse centrifuged (Prism Mini Centrifuge, Labnet International,Inc., New Jersey, United

States). The Gel-Dye mix was transferred to a spin filter and centrifuged (Prism Centrifuge, Labnet International,Inc., New Jersey, United States) at 2240 x *g* for ten minutes and the spin filter was discarded, the 2 ml microcentrifuge tube (Thermo Scientific, QSP, New Hampshire, United States) was protected from light by covering it with foil.

A new high sensitivity DNA chip (Agilent Technologies, California, United States) was placed on the chip priming station where the Gel-Dye mix (9 µl) was dispensed at the bottom of the well, labelled capital 'G' (located on the third row, the fourth column from the top right side of the chip). A sterile syringe was also placed on the priming station with the plunger positioned at 1 ml. The pressure was applied on the plunger until it was held in place by the clip, followed by a waiting period of 60 seconds. The plunger was released from the clip until it reached a 0.3 ml mark and it was pulled back to the 1 ml mark after five seconds. The Gel-Dye mix (9 µl) was dispensed into the rest of the wells marked G (located on the fourth column on the right) on the high sensitivity DNA chip (Agilent Technologies, California, United States). A volume of 5 µl of the high sensitivity DNA marker (Agilent Technologies, California, United States) was added into a well, marked with a ladder and also the 11 sample wells.

A volume of 1 µl of the high sensitivity DNA ladder (Agilent Technologies, California, United States) was dispensed in the well, marked with the ladder symbol. Subsequently, each DNA library (1 µl) was added to the 11 sample wells. The high sensitivity DNA chip (Agilent Technologies, California, United States) was placed horizontally on an IKA MS3 vortex mixer (IKA®-Werke GmbH & Co. KG, Staufen, Germany) and vortexed for 60 seconds at 16 *x g*. The chip was placed on an Agilent Technology 2100 Bioanalyzer (Agilent Technologies, California, United States) and the assay was run for an hour . The results were noted and used to covert ng/ µl to nM. The Agilent Technology 2100 Bioanalyzer results were illustrated in the form of a gel image and an electropherogram to show the library size distribution (Figures 3.4 and 3.5).

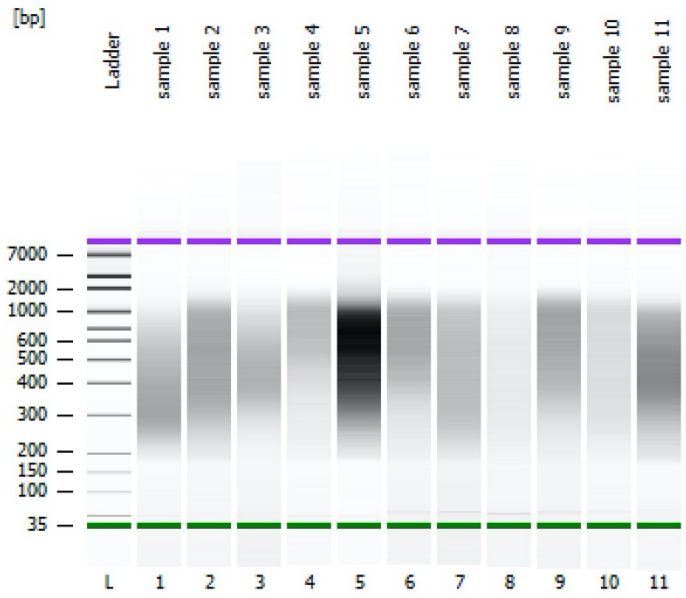
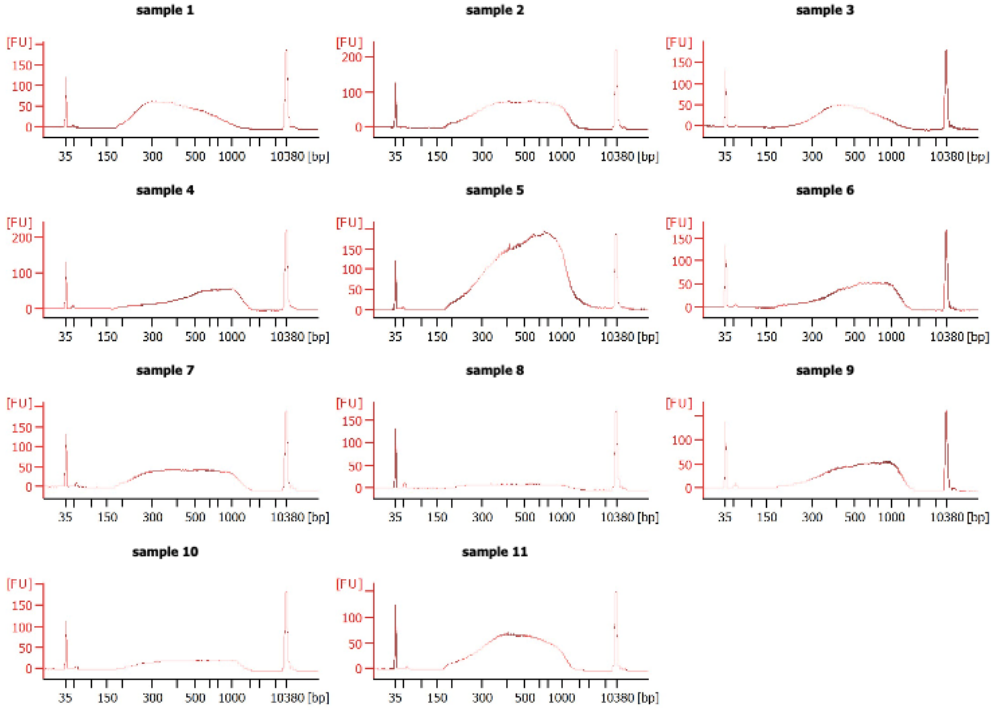


Figure 3.4: Bioanalyzer gel image showing the library size distribution.

A



B

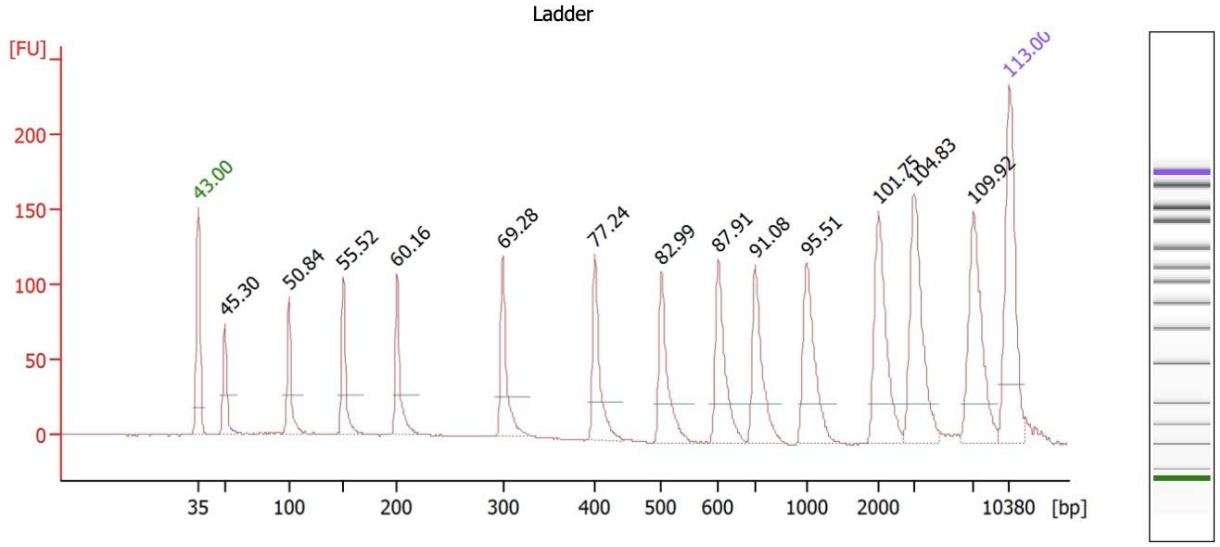


Figure 3.5: A) Bioanalyzer electropherogram showing library size distribution determined by high sensitivity dsDNA assay. **B)** Electropherogram of the ladder depicting the lower (green) and the upper (purple) markers used as a control.

3.6.4. Library pooling and denaturation

In preparation for sequencing, libraries with equal concentrations were pooled together by adding 5 µl of each library in a 1.5 ml microcentrifuge tube (Thermo Scientific, QSP, New Hampshire, United States), denatured with 0.2 N Sodium hydroxide, NaOH, (Sigma Aldrich, Missouri, United States) and then diluted to a final concentration of 20 pM at a volume of 600 µl. Briefly, 0.2 N NaOH (Sigma Aldrich, Missouri, United States) was prepared by adding 11 µl of NaOH (Sigma Aldrich, Missouri, United States) into a tube containing 250 µl molecular grade water, ultimately adding 739 µl of molecular grade water to a final volume of 1 ml. A volume of 5 µl of the freshly prepared 0.2 N NaOH (Sigma Aldrich, Missouri, United States) was added to a 1.5 ml microcentrifuge tube (Thermo Scientific, QSP, New Hampshire, United States) containing the 5 µl pooled libraries to a final volume of 10 µl. The blend was vortexed (Labnet International,Inc., New Jersey, United States) briefly and centrifuged (Prism Microcentrifuge, Labnet International,Inc., New Jersey, United States) at 280 x *g* for one minute. Furthermore, the blend was diluted to 20 pM by adding 990 µl of the prechilled Hybridization Buffer 1, HT1, (Illumina Inc., California, United States). The 20 pM denatured library was further constituted to 8 pM by transferring 240 µl of the denatured library to a new tube along with 360 µl prechilled HT1 buffer (Illumina Inc., California, United States).

The PhiX (Illumina Inc., California, United States), a bacteriophage genome, was used as a sequencing control. The PhiX (Illumina Inc., California, United States) was also diluted to 20 pM to produce an optimal cluster density using the Illumina MiSeq v3 kit, 600 cycles, (Illumina Inc., California, United States). Briefly, the 10 nM PhiX (Illumina Inc., California, United States) was diluted to 4nM. This was achieved by adding 2 µl of PhiX (Illumina Inc., California, United States) to a 1.5 ml microcentrifuge tube (Thermo Scientific, QSP, New Hampshire, United States) containing 3 µl of 10 mM Tris-Cl, pH 8.5 with 0.1% Tween 20, (Merck, Darmstadt, Germany). The 4 nM PhiX library (5 µl) was added to a tube containing 5 µl of 0.2 N NaOH (Sigma Aldrich, Missouri, United States) to denature the library. The blend was centrifuged (Prism Microcentrifuge, Labnet International,Inc., New Jersey, United States) at 280 x *g* for one minute and incubated at room temperature for five minutes. The denatured PhiX library (10 µl) was added to a tube containing 990 µl of the pre-chilled HT1 (Illumina Inc., California, United States) to dilute the PhiX library to 20 pM. Lastly, 30 µl of the 20 pM PhiX control was added to a tube containing 570 µl of the 8 pM library and the blend was heat-denatured for two minutes at 96°C on a heating block (AccuBlock™ Digital Dry Bath, Labnet International,Inc., New Jersey, United States) and immediately incubated on ice. The final library (660 µl) was loaded on an Illumina MiSeq v3 kit, 600 cycles, (Illumina, Inc., California, United States) and sequenced on an Illumina MiSeq platform (Illumina, Inc., California, United States) to generate 2 x 300 bp paired-end reads.

3.7. Data analysis

The data retrieved from the Illumina MiSeq platform (Illumina, Inc., California, United States) was analysed using various bioinformatics tools. Genome assembly and mapping of reads to reference-based sequences were conducted using Geneious Prime software v11.1.5 (Kearse et al., 2012, https://www.geneious.com/prime/) and CLC genomics Workbench v11 (CLC Bio, Qiagen, Hilden, Germany) as a complementary tool. The assembled contigs were used as query sequences in Rota C v2.0 (Maes et al., 2009, http://rotac.regatools.be/) and the Nucleotide Basic Local Alignment Search Tool (BLASTn), (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the genotype of each gene and their full-length nucleotide sequence. Reference strains were obtained from GenBank (Benson et al., 2000, http://www.ncbi.nlm.gov/genbank). Molecular Evolutionary Genetics Analysis 6 (MEGA 6), (Tamura et al., 2013, https://www.megasoftware.net) was used to construct the phylogenetic trees by aligning the sequences (Multiple alignments) using the MUSCLE algorithm, identifying the optimal evolutionary

models that best fits the sequence datasets by conducting a DNA Model Test and constructing the trees using Maximum Likelihood trees with 1000 bootstrap replicates to estimate branch support. Genetic distance matrixes for the nucleotide and amino acid sequence levels were calculated using the *p*-distance algorithm (Tamura et al., 2013). Swiss-Model protein structure homology-modelling server (Waterhouse et al., 2018, https://swissmodel.expasy.org) was used for protein modelling and the structures were modified using UCSF Chimera (Pettersen et al., 2004) and PyMoL v2. The structures were validated by YASARA (Krieger et al., 2002) and verify 3D (https://servicesn.mbi.ucla.edu/Verify3D/).

CHAPTER FOUR: WHOLE-GENOME AND *IN-SILICO* ANALYSES OF G1P[8] ROTAVIRUS STRAINS FROM PRE- AND POST-VACCINATION PERIODS IN RWANDA.

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4.1. Abstract

Rwanda was the first low-income African country to introduce RotaTeq® vaccine into its Expanded Programme on Immunization in May 2012. To gain insights into the overall genetic make-up and evolution of Rwandan G1P[8] strains pre- and post-vaccine introduction, rotavirus positive faecal samples collected between 2011 and 2016 from children under the age of five years as part of ongoing surveillance were genotyped with conventional RT-PCR based methods and whole-genome sequenced using the Illumina MiSeq platform. From a pool of samples sequenced (n=158), 36 were identified as G1P[8] strains (10 pre-vaccine and 26 post-vaccine), of which 35 exhibited a typical Wa-like genome constellation. However, one post-vaccine strain, RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU442/2012/G1P[8], exhibited a RotaTeq® vaccine strain constellation of G1-P[8]-I2-R2-C2-M2-A3-N2-T6-E2-H3, with most of the gene segments having a close relationship with a vaccine-derived reassortant strain, previously reported in the USA in 2010 and Australia in 2012. The study strains segregated into two lineages, each containing a paraphyletic pre- and post-vaccine introduction sub-lineages. In addition, the study strains demonstrated a close relationship amongst each other when compared with globally selected RVA G1P[8] reference strains. For VP7 neutralization epitopes, amino acid substitutions observed at positions T91A/V, S195D, and M217T in relation to the RotaTeq® vaccine were radical in nature and resulted in a change in polarity from a polar to a non-polar molecule, while for the VP4, amino acid differences at position D195G was radical in nature and resulted in a change in polarity from a polar to a non-polar molecule. The polarity change at position T91A/V of the neutralizing antigens might play a role in generating vaccine-escape mutants, while substitutions at positions S195D and M217T may be due to natural fluctuation of the RVA. Surveillance of RVA at the whole-genome level will enhance further assessment of vaccine impact on circulating strains, the frequency of reassortment events under natural conditions and epidemiological fitness generated by such events.

Keywords: rotavirus, Rwanda, genome constellation, epitope region, vaccine-derived strain

4.2. Introduction

Group A rotavirus is a significant viral etiological agent of AGE, resulting in approximately 125 000 deaths annually in children under five years worldwide (Troeger et al., 2018b). Rwanda rolled out the RotaTeq® (Merck and Co., Whitehouse Station, NJ, USA) vaccine into her Expanded Program on Immunization in May 2012. The vaccination coverage rate (>95%) has been consistently high over the years since 2013, resulting in a significant reduction in RVA-associated hospitalization among children younger than five years old (Ngabo et al., 2016b). Specifically, in 2013 and 2014 post introduction of the vaccine, 49% and 48% decreases in gastroenteritis cases requiring hospitalization, respectively, and 61% and 70% decreases in RV-specific diagnosis were observed, respectively (Vesikari, 2016).

The RVA is dsRNA viruses with a segmented genome enclosed within a triple-protein layer. The 11 gene segments code for six structural proteins (VP1-VP4, VP6, and VP7) and depending on the strain, five or six non-structural proteins (NSP1-NSP5/6) (Estes and Greenberg, 2013). A more complex RVA classification is in use based on the genotype properties of all the 11 gene segments as Gx-Px-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, which encodes for VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 proteins (x represents the genotype number) (Matthijnssens et al., 2011). The segmented nature of RVA enables reassortment events that drive RVA evolution along with genomic rearrangement, genetic drift, deletion of gene sequences, and zoonotic transmission (Bányai et al., 2011; Dóró et al., 2015; Kirkwood, 2010). Globally, the majority of the human RVA strains possess either the Wa-like genotype constellation (I1-R1-C1-M1-A1-N1-T1-E1-H1) or the DS-1-like genotype constellation (I2-R2-C2-M2-A2-N2-T2-E2-H2) (Bányai et al., 2012; Gentsch et al., 2005; J. Matthijnssens et al., 2008). Currently, 36 G, 51 P, 26 I, 22 R, 20 C, 20 M, 31 A, 22 N, 22 T, 27 E and 22 H genotypes have been approved by the Rotavirus Classification Working Group (https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification). Of the six common RVA genotypes (G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8]) circulating globally, the G1P[8] genotype constitutes most of the human RVA infections (Dóró et al., 2014). In Africa, the G1P[8] genotype accounts for approximately 29% of all the circulating RVA strains (Dóró et al., 2014).

To date, four oral live attenuated RVA vaccines, Rotarix® (GlaxoSmithKline, Rixensart, Belgium), RotaTeq® (Merck and Co., Whitehouse Station, NJ, USA), Rotavac® (Bharat Biotech, India) and Rotasil® (Serum Institute of India Pvt. Ltd., India) have been prequalified for global use by WHO (Kirkwood and Steele, 2018). All these vaccines are reported to be safe and highly efficacious based on clinical trial evaluations. In 2009, WHO recommended routine immunization of the RVA vaccine to all infants to reduce the high mortality associated with RVA infections (WHO, 2009). Rwanda was the first low-income country to introduce RotaTeq® vaccine in May 2012 but switched to Rotarix® in April 2017 (Seheri et al., 2018). RotaTeq® is an oral live attenuated RV vaccine comprising five bovine-human RVA reassortant strains (G1P[5], G2P[5], G3P[5], G4P[5] and G6P[8]) with a WC3 bovine backbone. The genotypes G1, G2, G3, G4 and P[8] are human rotavirus derived, while genotypes G6 and P[5] are bovine RVA derived (Dennehy, 2008). RotaTeq® is co-administered with other routine childhood vaccines in a three-dose schedule recommended at 6, 10, and 14 weeks of age (Heaton and Ciarlet, 2007).

The rationale for the RVA vaccine introduction was to combat the high morbidity and mortality of RVA-associated disease burden in Rwanda (Ngabo et al., 2014). Rotavirus strain G1P[8] is the most prevalent genotype globally, several countries have analyzed the full genome of G1P[8] strains; however, Rwanda lacks such reports (Arora and Chitambar, 2011; Bányai et al., 2011; Magagula et al., 2015; Rahman et al., 2010; Santos et al., 2019; Shintani et al., 2012; Zeller et al., 2015). Amongst these studies, one investigated the whole gene sequences of G1P[8] RVA strains collected from the pre- and post-vaccination era in South Africa and the second investigated only G1P[8] strains collected during the post-vaccination era in Brazil (Magagula et al., 2015; Santos et al., 2019). Magagula and colleagues concluded that all the G1P[8] strains analyzed exhibited a Wa-like genetic constellation and shared a moderate nucleotide identity of 89-96% and 93-95% to Rotarix® and RotaTeq® G1P[8] vaccine components, respectively. In the second study, Santos and colleagues, reported double reassortments between Wa- and DS-1-like genogroups and also, Wa- and AU-1-like genogroups. A comparison of the amino acid residues present in the antigenic epitopes of VP7 and VP4 indicated differences in the electrostatic charge distribution, between the Brazilian G1P[8] wild-types strains and Rotarix® and RotaTeq® G1P[8] vaccine strains. The availability of RVA strains collected from 2011 to 2016 in Rwanda during the pre- and post-vaccination periods with the RotaTeq® vaccine presented an opportunity to assess vaccine impact on the molecular diversity of circulating strains. To gain insight into the overall genetic makeup and evolution of the G1P[8] strains from Rwanda, whole-genome analyses was conducted and the data was compared with RVA strains from other parts of

the world. To our knowledge, this study is the first to analyze circulating RVA strains in Rwanda on a whole-genome level as compared to the traditional binary classification into G /P genotypes.

4.3. Results

4.3.1. Whole-genome constellation analyses.

A pool of 158 randomly selected Rwandan stool samples was sequenced and only 36 were genotype G1P[8], which was the inclusion criteria for this study. The genome constellations of the 35 pre- and post-vaccine G1P[8] strains described in this study were the typical G1-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1. One strain was a reassortant G1P[8] with the genetic backbone of I2-R2-C2-M2-A3-N2-T6-E2-H3 (Figure 4.1). The reassortant G1P[8] Rwandan strain was detected from a two months old infant collected four days after vaccination with the first dose of RotaTeq® vaccine. Almost all gene segments of the reassortant G1P[8] strain shared a near absolute nucleotide and amino acid identity to cognate gene sequences of the RotaTeq® vaccine G1P[8] strain. This suggested that the reassortant G1P[8] strain detected in a Rwandan child with diarrhoea was a RotaTeq® vaccine-derived G1P[8] (vdG1P[8]) strain. The lengths of the open reading frame (ORFs) for segments 1-11 for all 35 typical G1P[8] strains were 3264 bp, 2637 bp, 2505 bp, 2325 bp, 1458 bp, 1194 bp, 930 bp, 951 bp, 978 bp, 525 bp, and 594 bp, respectively. The ORF sequences for all 11 genes of these 35 Rwandan G1P[8] were sequenced and deposited in GenBank under accession numbers MN632673-MN633067 for all the gene segments.

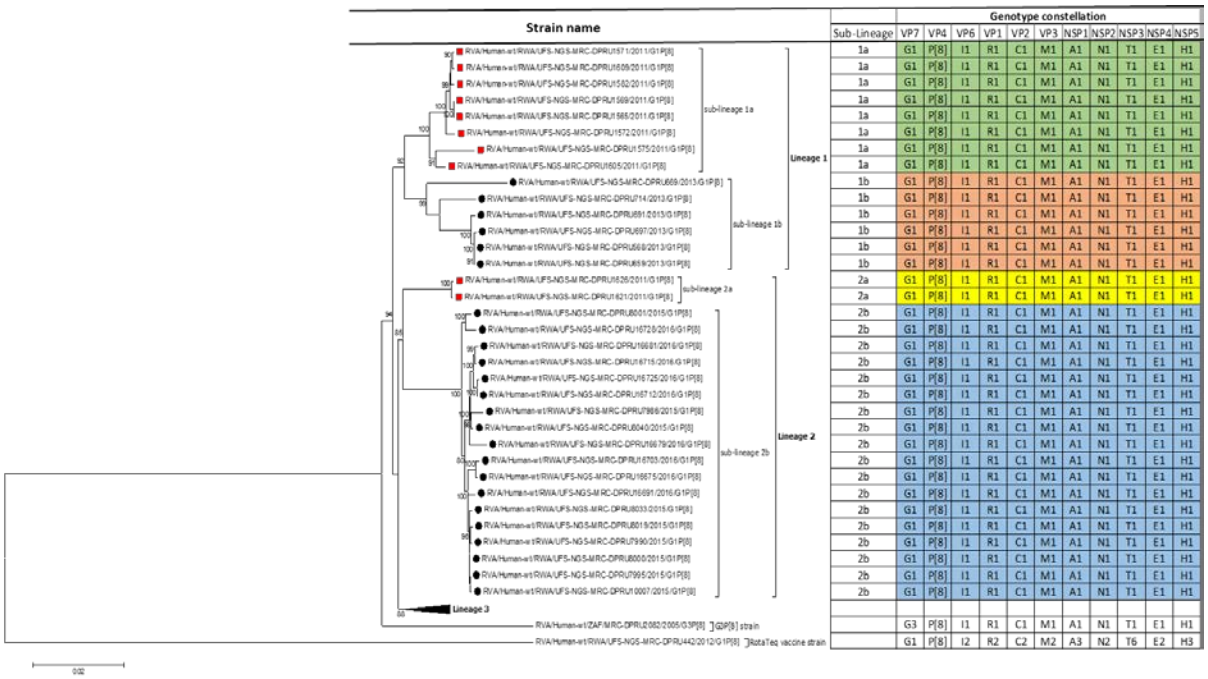

Figure 4.15: Maximum likelihood phylogram revealing the genetic relatedness of the concatenated, whole-genome ORF sequences (17,492 base pairs) for the 25 post-vaccine G1P[8] and 10 pre-vaccine G1P[8] RVA study strains characterized by whole-gene analysis. The strain-specific genotype constellations are included to the right of each strain and were identified using the assembled ORF sequences. Black circle denotes post-vaccine strains, while red square denotes pre-vaccine strains. Bootstrap values ≥ 70% are indicated at branch nodes where applicable and the scale bar shows the number of nucleotide substitutions per site. On the table, coloured in yellow and sky blue are lineage 2 pre- and post-vaccine strains belonging to sub-lineage 2a and sub-lineage 2b, respectively. Coloured in green and brown are lineage 1 pre- and post-vaccine strains belonging to sub-lineage 1a and sub-lineage 1b, respectively.

4.3.2. Phylogenetic and sequence analyses.

The concatenated ORF sequence analyses revealed the clustering of the Rwandan pre- and post-vaccine RVA strains into two lineages (Figure 4.1). Within each lineage there were two paraphyletic sub-lineages with pre-vaccine introduction strains occupying one of the sub-lineages and post-vaccine introduction strains occupying the other (Figure 4.1). Overall, the 35 typical pre- and post-vaccine introduction G1P[8] strains demonstrated a close genetic relationship amongst themselves as compared with other RVA G1P[8] strains circulating on a global scale (Figure 4.2). Even though the Rotarix® vaccine was not used in Rwanda at the time of the study, we included the Rotarix® vaccine gene sequences in all the analyses.

4.3.3. Phylogenetic and sequence analyses of the VP7 gene.

Phylogenetically, RVA G1 reference genotypes from human and animal RVA strains utilized in this analyses mapped into the seven known lineages (I-VII) and 35 of 36 Rwandan pre- and post-vaccine introduction G1P[8] strains clustered into lineage I, which consisted of a global collection of G1 strains detected during 2003-2015 RVA seasons (Le et al., 2010; Magagula et al., 2015). The lineage I strains were further segregated into sub-lineages usually by year and in some cases by vaccination status. Within this lineage I, the G1 strains segregated into two sub-lineages: one consisted of 19 G1 strains detected in the post-vaccination era and the other was a mixture of both pre-vaccine (ten G1) and post-vaccine (five G1) introduction strains (Figure 4.2). The nucleotide (nt) and amino acid (aa) identities of the 19 G1 post-vaccine introduction strains amongst themselves was in the range of 99.2-100% and 98.0-100%, respectively, whereas the nt (aa) similarities of the five post- and ten pre-vaccine introduction mixed G1 sub-lineage was 94.7-100% (92.7-100%). Further comparison of the G1 strains in the typical post-vaccine sub-lineage with those in the mixed pre- and post-vaccine sub-lineage revealed a nt (aa) similarities of 92.7-96.3% (90.7-96.0%). The VP7 gene of Rwandan post-vaccination introduction strain RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU669/2013/G1P[8] was an orphan gene that did not cluster in either the typical post-vaccine sub-lineage or mixed pre-and post-vaccine introduction sub-lineage (Figure 4.2). The VP7 gene sequence of the RotaTeq® vdG1P[8] strain, RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU442/2012/G1P[8], clustered into lineage III (Figure 4.2) alongside cognate gene sequence of the RotaTeq® vaccine strain RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P7[5] (Figure 4.2). The VP7 gene sequences of strain RVA/Human-wt/RWA/UFS-NGS-MRCDPRU442/2012/G1P[8] displayed absolute identity with the VP7 gene of RotaTeq® reassortant strain, RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P7[5] and appeared to be a RotaTeq VP7 gene.

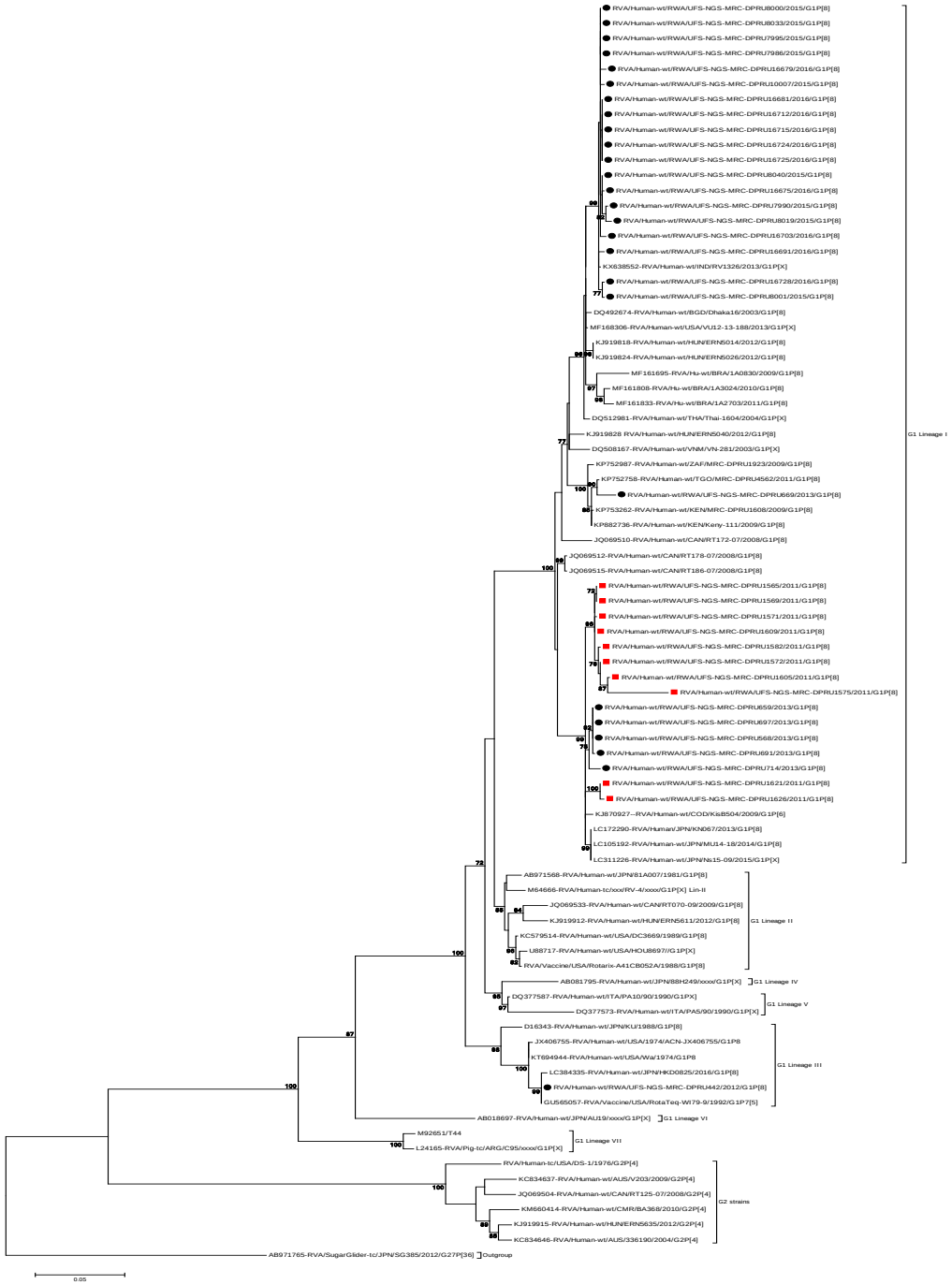

Figure 4.16: Phylogenetic relatedness of rotavirus group A species based on VP7 of the study strains from Rwanda with representatives of known human and animal rotavirus genotypes. Pre- and post-vaccine strains are indicated with red squares and black circles, respectively. Bootstrap values ≥ 70% are indicated at each branch node. Scale bars represent substitutions per nucleotide site.

4.3.4. Comparative analyses of neutralizing antigenic epitopes in the VP7 proteins of Rwandan G1P[8] and vaccine strains of RVA.

Structurally, the VP7 gene contains two defined neutralization epitopes: 7-1 and 7-2 (Aoki et al., 2009). Aoki and colleagues further subdivided 7-1 epitopes into 7-1a and 7-1b (Aoki et al., 2009). These three antigenic epitopes comprise 29 amino acid residues (14 residues in 7-1a; 6 residues in 7-1b and 9 residues in 7-2). Using the G1 alignment for the VP7 gene, we identified amino acid differences in these neutralization epitopes between the wild-type Rwandan G1 RVA strains and the RotaTeq® and Rotarix® vaccine G1 strain (Figure 4.3A). Out of these 29 amino acid residues, 20 (amino acids G96, W98, K99, Q104, V129 and D130 in 7-1a region, all positions in 7-1b region and K143, D145, Q146, N221, G264 in the 7-2 region) were completely conserved among all the Rwandan G1 strains. Relative to the G1 component of the RotaTeq® vaccine, the Rwandan G1 strains showed up to 10-11 differences (T87I, T91A/V, N94S, D97E, D100E, S123N, K291R, S147N, L148F, and M217T) located on the surface of the protein structure RotaTeq® (Figure 4.3A and 4.3B). Among these changes seen relative to RotaTeq® vaccine G1 strain, only the T91A/V, S190D, and M217T substitution at positions 91, 190, and 217 involve a change from a polar to a non-polar molecule, polar to a negative molecule, and non-polar to a polar molecule, respectively, were radical in nature. The remainder of the changes seen were neutral or conservative in nature.

A

		7-1a													7-1b						7-2							
		•	•	•	•	•	•	•	•	•					•	•		•	•			•		•	•	•		•
	Lineage	87	91	94	96	97	98	99	100	104	123	125	129	130	291	201	211	212	213	238	242	143	145	146	147	148	190	217	221	264
RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P1A[8]	II	T	T	N	G	E	W	K	D	Q	S	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	M	N	G
RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P7[5]	III	T	T	N	G	D	W	K	D	Q	S	V	V	D	K	Q	N	V	D	N	T	K	D	Q	S	L	S	M	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU442/2012/G1P[8]	**III**	T	T	N	G	D	W	K	D	Q	S	V	V	D	K	Q	N	V	D	N	T	K	D	Q	S	L	S	M	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16675/2016/G1P[8]	**I**	T	A	S	G	E	W	K	D	Q	N	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16679/2016/G1P[8]	**I**	T	A	S	G	E	W	K	D	Q	N	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16681/2016/G1P[8]	**I**	T	V	S	G	E	W	K	D	Q	N	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16691/2016/G1P[8]	**I**	T	T	S	G	E	W	K	D	Q	N	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16703/2016/G1P[8]	**I**	T	A	S	G	E	W	K	D	Q	N	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16712/2016/G1P[8]	**I**	T	V	S	G	E	W	K	D	Q	N	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16715/2016/G1P[8]	**I**	T	V	S	G	E	W	K	D	Q	N	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16724/2016/G1P[8]	**I**	T	V	S	G	E	W	K	D	Q	N	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16725/2016/G1P[8]	**I**	T	V	S	G	E	W	K	D	Q	N	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16728/2016/G1P[8]	**I**	T	T	S	G	E	W	K	D	Q	N	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU7986/2015/G1P[8]	**I**	T	A	S	G	E	W	K	D	Q	N	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU7990/2015/G1P[8]	**I**	I	A	S	G	E	W	K	D	Q	N	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU7995/2015/G1P[8]	**I**	T	A	S	G	E	W	K	D	Q	N	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU8000/2015/G1P[8]	**I**	T	A	S	G	E	W	K	D	Q	N	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU8001/2015/G1P[8]	**I**	T	T	S	G	E	W	K	D	Q	N	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU8019/2015/G1P[8]	**I**	T	V	S	G	E	W	K	D	Q	N	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU8033/2015/G1P[8]	**I**	T	A	S	G	E	W	K	D	Q	N	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU8040/2015/G1P[8]	**I**	T	A	S	G	E	W	K	D	Q	N	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU10007/2015/G1P[8]	**I**	T	A	S	G	E	W	K	D	Q	N	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU568/2013/G1P[8]	**I**	T	T	S	G	E	W	K	E	Q	N	V	V	D	R	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU659/2013/G1P[8]	**I**	T	T	S	G	E	W	K	E	Q	N	V	V	D	R	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU669/2013/G1P[8]	**I**	T	T	S	G	E	W	K	D	Q	N	V	V	D	R	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU691/2013/G1P[8]	**I**	T	T	S	G	E	W	K	E	Q	N	V	V	D	R	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU697/2013/G1P[8]	**I**	T	T	S	G	E	W	K	E	Q	N	V	V	D	R	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU714/2013/G1P[8]	**I**	T	T	S	G	E	W	K	E	Q	N	A	V	D	R	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1565/2011/G1P[8]	**I**	T	T	S	G	E	W	K	E	Q	N	V	V	D	R	Q	N	V	D	N	T	K	D	Q	N	F	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1569/2011/G1P[8]	**I**	T	T	S	G	E	W	K	E	Q	N	V	V	D	R	Q	N	V	D	N	T	K	D	Q	N	F	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1571/2011/G1P[8]	**I**	T	T	S	G	E	W	K	E	Q	N	V	V	D	R	Q	N	V	D	N	T	K	D	Q	N	F	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1572/2011/G1P[8]	**I**	T	T	S	G	E	W	K	E	Q	N	V	V	D	R	Q	N	V	D	N	T	K	D	Q	N	F	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1575/2011/G1P[8]	**I**	T	T	S	G	E	W	K	E	Q	N	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	F	D	I	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1582/2011/G1P[8]	**I**	T	T	G	G	E	W	K	E	Q	N	V	V	D	R	Q	N	V	D	N	T	K	D	Q	N	F	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1605/2011/G1P[8]	**I**	T	T	S	G	E	W	K	E	Q	N	V	V	D	R	Q	N	V	D	N	T	K	D	Q	N	F	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1609/2011/G1P[8]	**I**	T	T	S	G	E	W	K	E	Q	N	V	V	D	R	Q	N	V	D	N	T	K	D	Q	N	F	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1621/2011/G1P[8]	**I**	T	T	S	G	E	W	K	E	Q	N	V	V	D	R	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1626/2011/G1P[8]	**I**	T	T	S	G	E	W	K	E	Q	N	V	V	D	R	Q	N	V	D	N	T	Q	D	Q	N	L	S	T	N	G
RVA/Human-wt/TGO/MRC-DPRU4562/2011/G1P[8]	I	T	T	S	G	E	W	K	D	Q	N	V	V	D	R	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/JPN/MU14-18/2014/G1P[8]	I	T	T	S	G	E	W	K	E	Q	N	V	V	D	R	Q	N	V	D	N	T	K	D	Q	N	L	S	T	N	G
RVA/Human-wt/CAN/RT070-09/2009/G1P[8]	II	T	T	N	G	E	W	K	D	Q	S	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	M	N	G
RVA/Human-wt/USA/Wa/1974/G1P[8]	III	T	T	N	G	D	W	K	D	Q	S	V	V	D	K	Q	N	V	D	N	T	K	D	Q	S	L	S	M	N	G
RVA/Human-wt/JPN/88H249/xxxx/G1P[X]	IV	T	T	S	G	E	W	K	D	Q	N	V	V	D	R	Q	N	V	D	N	T	K	D	Q	N	L	S	M	N	G
RVA/Human-wt/ITA/PA10/90/1990/G1P[X]	V	T	T	N	G	E	W	K	D	Q	S	V	A	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	M	N	G
RVA/Human-wt/JPN/AU19/xxxx/G1P[X]	VI	I	N	N	G	E	W	K	D	Q	S	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	I	D	G
RVA/Pig-tc/ARG/C95/xxxx/G1P[X]	VII	V	N	N	G	E	W	K	D	Q	N	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	I	N	G

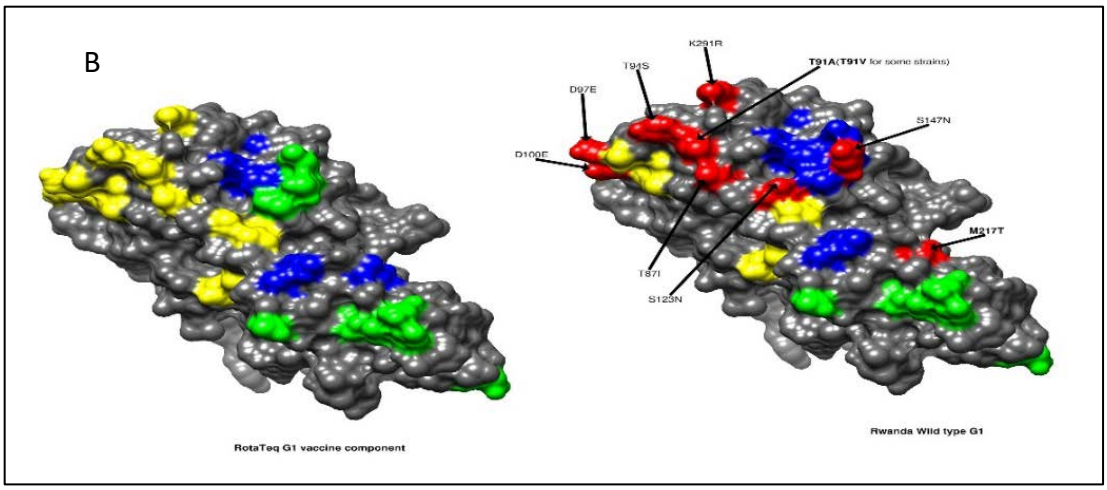

Figure 4.17: A) The alignment of the G1 component of Rotarix® and RotaTeq® vaccines and Rwandan wildtypes circulating from 2011- 2016 RVA seasons, based on the three VP7 antigenic residues (7-1a, 7-1b, and 7-2). Amino acids residues at positions 97 and 147 differ between Rotarix® and RotaTeq® and are indicated in boldface. Study strains amino acid residues highlighted in sky blue differs from both Rotarix® and RotaTeq®, while the green and brown coloured residues are different from Rotarix® and RotaTeq®, respectively. The black dot indicates changes in the residues associated with escape neutralization with monoclonal antibodies. Post-vaccine and pre- vaccine G1P[8] study strains are bolded in black and red, respectively. **B)** Location of surface-exposed amino acids differences between the VP7 protein of RotaTeq® G1 vaccine component versus a G1 wild-type strain from Rwanda (indicated in red). Antigenic epitopes in 3B are coloured in yellow (7-1a), green (7-1b), and blue (7-2) (created with PyMoL, 4 June 2019)

4.3.5. Comparative analyses of cytotoxic T lymphocytes epitopes of the G1 proteins of Rwandan and vaccine strains of RVA.

Cytotoxic T-lymphocytes have been linked to RV infection clearance, resulting in complete short-term and partial long-term protection against re-infection (Franco et al., 1997). Two linear CTL epitopes responsible for this activity have been reported on VP7 protein at amino acid positions 16-28 and 40-52 (Morozova et al., 2015; Wei et al., 2009). An analyses of the Rwandan G1 pre- and post-vaccine introduction strains showed three amino acid differences at positions T41F/S, V42M, and A46V with G1 component of the RotaTeq® and Y41F/S, V42M and A46V to Rotarix® vaccines (Figure 4.4). Amongst these amino acid differences, the change at position T41F relative to the RotaTeq® G1 gene sequence was radical in nature and resulted in a change in polarity from polar to non-polar, while the other two substitutions were conservative in nature. Twenty-three out of the 26 amino acids that comprise the T-cell antigen epitopes were completely conserved.

		T-cell epitope 16-28 aa													T-cell epitope 40-52 aa											
	Lineage	16	17	18	19	20	21	22	23	24	25	26	27	28	40	41	42	43	44	45	46	47	48	49	50	51	52
RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P1A[8]	II	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	Y	V	A	L	F	A	L	T	R	A	Q	N
GU565057-RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P7[5]	III	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	T	V	A	L	F	A	L	T	R	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16675/2016/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	F	M	A	L	F	V	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16679/2016/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	F	M	A	L	F	V	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16681/2016/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	F	M	A	L	F	V	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16691/2016/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	F	M	A	L	F	V	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16703/2016/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	F	M	A	L	F	V	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16712/2016/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	F	M	A	L	F	V	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16715/2016/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	F	M	A	L	F	V	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16724/2016/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	F	M	A	L	F	V	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16725/2016/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	F	M	A	L	F	V	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16728/2016/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	F	M	A	L	F	V	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU10007/2015/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	F	M	A	L	F	V	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU7986/2015/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	F	M	A	L	F	V	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU7990/2015/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	F	M	A	L	F	V	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU7995/2015/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	F	M	A	L	F	V	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU8000/2015/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	F	M	A	L	F	V	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU8001/2015/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	F	M	A	L	F	V	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU8019/2015/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	F	M	A	L	F	V	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU8033/2015/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	F	M	A	L	F	V	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU8040/2015/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	F	M	A	L	F	V	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU442/2012/G1P[8]	III	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	T	V	A	L	F	A	L	T	R	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU568/2013/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	S	V	A	L	F	A	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU659/2013/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	S	V	A	L	F	A	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU669/2013/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	F	V	A	L	F	A	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU691/2013/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	S	V	A	L	F	A	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU697/2013/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	S	V	A	L	F	A	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU714/2013/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	S	V	A	L	L	A	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1565/2011/G1P[8]	I	I	I	L	I	N	Y	I	L	K	S	V	T	R	I	S	V	A	L	F	A	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1569/2011/G1P[8]	I	I	I	L	I	N	Y	I	L	K	S	V	T	R	I	S	V	A	L	F	A	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1571/2011/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	S	V	A	L	F	A	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1572/2011/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	S	V	A	L	F	A	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1575/2011/G1P[8]	I	I	I	L	I	N	Y	I	L	K	S	V	T	R	I	S	V	A	L	F	A	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1582/2011/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	S	V	A	L	F	A	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1605/2011/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	S	V	A	L	F	A	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1609/2011/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	S	V	A	L	F	A	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1621/2011/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	S	V	A	L	L	A	L	T	K	A	Q	N
RVA/Human-wt/TGO/MRC-DPRU4562/2011/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	F	V	A	L	F	A	L	T	K	A	Q	N
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1626/2011/G1P[8]	I	I	I	L	L	N	Y	I	L	K	S	V	T	R	I	S	V	A	L	L	A	L	T	K	A	Q	N

Figure 4.18: Alignment of antigenic residues in T-cell antigen epitopes of the G1 vaccine component contained in Rotarix® and RotaTeq® compared to Rwanda G1 wild type strains circulating from 2011–2016 RVA seasons. Amino acid changes are highlighted in sky blue.

4.3.6. Phylogenetic and sequence analyses of VP4 genes.

The VP4 gene sequences of the 35 Rwandan strains and a single RotaTeq® vdG1P[8] strain collected in the pre- and post-vaccination periods were compared with representative human RVA from the four established VP4 P[8] genotype lineages (I to IV) (Arista et al., 2006; Le et al., 2010). Except for RotaTeq® vdG1P[8] strain RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU442/2012/G1P[8] which clustered in P[8]-lineage II with the P[8] component of RotaTeq® vaccine strain RVA/vaccine/USA/RotaTeq-W-179-4/1992/G6P1A[8], the other 35 study strains clustered in P[8]-lineage III (Figure 4.5). Within P[8]-lineage III cluster, the Rwandan strains segregated into three typical and one mixed sub-lineages (Figure 4.5). The first sub-lineage consisted of eight pre-vaccine introduction P[8] and a single post-vaccine introduction P[8] strains and they shared nt (aa) identities in the range of 99.7-100% (99.4-100%) amongst themselves (Figure 4.5). The second sub-lineage comprised of only two pre-vaccine introduction strains detected in 2011 RVA season and shared an absolute identity with each other (Figure 4.5). The third sub-lineage was characterised of five post-vaccine introduction strains circulating in the 2013 RVA season and shared a nt (aa) similarities amongst themselves ranging from 99.4-99.9% (98.6-100%) (Figure 4.5) and the fourth sub-lineage comprised of 19 post-vaccine introduction P[8] strains and shared nt (aa) identities amongst themselves in the range of 98.8-100% (98.0-100%) (Figure 4.5). Comparison of pre-vaccine introduction strains in the mixed sub-lineage to the post-vaccine introduction strains in the third and fourth sub-

lineages revealed moderate nt (aa) similarities in the range of 95.7-97.8% (89.5-94.5%). The VP4 P[8] component of the RotaTeq® vdG1P[8] strain RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU442/2012/G1P[8] clustered distinctly in P[8]-lineage II together with the RotaTeq® vaccine strain RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P1A8 and G3P[8] strains detected in 1976 in the US, G1P[8] strains detected in 2008 in Australia and G1P[8] detected in 2016 in Japan (Figure 4.5).

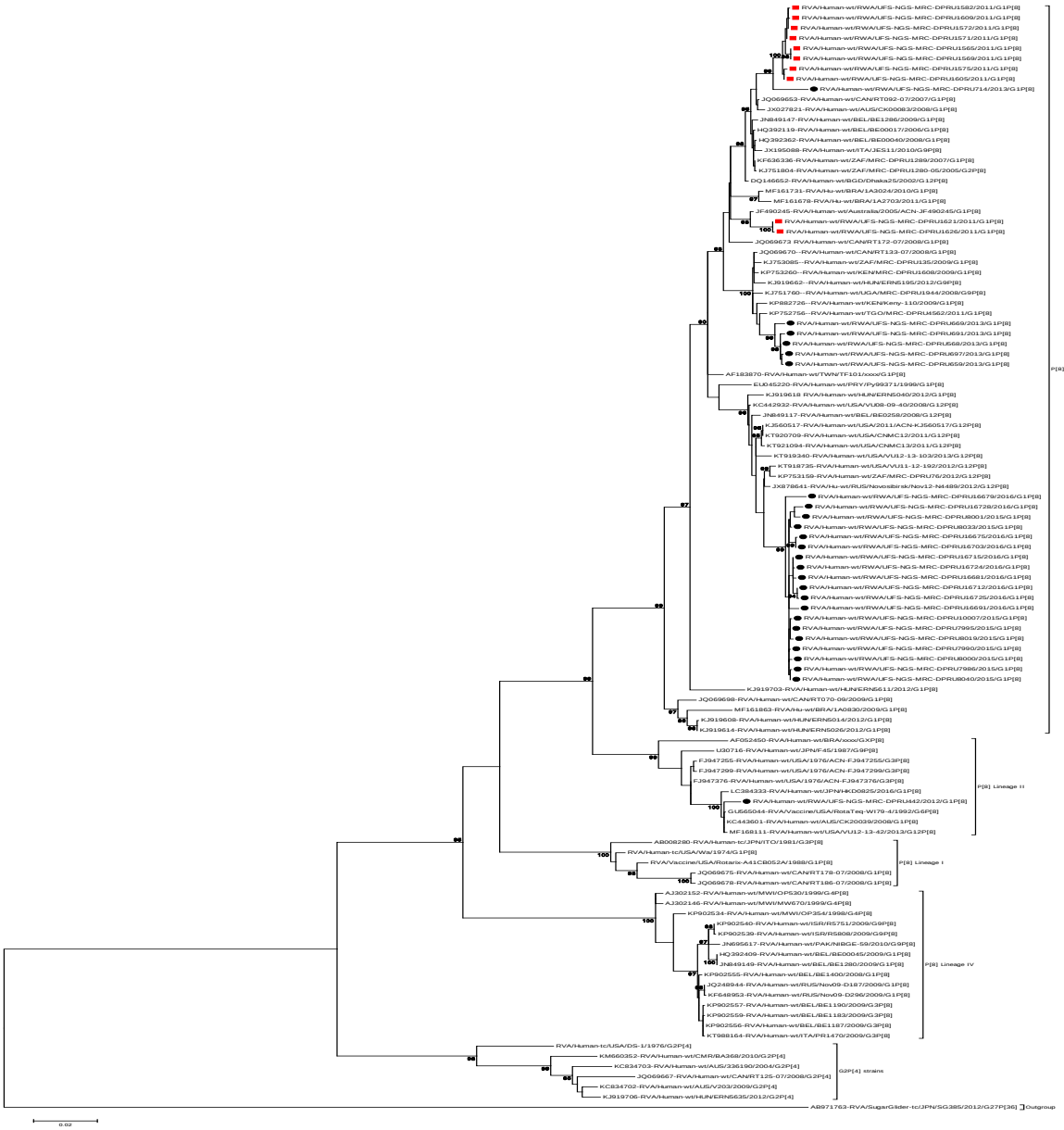

Figure 4.19: Phylogenetic relatedness of rotavirus group A species base on VP4 of the study strains from Rwanda with representatives of known human and animal rotavirus genotypes. Pre- and post-vaccine strains are indicated with red squares and black circles, respectively. Bootstrap values ≥ 70% are indicated at each branch node. Scale bars represent substitutions per nucleotide site.

4.3.7. Comparative analyses of neutralizing antigenic epitopes in the VP4 protein of Rwandan G1P[8] and vaccine strains of RVA.

The VP4 protein, which in this study represented P[8] strains, was divided into the VP8* and VP5* regions for comparison. The VP8* region contains four (8-1 to 8-4) neutralizing antigenic epitopes, while the VP5* region has five (5-1 to 5-5) (Dormitzer et al., 2002). These two epitopes contain 37 amino acid residues, 25 in the VP8* and 12 in the VP5* antigenic epitope regions. Out of these 37 amino acid residues that spans the neutralization epitopes, those at positions 100, 146, 148, 150, 188, 190 and 194 (8-1 region), 180 and 183 (8-2 region), 114, 116, 132, 133, and 135 (8-3 region), 87, 88, and 89 (8-4 region), 384, 386, 388, 393, 394, 398, 440, and 441 (5-1 region), 434 (5-2 region), 459 (5-3 region), 429 (5-4 region), and 306 (5-5 region), are known neutralization escape mutation sites (Fig. 6A) (Dormitzer et al., 2004; Monnier et al., 2006). A comparison of the Rwandan P[8] strains to the P[8] component of RotaTeq® and Rotarix® vaccines showed 29 identical amino acid residues distributed through the antigenic epitopes of the VP4 gene (Figure 4.6A). The differences between the P[8] study strains and vaccine P[8] component of RotaTeq® and Rotarix® were mostly contained in VP8* epitopes 8-1 and 8-3. Relative to the RotaTeq®, the P[8] component of the Rwandan G1P[8] strains exhibited 3-4 amino acid differences. The changes identified relative to the RotaTeq® P[8] vaccine strain were seen at positions E150D and D195G in the neutralization epitope 8-1 region (Figure 4.6A-4.6B) and were all located on the surface of the protein structure. Overall, the amino acid changes identified at positions N113D, S131R and N135D, resulted in a change in charge from a polar molecule with a potential side chain with five hydrogen bonds to a negatively charged molecule with four hydrogen bonds. Finally, amino acid substitutions at position N195G for both vaccines, resulted in a change in polarity.

A		VP8*																								VP5*											
		8-1											8-2		8-3									8-4			5-1								5-2	5-3	5-4	5-5
		•	•	•	•	•	•			•			•	•		•		•			•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	Lineage	100	146	148	150	188	190	192	193	194	195	196	180	183	113	114	115	116	125	131	132	133	135	87	88	89	384	386	388	393	394	398	440	441	434	459	429	306
RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P1A[8]	P[8]-I	D	S	Q	E	S	T	N	L	N	**N**	I	T	A	N	P	V	D	**S**	**S**	N	D	**N**	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P1A[8]	P[8]-II	D	S	Q	E	S	T	N	L	N	**D**	I	T	A	N	P	V	D	**N**	**R**	N	D	**D**	N	T	N	Y	F	L	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU442/2012/G1P[8]	P[8]-II	D	S	Q	E	S	T	N	L	N	D	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	L	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16675/2016/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16679/2016/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16681/2016/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	S	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16691/2016/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16703/2016/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16712/2016/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	S	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16715/2016/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	S	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16724/2016/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	S	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16725/2016/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	S	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU16728/2016/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU10007/2015/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU7986/2015/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU7990/2015/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU7995/2015/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU8000/2015/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU8001/2015/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU8019/2015/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU8033/2015/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU8040/2015/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU568/2013/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	D	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU659/2013/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	D	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU669/2013/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	D	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU691/2013/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	D	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU697/2013/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	D	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU714/2013/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1565/2011/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1569/2011/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1571/2011/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1572/2011/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1575/2011/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1582/2011/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1605/2011/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1609/2011/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1621/2011/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	D	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1626/2011/G1P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	D	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-tc/USA/Wa/1974/G1P[8]	P[8]-I	D	S	Q	E	S	T	N	L	N	N	I	T	A	N	P	V	D	S	S	N	D	N	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-tc/JPN/ITO/1981/G3P[8]	P[8]-I	D	S	Q	E	S	T	N	L	H	H	I	T	A	N	P	V	D	S	S	N	D	N	N	T	N	Y	F	L	W	P	G	R	T	P	E	L	R
RVA/Human-wt/JPN/F45/1987/G9P[8]	P[8]-II	D	S	Q	E	S	T	N	L	N	N	I	T	A	N	P	V	D	N	R	N	D	N	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/ZAF/MRC-DPRU76/2012/G12P[8]	P[8]-III	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	I	W	P	G	R	T	P	E	L	R
RVA/Human-wt/MWI/OP354/1998/G4P[8]	P[8]-IV	D	S	Q	E	S	T	D	L	T	S	I	T	A	D	P	V	D	S	R	N	D	N	N	T	N	Y	F	L	W	P	G	R	T	P	D	L	R

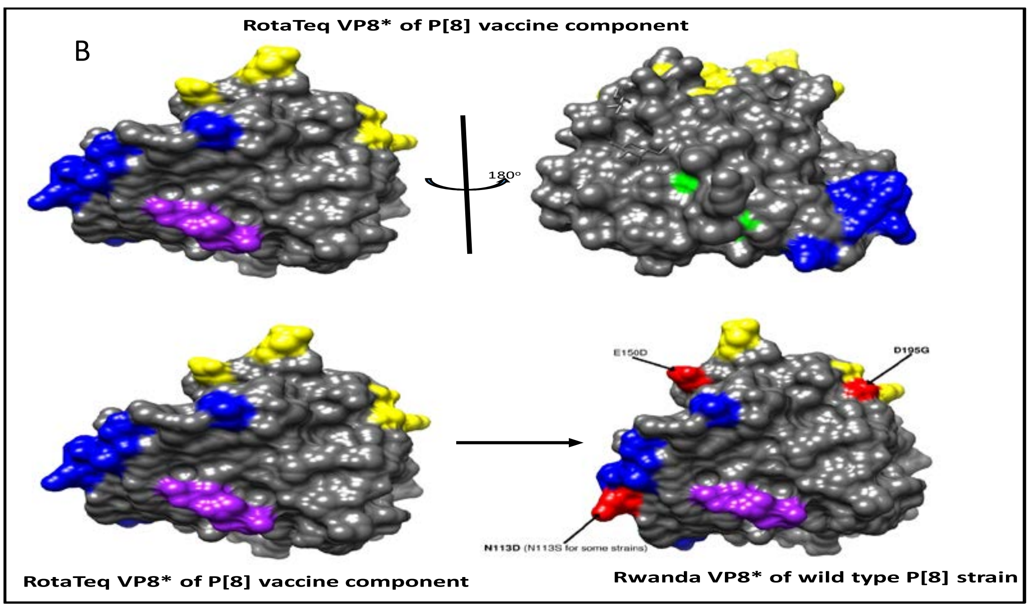

Figure 4.20: A) The alignment of the P[8] component of Rotarix® and RotaTeq® vaccines and wild type P[8] strains circulating in Rwanda from 2011–2016 RVA seasons, based on the antigenic residues in VP4. VP4 is divided into two regions VP8* (antigenic epitope 8-1, 8-2, 8-3, and 8-4) and VP5* (antigenic epitope 5-1, 5-2, 5-3, 5-4, and 5-5). The black dot indicates changes in the residues associated with escape neutralization with monoclonal antibodies. Amino acids differences between Rotarix® and RotaTeq® are shown at positions 195 (antigenic epitope 8-1), 125, 131, and 135 (antigenic epitope 8-3) and are indicated in boldface. Study strains amino acid residues highlighted in green and brown are residues that are different from Rotarix® and RotaTeq®, respectively. Also, amino acids differences between study strains and Rotarix® and RotaTeq® are highlighted in sky blue. Post-vaccine and pre- vaccine G1P[8] study strains are bolded in black and red, respectively. **B)** Location of surface-exposed amino acids differences between

VP8* protein of RotaTeq® P[8] vaccine component versus a wild-type P[8] strain from Rwanda (indicated in red). Antigenic epitopes in (B) are coloured in yellow (8-1), green (8-2), blue (8-3), and purple (8-4) (created with PyMoL, 4 June 2019)

4.3.8. Phylogenetic and sequence analyses of VP1-VP3, NSP1, NSP4, and NSP5.

These six genes segregated into typical pre- and post-vaccine introduction clusters, hence presented together. Phylogenetic analyses based on VP1-VP3, NSP1, NSP4, and NSP5 nucleotide sequences of each Rwandan G1P[8] strain showed that each gene (exception of RotaTeq® vdG1P[8] strain) segregated and clustered with cognate gene sequences of Wa-like strains belonging to genotype 1 (Figure 4.7A-4.7F). The VP1 gene sequences of the 35 typical Rwandan G1P[8] strains segregated into a single pre-vaccine introduction sub-lineage exhibiting nt (aa) similarities in the range of 99.9-100% (99.8-100%) and two post-vaccine introduction sub-lineages with nt (aa) identities in the range of 96.8-98.0% (98.7-99.5%). (Figure 4.2A). Four pre-vaccine introduction orphan strains that did not cluster in either of the three sub-lineages were also observed (Figure 4.7A). For VP2, the C1 genes segregated into three typical sub-lineages (one typical pre- and two post-vaccine introduction) and five orphan strains which did not cluster into either of the three sub-lineages (Figure 4.7B). The two post-vaccine introduction sub-lineage consisting of five and 19 strains shared nt (aa) similarities in the range of 99.6-100% (99.6-100%) and 99.3-100% (99.7-100%), respectively. Furthermore, the typical pre-vaccine introduction sub-lineage incorporated strains (n=6) detected in the 2011 RVA season and with nt (aa) sequence identities amongst themselves in the range of 99.9-100% (99.8-100%).

Phylogenetically, the VP3 gene sequences of the Rwandan pre- and post-vaccine introduction strains separated into three typical sub-lineages and three orphan strains were observed (Figure 4.7C). The first post-vaccine sub-lineage consisted of 19 strains and the second one had five strains that shared nt (aa) similarities ranging from 99.2-100% (98.9-100%) and 99.4-100% (99.3-100%), respectively. The pre-vaccine introduction sub-lineage contained strains that shared nt (aa) identities of 99.3-100% (98.5-100%). The NSP1 gene sequences of the typical Rwandan pre- and post-vaccine introduction G1P[8] strains were grouped into a single sub-lineage consisted of all ten pre-vaccine introduction strains and three typical post-vaccination introduction sub-lineages (Figure 4.7D). The pre-vaccine strains shared nt (aa) similarities that ranged from 99.0-99.9% (97.5-99.8%). The three post-vaccine introduction NSP1 genes that segregated into three sub-lineages revealed nt (aa) identities of 98.7-99.9% (98.1-99.8%), 99.0-99.9% (98.4-99.8%) and 98.2-99.9% (98.1-99.8%), respectively.

The NSP4 gene sequences of the Rwandan strains were also segregated into two typical post-vaccination sub-lineages and a single pre-vaccination sub-lineage (Figure 4.7E). Six orphan Rwandan E1 genotypes did not cluster into either the pre- or post-vaccine introduction sub-lineages (Figure 4.7E). The first typical post-vaccine introduction strain sub-lineage comprised of 19 NSP4 genes and the second one had five E1 strains detected in 2011 RVA season which exhibited nt (aa) identities in the range of 99.4-100% (99.4-100%) and 99.4-100% (99.4-100%), respectively. The pre-vaccination sub-lineage is comprised of five NSP4 strains with absolute gene identities among themselves. The NSP5 gene did not resolve phylogenetically, and the bootstrap support was not enough to separate the strains into distinct sub-lineages. On the other hand, a homologous relationship was observed for the VP1, VP2, VP3, NSP1, NSP4, and NSP5 gene segments of strain RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU442/2012/G1P[8] with cognate gene sequences of RVA/Vaccine/USA/Rotateq-W179-4/1992/G6P1A8.

4.7A VP1


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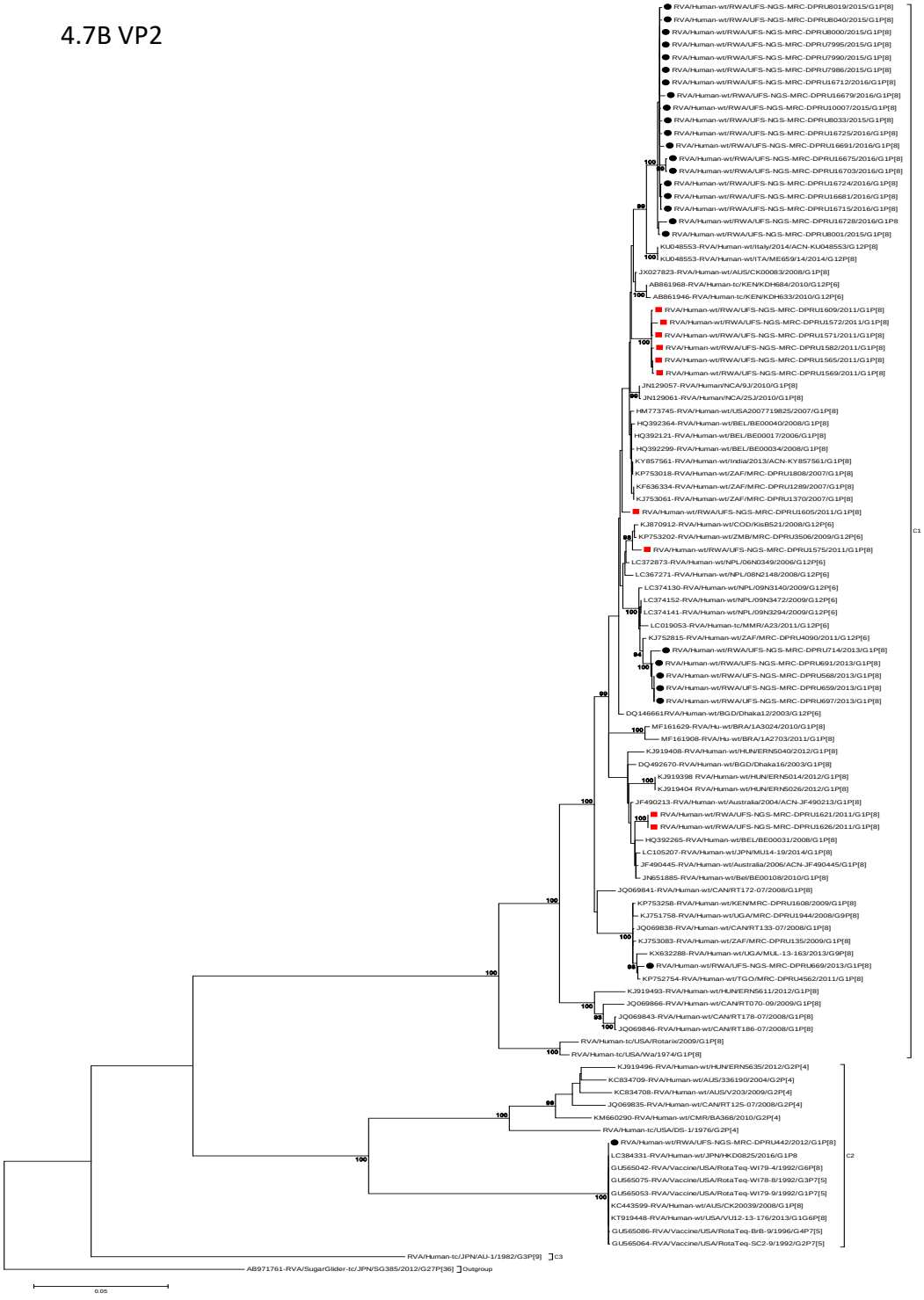
4.7B VP2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C2
C3
Outgroup
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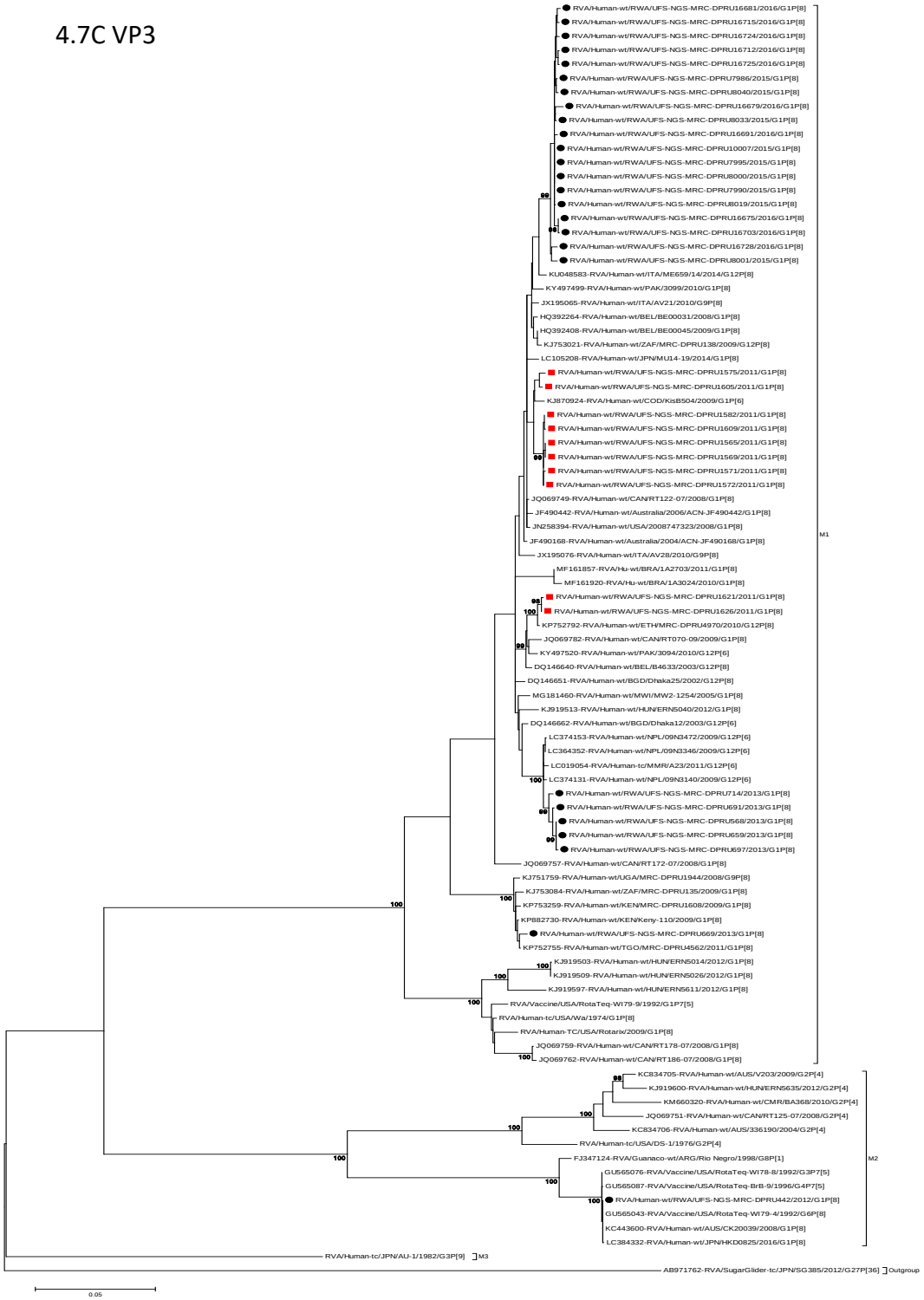
4.7C VP3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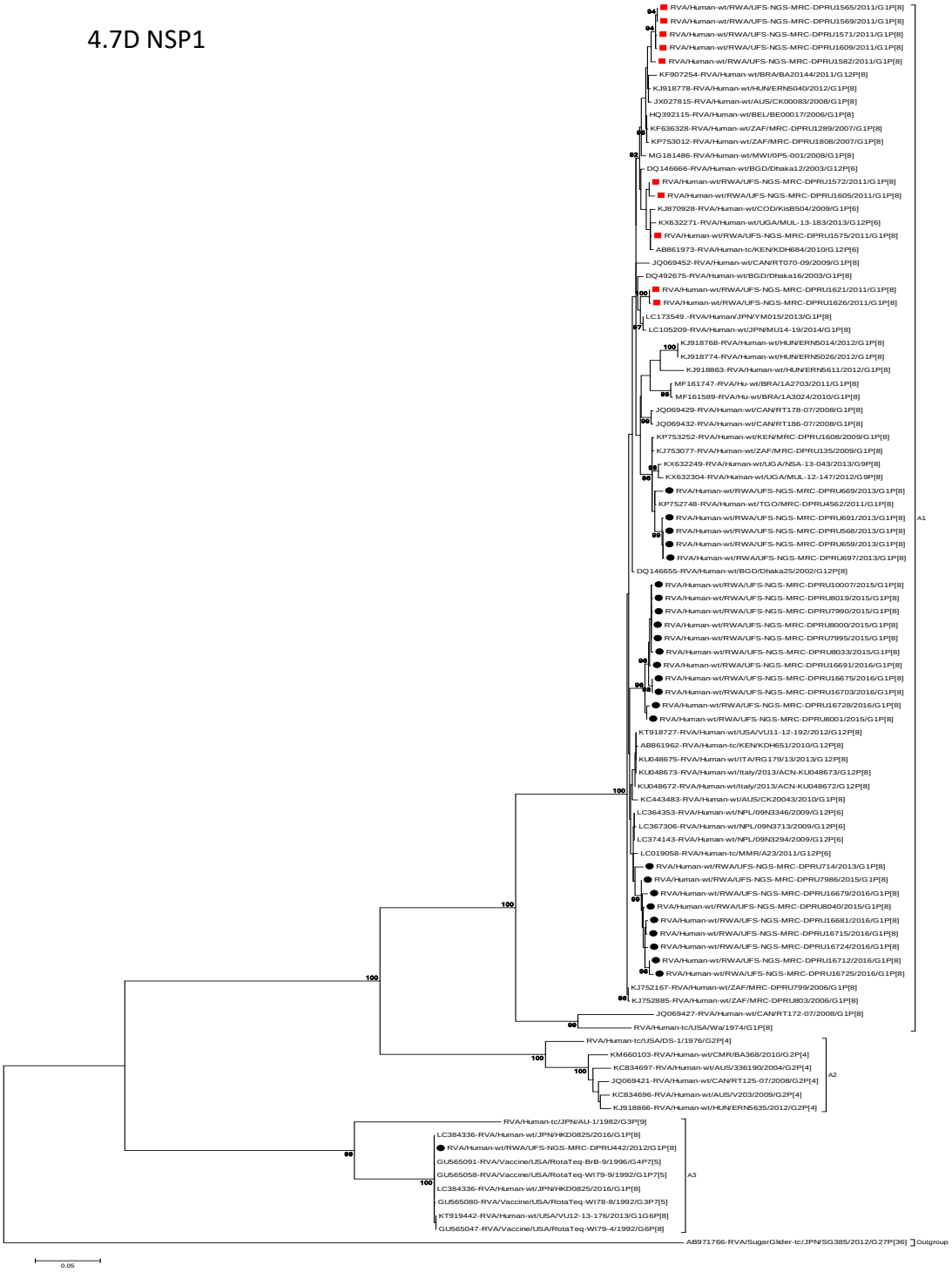
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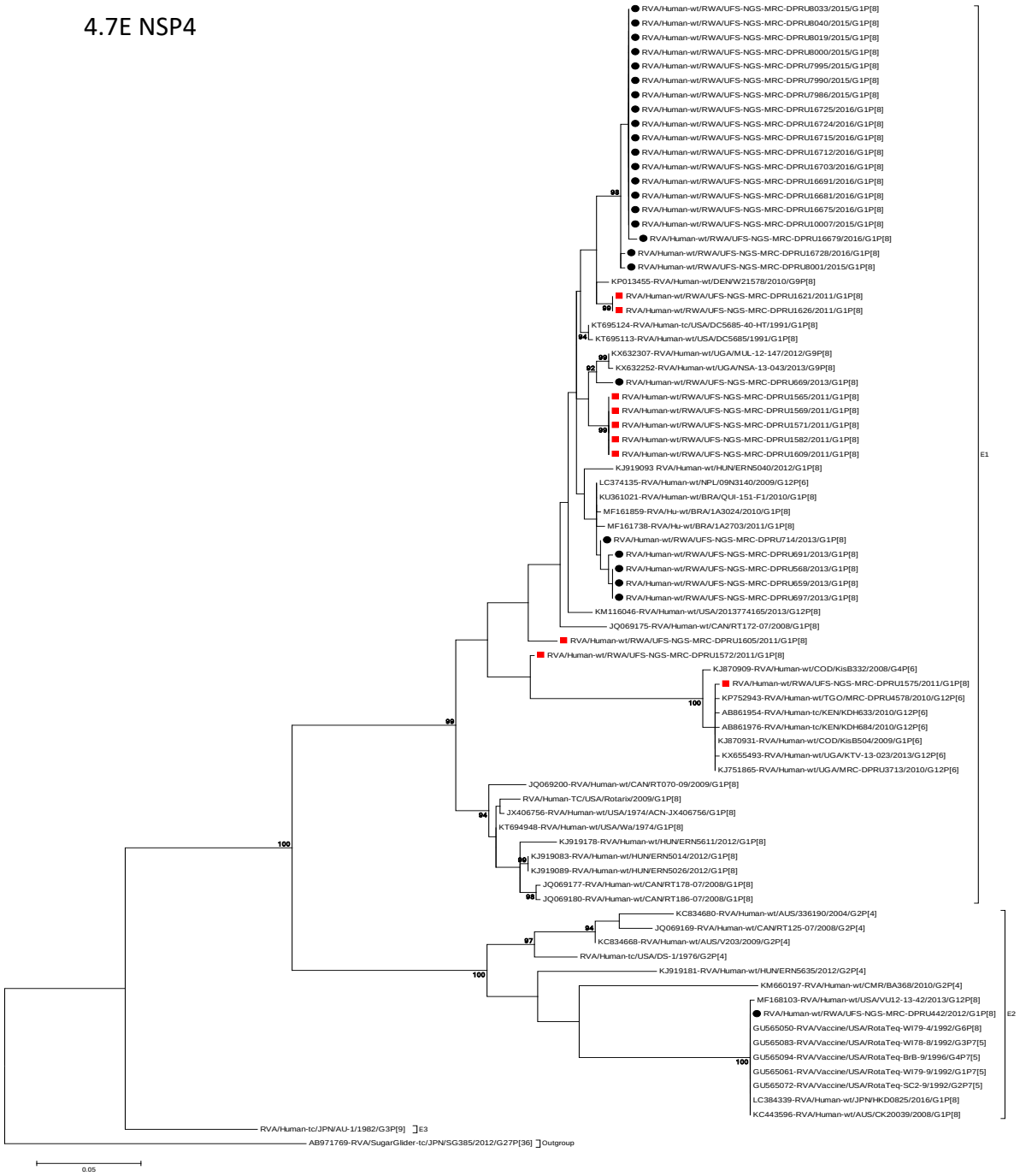
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4.7F NSP5

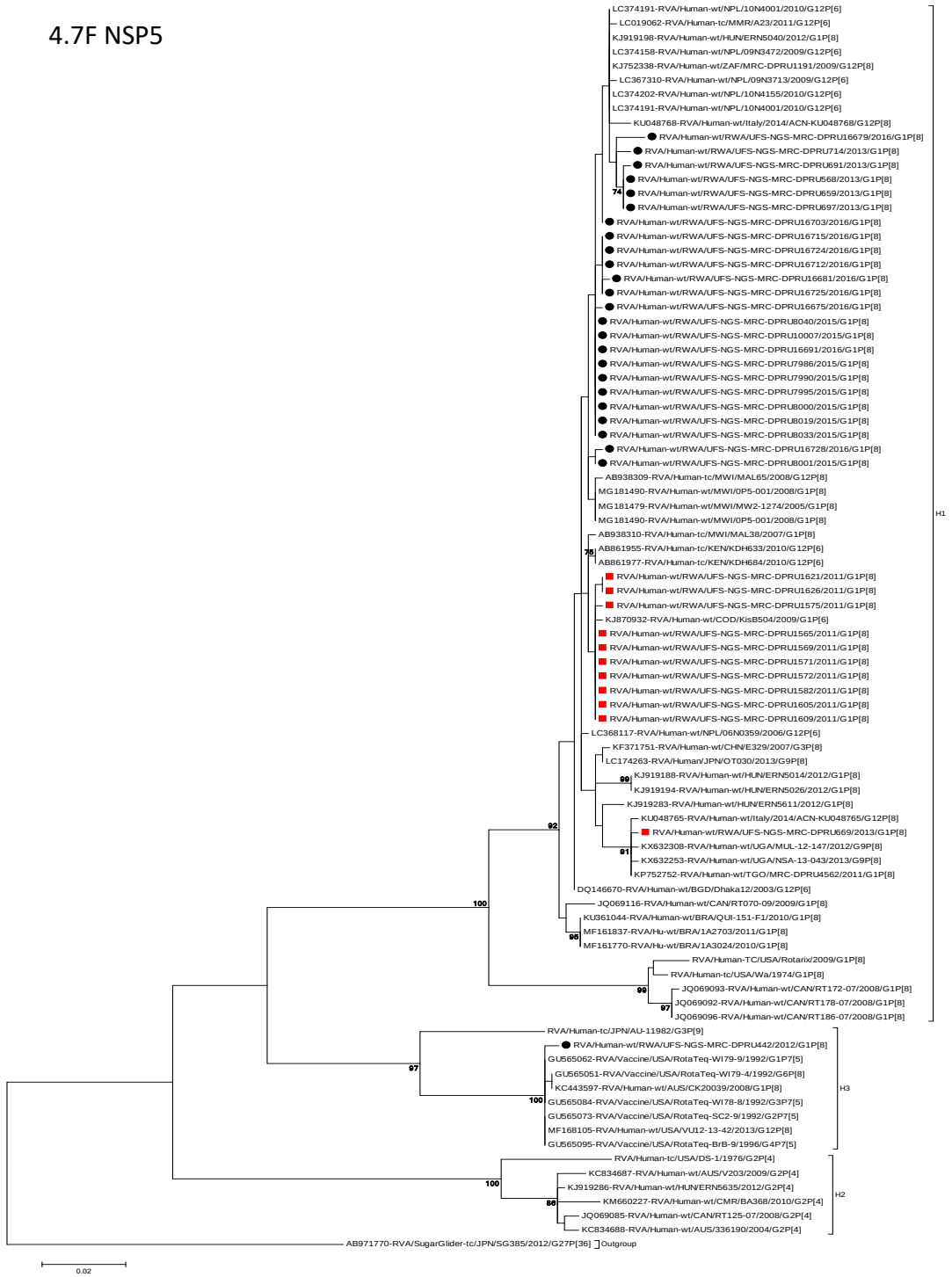


Figure 4.21: A) – **F)** Phylogenetic relatedness of rotavirus group A species base on **A)** VP1, **B)** VP2, **C)** VP3, **D)** NSP1, **E)** NSP4 and **F)** NSP5 of the study strains from Rwanda with representatives of known human and animal rotavirus genotypes. Pre- and post-vaccine strains are indicated with red squares and black circles, respectively. Bootstrap values ≥ 70% are indicated at each branch node. Scale bars represents substitutions per nucleotide site.

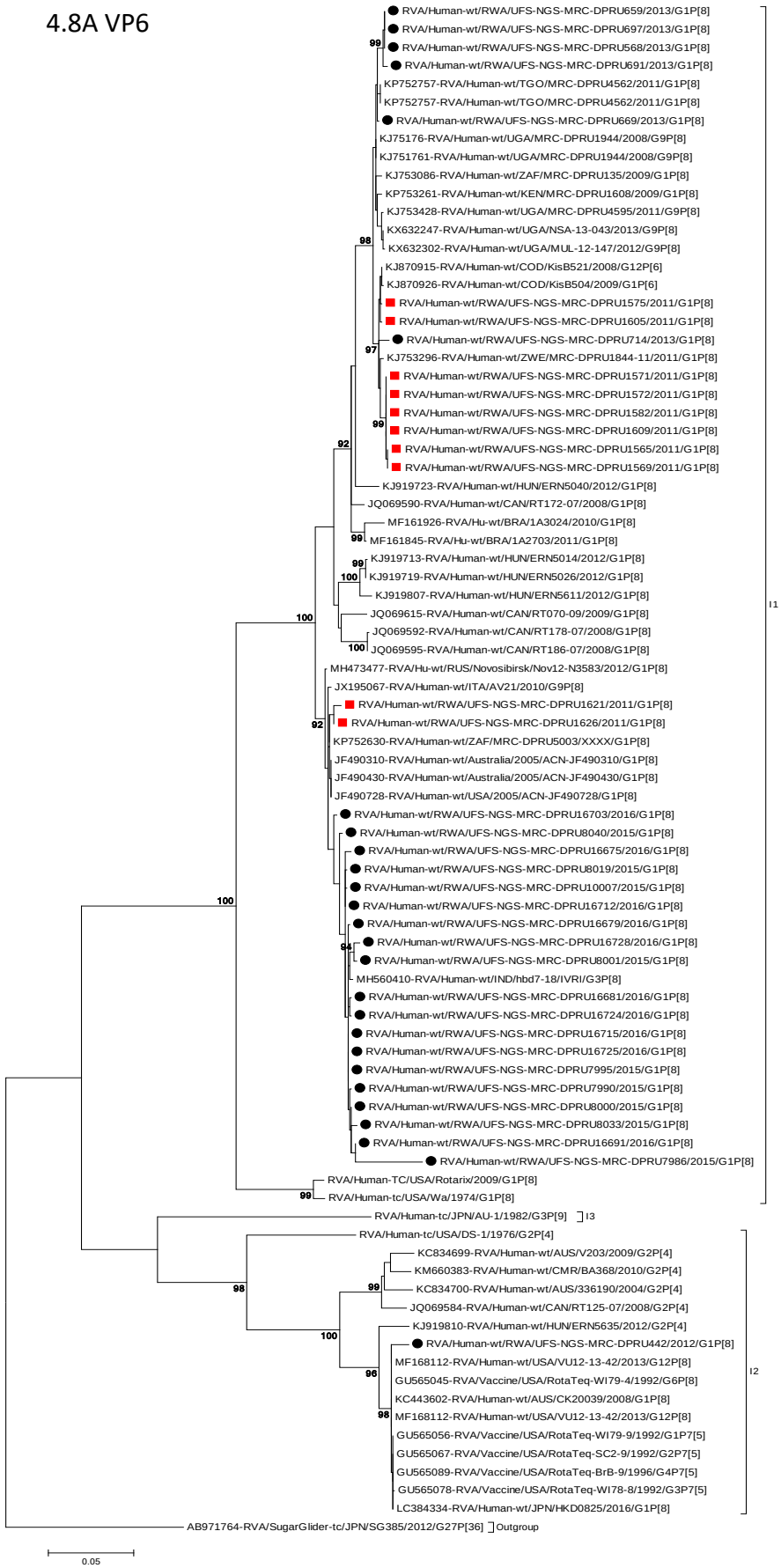
4.3.9. Phylogenetic and sequence analyses of VP6, NSP2, and NSP3.

Phylogenetically, the VP6, NSP2 and NSP3 gene segments of each Rwandan G1P[8] strain, except for RotaTeq® vdG1P[8] strain, segregated or grouped with cognate gene sequences of Wa-like strains belonging to genotype 1 (I1, N1, and T1, respectively) (Figure 4.8A-4.8C), while those of the RotaTeq® vdG1P[8] clustered together with genotype I2, N2, and T6 strains, respectively. Phylogenetic analyses of the VP6, NSP2, and NSP3 nucleotide sequences of the pre- and post-vaccine introduction G1P[8] strains from Rwanda showed segregation into typical sub-lineages and mixed sub-lineages (consisting of both pre- and post-vaccine introduction strains) (Figure 4.8A-4.8C). For the VP6 gene, the study strains separated into a post-vaccine sub-lineage and two mixed sub-lineages, each consisting of pre- and post-vaccine introduction VP6 genes (Figure 4.8A). The nt (aa) similarities amongst the five VP6 genes in the typical post-vaccine introduction strains sub-lineage ranged from 99.3-100% (99.7-100%). The nine I1 genes that grouped in the first mixed pre- and post-vaccine introduction strains sub-lineage revealed gene identities of 98.9-100% (99.5-100%), while the nt (aa) identities of the 21 strains that belonged to the second mixed pre- and post-vaccine introduction strains sub-lineage was in the range of 95.1-100% (93.7-100%).

The NSP2 genes segregated into three sub-lineages, a pre-vaccine sub-lineage, a mixed pre- and post-vaccine sub-lineage, and a post-vaccine sub-lineage (Figure 4.8B). Two orphan strains, RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1575/2011/G1P[8] and RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU669/2013/G1P[8] were also observed (Figure 4.8B). The seven genotype N1 strains that clustered in the pre-vaccine introduction strains sub-lineage exhibited nt (aa) similarities of 99.7-100% (100%) amongst each other, while the mixed pre- and post-vaccine introduction strains sub-lineage demonstrated gene identities that ranged from 98.7-100% (98.7-100%). The nt (aa) similarities of the five post-vaccine introduction strains detected in the 2013 RVA season and grouped in a single sub-lineage were 99.7-100% (99.7-100%). For the NSP3, gene sequences of the study strains segregated into one typical post-vaccine introduction strains sub-lineage and one mixed pre- and post-vaccine introduction RVA strains sub-lineage comprising of eight pre- and five post-vaccine introduction study strains (Figure 4.8C). The nt (aa) similarities of the NSP3 genes in the typical post-vaccine introduction strains sub-lineage was 99.5-100% (99.1-100%), while those in the mixed sub-lineage had gene identities ranging from 98.4-100% (97.7-100%). Three orphan strains RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1621/2011/G1P[8], RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU1626/2011/G1P[8] and RVA/Human-wt/RWA/UFS-NGS-MRC-

DPRU669/2011/G1P[8] that did not cluster in either of the typical or mixed sub-lineages were observed (Figure 4.8C). The VP6, NSP2 and NSP3 gene sequences of the single RotaTeq® vdG1P[8] strains shared nt (aa) homology of 98.0%, ≥99.7% and 100% with cognate gene sequence of RotaTeq® vaccine strains, respectively.

4.8A VP6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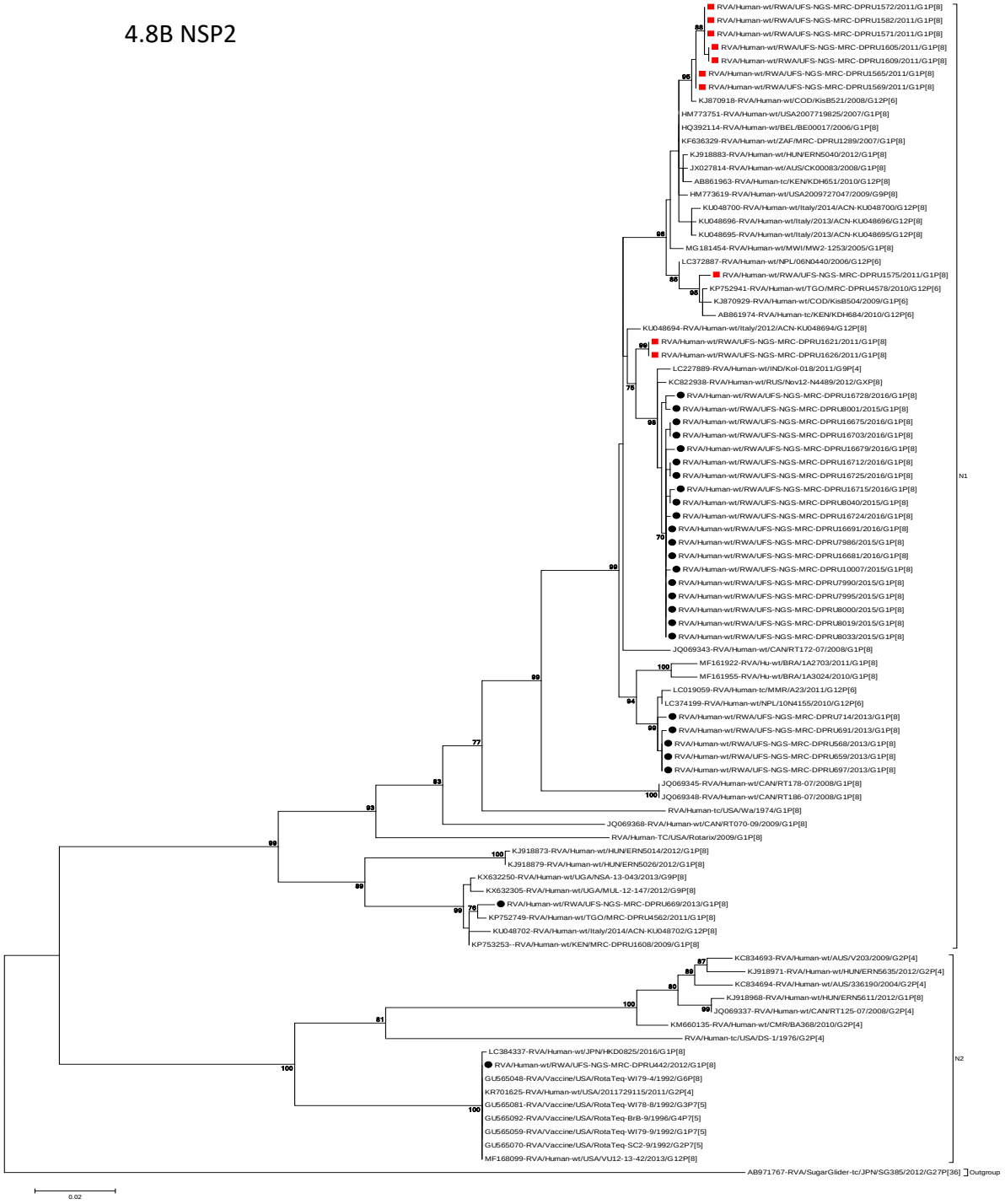
4.8B NSP2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N1
N2
Outgroup
0.02

4.8C NSP3

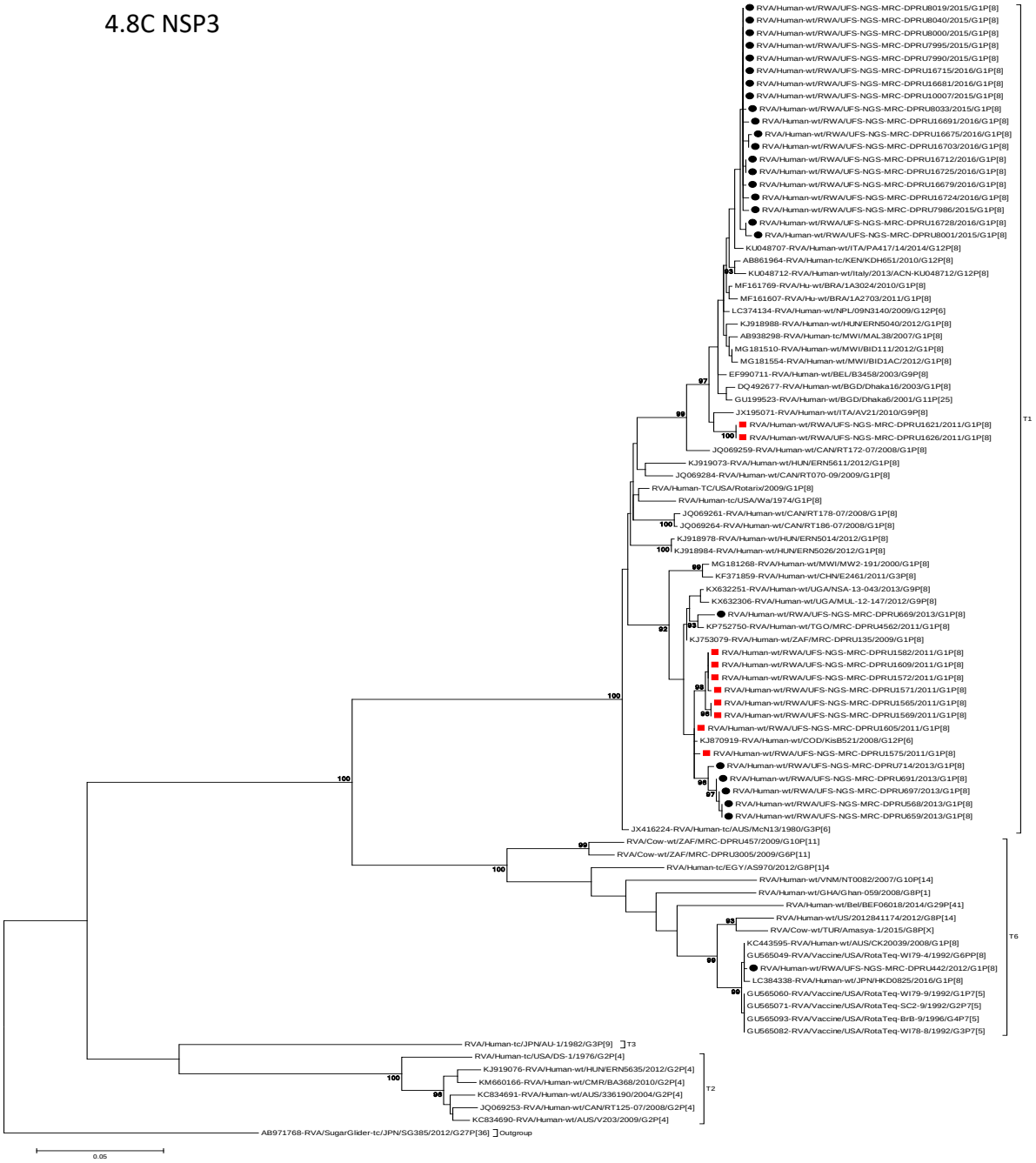


Figure 4.22: A) – **C)** Phylogenetic relatedness of rotavirus group A species base on **A)** VP6, **B)** NSP2 and **C)** NSP3 of the study strains from Rwanda with representatives of known human and animal rotavirus genotypes. Pre- and post-vaccine strains are indicated with red squares and black circles, respectively. Bootstrap values ≥ 70% are indicated at each branch node. Scale bars represent substitutions per nucleotide site.

4.4. Discussion

The introduction of the rotavirus vaccines has resulted in the reduction of the global burden of RVA associated diarrhoea diseases and hospitalization (Troeger et al., 2018b). Hence, continuous RVA surveillance in different settings remains vital to document and characterize post-vaccine licensure era RVA strains; possible vaccine-derived strains and inter-genogroup reassortant strains as part effort to document the impact of rotavirus vaccination. Therefore, in the present study, the whole gene analyses of 35 Rwandan G1P[8] and a single RotaTeq® vdG1P[8] strains collected from children less than five years of age during pre- and post-vaccination with RotaTeq® showed that all 35 of the strains possessed a typical Wa-like genotype constellation, while the single RotaTeq® vdG1P[8] strain RVA/Human-wt/RWA/UFS-NGS-MRC-DPRU442/2012/G1P[8], was apparently produced by a reassortment event between vaccine strains, RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P1A[8] and RVA/Vaccine/USA/Rotateq-W179-9/1992/G1P7[5]. However, the VP4 and VP6 genes may have originated from other circulating vaccine strains as their homologies with vaccine strain RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P1A[8] and RVA/Vaccine/USA/Rotateq-W179-9/1992/G1P7[5] were lower than other genes. Whole gene analyses confirmed that the VP7 and VP4 genes were vaccine-derived and the RotaTeq® vaccine WC3 bovine genetic backbone was confirmed to be G1-P[8]-I2-R2-C2-M2-A3-N2-T6-E2-H3. Though reassortment events between RotaTeq® vaccine strains which generated vaccine-derived G1P[8] reassortant have been reported previously in Australia, South Korea, Finland, and the USA, this is the first reported RotaTeq® vdG1P[8] from the African continent (Donato et al., 2012; Markkula et al., 2015; Payne et al., 2010; Than et al., 2015). Previous studies have shown that reassortment may occur more frequently between the VP7 gene of RotaTeq vaccine strain RVA/Vaccine/USA/Rotateq-W179-9/1992/G1P7[5] and VP4 gene of RotaTeq vaccine strain RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P1A[8] than between other genotypes (Donato et al., 2012; Than et al., 2015). The likelihood that this vdG1P[8] may possess increased virulence and cause AGE exists (Donato et al., 2012). The combination of the two human outer capsid proteins could potentially enhance cell binding and entry into enterocytes, hence an increase in virulence of this vdG1P[8] strain (Donato et al., 2012).

Although inter-genogroup reassortant G1P[8] strains have been reported globally, generally the G1P[8] strains are usually associated with the Wa-like backbone (Fujii et al., 2014; Jere et al., 2018; Kuzuya et al., 2014; Luchs et al., 2019; Matthijnssens et al., 2011). Phylogenetically, the 11 gene segments of the Rwanda pre- and post-vaccine introduction G1P[8] strains, with the exception of the RotaTeq® vdG1P[8],

were highly similar and clustered together with cognate gene segments of Wa-like strains (Fig. 1-8) and further segregated into either typical sub-lineages consisted of only pre-vaccine or post-vaccine introduction strains or mixtures of pre- and post-vaccine introduction strains. The segregation of pre- and post-vaccine introduction G1P[8] strains into different lineages and/or sub-lineages have been previously described in South Africa (Magagula et al., 2015). In each gene, we observed the segregation of strains in terms of vaccination status and year of detection. With the exception of the NSP1 gene which showed 2013, 2015 and 2016 post-vaccine strains in three different sub-lineages, the post-vaccine introduction G1P[8] strains detected during the 2015-2016 RVA seasons always clustered together within the post-vaccine introduction sub-lineages. The VP1-VP3, NSP1, NSP4, and NSP5 gene sequences of the pre-vaccine introduction strains always clustered together. Analyses of the VP7, VP4, VP6, NSP2, and NSP3 gene sequences, however, showed mixed sub-lineages consisting of both the pre-vaccine strains detected in the 2011 RVA season and post-vaccine introduction strains detected in 2013 (VP7, VP4, and NSP3), and 2015-2016 (VP6 and NSP2) seasons. In addition, within the typical pre- and post-vaccine introduction sub-lineages of all 11 gene segments, the sequence similarity was extremely high ≥ 99.9% and between sub-lineages the nt (aa) similarities were moderately high in the range of 96-98 % (98.1-99.2%). The high genetic relationship between gene sequences of the Rwandan pre- and post-vaccine introduction G1P[8] strains is an indication that the same strains that were circulating before vaccine introduction in Rwanda, are the same strains or their progeny that are causing RVA-associated diarrheal diseases and hospitalization after vaccination.

Globally, at least seven lineages have been previously described for G1 strains collected from different geographical locations (Arista et al., 2006; Bányai et al., 2009; Bucardo et al., 2012; Le et al., 2010). The emergence of distinct lineages or sub-lineages is attributed to the diverse evolutionary mechanisms such as mutation, recombination, and reassortments (Kirkwood, 2010). Based on the VP7 gene, the 35 Rwandan G1 strains which consisted of both pre- and post-vaccine introduction strains, clustered in lineage I and were comparable to the observation reported previously (da Silva et al., 2015; Magagula et al., 2015; Santos et al., 2019). On the other hand, the VP4 plays a role as an antibody-neutralization protein (Offit et al., 1986; Ward et al., 1993). Diversity of the P[8] genotypes has been demonstrated through four described distinct lineages (Arista et al., 2006; Cho et al., 2013). The clustering of the 35 P[8] strains in lineage III in this study is consistent with previous observations (Arora and Chitambar, 2011; da Silva et al., 2015; Magagula et al., 2015; Santos et al., 2019). However, despite the clustering of all VP7 and VP4 genes of the study strains in Lineage I (Figure 4.2) and lineage III (Figure 4.5), respectively, antigenic

variation seen at the neutralization epitope sites of the VP7 and VP4 proteins of the study strains have been previously described in Belgium and Australia (Diwakarla and Palombo, 1999; Zeller et al., 2012). Amino acid substitutions with effect on polarity changes were detected on Rwandan VP7 neutralization epitopes at positions T91A/V, S190D and M217. Amino acid substitutions on positions 94, 96, 147, 148, 190, 208, 211, 213 and 217 are critical and have been reported to alter rotavirus antigenicity (Ahmed et al., 2007; Trinh et al., 2007). Therefore, the detected substitutions may be involved in antigenic drift in Rwandan G1 strains. The resultant alteration in charge and polarity observed at position T91A/V on the VP7 neutralization epitope may play a role in escaping host immunity as it was distinctively observed post-vaccine introduction (Zeller et al., 2015, 2012). Furthermore, the change in polarity at position S190D and M217 could be due to natural fluctuation of RVA as they were observed during the pre-vaccine era and consistently before and after vaccine introduction, respectively (Jere et al., 2018). The presence of amino acid differences in CTL epitope positions Y41F/S, V42M, and A46V in Rwandan G1 strains possibly can result in greater host-immunity escape effects (Wei et al., 2009). In the intestine, trypsin-like proteases cleave the VP4 spike protein into two structural domains (VP8* and VP5*) (Estes and Greenberg, 2013). Four surface-exposed antigenic epitopes (8-1 to 8-4) have been described in the VP8* region, while five antigenic epitopes (5-1 to 5-5) in the VP5* region have been documented (Zeller et al., 2012). The amino acid changes identified at positions N113D, S131R and N135D in this study, resulted in polarity changes and played a role in escaping host-immunity (Dormitzer et al., 2004; Monnier et al., 2006).

Overall, full genomic analyses of 35 Rwandan G1P[8] strains revealed the predominance of G1-I and P[8]-III, which is consistent with what has been reported previously in Belgium (Zeller et al., 2012). The global prevalence of Wa-like human strains is hypothesized to be due to the ease of disseminating this genetic backbone in the human host (Ghosh and Kobayashi, 2011; Rahman et al., 2010). Although this study was insightful in reporting the whole gene composition of the circulating G1P[8] strains in Rwanda, it was limited to a few years (just one year) pre- and (five years) post-vaccination samples were included. Another limitation was that, for the sample where RotaTeq® vaccine shedding was observed, other plausible alternate aetiologies such as adenovirus and/or norovirus that might have led to the diarrhoea symptoms and possible hospitalization were not evaluated. Cell culture was not used to confirm the reassortment events as the project was mainly focused on *in-silico* work.

In conclusion, this is the first study to describe full genomic analyses of G1[P8] RVA strains in Rwanda. The detection of RotaTeq® vdG1P[8] strain from RVA positive child hospitalized with AGE symptoms was unexpected. Rotavirus group A strain surveillance at the whole gene level will enhance further assessment of vaccine impact on circulating RVA strains and the frequency along with the epidemiological fitness of reassortant strains.

4.5. Methods

4.5.1. Sample collection

RVA positive faecal samples (n=158) were obtained from children less than five years old who were hospitalized with AGE in Rwanda as part of the ongoing WHO/AFRO RVA surveillance program. The samples were conventionally genotyped into G and P types at the Diarrhoeal Pathogens Research Unit (DPRU), a WHO Rotavirus Reference Laboratory in South Africa (WHO RRL-SA). The samples were collected during the pre- (2011-2012) and post- (2012-2016) RVA vaccination periods with RotaTeq® vaccine in Rwanda. All the samples were stored at the DPRU at -20°C and G1P[8] strains were selected for whole-genome sequencing at the UFS-NGS Unit.

4.5.2. Double-stranded RNA extraction and purification

The extraction of dsRNA was performed at the DPRU and involved a method previously described by Nyaga et al. (2018). Briefly, a 100 mg stool sample was added to 200 µL freshly made phosphate-buffered saline (PBS, Sigma-Aldrich, St. Louis, USA). A 900 µL volume of TRI-reagent (Molecular Research Center, Inc, Cincinnati, OH, USA) was added to the suspended stool sample to homogenize as well as lyse the cells and cell components. A volume of 300 µL chloroform (Sigma-Aldrich, St. Louis, USA) was then added and centrifugation (16 000 x *g* for 20 minutes at 4°C) was done in a temperature-controlled microcentrifuge (Eppendorf centrifuge 5427R, Hamburg, Germany). The supernatant containing the total RNA was precipitated by the addition of 700 µL isopropanol (Sigma-Aldrich, St. Louis, USA) and by centrifugation at 16000 x *g* for 30 minutes at room temperature. The resulting pellet was re-dissolved by the addition of 90 µL of double-distilled water ,ddH_20, (Merck KGaA, Germany). A concentration of 8M $LiCl_2$ (Sigma-Aldrich, St. Louis, USA) was used to remove ssRNA through precipitation for 16 hours after which further centrifugation was done for 30 minutes at 16000 x *g*. The extracted dsRNA was purified by utilizing the

MinElute gel extraction kit (Qiagen, Hilden, Germany) and the integrity and enrichment of the dsRNA was verified via agarose gel electrophoresis.

4.5.3. cDNA synthesis

Complementary DNA was generated from the extracted viral RNA utilizing Maxima H Minus Double-Stranded cDNA Synthesis Kit (Thermo Fischer Scientific, Waltham, MA, USA) with minor modifications. Briefly, the extracted total RNA was denatured at 95^{o}C for five minutes and then 1 µL random hexamer primers (Thermo Fischer Scientific, Waltham, MA, USA) were added. The hexamer primers (Thermo Fischer Scientific, Waltham, MA, USA) were allowed to anneal at 65^{o}C for five minutes. A volume of 5 µL of first strand reaction mix (Thermo Fischer Scientific, Waltham, MA, USA) and 1 µL of first strand enzyme mix (Thermo Fischer Scientific, Waltham, MA, USA) was then added. The solution was then incubated at 25^{o}C, 50^{o}C and 85^{o}C for 10, 120 and five minutes, respectively. The tubes were removed from the thermocycler (Merck, Darmstadt, Germany) and second-strand synthesis was performed by adding 55 µL nuclease-free water, followed by the addition of 20 µL of 5x second strand reaction mix (Thermo Fischer Scientific, Waltham, MA, USA) and 5 µL of second strand enzyme mix (Thermo Fischer Scientific, Waltham, MA, USA). Subsequently, the solution was incubated at 16^{o}C for one hour and the reaction was stopped with 6 µL 0.5M EDTA (Thermo Fischer Scientific, Waltham, MA, USA). Residual RNA was removed with 10 µL RNase I (Thermo Fischer Scientific, Waltham, MA, USA) and the synthesized cDNA was incubated at room temperature for five minutes.

4.5.4. DNA library preparations and whole-genome sequencing

DNA libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina, Inc., California, USA) following the manufacturer's instructions. Briefly, DNA library preparation entailed tagmentation of the generated DNA, indexing using unique barcodes and amplification of tagmented DNA and clean-up of the amplified DNA. The library quality and size were assessed using an Agilent 2100 BioAnalyzer (Agilent Technologies, California, United States) according to the manufacturer's specified protocol. The Illumina custom protocol was utilized to normalize the libraries to 4 nM. All the normalized libraries were then pooled together into a single tube by combining 5 µL of each barcoded library. The pooled libraries were subjected to chemical denaturation using 0.2 N NaOH (Sigma Aldrich, Missouri, United States). After denaturation, 990 µL of pre-chilled HT1 buffer (Illumina, Inc., California, USA) was added to the 10 µL of the 4 nM denatured DNA library to dilute to 20 pM. A further dilution of the denatured library was

performed to get the desired final concentration of 8 pM. A PhiX control (Illumina, Inc., California, USA) spike-in of 20% was used. Whole-genome sequencing was performed for 600 cycles (301 x 2 paired-end) on a MiSeq benchtop sequencer (Illumina, Inc., California, USA) using Illumina MiSeq v3 reagent kit (Illumina, Inc., California, USA) at the UFS-NGS Unit, Bloemfontein, South Africa.

4.5.5. Genome assembly

Illumina sequence reads were analysed using Geneious software v11 (Kearse et al., 2012; https:www.geneious.com) and CLC Genomics Workbench v11 (CLC Bio, Qiagen, Hilden, Germany) which entailed genome assembly and mapping the reads to reference-based sequences to obtain the full-length genomes.

4.5.6. Identification of genotype constellations

Genotyping was performed by utilizing RotaC v 2.0 (Maes et al., 2009), an automated online genotyping tool for group A RV strains. Genome constellations were generated by assigning genotypes to each genome segment.

4.5.7. GenBank accession numbers

The sequences were deposited into GenBank under the accession number MN632673-MN633067.

4.5.8. Phylogenetic, sequence analyses and protein modelling

For each gene segment, the ORF were aligned and subjected to sequence comparisons as described previously (Esona et al., 2018, 2017; Ward et al., 2016). Briefly, multiple alignments were made using the MUSCLE algorithm implemented in MEGA 6 software (Tamura et al., 2013; http://www.megasoftware.net/). Once aligned, the DNA Model Test program implemented in MEGA version 6 was used to identify the optimal evolutionary models that best fit the sequence datasets. Using the Corrected Akaike Information Criterion (AICc), the model GTR-G-I was found to best fit the sequence data for each gene segment. With this model, maximum likelihood trees were constructed using MEGA 6 with 1000 bootstrap replicates to estimate branch support. The nucleotide and deduced amino acid sequence identities among strains were calculated for each gene using distance matrices prepared using

the *p*-distance algorithm in MEGA 6 software (Tamura et al., 2013). Protein modelling was performed on amino acid sequences for each strain and reference strain using the Swiss-Model protein structure homology-modelling server (Waterhouse et al., 2018; https://swissmodel.expasy.org). The structures were modified using UCSF Chimera (Pettersen et al., 2004).

CHAPTER FIVE: WHOLE-GENOME ANALYSES IDENTIFIES MULTIPLE REASSORTANT ROTAVIRUS STRAINS IN RWANDA POST-VACCINE

INTRODUCTION

This chapter is under internal review for submission to Frontiers in Microbiology

5.1. Abstract

Children in middle and low-income countries, like Rwanda, have a higher prevalence of rotavirus disease relative to those in developed countries. Following the implementation of the RotaTeq® vaccine in Rwanda in 2012, a drastic decline in rotavirus-induced diarrhoea was documented during the post-vaccine introduction impact evaluation. Evolutionary mechanisms leading to multiple reassortant rotavirus strains influence the diversity and evolutionary dynamics of novel rotaviruses over time. Comprehensive rotavirus whole-genome analysis was conducted on 158 RVA specimens collected pre- and post-vaccine introduction in children less than five years in Rwanda. Of these RVA positive specimens, five strains with the genotype constellations G4P[4]-I1-R2-C2-M2-A2-N2-T1-E1-H2 (n=1), G9P[4]-I1-R2-C2-M2-A1-N1-T1-E1-H1 (n=1), G12P[8]-I1-R2-C2-M1-A1-N2-T1-E2-H3 (n=2) and G12P[8]-I1-R1-C1-M1-A2-N2-T2-E1-H1 (n=1), with single, double and triple gene reassortant rotavirus strains were identified. Phylogenetic analysis revealed a close relationship between the Rwandan strains and cognate human RVA strains circulating on a global scale as well as the RotaTeq® vaccine strains in the VP1, VP2, NSP2, NSP4 and NSP5 gene segments. The VP2, VP3, and NSP1 genes of strain RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU566/2013/G9P[4] and RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU6235/2014/G4P[4] formed distinct clusters from similar genes of strains circulating globally. Pairwise analyses revealed considerable differences in the VP7 and VP4 antigenic regions of the representative Rwandan study strains and the RotaTeq® vaccine strain. Although the impact of such amino acid changes on the effectiveness remains unclear, this analysis underlines the potential of rotavirus whole-genome analysis program by enhancing knowledge on inter-genogroup reassortant strains circulating in Rwanda post-RotaTeq® vaccine introduction.

Keywords: rotavirus, Rwanda, reassortment, whole-genome based surveillance, epitopes, RotaTeq®

5.2. Introduction

Group A rotavirus infections are commonly associated with severe diarrhoea in young children less than five years of age, accounting for over 128 500 deaths globally in 2016 (Troeger et al., 2018b). Children living in third-world countries have been documented to experience a higher rotavirus disease burden attributed to lack of adequate sanitation (Sindhu et al., 2017). Prior to RotaTeq® (RV5, Merck & Co. Inc., USA) vaccine introduction in Rwanda, approximately 3500 RVA related mortality was reported in children annually, accounting for 8.8% of all childhood mortality in Rwanda (GAVI, 2012). RotaTeq® was introduced into the Rwandan market in May 2012 with a vaccine coverage of 99% within a year after introduction in 2013 (Gatera et al., 2016; WHO, 2020). During the first three years post RotaTeq® introduction, a decrease of total diarrheal hospitalization (25-44%) among children less than five years in the Eastern Province of Rwanda was documented (Sibomana et al., 2018).

Structurally, rotaviruses possess an icosahedral symmetry and consist of an 11-segmented dsRNA genome which encodes for both structural and non-structural proteins (Estes and Greenberg, 2013; Desselberger, 2014). The VP7 and VP4 proteins are generally used to distinguish RVA strains into G- and P-genotypes, respectively, based on their antigenic properties. Furthermore, the advancements of NGS techniques have enabled the characterization of all the 11-segments which has turned out to provide essential information that can be used to determine the evolutionary dynamics of the virus (Matthijnssens et al., 2008; Matthijnssens et al., 2011; Nyaga et al., 2020).Three human RVA genogroups, the Wa-like and DS-1-like (major genogroups) and the AU-1 like (minor genogroup) have been identified based on the rotavirus whole-genome (Ide et al., 2015; Komoto et al., 2016; Matthijnssens et al., 2008; Matthijnssens and Van Ranst, 2012). The majority of strains that possess the Wa-like (G1-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1) constellation are from porcine origin, while DS-1-like (G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2) and AU-1-like (G3-P[9]-I3-R3-C3-M3-A3-N3-T3-E3-H3) strains are from bovine and canine/feline origin, respectively (Matthijnssens et al., 2008).

Over 107 countries have implemented RVA vaccines as of April 2020 to curb RVA infections in young children. Based on the safety and efficacy studies, four RVA vaccines, Rotarix® (RV1, GlaxoSmithKline Biologicals, Belgium), RotaTeq® (RV5, Merck & Co. Inc., USA), Rotavac® (nHRV, Bharat Biotech International Limited India) and Rotasil® (BRV-PV, Serum Institute of India, India), have been prequalified by the WHO for global use (ROTA Council, 2016; WHO, 2018). RotaTeq® is a live-attenuated pentavalent

vaccine-derived from bovine and human reassortant parent strains (G1 [W179-9], G2 [SC2-9], G3 [W178-8], G4 [BrB-9] or P[8] [W179-4] of human strains and WC3, G6P[5] of the bovine strain) (Matthijnssens et al., 2010). In Africa, the RotaTeq® vaccine was introduced in only five countries; Burkina Faso (2013), Libya (2013), Morocco (2010), Rwanda (2012), and the Gambia (2013) (PATH, 2016). Moreover, Rwanda was the first low-income country to introduce the RotaTeq® vaccine. Different African countries have reported the detection of diverse GXP[X] combinations over the years, with G1P[8], G2P[4], G3P[8], G9P[8], G1P[6], G2P[6], G3P[6], G8P[4] and G9P[6] being the most prevalent combinations in Africa (Mwenda et al., 2010; Seheri et al., 2014; Steele et al., 2003a; Steele et al., 2003b; Todd et al., 2010). In Rwanda, G1P[8] was the predominant strain circulating in the pre-vaccine era in 2011. However, there was an immediate shift to G8P[4] (53% in 2013), G12P[8] (39.4% in 2014) and G4P[8] (36.6% in 2014) and were subsequently replaced by the re-emergence of G1P[8] (51.6%) in 2015 (Seheri et al., 2018).

Group A rotaviruses are constantly evolving through numerous evolutionary mechanisms influenced by the segmented nature and error-prone RdRp characteristic of the RNA virus (Bányai et al., 2011; Seheri et al., 2018; Taniguchi and Urasawa, 1995). Intra-genogroup and inter-genogroup reassortment events are generally influenced by co-infection with multiple RVA strains (Cowley et al., 2016; Dennis et al., 2014; Estes and Greenberg, 2013; Heylen et al., 2014; Nakagomi et al., 2013). The RVA strains derived from inter-genogroup reassortment events both in the pre- and post-vaccine period have been documented globally (Cowley et al., 2016; Hoa-Tran et al., 2016; Komoto et al., 2017; Mwangi et al., 2020). However, strains having pure Wa-like or pure DS-1-like genotype constellations are transmitted frequently across the human population as compared to these reassortant strains (Heiman et al., 2008; McDonald et al., 2009). The aim of this study is to report on five inter-genogroup reassortant strains observed post-vaccine introduction in Rwanda between 2013 and 2015 using whole-genome sequencing approach.

5.3. Results

5.3.1. Genome genotypes

The genetic relatedness and variability of Rwandan strains in comparison to selected reference RVA strains circulating globally, was determined by sequencing 158 Rwandan samples from the pre- (2011) and post- (2012 and 2016) vaccination period. From that dataset pool, only five samples from the pool possessed reassortant constellations (Table 5.1). The length size of contigs and the number of reads post-assembly are given (Table 5.1). All the five reassortant strains were observed post-vaccine introduction. Strain RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU6235/2014/G4P[4] and RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU566/2013/G9P[4] exhibited both the Wa-like and DS-1-like genotype constellation: G4P[4]-I1-R2-C2-M2-A2-N2-T1-E1-H2 and G9P[4]-I1-R2-C2-M2-A1-N1-T1-E1-H1, respectively. Two G12P[8] strains (RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU8020/2015/G12P[8] and RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU9995/2015/G12P[8]) possessed an identical genome constellation across the backbone (G12P[8]-I1-R2-C2-M1-A1-N2-T1-E2-H3), comprised of typical Wa-like, DS-1-like and AU-1-like genotype constellation. On the other hand, strain RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU6212/2014/G12P[8] exhibited both the Wa-like and DS-1-like genotype constellation G12P[8]-I1-R1-C1-M1-A2-N2-T2-E1-H1.

Table 5.6: Full genotype constellations of five Rwandan strains detected post-vaccine introduction (2013-2015) along with the contig lengths and number of reads mapped to each contig.

Strain Nomenclature		VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU6235/2014/G4P[4]	Genome constellations	G4	P[4]	I1	R2	C2	M2	A2	N2	T1	E1	H2
	Contig length	1065	2359	1353	3303	2729	3302	3302	1044	1073	750	673
	reads mapped to contigs	4013	16451	6652	17536	13647	19920	7673	3907	5391	3371	1892
RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU566/2013/G9P[4]	Genome constellations	G9	P[4]	I1	R2	C2	M2	A1	N1	T1	E1	H1
	Contig length	1061	2359	1352	3302	2726	2591	1567	1059	1074	748	664
	reads mapped to contigs	4324	7950	7548	19078	11493	11132	4451	3604	4152	3879	722
RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU8020/2015/G12P[8]	Genome constellations	G12	P[8]	I1	R2	C2	M1	A1	N2	T1	E2	H3
	Contig length	1062	2359	1360	3304	2707	3302	3302	1059	1074	751	667
	reads mapped to contigs	3888	6742	5761	20328	12815	11949	12849	3172	3484	2600	1170
RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU9995/2015/G12P[8]	Genome constellations	G12	P[8]	I1	R2	C2	M1	A1	N2	T1	E2	H3
	Contig length	1062	2359	1356	3302	2687	3302	3302	1059	1074	751	668
	reads mapped to contigs	25828	45020	42925	92061	65575	26672	18505	20815	12752	7205	11688
RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU6212/2014/G12P[8]	Genome constellations	G12	P[8]	I1	R1	C1	M1	A2	N2	T2	E1	H1
	Contig length	1061	2359	1356	3301	2735	2591	3302	1059	1066	749	669
	reads mapped to contigs	22656	60092	26846	62970	55548	59218	26988	15855	20750	24216	4705

Wa-like genogroups is represented by the green colour, DS-1like is represented by the red colour and AU-1-like is represented by the yellow colour.

5.3.2. The VP4 and VP7 antigenic region analyses

The antigenic differences between the RotaTeq® vaccine and the Rwandan study strains were compared by analysing the amino acid composition of the VP7 and VP4. Two structurally defined antigenic epitope regions: 7-1 and 7-2 made up of 29 amino acid residues are located on the VP7 protein (Aoki et al., 2009; Zeller et al., 2015). The 7-1 epitope is further subdivided into 7-1a and 7-1b. The VP7 epitopes of the Rwandan G4 strain was compared to the G4 VP7 protein of strain RVA/Vaccine/USA/RotaTeq-BrB-9/1996/G4P75, which showed 27 amino acid differences distributed across the VP7 epitope regions (Figure 5.1A and 5.1B). Only two residues in position 190 (within the 7-2 region) and 291 (within the 7-1a region) were conserved between the RotaTeq® vaccine strains and the Rwandan study strain (Figure 5.1A). Amino acid substitutions from the uncharged polar molecules to the charged polar molecules were observed in three positions, T96D, T217E, and S221D. Furthermore, amino acid substitution from the charged polar molecule to the uncharged polar molecule was also observed at position E97T and D211T.

Generally, under trypsin activation of viral particles, the VP4 is cleaved into two domains, the VP8* (8-1 to 8-4) and the VP5* (5-1 to 5-5) made up of 37 amino acid residues in the antigenic epitope regions (Dormitzer et al., 2002; Zeller et al., 2012). The VP4 epitopes of the Rwandan strains were compared to the P[8] VP4 protein of strain RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P1A8 (Figure 5.2). The Rwandan study strains differed from the RotaTeq® vaccine strain in only three positions, E150D and D195G at 8-1 epitope region and L388I at 5-1 epitope region, while the rest of the residues in the epitope region were conserved (Figure 5.2). At position 150, the amino acid changed from glutamic acid to aspartic acid. While the change at position 195 was from an aspartic acid (charged polar molecule) to a glycine molecule (nonpolar molecule), the change at position 388 was from leucine to isoleucine.

A

		7-1a													7-1b						7-2								
		*	*	*	*	*	*	*	*	*					*	*		*	*	*					*		*	*	*	*	*
	Lineage	87	91	94	96	97	98	99	100	104	123	125	129	130	291	201	211	212	213	238	242	143	145	146	147	148	190	217	221	264
GU565090-RVA/Vaccine/USA/RotaTeq-BrB-9/1996/G4P7[5]	I	S	T	S	T	E	W	K	D	Q	N	L	I	D	K	Q	D	T	A	D	T	R	A	S	G	E	S	T	S	G
RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU6235/2014/G4P[4]	I	P	P	I	D	T	E	W	K	S	S	V	S	I	.	T	T	N	T	L	S	I	F	T	S	G	.	E	D	V
JQ069531-RVA/Human-wt/CAN/RT039-09/2009/G4P[8]	I	P	P	I	D	T	E	W	K	S	S	V	S	I	.	T	T	N	T	L	S	I	F	T	S	G	.	E	D	V
DQ904524-RVA/Human-wt/JPN/J-4623/2003/G4P[X]	I	P	P	I	D	T	E	W	K	S	S	V	S	I	.	T	T	N	T	L	S	I	F	T	S	G	.	E	D	V
LC311232-RVA/Human-wt/JPN/OH529/2003/G4P[X]	I	P	P	I	D	T	E	W	K	S	S	V	S	I	.	T	T	N	T	L	S	I	F	T	S	G	.	E	D	V
KC841471-RVA/Human-wt/GR/Ath113/2009/G4P[8]	I	P	P	I	D	T	E	W	K	S	S	V	S	I	.	T	T	N	T	L	S	I	F	T	S	G	.	E	D	V
MH591291-RVA/Human-wt/LBNNG201/2013/G4P[8]	I	P	P	I	D	T	E	W	K	S	S	V	S	I	.	T	T	N	T	L	S	I	F	T	S	G	.	E	D	V
MH591282-RVA/Human-wt/LBNH183/2012/G4P[8]	I	P	P	I	D	T	E	W	K	S	S	V	S	I	.	T	T	N	T	L	S	I	F	T	S	G	.	E	D	V
KM288577-RVA/Human-wt/RUS/NN/118/2013/G4P[8]	I	P	P	I	D	T	E	W	K	S	S	V	S	I	.	T	T	N	T	L	S	I	F	T	S	G	.	E	D	V
KJ919840-RVA/Human-wt/HUN/ERN5063/2012/G4P[8]	I	P	P	I	D	T	E	W	K	S	S	V	S	I	.	T	T	N	T	L	S	I	F	T	S	G	.	E	D	V
JN849134-RVA/Human-wt/BEL/BE1113/2009/G4P[8]	I	P	P	I	D	T	E	W	K	S	S	V	S	I	.	T	T	N	T	L	S	I	F	T	S	G	.	E	D	V
KJ752863-RVA/Human-wt/ZWE/MRC-DPRU1850/2011/G4P[8]	I	P	P	I	D	T	E	W	K	S	S	V	S	I	.	T	T	N	T	L	S	I	F	T	S	G	.	E	D	V
MG969472-RVA/Hu-wt/RUS/Novosibirsk/Nov12-N3676/2012/G4P[8]	I	P	P	I	D	T	E	W	K	S	S	V	S	I	.	T	T	N	T	L	S	I	F	T	S	G	.	E	D	V
KF006890-RVA/Hu-wt/RUS/Novosibirsk/Nov10-N458/2010/G4P[8]	I	P	P	I	D	T	E	W	K	S	S	V	S	I	.	T	T	N	T	L	S	I	F	T	S	G	.	E	D	V
M86832-RVA/Human-wt/ITA/PV5257/XXXX/G4P[X]	II	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	N	V	.	.	.	K	.	.	.	.	.	M	.	.
DQ683522-RVA/Human-wt/THA/CMP121/XXXX/GAP[X]	V	T	.	N	N	.	.	.	.	.	.	.	V	.	.	.	N	A	N	.	.	.	T	.	.	Q	.	.	G	.
AJ488586-RVA/Humam-wt/MEX/D151/19988-01/G4P[6]	VI	T	.	.	.	.	.	.	.	.	.	.	.	.	R	.	N	.	G	.	.	.	V	A	.	.	.	M	N	.
AF192267-RVA/Human-wt/BRA/ICB185/XXXX/G4P[X]	VII	T	.	N	N	.	.	.	.	.	.	.	.	.	R	.	N	V	G	.	.	.	T	A	.	Q	.	V	N	.

B

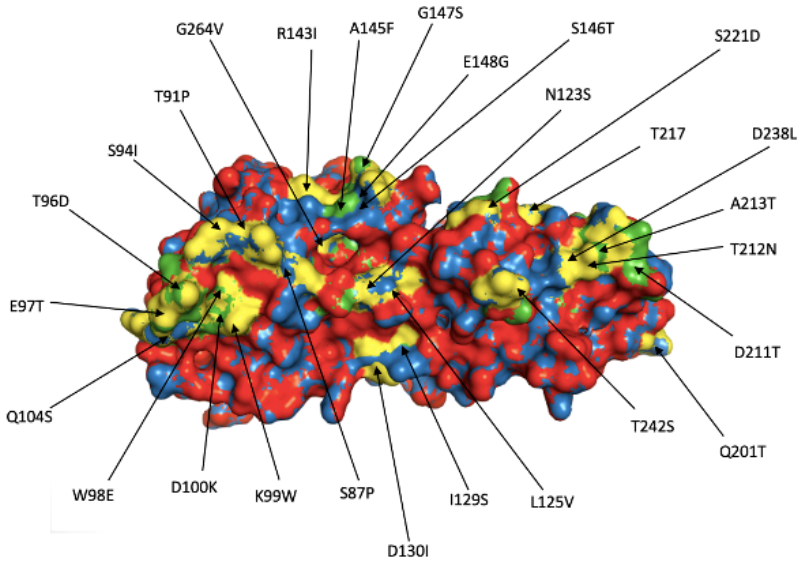


Figure 5.1: A) The alignment of the G4 VP7 component of the RotaTeq® vaccine strain and G4 Rwandan study strain based on the three surface exposed epitope regions (7-1a, 7-1b, and 7-2). The asterisk represents the amino acid position of residues associated with escape neutralization with monoclonal antibodies. **B)** Surface representation of the VP7 protein. The structure has the root mean square deviation (RMSD) of 0.044 Å. The RotaTeq® vaccine is represented with the red colour, while the Rwandan strain is indicated with the blue colour. The green colour represents the amino acid change observed on the Rwandan study strain as compared to the RotaTeq® vaccine strain (yellow) (Created with PyMoL v2, 15 July 2020)

		VP8*												
		8-1											8-2	
		*	*	*	*	*	*			*			*	*
	Lineage	100	146	148	150	188	190	192	193	194	195	196	180	183
GU565044-RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P1A[8]	II	D	S	Q	E	S	T	N	L	N	D	I	T	A
RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU8020/2014/G12P[8]	III	.	.	.	D	.	.	.	.	.	G	.	.	.
RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU6212/2014/G12P[8]	III	.	.	.	D	.	.	.	.	.	G	.	.	.
RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU9995/2014/G12P[8]	III	.	.	.	D	.	.	.	.	.	G	.	.	.
HM773747-RVA/Human-wt/USA2007719825/2007/G1P[8]	III	.	.	.	D	.	.	.	.	.	G	.	.	.
JN849147-RVA/Human-wt/BEL/BE1286/2009/G1P[8]	III	.	.	.	D	.	.	.	.	.	G	.	.	.
KJ753695-RVA/Human-wt/ZAF/MRC-DPRU1554/2010/G12P[8]	III	.	.	.	D	.	.	.	.	.	G	.	.	.
KF636171-RVA/Human-wt/ZAF/MRC-DPRU2306/2009/G1P[8]	III	.	.	.	D	.	.	.	.	.	G	.	.	.
KJ753792-RVA/Human-wt/ZAF/MRC-DPRU1195/2009/G2P6P[8]	III	.	.	.	D	.	.	.	.	.	G	.	.	.
KJ753121-RVA/Human-wt/ZAF/MRC-DPRU2489/2008/G1P[8]	III	.	.	.	D	.	.	.	.	.	G	.	.	.
HQ392119-RVA/Human-wt/BEL/BE00017/2006/G1P[8]	III	.	.	.	D	.	.	.	.	.	G	.	.	.
JX195088-RVA/Human-wt/ITA/JES11/2010/G9P[8]	III	.	.	.	D	.	.	.	.	.	G	.	.	.
KF636336-RVA/Human-wt/ZAF/MRC-DPRU1289/2007/G1P[8]	III	.	.	.	D	.	.	.	.	.	G	.	.	.
JX027821-RVA/Human-wt/AUS/CK00083/2008/G1P[8]	III	.	.	.	D	.	.	.	.	.	G	.	.	.
JX406750-RVA/Human-tc/USA/Wa/1974/G1P[8]	I	.	.	.	.	.	.	.	.	.	N	.	.	.
AB008280-RVA/Human-tc/JPN/ITO/1981/G3P[8]	I	.	.	.	.	.	.	.	.	H	H	.	.	.
U30716-RVA/Human-wt/JPN/F45/1987/G9P[8]	II	.	.	.	.	.	.	.	.	.	N	.	.	.
KP753159-RVA/Human-wt/ZAF/MRC-DPRU76/2012/G12P[8]	III	.	.	.	D	.	.	.	.	.	G	.	.	.
KP902534-RVA/Human-wt/MWI/OP354/1998/G4P[8]	IV	.	.	.	.	.	.	D	.	T	S	.	.	.

	VP8*												VP5*											
	8-3									8-4			5-1								5-2	5-3	5-4	5-5
		*		*			*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	113	114	115	116	125	131	132	133	135	87	88	89	384	386	388	393	394	398	440	441	434	459	429	306
GU565044-RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P1A[8]	N	P	V	D	N	R	N	D	D	N	T	N	Y	F	L	W	P	G	R	T	P	E	L	R
RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU8020/2014/G12P[8]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU6212/2014/G12P[8]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU9995/2014/G12P[8]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
HM773747-RVA/Human-wt/USA2007719825/2007/G1P[8]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
JN849147-RVA/Human-wt/BEL/BE1286/2009/G1P[8]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
KJ753695-RVA/Human-wt/ZAF/MRC-DPRU1554/2010/G12P[8]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
KF636171-RVA/Human-wt/ZAF/MRC-DPRU2306/2009/G1P[8]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
KJ753792-RVA/Human-wt/ZAF/MRC-DPRU1195/2009/G2P6P[8]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
KJ753121-RVA/Human-wt/ZAF/MRC-DPRU2489/2008/G1P[8]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
HQ392119-RVA/Human-wt/BEL/BE00017/2006/G1P[8]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
JX195088-RVA/Human-wt/ITA/JES11/2010/G9P[8]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
KF636336-RVA/Human-wt/ZAF/MRC-DPRU1289/2007/G1P[8]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
JX027821-RVA/Human-wt/AUS/CK00083/2008/G1P[8]	.	L	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
JX406750-RVA/Human-tc/USA/Wa/1974/G1P[8]	.	.	.	.	S	S	.	.	N	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
AB008280-RVA/Human-tc/JPN/ITO/1981/G3P[8]	.	.	.	.	S	S	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
U30716-RVA/Human-wt/JPN/F45/1987/G9P[8]	.	.	.	.	.	.	.	.	N	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
KP753159-RVA/Human-wt/ZAF/MRC-DPRU76/2012/G12P[8]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
KP902534-RVA/Human-wt/MWI/OP354/1998/G4P[8]	D	.	.	.	S	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.

Figure 5.2: The alignment of the P[8] VP4 component of the Rotateq vaccine strain and P[8] Rwandan study strain based on the two VP4 domains, the VP8* (8-1 to 8-4) and VP5 (5-1 to 5-5). The asterisk represents the amino acid position of residues associated with escape neutralization with monoclonal antibodies.

5.3.3. Phylogenetic analysis of the VP7 gene of G4, G9 and G12

Phylogenetic trees were constructed for each of the 11 segments of the five Rwandan RVA reassortant strains in comparison with global reference strains from GenBank (Figures 5.3-5.6). The three Rwandan G12P[8] strains clustered in G12 lineage III, and shared 87.6-99.3% nt similarity with other lineage III G12 strains (Figure 5.3). The G12 strains clustered together and shared 99.8-100% nt similarity amongst themselves. On the other hand, the Rwandan G9 strain clustered in G9 lineage III distantly from globally circulating G9 strains (81.7-92.1% nt similarity). The G4 strain clustered in G4 lineage I with circulating G4 global strains and exhibited 86% nt similarity with a RotaTeq® vaccine strain.

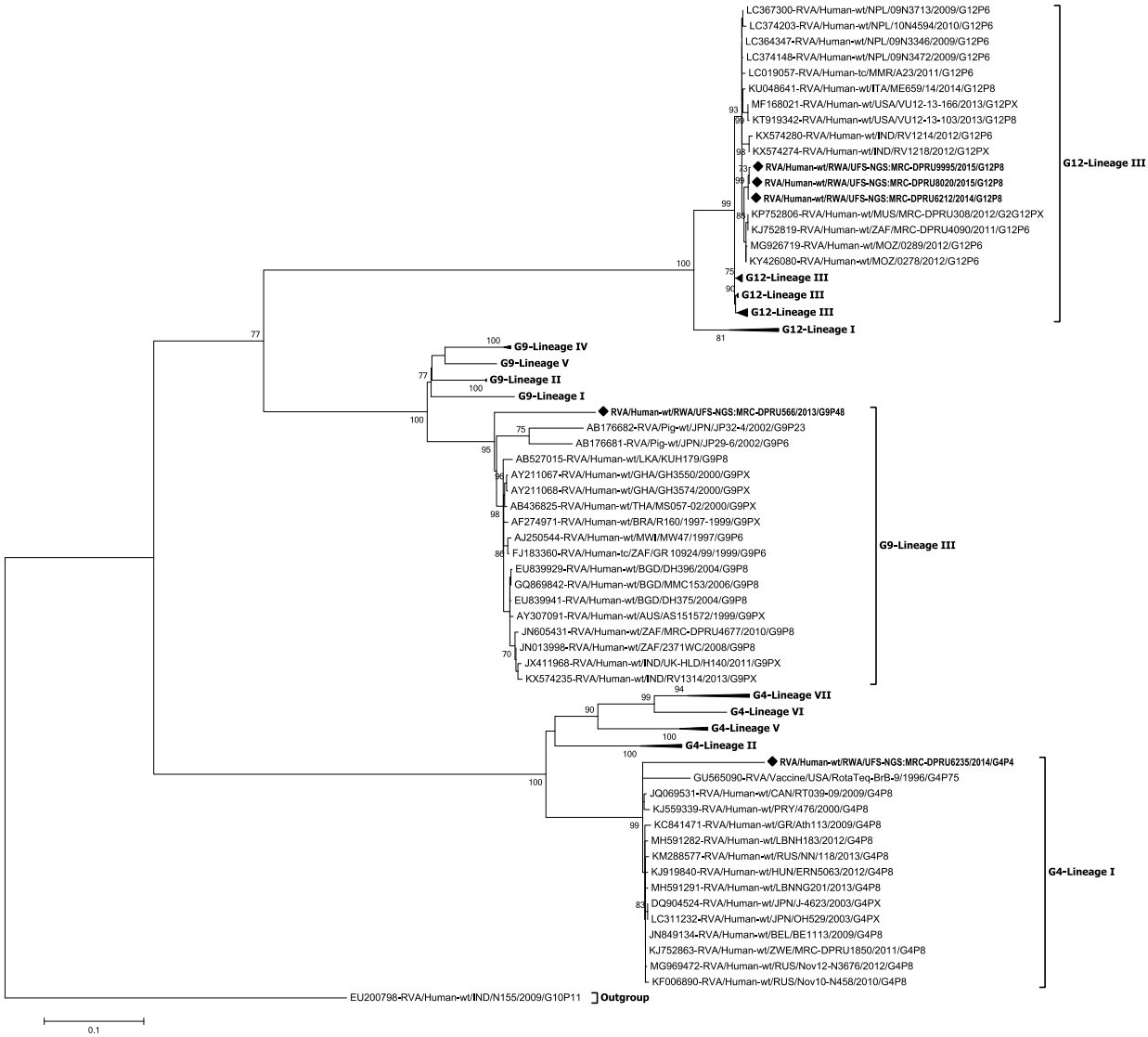


Figure 5.3: Phylogenetic tree of RVA strains based on the full length of the VP7 (G4, G9 and G12) gene displaying the relatedness of the study strains (◆) and reference strains from GenBank. Bootstrap values ≥ 70% are indicated at each branch node.

5.3.4. Phylogenetic analysis of the VP4 gene of P[4] and P[8]

The VP4 gene of the two Rwandan P[4] strains and three Rwandan P[8] strains clustered in P[4]-lineage II and P[8]-lineage III, respectively (Figure 5.4). Strain RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU6235/2014/G4P[4] and RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU566/2013/G9P[4] clustered amongst strains from Kenya, South Africa, Tanzania, and Uganda that circulated between 2011–2013 and shared 91.9-99.4% nt similarity. In contrast, the three Rwandan P[8] strains shared 99.3-100% nt similarity amongst themselves and clustered separately from other P[8] strains circulating globally.

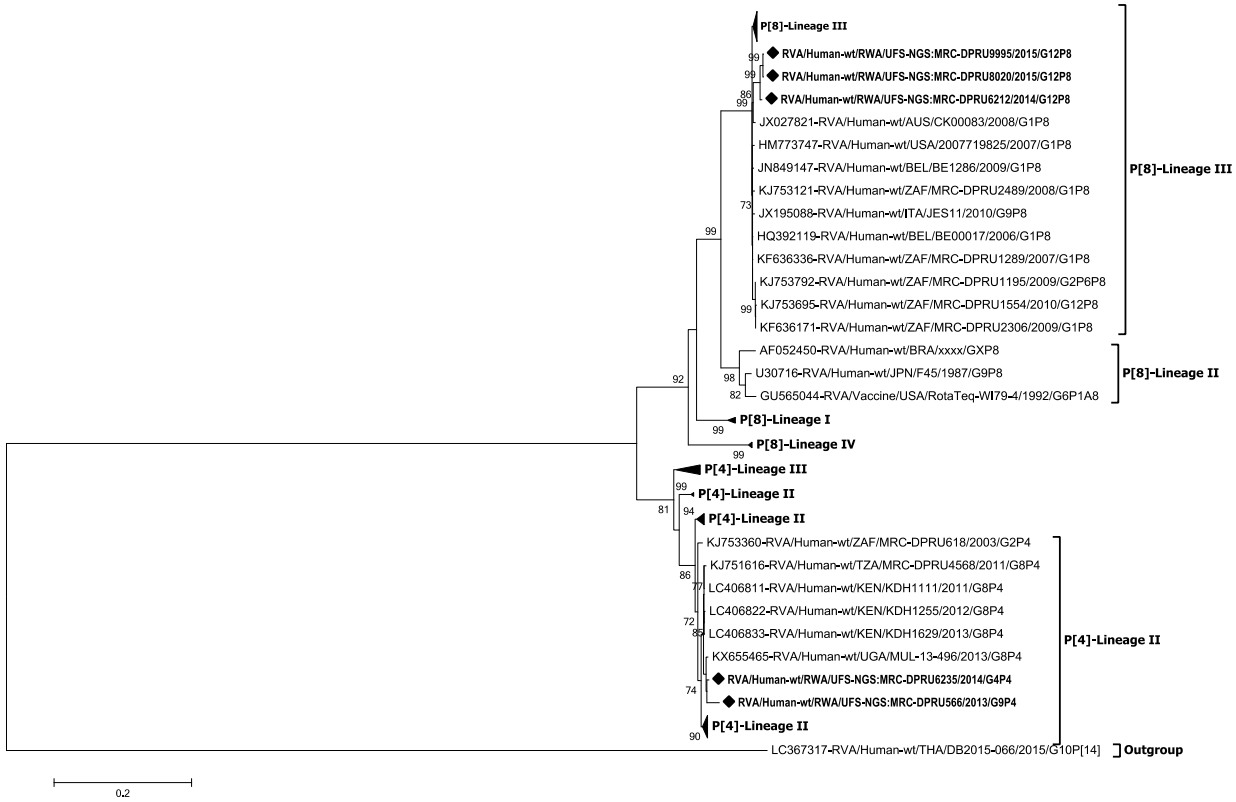
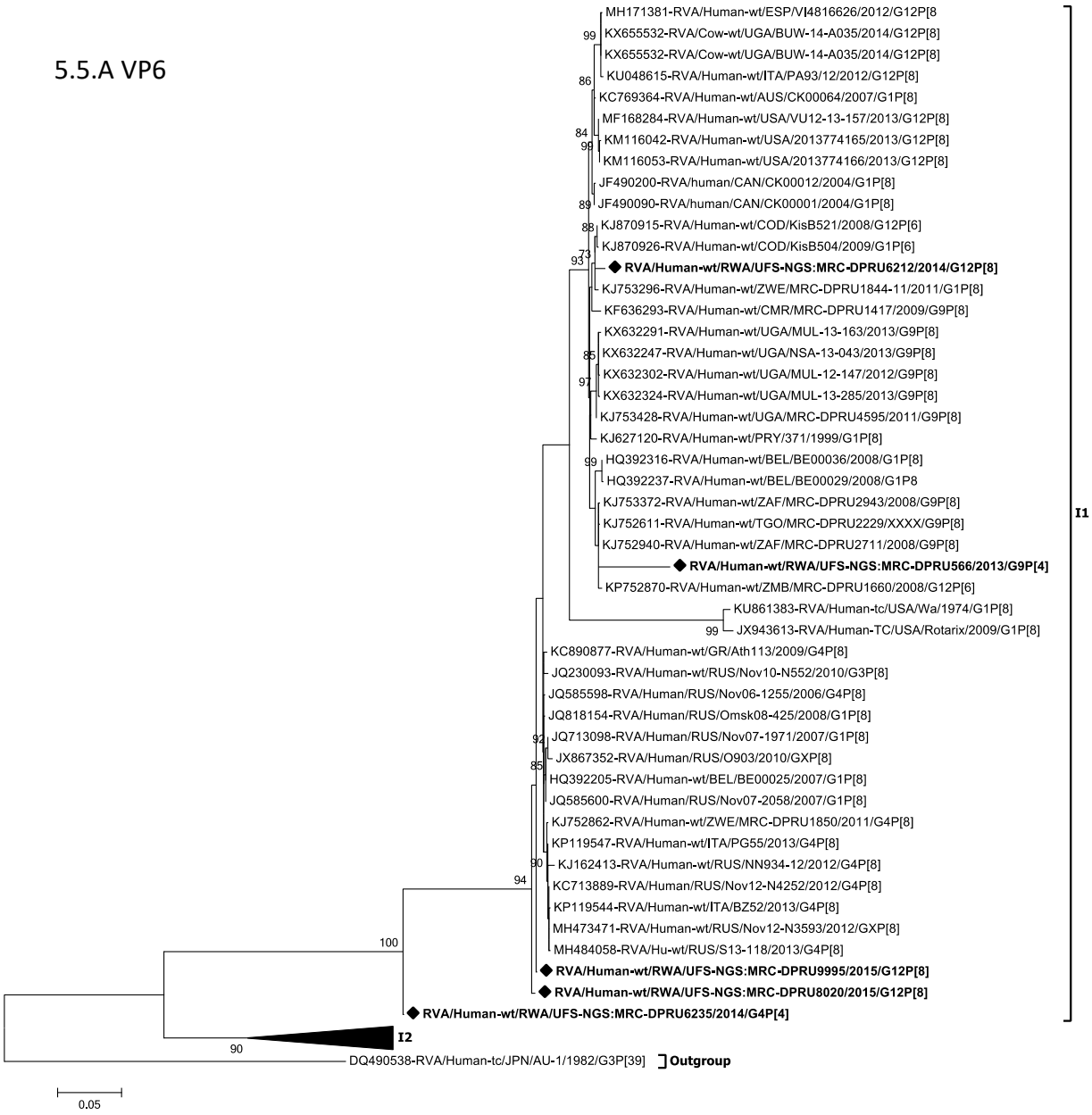


Figure 5.4: Phylogenetic tree of RVA strains based on the full length of the VP4 (P[4] and P[8]) gene displaying the relatedness of the study strains (◆) and reference strains from GenBank. Bootstrap values ≥ 70% are indicated at each branch node.

5.3.5. Phylogenetic analyses of the VP1-VP3 and VP6 genes

The VP6 gene of all the five Rwandan study strains clustered separately within lineage I1 and formed multiple sub-clusters (Figure 5.5A). Strain RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU8020/2015/G12P[8], RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU9995/2015/G12P[8] and RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU6235/2014/G4P[4] formed distinct branches that clustered separately from globally circulating strains. Furthermore, the VP1 gene of the two G12P[8] (RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU8020/2015/G12P[8] and RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU9995/2015/G12P[8]), G4P[4] and G9P[4] strains clustered in lineage R2, while strain RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU6212/2014/G12P[8] clustered in lineage R1 (Figure 5.5B). The two G12P[8] strains that clustered in lineage R2 clustered amongst human, animal and RotaTeq® vaccine strains were homologous. The VP2 gene separated into lineage C1 and lineage C2 (Figure 5.5C). RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU8020/2015/G12P[8] and RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU9995/2015/G12P[8] showed a similar clustering pattern as seen with the VP1 gene by clustering with both human and RotaTeq® vaccine strains exhibiting homologous similarity. RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU6212/2014/G12P[8] clustered with strains from Nepal and Myanmar that shared 99.5-99.6% nt similarity, while the G4P[4] and G9P[4] strains formed a distinct branch from strains circulating globally. The VP3 gene of the three G12P[8] strains clustered in lineage M1, while strain G4P[4] and G9P[4] clustered in lineage M2 (Figure 5.5D). RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU566/2013/G9P[4] and RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU6235/2014/G4P[4] formed distinct branches separate from globally circulating strains, while strain RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU6212/2014/G12P[8] clustered in a distinct sub-cluster to RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU8020/2015/G12P[8] and RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU9995/2015/G12P[8].

5.5.A VP6


MH171381-RVA/Human-wt/ESP/VI4816626/2012/G12P[8
KX655532-RVA/Cow-wt/UGA/BUW-14-A035/2014/G12P[8]
KX655532-RVA/Cow-wt/UGA/BUW-14-A035/2014/G12P[8]
KU048615-RVA/Human-wt/ITA/PA93/12/2012/G12P[8]
KC769364-RVA/Human-wt/AUS/CK00064/2007/G1P[8]
MF168284-RVA/Human-wt/USA/VU12-13-157/2013/G12P[8]
KM116042-RVA/Human-wt/USA/2013774165/2013/G12P[8]
KM116053-RVA/Human-wt/USA/2013774166/2013/G12P[8]
JF490200-RVA/human/CAN/CK00012/2004/G1P[8]
JF490090-RVA/human/CAN/CK00001/2004/G1P[8]
KJ870915-RVA/Human-wt/COD/KisB521/2008/G12P[6]
KJ870926-RVA/Human-wt/COD/KisB504/2009/G1P[6]
RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU6212/2014/G12P[8]
KJ753296-RVA/Human-wt/ZWE/MRC-DPRU1844-11/2011/G1P[8]
KF636293-RVA/Human-wt/CMR/MRC-DPRU1417/2009/G9P[8]
KX632291-RVA/Human-wt/UGA/MUL-13-163/2013/G9P[8]
KX632247-RVA/Human-wt/UGA/NSA-13-043/2013/G9P[8]
KX632302-RVA/Human-wt/UGA/MUL-12-147/2012/G9P[8]
KX632324-RVA/Human-wt/UGA/MUL-13-285/2013/G9P[8]
KJ753428-RVA/Human-wt/UGA/MRC-DPRU4595/2011/G9P[8]
KJ627120-RVA/Human-wt/PRY/371/1999/G1P[8]
HQ392316-RVA/Human-wt/BEL/BE00036/2008/G1P[8]
HQ392237-RVA/Human-wt/BEL/BE00029/2008/G1P8
KJ753372-RVA/Human-wt/ZAF/MRC-DPRU2943/2008/G9P[8]
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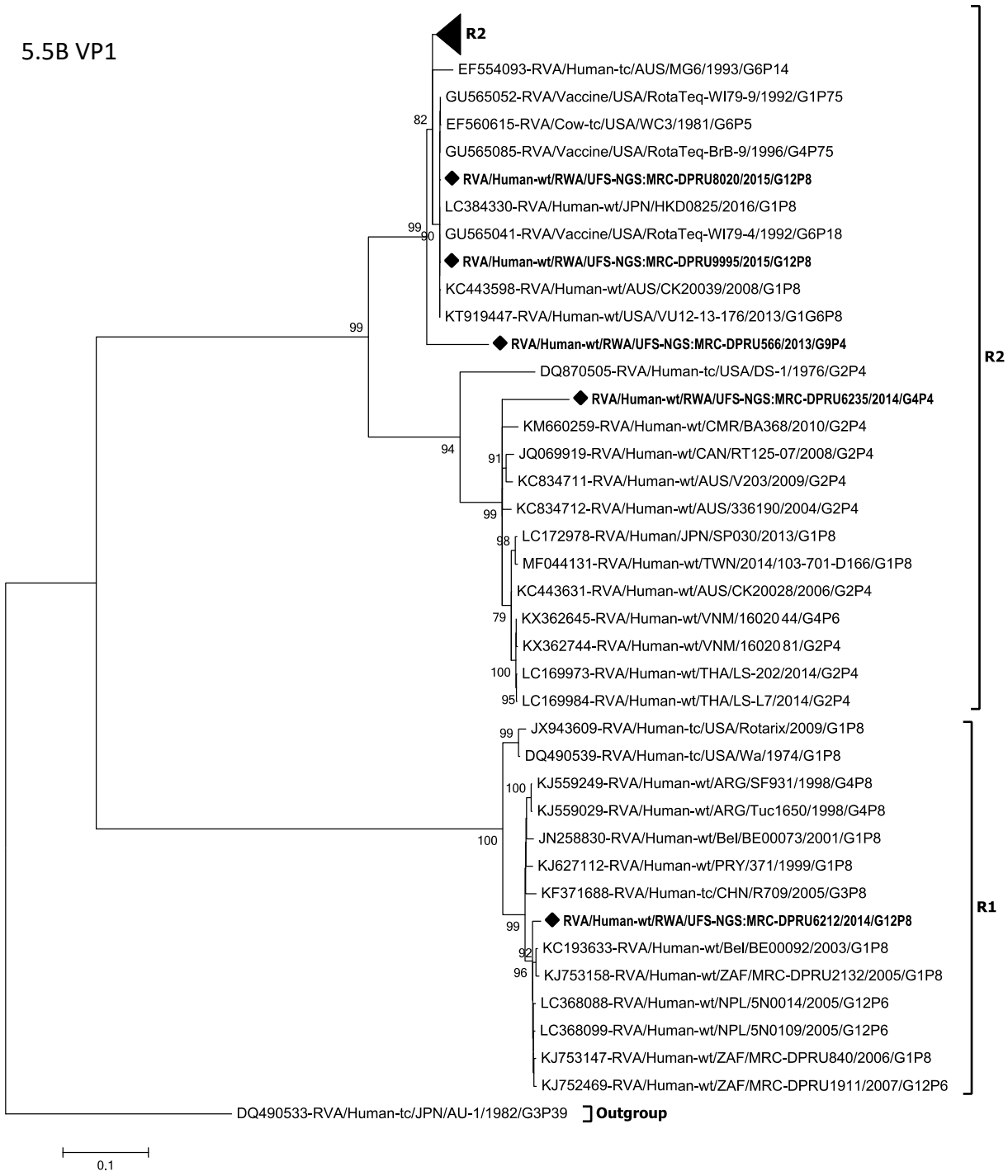
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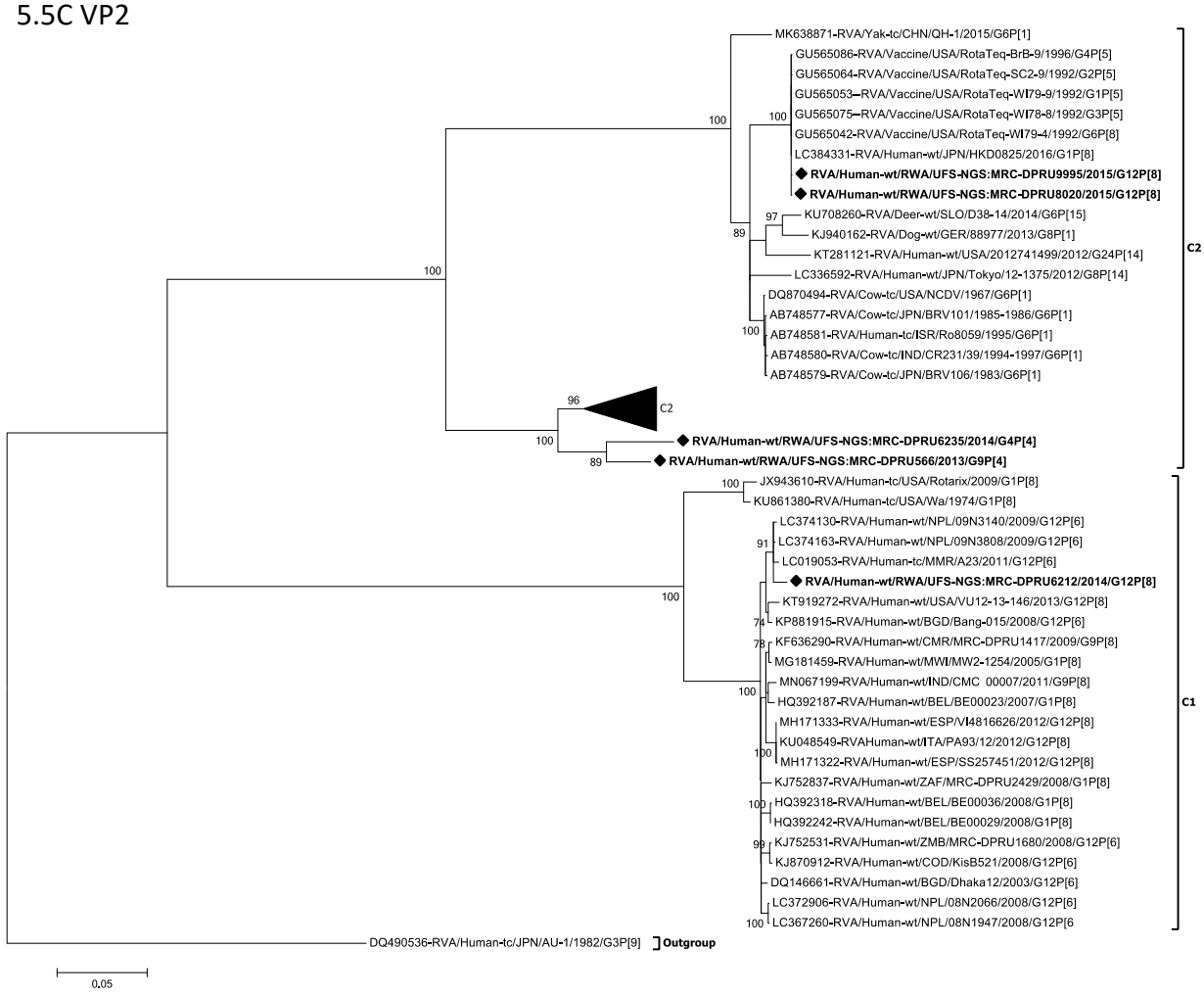
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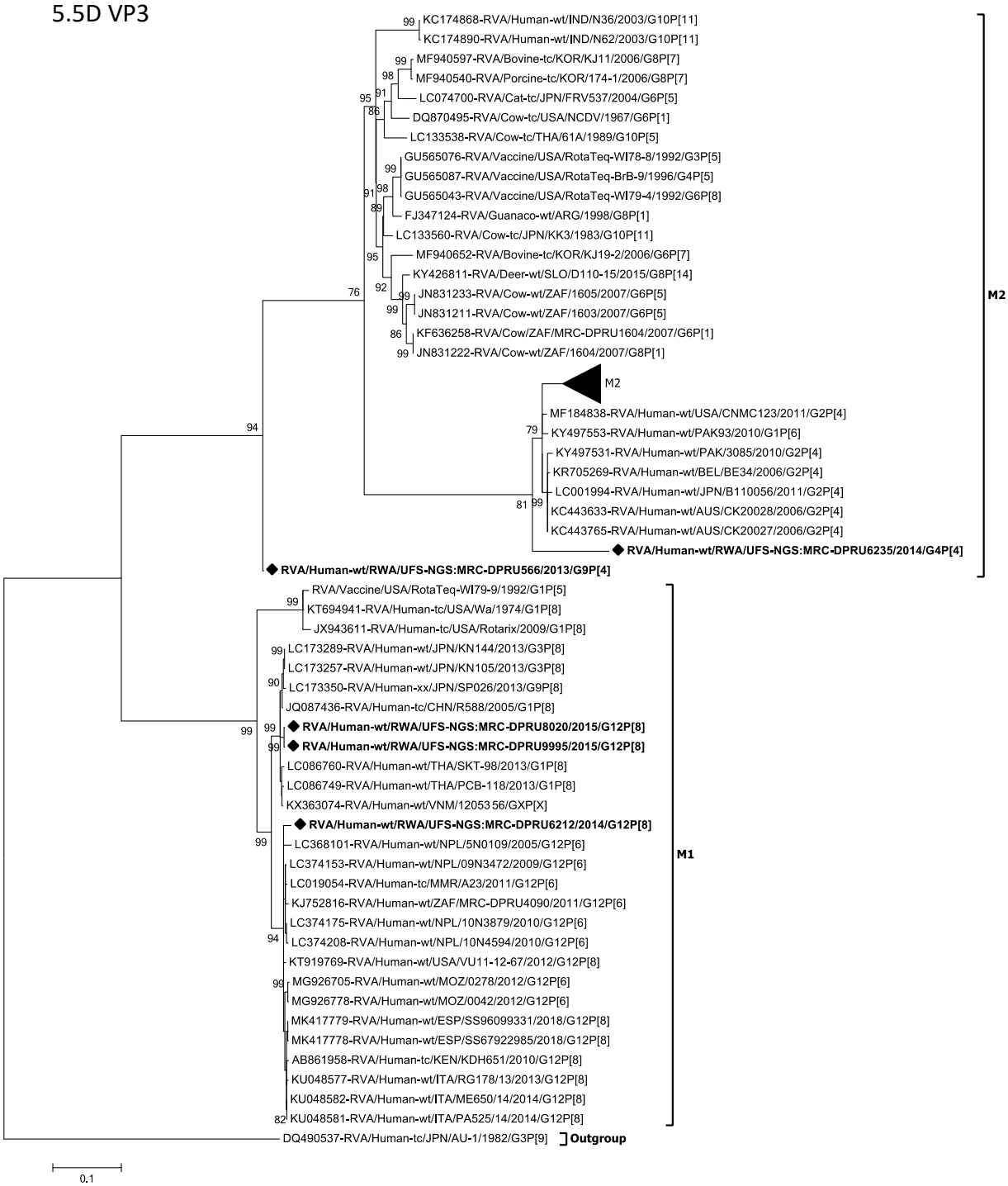
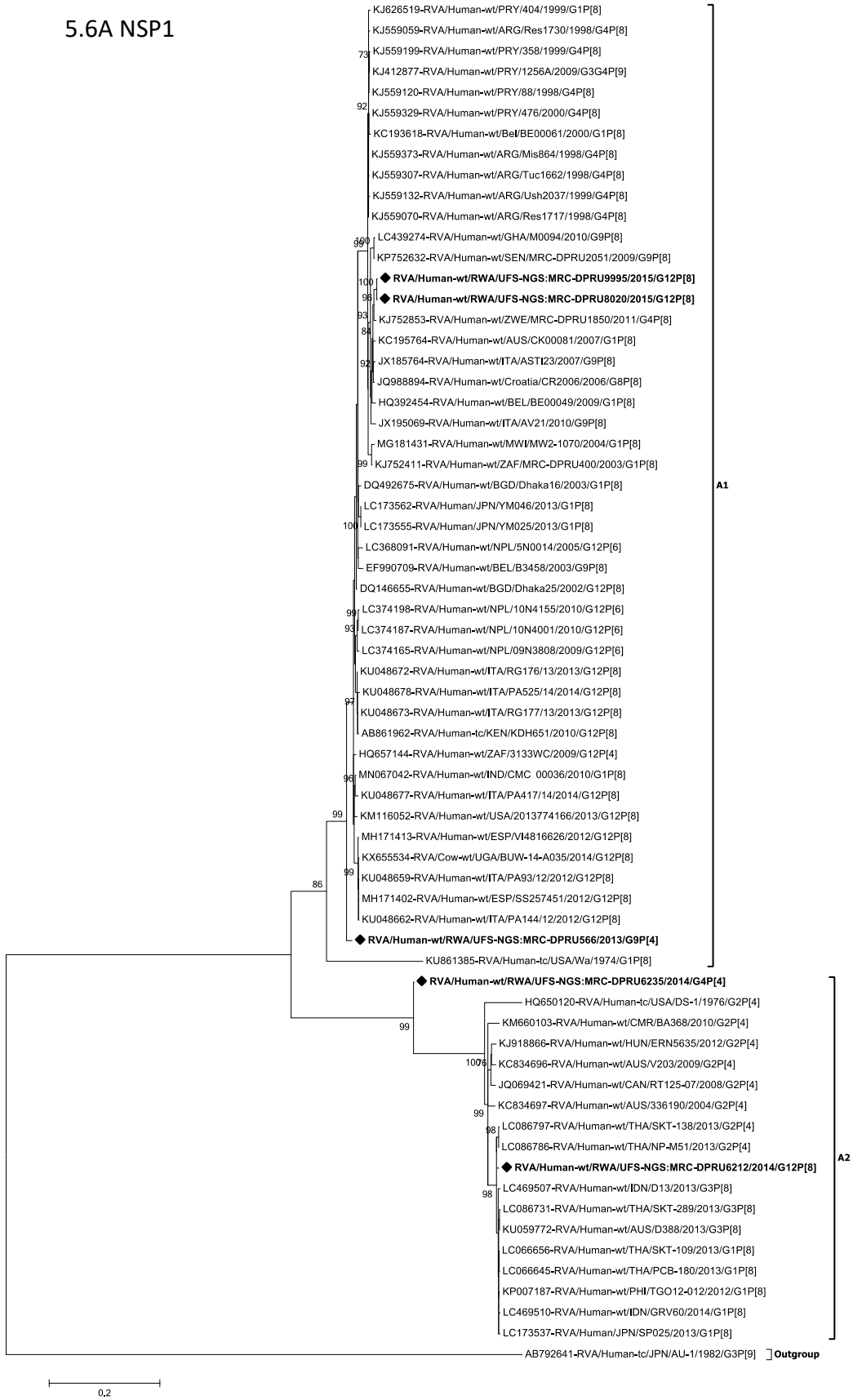


Figure 5.5: **A)** – **D)** Phylogenetic tree of RVA strains based on the full length of the **A)** VP6 **B)** VP1 **C)** VP2 **D)** VP3 genes displaying the relatedness of the study strains (◆) and reference strains from GenBank. Bootstrap values ≥ 70% are indicated at each branch node.

5.3.6. Phylogenetic analyses of the NSP1-NSP5 genes

The NSP1-NSP5 gene of study strain RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU8020/2015/G12P[8] and RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU9995/2015/G12P[8] phylogenetically clustered closely with reference strains circulating globally in previously established genotypes A1, N2, T1, E2 and H3 (Figure 5.6A-5.6E). The two G12P[8] strains also clustered among human and RotaTeq® vaccine strains in the NSP2 (homologous), NSP4 (homologous), and NSP5 (99.5-99.8% nt similarity) genes (Figure 5.6B, 5.6D and 5.6E). Furthermore, the NSP1 gene separated into lineage A1 and lineage A2 (Figure 5.6A). Strains G9P[4] and G4P[4] branched separately from globally circulating strains observed in lineage A1 and lineage A2, respectively. RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU6212/2014/G12P[8] clustered amongst strains from Thailand that shared 99.2% nt similarity. The NSP2 gene of the three G12P[8] strains and the G4P[4] strain clustered in lineage N2, while the G9P[4] strain clustered in lineage N1 (Figure 5.6B). RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU6212/2014/G12P[8] and RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU6235/2014/G4P[4] shared 95.9% nt similarity, while strain RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU566/2013/G9P[4] shared 97.1% nt similarity with a strain from the United States. The NSP3 gene of the two G12P[8] (RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU8020/2015/G12P[8] and RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU9995/2015/G12P[8]) strains, G4P[4] and G9P[4] clustered in lineage T1, while strain RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU6212/2014/G12P[8] clustered in lineage T2 (Figure 5.6C). RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU6212/2014/G12P[8] was homologous to a Ugandan strain. The NSP4 gene of the G4P[4] strain clustered closely with strains from Denmark, Russia and Japan that showed 99.4-99.6% nt similarity (Figure 5.6D). The G12P[8] and G9[4] strains clustered in lineage E1 and shared 99% nt similarity with Japan and Indian strains, respectively. The NSP5 gene separated into lineage H1, H2 and H3 (Figure 5.6E). The G12P[8] strain in lineage H1 clustered closely with strains from Nepal and Myanmar and shared 99.1-99.3% nt similarity, while the G9P[4] strain clustered closely with a strain from the United States (99.5% nt similarity). In contrast, the G4P[4] strain shared 99.5% and 99.8% similarity with a South African and Zimbabwean strain, respectively.

5.6A NSP1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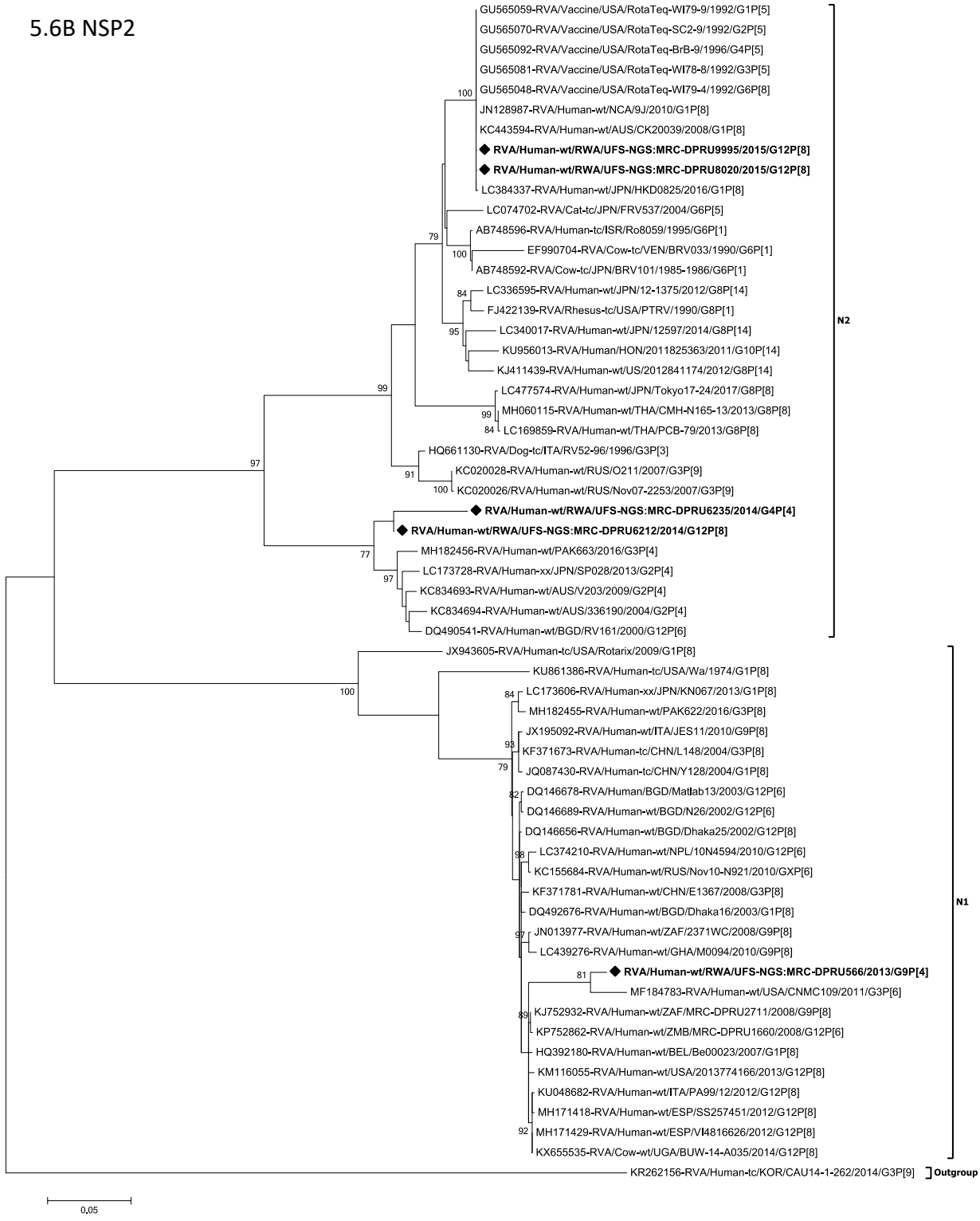
5.6B NSP2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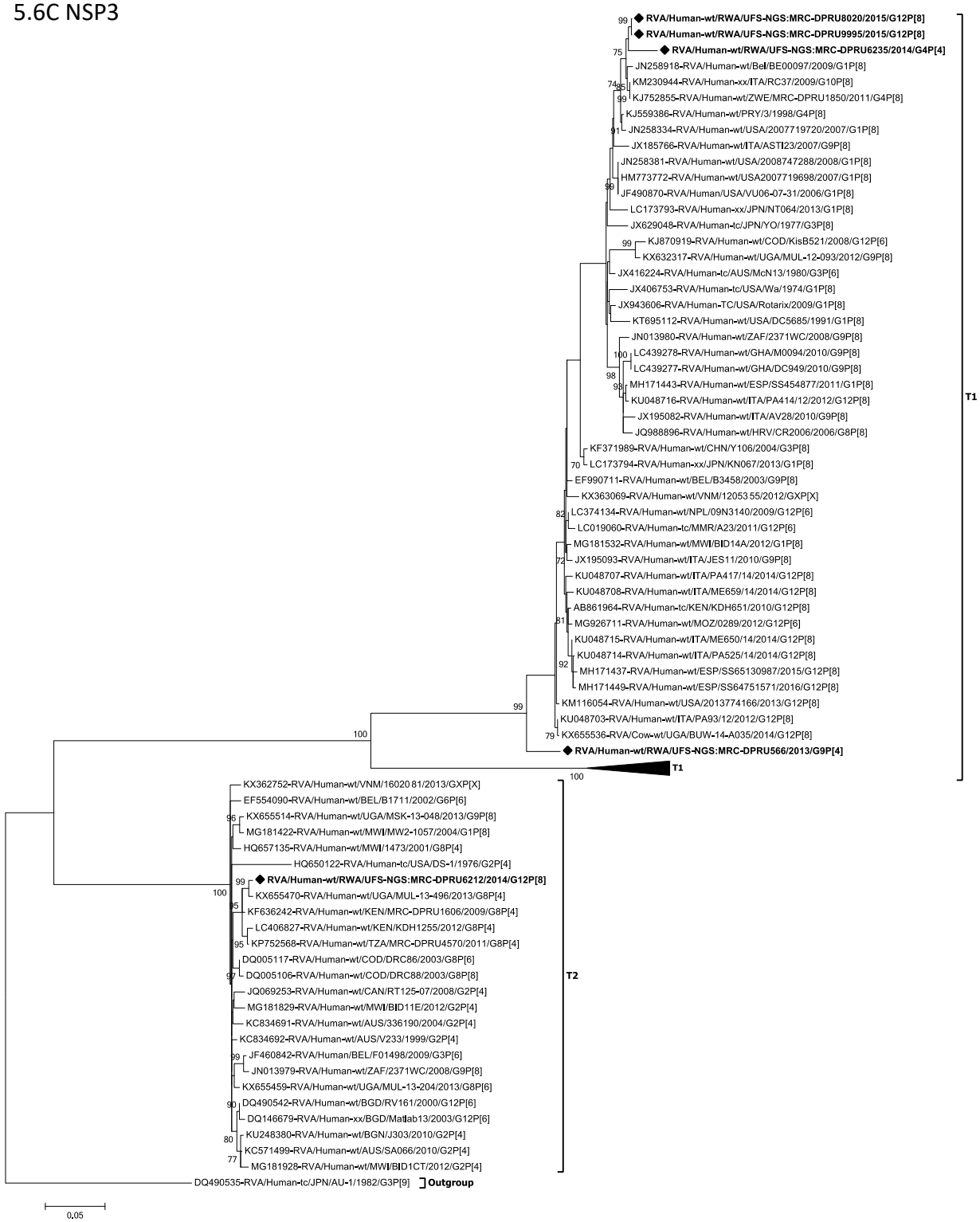
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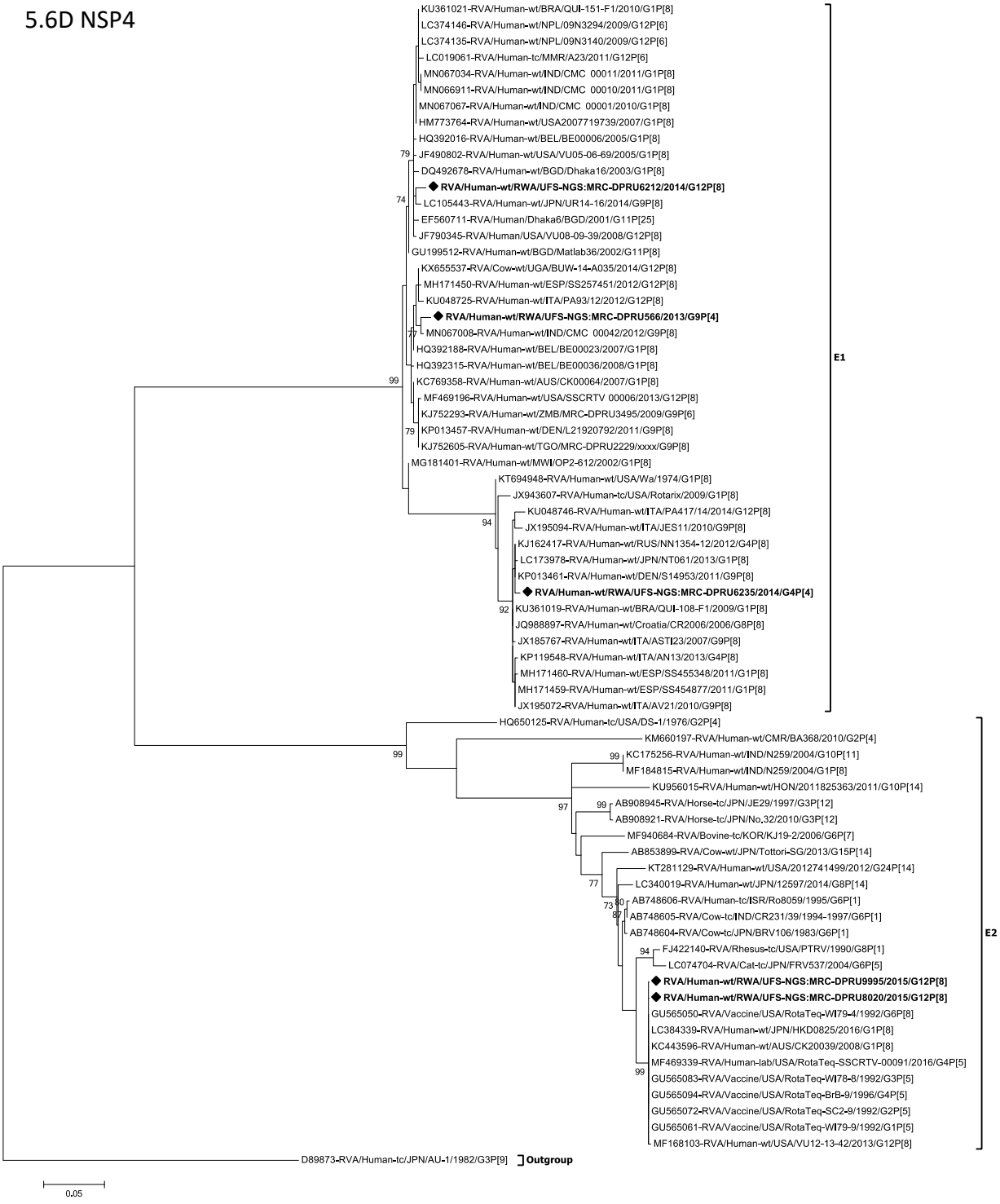
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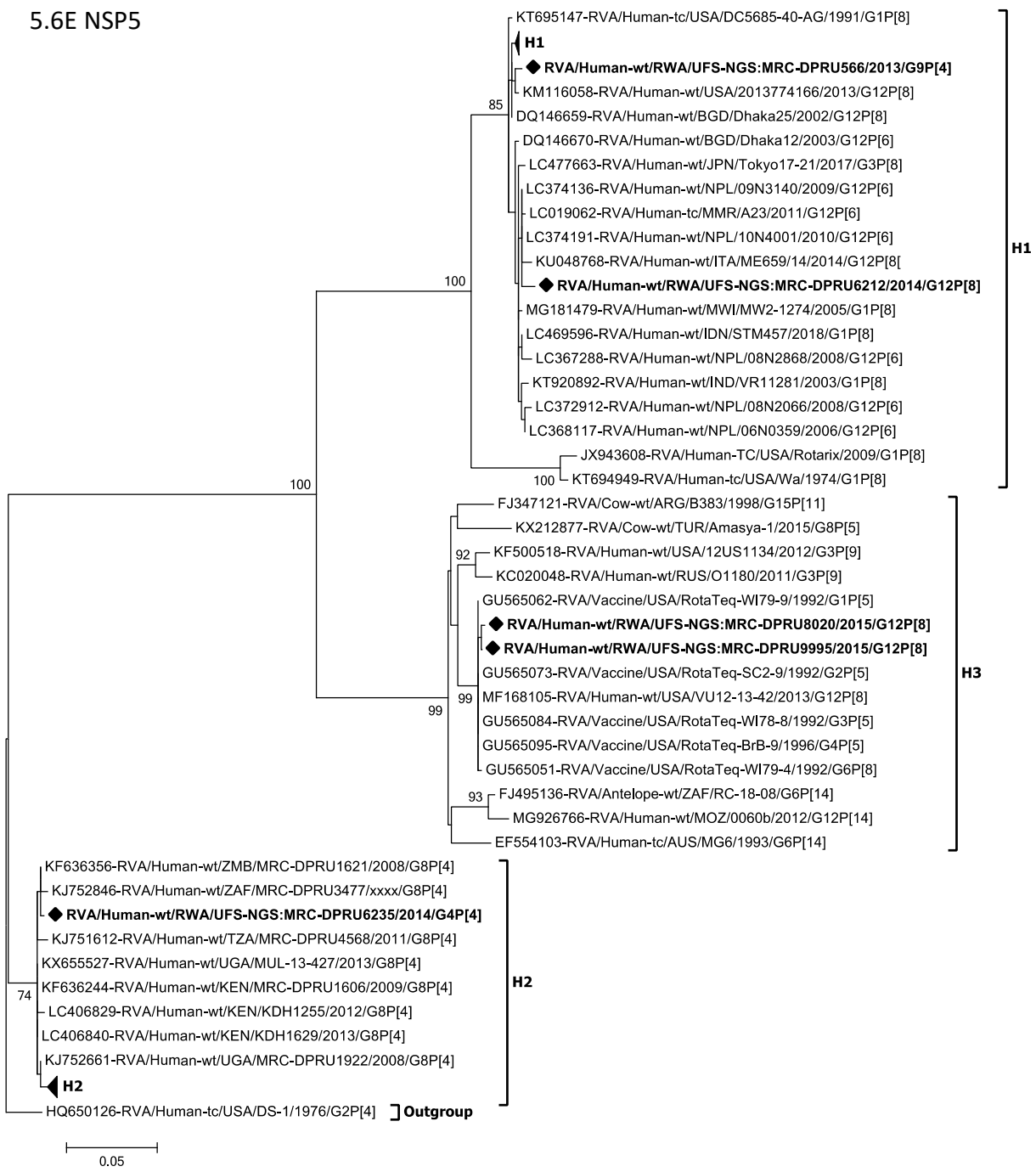

Figure 5.6: A) – **E)** Phylogenetic tree of RVA strains based on the full length of the **A)** NSP1 **B)** NSP2 **C)** NSP3 **D)** NSP4 **E)** NSP5 genes displaying the relatedness of the study strains (◆) and reference strains from GenBank. Bootstrap values ≥ 70% are indicated at each branch node.

5.4. Discussion

Five Rwandan rotavirus strains that have undergone inter-genogroup reassortment were identified in this study as part of WHO supported on-going rotavirus whole-genome sentinel surveillance. Strains RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU6235/2014/G4P[4], RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU566/2013/G9P[4] and RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU6212/2014/G12P[8] showed genotype constellations involving the Wa-like and DS-1-like genogroups while RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU8020/2015/G12P[8] and RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU9995/2015/G12P[8] exhibited triple-gene reassortment of all three human genogroups, Wa-like, DS-1-like, and AU-1-like. Bányai and colleagues (2011) stated that most atypical RVA strains are the result of natural inter-genogroup reassortment between the Wa-like and DS-1-like strains due to the segmented nature of RVA. The detection of these inter-genogroup reassortant strains in this study may be attributed to either the lack of RNA polymerase proofreading ability or co-infection of multiple strains from various human RVA strains (Estes and Greenberg, 2013; Jain et al., 2001; Kirkwood, 2010; Matthijnssens et al., 2008; Medici et al., 2007). Co-infections have been reported in high frequencies in several RVA strains across Africa (Dóró et al., 2014; Nyaga et al., 2018; Seheri et al., 2014; Todd et al., 2010).

The detection of the unusual G9P[4] strain (RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU566/2013/G9P[4]) in Rwanda is noteworthy as it is more prevalent in South-East Asia, Japan and Central America and detected in very low frequency (2%) in Africa (Afrad et al., 2013; Doan et al., 2017; João et al., 2020; Lartey et al., 2018; Pradhan et al., 2016; Quaye et al., 2013; Seheri et al., 2018; Yamamoto et al., 2015). Phylogenetic analysis revealed that the five Rwandan strains clustered mostly with strains circulating globally, suggesting a direct importation of these variants from abroad rather than local emergence through multiple reassortment events between locally circulating strains. It is evident that the reassortment events of the five Rwandan strains mostly occurred with contemporary human rotavirus strains as they did not show sufficient evidence of animal/human reassortment. Furthermore, RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU8020/2015/G12P[8] and RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU9995/2015/G12P[8] exhibit a high similarity amongst each other across the genome and both human and RotaTeq® vaccine strains in the VP2, NSP2, NSP4, and NSP5 gene segment. This finding suggests a reassortment between both human and RotaTeq® vaccine strains might have also transpired. This observation is consistent with the findings of Rose and co-workers, who have reported that such reassortment events are expected considering the attenuation of RotaTeq® vaccines and the segmented nature of the RVA genome (Rose et al., 2013b).

In the construction of the RotaTeq® vaccine in the 1980s, the G4 and P[8] components were included in the composition of this pentavalent vaccine (Matthijnssens et al., 2010). When comparing the G4 and P[8] components of the Rwandan strains and the RotaTeq® vaccine strains, we observed that the Rwandan G4 strain clustered closely to the RotaTeq® vaccine strain in G4-lineage I while the Rwandan P[8] strains clustered in P[8] lineage III distantly from the RotaTeq® vaccine strain in P[8]-lineage II. This phenomenon can be attributed to the constant evolutionary changes that rotaviruses undergo. Hence the currently circulating RVA strains may cluster in VP7 and VP4 lineages different from the RVA vaccine strains (Zeller et al., 2012). Such changes may influence a varying selective pressure against these VP7 and VP4 lineages ultimately reducing vaccine effectiveness over time. The VP1,VP3, NSP1 and NSP3 genes of strain RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU6235/2014/G4P[4] were phylogenetically distinct from strains circulating in other places thus suggesting that these gene segments may be unique.

The amino acid sequence comparison of the G4P[4] and G12P[8] Rwandan strains and the RotaTeq® vaccine strain showed that none of the Rwandan strains were identical to the RotaTeq® vaccine strain. Amino acid substitutions were observed throughout the VP7 epitope regions excluding position 291 and 190, while the VP4 exhibited only three amino acid substitutions at position 150, 195 and 388. The amino acid substitution at position 96, 97, 211, 217 and 221 on the VP7 epitope region suggests a radical change in polarity. McDonald et. al. (2009) suggested that such substitutions do not change the genotype specificity of the rotavirus strain. However, they may influence the binding of neutralizing antibodies thus affecting viral fitness through selection pressure. Despite the distant clustering between the Rwandan P[8] strains and the vaccine strain, only three changes were observed in the VP4 neutralizing epitope regions. Antigenic variations between rotavirus strains and the vaccine strains are usually implicated in the decreased effectiveness of rotavirus vaccines in low-income countries such as Rwanda (Narang et al., 2009; Taneja and Malik, 2012; Zaman et al., 2010). There is no evidence that the amino acid changes on the VP4 epitope regions are due to vaccination and could have occurred as a natural evolutionary process as they were observed in other strains circulating globally over the years.

5.5. Conclusion

The detection of five inter-genogroup reassortant Rwandan rotavirus strains from the whole-genome analysis further emphasises the ubiquitous nature and diversity of RVA strains in circulation. Whether vaccine introduction is responsible for the observed reassortment events or not remains enigmatic as several natural factors can be attributed to the evolution of these RVA strains. Amino acid substitutions observed in the epitope regions in the neutralizing epitope of the VP7 and VP4 proteins of the Rwandan strain when compared with the RotaTeq® vaccine strain may be implicated in the effectiveness of rotavirus vaccines being low in low-income countries. Continuous surveillance at the whole-genome level is highly recommended to monitor any possible changes due to vaccine pressure as part efforts to monitor the impact of vaccines on the circulating rotavirus strains especially in African countries.

5.6. Methods

5.6.1. Ethics statement

This study was reviewed and approved by the HSREC of the UFS and assigned an ethics number (UFS-HSD2019/1601/2810). Patients information were anonymized to ensure confidentiality.

5.6.2. Sample collection

One hundred and fifty-eight stool specimens were collected from hospitalized children presenting with acute gastroenteritis between 2012 and 2016 in Rwanda as part of the WHO/AFRO supported rotavirus surveillance program. These samples were retrieved from “the African stool repository” established to archive stool samples as part of the WHO supported African rotavirus surveillance network maintained at the DPRU, WHO RRL-SA. Only five samples presented the reassortant genome constellations, which fit the criteria for this study. Two samples were collected from unvaccinated children aged 14 months (female) and 36 months (male) from the South and East Province of Rwanda, respectively. The remaining three samples were collected from children (males) who received all three doses of the RotaTeq® vaccine, one aged 12 months (from the North Province) and two aged 24 months (both from the East Province). The samples were sequenced and whole-genome analysis was performed at the UFS-NGS Unit. The Rwandan strains described in this study were deposited in the GenBank database under accession numbers MT163179-MT163266.

5.6.3. Sample preparation for rotavirus whole-genome sequencing

The dsRNA was extracted using a protocol previously described by Nyaga et al. (2018) and purified using the Qiagen MinElute gel extraction kit (Qiagen, Hilden, Germany). The quantity of the purified dsRNA was thereafter verified by 1% agarose gel electrophoresis prior to quantification using a BioDrop-μLITE spectrophotometer (Biodrop, Cambridge, United Kingdom). For the cDNA synthesis, the Maxima H Minus Double-Stranded cDNA Synthesis Kit (ThermoFisher Scientific, Waltham MA, USA) was used according to the manufacturer's instructions with minor modifications. The modification included the denaturation of the dsRNA at 95°C for five minutes prior to synthesizing the first strand for 2 hours at 50 °C in a thermocycler (Merck, Darmstadt, Germany).

5.6.4. DNA library preparations and whole-genome sequencing

The Nextera® XT DNA library preparation kit (Illumina Inc., California, United States) was used in preparation for sequencing on an Illumina Miseq (Illumina Inc., California, United States) platform. Following the manufacturer's instructions, indexes were used to barcode the DNA and purified using AMPure XP magnetic beads (Beckman Coulter, Indiana, United States) while simultaneously selecting 300bp DNA fragments and removing short library fragments. The library was validated using a Bioanalyzer (Agilent Technologies, California, United States) and Qubit (Life Technologies, California, United States), and the samples were normalized to 8pM and pooled in a single tube in readiness for sequencing on an Illumina MiSeq (Illumina Inc., California, United States) platform. An Illumina v3 reagent kit (Illumina Inc., California, United States) was used for 600 cycles (301 x 2 paired ends) sequencing. A PhiX (20pM; Illumina Inc., California, United States), a spike of 20% was used as a control.

5.6.5. Computational analysis

The raw reads were assembled in Geneious Prime v11.1.5 (Kearse et al., 2012) and CLC Genomics Workbench v11 (CLC Bio, Qiagen, Hilden, Germany) as complementary tools. A reference-based assembly was carried out for the samples and the resulting contigs were used as query sequences in the RotaC v2.0 (Maes et al., 2009, http://rotac.regatools.be/) and the Nucleotide Basic Local Alignment Search Tool (BLASTn, https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the genotype of each gene and their full-length nucleotide sequence. Phylogenetic trees were constructed using the MEGA 6 software package (Tamura et al., 2013) with a Maximum Likelihood method-based model supported by bootstrap analysis with 1000 replicates. The *p*-distance algorithm was used to calculate nucleotide distances. The VP7 and VP4 protein structure was constructed using the Swiss-model

protein structure server (Waterhouse et al., 2018) and PyMOL v2. The validity of the structures were confirmed using YASARA (Krieger et al., 2002) and verify 3D (https://servicesn.mbi.ucla.edu/Verify3D/).

CHAPTER SIX: GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

6.1. Study summary

Over the years, RVA surveillance studies were primarily based on the outer capsid proteins (VP7 and VP4), which revealed great diversity in circulating strains with significant differences in prevalent strains, seasonally and geographically (Mwenda et al., 2010; Seheri et al., 2018). African countries are reported to have the greatest rotavirus diversity according to WHO global surveillance data. Since rotaviruses consist of 11 segments within the genome, information based solely on the outer capsid proteins may not be sufficient in providing conclusive data on the correct origin and overall genetic diversity of the strains (Ghosh and Kobayashi, 2011). The segmented RVA genome is vulnerable to reassortments, recombination and genomic rearrangements. Moreover, zoonotic transmissions are also possible and can only be confirmed through whole-genome analysis (Martella et al., 2010; Santos and Hoshino, 2005). Whole-genome sequencing has thus granted a platform to gain insight into the whole-genome of rotavirus strains circulating globally. Due to the lack of surveillance data on these strains with regards to the whole-genome, the WHO in partnership with the UFS-NGS Unit rolled out a pilot study to contribute to surveillance at whole-genome level, of several African countries, among them, Rwanda. To our knowledge, this is the first RVA whole-genome study involved in a surveillance study in Africa. The focus of our study was on G1P[8] and reassortant strains identified in Rwanda, highlighted in Chapter one and five, from a pool of 158 samples collected from hospitalized children presenting with gastroenteritis. The samples not analysed here (n=117), will be disseminated elsewhere.

Rotavirus dsRNA was successfully extracted from stool samples using TRI-reagent (Molecular Research Center, Inc, Cincinnati, OH, USA). All the dsRNA rotavirus samples collected from children presenting gastroenteritis were converted to cDNA using the maxima H minus ds cDNA synthesis kit (ThermoFisher Scientific, Waltham MA, USA) that was modified specifically for rotavirus as per the UFS-NGS Unit cDNA synthesis SOP. The qualities of the synthesized rotavirus cDNA were good enough to proceed with subsequent steps required prior to whole-genome sequencing and sufficiently covered objective one. However, some samples were excluded from sequencing due to low DNA concentrations which could be attributed the loss of nucleic material during the dsRNA extraction step, the cDNA purification step, and library preparation. Whole-genome characterization of rotavirus strains circulating in Rwanda was successfully achieved using an Illumina MiSeq platform.

The second and third objectives were addressed in chapter four and chapter five, respectively. Briefly, in chapter four, G1P[8] strains were analysed to determine the phylogenetic features pre- and post-vaccination in Rwanda. Data analysis of these strains revealed that the G1P[8] strains from Rwanda

are highly diverse compared to other circulating strains globally. The strains showed clear distinctive features between pre- and post-vaccine introduction strains by clustering separately in most of the phylogenetic trees. Furthermore, one of the G1P[8] strains was determined to be a RotaTeq® vaccine-derived strain as it was homologous to a RotaTeq® vaccine strain and clustered amongst vaccine strains throughout the entire backbone. The strain was shed from a two month-old infant, four days post-vaccine introduction. A representative G1P[8] strain was also compared to the RotaTeq® and Rotarix® vaccine strains, where amino acid changes in the neutralizing epitope regions and the CTL epitope region were observed. Some of the observed changes did not affect the polarity of the amino acids while others changed the polarity and were radical in nature. The changes were suggested to have occurred naturally as they were observed prior to vaccine introduction, while the other changes were observed post-vaccine introduction and are suspected of playing a role in the escape of host immunity (Rasebotsa et al., 2020).

In chapter 5, five reassortant strains were analysed to explore the probable evidence of rotavirus vaccine pressure in Rwanda. These strains were collected from two unvaccinated children (14 months female and 36 months male) and three children (12 months male and two 24 months old males) vaccinated with three doses of RotaTeq® vaccine. Two strains exhibited a triple gene reassortment (Wa-like, DS-1-like, and AU-1-like) while the other three strains showed a double gene reassortment (Wa-like and DS-1-like). The strains were all detected post-vaccine introduction from hospitalized children who presented episodes of diarrhoea and vomiting. Despite this observation, the reassortant strains were suggested to have occurred naturally as there was no evidence of vaccine-induced pressure. This was justified by observing how these strains were clustering in the phylogenetic trees. The strains were clustering with globally cognate circulating human RVA strains, with the exception of a few segments clustering with the RotaTeq® vaccine strain. Furthermore, comparative analysis of the antigenic region of our study strains and the RotaTeq® vaccine strain revealed the VP7 protein with multiple changes in the antigenic epitope region while the VP4 protein contained only two changes in the VP8* domain and only one change in the VP5* domain. These observations are consistent with changes observed on the antigenic regions of other strains circulating globally, suggesting a non-isolated event in Rwanda. These changes are however, worth noting as they may pose new challenges with regards to the effectiveness of currently available vaccines, especially in low-income countries such as Rwanda. Moreover, the observations in this project highlight that continuous whole-genome surveillance of circulating RVA strains is important in providing insight on the evolutionary changes occurring over the years, the impact of vaccines on circulating strains and also providing relevant information required for a rational design of future RVA vaccines.

The use of the Illumina Miseq platform increased throughput compared with less automated sequencing technologies thus providing quality data with a Q-score value of 30. The platform provided an opportunity to sequence multiple samples within a single run therefore decreasing the amount of time and money that would have been required when using the traditional sequencing method. This surveillance study is highly valuable to the Rwandan community as it successfully deciphered the whole-genome sequences of circulating rotavirus strains in Rwanda, thus expanding on the information already available from observing the outer capsid proteins. We established that the G1P[8] strains circulating in Rwanda have a stable Wa-like genome constellation such information will aid in understanding the transmission dynamics of G1P[8] strains in Rwanda. Furthermore, the whole-genome data from this study will contribute in addressing the evolutionary dynamics of rotavirus strains in Rwanda.

6.2. Limitations and recommendations

The principal limitation to this study was the number of years included in pre- and post-vaccine introduction thus it is not sufficient enough to conclusively determine the genetic variability of strains circulating in Rwanda as part of the pilot WHO whole-genome surveillance study. The data presented was for one year pre-vaccine introduction (2011) and five years post-vaccine introduction (2012-2016), where the ideal sample set could be in the long-term of at least seven years pre- and seven years post-vaccination. The study was principally *in-silico* based, thus cell culture was not attempted to provide supporting information on our findings. Nevertheless, whole-genome analysis is highly reliable in providing conclusive data on the evolutionary dynamics of circulating RVA strains.

In this study, we only analysed 41 samples out of 158 samples sequenced thus there is still enough room to explore the diversity and evolutionary patterns of rotavirus strains circulating in Rwanda. We also recommend conducting cell cultures to confirm conclusions made based on *in-silico* analysis. We highly recommend the use of NGS platforms for surveillance in Africa to decipher novel information that may be otherwise missed when looking only at the outer capsid proteins. Rwanda has recently switched from the use of the RotaTeq® vaccine (2012-2017) to the Rotarix® vaccine (2018); thus it will be interesting to see if the switch will have any effect on the strains circulation in Rwanda in the coming years.

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APPENDICES

Appendix A

Letter of ethics approval from the Health Sciences Research Ethics Committee

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

Health Sciences Research Ethics Committee

15-Oct-2019

Dear **Miss Sebotsana Rasebotsa**

Ethics Clearance: **Whole genome analyses of rotavirus strains circulating pre- and post RotaTeq™ vaccine introduction in Rwanda.**

Principal Investigator: **Miss Sebotsana Rasebotsa**

Department: **Medical Microbiology Department (Bloemfontein Campus)**

APPLICATION APPROVED

Please ensure that you read the whole document

With reference to your application for ethical clearance with the Faculty of Health Sciences, I am pleased to inform you on behalf of the Health Sciences Research Ethics Committee that you have been granted ethical clearance for your project.

Your ethical clearance number, to be used in all correspondence is:**UFS-HSD2019/1601/2810**

The ethical clearance number is valid for research conducted for one year from issuance. Should you require more time to complete this research, please apply for an extension.

We request that any changes that may take place during the course of your research project be submitted to the HSREC for approval to ensure we are kept up to date with your progress and any ethical implications that may arise. This includes any serious adverse events and/or termination of the study.

A progress report should be submitted within one year of approval, and annually for long term studies. A final report should be submitted at the completion of the study.

The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

For any questions or concerns, please feel free to contact HSREC Administration: 051-4017794/5 or email EthicsFHS@ufs.ac.za.

Thank you for submitting this proposal for ethical clearance and we wish you every success with your research.

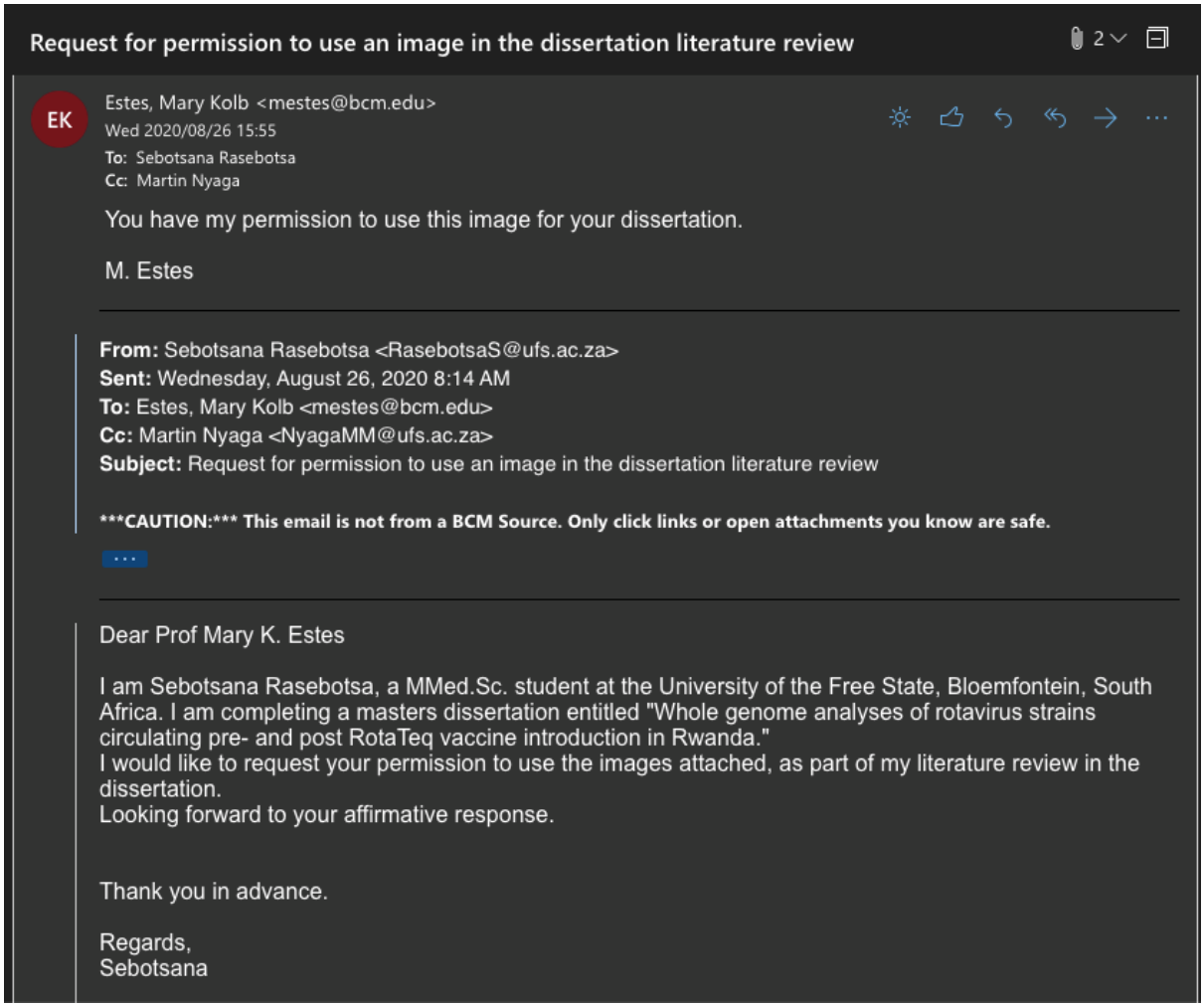
Yours Sincerely

Dr. SM Le Grange
Chair : Health Sciences Research Ethics Committee

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | E: ethicsfhs@ufs.ac.za
IRB 00006240; REC 230408-011; IORG0005187; FWA00012784
Block D, Dean's Division, Room D104 | P.O. Box/Posbus 339 (Internal Post Box G40) | Bloemfontein 9300 | South Africa

Appendix B

Permission to use images in figure 2.1 and 2.2 from an author



Request for permission to use an image in the dissertation literature review

EK

Estes, Mary Kolb <mestes@bcm.edu>
Wed 2020/08/26 15:55
To: Sebotsana Rasebotsa
Cc: Martin Nyaga

You have my permission to use this image for your dissertation.

M. Estes

From: Sebotsana Rasebotsa <RasebotsaS@ufs.ac.za>
Sent: Wednesday, August 26, 2020 8:14 AM
To: Estes, Mary Kolb <mestes@bcm.edu>
Cc: Martin Nyaga <NyagaMM@ufs.ac.za>
Subject: Request for permission to use an image in the dissertation literature review

CAUTION: This email is not from a BCM Source. Only click links or open attachments you know are safe.**

Dear Prof Mary K. Estes

I am Sebotsana Rasebotsa, a MMed.Sc. student at the University of the Free State, Bloemfontein, South Africa. I am completing a masters dissertation entitled "Whole genome analyses of rotavirus strains circulating pre- and post RotaTeq vaccine introduction in Rwanda."
I would like to request your permission to use the images attached, as part of my literature review in the dissertation.
Looking forward to your affirmative response.

Thank you in advance.

Regards,
Sebotsana

Appendix C

Supporting manuscript for chapter four.

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Whole genome and in-silico analyses of G1P[8] rotavirus strains from pre- and post-vaccination periods in Rwanda

Sebotsana Rasebotsa[1,6], Peter N. Mwangi[1,6], Milton T. Mogotsi[1], Saheed Sabiu[1], Nonkululeko B. Magagula[2], Kebareng Rakau[2], Jeannine Uwimana[3], Leon Mutesa[3], Narcisse Muganga[3], Didier Murenzi[3], Lisine Tuyisenge[3], Jose Jaimes[4], Mathew D. Esona[4], Michael D. Bowen[4], M. Jeffrey Mphahlele[2], Mapaseka L. Seheri[2], Jason M. Mwenda[5] & Martin M. Nyaga[1]✉

Rwanda was the first low-income African country to introduce RotaTeq vaccine into its Expanded Programme on Immunization in May 2012. To gain insights into the overall genetic make-up and evolution of Rwandan G1P[8] strains pre- and post-vaccine introduction, rotavirus positive fecal samples collected between 2011 and 2016 from children under the age of 5 years as part of ongoing surveillance were genotyped with conventional RT-PCR based methods and whole genome sequenced using the Illumina MiSeq platform. From a pool of samples sequenced (n = 158), 36 were identified as G1P[8] strains (10 pre-vaccine and 26 post-vaccine), of which 35 exhibited a typical Wa-like genome constellation. However, one post vaccine strain, RVA/Human-wt/RWA/UFS-NGS:MRC-DPRU442/2012/G1P[8], exhibited a RotaTeq vaccine strain constellation of G1-P[8]-I2-R2-C2-M2-A3-N2-T6-E2-H3, with most of the gene segments having a close relationship with a vaccine derived reassortant strain, previously reported in USA in 2010 and Australia in 2012. The study strains segregated into two lineages, each containing a paraphyletic pre- and post-vaccine introduction sub-lineages. In addition, the study strains demonstrated close relationship amongst each other when compared with globally selected group A rotavirus (RVA) G1P[8] reference strains. For VP7 neutralization epitopes, amino acid substitutions observed at positions T91A/V, S195D and M217T in relation to the RotaTeq vaccine were radical in nature and resulted in a change in polarity from a polar to non-polar molecule, while for the VP4, amino acid differences at position D195G was radical in nature and resulted in a change in polarity from a polar to non-polar molecule. The polarity change at position T91A/V of the neutralizing antigens might play a role in generating vaccine-escape mutants, while substitutions at positions S195D and M217T may be due to natural fluctuation of the RVA. Surveillance of RVA at whole genome level will enhance further assessment of vaccine impact on circulating strains, the frequency of reassortment events under natural conditions and epidemiological fitness generated by such events.

Group A rotavirus is a significant viral etiological agents of acute gastroenteritis (AGE) resulting in approximately 125,000 deaths annually in children under 5 years worldwide[1]. Rwanda rolled out the RotaTeq (Merck and Co., Whitehouse Station, NJ, USA) vaccine into her Expanded Program on Immunization in May 2012 and the vaccination coverage rate has been consistently high over the years > 95% since 2013, resulting in significant

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