INSTITUTO DE HIGIENE E MEDICINA TROPICAL
DESDE 1902

UNIVERSIDADE NOVA DE LISBOA

Universidade Nova de Lisboa

Instituto de Higiene e Medicina Tropical

Genetic characterization of rotavirus strains in Mozambique, 2012-2018: identification of rotavirus genotypes and full genome sequencing by next generation sequencing

Eva Dora da Cruz João

PHD DISSERTATION IN HUMAN GENETICS AND INFECTIOUS DISEASES

(AUGUST, 2019)

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Dissertation presented to fulfil the requirements necessary to obtain a PhD in Human Genetics and Infectious Diseases

Financial support by Calouste Gulbenkian Foundation, European Foundation Initiative into African Research in Neglected Tropical Diseases (EFINTD), World Health Organization, Regional Office for Africa (WHO / AFRO)

Articles

Published articles related to doctoral work

1 - Eva Dora João, Amy Strydom, Hester G. O'Neill, Assa Cuamba, Marta Cassocera, Sozinho Acácio, Inácio Mandomando, Lithabiso Motanyane, Nicola Page, Nilsa de Deus. Rotavirus A strains obtained from children with acute gastroenteritis in Mozambique, 2012-2013: G and P genotypes and phylogenetic analysis of VP7 and partial VP4 genes. 2018. Arch Virol 163(1):153-165.

2 - Eva Dora João, Benilde Munlela, Assucênio Chissaque, Jorfélia Chilaúle. Jerónimo Langa, Orvalho Augusto, Simone Boene, Elda Anapakala, Júlia Sambo, Esperança Guimarães, Diocreciano Bero, Marta Cassocera, Idalécia Cossa-Moiane, Jason M. Mwenda, Isabel Maurício, Hester G. O'Neill and Nilsa de Deus. Molecular Epidemiology of Rotavirus A Strains Pre- and Post-Vaccine (Rotarix®) Introduction in Mozambique, 2012–2019: Emergence of Genotypes G3P[4] and G3P[8]. 2020. *Pathogens 9*, 671.

Other related publication

1- Amy Strydom, Eva Dora João, Lithabiso Motanyane, Martin Nyaga, Cristiaan Potgieter, Assa Cuamba, Inácio Mandomando, Marta Cassocera, Nilsa de Deus, and Hester O'Neill. 2019. Whole Genome Analyses of DS-1-like Rotavirus A Strains Detected in Children with Acute Diarrhoea in Southern Mozambique Suggest Several Reassortment Events. Infection, Genetics and Evolution 69 (April): 68–75.

2 - Nilsa de Deus, Eva Dora João, Assa Cuamba, Marta Cassocera, Leopoldina Luís, Sozinho Acácio, Inácio Mandomando, Orvalho Augusto e Nicola Page. 2018. Epidemiology of Rotavirus Infection in Children from a Rural and Urban Area, in Maputo, Southern Mozambique, before Vaccine Introduction. Journal of Tropical Pediatrics, 1–5.

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Conferences presentations and contribution

Related to doctoral work

1 - Eva Dora João, Amy Strydom, Benilde Munlela, Martin Nyaga, Jorfélia Chilaúle, Jerónimo Langa, Ezequias Sitoe, Assucênio Chissaque, Elda Anapakala, Júlia Sambo, Esperança Guimarães, Diocreciano Bero, Marta Cassocera, Lena Manhique, José Paulo Langa, Idalécia Cossa-Moiane, Isabel Maurício, Hester G. O'Neill & Nilsa de Deus. Whole genome characterization of rotavirus strains from National Rotavirus Surveillance of Acute Diarrhea in Mozambique. 13th International dsRNA Virus Symposium 24 - 29 September 2018, Houffalize, Belgium. Poster.

2 - Eva Dora João, Amy Strydom, Benilde Munlela, Jorfélia Chilaúle, Jerónimo Langa, Elda Anapakala, Isabel Maurício, Hester G. O'Neill, Equipe VINADIA, & Nilsa de Deus. *Caracterização do Genoma completo de Estirpes de Rotavírus da Vigilância Nacional de Diarreia Aguda em Moçambique*. XVI *Jornadas de Saúde*, 17 - 20 September 2018, *Instituto Nacional de Saúde*, Maputo, Mozambique. Oral presentation.

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3 - Simon Boene, Eva Dora João, Amy Strydom, Benilde Munlela, Elvino Nabetse, Dalili Latifo, Aida Cala, Lourenço Mapaco, Hester O'Neill & Nilsa de Deus. Whole genome characterization of porcine rotavirus A collected in southern Mozambique, reveals G9P[13] and G4P[6] strains with a Wa-like backbone. 12th African Rotavirus Symposium, 30 July – 01 August 2019. Johannesburg, South Africa. Oral presentation.

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Dedication

To my daughter Maggui

Acknowledgements

The present work should not be concluded before I pronounce my gratitude to:

My supervisor Prof. Nilsa de Deus. We started this journey in 2012 and decided to continue until today. Thank you for all the unconditional support you provided.
My co-supervisor Prof. Isabel Maurício, who during this journey encouraged me to have energy and believe that I can finish this project.
My co-supervisor Prof. Hester O´Neill. We started this journey in 2013 and we are still together today, always ready to help unconditionally.

ViNaDiA team, which allowed this work to be done, especially Jorfelia and Elda, who provided all the materials to conduct this work.
Benilde Munlela, Simone Boene, Jerónimo Langa and Assucenio Chissaque, team from ViNaDiA for rotavirus detection and genetic characterization, we made a great team together.
Amy Strydom, thank you for all your time for training me on rotavirus full genome sequencing by Next Generation Sequencing.
Doctor Martin Nyaga, thank you for sequencing our rotavirus strains in your unit.
Marta Cassossera and Miguel Bambo who provided help for data management and to Idalécia Cossa-Moiane that opened to ViNaDiA and to me her laboratory.

My partner Milton Mapatse, always patient, waiting for me.
My mother Rosa Florência and my brother Miguel Tafula, who always kept my motivation up.
My friend and mother in Lisbon, Maria da Luz. I learned with you to be a better person.
Tio Rondane and Tia Mila, family in Lisbon, always ready to help me.
My friends Anabela Freitas and Liliana Santos from Lisbon, always ready to help me.
Margarida Cunha, from Calouste Gulbenkian Foundation, always ready to support me with all questions related to my PhD scholarship.

S**ponsors:**

This work was financed by:

- PhD scholarship from Calouste Gulbenkian Foundation (Process number 135630, reference 117/SBG/14).
- European Foundation Initiative into African Research in Neglected Tropical Diseases (EFINTD).
- World Health Organization, Regional Office for Africa (WHO / AFRO).
- Antigens and Reassortant Strains for Rotaviruses Circulating in Africa-*AFRota* (Deutsche Forschungsgemeinschaft -DFG; JO369/5-1)

Resumo

Os rotavirus do grupo A são uma das principais causas de diarreia aguda grave em crianças menores de 5 anos de idade em todo o mundo. Os vírus também causam infecção em animais jovens de variadas espécies de mamíferos e aves. Existem vários genótipos de rotavirus que causam infecção em crianças, por isso, as pesquisas sobre a circulação e prevalência de estirpes de genótipos de rotavirus circulantes e o sequenciamento do genoma completo são importantes para caracterizacção genética do vírus no país, auxiliando assim na avaliação do impacto da vacina contra rotavirus introduzida no programa alargado de vacinação em Setembro de 2015. Neste contexto, o principal objetivo deste projeto foi caracterizar geneticamente as estirpes de rotavirus circulantes entre 2012 e 2018 em Moçambique, incluindo uma comparação antes e após a introdução da vacina contra rotavirus no país.

As estirpes de rotavirus foram detectadas em crianças menores de cinco anos de idade que apresentaram diarreia aguda grave. A caracterização genética de rotavirus foi realizada utilizando RT-PCR, sequenciação de moléculas de DNA pelo método de paragem da polimerização por incorporação de didesoxirribonucleótidos (método de Sanger) e sequenciação do genoma completo da segunda geração (*Next Generation Sequencing*).

Os resultados de RT-PCR mostraram a alta diversidade de genótipos circulantes com variações anuais no período pré e após a introdução da vacina, conforme reportado anteriormente por vários estudos a nível mundial. Antes da introdução da vacina (2012-2013), os genótipos de rotavirus mais frequentes foram G2P[4] e G12P[6] e durante 2014-2015 foram G1P[8] e G9P[8]. Após a introdução da vacina (2016-2018), observou-se o surgimento dos genótipos emergentes como G9P[4], G3P[4] e G3P[8] que não tinham sido detectados antes. O surgimento de novos genótipos de rotavirus no período pós-vacinal reforça a necessidade de uma vigilância contínua em todo o país para monitorizar se o aparecimento de outros genótipos foi devido a vacina.

A análise do genoma completo das estirpes dos genótipos G1P[8] e G9P[8] de 2014 e 2016 revelou a circulação de estirpes com um *backbone Wa-like* típico. Com as análises do genoma completo, foi possível fornecer evidências da diversidade genética do genótipo P[8] em Moçambique ao longo dos anos e foram observadas várias substituições nas

regiões antigénicas de rotavirus, sendo importante continuar a vigilância das genótipos para monitorizar o efeito dessas mutações.

A análise do genoma completo das estirpes dos genótipos G2P[6] circulantes em 2015 e 2016 revelou um *backbone DS1-like* típico com alta identidade com estirpes moçambicanas que circularam em 2012 e 2013, entretanto, foram detectados possíveis eventos de *reassortments*.

No presente estudo, apesar da análise do genoma completo de rotavirus ter sido efectuada em estirpes provenientes de diferentes locais de Moçambique, os resultados ilustraram que no país estão em circulação estirpes com o mesmo *genetic makeup.*

Esta tese caracterizou estirpes de rotavirus do grupo A em crianças menores de 5 anos de idade e mostrou a existência de alta diversidade de estirpes circulantes no país. A análise do genoma completo evidenciou que estirpes G1P[8] e G9P[8] têm um *backone Wa-like* típico e estirpes G2P[6] têm um *backbone DS1-like* típico. No entanto, é importante a vigilância contínua, como forma de monitorizar os possíveis eventos de *reassortment* que derivam da pressão da vacina.

Palavras chaves: Rotavirus, Moçambique, genótipos, sequenciação, nova geração

Abstract

Rotavirus group A were confirmed as a major cause of life-threatening diarrhoea in infants and children <5 years of age worldwide and in the young of many mammalian and avian species. The virus is known to have several rotavirus strains causing infection in children, due to that, the assessment of information on the prevalence of circulating rotavirus strains as well the complete genetic makeup is important in the assessment of the likely impact of vaccine. In this context the main aim of this project was to genetically characterize the rotavirus strains circulating between 2012-2018 in Mozambique, including a comparison before and after rotavirus vaccine implementation in the country. The strains were from children under five years of age and suffering from moderate-to-severe acute diarrhoea. The genetic characterization was done using RT-PCR, dideoxynucleotide chain termination sequencing (Sanger method) and next generation sequencing of rotavirus genome.

The results by RT-PCR showed diversity of genotypes with yearly fluctuations of strains, prior and post vaccine introduction. Prior to vaccine introduction, during 2012-2013, the most frequent rotavirus genotypes were G2P[4] and G12P[6] and during 2014-2015 were G1P[8] and G9P[8]. Post vaccine-introduction (2016-2018) was observed emergence of genotypes G9P[4], G3P[4] and G3P[8]. The emergence of other rotavirus strains in the post-vaccination period supports the need for continuous surveillance across the country to understand if the emergence of the strains is vaccine derived and their impact in human health.

The full genome analysis of G1P[8] and G9P[8] strains from 2014 and 2016 revealed circulation of strains with typical Wa-like genome backbone. With full genome analyses, it was possible to provide evidence of genetic diversity within genotype P[8] in Mozambique throughout the years and several substitutions in rotavirus antigenic regions were observed. It is, thus, important to continue surveillance of rotavirus strains to monitor the implication of these mutations.

The full genome analysis of G2P[6] strains from 2015 and 2016 revealed a DS1-like typical genome backbone with high identity with Mozambican strains from 2012-2013, however, with possible occurrence of reassortment events.

The rotavirus genome of strains from different sites from Mozambique analysed in the present study showed, in general, the same genetic makeup of rotavirus strains in the country.

This thesis characterized the RVA strains from children less than five years of age and showed high diversity of strains in Mozambique. The full genome analyses showed Wa-like with typical genome backbone in strains G1P[8] and G9P[8], and DS1-like with typical genome backbone in G2P[6] strains. There is a need to continue the surveillance in order to monitor possible reassortment events derived from vaccine pressure.

Key-words: Rotavirus, Mozambique, genotypes, next generation sequencing

List of abbreviations

AGE - Acute gastroenteritis

BLAST- Nucleotide Basic Local Alignment Search Tool

DLP – Double layer virus particle

ELISA- Enzyme-linked immunosorbent assay

GEMS – The Global Enteric Multicenter Study

GRSN – The Global Rotavirus Surveillance Network

HBGA – Histo-blood group antigen

FUT – Fucosyltransferases

LSD – Less-severe diarrhoea

MSD – Moderate-to-severe diarrhoea

NSP – Non-structural protein

NGS – Next Generation Sequencing

ORF – Open reading frame

PABP – Poly (A)-binding protein

RCWG – Rotavirus Classification Working Group

RNA – Ribonucleic acid

RV – Rotavirus

RVA – Rotavirus group A

(+)ssRNA- Positive-sense single strand RNA viruses

(-)ssRNA- Negative-sense single strand RNA viruses

TLP – Triple layer virus particle

VP – Viral protein

ViPR- Database and Analysis Resource

ViNaDiA- National Surveillance of Acute Diarrhoea

WHO – World Health Organization

INDEX

CHAPTER 1 - Introduction

1.1. General introduction

Estimates in 2016 by the Global Burden Group of Diseases (GBD) reported that diarrhoeal diseases were responsible for 1.65 million deaths in all ages. Among children younger than 5 years of age, diarrhoeal diseases were responsible for 446 000 deaths (390 894 – 504 613) [GBD, 2018]. Diarrhoea was the eighth leading cause of death among all ages and the fifth leading cause among children younger than 5 years of age, behind preterm birth complications, neonatal encephalopathy, and lower respiratory infections [GBD, 2018]. Mortality from diarrhoea varied by location, being the highest in sub-Saharan Africa and South Asia [GBD, 2018] .

In Mozambique, 13.105 deaths occur each year in children under 5 years of age due to diarrhoea [Black et al., 2010]. Previous study from southern Mozambique, based on verbal autopsy under 15 years of age reported diarrhoea as the fourth highest cause of mortality in 1997 and 2006 in Manhiça district [Sacarlal et al., 2009] and as third cause in Maputo city in 1994 [Dgedge et al., 2001].

A recent published study aimed to determine the possible risk factors associated to death among children aged 0–59 months presenting with moderate-to-severe diarrhoea (MSD) in Manhiça district highlighted diarrhoea as the main cause of death [Acacio et al., 2019].

The etiological diarrheal pathogens can be viruses, bacteria and parasites, however, rotaviruses (RV), more specifically rotavirus group A (RVA), were confirmed as a major cause of life-threatening diarrhoea in infants and children <5 years of age worldwide and in the young of many mammalian and avian species [Crawford et al., 2017; Kotloff et al., 2019; Tate et al., 2016a]. Because of that, the World Health Organization (WHO) recommended the inclusion of RV vaccines in the national immunization program of highly affected countries [WHO 2013].

Mozambique participated in the global enteric multicenter case-control study (GEMS) on diarrhoea aetiologies, involving sub-Saharan countries such as Gambia, Mali and Kenya as well as several Asian countries (India, Bangladesh and Pakistan). In this study, Mozambique was the country that registered the highest attributable fraction of diarrhoea caused by RV in the age group from 0 to 11 months [Kotloff et al., 2013]. Recently, GEMS published data reinforced RV as the most common pathogen associated with non-dysentery

MSD and less-severe diarrhoea (LSD) in most of sites, including Manhiça district in Mozambique [Kotloff et al., 2019]. Of note, at the time of this study Mozambique had not introduced rotavirus vaccine.

After the introduction of monovalent rotavirus vaccine (Rotarix®) in 2015, the early impact study reported a reduction of positivity rate of rotavirus infection among children [de Deus et al., 2018a].

RVA strains are classified based on the gene segments encoding the viral proteins VP7 and VP4. However, analysis of the whole genome (11 gene segments) becomes more appropriate to do a full and conclusive analysis of the strains in terms of evolution, their origin and the role of other genetic segments [Matthijnssens and Van Ranst, 2012]. The segmented nature of the genome, increases the chances of reassortment between strains during co-infection and also promote interspecies transmission between animal and human strains [Iturriza-Gomara et al., 2001].

This project, through the genetic characterization analysis of human RV in Mozambique, may contribute with data about molecular epidemiology of RVA in the country and therefore help to monitor the prevalence of rotavirus strains in circulation and also can be useful to evaluate the impact and effectiveness of rotavirus vaccination introduction in September 2015.

1.2. Rotavirus particle

The RV infection particles (virions) are non-enveloped and possess a three concentric layers icosahedral protein capsid, approximately 75 nm in diameter, composed of an outer layer, an inner layer, and a core layer corresponding to a triple layer particle (TLP) [Cunliffe et al., 2002; Estes and Cohen, 1989]. The outer layer is composted by the VP7 and VP4 proteins. The inner layer is composed by VP6 and the core layer by VP2 proteins that encloses genome of 11 segments of double strand RNA (dsRNA) as well as the viral RNA dependent RNA polymerase (RdRp), VP1 and the capping enzyme, VP3 [Estes and Cohen, 1989; Mathieu et al., 2001; Pesavento et al., 2006] (Figure 1).

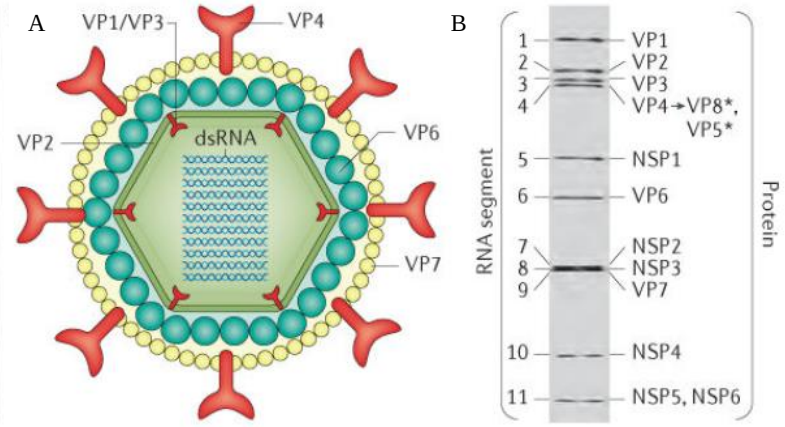

Figure 1. Rotavirus structure. Adapted from Crawford et al. (2017)

1.3. Rotavirus genome

The virus genome consists of 11 segments dsRNA, encoding six viral structural proteins (VP1, VP2, VP3, VP4 and VP7) and six non-structural viral proteins (NSP1, NSP2, NSP3, NSP4, NSP5/6), with the exception of the eleven gene segment that encodes two proteins (NSP5 and NSP6) [Cunliffe et al., 2002; Estes and Cohen, 1989] (Figure 1). The segments range in size from 667 (segment 11) to 3,302 base pairs (segment 1), with the total genome containing approximately 18,522 base pairs (Table 1) [Estes and Cohen, 1989].

Each RNA segment starts with a 5' guanidine followed by a set of conserved sequences that are part of the 5' noncoding sequences; an open reading frame (ORF) coding for the protein

product follows, and another set of noncoding sequences, which contains a different subset of conserved 3'-terminal sequences and ends with a 3'-terminal cytidine, is found after the stop codon [Estes and Cohen, 1989] (Figure 2 and 3).

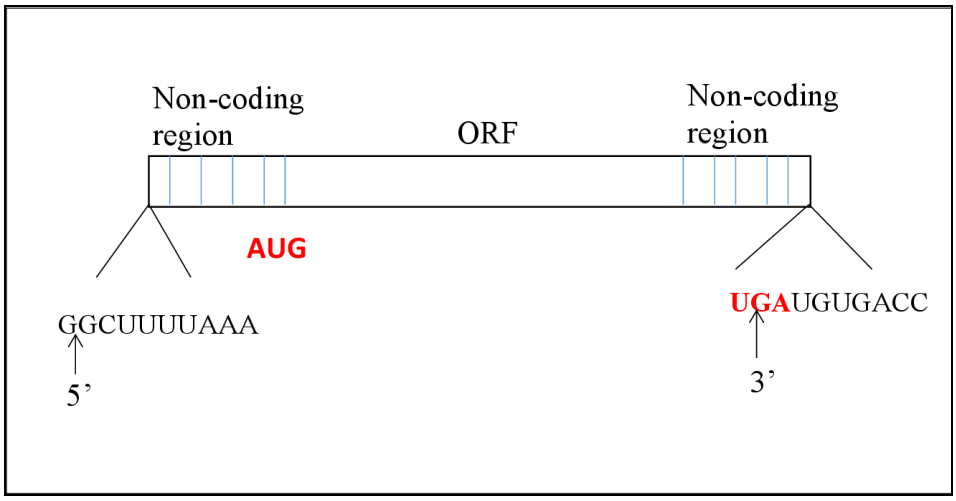

Figure 2. General rotavirus genome segment representation [Estes and Greenberg, 2013].

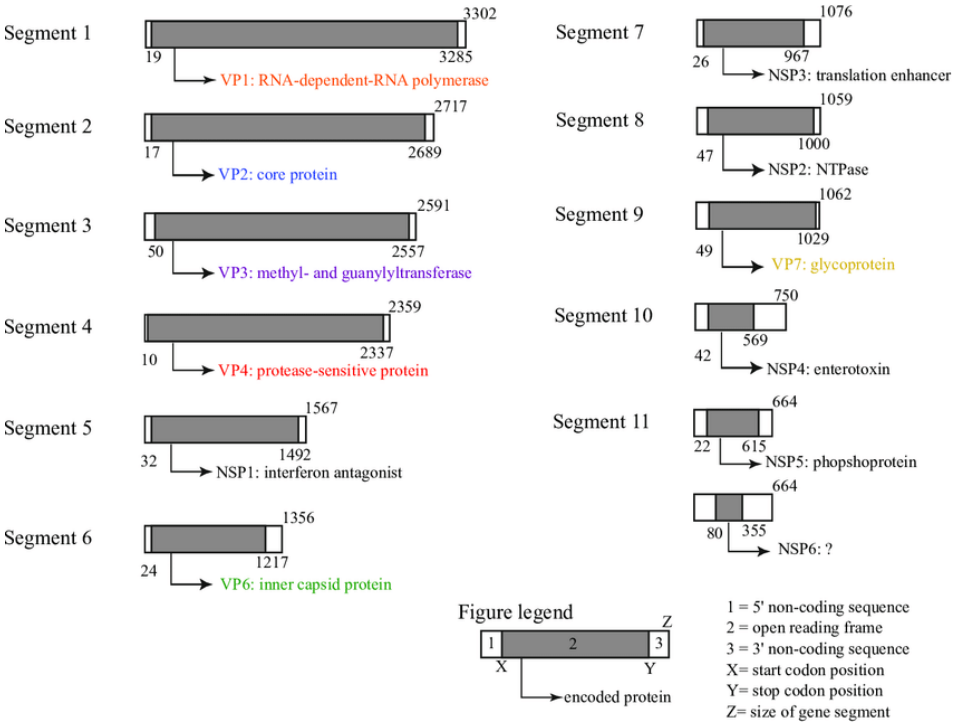

Figure 3. Rotavirus genome organization based on porcine reference strain Gottfried [Theuns, 2015].

All of the RV gene sequences are A+T rich (58% to 67%), and the codon usage is biased against CGN and NCC codons, as in many eukaryotic and other viral genes [Estes and Cohen, 1989].

1.4. Rotavirus gene assignment proteins

Protein functions are summarized in Table 1.

Table 1. Rotavirus gene-protein assignments, protein localization and protein-function assignment adapted to bovine rotavirus strain (G6P6[1])

Genome segment	Size (pb)	Encoded protein	Location in virion	Functions
1	3302	VP1	Core	Functions as RdRp; ssRNA binding and form transcription complex with VP3 [Patton, 1996; Zeng et al., 1996].
2	2687	VP2	Core	Has ability to non-specifically bind to messenger RNA (mRNA)) and are required for RdRp activity [Patton, 1996; Zeng et al., 1996].
3	2592	VP3	Core	Has the viral capping enzyme with phosphodieste-rase, guanylyltransferase and methylase activities and form transcription complex with VP1 [Patton, 1996; Zeng et al., 1996].
4	2362	VP4	Outer layer	It is a nonglycosylated outer capsid protein [Estes and Cohen, 1989] . Function as attachment protein; protease enhanced infectivity and virulence; fusion with cell membrane [Anthony et al., 1991; Estes and Cohen, 1989] . It is an antigen inducing host neutralizing antibody production and establishes the classification of genotype P [Coluchi et al., 2002; Estes and Cohen, 1989] .
5	1581	NSP1	Non-structural	A key function of NSP1 is its ability to act as an antagonist of the innate immune response, being interferon antagonist [Qin et al., 2011].
6	1356	VP6	Middle layer	It plays a key role in maintaining the three-dimensional structure of the virus [Mathieu et al., 2001] . Is immunogenic and antigenic, and it is the most frequently targeted protein in diagnostic assays to detect virus particles [Estes and Cohen, 1989]. This protein is required for transcription [Estes and Cohen, 1989].
9	1062	VP7	Outer layer	It is a glycoprotein considered to be the major host-specific neutralizing antibody-inducing antigen [Angel et al., 2007; Estes and Cohen, 1989; Hoshino et al., 1985]. Also used for classification into G genotypes [Angel et al., 2007; Estes and Cohen, 1989; Hoshino et al., 1985].

8	1059	NSP2	Non-structural	It has nucleoside triphosphatase (NTPase) - associated activity that provides energy needed for dsRNA synthesis [Taraporewala et al., 1999] through non-specific single-stranded RNA binding functions [Patton, 1996]. It is essential for viroplasm formation [Patton, 1996].
7	1074	NSP3	Non-structural	May act as a translation enhancer for viral gene expression [Poncet et al., 1997] . Binds to: 3 terminus of viral positive-sense single strand RNA [(+)ssRNA], translation factor eIF4G, Hsp90; displaces poly A binding protein (PABP); inhibits host protein translation [Poncet et al., 1997].
10	751	NSP4	Non-structural	It is endoplasmatic reticulum transmembrane glycoprotein; viroporin; intracellular receptor for DLPs; interacts with viroplasms and autophagy pathway; modulates intracellular Ca^{2+} and RNA replication; enterotoxin (secreted); virulence [Ball et al., 1996; Zhang et al., 2013].
11	666	NSP5	Non-structural	It is a glycosylated and phosphorylated protein with autokinase functions [Campagna et al., 2005; Poncet et al., 1997]. It is essencial in viroplasmas formation [Campagna et al., 2005; Poncet et al., 1997] .
		NSP6	Non-structural	Interaction with NSP5, localized in viroplasm [Campagna et al., 2005; Poncet et al., 1997].

Table adapted from Desselberger et al. (2014)

1.5. Rotavirus classification

1.5.1. Rotavirus groups

Rotavirus belongs to the family *Reoviridae*, genus *Rotavirus* of dsRNA viruses. According to the sequence and antigenic differences of VP6 [Matthijnssens et al., 2012b], nine different groups, also termed species, have been reported. These groups are differentiated in the RVA-RVJ (https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg; https://talk.ictvonline.org/taxonomy/) (Figure 4). RVA is the major cause of severe dehydrating diarrhoea in children in the world [Matthijnssens et al., 2012b] and also affect a large group of mammals species and birds [Martella et al., 2009].

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1.5.2. Dual classification system

RVA are classified into different genotypes based on sequence differences in genome segments 9 and 4 encoding VP7 (Glycoprotein-G) and VP4 (protease sensitive-P) proteins respectively, which form the basis of the dual nomenclature system used for RVA [Desselberger, 2014]. To date, 32 G genotypes and 50 P genotypes of RVA have been identified (Table 2) (https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg).

1.5.3. Full genome rotavirus classification

A comprehensive, nucleotide sequence-based classification comprising the complete genome has been introduced for RVAs, in which the VP7–VP4–VP6–VP1–VP2–VP3–NSP1–NSP2–NSP3–NSP4–NSP5/6 corresponding to Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, respectively. It is based on nucleotide homology cutoff ORF values for each genome segment which are used to classify rotavirus strains [Matthijnssens et al., 2008b]. The nine non-G and non-P genotypes represent the genotype backbone. Table 2 shows the number of RVA genotypes co-circulating in different population groups and animals at various times as well as the origin of the genotype name (Desselberger, 2014; https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg).

Table 2. Number of genotypes of eleven genome segments of RVA strains

RV protein	Percent identity[A]	Number of genotypes[B]	Genotype (acronym underlined)
VP7	80	36 G	Glycosylated
VP4	80	51 P	Protease sensitive
VP6	85	26 I	Inner capsid
VP1	83	21 R	RdRP
VP2	84	19 C	Core protein
VP3	81	19 M	Methyltransferase
NSP1	79	30 A	Interferon Antagonist
NSP2	85	20 N	NTpase
NSP3	85	21 T	Translation enhancer
NSP4	85	26 E	Enterotoxin
NSP5/6	91	21 H	PHosphoprotein

Table adapted from Desselberger et al. (2014). A: Nucleotide percent identity cutoff value defining genotypes was based on Matthijnssens et al. (2008b). B: Data of genotypes updated based on. https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg.

1.6. Rotavirus strain nomenclature

A standardized RV strain nomenclature system was proposed by the Rotavirus Classification Working Group (RCWG). The RV strains are named as follows: RV group/species; origin/country; identification/common name/year; identification/G- and P type [Matthijnssens et al., 2011a]. As examples, human RVA reference strain Wa is named as follows: RVA/Human-tc/USA/Wa/1974/G1P[8] and DS1, RVA/Human-tc/USA/DS-1/1976/G2P[4].

1.7. Rotavirus evolution

In general, rotavirus evolution is determined by the accumulation of point mutations over time, but also intragenic, recombination, interspecies transmission and gene reassortment [Matthijnssens et al., 2009b]. The accumulation of point mutations results in genetic and

antigenic drift, whereas reassortment of gene segments can occur after dual infection of individual cells by different RVA strains, leading to viral progeny with combinations of the parental genomes [Crawford et al., 2017]. Reassortment is the major cause of genetic diversity. It occurs between homologous or heterologous hosts and sometimes appears with novel constellation of RV gene segments [Mijatovic-Rustempasic et al., 2016]. Unusual genotypes G2P[8], G9P[4], G9P[6], and G12P[6] are said to have evolved by reassortment [Sadiq et al., 2018].

Interspecies transmission involves transfer of a gene segment from one species to another [Matthijnssens et al., 2009b]. For example, circulation of human G9 and G12 genotypes in the population is due to transfer of porcine genes into human RV genome [Rahman et al., 2007]. Reassortment between porcine RV and human Wa-like strain allows the detection of G11 genotype in humans and G8 evolved because of reassortment between bovine and human RV [Sadiq et al., 2018] . There is evidence of interspecies transmission, animal and human reassortment events from a wide variety of animal host species, such as artiodactyls (ruminants and camelids), cats, dogs, pigs, rabbits, and even wildlife (monkeys), in developing and underdeveloped world have been reported [Matthijnssens et al., 2011b].

The VP4 encoding protein is involved in several structural and functional interactions such as viral particle maturation in the endoplasmic reticulum, cell attachment, and cell membrane penetration and genetic variability of this protein in humans is more restricted than that found in VP7 [Trask et al., 2012].

The whole genome-based analysis is a reliable method for obtaining precise information on the origin of a given RV strain, and for tracing its evolutionary pattern [Matthijnssens and Van Ranst, 2012].

1.8. Viral entry and genome replication

RV infects and replicates in the mature, non-dividing enterocytes in the middle and tip of the villi and in enteroendocrine cells in the small intestine [Lundgren and Svensson, 2001].

1.8.1. Attachment and cell entry

RV attach to different glycan receptors on the host cell surface, depending on the virus strain, through interaction with the viral protein VP8* domain of VP4. For decades,

sialoglycans (gangliosides such as GM1 and GD1a) were considered the key cellular glycan receptor for VP8* [Lopez and Arias, 2004]. Although this remains true for animal rotavirus strains, VP8* of many human RV strains binds to non-sialylated glycoconjugates, called histo-blood group antigens (HBGAs). For the biosynthesis of HBGA the FUT2 gene (secretor) encoding the enzyme α1,2 fucosyltransferase, responsible for adding a residue (α1,2fucose) a type 1 chain precursor (Galβ3GlcNAcβ) for producing the H antigen type 1. Therefore, a child without a functional allele may have a protective effect on infection against RV since the virus can not bind to the HBGAs receptors, thereby prevents infection [Imbert-Marcille et al., 2013; Nordgren et al., 2014]. Other proposed receptors for RV cell entry include integrins [Fleming et al., 2010], heat shock protein 70 [Guerrero et al., 2002] and junctional proteins such as junctional adhesion molecule A, occludin and tight junction protein ZO-1 [Torres-Flores et al., 2015].

After initial binding, VP7 and the VP5* domain of VP4 can interact with several of these co-receptors listed above, which are concentrated at lipid rafts to mediate viral entry. The virus is internalized into cells by endocytic pathways [Diaz-Salinas et al., 2013]. The low calcium levels inside the endosome trigger the removal of the outer capsid layer, which releases the transcriptionally active double-layered particle (DLP) into the cytoplasm. The DLPs are transported to the cytoplasm via pores on capsid (Figure 5).

1.8.2. Transcription and replication

In the cytoplasm, DLPs become transcriptionally active molecules. The transcriptional complex of RV is composed by VP1, RdRp, and VP3 (viral capping enzyme) complexes with 11 segments of viral dsRNA [Trask et al., 2012]. Rotavirus (+)ssRNA transcripts are produced from the negative strand of genomic RNA [(-)ssRNA] in RV DLPs in cytoplasm [Ayala-Breton et al., 2009]. After several rounds of transcription, the newly synthesized (+)ssRNA are extruded out of DLPs to the cytosol [Ayala-Breton et al., 2009] (Fig. 5).

1.8.3. Virion assembly and release

The viral proteins produced accumulate in viroplasms where assembly of RV RNA and viral structural proteins takes place, followed by DLP formation [Lu et al., 2008]. The

DLPs bud from viroplasm through the endoplasmic reticulum by the coordinated action of NSP4 and VP6 [Pastor et al., 2014]. The assembly of outer capsid protein (VP7 and VP4) takes place with DLPs, resulting in the formation of fully infectious triple layered rotavirus particles (TLPs). The TLPs are released through cell by lysis or by release from the apical surface. The exact mechanisms of RV exit from cell are still unknown [Pastor et al., 2014].

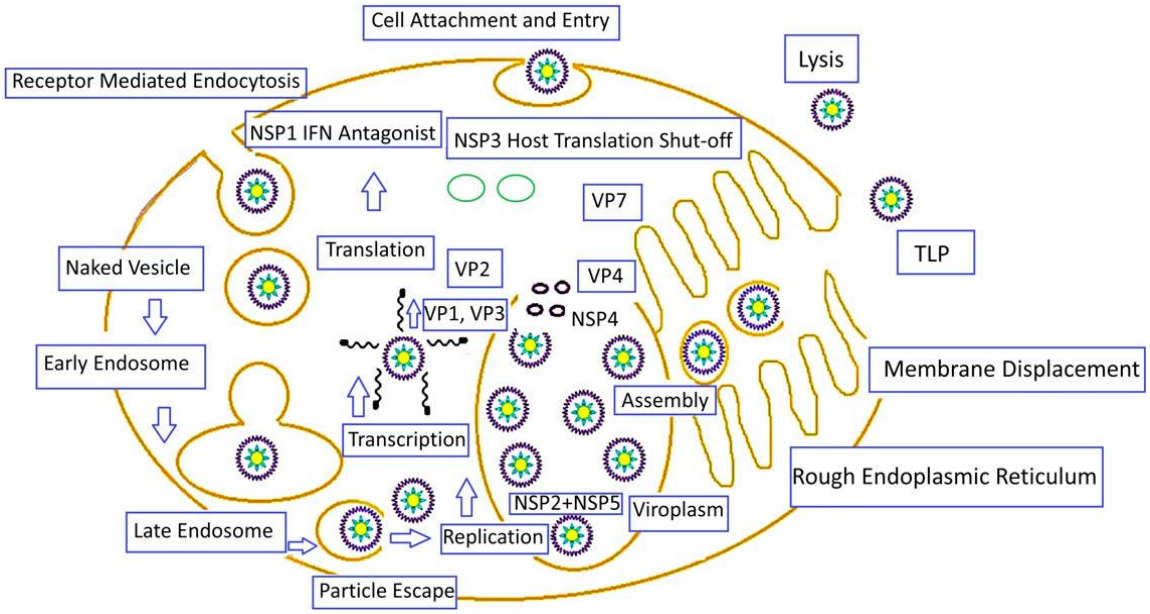


Figure 4. Rotavirus life cycle. Attachment of rotavirus to different glycan receptors on the host cell surface, mediated by VP7 and the VP8* domain of VP4. VP7 and the VP5* domain of VP4 can interact with several co-receptors and mediate viral entry. The virus is internalized into cells by several endocytic pathways. The low calcium levels inside the endosome trigger the removal of the outer capsid layer, which releases the transcriptionally active DLP into the cytoplasm. The transcription of (+)ssRNA in cytosol is mediated by VP1, VP2, and VP3 proteins, followed by translation of proteins. Replication using (-)ssRNA and formation of rotavirus DLP particles and assembly in viroplasm. Maturation and release of virus particles [Sadiq et al., 2018].

1.9. Pathogenesis and pathology

Diarrhoea caused by RV is multifactorial and may be due to enterocyte damage and death, reduced epithelial absorptive function, the NSP4 enterotoxin and activation of the nervous system.

1.9.1. Enterocyte damage and death

RV infection and replication in the duodenal mucosa of infants has been shown to cause disruption to normal cellular homeostasis, resulting in shortening and atrophy of villi, loss of microvilli, mononuclear cell infiltration, distended endoplasmic reticulum and mitochondrial swelling in enterocytes [Davidson and Barnes, 1979]. As shown in studies in piglets and mice, symptoms of rotavirus infection can occur before histological changes, indicating that these changes do not solely explain clinical presentation [Ward et al., 1996].

1.9.2. Reduced epithelial absorptive function

Several mechanisms underlying the reduction of epithelial absorptive function, which contributes to rotavirus-induced diarrhoea, have been proposed. These mechanisms include the loss of infected enterocytes and NSP4-mediated impairment of sodium-coupled solute symporters that are involved in the reabsorption of large volumes of water under physiological conditions [Crawford et al., 2017]. However, the contribution of the reduced epithelial absorptive function to rotavirus-induced diarrhoea is unclear.

The destruction of the intestinal epithelium induces the deficient absorption capacity of glucose, sodium, water, and other nutrients accompanied by a marked decrease in the levels of intestinal enzymes such as lactase, maltase, sucrose and alkaline phosphatase [Brunet et al., 2000; Carter, 2005], which induce the reduction of digestion, resulting in the transit of mono and disaccharides, fats, and proteins, not digested to the colon. Because the colon is incapable of absorbing enough water, it leads to osmotic diarrhoea [Ramig, 2004].

1.9.3. NSP4-Enterotoxin and activation of the nervous system

Early on, 16-36 hours post-infection, the virus produces a potent enterotoxin, NSP4, that can cause secretory diarrhoea [Tian et al., 1996]. The NSP4 secreted from cells infected with RV binds to intestinal epithelial cells [Seo et al., 2008] and signals through phospholipase C to increase cytoplasmic calcium levels, which activates calcium-dependent chloride channels [Hyser et al., 2010]. Activation of these channels causes excessive secretion of chloride ions into the intestinal lumen, creating an osmotic gradient that

facilitates the transport of water into the lumen, leading to secretory diarrhoea [Tian et al., 1996].

Rotavirus infection and NSP4-dependent 5-HT release can also activate vagal nerves that project to regions of the brain associated with nausea and vomiting [Hagbom et al., 2011].

1.10. Clinical symptoms

In contrast to gastroenteritis caused by bacterial pathogens, RV infections cause non-bloody diarrhoea that lasts for a relatively short duration and is associated with a limited inflammatory response [Lundgren and Svensson, 2001]. In children, the manifestation of RV disease ranges from no symptoms to mild, watery diarrhoea of short duration, and to severe diarrhoea with vomiting and fever that can result in rapid dehydration with shock, electrolyte imbalance and death [Glass et al., 1996; Gurwith et al., 1981]. RV can also cause limited disease in older children and adults, especially parents and caretakers of children with RV diarrhoea, immunocompromised individuals, travellers and elderly individual [Nakajima et al., 2001].

1.11. Epidemiology

1.11.1. Global burden

In 2013, RV were associated with an estimated >200,000 fatalities in children <5 years of age globally [Tate et al., 2016a]. Although the prevalence of RV infection in children hospitalized with diarrhoea is similar worldwide (~30–50%), >90% of children with fatal RV infections live in low-income countries globally [Tate et al., 2016a] (Fig. 6). Diarrhoea caused by RV infection is of more than average severity; the proportion of diarrhoea episodes is lowest in patients in the community who require only home care (5–10%) compared with those who require outpatient care (15–20%) or inpatient care (30–50%) globally [Tate et al., 2016a].

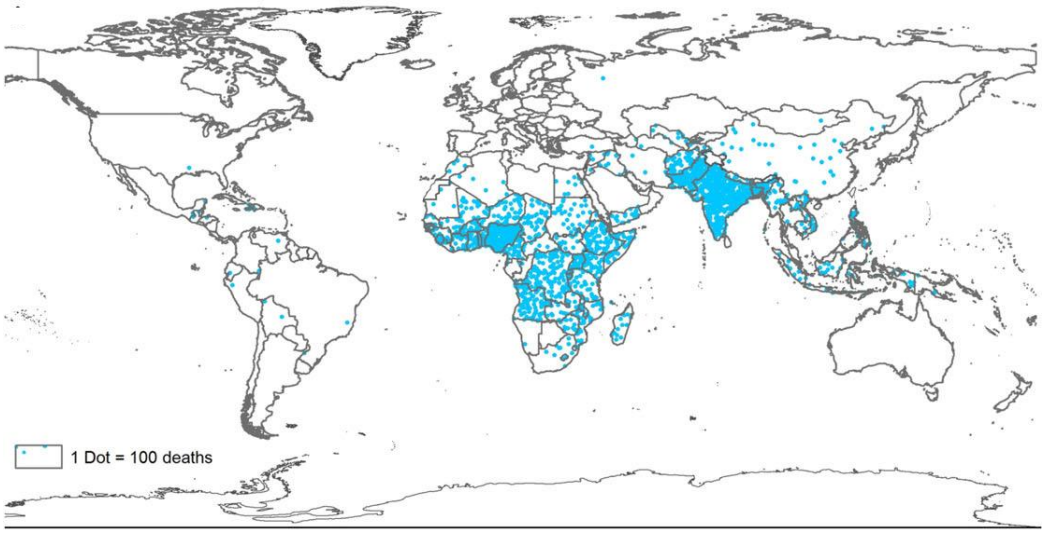


Figure 6. Number of rotavirus deaths among children <5 years of age, by country, 2013 [Tate et al., 2016a].

A reduction in the burden of RV disease has been observed in many countries following the introduction of RV vaccines. The Global Rotavirus Surveillance Network (GRSN) reported that RV prevalence (2008-2016) among children younger than 5 years of age admitted with acute gastroenteritis to hospitals or emergency units, decreased by nearly 40% in countries after introduction of RV vaccines into their national immunisation programmes. In contrast no such reduction was observed in countries and regions where it was not introduced [Aliabadi et al., 2019].

In countries that introduced the Rotarix® monovalent vaccine, such as Mozambique, South Africa, Malawi, Botswana, Ghana, impact analyses of vaccine have shown a decline in RV-related gastroenteritis hospitalization of 23-65% [Armah et al., 2016; Bar-Zeev et al., 2016; de Deus et al., 2018a; Enane et al., 2016; Groome et al., 2016]. With regards to RV vaccine effectiveness in some African countries, such as Ghana, Malawi, Zambia and Botswana, the vaccine effectiveness against RV hospitalization was 60%, 70%, 56% and 54%, respectively [Armah et al., 2016; Bar-Zeev et al., 2016; Beres et al., 2016; Enane et al., 2016].

1.11.2. Rotavirus and age distribution

The demographics of RV disease has changed since the introduction of vaccinations into national immunization schedules. Globally among RV gastroenteritis cases in the pre-vaccine period, the median age of RV gastroenteritis cases was 12 months (IQR 7–20), whereas after vaccine introduction, the median age was 15 months [Aliabadi et al., 2019] .

In some African countries, such as Malawi, RV hospitalization incidence did not decline in older children. In Swaziland, the average age of rotavirus infection after vaccine introduction increased to 13.5 months *vs* 10.0 months before vaccine introduction [Maphalala et al., 2017]. In South Africa, lower incidence reductions (39.8%–49.4%) were observed among children aged 12–24 months from the second year post–vaccine introduction onward [Groome et al., 2016]. In Rwanda, the cumulative age distribution of RV gastroenteritis cases showed a rightward shift, with 56% of RV gastroenteritis hospital admissions occurring among infants in the pre-vaccine period compared with 31% after rotavirus vaccine introduction [Tate et al., 2016b]. In Mozambique, in the early impact analyses, an increase of age post-vaccine introduction was not observed [de Deus et al., 2018a].

1.11.3. Seasonality

The seasonality correlates with the income level of a country, as more-seasonal disease occurs in high-income countries than in low-income countries [Patel et al., 2013]. However, changes in the seasonal pattern of RV disease have also been observed after vaccine introduction, including delays in the start of the RV season, a shorter duration of seasons

and blunting of seasonal peaks [Aliabadi et al., 2019]. This phenomenon was also reported from southern African countries such as South Africa, Swaziland and Mozambique [de Deus et al., 2018a; Groome et al., 2016; Maphalala et al., 2017].

In the United States of America, the regular annual seasonal pattern of rotavirus disease that was observed before rotavirus vaccine introduction has shifted to biennial increases in circulating rotavirus disease. This phenomenon might be due to the accumulation of unvaccinated children over two successive rotavirus seasons due to moderate levels of vaccine coverage (60–80%), leading to a larger epidemic every alternate year [Crawford et al., 2017].

1.11.4. Global rotavirus strains

G/P genotypes

To date, 36 G genotypes and 51 P genotypes of RVA have been identified (https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg), although globally, six G types (G1, G2, G3, G4, G9 and G12) and three P types (P[4], P[6] and P[8]) predominate [Banyai et al., 2012; Gentsch et al., 2005; Leshem et al., 2014; Matthijnssens et al., 2009a]. There are six most common genotype combinations accounting for 90% for globally circulating strains: G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8] [Banyai et al., 2012; Gentsch et al., 2005; Leshem et al., 2014; Matthijnssens et al., 2009a]. Geographical differences of RVA strain distribution have been observed, with more strains causing rotavirus disease in children in low-income countries than in children in high-income countries [Crawford et al., 2017]. For example in Africa, Asia and South America, uncommon genotypes such as G5, G6, G8, have been reported [Todd et al., 2010]. The genotype combinations G9P[6] and G12P[6] are mostly known to circulate in south Asia [Miles et al., 2012] while G2P[6] and G8P[6] are mostly found in Sub-Saharan Africa [Heylen et al., 2014]. Recently, phylogenetic analyses showed that P[6] is endemic in Africa, characterized by an extensive genetic diversity and long-time local evolution of the viruses [Nyaga et al., 2018].

In general, unusual strains are associated with 14% of cases in Asia, 37% in Africa, 11% in South America, 5% in North America, 1.4% in Europe, and 0.1% in Australia [Chakraborty et al., 2016; Dhital et al., 2017; Iturriza-Gomara et al., 2011; Todd et al., 2010].

With regards to circulation of RV strains after vaccine introduction, although initial differences in the genotypes of circulating rotavirus strains have been described in some countries after vaccine introduction, there is little evidence that this is due to selective pressure from vaccination [Leshem et al., 2014; Markkula et al., 2017].

Rotavirus strain diversity in Eastern and Southern African (ESA) countries of African Rotavirus Surveillance Network (2010-2015) period demonstrated that the most prevalent strains were G1P[8], followed by G2P[4], G9P[8], G12P[8], G2P[6] and G3P[6] in 15 ESA countries [Seheri et al., 2017]. In the same period of analysis no difference in circulating strains pre- and post-rotavirus vaccine introduction with yearly fluctuation of strains observed over time [Seheri et al., 2017].

Rotavirus genome backbone

The introduction of a RVA classification system encompassing all 11 RVA gene segments in combination with the development of next generation sequencing methods, have facilitated the exponential growth of complete RVA genome data during recent years [Matthijnssens and Van Ranst, 2012]. On the basis of complete RVA genome sequence comparisons, two major epidemiologically significant genotype backbone represented by Wa-like (I1-R1-C1-M1- A1-N1-T1-E1-H) and DS1-like (I2-R2-C2-M2-A2-N2- T2-E2-H2), have been shown to circulate worldwide among humans [Matthijnssens and Van Ranst, 2012] (Table 3). Human Wa-like RVA strains are believed to have a common ancestor with porcine RVA strains, whereas human DS-1-like strains are believed to have several gene segments, which have a common ancestor with bovine RVA strains [Matthijnssens et al., 2008a]. Additionally, a small third human genotype backbone, referred to as AU- 1-like (I3-R3-C3-M3-A3-N3-T3-E3-H3) is hypothesised to have originated from cats or dogs [Matthijnssens and Van Ranst, 2012; Nakagomi and Nakagomi, 1991] (Table 3).

The Wa-like strains are characterized by non-G/P genotype 1, and tend to have G/P genotype combinations G1P[8], G3P[8], G4P[8], G9P[8], G12P[6], and G12P[8], whereas the DS-1-like strains are characterized by non-G/P genotypes that tend to have G/P genotype combinations, such as G2P[4], G2P[6], G8P[4], G9P[4] [Matthijnssens and Van Ranst, 2012] (Table 3).

Table 3. Rotavirus genome backbones

Genotype backbone name	Proteins	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5/6
	Rotavirus genome segments	9	4	6	1	2	3	5	8	7	10	11
	Human Wa-like	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
		G3	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
		G4	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
		G9	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
		G12	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
		G12	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
		G1P[8]-Rotarix vaccine*		I1	R1	C1	M1	A1	N1	T1	E1	H1
	Human DS1-like	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
		G2	P6	I2	R2	C2	M2	A2	N2	T2	E2	H2
		G8	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
	Human AU-1-like	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3

Table 3 adapted from Matthijnssens and Van Ranst (2012).*Strain: RVA/Vaccine/USA/Rotarix-RIX4414/1988/G1P1A[8].
Green, red and yellow background represent Wa-like, DS1-like and AU-like backbones, respectively.

1.11.5. Epidemiology in Mozambique

Before the introduction of the vaccine, few studies reported a high burden of RV infection in Mozambique, particularly in the southern region. As the GEMS mentioned in the introduction of this Chapter, additional studies also assessed the epidemiology of RV in children under five years of age from urban (Maputo City) and rural (Manhiça District) areas in 2012 and 2013, documented a prevalence of RV infection higher than 40% [de Deus et al., 2018b]. In contrast, lower prevalence of RV (24%) was found in Gaza province [Langa, 2016], and data from National Surveillance of Acute Diarrhoea showed positivity rate for RV infection before vaccine introduction of 40.2% and 38.3% in 2014 and 2015 respectively [de Deus et al., 2018a]. The Rotarix® (GlaxoSmithKline, Belgium) monovalent vaccine was introduced into the National Program of Immunization in Mozambique in September 2015. Since then, its impact has been monitored suggesting a substantial reduction of the prevalence of RV infection to 12.2% and 13.5% in 2016 and 2017, respectively [de Deus et al., 2018a]. The introduction of the RV vaccine also affected all-cause acute gastroenteritis (AGE) hospitalization. Prior to vaccination introduction, the proportion of all-cause AGE hospitalizations at Mavalane General Hospital in 2014 and 2015 of children aged 0–11 months were responsible for 51.6% and 50.3%, respectively. In comparison to after vaccination introduction, in 2016, the proportion of all-cause AGE hospitalizations was 37.0% in the age group of 0–11 months [de Deus et al., 2018a]. Additionally, a greater reduction in the proportion of RV infection was observed in the age group 0–11 months than in older age groups. The RV hospitalizations peaked annually prior to vaccine introduction, between June and September coinciding with Winter. After vaccine introduction, RV occurred throughout the year, however, the peak moved to August - December in 2016 and was substantially blunted [de Deus et al., 2018a].

Regarding the genotypes circulating in Mozambique, few studies have been published. In 2011, in Gaza province the G12P[8] combination was reported as the most frequent [Langa, 2016] and a published study, described in Chapter 2, reported detection of G12P[6] and G2P[4] in 2012 and 2013, respectively, as the most frequent genotypes.

The whole genome sequences of strains from 2012 and 2013 detected G2P[4] with DS1-like backbone with several reassortment events [Strydom et al., 2019a], and G12P[6] and

G12P[8] with Wa-like backbone. However, one Wa-like strain in co-infection with DS1-like backbone from animal origin were detected [Strydom et al., 2019b].

1.12. Transmission

Few RV virions are needed to cause disease in susceptible hosts. A minimum intake of 10 to 100 particles is sufficient for transmission between humans [Glass et al., 2006]. The virus transmission occurs primarily via the oro-faecal route, through direct contact with infected persons, and can also be through contaminated fomites (objects that can enable the transmission of pathogens), water, food or by touching contaminated hands or surfaces (Figure 7) [Dennhy, 2000]. In addition, in developed countries, about 11% to 32% of RV infections are hospital acquired, suggesting that nosocomial infections are responsible for great burden of disease [Frühwirth et al., 2001].

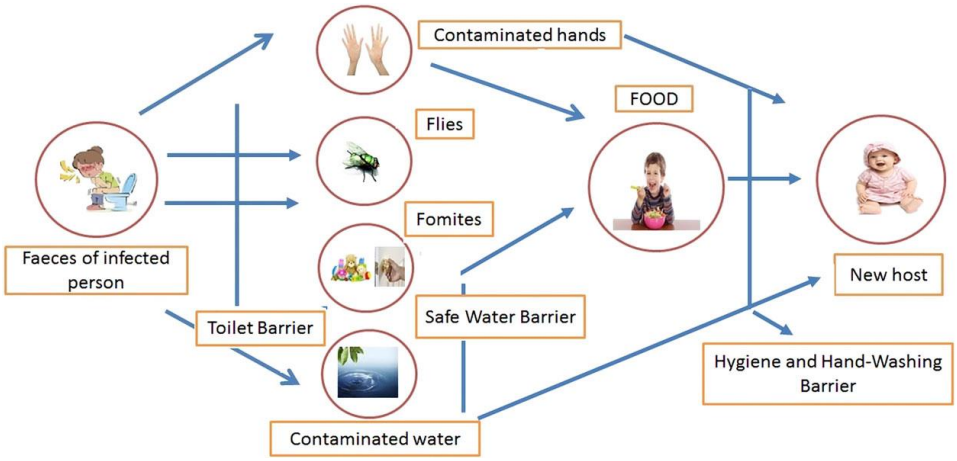


Figure 7. Rotavirus transmission. The primary mode of transmission is the transfer of the virus in stool to the mouth of another child (faecal oral route). Other possible means of transmission are fomites, contaminated food and contaminated water. The vertical lines (blue) show barriers against transmission, that is, toilet barrier, clean water barrier, hands washing, and proper hygiene barrier [Sadiq et al., 2018].

1.13. Diagnosis and screening

Although rotavirus-induced disease is clinically indistinguishable from diarrhoeal diseases caused by other gastroenteritis-inducing infectious agents, several factors can indicate RV

infection [Crawford et al., 2017]. For example, RV infections are often more severe than diarrhoeal diseases caused by other infectious agents. In addition, the seasonality of infection can indicate rotavirus as the main causative agent in some regions [Crawford et al., 2017].

When a laboratory-confirmed diagnosis of RV infection is requested by physicians in the clinical setting, this is based on antigen detection in stools using enzyme-linked immunosorbent assay (ELISA) or immunochromatography. The ELISA assay is the most appropriate enzyme immunoassay for large-scale studies [WHO, 2009].

The window for the detection of viral shedding using ELISA usually ends within 1 week after the onset of illness, but the virus can be detected for longer periods by more-sensitive assays, such as reverse transcription real time PCR (RT-qPCR) and conventional RT-PCR [Richardson et al., 1998]. Viral shedding ranges from 4 to 57 days [Richardson et al., 1998]. Importantly, up to 29% of healthy children <1 year of age have asymptomatic RV infection detected by RT-PCR [Richardson et al., 1998].

1.14. Prevention

Improvements in hygiene or sanitation, can be used to prevent rotavirus infections, however, vaccination against RV is the best measure to prevent RV disease [WHO, 2013]. In 2009, WHO recommended that RVs vaccines to be included in all national immunization programs, including low-resource settings in Africa and Asia [WHO, 2013].

In 2006, pivotal clinical trials of two live oral RV vaccines a pentavalent bovine-human reassortant vaccine (RV5) given in a 3-dose schedule (RotaTeq, Merck), and a monovalent human vaccine (RV1) given in a 2-dose schedule (Rotarix, GSK Biologicals) demonstrated good efficacy (85%–98%) in preventing severe rotavirus gastroenteritis [Parashar et al., 2016]. Recently, a monovalent vaccine, Rotavac® (Bharat Biotech) and Rotasiil (Serum Institute of India Ltd.) were pre-qualified for use globally [Soares-Weiser et al., 2019].

Although clinical trials of RV5 and RV1 demonstrated high efficacy (generally >85%) against severe rotavirus disease in high-income settings, trials performed in resource-poor settings have found substantially lower efficacies (40-60%) [Parashar et al., 2016]. The reasons for reduced oral vaccine efficacy in countries with higher child mortality rates are

unknown. Various factors have been proposed, including interference by maternal antibodies, co-administration with oral poliovirus vaccine, histoblood group antigen, diverse rotavirus strain types, micronutrient deficiencies, endemic infections such as malaria, tuberculosis, or HIV, concomitant enteric infections, gut inflammation, and altered gut microbiota [Czerkinsky and Holmgren, 2015].

Rotavirus vaccine had been introduced in 101 countries worldwide by the end of 2018. Global coverage was estimated at 35%. RV has now been introduced in 34 of 47 countries in the African Region of WHO (https://www.who.int/news-room/fact-sheets/detail/immunization-coverage).

1.15. Treatment

Due the lack of effective antiviral drugs, the infection treatment is based on symptom relief and oral or intravenous rehydration therapy to prevent and treat dehydration due to diarrhoea in infants and young children [King et al., 2003; Kotloff et al., 2013]. Clinical scales that consider the presence of signs and symptoms are available and are usually used to assess dehydration [Ruuska and Vesikari, 1990]. A thirsty, restless or fatigued child with a dry mouth should alert caretakers to ongoing dehydration [Ruuska and Vesikari, 1990].

1.16. Immunology

Rotavirus infection or vaccination induces both innate and adaptive immunity. The innate immune system is mediated by macrophages and produces type I and type III interferons (IFNs) and other cytokines in response to virus infection [Angel et al., 2014]. The adaptive immunity stimulates both humoral and cell mediated immune responses and is responsible for antigen specificity and memory [Angel et al., 2014; Mesa et al., 2010].

Although children can have many recurrent RV infections, they progressively diminish in severity. Levels of total serum rotavirus-specific immunoglobulin A (IgA) antibodies, measured after infection, are currently the best marker of protection against RV disease, and the presence of anti-rotavirus IgA antibodies in serum is widely used as a marker of vaccine take [Franco et al., 2006].

It has been reported that production of intestinal IgA antibodies is largely dependent on the activation of CD4+ T cells, therefore children with acute RV gastroenteritis have very low

levels of rotavirus-specific CD4+ T cells, which almost exclusively produce IFNγ [Mesa et al., 2010; Parra et al., 2014].

1.17. General objective

The main aim of this project was to genetically characterize the rotavirus strains circulating between 2012-2018 in Mozambique, including a comparison before and after rotavirus vaccine implementation in the country.

CHAPTER 2 Rotavirus A strains obtained from children with acute gastroenteritis in Mozambique, 2012-2013: G and P genotypes and phylogenetic analysis of VP7 and partial VP4 gene

Declaration: The content of the present Chapter is similar to the original published article. However, only part of data was included.

2.1. Introduction

Rotaviruses are one of the leading causes of severe-dehydrating diarrhoea in infants and young children. The number of global deaths due to rotavirus infection in children under the age of five was estimated to be 215 000 in 2013; of these deaths, 56% occurred in Sub-Saharan Africa [Tate et al., 2016a]. Rotaviruses are taxonomically classified within a genus of the *Reoviridae* family and contain an 11-segment double stranded RNA (dsRNA) genome. The dsRNA segments encode six structural (VP1–VP4, VP6 and VP7) and six nonstructural (NSP1–NSP6) proteins. The structural viral proteins (VPs) are assembled in three concentric layers enclosing the genomic segments, the viral RNA-dependent RNA polymerase (VP1) and the viral capping enzyme (VP3). The three capsid layers consist of 60 dimers of the inner capsid protein, VP2, 260 trimers of the middle layer protein, VP6, and 780 monomers of the glycosylated VP7 protein. Spike proteins, formed by 60 trimers of the protease-sensitive VP4 protein, protrude on the surface of the virion [Greenberg and Estes, 2009].

Rotaviruses are classified into several groups (A – H) based on serotyping and/or genotyping of VP6 [Matthijnssens et al., 2012b]. Recently, a classification of strains for rotavirus group I and group J has also been proposed [Banyai et al., 2016; Mihalov-Kovacs et al., 2015]. A dual typing system, based on the genome segments encoding the VP4 (P genotypes) and VP7 (G genotypes) proteins, is commonly used in surveillance studies of type A rotaviruses. Thus far, 37 P types and 27 G types have been described globally [Matthijnssens and Van Ranst, 2012; Trojnar et al., 2013], but another 13 P and 7 G types have also been proposed, as of April 2017 (https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification). The most prevalent rotavirus A strains found in humans are the G1, G2, G3, G4, G9 and G12 genotypes in combination with P[4], P[6] and P[8] [8, 9]. Unlike infections in developed countries, where G1P[8] strains cause almost 70% of all rotavirus infections, wide strain diversity is associated with infections in African countries [Todd et al., 2010]. Mozambique has a high diarrhoeal disease burden with more than 13 000 deaths occurring annually in children under 5 years of age [Black et al., 2010]. This country participated in the global enteric multi-centre study (GEMS) to establish the burden of diarrhoea and disease aetiologies in sub-Saharan Africa

and Asian countries. Results from the GEMS study showed that in Mozambique rotavirus group A (RVA) was the most significant cause of acute diarrhoea in infants from 0 to 11 months [Kotloff et al., 2013]. However, no genetic characterization of the rotavirus strains was performed during this study. The first description of RVA G- and P-genotypes in Mozambique was recently published, describing strains circulating in Chókwè, southern Mozambique in 2011 [Langa et al., 2016].

Between 2012 and 2013, an epidemiological study in children under 5 years of age in both an urban area (Mavalane, Maputo) and a rural district (Manhiça) was initiated, with RVA detected in 42.4% (163/384) of hospitalized diarrhea cases [de Deus et al., 2018b]. Considering that vaccination against RVA was introduced in Mozambique in 2015, that study provides baseline data on pre-vaccination RVA strains circulating in Mozambique.

2.2. Objectives

The aim of the work described in this Chapter is to report the G- and P-genotypes of the RVA strains detected in the 2012 and 2013 study and describe the molecular epidemiology of the VP7 and VP8* encoding genes of the selected strains.

2.3. Materials and methods

In this Chapter the main activities done by the student were:

- Stool sample collection with support from hospital staff;
- Enzime immunoassay test (EIA);
- RNA extraction;
- cDNA preparation;
- Caracterization of rotavirus G- and –P genotypes using convencional PCR;
- Selection of samples for sequencing in South Africa;
- Sequencing of all selected samples were done by South Africa team (University of Free State, Department of Microbial, Biochemical and Food Biotechnology);
- Data analysis was done by South Africa team and student.

2.3.1. Rotavirus strains

Between February 2012 and September 2013, a cross-sectional study was conducted at two sites in Mozambique: Mavalane General Hospital (MGH) in Maputo and Manhiça District Hospital (MDH) in the Manhiça district (Supplementary Material 1). Faecal samples were collected from children under five years of age that had been hospitalized with acute diarrhoea (duration of less than 7 days), defined by three or more looser-than-normal stool passages or watery diarrhoea in the 24 hours prior to the hospital visit. A total of 163 rotavirus positive specimens were detected by EIA (Oxoid, UK) and kept for further analysis [de Deus et al., 2018b].

2.3.2. RNA extraction and RT-PCR genotyping

RNA extraction was performed from 10% faecal sample suspensions in distilled water using the QIAamp Viral RNA Kit (Qiagen, USA), following the manufacturer's instructions. Total RNA was eluted in 60 μL AVE buffer, which contains RNase-free water with 0.04% NaN_3 (Sodium azide). The extracted RNA was amplified in a reverse-transcription polymerase chain reaction (RT-PCR) with AMV reverse transcriptase (Promega, USA) and Taq DNA polymerase (Promega, USA). The reactions targeted the full VP7 encoding gene (sBeg9/End9; 1062 bp) and the partial VP4 encoding gene for amplification (VP8*; Con3/Con2; 876 bp) [Gentsch et al., 1992; Gouvea et al., 1990] as described previously. G genotyping was carried out by semi-nested, type specific, multiplex

PCR as described previously [Banerjee et al., 2007; Gouvea et al., 1990]. Amplicons from the first round of RT-PCR were added to a second round multiplex PCR containing primer RVG9, as well as primers aBT1, aCT2, mG3, aDT4, aAT8v, mG9, G10, and G12b, specific for G types 1, 2, 3, 4, 8, 9, 10, and 12, respectively.

A similar approach was followed for P genotyping. The first round amplification with primers Con2 and Con3 was added to a reaction containing primer Con3 and primers 1T-1D, 2T-1, 3T-1, 4T-1, 5T-1, mP11 and p4943, which are specific for types P[8], P[4], P[6], P[9], P[10], P[11] and P[14] respectively [Gentsch et al., 1992; Iturriza-Gomara et al., 2000]. The amplicon size generated in the genotyping reactions denotes the respective G and P type and was determined by analysis on a 1.5% agarose gel, as specified previously [Banerjee et al., 2007; Gentsch et al., 1992; Gouvea et al., 1990; Iturriza-Gomara et al., 2000].

2.3.3. Nucleotide sequencing

Nucleotide sequencing of selected VP7 and VP8* encoding genes was performed using the dideoxynucleotide chain termination method (Supplementary Material 2). Specifically, AMV reverse transcriptase (Thermo Scientific) and KAPA HiFi polymerase (Kapa Biosystems) were used in RT-PCR reactions to amplify the VP7 (sBEG/End9)[Gouvea et al., 1990] and VP8* (Con3/Con2) [Gentsch et al., 1992] encoding genes. PCR amplicons were purified using the NucleoSpin® PCR clean-up and Gel extraction kit (Macherey-Nagel, Germany), according to the manufacturer's instructions. The nucleotide sequences of these amplicons were determined using the same forward and reverse primers used for amplicon generation and the BigDye terminator v.3.1 kit (Applied Biosystems), according to the manufacturer's instructions. Sequencing products were analysed on an ABI 3130 Genetic Analyzer (Applied Biosystems) and the resulting electropherograms were edited in CLC Bio (Qiagen).

2.3.4. Data analyses

Sequences were confirmed to be those expected, and the most similar sequences in GenBank found, using the Nucleotide Basic Local Alignment Search Tool (BLASTn) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and genotypes were confirmed with the online

database Virus Pathogen Database and Analysis Resource (ViPR) [Pickett et al., 2012]. Sequence alignments and phylogenetic analyses were carried out using MEGA 7.0.14 [Kumar et al., 2016]. A MUSCLE alignment was performed to align the Mozambican sequences with relevant nucleotide sequences obtained from GenBank. Phylogenetic trees were built with the Maximum Likelihood method using 1000 bootstrap replicates. The Tamura 3 correction parameter was determined as the best substitution model [Tamura, 1992] for the VP7 encoding sequences and the Hasegawa-Kishino-Yano model [Hasegawa et al., 1985] for the VP8* encoding sequences.

2.3.5. Compliance with Ethical Standards

Informed consent was obtained from the parents or guardians of the children and the ethical principles of Helsinki Declaration guided all procedures. The study was approved by the National Committee on Bioethics from Mozambique in 2010 (IRB 00002657) and by the Ethical Committee of University of Free State (UFS) (201/2013).

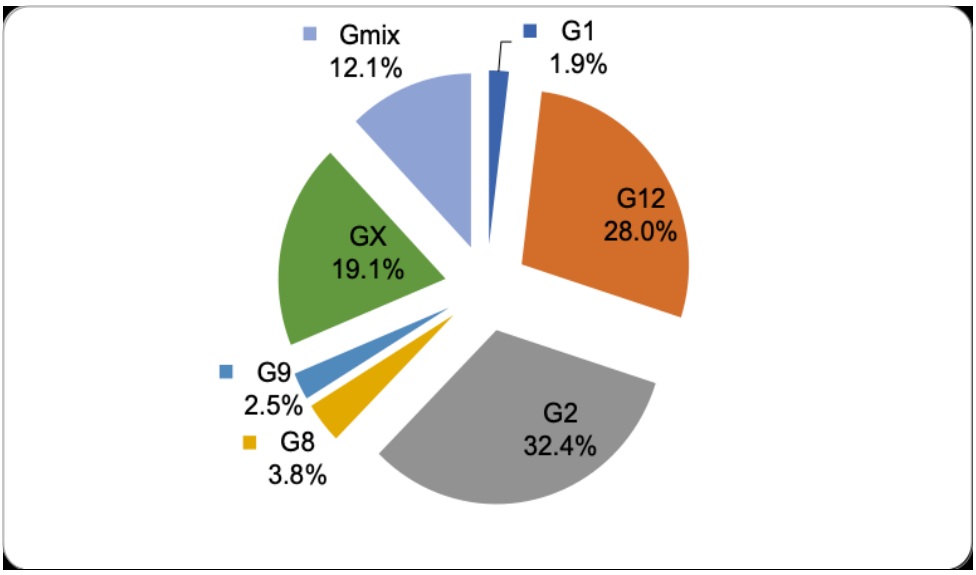
2.4. Results

It was possible to obtain typing results for at least the G or P genotypes from 157 of the 163 (89.2 %) RVA ELISA-positive samples. Both G and P genotypes could be determined for 70.7% (111/157) of the genotyped specimens. In 8.3% (13/157) of the samples only the G genotype was determined and in the remaining 10.2% (16/157) only the P genotype was obtained.

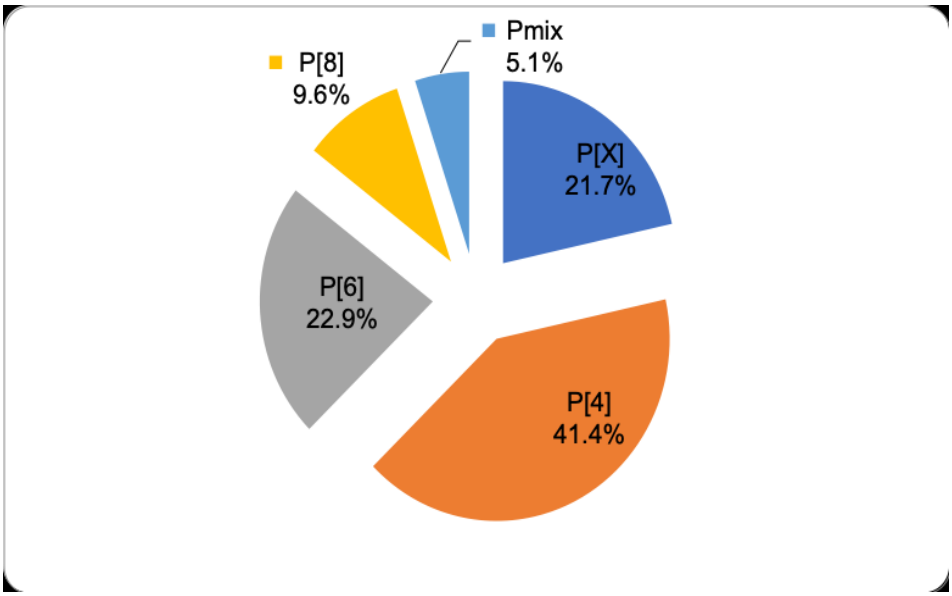
The most common G genotype was G2 (32.4%; 51/157), followed by G12 (28.0%; 44/157), representing 60.4 % of the total, but mixed types were found in 12.1% (19/157), including G9G2, G12G9 and G12G8 (Figure 1A) and 19.1% were nontypeable for G (Gx). Genotypes G8 (3.8%; 6/157), G9 (2.5%; 4/157) and G1 (1.9%; 3/157) were identified in lower frequencies. The most frequently detected P genotypes were P[4] at 41.4% (65/157) and P[6] at 21.7% (36/157; Figure 1B), making up 63 % of all genotypes. The P[8] genotype was determined at a frequency of 9.6% (15/157). A total of 21.7% (34/157) of P genotypes could not be determined (P[X]).

Among fully typed samples (n = 111), 19 different G and P combinations were observed. The most common G/P combinations detected were G2P[4] at a frequency of 42.3% (47/111) and G12P[6] at 28.8% (32/111; Figure 1C) in a total of 71.1 % of all genotypes. Other genotypes detected at lower frequencies included G8P[4] (4.5%; 5/111) and G12P[8] (5.4%; 6/111). Seven samples had mixed G genotypes, three samples had mixed P genotypes and three samples had both mixed G and P genotypes (Figure 1C).

A)


Gmix
12.1%
G1
1.9%
G12
28.0%
GX
19.1%
G9
2.5%
G8
3.8%
G2
32.4%

B)


Pmix
5.1%
P[8]
9.6%
P[X]
21.7%
P[6]
22.9%
P[4]
41.4%

C)

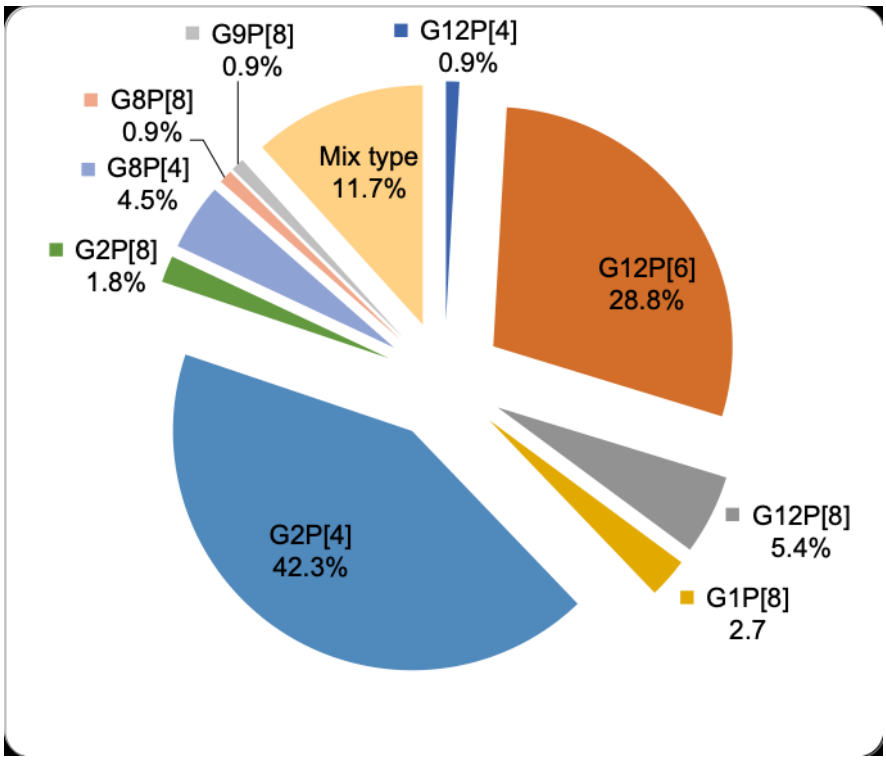


Figure 1. Frequency of partial G (A), P (B) and G/P (C) types determined from rotavirus positive samples from children under five years of age with acute diarrheal in Manhiça District Hospital and Mavalane General Hospital in Mozambique from February 2012 to September 2013. A): G partially typed samples. B): P partially typed samples. C): G/P fully typed samples. X refers to strains that were non-typeable for G or P. Gmix, Pmix and mix type represent the samples with more than one G or P type in the same samples.

The most prevalent genotype varied geographically: G2P[4] was the most prevalent (39.4%, 26/66) in Manhiça (rural area) (Table 1) while in Mavalane (urban area) (Table 1), G12P[6] strains (28.6%, 26/91) predominated. Mavalane also showed higher strain diversity with more mixed genotypes detected.

Table 1. Frequency of G/P genotype combinations detected in Manhiça (rural) and Mavalane (urban) areas.

Genotypes	Study area n (%)	
	Manhiça[1]	**Mavalane[2]**
G12P[4]	0	1 (1.1)
G12P[6]	6 (9.1)	26 (28.6)
G12P[8]	1 (1.5)	5 (5.5)
G12P[X]	2 (3.0)	1 (1.1)
G1P[8]	1 (1.5)	2 (2.2)
G2P[4]	26 (39.4)	21 (23.1)
G2P[8]	1 (1.5)	1 (1.1)
G2P[X]	0	2 (2.2)
G8P[4]	0	5 (5.5)
G8P[8]	0	1 (1.1)
G9P[8]	0	1 (1.1)
G9P[X]	2 (3.0)	0
GXP[4]	7 (10.6)	2 (2.2)
GXP[6]	1 (1.5)	0
GXP[8]	0	1 (1.1)
[a]Gmix-P[X]	8 (12.1)	1 (1.1)
[b]GX-Pmix	0	2 (2.2)
[c]Gmix-P	0	7 (7.7)
[d]G-Pmix	0	3 (3.3)
[e]Gmix-Pmix	0	3 (3.3)
NT	11 (16.7)	6 (6.6)
Total	66 (100)	91 (100)

Legend: X refers to strains that where non-typeable for G or P, NT refers to strains not typed for either G and P. Gmix and Pmix represents the samples with more than one G or P type in the same sample. [1]Manhiça. [a]Gmix-P[X]: G12G8P[X] (12.1%). [2]Mavalane. [a]Gmix-P[X]: G9G8G2P[X] (1.1%). [b]GX-Pmix: GXP[6]P[4] (1.1%), GXP[8]P[6] (1.1%). [c]Gmix-P: G12G8P[4] (2.2%), G12G8P[6] (1.1%), G12G9P[6] (1.1%), G9G2P[4] (1.1%), G9G2P[6] (1.1%), G9G2P[8] (1.1%). [d]G-Pmix: G9P[8]P[4] (1.1%), G12P[8]P[6] (2.2%). [e]Gmix-Pmix: G12G8P[6]P[4] (1.1%), G12G9P[8]P[6] (2.2%).

In an analysis of the temporal distribution of fully typed strains (n = 111), in 2012 G12P[6] was most prevalent (47.8%; 32/67), followed by mixed infections (19.4%; 13/67; Figure 2). In addition, G12 was detected in combination with P[8] (9.0%; 6/67) and G8 in combination with P[4] (7.5%; 5/67). All mixed infections were detected only in 2012. In 2013, the predominant strain was G2P[4] (97.7%; 43/44). A low frequency of G2P[8] (2.3%; 1/44) was also detected (Figure 2). In addition, greater strain diversity was observed in 2012, when compared to 2013 (Figure 2).

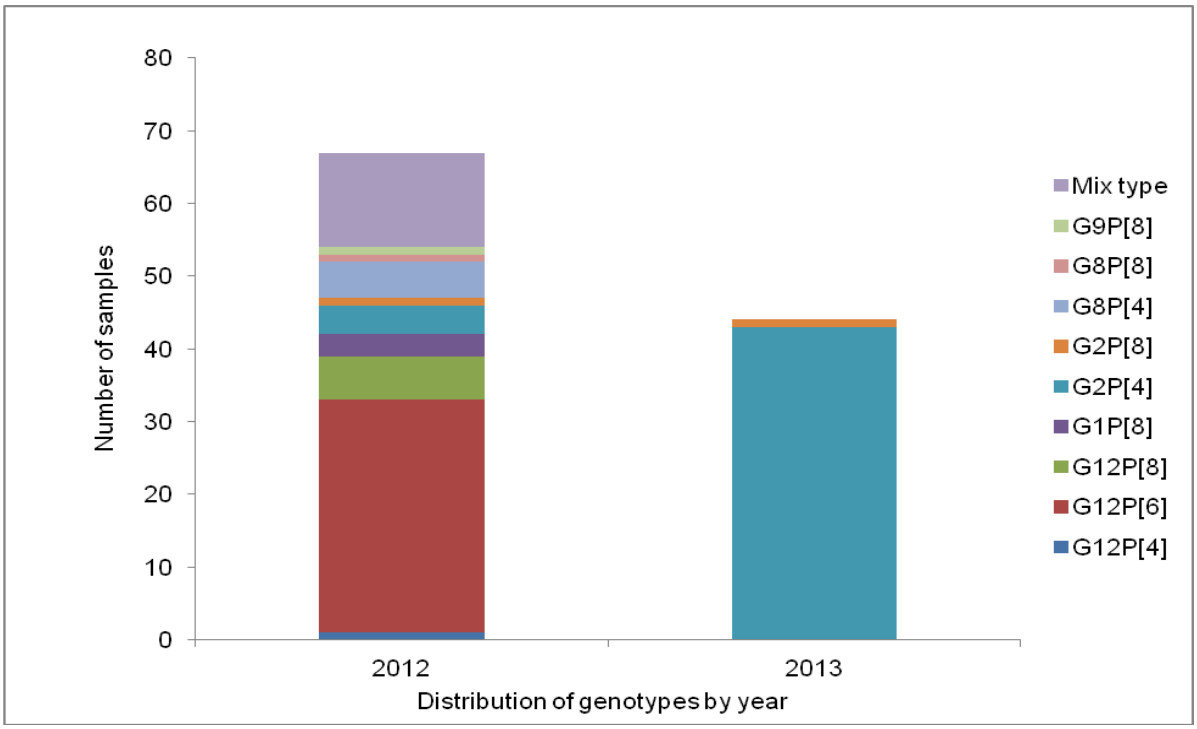


Figure 2. Frequency of G/P genotype combinations detected in rotavirus positive samples from children under 5 years of age with acute diarrhoea distributed by year.

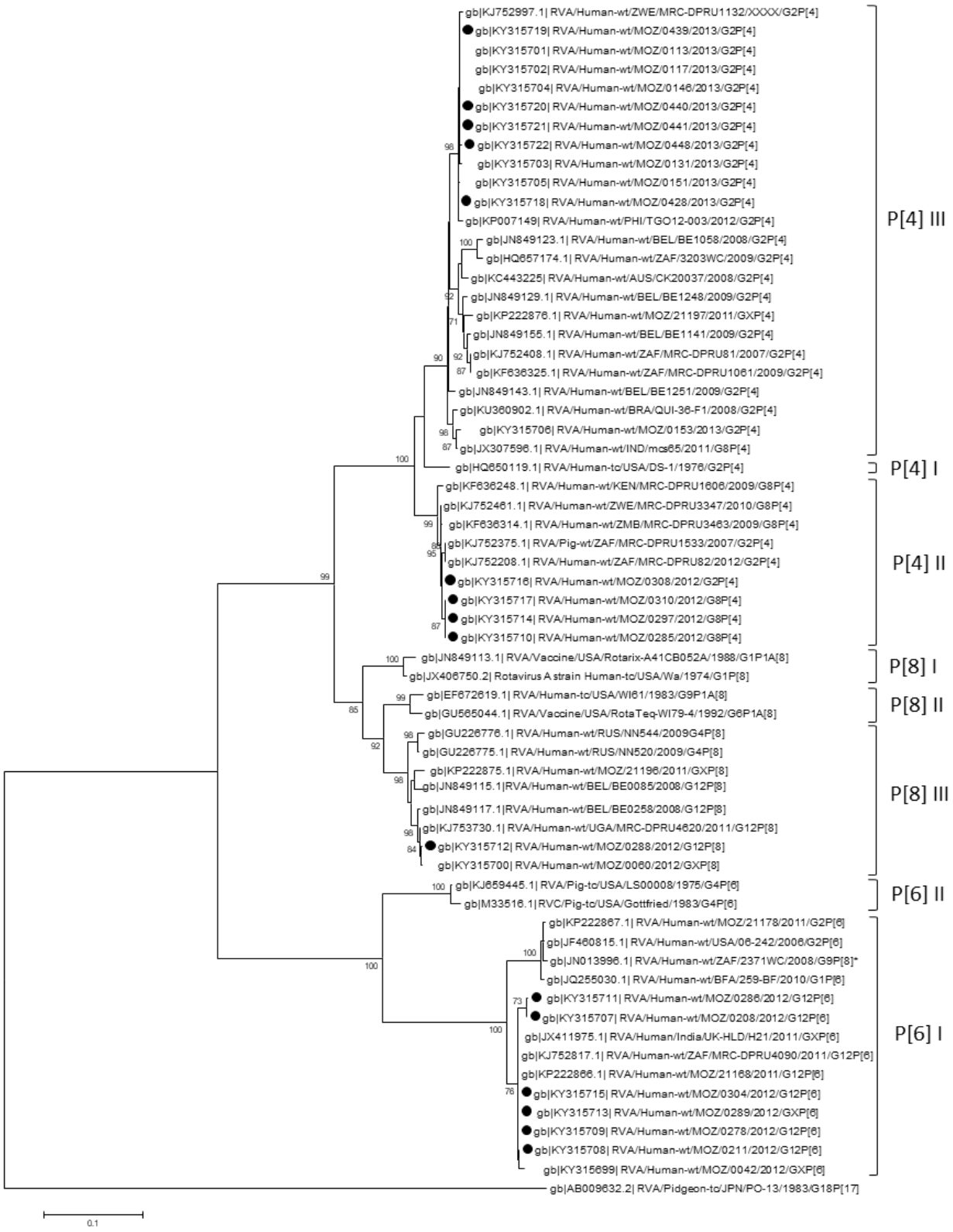
The nucleotide sequences of the 23 analysed Mozambican RVA strains were submitted to GenBank with accession numbers KY315699- KY315722 for VP8* and KY426071-KY426094 for VP7 encoding sequences, respectively. A total of 24 G and 23 P genotypes were sequenced and the sequencing results correlated with the genotyping results, apart from the P genotype for sample RVA/Humanwt/MOZ/0042/2012/GXP[6] (Supplementary Material 2). The partial VP4 encoding gene was identified as P[6] by sequencing and as

P[8] by genotyping PCR. Five G2 strains (RVA/Human wt/MOZ/0113/2013/ G2P[4], RVA/Human-wt/MOZ/0117/2013/G2P[4], RVA/ Human-wt/MOZ/0151/2012/G2P[4], RVA/Human-wt/MOZ/0153/2012/G2P[4] and RVA/Human-wt/ MOZ/0412/201/G2P[X]), which could not be typed by PCR, were identified by sequencing. Likewise, the P genotype for two samples (RVA/Human wt/MOZ/0060/2012/GXP[8] and RVA/Human-wt/MOZ/0441/2013/G2P[4]) were elucidated by sequencing and were identified as P[8] and P[4], respectively. Conversely, the G genotyping PCR results for two samples (RVA/Human-wt/MOZ/0060/2012/GXP[8] and RVA/Human-wt/MOZ/0289/2012/GXP[6] could not be confirmed as G12 with nucleotide sequencing. The VP8* encoding gene of RVA/Human-wt/MOZ/0277/2012/G12P[X] could not be confirmed as P[6]. Since the G2 genotype was missing for several samples (by genotyping PCR), the primer binding site of the G2 forward genotyping primer (aCT2) was analyzed (Supplementary Material 3). Although differences were observed between the last three nucleotides of the aCT2 primer and the G2 sequences of Mozambican strains, most Mozambican G2 strains could be genotyped using the aCT2 primer (RVA/ Human-wt/MOZ/0113/2013/G2P[4], RVA/Human-wt/ MOZ/0117/2013/G2P[4], RVA/Human-wt/MOZ/0151/2012/ G2P[4], RVA/Human-wt/MOZ/0153/2012/G2P[4] and RVA/Human-wt/MOZ/0412/201/G2P[X]; Supplementary Material 3). The incorrect typing of strain RVA/Human-wt/ MOZ/0042/2012/GXP[6] as P[8] was also investigated by comparing the primer binding regions for the respective genotypes (Supplementary Material 3). Again, it was not clear why the P[6] primer (3T-1) failed to detect the P[6] genotype using the genotyping PCR. Fifteen of the 18 base pairs in the P[8] genotyping primer (1T-1D) did not match the sequence of RVA/Human-wt/MOZ/0042/2012/GXP[6] and it is therefore unclear why the RVA/Human-wt/MOZ/0042/2012/ GXP[6] strain was incorrectly genotyped as P[8].

Nucleotide-based phylogenetic trees were built for the VP8* and VP7 encoding genes (Figure 3A and B). Ten of the 15 Mozambican P[4] strains clustered together with strains from the Philippines and Zimbabwe in lineage III. These Mozambican strains were detected in 2013 in both Manhiça and Mavalane (Supplementary Material 2). Another P[4] strain (RVA/Human-wt/MOZ/0153/2013/G2P[4]), detected in Manhiça, clustered with strains from India and Brazil in a separate sub-cluster in lineage III. The remaining four P[4]

Mozambican strains, detected in 2012 in Mavalane, formed a sub-cluster with Southern African strains and a South African porcine strain, in lineage II. The two Mozambican P[8] strains formed a sub-cluster in lineage III with a Ugandan strain and a Belgium strain. Another Belgium strain (RVA/Human-wt/BEL/BE0058/2008/G12P[8]) and a strain detected in Chókwè, Mozambique in 2011 (RVA/ Human-wt/MOZ/21196/2011/GXP[8]), clustered separately from the Mozambican strains described in this study (Figure 3A, Supplementary Material 1). The the seven Mozambican P[6] strains, all detected in 2012, clustered with lineage I. RVA/Human-wt/MOZ/0208/2012/G12P[6] and RVA/ Human-wt/MOZ/0286/2012/G12P[6] formed a sub-cluster in lineage I, whereas the remainder of the P[6] strains clustered with a South African strain detected in 2011 (P[6] encoding sequence of mixed infection, RVA/Human-wt/ ZAF/2371WC/2008/G9P[8] [24]), a strain from India and a strain detected in Chókwè, Mozambique (Figure 3A). All eight Mozambican G12 strains were detected in 2012 and clustered in lineage III (Figure 3B). Seven of these strains clustered with strains from Chókwè, Mozambique. The remaining G12 strain (RVA/Human-wt/MOZ/0288/2012/ G12P[8]) clustered with two Chókwè strains and a Belgian strain. The G12 genotype strain RVA/Human-wt/MOZ/0288/2012/G12P[8] associated with the P[8] genotype and also clustered with strains in which the G12 genotype was seen to associate with P[8]. The three Mozambican G8P[4] strains, detected in 2012 in Mavalane, clustered in lineage II with a Ugandan and a Zimbabwean strain. Finally, the 12 G2P[4] genotypes, isolated in 2013, clustered in lineage IV. The Mozambican strain (RVA/Humanwt/MOZ/0308/2012/G2P[4]) detected in Mavalane formed a separate sub-cluster, also in lineage IV with a South African strain (RVA/Human-wt/ZAF/MRC-DPRU82/2012/G2P[4]).

A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gb|KY315719| RVA/Human-wt/MOZ/0439/2013/G2P[4]
gb|KY315701| RVA/Human-wt/MOZ/0113/2013/G2P[4]
gb|KY315702| RVA/Human-wt/MOZ/0117/2013/G2P[4]
gb|KY315704| RVA/Human-wt/MOZ/0146/2013/G2P[4]
gb|KY315720| RVA/Human-wt/MOZ/0440/2013/G2P[4]
gb|KY315721| RVA/Human-wt/MOZ/0441/2013/G2P[4]
gb|KY315722| RVA/Human-wt/MOZ/0448/2013/G2P[4]
gb|KY315703| RVA/Human-wt/MOZ/0131/2013/G2P[4]
gb|KY315705| RVA/Human-wt/MOZ/0151/2013/G2P[4]
gb|KY315718| RVA/Human-wt/MOZ/0428/2013/G2P[4]
gb|KP007149| RVA/Human-wt/PHI/TGO12-003/2012/G2P[4]
gb|JN849123.1| RVA/Human-wt/BEL/BE1058/2008/G2P[4]
gb|HQ657174.1| RVA/Human-wt/ZAF/3203WC/2009/G2P[4]
gb|KC443225| RVA/Human-wt/AUS/CK20037/2008/G2P[4]
gb|JN849129.1| RVA/Human-wt/BEL/BE1248/2009/G2P[4]
gb|KP222876.1| RVA/Human-wt/MOZ/21197/2011/GXP[4]
gb|JN849155.1| RVA/Human-wt/BEL/BE1141/2009/G2P[4]
gb|KJ752408.1| RVA/Human-wt/ZAF/MRC-DPRU81/2007/G2P[4]
gb|KF636325.1| RVA/Human-wt/ZAF/MRC-DPRU1061/2009/G2P[4]
gb|JN849143.1| RVA/Human-wt/BEL/BE1251/2009/G2P[4]
gb|KU360902.1| RVA/Human-wt/BRA/QUI-36-F1/2008/G2P[4]
gb|KY315706| RVA/Human-wt/MOZ/0153/2013/G2P[4]
gb|JX307596.1| RVA/Human-wt/IND/mcs65/2011/G8P[4]
gb|HQ650119.1| RVA/Human-tc/USA/DS-1/1976/G2P[4]
gb|KF636248.1| RVA/Human-wt/KEN/MRC-DPRU1606/2009/G8P[4]
gb|KJ752461.1| RVA/Human-wt/ZWE/MRC-DPRU3347/2010/G8P[4]
gb|KF636314.1| RVA/Human-wt/ZMB/MRC-DPRU3463/2009/G8P[4]
gb|KJ752375.1| RVA/Pig-wt/ZAF/MRC-DPRU1533/2007/G2P[4]
gb|KJ752208.1| RVA/Human-wt/ZAF/MRC-DPRU82/2012/G2P[4]
gb|KY315716| RVA/Human-wt/MOZ/0308/2012/G2P[4]
gb|KY315717| RVA/Human-wt/MOZ/0310/2012/G8P[4]
gb|KY315714| RVA/Human-wt/MOZ/0297/2012/G8P[4]
gb|KY315710| RVA/Human-wt/MOZ/0285/2012/G8P[4]
gb|JN849113.1| RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P1A[8]
gb|JX406750.2| Rotavirus A strain Human-tc/USA/Wa/1974/G1P[8]
gb|EF672619.1| RVA/Human-tc/USA/WI61/1983/G9P1A[8]
gb|GU565044.1| RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P1A[8]
gb|GU226776.1| RVA/Human-wt/RUS/NN544/2009G4P[8]
gb|GU226775.1| RVA/Human-wt/RUS/NN520/2009/G4P[8]
gb|KP222875.1|RVA/Human-wt/MOZ/21196/2011/GXP[8]
gb|JN849115.1|RVA/Human-wt/BEL/BE0085/2008/G12P[8]
gb|JN849117.1|RVA/Human-wt/BEL/BE0258/2008/G12P[8]
gb|KJ753730.1| RVA/Human-wt/UGA/MRC-DPRU4620/2011/G12P[8]
gb|KY315712| RVA/Human-wt/MOZ/0288/2012/G12P[8]
gb|KY315700| RVA/Human-wt/MOZ/0060/2012/GXP[8]
gb|KJ659445.1| RVA/Pig-tc/USA/LS00008/1975/G4P[6]
gb|M33516.1|RVC/Pig-tc/USA/Gottfried/1983/G4P[6]
gb|KP222867.1| RVA/Human-wt/MOZ/21178/2011/G2P[6]
gb|JF460815.1| RVA/Human-wt/USA/06-242/2006/G2P[6]
gb|JN013996.1| RVA/Human-wt/ZAF/2371WC/2008/G9P[8]*
gb|JQ255030.1| RVA/Human-wt/BFA/259-BF/2010/G1P[6]
gb|KY315711| RVA/Human-wt/MOZ/0286/2012/G12P[6]
gb|KY315707| RVA/Human-wt/MOZ/0208/2012/G12P[6]
gb|JX411975.1| RVA/Human/India/UK-HLD/H21/2011/GXP[6]
gb|KJ752817.1| RVA/Human-wt/ZAF/MRC-DPRU4090/2011/G12P[6]
gb|KP222866.1| RVA/Human-wt/MOZ/21168/2011/G12P[6]
gb|KY315715| RVA/Human-wt/MOZ/0304/2012/G12P[6]
gb|KY315713| RVA/Human-wt/MOZ/0289/2012/GXP[6]
gb|KY315709| RVA/Human-wt/MOZ/0278/2012/G12P[6]
gb|KY315708| RVA/Human-wt/MOZ/0211/2012/G12P[6]
gb|KY315699| RVA/Human-wt/MOZ/0042/2012/GXP[6]
gb|AB009632.2| RVA/Pidgeon-tc/JPN/PO-13/1983/G18P[17]
0.1
P[4] III
P[4] I
P[4] II
P[8] I
P[8] II
P[8] III
P[6] II
P[6] I

B

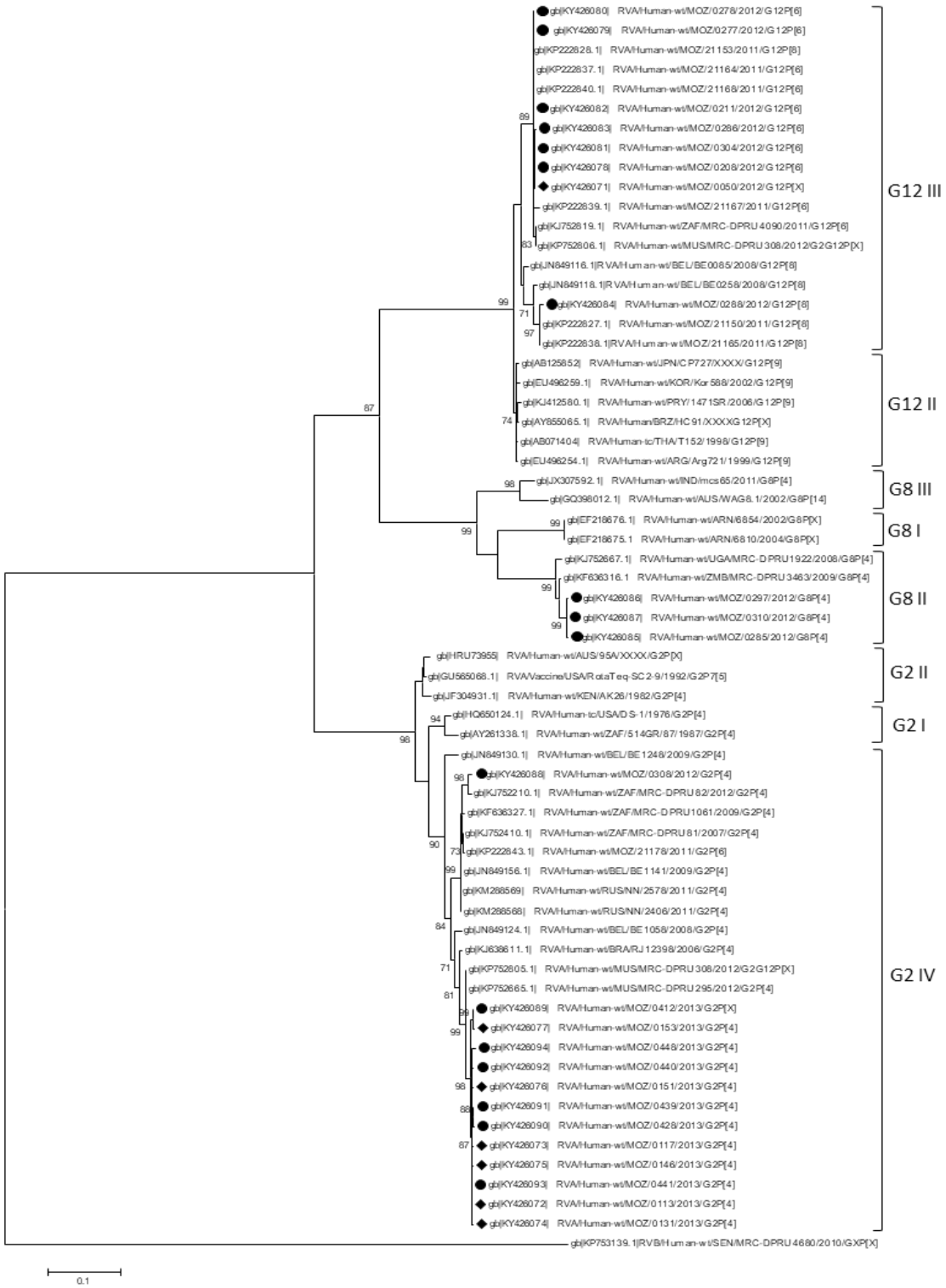


Figure 3. Phylograms based on nucleotide sequences encoding for VP8* (A) and VP7 (B). Mozambican strains from Mavalane are indicated with black ovals and those from Manhiça are indicated with black diamonds. Accession numbers

are included for all strains. The evolutionary history was inferred using the Maximum Likelihood method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches and values <70 are not shown. The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. A): Phylogenetic tree of VP8* encoding sequences. *RVA/Human-wt/ZAF/2371WC/2008/G9P[8] detected in South Africa contained multiple genotypes and the sequence in this analysis is based on the P[6] genotype. B): Phylogenetic tree of VP7 encoding sequences.

2.5. Discussion

The present study reports the genotyping and molecular characterization of rotavirus strains from southern Mozambique during 2012-2013. Although, worldwide, the most common G and P rotavirus strains are G1-4, G9 and G12 as well as P[4], P[6] and P[8][Banyai et al., 2012], in this study, G2P[4] was the prevalent genotype, accounting for 42.3% of strains detected in 2013. This differed from RVA genotypes detected in 2011 in Chókwè, a site situated closely to the area reported in this study. The G2P[4] genotype has been reported worldwide [Mwenda et al., 2014] and was also reported to be the predominant type (47.0%) circulating in South African in 2013 [Page et al., 2013]. However, it is important to highlight that Mozambique had not yet introduced rotavirus vaccines during the study period while South Africa introduced Rotarix® into their Expanded Programme on Immunisation in 2009. The second most frequent genotype was G12P[6] (28.8%).The G12 genotype was also detected at lower frequencies with P[8] and P[4] types. Previously, G12P[8] strains were predominant (57.0%) in Mozambique in 2011 [Langa et al., 2016]. The African Rotavirus Surveillance Network also reported the detection of G12P[6] and G12P[8] in 2012, but at much lower frequencies of 6.0% and 12.0%, respectively [Mwenda et al., 2014].

We also detected uncommon genotypes such as G8P[4] and G8P[8], albeit at low rates (4-5.0%). The G8 genotype is known to cause infection in cattle and has been responsible for rotavirus infection in humans, mainly in African countries [Cunliffe et al., 2010; Mwenda et al., 2010]. Recently it was reported that G8P[6] was the predominant type in São Tome & Príncipe in 2011, being responsible for 71.1% of rotavirus cases [Istrate et al., 2015].

Other genotypes detected in low frequencies were G1P[8] and G9P[8]. In contrast to this study, genotype G1P[8] strains were the second most common genotype detected in the

Chókwè study [Langa et al., 2016]. However, the G9 genotype was detected only at low frequencies in the current study and the study carried out in Chókwè.

In our study, we observed a difference in distribution of rotavirus strains between the two years. In 2012, G12P[6] was predominant and more rotavirus strain diversity was noted. Genotype G2P[4] was prevalent in 2013 and lower genotypic diversity was observed compared to 2012. Variation in circulating rotavirus genotypes has been reported to occur yearly [Mwenda et al., 2014], with the change due to the natural variability of rotavirus strains over time. It has been suggested that rotavirus vaccines may influence the distribution of genotypes [Matthijnssens et al., 2012a], and, indeed, a study carried out in Belgium reported a higher prevalence of G2P[4] in vaccinated rotavirus gastroenteritis patients than in unvaccinated rotavirus gastroenteritis patients [Matthijnssens et al., 2014]. So, in Mozambique, the prevalence of G2P[4] will require continuous monitoring, especially because of the introduction of rotavirus vaccines in 2015.

Geographical differences in genotype distribution within the same country or region can occur [Banyai et al., 2012]. We also observed differences in the geographical distribution of genotypes in Mozambique. In Mavalane, an urban area, G12P[6] (28.6%) was the most prevalent, while in contrast to Manhiça, a rural area, G2P[4] (39.4%) was predominant. During the study 10.8% (17/157) of the samples were untypeable for G or P, which is comparable to the rate of untypeable samples previously reported from Sub-Saharan countries (8.6-14.6%) [Mwenda et al., 2014]. It was noted that in Manhiça a higher number of untypeable strains (16.7%) was observed, when compared to Mavalane, the urban area (6.6%).

In our study, mixed genotypes were observed in 11.7% (13/111) of the samples typed for G and P. This is similar to the 12-14% mixed genotypes reported by the African Rotavirus Surveillance Network [Mwenda et al., 2014].

The phylogenetic analyses provided some evidence on the widespread circulation of rotavirus strains in Mozambique, with similar strains detected in Manhiça, Mavalane and Chókwè (Supplementary Material 1). The Chókwè G12 and P[6] strains phylogenetically clustered with strains detected in the current study indicating that these strains circulated in

southern Mozambique from 2011 to 2013 (Figure 3A, B). Interestingly, no G8 strains were identified in the Chókwè study during 2011 [Langa et al., 2016].

The genotypes of the Mozambican strains clustered mostly with those of strains detected in other African countries, specifically South Africa (G12, G2, P[6]), Uganda (P[8]), Zambia (G12), and Zimbabwe (P[4]; Figure 3A, B). South Africa, Zambia and Zimbabwe share borders with Mozambique and the circulation of similar rotavirus strains between these countries is not unusual. The South African strains (RVA/Human-wt/ZAF/MRC-DPRU4090/2011/G12P[6] and RVA/Human-wt/ZAF/MRC-DPRU82/2012/ G2P[4]) are closely related to the strains from southern Mozambique (Figure 3A, B). Strains from other countries, which also grouped with Mozambican strains, were from India (P[4], P[6]), Mauritius (G12) and the Philippines (P[4]; Figure 3A, B).

One exception, where the Mozambican strains did not cluster close together, is RVA/Human-wt/MOZ/0153/2013/G2P[4]. This strain clustered with an Indian (RVA/Humanwt/ IND/mcs65/2011/G8P[4]) and a Brazilian (RVA/Humanwt/ BRA/QUI-36-F1/2008/G2P[4]) strain in the P[4] lineage III. However, RVA/Human-wt/MOZ/0153/2013/G2P[4] grouped with the other Mozambican strains in lineage G2 IV. The sequencing of the VP8* encoding gene suggests that RVA/Human-wt/MOZ/0153/2013/G2P[4] is a reassortant strain.

Strain RVA/Human-wt/MOZ/0308/2012/G2P[4] is of particular interest. This strain was detected in 2012 in Mavalane and clustered with G8P[4] Mozambican strains and two South African strains (RVA/Human-wt/ZAF/MRC-DPRU82/2012/G2P[4] and RVA/Pig-wt/ZAF/ MRC-DPRU1533/2007/G2P[4]) in P[4] lineage II. However, in the nucleotide tree of segment 9, RVA/Human-wt/ MOZ/0308/2012/G2P[4] clustered with the same South African strain (RVA/Human-wt/ZAF/MRC-DPRU82/2012/ G2P[4]), separate from the G2 Mozambican strains that circulated in 2013. This grouping of the G2P[4] strain with G8P[4] strains in lineage II of P[4] was previously reported and might indicate a reassortment event or recombination in the VP4-encoding genome segment [Nyaga et al., 2014].

Lastly, RVA/Human-wt/MOZ/0288/2012/G12P[8] clustered separately from the other G12 Mozambican strains described in this study. However, this strain clustered with two strains

detected in Chókwè, Mozambique in 2011[Langa et al., 2016]. Segment 4 of this strain grouped with the other Mozambican P[8] strains. Therefore, RVA/Human-wt/MOZ/0288/2012/ G12P[8] is also a possible reassortant strain. However, confirmation of reassortment would have to be performed by whole genome sequencing of these viruses [Jere et al., 2012; Jere et al., 2011a; Jere et al., 2011b; Nyaga et al., 2014].

2.6. Conclusion

The current study, carried out during 2012 and 2013, provides valuable insight into the circulating rotavirus genotypes and strain diversity in southern Mozambique prior to vaccine introduction. The study showed that G2P[4] was prevalent although the study was not carried out for a full two years, which can bias the temporal distribution of genotypes. Phylogenetic analysis indicated that the Mozambican strains were related with a few potential reassortment events noted. There is evidence for strain movement between countries in southern Africa with common strains circulating in southern Mozambique as well as South Africa, Zambia and Zimbabwe.

CHAPTER 3 Molecular epidemiology of rotavirus A prior to and following Rotarix® introduction in Mozambique, 2014 – 2018: Emergence of genotype G3P[4] and G3P[8]

3.1. Introduction

In 2009 the World Health Organization (WHO) recommended the introduction of rotavirus vaccines in the national immunization program globally, particularly in countries with a high under-five mortality rate [WHO, 2013]. WHO has been coordinating and supporting the Global Network of Rotavirus Surveillance (GNRS) since 2006 to support countries to generate data to drive vaccine introduction [Mwenda et al., 2014]. Mozambique has actively participated in the African Surveillance Rotavirus Network, sharing the data from the National Surveillance of Acute Diarrhoea (ViNaDiA) implemented in the country in 2014. Data from ViNaDiA showed prevalence of 40.2% and 38.3% in 2014 and 2015 respectively, prior to vaccine introduction [de Deus et al., 2018a]. The Rotarix® (GlaxoSmithKline, Belgium) monovalent vaccine was introduced into the National Program of Immunization of Mozambique, in September 2015. Since then, substantially lower rotavirus prevalence has been reported at 12.2% and 13.5% in 2016 and 2017, respectively [de Deus et al., 2018a], representing 30-35% of the previous prevalence, thus suggesting that the vaccine has had a considerable impact.

Due to the dynamics on the epidemiology of strains in circulation, the implementation of continuous surveillance programs prior and post vaccine introduction is important to monitor strain diversity; to evaluate the effectivness of vaccine and evaluate the likelihood of vaccine-derived reassortment and re-emergence of unusual strains [Jere et al., 2018; Matthijnssens et al., 2012a].

3.2. Objective

To evaluate rotavirus strain diversity in Mozambique prior (2014 - 2015) and post (2016 - 2018) rotavirus vaccine introduction, among children less than five years of age with moderate-to-severe acute diarrhoea**.**

3.3. Materials and Methods

3.3.1. Study design

This doctoral thesis was carried out within the scope of a project entitled "The effect of rotavirus vaccine in gastroenteritis hospitalization and rotavirus evolution in Mozambique (pre- and post- vaccination introduction study)" - PREPOS. To this end, a National Surveillance of Acute Diarrhea (ViNaDia) in children was implemented by the *Instituto Nacional de Saúde* (INS), which aims to determine the burden of acute diarrhea in children aged 0 to 14 years, and the associated etiological agents (including rotavirus), in health units in four provinces of the country, between 2014 and 2018.

Clinical samples from patients in the surveillance system were collected by medical, nursing or laboratory staff trained in accordance with standard national procedures. Sociodemographic, clinical and epidemiological data were collected by trained medical and nursing staff. Rotavirus antigen detection was done by surveillance laboratory technicians.

For the work of this thesis, surveillance samples from four sentinel hospitals collected between 2014 to 2018 were included: positive samples from the pre-vaccination (2014-2015) and post-vaccination (2017-2018) periods. Rotavirus positive samples were used to determine which rotavirus genotypes strains were in circulation in the indicated period above, therefore, characterization of rotavirus G- and –P genotypes were done.

In this Chapter the main activicties done by student were:

- Organization of all positive samples for rotavirus caracterization;
- RNA extraction of rotavirus;
- cDNA preparation;
- Caracterization of rotavirus G- and –P genotypes using convencional PCR;
- Data analysis.

3.3.2. Study population and stool sample collection

RVA positive samples, screened by enzyme-linked immunosorbent assay (ELISA), from children under five years of age and suffering from moderate-to-severe acute diarrhoea

were included from the ongoing hospital-based diarrhoea surveillance (National Surveillance of Acute Diarrhoea - May 2014 and December 2018).

The National Surveillance of Acute Diarrhoea in children led by the "*Instituto Nacional de Saúde* (INS)" includes children from 0 to 14 years of age as target group. It has been conducted since May 2014 in Mavalane General Hospital (HGM, first sentinel site) in Maputo province, southern region, and was expanded to include other sentinel sites (Fig. 1). In March of 2015, José Macamo General Hospital (HJM), in Maputo Province, and Nampula Central Hospital (HCN), in Nampula province in north region, were added. In June of 2015, two additional sentinel sites were included in the surveillance programme: Beira Central Hospital (HCB) in Sofala Province, and Quelimane Hospital (HPQ), in Zambézia province for central region (Figure 1).

Enrolment in the surveillance was conducted from Monday to Friday, and a stool specimen was collected within 48 hours after admission.

At HGM and HJM, samples were collected and immediately transferred to the INS referral laboratory, while in HCB, HCN and HPQ, samples were collected and stored at -20˚C, for a maximum of 7 days, until samples were shipped on dry ice on a weekly basis to INS for analysis. Samples were stored at -70°C as previously described [de Deus et al., 2018a].

3.3.3. Ethical approval

The protocol of the National Surveillance of Acute Diarrhoea in children was approved by Mozambican National Committee on Bioethics for Health (CNBS) (reference No: 348/ CNBS/13; IRB00002657).

Figure 1. Mozambique Map, the yellow stars represent the surveillances sites

3.3.4. RNA extraction, cDNA synthesis and genotyping PCR

The RNA extraction, cDNA synthesis and genotyping RT-PCR were performed as described in Chapter 2.

3.3.5. Statistical analysis

For this analysis, the pre-vaccination period was defined as May 2014 to December 2015. The post-vaccination period was defined as January 2016 to December 2018.

The genotyping data from Mavalane General Hospital was analyzed separately from other sites because it was the first sentinel site and, thus, had data since 2014 whilst the other sites only had data since 2015.

The frequencies of identified genotypes were calculated and the unadjusted odds ratios (OR) and their 95% confidence intervals (95CI) were used to assess the magnitude of change in genotype proportions between the pre-vaccine and the post-vaccine periods. All statistical analyses were conducted using Stata software version 15.0 (Stata Corp., College Station, TX, USA). A p-value of < 0.05 was considered statistically significant.

3.4. Results

From May 2014 to December 2018 a total of 1573 diarrheal samples were collected. Of these, 443 (28.1%) were positive for group A rotavirus by ELISA, of which 94.8% (420/443) were genotyped. The 22 (5.0%) samples that could not be genotyped were due to insufficient amount of stool sample for molecular analysis. Among all samples, 246 (58.4%) samples were from the pre-vaccination period (2014 - 2015), and 174 (41.4%) from the post-vaccination period (2016 - 2018) (Table 1).

Table 1. Total number of stool samples collected at sentinel sites in Mozambique during surveillance from 2014 to 2018

Period	Year	Total stool samples	Rotavirus positive		Genotyped samples	
			n	%	n	%
Pre-vaccine	*2014	97	39	40.2	33	84.6
	2015	552	225	40.8	213	94.7
Subtotal	-	649	264	40.7	246	93.2
Post-vaccine	2016	358	41	11.5	39	95.1
	2017	344	104	30.2	104	100.0
	2018	222	34	15.3	31	91.2
Subtotal	-	924	179	19.4	174	97.2
Total	-	1573	443	28.1	420	94.8

*****only HGM has data

3.4.1.Comparison of Rotavirus G- and P-Types in Mozambique Pre- and Post-Vaccine Introduction

The global analysis of all surveillance sites combined, in the pre-vaccination period (2015, n=213), showed that G9 and G1 were the most frequent G types with 49.3% and 31.5%, respectively (Table 2), while in the post-vaccination period (2016-2018, n=174), G9 (28.2%), G1 (22.4%) and G3 (21.8%) were the most commonly circulating G genotypes (Table 2).

Table 2. Frequency of G types in Mozambique during surveillance pre- and post- vaccine introduction

G types	*Pre-vaccine 2015		Post-vaccine 2016-2018		[1]OR (95% CI)	P-value
	n	%	N	%		
G1	67	31.5	39	22.4	0.63 (0.38 - 1.01)	0.04
G12	2	0.9	2	1.1	1.22 (0.09 - 17.08)	0.83
G2	10	4.7	11	6.3	1.37 (0.51 -3.7)	0.48
G3	0	0.0	38	21.8	-	-
G8	0	0.0	3	1.7	-	-
G9	105	49.3	49	28.2	0.40 (0.26 - 0.63)	0.0001
[2]Gx	29	13.6	20	11.5	0.82 (0.42 - 1.57)	0.53
[3]Mix G	0	0	12	6.9	-	-
Total	213	100.0	174	100.0	-	-

[1]Odds-ratio was not calculated in cells with 1 ≥
[2]x: refers to strains that were non-typeable for G
[3]Mix G: 2016-2018 - G12G3P[4] (0.6%),G2G1P[8] (0.6%), G3G1P[8] (2.3%), G9G3P[6] (3.4%)

For P type, P[8] was the most frequent genotype, accounting for 85.4% (Table 3). However, this high frequency was largely reduced in the post-vaccination period when the three human P types, P[4], P[8] and P[6], were recorded at more comparable frequencies: 39.7%, 34.5% and 21.3%, respectively (Table 3).

Table 3. Frequency of P types in Mozambique during surveillance pre- and post- vaccine introduction

P types	Pre-vaccine 2015		Post-vaccine 2016-2018		[1]OR (95% CI)	P-value
	n	%	n	%		
P[4]	0	0.0	69	39.7	–	–
P[6]	10	4.7	37	21.3	5.48 (2.57 – 12.73)	0.0001
P[8]	182	85.4	60	34.5	0.09 (0.053 - 0.15)	0.0001
[2]P[x]	20	9.4	8	4.6	0.47 (0.17 - 1.14)	0,07
Total	213	100.0	174	100.0	–	–

[1]Odds-ratio was not calculated in cells with 1 ≥
[2]x: refers to strains that were non-typeable for P

Mavalane General Hospital was considered the sentinel site model. In the pre-vaccination period (n=110), the most prevalent G types were also G9 and G1 with 53.6% and 30.0%, respectively (Table 4). While in the post-vaccination period (n=35) were genotypes G3 and G1 with 40.0% and 25.7%, respectively (Table 4).

Table 4. Frequency of G types at Mavalane General Hospital pre- and post- vaccine introduction in Mozambique

G types	Pre-vaccine 2014-2015		Post-vaccine 2016-2018		[1]OR (95% CI)	P-value
	n	%	n	%		
G1	33	30.0	9	25.7	0.80 (0.29 - 2.02)	0.62
G12	1	0.91	1	2.9	-	-
G2	2	1.8	1	2.9	-	-
G3	0	0.0	14	40.0	-	-
G8	0	0.0	1	2.9	-	-
G9	59	53.6	4	11.4	0.11 (0.02 - 0.35)	0.0001
[2]Gx	15	13.6	4	11.4	0.81 (0.18 - 2.8)	0.73
[3]Mix G	0	0.0	1	2.9	-	-
Total	110	100.0	35	100.0	-	-

[1]Odds-ratio was not calculated in cells with 1 ≥
[2]x: refers to strains that were non-typeable for G
[3]Mix G: 2016-2018 - G3G12 (2.9%)

P[8] was the most predominant P type (88.2%). And in post-vaccination period a similar proportion of genotypes P[4] (45.7%) and P[8] (40.0%) was observed (Table 5).

Table 5. Frequency of P types at Mavalane General Hospital pre- and post- vaccine introduction in Mozambique

P types	Pre-vaccine 2014-2015		Post-vaccine 2016-2018		[1]OR (95% CI)	P-value
	n	%	n	%		
P[4]	0	0.0	16	45.7	-	-
P[6]	3	2.7	3	8.6	3.3 (0.42 - 25.9)	0.13
P[8]	97	88.2	14	40.0	0.08 (0.03 - 0.23)	0.0001
[2]P[x]	10	9.1	2	5.7	0.60 (0.61 - 3.07)	0.52
Total	110	100.0	35	100.0	-	-

[1]Odds-ratio was not calculated in cells with 1 ≥
[2]x: refers to strains that were non-typeable for P

Analyses for all sentinel sites and Mavalane separately showed a decrease in the odds of G1 genotype from pre-vaccine to the post-vaccine period of 37% (OR = 0.63, 95CI = 0.38 to 1.01; P < 0.04) and 20% (OR = 0.80, 95CI = 0.29 to 2.02, P > 0.62) respectively (Tables 2 and 4). A significant decrease of the odds for G9 genotype was also observed for the analyses including all sites combined at 60% (OR= 0.40, 95CI = 0.26 to 0.63, P < 0.0001) and 89% in Mavalane (OR = 0.11, 95CI = 0.02 to 0.35, P < 0.0001) (Table 2 and 4).

In all sites under surveillance and in Mavalane the odds for genotype P[8] significantly decreased by 91% (OR=0.09, 95CI = 0.05 to 0.15, P < 0.0001 and 92% (OR= 0.08, 95CI= 0.03 - 0.23, P < 0.0001), respectively (Table 3 and 5).

Untypeable strains for G and P types was observed for all sentinel sites as well as in Mavalane General Hospital (Table 3 and 5) in pre- and post-vaccination period.

3.4.2.Comparison of G/P Genotype Combinations in Mozambique Pre- and Post-Vaccine Introduction

The most frequent G/P combinations observed for all surveillance sites combined during the pre-vaccination period were G9P[8] and G1P[8] at 46.0% and 31.0%, respectively. These combinations comprised a total of 76.9% of all genotypes analysed. In the post-vaccination period, G1P[8] remained the most frequent G/P combination, but at a reduced

frequency of 21.3%. Other unusual genotype combinations such as G3P[4] (15.5%), G9P[4] (13.8%) and G9P[6] (9.8%), G3P[8] (4.0%) not detected before were observed. Mixed infections, as determined with RT-PCR, were detected for 6.9% of the samples during the pre-vaccination period (Table 6). Atypical genotypes such as G2P[6] were also detected at lower frequencies during the pre- and post-vaccine period (4.2% VS 4.6%). Additionally, other genotypes such as G12P[4], G1P[4], G1P[6] and G2P[4] were detected during surveillance at a rate ranging 0.3 – 2%.

Table 6. G/P type combinations frequency in Mozambique during surveillance pre and post- vaccine introduction

G/P types combination	Pre-vaccine 2015		Post-vaccine 2016-2018		[1]OR (95% CI)	P-value
	n	%	n	%		
G1P[8]	66	31.0	37	21.3	0.60 (0.37 - 0.98)	0.03
G3P[4]	0	0.0	27	15.5	–	–
G3P[6]	0	0.0	3	1.7	–	–
G3P[8]	0	0.0	7	4.0	–	–
G8P[4]	0	0.0	3	1.7	–	–
G9P[4]	0	0.0	24	13.8	–	–
G9P[6]	0	0.0	17	9.8	–	–
G2P[6]	9	4.2	8	4.6	1.09 (0.36 - 3.27)	0.85
G9P[8]	98	46.0	7	4.0	0.049 (0.02 - 0.11)	0.0001
Untypeable	13	6.1	6	3.4	0.55 (0.17 - 1.59)	0.22
[2]Other genotypes	4	1.9	7	4.0	2.19 (0.54 - 10.36)	0.21
[3]Mixed type	0	0.0	12	6.9	–	
Partial G/P types	23	10.8	16	9.2	0.84 (0.39 - 1.72)	0.60
Total	213	100.0	174	100.0		

[1]Odds-ratio was not calculated in cells with 1 ≥
[2]Other genotypes: 2015 - G12P[8] (0.9%),G1P[6] (0.5%), G2P[4] (0.5%); 2016-2018: G12P[4] (0.6%), G12P[8] (0.6%),G1P[4] (1.1%), G2P[4] (1.7%)
[3]Mixed type: 2016-2018 - G12G3P[4] (0.6%),G2G1P[8] (0.6%), G3G1P[8] (2.3%), G9G3P[6] (3.4%)

For Mavalane the most predominant combinations in pre-vaccination period were also G9P[8] (50.9%) and G1P[8] (30.0%), comprising a total of 80.9 % of all genotypes analysed. And in the post-vaccination period the genotypes G1P[8] and G3P[4] were the most predominant, both with 22.9.% (Table 7). Similar to the rest of the country, combinations such as G3P[8] (11.4%), G9P[4] and G9P[6] both with (5.4%) not detected before were observed. Mixed infections were detected at 2.9% (Table 7).

Table 7. G/P type combinations frequency at Mavalane General Hospital pre- and post-vaccine introduction in Mozambique

G/P types combination	Pre-vaccine 2014-2015		Post-vaccine 2016-2018		[1]OR (95% CI)	P-value
	n	%	n	%		
G1P[8]	33	30.0	8	22.9	0.69 (0.24 - 1.78)	0.41
G3P[4]	0	0.0	8	22.9	–	–
G3P[6]	0	0.0	1	2.9	–	–
G3P[8]	0	0.0	4	11.4	–	–
G8P[4]	0	0.0	1	2.9	–	–
G9P[4]	0	0.0	2	5.7	–	–
G9P[6]	0	0.0	2	5.7	–	–
G2P[6]	2	1.8	0	0.0	–	–
G9P[8]	56	50.9	1	2.9	–	–
Untypeables	7	6.4	2	5.7	0.89 (0.86 - 5.00)	0.88
[2]Other genotypes	1	0.9	3	8.6	3.3 (0.42 - 25.00)	0.13
[3]Mixed type	0	0.0	1	2.9	–	–
Partial G/P types	11	10.0	2	5.7	0.54 (0.05 - 2.70)	0.43
Total	110	100.0	35	100.0	–	–

[1]Odds-ratio was not calculated in cells with 1 ≥
[2]Other genotypes: 2015 - G12P[8] (0.9%), G2P[6]; 2016-2018:G12P[4], G1P[4] (2.9%), G2P[4] (2.9%)
[3]Mixed type: 2016-2018 - G12G3P[4] (2.9%)

Analyses for all sentinel sites, showed a decrease of odds of G1P[8] in 40% (OR = 0.60, 95CI = 0.37 to 0.98, P < 0.03) and G9P[8] in 95.1% (OR = 0.049, 95CI = 0.02 to 0.11, P < 0.0001) . In Mavalane a decreased odds ratio for G1P[8] in 31% (OR = 0.69, 95CI = 0.24 to 1.78, P > 0.41) was also observed (Table 6 and 7).

3.4.3.Distribution of rotavirus genotypes by year

In the combined data for all sites, in 2015 the most frequent G/P combination was G9P[8] (46.0%) while in 2016 G1P[8] (43.6%) was the most frequent G/P combination detected (Table 8). The emergence of new genotype combinations, such as G9P[6] (12.8%) and G9P[4] (7.7%) was observed. In 2017, two genotype, G1P[8] and the emerging combination G9P[4], both at a frequency of 19.2%, was observed as the most common G/P combinations. In the same year the emergence of G3P[4] at 13.5% was also observed (Table 8). In 2018, the G3P[4] became the most frequent genotype combination (37.5%) and was also observed in combination with P[8] (21.9%) and P[6] (3.1%) (Table 8).

Table 8. Frequency of G/P type combinations in Mozambique during surveillance by year

G/P types combination	Pre-vaccine		Post-vaccine					
	2015		2016		2017		2018	
	n	%	n	%	n	%	n	%
G1P[8]	66	31.0	17	43.6	20	19.2	0	0.0
G3P[4]	0	0.0	1	2.6	14	13.5	12	37.5
G3P[6]	0	0.0	0	0.0	2	1.9	1	3.1
G3P[8]	0	0.0	0	0.0	0	0.0	7	21.9
G8P[4]	0	0.0	0	0.0	1	1.0	2	6.3
G9P[4]	0	0.0	3	7.7	20	19.2	1	3.1
G9P[6]	0	0.0	5	12.8	9	8.7	3	9.4
G2P[6]	9	4.2	7	18.0	0	0.0	1	3.1
G9P[8]	98	46.0	0	0.0	6	5.8	1	3.1
Untypeables	13	6.1	1	2.6	5	4.8	0	0.0
[1]Other genotypes	4	1.9	3	7.7	3	2.9	1	3.1
[2]Mixed type	0	0.0	2	5.1	10	9.6	0	0.0
Parcial G/P types	23	10.7	0	5.1	14	11.5	2	6.3
Total	213	100.0	39	100.0	104	100.0	31	100.0

[1]Other genotypes: 2015 - G12P[4] (2.6%), G12P[8] (0.9%), G1P[6] (0.5%) G2P[4] (0.5%)
[2]Mixed type: 2016 - G12G3P[4] (2.6%), G2G1P[8] (2.6%); 2017: G3G1P[8] (3.9%), G9G3P[6] (5.8%)

In Mavalane, similar results were observed, with the exception of 2014. In 2015 and 2016, the most frequent genotype combination was G9P[8] (73.7%), and G1P[8] (66.7%) respectively. The emergence of genotypes was observed mostly from 2017, where G3P[4] was the most prevalent (25.0%) and G1P[8] was the second most common (18.8%). In 2018, G3P[8] and G3P[4] became the most prevalent genotype combinations with 33.3% and 25.0%, respectively, and G1P[8] was not detected (Table 9).

Table 9. Frequency of G/P type combinations at Mavalane General Hospital in Mozambique by year

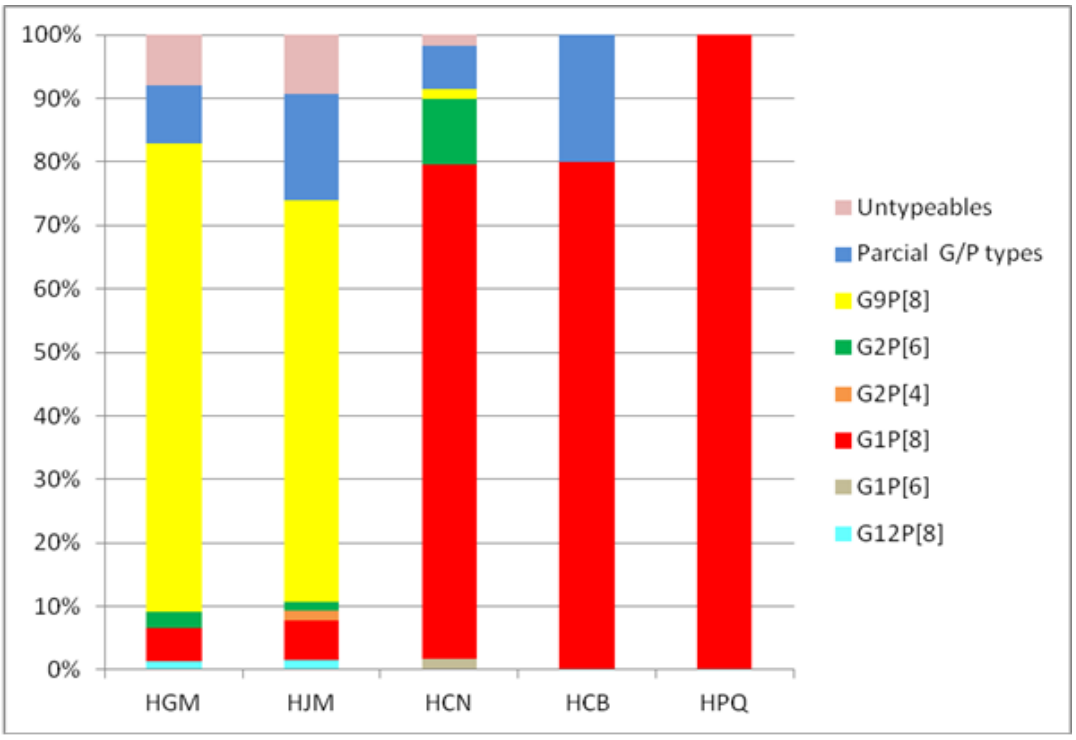
G/P types combination	Pre-vaccine						Post-vaccine			
	2014		2015		2016		2017		2018	
	n	%	n	%	N	%	n	%	n	%
G1P[8]	28	84.8	4	5.3	6	66.7	3	18.8	0	0.0
G3P[4]	0	0.0	0	0.0	1	11.1	4	25.0	3	25.0
G3P[6]	0	0.0	0	0.0	0	0.0	1	6.3	0	0.0
G3P[8]	0	0.0	0	0.0	0	0.0	0	0.0	4	33.3
G8P[4]	0	0.0	0	0.0	0	0.0	0	0.0	1	8.3
G9P[4]	0	0.0	0	0.0	0	0.0	2	12.5	0	0.0
G9P[6]	0	0.0	0	0.0	0	0.0	0	0.0	2	16.7
G2P[6]	0	0.0	2	2.6	0	0.0	0	0.0	0	0.0
G9P[8]	0	0.0	56	73.7	0	0.0	1	6.3	0	0.0
Untypeables	1	3.0	6	7.9	0	0.0	2	12.5	1	8.3
[1]Other genotypes	0	0.0	1	1.3	1	11.1	1	6.3	1	8.3
[2]Mixed type	0	0.0	0	0	1	11.1	0	0.0	0	0.0
Partial G/P types	4	12.1	7	9.2	0	0	1	6.3	0	0.0
Total	33	100.0	76	100.0	9	100.0	16	100.0	12	100.0

[1]Other genotypes: 2015-G12P[8] (1.3%), 2016: G12P[4] (11.1%); 2017: G1P[4] (6.3%); 2018: G2P[4] (8.3%)
[2]Mixed type: 2016 - G12G3P[4] (11.1%)

3.4.4. Geographical distribution of rotavirus genotypes

In general, G1P[8] was detected in all regions during the surveillance period and the emerging genotype combinations such as G9P[4], G9P[6], G3P[4] and G3P[8] were observed in all regions after vaccine introduction, except G3P[8] that was not detected in the north region (Figure 1A and B).

A



B

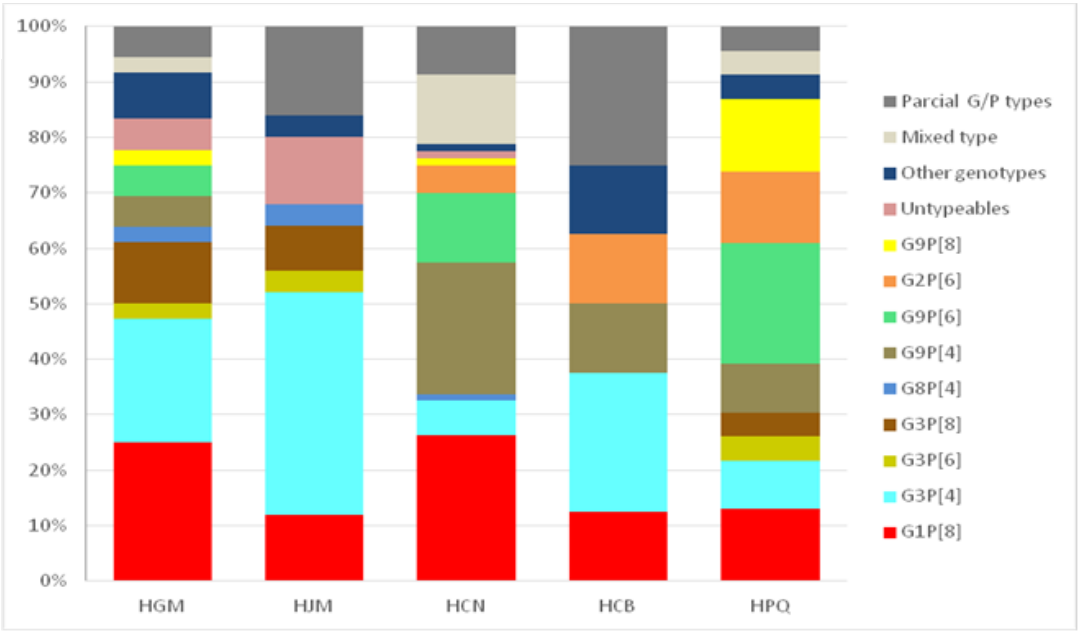


Figure 1. Distribution of genotypes by sites in pre-vaccine (A) and post-vaccine period (B) among surveillance sites in Mozambique. Mavalane General Hospital (HGM), José Macamo General Hospital (HJM), Nampula Central Hospital (HCN), Quelimane Provincial Hospital (HPQ), Beira Central Hospital (HCB).

3.5. Discussion

To our knowledge, this is the first report in Mozambique that analyses the rotavirus genotypes circulating across the country, providing evidence of high genetic diversity and time variation in genotypes circulating over the years.

The most common worldwide strains combinations were G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8] [Banyai et al., 2012]. In Mozambique, 57.4% of the analyzed samples in the present study contained the same strains. The 37.0% of uncommon strains such as G4P[6], G8P[6], G5P[8] and G3P[9] had been previously reported in Africa [Todd et al., 2010]. In the surveillance analysed here were detected further and uncommon combination such as G9P[4], G9P[6] and G3P[4].

We observed that the proportion of G1 and G9 and of P[8], P[4] and P[6] varied between pre and post-vaccination periods. The decreased proportion of genotypes G1, G9, and P[8], was accompanied by an increase of the proportion of genotypes G3, P[4] and P[6]. These results were similar in the combined analyses for all sites under surveillance and also in the analysis of Malavane site alone. These results suggest that genotype prevalence can vary from year to year pre- and post-vaccination periods in Mozambique.

When comparing the most predominant G/P combinations between pre and post-vaccine introduction, G9P[8] was the most predominant in the pre-vaccination period, while G1P[8] was most prevalent genotype combination in the post-vaccination period. However, the surveillance also showed decreased odds of G1P[8] post-vaccine introduction accompanied with emergence of genotype combinations that were not observed before, such as G3P[4], G9P[4], G9P[6] and G3P[8]. Additionally, mixed infections were observed only in the post-vaccination period. These results showed that in this early phase of rotavirus strain surveillance, it is not clear whether these variations in genotype combinations between both periods were due to the rotavirus vaccine or simply natural variation in genotype frequency. Our results are consistent with previously published studies, as a number of countries from Africa, Europe and America reported a variation in the strain diversity between the two periods [Bar-Zeev et al., 2016; Hungerford et al., 2019; Lartey et al., 2018; Luchs et al., 2015; Matthijnssens et al., 2014; Page et al., 2017; Seheri et al., 2017].

Countries that introduced the monovalent *Rotarix*® vaccine similar to Mozambique, reported a decline of genotype G1P[8] with a concurrent rise in other combinations in the post-vaccination period. For example, South Africa reported an increase in non-G1P[8] strains [Page et al., 2017]. In contrast, in Malawi, the reduction of G1P[8] was not significant [Bar-Zeev et al., 2016]. In Ghana, G1P[8] returned as one of the dominant strains in the fourth year post-vaccine introduction [Lartey et al., 2018]. In other studies, reported from England, Brazil, Belgium and Scotland, a decline in the proportion of G1P[8] with a rise in the proportion of heterotypic strains, such as G2P[4], was observed [Hungerford et al., 2019a; Luchs et al., 2015; Matthijnssens et al., 2014; Mukhopadhya et al., 2016].

Regarding the variation in the prevalence of some uncommon genotypes (e.g., G9P[4] G9P[6], G3P[4], G3P[6]) detected post-vaccine introduction in Mozambique, it is important to mention that a number of studies in Africa [Cunliffe et al., 2010; Page et al., 2017; Seheri et al., 2017] and Asia (India and Japan) also reported these uncommon genotypes before vaccine introduction in low frequency [Giri et al., 2019; Yamamoto et al., 2015]. However, a study conducted in Ghana reported the emergence of G9P[4] at a low frequency only during the fourth rotavirus season after vaccine introduction [Lartey et al., 2018].

The emergence of the genotype combinations G3P[4], detected in 2016, 2017, 2018, and G3P[8] in 2018 was observed in Mozambique. These strains were also reported in the same period in Botswana after vaccine introduction in 2012 [Mokomane et al., 2019]. In addition, several countries reported G3 in combination with P[4] and P[8] during the 12th African Rotavirus Symposium 2019 (http://ars.samrc.ac.za/programme.htm): Malawi (introduced vaccine in 2012, reported G3P[8] in 2018), South Africa (introduced vaccine in 2009, reported G3P[4] in 2015–2016), Kingdom of Eswatini (introduced vaccine in 2015, reported G3P[8] in 2018). These observations suggest that G3 strains were circulating in Southern Africa during 2015–2018, with a sharp increase in 2018. Around the world, the emergence of genotype G3P[8] and equine-like G3P[8] in 2013 in Australia and re-emergence of G3P[8] were observed in Brazil in the post-vaccine introduction [Carvalho-Costa et al., 2019; Cowley et al., 2015; Roczo-Farkas et al., 2018]. The European Rotavirus

Network (EuroRotaNet) reported 2017–2018 for the first time since inception, G3P[8] as the most prevalent strain [Hungerford, 2019b].

Temporal variation of rotavirus strains was observed in Mozambique, in particular in the Mavalane sentinel site, which is a model site, because data is available from 2012 to 2018. The baseline data from the cross-sectional study in urban and rural areas showed circulation of G12P[6] in 2012, and G2P[4] in 2013 [Joao et al., 2018]. In the period studied after the introduction of national surveillance, in 2014 and 2015, mostly G1P[8] and G9P[8] strains were detected. In the post-vaccine period (2016-2018), G9P[8] was replaced by G1P[8] in 2016, while in 2017, G1P[8] was detected in similar proportion with G3P[4]. Finally, in 2018 G1P[8] strains were not detected; instead only genotypes G3P[8] and G3P[4] were detected. These results confirmed the yearly variations of rotavirus strains over time reported by several studies globally [Banyai et al., 2012; Mwenda et al., 2010; Page et al., 2017; Seheri et al., 2017]. The variations can be explained by natural rotavirus fluctuation or by vaccine pressure in Mozambique as speculated in other countries such as Brazil, South Africa, Australia and Belgium [Banyai et al., 2012; Carvalho-Costa et al., 2019; Hungerford et al., 2019; Luchs et al., 2015; Matthijnssens et al., 2012a; Page et al., 2017; Roczo-Farkas et al., 2018].

In the post vaccination period it was observed, in general, an increase in strains that are probably the result of reassortment events such as G9P[4], G3P[4] [Matthijnssens and Van Ranst, 2012]. These reassortment events had previously been reported in Mozambique by Joao et al., (2018) and were confirmed through full genome characterisation [Strydom et al., 2019a]. In 2018, G1P[8] was not detected in Mozambique, but it is important to monitor its possible re-emergence later on. In Malawi, the circulation of G1P[8] with DS1-like backbone was reported after introduction of monovalent rotavirus vaccine [Jere et al., 2018].

Interestingly, the geographical distribution of rotavirus strains from sites under surveillance showed consistence of circulation of genotype G1P[8] across sites from different regions in the country. In general, it was observed in all regions under surveillance the circulation of emerged genotype combinations G9P[4], G9P[6] and G3P[4], suggesting that the increase

of new genotype combinations was not particularly localized in specific regions after vaccine introduction but country wide.

In addition, other atypical combinations, such as G2P[6], G1P[4], G1P[6], were also detected in low proportion as reported previously in African Rotavirus Surveillance Network [Seheri et al., 2017].

Various challenges and limitations were experienced during the study. These include logistical issues, which led to a delay in the start of surveillance at some sentinel sites. The study was limited by its small sample size; therefore, it was not possible to perform in-depth temporal analyses by site to assess the genetic variability of strains. Furthermore, bias in strain diversity is possible since a low number of strains were characterized at some sentinel sites. Extended pre-vaccine genotyping data (two years) was available for only one sentinel site, whereas only one year genotyping data were available for the remaining of the sentinel sites.

3.6. Conclusions

This is the first countrywide report showing a high diversity of rotavirus genotypes circulating in Mozambique including common and uncommon strains over time. Our results suggest changes of rotavirus genotypes over time, as previously reported by several studies globally. The emergence of new rotavirus strains in the post-vaccination period, supports the need for continuous surveillance across the country to understand if the emergence of new strains is due to vaccine derived and their impact in human health.

CHAPTER 4 Rotavirus A whole genome characterization of G1P[8] and G9P[8] Wa-like strains detected in Mozambique between 2014 and 2016

4.1. Introduction

The genotypes, G1P[8], G3P[8], G4P[8], G9P[8] and G12P[8] of RVA are the most common in circulation worldwide [Banyai et al., 2012; Gentsch et al., 2005; Todd et al., 2010]. These genotypes are associated predominantly with Wa-like genome backbone (-I1-R1-C1-M1-A1-N1-T1-E1-H1). The Rotarix® vaccine possess a Wa-like genome backbone [Matthijnssens and Van Ranst, 2012]. In humans, the Wa-like genome backbone is the most important and over 90% of all infections are caused by rotaviruses belonging to this backbone [Matthijnssens and Van Ranst, 2012]. Uncommon genotype combinations such as G5P[8], G10P[8], G11P[8] have been described, also with Wa-like genome backbones [Matthijnssens et al., 2008b; Matthijnssens and Van Ranst, 2012].

In recent years, several studies have reported genotypes typically associated with Wa-like genome backbone presenting DS-1-like genome backbone. Reports from Asia described a G1P[8] with a DS1-like genome backbone in Japan [Komoto et al., 2015; Kuzuya et al., 2014; Yamamoto et al., 2014], Thailand [Komoto et al., 2015] and Vietenam [Nakagomi et al., 2017]. Such strains have also been detected in Malawi [Jere et al., 2018]. Genotype G3P[8] genotype were also reported with DS1-like genome backbone in Japan [Komoto et al., 2018].

In Mozambique full genome characterization of G12P[6] and G12P[8] were detected with a typical Wa-like genome backbone in strains circulated in 2012. However, one sample contained a mixture of a G12P[8] Wa-like strain and a GXP[14] DS-1-like strain [Strydom et al., 2019b]. The same study also suggested that VP4 and VP7 encoding genes of the G12P[6] strains were reassortants containing backbones that are similar to that of the G12P[8] strain [Strydom et al., 2019b].

To our knowledge, in Mozambique there are no full genome sequences for G1P[8] and G9P[8] strains describing the genome backbone thereof being important to characterize these strains.

4.2. Objectives

- Characterize the complete genome of G1P[8] strains detected in 2014 and 2016 in Maputo , Nampula and Quelimane city;
- Characterize the complete genome of G9P[8] strains detected in 2015 in Maputo city;
- Compare the antigenic regions of outer proteins (VP7 and VP4) of Mozambican G1 and P[8] strains in relation to the Rotarix® vaccine strain.

4.3. Materials and Methods

In this Chapter the main activities done by the student were:

- Selection of rotavirus strains for whole genome characterization in Mozambique;
- RNA extraction, primer ligation and cDNA preparation in South Africa;
- Prepararation of libraries and other procedures for NGS was done by the UFS NGS Unit, and the student only observed;
- Data analysis was done by the student, after training in South Africa.

4.3.1. Rotavirus strains

Chapter 3 reported the RVA genotypes identified among years based on the National Acute Diarrheal Surveillance. Selected samples were shipped in dry ice to the University of the Free State in South Africa for full genome analyses at Next Generation Sequencing Unit.

The G1P[8] genotype combination in pre-vaccine and post vaccine period was detected in 30.9% and 21.3% respectively. And the G9P[8] genotype combination in pre and post vaccine period was detected in 46.0% and 4.0%, respectively.

The representative RVA strain genotypes were selected based on following criteria:

- Type and frequency of genotypes detected;
- Period of detection of genotypes (prior and post vaccine introduction);
- Samples from different regions under surveillance in Mozambique;
- Have appropriate amount of sample to repeat all tests;
- Amount of RNA available per sample and quality of the RNA.

In total 12 samples were selected, of which, three were from Jose Macamo General Hospital (HJM) and five from Mavalane General Hospital (HGM), both hospitals located in Maputo, southern region; three samples were from Nampula Central Hospital (HCN) in Nampula city (north region) and one sample was from Quelimane Provincial Hospital (HPQ) in Quelimane (center region) (Table 1).

Table 1. List of strains selected for whole genome sequencing

Sample	Year of collection	Hospital-City	Genotyping by PCR
48	2014	Mavalane General Hospital-Maputo	G1P[8]
59	2014	Mavalane General Hospital-Maputo	G1P[8]
154	2015	Nampula Central Hospital-Nampula	G1P[8]
338	2015	J. Macamo General Hospital-Maputo	G1P[8]
1336	2016	Nampula Central Hospital-Nampula	G1P[8]
1265	2016	Mavalane General Hospital-Maputo	G1P[8]
1152	2016	Quelimane Provincial Hospital-Quelimane	G1P[8]
1011	2016	Nampula Central Hospital Nampula	G1P[8]
353	2015	Mavalane General Hospital-Maputo	G9P[8]
389	2015	Mavalane General Hospital-Maputo	G9P[x]
643	2015	J. Macamo General Hospital-Maputo	G9P[8]
644	2015	J. Macamo General Hospital-Maputo	G9P[x]

4.3.2. Ethical approval

The study was approved by the National Committee of Bioethics from Mozambique in 2013 (IRB 0000265, reference No: 348/CNBS/13).) and by the Health Sciences Research Ethics Committee of the University of Free State (ECUFS NR 201/2013).

4.3.3. Extraction of dsRNA and cDNA synthesis for rotavirus whole genome

Total viral RNA was extracted from stool samples with TRI-reagent (Sigma, Germany) and single-stranded RNA was precipitated with lithium chloride (Sigma, Germany) to remove single strand RNA as described previously [Potgieter et al., 2009]. To obtain a full length nucleotide sequences of genes segments encoding VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 an anchor primer (PC3-T7loop; Integrated DNA Technologies) was ligated to the double strand RNA (dsRNA) in all samples as previously described before [Potgieter et al., 2009]. The dsRNA was analysed on a 1% agarose gel for verification of segments and purified with MinElute Gel Extraction kit, according to the manufacturer's instructions (Qiagen, United States).

Complementary DNA (cDNA) was synthesised using the Maxima H Minus Double Stranded cDNA kit (ThermoFisher Scientific, United States). The manufacturer's instructions were followed with minor modifications. Briefly, the dsRNA was denatured at

95 ℃ for 5 minutes and then, the first strand cDNA synthesis which includes 1 µl random hexamer primer, 5 µl 4X first strand reaction mix and 1 µl first strand enzyme mix was carried out for 25 ºC for 10 min and two hours at 50 ºC. The reaction was terminated by heating at 85 ℃ for 5 minutes. The second strand cDNA synthesis continued immediately and was added previous mixture reaction, 55 µl of nuclease-free water, 20 µl 5X second strand reaction mix, 5 µl second strand enzyme mix and was incubated at 16 ℃ for 60 minutes. The reaction was stopped by adding 6 µl 0.5 M EDTA, pH 8.0, and was added 1 or 2 µl of RNase to remove residual RNA from cDNA preparation. The cDNA was purified by Invisorb Fragment CleanUp kit, according to the manufacturer's instructions (Stratec Molecular, United States).

4.3.4. cDNA library construction and Illumina MiSeq sequencing

The cDNA was quantified with Qubit using a dsDNA High Sensitivity Assay kit according to the manufacturer's instructions. Only samples that had concentration of ≥ 2 ng/µl were used. Samples with high concentration of dsDNA were diluted to 0.3 ng/ µl and normalized to 2 nM. The library was prepared using Nextera XT DNA Library Preparation kit (Illumina, Inc.) following the manufacturer's instructions. Briefly, the genomic DNA were tagged, fragmented and adapter sequences (P5 and P7) were added to the ends simultaneously by Nextera XT transposome and incubated at 55 ℃ for 5 minutes. Tagmented DNA were submitted to a PCR amplification, using Nextera PCR master mix, index 1 (i7) and index 2 (i5) primers and sequencing primer. The PCR was carried out in follow conditions: 72 ℃ for 3 minutes, 95 ℃ for 30 seconds and 12 cycles of: 95 ℃ for 10 seconds, 55 ℃ for 30 seconds , 72 ℃ for 30 seconds and 72 ℃ for 5 minutes . The PCR products were purified using Ampure XP beads (Illumina, Inc.) and fresh 80% ethanol. After clean up, the library DNA was quantified and validated using a Bioanalyzer 2100 (Agilent Technologies, United States).

Equal volumes of normalized library were combined, denatured with NaOH prior to MiSeq sequencing and diluted to 8 pM in hybridization buffer. Finally, the pooled libraries were loaded and sequenced using MiSeq Reagent Kit V3 (600 cycles) with 301 bp paired-end

reads in the MiSeq system. The pooled libraries each had a different index in order to separate the data again at the end of run.

4.3.5. Sequence assembly and determination of rotavirus genotypes

The sequence reads from Illlumina MiSeq were assembled in CLC Genomics Workbench version 9.0 (Qiagen). The reads were trimmed, assembled by denovo assembly and contigs were identified in BLAST. The trimmed reads were then assembled in reference mapping and results from denovo and reference mapping were compared to confirm the correct consensus was determined for each segment. Specific genotype data results were obtained from the Pathogen Database and Analysis Resource tool (ViPR).

4.3.6. Sequence alignments and Maximum Likelihood phylogeny construction

The multiple sequence alignment of the study strains and reference strains, obtained from GenBank was done with the MUSCLE algorithm in MEGA version 7.2.1 [Kumar et al., 2016]. The DNA Model Test program implemented in MEGA was used to identify the optimal evolutionary models that best fit the sequence datasets of all of rotavirus genome segments. The following models were determined for ORF of the eleven genes encoding proteins: NSP1, VP2, VP3 (GTR+G+I), NSP2, NSP4, NSP5, VP4-P[8], VP7-G1, VP7-G9 (T92+G); NSP3, VP6 (T92+G+I); VP1 (TN93+G+I). Maximum Likelihood trees were constructed in MEGA program above mentioned with 500 bootstrap replicates to estimate branch support.

Nucleotide distance matrixes based on pairwise comparisons were analyzed using the p-distance algorithm in MEGA. In order to understand whole genomic relatedness of the Mozambican G1P[8] and other closest G1P[8] for which the full genome data are available in GenBank, 22 strains (only closest strains in phylogenetic analyses of individual rotavirus genome segments) were analyzed by phylogenetic tree constructed using the concatenated ORF nucleotide sequences for each strain. Strain 0059 was excluded since there was not a complete data set for the 11 genome segments. The best model used to construct the phylogenetic tree was GTR+G+I. The alignment and concatenation was done in BioEdit Program version 7.1.9. [Hall, 1999].

4.3.7. VP7 and VP4 rotavirus antigenic analyses

Amino acid sequences were aligned with MUSCLE in MEGA program version 7.2.1 and epitopes in proteins VP7 and VP4 were identified as described elsewhere [Aoki et al., 2009; Dyall-Smith et al., 1986; Kirkwood et al., 1993; Magagula et al., 2015; Zeller et al., 2012]. Amino acid sequences of the Mozambican VP7 (for G1P[8] strains) and VP4 (for G1P[8] and G9P[8] strains were compared to vaccine strain Rotarix® (A41CB052A) G1P[8].

4.4. Results

4.4.1. Genotype constellation of G1P[8] and G9P[8]Mozambican strains

The paired end reads trimmed data for each strain ranged from 82.098 to 36,405.94 reads, and the average coverage of segments per strain from 229 to 47.414 (Supplementary Table 1). Eight G1P[8] strains [RVA/Human-wt/MOZ/HGM0048/2014/G1P[8] (HGM0048), RVA/Human-wt/MOZ/HGM0059/2014/G1P[8] (HGM0059), RVA/Human-wt/MOZ/HCN154/2015/G1P[8] (HCN154), RVA/Human-wt/MOZ/HJM338/2015/G1P[8] (HJM338), RVA/Human-wt/MOZ/HCN1336/2016/G1P[8] (HCN1336), RVA/Human-wt/MOZ/HGM1265/2016/G1P[8] (HGM1265), RVA/Human-wt/MOZ/HPQ1152/2016/G1P[8] (HPQ1152), RVA/Human-wt/MOZ/HCN1011/2016/G1P[8] (HCN1011)] and four G9P[8] [RVA/Human-wt/MOZ/HGM353/2015/G9P[8] (HGM353), RVA/Human-wt/MOZ/HJM643/2015/G9P[8] (HGM643), RVA/Human-wt/MOZ/HJM644/2015/G9P[8] (HJM644), RVA/Human-wt/MOZ/HGM389/2015/G9P8 (HGM389)] strains had a typical Wa-like genetic backbone for all 9 segments (-I1-R1-C1-M1-A1-N1-T1-E1-H1) (Table 2). For only one strain G1P[8] (RVA/Human-wt/MOZ/HGM0059/2014/G1P[8] [(HGM0059)]) it was not possible to determine VP2 and VP3 and presented with a -I1-R1-Cx-Mx-A1-N1-T1-E1-H1 backbone (Table 2).

Table 2. Genome constellations of human G1P[8] and G9P[8] rotavirus strains circulating in Mozambique between 2014 and 2016

		Proteins* % Identity in ViPR										
Strain name	**Genotype by PCR**	**VP7**	**VP4**	**VP6**	**VP1**	**VP2**	**VP3**	**NSP1**	**NSP2**	**NSP3**	**NSP4**	**NSP5/6**
Genetic segment		9	4	6	1	2	3	5	8	7	10	11
RVA/Human-wt/MOZ/HGM0048/2014/G1P[8]	G1P[8]	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
		93.3	97.2	99.0	98.8	98.5	98.0	97.7	98.4	97.5	98.7	99.2
RVA/Human-wt/MOZ/HGM0059/2014/G1P[8]	G1P[8]	G1	P[8]	I1	R1	Cx	Mx	A1	N1	T1	E1	H1
		93.3	97.2	99.0	98.7	_	_	97.7	98.5	97.4	98.7	99.2
RVA/Human-wt/MOZ/HCN154/2015/G1P[8]	G1P[8]	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
		93.0	98.7	99.0	98.7	99.0	98.0	97.7	98.6	97.6	98.7	99.0
RVA/Human-wt/MOZ/HJM338/2015/G1P[8]	G1P[8]	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
		93.2	97	99.0	98.6	98.4	98.0	97.6	98.4	97.0	98.5	99.2
RVA/Human-wt/MOZ/HCN1336/2016/G1P[8]	G1P[8]	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
		97.3	97.8	99.0	98.5	98.3	97.8	97.5	98.4	97.4	98.5	99.2
RVA/Human-wt/MOZ/HGM1265/2016/G1P[8]	G1P[8]	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
		93.0	97.0	99.0	98.5	98.3	97.7	97.5	97.3	98.3	98.5	99
RVA/Human-wt/MOZ/HPQ1152/2016/G1P[8]	G1P[8]	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
		93.2	97	99.0	98.6	98.4	97.8	97.7	98.4	97.3	98.5	98.6
RVA/Human-wt/MOZ/HCN1011/2016/G1P[8]	G1P[8]	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
		93.4	97.0	99.0	98.5	98.4	98.0	97.5	98.6	97.4	98.5	99.2
RVA/Human-wt/MOZ/HGM353/2015/G9P[8]	G9P[8]	G9	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
		93.0	97.6	98.4	98.8	97.8	97.8	97.7	98.8	99.4	98.3	99.5
RVA/Human-wt/MOZ/HGM389/2015/G9P8	G9P[x]	G9	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
		93.0	97.5	98.3	98.8	97.8	97.8	97.7	99.0	99.2	98.3	99.5
RVA/Human-wt/MOZ/HJM643/2015/G9P[8]	G9P[8]	G9	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
		92.8	97.5	98.2	98.8	97.8	97.8	97.7	99.0	99.2	98.0	99.5
RVA/Human-wt/MOZ/HJM644/2015/G9P[8]	G9P[x]	G9	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
		93.5	89.2	99.0	99.0	98.3	98.2	98.2	99.0	99.0	99.0	99.8

Legend to Table 2: *VP- structural protein; NSP- nonstructural protein; Wa-backbone genotypes are indicated in green; Cx and Mx- were not possible to determine these genotypes. Nucleotide identities were determined in ViPR.

4.4.2. Phylogenetic analyses

Maximum likelihood trees based on phylogenetic analysis of complete reading frame nucleotide sequences of 11 genome segments of Wa-like G1P[8] strains from 2014 and 2016 and Wa-like G9P[8] strains from 2015, were constructed (Figure 1A-L). One G12P[8] and four G12P[6] Mozambican strains from another study with Wa-like backbone were included in the analysis.

4.4.2.1. VP7 encoding nucleotide sequences (genome segment 9, genotype genotypes G1 and G9)

In general, all eight Mozambican G1 strains detected between 2014 and 2016 had a nucleotide identity ranging from of 99.18 and 100% between them (Supplementary Table 2I). The strains from 2014 all presented with a 100% identity (Supplementary Table 2I). Phylogenetic analysis of G1 strains (Figure 1A) was grouped in seven lineages [Arista et al., 2006; Magagula et al., 2015]. All strains from Mozambique grouped together and clustered in lineage II. The closest relatives were human strains from India (RVA/Human/IND/RV1305/2013/G1P8, RVA/Human/IND/ RV1327/2013/G1P8, RVA/Human/IND/RV1302/2013/G1P8 and one G1 bovine strain (RVA/Bovine/IND/HR//B91/2011/G1Px) (Figure 1A, Supplementary Table 2I).

The four Mozambican G9 strains had high identity among them. However, strains HGM353, HGM389, HJM643 were more closely related (99.69 to 99.80%) (Supplementary Table 3I) and also clustered together in the phylogenetic analysis (Fig. 1B). Strain HJM644 grouped separately with an identity of 99.08 - 99.18% (Supplementary Table 3I). The G9 strains clustered in monophyletic group at lineage III [Dian et al., 2017] that included strains from South African, Japan, China, United States and Zimbabwe from 2013 and 2016. However, three G9 strains (HGM353, HGM389 and HJM643) grouped in a small cluster with Chinese strain RVA/Human-wt/CHN/km15105/2015/G9P8 and strain HJM644 grouped separately with RVA/Human-wt/CHN/Hu/JS2015/2015/G9P8, RVA/Human-wt/JPN/MI1128/2016/G9P8, RVA/Human-wt/JPN/IS1080/2016/G9P8 and RVA/Human-wt/JPN/CH1023/2016/G9P8 (Figure 1B, Suplementary Table 3I).

A: VP7 (genome segment 9) -genotype G1

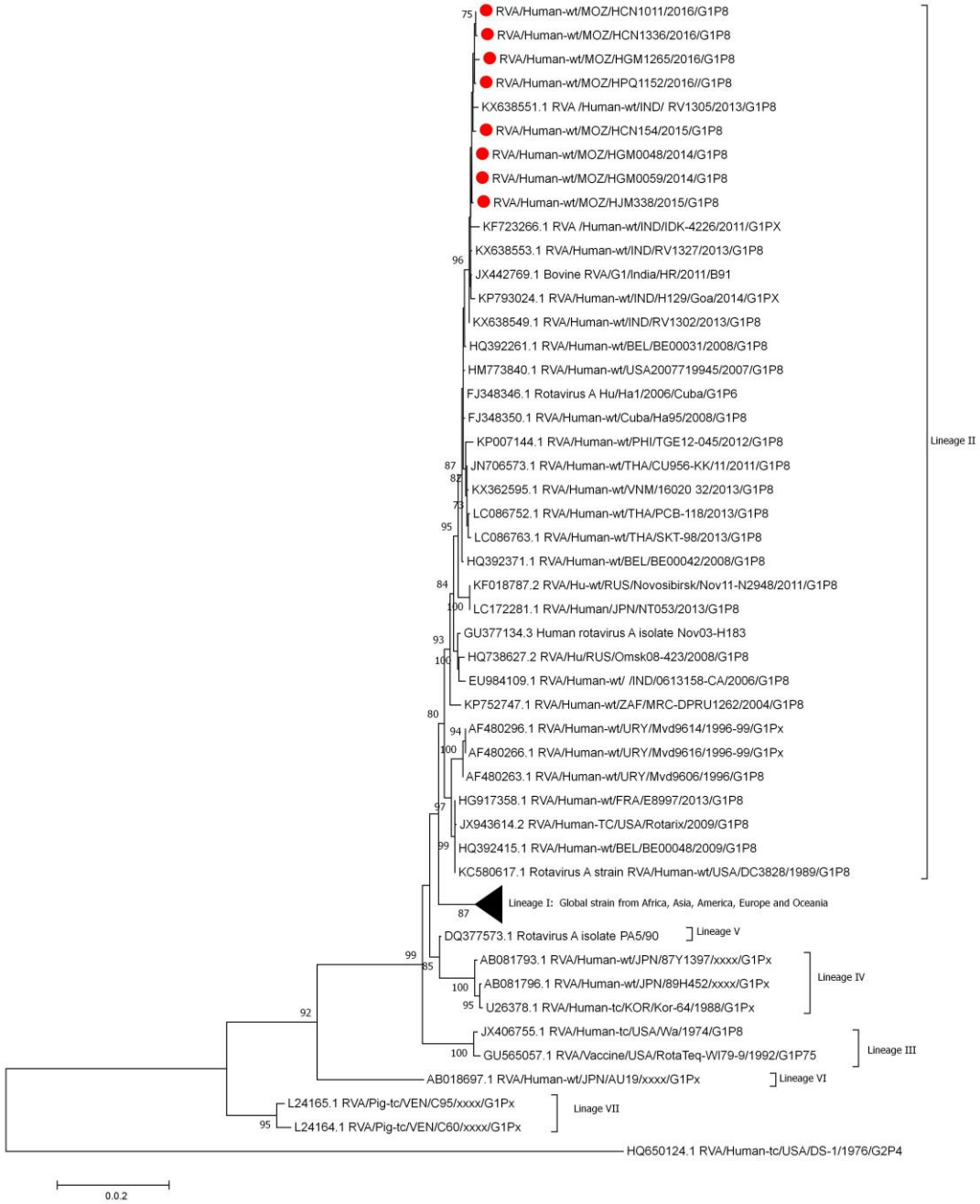
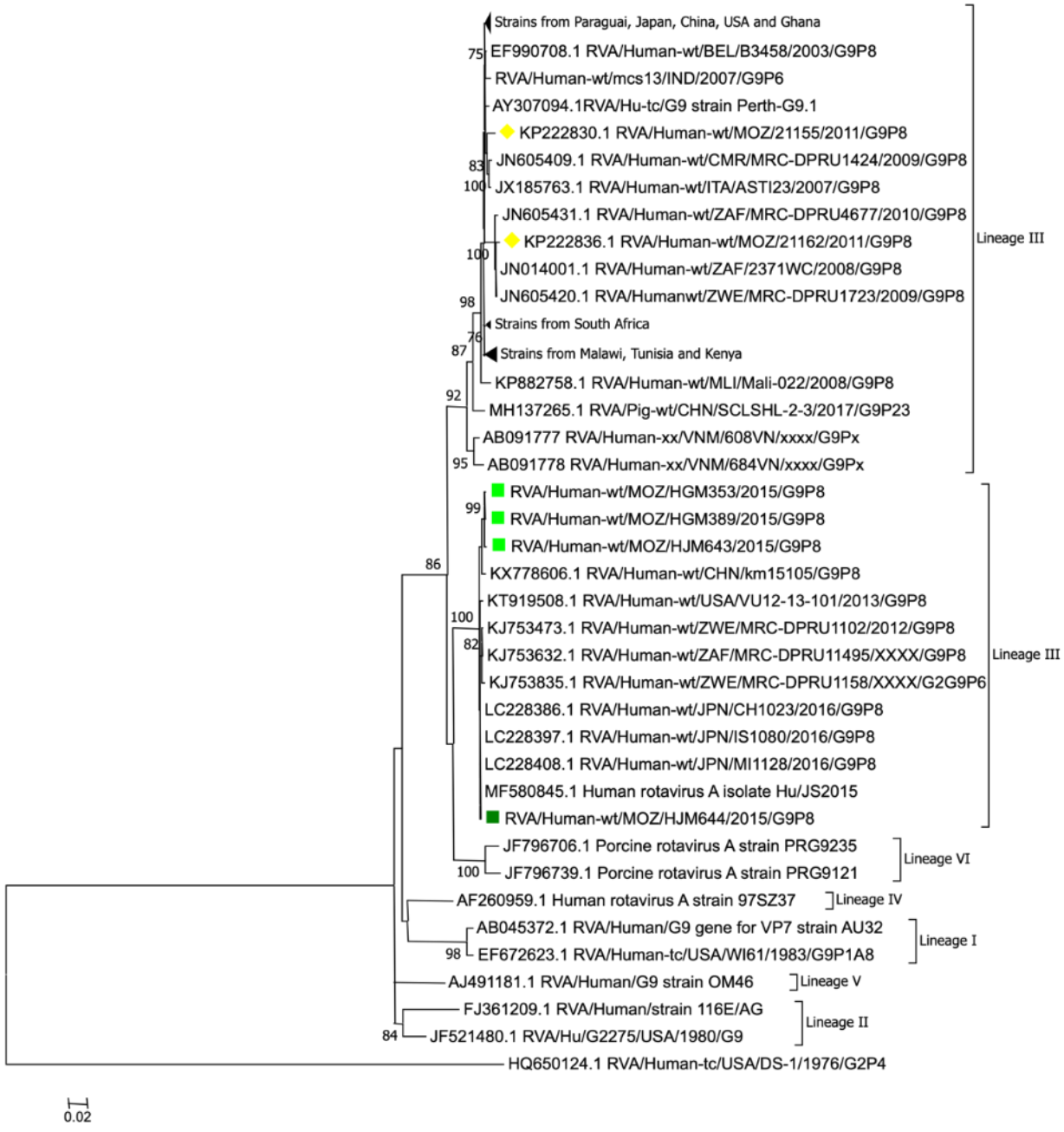


Figure.1: Molecular phylogenetic analysis by Maximum Likelihood Method of complete ORF nucleotide sequences of human G1P[8] and G9P[8] strains encoding VP7-G1 (A), VP7-G9 (B), VP4 (C), VP1 (D), VP2 (E), VP3 (F), VP6 (G), NSP1 (H), NSP2 (I), NSP3 (J), NSP4 (K), NSP5/NSP6 (L). Trees were constructed in MEGA 7.2.1. Only bootstrap ≥70% or greater are shown. The G1P[8] are labeled with filled red circles and G9P[8] are labeled with filled green box, one G9 strain is dark green. Other Mozambican G12P[6] and G1P8], strains circulated in 2011 and 2012 are represented by yellow diamond. The tree is drawn to a scale, with branch lengths measured in the number of substitutions per site.

B: VP7 (genetic segment 9)-Genotype G9

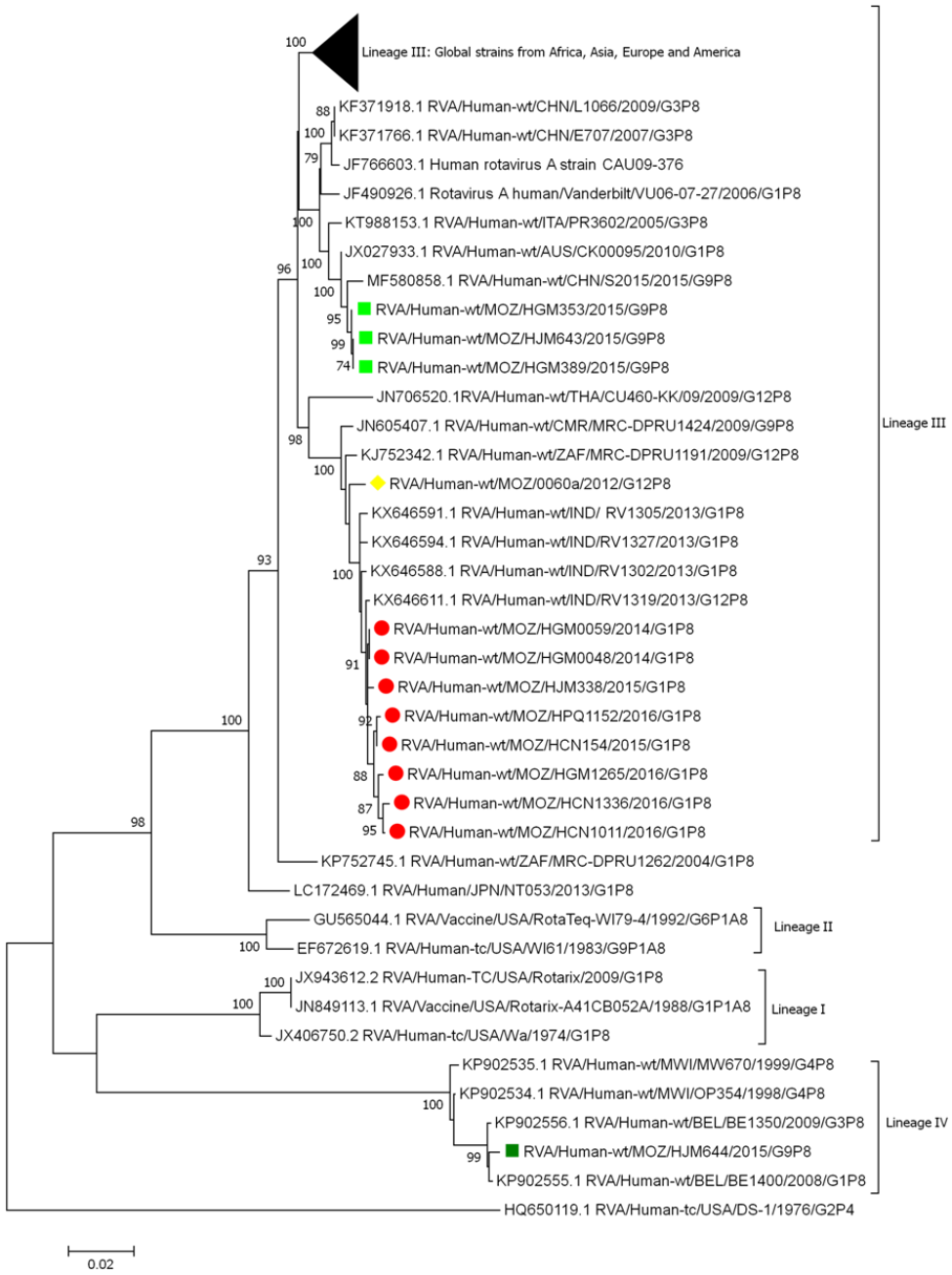


4.4.2.2. VP4 encoding nucleotide sequence (genome segment 4, genotype P[8])

The P[8] study strains were divided in four lineages [Magagula et al., 2015, Zeller et al., 2015, Damanka et al., 2018]. In general, the Mozambican P[8] strains from G1P[8] and G9P[8] grouped in different clusters. The P[8] strains from 2014 (HGM0048 and HGM0059) had a 100% of identity among them. The P[8] Mozambican strains from G1P[8] grouped in lineage III in cluster containing Indian strains from 2013 (RVA/Human-wt/IND/RV1319/2013/G12P8, RVA/Human-wt/IND/ RV1302/2013/G1P8, RVA/Human-wt/IND/RV1305/2013/G1P8, RVA/Human-wt/IND/RV1327/2013/G1P8) that had an identity of 99.83 - 98.93% (Supplementary Table 2D). A G12P[8] Mozambican strain from 2012 shared identity of 98.5-99.01%, (RVA/Human-wt/MOZ/0060a/2012/G12P8), however, this strain diverge from current study strains.

Three P[8] strains from G9P[8] (HGM353, HGM389, HJM643) clustered also in lineage III with strain from China (RVA/Human-wt/CHN/S2015/2015/G9P8) and Australia (RVA/Human-wt/AUS/CK00095/2010/G1P8). Strain HJM644 only had an average identity of 88.21% with all current study strains (Supplementary Table 3D) and clustered separately in lineage IV with strains from Belgium (RVA/Human-wt/BEL/BE150/2009/G1P8, RVA/Human-wt/BEL/BE1400/2008/G1P8) and Malawi (RVA/Human-wt/MWI/MW670/1999/G4P8, RVA/Human-wt/MWI/OP354/1998/G4P8 (Figure 1C).

C: VP4 (genome segment 4)-genotype P[8]



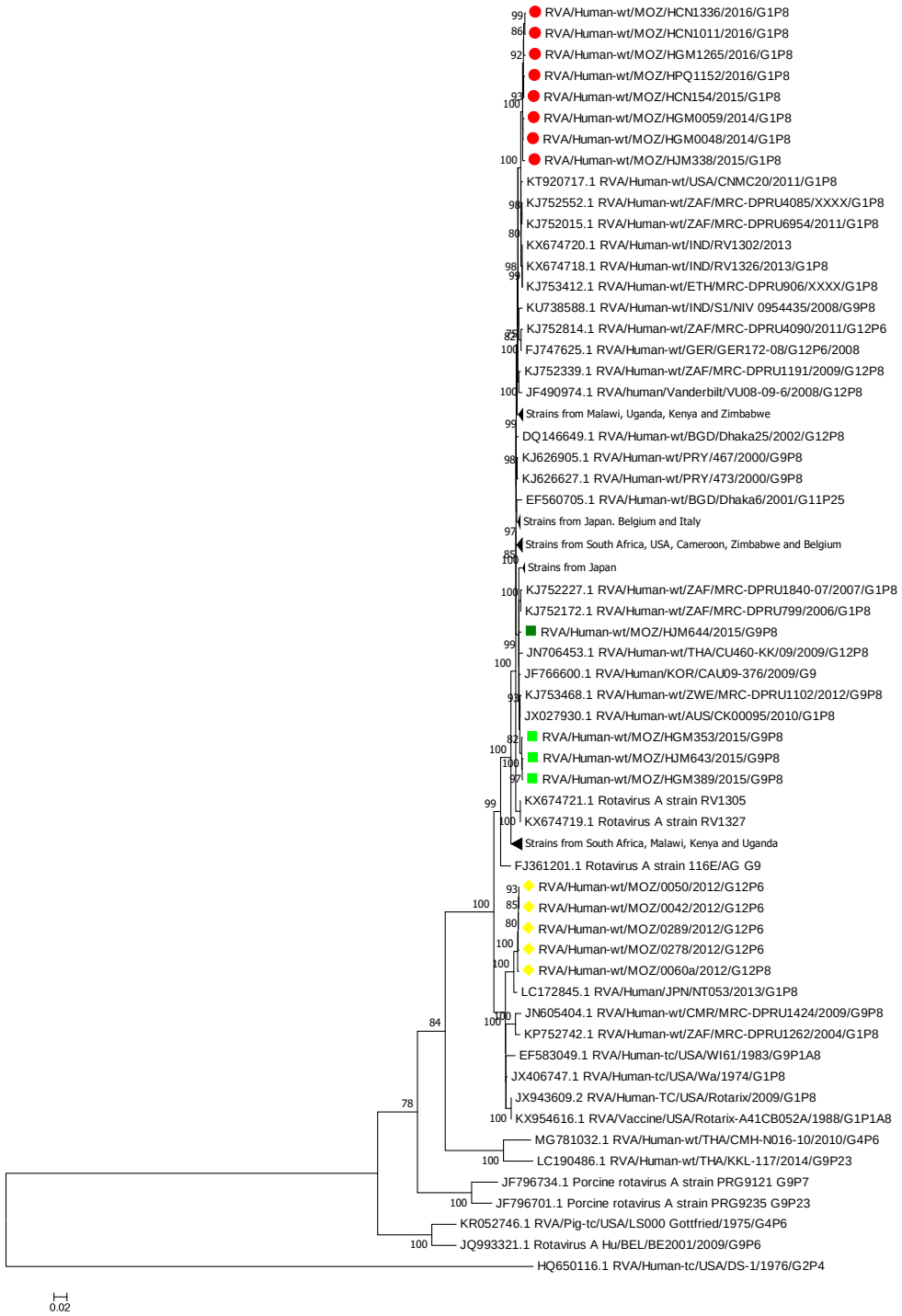
4.4.2.3. VP1-VP3 and VP6 encoding nucleotide sequences (genome segments 1, 2, 3 and 6)

The Mozambican VP1-VP3 and VP6 encoding nucleotide sequences (genome segments 1 to 3 and 6), correspond to genotypes R1, C1, M1 and I1, respectively (Table 2). The Wa-like G1P[8] and G9P[8] strains of current study grouped separately from each other in phylogenetic tree. The sequences encoding VP1, VP2, VP3 and VP6 of G1P[8] Mozambican strains clustered together and the closest relatives were RVA/Human-wt/ZAF/MRC-DPRU6954/2011/G1P8 (average identity 99.33%), RVA/Human wt/USA2009727036/2009/G9P8 (average identity 98.73%), RVA/Human-wt/USA/CNMC20/2011/G1P8 (average identity 99.17%), and RVA/Human-wt/INDRV1302/2013/G1P8 (average identity 99.24%), respectively (Supplementary Table 2A-C, F, Figure 1D-G).

The VP1-VP3 encoding nucleotide sequences from Mozambican G9P[8] strains grouped together, except for VP2 of strain HJM644 that had an identity, ranging 98.47 – 98.53% with others (Supplementary Table 3A-C, Figure 1E) and formed a small cluster with strain RVA/Human-wt/ZWE/MRC-DPRU1102/2012/G9P8 (Supplementary Table 3A-C, Figure 1D-F). And other three strains (HGM353, HGM389 and HJM643) formed clusters with strains from Australia, Japan, China, India, Zimbawe, South Africa, Belgium and Italia, however, the closest relative was an Australian strain (RVA/Human-wt/AUS/CK00095/2010/G1P8) (Figure 1D-F).

The VP6 encoding nucleotide sequence of strains HGM353, HGM389 and HJM643 formed a monophyletic group with strains from China and Japan and the closest relative was strain RVA/Human-wt/CHN/km15105/2015/G9P8. Moreover, HJM644 strain shared identity with others three strains ranging 98.49 – 98.66% and the close relative were RVA/Human wt/BGD/Dhaka6/2001/G11P25 and Belgium strain RVA/Human-wt/BEL/B3458/2003/G9P8 (Supplementary Table 3F, Figure 1G).

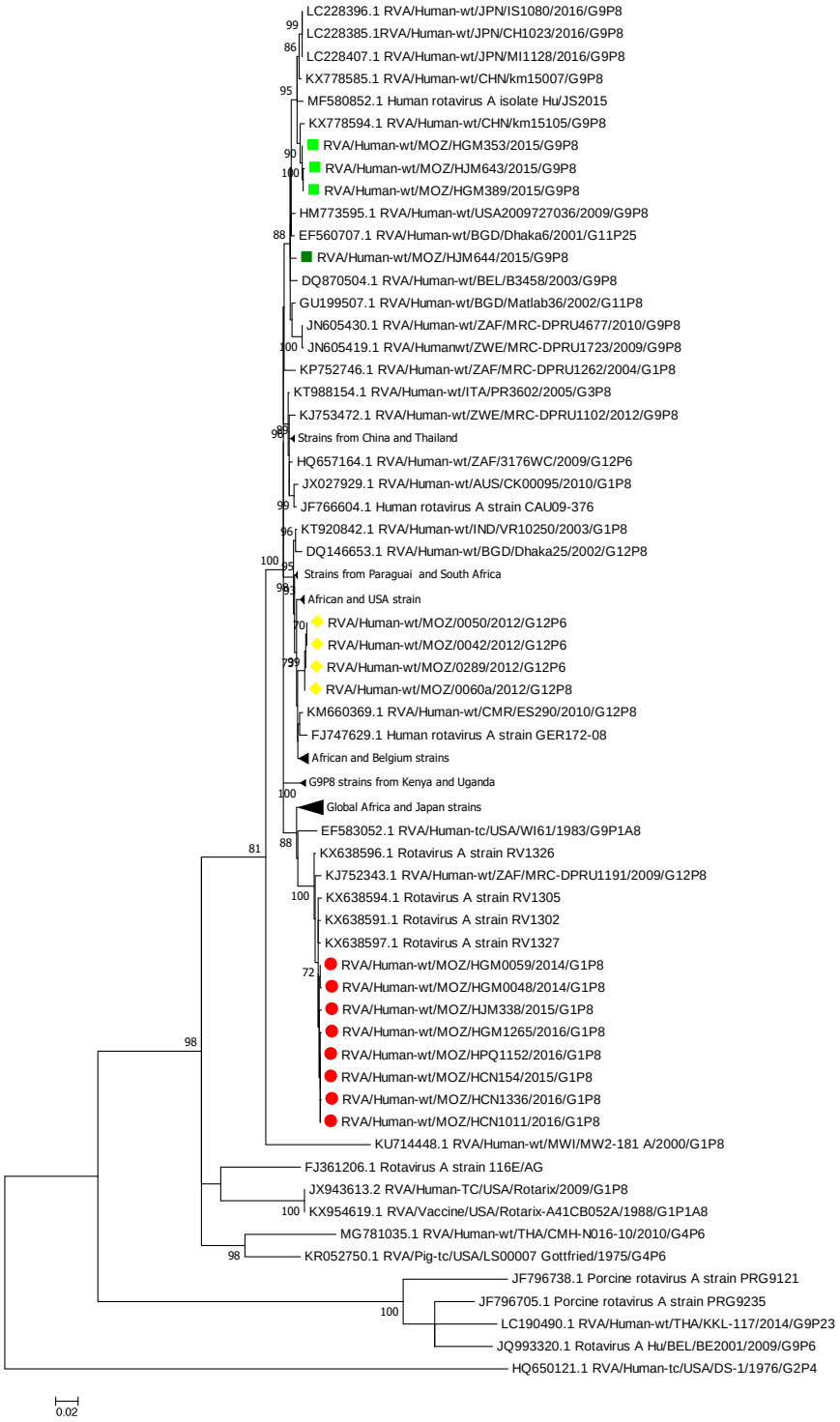
D: VP1 (genome segment 1)-genotype R1



E: VP2 (genome segment 2) - genotype C1

F: VP3 (genome segment 3)-genotype M1

G: VP6 (genome segment 6)-genotype I1



4.4.2.4. NSP1-NSP5/NSP6 encoding nucleotide sequences (genome segments 5, 8, 7, 10 and 11)

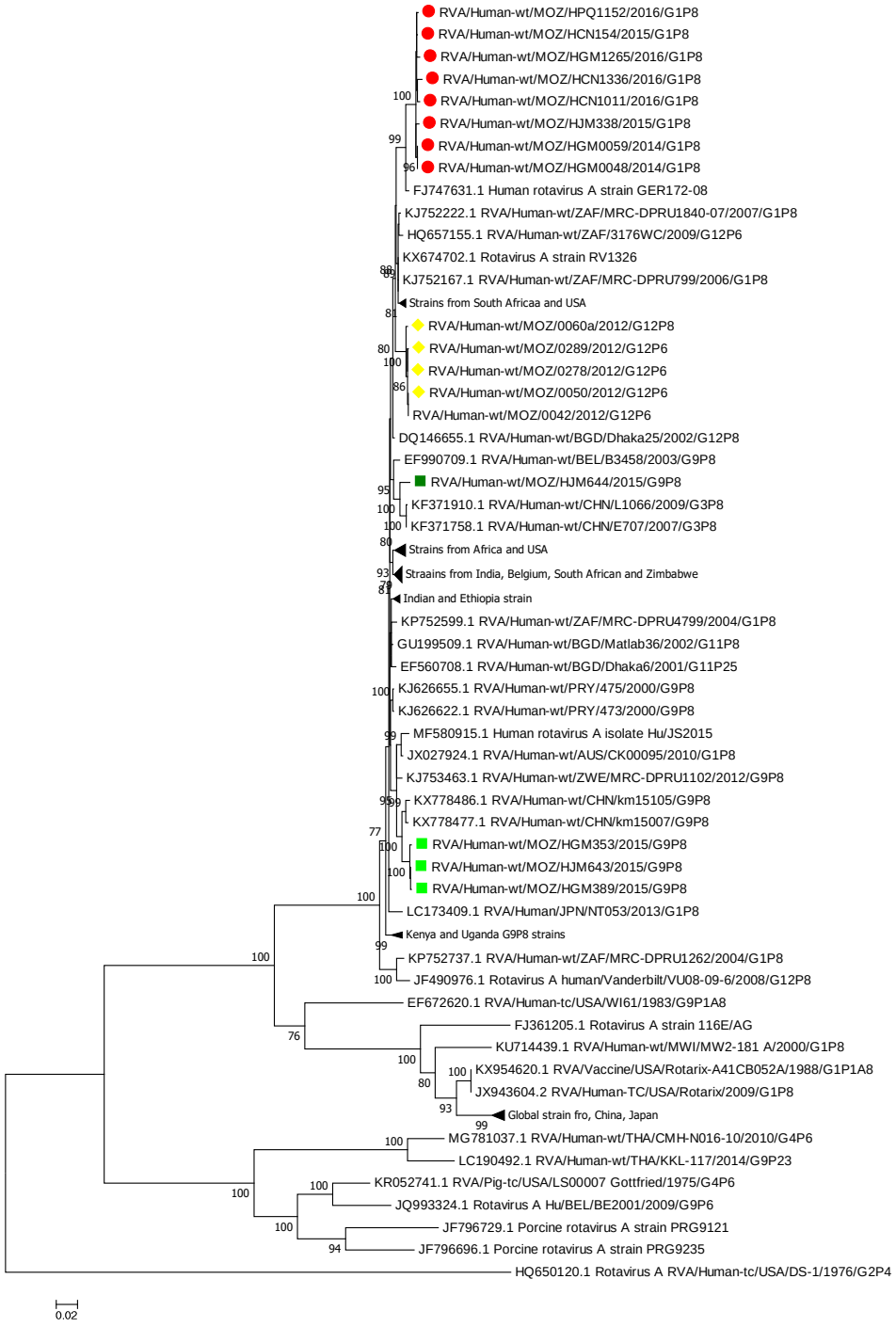
With regard of NSP1-NSP5/NSP6 encoding nucleotide sequences, the Mozambican G1P[8] and G9P[8] strains also grouped separately. The Mozambican NSP1 encoding nucleotide sequence (genome segment 5, genotype A1) formed a separated monophyletic group and had an identity ranging 99.79 -100%, however, grouped in the major cluster that included G1P[8], G12P[6] strains from 2006 and 2013, including G12P[6] from Mozambique (Supplementary Table 2E, Figure 1H).

NSP2 and NSP5/6 encoding nucleotide sequences grouped together, had high identity (99.48 - 100%) (Supplementary Table 2H, K). These sequences grouped in a major cluster more closely to strains from India (RVA/Human-wt/IND/RV1305/2013/G1P8 and RVA/Human-wt/IND/RV1327/2013 (Figure1 I, L). The NSP3 encoding nucleotide sequence was more closely related to strain from Cameroon strain RVA/Human-wt/CMR/MRC-DPRU1424/2009/G9P8 (Supplementary Tabel 2G, Fig.1J).

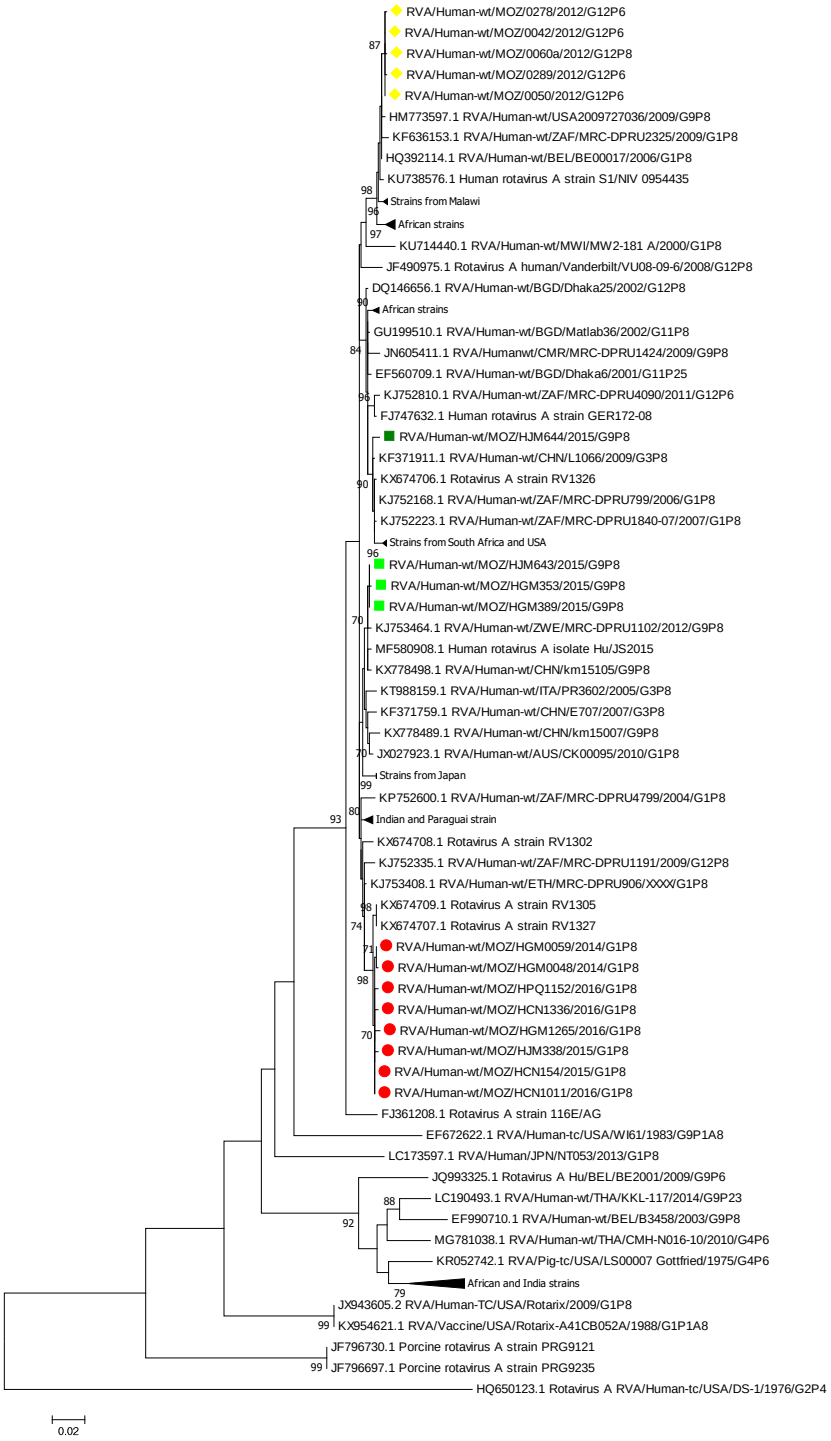
The G1P[8] NSP4 encoding nulcoetide sequence also gruped together and had a high identity (99.62 – 100%) and grouped in major clustel more closely related to a strain from India RVA/Human-wt/IND/RV1302/2013/G1P8 (identity 99.14%). These strains, however, also had an identity of 97.9% with Mozambican strain G12P[8] that circulated in 2012 (except for strains HGM0048 and HGM0059 from 2014, HCN0154 from 2015) (Supplementary Table 2J, Figure1K).The NSP1, NSP2 and NSP5 encoding nucleotide sequences from G9P[8] strains of three samples (HGM353, HGM389 and HJM643) are close relative to strains from China, particularly to strain RVA/Human-wt/CHN/km15105/2015/G9P8. Strain HJM644 clustered separately with RVA/Human-wt/CHN/L1066/2009/G3P8 for NSP1 and NSP2 encoding nucleotide sequences. During analysis for NSP5 encoding sequence, HJM644 clustered with Mozambican G12P[6] strains (Figure1H, I, L). The G9P[8] strains had an identity with G12P[6] Mozambican strains of 99.83% (HJM644) and 99.49% (HGM353, HGM389 and HJM643) (Supplemental Table 3, Figure1M). The NSP3 and NSP4 encoding nucleotide sequences formed a cluster with a strain from Australian (RVA/Human-

wt/AUS/CK00095/2010/G1P8), except for NSP4 where strain HJM644 only had an average identity of 98.59% with others strains (Supplementary Table 3G, J, Figure 1J-K).

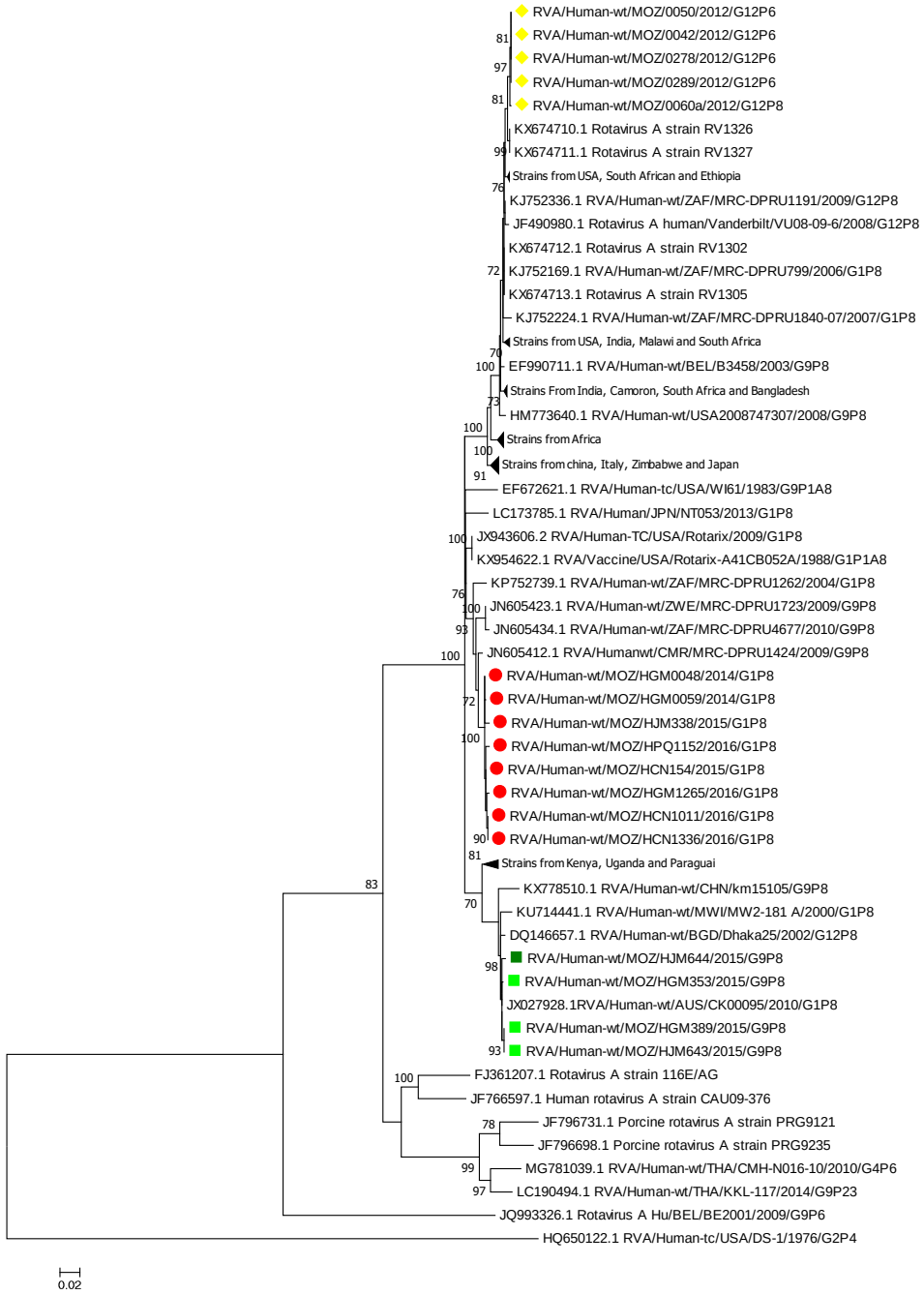
H: NSP1 (genome segment 5) - genotype A1



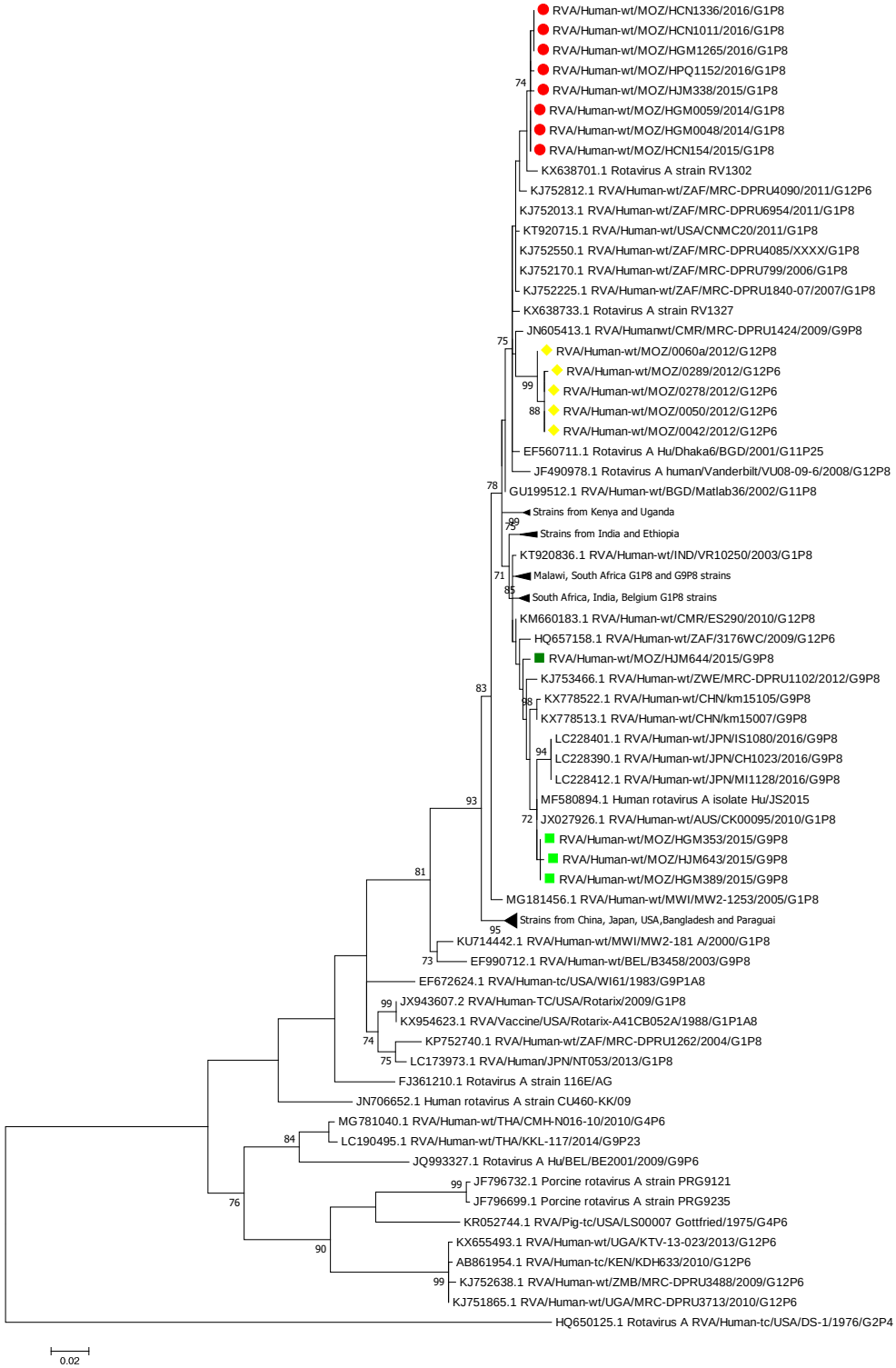
I: NSP2 (genome segment 8) - genotype N1



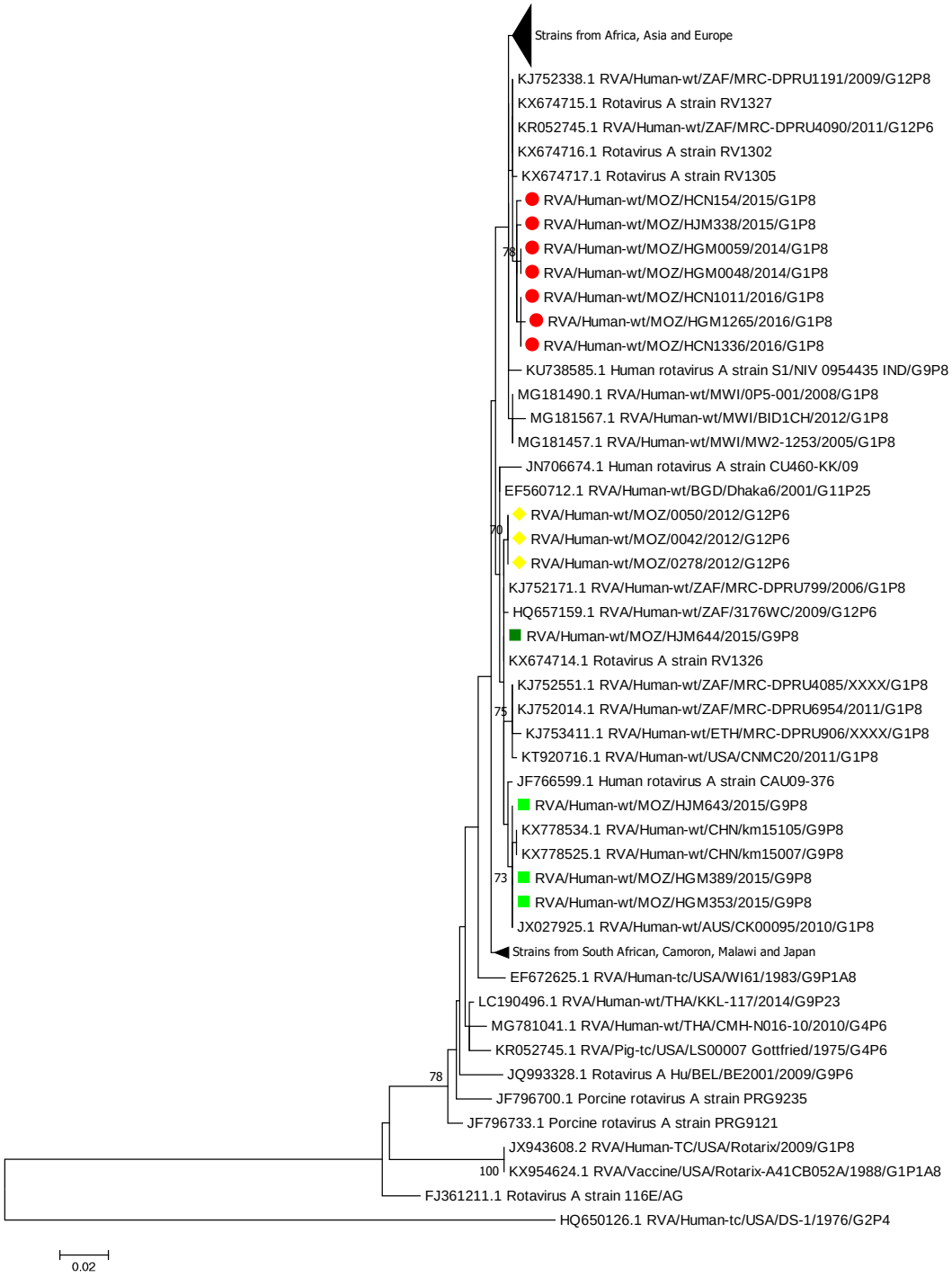
J: NSP3 (genome segment 7) - genotype T1



K: NSP4 (genome segment 10) - genotype E1



L: NSP5/NSP6 (genome segment 11) - genotype H1



4.4.2.5. Phylogenetic tree with concatenate sequences of G1P[8] genome

With concatenated sequences of all 11 genome segments, the G1P[8] Mozambican genomes clustered in group that contain Asian strain, being more close related to India strain from 2013. (Fig. 2).

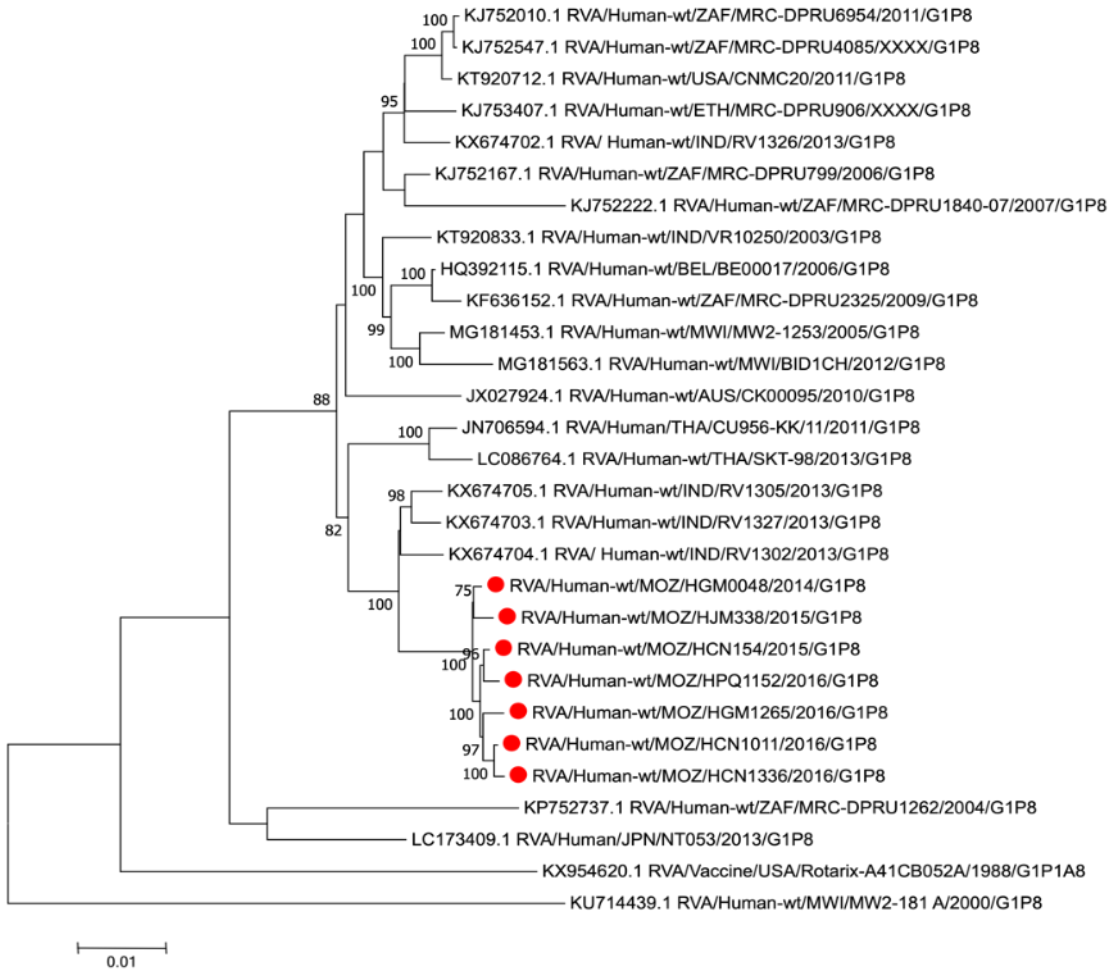


Figure 2: Molecular Phylogenetic analysis by Maximum Likelihood method of concatenated sequence of all ORF of 11 genetic segments of G1P[8] genomes conduted in MEGA 7.2.1. The Mozambican genome was marked in filled red circles. Bootstrap ≥70% are shown. The tree is drawn to scale, with branch lengths measured in number of substitutions per site.

4.4.3. Comparison of VP7 and VP4 amino acid sequences of Mozambican strains to Rotarix® vaccine

The Mozambican G1P[8] and G9P[8] (only for P[8]) strains were selected for molecular analysis of outer proteins, glycoprotein VP7 (G) and the protease sensitive VP4 (P) (fragment VP8* and VP5*) both carry the antigens epitopes comparing with Rotarix® vaccine strain (A41CB052A), this vaccine was introduced in 2015 in Mozambique (Figure. 3 and Figure. 4).

For VP7 gene, six antigenic regions have been described before and defined as A (aa 87–101), B (aa 141–150), C (aa 208–224), D (aa 291), E (aa 189), and F (aa 235–245). Antigenic regions A, B, C, and F correspond to VR-5, VR-7, VR-8, and VR-9, respectively were analyzed in the present study.

Comparison of the deduced amino acid sequence from Rotarix® G1 vaccine component with sequences from the Mozambican G1 strains revealed only amino acid differences in region B (VR-7) and C (VR-8). Mozambican G1 strains had different amino acid at one among the 10 positions in antigenic region B (VR-7, aa 141-150), where was observed amino acid substitution in position 147, aspargine was replaced by aspartic acid (N147D) in seven strains (HCN1011, HCN1336, HCN154, HJM338, HGM0048, HGM0059 and HPQ1152).

In the antigenic site C (VR-8, aa 208-224) from 17 positions were observed substitution in position 217 where methionine was replaced by isoleucine (M217I) in one strain (HCN154) from pre-vaccine period and four strains (HCN1011, HCN1336, HPQ1152 and HGM1265) from post-vaccine periods (Figure. 2)

The antigenic region A (VR-5, aa 87-100) and F (VR-9, aa 235-245) are conserved among G1 Mozambican strains (Figure. 3).

The VP4 protein is comprised of two structurally distinct regions (VP8* and VP5*). The VP8* region contains four antigenic domain (8–1 to 8–4), while VP5* contains five antigen domain (5–1 to 5–5).. In the VP8* region, the antigenic domain 8-1 in the position 146 for strain HJM338, was noted amino acid substitution of serine by aspargine (S146N); in eleven strains were observed in position 150 differences, glutamic acid were replaced by aspartic acid (E150D), except for strain HJM644 that had N194T substitution (Figure. 4).

In the position 195, was found that aspargine was replaced by glycine (N195G) in eight strains (HGM0048, HGM0059, HCN154, HJM338, HPQ1152, HGM353, HGM389, HJM643), while strains HCN1011, HCN1336 and HGM1265 had aspargine replaced by aspartic acid (N195D); and strain HJM644 presented N195S.

In the antigen domain 8-3, only P[8] strains from G9P[8] (HGM353, HGM389, HJM643 and HJM644) differ from vaccine in position 113, presenting aspartic acid instead of asparagine (N113D) . In the position 125 and 135 eleven strain are different to Rotarix®, presenting serine replaced by aspartic acid (S125N) and aspartic acid replaced by aspartic acid (N135D), respectively. In the position 131 all strains differ from the vaccine, presenting substitution S131R. The antigenic domains 8-2 and 8-4 of VP8* are more conserved.

With regard to the VP5* region, domain 5-1 in position 385 of all strains had Y385D, tyrosine were replaced by aspartic acid, in position 387 only strain HJM644 observed substitution S387T. Domains 5.2 and 5.5 are more conserved.

The P[8] strain HJM644 presented unique amino acid profile in some positions, supporting the fact that clustered in the lineage IV in the phylogenetic tree (Figure. 4).

Figure 3. Alignment of amino acid residues of Rotarix™ and Mozambican strains of VP7 neutralization antigenic region

VP7 antigenic regions — VR-5/antigenic site A (87-100)

Strain nomenclature	87	88	89	90	91	92	93	94	95	96	97	98	99	100
Rotarix-A41CB052A/1988/G1P1A	T	E	A	S	T	Q	I	N	D	G	E	W	K	D
Pre-vaccine period														
HCN154/2015/G1P8	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HJM338/2015/G1P8	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HGM0048/2014/G1P8	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HGM0059/2014/G1P8	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Post-vaccine period														
HGM1265/2016/G1P8	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HCN1011/2016/G1P8	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HCN1336/2016/G1P8	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HPQ1152/2016/G1P8	.	.	.	.	.	.	.	.	.	.	.	.	.	.

VR-7/antigenic site B (141-150)

Strain nomenclature	141	142	143	144	145	146	147	148	149	150
Rotarix-A41CB052A/1988/G1P1A	L	M	K	Y	D	Q	N	L	E	L
Pre-vaccine period										
HCN154/2015/G1P8	.	.	.	.	.	.	D	.	.	.
HJM338/2015/G1P8	.	.	.	.	.	.	D	.	.	.
HGM0048/2014/G1P8	.	.	.	.	.	.	D	.	.	.
HGM0059/2014/G1P8	.	.	.	.	.	.	D	.	.	.
Post-vaccine period										
HGM1265/2016/G1P8	.	.	.	.	.	.	.	.	.	.
HCN1011/2016/G1P8	.	.	.	.	.	.	D	.	.	.
HCN1336/2016/G1P8	.	.	.	.	.	.	D	.	.	.
HPQ1152/2016/G1P8	.	.	.	.	.	.	D	.	.	.

VR-8/antigenic site C (208-224)

Strain nomenclature	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224
Rotarix-A41CB052A/1988/G1P1A	Q	T	T	N	V	D	S	F	E	M	V	A	E	N	E	K	L
Pre-vaccine period																	
HCN154/2015/G1P8	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.
HJM338/2015/G1P8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HGM0048/2014/G1P8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HGM0059/2014/G1P8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Post-vaccine period																	
HGM1265/2016/G1P8	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.
HCN1011/2016/G1P8	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.
HCN1336/2016/G1P8	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.
HPQ1152/2016/G1P8	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.

VR-9/antigenic site F (235-245)

Strain nomenclature	235	236	237	238	239	240	241	242	243	244	245
Rotarix-A41CB052A/1988/G1P1A	H	K	I	N	L	T	T	T	T	C	T
Pre-vaccine period											
HCN154/2015/G1P8	.	.	.	.	.	.	.	.	.	.	.
HJM338/2015/G1P8	.	.	.	.	.	.	.	.	.	.	.
HGM0048/2014/G1P8	.	.	.	.	.	.	.	.	.	.	.
HGM0059/2014/G1P8	.	.	.	.	.	.	.	.	.	.	.
Post-vaccine period											
HGM1265/2016/G1P8	.	.	.	.	.	.	.	.	.	.	.
HCN1011/2016/G1P8	.	.	.	.	.	.	.	.	.	.	.
HCN1336/2016/G1P8	.	.	.	.	.	.	.	.	.	.	.
HPQ1152/2016/G1P8	.	.	.	.	.	.	.	.	.	.	.

Legend: Dots indicate an amino acid identical to Rotarix™. T-threonine, E-glutamic acid, A-alanine, S-serine, Q-glutamine, I-isoleucine, N-aspargine, D-aspartic acid, G-glycine, W-tryptophan, K-lysine, Y-tyrosine, L-leucine, V-valine, F-phenylalanine, M-methionine, H-histidine.

Figure 4.Alignment of amino acid residues of Rotarix® and Mozambican strains of VP4 neutralization antigenic region

Strains nomenclature	VP8*																										VP5*									
	8_1												8_2		8_3										8_4			5_1								5_2	5_3	5_4	5_5
	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	383	385	387	392	393	397	439	440	433	458	428	305
Rotarix-A41CB052A/1988/G1P1A8	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	S	Y	S	A	W	N	L	R	E	N	S	L
Pre-vaccine period																																						
HGM0048/2014/G1P8	.	.	.	D	.	.	.	.	.	G	.		.	.	.	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.
HGM0059/2014/G1P8	.	.	.	D	.	.	.	.	.	G	.		.	.	.	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.
HCN154/2015/G1P8	.	.	.	D	.	.	.	.	.	G	.		.	.	.	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.
HJM338/2015/G1P8	.	N	.	D	.	.	.	.	.	G	.		.	.	.	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.
HGM353/2015/G9P8*	.	.	.	D	.	.	.	.	.	G	.		.	.	D	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.
HGM389/2015/G9P8	.	.	.	D	.	.	.	.	.	G	.		.	.	D	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.
HJM643/2015/G9P8	.	.	.	D	.	.	.	.	.	G	.		.	.	D	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.
HJM644/2015/G9P8	.	.	.	.	.	.	D	.	T	S	.		.	.	D	.	.	.	.	R	.	.	.	.	.	.	.	D	T	.	.	.	.	.	.	.	.	.
Post-vaccine period																																						
HCN1011/2016/G1P8	.	.	.	D	.	.	.	.	.	D	.		.	.	.	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.
HPQ1152/2016/G1P8	.	.	.	D	.	.	.	.	.	G	.		.	.	.	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.
HGM1265/2016/G1P8	.	.	.	D	.	.	.	.	.	D	.		.	.	.	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.
HCN1336/2016/G1P8	.	.	.	D	.	.	.	.	.	D	.		.	.	.	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.

Legend: Dot illustrate the amino acid identical to Rotarix®. D-aspartic acid, S- serine, Q- glutamine, E- glutamic acid, T- threonine, N- aspargine, I- isoleucine, A- alanine, P-proline, V-valine. Y- tyrosine, W- tryptophan, L- leucine, R-arginine, G-glicine.. *Strains of G9P[8] for post-vaccine period were not sequenced.

4.5. Discussion

In this Chapter, whole genome characterization was performed in eight Wa-like G1P[8] and four G9P[8] strains from Mozambique, from 2014 and 2016, originating from different regions. All G1 strains belonged to lineage II and were closely related to India strains (RVA/Human/IND/RV1305/2013/G1P8, RVA/Human/IND/ RV1327/2013/G1P8, RVA/Human/IND/RV1302/2013/G1P8 and (RVA/Bovine/IND/HR//B91/2011/G1Px). However, five G1 strains from the 2011 study in Gaza province in southern Mozambique, which determined partial VP7 and VP4 (VP8*) genes, grouped in lineage I [Langa et al., 2016]. Characterized G1P[8] strains from South African vaccinated and non-vaccinated children, also clustered mostly in lineage I, except one strain (RVA/Human-wt/ZAF/MRC-DPRU1262/2004/G1P8) which clustered in lineage II [Magagula et al., 2015]. Interestingly, a describing the evolution of G1P[8] strains in India reported that most of them belong to lineage II [Pradhan and Chitambar, 2018]. On the other hand, the Mozambican G9 strains all clustered in lineage III with other Mozambican [Langa et al., 2016], Zimbabwean, South African, and Kenyan [Bwogi et al., 2017; Jere et al., 2011a; Nyaga et al., 2013] strains. Asian countries, such as India and China, have reported the circulation of this lineage [Dian et al., 2017; Mullick et al., 2014]. The G9 lineage III was detected in surveillance networks around the world [Matthijnssens et al., 2010].

Of the 12 P[8] strains characterized, 11 grouped in lineage III and one in lineage IV. A similar result was observed by Langa et al., (2016), who reported strains belonging to lineage III and one strain to lineage IV.

P[8] is the most common P genotype detected in children with diarrhoea and lineage III is the most common globally [Zeller 2012]. However, a genetically distinct lineage of P[8] genotype also known as OP354-like P[8] or lineage IV has been detected in different parts of Europe, Africa and Asia [Zeller et al., 2015]. An inferred evolutionary history of OP354-like P[8] rotavirus strains globally showed that these strains emerged relatively recently, spreading from South and East Asia to Europe, Sub-Saharan Africa and North America [Zeller et al., 2015]. In fact, the spreading of this rare lineage was illustrated in the present study.

A study in Ghana, with the aim to genotype previously non-typeable rotavirus VP4 genes, identified 10.4% of rare OP354-like P[8] subtype by partial sequencing of VP4 gene [Damanka et al., 2016] and the Ghanaian follow-up study confirmed by full genome sequence the circulation of this rare strain [Damanka et al., 2019]. Other countries such as Bangladesh, India and Malawi also reported this rare strain [Cunliffe et al., 2001; Nagashima et al., 2009]. Interestingly, the one Mozambican strain detected in lineage four in the present study was unable to detect by RT-PCR using recommended primers presently available within the WHO manual of rotavirus characterization method, being nontypable for P genotype.

Phylogenetic analysis of VP1-3 and VP6 encoding sequences of G1P[8] strains showed that these individual genes had a high identity with strains from Zambia (RVA/Human-wt/ZAF/MRC-DPRU6954/2011/G1P8), USA (RVA/Human wt/USA2009727036/2009/G9P and RVA/Human-wt/USA/CNMC20/2011/G1P8) and India (RVA/Human-wt/INDRV1327/2013/G1P8 and RVA/Human-wt/INDRV1302/2013/G1P8).

Regarding the NSP1-NSP5/6 from Wa-like strains G1P[8], it was observed that the NSP1 and NSP4 encoding nucleotide sequences were the only two individual genome segments more closely related to Mozambican strains Wa-like G12P[6] and G12P[8], which circulated in 2012. This similarity in only two genome segments can suggest diversity of Wa-like backbone in Mozambican strains. Phylogeny of concatenated sequences of the complete genome ORF of G1P[8] showed that strains from Mozambique were similar to Indian strains. The fact, in the analysis of individual genome segments, with the exception of NSP3 encoding nucleotide sequence, all 10 genes grouped in clusters that contain at least one or more Indian strains. The NSP3 is one encoding sequence that grouped with strains from Cameroon, South Africa and Zimbabwe. This result can suggest that G1P[8] strains were introduced from India, rather than global population strains, or from a country with related viruses in circulation, given the limitation in the available sequence data from many neighbouring countries.

The VP1-VP6 and NSP1-NSP5 encoding sequences of G9P[8] Wa-like strains, three (HGM353, HGM389 and HJM643) grouped with contemporaneous strains in all genome

segments and had a high identity with Australian and China strains. However, one strain (HJM644) grouped separately from others in genome segments encoding VP2, VP6, NSP1, NSP2 proteins that clustered with different strains from Zimbabwe, Bangladesh, and Belgium. Interestingly, the NSP5 encoding sequence was the only genome segment that grouped in a major cluster with Mozambican G12P[6] from 2012 and had a high degree of identity, however, also have high similarities with other global strains, suggesting that it is not a local strain.

When the antigenic regions of outer proteins of rotavirus were analysed, differences of pattern of substitutions in VP7 antigen region were observed, while in region A are conserved in Mozambique G1 strains. Magagula et al., (2015), reported several substitutions; region B is conserved in South Africa strains while in Mozambican strains presented several substitutions. In region C, Mozambican strains had a mutation in position 217, mostly in strains after introduction of rotavirus vaccine than in strains before vaccine introduction.

On the other hand, for VP4, in VP8* region, the same substitution patterns were reported in previous studies from Mozambique and South Africa [Joao et al., 2018; Magagula et al., 2015]. In regionVP5* substitutions in Y385D were similar, as well as the conserved region found by Magagula et al., (2015). Despite these substitutions detected in antigenic regions in Mozambican G1P[8] strains, a reduction of burden of acute gastroenteritis by rotavirus was observed in Mozambique early after the introduction of rotavirus vaccine [de Deus et al., 2018a; Joao et al., 2018; Magagula et al., 2015; Zeller et al., 2015].

In the present study, it was not possible to determine the full genome backbone of strain HGM059 due to the low coverage for VP2 and VP3 encoding sequences. Other limitations include the number of strains per year sequenced in pre-vaccine and post-vaccine period for phylogeny and the analyses of antigenic regions did not allow to draw conclusion regarding rotavirus diversity and evolution along the years.

4.6. Conclusion

The present study studied the genetic makeup and relatedness of the full genome of G1P[8] and G9P[8] strains from Mozambique, where it was observed the circulation of typical Wa-like backbone. Evidence of genetic diversity of P[8] in Mozambique in different years was uncovered, with several substitutions in rotavirus antigenic regions, which stress the importance of the continued surveillance of rotavirus strains to monitor the implication of these mutations. Although, G1P[8] strains came from different regions of Mozambique, these were closely related, meaning that there are circulating strains with similar genetic makeup in Mozambique, and that importation is not significant.

CHAPTER 5 Whole genome characterization of RVA G2P[6] DS1-like strains

5.1. Introduction

G2P[4] is one of the globally prevalent strains [Banyai et al., 2012], and, although the global rate of prevalence is lower than G1P[8] strains, can frequently reach epidemiological predominance in some geographical areas. Report of distribution of the rotavirus strains in 15 Eastern and Southern Africa (ESA) countries from 2010 – 2015 as part of active WHO rotavirus surveillance demonstrated that G2P[4] was the second most detected with 11.8% and, with lower frequency, was also detected the uncommon combination G2P[6] in 4.6% [Seheri et al., 2017]. In Mozambique an epidemiological cross-sectional study in southern Mozambique during 2012 and 2013 indicated a predominance of G2P[4] strains (42.3%) (Chapter 2) [Joao et al., 2018]. In the 2011 study from Gaza province in southern Mozambique, the combination G2P[4] was not detected and G2P[6] was detected in 1.8% [Langa et al., 2016].

The P[6] genotype is higly prevalent in sub-Sahara Africa and has been reported to have extensive genetic diversity and was reported as a genotype with porcine-origin [Nyaga et al., 2018; Seheri et al., 2014]. Usually, G2P[6] strains, as well as G2P[4], belong to the DS1-like genome backbone. However, combination genotypes that usually had Wa-like backbone have been reported to have DS1-like backbones, such as G1P[8] [Jere et al., 2018], G3P[8] [Arana et al., 2016] and G8P[8] [Hoa-Tran et al., 2016]. Unlike the genotype P[4] strains, which are mostly associated with DS-1-like genome backbone, and genotype P[8] strains with Wa-like genome backbone, the P[6] strains are associated with both the DS-1-like and Wa-like genome backbones [Matthijnssens and Van Ranst, 2012].

In Mozambique, strains from 2012 and 2013 (G2P[4] and G8P[4]) were reported to have a typical DS1-like genome backbone, however, were identified in those strains on a DS-1-like genome backbone various possible reassortment events [Strydom et al., 2019b].

To our knowledge there are no genome characterization of DS1-like strains (G2P[6]), particularly in north and center regions of Mozambique. The characterization of these strains will reinforce the previous information on DS1-like genome backbone, once the previously were only characterized G2P[4] and G8P[4] in southern region and not G2P[6].

5.2. Objectives

Characterize the genetic diversity of DS1-like strains from Nampula (north region) and Quelimane (center region) in 2015 and 2016.

5.3. Materials and Methods

In this Chapter the main activities done by the student were:

- Selection of rotavirus strains for whole genome characterization in Mozambique;
- RNA and cDNA preparation of samples in South Africa;
- Prepararation of libraries and other procedures for NGS was done by the UFS NGS Unit, and the student only observed;
- Data analysis was done by the student, after training in South Africa.

5.3.1. Rotavirus strains

For the analyses of DS1-like strains, a total of five samples shipped in dry ice were selected for full genome analyses at the Next Generation Sequencing Unit at the University of the Free State in Bloemfontein, South Africa (Table 1). These samples were identified based on the National Acute Diarrheal surveillance described in Chapter 3. The G2P[6] genotype combination in the pre and post vaccine period was detected in 4.2% and 4.6% respectively (Chapter 3).

The criteria for sample selection was: to be the uncommon strain combination, region, and enough amount of DNA of adequate quality.

Table 1. List of strains selected for whole genome sequencing

Sample	Year of collection	Hospital-city	Genotyping PCR
561	2015	Quelimane Provincial Hospital-Quelimane	G2P[6]
738	2015	Nampula Central Hospital-Nampula	G2P[6]
1313	2016	Nampula Central Hospital - Nampula	G2P[6]
1208	2016	Quelimane Provincial Hospital-Quelimane	G2P[6]
1328	2016	Nampula Central Hospital - Nampula	G2P[6]

5.3.2. Ethical approval

The study was approved by the National Committee of Bioethics from Mozambique in 2013 (IRB 00002657, reference No: 348/CNBS/13).) and by the Health Sciences Research Ethics Committee of the University of Free State (ECUFS NR 201/2013).

5.3.3. Experimental procedures and data analyses

RNA extraction, cDNA synthesis and next generation sequencing using the Illumina MiSeq platform, were performed as described in Chapter four. Genotyping were determined using ViPR and BLASTn analysis.

Multiple sequence alignments and phylogeny were performed in MEGA version 7.2.1 as described in Chapter four, the DNA Model Test program implemented in MEGA was used to identify the optimal evolutionary models that best fit the sequence datasets of all rotavirus ORF region genome segments. The following models were determined for the eleven genome segments: VP1 (GTR+G+I), VP2 (T93+I), VP3, VP4, NSP2 and NSP4 (T92+G+I), VP6 and NSP1 (GTR+G) and VP7, NSP3 and NSP5 (T92+I). Nucleotide distance matrixes were analyzed using the p-distance algorithm in MEGA version 7.2.1.

5.4. Results

5.4.1. Genotype constellation of G2P[6] Mozambican strains

The paired-end reads trimmed data of strains ranged from 91.518 and 259. 310 reads, and the average coverage of segments ranged from 426 and 4.331 (Supplementary Table 1). The five G2P[6] strains, RVA/Human-wt/MOZ/HPQ561/2015/G2P[6] (HPQ561), RVA/Human-wt/MOZ/HCN738/2015/G2P6 (HCN738) RVA/Human-wt/MOZ/HPQ1208/2016/G2P6 (HPQ1208) and RVA/Human-wt/MOZ/HCN1313/2016/G2P[6] (HCN1313) had a typical DS1-like constellation (-I2-R2-C2-M2-A2-N2-T2-E2-H2) (Table 2). For one strain, HCN738, it was not possible to determine the consensus sequence for genome segment 5 encoding NSP1 and presented a –I2-R2-C2-M2-Ax-N2-T2-E2-H2 constellation.

Table 2. Genome constellations of human G2P[6] strains circulating in Mozambique between 2015 and 2016

		Proteins (% identity*)										
Strain name	**Genotype by PCR**	**VP7**	**VP4**	**VP6**	**VP1**	**VP2**	**VP3**	**NSP1**	**NSP2**	**NSP3**	**NSP4**	**NSP5/6**
Segment		**9**	**4**	**6**	**1**	**2**	**3**	**5**	**8**	**7**	**10**	**11**
RVA/Human-wt/MOZ/HPQ561/2015/G2P[6]	G2P[6]	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2
		95.8	96.7	96.7	95.3	97.8	89.0	97.5	97.4	97.5	91.3	99.8
RVA/Human-wt/MOZ/HCN0738/2015/G2P6	G2P[6]	G2	P[6]	I2	R2	C2	M2	Ax	N2	T2	E2	H2
		95.7	96.7	97.5	95.2	98.0	89.0	-	97.5	97.8	91.0	99.7
RVA/Human-wt/MOZ/HPQ1208/2016/G2P6	G2P[6]	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2
		95.7	99.0	96.4	97.8	97.7	97.0	97.0	97.2	98.9	91.0	99.3
RVA/Human-wt/MOZ/HCN1328/2016/G2P6	G2P[6]	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2
		95.4	96.5	97.6	95.2	97.8	89.0	97.4	97.4	97.4	91.0	99.0
RVA/Human-wt/MOZ/HCN1313/2016/G2P[6]	G2P[6]	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2
		95.6	96.5	96.5	95.3	97.8	89.0	97.0	97.4	97.5	91.3	99.5

*VP- structural protein; NSP- nonstructural protein; DS1-backbone genotypes are indicated in red; Ax - was not possible to determine the complete ORF of this genotype; nucleotide identities used in the table were determined in ViPR.

5.4.2. Phylogenetic analyses

Maximum likelihood gene trees based on phylogenetic analysis of the complete ORF of the 11 genome segments for all the DS1-like G2P[6] strains were constructed (Fig. 1 A to K). Mozambican G2P[4] and G8P[4], both with five strains also with a DS1-like backbone detected during 2012 and 2013, were included in the analyses [Strydom et al., 2019a].

5.4.2.1. VP7 encoding nucleotide sequences (genome segment 9, genotype G2)

Phylogenetic analysis of G2 strains (Figure 1A) was divided into seven lineages [Doan et al., 2017; Strydom et al., 2019a]**.** All strains from Mozambique, analysed in this study, clustered with high identity (ranging 99.07-99.9%) (Supplementary Table 2I) in lineage IV, sub-lineage 4a-3. These strains were closely related with a high identity (an average of 99.43%) to three G2 Mozambican strains (RVA/Human-wt/MOZ/0440/2013/G2P4, RVA/Human-wt/MOZ/0144/2013/G2P4 and RVA/Human-wt/MOZ/0126/2013/G2P4) detected in 2013 from southern Mozambique and with G2 strains from Zambia and Zimbabwe (Supplementary Table 2I, Figure 1A).

A: VP7 (genome segment 9) - genotype 2

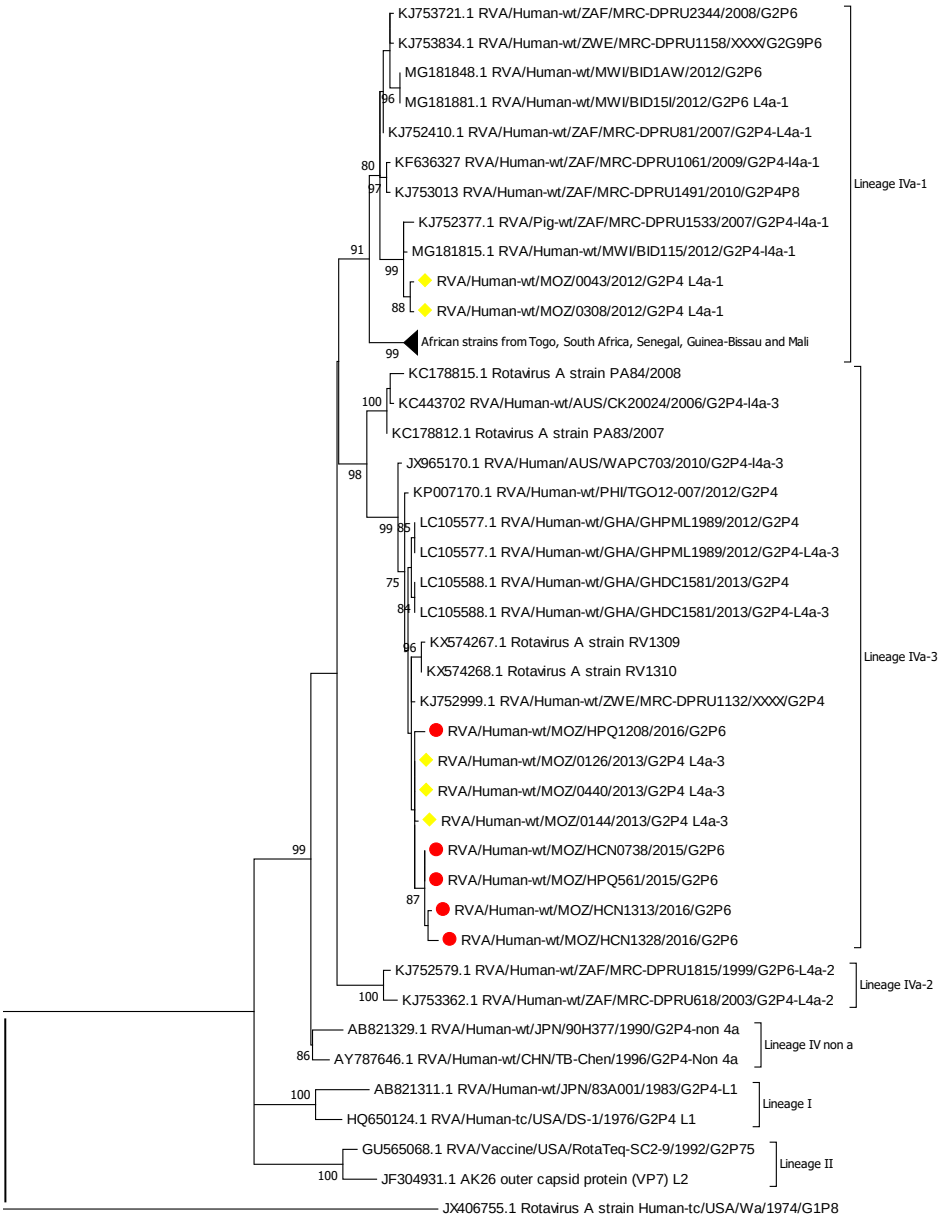


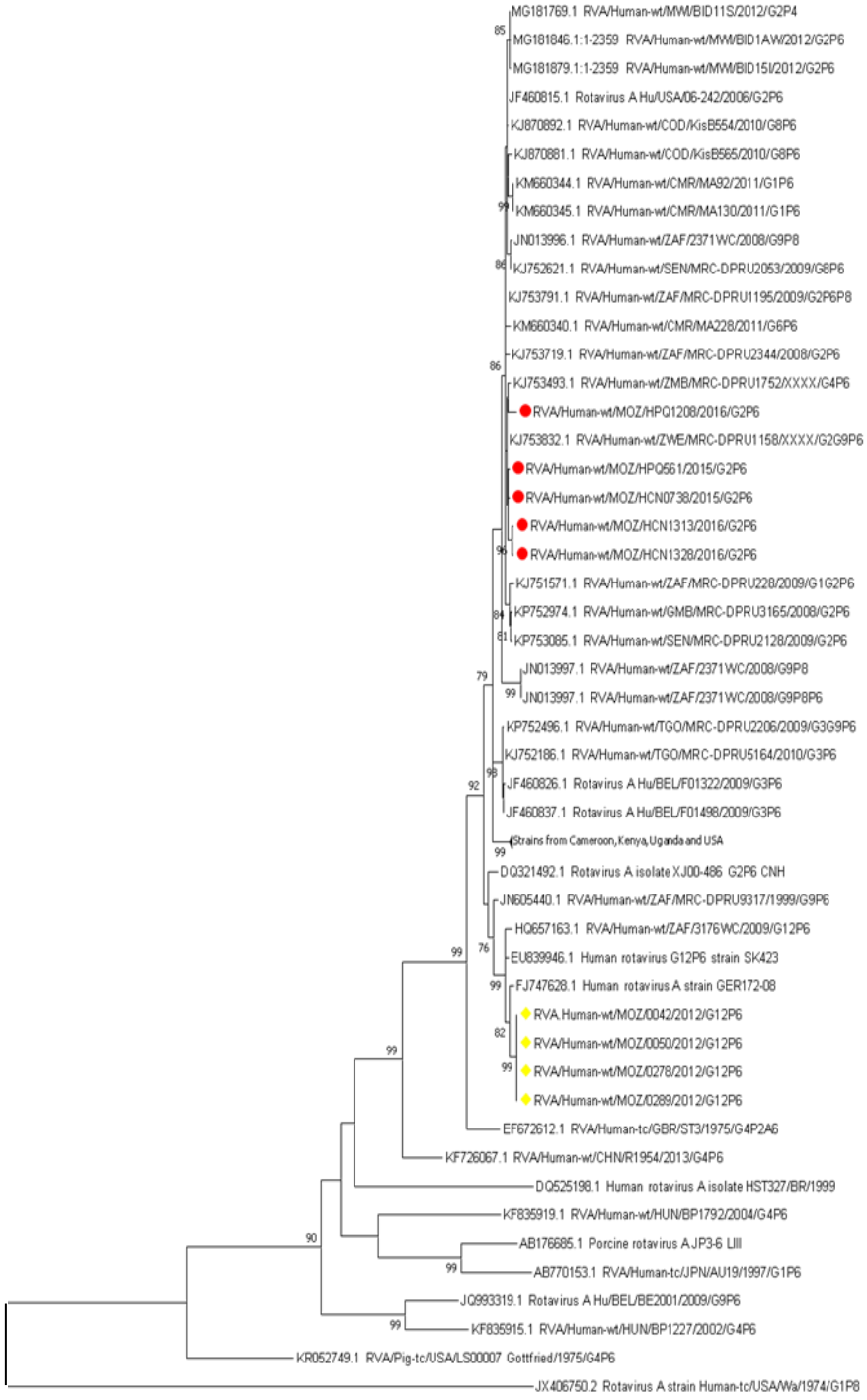
Figure 1: Molecular phylogenetic analysis by Maximum likelihood method of complete ORF nucleotide sequences genes of human G2P[6] strains encoding VP7-G2 (A), VP4 (B), VP1 (C), VP2 (D), VP3 (E), VP6 (F), NSP1 (G), NSP2 (H), NSP3 (I), NSP4 (J), NSP5/NSP6 (L). Trees were constructed in MEGA 7.2.1. Only bootstrap ≥70% or greater are shown. The G2P[6] are labeled with filled red circles. Other Mozambican

G2P[4] and G8P[4,] and G12P[6] strains circulated in 2012 and 2013 are represented in yellow diamond. The tree is drawn to a scale, with branch lengths measured in the number of substitutions per site.

5.4.2.2. VP4 encoding nucleotide sequence (genome segment 4, genotype P[6])

The Mozambican P[6] strains were closely related to each other with an of identity 99.12-99.91% (Supplementary Table 2D, Figure 1B. The closest relative strains were from Zimbabwe, RVA/Human-wt/ZWE/MRC-DPRU1158/XXXX/G2G9P6 and Zambia, RVA/Human-wt/ZMB/MRC-DPRU1752/XXXX/G4P6 (Supplementary Table 2D, Figure 1B). Mozambican P[6] strains detected in 2012 derived from the G12P[6] Wa-like backbone grouped in a separate cluster (Supplementary Table 2D, Figure 1B).

B: VP4 (genome segment 4) - genotype P[6]



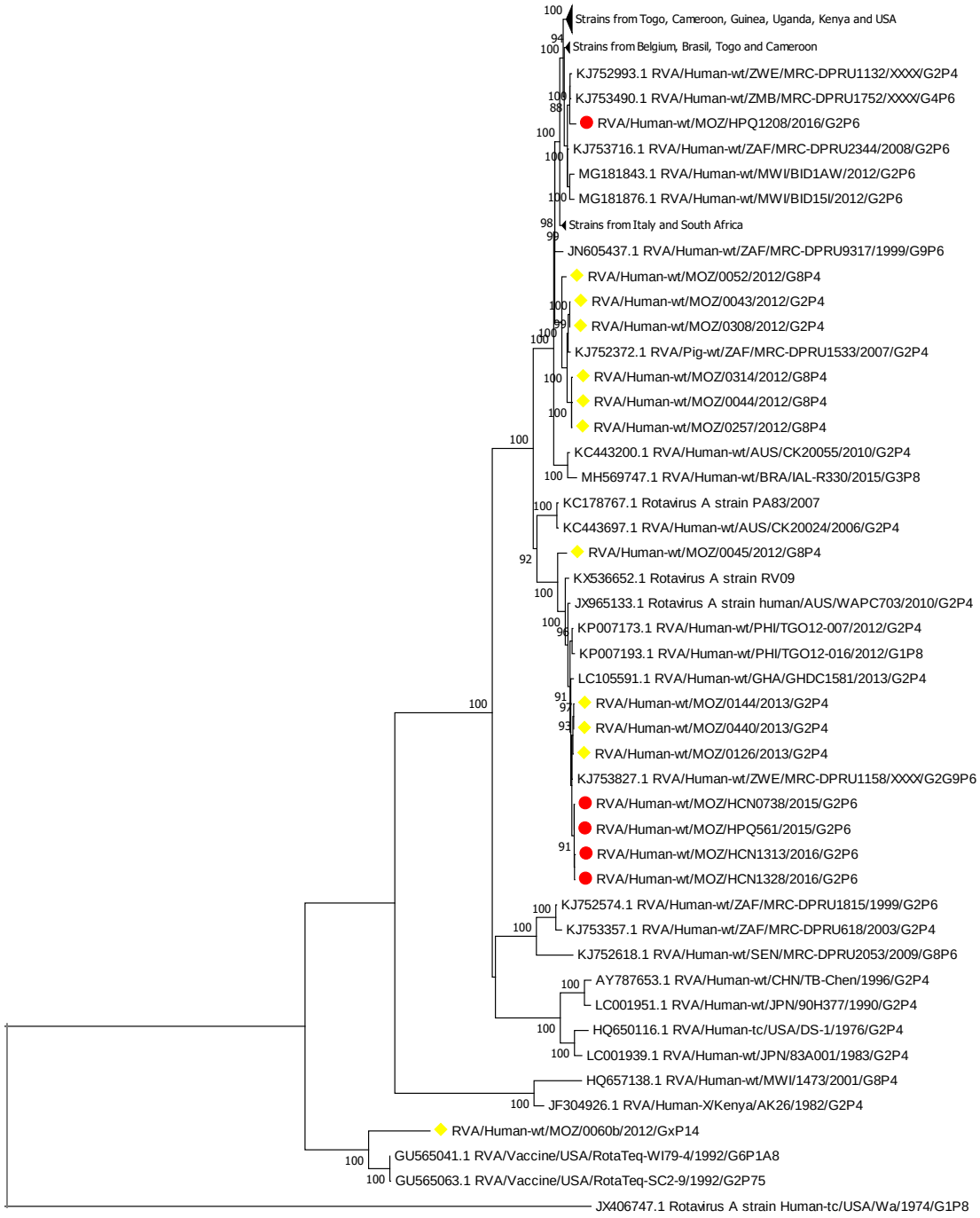
5.4.2.3. VP1-VP3 and VP6 encoding nucleotide sequences (genome segments 1, 2, 3 and 6)

The Mozambican VP1-VP3 and VP6 encoding nucleotide sequences (genome segments 1 to 3 and 6), correspond to genotypes R2, C2, M2 and I2, respectively (Table 2). Phylogenetic analysis for the R2, M2 and I2 genotypes showed that the four DS1-like strains (HCN738, HPQ561, HCN1313, and HCN1328) clustered together with the exception of strain HPQ1208 that clustered separately. The four strains had a high identity with an average identity of more than 99% for all three genotypes (Supplementary Table 2A-C, F, Figure1 C-F). The closest relatives were a Zimbabwean strain (RVA/Human-wt/ZWE/MRC-DPRU1158/XXXX/G2G9P6) and the three G2P[4] Mozambican strains (RVA/Human-wt/MOZ/0144/2013/G2P4, RVA/Human-wt/MOZ/0440/2013/G2P4 and RVA/Human-wt/MOZ/0126/2013/G2P4) had an average of identity above 99% with the current Mozambican strains analysed (Supplementary Table 2A-C, F, Figure 1C-F).

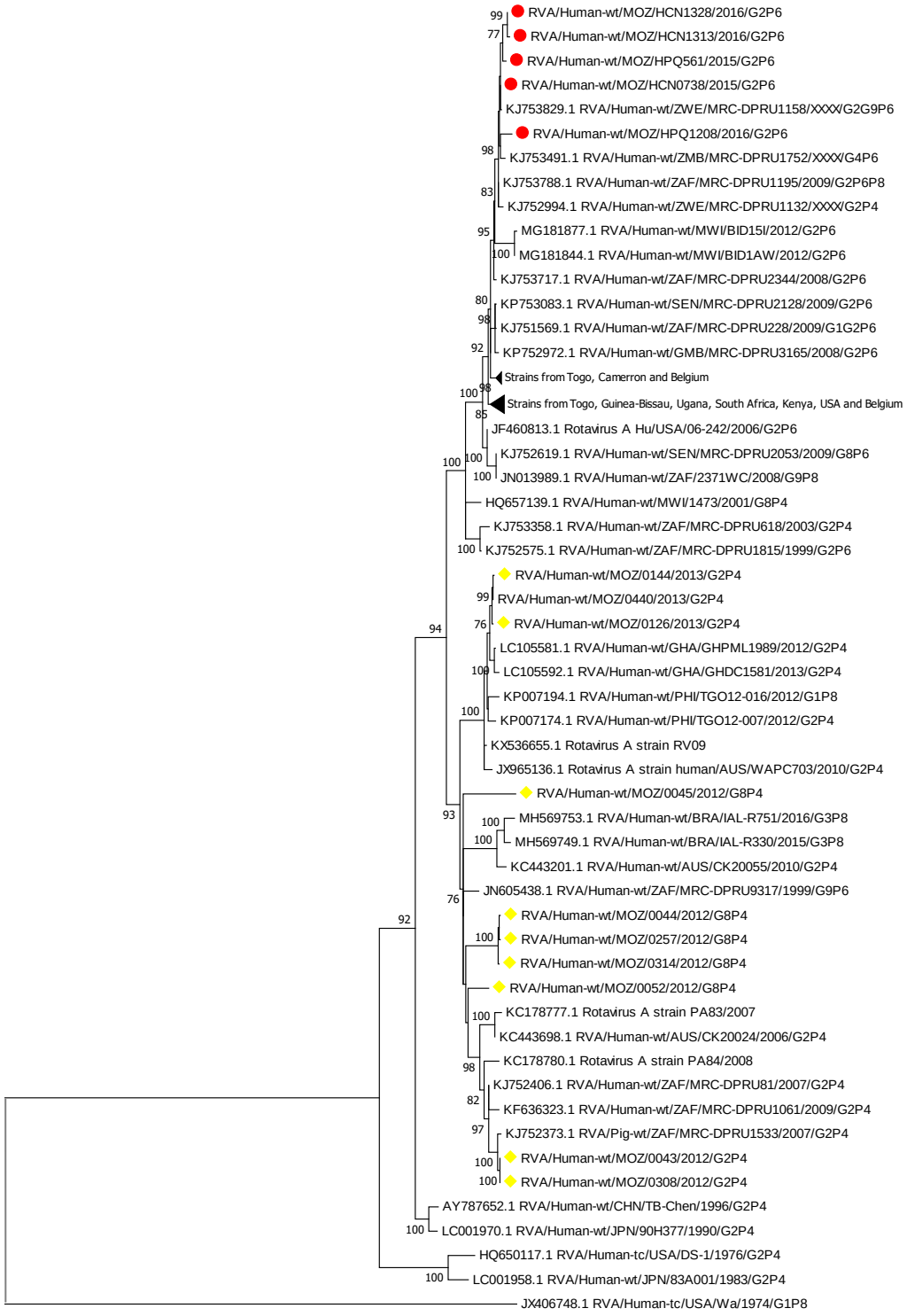
The strain that grouped separately, HPQ1208, had an average nucleotide identity of 94.28%, 87.35, and 96.73% with other strains encoding VP1, VP3 and VP6, respectively (Supplemental Table 2A-C, F, Figure 1 C, E-F). The closest relative was a Zimbabwean G4P[6] strain (RVA/Human-wt/ZMB/MRC-DPRU1752/XXXX/G4P6) and a Zambian G2P[4] strain (RVA/Human-wt/ZWE/MRC-DPRU1132/XXXX/G2P4) during analysis for R2 (VP1) and M2 (VP3) genotypes (Figure1C, E). During analysis for the I2 (VP6) genotype, this strain also grouped with three Mozambican strains (RVA/Human-wt/MOZ/0044/2012/G8P4, RVA/Human-wt/MOZ/0257/2012/G8P4 and RVA/Human-wt/MOZ/0314/2012/G8P4) and had a nucleotide identity of 99.66% with this strain (Supplementary Table 2A-C, F, Figure 1C-F) .

On the other hand the VP2 encoding G2P[6] Mozambican strains all clustered together with an identity of 99.22-99.92% and were closely related to the Zimbabwean RVA/Human-wt/ZWE/MRC-DPRU1158/XXXX/G2G9P6 and Zambian (RVA/Human-wt/ZMB/MRC-DPRU1752/XXXX/G4P6) strains (Supplementary Table 2B, Figure1D).

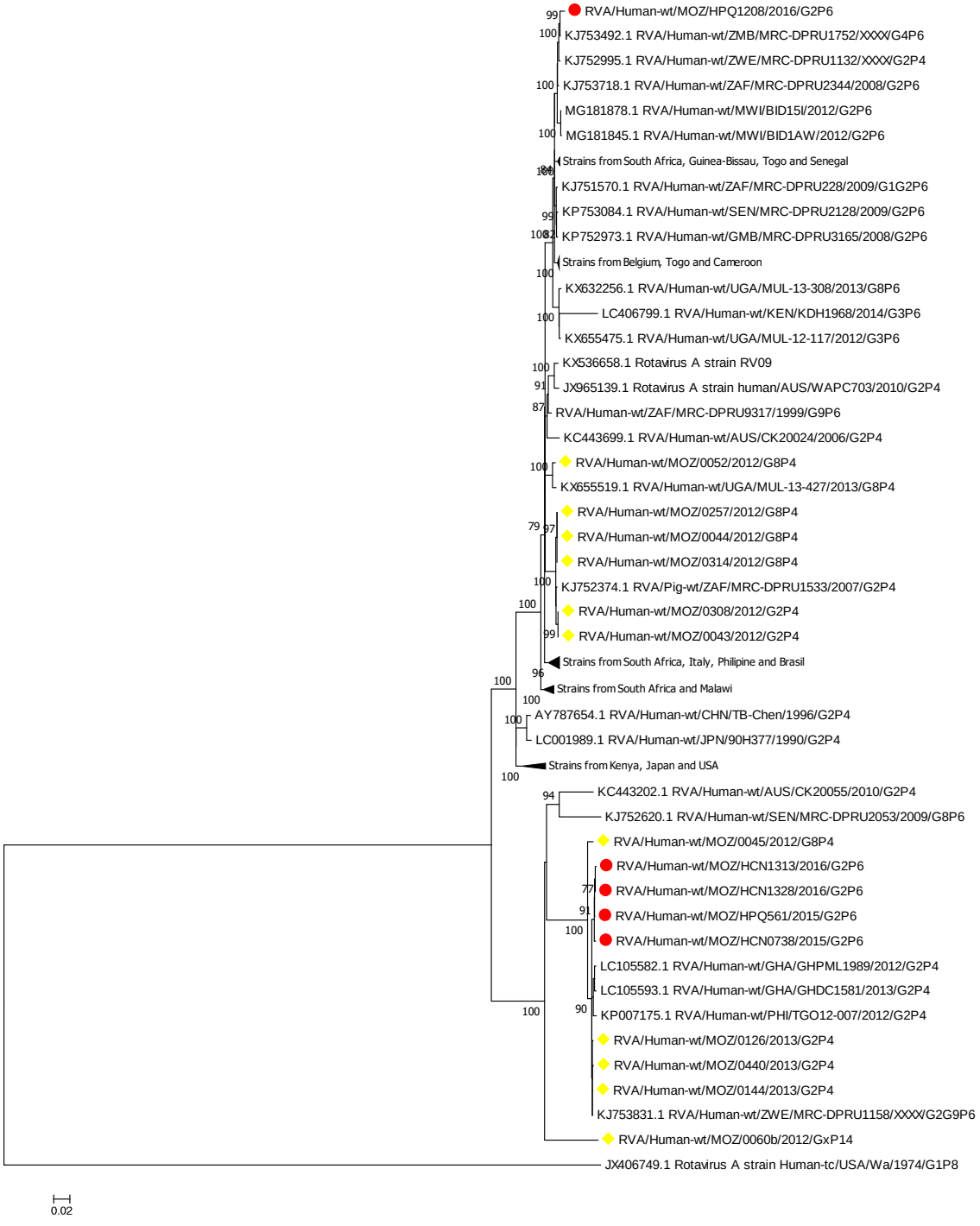
C: VP1 (genome segment 1)- genotype R2



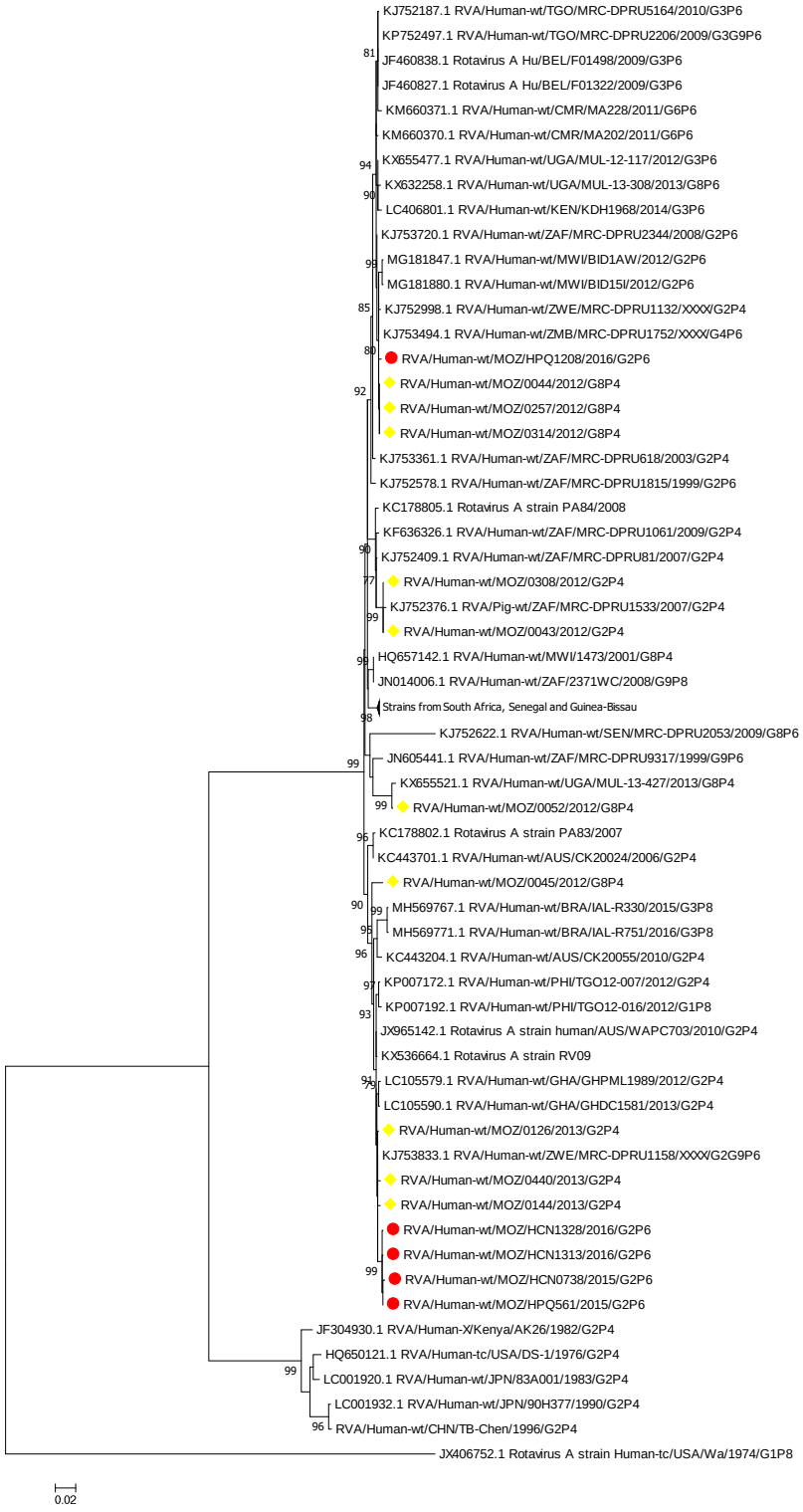
D : VP2 (genome segment 2)-genotype C2



E: VP3(genome segment 3)-genotype M2



F: VP6 (genome segment 6)- genotype E2



5.4.2.4. NSP1-NSP5/NSP6 encoding nucleotide sequences (genome segments 5, 8, 7, 10 and 11)

In the phylogenetic analysis for genome segments 5 and 7, encoding NSP1 and NSP3, respectively, for the DS1-like G2P[6] Mozambican strains, all strains grouped together with the exception of HPQ1208. The strains had a high nucleotide identity of an average of 99.52% (Supplemental Table 2E, G, Figure 1G, I). Their closest relatives were strains from Zambia (RVA/Human-wt/ZMB/MRC-DPRU1752/XXXX/G4P6) and Zimbabwe (RVA/Human-wt/ZWE/MRC-DPRU1132/XXXX/G2P4 and RVA/Human-wt/ZWE/MRC-DPRU1158/XXXX/G2G9P6) (Figure1 G, I). Strain HPQ1208 had an identity of 96.43% and 96.77% for NSP1 and NSP3 encoding nucleotide sequences, respectively (Supplementary Table 2E, G, Figure 1 G, I). This strain also clustered with three G2P[4] Mozambican strains (RVA/Human-wt/MOZ/0440/2013/G2P4, RVA/Human-wt/MOZ/0126/2013/G2P4, RVA/Human-wt/MOZ/0144/2013/G2P4) previously determined (Fig.1G, I, Supplementary Table 2E, G) .

The NSP2, NSP4 and NSP5 encoding nucleotide sequences of the Mozambican G2P[6] strains grouped together. However, the NSP2 encoding nucleotide sequence of strain HPQ1208 only had an identity of 98.77% with other G2P[6] Mozambican strains. This genome segment encoding NSP2 also had a high identity with strains from Mozambique (RVA/Human-wt/MOZ/0144/2013/G2P4, RVA/Human-wt/MOZ/0440/2013/G2P4, RVA/Human-wt/MOZ/0126/2013/G2P4) and a Zimbabwean strain (RVA/Human-wt/ZWE/MRC-DPRU1158/XXXX/G2G9P6) (Figure 1H, Supplementary Table 2H).

For the NSP4 encoding nucleotide sequences the nucleotide identity ranged from 99.16% to 100% (Supplementary Table 2J). The strains were more closely related to a Zambian strain RVA/Human-wt/ZMB/MRC-DPRU1752/XXXX/G4P6. Interestingly, in the same cluster these G2P[6] strains also grouped with other Mozambican strains containing a DS1-like backbone, one G2P[4] strain at an identity of 98.33-98.54% and four G8P[4] strains at an identity between 98.54-98.95% (Figure 1J, Supplementary Table 2).

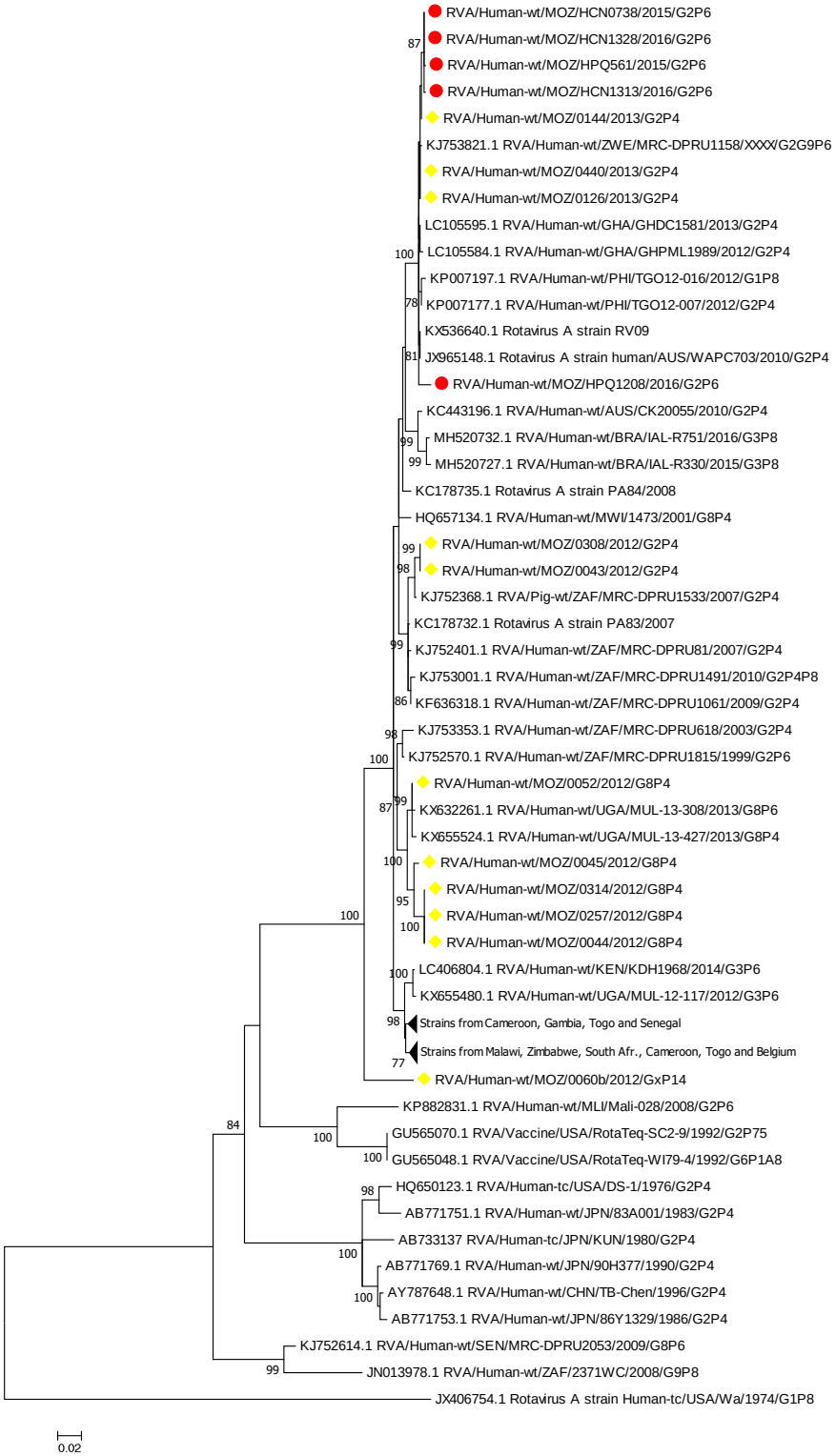
Finally, the NSP5 encoding nucleotide sequences from this study grouped separately, four strains (HPQ561, HCN0738, HCN1328 and HCN1313) had an identity of 99.46% clustered in the major group that contain a Mozambican strain 0308 from 2012 and other strains from

Zimbabwe, South Africa, Malawi and Italy. In this larger group strains HPQ561, HCN0738 and HCN1313 grouped together were closely related to RVA/Human-wt/ZMB/MRC-DPRU1752/XXXX/G4P6, RVA/Human-wt/ZAF/MRC-DPRU1491/2010/G2P4P8 and RVA/Human-wt/ZWE/MRC-DPRU1132/XXXX/G2P4, although strains HCN1313 are isolated from others and strains HCN1328 formed a small cluster within the large cluster and a closely related to strain RVA/Human-wt/ITA/PA84/2008/G2P4 (Supplementary Table 2K, Figure 1K).

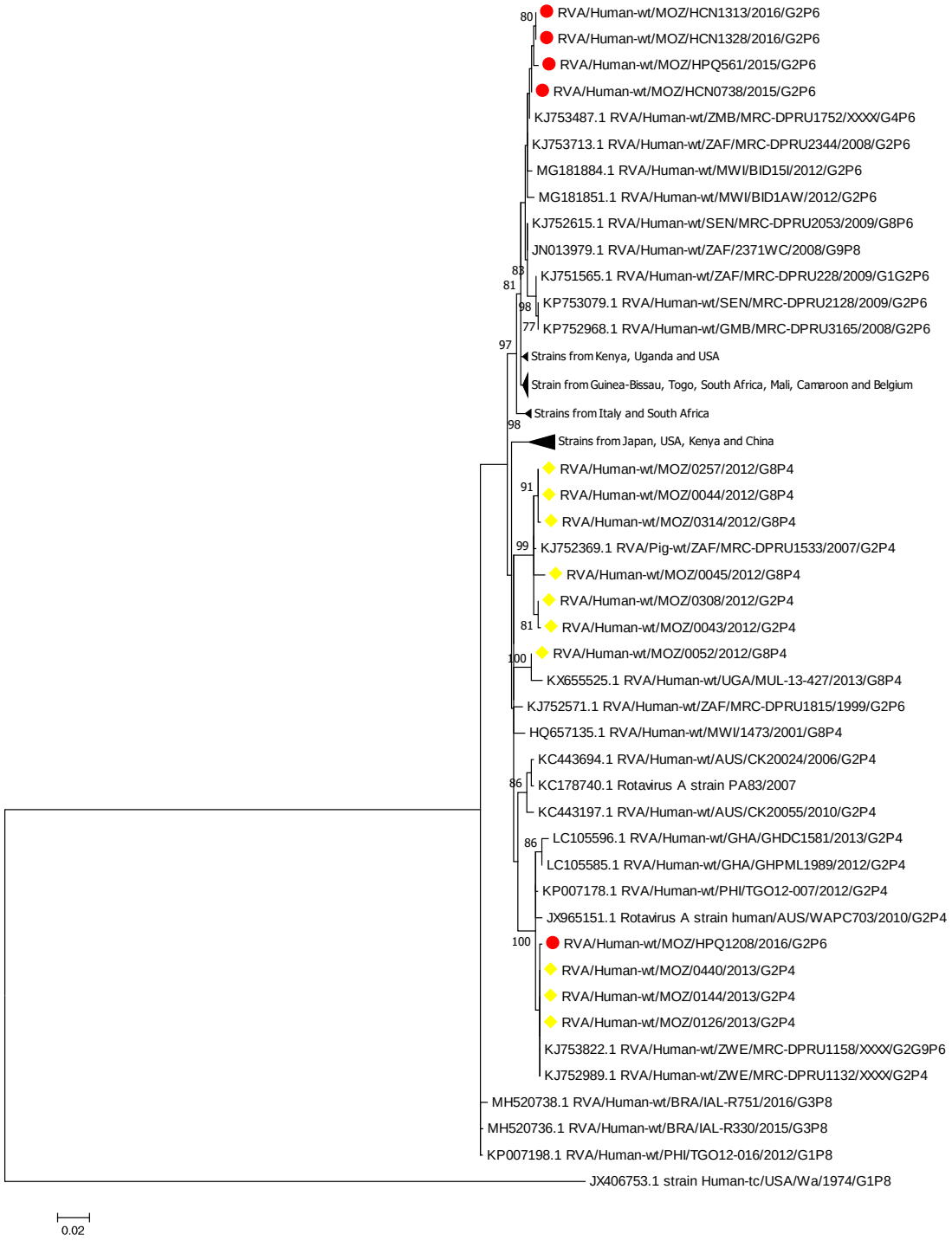
Strain HPQ1208 clustered separately to other G2P[6] strains and only had an average identity of 98.39% with other four strains of current study. This strain hdad an identity of 99.67% with Mozambican strains 0126 and 0440 from 2013 (Supplementary Table 2K, Figure 1K).

G: NSP1 (genome segment 5)-genotype A2

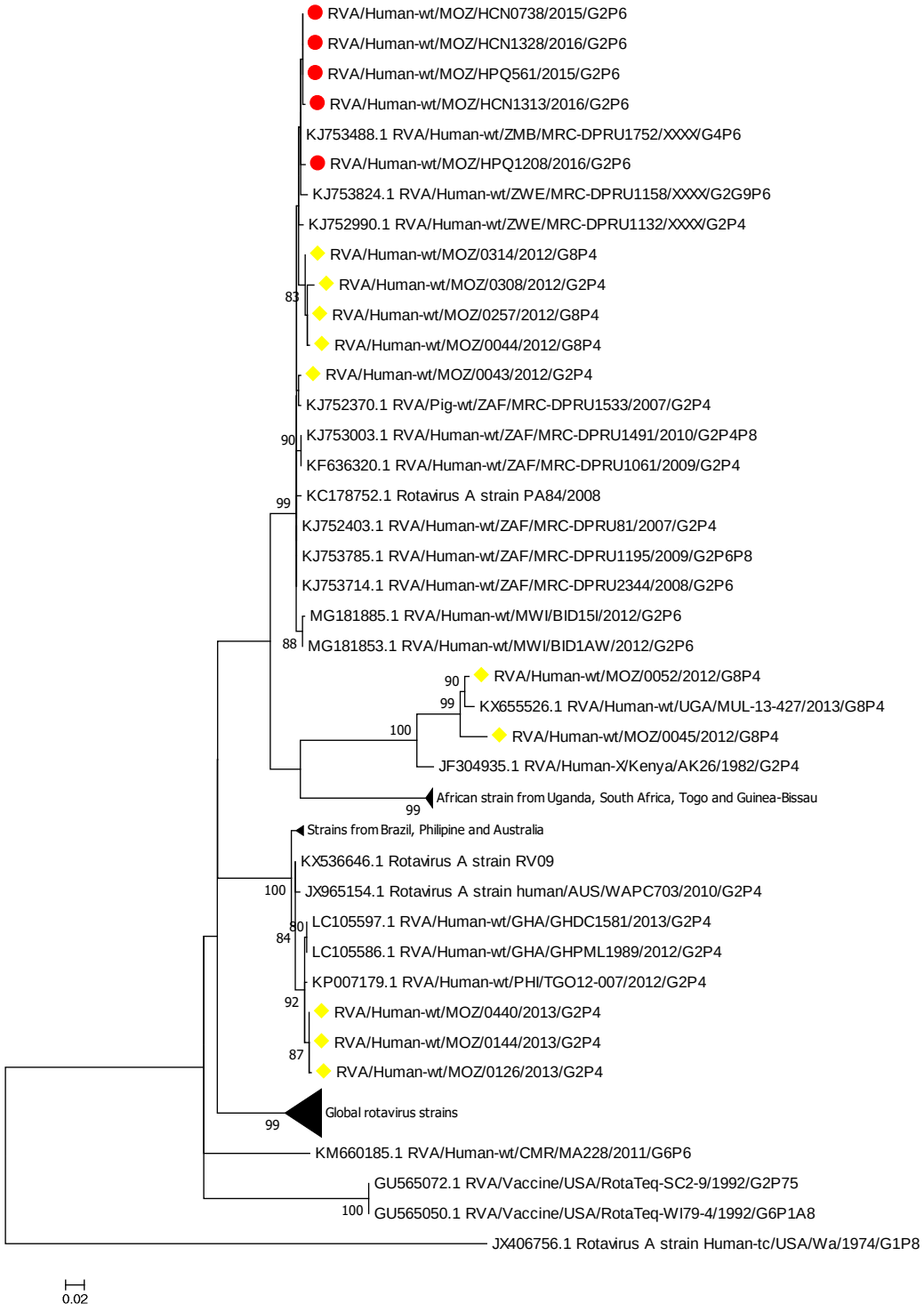
H: NSP2 (genome segment 8)-genotype N2



I: NSP3 (genome segment 7)-genotype T2



J: NSP4 (genome segment 10)-genotype E2



K: NSP5/6 (genome segment 11)-genotype H2

5.5. Discussion

The study described in Chapter 2, reported detection in high frequency of G2 in combination with P[4] in 2013 [Joao et al., 2018], however, in recent years this genotype disappeared and appeared in combination with P[6] in the National Acute Diarrheal Surveillance at low frequency since 2015. The whole genome sequence of G2P[4] strains from 2013 was determined, revealing typical genome DS1-like backbone and high diversity with several genome reassortment events [Strydom et al., 2019a]. In the current analyses the G2P[6] Mozambican strains from 2015 and 2016 circulated in the central (HPQ1208 and HPQ561) and northern (HCN1313, HCN1328 and HCN738) regions of Mozambique also had the typical DS1-like genome backbone as strains described previously [Strydom et al., 2019a]. No association was observed between strain and area where the sample was collected, suggesting that the G2P[6] circulating in the northern and central regions are the same.

VP7 encoding nucleotide sequences (genotype G2 Mozambican strains) from the current analysis are related to three G2 detected in 2013 from the southern region, suggesting an unique introduction or the circulation of strains with same genome makeup in country. This finding was reinforced by the lineage in which all clustered in the same lineage IV, sub-lineage 4a-3, described previously [Strydom et al., 2019a]. However, the Mozambican G2 strains also clustered with Zimbabwean strain (RVA/Human-wt/ZWE/MRC-DPRU1132/XXXX/G2P4), suggesting the circulation of the same strain between the two neighbouring countries.

The lineage IV of G2 has been reported to dominate globally since 2000 with three distinct sub lineages [Doan et al., 2017]. The circulation of lineage IVa-3 has also been reported in Ghana [Agbemabiese et al., 2016]. However, G2 strains belonging to lineage II were reported from South Africa and Togo [Nyaga et al., 2014].

The P[6] Mozambican strains grouped together with high identity and are related to Zimbabwean strain (RVA/Human-wt/ZWE/MRC-DPRU1158/XXXX/G2G9P[6] and Zambian strains RVA/Human-wt/ZMB/MRC-DPRU1752/XXXX/G4P[6]. The P[6] strains in the present study were not related to P[6] strains detected in 2012 [Strydom et al., 2019b]

showing the diversity pathways of evolution of this genotype. In Africa this genotype has been reported as endemic [Nyaga et al., 2018].

The VP1-3, VP6 and NSP1-NSP5/6 encoding nucleotide sequences of Mozambican G2P[6] strains from 2015 and 2016 were, in general, closely related to each other, with the exception of one strain. The closest relatives were Mozambican DS1-like strains (G2P[4] and G8P[4]) that circulated between 2012 and 2013 [Strydom et al., 2019a], and reinforced again that similar DS1-like strains persist in circulation in country. With regards to other countries, the African strains grouped mostly with high identities among several clusters, particularly, with neighbouring countries such as Zimbabwe (strain RVA/Human-wt/ZWE/MRC-DPRU1158/XXXX/G2G9P6, RVA/Human-wt/ZWE/MRC-DPRU1132/XXXX/G2P4) and Zambia (strain RVA/Human-wt/ZMB/MRC-DPRU1752/XXXX/G4P6), suggesting a similar genetic makeup for these strains circulating in these countries. However, the Mozambican strains also clustered with strains from other countries, such as Philippines, Ghana, Italy and Australia.

In the phylogenetic analyses it was observed that in general most of segments grouped more closely with G2P[4] and G4P[6] strains than with G2P[6], are part for segments VP6, NSP3, NSP4 and NSP5 encoding sequences that also grouped with G8P[4] strains. The fact that VP6 (strain HPQ1208), NSP4, NSP5 encoding sequences grouped with strains previously reported as possible reassortants (eg. strains 0308, 0257, 0044 and 0314) [Joao et al., 2018; Strydom et al., 2019a], reinforces the idea that possible reassortment events are still occurring in DS1-like Mozambican strains. This was observed by Nyaga et al., (2018) in South Africa strains. However, to confirm this further analyses are need.

This analysis had some limitations, namely the low number of strains characterized for whole genome that could not allow conclusive analysis of the genetic diversity.

5.6. Conclusion

The G2P[6] strains isolated in Mozambique in 2015 and 2016 showed a typical DS1-like genome backbone of strains and the high identity with Mozambican strains detected in 2012 and 2013, showing that DS1-like strains with same genetic makeup are still circulating in Mozambique. The analysis suggests the continued occurrence of diverse

reassortment events between DS1-like strains in Mozambican. The study underscored the importance of monitoring the emergence of DS1-like strains in Mozambique taking into account that the vaccine introduced was the monovalent containing a G1P[8] strain.

CHAPTER 6 General discussion and conclusions

6.1. General discussion

Rotavirus strains surveillance and caracterization of rotavirus strains based on the G (VP7) and P (VP4) genotypes

Before the vaccine introduction in Mozambique (2011-2015), RVA surveillance and studies in southern region reported a prevalence of RVA ranged in 24-40% [de Deus et al., 2018a; de Deus et al., 2018b; Langa et al., 2016]. After the introduction of the vaccine in September 2015, its impact has been monitored suggesting a substantial reduction in the prevalence of RVA infection to 12.2% and 13.5% in 2016 and 2017, respectively [de Deus et al., 2018a]. The number of rotavirus strains characterized here in the post vaccination period was lower compared to pre-vaccination period. This was due to the impact of vaccine in reducing the number of rotavirus infections in children [de Deus et al., 2018a]. Additionally, it is important to mention that Rotarix®, despite being a monovalent vaccine (G1P[8] strain), induces cross-immunity against other rotavirus strains. Similar cross-immunity has been observed for RotaTeq®, a pentavalent vaccine (G1, G2, G3. G4 and P[8] strains). Both have been reported with similar efficacy and effectiveness [Parashar et al., 2016].

The information about which rotavirus strains are circulating in Mozambique helps to understand the diversity of RVA, important to guide post-vaccine introduction monitoring and in understanding reasons for any program failure that may occur [WHO, 2002]. For example the surveillance of RVA strains can detect if the expanded immunization program in Mozambique influences the distribution of RVA strains and thus monitor the possible selective pressure induced by the vaccine [Banyai et al., 2012; Matthijnssens et al., 2012a].

In this thesis, the genetic characterization of RVA strains was done using a dual system of typing of RV strains by RT-PCR of VP7 (G) and VP4 (P) [Gentsch et al., 1992; Gouvea et al., 1990] described in Chapter 2 and 3. In addition, complete genome characterization for each of the eleven genome segments for selected strains was determined (Chapters 4 and 5).

The dual characterization was determined with high accuracy in Mozambique, with a high percentage of strains detected by RT-PCR confirmed by sequencing results reported in

Chapters 2, 4 and 5. The Sanger sequencing method as well as the NGS method elucidated some partial genotypes and full genome sequences of strains that could not be typed using RT-PCR. These results suggested high sensitivity of these methods for genetic characterization of strains [Dung et al., 2017]. However, in the Chapter 2 some strains detected by RT-PCR failed in sequencing using Sanger method, which can be explained by the low quality of DNA sample used [Dung et al., 2017].

Partial typing or the inability to type certain strains using RT-PCR (Chapter 2 and 3), can be due to incorrect annealing of the genotyping primer, presence of inhibitors in the extracted RNA that prevents the functioning of used enzymes in RT-PCR and low amount of RNA in the samples [Hungerford, 2019b].

Data from 2012 and 2013 in the initial RVA epidemiological study (Chapter 2) accessed the characterization of strains in urban and rural area, reported high frequency of genotype G2P[4]. The characterization of RVA strains from the National Surveillance of Acute Diarrhoea in children between 2014 and 2018 led by the "*Instituto Nacional de Saúde* (INS), which included several sites (Chapter 3) detected several rotavirus strains circulating in Mozambique such as G1P[8], G9P[8], G9P[4], G9P[6] G3P[4], G3P[8] and others strains detected in less frequency such as G2P[6]. During the surveillance, in general, the genotype combinations G9P[8] and G1P[8] were the most prevalent.

Analysing the data generated from Chapters 2 and 3 and considering Mavalane General Hospital as a model site, because data for this site was available from 2012 and 2018, it was observed that the RVA presented yearly variations on prevalence of strains prior to vaccine introduction (2012-2015), where it was detected circulation of genotypes G2P[4] and G12P[6] (2012-2013); G1P[8] and G9P[8] (2014-2015); and in post vaccine introduction (2016-2018) it was observed the emergence of genotypes such as the uncommon rotavirus strain combination G3P[4] and a common combination G3P[8], however rarely detected in Mozambique. It is not clear if in post-vaccine period, the emergence of strains was due the natural seasonal variation of RV previously reported worldwide [Banyai et al., 2012; Page et al., 2017; Yang et al., 2008] or due to vaccine pressure. However, coincidently, these emerging strains are also circulating in other Southern African countries such as Malawi, South Africa, Kingdom of Eswatini, Botswana (13th African Rotavirus Symposium, 2019;

http://ars.samrc.ac.za/programme.htm). The circulation of the same RVA strains in these Africa countries can be due to shared borders between these countries, however, there is a need to analyse the strains using full genome sequencing to confirm this hypothesis. Additionally, these observations suggest that G3 strains were circulating in Southern Africa during 2015–2018, with a sharp increase in 2018.

In general, the results presented here are consistent with previously published studies, as a number of countries from Africa, Europe and North America reported a variation in the strain diversity between the periods pre- and post-vaccination [Bar-Zeev et al., 2016; Hungerford et al., 2019; Lartey et al., 2018; Luchs et al., 2015; Matthijnssens et al., 2014; Page et al., 2017; Seheri et al., 2017], emphasising the importance to continue surveillance of rotavirus strains across country.

Caracterization of rotavirus strains based on 11 genome segments

Due to the segmented nature of the RV genome [McDonald et al., 2016], it is likely to occur several ressortment events (the main evolution RV mechanism) [McDonald et al., 2016]. Therefore, performing genetic characterization only using dual classification can be insufficient, and as a result whole genome characterization has been used for rotavirus strains [Matthijnssens and Van Ranst, 2012].

The whole genome characterization of G1P[8] and G9P[8] in this work in Chapter 4 in several Mozambican regions revealed the Wa-like strains with typical genome backbone circulated in the country. However, the P[8] strains revealed high diversity including the detection of a rare P[8] lineage in circulation in Mozambique. Additionally, analyses on the antigenic regions from Mozambican strains compared to Rotarix® vaccine revealed several substitutions.

Analysis of the G2P[6] strains in Chapter 5 revealed also DS1-like with typical genome backone, however, with some possible reassortment events reported previously in a related study that characterized G2P[4] and G8P[4] DS1-like strains [Strydom et al., 2019a], although there is a need to do more analyses.

The full genome analyses showed Wa-like with typical genome backbone from strains G1P[8] and G9P[8] and DS1-like with typical genome backone from G2P[6] strains. There

is a need to continue the surveillance in order to monitor possible reassortments events derived from vaccine pressure.

Rotavirus characterization based on two genome segments VS eleven segments and methods for characterization

The dual G and P typing has historically been considered to be adequate for rotavirus surveillance, epidemiology and for the identification of escapees during vaccine efficacy studies [Leshem et al., 2014; Patel et al., 2016]. However, sequencing of just 2/11 genome segments disregards approximately 81% (15,138 bp/ 18,550 bp) of the genome that exists outside these capsid genes, thus limiting the phylogenetic understanding of the other genome segments and the evolutionary processes that may be active across the rotavirus genome. To address this limitation, a more complex rotavirus classification system using all eleven genomic segments has been established [Dung et al., 2017; Matthijnssens et al., 2011a; Matthijnssens et al., 2008a].

Despite the introduction of a whole genome classification system for RV, whole genome sequencing is yet to be universally adopted for routine identification, surveillance (including in Mozambique) and phylogenetic studies. For whole genome sequencing of rotavirus strains most groups perform amplification of individual segments which contributes to bias introduced by PCR [Dung et al., 2017; Matthijnssens et al., 2008a; Matthijnssens et al., 2006; Ramani et al., 2009]. This is also time consuming as individual segments have to be amplified. It is often also not possible to determine the entire genome segment sequence, especially non-coding regions established [Dung et al., 2017; Matthijnssens et al., 2008a; Matthijnssens et al., 2006; Ramani et al., 2009]. Large gene segments have to be coupled with primer-walking to complete sequencing PCR [Matthijnssens et al., 2008a].

Whole genome amplification combined with next generation sequencing methods can be utilized to overcome many of the complications that prevent the generation of adequately characterized RV gene sequences representative of the complete diversity present in a single sample [Dung et al., 2017; Potgieter et al., 2009; Nyaga et al., 2014, Strydom et al.,

2019a]. These more advanced and sequence-independent methods which have reduced bias introduced during PCR and specific primers were successfully applied in this study.

On the other hand, the use of Sanger sequencing methods, which generate a single consensus sequence based on nucleotide abundance, does not allow easily detection of complete genetic variation, the presence of mixed infection within a sample it is not easy to interprete, which may bias inference related to RV infection and evolution [Dung et al., 2017].

Additionaly, third-generation sequencing, such as nanopore technology, can directly sequence single DNA molecules without aplification. Nanopore technology is a method for determining the order and modifications of DNA/RNA nucleotides by detecting the electric current variations when DNA/RNA oligonucleotides pass through the nanometer-sized hole (nanopore) (https://nanoporetech.com). Nanopore sequencing has beeen adapted to a portable and affordable instrument, called MinION (Oxford Nanopore Technologies). This technology might be a useful and affordable diagnostic tool with rapid sample preparation and real-time sequence analysis (https://nanoporetech.com). In the field of virology, this technology has mainly been applied in human medicine. Using nanopore sequencing, for example, it has been possible to distinguish three poxviruses with 98% nucleotide similarity at strain level [Kilianski et al., 2015]. It has been used as a diagnostic tool during the recent Ebolavirus outbreaks in West Africa, allowing fast on-site characterization of circulating strains [Hoenen et al., 2016]. In the veterinary field, it has been tested as a rapid and easy-to-use diagnostic tool in pig health management for diagnosis of viral enteric disease complexes [Theuns et al., 2018]. However, for rotavirus strains characterizations no data are available using this technology, therefore, such new methodology could be in the near future a promising technology, but more extensive validations shoud be done.

In the context of Mozambique, rotavirus characterization by dual typing of RV strains using RT-PCR of VP7 (G) and VP4 (P) was well established. To the author's knowledge, before this work, sequencing methods for rotavirus characterization had not been introduced in Mozambique. In the first phase, Sanger sequencing can be introduced as part of strain surveillance method to elucidate partial typeable strains, untypeable strains, uncommon rotavirus strains and suspect vaccine strain shedding cases due to Rotarix® (vaccine derived

strains) [Roczo-Farkas et al., 2017]. This technology for Mozambique can be a preffered cost-effective choice when interrogating a small region of DNA on a limited number of samples or genomic targets. Although time consuming, individual gene characterization of rotavirus strains using specific primers can be done using Sanger sequencing and performing primer walking to cover the complete sequences of segments on both strands [Matthijnssens et al., 2008a] for regions that are not amplified using available primers.

Due to the training received, preparation of cDNA can now be performed in Mozambique which can then be shipped to a suitable service laboratory such as the UFS NGS Unit for complete characterization by NGS of Mozambican strains whenever the need is justified.

Limitations

A low number of strains were characterized at some sentinel sites (eg. Beira Central Hospital and Quelimane Provincial Hospital, both in center region), which can introduce bias in the analysis of the strain diversity, and consequently influence the lack of strain representation by region for whole genome characterization of rotavirus;

Only one sentinel site (Mavalane General Hospital, southern region) had two year genotyping data from pre-vaccination period, whereas only one year genotyping data were available for the remaining of the sentinel sites, therefore no strains representative from pre-vaccination period of all regions from National Surveillance of Acute Diarrhea was possible to include in genome characterization of rotavirus;

The low number of selected strains for whole genome characterization of Wa-like and DS1-like rotavirus strains could not allow robust conclusions regarding genomic diversity.

6.2. Conclusions

With this thesis it was possible to draw the following conclusions:

- Rotavirus strain diversity was observed in Mozambique, including circulation of common and uncommon genotype combinations;
- Yearly variations on prevalence of rotavirus genotypes were observed prior and post-vaccine introduction;
- The complete genome characterization of rotavirus strains from Mozambique revealed circulation of G1P[8]/G9P[8] and G2P[6] with typical Wa-like and DS1-like genome backbones, respectively;
- The Wa-like strains are mostly related to Asian strains (India, China and Japan) and DS1-like strains are mostly related to other previously Mozambican characterized strains and strains from neighbouring countries (manly Zimbabwe and Zambia);
- The DS1-Like strains revealed occurrence of possible reassortment events;
- The strains characterized from different sites from Mozambique had similar genome makeup around the country.

7. References

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APPENDICES

APPENDIX A: Ethical letters

REPÚBLICA DE MOÇAMBIQUE

MINISTÉRIO DA SAÚDE

COMITÉ NACIONAL DE BIOÉTICA PARA A SAÚDE
IRB00002657

Exma Senhora
Drª Nilsa de Deus
INS

Ref: 348/CNBS/13 **Data 26 de Novembro de 2013**

Assunto: Parecer do Comité Nacional de Bioética para Saúde (CNBS) sobre o estudo: "*Vigilância Nacional de Diarreias Agudas em Crianças (ViNaDiA)*"

O Comité Nacional de Bioética para Saúde (CNBS) analisou as correcções efectuadas no protocolo intitulado: "***Vigilância Nacional de Diarreias Agudas em Crianças (ViNaDiA)***", conforme os requisitos da Declaração de Helsínquia,

Não havendo nenhum inconveniente de ordem ética que impeça a realização do estudo, o CNBS dá a sua devida aprovação.

Todavia, o CNBS recomenda aos investigadores que o mantenham informado do decurso do estudo, salientando que esta aprovação ética não substitui a autorização administrativa.

Com as nossas mais cordiais saudações.

O Presidente

Dr. João Fernando Lima Schwalbach

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HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

IRB nr 00006240
REC Reference nr 230408-011
IORG0005187
FWA00012784

01 August 2016

DR HG O'NEILL
DEPT OF MICROBIAL, BIOCHEMICAL AND
FOOD BIOTECHNOLOGY
FACULTY OF NATURAL AND AGRICULTURAL SCIENCES
UFS

Dear Dr O'Neill

ECUFS NR 201/2013
DR HG O'NEILL DEPT OF MICROBIAL, BIOCHEMICAL AND FOOD BIOTECHNOLOGY
PROJECT TITLE: WHOLE GENOME CONSENSUS SEQUENCE DETERMINATION OF MOZAMBICAN ROTAVIRUS STRAINS.

1. You are hereby kindly informed that, at the meeting held on 26 July 2016, the Health Sciences Research Ethics Committee (HSREC) took note with approval of the following:

   - *Protocol Amendment:*
     - *Detailed protocol including budget with track changes and final version*
     - *Completed staff addition form*
     - *CV of post-doctoral fellow*

2. 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.

Yours faithfully

.....

PROF WJ STEINBERG
VICE CHAIR: HEALTH SCIENCES RESEARCH ETHICS COMMITTEE

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2013-11-28

REC Reference nr 230408-011
IRB nr 00006240

DR HG O'NEILL
DEPT OF MICROBIAL, BIOCHEMICAL AND
FOOD BIOTECHNOLOGY
FACULTY OF NATURAL AND AGRICULTURAL SCIENCES
UFS

Dear Dr O'Neill

ECUFS NR 201/2013
DR HG O'NEILL DEPT OF MICROBIAL, BIOCHEMICAL AND FOOD BIOTECHNOLOGY
PROJECT TITLE: WHOLE GENOME CONSENSUS SEQUENCE DETERMINATION OF MOZAMBICAN ROTAVIRUS STRAINS.

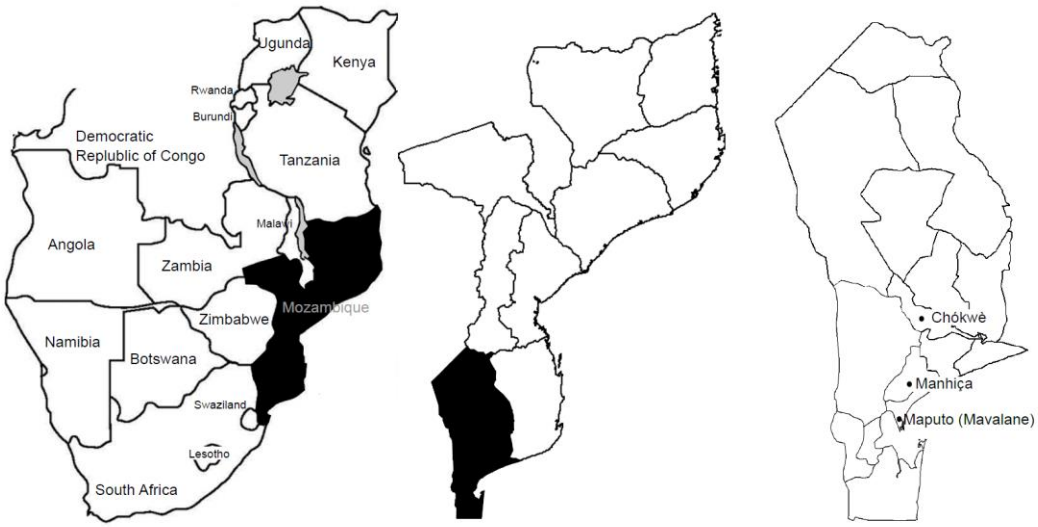
- You are hereby kindly informed that the Ethics Committee approved the above project at the meeting held on 26 November 2013.
- Committee guidance documents: Declaration of Helsinki, ICH, GCP and MRC Guidelines on Bio Medical Research. Clinical Trial Guidelines 2000 Department of Health RSA; Ethics in Health Research: Principles Structure and Processes Department of Health RSA 2004; Guidelines for Good Practice in the Conduct of Clinical Trials with Human Participants in South Africa, Second Edition (2006); the Constitution of the Ethics Committee of the Faculty of Health Sciences and the Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines.
- Any amendment, extension or other modifications to the protocol must be submitted to the Ethics Committee for approval.
- The Committee must be informed of any serious adverse event and/or termination of the study.
- All relevant documents e.g. signed permission letters from the authorities, institutions, changes to the protocol, questionnaires etc. have to be submitted to the Ethics Committee before the study may be conducted (if applicable).
- A progress report should be submitted within one year of approval of long term studies and a final report at completion of both short term and long term studies.
- Kindly refer to the ETOVS/ECUFS reference number in correspondence to the Ethics Committee secretariat.

Yours faithfully

.....
FOR CHAIR: ETHICS COMMITTEE

APPENDIX B: Chapter 2

Supplementary material 1. Map indicating the sampling sites relative to each other and other southern and eastern African countries.



A B C

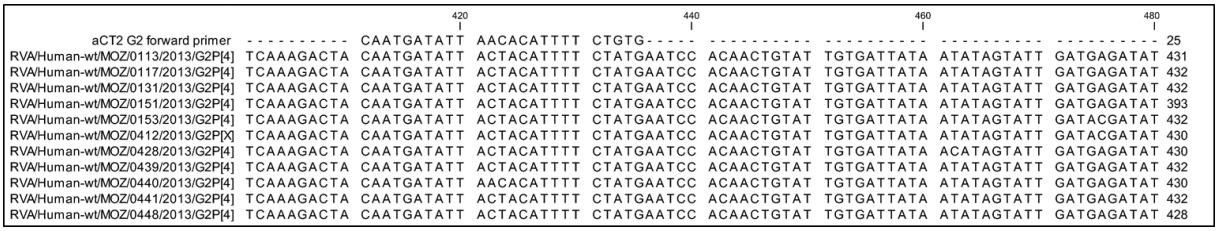
A: Southern and Eastern Africa with Mozambique highlighted; **B**: Mozambique with the southern Mozambican region highlighted; **C**: Southern Mozambique with study sites (Manhiça and Mavalane) highlighted. Chókwè, in the Gaza province, where a previous study by Langa and co-workers were carried out, is also indicated. The distance between Manhiça and Mavalane is 81 km.

Supplementary material 2: Comparison of G and P typing results using the genotyping PCR and Sanger sequencing

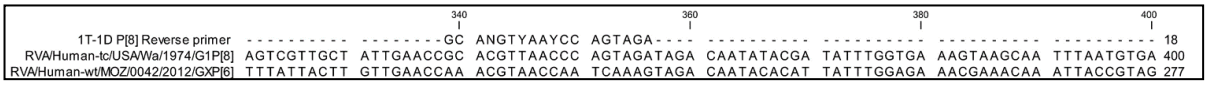
	Sample	Date	Area	Genotyping PCR	Sanger sequencing
1	0042	2012	Manhiça	GXP[8]	GXP[6]
2	0050	2012	Manhiça	G12P[X]	G12P[X]
3	0060	2012	Manhiça	G12P[X]	GXP[8]
4	0113	2013	Manhiça	GXP[4]	G2P[4]
5	0117	2013	Manhiça	GXP[4]	G2P[4]
6	0131	2013	Manhiça	G2P[4]	G2P[4]
7	0146	2013	Manhiça	G2P[4]	G2P[4]
8	0151	2013	Manhiça	GXP[4]	G2P[4]
9	0153	2013	Manhiça	GXP[4]	G2P[4]
10	0208	2012	Mavalane	G12P[6]	G12P[6]
11	0211	2012	Mavalane	G12P[6]	G12P[6]
12	0277	2012	Mavalane	G12P[6]	G12P[X]
13	0278	2012	Mavalane	G12P[6]	G12P[6]
14	0285	2012	Mavalane	G8P[4]	G8P[4]
15	0286	2012	Mavalane	G12P[6]	G12P[6]
16	0288	2012	Mavalane	G12P[8]	G12P[8]
17	0289	2012	Mavalane	G12P[6]	GXP[6]
18	0297	2012	Mavalane	G8P[4]	G8P[4]
19	0304	2012	Mavalane	G12P[6]	G12P[6]
20	0308	2012	Mavalane	G2P[4]	G2P[4]
21	0310	2012	Mavalane	G8P[4]	G8P[4]
22	0412	2013	Mavalane	NT	G2P[X]
23	0428	2013	Mavalane	G2P[4]	G2P[4]
24	0439	2013	Mavalane	G2P[4]	G2P[4]
25	0440	2013	Mavalane	G2P[4]	G2P[4]
26	0441	2013	Mavalane	G2P[X]	G2P[4]
27	0448	2013	Mavalane	G2P[4]	G2P[4]

Supplementary material 3. Comparison of binding sites of genotyping primers to nucleotide sequences of Mozambican strains.

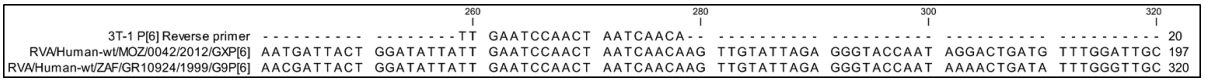
A



B



C



A: The VP7 encoding sequence of RVA/Human-wt/MOZ/0113/2013/G2P[4], RVA/Human-wt/MOZ/0117/2013/G2P[4], RVA/Human-wt/MOZ/0151/2013/G2P[4], RVA/Human-wt/MOZ/0153/2013/G2P[4] and RVA/Human-wt/MOZ/0412/2013/G2P[4] could only be only identified with nucleotide sequencing. The majority of Mozambican strains were similar to the primer sequence of aCT2 apart from position 422, 423 and 433. RVA/Human-wt/MOZ/0440/2013/G2P[4] were identical to the primer sequence apart from position 433. B: Alignment of the 1T-1D primer (P[8]) with RVA/Human-tc/USA/Wa/1974/G1P[8] and RVA/Human-wt/MOZ/0042/2012/GXP[6]. C: Alignment of reverse primer 3T-1 (P[6]) to RVA/Human-wt/ZAF/GR10924/1999/G9P[6] and strain RVA/Human-wt/MOZ/0042/2012/GXP[6] showed perfect conservation of the primer binding region.

APPENDIX C: Chapter 4

Supplementary Table 1. Genome assembly of Wa-like Mozambican rotavirus strains detected between 2014 and 2016

Strain name	Trimmed reads		VP1	VP2	VP3	VP4	VP6	VP7	NSP1	NSP2	NSP3	NSP4	NSP5/6
RVA/Human-wt/MOZ/HGM0048/2014/G1P[8]	92,448	% ORF	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		% length	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		Average coverage	606.0	542.0	735.0	901.0	1367.0	1658.0	1191.0	1382.0	1663.0	1758.0	1777.0
		% Identity (ViPR)	98.8	98.5	98.0	97.2	99.0	93.3	97.7	98.4	97.5	98.7	99.2
RVA/Human-wt/MOZ/HGM0059/2014/G1P[8]	82,098	% ORF	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		% length	100.0	–	–	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		Average coverage	450.0	-	-	637.0	1165.0	1483.0	799.0	967.0	1180.0	1962.0	1895.0
		% Identity (ViPR)	98.8	–	–	97.2	99.0	93.3	97.7	98.5	97.4	9.7	99.2
RVA/Human-wt/MOZ/HCN154/2015/G1P[8]	209,339	% ORF	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		% length	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		Average coverage	921.0	1758.0	654.0	908.0	814.0	904.0	962.0	995.0	1166.0	1294.0	2223.0
		% Identity (ViPR)	98.7	99.0	98.0	98.7	99.0	93.0	97.7	98.6	97.6	98.7	99.0
RVA/Human-wt/MOZ/HJM338/2015/G1P[8]	137,395	% ORF	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		% length	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		Average coverage	1577.0	3671.0	1292.0	1720.0	2045.0	2037.0	2317.0	1915.0	2193.0	2570.0	2957.0
		% Identity (ViPR)	98.6	98.4	98.0	97.0	99.0	93.2	97.6	98.4	97.0	98.5	99.2
RVA/Human-wt/MOZ/HCN1011/2016/G1P[8]	346,985	% ORF	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		% length	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		Average coverage	1681.0	1368.0	2231.0	2008.0	3826.0	4579.0	3351.0	3162.0	3824.0	5922.0	4778.0
		% Identity (ViPR)	98.5	98.4	97.9	97.0	99.0	93.4	97.5	98.6	97.4	98.5	99.2
RVA/Human-wt/MOZ/HPQ1152/2016/G1P[8]	259,362	% ORF	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		% length	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		Average coverage	1155.0	967.0	1526.0	1568.0	2564.0	3047.0	2342.0	1997.0	2196.0	3371.0	2682.0

		% Identity (ViPR)	98.6	98.4	97.8	97.0	99.0	93.2	97.7	98.4	97.3	98.5	98.6
RVA/Human-wt/MOZ/HGM1265/2016/G1P[8]	300,402	% ORF	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		% length	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		Average coverage	2014.0	1597.0	2673.0	2633.0	3978.0	4971.0	3701.0	3603.0	4153.0	5110.0	3601.0
		% Identity (ViPR)	98.5	98.3	97.7	96.9	93.0	99.0	97.5	98.3	97.3	98.5	99.0
RVA/Human-wt/MOZ/HCN1336/2016/G1P[8]	354,697	% ORF	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		% length	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		Average coverage	4125.0	3570.0	5048.0	4954.0	3165.0	2850.0	4913.0	2521.0	2698.0	2108.0	1767.0
		% Identity (ViPR)	98.5	98.3	97.8	97.8	99.0	93.3	97.5	98.4	97.4	98.5	99.2
RVA/Human-wt/MOZ/HGM353/2015/G9P[8]	331,080	% ORF	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		% length	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		Average coverage	1663.0	1619.0	2094.0	2525.0	4155.0	5153.0	3360.0	3819.0	4372.0	5595.0	4913.0
		% Identity (ViPR)	98.8	97.8	97.8	97.6	98.4	93.0	97.7	98.8	99.4	98.3	99.5
RVA/Human-wt/MOZ/HGM389/2015/G9P8	98,413	% ORF	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		% length	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		Average coverage	604.0	632.0	749.0	884.0	925.0	1004.0	809.0	661.0	1051.0	1096.0	831.0
		% Identity (ViPR)	98.8	97.8	97.8	97.5	98.3	93.0	97.7	99.0	99.3	98.3	99.5
RVA/Human-wt/MOZ/HJM643/2015/G9P[8]	389,259	% ORF	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		% length	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		Average coverage	1738.0	1647.0	2664.0	2608.0	4406.0	5584.0	4104.0	4345.0	5188.0	6844.0	5911.0
		% Identity (ViPR)	98.8	97.8	97.8	97.5	98.2	92.8	97.7	99.0	99.2	98.0	99.5
RVA/Human-wt/MOZ/HJM644/2015/G9P[8]	3,640,594	% ORF	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		% length	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		Average coverage	25787.0	24062.0	33743.0	34250.0	38521.0	45589.0	40564.0	30829.0	44790.0	47414.0	34716.0
		% Identity (ViPR)	99.0	98.3	98.2	89.2	99.0	93.5	98.2	99.0	99.0	99.0	99.8

Supplementary Table 2 (A-K). Pairwise distance (G1P[8] strains)

A. Genome segment 1 (VP1)

	Strains	1	2	3	4	5	6	7	8	9	10	11	12	13
1	RVA/Human-wt/MOZ/HGM0048/2014/G1P8													
2	RVA/Human-wt/MOZ/HGM0059/2014/G1P8	0.0015												
3	RVA/Human-wt/MOZ/HCN154/2015/G1P8	0.0021	0.0031											
4	RVA/Human-wt/MOZ/HJM338/2015/G1P8	0.0034	0.0043	0.0043										
5	RVA/Human-wt/MOZ/HCN1011/2016/G1P8	0.0037	0.0049	0.0031	0.0061									
6	RVA/Human-wt/MOZ/HPQ1152/2016/G1P8	0.0031	0.0040	0.0015	0.0052	0.0040								
7	RVA/Human-wt/MOZ/HGM1265/2016/G1P8	0.0040	0.0043	0.0031	0.0061	0.0043	0.0040							
8	RVA/Human-wt/MOZ/HCN1336/2016/G1P8	0.0040	0.0052	0.0034	0.0064	0.0009	0.0043	0.0040						
9	KT920717.1_RVA/Human-wt/USA/CNMC20/2011/G1P8	0.0058	0.0067	0.0067	0.0080	0.0086	0.0077	0.0086	0.0089					
10	KJ752015.1_RVA/Human-wt/ZAF/MRC-DPRU6954/2011/G1P8	0.0049	0.0058	0.0058	0.0070	0.0077	0.0067	0.0077	0.0080	0.0034				
11	KX674718.1_RVA/Human-wt/IND/RV1326/2013/G1P8	0.0055	0.0064	0.0064	0.0077	0.0083	0.0073	0.0083	0.0086	0.0040	0.0031			
12	KJ753412.1_RVA/Human-wt/ETH/MRC-DPRU906/XXXX/G1P8	0.0055	0.0064	0.0064	0.0077	0.0083	0.0073	0.0083	0.0086	0.0040	0.0031	0.0000		
13	KX674720.1_RVA/Human-wt/IND/RV1302/2013	0.0055	0.0064	0.0064	0.0077	0.0083	0.0073	0.0083	0.0086	0.0040	0.0031	0.0000	0.0000	
14	KJ752552.1_RVA/Human-wt/ZAF/MRC-DPRU4085/XXXX/G1P8	0.0052	0.0061	0.0061	0.0073	0.0080	0.0070	0.0080	0.0083	0.0037	0.0003	0.0034	0.0034	0.0034

B. Genome segment 2 (VP2)

	Strains	1	2	3	4	5	6	7	8	9	10	11
1	RVA/Human-wt/MOZ/HPQ1152/2016/G1P8											
2	RVA/Human-wt/MOZ/HJM338/2015/G1P8	0.0037										
3	RVA/Human-wt/MOZ/HGM1265/2016/G1P8	0.0048	0.0048									
4	RVA/Human-wt/MOZ/HGM0048/2014/G1P8	0.0037	0.0030	0.0041								
5	RVA/Human-wt/MOZ/HCN154/2015/G1P8	0.0030	0.0037	0.0041	0.0030							
6	RVA/Human-wt/MOZ/HCN1336/2016/G1P8	0.0048	0.0041	0.0060	0.0041	0.0048						
7	RVA/Human-wt/MOZ/HCN1011/2016/G1P8	0.0048	0.0041	0.0052	0.0034	0.0041	0.0007					
8	KY857563.1_Rotavirus_A_strain_G1P8_isolate_RV1327	0.0130	0.0130	0.0142	0.0115	0.0130	0.0142	0.0134				
9	KY857560.1_Rotavirus_A_strain_G1P8_isolate_RV1302	0.0130	0.0130	0.0142	0.0115	0.0130	0.0142	0.0134	0.0000			
10	KU738591.1_RVA/Human-wt/S1/NIV/0954435/2009/G9P8	0.0164	0.0156	0.0168	0.0142	0.0156	0.0160	0.0153	0.0123	0.0123		
11	HM773591.1_RVA/Human-wt/USA2009727036/2009/G9P8	0.0127	0.0127	0.0130	0.0112	0.0127	0.0138	0.0130	0.0078	0.0078	0.0104	
12	AB861946.1_RVA/Human-tc/KEN/KDH633/2010/G12P6	0.0149	0.0142	0.0160	0.0134	0.0149	0.0153	0.0153	0.0101	0.0101	0.0142	0.0104

C. Genome segment 3 (VP3)

	Strains	1	2	3	4	5	6	7	8	9	10	11
1	RVA/Human-wt/MOZ/HPQ1152/2016/G1P8											
2	RVA/Human-wt/MOZ/HJM338/2015/G1P8	0.0072										
3	RVA/Human-wt/MOZ/HGM1265/2016/G1P8	0.0068	0.0068									
4	RVA/Human-wt/MOZ/HGM0048/2014/G1P8	0.0060	0.0028	0.0056								
5	RVA/Human-wt/MOZ/HCN154/2015/G1P8	0.0040	0.0040	0.0036	0.0028							
6	RVA/Human-wt/MOZ/HCN1336/2016/G1P8	0.0056	0.0056	0.0052	0.0044	0.0024						
7	RVA/Human-wt/MOZ/HCN1011/2016/G1P8	0.0052	0.0052	0.0048	0.0040	0.0020	0.0012					
8	KX674725.1_Rotavirus_A_strain_RV1305	0.0120	0.0088	0.0116	0.0076	0.0088	0.0104	0.0100				
9	KJ753414.1_RVA/Human-wt/ETH/MRC-DPRU906/XXXX/G1P8	0.0120	0.0088	0.0116	0.0076	0.0088	0.0104	0.0100	0.0000			
10	KJ752554.1_RVA/Human-wt/ZAF/MRC-DPRU4085/XXXX/G1P8	0.0128	0.0096	0.0124	0.0084	0.0096	0.0112	0.0108	0.0040	0.0040		
11	KT920719.1_RVA/Human-wt/USA/CNMC20/2011/G1P8	0.0112	0.0080	0.0108	0.0068	0.0080	0.0096	0.0092	0.0024	0.0024	0.0032	
12	KJ752017.1_RVA/Human-wt/ZAF/MRC-DPRU6954/2011/G1P8	0.0140	0.0108	0.0136	0.0096	0.0108	0.0124	0.0120	0.0052	0.0052	0.0012	0.0044

D. Genome segment 4 (VP4)

	Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	RVA/Human-wt/MOZ/HJM644/2015/G9P8																
2	RVA/Human-wt/MOZ/HJM643/2015/G9P8	0.1168															
3	RVA/Human-wt/MOZ/HGM353/2015/G9P8	0.1173	0.0004														
4	RVA/Human-wt/MOZ/HGM389/2015/G9P8	0.1168	0.0000	0.0004													
5	RVA/Human-wt/MOZ/HGM0059/2014/G1P8	0.1173	0.0322	0.0318	0.0322												
6	RVA/Human-wt/MOZ/HGM0048/2014/G1P8	0.1173	0.0322	0.0318	0.0322	0.0000											
7	RVA/Human-wt/MOZ/HCN154/2015/G1P8	0.1181	0.0348	0.0344	0.0348	0.0034	0.0034										
8	RVA/Human-wt/MOZ/HJM338/2015/G1P8	0.1181	0.0339	0.0335	0.0339	0.0026	0.0026	0.0052									
9	RVA/Human-wt/MOZ/HCN1011/2016/G1P8	0.1173	0.0352	0.0348	0.0352	0.0056	0.0056	0.0039	0.0073								
10	RVA/Human-wt/MOZ/HPQ1152/2016/G1P8	0.1177	0.0352	0.0348	0.0352	0.0047	0.0047	0.0013	0.0064	0.0052							
11	RVA/Human-wt/MOZ/HGM1265/2016/G1P8	0.1181	0.0357	0.0352	0.0357	0.0052	0.0052	0.0034	0.0069	0.0030	0.0047						
12	RVA/Human-wt/MOZ/HCN1336/2016/G1P8	0.1194	0.0365	0.0361	0.0365	0.0069	0.0069	0.0052	0.0086	0.0021	0.0064	0.0043					
13	RVA/Human-wt/MOZ/0060a/2012/G12P8	0.1203	0.0305	0.0301	0.0305	0.0099	0.0099	0.0125	0.0116	0.0137	0.0137	0.0133	0.0150				
14	KX646611.1_Rotavirus_A_strain_RV1319	0.1164	0.0314	0.0309	0.0314	0.0017	0.0017	0.0043	0.0034	0.0064	0.0056	0.0060	0.0077	0.0099			
15	KX646588.1_Rotavirus_A_strain_RV1302	0.1173	0.0314	0.0309	0.0314	0.0034	0.0034	0.0060	0.0052	0.0073	0.0073	0.0069	0.0086	0.0090	0.0034		
16	KX646591.1_Rotavirus_A_strain_RV1305	0.1168	0.0318	0.0314	0.0318	0.0047	0.0047	0.0073	0.0064	0.0086	0.0086	0.0082	0.0099	0.0095	0.0047	0.0039	
17	KX646594.1_Rotavirus_A_strain_RV1327	0.1186	0.0326	0.0322	0.0326	0.0056	0.0056	0.0082	0.0073	0.0095	0.0095	0.0090	0.0107	0.0103	0.0056	0.0047	0,0052

E. Genome segment 5 (NSP1)

	Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	RVA/Human-wt/MOZ/HPQ1152/2016/G1P8																
2	RVA/Human-wt/MOZ/HJM338/2015/G1P8	0.0048															
3	RVA/Human-wt/MOZ/HGM1265/2016/G1P8	0.0041	0.0062														
4	RVA/Human-wt/MOZ/HGM0059/2014/G1P8	0.0034	0.0041	0.0048													
5	RVA/Human-wt/MOZ/HGM0048/2014/G1P8	0.0034	0.0041	0.0048	0.0000												
6	RVA/Human-wt/MOZ/HCN154/2015/G1P8	0.0021	0.0041	0.0034	0.0027	0.0027											
7	RVA/Human-wt/MOZ/HCN1336/2016/G1P8	0.0062	0.0082	0.0075	0.0068	0.0068	0.0055										
8	RVA/Human-wt/MOZ/HCN1011/2016/G1P8	0.0041	0.0062	0.0055	0.0048	0.0048	0.0034	0.0062									
9	RVA/Human-wt/MOZ/0289/2012/G12P6	0.0274	0.0281	0.0287	0.0267	0.0267	0.0267	0.0294	0.0274								
10	RVA/Human-wt/MOZ/0278/2012/G12P6	0.0281	0.0287	0.0294	0.0274	0.0274	0.0274	0.0301	0.0281	0.0007							
11	RVA/Human-wt/MOZ/0060a/2012/G12P8	0.0281	0.0287	0.0294	0.0274	0.0274	0.0274	0.0301	0.0281	0.0034	0.0027						
12	RVA/Human-wt/MOZ/0050/2012/G12P6	0.0281	0.0287	0.0294	0.0274	0.0274	0.0274	0.0301	0.0281	0.0014	0.0007	0.0034					
13	RVA/Human-wt/MOZ/0042/2012/G12P6	0.0281	0.0287	0.0294	0.0274	0.0274	0.0274	0.0301	0.0281	0.0014	0.0007	0.0034	0.0000				
14	KJ752809.1_RVA/Human-wt/ZAF/MRC-DPRU4090/2011/G12P6	0.0287	0.0294	0.0301	0.0281	0.0281	0.0281	0.0322	0.0287	0.0205	0.0198	0.0198	0.0198	0.0198			
15	KJ752222.1_RVA/Human-wt/ZAF/MRC-DPRU1840-07/2007/G1P8	0.0233	0.0240	0.0246	0.0226	0.0226	0.0226	0.0267	0.0233	0.0151	0.0144	0.0144	0.0144	0.0144	0.0096		
16	HQ657155.1_RVA/Human-wt/ZAF/3176WC/2009/G12P6	0.0240	0.0246	0.0253	0.0233	0.0233	0.0233	0.0274	0.0240	0.0171	0.0164	0.0164	0.0164	0.0164	0.0116	0.0048	
17	FJ747631.1_Human_rotavirus_A_strain_GER172-08	0.0137	0.0144	0.0151	0.0130	0.0130	0.0130	0.0171	0.0151	0.0233	0.0226	0.0226	0.0226	0.0226	0.0205	0.0137	0.0157

F. Genome segment 6 (VP6)

	Strains	1	2	3	4	5	6	7	8	9	10	11	12
1	RVA/Human-wt/MOZ/HPQ1152/2016/G1P8												
2	RVA/Human-wt/MOZ/HJM338/2015/G1P8	0.0025											
3	RVA/Human-wt/MOZ/HGM1265/2016/G1P8	0.0008	0.0034										
4	RVA/Human-wt/MOZ/HGM0059/2014/G1P8	0.0017	0.0025	0.0025									
5	RVA/Human-wt/MOZ/HGM0048/2014/G1P8	0.0025	0.0034	0.0034	0.0008								
6	RVA/Human-wt/MOZ/HCN154/2015/G1P8	0.0000	0.0025	0.0008	0.0017	0.0025							
7	RVA/Human-wt/MOZ/HCN1336/2016/G1P8	0.0008	0.0034	0.0017	0.0025	0.0034	0.0008						
8	RVA/Human-wt/MOZ/HCN1011/2016/G1P8	0.0000	0.0025	0.0008	0.0017	0.0025	0.0000	0.0008					
9	KX638597.1_Rotavirus_A_strain_RV1327	0.0034	0.0042	0.0042	0.0034	0.0042	0.0034	0.0042	0.0034				
10	KX638596.1_Rotavirus_A_strain_RV1326	0.0059	0.0067	0.0067	0.0059	0.0067	0.0059	0.0067	0.0059	0.0059			
11	KX638594.1_Rotavirus_A_strain_RV1305	0.0042	0.0050	0.0050	0.0042	0.0050	0.0042	0.0050	0.0042	0.0042	0.0067		
12	KX638591.1_Rotavirus_A_strain_RV1302	0.0034	0.0042	0.0042	0.0034	0.0042	0.0034	0.0042	0.0034	0.0034	0.0059	0.0042	
13	KJ752343.1_RVA/Human-wt/ZAF/MRC-DPRU1191/2009/G12P8	0.0092	0.0084	0.0101	0.0092	0.0101	0.0092	0.0101	0.0092	0.0092	0.0067	0.0101	0.0092

G. Genome segment 7 (NSP3)

	Strains	1	2	3	4	5	6	7	8	9	10	11
1	RVA/Human-wt/MOZ/HPQ1152/2016/G1P8											
2	RVA/Human-wt/MOZ/HJM338/2015/G1P8	0.0086										
3	RVA/Human-wt/MOZ/HGM1265/2016/G1P8	0.0043	0.0086									
4	RVA/Human-wt/MOZ/HGM0059/2014/G1P8	0.0054	0.0054	0.0054								
5	RVA/Human-wt/MOZ/HGM0048/2014/G1P8	0.0043	0.0043	0.0043	0.0011							
6	RVA/Human-wt/MOZ/HCN154/2015/G1P8	0.0032	0.0054	0.0032	0.0021	0.0011						
7	RVA/Human-wt/MOZ/HCN1336/2016/G1P8	0.0054	0.0075	0.0032	0.0043	0.0032	0.0021					
8	RVA/Human-wt/MOZ/HCN1011/2016/G1P8	0.0054	0.0075	0.0032	0.0043	0.0032	0.0021	0.0000				
9	KP752739.1_RVA/Human-wt/ZAF/MRC-DPRU1262/2004/G1P8	0.0257	0.0257	0.0257	0.0225	0.0214	0.0225	0.0247	0.0247			
10	JN605434.1_RVA/Human-wt/ZAF/MRC-DPRU4677/2010/G9P8	0.0247	0.0247	0.0247	0.0214	0.0204	0.0214	0.0236	0.0236	0.0268		
11	JN605423.1_RVA/Human-wt/ZWE/MRC-DPRU1723/2009/G9P8	0.0214	0.0214	0.0214	0.0182	0.0171	0.0182	0.0204	0.0204	0.0236	0.0032	
12	JN605412.1_RVA/Humanwt/CMR/MRC-DPRU1424/2009/G9P8	0.0139	0.0139	0.0139	0.0107	0.0096	0.0107	0.0129	0.0129	0.0182	0.0171	0.0139

H. Genome segment 8 (NSP2)

	Strains	1	2	3	4	5	6	7	8	9	10	11	12
1	RVA/Human-wt/MOZ/HPQ1152/2016/G1P8												
2	RVA/Human-wt/MOZ/HJM338/2015/G1P8	0.0042											
3	RVA/Human-wt/MOZ/HGM1265/2016/G1P8	0.0052	0.0052										
4	RVA/Human-wt/MOZ/HGM0059/2014/G1P8	0.0031	0.0031	0.0042									
5	RVA/Human-wt/MOZ/HGM0048/2014/G1P8	0.0042	0.0042	0.0052	0.0010								
6	RVA/Human-wt/MOZ/HCN154/2015/G1P8	0.0021	0.0021	0.0031	0.0010	0.0021							
7	RVA/Human-wt/MOZ/HCN1336/2016/G1P8	0.0042	0.0042	0.0052	0.0031	0.0042	0.0021						
8	RVA/Human-wt/MOZ/HCN1011/2016/G1P8	0.0021	0.0021	0.0031	0.0010	0.0021	0.0000	0.0021					
9	KX674709.1_Rotavirus_A_strain_RV1305	0.0052	0.0052	0.0063	0.0042	0.0052	0.0031	0.0052	0.0031				
10	KX674708.1_Rotavirus_A_strain_RV1302	0.0157	0.0157	0.0168	0.0147	0.0157	0.0136	0.0157	0.0136	0.0147			
11	KX674707.1_Rotavirus_A_strain_RV1327	0.0052	0.0052	0.0063	0.0042	0.0052	0.0031	0.0052	0.0031	0.0000	0.0147		
12	KJ753408.1_RVA/Human-wt/ETH/MRC-DPRU906/XXXX/G1P8	0.0094	0.0094	0.0105	0.0084	0.0094	0.0073	0.0094	0.0073	0.0084	0.0084	0.0084	
13	KJ752335.1_RVA/Human-wt/ZAF/MRC-DPRU1191/2009/G12P8	0.0147	0.0147	0.0157	0.0136	0.0147	0.0126	0.0147	0.0126	0.0136	0.0136	0.0136	0.0073

I. Genome segment 9 (VP7)

	Strains	1	2	3	4	5	6	7	8	9	10	11	12	13
1	RVA/Human-wt/MOZ/HPQ1152/2016//G1P8													
2	RVA/Human-wt/MOZ/HJM338/2015/G1P8	0.0041												
3	RVA/Human-wt/MOZ/HGM1265/2016/G1P8	0.0061	0.0082											
4	RVA/Human-wt/MOZ/HGM0059/2014/G1P8	0.0031	0.0010	0.0071										
5	RVA/Human-wt/MOZ/HGM0048/2014/G1P8	0.0031	0.0010	0.0071	0.0000									
6	RVA/Human-wt/MOZ/HCN154/2015/G1P8	0.0041	0.0041	0.0082	0.0031	0.0031								
7	RVA/Human-wt/MOZ/HCN1336/2016/G1P8	0.0031	0.0051	0.0071	0.0041	0.0041	0.0051							
8	RVA/Human-wt/MOZ/HCN1011/2016/G1P8	0.0020	0.0041	0.0061	0.0031	0.0031	0.0041	0.0010						
9	KX638553.1_Rotavirus_A_strain_RV1327	0.0051	0.0031	0.0071	0.0020	0.0020	0.0051	0.0061	0.0051					
10	KX638551.1_Rotavirus_A_strain_RV1305	0.0061	0.0061	0.0082	0.0051	0.0051	0.0061	0.0071	0.0061	0.0051				
11	KX638549.1_Rotavirus_A_strain_RV1302	0.0051	0.0031	0.0071	0.0020	0.0020	0.0051	0.0061	0.0051	0.0020	0.0051			
12	KP793024.1_Human_rotavirus_A_isolate_H129/Goa/2014	0.0092	0.0071	0.0112	0.0061	0.0061	0.0092	0.0102	0.0092	0.0061	0.0092	0.0061		
13	KF723266.1_Rotavirus_A_strain_IDK-4226/2011	0.0122	0.0102	0.0143	0.0092	0.0092	0.0122	0.0133	0.0122	0.0092	0.0122	0.0092	0.0112	
14	JX442769.1_Bovine_RVA/G1/India/HR/2011/B91	0.0061	0.0041	0.0082	0.0031	0.0031	0.0061	0.0071	0.0061	0.0031	0.0061	0.0031	0.0041	0.0082

J. Genome segment 10 (NSP4)

	Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	RVA/Human-wt/MOZ/HPQ1152/2016/G1P8															
2	RVA/Human-wt/MOZ/HJM338/2015/G1P8	0.0038														
3	RVA/Human-wt/MOZ/HGM1265/2016/G1P8	0.0038	0.0038													
4	RVA/Human-wt/MOZ/HGM0059/2014/G1P8	0.0019	0.0019	0.0019												
5	RVA/Human-wt/MOZ/HGM0048/2014/G1P8	0.0019	0.0019	0.0019	0.0000											
6	RVA/Human-wt/MOZ/HCN154/2015/G1P8	0.0019	0.0019	0.0019	0.0000	0.0000										
7	RVA/Human-wt/MOZ/HCN1336/2016/G1P8	0.0038	0.0038	0.0000	0.0019	0.0019	0.0019									
8	RVA/Human-wt/MOZ/HCN1011/2016/G1P8	0.0038	0.0038	0.0000	0.0019	0.0019	0.0019	0.0000								
9	RVA/Human-wt/MOZ/0289/2012/G12P6	0.0265	0.0265	0.0265	0.0246	0.0246	0.0246	0.0265	0.0265							
10	RVA/Human-wt/MOZ/0278/2012/G12P6	0.0246	0.0246	0.0246	0.0227	0.0227	0.0227	0.0246	0.0246	0.0019						
11	RVA/Human-wt/MOZ/0060a/2012/G12P8	0.0208	0.0208	0.0208	0.0189	0.0189	0.0189	0.0208	0.0208	0.0057	0.0038					
12	RVA/Human-wt/MOZ/0050/2012/G12P6	0.0246	0.0246	0.0246	0.0227	0.0227	0.0227	0.0246	0.0246	0.0019	0.0000	0.0038				
13	RVA/Human-wt/MOZ/0042/2012/G12P6	0.0246	0.0246	0.0246	0.0227	0.0227	0.0227	0.0246	0.0246	0.0019	0.0000	0.0038	0.0000			
14	KX638701.1_Rotavirus_A_strain_RV1302	0.0095	0.0095	0.0095	0.0076	0.0076	0.0076	0.0095	0.0095	0.0284	0.0265	0.0227	0.0265	0.0265		
15	KJ752812.1_RVA/Human-wt/ZAF/MRC-DPRU4090/2011/G12P6	0.0114	0.0114	0.0114	0.0095	0.0095	0.0095	0.0114	0.0114	0.0227	0.0208	0.0170	0.0208	0.0208	0.0095	
16	KJ752013.1_RVA/Human-wt/ZAF/MRC-DPRU6954/2011/G1P8	0.0095	0.0095	0.0095	0.0076	0.0076	0.0076	0.0095	0.0095	0.0170	0.0152	0.0114	0.0152	0.0152	0.0114	0.0057

K. Genome segment 11 (NSP5/6)

	Strains	1	2	3	4	5	6	7	8	9	10	11
1	RVA/Human-wt/MOZ/HJM338/2015/G1P8											
2	RVA/Human-wt/MOZ/HGM1265/2016/G1P8	0.0051										
3	RVA/Human-wt/MOZ/HGM0059/2014/G1P8	0.0034	0.0051									
4	RVA/Human-wt/MOZ/HGM0048/2014/G1P8	0.0034	0.0051	0.0000								
5	RVA/Human-wt/MOZ/HCN154/2015/G1P8	0.0034	0.0051	0.0034	0.0034							
6	RVA/Human-wt/MOZ/HCN1336/2016/G1P8	0.0034	0.0017	0.0034	0.0034	0.0034						
7	RVA/Human-wt/MOZ/HCN1011/2016/G1P8	0.0034	0.0017	0.0034	0.0034	0.0034	0.0000					
8	KX674717.1_Rotavirus_A_strain_RV1305	0.0051	0.0067	0.0051	0.0051	0.0051	0.0051	0.0051				
9	KX674716.1_Rotavirus_A_strain_RV1302	0.0034	0.0051	0.0034	0.0034	0.0034	0.0034	0.0034	0.0017			
10	KX674715.1_Rotavirus_A_strain_RV1327	0.0034	0.0051	0.0034	0.0034	0.0034	0.0034	0.0034	0.0017	0.0000		
11	KR052745.1_RVA/Human-wt/ZAF/MRC-DPRU4090/2011/G12P6	0.0034	0.0051	0.0034	0.0034	0.0034	0.0034	0.0034	0.0017	0.0000	0.0000	
12	KJ752338.1_RVA/Human-wt/ZAF/MRC-DPRU1191/2009/G12P8	0.0034	0.0051	0.0034	0.0034	0.0034	0.0034	0.0034	0.0017	0.0000	0.0000	0.0000

Supplemental Table 3 (A-K). Pairwise distance (G9P[8] strains)

A. Genome segment 1 (VP1)

	Strains	1	2	3	4	5	6	7	8	9	10	11	12
1	RVA/Human-wt/MOZ/HGM353/2015/G9P8												
2	RVA/Human-wt/MOZ/HGM389/2015/G9P8	0.0018											
3	RVA/Human-wt/MOZ/HJM643/2015/G9P8	0.0024	0.0012										
4	RVA/Human-wt/MOZ/HJM644/2015/G9P8	0.0080	0.0080	0.0086									
5	LC105403.1_RVA/Human-wt/JPN/To14-38/2014/G1P8	0.0073	0.0073	0.0080	0.0037								
6	LC105217.1_RVA/Human-wt/JPN/MU14-21/2014/G1P8	0.0073	0.0073	0.0080	0.0037	0.0006							
7	JF766600.1_RVA/Human/KOR/CAU09-376/2009/G9	0.0058	0.0064	0.0070	0.0052	0.0046	0.0046						
8	KT920926.1_RVA/Human-wt/USA/CNMC30/2011/G1P8	0.0052	0.0052	0.0058	0.0052	0.0046	0.0046	0.0037					
9	JX027930.1_RVA/Human-wt/AUS/CK00095/2010/G1P8	0.0043	0.0043	0.0049	0.0049	0.0043	0.0043	0.0034	0.0015				
10	KJ753468.1_RVA/Human-wt/ZWE/MRC-DPRU1102/2012/G9P8	0.0052	0.0052	0.0058	0.0058	0.0052	0.0052	0.0043	0.0024	0.0015			
11	JN706453.1_RVA/Human-wt/THA/CU460-KK/09/2009/G12P8	0.0095	0.0089	0.0095	0.0083	0.0077	0.0077	0.0073	0.0073	0.0070	0.0080		
12	KJ752227.1_RVA/Human-wt/ZAF/MRC-DPRU1840-07/2007/G1P8	0.0092	0.0089	0.0095	0.0073	0.0067	0.0067	0.0064	0.0064	0.0061	0.0070	0.0092	
13	KJ752172.1_RVA/Human-wt/ZAF/MRC-DPRU799/2006/G1P8	0.0077	0.0077	0.0083	0.0058	0.0052	0.0052	0.0049	0.0049	0.0046	0.0055	0.0080	0.0015

B. Genome segment 2 (VP2)

	Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	RVA/Human-wt/MOZ/HGM353/2015/G9P8														
2	RVA/Human-wt/MOZ/HGM389/2015/G9P8	0.0004													
3	RVA/Human-wt/HJM643/2015/G9P8	0.0011	0.0007												
4	RVA/Human-wt/MOZ/HJM644/2015/G9P8	0.0150	0.0146	0.0153											
5	DQ870502.1_RVA/Human-wt/BEL/B3458/2003/G9P8	0.0258	0.0262	0.0269	0.0236										
6	EF583050.1_RVA/Human-tc/USA/WI61/1983/G9P1A8	0.0453	0.0449	0.0456	0.0430	0.0501									
7	KJ753413.1_RVA/Human-wt/ETH/MRC-DPRU906/XXXX/G1P8	0.0296	0.0292	0.0299	0.0258	0.0329	0.0505								
8	KJ753469.1_RVA/Human-wt/ZWE/MRC-DPRU1102/2012/G9P8	0.0161	0.0157	0.0165	0.0056	0.0262	0.0430	0.0269							
9	KT920916.1_RVA/Human-wt/USA/CNMC23/2011/G1P8	0.0037	0.0034	0.0041	0.0142	0.0266	0.0441	0.0288	0.0153						
10	KX778549.1_R_RVA/Human-wt/CHN/km15007/G9P8	0.0079	0.0075	0.0082	0.0168	0.0284	0.0468	0.0307	0.0180	0.0064					
11	LC228382.1_RVA/Human-wt/JPN/CH1023/2016/G9P8	0.0146	0.0142	0.0150	0.0131	0.0254	0.0453	0.0273	0.0142	0.0138	0.0165				
12	LC228393.1_RVA/Human-wt/JPN/IS1080/2016/G9P8	0.0150	0.0146	0.0153	0.0135	0.0258	0.0456	0.0277	0.0146	0.0142	0.0168	0.0004			
13	LC228404.1_RVA/Human-wt/JPN/MI1128/2016/G9P8	0.0150	0.0146	0.0153	0.0135	0.0258	0.0456	0.0277	0.0146	0.0142	0.0168	0.0004	0.0007		
14	JX027931.1__RVA/Human-wt/AUS/CK00095/2010/G1P8	0.0034	0.0030	0.0037	0.0138	0.0262	0.0438	0.0284	0.0150	0.0019	0.0060	0.0135	0.0138	0.0138	
15	MF580873.1_Human_rotavirus_A_isolate_Hu/JS2015_G9P8	0.0041	0.0037	0.0045	0.0138	0.0262	0.0453	0.0284	0.0150	0.0026	0.0067	0.0142	0.0146	0.0146	0.0022

C. Genome segment 3 (VP3)

	Strains	1	2	3	4	5	6	7	8	9	10	11	12	13
1	RVA/Human-wt/MOZ/HGM353/2015/G9P8													
2	RVA/Human-wt/MOZ/HGM389/2015/G9P8	0.0024												
3	RVA/Human-wt/MOZ/HJM643/2015/G9P8	0.0024	0.0000											
4	RVA/Human-wt/MOZ/HJM644/2015/G9P8	0.0144	0.0144	0.0144										
5	DQ870503.1_RVA/Human-wt/BEL/B3458/2003/G9P8	0.0220	0.0220	0.0220	0.0180									
6	EF583051.1_RVA/Human-tc/USA/WI61/1983/G9P1A8	0.1346	0.1338	0.1338	0.1338	0.1258								
7	JX027932.1_RVA/Human-wt/AUS/CK00095/2010/G1P8	0.1146	0.1138	0.1138	0.1126	0.1094	0.1130							
8	KT920917.1_RVA/Human-wt/USA/CNMC23/2011/G1P8	0.0064	0.0064	0.0064	0.0088	0.0164	0.1306	0.1130						
9	KX778561.1_RVA/Human-wt/CHN/km15007/G9P8	0.0088	0.0088	0.0088	0.0104	0.0188	0.1322	0.1154	0.0032					
10	LC105060.1_RVA/Human-wt/JPN/HK14-10/2014/G1P8	0.0124	0.0124	0.0124	0.0048	0.0172	0.1326	0.1134	0.0068	0.0084				
11	LC105093.1_RVA/Human-wt/JPN/MU14-10/2014/G1P8	0.0120	0.0120	0.0120	0.0044	0.0168	0.1322	0.1130	0.0064	0.0080	0.0004			
12	LC105472.1_RVA/Human-wt/JPN/UR14-19/2014/G9P8	0.0068	0.0068	0.0068	0.0108	0.0184	0.1306	0.1138	0.0028	0.0052	0.0088	0.0084		
13	LC105483.1_RVA/Human-wt/JPN/UR14-20/2014/G9P8	0.0072	0.0072	0.0072	0.0112	0.0188	0.1302	0.1134	0.0032	0.0056	0.0092	0.0088	0.0004	
14	LC105516.1_RVA/Human-wt/JPN/UR14-25/2014/G9P8	0.0072	0.0072	0.0072	0.0112	0.0188	0.1302	0.1134	0.0032	0.0056	0.0092	0.0088	0.0004	0.0000

D. Genome segment 4 (VP4)

	Strains	1	2	3	4	5	6
1	RVA/Human-wt/MOZ/HGM353/2015/G9P8						
2	RVA/Human-wt/MOZ/HGM389/2015/G9P8	0.0004					
3	RVA/Human-wt/MOZ/HJM643/2015/G9P8	0.0004	0.0000				
4	RVA/Human-wt/MOZ/HJM644/2015/G9P8	0.1173	0.1168	0.1168			
5	MF580858.1_Human_rotavirus_A_isolate_Hu/JS2015	0.0056	0.0060	0.0060	0.1177		
6	KP902534.1_RVA/Human-wt/MWI/OP354/1998/G4P8	0.1181	0.1177	0.1177	0.0137	0.1186	
7	JX027933.1_RVA/Human-wt/AUS/CK00095/2010/G1P8	0.0326	0.0331	0.0331	0.1233	0.0365	0.1241

E. Genome segment 5 (NSP1)

	Strains	1	2	3	4	5	6	7	8
1	RVA/Human-wt/MOZ/HJM644/2015/G9P8								
2	RVA/Human-wt/MOZ/HJM643/2015/G9P8	0.0344							
3	RVA/Human-wt/MOZ/HJM338/2015/G1P8	0.0398	0.0418						
4	RVA/Human-wt/MOZ/HGM389/2015/G9P8	0.0351	0.0007	0.0425					
5	RVA/Human-wt/MOZ/HGM353/2015/G9P8	0.0351	0.0020	0.0425	0.0027				
6	KX778486.1_RVA/Human-wt/CHN/km15105/G9P8	0.0351	0.0142	0.0412	0.0148	0.0148			
7	KX778477.1_RVA/Human-wt/CHN/km15007/G9P8	0.0310	0.0128	0.0412	0.0135	0.0135	0.0054		
8	KF371910.1_RVA/Human-wt/CHN/L1066/2009/G3P8	0.0148	0.0331	0.0358	0.0337	0.0337	0.0324	0.0310	
9	KF371758.1_RVA/Human-wt/CHN/E707/2007/G3P8	0.0142	0.0324	0.0351	0.0331	0.0331	0.0317	0.0304	0.0007

F. Genome segment 6 (VP6)

	Strains	1	2	3	4	5	6	7	8	9
1	RVA/Human-wt/MOZ/HJM644/2015/G9P8									
2	RVA/Human-wt/MOZ/HJM643/2015/G9P8	0.0151								
3	RVA/Human-wt/MOZ/HGM389/2015/G9P8	0.0142	0.0008							
4	RVA/Human-wt/MOZ/HGM353/2015/G9P8	0.0134	0.0017	0.0008						
5	MF580852.1_Human_rotavirus_A_isolate_Hu/JS2015	0.0142	0.0109	0.0101	0.0092					
6	KX778594.1_RVA/Human-wt/CHN/km15105/G9P8	0.0151	0.0067	0.0059	0.0050	0.0109				
7	KX778585.1_RVA/Human-wt/CHN/km15007/G9P8	0.0126	0.0092	0.0084	0.0075	0.0084	0.0092			
8	HM773595.1_RVA/Human-wt/USA2009727036/2009/G9P8	0.0092	0.0126	0.0117	0.0109	0.0134	0.0126	0.0117		
9	EF560707.1_RVA/Human-wt/BGD/Dhaka6/2001/G11P25	0.0084	0.0134	0.0126	0.0117	0.0126	0.0134	0.0109	0.0059	
10	DQ870504.1_RVA/Human-wt/BEL/B3458/2003/G9P8	0.0109	0.0176	0.0168	0.0159	0.0168	0.0142	0.0151	0.0101	0.0092

G. Genome segment 7 (NSP3)

	Strains	1	2	3	4	5	6	7
1	RVA/Human-wt/MOZ/HGM353/2015/G9P8							
2	RVA/Human-wt/MOZ/HGM389/2015/G9P8	0.0032						
3	RVA/Human-wt/MOZ/HJM643/2015/G9P8	0.0032	0.0000					
4	RVA/Human-wt/MOZ/HJM644/2015/G9P8	0.0043	0.0054	0.0054				
5	DQ146657.1_RVA/Human-wt/BGD/Dhaka25/2002/G12P8	0.0064	0.0075	0.0075	0.0086			
6	JX027928.1RVA/Human-wt/AUS/CK00095/2010/G1P8	0.0011	0.0021	0.0021	0.0032	0.0054		
7	KU714441.1_RVA/Human-wt/MWI/MW2-181_A/2000/G1P8	0.0129	0.0139	0.0139	0.0150	0.0129	0.0118	
8	KX778510.1_RVA/Human-wt/CHN/km15105/G9P8	0.0204	0.0214	0.0214	0.0204	0.0247	0.0193	0.0311

H. Genome segment 8 (NSP2)

	Strains	1	2	3	4	5	6	7	8	9	10
1	RVA/Human-wt/MOZ/HGM353/2015/G9P8										
2	RVA/Human-wt/MOZ/HGM389/2015/G9P8	0.0010									
3	RVA/Human-wt/MOZ/HJM643/2015/G9P8	0.0010	0.0000								
4	RVA/Human-wt/MOZ/HJM644/2015/G9P8	0.0199	0.0189	0.0189							
5	KF371911.1_RVA/Human-wt/CHN/L1066/2009/G3P8	0.0168	0.0157	0.0157	0.0052						
6	KJ752168.1_RVA/Human-wt/ZAF/MRC-DPRU799/2006/G1P8	0.0168	0.0157	0.0157	0.0052	0.0021					
7	KJ752223.1_RVA/Human-wt/ZAF/MRC-DPRU1840-07/2007/G1P8	0.0157	0.0147	0.0147	0.0063	0.0031	0.0010				
8	KJ753464.1_RVA/Human-wt/ZWE/MRC-DPRU1102/2012/G9P8	0.0042	0.0031	0.0031	0.0199	0.0168	0.0168	0.0157			
9	KX674706.1_Rotavirus_A_strain_RV1326	0.0178	0.0168	0.0168	0.0063	0.0031	0.0010	0.0021	0.0178		
10	KX778498.1_RVA/Human-wt/CHN/km15105/G9P8	0.0042	0.0031	0.0031	0.0199	0.0168	0.0168	0.0157	0.0042	0.0178	
11	MF580908.1_Human_rotavirus_A_isolate_Hu/JS2015	0.0042	0.0031	0.0031	0.0199	0.0168	0.0168	0.0157	0.0042	0.0178	0.0042

I. Genome segment 9 (VP7)

	Strains	1	2	3	4	5	6	7	8	9	10	11	12
1	RVA/Human-wt/MOZ/HGM353/2015/G9P8												
2	RVA/Human-wt/MOZ/HGM389/2015/G9P8	0.0020											
3	RVA/Human-wt/MOZ/HJM643/2015/G9P8	0.0031	0.0031										
4	RVA/Human-wt/MOZ/HJM644/2015/G9P8	0.0082	0.0082	0.0092									
5	KJ753473.1_RVA/Human-wt/ZWE/MRC-DPRU1102/2012/G9P8	0.0112	0.0112	0.0122	0.0031								
6	KJ753835.1_RVA/Human-wt/ZWE/MRC-DPRU1158/XXXX/G2G9P6	0.0133	0.0112	0.0143	0.0051	0.0041							
7	KX778606.1_RVA/Human-wt/CHN/km15105/G9P8	0.0082	0.0082	0.0092	0.0082	0.0112	0.0133						
8	LC228386.1_RVA/Human-wt/JPN/CH1023/2016/G9P8	0.0082	0.0082	0.0092	0.0000	0.0031	0.0051	0.0082					
9	RVA/Human-wt/USA/VU12-13-101/2013/G9P8	0.0112	0.0112	0.0122	0.0031	0.0061	0.0082	0.0112	0.0031				
10	KJ753632.1_RVA/Human-wt/ZAF/MRC-DPRU11495/XXXX/G9P8	0.0112	0.0112	0.0122	0.0031	0.0020	0.0041	0.0112	0.0031	0.0061			
11	LC228397.1_RVA/Human-wt/JPN/IS1080/2016/G9P8	0.0082	0.0082	0.0092	0.0000	0.0031	0.0051	0.0082	0.0000	0.0031	0.0031		
12	LC228408.1_RVA/Human-wt/JPN/MI1128/2016/G9P8	0.0082	0.0082	0.0092	0.0000	0.0031	0.0051	0.0082	0.0000	0.0031	0.0031	0.0000	
13	MF580845.1_Human_rotavirus_A_isolate_Hu/JS2015	0.0082	0.0082	0.0092	0.0000	0.0031	0.0051	0.0082	0.0000	0.0031	0.0031	0.0000	0.0000

J. Genome segment 10 (NSP4)

	Strains	1	2	3	4	5	6	7	8	9	10	11	12
1	RVA/Human-wt/MOZ/HGM353/2015/G9P8												
2	RVA/Human-wt/MOZ/HGM389/2015/G9P8	0.0000											
3	RVA/Human-wt/MOZ/HJM643/2015/G9P8	0.0019	0.0019										
4	RVA/Human-wt/MOZ/HJM644/2015/G9P8	0.0133	0.0133	0.0152									
5	JX027926.1_RVA/Human-wt/AUS/CK00095/2010/G1P8	0.0019	0.0019	0.0038	0.0114								
6	MF580894.1_Human_rotavirus_A_isolate_Hu/JS2015	0.0019	0.0019	0.0038	0.0114	0.0000							
7	LC228412.1_RVA/Human-wt/JPN/MI1128/2016/G9P8	0.0095	0.0095	0.0114	0.0152	0.0076	0.0076						
8	LC228401.1_RVA/Human-wt/JPN/IS1080/2016/G9P8	0.0095	0.0095	0.0114	0.0152	0.0076	0.0076	0.0000					
9	LC228390.1_RVA/Human-wt/JPN/CH1023/2016/G9P8	0.0095	0.0095	0.0114	0.0152	0.0076	0.0076	0.0000	0.0000				
10	KX778522.1_RVA/Human-wt/CHN/km15105/G9P8	0.0114	0.0114	0.0133	0.0133	0.0095	0.0095	0.0170	0.0170	0.0170			
11	KX778513.1_RVA/Human-wt/CHN/km15007/G9P8	0.0095	0.0095	0.0114	0.0114	0.0076	0.0076	0.0152	0.0152	0.0152	0.0019		
12	KJ753466.1_RVA/Human-wt/ZWE/MRC-DPRU1102/2012/G9P8	0.0133	0.0133	0.0152	0.0114	0.0114	0.0114	0.0189	0.0189	0.0189	0.0133	0.0114	
13	HQ657158.1_RVA/Human-wt/ZAF/3176WC/2009/G12P6	0.0170	0.0170	0.0189	0.0114	0.0152	0.0152	0.0189	0.0189	0.0189	0.0170	0.0152	0.0152

K. Genome segment 11 (NSP5/6)

	Strains	1	2	3	4	5	6	7	8	9	10	11
1	RVA/Human-wt/MOZ/HGM353/2015/G9P8											
2	RVA/Human-wt/MOZ/HGM389/2015/G9P8	0.0000										
3	RVA/Human-wt/MOZ/HJM643/2015/G9P8	0.0000	0.0000									
4	RVA/Human-wt/MOZ/HJM644/2015/G9P8	0.0034	0.0034	0.0034								
5	RVA/Human-wt/MOZ/0050/2012/G12P6	0.0051	0.0051	0.0051	0.0017							
6	RVA/Human-wt/MOZ/0042/2012/G12P6	0.0051	0.0051	0.0051	0.0017	0.0000						
7	HQ657159.1_RVA/Human-wt/ZAF/3176WC/2009/G12P6	0.0051	0.0051	0.0051	0.0017	0.0034	0.0034					
8	JX027925.1_RVA/Human-wt/AUS/CK00095/2010/G1P8	0.0000	0.0000	0.0000	0.0034	0.0051	0.0051	0.0051				
9	KJ752171.1_RVA/Human-wt/ZAF/MRC-DPRU799/2006/G1P8	0.0034	0.0034	0.0034	0.0000	0.0017	0.0017	0.0017	0.0034			
10	KX674714.1_Rotavirus_A_strain_RV1326	0.0034	0.0034	0.0034	0.0000	0.0017	0.0017	0.0017	0.0034	0.0000		
11	KX778525.1_RVA/Human-wt/CHN/km15007/G9P8	0.0017	0.0017	0.0017	0.0051	0.0067	0.0067	0.0067	0.0017	0.0051	0.0051	
12	KX778534.1_RVA/Human-wt/CHN/km15105/G9P8	0.0017	0.0017	0.0017	0.0051	0.0067	0.0067	0.0067	0.0017	0.0051	0.0051	0.0000

APPENDIX D: Chapter 5

Supplementary Table 1. Genome assembly of DS1-like Mozambican rotavirus strains detected in 2015 and 2016**.**

Strain name	Trimmed reads		VP1	VP2	VP3	VP4	VP6	VP7	NSP1	NSP2	NSP3	NSP4	NSP5/6
RVA/Human-wt/MOZ/HPQ561/2015/G2P[6]	259,310	% ORF	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
		% length	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
		Average coverage	1315.00	1336.00	1797.00	1842.00	2832.00	3316.00	1737.00	2487.00	2962.00	4331.00	3126.00
		% Identity (ViPR)	95.30	97.80	89.10	96.70	97.60	95.80	97.50	97.40	97.40	91.00	99.80
RVA/Human-wt/MOZ/HCN1313/2016/G2P[6]	218,774	% ORF	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
		% length	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
		Average coverage	1216.00	1362.00	1768.00	1325.00	3325.00	3619.00	2188.00	3011.00	3472.00	4920.00	4310.00
		% Identity (ViPR)	95.30	97.80	89.00	96.50	97.50	95.60	97.00	97.40	97.50	91.30	99.50
RVA/Human-wt/MOZ/HCN0738/2015/G2P6	215,731	% ORF	100.00	100.00	100.00	100.00	100.00	100.00	X	100.00	100.00	100.00	100.00
		% length	100.00	100.00	100.00	100.00	100.00	100.00	X	100.00	100.00	100.00	100.00
		Average coverage	554.00	648.00	663.00	805.00	1184.00	909.00	X	1282.00	960.00	1793.00	1002.00
		% Identity (ViPR)	95.20	98.00	89.00	96.70	97.50	95.70	X	97.50	97.80	91.00	99.70
RVA/Human-wt/MOZ/HPQ1208/2016/G2P6	226,443	% ORF	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
		% length	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	99.00	100.00
		Average coverage	855.00	825.00	1034.00	1108.00	1400.00	1235.00	763.00	1058.00	969.00	1442.00	984.00
		% Identity (ViPR)	97.80	97.70	97.00	96.40	99.00	95.70	97.00	97.20	98.90	91.00	99.30
RVA/Human-wt/MOZ/HCN1328/2016/G2P6	91,518	% ORF	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
		% length	99.70	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
		Average coverage	460.00	483.00	561.00	536.00	773.00	666.00	426.00	662.00	653.00	978.00	561.00
		% Identity (ViPR)	95.20	97.80	88.90	96.50	97.60	95.40	97.40	97.40	97.40	91.00	99.00

Supplementary Table 2 (A-K). Pairwise distance (G2P[6] strains)

A. Genome segment 1 (VP1)

	Strains	1	2	3	4	5	6	7	8	9	10	11	12	13
1	RVA/Human-wt/MOZ/HPQ561/2015/G2P6													
2	RVA/Human-wt/MOZ/HPQ1208/2016/G2P6	0.0572												
3	RVA/Human-wt/MOZ/HCN1328/2016/G2P6	0.0006	0.0579											
4	RVA/Human-wt/MOZ/HCN1313/2016/G2P6	0.0009	0.0575	0.0009										
5	RVA/Human-wt/MOZ/HCN0738/2015/G2P6	0.0003	0.0575	0.0009	0.0012									
6	RVA/Human-wt/MOZ/0440/2013/G2P4	0.0025	0.0575	0.0031	0.0034	0.0028								
7	RVA/Human-wt/MOZ/0144/2013/G2P4	0.0028	0.0579	0.0034	0.0037	0.0031	0.0003							
8	RVA/Human-wt/MOZ/0126/2013/G2P4	0.0025	0.0579	0.0031	0.0034	0.0028	0.0012	0.0016						
9	RVA/Human-wt/MOZ/0045/2012/G8P4	0.0212	0.0579	0.0218	0.0215	0.0215	0.0218	0.0221	0.0218					
10	LC105591.1_RVA/Human-wt/GHA/GHDC1581/2013/G2P4	0.0056	0.0591	0.0062	0.0065	0.0059	0.0050	0.0053	0.0050	0.0218				
11	KP007193.1_RVA/Human-wt/PHI/TGO12-016/2012/G1P8	0.0078	0.0594	0.0084	0.0087	0.0081	0.0072	0.0075	0.0072	0.0227	0.0065			
12	KJ753827.1_RVA/Human-wt/ZWE/MRC-DPRU1158/XXXX/G2G9P6	0.0025	0.0566	0.0031	0.0034	0.0028	0.0019	0.0022	0.0019	0.0205	0.0037	0.0059		
13	KJ753490.1_RVA/Human-wt/ZMB/MRC-DPRU1752/XXXX/G4P6	0.0554	0.0062	0.0560	0.0557	0.0557	0.0557	0.0560	0.0560	0.0560	0.0572	0.0569	0.0547	
14	KJ752993.1_RVA/Human-wt/ZWE/MRC-DPRU1132/XXXX/G2P4	0.0563	0.0072	0.0569	0.0566	0.0566	0.0566	0.0569	0.0569	0.0566	0.0582	0.0585	0.0557	0.0022

B. Genome segment 2 (VP2)

	Strains	1	2	3	4	5	6	7	8	9	10	11
1	RVA/Human-wt/MOZ/HPQ561/2015/G2P6											
2	RVA/Human-wt/MOZ/HPQ1208/2016/G2P6	0.0066										
3	RVA/Human-wt/MOZ/HCN1328/2016/G2P6	0.0027	0.0070									
4	RVA/Human-wt/MOZ/HCN1313/2016/G2P6	0.0035	0.0078	0.0008								
5	RVA/Human-wt/MOZ/HCN0738/2015/G2P6	0.0019	0.0047	0.0023	0.0031							
6	KJ753829.1_RVA/Human-wt/ZWE/MRC-DPRU1158/XXXX/G2G9P6	0.0023	0.0051	0.0027	0.0035	0.0004						
7	KJ753491.1_RVA/Human-wt/ZMB/MRC-DPRU1752/XXXX/G4P6	0.0043	0.0055	0.0047	0.0055	0.0023	0.0027					
8	KJ753788.1_RVA/Human-wt/ZAF/MRC-DPRU1195/2009/G2P6P8	0.0027	0.0047	0.0031	0.0039	0.0008	0.0012	0.0023				
9	KJ752994.1_RVA/Human-wt/ZWE/MRC-DPRU1132/XXXX/G2P4	0.0043	0.0062	0.0047	0.0055	0.0023	0.0027	0.0039	0.0016			
10	RVA/Human-wt/MOZ/0144/2013/G2P4	0.0323	0.0335	0.0320	0.0327	0.0304	0.0308	0.0304	0.0296	0.0312		
11	RVA/Human-wt/MOZ/0126/2013/G2P4	0.0320	0.0331	0.0316	0.0323	0.0300	0.0304	0.0300	0.0292	0.0308	0.0012	
12	RVA/Human-wt/MOZ/0045/2012/G8P4	0.0374	0.0394	0.0374	0.0382	0.0355	0.0359	0.0362	0.0347	0.0362	0.0296	0.0296

C. Genome segment 3 (VP3)

	Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	RVA/Human-wt/MOZ/HPQ561/2015/G2P6														
2	RVA/Human-wt/MOZ/HPQ1208/2016/G2P6	0.1265													
3	RVA/Human-wt/MOZ/HCN1328/2016/G2P6	0.0012	0.1277												
4	RVA/Human-wt/MOZ/HCN1313/2016/G2P6	0.0020	0.1269	0.0008											
5	RVA/Human-wt/MOZ/HCN0738/2015/G2P6	0.0024	0.1265	0.0028	0.0036										
6	RVA/Human-wt/MOZ/0440/2013/G2P4	0.0056	0.1261	0.0060	0.0068	0.0064									
7	RVA/Human-wt/MOZ/0144/2013/G2P4	0.0048	0.1261	0.0052	0.0060	0.0056	0.0008								
8	RVA/Human-wt/MOZ/0126/2013/G2P4	0.0048	0.1257	0.0052	0.0060	0.0056	0.0024	0.0016							
9	RVA/Human-wt/MOZ/0045/2012/G8P4	0.0161	0.1265	0.0165	0.0173	0.0169	0.0136	0.0128	0.0136						
10	LC105593.1_RVA/Human-wt/GHA/GHDC1581/2013/G2P4	0.0088	0.1273	0.0092	0.0100	0.0096	0.0072	0.0064	0.0064	0.0128					
11	LC105582.1_RVA/Human-wt/GHA/GHPML1989/2012/G2P4	0.0088	0.1273	0.0092	0.0100	0.0096	0.0072	0.0064	0.0064	0.0136	0.0032				
12	KP007175.1_RVA/Human-wt/PHI/TGO12-007/2012/G2P4_M	0.0084	0.1285	0.0088	0.0096	0.0092	0.0076	0.0068	0.0068	0.0132	0.0068	0.0060			
13	KJ753831.1_RVA/Human-wt/ZWE/MRC-DPRU1158/XXXX/G2G9P6	0.0036	0.1261	0.0040	0.0048	0.0044	0.0020	0.0012	0.0012	0.0124	0.0052	0.0052	0.0056		
14	KJ752995.1_RVA/Human-wt/ZWE/MRC-DPRU1132/XXXX/G2P4	0.1281	0.0084	0.1293	0.1285	0.1281	0.1277	0.1277	0.1273	0.1281	0.1281	0.1281	0.1301	0.1277	
15	KJ753492.1_RVA/Human-wt/ZMB/MRC-DPRU1752/XXXX/G4P6	0.1277	0.0056	0.1289	0.1281	0.1277	0.1273	0.1273	0.1269	0.1277	0.1285	0.1285	0.1297	0.1273	0.0036

D. Genome segment 4 (VP4)

	Strains	1	2	3	4	5	6	7	8	9	10	11	12	13
1	RVA/Human-wt/MOZ/HPQ561/2015/G2P6													
2	RVA/Human-wt/MOZ/HPQ1208/2016/G2P6	0.0069												
3	RVA/Human-wt/MOZ/HCN1328/2016/G2P6	0.0022	0.0082											
4	RVA/Human-wt/MOZ/HCN1313/2016/G2P6	0.0026	0.0087	0.0013										
5	RVA/Human-wt/MOZ/HCN0738/2015/G2P6	0.0009	0.0069	0.0022	0.0026									
6	KJ753832.1_RVA/Human-wt/ZWE/MRC-DPRU1158/XXXX/G2G9P6	0.0017	0.0061	0.0030	0.0035	0.0017								
7	KJ753493.1_RVA/Human-wt/ZMB/MRC-DPRU1752/XXXX/G4P6	0.0035	0.0061	0.0048	0.0052	0.0035	0.0026							
8	KJ753719.1_RVA/Human-wt/ZAF/MRC-DPRU2344/2008/G2P6	0.0061	0.0104	0.0074	0.0078	0.0061	0.0052	0.0069						
9	KM660340.1_RVA/Human-wt/CMR/MA228/2011/G6P6	0.0108	0.0152	0.0121	0.0126	0.0108	0.0100	0.0117	0.0082					
10	KJ753791.1_RVA/Human-wt/ZAF/MRC-DPRU1195/2009/G2P6P8	0.0026	0.0069	0.0039	0.0043	0.0026	0.0017	0.0035	0.0035	0.0082				
11	RVA/Human-wt/MOZ/0289/2012/G12P6	0.0420	0.0464	0.0433	0.0438	0.0420	0.0420	0.0420	0.0412	0.0425	0.0412			
12	RVA/Human-wt/MOZ/0278/2012/G12P6	0.0425	0.0468	0.0438	0.0442	0.0425	0.0425	0.0425	0.0416	0.0429	0.0416	0.0013		
13	RVA/Human-wt/MOZ/0050/2012/G12P6	0.0420	0.0464	0.0433	0.0438	0.0420	0.0420	0.0420	0.0412	0.0425	0.0412	0.0009	0.0013	
14	RVA.Human-wt/MOZ/0042/2012/G12P6	0.0425	0.0468	0.0438	0.0442	0.0425	0.0425	0.0425	0.0416	0.0429	0.0416	0.0013	0.0009	0.0004

E. Genome segment 5 (NSP1)

	Strains	1	2	3	4	5	6	7	8	9	10	11	12
1	RVA/Human-wt/MOZ/HPQ561/2015/G2P6												
2	RVA/Human-wt/MOZ/HPQ1208/2016/G2P6	0.0343											
3	RVA/Human-wt/MOZ/HCN1328/2016/G2P6	0.0021	0.0350										
4	RVA/Human-wt/MOZ/HCN1313/2016/G2P	0.0050	0.0378	0.0071									
5	RVA/Human-wt/MOZ/0440/2013/G2P4	0.0343	0.0014	0.0350	0.0378								
6	RVA/Human-wt/MOZ/0144/2013/G2P4	0.0335	0.0007	0.0343	0.0371	0.0007							
7	RVA/Human-wt/MOZ/0126/2013/G2P4	0.0335	0.0007	0.0343	0.0371	0.0007	0.0000						
8	LC105594.1_RVA/Human-wt/GHA/GHDC1581/2013/G2P4	0.0350	0.0107	0.0357	0.0385	0.0107	0.0100	0.0100					
9	LC105583.1_RVA/Human-wt/GHA/GHPML1989/2012/G2P4	0.0328	0.0086	0.0335	0.0364	0.0086	0.0079	0.0079	0.0021				
10	KP007176.1_RVA/Human-wt/PHI/TGO12-007/2012/G2P4	0.0335	0.0093	0.0343	0.0371	0.0093	0.0086	0.0086	0.0086	0.0064			
11	KJ753818.1_RVA/Human-wt/ZWE/MRC-DPRU1158/XXXX/G2G9P6	0.0036	0.0343	0.0057	0.0086	0.0343	0.0335	0.0335	0.0350	0.0328	0.0335		
12	KJ753485.1_RVA/Human-wt/ZMB/MRC-DPRU1752/XXXX/G4P6	0.0036	0.0343	0.0057	0.0086	0.0343	0.0335	0.0335	0.0350	0.0328	0.0335	0.0029	
13	KJ752986.1_RVA/Human-wt/ZWE/MRC-DPRU1132/XXXX/G2P4	0.0043	0.0350	0.0064	0.0093	0.0350	0.0343	0.0343	0.0357	0.0335	0.0343	0.0036	0.0036

F. Genome segment 6 (VP6)

	Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	RVA/Human-wt/MOZ/HPQ561/2015/G2P6														
2	RVA/Human-wt/MOZ/HPQ1208/2016/G2P6	0.0327													
3	RVA/Human-wt/MOZ/HCN1328/2016/G2P6	0.0017	0.0327												
4	RVA/Human-wt/MOZ/HCN1313/2016/G2P6	0.0017	0.0327	0.0017											
5	RVA/Human-wt/MOZ/HCN0738/2015/G2P6	0.0017	0.0327	0.0034	0.0034										
6	RVA/Human-wt/MOZ/0440/2013/G2P4	0.0077	0.0301	0.0077	0.0077	0.0095									
7	RVA/Human-wt/MOZ/0314/2012/G8P4	0.0310	0.0034	0.0310	0.0310	0.0327	0.0284								
8	RVA/Human-wt/MOZ/0257/2012/G8P4	0.0310	0.0034	0.0310	0.0310	0.0327	0.0284	0.0000							
9	RVA/Human-wt/MOZ/0144/2013/G2P4	0.0077	0.0301	0.0077	0.0077	0.0095	0.0052	0.0284	0.0284						
10	RVA/Human-wt/MOZ/0126/2013/G2P4	0.0060	0.0284	0.0060	0.0060	0.0077	0.0034	0.0267	0.0267	0.0034					
11	RVA/Human-wt/MOZ/0044/2012/G8P4	0.0310	0.0034	0.0310	0.0310	0.0327	0.0284	0.0000	0.0000	0.0284	0.0267				
12	LC105579.1_RVA/Human-wt/GHA/GHPML1989/2012/G2P4	0.0095	0.0301	0.0095	0.0095	0.0112	0.0069	0.0284	0.0284	0.0052	0.0052	0.0284			
13	KJ753494.1_RVA/Human-wt/ZMB/MRC-DPRU1752/XXXX/G4P6	0.0301	0.0026	0.0301	0.0301	0.0318	0.0275	0.0009	0.0009	0.0275	0.0258	0.0009	0.0275		
14	KJ753833.1_RVA/Human-wt/ZWE/MRC-DPRU1158/XXXX/G2G9P6	0.0052	0.0275	0.0052	0.0052	0.0069	0.0026	0.0258	0.0258	0.0026	0.0009	0.0258	0.0043	0.0249	
15	KJ752998.1_RVA/Human-wt/ZWE/MRC-DPRU1132/XXXX/G2P4	0.0318	0.0043	0.0318	0.0318	0.0335	0.0292	0.0026	0.0026	0.0292	0.0275	0.0026	0.0292	0.0017	0.0267

G. Genome segment 7 (NSP3)

	Strains	1	2	3	4	5	6	7	8	9	10	11	12	13
1	RVA/Human-wt/MOZ/HPQ561/2015/G2P6													
2	RVA/Human-wt/MOZ/HCN1313/2016/G2P6	0.0037												
3	RVA/Human-wt/MOZ/HCN0738/2015/G2P6	0.0037	0.0025											
4	RVA/Human-wt/MOZ/HPQ1208/2016/G2P6	0.0335	0.0322	0.0297										
5	RVA/Human-wt/MOZ/HCN1328/2016/G2P6	0.0050	0.0012	0.0037	0.0335									
6	RVA/Human-wt/MOZ/0440/2013/G2P4	0.0347	0.0335	0.0310	0.0012	0.0347								
7	RVA/Human-wt/MOZ/0144/2013/G2P4	0.0347	0.0335	0.0310	0.0012	0.0347	0.0000							
8	RVA/Human-wt/MOZ/0126/2013/G2P4	0.0347	0.0335	0.0310	0.0012	0.0347	0.0000	0.0000						
9	KJ753487.1_RVA/Human-wt/ZMB/MRC-DPRU1752/XXXX/G4P6	0.0050	0.0037	0.0012	0.0285	0.0050	0.0297	0.0297	0.0297					
10	KJ753822.1_RVA/Human-wt/ZWE/MRC-DPRU1158/XXXX/G2G9P6	0.0347	0.0335	0.0310	0.0012	0.0347	0.0000	0.0000	0.0000	0.0297				
11	KJ752989.1_RVA/Human-wt/ZWE/MRC-DPRU1132/XXXX/G2P4	0.0347	0.0335	0.0310	0.0012	0.0347	0.0000	0.0000	0.0000	0.0297	0.0000			
12	KJ753713.1_RVA/Human-wt/ZAF/MRC-DPRU2344/2008/G2P6	0.0074	0.0062	0.0037	0.0260	0.0074	0.0273	0.0273	0.0273	0.0025	0.0273	0.0273		
13	MG181884.1_RVA/Human-wt/MWI/BID15I/2012/G2P6	0.0099	0.0087	0.0062	0.0285	0.0099	0.0297	0.0297	0.0297	0.0050	0.0297	0.0297	0.0025	
14	MG181851.1_RVA/Human-wt/MWI/BID1AW/2012/G2P6	0.0124	0.0112	0.0087	0.0285	0.0124	0.0297	0.0297	0.0297	0.0074	0.0297	0.0297	0.0050	0.0074

H. Genome segment 8 (NSP2)

	Strains	1	2	3	4	5	6	7	8	9	10	11	12	13
1	RVA/Human-wt/MOZ/HPQ561/2015/G2P6													
2	RVA/Human-wt/MOZ/HPQ1208/2016/G2P6	0.0128												
3	RVA/Human-wt/MOZ/HCN1328/2016/G2P6	0.0011	0.0117											
4	RVA/Human-wt/MOZ/HCN1313/2016/G2P6	0.0021	0.0128	0.0011										
5	RVA/Human-wt/MOZ/HCN0738/2015/G2P6	0.0011	0.0117	0.0000	0.0011									
6	RVA/Human-wt/MOZ/0440/2013/G2P4	0.0043	0.0107	0.0032	0.0043	0.0032								
7	RVA/Human-wt/MOZ/0144/2013/G2P4	0.0032	0.0096	0.0021	0.0032	0.0021	0.0011							
8	RVA/Human-wt/MOZ/0126/2013/G2P4	0.0043	0.0107	0.0032	0.0043	0.0032	0.0000	0.0011						
9	KJ753821.1_RVA/Human-wt/ZWE/MRC-DPRU1158/XXXX/G2G9P6	0.0053	0.0117	0.0043	0.0053	0.0043	0.0011	0.0021	0.0011					
10	LC105595.1_RVA/Human-wt/GHA/GHDC1581/2013/G2P4	0.0064	0.0107	0.0053	0.0064	0.0053	0.0021	0.0032	0.0021	0.0032				
11	LC105584.1_RVA/Human-wt/GHA/GHPML1989/2012/G2P4	0.0085	0.0128	0.0075	0.0085	0.0075	0.0043	0.0053	0.0043	0.0053	0.0021			
12	KX536640.1_Rotavirus_A_strain_RV09	0.0064	0.0107	0.0053	0.0064	0.0053	0.0021	0.0032	0.0021	0.0032	0.0021	0.0043		
13	KP007197.1_RVA/Human-wt/PHI/TGO12-016/2012/G1P8	0.0107	0.0149	0.0096	0.0107	0.0096	0.0064	0.0075	0.0064	0.0075	0.0064	0.0085	0.0064	
14	KP007177.1_RVA/Human-wt/PHI/TGO12-007/2012/G2P4	0.0075	0.0117	0.0064	0.0075	0.0064	0.0032	0.0043	0.0032	0.0043	0.0032	0.0053	0.0032	0.0032

I. Genome segment 9 (VP7)

	Strains	1	2	3	4	5	6	7	8	9	10	11	12
1	RVA/Human-wt/MOZ/HPQ561/2015/G2P6												
2	RVA/Human-wt/MOZ/HPQ1208/2016/G2P6	0.0072											
3	RVA/Human-wt/MOZ/HCN1328/2016/G2P6	0.0041	0.0093										
4	RVA/Human-wt/MOZ/HCN1313/2016/G2P6	0.0021	0.0093	0.0041									
5	RVA/Human-wt/MOZ/HCN0738/2015/G2P6	0.0010	0.0082	0.0051	0.0031								
6	RVA/Human-wt/MOZ/0440/2013/G2P4	0.0031	0.0041	0.0072	0.0051	0.0041							
7	RVA/Human-wt/MOZ/0144/2013/G2P4	0.0041	0.0051	0.0082	0.0062	0.0051	0.0010						
8	RVA/Human-wt/MOZ/0126/2013/G2P4	0.0031	0.0041	0.0072	0.0051	0.0041	0.0000	0.0010					
9	LC105588.1_RVA/Human-wt/GHA/GHDC1581/2013/G2P4	0.0103	0.0093	0.0123	0.0123	0.0113	0.0072	0.0082	0.0072				
10	LC105577.1_RVA/Human-wt/GHA/GHPML1989/2012/G2P4	0.0093	0.0103	0.0134	0.0113	0.0103	0.0062	0.0072	0.0062	0.0031			
11	KX574268.1_Rotavirus_A_strain_RV1310	0.0082	0.0093	0.0123	0.0103	0.0093	0.0051	0.0062	0.0051	0.0082	0.0072		
12	KX574267.1_Rotavirus_A_strain_RV1309	0.0082	0.0093	0.0123	0.0103	0.0093	0.0051	0.0062	0.0051	0.0103	0.0093	0.0021	
13	KJ752999.1_RVA/Human-wt/ZWE/MRC-DPRU1132/XXXX/G2P4	0.0051	0.0062	0.0093	0.0072	0.0062	0.0021	0.0031	0.0021	0.0072	0.0062	0.0051	0.0051

J. Genome segment 10 (NSP4)

	Strains	1	2	3	4	5	6	7	8	9	10	11
1	RVA/Human-wt/MOZ/HPQ561/2015/G2P6											
2	RVA/Human-wt/MOZ/HPQ1208/2016/G2P6	0.0063										
3	RVA/Human-wt/MOZ/HCN1328/2016/G2P6	0.0000	0.0063									
4	RVA/Human-wt/MOZ/HCN1313/2016/G2P6	0.0021	0.0084	0.0021								
5	RVA/Human-wt/MOZ/HCN0738/2015/G2P6	0.0000	0.0063	0.0000	0.0021							
6	RVA/Human-wt/MOZ/0314/2012/G8P4	0.0105	0.0126	0.0105	0.0126	0.0105						
7	RVA/Human-wt/MOZ/0308/2012/G2P4	0.0146	0.0167	0.0146	0.0167	0.0146	0.0084					
8	RVA/Human-wt/MOZ/0257/2012/G8P4	0.0126	0.0146	0.0126	0.0146	0.0126	0.0021	0.0063				
9	RVA/Human-wt/MOZ/0044/2012/G8P4	0.0146	0.0167	0.0146	0.0167	0.0146	0.0042	0.0084	0.0021			
10	RVA/Human-wt/MOZ/0043/2012/G2P4	0.0126	0.0146	0.0126	0.0146	0.0126	0.0105	0.0188	0.0126	0.0146		
11	KJ753824.1_RVA/Human-wt/ZWE/MRC-DPRU1158/XXXX/G2G9P6	0.0084	0.0105	0.0084	0.0105	0.0084	0.0146	0.0188	0.0167	0.0188	0.0167	
12	KJ753488.1_RVA/Human-wt/ZMB/MRC-DPRU1752/XXXX/G4P6	0.0021	0.0042	0.0021	0.0042	0.0021	0.0084	0.0126	0.0105	0.0126	0.0105	0.0063

K. Genome segment 11 (NSP5/6)

	Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	RVA/Human-wt/MOZ/HPQ561/2015/G2P6														
2	RVA/Human-wt/MOZ/HPQ1208/2016/G2P6	0.0133													
3	RVA/Human-wt/MOZ/HCN1328/2016/G2P6	0.0066	0.0199												
4	RVA/Human-wt/MOZ/HCN1313/2016/G2P6	0.0033	0.0133	0.0066											
5	RVA/Human-wt/MOZ/HCN0738/2015/G2P6	0.0017	0.0149	0.0083	0.0050										
6	RVA/Human-wt/MOZ/0440/2013/G2P4	0.0100	0.0033	0.0166	0.0100	0.0116									
7	RVA/Human-wt/MOZ/0308/2012/G2P4	0.0050	0.0182	0.0116	0.0083	0.0066	0.0149								
8	RVA/Human-wt/MOZ/0126/2013/G2P4	0.0100	0.0033	0.0166	0.0100	0.0116	0.0000	0.0149							
9	MG181776.1_RVA/Human-wt/MWI/BID11S/2012/G2P6	0.0017	0.0149	0.0083	0.0050	0.0033	0.0116	0.0066	0.0116						
10	KP007158.1_RVA/Human-wt/PHI/TGO12-003/2012/G2P4	0.0116	0.0083	0.0182	0.0116	0.0133	0.0050	0.0166	0.0050	0.0133					
11	KJ753715.1_RVA/Human-wt/ZAF/MRC-DPRU2344/2008/G2P6	0.0017	0.0149	0.0083	0.0050	0.0033	0.0116	0.0066	0.0116	0.0033	0.0133				
12	KJ753489.1_RVA/Human-wt/ZMB/MRC-DPRU1752/XXXX/G4P6	0.0000	0.0133	0.0066	0.0033	0.0017	0.0100	0.0050	0.0100	0.0017	0.0116	0.0017			
13	KJ753004_RVA/Human-wt/ZAF/MRC-DPRU1491/2010/G2P4P8	0.0000	0.0133	0.0066	0.0033	0.0017	0.0100	0.0050	0.0100	0.0017	0.0116	0.0017	0.0000		
14	KJ752991.1_RVA/Human-wt/ZWE/MRC-DPRU1132/XXXX/G2P4	0.0000	0.0133	0.0066	0.0033	0.0017	0.0100	0.0050	0.0100	0.0017	0.0116	0.0017	0.0000	0.0000	
15	KC178761.1_RVA/Human-wt/ITA/PA84/2008/G2P4	0.0017	0.0149	0.0050	0.0050	0.0033	0.0116	0.0066	0.0116	0.0033	0.0133	0.0033	0.0017	0.0017	0.0017