PREVALENCE OF HUMAN BOCAVIRUS IN CHILDREN WITH ACUTE GASTROENTERITIS

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PREVALENCE OF HUMAN BOCAVIRUS IN CHILDREN WITH ACUTE GASTROENTERITIS IN LIMPOPO PROVINCE OF SOUTH AFRICA

by

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DECLARATION

I, Mpumelelo Casper Rikhotso (student number 11606599), declare that the thesis hereby submitted to the University of Venda for the degree PhD (Microbiology) and the work contained therein is my own original work and has not previously, in its entirely or in part, been submitted to any university or higher institution for a degree. I certify that all sources of information used in this thesis have been duly acknowledged.

Rikhotso M.C

Rikhotso Mpumelelo Casper

2 February 2020

Date

DEDICATION

I dedicate this work to my family

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ABSTRACT

Background: Acute gastroenteritis (AGE) is a major cause of morbidity and mortality in children globally. Several reports have indicated that diarrheal diseases caused by viruses, bacteria and parasites are associated with unsafe drinking water, poor sanitation and hygiene practices which leads to infection in vulnerable individuals.

Human Bocavirus (HBoV) have been reported globally in numerous studies as an emerging viral pathogen involved in AGE. However factors contributing to the infection, the genetic diversity and the transmission of the virus are poorly understood globally. There is currently limited data for HBoV prevalence, genetic diversity and possible transmission routes of the virus in South Africa, especially in rural communities where there is still challenges of poor water and sanitation infrastructure.

Even though HBoV have been extensively reported globally, to date most of the reports have been reported in developed countries. Therefore, given the excessive burden of diarrheal diseases in developing countries, it is important to investigate the role of HBoV in diarrhea in a developing country such as South Africa.

Objective: To determine the prevalence of HBoV in children with acute gastroenteritis and investigate the genetic diversity of strains circulating in the rural Vhembe district, Limpopo province, SA.

Methods: In order to support the rationale of this research study, a systematic literature review which assessed the role of HBoV in diarrheal diseases in Africa, other

developing countries and worldwide was carried out. Studies were selected which met the inclusion criteria: (i) Studies performed in Africa/other developing countries/worldwide between year 2005 and 2016. (ii) Studies for the detection of HBoV in patients with/without diarrhea and respiratory tract symptoms. (iii) Studies using standardized laboratory techniques for detection of HBoV including PCR, real-time-PCR, and Multiplex PCR (m-PCR).

To determine the prevalence of HBoV genotypes in children (≤5 years) from rural communities in SA suffering from AGE, a study which investigated the prevalence of HBoV in children with AGE was done between 2017 and 2018 in rural communities in the Vhembe district municipality of the Limpopo province. A total of 141 stool samples were collected from children ≤5 years with AGE and the prevalence of HBoV was determined using a real-time multiplex PCR. Genetic characterization of HBoV was achieved through Sanger DNA sequencing, where the NS1 gene was used to confirm circulating HBoV genotypes. The genotypes were compared with those of reference strains available in NCBI GenBank circulating globally and phylogenetic trees were constructed using the Molecular Evolutionary Genetics Analysis (MEGA) 7 program.

Results and Discussion: The literature search on HBoV prevalence yielded a total of 756 studies of which 70 met the inclusion criteria which included 11 studies from African countries and 59 studies from other developing countries and worldwide. The review showed that the prevalence rate of HBoV in Africa was 13%. Furthermore, revealed that HBoV infections are most likely to be underreported in Africa.

HBoV was detected in 8 (5.7%) stools from the 141 children with AGE and mostly in children between 1-24 months of age. HBoV1 and HBoV3 genotypes were each detected in 3 (37.5%) stool samples and HBoV2 in 2 (25%) stool samples.

Phylogenetic analyses were performed to compare identified HBoV genotypes to global circulating strains. Co-infection with other enteric viruses were also seen with Rotavirus (3/8; 37.5%); Adenovirus (3/8; 37.5%); Norovirus (2/8; 25%) and Astrovirus (1/8; 12.5%) in this study.

Conclusion: The findings highlighted the prevalence and genetic diversity of HBV strains circulating in a rural area with little or no water and sanitation infrastructure. To our knowledge this is the first study in SA showing circulating HBoV genotypes in rural communities. More surveillance of individuals suffering from infections in South Africa is required to monitor the prevalence of HBoV and help understand the role of HBoV in individuals suffering from gastroenteritis with/without respiratory tract infection.

Keywords: Human Bocavirus, Acute gastroenteritis, Children, Rural communities, Africa.

LIST OF ABBREVIATIONS

HBOV	-	HUMAN BOCAVIRUS
RTI	-	RESPIRATORY TRACT INFECTIONS
HCOV	-	HUMAN CORONAVIRUSES
ARTIS	-	ACUTE RESPIRATORY TRACT INFECTIONS
NPAS	-	NASOPHARYNGEAL ASPIRATES
VP	-	VIRAL PROTEIN
PBS	-	PHOSPHATE BUFFERED SALINE
M –PCR	-	MULTIPLEX REAL-TIME PCR
SA	-	SOUTH AFRICA
DNA	-	DEOXY RIBONUCLEIC ACID
RNA	-	RIBONUCLEIC ACID
TEMP	-	TEMPERATURE
ML	-	MICROLITER
USA	-	UNITED STATES OF AMERICA
WHO	-	WORLD HEALTH ORGANIZATION
%	-	PERCENTAGE
SSA	-	STATISTICS SOUTH AFRICA
D	-	DIARRHEA
RT-PCR	-	REVERSETRANSCRIPTASE POLYMERASE CHAIN REACTION
L	-	LITRE
IC	-	INTERNAL CONTROL

MM	-	MICRO-METER
UN	-	UNITED NATIONS
UK	-	UNITED KINGDOM
U	-	UNIT (S)
V	-	VOLUME
ORFS	-	OPEN READING FRAMES
NS	-	NONSTRUCTURAL PROTEIN
VPS	-	VIRAL PROTEINS
C	-	CELSIUS
AGE	-	ACUTE GASTROENTERITIS
BP	-	BASE PAIR
CDNA	-	COMPLEMENTARY DNA
EIA	-	ENZYME IMMUNOASSAY
ELISA	-	ENZYME-LINKED IMMUNOSORBENT ASSAY
MRNA	-	MESSENGER RNA
SARS	-	SEVERE ACUTE RESPIRATORY SYNDROME
BCV	-	BOVINE CORONAVIRUS
HAE	-	HUMAN AIRWAY EPITHELIUM
VLP	-	VIRUS-LIKE PARTICLE
TH	-	T-HELPER
AOR	-	ADJUSTED ODDS RATIO
IL	-	INTERLEUKIN

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Chapter 1

GENERAL INTRODUCTION

1.1. INTRODUCTION

Acute gastroenteritis (AGE) is considered as a leading cause of death in young children globally (WHO, 2016; Liu et al., 2012). Several reports worldwide have ranked AGE as one of the top leading cause of mortality in young children less than 5 years old, in Africa and other low-income countries (Misigo et al., 2014; Niang et al., 2012; Arden et al., 2010). The World Health Organization (WHO) has estimated that about 2.2 million deaths occurring annually are caused by AGE in children ≤5 years and that majority of the cases relates to poor sanitation and hygiene practices (WHO, 2016).

While deaths caused by AGE have declined notably in children over the past two decades in high-income countries worldwide (Abdel-Moneim et al., 2016; Glass et al., 2000), the occurrence of childhood diarrheal cases in low-income countries haven't decreased significantly (Liu et al., 2012). Viruses play a big role in AGE particularly in young children and they include Rotaviruses, Noroviruses, Astroviruses and Adenoviruses (Guerrant et al., 2013), and very recently, the Human Bocavirus (HBoV) (Schildgen et al., 2012; Bulkow et al., 2012; Arden et al., 2010; Garcia-Garcia et al., 2008; Vicente et al., 2007; Soares et al., 2007; Volotao et al., 2006; Bastien et al., 2006; Kaplan et al., 2006).

Human Bocavirus was isolated first in young children from Sweden with infections of the respiratory tract (Allander et al., 2005), and later on, in young children with AGE (Zhao et al., 2013; Niang et al., 2012; Maggi et al., 2007). Human Bocavirus forms part

of the *Parvoviriadae* family, *Parvovirinae* subfamily, and genus of *Bocavirus* (Lindner et al., 2008; Chieochansin et al., 2007; McIntosh et al., 2006). The virus is small, non-enveloped, and icosahedral, approximately 5.3 kb ss-DNA with 3 (ORF): 1st ORF, at the 5', is NS1, a nonstructural protein (Gurda et al., 2010). 2nd ORF, is NP1, a second nonstructural protein. 3rd ORF, at the 3' end, the 2 structural capsid viral proteins (VP), namely VP1/VP2 (Zhang et al., 2012; Kumar et al., 2011). HBoV prevalence in young children has been investigated and reported in Europe (Regamey et al., 2007; Foulongne et al., 2006), America (Bastien et al., 2006; Kesebir et al., 2006), Asia (Lin et al., 2007; Ma et al., 2006), Australia (Arden et al., 2006; Sloots et al., 2006), Africa (Smuts and Hardie, 2006), and the Middle East (Kaplan et al., 2006). Generally the prevalence of Human Bocavirus is reported ranging between 1.5%-19.3% (Bonzel et al., 2008; Bastien et al., 2006).

Infection with Human Bocavirus have been reported mostly in young children ranging from 6 - 24 months (Zheng et al., 2010; Chieochansin et al., 2008; Ma et al., 2006). However, HBoV can also infect older children and adults (Chow and Esper, 2009; Allander et al., 2005). There is no treatment/vaccine for HBoV (Khamrin et al., 2012; Jartti et al., 2011; Arthur et al., 2009). At least, four HBoV genotypes are currently recognized and include: HBoV1, HBoV2, HBoV3, and HBoV4 (Arden et al., 2010; Kantola et al., 2010; Arthur et al., 2009; Tozer et al., 2009; Allander et al., 2005). Several reports have indicated that HBoV genotypes 2, 3 and 4 are mainly involved in AGE while genotype 1 has largely been associated with infections of respiratory tract (Jartti et al., 2011; Jin et al., 2011; Arnold et al., 2010).

Several methods are used for HBoV detection worldwide. However, HBoV detection has been mainly carried through conventional PCR (Arden et al., 2006; Bastien et al.,

2006; Kesebir et al., 2006; Allander et al., 2005) and real-time PCR (Allander et al., 2007; Esposito et al., 2007; Choi et., 2006; Manning et al., 2006). Other methods include ELISA, however, limited data is available on ELISA (Lin et al., 2008).

While HBoV epidemiological reports have demonstrated extensive exposure to HBoV, however the causative role of Human Bocavirus in AGE is undergoing investigation (Schildgen et al., 2012). Studies have provided evidence indicating difficulties in obtain the causative role of the virus without a proper *in vitro* culture system and a working animal model. This is supported by the fact that it is impossible to fulfill Koch's postulates for HBoV due to technical restrictions, *i.e*., currently, neither a versatile cell culture system nor an animal model has been established, nor have there been documented cases of the human-to-human transmission of HBoV. (Hustedt et al., 2012; Kapoor et al., 2011; Chen et al., 2010; Allander et al., 2005).

1.2. PROBLEM STATEMENT

Several rural communities in South Africa (SA), face challenges of poor sanitation and hygiene practices which plays a role in fecal-oral-route transmission of pathogens which results in acute gastroenteritis (AGE), especially in young children. The Vhembe District in Limpopo province of SA is one such a region that still faces such challenges. Factors including poor and unsafe water use, sanitation and hygiene practices expose vulnerable individuals to viral pathogens which results in AGE and leads to deaths globally. AGE is considered one of the foremost cause of mortality in young children ≤5 years both in South Africa and globally (StatsSA, 2012; Bradshaw et al, 2003).

Prevalence of HBoV in South Africa has been previously reported in children suffering from respiratory infections (Nunes et al., 2014; Smuts et al., 2008; Smuts and Hardie, 2006) and recently in children with AGE (Netshikweta et al., 2019). There is currently

no study that has investigated the prevalence of HBoV from children suffering with AGE in South African rural communities where there is little or no water and adequate sanitation infrastructures. Therefore, this study aimed to determine the prevalence of Human Bocavirus in young children with AGE and investigate the genetic diversity of HBoV (strains) circulating in Vhembe district rural communities, Limpopo province (SA).

1.3. OBJECTIVES OF THE STUDY

1.3.1. Aim of the study

- To study the prevalence of HBoV in children with acute gastroenteritis and investigate the genetic diversity of strains circulating in the rural Vhembe district, Limpopo province (SA).

1.3.2. Objectives of the study

Objectives of the study were to:

- Review studies on the prevalence of HBoV in individuals with acute gastroenteritis from Africa, other developing countries and worldwide.
- Determine the prevalence of HBoV genotypes in stool of children ≤5 years old with AGE using real-time multiplex PCR.
- Assess the relationship of HBoV strains circulating in the study area to strains circulating worldwide and show genetic diversity.

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Chapter 2

LITERATURE REVIEW

2.1. INTRODUCTION

More than 800 000 children die due to diarrheal diseases annually, worldwide (Liu et al., 2012). Diarrhea is a big contributor to mortality in young children in Africa and low-income countries (Misigo et al., 2014). Viruses are known to play a crucial role in acute gastroenteritis (AGE), affecting young children mostly, furthermore viral pathogens associated with AGE in human have increased progressively (Chow et al., 2010).

These viruses include *Rotaviruses*, *Noroviruses* and enteric *Adenoviruses* which are associated with AGE in human (Guerrant et al., 2013). Other viruses including Human *Bocavirus*, *Aichi* virus, *Toroviruses, Picobirnaviruses* and the *Coronaviruses* are to an increasing extent being recognized as potential causative agents of diarrhea (Bulkow et al., 2012; Dennehy, 2011; Arden et al., 2010).

Human Bocavirus was discovered in young children suffering from respiratory infection *(*Allander et al., 2005), and later isolated in stools of young children with diarrhea, which suggested that infection with the virus in diarrheal disease was possible (Lindner et al., 2008; Vicente et al., 2007). There is currently four known HBoV genotypes which include HBoV1 to HBoV4 and all have been identified globally in human feces and respiratory secretions (Jacobsen, 2018; Kantola et al., 2010; Allander et al., 2007).

2.2. HUMAN BOCAVIRUSES (HBoV)

2.2.1. Background

Human Bocavirus (HBoV) has been reported as a viral pathogen and reported globally in several investigations (studies) being a causal agent of diarrhea and respiratory tract infections (Albuquerque et al., 2007; Lau et al., 2007; Lee et al., 2007; Vicente et al., 2007; Allander et al., 2005). HBoV was first isolated in young children showing symptoms of respiratory infections. Later on, HBoV was reported as a possible cause of diarrhea (Bulkow et al., 2012; Arden et al., 2010;Zheng et al., 2010; Chieochansin et al., 2008; Ma et al., 2006;Chow and Esper, 2009; Allander et al., 2005). Treatment or vaccine is not available for HBoV (Khamrin et al., 2012; Jartti et al., 2011; Arthur et al., 2009). HBoV was mainly associated with respiratory infections initially, especially HBoV1. However, reports have indicated the main connection of HBoV2, HBoV3 and HBoV4 in gastroenteritis (Cashman & O'Shea, 2012; Jartti et al., 2011; Jin et al., 2011).

2.2.2. Classification and Structure

Human Bocavirus is part of the *Parvoviriadae* family, the *Parvovirinae* subfamily and genus *Bocavirus* (Lindner et al., 2008; Chieochansin et al., 2007). *Parvoviridae* comprise of small viruses which are non-enveloped and icosahedral approximately 5.3 kb ssDNA with 3 (ORF), 1st one, at the 5' end, NS1, a nonstructural protein (Figure 2.1) (Gurda et al., 2012). 2nd one, NP1, a second nonstructural protein. 3rd one, at the 3' end, the capsid viral proteins, VP1/ VP2 (Figure 2.1) (Schildgen & Qiu, 2012). The capsid is similar to that of *Parvovirus* B19 (Schildgen and Qiu, 2012). There are four HBoV species recognized globally recognized as: HBoV1, HBoV2, HBoV3, and

HBoV4 (Figure 2.1) (Guo et al., 2012; Cashman & O'Shea, 2012; Koseki et al., 2012; Kantola et al., 2011; Kapoor et al., 2010).

Looking at HBoV structure, little information is available on the proteins interactions with one another, mainly due to the other reagents involved, such as protein-specific antibodies of HBoV and adaptable system of cell culture being unavailable. The work published by Huang et al., (2012) can be a practical tool for analyzing the functions of the human Bocavirus proteins.

The overexposed viral protein 2 protein is capable of forming capsid-like composition that highly look like viral particles (Gurda et al., 2012). VP2 particles have been used to study T-helper (Th) cell immunity through the assessment of HBoV T-cell proliferation in T-cells extracted from older patients (Kumar et al., 2011). In contrary to *Parvovirus* B19, the Human Bocavirus bring about little varying *Th* response regarding the rapid reproduction. Zhang et al., (2012), reported NP1 can indirectly block the IFN-β promoter and hence hinder the making of interferon beta (Zhang et al., 2012).

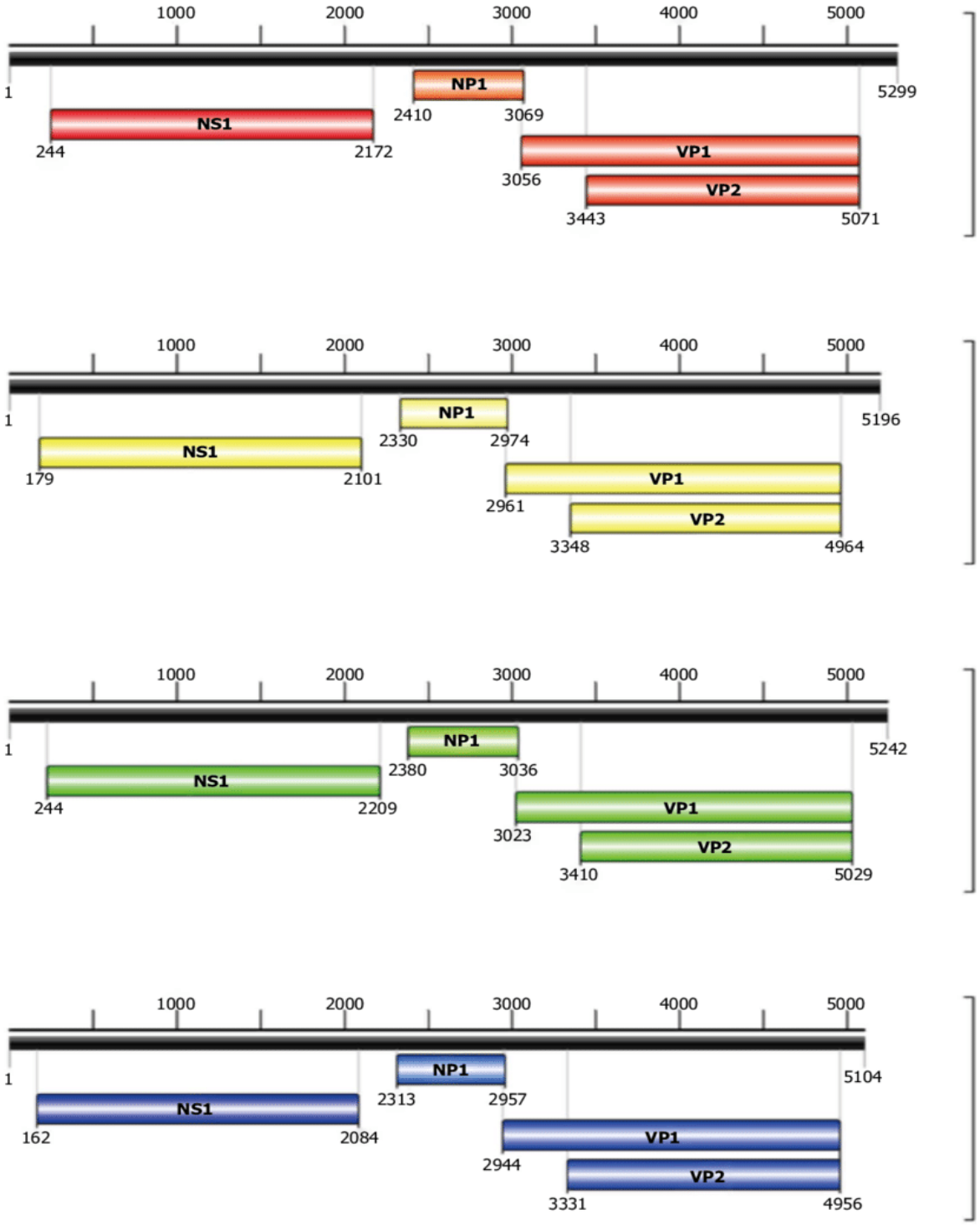

Figure 2.1. Genomic organization of Human Bocavirus. Schematic maps of the HBoV genomes with Gene Bank references (HBoV1, NC_007455; HBoV2, NC_012042; HBoV3, NC_012564; HBoV4, NC_012729). The genes encoding the protein NS1 (non-structural protein), NP1 and VP1/VP2 (capsid proteins) and their nucleotide positions are shown (Guido et al., 2016).

2.2.3. Pathogenesis

Human Bocavirus infection can lead to damage of the epithelium (respiratory system) by affecting the tight cell junctions, with the loss of cilia and the hypertrophy of the epithelial cells (Sun et al., 2013). During HBoV infection, the IgM-HboV antibodies can be detected in the serum. This emphasize the possibility of systemic infection in the host. Furthermore, the infection induces an immune response with the secretions by the *Th1*/ *Th2* (Sun et al., 2013) as shown in Figure 2.2. HBoV can persist in the host for a period of up to 4 to 5 months, most likely through persistence and replication. Reports have indicated that this could explain the high frequency of HBoV being detected in co-infections with other pathogens (Huang et al., 2012; Luo et al., 2011).

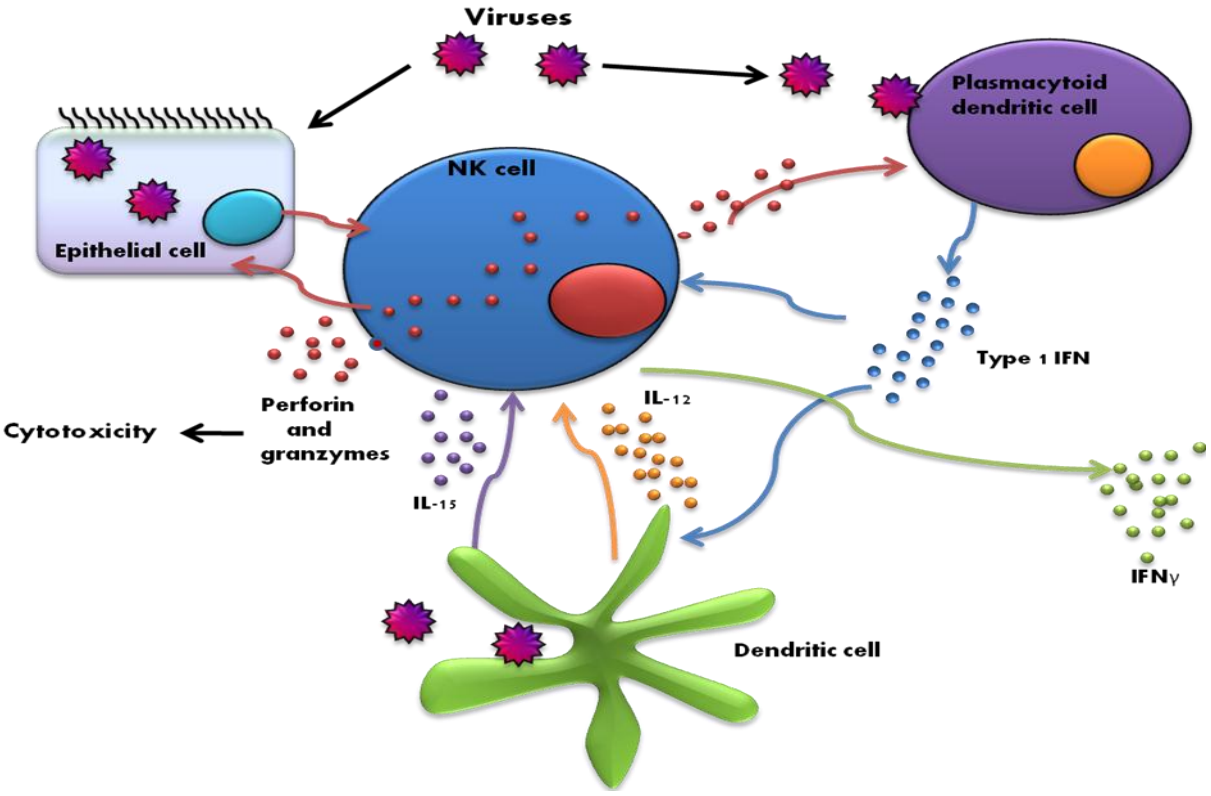

Figure 2.2. Pathogenesis of viral respiratory infection (Manjarrez-Zavala et al., 2013).

Pathogenesis of viral respiratory infection (Figure 2.2). During the infection cycle the viral pathogen triggers IFN-1 production through plasmacytoid dendritic cells and cytokines IL – 12. The IL – 12 and IFN then induce IL – 15 production by dendritic cells. Then IL – 15 is introduced to the NK cells, and NK are activated. Furthermore, the IL – 15 triggers other inflammatory cytokines, which may include the secretion of IFN – γ by NK cells or release of perforin and grazymes resulting in cytotoxicity (Figure 2.2) (Manjarrez-Zavala et al., 2013).

2.2.4. Diagnostic methods

To date, HBoV detection is through PCR (Arden et al., 2006; Bastien et al., 2006; Kesebir et al., 2006; Kupfer et al., 2006; Manning et al., 2006; Sloots et al., 2006; Allander et al., 2005) and Real-Time PCR (Allander et al., 2007; Esposito et al., 2007; Qu et al., 2007; Choi et al., 2006; Manning et al., 2006; Smuts and Hardie, 2006).

Real-time PCR gives high precision, high specificity, and offers a closed system, reducing the chances of wrong positive results from contamination during sample preparation and analysis (Chieochansin et al., 2008). The platform again provide quick turnaround times with the benefit of being able to test for several genes targeted through a multiplex system. There is little data on the method of ELISA used in detection of HBoV. A study conducted in China have reported the use of Elisa (VP2 virus-like particles), they detected anti-HBoV antibodies in children sera (HBoV) (Lin et al., 2008). Detection was seen in (36%) children which were less than 9 years old (Lin et al., 2008).

2.2.5. Epidemiology

Human Bocavirus has been isolated in individuals with both respiratory and diarrheal infection (Bulkow et al., 2012). All four HBoV genotypes are circulating globally, with no regional, geographic, or border limitation (Kapoor et al., 2010). Following its first detection, HBoV have been reported in Europe (Modrow et al., 2011; Fabbiani et al., 2009; Soderlund et al., 2009; Bonzel et al., 2008; Garcia et al., 2008; Longtin et al., 2008; Allander et al., 2007; Kleines et al., 2007; Terrosi et al., 2007; Volz et al., 2007; Weissbrich et al., 2006), North (Albuquerque et al., 2009; Longtin et al., 2008; Bastien et al., 2006) and South America (Ghietto et al., 2012; Flores et al., 2011; Pilger et al., 2011; Salmon et al., 2011), Africa (Carrol et al., 2011; Smuts et al., 2008), Asia (Khamrin et al., 2012; Pham et al., 2011; Chieochansin et al., 2008; Lau et al., 2007) and Australia (Arden et al., 2010; Arthur et al., 2009; Tozer et al., 2009; Arden et al., 2006; Sloots et al., 2006).

Reports have shown that HBoV1 prevalence in symptomatic individuals range from 1.5 to 16% globally (Arnott et al., 2012; Do et al., 2011), between 21-26% in HBoV2 (Kapoor et al., 2010), 1% in HBoV3 (Cashman & O'Shea, 2012), and 0.6% in HBoV4 (Koseki et al., 2012). A study from Ireland reported on detection of HBoV from children with AGE and suggested that HBoV genotypes are highly recombinant among one another, and that some of the HBoV genotypes originate from the recombination of the other two HBoV genotypes (Cashman & O'Shea, 2012).

Studies globally suggest that HBoV prevalence is dependent considerably on the age of patient population and the prevalence ranges between 0 to 40% in children within 18 to 23 months old up and children ≥2years, with an average of 76.6% in young children and 96% in adults globally (Hustedt et al., 2012; Kantola et al., 2011).

Clinical studies have proven that severe infections (*i.e.*, clinically relevant infection, require hospitalization, receiving standardized diagnosis) usually constitute more than one pathogen infection and occur with numerous other pathogens in an individual (Arnott et al., 2012; Babady et al., 2012; Loeffelholz et al., 2011; Balada et al., 2011; Do et al., 2011; Lassauniere et al., 2010; De Vos et al., 2009). The scale of co-infections of pathogens that appear at one and the same time with HBoV is between 60 to 90% (Arnott et al., 2012). Studies have speculated that the high co-infection rate can be due to HBoV being shed in asymptomatic patients and through persistence (Kapoor et al., 2011; Lusebrink et al., 2011; Martin et al., 2010). Clinical symptoms usually seen in patients infected with HBoV include fever, cough, respiratory symptoms, and diarrhea (Maggi et al., 2007; Weissbrich et al., 2006).

Available studies thus far in South Africa, have focused mainly on the isolation of Human Bocavirus in individuals, particularly young children suffering from respiratory infection. One such study conducted by Smuts and Hardie (2006), took nasopharyngeal and Broncho alveolar lavage samples from young children (≥ 2 days to 12 years) hospitalized with respiratory infections in the year 2004 in Cape Town, SA. HBoV1 was detected in 11% of children, all of the positive cases were in ≤2 years of age. Infections were seen in all seasons, however high positive cases were seen in the autumn/winter season with 63% compared to the rest of the year of 37% of cases observed. The findings further suggested that HBoV may play a role in respiratory tract infections in young children who require hospitalization (Smuts and Hardie, 2006).

High prevalence of HBoV in South Africa was observed by Smuts and Hardie (2008), who investigated novel viruses involved in respiratory infections from 238 children.

The viruses were detected in 44 (18.2%) children. HMPV in 20 (8.3%), HBoV 18 (7.4%), and HCoV NL63 in 6 (2.4%) cases (Smuts and Hardie, 2008).

Very recently only one study from South Africa have reported the isolation of HBoV in young children with AGE from urban area in Gauteng province (Netshikweta et al., 2019), The virus was identified in 5.63% patients, and majority of positives were seen in ≤2 years children (92%), infections occurred during summer season and autumn (60%). The study investigated co-infections and found that bacteria (adjusted odds ratio [aOR] = 2.20; 95% confidence interval [CI], 1.41- 3.45; P = .001) and Sapovirus (aOR = 2.05; 95% CI, 1.08- 3.86; P = .027) were highly related with HBoV in a multivariate statistical analysis. The study successfully genotyped HBoV in 191 out of the 212 cases with HBoV1 genotype being the most prevalent (79.6%; 152 of 191) which was followed by HBoV3 (13.6%; 26 of 191), HBoV2 (5.2%; 10 of 191), and HBoV4 (1.6%; 3 of 191) (Netshikweta et al., 2019).

A study carried out in South Africa by Nunes et al., (2014), used a multiplex real-time RT-PCR and investigated stored respiratory samples from children infected with HIV and a health group which were hospitalized for Lower respiratory tract infection, who were previously assessed for RSV, hMPV, HPIV1-3, Adenovirus and Influenza A/B. Overall, one of the viruses had been detected before in 274 (53.0%) and in 509 (54.0%) in HIV infected/ uninfected young children in given order. The highest detection in HIV infected (31.7%) was Human Rhinovirus compared to healthy group (32.0%), which were followed by human Coronaviruses-OC43 with (12.2%) and the Human Bocavirus (9.5%) in HIV infected children. The infection with HBoV in HIV uninfected were HBoV1 (13.3%) and Polyomavirus-WU WUPyV (11.9%), Nunes et al., (2014).

Another study in South Africa conducted by Madhi et al., (2015) investigated bacterial and respiratory viral interactions in infected and uninfected children with HIV. Overall, viral pathogens were identified in 74.2% of children, which included Human Rhinovirus, Adenovirus and HBoV, (37.7%), (14.2%), and (11.5%) respectively irrespective of their HIV status (Madhi et al., 2015).

2.2.6. Transmission

There are different possible routes of transmission for HBoV. The presence of the virus in respiratory infection, diarrhea, and the environment indicates the possible routes of transmission of the virus, which is similar to other viruses involved in respiratory and diarrheal infections (Guido et al., 2016; Bonvicini et al., 2006). Furthermore, fecal-oral route transmission of viruses have certainly proven to be systemic in the transmission of viruses (Guido et al., 2016; Bulkow et al., 2012; Bonvicini et al., 2006).

A study in USA conducted by Kesebir et al., (2006) reported three infants (14%) out of twenty-two (100%) with presumed nosocomial HBoV infection (Kesebir et al., 2006). The infected infants were one, four, and six months old and were hospitalized from birth during the investigation, samples of nasopharyngeal aspirates were collected during the study. From the three infants two were HBoV positive within a period of four days and were cared for in the same ward. Phylogenetic analysis revealed identical sequences in the NP1 and VP1/VP2 genes (Kesebir et al., 2006).

Another study by Kleines et al., (2007) reported 3 HBoV positive children out of 12 in Germany that developed symptoms of acute respiratory tract infections after four weeks of being hospitalized. Unfortunately, the waiting period of HBoV infection unknown, therefore it is not easy if this was a nosocomial transmission (Kleines et al., 2007). HBoV presence in the blood and its persistence could have implications in

transfusion of organs or blood related products coming from infected donors as a sources of infection (Allander et al., 2007; Qu et al., 2007).

2.2.7. Treatment

No prescribed treatment is available for infection with Human Bocavirus (Jartti et al., 2011). The treatment approaches for infection with HBoV are comparable to those used for other enteric viruses, in research labs, HBoV is “treated” in the same manner as other *Parvoviruses* (family) using methods such as fluid and electrolyte replacement therapy and antibiotics which are essential in treating gastrointestinal infections (Dennehy, 2005). HBoV infections have been self-limiting and generally uncomplicated, requiring basic treatment (Jartti et al., 2011; Lee et al., 2007).

One study reported a case in which a treatment of antiviral approach was connected with the non-detection of Human Bocavirus later on in a boy infected with both HBoV and Herpes viruses (HHV-6) (Streiter et al., 2011). The case was of a boy who had immunodeficiency, who lost abilities of establishing antibody based immunity. The treatment was of Cidofovir aimed to treat Herpes which resulted in the HHV-6 viremia decrease and simultaneously the HBoV was undetected in the process. The end results of this study were similar to another study by Klinkenberg and colleagues, which also observed HBoV viremia during treatment (Klinkenberg et al., 2012).

The human Bocavirus has been classified as a level two biosafety agent, this was declared by the German national Biological committee, the Zentralkommission für Biologische Sicherheit (Schildgen O., 2013).

2.3. SUMMARY OF LITERATURE REVIEW

Human Bocavirus (HBoV) which is recognized as an emerging pathogen involved in both respiratory and diarrheal infections. Several reports relating to the epidemiology and pathogenesis of the virus have been provided globally (Jacobsen, 2018; Allander et al., 2005), in both respiratory infections and diarrhea globally (Chow et al., 2010; Yu et al., 2008; Lau et al., 2007). Reports indicate HBoV2, HBoV3 and HBoV4 genotypes are involved in gastroenteritis (Jartti et al., 2011; Jin et al., 2011). Clinical observations of infection with HBoV are respiratory symptoms, including cough, fever and diarrhea (Maggi et al., 2007; Weissbrich et al., 2006). Even though studies have investigated the epidemiology of HBoV and show evidence of the great exposure to the virus globally, there is not enough information on the causative role of the virus in both respiratory and diarrheal diseases and furthermore the role is still under investigation (Schildgen et al., 2012). It has been difficult to fully understand the role of the virus without an animal model and *in vitro* culture system (Hustedt et al., 2012; Kapoor et al., 2011; Chen et al., 2010; Allander et al., 2005).

Although several studies have reported on HBoV infection in respiratory and AGE cases in South Africa, there is no study that have reported the prevalence of HBoV in young children with AGE in South African rural communities. Therefore, the aim of the study was to determine the prevalence of HBoV genotypes in young children with AGE in rural communities from South Africa.

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Chapter 3

ENCLOSED ARTICLES

3.1. OBJECTIVE 1: TO REVIEW STUDIES ON THE PREVALENCE OF HUMAN BOCAVIRUS IN INDIVIDUALS WITH ACUTE GASTROENTERITIS FROM AFRICA, OTHER DEVELOPING COUNTRIES AND WORLDWIDE.

In order to assess global prevalence of HBoV, a systematic review which assessed the prevalence of Human Bocavirus in individuals with acute gastroenteritis from Africa, other developing countries and worldwide was carried out.

ARTICLE 1: REVIEW ARTICLE

Title: **"PREVALENCE OF HUMAN BOCAVIRUS IN AFRICA AND OTHER DEVELOPING COUNTRIES BETWEEN 2005 AND 2016: A POTENTIAL EMERGING VIRAL PATHOGEN FOR DIARRHEA"**

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Review Article

Prevalence of Human Bocavirus in Africa and Other Developing Countries between 2005 and 2016: A Potential Emerging Viral Pathogen for Diarrhea

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Background. Human Bocavirus (HBoV) is an emerging virus discovered in 2005 from individuals suffering gastroenteritis and respiratory tract infections. Numerous studies related to the epidemiology and pathogenesis of HBoV have been conducted worldwide. This review reports on HBoV studies in individuals with acute gastroenteritis, with and without respiratory tract infections in Africa between 2005 and 2016. *Material and Method.* The search engines of PubMed, Google Scholar, and Embase database for published articles of HBoV were used to obtain data between 2005 and 2016. The search words included were as follows: studies performed in Africa or/other developing countries or/worldwide; studies for the detection of HBoV in patients with/without diarrhea and respiratory tract infection; studies using standardized laboratory techniques for detection. *Results.* The search yielded a total of 756 publications with 70 studies meeting the inclusion criteria. Studies included children and individuals of all age groups. HBoV prevalence in Africa was 13% in individuals suffering gastroenteritis with/without respiratory tract infection. *Conclusion.* Reports suggest that HBoV infections are increasingly being recognized worldwide. Therefore, surveillance of individuals suffering from infections in Africa is required to monitor the prevalence of HBoV and help understand the role of HBoV in individuals suffering from gastroenteritis with/without respiratory tract infection.

1. Introduction

Diarrhea is a leading cause of morbidity and mortality in children worldwide [1, 2]. Diarrhea is the third major cause of childhood mortality in children less than 5 years of age especially in Africa and developing countries [3–5]. The modes of transmission include ingestion of contaminated food or water (e.g., via flies, inadequate sanitation facilities, sewage and water treatment systems, and cleaning food with contaminated water), direct contact with infected feces (fecal-oral route), person-to-person contact, and poor personal hygiene [6, 7].

According to WHO [1], approximately 90% of the estimated 2.2 million of deaths caused by diarrheal infections in children less than 5 years of age are related to poor sanitation and hygiene behaviors worldwide. While the mortality due to diarrheal diseases has declined significantly in children over the past twenty years in developed countries [8, 9], the incidence of childhood diarrhea in developing countries has not decreased [1, 2]. Those who survive these illnesses have repeated infections by enteric pathogens which remains a critical factor leading to serious lifelong health consequences [10] and eventually result in death [11]. Viruses are recognized as major cause of gastroenteritis, particularly in children, and the number of viral agents associated with diarrheal disease in humans has increased progressively. Viruses such as rotavirus, norovirus, astrovirus, and adenovirus that cause diarrhea have been reported worldwide [11].

The Human Bocavirus (HBoV) is a viral agent that has been reported worldwide in various studies as a potential

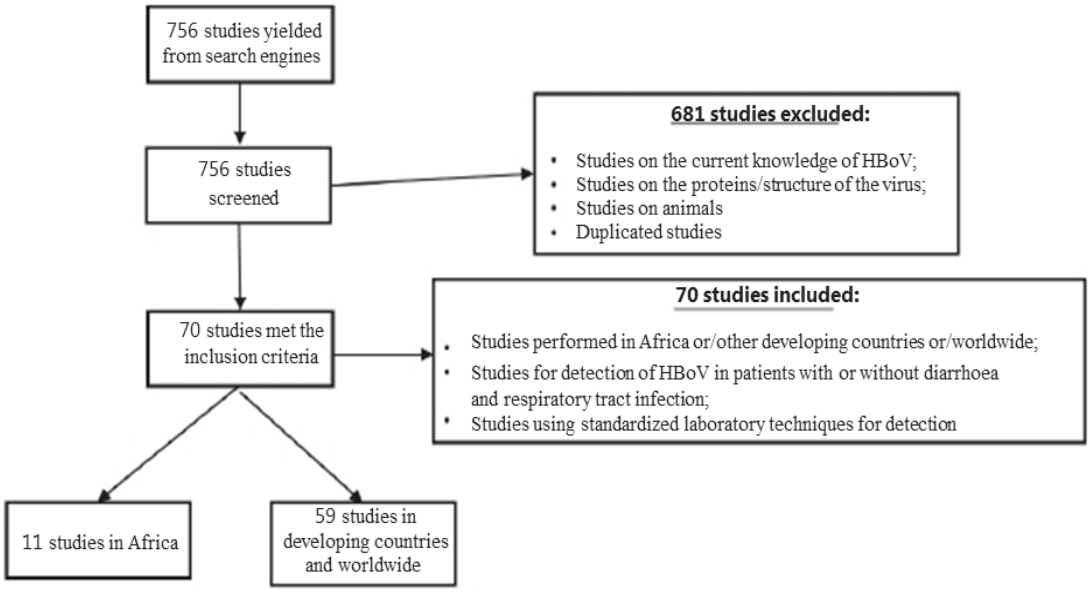

Figure 1: Schematic presentation of search engine used.

cause of diarrhea outbreaks [5, 12–18]. The HBoV is a member of the Parvoviridae family, Parvovirinae subfamily, and the genus of *Bocavirus* [19–21]. The family Parvoviridae includes small, nonenveloped, icosahedral viruses with 5.3 kb single stranded DNA genome containing three open reading frames (ORFs); the first ORF, at the 5□ end, encodes NS1, a nonstructural protein [22]. The second ORF encodes NP1, a second nonstructural protein. The third ORF, at the 3□ end, encodes the two structural capsid viral proteins (VPs), VP1 and VP2 [23, 24]. There are currently four Bocavirus species identified worldwide, namely, HBoV1, HBoV2, HboV3, and HBoV4 [5, 25–28]. HBoV was first discovered in 2005 in children with acute respiratory tract infection [28]. In 2007, HBoV was detected in children suffering from gastroenteritis with and without symptoms of respiratory tract infections [14, 29–31]. Primary infection with HBoV occurs early in life in children between 6–24 months of age [32–34]; however, older children and adults can also be infected [28, 35]. Currently there is no specific approved treatment or vaccine for HBoV infection [25, 36, 37]. Since its discovery, the virus was mainly associated with respiratory tract infections, but recent studies have revealed the involvement of the virus in gastroenteritis. These studies indicate that only HBoV2, HBoV3, and HBoV4 strains of the virus are mainly involved in gastroenteritis [37–39]. Currently, there is limited data on ELISA method for the detection of the virus. HBoV detection has been done by conventional PCR [17, 28, 40, 41] and real-time PCR [42–45]. While HBoV epidemiological studies have shown evidence for widespread exposure to the virus, the causative role of HBoV in respiratory tract disease and gastroenteritis is still under investigation [46]. Proven evidence is difficult to obtain without an *in vitro* culture system and animal model [28, 47–49].

The prevalence of HBoV has been reported in Europe [50, 51], America [17, 41], Asia [34, 52], Australia [40, 53], Africa [54], and the Middle East [18], ranging from 1.5% to 19.3% [17, 55]. This review is a summary of reported HBoV studies in individuals with acute gastroenteritis, with and without respiratory tract infections looking specifically to studies in Africa to determine the role of HBoV in diarrheal outbreaks.

2. Materials and Methods

2.1. Search Strategy. A literature search of selected studies that investigated HBoV in Africa, in other developing countries and worldwide was performed using the following terms: Human + Bocavirus + Africa + Developing countries + Worldwide on PubMed, Google scholar and Embase. This search yielded 756 publications (Figure 1). The first search was performed for HBoV + Africa, the second search was HBoV + other developing countries, and the third search was HBoV + worldwide. Keywords used included Human Bocavirus, Bocavirus, and Human parvovirus combined for each (Africa; Developing country; Worldwide). To avoid leaving out any studies not found in major scientific databases, Google search was also used. After reviewing each article, studies were selected if they met the following inclusion criteria:

(i) Studies performed in Africa/other developing countries/worldwide between 2005 and 2016.

(ii) Studies for the detection of HBoV in patients with or without diarrhea and respiratory tract symptoms. Diarrhea defined as the passage of loose or watery stools, at least three times in a 24-h period [56].

(iii) Studies using standardized laboratory techniques for detection of HBoV including PCR, real-time-PCR, and Multiplex PCR (m-PCR).

3. Data Extraction

Information extracted from the inclusion studies included country where study was done, time period of study, age range of participants, study setting (rural/urban/periurban), sampled population (number of included samples), method used for detection, clinical symptoms, sample type, and HBoV subtype.

4. Statistical Analysis

All analysis were conducted using R programming environment for data analysis and graphics Version 3.5.0 [57] to calculate random and fixed effects. Function "rma" from the package Metafor [58] was used to calculate heterogeneity between studies and generate a forest plot. Heterogeneity was assessed by Cochran's Q test.

5. Results and Discussion

Between 2005 and 2016 a total of 756 studies were published in Africa, other developing countries and worldwide. From these studies, 70 studies met the inclusion criteria of which 11 studies were from African countries and 59 studies combined were for other developing countries and worldwide. None of the studies reported on outbreaks (Tables 1, 2, and 3).

All 70 studies were reports on children ≤5 years of age (33%; 23/70) and children and individuals of all ages, ≥5 years (67%; 47/70). The majority of the studies (78%; 55/70) were reports on patients suffering from respiratory tract infection and 21.4% (15/70) were reports on patients suffering from diarrheal disease. Fifty-four studies (77%; 54/70) were done in urban settings and 23% (16/70) were done in rural settings (Table 1). The most reported HBoV subtype was HBoV1 (100%; 70/70), followed by HBoV2 (16%; 11/70), HBoV3 (13%; 9/70), and HBoV4 (7%; 5/70) (Table 1). A total of 54% (36/67) studies were done on samples collected from nasal swabs, 7% (5/67) were done on samples collected from throat swabs, 22% (15/67) were done on stool samples, 4% (3/67) were combined nasal/throat samples, 3% (2/67) were combined stool/nasal samples, 6% (4/67) were combination of nasal/stool/serum samples, and 3% (3/67) were a combination of nasal/serum samples.

Meta-analysis was done to provide transparent, objective, and replicable summaries of the study findings. From all the 70 studies, 66 had sufficient information to enable statistical analysis. As shown in Figure 2 with the dispersion in study prevalence, there was a low heterogeneity among the studies (Cochran Q = 12.2800 [df = 65] P-Val = 1). Apart from the observed increase in the prevalence of HBoV, none of the other drivers (including age, setting, symptoms, method of detection, and hospitalization) achieved statistical significance. The test for overall effect was Z = 13.29 (P<0.0001) which was highly significant in the findings.

Ten studies were from other developing countries of which eight studies (80%) reported on patients suffering from respiratory tract infection and two studies (20%) reported on patients suffering from diarrheal disease. All ten of the studies focused only on children (100%; 10/10) (Table 2). All studies in other developing countries worked on hospitalized patients (Table 2). A total of 70% (7/10) of the studies collected nasal swabs, 20% collected throat swabs, and 20% of the studies collected stool samples. The most sampled population was children ≤ 6 years of age (Table 2). Majority 60% (6/10) were done in urban setting in other developing countries while 40% (4/10) were done in rural settings. Eight (80%) of the studies reported on patients suffering from respiratory tract infections (Table 2). In other developing countries, HBoV was reported in Argentina 10% (1/10), Cambodia 10% (1/10), China 40% (4/10), India 10% (1/10), Jordan 10% (1/10), and the Philippines 10% (1/10).

In Africa, the majority of studies (82%; 9/11) were done in urban settings while 18% (2/11) were done in rural settings. Ten (91%) of the studies reported on patients suffering from respiratory tract infections and one study (9%) reported on patients suffering from gastroenteritis. In these studies, a total of 383 (10.4%) samples tested positive for HBoV (Table 3). More studies reported HBoV in children less than five years of age (54%; 6/11) compared to children above the age of 5 and adults 45% (5/11) (Table 3).

Countries that reported on HBoV in Africa included Kenya 18% (2/11), South Africa 36% (4/11), Egypt 18% (2/11), Cameroon 9% (1/11), and Senegal 18% (2/11). Five of the 11 studies in Africa focused on hospitalized patients and 36% (4/11) studies focused on outpatients, while 18% (2/11) studies focused on both hospitalized and outpatients. Eight (73%; 8/11) of the studies in Africa collected nasal swabs, two studies 18% (2/11) collected throat swabs, and one (9%; 1/11) study collected stool samples.

The prevalence of HBoV in Africa was 13% in individuals suffering from gastroenteritis with and without respiratory tract symptoms. The high detection rate of HBoV in Africa was consistent with the global increase of HBoV in children less than 5 years of age [4, 59]. Children of all age group are most likely to experience HBoV infection as a result of poor sanitation and hygiene practices [59]. The most predominant HBoV subtype identified in Africa was HBoV1, which was detected in all the studies. Only one study (9%), from Kenya detected all subtypes (HBoV1-4) from children of all age group (Table 3). Not all the studies tested for all HBoV subtypes; this may be due to the fact that other HBoV subtypes have just been recently discovered compared to HBoV1 [37]. The most predominant HBoV subtype identified in other developing countries was HBoV1, which was isolated in all the studies. Only 10% (1/10) of the studies from china detected HBoV2 in the study population (Table 2).

The results in Africa indicated that HBoV in children less than five years of age was high, 54% (6/11) compared to children above 5 years of age and adults 45% (5/11) (Table 3). Schildgen and colleagues [60] showed that all age groups can be affected by HBoV, although severe infections requiring hospitalization occur primarily in patients with an underlying disease and children under 5 years of age [61–64].

Table 1: Human Bocavirus globally, studies published between 2005 and 2016.

Country	Study period	Setting	Age range	Sampled population	Tested samples	Positive samples (%)	Hospitalized	Outpatient	Sample type	Symptoms	Detection method	HBoV type	Reference
Australia	2001	-	-	children	197	125 (63.5%)	197	-	Stool	Diarrhea	NestedPCR	1,2,3	[25]
	2003-2004	Urban	≤ 5 years	children	700	41 (6%)	604	96	Nasal–throat, stool,whole blood	Respiratory infection/diarrhea	Real-time PCR	1	[26]
America	2004	Urban	< 2 years	children	1271	22 (1.7%)	1271	-	Nasal	Respiratorytract infection	PCR	1	[41]
	-	Peri-urban	All	children	641	101 (16%)	-	-	Stool	Diarrhea	PCR	1,2,3,4	[74]
	2007-2008		2-11 years	children	149	7 (5%)	-	149	Throat, Nasal	Respiratorytract infection	Real-time PCR	1	[66]
Argentina	2011	Peri-urban	≤ 2 years	children	222	15 (7%)	222	-	Nasal	Respiratorytract infection	PCR	1	[76]
Argentina,Nicaragua,Peru	-	-	< 6 years	children	568	61 (11%)	568	-	Nasal	Respiratorytract infection	Real time PCR	1	[77]
Brazil	2004-2007	Peri-urban	≤ 2 years	children	397	3 (0.76%)	-	397	Nasal, throat	Respiratorytract infection	PCR	1	[62]
	2003-2005	Peri-urban	<15 years	children	705	14 (2%)	285	420	Stool	Diarrhea	PCR	1	[15]
	1998-2004	Urban	< 5 years	children	762	44 (5.8%)	762	-	Stool	Diarrhea	PCR	1,3	[78]
	2008	Urban	<2 years	children	511	55 (11%)	511	-	Nasal	Respiratorytract infection	PCR	1,2,3	[79]
	2010-2012	Urban/rural	≤18	children	200	67 (33.9%)	200	-	Nasal	Respiratorytract infection	Real-time (RT-PCR)	1	[80]
	2010-2011	Urban/rural	1-14 years	children	121	36 (29.8%)	121	-	Nasal	Respiratorytract infection	Real-time PCR	1	[81]
	2005-2007	Urban/rural	All	Children/adults	1015	49 (4.8%)	1015	-	Nasal	Respiratorytract infection	PCR	1	[82]
	2008-2009	Urban	< 5 years	children	407	77 (19%)	407	-	Nasal	Respiratorytract infection	PCR	1	[83]
	2006-2007	Urban/rural	All	Children/adults	90	2 (2%)	90	-	Stool	Diarrhea	PCR	1	[84]
Cambodia	2009-2010	Urban	All	Children/adults	292	162 (55%)	292	-	Nasal, throat	Respiratorytract infection	Multiplex PCR	1	[85]
Cameroon	2011-2013	Urban	≤15 years	children	347	37 (11%)	347	-	Throat	Respiratorytract infection	Multiplex PCR	1	[86]

Table 1: Continued.

Country	Study period	Setting	Age range	Sampled population	Tested samples	Positive samples (%)	Hospitalized	Outpatient	Sample type	Symptoms	Detection method	HBoV type	Reference
China	2009-2013	Urban/rural	All	Children/adults	29248	551 (2%)	29 248	-	Nasal	Respiratorytract infection	Real-time PCR	1	[87]
	2004-2005	Urban	< 18 years	children	203	83 (40%)	203	-	Stool,Nasal	Respiratory infection/diarrhea	PCR	1	[88]
	2007-2008	Urban	≤15 years	children	235	21 (9%)	235	-	Nasal	Respiratorytract infection	PCR	1,2	[89]
	2009-2012	Urban/rural	All	Children/adults	14237	180 (1.26%)	14237	-	Nasal	Respiratorytract infection	PCR	1	[23]
	2012-2013	Urban/rural	<14 years	children	4130	(16.7%)	4130	-	Throat	Respiratorytract infection	Real-time PCR	1	[90]
	2012	Peri-urban	≤ 5years	children	122	-	-	122	Stool	Diarrhea	Multiplex real-timePCR	1	[91]
	2009-2014	-	All	Children/adults	12502	225 (2%)	12502	-	Nasal	Respiratorytract infection	PCR	1	[92]
	2009-2014	Urban	<14 years	children	4242	125(3%)	4242	-	Nasal	Respiratorytract infection	Real time PCR	1	[93]
	2012-2013	Urban	< 6years	children	346	60 (17.34%)	346	-	Stool	Diarrhea	PCR	1,2	[94]
Egypt	2013-2014	Urban	≤ 36 months	children	95	54 (56.8%)	95	-	Nasal	Respiratorytract infection	Real-time PCR	1	[8]
	2013-2015	Urban	1 month-2 years	children	100	2(2%)	100	-	Stool	Diarrhea	PCR	1	[95]
Finland	2000-2002	Urban	3 months-15 years	children	117	24 (49%)	117	-	Nasal, serum	Respiratorytract infection	Qualitative PCR	1	[96]
	2010	Urban	All	Children/adults	250	4 (1.6%)	250	-	Stool	Diarrhea	Multiplex real-time quantitative PCR	1,2,3,4	[27]
France	2003-2004	-	< 5years	children	589	9 (1.5%)	589	-	Nasal	Respiratorytract infection	PCR	1	[51]
	2010-2011	Urban	All	Children/adults	1465	5 (0.3%)	1465	-	Nasal	Respiratorytract infection	Multiplex PCR	1	[97]

Table 1: Continued.

Country	Study period	Setting	Age range	Sampled population	Tested samples	Positive samples (%)	Hospitalized	Outpatient	Sample type	Symptoms	Detection method	HBoV type	Reference
Germany	2007	Urban	-	children	834	115 (14%)	834	-	Stool,nasal, serum	Respiratorytract infection	Real-time PCR	1	[98]
HongKong	2004-2005	Periurban	-	children	1178	12 (1%)	1178	-	Nasal	Respiratorytract infection	Real time PCR; PCR	1	[99]
	2004-2005	Urban	<18years	children	3035	103 (3.4%)	3035	-	Nasal	Respiratorytract infection	PCR	1	[88]
Iran	2009-2011	Peri-urban	2-108 months old	children	80	6(8%)	80	-	Stool	Diarrhea	Real-time PCR	1	[100]
	2010-2011	Urban	<4 years	children	200	16 (8%)	200	-	Stool	Diarrhea	Real-time PCR	1	[101]
Istanbul	2014-2015	Urban/rural	All	Children/adults	845	91 (11%)	845	-	Nasal	Respiratorytract infection	Real time PCR	1	[102]
Italy	2005-2006	Urban	-	Children/adults	426	42 (9.9%)	426	-	Nasal	Respiratorytract infection	PCR	1	[103]
	2000-2006	Urban	All	Children/adults	355	4.5%,	355	-	Nasal	Respiratorytract infection	PCR	1	[30]
	2004-2007	Urban/rural	<14 years	children	415	34 (8.2%)	415	-	Nasal	Respiratorytract infection	PCR	1	[104]
	2011-2012	Urban	All	Children/adults	689	14 (2%)	689	-	Stool	Diarrhea	Real time PCR	1	[105]
India	2010-2011	Rural/Peri-urban	0–6 years	children	300	2 (0.67%)	300	-	Throat	Respiratorytract infection	PCR	1	[106]
Jordan	2003-2006	Peri-urban	< 5years	children	326	57 (17%)	326	-	Nasal	Respiratorytract infection	PCR	1	[18]
	2007	Peri-urban	≤13 years	children	220	20 (9%)	220	-	Nasal	Respiratorytract infection	PCR/ real-timePCR	1	[107]
	2003-2004	Urban	≤ 5years	children	326	57 (17%)	326	-	Nasal	Respiratorytract infection	Real time PCR	1	[108]
Japan	2005-2011	Peri-urban	0-136 months	children	850	132 (15.5%)	850	-	Nasal	Respiratorytract infection	NestedPCR	1,2,3,4	[73]
	2007–2009	Urban/rural	<2 years	children	402	34 (8.5%)	402	-	Nasal	Respiratorytract infection	PCR	1	[109]

Table 1: Continued.

Country	Study period	Setting	Age range	Sampled population	Tested samples	Positive samples (%)	Hospitalized	Outpatient	Sample type	Symptoms	Detection method	HBoV type	Reference
Kenya	2013	Rural	≤5 years.	children	125	21 (17%)	125	-	Throat	Respiratorytract infection	PCR	1	[59]
	2007-2009	Urban	All age group	Children/adults	384	7 (1.8%)	-	384	Nasal	Respiratorytract infection	PCR	1,2,3,4	[3]
UnitedKingdom	1993–1996	Urban/rural	All	Children/adults	4380	324 (7.4%)	4380	-	Stool	Diarrhea	Real time PCR	1,2,3	[110]
Malaysia	2012	Urban/rural	Children	children	1	1 (99%)	1	-	Nasal	Respiratorytract infection	PCR	1	[111]
Netherland	2005-2006	Peri-urban	3 months-6 years	children	257	4 (1.6%)	-	257	Nasal	Respiratory infection/diarrhea	Real time PCR	1	[112]
Pakistan	2008	Rural	-	Children/adults	98	-	-	-	Stool	Diarrhea	PCR	1,2	[113]
Philippines	2008-2009	Urban	8 days to 13 years	children	1242	2 (0.16%)	1242	-	Nasal	Respiratorytract infection	PCR	1	[114]
Senegal	2009-2011	Urban	All age group	Children/adults	232	1 (0.4%)	232	-	Nasal	Respiratorytract infection	Real-Time PCR	1	[115]
	2007	Rural	≤5	children	82	1 (1.2%)	82	-	Nasal	Respiratorytract infection	PCR	1	[4]
South Africa	1998-2000	Urban	<2	children	1460	332 (22.8%)	1460	-	Nasal	Respiratorytract infection	RT-PCR	1	[116]
	2004	Urban	2 days–12 years	children	341	13 (37%)	341	-	Nasal	Respiratorytract infection	PCR	1	[54]
	2004-2005	Urban	2 months to 6 years	children	242	18 (7.4%)	242	-	Nasal	Respiratorytract infection	NestedPCR	1	[117]
	2009-2010	Rural	3 months to <5 years	children	260	30 (11.5%)	-	260	Nasal	Respiratorytract infection	PCR	1	[118]
Shanghai	2009-2012	Peri-urban	≤ 5 years	children	554	39 (7.0%)	554	-	Nasal, stool, whole blood	Respiratorytract infection	Real time PCR/ PCR	1	[29]
Spain	2005-2006	Urban	<3 years	children	527	48 (9.1%)	527	-	Stool,Nasal	Respiratorytract infection/diarrhea	PCR	1	[14]
Sweden	2000-2002	Urban	3 months to 15 years	children	259	49 (19%)	259	-	Nasal, serum	Respiratorytract infection	Real-time PCR	1	[42]
Taiwan	2008-2009	Peri-urban	5 months-9 years	children	705	35 (5%)	-	705	Throat	Respiratorytract infection	PCR	1	[119]
Thailand	2006	Urban	1 month-9 years	children	252	18 (7%)	252	-	Nasal	Respiratorytract infection	PCR	1	[20]
	2005-2007	Peri-urban	2 months-5 years	children	427	2 (0.4%)	225	202	Stool	Diarrhea	PCR	1	[33]
Turkey	2015	Urban	Five months	children	1	1 (99%)	1	-	Nasal, stool	Respiratorytract infection/diarrhea	Multiplex PCR	1,2,3,4	[120]

Table 2: Human Bocavirusstudies in other developing countries between 20005 and 2016.

Country	Study period	Setting	Age range	Sampled population	Tested samples	Positive samples (%)	Hospitalized	Outpatient	Sample type	Symptoms	Detection method	HBoV type	Reference
Argentina, Nicaragua and Peru	-	-	< 6 years	children	568	132 (23%)	568	-	Nasal	Respiratory tract infection	Real time PCR	1	[77]
Brazil	2007	Rural	< 3 years	children	260	27 (10.4)	260	-	Nasal	Respiratory tract infection	Real-time PCR	1	[121]
Cambodia	2009-2010	-	All	Children/adults	292	9 (3%)	292	-	Throat swabs, nasal	Respiratory tract infection	Multiplex real-time PCR	1	[85]
China	2012	Peri-urban	≤ 5 years	children	122	-	-	-	Stool	Diarrhea	Multiplex real-time PCR	1	[91]
	2009-2014	-	All	Children/adults	12502	225 (2%)	12502	-	Nasal	Respiratory tract infection	PCR	1	[92]
	2009-2014	Urban	< 14 years	children	4242	125 (3%)	4242	-	Nasal	Respiratory tract infection	Real time PCR	1	[98]
	2012-2013	Urban	< 6 years	children	346	60 (17.34%)	346	-	Stool	Diarrhea	PCR	1,2	[94]
India	2010-2011	Rural/Peri-urban	0–6 years	children	300	2 (0.6 %)	300	-	Throat swabs	Respiratory tract infection	PCR	1	[106]
Jordan	2003-2004	Urban	≤ 5 years	children	326	57 (17%)	326	-	Nasal	Respiratory tract infection	Real time PCR	1	[108]
Philippines	2008-2009	Urban	8 days to 13 years	children	1242	2 (0.16)	1242	-	Nasal	Respiratory tract infection	PCR	1	[114]

Table 3: Human Bocavirus studies in Africa between 2005 and 2016.

Country	Study period	Setting	Age range	Sampled population	Tested samples	Positive Samples (%)	Hospitalized	Outpatient	Sample type	Symptoms	Detection method	HBoV type	Reference
Cameroon	2011-2013	Urban	Children aged ≤15 years	children	347	37 (10.6%)	347	-	Throat	Respiratory tract infection	Multiplex PCR	1	[86]
Egypt	2013-2015	Urban	1 month-2 years	children	100	2 (2%)	40 (40%)	60 (60%)	Stool	Diarrhea	PCR	1	[95]
	2013-2014	Urban	≤ 36 months	children	95	54 (56%)	11 (40%)	43 (63%)	Nasal	Respiratory tract infection	Real-time PCR	1	[8]
Kenya	2013	Urban	≤5	children	125	21 (16.8%)	125	-	Throat	Respiratory tract infection	PCR	1	[59]
	2007-2009	Urban	All age group	Children/adults	384	7 (1.8%)	-	384	Nasal	Respiratory tract infection	PCR	1,2,3,4	[3]
Senegal	2007	Rural	≤5	children	82	1 (1.2%)	-	82	Nasal	Respiratory tract infection	PCR	1	[4]
	2009-2011	Urban	All age group	Children/adults	232	1 (0.43%)	-	232	Nasal	Respiratory tract infection	Real-Time PCR	1	[115]
South Africa	1998-2000	Urban	<2	children	1460	174 (22.8%)	1460	-	Nasal	Respiratory tract infection	RT-PCR	1	[116]
	2004	Urban	2 days–12 years	children	341	38 (11%)	341	-	Nasal	Respiratory tract infection	PCR	1	[54]
	2004-2005	Urban	2 months to 6 years	children	242	18 (7.4%)	242	-	Nasal	Respiratory tract infection	Nested PCR	1	[117]
	2009-2010	Rural	3 months to <5 years	children	260	30 (11.5%)	-	260	Nasal	Respiratory tract infection	PCR	1	[118]

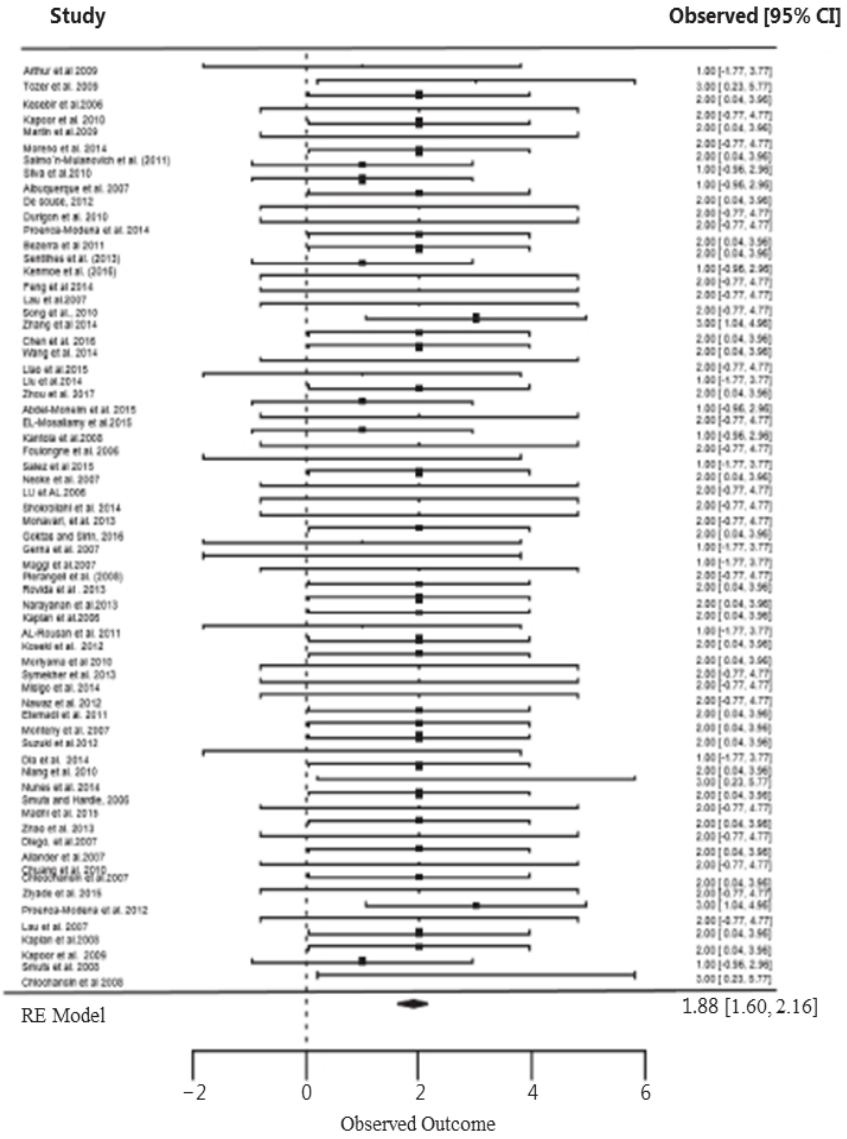

Figure 2: Forest plot for prevalence studies in detection of Human Bocavirus.

Severe clinical cases (such as destruction of the epithelium of the respiratory system) have been described in children [61, 64–66] and adults with immunodeficiency [64] and other risk groups [67]. Studies in Africa (13%; 9/70) were mostly done in urban setting compared to other developing countries/worldwide (87%; 61/70) (Tables 1, 2, and 3). This could be due to the lack of laboratory resource capacity and technology for the detection of HBoV in rural settings.

The methods used for detecting HBoV have been conventional PCR [17, 28, 40, 41, 45, 53, 64] and real-time PCR [42–45, 54, 68], due to the limited success of serological and viral culture techniques. Real-time PCR is more sensitive and offers greater sensitivity, increased specificity with the addition of oligoprobes, and the added benefit of a closed detection system, reducing the likelihood of false positive results due to contamination with amplicon [33]. In Africa, 63% (7/11) of studies used conventional PCR for detection, 27% (3/11) used real-time PCR, and 9% (1/11) used Multiplex PCR which is also conventional PCR (Table 3).

In all eleven African studies, HBoV1 was detected (Table 3), similarly in other developing countries HBoV1 was detected in all the studies. HBoV Subtype 1 is mainly associated with respiratory diseases but can also be found in stool samples from patients suffering from diarrhea. Previous studies have reported prevalence of HBoV in symptomatic patients 1.5–16% worldwide [69, 70]. Several studies have isolated HBoV from children with respiratory tract infection worldwide, and the prevalence of HBoV in these children was 1.5%–19% [32, 33, 35].

The reports on HBoV in Africa, other developing countries, and worldwide in individuals suffering from respiratory tract infection 78% (55/70) were higher compared to those suffering from gastroenteritis 21% (15/70) (Tables 1, 2, and 3). This could be due to the fact that most studies focused

on HBoV in respiratory tract infection since its discovery in 2005 [28, 33, 36, 71]. However recent studies are increasingly detecting HBoV in individuals suffering from diarrheal diseases due to the presence of the virus in stool samples of individuals suffering from gastroenteritis [37, 72–74].

Although the number of studies in Africa is limited, the HBoV prevalence rate of 13% indicates that this virus is one of the emerging viral agents in those suffering from diarrhea with and without respiratory tract infections. Currently there is no available reporting system for HBoV infection in the primary healthcare systems in Africa, suggesting that diarrheal cases with and without respiratory tract infection are likely to be underreported [47]. The high frequency of HBoV in children raises a potential health risk as these children may act as reservoir for other emerging epidemic HBoV strains [27, 37, 75].

6. Conclusion

More studies are required in Africa, especially in rural settings to monitor the prevalence of HBoV and help understand the role of HBoV in individuals suffering from gastroenteritis with/without respiratory tract infection. HBoV infections are likely to be underreported in Africa considering the costs of testing for the virus. This review was done to shed light on HBoV and its possible role in diarrheal incidence.

Conflicts of Interest

The authors declare no conflicts of interest.

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3.2. OBJECTIVE 2: TO DETERMINE THE PREVALENCE OF HBOV GENOTYPES IN STOOL OF CHILDREN ≤5 YEARS OF AGE WITH ACUTE GASTROENTERITIS USING REAL-TIME MULTIPLEX PCR.

ARTICLE 2: RESEARCH ARTICLE

Title**: "PREDOMINANCE OF HUMAN BOCAVIRUS GENOTYPE 1 AND 3 IN OUTPATIENT CHILDREN WITH DIARRHEA FROM RURAL COMMUNITIES IN SOUTH AFRICA, 2017-2018"**

Running Head: Human Bocavirus in children with AGE

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Predominance of Human Bocavirus Genotype 1 and 3 in Outpatient Children with Diarrhea from Rural Communities in South Africa, 2017–2018

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Abstract: Human bocavirus (HBoV) is an emerging virus globally associated with diarrhea in young children. This study aims to investigate the prevalence of HBoV genotypes in children (≤5 years) from rural communities in South Africa (SA) suffering from acute gastroenteritis (AGE). A total of 141 fecal samples of children ≤5 years with acute gastroenteritis (AGE) were collected from rural primary health care facilities in the Vhembe district of SA between June 2017 and July 2018. Clinical symptoms and demographic data were also recorded. A total of 102 (72%) were outpatients, and 39 (28%) were hospitalized patients. Human bocavirus (HBoV) genotypes were determined using real-time multiplex PCR. DNA extracts of positive samples were confirmed by conventional PCR targeting the NS1 gene. Co-infection with other enteric viruses were determined in HBoV-positive samples using real-time PCR. HBoV was detected in eight (5.7%) children with AGE, of which three (37.5%) were HBoV1, three (37.5%) were HBoV3, and two (25%) were HBoV2. The majority of positive cases were identified in outpatients (62%) between the ages of 1 and 24 months. Co-infection in HBoV-positive samples with other enteric viruses included rotavirus (37.5%), adenovirus (37.5%), norovirus (25%), and astrovirus (12.5%). HBoV infections could be seen as a potential emerging diarrheal pathogen in South Africa. However, more studies are needed to understand the role of HBoV infections in children with AGE.

Keywords: human bocavirus; acute gastroenteritis; rural communities; children

1. Introduction

Acute gastroenteritis (AGE) is recognized as one of the major causes of mortality in children ≤5 years of age in Africa and other developing countries [1,2]. AGE can be caused by several viral pathogens, including human bocavirus (HBoV), which is an emerging viral agent reported as a potential cause of diarrhea, especially in young children [3,4].

HBoV is a member of the Parvoviriadae family, Parvovirinae subfamily, and the genus Bocavirus [4]. Human bocavirus are small, nonenveloped, icosahedral viruses with an approximately 5.3 kb single-stranded DNA genome containing three open reading frames. (ORFs): the first ORF encodes NS1, the second ORF encodes NP1, and the third ORF encodes the viral proteins VP1 and VP2 [5]. There are currently four bocavirus genotypes identified globally, namely human bocavirus genotypes 1 to 4 [4,6].

HBoV investigations in South Africa (SA) have previously reported on the detection of HBoV genotypes in children with respiratory tract infections and AGE [7–10]. There is no published work on

the detection of this virus from stools in children with AGE or children living in rural communities with poor water and sanitation infrastructure in South Africa. This study aimed to determine the prevalence and genetic diversity of HBoV in children with AGE from rural communities in Limpopo, South Africa.

2. Materials and Methods

2.1. Study Design

The cross-sectional study was carried out between June 2017 and July 2018. Stool samples were randomly collected from patients at different clinics (outpatients) and hospitals (inpatients) from rural communities in the Vhembe region, Limpopo province of South Africa. Clinics and hospitals were both chosen as collection points because most cases of AGE in South Africa are seen by primary health care centers (clinics). These are situated in rural communities where the clinic refers only severe cases of AGE (with dehydration) to the hospital, where they take over the treatment plan. In total, 14 clinics and three district hospitals were visited during the study period.

2.2. Ethical Clearance and Consent

The study protocol was reviewed, approved, and registered by the ethics committee at the University of Venda (Ref. SMNS/17/MBY/03). Ethical clearance was obtained from the provincial Department of Health (Limpopo), South Africa (Ref: 4/2/2). Written, informed consent was given to the parent/guardian of the child to grant permission before participation and the collection of a stool sample from the child.

2.3. Sampling

In total, 141 stool samples were collected from children ≤5 years of age with AGE, of which 102 stool specimens were collected from clinics and 39 from hospitals. Only children who fit the criteria for acute gastroenteritis (diarrhea/vomiting/fever/cramping/dehydration) were recruited. Samples were transported to the laboratory after collection at 4 °C and stored at −20 °C until further analysis.

2.4. Data Collection

Personal information such as age and sex was collected from patients, including consultation details, parental status, family living conditions, water source, and type of latrine facility used at home. Clinical symptoms, including symptoms of fever, vomiting, cough, diarrhea, and dehydration were recorded.

2.5. DNA Extraction and Amplification

The Boom method was used for DNA extraction, as previously described [11]. The method is based on the lysing and nuclease inactivating properties of the chaotropic agent guanidinium thiocyanate, together with the nucleic acid-binding proprieties of silica particles. The extracted DNA was stored at −20 °C until further analysis.

A HBoV real-time PCR commercial kit was used to detect HBoV1-4 genotypes following the manufacturer's instructions (Liferiver, Shanghai, China). Amplification reactions were performed in a volume of 40 µL containing 35 µL reaction mix, 0.4 µL enzyme mix, 1 µL internal control, and 4 µL extracted DNA according to the manufacturer's instructions. The real-time m-PCR was performed using the Corbett Research Rotor-Gene 6000 with the following conditions: 2 min at 37 °C, 2 min at 94 °C and 40 cycles of 15 s at 93 °C, and 1 min 60 °C. Positive samples were confirmed by sequencing, targeting the NS1 partial sequence.

2.6. Genotype Analysis

All positive samples from the real-time PCR assay were subjected to subsequent PCR targeting the NS1-nonstructural protein gene using the primers listed in Table 1.

Table 1. Primers for human bocavirus (HBoV) genotyping.

HBoV Genotype	Target Gene	Primer	Sequence (5–3)	Fragment Length (bp)	Reference
HBoV-1	NS1	188F 542R	GACCTCTGTAAGTACTATTAC CTCTGTGTTGACTGAATACAG	354	[4]
HBoV-2/4	NS1	HBoV2-sf1 HBoV2-sr1	AACAGATGGGCAAGCAGAAC AGGACAAAGGTCTCCAAGAGG	454	[12]
HBoV-3	NS1	P5P6	CAGAAGCATCGGAAGTGGGTGT ATGTGAGGCTTTATGCTGGCTGA	440	[13]

The TopTaq Master Mix Kit (Qiagen) was used for the amplification of all positive HBoV samples following the manufacturer's instructions. Amplification reactions were performed in a volume of 50 µL containing 25 µL master mix, 5 µL each of primer F and R, 15 µL RNase-free water, and 5 µL DNA. Amplification conditions for HBoV1 were initial denaturation for 10 min at 94 °C and 35 cycles of amplification (94 °C for 1 min, 54 °C for 1 min, and 72 °C for 2 min); the expected product size was 354 bp. HBoV2/4 amplification was initiated with a denaturation step of 15 min at 95 °C followed by 40 cycles of 94 °C for 15 s, 53 °C for 30 s, and 72 °C for 1 min; the expected product size was 454 bp for HBoV2/4. Human bocavirus 2 and 4 used the same antisense primers devised by Kapoor et al. [12], which were unable to differentiate between HBoV2 and 4 genotypes. Therefore, genotypes were further confirmed through sequencing. The amplification of HBoV3 consisted of 1 step of 95 °C for 15 min followed by 45 cycles of 94 °C for 20 s, 52 °C for 20 s, and 72 °C for 40 s, followed by an extension step at 72 °C for 10 min; the expected product size for HBoV3 was 440 bp [13].

All PCR products were visualized on 2% agarose gel stained with ethidium bromide. All positive samples were sequenced. The Sanger DNA sequencing was performed on the ABI 3500XL Genetic Analyzer POP7TM (Thermo-Scientific). The nucleotide sequences were compared with those of the reference strains available in the NCBI GenBank using BLAST tool available at http://www.ncbi.nlm.nih.gov/blast and then analyzed for their respective genotypes.

2.7. Co-Infection Viruses Detected

Co-infection with other enteric viruses was determined using a CFX96 (Bio-Rad) real-time PCR. The Allplex gastrointestinal panel virus assays (Seegene Technologies Inc., California, USA) were used to determine the prevalence of other enteric viruses in the stool samples, following the manufacturer's instructions.

3. Results

3.1. Study Population Characteristics

A total of 141 children with AGE were recruited in this study, of which 66 (47%) were males and 75 (53%) were females. A total of 83 (59%) children were aged between 1 and 12 months, 36 (25%) children were aged between 13 and 24 months, 10 (7%) children were aged between 25 and 36 months, nine (6%) children were aged between 37 and 48 months, and three (2%) children were aged between 49 and 60 months. All the children presented with symptoms of diarrhea (141; 100%). Fever (48; 27%), vomiting (47; 27%), dehydration (28; 16%), respiratory tract infection (26; 15%), and abdominal pain (27; 15%) were other observed symptoms. The majority (128/144; 90%) of the participants used tap water at home, and 119 (84%) used pit latrines.

3.2. Detection and Genotyping of HBoV Isolates

The general characteristics of HBoV-positive patients are summarized in Table 2. The prevalence of HBoV genotypes 1 to 4 in stool samples of children with AGE was determined using real-time PCR. In total, eight (5.7%) samples were found positive for human bocavirus. HBoV1 was detected in three female patients with a mean age of 9 months; HBoV2 was detected in two patients of which one was male and one female, with a mean age of 9.5 months; HBoV3 was detected in three patients (37%) of which two were female and one was male, with a mean age of 19 months. HBoV4 was not detected in any of the 141 patients. Five (62%) of the positive cases were from clinics, and three (37%) of the positive cases were from hospitals (Table 2). Genotypes were determined through Sanger DNA sequencing and confirmed by comparison with reference genotypes available in the NCBI GenBank using BLAST tool. HBoV Sequences from the current study were deposited into GenBank under accession numbers MN072357-MN072360, MN082386, and MN082387.

Human bocavirus genotypes 1 and 3 were each detected in three (37%) stool samples, and genotype 2 was detected in two (25%) stool samples (Table 2) of patients. Among the eight positive HBoV samples, co-infection with other enteric viruses was found in seven out of eight (87.5%) patients, while infection with HBoV alone was detected in one out eight (12.5%) of the HBoV-positive patients. Mixed infections with rotavirus (three out of eight patients; 37.5%); norovirus (two out of eight patients; 25%); adenovirus (three out eight patients; 37.5%) and astrovirus (one out of eight patients; 12.5%) were observed in this study population (Table 2).

General characteristics of the eight HBoV-positive patient's symptoms observed in this study were as follows: Among the three HBoV1 patients, one child (33.3%) had diarrhea, two children (66.6%) presented with diarrhea, fever, abdominal pain, vomiting, dehydration, and one patient had respiratory symptoms (Table 2). In HBoV2 patients, both children (100%) had diarrhea, while only one child (50%) had diarrhea and fever (Table 2). All HBoV3 positive children had diarrhea (100%), from which only one child (33.3%) had diarrhea, fever, vomiting, dehydration, and abdominal pain (Table 2).

HBoV 1 was observed in February (one out of eight patients; 12.5%), June (one out of eight patients; 12.5%), and July (one out of eight patients; 12.5%) (Figure 1). HBoV2 was only observed in June (one out of eight patients; 12.5%) and December (one out of eight patients; 12.5%), HBoV 3 was observed in March (one out of eight patients; 12.5%), July (one out of eight patients; 12.5%), and December (one out of eight patients; 12.5%) (Figure 1).

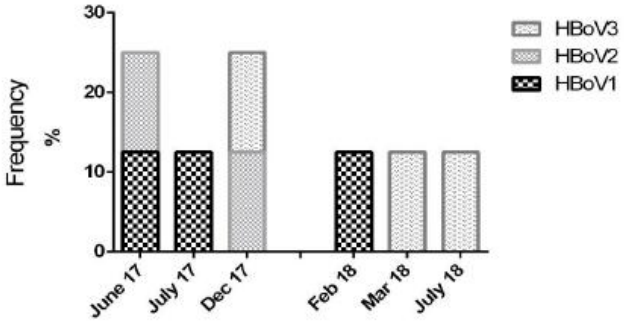

Figure 1. Human bocavirus genotype distribution (2017–2018).

Table 2. Characteristics of children showing positive detection of HBoV in fecal specimens.

Stool Specimen Collected (n = 141)	Positive Cases (n = 8)	Sample Code	Age (Months)	Sex	Symptoms						HBoV C_T Values	HBoV Genotype	Co-Infection with Other Enteric Viruses
					Diarrhea	Fever	Vomiting	Dehydration	Abdominal Pain	Respiratory			
Clinics 102 (72%)	5 (62%)	18	8	Female	1 (12.5%)	-	-	-	-	-	29.94 C_T	HBoV1	Rotavirus, Astrovirus
		26	5	Female	1 (12.5%)	-	-	-	-	-	33.46 C_T	HBoV2	Rotavirus
		119	13	Female	1 (12.5%)	-	-	-	-	-	17.94 C_T	HBoV3	Adenovirus F
		105	14	Male	1 (12.5%)	1 (25%)	-	-	-	-	38.63 C_T	HBoV2	-
		268	24	Female	1 (12.5%)	-	-		-	-	31.01 C_T	HBoV3	Adenovirus F
Hospitals 39 (28%)	3 (37%)	11	20	Male	1 (12.5%)	1 (25%)	1 (33.3%)	1 (33.3%)	1 (50%)	-	34.04 C_T	HBoV3	Norovirus, Adenovirus F
		40	7	Female	1 (12.5%)	1 (25%)	1 (33.3%)	1 (33.3%)	-	1 (100%)	33.39 C_T	HBoV1	Rotavirus
		55	12	Female	1 (12.5%)	1 (25%)	1 (33.3%)	1 (33.3%)	1 (50%)	-	8.06 C_T	HBoV1	Norovirus GII

HBoV = Human Bocavirus; C_T = Cycle threshold.

4. Discussion

Several studies have reported human bocavirus in children with respiratory tract infections and AGE in South Africa [7–10]. However, no data are available on the prevalence of HBoV and other enteric pathogens in children with AGE from rural communities, suggesting that cases from these rural areas are most likely to be underinvestigated and under-reported [14,15]. This study, for the first time, investigated the prevalence of HBoV in children with AGE from rural communities in South Africa.

Altogether, a total of 141 stool samples from children with AGE were assessed for the presence of HBoV. The prevalence of 5.7% for HBoV in this study was comparable to other studies that have investigated HBoV in children with diarrhea worldwide [16–18]. Several of these studies have indicated that HBoV prevalence in patients with AGE ranges between 0.8% to 42% [16,17]. The high detection of HBoV in children from the clinics (outpatient) in the current study suggested that HBoV could be associated with mild to moderate diarrheal cases and asymptomatic cases [19]. Results from this study further suggest that HBoV could be seen as an emerging viral pathogen in the rural communities of South Africa. Although HBoV is considered a potential cause of diarrhea, available evidence supporting the causative role of the virus in acute gastroenteritis is inconclusive [20]. The presence of HBoV in asymptomatic individuals raises questions regarding the role of the virus in gastrointestinal infections as a pathogen or just as a bystander [20]. This is due to limited available studies investigating the pathogenesis of HBoV due to the lack of cell culture systems or animal models [12,21,22].

Reports worldwide have indicated that young children are prone to HBoV infections [23]. In this study, HBoV was detected in children between the ages of 1 and 24 months (≤24 months) (Table 2). No study to date has confirmed the association of HBoV infection with a specific age group of children affected. The virus could be infecting young children via fecal–oral-route transmission. A study in China [19] has reported that the transmission of HBoV was through ingestion of contaminated food/water (e.g., via flies, inadequate sanitation facilities, inadequate sewage and water treatment systems, and the cleaning of food with contaminated water), direct contact with infected feces (fecal–oral-route), person-to-person contact, and poor personal hygiene. In this study, the patients came from rural communities with no or inadequate water and sanitation infrastructure and poor hygiene practices [24]. The majority (128/144; 90%) of the participants used tap water in this study. From the eight positive cases of HBoV in this study, seven (87%) used tap water as a source of water, and only one (12%) used spring water as a source of water at home. Rural communities in South Africa usually collect water in storage tanks for both domestic and sanitation use due to scarcity of water in rural areas, and this results in the contamination of water through fecal–oral-route transmission [24]. Waterborne viruses represent a major health risk to the population worldwide [25]. There are currently limited data worldwide exploring the circulation of HBoV from environmental samples. Some studies have detected human bocavirus in river water [25,26] and wastewater samples [27–29]. Even though the role of HBoV in gastrointestinal infections is not fully understood, the risk of infection via contaminated water should be taken into consideration since many rural communities still face challenges of poor sanitation and hygiene practices [15]. In addition, the majority (119/144; 84%) of the participants in this study used pit latrines at home. All eight HBoV-positive cases used a pit latrine at home. The use of a pit latrine plays a role in the transmission of pathogens since the latrine lacks a handwashing facility and usually attracts flies which move between the facility and the house [24].

From the four known genotypes, only HBoV1, HBoV2, and HBoV3 were detected in this study. HBoV1 and HBoV3 were detected in three (37%) stool samples each, while HBoV2 was detected in two (25%) stool samples. Some studies have indicated that HBoV2, HBoV3, and HBoV4 are highly associated with gastroenteritis [30,31]. The widespread distribution of HBoV1 and HBoV3 in comparison to HBoV2 could presumably be due to differences in pathogenesis that may influence their transmission route and ability to establish persistence [32]. A study in Thailand [33] also only detected HBoV1, HBoV2, and HBoV3. Likewise, studies from Finland and Pakistan also detected genotypes HBoV1 to 3 [34,35]. In this study, HBoV4 was not detected. This was also the case in several other recent studies [6,34,36,37], which makes the role of HBoV4 genotype unclear. The predominance of

HBoV1 and HBoV3 in children with AGE in this study is similar to a previous report in SA, which also observed a high prevalence of HBoV1 and HBoV3 [10]. However, the study only investigated HBoV in hospitalized children with AGE from an urban area, compared to this study which investigated both hospitalized and outpatients.

HBoV co-infection with other enteric viruses has been reported worldwide. In this study, HBoV was co-detected with other enteric viruses that are involved in acute gastroenteritis, including adenovirus F, which was detected in three out of eight (37.5%) samples. In comparison, rotavirus was detected in three (37.5%), norovirus in two (25%), and astrovirus in one (12.5%) out of eight samples. In only one out of eight (12.5%) of the human bocavirus positive samples, HBoV was detected alone without co-infection with other enteric viruses. A study in China [36] found HBoV co-infection with rotavirus was the most commonly detected (45.3%), followed by human coronavirus (10.1%), astrovirus (4.9%), and adenovirus (4.7%). Another study in Gabon [38] co-detected HBoV with rotavirus (33.3%), sapovirus (33.3), and adenovirus/norovirus (33.3%) in children with diarrhea. In this study, rotavirus (37.5%) and norovirus (37.5%) were the most commonly detected, followed by adenovirus (25%), and astrovirus (12.5%). Previous reports have indicated that HBoV co-infection with other enteric viruses is common. Studies from China, Thailand, Japan, Brazil, and Pakistan also reported that co-infection was very high, while rotavirus and norovirus were the most predominant co-infections [17,33,39,40].

A study in China [36] suggested that differences in the prevalence of certain HBoV genotypes might be due to regional differences in viral epidemiology. Some studies have suggested that HBoV has a seasonal peak during the spring months, while other studies suggested that the winter months, the geographic location, and seasonality [41,42] play an important role in HBoV prevalence. In this study, samples were collected over a period of twelve months. However, samples were not available for collection during each calendar month. Therefore, overall HBoV prevalence was inconclusive concerning seasonal patterns.

Limitations of the study included the absence of a control group (asymptomatic cases). Another limitation was the small number of stool samples collected. Human bocavirus infections are increasingly being recognized globally as a newly emerging virus associated with diarrhea. Therefore, surveillance of HBoV is crucial to monitor the prevalence and to help understand the role of this virus in individuals with AGE. The involvement of HBoV in children with AGE from rural communities in South Africa is most likely to be underinvestigated and under-reported. To the best of our knowledge, this study was the first to report on the prevalence and genetic characterization of human bocavirus in children with AGE from rural communities in Limpopo, South Africa.

Author Contributions: All authors have contributed equally in the conceptualization, methodology, validation, formal analysis, investigation, resources, data curation, writing—original draft preparation, writing—review and editing, visualization, supervision, project administration, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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

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3.3. OBJECTIVE 3: ASSESS THE RELATIONSHIP OF HUMAN BOCAVIRUS STRAINS CIRCULATING IN THE STUDY AREA TO STRAINS CIRCULATING WORLDWIDE AND SHOW GENETIC DIVERSITY.

ARTICLE 3: RESEARCH ARTICLE

Title**: "PHYLOGENETIC ANALYSIS OF HUMAN BOCAVIRUS IN CHILDREN WITH ACUTE GASTROENTERITIS FROM RURAL COMMUNITIES IN SOUTH AFRICA"**

Running Head: Human Bocavirus in SA

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ABSTRACT

BACKGROUND: Prevalence of Human Bocavirus (HBoV) has been reported in children suffering from respiratory infections and only very recently in children suffering from acute gastroenteritis in South Africa. This study assessed the relationship of HBoV strains from stool samples in a rural area of South Africa to strains circulating worldwide.

MATERIALS AND METHOD: HBoV amplification was through Real-Time PCR and PCR products of the positives were assessed by targeting the NS1- nonstructural protein gene. Partial nucleotide sequences of HBoV1-3 were compared with global reference strains available in NCBI GenBank to determine relatedness. Phylogenetic tree constructions were performed using the Molecular Evolutionary Genetics Analysis (MEGA) program.

RESULTS: Six HBoV strains were sequenced and phylogenetic analysis were performed. Two (33.3%) samples were confirmed as HBoV1 strains, one (16.6%) sample was confirmed as HBoV2 strain and three samples (50%) were confirmed as HBoV3 strains. Comparisons with global HBoV circulating strains from stool/clinical samples, respiratory samples and environmental (water) samples showed that the strains identified from rural communities in South Africa where households do not have adequate water and sanitation conditions are closely related.

CONCLUSION: There are still various questions that needs to be answered on the role of HBoV in diarrhea and respiratory infections. More studies are also needed on the pathways and the role of WASH factors in the transmission of this virus.

Keywords: Human Bocavirus, Acute Gastroenteritis, Rural communities.

1. INTRODUCTION

Prevalence of Human Bocavirus (HBoV) has been reported in urban areas from South Africa, in 3 studies from children suffering from respiratory infections (1-3) and in 1 study from children suffering from AGE (4). No study has investigated the prevalence of HBoV from children suffering with acute gastroenteritis (AGE) in rural communities from South Africa with little or no water and sanitation infrastructures (5). However, several studies globally have reported the virus in children with respiratory tract infections (5-7). Recently, there is an increase in the number of studies reporting HBoV in acute gastroenteritis, its role, genetic diversity and distribution globally (8-11).

A recent review on HBoV studies has shown the prevalence rate of 13% from Africa in individuals with AGE with/without respiratory tract infections (5). The study further showed that HBoV infections are most likely to be underreported in Africa and children are most likely to experience HBoV infections as a result of poor sanitation and hygiene practices. The review also pointed out that more investigations are needed to determine the prevalence and role of HBoV in respiratory infections, gastroenteritis and environmental samples (5, 12).

This study assessed the prevalence of HBoV in children suffering from acute gastroenteritis from rural communities in South Africa. The sequences of HBoV strains from this study were used to determine their relationship to strains detected globally from respiratory infections, gastroenteritis and the environment using reference strains in the NCBI GenBank. The results of this study aimed to add knowledge of circulating HBoV strains from rural communities in SA to the global information on HBoV research.

2. MATERIALS AND METHODS

2.1. Study design

The study was conducted between June 2017 and July 2018. Stool samples were randomly collected from both outpatients and hospitalized patients with acute gastroenteritis from rural communities in Limpopo Province of South Africa. The study was approved at the University of Venda by the research ethical committee (Ref. SMNS/17/MBY/03) and the Department of Health Provincial level (Limpopo), South Africa (Ref: 4/2/2). In total, 141 stool were collected from young children ≤5 years and younger with symptoms relating to AGE. Patient data were provided by the parents/guardian (after signing an informed consent) and the Health Care Centers where the children were treated.

2.2. Amplification of HBoV1, HBoV2 and HBoV3

All HBoV positive samples detected by Real-Time PCR assay were subjected to subsequent PCR targeting the NS1-nonstructural protein gene using the primers listed in Table 1. The TopTaq Master Mix Kit (Qiagen) was used for the amplification of all positive HBoV samples following the manufactures instructions. Reactions of amplification were performed in a volume of 50 µl containing 25 µl master mix, 5 µl each of the F and R primer, 15 µl Free-RNase water and 5 µl DNA. Amplification condition for HBoV1 were initial denaturation for 10 min at 94°C, 35 cycles of amplification (94°C for 1 min, 54°C for 1 min, and 72°C for 2 min) and the expected product size was 354 bp. HBoV2/4 amplification was initiated with a denaturation step of 15 min at 95ºC followed by 40 cycles of 94ºC for 15 sec, 53ºC for 30 sec, and 72ºC for 1 min, with an expected product size of 454 bp. Human Bocavirus 2 and 4 used the same antisense primers (13), which was unable to differentiate between the two

(HBoV2/4) genotypes. Therefore the genotypes were confirmed through sequencing. The amplification of HBoV3 consisted of 1 step of 95ºC for 15 min followed by 45 cycles of 94ºC for 20 sec, 52ºC for 20 sec, and 72ºC for 40 sec, followed by an extension step at 72ºC for 10 min, with the expected product size of 440 bp. All PCR products were visualized on 2% agarose gel electrophoresis and ethidium bromide staining using a 1000 bp molecular ladder.

Table 1: Primers used for HBoV amplification and sequencing

Virus	Target gene	Primer	Sequence (5'–3')	Fragment length (bp)	Reference
HBoV-1	NS1	188F 542R	GACCTCTGTAAGTACTATTAC CTCTGTGTTGACTGAATACAG	354	(Allander et al. 2005) (6)
HBoV2/4	NS1	HBoV2-sf1 HBoV2-sr1	AACAGATGGGCAAGCAGAAC AGGACAAAGGTCTCCAAGAGG	454	(Kapoor et al. 2009) (13)
HBoV-3	NS1	P5 P6	CAGAAGCATCGGAAGTGGGTGT ATGTGAGGCTTTATGCTGGCTGA	440	(Santos et al. 2010) (14)

2.3. Sequence analysis: All amplified HBoV1, HBoV2 and HBoV3 positive PCR products were purified, using specific primers (Table 1). Sequencing was performed with the ABI 3500XL Genetic Analyzer POP7TM (Thermo-Scientific). The NS1 partial sequence results were edited with FinchTV for viewing of trace raw data from the Sanger DNA sequencer.

2.4. Phylogenetic analysis of HBoV: Sequences of HBoV strains were compared with those of the reference nucleotide sequences available in the NCBI GenBank using BLAST tool at http://www.ncbi.nlm.nih.gov/blast. Sequence alignments and genetic distance estimates were performed using Clustal W in the Molecular Evolutionary Genetic Analysis (MEGA) (14). The phylogenetic tree construction were performed using the Molecular Evolutionary Genetics Analysis (MEGA) version 7

program (15) and the neighbour-joining method was used to build the phylogenetic trees.

3. RESULTS AND DISCUSSION

HBoV was detected in 8 (5.7%) children with AGE from 141 stool samples. Among the patients 3 (37.5%) stool samples each were positive for HBoV1 and HBoV3 (37.5%) respectively and 2 (25%) stool samples were positive for HBoV2. HBoV4 were not detected in the current study. Only 6/8 (75%) of the strains were successfully sequenced. Phylogenetic analysis using the partial nucleotide sequences was done by comparing global reference sequences from NCBI GenBank to the 6 HBoV sequences.

3.1. Relatedness of identified HBoV genotypes to global HBoV strains detected in children suffering from AGE.

Comparative results showed that HBoV1 (MN072357, MN072359) sequences in this study were close (79%) to the strain previously identified in Gabon (MF784510) (Figure 1A), furthermore, HBoV1 sequences in this study were closely related (74%) to a strain from Sudan (16), which demonstrated the spread of this strain in Africa (Figure 1A). HBoV1 strains in this study were closely related (95%) to strains previously reported in South Africa (MK387166, MK387169). This suggested the circulation of the same strain with close genetic relatedness in Africa (17). The HBoV2 strain in this study (MN072358) showed close relation (98%) with sequences (GQ281572, GQ281571) previously identified in South Korea (18), strains from South Africa (MK387164, MK387168 and MK387158) with close (100%) relatedness (4), followed by sequence (JX257046) previously identified (100%) in China (19) (Figure 1B).

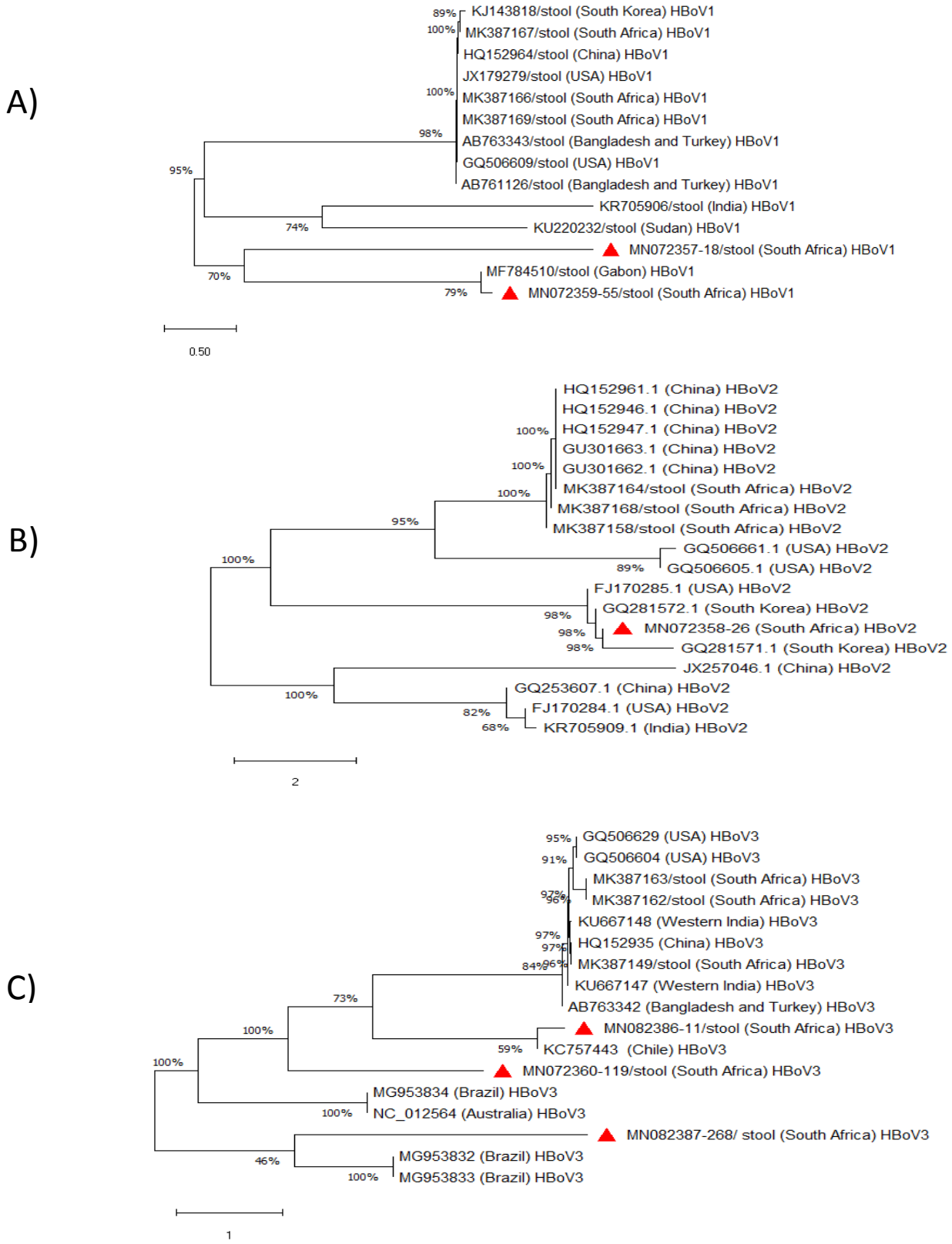


Figure 1. Phylogenetic analysis of the partial nucleotide sequences of non-structural protein (NS1) from South African HBoV1 (A) genotypes (MN072357/MN072359), HBoV2 (B) genotypes (MN072358) and HBoV3 (C) genotypes (MN072360/

MN082386/ MN082387) from stools compared to others reported in AGE. The phylogenetic tree with 1,000 bootstrap replicates using Clustal W in the Molecular Evolutionary Genetic Analysis 7 software package.

Two of the HBoV3 sequences (MN072386, MN072360) in this study demonstrated a close (73%) relationship to a sequences previously identified in South Africa (MK387163, MK387162 and MK387149), China (HQ152935), USA (GQ506629) and western India (KU667147) (Figure 1C). A close relation (100%) between HBoV3 sequence (MN082360) to sequences from Brazil and Australia (MG953834, NC_0125864) in children with AGE.

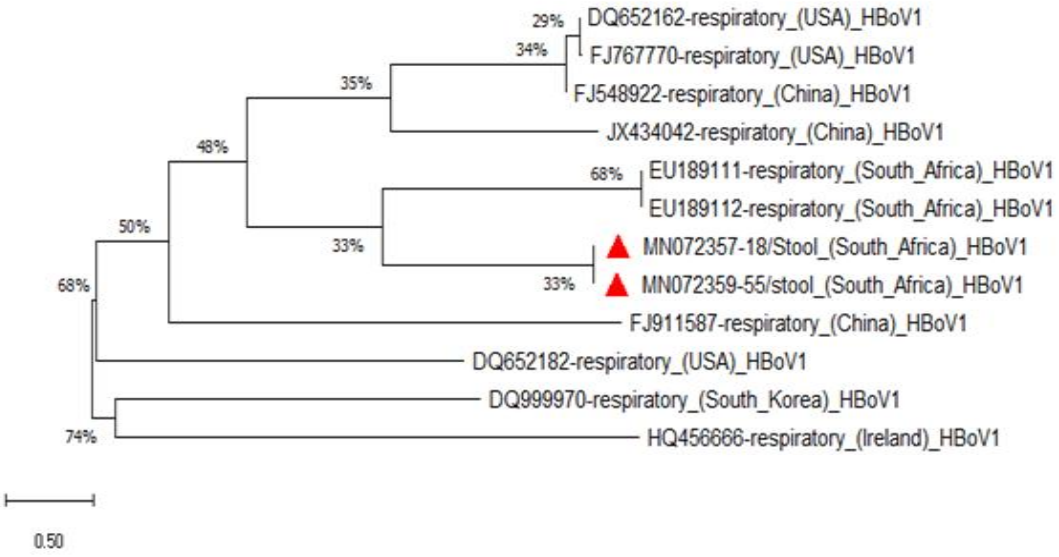
The close relation between the sequences suggested the circulation of closely related HBoV species from the South American region and Africa (8). HBoV3 sequence (MN072360) in this study was closely (71%) related to strains from India (KU667148) and Australia (NC_012564), which demonstrated the distribution of this genotype in South Asia, Australia and Africa (9, 20) (Figure 1).

Studies have shown that HBoV1 strains have very few variations among them, and the sequences are conserved (21, 22). This corroborated with our results which showed little variations within the HBoV1 sequences in this study compared to others globally. Studies have suggested that global travelling could be a reason for closely related strains and while mutations could be responsible for large variations in relatedness of strains (5, 18, 23, 24). While other studies suggest that there are geographical differences between HBoV strains circulating worldwide and herd immunity of the population could be a factor influencing the distribution of HBoV (10).

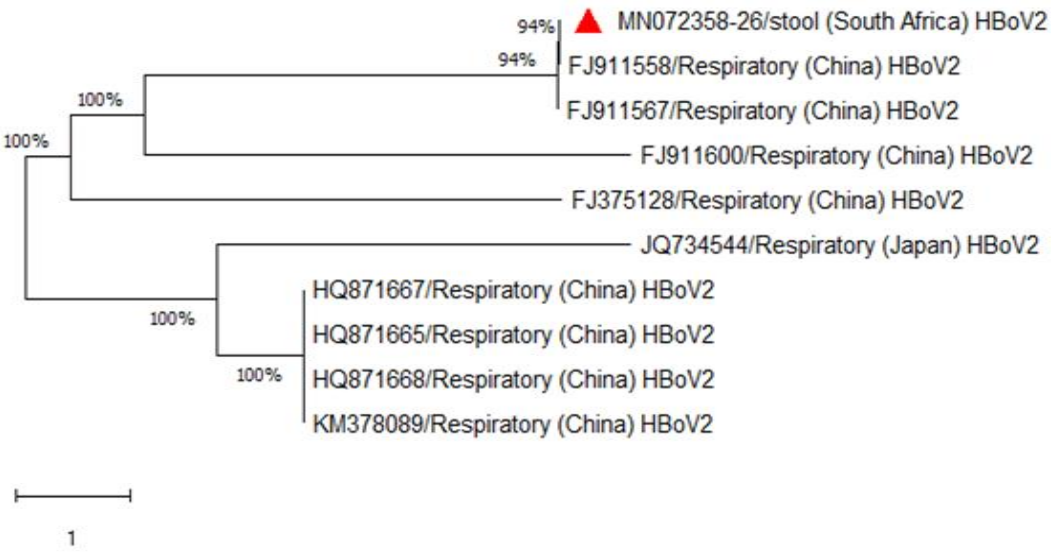
3.2. Relatedness of identified HBoV genotypes to global HBoV strains reported in children suffering from respiratory tract infections.

HBoV1 sequences (MN072357, MN072359) in AGE showed close relation to sequences (EU189111/EU189112) previously identified in South Africa from respiratory tract infections (1, 2) (Figure 2A).

A)



B)



C)

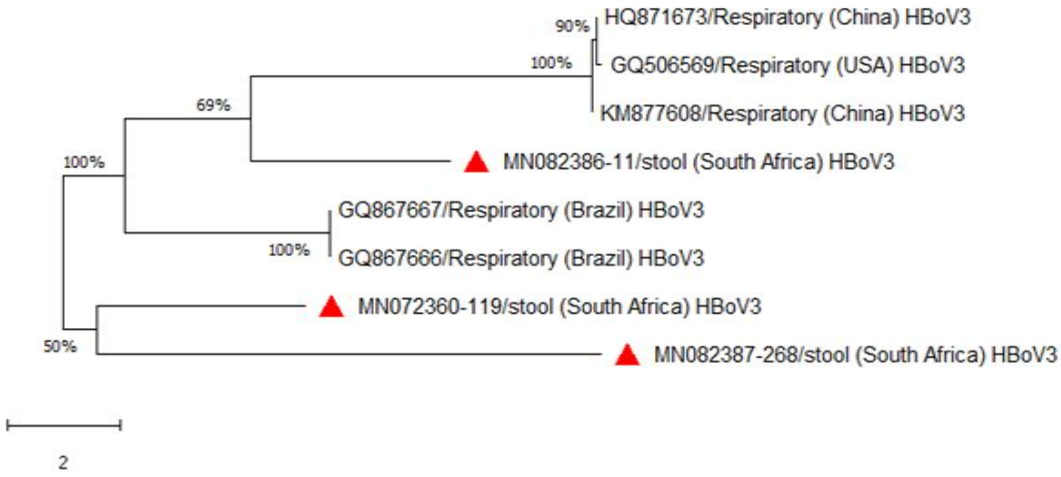


Figure 2. Phylogenetic analysis of the partial nucleotide sequences of non-structural protein (NS1) from South African HBoV1 (A) genotypes (MN072357/MN072359), HBoV2 (B) genotypes (MN072358) and HBoV3 (C) genotypes (MN072360/MN082386/MN082387) in stools compared to others reported in respiratory tract infections. The phylogenetic tree with 1,000 bootstrap replicates using Clustal W in the Molecular Evolutionary Genetic Analysis 7 software package.

Phylogenetic analysis of the HBoV2 (MN072358) strain in this study showed relatedness (94%) to FJ911558 strain and FJ911567 strain from China isolated in respiratory tract infection (Figure 2B) (25). The HBoV3 sequence (MN082386) from this study was closely (69%) related to strains (HQ871673/ GQ506569/ KM877608) from China and USA in children with respiratory tract infections (11, 26) (Figure 2C).

HBoV has long been reported in individuals with respiratory tract infections (6, 7, 27). Furthermore, several studies have shown that HBoV may also be present in stool of children with respiratory infections (28). Other studies have suggested that HBoV first causes respiratory tract infections and penetrates later to the gastrointestinal tract resulting in diarrhea (21, 29). Even though HBoV has been detected in stools of children suffering from respiratory tract infections, there are no studies that have established the correlation between the strains involved in the respiratory tract infection to the strains found in AGE (17, 21).

The results of this study showed that strains involved in AGE and respiratory infections have few variations among and are closely related globally. It is reasonable to assume that HBoV virus have a causative role in AGE and may be either shed from the respiratory tract or infecting the intestinal tract with no pathogenic role in AGE (28). More studies are required to simultaneously test for respiratory and stool samples

together to clarify the assumptions, pathogenesis and give answers to the possible association of HBoV to AGE and respiratory tract infection respectively.

3.3. Relatedness of identified HBoV genotypes to global HBoV strains reported in the environment.

Many rural communities in South Africa still face challenges of access to clean drinking water and proper sanitation which poses a serious health risk for vulnerable individuals such as young children (30). Studies investigating HBoV in the environment are very limited globally (31). Only one study on HBoV strains was recently published on the prevalence of HBoV in raw sewage and mussels samples in South Africa (12).

In this study, HBoV1 sequences (MN072357/MN072359) from stools were closely related (100%) to sequences (KX962126/ GQ129127/ KX962125/ GQ129129/ KX962122/ MG383450) that were previously reported in water samples from Egypt, Germany and Ethiopia respectively (31-33) (Figure 3A). HBoV2 sequence (MN072358) in stool from children were closely (100%) related to sequences (KX962156/KX962158/ KX962154/ KX962141) reported in Egypt from water samples (Figure 3B). HBoV3 Sequences (MN072360/ MN082386/ MN082387) in stools of children from South Africa were closely (96%) related to sequences from Egypt (KX962110/KX962114/KX962113/KX962116) in water samples (Figure 3C).

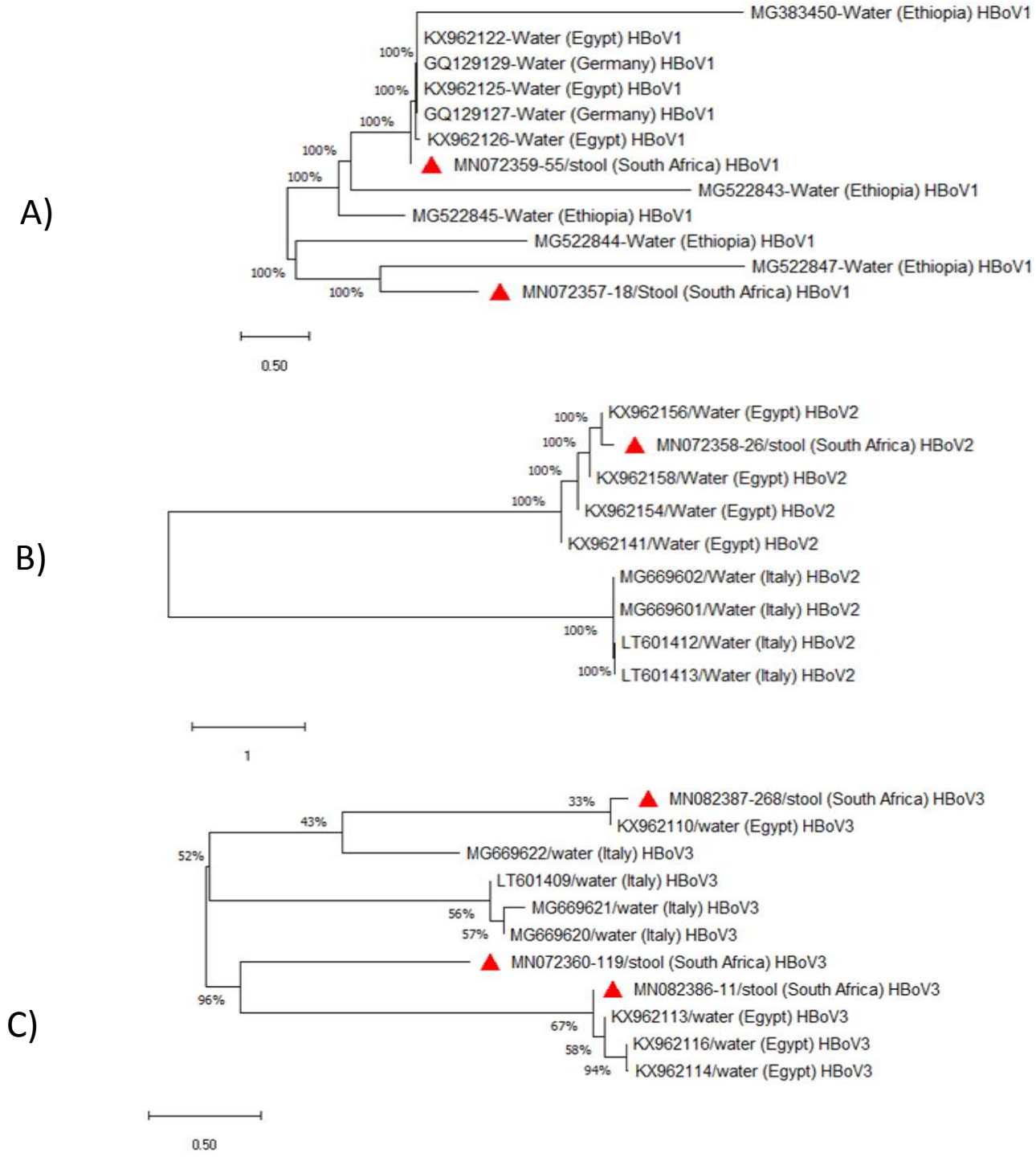


Figure 3. Phylogenetic analysis of the partial nucleotide sequences of non-structural protein (NS1) from South African HBoV1 (A) genotypes (MN072357/MN072359), HBoV2 (B) genotypes (MN072358) and HBoV3 genotypes (MN072360/MN082386/MN082387) in stools compared to other HBoV3 in the

environment. The phylogenetic tree with 1,000 bootstrap replicates using Clustal W in the Molecular Evolutionary Genetic Analysis 7 software package.

The results from this study showed relatedness between strains in this study to global environmental strains. The close relation between strains in this study to strains identified globally, could imply that HBoV is a possible pathogen for waterborne infection in South Africa (SA). The presence of viral pathogens in the environment depends on infection rates and shedding within the host population (31).

4. CONCLUSION

The results from this study has shown that the HBoV strains from children with AGE circulating from rural communities in South Africa are closely related to strains isolated from children suffering with respiratory infections, children suffering from AGE and also strains isolated from the environment in water samples globally. However, a clear conclusion cannot be made on the role of HBoV virus in infections and their specific pathways in transmission, and more studies will have to be done to give answers to these questions. This study provides additional proof that the virus is circulating in the environment and is also part of clinical infections (respiratory and diarrhea) and might be playing a big role in young children's health.

5. ACKNOWLEDGEMENTS

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6. CONFLICT OF INTEREST

The authors declare no conflict of interest

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Chapter 4

SUMMATIVE COMMENTS AND RECOMMENDATIONS

4.1 SUMMATIVE COMMENTS

The main objective of the study was to investigate the prevalence of HBoV in children with acute gastroenteritis and the genetic diversity of stains circulating in rural settings from Vhembe district, Limpopo province of South Africa. The objectives were to study the prevalence of HBoV in children with acute gastroenteritis and investigate the genetic diversity of strains circulating in Vhembe district, Limpopo province (SA), and to assess the relationship of HBoV genotypes circulating in the study area to the ones circulating worldwide and their genetic diversity.

Human Bocavirus has been recognized as a viral pathogen and reported worldwide in several studies as being responsible for diarrhea (Kapoor et al., 2012). Africa is one of the most affected by diarrheal related diseases, there is currently limited data reporting the contribution of HBoV to gastroenteritis (Rikhotso et al., 2018). No health reporting system is currently in place reporting emerging pathogens including HBoV infections, thus suggesting that infections are most probably underreported and the prevalence rate obviously underestimated.

To motivate the rationale of the research study, we performed a systematic review of the PubMed, Google Scholar and EMBASE databases for published articles of HBoV in Africa between 2005 and 2016, and we found that HBoV prevalence in Africa was 13% in individuals suffering gastroenteritis with/without respiratory tract infection

(Rikhotso et al., 2018). The study showed that systemic surveillance of HBoV infection is needed to estimate the burden of HBoV in children with AGE in Africa.

The prevalence of HBoV in children with AGE was investigated in this study and a detection rate of 5.7% in children with AGE was observed. The detection of HBoV reveals the substantial exposure of children to HBoV in rural communities with poor environmental living conditions. Children aged between 1 to 24 months old had high 62% rates of infection with HBoV compared to the other age groups. Findings were similar to previous studies which indicated that young children usually become more active and interact more with their surroundings which may expose them to be contaminated by HBoV infected environments (Hamza et al., 2017; Risku et al., 2012; Jin et al., 2011).

This study determined HBoV genetic diversity among children less than 5 years of age with AGE, from hospitals and clinics in rural communities in South Africa. Considering the fact that not all the children with AGE will consult at any healthcare facilities when infected, what this study found and have reported could be far less than what the actual infection rate of HBoV is concerned in rural communities from South Africa. Findings of HBoV1-3 genotypes in the current study, which have been largely associated with HBoV infections in respiratory tract infections and acute gastroenteritis suggest that there is HBoV involvement during infections in the study area. Unfortunately, this has not been reported before due to the lack of surveillance in South Africa. Furthermore, this may probably relate to other low-income countries where there is currently no surveillance system in place. The characterization of HBoV genotypes may be useful to assess the role of HBoV in diarrheal disease in Africa compared to other developed countries, their distribution and diversity.

HBoV strains identified in this study have been previously reported globally in respiratory tract infections, diarrhea and the environment (water) (Lasure and Gopalkrishna et al., 2017; Lee et al., 2016; Levican et al., 2013). This study assessed the similarities of HBoV genotypes identified in this study related to other reference genotypes in NCBI GenBank, the results from this study has shown that the HBoV strains from children with AGE circulating from rural communities in South Africa are related to strains isolated previously in South Africa from children suffering with respiratory infections. The identified strains in this study were related to strains from the environment in water samples identified globally (Onosi et al., 2019; Hamza et al., 2017). The close relation between strains in this study to strains identified in the environment could imply that HBoV is a possible pathogen for waterborne infection in South Africa (SA).

This study investigated the prevalence of HBoV in children with acute gastroenteritis and the genetic diversity of stains circulating in rural communities of Vhembe district, Limpopo province of South Africa. Studies from Africa have extensively reported in urban setting (Hamza et al., 2017; Rikhotso et al., 2016) and therefore, we speculate that the poor living conditions found in rural setting may impact the prevalence and genetic diversity of circulating HBoV strains. This study has some limitations which includes the fact that we were not able to work on environmental samples due to cost limit. Second, we were not able to perform the whole genome sequencing that would help to entirely assess the HBoV genetic diversity. Third, the study sample size was small. However, HBoV genotypes were identified and the results suggested HBoV diversity in the study area. Despite limitations of the study, we did achieve the objectives of the study which were to determine the prevalence of HBoV in children with acute gastroenteritis and the genetic diversity of stains circulating in rural

communities of Vhembe district, Limpopo province of South Africa, and to assess the relationship of HBoV (strains) circulating in the study area to the ones circulating worldwide and their genetic diversity. This study provides additional proof that the virus may be circulating in the environment and is also part of clinical infections (respiratory and diarrhea). Further investigations combining clinical and environmental samples are needed to understand the role of HBoV infection in young children with AGE from rural communities in South Africa.

4.2 RECOMMENDATIONS

Based on the research findings, the study would like to make the following recommendations:

- A systemic surveillance of HBoV infections associated with acute gastroenteritis in young children from rural communities in South Africa (SA) is needed. This can help to monitor the emerging and trends of HBoV infection in SA to better prevent and manage infections.

- Continued monitoring of the strain distribution within the population can help in identification of HBoV disease causing strains and their role during infection.

- Studies investigating HBoV prevalence in the environment are warranted to ascertain the environmental transmission route of HBoV from rural communities in SA. This is crucial in understanding the circulation of this virus in the environment especially in rural areas where most people lack adequate water and sanitation infrastructure.

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APPENDIX A

A.1 APPROVAL OF RESEARCH STUDY FROM THE UNIVERSITY OF VENDA

UNIVERSITY OF VENDA

OFFICE OF THE DEPUTY VICE-CHANCELLOR: ACADEMIC

TO : MR/MS M.C RIKHOTSO

SCHOOL OF MATHEMATICAL AND NATURAL SCIENCES

FROM: PROF J.E. CRAFFORD

DEPUTY VICE-CHANCELLOR: ACADEMIC

DATE : 03 FEBRUARY 2017

DECISIONS TAKEN BY UHDC OF 24TH JANUARY 2017

Application for approval of Thesis research proposal in Mathematical and Natural Sciences: **M.C Rikhotso (11606599)**

Topic: "Prevalence of Human Bocavirus in Children with Acute Gastroenteritis in Limpopo Province of South Africa."

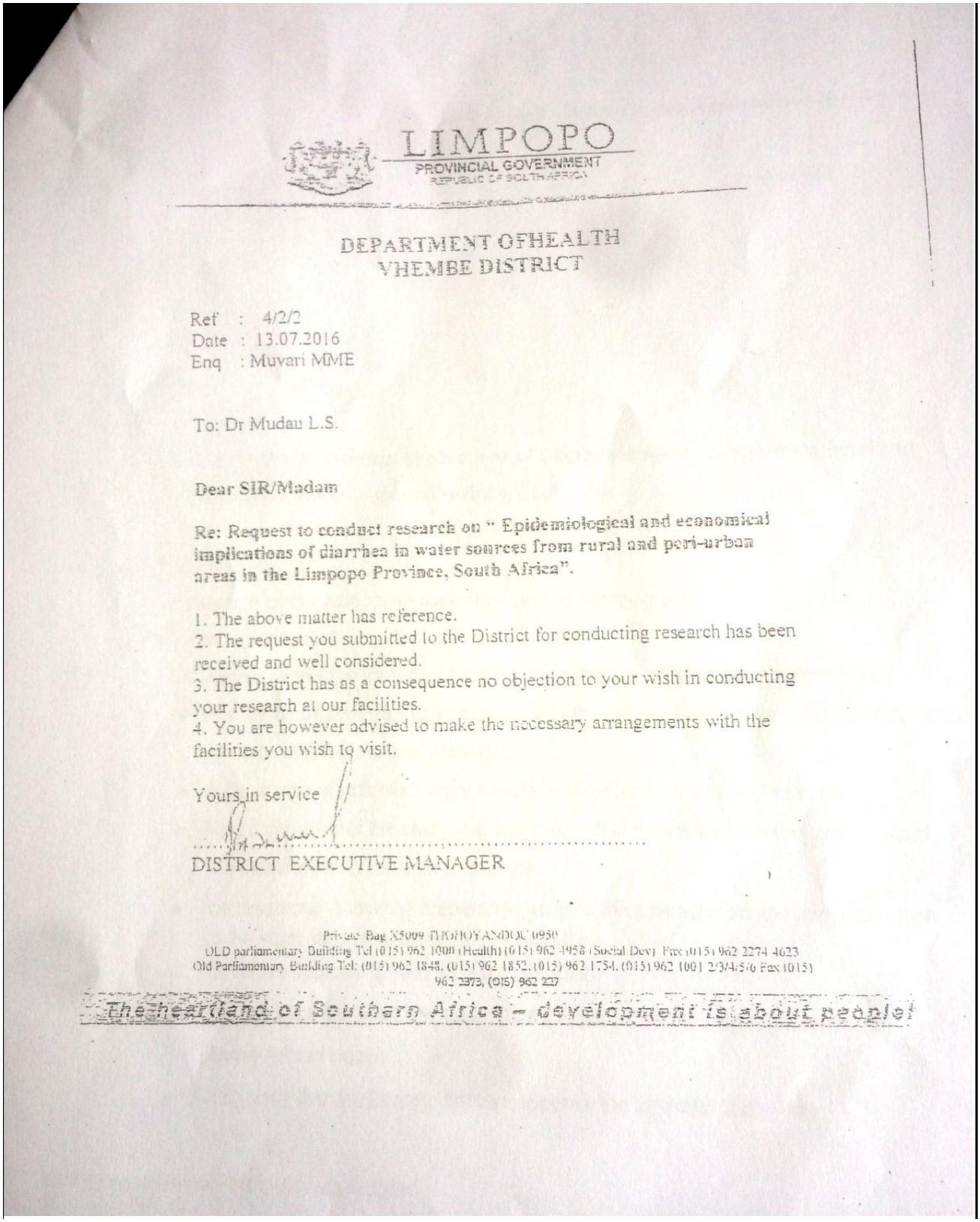
Promoter	UNIVEN	Prof. N. Potgieter
Co-promoter	UNIVEN	Dr. A.N Traoré

UHDC approved Thesis proposal

Prof J.E. CRAFFORD

DEPUTY VICE-CHANCELLOR: ACADEMIC

A.2 APPROVAL OF RESEARCH STUDY FROM DEPARTMENT OF HEALTH DISTRICT OF VHEMBE REGION, LIMPOPO, SOUTH AFRICA



LIMPOPO
PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OFHEALTH
VHEMBE DISTRICT

Ref : 4/2/2
Date : 13.07.2016
Enq : Muvari MME

To: Dr Mudau L.S.

Dear SIR/Madam

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

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

Yours in service

.....
DISTRICT EXECUTIVE MANAGER

Private Bag X5009 THOHOYANDOU 0950
OLD parliamentary Building Tel (015) 962 1000 (Health) (015) 962 4958 (Social Dev) Fax (015) 962 2274 4623
Old Parliamentary Building Tel: (015) 962 1848, (015) 962 1852, (015) 962 1734, (015) 962 1001 2/3/4/5/6 Fax (015) 962 2373, (015) 962 227

The heartland of Southern Africa – development is about people!

A.3 APPROVAL OF RESEARCH STUDY FROM DEPARTMENT OF HEALTH PROVINCIAL GOVERNMENT, LIMPOPO, SOUTH AFRICA

LIMPOPO
PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA

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

Potgieter N
University of Venda
Private Bag X5050
Thohoyandou
0950

Greetings,

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

The above matter refers.

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

Your cooperation will be highly appreciated.

Head of Department

15/06/2016
Date

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

A.4 CONSENT FORM AND QUESTIONNAIRE USED IN THIS STUDY

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT: PREVALENCE OF SELECTED VIRUSES IN CHILDREN WITH ACUTE GASTROENTERITIS IN LIMPOPO PROVINCE OF SOUTH AFRICA.

Investigators:

Mr. Rikhotso MC (PhD student)

Prof Natasha Potgieter (Promoter)

Prof A.N Traoré-Hoffman (Co-promoter)

Dr Kabue Jean Pierre (Co-promoter)

Address:

Department of Microbiology

Life Science Building

School of Mathematical and Natural Sciences

University of Venda

Contact number: 015 962 8107

Dear participant, you and/or 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(s). Please ask the study staff any questions about any part you do not fully understand. Your participation is **entirely voluntary and you are free to decline to participate**.

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

What is this research project study all about?

- The study will include stools from young children under 5 years of age with diarrhoea.
- The project(s) aimed to investigate the diversity of Bocavirus circulating in the rural communities of the Limpopo province (SA).
- This information will help decisions making in public prevention strategies against diarrhoea disease particularly in Bocavirus infections also in the improvement of sanitary environments in rural communities. The findings of this study will also provide information on Bocavirus diversity with implications on vaccine development.
- General information will be taken from you, including contact details, age, gender, use of toilet, date of diarrhoea, HIV status and other illnesses, etc. A total of 10g of stools will be collected from the participant and will be transported to the university laboratory for analysis.

Why have been invited to participate?

You and/or your baby was selected for this study because of suffering from AGE.

What will your responsibility be?

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

Will you benefit from taking part in this research project?

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

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

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

Who will have access to your medical records?

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

Is there anything else that you should know?

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

Declaration by participant

By signing below, I …….. agree to take part in the research study entitled "**Prevalence of Human Bocavirus in children with acute gastroenteritis in Limpopo province of South Africa**".

I declare that:

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

Signed at (place) ……... on (date) …….

_____ _____

Signature of Participant Signature of Witness

Declaration by investigator(s):

I …… declare that:

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

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

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

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

Signed at (place) …… on (date) ……..

_____ _____

Signature of Investigator Signature of Witness

Declaration by interpreter:

I ……..declare that:

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

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

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

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

Signed at (place) …… on (date) …….

_____ _____

Signature of interpreter Signature of Witness

A.5 DATA CAPTURE FORM/ QUESTIONNAIRE USED IN THIS STUDY

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

Symptomatic patient Subject Number……

Consultation details			
Date:	Visit Number:	Hospital/Clinic name:	
Patient information			
Name _____	Date of birth _____	Gender M ☐ F ☐	Contact details: _____
Parental status:	Unemployed ☐	Employed ☐	Self-employed ☐
Family condition			
Water source:	Tap ☐	Spring/wells ☐	Boreholes ☐ River ☐
Sanitation:	VIP/Pit latrine ☐	Flush toilet ☐	
Other :	Livestock ☐	Breastfeeding ☐	
Medical History			
Clinical symptoms:	Diarrhea ☐ Fever ☐	Vomiting ☐	Dehydration ☐
	Respiratory tract infection ☐	Immunodeficiency ☐	Dehydration ☐
	Abdominal pain/cramps ☐		
Date of Onset: _____	***Rota Vaccine dose received*** ☐		
How many days of presenting with diarrhea before consulting:			
Sample collection			
Date of collection:	_____		
Type of sample:	Type of Stool: Watery ☐ Sausage ☐ Mushy ☐		
Treatment			
Current :			
Previous :			
Laboratory Results			
PCR:			
Sequencing:			