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MOLECULAR CHARACTERIZATION OF GROUP A ROTAVIRUSES DETECTED FROM ASHAIMAN IN THE GREATER ACCRA REGION OF GHANA (2014-2016)

BY

VICTOR LETSA
(10281491)

THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF MPHIL. MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASES DEGREE

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DEDICATION

I dedicate this work to the Almighty God for His steadfast love which saw me through my academic journey. I also dedicate this work to my family for their prayers and financial supports.

DECLARATION

I, Victor Letsa, do hereby declare that except for references from other peoples' work which have been duly acknowledged, this thesis report is my own work carried out at University of Ghana, Legon under the supervision of Dr. Osbourne Quaye. This work has not been submitted in whole or in part for any degree in this or any other university.

Signature ……. ……..

Victor Letsa Date

(Student)

Signature ……. ……..

Dr. Osbourne Quaye Date

(Supervisor)

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Abstract

Group A Rotaviruses (RVAs) are the most important etiological agents of acute gastroenteritis (AGE) in children less than 5 years of age. Mortality resulting from RVA gastroenteritis is higher in developing countries than in developed ones, causing a huge public health burden in global regions like Africa and South East Asia. The aim of this study was to investigate the strains of RVAs causing AGE in children less than 5 years of age in Ashaiman after the introduction of the Rotarix™ vaccine into the Expanded Programme on Immunization. Stools samples were collected from children less than 5 years of age who visited Ashaiman Polyclinic with AGE. The samples were tested by enzyme immunoassay (EIA) and the EIA negative samples were subjected to non-structural protein 3 (NSP3) qRT-PCR. One-Step multiplex RT-PCR was performed on EIA and NSP3 positive samples for gel based binomial genotyping. Sanger sequencing was performed to confirm the gel based genotyping results. RVA strains were selected from the gel based genotyping for full genome characterization (FGC) using Illumina MiSeq. Maximum likelihood trees were drawn for VP7 and VP4 genes using Tamura 3 parameter evolutionary model for 1000 bootstraps using MEGA 6.0 software. RVA prevalence was found to be 39% (145/369). Five VP7 (G1,G3,G9, G10 and G12) and three VP4 (P[4], P[6] and P[8]) genotypes which were phylogenetically identical within genotypes and more related to African strains were detected. There were 8 G/P combinations and 7 mixed infections. FGC result gave Wa-like, Ds-1 like and Wa-/ Ds-1 like constellations which suggests zoonotic transmission. For the first time, a unique strain; RVA/Human-wt/GHA/020/2015/G10P[8]-I1-R5-C2-M2-A3-N2-T1-E1-H2 has been characterized from this study and justify the need for full genome sequencing of rotaviruses in surveillance studies. The findings thus confirmed that,

insanitary setting could create a conducive environment for RV co-infections and the upsurge of RV diversity.

Abbreviations

AGE	: Acute gastroenteritis
BLAST	: Basic Local Alignment Search Tool
Bp	: Base pair
cDNA	: complimentary Deoxyribonucleic Acid
DLP	: Double-layered particle
DsRNA	: single stranded ribonucleic acid
EIA	: Enzyme-immuno assay
EM	: Electron microscopy
ER	: Emergency Room
EPI	: Expanded programme on immunization
FGC	: Full genome constellations
GITC	: Guanidinium Isothiocyanate
IgA	: Immunoglobulin A
NaOAc	: Sodium acetate
NSP	: Non-structural protein
NT	: Non-typeable
NTC	: No template control
OD	: Optical density
PCV1	: Porcine circovirus 1
PAGE	: Polyacrylamide gel electrophoresis

RFPL	: Restriction fragmented length polymorphism
REST	: Rotavirus efficacy and safety trials
RTth	: *Thermus thermophiles*
RT-PCR	: Reverse-transcriptase polymerase chain reaction
RV	: Rotavirus
RVA	: Group A Rotaviruses
SDS	: sodium dodecyl sulfate
ssRNA	: single stranded ribonucleic acid
TLP	: Triple-layered particle
VP	: Viral protein
WHO	: World Health Organization

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CHAPTER ONE

1.0 Introduction

Rotavirus infection is the leading cause of acute gastroenteritis in children under the age of 5 years and a threat to pediatric health (Jones et al., 2016). Group A rotaviruses (RVs) are the single most important etiological agents of viral gastroenteritis in children less than five years of age (Jonesteller et al., 2017; Operario et al., 2017). Mortality resulting from rotavirus gastroenteritis is higher in developing countries than in developed ones, leading to a huge public health burden in regions like Africa and South East Asia (Breurec et al., 2016; Burnett et al., 2017; Clark et al., 2017; Hungerford et al., 2017).

Group A rotaviruses belong to the *Reoviridae* family of non-enveloped dsRNA-containing viruses (Gonzalez-Ochoa et al., 2017; Saxena et al., 2016). They are members of the genus *Rotavirus* with 11-segmented genome encased within three icosahedral protein shells and are referred to as triple-layered viruses (Shao et al., 2016). The virion consists of an inner VP2 (viral protein 2) protein layer enclosing the double stranded RNA segments. The inner core also contains VP1 and VP3 proteins which make the endogenous viral RNA-dependent RNA polymerase and the 5' capping enzyme respectively forming the polymerase complex. The polymerase complex is used for the synthesis of viral transcripts. The middle VP6 protein capsid is required for transcription. The outer layer capsid containing glycosylated VP7 capsid and VP4 protease-sensitive protein spikes embedded in the glycosylated VP7 capsid. The protease-sensitive protein spikes, VP4 capsid serves as neutralizing antigens and for cell attachments (Deal et al., 2010). The capsid proteins are responsible for many of the serological properties of

Group A rotaviruses (Fajardo et al., 2017; Long & McDonald, 2017). Host antibodies to the VP6 protein define the rotavirus group antigen, whereas antibodies to VP7 and VP4 define G and P serotypes, respectively (Deal et al., 2010). The two outer capsid proteins VP4 (P-type specificity) and VP7 (G-type specificity) stimulate human immunological response and are targets for the development of rotavirus vaccines (Deal et al., 2010). Results from studies of animal models suggest that antibodies to VP6 and a viral nonstructural protein 4 (NSP4) might also be involved in generation of protective immunity (Deal et al., 2010). For the reason that VP7 and VP4 proteins are neutralizing antigens, the genes which encode their respective proteins form the basis for the traditional binomial classification of rotaviruses into G/P combinations.

Currently, 27 G and 37 P genotypes have been identified in humans and animals (Desselberger, 2014). The double-stranded RNA virus is classified into 8 groups (A – H) based on antigenicity and nucleotide sequence identities of the VP6 gene (Desselberger, 2014). Global epidemiological surveys have identified G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12[P8] as the most common circulating strains associated with diarrhea in humans (Desselberger, 2014). However, due to mutations, genome reassortments and zoonotic transmission of strains, recent studies especially those from the developing countries have shown greater diversity of rotavirus genotypes with several novel combinations (Azaran et al., 2017; Doll et al., 2015; Leite et al., 2017; O'Ryan, 2017; Ouermi et al., 2017; Rahajamanana et al., 2017; Wandera et al., 2017; Zaraket et al., 2017). In rotaviruses, all the gene segments are capable of genetic reassortment, and this therefore has led a new classification method, which looks at all the 11 gene segments. Based on nucleotide sequence identity, three main Geno groups or genotype constellations have

been described to understand the genotype combinations of strains responsible for the RV disease burden (Theuns et al., 2016).

The most preventive measure of reducing the burden due to rotaviruses is by the use of vaccines (Costa et al., 2016; Desselberger et al., 2009; Du et al., 2016; Vesikari, 2016). Rotavirus vaccine development strategies against rotaviruses are based on the engineering of the global circulating strains into monovalent or polyvalent live-attenuated vaccines. Such vaccines elicit serotype-specific or heterotypic immunity to the most common rotavirus serotypes (Heylen et al., 2015). The development of rotavirus vaccines, Rotateq® (Merck) and Rotarix™ (GSK), was a welcome news world-wide, and not surprising that a highly successful clinical trials, resulted in WHO recommending the global use of the vaccines to reduce the disease burden. The advent of the rotavirus vaccines has led to significant reduction in diarrheal disease burden in children under 5 years of age globally (Burnett et al., 2017; Das et al., 2016; Vesikari, 2016). However, post-vaccine era surveillance studies suggest a stark disparity in vaccine effectiveness between developed and developing countries (Pringle et al., 2016). As high vaccine efficacies have been recorded for both vaccines [Rotateq® (Merck) and Rotarix™ (GSK)] when used in developed countries. Africa and countries in Southeast Asia have however shown less vaccine efficacies for both vaccines (Patel et al., 2016; Tate et al., 2016; Young et al., 2015; Zhen et al., 2015).

1.1 Problem statement

Ashaiman municipality is a one-town municipality with overcrowded satellite settlements. The 2010 population and housing census in Ghana revealed that the total population of the municipality was 190,972 (GSS, 2014). The municipality is located about 4km to the North of

Tema and about 30km from Accra, the capital of Ghana. Whiles Tema is situated on the Greenwich Meridian on the Longitude 00, Ashaiman falls within Latitude 5° 42' North and Longitude 0° 01'west. Ashaiman shares boundaries to the North and East with Kpone-Katamanso District and to the South and West with Tema metropolis. The age group 0-4 years has the highest population representing 12.3% of the total population. Since rotaviruses are highly contagious and are transmissible through the respiratory route, the large population of children under five years of age in the midst of overcrowded settlements may promote the disease burden.

Public toilet is the most dominant toilet facility in the municipality (63.5%) (GSS, 2014) which implies that, majority of houses within the municipality do not have toilet facilities. The high dependence on public toilet may promote, indiscriminate open defecation and the disposal of human excreta into public drains and unto refuse dumps. Also animal rearing is one of the major occupations of the people in the municipality where animals are allowed to stray in search for food. Poor disposal of human excreta, high frequency of human-animal interactions and poor personal hygiene practices may promote mixed infections and the possible cause of the diverse strains of rotaviruses detected in developing countries than the developed countries.

In-patient and out-patient reports for Ashaiman Polyclinic from 2010 to 2013 revealed that, diarrheal disease was prevalent in children under five years of age surpassed by malaria and acute respiratory tract (ART) infections even in the post-vaccine introduction era. Hence, the need to ascertain RV genotypes responsible for diarrheal diseases at the study area.

1.2 Justification

Rotaviruses are transmitted through both the respiratory and fecal-oral routes and are shed in saliva and stools before the onset of symptoms as such, they are enormously infectious (Mayanskiy et al., 2015; Pikul et al., 2017). The risk of transmitting rotaviruses can be reduced by frequent hand washing and treatment of contaminated surfaces in high temperatures above 50 ^{o}C. The virus can also be inactivated with disinfectants especially, 95% ethanol which exerts its effect by removing the outer protein capsid of the virus (Estes & Greenberg, 2013). Unfortunately, children under 5 years of age may find it difficult to adhere to preventive standards. Also, disinfectants may be too strong to use on children under 5 years of age.

Several studies have shown that, the most effective method of reducing the rotavirus disease burden is by the use of vaccines (Mujuru et al., 2017; O'Ryan, 2017; Shah et al., 2017). In Ghana, the monovalent rotavirus vaccine Rotarix™ was introduced into the national Expanded Programme on Immunization (EPI) in May 2012 to manage diarrhea due to rotaviruses in children under five years of age. However, there is paucity of data on circulating rotavirus strains in the post-vaccine era. Continuous strain surveillance is therefore crucial for establishing the impact of the vaccine on rotavirus strain diversity in the country after the vaccine introduction.

1.3 Aim

The aim of this study was to investigate the strains of rotaviruses causing AGE in children less than 5 years of age in Ashaiman after the introduction of the Rotarix™ vaccine into the Expanded Programme on Immunization.

1.4 Objectives of the study

1. To screen, detect and genotype RVAs in stool samples
2. To determine full-genome constellation of selected RVA strains
3. To determine the genetic relatedness of the strains detected in this study to cognate gene segments of global strains
4. To investigate the genetic diversity due to reassortment or recombination between human and animal strains or wild-type and vaccine RVA strains

1.5 Significance of the study

This study will provide information on strain diversity, as well as on genetic diversity due to reassortment or recombination between human and animal strains, or wild-type and vaccine RVA strains. The study will also provide evidence to ascertain the impact of increased human-animal contacts and poor sanitation on strain and genetic diversity of rotaviruses. Finally, recommendations from this study will help public health officials to formulate policies to reduce high human-animal contacts as well as improve sanitation in order to reduce rotavirus disease burden at the study area and in the county as a whole.

CHAPTER TWO

2.0 Literature review

2.1 General characteristics of rotaviruses

Group A rotaviruses (RVAs) are the single most important etiological agents of acute gastroenteritis in children under 5 years of age worldwide, and are responsible for 215,757 rotavirus-associated deaths annually, of which the majority occur in countries of sub-Saharan Africa and South East Asia (Clark et al., 2017).

The viruses are members of the genus rotavirus within the family *Reoviridae* with 11 double-stranded RNA segments serving as the viral genome. The genome is surrounded by three protein capsids: the inner capsid being VP2, the middle capsid, VP6 and the outer capsid, VP7 forming a triple-layered icosahedral structure (Fig 2.1) and about 70nm in diameter (Lever & Desselberger, 2016). When viewed under the electron microscope, the VP4 is seen as 60 protein spikes extending from the smooth surface of the outer shell (VP7). It has been shown that, the integrity of the protein capsids require calcium, as a result, calcium ions imbalance in the host is implicated in the pathogenesis of the virus (Biswas et al., 2014; Greenberg & Estes, 2009; Yin et al., 2015). The 11 double-stranded RNA segments encode 6 viral structural proteins (VP1-VP4, VP6 and VP7) and 6 non-structural proteins (NSP1-NSP6) with different molecular weights, play different roles in viral replication (Table 2.1).

There are eight distinct groups, A-H of rotaviruses of which, group A, B, C and H rotaviruses are found in both humans and animals, whereas groups D, E, F and G have been found in animals to date (Desselberger, 2014).

Rotaviruses invade the enterocytes and as members of the *Reoviridae* family, they do not depend on hot's enzyme for replication (Fajardo et al., 2017; Long & McDonald, 2017). They are well endowed with endogenous RNA-dependent RNA polymerase complex for the synthesis of RNA transcripts (Komoto et al., 2017). Virus replication occurs in the cytoplasm of infected enterocytes and rotaviruses of the same group of different strains co-infecting a cell are capable of genetic reassortment (Gluck et al., 2017). Virus particles are formed by budding into the endoplasmic reticulum of infected enterocyte leading to transient formation of enveloped particle in rotavirus morphogenesis. Matured particles are non-enveloped and virions are liberated from infected cells by lysis or by non-classic vesicular transport in polarized epithelial cells (Desselberger, 2014; Greenberg & Estes, 2009).

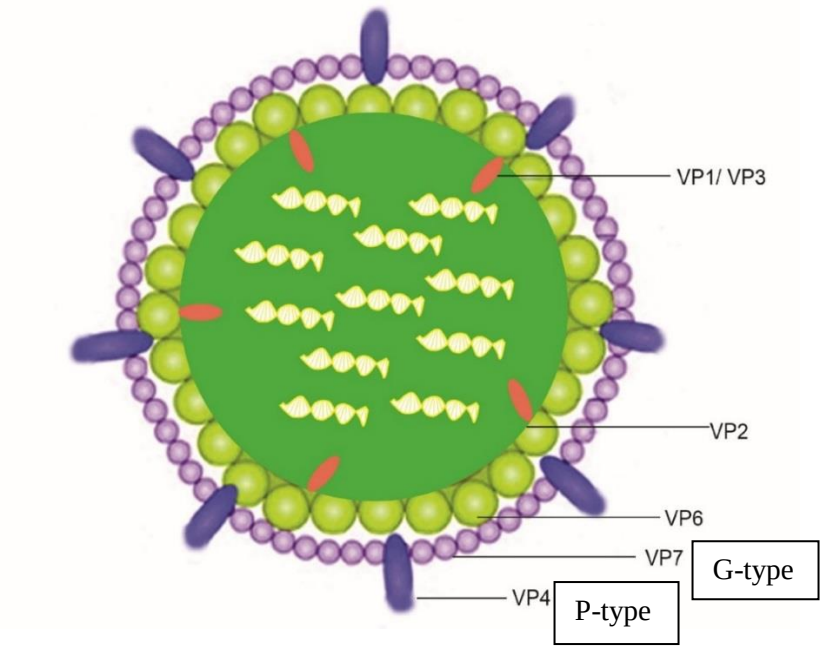


Fig 2.1 A schematic illustration of rotavirus structure—the virion entails an inner VP2 protein capsid surrounding the RNA segments and several molecules of VP1 and VP3 proteins, a central VP6 protein capsid, and an external capsid containing VP4 protein spikes embedded in a VP7 protein capsid. Image modified from (Lepault et al., 2001)

Table 2.1: Rotavirus gene segments with their respective sizes, weights, locations and functions

Gene Segment	Protein	Size (bp)	Weight (kDa)	Location	Function
1	VP1	3302	125	Core	Forms complex with VP3 for mRNA transcription.
2	VP2	2690	102	Core	RNA binding, required for VP1 replicase functioning
3	VP3	2591	88	Core	Forms polymerase complex with VP1, mRNA transcription
4	VP4 (VP5 +VP8)	2362	87	Outer capsid	Cell attachment, hemagglutinin, neutralizing antigen
5	NSP1	1611	59	Nonstructural	Interferon antagonist
6	VP6	1356	45	Inner capsid	Required for transcription, subgroup antigen (main target for IgA)
7	NSP3	1104	37	Nonstructural	Inhibits host translation
8	NSP2	1059	35	Nonstructural	Forms viroplasm with NSP5
9	VP7	1062	37	Outer capsid	Neutralizing antigen
10	NSP4	751	20	Nonstructural	Helps in outer capsid assembly, enterotoxin
11	NSP5+NSP6	667	22	Nonstructural	Forms viroplasm with NSP2

RV structural/viral proteins (VP) and non-structural proteins (NSP) locations, weights, sizes positions and functions (Estes & Greenberg, 2013).

2.2 Rotavirus detection

The clinical and epidemiological indicators of rotavirus illnesses (severe diarrhea, vomiting, dehydration, fever in young children, and seasonality in non-tropical areas) are not sufficiently unique to permit diagnosis even during a rotavirus "season". The precise diagnosis, therefore, requires the detection of the virus or viral antigen, or a virus-specific serologic response or even the gene fragments of the virus. The conventional epidemiological manifestations of rotavirus disease at any one time may be useful in the diagnosis of the disease, however, laboratory confirmation is prerequisite for the confirmation of the rotavirus infection (Waku-Kouomou et al., 2016). In view of that, many biochemical and molecular assays have been developed for the detection of rotaviruses in stool specimen. Some of the detection methods include; electron microscopy, antigen detection – enzyme immunoassay (EIA), spectrophotometry, immuno-chromatography, nucleic acid detection (PAGE and RT-PCR), among others.

2.2.1 Microscopic detection

The morphological appearance of rotavirus particle is distinctive and makes it possible for the identification of the virus microscopically. Direct visualization of stool specimen by electron microscope (EM) has advantage of high specificity due to its distinct morphological appearance from other entero-pathogens (Greenberg & Estes, 2009). The complete particles resemble a wheel with short spokes and a well-defined smooth outer rim. The name rotavirus (from the Latin rota, meaning "wheel") was derived from its morphological appearance forming the basis of rotavirus detection microscopically by the use of electron microscope (EM) (Estes & Greenberg, 2013; Jiang et al., 2006). The virions are also known as triple-layered particles due to three protein coats covering the double stranded RNA segments (Jiang et al., 2006). The double-

layered particle lacking the outer layer are also described as rough particles because their periphery exhibit projecting trimeric subunits of the inner capsid (Prasad et al., 2001). The single –layered are seen rarely usually lacking genomic RNA and often aggregated. However, the three dimensional reconstructions using cryo-electron microscopy and image processing techniques have been useful in studying the triple- layered, the double-layered and the single-layered rotavirus particles (Boudreaux et al., 2015; Salgado et al., 2017). EM continues to be important in the diagnosis of rotaviral diseases and frequently used to resolve disparities in results from other techniques and when only few specimens are to be examined for rotaviruses**.** The method is too labor intensive for routine detection of rotavirus in large numbers of stool specimens. In addition, EM requires an expensive instrument and highly trained personnel and cannot distinguish between rotaviruses of different groups since rotaviruses of different groups are not unique by appearance under the electron microscope (Boudreaux et al., 2015).

2.2.2 Antigen detection

Various automated methods are available for the detection of rotaviruses in stool specimen. The most widely used methods for rotavirus diagnosis are based on detection of protein antigens on rotavirus particles in stool specimens (Desselberger, 2014). The most appropriate antigen detection format for large-scale surveillance studies is the enzyme immuno-assay (EIA) techniques that use rotavirus specific antibodies to capture antigen onto wells of plastic plates. The antigen is then detected in a colorimetric reaction using a second rotavirus-specific antibody coupled to a detector enzyme (Zhao et al., 2015). The EIA format is highly sensitive and specific and is adaptable to large sample volumes in the 96-well plate format. The optical density (OD) results can be easily recorded with a standard plate reader, permitting analysis of results with

standard computer programs. EIA has traditionally been the method of choice for clinical detection of group A rotaviruses in stool specimen because it is sensitive, does not require any special equipment, has in-built controls for non-specific reactions and defines the etiology of rotavirus-induces gastroenteritis (Chung et al., 2015; Waku-Kouomou et al., 2016; WHO, 2009). In the recent times, the EIA techniques have been modified into rapid diagnostic cassettes which makes EIA techniques more appropriate for easy detection of rotaviruses and the best diagnostic tool at hospitals (Ope et al., 2017). The rapid detection method could easily permit the detection of rotaviruses in the consulting rooms. The cassette is coated with anti-rotavirus antibodies, which operates on immune-chromatographic techniques (Waku-Kouomou et al., 2016). Due to the importance of rotaviruses in clinical settings, many antigen detection methods have been commercialized, and data are available on their sensitivity and specificity (Esona & Gautam, 2015).

2.2.3 Nucleic acid detection

Nucleic acid detection technique is based on either the detection of the viral nucleic acid by polyacrylamide gel electrophoresis (PAGE) just after RNA extraction or by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) method. RT-PCR detection method can either be detecting the nucleic acid in real time using quantitative real time polymerase chain reaction (qRT-PCR) assay or using the conventional RT-PCR assay where the amplicons are detected by agarose gel electrophoresis (Gautam et al., 2016). RT-PCR/qRT-PCR detection is more sensitive than electrophoresis however, more expensive than PAGE detection (Montes & Iturriza-Gomara, 2008). Because of the presence of large quantities of rotavirus particles in stool samples from children suffering from rotavirus-gastroenteritis, the viral nucleic acid segments

can be visualized directly after extraction from virus particles, by electrophoresis on acrylamide gels, and staining with ethidium bromide or silver nitrate (WHO, 2009). After electrophoresis, human rotavirus Groups A, B, and C have distinct patterns of gene-segment distribution and designated electropherotypes techniques can be used in rotavirus surveillance (Desselberger, 2014). The results of electropherotyping correlate with the presence of viruses of a specific group as shown by using other methods. Thus, the presence of distinct electropherotype patterns has long been considered a good detection of individual rotaviruses of Groups A, B, and C (Doan et al., 2016; Estes & Greenberg, 2013). For Group A rotaviruses, most samples that are positive for rotavirus by EIA will be positive for the characteristic pattern of rotavirus RNA segments after electrophoresis and silver staining (WHO, 2009). Consequently, the polyacrylamide gel electrophoresis (PAGE) method has sometimes been used to categorize Group A rotavirus into their various electropherotypes for surveillance studies (Esona & Gautam, 2015).

2.2.3.1 Nucleic acid detection by PAGE

Rotavirus dsRNA can be detected in clinical specimens by extraction of the viral RNA and analysis by electrophoresis on a polyacrylamide gel followed by silver staining (WHO, 2009). Rotavirus dsRNA has 11 segments. The dsRNA of rotavirus is negatively charged and during electrophoresis through the acrylamide gel, these negatively charged macromolecules towards the positive pole and are separated according to size. The patterns of dsRNA can then be visualized in the gel by staining with silver nitrate (Estes & Greenberg, 2013). Silver staining is a sensitive procedure to detect small amounts of nucleic acid in polyacrylamide gels because, silver ions form a stable complex with nucleic acids (WHO, 2009), Silver stained gels can be dried and stored for future use. The dsRNA extracted from Group A rotaviruses can be split into

four size classes: four large segments, two medium-sized segments, three small segments, and the two smallest segments indicating the 4:2:3:2 pattern as a characteristic migration pattern of group A rotaviruses (Desselberger, 1996). Group A human and animal rotaviruses also display two electropherotypes: “long” and “short. Short electrophoretic patterns exhibit a larger segment (encoding NSP5) that migrates more slowly and is located between gene segments 9 and 10 (Estes & Greenberg, 2013).

Although most Group A rotaviruses have either a short or a long pattern, super-short electropherotypes have been documented (Desselberger, 1996). These correlations between RNA patterns and serotypes have been maintained and have become a useful epidemiologic tool in rotavirus surveillance (Waku-Kouomou et al., 2016). Detailed descriptions of the correlations between electropherotypes and viral antigenic and genetic properties have been described in several rotavirus studies (Kirkwood et al., 2015; Kowalzik et al., 2016; Nirwati et al., 2016; Pellegrinelli et al., 2015).

Although this method is relatively time-consuming, it also requires a trained person who can do the analysis accurately. The main advantage of PAGE is the lack of ambiguity in the results. The genome pattern obtained from a Group A rotavirus can be readily distinguished from, for example, a Group C rotavirus genome pattern. The sample is positive for Group A rotavirus if 11 segments of dsRNA are visible and the pattern is similar to Group A rotavirus control RNA. Uncommon patterns can be tested against Group B and Group C rotavirus for confirmation. Reports from different parts of the world have shown electrotyping as a potential tool for studying the molecular epidemiology of human rotavirus infections (Waku-Kouomou et al.,

2016). Even though the sensitivity of PAGE assay is comparable to EM and ELISA nevertheless, it is labour intensive and time consuming, thus not routinely used to detect rotavirus in stool samples in the recent times (Esona & Gautam, 2015).

2.2.3.2 RT-PCR for Rotavirus detection

A variety of sensitive conventional or real-time reverse-transcription polymerase chain reaction methods have been developed based on primers specificity for rotavirus genes (Tong et al., 2015; Waku-Kouomou et al., 2016). The reverse transcriptase polymerase chain reaction (RT-PCR) method for rotavirus detection has been particularly useful in detecting rotavirus in extra-intestinal tissues, in studies of the duration of viral shedding in stool and the correlation between disease severity and viral load (Fumian et al., 2016; Li et al., 2015; Zhou et al., 2016). The RT-PCR assays can detect rotavirus in stool samples at relatively lower concentration (1000 times less) than those required for detection by EM and EIA (Esona & Gautam, 2015). The rotavirus VP6 gene encodes a group-reactive protein and is highly conserved among all RVAs. VP6 gene–specific RT-PCR assay designed in the conserved region of the VP6 gene followed by gel electrophoresis is performed routinely to detect a wide range of rotavirus strains in stool samples in strain surveillance studies (Oni et al., 2017). Also, because the RT-PCR product could be viewed on an agarose gel prior to genotyping, its serves as a useful verification of RNA extracts integrity prior to genotyping.

Rotavirus characterization by PCR is based on initially converting the single stranded RNA (ssRNA) of the rotavirus into cDNA with a subsequent amplification with genotyping primers in either singleplex or multiplex reactions, in combination with gel electrophoresis, probe

hybridization, or capturing real time fluorescence. In real time assays, the protocols are made to target a highly conserved region of the genome across a particular species of the virus (Esona et al., 2015).

The 3' end of NSP3 gene is a highly conserved region compared to the other regions of the genome; thus, it is the best target for the detection of a wide variety of RVA genotypes (Estes & Greenberg, 2013). Multiple qRT-PCR assays have targeted a highly conserved region near the 3' end of the NSP3 gene (Gautam et al., 2016). One-step qRT-PCR assays have been developed for detection of the dsRNA RVA NSP3 gene using the recombinant thermostable *Thermus thermophiles* (rTth) polymerase enzyme which are validated on a large number of stool samples positive for rotavirus (Bubba et al., 2015; Trang et al., 2015). By using rTth polymerase enzyme, the denaturation of an antecedent dsRNA step could be included in the thermal cycling program, thus reducing the possibility of sample cross-contamination and requiring less hands on time (Zhou et al., 2016).

The sensitivity and specificity of NSP3 qRT-PCR assay were determined to be 100% and 86%, respectively, compared with conventional RT-PCR assay (Waku-Kouomou et al., 2016). The efficiency of the NSP3 qRT-PCR assay was determined to be 94% with a limit of detection of 1 copy (Waku-Kouomou et al., 2016). Using a dsRNA transcript for NSP3 gene, the NSP3 qRT-PCR assay was made quantitative to determine the viral load in the samples (Gautam et al., 2016; Zhou et al., 2016). NSP3 qRT-PCR assay is a highly sensitive, specific, and quantitative tool to detect a broad spectrum of RVA genotypes in RV genotype surveillance studies, in serum, saliva or cerebrospinal fluid samples in cohort studies (Cowley et al., 2017; Gautam et al., 2016; Gratia

et al., 2016; Jeong et al., 2016; Tacharoenmuang et al., 2015). However, because PCR assays are relatively expensive and labor intensive and detects low copy numbers of RNA, they are not suitable for hospital based diagnosis (Gautam et al., 2016; Waku-Kouomou et al., 2016). Also, because rotaviruses are ubiquitous and stools which contain low viral loads and could not be detected by EIA or PAGE could be detected by PCR (Estes & Greenberg, 2013), PCR detection assays are not suitable for distinguishing between symptomatic and asymptomatic individuals. In the view of that, EIA detection methods remain the gold standard for describing the etiology of rotavirus-induced gastroenteritis in clinical settings (Waku-Kouomou et al., 2016).

2.3 Rotavirus characterization

The segmented nature and replication process of rotaviruses, make them undergo rotavirus nucleic acid reassortment, genome rearrangements and genomic point mutations (Patton, 2001; Tacharoenmuang et al., 2015). These proposed mechanisms of rotavirus evolution in nature, have been proven to permit several genotype combinations among rotaviruses (Estes & Greenberg, 2013). The replication of the virus in the enterocytes of the infected host permits the production of genetic variants forms of the rotavirus progeny (Cowley et al., 2016; Komoto et al., 2015; Komoto et al., 2016; Tacharoenmuang et al., 2015). Due to several variants forms of the virus, there is the need to develop effective characterization methods for genotyping rotaviruses in rotavirus surveillance (Waku-Kouomou et al., 2016). Sequencing, PCR, serotyping, southern blot, northern blot, reverse line blot hybridization, polymerase chain reaction- enzyme-linked immunosorbent assay (PCR-ELISA), and restriction fragmented length polymorphism (RFLP) are some of the genotyping methods (WHO, 2009).

The genes which encode the antigenic variant forms of the VP7 and VP4 viral proteins underpin the reason for characterizing rotaviruses by PCR into G-genotypes and P-genotypes respectively (Desselberger, 2014). There are several PCR techniques used in genotyping rotaviruses. However, the principle behind the methods are similar such that, in some methods, the cDNA synthesis is done separately from the genotyping (2 steps RT-PCR) whilst others involve cDNA synthesis and genotyping in the same synthetic environment (1 step RT-PCR) (Gautam et al., 2016). Among the RT-PCR genotyping methods are; the semi-nested multiplex-RT-PCR, one step quantitative duplex RT-PCR, SYBR Green real time RT-PCR, multiplex real time RT-PCR (Bubba et al., 2015; Fumian et al., 2016; Tong et al., 2015; Waku-Kouomou et al., 2016).

2.4. Limitations in PCR genotyping

Studies have shown that, regardless of rotavirus primers used in the characterization, a fraction of strains cannot be typed for rotavirus P or G genotype or for both P and G genotypes (Azaran et al., 2017; Esona & Gautam, 2015; Gautam et al., 2016). The genetic variations in the VP4 and VP7 genes of the globally common rotaviruses have led to observed increase in rotavirus non-typeable (NT) strains (Esona et al., 2017). This inability to genotype rotaviruses has been manifested by the detection of strains that produce a high yield of the consensus PCR product in the first-round PCR but do not yield a genotyping PCR product during the second round of amplification (Iturriza-Gomara et al., 2009).

Sequencing of the VP4 and/or VP7 genes of some of these NT strains showed that they contained several sequence changes in the region corresponding to for instance, the P[8] or G1 primer binding sites that prevented amplification with the original genotyping primers (Gautam

et al., 2016; WHO, 2009). New primers based on the variant sequence of the NT strains were designed at the same genome position as the original primer and subsequently used to genotype the remaining strains, suggesting that the variant strains belonged to the same P[8] or G1 genotype (Esona et al., 2010). These studies show the importance of considering genetic variation when using RT-PCR for strain genotyping (Esona & Gautam, 2015).

Investigators setting up genotyping methods using the originally published genotyping primers are likely to encounter NT strains and will need to develop a strategy (e.g., use of alternative primers) to reduce the number of NT strains detected (Waku-Kouomou et al., 2016; WHO, 2009). If the available primers do not work well for given specimen collections, genotyping primers might also need to be tailored to strains circulating in certain countries or regions (Gautam et al., 2015). Local laboratories might consider collaborating with Regional laboratories to obtain sequence information on circulating rotavirus strains to facilitate modification of primers (WHO, 2009). All redesigned primers need to be tested against a variety of field isolates and standard strains bearing common genotypes to detect cross-reactivity. In addition, results obtained with new primer sets will need to be selectively confirmed. Finally, because new rotavirus variants might co-circulate with parental strains, the design of degenerate primers capable of binding to both strains should be considered (WHO, 2009).

2.4.1 Non-typeable strains resulting from the presence of novel strains

Although NT strains are often genetic variants of common genotypes that no longer bind to the original genotype-specific primers, further characterization of NT strains, often by nucleotide sequencing, has demonstrated the presence of novel rotaviruses in which examples include the

detection of types G5, G6, G10, G12, P[7], P[11] and P[14] among NT strains (Esona et al., 2009; WHO, 2009). The availability of sequence data permitted the design of a specific primer or primer pairs for the novel strains that subsequently could be used in monoplex or multiplex PCR to genotype related strains (Waku-Kouomou et al., 2016). Although the prevalence of novel strains are relatively low as compared to the usual circulating strains, there is the need to characterize all detected rotaviruses in fecal specimen (Mijatovic-Rustempasic et al., 2016; WHO, 2009).

2.4.2 Other reasons for strains to be non-typeable

Stool specimen which have been positive for rotavirus antigen might fail to produce any PCR products after amplification with G and P consensus primers (Esona & Gautam, 2015). Genotyping primers might not bind to non-typeable samples as a result of a false-positive EIA, insufficient or degraded RNA, the presence of residual PCR inhibitors in the RNA extract, the presence of novel strains, or technical problems with the assay itself. If non-typeable samples represent a significant percentage of the analyzed strains, it is important to design a strategy to identify them (WHO, 2009).

A possible first step might be to confirm the presence of rotavirus particles by electron microscopy or rotavirus antigen and RNA by one of the several methods, including a repeat of the antigen EIA and subsequent PAGE analysis, or the use of a detection RT-PCR with consensus primers (Mijatovic-Rustempasic et al., 2016). If rotavirus is detected or RNA is present by PAGE, then a repetition of the RNA extract might be considered followed by a repeat of the typing procedure (Waku-Kouomou et al., 2016). If RNA is absent by PAGE and/or RT-

PCR, then the samples should be categorized as "RNA not detected" rather than NT. If these additional steps fail to identify a sample with intact RNA, then characterization of such strains might require testing a variety of primers to obtain products for sequencing or using advanced methods (WHO, 2009).

2.4.3 Confirmation of results

Although the gel based genotyping methods have been shown to be more than 90% accurate, misidentification by RT-PCR methods does occur, to ensure the accuracy of results, selective confirmation of genotype assignments should be carried out (WHO, 2009). Even though several confirmation methods have been described (e.g., Southern hybridization with cDNA and oligonucleotide probes or serotyping methods), sequence analysis has become the standard for both confirmation and identification of NT strains in gel based genotyping (Gautam et al., 2015). For confirmation, sequencing can be performed either on the genotype specific products or on a fragment of the VP7 or VP4 gene after amplification. The advantage of sequencing the genotype-specific PCR products is the ability to confirm infections by purifying and sequencing different sized products isolated from an agarose gel (Esona & Gautam, 2015). For the VP7 gene, a variety of consensus primer-pairs have been described including beg9/end9 and VP7-F/VP7-R and degenerate versions, 9con1/9con2, and 9con1-L/VP7-R deg. Consensus primers for VP4 gene fragments include con2/ con3, HumCom5/HumCom3 and VP4-F/VP4-R (Esona & Gautam, 2015). After sequencing, the strain genotype can be determined by comparing the genes of strains with known VP4 or VP7 types from the GenBank database. In some cases, the PCR products might need to be cloned before sequence analysis (WHO, 2009). An advantage of

cloning is that only a small amount of PCR product is required, thereby enabling sequencing of strains even when the concentration of product is too low for direct sequencing (WHO, 2009).

2.4.4 Other methods used for the characterization of rotaviruses

Some strains might require additional characterization techniques. An unusual level of stool inhibitors or a low level of intact virus in some samples might make it difficult to identify strain genotypes by RT-PCR or sequencing, and the samples might not be typeable by EIA (Waku-Kouomou et al., 2016). The presence of novel strains might also impede characterization by routine methods. Additional characterization techniques include cultivation in cell culture to amplify the amount of virus present and dilute out stool inhibitors, followed by repetition of routine methods or sequencing (WHO, 2009).

If the sequences of the VP4 and/or VP7 genes suggest a novel serotype, it might be necessary to prepare hyper-immune sera to the strains and conduct cross-neutralization tests to determine if the strains are antigenically distinct from known rotavirus serotypes (WHO, 2009). Such studies have been used in the past to define new rotavirus serotypes (Santos & Hoshino, 2005). If the genotype of the strain is novel or shows relationships to animal strains, additional studies can be carried out to define the potential origin of the strain. These studies might include sequencing additional genes, with comparison to human and animal rotaviruses, or conducting whole-genome hybridization studies to define the relationships to common rotaviruses of animals and humans. These types of studies have helped to demonstrate that some rare human rotaviruses

arose through interspecies transmission of an animal rotavirus to humans. Such studies also suggested that some strains, both common and uncommon, probably arose through reassortment between human and animal rotaviruses (Agbemabiese et al., 2017; Dennis et al., 2014; Ianiro et al., 2017). Thus, the analysis of non-typeable rotavirus strains from surveillance studies has been important in defining the genetic diversity and possible origin of many human rotaviruses and the techniques require a variety of molecular analyses. Collaborations between Regional laboratories and surveillance sites are recommended (WHO, 2009).

2.4.5 Full-genome sequencing

Next-generation sequencing (NGS) technology provides high-throughput sequencing of all the 11 gene segments of rotaviruses in a cost-effective and efficient way. NGS technology produces millions of sequences in repeats from the same sample (Waku-Kouomou et al., 2016). There are many commercially available NGS platforms, such as the Roche 454 (Roche Diagnostic, Indianapolis, IN), Ion Torrent Personal Genome Machine (Life Technologies, Carlsbad, CA), SMRT (single-molecule real-time) (Pacific Biosciences, Menlo Park, CA), Illumina HiSeq and Illumina MiSeq (Illumina, San Diego, CA) sequencers. The sequencing technology in MiSeq makes use of clustering and sequencing by synthesis using a fixed position on the flow cell instead of transporting through a gel (Gautam et al., 2015). For NGS of rotavirus genome, dsRNA is extracted from the stool samples and libraries for NGS sequencing are then prepared using reagents and kits specific for NGS sequencing technique of the manufacturer (Esona & Gautam, 2015). The quality and quantity of the genomic library are assessed on Bioanalyzer (Agilent Technologies, Santa Clara, CA), LabChip (PerkinElmer, Waltham, MA), or Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA) followed by sequencing on an NGS platform available using instrument-specific kits. Data analysis is performed by the reporter program

integrated in the instrument to generate FASTQ formatted sequence data. Contigs are assembled from the obtained sequence reads using de novo assembly command or map to reference in the CLC Genomics Workbench (CLC bio, Boston, MA) (Esona & Gautam, 2015; Esona et al., 2017; Gautam et al., 2015). The assembled consensus sequences of each gene are used to query the available RVA nucleotide database in the GenBank using nucleotide BLAST or by submitting the sequences to RotaC for genotype characterization (Esona & Gautam, 2015). Finally, the complete genome for each sample is built from nucleotide sequences of 11 genome segments by MEGA version software (Nyaga et al., 2015).

2.5 Rotavirus vaccines

The primary purpose of a rotavirus vaccine is to preclude the severity of rotavirus gastroenteritis during the early years of life. Even though rotavirus disease severity is significantly reduced during secondary and subsequent infections, natural infection does not provide complete protection against a subsequent reinfection and disease (Deal et al., 2010). Results. Considerable evidence from studies in animals indicates that the presence and quantity of rotavirus specific antibody in the lumen of the gut plays a critical role in resistance to rotavirus disease (Kang et al., 2015). Studies have shown that, children with elevated levels of fecal IgA in response to rotavirus infection had resistance to rotavirus illness (Chilengi et al., 2016; Hungerford et al., 2015; Marlow et al., 2015). These observations suggest that the effectiveness of a rotavirus vaccine largely depends on its ability to stimulate transport of antibodies into the gut lumen or to stimulate local production of antibodies (Pringle et al., 2016).

Currently, perhaps the most reliable and well-documented method of stimulating local intestinal immunity is thought to be infection or immunization in the intestine (O'Ryan, 2017). For this reason, most efforts to date have focused on live attenuated vaccines that are administered orally (Lazarus et al., 2017; Leino et al., 2017; Mo et al., 2017; Sow et al., 2017; Yu et al., 2017). Longitudinal studies have thrown more light on the resistance to disease induced by previous, naturally acquired rotavirus infection. Reinfection within the first few years of life is common, and an infant who had a primarily rotavirus infection, whether severe, mild, or asymptomatic, usually experiences a milder illness during reinfection (Dou et al., 2017; Harris et al., 2017; Lewnard et al., 2017). The level of protection induced by primary and subsequent reinfections may be less in some poor countries than in the developed world (Desselberger, 2014; Estes & Greenberg, 2013; Harris et al., 2017; Pringle et al., 2016) which could be influenced by several factors such as; malnutrition, intestinal microbiota, frequent infection with other intestinal pathogens, or other health factors of the children in those poorer countries (Estes & Greenberg, 2013).

The significant global burden of diarrhea disease associated with rotaviruses has led to the recommendation by the World Health Organization (WHO) for the necessity for vaccine development to reduce the severity of diarrhea due to rotaviruses. Because rotaviruses exhibited protective immunity from recurrent infections (Greenberg & Estes, 2009), it is therefore rationale to exploit immunology to develop vaccines to alleviate diarrhea burden due to rotaviruses. The first vaccine trial began in 1983, investigating the effectiveness of an oral vaccine derived from a bovine strain (RIT 4237) (Hull et al., 2011). Findings from vaccine trials demonstrated that, live oral vaccines were effective in protecting infants from the severity of rotavirus infections

(Vesikari et al., 1984) and that, protection was greatest against the most severe cases, and vaccines derived from animal strains were able to elicit immune response against human strains in vaccine trials. However, the vaccine failed to show consistency in efficacy in several countries which led to the delay of the development of the vaccine (Greenberg & Estes, 2009). It took fifteen more years to license a vaccine in which Rotashield® (Wyeth) was introduced in 1998 as a tetravalent rhesus vaccine containing G1-G4 strains. Clinical trials occurred in parts of the US, Finland and Venezuela and demonstrated an 80-100% efficacy in preventing severe diarrhea (Kirkwood et al., 2017). The vaccine was recommended for routine use in the US and was given to more than 60,000 infants in the first nine months; however, in July 1999 Rotashield® was removed, due to reports of a heightened menace of intussusception within the first two weeks of the first dose being administered (Lynch et al., 2006). Although there was 1 intussusception case out of 10,000 vaccinated children, the actual risk was unclear hence, the vaccine remained withdrawn for further use (Greenberg & Estes, 2009).

Two vaccines were later developed after Rotashield® was taken off the market and provided two different approaches to their development. Rotateq® (Merck) used a combination of five different bovine human re-assortment strains and was meant to be administered in three doses at 2, 4 and 6 months of age, not to exceed 8 months. Due to the keen concerns from Rotashield®, the Rotavirus Efficacy and Safety Trial (REST) study team recruited more than 60,000 infants to determine efficacy and intussusception rates for Rotateq®. During this trial, 12 cases of intussusception occurred in the vaccine group and 15 in the placebo group within one year after the first dose. Results demonstrated a vaccine efficacy of 98% against severe gastroenteritis and a reduction of hospitalizations and ER visits by 94.5% (Vesikari et al., 2006). From these

findings, Rotateq® was licensed in the US in February 2006 and was recommended for routine use in children (Bowen et al., 2016). The second vaccine, Rotarix™ (GlaxoSmithKline), is a human, live attenuated oral vaccine, and is based on the idea of protective immunity. This vaccine is administered in two doses and is recommended at 2 and 4 months of age, not to exceed 8 months (Vesikari et al., 2010). Rotarix™ also went through intense safety and efficacy trials, with over 60,000 infants. Results indicated that this vaccine was 85% effective against rotavirus hospitalizations and 100% effective against severe rotavirus. Intussusception rates were also noted and had six cases in the vaccine group and seven in the placebo. It was concluded from these trials that Rotarix™ was efficacious and had no association with increased risk of intussusception. This vaccine was first licensed in Mexico and the Dominican Republic in 2004 and was later introduced into thirty five countries around Europe and licensed in the US in June 2008 (Vesikari et al., 2010). In May 2010, Rotarix™ was temporarily suspended, due to identification of porcine circovirus1 (PCV1) in the vaccine, even though there was no health risk for humans associated with PCV1 (Kirkwood et al., 2017).

Testing was performed on both vaccines and it was determined that Rotateq® also had traces of PCV1, although suspension was not done on this vaccine. The suspension on Rotarix™ was lifted later in that same year after no risks were identified (Estes & Greenberg, 2013). In May, 2012, Ghana introduced the monovalent rotavirus vaccine Rotarix™ into the national Expanded Programme on Immunization (EPI) to manage diarrhea due to rotaviruses

Several studies have observed major declines in hospitalization rates due to rotavirus in high and middle income countries, where vaccines have been introduced (Harris et al., 2017). Vaccine

efficacy (VE) studies in the US have demonstrated a Rotateq® VE between 89- 94%, (Vesikari, 1999). Rotarix™ has also shown high efficacy in the US, with a VE of 91%. Developing countries however, have seen more variability in VE for Rotarix™ with a VE ranging from 39-77% (Beres et al., 2016; Burnett et al., 2016; Jonesteller et al., 2017; Mpabalwani et al., 2017; Vesikari, 2012).

2.6 Genotype distribution in vaccine era

Before the global introduction of the rotavirus vaccines for routine immunization of infants worldwide, rotavirus strain surveillance studies have been carried out in several regions to make available, useful information on rotavirus strains that were circulating in the pre-rotavirus vaccine era and an insight to examine the impact of rotavirus vaccine on future strain prevalence (Banerjee et al., 2006; Vesikari et al., 2006; Vesikari et al., 2007). After the introduction of rotavirus vaccines, surveillance on rotavirus strains is necessary to monitor the effect of rotavirus vaccines on genotype distribution. Studies have been carried out in many countries in monitoring the effect of rotavirus genotype distribution after the introduction of the rotavirus vaccines (Boula et al., 2014; Bruun et al., 2016; Estes & Greenberg, 2013).

Before the universal mass introduction of the rotavirus vaccine in Belgium, the G2P[4] genotype was found to account for less than 5% of all rotavirus induced gastroenteritis cases, but there had been rapid increase in the G2P[4] genotype, after three years of the rotavirus vaccine introduction, the strain accounted for 30-40% of all rotavirus induced gastroenteritis cases in the country (Pitzer et al., 2015). Also, in later years, it was revealed that, the prevalence of G2P[4] strains was associated with the disappearance of other rotavirus strains in the post-vaccine era

(Mandile et al., 2014). Although the strong re-emergence of G2P[4] strains was initially hypothesized to be related to the mass use of vaccine, however, the G2P[4] genotype is phylogenetically further than the other common human rotavirus genotypes (Do et al., 2015). Consequently, the causal relationship is unclear, since the emergence of G2P[4] had already been identified before the introduction of the rotavirus vaccines (De Donno et al., 2009; Paul et al., 2009).

In Australia, both vaccines, RotaTeq® and Rotarix™ are licensed and in use in different Australian states. Interestingly, with an overall vaccine coverage of more than 80% of the population, during the first two years after the vaccines were introduced, in states using Rotarix™ the G2P[4] became the most predominant rotavirus genotype, while in states using RotaTeq® G1P[8] and G3P[8] accounted for the majority of rotavirus infections (Hull et al., 2011). However, studies conducted have shown that the predominant rotavirus genotypes in states using both the Rotarix™ and RotaTeq® respectively follow different trend (Bowen et al., 2016) in suggestive of, seasonal fluctuations instead of vaccine-induced selective pressure.

In the United States, RotaTeq® and Rotarix™ were approved for immunization in 2006 and 2008, respectively and before the introduction of the vaccines, G1P[8] was the prevalent genotype for several years, but soon after the introduction of RotaTeq®, the proportional role of G1P[8] decreased to 30.7% while G3P[8] became the predominant genotype at 36.3% (Bowen et al., 2016). In addition to shifts in the genotype distributions, since the introduction of both rotavirus vaccines, emergence of previously uncommon genotypes such as G9 and G12 have been observed worldwide (Matthijnssens et al., 2008).

Several studies have detected novel strains of the rotavirus globally in the post vaccine era (Arana et al., 2016; Day & Zsak, 2016; De Grazia et al., 2015; Ianiro et al., 2017; Jere et al., 2014; Komoto et al., 2015; Masuda et al., 2014; Navarro et al., 2013; Niira et al., 2016; Rojas et al., 2017; Stupka et al., 2012). This might be an indication for future increase in unusual rotavirus genotypes (Flerlage et al., 2017; Ouermi et al., 2017). As the introduction of the vaccines has taken place within a short period of time, it is still not clear to speculate as to which of the changes in genotype distribution are due to natural fluctuation of genotypes over a period of time or as a result of vaccine-induced selection pressure.

2.7 Genotype distribution and seasonal patterns

Prior to the global introduction of rotavirus vaccines, rotaviruses displayed a marked seasonal pattern of infection in developed countries, with epidemic peaks occurring in the cooler months of each year (Gonzalez Chavez, 2015). The reason for the seasonal pattern observed in the developed countries has not been proven, as such, not known, but the influence of low relative humidity in the home has been suggested as a factor facilitating the survival of rotaviruses on surfaces (Estes & Greenberg, 2013). The usual seasonal pattern of rotavirus infection observed in the temperate climates does not occur uniformly in other areas of the world. Many locations in the tropics show no, or a diminished, seasonal trend and majority of rotavirus related deaths occur in developing countries where access to health care is limited (Desselberger, 2014). Each child is normally infected at least once before the age of five, with majority of them before 2-years of age.

Recent modeling studies have suggested that the relative lack of rotavirus seasonality observed in many tropical countries may be due to the high birth rates and transmission rates typical of developing countries (Esona & Gautam, 2015). Rotaviruses have been isolated from hospitalized children with acute gastroenteritis throughout the world and the disease burden has been considered a pandemic disease with a global public health concern. These viruses consistently constitute the major etiological agents of severe infantile diarrhea in every country where this disease has been studied (Clark et al., 2017; Kotirum et al., 2017; Marchetti et al., 2017; Sow et al., 2017; Tiku et al., 2017).

2.8 Rotavirus strain diversity in Ghana

To monitor the effect of rotavirus vaccines, systematic research into the changes in circulating rotavirus genotypes has been carried out in some countries including Africa (Mbuh et al., 2015) especially in countries that exclusively use the Rotarix™ vaccine, such as Ghana. Prior to rotavirus vaccine introduction in Ghana, studies have been carried out on circulating strains in the country in predictive of the impact the strains on vaccine efficacies.

An illustration of a pre-vaccine era survey was an epidemiological survey conducted on children less than 5 years with acute gastroenteritis in the rural Upper Eastern Region of Ghana during 1998 to detect the various circulating rotavirus strain in Ghana. In that study, rotavirus genotypes G1, G2, G3, P[4], P[6] and P[8] were detected. The G3 genotypes were found to be the predominant strain, followed by G2 and G1 among the VP7 genotypes (Asmah et al., 2001).

Another work carried out in northern Ghana reported G2P[6], G3P[4] and G9P[8] as the major circulating rotavirus strains (Binka et al., 2003). One of the most recent studies carried out before the introduction of the Rotarix™ vaccine in southern Ghana identified 8 G genotypes (G1, G2, G3, G4, G8, G9, G10, G12) and 3 P genotypes (P[4], P[6], P[8]) with G1 being the most prevalent genotype (50.9%) among the G-genotypes. Whilst the P[8] (36.1%), was the most prevalent genotype among the P-genotypes the most prevalent G/P genotype combination was G1P[8] (28%) followed by G3P[6] and G2P[6] respectively. Fifteen percent of the samples were partially characterized and were referred to as others (Fig 2.2). According to their findings, the others may be novel strains which could contribute the diversity of rotaviruses in Ghana (Enweronu-Laryea et al., 2013). Mixed G and P genotypes were also detected with their respective proportions as G-mixed (7.3%) and P-mixed (24.2%) (Enweronu-Laryea et al., 2013).

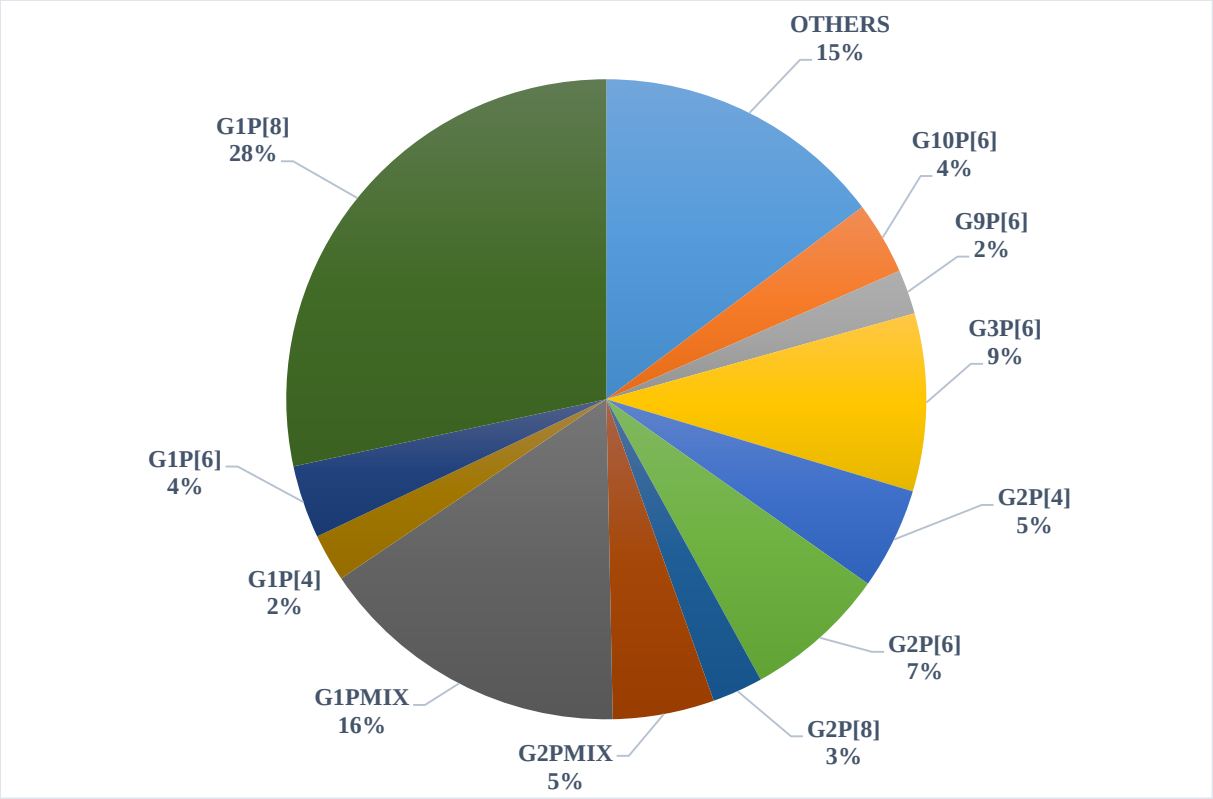


Fig 2.2 prevalence (%) of rotavirus genotypes among 624 children hospitalized with acute gastroenteritis in Ghana: 2007-2011. Image modified from (Enweronu-Laryea et al., 2013)

The G8 genotype is common in bovine rotaviruses (Dennis et al., 2014), and was previously detected sporadically in humans, in which zoonotic transmission was postulated (Gautam et al., 2015). The prevalence of G8 strains has been speculated to be the result of interspecies transmission of rotaviruses between humans and cattle (Dennis et al., 2014; Komoto et al., 2016; Ribeiro et al., 2016) due to poor sanitation in Africa coupled with high rate and chance of human-animal contacts. Even though it has not been shown that, poor sanitation influence the selection of unusual rotavirus strains leading to the observed diversity of rotaviruses in Africa, it has been shown that, there is interspecies transmission and reassortment events between circulating cattle/sheep/goat rotaviruses and human DS-1-like RVA strains in Ghana (Dennis et

al., 2014). In addition, there are several non-typeable strains detected in Ghana (Enweronu-Laryea et al., 2013). These non-typeable strains contribute to the diverse strains of rotaviruses (Desselberger, 1996; Pesavento et al., 2006) detected in the country and may be the cause of the low vaccine efficacies recorded in the country.

2.9 Rotavirus transmission

Rotaviruses are highly contagious with as little as one tissue culture infectious dose being able to cause illness in a susceptible host (Dennehy, 2000; Desselberger, 2014; Greenberg & Estes, 2009; Phillips et al., 2010; Prasad et al., 2001). Aside being highly contagious, the viruses are very stable in the environment and are shed in very large quantities in the feces (Desselberger et al., 2009) enhancing their transmission potentials. Rotaviruses are highly infectious and are transmitted via the fecal-oral route or respiratory droplets (Leung et al., 2005). Work done to ascertain the infectious dose and serological response to infection revealed that, children start shedding rotaviruses in their stools before the onset of symptoms and may pass out more than one thousand rotavirus particles per gram of feces in their stools, however, fewer than one hundred particles are required to infect new hosts this makes the virus highly contagious and effective in transmission. The risk of transmitting rotavirus can be lowered by frequent hand washing and treating contaminated materials in high temperatures (over 50 °C). The virus can be inactivated by several disinfectants, especially 95% ethanol, which exerts its effect by removing the outer-most layer (Dennehy, 2000; WHO, 2009).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

Children under the age of 5 years of age who report with diarrhea and/or emesis to the Emergency and Out-patient Departments of the Ashaiman Polyclinic in the greater Accra region of Ghana, and whose parents/guardians gave their consent to participate in the study were enrolled. Demographic, clinical and epidemiological data about the onset and duration of diarrhea, emesis, fever and treatment were obtained. Collected stool samples were stored at -20 ^{o}C at the hospital and later transferred into sterile containers by using microbiologically approved aseptic techniques and transported to the department of Biochemistry, Cell and Molecular Biology of University of Ghana, Legon. The collection was done during two Rotavirus seasons (2014-2015 and 2015-2016). In all, 369 diarrhea stools were collected for the study.

The samples were screened for Group A rotaviruses (RVA) using Premier^{TM} Rotaclone$^{®}$ enzyme immunoassay (EIA) kit. Samples which tested positive by EIA were processed in the West African Regional Rotavirus Reference Laboratory at Noguchi Memorial Institute for Medical Research, Legon for gel-based genotyping. Total RNA was extracted from all stool samples collected (both EIA positive and EIA negative samples) and were sent to the Centers for Disease Control and Prevention, Atlanta, Georgia, USA for the confirmation of the gel-based genotyping results by Sanger sequencing. Subsequently, full genome characterization using Next Generation Sequencing (NGS) techniques was done on selected strains from the gel-based genotyping

results and phylogenetic and full genome RVA nomenclature data were obtained from the NGS data.

3.2 Flow chart of the study design

The flow chart below (Fig 3.1) outlines the main steps of the study design. There were 6 main steps in the design which were; ethical clearance acquisition, sample collection, screening for RVA, gel based genotyping, genotyping by sequencing assays and data analysis.

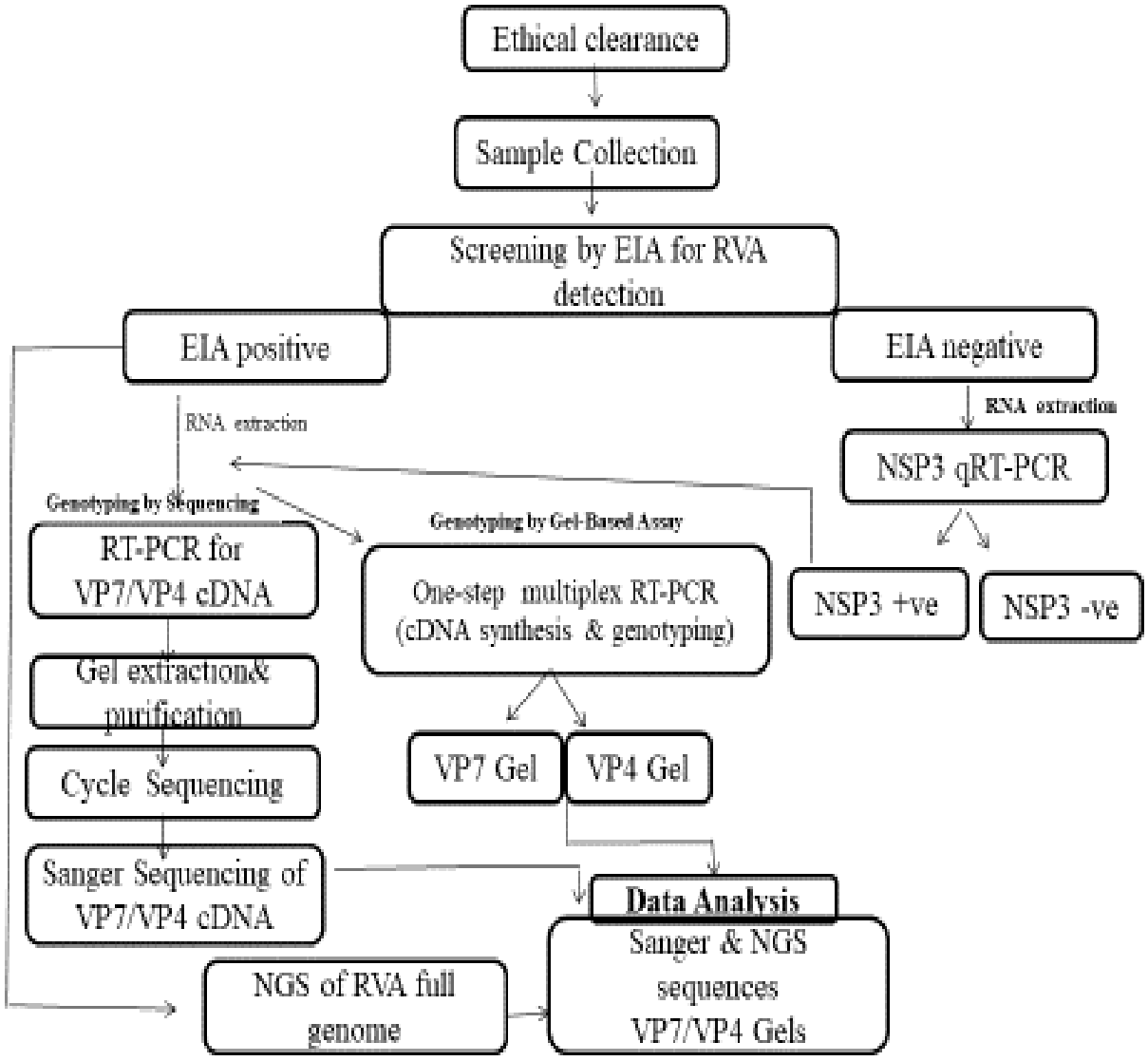


Fig 3.1 A schematic representation of the study design. The arrows direct the flow of methods used in this study.

3.3 Ethical clearance

This study involved the characterization of group A rotaviruses obtained from stool samples collected from the study area for rotavirus surveillance studies in Ghanaian children. The study was approved by a licensed and authorized ethical clearance board; the Institutional Review Board of Noguchi Memorial Institute for Medical Research, Legon, Ghana. A written informed

consent for the collection, testing and processing of stool samples for rotaviruses and characterization of identified rotavirus strains was obtained from children's parents and or guardians prior to sample collection.

3.4 Methods

3.4.1 RVA detection

3.4.1.1 ELISA detection by Premier™ Rotaclone® EIA kit

The diarrhea stools were retrieved from storage and thawed to room temperature after which they were vortexed briefly and 10% phosphate buffered saline (PBS) stool suspension was made for each sample by adding 100 µl of stool to 900 µl of PBS in a 1.5 ml Eppendorf tube and were vortexed briefly. After vortexing, 100 µl of the 10% stool suspension was pipetted into the 96 wells. The last two wells were filled with the negative (100 µl of PBS) and positive controls (2 drops) respectively. Two drops of the RotaClone enzyme conjugate was added to each sample including the controls and mixed gently by swirling. The sample-conjugate mixture was incubated at room temperature for 60 min. After incubation, the liquid mixture was discarded from the wells followed by banging the wells downwards on absorbent paper to ensure complete removal of unbound particles from the wells. The wells were filled with deionized water and rewashed as described above. Washing procedure was repeated for 4 more times. Two drops of RotaClone substrate A was added to each of the washed wells which was followed by adding 2 drops of the substrate RotaClone B. The complex was incubated at room temperature for 10 min. This was then followed by adding 2 drops of stop solution (H_2SO_4) for photometric determination. Plates were read within 30 min. on an EIA plate reader (Titertek MultiscanMCC/340 MKII) using a 450-nm filter. Positive samples were determined as: P - N

>0.07, where P was absorbance of the well coated with positive control and N was the absorbance of the well coated with PBS.

3.4.1.2 Extraction of RV dsRNA from EIA negative samples using Kingfisher Flex 96 Extraction

In an RNA extraction room, autoclaved 96 extraction wells were labeled according to the extraction order on the extraction sheet. Kingfisher RV extraction reagents: lysis buffer, binding beads, wash solution 1 and 2 and elution buffer were retrieved from storage and allowed to thaw to room temperature. Stool samples (10% PBS suspension) were also retrieved from storage and allowed to thaw after which, 130 µl of the lysis buffer and 25 µl of the bead matrix were added to each of the samples in the 96 wells for cell lyses and binding of the template to beads matrix respectively. In a separate 96 wells, 150 µl of the wash solution 1 and 2 were pipetted into their respectively labelled plates according to the extraction order on the extraction sheet. This was repeated to obtain 2 plates each filled with wash solution 1 and 2. The plates containing sample matrix, wash solutions and elution buffer in their respective wells were loaded into the Kingfisher Flex 96 Extraction machine. After 25 min, the extracted total RNA was eluted into the elution wells and the plates were covered and stored at -65 ^{o}C.

3.4.1.3 NSP3 qRT-PCR on EIA negative samples using ABI 7500 Fast DX instrument

In the clean PCR preparation room all surfaces such as: ice bucket, cooling chamber and pipettes were wiped using 10% bleach solution, followed by 70% ethanol, and UV light exposure of 30 min. Also, in the RNA work area, all surfaces such as: ice bucket, cooling chamber and pipettes

were wiped using 10% bleach solution, followed by 70% ethanol, and UV light contact for 30 min.

After the application of the above aseptic techniques, the ABI 7500 Fast Real-Time Instrument lamp was turned on to warm up. Real time reagents were allowed to thaw (master mix reagents kept at -20 °C; primer/probe working stocks at 4 °C). Samples to be tested and positive control, were also removed from sample storage freezer, and were allowed to thaw in refrigerator. In the clean room, a 96 well Fast optical reaction plate was prepared and labeled since one well was needed for each sample being tested, plus the controls. In the clean PCR preparation room, the master mix was prepared to give a total volume of 4991 µl for 217 reactions as shown in table A 1.1. The reaction mix was kept on ice and 23 µl of master mix was aliquoted into each well of an ABI Fast plate with the plate being loosely sealed and the reaction plate containing master mix was transferred to RNA work area whilst on ice, 2 µl of the sample RNA, positive controls, and negative controls were respectively added to the 96 well plates. Samples were processed to run in duplicates and the plates were sealed**.** The plates were spun in micro-centrifuge for 10 sec. at 13,000 rpm, and returned to ice. Whilst on ice, the plates were transferred to the real-time instrument room. The thermal cycler protocol was sequentially programmed to run at initial dsRNA denaturation at 95 ^{o}C for 1 cycle, reverse transcription for 5 min for at 50b ^{o}C, RNA – cDNA denaturation for 45 cycles at 95 ^{o}C for 15 sec., 2-step amplification; quantification at 60 ^{o}C for 60 sec. The program was run for approximately, 2 hours and the results were obtained in real time.

3.4.2 Gel-based genotyping of VP7 and VP4 genes

3.4.2.1 Extraction of dsRNA from EIA positive samples using phenol/chloroform/RNAID

In a clean extraction room, 10% sodium dodecyl sulfate (SDS) solution was prepared by dissolving 10 g of salt in 100 ml of distilled water placed in 65 ^{o}C water bath. Also, in preparation of 1 M sodium acetate (NaOAc) / SDS solution, 8.2 g of the NaOAc crystal was dissolved in 60 ml distilled water followed by adding 1 ml of the 10% SDS stock and then adjusting the pH to 5.0 using glacial acetic acid with the final volume made to 100 ml by adding distilled water. Guanidinium isothiocyanate (GITC) solution of concentration 6 M was prepared prior to use by dissolving 7.09 g of the GITC salt in 10 ml distilled water followed by heating in 56 ^{o}C water bath for 15 min. The temperature of the 1M NaOAc containing SDS at pH of 5 was raised to 37 ^{o}C in a water bath after which, 50 µl of it was added to 500 µl of the 10% stool suspension and was vortexed for 10 sec. The reaction mixture (NaOAc/SDS and stool suspension) was incubated at 37 ^{o}C for 15 min to break the protein capsids and to release the dsRNA into solution. After the 15 min. incubation to break protein capsid, 500 µl of phenol/chloroform was added, vortexed for 1 min. and incubated at 56 ^{o}C for 15 min. after which the tubes were opened and resealed to remove inbuilt pressure. After the release of inbuilt pressure, the mixture was again vortexed for 1 min. and centrifuged at 12000 rpm for 3 min.

The supernatant which contained the dsRNA was carefully removed and placed in clean Eppendorf tubes. The extraction was repeated with 250 µl of phenol/chloroform as described above to maximize yield. For the purification of the extracted dsRNA, 500 µl of 6 M GITC was added to the recovered suspension and vortexed for 30 sec. and then, centrifuged at 12000 rpm for 5 min. after which the mixture was transferred into clean Eppendorf tubes. The RNAID matrix was vortexed for 1 min. prior to use and 10 µl of it was added to each sample, vortexed

for 10 sec. and incubated on a rocker at room temperature for 15 min. The mixture was centrifuged at 5000 rpm for 20 sec. after which the supernatant was discarded. After discarding the supernatant, 400 µl of RNAID wash buffer was added to the pellet and gently suspended with pipette. The mixture was then centrifuged for 30 sec. at 12000 rpm to discard the supernatant. For washing, 100 µl of RNAID wash buffer was added to the pellet and gently suspended with pipette. The mixture was then centrifuged for 60 sec. at 12000 rpm to discard the supernatant and then, blotted well to get rid of excess ethanol. Pellet was re-suspended in 50 µl diethylpyrocabonate (DEPC) treated water to deactivate any RNase and was incubated on water bath at 56 ^{o}C for 10 min. to elute RNA from beads. The mixture was centrifuged at 12000 rpm for 3 min. to separate the RNA from the beads. The supernatant which contained the dsRNA was carefully and gently transferred to a sterile Eppendorf tubes and stored at – 20 ^{o}C.

3.4.2.2 VP7/VP4 genotyping by multiplexed one-step RT-PCR using ABI 7500 Fast DX instrument

For each reaction, primer mix was made for VP7 reactions by adding 1.5 µl of 120 µM VP7 forward (VP7uF) and 0.25 µl of each 120 µM reverse primer (G1-R4, G2-R1, G3-R1, G4-R2, G9-R2 and G12-R2) whilst working on ice in a biosafety cabinet. Qiagen one-step RT-PCR master mix for VP7 was prepared (Table A 1.2). Also, VP4 primer mix was made by adding 1.5 µl of 100 µM VP4 forward (VP4uF) and 0.3 µl of each 100 µM reverse primer (P[4]-R5, P[6]-R2, P[8]-R2, P[9]-4T-1 and P[10]-5T-1)**.** Qiagen one-step RT-PCR master mix was also prepared for the VP4 reactions (Table A 1.3). After primer mix and master mix preparations for VP7 and VP4 reactions, 2 µl of the respective primer mix was added to each PCR wells according to the worksheet and was followed by adding 5 µl of the dsRNA template. The PCR

reaction tubes were spun at 13000 rpm for 15 sec. to ensure that all RNA and primers were at the bottom of the reaction tubes. The RNA and primers were together denatured in 0.5 ml tubes at 97 ^{o}C for 4 min. The tubes were immediately placed on ice for 1min. after which they were centrifuged at 13000 rpm for 10 sec. to flush down the samples. After flushing down the denatured template and primers, 23 µl of the master mix was added to the denatured templates immediately and was centrifuged at 13000 rpm for 10 sec. again to flush down the samples. The PCR tubes were transferred to the pre-heated thermal cycler designated for the one-step multiplex reaction. The reaction was carried out with an initial denaturation at 94 ºC for 2 min followed by 30 cycles of amplification (denaturation at 94 ºC for 1 min, annealing at 42 ºC for 2 min and extension at 72 ºC for 3 min) and a final extension at 72 ºC for 7 min and the reaction was held at 4 ^{o}C.

3.4.2.3 Electrophoresis of one-step multiplex RT-PCR VP7/VP4 products

Electrophoresis buffer was prepared by diluting 10X TAE 1:10 in deionized water (1000 ml TAE was added to 10000 ml deionized water). Also, 3% agarose gel was prepared by dissolving 117 g of SeaKem ME agarose per 300 ml of 1X TAE Buffer. The agarose buffer solution was heated in a microwave for 5 min. to boil and dissolve the agarose and 30 µl of Gel Red was added and mixed prior to pouring of the gel. The gel was then poured into a casting tray with no bubbles and was made to stand for 30 min to solidify. After the solidification of the gel, 2 µl of loading dye was mixed with 10 µl of PCR product by pipetting after which, 10 µl of the mixture was loaded into the wells of the gel**.** Electrophorese in 1X TAE buffer was made at 100 volts for 1 hr. where the dye indicator was made to run 90% of the gel length. The gels were taken to the dark room for UV trans-illumination.

3.4.3 Confirmation of VP7/ VP4 genotypes by Sanger Sequencing

3.4.3.1 VP7/VP4 RT-PCR for cDNA synthesis using ABI 7500 Fast DX instrument

An autoclaved 96 well Fast optical reaction plate was labeled according to the worksheet. After labelling, primer mix was made for VP7 by adding 1.5 µl, 20 µM of VP7 forward (9con1L) and 1.5 µl 20 µM of VP7 reverse (VP7RDeg). Primer mix was made for VP4 by adding 1.5 µl, 20 µM of VP4 forward (Con3) and 1.5 µl 20 µM of VP4 reverse (Con2). Reaction master mix was prepared to give a total volume of 1922 µl for 84 reactions (Table A 1.4). The PCR reaction was then programmed as described above in section 3.4.2.2. After the cDNA synthesis, the products were separated on a 1% agarose gel as described previously in section 3.4.2.3.

3.4.3.2 QIAquick Gel extraction and purification

Under the UV light, the cDNA was excised from the agarose gel using a clean scalpel. In order to get 10 µg weight of the gels, the tubes were pre-weighed and then subtracted from the weight of the gel plus that of the tube. A 1/30 W/V solution was made by adding 300 µl of the Qiagen QG buffer to the previously weighed 10 µg agarose gel. The tubes were incubated on a heating block at 50 ^{o}C and were occasionally vortexed in between until the gel dissolved completely after 10 min. The colour of the dissolved gel was examined to be yellow after the 10 min. incubation to ensure the optimum pH of the mixture before proceeding to the next step. The QIAquick spin column was placed in a 2 ml tube and samples were applied to the QIAquick spin column. The tubes were spun twice at 13000 rpm for 1 min. each to get rid of unwanted effluent from the tube. After getting rid of effluent, 500 µl of buffer QG was added to the column and

was spun at 13000 rpm for 1 min. after which the flow was discarded. For washing out unbound materials in column, 750 µl of Qiagen buffer PE was added to the column and spun for 1min. at 13000 rpm and the flow was discarded. The column was finally placed in a labeled 1.5 ml micro-centrifuge tube and 50 µl of Qiagen buffer EB (10 mM Tris-HCl, pH 8.5) was placed at the center of the column membrane and the column was left to stand for 1 min. Finally, the column was spun at 13000 rpm for 1 min. to elute the cDNA into labeled 1.5 micro-centrifuge tubes.

3.4.3.3 Sanger sequencing of VP7 and VP4 cDNA

3.4.3.3.1 Cycle sequencing using ABI 7500 Fast DX Instrument

In the clean PCR reaction room, 20 µM of the PCR primers were diluted to 2 µM with DEPC treated water for cycle sequencing. A master mix was made for 150 reactions to give a total volume of 1200 µl as (Table A 1.5). After master mix preparation, 2 µl of purified cDNA was placed in a 1.5 ml reaction plate and was labeled according to the template sheet of the plate after which 2 µl of the 2 µM primer was added to the templates. After the addition of 2 µM to the templates, 6 µl of the master mix was then added to make a total volume of 10 µl. The samples in the tubes were spun for few seconds to flash down. The samples were then placed in the thermal cycler and was run and the reaction was carried out with an initial denaturation at 96ºC for 10 sec. followed by 25 cycles of amplification (denaturation at 96 ºC for 10 sec. annealing at 50 ºC for 5 sec and extension at 60 ºC for 4 minutes) and the reaction was held at 4 ^{o}C.

3.4.3.3.2 Cycle Sequencing Product Purification using BioMag® Carboxyl Beads and Sanger sequencing using ABI 3030xl Genetic analyzer

BioMag® beads stock solution was prepared by adding 1ml of the BioMag® Carboxyl beads to the 1.5 ml Eppendorf tubes. The tubes were then placed in a magnetic separator for 2 min after which the supernatant was poured off and 0.5 ml EDTA was added and the beads re-suspended using pipette and then returned to the magnetic separator for 2 min. The supernatant was then discarded with the beads re-suspended again with 0.5 ml of 0.5M EDTA. After re-suspension of the beads, 19.5 ml of 5.0 M NaCl was then added to make the stock solution ready for use. BioMag® Carboxyl beads stock was well shaken to fully re-suspend after which 10 µl of the cycle sequenced product was added and 42 µl of 85% ethanol was added to the 10 µl cycle sequenced product. This was followed by pipette mixing to homogenize the solution.

The reaction plate was then placed onto SPRIPlate 96R ring magnetic plate for 5 min. for the solution to become clear after separation from magnetic beads. The separated beads formed a circle at one corner of the well. Whilst working on the magnet, the tubes were inverted onto a lint-free wipe by tapping it gently on hard surface few times. After which 200 µl of 85% ethanol was dispensed into each well containing the beads. After the dispensation of 85% ethanol, there was a waiting time of 30 sec. to allow the beads to settle before proceeding to the next step. Whilst working on magnet, the plates were inverted onto a lint-free wipe by tapping it gently on a hard surface for few times to remove as much supernatant as possible. The washing process was repeated with the 200 µl of the 85% ethanol as described previously. The plates were inverted onto a lint-free wipe, tapping it gently on a hard surface for few times to remove as much supernatant as possible as it possibly contained excess fluorescent dye and contaminants. Finally, whilst the plates were on magnet, they were centrifuged at 500 rpm for 10 sec. to evaporate all ethanol. Whilst working on magnet, 40 µl of DEPC treated water was added to the

dry wells. The direct inject magnet was placed into the base of the sequencer alongside with the magnet. The sequencer was allowed to run for 24 hrs. in ABI 3030xl Genetic analyzer.

3.4.4 Library preparation for Next-Generation Sequencing (NGS)

3.4.4.1 Total RNA extraction from 30% stool suspension and Lithium chloride (LiCl) precipitation of ssRNA from dsRNA

Total RNA was extracted from 30% stool suspension as described previously in section 3.4.1.2 for the NGS library preparations and was followed by LiCl precipitation of ssRNA.

In a 0.6 ml micro-centrifuge tube, 45 µl RNA sample extracted, 5 µl of 10X DNase I buffer and 1 µl of rDNase I (2U) were added and made to mix by quick spun and vortexing. The reaction plate was transferred to a thermal cycler and incubated at 37 ^{o}C for 30 min. After 30 min. incubation, the DNAse I enzyme was deactivated by adding 10.2 µl of 25 mM EDTA to each reaction tube and was incubated at 75 ^{o}C for 30 min. in a thermal cycler. Freshly prepared 8 M LiCl of volume, 20.4 µl was added to the DNAse treated sample and was mixed by vortexing briefly and left at room temperature for 15 min. After the 15 min. room temperature incubation, the mixture was subjected to a longer incubation of 16 hrs. at 4 ^{o}C. After the 16 hrs. incubation, the samples were centrifuged at 12,000 rpm on Eppendorf Centrifuge at 4 ^{o}C for 30 min. The supernatant which contained the dsRNA was transferred into 1.7 ml micro-centrifuge tubes.

3.4.4.2 Purification of dsRNA using MinElute Gel Extraction kit

In a clean extraction room, 300 µl of buffer QG was added to each sample followed by adding 100 µl of isopropanol to each sample and mixed by inverting the tubes. The MinElute spin columns were placed in a column provided in 2 ml collection tube. The samples in the tubes were transferred into the MinElute spin column and were spun at 13,000 rpm for 1 min. The flow was discarded through and the MinElute columns and were placed back into the tubes. After placing the MinElute columns back into the tubes, 750 µl of buffer PE was added to the MinElute column and centrifuged at 13,000 rpm for 1 min to discard the flow. The MinElute column was centrifuged one more time at 13,000 for 1 min. Each MinElute column was placed into a clean 1.5 ml micro-centrifuge tubes. After placing the MinElute columns back into the tubes, 30 µl of buffer EB was added to the columns and were made to stand for 1 min. and the columns were spun at 13,000 rpm for 1 min. to elute the dsRNA into clean 1.5 ml micro-centrifuge tubes.

3.4.4.3 RNA fragmentation by NEBNext® Magnesium and random priming

RNA fragmentation reagent (NEBNext® buffer (5X)), nuclease free water and random primers were retrieved from storage and placed on ice in a biosafety cabinet. Whilst working on ice, 5 µl of purified dsRNA was pipetted into reaction wells whilst 5 µl of nuclease free water was added to no template control (NTC) well**.** Master mix was prepared by adding 104 µl of NEBNext® buffer (5X) and 26 µl random primers to give a total volume of 135ul for 26 reactions as shown in Table A 1.6. After master mix preparation, 5 µl of the master mix was added to each well containing the specific dsRNA and the NTC to give a total volume of 10 µl in each well. The thermal cycler was set to concurrently fragment RVA dsRNA to the 11 different fragments and to denature fragmented RNA for random priming by incubating at 94 ^{o}C for 5 min. in a thermal

cycler. The samples were retrieved from the cycler and were immediately transferred onto ice for cDNA synthesis of dsRNA fragments.

3.4.4.4 First and second strands cDNA synthesis

A master mix for first strand synthesis was prepared to give a total volume of 260 µl for 26 reactions as shown in table A 1.7. The mixture was mixed by gently pipetting and 10 µl of the master mix was added to the 10 µl of the fragmented and primed RNA template and NTC to make a total volume of 20 µl in each well. The samples were incubated in ABI thermal cycler as follows: 25 ^{o}C for 10 min. followed by 42 ^{o}C for 30 min. and then 70 ^{o}C for 15 min. and was held at 4 ^{o}C. Immediately after first strand synthesis, the second round reaction was prepared as below.

Master mix was prepared for 26 reactions to give a total volume of 1560 µl for the second strand synthesis (Table A 1.8). The master mix was mixed thoroughly by gentle pipetting and 60 µl of the master mix was added to the first round product to give 80 µl total volume. The mixture was incubated in a thermal cycler for 1 hour at 16 ^{o}C with the heated lid of the thermal cycler set at 40 ^{o}C.

3.4.4.5 Purification of fragmented amplicons using 1.8X AMPure XP Beads

In a clean NGS room, 144 (1.8X) ml of re-suspended AMPure XP beads was added to the second strand synthesized products and was mixed well by pipette mixing followed by room temperature incubation for 5 min. The mixture was transferred from 0.65 PCR plates to 96 deep

well plate and 200 µl of freshly prepared 80% ethanol was added to each well containing the samples as the plate was on a magnetic rack with an incubation period of 30 sec. The supernatant was carefully discarded. The washing was repeated for one more time with 200 µl of the 80% freshly prepared ethanol as described above. The beads were air dried while the plate was still on magnetic rack. The plate was removed from magnetic rack and 60 µl 0.1 X TE buffer was added to the wells to elute the DNA from the beads. The beads were re-suspended in the elution buffer by pipette mixing and then placed back on the magnetic rack until the solution became clear. After elution, 55.5 µl of the supernatant was transferred to a clean 96 well PCR plate.

3.4.4.6 End Repair/ dA-tail of cDNA Library

End repair dA- tailing of cDNA reagents were retrieved from storage and placed on ice in a biosafety cabinet. Master mix was prepared to give a total volume of 247 in a 1.7 ml Eppendorf tube for 26 reactions as shown in Table A 1.9. After the master mix preparation, 9.5 µl of the master mix was added to the 55.5 µl purified double stranded cDNA strand to give a total volume of 65 µl in each well**.** The reaction was incubated at 20 ^{o}C for 30 min. followed by 65 ^{o}C for 30 min. and was held at 4 ^{o}C. After the end repair dA- tailing of cDNA, the reaction was processed immediately for adaptor ligation.

3.4.4.7 Adapter ligation and size selection of adaptor ligated-DNA

The NEBNext Adaptor was retrieved from storage at 15 µM and was diluted with 10 mM NaCl to 1.5 µl. Master mix was prepared to give a total volume of 455 µl for 26 reactions as shown in Table A 1.10. A volume of 17.5 µl of the master mix was added to the dA-Tailed cDNA and 1 µl

of diluted adaptor which made up the volume to 83.5 µl in each well. This was followed by pipette mixing and a quick span to homogenize and flush down the mixture. The reaction was incubated in a thermal cycler for 15 min. at 20 ^{o}C. After the 15 min. incubation period, 3 µl of USER Enzyme was added to each well of the ligated product to make a total volume of 86.5 µl per well and was proceeded by pipette mixing and brief spinning to homogenize and subsequently flush down the mixture. The ligated product was incubated at 37 ^{o}C for 15 min. to denature the USER Enzyme after the enzyme had completed its task and was prepared for size selection.

The adaptor ligated-DNA was purified from solution using the AMPure XP beads as described above in section 3.4.4.5. The plate was removed from magnetic rack and 22 µ 0.1 X TE buffer was added to the wells to elute the DNA from the beads and 20 µl of the supernatant was transferred to a clean 96 well PCR plate.

3.4.4.8 PCR Library Enrichment

PCR library enrichment reagents were retrieved from storage and placed on ice in a biosafety cabinet. Master mix was prepared by for a volume of 715 µl for 26 reactions as shown in Table A 1.11. After the master mix preparation, 27.5 µl of it was added to the 20 µl adaptor ligated-DNA and NTC, followed by adding 2.5 µl index primers of concentration,10 µM to make a total volume of 50 µl in each well. The mixture was vortexed and briefly spun to mix and the mixture was flushed down. The PCR reaction was carried out with an initial denaturation at 98 ºC for 30 seconds followed by 15 cycles of amplification (denaturation at 98 ºC for 10 sec., annealing at 65

ºC for 75 sec. and extension at 65 ºC for 75 sec.) and a final extension at 65 ºC for 75 sec. and the reaction was held at 4 ^{o}C with the adaptor ligated DNA barcoded with index primers.

The barcoded DNA was purified from solution using the AMPure XP beads as described above in section 3.4.4.5 and 20 µl of the supernatant was transferred to a clean 96 well PCR plate.

3.4.4.9 Normalization of DNA library

In order to standardize the concentration of the prepared library prior to NGS, Qubit photometric normalization technique was employed. The concentrations of the NGS library for each sample was determined and in order to normalize each concentration to 0.02 ng/µl, serial dilutions were made with nuclease free water after which, the concentrations were confirmed with the photometer.

The DNA library was pooled together by adding 5 µl of sample from each well into a single Eppendorf tube and then vortexed and spun briefly. After spinning, 5 µl of the well mixed pooled library was pipetted into another Eppendorf tube and 10 µl of 10N NaOH solution was added and followed by adding 800 µl of nuclease free water. The mixture was vortexed and spun briefly after which, 5 min. room temperature incubation period was allowed to denature the DNA. A volume of 960 µl of chilled HT1 buffer was added to the denatured DNA. In order to denature the PhiX control, 5 µl of 2nM PhiX was aliquoted into a separate Eppendorf tube and 5 µl of freshly prepared 0.2 N NaOH was added. The mixture was briefly vortexed and spun and incubated for 5 min at room temperature to denature the DNA into single strands and was

followed by adding 990 µl of chilled HT1 buffer to the denatured PhiX DNA. For the spiking of 20% PhiX, 480 µl of the denatured template library was placed in a separate Eppendorf tube and 120 µl of the denatured PhiX was added. For the spiking of 5% PhiX, 570 µl of the denatured template library was placed in a separate Eppendorf tube and 30 µl of the denatured PhiX was added. Finally, a volume of 600 µl of 12 pM denatured library spiked with PhiX was loaded onto the MiSeq reagent cartridge and was made to run for 72 hrs.

3.5 Data analysis

3.5.1 Analysis of VP7 and VP4 gels

VP7 and VP4 amplicons were matched to the molecular weight marker and the sizes of each band was compared to referenced sizes (tables A 1.12 and 1.13) to determine genotypes. Frequency distribution charts were drawn for the various G, P and G/P genotypes using Microsoft excel.

3.5.2 Analysis of VP7 and VP4 Sanger sequences

The raw sequences were retrieved from the Crick Sanger Sequencing machine and were loaded in Sequencher 5.0 software. The sequences were sorted according to quality and size. Sequences with low quality less than 70% in quality and less than 250 bp were deleted. Sequences were then sorted by kind in order to bring together the consensus (forward and reverse) sequences. Contigs were formed from the consensus sequences and the chromatogram of the individual Contigs were perused to correct mismatches. The Contigs were merged into one sequence where

they were converted into FASTA files. The FASTA files were blasted in NCBI to obtain the genotypes.

3.5.3 Phylogenetic trees construction for VP7 and VP4 genes

The genotypes for the various gene segment were determined by RotaC version 2.0. Multiple sequence alignment was made for each gene using MUSCLE algorithm implemented in MEGA 6.0. Maximum likelihood trees were constructed for each gene segment in PhyML 4.0 using the optimal model. Selection analysis was performed using a combination of Single Likelihood Ancestor Counting, Fixed Effect Likelihood and Random Effect Likelihood analysis. Substitution in the VP8*, VP5* and VP7 regions were mapped to crystal structures available in PDB. For VP5* RRV crystal 2B41 was used, for VP8* the Wa crystal 2DWR was used and for VP7 RRV crystal 3FMG was used and the best model selection was done based on Akaike information criterion which were general time reverse. Approximate likelihood ratio statistical tests were computed for branch support sub genetic clusters were also computed to categorize strains into lineages. Strains were represented according to the strain representation criteria proposed by the Rotavirus Classification Working and were labeled for easy visualization on their respective trees.

3.5.4 Full genome constellation of NGS samples

The MiSeq reporter programme generated FASTQ formatted sequence data for each sample. Sequences were analyzed using CLC Genomics Workbench Software v6.5.1 (CLC Bio, Aarhus, Denmark). Contigs were assembled from obtained sequence reads by de novo assembly. Subsequently, assembled contigs sequences were used to query the non-redundant nucleotide database in GenBank employing the Basic Local Alignment Search Tool (BLAST). Genotypes of confirmed RVA sequences were then determined according to the recommendations of the Rotavirus Classification Working Group using the automated genotyping tool for Group A rotaviruses, RotaC v2.2b. Nucleotide sequences of successfully characterized RVA genes were aligned with cognate gene sequences available in GenBank using the ClustalX2.1

The complete genotype descriptor Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx ("x" indicating the genotype number) represents the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5- encoding gene segments, respectively giving either Wa-like (genotype 1), DS-1-like (genotype 2), or AU-1-like genotype constellations (Matthijnssens et al., 2011). Based on the complete RVA genome sequence comparisons, the genotype constellations of the non-G, non-P genes were characterized for each NGS sample.

CHAPTER FOUR

RESULTS

4.1 Screening of samples for RVA

Children under the age of 5 years of age who presented signs and symptoms of AGE at Ashaiman Polyclinic between November, 2014 to May, 2015 and December, 2015 to June, 2016 whose parents or guardians gave their consent to participate in the study were enrolled. Diarrheic stool samples were collected from the hospitalized children who presented signs and symptoms of AGE. In all, 369 stools samples were obtained from the children, all of which were screened for the presence of RVA antigens using Premier™ Rotaclone® EIA kit. Of the 369 samples screened, 145 (39%) were positive for RVA and the remaining 224 (61%) samples were negative for RVA (Fig 4.1).

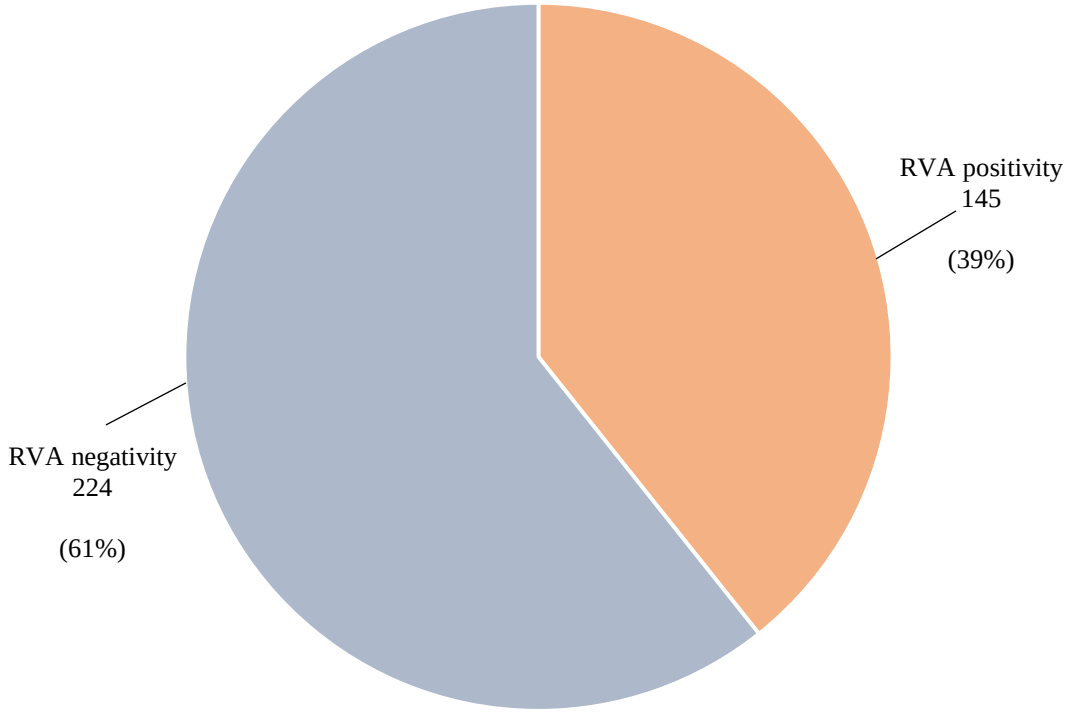


Fig 4.1: Prevalence of RVA infection among 369 diarrheic children from Ashaiman: 2014-2016.

4.2 Demographic and clinical characteristics of study participants.

Demographic and clinical data about vaccination status, fever, the onset and duration of diarrhea were obtained for the 145 children who tested positive for RVA by EIA. Table 4.1 below shows the relationship between demographic characteristics and RVA clinical presentations of the children. The ages of the 145 RVA-EIA positive children ranged from 0 to 24 months. Age distribution of the children has shown that, majority (101/145) have their ages between 7 to 12 months followed by the age group 13 to 18 months, 19 to 24 months and 0 to 6 months with their respective prevalence of (39/145), (3/145) and (2/145). Sex distribution revealed that, majority (109) were males and the rest (36) were females. As many as 143 received the Rotarix vaccine whilst only 2 did not receive the vaccine. Thirty-seven of the children passed between 3 to 5 diarrhea stools per day during their hospitalization, 107 passed between 6 to 8 diarrhea stools per day and the remaining 1 person passed more than 8 diarrhea stools per day. Finally, clinical presentations of fever revealed that, 40 had fever whilst the remaining 105 had normal body temperature readings (no fever).

4.3 VP7 /VP4 gel-based genotyping

The number of stool samples which tested positive for RVAs by EIA were 145 and 224 tested negative. The 224 EIA negative samples were subjected to NSP3 qRT-PCR of which, 17 were positive for RVA. A total of 162 (145 EIA and 17 NSP3) RVA positive samples were processed by the traditional VP7/VP4 gel-based genotyping. Qiagen one-step RT-PCR for VP7 and VP4 genes using universal primers. The amplicons were run on 3% agarose gel with 100 bp molecular weight marker for visual observations (Fig 4.2) and genotypes were subsequently identified by comparing amplicon size on the gel with reference molecular size of genotypes (Table A 1.12

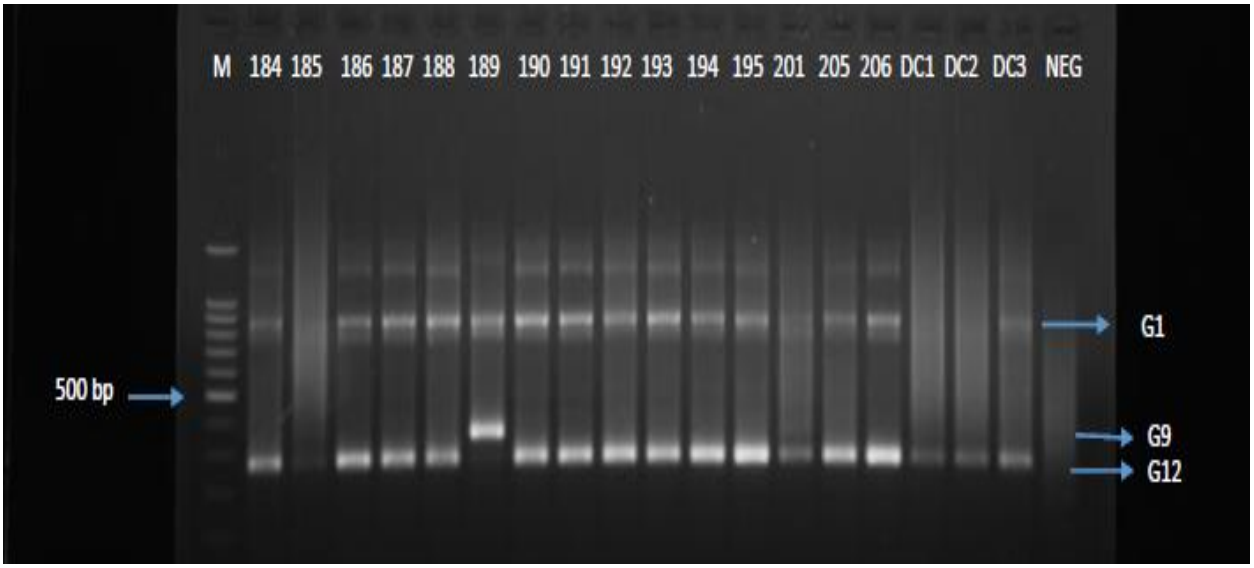
and A 1.13). Out of the 162 RVA positive samples, 136 were fully or partially genotyped by the VP7/VP4 gel-based genotyping assay.

Table 4.1 Demographic and clinical characteristics of EIA-RVA positive children

Age (Months)	No. of patients	Sex		Vaccination status		Frequency of diarrhea per day			Fever	
		Male	Female	Yes	No	3-5	6-8	> 8	Yes	No
0-6	2	1	1	0	2	1	1	0	0	2
7-12	101	76	25	101	0	15	84	2	7	94
13-18	39	29	10	39	0	10	21	8	30	9
19-24	3	3	0	3	0	2	1	0	3	0
Total	**145**	**109**	**36**	**143**	**2**	**37**	**107**	**1**	**40**	**105**

Table 4.1: Demographic and clinical characteristics hospitalized children with RVA infection. >8 implies children who passed more than eight diarrhea stools per day. In vaccination status, yes means vaccinated children and no means unvaccinated children. Under fever; yes, means children with fever and no means children without fever.

A



B

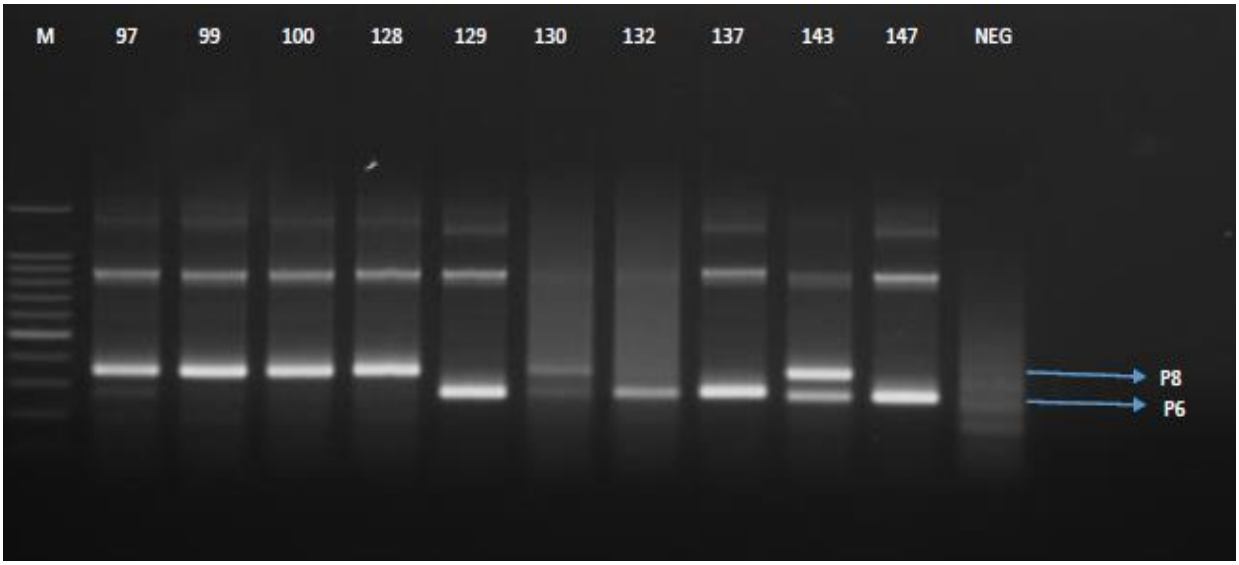


Fig. 4.2: Multiplexed one-step RT-PCR genotyping of RVA. (A): VP7gel-based genotyping using VP7uF and genotype-specific reverse primers for G1, G2, G3, G4, G9, G10 and G12. Approximate amplicon sizes of 825 bp 350 bp and 280 bp represented G1, G9 and G12 genotypes respectively. (B): VP4 gel-based genotyping using VP4uF and genotype-specific reverse primers for P[8], P[4], P[6],P[9] and P[10]. Approximate amplicon sizes of 210 bp and 350 bp represented P[6] and P[8] genotypes respectively whilst 850 bp represented ungenotyped VP4 first round products during the one-step amplification. M, was a 100 bp marker. NEG lane represented negative controls and lanes with numbers were containing samples. DC1, DC2 and DC3 were cases referred from Dabem Clinic (DC), Ashaiman to Ashaiman Polyclinic.

4.4 Distribution of VP7 genotypes

In all, 136 samples were fully or partially characterized by the gel-based genotyping assay and five G genotypes (G1, G3, G9, G10 and G12) were identified. The most prevalent G genotypes G3, G12, G1 and G9 accounted for 53/136 (39%), 21/136 (15%), 19/136 (14%) and 14/136 (10%) of all the 136 cases respectively. Mixed G genotypes were detected with prevalence of 5/136 (4%) and the G non-typeable rotaviruses comprised 21/136 (15%) of all G genotypes (Fig 4.3).

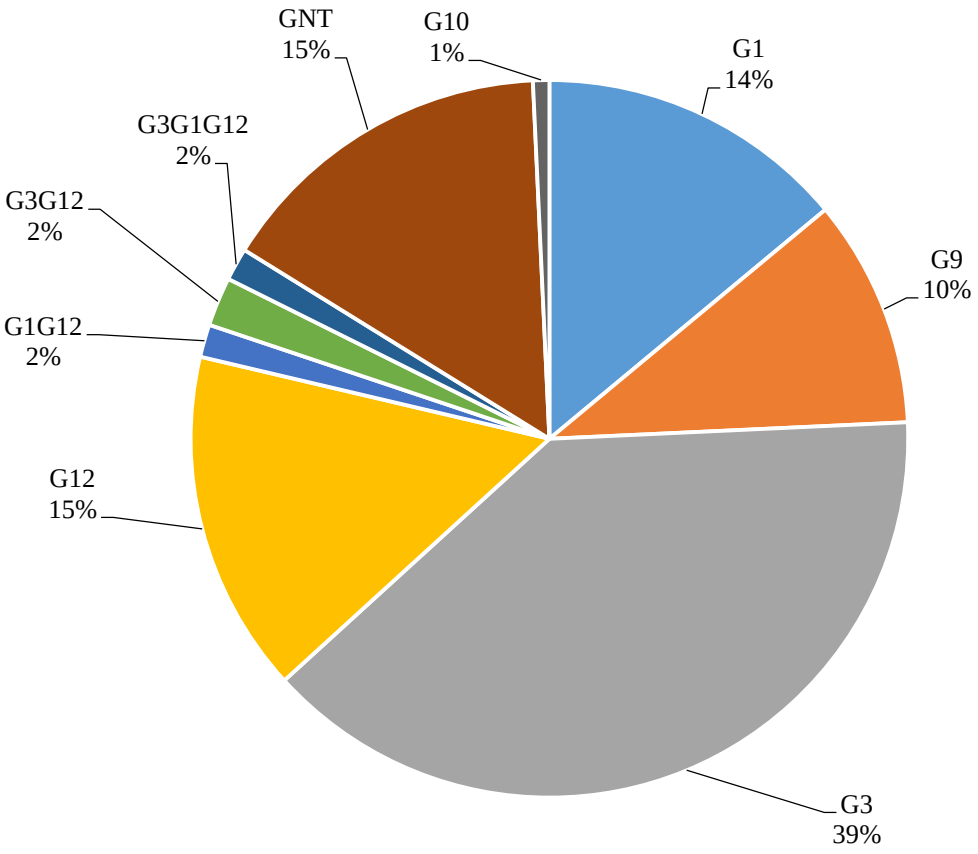


Fig. 4.3: Frequency (%) distribution of characterized 136 RVA G genotypes among 369 diarrheic samples from Ashaiman: 2014-2016

4.5 Distribution of VP4 (P) genotypes

Three P genotypes (P[4], P[6] and P[8]) were identified among the 136 samples and accounted for 13/136 (9.5%), 69/136 (51%) and 38/136 (28%) of P genotypes respectively characterized. Mixed P genotypes were detected with prevalence of 12/136 (9%). The non-typeable rotaviruses comprised 5/136 (3%) for P genotypes (Fig 4.4).

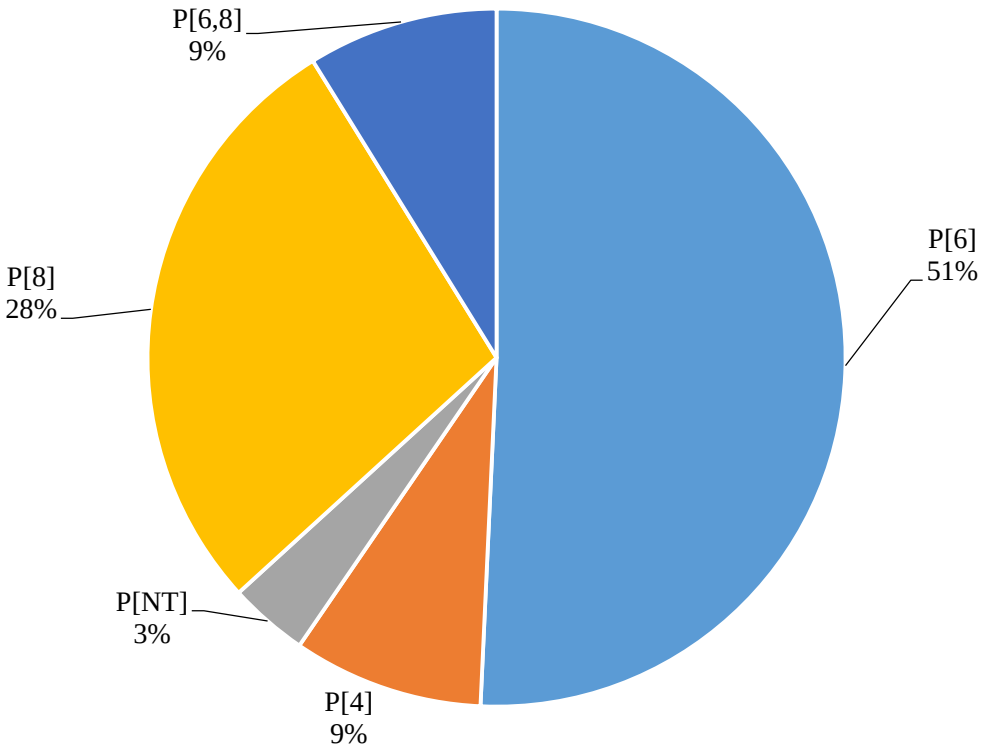


Fig 4.4: Frequency (%) distribution of P genotypes among 136 RVA genotypes detected from Ashaiman: 2014-2016

4.6 Distribution of VP7/VP4 (G/P) genotypes

In order to determine the RVA VP7/VP4 strains from this study, 136 specimens were assigned G/P genotype combinations. The G/P genotype nomenclature identified 8 samples in single infections (G1P[8], G1P[6], G3P[6], G3P[8], G9P[4], G9P[8], G10P[8], and G12P[8]), 7 were

detected in mixed infections (G1P[6,8], G1G12P[8], G3G12P[6,8], G12P[6,8], G3G12P[8], G1G3G12P[6] and G1G3G12P[6,8]) and 3 were partially characterized (GNTP[6], GNTP[6,8] and G3P[NT]). The genotype distribution of all characterized strains; G3P[6], G12P[8], G1P[8] and G9P[4] were the most prevalent strains and were responsible for 68% of all detected RVA cases (Fig 4.5) in children less than five years of age in Ashaiman in 2014 and 2016 of the post-vaccine introduction era. The partially characterized strains accounted for 19% of all cases whilst 12% of cases were due to mixed-infections. The remaining 1% was due to an unusual G10P[8] detected in this study.

4.7 Phylogenetic analysis of the G1 genotype

In an attempt to establish any genetic relatedness of the detected G1 strains from this study to the global G1 strains as well as the G1 of Rotarix and Rotateq vaccines strains, a phylogenetic tree was constructed. The two G1 genotypes used for the construction of the tree were in combinations with P[6] and P[8] respectively. They exhibited close phylogenetic relationship on the tree and were found to belong to lineage 1. They subsequently clustered with Cameroonian strain RVA/Human-wt/CAM/ MA130/2011/G1P6 on the phylogram but were however divergent to the G1 of Rotarix; RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P75 and RotaTeq; RVA/Vaccine/USA/Rotarix-RIX4414/1988/G1P1A8 vaccine strains. The G1 of Rotarix and Rotateq respectively clustered with lineage 2 and 3 G1 strains instead on the phylogram (Fig 4.6).

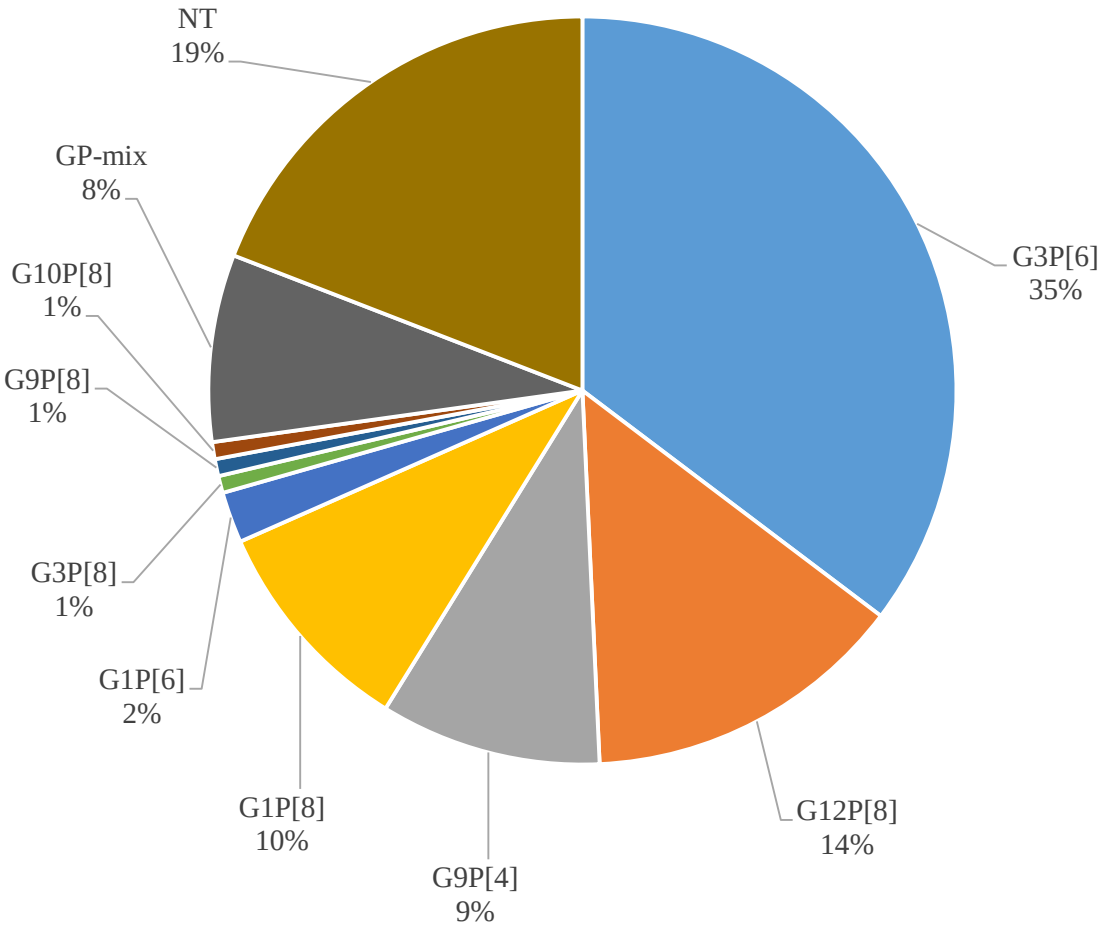


Fig 4.5: Frequency (%) distribution of G/P combinations among 136 RV genotypes detected from Ashaiman: 2014-2016. NT represents GNTP[6], GNTP[6,8] and G3PNT accounting for 19%. GP-mix represents G1P[6,8], G12P[6,8], G1G12P[8], G3G12P[6,8], G3G12P[8], G3G1G12P[6] and G3G1G12P[6,8] accounting for 8% of all cases**.**

4.8 Phylogenetic analysis of the G3 genotypes

The G3 was the most prevalent characterized genotype in this study. Due to their high prevalence, 6 of them were selected for the construction of the maximum likelihood tree. Rotateq

vaccine is a reassortant with G3 genotype hence, the G3 genotype of the vaccine strain; RVA/Vaccine/USA/RotaTeq-WI79-29/1992/G1P75 was included in the construction of the G3 phylogram. The selected G3 genotypes from this study were in combinations with P[4], P[6] and P[8]. Four of them were in combinations with P[6], 1 was in combination with P[4] and the other 1 was in combination with P[8]. The phylogram revealed that, all the 6 G3 gene segments were monophyletic on the tree and clustered with lineage 1 G3 strains. The vaccine G3 thus, clustered with linage 3 strains on the phylogram and exhibited nucleotide sequence divergence with all the 6 G3 genotypes characterized by this study (Fig 4.7).

4.9 Phylogenetic analysis of the G9 genotypes

Three G9 genes which were in combination with P[4] were selected for the construction of the G9 tree. They all clustered with the Cameroonian strain; RVA/Human-wt/CMR/6805/1999/G9P, the Nigerian strain; RVA/Human-wt/NGA/Bulumkutu/XXXX/G9PX, the Brazilian strain; RVA/Human-wt/BRA/ac11824/06/2006/G9P8, the Chinese strain; RVA/Human-wt/CHN/XJ99-468/1999/G9P6 and the USA strain; RVA/Human-tc/USA/US1205/1997/G9P6 on lineage 1. They were however more related to the African strains than the Asian and European strains on the phylogram (Fig 4.8).

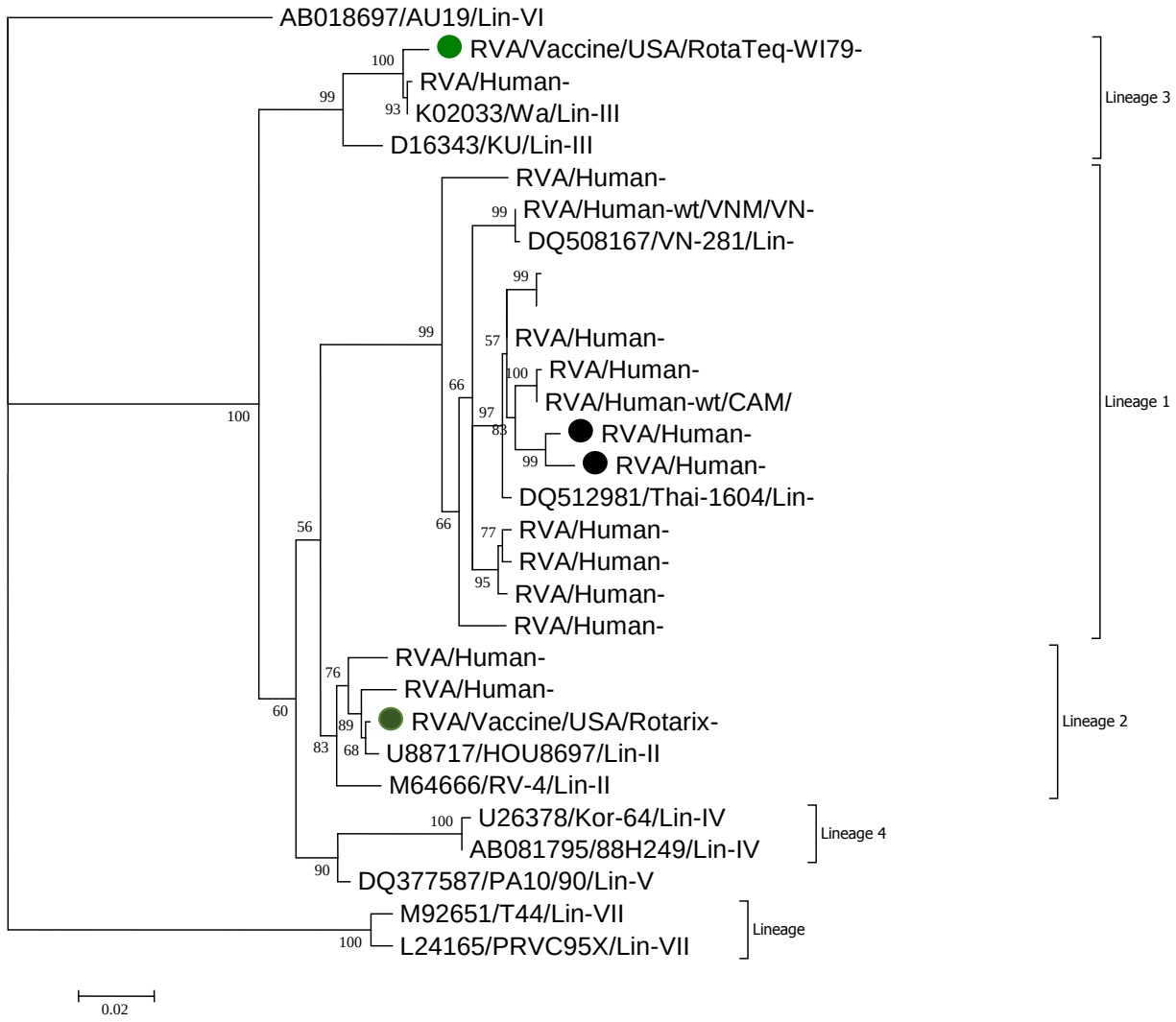


Fig 4.6: Phylogenetic analysis of RVA G1 genotypes. Maximum likelihood tree for the detected G1 strains from this study were labeled with black dots and that of the G1 of Rotateq and Rotarix strains were labeled with green dots.

Fig 4.7: Phylogenetic analysis of RVA G3 genotypes. Maximum likelihood tree for the detected G3 strains from this study were labeled with black dots and that of the Rotateq G3 strain was labelled with a green dot.

4.10 Phylogenetic analysis of the G12 genotypes

The G12 represented the second most common VP7 genotype in this study. In order to determine their phylogeny, 6 of them were selected for the analysis. The 6 G12 genotypes chosen for the construction of the G12 phylogram were in combinations with the P[8] genotype. The 6 G12 gene segments were seen to exhibit close genetic relatedness to each other. They subsequently clustered with lineage 1 G12 strains on the phylogenetic tree (Fig 4.9).

4.11 Phylogenetic analysis of the P[4]genotypes

Three P[4] strains from this study were selected for the constructions of the P[4] phylogenetic tree. The 3 selected P[4] strains were all in combination with the G9 genotype. Phylogenetic analysis on the 3 P[4] gene segments revealed a close genetic relatedness as they belong to the same clade on the phylogram. Also, they were found to clustered with DS-1 like P[4] strains and lineage 1 P[4] VP4 strains on the phylogram (Fig 4.10).

4.12 Phylogenetic analysis of the P[6] genotypes

Five P[6] genotypes which were in combination with G1 and G3, VP7 genotypes were selected for the P[6] tree construction. Four of the P[6] strains were in possessing VP7, G3 gene segments and 1 was possessing VP7, G1 gene segment. All the 5 P[6] genes however, belonged to the same clade and clustered on the tree with the P[4] DS-1 lineage 2 strains (Fig 4.11).

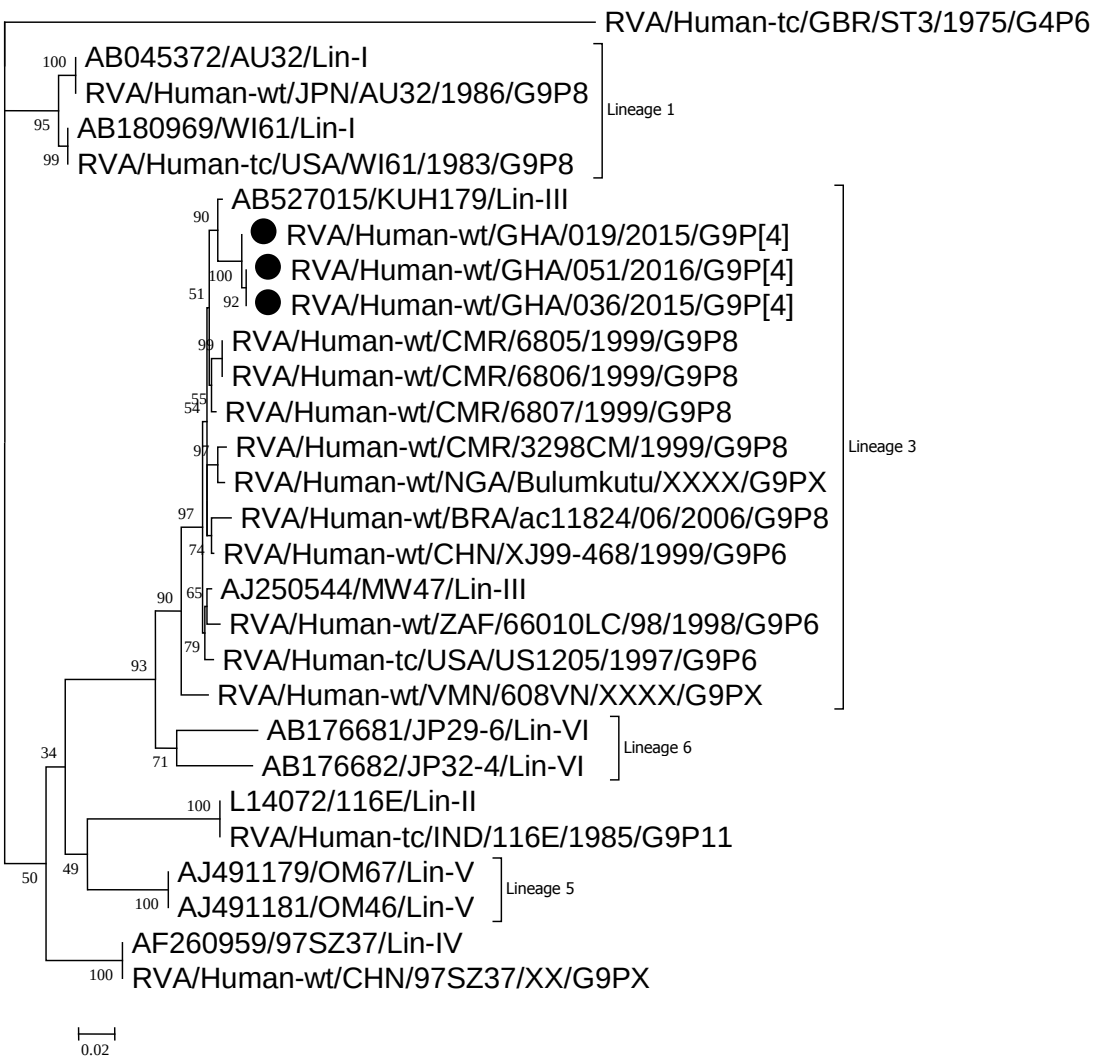


Fig 4.8: Phylogenetic analysis of RVA G9 genotypes. Maximum likelihood tree for the detected G9 strains from this study were labelled with black dots.

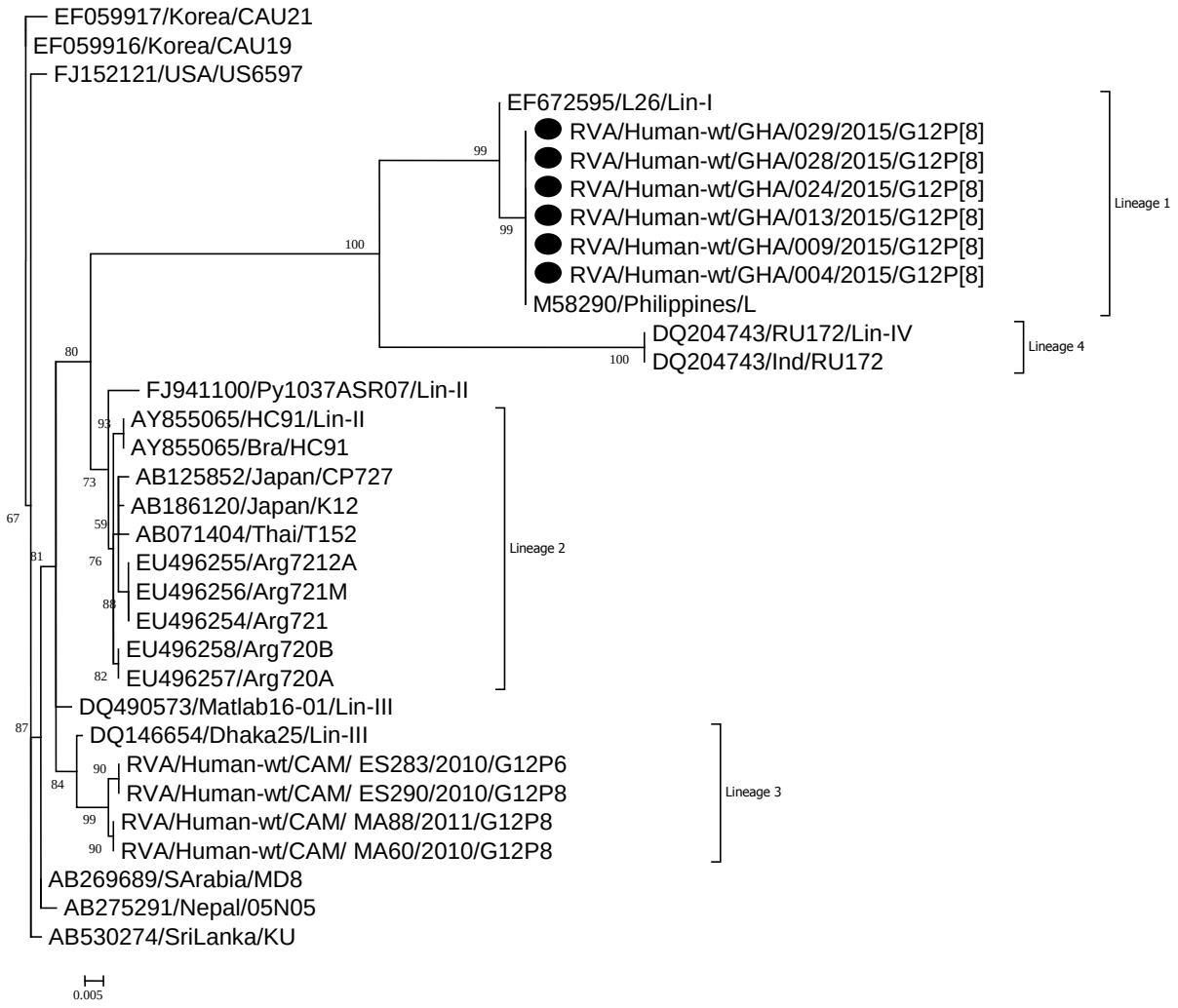


Fig 4.9: Phylogenetic analysis of RVA G12 genotypes. Maximum likelihood tree for the detected G12 strains from this study were labeled with black dots.

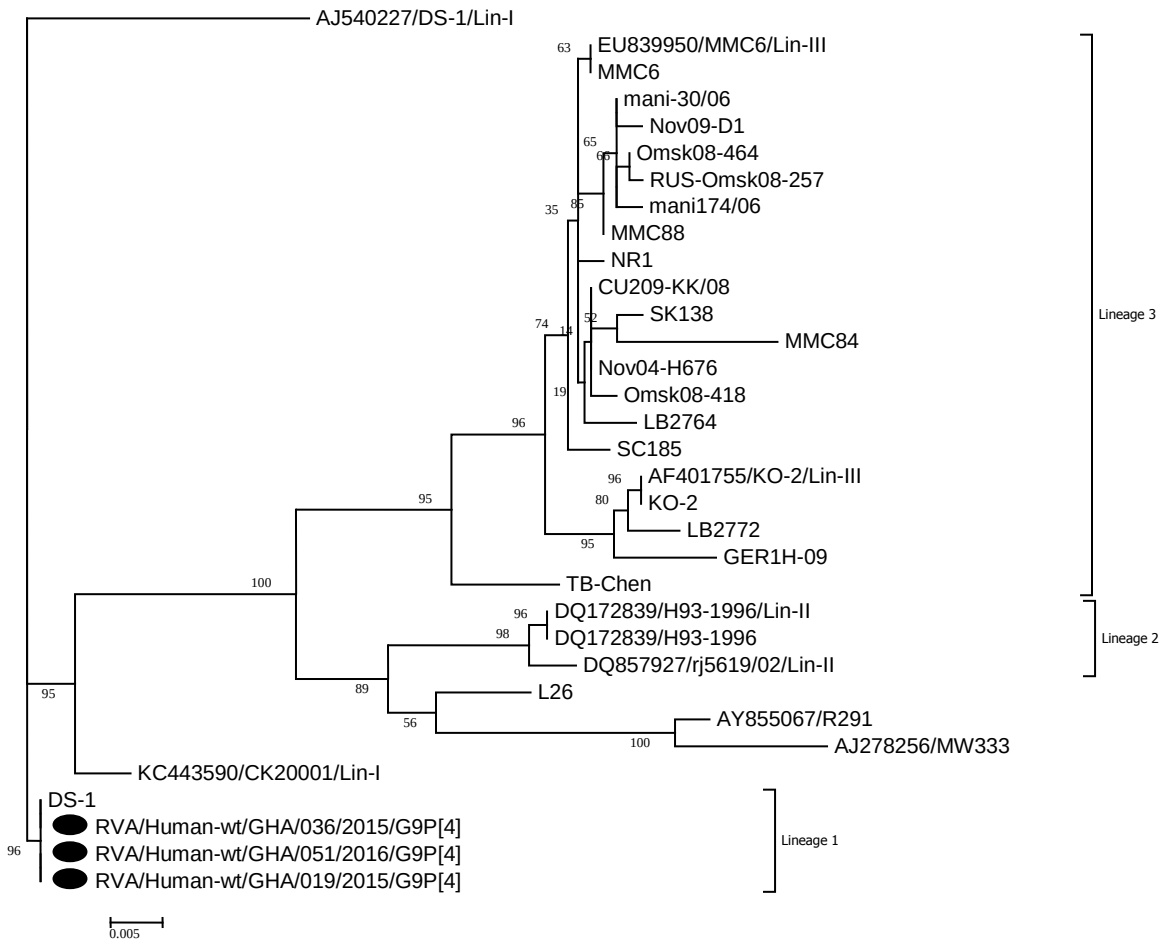


Fig 4.10: Phylogenetic analysis of RVA P[4] genotypes Maximum likelihood tree for P[4] strains detected from the study were labeled with black dots.

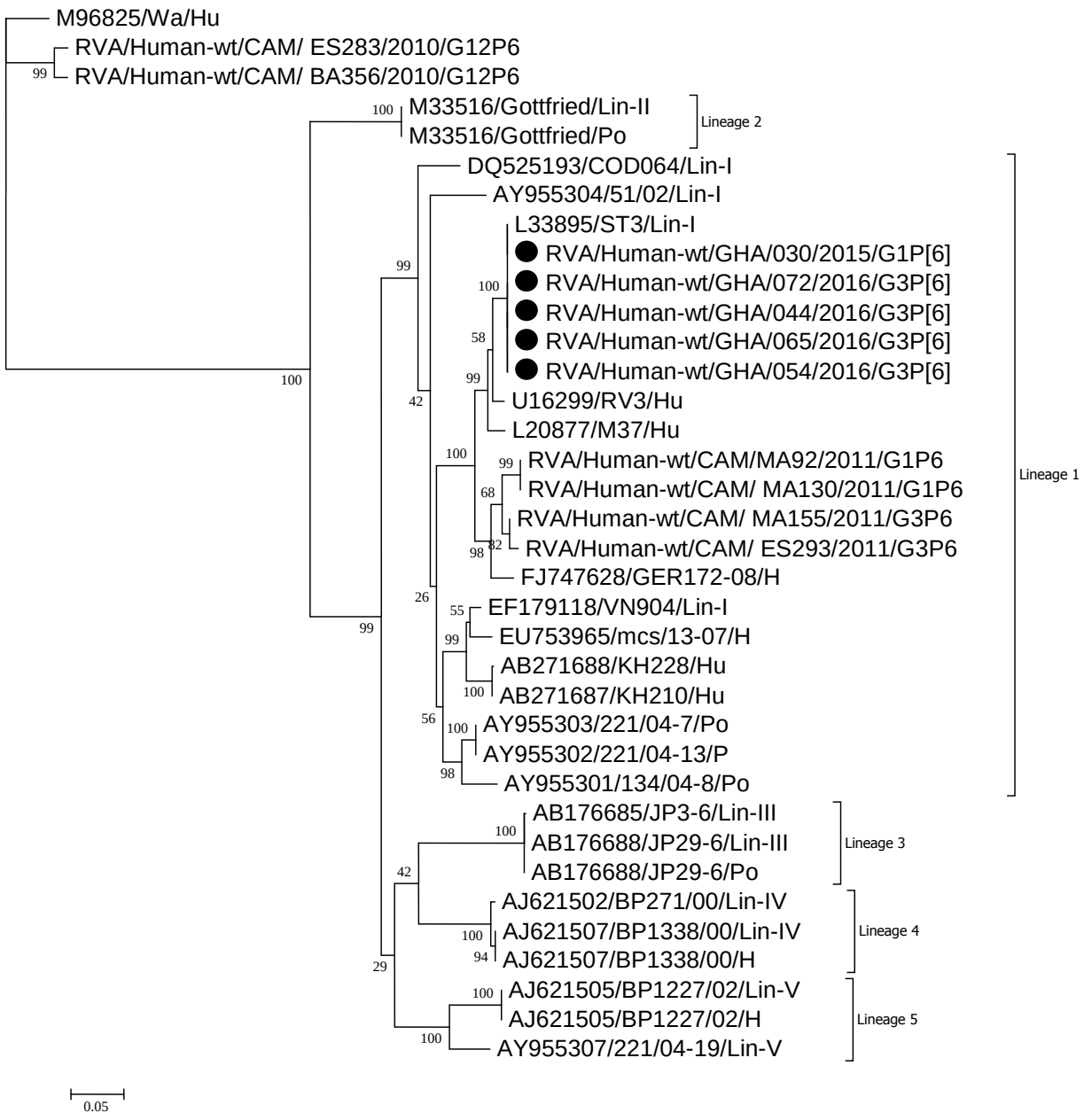


Fig 4.11: Phylogenetic analysis of RVA P[6] genotypes. Maximum likelihood tree for P[6] strains detected from this study were labeled with black dots.

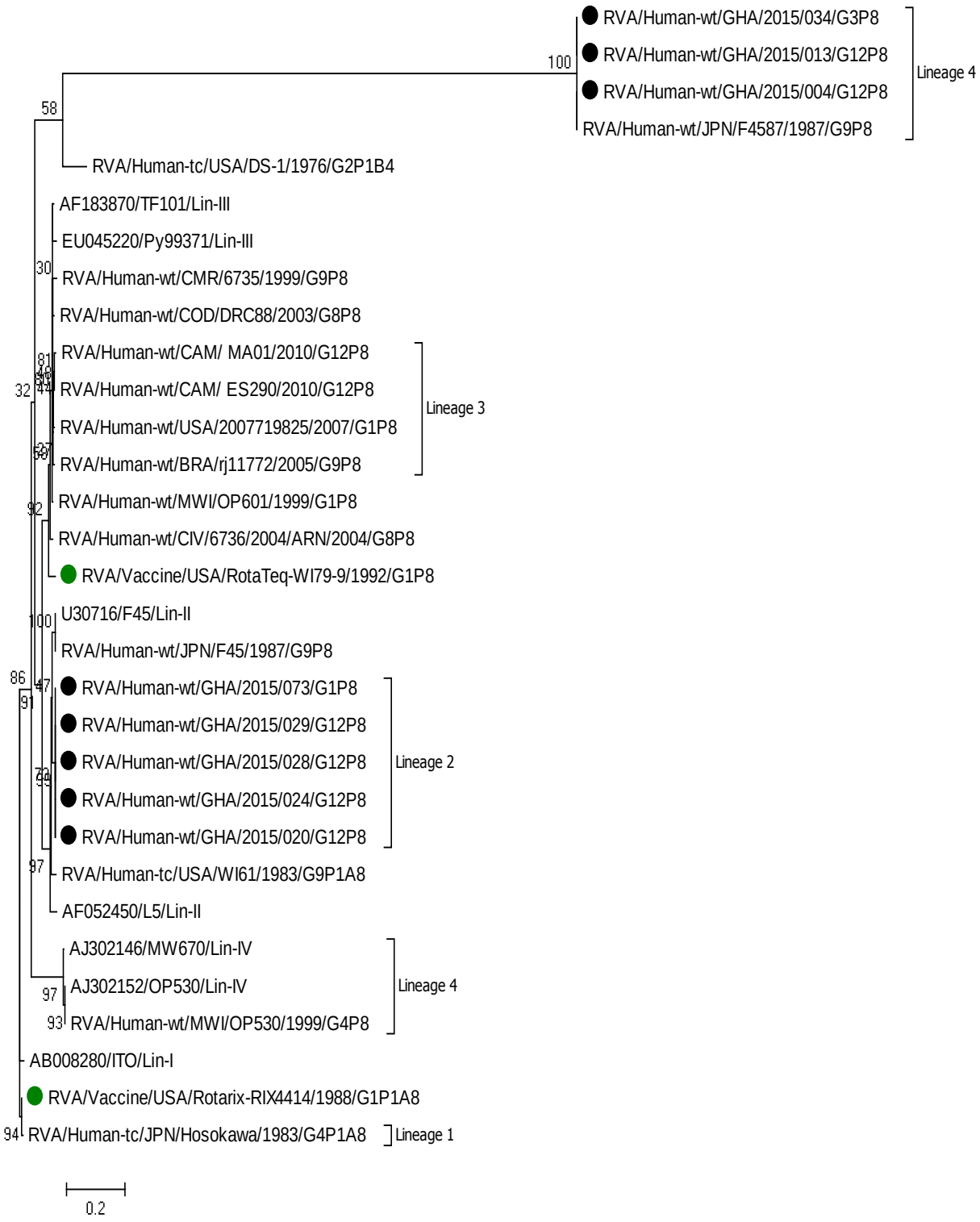


Fig 4.12: Phylogenetic analysis of RVA P[8] genotypes Maximum likelihood tree for P[8] strains detected from this study were labelled with black dots. That of the vaccine strains were labelled with green dots.

4.13 Phylogenetic analysis of the P[8] genotypes

Among the characterized VP4 genotypes by this study, P[8] was the most prevalent as such, 8 of them were selected for the construction of the tree. Also, because Rotarix and Rotateq vaccines contains the P[8] genotype, the P[8] of the two vaccine strains labeled with green dots were also included in the tree construction. Five of the P[8] genotypes were in combinations with G12, two were in combinations with G1 and the remaining one was in combination with G3. Three of the P[8] genotypes exhibited similar nucleotide identity and clustered lineage 4 Japanese strain; RVA/Human-wt/JPN/F4587/1987/G9P8. The remaining 5 P[8] genotypes also clustered with the Japanese strain; RVA/Human-wt/JPN/F45/1987/G9P8 of lineage 2 on the phylogram. Whilst the P[8] of the Rotarix strain clustered with lineage 1, that of Rotateq clustered with lineage 3. The P[8] of the two vaccine strains were however divergent from the 8 P[8] strains detected from this study (Fig 4.12).

4.14 Full genome constellations of 17 selected strains

In this study, 136 samples were fully or partially characterized by the traditional binomial (G/P) classification of which, 17 (one G1P[6], one G1P[8], four G3P[6], one G3P[8], three G9P[4], one G10P[8], five G12P[8] and one G12P[6]) were selected for full genome characterization. The selection was based on representation of individual genotypes and availability of sufficient stool material for Illumina MiSeq library preparation. After the full genome characterization, the G1P[6] and G1P[8] exhibited the Wa-like genotype constellation without any reassortment. The four G3P[6] however, exhibited DS-1 like genotype constellations with one having reassortments gene segment at the NSP4 gene segment giving an E6 for the NSP4 instead of the

usual E2. Also, the one G3P[8], exhibited a Wa-like backbone with reassortment at NSP1 and NSP5 genes giving an A2 and H2 respectively instead of the usual A1 and H1. Among the three G9P[4], they all exhibited DS-1 like backbone however, with reassortments. One of them exhibited reassortment at the NSP4 gene giving E6 instead of E2 and the other two also exhibited reassortment at the NSP2 and NSP4 genes giving N1 and E6 respectively instead of N2 and E2. The one G10P[8], exhibited Wa-like/ DS-1 like genotype constellation. The five G12P[8] exhibited Wa-like backbone with no reassortment and also, the one G12P[6] exhibited Wa-like backbone with no reassortment (Table 4.2)

Table 4.2: Full genome constellations of 17 selected strains

Genogroup	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
I (Wa-like)	G1	P8	I1	R1	C1	M1	A1	N1	T2	E2	H1
I (Wa-like)	G12	P[4]	I1	R1	C1	M1	A1	N1	T1	E1	H1
I (Wa-like)	G12	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
I (Wa-like)	G12	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
Wa-Ds-1	G10	P8	I1	R5	C2	M2	A3	N2	T1	E1	H2
I (Wa-like)	G12	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
I (Wa-like)	G12	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
I (Wa-like)	G12	P8	I1	R1	C2	M1	A1	N1	T1	E1	H1
I (Wa-like)	G1	P6	I1	R1	C1	M1	A1	N1	T1	E1	H1
I (Wa-like)	G3	P8	I1	R1	C1	M1	A2	N1	T1	E1	H2
II (DS-1)	G9	P4	I2	R2	C2	M2	A2	N1	T2	E6	H2
II (DS-1)	G9	P4	I2	R2	C2	M2	A2	N2	T2	E6	H2
II (DS-1)	G9	P4	I2	R2	C2	M2	A2	NA	T2	E6	H2
II (DS-1)	G3	P6	I2	R2	C2	M2	A2	N2	T2	E6	H2
II (DS-1)	G3	P6	I2	R2	C2	M2	A2	N2	T2	E2	H2
II (DS-1)	G3	P6	I2	R2	C2	M2	A2	N2	T2	E2	H2
II (DS-1)	G3	P6	I2	R2	C2	M2	A2	N2	T2	E2	H2

Table 4.2: Full genome constellations of 17 selected strains: Wa-like genogroup genotypes were represented in green. DS-1 genogroup genotypes were represented in blue. Reassorted gene segments were indicated in red.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Introduction

Group A Rotaviruses are the single most important cause of acute gastroenteritis in children less than 5 years of age. In the midst of rotavirus disease burden, highly successful clinical trials which precluded any side effects has led to WHO recommending the global use of two vaccines; Rotateq® (Merck), a pentavalent vaccine and Rotarix™ (GSK), a monovalent vaccine to reduce the disease burden. The advent of the two vaccines was a welcome news world-wide leading to their global introductions into the primary health care system to manage infantile diarrhea. In Ghana, the monovalent vaccine, Rotarix™ was introduced into the Expanded Programme on Immunization in May, 2012 to reduces rotavirus associated morbidities and mortalities in children less than 5 years. Post-vaccine era surveillance studies however, have shown stark disparity in vaccine effectiveness between developed and developing countries and mortalities due to RVA gastroenteritis is higher in developing countries than the developed ones, instigating a huge public health threat in global regions like Africa and South East Asia. The aim of this study was to investigate the strains of RVAs causing AGE in children less than 5 years of age in Ashaiman after the introduction of the Rotarix™ vaccine into the Expanded Programme on Immunization.

5.2 Discussion

In this study, RVAs, were screened using EIA which gave a prevalence of 39%. The reported RVA prevalence in this study was relatively high as compared to other studies in the post-vaccine era (Ouermi et al., 2017; Rahajamanana et al., 2017; Yu et al., 2017; Zaki et al., 2017;

Zaraket et al., 2017). However, the sample size of this study was much less and serves a limitation for such comparisons. The 2010 population and housing census at the study area revealed that, grouping ages with intervals of 4 years, the age group (0-4) years represented the majority of the population. The dominance of children less than 5 years of age may lead to overcrowding at schools and homes and thus, increase their risk to RVA infections.

Demographic and clinical data on the 39% of the children with RVA infections were retrieved. The ages of the children ranged from 0 to 24 months. Majority, 70% (101/145) of the children had their ages ranging from 7 to 12 months. This data corresponds to findings from previous studies (Andersson & Lindh, 2017; Bowen et al., 2016; Omore et al., 2016; Zaraket et al., 2017), which also reported the age group, 7 to 12 months as the majority with RVA infections. The study further revealed that, the 0 to 6 months age group recorded only 2 cases, 19 to 24 months group also recorded 3 cases and as many as 101 children with RVA infections were within the age group 7 to 12 months. Cohort studies have shown that, maternal antibodies provide some level of protection against rotavirus infections (Das et al., 2016; Laucirica et al., 2017; Murphy, 2017) and could account for only 2 cases being recorded for 0 to 6 months age group. As exclusive breastfeeding in infants stops after the age of 6 months, maternal protection reduces and thus, render them more susceptible to the infection. Also, subsequent exposures to the viruses lead to a milder form of illness as a result of immunity to the disease after reinfections. As the children become older than 2 years, their susceptibility to RVA-induced gastroenteritis reduces significantly as compared to their counterparts less than 2 years (Aupiais et al., 2009; Cunliffe et al., 2014; Prameela & Vijaya, 2012). This could explain why as many as 70% of the

cases was recorded for the 7 to 12 months age group, 3 cases were recorded for the 19 to 24 months age and no child with the infection was older than 24 months.

The projected populations of children less than 5 years of age at the study area for the year 2014, 2015 and 2016, revealed that, female children were more for the mentioned years than their male counterparts (GSS, 2014) however, majority (109/104) of the children from this study with RVA infections were males. The higher infection rate recorded among male infants may be due to the general activeness of male children than their female counterparts. After 6 months of age, infants became very active and more explorative of their environment than females. The more active a child is, the more it interacts with its environment and hence, the higher risk of RVA infection (Esona & Gautam, 2015).

Vaccination status of the children with RVA infections had shown that, as many as 143 out of the 145 were vaccinated with Rotarix vaccine yet, were infected with rotaviruses whilst only 2 did not receive the vaccine. This findings highlights on the inability of the rotavirus vaccines to provide 100% protection among the vaccinated individuals. Studies have shown that, the most effective measure of controlling rotavirus infections is by the use of vaccines to reduce the severity of the infection when exposed to the virus for the first time (Burnett et al., 2016; Doll et al., 2015; Ebrahim, 2008; Gray, 2011; O'Ryan, 2017; Vesikari et al., 2010; Ward et al., 2008). Even though both vaccines have helped in the reduction of diarrhea related morbidities and mortalities in children under the age of 5 years globally, both vaccines have been more effective in the developed countries than the developing ones causing a huge public health burden in developing countries (Burnett et al., 2017; Gruber et al., 2017; Kotirum et al., 2017; Mujuru et

al., 2017; Shah et al., 2017) hence, the need for continues RVA strain surveillance especially, in the developing countries for further vaccine developments against the emerging strains.

Clinical presentations of the children revealed that, more than one-half of the population with RVA infection had normal body temperature readings with diarrhea episodes ranging from 3 to 8 times per day, a typical of rotavirus infections (Alkali et al., 2015; Flerlage et al., 2017; Staat et al., 2002; Zvizdic et al., 2004).

RVA pre-vaccine introduction era surveillance reports including those from Ghana have flagged G1 as the globally common VP7 genotype (Diez-Domingo et al., 2011; Enweronu-Laryea et al., 2013; Esteves et al., 2016; Kirkwood et al., 2008; Payne et al., 2008). In the post-vaccine introduction era however, several studies have reported the emergence of other predominant VP7 genotypes which have substituted the global dominance of the G1 genotype (Bowen et al., 2016; Estes & Greenberg, 2013; Luchs et al., 2015; Mullick et al., 2014). In this study, the detected RVAs from children less than 5 years of age with acute gastroenteritis were characterized by molecular methods. The most prevalent VP7 genotype was G3 (39%), followed by G12 (15%) and the third common genotype was G1 (14%). The prevalence of the G3 genotype has dominated the VP7 genotypes in other reports from Ghana (Asmah et al., 2001; Binka et al., 2003) and may account for their dominance in this study. Three mixed infections, G3,G12, G1,G12 and G1,G3,G12 were detected from this study. The mixed G-types were due to rotaviruses with different G-types co-infecting the same host (Desselberger, 2014; Estes & Greenberg, 2013). Mixed RV G-types infections may reassort to give rise to new strains (Desselberger et al., 2001; Estes & Greenberg, 2013) which may increase G-types in the study

area in the future. As many as 15% of the samples could not be genotyped (GNT). The GNT detected in this study were samples that could not be amplified during the second round PCR reaction. Unfortunately, those samples were not having adequate stool material for further characterization by next generation sequencing techniques. The study reported the circulation of 3 VP4 genotypes: P[4], P[6] and P[8]. The P[6] genotype was the dominant (51%) VP4 genotype. Studies from Ghana in the past have reported similar trends with the dominance of P[6] genotype (Armah et al., 2001; Binka et al., 2011; Damanka, Adiku, et al., 2016).

Strains with the binomial VP7/VP4 genotypes gave 8 G/P combinations and 7 mixed- infections. The genotypes: G3P[6], G12P[8], G1P[8] and G9P[4] were the most prevalent and accounted for 68% of all characterized strains. Earlier reports from Ghana in the pre-vaccine era have identified G2P[6], G3P[4] and G9P[8] as the most prevalent strains in northern Ghana (Binka et al., 2003) and G1P[8], G3P[6] and G2P[6] as the most prevalent strains in southern Ghana (Enweronu-Laryea et al., 2013).The global explosion of the G12 strains in the post-vaccine introduction era (Ategbo et al., 2015; Bowen et al., 2016; Bucardo et al., 2015; da Silva et al., 2017; Ndze et al., 2013; Pacilli et al., 2015; Wylie et al., 2016) probably explains the emergence of the G12P[8] in this study. The unusual G9P[4] was detected only once in previous studies from Ghana (Armah et al., 2003). G9 and G10 have been shown to be more commonly associated with RV infections in animals (G. E. Armah et al., 2010; Chandler-Bostock et al., 2014; Esona et al., 2011; Quaye et al., 2013; Wu et al., 2017). The unusual G9P[4] and the G10P[8] detected may be due to the straying of animals which led to their introduction into the human population. Open defecation was a common practice at Ashaiman as 63.5% of houses lack toilet facilities and 28.2 % of households into agriculture in the municipality were involved

in livestock rearing, thus promoting the straying of animals in the municipality (GSS, 2014). Lack of toilet facility at home is likely to promote open defecation and straying of animals in communities could possibly encourage the surge of animal fecal matter within the communities thus, the risk of RV co-infections. The findings hypothesized that, insanitary setting could create a conducive environment for RV co-infections and the upsurge of RV diversity.

Even though the study reported much diversity of rotavirus strains at the study area, phylogenetic analysis of the VP7 and VP4 gene segments exhibited close genetic relatedness among strains of the same genotype. For instance, rotavirus strains of genotype G1 were closely related to each other, and those of genotype G12 were also closely related and so on. The genetic relatedness of strains at the study area suggests the transmission of identical strains and supports the theory of strain survival under vaccine induced selective pressure (Afrad et al., 2014; Almeida et al., 2017; Bucardo & Nordgren, 2015; Kaplon et al., 2013; Zeller et al., 2015) however, one period surveillance study was not enough for such conclusions. As strains of same genotypes were closely elated to each other, they were also found to be more related to strains from African in exception to the strains with P[8] genotype which seem to be more related to the Japanese strains on the P[8] tree. Up to date, rotaviruses with the P[8] genotype remain the major cause of acute infantile diarrhea (da Silva et al., 2017; Dulgheroff et al., 2016; Groome et al., 2017; Zaraket et al., 2017). The worldwide dominance of the P[8] could easily promote cross-continent genotype transmission. Ashaiman is a densely populated municipality with several business activities and markets which serve the surrounding communities like Tema. Tema is a major industrial city in Ghana with several foreigners. The detected strains with P[8] genotype being more related to the Japanese strains may be as a result an imported case.

Previous work have reported G9P[4] strains with E6 constellation for the NSP4 gene segments. All of which were from Bangladesh, India, Honduras, Guatemala and Mexico (Lewis et al., 2014; Matthijnssens et al., 2008; Quaye et al., 2013; Rahman et al., 2007; Sharma et al., 2009). Three of the G9P[4] strains from this study exhibited reassortments of E6 and the remaining one exhibited reassortment with N1 gene segment (Table 4.2), the first ever to be detected in Africa and for that matter, Ghana. It has been postulated that, the G9P[4] genotype combination was due to reassortment between frequently occurring genotypes (e.g., G2P[4] and G9P[6] genotype (Rahman et al., 2008). The parental strains, G2P[4] and G9P[6] have been previously detected in southern Ghana but however, were in low circulation (Enweronu-Laryea et al., 2013). A study has detected G9 strains in pigs and proposed zoonotic transmission of the G9 strains (Chandler-Bostock et al., 2014). Also, three G3P[6] strains of Wa-like backbone E6 reassortments, one G3P[8] with Wa-like backbone and A2/H2 reassortments have been detected. Nevertheless, one G10P[8], exhibited Wa-like/ DS-1 like genotype constellation. A study carried out to explicate the genetic and evolutionary relatedness of G10 rotavirus strains from West Africa, revealed a 97% to100% nucleotide similarity of all the G10 gene segments included in that study (Esona et al., 2011). P[8] is a globally common genotype and could possibly combine with any VP7 genotype (da Silva et al., 2017; Damanka, Dennis, et al., 2016; Groome et al., 2017). RVA surveillance study concluded that, rotavirus G10 genotype was one of the main rotaviruses circulating in cattle throughout the world. The study further hypothesized that, the G10 genotype was acquired through zoonotic transmission and associated the G10 genotype to rotavirus zoonosis (Steyer et al., 2010). In this study, full genome constellation for one G10P[8] genotype detected was characterized; RVA/Human-wt/GHA/020/2015/G10P[8]-I1-R5-C2-M2-

A3-N2-T1-E1-H2 which was found to be novel. Further analysis is ongoing to establish genetic relatedness of the individual gene segments to the gene segments of already circulating RVA G10P[8] strains.

5.2 Conclusion

Findings from this study has reported RVA prevalence of 39% and then suggested the dominance of G3P[6] and the emergence of G12P[8] and G9P[4] at the study area. The study has described much diversity in RVA genotype combinations which is in assertion with previous studies from Ghana (Armah et al., 2001; Enweronu-Laryea et al., 2013) and other African countries (Agutu et al., 2017; Clark et al., 2017; Nyaga et al., 2015; Operario et al., 2017; Ouermi et al., 2017; Park et al., 2017; Rahajamanana et al., 2017; Scott-Jupp, 2017; Sow et al., 2017) and in disparity to data from developed countries (da Silva Soares et al., 2014; Roczo-Farkas et al., 2016; Yandle et al., 2017).

Even though the study reported much diversity in RVA genotypes, phylogenetic analysis of the VP7 and VP4 gene segments suggested close genetic relatedness among strains and to those from Africa.

No vaccine RVA reassortants was identified but human-human and human-animal reassortants were detected which suggests zoonotic transmission at the study area and for the first time, a unique strain G10P[8]-I1-R5-C2-M2-A3-N2-T1-E1- H1 has been characterized from this study. The findings thus hypothesized that, insanitary setting could create a conducive environment for RV co-infections and the upsurge of RV diversity.

This study was carried out within two rotavirus seasons and thus, the short duration of this study, becomes a limitation to understanding the survival and transmission of genetically related rotavirus strains in the post-vaccine era at the study area. Also, because most of the strain included in the phylogenetic analysis were laboratory, vaccine strains and African strains, the study could not compare how related the strains were to globally circulating rotavirus strains.

5.3 Recommendations

Continuation of rotavirus surveillance for a longer period at the study area is needed to certainly describe the circulating strains at the study area, and may also help to understand the effectiveness of the vaccine after its introduction in Ashaiman Municipality.

Full genome characterization is highly recommended for all rotavirus surveillance work to clearly define reassortment and recombination events, especially between the wild type and vaccine strains and between the wild type and animal strains.

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Appendix 1

Table A 1.1 PCR master mix on NSP3 qRT-PCR on EIA negative samples using (ABI 7500 Fast DX Instrument)

Reagent	**Volume (µl)**	**217 Reactions volume (µl)**
Nuclease free water	6.75	1464.75
5X EZ buffer	5	1085
dNTPs	6	1302
25mM $MnOAc_2$	2.5	542.5
rTth polymerase	1	217
NSP3 forward primer (40 µM)	0.3125	67.8
NSP3 reverse primer (40 µM)	0.3125	64.8
NSP3 Probe (10 µM)	0.375	81.4
Xeno or MS2 Forward primer (40 µM)	0.25	54.25
Xeno or MS2 Reverse primer (40 µM)	0.25	54.25
Xeno or MS2 Probe (10 µM)	0.25	54.25
Total volume	23	4991

Table A 1.2 Master-mix for VP7 Genotyping by multiplexed one-step RT-PCR using (ABI 7500 Fast DX Instrument)

Reagent	**Amount per 150 Reaction (µl)**
H_2O	80
5X Buffer	25
dNTP mix	5
One-Step RT-PCR enzyme	5

Table A 1.3 Master-mix for VP4 Genotyping by multiplexed one-step RT-PCR using (ABI 7500 Fast DX Instrument)

Reagent	**Amount per 150 Reaction (µl)**
H_2O	80
5X Buffer	25
dNTP mix	5
One-Step RT-PCR enzyme	5

Table A 1.4 Master-mix for VP7/ VP4 Qiagen-one step RT PCR

Reagent	**1 Reaction (µl)**	**84 ReactionsVP7 (µl)**	**84 ReactionsVP4 (µl)**
Water	16	1334	1334
5x buffer	5	420	420
dNTP mix	1	84	84
One-step RT-PCR enzyme	1	84	84

Table A 1.5 Master-mix for cycle sequencing using (ABI 7500 Fast DX Instrument)

	Per reaction	**Total for master mix**
		150 reactions
Water	2 µl	300 µl
Big Dye (V.3)	2 µl	300 µl
5X seq. Buffer	2ul	300ul

Table A 1.6 Master- mix for RNA fragmentation and priming

Reagents	Volume/reaction	N=24 reactions + 2 (26)
Purified dsRNA (10-100) ng	5 µl	
NEBNext First Strand Synthesis Reaction buffer (5X)	4 µl	104 µl
Random primers	1 µl	26 µl
Total volume	10 µl	135 µl

Table A 1. 7 First strand cDNA synthesis

Reagents	Volume/reaction	N=24 reactions + 2 (26)
Murine RNase inhibitor	0.5 µl	13 µl
Protoscript II Reverse Transcriptase	1 µl	26 µl
Nuclease free water	8.5 µl	221 µl
Total volume	10 µl	Total volume = 260 µl

Table A 1.8 Second strand cDNA synthesis

Reagents	Volume/reaction	N=24 reactions + 2 (26)
Second strand synthesis buffer	8 µl	208 µl
Second strand synthesis enzyme mix	4 µl	104 µl
Nuclease free water	48 µl	1248 µl
Total volume	60 µl	Total volume = 1560 µl

Table A 1.9 Master- mix for End Repair/ dA-tail of cDNA Library

Reagents	Volume/reaction	N=24 reactions + 2 (26)
NEBNext End Reaction buffer (10X)	6.5 µl	169 µl
NEB End Prep Enzyme Mix	3 µl	78 µl
Total volume	9.5 µl	Total volume = 247 µl

Table A 1.10 Master- mix for Adapter ligation

Reagents	Volume/reaction	N=24 reactions + 2 (26)
Blunt/TA Ligase Master Mix	15 µl	390 µl
Nuclease free water	2.5 µl	65 µl
Total volume	17.5 µl	Total volume = 455 µl

Table A 1.11 Primer mix for PCR Library Enrichment

Reagents	Volume/reaction	N=24 reactions + 2 (26)
NEBNext Q5 Hot Start High PCR Master mix	25 µl	650 µl
Universal PCR Primer	2.5 µl	65 µl
Total volume	27.5 µl	Total volume = 715 µl

Table A 1.12 Primer sequence for VP7 genes

Primer	Sequence (5'-3')	Polarity	Gene	Genotype	Nucleotide Position	Approximate Amplicon size, bp
VP7-F	GGC TTT AAA ARM GAG AAT TTC CG	+	VP7	Generic	1-23	
G1-R4	ACA TTA GAR CCA CCA ACT TGT AT	-	VP7	G1	850-828	849
G2-R1	CAT TAT AAT CAC AAT ACA GTT G	-	VP7	G2	147-127	146
G3-R1	CGT CAG TAA TYA CTA RTT TYT CAG CTG	-	VP7	G3	733-707	732
G4-R1	GAG CAT TCG MTA ATA MTG ATA ATA C	-	VP7	G4	175-199	198
G9-R3	CAG AGT ATY YTT CCA TTC HGT ATC TCC	-	VP7	G9	328-354	353
G12-R2	GAA GTC ATA AAA YTY TCT TGT TG	-	VP7	G12	262-284	283

Table A 1.12 Primer sequence for VP7 genes: F, forward; R, reverse; VP, structural protein; bp, base pair

Table A 1.13 Primer sequence for VP4 genes

Primer	Sequence (5'-3')	Polarity	Gene	Genotype	Nucleotide Position strand	Approximate Amplicon size, bp
VP4uF	TGG YTT CVC TCA TTT ATA GAC A	+	VP4	Generic	11-32	
P[4]-R5	GCA TYC CTA CAA GTC TAT TAY TAG	-	VP4	P[4]	508-485	497
P[6]-R2	ACC ATC GAG TAC TGG YTC TAT YGT TG	-	VP4	P[6]	210-185	199
P[8]-R2	GYG GTT CAA YAG CAA CKA CT	-	VP4	P[8]	350-330	339
P[9]-4T-1	TGA GAC ATG CAA TTG GAC	-	VP4	P[9]	402-385	391
P[10]-5T-1	ATC ATA GTT AGT AGT CGG	-	VP4	P[10]	594-575	583

Table A 1.13 Primer sequence for VP4 genes:F, forward; R, reverse; VP, structural protein; bp, base pair