EPIDEMIOLOGY AND CHARACTERISATION OF ENTERIC DNA VIRUSES ASSOCIATED WITH GASTROENTERITIS IN CHILDREN IN SELECTED REGIONS OF SOUTH AFRICA

by

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DECLARATION

I, Rembuluwani Netshikweta, declare that this work was not copied or repeated from any other studies either from national or international publications. Where other people's work has been used, it has been properly acknowledged and referenced. Procedures were carried out in accordance with the ethical rules as prescribed by the Faculty of Health Science Research Ethics Committee, University of Pretoria.

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SUMMARY

Acute gastroenteritis (AGE) is a global public health problem causing considerable morbidity and mortality among infants and children, especially in low-income settings. Viruses including group A rotaviruses (RVA), noroviruses (NoV), adenoviruses (AdV), sapoviruses (SaV) and astroviruses (AstV) are widely acknowledged to be the most common cause of AGE in children. The importance of newly recognised viruses such as human bocavirus (HBoV) as an aetiological agent of AGE is becoming increasingly evident. The aim of this study was to investigate the molecular epidemiology of HAdV and HBoV in children aged ≤5 years hospitalised for AGE in South Africa (SA) from April 2009 to April 2015. Clinical and demographic data, along with stool specimens were collected from hospitalised children who presented with AGE. Real-time polymerase chain reaction (PCR) was used to screen for the presence of enteric DNA viruses. Genotyping was achieved by nucleotide sequence analysis or multiplex PCR. Whole genome sequencing was performed on selected strains to characterise their genetic variation and evolution. Between

April 2009 and December 2014, the prevalence of HAdV in hospitalised children with AGE in SA was 18.1% (656/3623); 62.3% of the HAdV-positive children were 7–24 months of age. Human AdV was detected year round. Co-infections were found in 76.3% (222/291) cases of the HAdV-positive specimens with full enteric screening and AstV was detected most frequently as a co-infecting pathogen. Prolonged hospital stay was observed in human immunodeficiency virus (HIV)-infected children with HAdV. Human AdV-F was the most common species identified (254/603, 42.1%), with almost equally distribution of -40 and -41. Recombination breakpoints of the five HAdV41 strains varied in the number and location, indicating different evolution origins. Between April 2009 and April 2015, the prevalence of HBoV in hospitalised children with AGE in SA was 5.6% (212/3765); the majority of which were from children ≤2-year of age (92%, 195/212). Viral co-infections were found in 67% (142/212) of HBoV cases, while in fully screened specimens (virus, bacteria and parasites), 83.1% (74/89) had evidence of co-infections. In all co-infections, only HAdV was significantly associated with HBoV (adjusted Odds Ratio (aOR))=1.68; (95% CI 1.10-2.52; p=0.015) in multivariate analysis. Human BoV infections were reported throughout the year. All four HBoV genotypes were detected with HBoV1 being the most prevalent (79.6% (152/191). The variation in total number of specimens screened for HAdV and HBoV is because HAdV screening was done until December 2014; while HBoV screening was done until April 2015. The current study highlights the genetic diversity of HAdV-40 and -41 strains circulating in SA and suggests possible evolution from inter-strain recombination. Furthermore, the present study highlights the wide spectrum of HBoV genotypes in children with AGE in SA. This study presents the most comprehensive recent data on HAdV diversity in SA, and new baseline data on a HBoV-associated gastroenteritis in a country where no previous report is available.

Keywords: Human adenovirus, human bocavirus, South Africa, epidemiology, prevalence; gastroenteritis, genotypes, species F

PRESENTATIONS AND PUBLICATIONS

Publications

Mans J, Murray TY, Nadan S, **Netshikweta R**, Page NA, Taylor MB. Norovirus diversity in children with gastroenteritis in South Africa from 2009 to 2013: GII.4 variants and recombinant strains predominate. Epidemiology and Infection 2016;144(5):907-919.

Page N, Groom MJ, Murray T, Nadan S, **Netshikweta R**, Keddy KH, Poonsamy B, Moyes J, Walaza S, Kahn K, Kuonza L, Taylor MB, Madhi SA, Cohen C. Sapovirus prevalence in children less than five years of age hospitalised for diarrhoeal disease in South Africa, 2009–2013 Journal of Clinical Virology 2016;78:82–88.

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Murray TY, Nadan S, Mans J, **Netshikweta R**, Page NA, Taylor MB. Genetic diversity of sapoviruses in hospitalised patients with gastroenteritis in South Africa [Presentation]. Fifth International Conference on Caliciviruses, 12-15 October 2013 Beijing, China.

Netshikweta R, Nadan S, Page N, Kruger T. Human bocavirus infections in paediatric patients hospitalised with acute gastroenteritis in South Africa between 2009 and 2013 [Poster]. 11th International Rotavirus Symposium 3-5 September 2014 in New Delhi, India.

Nadan S, McLaren B, **Netshikweta R**, Kruger T, DeMaayer T, Reubenson G, Page N. The detection of enteric viruses in malnourished paediatric patients from South Africa [Poster]. 11th International Rotavirus Symposium 3-5 September 2014 in New Delhi, India.

Murray TY, Nadan N, **Netshikweta R**, Page NA, Taylor MB. Prevalence and genetic diversity of sapoviruses in children hospitalised with gastroenteritis in South Africa [Poster]. Vaccines For Enteric Diseases (VED 2015), 8-10 July 2015, The Royal College of Physicians of Edinburgh, Edinburgh, UK.

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Netshikweta R, Nadan S, Kruger T, Page NA. Human Bocavirus in children with acute gastroenteritis in South Africa, 2009-2013 [Presentation]. Faculty Day, Faculty of Health Sciences, University of Pretoria 19-20 August 2014: HW Snyman Building, Pretoria.

Netshikweta R, Nadan S, Kruger T, Page N. Frequency of human adenovirus species in children hospitalised with acute gastroenteritis in South Africa [Poster]. Pathology Research and Development Congress 15 - 16 April 2015 Emperors Palace Johannesburg South Africa.

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A.2 ABSTRACT: Netshikweta R*, Nadan S, Kruger T, Page NA. Human Bocavirus in children with acute gastroenteritis in South Africa, 2009-2013 [Presentation].

A.3 ABSTRACT: Netshikweta R*, Nadan S, Page N, Kruger T. Human bocavirus infections in paediatric patients hospitalised with acute gastroenteritis in South Africa between 2009 and 2013 [Poster].

ABBREVIATIONS AND SYMBOLS

aa	amino acid
AdV	Adenovirus
AGE	Acute gastroenteritis
AIDS	Acquired immunodeficiency syndrome
ARTI	Acute respiratory infection
AstV	Astrovirus
Avp	Adenoviral protease
BPV	Bovine parvovirus
CAR	Coxsackie-AdV receptor
CDC	Centers for Disease Control and Prevention
CED	Centre for Enteric Diseases
CHBAH	Chris Hani Baragwanath Academic Hospital
CHERG	Child Health Epidemiology Reference Group
CI	Confidence interval
CPE	Cytopathic effect
CSF	Cerebrospinal fluid
DAEC	Diffusely-adherent *Escherichia coli*
DBS	Dried blood spot
DNA	Deoxyribonucleic acid
ds	Double-stranded
EAd	Enteric adenovirus
EAggEC	Enteroaggregative *Escherichia coli*
EDH	Edendale Hospital
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
EKC	Epidemic keratoconjunctivitis
EPEC	Enteropathogenic *Escherichia coli*
EPI	Expanded Programme of Immunization
ETEC	Enterotoxin-producing Escherichia coli
GBoV	Gorilla bocavirus
GEMS	Global Enteric Multicenter Study

HAdV	Human adenovirus
HAE	Human airway epithelial
HBoV	Human bocavirus
HIV	Human immunodeficiency virus
HI	Haemagglutination inhibition
HSPG	Heparan sulfate proteoglycans
HVR	Hypervariable regions
ICTV	International Committee on Taxonomy of Viruses
IFA	Indirect fluorescent antibody
IFN	Interferon
InDels	Insertions or deletions
IQR	Interquartile range
ITR	Inverted terminal repeats
kb	Kilobase
MAL-ED	Malnutrition and Enteric Disease Study
MAUVE	Multiple alignment of conserved genomic sequence with rearrangements
MHC	Major histocompatibility complex
min	Minute
MKH	Edendale Hospital
MLP	Major late promoter
MPH	Mapulaneng Hospital
MSD	moderate-to severe diarrhoea
MVC	Minute virus of canine
nm	Nanometre
NICD	National Institute for Communicable Diseases
NoV	Norovirus
NP	Nuclear phosphoprotein
NS	Non-structural protein
OR	Odds ratios
ORF	Open reading frame
PBoV	Porcine bocavirus
PCR	Polymerase chain reaction
pH	Potential of hydrogen

ppm	Parts per million
PtMPV	Pig-tailed macaque parvovirus
RATT	Rapid Annotation Transfer Tool
RhMPV	Rhesus macaque parvovirus
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RSSP	Rotavirus Sentinel Surveillance Program
RTI	Respiratory tract infection
RT	Reverse Transcriptase
RV	Rotavirus
SaV	Sapovirus
SA	South Africa
Sec	Second
SPV	Simian parvovirus
ss	Single stranded
ST	Heat stable toxin
TP	Terminal protein
US	United States
USA	United States of America
VP	Viral proteins
VLP	Virus-like particle
WHO	World Health Organization
%	Percentage
˚C	Degree Celsius
µℓ	Microlitre
µM	Micrometre

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CHAPTER 1

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Acute gastroenteritis (AGE) is one of the leading causes of morbidity and mortality among children worldwide, particularly in children age under five years of age (Naghavi et al., 2017; Troeger et al., 2018). Acute gastroenteritis defines a change in stool consistency to a loose or liquid form or an increase in the frequency of evacuations (3 or more in 24 hours) with or without fever or vomiting and lasting fewer than 14 days (Guarino et al., 2014). The burden of AGE is heaviest in low and middle income settings, areas that have a high prevalence of known risk factors for diarrhoeal disease, such as poor hygienic conditions, lack of access to treated drinking water, inadequate sanitation and low socio-economic status (Troeger et al., 2018). According to The Global Burden of Disease, diarrhoeal diseases caused more than 1.6 million deaths in 2016 among all ages worldwide, with more than a quarter (26.93%) in children younger than 5 years of age with approximately 90% (89.37%) of these deaths in South-East Asia and sub-Saharan Africa (Naghavi et al., 2017).

The aetiology of AGE includes viruses, bacteria, and parasites (Platts-Mills et al., 2013; Humphries and Linscott, 2015). Kotloff et al. (2013) in the original Global Enteric Multisite Study (GEMS), which investigated moderate-to-severe diarrhoea (MSD) in children <5 years at seven sites in Africa and Asia, found

that moderate to severe diarrhoea in children was mostly attributed to *Cryptosporidium*, enterotoxigenic *Escherichia coli*, *Shigella*, and rotaviruses (RVs) pathogens. When stool specimens from the original GEMS study were re-analysed using highly sensitive quantitative polymerase chain reaction (PCR) methods, it was found that the leading pathogens causing diarrhoea in young children were (in descending order), *Shigella*, RV, adenovirus (AdV) 40/41, heat stable toxin (ST)-positive enterotoxin-producing *E. coli* (ETEC), *Cryptosporidium* spp. and *Campylobacter* (Liu et al., 2016).

Viral aetiologies are responsible for the majority of AGE (Ciccarelli et al., 2013; Platts-Mills et al., 2018). The most frequently reported viruses include RV, noroviruses (NoV), AdVs, sapoviruses (SaV) and astroviruses (AstV) (Dennehy, 2011; Desselberger, 2014; World Health Organization - Child Health Epidemiology Reference Group [WHO-CHERG], 2014). In recent data from the Malnutrition and Enteric Disease Study (MAL-ED) investigating pathogen-specific burdens of community diarrhoea among children aged 0-2 years of age (November 2009 and February 2012), viruses were found to be responsible for 36.4% of overall diarrhoeal incidence, bacterial for 25.0%, and parasites for 3.5% (Platts-Mills et al., 2018).

Global estimates of the mortality due to AGE has decreased since 1990 by nearly 65% among children younger than five due to among other factors improvements in safe water and sanitation and reductions in undernutrition (Troeger et al., 2018). However, morbidity has not declined to a similar degree, diarrhoea disease continues to persist as a significant cause of childhood

morbidity globally (Troeger et al., 2018). South Africa (SA) implemented the RV vaccine in the national expanded programme on immunisation in 2009 (Madhi et al., 2010). In SA, the under five year mortality due AGE-associated disease has been decreasing over time, however, morbidity has not shown a parallel decline in comparison to mortality trends. For example, the mortality dropped from 7.0% in 2010/2011 to 2.2% in 2015/16 in children under-five, however, the number of diarrhoea admissions has increased since 2012/2013 (Massyn et al., 2016).

A substantial proportion (approximately 40%) of AGE is thought to remain undiagnosed, suggesting that additional pathogens for which diagnosis are unknown are likely to exist (Knox et al., 2012; Nicholson et al., 2016). The advances in molecular biological techniques have made it possible for previously known viruses to be more reliably identified and new or previously undescribed viruses to be identified (Khan and Bass, 2010). The newly discovered enteric viruses that contribute to AGE burden include Aichi virus, human parechovirus and human bocavirus (HBoV), of which the precise clinical relevance remains under debate (Khan and Bass, 2010; Ngan, 2011; Knox et al., 2012).

Human AdV (HAdV) and HBoV belong to two different families, *Adenoviridae* and *Parvoviridae*, respectively, and share several characteristics. They are both deoxyribonucleic acid (DNA) viruses and have the ability to cause acute infection in both the respiratory system and the gastrointestinal tract. The majority of previous studies in SA focus on RV as the major pathogen

associated with AGE and, therefore, comprehensive understanding of the aetiology of diarrhoea is lacking. Because information about viruses different from RV associated with AGE in SA is limited, the focus of this study is to highlight the epidemiology, molecular epidemiological characteristics of HAdV in children hospitalised with AGE in SA, and to determine the prevalence of HBoV in a country where no previous report is available.

1.2 ADENOVIRUS

1.2.1 Historical perspective

Adenovirus was first discovered in 1953 by Rowe and colleagues from cultured surgically removed tonsillar and adenoid tissues from children (Rowe at al., 1953). Shortly after, Hilleman and Werner isolated a related agent from tracheal cells of military recruits suffering from respiratory illness (Hilleman and Werner, 1954). This virus was initially named adenoid degeneration, adenoid-pharyngeal conjunctival and acute respiratory disease agents. It was not until 1956 that the virus was collectively called “adenoviruses”, coming from the Greek word for “gland”, after the original tissue from which it was originally isolated (Enders et al., 1956). Since then, further isolation of AdV was confirmed from tissues of different animal species including cattle, horses, turkeys and mice (Klein et al., 1959; Hartley and Rowe 1960; Ardans et al., 1973; Carlson et al., 1974).

1.2.2 Classification of adenoviruses

Human AdV belong to the genus *Mastadenovirus* of the family *Adenoviridae* (Harrach et al., 2012; Sidoti et al., 2015). This family includes genera, *Aviadenovirus* (infecting birds), *Mastadenovirus* (infecting mammals), *Siadenovirus* (infecting birds and frogs), *Atadenovirus* (infecting mammals, birds and reptiles) and *Ichtadenovirus* (infecting fishes) (Figure 1.1) (Lynch et al., 2011; Robinson et al., 2011; Harrach et al., 2012). Each genera is further divided into several species, each comprised of specific types.

Over 70 types of HAdVs have been identified which are classified in seven species, HAdV-A to -G, based on a variety of biochemical, immunological and genetic parameters (Matsushima et al., 2013; Kaján et al., 2017; Hashimoto et al., 2018; Khanal et al., 2018). Of these types, 51 HAdV were identified, characterised and classified based on traditional serological typing criteria and are referred to as serotypes. Classification of HAdV into species were based on their ability to agglutinate red blood cells, as only antisera against viruses of the same species will prevent the hemagglutination reaction, while novel serotypes were denoted based on the ability of the virus to resist neutralisation by antisera against known HAdV types.

Since 2007, serotyping has been replaced by genomic data analysis, which revealed over 80 types of HAdV (Ghebremedhin, 2014; Lion, 2014; Hashimoto et al., 2018). Currently the characterisation and typing of HAdV it is based on whole-genome sequence analysis as proposed by the Human Adenovirus Working Group (Seto et al., 2011). Recombination is considered as the major

driving forces in HAdV evolution (Robinson et al., 2013; Gonzalez et al., 2015). The significance of major capsid genes, namely the hexon, penton base, and fiber genes as hot spots for recombination has been described (Matsushima et al., 2012).

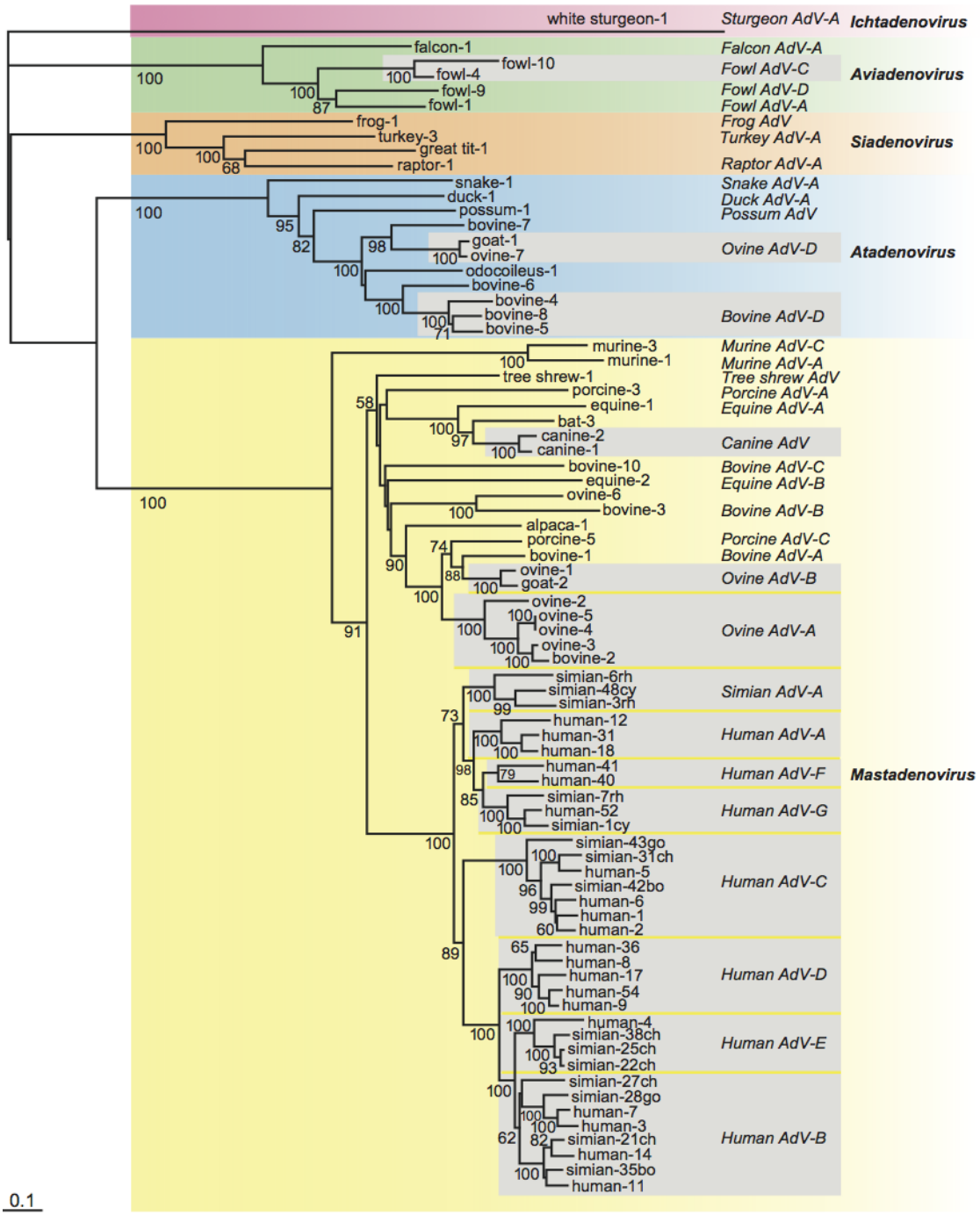

Figure 1.1: Phylogenetic tree of adenoviruses based on a distance matrix analysis of hexon amino acid sequences from: https://talk.ictvonline.org/ictv-reports/ictv_9th_report/dsdna-viruses-2011/w/dsdna_viruses/94/adenoviridae-figures: accessed 29.03.2019.

Most of the new types were identified as either sequence-divergent and/or recombinant viruses of previously recognised types (Kaneko et al., 2009; 2011; Zhou et al., 2012; Robinson et al., 2013; Uusi-Kerttula et al., 2015). Recombination can easily occur within the same AdV species rather than among different AdV species, and several intertypic recombinant HAdVs have been reported (Noda et al., 1991; Aoki et al., 2008; Lukashev et al., 2008; Walsh et al., 2010; Kaneko et al., 2011).

1.2.3 Structure

1.2.3.1 Adenoviral virion

Human AdV is a medium-sized (90–100 nanometres [nm]), non-enveloped virus with an icosahedral nucleocapsid. It is comprised of capsid proteins, minor proteins, and core proteins. (Figure 1.2). The virion consist of 252 capsomers (240 hexons and 12 pentons) and 12 fiber proteins (Allard and Vantarakis, 2017). These three proteins (hexons, pentons, and fibers) constitute the major antigens important in viral classification and disease diagnosis. The hexon is the main structural protein of the capsid and the most abundant AdV protein (Lion, 2014). Each penton consists of a penton base and a projecting fibre with a terminal knob that interacts with cellular receptors (Russell, 2009; Ghebremedhin, 2014). The fiber consist of three structural domains including the tail, the shaft and the knob (Figure 1.2) (Lion, 2014). The tail domain is the binding site for the penton base, the shaft extends the globular knob domain from the virion and the knob domain mediates binding to the host cell surface receptor (Lion, 2014). Unlike most members of the genus *Mastadenovirus*, HAdV-F and HAdV-G encode two fiber proteins (long and short) (Jones et al., 2007).

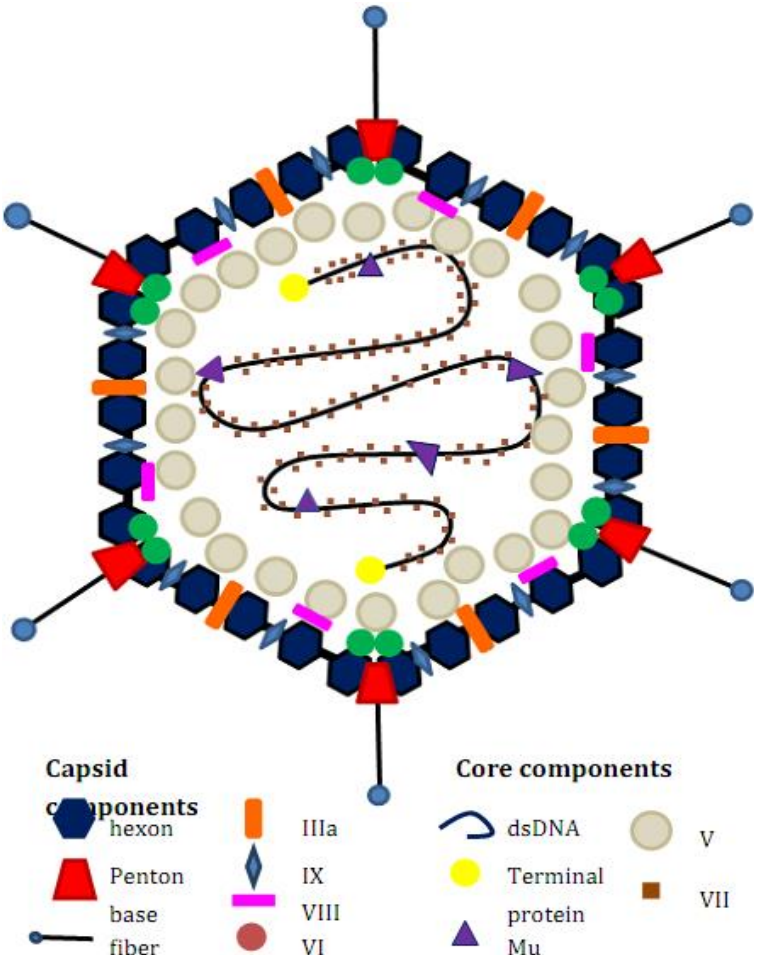

Figure 1.2: The structure of the adenovirus (Waye and Sing, 2010).

The capsid also contains a number of minor structural proteins such as: protein IIIa, protein VI, protein VIII, and protein IX which act as cement proteins by interacting with each other as well as hexon and core proteins, thus providing the virion structure stability (Figure 1.2) (Waye and Sing, 2010). The six core proteins (protein V, protein VII (pVII), protein Mu, protein IVa2, adenoviral protease [Avp]) and the terminal protein are associated with viral DNA and are important during DNA assembly and stabilisation (Kennedy and Parks, 2009). While the core proteins secure condensation and stability of the viral genome,

cement proteins and the major capsid proteins form an extra protective shell for the virus and facilitate virus attachment to the host cell receptors.

1.2.3.2 Adenovirus genome

The genome of HAdV type 2 (HAdV-2) and HAdV type 5 (HAdV-5) were the first to be fully sequenced and most of the structural knowledge on AdV genomes come from these serotypes (Saha et al., 2014). Human AdV comprise linear, non-segmented double-stranded (ds) DNA genome ranging from 34 to more than 37 kilo-base pairs (kb) and encode over 40 proteins (Lion, 2014). The genome has inverted terminal repeats (ITR) associated with each of the 5' ends of the linear dsDNA, which act as replication origins for DNA synthesis (Figure 1.3) (Robinson et al., 2011; Lion, 2014).

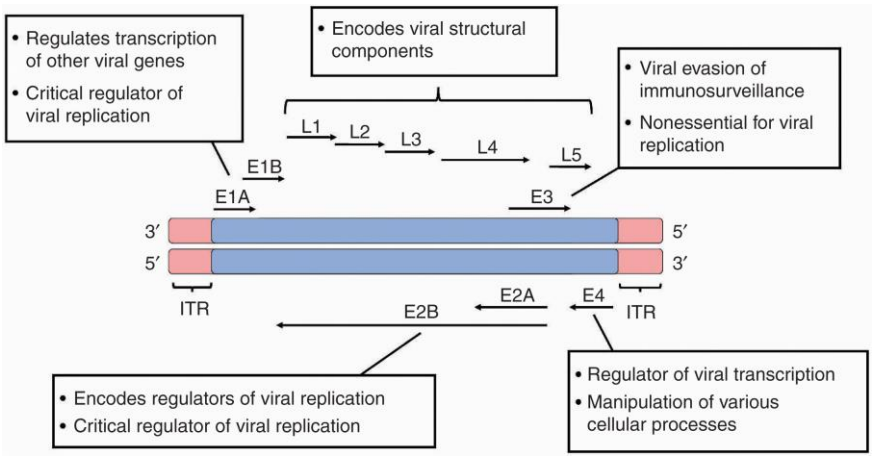

Figure 1.3: Schematic representation of the adenovirus genome (Afkhami et al., 2016). The early regions are noted as E1–E4 and late regions are noted L1–L5.

The genome coding regions are designated early (E1A, E1B, E2, E3, E4), intermediate (IX, Iva), and late (L1-L5) transcriptional units and are expressed in a step-wise manner and named in accordance with the timing of their

transcription and translation during the replication cycle (Figure 1.3) (Robinson et al., 2011; Lion, 2014).

1.2.4 Human adenovirus species and disease spectrum

Each species of HAdV has been associated with different spectra of diseases and severity of infection. The disease conditions associated with particular species and types of HAdVs are presented in Table 1.1.

Table 1.1. Classification of human adenoviruses into species and their respective main tropism and disease site (Chen and Tian, 2018).

Species	Hemagglutination group	Types	% GC	Associated disease
A	IV	12, 18, 31, 61	47~49	Cryptic enteric infection
B	I	B1: 3, 7, 16, 21, 50, 66, 68	50~52	Conjunctivitis; acute respiratory disease; hemorrhagic cystitis; central nervous system
		B2: 11, 14, 34, 35, 55, 79	50~52	
C	III	1, 2, 5, 6, 57	57~59	Endemic infection; respiratory symptoms
D	II	8~10, 13, 15, 17, 19, 20, 22~30, 32, 33, 36~39, 42~49, 51, 53, 54, 56, 58~60, 62~65, 67, 69, 70, 71, 73, 74, 75	57~60	Keratoconjunctivitis in immunocompromised and AIDS patients
E	III	4	57	Conjunctivitis; acute respiratory disease
F	III	40, 41	57~59	Infantile diarrhea
G	Unknown	52	58	Gastroenteritis
Unclassified	Unknown	72, 76, 77, 78	Unknown	Unknown

1.2.4.1 Species A

Human AdV species A, compromised of HAdV-12, -18, -31 and type-61, have a high oncogenic potential (Walsh et al., 2010). Human AdV type -12 was the first AdV shown to induce malignant tumors in rodents (Trentin et al., 1962). Some serotypes in HAdV-A have strong causative associations with childhood diarrhoea (Schmitz et al., 1983). A recent study in Brazil reported an outbreak of gastroenteritis linked with HAdV-12 (Portes et al., 2016). Human AdV-31 is has also been linked with gastroenteritis and is increasingly recognised as a

causative agent of fatal disseminated disease in immunocompromised individuals, particularly children following hematopoietic stem cell transplantation (Matsushima et al., 2011).

1.2.4.2 Species B

Human AdV-B is further divided in two subspecies (HAdV-B1 and HAdV-B2), exhibiting different tissue tropisms and levels of virulence (Russell, 2009). Members of HAdV-B1 predominantly infect the upper respiratory tract in children and military recruits (Wold and Horwitz, 2007; Charlton et al., 2019). Some serotypes of B1 have previously been associated with severe and even fatal outcomes (serotype -3, -7 and -21) (Kajon et al., 1996; Dean et al., 2002; Yamamoto et al., 2014). Members of species B2 (serotype -11, -14, -34 and -25) are frequently associated with urinary tract infections and to a lesser extent ocular and respiratory infections (Leen and Rooney, 2005; Russell, 2009).

1.2.4.3 Species C

Species C is comprised of HAdV types -1, -2, -5, -6, and -57 and are commonly associated with paediatric upper respiratory tract infections worldwide (Garnett, et al., 2002). This species is among the most frequently reported HAdV, found in approximately 60% of HAdV respiratory infections in children and young adults (Demian et al., 2014). More than 80% of the human population have been infected with species C in early life (Garnett et al., 2002). Infections with HAdV-C range from uncomplicated upper respiratory infections to severe pneumonia. Some members of HAdV-C may persist as latent infections for years in adenoids and tonsils and are characterised by a strong and prolonged

shedding in faeces for many months after recovery (Lion, 2014). In addition, some types of HAdV-C (types -1, -2, and -5) have been linked with diarrhoea (Hierholzer 1992; Li et al., 2005). Human AdV-C is also increasingly associated with intussusception (Minney-Smith et al., 2014; Kim et al., 2017). Moreover, species C predominates in HAdV disseminated disease in both adult and paediatric haematopoietic stem cell patients (Ganzenmueller et al., 2011).

1.2.4.4 Species D

Species D are the most divergent and the largest species of HAdV (Harrach et al., 2012). Unlike other HAdV species that are isolated from primates, species D exclusively contains HAdVs (Harrach et al., 2012). A large number of novel HAdV-D types were detected in HIV-positive patients (Echavarria, 2008; Robinson et al., 2011, 2013; Matsushima et al., 2013), where they are a common cause of keratoconjunctivitis (Lion, 2014). Some types of HAdV-D have also been implicated in inflammation of the cornea (Walsh et al., 2009), fatal pneumonia in a neonate, obesity in animals and humans (Arnold et al., 2010) and gastroenteritis in children (Matsushima et al., 2013). Human AdV-D36 has also been implicated with obesity (Esposito et al., 2012). Members of HAdV-D associated with diarrhoea include HAdVD-28, -29, -30, -32, -37, -43–46, -65 and -67 (Matsushima et al., 2012, 2013).

1.2.4.5 Species E

Species E has one serotype, HAdV-4, which is primarily responsible for acute respiratory disease but may also cause ocular disease (Gray et al., 2000; Chang et al., 2008). It is also one of the two HAdVs for which the vaccine has

been developed (Jones et al., 2007). Infections with HAdV-E are fairly common in adult populations (Madisch et al., 2005; Colloca et al., 2012). This species accounted for more than 95% of HAdV isolates from United States of America (USA) military recruits between 1999 and 2005 (Metzgar et al., 2007). All USA military recruits currently receive a licensed live oral type -4 and -7 AdV vaccine during basic training. However, HAdV-E is less frequently detected in the civilian population (Metzgar et al., 2007). This species has been previously associated with global outbreaks of epidemic conjunctivitis in Rome in 1974, Bristol in 1978, and Chicago in 1981 (Muzzi et al., 1975; Tullo and Higgins, 1980; Levandowski and Rubenis, 1981).

1.2.4.6 Species F

Human AdV-F, composed of two serotypes HAdV-40 and HAdV-4, are one of the major causative species of AGE in children worldwide (Pereira Filho et al., 2007). These types were first identified by electron microscopy (EM) from stool specimens of infants suffering from AGE (Flewett et al., 1975). They have been classified serologically using neutralisation assays and genetically using DNA hybridisation experiments, as a group of highly related strains causing AGE (van Loon et al., 1985). This species has been called enteric AdV because of its associated with AGE and the display of gut tropism *in vivo*. The possible explanation for HAdV-F tropism is the presence of two fiber proteins and the lack of a Arg-Gly-Asp (RGD) motif in the penton base which is responsible for virion internalisation. These ligands are used by AdV to enter a host cell (Kidd et al., 1993; Yeh et al., 1994; Albinsson and Kidd, 1999). These two serotypes have been related with AGE in variable frequency (ranging from 1 to 20%)

depending on the geographic location in young children globally and type of patients (Lynch et al., 2011; Allard and Vantarakis, 2017).

1.2.4.7 Species G

Species G was discovered relatively recently from patients with diarrhoea of unknown origin (Jones et al., 2007). Species G includes only HAdV-52 and identification in clinical specimens has rarely been reported (Jones et al., 2007). There has been controversy as to whether HAdV-G52 defines a new species of HAdV or if it is a distant member of type 41 (de Jong and Osterhaus, 2008). Although HAdV-G52 has been associated with AGE, infections seem to be rare.

1.2.5 Infectious cycle

Adenovirus life cycle studies have been carried out mainly on HAdV-2 and HAdV-5, due to the ease of their *in vitro* production (Berk, 2007). The fiber knob facilitates the attachment of the virus to host cell receptor (Figure 1.4) (Khanal et al., 2018). Except for species B and species D (serotypes 8 and 37), all other HAdV subgroups use the coxsackie-AdV receptor (CAR) for entry into cells (Hall et al., 2010; Smith et al., 2010; Beatty and Curiel, 2012).

The CD46 molecule has been shown to be the cellular receptor for species B AdVs (Hall et al., 2010; Uusi-Kerttula et al., 2015). Alternative binding targets include heparan sulfate proteoglycans (HSPG), CD80, CD86, desmoglein-2, GD1a glycan, sialic acid, major histocompatibility complex (MHC) molecule and scavenging receptors (Ranki and Hemminki, 2010; Uusi-Kerttula et al., 2015).

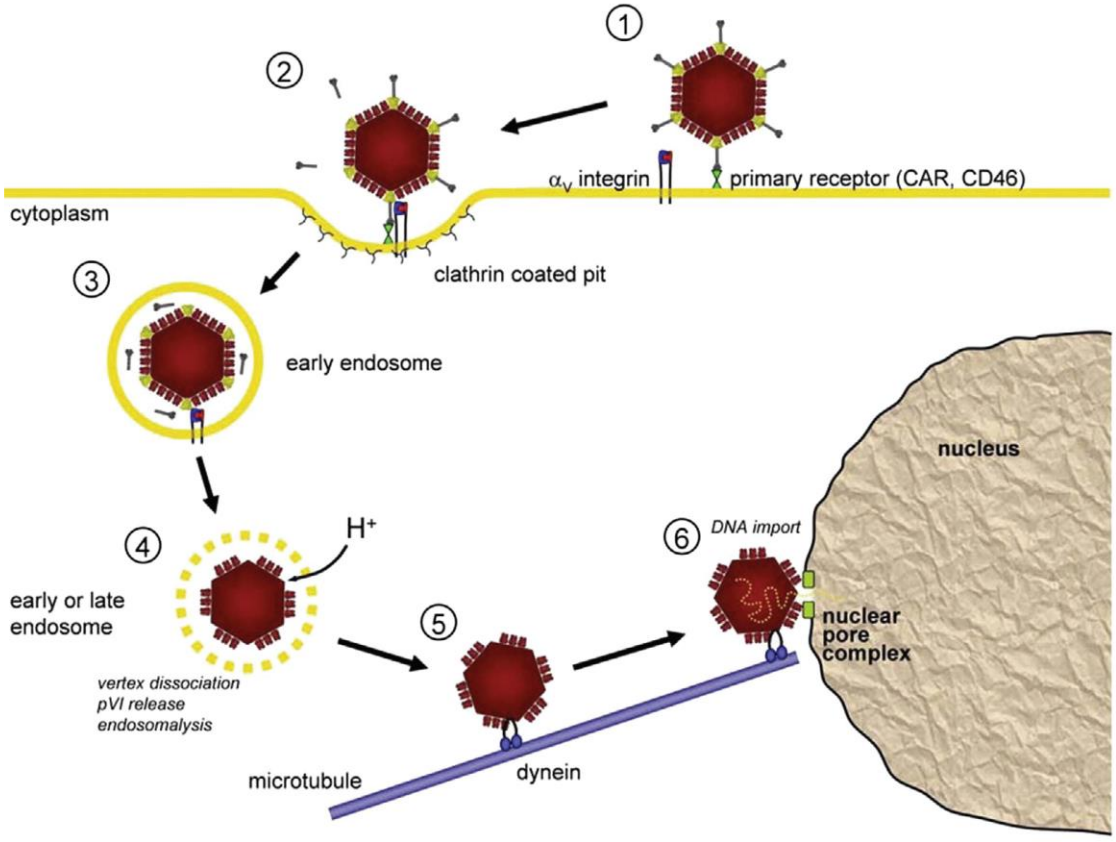

Figure 1.4. Human adenovirus attachment, internalization, intracellular transport and uncoating (Nemerow et al., 2009).

Following attachment to the cell surface, a second lower affinity interaction occurs between the penton base and the integrins, triggering internalization of the virus particles by endocytosis within clathrin coated pits (Khanal et al., 2018). Once the virus has successfully gained entry into the host cell, it begins to dissociate due to exposure to the mildly acidic conditions of the endosome, and releases the vertex proteins including pVI, which disrupt the endosomal membrane allowing the virus to escape to the cytosol (Waye and Sing, 2010). This is followed by the trafficking of virions along microtubules toward the nucleus where viral DNA is subsequently released and enters the nucleus via the nuclear pore providing material for DNA replication (Nemerow and Stewart, 2016). The AdV DNA replication cycle is divided into two stages: early phase

which involves expression of regulatory proteins and late phase which involves the production of mature virions (Waye and Sing, 2010; Robinson et al., 2011). Newly synthesized viral components are assembled and released from the cell as a result of virally induced cell lysis, to infect other cells and repeat the cycle (Harrach et al., 2012).

1.2.6. Human adenovirus disease spectrum

Human AdVs are widespread globally and are associated with a wide spectrum of disease presentations including gastroenteritis, fever, colds, pharyngitis (sore throat), conjunctivitis, bronchitis, pneumonia, acute haemorrhagic cystitis, meningoencephalitis, hepatitis, myocarditis and rarely life-threatening disseminated diseases (Tebruegge and Curtis, 2012; Lion, 2014; Cook and Radke, 2017; Khanal et al., 2018).

1.2.6.1 Respiratory tract involvement

Human AdV has long been acknowledged to be an aetiological agent of respiratory infections (Rowe at al., 1953). They have been associated with outbreaks of acute febrile respiratory illness and severe pneumonia (including several deaths) in military populations (Centers for Disease Control and Prevention [CDC], 2007; Metzgar et al., 2007). The most frequently reported HAdV serotypes associated with acute respiratory diseases in both military and civilian communities include species B1 (HAdV-3, HAdV-7, and HAdV-21), species C (HAdV-1, HAdV-2, HAdV-5, and HAdV-6) and species E (HAdV-4) (Echavarria, 2008; Allard and Vantarakis, 2017). The clinical presentations includes cough, nasal congestion, and fever, with rare progression to

pneumonia, respiratory failure, and death (Lynch and Kajon, 2016). Although community respiratory outbreaks have been reported (Lewis et al., 2009; Scott et al., 2016), HAdV respiratory infections are more common in enclosed settings such as military, hospitals, and long-term care facilities (Gerber et al., 2001; Calder et al, 2004; Hoke and Snyder, 2013). Severe respiratory disease in the very young, those with underlying lung disease, the elderly and the immunocompromised have been reported (Lynch et al., 2011). There is currently a licenced HAdV vaccine which is limited to military use US Food and Drug Administration, 2014). Human AdV accounts for 5-10% of respiratory illnesses in children and 1-7% of adult respiratory infections worldwide (Lynch and Kajon, 2016; Allard and Vantarakis, 2017). Adenoviral prevalence in children with pneumonia in SA has been reported to be 19-26% (Zampoli and Mukuddem-Sablay, 2017). Another study in SA in hospitalised children <5 years detected HAdV in 29% of children presenting with severe acute respiratory illness (Pretorius et al., 2016).

1.2.6.2 Gastrointestinal manifestations

Human AdV is a common cause of AGE in infants and young children in both low-income and high-income countries (Adhikary et al., 2011). They can cause sporadic outbreaks of AGE in the community, institutions such as childcare centres, hospitals and nursing homes (La Rosa et al., 2013; Khanal et al., 2018). The frequency of AdV diarrhoeal cases varies greatly between studies, depending on socioeconomic status or geographical location. Epidemiological data show that HAdV diarrhoea occurs worldwide and is present throughout the year with no seasonal variation (Adhikary et al., 2011).

Among the more than 50 types of HAdV, enteric AdV-F40/41 are responsible for the vast majority of HAdV diarrhoeal infections, contributing 1-20% of hospitalisations of childhood diarrhoea and 50% of all AdVs found in stool specimens (Lynch et al., 2011). Between the two serotypes of species F, HAdV-F41 may induce a longer illness duration compared to HAdV-F40. Other types of HAdV are also associated with diarrhoeal infections, such as types 12, 18 and 31 of species A; types 1, 2, 5 and 6 of species C; types 8, 9, 10, 28, 29, 30, 32, 37, 43, 46, 61, 64, 65, 67, 70 of species D and type 52 of species G (Matsushima et al., 2012; 2013; Allard and Vantarakis, 2017; Qiu et al., 2018).

1.2.6.3 Conjunctivitis

Conjunctivitis, characterised by redness, discomfort, discharge and inflammation of the thin membrane that covers the front of the eye, is the most frequent ocular disorder observed in ophthalmic clinics. Human AdVs are by far the leading cause of viral conjunctivitis worldwide (Pihos, 2013). Adenovirus conjunctivitis is associated with species D, including types 8, 19, and 37, but also with HAdVs -E4, -C5, -B3, -B7, -B11, and -B14 (Lynch et al., 2011; Allard and Vantarakis, 2017). Three types of adenoviral conjunctivitis are noted: pharyngoconjunctival fever, epidemic keratoconjunctivitis (EKC) and non-specific conjunctivitis (Lynch et al., 2011; Pihos, 2013).

1.2.6.4 Urinary tract involvement

Less frequently, HAdV may cause infections in the urinary tract, especially among hematopoietic stem cell and solid organ transplant recipients (Lynch et al., 2011). Type 11 and 21 of species B are typically associated with urinary

tract infections (Khanal et al., 2018). Adenovirus urinary tract infections can be asymptomatic or be present with dysuria, hematuria, haemorrhagic cystitis and renal allograft dysfunction (Lynch et al., 2011; Walls et al., 2014).

1.2.6.5 Disseminated disease

Although most adenoviral diseases are self-limiting, disseminated infections with multi-organ failure have been reported both in immunocompetent and immunocompromised patients (Lynch et al., 2011; Walls et al., 2014). Patients with impaired immunity including infection with human immunodeficiency virus (HIV), malignant neoplasms or bone marrow and solid organ transplant recipients are at higher risk of HAdV disseminated infections (Echavarria, 2008; Martín, 2012; Lion, 2014). Serotypes in species C and B are more commonly associated with disseminated disease (Lion et al., 2003). Human AdV-A31 has also been reported as a causative agent of dissemination disease in immunosuppressed children after allogenic haematic stem cell transplants. Adenovirus disseminated diseases, documented by a rise of AdV DNA loads in plasma, are associated with fatal outcomes, with up 80% mortality for both children and adults (Lion, 2014; Sandkovsky et al., 2014).

1.2.6.6 Human adenovirus infections in AIDS patients

Human AdV are common opportunistic pathogens in immunocompromised individuals including those with HIV/acquired immunodeficiency syndrome (AIDS) (Echavarria, 2008; Lynch et al., 2011). Adenovirus clinical syndromes that are rare in healthy individuals such as hemorrhagic cystitis, prostatitis, nephritis, hepatitis, cholecystitis, parotitis, and encephalitis are reported in

HIV/AIDS patients (Janner et al., 1990; Krilov et al., 1990; Anders et al., 1990-1991; Shintaku et al., 1993; Gelfand et al., 1994; Green et al., 1994; Schnurr et al., 1995; Hedderwick et al., 1998; Ghez et al., 2000; Dikov et al., 2005). However, these clinical manifestations have become less likely due to the availability of effective antiretroviral treatment (Lion, 2014). Human AdV infections in HIV-positive individuals are mostly associated with acute diarrhoea (Lion, 2014). Human AdV infections occur in up to 28% of human HIV-positive individuals, and approximately 45% of these infections result in death (Kolawole et al., 2014). Prolonged adenoviral shedding is common in this group, which may result in asymptomatic carriage and transmission of mutated strains and recombinant serotypes (Echavarria, 2008). Patients with HIV are the source of many of the newly described HAdV types, especially species D, suggesting a possible role of immunocompromised hosts in the genesis of new HAdV types (Echavarria, 2008).

1.2.7 Laboratory diagnosis

Clinical specimens such as nasopharyngeal, throat and conjunctival swabs, urine, cerebrospinal fluid, and stool can be used for laboratory diagnosis, depending on the syndrome being investigated (Lion, 2014).

1.2.7.1 Viral isolation

The classical method for diagnosis of HAdV infections has been virus isolation in cell culture from clinical specimens. With the exception of types 40 and 41 (species F), most HAdVs grow readily in cell culture (Ghebremedhin, 2014). The common cell lines used for isolation of AdV include A549 (lung epithelial

carcinoma), HeLa (cervical epithelial adenocarcinoma), KB (nasopharyngeal carcinoma) and HEp-2 (hepatocellular carcinoma) (Echavarria, 2008). The cytopathic effect (CPE) produced by HAdV is characterised by clumping, nuclear inclusions and netting and may take up to 21 days to manifest (Echavarria, 2008; Lynch et al., 2011; Ghebremedhin, 2014). The limiting factor using viral isolation for diagnosis is that CPE and identification takes several days and enteric HAdVF-40/41 are difficult to isolate as they do not produce clear and consistent CPE in routinely used cell cultures (Echavarria, 2008).

1.2.7.2 Serology

Serological methods can be used for indirect detection of HAdV by identifying a significant rise in AdV-specific antibodies following infection (Ghebremedhin, 2014). All HAdV types share common group-specific hexon antigens, which can be used in group-specific serological tests. Serological methods have included indirect fluorescent antibody (IFA), enzyme immunoassays (EIAs) and radioimmunoassay (RIA) (Meurman et al., 1983). Enzyme immunoassays and RIA are more sensitive and can differentiate between the various antibody classes. Enzyme immunoassays can detect both group-specific and type-specific antibodies to HAdV, while serum neutralisation and/or hemagglutination inhibition can be used for HAdV type−specific identification (Bil et al., 2008). Serologic techniques are of little value because of their low sensitivity, low specificity and false negative results during the window period as well as difficulties arising from virus variability (Bil et al., 2008).

1.2.7.3 Viral detection

1.2.7.3.1 Direct antigen detection

Direct antigen detection methods for HAdV include IFA, EIAs, immunochromatography and latex agglutination (Echavarria, 2008). Direct antigen detection from clinical specimens may be useful for acute infections and also provide rapid results, but these methods are less sensitive than molecular detection (Ghebremedhin, 2014).

1.2.7.3.2 Electron microscopy

The use of EM allows for the direct observation of HAdV particles without the need for further identification. This technique has been mainly used for identification of HAdV AGE and acute respiratory infections (Echavarria, 2008). The EM technique is rarely used in diagnostic laboratories as it requires high viral load for visualisation (Echavarria, 2008).

1.2.7.3.3 Molecular assays

Molecular detection methods, primarily the polymerase chain reaction (PCR) have become a popular method for the detection of viruses and are gradually replacing classic techniques such as cell culture and EIA. The PCR is based on the sequence-specific amplification of small genetic material, combining specificity and sensitivity in detecting a pathogen. Detection of the amplified sequence may be done at the reaction endpoint, or by continuous monitoring (real-time PCR).

The first HAdV PCR assays were introduced in the late 1990s (Allard et al., 1990, 1992; Pring-Akerblom et al., 1997; Castignolles et al., 1998) and since then, several PCR methods for HAdV have been described including conventional PCR (Avellón et al., 2001; Rohayem et al., 2004; Lu and Erdman 2006; Kuo et al., 2009), real-time PCRs using degenerate/consensus primers and probes (Lion et al, 2003; Heim et al, 2003; Miura-Ochiai et al, 2007; Buckwalter et al., 2012), or multiplexing of multiple primers and probes into one or two different reactions for the differential detection of HAdV species and types (Xu et al., 2000; Gu et al., 2003; Chmielewicz et al., 2005; Ebner et al., 2005, 2006).

The PCR assays have been successfully used for the detection of HAdV in a variety of samples including those with low viral concentration in cerebrospinal fluid and blood (Lion et al., 2003; Jalal et al., 2005; Kampmann et al., 2005; Buckwalter et al., 2012), and in environmental samples such as swimming pool water (Papapetropoulou and Vantarakis, 1998; van Heerden et al., 2005a), coastal waters (Jiang et al., 2001), river waters (Castignolles et al., 1998; van Heerden et al., 2003; 2004; 2005b; Sibanda and Okoh, 2012), sewage and shellfish (Pina et al., 1998).

The major hexon and fiber proteins are highly conserved among serotypes, and there are group- and type-specific epitopes on both hexons and fibers. Thus, many PCR assays developed for HAdV detection and identification rely on the hexon gene (Avellón et al., 2001; Heim et al., 2003; Lion et al., 2003; Rohayem et al., 2004; Sarantis et al., 2004; Shimada et al., 2004; Banik et al., 2005;

Frantzidou et al., 2005; Lu and Erdman 2006; Miura-Ochiai et al, 2007; Kuo et al., 2009) or the fiber gene (Kidd and Tiemessen 1993; Xu et al., 2000). Other genes that were found to be conserved includes sequences in the virally associated RNA I and II genes (Kidd and Tiemessen 1993), the pIX genes (Akalu et al., 1998) or the E3 gene (Dou et al., 2018).

1.2.8 Epidemiology of human adenovirus in diarrhoeal disease

Among HAdV species, HAdV-F serotypes 40 and 41 have been found to be frequent causes of diarrhoea in children. Recent studies have reported the predominance of HAdV-F41 compared to HAdV-F40 (Li et al., 2005; Shimizu et al., 2007; Lion, 2014; Zhang et al., 2016; Lu et al., 2017). This could be due to antigenic drift of HAdV-F41, resulting in the escape of this serotype from acquired immunity resulting in an increase of HAd41 infection within the community (Shimizu et al., 2004; 2007) or just regional divergence leading to a higher prevalence of HAdV-41 than HAdV-40. In contrast, some studies have still shown the predominance of HAdV-F40 (Dey et al., 2009).

Enteric AdV infections have been observed throughout the year, mostly affecting children under two years of age (Allard and Vantarakis, 2017). Predominantly spread via the faecal oral route, HAdV have an incubation period of 8 to 10 days (Wilhelmi et al., 2003; González et al., 2011; Allard and Vantarakis, 2017). Symptoms usually manifest as low grade fever, vomiting, abdominal pains, and dehydration (González et al., 2011; Allard and Vantarakis, 2017). They are highly transmissible during the first few days of acute illness; persistent infections and prolonged shedding are common, as are

asymptomatic infections and re-infections (Dennehy, 2011). A distinct feature of enteric AdV is the ability to cause prolonged diarrhoea which may contribute to infant dehydration and malnutrition especially in low-income countries (Al-Sayidi et al., 2014).

Epidemiological data from different countries show a wide variation in the prevalence pattern of enteric AdV among children <5 years of age with diarrhoea. The prevalence of enteric AdV infection ranged from 1,6% to 29.1% among analysed studies (Table 1.2). The highest prevalence has been reported from Malawi (Iturriza-Gómara et al., 2019). These prevalence rates disparities could be due to differences in study design and diagnostic methodologies.

Table 1.2. Enteric adenovirus studies done in children <5 years with diarrhoea

Reference	**Country**	**EAdV%**	**Age in Month**
Fabiana et al., 2007	Albania	9.8%	<60 months
Khan et al., 1993	Bangladesh	2.8%	<60 months
Mladenova et al., 2015	Bulgaria	7.5%	<60 months
Bonkoungou., 2013	Burkina Faso	5.0%	<60 months
Allayeh et al., 2018	Egypt	6.7%	<60 months
Lekana-Douki et al., 2015	Gabon	4.1%	<60 months
Dashti et al., 2016	Iran	5.18%	<60 months
Shimizu et al 2007	Japan	8.0%	<60 months
Iturriza-Gómara et al., 2019	Malawi	29.1%	<60 months
Nhampossa et al., 2015	Mozambique	1.9%	<60 months
Aminu et al., 2007	Northern Nigeria	8.0%	<60 months
Kabayiza et al., 2014	Rwandan	6.4%	<60 months
Kidd et al., 1986	South Africa	6.5%	<60 months
Moore et al, 1998	South Africa	3.4%	<60 months
Marx et al., 1998	South Africa	3.7%	<60 months
Rossuow, 2004	South Africa	1.6%	<60 months
Moyo et al., 2014	Tanzania	1.8%	<24 months
Fodha et al., 2006	Tunisia	3.0%	<60 months

Molecular-based assays are now used widely and have demonstrated that traditional detection methods underestimate the enteric AdV-F burden (Liu et al., 2016; Platts-Mills et al., 2018). When stool specimens from the original GEMS study were re-analysed using PCR, the AdV-F incidence was five times higher than previously reported, shifting this enteropathogen to become among the top six diarrhoea attributable pathogens (Kotloff et al., 2013; Liu et al., 2016). Similar trends were observed in the Malnutrition and Enteric Disease Study (MAL-ED) study which was a longitudinal community-based birth cohort study that analysed pathogen-specific diarrheal burden in children <2 years in eight sites in Africa, Latin America and Asia. In this latter study enteric AdV-F was responsible for a considerable proportion of diarrhoea, predominantly found in the first year of life (Platts-Mills et al., 2018).

1.2.8.1 Transmission of human adenovirus

Human AdV can be transmitted via inhalation of aerosolised droplets or direct conjunctival inoculation, faecal-oral spread, including contact with recreational, freshwater or tap water, exposure to infected tissue or blood, airflow filters, or environmental surfaces (La Rosa et al., 2013; Lion, 2014; Khanal et al., 2018). Faecal-oral transmission is the most common route of spread because of high viral loads in stool of infected individuals compared to other clinical specimens (Jeulin et al., 2011). Asymptomatic carriers can shed the virus for weeks or months (Echavarria, 2008; Hoffman, 2009; Lynch et al., 2011).

Crowding and poor hygiene may facilitate further spread of HAdV. Individuals who are in close living quarters such as hospitals, neonatal nurseries,

psychiatric or long term care facilities, job training centers, boarding schools or dormitories, children's homes and orphanages, are at greater risk of HAdV infections (Lynch et al., 2011). Even though HAdVs rarely causes nosocomial infections, outbreaks of HAdV in haematology or transplant wards resulting in closures have been reported (Jalal et al., 2005; Leruez-Ville et al., 2006; Mattner et al., 2008; CDC, 2013; Lion, 2014).

1.2.8.2 Stability and inactivation

Human AdV is stable and can survive for several weeks in the environment (Khanal et al., 2018). They can maintain their infectivity at room temperature for up to 3 weeks (Echavarria, 2008; Lion, 2014). They are stable over the potential of hydrogen (pH) range 4-8, and therefore are able to survive in the acidic environment of the stomach and gastrointestinal tract. In transplant intensive care units, efficient decontamination of surfaces is important to prevent transmission to immunocompromised patients (Lion, 2014).

Physically, HAdV can be de-activated by heating to 56°C for 30 min or 60°C for 2 min. Autoclaving for 30 minutes at 121°C in laboratory environments can deactivate HAdV. In homes, surface disinfection with 10% sodium hypochlorite (bleach) can be used to de-activate HAdV. Wiping down of stainless steel with 70% ethanol after bleach is recommended to avoid corrosion. Sodium hypochlorite (final concentration 10%) can also be used to de-activate AdV in liquid waste by exposing for 15 min before disposing into sink (Allard and Vantarakis, 2017).

Preventing HAdV infections is based on common practices of good hygiene, including handwashing with soap and water, especially after contact with a person with any kind of flu-like illness, covering one's mouth and nose when coughing or sneezing, avoiding touching one's eyes, nose, or mouth with unwashed hands, avoiding close contact with people who are sick and staying home when people are sick themselves (Khanal et al., 2018).

1.2.8.3 Treatment of HAdV

Currently, no specific antiviral treatment against HAdV infection is available. Treatment of HAdV-associated infections includes supportive measures (Tebruegge and Curtis, 2012). Most HAdV infections are mild and require no therapy, but treatment may be required in immunocompromised patients, keratoconjunctivitis and paediatric lower respiratory tract infections. Successful therapy of adenoviral infections with cidofovir has been reported, while drugs such ganciclovir, ribavirin, vidarabine and foscarnet have failed to show consistent anti-viral activity *in vitro* (Khanal et al., 2018).

Diarrhoeal disease caused by HAdV in healthy individuals normally require no treatment, and in most cases resolves spontaneously without the need for additional interventions. Young children and the elderly may require hospitalisation with intravenous fluids administration to correct consequences of diarrhoea (Uhnoo et al., 1984). Oral rehydration therapy can be effective in the treatment of diarrhoea in children (Elliot, 2007).

Previously available HAdV vaccines against types 4 and 7 were discontinued in 1996 (Lion, 2014). However, the US Food and Drug Administration in March 2011 approved a new live, oral vaccine against HAdV type 4 and 7 for USA military personnel age from 17 to 50 years old (Allard and Vantarakis, 2017). The vaccine is currently not available to the general population to prevent acute respiratory disease (Liu et al., 2018). Immunotherapy such as intravenous immunoglobulin, adenovirus-specific T cells, and donor lymphocytes infusions have been used as an alternative treatment of HAdV infections (Leen et al., 2006; Lenaerts and Naesens, 2006; Lindemans et al., 2011).

1.3 HUMAN BOCAVIRUS

1.3.1 History

Human bocavirus was first described in 2005 following its detection from pools of nasopharyngeal aspirates obtained from individuals with respiratory tract infections (Allander et al., 2005). It was discovered using a molecular virus-screening technique based on random amplification, cloning, sequencing and phylogenetic analysis of two chronologically distinct pools of nasopharyngal aspirates (Malecki et al., 2011). Phylogenetic analyses of the complete genome of HBoV revealed that the virus is most closely related to the minute virus of canine (MVC) and bovine parvovirus (BPV), two related members of *Parvoviridae* family, subfamily *Parvovirinae*, genus *Bocaparvovirus* (Guido et al., 2016). The name bocavirus was derived from bovine (‘’Bo’’) and canine (‘’ca’’) parvoviruses (Cotmore et al., 2014; Guido et al., 2016). Minute virus of canine and BPV are known to cause gastrointestinal infections in dogs and

calves, respectively. Since the first detection of human bocavirus 1 (HBoV-1) in 2005, three additional HBoV species, namely HBoV-2, HBoV-3 and HBoV-4, have been described (Arthur et al., 2009; Kapoor et al., 2009, 2010). Human BoV2 was detected in the stool of children with non-polio acute flaccid paralysis in Pakistan (Arthur et al., 2009), HBoV-3 in stool of Australian children with diarrhoea (Kapoor et al., 2009) and HBoV-4 in stool samples from Nigeria, Tunisia, and USA (Kapoor et al., 2010).

1.3.2 Classification

Human bocavirus belongs to the family *Parvoviridae* (Cotmore et al., 2014; Guido et al., 2016). This family is divided into two subfamilies Parvovirinae and Densovirinae (Figure 1.5) (Cotmore et al., 2014). The *Parvovirinae* infects vertebrates and is subdivided into eight genera; namely *Amdoparvovirus, Aveparvovirus, Bocaparvovirus, Copiparvovirus, Dependoparvovirus, Erythroparvovirus, Protoparvovirus* and *Tetraparvovirus* (Figure 1.5) (Guido et al., 2016; Qiu et al., 2017). These genera can be distinguished by their genetic diversity, host range, and pathogenicity (Cotmore et al., 2014, Kailasan et al., 2015). All viruses in a genus are usually monophyletic and encode NS1 proteins that are generally <30% identical to each other at the amino acid sequence level but >30% identical to those of other genera as determined by pairwise alignments (Cotmore et al., 2014). The International Committee for Taxonomy of Viruses (ICTV) recently renamed the genus *Bocavirus* to *Bocaparvovirus* (Cotmore et al., 2014). Different species of the genus *Bocaparvovirus* should show amino acid similarity in the NS1 gene of at least 85% while diverging by 15% from viruses in other species (Cotmore et al., 2014).

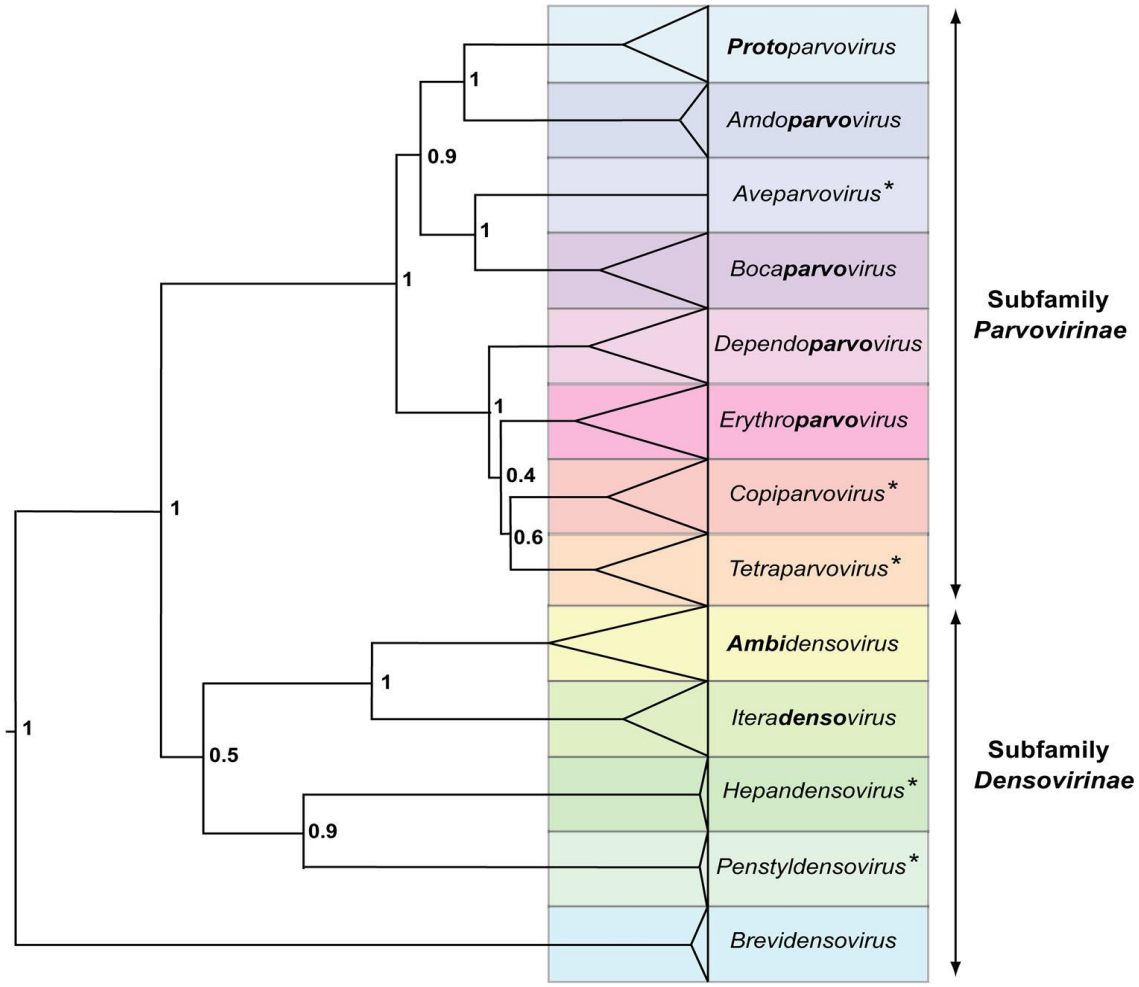

Figure 1.5: Phylogenetic tree showing genera in the family *Parvoviridae* (Cotmore et al., 2014). The Bayesian tree is based on the amino acid sequence of the viral replication initiator protein, NS1, and is displayed in the ultrametric format on an arbitrary scale, with posterior probability scores indicated at statistically significant nodes

The genus *Bocaparvovirus* includes human bocaparvovirus, chimpanzee and gorilla bocavirus (GBoV), and Erythroparvovirus encompasses human parvovirus (B19), simian parvovirus (SPV), pig-tailed macaque parvovirus (PtMPV), and rhesus macaque parvovirus (RhMPV) (O'Sullivan et al., 1994; Brown et al., 1995; Kapoor et al., 2010). Based on genetic variability of viral capsid protein 1 (VP1) region, HBoV are divided into 4 species: HBoV-1 to HBoV-4 (Jartti et al., 2012, Ong et al., 2016).

1.3.3 Structure of human bocavirus

1.3.3.1 Human bocavirus virion

Parvoviruses are among the smallest viruses known. The structure of HBoV-1 is typical for *Parvovirinae,* it is a non-enveloped virus with icosahedral symmetry, a diameter of 18-26 nm and a capsid formed by approximately 60 capsomeres (Lüsebrink et al., 2009; Guido et al., 2016). Although the capsid structure of HBoV is a mixture of the features of members of *Parvoviridae* family, it is most closely related to parvovirus B19. The icosahedral capsid is composed of proteins VP2 and VP1 in a ratio of 95% and 5%, respectively (Gurda et al., 2010; Gallinella, 2018). This capsid protein has a highly conserved secondary structure with core elements that are shared even between parvovirus genera while the variable surface loops confer host specific tropism and diverse antigenic properties. The virus has no lipid envelope, which makes it resistant to most of the antiviral procedures such as solvent and heat treatment (Heegaard and Brown, 2002).

1.3.3.2 Genome and proteins

The genome of HBoV is closely related to the BPV and MVC, both of which are members of the genus *Bocaparvovirus*, family *Parvoviridae.* The viral genome is non-segmented, composed of a single-stranded DNA of both positive and negative sense and is approximately 5.3 Kb with hairpin at both ends which are necessary for viral genome replication (Schildgen, 2013). The centre of the viral genome contains a small open reading frame (ORF) which encodes two forms of the nonstructural protein (NS1), nuclear phosphoprotein (NP1) and viral capsid proteins (VP1 and VP2) (Guido et al., 2016). Except for an additional

amino terminal domain of 129 amino acids in VP1, VP1 and VP2 have identical sequences (Allander et al., 2005). Unlike other members of the family *Parvoviridae*, the NP1 is unique to the genus *Bocaparvovirus* and its function is unknown (Figure 1.6) (Allander et al., 2005; Lüsebrink et al., 2009; Schildgen, 2013).

The first ORF encodes for a NS1, a multifunctional protein with the most conserved sequence. Open reading frame 1 is essential for transcription, DNA replication of the viral single-stranded DNA genome and packaging (Malecki et al., 2011). The second ORF encodes for VP1 and VP2, which overlap in the genome (Malecki et al., 2011). The major antigen of HBoV is localised on the VP2 protein and can form empty virus-like particles (VLPs). The third ORF encodes for a non-structural protein, NP1, and shares 47% amino acid identity to NP1 of MCV and BPV (Malecki et al., 2011). However, NP1 is believed to play a role in virus replication, viral RNA processing, arrest of host cell cycles, and induction of apoptotic cell death and disruption of interferon (IFN) responses (Guido et al., 2016). Compared to VP1 and VP2, the NS1 and NP1 genes are the most conserved areas with the genetic variability of known HBoV strains being low (Allander et al., 2005). Phylogenetic analysis indicates the possibility that HBoV-3 may have originated from a recombination event between HBoV-1 and HBoV-2, whereas HBoV-4 may have originated from recombination between HBoV-3 and HBoV-2. The non-coding regions in the genomic termini contain palindromic sequences, commonly known as inverted terminals, which play an important role in the viral replication (Babkin et al., 2013).

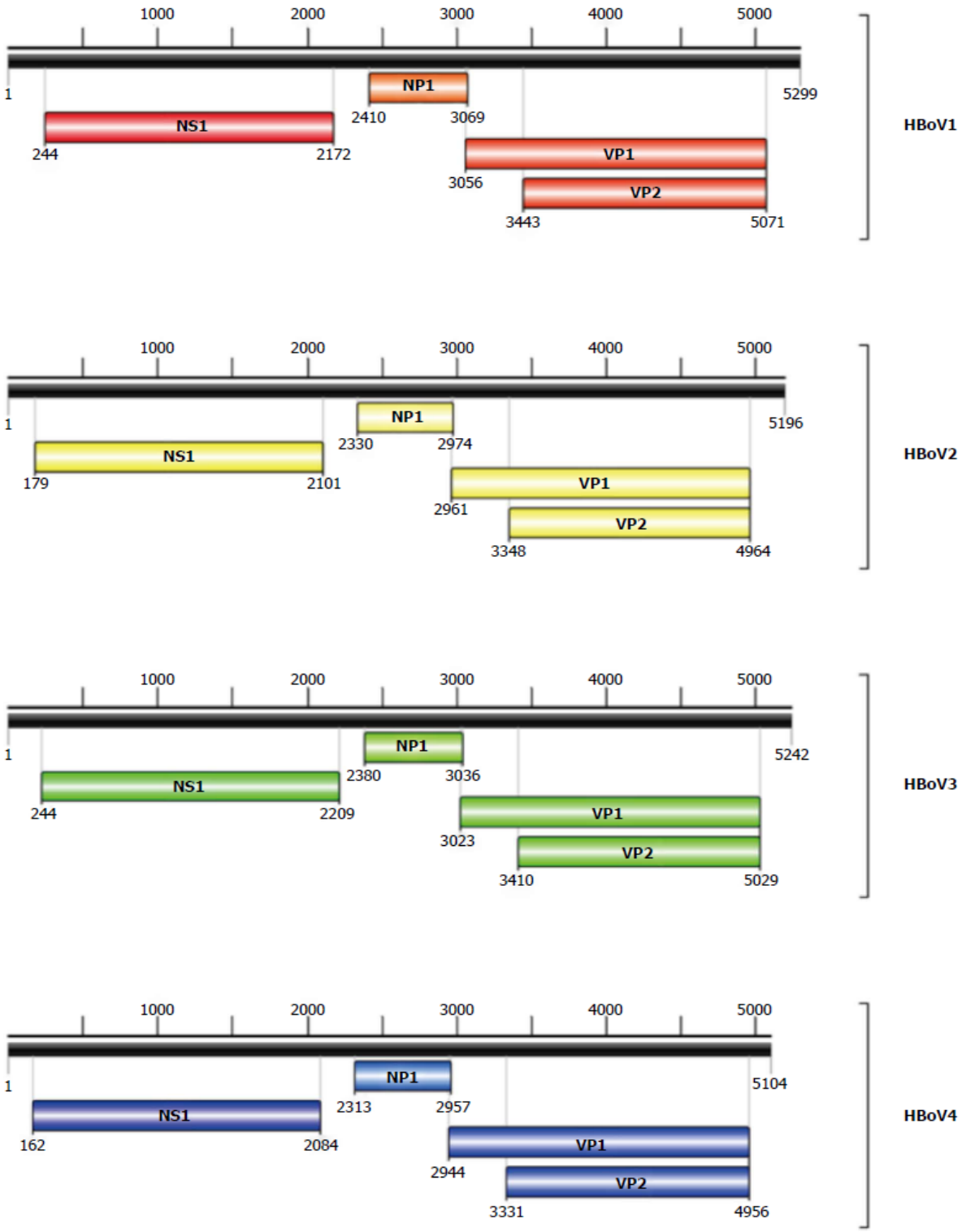

Figure 1.6. The genome of human bocavirus with major open reading frames and proteins. Viral (structural) protein (VP); nonstructural protein (ns); nuclear phosphoprotein (NP)(Guido et al., 2016).

1.3.4 Epidemiology of human bocavirus

Since its initial detection in human respiratory tract specimens in 2005, HBoVs are widely distributed and have been reported to cause various clinical manifestations including gastroenteritis and respiratory tract infections (Jartti et al., 2011; Schildgen, 2013). It is frequently found in children younger than two years old with low prevalence among the children younger than six months, predominantly during the winter season (Allander et al., 2005; Allander, 2008). Although primarily reported in young children, a few reports have described HBoV infections in adults (Kupfer et al., 2006; Longtin et al., 2008). Human bocavirus infections occur all year, and is more likely to be detected in winter and spring (Guido et al., 2016).

Serologic studies indicate that approximately 40% of children between 18 and 23 months have been infected with HBoV, with prevalence increasing to 100% by seven years of age. On average, 76% of children and 96% of adults are seropositive for HBoV, indicating a high rate of exposure to the virus (Schildgen, 2013). In infants under two months, seropositivity is common due to vertical antibody transfer. The seroprevalance is highest for HBoV-1 (66.6-96%), followed by HBoV-2 (34-49.3%), HBoV3 (10-38.7%) and the seroprevelance for HBoV-4 was found to be much lower (0.8-5%) among 0-70 years old from Beijing, China and Pakistan (Schildgen, 2013).

Human bocavirus type 1 was originally discovered in hospitalised children with a respiratory tract infection and has since been linked with respiratory diseases (Jartti et al., 2011). They can be detected throughout the year, but are most

common in winter and spring months (Peltola et al., 2013; Guido et al., 2016). Human bocavirus type 1 is common in young children aged 6-24 months, and detection in the respiratory specimens of immunocompetent adults and the elderly is still rare. The clinical symptoms associated with HBoV-1 includes common cold-like complaints, wheezing, bronchiolitis and pneumonia (Jartti et al., 2012). Similar to HBoV, the detection of HBoV-1 is commonly in winter and spring (Jartti et al., 2012).

The prevalence rate of HBoV-1 in respiratory tract secretions is approximately 2-19% in children in North and South America, Africa, Asia, Europe, and Australia (Bastien et al., 2006; Allander et al., 2007; Fry et al., 2007; Jartti et al., 2012). Approximately 90% of HBoV-1 cases occur with other pathogens present, which complicates the description of the role of HBoV-1 in respiratory infections.

While HBoV-1 has been associated with respiratory disease, HBoV-2 to HBoV-4 are mainly detected in enteric infections and are considered enteric viruses (Guido et al., 2016). The definite causal relation between HBoVs and AGE remains to be established. Although considered enteric, HBoV2-4 are not entirely absent from respiratory specimens, HBoV-2 was reported in 4%, 2%, and 0.5% of specimens from children with respiratory tract disease from China, Korea and Japan, respectively (Han et al., 2009; Song et al., 2010; Koseki et al., 2012). Several studies worldwide reported the detection of HBoV in faecal samples of patients with AGE infections including Spain, Germany, Hong Kong, Korea, China, Japan, and Brazil (Ricour and Goubau, 2008; Pham et al., 2011;

Noorbakhsh et al., 2013). The prevalence of HBoV-2 has been reported in approximately 0.8-9.1% of children with diarrhoeal disease. Based on the number of studies globally, HBoV-2 is the most prevalent with detection rate of approximately 26%, followed by HBoV-3 (5%), and HBoV-4 (2%) (Jartti et al., 2011). The detection of HBoV-2 have been found throughout the year without a clear seasonal variation (Xu et al., 2011). Like HBoV-1, HBoV-2 to -4 has been frequently detected simultaneously with other viral pathogens, thus, it has been challenging to demonstrate the clinical importance of HBoV detection in AGE (Lüsebrink et al., 2009).

1.3.5 Transmission

Because HBoV is a newly discovered virus, the transmission of this virus remains poorly understood. It is assumed that it is similar to other parvoviruses with transmission by inhalation or contact of sputum, faeces, or urine (Guido et al., 2016). In addition, HBoV has also been detected in blood and environmental samples such river water and sewage (Guido et al., 2016). Because of a high level of immunity among pregnant woman, intrauterine infection is uncommon (Jartti et al., 2011).

1.3.6 Human bocavirus genotypes and disease spectrum

1.3.6.1 Human Bocavirus 1

Human bocavirus type 1 is predominantly detected in the upper airways of young children with respiratory tract infections (Kesebir et al., 2006; Allander et al., 2007; Fry et al., 2007; Jartti et al., 2012; Paloniemi et al., 2014; Martin et al., 2015; Guido et al., 2016). This genotype have also been identified in children

with AGE. Several additional studies conducted across the world showed that HBoV is truly a respiratory pathogen. Human bocavirus type 1 DNA has been detected in the respiratory specimens of completely asymptomatic individuals and individuals with non-respiratory symptoms. Co-infection with other respiratory viruses (34.6-90%) is common and detection in asymptomatic children has resulted in questioning the role of HBoV-1 as a respiratory pathogen. However, high viral load, viremia and detection of HBoV-1 alone in respiratory infections in children support HBoV as a respiratory pathogens (Christensen et al., 2010). Human BoV-1 has been detected in sporadic cases associated with life threatening respiratory infection in children, particularly children aged 6-24 months (Edner et al., 2012; Ursic et al., 2011; Korner et al., 2011; Pekcan et al., 2014; Guido, 2016). Data are scarce on the occurrence of HBoV-1 infection among the elderly, however, a HBoV-1 case was reported in a 74 years old man requiring intensive care (Krakau et al., 2015).

It has been suggested HBoV-1 may also play a role in gastroenteritis because diarrhoea was common in patients with high HBoV-1 viral loads in respiratory specimens compared to those with lower viral load (Proença-Modena et al., 2011). However, other studies showed no association between high HBoV-1 viral load in respiratory specimens and diarrhoea (Kim et al., 2011). The presence of HBoV-1 DNA in stool and urine specimens may reflect swallowed virus or virus filtered from the blood in the kidneys, rather than active shedding in either secretory pathway. This passive spread of HBoV-1 from the respiratory to the gastrointestinal tract is supported by the consistently low levels of viral DNA in stool (Yu et al., 2008).

1.3.6.2 Human Bocavirus 2

Since its discovery in stool specimens in 2009, HBoV-2 have been detected in 1.4-24% of children with AGE. Co-infection of HBoV-2 with other enteric pathogens has been reported in 0.2-100% of cases (Arthur et al., 2009; Jin et al., 2011; Nawaz et al., 2012). It has also been detected in 0.6%-4.3% of respiratory specimens from children with acute respiratory infections (ARTI) and in stool specimens of children with ARTI (Han et al., 2009; Shan et al., 2009; Song et al., 2010; Koseki et al., 2012). Although the exact role of HBoV2 needs to be further investigated, HBoV has also been detected in cerebrospinal fluid (CSF) of children with encephalitis, demonstrating that it is capable of crossing blood brain barrier (Mitui et al., 2012). A fatal subacute myocarditis case associated with HBoV-2 in a 13 month old baby has been previously reported (Brebion et al., 2014). The detection of HBoV-2 occurs throughout the year and it is the second most prevalent after HBoV-1 (Peltola et al., 2013). Similar to HBoV1, the prevalence of HBoV-2 appears to be relatively common among young children.

1.3.6.3 Human Bocavirus 3

It is the third most common type of HBoVs and it was discovered during the screening of stool specimens for HBoV-2 (Arthur et al., 2009). The prevalence of HBoV-3 in stool specimens varies between 0.5-2.7% and the co-infection rate of HBoV-3 varies from 50-100% (Jin et al., 2011; Khamrin et al., 2012; Wang et al., 2014). With the exception of 0.4% prevalence rate reported in Japan, detection of HBoV-3 in respiratory specimens are rare (Koseki et al., 2012).

1.3.6.4 Human Bocavirus 4

It is the least common HBoV type, which was discovered in stool in 2010. In comparison to HBoV1-3, HBoV-4 DNA detection appears to be rare in stool specimens. The reported prevalence rate of HBoV-4 in healthy children under 14 years is 0.8% and in healthy adults is 1.4% (Guo et al., 2012). Human bocavirus type 4 has also been detected in 0.6% of respiratory samples of children with ARTI (Koseki et al., 2012).

1.3.6.5 HBoV in the immunocompromised

Even though HBoVs have been detected in immunocompromised individuals, prevalence and potential pathogenicity of HBoV in these patients remains poorly understood and poorly investigated (Allander, 2007; de Vries et al., 2009).

1.3.7 Pathogenesis

Owing to the lack of permissive cell lines for virus propagation, limited information is available on the pathogenesis of HBoV (Guido et al., 2016). Previous attempts with other common cell lines to grow HBoV such as Hep-2, Vero, and MRC-5 were unsuccessful mainly due to lack of expression of a cellular receptor (Guido et al., 2016). The pathogenic mechanism of only HBoV-1 infection has been characterised (Huang et al., 2012). Of the few HBoV-1 *in vitro* models, cultivation that has been developed for differentiated human airway epithelial (HAE) cells and a reverse genetics system using a plasmid clone of HBoV-1 and human embryonic kidney 293 cells (Dijkman et al., 2009; Huang et al., 2012). In these models, HBoV-1 infection causes disruption of the

tight junction barrier, epithelial cell hypertrophy, and the loss of cilia, indicating signs of respiratory tract damage.

1.3.8 Diagnosis of human bocavirus

Human BoV has been found in variety of specimens, including serum, faeces, urine, saliva, and cerebrospinal fluid (Pozo et al., 2007, Chieochansin et al., 2008, Tozer et al., 2009, Söderlund-Venermo et al., 2009, Martin et al., 2009, Kapoor et al., 2010, Wang et al., 2010, Christensen et al., 2010, Mitui et al., 2012).

1.3.8.1 Viral isolation

Similar to many newly discovered viruses, HBoV is difficult to cultivate *in vitro* (McIntosh, 2006). The delay in the discovery was in part due to the difficulty of isolation in virus culture. Detection of HBoV has been exclusively done by probing for the presence of the viral genome (Mahony, 2008).

1.3.8.2 Election microscopy

Although it has been impractical for routine diagnostic, EM is the most valuable technique in the study of virus particle structure. The structural characteristics of HBoV resemble that of other members of the *Parvoviridae* family (Figure 1.7)(Brieu et al., 2007).

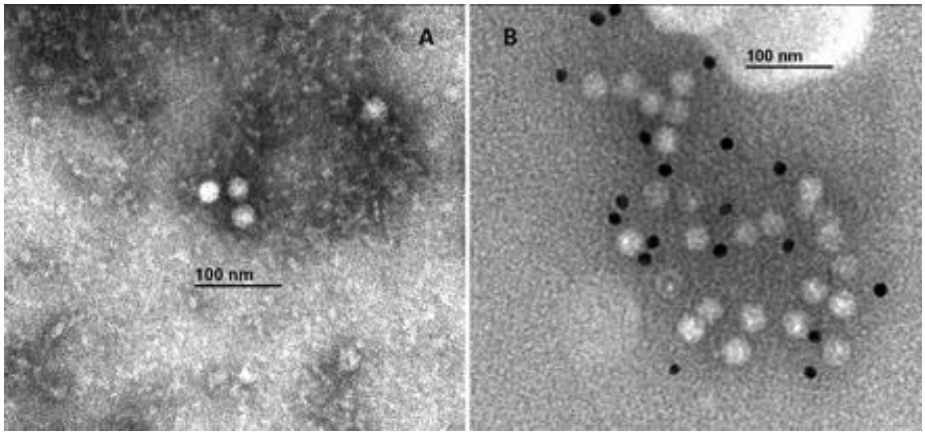

Figure 1.7. Electron microscopy image of HBoV in a direct preparation from a nasopharyngeal swab suspension (Ursic et al., 2011).

1.3.8.3 Molecular detection of human bocavirus

Advances in molecular biology have greatly improved the detection of viral pathogens (Sloots et al., 2008). Molecular methods led to the discovery of HBoV as a potential pathogen in humans (Allander et al., 2005). The routine laboratory diagnostics of HBoV infections almost exclusively relies on PCR systems targeting the VP1/2, NS1 and NP1 genes (Peltola et al., 2013; Guido et al., 2016). There are several reports of detection of HBoV by PCR. Therefore, most of the epidemiological studies have been done using the NS1, NP1 and VP1/2 gene for HBoV. Human bocavirus can be divided into genogroups based on the variations in VP1 and VP2 genes (Smuts and Hardie, 2006). The NS1 and NP1 genes have been reported to be the most conserved regions, and cannot detect differences between HBoVs, and therefore can only be utilised for HBoV detection (Chieochansin et al., 2007). Currently the detection of HBoV has also been included in a number of commercially available approved multiplex PCR panels for the detection of respiratory viruses (Guido et al., 2016). Multiplex PCR for the detection of different types of HBoV have also been reported (Guido et al., 2016). The frequent detection of HBoV in

asymptomatic persons has made the sole use of this method problematic (Bonvicini et al., 2011; Li et al., 2015). Acute infection is generally associated with a high HBoV DNA load (>10,000 copies/ml) in the nasopharynx and viremia (Söderlund-Venermo et al., 2009).

1.3.8.4 Serology

Serological diagnosis is important in determining whether the infection is acute or past. These tests can be used for indirect detection of HBoV via detection of antibodies to HBoV by utilising recombinant capsid antigens or VLPs from HBoV VP1 or VP2 protein to detect HBoV specific IgM and IgG antibodies (Lüsebrink et al., 2009). Synthesised VLPs can be used as the antigenic component for an enzyme-linked immunosorbent assay (ELISA)-based serologic assay (Endo et al., 2007; Kantola et al., 2008; 2011; Lindner and Modrow, 2008; Guo et al., 2012; Jartti et al., 2012; Fang et al., 2014). The use of serodiagnostics for HBoVs has been complicated by the cross-reactivity of past-immunity antibodies toward HBoV-2 to HBoV-4 with HBoV-1 (Kantola et al., 2011; Fang et al., 2014).

1.3.9 Treatment of human bocavirus

Human bocavirus disease is mostly self-limiting in healthy children, and the majority may be cared for at home. There are no specific treatment for HBoV infections and no vaccine is available for the prevention of disease in humans. Therefore, the treatment of neonatal HBoV-induced infection are still primarily based on supportive care. Preventing HBoV infections is based on common practices of good hygiene, which are also recommended for other

gastrointestinal pathogens. Owing the lack of cultivability, and a high rate of co-infection with other pathogens, a specific treatment for HBoV might not be available in the near future (Milder and Arnold, 2009).

1.3.10 Human bocavirus in Africa

The detection of HBoV in respiratory specimens has been reported in many African countries including Kenya (Symekher et al., 2013), Malawi (Carrol et al., 2011), Nigeria (Akinloye et al., 2011), Egypt (Shafik et al., 2012), Cameroon (Kenmoe et al., 2017) and Senegal (Fall et al., 2017). In SA, the prevalence of HBoV was 11% in children <2 years diagnosed with pneumonia (Smuts and Hardie, 2006). Subsequently, HBoV was also detected in 9.5% of HIV-infected and 13.3% of HIV-uninfected children hospitalised with respiratory infections (Nunes et al., 2014). Another study demonstrated the presence of HBoV in 11.5% of children ≥ 3 months to <5 years of age with acute otitis media (Madhi et al., 2015).

Though many studies have identified HBoV as a potential cause of AGE in infants and children, there are few recent studies providing valuable data regarding regional epidemiology of HBoV in children with AGE. In the few studies conducted in Africa, EL-Mosallamy et al., (2015) reported an HBoV infection rate of 2% in children (1 month to 2 years) with AGE from Egypt. This prevalence rate was somewhat comparable to the 2.2% in Gabon among children under 5 years of age with diarrhoea (Lekana-Douki et al., 2018). Although variable rates of HBoV infections and their genotypic distribution in AGE cases have been reported worldwide, no such reports are available from

SA. More data are needed to gain better insight into the epidemiology of HBoV in Africa.

1.4 PROBLEM STATEMENT

In low income-countries, AGE is a major source of morbidity and mortality among young children. Since RVs are the most frequently identified aetiological agents of AGE in infants and children worldwide, past investigations of enteric viral infections of AGE have focused on the epidemiological control of RV (Knox et al., 2012). Thus, RV has received much attention during the last decade, which has led to the development and licencing of two RV vaccines. Less attention has been given to other gastroenteritis viruses. Studies have reported that after the introduction of RV vaccines, there has been a dramatic reduction in RV hospital admissions in countries that have included the vaccine in their national immunisation programs (Desai et al., 2012; Msingmang et al., 2013). In 2009, RV vaccine was included in the national immunisation program in SA (Steele and Glass, 2011; Knox et al., 2012). According to The District Health Barometer survey, diarrhoeal diseases accounted for 2.2% of deaths in children under 5 years of age in 2015/2016 in SA (Massyn et al., 2016).

Human AdV is a common enteric pathogen that can cause diarrhoea in children. With the use of molecular assays such PCR, the detection rates of AdV in diarrhoeal cases have increased (Liu et al., 2016). This has prompted renewed interest in study of the epidemiology of HAdV infections. Previous studies on HAdV in children with diarrhoea in SA have been either small,

conducted in selected populations, done before the era of PCR diagnostics, or done before the introduction of RV vaccine that may contribute substantially to the prevalence rate of this virus.

The limitations extend beyond the epidemiology of HAdV and detailed information on viral genotypes and phylogenetic distribution is also lacking in SA. In addition, due to the short fragment of hexon region used in the Magwalivha and colleagues study, results obtained could not be compared with existing recombinant AdVs and intermediate strains could not be clearly identified (Magwalivha, 2009). While most phylogenetic studies of AdV are based upon a small segment of the viral genes, full-length sequences of these enteric viruses from middle-income countries like SA will provide different insights on the classification and characterisation of these viruses and could result in identification of novel genogroups or genotypes.

New viruses such as HBoV have been discovered as a result of improved diagnostic tests as possible etiological agent of gastroenteritis and its epidemiology has been investigated in many countries across the globe. There is limited data on the prevalence of HBoV among patients presenting with AGE in Africa. Data on the distribution of enteric viruses among patients with AGE is important to guide appropriate case management and inform preventive strategies. Thus, studies are needed to determine what fraction of the human population has been infected with this novel virus and when initial exposure occurs.

1.5 AIM OF THE STUDY

The aim of this study is to provide updated epidemiology, genetic diversity and dynamics of circulation of human enteric DNA viruses in hospitalised children with AGE in SA.

1.6 OBJECTIVES

1. To determine the epidemiology of enteric DNA viruses including prevalence, age-related distribution, seasonal patterns and clinical features in children hospitalised with AGE in SA.
2. To investigate the molecular epidemiology of South African HAdV strains detected from paediatric patients with AGE.
3. To characterise genotypes of HBoV and to describe the distribution of these genotypes.
4. To perform phylogenetic analyses on HBoV strains
5. To study the full-length genome of selected enteric HAdV and HBoV detected in SA, by next generation sequencing and phylogenetic analysis.
6. To develop or optimise species-specific multiplex PCR method based on hexon, penton and fiber genes for the identification of enteric HAdV species circulating in SA.
7. To develop or optimise species-specific multiplex PCR method based on NS1 and NP genes for the identification of the HBoV species, which are circulating in SA.

CHAPTER 2

EPIDEMIOLOGY OF HUMAN ADENOVIRUS INFECTIONS IN CHILDREN HOSPITALISED WITH ACUTE GASTROENTERITIS IN SOUTH AFRICA, 2009 TO 2014

The Editorial Style of the journal "Diagnostic Microbiology and Infectious Disease" was followed in this Chapter. Aspects of Objective 1, 2 and 6 are addressed in this Chapter.

Abstract

Human adenovirus (HAdVs) are associated with acute gastroenteritis (AGE). The objective of this study was to describe the epidemiology and clinical features of HAdV among children with AGE in South Africa (SA). From April 2009 to December 2014, clinical, demographic and environmental data, and stool specimens were collected from children <5 years hospitalised for AGE from four sentinel surveillance sites in South Africa. Human adenovirus DNA was detected by PCR followed by genotyping using multiplex PCR. Analysis for other enteric viruses, enteropathogenic bacteria and parasites was also performed. Clinical and demographic data were collected. Human adenovirus was detected in 18.1% (656/3623) of the stool specimens; 62.3% of the HAdV-positive children were 7–24 months of age. Human adenoviruses were detected year round although slightly less frequent between July and September. Co-infections were found in 76.3% (222/291) cases of the HAdV-positive specimens with full enteric screening; human astrovirus was detected most frequently as a co-infecting pathogen. No significant difference in clinical presentation was found between children with HAdV single infection and co-infections. Human adenovirus F was

the most common species identified (254/603, 42.1%). The clinical characteristics did not differ among the HAdV species detected. Overall HAdV detection in children with AGE in SA has increased in recent years with enteric HAdV-F remaining predominant. However the HAdV-F prevalence remained similar to previous reports in SA. A distinct distribution pattern for HAdV-F40 and HAdV-F41 was observed. This is the main HAdV species associated with human enteric infections worldwide and is therefore of public health importance.

Keywords: Human adenovirus; South Africa, epidemiology; prevalence; acute gastroenteritis; risk factors

2.1 Introduction

Acute gastroenteritis (AGE) is a major public health problem in low-income countries and is characterised by high mortality and morbidity, particularly among children younger than five years of age (Iturriza Gómara et al., 2008; Desselberger, 2014; Malik and Matthijnssens, 2014; Naghavi et al., 2017; Troeger et al., 2017). Nearly 90% of AGE deaths in children under five years are in low-income countries (Naghavi et al., 2017). The aetiologies of AGE in children includes bacteria, parasites and viruses (Dennehy, 2011). Viruses are widely acknowledged to be the most common cause of AGE in children (Sidoti et al., 2015). According to the multisite birth cohort study investigating pathogen-specific burdens of community diarrhoea (MAL-ED) among children aged 0-2 years of age (November 2009 and February 2012), viruses were found to be responsible for 36.4% of overall diarrhoeal incidence, bacterial for 25.0%, and parasites for 3.5% (Platts-Mills et al., 2018). Besides group A rotaviruses (RV),

which are the leading cause of paediatric diarrhoea (Patel and Glass, 2009), noroviruses (NoV), adenoviruses (AdV), sapoviruses (SaV) and astroviruses (AstV) are frequent causes of AGE in children (Dennehy, 2011; Desselberger, 2014; World Health Organization - Child Health Epidemiology Reference Group [WHO-CHERG], 2014). Human adenoviruses (HAdV) are ubiquitous pathogens, causing infections in immunocompetent and immunocompromised individuals (Echavarría, 2009; Ghebremedhin, 2014; Lion, 2014). In addition to keratoconjunctivitis, hepatitis, pancreatitis, haemorrhagic cystitis, upper and lower respiratory tract infections, HAdVs are recognized as an important cause of infectious AGE in paediatric patients (Echavarría, 2009, Desselberger, 2014; Liu et al., 2016, Khanal et al., 2018).

Over 70 types of HAdV have been identified and these are classified in the family *Adenoviridae* genus *Mastadenovirus* which is divided into seven species (Human mastadenovirus-A to G), with further subdivision into subspecies (Harrach et al., 2012; Lion, 2014; Hage et al., 2015; Kosulin and Lion, 2016). The majority of HAdV diarrhoeal infections are caused by species F serotypes 40/41(HAdV-F) (Dennehy, 2011; Desselberger, 2014) with other serotypes occasionally implicated, including serotypes 12, 18 and 31 of species A; serotypes 1, 2, 5 and 6 of species C; serotypes 8, 9, 10, 28, 29, 30, 32, 37, 43, 46, 61, 64, 70 of species D and type 52 of species G (Echavarría, 2009; Qiu et al., 2018).

The incidence of HAdV-associated diarrhoeal disease varies considerably and is generally more prevalent in children under 4 years of age (Wold and Ison, 2013). The role of enteric HAdVs as a causative agent of diarrhoeal disease is similar

worldwide (Wold and Ison, 2013) and HAdVs are responsible for approximately 1.5%-5.4% diarrhoeal cases in children younger than 2 years of age (Oude Munnick and van der Hoek, 2016). In Africa, however, prevalence rates ranging from 3.5% in Tanzania (Moyo et al., 2014) to 19.6% in Gabon (Lekana-Douki et al., 2015), 20% in Egypt (El Sayed Zaki and El Kheir, 2017), 31.2% in Burkina Faso (Ouédraogo et al., 2016) and 39.7% in Rwanda (Kabayiza et al., 2014), have been recorded in the last 6 years for children younger than 5 years of age. Re-analysis of stool specimens from the GEMS study using PCR methods showed that AdV-F incidence was five times higher than previously reported using enzyme immunoassays, shifting this enteropathogen to become among the top six diarrhoea attributable pathogens in children <5 years at seven sites in Africa (Gambia, Kenya, Mali and Mozambique) and Asia (Bangladesh, India and Pakistan) (Kotloff et al., 2013; Liu et al., 2016). In one of the first studies on HAdV-associated childhood gastroenteritis in South Africa (SA), an average prevalence of 6.5% (40/616) for HAdV-F was recorded, with a midsummer peak of 10.1% (Kidd et al., 1986). In subsequent studies, the prevalence rates of HAdV-F infection in children under 5 years of age varied from 1.6% (25/1575) to 13.2% (41/310) (Tiemessen et al., 1989; Marx et al., 1998; Moore et al., 1998; Rossouw, 2004; Magwalivha, 2009).

In many countries, the introduction of the rotavirus (RV) vaccines has reduced the number of hospital admissions and mortality due to severe RV-associated diarrhoeal disease in children (Groome et al., 2016; Platts-Mills et al., 2017; Platts-Mills and Steele, 2018; Steele et al., 2019), but the introduction of RV vaccines may favour the circulation of other AGE-associated viruses (Reis et al.,

2016; Kim et al., 2017). In SA, RV vaccines were introduced into the Expanded Programme of Immunization (EPI) in August 2009 (Seheri et al., 2012), and while there have been previous reports on the prevalence of HAdV in children with AGE in SA, there have been no HAdV epidemiological studies performed in the post-RV vaccine era using molecular diagnostics. This study aims to describe the prevalence, seasonality and associated factors of HAdV infection in hospitalised children younger than 5 years old with AGE post-RV vaccine introduction in SA.

2.2 Materials and methods

2.2.1 Study setting and design

The study formed part of the prospective Rotavirus Sentinel Surveillance Program (RSSP). The study population comprised children less than 5 years of age with AGE admitted to four hospitals, namely Chris Hani-Baragwanath Academic Hospital (CHBAH) in an urban area of Gauteng Province; Edendale Hospital (EDH) in a peri-urban area of KwaZulu-Natal Province, and Matikwane (MKH) and Mapulaneng Hospitals (MPH) in rural areas of Mpumalanga Province, SA. Children were enrolled between April 2009 and December 2014, and were recruited as previously described (Page et al., 2016; 2017). Acute gastroenteritis was defined as three looser than normal stools within a 24 h period with ≤ 7 days duration. Demographic, socioeconomic and associated factor data, clinical presentation and medical history were collected by surveillance officers using standard questionnaires. Stool samples were collected within 48 h of admission and transported on ice to the Virology Division, Centre for Enteric Diseases (CED) at the National Institute for Communicable Diseases (NICD) where they were stored at 4-8˚C until processed. For those patients with unknown human

immunodeficiency virus (HIV) status, dried blood spots were collected for anonymised testing of HIV after consent had been obtained from the parents.

2.2.2 Laboratory investigations

Viral nucleic acid was extracted from 160 µℓ faecal suspensions (10% w/v in nuclease-free water) using the QIAamp® Viral RNA Mini Kit (Qiagen Inc, Hilden, Germany) according to manufacturer's instructions for automatic extraction in the QIAcube extractor (Qiagen). The extracted nucleic acid was eluted in 60 µℓ of buffer and stored at -40˚C. The faecal specimens were screened for HAdV by real-time polymerase chain reaction (rtPCR) using 5 µℓ DNA, the LightCycler® 480 Probe Master Kit (Roche Diagnostics GmbH, Mannheim Germany) and published primers and probe detecting the hexon gene (Table 2.1) (Heim et al., 2003). Thermal cycling was done on the Biorad CFX-96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) under the following conditions: 95 °C for 10 min, then 40 cycles of 95 °C for 15 s, 58 °C for 10 s, and 65 °C for 45 s. Analysis for other enteric viruses, namely RVA, NoV, SaV, human AstV (HAstV) and human bocavirus (HBoV), enteropathogenic bacteria and parasites was performed as previously described (Samra et al., 2013; Groome et al., 2014; Page et al., 2016; 2017).

Dried blood spots (DBS) were tested for HIV DNA using the using the qualitative COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 QualitativeTest, v2.0 (Roche Molecular Systems, Inc., Branchburg, NJ) according to the manufacturer's instructions The real-time PCR assay detects HIV-1 proviral DNA on whole blood

or DBS specimens. The HIV tests were performed by the Centre of HIV and Sexually Transmitted Infections, NICD.

Table 2.1. Oligonucleotide primers used for PCR amplification of adenoviruses

Source	Primers	Gene	Sequence (5'-3')	Amplicon (bp)
Heim et al., 2003	AQ1	Hexon	GCCACGGTGGGGTTTCTAAACTT	131
	AQ2	Hexon	GCCCCAGTGGTCTTACATGCACATC	
	AP	Hexon	Texas Red-TGCACCAGACCCGGGCTCAGGTACTCCGA-TAMRA	
Casa et al., 2005	ADHEX1F ADHEX1R	Hexon	CAACACCTAYGASTACATGAA KATGGGGTARAGCATGTT	475
	ADHEX2F ADHEX2R	Hexon	CCCITTYAACCACCACCG ACATCCTTBCKGAAGTTCCA	
Xu et al., 2000	AdA1 AdA2	Fiber	GCTGAAGAAMCWGAAGAAAATGA CRTTTGGTCTAGGGTAAGCAC	1444-1537
	AdB1 AdB2	Fiber	TSTACCCYTATGAAGATGAAAGC GGATAAGCTGTAGTRCTKGGCAT	670-772
	AdC1 AdC2	Fiber	TATTCAGCATCACCTCCTTTCC AAGCTATGTGGTGGTGGGGC	1988-2000
	AdD1 AdD2	Fiber	GATGTCAAATTCCTGGTCCAC TACCCGTGCTGGTGTAAAAATC	1205-1221
	AdE1 AdE2	Fiber	TCCCTACGATGCAGACAACG AGTGCCATCTATGCTATCTCC	967
	AdF1 AdF2	Fiber	ACTTAATGCTGACACGGGCAC TAATGTTTGTGTTACTCCGCTC	541-586

The HAdV species diversity was evaluated by either nucleotide sequence analysis of the hexon gene fragment from a nested-PCR (Casas et al., 2005) or the use of HAdV species-specific conventional PCR assays (Xu et al., 2000).

Amplification reactions were performed using published primers (Table 2.1) and the Go Taq ® Hot Start Polymerase kit (Promega Corp., Madison WI).

For sequencing, amplicons were purified either directly using ExoSAP-it (USB Corporation, OH) or MinElute Gel Extraction Kit (Qiagen). Purified PCR products were sequenced in both directions on an Applied Biosystems 3500 automated genetic 6analyzer (Life Technologies) with a BigDye v3.1 terminator cycle sequencing kit (Life Technologies). Sequences were analysed using Sequencher® 4.10 (Gene Codes Corporation, Ann Arbor, MI) and BioEdit sequence Alignment Editor (V.7.0.9.0). The nucleotide sequences were compared to reference strains from GenBank using BLAST search (http://blast.ncbi.nim.nih.gov/).

2.3 Statistical analysis

Statistical analysis was performed using Stata software package, version 11.1 (Stata Corp., College Station, TX). For categorical variables, frequencies and percentages were reported. Chi-square and Wilcoxon rank-sum tests were used to compare means and medians, respectively, while bivariate and stepwise multivariable logistic regression were used to identify characteristics that were associated with HAdV infections. For continuous variables, median and interquartile ranges (IQR) were presented. Variable with p-value <0.2 in Bivariate analysis were used in multivariate regression model with forward selection. The study results were presented as odds ratios (OR) with (95% confidence interval [CI]) and p-values. Missing data were accounted for in the analysis by pairwise deletion; therefore, the sample size varied slightly with regard to a particular

variable due to missing data. The results were considered statistically significant at p-value ≤ 0.05. A specimen was considered to be “fully screened” when all viral, bacterial and parasite testing had been done. Mixed infections were defined as specimens in which HAdVs were detected together with one or more other enteric viral and/or bacterial and/or parasitic pathogens.

2.4 Ethical approval

This project was approved by the Human Research Ethics Committee (Medical), University of Witwatersrand (M091018) and Faculty of Health Sciences Research Ethics Committee, University of Pretoria (383/2015).

2.5 Results

2.5.1 Patient demographic data

From April 2009 to December 2014, a total of 3623 children with AGE were enrolled at the four sentinel sites; CHBAH (n=2103, 58%), MPH (n=429, 12%), MKH (n=719, 20%) and EDH (n=372, 10%). All demographic, clinical, and laboratory data for children with HAdV infection were summarised in Table 2.2. The overall median age of children enrolled was nine months (interquartile range [IQR]: 4-15 months).

Table 2.2: Bivariate and multivariable analysis of demographic factors associated with human adenovirus infection. Only variables with p-values <0.2 in the bivariate analysis were reported and included in the multivariable model

Parameter	Adenovirus prevalence n/N (%)	Bivariate analysis		Multivariate analysis	
		Odds Ratio (OR; 95% Confidence Interval (CI)	p-value	Adjusted OR (aOR; 95% CI)	p-value
Demographic characteristics					
Age (in months)					
0-6	196/1363 (14.38)	ref	ref	ref	
7-12	234/1081 (21.62%)	1.644 (1.33-2.02)	0.000	1.61 (1.29-2.00)	0.000
13-18	121/531 (22.79%)	1.75 (1.36-2.26)	0.000	1.68 (1.29-2.20)	0.000
19-24	54/283 (19.08%)	1.40 (1.00-1.95)	0.046	1.46 (1.04-2.06)	0.028
≥25	50/360 (13.89%)	0.96 (0.68-1.34)	0.813	0.86 (0.60-1.22)	0.408
Unknown	1/5 (20%)				
Year					
2014	76/519 (14.64%)	ref	ref	Ref	
2013	105/538 (19.52%)	1.41 (1.02-1.95)	0.036	1.42 (1.01-1.99)	0.039
2012	57/459 (12.42%)	0.82 (0.57-1.19)	0.31	0.77 (0.52-1.14)	0.195
2011	76/553 (13.74%)	0.92 (0.65-1.30)	0.673	1.00 (0.70-1.44)	0.958
2010	195/919 (21.22%)	1.56 (1.17-2.09)	0.002	1.53 (1.13-2.08)	0.005
2009	147/635 (23.15%)	1.75 (1.29-2.38)	0.000	2.47 (1.76-3.48)	0.000
Sentinel site (n/N)					
CHBAH	378/2103 (17.97%)	ref	ref		
MPH	75/429 (17.48%)	0.96 (0.73-1.27)	0.809		
MKH	144/719 (20.03%)	1.14 (0.92-1.41)	0.221		
EDH	59/372 (15.86%)	0.86 (0.63-1.16)	0.325		
Seasonality (All)					
Jan	53/277 (19.13%)	ref	ref	ref	
Feb	85/412 (20.63%)	1.099 (0.74-1.61)	0.630	0.99 (0.67-1.48)	0.990
Mar	85/327 (25.99%)	1.48 (1.00-2.18)	0.046	1.34 (0.90-2.00)	0.146
Apr	51/231 (22.08%)	1.19 (0.77-1.84)	0.413	0.93 (0.59-1.46)	0.778
May	66/335 (19.70%)	1.03 (0.69-1.55)	0.860	0.87 (0.57-1.34)	0.553
Jun	72/473 (15.22%)	0.75 (0.51-1.12)	0.166	0.65 (0.43-0.99)	0.048
Jul	50/380(13.16%)	0.64 (0.42-1.97)	0.04	0.60 (0.38-0.94)	0.028
Aug	42/310 (13.55%)	0.66 (0.42-1.25)	0.068	0.60 (0.38-0.97)	0.039
Sept	37/235 (15.74%)	0.78 (0.49-1.25)	0.316	0.70 (0.43-1.15)	0.169
Oct	47/235 (20.00%)	1.05 (0.68-1.63)	0.805	0.88 (0.55-1.41)	0.619
Nov	46/267 (17.23%)	0.78 (0.56-1.36)	0.565	0.80 (0.51-1.27)	0.361
Dec	22/141 (15.60%)	0.78 (0.45-1.34)	0.375	0.68 (0.38-1.21)	0.197

Table 2.2 *continued*:

Parameter	Adenovirus prevalence n/N (%)	Bivariate analysis		Multivariate analysis	
		Odds Ratio (OR; 95% Confidence Interval (CI)	p-value	Adjusted OR (aOR; 95% CI)	p-value
Clinical characteristics					
Fever duration (in days)					
1	50/384 (13.02%)	Ref	ref		
2	88/486 (17.64%)	1.47 (1.03-2.15)	0.042		
≥3	226/1369 (16.51%)	1.43 (0.98-2.08)	0.062		
Fever					
No	148/1046 (14.15%)	ref	ref		
Yes	235/1403 (16.75%)	1.22 (0.97-1.52)	0.080		
Anemia (before adjusted for shigella)					
No	616/3429 (17.96%)	ref	ref		
Yes	23/80 (28.75%)	1.84 (1.12-3.01)	0.015		
Anemia (after adjusted for shigella)					
No	169/843 (20.05%)	ref	ref		
Yes	4/19 (21.05%)	1.06 (0.34-3.24)	0.914		
Cough duration (in days)					
1-2	50/391 (12.79%)				
3	33/203 (16.26%)	1.32 (0.82-2.13)	0.249		
≥4	60/337 (17.80%)	1.47 (0.98-2.22)	0.061		
Maximum number of stools in 24 hours prior hospitalisation					
≤3	122/728 (16.76%)				
4-5	351/1798 (19.52%)	1.20 (0.96-1.51)	0.107		
≥6	151/912 (16.56%)	0.98 (0.75-1.28)	0.913		
Maximum number of vomits in 24 hours					
1-2	158/719 (21.97%)	ref	ref		
3	104/65 (16.12%)	0.68 (0.51-0.89)	0.006		
≥4	142/855 (16.61%)	0.70 (0.54-0.90)	0.007		
Dehydration					
No	118/754 (15.65%)	ref	ref		
Yes	446/2376 (18.90%)	1.24 (0.99-1.55)	0.052		
Diarrhoea duration (in days)					
1-4	526/2951 (17.82)				
5	71/298 (23.83%)				
≥6	35/226 (15.49%)				
Number of people that sleep in the same room as child					
0-2	200/1191 (16.79%)	ref	Ref		
3	82/575 (14.26%)	0.82 (0.62-1.08)	0.174		
≥4	104/701 (14.84%)	0.86 (0.66-1.11)	0.263		

Table 2.2 *continued*:

Parameter	**Adenovirus prevalence n/N (%)**	**Bivariate analysis**		**Multivariate analysis**	
		Odds Ratio (OR; 95% Confidence Interval (CI)	**p-value**	**Adjusted OR (aOR; 95% CI)**	**p-value**
Adenovirus mixed pathogen infections					
Adenovirus only	69/702 (9.83%)	ref	ref	**Not included in the analysis**	
Adenovirus + 1 pathogen	128/406 (31.53%)	3.51 (2.86-4.32)	0.00		
Adenovirus + 2 or more	94/175 (53.7%)	7.97 (6.09-10.43)	0.00		
Adenovirus & Rotavirus					
Negative	476/2445 (19.47%)	ref	ref	ref	ref
Positive	180/1176 (15.31%)	0.74 (0.61-0.90)	0.002	0.67 (0.53-0.84)	0.0001
Adenovirus & NoV GI					
Negative	631/3519 (17.93%)	ref	ref		
Positive	25/104 (24.04%)	1.44 (0.91-2.28)	0.113		
Adenovirus & NoV GII					
Negative	595/3202 (18.58%)	ref	ref	ref	ref
Positive	61/421 (14.49%	0.74 (0.55-0.98)	0.041	0.70 (0.51-0.96)	0.027
Adenovirus & Astrovirus					
Negative	592/3369 (17.57%)	ref	ref	ref	ref
Positive	64/254 (25.20%)	1.58 (1.17-2.12)	0.003	1.50 (1.10-2.04)	0.010
Adenovirus & Bocavirus					
Negative	607/3426 (17.72%)	ref	ref		
Positive	49/197 (24.87%)	1.53 (1.10-2.14)	0.012		
Adenovirus & Bacteria					
Negative	361/2168 (16.65%)	ref	ref		
Positive	185/629 (19.91%)	1.24 (1.02-1.51)	0.029		
Adenovirus & Parasite					
Negative	290/1658 (17.49%)	ref	ref		
Positive	56/226 (24.78%)	1.55 (1.11-2.15)	0.008		

Table 2.2 *continued*:

Parameter	**Adenovirus prevalence n/N (%)**	**Bivariate analysis**		**Multivariate analysis**	
		Odds Ratio (OR; 95% Confidence Interval (CI)	**p-value**	**Adjusted OR (aOR; 95% CI)**	**p-value**
Outcome					
Died	4/89 (4.49%)	ref	ref	ref	
Discharged	630/3401 (18.82%)	4.83 (1.7-13.21)	0.002	4.93 (1.79-3.60)	0.002
Environmental features					
Water source					
In-door water	309/1613 (19.16%)	ref	ref		
Others	324/1884 (17.20%)	0.87 (0.73-1.04)	0.87 (0.73-1.04)	0.134	

2.5.2 HAdV detection

In total, 18.1% (656/3623) of the stool specimens collected over the study period (635 in 2009, 919 in 2010, 553 in 2011, 459 in 2012, 538 in 2013 and 519 in 2014) tested positive for HAdV. The prevalence of HAdV infection did not differ significantly between study sites (Table 2.2). There was a heterogeneity in prevalence of HAdV detected in different years, with the highest prevalence in 2009 (23.15%, 147/635) and the lowest prevalence in 2012 (12.42%, 57/549) (Fig. 2.1) (Figure 2.1; Table 2.2). Compared to 2014, the detection of HAdV was significantly higher in 2009, 2010 and 2013 (2009: adjusted OR (aOR) =2.47 (95% CI 1.76-3.48) p=0.000; 2010: aOR=1.53 (95%CI 1.13-2.08); p=0.005; 2013: aOR=1.42 (95%CI 1.01-1.99); p=0.039) (Table 2.2).

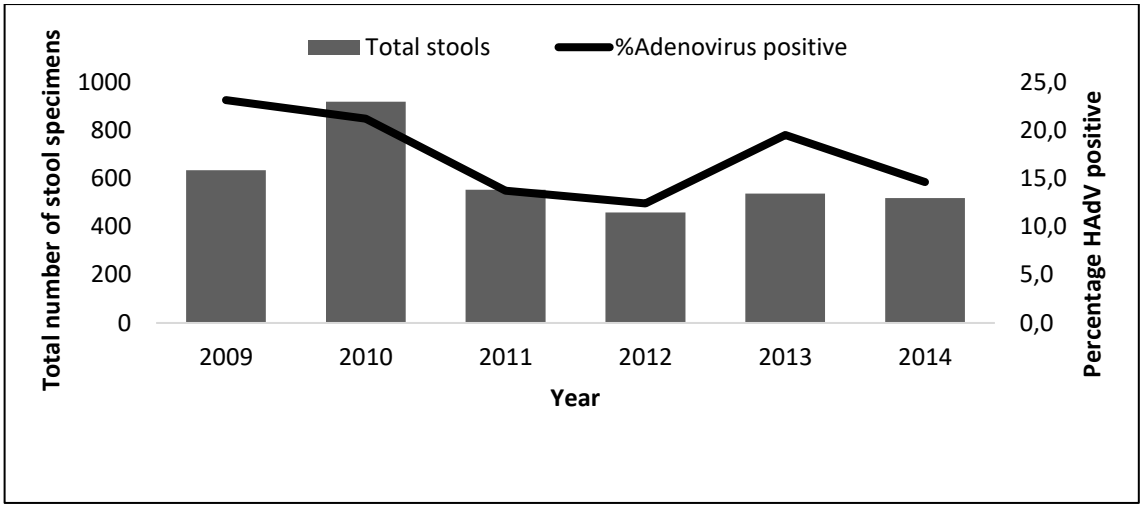

Figure 2.1: Yearly distribution of HAdV from 2009 to 2014

The detection rates of HAdV in males and females were 57.5% (377/656) and 42.5% (279/656), respectively. However, the differences observed in the prevalence by gender was not statistically significant ($p > 0.05$). From Table 2.2 it is evident most (92%: 605/656) HAdV positive stools were from children under two years of age. Analysis by age-group showed that HAdV was highest in children 6 and 12 months (OR=1.6 (95%CI 1.2-2.0); p=0.001)), 13 to 18 months (OR=1.6 (95%CI 1.2-2.2); p=0.001)) and 19 to 24 months (aOR=1.4 (95%CI 1.0-2.0); p=0.02) compared to children six months of age and younger. A decline in the detection rate with age was observed.

The monthly distribution of HAdV across the study period is depicted in Table 2.2 and Fig 2.2. The detection rates of HAdV in autumn (March-May) were 22.62% (202/893), summer (December to February) were 19.62% (160/830), spring (September to November) were 17.63% (130/737) and winter (June-August) were 14.1% (164/1163), respectively. However, the detection rate did not differ

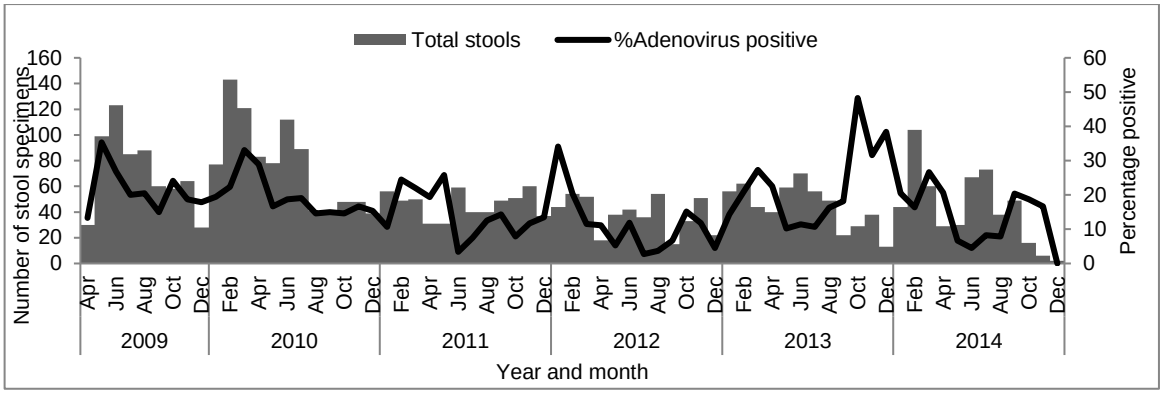

Figure 2.2: Monthly distribution of HAdVs in stools specimens from hospitalised children with AGE between April 2009 and December 2014.

significantly (p>0.05) (Fig. 2.2) (Table 2.3). The monthly distribution of HAdV-positive cases was highest in June (13.1%; 473/3623), followed by in February (11.4%; 412/3623) (Table 2.2). When compared to the January, the prevalence of HAdV dropped significantly in winter months (June; aOR=0.65 (95% CI 0.4-0.9); p=0.048, July; aOR=0.6 (95%CI 0.38-0.94); p=0.028 and August; aOR=0.6 (95%CI 0.38-0.97); p=0.03)) (Table 2.2).

2.5.3 Co-infections

Of the 656 HAdV-positive specimens, 291 were fully screened for viral, bacterial and parasitic pathogens. Among the 291 HAdV-positive cases, 23.7% (69/291) were infected only with HAdV and 76.3% (222/291) were co-infected with other pathogens (Table 2.2). Among the observed HAdV co-infections, 44% (128/291) was HAdV with a single co-pathogen and 32.3% (94/291) was with two or more co-infecting pathogens. Of all enteric pathogens, HAdV-infected children were more likely to be co-infected with HAstV, (aOR=1.53; 95% CI 1.10-2.04; p=0.010) than with RVA (aOR=0.67; 95% CI 0.53-0.84; p=0.001) and NoV GII (aOR=0.70; 95% CI 0.51-0.96; p=0.027). Gender or age-specific differences in single and

mixed infections was not observed. Similarly, no significant difference in clinical symptoms was observed between cases with mixed and single HAdV infections ($p>0.05$) (data not shown).

2.5.4 Clinical characteristics

There were no differences in the clinical features between HAdV-positive and HAdV-negative children (Table 2.2). Only 97.4% (3530/3623) of hospitalised children had available outcome data, of whom 2.5% (89/3530) died during the study period. The highest number of children that died was recorded in 2011, (3.6%; 20/548) followed by 3% (27/900) in 2010, 2.5% (11/439) in 2012, 1.96% (10/510) in 2014, 1.95% (10/514) in 2013 and 1.77% (11/619) in 2009. Amongst children who died, 4.5% (4/89) tested positive for HAdV. Only one of the four children had full screening for all enteric pathogens. In this case, none of the enteric pathogens tested for were detected. All four children who died were under 12 months of age and were from different study sites. Only two of the deceased children were found to have HIV results available, one HIV negative and one HIV positive. However, they were all from households where outdoor/river water was used as main source of water.

2.5.5 Association between HAdV infection and HIV status

Information on HIV status was available for 84.52% (3062/3623) of children enrolled (Table 2.3). The children's HIV status was obtained from medical records, laboratory reports and maternal verbal reports in 59.1% (1810/3062) of the children with AGE during admission and 40.9% (1252/3062) of children were tested after admission. The annual prevalence of HIV-positive diarrhoeal cases

Table 2.3: Bivariate and multivariable analysis of demographic data, clinical characteristics and environmental features associated with HAdV detection in HIV-infected and HIV-uninfected children.

Parameter	**Adenovirus prevalence n/N (%)**	**Bivariate analysis**		**Multivariate analysis**	
		Odds Ratio (OR; 95% Confidence Interval (CI)	**p-value**	**Adjusted OR (aOR; 95% CI)**	**p-value**
Demographic characteristics					
Gender					
Female	29/236(12.29%)	ref	ref		
Male	28/318 (8.81%)	0.68 (0.39-1.19)	0.184		
Year					
2014	1/32 (3.13%)	ref	ref		
2013	7/102 (6.86%)	2.28 (0.27-19.30)	0.448		
2012	3/54 (5.56%)	1.82(0.18-18.31)	0.610		
2011	5/76 (6.58%)	2.18 (0.24-19.46)	0.484		
2010	31/174 (17.82%)	6.72 (0.88-51.10)	0.066		
2009	10/116 (8.62%)	2.92 (0.36-23.74)	0.315		
Sentinel site (n/N)					
CHBAH	18/302 (5.96%)	ref	ref		
MPH	10/67 (14.93%)	2.76 (1.21-6.30)	0.015		
MKH	25/132 (18.94%)	3.68 (1.93-7.02)	0.000		
EDH	4/53 (10.29%)	1.28 (0.41-3.96)	0.659		
Monthly					
Jan	3/47 (6.38%)	ref	ref		
Feb	11/66 (16.67%)	2.93 (0.77-11.16	0.115		
Mar	6/70 (8.57%)	1.37 (0.32-5.79)	0.664		
April	5/42 (11.90%)	1.98 (0.44-8.85)	0.370		
May	6/55 (10.91%)	1.79 (0.42-8.85)	0.427		
Jun	5/61 (8.20%)	1.30 (0.29-5.78)	0.722		
Jul	3/33 (7.89%)	1.25 (0.23-6.61)	0.787		
Aug	2/37 (5.41%)	0.83 (0.13-5.29)	0.851		
Sept	3/30 (10.00%)	1.62 (0.30-8.66)	0.567		
Oct	5/46 (10.87%)	1.78 (0.40-7.96)	0.445		
Nov	6/41 (14.63%)	2.51 (0.58-10.77)	0.214		
Dec	2/21 (9.52%)	1.54 (0.23-10.00)	0.649		
Nursery school attendance					
No	30/280 (10.71%)	ref	ref		
Yes	1/63 (1.59%)	0.13 (0.01-1.00)	0.051		

Table 2.3. *continued:*

Parameter	**Adenovirus prevalence n/N (%)**	**Bivariate analysis**		**Multivariate analysis**	
		Odds Ratio (OR; 95% Confidence Interval (CI)	**p-value**	**Adjusted OR (aOR; 95% CI)**	**p-value**
Clinical characteristics					
Maximum number of vomits in 24 hours					
1-2	11/84 (13.10%)	ref			
3	13/121 (10.74%)	0.79 (0.33-1.88)			
≥4	14/181 (7.73%)	0.55 (0.24-1.28)			
Dehydration					
No	17/106 (16.04%)	ref			
Yes	34/376 (9.04%)	0.52 (0.27-0.97)			
Term status					
Term	12/119 (10.08%)	ref	ref		
Preterm (<37 weeks)	7/35 (20.00%)	2.22 (0.80-6.18)	0.124		
Child's birth weight					
≤2.49kg	23/272 (8.46%)	ref	ref		
≥2.5kg	7/45 (15.56%)	1.99 (0.80-4.96)	0.138		
Number of days admitted to hospital					
≤3 days	8/254 (3.15%)	ref	ref	ref	ref
≥4 days	47/290 (16.21%)	5.94 (2.75-12.84)	0.000	5.94 (2.75-12.84)	**0.000**
Co-infections in adenovirus-positive specimens					
Adenovirus & rotavirus					
No	49/407 (12.04%)	ref	ref		
Yes	8/147 (5.44%)	0.42 (0.19-0.91)	0.028		
Adenovirus & Bacteria					
No	27/314 (8.60%)	ref	ref		
Yes	25/164 (15.24%)	1.91 (1.06-3.41)	0.029		
Sanitation					
Flushing toilet					
No	18/268 (6.72%)	ref	ref		
Yes	39/279 (13.98%)	2.25 (1.25-4.05)	0.006		

Tabble 2.3. continued:

Parameter	Adenovirus prevalence n/N (%)	Bivariate analysis		Multivariate analysis	
		Odds Ratio (OR; 95% Confidence Interval (CI)	p-value	Adjusted OR (aOR; 95% CI)	p-value
Housing material					
Bricks	49/429 (11.42%)	ref	ref		
Others	7/120 (5.83%)	0.48 (0.21-1.09)	0.079		
Number of people that sleep in the same room as child					
≤2	17/182 (9.34%)	ref	ref		
3	2/77 (2.60%)	0.25 (0.05-1.14)	0.075		
≥4	13/87 (14.94%)	1.70 (0.78-3.69)	0.176		
Environmental features					
Water source					
In-door water	21/262 (8.02%)	ref	ref		
Others	35/285 (12.28%)	1.60 (0.90-2.83)	0.103		

were 10.15% (47/463) in 2009, 16.88% (143/847) in 2010, 11.76% (64/544) in 2011, 11.96% (53.443) in 2012, 9.28% (49/528) in 2013, and 5.91% (14/237) in 2014, respectively. Of those patients with known HIV status during admission (7.95%; 144/1810) were HIV positive. Of the HIV positive patients 44.4% (64/144) were on treatment, 10.4% (15/144) not on treatment and for 45.1% (65/144) the treatment status was unknown. Hospital stay was longer in HIV-infected compared (≥4 days) to HIV-uninfected (≤3 days) children with HAdV (OR=5.94; 95% CI 2.75-12.84; p<0.000) (Table 2.3). No additional stastical significant differences in the demographic, clinical symptoms or laboratory findings between HIV-infected and HIV-uninfected children with HAdV was observed.

2.5.6 Human AdV species

Six hundred and three of 656 (91.9%) HAdV strains detected in the stool specimens were successfully genotyped, of which 42.1% (254/603) were HAdV-

F, followed by HAdV-C (26.7%; 161/603), HAdV-B (12.4%; 75/603), HAdV-A (8.6%; 52/603) and HAdV-D (7.8%; 47/603) (Table 2.4). The least frequently detected species was HAdV-E, accounting for 1.0% (6/603) of the HAdV infections. Co-infection with more than one HAdV species was observed in 1.3% (8/603) of the cases. Mixed species infection comprised seven combinations in different proportions including - HAdV-A+B [n=1]; HAdV-A+C [n=1]; HAdV-B+C [n=1]; HAdV-B+D [n=1]; HAdV-C+D [n=1]; HAdV-C+F [n=1] and HAdV-D+F [n=2]. Sequence analyses of 157 HAdV-F positive specimens between 2009 and 2012 revealed almost equal distribution of HAdV-40 and HAdV-41, 48.4% (76/157) and 51.6% (81/157), respectively. The co-infection rate of different species HAdV with other enteric pathogens are shown in Table 2.5. Of the 103 HAdV-F with full enteric screening, 32.0% (33/103) were the only pathogen present detected and 68.0% (70/103) were with other enteric pathogens. Similarly, 32.3% (10/31) of HAdV-B were single infection and 67.7% (21/31) had other enteric pathogens present. Among 199 HAdV-F sequenced, 49.2% (98/199) were HAdV-40 and 50.8% (101/199) HAdV-41.

Table 2.4. Number of HAdV species as a single or co-infection

Species	Total positive	Single N (%)	Co-infection N (%)
HAdV-A	27	3 (11.1)	24 (88.9)
HAdV-B	31	10 (32.3)	21 (67.7)
HAdV-C	79	11 (13.9)	68 (86.1)
HAdV-D	25	6 (24.0)	19 (76.0)
HAdV-E	4	1 (25.0)	3 (75.0)
HAdV-F	103	33 (32.0%)	70 (68.0)
HAdV-Mix	3	0 (0.0)	3 (100)

2.5.7 Distribution of HAdV-F

There were no significant differences in either sex or age distributions in children with HAdV-F (p>0.05) (Table 2.5). The yearly detection rates of HAdV-F varied from 4.8% to 9.1% with a mean of 7%, but there was no significant difference in the yearly distribution of HAdV-F. The prevalence of HAdV-F was significantly higher at MKH (aOR=2.36; 95% CI 1.52-3.666; p=0.000) compared to CHBAH (Table 2.6). A significantly less prevalence of HAdV-F was observed in July (aOR=0.26; 95% CI 0.94-0.74; p=0.012), August (aOR=0.29; 95% CI 0.10-0.83; p=0.022), and September (aOR=0.21; 95% CI 0.22-2.06; p=0.049) compared to January (Table 2.5). The detection of HAdV-F was significantly associated with 4-5 diarrhoeal episodes per day when compared to children without HAdV-F infection (aOR=1.94; 95% CI 1.13-3.34; p=0.016).

2.6 Discussion

The present study is the first to estimate the prevalence (18.1%, 656/3623) of HAdV in SA post-RV vaccine introduction. The current study detected a higher prevalence of HAdV than previously reported in young children with AGE in SA, which varied from 4.6% to 9.8% (Kidd et al., 1986; Moore et al, 1998; Rossuow, 2004; Magwalivha, 2009). The reasons for the higher HAdV detection rate are not clear. However, it is important to note that the HAdV PCR assay used in this study detects all AdV types and not just the HAdV-F40 and HAdV-F41. The possible limitation of the previous studies were the use of a less sensitive detection methods (enzyme immunoassay (EIA) and hybridisation assay) as compared to PCR that has shown improved sensitivity in the detection of HAdV, for example in the GEMS study (Liu et al., 2016).

Table 2.5: Bivariate and multivariable analysis of demographic data, clinical characteristics and environmental features associated with enteric AdV-F detection in children with AGE.

Parameter	HAdV-F prevalence n/N (%)	Bivariate analysis		Multivariate analysis	
		Odds Ratio (OR; 95% Confidence Interval (CI)	p-value	Adjusted OR (aOR; 95% CI)	p-value
Demographic characteristics					
Age in months					
0-6	99/1363 (7.26%)	ref	ref		
7-12	84/1081 (7.77%)	1.07 (0.79-1.45)	0.636		
13-18	44/531 (8.29%)	1.15 (0.79-1.67)	0.449		
19-24	13/283 (4.59%)	0.6 (0.33-1.11)	0.108		
>24	13/360 (3.61%)	0.47 (0.26-0.86)	0.014		
Collection year					
2014	31/519 (5.97%)	ref	ref		
2013	49/538 (9.11%)	1.57 (0.98-2.51)	0.056		
2012	35/459 (7.63%)	1.29 (0.78-2.14)	0.305		
2011	27/553 (4.88%)	0.80 (0.47-1.37)	0.431		
2010	71/919 (7.73%)	1.31 (0.85-2.03)	0.215		
2009	41/635 (6.46%)	1.08 (0.67-1.75)	0.735		
Sentinel site (n/N)					
CHBAH	133/2103 (6.3%)	ref	ref	ref	ref
MPH	30/429 (6.99%)	1.11 (0.73-1.67)	0.607	1.38 (0.73-2.59)	0.309
MKH	67/719 (9.32%)	1.52 (0.12-2.06)	0.007	2.36 (1.52-3.66)	0.000
EDH	24/372 (6.45%)	1.02 (0.65-1.60)	0.926	0.86 (0.38-1.96)	0.735
Seasonality					
Spring	39/737 (5.29%)	ref	ref	ref	ref
Winter	54/1162 (4.65 %)	0.87 (0.57-1.33)	0.526	Not included in the analysis	
Autumn	70/893 (7.84%)	1.52 (1.01-1.33)	0.042		
Summer	91/830 (10.96%)	2.20 (1.49-3.25)	0.000		
Mom education					
None	7/53 (13.21%)	ref	ref		
Primary	12/202 (5.94%)	0.41 (0.15-1.11)	0.081		
Secondary	139/2050 (6.78%)	0.47 (0.21-1.07)	0.075		
Tertiary	8/138 (5.80%)	0.40 (0.13-1.17)	0.097		

Table 2.5 *continued:*

Parameter	HAdV-F prevalence n/N (%)	Bivariate analysis		Multivariate analysis	
		Odds Ratio (OR; 95% Confidence Interval (CI)	p-value	Adjusted OR (aOR; 95% CI)	p-value
Demographic characteristics					
Seasonality					
Jan	37/277 (13.36%)	ref	ref	ref	ref
Feb	43/412 (10.44%)	0.75 (0.47-1.20)	0.242	0.98 (0.44-2.20)	0.976
Mar	33/327 (10.09%)	0.72 (0.44-1.19)	0.213	1.66 (0.75-3.64)	0.204
Apr	18/231 (7.79%)	0.54 (0.30-0.99)	0.047	0.65 (0.25-1.72)	0.395
May	19/335 (5.67%)	0.39 (0.21-0.69)	0.001	0.73 (0.30-1.79)	0.504
Jun	24/473 (5.07%)	0.34 (0.20-0.59)	0.000	0.55 (0.23-1.32)	0.187
July	18/379 (4.75%)	0.32 (0.17-0.58)	0.000	0.26 (0.94-0.74)	0.012
Aug	12/310 (3.87%)	0.26 (0.13-0.51)	0.000	0.29(0.10-0.83)	0.022
Sept	10/235 (4.26%)	0.28 (0.14-0.59)	0.001	0.21(0.22-2.06)	0.049
Oct	14/235 (5.96%)	0.41 (0.21-.078)	0.007	0.67 (0.22-2.06)	0.493
Nov	15/267 (5.62%)	0.38 (0.20-0.72)	0.003	0.79 (0.32-1.93)	0.611
Dec	11/141 (7.80%)	0.54 (0.27-1.11)	0.096	1.07 (0.38-2.96)	0.889
Clinical characteristics					
Anemia					
No	237/3429 (6.91%)	ref	ref		
Yes	11/80 (13.75%)	2.14 (1.12-4.11)	0.021		
Blood in stool					
No	227/3113 (2.29%)	ref	ref		
Yes	19/369 (5.19%)	0.69 (0.42-1.11)	0.131		
Maximum number of episodes of diarrhoea per day					
≤3	45/728 (6.18%)	ref	ref	ref	ref
4-5	138/1798 (7.68%)	1.26 (0.89-1.78)	0.190	1.94 (1.13-3.34)	0.016
≥6	58/912 (6.36%)	1.03 (0.68-1.54)	0.882	1.37 (0.72-2.62)	0.334
Duration of fever					
1 day	24/417 (5.76%)	ref	ref		
≥ 2 days	77/985 (7.82%)	1.38 (0.86-2.22)	0.174		
Admin temperature					
≤ 37.9	127/1695 (7.49%)	ref	ref		
≥ 38	153/2149 (5.73%)	0.75 (0.48-1.15)	0.195		

Table 2.5. *continued:*

Parameter	HAdV-F prevalence n/N (%)	Bivariate analysis		Multivariate analysis	
		Odds Ratio (OR; 95% Confidence Interval (CI)	p-value	Adjusted OR (aOR; 95% CI)	p-value
Clinical characteristics					
Days admitted in hospital					
≤ 2	111/1852 (5.99%)	ref	ref		
≥ 3	143/1771 (8.07%)	1.37 (1.06-1.78)	0.015		
Environmental features					
Housing material					
Bricks	196/2624 (7.47%)	ref	ref		
Others	50/875 (5.71%)	0.75 (0.54-1.03)	0.088		
Toilet/ Sanitation					
Flush toilet	117/1833 (6.38%)	ref	ref		
Pit latrine	117/1525(7.67%)	1.21 (0.93-1.58)	0.145		
Bucket system	3/61 (4.92%)	0.75 (0.23-2.45)	0.645		
None/ outdoor	9/80 (11.25%	1.85 (0.90-3.81)	0.091		
Adenovirus mixed pathogen infections					
AdV-F only	100/1554 (6.44%)	ref	ref	Not included in the analysis	
AdV + 1 Pathogen	104/839 (12.40%)	2.05 (1.54-2.74)	0.000		
AdV + 2 or more	50/308 (16.23%)	1.81 (1.95-4.05)	0.000		
Adenovirus & rotavirus					
No	197/2445 (80.6%)	ref	ref		
Yes	87/1176 (4.85%)	0.58 (0.42-0.78)	0.00		
Adenovirus & Norovirus GI					
No	242/3519 (6.88%)	ref	ref		
Yes	12/104 (11.54%)	1.76 (0.95-3.26)	0.070		
Adenovirus & Norovirus GII					
No	239/3202 (7.46%)	ref	ref		
Yes	15/421 (3.56%)	0.45 (0.26-0.77	0.004		
Adenovirus & Bacteria					
No	141/2168 (6.50%)	ref	ref		
Yes	73/929 (7.86%)	1.22 (0.91-1.64)	0.174		
Adenovirus & Parasites					
No	113/1658 (6.82%)	ref	ref	ref	ref
Yes	9/226 (3.98%)	0.56 (0.28-1.13)	0.109	0.41 (0.20-0.83)	0.014

Table 2.5. *continued:*

Parameter	HAdV-F prevalence n/N (%)	Bivariate analysis		Multivariate analysis	
		Odds Ratio (OR; 95% Confidence Interval (CI)	p-value	Adjusted OR (aOR; 95% CI)	p-value
Adenovirus mixed pathogen infections					
Adenovirus & Norovirus GI					
No	242/3519 (6.88%)	ref	ref		
Yes	12/104 (11.54%)	1.76 (0.95-3.26)	0.070		
Adenovirus & Norovirus GII					
No	239/3202 (7.46%)	ref	ref		
Yes	15/421 (3.56%)	0.45 (0.26-0.77	0.004		
Adenovirus & Bacteria					
No	141/2168 (6.50%)	ref	ref		
Yes	73/929 (7.86%)	1.22 (0.91-1.64)	0.174		
Adenovirus & Parasites					
No	113/1658 (6.82%)	ref	ref	ref	ref
Yes	9/226 (3.98%)	0.56 (0.28-1.13)	0.109	0.41 (0.20-0.83)	0.014

The prevalence of HAdV in this study is comparable to other studies performed in African countries using PCR method, 19.6% in Gabon (Lekana-Douki et al., 2015), 20% in Egypt (El Sayed Zaki and El Kheir, 2017), but lower than 31.2% in Burkina Faso (Ouédraogo et al., 2016) and 39.7% in Rwanda (Kabayiza et al., 2014). In other parts of the world, a lower prevalence of 6.29% in Chagqing, China (Ren et al., 2013), and 16% in Southern Brazil (Raboni et al., 2014) have been reported. However, a higher prevalence of 23.2% in Albania (La Rosa et al., 2015) has been reported. The differences in prevalence could be due to differences in demographical, geographical, and methodologies used for estimating HAdV. The detection of HAdV throughout the study sites was not a surprise, since they are associated with different clinical illnesses. The results indicate that the prevalence of HAdV in children with diarrhoea is higher than

previously thought in SA, indicating that the disease could be being underreported.

Previous studies showed that co-infections with other enteric pathogens is common in HAdV-infected patients, detected in 34.8 - 85% of children with AGE (Koh et al., 2008; Lekana-Douki et al., 2015; Imade and Eghafona, 2015). In agreement with these studies, 76.3% of children with HAdV were co-infected with other enteric pathogens. The high co-infection rate could be explained by simultaneous infections with prolonged viral shedding, as well as asymptomatic carriage of one or more pathogen. It has been previously shown that HAdV could establish latent infections in tonsillar and adenoid tissue and can be found in stool specimens of children without active symptoms (Garnett et al., 2002; Wold and Horwitz, 2007; Garnett et al., 2009; Moyo et al., 2014). In addition, the high co-infection rate may also be due to the broader screening with a larger panel of enteric pathogens as well as more sensitive molecular techniques. Human AstV was significantly associated with HAdV. The high frequency of HAdV co-infections detected together with other enteric pathogens raises questions about whether HAdV was a true pathogen or just a bystander. This study did not show an association between disease severity and HAdV co-infections (data not shown).

In this study, 92.2% of the children positive for HAdV were under 2 years of age, with the highest prevalence between 7-24 months, emphasising the fact that HAdV infection occurs early in life. This is similar to the findings of many studies

worldwide (Pereira Filho et al., 2007; Verma et al., 2009; Dashti et al., 2016; Jaff et al., 2016; Platts-Mills et al., 2018), and makes this age group an important target for interventions such as vaccination. The lower prevalence of HAdV in the 0-6 months age-group may be attributed to partial protection from maternal antibodies. The increased prevalence of HAdV infections among children aged ≥6 months may be explained by increased exposure or increased susceptibility due to waning maternal antibodies. Similar to other studies, there was no significant difference between gender and HAdV infection (Oh et al., 2003; Ouyang et al., 2012; Liu et al, 2014, Babalola et al., 2015). Nevertheless, studies from elsewhere have shown evidence that males were more susceptible to HAdV, a phenomenon not observed in this study (Cruz et al., 1990).

During the study period, there was no clear seasonal pattern and HAdV circulation was year-round, similar to previous reports in SA (Kidd et al., 1986; Tiemessen et al., 1989). However, the lowest detection of HAdV was observed in winter months. This period is characterised by low temperatures with little or no rainfall in the region, and the observation on HAdV prevalence is contrary to that for RV, which is more prevalent in the cooler months (WHO, 2013). Similar results were reported in other countries (Pereira Filho et al., 2007; Raboni et al., 2014; Tripathi et al., 2016). Efforts to prevent HAdV should be considered throughout the year in SA. The study performed in Iranian children under 6 years old showed HAdV in stools specimens during the autumn and winter months (Hamedi et al., 2010). The conflicting results may be due to geographical differences.

The annual prevalence of HAdV was 23.15% in 2009 and 21.22% in 2010, which has fallen in subsequent years to 13.74% in 2011 and 12.42% in 2012 (the lowest number recorded during the study period). These were early years of RV vaccine introduction in SA, when there may be still a low vaccination coverage. The increased RV vaccine coverage rates reduced diarrheal hospitalisations over the years (Groome et al., 2016). Interestingly, a noticeable increase in the prevalence of HAdV in 2013 (19.52%) was observed, which was then dropped in 2014 (14.64%). The increase prevalence in 2013 could be partially attributed to an increase in the number of diarrhoeal cases recorded in that period (Page et al., 2015). This uneven HAdV detection rate could also be due to the variation in the specimens number for different years rather than reduction in HAdV circulation.

No difference was observed in the clinical symptoms between the HAdV-positive and HAdV-negative children. This study forms part of the sentinel diarrhoea surveillance programme established in SA in 2009 to monitor diarrhoeal diseases in hospitalised children after RV vaccine introduction into the national immunisation program. These data suggest that in children HAdV infections are likely to be mild. Similar findings were reported where poor or non-existent links between HAdV infection status and gastrointestinal symptoms (Liste et al., 2000; Magwalivha et al., 2010; Moyo et al., 2014). Moyo et al. (2014) reported a HAdV prevalence rate of 3.5% in diarrhoeic children as well as in 2.4% of non-diarrhoeic children in Tanzania, emphasizes the importance of asymptomatic patients in HAdV transmission where hygiene and sanitation are poor. Alternatively, clinical features associated with HAdV may be milder but otherwise indistinguishable from those of other viral causes of AGE.

A low mortality (4.5%) was observed in HAdV-positive children. The death of four children infected with HAdV during the study period needs to be interpreted with caution. Among the four children who died, it is possible that even though the patients were hospitalised for severe diarrhoea, admission may have been too late to respond to rehydration, since hospitalised children infected with HAdV were less likely to die. Therefore, early seeking of care or hospital admission is important in preventing unnecessary deaths. All four deaths occurred in children less than one year old, highlighting that particular attention should be paid to these children.

This is the first study to describe the prevalence of HAdV among HIV-infected children with AGE in SA. There are limited HAdV data on HIV infected children with AGE in Africa. No associations were found between HAdV in HIV-infected children and clinical symptoms in this study. Previous studies in Africa found no association between HAdV and HIV (Magwalivha et al., 2010; Moyo et al., 2014). Another study reported similar findings in Venezuela (Liste et al., 2000). The association between HIV-infection and long hospital stay was observed in this study. However, the current study was not designed to assess the impact of HIV on diarrhoeic children with HAdV infection. However, the association of HAdV and HIV infection in children with AGE do not appear to be straightforward and warrant further investigation

According to Pereira Filho et al. (2007), HAdV-F accounts for approximately 50% of all HAdV found in stool specimens in both hospitalised and outpatients. Magwalivha, (2009) reported HAdV-F as the most common species (77.8%, 7/9)

found in children with AGE in SA. This study supported these findings, as HAdV-F was also the predominant species identified, representing 42.1% of the total HAdV detected. This figure is higher than 20.3% found in children ≤5 years in Burkina Faso (Ouédraogo et al., 2016). In contrast, HAdV-C was dominant in Gabonese children ≤5 years with diarrhoea (Lekana-Douki et al., 2015). In other countries, HAdV-F positive rates in children with AGE were reported at 65% in Brazil in hospitalised children ≤5 years (Pereira Filho et al., 2007), 60.9% among children ≤5 years of age in Kolkata, India (Dey et al., 2011) and 72.2% in Korea in children ≤5 years (Lee et al., 2012).

Recent studies have demonstrated the decline in HAdV-F40 and elevation of HAdV-F41 (Li et al, 2005; Fukuda et al., 2006; Lennon et al., 2007; Verma et al., 2009; Dey et al., 2011; Ouyang et al., 2012; Lu et al., 2017). In the current study, a distinct distribution pattern for HAdV-F40 and HAdV-F41 was observed. The distribution of this strain among hospitalised children with AGE in SA was almost equal. Only a few studies have observed strain-distribution patterns of HAdV-F in Africa. This pattern is consistent with the findings of Moyo et a. (2014) in hospitalised children ≤2 years of age with AGE in Tanzania. However, Lekana-Douki et al (2015) reported the predominance of HAdV-F41 in Gabon.

Human AdV-F (40/41) can cause up to 20% diarrhoea in children, with a widely varying prevalence (Borkakoty et al., 2016). The detection rate in this study was 7% (254/3623), accordance with previous reports in SA, which ranged from 1.6% to 13.2% (41/310) (Kidd et al., 1986; Moore et al., 1998; Tiemessen et al., 1989; Marx et al., 1998; Rossouw, 2004; Magwalivha, 2009). A lower HAdV-F

prevalence of 1.8% in Tanzania (Moyo et al., 2014), 1.77% in Northern Nigeria (Aminu et al., 2007), 2% in Botswana (Basu et al., 2003), 3.1% in Tunisia (Fodha et al., 2006), 4.1% in Gabon (Lekana-Douki et al., 2015) have been reported. However, a higher prevalence of 10.4% reported in Egypt (Kamel et al., 2009), 10.2% in Burkina Faso (Ouédraogo et al., 2016) and 18% in Owo, Ondo State in Nigeria (Babalola et al., 2015) have also been reported. Studies from other parts of the world include, 2.8% in Bangladesh (Khan et al., 1993), 9.8% China (Liu et al., 2014), 5.18 Iran (Dashti et al., 2016), 7.5% in Bulgaria (Mladenova et al., 2015), 8% in Japan (Shimizu et al., 2007), 10.9% in Northeast India (Borkakoty et al., 2016) and 9.8% in Albania (Fabiana et al., 2007). Detection of HAdV-F was less frequent in the winter season. All the studies above show that HAdV-F plays an important role in AGE worldwide and detection rates differ between countries.

Similar to other studies, HAdV-F detection was year round, more frequent in warmer months (Borkakoty et al., 2016). The prevalence of HAdV-F in MKH was significantly higher than that from CHBAH among comparably aged children, indicating that this is an area of high endemicity. This difference may be explained by differences in the distribution of risk factors across the study sites, such as living conditions and socioeconomic status. Chris Hani-Baragwanath Academic Hospital is found in an urban area of Gauteng Province. Although independent risk factors couldn't be identified in this study, improved sanitation in urban areas may reduce diarrhoeal risk by 22% to 36% (Baker et al., 2016). No HAdV-F specific pattern of clinical illness was observed, except that children with HAdV-F had 4-5 maximum number of diarrhoeal episode per day. In other studies

including the MAL-ED, HAdV-F infection was found to be significantly associated with dehydration (Borkakoty et al., 2016; Platts-Mills et al., 2018).

Limitations

The study focussed on hospitalised children with severe AGE while children with mild AGE were not included, therefore the results do not reflect the overall burden of HAdV in the general paediatric population. In addition, the current study did not include an age-matched control group to compare the prevalence of HAdV circulating in symptomatic children with severe AGE and asymptomatic healthy children. Consequently, the data may not be representative of the overall HAdV prevalence and species diversity circulating in the paediatric population in SA. The high number of co-infections with other potential enteric pathogens limited our ability to determine whether or not specific clinical symptoms were associated with HAdV infections.

2.7 Conclusion

This study provides the prevalence of HAdV from hospitalised children with AGE between 2009 and 2014 in SA. Human adenovirus infection occurred throughout the year, particularly in children aged < 2 years, and was frequently detected with other enteric pathogens. No relationship between the presence of HAdV in viral co-infections and severity of disease was observed. As a higher frequency of HAdV infections was noted in the warmer months, clinicians must be alerted to the potential aetiology and possibility of co-infections which may impact on the laboratory diagnosis of AGE. A wide variety of HAdV species were circulating during the study period, HAdV-F was the most common species circulating in

children with AGE In SA. Overall HAdV detection in children with AGE in SA has increased in recent years, enteric HAdV-F remained predominant, but at a low and or similar prevalence compared to previous reports in SA. This is the main HAdV species associated with human enteric infections worldwide, and is therefore of public health importance.

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2.9 Competing interests

The authors declare that they have no competing interests.

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2.11 Author contribution

Rembuluwani Netshikweta: laboratory analysis, data analysis and writing of manuscript

Lizyben Chidamba: data analysis, co-wrote and revision of manuscript

Sandrama Nadan: review of manuscript

Nicola A Page: designed study and edited manuscript

Maureen B Taylor: reviewed and edited manuscript

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CHAPTER 3

WHOLE GENOME CHARACTERISATION OF HUMAN ADENOVIRUS F FROM CHILDREN UNDER 5 YEARS WITH ACUTE GASTROENTERITIS IN SOUTH AFRICA

The Editorial Style of the journal "Infection, Genetics and Evolution" was followed in this Chapter. Objective 5 was addressed in this Chapter.

Abstract

Enteric adenovirus (EAd) serotypes 40 and 41 belonging to species F, are a major causative agent in acute gastroenteritis (AGE) caused by EAds in children worldwide. Most studies in EAd-F have reported on EAd-41 diversity and epidemiological characteristics with a few focussing on serotype 40. In this study, screening of 3623 faecal specimens from children with AGE admitted to four provincial hospitals in South Africa (SA) showed 603 human adenovirus positive cases of which 254 were EAd-F. Ten EAd-F positive faecal specimens were selected for DNA isolation, sequencing, assembly and analysis of EAd-F genomes. Full genome analysis of the ten strains that were characterised showed the identities of the EAd-F species to be serotypes 40 and 41 in equal distribution. Low-level genetic variations (99.94-99.99% nucleotide identity) were observed in EAd-40 strains which were highly similar (99.71-99.72% nucleotide identity) to the Dugan reference strain. However, EAd-41 strains showed divergence with

98.77-99.99% nucleotide identity within genotype and could be divided into two clusters with subcluster identity >99.9%. Phylogenetic analysis showed all EAd-40 to be highly similar and were clustered together in all the analysis including for whole genome, DNA polymerase, long fibre, short fibre and the hexon gene, including the partial sequence of the hexon gene hypervariabele regions. Diversity of EAd-41 strains showed two genome type clusters that were highly similar in trees of whole genomes and individual genes. Inference of recombination events with the Recombination Detection Program showed a single event in all EAd-40 strains. The recombination breakpoints of the five EAd-41 isolates varied in number and location, indicating different evolution origins. These findings highlight the genetic diversity of EAd-40 and -41 isolates circulating in SA and suggests possible evolution from inter-strain recombination.

Key words: adenovirus; acute gastroenteritis, infants, mutation; recombination

3.1 Introduction

The Global Burden of Diseases 2017 (GBD 2017) estimated that diarrhoeal diseases caused more than 1.6 million deaths in 2016 among all ages worldwide, with more than a quarter (26.93%) in children younger than 5 years of age (Naghavi et al., 2017). A number of enteric viruses including rotaviruses,

astroviruses, enteric adenoviruses (EAds) (serotypes 40 and 41), and caliciviruses (norovirus and sapovirus) (Clark and McKendrick, 2004; Kotloff et al., 2013) have been reported to cause gastroenteritis. However, higher proportions of AdV infections have been reported in low income countries from epidemiological studies in acute gastroenteritis (AGE) among children.

Human adenoviruses (HAdVs) belong to the family *Adenoviridae* with a non-enveloped capsid and double-stranded DNA, and are classified within the genus *Mastadenovirus* (Khanal et al., 2018). The HAdV genotypes in the *Mastadenovirus* genus number more than 70 and can be grouped into species HAdV-A to -G (Hage et al., 2015; Kaján et al., 2017; Khanal et al., 2018). A number of the AdVs, including HAdV-types 2, -5, -12, -18 and -31, have been reported in faecal samples, but only EAd-40 and EdA-41, the sole members species F, have specifically been implicated in childhood gastroenteritis, including acute sporadic diarrhoea and occasional outbreaks (Akihara et al., 2005; Aminu et al., 2007; Shimizu et al., 2007; Filho et al., 2007; Chen et al., 2015; Banerjee et al., 2017). Human adenovirus type 52, belonging to species G, has also been identified among gastroenteritis cases but only in one study (Jones et al., 2007).

The genome of EAd-F is approximately 35 kb in length, and compared with other HAdVs, EAds contain two distinct fiber genes. One fiber gene encodes for the long fiber and the other encodes a short fiber which form homotrimers that protrude externally from different vertices of the virion (Kidd et al., 1993; Yeh et al., 1994). The penton base of EAd lacks a RGD motif (Albinsson and Kidd, 1999) which together with the fibres are the primary and the secondary ligands that AdVs use to enter a host cell, a key difference for observed tropism between EAd and other AdVs. In addition, EAd-41 is acid resistant (Favier et al., 2004; Lu et al., 2009), and can bind phospholipids and several sphingolipids, which are rich in gastrointestinal mucosa (Favier et al., 2004).

The distribution and prevalence of EAd types varies by geographical region and even in different areas of the same country. Enteric Ad-41 is currently estimated to account for up to twenty percent of gastroenteritis reported in children and is the second most common etiological agent after rotavirus (Li et al., 2004; Shimizu et al., 2007; Reis et al., 2016). A study in Kolkata, Eastern India reported co-circulation of both EAd-40 and -41 with serotype 41 being the dominant genotype among children below 5 years of age (Dey et al., 2011). However, a follow-up study in the same area reported EAd-40 as more prevalent over EAd-41 (Banerjee et al., 2017). Continuous antigenic changes overtime have been

reported to promote shifts in observed serotype dominance due to immune selective pressure and may explain reported increases in EAd-40 strain dominance over EAd-41 (Ismail et al., 2016). In general, genetic diversity in AdVs is dynamic and is strongly driven by recombination which contributes to the evolution and emergence of new circulating strains (Robinson et al., 2011; 2013; Cook and Radke, 2017).

However, detailed information on the genetic diversity of EAd-40 and 41 is still limited and no studies, to our knowledge, have detailed complete genome sequences of EAd-F in South Africa (SA). Moreover, at the worldwide scale, detailed information on the genetic diversity of EAd-F, and in particular EAd-40, is limited and only a few complete genome sequences of EAd-41 are currently available. This study aimed to investigate genome and evolutionary characteristics of EAd-40 and -41 strains from children hospitalised with AGE, data of which could be important for disease control and future vaccine development.

3.2 Methods and Materials

3.2.1 Selection of EAd-F positive sample for whole genome sequencing

A total of 603 HAdV positive cases (17%) were detected from 3623 faecal specimens collected from children with AGE admitted to four provincial hospitals in SA between April 2009 and December 2014. Ten EAd-F strains were randomly selected for whole genome sequencing from 254 EAd-F positive stool specimens (42%; 254/603) detected during the study (refer Chapter 2).

3.2.2 Whole genome sequencing and comparative analysis.

Viral nucleic acid was extracted from 160 µℓ faecal suspensions (10% w/v in nuclease-free water [Promega Corp.]) using the QIAamp® Viral RNA Mini Kit (Qiagen Inc, Hilden, Germany) according to manufacturer's instructions for automatic extraction in the QIAcube extractor (Qiagen). The extracted nucleic acid was eluted in 60 µℓ of buffer and stored at -40˚C. The MspJI enzymes (New England Biolabs, Inc. Beverly, MA) have been used to selectively degrade human DNA in EAd clinical specimens at 37°C for 8 h. Purified DNA was used to prepare libraries with Nextera XT DNA sample. Libraries were sequenced on Illumina MiSeq platform (Illumina), after which low quality bases were filtered and adapters trimmed, before the resultant paired-end reads were compiled into complete genomes by *de novo* assembly using the VirusTAP analysis tool. Contigs were aligned against reference genomes from Genbank with the Multiple Alignment of Conserved Genomic Sequence with Rearrangements (MAUVE) tool

(Darling et al., 2004). Contigs were aligned against reference EAd-40 (KU162869, Hovi X) and EAd-41 (DQ315364, Tak) genomes before comparative analysis against the prototype strains as well as previously reported strains of EAd-40 and 41. The Genome Annotation Transfer Utility (GATU) (Tcherepanov et al., 2006) was used for annotation of whole genomes based on reference sequences of HAdV-40 (Dugan) and HAdV-41 (Tak) strains and were manually checked (Otto et al., 2011). The nucleotide sequences from HAdV strains from this study and those from NCBI were subjected to comparative genomic analysis to identify nucleotide changes.

3.2.3 Phylogenetic and recombinant analysis

Nucleotide sequences of the complete DNA polymerase, fiber and, hexon genes and genomes of EAd-F from this study and representative sequences downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/nucleotide/) were analysed to understand genome-type clusters (Li *et al*., 2004). The AdV reference strains used for comparative purpose are presented in Table 3.1. Phylogenetic analysis of individual genes and whole genomes was performed on multiple-sequence alignments (MAFFT version 6) (Katoh and Toh, 2008).

Table 3.1. Representative HAdV strains from the rest of the world.

Type/ Serotype	Accession number and Country	Type/ Serotype	Accession number and Country
A12	X73487 (USA)	F41	AB610528 (Japan)
B3	KF268311 (China)		AB610529 (China)
C5	USA/CL_42/1988/5[P5H5F5] (USA)		KF303070 (USA)
D38	KF268312 (German)		KF303071 (USA)
E4	KX384948 (USA)		KX868523 (Sweden)
F40	KU162869 (Finland)		MG925783 (Iraq)
	L19443 (UK)		DQ315364 (Netherlands)
	X51782 (UK)		HM565136 (China)
	AB330121 (Japan)		MH465394 (China)
	M28822 (Sweden)		KY316161 (China)
	AB361416 (Japan)		KY316162 (China)
F41	AB728839 (Japan)		AB610523 (Japan)
	AB610545 (Thailand)		HQ005289 (India)
	AB610520 (China)	G52	DQ923122 (USA)

USA: United Sates of America; UK: United Kingdom

Phylogenetic trees were constructed using the neighbour-joining (NJ) method on the Molecular Evolutionary Genetic Analysis program (MEGA6) with 1000 bootstraps (Tamura et al., 2013).

Recombination events were assessed among the different EAd-F strains with the Recombination Detection Program (RDP4) suite (Martin et al., 2017) based on nucleotide sequence alignments of individual genes and genomes used in the phylogenetic analysis. Potential recombination events between sequences were predicted using multiple default mode methods including, 3Seq (Boni et al., 2007), BootScan (Martin et al., 2005) Chimaera (Posada and Crandall, 2001),

GENECONV (Padidam et al., 1999), MaxChi (Maynard Smith, 1992), PhylPro (Weiller, 1998), RDP (Martin and Rybicki, 2000), SiScan (Gibbs et al., 2000) and LARD (Holmes et al., 1999). Recombinant events were considered significant, only when supported by a minimum of two of the nine methods and were visualised on SimPlot 3.5.1 with default settings (Lole et al., 1999)

3.3 Results

3.3.1 Human adenovirus type F identification and genome sequencing.

Whole genome sequencing of the EAd strains from the randomly selected EAd-F positive faecal specimens showed their identities to be EAd-40 and 41 in equal distribution. The strains (5 EAd-40 and 5 EAd-41) were from different study sites, different years with real-time polymerase chain reaction (PCR) threshold cycle (Ct) values of <20. Illumina sequencing and processing with VirusTap including adapter trimming, and removal of RNA, bacteria and host genome, assembly and non-viral filtering of reads generated 2-7 contigs ranging in size on average from 1,681 bp-32,125 bp and totalling 33,346 bp – 33,921 bp per strain.

3.3.2 Comparative whole genome analysis.

A whole-genome phylogenetic tree of the ten strains from this study, together with representative HAdV species is shown in Figure 3.1. The South African EAd-40 strains showed 99.94-99.99% nucleotide similarity to each other and 99.71-

99.72% nucleotide similarity to the Dugan reference strain. In contrast, the EAd-41 strains displayed 98.77-99.99% nucleotide sequence similarity among themselves, with two distinct clusters. Strains SA13026 and SA6749 showed a 99.93% nucleotide similarity to each other and strains SA7335, SA13020 and SA12680 had a 99.96-99.99% nucleotide identity. Strains SA13026 and SA6749 clustered with strain MU35 from Iraq with 99.68-99.7% nucleotide identity. The other three (SA7335, SA13020 and SA12680) EAd-41 strains clustered together with strains NY/2010/4845 and 10-4851 (USA), GyK253 (Sweden) and SaP3-3F (Japan) with a >99.82% within group nucleotide identity. The reference strain Tak (Netherlands) and the four strains NIVD103, Anhui/201, SH/2015/D240 and SH/2015/D16 (China), clustered separately with a >99.4% within group identity. Between group nucleotide identities for the observed three clusters ranged from 98.77 to 99.12%.

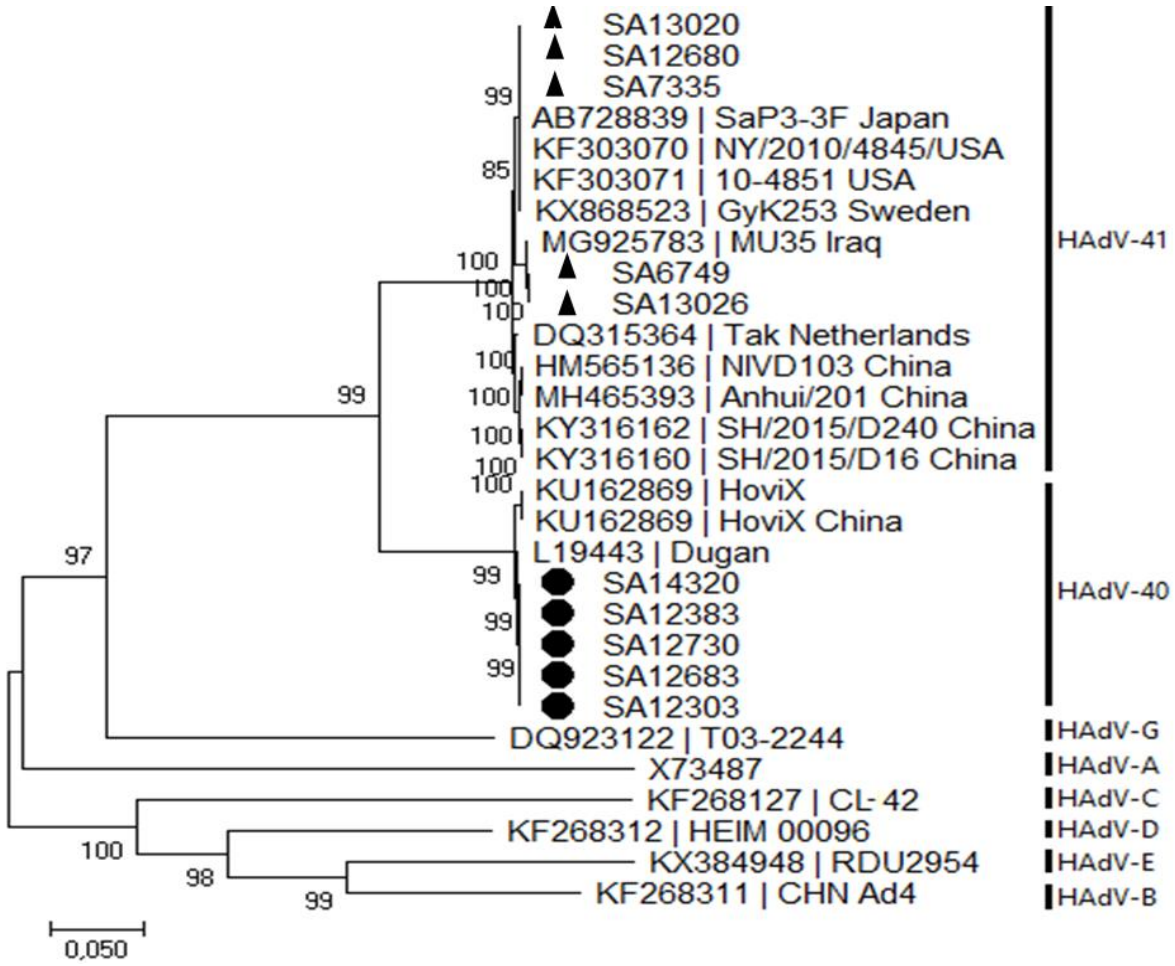

Figure 3.1: Phylogenetic tree of EAd whole genome from South Africa (SA) and published strains. The EAd-40 from SA are labelled with black triangle and EAd-41 are labelled with black circle. The tree was constructed using the whole genome with the neighbour-joining method and 1,000 bootstrap replicates. Groupings with bootstrap support >70% were considered significant. Reference strains are labelled by their GenBank accession numbers, strain names and country of origin. The scale bar indicates an evolutionary distance of 0.05 nucleotides per position in the sequence.

Most of variations observed in EAd-40 strains were single nucleotide mutations and located mainly in the coding regions. However, the long fibre, short fibre, hexon and DNA polymerase genes of our isolates showed some insertions or deletions (InDels) compared to that of the reference strains (Figure 3.2). The InDel patterns of EAd-41 suggest two genome type clusters (Figure 3.2). The two

strains of EAd-41 (SA13026 and SA6749; Figure 3.2) shared unique nucleotide substitution similarities, which distinguished them from other EAd-41 sequences.

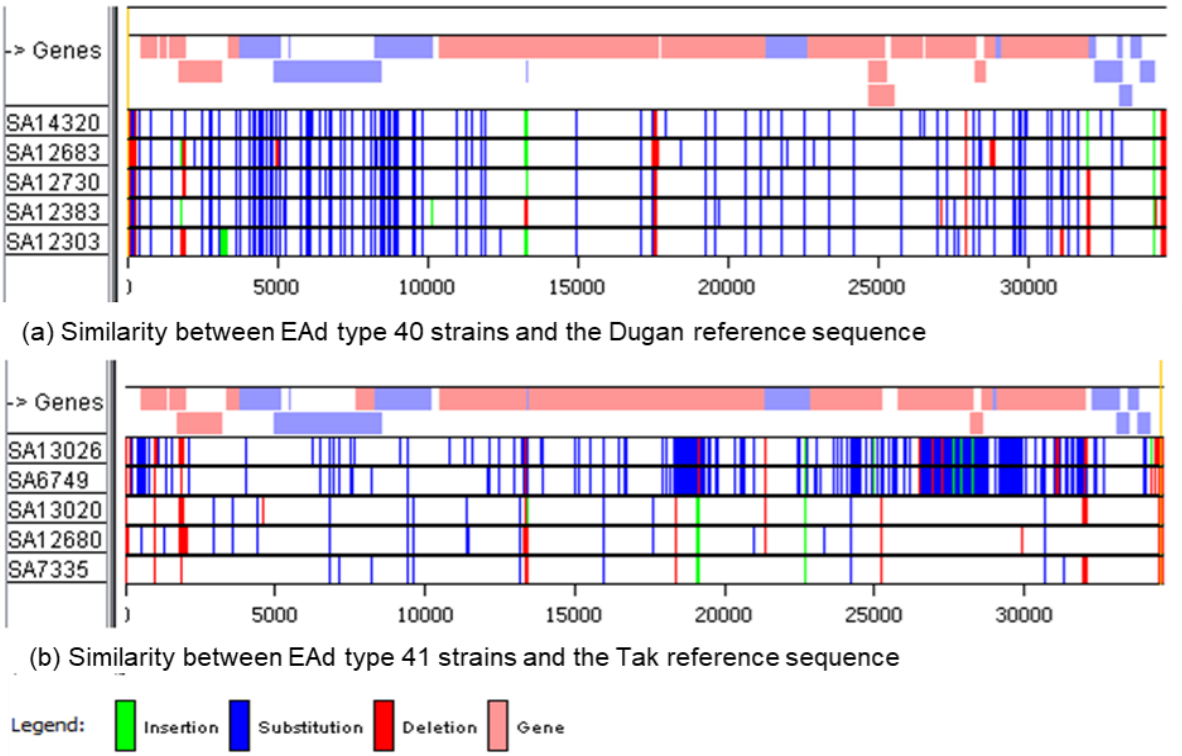

Figure 3.2: Overview summary of (a) EAd-40 strain nucleotide similarity to reference sequence NCBI accession L19443, strain Dugan and (b) EAd-41 strain nucleotide similarity to reference sequence NCBI accession KF303070, strain NY/2010/4845

3.3.3 Molecular characterisation and phylogenetic analysis of hexon, fiber and DNA polymerase genes

The nucleotide sequences of the DNA polymerase, hexon, long fiber and short fiber gene of the ten EAd strains and those of representative strains were analysed. The hexon gene was analysed both as a whole gene and targeting of the six hypervariable regions (HVRs) (HVR1, HVR2, HVR3, HVR4, HVR5, HVR6). The phylogenetic tree based on the HVRs of the hexon gene is shown in Figure 3.3.

The hexon gene nucleotide sequences of EAd-40 strains SA14320, SA12383, SA12730, SA12683 and SA12303 showed 99.69-99.86% nucleotide identity to each other and 99.49-99.53% nucleotide identity to reference strains FL19443 and AB330121 (Dugan).

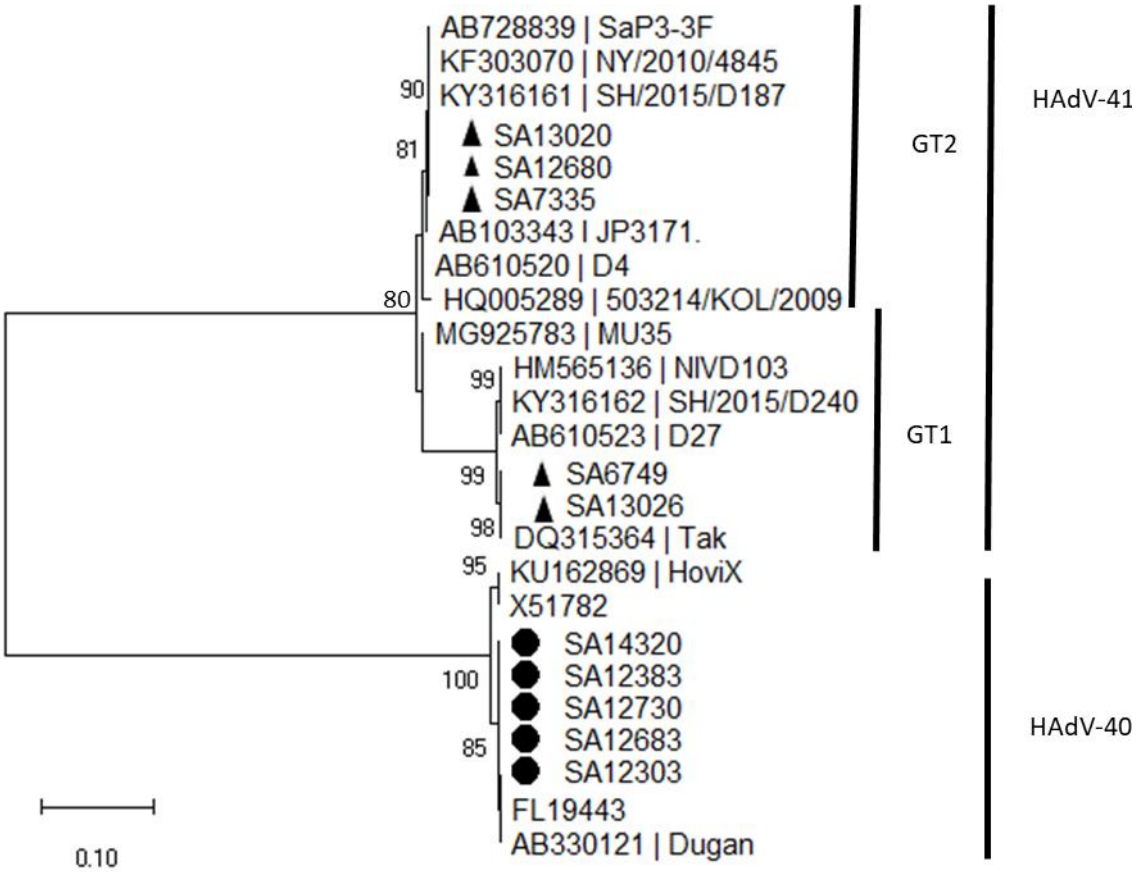

Figure 3.3: Phylogenetic tree based on the hypervariable region (HVR1–HVR6) of the hexon gene of the EAd-40 (black circle) and EAd-41 (black triangle) strains isolated from children <5 years of age in South Africa. Two genomic type clusters of EAd-41 are evident. The tree was constructed using the neighbour-joining method and 1,000 bootstrap replicates. Groupings with bootstrap support >70% were considered significant.

The five EAd-41 strains formed two distinct genomic clusters (GTCs) (Figure 3.3). In the GTC1 group, strains SA6749 and SA13026 clustered with the prototype strain Tak (AB330122), NIVD103 (HM565136) and SH/2015/D240 (KY316162).

The nucleotide identity of sequences within the GTC1 group ranged from 99.21 to 99.96%. The other three South African EAd-41 strains, namely SA7335, SA13020 and SA12680, clustered together with reference strains 503214/KOL/2009 (HQ005289), D4 (AB610520) in the second GTC (GTC2) with a nucleotide identity of 99.75-100% within the cluster.

Phylogenetic analysis of the DNA polymerase, long and short fiber genes consistently clustered the five EAd-40 strain in a single group separate from the reference strains (Fig. 3.4). For all three genes, there was >99.86% nucleotide identity between the South African strains, with 99.03-99.78% nucleotide identity to the reference strains. Clustering of the five EAd-41 isolates with respect to the DNA polymerase, long and short fiber genes showed consistent grouping into two clades similar to that for hexon genes. However, less similarity was observed for the five isolates in the DNA polymerase gene, with isolates not clearly branching out as two distinct clusters and having >99.75% within group nucleotide identity (Figure. 3.4). The entire hexon gene phylogenetic analysis (Figure. 3.4) was in accordance with the HVRs-based tree (Figure. 3.3). Trees of the long and short fiber genes also formed two clusters but different from that of hexon gene (Figure. 3.4).

3.3.4 Comparative antigenic analysis of the fiber protein

Comparative analysis of the short fiber gene amino acid sequences in the shaft region of EAd-41 strains and reference strains showed a 15 amino acid deletion in two of the South African strains (SA6749 and SA13026) and the reference

strains D26, D31, MU35, D31 and 503221/KOL/2009 in the 15th repeat motif (Fig. 3.5).

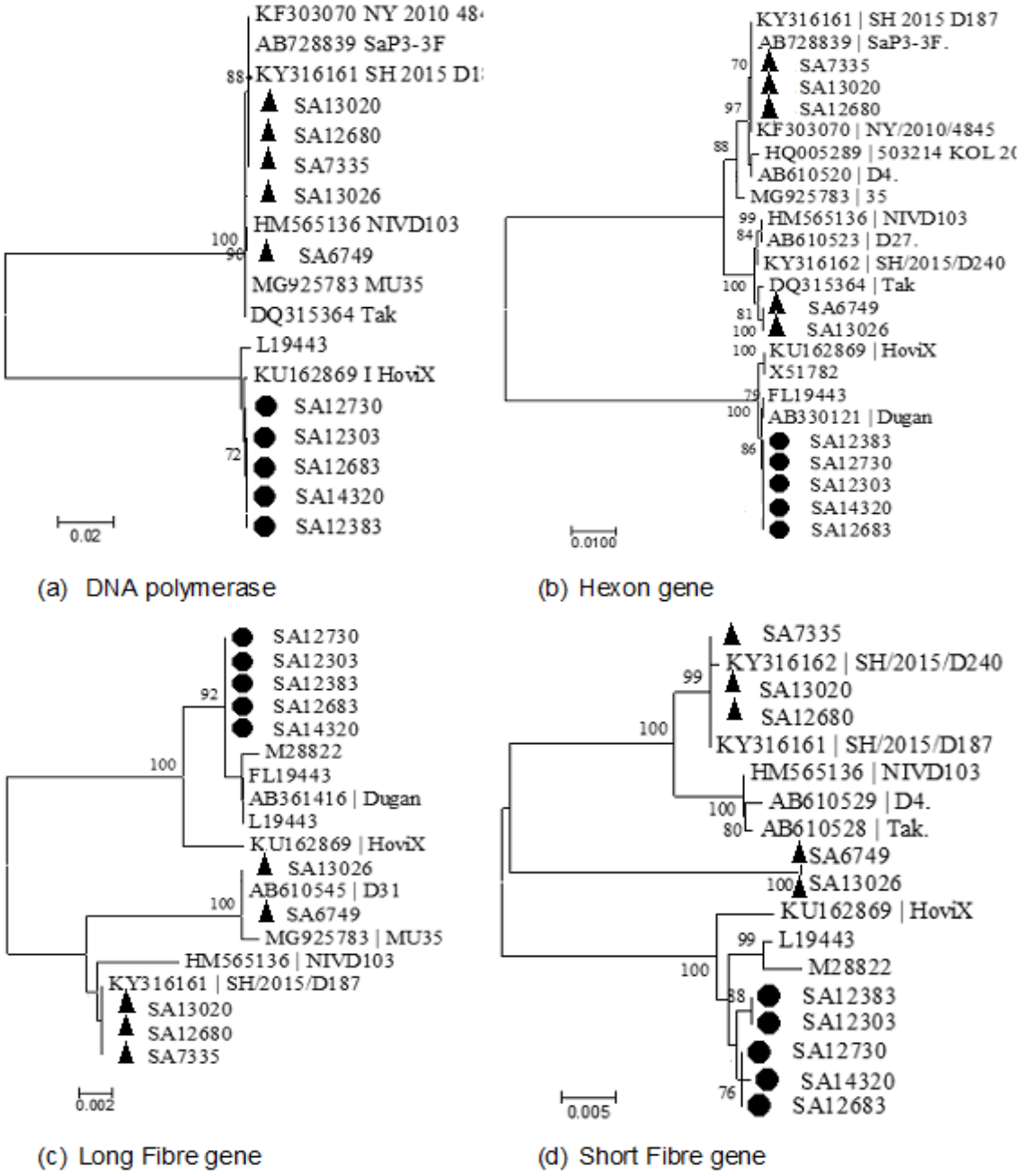

Figure 3.4: Neighbour-Joining phylogenetic relationship of five EAd-40 (black circle) and five EAd-41 (black triangle) isolates including reference sequences inferred from (**a**) DNA polymerase, (**b**) hexon gene, (**c**) long fiber gene, and (**d**) short fiber gene. The tree was constructed using the neighbour-joining method and 1,000 bootstrap replicates. Groupings with bootstrap support >70% were considered significant.

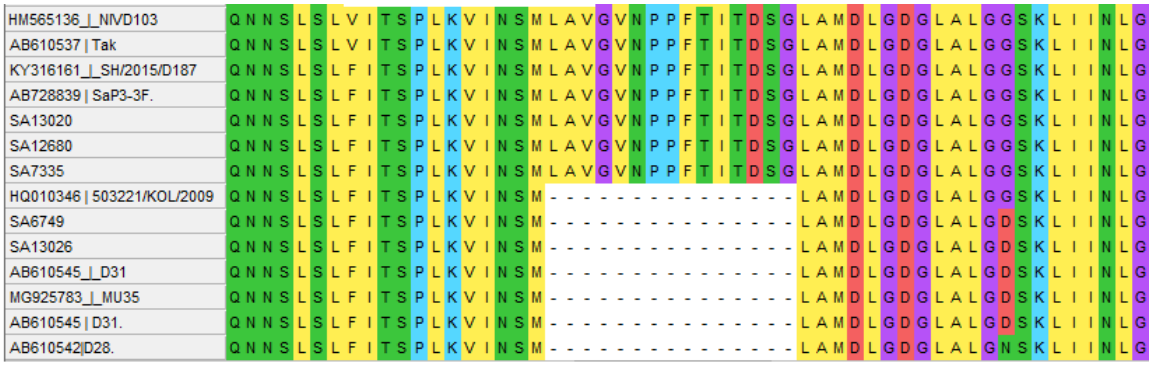

Figure 3.5: Alignment of the deduced amino acid sequences of the fibre gene shaft region from nine representative and the five South African EAd-41 strains. Five of the nine representative strains and two of the South African strains (SA6749, SA13026) show a 15 amino acid deletion compared to four representative isolates and three South African strains (SA13020, SA12680, SA7335).

3.3.5 Recombination analysis

A single recombination event was observed for the five South African EAd-40 strains. The recombination breakpoint of which was located in the Iva2 and pol gene region and originated from a recombination between HoviX and Dugan (Figure. 3.6). Recombination in EAd-41 divided the strains into two groups similar to those observed for the hexon GTCs. The strains SA6749 and SA13026 in GTC1 had a total of six recombination events of varying strength while the GTC2 strains (SA7335, SA13020 & SA12680) had four (Table 3.1). Analysis of individual genes for recombination showed single events in the hexon. No signals of recombination were detected in the DNA polymerase, long fibre and short fiber genes using RDP4 software. The SimPlot software was used to further analyse, putative recombinants with previously detected recombinants used as query, while the Major parent and Minor parent were used as references. The bootscan analysis results further confirmed the recombination results of RDP analysis (Figure. 3.6).

Table 3.2. Recombination events detected with RDP4 from the alignment of whole genomes for EAd-40 and EAd-41. The average P-value of detection methods are indicated.

			Detection Methods						
Breakingpoints	Minor parent	Major parent	RDP	GENECONV	Bootscan	Maxchi	Chimaera	SiSscan	3Seq
Relative to Tak			Whole genome recombinant events in EAd41 strains 13026 & 6749						
7749* - 12762*	NIVD103	NIVD103	5,36E-03	1,45E-03	NS	5,08E-08	1,99E-03	7,59E-09	6,91E-08
29552* - 30831*	Tak	unkown	1,90E-03	NS	2,38E-03	1,05E-06	4,68E-04	NS	1,60E-05
18082* - 30886*	NIVD103	SA12680	4,06E-04	NS	2,94E-02	2,04E-08	3,03E-04	1,05E-23	3,10E-04
31295 - 31739*	Tak	unkown	1,75E-06	4,26E-07	3,12E-07	9,34E-03	3,34E-02	NS	2,31E-05
31763 -33607	Tak	SA12680	2,77E-20	5,10E-21	3,81E-21	9,22E-13	2,12E-10	3,82E-18	1,26E-15
33608* - 2544*	SA12680	SA12680	6,10E-05	1,41E-03	5,01E-05	2,86E-02	NS	NS	NS

Table 3.2 continued

Relative to Tak		Whole genome recombinant events in EAd41 strains SA7335, 13020 &12680							
26334 -29582	SA6749	NIVD103	1,75E-37	4,50E-40	1,18E-24	1,10E-24	1,56E-23	4,09E-27	6,96E-33
34048 -13169	unkown	Tak	1,34E-18	1,71E-12	NS	6,70E-13	7,66E-13	1,18E-17	2,22E-15
19771 -23401	unkown	NIVD103	1,37E-05	1,98E-02	1,51E-02	4,34E-10	1,41E-07	NS	1,88E-05
13148* -17886	unkown	NIVD103	5,02E-10	2,50E-05	1,20E-07	4,38E-05	1,06E-05	2,20E-24	1,03E-09
Relative to SA14320		Whole genome recombinant event in EAd40 stains SA12683, SA14320, SA12730, SA12383 & SA12303							
3475-8956	HoviX	Dugan	1,78E-11	5,53E-07	7,39E-05	2,34E-08	6,54E-09	NS	2,65E-06
Relative to NIVD103		Hexon geno recombinant event in EAd41 stains 13026 & 6749							
394_ 1913	unknown	NIVD103	NS	NS	NS	2,81E-02	NS	NS	1,24E-03

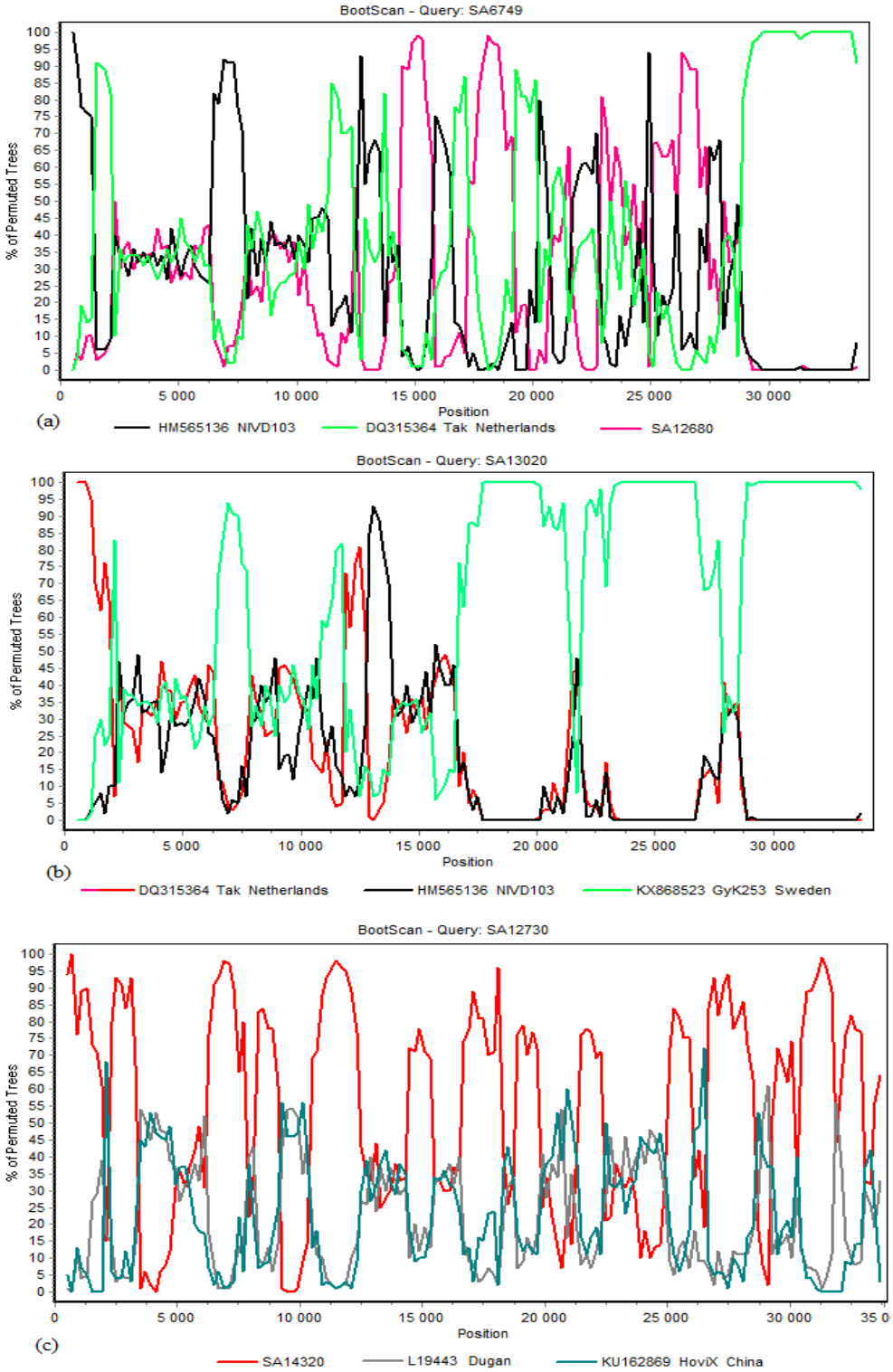

Figure 3.6: Whole genome sequences bootscan plots of the EAd-41 recombinant strains (a) EAd SA6749 & SA13026, (b) SA7335, SA13020 & SA12680 and (c) EAd-40 strains SA12683, SA14320, SA12730, SA12383 and SA12303. Single representative query sequence were used for each group of similar sequences. Genome analysis was set with a 1000 bp window size and 200 bp step size. Reference sequences are coloured accordingly in the colour schemes below the figure.

3.4 Discussion

Human adenoviruses are responsible for a wide range of diseases affecting various organ systems with EAd-F (type 40/41) being the second most important cause of viral gastroenteritis in infants (Dey et al., 2011; Oude Munnink and van der Hoek, 2016). Although molecular epidemiological studies have been reported for EAds in SA, no data has been provided for whole genome studies. In this study, whole-genomes of ten EAd strains that were randomly selected from 254 EAd-F positive specimens from children with acute diarrhoea sequenced and analysed. Evaluation of complete genome and individual gene sequences showed one monophyletic cluster of EAd-40 and two different genetic clusters EAd-41 to be circulating. Previous research has shown varying prevalence in EAd-F (type 40/41) species. A study in Shanghai, China reported EAd-41 as the prevalent HAdV genotype causing gastroenteritis in children (Lu et al., 2015). Whereas in Kolkata, India, a study (2013–2014) in children seeking health care with gastroenteritis showed EAd type 40 to be the prevalent genotype (Banerjee et al., 2017). Continued monitoring for types 40/41 in the same population where EAd is endemic may provide an answer concerning serotype drifts, if any exist; which could establish molecular evolutionary trend of EAd.

The HVRs, have traditionally been used for EAd genome typing and relationship analysis (Dey et al., 2011; Ismail et al., 2016; Banerjee et al., 2017). Li et al. (2004) reported two GTCs of EAd-41, GTC1 and GTC2, based sequencing of the hexon gene in Vietnamese children. In this study, analysis of the entire hexon gene of EAd-41 divided the South African strains together with the reference strains into GTC1 and GTC2 as previously described (Li et al., 2004; Lee et al.,

2017). However, it was interesting to note that clustering of SA strains were similar for hexon gene and HVRs. Previous studies have reported small incongruence between phylogenetic tree of HVRs and whole hexon genes among closely related isolates, and suggested the importance of variants of non-HVR regions in genome typing (Li et al., 2017). This existence of two GTCs of EAd-F41 indicate accumulation of amino acid mutations in the HVRs of the hexon gene (Dey et al., 2011).

Interestingly, analysis of the amino acid sequences of the fiber gene revealed deletion of 15 amino acids in the two SA strains in the 15th repeat motif of EAd-41. This deletion of the 15th repeat motif from novel EAd-41 strains decreases the length of the fiber. However, this deletion has not been conserved in the strains circulating in SA as only two of the five EAd-41 strains has the deletion. In India, Dey et al (2011) reported conserved sequence deletion among all EAd-41 strains detected among children <5 years of age.

Phylogenetic relationship among our isolates remained clearly resolved as per the hexon GTC clusters in the long and short fibre protein trees although the resolution was poor for the DNA polymerase gene. However, varied clustering was observed with respect to the short fibre gene as has been previously reported, with isolates forming subclades and relatively greater similarity distances in sub clusters (Li et al., 2017).

Recombination is the major contributor to the observed genetic diversity of adenovirus and drives their evolution (Robinson et al., 2011; 2013). Phylogenetic

analysis of the individual genes showed two clades for EAd-41 and one for EAd-40 similar to the observations in recombinant analysis. The observed variation in in frequency and size of recombination events among the isolates may suggests evolution during different periods pressure, most probably sharing a common ancestor as suggested by inter-strain recombination events. Although an increasing number of reports have cited EAd-40 and -41 with increasing prevalence in acute gastroenteritis, the majority of complete genomes are mostly those for EAd-41 and we could only verify three for the EAd-40 to be available in the GenBank database. Complete genome sequences in particular those for EAd are needed to improve our understanding on genomic epidemiology and pathogenesis of EAd-40 and -41. Findings from this study have shown further evidence on the genetic diversity and recombination of EAd-40 and -41 circulating in SA. This study has further shown the importance of whole-genome sequencing in unravelling genetic variation and the role of recombination, in understanding adenovirus evolution which is crucial in future strategies in vaccine development and vaccination.

3.5 Ethical Approval

This project was approved by the Human Research Ethics Committee (Medical), University of Witwatersrand (M091018) and Faculty of Health Sciences Research Ethics Committee, University of Pretoria (383/2015).

3.6 Acknowledgements

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CHAPTER 4

MOLECULAR EPIDEMIOLOGY OF HUMAN BOCAVIRUS INFECTION IN CHILDREN HOSPITALISED WITH ACUTE GASTROENTERITIS IN SOUTH AFRICA, 2009 TO 2015

The Editorial Style of the "Journal of Medical Virology" was followed in presentation of this Chapter. Aspects of Objective 1, 3, 4 and 5 are addressed in this Chapter

Abstract

Human bocavirus (HBoV) is known to be associated with a variety of clinical manifestation including acute gastroenteritis (AGE). Despite their global prevalence, no data is available on the epidemiology of HBoV AGE in South Africa. Beginning April 2009 to April 2015, a total of 3765 stool specimens were collected from children <5 years of age hospitalized for diarrhoea and screened for selected enteric viruses, bacteria and parasites. HBoV was detected in 5.63% (212/3765) of stool specimens; the majority of which were from children ≤2-year of age (92%, 195/212). Viral co-infections were found in 67% (142/212) of HBoV cases, while in fully screened specimens (virus, bacteria and parasites), 83.1% (74/89) had evidence of co-infections. In all co-infections, only HAdV was significantly associated with HBoV (adjusted Odds Ratio (aOR))=1.68; (95% CI 1.10-2.52; p=0.015) in multivariate analysis. Although HBoV infections were reported throughout the year, most cases were detected between February and May of each year. Human bocavirus 1 was the most prevalent genotype observed (79.6% (152/191)) followed by HBoV-3 (13.6% (26/191)), HBoV-2 (5.2% (10/191)) and HBoV-4 in 1.6% (3/191). This first molecular epidemiological study

of HBoV in diarrhoel specimens in SA reports prevalence data which correlates with worldwide reports, and a considerable HBoV genotype diversity was noted. However no association between HBoV and AGE was observed.

Keywords: Human bocavirus, South Africa, epidemiology; prevalence; diarrhoea, genotypes

4.1 Introduction

Diarrhoea is a leading cause of morbidity and mortality in children under 5 years of age, particularly in Africa and other low-income countries.[1–5] Despite the decline in mortality from diarrhoea in many countries in recent years, mainly due to environmental improvements and advances in health care, vaccination and treatment, diarrhoea remains a common cause of death among children.[6] Diarrheal disease was responsible for nearly 500 000 deaths among children younger than 5 years in 2015 and was the fourth leading cause of death in this age group[1,7] which is equivalent to 9% of all deaths in this age group.[8] Among the causes of diarrhoeal disease, viruses including rotavirus (RV), norovirus (NoV), astrovirus (AstV), and adenovirus (AdV), are recognised as the major contributors, particularly in children.[9–12]

Human bocaviruses (HBoVs), first described in 2005[13] are members of the family *Parvoviridae*, subfamily *Parvovirinae*, genus *Bocaparvovirus* and have been reported worldwide in various studies as a potential cause of diarrhoea.[14–16] Since 2005, four HBoV genotypes (HBoV-1-HBoV-4) have been reported and are characterised by a 5.3 kb single-stranded DNA genome encapsulated in a non-

enveloped icosahedral capsid protein coat.[17] The genome has three open reading frames (ORFs), with non-structural proteins, NS1 and NP1 encoded in the first and second frames, respectively, while the third ORF encodes the viral capsid proteins VP1/VP2.[15]

Human BoV-1 was the first genotype to be described and has a prevalence rate of approximately 4.4–25% in symptomatic children with upper and lower respiratory tract infections.[13,15] The other genotypes (HBoV-2, 3 and 4) have been detected in stool specimens of children with acute gastroenteritis (AGE) at a prevalence of 1.4–24.6%, 0.5-2.7% and 0-0.5%, respectively.[18–20] Reports on HBoV in Africa have mainly focused on respiratory infections,[4,21–28] with little done in AGE. The few African studies on HBoV in AGE cases include detection rates of 2% for children under two years of age from Egypt[29] and 2.2% for children under 5 years of age from Gabon.[30] Large-scale epidemiological studies of HBoV in children with AGE are lacking in Africa and little is known about molecular epidemiology of HBoV in children with AGE. HBoV detection rates of 29% (28/96) in Nigeria and 33% (32/96) in Tunisia have also been reported in stool specimens from children aged between 4 months and 15 years with non-polio acute flaccid paralysis.[31]

In South Africa (SA), HBoV have been described in respiratory infections, with prevalence of 11% in children <2 years diagnosed with pneumonia,[21] and in 9.5% of HIV-infected and 13.3% of HIV-uninfected children hospitalised with respiratory infections.[25] A further study reported 11.5% HBoV in children ≥ 3 months to < 5 years of age with acute otitis media.[26] Although the role of HBoV

appears to have been established in respiratory infections, its significance in AGE is still unclear as the virus is frequently detected in co-infections with other enteric pathogens and have also been reported in healthy individuals.[31–34] Additional evidence and studies are, therefore, needed to gain a better national and global understanding of HBoV significance in AGE. In this study, diarrhoeal stool specimens from children under 5 years of age, who were hospitalised for AGE were collected in SA from April 2009 to April 2015 and analysed for HBoV prevalence, epidemiology and molecular diversity.

4.2. Materials and methods

4.2.1 Ethical approval

Ethical approval for this study was granted by the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, South Africa: Number (383/2015). The parent Rotavirus Sentinel Surveillance Program has been approved by the University of Witwatersrand (Approval No.M091018).

4.2.2 Study participants and sites

The study was conducted as a part of the SA Rotavirus Sentinel Surveillance Program (RSSP) which commenced in April 2009. Between April 2009 and April 2015, diarrhoeal stool specimens were collected from children under 5 years of age, who were hospitalised for AGE, defined as passing of three or more liquid or loose stools within 24 hours, for 7 days or less. Patients were recruited from four different hospitals including Chris Hani-Baragwanath Academic Hospital (CHBAH) in an urban area of Gauteng Province, Edendale Hospital (EDH) in a

peri-urban area of KwaZulu-Natal Province and, Matikwana (MKH) and Mapulaneng Hospitals (MPH) in rural areas of Mpumalanga Province. A standardised questionnaire was used to record information on demographics, clinical presentation, medical history, socioeconomic and associated factors for AGE. All stool samples were collected within 48 h of admission and transported on ice to the Virology Division, Centre for Enteric Diseases (CED) at the National Institute for Communicable Diseases (NICD) where they were stored at 4-8˚C until processed.

4.2.3 Specimens processing and nucleic acid extraction

Faecal suspensions (10% w/v in distilled water) were prepared and clarified by centrifugation. Viral nucleic acid were extracted using QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions for automatic extraction in QIAcube extractor (Qiagen, Hilden, Germany) and eluted in 60 µℓ of buffer before storage at -40˚C.

4.2.4 Pathogens screening

All specimens were screened for HBoV by real-time PCR targeting the highly conserved nonstructural proteins 1 (NS1) region.[35] Specimens were also tested for the presence of five other enteric viruses: namely RV, NoV, SaV, AstV and human adenovirus (HAdV) using enzyme-linked immunosorbent assay (ELISA), conventional RT-PCR and/or real-time PCR based on the method described by Page et al., (2016).[36] Enteric bacteria and parasitic screening were also performed as described by Page et al. (2016). Commercially available nucleic acid amplification COBAS AmpliPrep/COBAS TaqMan HIV-1 test (Roche

Diagnostic Systems, Branchburg, NJ, USA) was used to test for HIV from dried blood spots collected from children at admission. The COBAS AmpliPrep instrument enabled automated specimen preparation and COBAS TaqMan analyzer allowed automated amplification and detection for HIV.

4.2.5 Nucleotide Sequencing, Genotyping, and Phylogenetic Analysis

For genotyping HBoV strains from all HBoV-positive stool specimens were subjected to nested-PCR targeting the partial region of VP1 gene as described previously.[31] The first-round of the nested-PCR was carried out using the AK-VP-F1 (5'-CGCCGTGGCTCCTGCTCT-3') and AK-VP-R1 (5'-TGTTCGCCATCACAAAAGATGTG-3') primers set, and the second round using the AK-VP-F2 (5'-GGCTCCTGCTCTAGGAAATAAAGAG-3') and AK-VP-R2 (5'-CCTGCTGTTAGGTCGTTGTTGTATGT-3'). Briefly, 3 µl of DNA was added to 22 µl of the first-round PCR reaction mixture consisting of 20 pmol of both DNA sense primers (University of Cape Town), 2.0 mM MgCl2, 0.25 mM of each deoxynucleoside triphosphate (dNTP), 1 X reaction buffer and 5 U GoTaq® Hot Start polymerase. Unless stated otherwise, all reagents used were obtained from Promega (Promega Corp., Madison WI). For the second PCR, 1 µl of the first-round product was used as the template in the PCR mixture as described above containing the second round primers. Amplicons were purified either directly using ExoSAP-it (USB Corporation, OH) or QIAquick PCR purification kit (Qiagen). Purified PCR products were sequenced in an Applied Biosystems 3500 automated genetic analyzer (Life Technologies) with a BigDye v3.1 terminator cycle sequencing kit (Life Technologies). The forward and reverse sequences were assembled by DNA Baser Sequence Assembler v5.15 (Heracle BioSoft

SRL Romania, http://www.DnaBaser) and subjected to BLAST search (http://www.ncbi.nlm.nih.gov) to confirm their identity. Reference nucleotide sequences of HBoV strains obtained from GenBank were aligned together with South African strains using MAFFT version 6.[37] Phylogenetic relationships among strains were inferred using the maximum-likelihood statistical method. Phylogenetic analyses were carried out using MEGA 7 software version 7.0.14 (MEGA, PA, USA)[38] based on the Jukes-Cantor model.[39] Eight HBoV positive stool specimens were selected for whole genome sequencing (WGS) from 212 HBoV positive specimens. Whole genome sequencing was done as described previously (refer: Section 3.2.2).

4.2.6 Statistical analysis

All statistical analysis were performed using Stata software package, version 11.1 (Stata Corp., College Station, TX). Categorical variables were described using frequency and percentages. Demographic data, clinical and environmental factors associated with HBoV detection were compared in children with or without HBoV infection. Chi-square and Wilcoxon rank-sum tests were used to compare means and medians, respectively, while bivariate and stepwise multivariable logistic regression were used to identify characteristics that were associated with HBoV infections. Variables with a *p*-value <0.2 according to a bivariate analysis were included in the multivariate analysis. The p-value <0.2 was used for elimination because most predictors in the data set are variables that could be relevant. However, if the predictor has a p-value greater than 0.2 in a univariate analysis it is highly unlikely that it will contribute anything to a model. Differences within groups were calculated as odds ratios (OR) and *p*-values <0.05 were

considered statistically significant. A modified Vesikari scoring system was used to calculate the clinical severity of diarrhoeal infections using the clinical features recorded. An episode was considered mild for a Vesikari score 5 or less and moderate to severe for 6 or more. Missing data were accounted for in the analysis by pairwise deletion; therefore, the sample size varies slightly with regard to a particular variable due to missing data.

4.3 RESULTS

4.3.1 Prevalence of HBoV

Beginning April 2009 to April 2015, a total of 3765 stool specimens were collected from hospitalised children with AGE, of which 5.63% (212/3765) tested poitive for HBoV. Annual detection rates ranged from 4.16 to 7.6 % (mean = 5.4%) between 2009 and 2014, and with a doubling of the rate (10.5%) in 2015 (Figure 4.1; Table 4.1). Human BoV detection was similar across the sites throughout the study period (data not shown). Detection of HBoV among different ages increased from 4.70% (67/1407) in 0-6 months olds to 6.61% (74/1119) for 7-12 months old, 6.40% (36/564) for 13-18 months old and 6.55% (19/290) for 19-24 months old. The lowest detection rate (4.47%) was observed in children older than 25 months (Table 4.1). The median age of children with HBoV was 10 months [interquartile range ([IQR]: 5.6-15). Bivariate analysis showed that children aged 7-24 months had higher prevalence of HBoV infection than those aged > 6 months or older than 25 months (Table 4.1).

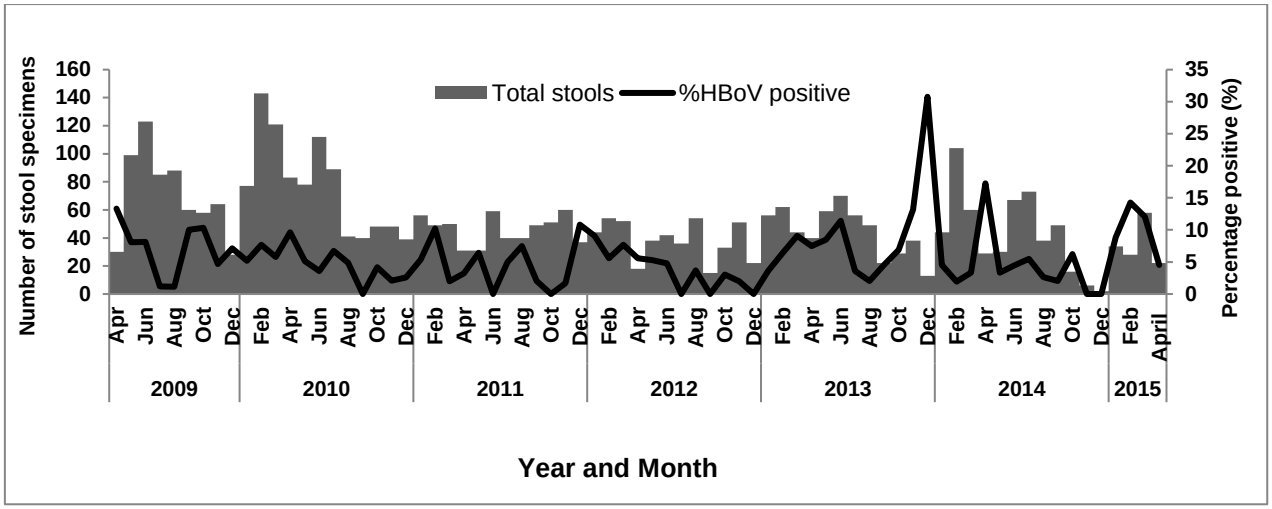

Figure 4.1: Monthly distribution and percentage positive of HBoV detected in children with acute gastroenteritis, April 2009–April 2015.

The monthly and seasonal prevalence of HBoV detection showed circulation throughout the year, although HBoV was more prevalent in summer (December-February) (6.50%; 58/892) and autumn (March–May) (7.19%; 70/973) compared to winter (June–August) (4.47%; 52/1163) and spring (September-November) (4.34%; 32/737) (Figure 4.1; Table 4.1). Bivariate analysis showed attending nursery school, Vesikari score, and co-infections with bacteria, SaV, parasites and HAdV to be associated with HBoV infection (Table 4.1). However, only nursery school attendance (aOR=1.69 (95% CI 1.13-2.54; p=0.011) and HAdV co-infection (aOR=1.69 (95% CI 1.13-2.54); p=0.011) were significant predictors in multivariate analysis of HBoV among the children. Evaluation of the link between specific AGE symptoms, including the role of HBoV in HIV-positive patients showed no significant association with HBoV infections (Table 4.1).

Table 4.1: Bivariate and multivariable analysis of demographic, clinical characteristics and environmental features associated with HBoV detection in children with AGE. Only variables with p-values <0.2 in the bivariate analysis were reported and included in the multivariable model.

Parameter	**Bocavirus prevalence n/N (%)**	**Bivariate analysis**		**Multivariate analysis**	
		Odds Ratio (OR; 95% Confidence Interval (CI)	**p-value**	**Adjusted OR (aOR; 95% CI)**	**p-value**
Demographic characteristics					
Age (in months)					
0-6	66/1407 (4.70%)	ref	ref		
7-12	74/1119 (6.61%)	1.43 (1.02-2.02)	0.037		
13-18	36/564 (6.40%)	1.38 (0.91-210)	0.127		
19-24	19/290 (6.55%)	1.42 (0.84-2.41)	0.127		
≥25	17/380 (4.47%)	0.95 (0.55-1.64)	0.858		
Unknown	0/4 (0.00%)				
Gender					
Male	81/1620 (5.0%)	ref	ref		
Female	131/2144 (6.11%)	1.26 (0.95-1.68)	0.107		
Unknown	0/1 (0.00%)				
Year of collection					
2015	15/142 (10.52%)	ref	ref	ref	ref
2014	22/519 (4.24%)	0.37 (0.188-0.74)	0.005	0.43 (0.19-0.96)	0.041
2013	41/538 (7.62%)	0.69 (0.37-1.30)	0.259	0.66 (0.31-1.41)	0.294
2012	20/459 (4.36%)	0.38 (0.19-0.77)	0.007	0.39 (0.17-0.90)	0.028
2011	23/553 (4.16%)	0.36 (0.18-0.72)	0.004	0.38 (0.17-0.84)	0.018
2010	50/919 (5.44 %)	0.48 (0.260.89)	0.020	0.40 (0.17-0.89)	0.026
2009	41/635 (6.46%)	0.58 (0.31-1.08)	0.090	Omitted	Omitted
Monthly					
Jan	18/311 (5.79%)	ref	ref		
Feb	29/440 (6.59%)	1.14 (0.62-2.10)	0.655		
Mar	25/385 (6.49%)	1.13 (0.60-2.11)	0.701		
Apr	23/253 (9.09%)	1.62 (0.85-3.08)	0.136		
May	22/335 (6.57%)	1.44 (0.60-2.17)	0.681		
Jun	27/473 (5.71%)	0.98 (0.83-1.82)	0.963		
Jul	15/379 (3.96%)	0.67 (0.33-1.35)	0.379		
Aug	10/310 (3.23%)	0.54 (0.24-1.19)	0.129		
Sept	9/235 (3.83%)	0.64 (0.28-1.46)	0.299		
Oct	12/235 (5.11%)	0.87 (0.41-1.85)	0.730)		
Nov	11/267 (4.12%)	0.69 (0.32-1.50)	0.362		
Dec	11/141 (7.80%)	1.37 (0.632-.99)	0.420		

Table 4.1 *continued*:

+Parameter	Bocavirus prevalence n/N (%)	Bivariate analysis		Multivariate analysis	
		Odds Ratio (OR; 95% Confidence Interval (CI)	p-value	Adjusted OR (aOR; 95% CI)	p-value
Demographic characteristics					
Season					
Autumn	70/973 (7.19%)	ref	ref	**Not included in the analysis**	
Winter	52/1162 (4.47%)	0.60 (0.41-0.87)	0.008		
Spring	32/737 (4.34%)	0.58 (0.38-0.89)	0.015		
Summer	58/892 (6.50%)	0.89 (0.62-1.28)	0.555		
Nursery school attendance					
No	97/2101 (4.62%)	ref	ref	ref	ref
Yes	35/441 (7.94%)	1.78 (1.9-2.65)	0.005	1.69 (1.13-2.54)	0.011
Number of people that sleep in the same room as child					
≤2	59/1251 (4.72%)	ref	ref		
3	31/593 (5.23%)	1.11 (0.71-1.74)	0.634		
≥4	45/707 (6.36%)	1.37 (0.92-2.04)	0.119		
Clinical characteristics					
Maximum number of stools per 24 hours					
≤3	35/742 (4.72%)	ref	ref		
4-5	106/1820 (5.82%)	1.24 (0.84-1.84)	0.266		
≥6	60/956 (6.28%)	1.35 (0.88-2.07)	0.167		
Vesikari Score System (Points)					
Mild/moderate (≤10)	13/444 (2.93%)	ref	ref		
Severe (≥10)	71/1094 (6.49%)	2.30 (1.26-4.20)	0.007		
Supplementary oxygen given					
No	112/1992 (5.62%)	ref	ref		
Yes	1/100 (1.0%)	0.16 (0.02-1.22)	0.079		
Child's HIV status					
Negative	144/2674 (5.39%)	ref	ref		
Positive	13/161 (8.07%)	1.54 (0.85-2.78)	0.150		
Environmental features associated with HAdV					
Electricity in the house					
No	90/1379 (6.53%)	ref	ref		
Yes	114/2215 (5.15%)	0.77 (0.58-1.03)	0.083		
Outcome					
Died	2/92 (2.17%)	ref	ref		
Discharged	201/3537 (5.68%)	2.71 (0.66-11.08)	0.165		

Table 4.1 *continued*:

Parameter	Bocavirus prevalence n/N (%)	Bivariate analysis		Multivariate analysis	
		Odds Ratio (OR; 95% Confidence Interval (CI)	p-value	Adjusted OR (aOR; 95% CI)	p-value
Outcome					
Died	2/92 (2.17%)	ref	ref		
Discharged	201/3537 (5.68%)	2.71 (0.66- 1.08)	0.165		
Human bocavirus mixed pathogen infections					
Co-infections					
HBoV only	15/72 (2.08%)	ref	ref		
HBoV + Sapovirus	13/274 (4.74%)	2.33 (1.25-4.33)	0.007		
HBoV + Adenovirus	24/303 (7.92%)	1.70 (1.04-2.76)	0.032	1.66 (1.10-2.52)	0.015
HBoV + Bacteria	43/527 (8.16%)	2.08 (1.35-3.20)	0.001		
HBoV + Parasite	17/196 (8.67%)	1.82 (1.05-3.17)	0.032		
HBoV + 1 pathogen	28/412 (6.80%)	3.43 (1.81-6.50)	0.000	**Not included in the analysis**	
HBoV + 2 or more	46/177 (25.99%)	16.52 (8.96-30.47)	0.000		

4.3.2 Co-infections of HBoV with other enteric pathogens

Evaluation of single and multiple viral infections in the 212 HBoV positive specimens showed a high prevalence of co-infection (67% (142/212)), with single virus (48.6% (103/212)) and two or more (18.4% (39/212)) co-infecting viruses detected (Figure 4.2). Only 42% (89/212) of the HBoV positive specimens were fully screened for enteric viruses, bacteria and parasites. In these specimens, HBoV only infections accounted for 16.8% (15/89) cases, with 51.7% (46/89) and 31.5% (28/89) having single and two or more co-infecting pathogens, respectively (Figure 4.2). The three gastroenteritis pathogens most often identified in dual infections were RV (10.1%; (9/89), bacteria (10.1%; (9/89) and HAdV (7.9% (7/89). In bivariate analysis, HAdV (OR=1.70 (95% CI 1.04-2.76); p=0.032), SaV (OR=2.33 (95%CI 1.25-4.33); p=0.007), bacteria (including 3 Enteroaggregative *Escherichia coli* (EAggEC), 3 diffusely-adherent *E.coli* (DAEC), 2

Enteropathogenic *E. coli* (EPEC) and 1 *Campylobacter jejuni*) (OR=2.08 (95%CI 1.35-3.20); p=0.001) and parasites (*Cryptosporidium*)(OR=1.82 (95%CI 1.05-3.17); p=0.032) were significantly associated with HBoV (Table 4.1). However, only HAdV co-infections were significantly associated with HBoV (adjusted OR (aOR)=1.68; (95% CI 1.10-2.52; p=0.015) in multivariate analysis.

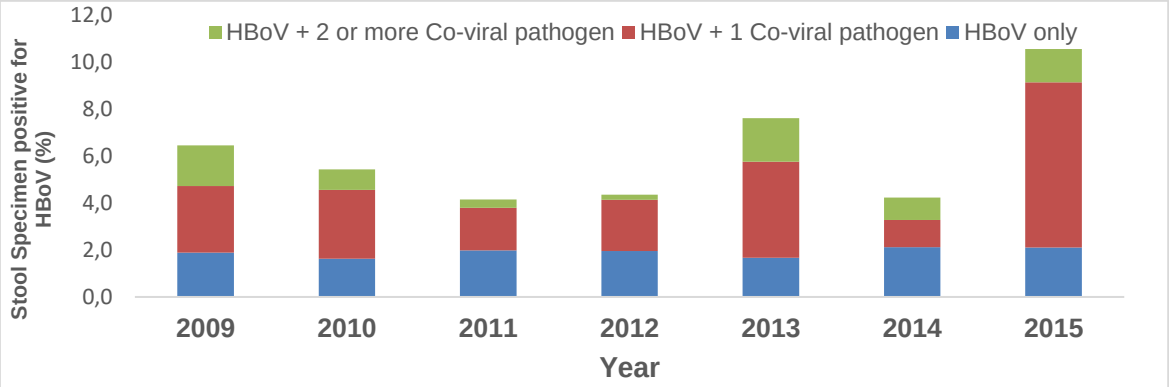

Figure 4.2: Yearly distribution of HBoV detected as a single virus or in combination with other enteric viruses

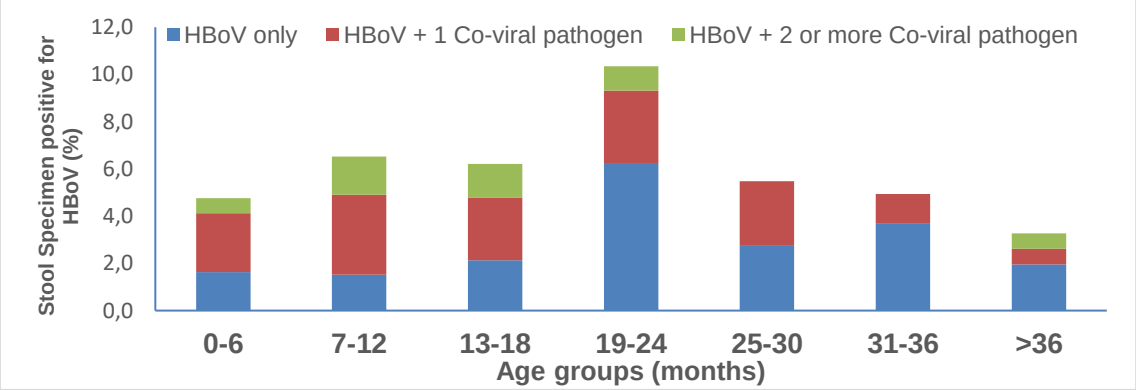

Figure 4.3: Age distribution of HBoV detected as a single virus or in combination with other enteric viruses

4.3.3 Distribution of HBoV genotypes

Out of 212 specimens positive for HBoV, 90.1% (191) were successfully genotyped by sequencing of the VP1/VP2. Among the four genotypes identified, HBoV-1 was the most frequently observed and accounted for 79.6% (152/191) of all typed HBoVs. The three other genotypes appear to circulate at lower frequency, and included HBoV-3 detected in 13.6% (26/191), HBoV-2 in 5.2% (10/191) and HBoV-4 in 1.6% (3/191) of cases. Human BoV1 and HBoV-3 co-circulated in most months of surveillance, while HBoV-2 and HBoV-4 occurred only rarely and sporadically throughout the study period. Numbers of HBoV-1 cases were high in 2009, 2010, and again in 2013; while HBoV-3 cases were high in 2010. There was no trend found in the distribution of HBoV genotypes among various age groups. While HBoV-1 and 2 were present in all age groups, HBoV-3 and 4 were absent from children aged older than 24 months. However, this relationship was, not statistically significant. There were also no statistical differences in genotype variability between the different clinical symptoms. No significant difference was observed in terms of the distribution of HBoV types per study site. This type distribution did not differ significantly by age, gender, clinical or environmental factors (data not shown).

4.3.4 Genetic diversity of HBoV genotypes and whole genome sequencing

To investigate divergence in the HBoV genome, a 565-bp DNA fragment representing the VP1/2 capsid region was successfully amplified and sequenced in 122 specimens of the 191 that were genotyped. All sequences clustered into the concordant genotypes (HBoV-1-4) assigned by the capsid gene (Figure 4.4). Phylogenetic analysis, showed high nucleotide identity (97.9–

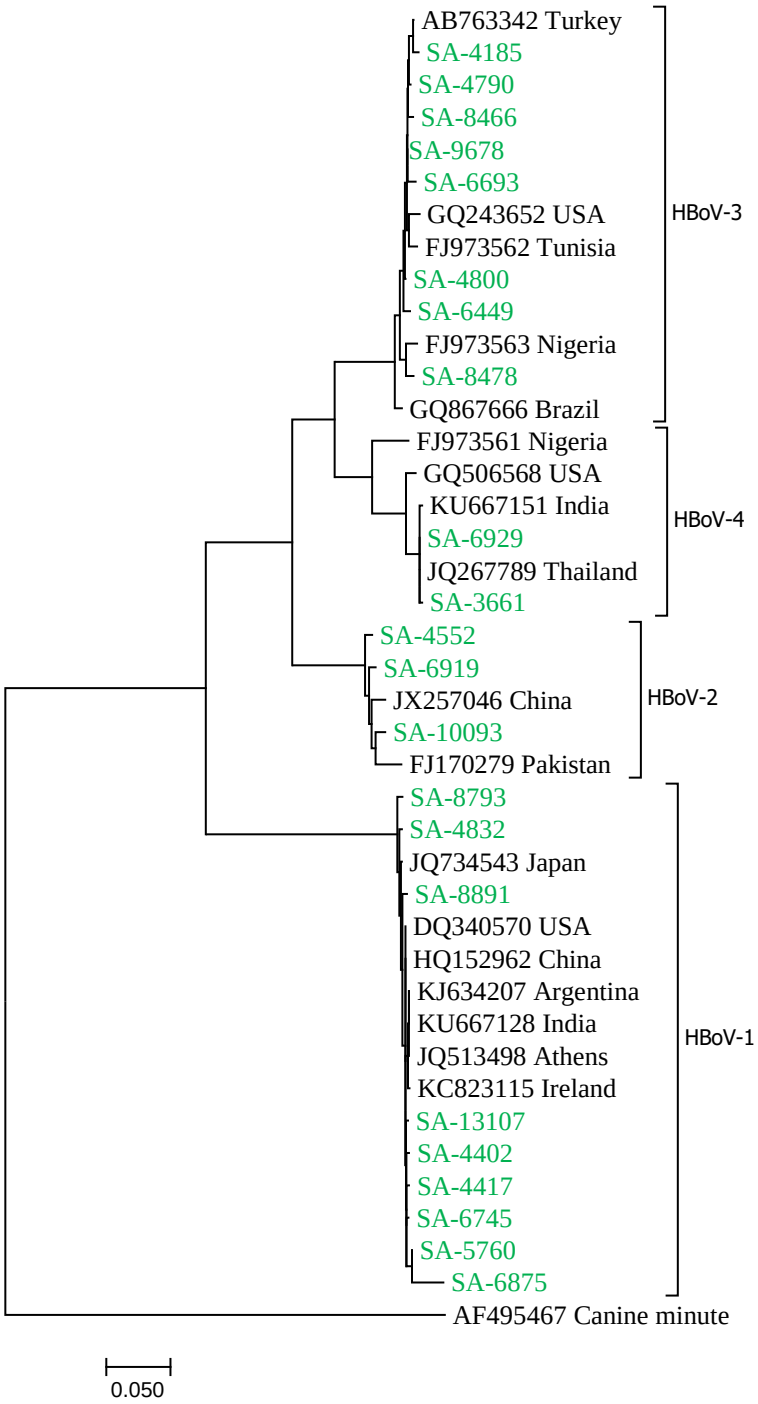

Figure 4.4. Phylogenetic analysis of partial VP1/VP2 gene sequences from South African (green) and reference human bocavirus (HBoV) strains. Phylogeny was constructed using the neighbour-joining method with 1000 bootstrap replicates. Genotypes indicated are human bocavirus (HBoV) 1 to 4.

100%) within the four genotypes with most of the nucleotide variations conserved at the amino acid level, giving a 99.5–100% sequence identity. In HBoV-1, nucleotide differences were observed at 12 sites, while HBoV-2 with 3 strains had

15 and HBoV-3 with 17 strains had 17, the two HBoV-4 strains had two sites only. However, amino acid substitution were similar with HBoV-1 having three and HBoV-2-4 having two amino acid substitutions in each genotype cluster. The mean diversities within the four genotypes, calculated using MEGA 7 (maximum composite likelihood model) ranged from 0.002 to 0.007 base substitutions per site. The constructed tree in the VP1/VP2 region showed that all HBoV identified in circulation in SA clustered with respective prototype strain (Figure 4.4). Full genome analysis of HBoV-3 (6003) confirmed the identity of the HBoV. This strain was highly similar to reference strains from GenBank.

4.4 DISCUSSION

Human bocavirus, first described in 2005, has been associated with upper and lower-respiratory tract infections and gastrointestinal illness throughout the world.[18,40,41] Little epidemiological and molecular data are available on HBoV in SA, as in most other African countries. This paper presents the first molecular epidemiological study of HBoV in children with AGE in SA, as a step to a better understanding of HBoV epidemiology in Africa. Evaluation of HBoV over a six-year period in South African children aged 5 years and under, who had been admitted with AGE showed a prevalence rate of 5.63%, similar to previous reports.[15] The observed prevalence of HBoV was higher than that reported in earlier studies in Africa including one by EL-Mosallamy et al. (2015) at 2% in children under 2 years in Egypt and Lekana-Douki et al. (2018) at 2.2 % in children under 5 years in Gabon. This difference could be due to shorter study durations combined with the smaller sample sizes of the previous studies.

However, HBoV prevalence rate has been reported in diarrhoeal cases in a range from 0.8% to 42%.[42–45]

The HBoV infections in this study were mostly in children under two years of age, with a peak prevalence between the ages of 7-24 months, similar to previous reports.[16,42,46] A possible explanation is the protection by maternal antibodies, which decline and are lowest at age 6–24 months.[47] However, although our findings showed a similar trend, the significance of the associations between HBoV infection and age could not be confirmed with multiple logistic regression.

Seasonal peaks of HBoV infection have been reported to vary among different countries and regions, with most studies suggesting higher detection rate in winter.[42,48–50] Although a previous South African study on HBoV in children with respiratory tract infection observed higher incidences in autumn-winter period,[21] there was no clearly pronounced seasonal pattern in this study. Higher detection of HBoV in 2015 need to be interpreted with caution as this was not a full year of screening and could be seasonal differences, instead of annual.

Children who tested HBoV-positive did not significantly differ from those who tested HBoV-negative with regard to demographic variables, clinical symptoms, or length of hospitalisation. The discrepancy in associating HBoV with AGE in the current study may be as a result of the case definition used, selecting only severe cases of diarrhoea. Day care attendance is known to have substantial negative influence on children's health as children share everything and in this study were predictive of HBoV infection.[51] It may be necessary that future studies combine

known risk factors of AGE with variables related to the day care conditions, including the size of premises, number of children and hygiene policies.

Published information on HBoV genotype distribution in children with AGE has been shown to vary with country, study period, type of specimen/sample and study population.[16] The distribution of HBoV genotypes in Africa is not well described although there are some reports on the overall prevalence of HBoV.[29,30] The current study documents circulation of heterogeneous HBoV genotypes in children under 5 years of age in SA. No differences in clinical or epidemiological variables were found beween different HBoV genotypes. The dominance of HBoV-1 in AGE cases, primarily associated with respiratory infections, suggests a respiratory tract infection rather than a gastrointestinal one.[15,16,44,51–53] However, clinical presentation of HBoV-1, has been reported to include gastroenteritis, and is cited as one of the reasons for seeking health care services in 10% of children with acute respiratory infection.[54] Although this has led to the suggestion that HBoV-1 may be a pneumoenteric virus, whose infection starts on respiratory surfaces before spreading to the intestines, it has not been linked to diarrhoea episodes.[4,51]

The HBoV-3 genotype was the second most common strain in this study, contrary to previous reports in which lower detection rates were reported, with HBoV-2 being more dominant.[16,31] In contrast to HBoV-1, HBoV-2-4 are considered enteric viruses because of their detection in AGE stools but not in respiratory specimens[18,40,41] Few studies have reported the detection of HBoV-4 in children with AGE, with prevalence rates ranging from 0-0.5%, which is lower than the

rate in this study.[16,32,41,55] One other study in Western India has similarly reported higher HBoV-4 prevalence rates of 12% (3/24) in children ≤ 5 years hospitalised with AGE.[16]

The high rate of co-infections observed in this study is in accordance with previous reports which cited co-infection rates as high as 83% for respiratory specimens and 100% for faecal specimens.[44,56–58] Similarly, co-infections were high in this study, and occurred in 83.1% (74/89) of children with fully screened specimens. It has been suggested that the high rate of co-infection is because, HBoV is either a helper virus, facilitating replication of other pathogens or requires a helper virus to facilitate productive infection.[59] Our findings show HAdV, a known AGE agent, as the most common pathogen in co-infections, making it challenging to demonstrate the clinical importance of HBoV.[60] Concurrent detection of HBoV and HAdV infection has been reported with high frequency in some studies of respiratory infections.[61,62] One possibility here is that HBoV infections may reactivate HAdV, leading to disease. Additional studies are required to clarify this relationship. Moreover, the effect of co-infections on disease severity could not be established similar to previous reports.[63] .

Phylogenetic analysis in this study showed HBoV-1 to be highly conserved with a very low degree of genetic variability considering that 80 of the 98 HBoV-1 strains were identical. All the sequences clustered along with previously reported strains from GenBank from different parts of the world for the four genotypes although HBoV-2-4 were less similar than HBoV-1. This result also emphasises the worldwide circulation of HBoV strains. Previous studies have reported higher

diversity in HBoV-2-4 compared to HBoV-1 which is comparable to our findings.[13,48] Seven of the eight randomly selected HBoV specimens for WGS could not be sequenced. There are many possible reasons for this failure. Firstly, the NGS sequencing protocol was optimised for HAdV (dsDNA), applied without further optimisation; it is plausible that this protocol may not be equally optimal for HBoV (ssDNA). Secondly, the failure could also be due to poor template DNA quality or quantity; specimens with none or few co-infecting pathogens were selected irrespective of their real-time cycle threshold (ct) value. Whole genome sequencing results from one specimen was consistent with previous results on the strain identification and genetic characterisation of HBoV genome based on partial sequence analysis. No evidence was seen for recombination events.

Strengths of this study include that this is the largest study to date on the epidemiology of HBoV in children with AGE in Africa and the integration of molecular epidemiological data. This study also has several limitations. First, the study focussed on hospitalised children with severe AGE while children with mild AGE were not included, therefore the results do not reflect the overall burden of HBoV in the general paediatric population. In addition, the current study did not include an age-matched control group to compare the prevalence of HBoV circulating in symptomatic children with severe AGE and asymptomatic healthy children. Consequently, the data may not be representative of the overall HBoV prevalence and species diversity circulating in the paediatric population in SA. The high number of co-infections with other potential enteric pathogens limited our ability to determine whether or not specific clinical symptoms were associated with HBoV infections.

Numerous studies that have examined the role of HBoV in diarrhoeal diseases, have either supported or dismissed the significance of HBoV as a pathogen in AGE[41,64] The HBoV prevalence in children with AGE in SA is similar to those found in other countries. The lack of clear disease associations indicate that HBoV is not a major pathogen in children presenting with AGE. The high frequency of co-infections with other enteric pathogens illustrates the complexities in attributing causality of HBoV to AGE.

Author's contribution

R.N. performed laboratory work, data analysis and co-wrote manuscript

L.C. data analysis and writing

N.A.P. designed study, edited manuscript, supervisor lead author

M.B.T. reviewed and edited manuscript, co-supervisor lead author

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Competing interests

The authors declare that they have no competing interests.

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CHAPTER 5

GENERAL DISCUSSION

Acute gastroenteritis causes a substantial burden of morbidity and mortality worldwide, particularly in children age under five years of age in low-income settings. The importance of viruses as the leading cause AGE in children has been underlined in several reports worldwide. Enteric viruses, mainly RV, NoV, AdV, SaV and AstV, have been noted to cause a significant proportion of AGE in infants and young children. Additionally, newly recognised viruses such as HBoV are suggested to play an important role in AGE. Understanding the prevalence and risk factors associated with these viruses as well as the circulating genotypes may aid in the control and prevention of these infections. The major goals of this study were to investigate the prevalence, clinical outcomes, risk factors and molecular diversity of HAdV and HBoV from stool specimens collected from children aged ≤5 years hospitalised for AGE in SA from April 2009 to May 2015. Testing for HAdV was conducted from April 2009 to December 2014, while testing for HBoV was conducted from April 2009 to May 2015, hence the different in total number of specimens screened for these enteric pathogens.

The first objective of the study was to address the epidemiology of enteric DNA viruses, namely HAdV and HBoV, in children hospitalised with AGE in SA. Human AdV are among the most prevalent disease-causing viruses in children and elicit a wide range of diseases including AGE. Between April 2009 and December 2014, the prevalence of HAdV in hospitalised children with AGE in SA was 18.1%,

(656/3623). This prevalence is much higher than the previous HAdV frequencies found in SA which ranged between 4.6% to 9.9% (Kidd et al., 1986; Moore et al., 1998; Rossouw, 2004; Magwalivha, 2009). The high HAdV detection rates observed in this study most probably have been influenced by the use of PCR detection method that has shown improved sensitivity in the detection of HAdV (Liu et al., 2016), compare to previous studies where they used less sensitive EIA and hybridisation methods. This results confirm that HAdV is commonly found in faecal specimens from children with AGE in SA. Human AdV prevalence rates have been reported in other African countries using PCR method in child diarrhoeal cases to range from 19.6% to 39.7% (Kabayiza et al., 2014; Lekana-Douki et al., 2015; Ouédraogo et al., 2016; El Sayed Zaki and El Kheir, 2017). Similar to previous reports in SA (Kidd et al., 1986; Tiemessen et al., 1989), HAdV circulation was year-round with no clear seasonal pattern. The prevalence of HAdV was particularly more frequent in the age group 7-24 months, making this age group an important target for interventions such as vaccination. Furthermore, HIV-infected children with HAdV had a longer length of hospital stay compared to HIV-uninfected children with HAdV, suggesting high morbidity associated with this infection particularly in terms of severity. A previous study in Tunisian children with AGE showed no significant association between HIV and HAdV (Moyo et al., 2014). Future studies are required to analyse the associations between immunosuppression and HAdV infection among different age groups.

Human bocavirus are known to cause various clinical manifestations including AGE. Although HBoV infections and their genotypes have been detected in human patients worldwide, no such reports are available from SA to ascertain the

association of HBoVs in AGE. Between April 2009 and April 2015, the prevalence of HBoV in hospitalised children with AGE in SA was 5.6% (212/3765). This prevalence is higher than that reported in earlier studies in African countries reporting 2-2.2% HBoV positivity cases of AGE (EL-Mosallamy et al., 2015; Lekana-Douki et al., 2018). However, the HBoV prevalence rate has been reported in other countries in diarrhoeal cases in a range from 0.8% to 42% (Lee et al., 2007; Cheng et al., 2008; Alam et al., 2015; Campos et al., 2016). The difference in the HBoV prevalence seen in the literature might reflect the discrepancies of the studies conducted based on geographical, study design, as well as the testing methods. In agreement with the previous studies, nearly all (92%, 195/212) the infections occurred in the first 2 years of life, and a larger proportion were between 7 to 12 months (Lee et al., 2007; Cashman and O'Shea, 2012; Lasure and Gopalkrishn, 2017). Co-infections with other enteric pathogens were found in 83.1% (74/89) of stool specimens with full enteric screening (bacteria and parasites). This epidemiological profile of the co-infections was similar to the previous findings, suggesting that co-infection is common for HBoV (Lindner and Modrow, 2008; Han et al., 2009; Kantola et al., 2010; Alam et al., 2015). The occurrence of co-infections did not influence the clinical outcome toward more severe disease. In 2015, screening for HBoV was only done from January to April. Thus, the increase in HBoV cases observed in 2015 is most likely attributed to the variation in the specimens number for 2015 rather than increase in HBoV circulation. Day care attendance is known to have substantial negative influence on children's health as children share everything and in this study were predictive of HBoV infection (Bailey, 2013). The role of HBoV in causing AGE is yet to be determined. Although some studies report the lack of

association, some studies found association between HBoV infection and diarrhoea disease, no conclusive data are available to date (Jin et al., 2011; Nawaz et al., 2012; Chhabra et al., 2013). While in the current study (without diarrhoea-free controls), the clinical profiles of the children with HBoV infected were not significantly different from the cases negative for HBoV. This once again suggested that HBoV might not be a causal agent for AGE.

The second objective was to address the molecular epidemiology of South African HAdV strains detected in the paediatric patients with AGE. This objective was linked to objective six which was to develop or optimise species-specific methods for the identification of the detected HAdVs. Positive HAdV stool specimens from 2009 to 2012 were subjected to sequencing using published set of primers targeting a conserved hexon gene to determine HAdV species and strains (Casas et al., 2005). Furthermore, a published multiplex-PCR (m-PCR) was adapted, optimised and applied for rapid differential detection of HAdV species from previously HAdV-positive stool specimens (Xu et al., 2000). The m-PCR analysis combined with sequence analysis permitted determination of HAdV species and strains from previous HAdV positive stool specimens. Six species of HAdV (HAdV A-F) were found circulating in children with AGE in SA, which suggests circulation of heterogeneous HAdV species in the country. The predominant species detected over the study period was HAdV-F, was identified in each year. This species accounted for 42.1% (254/603) of all HAdV positive specimens and 7% (254/3623) in total diarrhoeal specimens tested. This figure is comparable with previous studies conducted in children with AGE in SA, where the prevalence ranged from 1.6% to 13.2% (Kidd et al., 1986; Moore et al., 1998;

Tiemessen et al., 1989; Marx et al., 1998; Rossouw, 2004; Magwalivha, 2009). This is the main HAdV species associated with human enteric infections worldwide, and is therefore of public health importance (Liu et al., 2016; Platts-Mills et al., 2018). The obtained sequences were further used for identification of circulating HAdV strains during 2009–2012 period. The results demonstrate that diverse strains of HAdV were circulating in SA. Studies reported from other countries have shown HAdV-F-41 as the predominant type in children with AGE (Lion, 2014). The circulation of AdV-40 and -41 strains as revealed by our study in SA shown almost equally distribution of the two strains. At present, the reasons for this observation in SA are not known. However, climatic differences, and human activities among the local environments may contribute to the different.

Following on objective two, objective five addressed the molecular characterisation of HAdV strains by WGS. Ten faecal specimens were chosen from 254 HAdV-F positive specimens for detailed whole genome analysis in order to characterise their genetic variation and evolution. Low-level genetic variations were observed in HAdV-40 strains and were highly similar to the reference strain, while HAdV-41 strains showed divergence with within group identity and could be divided into two groups. Phylogenetic analysis showed all HAdV-40 to be highly similar and were clustered together in all the analysis, while diversity of HAdV-41 strains showed two genome type clusters that were highly similar in trees of whole genomes and individual genes. Furthermore, the recombination breakpoints of the five HAdV-41 strains varied in the number and location, indicating different evolution origins. Recombination is a recognised feature of HAdV which may lead to the emergence of new types and subtypes. These findings highlight the genetic

diversity of HAdV-40 and -41 strains circulating in SA and suggests possible evolution from inter-strain recombination which is crucial information in future strategies in vaccine development and vaccination.

Objectives three and four were to characterise and perform phylogenetic analysis on HBoV strain detected in the stool specimens. A total of 90.1% (191/212) HBoV strains were genotyped by nucleotide sequence analysis of the VP1/VP2 region, which comprised all four different HBoV genotypes, suggesting circulation of heterogeneous HBoV genotypes among hospitalised children with AGE in SA. Of these, HBoV1 was the most predominant genotype, accounted for 79.6% (152/191). The dominance of HBoV1 in AGE cases, primarily associated with respiratory infections, may suggests a respiratory tract infection rather than a gastrointestinal one (Campe et al., 2008; Chow and Esper, 2009; Alam et al., 2015; Guido et al., 2016; Lasure and Gopalkrishna, 2017). The SA strains detected in this study predominantly cluster with other global reference strains, suggesting HBoV strains from SA were closely related to HBoVs identified elsewhere in the world. The low divergence observed in HBoV strains may be attributable to the low frequency of recombination observed in this genotype. The phylogenetically close relationship between SA strains and different HBoV strains from around the world underline the global nature of HBoV distribution. Seven of the eight randomly selected HBoV strains for WGS could not be sequenced. Whole genome sequencing results from one specimen was consistent with previous results on the strain identification and genetic characterisation of HBoV genome based on partial sequence analysis. No evidence was seen for recombination events. As all HBoV strains could be typed by Sanger nucleotide

sequence analysis, and the fact that HBoVS were found not to be aetiologically associated with AGE, the development and optimisation of species-specific typing methods as outlined in objective seven was not performed.

A number of limitations were identified in the study. The investigations focussed on hospitalised children with severe AGE while children with mild AGE were not included, therefore the results do not reflect the overall burden of enteric DNA viruses in the general paediatric population. In addition, the current study did not include an age-matched control group to compare the prevalence of enteric DNA viruses circulating in symptomatic children with severe AGE and asymptomatic healthy children. Consequently, the data may not be representative of the overall prevalence and species diversity circulating in the paediatric population in SA. The high number of co-infections with other potential enteric pathogens limited our ability to determine whether or not specific clinical symptoms were associated with enteric DNA viral infections.

In conclusion, the current study provides an important contribution to the scarce data on HAdV epidemiology in SA, and to that of HBoV in a country where no previous report is available. The large sample size of the study, allowed epidemiological analysis of enteric DNA viruses in greater detail than previously undertaken. This study has demonstrated a high prevalence of enteric DNA viruses in the stools from hospitalised children with AGE in SA, with a considerable proportion of co-infections. There was no seasonal pattern observed throughout the study period. We report evidence of equal distribution of HAdV-40 and -41 strains of HAdV in stool specimens in SA. The current study

highlights the genetic diversity of HAdV-40 and -41 strains circulating in SA and suggests possible evolution from inter-strain recombination. Furthermore, the present study highlights the detection and characterisation of a wide spectrum of HBoV genotypes in AGE children for the first time from SA. The high prevalence of HBoV 1 genotype, a known respiratory infection virus and the association between HBoV positive specimens with already established enteric pathogens from AGE cases, suggests HBoV may play a limited role in diarrhoea diseases. Additional research, including case–control studies will help to determine their association with AGE.

CHAPTER 6

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APPENDIX A

A1: Nadan S, McLaren B, **Netshikweta R**, Kruger T, DeMaayer T, Reubenson G, N Page N. The detection of enteric viruses in malnourished paediatric patients from South Africa [Poster]. 11th International Rotavirus Symposium 3-5 September, 2014 in New Delhi, India.

ABSTRACT: Diarrhoea increases the odds of death in children with severe acute malnutrion (SAM), irrespective of other factors. Identifying the causes of the diarrhoea is critical of the management, treatment and ultimately the reversal of this risk. Between 1 December 2012 and 30 November 2013, 81 stool samples were collected from hospitalized children who were diagnosed with SAM, and had presented with or without accompanying diarrhoea. Severe acute malnutrition was defined by weight for Z scores of less than -3 standard deviations and a mid-upper arm circumference (MUAC) value of less than 115mm (for the age group 6 to 60 months). Screening for the following enteric viruses was conducted: rotavirus group A, norovirus GI, norovirus GII, human astrovirus, sapovirus, human adenovirus and human bocavirus. Reverse transcriptase (RT) PCR was performed for genotyping of rotavirus using standardized methods and all other viruses were detected by real-time PCR. The weight for height range of the study population was 1.9 to -9.1 standard deviations and MUAC values were from 141mm to 76mm. The ages of the patients were between 6 months and 38 months. Enteric viruses were detected in 39.5% (32/81) of the stool samples. Single viral infections were identified as follows: rotavirus group A (7/81, 8.6%), norovirus G (2/81, 2.5%), norovirus GII(4/81, 5%), Sapovirus (1/81, 1.2%), human adenovirus (3/81, 3.7%) and human bocavirus (2/81, 2.5%). Human astroviruses were only present twice from a total of 116% (13/81) mixed infections. No viral enteric pathogens could be detected in 60.5% (49/81) of the total samples. This is the first study from South Africa to report on the detection of enteric viruses in malnourished children. Although no viral enteric pathogens could be detected in the majority of the samples (60.5%), this does not exclude the role of other enteric viruses as important causes of morbidity among malnourished children. It can be concluded that for this cohort, from the viruses screened for, rotavirus posed the greatest risk for diarrhoea disease whereas astroviruses were only present when co-infecting with other enteric viruses. This study provides an insight of the susceptibility of malnourished children to enteric viruses. These data will help with implementing prevention and treatment measures, for reducing further deaths resulting from diarrhoeal disease, among malnourished children.

A.2: Netshikweta R, Nadan S, Kruger T, Page NA. Human Bocavirus in children with acute gastroenteritis in South Africa, 2009-2013 [Presentation]. Faculty Day, Faculty of Health Sciences, University of Pretoria 19-20 August 2014: HW Snyman Building, Pretoria.

ABSTRACT: Viruses are among the most common causes of acute gastroenteritis (AGE). A wide range of enteric viruses, including rotavirus, calicivirus, adenovirus and astrovirus, have repeatedly been detected in AGE. However, aetiology is still undefined in a significant proportion of AGE. Human bocavirus (HBoV) is a novel parvovirus associated with respiratory illness and gastrointestinal illness worldwide. Previous studies indicated that HBoV prevalence in AGE is between 0.8% - 9.1%. To date, there are no reported studies on the prevalence of HBoV in stool specimens of children suffering from AGE in South Africa (SA). This study was carried out in order to gain new insight into HBoV infection among hospitalised children with AGE in selected regions in SA. Methods: During April 2009 to December 2013, a total of 5422 faecal specimens were collected from paediatric patients hospitalised with AGE as part of the sentinel surveillance of rotavirus diarrhoea in SA. The presence of HBoV was investigated by real-time PCR according to published methods. Human bocavirus PCR- positive samples were sequenced to identify genotypes. Other enteric viral pathogens were also detected by RT-PCR or real-time PCR. Results: In total, 333 of 5422 specimens were positive for HBoV, indicating an annual prevalence of 6.1%. Only 280 of 333 (84%) were genotyped by sequencing and genotype distribution was 77%, 6%, 16% and 1% for HBoV1, HBoV2, HBoV3 and HBoV4, respectively. Human bocavirus circulated throughout the year and showed a slight peak during the autumn-winter season. Co-infections of HBoV with other enteric viruses were detected in 65.5% (218/333) of samples. Conclusion: This is the first detailed report contributing critical preliminary data on the epidemiology of HBoV associated with AGE in SA. The presence of all 4 HBoV genotypes was confirmed, HBoV1 and HBoV3 were the predominant genotypes circulating. These can provide baseline information for designing control strategies as well as the determination of the source of infection. The results are comparable to published data for the prevalence of HBoV in other countries, providing supportive evidence that HBoV is a relatively common pathogen of children with AGE and is often associated with other common enteric viruses.

A.3: Netshikweta R, Nadan S, Page N, Kruger T. Human bocavirus infections in paediatric patients hospitalised with acute gastroenteritis in South Africa between 2009 and 2013 [Poster]. 11th International Rotavirus Symposium 3-5 September, 2014 in New Delhi, India.

ABSTRACT: Viruses are among the most common causes of acute gastroenteritis (AGE). A wide range of enteric viruses, including rotavirus, calicivirus, adenovirus, and astrovirus, have repeatedly been detected in AGE. However, aetiology is still undefined in a significant proportion of AGE. Human bocavirus (HBoV) is a newly discovered pathogen thought to be associated with both respiratory and enteric infections and are frequently found associated with other common respiratory or enteric viruses. Previous studies indicated that HBoV prevalence in AGE is between 0.8% - 9.1%. To date, there are no reported studies on the prevalence of HBoV in stool specimens of children suffering from AGE in South Africa (SA). This study was performed to gain new insight into HBoV infection among hospitalised children with AGE in selected regions in SA. Methods: A total of 5422 faecal specimens were collected from hospitalised paediatric patients admitted for acute gastroenteritis during April 2009 to December 2013 as part of the sentinel surveillance of rotavirus diarrhoea in SA. The presences of HBoV were investigated by real-time PCR according to published methods. Other viral enteric viruses were also detected by RT-PCR or real-time PCR according to standardised methodology. Results: In total, 333 of 5422 specimens were positive for HBoV, indicating an annual prevalence of 6.1%. Human bocavirus circulated throughout the year and showed a slight peak during the autumn-winter season. Co-infection of bocavirus with other enteric viruses was relatively common (65.5%, 218/333), and included co-infections with rotavirus (17.7%, 59/333), adenovirus (16.2%, 54/333), norovirus GII (8.4%, 28/333), sapovirus (6.9%, 23/333), astrovirus (4.5%, 15/333) and norovirus GI (0.6%, 2/333). Conclusion: Results from this study confirmed that HBoV is a relatively common pathogen of children with AGE and is often associated with other common enteric viruses. Similar to other investigations the predominant pathogen co-infecting with HBoV was rotavirus, confirming the importance of rotavirus as the main viral aetiological agent of childhood diarrhoea. This raises questions around bocavirus and the causation of diarrhoea. Further studies will be needed to determine causality and clarify if co-infection of HBoV with other enteric viruses leads to longer hospitalisation or more severe clinical outcome. To our knowledge, this is the first report of HBoV infections in a paediatric population with AGE in SA.

A.4: Netshikweta R, Nadan S, Kruger T, Page N. Frequency of human adenovirus species in children hospitalised with acute gastroenteritis in South Africa [Poster]. Pathology Research and Development Congress 15 - 16 April 2015. Emperors Palace Johannesburg South Africa.

ABSTRACTS: Acute gastroenteritis (AGE) is one of the leading causes of morbidity and mortality in young children, especially in developing countries. Viruses remain by far the most common cause of AGE in children. Human adenoviruses (HAdV) are classified into seven species (A-G) which are often associated with unique clinical outcomes and epidemiological features. The importance of HAdV as a cause of gastroenteritis is already well-define, particularly HAdV-F. Objectives: To evaluate the prevalence and seasonality of HAdV species in children <5 years old hospitalised with AGE in selected sentinel sites in South Africa (SA) in 2011 and 2012. Methods: Between January 2011 to December 2012, 2251 faecal specimens were collected from hospitalised paediatric patients admitted for AGE as part of the sentinel surveillance of rotavirus diarrhoea in SA. The presence of HAdV were investigated by real-time PCR and classified further into species by nucleotide sequencing of hexon gene. Other enteric viruses were also detected by RT-PCR or real-time PCR. Results: Human adenoviruses were detected in 14.3% (322/2251) of specimens. The majority of cases (99%; 319/322) occurred in children between 0-36 months of age. While HAdV was detected year round, frequency peaked during late summer and early autumn. A total of 295 HAdV infections were further classified into species. These included 40.7% (120/295) HAdV-F, 23.1% (68/295) HAdV-B, 19% (56/295) HAdV-C, 8.5% (25/295) HAdV-D, 8.1% (24/295) HAdV-A and 0.7% (2/295) HAdV-E. Co-infection with other enteric viruses was observed in 41.3% (133/322) of HAdV positive specimens. Conclusion: The results confirm the endemism of HAdV among children < 3 years with AGE in 2011 and 2012. Diverse HAdV species were detected during the study period, predominantly HAdV-F in AGE cases. This study provides important information on the epidemiology and species distribution of HAdV in the paediatric population hospitalised with AGE post rotavirus vaccine introduction in SA.

A.5 Netshikweta R, Nadan S, Kruger T, Page P. Human adenovirus species in children hospitalised with acute gastroenteritis in South Africa [Poster]. Faculty Day, Faculty of Health Sciences, University of Pretoria 18-19 August 2015: HW Snyman Building, Pretoria.

ABSTRACT: Human adenovirus (HAdV) infections are common worldwide. The virus consists of seven known species, ranging from HAdV-A to HAdV-G. They can cause a wide variety of clinical diseases including respiratory infection, gastroenteritis, conjunctivitis and urinary tract infections. These diseases range from mild and self-limited, severe and even fatal, in both immunocompetent and immunocompromised patients. Aim: To evaluate the prevalence of HAdV species in children <5 years old hospitalised with acute gastroenteritis (AGE) in selected sentinel sites in South Africa (SA) from 2009 to 2013. Methods: Stool specimens were collected from hospitalised children with AGE during 2009-2013 as part of the sentinel surveillance of rotavirus diarrhoea in SA. Real-time polymerase chain reaction (PCR) was used to identify the presence of HAdV, and HAdV-positive specimens were classified further into species by nucleotide sequencing of the hexon gene or multiplex PCR targeting the fiber gene. Other enteric viruses were also detected by reverse transcriptase (RT)-PCR or real-time PCR. Results: Of the 5507 stool specimens obtained, HAdV was detected in 17.5% (966/5507). Human adenovirus were most frequently detected in children who were 1 year of age or younger [65.6% (634/966)] than those over 1 year of age [34.4% (332/966)]. A total of 843 HAdV positive specimens were further classified into species. These included HAdV-F [38% (320/843)], HAdV-C [25% (209/843)], HAdV-B [18% (154/843)], HAdV-A [9% (80/843)], HAdV-D [9% (72/843)] and HAdV-E [1% (8/843)]. Co-infection with other enteric viruses was observed in 50% (484/966) of HAdV positive specimens. While HAdV was detected all year round, frequency peaked during late summer and early autumn. Discussion/Conclusion: The results confirm wide distribution of HAdV among children < 1 year of age. Multiple HAdV species were detected during the study period, and species F was by far the most frequent. This species is often associated with diarrhea, mostly in children under 3 years of age. This study provides important information on the epidemiology of HAdV in hospitalised children with AGE post rotavirus vaccine introduction in SA.

APPENDIX B

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

1/10/2015

Approval Certificate
New Application

Ethics Reference No.: 383/2015

Title: Epidemiology and characterisation of enteric DNA viruses associated with gastroenteritis in children in selected regions of South Africa

Dear Rembuluwani Netshikweta

The **New Application** as supported by documents specified in your cover letter dated 13/08/2015 for your research received on the 13/08/2015, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 30/09/2015.

Please note the following about your ethics approval:
- Ethics Approval is valid for 4 years
- Please remember to use your protocol number (**383/2015**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

*** Kindly collect your original signed approval certificate from our offices, Faculty of Health Sciences, Research Ethics Committee, H W Snyman South Building, Room 2.33 / 2.34.*

Dr R Sommers; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

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Faculty of Health Sciences

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

15 March 2019

Approval Certificate
Annual Renewal

Ethics Reference No.: 383/2015
Title: Epidemiology and characterisation of enteric DNA viruses associated with gastroenteritis in children in selected regions of South Africa.

Dear Mr R Netshikweta

The **Annual Renewal** as supported by documents received between 2019-02-08 and 2019-03-13 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2019-03-13.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2020-03-15.
- Please remember to use your protocol number (383/2015) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

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