CHARACTERIZATION OF HUMAN ASTROVIRUS IN PEDIATRIC PATIENTS WITH DIARRHEA FROM RURAL COMMUNITIES OF LIMPOPO SOUTH AFRICA

by

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DECLARATION

I, Khumela R (student number: 11632210), declare that this dissertation for the award of MSc. Master's Degree in Microbiology of the University of Venda has not previously been submitted for a degree at this or any other institution and that all reference materials contained herein have been duly acknowledged.

…………………………   11 june 2020 ……………

Signature   Date

DEDICATION

I dedicate this research to my parents for showing me their endless support all my life; to my sisters: you can achieve whatsoever you put your mind to, to my supervisors for giving me the opportunity to work with them on this project, and to my Lord and Savior, Jesus Christ for giving me the potential to acquire all things. Lastly, I dedicate this to myself for not giving up through it all and cheers to more that is to come!!!!!

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TABLE OF CONTENTS   **PAGE NO.**

CHAPTER 1: GENERAL INTRODUCTION

CHAPTER 2: LITERATURE REVIEW

University of Venda

CHAPTER 3: MATERIALS AND METHODS

CHAPTER 4: RESULTS

CHAPTER 5

University of Venda

LIST OF ABREVIATIONS

%	-	Percentage
˚C	-	Degree Celsius
3'	-	3 Prime
5'	-	5 Prime
AGE	-	Acute Gastroenteritis
bp	-	Base pair
CDC	-	Centers for Disease Control and Prevention
CT	-	Cycle threshold
DNA	-	Deoxyribonucleic acid
EDTA	-	Ethylenediamine tetra acetic acid
EIA	-	Enzyme Immuno Assays
et al	-	(et al) and others
g	-	gram
HAstV/s	-	Human Astrovirus/es
HEK293	-	Human embryonic kidney 293 cells
ICR	-	Internal control RNA
Kb	-	Kilobase
Kbp	-	Kilobase pair
kDa	-	Kilodaltons
mM	-	Micro Molar
min(s)	-	Minute(s)
MLB	-	Melbourne
NGS	-	Next generation sequencing
nm	-	Nanometer

ORF(s)	-	Open Reading Frame(s)
ORF1, ORF2	-	Open reading frame 1, open reading frame 2
PCR	-	Polymerase Chain reaction
RdRp	-	RNA-dependent RNA polymerase
RNA	-	Ribonucleic acid
RT- PCR	-	Real Time Polymerase Chain reaction
TAE	-	Tris-Acetate EDTA buffer
VA/HMO	-	Virginia/Human-Mink-Ovine-like
VLPs	-	Virus-like particles
VP70	-	Viral protein 70
VP90	-	Viral protein 90
W/V	-	Weight per volume
WHO	-	World Health Organization
µg	-	Microgram

LIST OF FIGURES AND PAGE NUMBERS

LIST OF TABLES AND PAGE NUMBERS

University of Venda

ABSTRACT

CHARACTERIZATION OF HUMAN ASTROVIRUS IN PEDIATRIC PATIENTS WITH DIARRHEA FROM RURAL COMMUNITIES OF LIMPOPO SOUTH AFRICA

Background: Globally, approximately 7,600,000 children under the age of 5 die annually due to diarrhea caused by viruses, bacteria and parasites. Human Astrovirus (HAstVs) has been identified as a causative agent of diarrheal disease worldwide especially in young children under five years of age. Recent reports in South Africa demonstrated HAstVs as a potential pathogen associated with diarrhea. The aim of this study was therefore to investigate the genetic characteristics of HAstVs in young children with diarrhea in rural communities of Vhembe District, Limpopo Province.

Methodology: A total of 141 archived RNA, extracted from stool samples, were retrieved from -20°C storage. Using questionnaire, clinical data useful in the analysis of results were captured. The RIDA®GENE Viral Stool Panel I (PG1325) multiplex real-time RT-PCR assay was used for the detection of Astrovirus. Positive Astrovirus extracts were amplified by one-step ahead RT-PCR (cat no: 220213, (QIAGEN)) and one-step RT-PCR kit (cat no: 210212, (QIAGEN)) using specific primers targeting the viral capsid and polymerase regions. Amplified fragments were sequenced, and phylogenetic trees constructed by the neighbor-joining method using MEGA X (10.0.5) software.

Results: HAstVs was detected in 10 (7%) of the 141 stool samples. A total of 9/10 (90%) HAstVs cases were from outpatients. The sequence analyses revealed HAstV genotype 1 and 2. A putative recombinant strain was found. Phylogenetic analysis revealed strain's relatedness to others circulating in the African continent.

Conclusion: This is the first study to characterize HAstVs from pediatric stool sample in the Vhembe district of Limpopo/South Africa. The study findings revealed the presence of HAstV type 1 and 2 in young children in the rural communities of the Vhembe District. Human Astrovirus genotype 1 and 2 are globally associated with diarrhea. Systematic surveillance to monitor HAstV strains circulation will help to understand the role of the pathogen in the study area.

KEYWORDS: Acute gastroenteritis (AGE), Children, Diarrhea, Human astrovirus.

CHAPTER 1
GENERAL INTRODUCTION

1.1 BACKGROUND

The World Health Organization (WHO) defines diarrhea as the passing of at least three or more loose stools within 24 hours or more frequently than normal for an individual (WHO., 2005). Each year, approximately 7,600,000 children under the age of five die globally due to diarrhea (Misgna et al., 2019). Reports of diarrheal cases of about 1.7 billion are made annually (Sreeramareddy et al., 2017). Diarrhea kills young children more than the combination of already known as "deadly diseases", namely, acquired immunodeficiency syndrome (AIDS), malaria and measles (Misgna et al., 2019; Mengistie et al., 2012).

In Africa, children under five years on average get to experience five episodes of diarrhea yearly and most of these cases are concentrated in the sub-Saharan African countries (Misgna et al., 2019; WHO, 2009). The WHO reported that in Africa and South East Asia, more than 70% of deaths that occur are due to diarrhea (WHO, 2018; WHO, 2012). The pediatric population of less than five years of age is primarily affected by these diarrheal cases (WHO, 2018).

Industrialized or high-income countries have lower incidences of infectious diarrhea as compared to very high rates observed in low-income countries (Lekana-Douki et al., 2015). Sanitary water, good hygiene practice and adequate waste disposal are still a challenge in low- and middle-income countries. Among others, these factors contribute to contaminated foods and water, and combined, they introduce high-risk of infectious diarrhea (WHO, 2018; WHO, 2013).

Gastroenteritis is a common disease in children, characterized by diarrhoea, vomiting, abdominal pain and fever (Bergallo et al., 2018). Approximately 75% of reported acute childhood diarrhea or acute gastroenteritis (AGE) is associated with viruses as etiological agents (Jacobsen et al., 2018; Grytdal et al., 2016; Ren et al., 2013; WHO, 2013). It has been reported that the global prevalence of enteric viruses in children suffering from AGE ranges between 29.6% and 85.6% (Biscaro et al., 2018; Ren et al., 2013). Commonly recognized viral pathogens that account for a significant number

of diarrheal or AGE cases are Rotavirus (RV), Norovirus (NoV), Adenovirus (Adv), Astrovirus (AstVs) and Sapovirus (Jacobsen et al., 2018; Grytdal et al., 2016).

Generally, Rotavirus is regarded as the number one cause of acute gastroenteritis (AGE) in children worldwide (Burnett et al., 2017; Tate et al., 2016; Ren at al., 2013). However, since the introduction of the Rotavirus vaccine, a decline in cases of Rotavirus has been observed (Atchison and Hassounah, 2015; Paternina-Caicedo et al., 2015). Norovirus has been shown lately as the predominant cause of AGE in countries where the Rotavirus vaccine has been introduced (Nguyen et al., 2017; McAtee et al., 2016).

Astroviruses are among the least studied enteric RNA viruses which have been associated with gastroenteritis and diarrhea (Cortez et al., 2017; Guix et al., 2002). These RNA viruses are relatively frequent etiologic agents of pediatric AGE (Johnson et al., 2017; Vu et al., 2017). They are thought to take the third spot after Rotavirus and Norovirus infections as the common cause of AGE, particularly diarrhea (Kumthimp et al., 2018; Bosch et al., 2014; Mendez and Andrias., 2013). Outbreaks of Astrovirus causing diarrhea are increasingly being reported worldwide (Olortegui et al., 2018; Johnson et al., 2017; Tseng et al., 2012).

1.2 STUDY RATIONALE

Despite the excessive burden of diarrheal disease in developing countries, enteric virus outbreaks have so far been mainly reported in developed countries (Liu et al., 2015; UNICEF, 2011). In South Africa, limited data are available to estimate the actual prevalence of circulating enteric viruses across the country (Olortegui et al., 2018; Kabue et al., 2016; Platts-Mills et al., 2015; Mans et al., 2010).

Although most viral gastroenteritis outbreaks worldwide are caused by Noroviruses (Lopman et al., 2016), considerable human Astrovirus (HAstVs) outbreaks have also been documented globally (Vu et al., 2017; Jarchow-Macdonald et al., 2015; Aragão et al., 2010; Svraka et al., 2007; Marshall et al., 2007). In the past years in selected areas of South Africa, HAstVs was shown as the second most important cause of viral diarrhea in young children (Nadan et al., 2003; Marx et al., 1998; Steele et al., 1998).

Recent reports in South Africa still demonstrate HAstVs as an important pathogen of diarrhea in young children (Nadan et al., 2019; Olortegui et al., 2018).

Data presenting the epidemiology of HAstV, particularly in rural areas of South Africa are scarce. Olortegui et al. (2018) reported that among other etiological agents of gastroenteritis, the burden of HAstVs was poorly recognized (Olortegui et al., 2018). A recent report showed a high prevalence of Norovirus infections among children under the age of five years in rural communities of Vhembe demonstrating frequent exposure of young children to enteric pathogens (Kabue et al., 2016). This suggests a need for more elaborate investigations on the prevalence and risks of enteric viruses circulating in the area.

A high risk of HAstVs infections is mostly observed in children less than five years of age, with HAstV-1 as the most prevalent among the eight known genotypes (Bosch et al., 2014). However, elderly and immune-compromised people are also affected (Nadan et al., 2019; Johnson et al., 2017). More studies on HAstVs are needed to determine the role of HAstVs in AGE, to contribute to epidemiological investigations as well as a potential target for vaccine development. Detection and characterization of HAstVs within rural communities of Vhembe District of Limpopo will be useful to confirm their role in diarrheal diseases and help in public prevention strategies against diarrheal disease transmission. This study aimed to investigate the prevalence of HAstVs and characterize the strains circulating in the Vhembe District.

1.3 OBJECTIVES OF THE STUDY

1.3.1 PRIMARY OBJECTIVE

- To investigate the genetic characteristic of Human Astrovirus in children under five years with diarrhea in rural communities at Vhembe District, Limpopo Province.

1.3.2 SECONDARY OBJECTIVES

- To determine the prevalence of Human Astrovirus in rural communities of the Vhembe District using real-time one-step RT-PCR.
- To determine the genotype strain of Human Astrovirus circulating in the rural communities of Vhembe District using conventional RT-PCR and the Sanger Sequencing method.
- To assess the relatedness of Human Astrovirus strains from rural communities in the Vhembe District to the global circulating strains using the BLAST tool and the MEGA X (10.0.5) software.

CHAPTER 2
LITERATURE REVIEW

2.1 INTRODUCTION

The etiology of gastroenteritis remained unknown for most cases until in the late 1940s when viruses were the suspected causative agents of gastroenteritis (Wilhelmi et al., 2003; Kapikian., 1996). After a diarrheal outbreak in 1972, Norwalk virus (also known as Norovirus) was the first virus identified in stool samples as a cause of gastroenteritis (Kapikian., 1996). Rotavirus was isolated a year later from children with gastroenteritis by Bishop et al. in 1973. and Astrovirus in the following year. Since then, the number of viruses associated with acute gastroenteritis has steadily increased (Wilhelmi et al., 2003). Acute gastroenteritis (AGE) in children represents one of the most deadly diseases reported worldwide, resulting in morbidity and mortality (Alcalá et al., 2018; Liu, 2016). Human enteric viruses are the leading cause of acute gastroenteritis, especially in children (Vu et al., 2016; Kotloff et al., 2013; Ren et al., 2013). Astrovirus infection is known as the third common cause of AGE in children, after rotavirus and norovirus (Bosch et al., 2014).

2.2 ASTROVIRUS

2.2.1 DISCOVERY OF ASTROVIRUS

The discovery of Astroviruses occurred in the year 1975 when Appleton and Higgins reported the presence of 28-30 nm particles in the stool samples of children suffering from mild diarrhea and vomiting (Appleton and Higgins., 1975). In the same year, Madeley and Cosgrove described the small round viruses with a star-like appearance as Astrovirus (**Figure 2.1**), coming from the Greek word *Astron* which originally means star (Risco et al., 1995; Madeley and Cosgrove., 1975). Astroviridae was proposed as their family and established in 1995. This family separates them from hairy viruses with rough irregular edges, such include caliciviruses (Monroe et al, 1993; Madeley., 1988).
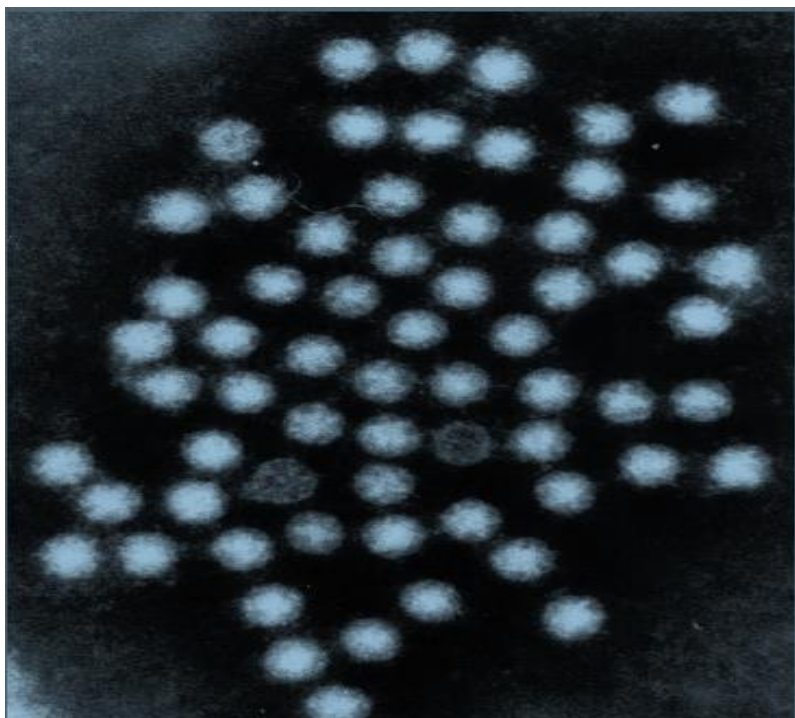
Figure2.1: Astrovirus particles under immunoelectron microscopy, 50 nm (http://naturalsciencenews.com/wp-content/uploads/2016/11/Astrovirus-741x406.jpg)

2.2.2 GENOME ORGANIZATION AND CLASSIFICATION OF ASTROVIRUS

Due to their broad host range, two Astrovirus genera have been proposed (Mamastrovirus and Avastrovirus). Mamastroviruses infect mammals, including humans, whereas Avastrovirus, infects poultry and other birds (Johnson et al., 2017; Vu et al., 2017). Human Astrovirus (HAstV) type 1 was the first genotype to be described in 1975 (Madeley and Cosgrove), and today, eight genotypes are described as HAstVs 1-8. These are commonly known as classical or canonical Human Astroviruses.

Many other types have been described in humans and animals (Bosch et al., 2014). The field of Astrovirus recently changed dramatically after metagenomic surveillance studies led to the discovery of numerous highly divergent novel Astroviruses with the ability to infect various animal species including humans and are not related to the previously described eight serotypes of Astrovirus (Bosch et al., 2014; Finkbeiner et al., 2009a; Finkbeiner et al., 2008). Since 2008, two novel groups of Astroviruses named MLB (Melbourne) and VA/HMO (Virginia/Human-Mink-Ovine-like) have been identified in human stools with diarrhea using next-generation sequencing (NGS)

(Finkbeiner et al., 2009a; Finkbeiner et al., 2008). The HAstV-MLB clade contains at least three strains (MLB1, MLB2, MLB3) and the HAstV-VA/HMO clade contains at least five strains (VA1, VA2, VA3, VA4, VA5) (Johnson et al., 2017). The HAstV-MLB and HAstV-VA/HMO viruses have been designated as non-canonical human genotypes and their prevalence varies based on geographical location (Vu et al., 2016). Both classic but especially novel HAstVs have also been implicated in infections of the central nervous system suggesting that they can bypass the gastrointestinal tract to infect other vulnerable organs and tissues (Vu et al., 2017; Vu et al., 2016).

Astroviruses are characterized by a genome containing three open reading frames (ORFs) named from 5' to 3' end as ORF1a, ORF1b, and ORF2 (Donato and Vijaykrishna., 2017). The ORF1a region encodes a non-structural polyprotein (serine protease) while, ORF1b encodes a polyprotein including the RNA-dependent RNA polymerase (RdRp), and altogether are involved in RNA transcription and replication (Bosch et al., 2014). ORF2 encodes the viral capsid protein expressed from sub genomic RNA (Donato and Vijaykrishna., 2017; Bosch et al., 2014). In the ORF2 region of the genome, there is a highly conserved N-terminal domain with amino acids (1–424), a hypervariable domain with amino acids (425–688) which is believed to form the capsid spike and contributes to receptor binding, and a highly acidic C-terminal domain (Dong et al., 2011). In the classic and other mammalian astroviruses (AstVs), a new ORF named OFRX which overlaps the 5' end of ORF2 in the +1 reading frame has been previously described, (**Figure 2.2**); (Bosch et al., 2014). Located at the ends of AstVs genome are the two untranslated regions (UTR)s 5' UTR and 3' UTR comprised of 11-85 and 80-85 bases (Bosch et al., 2014).

There are four genotypic species or phylogenetic clades identified from human stools. The eight most common serotypes (HAstVs 1-8) belong to MAstVs 1; other human viruses belong to genotype MAstV 6, 8, and 9 (Bosch et al., 2014). Classic HAstVs (MAstV 1) and HAstV-MLB (MAstV 6) form a monophylogenetic group with viruses from pigs, cats, dogs, rabbits, California sea lions and dolphins while MAstV8 and MAstV9 are genetically related to each other and viruses from mink, sheep, California sea lions, bats, cattle, pigs and mice (Bosch et al., 2014).
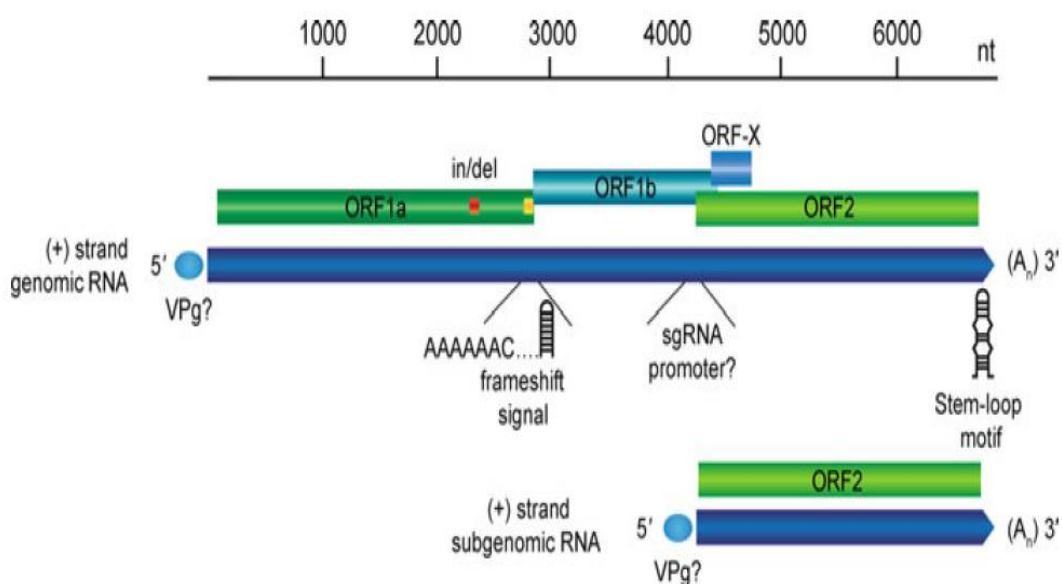
Figure 2.2 Genome organization of Human Astrovirus (Bosch et al., 2014)

2.2.3 GENETIC DIVERSITY, INTERSPECIES TRANSMISSION AND RECOMBINATION

The ability of Astroviruses to generate genetic variability and evolve rapidly equips them to adapt quickly to novel niches (De Benedictis et al., 2011). Several factors contribute to the high genetic diversity of the Astrovirus population, including high mutation rate, interspecies transmission and recombination. Cross-species transmissions are important events for the evolutionary history of RNA viruses (Wohlgemuth et al., 2019; De Benedictis et al., 2011; Parrish et al., 2008). Genetic diversity of Mamastrovirus as a result of extensive interspecies transmission occurs between humans, domestic and wild animals (Donato and Vijaykrishna., 2017; De Grazia et al., 2013; Wolfaardt et al., 2011). Both intra-species and inter-species recombination can rapidly generate novel, divergent viruses (Donato and Vijaykrishna., 2017). Astrovirus strains have been identified in over 80 different host species. These strains could either infect one or only a few of related species (Mendenhall et al., 2015), but a single host can be infected by a wide range of Astrovirus strains (Wohlgemuth et al., 2019). Astroviruses are stable for a long time in the environment due to their lack of a lipid envelope and also are very prevalent in several animal species which means they could easily infect livestock (cattle and pigs) as well as survive recreational and drinking waters (Fischer et al., 2017). It has been

discovered that novel HAstVs phylogenetically cluster more closely with animal Astrovirus strains than the classic HAstV-1 strains (Finkbeiner et al., 2009b, Kapoor et al., 2009). The Astrovirus receptor is still unknown; therefore, cross-species transmission is based on the homology of old and new host receptor molecule meaning that to infect a new host the virus would most likely evolve (Wohlgemuth et al., 2019).

Recombination describes the co-infection of one cell by two different strains (either two unique human strains or one human strain and another one acquired as a result of inter-species transmission). This kind of event is common to most RNA viruses (Su et al., 2016). Genetic variation can also be generated by recombination among related strains of Astrovirus (Donato and Vijaykrishna., 2017). Two theories describe the evolution of recombination among Astroviruses. The first one states that this event allows for the removal of "deleterious" genes and alleles (Simon-Loriere and Holmes, 2011; Chao et al., 1997). The second theory states that recombination allows for the production of advantageous alleles (Vuilleumier and Bonhoeffer, 2015). Analyses of genome sequences have revealed that multiple strains that undergo recombination are usually located in the ORF1b and ORF2 region. Recombination on ORF1a has also been identified (Wolfaardt et al., 2011).

Contributing to the rate of mutation is the RNA polymerase and kinetics of virus replication. Unlike most DNA viruses, RNA viruses consist of RdRp (RNA dependent RNA polymerase) enzymes which lack proofreading functions and are prone to error. Infectious particles of Astroviruses can be detected as soon as eight hours after the infection. The quick generation time contribute to their genetic variability (Mendez et al., 2013). The most heterogeneous region of Astroviruses is the ORF2 which encodes the capsid genome. ORF2 is exposed to most host immune system epitopes and, therefore, expected to have high positive selection pressure to evolve the epitopes (Wohlgemuth et al., 2019).

2.2.4 EMERGENCE OF ASTROVIRUS

Since the discovery of HAstVs, infections were thought to be limited only to the eight well known genotypes (HAstVs 1-8). Advanced technologies for sequencing and metagenomics studies have revealed many other genotypes, among them two clades affecting human are characterized. Primers targeting the highly conserved RdRp region (pan-Astrovirus RT-PCR primers) are designed to improve identification of astrovirus (Chu et al., 2008), and identification of novel strains (Finkbeiner et al. 2009a). Astroviruses are not species-specific and have high genetic diversity which now demonstrates the probability of emergence of novel HAstVs strains. Wohlgemuth et al. (2019) reported two principal ways in which novel HAstVs emerge (cross-species transmission to humans and co-infection with different strains), both requiring more research to better understand potential risks presented by such events.

2.2.5 HUMAN ASTROVIRUS REPLICATION

The main cellular receptor for HAstVs is still being investigated, and so far it is known that different cell lines are susceptible to different HAstVs serotypes, which implies that they may have multiple receptor molecules, such as polysaccharides, that may be found in all HAstVs (Bosch et al., 2014; Dong et al., 2011; Brinker et al., 2000). In the surface structure of HAstVs, a putative receptor binding site that recognizes polysaccharide molecules and is conserved in all HAstVs serotypes was identified (Dong et al., 2011). Dong et al (2011) reported that dextran/heparin sulphate and heparin might partly play a role in blocking infectivity of HAstVs. Involvement of sialic acid virus in cell recognition was not observed.

As confirmed on CaCo-2 (Mendez et al., 2014) and HEK293 (Donelli et al., 1992) cells, HAstVs use a clathrin-mediated endocytosis pathway as a mechanism of entry (Bosch et al., 2014). Entry also depends on the acidification and maturation of endosomes where membrane permeabilization and RNA un-coating would occur. The estimated that half-time of virus binding to the cell surface is about 10 minutes, and virus un-coating takes about 130 minutes (Mendez et al., 2014). For effective binding and infection, HAstVs interact with cells to activate extracellular signal-regulated kinase (ERK1/2) and phosphoinositide 3-kinase (PI3K) pathways (Bosch et al., 2014).

After the virus is bound to one or more cellular receptors, it is taken in through clathrin-mediated endocytosis. The following step would be un-coating, which is a result of pH drop. The mature non-structural proteins which are needed for genome replication are formed by translation of two main non-structural polyproteins (nsP1a and nsP1b) from the VPg-linked genomic RNA, which are then further cleaved by viral and cellular proteases (Speroni et al., 2009). Replication complexes assemble in close association with intracellular membranes. Synthesis of RNA strands (positive or negative sense) and sub-genomic RNA may be regulated by nsp1a/4 protein after its interaction with RdRp protein. The protein (nsP1a/4) phosphorylation status may contribute to the regulation step (Fuentes et al., 2012). Production of sub-genomic RNAs is in large quantities, and they are used for capsid proteins expression. The structural VP90 polyprotein first assembles into immature virions (in association with intracellular membranes) then several cellular caspases further cleave these VP90 polyproteins once they have dissociated from membranes, resulting in VP70 immature viral capsids (Fuentes et al., 2012). VP70 particles are then released into the medium, and virions mature extracellularly by the action of trypsin (Bosch et al., 2014; Mendez et al., 2013, Guix et al., 2005).

2.6   PATHOGENESIS OF ASTROVIRUS AND HOST RESPONSE.

2.6.1 CLINICAL DISEASE CORRELATES

Human Astroviruses (HAstVs) are classified as the second or third most common cause of diarrhea in young children worldwide (Mendez and Arias, 2013). Most young people who get infected by HAstVs develop antibodies in the early ages, which help to fight the infection in the later stage, leaving the immune-compromised and elderly individuals as the group at high risk. HAstVs induce watery diarrhea associated with vomiting, fever and abdominal pain that last for at least two to three days. Available data indicate that the mean incubation period for HAstVs is four to five days, which is longer than that of Rotavirus, HAstVs diarrhea is milder than that caused by Rotavirus and Norovirus (Bosch et al., 2014). The prevalence of asymptomatic cases is yet to be well documented because such cases have been reported (Jeong et al., 2012). Astrovirus infection has also been associated with other disease conditions besides diarrhea (respiratory illness and encephalitis in immune-compromised people) (Vu et al., 2016). Two novel clades (MLB and VA) are also generally associated with

gastroenteritis, but further research is required to know more about their etiological role.

2.6.2 MECHANISM OF PATHOGENESIS AND INDUCTION OF DIARRHOEA BY ASTROVIRUSES

Progress has been made in the identification of HAstV genotypes thus far, but little is known about HAstVs pathogenesis, especially among these different HAstV genotypes (Johnson et al., 2017). Moser et al. (2007) demonstrated that HAstV increase the permeability of epithelial cell by disrupting tight cellular junctional complexes. Tight junctions help the intestinal tract to separate the lumen from the basal lamina. Loosening of such may increase the exchange of water, solute and ion across the compartments. The intestines therefore lose the ability to reabsorb water and nutrients, resulting in diarrhea (Johnson et al., 2017). It was already indicated that HAstVs infection lead to osmotic diarrhea resulting in disruption of intestinal absorption (Thouvenelle et al., 1995). Astroviruses causing other extra-gastrointestinal diseases in humans and animals have been reported (Li et al., 2013; Quan et al., 2010).

2.6.3 HOST IMMUNE RESPONSE

Astrovirus has been noted to be age-dependent upon infection of different species. It is commonly found in children under the age of two to five years. Although the immune response towards HAstVs is not fully understood, it is believed that immunity against the infection is developed after the initial encounter (Burbelo et al., 2011; Koci., 2005). The Humoral immune response plays a role against the presence of HAstVs and studies have shown that young, healthy people have antibodies against the most prevalent strains of classic HAstVs as well as against some novel strains (Bosch et al., 2014; Koopmans et al., 1998). Other findings from sero-prevalence studies showed the presence of serotype-specific neutralizing antibodies.

CD4^{+} and CD8^{+} T cells that are specific for human astrovirus have also been identified in normal human tissues (Burbelo et al., 2011). It can, therefore, be concluded that both humoral and adaptive immune responses play a role against infection in healthy adults. Innate immunity does play a role as well in limiting the infection (Mauriello et

al., 2013), however the capsid of HAstVs inhibits activation of the complement system which is essential for the innate immune response (Stoermer and Morrison, 2011).

2.7 EPIDEMIOLOGY OF ASTROVIRUS

Astrovirus is an overlooked cause of diarrhea among vulnerable children worldwide (Olortegui et al., 2018). Human Astroviruses (HAstVs) predominantly affect the pediatric population, although older adults and immune-compromised patients, as well as healthy individuals, are also affected (Bosch et al., 2014; Mendez and Arias., 2007). Classic HAstVs are ubiquitous, however in developing countries it is important to note their burden given that the poor living conditions may add to the level of exposure (Vu et al., 2016). HAstVs circulate throughout the year, although distribution of classic HAstVs in temperate regions is high during cold periods and low in warmer periods because they are more stable in lower temperatures (Mendez and Arias., 2007; Abad et al., 2001). Currently, epidemiological surveillance including the newly identified MLB and VA in association with gastroenteritis in humans is limited (Kumthip et al., 2018). More studies are needed to show seasonality of non-classic HAstVs.

Global epidemiological investigations showed that the prevalence of HAstVs varies from 2 to 16.5% in cross-sectional studies of young children treated for gastroenteritis (outpatients or hospitalized) (Vu et al., 2017; Espul et al., 2004; Glass et al., 1996), and from gastroenteritis outbreaks in schools, day-care centers, and other closed populations (Espul et al., 2004; Mitchell et al., 1999). On a worldwide range, classic HAstVs are associated with non-bacterial acute diarrhea in children in about 2 to 9% of reported cases (De Benedictis et al., 2011).

In Africa, studies have shown low prevalence (approximately 4%) of HAstVs in patients with diarrhea (Monastiri et al., 2015; Lekana-Douki et al., 2015; Phan et al., 2014). In South Africa, the concurrent presence of identical HAstVs strains in wastewater samples and hospitalized patients led to suggestions that HAstVs present in the environment pose a potential risk to communities in which faecally contaminated water is used for recreational and domestic purposes (Nadan et al., 2003). Nadan et al. (2019) reported the presence of Astrovirus in 7.0% (234/3340) of cases and most frequently in ages of 7 to 12 months (9.2%, 90/975). More HAstVs were detected in

children from homes that used outdoor water sources (7.6%) compared to indoor sources (5.7%). The study concluded that more investigations needed to be done in rural communities (Nadan et al., 2019).

2.8 HUMAN ASTROVIRUS TRANSMISSION

Astroviral-dependent gastroenteritis is primarily manifested by diarrhea but can also be accompanied by vomiting and fever. The infection is essentially transmitted by the fecal-oral route, contaminated foods and water (Cortez et al., 2017; Johnson et al., 2017; Bosch et al., 2014; Soares et al., 2008; Nadan et al., 2003; Walter and Mitchell., 2000). Several outbreaks have been associated with the consumption of contaminated foods, poor hygiene and improper food handling especially in less developed settings (Koopmans et al., 2008; Bosch, 2007). Bosch et al (2014) demonstrated an overview of potential environmental routes of HAstVs transmission (**Figure 2.3**).
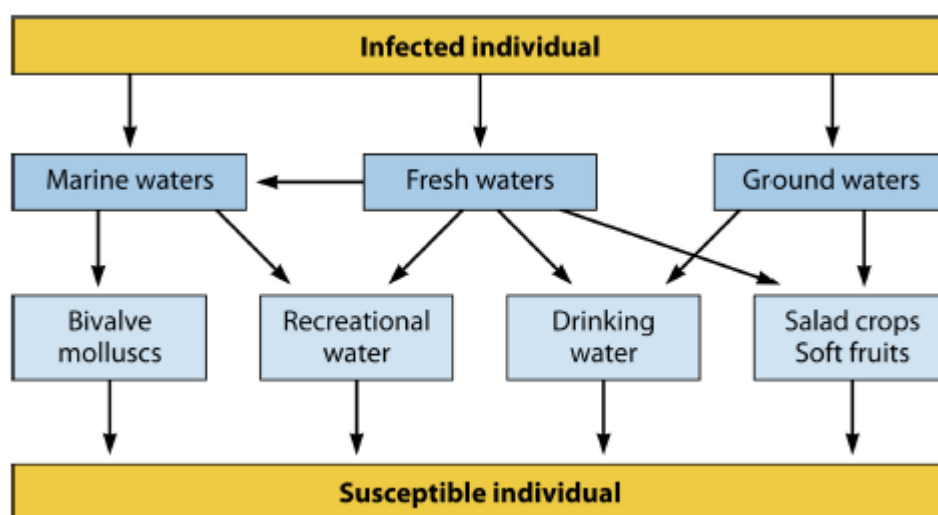
Figure 2.3: Flowchart of potential routes of environmental transmission of HAstVs (Bosch et al., 2014)

Treated and untreated wastewater are major contaminants of the environment as they may affect fresh water, ground and marine waters (Pinto et al., 2001), thus affecting foods that grow depending on these water sources and are minimally processed before consumption. Stools of individuals infected may carry genome copies of up to 10^{13} per gram (Caballero et al., 2003). HAstVs have been isolated in tap water in Ghana (Dongdem et al., 2011).

2.9 SYMPTOMS OF HUMAN ASTROVIRUS INFECTION

Watery diarrhea that may be accompanied by fever, headaches, abdominal pain, and anorexia that last two to four days are usually the signs associated with HastVs infections (Liu., 2016; Meliopoulos and Schultz-Cherry., 2013; Glass et al., 1996). Symptoms such as vomiting, fever and dehydration were demonstrated in patients by Siqueira et al (2017). The average length of hospitalization due to the virus was six days. However, many of the infections in healthy children and adults tend to be asymptomatic (Jeong et al., 2012). Asymptomatic individuals that serve in food industries have been implicated in acute gastroenteritis outbreaks than symptomatic individuals (Vu et al., 2017; Bosch et al., 2014). The occurrence of asymptomatic individuals makes it difficult to trust the estimated HAstVs prevalence since many studies focus on symptomatic patients. However, HAstVs infections are of clinical concern in the immunocompromised population due to their increased severity of symptoms and extra-gastrointestinal involvement (Siqueira et al., 2017).

2.10 LABORATORY DIAGNOSTICS OF ASTROVIRUS

HAstVs were first described using electron microscopy (EM), (Appleton and Higgins., 1975). Sensitivity to EM requires a higher concentration of stool (Glass et al., 1996). Enzyme immunoassay (EIA) and reverse transcription polymerase chain reaction (RT-PCR) are improved methods that have led to the perceived significance of HAstV-associated gastroenteritis (Aragão et al., 2010; Espul et al., 2004). As a way to meet a demand for high specificity and sensitivity, many PCR protocols have been developed from conventional PCR. Real-time RT-PCR (RTqPCR) compared to conventional PCR is a more sensitive and excellent tool for the rapid sensitive detection and quantification of viruses, where the amplified product is quantified either using SYBR Green or by various fluorescent probe chemistries (Malik et al., 2019).

A combination of EIA, culture and RT-PCR showed that RT-PCR allowed the detection of about one-quarter of the strains not detected by EIA (Espul et al., 2004). Perot et al. (2017) concluded that a predominant method for diagnosis in a clinical setting remains RTqPCR due to its high sensitivity and specific nature. RT-PCR is of low cost compared to advanced molecular methods and the gives the ability to multiplex with other targets of interest (Perot et al., 2017). Currently, to understand molecular

characterization, epidemiology and transmission of pathogens, Next-generation DNA sequencing is being used increasingly. A single test using this method can detect large deposits of genes available in the clinical which makes it more resourceful than Sanger sequencing (Malik et al., 2019). Sanger sequencing, also known as chain termination method, has been widely used although it only sequences a single DNA fragment at a time.

2.11 PREVENTION AND TREATMENT OF ASTROVIRUS

Currently, there is no vaccine and treatment against Astrovirus. The prevention of human Astrovirus is based on the control of the transmission routes and the use of hygiene measures including disinfection of contaminated fomites. Steps used to control the virus transmission comprise the detection and inactivation of viral particles in water and food (Vu et al., 2017; Bosch et al., 2014).

2.12 ASTROVIRUS IN WATER AND FOOD

Although there is progress in environmental sanitation and potable water compared to the last century, there is still an alarming rate of diarrheal diseases due to the consumption of contaminated water and food. Gastroenteritis outbreaks due to water-borne or food-borne transmission of HAstVs have been documented globally (Scarcella et al., 2009; Smith et al., 2006). In early 2009, the possible zoonotic transmission of Astroviruses from cows was proposed (Kapoor et al., 2009). In South Africa, the most recent outbreak of gastroenteritis was reported in various branches of a childcare facility in Gauteng Province. This outbreak was associated with HAstVs. The main route of transmission in the astrovirus outbreak was the food catered (Sekwadi et al., 2018).

2.13 SUMMARY OF LITERATURE REVIEW

Human Astroviruses (HAstVs) are single-stranded RNA viruses that are non-enveloped and belong to the Astroviridae family (Vu et al., 2017). Classical HAstVs infections are a major cause of viral diarrhea and mainly affect the pediatric population (Parashar et al., 2003). Limited data reporting on the involvement of HAstVs in gastroenteritis have been published in Africa. More studies are needed in South Africa especially in rural communities to determine the prevalence of this virus since diarrhea

is a major health problem. More recently, real-time polymerase chain reaction (RTqPCR) has been used as a highly sensitive method to detect Astroviruses in stool samples (Perot et al., 2017). For further screening and isolate typing, conventional RT-PCR methods are broadly used. Primers Mon269/Mon270, as described by Noel et al. (1995), detect eight serotypes of classical HAstV and target the conserved 5' end of ORF2, generating amplicons for partial sequencing. Novel strains can be identified using primers SF0073/SF0076, which also detect classical strains on the polymerase gene fragment (Finkbeiner et at., 2009b). This study was therefore set to detect and characterize human astrovirus in pediatric patients in the rural communities of Vhembe District in the Limpopo Province of South Africa.

Chapter 3

MATERIALS AND METHODS

3.1. INFORMED AND ETHICAL CONSENT

The study protocol and consent procedures were approved by the ethics committees of the Department of Health in the Limpopo Province (Ref. 4/2/2) and the Research Directorate of the University of Venda (Ref.SMNS/12/MBY/07) (Appendices 1 and 2). Written, informed consent was given by the parents or child guardians before stool sample collection (Appendix 3).

3.2. DEMOGRAPHICS OF PATIENTS

This study included patients between the age of 1 day and 72 months (≤5 years old). The parents or guardian were interviewed by nurses using a questionnaire (Appendix 4) to gather personal information such as date of birth, gender, date of onset of diarrhea and symptoms associated with the illness such as abdominal pain (AP), fever and dehydration. Living conditions of the patients such as the type of water source used, the presence of livestock, type of toilet used and employment status of the parents were also documented.

3.3. SAMPLE COLLECTION

This study was a cross-sectional clinic-based investigation of children younger than five years of age. The study was part of an on-going project in which, stool samples of patients (n=141) were already collected in different primary health care centers situated within the rural communities in the Vhembe District of Limpopo in South Africa (**Figure 3.1**). Specimens were stored at - 20˚C prior to RNA extraction. The collection period was between January 2017 and November 2018.
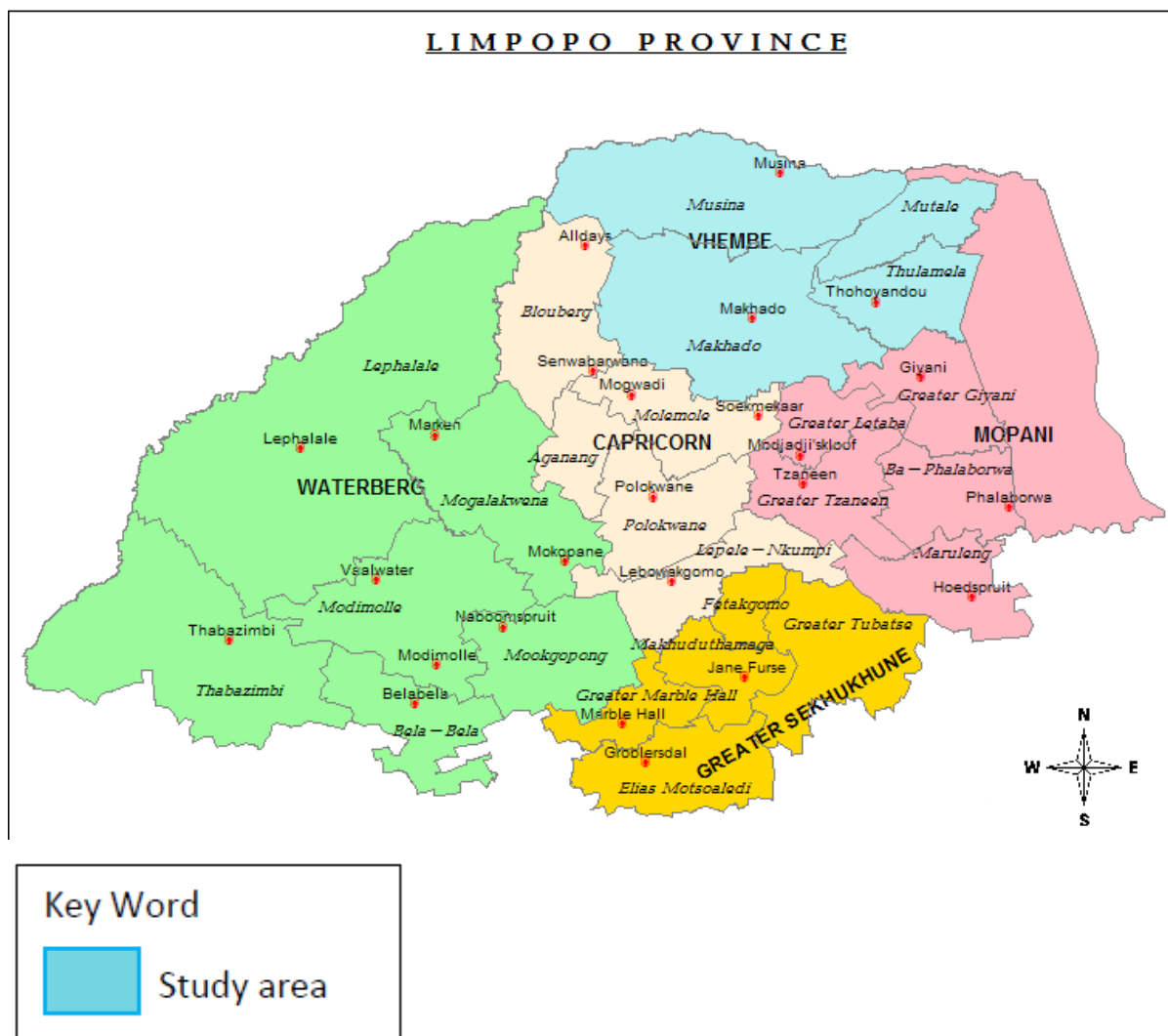
Figure 3.1: Map of the Limpopo Province of South Africa, indicating the Vhembe District.

http://www.capeinfo.com/blogs/wp-ntent/blogs.dir/akela/files/2009/05/limpopo_dlghgovza.gif

3.4 RNA EXTRACTION (BOOM METHOD)

RNA extraction was performed on the fecal suspension using the adapted version of the guanidium thiocyanate/silica method reported by Boom et al. (1990) (Appendix 5). The method is based on lysing and nuclease inactivation properties of the chaotropic agent guanidium thiocyanate, together with the nucleic acid binding properties of silica particles. Before the RNA extraction, samples were processed as follows, each raw specimen was diluted 1:10 in phosphate buffer saline (PBS, 0.01 M, pH 7.2) (Thermo-Fisher Scientific, Waltham, Massachusetts, United States) and thoroughly vortexed to allow proper mixing. Extracts were stored at -20°C.

3.5 Real-time PCR METHOD FOR ASTROVIRUS DETECTION

The RIDA®GENE Viral Stool Panel I (PG1325) multiplex real-time RT-PCR (r-BiopharmAG, Darmstadt, Germany) assay was used for the direct, qualitative detection and differentiation of Astrovirus. This kit has been recently evaluated for the detection of Astrovirus as reported by Redli et al. (2020). Amplification of the gene fragments specific for Astrovirus was done using the archived isolated RNA. The RIDA®GENE Viral Stool Panel I assay contains an Internal Control RNA (ICR) as an internal control of the sample preparation procedure and to determine possible PCR-inhibition. The manufacturer does not disclose primers and probes used to target Astrovirus on real-time RT-PCR. The Ridagene kit detects positive samples with ≤50 copies per reaction.

The test was carried out in a one-step real-time RT-PCR format in which the reverse transcription of RNA is followed by the PCR in the same tube. The real-time PCR program was performed on a Corbett Research Rotor Gene 6000 (Corbett Life Sciences, Concorde, Australia) with the following cycling conditions: reverse transcription for 10 min at 58°C; initial denaturation step for 1 min at 95°C followed by 45 cycles of 95°C for 15s and 55°C for 30s continuous fluorescence reading. To minimize the risk of amplicons carry-over and contamination of samples, pre- and post-amplification steps were carried out in separate rooms. To acquire and analyze data, validate and interpret, the Rotorgen 6000 Corbet software was used.

3.6 ASTROVIRUS RT-PCR AMPLIFICATION

RNA extracts that tested positive by One step Real-time RT-PCR were amplified using one step ahead RT-PCR cat: 220213 (QIAGEN). The primer sequences used for RT-PCR amplification were obtained from Noel et al. (1995) and Finkbeiner et al. (2009b) listed in **Table 3.1**. Primer specificity was checked using NCBI nucleotide blast and primer sequences gave no self-complementarity or hairpins. Primer MON269 & MON270 amplify a capsid region of size 449 bp. Primers targeting the polymerase region SF0073 and SF0076 amplify a size 409 bp. During One step Ahead RT-PCR amplification, the following conditions were used for both sets of primers (Hold 1: 42°C for 30 minutes; Hold 2: 95°C for 15 minutes; Cycles (40X): Denaturation - 95°C for 1

minute; Annealing - 56°C for 1 minute; Extension - 72°C for 1 minute and a final extension at 72°C for 10 minutes). Each primer (final concentration, 1µM) was included in a final reaction volume of 25 µl. For the best yield of Astrovirus amplicons able to successfully generate the sequences, one step RT-PCR cat: 210212 was used under the following conditions: Hold 1: 50°C for 30 minutes; Hold 2: 94°C for 15 minutes; Cycles (40X): Denaturation - 94°C for 1 minute; Annealing - 50°C for 1 minute; Extension - 72°C for 1 minute and final extension at 72°C for 10 minutes.

Table 3.1: Primer sequences used for RT-PCR amplification

Species	Code	Primers (3'-5')	Reference
HAstVs (Classical)	MON 270	TCAGATGCATTGTCATTGGT	Noel et al., 1995
HAstVs (Classical)	MON 269	CAACTCAGGAAACAGGGTGT	Noel et al., 1995
HAstVs (Classical & Novel)	SF0073	ATTGGACTCGATTTGATGG	Finkbeiner et al. 2009b
HAstVs (Classical & Novel)	SF0076	CTGGCTTAACCCACATTCC	Finkbeiner et al. 2009b

3.7 GEL ELECTROPHORESIS

PCR products were then analyzed using a 2% (w/v) agarose gel in TAE buffer (40 mM Tris; 20 mM acetic acid; 1mM EDTA; pH 8.3) stained with ethidium bromide. The relative sizes of the RNA fragments were estimated by comparing their electrophoretic mobility with that of the molecular marker run with the samples on each gel.

3.8 GENOTYPING AND PHYLOGENETIC ANALYSIS OF ASTROVIRUS

The RT-PCR products of the amplified fragments were purified with Zymoclean™ Gel DNA recovery kit following the manufacturer's instructions. Using the same specific primers, Sanger sequencing was performed on ABI 3500XL Genetic Analyzer POP7™

(Thermo-Scientific). Raw sequence reads were edited with Finch TV v1.4 (Geospiza, Seattle, USA). Nucleotide sequences of HAstVs obtained were compared with reference strains obtained in the NCBI GenBank using BLAST tool available at https://**www.ncbi.nlm.nih.gov/blast** followed by the construction of phylogenetic tree using MEGA X (10.0.5) software (Kumar et al., 2018). Reference strains from GenBank which were selected among the BLAST hits had ≥80% similarities with the query sequence of the strains identified. The neighbor-joining method (Saitou and Nei., 1987) was used to build the phylogenetic tree, and the reliability of different phylogenetic groupings was evaluated by bootstrap analysis (Felsenstein., 1985). Evolutionary distance was computed using p-distance method (Nei and Kumar., 2000).

Chapter 4

RESULTS

4.1. PREVALENCE OF ASTROVIRUS

A total of 141 non bloody diarrheal samples (64 males/77 females) were collected between January 2017 and November 2018, from children residing within the Vhembe District, Limpopo, South Africa. All the samples were screened for the presence of Astrovirus using real-time RT-PCR. The age of all participants ranged from 2 weeks to 5 years. Demographic data and clinical features of participants are shown in **Table 4.1** and **Table 4.2**. Out of 10 (7%) Astrovirus positives sample, 6 were males and 4 were females. The highest detection rate was found in female children of age group 0-12 months (6/68; 9%).

Table 4.1: Demographic profile of Astrovirus tested participants under the age of 5 living in rural communities of Vhembe District, Limpopo, South Africa.

Variable	Diarrheal stools (n)	Astrovirus positive n (%)	Astrovirus negative n (%)
TOTAL	141	10 (7%)	131 (93%)
GENDER			
Females	77	4 (5%)	73 (95%)
Males	64	6 (9%)	58 (91%)
AGE GROUP (MONTHS)			
[0-12]	68	6 (9%)	62 (91%)
[13-24]	42	2 (5%)	40 (95%)
[25-36]	16	2 (13%)	14 (88%)
[37-48]	10	0	0
[49-60]	2	0	0
Unknown	3	0	0

Internal Control (IC) was detected in 100% of all tested samples including the positives on real-time RT-PCR. Sample 44 (blue curve) displayed alongside the positive control

curve (green) in **Figure 4.1** is an exemplary overview of what is observed after a complete cycle for a positive Astrovirus sample. Quantitative data for positive sample(s) generated by the real-time PCR machine displays the curve of the positive sample rising above the threshold (0.03). Astrovirus negative samples are represented by all the colors that did not rise above the red line (threshold = 0.03)
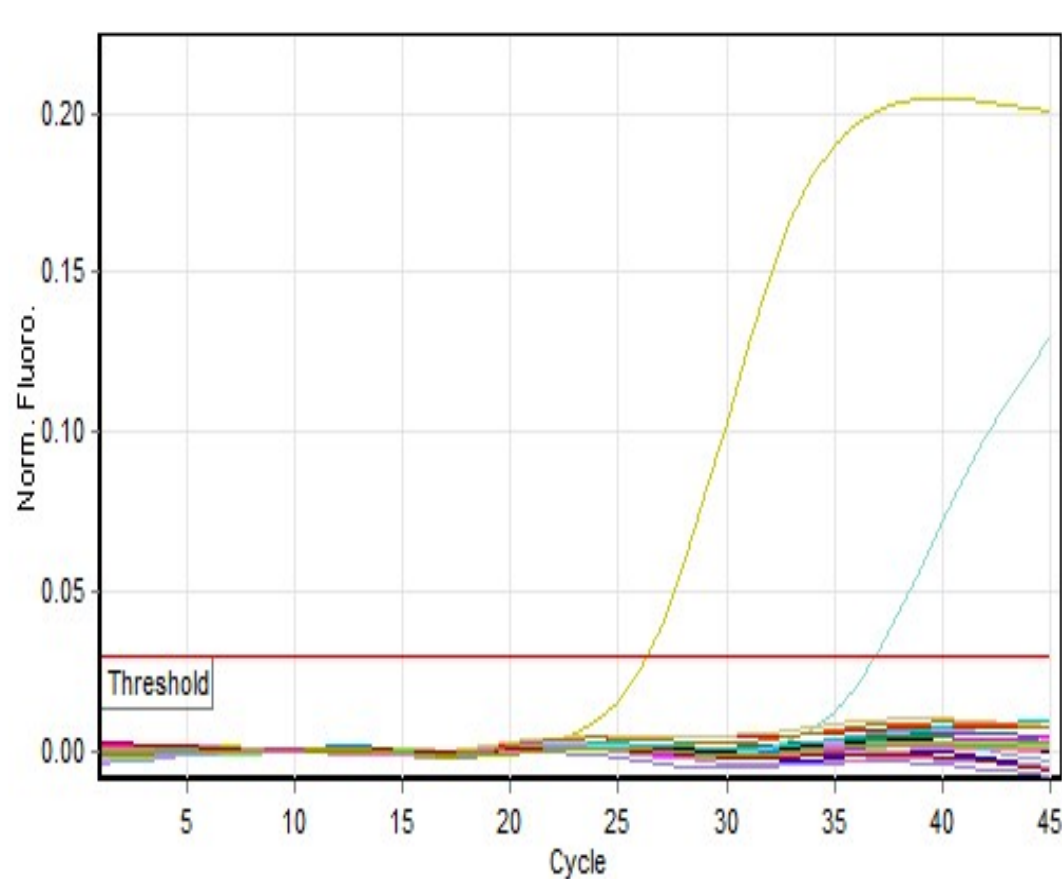
Figure 4.1: Quantitative data of Cycling A. Red for Astrovirus. Green curve – Positive control with Ct Value = 26.35; Blue curve – Sample 44 with Ct Value = 36.93; Red straight line – Threshold = 0.03. (Other colors visible below the red straight line of the threshold are Astrovirus negative samples).

During detection, the Ct value for each HAstV positive samples was recorded, data obtained is presented on **Figure 4.2**. The positive control Ct value remained constant between 26 and 27. Approximately 50% of HAstV positive samples displayed Ct value ranging from 30 to 45. Values too low compared to positive control were observed in only 2 samples (**G and J in Figure 4.2**).
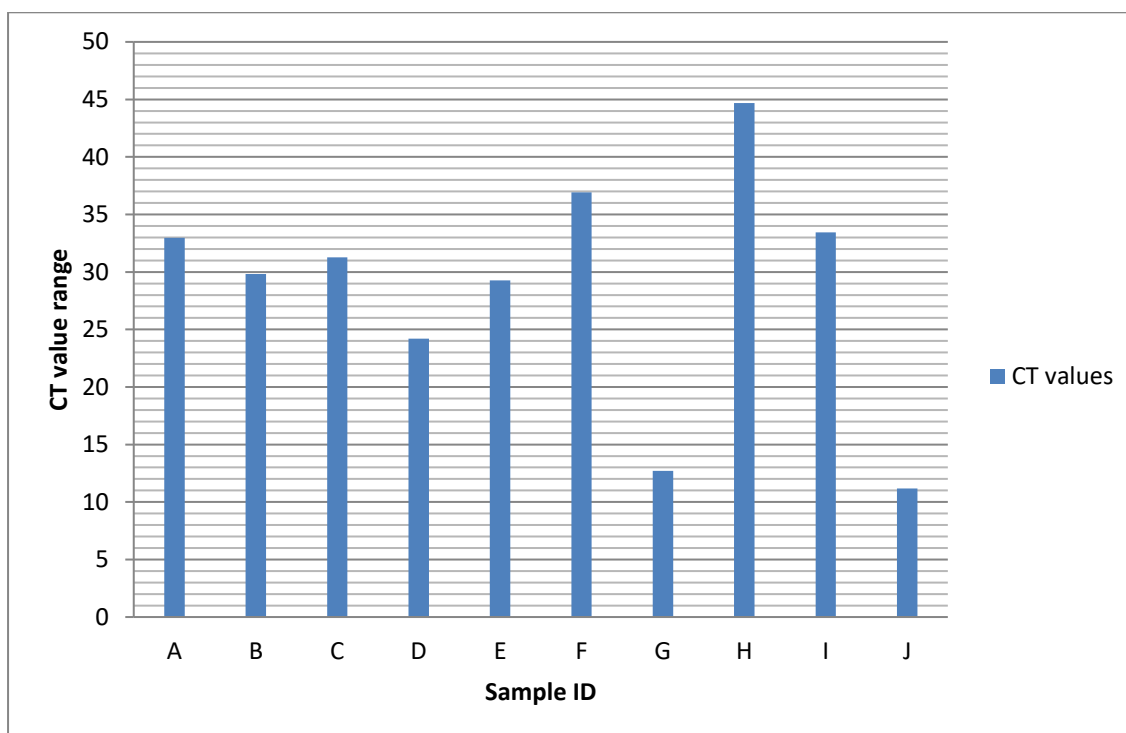
Figure 4.2: Ct values for all Astrovirus positive samples collected in the Vhembe District of Limpopo, South Africa presented in blue bars. Key: A (sample-4), B (sample-18), C (sample-23), D (sample-24), E (sample-41), F (sample-44), G (sample-48), H (sample-92), I (sample-105) and J (sample-254).

Clinical features of children included in the study in Vhembe district, Limpopo, South Africa

Most commonly associated symptoms of gastroenteritis included diarrhea 46 (13%) and vomiting 24 (8%). Out of the 141 samples, 68 came from children who had had mixed symptoms with no HAstV detected. A high number of samples was collected from outpatients. **Table 4.2** show all symptoms associated with diarrhea and HAstV detection together with the clinical or health setting from which all the samples where obtained.

Table 4.2: Clinical features of study participants under the age of 5 from the rural communities of Vhembe District, Limpopo, South Africa.

Variable	Diarrheal stool samples (n)	Astrovirus positive n (%)	Astrovirus negative n (%)
TOTAL	141	10 (7%)	131 (93%)
HEALTH SETTING			
Hospitalized	39	1 (3%)	38 (97%)
Outpatients	102	9 (9%)	93 (91%)
SYMPTOMS			
D only	46	6 (13%)	40 (87%)
D and V	24	2 (8%)	22 (92%)
D, V and AP	2	1 (50%)	1 (50%)
D and R	1	1 (50%)	1 (50%)
Others	68	0	68 (100%)

Key: D=diarrhea, V=Vomiting, AP=Abdominal pain, RI=Respiratory infection, Others include mixed symptoms.

Seasonal distribution of AGE and Astrovirus prevalence

The overall distribution of gastroenteritis among children below five years in rural communities of Vhembe District was evaluated seasonally using a bar graph (**Figure 4.3**). Data revealed the occurrence of gastroenteritis throughout the year covering all seasons. The peak season was winter with 4/10 (40%) positive samples.
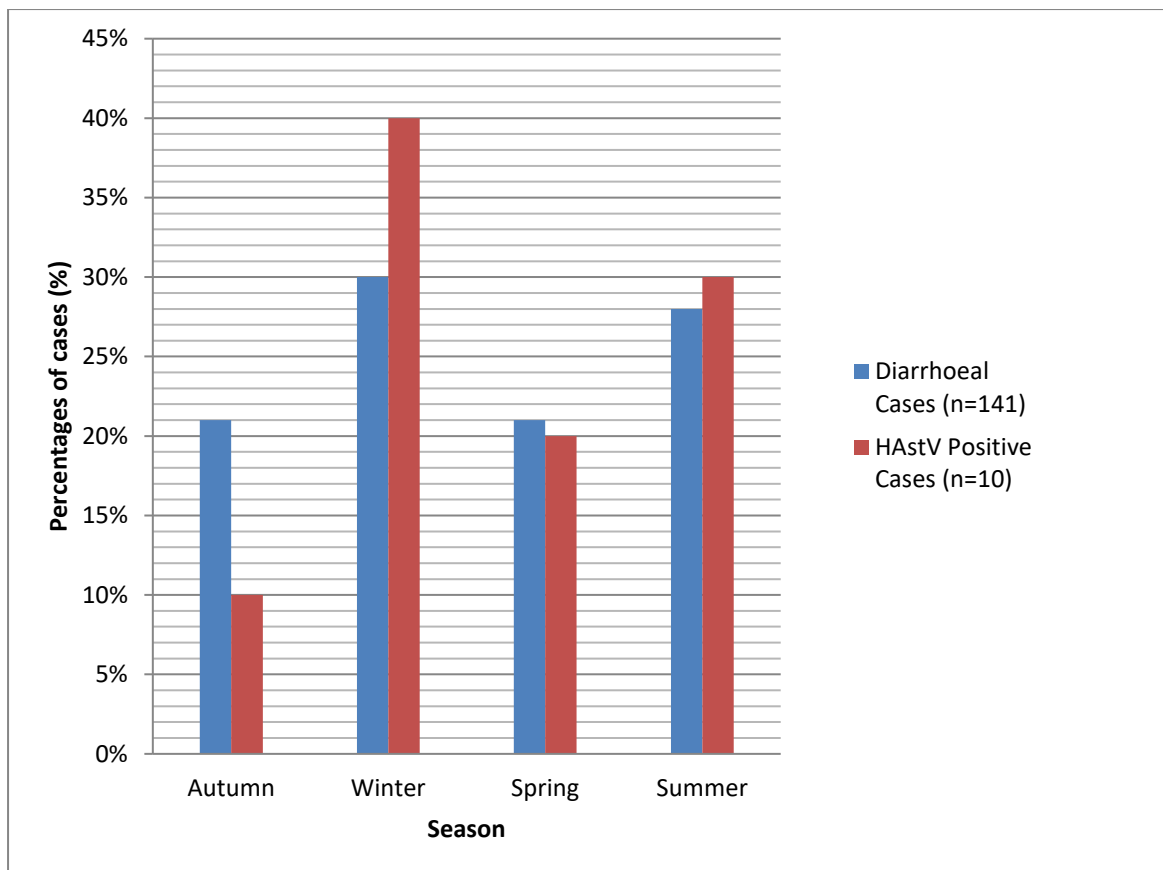
Figure 4.3: Seasonal distribution of gastroenteritis and Astrovirus prevalence (in %) among 141 children below five years of age in the Vhembe District of Limpopo, South Africa between 2017 and 2018. The letter n stands for the total number of samples collected per season.

Living conditions of study participants from Vhembe District; Limpopo Province, South Africa

Additional data was obtained using a questionnaire to have an idea of the study participants' living conditions in the rural communities of Vhembe District, Limpopo South Africa. A total of 118/141 (84%) participants were from households that still used pit toilets. Other participants use rivers as their main source of water. Also, 132/141 (94%) children were coming from unemployed families. **Table 4.3** summarizes all data captured during questionnaire interviews.

Table 4.3: Living conditions of study participants under the age of five living in rural communities of Vhembe District, Limpopo, South Africa.

Variable	Diarrheal stool samples (n)	Astrovirus positive n (%)	Astrovirus negative n (%)
TOTAL	141	10 (7%)	131 (93%)
LIVING			
CONDITIONS			
WATER SOURCE			
Tap	53	6 (11%)	47 (89%)
Borehole	7	2 (29%)	6 (71%)
Spring/Well	5	1 (20%)	4 (80%)
River	1	0	1 (100%)
Unknown	1	0	1 (100%)
TYPE OF TOILET			
USED			
Pit latrine	118	10 (8%)	108 (92%)
VIP latrine	18	0	18 (100%)
Flush toilet	4	0	4 (100%)
BREASTFEEDING			
STATUS			
Yes	90	5 (6%)	85 (94%)
No	44	4 (9%)	40 (91%)
Unknown	7	1 (14%)	6 (86%)
PRESENCE OF			
LIVESTOCK			
Yes	34	2 (6%)	32 (94%)
No	107	8 (7%)	99 (93%)
EMPLOYMENT			
STATUS			
Yes	9	0	9 (100%)
No	132	10 (8%)	102 (92%)

4.2 GENOTYPING AND PHYLOGENETIC ANALYSIS OF ASTROVIRUS

4.2.1 AMPLIFICATION AND GENOTYPING RESULTS

The 10 RNA extracts in which HAstV was detected by real-time RT-PCR were amplified using conventional RT-PCR targeting both capsid and polymerase gene fragments. Out of the 10 positive samples, 4 (40%) were successfully amplified and generated the amplicons needed for sequencing. The amplicons subjected to gel electrophoresis display the target bands in **Figure 4.4** and **Figure 4.5**.
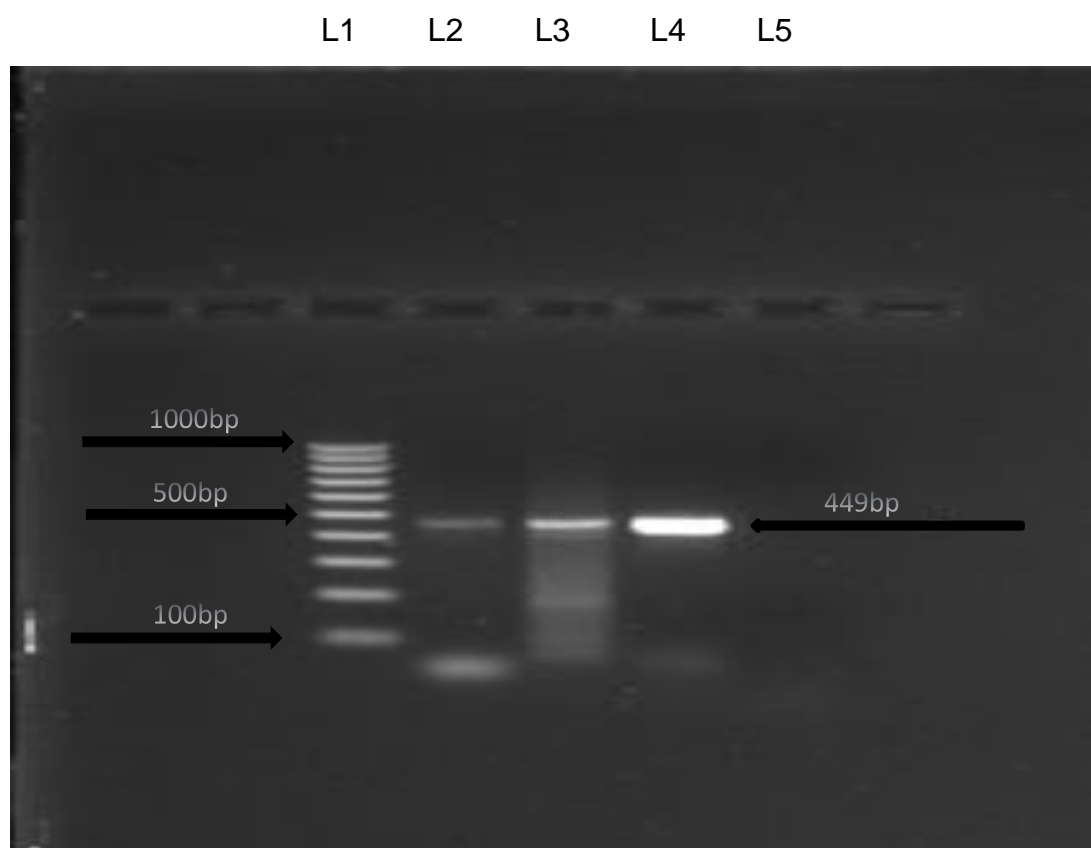
Figure 4.4: Gel electrophoresis results of Astrovirus capsid gene fragment (449bp) PCR products. L1-100bp DNA ladder; L2-sample 4; L3-sample 18; L4-sample 44; L5-Negative Control (NC).
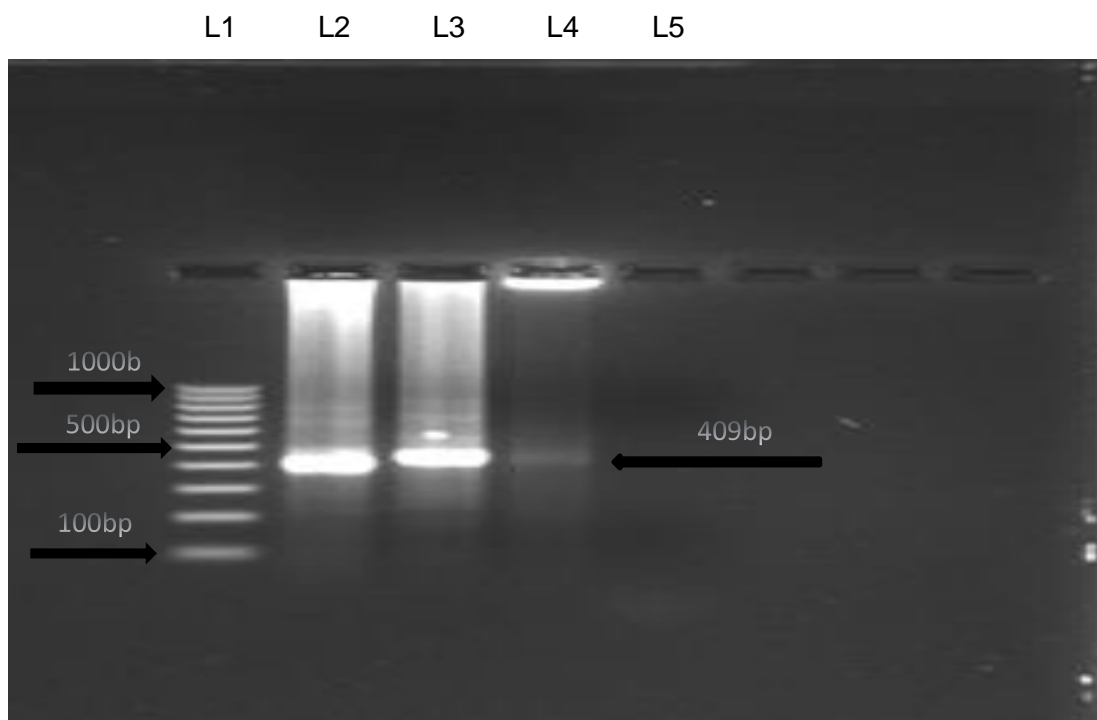
Figure 4.5: Gel electrophoresis results of Astrovirus polymerase gene fragment (409bp) PCR products. L1-100bp DNA ladder; L2-sample 4; L3-sample 23; L4-sample 44; L5-Negative Control (NC).

Only 3 samples were successfully sequenced, on polymerase gene fragment (1) and capsid gene fragment (2). The FASTA format of the sequences obtained were blasted on GenBank repository to get the best hits and identify the primary sequences. The similarity with the selected reference strains ranged between 80-97%. Sequence Blast of the RdRp gene fragment revealed the genotype of Classical Human Astrovirus type 1(HAstV1). The GenBank accession number for this sequence is MN656978. Partial capsid gene fragment Blast resulted in Classical Human Astrovirus 2 in the remaining 2 samples. The accession numbers for these sequences are MT157247 and MT157248.

4.2.2 PHYLOGENETIC ANALYSIS

The phylogenetic tree was constructed to evaluate the genetic relationship of the identified Human Astrovirus strains with other related strains based on the partial nucleotide sequence of RdRp gene fragment (**Figure 4.6**) and partial nucleotide sequence of capsid fragment (**Figure 4.7**). The similarity amongst selected strains ranged from 80-97%. Identified strains were related to others circulating in the Africa.
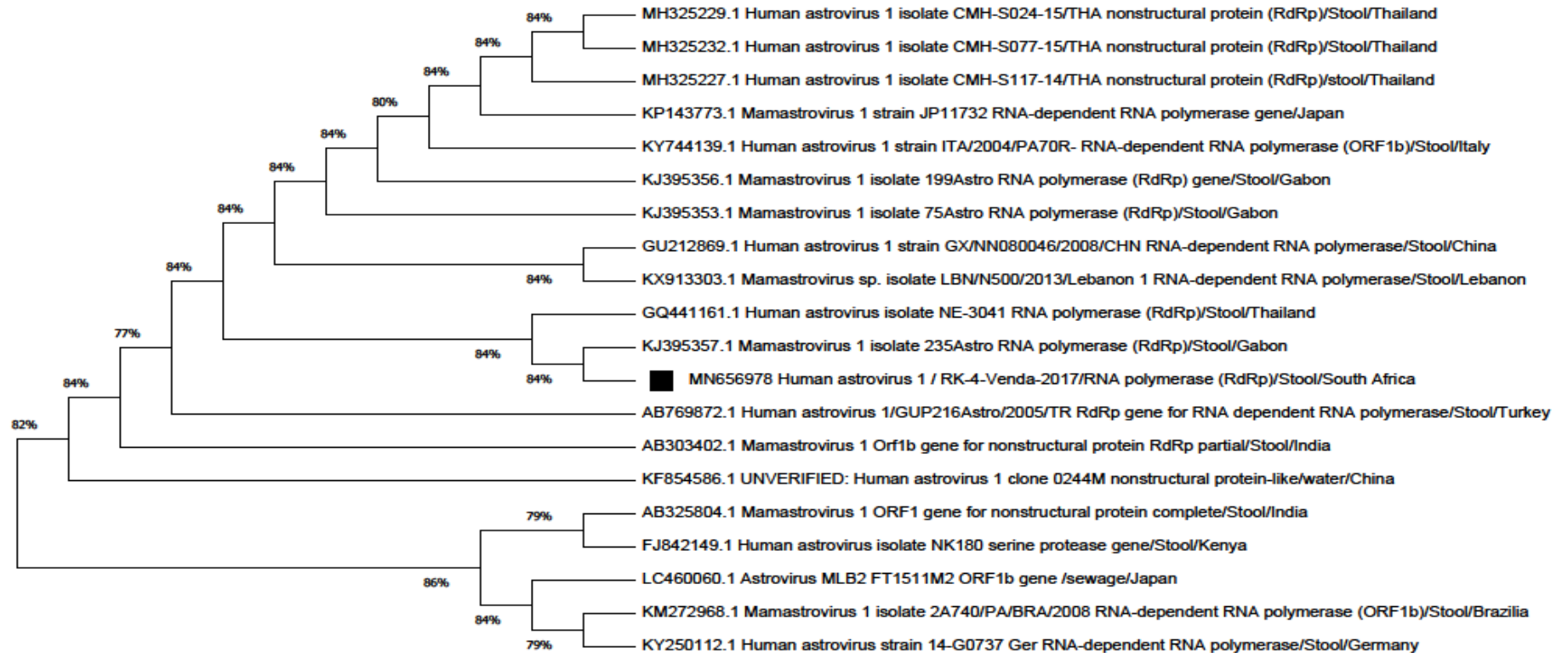
Figure 4.6: Phylogenetic tree based on 409-nucleotide sequence of HAstVs 1 polymerase gene fragment. The Neighbor-Joining tree of HAstV strain circulating between January 2017 and November 2018 in the rural communities of Vhembe district, Limpopo Province, South Africa. The analysis involved 20 nucleotide sequences randomly selected from GenBank with their respective accession numbers. Squared black dot indicates HAstV1 genotyped on this study. All positions containing gaps and missing data were eliminated. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. Evolutionary analyses were conducted in MEGA X (10.0.5) and bootstrap tests (1000 replicates) based on the Kimura two-parameter model.
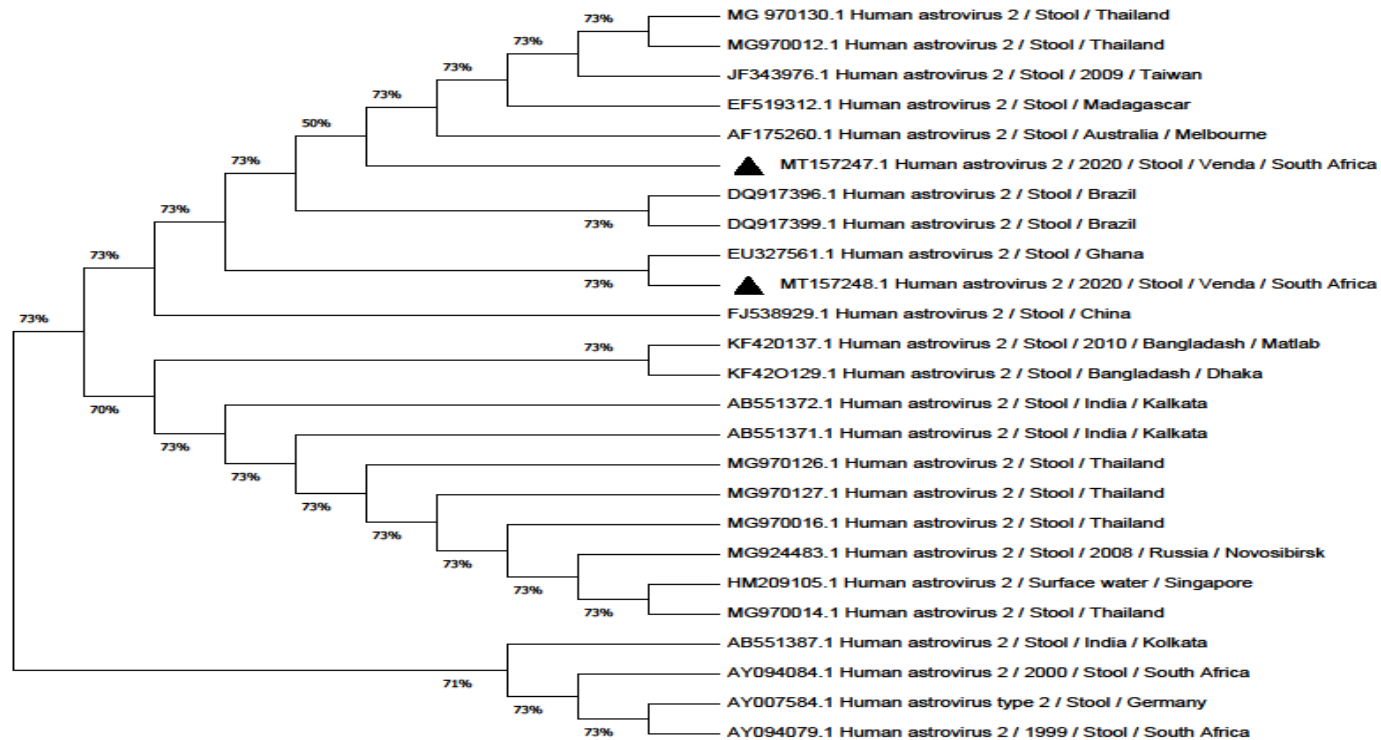
Figure 4.7: Phylogenetic tree based on the 449-nucleotide sequence of HAstVs 2 capsid gene fragment. The Neighbor-Joining tree of HAstV strains which were circulating between January 2017 and November 2018 in the rural communities of Vhembe district, Limpopo province/South Africa. A total of 23 nucleotide sequences randomly selected from GenBank with their respective accession numbers were used to set the tree. Black triangles indicate two HAstV type 2 genotyped in this study. All positions containing gaps and missing data were eliminated. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. Evolutionary analyses were conducted in MEGA X (10.0.5) and bootstrap tests (1000 replicates) based on the Kimura two-parameter model.

Chapter 5

DISCUSSION

Human Astrovirus is one of the important causes of AGE in children under five years of age, with a global distribution (Vu et al., 2017). There is limited data about the prevalence of HAstVs in rural areas in the Vhembe region of Limpopo (Mans et al., 2014). This study aimed to detect and characterize HAstVs in young children of Vhembe District, Limpopo, South Africa. The study results revealed the occurrence of HAstVs in children of this region (**Table 4.1**), Astrovirus type 1 and 2 were identified, and one positive recombinant strain was found. PCR inhibition was monitored by IC control during RNA extraction and real-time PCR run. The Ct value range for positive samples was from 11.18 to 44.69, while the positive control showed a consistent range between 26 and 27.86.

Human Astrovirus prevalence had already been previously reported in other studies done in South Africa in stool samples (Nadan et al., 2019; Olortegui et al., 2018; Sekwadi et al., 2018; Nadan et al., 2003; Steele et al., 1998). The prevalence obtained in this study (7%) is within the common range of Astrovirus prevalence found in other South African studies. Previously, the MAL-ED surveillance study reported 2.7% (0-11 months) and 4.2% (12-24 months) of HAstV cases (Olortegui et al., 2018). According to literature, about 2.9 to 10.3 % of gastroenteritis cases are associated with Astrovirus (Jacobsen et al., 2018; Siqueira et al., 2017). A recent publication in South Africa presented 7% of HAstVs (Nadan et al., 2019) on hospitalized cases only. High prevalence of HAstVs has been reported in children in other countries like Chile and Nigeria (Ayolabi et al., 2012; Gaggero et al., 1998). Studies conducted globally have reported prevalence ranging from 2% to 29% (Arowolo et al., 2019; Vu et al., 2017). Up to 90% of HAstVs infected children in this study were outpatients, this is not surprising since it is known that HAstVs usually causes mild diarrhea (Vu et al., 2017; Bosch et al., 2014).

Human Astrovirus affects mostly children, elderly people and immunocompromised individuals (Kumthip et al., 2018). While adult gastroenteritis due to Astrovirus has been reported, a high number of cases predominantly affects young children (Vu et

al., 2017). The age of HAstV-infected children is variable, ranging from new-born up to five years old (Guix et al., 2002) but infections are more common in children below two years. The study results demonstrated a similar trend; children in the lowest age group (0-24 months) were the most infected group (**Table 4.1**). Our results are consistent with the MAL-ED surveillance findings (Olortegui et al., 2018). Although these results are consistent with our results, EIA based methods were used contrary to the PCR method used in our study. Variations in the number of Astrovirus detected in different countries may be due to differences methods used. For example, 50 extra Astrovirus positive samples were identified using RT-PCR in a study done in Spain (Dalton et al., 2002). The same additional samples were negative for Astrovirus when using EIA suggesting that the EIA could lead to the underestimation of HAstVs rates (Zaraket et al., 2017; Dalton et al., 2002). The real time PCR is known for its higher sensitivity, even when compared to conventional PCR (Malik et al., 2019; Perot et al., 2017).

Other factors such as low viral load may play a role in the detection of HAstVs. Although HAstVs were detected in 10 RNA extracts using real-time RT-PCR, about 50% of these extracts had high Ct value (low viral load). RNA degradation may have occurred due to load-shedding of electricity, causing unstable storage conditions in the refrigerators, consequently leading to low viral load (Vinjé et al., 2004). Since archived RNA were used in this study, the time interval between storage of RNA extracts and amplification could also have influenced the amplification results (Vinjé et al., 2004). Only 4 samples were successfully amplified using conventional RT-PCR out of 10 that were detected by real-time RT-PCR. This could be explained by the difference in the target region, RIDAGENE real-time RT-PCR targets the ORF1/ORF2 junction whereas conventional PCR uses primers targeting ORF1a, ORF1b and ORF2 (Kanwar et al., 2018).

A high number of diarrhea cases was observed during the winter and summer seasons. Geographical location may play a role in the controversial seasonality of HAstVs infection. It is dry during winter in the study area and rainy in summer. The trend of HAstV prevalence in Vhembe District correlates with other findings that observed peaks during cold winter seasons and hot summer months (Jacobsen et al.,

2018; Kumthip et al., 2018; Malasao et al., 2012). Human Astrovirus occurred throughout the year.

Data showing the poor living conditions of study participants (**Table 4.3**) suggest a lack of good hygiene practices possibly facilitating the spread of enteric viral pathogens. In 2016, a high exposure to Norovirus was reported in the same study area (Kabue et al. 2016). About 90% of the global gastroenteritis burden affecting the pediatric population is estimated to be caused by the consumption of unsafe water and poor hygiene and sanitation (WHO, 2017). Astrovirus diarrheal infection is essentially transmitted by the fecal-oral route, contaminated foods and water and is quickly spread by human-to-human contact as well as through fomites and airborne droplets (Bosch et al., 2014; Estes and Greenberg, 2013).

Some of the babies infected by HAstVs in this study were still being breastfed; such close contact may have played a role in viral transmission (Estes and Greenberg, 2013), since adults (mothers of the babies) can also be infected by HAstVs (De Benedictis et al., 2011). Several outbreaks have been associated with the consumption of contaminated foods, poor hygiene and improper food handling especially in less-developed settings (Sekwadi et al., 2018; Koopmans et al., 2008; Todd et al., 2007). In this study, 30% of infected children came from families dependent on river and borehole water. Although water quality was not tested in the current study, this may be associated with HAstVs infection. Dongdem et al. (2011), detected Human Astrovirus in tap water. Most positive samples came from households using tap water, we do not directly implicate the association of tap water with infection. However, the findings of this study do not directly implicate the association of tap water with infection.

The present study detected and characterized HAstV type 1, which is known as the predominantly distributed strain worldwide. Human Astrovirus type 1 was the first genotype to be described in the stool of a child suffering from diarrhea in 1975 (Madeley and Cosgrove). Years following its discovery, eight genotypes were described as HAstVs 1-8 (Bosch et al., 2014). HAstV 1 is still the most prevalent strain reported worldwide (Arowolo et al., 2019; Kumthip et al., 2018; Bosch et al., 2014). The phylogenetic tree (**Figure 4.6**) revealed that this strain is closely related to other

strains circulating in Africa (Gabon, Lebanon, and Kenya) (Zaraket et al., 2017; Lekana-Douki et al., 2015; Wolfaardt et al., 2011). Population movement may facilitate the importation of the strain within the continent.

Human Astrovirus 2 was also detected and characterized in this study. The prevalence of type 2 HAstV is variable in different countries (Medici et al., 2012; Ahmed et al., 2011; Guo et al., 2010) and has reached epidemiological importance in some settings (Gabbay et al., 2007; Dalton et al., 2002). Among the HAstVs genotypes (1-8), 1 to 5 and 8 are commonly detected. Only 6 and 7 genotypes are the most rarely detected (Afrad et al., 2013; Mendez-Toss et al., 2000). The phylogenetic tree (**Figure 4.7**) revealed that Human Astrovirus type 2 strains obtained in this study were not closely related to the previously described type 2 strains in South Africa. This suggests a change in nucleotides over time or evolution. The strain is however related to those circulating in Ghana (Silva et al., 2008) and Asian countries, implying the role of population movement in the distribution of related strains.

Interestingly, HAstV 2 (capsid genotype) was identified in the same sample that was positive for HAstV 1 (RdRp genotype) suggesting the possible occurrence of a recombination event. Putative recombination sites within ORF1b and ORF2 have been reported previously (De Grazia et al., 2012). It is common for ssRNA viruses to exchange genome fragments in highly conserved regions increasing the prevalence of the virus, affecting its phylogenetic grouping and vaccine development (Bull et al., 2007). The recombination of strains is an open door to mutations that may mislead future epidemiological investigations.

Chapter 6

CONCLUSION

The importance of Astroviruses as human pathogens has increased with the widespread use of molecular techniques. With the use of real-time RT-PCR, this study confirms the occurrence of Human Astrovirus in young children of Vhembe District, Limpopo, South Africa. This is the first study to characterize Human Astrovirus in the Vhembe District of Limpopo, South Africa looking specifically at pediatric diarrheal stool samples. The prevalence of HAstV shows that this virus may be associated (contributing) to AGE in the study area. The strains circulating in this area are similar to others circulating in Africa and other continents. These findings highlight the need to consider HAstVs as important pathogens when developing preventive strategies against diarrhea. Sequences revealing the genotypes characterized in this study have been submitted to the GenBank, this information will help in future epidemiologic studies and potential vaccine development.

STUDY LIMITATIONS

-The study covered a small samples size collected in a specific year. Generally, people are reluctant to give consent to giving bodily fluid/stool samples due to their cultural beliefs.

-Low viral load in most samples that tested positive for Human Astrovirus on real-time but could not be amplified on conventional PCR to have an amplicon that will generate a sequence have hindered the knowledge of strains that are circulating in this area.

-Only partial genomic sequences were analyzed in this study. Full genome sequence analysis provides more data on the viral evolutionary information.

RECOMMENDATIONS

The following recommendation are made based on the findings of the current study:

- A systematic surveillance to monitor the circulating Astrovirus strains in the study area.

-Good hygiene practice, provision of quality water and toilets can minimize AGE cases in the area.

-Astrovirus should be considered as a potential candidate for vaccine development in the near future.

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APPENDIX 1

LIMPOPO
PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA

Enquiries: Latif Shamila (015 293 6650)   Ref:4/2/2

Potgieter N
University of Venda
Private Bag X5050
Thohoyandou
0950

Greetings,

RE: Epidemiological and Economical Implications of Diarrhea in water sources from Rural and Peri-Urban communities in the Limpopo Province, South Africa

The above matter refers.

1. Permission to conduct the above mentioned study is hereby granted.
2. Kindly be informed that:-
   - Research must be loaded on the NHRD site (http://nhrd.hst.org.za) by the researcher.
   - Further arrangement should be made with the targeted institutions, after consultation with the District Executive Manager.
   - In the course of your study there should be no action that disrupts the services.
   - After completion of the study, it is mandatory that the findings should be submitted to the Department to serve as a resource.
   - The researcher should be prepared to assist in the interpretation and implementation of the study recommendation where possible.
   - The above approval is valid for a 3 year period.
   - If the proposal has been amended, a new approval should be sought from the Department of Health.
   - Kindly note, that the Department can withdraw the approval at any time.

Your cooperation will be highly appreciated.

Head of Department   15/06/2016
                                                             Date

18 College Street, Polokwane, 0700, Private Bag x9302, POLOLKWANE, 0700
Tel: (015) 293 6000, Fax: (015) 293 6211/20 Website: http//www.limpopo.gov.za

LIMPOPO
PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF HEALTH
VHEMBE DISTRICT

Ref : 4/2/2
Date : 13.07.2016
Enq : Muvari MME
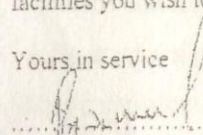
To: Dr Mudau L.S.

Dear SIR/Madam

Re: Request to conduct research on " Epidemiological and economical implications of diarrhea in water sources from rural and peri-urban areas in the Limpopo Province, South Africa".

1. The above matter has reference.
2. The request you submitted to the District for conducting research has been received and well considered.
3. The District has as a consequence no objection to your wish in conducting your research at our facilities.
4. You are however advised to make the necessary arrangements with the facilities you wish to visit.

Yours in service

....
DISTRICT EXECUTIVE MANAGER

Private Bag X5009 THOHOYANDOU 0950
OLD parliamentary Building Tel (015) 962 1000 (Health) (015) 962 4958 (Social Dev) Fax (015) 962 2274 4623
Old Parliamentary Building Tel: (015) 962 1848, (015) 962 1852, (015) 962 1754, (015) 962 1001 2/3/4/5/6 Fax (015)
962 2373, (015) 962 227

The heartland of Southern Africa – development is about people!

APPENDIX 3a

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

Molecular characterization of enteric viruses circulating in the rural communities of Limpopo Province, South Africa.

Investigators:

Miss R Khumela (MSc student)

Prof Natasha Potgieter (Promoter)

Address:

Department of Microbiology

Life Science Building

School of Math and Natural Sciences

University of Venda

Contact number

You and/or your baby is being invited to take part in this research project(s). Please take some time to read the information presented here, which will explain briefly the project(s). Please ask the study staff any questions about any part you do not fully understand. Your participation is **entirely voluntary and you are free to decline to participate**.

This study has been approved by the committee for Human Research at the University of Venda. And will be conducted according to the ethical guidelines and principles of the International Declaration of Helsinki, South African Guidelines for Good Practice and the Medical Research Council (MRC) Ethical Guidelines for research.

What are this research projects study all about?

▪ The studies will include stools from young children under 5 years and older participants with diarrhea, and respiratory samples from participants with respiratory infection. About 500 children will take part in this project.

▪ The project(s) aimed to investigate the diversity of Astrovirus circulating in the rural communities of the Limpopo province.

- This information will help decisions making in public prevention strategies against diarrhea disease and respiratory infection particularly in Astrovirus infections also in the improvement of sanitary environments in rural communities. The findings of this study will also provide information on Astrovirus diversity with implications on vaccine development.

- General information will be taken from you, including contact details, age, gender, use of toilet, date of diarrhea, HIV status and other illnesses, etc. A total of 10g of stools will be collected from the participant and will be transported to the laboratory for analysis.

Why have been invited to participate?

You and/or your baby was selected for this study because of sufferings from diarrhea.

What will your responsibility be?

Participation in this study is completely voluntary. You may refuse to provide information or sample(s).

Will you benefit from taking part in this research project?

No monetary compensation is offered for your participation. But you will be receiving the results of bacteriological and virological analysis if positive.

Are there risks involved in your taking part in this research?

There are no risks involved in participating. Collection of stools will be done after or when the participant is eliminating waste during diarrhea episodes. Respiratory swabs will be collected when the participant is coughing.

Who will have access to your medical records?

Only the medical doctor/nurse and the research team will have access to your medical information. The participant's identity will not be made public and if the results are published or presented, a participant will only be referred to by a code number. The participant's identity will be strictly kept confidential.

Is there anything else that you should know?

You may contact Prof Natasha Potgieter (University of Venda/ Life Science offices) at Tel. 0159628256 if you have any further queries or encounter any problems.

Declaration by participant

By signing below, I …………………………………………. agree to take part in the research study entitled "**Molecular characterization of human Astrovirus circulating in the rural communities of Limpopo province, South Africa**".

I declare that:

➢ I have read or was read to me this information and consent form and it is written in a language with which I am fluent and comfortable.

➢ I have had a chance to ask questions and all my questions have been adequately answered.

➢ I understand that taking part in this study is **voluntary** and I have not been pressurized to take part.

Signed   at   (place)   …………………………………...   on   (date) …………………………….

_____   _____

Signature of Participant   Signature of Witness

Declaration by investigator(s):

I ………………………………………………………… declare that:

✓ I explained the information in this document to the participant.

✓ I encouraged the participant to ask questions and took adequate time to answer them.

✓ I am satisfied that the participant adequately understands all aspects of the research, as discussed above

✓ I did/ did not use an interpreter (If an interpreter is used then the interpreter must sign the declaration below).

Signed   at   (place)   ……………………………   on   (date) …………………………

_____   _____

Signature of Investigator   Signature of Witness

Declaration by interpreter:

I …………………………………………………… declare that:

✓ I assisted the investigator (name) …………………………………… to explain the information in this document to (name of participant) ………………………………… Using the language medium of Venda/Tsonga

✓ We encouraged the participant to ask questions and took adequate time to answer them.

✓ I conveyed a factually correct version of what was related to me.

✓ I am satisfied that the participant fully understands the content of this informed consent document and has all the question satisfactorily answered.

Signed   at   (place)   …………………………………   on   (date) ……………………………

_____   _____

Signature of interpreter   Signature of Witness

APPENDIX 3b

FOMO YA THENDELO NA BAMBIRI ḼA ZWIDODOMBEDZWA ZWA MUDZHENELELI

ṰHOHO YA THANDELA YA ṰHOḒISISO:

THODISISO YA MOLUKHULI DZA SAPOVIRUS DZINE DZA KHOU MONOLODZA VHUPONI HA MAHAYANI KHA VUNDU LA LIMPOPO, AFURIKA TSHIPEMBE.

Nomboro ya Referentsi:

Vhasedzulusi: Miss R Khumela (Mutshudeni wa MSc)
Prof. Natasha Potgieter (Muṱoli)

ḒIRESI: Department of Microbiology
Life Science Building
School of Math and Natural Sciences
University of Venda
Nomboro ya lutingo:

Ṅwana wavho kana vhone mubebi vha khou humbelwa u dzhenelela kha u vha tshipiḓa kha thandela hei ya ṱhoḓisiso. Vha khou humbelwa uri vha dzhie tshifhinga vha vhale mafhungo o ṋetshedzwaho hafha, ane a ḓo ṱalutshedza nga u pfufhifhadza thandela hei. Kha vha vhudzise vhashumi vha ngudo idzi mbudziso dziṅwe na dziṅwe nga ha tshipiḓa tshine vha sa khou tshi pfesesa zwavhuḓi. U dzhenelela havho **ndi ha u tou funa nahone vha a tendelwa u hana arali vha sa funi u dzhenelela.**

Ngudo hedzi dzo tendelwa nga komiti ya Human Research Yunivesity ya Venḓa nahone i ḓo itwa ho sedzwa maitele na milayo ino tea u tevhedzwa nga vha International Declaration of Helsinki, na nga maitele a mashumele avhuḓi a Afrika Tshipembe na maitele a ṱhoḓisiso ano fanela u tevhedzwa a Medical Research Council (MRC).

Thandela iyi ya ṱhoḓisiso ndi ya mini?

- Ngudo hei i ḓo katela mafhambuwa a vhana vha minwaha ire fhasi ha miṅwaha miṱanu vhane vha khou dinwa nga u tshuluwa na u hotola. Hu do dovha ha todiwa sambula na kha vhahulwane vha vhukale hono bva kha minwaha mitanu. Vhana vhafhasi ha minwaha mitanu vha 500.

- Ṱhoḓisiso hei yo livhiswa kha u ṱoḓulusa tshakha dza virasi dza Astrovirus dzi no khou monolodza vhuponi ha mahayani kha Vundu ḽa Limpopo.

- Mafhungo haya a ḓo thusa hu tshi dzhiwa tsheo kha maitele a tshitshavha a u thivhela phiriselo ya vhulwadze ha u tshuluwa nga maanḓa kha u kavhiwa nga virasi ingaho Astrovirus nga u khwinifhadza fhethu hune ha vha na mabunga a kha zwitshavha zwa mahayani.

- Mawanwa a ngudo iyi a ḓo ṋetshedza mafhungo nga ha u phaḓalala ha virasi ya Astrovirus ho sedzwa kha u bveledza dzilafho lo livhanywaho na utshuluwa ha vhana.

- Mafhungo oṱhe ane a khou ṱoḓea a ḓo waniwa kha vhone, zwi tshi katela na nḓila ine ra nga vha kwama ngayo, vhukale, mbeu, ḓuvha ḽa u tshuluwa, tshiimo tshavho tsha HIV na maṅwe malwadze. Mafhambuwa a linganaho 10g, a ḓo dzhiwa kha ṅwana wavho mutuku kana muhulwane, zwenezwo zwi ḓo iswa laborothari u senguluswa.

Ndi ngani vhone vho humbelwa u dzhenelela?

Ṅwana wavho mutuku na muhulwane kana vhone vho nangiwa kha ngudo iyi ho sedzwa u tshuluwa na u hotola hune ha khou dina tshitshavha.

Vhone vha fanela u ita zwifhio?

U dzhenelela kha ngudo iyi ndi zwa u tou funa nga iwe muṋe. Vha nga hana u ṋekedza mafhungo kana mafhambuwa.

Vha ḓo vhuelwa nga u vha tshipiḓa tsha thandela iyi ya ṱhoḓisiso?

A huna ndiliso ya tshelede ine vha ḓo ṋewa nga u dzhenelela havho. Fhedzi zwa konadzea vha ḓo wana mvelelo dza tsenguluso ya zwitshili zwo waniwaho.

Hu na khombo dzine vha nga dzi wana nga u vha tshipida kha ṱhoḓisiso iyi?

A huna khombo dzine vha nga dzi wana nga u dzhenelela havho. U kuvhanganya mafhambuwa zwi ḓo itwa nga murahu ha musi ṅwana kana uyo o dzhenelelaho a tshi khou bvisa malaṱwa nga tshifhinga tsha u tshuluwa.

Ndi nnyi ane a ḓo kona u swikelela rekhodo yavho ya dzilafho?

Mafhungo avho nga ha dzilafho ḽavho zwi ḓo kona u swikelelwa fhedzi nga dokotela, nese na tshigwada tsha ṱhoḓisiso.

Madzina avho ha nga anḓadzwi tshitshavhani nahone arali mawanwa a ngudo iyi a tshi khou tea u anḓadzwa kana u ṋetshedzwa, mafhungo avho a ḓo vha o ṱalulwa nga nomboro kana khoudu. Madzina avho a ḓo vhulungwa lwa tshiphiri.

Arali hu na zwiṅwe-vho zwine vha ṱoḓa u zwi ḓivha kana u zwi ita?

Vha nga kwamana na Prof. Natasha Potgieter (Department of Microbiology / University of Venḓa) Luṱingo: 015 962 8256 arali vha na dziṅwe mbudziso dzine vha vha nadzo kana musi vha tshi khou ṱangana na thaidzo.

Muano nga mudzheneleli:

Nga u saina hafha fhasi, Nṋe …………………………………. ndo tenda u dzhenelela kha ngudo ya ṱhoḓisiso ya ṱhoho heyi: **"Thodisiso ya molukhuli dza Astrovirus dzine dza khou monolodza vhuponi ha mahayani kha vundu la Limpopo, Afurika Tshipembe".**

Ndi khou bula zwauri:

- ✓ Ndo vhala kana ndo vhalelwa mafhungo haya na fomo ya thendelo yo ṅwalwa nga luambo lune nda luamba na u lu pfa zwavhuḓi.
- ✓ Ndo vha na tshifhinga tsha u vhudzisa dzimbudziso nahone mbudziso dzanga dzoṱhe dzo fhindulwa zwavhuḓi.
- ✓ Ndo zwi pfesesa uri u dzhenelela kha ngudo iyi ndi **u tou funa iwe muṋe** nahone

  a tho ngo tou kombetshedzwa u vha tshipiḓa kha ngudo hei.

Tsaino yo itwa (Fhethu)…………………………… nga ḽa (datumu)………………..20….

_____

_____

Tsaino ya mudzheneleli   **Tsaino ya ṱhanzi**

Muano nga musedzulusi:

Nṋe (dzina) …………………………………………………………………. ndi khou
ana uri:

- ✓ Ndo ṱalutshedza mafhungo a re kha liṅwalo heḽi.
- ✓ Ndo vha ṱuṱuwedza uri vha vhudzise mbudziso, nahone nda dzhia tshifhinga tsho teaho u dzi fhindula.
- ✓ Ndo fushea ngauri vho pfesesa zwipiḓa zwoṱhe zwa ṱhoḓisiso sa zwe zwa ṱalutshedzwa afho nṱha.
- ✓ Ndo shumisa / kana a thongo shumisa muḓologi. (Arali muḓologi o shumiswa, muḓologi u fanela u saina muano u re afho fhasi.

Tsaino   yo   itwa
(fhethu)………………………………………………………………….   nga   ḽa
(datumu)…………………………………20….

_____

_____

Tsaino ya musedzulusi   **Tsaino ya ṱhanzi**

Muano nga muḓologi:

Nṋe(dzina)……………………………………………………………………ndi khou ana uri:

- ✓ Ndo   thusedza   musedzulusi
  (dzina)…………………………………………………u ṱalutshedza mafhungo
  are   kha   liṅwalo   heḽi   kha   (dzina   ḽa
  mudzheneleli)……………………………………….………hu   tshi
  khou shumiswa luambo lwa Tshivenḓa.
- ✓ Ro muṱuṱuwedza u vhudzisa dzimbudziso ra dzhia tshifhinga tsho teaho u dzi fhindula.
- ✓ Ndo ṋekedza mafhungo one one o teaho a sina tshiṱahe kha zwe zwa vha zwi tshi khou elana na nṋe

✓ Ndo fushea ngauri mudzheneleli o pfesesa nga vhuḓalo zwine zwa vha kha liṅwalo ḽa thendelo ḽo tevhelaho maga a mulayo o ṋetshedzwaho naho ne mbudziso dzawe dzoṱhe dzo fhindulwa lu fushaho

Tsaino yo itwa (fhethu)…………………………………. nga ḽa (datumu)…...……..20….

_____

_____

Tsaino ya Muḓologi   **Tsaino ya ṱhanzi**

APPENDIX 4a

DEPARTMENT OF MICROBIOLOGY, SCHOOL OF MATHEMATICAL AND NATURAL SCIENCES, UNIVERSITY OF VENDA

Research project data capture form: **Symptomatic patient**

Subject Number………………………

Consultation details		
Date:	Visit Number:	Hospital/Clinic name:

Patient information			
Name _____	Date of birth _____	Gender M ☐ F ☐	Contact details: _____

Parental status: Unemployed ☐   Employed ☐   Self-employed ☐

Family condition

Water source: Tap ☐   Spring/wells ☐   Boreholes ☐   River ☐

Sanitation: VIP/Pit latrine ☐   Flush toilet ☐

Other: Livestock ☐   Breastfeeding ☐

Medical History

Clinical symptoms: Diarrhea ☐   Fever ☐   Vomiting ☐   Dehydration ☐

Respiratory tract infection ☐   Immunodeficiency ☐   Dehydration ☐

Abdominal pain/cramps ☐

Date of Onset: _____   **Rota Vaccine dose received** ☐

How many days of presenting with diarrhea before consulting?

Sample collection

Date of collection: _____

Type of sample: Type of Stool: Watery ☐   Sausage ☐   Mushy ☐

Respiratory Swab: Nasal ☐   Throat ☐

Treatment

Current:

Previous:

Laboratory Results

PCR:

Sequencing:

APPENDIX 4b
DEPARTMENT OF MICROBIOLOGY, SCHOOL OF MATHEMATICAL AND NATURAL SCIENCES, UNIVERSITY OF VENDA

Research project data capture form: **Asymptomatic patient**

Subject Number………………………

Consultation details		
Date:	Visit Number:	Hospital/Clinic name:

Patient information		
Name _____	Date of birth _____	Gender: M ☐ F ☐ Contact details _____

Parental status: Unemployed ☐   Employed ☐   Self-employed ☐

Family condition

    Water source: Tap ☐   Spring/wells ☐   Boreholes ☐   River ☐

    Sanitation: VIP/Pit latrine ☐   Flush toilet ☐

    Other   Livestock ☐   Breastfeeding ☐

Medical History

No Clinical symptoms

    Rota Vaccine dose received ☐

Sample collection

    Date of collection: _____

    Type of sample: Type of stool: Soft ☐   Sausage ☐   Mushy ☐

    Respiratory Swab: Nasal ☐   Throat ☐

Treatment
Current:
Previous:

Laboratory Results
PCR:
Sequencing:

(BOOM METHOD FOR NUCLEIC ACID EXTRACTION)

Pea-sized amount faeces in 500 µl PBS (or less if small volume)

Centrifuge for 15 sec @ 12,000 rpm

Transfer supernatant in 1.5 ml sterile micro-tube + 900 µl L6 buffer

Vortex for 1 min and centrifuge for 15 sec @ 12,000 rpm

Transfer the supernatant to a new 1.5 ml tube

Add extraction matrix (100 µl Silica beads)

{note: Vortex the extraction matrix thoroughly before use}

Vortex the solution for 15 sec and shake softly for 15 min (or on Rocking platform)

Centrifuge the tube @ 2,000 rpm for 15 sec and discard the supernatant

Add 500 µl of L2 buffer and mix

Centrifuge @ 2,000 rpm for 15 sec and discard the supernatant (X2)

Add 500 µl of 70% Ethanol and mix

Centrifuge @ 2,000 rpm for 15 sec and discard the supernatant (X2)

Add 500 µl of Acetone and mix

Centrifuge @ 2,000 rpm for 15 sec and discard the supernatant (X1)

Dry the Silica pellet by placing the opened tube in a heat block @ 50 °C for 5 min

Re-suspend the Nucleic acid with 150µl PCR grade water, Mix well & heat@56°C for 5 min

Centrifuge @ Maximum speed for 20 min

Change the glove and collect carefully 100 µl of supernatant

(avoid collecting the silica pellet)

Store the extracted Nucleic acid @ -80°C (or -20°C).