MOLECULAR CHARACTERIZATION OF HUMAN SAPOVIRUSES CIRCULATING IN THE RURAL COMMUNITIES OF LIMPOPO PROVINCE, SOUTH AFRICA

by

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DECLARATION

I, Mpho Magwalivha (student number 11500730), declare that I am the author of this thesis and hereby submit to the Department of Microbiology and Biochemistry, University of Venda. This work was not copied or repeated from any other studies either from national and international papers. I certify that, to the best of my knowledge, my work will not infringe upon anyone's copyright and that any ideas, techniques, quotations, or any other material from the work of other people included in this thesis, published or otherwise, are fully acknowledged.

05 September 2022

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Magwalivha Mpho Date

DEDICATION

I dedicate this thesis in motivating my family, relatives, and you reading this document, to never lose the FOCUS....

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- ✓ I give thanks to God for wisdom and strength that helped me through-out my study.
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ABSTRACT

Background: Viral diarrhoea is a common cause of mortality among children less than five years of age in developing countries. Sapovirus (SV), one of the enteric viruses has been reported to be associated with viral diarrhoea worldwide. Reported studies on SVs in selected provinces of South Africa (SA) have been published based on the patients admitted in the hospitals located in the urban areas. There is a need for continuous epidemiological studies of SVs from the rural based regions within SA, especially from outpatients reporting in rural health care centers.

Objective: To determine the genotypes, characterize, and analyze a capsid protein of the detected human Sapovirus strains associated with diarrhoea in children less than 5 years of age from rural communities in SA, and also compare the detected SV strains in this study with other strains reported elsewhere around the world.

Method: A review article on the prevalence of human SV in developing countries was compiled to support the rationale of this study. To find the SV genotypes, characterizing, and comparing them with previously reported SV strains, an investigation on the "Prevalence and genetic characterisation of human sapovirus from children in the rural areas of Vhembe district" was conducted. A total of 284 stool samples were collected from children under 5 years of age suffering with diarrhoea (n=228) and without diarrhoea (n=56). Samples were screened for SV using real-time PCR. Sapovirus positive samples were further analysed for genogrouping by a One-Step Ahead RT-

PCR, and SV Strains were genotyped using Sanger sequencing. A polyprotein (partial capsid protein) was successfully amplified using One-Step RT-PCR from 25% (10/40) positive samples, and further sequenced using Sanger method.

Results: From a review report, 6.5% prevalence rate for SV in the low and middle income countries was determined, with significance difference of SV prevalent rate seen between low income and middle income countries. This study reports 14.1% (40/284) SV detection from stool samples [16.7% (38/228) of diarrhoeal and 3.6% (2/56) of non-diarrhoeal samples]. Genogroup-I was found as the most prevalent strain comprising 68.75% (11/16), followed by SV-GII 18.75% (3/16), and SV-GIV 6.25% (1/16), with GI and GII detected in 6.25% (1/16) of the sample. Significant correlation between SV positive cases and water sources was noted (Chapter 4). A partial VP1 was successfully sequenced from 10/16 amplicons, and results showed genotype GI.1 to be the most prevalent (60%; 6/10), followed by 20% of SV-GII.1, and 10% of each SV-GI.3 and SV-GII.3 (Chapter 5). The relatedness of strains detected from non-human host with the detected strains from this study was noted with a concern (Chapters 4 and 5).

Conclusion: The presence of SV, and substantial evidence of SV associated with diarrhoeal disease in low income regions was determined. This study defined human SV strains in rural communities from Vhembe district, and therefore outpatients in rural settings are possibly at a risk of the burden of diarrhoeal disease triggered by enteric-viruses among other pathogens. However, reports on SV as an emerging diarrhoeal causative agents in the developing regions are limited. Investigations on the analysis and surveillance of human SV strains in rural settings (at a community or household level) is essential to assess burden of diseases.

Keywords: Children, Diarrhoea, Human Sapovirus, Outpatients, Rural communities.

LIST OF ABBREVIATIONS

AGE	Acute gastroenteritis
bp	Base pairs
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscope
Kb	kilobyte
min	Minute
mg	Milligram
ml	Millilitre
N	Number
nm	nano-meter
NoV	Norovirus
NS	Non-structural protein
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RdRp	Protease-RNA-dependent-RNA-polymerase
RNA	Ribonucleic acid
rpm	revolutions per minute
RT-PCR	real-time polymerase chain reaction
SA	South Africa
SV	Sapovirus
sec	Second
WHO	World Health Organisation

µl	Microlitre
<	Less than
>	Greater than
~	Approximately
ºC	Degree Celsius
%	Percentage
α	Alpha
β	Beta

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CHAPTER 1

GENERAL INTRODUCTION

1.1 BACKGROUND

Although diarrhoeal disease can be preventable and treatable, an estimation of 1.7 billion cases of childhood diarrhoeal disease were reported yearly around the world (WHO, 2017). In addition, this disease is labelled as the second leading cause of death in children under the age of five years old (WHO, 2017). However, Acute gastroenteritis (AGE) is known to be one of the major causes of death in children under the age of five years worldwide (Sisay et al., 2016), previously associated with fatality of estimated 1.87 million cases in developing countries (Boschi-Pinto et al., 2008). Around the year 2013, 6.3 million fatality cases of young children less than 5 years were reported worldwide (Liu et al., 2015). Global deaths from diarrhoeal disease remain a major health concern particularly in Africa, though it has been reported to be decreased from 2.6 million to 1.3 million in the past years (Shioda et al., 2016).

Diarrhoea is one of the clinical symptoms resulting from an infection in the host's intestinal tract, of which the causative agents might include pathogens like viruses, bacteria or parasites (WHO, 2017). Among other diarrhoeal-causative viruses, Sapovirus (SV) is one of the enteric-viruses that causes AGE in humans. Epidemiological data from SV gastroenteritis cases, the incubation period of this virus ranges from less than 1 day to 4 days (Oka et al., 2015; Lee et al., 2013; Kobayashi et al., 2012; Iizuka et al., 2010; Yamashita et al., 2010).

Co-infections of SVs and multiple enteric viruses (such as noroviruses (NoVs), rotaviruses (RVs), astroviruses (AstVs), adenoviruses (AdVs), enteroviruses (EVs), kobuviruses (KbVs)) have also been noticed among human AGE outbreaks (Iritani et al., 2014; Iizuka et al., 2010; Rasanen et al., 2010). There is evidence on genetically indistinguishable SVs constructed on partial virus genome sequences detected in human clinical specimens that have also been detected from shellfish (clams and oysters) (Oka et al., 2015; Iizuka et al., 2013; 2010) and environmental water samples (river water and wastewater) (Murray et al., 2013a; Ng et al., 2012; Kitajima et al., 2011; Sano et al., 2011). Sapoviruses are known to show a high level of diversity, thus, their prevalence has also been reported from multiple mammalian species including: chimpanzees, dogs, mink, pigs, rats, and sea lions (Oka et al., 2016).

1.2 STUDY RATIONALE

The associated effects of human waste disposal, water quality and health status, and diseases especially transmitted through faecal-oral route, are well predictable. Hence, the survival and development of children is dependent on clean water, basic toilets, and good hygiene practices. Diarrhoeal disease is known as an important cause of morbidity and mortality in low- and middle income countries and reported to be the third most frequent cause of death and greatest contributor to the burden of disease in children younger than 5 years of age (Govender et al., 2011). Additionally, due to infection, diarrhoea disease is widespread throughout the developing countries (GBD, 2018; WHO, 2017). The enteric pathogens are presumed to be transmitted at household level through a complex set of interdependent pathways such as: contaminated food and water, poor waste disposal that contaminates living spaces, and complex household and community person-to-person

interactions (Govender et al., 2011; Eisenberg et al., 2007), which is a customary routine among most rural communities or disadvantaged households.

Within the Caliciviridae family, NoV has been the most targeted genus whilst SV has been overshadowed (Lee et al., 2012). Sapovirus infections have been identified as a public health problem, and their cause of AGE in people of all ages in both outbreaks and sporadic cases worldwide, and outbreaks occurring in the semi-closed settings have been reported (Oka et al., 2015). Sapoviruses have been identified as possible viruses of human faecal origin discharged into environmental waters, and accumulated in shellfish (including clams or oysters) (Oka et al., 2015). Of importance, it is indicated that sudden risks of illness are likely to be caused by emerging and re-emerging viruses, with the reference to a newly widespread distribution of the emerging SV-GII.8 (Xue et al., 2019). The detection of SVs has been noted worldwide with few reports in South Africa (SA) giving the baseline data on the prevalence of SV (Murray et al., 2016; Page et al., 2016; Murray and Taylor, 2015; Mans et al., 2014; Murray et al., 2013b), thus suggesting a limitation on the genetic information of the SVs distribution within the rural-based communities of SA.

1.3 PROBLEM STATEMENT

Sapoviruses, like other enteric viruses, play an important role in the burden of disease around the globe (GBD, 2018). Although there are reports on the prevalence of SV in SA (Murray et al., 2016; Murray and Taylor, 2015; Murray et al., 2013b), such studies focused on selected provinces within the country, and therefore there is a need to investigate more on the prevalence and molecular diversity of SV in the secluded rural communities that are struggling with poor water infrastructure and sanitation conditions. To date, the detection of

SVs from human stool samples in SA has been mostly reported on hospitalised patients, which could be different from sporadic infections in out-patients-children living in the poor rural communities. There is a need to fill the gap that exists in studies done on the circulation of SVs in the rural areas, which can also address the issue of sanitation and hygiene practices.

Research question:

Can conditions in rural settings influence the genetic characterization and transmission of circulating strains of SV in rural communities of the Limpopo Province, South Africa?

1.4 AIM AND OBJECTIVES OF THE STUDY

1.4.1. Aim

To determine the genetic characterization of human Sapovirus strains circulating in the rural communities of the Vhembe region (Limpopo Province).

1.4.2. Objectives

- ✓ To determine the genotypes, and characterize the human Sapovirus strains associated with diarrhoea in children less than 5 years of age from rural communities in SA;
- ✓ To compare the detected SV strains circulating with the reference strains reported elsewhere in SA and developing countries and
- ✓ To analyse the capsid protein (VP1) of the detected SVs in this study.

Outline of the Objectives

➢ Objectives 1 and 2, were incorporated and written as a manuscript for peer-reviewed publication. The Research article, “Prevalence and Genetic Characterisation of Human Sapovirus from Children with Diarrhoea in the Rural Areas of Vhembe District, South Africa, 2017-2020”, was published by Viruses (MDPI Journals), March 2021.

- Magwalivha Mpho - Performed the laboratory analysis and drafting of the manuscript; Kabue Jean-Pierre - Revised the manuscript; Traore A. Ndama - performed statistical analysis and revised the manuscript; Potgieter Natasha - conceived the idea and revised the manuscript.

➢ Objective 3 has been written as a manuscript for peer-reviewed publication. The Research article, “Partial analysis of the capsid protein (VP1) of Human Sapovirus isolated from children with diarrhoea in rural communities of South Africa”, was published by Advances in Virology (Hindawi Journals), June 2022.

- Magwalivha Mpho - Performed the laboratory analysis and drafting of the manuscript; Kabue Jean-Pierre - Revised the manuscript; Traore A. Ndama - Revised the manuscript; Potgieter Natasha - Revised the manuscript.

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CHAPTER 2

LITERATURE REVIEW

2.1 HISTORY

Members of the Caliciviridae family are capable of infecting humans and other range of animals, including birds, fishes, reptiles and domestic animals (Oka et al., 2016). This family is currently divided into five genera based on phylogenetic differences, namely: Lagovirus, Nebovirus, Norovirus, Sapovirus, and Vesivirus (Goodfellow and Taube, 2016). As scientist have a practice of naming pathogens by the location of its discovery, in 1972 a viral particle sized 27 nano-meter (nm), was detected and reported as a causative agent of gastroenteritis outbreak in Norwalk (Ohio, USA) hence named Norwalk virus (Kapikian et al., 1972). Likewise, a related viral particle was detected, currently known as Sapovirus, following its discovery in an outbreak of gastroenteritis in a home for infants in Sapporo (Japan) in October 1977 (Chiba et al., 2000; 1979). As a group, Norwalk and Sapporo-like viruses, were then identified as the Norwalk-like viruses or small round structured viruses (SRSVs) (Oka et al., 2015).

Sapoviruses are classified as diarrhoeal-causative agents of AGE in the mammalians and also distinguished as the second major gastroenteritis after NoV in humans (Varela et al., 2019). Human SVs has been associated with human gut infection in all ages, and reported to cause outbreaks and sporadic cases globally. Outbreaks caused by SVs were previously observed in semi-closed settings such as old-aged homes and orphanages, and associated with foodborne transmission (Oka et al., 2015). Diarrheic diseases are one of the leading causes of death in children, with an estimation of 525,000 deaths in children under the age

of five years worldwide (Quintero-Ochoa et al., 2019; Sisay et al., 2016), leading to an estimated 1.87 million fatalities annually in developing countries (Boschi-Pinto et al., 2008).

2.2 STRUCTURAL PROPERTIES

As shown in the Figure 2.1, Sapovirus is a small non-enveloped virus, with a typical cup-shaped indentation on its surface and about 10 spikes on the exterior. This virus is comprised of capsid typically described as 27 - 40 nm in diameter, with T = 3 icosahedral symmetry. In addition, their capsid is composed of 180 capsid proteins.

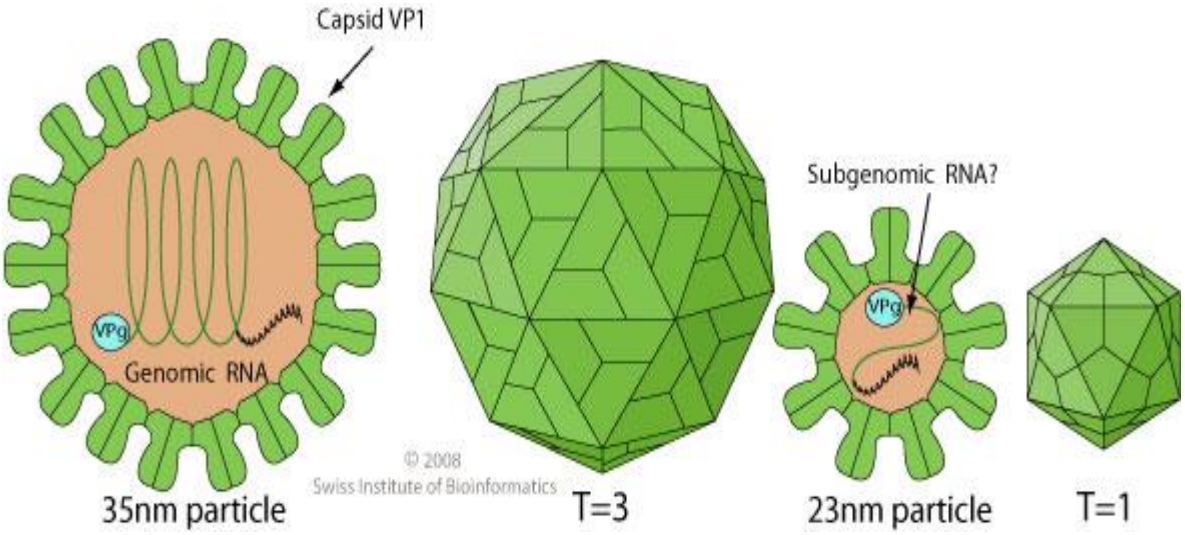

Figure 2.1: This figure shows a virion structure of a Sapovirus (Taken from viralzone.expasy.org/all_by_species/196.html#tab6).

Sapovirus, is a viral particle which has a positive-sense, single-stranded ribonucleic acid (RNA) genome of approximately 7.1 to 7.7 kilobyte (kb) in size with a poly (A) tail at the 3'-end (Oka et al., 2016; Green, 2007; Chang et al., 2005). As shown in Figure 2.2, there are three open reading frames (ORFs), which are however reported differently, with either two

or three reading frames (which are ORF1, ORF2 and ORF3). The ORF1 region is labelled to encode for a large polyprotein containing at least six viral non-structural proteins (NSs) (helicase, protease, VPg [NS5], and fused protease-RNA-dependent-RNA-polymerase [RdRp] [NS6-NS7]), and a major capsid protein, VP1. While ORF2 is proposed to encode for a minor structural protein VP2 (Oka et al*.,* 2016; 2015; Ishida et al*.,* 2008), and ORF3 is proposed for unknown functions in several SV strains, although is identified in some human and bat strains but with unknown function (Yinda et al., 2019; Okada et al., 2006).

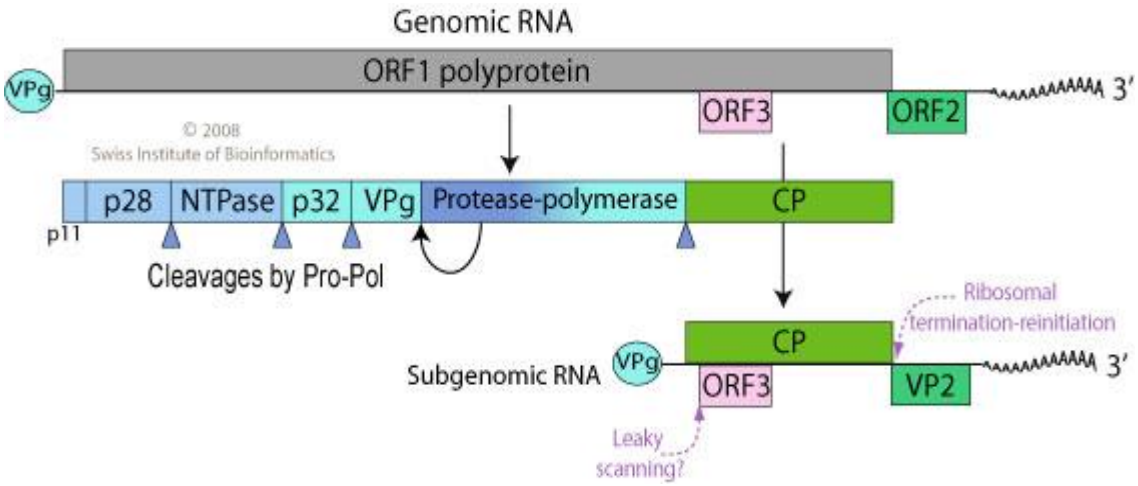

Figure 2.2: This figure shows a genomic structure: monopartite, linear ssRNA (+) genome. The 5'-terminus is linked to a VPg protein and the 3'-terminus has a poly (A) tract (adopted from viralzone.expasy.org/all_by_species/196.html#tab6).

The genomic organization of SVs is assumed to be like that of NoVs, with the exception that the polyprotein of SVs and capsid ORFs are contiguous and fused. The lack of a convenient *in vitro* propagation system, and antigenic characterization of SV is also described to be less straightforward. The phylogenetic trees based on capsid sequence have a slightly different structure from those of polymerase structure, likely due to the established amino

acid homology in the capsid region which is less counted than that of polymerase region (Bennett et al*.,* 2014)**.**

2.3 VIRAL STABILITY

Members of the Caliciviridae family are likely to be vulnerable to proteolysis, and to lose their capsid definition on purification or storage. In addition, other virus was reported sensitive to 60% and 70% ethanol treatments at room temperature for 30 seconds, inactivated by high temperature (56 °C) for a period of 2 hours, and inactivated by sodium hypochlorite of 200 mg/liter at room temperature for 30 minutes (Oka et al., 2015; Wang et al., 2012). In contrary, the virus resistance was reported against different concentrations (2.5 mg/liter and 10.0 mg/liter) of chlorine, stable at low pH (3.0) and high pH (8.0) at room temperature for 60 minutes (Wang et al., 2012). Takagi and colleagues (2020), suggested from their findings that it is unlikely to completely inactivate human SV by exposing it to 60 °C for about 30 minutes, as this virus was stable after exposed to heat treatment. In addition, they reported that SV particles contained in stool samples are resistant to UV irradiation.

2.4 CLASSIFICATION

Sapoviruses were previously called “typical human Caliciviruses” or “Sapporo-like viruses”. In 2002, Sapporo-like virus species, with other genera like Norovirus, Lagovirus, Vesivirus and Nebovirus, were assigned in the family Caliciviridae by the International Committee on the Taxonomy of Viruses (Mayo, 2002). During its first detection in human diarrheic stool samples using electron microscopy (EM) (Oka et al*.,* 2015; Madeley and Cosgrove, 1976), SV was from then recognized as a new gastroenteritis pathogen (Oka et al*.,* 2015; Chiba et

al., 2000; Nakata et al., 2000). However, the prototype strain of the SV genus was identified from another outbreak in Sapporo, Japan, in 1982 (strain Hu/SV/Sapporo/1982/JPN), after extensive studies for SV virological and genetic characteristics (Oka et al., 2015). Sapoviruses are known to be highly diverse in their genetic arrangement, for this reason, the 14 SVs genogroups (GI–GXIV) are classified based on the complete VP1 amino acid sequencing (Matussek et al., 2015; Oka et al., 2012).

2.4.1 Species designation and clinical significance

The entire VP1 region is vital for the designation of new or existing genogroups and or genotypes. There are five prominent genogroups (designated, GI to GV) of SV currently known, among which GIII is well known to infect porcine species (Oka et al., 2015; Hansman et al., 2007; 2005), while GI, GII, GIV, and GV are known to infect humans (Oka et al., 2012). However, two genotypes of GV (namely: GV.3 and GV.4) are detected from pigs and sea lions, respectively (Oka et al., 2015). The human associated SV genogroups are further subdivided in one or multiple genotypes, of which currently there are 18 genotypes based on phylogenetic analysis of the complete capsid gene or full-length nucleotide sequence of VP1. Genogroup I is subdivided into seven genotypes which are GI.1–GI.7, and genogroup II subdivided into eight genotypes, namely and GII.1–GII.8 (Xue et al., 2019; Liu et al., 2016). Whereas genogroup IV has a single genotype which is GIV.1, and genogroup V divided into two genotypes which are GV.1 & GV.2 (Xue et al., 2019; Dey et al., 2018; Oka et al., 2015; Oka et al., 2012). Genogroup I is often predominantly detected and associated with severe symptoms (Matussek et al., 2015; Bucardo et al., 2014). Below is a table (Table 2.1) presenting a brief on the SV strains with the clinical symptoms associated with their infection.

Table 2.1: A summary on identified Sapovirus strains

Virus	Genogroup (G)	Genotype	Clinical characteristics	References
Human Sapovirus	Genogroup I	GI.1 to GI.7	Diarrhoea, Vomit, Fever, Dehydration, Abdominal pain, Headache, Myalgia, Respiratory.	Oka et al., 2015; Oka et al., 2012; Farkas et al., 2004.
	Genogroup II	GII.1 to GII.8		
	Genogroup IV	GIV.1		
	Genogroup V	GV.1 and GV.2		
Animal Sapovirus	Genogroup III	GIII	Diarrhoeal manifestation	Lauritsen et al., 2015
	Genogroup V	GV.3 and GV.4		Oka et al., 2015; Lauritsen et al., 2015; Scheuer et al., 2013
Other Sapovirus	Genogroup VI – GXIV	Not defined		

There are reports on other animals associated with SVs infections, of which most studies are on the detection of SV from pigs with and without diarrhoea. Porcine has been proposed as a primary host associated with SV-GIII infection, including a wider range of SV genogroups, namely: GVI to GXI (Lauritsen et al., 2015; Scheuer et al., 2013). The close relatedness of human genogroups to the swine genogroups have been predictable based on the sequence of the RNA polymerase region of SV (Lauritsen et al., 2015). In addition, the prevalence of SVs has been reported in variety of animals like dogs, of which their large population and interactions with humans can be considered as a potential risk factor for zoonotic virus transmission (Oka et al., 2016; Li et al., 2011).

The antigenicity of SVs differs among its genogroups and distinct amongst genotypes especially within SV-GI and SV-GII. The distinct antigenicity is presented by using virus-like particles (VLP) or recombinant VP1 proteins, and this antigenicity is somewhat determined by VP1 (Oka et al., 2015). Although SVs genogroups show a high level of diversity (Oka et al., 2016), there are reports on genetically indistinguishable SVs (that is, similarities based on their partial genome sequences) detected in human, ready to eat food like shellfish (Oka et al., 2015; Iizuka et al., 2013; 2010) and environmental waters (Murray et al., 2013a; Ng et al., 2012; Kitajima et al., 2011; Sano et al., 2011).

2.4.2 Recombinant strains

Genetic recombination appears to be one of the evolutionary mechanisms of SV and other members of Caliciviridae family like NoV (Kumthip et al., 2018). The inconsistent grouping within the RdRp region (amongst other nonstructural protein-encoding region) and VP1 encoding region of SVs are designated recombinant strains, with both intragenogroups and intergenogroups recombination showing characters of molecular evolution (Dey et al., 2018; Oka et al., 2015). Analyzed on the phylogenetic trees, SV strains are clustered differently based on VP1 and RdRp encoding regions. This intergenogroup recombinant was observed when the detected SV strain was grouped with GIV based on VP1 sequence, but clustering with GII based on RdRp region (Oka et al., 2015; Chanit et al., 2009; Hansman et al., 2007; 2005). Dey and colleagues (2018) identified the SV GI.1/2 “intragenogroup” recombination, as a highly recombinant strain, wherein GI.1 and GI.2 were determined by the nonstructural protein-encoding and VP1-encoding regions, respectively.

2.4.3 Clinical infection

2.4.3.1 Pathogenesis

Childhood diarrhoea is assumed to be primarily caused by viruses (Ren et al., 2013). Moreover, viral gastroenteritis is seen most frequently in infants of one to eleven months of age, where the virus is suspected to attack the epithelial cells of the upper intestinal villi causing mal-absorption, impairment of sodium transport, and diarrhoea (Willey et al., 2011). Additionally, Ren and colleagues (2013) suggested that a viral infection can lead to a secondary lactose intolerance which might cause persistent diarrhoea.

Clinical manifestation is known to typically range from asymptomatic to a relatively mild diarrhoea with headache and fever, to a severe, watery, and non-bloody diarrhoea with abdominal cramps. A clinical symptom of fatal dehydration has been documented to be the most common in children (Willey et al., 2011). Major clinical symptoms including vomiting with additional symptoms such as nausea, chills, myalgia, or malaise are also reported. A manifestation of a diarrhoea that usually resolves within 1 week has been reported (Kobayashi et al., 2012; Lee et al., 2012; Mikula et al., 2010), as well as the cases of individuals showing symptoms from over a week to up to 20 days (Oka et al., 2015; Wu et al., 2008; Cubitt et al., 1981). Lee and colleagues (2012) conducted a study from an outbreak that occurred in a long-term-care facility for the elderly and noted that mortality was rare.

2.4.3.2 Syndromes

The most common symptoms associated with SV infection amongst children are diarrhoea, vomit, fever. In addition, symptoms from children of less than 2 years of age infected by SV,

are usually a frequent vomiting and a fever which rarely manifest in some cases (Liu et al., 2016).

It is well documented that gastroenteritis symptoms are usually self-limiting, and that some patients usually recover within a couple of days; however, it has been noted that symptoms, severity and duration of disease are also dependent on the individual. Nevertheless, SV infection is sometimes known to lead to hospitalization (Medici et al*.,* 2012; Park et al*.,* 2012; Trang et al*.,* 2012; Lorrot et al*.,* 2011). Moreover, treatment against viral gastroenteritis is designed to provide relief using oral fluid replacement with isotonic liquids, analgesics, and antiperistaltic agents. Recovery after infection is known to often results in protective immunity to subsequent infection (Willey et al*.,* 2011).

2.5 EPIDEMIOLOGY

Viruses are ubiquitous and plentiful in the microbial ecosystems of water, soil and sediment (Ganesh and Lin, 2013; Wommack et al. 1995). Thus, hygiene practices, eating habits and environmental conditions are likely to play a role in the infection frequency of individuals in different settings (Hansman et al., 2007). The epidemiology of SVs has been studied but less than NoV. The SVs infection in adults has been reported in outbreaks, including long-term care facilities (Lee et al*.,* 2012). Additionally, most outbreaks have been correlated with drinking contaminated water, swimming in contaminated pools or lakes in which infected individuals have been swimming, and it was noted that SV seem to be relatively resistant to inactivation by chlorine (Wang et al., 2012). Contamination of food stuffs has been traced to both pre-symptomatic and post-symptomatic food handlers (Bennett et al*.,* 2014).

Detection rate of SV differs for various countries with milder illness. The emergence of human SVs in European countries, and the dynamic changes of SV strains detected from gastroenteritis patients is known (Oka et al., 2015; Harada et al., 2013; 2012), hence, Oka and colleagues (2015) emphasized the importance of analyzing and characterizing the circulating SVs within populations.

Incubation periods of SV is generally 24 to 48 hours, although reported observation that ranges from 18 to 72 hours have been speculated. It has been observed that virus shedding in stools was about over the first 24 to 48 hours after illness. Sapovirus shedding levels in clinical stool specimens is assumed to range from 1.32 x 10^5 to 1.05 x 10^{11} genomic copies/gram of stool (Harada et al*.,* 2012; Mikula et al*.,* 2010; Yamashita et al*.,* 2010; Iwakiri et al*.,* 2009), and that SV RNA shedding levels in faeces gradually decreased after onset of illness (Iwakiri et al*.,* 2009). It is also reported that detection of viruses can be for up to 3 weeks after illness by using polymerase chain reaction (PCR) techniques. The clinical implication of the prolonged detection of SV in stools is unclear, but epidemiologic data have implicated individuals who are not symptomatic in the transmission of illness (Oka et al., 2015; Yoshida et al., 2009).

2.6 TRANSMISSION

Health effects associated with the presence of enteric viruses in the environment (e.g. water) include paralysis, meningitis, hepatitis, respiratory illness and diarrhoea (Ganesh and Lin, 2013; Hewitt et al. 2007). As remarked, SVs amongst other viruses are significant viral particles associated with intestinal infections leading to gastroenteritis. Figure 2.3 shows the patterns of SVs transmission from person to person via contact with sapovirus-positive

faeces, vomitus, sharing cups and utensils with infected people or via contaminated food and drinking water as previously reported (Kumthip et al., 2018; Silva et al., 2018; Kobayashi et al., 2012; Lee et al., 2012; Kitajima et al., 2011; Iizuka et al., 2010; Rasanen et al., 2010; Yamashita et al., 2010). In addition, communal surface area can be contaminated and play a role in harboring airborne virus that might settle, especially on surfaces like carpets (Yeargin et al., 2016). Furthermore, transmission of viral particles can also be via inhalation of contaminated aerosol (Kumthip et al., 2018; Silva et al., 2018).

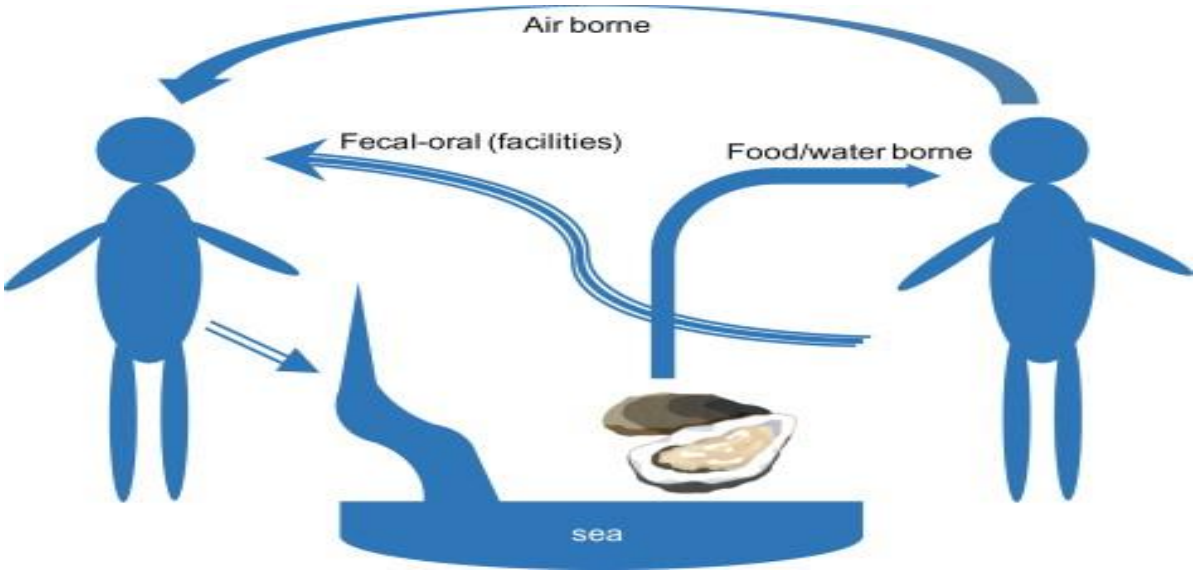

Figure 2.3: A figure showing the transmission patterns of viral particles (adopted from https://ars.els-cdn.com/content/image/3-s2.0-B9780124169753000169-f16-01-9780124169753.jpg)

2.6.1 Person-to-person transmission

Access to adequate sanitation can reduce the incidence of disease, thereby enhancing the quality of life (Sibiya and Gumbo, 2013). The lack of sanitation and hygiene is part of the key risk factor for diarrhoeal disease (Lewin et al., 2007). The experimental induction of illness in normal volunteers has suggested that the major route of person-to-person

transmission is fecal-oral. Airborne transmission has also been suggested, but with unsuccessful limited attempts to experimentally transmit virus with nasopharyngeal washings from an ill volunteer (Lee et al., 2012). However, SV was lately detected from nasopharyngeal swabs from children in Brazil (Silva et al., 2018).

2.6.2 Foodborne transmission

A major quantity of viral disease can be contracted through contaminated food. Outbreaks associated with food-borne, caused by SV is rare and much less frequent than that of NoV, but is suggested to be caused by contaminated or infected food-handlers (Usuku et al., 2008; Hansman et al., 2007; Noel et al., 1997). Moreover, pathogenic viruses causing a variety of diseases such as gastroenteritis can survive and be transmitted through foods (like vegetables) if they have been contaminated during irrigation, during preparation and post-preparation (Bidawid et al., 2009). Contamination of foodstuffs of animal origin is highly possible, and their consumption may cause human infection if the pathogenic agent are not inactivated during food processing (Rodrıguez-Lazaro et al., 2012).

2.6.3 Waterborne transmission

Water is an essential source to health and development in life, and diseases related to contamination of drinking-water constitutes a major burden on human health (WHO, 2011). The outbreak of waterborne linked to viral diseases has been reported extensively worldwide (Ganesh and Lin, 2013; Ye et al. 2012), regularly caused by the enteric viruses shed in high numbers from the faeces of infected individuals (Grabow, 2007). Thus, the continuous surveillance for the presence of enteric viruses in water samples becomes an important indicator of the level of human faecal pollution in various countries (Ganesh and

Lin, 2013). It has been illustrated that despite a low-level detection of enteric viruses in various matrices, its pollution can cause a significant risk of infection in vulnerable individuals (Teunis et al. 2008; Fong and Lipp, 2005). Of which the presence of these viruses in water are of most important concern of water quality, and have a primary public health and socioeconomic impact. In addition, enteric viruses influenced by faecal contamination are the important pathogens frequently detected from surface waters and have been associated with many waterborne outbreaks (Ganesh and Lin, 2013).

The effects associated with human health status, quality of water used, improper human waste disposal, and disease transmitted through fecal-oral route are anticipated (Govender et al., 2011). Viral pathogens in vomit and faeces of infected individuals can contaminate the marine environment, fresh water, and groundwater through rain run-offs or directly from an infected individual. The contamination of drinking water sources, recreational and irrigation waters, enables these viruses to be transmitted from person-to-person and surface-to-person thereby causing infections (Ganesh and Lin, 2013; Griffin et al. 2003). Therefore, assessment of water sources used for domestic purposes is vital, as high incidence of enteric viruses in water is constantly reported (Ganesh and Lin, 2013; Kiulial et al. 2010).

2.7 LABORATORY DIAGNOSIS

In the microbiological laboratories, diagnostic methods including: Viral culture, electron microscopy, and rapid latex agglutination tests have been used in diarrhoeal disease to detect viral pathogens. However, there are some viruses that are not easily detected using some of the above-mentioned tests, as in the case of SV that has been difficult to grow in

cell lines (Oka et al., 2018; 2015). Although SV can be detected using the electron microscope, PCR based techniques are widely used for SV detection from clinical and environmental samples, because of its rapidness, sensitivity and specificity (Oka et al., 2015).

2.7.1 Electron microscopy

From past studies, observation of viruses using EM has been known to be relatively insensitive, but useful for direct detection in faecal samples based on the morphological characteristics of the viral particles in stool specimen (Dufkova et al*.,* 2011). However, EM is highly specific, but can successfully detect viral diarrhoea during the first 48 hours wherein there must be 1 million viruses per milliliter of stool. This microscope can scan a ground of roughly 1 millionth of a milliliter (LaBaron et al., 1990). Figure 2.4 shows the captured SV particles during observations under EM. The “Star of David” surface morphology of SV, makes it distinguishable from other enteric viruses when observed under EM (Oka et al., 2015; Madeley, 1979).

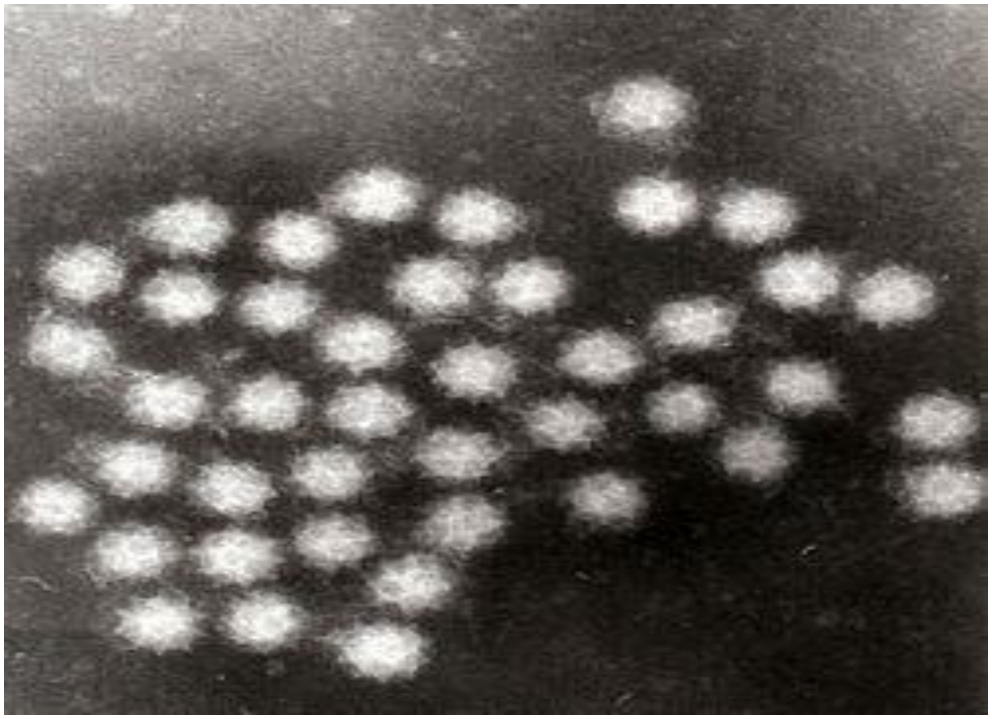

Figure 2.4: A figure showing the structure of Sapporo viruses under electron micrograph (adopted from https://en.wikipedia.org/wiki/Sapovirus)

2.7.2 Antigen detection

Although molecular based techniques are largely more sensitive, enzyme-linked immunosorbent assay (ELISA) have some usefulness. In the testing of high-volume setting, ELISA tests are the most suitable diagnostic tool in terms of convenience, cost-effectiveness, and timesaving (Hansman et al., 2006; Jiang et al., 2000). For serological detection method, Hansman and colleagues (2006) developed a novel antigen ELISA system that was based on hyper-immune rabbit and guinea pig antisera raised against SV genogroup I virus-like particles. This ELISA was reported to have 100% specificity and a sensitivity of 60% (compared to single-round PCR) and 25% (compared to nested PCR), in detecting SV GI antigens in clinical stool specimens. Furthermore, it was remarked that the development of an ELISA system with a higher sensitivity and a broader-range of detection, needs a raise of the antisera against other SV strains (Oka et al., 2015).

2.7.3 Cell culture detection

The cell culture method is ranked as the gold standard which can monitor viability of viruses, but earlier studies have shown an explosive increase in detection rates by using methods that targets the nucleic acid of the wanted viruses. Due to the largely use of new molecular detection technologies, the role of other (mostly difficult-to-culture) viruses, such as SV, is no more been overlooked (Wolffs et al*.,* 2011).

An attempt of growing human SV in cell cultures like green monkey kidney cells and primary human embryo kidney cells was made, although there are currently no reports of secondary attempt to validate the procedure for further use (Oka et al., 2015). In addition, it has been identified that human SV's expression of a recombinant sub-genomic-like construct or VP1 alone in insect or mammalian cells, results in the formation of virus-like particles (VLPs) which were reported to be morphologically similar to native SV (Hansman et al*.,* 2007; Oka et al*.,* 2006). In contrast, Oka et al (2018) attempted to test the possibility of growing human SV with other viral types or strain(s) in different cells and culture conditions with no success. Thus, human SV unlike porcine SV strains are currently known to be uncultivable.

2.7.4 Viral nucleic acid detection

Reverse transcription polymerase chain reaction (RT-PCR), including real-time PCR, for the past decades till to-date is an important assay in research and clinical diagnosis because of its reactivity, rapidity, sensitivity, specificity and its usefulness in genetic analysis (Oka et al., 2015). This method is widely employed for genetic analysis in different studies involving plant, animals and microorganisms. Additionally, it has been used for successful detection of almost all strains of SV in a variety of samples, such as stools (Makhaola et al., 2022;

Magwalivha et al., 2021; Platts-Mills et al., 2018; Ouédraogo et al., 2016; Page et al., 2016; Matussek et al., 2015; Mans et al., 2014; Harada et al., 2013; Sdiri-Loulizi et al., 2011; Chanit et al., 2009; Okada et al., 2006) and waters (Murray and Taylor 2015; Murray et al., 2013a; 2013b; Ng et al., 2012; Kitajima et al., 2011; Sano et al., 2011). More recently, RIDA GENE real-time PCR assay have been developed for detection and differentiation of bacteria, parasites, and viruses including SV. Hence, reported to be useful for enteric viruses' detection (Redli et al., 2020).

Successful detection of viruses from samples needs the identification of a targeted genome, thereby synthesizing a short oligonucleotide or primers to bind at the defined area on the genome of the virus. A variety of primers have been designed and tested for the detection of SVs, and detection of human SVs by RT-PCR methods has been reported around the globe (Oka et al., 2015). Efforts have been achieved to increase the sensitivity of SV detection, as some assays were recognized to be less-sensitive to detect almost all genogroups because of lack of nucleotide sequences information (Oka et al., 2015). Okada et al. (2006) reported on their developed genogroup-specific primers that generated PCR products of different lengths, allowing the genogrouping of the four human SVs without nucleotide sequencing.

2.7.4.1 Target regions for SV detection

The complete viral structure of SV has VP1 as a major constituent, which is approximately 60 kilodalton (kDa) protein, encoded on ORF1 and fused to non-structural genes (Figure 2.5). This protein is assumed to be produced by both or either cleaving from the ORF1-encoded polyprotein, and/ or by translating from a sub-genomic RNA. VP1 is separated into

various regions which are: N-terminal variable region (NVR), N-terminal (N), central variable region (CVR), and C-terminal (C). The N and CVR junction are predicted to contain a conserved amino acid motif identified as GWS, of which “G” is conserved within the caliciviruses (Oka et al., 2015; 2009). Other protein like VP2 have not been defined, although its expression has been detected from both the porcine SV full length genomic cDNA construct and infected cells (Oka et al., 2015; Chang et al., 2005).

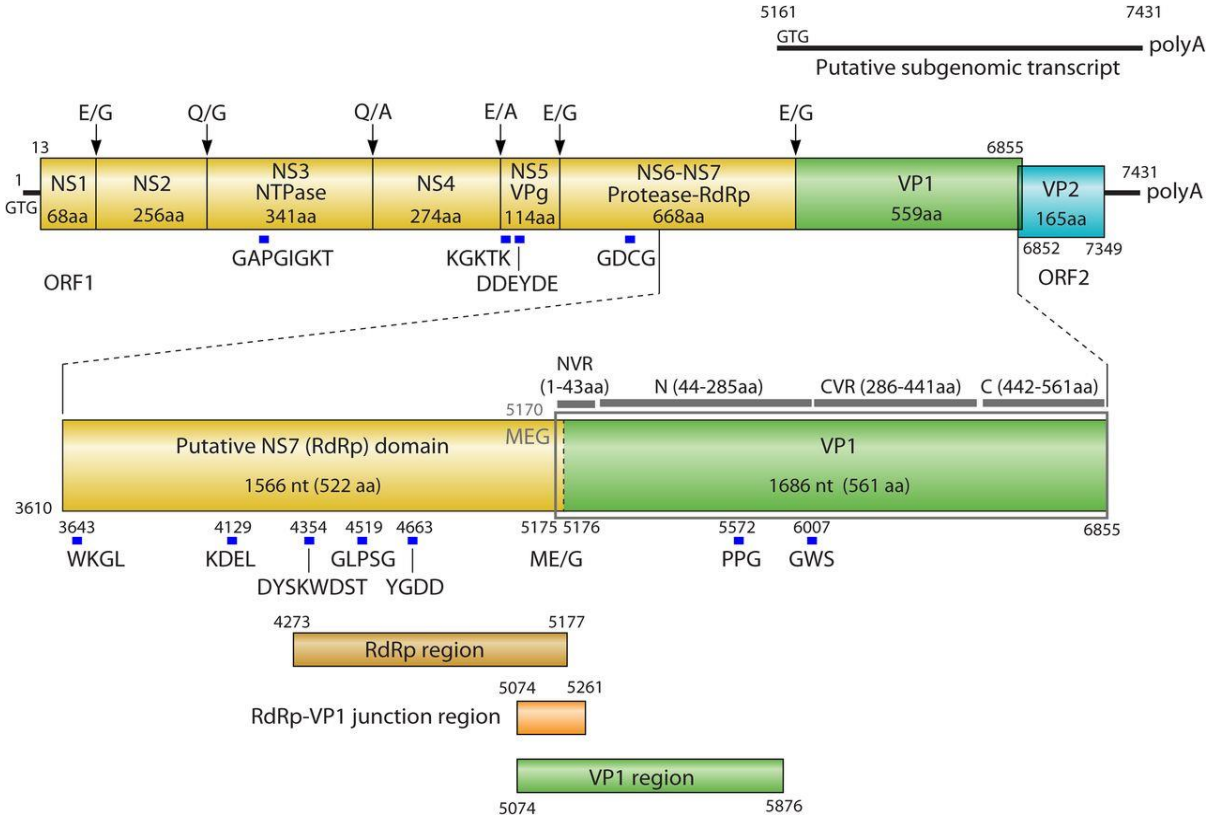

Figure 2.5: This figure shows a genomic organization of SV and its PCR target regions (adopted from Oka et al., 2015)

Figure 2.5 shows a schematic diagram of the SV displaying the genomic organization, including the putative subgenomic transcript segment, open reading frame 1 and 2, the predicted viral nonstructural proteins, protease-RNA-dependent RNA polymerase (RdRp),

and structural proteins VP1 and VP2. The putative first amino acids of VP1 from subgenomic transcript “MEG” and the cleavage site of this motif (ME/G; the slash indicates the cleavage site) from the ORF1 polyprotein are shown with their nucleotide positions. The subdomains in the VP1 from the subgenomic transcript (N-terminal variable region [NVR] [1 to 43]), N-terminal region [N] [44 to 285]), central variable region [CVR] [286 to 441]), and C-terminal region [C] [442 to 561]) are also labelled (Oka et al*.,* 2015).

For a successful analysis of SVs, partial VP1 region or partial RNA-dependent RNA polymerase (RdRp), or both these regions are commonly used to partially characterize SVs, and to investigate similarities of SV for surveys. Nevertheless, VP1 sequence is targeted for genetic classification as it correlates with the viral antigenicity and more diverse (Oka et al., 2015; Hansman et al., 2005; Farkas et al., 2004). In addition, due to SV’s high genetic diversity, most protocols use multiplex assay or degenerate primers for a successful detection (Redli et al., 2020; Varela et al., 2018; Okada et al., 2006).

2.8 TREATMENT AND PREVENTION

Viral gastroenteritis causes the morbidity and mortality which characterize a significant economic and public health burden (Das et al., 2014). During diarrhoeal manifestation, the host’s body loses a lot of fluid. Diarrhoea occurring continuously can cause a very rapid decrease of water and sodium in the body, which can also lead to a life-threatening condition (Kang, 2016).

Generally, the approach against diarrhoea with no or mild dehydration is oral rehydration therapy, which is a simple treatment that also permits management of uncomplicated cases

of diarrhoea at home, regardless of aetiology (Duggan et al., 1999). Oral rehydration with an electrolyte containing solution (usually glucose and table salt) is one of the most important mechanism, as sodium ions in these extracellular fluid is critical for normal metabolism of the body system (Kang, 2016).

Vomiting is also commonly known as a presenting and common feature of viral gastroenteritis which can result in failure to rehydrate orally, therefore medically the use of antiemetic agents is suggested to patients in some settings. It is scientifically and generally known that antibiotics have no role to play in viral gastroenteritis. In addition, specific antiviral agents directed against the causes of gastroenteritis have not been circulated or established (Kang, 2016; Farthing et al., 2008; Freedman, 2007). However, providing hygiene information on diarrhoeal disease to the communities (especially the caregivers of children), can contribute to reducing transmission of diarrhoeal infection within the populations.

2.9 DIARRHOEAL DISEASE IN AFRICA

Diarrhoeal disease is the second leading cause of death in children under five years old yearly, and remain a high burden disease (WHO, 2017). In Africa, diarrhoea is estimated to be responsible for as much as 7.7% of all deaths. Although, world-wide around 1.1 billion people lack access to improved water sources and 2.4 billion have no basic sanitation. The occurrence of diarrhoea cases might be presumably rare in the developed countries where sanitation is available, with high access to safe water and a relatively good domestic hygiene. However, diarrhoea due to infection is reported to be prevalent throughout the developing world (https://rehydrate.org/diarrhoea/). Is reported that poor quality of care at

health facilities represents an enormous missed opportunity to prevent unnecessary child deaths due to diarrhoea. Consequently, is noted that children are not receiving acceptable care for diarrhoea in high-burden sub-Saharan African countries, including those seen in health facilities (Carvajal-Vélez et al., 2016).

The detection of SV in the African regions has been reported in few studies. A study by MAL-ED reported SV amongst other detected pathogen, as a second highest causative pathogen of diarrhoea within the participating rural community in SA (Platt-Mills et al., 2018). However, the underreporting of diarrhoeal causative agent such as enteric viruses (especially SV) in the developing or underdeveloped regions is evident (Magwalivha et al., 2018). According to published data in the African continent (Figure 2.6), SVs has been detected mostly from stool specimens of hospitalized children younger than 5 years of age (Makhaola et al., 2022; Ouédraogo et al., 2016; Page et al., 2016; Matussek et al., 2015; Sdiri-Loulizi et al., 2011; Mans et al., 2010). However, in SA, a study has reported the detection of SVs from both young and adults age groups (Mans et al., 2014). In addition, other studies have also reported on the occurrence of SV on environmental samples (Murray and Taylor 2015; Murray et al., 2013a; 2013b).

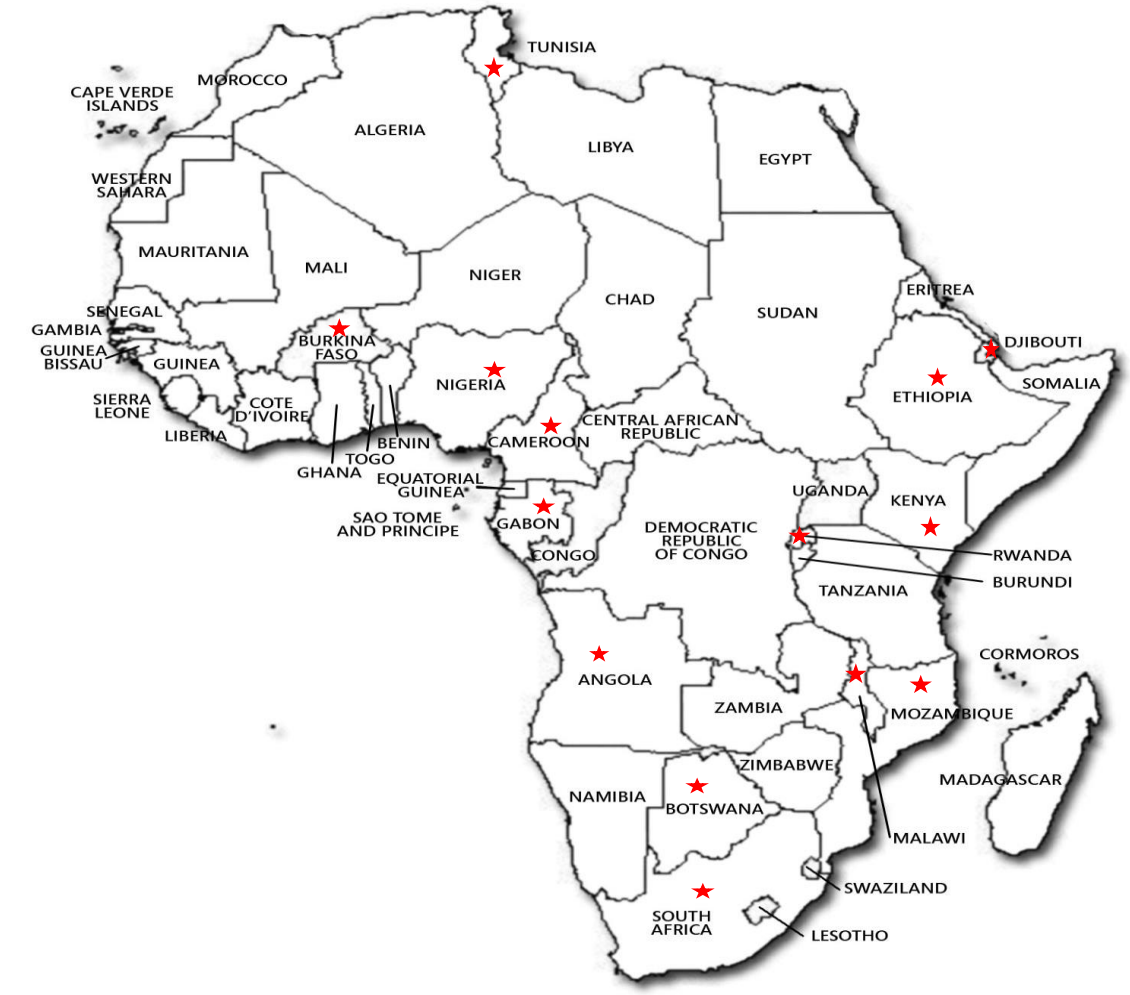

Figure 2.6: African continent map showing countries that have conducted or published studies on Human SV. The detection/prevalence of human SV in Africa, reported only from the marked (Star) areas, namely: Angola, Burkina Faso, Botswana, Cameroon, Ethiopia, Gabon, Kenya, Malawi, Mozambique, Nigeria, South Africa, and Tunisia. The position of a star is not an indication of the geographic position where a study was done in such a country.

As shown in Figure 2.6, the scarcity of reports on human SV circulating within the regions of Africa is evident. Hence, identification of circulating human SV in such developing regions is important for epidemiological surveillance, understanding of SV pathogenesis and exposure of new or recombinant strains. Thus, a broad spectrum for the success of

surveillance may require studies in African regions to include gastroenteritis cases reported by outpatients and hospitalization patients.

2.10 SUMMARY OF LITERATURE REVIEW

Viral diarrhoea, caused by enteric viruses, is associated with mortality rate among children less than five years of age in developing countries. Enteric viruses are an assorted group of viruses that generally causes AGE and are detected in both symptomatic and asymptomatic individuals. Their transmission among individuals commonly occurs through faecal-oral route. Enteric viruses like Rotaviruses, Noroviruses, Adenoviruses, Sapoviruses, and Astroviruses are reported to be associated with viral diarrhoea worldwide. Sapoviruses amongst others are likely viruses of human faecal origin discharged into environmental waters and accumulated in oysters or clams. Hence, their diverse genetic existence in different matrices like human clinical specimens, shellfish, and environmental water samples have been reported worldwide (Oka et al., 2015).

Clinical manifestation on humans as a result of enteric viral infection, typically ranges from asymptomatic to a moderately mild diarrhoea with fever and headache, to a severe watery, abdominal cramps with non-bloody diarrhoea. Fatal dehydration has been documented to be the most common in children. A manifestation of a diarrhoea usually resolves within 1 week, although cases of individuals show symptoms from over a week to up to 20 days. Data on the detection of SVs in SA has been reported mostly among the hospitalized patients (Murray et al., 2016; Page et al., 2016; Mans et al., 2014;), and in a follow-up investigation among the selected rural community in South Africa (Platts-Mills et al., 2018). However, there is limited data on the occurrence of SV circulating strains within the rural-

based communities of SA. Hence, the aim of this study was to determine the genetic characterization of human Sapovirus strains circulating in the rural communities of the Vhembe region (Limpopo Province, South Africa).

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CHAPTER 3

REVIEW ARTICLE

THE PREVALENCE OF SAPOVIRUS IN DEVELOPING COUNTRIES

Sapovirus infection is a public health concern, causing AGE in people of all ages in both outbreaks and sporadic cases worldwide with reports on the outbreaks occurring in the semi-closed settings. To assess the prevalence of SV in the disadvantageous or under developed countries as part of this study, a literature review on human sapovirus studies reported in such regions was carried out.

Prevalence of Human Sapovirus in Low and Middle Income Countries

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Review Article

Prevalence of Human Sapovirus in Low and Middle Income Countries

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Background. Sapovirus (SV) infection is a public health concern which plays an important role in the burden of diarrhoeal diseases, causing acute gastroenteritis in people of all ages in both outbreaks and sporadic cases worldwide. *Objective/Study Design.* The purpose of this report is to summarise the available data on the detection of human SV in low and middle income countries. A systematic search on PubMed and ScienceDirect database for SV studies published between 2004 and 2017 in low and middle income countries was done. Studies of SV in stool and water samples were part of the inclusion criteria. *Results.* From 19 low and middle income countries, 45 published studies were identified. The prevalence rate for SV was 6.5%. A significant difference ($P=0$) in SV prevalent rate was observed between low income and middle income countries. Thirty-three (78.6%) of the studies reported on children and 8 (19%) studies reported on all age groups with diarrhoea. The majority (66.7%) of studies reported on hospitalised patients with acute gastroenteritis. Sapovirus GI was shown as the dominant genogroup, followed by SV-GII. *Conclusion.* The detection of human SV in low and middle income countries is evident; however the reports on its prevalence are limited. There is therefore a need for systematic surveillance of the circulation of SV, and their role in diarrhoeal disease and outbreaks, especially in low and middle income countries.

1. Introduction

An estimated number of 6.3 million deaths of children under the age of 5 years suffering from diarrhoea have been reported worldwide [1, 2]. In Africa, death due to diarrhoeal disease remains a major health concern, though it has decreased from 2.6 million to 1.3 million between 1990 and 2013 [3]. Diarrhoeal disease is the important cause of morbidity and mortality in low and middle income countries, also the third most frequent cause of death and greatest contributor to the burden of disease in children younger than 5 years of age [4]. The infection of human intestinal tract occurs through transmission at the household level due to different pathways such as ingestion of contaminated food and water, poor waste disposal, and person-to-person interactions in the households and community [4, 5]. Low and middle income countries still face challenges like inadequate human waste disposal, poor water quality, poor health status, and disease transmission through faecal-oral route [6].

Amongst diarrhoeal causing agents, Sapovirus (SV) is one of the enteric viruses that cause acute gastroenteritis in humans and animals. Sapoviruses were previously called "typical human Caliciviruses" or "Sapporo-like viruses" in the family Caliciviridae [7]. They are identified as nonenveloped, positive-sense, single-stranded ribonucleic acid (RNA) genome of approximately 7.1 to 7.7 kb in size with a poly(A) tail at the 3'-end [8–10]. Amongst the five designated genogroups (GI to GV), GIII infects porcine species [11–14], while GI, GII, GIV, and GV infect humans [15]. Currently, human SV genogroups are classified into 16 genotypes (comprising seven genotypes for GI and GII, respectively, and one genotype each for GIV and GV) through phylogenetic analysis of the complete capsid gene [15, 16]. Coinfections of SVs with other enteric viruses (such as

noroviruses [NoVs], rotaviruses [RVs], astroviruses [AstVs], adenoviruses [AdVs], enteroviruses [EVs], and kobuviruses [KbVs]) have been noted in acute gastroenteritis outbreaks in humans [17–19].

This review summarises reports on SV detection and typing in low and middle income countries. In addition, it highlights the need to establish the relatedness of circulating SV strains in environmental (water) samples and clinical samples from communities in low and middle income countries (particularly rural settings). The time-frame chosen was 2004 to 2017 because of the availability of published data on human SV within the low and middle income countries.

2. Methodology

Two literature searches were carried out. The first literature search was performed using the terms: calicivirus, sapovirus, and developing countries, as listed by National Institutes of Health PUBMED library and ScienceDirect. A second literature search was independently done for each of the 139 "developing" countries accessed from the list published by the Society for the Study of Reproduction (http://www.ssr.org). Furthermore, the identified countries were then assessed according to the 2018 World Bank analytical classification report (http://datahelpdesk.worldbank.org/knowledgebase/articles/906519). For a successful search, each of the countries' names was combined with the following keywords: calicivirus, sapovirus, enteric viruses, and gastroenteritis. Studies identified by the search terms were selected for inclusion in the review based on the following inclusion criteria:

(a) Studies limited to human SV detected in clinical specimen and environmental water samples, reported in the 21st century.

(b) SV studies using laboratory molecular techniques including nested-PCR (nPCR), real time-PCR (RT-PCR), and RT-multiplex PCR.

Studies were excluded from the review if SV was detected in other mammalian species or animals or if the study was conducted in high income countries. In case of duplication of studies by authors, only one article was included.

Data was extracted from each selected study when provided: country name and its economic status (i.e., low income, lower, and upper middle income) as per the analytical classification report by World Bank, study setting (hospitalised, outpatient, and environment), study population (age group), population size, duration of the study, diagnostic method used, number of samples tested for SV (including their genogroups and genotypes), first author, and year of publication (Tables 1, 2 and 3).

The difference of SV data in middle and low income countries was analysed for statistical significance by Student's t-test using the simple interactive statistical analysis (SISA) at http:home.clara.net/sisa. Result with $P < 0.05$ was considered significant.

3. Results

A total of 138 articles published from 2004 to 2017 were identified from 19 low and middle income countries. After selection based on the selection criteria (Figure 1), a total of 45 studies met the inclusion criteria. From 45 publications, 41 reported on clinical (stool) samples, 3 on environmental (water) samples, and 1 on both. Of the 42 studies conducted on clinical specimens, 66.7% (n=28) were done in hospitalised patients, 23.8% (n=10) in outpatients, and 9.5% (n=4) in both hospitalised and outpatient settings.

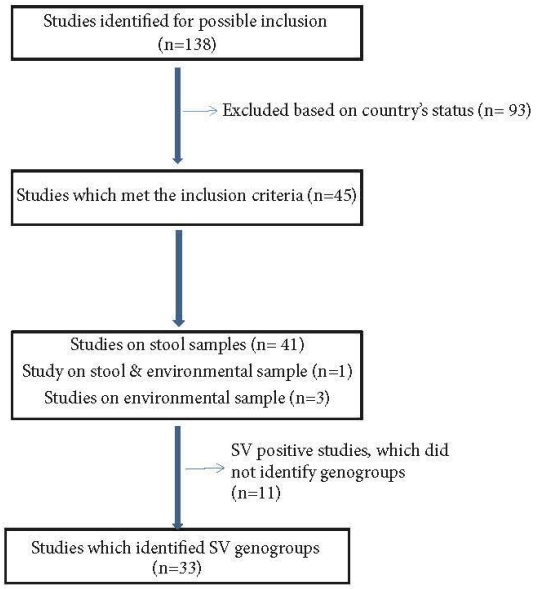

Figure 1: Schematic diagram showing search process for selection of studies reported.

3.1. SV Age Distribution in Human Populations. The majority of studies (78.6%; 33/42) investigated SV in children less than 5 years of age and a further 19% (8/42) included all ages. However, only a single study investigated SV in adults with diarrhoea or acute gastroenteritis.

3.2. Seasonality. The detection of SV from clinical samples based on seasonality was reported in only 14.3% (6/42) of the studies. The majority (42.9%, 18/42) of the studies did not report on the time-frame of detection, 38% (16/42) of the studies showed inconsistent time-frame of detection, and 4.8% (2/42) of the studies showed detection throughout the year. Studies investigating SV in water sources in South Africa (SA) did not detect any seasonal peaks.

Five studies reported on samples collected within a period of 2 to 4 months, and these cases were not defined as outbreaks, while the duration period of sample collection for other 40 studies ranged over periods from 1 year to 5 years.

3.3. Sapovirus Detection and Genotyping. From the 42 included studies, 41 of these reported SV positive cases while only one study on adults reported negative results (Tables 1 and 2). Mixed infection of SV with bacteria and/or other enteric viruses was identified in 19.5% (8/41) of the studies, a

Table 1: Summary of human SV detection from 33 studies (stool samples) conducted in 14 non-African low and middle income countries.

Country	World Bank Classification as of year 2018	Study setup			Duration of study	Prevalence (seasons or defined period of incidence)	Method used	Rate of Detected Genotypes	Reference
		Study population	Population size	Study setting					
Bangladesh	Lower middle income	Infants/ Children	917	HP with AGE	From 2004 to 2005	Oct 2004 – Jan 2005, Sept 2005	RT-PCR	2.7 % SV (***All in <3 yrs of age***) SV-GI.1, GI.2	Dey et al [20]
Brazil	Upper middle income	Children	305	HP severe GE	From March to September 2003	March, May - September	RT-PCR	15/305 (4.9%), mixed infection of SV and Astv in 1 sample SV-GII.1, SV-GI.1, SV-GI.2	Aragao et al [21]
		Children (0 – 10 yrs	159	OP (81 = diar; 78 = non-diar)	From April 2008 to July 2010	February, April	RT-PCR	2 of 81: 2.5% SV (GI.1, GII.2)	Aragao et al [22]
		Children (6-55 mn old)	539	Day Care (Healthy)	From October 2009 to October 2011	Not defined	RT- multiplex PCR	25/539 (4.6%) SV, SV-GI.1, GI.3	de Oliveira et al [23]
		Children, outpatients	212 129	HP OP With AGE	From 2012 to 2014	Not defined	Quantitative real-time PCR (qPCR)	12/341 (3.5%) **[9/12 – HP, 3/12 – OP].** SV-GI.1 dominant, GI.2, GI.6, GII.1, GV.1	Fioretti et al [24]
		Children < 10yrs	426 (156 of <3yrs tested)	HP with AGE	From January 2010 to October 2011	Aug & Sept	RT-PCR	6/156 (3.8%), SV-GI.1, GI.2, GII.2, GII.4	Reymao et al [25]
		Children	172	Community	From 1990 t0 1992	Not defined	Nested PCR	9/172 (5.2%) SV-GI.1, GI.7, GII.1, GV.2	Costa et al [26]
China	Upper Middle income	Children <5yrs old	500	OP with acute (477)/ persistent (23) diar	From August to November 2010	Aug – Nov 2010	RT-PCR	9/477: 1.89% SV ***(<24 month children)***, mixed infection of SV & AdV in 1 sample, SV-GI dominant, SV-GII & SV-GIV	Ren et al [27]
		Patients (1mn – 78yrs)	412	HP & OP with AGE	From August 2014 to September 2015	Not defined	RT-PCR	[9/412] 2.2% SV single infection, Co-infection: 2/412 ETEC with SV, 1/412 Salmonella sp with SV, 1/412 Salmonella sp with SV & AdV **Genogroups not defined**	Shen et al [28]

Table 1: Continued.

Country	World Bank Classification as of year 2018	Study population	Study setup Population size	Study setting	Duration of study	Prevalence (seasons or defined period of incidence)	Method used	Rate of Detected Genotypes	Reference
India (New Delhi)	Lower middle income	Children <10yrs	226	HP with AGE	From August 2000 to December 2001	Not defined	Multiplex two-step RT-PCR	23/226 (39%), mixed infection in 5 samples {NV-GII and SV-GI} SV-GI [22], GII [1]	Rachakonda et al [29]
Iran	Upper middle income	Children	200	HP with AGE	From 2008 to 2009	Winter and in fall	RT-PCR	6/200 (3%), SV-GII	Parsa-Nahad et al [30]
		Patients (3 mn - 69yrs; mean 15.3yrs	42	HP with AGE	From May to July 2009	May – July 2009	RT-PCR	11.9% SV (***patients with <5yrs of age***) SV-GI.2	Romani et al [31]
Mongolia	Lower middle income	Infants	36	households	From July to August 2003	Jul – Aug 2003	RT-PCR	1/36 (2.8%) pos for SV SV-GI	Hansman et al [11, 12]
Nicaragua	Lower middle income	Children <5yrs	330	(175 HP; 155 OP), with AGE /diar	From September 2009 to October 2010	Nov 2009- Feb/Mar 2010, May-Aug/Sept 2010	Real-time PCR	57/330 (17%): **HP = 15% [27/175], OP = 19% [30/155].** SV-GI, GII, GIV {HP: GI.1, GI.2; OP: GII.2, GII.3	Bucardo et al [32]
Pakistan	Lower middle income	Infants <6 to >35 mn	122 Pos: Enteric Viruses	HP with AGE	From 1990 to 1994	Mar, Aug - Oct	RT-PCR	13.9% SV detection (12.3% SV mono-infections, 1.6 mixed infection – AstV & SV), SV-GI	Phan et al [33]
		Infants & children <1 mn – 5yrs	517	HP with AGE	From 1990 to 1994	1990: Aug, Sept, Oct 1991: Jan, May, Jul, Oct 1992: Mar, Aug, Sep 1993: Sep 1994: Apr, July	RT-PCR	3.2 % SV SV-GI dominated, followed by GII, and GIV	Phan et al [34]
Papua New Guinea (Goroka)	Lower middle income	Children <5yrs	199	HP with AGE	From August 2009 to November 2010	Not defined	RT-PCR	4/199 (2%) SV, **Genogroups not defined**	Soli et al [35]

Table 1: Continued.

Country	World Bank Classification as of year 2018	Study population	Study setup: Population size	Study setup: Study setting	Duration of study	Prevalence (seasons or defined period of incidence)	Method used	Rate of Detected Genotypes	Reference
Peru	Upper middle income	Children <2yrs	599	300 non-diar, 299 diar	From 2007 to 2010	Four seasons	Quantitative reverse transcription-real-time PCR (qPCR)	9.0% overall: ∗**12.4% [37/299] diarrhoeal** – SV-GI/1/2/6/7, GII.1/2/4/5, GIV, GV/1; ∗**5.7% [17/300] non-diarrhoeal** – SV-GII.5, GIV	Liu et al [36]
Philippines	Lower middle income	Children <5yrs	417	HP with AGE	From June 2012 to August 2013	Not defined	Real-time PCR	29/417 (7%) detection, (co-infection in 10/29: 6/10 with RV, 2/10 with NV, 2/10 with AstV). SV-GI.1, GI.2, GII.1, GII.4 & GV	Liu et al [1, 2]
Thailand	Upper middle income	Infants	80 randomly selected	HP with AGE	From November 2002 to April 2003	Nov 2002 – April 2003	RT-PCR	15%: 11% single infection, 4% mixed infection – NoV & SV), SV-GI	Guntapong et al [37]
		Children <5yrs	248	HP with AGE	From 2002 to 2004	Not defined	RT-PCR	3/248 (1.2%) SV- single infections SV-GI [GI.1 &GI.2], GIV	Khamrin et al [38]
		Children	296	HP with AGE	From May 2000 to March 2002	Jun-Jul, Jan-Mar, May-Jul, Mar.	RT-PCR	25%, mixed infection I 1 sample (NV-GI and SV) SV-GI.1, GI.4, GI.5, GII.1, GII.2	Malasao et al [39]
		All age groups	273	HP with AGE/diar	From January 2006 to February 2007	Early summer: March & April	RT-PCR	0.8% SV SV-GII/3	Kittigul et al [40]
		Children (Neonate to 5yrs old)	147	HP with AGE/watery	January to December 2005	Not defined	RT-PCR	5/147 (3.4%) SV SV-GI [GI.2, GI.1, GI.5] dominating, SV-GII.3	Khamrin et al [41]
		Pediatric patients	160	HP with AGE	January to December 2007	Throughout the year	RT-multiplex PCR	5/160 (3.1%) SV **Genogroup not defined**	Chaimongkol et al [42]
		Children <5yrs	567	HP with AGE	In 2007, and from 2010 to 2011	2007: Feb, Sept, Oct. & 2010: Dec	Semi-nested RT-PCR	7/567 (1.2%), SV-GI.1	Chaimongkol et al [43]
		Adult (15yrs – 90yrs)	332	HP with diar	Year 2008	Not defined	RT- multiplex PCR	***No SV detected***	Saikruang et al [44]
		Patients	1141	HP with AGE	From 2006 to 2008	May - July	RT-PCR	1.1% SV, mixed infection of NoV-GII & SV in 2 samples **Genogroup not defined**	Pongsuwanna et al [45]

Table 1: Continued.

Country	World Bank Classification as of year 2018	Study population	Study setup: Population size	Study setup: Study setting	Duration of study	Prevalence (seasons or defined period of incidence)	Method used	Rate of Detected Genotypes	Reference
		Children	448	HP with acute sporadic gastroenteritis	From December 1999 to November 2000	Not defined	RT-PCR	1/448 (0.2%) SV SV-GI	Hansman et al [46]
		Paediatric patients	1010	HP with viral AGE	From October 2002 to September 2003	Oct 2002 – Sep 2003, Rainy season (July)	RT-PCR	0.8% SV (0.4% monoinfection, 0.4% coinfection), **Genogroup not defined**	Nguyen et al [47]
Vietnam	Lower middle income	Pediatric	502	HP with AGE	From December 2005 to November 2006	Dry season	RT-PCR	1.2% SV	Nguyen et al [48]
		Children <5yrs	501	HP with AGE	From November 2007 to October 2008	Cooler months (Oct – Feb)	Real-time RT-PCR	1.4% SV SV-GI and SV-GII Co-infection of (NoV & SV) in 1 sample, of (NoV, SV, and RV) in 1 sample	Trang et al [49]
Independent States of the former ***Soviet Union***	See information below describing the States	Children	495	HP with AGE	From January to December 2009	Jan - Mar, May – Aug	Real-time PCR	16/495 (3.2%) SV-GI.1 dominating	Chhabra et al [50]

HP = hospitalised patient; OP = outpatient; AGE = acute gastroenteritis; mn = month; yr(s) = year(s); diar = diarrhoea; SV = Sapovirus; G (I-IV) = genogroup (I-IV)
Independent States of the former Soviet Union refers to* ***Armenia, Azerbaijan & Belarus* *(upper middle income status),* ***and Georgia, Republic of Moldova & Ukraine*** *(lower middle income status).*

Table 2: Summary of human SV detection from 9 studies (stool samples) conducted in 5 African countries.

Country	World Bank Classification as of year 2018	Study population	Study setup		Duration of study	Prevalence (seasons or defined period of incidence)	Method used	Rate of Detected Genotypes	Reference
			Population size	Study setting					
Burkina Faso	Low income	Children	263 diarrhoeal, 50 non-diarrhoeal	Urban area (HP & OP)	From November 2011 to September 2012	Not defined	Real-time RT-PCR	9%: 27/263 (10.3%) [***5/27*** = ***hospitalised, 22/27*** = ***non-hospitalised***]& 3/50 (6%) SV-GII [GII.2, GII.1, GII.3], SV-GI.2	Ouedraogo et al [51]
		Children <5yrs	309 diarrhoeal	Not defined	From May 2009 to March 2010	Not defined	Real-time PCR	56/309 (18%) [mixed infection: with RV 25/56, with NV 5/56; single infection 20/56] Genogrouping [34/56]: SV-GI [GI.1, GI.4], GII [GII.1, GII.4, GII.6], GIV.1 & GV.1	Matussek et al [16]
Ethiopia	Low income	All age groups	213 diarrheic samples	Government Health Care Centre	From June to September 2013	June-sept 2013	RT-PCR	9/213 (4.2%) One sequenced (SV-GII.1)	Sisay et al [52]
Kenya	Lower middle income	All age groups	334-Lwak & 524-Kibera.	Clinics with diar	From June 2007 to October 2008	Not defined	RT-PCR	5%: 13/334 (4%) and 31/524 (6%) SV **Genogroups not defined**	Shioda et al [3]
South Africa	Upper middle income	Paediatric <13yrs	245	HP gastroenteritis	Year 2008	Not defined	Real-time RT-PCR	10/245 (4.1%) incl. one Mixed infection with NV **Genogroups not defined**	Mans et al [53]
		Patients 1mn to 87yrs mean 14yrs	190 94 diar 93 non-diar 3 unknown	Bio-wipes from rural households	From July 2007 to December 2008	Not defined	Real-time RT-PCR	16/190 (8.4%): (1 - 62yrs: mean 24yrs) **Genogroups not defined**	Mans et al [54]
		Children	Selected) 296 of 477 SV-Pos (for characterisation)	HP with gastroenteritis	From April 2009 to December 2013	Not defined	Nested PCR	221 were characterised (genotyped) SV-GI [GI.1 – GI.3, GI.5, GI.6, GI.7], SV-GII [GII.1 – GII.7], SV-GIV	Murray et al [55]
		Children <5yrs	3103	HP diar	From 2009 to 2013	Higher in Summer & Autumn (Nov to Apr)	Real-time PCR	238/3103 (7.7%) SV **Genogroups not defined**	Page et al [56]
Tunisia	Lower middle income	Children	788 [408 HP, 380 OP]	Consulting for AGE	From January 2003 to April 2007	Not defined	RT-PCR Primer Noel, 1997	6/788 (0.8%) [Mixed infection: with RV 2/6; single infection 4/6]. Positive from OP samples SV-GI.1	Sdiri-Loulizi et al [57]

HP = hospitalised patient; OP = outpatient; AGE = acute gastroenteritis; mn= month; yr(s) = year(s); diar = diarrhoea; SV = Sapovirus; G (I-IV) = genogroup (I-IV).

Table 3: Summary of human SV detection from 4 studies (water samples) conducted in low and middle income countries.

Country	World Bank Classification as of year 2018	Samples Type	Samples Size	Duration	Prevalence (season)	Method used	Rate of detection	Reference
Brazil	Upper middle income	Wastewater	156	From 2012 to 2014	Summer and Autumn	Quantitative real-time PCR (qPCR)	51/156 (33%)	Fioretti et al [24]
South Africa	Upper middle income	River water	99	From 2009 to 2010	May, Aug, Nov (2009); Jan, April (2010)	RT-PCR	48/99 (48.5%)	Murray et al [58]
		Wastewater	51	From August 2010 to December 2011	August (2010), June, July (2011)	Real-Time qPCR	37/51 (72.5%)	Murray et al [59]
		Water (various source)	10	January and March 2012	January and March 2012	Real-Time PCR	8/10 (80%)	Murray and Taylor [60]

SV single strain was identified in 36.6% (15/41) of the studies, and mixed strains of SV were identified in 43.9% (18/41) of the studies. From the 41 studies, only 31 studies reported SV detection with identification of the genogroups/genotypes. Overall detection of SV strains showed SV-GI.1 and GI.2 as the most dominant [90% (28/31)] strain from different settings of studies, followed by SV-GII.1, GII.2, GII.3, and GII.4 with the least detection of SV-GIV strain and –GV (GV.2) strain. No study showed the occurrence of SV-GIV as a single detection but only in mixed infection cases.

The prevalence rate of SV from the 41 documented studies in low and middle countries was 6.19% with a range from 0.2% to 39%. Further breakdown showed significant difference (P =0) in SV prevalence rate between low income (10.40%) and middle income (5.86%) countries. Although data on the prevalence of SV in African countries is limited, thus far, eight studies have been conducted in urban settings. Detection of SV from children in Africa is recorded with different incidence rates: in Tunisia [0.8%] [57], Burkina Faso [18%, 10.3%, respectively] [16, 51], and South Africa [4.1%, 7.7%, respectively] [53, 56]. The prevalence of SV in all ages was reported from South Africa [8.4%] [54], Ethiopia [4.2%] [52], and Kenya [4%] [3]. A predominance of SV-GIV (53/221, 24%) was noted in the South African study done on stool samples from hospitalised children with gastroenteritis [55].

Only 8.9% of studies reported SV in the environmental and waste water samples from low and middle income countries. The detection of SV-GI, SV-GII, and SV-GIV has been reported from polluted water sources by wastewaters and also on samples collected from treatment plants within selected areas of SA [58–60]. Sapovirus genogroups I and II were identified from river water samples, with detection rate of 48.5% (48/99) [58], while, in Brazil, SV-GI (genotypes 1 and 2) were detected (33%, 51/156) from the wastewaters [22], Table 3.

4. Discussion

This review provides a summary of studies conducted in developing countries on the detection of human SV. Only 45 (41 stool samples, 3 water samples, and 1 both stool and water sample) studies satisfied the inclusion criteria of this review highlighting the importance for systematic surveillance monitoring human SV circulating in developing countries (rural and urban communities). Very little is known about the contribution of human SV to diarrhoeal disease in developing countries; this is reflected in the fact that reported studies were only from 19 identified countries which include 5 African countries, namely, Burkina Faso, Ethiopia, Kenya, South Africa, and Tunisia (Table 2). A total of 78.6% (33/42) studies reported on children ≤5 years of age from the collected data, highlighting the role of SV in diarrhoeal disease amongst children in the developing countries. Hence, SV and other emerging enteric viruses, being underappreciated, can be an important cause of Norovirus negative outbreaks as reported by Lee and colleagues [61]. In addition, since it is difficult to culture human SV on cell lines [13], specialised molecular laboratories are needed for the investigation of such virus in the developing countries. Because of lack of funding and skills, the prevalence of enteric viruses is underreported in Africa and other developing countries [62]

Most of the studies (66.7%; 28/42) were done in hospitalised patients, and this might be due to the fact that SV infection sometimes leads to hospitalisation as illustrated from other studies [49, 63]. GEMS study reported SV amongst other enteric pathogens to have been associated with moderate to severe diarrhoea in developing countries [64]. The Millennium Development Goals (MDG) 2015 report shows disadvantaged settings being vulnerable as compared with the advantaged or developed settings, highlighting the effectiveness and affordability of treatments, and improved service delivery and political commitment playing a role in such settings. The statistical analysis of this review similarly

showed a significant difference in the prevalence of SV in low income than in middle income countries (P=0).

The circulation of SV genogroups shows variability, with SV-GI and SV-GII detected frequently, while SV-GIV and SV-GV are rarely detected comparing to other genogroups [16]. An African study (Burkina Faso) reported SV-GII as the predominated strain, mostly in outpatients with diarrhoea (81.5%: 22/27), suggesting that this genogroup may be less virulent and require fewer hospital admissions. However, additional studies on outpatients will have to be conducted to confirm this observation. Although the detection of SV-GII is seen in diarrhoeal samples, it might be less virulent to cause severe symptoms leading to hospitalisation of patients, unlike SV-GI which is commonly known to be associated with severe symptoms and frequently detected in patients presenting with gastroenteritis [16, 32]. The detection of SV (GI, GII, GIV, and GV) in gastroenteritis outbreak cases has been reported in high income countries, however with less detection rate of SV-GII in both cases [14, 17, 61, 65].

Human SV infections cases relating to acute gastroenteritis in people of all ages have been identified worldwide [14]. Notwithstanding the potential selection biases present based on the studies available for inclusion, this review shows that the prevalence in children may be higher than in adults in low and middle income countries. In addition, the GEMS study in low and middle income countries highlights diarrheal disease in children as a leading cause of illness and death and also increasing the risk of delayed physical and intellectual development [66]. It has been reported that sporadic and outbreak cases caused by enteric viruses spread mainly by person-to-person contact, contaminated surfaces or objects, and contaminated water or food [67]. Therefore children are more vulnerable than adults within such exposed environment, probably because of immune system development. However, previous studies noted that gastroenteritis symptoms are usually self-limiting, and patients usually recover within a couple of days depending on the individual immune's response [49, 63]. Adults are likely to consider self-treatment by oral rehydration solution (ORS) which is the safe, effective, and low cost therapeutic option preventing dehydration [68], hence not consulting in healthcare facilities or likely due to self-respect.

Sapoviruses, like other enteric viruses, play an important role in the burden of disease worldwide. The GEMS conducted a three-year study in selected low and middle income countries, amongst children aged 0 to 59 months, and reported the detection of SV (3.5%) associated with diarrhoea [64]. However, there is no surveillance system on SV infection and prevalence in low and middle income countries, which means underreporting of sporadic cases of human SV and its epidemic are underestimated. Nevertheless, detection and comparison of the SV strains circulating in low and middle income countries (especially Africa) are currently underreported and this could be due to various techniques used for sampling and detection, including study site conditions.

Information on seasonality, patient history, area settings, and predicated pattern of transmission of viruses within the community provides knowledge needed to implement public health intervention strategies. Furthermore, detection of enteric viruses (such as SV) in environmental samples gives awareness of the circulation of infectious viral particles within the population and health-hazards which might be associated with the environment. The predictable effects of human waste disposal, water quality, and high rate of immunocompromised society have been a big concern in low and middle income countries, but there are still few documented reports on the detection of SV from environmental samples. This is highlighted by the finding of this study with high prevalence of SV in low income countries. The survival and development of children depend on good hygiene practices and use of clean drinking and domestic water on daily basis [4]. Monitoring of genetic diversity of the current circulating or emerging SV genogroups, possible water-borne transmission, and possible zoonotic infections amongst the communities is critical, and studies which can show the transmission of SV between the environment(s) (especially river water), domestic animals, and human should be considered, and the role that SV plays in diarrhoeal diseases [69].

5. Conclusion

This review found substantial evidence of SV proportion associated with diarrhoeal disease in low and middle income countries. However there is limited data reporting the detection of circulating SV strains. Therefore systematic surveillance of SV circulation within the communities in low and middle income countries is needed to assess sufficiently its role in diarrhoea disease.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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CHAPTER 4

RESEARCH ARTICLE

OBJECTIVE 1: To determine the genotypes, and characterize the human Sapovirus strains associated with diarrhoea in children less than 5 years of age from rural communities in SA

OBJECTIVE 2: To compare the detected SV strains circulating with the reference strains reported elsewhere in SA and developing countries

The genetic diversity of SVs is established, and the detection of SV strains varies around the globe. However, utmost reports of SV is based on urban or developed areas and hospitalized patients, especially in South Africa. Hence, this study was designed focusing on the rural communities from the Vhembe district located in the far-North of South Africa. The study reported on detected genotypes, and characterized the human SV strains circulating in rural communities, and compared the detected strains with other stains reported elsewhere.

Prevalence and Genetic Characterisation of Human Sapovirus from Children with Diarrhoea in the Rural Areas of Vhembe District, South Africa, 2017-2020

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Prevalence and Genetic Characterisation of Human Sapovirus from Children with Diarrhoea in the Rural Areas of Vhembe District, South Africa, 2017–2020

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Abstract: Diarrhoeal disease is considered an important cause of morbidity and mortality in developing areas, and a large contributor to the burden of disease in children younger than five years of age. This study investigated the prevalence and genogroups of human sapovirus (SV) in children ≤5 years of age in rural communities of Vhembe district, South Africa. Between 2017 and 2020, a total of 284 stool samples were collected from children suffering with diarrhoea (n = 228) and from children without diarrhoea (n = 56). RNA extraction using Boom extraction method, and screening for SV using real-time PCR were done in the lab. Positive samples were subjected to conventional RT-PCR targeting the capsid fragment. Positive sample isolates were genotyped using Sanger sequencing. Overall SV were detected in 14.1% (40/284) of the stool samples (16.7% (38/228) of diarrhoeal and 3.6% (2/56) of non-diarrhoeal samples). Significant correlation between SV positive cases and water sources was noted. Genogroup-I was identified as the most prevalent strain comprising 81.3% (13/16), followed by SV-GII 12.5% (2/16) and SV-GIV 6.2% (1/16). This study provides valuable data on prevalence of SV amongst outpatients in rural and underdeveloped communities, and highlights the necessity for further monitoring of SV circulating strains as potential emerging strains.

Keywords: hospitalized patients; outpatients; rural communities; sapovirus

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1. Introduction

Diarrhoeal diseases are recognized as the third leading cause of death among children under five years of age in South Africa (SA) [1,2]. The effects of poor sanitation and hygiene practices, quality of supplied water may play an important role in the burden of diarrhoeal disease which is a major concern in developing countries [1,3,4]. Viral infections may present from asymptomatic to relatively mild diarrhoea with a headache and fever, to severe watery diarrhoea accompanied with abdominal cramps [5].

Sapovirus is an enteric virus, and is recognized as a public health problem causing acute gastroenteritis in people of all age groups globally, and it also causes outbreaks in semi-closed settings, like orphanages and elderly care facilities [6]. Sapovirus has been associated with persistence vomiting suggested to possibly cause gastroenteritis in humans [7]. The increase of acute gastroenteritis associated with sapovirus (SV) has been reported and recognized as a major public health problem particularly in developing countries [8,9]. It is documented that after the successful deployment of the Rotavirus vaccine, SVs have emerged as the second most commonly etiological virus behind Norovirus in children with acute diarrhoea [9]. In addition, a longitudinal study by MAL-ED reported SV as a notable second highest attributable incidence of diarrhoea within the enrolled rural community in South Africa [10].

Sapovirus is a single-stranded, positive-sense RNA virus, with three open reading frames (ORFs) identified as ORF1, ORF2, and ORF3, of which ORF1 region is labelled to encode among other proteins, a major capsid protein (VP1) [11,12]. Sapovirus display a high level of diversity, currently with four genogroups (e.g., GI, GII, GVI, and GV) associated with human gastroenteritis infection [6,13–15]. Viral particles spread from person to person through faecal–oral route by consuming contaminated food and drinking water, and/or handling sapovirus-positive faeces [16–20].

Prevalence of Sapovirus varies in different countries possibly due to environmental conditions and hygiene practices, which are likely to play a role in the infection frequency of individuals in different settings [21]. Only a few studies in SA have reported on the prevalence of SV from hospitalized patients [3,22–24], and a longitudinal investigation [10]. This study aims to report on human SV circulating among children of less than five years of age residing in the rural settings of the Vhembe region in Limpopo, SA.

2. Results

2.1. Study Population

Out of 284 participants enrolled, 68% (193/284) were outpatients and 32% (91/284) were hospitalized. Amongst the participants, children less than 12 months age group, both in various settings, namely: symptomatic outpatients (66/137 : 48.2%), asymptomatic outpatients (38/56 : 67.9%), and hospitalized (46/91 : 50.5%) were the most enrolled, with the least enrolled age groups from 13 to 60 months in all settings (Table 1).

Table 1. Children presenting with diarrhoea and non-diarrhoea enrolled in the study.

Clinical Samples Collected	Outpatients from Clinics					Inpatients from Hospitals					Total Overall
	n	Age (Months)		Gender		*n*	Age (Months)		Gender		*n*
				M	F				M	F	
Children with diarrhoea (symptomatic)	137 (71%)	0–12	64(46.7%)	31	34	91 (100%)	0–12	46(50.5%)	28	18	228
		13–24	42(30.7%)	23	19		13–24	29(31.9%)	12	17	
		25–36	14(10.2%)	7	7		25–36	12(13.2%)	7	5	
		37–48	12(8.8%)	3	9		37–48	–	–	–	
		49–60	2(1.5%)	0	2		49–60	–	–	–	
		Unknown	3(2.2%)	1	2		Unknown	4(4.4%)	2	2	
Children without diarrhoea (asymptomatic)	56 (29%)	0–12	38(67.9%)	15	23	0 (0%)	0–12	–	N/A	N/A	56
		13–24	13(23.2%)	5	8		13–24	–			
		25–36	4(7.1%)	2	2		25–36	–			
		37–48	–	–	–		37–48	–			
		49–60	–	–	–		49–60	–			
		Unknown	1(1.8%)	0	1		Unknown	–			
Total	*n* = 193 (100%)					*n* = 91 (100%)					284

2.2. Sapovirus Detection

The RIDA®GENE Sapovirus kit (R-Biopharm AG, Darmstadt, Germany) showed evidence of proficiency for all tested samples. The Ct value of the reactions ranged from 14.10 to 40.43 (mean = 30.89) and was determined from a threshold of 0.03.

Of the 284 collected samples, 40 (14.1%) were positive for human SV by RIDA®GENE test kit, with most samples detected at a low viral concentration (C_t range of 34.12–42.22). Among these positive samples, 13.2% (12/91) were of the hospitalized cases and 14.5% (28/193) from outpatient cases, with insignificant statistical difference (p = 0.765, Pearson Chi-Square, 2-sided). Out of 28 outpatients, 2 (7.1%) were patients without diarrhoea, and 26 (92.9%) were patients with diarrhoea.

2.3. Clinical Manifestation

Table 2 shows the manifestation of symptoms stated. In positive cases, diarrhoea as a single symptom was observed in 57.9% (22/38) and also in 42.1% (16/38) with other symptom(s), amongst which vomiting was specified in 29.7% (11/38) cases, fever in 21.6% (8/38) cases, abdominal pains in 10.8% (4/38) cases, and dehydration in 8.1% (3/38) cases. Overall reporting to the health facilities within three days of symptoms manifestation was noted in 63.2% (144/228) cases, while 36.4% (83/228) reported after three days.

Table 2. Clinical features of study participant children under 5 years of age.

Parameters	Case Patients (*n* = 228)		Controls (*n* = 56)	
	SV Positives (%) *n* = 38 (16.7%)	SV Negatives (%) *n* = 190 (83.3%)	SV Positives (%) *n* = 2 (3.6%)	SV Negatives (%) *n* = 54 (96.8%)
Symptoms				
Diarrhoea only	22 (57.9%)	68 (35.8%)	None	
Diarrhoea with other symptoms	16 (42.1%)	121 (63.7%)		
Unknown	–	1 (0.5%)		
Other symptoms				
Vomiting	11 (29.7%)	91 (47.6%)	N/A	
Fever	8 (21.6%)	60 (31.4%)		
Abdominal pain	4 (10.8%)	27 (14.1%)		
Dehydration	3 (8.1%)	24 (12.6%)		
Interval *				
≤3 days	22 (57.9%)	122 (64.2%)	N/A	
≥3 days	16 (42.1%)	67 (35.3%)		
Not defined	–	1 (0.5%)		

* Between the onset of diarrhoea and collection of sample.
SV = Sapovirus.

Most cases of other symptoms accompanying diarrhoea manifested in multiple (data not shown). Table 3 present the clinical manifestation of symptoms on patients reporting to clinics and those admitted in hospitals. Diarrhoeal symptom was mostly noted in clinic settings as previously emphasized (methods, study population). Of all case patients, only 11.1% of dehydration was recorded in Hospital settings.

Table 3. Symptoms shown by children in Clinics versus Hospital settings.

Parameters	Case Patients (*n* = 228)	
	Clinics (*n* = 137) (Positives/No. of Cases (%))	Hospitals (*n* = 91) (Positives/No. of Cases (%))
Diarrhoea only	17/69 (24.6%)	5/21 (23.8%)
Diarrhoea with other symptoms	9/68 (13.2%)	7/69 (10.1%)
Unknown	None	1 (Neg)
Other symptoms		
Vomiting	7/45 (15.6%)	4/55 (7.3%)
Fever	4/22 (18.2%)	2/32 (6.3%)
Abdominal pain	2/13 (15.4%)	4/28 (14.3%)
Dehydration	0	3/27 (11.1%)

2.4. Household Setting and SV Distribution

Data on household setting as presented on Table 4, was recorded during an interview prior to sample collection. Notable number of SV positive cases were likely associated with the use of latrine and water sources used, and further correlated by Bayesian linear regression. The correlation between the positive cases and water source showed a statistical significance (p = 0.006), whereas there was no significance in the correlation between positive cases and usage of latrine (p = 0.067). Children breastfeeding were also observed to be the most infected by the virus but this was not statistical significant (p = 0.930). The distribution of SV, was high among children <12 months of age by 47.5% (19/40), followed by 35% (14/40) detection from children aged 13 to 24 months, with the least detection of 10% (4/40) from children aged 25 to 36 months, and 7.5% (3/40) in children 37–48 months of age (Table 4).

Table 4. Household settings of the participants and distribution of sapovirus (SV) positive cases.

Household Settings	SV Positives v/s Enrolled Cases (%)	Patients Age Group (Month) and SV Positive Cases					
		0–12 Months	13–24 Months	25–36 Months	37–48 Months	49–60 Months	Unknown
		19 pos	14 pos	4 pos	3 pos	0	0
Latrine							
Used	21/187 (11.2%)	9/96	7/53	2/24	3/10	0/2	0/2
Not used	19/95 (20%)	10/51	7/32	2/7	0/1	0	0/4
Unknown	0/2 (0%)	0	0	0/2	0	0	0
Water sources							
Tap	31/244 (12.7%)	14/125	11/72	4/30	2/10	0/1	0/6
Borehole	5/26 (19.2%)	3/16	1/6	0/2	1/1	0/1	0
River	2/2 (100%)	1/1	1/1	0	0	0	0
Spring	2/9 (22.2%)	1/5	1/4	0	0	0	0
Unknown	0/3 (0%)	0	0/3	0	0	0	0
Breastfeeding							
Yes	25/185 (13.5%)	16/119	6/45	2/9	1/7	0/1	0/4
No	14/92 (15.2%)	3/28	8/37	2/21	1/3	0/1	0/2
Unknown	1/7 (14.3%)	0/1	0/3	0/2	1/1	0	0
Livestock							
Present	9/88 (10.2%)	4/39	3/32	1/10	1/3	0	0/4
Absent	31/196 (15.8%)	15/106	11/56	3/22	2/8	0/2	0/2
Unknown	0/1 (0%)	0/1	0	0	0	0	0

SV = Sapovirus.

As presented in Figure 1, seasonal distribution of SV in this study differed between the sampling periods. Sapovirus was commonly detected in summer seasons during all these years. A high detection rate was noticed during winter in 2017, autumn season in 2018, and spring season in 2019.

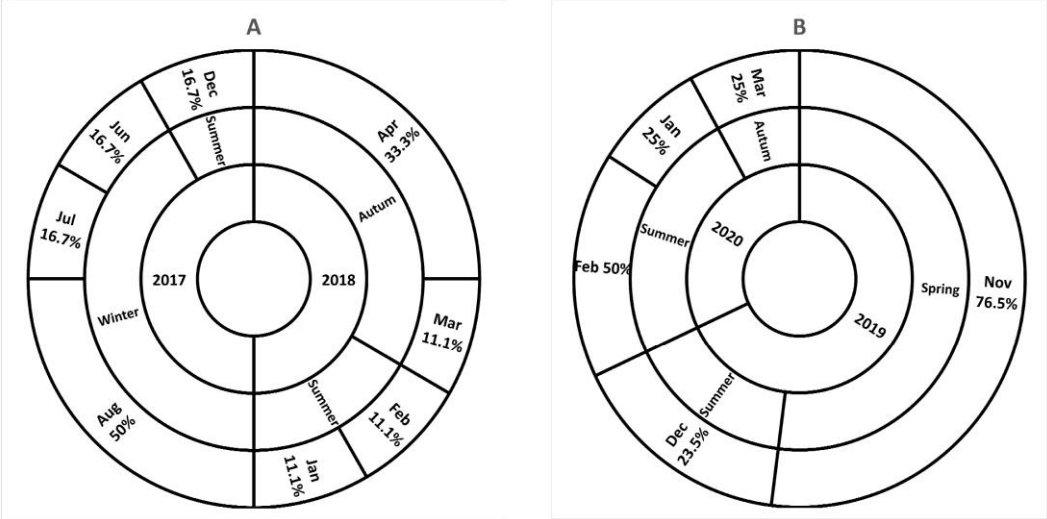

Figure 1. Seasonal distribution of detected SV from 2017 to 2018 (**A**), and from 2019 to 2020 (**B**). Inner circle present a year of sample collection; middle circle present seasons of the year; and the outer circle present the SV detection rate per month.

2.5. Molecular Characterization

Further molecular analysis on the identified SV positive cases was done to determine the SV genogroups, and 16 (40%) of the identified positives were successfully amplified for genogrouping. With note, nine of these samples had a low RNA concentration (C_t > 34.12). SV-GI (13/16: 81.3%) was predominately detected followed by SV-GII (2/16: 12.5%) and SV-GIV (1/16: 6.2%). Furthermore, three randomly selected samples (SV-G1-R/SaV124F amplicons of samples number Z01 (C_t = 23.42), Z22 (C_t = 22.21), and Z31 (C_t = 32.85); from different clinics) were subjected to sequencing analysis to determine the SV genotypes. A BLAST search gave a 95–99% sequence identity to the most closely related human SV strains in GenBank (accession number: MT741940, MT741941, and MT741942). Noronet genotyping tool [25] confirmed these sequences as the following SV genotypes: one as G1.1, and two G1.5.

The identified SV genotypes from this study, SVG1.1 (MT741940) showed a 98% identity by clustering with strain detected from a chimpanzee in Congo (KJ858686.1), and 96% identity with strains detected from human stool samples in South Africa (KP196476.1; KP196437.1). The identified SV genotypes from this study SV-G1.5 strains (MT741941 and MT741942) showed 98% identity to a human strain reported in India (KU317439.1) and 96% identity to the strain detected from food (ruditape) in Japan (AB765970.1), which also clustered closely. Other strains which gave identity of between 90% and 95% on GenBank, showed distinct clusters from the strains detected in this study when rooted by a porcine SV strain (DI203382.1), as presented in Figure 2.

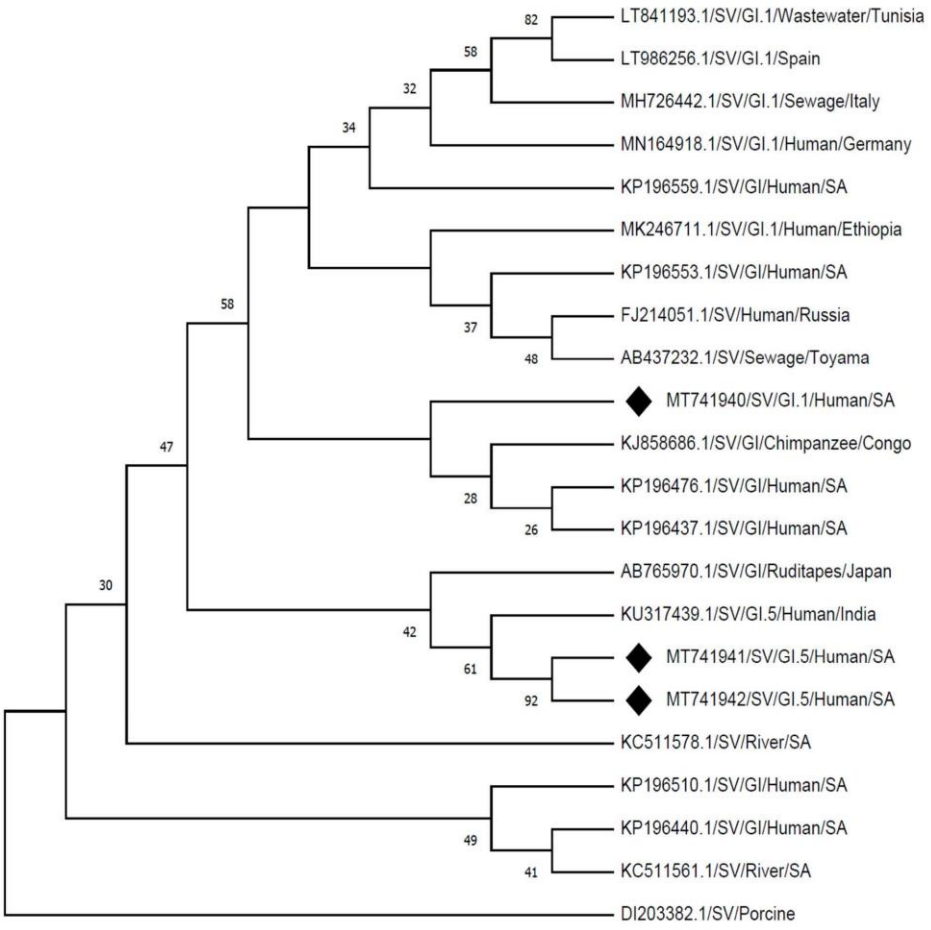

Figure 2. Phylogenetic analysis of the partial nucleotide sequences of sapovirus detected (MT741940, MT741941, and MT741942) in Vhembe district (South Africa), and reference strains of human sapovirus were selected from GeneBank database. Phylogenetic tree was deduced by the Neighbour-Joining method using MEGA X [26], based on a 360 base-pair fragment of the capsid (VP1) region showing the relationships within SV-G1 strains. The porcine SV (DI203382.1) was selected as an outgroup strain.

3. Discussion

The occurrence of SV in outpatients and hospitalised settings has been reported with different rates in various developing countries [3]. In this study, the prevalence rate (14.1%) is high compared to a 7.7% SV detection rate previously reported in SA from hospitalised patients [27], 5.2% [28], and 2.5% [29] reported in Brazil. However comparable detection rate (13.9%) was observed in hospitalized patients from Pakistan [30]. Furthermore, a study in Burkina Faso reported a 9% detection rate from hospitalized and outpatients [31]. A longitudinal investigation by MAL-ED found a high SV detection rate of 22.8% (range of 18.9–27.5%) from around the world, including South Africa [9].

This cross-sectional study report human SV prevalence rate of 14.1% within the rural communities of South Africa, with no evident outbreaks throughout the period of study. SV were detected in both asymptomatic (5%; 2/40) and symptomatic (95%; 38/40) children. A manifestation of more than one symptoms per individual(s) was noted, although it

could be as a result of other factors or causative pathogens. As shown in Table 3, most cases of diarrhoea were recorded in the clinics as compared to hospitals. It is assumed that most patients report to the clinics and possibly drink oral rehydration solution for self-treatment, which is effective and less costly [32]. Hence, cases of multiple symptoms including dehydration were seen in hospitals as severe cases.

Evidence of SV associated with diarrhoea amongst children less than five years of age has been observed around the globe [10,33], with viral gastroenteritis frequently seen in infants less than one year of age [5]. This study demonstrate a 17.1% (31/181) of SV detection in children less than two years of age presenting with diarrhoea (Table 1), comparable to a study reported in Peru which documented a 12.4% (37/299) SV detection rate among similar cohorts [9]. The findings of high proportion of SV detection amongst children <1 year of age is of concern in rural settings because of limited health infrastructure, and that children are more vulnerable due to development of their immune [5].

The variability of SV's prevalence in different regions of the world is evident, although SV-GI is the genogroup that is most associated with severe gastroenteritis cases [34]. In this study, SV-G1 predominance among the patients presenting with diarrhoea was noticeable and highlighted a possible threat among children in the rural communities. The detected SV-G1.1 strain showed identity with those detected from the hospitalized patients elsewhere within South Africa (Figure 2: KP196476.1 and KP196437.1). In this study, a high rate of SV detection in outpatient children compared to the hospitalised children was observed, as previously reported in Nicaragua [35]. This could imply that SVs circulating within rural communities are more likely associated with moderate diarrhoea which does not require hospitalisation. However, Table 2 presents a notable number of patients reporting late to the health care facilities which might lead to limited data documented. There is globally a noted concern of high prevalence rates of SV in low-income countries, but little data on the frequency of human SV in developing countries have been documented to date [3].

Among other provinces in SA, Limpopo is predominantly rural and one of the poorest provinces, with scarce water resources and sanitation [36]. From data collected in this study, the results showed that most households used pit-latrines and municipal tap water as source of water (Table 4). Positive cases associated with water sources (variables: municipal tap water, borehole, river, and spring) accessible by people for drinking, food preparation, bathing and other daily household tasks was established with statistical significance. Poor microbial quality on piped-water (tap water) in a low socio-economic setting, and high level of indicator micro-organism counts in water storage containers compared to the indoor tap water have been previously reported [1,37]. The use of pit-latrines might potentially play a role in viral transmission, directly or indirectly through flies as vectors, as previously stated [38]. Positive cases linked with children breastfeeding in this study is of concern, since risk factors such as poor hygiene practice and overcrowding of people living in a household leading to close contact of persons have been identified as some of the factors associated with diarrhoeal disease [1,39]. In this study occurrence of SV varied throughout the seasons; however, there was an evidence of persistent SV's manifestation in summer season, which is considered a rainy season in the Vhembe region of South Africa. Furthermore, many rural dwellers use untreated water for domestic use and are more at risk of the devastating effects of diarrhoea since causative pathogens may be transmitted as a result of poor quality of water, inadequate sanitation, and hygiene [1,40].

4. Materials and Methods

4.1. Study Population

This is a cross-sectional study, conducted between 2017 and 2020. The participants were children ≤5 years of age (with and without diarrhoea) residing in rural communities

of Vhembe district in Limpopo Province, South Africa. In South Africa, most cases of intestinal gastroenteritis are seen by the primary health care centres (clinics) situated in the rural communities and only the severe cases (with dehydration) are referred to the hospitals [41].

4.2. Sample Collection

To exclude the chances of nosocomial infections from hospitalized participants, samples were collected within the first two days of admission. Additionally, only patients admitted due to the diarrhoeal case were considered for this study. The World Health Organisation [42] definition for diarrhoea was used to include patients in the study. A total of 284 of stool samples (228 diarrhoeal and 56 non-diarrhoeal) were collected from participants at their respective local primary health care centres (20 clinics and 4 hospitals). Availability of samples was dependent on the willingness of participants to provide a sample and be included in the study. The samples collected were kept in closed stool bottles at +4 °C, and transported to the laboratory.

4.3. Quality Control

All sample analysis protocol and storage were done in separate rooms to avoid cross-contamination and PCR inhibition. Internal control was used to monitor inhibition and contaminations. For RIDA®GENE test runs, C_t values for internal control and positive control were in range as following the Quality Assurance Certificate.

4.4. Molecular Detection and Genogrouping of Sapovirus

4.4.1. Nucleic Acid Extraction

Prior to the nucleic acid extraction by Boom extraction reagents (Severn Biotech, Worcestershire, UK), the stool specimens were diluted to 10% suspension in Phosphate-Buffered Saline solution (pH = 7.0, Lasec SA (Pty) Ltd, Cape town, SA.) and stored at −20 °C. The viral RNA was extracted using the boom extraction method [43], with internal control added during extraction for quality control [41]. Briefly: A 500 µL of 10% suspension stool was centrifuged for 15 s at 13.3 x 1000 g. Then, 900 µL of L6 buffer was added to the supernatant in a sterile 1.5 mL tube, mixed by vortex for 1 min, 20 µL of internal control was added, centrifuged for 15 s at 13,300× *g*. Into a sterile 1.5 mL tube, 100 µL of Silica beads (Severn Biotech, Worcestershire, UK) was added to the transferred supernatant, mixed by vortex for 15 s and shaken softly for 15 min. Tube was centrifuged at 300× *g* for 15 s, and supernatant discarded. The pellet was re-suspended in 500 µL of L2 buffer, centrifuged at 300× *g* for 15 s and supernatant discarded. The pellet was re-suspended in 500 µL of 70% Ethanol, centrifuged at 300× *g* for 15 s and supernatant discarded. The pellet was re-suspended in 500 µL of Acetone, centrifuged at 300× *g* for 15 s, supernatant discarded. The opened tube was placed in a heat block at 50 °C for 5 min, to dry the silica pellet. The pellet was re-suspended in 150 µL PCR grade water, and heated at 56 °C for 5 min, centrifuged at maximum speed for 20 min. Finally, 100 µL of supernatant containing RNA was transferred to sterile closed 0.5 mL tube, stored at −20 °C until further analysis.

4.4.2. mPCR Detection of Sapoviruses from Stools

The RIDA®GENE Sapovirus, real-time RT-PCR kit (R-Biopharm AG, Darmstadt, Germany) for the direct detection of SV was used. This is a designed multiplex real-time RT-PCR for direct qualitative detection of human SV (Genogroup I, II, IV, and V), targeting the ORF1 region with fluorogenic target-specific hydrolysis probes. Reagents for the assay are provided with the kit including the internal control RNA which monitor PCR inhibition and reagent integrity. Prior PCR reaction, a 0.1 mL sterile tube with a total volume of 25 µL: containing 5 µL of RNA and 20 µL of Master Mix (19.3 µL of reaction mix, 0.7 µL of enzyme mix), 1 µL of internal control RNA was added to the negative and

positive controls. The real-time PCR program was performed on a Corbett Research Rotor Gene 6000 with the following cycling conditions: Reverse transcription for 10 min at 58 °C; initial denaturation for 1 min at 95 °C followed by 45 cycles of 95 °C for 15 s and 60 °C for 60 s with continuous fluorescence reading, as per the manufacturer. To minimize the risk of sample contamination and amplicon carry-over, separate rooms were used for the pre- and post-amplification steps.

4.4.3. Sapovirus Genogrouping and Sequencing

Positive samples for SV were further analyzed using One-Step Ahead RT-PCR kit (QIAGEN Co., Hilden, Germany) using previously published primers [6] to determine specific SV genogroups. The One Step Ahead RT-PCR utilizes a pair of specific oligonucleotide primers, namely: SV-G1-R/SaV124F to amplify GI capsid fragment, SV-G2-R/SaV124F to amplify GII capsid fragment, SV-G4-R/SaV124F to amplify GIV capsid fragment, and SV-G5-R/1245Rfwd to amplify the GV capsid fragment. Three randomly selected samples (SV-G1-R/SaV124F amplicons: Z01 [C_t = 23.42], Z22 [C_t = 22.21] and Z31 [C_t = 32.85]) were subjected to sequencing analysis. The PCR products of the amplified fragments were directly purified with a master mix of ExoSAP (Nucleics Pty Ltd., Woollahra, Australia). Using the same specific primers, Sanger sequencing was performed on the ABI 3500XL Genetic Analyzer POP7TM (Thermo-Scientific Inc., MA, USA). The nucleotide of the successful sequences were compared with those of the reference strains available in the NCBI GenBank using BLAST tool [44]. Since Sapovirus is closely related to Norovirus, Noronet typing tool was used to determine the SV genotypes [25].

4.5. Statistical Analyses

Bayesian linear regression (ANOVA analysis) and Descriptive (Pearson χ^2, 2-sided analysis) methods were performed for data analysis using IBM SPSS 26 software (IBM, Sandton, SA). Tests were used to determine statistical significance ($p < 0.05$).

5. Conclusions

The presence of SV in developing settings of the Vhembe region is evident. This is the first cross-sectional study to report on defined human SV strains in rural communities from South Africa. Outpatients in rural settings are potentially exposed to possible risk of the burden of diarrhoeal disease triggered by SVs among other pathogens and several factors including water, sanitation and hygiene practices. However, scientific data from Africa to report on enteric viruses as diarrhoeal causative agents are scant. Further investigation on the analysis and surveillance of human SV strains in rural settings (community or household level) is essential to assess burden of diseases.

Author Contributions: Performed the laboratory analysis and drafting of the manuscript, M.M.; revised the manuscript, J.-P.K.N.; performed statistical analysis and revised the manuscript, A.N.T.; conceived the idea and revised the manuscript, N.P. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of University of Venda (SMNS/18/MBY/02 and 01/2017), and permission to use health facilities was obtained from the Limpopo Provincial Department of Health and the District (Ref:4/2/2).

Informed Consent Statement: Informed consent was obtained from all parents or guardians of the children involved in the study.

Data Availability Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER 5

RESEARCH ARTICLE

Objective 3: Analysis of the capsid protein (VP1) of the detected human Sapovirus

Scientific data on sapovirus capsid proteins (VP1) is worth reporting, as it may bring insight on vaccine production. VP1 as a major protein of SV, carries vital amino acid sequences responsible for coding structural proteins intended for the functioning of this virus. Analysis of full capsid protein from the detected human SV was attempted, and was unsucceful. Therefore, this study reported on the analysis of a partial capsid protein of human SVs.

Partial analysis of the capsid protein (VP1) of Human Sapovirus isolated from children with diarrhoea in rural communities of South Africa.

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Research Article

Partial Analysis of the Capsid Protein (VP1) of Human Sapovirus Isolated from Children with Diarrhoea in Rural Communities of South Africa

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Background. Viral diarrhoea is a concern in acute gastroenteritis cases among children younger than 5 years of age. Sapovirus has been noted as an emerging causative agent of acute gastroenteritis worldwide. *Objective/Study Design*. The aim of this study was to characterize human sapoviruses targeting the VP1 (NVR and N-terminal) region. Twenty-five samples were randomly selected from 40 sapovirus-positive samples previously detected and analyzed for the VP1 region using the One-Step RT-PCR assay. The PCR products were subjected to Sanger sequencing analysis. *Results*. The polyprotein segment (NVR and N-terminal) was successfully amplified from 10/25 samples. Sapovirus GI.1 was the most predominant strain (6/10; 60%), followed by SV-GII.1 (2/10; 20%) and 10% of each GI.3 and GII.3. *Conclusion*. Through the partial analysis of the VP1 region, this study provides more data to add on the human sapovirus genetic characterization of circulating strains in South Africa, with the proposition of further analysis of sapovirus VP1 fragments for the viral structure and function.

1. Introduction

Human sapovirus (SV) as one of the leading causative agents of diarrhoea in young children is becoming notable worldwide [1, 2]. Information on the distribution of SV genotypes can give insights into the patterns of probable transmission, immunity amongst exposed people, relevant diagnosis of the circulating strains, and development of vaccine to regulate or eradicate virulent strains [3]. Human SV has four well-known genogroups, namely, genogroups I, II, IV, and V. Although SV-GI is the most detected genogroup followed by SV-GII, these genogroups are commonly associated with acute gastroenteritis across age groups and often detected in infants [2, 4–8]. There are few studies on the detection of human SV in South Africa which have been reported in different settings, that is, on rural outpatients [9], in a longitudinal study [10], on urban hospitalized children [11, 12], and on all age groups [13].

Human SV, a single-stranded positive-sense RNA virus classified in the *Caliciviridae* family, has a genome estimated to be 7.7 kb in size. Amongst the three open reading frames (ORF-1, 2, and 3) documented, ORF-1 amongst other proteins contains the major capsid protein (VP1). The VP1 region is used for the classification of sapoviruses [14], and it is the most common targeted area for detection of this virus. Moreover, VP1 contains a segment which correlates with the genetic diversity and antigenicity of SV [4, 15, 16].

A variety of primers have been designed from previously accessible nucleotide sequences and tested for the detection of SVs [1, 15]. The capsid protein is described to have four regions, namely, *N*-terminal variable region (NVR) with 1–43 amino acid aa sequence containing 6.1% conserved residues, followed by the well-conserved section of the *N*-

Table 1: Primers for amplification of VP1 segments [17].

PCR type	Primer	Sequence	Location	Product size (bp)
One-Step RT-PCR	SV-F11	GCY TGG TTY ATA GGT GGT AC	5098–5117	781
	SV-R1	CWG GTG AMA CMC CAT TKT CCA T	5857–5878	
	KSV-F8	ATG GAM AAT GGK GTK TCA CCW G	5857–5878	632
	KSV-R8	AGC CAG TGT GGC TGT GA	6473–6488	
	KSV-F9	GAC TTT GAC ACY AGT GGY TTT GC	6379–6401	670
	KSV-R9	CCA TTR ATG GAG AGG TCY CG	7029–7048	

terminal region (*N*) with 44–285 aa sequence containing 40.5% conserved residues, a central variable region (CVR) with 286–441 aa sequence containing 5.9% conserved residues, and a last quarter of the *C*-terminal region (*C*) with 442–561 aa sequence containing 27.3% conserved residues [16].

The conserved amino acid motifs of SV are predicted to be positioned on VP1. Therefore, analysis of this protein may play a role in proper understanding of the diversity of strains that are circulating, for epidemiological surveillance to monitor emerging strains and controlling of pathogens posing serious illness among people. The detections of human SV have been reported worldwide, but mostly targeting a short sequence at the conserved RdRp/VP1 junction region of these viruses [2, 15]. This study aimed at reporting on the identification of human SV targeting a larger conserved region of the capsid protein (VP1).

2. Methods

2.1. Ethical Clearance and Consent. Ethical clearance for this study was obtained from the UNIVEN Research Ethics Committee (SMNS/18/MBY/02), and permission to collect samples was obtained from the Limpopo Provincial Department of Health and the District (Ref: 4/2/2). Written informed consent was obtained from all parents or guardians of participating children before stool sample collection.

2.2. Sample Selection. Twenty-five (62.5%) randomly selected and extracted RNA samples, from 40 samples which were previously identified as SV-positive [9], were subjected to further RT-PCR amplification targeting the fragments which form the capsid protein (VP1).

2.3. Amplification of Partial VP1 by RT-PCR. The One-Step RT-PCR (QIAGEN, Germany) kit was used for amplification of the selected SV-positive samples. Selected primers (Table 1) were used on the attempt to target region of the VP1 sequence [17]. The reagent mixture for SV amplification were as follows: 25 μl of reaction volume containing 5 μl of 5X One-Step RT-PCR buffer, 1 μl of One-Step RT-PCR enzyme mix, 1 μl of dNTP mix (containing 10 mM of each dNTP), 1.5 μl of 0.6 μM of each primer, 10 μl of RNase-free water, and 5 μl of the RNA sample. Amplification was done using the following conditions set on a T100 Thermal cycler (Bio-Rad, USA): reverse transcription at 50°C for 30 min; followed by initial PCR activation at 95°C for 15 min; then 39 cycles of three-step cycling (denaturation for 30 sec at 94°C, annealing for 30 sec at 53.5°C, and extension for 60 sec at 72°C); and final extension at 72°C for 10 min.

The location of part of the genome targeted for PCR amplification is important to understand the diversity and virulence of pathogens which are detected. Figure 1 displays the targeted locations by selected primers aligned against the VP1 segment, for amplification of human sapoviruses. In previous studies, the most reported target region of the SV genome has been the RdRp/VP1 junction [15].

2.4. Sequencing. PCR products of the amplified fragments were directly purified with a master mix of ExoSAP (Nucleics, Australia). Using the same primers (Table 1), Sanger sequencing was performed on the ABI 3500XL Genetic Analyzer POP7TM (Thermo-Scientific). The obtained nucleotide of the successful sequenced amplicons were compared against those of the reference strains available in the NCBI GenBank, using BLAST available at https://www.ncbi.nlm.nih.gov/blast [18]. The reference strains with sequences of ≥709 nucleotides (SV-GI) and ≥644 (SV-GII) nucleotides were randomly selected among BLAST hits with >85% similarities on the query sequences of SV strains detected from this study. For confirmation of SV genotypes, the human calicivirus typing tool available at https://norovirus.ng.philab.cdc.gov [19] was used. Phylogenetic analysis was performed to check for close relatedness of human SV strains using MEGA 11 [20]. The confirmed nucleotide sequences were submitted to GenBank under the accession numbers OK180480–OK180489.

3. Results

3.1. RT-PCR Amplification. This study reports on 40% (10/25) successful amplification of partial VP1 using SV-F11 and SV-R1 primers, while other 15 samples failed to amplify. In addition, other pairs of primers used failed to amplify in all selected samples. The unsuccessful amplifications are suggested to be as a result of degraded or limited RNA genome copies. Sixty percent (6/10) of the results was identified as SV-GI.1, followed by 20% (2/10) SV-GII.1 and 10% of each GI.3 and GII.3 (Table 2). Of these 10 amplified samples, 60% (6) were obtained from outpatient children with diarrhoea and 40% (4/10) from hospitals based in the rural communities of Vhembe district, South Africa.

3.2. Sequence and Phylogenetic Analysis. The sequences generated from this study were identified as the polyprotein segment. The human calicivirus typing tool gave a BLAST score of 75–99% capsid protein identity. A BLAST search

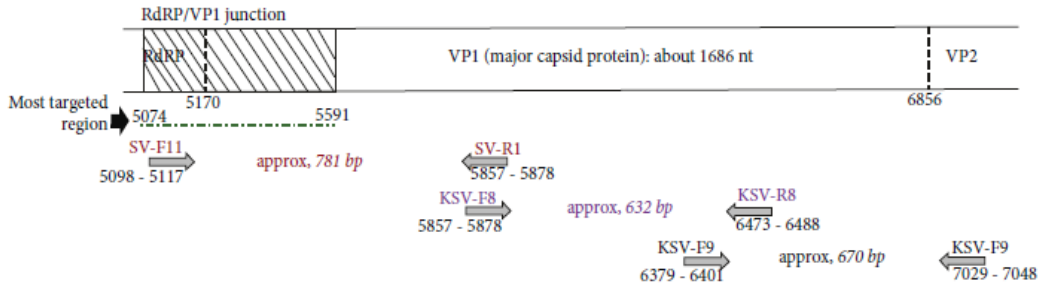

FIGURE 1: Schematic diagram of human sapovirus partial genomic organization and RT-PCR target regions. The diagram presents the most targeted RdRp/VP1 junction by RT-PCR pointed by a black arrow, and the location of primers targeting the complete VP1 segment shown by grey arrows on the binding location.

TABLE 2: Positive samples by partial VP1 analysis.

Sample ID (admitted)	Collection date	Place coordinates	Sapovirus genotype	Accession numbers
R17 (Mph.clinic)	2019/11/29	22°38′58.6″S 30°49′48.0″E	GI.1	OK180480
R77 (Tsh.hospital)	2020/02/26	22°59′42.0″S 30°24′52.7″E	GI.1	OK180481
R95 (Sil.hospital)	2020/10/03	22°54′03.5″S 30°11′37.3″E	GI.1	OK180482
R26 (Tsh.hospital)	2019/12/18	22°59′42.0″S 30°24′52.7″E	GI.1	OK180483
R102 (Eli.hospital)	2020/05/03	23°09′16.6″S 30°03′20.2″E	GI.1	OK180484
Z01 (Xig.clinic)	2018/02/11	22°55′29.0″S 30°43′21.7Ë	GI.1	OK180485
R21 (Maj.clinic)	2019/11/20	23°13′17.6″S 30°20′08.8″E	GII.1	OK180486
R80 (Sil.clinic)	2020/02/28	22°54′03.2″S 30°11′37.2″E	GII.3	OK180487
R24 (Maj.clinic)	2019/04/12	23°13′17.6″S 30°20′08.8″E	GII.1	OK180488
Z31 (Mal.clinic)	2018/11/03	23°00′47.9″S 30°42′05.2″E	GI.3	OK180489

gave a hint of 87–98% similarities of all featured sequences on a phylogenetic tree (Figures 2 and 3).

The phylogenetic tree (Figure 2) presents two distinctive clusters of genotypes, SV-GI.1 and SV-GI.3, showing relatedness of their strains. The detected SV-GI.1 genotypes (OK180480–OK180485) from this study presented an isolated cluster showing their close relatedness and a slight relatedness to a strain detected in Congo from a chimpanzee (KJ858686), with a common ancestor. The GI.1 strains detected in this study showed similarities of between 93 and 96.43% with KJ858686 strain on BLAST. The detected SV-GI.3 (OK180489) from this study clustered with a strain (MN102410) detected in Taiwan, and this strain had 97.38% identity on BLAST. Although other reference strains had similarities of between 90 and 98% (BLAST) with the detected strains from this study, they showed distinct clusters when rooted by a porcine SV strain (MF766258).

The phylogenetic tree (Figure 3) displays two distinctive clusters of genotypes, SV-GII.1 and SV-GII.3, showing their strains' relatedness. The detected GII.1 strains (OK180486 and OK180488) from this study did not cluster with any of the reference strains although sharing a common ancestor and similarity hints of >85% on BLAST. Within a GII.3 cluster, relatedness of a strain (OK180487) detected in this study with a strain (MF944258) detected in China is shown by a distinct cluster and these strains had a similarity of 89.62% on BLAST. Reference strains used on the phylogenetic tree (Figure 3) gave similarity hints of between 85% and 92.55% (BLAST) to the detected strains from this study, although they present distinct clusters from strains reported in this study.

4. Discussion

The detection of circulating human SVs has been previously reported based on the analysis of small fragments (especially the RdRp/VP1 junction) on the ORF1 segment of the viral genome [15]. In this study, the successful amplification of partial VP1 provides valuable data on circulating SV strains in South Africa.

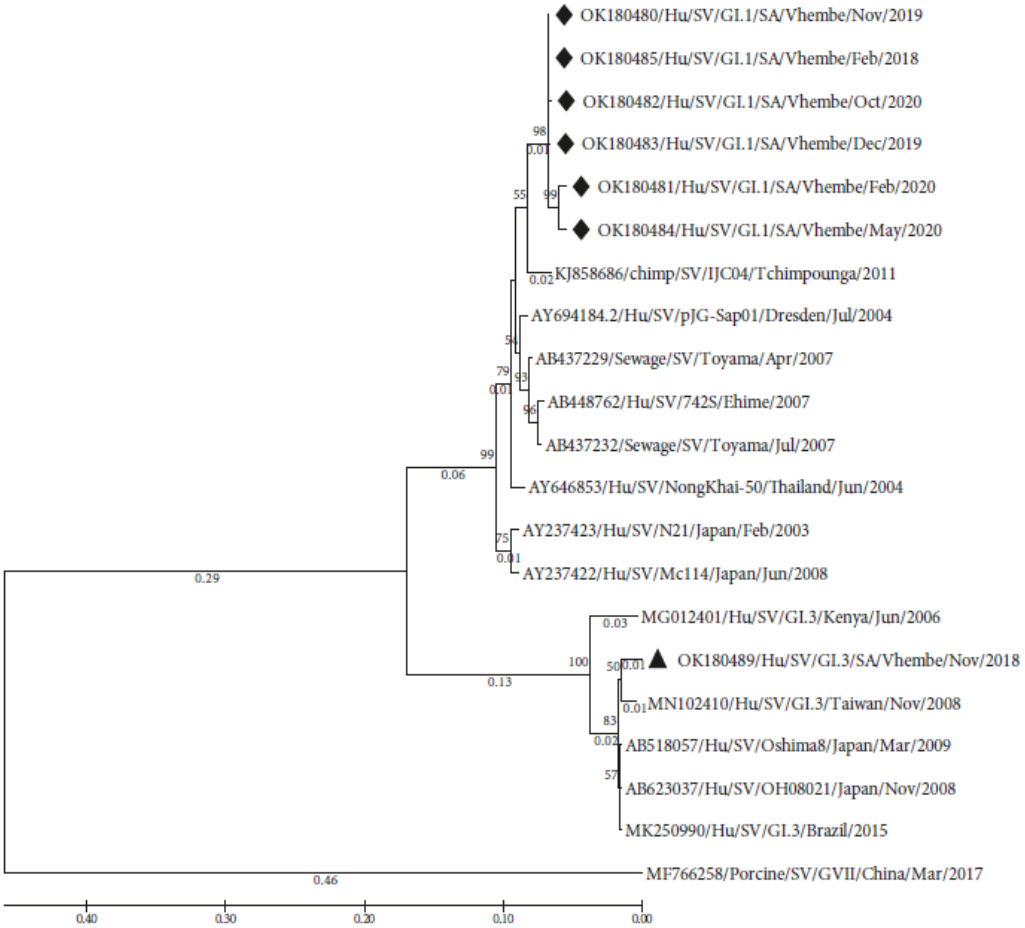

FIGURE 2: Phylogenetic analysis of the partial polyprotein of human SV-GI detected in Vhembe district (South Africa) and reference strains selected from the GenBank database. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. A bar scale representing a genetic distance scale. The phylogenetic tree was deduced by the maximum likelihood method and the Kimura 2-parameter model using MEGA 11 [20, 21], based on a 709-nucleotide sequence fragment of the polyprotein (a VP1 segment) showing relationships of SV strains. The porcine SV (MF766258) was selected as an outgroup strain.

This study reports on 40% (10/25) partial sequencing of the VP1 (polyprotein) fragment of the human SV strain circulating in the rural areas of South Africa. It has been previously reported that single-stranded RNA is generally known to be very unstable, which may lead to difficulties of generating positive results [21]. This may explain the reason that some amplicons failed to generate successful sequence in this study.

The SV GI.1, reportedly associated with gastroenteritis [23], was noted as a dominate genotype (60%: 6/10) from this study. SV-GI.1 strains were detected from 66.7% (4/6) cases of patients admitted in hospitals and 33.3% (2/6) cases of patients in clinics (Table 2). However, other strains (SV-GII.1 (20%: 2/10), SV-GI.3 (10%: 1/10), and SV-GII.3 (10%: 1/10)) were detected from patients aided in clinics. SV-GI.1 seems to be the most widespread genotype, since it was also reported as the predominant strain in a study from Brazil [4], with other genotypes detected at a low rate similar to the findings of this study.

The ORF1 of SV is proposed to encode a polyprotein that is processed by the viral protease, resulting in manifestation of proteins needed for the viral genome's replication [14, 24]. In this study, the analysis of long amino acid sequences of NVR and N terminals was performed. It has been proposed that analysis of viral polyproteins provides understanding in mechanisms and clues leading to the drug design against viral diseases [24]. Moreover, the NVR is reportedly common to all SV genotypes [15, 16], and *N*-terminal has been identified as an area which can undergo significant conformational variation [14]. The analysis of the capsid protein region (VP1) is very crucial, since genotyping of SV is based on the capsid, which strongly correlates with the viral antigenic properties [16].

Sapovirus GI is known to be associated with severe diarrhoeal disease. Furthermore, GI.1 strains have previously been detected in acute gastroenteritis cases in studies

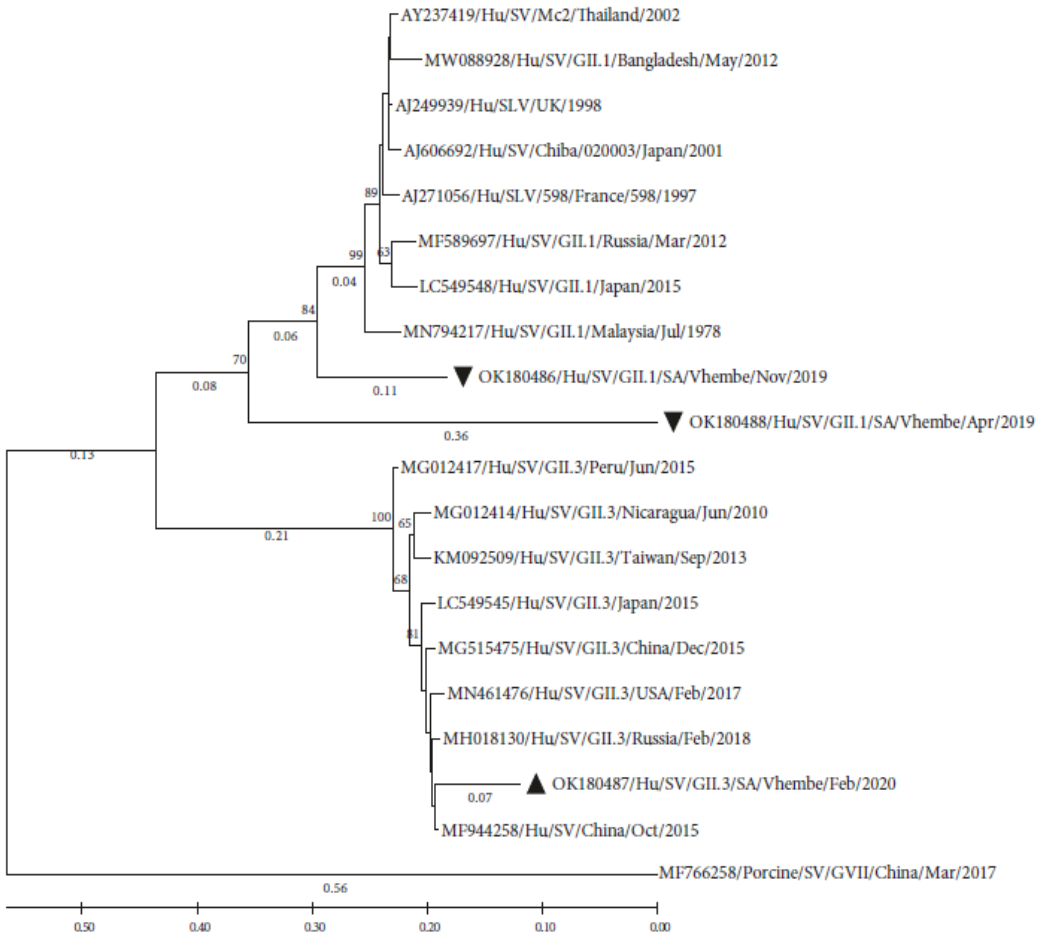

FIGURE 3: Phylogenetic analysis of the partial polyprotein of human SV-GII detected in Vhembe district, South Africa, and reference strains selected from the GenBank database. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. A bar scale representing a genetic distance scale. The phylogenetic tree was deduced by the maximum likelihood method and the Kimura 2-parameter model using MEGA 11 [20, 21], based on a 644-nucleotide sequence fragment. The porcine SV (MF766258) was selected as an outgroup strain.

conducted in Africa [23, 25, 26]. The phylogenetic analysis on this study showed a variability of strains detected. A discrete cluster of the detected strains (OK180480–OK180485) within genotype GI.1 suggests slight mutations on strains circulating in the Vhembe rural communities, South Africa (Figure 2). In addition, a slight relation of the cluster of GI.1 strains from this study with a strain (KJ858686) detected from a nonhuman host proposed a possibility of zoonotic transmission. Although close relatedness of human genogroups to the nonhuman genogroups have been predictable [27], more analysis on SV strains detected from human and other mammalians should be performed to confirm zoonotic transmission.

Sapovirus-GII is mostly associated with nonsevere diarrhoea, and it has been reportedly detected in diarrhoea cases in Africa [23, 25, 28]. Among other strains of genogroup-II, the most commonly detected is GII.1 as also reported in South Africa by Murray et al. [25]. The identified SV-GII.1 in this study clustered away from each other and reference strains, which could suggest possible mutations based on the occurred number of substitutions per site measured by the branch lengths (Figure 3).

A more successful detection rate of SVs has been commonly achieved by targeting the RdRp/VP1 junction [29, 30]. However, the analyzed segment in this study is reliable for accurate strain identification, since it is most variable and contains the maximum conserved residues on the VP1 sequence [15, 29]. From BLAST search list, there were no available data on SV strains previously reported in South Africa that could be used for phylogenetic relatedness. Moreover, analysis of a larger fragment on SV should be considered, as it creates a better possibility of genetic characterization by sequence analysis and comparison of strains around the globe. To our knowledge, this is the first study in South Africa to report on analysis of the large fragment (≥644 nucleotide long) of human sapovirus VP1.

5. Conclusion

Our study reports on partial analysis of VP1 protein (polyprotein), suggested to be responsible for viral protein folding, dimer formation, and viral particle assembly. Predominance of SV-GI.1 was determined in this study. Studies on the antigenicity and VP1 variations of SV are needed for accessing the role of SV as an emerging virulent agent associated with diarrhoeal diseases in young children. Analysis of SV's capsid protein should be carried out for epidemiological reference, to identify the diversity of virulent proteins produced and possibly to give insight into vaccine proposition.

Data Availability

All data supporting this results are included within the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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CHAPTER 6

SUMMATIVE COMMENTS AND RECOMMENDATIONS

6.1 SUMMATIVE COMMENTS

Diarrhoeal diseases remains one of the most important problems for child health, and is known as a cause of serious economic burden in developed countries (Okitsu et al., 2020). Moreover, the most common illness affecting young children around the world is viral diarrhoea (Kim et al., 2020). Sapoviruses are increasingly recognized as an important pathogen associated with sporadic AGE in young children (Li et al., 2020), and as a causative agent of outbreaks involving person-to-person transmission (Hergens et al., 2017). There are reports highlighting that SV infection may cause mild, self-limiting illness, whereas, other studies reports that SVs infections can lead to severe dehydration, which may require medical attention or hospitalization (Wu et al., 2020; Matussek et al., 2015; Lee et al., 2012; Svraka et al., 2010).

This study was designed with the intension of assessing if conditions in rural settings can influence the genetic characterization and transmission of SV. Therefore, the study investigated the prevalence and genetic characterization of human sapovirus strains circulating in the rural communities found in the Vhembe district of Limpopo province, South Africa. This aim was achieved through defining the genotypes, and characterizing the detected human SV strains by analyzing the capsid protein region; and also comparing the detected SV strains with the reference strains reported elsewhere around the world.

Area of study, Vhembe district, is one of the 5 districts that are predominantly rural found in Limpopo province, one of the disadvantaged provinces in South Africa. Most disadvantageous households are found in such rural settings where the probabilities of limited knowledge on pathogens causing diarrhoeal diseases are high. Households in rural areas are likely to have an overcrowded family setup with untreated domestic animals present, susceptible to poor hygiene practices and daily usage of untreated water especially from rivers or springs (Magwalivha et al., 2021). Moreover, vulnerability of these disadvantageous households (United Nations Millennium Development Goals report, 2015) may lead to children being more exposed to burden of diseases including severe viral gastroenteritis. Although, incidences of human SV in the developing regions have dominantly been reported on hospitalised patients in urban areas. A review report from this study highlighted the significance of SV been more prevalent within the low-income regions (10.40%) as compared to middle income regions (5.86%) (Magwalivha et al., 2018).

This cross-sectional study presents a 14.1% prevalence rate of human SV from the rural communities of Vhembe region in Limpopo province, SA. From this study's analysis, a high positivity rate of 70% (28/40) from outpatients and 30% (12/40) from the hospitalised patients was observed. With note, one hospitalised positive case was of a child admitted after 10 days of presenting with diarrhoea without medical attention prior hospitalisation (data not shown). This study also reported on a high SV detection in children <2 years of age presenting with diarrhoea (Magwalivha et al., 2021). As previously highlighted, the findings of high percentage of SV detection amongst children <1 year of age alert for concern in rural settings, because this areas have limited affordability of treatments, poor service delivery and lack of political commitment in such settings (United Nations Millennium

Development Goals, 2015), and that children are more vulnerable to infections due to development of their immune system (Willey et al., 2011).

A notable rate detection by real-time RT-PCR was accomplished, but amplification to acquire sequences for comparison analysis from this positive samples was limited. Viral load is generally known to provide the greatest analytic accuracy for identifying cases. Although in this study some of the real-time RT-PCR detection happened at high Ct value (i.e. low viral load), this method proved to be highly sensitive, providing fast and high throughput detection (Kralik and Ricchi, 2017). Additionally, it is suggested that use of real time PCR assays possess the potential of yielding a significant high detection rate, because of the addition of fluorescent probes in the assay which seems to increase the diagnostic specificity, as compared to using a conventional PCR assays (Diez Valcarce et al., 2021; Phillips et al., 2010).

The limited outcomes from amplification of larger fragment from positive samples in this study could have been due to encountered external factors including: late reporting of infections and collection of samples which might have led to getting very low viral load for analysis, and prolonged power cuts affecting sample storage leading to high chance of degradation of viral genome which contributed in limited analysis or poor outcomes. Viral genome template like single stranded RNA are generally known to be very unstable which may lead to the difficulties of generating positive results (Kralik and Ricchi, 2017). Furthermore, RNA viruses is known to generate high mutational changes, having small multi-segmented genomes, because of lack of DNA polymerase which have the proofreading ability for repairing damaged genetic material (Liu, 2011).

In this study, SV-GI was predominately (32.5%: 12/40) detected among patients presenting with diarrhoea. Previous studies have documented SV-GI to likely cause severe symptoms and frequently detected in severe acute gastroenteritis cases (Matussek et al., 2015; Bucardo et al., 2014). In most cases of severe dehydration, medical attention and hospitalization is essential. Sapovirus-GII was detected in 10% (4/40) diarrheoal samples, of which 1 of these was seen from a non-diarrhoeal sample. In contrast, Sapovirus GII has been previously detected in outpatients presenting with diarrhoea, although suspected to be less virulent as patients infected were often not hospitalised (Matussek et al., 2015; Bucardo et al., 2014). Sapovirus-GIV was only detected in 2.5% (1/40) of the samples, however, further analysis (sequencing) was not successful. Of the 16 samples amplified by RT-PCR, SV-GI and GII genogroups were detected in one sample, although, only GII was successfully sequenced from this sample.

Human SV strains identified in this study were compared to other SV strains reported around the world. Phylogenetic analysis showed some distinct clusters of genogroup 1 strains detected. The relatedness of the detected GI.1 genotypes from this study with a strain detected from a chimpanzee in Congo (KJ858686.1), and the relatedness of SV-G1.5 strains with a strain detected from food (ruditape) in Japan (AB765970.1) as documented (Magwalivha et al., 2021), were highly noted. These findings suggest the possibilities that conditions in rural settings can influence the genetic characterization of SV strains circulating in rural communities. The detection of polyprotein particles particularly of the SV-GI strains supports chances of SV's association with diarrhoeal infection in this case study,

since other enteric viruses including Norovirus, Bocavirus and Astrovirus from collaborative studies were not detected on the samples used.

Water used in the rural communities for daily purposes can be considered as a factor that might pose a threat of viral transmission, as supported by the reported significance of SV positive cases associated with water sources (Magwalivha et al., 2021). Department of Water and Sanitation (2015) in South Africa, reported on challenges of scarcity of water resources and sanitation in some provinces including Limpopo. Consequently, it is known that diarrhoeal causative pathogens may be transmitted through poor quality of water, untreated water storage or untreated water from the source. Moreover, most people in low income settings uses piped-water, stored or untreated water for domestic chores which lead to their vulnerability to a risk of the devastating effects of diarrhoea (Kapwata et al., 2018; Bessong et al., 2009; Potgieter et al., 2009). Detection of SV in environmental samples gives alert of the circulating transmissible viral particles within the communities.

The substantial detection of human SV from outpatient children in Vhembe region of SA is evident, and objectives of this study were attained. This study reports a high rate of SV detection in outpatient children as compared to the hospitalised children. Late or delayed consultation showed a sign of lack of awareness to communities, concerning the threat posed by diarrhoeal disease and their causative pathogens. Association of high detection of SV positive cases against water used, use of latrine, and breastfeeding, suggest that rural setting conditions can possibly influence the transmission of SV strains circulating in rural communities, possibly due to poor hygiene practices. More data on the burden of diarrhoeal disease is needed to understand the role of enteric viruses (especially SV) on acute

gastroenteritis cases, and for prevention and control of such pathogens in the underdeveloped rural settings.

6.2 RECOMMENDATIONS

The following recommendations were deduced based on the outcomes and limitations seen in this study:

- ✓ Studies presenting the defined human SV strains circulating, and relatedness of viral particles from environmental samples must be piloted to bring valuable data of viral transmission pathway(s) within the rural communities.
- ✓ Analysis of larger segment (especially VP1) or whole genome should be considered to get some wide-ranging outcomes.
- ✓ Investigations on the analysis and surveillance of enteric viruses including human SV strains is essential to assess burden of diseases in rural settings at a community or household level.
- ✓ Potential outbreaks of gastroenteritis should be monitored, in rural areas where poor sanitation infrastructure and water supply are identified.
- ✓ Educational programs on pathogens' transmission or contaminations should be frequently conducted with parents or guardians, as people gave the impression of underestimating diarrhoeal manifestation up until associated severe symptoms were manifested.

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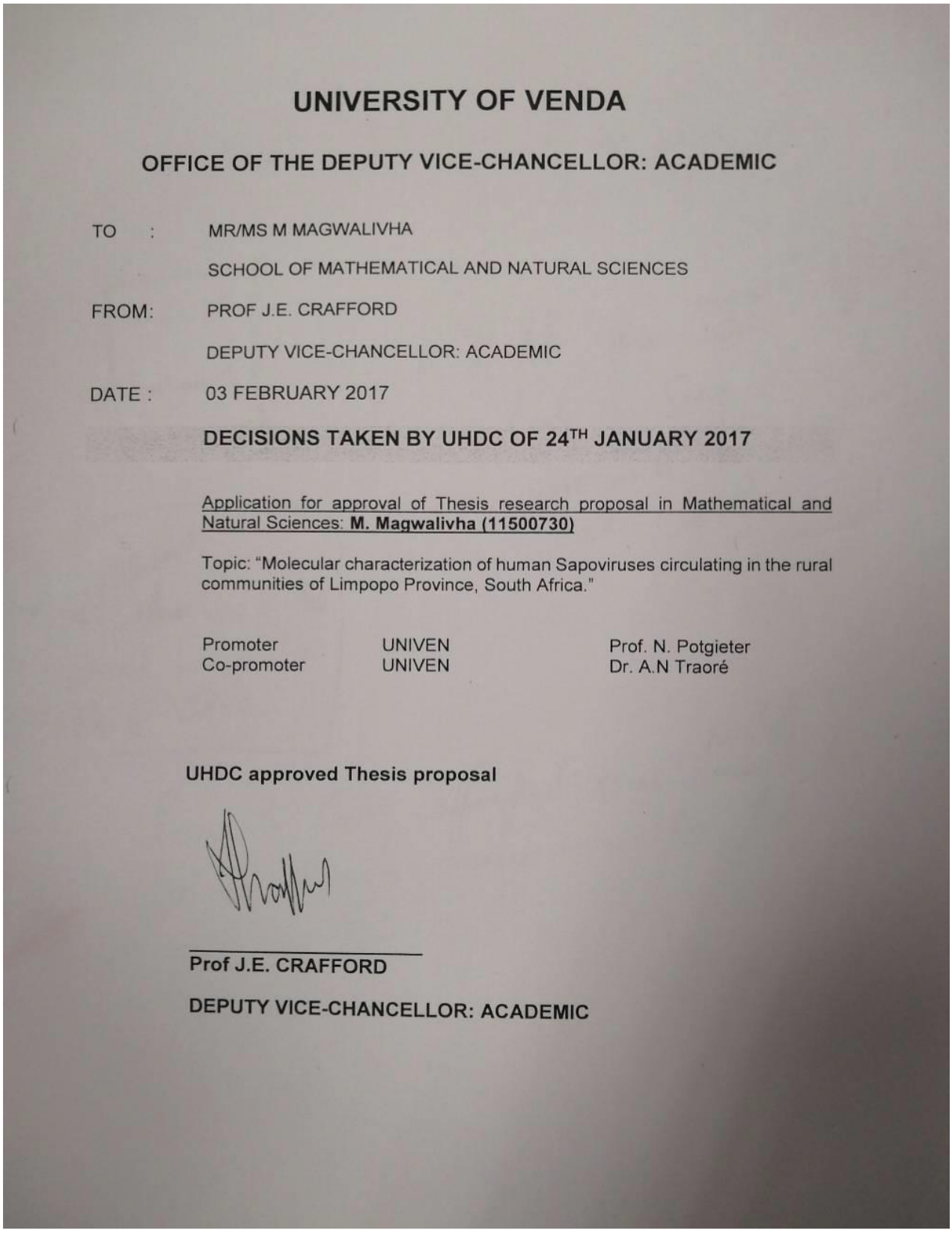
APPENDIX

A.1. Approval of Research study from University of Venda



UNIVERSITY OF VENDA

OFFICE OF THE DEPUTY VICE-CHANCELLOR: ACADEMIC

TO : MR/MS M MAGWALIVHA

SCHOOL OF MATHEMATICAL AND NATURAL SCIENCES

FROM: PROF J.E. CRAFFORD

DEPUTY VICE-CHANCELLOR: ACADEMIC

DATE : 03 FEBRUARY 2017

DECISIONS TAKEN BY UHDC OF 24TH JANUARY 2017

Application for approval of Thesis research proposal in Mathematical and Natural Sciences: **M. Magwalivha (11500730)**

Topic: "Molecular characterization of human Sapoviruses circulating in the rural communities of Limpopo Province, South Africa."

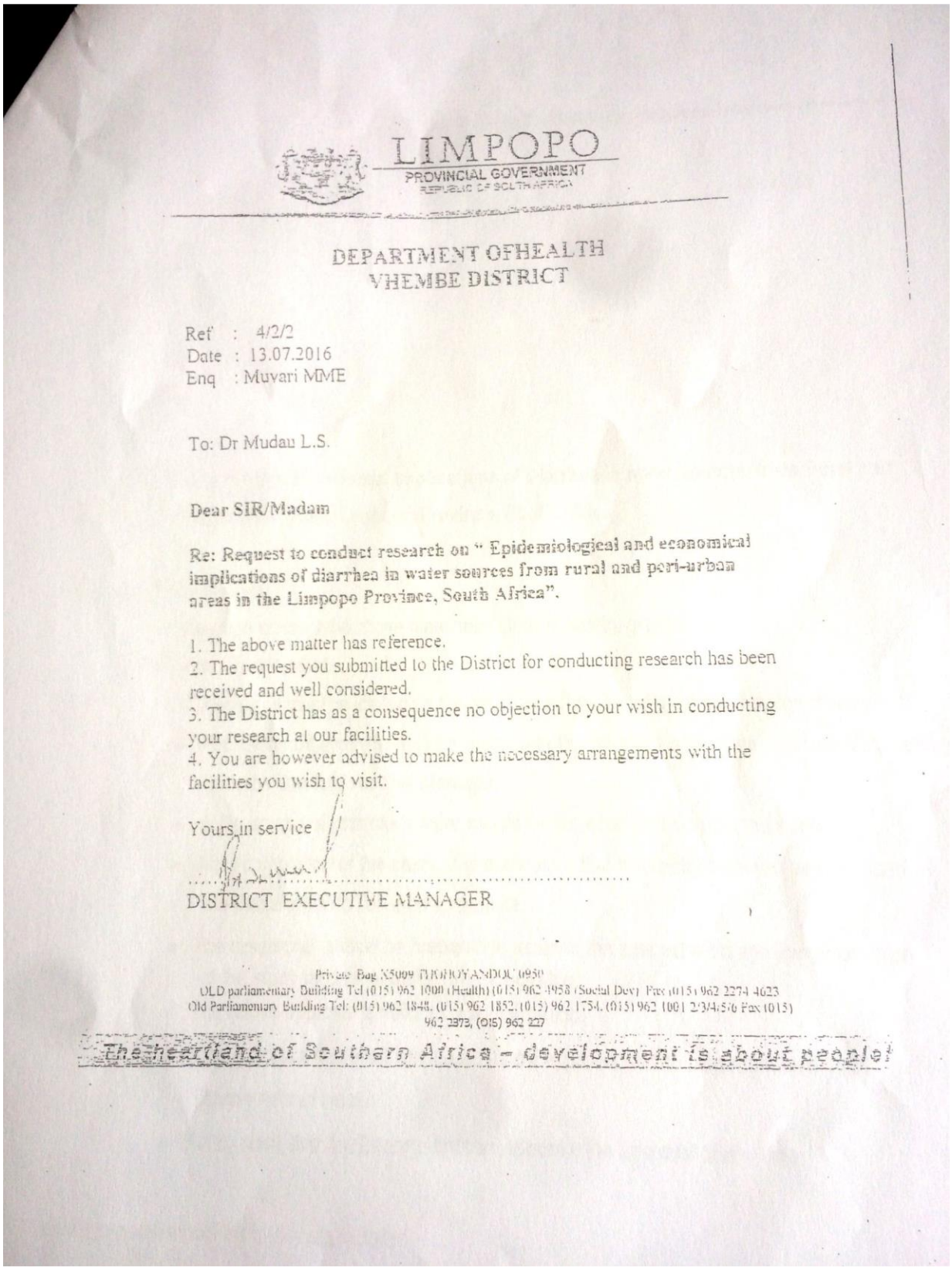
Promoter	UNIVEN	Prof. N. Potgieter
Co-promoter	UNIVEN	Dr. A.N Traoré

UHDC approved Thesis proposal

Prof J.E. CRAFFORD

DEPUTY VICE-CHANCELLOR: ACADEMIC

A.2. Approval of Research study from Department of Health district of Vhembe region, Limpopo province, South Africa



LIMPOPO
PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OFHEALTH
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.

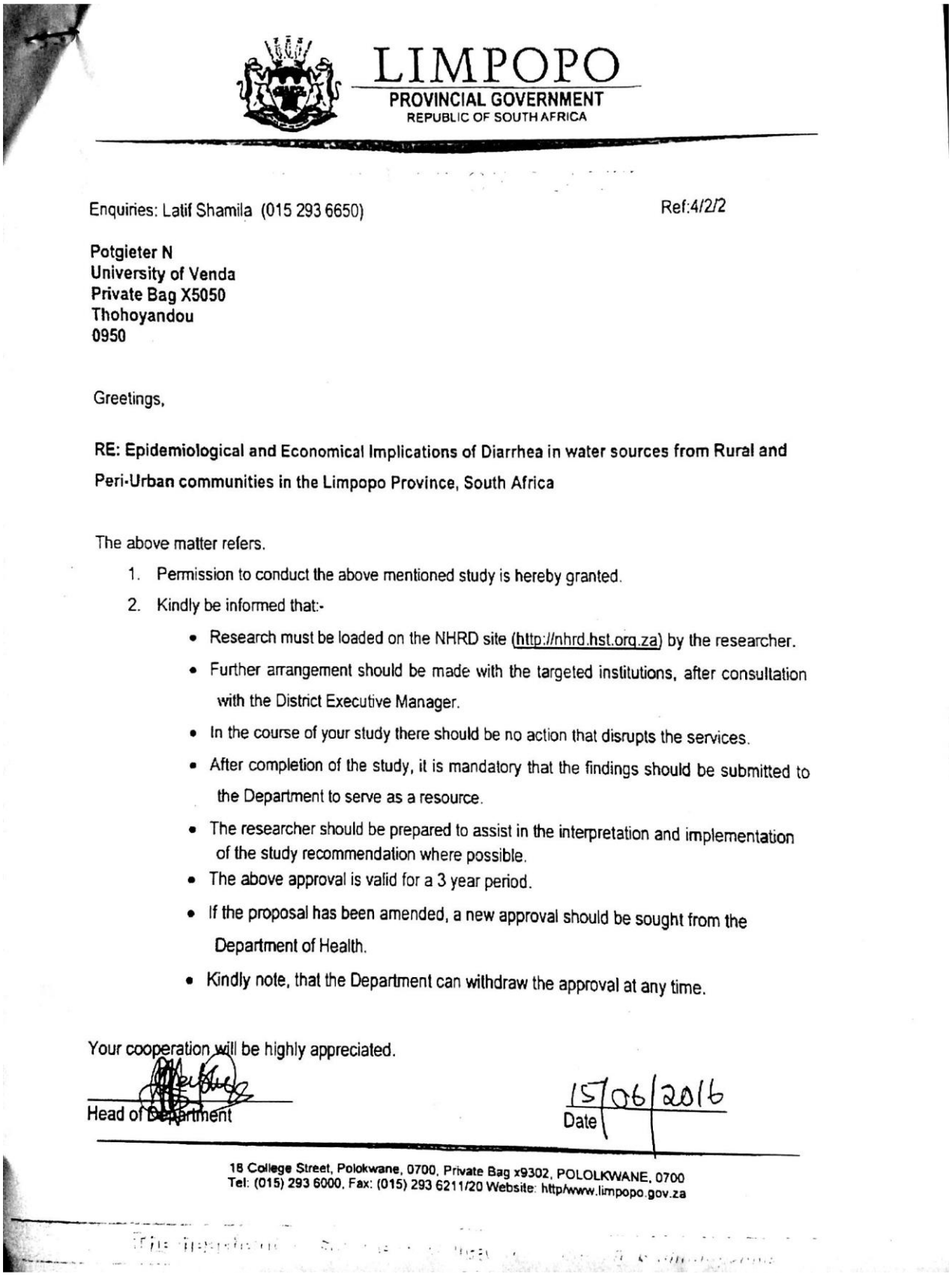
Yours in service

.....
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 2873, (015) 962 227

The heartland of Southern Africa – development is about people!

A.3. Approval of Research study from Department of Health Provisional government, Limpopo province, South Africa



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, POLOKWANE, 0700
Tel: (015) 293 6000, Fax: (015) 293 6211/20 Website: http/www.limpopo.gov.za

A.4. Consent form used in this study (English version)

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

Molecular characterisation of human Sapoviruses circulating in the rural communities of Limpopo province, South Africa.

Investigators:

Mr M Magwalivha (PhD student)

Prof Natasha Potgieter (Promoter)

Prof AN Traore (Co-promoter)

Address:

Department of Microbiology

Life Science Building

School of Maths 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 studies 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 diarrhoea, 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 Sapovirus circulating in the rural communities of the Limpopo province.
- This information will help decisions making in public prevention strategies against diarrhoea disease and respiratory infection particularly in Sapovirus infections also in the improvement of sanitary environments in rural communities. The findings of this study will also provide information on Sapovirus diversity with implications on vaccine development.
- General information will be taken from you, including contact details, age, gender, use of toilet, date of diarrhoea, 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 diarrhoea.

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 diarrhoea episodes. Respiratory swabs will 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 characterisation of human Sapoviruses 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 pressurised 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 understand 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

A.5. Consent form used in this study (Tshivenda version)

FOMO YA THENDELO NA BAMBIRI ḼA ZWIDODOMBEDZWA ZWA MUDZHENELELI

ṰHOHO YA THANDELA YA ṰHOḌISISO:

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

Vhasedzulusi: Mr Magwalivha M (Mutshudeni wa PhD)

Prof. Natasha Potgieter (Muṱoli)

Prof. Afsatou N. Traore (Muthusa-mutoli)

ḌIRESI: Department of Microbiology

Life Science Building

School of Maths 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 na vhahulwane vha 250 vha do lavheleswa kha ino thandela.
- Ṱhoḓisiso hei yo livhiswa kha u ṱoḓulusa tshakha dza virasi dza Sapo 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 Sapo 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 Sapo 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 Sapovirus 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 ḽiṅwalo 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**

<u>Muano nga muḓologi:</u>

Nṋe(dzina)……..ndi khou ana uri:

- ✓ Ndo thusedza musedzulusi (dzina)…… …u ṱalutshedza mafhungo are kha ḽiṅwalo heḽi kha (dzina ḽa mudzheneleli)……hu tshi khou shumiswa luambo lwa Tshivenḓa.

- ✓ Ro mu̱tu̱tu̱wedza u vhudzisa dzimbudziso ra dzhia tshifhinga tsho teaho u dzi fhindula.
- ✓ Ndo n̤ekedza mafhungo one one o teaho a sina tshi̱tahe kha zwe zwa vha zwi tshi khou elana na nn̤e
- ✓ Ndo fushea ngauri mudzheneleli o pfesesa nga vhu̱dalo zwine zwa vha kha l̤iṅwalo l̤a thendelo l̤o tevhelaho maga a mulayo o n̤etshedzwaho nahone mbudziso dzawe dzo̱the dzo fhindulwa lu fushaho

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

_____ _____

Tsaino ya Mu̱dologi **Tsaino ya t̤hanzi**

A.6. Data capture forms used in this study

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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:	Diarrhoea ☐	Fever ☐	Vomiting ☐ Dehydration ☐
	Respiratory tract infection ☐	Immunodeficiency ☐	Dehydration ☐
	Abdominal pain/cramps ☐		
Date of Onset: _____	***Rota Vaccine dose received*** ☐		
How many days of presenting with diarrhoea before consulting:			
Sample collection			
Date of collection:	_____		
Type of sample:	Type of Stool: Watery ☐	Sausage ☐	Mushy ☐
Treatment			
Current :			
Previous :			
Laboratory Results			
PCR:			
Sequencing:			

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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 ☐			
Treatment			
Current :			
Previous :			
Laboratory Results			
PCR:			
Sequencing:			

A.7. Extraction method used in this study

Boom method for Nucleic acid extraction

1. Pea-sized amount faeces in 500 µl PBS (or less if small volume)
2. Centrifuge for 15 sec @ 12,000 rpm
3. Transfer supernatant in 1.5 ml sterile, add 900 µl L6 buffer (please wear nitrile gloves)
   modification: *add 20 µl of Internal control*
4. Vortex for 1 min and centrifuge for 15 sec @ 12,000 rpm
5. Transfer the supernatant to a new 1.5 ml tube
6. Add extraction matrix (100 µl Silica beads) {***note*: Vortex** ~1 min **the extraction matrix before use**}
7. Vortex the solution for 15 sec and shake softly for 15 min (or on Rocking platform)
8. Centrifuge tube @ 2,000 rpm for 15 sec and discard the supernatant
9. Add 500 µl of L2 buffer and mix, Centrifuge @ 2,000 rpm for 15 sec and discard the supernatant (**repeat this step: X2**)
10. Add 500 µl of 70% Ethanol and mix, Centrifuge @ 2,000 rpm for 15 sec and discard the supernatant (**repeat this step: X2**)
11. Add 500 µl of Acetone and mix, Centrifuge @ 2,000 rpm for 15 sec and discard the supernatant (**repeat this step: X2**)
12. Dry the Silica pellet by placing the opened tube in a heat block @ 50 °C for 5 min
13. Re-suspend the Nucleic acid with 150 µl PCR grade water, Mix well and heat @ 56 °C for 5 min
14. Centrifuge @ ***Maximum speed for 20 min***
15. 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).