NOROVIRUS-HOST INTERACTION STUDIES: HBGA PHENOTYPIC AND GENOTYPIC PROFILES OF PAEDIATRIC PATIENTS WITH DIARRHOEA FROM RURAL COMMUNITIES OF LIMPOPO SOUTH AFRICA

by

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DECLARATION

I, **Ronewa Khumela (11632210)** declare that the thesis titled: **Norovirus-host interaction studies: HBGA phenotypic and genotypic profiles of paediatric patients with diarrhoea from rural communities of Limpopo South Africa,** hereby submitted to the University of Venda for the PhD degree in microbiology has not been previously submitted by me to this or any other University and that the work contained here is my own work in design and execution, and that all materials used here are duly acknowledged.

Signature……  …… Date…….21/02/2024……

DEDICATION

I dedicate this work to my family (My Daughter Uzwothe, My Mom, Dad, and Sisters).

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EXECUTIVE SUMMARY

Human norovirus (HNoV) are the leading aetiological agents of viral acute gastroenteritis (VAGE) worldwide. Approximately 685 million cases of VAGE and 219 000 deaths associated with norovirus infection are recorded each year. The burden is more severe in developing nations such as those in Africa, and children below the age of five are the most vulnerable. Noroviruses are commonly transmitted from person-to-person via the faecal oral route and contaminated fomite, food and water. Norovirus are extremely prevalent and exhibit great genetic diversity and rapid rates of evolution.

Studies have shown that histo-blood group antigens are essential genetic susceptibility factors for HNoV infection. Histo-blood group antigens (HBGAs) are a class of carbohydrates antigens found in the surface of red blood cells and bodily fluids or tissues. HNoVs recognize and bind to HBGAs for attachment to human epithelial cells in the gastrointestinal tract. The synthesis of HBGAs is mediated by fucosyltransferase and glycosyltransferase, which are genetically regulated by FUT2 (Secretor), FUT3 (Lewis) and ABO (H) genes. Individuals with functional FUT2 gene are termed secretors; they express HBGAs in gut epithelial cell surface and body fluids. Natural variability and inability to express HBGAs (due to mutations) plays a role in population genetic susceptibility to norovirus infection and genotype distribution.

Approximately 20% of VAGE are caused by norovirus. Recently, global prevalence of norovirus was reported at 16%. In African countries, the range of reported prevalence was between 13% and 20%. However, higher prevalence has been reported, particularly in Ghana and South Africa. Previous data in South Africa have demonstrated the occurrence of norovirus, with higher number of cases reported in rural communities. There are limited or no studies to explain the elevated prevalence of enteric HNoVs in rural communities of Limpopo, South Africa.

Various factors including genetic, geographical, meteorological and socio-economic may play a role in the epidemiology of enteric viruses. A potential interaction between human and viral genetic diversity has been demonstrated via association of population secretor profiles and viral genotype susceptibility and distribution. Therefore, this study, aimed to investigate the prevalence and norovirus host genetic susceptibility

profiles in paediatric population under five years, within rural communities of South Africa.

To achieve the aim of this study, the following objectives were set: 1) To determine molecular characteristics of HNoV strains circulating in the rural communities of Vhembe district, South Africa; 2) To determine host phenotypic profiles of HBGAs (ABO/H and Lewis system) in HNoV infections among children from rural communities of Vhembe district, South Africa; 3) To determine the host genotypic profiles of HBGAs (FUT2/FUT3) in HNoV infections among children from rural communities of Vhembe district, Limpopo Province (South Africa) and 4) To assess the correlation between circulating HNoV genotypes and HBGAs profiles in young children from rural communities of Vhembe district, Limpopo Province (South Africa).

To achieve the study objectives, a cross-sectional survey in children with (200) and without (100) diarrhoea, below the age of five was performed. Co-paired stool (300) and saliva (300) samples were collected from October 2019 to September 2021, in South African healthcare institutions (clinics and hospitals) within the Vhembe district of Limpopo province.

Objective 1: HNoVs were detected from stool samples using real-time RT-PCR, then amplified by conventional RT-PCR, and further genotyped by Sanger sequencing. Norovirus strains from this investigation were compared to the worldwide circulating strains by phylogenetic analysis using MEGA11. ClustalW software was used to compare nucleotide similarity among strains genotyped in this study and those previously obtained within the same study region.

Objective 2: HBGAs phenotypic profiles were determined from saliva samples by ELISA assays. Monoclonal antibodies including A, B, Le-a, and Le-b were used to determine HBGAs phenotypic profiles in saliva samples. In samples where the phenotypic status was inconclusive, the secretor and non-secretor phenotypes were further confirmed using the *Ulex europaeus* agglutinin (UEA-1), which is specific for Fucα1-2Gal-R present in secretor, but not in non-secretor saliva.

Objective 3: Genotypic characterization of HBGAs were determined by Touch-Down-PCR using the Platinum Taq DNA Polymerase High Fidelity enzyme. The primers

used to detect SNPs were also used for nucleotide sequencing of both FUT2 and FUT3 partial genes of selected samples (100).

Objective 4: Statistical analysis was performed using Graphpad 10.0 for the association of HNoV prevalence and HBGAs profiles.

The results indicate that HNoVs were still highly prevalent in children from Vhembe district, with a confirmed significant difference ($p < 0.0001$) between symptomatic (37%) and asymptomatic (14%). Genogroup II noroviruses predominated children with AGE (80%), whereas genogroup I norovirus were high in children without AGE. Genotype GII.4 Sydney 2012 [P31] were dominant throughout the study period. The detected strains were phylogenetically closely related to the worldwide circulating norovirus strains but showed low nucleotide similarity when compared to other HNoV strains previously characterized in Vhembe region.

Elisa assay findings showed diversity of HBGA phenotypic profiles including Lewis and ABO type amongst study participants. Both Lewis negative and positive phenotypes were identified with the latter being the predominant and mostly infected with HNoV. All ABO phenotypes were also identified within the study population. The highest occurring was type O, which also represented the primarily norovirus infected group, followed by type A. Overall, majority of children had secretor phenotypes (81%) over non-secretors (19%), similarly, higher cases on HNoV were detected in children with secretor phenotype (93%).

Amongst the selected samples for FUT2 genotyping, only 42 could be amplified and of those, 36 (36%) were successfully genotyped. Majority of FUT2 secretors were homozygous mutations (64%) compared to heterozygous (28%). However, only 8% were determined as non-secretor with homozygous nonsense mutation on the G428A position. FUT3 genes were also associated with norovirus positivity. The G508A SNP was the most common and predominant among positive norovirus cases. Interestingly, 2 mutant SNPs were detected. Although non-secretor was the lowest in HBGAs genotypic profiles and is often related to resistance to infection, norovirus GII.4 Sydney 2012 [P31] were identified in two samples from hospitalized children.

Overall, the data revealed correlation between the presence of HNoV and the population HBGAs profiles. Individuals expressing HBGAs of secretor status were

highly infected with norovirus, whereas non-secretor individuals demonstrated protection or less susceptibility towards norovirus infection. The GII.4 Sydney 2012 [P31] recombinant strain was dominant, followed by the GII.4 Sydney 2012 variant in all the secretor profiles.

This study concludes that the prevalence and susceptibility of human norovirus infection in symptomatic and asymptomatic children from rural communities of Vhembe District is related to their HBGAs genetic profiles. The dominance of GII.4 Sydney 2012 [P31] in AGE children may be explained by the strain's epidemiological fitness, and genetic susceptibility related to the prevalence of secretor profiles. Population's genetic profiles may potentially drive human norovirus genetic diversity and evolution. This has implications for vaccine formulation in the current era of vaccine development. Further surveillance on impoverished communities where preventive strategies will most likely have greater impact is needed to monitor distribution of the virus and overall population HBGAs status.

Keywords: Human norovirus (HNoV), Viral acute gastroenteritis (VAGE), Histo-blood group antigens (HBGA), secretor, Non-secretor, Susceptibility

LIST OF ABBREVIATIONS

AGE	Acute gastroenteritis
EIAs	Enzyme Immunoassays
FBS	Fetal bovine serum
FUT	Fucosyltransferase
GI	Genogroup I
GII	Genogroup II
HBGAs	Histo-blood group antigens
HNoV	Human norovirus
NGS	next-generation sequencing
NoV	Norovirus
nSe	non-secretors
ORF	Open reading frame
PBS	Phosphate-Buffered Saline
PBST	Phosphate-Buffered Saline Tween
PC	polymerase-capsid
SA	South Africa
Se	Secretors
SNPs	Single nucleotide polymorphisms
TMB	Tetramethylbenzidine
UEA-1	Ulex europaeus agglutinin

VAGE	Viral acute gastroenteritis
VLP	viral like particle
WASH	water quality, sanitation and hygiene
WHO	World health Organisation

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Acute gastroenteritis (AGE), which is the condition leading to the inflammation of the gastrointestinal tract, is a globally frequent ailment affecting people of all ages and socio-economic backgrounds (Unicef, 2013). This condition is commonly accompanied by symptoms such as diarrhoea, vomiting, abdominal cramps, and sometimes fever (Graves, 2013). Acute diarrheal disease typically improves on its own over a short period of time, although it can cause serious complications for young, elderly or immunocompromised individuals (King et al., 2003). The burden of AGE including diarrheal disease is significantly felt globally, especially in underdeveloped nations where access to clean water and sanitary facilities is scarce (WHO, 2023).

There have been substantial improvements in several parts of the world, however, billions of people still live in conditions with inadequate drinking water quality, sanitation and hygiene (WASH) services, which poses a major health concern (Wolf et al., 2023). The risk of diarrheal diseases is significantly increased with poor WASH conditions. According to recent World health Organisation (WHO) estimates, 60% (54-65%) of deaths occurring in the low-and-middle income countries are attributed to poor WASH services and practice (WHO, 2019). Annually, approximately 829 000 deaths in these nations are due to unsuitable drinking-water (35%), sanitation (31%) and hygiene (12%) (WHO, 2019).

A variety of aetiological agents can lead to AGE including bacteria, viruses and parasites, however, majority of AGE are caused by viral pathogens (Elliott, 2007). Viral gastroenteritis is one of the leading causes of death globally and is amongst the most common cause of morbidity from infectious diseases (Bányai et al., 2018, Liu et al., 2015, Fischer Walker et al., 2012). Enteric viruses are the most common aetiological agents of viral acute gastroenteritis, contributing about 70% of cases since their discovery in the early 1970s (Bányai et al., 2018). Viruses frequently linked to acute gastroenteritis include rotavirus, norovirus, sapovirus, astrovirus and adenovirus (Flynn et al., 2024, Bányai et al., 2018, Chhabra et al., 2013). Although there are

various routes of transmission, enteric viruses are mainly transmitted via the faecal oral route (Bányai et al., 2018, Bishop and Kirkwood, 2014).

In countries where rotavirus vaccine coverage is high, norovirus has surpassed rotavirus as the leading cause of paediatric AGE (Stuempfig and Seroy, 2018, McAtee et al., 2016). Currently, human norovirus (HNoV) is the main leading cause of sporadic and epidemic viral acute gastroenteritis (VAGE) in both community and healthcare settings worldwide (Winder et al., 2022, Robilotti et al., 2015). Norovirus outbreaks in people of all ages have been reported around the world in different settings.

The most vulnerable population with reported severe cases is primarily children under the age of five years followed by immunocompromised adults (Riera-Montes et al., 2018, Lindsay et al., 2015). Globally, approximately 20% of viral diarrheal diseases, 685 million cases and 219 000 deaths are associated with HNoV infection (Farahmand et al., 2022, Troeger et al., 2017). Higher proportions of children's death due to diarrheal diseases are reported in the Sub-Saharan Africa and South-Asia, as compared to developed countries (Elbashir et al., 2022, Mans, 2019).

The emergence and prevalence of viruses among populations is dependent on many factors including mutation, recombination, replication efficiency and host interactions (LaTourrette and Garcia-Ruiz, 2022, Domingo, 2010). Research has advanced our knowledge about the influence of host factors on the prevalence of NoV. Norovirus recognize polymorphic carbohydrates known as histo-blood group antigens (HBGAs) for binding during attachment step (Nordgren and Svensson, 2019, Kambhampati et al., 2016). HBGAs are known as HNoV receptors that play a huge role in genetic susceptibility of the virus. Human population genetic makeup and diversity impact the expression of HBGAs phenotypes on enteric epithelial cells, based on the individual HBGAs genotypic profile (Le Pendu and Ruvoën-Clouet, 2020).

1.2 PROBLEM STATEMENT

Data shows that Sub-Saharan Africa and South Asia accounted for the slowest progress in decline of child mortality, according to the "all-cause and cause-specific mortality findings from the Global Burden of Disease Study in 2019" (Paulson et al., 2021). Diarrhoea is responsible for the deaths of more than 80% of young children in African and South Asian populations (Liu et al., 2015). In South Africa (SA),

approximately 20% of deaths in children below the age of five years is attributed to diarrhoea (Lehohla, 2015). Furthermore, water related diseases including diarrhoea are a major health concern in young children, especially in rural communities where previously, an estimated 9.8% children death rate was caused by poor WASH conditions (Lewin et al., 2007). According to a recent study, poor WASH conditions still persist even in healthcare facilities (Potgieter et al., 2021). The difference in access to clean water is associated with high risk of norovirus gastroenteritis. Water-related diseases have a significant impact on human health on a global scale.

According to a previous report, norovirus mortality in young children from developing countries ranges up to 212 000 deaths per year (Pires et al., 2015). Despite a rise in norovirus gastroenteritis research in developing countries, developed nations provide the vast majority of the data used for estimating the global prevalence of HNoV (Nguyen et al., 2017). Several investigations have demonstrated the occurrence of norovirus in acute gastrointestinal cases reported in South Africa in both urban and rural communities (Page et al., 2017, Kabue et al., 2016). However, there is still limited data to estimate the actual prevalence of human norovirus circulating within the country.

Factors that govern genetic diversity, transmission, and persistence of norovirus (NoV) infection are not all well understood. Existing evidence suggests that the contribution of different pathogens to diarrhoea varies significantly across regions, depending on local meteorological, geographic, ethnicity, socioeconomic conditions, as well as host genetic susceptibility and immunologic factors (Nordgren and Svensson, 2019, Nordgren et al., 2013, Bruggink and Marshall, 2010, Reither et al., 2007). Pathogens including norovirus, are selective in host cell molecules recognized during infection, and most of these molecules are oligosaccharides synthesized by glycosytransferase (Ferrer-Admetlla et al., 2009).

The interaction of NoV with HBGAs has been established as a factor that increase susceptibility to infection, however majority of studies have been performed primarily on Caucasians (Bucardo et al., 2009). Interestingly, a directly proportional relationship between the number of individuals with HBGAs secretor status on a country-level and norovirus prevalence has been established (Arrouzet et al., 2020). Different populations are composed of different genetic polymorphisms that are involved in

determining the presence or absence of HBGAs. Histo-blood group antigens, particularly FUT2, FUT3 and ABO genes restrict or advance norovirus infection, even to the extent of genotypic strain selection (Nordgren and Svensson, 2019).

There is limited data on HBGAs as NoV susceptibility factors, specifically in South Africa (Rossouw et al., 2021), and only a small number of studies have attempted to explore how this could influence susceptibility to NoV infection in African populations (Nordgren et al., 2013).

1.3 STUDY RATIONALE

In the post rotavirus vaccine era, norovirus (NoV) plays an increasingly important role in epidemic and sporadic non-bacterial gastroenteritis among children. Previously it was thought that the clinical outcome of an infection was mainly due to the virulence factors of the microorganism, however recent knowledge acknowledges the role of host genetic factors in enhancing NoV infection (Nordgren and Svensson, 2019). Several findings demonstrate that HBGAs comprising (ABO, Hh, secretor and Lewis systems) play an essential role as host genetic factors associated with susceptibility to human NoV and other enteropathogens (Kambhampati et al., 2016, Shirato, 2011).

Due to the well-established disease and economic burden of norovirus in high and low-and-middle income countries, the need for an effective vaccine has become more mandatory (Winder et al., 2022). A successful norovirus vaccine design would require understanding the prevalence of norovirus disease worldwide, as well as the genotype diversity, dominant strains, and patterns of strain replacement. Furthermore, to better direct these developments, a comprehensive understanding of the relationship that exists between variables facilitating the severity and susceptibility is crucial (Armah et al., 2023). Thorough research on the interactions between viral pathogens and histo-blood group antigens (HBGAs) in different populations could prove more effective on targeted and regionalized interventions (Arrouzet et al., 2020).

Norovirus is a notorious pathogen that exhibits high infectivity around the globe infecting various hosts (Chhabra et al., 2019). Studies have shown that susceptibility to norovirus is genotype dependent and despite this, some human hosts demonstrate resistance to norovirus infection (Reyes et al., 2022, Nordgren and Svensson, 2019) . Genetic susceptibility is largely mediated by presence or absence of HBGAs genes,

which are significantly diverse amongst different populations. Furthermore, conflicting reports indicate the resistance of non-secretors to GII.4 strains, whereas others demonstrate secretor independence during infection by the same strain (Jin et al., 2013, Carlsson et al., 2009). Similarly, more data is still needed to establish the pattern of norovirus infection with regards to Lewis status.

South Africa is a multicultural country with different ethnic groups, therefore viral and population genetic studies are crucial to explain the pattern of norovirus occurrence within the country. Previous report demonstrated high prevalence of HNoV in rural communities of Limpopo, S.A (Kabue et al., 2016). More recently, our study showed that after approximately five years, norovirus was still predominantly circulating amongst children from rural Limpopo (Khumela et al., 2023). Rossouw and colleagues associated norovirus infection with FUT2 secretor status, in hospitalized children from South African urban areas (Rossouw et al., 2021). However, no study has investigated the reason for elevated norovirus prevalence in rural Vhembe. Extensive research is required to determine the role and source of viral agents linked to diarrhoea in various African settings, as well as the effect of HBGAs interaction in NoV infection.

Various expressions of blood group antigens contribute to the increased or decreased rate of infection. Several phenotypes are associated with host genetic resistance to infection, furthermore, point mutations in the genes are population specific (Ferrer-Admetlla et al., 2009). The high degree of genetic diversity amongst RNA viruses accounts for their adaptability and widespread presence (De Graaf et al., 2016, Barr and Fearns, 2016).

1.4 OBJECTIVES OF THE STUDY

The primary objective of this study was to investigate the host phenotypic and genotypic profiles of HBGAs in NoV infections among children less than 5 years of age from rural communities of Vhembe district, Limpopo Province (South Africa). This was further subdivided into 4 secondary objectives which are further explained.

1.4.1 OBJECTIVE 1

- **To determine molecular characteristics of NoV strains circulating in the rural communities of Vhembe district, South Africa**

For the past 3 decades, norovirus GII.4 strains and variants have been the most predominant and responsible for multiple pandemics (Bucardo et al., 2017). Recombinant strain GII.4 Sydney 2012 [P31] strains has been increasingly reported in the past years (Ai et al., 2021, Duan et al., 2021, Zhu et al., 2021). The characteristic of dominance by a viral strain or variant in a geographical area is known as epidemiological fitness (Domingo et al., 2019, Wargo and Kurath, 2012, Domingo, 2010). Some of the features that defines the epidemiological fitness of a strain encompass viral and host characteristics, and overall they cannot be easily quantified (Wargo and Kurath, 2012). Observational data such as change in prevalence, distribution and composition of viral genotypes may be used to quantify epidemiological fitness of a virus (Domingo, 2010).

Recently, GII.4 Sydney 2012 [P31] strain was reported in children hospitalized with AGE from a South African urban area (Rossouw et al., 2021). Similarly, Kabue et al. (2016) described high prevalence of norovirus GII.4 strains in outpatients from a rural area in South Africa. Rural communities are still faced with poor WASH (water, sanitation, and hygiene) facilities (Potgieter et al., 2021), which may aid the spread of pathogens. Understanding epidemiological fitness of prevalent viral genotypes is critical for therapeutic interventions and preventive strategies to minimize the spread. Therefore, continuous surveillance is critical in this era of vaccine development to provide up-to-date genetic information and understanding of norovirus epidemiology in areas with history of high norovirus prevalence where preventive measures would have enormous impact (Tan, 2021). The study aimed to give an update on norovirus prevalence and genetic characteristics in children within the rural communities of the Vhembe district in South Africa.

1.4.2 OBJECTIVE 2

- **To determine host phenotypic profiles of HBGAs (ABO/H and Lewis system) in NoV infections among children from rural communities of Vhembe district, Limpopo Province (South Africa).**

A vital step for a successful infection of cells by viruses is recognition of cell surface glycans for viral attachment (Tenge et al., 2021). HBGAs phenotypes including ABO (H), secretor (FUT2) and Lewis antigens (FUT3) secreted in blood, saliva and epithelial cells are important for norovirus infection as either ligands or restriction factors (Nordgren et al., 2013, Lindesmith et al., 2003, Marionneau et al., 2002) . The expression of HBGAs on the cell surface is determined by the fucosyltransferase 2 (FUT2) gene which encode alpha (1,2) fucosyltransferase to generate H-antigen. The H-antigen produced will then be catalysed to generate A or B blood group antigen (Kambhampati et al., 2016).

Individuals carrying functional FUT 2 are termed secretors, hence can express the A and B blood group antigen as well as Lewis b (Leb) antigen on mucosa and in secretion. Studies have shown high norovirus susceptibility in secretors worldwide (Sharma et al., 2020, Lindesmith et al., 2003). Individuals without functional FUT 2 gene are referred to as non-secretors, meaning they do not secrete or express HBGAs (Shirato, 2011, Atmar et al., 2008)Atmar, 2010). FUT 3 enzymes catalyse the expression of Lewis antigens including Lewis a (for non- secretor), Lewis b (for secretors) (Henry et al., 1995). Non-secretors are generally known to be partially protected from NoV (Tan et al., 2008; Thorven et al., 2005).

Population ethnicity and host genetics drive the diversity of HBGAs profiles, and consequently the evolution and diversity of norovirus (Arrouzet et al., 2020). Different studies have shown diversity of HBGAs and norovirus in various populations (King et al., 2018; Nordgren et al., 2016; Currier et al., 2015). Recently, a study in South Africa demonstrated norovirus susceptibility in HBGA secretors and non-secretor, with the later less infected (Rossouw et al., 2021). High norovirus prevalence has been reported in South African rural communities of Vhembe district (Khumela et al., 2023; Kabue et al., 2016), however data on host genetic factors involved in the expression of pathogen-specific receptors are still missing. Therefore, this study aimed to assess

interaction between host population HBGA phenotypes and norovirus infection in rural communities of Vhembe district, South Africa.

1.4.3 OBJECTIVE 3

- **To determine the host genotypic profiles of HBGAs (FUT2/FUT3) in NoV infections among children from rural communities of Vhembe district, Limpopo Province (South Africa).**

The susceptibility and possible distribution of norovirus strains is determined by population genetic polymorphisms. It has been established that the single nucleotide genetic polymorphisms of HBGAs are mediated by FUT2 gene (Lindesmith et al., 2020). The polymorphisms in FUT2 gene are significantly associated with ethnic specificity (Ferrer-Admetlla et al., 2009). Furthermore, the prevalence of HBGAs in specific geographic regions or demographic groups may influence the regional burden of norovirus and the occurrence of outbreaks. The outcomes of pandemics and the transmission of viruses within the human population are determined by host genetic diversity and pre-exposure histories (Lindesmith et al., 2020). Variations in HBGAs among individuals contribute to differences in susceptibility to norovirus infection, affecting the prevalence and impact of the virus within human populations.

1.4.4 OBJECTIVE 4

- **To assess the correlation between circulating NoV genotypes and HBGAs profiles in young children from rural communities of Vhembe district, Limpopo Province (South Africa).**

The correlation between circulating norovirus (NoV) genotypes and histo-blood group antigens (HBGAs) profiles is a complex and dynamic interaction that plays a crucial role in the susceptibility, transmission, and epidemiology of norovirus. Norovirus, particularly genogroups I and II, exhibits strain-specific interactions with HBGAs. Different NoV genotypes may preferentially bind to specific HBGAs on the surface of host cells. Each NoV genotype may display distinct preferences for certain HBGAs. This variability contributes to the diversity in norovirus binding and infection patterns. The correlation between NoV genotypes and HBGAs profiles has implications for the dynamics of norovirus outbreaks. Understanding the prevalent HBGAs in a population

can help predict the likelihood and severity of outbreaks associated with particular NoV genotypes. Statistical methods are used to investigate the correlation or association between categorical variables, such as circulating norovirus (NoV) genotypes and histo-blood group antigens (HBGAs) profiles.

1.5 RESEARCH QUESTION AND HYPOTHESIS

Are there any associations between NoV infections and HBGAs in young children from rural communities of Vhembe district, Limpopo Province, (South Africa)?

H_0: There is no association between host genetic receptors of children from Vhembe, and norovirus infection.

H_1: The expression of host genetic receptors in young children from rural communities of Vhembe district is associated with the susceptibility of NoV infection.

1.6 RESEARCH APPROACH

This was a cross-sectional research study. Data was gathered after phenotyping and genotyping HBGAs in saliva samples, norovirus detection and genotyping in stool samples for statistical analysis to confirm our hypothesis.

CHAPTER 2

LITERATURE REVIEW

2.1 BACKGROUND

Norovirus is responsible for numerous acute gastroenteritis pandemics (Bull et al., 2010), despite this, norovirus biology is poorly understood due to difficulties in developing in-vitro models (Ludwig-Begall et al., 2021) and viral diversity (van Beek et al., 2018). Improvement in the diagnostic and analytical techniques has led to the notable advances in norovirus research. A few of those include updated classification, insights in the role of viral proteins and host attachment factors, potential reservoirs and transmission routes, understanding immune response as well as the progress in vaccine development stages (Lucero et al., 2021, Ludwig-Begall et al., 2021) which will be discussed in this chapter.

2.2 EVOLUTIONARY HISTORY AND STRUCTURE OF NOROVIRUS

Human norovirus was first described as a causative agent of an illness they called “winter vomiting illness” in 1929, due to its association with cases of vomiting occurring during winter season (Zahorsky, 1929). Later in 1968, norovirus was the first enteric virus identified as an aetiological agent of viral gastroenteritis (Kapikian et al., 1972). This follows the occurrence of AGE outbreak in Norwalk, Ohio, hence the virus was initially known as the Norwalk virus. Viral particles visualization was first made possible by the use of immunoelectron microscope, which led to the description of a 27nm round particle (**Figure 2.1**) (Kapikian et al., 1972). Norovirus description was limited to physical characteristics as a result of difficulties in in-vitro propagation (Robilotti et al., 2015).

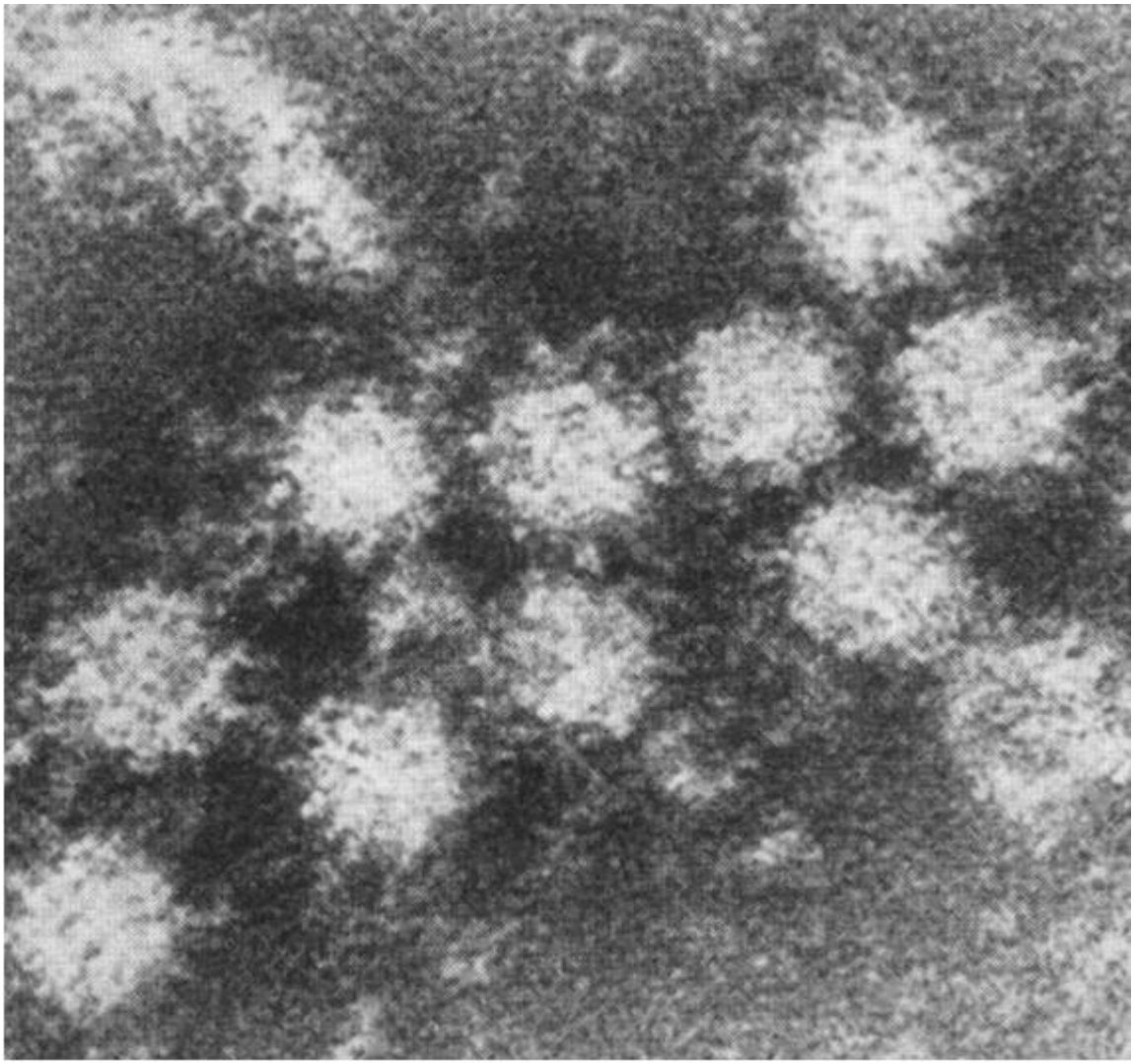

Figure 2.1 Norovirus round particles first visualized under electron microscopy by Kapikian and colleagues at the size of 27nm per each particle (Kapikian et al., 1972).

The genus norovirus belongs to the *Caliciviridae* family, which is composed of small non-enveloped, positive sense, single stranded RNA viruses infecting a wide range of species (Knipe et al., 2013). The family has eleven genera, including norovirus (**Figure 2.2; Figure 2.4**). Previously, norovirus was classified into six genogroups, and based on the VP1 amino acid clustering they were further characterized into more than forty genotypes (Kroneman et al., 2013). Currently, the classification of norovirus has been updated to ten genogroups (GI-GX) and forty-nine genotypes (Chhabra et al., 2019). The genogroups which infect humans are shown in (**Figure 2.2 and Figure 2.4**). Additionally, two tentative genogroups (GNA1 and GNA2) and three genotypes were proposed. Dual typing nomenclature which considers the recombination of ORF1 and

ORF2 is now names the virus by first listing capsid genotype, then the p-type group in put into brackets (Chhabra et al., 2019).

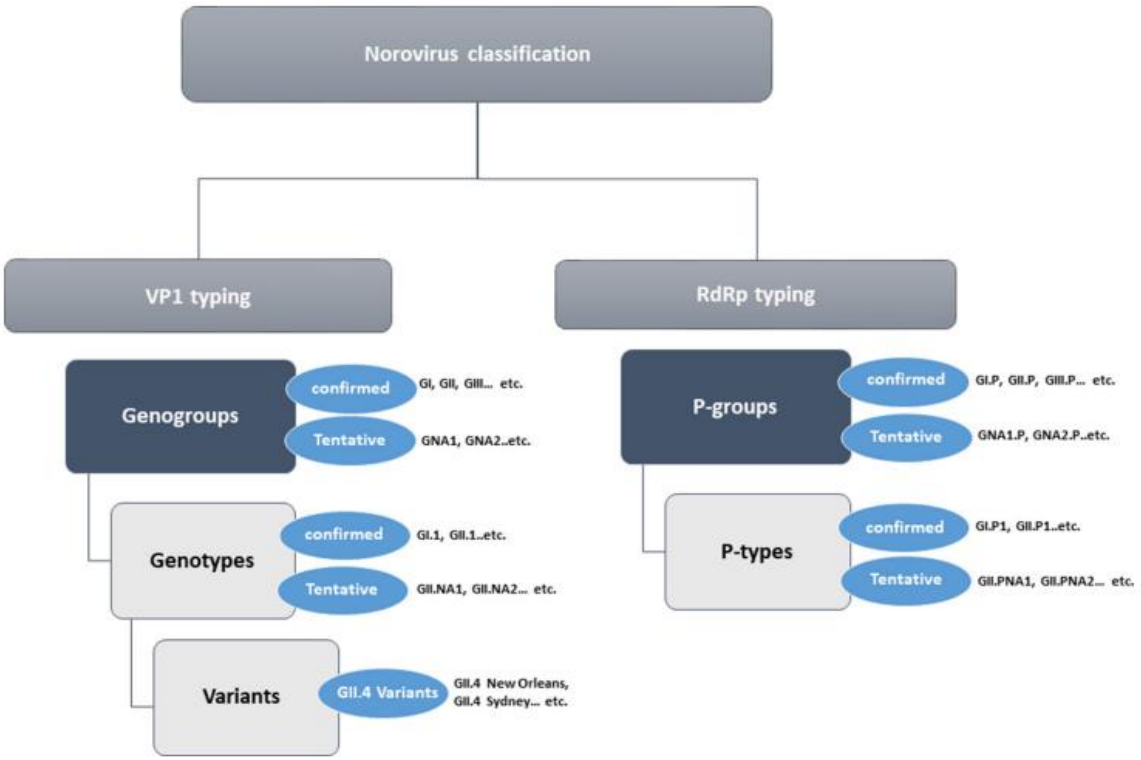

Figure 2.2 Evolution of norovirus updated genogroups and genotypes including both VP1 and RdRp types (Chhabra et al., 2019).

2.3 GENOME ORGANIZATION, GENETIC DIVERSITY AND EVOLUTION OF HUMAN NOROVIRUS

Human norovirus genome comprises a linear, positive-sense and single stranded RNA genome that is approximately 7.5 kb in length. Along with the sub-genomic RNA found in viral particles, they are covalently linked to a viral protein (VPg) at the 5' end and polyadenylated at the 3' end (Knipe et al., 2013). Untranslated regions (UTRs) are on the edges of norovirus genomes (Knipe et al., 2013). The single stranded RNA genome consists of 3 open reading frames, ORF1, ORF2 and ORF3, however, murine noroviruses contain a fourth ORF (Lee et al., 2019); **Figure 2.3**). ORF1 encodes a large polyprotein that is processed by a virus encoded protease into 6 non-structural proteins (NS1/2 - 7). The non-structural proteins are involved in various functions including formation of replication complex by NS1/2, NS3 and NS4, genome linkage by NS5 and VPg, NS6 is involved in processing the polyprotein, lastly NS7 and RdRp in genome replication (Thorne and Goodfellow, 2014, Thorne et al., 2012).

The structural components of the virion are encoded by ORF2 and ORF3. ORF2 encodes the major structural protein (VP1). VP1 contains the shell (S) and the protruding P domains (P1 and P2) wherein the P domain, particularly P2 is linked to the S domain by a flexible hinge. Viral RNA is surrounded by shell domain (Bányai et al., 2018, Knipe et al., 2013). The P2 subdomain is highly variable and harbours major neutralization epitopes, furthermore it is where interaction with HBGAs takes place (Donaldson et al., 2010, Hutson et al., 2003). VP2 is the minor structural protein encoded by ORF3. It is found inside the viral particle and has been suggested to be involved in capsid assembly and genome encapsulation (Kroneman et al., 2013).

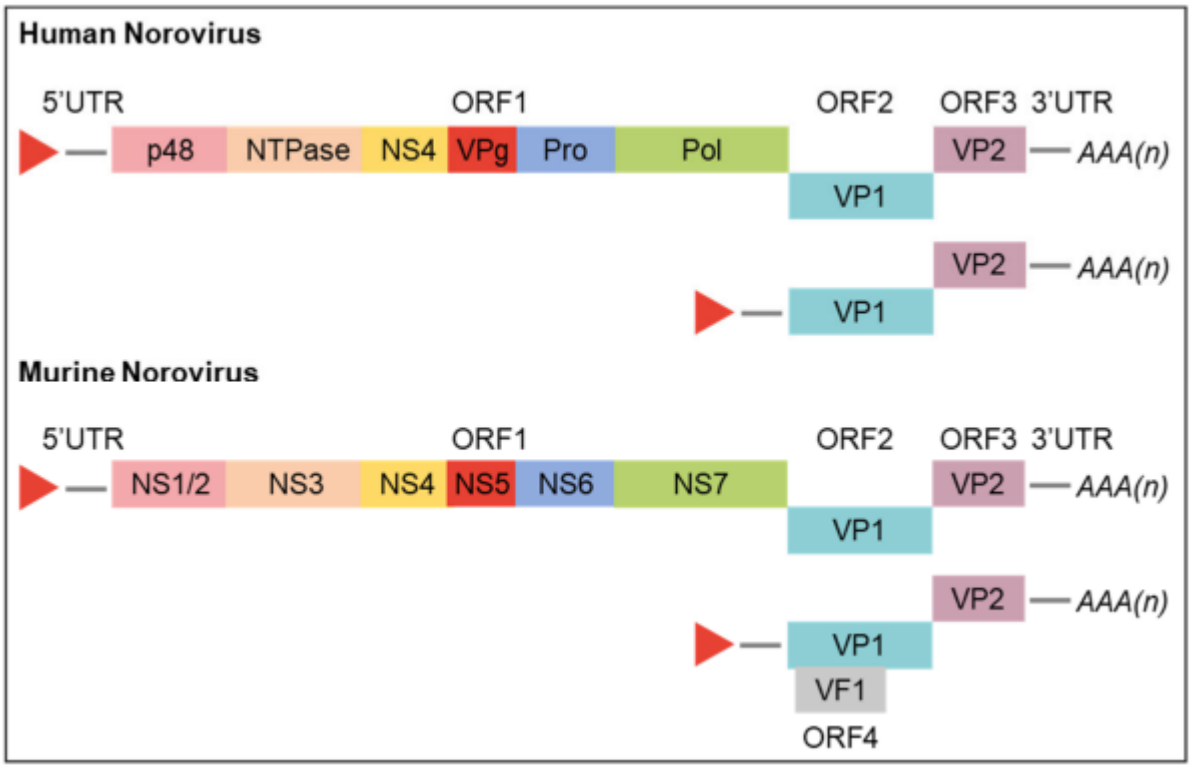

Figure 2.3 Genome structure and organization of human and murine norovirus, (Thorne and Goodfellow, 2014; Ludwig-Begall et al., 2021)

Human norovirus are genetically and antigenically diverse pathogens infecting a wide range of species. Recombination in ORF1 and ORF2 of norovirus is common and increases diversity of the virus. GII.4 variants have been predominantly circulating for the past two decades, with eight variants replacing one after the other (van Beek et al., 2018). The emerging norovirus strains are antigenically divergent due to immunogenic pressures, indicating that it plays a role in evolutionary journey of the

strains (Donaldson et al., 2010, Lindesmith et al., 2008). The newly emerged strains are characterized by high rates of mutation and evolution (Lindesmith et al., 2008).

Various pandemics caused by GII.4 strains have been reported since the mid-90s including US 1995/1996, Farmington Hills 2002, Hunter 2004, Den Haag 2006b, New Orleans 2009 and the currently circulating Sydney 2012 (van Beek et al., 2018, Lindesmith et al., 2008). Amino acid mutations are able to change norovirus binding specificity, which occurs on the P2 subdomain with HBGAs thus leading to increased prevalence of GII.4 strains (De Graaf et al., 2016). Norovirus strains adapt to diverse pressures in different host populations such as pre-existing immunity, health and age leading to possibly unique epidemiology trends in each community that may influence norovirus genotype evolution (De Graaf et al., 2016).

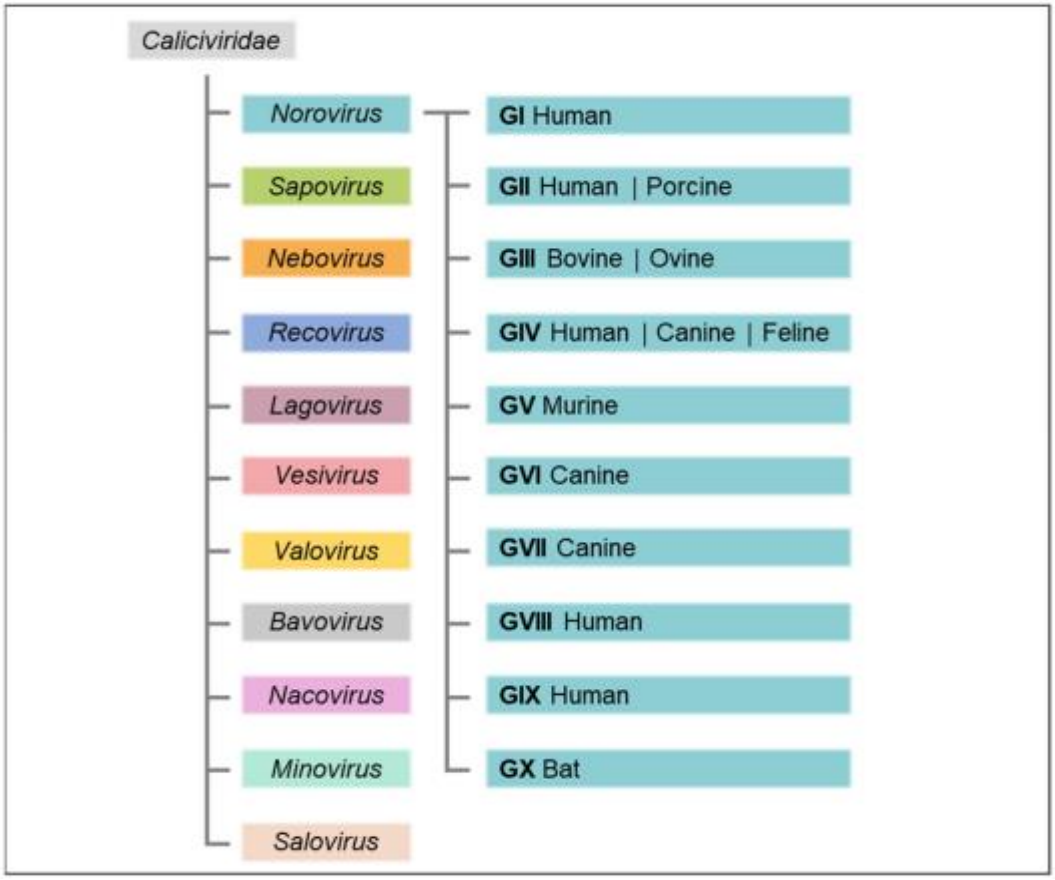

Figure 2.4 The Caliciviridae genera including norovirus genus with respective genogroups and their hosts (Ludwig-Begall et al., 2021).

2.4 CLINICAL FEATURES OF HUMAN NOROVIRUS INFECTION

Norovirus pathogenesis involves its entry into the body through ingestion or contact, followed by attachment and invasion of gastrointestinal cells (Karst, 2010). The virus replicates within these cells, causing damage and triggering inflammation, leading to symptoms such as vomiting, diarrhea, and abdominal discomfort (Donaldson et al., 2008). Norovirus infection is usually demonstrated by acute watery non-bloody diarrhoea and vomiting which generally takes a short period of time (Shane et al., 2017). Typically, norovirus incidents levels peaks in cold and dry seasons (Ahmed et al., 2013, Lopman et al., 2009). Clinical symptoms normally show up between 24 to 48 hours of incubation and typically last two to three days (Shane et al., 2017). Severe and prolonged cases are not common in healthy individuals as in younger children and immune-compromised victims, but may occur occasionally (Robilotti et al., 2015). Immunocompromised people tend to experience more of persistent NoV infection, they may continue to shed the virus for years (Davis et al., 2020). Normally, individuals infected with norovirus shed the virus for at least four weeks post clinical manifestations (Atmar et al., 2008).

There are other symptoms that have been associated with NoV infection including abdominal pain, fever and headaches (Tseng et al., 2011). Asymptomatic norovirus infection is a common issue in both developed and developing world, especially in children (Khumela et al., 2023, Kabue et al., 2016, Frange et al., 2012, Bucardo et al., 2010, García et al., 2006). Epidemiological studies have shown that clinically healthy individuals shed detectable amounts of norovirus, just as much as the symptomatic patients. Outbreaks which involve GII.4 strains are mostly associated with severe outcomes including mortality (Burke et al., 2019, van Beek et al., 2018, Desai et al., 2012).

2.5 EPIDEMIOLOGY OF HUMAN NOROVIRUS

Due to improvements made with sensitive diagnostic molecular methods and introduction of rotavirus vaccine, acute gastroenteritis epidemics caused by norovirus have been well-documented on a global scale and in vastly diverse geographical regions (Lecero et al., 2021; Robilotti et al., 2015). At least 20% of all viral acute diarrheal diseases have been linked to norovirus infection. Nearly 685 million cases

and 219 000 deaths occur due to the virus, annually (Farahmand et al., 2022). In high-income countries such as the United States (US) with good surveillance systems, approximately 19 to 21 million norovirus morbidity rates are documented yearly including 900 mortalities, 103 000 hospital admissions, 460 000 emergency departments consultations and 2.6 million consultations by outpatients (Burke et al., 2021).

Although majority of norovirus infections predominantly affect young children under the age of five years old, norovirus can occur in people of all ages. Individuals at the extremes of age are mainly affected by morbidity and mortality (Riera-Montes et al., 2018, Lindsay et al., 2015, Green, 2014). Norovirus affect people of all socioeconomic standards in both low-and-middle income countries and high-income countries, however, the risk of infection rate and severity is mostly beard by the low socioeconomic population (Lucero et al., 2021, Nguyen et al., 2017).

The global pooled prevalence rate of norovirus has been reported at a range between 16% and 18% (Farahmand et al., 2022, Liao et al., 2021, Ahmed et al., 2014). Similarly, approximately 17% of norovirus was detected in AGE cases reported in developing countries (Nguyen et al., 2017). In another study, norovirus prevalence ranged 11% to 15% in low-income countries and low-and-middle income countries amongst symptomatic patients, and 8% to 9% in asymptomatic individuals(Mans, 2019). High norovirus was reported particularly in Africa, pooled rates within the continent reaching up to 20.2% (Afework et al., 2022). Furthermore, prevalence of norovirus over 35% has been reported in Africa (Lartey et al., 2020) and South Africa particularly (Khumela et al., 2023, Kabue et al., 2016).

Circulating norovirus strains have demonstrated high genetic diversity in various populations and study locations, however, the widely distributed genogroup is GII followed by GI (Cannon et al., 2021, Li et al., 2021, Mathew et al., 2019). Globally, norovirus genogroup II genotype 4 (GII.4) are the most prevalent cluster, which evolves every year (Cannon et al., 2021). They are thought to modify their binding interactions with different types of HBGAs (Lindesmith et al., 2012, Lindesmith et al., 2008). The GII.4 noroviruses have dominated outbreaks over the past decade, which may be explained by their strong HBGA binding mechanisms and fondness for Lewis

HBGAs (Le Pendu and Ruvoën-Clouet, 2020). They are responsible for the most severe outcomes and hospitalizations worldwide (Calderwood et al., 2022).

2.6 DIAGNOSIS OF NOROVIRUS INFECTION

Norovirus samples require proper collection and handling for quality diagnostic interpretation. For example, the optimal specimen is diarrhoeal stool, which must be collected within 48 hours of symptoms and kept in a closed container at 4°C or -20°C depending on time for testing (Hall et al., 2011). Norovirus can be identified in other matrices including food (Schultz and Myrmel, 2013), water (Hill et al., 2010) or environmental specimen (Park et al., 2017).

Enzyme Immunoassays (EIAs) which have demonstrated specificity and sensitivity are commercially available for the detection of NoV GI/GII antigens in stool samples. Studies have shown that EIA methods are more effective when used in outbreaks than in sporadic cases (Costantini et al., 2010, Gray et al., 2007). Factors such as viral load and type of genotypes present in specimen play a role in these tests. Although EIAs may be easier to perform, there is a global increase in use of molecular methods.

Conventional RT-PCR is still widely used. Vinje and colleagues demonstrated some challenges regarding primer design which would be inclusive of NoV genotypes in note of NoV diversity (Vinjé et al., 2003). Most of the limitations were covered by the introduction of real-time RT-PCR. Both conventional and real-time PCR use specific primers and probes to typically target the conserved junction region of ORF1/ORF2 in norovirus (Stals et al., 2012). Moreover, PCR assays are multiplexed, able to subsequently detect multiple genogroups at the same time.

Sequencing (Sanger sequencing/next-generation sequencing (NGS)) has revealed that the conserved region at the polymerase-capsid junction of NoV (ORF1-ORF2) is an effective target for detection (Kageyama et al., 2003). RT-PCR assays are now widely available and are currently the most sensitive methods for detection of NoV in clinical samples. Although PCR assays are able to rapidly detect the amount of nucleic acid in a sample, they do not indicate viability of the tested viral particles. While they are still widely used, improved PCR assays that will include the assessment of viral viability test prior detection of nucleic acid have been proposed (Razafimahefa et al.,

2021, Karim et al., 2015). This will give a more accurate description of norovirus infection burden.

2.7 TRANSMISSON OF NOROVIRUS

Human NoV infection transmission occurs by three general routes: person to person, foodborne, and waterborne. Person to person transmission might occur directly through the faecal oral route by ingestion of aerosolized vomitus or indirect exposure via fomites or contaminated environmental surfaces (Lopman et al., 2012). Norovirus particles are mainly shed from stool or vomit of both symptomatic and asymptomatic individuals for a period of seven days (median) to at least a month, however in immunocompromised patients shedding may continue for years. Recently, Ghosh et al. demonstrated that enteric viruses replicate in salivary glands and are able to cause infection through saliva (Ghosh et al., 2022). On different dosages, norovirus circulate between the hosts and environment via person to person contact or consumption of contaminated food or water (de Graaf et al., 2017).

Norovirus are highly resistant to most virucidal treatments and remain infectious for at least a month after being exposed to the environment (Zonta et al., 2016). High norovirus prevalence is dispersed through improperly treated sewage water aided by floods to other water bodies such as coast areas (Hassard et al., 2017). Water bodies are also linked with contamination of food which leads to foodborne outbreaks.

Foodborne related outbreaks are a great threat to public health. In these incidences, majority of healthy individuals are exposed to the virus. In 2015, WHO reported norovirus as the number one cause of foodborne outbreaks (WHO, 2015). Food production and food preparations are the two major steps which have been implicated in norovirus contamination. Consumption of raw food (fruits, vegetables, and shellfish) imposes a high risk of pathogen transfer primarily from water sources (Tian et al., 2011). Approximately 61% of food related outbreaks were associated with seafood (Hardstaff et al., 2018). Moreover, seafood such as molluscs contain carbohydrate structures that aid in norovirus attachment subsequently introducing the pathogen to consumers. Food may directly be exposed to faecal matter or lack of hygiene by food handlers as well as ill food handlers contributes to food contamination (Kuo et al., 2009).

There are some NoV strains which have been linked with particular routes of transmission, such include GII.4, which seemed to be most likely associated with person to person especially healthcare facilities. Nosocomial transmission of norovirus is of concern to hospitalized patients who may develop chronic infection since they are immunocompromised (Sukhrie et al., 2012). Although some inpatients become asymptomatic shedders, majority of nosocomial norovirus transmission are caused by symptomatic shedders, who consequently if they become chronically ill, they harbour various strains and variants and act as reservoirs (Sukhrie et al., 2012, Sukhrie et al., 2010). GII.12 and GI.7 were linked with foodborne diseases (Vega et al., 2014). GI strains have been mostly associated with waterborne transmission that GII (Lysén et al., 2009).

Human norovirus strains have been detected in different host species including cattle, pigs, goats and dogs, suggesting transmission between hosts (Matamoros et al., 2023, Summa et al., 2018, Di Felice et al., 2016). Furthermore, human norovirus GII.4 strain was thought to be the cause of gastroenteritis in dogs, due to strain similarity to HNoV and close relationship between the two hosts (Matamoros et al., 2023, Summa et al., 2012). Moreover, human and canine seroprevalence to HNoV are related (Caddy et al., 2015). However, no evidence of animal norovirus infecting humans have been produced thus far. Interestingly, veterinarians who are often in close contact with animals have been found with high content of serum antibodies against canine and bovine norovirus compared to general population (Widdowson et al., 2005). Recently, norovirus with high viral load were detected in stool samples of birds and rodents, suggesting that may be new carriers involved in transmission of the virus (Summa et al., 2018).

2.8 NOROVIRUS IMMUNITY, CONTROL AND PREVENTION

There is not a specific treatment or vaccine for norovirus and as such, humans are primarily dependent on their immune system to restrict pathogenic infections. Innate immune response is able to partially restrict the replication process of human norovirus (Hosmillo et al., 2020). The innate immune response involves the production of proinflammatory and anti-inflammatory cytokines that limit norovirus replication in human intestinal epithelial cells (Mboko et al., 2022). Adaptive immunity has been shown to last for up to 2 years in human challenge studies, however, available data

indicate that seropositivity for norovirus does not reflect full protection as most of these antibodies only partially neutralize the virus (Debbink et al., 2012).

Primary treatment given to patients with NoV gastroenteritis is supportive, for example rehydration and electrolyte replenishing are used to help reverse dehydration effect (Robilotti et al., 2015). There is presently no specific therapy for NoV gastroenteritis, however, administration of nitazoxanide was linked with reducing the time of illness on patients with viral gastroenteritis including NoV infected individuals (Rossignol and EL-GOHARY, 2006). Antiviral therapies target stages of viral replication, furthermore, efforts are directed into preventing viral attachment and entry steps wherein the targeted steps include inhibition of HBGA binding and use monoclonal antibodies for passive immunotherapy (Chen et al., 2013).

In 2011, a guideline for healthcare facilities in prevention and control NoV gastroenteritis outbreaks was published (MacCannell et al., 2011, Hall et al., 2011). An appropriate measure for hand hygiene is a primary step to minimise the spread of norovirus. It is recommended that patients showcasing symptoms of diarrhoea and vomiting should be in isolated rooms to minimise contact with other patients (Robilotti et al., 2015, Kambhampati et al., 2015).

2.9 VACCINE DEVELOPMENT

Norovirus is a major health and economic burden. It has been implicated in both sporadic and outbreaks settings (Hall et al., 2013). Due to its public health importance, norovirus vaccines are under development. There have been challenges with vaccine development due to norovirus genetic diversity leading to periodically mutated strains and emerging variants (Zhou et al., 2021). Other challenges are the lack of proper cell lines and animal model to advance norovirus research. It is important to note populations with higher risk of infection transmission when developing vaccines (Aliabadi et al., 2015). Younger children are primarily affected by NoV, however, elderly people are also vulnerable (Mans, 2019).

Norovirus are genetically diverse but majority of cases globally are caused by GII.4 with variants that emerge and replace the previous one every 2 to 4 years (Singh et al., 2015). Changes are induced by mutation and recombination (Dingle, 2004). Studies show that antibodies may be protective against NoV infection. The presence

of antibodies increases with age as demonstrated by (Nurminen et al., 2011) which should be considered as there could be possibilities for robust response in adults. Protection duration is still to be determined.

Despite the challenges, interest in developing effective norovirus vaccines remains high, with three main types currently in development: nonreplicating virus-like particles (VLPs), recombinant adenoviruses, and P particles (Omatola et al., 2024). VLPs mimic wild-type viruses and can elicit specific antibody responses without infection risk, using cost-effective platforms like the Venezuelan equine encephalitis and baculovirus replicon systems, though these have some limitations (Esposito and Principi, 2020). P particles, derived from the norovirus's polymerized capsid domain, are highly immunogenic and trigger robust immune responses but are less balanced compared to VLPs (Omatola et al., 2024, Tan and Jiang, 2012). The recombinant adenovirus platform, expressing norovirus capsid proteins, induces specific immune responses when administered intranasally (Zhang et al., 2021). Various clinical trials have shown that oral recombinant adenovirus type-5 (rAd5) norovirus vaccine tablets can generate strong mucosal immune responses and potentially prevent norovirus entry into the host or lessen the severity of illness (Braun et al., 2023). This oral vaccine platform using rAd5 could significantly contribute to lowering the worldwide incidence of norovirus infections (Braun et al., 2023). While some vaccines have been discontinued in preclinical stages, many candidates are advancing through clinical trials.

Promising vaccine candidates are viral like particle (VLP) based. In numerous studies, doses of oral and intranasal preparations of GI.1 VLP vaccine were tested and found elevated IgG and IgA responses (El-Kamary et al., 2010). Phase I trial of a bivalent adjuvant GI.1/GII.4 intramuscular vaccine given to adults up to 49 years old also showed a robust antibody-secreting cell response (Sundararajan et al., 2015). Current vaccines under development have been tested in healthy adult populations with only modest protection from clinical infection and disease cases (Aliabadi et al., 2015). Recent development also showed results of safety in young children (Masuda et al., 2018). Data from other studies have shown potential for cross-protective activity of multivalent vaccines (Aliabadi et al., 2015). Further studies will need to clarify vaccine safety and efficacy in these latter age populations.

2.10 HOST SUSCEPTIBILITY FACTORS

2.10.1 HISTO-BLOOD GROUP ANTIGEN

The idea of susceptibility and resistance to norovirus infection came about a long time ago after a challenge study determined that although there is high infectivity and rapid transmission of norovirus, a subpopulation of individuals does not develop symptomatic disease or become infected after exposure (Parrino et al., 1977). It was later determined that different response to norovirus exposure was due to the presence of inherited histo-blood group antigens (HBGAs) mainly the types controlled by the FUT2 (Secretor), FUT3 (Lewis), and ABO genes (Nordgren and Svensson, 2019). Histo-blood group antigens are the complex carbohydrates receptors expressed on the surface of red blood cells and mucosal epithelium of respiratory, genitourinary and digestive tracts (Ravn and Dabelsteen, 2000). They can also be found in biological fluids including saliva, intestinal content, milk and blood.

HBGAs are determinants of both ABO and Lewis blood group system (Shirato, 2011). The expression of HBGAs on the cell surface is determined by the fucosyltransferase 2 (FUT2) gene which encode alpha (1, 2) fucosyltransferase in order to generate H-antigen. FUT 2 enzymes synthesize H-antigen from the precursor chain by the addition of a fucose group. The H-antigen produced will be then catalysed to generate A or B blood group antigen (Kambhampati et al., 2016). Individuals carrying functional FUT 2 (expression alpha 1, 2 fucosyltransferase 2) are termed secretors. They are able to express the A and B blood group antigen as well as Lewis b (Leb) antigen on mucosa and in secretion. Individuals without functional FUT 2 gene are referred to as non-secretors (Shirato, 2011, Atmar, 2010). FUT 3 enzymes catalyse the expression of Lewis antigens including Lewis a (for non- secretor), Lewis b (for secretors) (Cooling, 2015).

2.10.2 NOROVIRUS HOST INTERACTIONS

The most important step during microbial host infection is the ability of a pathogen to attach to host receptor cells, in the case of norovirus infection, they particularly target histo-blood group antigens (Heggelund et al., 2017). Sequence analysis of the NoV binding region indicates that the polymorphic human HBGAs are likely to have been a driving force for the divergence of human NoV genotypes (Tan and Jiang, 2010). The

major capsid protein VP1 is involved in the recognition of the host receptor (Tan and Jiang, 2010). The P2 ligand of the viral capsid is the natural binding site with human HBGA, which may be the point of initial viral attachment (Donaldson et al., 2008). There is now some evidence that small genetic change in the capsid region can have a big influence on NoV virulence; using an infectious cDNA clone of the attenuated virus, Bailey et al. (2008) restored murine NoV *in vivo* virulence by a glutamate-to-lysine substitution at position 296 in the capsid protein (VP1) (Bailey et al., 2008). Antigenic drift and receptor switching may act synergistically in GII.4 NoV infection to overcome human herd immunity (Bull and White, 2011, Lindesmith et al., 2008).

Norovirus (NoV) infection involves the lamina propria in the promixal portion of the small intestine (Hutson et al., 2002). Most NoV genotypes recognize the type 1 and 2 carbohydrates cores of the HBGAs (Shirato, 2011), expressed on the surfaces of red blood cells and commonly in the gut (Hutson et al., 2002). Thus, differing host HBGA genotypes can determine susceptibility of NoV infection (Lindesmith et al., 2003). The host genetic susceptibility to NoV infection may be strain specific (Kambhampati et al., 2016). The strain-specific binding and the variable expression of the HBGA receptors may explain the unpredictable host susceptibility observed in NoV outbreaks (Huang et al., 2005, Huang et al., 2003, Hutson et al., 2002) and confound investigation relating to the NoV immunity, since people without antibodies to a particular NoV may lack genetic susceptibility to the infection (Patel et al., 2009).

The interaction between HBGAs and human NoV was first observed in human volunteer studies in which the O individuals had higher infection rate compared to other blood type in a group of volunteers after challenge with Norwalk virus. The HBGA-binding profiles were comparable to the norovirus infection rates of the volunteers (Hennessy et al., 2003, Hutson et al., 2002). Lindesmith and colleagues reported a natural resistance of non-secretor to Norwalk virus challenge and an absence of binding of Norwalk VLPs (Virus like particles) to saliva sample from non-secretor (Lindesmith et al., 2003). In culture, G.II 4 strains which are the predominant strains worldwide have been found infecting enteroids derived from secretor but not non-secretor individuals (Ettayebi et al., 2016). The HBGAs binding interfaces with Human NoV have been identified in P domain of viral capsid, in which group of amino

acids interacts with distinct oligosaccharides residues of the HBGA receptors (Tan and Jiang, 2010, Cao et al., 2007).

The discovery of ABO blood groups and the potential role of blood groups in infectious diseases have spiked an on-going interest in epidemiological studies (Cooling, 2015). The genetic polymorphism of blood groups among different individuals and populations makes them a frequent target on current epidemiological investigations. Blood groups serve as ligands or restriction factors for many viruses, parasites, bacteria and toxins (Cooling, 2015).

2.10.3 ABO SEROLOGY

Two antigens (A and B) make up the ABO histo-blood group system, which consist of 4 phenotypic blood types expressions (A, B, AB and O). Antigens A and B are produced by ABO gene and are autosomal co-dominant (Reid et al., 2012). Phenotype O is autosomal recessive due to homozygous inheritance of null ABO alleles. There are no O antigens, and therefore, the O type phenotype expresses the H antigen, which is the biosynthetic precursor of A and B antigens (Dean et al., 2012). Distribution of ABO blood groups vary between different ethnic groups, but O type is commonly the most prevalent. ABH antigens are widely expressed in tissues and secretions including intestinal mucosa, endothelium, kidney, heart, and other organs (Cooling, 2015, Cooling et al., 2005).

ABO typing can be affected by several conditions, which may be genetic, developmental or clinical (Ewald and Sumner, 2016). This effect has epidemiological implications. The expression of ABO in epithelial tissues is heavily dependent on FUT2 gene/secretor (Azad et al., 2018, Ewald and Sumner, 2016).

2.10.4 ABO AND LEWIS GENETICS FUT1 AND FUT2 AND ABO (H) ANTIGEN SYNTHESIS

ABH are carbohydrate antigens expressed in glycoproteins. About a million of ABH antigens can be found in each normal mature red cell (Ravn and Dabelsteen, 2000). Synthesis of ABH antigens is based on sequential addition of carbohydrates to an oligosaccharide backbone (Mattos, 2016). H antigen is synthesized by the FUT1/H gene in red cells; α1,2fucosyltransferase adds a terminal fucose to lactosamine and form fucaα-2Galß1-4GlcNAc-R (Mattos, 2016, Cooling, 2015). The H antigen then

serves as a substrate for ABO. It can add either N-acetylgalactosamine (GalNAc) for the A antigen or galactose (Gal) for the B antigen in an α1,3 linkage to the same subterminal galactose (Yamamoto et al., 2003). ABH can be expressed in different oligosaccharide backbones that are tissue and species specific. The oligo backbone plays a role in recognition of ABH by antibodies or microorganisms. The difference between type 1 and type 2 structures is the galactose linkage (Reid et al., 2012). Type 1 structures are richly found in gastrointestinal epithelial cells and bodily fluids such as saliva, plasma and mucus. The synthesis of type 1 chain is dependent on FUT2 gene (Nordgren and Svensson, 2019).

Both FUT1 and FUT2 genes are located in chromosome 19q13 due to gene duplication (Matzhold, 2010). FUT1 is relatively specific for type 2 chain substrates and responsible for the expression of ABH in erythrocytes. FUT1 mRNA is widely found in most tissues except in salivary and parotid glands which only express FUT2. FUT2 is responsible for synthesis of type 1 chain ABH and Le(b) antigens(Cooling, 2015). FUT2 is highly expressed in salivary glands and intestinal mucosa. Individuals that inherit at least one functional FUT2 allele are termed secretors because they express ABH substances in saliva, milk, blood and other body fluids (Nordgren and Svensson, 2019, Nordgren et al., 2013).

Homozygous individuals that inherit FUT2-null alleles are non-secretors and that exhibit only Le(a) in their secretions. In Caucasians, *se428* (Trp143stop) is the most common non-secretor allele and is often used in genomic studies (G428A; rs601338) (Azad et al., 2018). The *se428* allele is also common among Africans, Iranians, and Turks (Koda et al., 2003). FUT3 is called the Lewis gene, it is an _1,3/4-fucosyltransferase capable of utilizing both type 1 and type 2 chain substrates to generate Lea, Leb (Matzhold, 2010). This gene is located on chromosome 19p13.3. *FUT3* is tissue restricted and correlates fairly well with *FUT2* expression. The strongest *FUT3* mRNA expression is observed in the trachea, intestine, bladder, and lower female reproductive tract (Matzhold, 2010).

2.11 SUMMARY OF LITERATURE REVIEW

The literature review provides an extensive overview of various aspects of norovirus, covering its biology, epidemiology, diagnosis, transmission, immunity, prevention, and

vaccine development. Norovirus is a significant cause of acute gastroenteritis and presents challenges in research due to limited in-vitro models and viral diversity (Ludwig-Begall et al., 2021). The genetic diversity of norovirus remains a highlight propelling continuous spread worldwide, particularly the currently predominant of GII.4 strains causing pandemics(van Beek et al., 2018, De Graaf et al., 2016). Norovirus infections typically manifest as acute gastroenteritis, with symptoms including vomiting, diarrhoea, and abdominal discomfort, impacting different age groups and socioeconomic populations (Lucero et al., 2021, Nguyen et al., 2017). Various diagnostic methods, including enzyme immunoassays (EIAs) and molecular techniques like RT-PCR and sequencing have improved norovirus research, however, there are still challenges in primer design and the need for improved assays (Pang and Lee, 2015). Norovirus typically spreads through person-to-person contact, contaminated food, and water and the mechanisms of transmission are enhanced by environmental persistence and foodborne outbreaks (De Graaf et al., 2016, de Graaf et al., 2017). Innate and adaptive immune responses to norovirus, as well as treatment options and preventive measures such as hand hygiene and isolation protocols, are employed to control norovirus disease (Lopman et al., 2016). Challenges in vaccine development are due to genetic diversity, however, promising vaccine candidates, including viral-like particle (VLP)-based vaccines development are underway (Tan, 2021). There is need for further research on safety and efficacy, particularly in high-risk populations. The role of histo-blood group antigens (HBGAs) in host susceptibility to norovirus infection, including ABO and Lewis blood group systems emphasize that genetic polymorphisms affect susceptibility and resistance to infection (Nordgren and Svensson, 2019).

The current study acknowledges previously published data highlighting high prevalence of enteric pathogens in the study area and explores the role of host genetics in susceptibility to norovirus. To the best of my knowledge, it is the first study to investigate host genetics' impact in susceptibility to norovirus in South African rural communities. Furthermore, there is high cultural diversity in S.A and limited data to explain how this may impact host susceptibility.

HBGAs possess population specific mutations that may drive epidemiology of pathogens (Arrouzet et al., 2020). Therefore, this study will provide insight with regards

to population level genetics potential in distribution of pathogens. Africa faces the highest disease burden and therefore, interventions to control and minimize the infectious diseases will have the most impact. HBGAs are potential drivers of norovirus genotype distribution, which may impact vaccine development to be population specific.

CHAPTER 3

METHODOLOGY

3.1 OVERVIEW OF THE METHODOLOGY

To reach the objectives of this study, a cross sectional investigation on children under five years of age was conducted. A pair of stool and saliva specimens were randomly collected from each participant from different healthcare centres (clinics and hospitals) situated within the rural communities of Vhembe district in Limpopo province, South Africa. At least 30 clinics and 4 hospitals (Tshilidzini, Elim, Siloam and Donald Fraser Hospital) were the designated sampling sites for this study. All samples were processed in the University of Venda microbiology laboratory for norovirus detection, HBGAs phenotyping and DNA extraction for genotyping of FUT2/3 genes from saliva specimen. Statistical analysis was performed using GraphPad prism 10, to assess the relationship between norovirus infection and HBGAs profile.

3.2 METHODOLOGY

3.2.1 STUDY ETHICS STATEMENT

The study protocol and consent procedures were approved by ethics committees of the department of Health in the Limpopo province (Ref: LP-2018-07-016; appendix D/E) and University of Venda (Ref: SMNS/18/MBY/07/2505; appendix F). Written, informed consent (appendix G, H, I) was obtained from the parents or child guardian before samples (stool & saliva) were collected.

3.2.2 STUDY POPULATION AND SAMPLE COLLECTION

Stool and Saliva samples.

The study population was subdivided into 2 groups including symptomatic (children with AGE symptoms) and asymptomatic (children without any AGE symptoms for at least one month). Inclusion criteria for AGE children was as follows: children passing at least 3 or more loose stools within 24 hrs and samples were collected between 2019 and 2021. Other symptoms considered were dehydration, fever, vomiting and abdominal pain. Non-bloody diarrhoeal stool samples (n=200) were randomly collected in different primary health care facilities (clinics and hospitals) within Vhembe

district. Additionally, 100 stools samples from healthy controls were simultaneously collected in the same facilities within the Vhembe region.

In a similar manner, saliva samples were obtained concurrently from each of the participants. A sterile cotton swab was used to collect saliva and epithelial cells from each study participant, provided he/she was not breastfed for an hour prior collection. The swab was rubbed inside of the cheek for at least a minute and then stored in PBS. Clinical data and personal details including living conditions (e.g. water source, presence of livestock) of the patients were obtained on pre-printed investigation forms. In hospitalized cases, only children admitted within 24 hours were considered for sampling this investigation. All specimens were transported to the University of Venda Microbiology Laboratory and stored at −20°C until further analysis.

3.2.3. STOOL SAMPLES ANALYSIS

3.2.3.1 RNA EXTRACTION AND HUMAN NOROVIRUS DETECTION

Viral nucleic acid extraction from stool samples was carried out using the Boom method (Boom et al., 1990). RNA extracts were stored at -20°C until norovirus detection. Norovirus genogroups I and II were detected and differentiated by RIDAGENE real-time RT-PCR kits, viral stool panel (PG1415) from r-Biopharm AG, Darmstadt, Germany, following the procedure outlined (Khumela et al., 2023). The assay is highly sensitive and specific at the estimated rate of 98% and includes an internal control for monitoring extraction efficiency and amplification inhibition. The Rotor-gene Corbett 6000 platform was used for quantitative PCR (qPCR). A threshold cycle value of ≤37 (Ct) was considered indicative of norovirus positivity for GII, while a threshold of ≤35 was for norovirus GI.

3.2.3.2 NOROVIRUS RT-PCR AND GENOTYPING, CLUSTALW ALIGNMENT AND PHYLOGENETIC ANALYSIS

The RNA extracts positive for norovirus were subjected to PCR amplification using a genogroup-specific polymerase-capsid (PC) assay, as previously outlined (Chhabra et al., 2019). The Qiagen one-step Reverse Transcription-PCR kit (Qiagen, Germantown, MD, USA) was used to generate amplicons for nucleotide sequencing. The primers previously established for GI: MON432/GISKR (579bp) were used (Kageyama et al., 2003, Kojima et al., 2002). PCR conditions were as follows: reverse

transcription at 42°C for 30 minutes, activation of Taq polymerase at 95°C for 15 minutes, and 40 cycles of PCR amplification at 95°C, 50°C, and 72°C for 1 minute each. The final step was extension at 72°C for 10 minutes and cooling down to 4°C. GII primers MON431/GIISKR (570bp) were used with a minor adjustment in the PCR conditions, specifically annealing at 56°C.

Norovirus-positive extracts that failed to amplify under initial conditions and primers underwent an additional round of amplification via semi-nested PCR approach. This involved the use of COG2F/G2SKR (GII) and COG1F/G1SKR (GI) primers, generating 390pb and 380pb fragments, respectively, as detailed (Cantelli et al., 2020). Subsequently, the amplicons that still did not produce an amplification band were further subjected to reverse transcription-polymerase chain reaction (RT-PCR) using GISKR/F and GIISKR/F primers. This step produced fragments of 330bp and 344bp, respectively.

The norovirus amplicons were electrophoresed on a 2% agarose gel stained with ethidium bromide to visualize specific-sized bands on a UV light transilluminator. All target sized amplicons size were selected for partial Sanger sequencing. The sequencing process was conducted using the 3730XL Genetic Analyzer POP7TM from Thermo-Scientific at Inqaba BiotecTM (SA). The identical primers employed in the conventional PCR were also used in the Sanger sequencing.

The obtained sequences were read and edited using Finch TV (version 1.4) from Geospiza in Seattle, USA. The edited nucleotide sequences were subsequently compared to reference strains from the NCBI Blast tool at http://www.ncbi.nlm.nih.gov. Genotyping of sequences was done using the online NoroNet platform at http://www.rivm.nlm/norovirus/typingtool and the Human Calicivirus Typing Tool (HuCaT) based in Atlanta, USA, at https://norovirus.ng.philab.cdc.gov.

All successfully genotyped sequences obtained during this investigation were submitted to NCBI Genbank and have been assigned the following accession numbers: OM948743-OM948745, OM961396-OM961399, OM970798-OM970802, OM985015-OM985018, OM993270-OM993272, ON005452, ON008179, OP257195-OP257196, OP600464-OP600468, and OQ048857-OQ048862.

We explored the nucleotide percent identity of nucleotide sequences using the ClustalW alignment tool. The comparison was between this study's sequences (n = 34) and previously documented norovirus sequences (n = 14) from the same study area. ClustalW provided a nucleotide similarity percentage representation of across all sequences.

To explore the genetic relationships between the norovirus strains identified in this study and those circulating globally, phylogenetic analysis was conducted. Reference strains from GenBank, showing the best hit of >90% similarity to the study's query sequences, were randomly selected for the analysis using MEGA 11. Additional strains were included based on their geographical locations to thoroughly investigate the relatedness of globally circulating strains over time.

3.2.4 SALIVA SAMPLES ANALYSIS

3.2.4.1 PHENOTYPING HBGAs IN SALIVA

Detection of HBGAs phenotypes including ABO(H) and Lewis in saliva samples was performed as previously described (de Moraes et al., 2019) with slight modifications. Briefly on day one, ELISA plate was coated with diluted saliva samples (1:100 in carbonate bicarbonate) then incubated for 2 hours at 37°C, followed by overnight incubation at 4°C. On each plate, four wells were coated with the same saliva sample.

On day two, the plate was washed 4 times with PBST (PBS containing 0.05% of tween 20). In 100 µL, monoclonal antibodies including α-A, α-B, α-Lea and α-Leb were applied each per well per sample. The anti-A, anti-Lea and anti-Leb mAbs were diluted 1:5000 and the anti-B mAbs were diluted 1:2500 in PBST containing 5% of fetal bovine serum (FBS). The plate was incubated for 1 h and 30 min at 37°C, washed as described previously. A dilution 1:10 000 of horseradish peroxidase conjugated goat anti-mouse IgG (heavy plus light chain) was added, and the plate was incubated for another 1 hr and 30 min at 37 °C. The plate washed again four times, the reaction was developed using 3,3′,5,5′-Tetramethylbenzidine (TMB) Substrate Systems (Sigma-Aldrich®, St.Louis, USA) and stopped by addition of 2 M H2SO4. The absorbance was measured at 450nm.

Samples which were negative for mAb anti-Lea, Leb, A and B were further tested for the detection of H antigen. H antigen is the precursor for ABO synthesis, and is

encoded by 1,2 fucosyltransferase. The dilution for H antigen was 1:1000. Various known HBGAs phenotypes were determined and categorized as follows, ABO (A, B, AB, O), H (H positive, H negative), secretor (secretor, non-secretor) and Lewis [Le(a+b−), Le(a−b+), Le(a+b+) and Le(a−b−)]. A sample was considered secretor if A, B, AB or Lewis B was positive, and non-secretor if Lewis A was positive. Weak secretors were defined as those able to secrete both Lewis A and B positive.

Individuals expressing O and Lewis negative secretory status was determined by detecting the presence of H antigen. HBGA synthesized from H type 1 precursor are present in saliva, other body fluids and tissues. FUT2 and FUT3 genetically control the expressions of these carbohydrates by individuals known as secretors. Non-secretors only express these antigen in blood cells.

After using H-antigen in Lewis-negative, weak secretors and type O saliva samples, results showed some discrepancy and were therefore inconclusive to establish secretor phenotype. To confirm the secretor phenotype, we further used lectin following the protocol described by (Nordgren et al., 2014), with established secretors and non-secretor samples as control. The *Ulex europaeus* agglutinin (UEA-1) is specific for the detection of Fucα1-2Gal-R present in secretor, but not in non-secretor saliva. ELISA plates (NUNC 96F Maxisorp; Thermo Fisher Scientific) were coated with these saliva samples, diluted at 1:500 in (0.1 M carbonate–bicarbonate buffer, pH 9.6) and then incubated for 2 h at 37°C followed by 4°C overnight.

On the following day the plates were washed as previously described and then blocked for 1 h at 37°C with 3% FBS in PBS, followed by the addition of HRP-conjugated *Ulex europaeus* agglutinin (UEA-I, Sigma Aldrich, Sweden) diluted 1:3200 (1mg/ml) and incubated for 1 h 30 minutes at 37°C. The reaction was developed using 3,3′,5,5′-Tetramethylbenzidine (TMB) Substrate Systems (Sigma-Aldrich®, St.Louis, USA) and stopped by addition of 2M H_2SO_4. With reference to the control samples, the cutoff value for secretor positive samples was taken as two times the absorbance value of the negative controls.

3.2.4.2 DNA EXTRACTION FROM SALIVA SAMPLES, DETECTION AND GENOTYPING OF FUT2/3

A total of 100 samples (symptomatic and asymptomatic including norovirus positive and negative) with various phenotypic profiles as determined by ELISA assay were randomly selected for FUT2 and FUT3 genotyping. Prior to this procedure, saliva samples were subjected to DNA extraction using Qiagen DNA mini kit as per manufacturer's instructions.

DNA extracts were amplified by Touchdown-PCR technique, using the platinum Taq DNA polymerase high fidelity enzyme (Thermo Fisher Scientific, Waltham, MA, USA). Conditions used during the TD-PCR were as follows: 65 °C -55 °C (annealing), 26+19 (cycles) and 68 °C (elongation), previously described for FUT2 (de Moraes et al., 2019) and FUT3 primers outlined (Nordgren et al., 2013, Elmgren et al., 1996). After TD-PCR, the amplicons were purified and sequenced using an ABI Prism BigDye Terminator 3.1 Cycle Sequencing Ready Reaction Kit and ABI Prism 3730 Genetic Analyser® (Applied Biosystems®, Foster City, CA, USA). The chromatograms and FUT2/3 gene encoding nucleotide sequences were analysed using the free tracer viewer Chromas 2.4® (Technelysium Pty Ltd.®, South Brisbane, QLD, AUS).

3.2.4.3 FUT2 AND FUT3 SNPs

For the analysis of FUT2 SNPs, the G428A position was analysed to investigate the homozygous (se/se or SESE) and heterozygous (SE/se) mutations. The G428A SNP is common amongst African populations for non-secretor status. FUT3 sequences were genotyped to analyse the most common mutations including C314T, G508A, T1067A, T59G and T202C.

The sequences were aligned using BioEdit software and the following reference sequences were used to identify the common and new SNPs (NM_000149 NG_007482).

3.2.5 STATISTICAL ANALYSIS

Data were captured on Microsoft excel spreadsheet. Pearson's correlation values and statistical significance including Chi-square and Fisher's exact test (p-values) were determined using Graphpad prism 10 (Inc, USA). A p-value ≤ 0.05 was considered

significant at 95% confidence level. Data management plan to ensures data integrity, facilitate data sharing, and enhance the reproducibility and transparency of this research findings is attached (appendix C).

CHAPTER 4

RESULTS

4.1 NOROVIRUS PREVALENCE

This investigation showed norovirus trends within in the rural communities of Vhembe District South Africa, between 2019 and 2021 (Table 1). There prevalence of HNoV was high ranging up to 37% (74/200) among symptomatic individuals and 14% (14/100) among asymptomatic individuals. Importantly, the difference in HNoV prevalence between symptomatic and asymptomatic cases was demonstrated to be statistically significant ($p < 0.0001$). Furthermore, HNoV had a notable impact on hospitalized patients, affecting 60% (44/74) significantly more than outpatients at 41% (30/74) during the investigation ($p = 0.0125$).

In terms of gender distribution, majority of study participants were males (58%) compared to females (42%), similarly, a higher prevalence of HNoV was recorded in males (66%) than in females (34%). Among different age groups, children aged 6–23 months were predominantly affected by HNoV (65%), followed by children aged 0–5 months (20%).

In the majority of children (76%), diarrhoea was manifested with other symptoms and consequently HNoV infection was mostly associated with diarrhoea and other symptoms. Amongst the symptoms, norovirus presence was predominantly characterized by dehydration (82%), vomiting (79%) and fever (49%). Norovirus associated diarrhoea typically lasted for 3 days and was mainly presented in watery than formed stools (Table 4.1).

Most of the rural community members now have access to municipal treated water, however a small portion still use untreated water sources to cover their basic needs (Table 4.1). Norovirus was detected in both these groups (65%; 71%) with an alarming high rate in people with access to clean water. However, the only source referred to for norovirus detection in this study was the stool sample. Some of the data included under consideration as living conditions were inconsistent, however, individuals with domestic animals in their yards had high chances of norovirus infection. The

distribution of norovirus in flush and pit toilets was slightly higher in people using pit toilets (Table 4.1).

Table 4.1 Demographic characteristics, clinical features, and HNoV prevalence among children with AGE from Vhembe district, South Africa in 2019-2021.

	Symptomatic		Asymptomatic	
	Total (%)	HNoV + (%)	Total (%)	HNoV + (%)
Detection rate (%)	n=200	n=74 (37)	n=100	n=14 (14)
Setting				
Outpatients	104 (52)	30 (41)	100 (100)	14 (14)
Hospitalized	96 (48)	44 (59)	0	0
Gender				
Males	116 (58)	49 (66)	53 (53)	8 (57)
Females	84 (42)	25 (34)	47 (47)	6 (43)
Age in month				
0-5	49 (25)	15 (20)	21 (21)	2 (14)
6-23	124 (62)	48 (65)	59 (59)	10 (71)
24-60	27 (14)	11 (15)	20 (20)	2 (14)
Symptoms				
Diarrhoea only	48 (24)	8 (11)		
Diarrhoea and other symptoms	152 (76)	66 (89)		
Other symptoms seen together with diarrhoea				
Dehydration	132 (66)	61 (82)		
Vomiting	119 (60)	54 (73)		
Fever	79 (40)	36 (49)		
Abdominal pain	29 (15)	15 (20)		
Stool appearance				
Watery	103 (52)	46 (62)		
Formed	97 (49)	28 (38)	100 (100)	14 (14)

Duration of diarrhoea				
3 days	163 (82)	62 (84)		
>3 days	36 (18)	12 (16)		
Interval				
1-3 days	161 (81)	61 (82)		
3 days +	39 (20)	13 (18)		
Living conditions				
Treated water	131 (66)	48 (65)	73 (73)	10 (71)
Untreated water	37 (19)	11 (15)	13 (13)	4 (29)
Mixed	32 (16)	15 (20)	14 (14)	
Pit toilets	111 (56)	41 (55)		
Flush toilets	89 (46)	33 (45)		
Breastfeeding	130 (65)	51 (69)	63 (63)	10 (71)
Not-breastfed	70 (35)	23 (31)	37 (37)	4 (29)
Livestock	67 (34)	29 (39)	32 (32)	3 (21)
No Livestock	21 (11)	9 (12)	7 (7)	1 (7)

Breastfeeding was common in the study population, intriguingly norovirus was predominantly detected in children who are breastfed than non-breastfed in both AGE and control groups (63%; 71%), respectively.

4.2 HNoV GENOGROUPS AND GENOTYPES DISTRIBUTION

There was a rise in the distribution of norovirus genogroup II (GII) infections over the years, increasing from 2019 (3/59), 2020 (15/59) to 2021 (41/59) among children with AGE. In overall range, GII infections were dominant, constituting 80% (59/74) in children with AGE, while GI infections accounted for 12% (9/74). Conversely, in children without AGE, GI infections were more prevalent at 64% (9/14) compared to GII at 36% (5/14) (Table 4.2). The dominance of GII was more pronounced in hospitalized patients (86%) than in outpatients (70%). Co-infection with both GI and GII was infrequent in this study.

The median Cycle threshold (Ct) values for GII was slightly different between symptomatic (32.25) and asymptomatic (35.02) cases. Similarly, the mean GI Ct value was 32.16 in the symptomatic group and 32.50 in the asymptomatic group. Additional data for CT values of all samples is attached (appendix A).

Among the HNoV GII cases identified in symptomatic children, we were able to successfully genotype 54% (32/59). In asymptomatic participants, genotyping was successful for 40% (2/5) of GII strains. Notably, GII.4 Sydney 2012 [P31] recombinants and capsid genotype GII.4 Sydney 2012 were the only genotypes identified. The GII.4 Sydney 2012 [P31] recombinants were dominant in both symptomatic (59%; 19/32) and asymptomatic HNoV infections (40%; 2/5). Moreover, the strain dominance was further observed among hospitalized children, wherein the GII.4 Sydney 2012 [P31] genotype account for 74% (14/19), compared to outpatients at 26% (5/19). GII.4 Sydney 2012 genotype prevalence between inpatients and outpatients were similar. Unfortunately, HNoV GI group and other GII positive samples could not be typed, possibly due to low viral load and the impact of electricity load shedding on laboratory storage facilities.

Table 4.2 Distribution of HNoV genogroup and genotype in children from Vhembe district, South Africa.

		Symptomatic		Asymptomatic
		Outpatients	In-patients	
	n=200 (%)	n=104 (%)	n=96 (%)	n=100 (%)
Total HNoV +	74 (37)	30 (41)	44 (60)	14 (14)
Genogroups				
GI	9 (12)	4 (12)	5 (11)	9 (64)
GII	59 (80)	21 (70)	38 (86)	5 (36)
Mixed	5 (7)	2 (7)	3 (7)	
GII Genotypes	32			
GII.4 Sydney 2012 [P31]	19 (59)	5 (26)	14 (74)	2 (40)
GII.4 Sydney 2012	13 (41)	6 (46)	7 (54)	

4.3 CLUSTALW ANALYSIS ON HNOV STRAINS CIRCULATING IN VHEMBE DISTRICT, SOUTH AFRICA.

The ClustalW percentage identity matrix (Figure 4.1) shows nucleotide similarity between aligned sequences at a given time. Comparison between norovirus strains documented in the study area previously (Kabue et al., 2017) and those identified in the current survey (Khumela et al., 2023) revealed a markedly low similarity, measuring less than 50% in nucleotide sequences. This implies that there has been notable change in nucleotide sequences over a period of approximately 4 to 5 years between the study periods. Upon analysis of HNoV sequences obtained from this investigation, it was observed that they exhibited high similarity, with a nucleotide percent identity surpassing 90%.

Percent Identity Matrix - created by Clustal2.1

1: KY548497.1	100.00	97.16	97.85	96.09	98.21	97.20	31.84	33.22	30.84	30.10	38.28	41.49	40.75	41.89	43.73	43.11	42.47	41.63	42.83	42.91	42.54	42.80	43.11	42.61	44.11	43.25	43.07	42.83	44.28	42.94	43.15
2: KY548500.1	97.16	100.00	98.33	95.49	96.99	97.12	31.94	32.89	31.72	31.12	37.80	38.95	40.07	42.79	42.32	42.32	41.16	40.87	41.91	41.91	41.88	42.29	42.12	40.94	42.80	42.70	42.70	42.12	42.90	42.12	41.52
3: KY548499.1	97.85	98.33	100.00	95.99	97.63	97.61	31.46	32.57	30.84	30.10	38.76	41.05	40.96	42.34	43.18	42.55	42.76	41.17	42.27	42.36	41.92	42.19	42.55	42.81	44.11	44.14	43.82	42.24	43.75	42.47	43.34
4: KY548496.1	96.09	95.49	95.99	100.00	96.31	96.51	32.32	33.55	31.28	30.61	38.28	41.40	40.61	43.24	43.09	43.09	42.42	41.77	42.89	42.89	42.50	42.92	43.09	42.47	43.73	43.10	42.70	42.83	43.75	43.17	43.00
5: KY548495.1	98.21	96.99	97.63	96.31	100.00	97.61	33.08	34.54	31.28	31.12	39.23	42.46	41.64	42.79	43.89	43.89	43.43	42.57	43.69	43.69	43.33	43.75	43.89	43.49	44.87	44.14	43.82	43.64	44.64	43.98	44.03
6: KY548498.1	97.20	97.12	97.61	96.51	97.61	100.00	32.68	34.24	31.28	30.61	38.76	42.11	41.64	43.24	44.06	44.06	43.43	43.02	43.61	43.84	42.96	43.20	43.61	43.15	44.87	44.14	43.82	43.78	44.64	44.16	44.03
7: KY012295.1	31.84	31.94	31.46	32.32	33.08	32.68	100.00	97.45	98.73	98.51	39.02	40.79	41.36	41.05	42.64	42.53	39.51	42.64	42.53	42.64	42.15	42.75	42.64	38.51	34.81	37.74	37.58	42.59	43.84	43.13	40.74
8: KY012294.1	33.22	32.89	32.57	33.55	34.54	34.24	97.45	100.00	99.15	99.01	37.50	41.24	37.25	39.39	41.50	41.91	38.24	41.18	41.58	40.85	41.91	41.12	41.50	37.93	33.33	35.82	35.68	40.66	41.22	40.79	37.75
9: KY548503.1	30.84	31.72	30.84	31.28	31.28	31.28	98.73	99.15	100.00	100.00	39.19	39.71	39.04	40.23	40.00	40.00	37.67	40.00	40.00	40.00	40.44	40.00	40.44	37.93	31.93	36.36	36.17	40.44	42.78	40.44	39.04
10: KY548502.1	30.10	31.12	30.10	30.61	31.12	30.61	98.51	99.01	100.00	100.00	38.67	39.86	39.19	40.34	40.31	40.31	37.84	40.31	40.31	40.31	40.84	40.31	40.84	38.10	32.23	36.55	36.36	41.36	42.86	41.36	39.19
11: MN473876.1	38.28	37.80	38.76	38.28	39.23	38.76	39.02	37.50	39.19	38.67	100.00	75.76	67.31	68.69	70.95	70.95	70.67	70.00	70.48	70.48	70.00	70.48	70.00	69.57	69.06	68.78	68.47	70.48	70.48	70.48	70.19
12: KY012285.1	41.49	38.95	41.05	41.40	42.46	42.11	40.79	41.24	39.71	39.86	75.76	100.00	75.26	79.57	78.12	78.12	77.85	77.70	78.12	78.47	76.95	77.32	78.47	78.40	78.65	78.05	77.07	77.82	77.85	78.40	78.40
13: OP600465.1	40.75	40.07	40.96	40.61	41.64	41.64	41.36	37.25	39.04	39.19	67.31	75.26	100.00	92.35	91.25	91.25	91.25	91.58	91.58	91.92	93.19	93.19	91.92	91.89	91.76	91.50	92.25	95.24	94.95	94.95	94.95
14: MN473875.1	41.89	42.79	42.34	43.24	42.79	43.24	41.05	39.39	40.23	40.34	68.69	79.57	92.35	100.00	95.54	95.54	94.90	94.64	94.64	94.64	94.64	95.09	94.64	93.85	94.08	94.30	94.24	95.09	94.64	95.09	94.39
15: OM985018.1	43.73	42.32	43.18	43.09	43.89	44.06	42.64	41.50	40.00	40.31	70.95	78.12	91.25	95.54	100.00	99.43	99.66	96.79	97.91	98.12	98.02	98.03	97.93	98.31	97.00	96.26	96.31	95.80	94.99	96.01	95.96
16: OM993272.1	43.11	42.32	42.55	43.09	43.89	44.06	42.53	41.91	40.00	40.31	70.95	78.12	91.25	95.54	99.43	100.00	99.66	97.52	98.48	98.29	98.61	98.62	98.48	98.31	97.00	96.26	96.31	96.74	94.99	96.56	95.96
17: OP600468.1	42.47	41.16	42.76	42.42	43.43	43.43	39.51	38.24	37.67	37.84	70.67	77.85	91.25	94.90	99.66	99.66	100.00	97.98	97.65	97.99	98.57	98.57	97.99	98.31	97.00	96.26	96.31	96.26	95.00	95.96	95.96
18: OM948743.1	41.63	40.87	41.17	41.77	42.57	43.02	42.64	41.18	40.00	40.31	70.00	77.70	91.58	94.64	96.79	97.52	97.98	100.00	97.71	97.36	97.82	97.83	97.55	98.65	96.63	95.58	95.94	95.80	95.56	96.20	96.30
19: OM961396.1	42.83	41.91	42.27	42.89	43.69	43.61	42.53	41.58	40.00	40.31	70.48	78.12	91.58	94.64	97.91	98.48	97.65	97.71	100.00	99.43	99.40	99.41	99.24	98.99	96.25	95.58	96.31	96.74	95.28	96.95	96.63
20: OM961398.1	42.91	41.91	42.36	42.89	43.69	43.84	42.64	40.85	40.00	40.31	70.48	78.47	91.92	94.64	98.12	98.29	97.99	97.36	99.43	100.00	99.01	99.41	99.44	99.32	96.63	95.92	96.31	96.56	95.58	97.15	96.97
21: OM985015.1	42.54	41.88	41.92	42.50	43.33	42.96	42.15	41.91	40.44	40.84	70.00	76.95	93.19	94.64	98.02	98.61	98.57	97.82	99.40	99.01	100.00	99.21	99.60	100.00	97.22	96.38	96.31	96.83	95.62	96.83	96.77
22: OM985016.1	42.80	42.29	42.19	42.92	43.75	43.20	42.75	41.12	40.00	40.31	70.48	77.32	93.19	95.09	98.03	98.62	98.57	97.83	99.41	99.41	99.21	100.00	99.61	99.28	97.22	96.38	96.31	97.05	95.62	97.24	96.77
23: OM970798.1	43.11	42.12	42.55	43.09	43.89	43.61	42.64	41.50	40.44	40.84	70.00	78.47	91.92	94.64	97.93	98.48	97.99	97.55	99.24	99.44	99.60	99.61	100.00	100.00	96.63	95.92	96.31	96.76	95.58	97.34	96.97
24: OQ048860.1	42.61	40.94	42.81	42.47	43.49	43.15	38.51	37.93	37.93	38.10	69.57	78.40	91.89	93.85	98.31	98.31	98.31	98.65	98.99	99.32	100.00	99.28	100.00	100.00	96.63	95.92	96.31	96.59	96.62	96.96	96.96
25: KY012284.1	44.11	42.80	44.11	43.73	44.87	44.87	34.81	33.33	31.93	32.23	69.06	78.65	91.76	94.08	97.00	97.00	97.00	96.63	96.25	96.63	97.22	97.22	96.63	96.63	100.00	98.50	98.80	96.63	96.25	96.25	96.25
26: KY012282.1	43.25	42.70	44.14	43.10	44.14	44.14	37.74	35.82	36.36	36.55	68.78	78.05	91.50	94.30	96.26	96.26	96.26	95.58	95.58	95.92	96.38	96.38	95.92	95.92	98.50	100.00	99.26	95.88	95.58	95.58	95.58
27: KY012277.1	43.07	42.70	43.82	42.70	43.82	43.82	37.58	35.68	36.17	36.36	68.47	77.07	92.25	94.24	96.31	96.31	96.31	95.94	96.31	96.31	96.31	96.31	96.31	96.31	98.80	99.26	100.00	95.94	95.94	95.94	95.94
28: OM970802.1	42.83	42.12	42.24	42.83	43.64	43.78	42.59	40.66	40.44	41.36	70.48	77.82	95.24	95.09	95.80	96.74	96.26	95.80	96.74	96.56	96.83	97.05	96.76	96.59	96.63	95.88	95.94	100.00	98.21	99.62	99.66
29: OP257195.1	44.28	42.90	43.75	43.75	44.64	44.64	43.84	41.22	42.78	42.86	70.48	77.85	94.95	94.64	94.99	94.99	95.00	95.56	95.28	95.58	95.62	95.62	95.58	96.62	96.25	95.58	95.94	98.21	100.00	98.22	99.66
30: OM993270.1	42.94	42.12	42.47	43.17	43.98	44.16	43.13	40.79	40.44	41.36	70.48	78.40	94.95	95.09	96.01	96.56	95.96	96.20	96.95	97.15	96.83	97.24	97.34	96.96	96.25	95.58	95.94	99.62	98.22	100.00	100.00
31: OQ048859.1	43.15	41.52	43.34	43.00	44.03	44.03	40.74	37.75	39.04	39.19	70.19	78.40	94.95	94.39	95.96	95.96	95.96	96.30	96.63	96.97	96.77	96.77	96.97	96.96	96.25	95.58	95.94	99.66	99.66	100.00	100.00
32: OM948744.1	42.16	41.49	41.52	42.20	43.00	43.51	41.98	40.13	39.56	39.79	70.95	77.51	93.60	95.09	95.83	96.38	95.65	97.53	96.58	96.77	96.63	97.05	96.96	96.96	97.00	95.92	96.31	97.13	96.76	97.34	97.98
33: ON005452.1	43.01	42.15	42.58	42.58	43.66	43.68	42.53	40.59	40.00	40.84	70.48	77.70	95.34	95.54	96.04	96.67	97.13	96.46	97.29	97.29	97.08	97.50	97.50	97.84	98.02	97.10	97.05	98.96	97.50	98.96	98.92
34: OP600467.1	42.81	41.52	43.00	42.66	43.69	43.69	39.51	36.76	37.67	37.84	69.71	77.35	93.94	93.88	95.62	95.62	95.62	95.96	95.96	96.30	96.42	96.42	96.30	96.28	96.25	95.24	95.57	98.64	98.65	98.65	98.65
35: OQ048861.1	43.99	42.39	44.00	43.67	44.67	44.67	39.13	36.45	37.24	37.41	69.57	77.85	93.92	94.36	96.30	96.30	95.33	96.62	96.97	97.31	97.12	97.12	97.31	97.30	96.25	95.58	95.94	98.63	98.33	98.99	98.99
36: OM970801.1	43.35	42.12	42.80	43.09	43.89	44.06	43.40	40.85	40.44	40.84	70.48	77.08	93.94	95.54	95.34	96.57	95.97	95.85	96.58	96.62	96.83	97.24	96.80	96.62	95.88	95.24	95.57	98.66	97.05	98.86	98.32
37: OM948745.1	42.83	42.12	42.19	42.60	43.60	43.51	42.15	40.59	40.00	40.31	70.00	77.16	93.60	94.64	96.01	96.57	95.65	97.14	96.58	96.77	96.63	97.04	96.96	96.62	97.00	95.92	95.94	97.89	97.06	97.71	98.32
38: OM970799.1	43.16	42.53	42.61	43.29	44.09	44.29	43.18	40.85	40.44	41.36	70.48	77.78	94.28	95.09	96.41	96.95	96.64	96.40	96.96	97.16	97.02	97.44	97.35	97.30	96.63	95.92	95.94	99.05	97.35	99.24	98.99
39: OQ048862.1	43.84	42.24	43.88	43.54	44.56	44.56	40.12	37.25	38.36	38.51	70.19	78.12	94.28	94.90	96.64	96.64	96.31	96.97	97.32	97.65	97.49	97.49	97.65	97.64	96.25	95.58	95.94	98.98	98.99	99.33	99.33
40: OQ048857.1	44.78	43.26	44.97	44.63	45.64	45.64	40.72	37.80	39.07	39.22	70.48	78.05	94.28	95.02	96.69	96.69	96.63	97.02	97.35	97.68	97.54	97.54	97.68	97.64	96.25	95.58	95.94	99.00	99.01	99.34	99.33
41: OQ048858.1	43.84	42.24	44.03	43.69	44.71	44.71	40.12	37.25	38.36	38.51	70.19	78.05	94.28	94.90	96.63	96.63	96.63	96.97	97.31	97.64	97.49	97.49	97.64	97.64	96.25	95.58	95.94	98.98	98.99	99.33	99.33
42: OM970800.1	43.14	42.53	42.58	42.89	44.09	44.06	42.75	40.46	40.00	40.84	70.48	78.12	94.61	95.54	96.20	96.76	96.31	96.20	96.77	96.96	96.83	97.24	97.15	96.96	97.00	96.26	96.31	99.43	97.94	99.43	99.33
43: OP600466.1	42.96	42.03	43.15	42.81	43.84	43.84	39.75	36.95	37.93	38.10	70.05	77.70	94.93	94.87	96.28	96.28	96.28	96.28	96.28	96.62	97.12	97.12	96.62	96.62	97.00	95.92	96.31	99.66	98.99	98.99	98.99
44: OP257196.1	44.65	43.27	44.51	44.51	45.43	45.43	42.42	39.58	41.21	41.30	70.48	77.70	94.95	95.54	96.40	96.40	95.96	96.70	96.70	97.00	97.46	97.46	97.00	96.62	97.00	95.92	96.31	99.09	98.50	99.10	98.99
45: OP600464.1	42.70	41.52	42.70	42.35	43.42	43.42	40.12	37.75	38.36	38.51	70.19	77.45	95.44	94.39	96.84	96.84	96.84	97.19	97.19	97.54	97.49	97.49	97.54	97.54	97.67	96.81	96.68	99.30	99.30	99.30	99.30
46: OM961397.1	42.36	42.32	42.20	42.69	43.09	43.61	43.40	40.85	40.89	41.36	69.52	78.47	92.59	96.88	96.82	97.33	96.31	96.42	97.15	97.37	97.42	97.64	97.56	96.62	97.00	96.94	97.42	97.14	95.87	97.53	96.97
47: OM993271.1	42.22	42.32	42.14	42.77	43.17	43.71	43.40	41.18	40.89	41.36	69.52	78.75	91.92	96.88	96.23	96.95	96.30	96.04	96.57	96.60	96.63	97.05	96.79	95.95	96.63	96.60	96.68	96.76	95.27	96.96	96.30
48: ON008179.1	43.41	42.50	42.95	42.95	43.64	43.44	41.76	40.26	40.00	40.31	70.48	77.32	94.98	95.09	96.48	96.92	97.49	96.92	97.14	97.14	96.92	97.36	97.36	98.20	97.62	96.74	96.68	97.58	97.19	97.58	98.57
49: OM961399.1	42.55	41.91	41.99	42.48	43.29	43.38	42.75	40.79	40.00	40.31	70.48	77.43	94.28	95.54	96.58	97.14	95.97	96.58	97.53	97.34	98.02	98.23	97.53	96.62	97.00	96.60	97.05	97.90	96.46	97.91	97.64
50: OM985017.1	42.80	42.29	42.19	42.92	43.75	43.91	42.75	40.46	40.00	40.31	70.48	76.58	95.70	95.09	96.46	97.04	96.06	96.65	97.44	97.44	97.22	97.64	97.64	96.76	96.83	96.01	95.94	98.62	97.81	98.82	99.28

Figure 4.1 ClustalW alignment for NoV sequence similarity analysis.

4.4 PHYLOGENETIC ANALYSIS

The nucleotide sequence identity of norovirus strains obtained through blast in this study demonstrated above 95% similarity, however other strains were included based on geographical location to evaluate possible relatedness. The phylogenetic analysis of HNoV GII.4 Sydney 2012 [P31] sequences in Vhembe revealed multiple clades, as illustrated in Figure 4.2. These clades predominantly clustered together, suggesting a close relationship among the circulating HNoV strains in the area. Within these clusters, we observed that the main ancestor is more related to the GII.4 Sydney 2012 [P31], which later gave rise to the rest of the strains including to the novel GII.4 San Francisco. The closely related strains to our strains within the clusters were mostly those from Brazil, China and Australia. In comparison to the novel GII.4 San Francisco, few South African strains showed close relatedness to strains from UK than those from Johannesburg South Africa. There was close relationship between strains from within other African countries.

In Figure 4.3, the main ancestor seemed to carry first strains that were previously in South Africa from 2013. In a cluster with multiple strains characterized during this investigation, most related strain was from Ghana and more interesting the novel GII.4 San Francisco from USA. Once more the novel South African strains were distant when compared to UK.

In contrast, norovirus from Vhembe showed close relatedness to novel GII.4 San Francisco strains, mostly the one characterized in South Africa then the USA strain found 2017 carried the ancestral branch (Figure 4.4). The nearby clades carried strains from other African countries, however, other distant strains were also from within the continent but clustered with those from countries like Japan and South Korea.

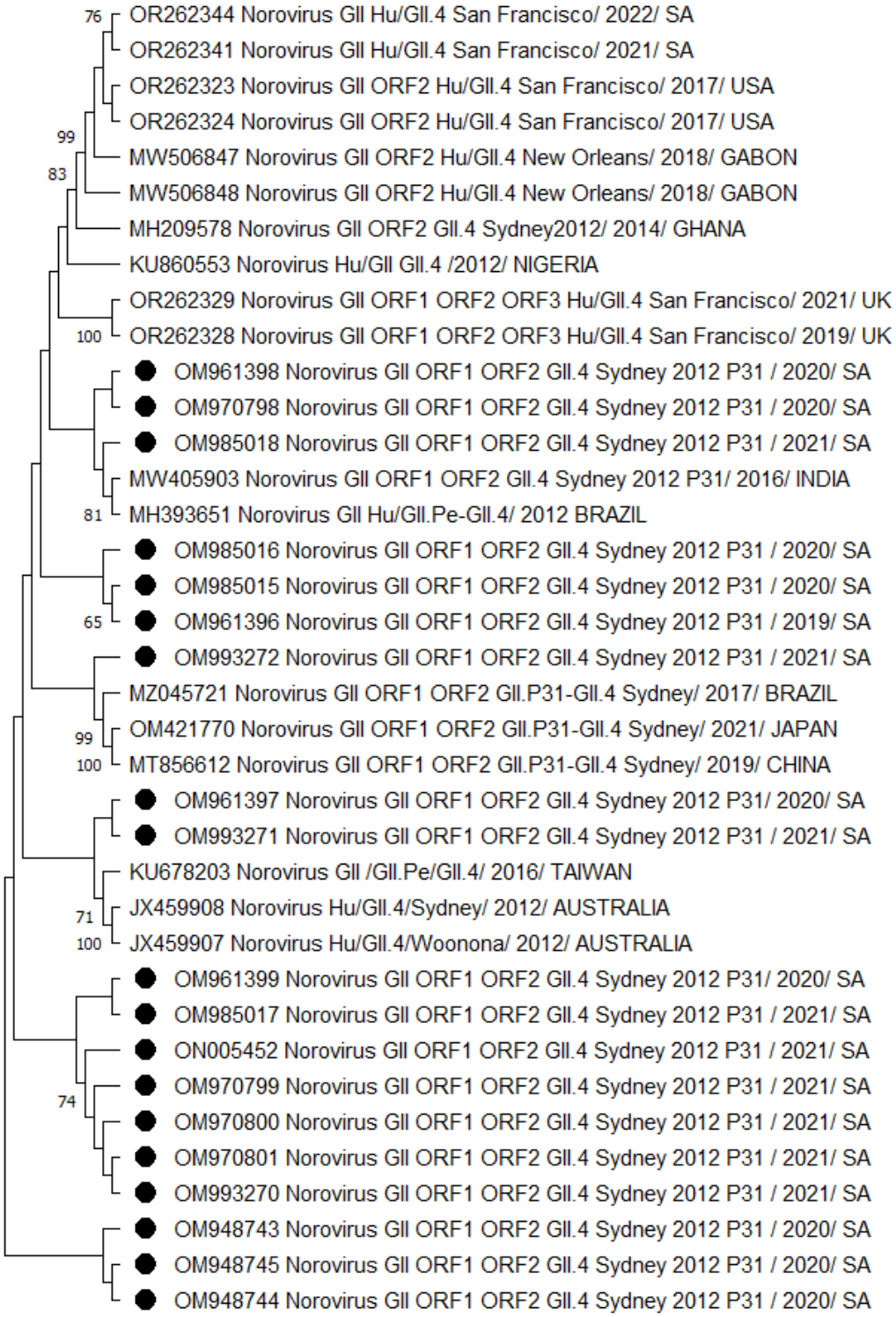

Figure 4.2 Phylogenetic analysis of HNoV nucleotide sequences circulating in the Vhembe district (South Africa) between 2019 and 2021, based on dual typing (polymerase and capsid junction) region, 570bp with [MON431/G2SKR].

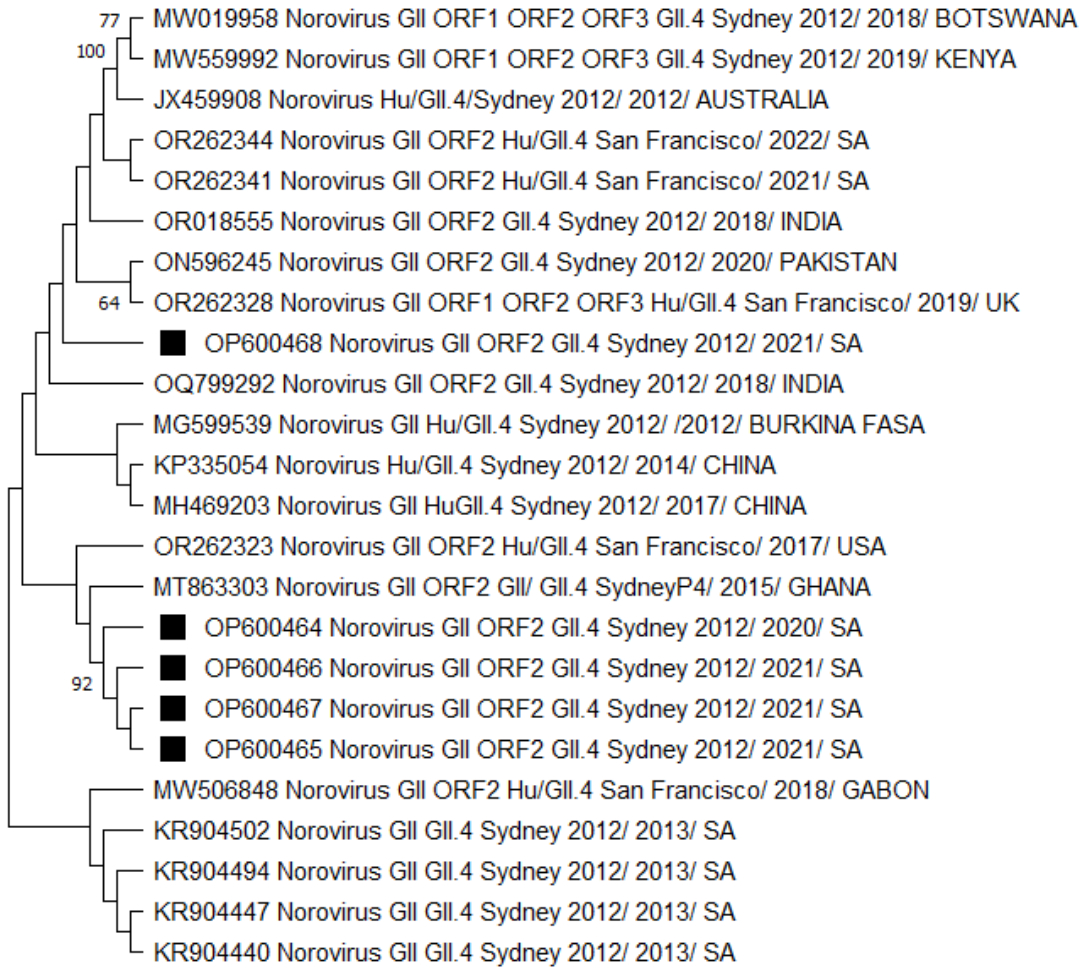

Figure 4.3 Phylogenetic analysis of the HNoV nucleotide sequences from the Vhembe district (South Africa) between 2019 and 2021, focusing on the partial typing of the 340bp capsid region with [G2SKF/R].

The phylogenetic trees were created through the Neighbor-Joining method. HNoV genotypes obtained during this study are represented by black circles, squares and triangles for Figures 4.2, 4.3 and 4.4, respectively. The reference sequences were randomly chosen from GenBank due to their high similarity with our study sequences. Each sequence is also described with respective accession numbers. Any positions containing gaps or missing data were excluded from the analysis. Evolutionary distances were calculated using the p-distance method and are expressed as the number of base differences per site. The analyses were conducted using MEGA 11 (10.0.5), and bootstrap tests (1000 replicates) were performed based on the Kimura two-parameter model. Only bootstrap values exceeding 65% are displayed.

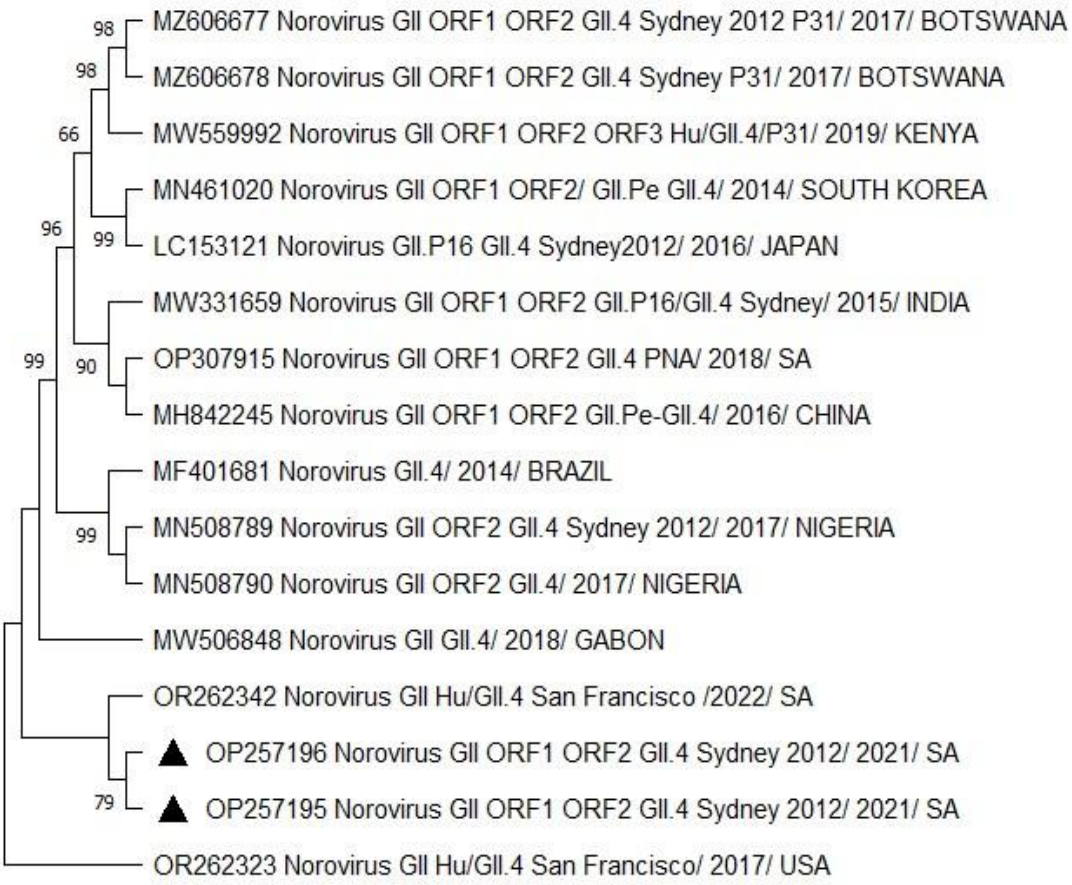

Figure 4.4 Phylogenetic tree based on 380bp [with COG2F/G2SKR] capsid/RdRp junction region of HNoV nucleotide sequence circulating in Vhembe district (South Africa) in 2019-2021.

4.5 HBGAS PHENOTYPE AND NOROVIRUS DISTRIBUTION

Amongst the study participants, distribution of Lewis positive phenotypes was higher than Lewis negative in both symptomatic and asymptomatic category (Table 4.3). Likewise, norovirus infections were predominant in Lewis positive children. Lewis phenotypes were grouped into (Lea-b+, Lea+b+, Lea-b- and Lea+b-). The most common Lewis phenotypes were Lea-b- (38%; 32%) and Lea-b+ (37.5%; 46%) in both symptomatic and asymptomatic children, and norovirus predominantly infected individuals with these phenotypes. The non-secretor phenotype Lea+b- was the least common with lowest norovirus infections for both groups.

In ABO phenotypic profiles, type O was the most frequent (59.5%; 45%), followed by A positive children (25.5%; 39%) in AGE and healthy participants, respectively. The most rare phenotype was AB (1.5%; 1%) followed by the relatively low B (13.5%; 15%). Similarly, norovirus infections were rare in AB profiles (0%; 7%) and higher on O (55%; 57%) and A (28%; 21%) phenotypes, in symptomatic and asymptomatic children, respectively.

Table 4.3 Summary of HBGA phenotypic profiles and distribution of norovirus observed in children in the Vhembe district, South Africa.

	Symptomatic		**Asymptomatic**	
Lewis phenotypes	**Frequency 200 (%)**	**NoV+ 74 (%)**	**Frequency 100(%)**	**NoV+ 14 (%)**
Lewis-	76 (38)	28 (38)	32 (32)	3 (21)
Lewis+	124 (62)	46 (62)	68 (68)	11 (79)
Le a/b profiles				
Lea-b+	75 (37.5)	35 (47)	46 (46)	8 (57)
Lea-b-	76 (38)	28 (38)	32 (32)	3 (21)
Lea+b+	35 (17.5)	10 (14)	10 (10)	2 (14)
Lea+b-	14 (7)	1 (1)	12 (12)	1 (7)
ABO phenotypes				
A	51 (25.5)	21 (28)	39 (39)	3 (21)
B	27 (13.5)	12 (16)	15 (15)	2 (14)
AB	3 (1.5)	0 (0)	1 (1)	1 (7)
O	119 (59.5)	41 (55)	45 (45)	8 (57)
HBGAs phenotypic summary				
nSe	32 (16)	5 (7)	16 (16)	1 (7)
Se	168 (84)	69 (93)	74 (74)	13 (93)

Overall distribution of secretor and non-secretor HBGAs profiles was similar in both symptomatic and asymptomatic. Likewise, norovirus positivity was higher in secretor individuals of both groups than non-secretors (Table 4.3).

4.6 HBGA GENOTYPES AND NOROVIRUS INFECTIONS

Of the 100 saliva samples randomly selected for FUT2 analysis, 36% successfully generated sequences used to further confirm secretor and non-secretor status based on the G428A mutation. Upon analysis, majority of FUT2 secretor sequences carried the homozygous GG Single Nucleotide Polymorphisms (SNPs) on the G428A position (23/36; 64%), followed by the heterozygous (GA) secretors (10/32; 28%). Only 8% (3/36) were non-secretors homozygous carriers of the FUT2 G428A nonsense mutation (AA).

Norovirus genogroup I infection was rare in FUT2 genotyped children while genogroup II was dominant. High prevalence of HNoVs was found in children with homozygous GG for FUT2 gene (64%), followed by the heterozygous GA (28%). Amongst the only 3 children genotyped as non-secretor, 2 were infected with norovirus GII.4 Sydney [P31] strain and only 1 was negative.

FUT3 sequences were genotyped to analyse the most common mutations including C314T, G508A, T1067A, T59G and T202C. Out of a total of 100 randomly selected samples for FUT3 analysis, 40 were successfully amplified and of those, 20 were successfully genotyped. Although not all selected samples were norovirus positive, norovirus GII and genotype GII.4 Sydney 2012 [P31] were the most commonly detected among positively genotyped FUT3 sequences. Based on this data, norovirus could be associated with the HBGAs Lewis profile in this study, and particularly those with G508A SNP (Table 4.4). The C314T and T1067A SNPs were not detected, and this could potentially be due to low quality sequence which could not yield conclusive results.

Table 4.4 FUT2/3 genes in norovirus infections

	FUT2 **n= 100**	**NoV +**	**GI**	**GII**	**GI/GII**	**GII.4 S2012**	**GII.4 P31**
FUT2 amplified	42						
FUT2 sequenced	36	27	4	19	4	3	10
Homo	23	17	2	13	2	3	5
Hetero	10	7	1	4	2	0	3
NSe	3	3	1	2	0		2
FUT3 amplified	40						
FUT3 sequenced	19	11	2	9	0	1	4
T59G	3	1	0	1	0	0	1
T202C	1	1	0	1	0	-	-
C314T	0	0	0	0	0	0	0
G508A	16	8	2	8	0	1	3
T1067A	0	0	0	0	0	0	0

Analysis of FUT3 SNPs showed that the most prevalent SNP was G508A, and was also the most commonly associated with infections that occurred in this study. Table 4.5 details the distribution of FUT3 SNPs profile. Majority of the FUT3 genes were homozygous wild types, followed by those with heterozygous SNPs. Mutant heterozygotes occurred in the G508A position and consisted of 1 TG and 2 GT nucleotides in the same position.

Table 4.5 FUT3 SNP profiles

FUT3 SNP	Homozygous wild type	Heterozygous	Mutant Heterozygous	Total
T59G	2	1	0	3
T202C	0	1	0	1
C314T	0	0	0	0
G508A	8	5	3	16
T1067A	0	0	0	0

4.7 HNOVS GENOGROUPS AND GENOTYPES IN HBGA PROFILES

We further investigated the distribution of norovirus genogroups and genotypes among the study participants based on HBGA susceptibility profiles (Table 4.6). The combined prevalence of all participating children with secretor status was (84%) to (16%) of non-secretors. Only (29%; 88/300) of this population was norovirus positive. Interestingly, 93% of these infected children were secretor positive compared to the 7% of non-secretors. Although detected, norovirus infection was less common in children with the non-secretor profiles. Overall distribution of norovirus genogroup showed higher prevalence of GII (74%) compared to GI (20%), mainly in children with Se status. Co-infection contributed only 6% in norovirus positivity and was only found in secretor profiles. Only 39% and GII strains in norovirus positive cases were successfully genotyped, and the recombinant GII.4 Sydney 2012 [P31] was the most prevalent (24%). In all HBGAs profiles, only two children with non-secretor status were infected with this recombinant strain. Our data is however limited to conclude on genotype-dependance based on HBGA profiles due to the minimal norovirus genotypes obtained.

Table 4. 6 Distribution of norovirus genogroups and genotypes in secretor and non-secretor children

		NoV +	GI	GII	GI/GII	GII.4 2012	GII.4 2012 [P31]
Total	300	88 (29)	18 (20)	65 (74)	5 (6)	13 (15)	21 (24)
Se	252 (84)	82 (93)	15 (83)	62 (95)	5 (100)	13 (100)	19 (90)
nSe	48 (16)	6 (7)	3 (17)	3 (5)	0 (0)	0 (0)	2 (10)

4.8 CORRELATION OF NOROVIRUS PRESENCE WITH HBGAS PROFILES

There was positive correlation between HBGAs secretor profiles (Lewis and ABO) and the distribution of norovirus genogroups and genotypes indicating the linear increase in both norovirus positivity (which depicts genogroup and genotype distribution) and secretor status. Although the P-value was not significant for both GI and GII (0.1580; 0.0747), the r-value representing more correlation was found in GII (r = 0.9253) than GI (r = 0.8420), meaning GII are strongly correlated with secretor status. Similarly, both GII.4 Sydney 2012 and GII.4 Sydney 2012 [P31] variant were positively correlated (r = 0.8665; 0.9374) with HBGAs secretor status. However the data regarding genotypes is limited due to most norovirus positive samples that could not be typed (Figure 4.5).

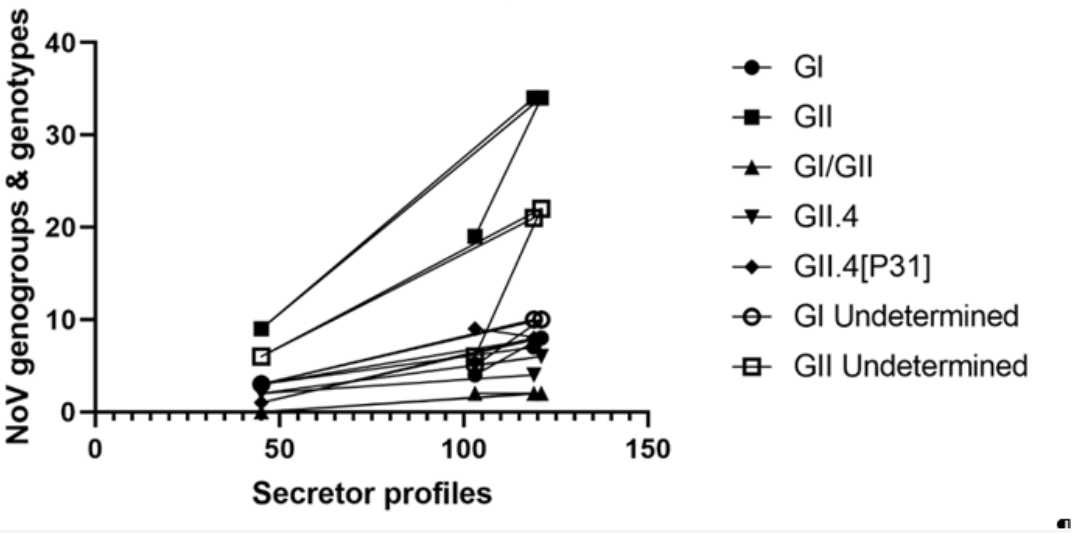

Figure 4.5 Correlation of HBGAs secretor profiles and norovirus

When comparing the non-secretor HBGAs profiles and norovirus distribution, the correlation was negative for both GI, GII and genotype distribution (r = -0.8441; r = -0.9991; r = -0.9991). Figure 4.6 demonstrate that the increase in non-secretor profiles is associated with low norovirus positivity and genogroup distribution. Although p-values for genogroup II and genotype GII.4 Sydney 2012 [P31] were statistically significant (0.0269; 0.0269), they were negatively correlated with the non-secretor profiles.

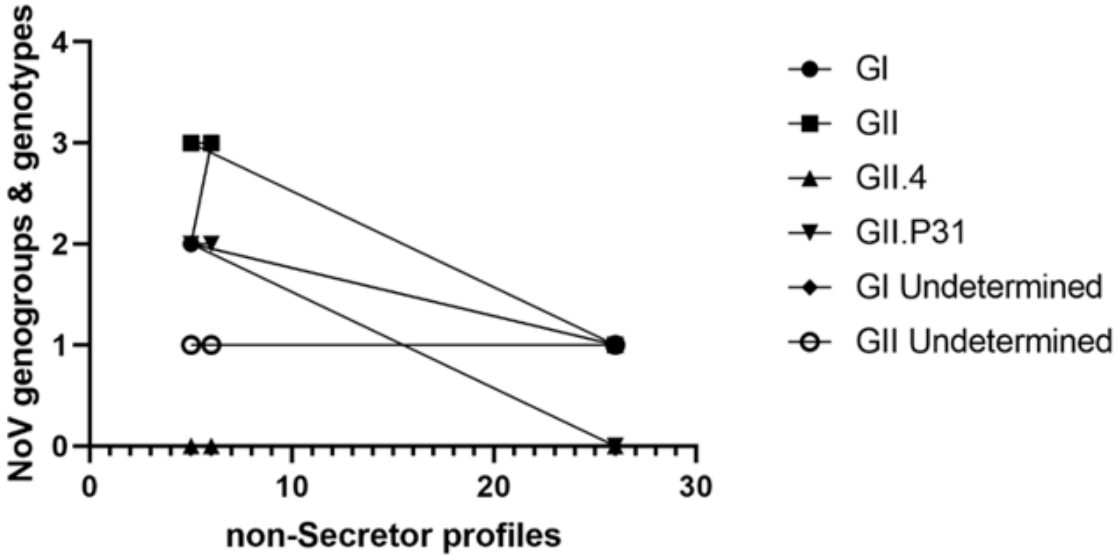

Figure 4.6 Correlation of HBGAs non-secretor profiles and norovirus

CHAPTER 5

DISCUSSION

Human norovirus is a global public health issue linked to diarrheal illness worldwide. In this work, we investigated the prevalence and genotype distribution of noroviruses in children with gastrointestinal diseases, in an effort to understand epidemiological fitness of different strains. Furthermore, we analysed the impact of local genetic profiles role in susceptibility and distribution of norovirus infections among South African children under five years of age.

In rural communities of Venda, South Africa, there has previously been evidence of high prevalence of HNoV (Kabue et al., 2016). After approximately 5 years since the previous report on norovirus, we present more recent data indicating that within the same study area, HNoV strains continue to circulate at a high rate (37%) (Khumela et al., 2023). Similarly high rate (36%) has been reported in an African country, particularly Ghana (Lartey et al., 2020). This prevalence rate is higher than the recently documented pooled prevalence of norovirus strains (20.2%) in Africa (Afework et al., 2022) which is as well higher than the global rate (Farahmand et al., 2022, Liao et al., 2021). This data emphasizes the increased and evidenced occurrence of diarrheal diseases and norovirus associated AGE in African countries, moreover in rural based areas. The burden in these hotspots is not well documented and as such overall estimates omit considerable amount of data that could direct interventions.

In South Africa, other studies have reported lower prevalence on norovirus ranging around 15% (Rossouw et al., 2021, Page et al., 2017). However, these studies focused only on hospitalised children and with participants based in urban areas. Our data includes both hospitalised and outpatients from rural community-based settings, which could explain the elevated prevalence rate. Recent reviews however indicate that the norovirus prevalence rate decreases from community based, outpatients to hospitalised (Afework et al., 2022, Liao et al., 2021). Interestingly, when the rate between hospitalised and outpatients was evaluated, inpatients had high norovirus infections (59%) compared to outpatients (41%). This may indicate an increased role of norovirus in the severity of diarrhoea disease. More studies including both rural and urban areas with varied settings are needed to establish a pattern.

The setting-based difference in infection was statistically significant in this study, highlighting the potential role of norovirus in the development of AGE symptoms and diarrhoea (P = 0.0129). Furthermore, among the crucial symptoms associated with HNoV infection, dehydration was on the lead. A recent study conducted in urban South Africa found that hospitalized children infected with norovirus presented severe diarrhoea (Rossouw et al., 2021). Similarly, we evaluated our study population and found a high vesikari score indicating the association of norovirus with severe AGE (Kabue et al., 2023). The severity and complications of norovirus have been previously documented in other nations, such as Qatar and Taiwan, respectively (Mathew et al., 2019, Wang et al., 2016). Co-infection with other pathogens that cause gastroenteritis may play a role in severity, however only the presence of norovirus was evaluated in this study.

The increased prevalence rate could be further explained by the fact that nowadays norovirus has become the primary cause of AGE globally (Ludwig-Begall et al., 2021), particularly in areas where rotavirus vaccination has been implemented (Godfrey et al., 2020). This study was carried out in South Africa's rural Vhembe district, where rotavirus vaccinations have been implemented since 2009.

The rate of HNoV infections varied significantly (P<0.0001) between the symptomatic (37%) and asymptomatic (14%), groups. There was no statistically significant difference between the symptomatic and asymptomatic subgroups in a prior norovirus survey conducted in this area (Kabue et al., 2016). This finding suggests that norovirus currently plays a significant role in AGE symptoms among children from the Vhembe district. Norovirus is actively involved with AGE in the area, which is consistent with observations from other low- and middle-income nations (Omore et al., 2023, Cohen et al., 2022).

During this study, male children exhibited a higher infection rate compared to females. Prior research has consistently shown that viral infections tend to be more prevalent among males than females (Duan et al., 2021, Jaillon et al., 2019). According to Jaillon and colleagues, females are believed to possess stronger immunity relative to males due to the presence of certain genes crucial for immune response located on the X chromosome (Jaillon et al., 2019).

HNoV was more common in children aged 6 to 23 months in this study (Table 4.1). According to recently released data, the majority of African countries' norovirus disease burden is experienced at this age range (Omore et al., 2023). The MALED group study reported a similar finding, indicating that norovirus infection peaked after six months (Rouhani et al., 2016). This is a crucial age for the development of the immune system in young individuals which comes with increased exposure. Data available indicated that children, particularly those under two years old, were highly susceptible to infection (Lartey et al., 2020, Rouhani et al., 2016). Children are believed to have lost their mother's antibody protection at this age (Papaventsis et al., 2007), and are more likely to engage in physical activities that could expose them to more environmental factors. Our results corroborate prior research suggesting that younger populations should be prioritised when developing vaccines.

The current study did not find any statistically significant protection against HNoV infection in breastfed children, despite the fact that breastfeeding has been linked to infection prevention (McCormick et al., 2022, North et al., 2022, Labayo et al., 2020) (P = 0.3732). The outcomes concur with current research (Vielot et al., 2022, Haddadin et al., 2021). However, Ghosh et al. have shown that during breastfeeding, enteric viruses can be transmitted from the baby's salivary glands to the mother's mammary glands, increasing the flow of maternal milk secretory IgA antibodies (Ghosh et al., 2022). While various viral pathogens have been identified in breastmilk (Diaz et al., 2018, Van de Perre et al., 2021), these pathogens seldom cause infections or diseases in infants (Henrick et al., 2017, Lanzieri et al., 2013). Since it is known that breastfeeding boosts an infant's immunity by introducing maternal antibodies, the World Health Organisation and UNICEF continue to recommend exclusive breastfeeding for the first six months of life in relation to AGE protection. A recent study recommending breastfeeding further stated that additional research is required to clearly emphasize risk or benefit ratio of breastfeeding for some known or emerging maternal infections and to analyze how the bioactive components cooperate in mediating the antiviral action of breastmilk (Francese et al., 2023).

While only the clinical samples underwent testing, the broader data on living conditions did not yield statistically significant findings. Nonetheless, the detection of HNoV in both pit/flush toilets, treated water, and among children residing with domestic animals

suggests inadequate hygiene practices within the study area. Previous studies conducted in the same area have revealed faecal contamination in various water sources, including tap water and household storage containers, which raises concerns about distribution networks and unhygienic behaviours (Luvhimbi et al., 2022). Additionally, Ayukekbong et al. documented the presence of norovirus in tap water in Cameroon (Ayukekbong et al., 2014), suggesting a potential resistance to chlorine treatment, as previously observed (Duizer et al., 2004).

Due to the lockdown restrictions imposed by the Covid-19 pandemic, seasonal distribution trends could not be included in the investigation. Nevertheless, throughout the study duration, HNoV infections displayed an annual increase, reaching the highest number of cases in 2021. This trend aligns with the forecasted surge in norovirus incidents following the easing of Covid-19 restrictions, (O'Reilly et al., 2021). Evidence exists regarding both the escalation and reduction of norovirus cases during the Covid-19 pandemic (Lu et al., 2022, Lennon et al., 2020). Additionally, norovirus outbreaks were reported in China and Japan during the Covid-19 pandemic (Pham et al., 2022, Zhu et al., 2021).

GII infections of HNoV were more prevalent among symptomatic cases, particularly among hospitalized children, which supports the notion of prioritizing them in vaccine development efforts (Table 4.2). Our results align with previous studies highlighting the significant role of GII in symptomatic infections (Mathew et al., 2019, Kreidieh et al., 2017, Page et al., 2017). Genogroup II strains have been linked to the majority of outbreaks globally (Cornejo-Sánchez et al., 2023, Robilotti et al., 2015). In contrast, the GI group was more commonly observed among control cases in our study. These findings are consistent with other research indicating that GI viruses tend to cause milder infections, whereas GII viruses are often associated with symptomatic illness (Kabue et al., 2016, Kitajima et al., 2012). However, a recent review found that norovirus GII was prevalent mainly in asymptomatic individuals (Wang et al., 2023). Symptomatic and asymptomatic individuals may shed the similar viral load and other shedding characteristics, and both have the potential to cause outbreaks (Utsumi et al., 2017, Teunis et al., 2015).

In this study, the GII.4 Sydney 2012 [P31] variant was prevalent, especially among symptomatic inpatients (Table 4.2). Initially identified in Australia, the GII.4 Sydney

2012 strain has since spread globally, with new variants emerging approximately every two to three years (van Beek et al., 2018). The majority of norovirus outbreaks are attributed to GII.4 genotypes, which led numerous pandemics since the mid-90s (Noel et al., 1999). In 2000 and 2004, the US95/96 strain was succeeded by two new GII.4 variants, Farmington Hills and the Hunter GII.4 variant (Widdowson et al., 2004, Fankhauser et al., 2002). Additional strains emerged in 2006 and 2009 (Parra et al., 2023).

The GII.4 Sydney 2012 [P31] variant has become predominant in many countries, including South Africa. Previously, Kabue et al. reported the predominance of GII.Pe/GII.4 Sydney 2012 variant between 2014 and 2015 in the study area (Kabue et al., 2017). Although without the data to account for the years between 2015 to 2019, we may speculate that the variant may have remained dominant to be the leading cause of AGE in this region. In Shanghai, this particular variant emerged as the dominant genotype from its onset in 2012 until 2019 (Lu et al., 2019), indicating the dominance characteristic since it has circulating. Most of the severe outcomes are in fact linked with GII.4 strains (van Beek et al., 2018, Desai et al., 2012). Similarly, our study findings indicate an increase in GII norovirus strains associated with dehydrating diarrhoea from 2019 to 2021.

The GII.4 Sydney 2012 [P31] strain, has emerged as the dominant and persistent epidemiological force, evolving through mutations and recombination (van Beek et al., 2018). The rapid evolution of GII.4 variants, particularly in the capsid region, contributes to their epidemiological fitness (Bull et al., 2012). Upon further examination of GII.4 Sydney 2012 [P31] nucleotide sequences in this study, substantial changes and potential mutations in the strains over time were observed. Comparing the nucleotide sequences of HNoV GII.4 strains obtained in this study to previously published data revealed less than 50% similarity (Figure 4.1). The genetic variation observed in HNoV strains over time underscores the ongoing impact of HNoV on vulnerable communities globally, with the potential emergence of new variants in the foreseeable future (Pham et al., 2022, Zhu et al., 2021). The variability in sequence nucleotides may elucidate the viral adaptability and epidemiological dominance in the region (Bull et al., 2010). Molecular analysis of GII.4 Sydney [P31] strains during an

outbreak in China suggested ongoing evolution, potentially leading to the emergence of novel variants (Zhu et al., 2021).

It remains unclear what factors might have specifically contributed to the epidemiological fitness and spread of the HNoV GII.4 Sydney 2012 [P31] strain during the Covid-19 pandemic in the study area. However, reports indicate the circulation of the same strain in Japan and China amidst the Covid-19 outbreak (Pham et al., 2022, Zhu et al., 2021). The characteristics determining epidemiological fitness are difficult to quantify (Wargo and Kurath, 2012). To comprehend the prevalence of the norovirus GII.4 Sydney 2012 [P31] strain, more extensive data encompassing various viral, host, and environmental factors are required. Recent data have highlighted the predominance of the GII.4 Sydney 2012 [P31] strain in norovirus-related diarrhoea (Ai et al., 2021, Duan et al., 2021), suggesting that this genotype may persist as the dominant strain for a considerable period due to its adaptability across different populations. We could not characterize other genotypes in this study, potentially due to the adverse effects of the South African national electricity load-shedding schedule on laboratory cold storage units, which may have favoured the survival of GII.4 Sydney 2012 [P31] and GII.4 Sydney 2012 strains. Furthermore, it is unclear how the heightened hygiene practices imposed during this pandemic may have impacted norovirus strains circulation.

Phylogenetic analysis unveiled the close relatedness among circulating norovirus GII.4 Sydney 2012 [P31] recombinants (Figure 4.2) within the study region. A similar pattern was observed for GII.4 Sydney 2012 strains (Figures 4.3 and 4.4), all of which demonstrated phylogenetic affinity to one another. Notably, the GII.4 Sydney 2012 [P31] strains showed close genetic links to norovirus strains circulating in Australia, India, Brazil, China, and Japan. In instances where norovirus strains exhibit close genetic relationships with strains circulating outside Africa, the distribution is likely due to population movement preceding the Covid-19 pandemic lockdown.

Sequence analysis indicated that GII.4 Sydney 2012 strains (Figure 4.3 and 4.4) are most closely related to HNoV strains from South African and countries within African continent. The circulation of closely related strains across different countries, particularly those within the same continent, may be attributed to border proximity.

GII.4 variants have become predominant in outbreaks since the early 1990s, continuing through the presently circulating GII.4 Sydney 2012 variant.

Of note, the GII.4 Sydney strain circulating in Vhembe, (particularly in figure 4.3 and 4.4) showed phylogenetic relatedness with the novel GII.4 San Francisco variant recently described (Chhabra et al., 2024). In their report they highlighted that novel strains when compared to the Sydney sequences in the 5'end of ORF2 exhibit maximum identities of up to 91-95%. Furthermore, the RdRp region of the novel strains were typed as GII.P31, this may explain the close phylogenetic relationship between our GII.4 strains and the novel norovirus variants. The dominance of the GII.4 Sydney 2012 [P31] may be influence by the fact that the emerging strains are similar. There is minimal data to determine the pandemic potential of the novel strain.

Over the past years, HBGA profiles have gained much attention as important viral attachment receptors that facilitate susceptibility to norovirus infection in different populations (Nordgren and Svensson, 2019). This study further investigated the association between norovirus prevalence and HBGA profiles among children less than five years. Our study confirms the role of population HBGAs profiles in norovirus infections. This study demonstrates genetic susceptibility in both symptomatic and asymptomatic groups (Table 4.3).

Study population consisted of more children with HBGAs secretor phenotypes compared to non-secretors. The distribution of secretor and non-secretor is variable among populations. Though most likely demonstrated for the first time in rural based population, the ratio of high secretor to non-secretor is not new in South African population (Rossouw et al., 2021, MacDonald et al., 2020) and oversees countries (Peña-Gil et al., 2021, Nordgren and Svensson, 2019, Cooling, 2015). In African populations, secretors are estimated to reach about 75-80%, whereas non-secretors can reach up to 30%, our trend is similar to this, although slightly higher most likely due to the limited number of samples used for this investigation. This is markedly different from the observations made in Asian populations, wherein non-secretors contribute approximately 5% (Parker et al., 2019).

In ABO groups, O positive children were highly infected followed by type A (Table 4.3). In symptomatic children, all GI infections were detected in children with type O

phenotype, as well as majority of asymptomatic NoV positive children. Group O phenotype has been associated with notably higher infectivity rate, particularly with GI (Nordgren et al., 2013, Rockx et al., 2005, Hutson et al., 2002). Contrary to this, another report demonstrated low infectivity of individuals with type O, however this was only based on norovirus GII strains (Tan et al., 2008). Predominant norovirus genogroup in ABO subgroups were GII, this could be explained by the fact that Genogroup II norovirus demonstrate no preference in blood type during infection (Rockx et al., 2005, Huang et al., 2003).

This study found considerable infection rate associated with type A phenotype. Contrary to this, a recent study could not establish a positive association between type A and norovirus infection (Liao et al., 2020). Moreover, the role of type A in HNoV infection could be considered neutral based on genetic characteristics of prevalent strains, binding patterns to histo-blood group antigens and the level of susceptibility across population and age group (Liao et al., 2020, Mallory et al., 2019, Chan et al., 2015).

The rate of susceptibility in blood type B was second to the least infected AB group. Studies have demonstrated partial protection to HNoV infection in people with blood type B and AB, especially with GI (Nordgren et al., 2010, Rockx et al., 2005, Marionneau et al., 2002). Thus, our results confirm this finding as no GI virus was detected in children in either B or AB phenotype. In-fact, only GII was detected at a low rate in symptomatic and in asymptomatic group. With note, not many individuals in the study population possess this phenotype. More recently, Liao and colleagues (2020) found no association in the presence of norovirus with type B (Liao et al., 2020). With regards to AB individuals, who are generally the smallest group in populations, the influence of blood type AB on norovirus shedding patterns could be affected by the overall sample size.

Based on this study's findings in Lewis antigens, it is concluded that Lea-b+ is common amongst all populations, and Lea-b- is generally common amongst African individuals, although thought to be rare in Caucasians (Mattos, 2016, Nordgren et al., 2013, Henry et al., 1995, Oriol, 1995). We however did not include Caucasians in the study population. The relatively low detection of Lea+b+ in both symptomatic and asymptomatic individuals in this study could be due to the fact that this phenotype is

mostly found in Asians, however a study in Brazil associated majority of participants with this profile and norovirus positivity in asymptomatic individuals (Tonini et al., 2020). We found high norovirus prevalence in children with Lea-b+ followed by Lea-b- profiles (Table 4.3), contradicting previous data suggesting that the presence of Lewis b is not necessary for norovirus infection. The suggestion was based on the high infectivity of Lewis b negative children (Nordgren et al., 2013, Bucardo et al., 2009).

Two of the non-secretor children were particularly infected by GII.4 Sydney 2012 [P31] recombinant strain. Our findings are comparable with recent data reporting infection of non-secretors with GII.4 (Tarris et al., 2022, Lindesmith et al., 2020). Tarris et al demonstrated that GII.4 strains are able to interact with Lea antigens. Furthermore, non-secretors are commonly found in Caucasian population, due to the homozygous nonsense mutation in the G428A (Kindberg and Svensson, 2009, Thorven et al., 2005).

Significant change of HBGA phenotypic expressions in different populations, which may explain norovirus intra-species diversity. However, there is limited data to substantially elaborate on the role of Lewis phenotype in norovirus susceptibility. Our results demonstrate GI and GII infections were predominantly found in Lewis negative children, whereas elsewhere, in an African population with similar distribution of HBGA profiles (Lewis negative), Nordgren et al. found GI infections only in Lewis positive profile (Nordgren et al., 2013). GI infection on Lewis positive was very rare in our study. More studies including larger African populations are required to accurately estimate the overall distribution of HBGAs, particularly Lewis profiles and their distinguished roles in NoV susceptibility.

FUT2 genotype results obtained during this research is consistent with known literature showing that individuals with functional FUT2 gene have increased susceptibility to norovirus (Hong et al., 2021, Ferrer-Admetlla et al., 2009). Furthermore, according to literature most of the non-secretor individuals have lower chances of susceptibility (Nordgren and Svensson, 2019). However, the GII.4 Sydney 2012 [P31] strain found in such individuals is known to show limited selectivity during infection due to constantly evolving binding strategies on the HBGAs binding epitopes (Zhu et al., 2021).

The FUT3 gene G508A SNP was the most prevalent in children from Vhembe. This is consistent with the data illustrating that it is one of the most common SNPs in the African continent (Soejima et al., 2009). Moreover, heterozygote and mutant heterozygotes were also detected in this particular SNP (Table 4.5), this is consistent with data indicating its high diversity in African population (Soejima et al., 2009). Second to this was the T59G, which is known to be less common than T202C in Africa (Olivares et al., 2021). Previously, the T59G was amongst the prevalent SNPs in a study conducted in Brazil (Olivares et al., 2021). However, it is possible that the inconsistency may as well be due to limited number of quality sequence that could be genotyped.

The predominant norovirus genotype in the study population was recombinant GII.4 Sydney 2012 [P31], followed by GII.4 Sydney 2012 (Table 4.2). There is notable increase in binding capabilities of GII.4 to various HBGA profiles. Several studies demonstrated that higher evolution rate and binding capabilities of norovirus with different Lewis HBGA enables them to infect the symptomatic and asymptomatic groups (Liang et al., 2021, Singh et al., 2015). Moreover, GII.4 strains exhibit epidemiological fitness and persistence over the Covid-19 pandemic, where most strains could not be obtained.

Pertaining to the HBGAs genotypes, the distribution of norovirus was strongly associated with homozygous GG secretor profile. According to literature, individuals with functional FUT2 gene are more prone to infection (Ferrer-Admetlla et al., 2009). Similarly, FUT3 genotypes were also associated with the presence of norovirus.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

HBGAs profiles and norovirus infection studies are of paramount importance and being increasingly studied due to their implications in development of preventive measures such as vaccines and potential treatments. Understanding molecular interactions of these molecules in populations mostly burdened is crucial for prevention and control of outbreaks. Noroviruses continue to be a primary cause of sporadic illnesses and outbreaks of acute gastroenteritis, even in industrialized areas. This underscores the notion that enhanced hygiene and sanitation measures alone may not entirely be sufficient in managing norovirus. This study provides valuable insight in the dominance of GII.4 Sydney 2012 [P31] genotype and its genetic variation over time. More efforts are needed in the control of norovirus circulation and spread, especially in highly affected rural communities with inadequate WASH conditions. It is important to monitor genetic changes, epidemiological fitness of HNoV strains and possible emergence of new variants in this era of norovirus vaccine development. Overall, all secretor profiles were positively correlated with norovirus genogroup and genotype distribution in HBGAs profiles of children within Vhembe district. In non-secretor population, Pearson's correlation revealed that a population with high non-secretors has low norovirus prevalence rate. Therefore, local population HBGAs profiles plays a role in the epidemiological patterns of norovirus in Vhembe district.

To answer the research question, data obtained during this investigation indicates that there is association between norovirus infection and HBGAs profiles in children from Vhembe district of Limpopo, South Africa. High prevalence of norovirus was associated with children who had secretor HBGAs profiles. Therefore, we reject the null hypothesis.

We recommend improved surveillance and targeted interventions. Systematic surveillance must be implemented in this area, continuous data will prove useful in the goals of lowering children death and diarrheal disease.

Thorough investigation in population genetics will advance the understanding of genetic susceptibility, epidemiology patterns and treatment development not only for norovirus AGE.

Improve WASH conditions and community awareness.

- Limitations of this study include: i) not able to provide seasonal distribution data of norovirus due to the Covid-19 lockdown restrictions during the study period that impacted sampling; ii) Other norovirus extracts could not be genotypes potentially due to minimal viral load, which may have been further enhanced by electrical power-cut in the laboratory cold storage units, due to national loadshedding; iii) This further impacted the provision of genetic diversity data; iv) Similarly, FUT2/3 genes could not be amplified in majority due to limited funds, and degradation of samples as a result of power-cut.

  Areas for Further Study

  - Longitudinal studies to track infection patterns over time.
  - Development of norovirus vaccines tailored to genetic profiles.

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APPENDICES

A. TABLE 1: CT-VALUES OF SYMPTOMATIC SAMPLES

Sample ID	Genogroup	CT-Value	Sample ID	Genogroup	CT-Value
2	G1	32.16	130	G2	33.92
6	G1	35.14	131	G2	36.29
12	G1	24.62	132	G2	34.90
16	G1	31.18	134	G2	35.62
18	G2	25.91	135	G2	35.26
24	G1	33.61	138	G1	32.27
30	G2	32.47	139	G2	26.33
32	G2	33.64	140	G2	36.68
37	G2	28.91	141	G2	36.91
40	G2	22.53	142	G2	34.98
42	G2	33.24	144	G1	27.52
51	G1	32.51	145	G2	36.14
52	G1	35.81	148	G2	35.87
66	G2	27.82	149	G1/G2	29.16/35.88
67	G2	22.20	154	G2	35.46
71	G2	29.87	157	G2	36.02
74	G2	25.29	158	G2	33.11
75	G2	26.25	159	G2	20.19
82	G2	27.21	160	G2	35.98
83	G2	35.17	163	G2	23.70
84	G2	29.50	167	G2	34.43
88	G1	35.28	169	G2	30.36
90	G1/G2	21.91/21.91	171	G2	36.76
96	G2	25.19	174	G2	35.18
97	G2	30.79	176	G2	35.09

98	G2	22.93	177	G2	27.66
103	G1	30.15	179	G2	35.33
116	G2	33.78	180	G2	36.51
117	G2	35.77	181	G2	30.47
118	G2	32.38	183	G1/G2	34.70/36.47
119	G2	34.43	184	G1/G2	30.27/34.49
120	G2	31.12	185	G2	18.86
121	G2	32.08	186	G2	36.21
122	G2	18.22	188	G2	32.25
123	G1/G2	35.39/17.81	192	G2	27.57
124	G2	31.35	194	G1	27.23
125	G2	32.12	200	G1	35.87
126	**G2**	31.79			
127	G2	34.82			
128	G2	34.96			
129	G1/G2	30.59/34.09			

B. TABLE 2: CT-VALUES FOR ASYMPTOMATIC SAMPLES

Sample ID	Genogroup	CT-Value	Sample ID	Genogroup	CT-Value
H002	G1	30.06	H070	G1	30.87
H023	G2	36.68	H073	G1	32.81
H029	G2	36.77	H080	G1	32.06
H034	G1	35.96	H082	G1	34.20
H039	G2	35.02	H085	G1	27.75
H048	G2	30.44	H087	G1	36.47
H053	G2	19.63	H101	G1	34.97
H054	G1	32.50	H102	G1	29.50

C. DATA MANAGEMENT PLAN

DATA MANAGEMENT PLAN
Project Title: NOROVIRUS-HOST INTERACTIONS STUDIES: HBGA PHENOTYPIC AND GENOTYPIC PROFILES OF PAEDIATRIC PATIENTS WITH DIARRHOEA FROM RURAL COMMUNITIES OF LIMPOPO SOUTH AFRICA

Principal Investigator: Ronewa Khumela
Institution: University of Venda
Date: 26/06/2024

1. Introduction
This Data Management Plan (DMP) outlines the procedures for handling data collected during the project " NOROVIRUS-HOST INTERACTIONS STUDIES: HBGA PHENOTYPIC AND GENOTYPIC PROFILES OF PAEDIATRIC PATIENTS WITH DIARRHOEA FROM RURAL COMMUNITIES OF LIMPOPO SOUTH."

2. Data Creation and Collection
2.1 Types of Data
Clinical data: Information about the clinical characteristics of each participant.
Phenotypic Data: Information on the phenotypic expression of HBGAs in children, secretor status.
Genotypic Data: DNA sequences related to HBGAs and norovirus susceptibility, obtained from saliva samples.
Epidemiological Data: Demographic information, health history, and environmental factors.
Laboratory Data: Results from assays and experiments, including PCR, qPCR, ELISA, and sequencing data.
2.2 Methods and Tools
Data Collection Instruments: Questionnaires, stool and saliva collection kits.
Software Tools: Microsoft excel for data entry and management, Graphpad prism 10 for statistical analysis, and bioinformatics tools for sequence analysis.
2.3 Metadata Standards and Documentation
Metadata: Includes variable names, definitions, data types, and units of measurement.
Documentation: Standard Operating Procedures (SOPs) for data collection, processing, and analysis.
Data Dictionary: Provided in an Excel spreadsheet, detailing variable names and coding schemes.

3. Data Storage and Security
3.1 Storage Locations
Short-term Storage: Data will be stored on secure institutional servers with regular backups.
Long-term Storage: Data will be archived in repositories.

3.2 Security Measures
Access Controls: Restricted access to authorized personnel only, using passwords and user permissions.
Physical Security: Secure access to physical storage locations for biological samples.
3.3 Backup Procedures
Regular Backups: Automated daily backups of digital data.
Off-site Storage: Copies of backups stored in a separate location to prevent data loss.

4. Data Sharing and Access
4.1 Data Sharing Policies
Internal Sharing: Data will be shared among project members through secure institutional platforms.
External Sharing: Data will be shared with third parties following ethical guidelines and data sharing agreements.
4.2 Access Control Mechanisms
Data Requests: External researchers must submit a data request form detailing their research questions and intended use of the data.
Approval Process: Data requests will be reviewed and approved by the Principal Investigator and relevant stakeholders.
Data Integrity Checks: Regular checks to ensure data has not been altered or corrupted.

5. Ethical and Legal Compliance
5.1 Ethical Considerations
Informed Consent: All participants will provide informed consent for data collection and sharing.
Privacy Protection: Personal identifiers will be removed or anonymized to protect participant privacy.
6.2 Legal Requirements
Compliance: Adherence to local and international regulations regarding data protection and privacy.

6. Conclusion
This Data Management Plan outlines the comprehensive approach to managing data for the project " NOROVIRUS-HOST INTERACTIONS STUDIES: HBGA PHENOTYPIC AND GENOTYPIC PROFILES OF PAEDIATRIC PATIENTS WITH DIARRHOEA FROM RURAL COMMUNITIES OF LIMPOPO SOUTH AFRICA." By following this plan, we aim to ensure data integrity, facilitate sharing and collaboration, and enhance the overall quality and impact of our research.

Signature:

Principal Investigator: _  _____
Date: _26/06/2024_____

D. ETHICAL CLEARANCE – PROVINCIAL

LIMPOPO
PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA
DEPARTMENT OF HEALTH

Enquiries: Stander SS (015 293 6650) Ref: LP_2018_07_016

Kabue M
University of Venda

Greetings,

RE: Genetic susceptibility to norovirus infections in young children from rural communities of Vhembe District, 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, or incur any cost on the Department.
   - 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

Date 03/09/2018

Private Bag X9302 Polokwane
Fidel Castro Ruz House, 18 College Street. Polokwane 0700. Tel: 015 293 6000/12. Fax: 015 293 6211.
Website: http/www.limpopo.gov.za

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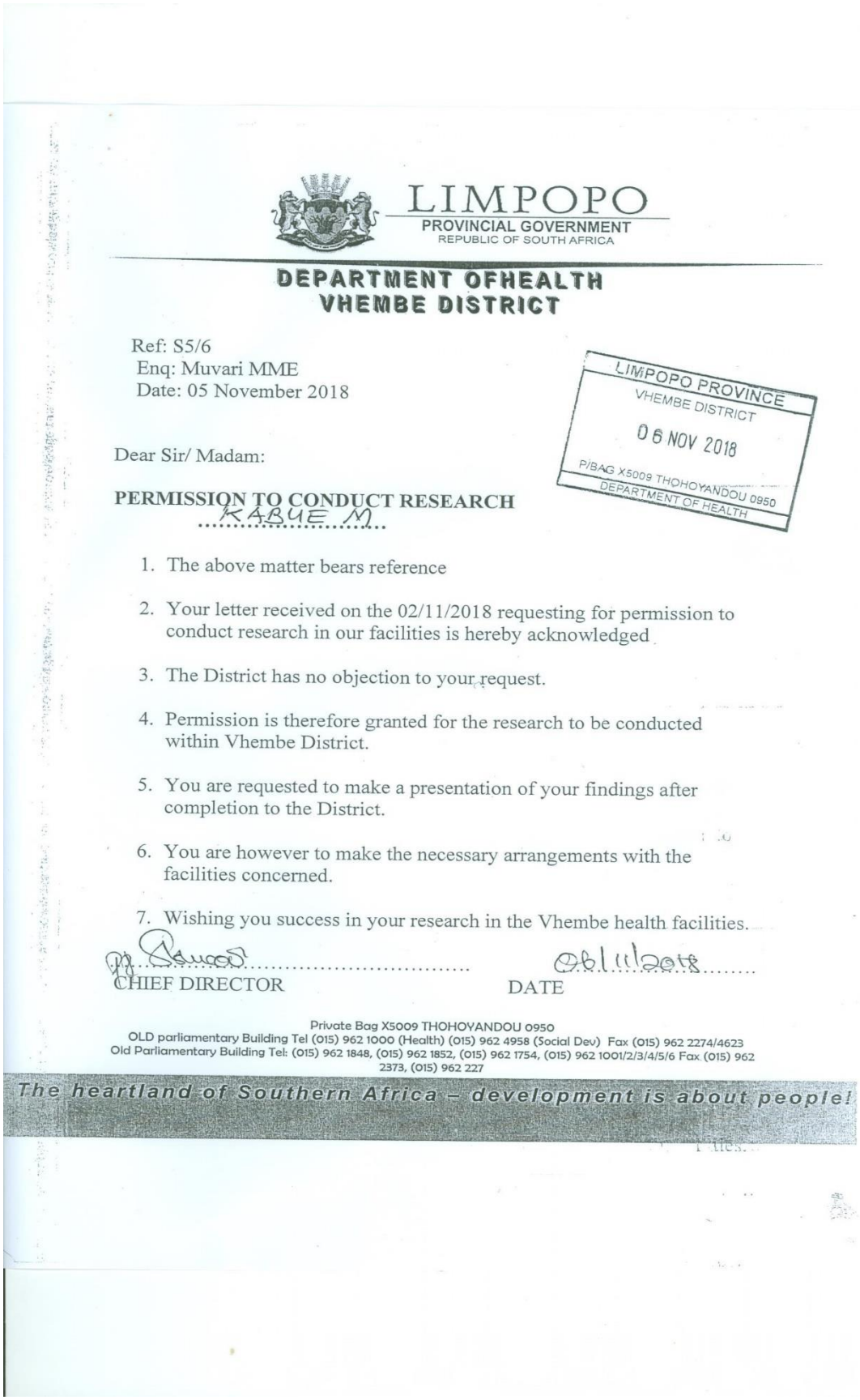
E. ETHICAL CLEARANCE – DISTRICT



LIMPOPO
PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OFHEALTH
VHEMBE DISTRICT

Ref: S5/6
Enq: Muvari MME
Date: 05 November 2018

LIMPOPO PROVINCE
VHEMBE DISTRICT
06 NOV 2018
P/BAG X5009 THOHOYANDOU 0950
DEPARTMENT OF HEALTH

Dear Sir/ Madam:

PERMISSION TO CONDUCT RESEARCH
KABUE M

1. The above matter bears reference
2. Your letter received on the 02/11/2018 requesting for permission to conduct research in our facilities is hereby acknowledged
3. The District has no objection to your request.
4. Permission is therefore granted for the research to be conducted within Vhembe District.
5. You are requested to make a presentation of your findings after completion to the District.
6. You are however to make the necessary arrangements with the facilities concerned.
7. Wishing you success in your research in the Vhembe health facilities.

CHIEF DIRECTOR ..... DATE 06/11/2018

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

The heartland of Southern Africa – development is about people!

F. ETHICAL CLEARANCE – UNIVERSITY OF VENDA

RESEARCH AND INNOVATION
OFFICE OF THE DIRECTOR

NAME OF RESEARCHER/INVESTIGATOR:
Mr M Kabue

Staff No:
6963

PROJECT TITLE: Genetic susceptibility to norovirus infections in young children from rural communities of Vhembe District, South Africa.

PROJECT NO: SMNS/18/MBY/07/2505

SUPERVISORS/ CO-RESEARCHERS/ CO-INVESTIGATORS

NAME	INSTITUTION & DEPARTMENT	ROLE
Mr M Kabue	University of Venda	Investigator
Prof N Potgieter	University of Venda	Co - Investigator
Prof A Ndama	University of Venda	Co - Investigator
De E Meader	University of Venda	Co - Investigator

ISSUED BY:
UNIVERSITY OF VENDA, RESEARCH ETHICS COMMITTEE

Date Considered: May 2018
Decision by Ethical Clearance Committee Granted
Signature of Chairperson of the Committee: .....
Name of the Chairperson of the Committee: Senior Prof. G.E. Ekosse

UNIVERSITY OF VENDA
DIRECTOR
RESEARCH AND INNOVATION
2018 -05- 28
Private Bag X5050
Thohoyandou 0950

University of Venda
PRIVATE BAG X5050, THOHOYANDOU, 0950, LIMPOPO PROVINCE, SOUTH AFRICA
TELEPHONE (015) 962 8504/8313 FAX (015) 962 9050
"A quality driven financially sustainable, rural-based Comprehensive University"

G. ENGLISH CONSENT FORM

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT:

"Genetic susceptibility to Norovirus infections in young children from rural communities of Vhembe district, South Africa"

Reference Number: **SMNS/18/MBY/07/2505**

Investigators:

Dr Jean Pierre KABUE NGANDU

Prof NATASHA POTGIETER

Address:
Department of Microbiology
School of Mathematical and Natural Sciences
University of Venda
Contact Number: 0159628256 / 0159628107/0813260163

Dear parent or guardian, thank you for showing interest in this research project.
Your baby is being invited to take part in a research project. Please take some time to read the information presented here, which will explain briefly the project. Please ask the study staff any questions about any part that you do not fully understand. Your participation is **entirely voluntary and you are free to decline to participate.**
This study has been approved by the committee for Human Research at The University of Venda. And will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Practice and the Medical Research Council (MRC) Ethical Guidelines for research.

What is this research project study all about?

. This study will include stool and saliva specimens from young children under 5 years with or without diarrhea. About 400 children will be included in this study.

. The project aimed to investigate the host genetic susceptibility to Norovirus infections among young children under 5 years of age from rural communities of Vhembe district, South Africa. This information will help decisions making in public prevention strategies against diarrhea disease transmission particularly in Norovirus infection also in the improvement of sanitary

1

environments in the rural communities. The findings of this study will also provide information on Norovirus diversity with implications on vaccine development.

General information will be taken from you, including contact details, age, gender, use of toilet, date of diarrhea, HIV status and others illnesses, ect. Saliva and stool specimens will be collected from your baby and will be transported to the laboratory for analysis.

Why have been invited to participate?

Your baby was selected for this study on the basis of the diarrhea he is suffering from.

What will your responsibilities be?

Participation on in this study is completely voluntary. You may refuse to provide information or stool.

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 virological analysis if positive.

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

There are no risks involved in participating. Collection of stool and saliva specimens will be done after or when the baby is eliminating waste during diarrhea episodes.

Who will have access to your medical records?

Only the medical doctor/nurse and research team will have access to your medical information. Your identity will not be made public and if the results of this study are published or presented, you will only be referred to by a code number. Your identity will be kept confidential.

Is there anything else that you should know or do?

You may contact Dr Jean-Pierre KABUE at 0813260163 / 0813044914 or Prof Natasha Potgieter at 0159628107 if you have any further queries or encounter any problems.

Declaration by participant:

By signing below, I .....agree to take part in a research study entitled **"Genetic susceptibility to Norovirus infections in young children from rural communities of Vhembe district, South Africa"**

I declare that:

- I have read or had to read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurized to take part.

Signed at (place).....on (date).....2019.

_____ _____

Signature of participant **Signature of witness**

Declaration by investigator:

I (name) .....declare that:

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

. I encouraged him/her to ask questions and took adequate time to answer them.

. I am satisfied that he/she adequately understands all aspects of the research, as discussed above

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

Signed at (place) .....on (date).....2019.

_____ _____

Signature of investigator **Signature of witness**

Declaration by interpreter:

I(name).....declare that:

. I assisted the investigator(name).....to explain the information in this document to (name of participant).....using the language medium of Venda.

. We encourage him/her 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 had all his/her question satisfactorily answered.

Signed at (place).....on (date).....2019.

_____	_____
Signature of Interpreter	**Signature of witness**

H. VENDA CONSENT FORM

FOMO YA THENDELO NA BAMBIRI ḼA ZWIDODOMBEDZWA ZWA MUDZHENELELI

ŢHOHO YA THANDELA YA ŢHOḌISISO:

"Dzofha li disaho khonadzeo ya vhulwadze ho vhangwaho nga Norovirus kha vhana vha dzulaho mahayani tshitirikini tsha Vhembe, Africa Tshipembe"

Nomboro ya Referentsi: SMNS/18/MBY/07/2505

Vhasedzulusi: Dr. Jean Pierre Kabue Ngandu

Prof. Natasha Potgieter

ḌIRESI: Department of Microbiology

School of Maths and Natural Sciences

University of Venda

Luţingo: 0813260163 / 015 962 8256 / 0159628107

Kha vhabebi/vhaundi, ri livhuwa u dzhenela havho kha eyi thodisiso.

Ṅwana wavho u khou humbelwa u dzhenelela kha u vha tshipiḓa kha thandela hei ya ţhoḓisiso. Vha khou humbelwa uri vha dzhie tshifhinga vha vhale mafhungo o ṉetshedzwaho hafha, ane a ḓo ţalutshedza nga u pfufhifhadza thandela hei. Kha vha vhudzise mushumi wa ngudo iyi 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.

1

Ngudo hei yo tendelwa nga komiti ya Human Research Yunivesity ya Venḓa nahone i ḓo itwa ho sedzwa maitele na milayo ine fanela 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 ṅwana wa vhukale ha miṅwaha miṱanu ane a khou dinwa nga u tshuluwa

. Ngudo iyi i ḓo katela vhana vha 400, zwifuwo zwa hayani zwa 400,

. Ṱhoḓisiso hei yo livhiswa kha u ṱoḓulusa vhushaka vhune ha vha hone kha virasi dza Noro dzi no wanala kha vhana vha minwaha ya fhasi ha mitanu (5) dzine dza 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 ya Noro na kha u khwinifhadza fhethu hune ha vha na mabunga kha zwitshavha zwa mahayani.

Mawanwa a ngudo iyi a ḓo ṋetshedza mafhungo nga ha u phaḓalala ha virasi ya Noro ho sedzwa kha u bveledza dzilafho.

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. Mare na Mafhambuwa zwiḓo dzhiwa kha ṅwana wavho zwenezwo zwi ḓo iswa laborothari u senguluswa.

Ndi ngani vhone vho humbelwa u dzhenelela?

Ṅwana wavho o nangiwa kha ngudo iyi ho sedzwa u tshuluwa hune a khou vha khaho.

Vhone vha fanela u ita zwifhio?

U dzhenelela kha ngudo iyi ndi zwa u tou funa nga iwe muṉe. Vha nga hana u ṉekedza mafhungo kana mafhambuwa na mare.

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

A huna ndiliso ya tshelede ine vha ḓo ṉewa nga u dzhenelela havho. Fhedzi zwa konadzea vha ḓo wana mvelelo dza tsenguluso ya zwisinisa na dza tsenguluso ya zwitshili.

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

A huna khombo dzine vha nga dzi wana nga u dzhenelela havho. U kuvhanganya mafhambuwa na mare zwi ḓo itwa nga murahu ha musi ṅwana 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 ya 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 Dr Jean-Pierre KABUE at 0813044914 kana 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 hei:
Ndi khou bula zwauri: **"Dzofha li disaho khonadzeo ya vhulwadze ho vhangwaho nga Norovirus kha vhana vha dzulaho mahayani tshitirikini tsha Vhembe, Africa Tshipembe"**

- 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).....2019.

_____	_____
Tsaino ya mudzheneleli	Tsaino ya ṱhanzi

Muano nga musedzulusi:

Nṋe (dzina) .....ndi khou ana uri:

. Ndo ṱalutshedza mafhungo a re nga ḽiṅwalo heḽi kha Vho - .....

. 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 thon go shumisa muḓologi. (Arali muḓologi o shumiswa, muḓologi u fanela u saina muano u re afho fhasi.

Tsaino yo itwa (fhethu).....nga ḽa

(datumu).....2019.

Tsaino ya musedzulusi

Tsaino ya ṱhanzi

Muano nga muḓologi:

Nṋe(dzina).....ndi khou ana uri:

- Ndo thusedza musedzulusi (dzina).....u ṱalutshedza mafhungo kha a re kha ḽiṅwalo heḽi kha (dzina ḽa mudzheneleli).....hu tshi khou shumiswa luambo lwa Tshivenḓa.
- Ro muṱuṱuwedza u vhudzisa dzimbudziso ra dzhia tshifhinga tsho teaho u dzi fhindula.
- Ndo ṋekedza mafhungo one one o teaho a sina tshiṱahe kha zwe zwa vha zwi tshi khou elana na nṋe
- Ndo fushea ngauri mudzheneleli o pfesesa nga vhuḓalo zwine zwa vha kha ḽiṅwalo ḽa thendelo ḽo tevhelaho maga a mulayo o ṋetshedzwaho nahone mbudziso dzawe dzoṱhe dzo fhindulwa lu fushaho

Tsaino yo itwa (fhethu)..... nga ḽa (datumu).....2019.

_____ _____

Tsaino ya Muḓologi Tsaino ya ṱhanzi

I. XITSONGA CONSENT FORM

VUXOKOXOKO BYA MULAVISISI NA FOMO YA MPFUMELO HI MUTSWARI/MUHLAYISI

NHLOKOMHAKA YA NDZAVISISO

"Genetic susceptibility to Norovirus infections in young children from rural communities of Vhembe district,South Africa."

Ndzavisiso wa swivangelo swa ku va vana lavantsongo lava kumekaka eka miganga ya Vhembe district eSouth Africa ,va beburiwa va tsanile no va swi olova ku va va hlaseriwa hi xitsongwatsongwana xa Norovirus.

Reference number:SMNS/18MBY/07/2505

INVESTIGATORS/VALAVUISISIA:

Dr Jean-Pierre KABUE NGANDU

Prof NATASHA POTGIETER

Address/Kherefu:

Department of Microbiology

School of Mathematical and Natural Sciences

University of Venda

Tinqingho:0159628256/0159628107/0813260163

Eka mutswari kumbe muhlayisi wa n'wana,ha khensa ku komba ka n'wina ntsakelo wa ndzavisiso/projeke leyi.Hi rhamba n'wana wa n'wina ku nghenelela eka nthirho lowu.Mi komberiwa ku tinyika nkarhi wo hlaya mahungu lama tsariweke eka tsalwa leri lama baka mavala ya yinghwe hi projeke leyi.Ma pfumeleriwa ku vutisa swivutiso eka valavisisi mayelana na swin'wana leswi mi nga swi twisisiki swinene eka riseche/projeke leyi.Ku nghenelela ka n'wina eka ntirho lowu **a hi xikolokolo, ma pfumeleriwa ku ala loko mi nga swi tsakeli.**

Ndzavisiso lowu wu pasisiwile hi komiti ya "Human Research"e yunivhesiti ya

Venda.Hikwalaho ka sweswo,riseche leyi yi ta endliwa hi ku landzelela milawu na maendlele ya"the international Declaration of Helsinki,South Africaan Guidelines and guidelines for Good practice and the Medical Research Council(MRC)Ethical Guidelines for research.

Ku ta tirhisiwa no landzeleriwa milawu ya maendlele ya riseche.

What is this research project study about?
Xana riseche/projeke leyi mayelelana no lavisisa yini?
*Ku ta laveka marhanyana na mahambukanyana ya vana lava nga hansi ka malembe ya ntlhanu, lava nga na nchuluko na la va nga chulukiki.Eka ndzavisiso lowu ku ta laveka vana vo ringana 400.

1

*Xikongomelo xa projeke leyi i ku lavisisa ndhawu leyi xitsongwatsongwana lexi xa Norovirus ,lexi hlaselaka vana va le hansi ka malembe ya Ntlhanu emigangeni ya le Vhembe district,South Africa xi kumekaka kona emmirhini.

-Mbuyelo wa reseche leyi wu ta pfuna eka ku kuma ndlela yo sivela ntlulelo wa ntungu wa nchuluko eka xixaka ngopfungopfu wo vangiwa hi xitsongwatsongwana xa Norovirus ,kasi na nkululo emigangeni wu ta antswisiwa.

-Vuxokoxoko lebyi nga ta laveka eka n'wina byi katsa,riqingo,rimbewu,minkarhi ya mayele ya n'wina exihambukelweni hi siku,siku leri mi veke na nchuluko,xiyimo xa n'wina xa HIV na man'wana mavabyi,swi.n.swi.

-Marha na mahambuka swi ta tekiwa eka n'wana wa n'wina swi rhumeriwa eka laboratari ku ya kamberiwa.

Why have you been invited to participate?
Hikwalaho ka yini mi komberiwile ku va eka reseche leyi?
-N'wana wa n'wina u hlawuriwile leswaku ku ta lavisisiwa xivangelo xa nchuluko lowu a nga na wona.

What will your responsibility be?
Xana vutihlamuleri bya n'wina byi ta va byihi eka projeke leyi?
-Munhu a nga boheki ku nghenelela eka riseche leyi,a nga boheki ku nyika tinhlamulo eka swivutiso hambi ku ri ku nyika mahambuka.

Will you benefit from taking part in this research?
Xana mi ta vuyeriwa hi ku nghenelela eka projeke leyi?
-A ku na mali leyi mi nga ta yi kuma eka ntirho lowu handle ka mbuyelo wa vulavisisi mayelana na nchuluko wa n'wana wa n'wina loko wu ri lowunene.

Are there risks involved in your taking part in this research project?
Xana ku nghenelela eka project leyi a swi na khombo ke?
*Ku hava khombo hikuva marha na mahambuka swi ta kumeka loko wu ri nkarhi wa n'wana wo swi humesa kumbe loko a hetile ku endla tano.

Who will have access to your medical records?
I vamani lava nga ta va na mfanelo wo vona mbuyelo wa riseche /projeke leyi?
*I vadokodela/manese na lava endlaka riseche ntsena lava nga na mpfumelelo wo vona mbuyelo wa vulavisisi lebyi nga ta endliwa.

-Loko mbuyelo wu huma,mavito ya vana a ma nga boxiwi,ku ta tirhisiwa ti code numbers.

Is there anything else that you should know or do?
Xana ka ha ri na swin'wana leswi mi lavaka ku swi tiva kumbe ku endla?
Mi nga khumbha Dr Jean-Pierre KABUE eka 0813260163/0813044914 kumbe Prof Natasha Potgieter eka 0159628107 loko mi ri na swivutiso kumbe ku tikeriwa kun'wana.

DECLARATION BY PARTICIPANT:

KU HLAMBANYA HI MUTSWARI/MUHLAYISI

Hi ku va ndzi siyinile laha hansi,Mina(Mutswari/Muhlayisi)---------- ndza pfumele ku nghenelela eka riseche leyi,ya nhlokamhaka"Ndzavisiso wa ku va vana lavantsongo va le migangeni ya Vhembe district va velekiwa va tsanile no va swi olova ku va va hlaseriwa hi xitsongwatsongwana xa Norovirus."

Ndza hlambanya leswaku:

-Ndzi hlayile/hlayeriwile mahungu lawa na fomo yo pfumela ku nghenelela eka projeke.

-I nyikiwile nkarhi wo vutisa swivutiso no kuma tiunhlamulo leti ti ndzi eneteke.

-Ndza swi twisisa leswaku ku nghenelela eka projeke leyi a swi bohi na leswaku a ndzi lo susumeteriwa hi munhu ku endla tano.

Signed at (Ndzhawu)----------hi(Siku)----------2019.

_____
_____ Signature of participant (Mutswari/Muhlayisi)
Signature of witness(Mbhoni)

DECLARATION BY INVESTIGATOR:

KU HLMBANYA HI MALAVISISI:

I(Vito)---------- Ndza hlambanya leswaku :

-Ndzi hlayile no hlamusela----------mahungu lama nga eka tsalwa leri.

-Ndzi n'wi nyikile nkarhi wo vutisa swivutiso/ndzi tlhela ndzi swi hlamula hi ku hetiseka.

-Ndza pfumela leswaku mutswari/muhlayisi loyi wa twisisa hinkwaswo swilaveko swo riseche leyi.

-A ndzi tirhisangi toloki.

Signed at Ndzhawu:----------on(Siku)---------- 2019

_____ _____

Signature of investigator Signature of witness

KU HLAMBANYA HI TOLOKI

I,(Vito)----------Ndza hlambanya leswaku:

-Ndzi pfunile mulavisisi----------ku hlamusela ----------Mutswari/Mupfuni mahungu lama nga eka tsalwa leri hi ririmi ra Xitsonga .

-Hi n'wi hlohleterile ku vutisa swivutiso no n'wi nyika tinhlamulo hi ku hetiseka.

-Ndzi hlamuserile hi ku hetiseka,hinkwaswo leswi kongomaneke na projeke leyi.

-Ndza enetiseka hi ndlela leyi mutswari/Muhlayisi a twisisaka ha kona mahungu lama nga eka tsalwa leri na hi ndlela leyi a hlamuleke swivutiso ha kona.

Signed----------(Ndzhawu)----------on(Date)---------- 2019.

Signature of interpreter

signature of witness

J. STUDY OUTPUTS

- One article based on objective one of this study has been published (Khumela, R., Kabue, J.P., Moraes, M.T.B.D., Traore, A.N. and Potgieter, N., 2023. Prevalence of Human Norovirus GII. 4 Sydney 2012 [P31] between 2019 and 2021 among Young Children from Rural Communities in South Africa. Viruses, 15(8), p.1682).

- For objective two and three, the following abstract has been accepted for oral presentation in the SASBMB Congress 2024 which will be held on the 7 - 10 July 2024.
  HBGAs Phenotypic and Genotypic Profiles in Norovirus Infections Among Children from Rural Communities in the Vhembe District.
  Authors: Ronewa Khumela, Jean-Pierre Kabue, Afsatou Ndama, Natasha Potgieter
  Presenter: Ronewa Khumela
  Norovirus is a major cause of acute gastroenteritis globally, with cell surface glycans such as histo-blood group antigens (HBGAs) playing a crucial role in viral attachment to cells. These HBGAs, including ABO, secretor (FUT2), and Lewis antigens (FUT3), act as either viral attachment ligands or restriction factors. The diversity of HBGAs profiles, influenced by population ethnicity and host genetics, impacts the evolution and diversity of norovirus. This study aimed to investigate HBGAs as susceptibility factors for norovirus infections in children in the Vhembe district. Stool and saliva specimens were collected from 200 children with acute gastroenteritis (AGE) and 100 without AGE. Stool samples were analyzed for norovirus using RT-PCR, RT-qPCR, and Sanger sequencing, while saliva specimens underwent HBGAs phenotyping and genotyping via ELISA and TD-PCR, respectively. Norovirus prevalence was significantly higher in symptomatic (37%) compared to asymptomatic (14%) individuals. GII infections were predominant (80%) in children with AGE, with GII.4 Sydney 2012 being the dominant genotype. High norovirus positivity was associated with secretor individuals compared to non-secretors. These findings confirm the influence of population HBGAs as susceptibility factors for norovirus infections in the study region.
  Keywords: Norovirus, Acute gastroenteritis (AGE), Histo-blood group antigens (HBGAs), susceptibility.

- Article number two is in progress, which will outline key insights based on overall objectives and answer the research question.

viruses

MDPI

Article

Prevalence of Human Norovirus GII.4 Sydney 2012 [P31] between 2019 and 2021 among Young Children from Rural Communities in South Africa

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Abstract: Acute gastroenteritis (AGE) accounts for considerable morbidity and mortality in the paediatric population worldwide, especially in low-income countries. Human norovirus (HNoV), particularly GII.4 strains, are important agents of AGE. This study aimed to detect and characterise HNoV in children with and without AGE. Between 2019 and 2021, 300 stool samples (200 AGE and 100 without AGE) were collected from children below 5 years of age referred to the healthcare facilities of the rural communities of Vhembe District, South Africa. After detection using real-time RT-PCR, HNoV positive samples were subjected to RT-PCR and Sanger sequencing. Partial nucleotide sequences (capsid/RdRp) were aligned using the Muscle tool, and phylogenetic analysis was performed using MEGA 11. The nucleotides' percent identity among HNoV strains was compared using ClustalW software. A significant difference in HNoV prevalence between AGE children (37%; 74/200) and non-AGE (14%; 14/100) was confirmed ($p < 0.0001$). Genogroup II (GII) HNoV was predominant in AGE children (80%; 59/74), whereas most non-AGE children were infected by the GI norovirus genogroup (64%; 9/14). GII.4 Sydney 2012 [P31] strains were dominant (59%; 19/32) during the study period. A phylogenetic analysis revealed a close relationship between the HNoV strains identified in this study and those circulating worldwide; however, ClustalW showed less than 50% nucleotide similarity between strains from this study and those from previously reported norovirus studies in the same region. Our findings indicate significant changes over time in the circulation of HNoV strains, as well as the association between high HNoV prevalence and AGE symptoms within the study area. The monitoring of HuNoV epidemiology, along with stringent preventive measures to mitigate the viral spread and the burden of AGE, are warranted.

Keywords: norovirus; acute gastroenteritis (AGE); symptomatic; asymptomatic; GII and GI genogroups; GII.4 Sydney 2012 [P31]; GII4 Sydney 2012

Citation: Khumela, R.; Kabue, J.-P.; Moraes, M.T.B.d.; Traore, A.N.; Potgieter, N. Prevalence of Human Norovirus GII.4 Sydney 2012 [P31] between 2019 and 2021 among Young Children from Rural Communities in South Africa. *Viruses* **2023**, *15*, 1682. https://doi.org/10.3390/v15081682

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1. Introduction

Viral acute gastroenteritis (VAGE) is a common disease in both high- and low-income countries [1]. Since its discovery, human norovirus (HNoV) has emerged and become the common cause of VAGE worldwide, accounting for over 700 million cases and 219,000 deaths annually [2–4]. Although norovirus affects people of all ages, it remains a significant cause of paediatric morbidity and mortality [1], especially in low-income countries [5,6]. The global prevalence of norovirus ranges from 12% to 20.2% [7–9]; however, a higher prevalence has also been reported in Africa [10,11]. The spread of norovirus is mainly facilitated by the faecal oral route and poor hygiene practice [12].

Norovirus is a genus in the *Caliciviridae* family composed of enveloped, positive sense, genetically diverse viruses with an approximately 7.5 kb single-stranded RNA genome [13,14]. The norovirus genome consists of three ORFs (open reading frames), including ORF1, which encodes a large polyprotein that is cleaved by viral protease to generate nonstructural proteins (NS1/2-7); ORF2, which encodes a major capsid protein VP1; and ORF3, which encodes a minor structural protein VP2 [13,15]. The capsid protein consists of the shell (S) domain and two protruding (P) subdomains. Norovirus vaccine candidates currently in trial are VP1-based [16]. Amongst the 10 genogroups (GI-GX) of norovirus, VAGE is mainly caused by GI, GII, and GIV, with GII being the most predominant genogroup worldwide. Based on VP1 amino acid sequences and partial RdRp nucleotide sequences, norovirus genogroups are subdivided into 48 capsid genotypes and 60 P-types [17]. GII.4 is the most common genotype infecting humans worldwide and has been implicated in serious pandemics for about three decades [18]. The majority of HNoV-related deaths are predominantly reported in low-income countries [5,6], and over the years, HNoV infections increasingly impose an enormous economic burden in developed countries [19].

The predominance of GII.4 Sydney 2012 [P31] strains has been increasingly reported [20–22]. The capacity of a viral strain or variant to remain dominant in a given geographical area is defined as epidemiological fitness [23–25]. The features involved can include viral and host characteristics, though they cannot be easily quantified [23]. Observational data, such as changes in the prevalence, distribution, and composition of viral genotypes, may be used to quantify the epidemiological fitness of a virus [24].

In South Africa, the GII.4 Sydney 2012 [P31] strain was detected in children hospitalised with AGE from an urban area [26]. Kabue et al. [27] worked on outpatients from a rural area in South Africa, who also showed a high prevalence of GII.4 norovirus strains. There is poor WASH (water, sanitation, and hygiene) practice in rural communities [28], which may aid the spread of pathogens. Understanding the epidemiological fitness of prevalent viral genotypes is critical for therapeutic interventions and preventive strategies to minimise the spread. Therefore, continuous surveillance is critical in this era of vaccine development to provide up-to-date genetic information and understanding of norovirus epidemiology in areas with a history of high norovirus prevalence where preventive measures would have an enormous impact [16]. This study aimed to give an update on norovirus prevalence and genetic characteristics in children within the rural communities of Vhembe District in South Africa.

2. Materials and Methods

2.1. Ethics

The study protocol and consent procedures were approved by the ethics committees of the Department of Health, Limpopo Province (LP-2018-07-016) and the Research Directorate of the University of Venda (Ref. SMNS/18/MBY/07/2505). Written, informed consents were given by the parents or children's guardians before sample collection.

2.2. Study Population and Sample Collection

Between 2019 and 2021, a cross-sectional study on children under five years of age living in the rural communities of Vhembe District, Limpopo, South Africa, was performed. The study population was subdivided into 2 groups, symptomatic (children with AGE symptoms) and asymptomatic (children without any AGE symptoms for at least one month) [12]. Inclusion for AGE children was based on the passing of at least 3 or more loose stools within 24 h [29]. Nonbloody diarrhoeal stool samples (n = 200) were randomly collected at different primary healthcare clinics and hospitals within the district. For healthy controls, 100 stools samples were collected in healthcare facilities within the same region. Information relating to the personal details, clinical symptoms, and living conditions (e.g., water source, presence of livestock) of the patients was obtained on preprinted investigation forms. In hospitalised cases, only children admitted within 24 h were considered for this

investigation. All specimens were transported to the University of Venda Microbiology Laboratory and stored at −20 °C for further analysis.

2.3. RNA Extraction and Human Norovirus Detection

Viral nucleic acid was extracted using Boom method [30] from pea-sized stools suspended in 500 µL of phosphate buffer saline (PBS). This method is based on the lysis and nuclease-inactivating properties of the chaotropic agent guanidium thiocyanate, together with the nucleic acid-binding properties of silica particles. RNA extracts were stored at −20 °C prior to norovirus detection. RIDAGENE real-time RT-PCR (r-Biopharm AG, Darmstadt, Germany) kit's viral stool panel (PG1415) was used to detect and differentiate norovirus genogroup I and II. This assay, according to the manufacturers, is thought to not cross-react with other common enteric pathogens. The assay has 98% sensitivity and specificity [31] and includes internal control to monitor the extraction efficiency and amplification inhibition. The platform used for qPCR was Rotor-gene Corbett 6000. Norovirus positivity threshold cycle value was considered up to ≤37 (Ct) for GII and ≤35 for GI, with display of sigmoid curve [32,33].

2.4. Norovirus RT-PCR and Genotyping, ClustalW Alignment, and Phylogenetic Analysis

Norovirus positive RNA extracts were amplified with genogroup-specific polymerase–capsid (PC) assay [32] using Qiagen OneStep Reverse Transcription-PCR kit (Qiagen, Germantown, MD, USA) for the purpose of nucleotide sequencing. Previously described primers [34,35] for GI MON432/GISKR (579 bp) were used for this assay under the following PCR conditions: reverse transcription at 42 °C for 30 min, activation of Taq polymerase at 95 °C for 15 min, and 40 cycles of PCR amplification at 95 °C, 50 °C, and 72 °C for 1 min each, followed by 10 min at 72 °C and cooling down to 4 °C. MON431/GIISKR (570 bp) for GII was used with one adjustment on the PCR conditions annealing at 56 °C. Norovirus positive extracts that could not be amplified using previous conditions were subjected to another round of amplification (seminested PCR) by the primers COG2F/G2SKR(GII) and COG1F/G1SKR (GI), which generate 390 pb and 380 pb fragments, respectively [34,36]. The amplicons that still could not generate a band were subjected to OneStep Ahead RT-PCR using GISKR/F and GIISKR/F primers that produce 330 bp and 344 bp, respectively [34]. The norovirus amplicons were separated using electrophoresis on 2% agarose gel stained with ethidium bromide and specific-sized band visualised using UV light transilluminator. All amplicons visualised at the expected size were sent for partial Sanger sequencing using 3730XL Genetic Analyzer POP7™ (Thermo-Scientific, Waltham, MA, USA) at Inqaba Biotec™ (Pretoria, South Africa). The same primers for conventional PCR were used during Sanger sequencing. Raw sequences were read and edited via Finch TV (version 1.4) (Geospiza, Seattle, WA, USA). The edited nucleotide sequences were then compared with reference strain accessed on NCBI blast tool (accessed on 13 March 2023) at http://www.ncbi.nlm.nih.gov/. Edited sequences were genotyped using the online noronet platform at http://www.rivm.nlm/norovirus/typingtool (accessed on 15 March 2023) and human calicivirus typing tool HuCaT (Atlanta, GA, USA) at https://norovirus.ng.philab.cdc.gov/ (accessed on 15 March 2023). The norovirus genotypes obtained in this study were submitted to NCBI GenBank and have been assigned the following accession numbers: OM948743-OM948745, OM961396-OM961399, OM970798-OM970802, OM985015-OM985018, OM993270-OM993272, ON005452, ON008179, OP257195-OP257196, OP600464-OP600468, and OQ048857-OQ048862.

The ClustalW alignment tool was used to compare aligned sequences' nucleotide percent identity between current genotyped norovirus (n = 34) and previously published norovirus sequences (n = 14) in the same study area [37]. After sequence input, ClustalW is able to show nucleotide similarities in percentages amongst all sequences.

Phylogenetic analysis was performed to investigate the genetic relationship between norovirus strains detected in this study and others circulating worldwide. The reference strains from GenBank with best hit of >80% similarity to the study's query sequences were

randomly selected for phylogenetic analysis using MEGA 11 [38]. Additional strains were selected based on location to fully investigate the relatedness of worldwide circulating strains over time.

2.5. Statistical Analysis

Data were captured on Excel spreadsheet. Statistical significance was determined by calculating *p*-values using Chi-square and Fisher's exact test on GraphPad Prism 9 (GraphPad Inc., San Diego, CA, USA).

3. Results

3.1. Human Norovirus Prevalence and Sample Characteristics

This study presented the prevalence of HNoV between 2019 and 2021 (Table 1) within the rural communities of Vhembe District. The prevalence of HNoV was 37% (74/200) in symptomatic and 14% (14/100) in asymptomatic individuals. The difference in symptomatic and asymptomatic HNoV prevalence was proven to be statistically significant ($p < 0.0001$). HNoV significantly ($p = 0.0125$) affected more hospitalised patients (60%; 44/74) than outpatients (41%; 30/74) during the investigation. The majority of study participants were males (58%) compared to females (42%), and a higher HNoV prevalence was recorded in males (66%) than females (34%). Children aged 6–23 months (65%) were predominantly infected by HNoV, followed by children aged 0–5 months (20%).

Table 1. Demographic characteristics, clinical features, and HNoV prevalence among children with AGE from Vhembe District, South Africa, in 2019–2021.

	Symptomatic		**Asymptomatic**	
	Total (%)	**HNoV+ (%)**	**Total (%)**	**HNoV+ (%)**
Detection rate (%)	*n* = 200	*n* = 74 (37)	*n* = 100	*n* = 14 (14)
Setting				
Outpatients	104 (52)	30 (41)	100 (100)	14 (14)
Hospitalised	96 (48)	44 (59)	0	0
Gender				
Males	116 (58)	49 (66)	53 (53)	8 (57)
Females	84 (42)	25 (34)	47 (47)	6 (43)
Age in months				
0–5	49 (25)	15 (20)	21 (21)	2 (14)
6–23	124 (62)	48 (65)	59 (59)	10 (71)
24–60	27 (14)	11 (15)	20 (20)	2 (14)
Symptoms				
Diarrhoea only	48 (24)	8 (11)		
Diarrhoea and other symptoms	152 (76)	66 (89)		
Other symptoms seen together with diarrhoea				
Dehydration	132 (66)	61 (82)		
Vomiting	119 (60)	54 (73)		
Fever	79 (40)	36 (49)		
Abdominal pain	29 (15)	15 (20)		
Stool appearance				
Watery	103 (52)	46 (62)		
Formed	97 (49)	28 (38)	100 (100)	14 (14)

Table 1. *Cont.*

	Symptomatic		Asymptomatic	
	Total (%)	**HNoV+ (%)**	**Total (%)**	**HNoV+ (%)**
Duration of diarrhoea				
3 days	163 (82)	62 (84)		
>3 days	36 (18)	12 (16)		
Interval				
1–3 days	161 (81)	61 (82)		
3 days +	39 (20)	13 (18)		
Living conditions				
Treated water	131 (66)	48 (65)	73 (73)	10 (71)
Untreated water	37 (19)	11 (15)	13 (13)	4 (29)
Mixed	32 (16)	15 (20)	14 (14)	
Pit toilets	111 (56)	41 (55)		
Flush toilets	89 (46)	33 (45)		
Breastfeeding	130 (65)	51 (69)	63 (63)	10 (71)
Not breastfed	70 (35)	23 (31)	37 (37)	4 (29)
Livestock	67 (34)	29 (39)	32 (32)	3 (21)
No Livestock	21 (11)	9 (12)	7 (7)	1 (7)

In addition, HNoV infections were mainly found in diarrhoea cases associated with other related symptoms (89%) than diarrhoea alone (11%) amongst the study participants. The most common norovirus infection symptoms included dehydration (82%), vomiting (73%), and fever (49%), as shown in Table 1. There was an increase in HNoV in children with dehydrating diarrhoea from 2019 (27%; 10/37) to 2020 (45%; 30/66) and 2021 (92%; 89/97). Children with watery diarrhoea had high infection (62%) compared to those releasing formed stools (38%). The majority of HNoV-positive (82%) children reported having diarrhoea that lasted no more than 3 days.

Children who reported coming from households using treated tap water showed a higher prevalence of HNoV (65%; 71%) compared to those who depend on untreated river water (15%; 29%) or mixed water sources (20%) in both the symptomatic and asymptomatic groups, respectively. There was a slight difference in HNoV positivity amongst participants who use pit toilets (55%) and flush toilets (45%). There was no statistical significance in HNoV-positive breastfed children with AGE (69%) compared to nonbreastfed children (31%) ($p = 0.3732$). Similarly, HNoV was detected in the majority of asymptomatic children who were breastfed (71%) compared to those not breastfed (29%) ($p = 0.4812$). The presence or absence of livestock data, as provided by participants, was inconsistent; however, people who lived with animals had a high HNoV positivity rate (39%; 21%) compared to those without (12%; 7%) in both the symptomatic and asymptomatic groups.

3.1.1. HNoV Genogroup and Genotype Distribution

We observed an increase in distribution of GII over the years from 2019 (3/59) to 2020 (15/59) and 2021 (41/59) in children with AGE. GII infections predominated (80%; 59/74) GI (12%; 9/74) in children with AGE. Children without AGE were infected with GI HNoV (64%; 9/14) more than GII (36%; 5/14) (Table 2). The predominance of GII was most common in hospitalised patients (86%) than in outpatients (70%). GI/GII coinfection was less common in this study. The median of GII Ct values between the symptomatic (32.25)

and asymptomatic (35.02) groups was slightly different. Similar findings were observed in the mean GI Ct values in the symptomatic (32.16) and asymptomatic (32.50) groups.

Table 2. Distribution of HNoV genogroup and genotype in children from Vhembe District, South Africa.

		Symptomatic		**Asymptomatic**
		Outpatients	**Inpatients**	
	n = 200 (%)	*n* = 104 (%)	*n* = 96 (%)	*n* = 100 (%)
Total HNoV+	74 (37)	30 (41)	44 (60)	14 (14)
Genogroups				
GI	9 (12)	4 (12)	5 (11)	9 (64)
GII	59 (80)	21 (70)	38 (86)	5 (36)
Mixed	5 (7)	2 (7)	3 (7)	
GII Genotypes	32			
GII.4 Sydney 2012 [P31]	19 (59)	5 (26)	14 (74)	2 (40)
GII.4 Sydney 2012	13 (41)	6 (46)	7 (54)	

Out of 59 cases of HNoV GII detected in symptomatic children, 54% (32/59) were successfully amplified and genotyped. In asymptomatic participants, 40% (2/5) of GII strains were genotyped. Only GII.4 Sydney 2012 [P31] recombinants and the capsid genotype GII.4 Sydney 2012 were successfully amplified and sequenced in this study. An analysis of the junction region of ORF1/2 revealed the predominance of GII.4 Sydney 2012 [P31] recombinants in symptomatic (59%; 19/32) and asymptomatic HNoV (40%; 2/5) infections. The GII.4 Sydney 2012 [P31] genotype was more common in hospitalised children (74%; 14/19) compared to outpatients (26%; 5/19). The distribution of the GII.4 Sydney 2012 genotype in inpatients and outpatients was similar. The remaining positive amplicons (41%; 13/32) could only be typed on the capsid fragment as GII.4 Sydney 2012 genotypes. The HNoV GI group and other GII positive samples could not be typed, possibly due to low viral load and the impact of electricity load shedding on our storage facilities.

3.1.2. Comparison of Nucleotide Sequences among HNoV Strains Circulating in Vhembe District, South Africa

The ClustalW percent identity matrix (Data S1) between norovirus strains previously reported in the study area [37] and those characterised from the current survey showed a significantly low similarity of less than 50% in nucleotide sequences. The change in nucleotide sequences was observed in a period of about 4 to 5 years. When comparing HNoV sequences obtained only from this investigation, we noted that they were all similar, with a nucleotide percent identity of more than 90%.

3.2. Phylogenetic Analysis

A phylogenetic analysis of HNoV GII.4 Sydney 2012 [P31] sequences from Vhembe showed multiple clades (Figure 1), clustering mostly among each other, indicating a close relatedness of the circulating HNoV strains in the area. Amongst these clusters, we observed HNoV strains from India, Cameroon, and China sharing a common ancestor; however, Australian strains from 2012 were in a very distant branch, indicating the existence of a considerable difference with those that have been circulating recently. The nucleotide sequence identity of norovirus strains obtained from this study showed 95% similarities. Figure 2 showed that the most closely related strains were those from China and previously characterised South African strains.

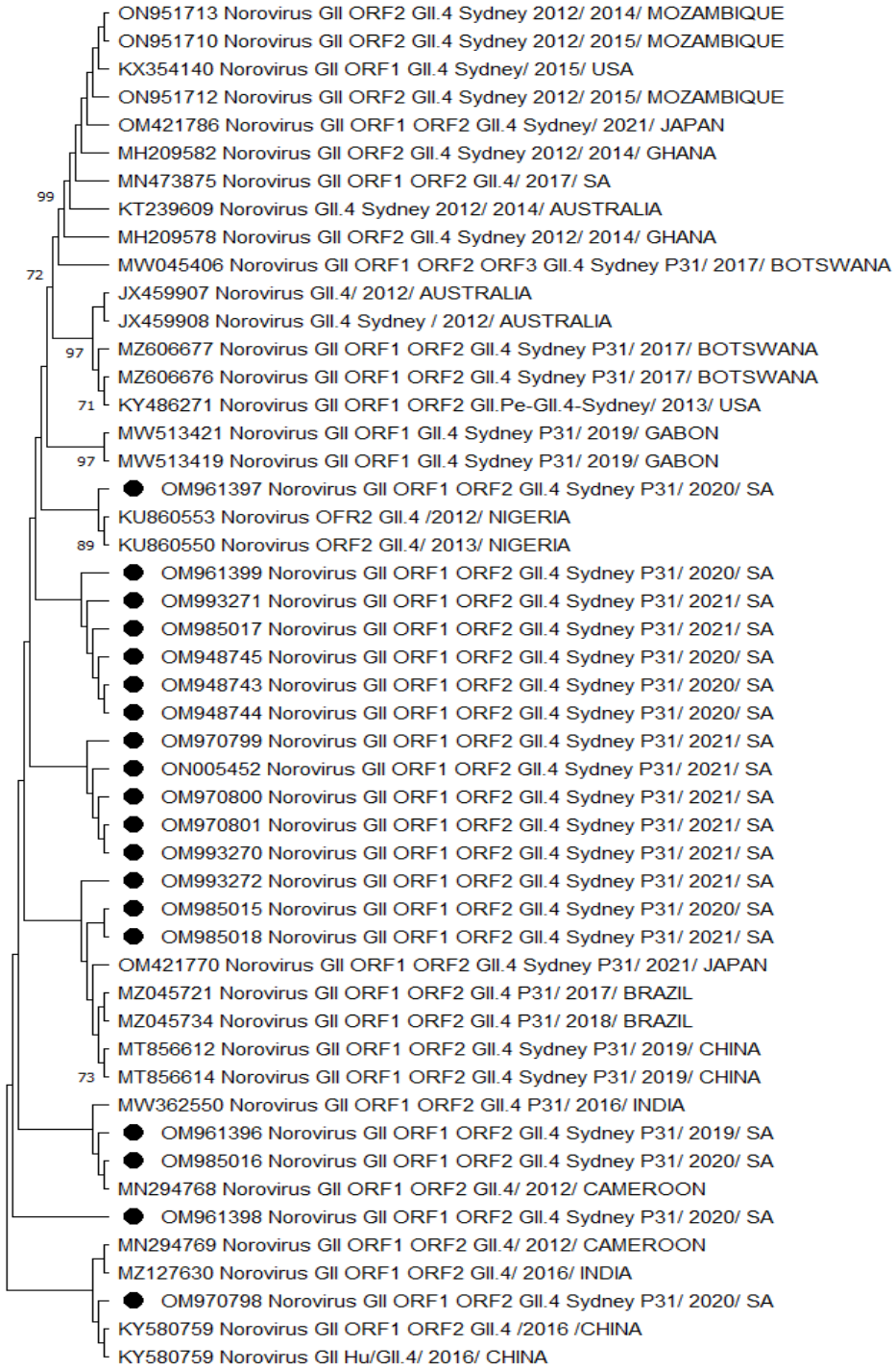

Figure 1. Phylogenetic analysis of the dual typed (polymerase and capsid junction region, 570 bp (with MON431/G2SKR)) HNoV nucleotide sequence circulating in Vhembe District (South Africa) in 2019–2021. Phylogenetic tree was set using neighbour joining method. Round black dots indicate HNoV genotyped in this study, and reference sequences were randomly selected from GenBank, with their respective accession numbers based on high similarity with our study sequences. All positions containing gaps and missing data were eliminated. The evolutionary distances were computed using the *p*-distance method and are in the units of the number of base differences per site. Evolutionary analyses were conducted in MEGA 11 (10.0.5) and bootstrap tests (1000 replicates) based on the Kimura two-parameter model. Only bootstrap values greater than 70% are shown.

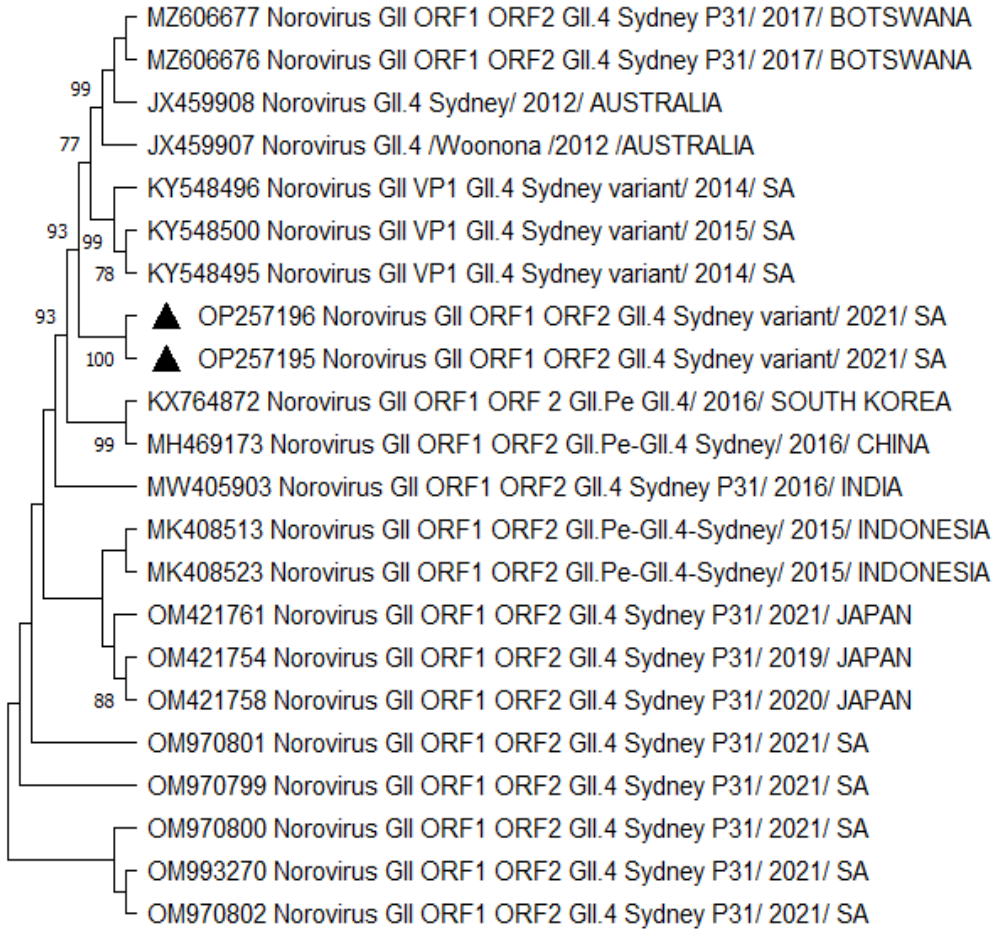

Figure 2. Phylogenetic tree based on 380 bp (with COG2F/G2SKR) capsid/RdRp junction region of HNoV nucleotide sequence circulating in Vhembe District (South Africa) in 2019–2021. Neighbour joining method was used in building the tree. Triangle black dots indicate the HNoV genotypes obtained during this study, and reference sequences were randomly selected from GenBank, with their respective accession numbers based on high similarity with our study sequences. All positions containing gaps and missing data were eliminated. The evolutionary distances were computed using the *p*-distance method and are in the units of the number of base differences per site. Evolutionary analyses were conducted in MEGA 11 (10.0.5) and bootstrap tests (1000 replicates) based on the Kimura two-parameter model. Bootstrap values higher than 70% are shown.

The GII.4 Sydney 2012 strains analysed in Figure 3 were mostly related to a norovirus strain from Ghana. The neighbouring clades consisted of norovirus strains from Burkina Faso and the other previously listed countries in Figures 1 and 2. There was no significant difference in the capsid- and polymerase-protein-based phylogenetic analysis (Figures S2 and S3) compared to the nucleotide-based trees. However, the capsid-protein-based tree confirmed that strains from neighbouring countries (Botswana and Mozambique) shared a common ancestor with the HNoV strains detected in this study (Figures S1 and S4), reflecting the role of border proximity.

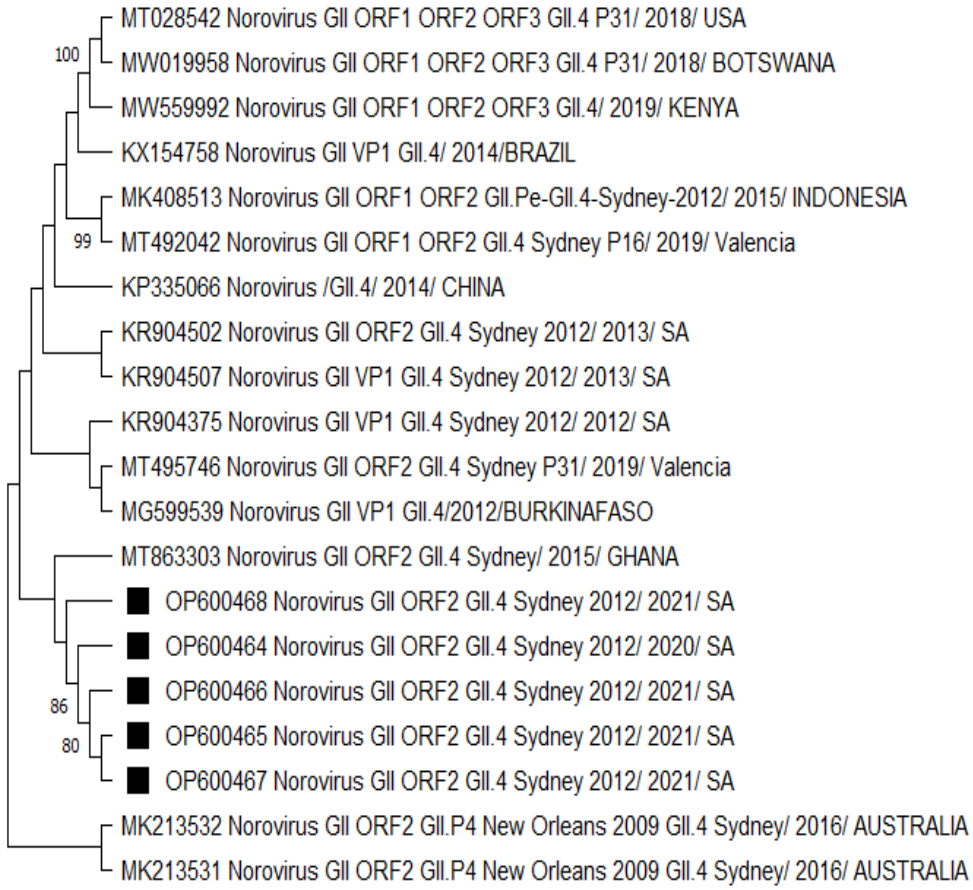

Figure 3. Phylogenetic analysis of the partial typed 340 bp (with G2SKF/R) capsid region of HNoV nucleotide sequence circulating in Vhembe District (South Africa) in 2019–2021. Phylogenetic tree was set using neighbour joining method. Squared black dots indicate HNoV genotyped in this study, and reference sequences were randomly selected from GenBank, with their respective accession numbers based on high similarity with our study sequences. All positions containing gaps and missing data were eliminated. The evolutionary distances were computed using the *p*-distance method and are in the units of the number of base differences per site. Evolutionary analyses were conducted in MEGA 11 (10.0.5) and bootstrap tests (1000 replicates) based on the Kimura two-parameter model. Only bootstrap values greater than 70% are shown.

4. Discussion

Human norovirus is a global public health problem associated with diarrhoea disease worldwide. In this study, we attempted to understand the epidemiological fitness of norovirus strains by analysing the change in prevalence, distribution, and composition of norovirus genotypes. A high prevalence of HNoV was previously reported in the rural communities of Venda, South Africa [27]. Table 2 shows that HNoV strains were still circulating at a high rate (37%) within the study area. This rate is higher than the pooled prevalence of norovirus strains in Africa [7] but comparable to what has been reported, particularly in Ghana [10]. Norovirus has now emerged as the leading cause of AGE worldwide [3], especially in regions where the rotavirus vaccine has been introduced [39]. This study was conducted in the rural communities of Vhembe District, South Africa, where rotavirus vaccines have been introduced since 2009.

There was a significant difference ($p < 0.0001$) in the HNoV infection rate between the symptomatic (37%) and asymptomatic (14%) groups. This observation indicated the considerable role of norovirus in the symptomatic presentations of AGE in Vhembe District. A previous norovirus survey in this study area did not find a statistical difference between symptomatic and asymptomatic subgroups [27]. The findings suggest that norovirus

is strongly associated with VAGE in this region, as reported in other low- and middle-income countries [40,41]. HNoV infections level were higher (59%) in hospitalised patients compared to outpatients (41%), suggesting that norovirus is actively involved in AGE symptoms and diarrhoea disease (p = 0.0129). In a recent South African study within urban areas, norovirus was associated with severe diarrhoea in hospitalised children [26]. Norovirus severity and complications have been previously described elsewhere in countries like Qatar and Taiwan, respectively [42,43]. However, our data did not include other gastroenteritis-causing pathogens.

In this study, HNoV predominated among children between 6 and 23 months old. Recently published data showed that norovirus disease burden is mostly experienced at this age range in African countries [40]. A similar finding was observed in the MALED group study, wherein norovirus infection peaked after 6 months [44]. This is a critical age for individual and immune system development with increased exposure. Available data showed high vulnerability to infection in children, especially those under 2 years [10,44]. At this age, children are thought to have lost maternal antibody protection [45] and are more engaged in increased physical activities, which could lead to more environmental exposure. The results in this study support other findings which suggested that vaccine development should prioritise and target the low age range around six months and above. Approximately 85% of children under 2 years could be protected with this implementation [10,44,45].

Although the breastfeeding of children has been associated with protection against infections [46–48], no statistically significant protection against HNoV infection was seen from the present study (p = 0.3732). The results agree with recent findings [49,50]. In the first six months of life, the WHO and UNICEF still recommend exclusive breastfeeding with its association to AGE protection because an infant's immunity is boosted by maternal antibodies through breastfeeding [51]. Furthermore, Ghosh et al. [52] recently demonstrated the transmission of enteric viruses from infants' salivary glands to mothers' mammary glands during suckling, leading to an increased flow of maternal milk secretory IgA antibodies.

Male children were more infected than females during this investigation. Previous studies indicated that prevalence of viral infections is typically higher in males than females [21,53]. Jaillon et al. [53] reported that females are thought to have stronger immunity compared to males, as some genes that aid during immune response are located in the X chromosome.

Although only the clinical samples were tested, the overall living conditions data provided were not statistically significant. However, the presence of HNoV in both pit/flush toilets, treated water, and in children living with domestic animals suggests poor hygiene practices in the study area. Previous data in the study area have shown faecal contamination of multiple water sources, including tap and household storage containers, with implications in distribution networks and unhygienic practices [54]. Furthermore, Ayukekbong et al. [55] reported norovirus presence in tap water in Cameroon, implying possible resistance to chlorine used in the treatment of water, as previously demonstrated [56].

Due to COVID-19 pandemic lockdown restrictions, no seasonal distribution data could be provided during the investigation. However, throughout the study period, HNoV infections showed a yearly increase, with the highest number recorded in 2021. This observation was consistent with the prediction of the rise in norovirus cases upon the relaxation of COVID-19 regulations, as demonstrated by O'Reilly et al. [57]. Data indicating both the rise and decline in norovirus cases during the COVID-19 pandemic were reported [58,59]. Furthermore, norovirus outbreaks occurred during the COVID-19 pandemic in China and Japan [22,60].

HNoV GII infections predominated in symptomatic cases, especially hospitalised children, supporting the view that they should be prioritised during vaccine development. Our findings are comparable with studies demonstrating the role of GII in symptomatic infections [42,61,62]. Genogroup II strains have been associated with the majority of outbreaks worldwide [15,63]. In contrast, the GI group was found to be more prevalent

amongst control cases in this study. These findings agree with the other studies outcomes suggesting that GI viruses are usually associated with mild infection, while GII viruses are mostly associated with disease symptoms [27,64].

Although the outcome of norovirus epidemiology studies may vary in each region, both GI and GII are epidemiologically important groups requiring continuous surveillance. There was no significant difference between the norovirus GI viral load in symptomatic and asymptomatic norovirus-positive samples. Similarly, in GII infections, the difference in NoV Ct values was insignificant, although a slightly higher median was observed in the asymptomatic group compared to the symptomatic group. Contrary to these findings, a previous investigation in the study area found a significant difference in Ct values between GI and GII groups [27].

The GII.4 Sydney 2012 [P31] variant predominated in this study, particularly among symptomatic inpatients. The GII.4 Sydney 2012 strain was first detected in Australia and later spread throughout the world, with new variants emerging almost every two to three years [65]. The majority of HNoV outbreaks are caused by GII.4 genotypes, which have led multiple pandemics since the mid-1990s [66]. In 2000 and 2004, US95/96 was replaced by two new GII.4 variants, Farmington Hills and Hunter GII.4 variants [67,68]. Other strains were also found in 2006 and 2009 [69]. The GII.4 Sydney variant dominates in most of countries, including South Africa. GII.4 strains have been associated with severe outcomes of norovirus infection and less asymptomatic infection [65,70]. Similarly, our study findings indicate the increase in GII norovirus strains in dehydrating diarrhoea from 2019 to 2020 and 2021.

Since 2012 to date, the Sydney variant, particularly the GII.4 Sydney 2012 [P31] strain, became epidemiologically dominant and persistent through mutations and recombination [65]. The ability of GII.4 variants to evolve at a faster rate on capsid accounts for their epidemiological fitness [71]. Further analysis of GII.4 Sydney 2012 [P31] nucleotide sequences in this study showed evidence of considerable change and possible mutations in strains over time. We observed less than 50% similarity in the nucleotide sequences of HNoV GII.4 strains obtained in this study compared to previously published data (Data S1). The genetic variation of HNoV strains over the years ascertains how HNoV may continue to affect vulnerable communities in different countries, with the possibility of the emergence of new variants in the near future [22,60]. The variation of sequence nucleotides could explain the viral adaptability and epidemiological fitness or dominance in the area [72]. The molecular characteristics of GII.4 Sydney [P31] strains during an outbreak in China suggested that the strains were undergoing evolution, which could lead to the emergence of new variants [22].

It is unclear what may have selectively facilitated the epidemiological fitness and spread of the HNoV GII.4 Sydney 2012 P31 strain amid COVID-19 in the study area. However, the same strain was reported to be circulating in Japan and China during COVID-19 [22,60]. The features of epidemiological fitness are not easily quantifiable [24]. More data, including a vast category of viral, host, and environmental characteristics, are needed to understand the dominance of the norovirus GII.4 Sydney 2012 [P31] strain. Recent data reported the predominance of the GII.4 Sydney 2012 [P31] strain in norovirus-associated diarrhoea [20,21], suggesting that this genotype may remain dominant for a while due to its fitness capacity in different populations. Other genotypes were not characterised in this study; this could be due to the devastating impact of the South African national electricity load shedding schedule on laboratory cold storage units, which may have selectively favoured the survival of GII.4 Sydney 2012 [P31] strains. Furthermore, the majority of samples were amplified using one-step RT-PCR for the purpose of dual typing, which may result in a high error rate during amplification compared to high-fidelity polymerases [73]. This could have limited our typing.

A phylogenetic analysis revealed the close relatedness among norovirus GII.4 Sydney 2012 [P31] recombinants (Figure 1) circulating within the study region. A similar observation was made for GII.4 Sydney 2012 strains (Figures 2 and 3), which were all phylo-

genetically related to each other. We noted the GII.4 Sydney 2012 [P31] strains were closely related to the NoV strains circulating in Nigeria, Gabon, Cameroon, China, and Japan. Norovirus GII.4 Sydney 2012 [P31] from neighbouring countries such as Mozambique and Botswana shared a common ancestor (Figures S1 and S4). The circulation of closely related strains in different countries, especially those within the same continent, may be attributed to border proximity. However, in some cases, norovirus strains were closely related to other circulating strains outside of Africa, which is thought to have been due to population movement prior to the COVID-19 pandemic lockdown. Sequence analysis showed that GII.4 Sydney 2012 strains (Figures 2 and 3) are most phylogenetically related to the HNoV strains recently reported in Japan [60] in sporadic cases and outbreaks and elsewhere in countries such as China [74], Botswana [75], and Ghana [10]. The GII.4 variants became predominant in outbreaks starting from the early 1990s until the current circulating GII.4 Sydney 2012.

Although the GII.4 Sydney strains shared a common ancestor, the data from the present study suggested that there has been a change over the years in the predominant GII.4 Sydney 2012 strains first detected in Australia (2012) compared to the recent circulating variants (Figures 1–3) by distant clusters. This phenomenon is confirmed by the nucleotide sequence variations observed in the present investigation between different norovirus strains over the years.

5. Conclusions

HNoV continues to be responsible for VAGE outbreaks affecting various populations worldwide. This study provides valuable insight into the dominance of the GII.4 Sydney 2012 [P31] genotype and its genetic variation over time. More efforts are needed in the control of norovirus circulation and spread, especially in highly affected rural communities with inadequate WASH conditions. It is imperative to monitor the genetic changes and epidemiology fitness of HNoV strains and the possible emergence of new variants in this era of norovirus vaccine development.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v15081682/s1, Data S1: Percent Identity Matrix created by Clustal2.1. Norovirus sequence numbers 1, 13–15, and 39–48 were obtained from previously conducted studies in Vhembe District. The rest of the sequences were produced during this investigation. Figures S1, S3 and S4: Capsid protein based phylogenetic tree of norovirus. Figure S2: RdRP proteins based phylogenetic tree of norovirus.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the department of health in Limpopo Province, South Africa, (Ref. LP_2018_07_016) and University of Venda (SMNS/18/MBY/07/2505).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data set, including parameters, considered during this investigation is available upon request to the corresponding author.

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