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THE MOLECULAR CHARACTERISATION OF HUMAN ADENOVIRUSES FROM HUMAN SPECIMENS AND ENVIRONMENTAL SAMPLES

BY

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SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

MAGISTER SCIENTIAE

MSC *(MEDICAL VIROLOGY)*

IN THE

DEPARTMENT OF MEDICAL VIROLOGY

FACULTY OF HEALTH SCIENCES

UNIVERSITY OF PRETORIA

PRETORIA

DECEMBER 2009

DECLARATION

I, Mpho Magwalivha, declare that this work was not copied or repeated from any other studies either from national and international papers. Confidentiality of patients' identity has been followed according to the ethical rules as prescribed by the Faculty of Health Science Research Ethics Committee, University of Pretoria.

Signature: …….

Date: ……

ACKOWLEDGEMENTS

I would like to give thanks to God for the strength and courage that helped me during the course of my study. I am grateful to the Poliomyelitis Research Foundation (PRF) for the financial support and Rand Water and City of Windhoek for the water samples. I am profoundly grateful to Professor Maureen B. Taylor (supervisor) and Dr. Walda B. van Zyl (co-supervisor), for their patient assistance, guidance and advice throughout my study, and for also giving the training on molecular techniques in the laboratory.

I also appreciate the assistance of Mr. Nicholas M. Kiulia for the samples collected in Kenya, Dr. Marianne Wolfaardt for helping and guiding me on gathering the data on the molecular level, Dr S Olorunju from the Medical Research Council for guidance in statistical analysis, my fellow post-graduate students in the lab for being there, may Almighty God bless you.

Finally, I like to appreciate the tolerance of my family who gave a huge support with love and care from the beginning.

This dissertation is dedicated to my mother, sisters and uncles for the total support they gave me, and to my life partner for the continuous encouragement and support. I also dedicate this mini-book in motivating my younger sisters and cousins to study….

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DEGREE: MAGISTER SCIENTIAE (MEDICAL VIROLOGY)

SUMMARY

Human adenoviruses (HAdVs) are non-enveloped DNA viruses, currently comprising 52 serotypes which are divided into seven species, designated A to G. The HAdVs are associated with a number of diseases affecting respiratory, urinary, gastrointestinal tracts and the eye. Human AdVs have increasingly been recognized as important pathogens in immunocompromised individuals. Human AdVs are ubiquitous in the environment resulting in the possible contamination of treated and untreated drinking water supplies by human secretions and excretions. As AdVs do not have an envelope, they are extremely resistant to inactivation, allowing for prolonged survival in the environment. The presence of AdVs in water sources is considered important, as they are exceptionally resistant to selected water treatment processes. Precise typing of HAdVs is, therefore, essential for epidemiological surveillance and the understanding of infection chains. The aim of this study was to determine the prevalence and genetic heterogeneity of HAdVs circulating in communities in selected regions of Africa compared to the rest

of the world. It is also important to determine the genetic relationship between HAdVs strains occurring in water sources and those detected in human clinical specimens, as this may give some indication as to whether or not water sources are a potential source of infection.

As part of ongoing surveillance in southern Africa of treated and untreated water sources for enteric viruses, 765 water samples were tested using a nested polymerase chain reaction (nPCR) for HAdVs. Of these samples, 65 (8.6%) water samples were positive for HAdVs, and selected samples were characterised. In the untreated water, HAdV-F was the dominant species (65.6%) and HAdV-D was second-most common (21.9%) species identified. Species HAdV–B, -A and –C were identified amongst the rest of the strains. From treated water, HAdV-D and –F were identified in one isolate each. Analysis of diarrhoeal stool specimens for HAdVs identified HAdV-F as the predominant species, comprising 77.8% of the identified strains, with species HAdV-C and –A less common, identified in 11.1% specimens. In the respiratory specimens from the same region, HAdV-C was identified in 28.6% of the specimens. Comparative genetic analysis of HAdVs from water sources and clinical specimens showed genetic relatedness between the strains. Water may therefore play an important role as source of infection in the surrounding communities.

In developing countries, diarrhoea is a major cause of morbidity and mortality and after rotaviruses HAdVs are considered to be the second-most important cause of viral infantile diarrhoea. Samples also were available from Kenya, where there are very little data on the prevalence and distribution of HAdV serotypes associated with diarrhoea in paediatric patients. From Kenya, 278 stool specimens were analysed, of which 104 (43 diarrhoea; 61 non-diarrhoea) were from an urban hospice for human immunodefiency virus (HIV)-seropositive children, 94 from selected urban clinics and 80 from the rural setting. From these, the detection of HAdVs in diarrhoeal and non-diarrhoeal stool specimens was 43.3% and 16.4%, respectively. In the urban hospice

setting, 43.3% of the stool specimens from HIV-seropositive children tested positive for HAdV. The overall detection of HAdVs species and genotypes in the stool specimens showed HAdV-D to predominate, being detected in 36.1% of specimens with HAdV-C (29.5%), HAdV-F (16.4%), HAdV-B (13.1%), and HAdV-A (6.5%) present in lower numbers. This study provided valuable new data on the prevalence and distribution of HAdV genotypes in diarrhoeal stool specimens in Africa.

In this study where nucleotide sequence comparison was used to determine the genetic relatedness of African HAdVs to those from the rest of the world, it was noted that in most cases the African strains differed from those from the rest of the world. The use of molecular techniques for the detection and characterisation of HAdVs, especially in Kenyan cohorts, was of importance, as it provided new baseline data for further burden of disease studies which are necessary for future prevention and treatment programmes.

PRESENTATIONS AND PUBLICATIONS

Publications

Magwalivha M, Wolfaardt M, Kiulia NM, van Zyl WB, Mwenda JM, Taylor MB. High prevalence of species D human adenoviruses in faecal specimens from urban Kenyan children with diarrhoea. Journal of Medical Virology 2010;82:77-84.

National presentations

Magwalivha M, Wolfaardt M, Taylor MB. Molecular characterisation of human adenoviruses from treated and untreated drinking water in Gauteng. [Presentation]. Faculty Day, Faculty of Health Science, University of Pretoria 14-15 August 2007: BMS Building, Prinshof Campus, Pretoria.

Magwalivha M, Wolfaardt M, Taylor MB**.** Molecular characterisation of human adenoviruses from treated and untreated drinking water in Gauteng. [Poster]. The 2007 MCBG symposium. Department of Medical Virology, University of Pretoria/Molecular and Cell Biology Group of South Africa. 17 October 2007: HW Snyman North, Faculty of Health Science, University of Pretoria, Pretoria

Magwalivha M, Wolfaardt M, Kiulia N, van Zyl W, Mwenda J, **Taylor MB.** Molecular characterisation of adenoviruses from Kenyan children with diarrhoea[Presentation]. HIV and AIDS Research Indaba. 26-27 February 2009: Moot Court Room, Faculty of Law, University of Pretoria.

Magwalivha M, Wolfaardt M, Kiulia NM, van Zyl WB, Mwenda JM, Taylor MB. High prevalence of species D human adenoviruses in faecal specimens from Kenyan children with diarrhoea [Poster]. Faculty Day, Faculty of Health Sciences, University of Pretoria 18-19 August 2009: HW Snyman Building, Pretoria.

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ABBREVIATIONS

AdVs = Adenoviruses
AIDS = Acquired immunodeficiency syndrome
APCs = Antigen-presenting cells
BMT = Bone marrow transplant
bp = Base pair
CAR = Coxsackie-adenovirus receptor
CPE = Cytopathic effect
d = Day
DBP = DNA binding protein
DNA = Deoxyribonucleic acid
dsDNA = Double stranded DNA
dNTP = Deoxynucleotide phosphate
EIA = Enzyme immunoassay
ELISA = Enzyme-linked immunosorbent assay
EM = Electron microscope
F = Female
g = Grams
GBEB = Glycine-beef-extract buffer
GONs = Groups-of-nine hexons
h = Hour
HAdVs = Human adenoviruses
HAstV = Human astrovirus
HAV = Hepatitis A virus
HIV = Human immunodeficiency virus
HRV = Human rotavirus
HVR = Hypervariable region
IC = Immunochromatography
IF = Immunofluorescence
ITR = Inverted terminal repeats
kDa = KiloDalton

ℓ	= Litre
LRTI	= Lower respiratory tract infection
M	= Male
MHC	= Major histocompatibility complex
min	= Minute
mg	= Milligram
$MgC\ell_2$	= Magnesium chloride
mℓ	= Millilitre
mo	= Month
mRNA	= Messenger RNA
neg	= Negative
NJ	= Neighbor-Joining
nm	= nano-meter
no	= Number
NAb	= Neutralising antibodies
NPA	= Nasophyrangeal asparate
nPCR	= Nested polymerase chain reaction
PCR	= Polymerase chain reaction
PEG	= Polyethylene glycol
pos	= Positive
RE	= Restriction enzyme
RESP	= Respiratory
RFLP	= Restriction fragment length polymorphism
RGD	= Arg-Gly-Asp sequence
RIA	= Radioimmunoassay
RNA	= Ribonucleic acid
rpm	= Revolutions per minute
SA	= South Africa
SCID	= Severe combined immunodeficiency disease
sec	= Second
ST	= Stool
TNF	= Tumour necrosis factor
TP	= Terminal protein

ts	= Temperature-sensitive
UK	= United Kingdom
URTI	= Upper respiratory tract infection
USA	= United State of America
UV	= Ultraviolet
yr	= Year
WHO	= World Health Organisation
µℓ	= Microlitre
ºC	= Degree Celsius

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CHAPTER 1

LITERATURE REVIEW

1.1 HISTORY

Adenoviruses (AdVs) are ubiquitous, have been isolated from nearly every class of vertebrate (Russell and Benkö, 1999; Benkö *et al.*, 2005), and are species-specific (Strauss and Strauss, 2002). Adenoviruses were named after adenoids, the lymphoid tissue from which they were first isolated (Rowe *et al.*, 1953). In 1962, observations revealed that AdV serotype 12 (AdV-12) of species A induced sarcomas in newborn hamsters and, as a result, AdV-12 has been used as a model of viral oncogenesis (Wadell, 1999; Echavarría, 2009). Although outbreaks of epidemic keratoconjuctivitis characteristic of AdVs were known among factory workers in Germany during the nineteenth century (Wadell, 1999), it was not until the 1950s that HAdVs were first identified in humans as the causative agent of epidemic febrile respiratory disease among military recruits (Fong and Lipp, 2005). Adenoviruses are now recognised as the aetiologic agent of a variety of clinical syndromes, but they are often also isolated from the pharynx and stool of asymptomatic children (Wadell, 1999). Adenoviruses are associated with both sporadic and epidemic disease that results in significant economic losses and morbidity, especially amongst populations in closed settings (Gompf and Oehler, 2005). In 1980, it was demonstrated that the enteric AdVs, associated with infantile diarrhoea, differed from other human adenoviruses (HAdVs) by being fastidious in their *in vitro* growth requirements. Because of this and other features, they were assigned to a separate species designated F (Wadell, 1999). Recently, a new AdV species, G, which is suggested to be associated with human gastroenteritis, was discovered and categorised as the seventh HAdV species (Jones *et al.*, 2007). The ability of AdV gene products to adjust in the host immune response seems to play a role in their latency/persistent state, as has

been noticed for species C which persist in tonsils for years (Garnett *et al.*, 2007; Echavarría, 2008).

1.2 VIROLOGY OF ADENOVIRUSES

1.2.1 Morphology and Classification

1.2.1.1 Morphology

Adenoviruses are non-enveloped virions 70-90 nanometers (nm) in diameter (Figure 1.1A) that contains double-stranded (ds) DNA. They display characteristic icosahedral capsid structure and are composed of 252 capsomeres: 240 subunits of the trimeric hexon protein (hexons) which form the facets and 12 pentons, comprising the pentameric penton base protein and the externally projecting trimeric fibre, forming the vertices (Figure1.1B) (Burnett, 1997; Benkö *et al.*, 2005; Seiradake and Cusack, 2005).

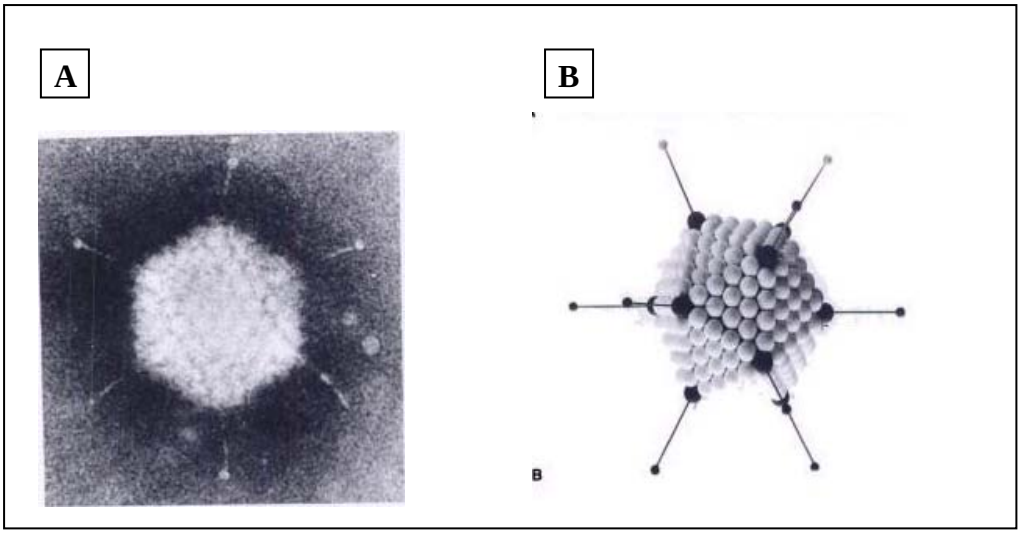

Figure 1.1: (A) Negatively stained preparation of an adenovirus showing the fibres projecting from the vertices; (B) Model of adenovirus showing the arrangement of capsomeres and fibres (Dimmock *et al*., 2001)

1.2.1.2 Classification

Adenoviruses have been isolated from placental mammals, marsupials, birds and amphibians (Seiradake and Cusack, 2005). A nomenclature for AdVs was agreed upon in 1956, and subsequently modified in 1999 (Benkö *et al.*, 2000;

Wold and Horwitz, 2007). The family Adenoviridae is comprised of four genera: Mastadenovirus, Aviadenovirus, Atadenovirus and Siadenovirus. Human adenoviruses are classified in the Mastadenovirus genus, which also includes simian, bovine, equine, porcine, ovine and canine AdVs (Seiradake and Cusack, 2005). Members belonging to the genus Mastadenovirus share common epitopes on the hexons (http://virology-online.com/viruses/Adenoviruses.htm). Serologically distinct viruses are defined as serotypes, and these are grouped into species. Species are defined depending upon a number of characteristics, including calculated phylogenetic distance, DNA hybridisation, restriction fragment-length polymorphism (RFLP) analysis, possibility of recombination, oncogenicity in rodents, host range (Benkö *et al.*, 2005), and degree of DNA homology. DNA homology of more than 50% occurs between members within a species and is less than 20% between members of different species (http://virology-online.com/viruses/Adenoviruses.htm).

1.2.2 Structure

1.2.2.1 Capsid proteins

The AdV particle is composed of more than 7200 polypeptide molecules, with a total mass of approximately 150×10^6 kilo Dalton (kDa). The hexon is the largest and most abundant of the structural proteins in the AdV capsid. The 720 hexon monomers present in each virion form 240 hexon homotrimers, which in turn form 20 capsid facets, each consisting of 12 hexon homotrimers (Stewart *et al.*, 1993). The other two major capsid proteins, fibre and penton base, form the penton complexes (three subunits of protein IV and five subunits of protein III) at each virion vertex. The position of the minor capsid protein IIIa has been mapped to the icosahedral edges, and hexamers of protein VI are positioned underneath the penton base (Figure 1.2) (Stewart *et al.*, 1991; 1993). The 240 hexons fill the facets and the 12 pentons are the vertices of the icosahedron; on the vertices are also the characteristics fibres (62 kDA), that range from 10 to 30 nm in different serotypes and act as attachment structures (one per cell) (Levy *et al.*, 1994).

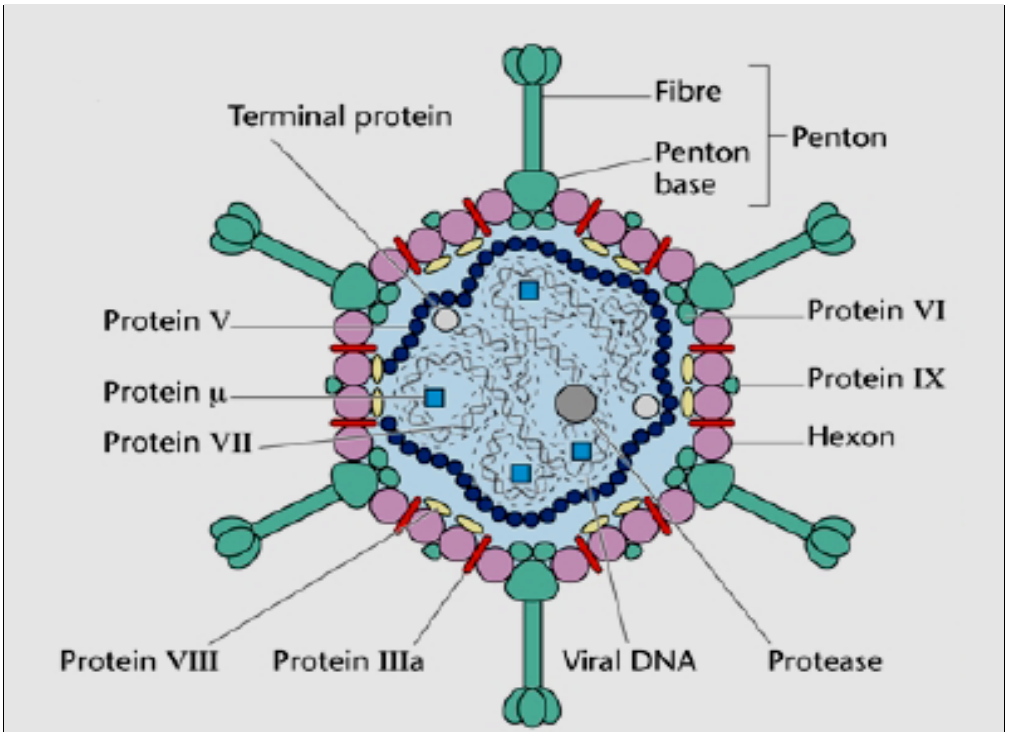

Figure 1.2: A section of a mastadenovirus particle depicting the adenoviral proteins (Flint and Shenk, 1997)

(a) Minor capsid proteins

Protein IIIa: the precursor of protein IIIa has a mass of 67 kDa and is cleaved at the N terminus during maturation of the virion to generate the 63.5 kDa protein IIIa. Protein IIIa is located at the surface of the particle and its modification may affect the tropism (Vellinga *et al*., 2005). The presence of the protein in the capsid is biologically significant, as temperature-sensitive (*ts*) mutants that affect protein IIIa (viz. H2*ts*4, H2*ts*112, H5*ts*58 and H2*ts*101) are defective in the assembly of virions and accumulate empty and protein IIIa-deficient particles at the non-permissive temperature (Vellinga *et al*., 2005).

Protein VI: the mature version of protein VI is of 22 kDa and is generated by cleavage from a larger precursor (pVI). Protein VI is positioned in the interior of the capsid, presumably adjacent to the hexons (Stewart *et al*., 1991; 1993; Greber *et al*., 1993). An important function of protein VI is to facilitate nuclear import of hexon proteins (Vellinga *et al*., 2005).

Protein VIII: the least-studied of the minor capsid proteins is the 15.3 kDa protein VIII. It is located at the inner surface of the triangular facets as dimers and interacts with hexons of adjacent facets. Analyses using mutant viruses suggest that protein VIII plays a role in the virion's structural stability (Vellinga *et al*., 2005).

Protein IX: the 14.3 kDa protein is the smallest of the minor capsid proteins. Protein IX is unique to the mastadenoviruses and is absent in the other adenovirus genera. Twelve molecules of protein IX are located at each of the 20 facets of the icosahedral capsid. The groups-of-nine hexons (GONs) form the central part of each of the facets of the icosahedral capsid (Rux and Burnett, 2004). In addition, protein IX affects the DNA-packaging capacity of human adenovirus, and it also affects transcriptional activity of several promoters (Stewart *et al*., 1991, 1993).

(b) Core protein

Two major polypeptides are associated with adenovirus cores, polypeptides V and VII. Polypeptide VII is the major core protein and accounts for about 10% of the protein mass of the virion (Soumitra *et al.*, 1998). It is synthesized as a precursor, polypeptide pVII, which is cleaved during virion maturation into the mature core protein, which has an estimated molecular weight of 19.4 kDa (Soumitra *et al.*, 1998). Polypeptide V is moderately basic, virus particle has an estimated molecular weight of 41.7 kDa, and is represented in the virus particle as 180 molecules of polypeptide V. Polypeptide V, unlike polypeptide VII, matures without proteolytic trimming, and it appears to be less tightly bound to the DNA than protein VII (Soumitra *et al.*, 1998).

1.2.2.2 Nucleic acid and genome properties

The genome of AdVs is a single, linear molecule of ds DNA that contains inverted terminal repeats (ITRs) (Russell and Benkö, 1999). Covalently linked to the 5'- end of each DNA strand is a virus-encoded terminal protein (TP) (Figure 1.3) (Benkö *et al*., 2005). The sizes of AdV genomes range between 26,163 and 45,063 base pairs (bp), with ITRs of 36 to 371 bp in

length; the G + C content varies between 33.7% and 63.8%. The genome of Mastadenoviruses (ranging between 30,288 and 36,521 bp) is smaller than that of Aviadenoviruses (ranging between 43,804 and 45,063 bp) (Russell and Benkö, 1999; Benkö *et al.*, 2005), with a G + C content ranging between 40.8% and 63.8% (Benkö *et al.*, 2005). The central part of the AdV genome is well conserved throughout the family, but the two ends show large variations in length and content (Benkö *et al.*, 2005). Furthermore, the DNA sequences that encode most virion structural proteins, and the proteins that are involved in replication of the viral DNA and assembly of virions, are highly conserved between all AdV genomes. The genome of HAdVs includes several repeats of cis-acting packaging sequence between the left terminal repeat and the first protein-coding region (Grable and Hearing, 1992; Berk, 2007).

The genome organisation of Mastadenoviruses are similar to that of the HAdVs. Sequence data for the animal AdVs however, indicates there is variation in the content of the E3 region (Figure 1.3) (Benkö *et al.*, 2005).

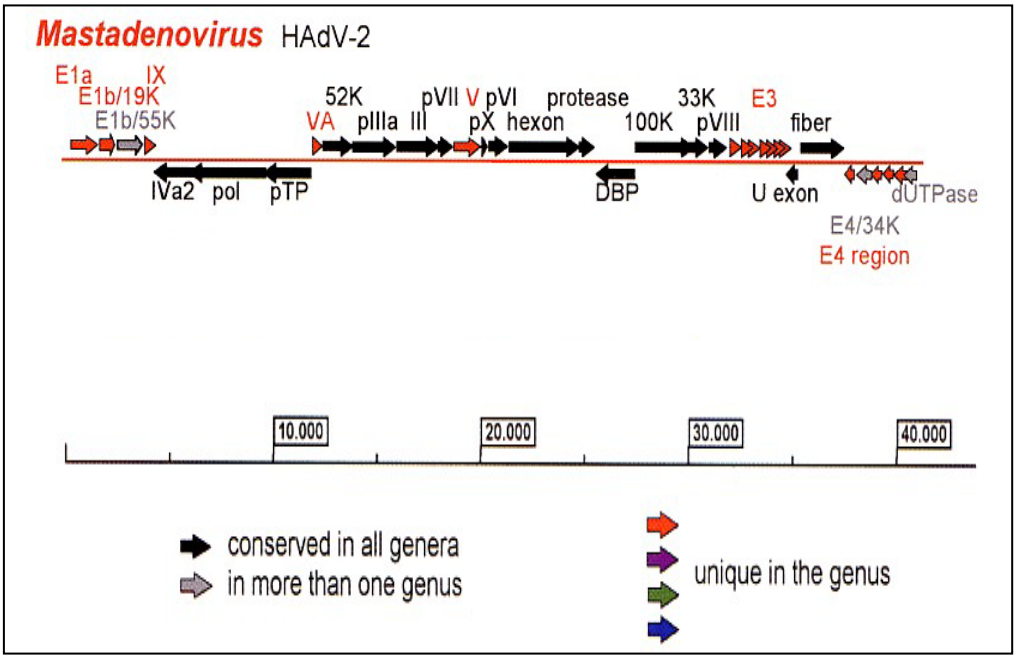

Figure 1.3: A schematic representation of the genomic organisation of human adenovirus (Benkö *et al.*, 2005).

The E3 region is known to have a different number and entity of genes even in the different serotypes of HAdVs (Benkö *et al.*, 2005). The E2 region, which is located between E1 and E3, is conserved throughout the family, while the length and content of E1, E3 and E4 (Figure 1.3) have great variability, even within the genera. The intermediate and late gene products of the replication cycle, which are L1 to L5, are concerned with the assembly and maturation of the virion (Benkö *et al.*, 2005).

1.2.3 Viral stability

Adenoviruses are stable at -70 ºC and are insensitive to lipid solvents (Benkö *et al*., 2005). Adenoviruses are relatively acid-stable (Russell and Benkö, 1999; Benkö *et al*., 2005; Echavarría, 2008). Heat sensitivity varies between viruses from different genera, but the Mastadenoviruses are inactivated by heating at 56ºC for 10 min (Benkö *et al*., 2005). Non-enveloped viruses with capsomeric lipophilicity such as AdVs are moderately sensitive to chemical disinfectants (Prince and Prince, 2001; Sauerbrei *et al.*, 2004). Adenovirus serotypes have been shown to possess an unusually variable spectrum of sensitivity to chemical biocides especially to peroxygen compounds and iodophors *in vitro* (Wutzler and Sauerbrei, 2000; Sauerbrei *et al.*, 2004). Human adenovirus serotypes 1, 8 and 29 are responsive to 0.05-2.5% liposomal povidone-iodine, while serotypes 2, 23, 25, 37, and 44, are resistant to inactivation by 0.05-2.5% liposomal povidone-iodine at exposure times of 5, 30, and 60 min (Sauerbrei *et al*., 2004). As AdVs do not have an envelope, a suggested reason for the different sensitivities is the differences in the viral capsid proteins (Sauerbrei *et al.*, 2004).

As viral pathogens are <100 nm in size, they are not removed by sand filtration during water treatment. This makes disinfection essential in order to ensure drinking water is safe for human consumption (Thurston-Enriquez *et al.*, 2005). Disinfection of AdVs is problematic because they are exceptionally resistant to some water treatment processes, such as chemical disinfection (including chlorine), physical agents and pH variation.

Adenoviruses have been shown to be up to 60 times more resistant to UV irradiation than RNA viruses, including enteroviruses and hepatitis A virus (HAV) (Enriquez *et al.*, 1995; Horwitz, 1996; Foy, 1997; He and Jiang 2005; Gerba *et al.*, 2008).

1.3 HUMAN ADENOVIRUSES

1.3.1 Species designation and clinical significance

The first HAdVs reported were isolated from explants of adenoid tissue (Rowe *et al.*, 1953). Human adenoviruses are divided into seven species (HAdV-A, HAdV-B, HAdV-C, HAdV-D, HAdV-E, HAdV-F and HAdV-G) comprised of 52 serotypes (Jones *et al.*, 2007). There is significant genetic drift within some genotypes of AdV, e.g. HAdV-F40 and HAdV-B1:7. Human AdVs are associated with a number of diseases, which affect the respiratory, urinary and gastrointestinal tracts and the eyes (Horwitz, 2001). They also can be recovered in healthy persons from the tonsils or adenoid glands (Levy *et al.*, 1994). Among human enteric viral pathogens, HAdV is the only DNA virus (Enriquez *et al.*, 1995; He and Jiang, 2005). The enteric AdVs were recognised in stool samples of infants with acute gastroenteritis. Adenovirus species A can be found in the gastrointestinal tract where it becomes latent in the crypts. They are distinct from other AdV serotypes, by serology and DNA restriction pattern (Desselberger and Gray, 2003). The HAdV species, their serotypes and common clinical manifestation in man is presented in Table 1.1.

1.3.1.1 Species A

Human adenovirus species A (types 12, 18, 31) is commonly detected exclusively from the gastrointestinal tract (Kidd *et al.*, 1996) but are also rarely isolated. Human adenoviruses species A is known to be highly oncogenic compared with other HAdV species. These viruses have recently been detected, by molecular-based assays, in the blood of immunocompromised patients (Kampmann *et al.*, 2005; Echavarría, 2008). Human adenovirus type 31 has been detected in stool, urine and throat

secretions from patients with severe combined immunodeficiency disease (SCID) and bone marrow transplant (BMT) patients with pneumonia, hepatitis, diarrhoea, and acute haemorrhagic cystitis (Hierholzer, 1992).

Table 1.1: Human adenovirus species and serotypes, and most common disease manifestations in immunocompromised patients (Leen and Rooney, 2004).

Species	***Serotypes***	***Common disease syndromes***
A	12, 18, 31	Gastroenteritis
B	3, 7, 11, 14, 16, 21, 34, 35, 50	Upper and lower respiratory infections, acute respiratory disease, pharyngoconjunctival fever
C	1, 2, 5, 6	Upper and lower respiratory infections in young children
D	8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49, 51	Epidemic keratoconjunctivitis, conjunctivitis
E	4	Acute respiratory disease, pharyngoconjunctival fever
F	40, 41	Gastroenteritis
G	52	Gastroenteritis

1.3.1.2 Species B

Human adenovirus species B has been divided into two sub-species B:1 and B:2, based upon tropism and restriction cleavage patterns (de Jong *et al*., 1999). The most common members of HAdV species B, types 3 and 7, have been reported to cause community-acquired epidemics of respiratory and ocular infections every few years. They are known to be severe respiratory pathogens in children younger than one year of age when infection is occasionally fatal and also the cause of permanent pulmonary damage (de Jong *et al*., 1999). Human adenovirus species B infections are reported to be the common cause of outbreaks of febrile respiratory tract infection and pneumonia in military recruits and responsible for 90% of recruit hospitalisations for pneumonia (Booth *et al*., 2004). They are also a significant cause of disease in immune-suppressed persons (Booth *et al*., 2004). Members of sub-species B:1, HAdV-3 and HAdV-7, are know to

account for 13% and 20%, respectively, of all AdVs isolates reported to the World Health Organisation (WHO), and they also show epidemic periodicity at four to five year intervals. There are three different patterns of HAdV-7 infection documented: the first pattern represents outbreaks, mainly during the winter season, the second pattern manifests as outbreaks of respiratory disease among school children, and the third pattern is known to constitute outbreaks among newly enlisted military recruits of HAdV-4, -7, -14 and -21. The last-mentioned outbreaks can be ascribed to the close contact among young men sleeping in crowded quarters (Kidd *et al*., 1996; Wadell, 1999; Carballal *et al*., 2002; Booth *et al*., 2004). The majority of sub-species B:2 HAdVs have been isolated from the urinary tract (Kidd *et al*., 1996). Persistent infection of the kidneys is caused by HAdV-B2:11, HAdV-B2:34 and HAdV-B2:35 and these types are the most common HAdVs isolated from the urine of patients with acquired immunodeficiency syndrome (AIDS) or BMT recipients (Wadell, 1999)

1.3.1.3 Species C

The common HAdV serotypes of species C, namely HAdV-1, -2, -5 and -6, reportedly cause approximately 5% of symptomatic upper respiratory tract and 15% of lower respiratory tract infections in children younger than 5 years of age, and these types are regarded as endemic worldwide (Garnett *et al.*, 2002). The infection of infants is known to be from older siblings shedding the virus after recrudescent infections for up to 2 years (Wadell, 1999). By the age of 4 years, at least 50% of children have antibodies against HAdV-1 or HAdV-2 (Garnett *et al.*, 2002). In addition to acute disease, HAdV-C serotypes establish persistent infections in immunocompetent hosts characterised by intermittent viral shedding. Although primary infections occur at the respiratory site, HAdV-C is shown to display prolonged faecal shedding for months, and even years, after virus is no longer detected in nasopharyngeal washings (Garnett *et al.*, 2002). Viruses from HAdV-C species account for 50% of all HAdV serotypes reported to the WHO (Wadell, 1999).

1.3.1.4 Species D

Species D is known to be comparatively rare in immunocompetent individuals, with the exception of HAdV-10 and -19 which are known to cause keratoconjuctivitis (Lord *et al*., 2000). Types HAdV-19 and -37 are the suspected cause of sporadic cases, as well as outbreaks of severe epidemic keratoconjuctivitis, particularly in dry climates or in densely populated areas. The latter two strains are also well known to cause nosocomial infections (Jernigan *et al*., 1993). All of the recently described HAdV-D species, HAdV-42 to HAdV-49, were originally isolated from patients with AIDS, but still have to be determined to cause disease (Lord *et al*., 2000).

1.3.1.5 Species E

Human adenovirus serotype 4 was originally isolated from a United States of America (USA) army trainee diagnosed with acute respiratory illness during an epidemic in the winter of 1952 to 1953 (Hilleman, 1956). Two distinct genome types, AdV-4 and AdV-4a, have been identified (Wadell, 1999). Serotype 4 cause epidemic outbreaks of respiratory disease among military recruits and acute viral conjunctivitis (Schepetiuk *et al.,* 1993; Purkayastha *et al.,* 2005). The AdV-4a genome type is described as being second only to AdV-8 as a cause of AdV-associated eye infections in Japan (Cooper *et al*., 1993; Erdman *et al*., 2002). Its evolutionary origins have been a subject of speculation. A the lack of genomic diversity among HAdV-4 strains (Cooper *et al*., 1993; Erdman *et al*., 2002) implies that HAdV-4a species may be the product of a relatively recent evolutionary event with HAdV-4 as the archetype (Purkayastha *et al*., 2005). Early restriction enzyme digestion analysis of HAdV-4 and two chimpanzee AdVs (Pan-7 and -9) suggests that HAdV-4 may be distantly related to these simian AdVs and studies suggest that the HAdV-E species may have originated from a chimpanzee-to-human zoonotic event (Purkayastha *et al*., 2005).

1.3.1.6 Species F

Species F are the common recognised enteric HAdVs (HAdV-40 and HAdV-41) and an important cause of infantile viral gastroenteritis worldwide. They are considered to be the second only to human rotaviruses (HRVs) as the cause of acute childhood diarrhoea (Scott-Taylor and Hammond, 1992). In contrast to HRVs, there is no significant seasonal variation in the pattern of infection. Enteric HAdVs are rarely detected in the stools of healthy individuals (Wadell, 1999). Human adenovirus types 40/41 have been detected in stool specimens from Africa, Asia, Europe, Latin America and North America (Wadell, 1999) and are fastidious in their ability to grow in cell culture (Grabow *et al.*, 1992; Tiemessen and Kidd, 1995).

1.3.1.7 Species G

A newly described HAdV detected from humans was identified and characterized as a new virus, after it was cultured from the stool of a patient presenting with gastroenteritis in 2003, at the Los Angeles Country Public Health Department. Human adenovirus type 52 represents a new type and a new species in the family Adenoviridae (Jones *et al.*, 2007).

1.3.1.8 Phylogeny of adenoviruses

The hexon protein is considered as the most important AdV protein for the classification and recognition of individual serotypes (Ebner *et al.*, 2006). The amplification of the hypervariable portion of the hexon gene, followed by DNA sequencing, also can be utilised to sequitype (Sarantis *et al.*, 2004). The amplification, sequence and phylogenetic analysis of the moderately conserved region, amino acids 540 to 662, of the hexon gene (Crawford-Miksza and Schnurr, 1996) are sufficient to allow HAdV speciation and, in most cases, genotype identification (Casas *et al.*, 2005). The remaining part of the hexon protein (765 amino acids) shows relatively little variability among different HAdV serotypes (Crawford-Miksza and Schnurr, 1996). The phylogenetic relationship of the HAdV serotypes and species, based on the conserved L1 region of the hexon gene, is presented Figure 1.4.

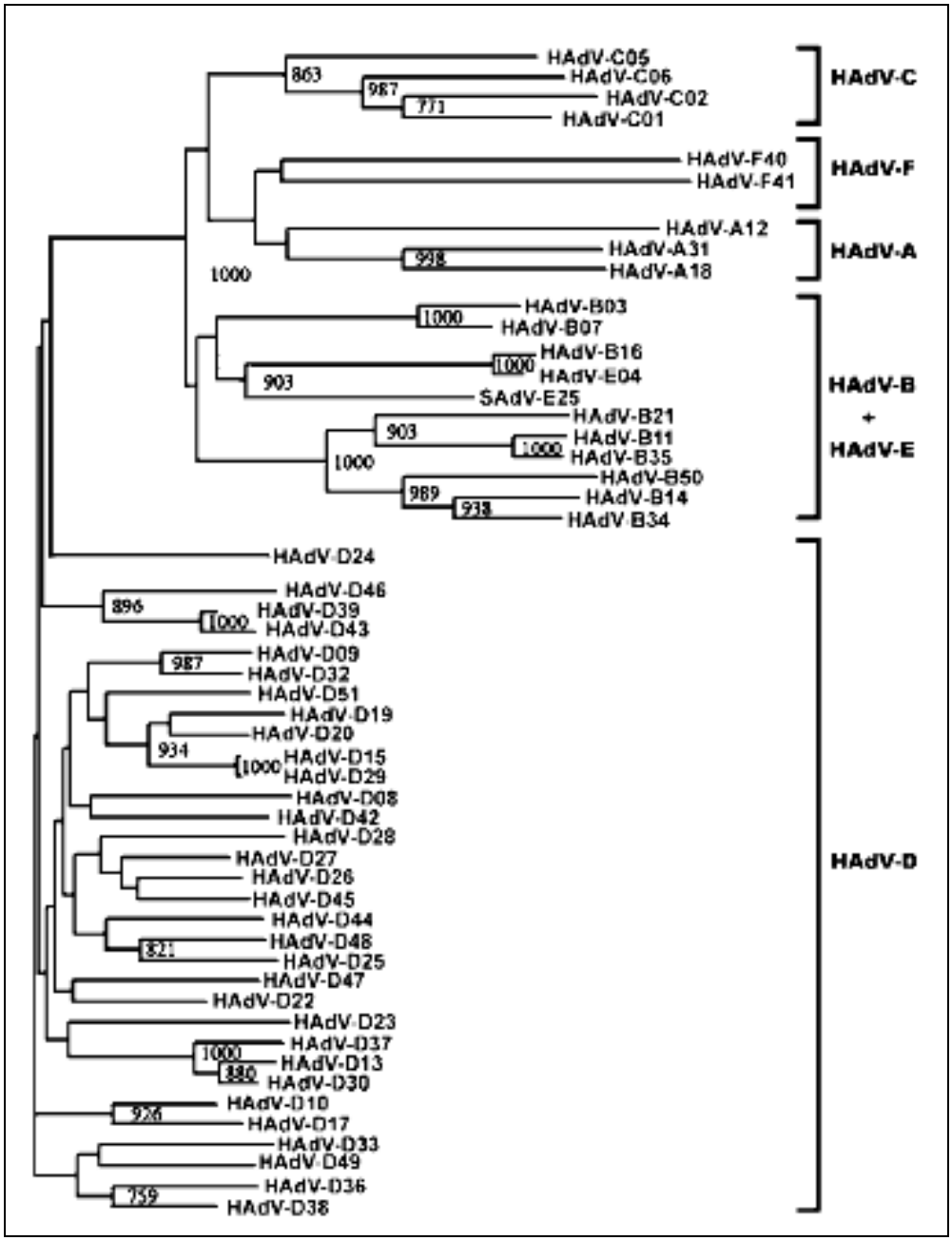

Figure 1.4: Phylogenetic analysis of nucleotide sequences of AdV hexon, based upon the conserved L1 region (Pring-Akerblom and Adrian, 1994)

1.3.2 Clinical infection

1.3.2.1 Infectious cycle

Adenovirus attachment is mediated by the interaction between the AdV fibre protein and a specific receptor on the target cell. Most AdVs are reported to utilize the coxsackie-adenovirus receptor (CAR) as their point of entry (Carson, 2001; Leen and Rooney, 2004), except for species B viruses which specifically interact with the CD46 membrane cofactor protein (Gaggar *et al.*, 2003; Sirena *et al.*, 2004) and species D serotype 37, which requires the presence of sialic acid rather than CAR on target cells (Arnberg *et al.*, 2000). Thereafter, replication occurs in three phases: an immediate-early phase, a delayed-early phase which precedes viral DNA replication and a late phase which is characterised by the expression of the structure proteins of the viral capsid. Replication of AdV is understood to occur in the nucleus and is semi-conservative, with each strand being elongated continuously without Okazaki intermediates. The early mRNAs appear during the first 8 to 10 hours, but they continue to be made throughout the infection cycle (Subramanian *et al.*, 2006).

Adenovirus particles assemble in the nuclei of infected cells and the replication cycle takes 32 to 36 hours (Echavarrìa, 2009). Although fibre-deficient mutants of AdV seem to have an altered viral morphogenesis, more recent data suggest that the fiber plays a role in capsid stabilisation, rather than in capsid assembly (Hong and Engler, 1996). Three different types of infection are caused by AdVs: a productive lytic infection predominantly in epithelial cells, and latent or persistent infections in the lymphoid mucosal tissue which may infrequently produce virus, or transformation predominantly in rodent cells. A schematic representation of an AdV lytic cycle is presented in Figure 1.5. The AdV lytic cycle is said to be efficient producing, 10,000 to over 1,000,000 virions per cell, with 1-5% being infectious (Echavarrìa, 2009).

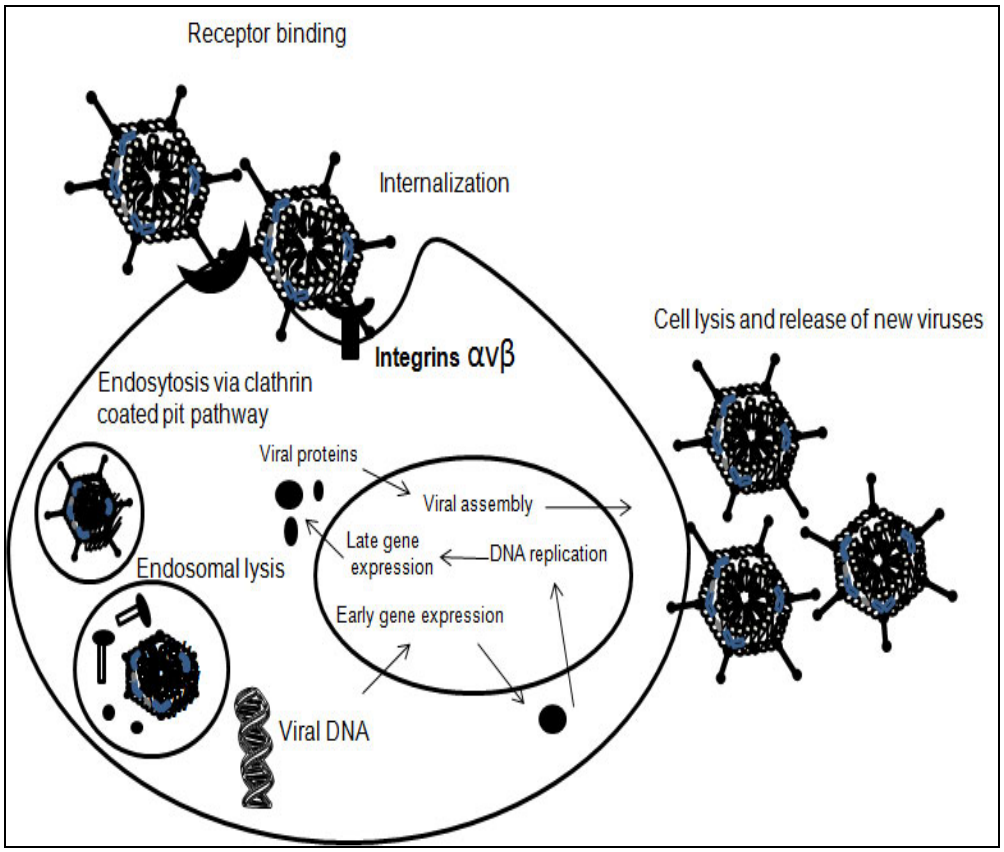

Figure 1.5: A schematic illustration of the adenovirus 5 infection pathway (Särkioja, 2008).

1.3.2.2 Pathogenesis

The pathogenicity of AdVs differs according to species and type, and organ specificity and disease patterns appear to occur within particular serotypes (Horwitz, 2001; Kojaoghlanian *et al.*, 2003). Adenovirus infections can be either localised to a single organ or can affect numerous organ systems which may lead to disseminated disease, with an especially high mortality rate (Gu *et al.*, 2003; Echavarría, 2008). The incubation period for AdV infection ranges from 2 to 21 days with an average of 7 to 13 days and the primary site of replication is the epithelial cells. The virus-mediated tissue damage is thought to result from direct cytotoxicity of the virus, but can also occur be due to the inflammatory cell infiltrate (Ruuskanen *et al.*, 2009).

Adenovirus infection results in an immune response that probably renders lasting protection against re-infection with the same virus type and against

reactivation of persistent infection. This acquired immunity is partly evaded through the evolution of a large number of serotypes, which induce no or only minor cross-protection (Desselberger and Gray, 2003). In the presence of a functional immune system, AdV infections are not associated with life-threatening disease, but there is evidence of latent infections with persistence of the viral genome, involving particularly species C (Garnett *et al.*, 2002).

Non-specific effector cells, such as natural killer cells and macrophages, and antigen-specific T- and B-lymphocytes, have been described to mediate immune responses against AdVs (Leen and Rooney, 2004). The innate immune system is said to recruit and activate antigen-presenting cells (APCs) that facilitate the development of an optimal adaptive immune response (Li *et al.*, 2001; Leen and Rooney, 2004).

Most patients infected with AdVs are known to develop both group- and type-specific antibodies to the infecting strain. Group-specific antibodies are generally non-neutralizing, while serotype-specific responses are generally directed against hypervariable regions on the virus capsid and can effectively neutralise extracellular virus, thus preventing virus spread (Crawford-Miksza and Schnurr, 1996; Horwitz, 2001; Leen and Rooney, 2004). Once inside the cell, AdV is likely to be protected from antibodies but becomes the target of cellular immune responses, usually resulting in eradication of the infected cell by $CD4^+$ and $CD8^+$ T cells (Lee *et al.*, 2004). In immunosuppressed patients, AdVs are one of the opportunistic pathogens that cause severe, prolonged and frequently fatal infections (Leen and Rooney, 2004).

1.3.2.3 Clinical syndromes

Adenovirus infections are found worldwide with primary infection occurring in the first few years of life. Multiple AdV infections are common because immunity is type-specific and persons are exposed to many types (Hierholzer, 1992; Wiedbrauk and Johnston, 1993).

(a) Enteric infection and gastroenteritis

The fastidious HAdVs, HAdV-F40 and HAdV-F41, cause diarrhoea in 4 to 15% of children in hospitals, out-patient clinics and child care centres. Other HAdV types commonly detected in stools are HAdV-C1, -C2, -B3, -C5, -B7 and -A31 (Ruuskanen *et al.,* 2009). The incubation period is about 8 to 10 days and the mean duration of diarrhoea is 3 to 11 days (Ruuskanen *et al.,* 2009). The stools are usually watery, non-bloody with no faecal leukocytes (Echavarría, 2009), and other symptoms include vomiting, mild fever, abdominal pain and dehydration, which occurs in less than one-fifth of patients (Wadell, 1999; Echavarría, 2009). Adenoviral diarrhoea occurs most often in children younger than 4 years of age and is clinically not distinguishable from HRV infection (Reina *et al.,* 1994; Wold and Horwitz, 2007). The enteric HAdVs cause longer gastroenteritis than non-enteric HAdVs and HAdV-F41-induced diarrhoea lasts longer than HAdV-F40-induced diarrhoea (Ruuskanen *et al.,* 2009). In most immunocompromised patients, AdV gastroenteritis is associated with HAdV-A31, HAdV-C2, HAdV-F40 and HAdV-F41, while in patients with AIDS, HAdV gastroenteritis is usually associated with HAdV-D serotypes 42 to 49 (Hierholzer, 1992). The faecal shedding of AdVs by patients with AIDS has been associated with poor prognosis and shorter survival (Sabin *et al.,* 1999).

Other documented enteric conditions associated with HAdVs include intussusception, acute mesenteric lymphadenitis and appendicitis (Wold and Horwitz, 2007; Echavarría, 2009). In one study, 20% of children with intussusception had non-enteric HAdVs in their stools in comparison with 4% of healthy controls (Ruuskanen *et al.,* 2009).

(b) Respiratory disease

Upper respiratory tract infections (URTI) caused by HAdVs cannot be distinguished from URTI infections induced by other viruses. Adenovirus-associated URTI include pharyngitis, pharyngoconjunctival fever, acute respiratory disease, common colds and tonsillitis. Most children are infected

early in life and these infections are usually mild and self-limited. Species C HAdVs, specifically types 1, 2, and 5 are mostly associated with URTIs. Viruses can be isolated from the oropharynx after the onset of symptoms after which AdV shedding in the stools may be prolonged for months (Wiedbrauk and Johnston, 1993; Echavarría, 2009).

Lower respiratory tract infections (LRTI), such as bronchitis, bronchiolitis and pneumonia, are caused by types B1:3, E4, B1:7 and B1:21. Type C5 infection has been associated with a pertussis-like syndrome and types E4 and B1:7 have been associated with acute respiratory disease in military recruits in the US (Swenson, 1999).

(c) Ocular disease

Eye infections caused by HAdVs include pharyngocnjunctival fever, keratoconjunctivitis and waterborne "swimming pool" conjunctivitis pharyngoconjunctival fever occurs predominately in children. Three known serotypes of HAdV species C (-1, -2 and -5) are endemic whereas two serotypes of species B1 (-3 and -7) cause epidemic outbreaks of pharyngoconjuctival fever. The pharyngeal conjunctival fever has been suggested to appear after a mean incubation period of 6 days. The initial conjunctivitis is followed by high fever and sore throat. Vomiting, diarrhoea and meningeal signs are found and temperature returns to normal after 7-8 days. Symptoms are usually more severe in children but sequelae are rare (Wadell, 1999).

Epidemic keratoconjunctivitis occurs mainly in adults and is typically caused by types D8, D19 and D37. Outbreaks of keratoconjunctivitis occur in ophthalmology clinics, hospitals, camps, military bases and industrial plants. The virus can remain viable for several weeks on wash basins and towels and is transmitted by contaminated ophthalmic solutions and instruments and by the sharing of bathrooms (Ruuskanen *et al.*, 2009). After incubation period of 8 to 10 days symptoms such as follicular conjunctivitis, headache,

photophobia and mild upper respiratory tract infections usually resolves within 2 weeks although focal subepithelial keratitis can develop and interfere with the patients' vision (Wiedbrauk and Johnston, 1993: Ruuskanen *et al.*, 2009).

(d) Urinary and Genital tract infections

Haemorrhagic cystitis is associated with an HAdV-B2:11 infection and in children occurs more often in boys than girls. In renal transplant and BMT patients female gender, seropositivity to AdV and graft-versus-host disease are risk factors for HAdV-B2:11 haemorrhagic cystitis (Ruuskanen *et al.*, 2009).

Adenoviruses types C2, D19 and D37 can cause herpes-like genital lesions are often associated with orchitis, cervicitis and urethitis (Weidbrauk and Johnston, 1993).

(e) Infections of the immunocompromised and transplant host

Disseminated AdV disease has been reported with increasing frequency in patients with AIDS or malignancies (Janner *et al.*, 1990; Krilov *et al.*, 1990; Hierholzer, 1992; Arola *et al.*, 1995; Jogler *et al.*, 2006) and in bone marrow or solid-organ transplant recipients (Cames *et al.*, 1992; Michaels *et al.*, 1992; Flomenberg *et al.*, 1994; Blanke *et al.*, 1995). In addition, the frequency of severe AdV disease is increasing in association with growing numbers of immunocompromised individuals, and fatality rates as high as 64% have been reported (Bruno *et al.*, 2003; Lion *et al.*, 2003; Leen and Rooney, 2004). In the immunocompromised host, however, HAdV, including species C infections are causative agents of severe localised disease including pneumonitis, colitis, haemorrhagic cystitis, hepatitis, nephritis, encephalitis, or disseminated AdV disease with multi-organ failure leading to death (Hierholzer, 1992; Blanke *et al.*, 1995; Carrigan, 1997; Jogler *et al.*, 2006). Infections with subspecies B:2 viruses tend to be more prevalent, especially when the kidney is involved (Hierholzer, 1992).

Typical HAdVs found in association with HIV/AIDS include types D9, D17, D20, D22, D23, D26, D27, and D42 to D51. It has been suggested that long-term infection characteristics of AIDS patients provides the opportunity for mutation to occur within a strain or for recombinational events between co-infecting serotypes to take place (Hierholzer, 1992). Most of the HAdVs infecting the gut of HIV-infected patients represent serotypes of the HAdV-D which are rarely encountered in the stools of immunocompetent patients or in patients suffering from other kinds of immunodeficiency (Khoo *et al.*, 1995; de Jong *et al.*, 1999).

Adenoviruses have been reported as the common viral pathogens which are responsible for significant levels of morbidity and mortality post-transplantation (Chakrabarti *et al.*, 2002; Lion *et al.*, 2003). Adenoviral infections are one of the major causes of morbidity and mortality, specifically HAdV-C1 and -C2 associated with multi-organ infection, and HAdV-A31 occasionally associated with gastroenteritis. In patients who receive a haematopoietic stem cell transplant from an unrelated or mismatched donor HAdV-B1:3 and –B1:7 have been associated with severe respiratory infection (Leen and Rooney, 2004). Allogeneic bone marrow and stem cell transplant patients appear to be especially high risk for HAdV infection, with some evidence of higher risk among recipients of unrelated or HLA-mismatched transplants as well as in paediatric age groups (Gu *et al.*, 2003). Human AdV infections are especially frequent and sometimes fatal in the BMT patients because of aggressive immunosuppressive treatments, with children of 0-18 years of age at high risk compared to adults (Flomenberg *et al.*, 1994; Howard *et al.*, 1999). Adenovirus enteritis has been reported from small bowel transplant patients (Parizhskaya *et al.*, 2001).

1.4 LABORATORY DIAGNOSIS

The definitive diagnosis of an AdV infection requires either the visualisation of the virus by electron microscopy (EM), isolation of the virus in cell culture,

demonstration of antigen-infected cells by immunofluorescence (IF), detection of virus antigen by enzyme immunoassay (EIA), the demonstration of viral nucleic acid by molecular-based assay or demonstration of IgM antibody or a four-fold rise in antibody titre in blood specimens (Swenson, 1999; Echavarría, 2009).

1.4.1 Serology

A group-specific serological diagnosis can be performed using EIA or complement fixation assays, while for serotype-specific diagnosis assays such as haemagglutination inhibition or neutralisation assays are required. Although these assays can give indication of recent or past infection, false negative results have been documented in children due to persistent infections and poor serological responses and false positive results due to heterotypic anamnestic responses that can occur (Wiedbrauk and Johnston, 1993; Echavarría, 2008).

1.4.2 Viral isolation

Due to the species specificity of AdVs cell cultures of human origin are usually used for the isolation of HAdVs (Swenson, 1999). For most HAdVs the characteristic cytopathic effect (CPE) is evident within 2 to 7 days post-inoculation. If the innoculum virus titer is low, then the CPE may take up to 14 days or longer to develop (Wold and Horwitz, 2007). Virus isolation can be confirmed in conventional tube cell culture by typical CPE (Malherbe and Strickland-Cholmley, 1980). Cell culture confirmation can also be done by fluorescent antibody staining in the shell vial assay (Swenson, 1999).

1.4.3 Viral detection

A variety of conventional and molecular methods are used for the direct detection of AdVs in clinical specimens.

1.4.3.1 Electron microscopy

A wide variety of viruses have been detected by direct EM examination of negatively stained faecal specimens. Previously the diagnosis of AdV-associated gastroenteritis was limited to EM examination of stool specimens as serotypes 40 and 41 are not easily isolated in cell culture (Martin and Kudesia, 1990). Electron microscopes however require major capital investment and they are expensive to maintain and their use has in most instances been superceded by more cost effective EIA or molecular-based assays (Madeley, 1997).

1.4.3.2 Viral antigen detection

A rapid diagnosis of AdV infection can be made by direct detection methods, such as EIA, radioimmunoassay (RIA), and direct IF antibody techniques (Takimoto *et al.*, 1991; Trabelsi *et al.*, 1992; Tsutsumi *et al.*, 1999). Although IF antibody techniques for the detection of AdVs in pharyngeal or conjuctival epithelial cells are moderately sensitive, they require special equipment and the assay takes at least 1 hour to complete. Consequently, these tests are not ideal for wider clinical application at the bedside or in an outpatient clinic (Tsutsumi *et al.*, 1999). For the routine detection of HAdV-F40/41 in stool specimens, species F specific EIAs are commonly applied (Marx *et al.*, 1998; Audu *et al.*, 2002a; Fodha *et al.*, 2006). Immunochromatographic assays have been widely used, and provide a rapid diagnostic test for detection of HAdV in stools, nasopharyngeal aspirates and conjunctival scrapings (Tsutsumi *et al.*, 1999; Weitzel *et al.*, 2007).

1.4.3.3 Molecular-based methods

Efforts have been implemented to increase the sensitivity of detection for AdVs and molecular approaches have emerged as the most promising methods. The polymerase chain reaction (PCR) has become an important method for the rapid, sensitive, and specific detection for bacterial and viral agents in the past decade (Puig *et al.*, 1994; Cho *et al.*, 2000) and has successfully been applied for the detection of AdV in a variety of samples and

specimens, namely stools (Reither *et al.,* 2007) and respiratory secretion (McAnerney *et al.,* 1994). Comparisons have shown that PCR is more sensitive and specific than previous detection methods, such as cell culture and EIA (Ruuskanen *et al.,* 2009). The application of PCR enabled AdVs to be detected in blood (Lion *et al.,* 2003; Jalal *et al.,* 2005; Kampmann *et al.,* 2005), 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), sewage and shellfish (Pina *et al.,* 1998), and which had previously been difficult. It must however be emphasized that detection of AdVs in environmental samples by PCR assay indicates the presence AdV DNA and does not provide any information on infectivity of the virus, which is directly related to the human health risk (Sobsey *et al.,* 1998; Jiang *et al.,* 2001; Kok *et al.,* 2003).

More recently, real-time quantitative PCR assays have been developed and proved invaluable for the management and treatment of AdV infections and establishing the extent of environmental contamination. By adding a fluorescently labelled probe in the real-time PCR methods, quantification of the target gene is facilitated (He and Jiang, 2005). Among the various quantitative PCR strategies available, those based upon the real-time monitoring of the amplification reaction are the most accurate (Kalinina *et al.,* 1997). Real-time PCR has also been described as the method that has enhanced the speed and scope of measuring viral strain and titre differences in patients displaying different syndromes due to varieties of the same virus (Furuta *et al.,* 2001). Other studies described the use of real-time PCR to provide insight into the role(s) some compounds have on PCR inhibition as well as shedding light on the efficiency of different nucleic acid extraction methods from a diverse range of sample types (Biel *et al.,* 2000; Niesters *et al.,* 2000). Additionally, the study of emerging viruses has been complimented by the use of real-time PCR as an essential tool to demonstrate

links between unique viral sequences and patient clinical signs and symptoms (Lanciotti *et al.*, 2000; Gibb *et al.*, 2001; Smith *et al.*, 2001).

1.5 EPIDEMIOLOGY

All age groups, but particularly young children are infected by AdVs. Most of the infections are sub-clinical and symptomatic infections are mild and self-limiting with occasional mortality in healthy individuals (Ryan *et al.*, 2001). Adenovirus infections occur worldwide as epidemic, endemic or sporadic infections (Desselberger and Gray, 2003). Infections are known to occur throughout the year, but outbreaks are more common in late winter, spring and early summer. Most respiratory HAdVs are both endemic and epidemic causing outbreaks in closed communities, namely boarding schools, day-care centres, military camps etc. This type of infection is known to show seasonal preference, i.e. winter and spring, in temperate climates (Desselberger and Gray, 2003). Enteric AdV infection is well known as a common cause of infantile diarrhoea in the day-care setting. However it is noted to be less a common cause than HRV infection and, in some settings, even less than common HAstV (Gompf and Oehler, 2005). Eye infections occur sporadically or cause disease in large groups of contacts. When the source of HAdV infection is a swimming pool or small lake, many children and young adults may develop symptoms (Wold and Horwitz, 2007).

1.5.1 Transmission

Human adenovirus are very contagious and local outbreaks are common (Horwitz, 1996). The mouth, nasopharynx, and ocular conjunctiva serve as the port of entry for HAdVs (Gu *et al.*, 2003). The pattern of infection often correlates with the viral serotype and the age (children or adults) of the susceptible population (Wold and Horwitz, 2007). Owing to the diverse epidemiology of the wide spectrum of HAdVs, exposure and infection are possible by a variety of routes (Figure 1.6). Person-to-person contact plays a major role in the transmission of the virus. Depending on the nature of illness,

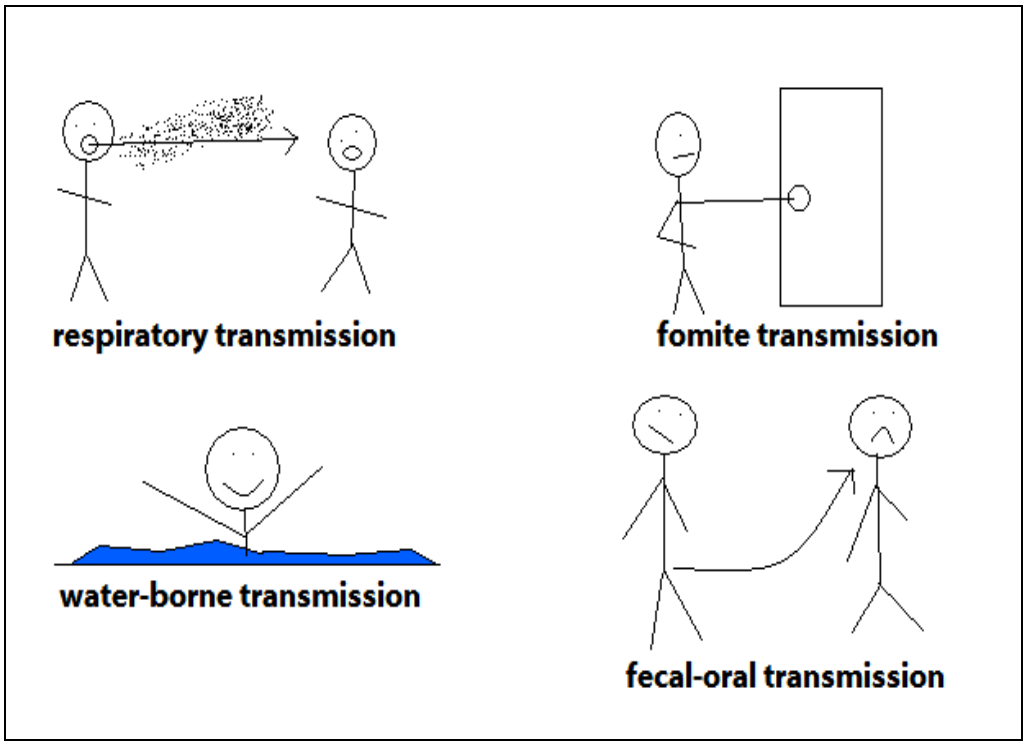

Figure 1.6: Stick figures depicting the transmission of HAdVs from one person to another (*Picture by Jeremy Schneider – based on Dr. Siegel's T-Shirt, 2005*) (http://www.stanford.edu/group/virus/adeno/2005/myvirus.html).

this can include faecal-oral, oral-oral and hand-eye contact transmission, as well as indirect transfer through contaminated surfaces or shared utensils (Puig *et al.*, 1994; Chapron *et al.*, 2000; Grabow *et al.*, 2001; WHO, 2004).

The duration shedding of HAdVs from different sites varies from 1 to 3 days from the throat in adults with the common cold presentation; 3 to 5 days from the nose, throat, stool, and eye from patients with pharyngoconjunctival fever; 3 to 6 weeks from throat or stool of children with respiratory or generalised illness; immune-compromised and immune deficient individuals may shed AdVs for many months in respiratory secretions, stool and urine and are therefore potentially a major source of infection (Wold and Horwitz, 2007).

1.5.1.1 Person-to-person transmission

(a) Faecal-oral and/or fomite route

During early childhood, infection by the oral route, as the consequence of shedding into stools occurs, prolonged carriage in the intestine makes the faeces a more common source during both the acute illness and intermittent recurrences of shedding (Wadell, 1999; Wold and Horwitz, 2007). Infection of the eye is suggested to occur through fomites as revealed by outbreaks from ophthalmologists' offices via tonometers as the common source of infection (Wadell, 1999). As AdVs are very stable in the environment and also being capable of surviving chemical and pH disturbances, they can be transmitted through direct contact by touching a contaminated surface and then touching the mucosa or eyes or mouth.

(b) Respiratory route

Respiratory transmission is known to occur usually through a cough or a sneeze. Adenoviruses infect the mucosa of the eyes or respiratory tract through aerosolised viral particles or through mucous droplets, though dissemination in swimming pools has also been implicated in epidemics of pharyngoconjuctival fever and conjunctivitis. Despite the large number and the worldwide distribution of HAdVs, their clinical importance is largely restricted to epidemics of acute respiratory disease, an influenza-like illness in military recruits, and to limited outbreaks among children (http://www.stanford.edu/group/virus/adeno/2005/transmission.html).

1.5.1.2 Waterborne transmission

Waterborne transmission of non-enteric serotypes of HAdVs have been associated with a number of swimming pool-related outbreaks of pharyngoconjuctivitis (Jiang, 2006), especially when insufficient chlorine has been used. The role of water in the transmission of enteric AdVs (HAdV-F40/41) is as yet undetermined (Wyn-Jones and Sellwood, 2001).

1.6 TREATMENT AND PREVENTION

There are many reports of the use of ribavirin and cidofovir for treatment of HAdVs. The data however shows that these agents are only partially effective (Wadell, 1999; Ruuskanen *et al.*, 2009). The prevention of AdV infection in community and institution settings is not yet possible (Echavarría, 2009). The prevention of AdV infection in hospital and eye clinics is dependant on proper cleansing, careful hand washing before and after contact with AdV-infected patients and the appropriate cleaning and disinfection of equipment (Ruuskanen *et al.*, 2009). For the prevention of keraconjunctivitis adequate chlorination of swimming pools is necessary (Echavarría, 2009).

1.7 HUMAN ADENOVIRUSES IN AFRICA

1.7.1 Seroprevalence

Selected studies on the African continent have focused upon determining the prevalence of neutralising antibodies (NAb) to HAdV-5 and -35 which are widely used vaccine vectors. In a study on the prevalence of NAb to adenoviral serotypes 5 and 35 from the selected patients' specimens from Gambia, South Africa (SA) and outside of Africa (USA). On both continents, NAb to HAdV-C5 was found to be both at low and high titer. The low prevalence NAb to HAdV-B2:35 led to a suggestion that HAdV-B2:35 can be used in future gene therapy and vaccine programs (Nwanegbo *et al.*, 2004). In comparable studies done in the Netherlands (Goossens *et al.*, 2001) and USA (Schulick *et al.*, 1997) similar results were obtained. A study related to the above was done in sub-Saharan Africa where NAb titers to HAdV-C5 and six rare HAdV serotypes (HAdV-B2:11, -B2:35, -B1:50, -D26, -D48, and -D49) were assessed in paediatric populations. Outcomes of the study showed the seroprevalence and NAb titers for serotypes HAdV-B2:11, -B2:35, -B1:50, -D26, -D48, and -D49 to be markedly lower than those for HAdV-C5 in paediatric populations, and that HAdV-C5 vectors for vaccine and gene

therapy can have maximal utility when delivered during a given period, i.e. from 6 months to 2 years (Thorner *et al.,* 2006).

1.7.2 Prevalence of adenoviruses

Investigations into the prevalence of HAdVs in Africa have focused predominantly on the gastroenteritis-associated enteric HAdVs types –F40 and –F41 (Kidd *et al.,* 1986; Marx *et al.*, 1998; Moore *et al.*, 1998) and in many cases have been secondary to epidemiological studies conducted by the Africa Rotavirus Network (Audu *et al.,* 2002a, b; Basu *et al.*, 2003; Rossouw, 2004; Fodha *et al.*, 2006; Aminu *et al.*, 2007). Early studies in SA showed the prevalence of HAdV-F40/41 in patients with acute summer gastroenteritis to be 6.5% being second to HRVs with a prevalence of 13.8% (Kidd *et al*., 1986). Subsequent studies revealed HAdV-F40/41 prevalences of 3.4% (Moore *et al.*, 1998), 3.7% (Marx *et al.*, 1998) and 1.6% (Rossouw, 2004). In other studies in Africa identifying viral agents causing paediatric diarrhoea a HAdV-F40/41 prevalence of 3% were recorded in Tunisia (Fodha *et al.*, 2006), 2% in Botswana (Basu *et al.*, 2003), 6.7% in Nigeria (Audu *et al.*, 2002a) and 1.8% in Malawi (Cunliffe *et al.,* 2002). In addition to the method of detection, namely EIA (Moore *et al.,* 1998), hybridisation (Kidd *et al.*, 1986), PCR (Reither *et al.,* 2007) and IC (Weitzel *et al.,* 2007). The variation in AdV species/genotypes detected could be due to regional differences such as climate, environmental and social factors. These studies all showed that HRVs were the most prevalent virus associated with paediatric diarrhoeal disease with HAdV or HAstV being the second most commonly identified viral agent. In a number of these studies the prevalence of all HAdV types in stool specimens was higher than that recorded for enteric HAdVs, namely 9.8% in SA (Moore *et al*., 1998), 5.5% in Tunisia (Fodha *et al.*, 2006), 7.8% in Botswana (Basu *et al.,* 2003) and 28% in Ghana (Reither *et al.,* 2007). This higher prevalence of non-HAdV-F40/41 types in stool specimens could be due to the presence of new serotypes, variants and intertypic strains with the potential for homologous recombination as postulated by various authors (Hierholzer, 1992; Moore *et al.*, 2000; Echavarría, 2008).

There are very few studies on the epidemiology of HAdVs in acute respiratory infections in children in Africa. Studies have identified HAdVs in 3.6% of respiratory specimens in Nairobi, Kenya (Hazlett *et al.*, 1988) and 11% in respiratory specimens in SA (McAnerney *et al.*, 1994)

1.7.3 Molecular epidemiology of adenoviruses

Identification of circulating HAdVs is critical for epidemiological surveillance, the detection of new strains and the understanding of HAdVs pathogenesis. The characterisation of HAdVs was previously dependant on neutralisation and haemagglutination inhibition assays (Swenson, 1999; Echavarría, 2008). The application of molecular typing methods such as multiplex PCRs, and restriction enzyme analysis and sequencing have overcome many of the problems associated with the serological change. Based on restriction enzyme analysis three genome variants of HAdV-F40 were identified in 1982-1983 in the Johannesburg-Soweto area (Kidd *et al.*, 1984), and HAdV-F41 strains were found to differ from strains from the northern hemisphere (Kidd *et al.*, 1984). Subsequent studies on gastroenteritis-associated HAdVs suggested that there were hexon variants of HAdV-F40/41 or emerging HAdV types circulating (Moore *et al.*, 1998). Strains HAdV-A31 and species C HAdVs were also identified in the diarrhoeal stool specimens. These results questioned the relevance of commercial diagnostic tests for the detection of enteric AdV infection in SA (Moore *et al.*, 2000). Further studies on enteric HAdV-F40/41 strains from SA and Botswana showed that the hexon loop 1 region of –F41 strains were hetergenous. A novel –F41 hexon variant was identified which was similar to a –F41 variant diagnostic escape mutant detected in Canada (Rossouw, 2004). Although the prevalence of HAdVs in other African regions has been documented few detailed molecular epidemiology studies have been reported. In a recent study in Ghana species -A (15.1%), -B (12.3%), -C (15.1%), -D (31.5%) and –F (23.3%) were identified by sequence analysis of the 5’ end of the HAdV hexon genome detected in the stool specimens (Silva *et al.*, 2008).

This diversity of HAdVs identified in the stool specimens could be due to the use of PCR assays for the detection (Reither *et al.*, 2007) rather than immunological based assays used in other African investigations (Marx *et al.*, 1998; Audu *et al.*, 2002a; Basu *et al.*, 2003; Fodha *et al.*, 2006). There is therefore a need for more in-depth studies in the molecular epidemiology of HAdVs circulating in Africa.

1.8 ADENOVIRUSES IN WATER

Worldwide, 1.1 billion people lack access to improved water supplies and 2.6 billion people lack adequate sanitation (Moe and Rheingans, 2006). It has been estimated that 1.7 million deaths per year are related to unsafe water, sanitation and hygiene (Grabow, 2007). Due to shortcomings in epidemiological studies, the impact of waterborne disease is difficult to assess and the health impact underestimated (Grabow, 2007). Viruses transmitted by the waterborne route are usually enteric viruses, i.e. viruses which primarily infect the cells of the gastrointestinal tract and are shed in high numbers in the faeces of infected individuals (Grabow, 2007). HAdVs are of major public health importance and have been associated with clinical infections such as gastroenteritis, respiratory disease and conjunctivitis (Echavarría, 2009; Ruuskanen *et al.*, 2009). Respiratory AdVs have been shown to be transmitted by recreational waters. There is therefore the potential for viruses that may not primarily replicate in the gastrointestinal tract to be transmitted by water (Gerba, 2007). Adenoviruses are frequently found in faecally polluted water (Puig *et al.*, 1994; Pina *et al.*, 1998; van Heerden *et al.*, 2003; 2005a) and it has been was suggested that AdVs be used as molecular index of the presence of human enteric viruses where infectivity need not be established (Pina *et al.*, 1998). Adenoviruses were included on the candidate contaminant list of the Environmental Protection Agency in the USA which is the reference for primary contaminants for the EPA's drinking water program (Jiang, 2006).

1.8.1 Epidemiology of adenoviruses in water

Human adenoviruses have been shown to occur in substantial numbers in raw water sources and treated drinking-water supplies (Puig *et al.*, 1994; Chapron *et al.*, 2000; Grabow *et al.*, 2001; WHO, 2004). Is also shown that they are ubiquitous in the environment where contamination by human faeces or sewage has occurred (Desselberger and Gray, 2003). The occurrence of HAdVs in treated drinking water and river water has previously been described (Puig *et al.*, 1994, Enriquez *et al.*, 1995; Muniain-Mujika *et al.*, 2000; Formiga-Cruz *et al.*, 2002; van Heerden *et al*., 2003), and the potential health risks constituted by AdVs in water sources are widely recognised (van Heerden *et al.*, 2005b). Risk based analysis indicates that infectious HAdV at levels of 1/100 – 1/1000 ℓ in drinking water could produce an illness rate between 8.3/1,000 and 8.3/10,000 (Jiang, 2006). However, recent figures from SA suggest that the estimated annual risk for infection due to drinking water is much higher and is between 10 and 17 infections per 100 consumers (van Heerden *et al.*, 2005b; Jiang, 2006). Drinking water is a major source of pathogens in developing regions (Ashbolt, 2004) and HAdVs have been detected in drinking-water supplies that met accepted specifications for treatment, disinfection and conventional indicator organisms (Puig *et al.*, 1994; Chapron *et al.*, 2000; Grabow *et al.*, 2001; van Heerden *et al.*, 2003; 2005a).

Several reports from SA (Grabow *et al.*, 2001; van Heerden *et al.*, 2003, 2004, 2005a) and Korea (Cho *et al.*, 2000; Lee and Kim 2002; Lee and Jeong 2004; Lee *et al.*, 2005) have indicated that infectious AdVs are often present in treated drinking water supplies. In Benin, West Africa, AdVs were deteced in 12.9% of the drinking water sources tested (Verheyen *et al.*, 2009). The detection of AdVs in drinking water sources is not limited to developing regions, as similar studies found AdV to be present in treated drinking water in New Zealand and California (Jiang, 2006). Adenoviruses have been detected at recreational beaches of the Great Lakes in the USA (Xagoraraki *et al.*,

2007) and have been responsible for a number of swimming pool-related outbreaks (Jiang, 2006; Mena and Gerba, 2009).

In most cases, HAdV-F40/41 are considered to the most important serotypes implicated in environmental contamination but there is very little information regarding characterisation, by sequence analysis, of environmental HAdV strains to show all the possible serotypes present. Wold and Horwitz (2007) reported that common–source waterborne outbreaks usually occur in summer and are caused by HAdV-B1:3 and -B1:7. In their investigation van Heerden and colleagues (2005a) were the first to report on the typing of HAdVs in treated drinking water and river water. In the treated drinking water species D predominated with HAdV-C2, HAdV-F40 and HAdV-F41 detected in single samples. In contrast HAdV-F40/41 predominated in the river water with HAdV-C2 and HAdV-Ds detected in single samples.

1.8.2 Recovery and detection of adenoviruses in water samples

The preservation of water quality and prevention of waterborne disease is a complicated task (Meinhardt, 2006). The analysis of water for enteric viruses is a two stage process. As viral particles in water are presumed to be in low number, the first step is to apply efficient recovery and concentration procedures (Köster *et al.,* 2003). Thereafter, a range of isolation and/or detection methods may be applied (Wyn-Jones and Sellwood, 2001). There are, however, no standard procedures for the recovery and detection of AdVs in water samples (Verheyen *et al.,* 2009).

Important criteria to be considered when concentrating viruses from water are: i) the technique should be easy to complete in a short time; ii) have high virus recovery rate; iii) should concentrate large range of viruses; iv) be less costly; v) be capable of processing large volumes of water; and vi) be repeatable within a laboratory (Wyn-Jones and Sellwood, 2001; Albinana-Gimenez *et al.*, 2009). For this reason, different methods have been used for the recovery of viruses from water in different studies. Based on the different properties of

the viruses four main principal approaches are used for viral recovery and concentration. Each method has its own variations, advantages and disadvantages.

(a) Adsorption-elution

The sample containing virus is brought into contact with an adsorbent such as, electron-charged glass-wool, filter membrane etc., to which the virus will adsorb under specific conditions of pH (usually pH 6-8) and ionic strength. Once adsorbed the virus is then released from the adsorbent by elution into a smaller volume of elution buffer (e.g. beef extract) at a high pH value of 9.5, so as to displace the virus from the adsorbent, though this volume is still too large for direct analysis (Wyn-Jones and Sellwood, 2001; Wyn-Jones, 2007).

(b) Entrapment

The sample containing the virus is passed though the filter leaving the virus bound principally by virtue of its size rather than by any charges on the particle (Wyn-Jones, 2007). Vortex flow filtration and tangential flow filtration are examples of ultrafiltration methods and are an alternative to adsorption-elution techniques as they have shown to be efficient in recovering viruses from water (Fong and Lipp, 2005). The advantage of these entrapment methods is that minimal manipulation of water is required and samples can be processed under natural pH with no elution step needed (Fong and Lipp, 2005). The disadvantage of these methods is that they tend to concentrate more PCR inhibitors with the viruses (Jiang *et al.*, 2001).

(c) Ultracentrifugation

This is a “catch-all” method for concentrating viruses from a variety of water sources (Wyn-Jones and Sellwood, 2001; Wyn-Jones, 2007). But the disadvantage is the limited water volumes that can be processed (Wyn-Jones, 2007).

(d) Immuno-affinity columns and magnetic beads

These methods are less used and are relatively new techniques which are useful from small volumes of water (Wyn-Jones and Sellwood, 2001; Hwang *et al.*, 2007). A number of these methods have been applied by different research groups for the recovery and concentration of AdVs from water samples. Glass wool adsorption-elution was used successfully by van Heerden *et al.* (2003; 2004; 2005a), Lambertini *et al.* (2008) and Albinana-Gimenez *et al.* (2009) while adsorption-elution using 1MDS cartridge filters was performed by Verheyen *et al.* (2009) and ultracentrifugation was one of the methods applied by Pina *et al.* (1998).

The methods used for the isolation and detection of viruses from water sources have in most instances been adapted from their clinical applications (Jiang, 2006). A variety of cell lines including Buffalo Green Monkey Kidney (BGM), human colonic adenocarcinoma (CaCo-2), human laryngeal epidermoid carcinoma (Hep-2), human oral epidermoid carcinoma (KB), human lung carcinoma (A549), human hepatoma (PLC/PRF/5) and human embryonic kidney (HEK 293) have been used for the isolation of HAdVs and the susceptibility of the cell lines was found to vary depending on the sample source (Jiang, 2006, Jiang *et al.*, 2009). Cell lines of human origin, namely, HEK 293, A549, PLC/PRF/5 and CaCo-2 were more efficient for the isolation of potential infectious AdVs from water sources than BGM even though BGM was often the cell line of choice (Jiang, 2006). In recent years molecular-based assays have replaced cell culture assays for the detection of viruses (Havelaar and Rutjes, 2008; Jiang *et al.*, 2009). The drawback of molecular detection methods is that they give no indication as to the infectivity of the virus. Molecular-based assays have facilitated faster more sensitive detection of viruses with the added benefit of detecting viruses which cannot be isolated in cell culture (Wyn-Jones, 2007). The PCR assay has been used extensively for the detection of HAdVs in water sources (Puig *et al.*, 1994; Pina *et al.*, 1998; Karamoko *et al.*, 2005; van Heerden *et al.*, 2003, 2004, 2005a). The nested PCR and primers described by Allard *et al.* (2001) are the most

commonly used for the detection of HAdVs in water and environmental sources (Jiang, 2006). With conventional PCR there is however a risk of carry-over contamination and results are qualitative (Heim *et al.*, 2003). The development and application of real-time PCR has further improved the sensitivity and specificity of HAdV detection (Jiang, 2006). In addition the HAdV viral load in a water sample can be quantified (Choi and Jiang, 2005; van Heerden *et al.*, 2005b). Results from quantitative real-time PCR assays in relation to public health risk must be interpreted with caution as high genome copy numbers have been detected in the absence of infectivity (Choi and Jiang, 2005).

1.9 PROBLEM STATEMENT AND HYPOTHESIS

With the genetic variability in AdVs and with the description of seven types, HAdV-D42 to -D49, exclusively from patients with HIV/AIDS, it is important to be vigilant and to routinely monitor for the emergence of new types and variants, especially in areas with a high percentage of immunocompromised individuals, namely SA and Africa in general. Adenoviruses have successfully been detected in water sources in SA (van Heerden *et al.*, 2003, 2004, 2005a), but to date there has been no comparative genetic analysis on clinical and environmental isolates in SA to other regions in Africa, e.g. Kenya. In addition there are no studies investigating the link between clinical and environmental strains. The importance and clinical relevance of environmental strains therefore needs further investigation.

The hypothesis for this study was as follows:

1. The HAdVs detected in water sources are genetically similar to those in clinical specimens suggesting that water is an important source of HAdV infection; and
2. There is an emergence of new serotypes and variants of HAdVs in immunocompromised individuals in Africa.

1.10 AIM/OBJECTIVES OF THE STUDY

The aim of this study was to determine the genetic heterogeneity of AdVs circulating in communities in selected regions of Africa. The specific objectives are:

(a) To establish which AdV serotypes are associated with specific clinical syndromes, namely gastroenteritis and respiratory infections, in selected patient cohorts in the Tshwane Metropolitan area of SA and other regions of Africa, namely Kenya;

(b) To characterise AdVs from different water sources to provide additional information regarding AdVs circulating in a given community, and to ascertain whether or not the same strains have been associated with human infection;

(c) To determine the genetic heterogeneity of the African AdV strains in relation to strains from other regions of the world.

CHAPTER 2

MOLECULAR DETECTION AND CHARACTERISATION OF ADENOVIRUSES FROM CLINICAL SPECIMENS AND ENVIRONMENTAL SAMPLES FROM SOUTH AFRICA

2.1 INTRODUCTION

Adenovirus infections reportedly occur worldwide as epidemic, endemic or sporadic infections. Infections are observed to occur throughout the year, with outbreaks being more common in late winter, spring, and early summer (Desselberger and Gray, 2003). Human adenoviruses are ubiquitous in the environment, where contamination of water sources with human faeces or sewage can occur (Formiga-Cruz *et al.*, 2002; van Heerden *et al.*, 2005b). Enteric HAdVs may pose a potential health risk in water intended for human consumption as they are typically known to be transmitted by the faecal–oral route (Godfree *et al*., 1990; Fewtrell, 1991; Kay and Jones, 1992; van Heerden *et al.*, 2005b). In developing countries, improper water quality has been reported to cause major public health problems affecting mortality rates in highly susceptible people, including small children and immunocompromised patients (Verheyen *et al.*, 2009). It is, therefore, important to determine the genetic relationship between HAdVs strains occurring in water sources and those detected in human clinical specimens, as this may give some indication as to whether or not water sources are a potential source of infection.

In contrast to other African countries, the epidemiology of HAdVs in clinical and environmental sources in SA has been well documented. As early as 1986, a HAdV-F40/41 prevalence of 6.5% (40/616) was noted in urban black children hospitalised with acute gastroenteritis compared to the prevalence of 13.8% (85/6165) recorded for HRV (Kidd et al., 1986). In two subsequent studies, HAdV-F40/41 prevalence of 3.7 to 9% in the Johannesburg area and

35% hospitalised black children in Soweto were recorded. In the latter study, species C and species A HAdVs were also associated with paediatric diarrhoea and this was the first report of HAdV-A12 in paediatric gastroenteritis in SA. In contrast, species C, D and E HAdVs were identified in ocular swabs from cases of suspected adenoviral conjunctivitis (Erasmus *et al.,* 2001). More recently, HAdV-F40/41 was detected in 4.6% of stool specimens from young children presenting with gastro-intestinal disease (Rossouw, 2004). The occurrence and types of HAdVs in treated and untreated water samples was reported by van Heerden *et al.* (2003, 2004, 2005a) and the risk has been quantified (van Heerden *et al.,* 2005b). Although the HAdVs that were detected in the clinical specimens and environmental samples were characterised, there are no studies exploring the possible connection between clinical and environmental HAdV strains in SA.

The objectives of this study were: i) to establish HAdV genotypes associated with specific clinical syndromes, i.e. gastroenteritis and respiratory infections, in selected patient cohorts in the Tshwane Metropolitan area of SA; ii) to characterise HAdVs from different water sources to provide additional information regarding AdVs circulating in the SA community; and iii) to ascertain whether or not the same strains were detected in both water sources and clinical specimens.

2.2 MATERIALS AND METHODS

2.2.1 Ethical approval

Ethical approval for this study was granted by the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, South Africa: Number S27/2008. There was no direct patient contact associated with this investigation and the results of this study had no impact on patient management or future lifestyle.

2.2.2 Study population, specimen collection and processing

2.2.2.1 Stool specimens

Stool specimens referred to the Diagnostic Laboratory, Department of Medical Virology, National Health Laboratory Service Tshwane Academic Division (NHLS TAD) for routine analysis for gastroenteritis viruses in 2006 were used for this investigation. Of the 242 specimens analysed 20 (8.3 %: ST1 to ST20) tested positive for HAdV-F40/41 by EIA (Premier Adenoclone® 40/41; Meridian Bioscience, Inc. OH) and were available for characterisation.

A 10% stool suspension was prepared in 1 mℓ nuclease-free water (Promega Corp. Madison, WI) and mixed by vortexing for 1 min to give a well defined suspension. The suspension was stored at -20ºC prior to nucleic acid extraction.

2.2.2.2 Respiratory specimens

Respiratory specimens (bronchial lavages and nasopharyngeal aspirates), referred to the Diagnostic Laboratory, Department of Medical Virology, NHLS TAD from 2005 to 2007 for routine analysis for respiratory viruses were included in this investigation. Specimens (n=22 [5.9%]: RESP1 to RESP22) found to be HAdV-positive by a real-time mutiplex RT-PCR in a separate study (MSc study of MM Lassauniere; Development of quantitative multiplex real-time PCR assays for detection of 14 conventional and newly discovered viruses with lower respiratory tract infections in children in South Africa) were used for HAdV characterisation. Depending on the viscosity of the specimen, nucleic acid was either extracted directly from the specimen or from a 1 mℓ suspension in nuclease-free water (Promega Corp.).

2.2.3 Water samples

2.2.3.1 Samples and virus recovery

From January 2006 to December 2007, treated (100 ℓ) and untreated (10 ℓ) water samples were drawn from water treatment plants in different geographical areas from SA and from a water purification and treatment plant

in Namibia. Included were untreated river water samples (10 ℓ) used by the lower socio-economic communities in informal settlements for domestic purposes and treated drinking water (10 ℓ – 100 ℓ) samples from two sampling sites in Gauteng. Water samples were collected and transported to the laboratory in sterile containers and stored in the laboratory at 4ºC until processing.

Viruses were recovered from the water samples using a glass wool adsorption-elution procedure based on the method of Vilaginès *et al.*, (1993) as described by Wolfaardt *et al.*, (1995), Grabow *et al.*, (1996), and Vivier *et al.*, (2004) and further modified and optimised by Venter (2004). Positively charged glass wool columns were prepared as follows: 3 × 5 g oiled sodocalcic glass wool (Glass wool Bourre 725 QN, Ouest Isol, Alizay, France) were teased and compressed into a column at a different angle to each other to a final density of 0.5 g/cm^3, with two steel sieve grids (pore size of ~ 1 mm^2) inserted between the glass wool sections. A metal or plastic coupling link was attached to each end of the column. The glass wool was soaked with sterile distilled water and pre-treated consecutively with 40 mℓ 1M HCℓ, 100 mℓ sterile distilled water, 40 mℓ 1M NaOH and 200 mℓ sterile distilled water to adjust the pH to pH 7.0. To remove the chlorine from treated water to be passed through a column, 15 g of hydrochlorex/ dechlorit tablets (USF Wallace and Tiernam, Günzburg, Germany) were added to the column above the glass wool.

Filtration or capturing of viruses from water samples was done either in-line (for 100 ℓ) or by passing a water sample from a 10 ℓ container through the positively charged glass wool columns by negative pressure at a rate of 10 ℓ/h. The negatively charged viruses, which adsorbed to the glass wool, were eluted with 100 mℓ of sterile glycine-beef-extract buffer (GBEB, pH 9.5) (3.754 g/ℓ glycine [Merck, Darmstadt, Germany], 5 g/ℓ beef extract powder [BBL^{TM} Becton Dickinson and Co., Sparks, MD]), which reverses the ionic charge of the viruses and releases them from glass wool. The eluting solution (GBEB)

was left in contact with the glass wool for 5 min before being passed through the filter under pressure, and thereafter the pH of the eluate was adjusted to pH 7.0 with 1M HCl (Vivier *et al.*, 2001).

A secondary concentration of the recovered viruses was done using a modification of the polyethylene glycol (PEG) 6000 [Merck] / NaCl [Merck] method as described by Vilaginès *et al.* (1997). PEG 6000 (14g/100 mℓ) and NaCl (1.17g/100 mℓ) was added to the 100 mℓ neutralised eluate, dissolved by gentle shaking and held for ~ 16 h at 4ºC. The solution was centrifuged at 6000 × g (Sorvall® Super T21, DuPont Co., DE) for 30 min at 4ºC, the resulting pellet was re-suspended in 20 mℓ of sterile phosphate buffered saline pH 7.4 (PBS) (Sigma-Aldrich, Inc., St Louis, MO). The resuspended pellet was sonicated (Soniprep 150, MSE) at 20 amplitude microns for 30 sec to dissociate any clumps and centrifuged for 10 min at 3000 rpm at 4°C. The supernatant was withdrawn into a clean container and 5 mℓ of the supernatant was used to resuspend the pellet. After decontamination with ~2 mℓ of chloroform (Merck) the suspension was centrifuged for 5 min at 3000 rpm at 4°C and 1-2 mℓ of the supernatant was used to determine the presence of HAdVs directly in the recovered virus suspension. The remainder of the re-extracted supernatant was mixed with the original supernatant, shaken well and 4 mℓ was used for the infection of cell cultures. The remaining supernatant was stored at -20ºC.

2.2.3.2 Propagation of adenoviruses in cell culture

(a) Cell cultures

PLC/PRF/5 human hepatoma cell line (American type culture collection [ATCC] CRL 8024). Adenoviruses usually infect cell cultures derived from the natural host range, e.g. humans, or closely related species. The *PLC/PRF/5* cell line has successfully been used to isolate HAdVs from water samples (van Heerden *et al.*, 2003, 2004; Jiang, 2006) and clinical samples (Erasmus *et al.*, 2001) and was used for the primary isolation of HAdVs from the recovered viral suspensions.

Vero African Green Monkey cell line (European collection of cell cultures ECACC 8411 3001). Infected *PLC/PRF/5* cell cultures were blind-passaged onto standard tube cultures of Vero cells to monitor for the development of a CPE and typical AdV inclusion bodies.

(b) Cell culture propagation and maintenance

Each cell line was propagated and maintained as described by Taylor *et al.* (2001), Nadan *et al.* (2003) and Venter (2004).

(c) Virus isolation and detection

For the infection of cell cultures with the recovered viruses from the treated water samples, 25 cm^2 flasks of *PLC/PRF/5* cell cultures were prepared and infected as previously described (Taylor *et al.*, 2001). After two blind-passages on *PLC/PRF/5* cell cultures, the infected cells were passaged onto standard tube cultures of Vero cells. The tube cultures were monitored by light microscopy for CPE. After a seven day incubation period, the cells were stained with haematoxylin and eosin and examined for AdV-specific inclusion bodies (Malherbe and Strickland-Cholmley, 1980).

2.2.4 Molecular detection of adenoviruses

2.2.4.1 Nucleic acid extraction

Genomic viral nucleic acid was extracted directly from 1 mℓ of the recovered virus suspension from the water samples, and from 200 µℓ of clinical specimens and harvested cell culture using the MagNA Pure Nucleic Acid Isolation Kit (large volume) (Roche Diagnostics GmbH, Mannheim, Germany) and MagNA Pure Nucleic Acid Isolation Kit (Roche Diagnostics), respectively, in a MagNA Pure LC instrument (Roche Diagnostics), following the manufacturer's instructions. This instrument has the advantage of a standardised procedure for extracting nucleic acid from up to 32 samples in parallel.

The principle of this isolation procedure is based on magnetic-bead technology, which is as follows: samples are lysed by incubation with a buffer that contain chemotropic salts and Proteinase K; free nucleic acid from lysates is bound to the surface of added magnetic glass particles and unbound substance are removed by several washing steps, and the purified nucleic acid is eluted in 50 µℓ to 100 µℓ elution buffer. Purified nucleic acid was aliquoted and stored at -70°C until use. Five to ten microlitres of the eluted nucleic acid was used for virus nucleic acid amplification/detection assays.

2.2.4.2 Detection of adenoviruses by PCR

Published degenerate primer pairs ADHEX1F and ADHEX2R, and ADHEX2F and ADHEX1R, which amplify a conserved region of the AdV hexon gene fragment and which reportedly detect all known serotypes of HAdVs (Casas *et al.*, 2005) were used in a nPCR, as described by Avellòn *et al.*, (2001) and modified by van Heerden *et al.*, (2003, 2004).

(a) Primers

The primers used for the detection of HAdV DNA in a conventional PCR, followed by nPCR, and their predicted amplicon sizes are presented in Table 2.1.

Table 2.1: Degenerate primers that target a hexon region of the adenovirus genome (Avellón *et al*., 2001)

Primary (first round) PCR		**Product size**
ADHEX1F	5’ CAA CAC CTA YgA STA CAT gAA 3’	473 bp
ADHEX2R	5’ ACA TCC TTB CKg AAg TTC CA 3’	
Nested (second round) PCR		
ADHEX2F	5’ CCC ITT YAA CCA CCA CCg 3’	168 bp
ADHEX1R	5’ KAT ggg gTA RAg CAT gTT 3’	

(b) Detection

The first-round reaction (primary amplification): 10 µℓ of extracted nucleic acid was added to 40 µℓ of a reaction mixture containing 5x green Go Taq

flexi buffer (5 µℓ), 25 mM $MgCl_2$ (8 µℓ), 10mM dNTPs (2 µl), 20 pmol/µl primer ADHEX1F (0.5 µl), 20 pmol/µl primer ADHEX2R (0.5 µl), 5 U/µℓ Go Taq DNA polymerase (0.5 µℓ), and nuclease-free water (23.5 µℓ). The PCR cycling parameters consisted of an initial denaturing step for 2 min at 94°C. Followed by denaturation for 1 min at 93°C, annealing for 1 min at 50°C, and extension for 1 min at 70°C for 30 cycles. Nested PCR (nPCR) (second amplification): 1 µℓ of first round amplification product was added to 49 µℓ of a reaction mixture containing 5x green Go Taq flexi buffer (5 µℓ), 25 mM $MgCl_2$ (8 µl), 10 mM dNTPs (1.5 µl), 20 pmol/µl primer ADHEX1F (0.5 µl), 20 pmol/µl primer ADHEX2R (0.5 µl), 5 U/µℓ Go Taq DNA polymerase (0.5 µℓ), and nuclease-free water (33 µℓ). The cycling parameters consisted of an initial denaturing step for 2 min at 94°C, followed by denaturation for 1 min at 93°C, annealing for 1 min at 54°C and extension for 1 min at 72°C for 30 cycles, with a final extension step for 7 min at 72°C. Unless stated to the contrary, the reagents were from Promega Corp.

(c) Visualisation

An aliquot (20 µℓ) of the amplified product was resolved by agarose gel (2% agarose) electrophoresis and visualised by staining with ethidium bromide (10 mg/mℓ) and illumination under UV light. The amplicons size was compared to the 100-bp molecular weight (MW) ladder (O'GeneRuler, Fermentas Life Sciences, Burlington, Ontario) and the positive control. Amplicons of the correct size were considered to be positive.

(d) Quality control

To exclude the possibility of cross-contamination, standard precautions were applied in all manipulation of samples. Reagents for the PCR were prepared and the nucleic acid was extracted in rooms separate from those used for the processing of the clinical specimens and water samples and for the analysis of the amplicons. The extraction controls, a negative nPCR control (nuclease-free water), and a positive control (nucleic acid from an appropriate well-

characterised cell-cultured adapted HAdV-C2 strain) were included in each PCR run to monitor for false-positive and false-negative reactions.

2.2.5 Molecular characterisation of adenoviruses

2.2.5.1 Sequencing of PCR amplicons

Selected AdV-positive nPCR products were purified and sequenced. The PCR products were purified using the DNA Clean & Concentrator ^{TM}-25 kit (Zymo Research, Orange, CA), and treated with a standard sequencing protocol with the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) in an automated sequencer, using primers ADHEX2F and ADHEX1R. For purification, two volumes of a DNA-binding buffer were added to each volume of DNA sample (~30µℓ) and mixed. The mixture was loaded onto a Zymo-spin column placed into a 2 mℓ collection tube. After centrifugation at full speed (≥10,000 × g) for 30 sec, the flow-through was discarded. A volume of 200 µℓ of wash buffer was added and spun for 30 sec; this step was repeated. The Zymo-spin column was placed into a sterile 1.5 mℓ micro-centrifuge tube, and 30 µℓ of RNAse-free water was added directly to the column matrix and spun to elute the DNA (ready to use).
If multiple bands were visible on a gel, the correct AdV amplicon was excised from the gel and purified with the aid of the Zymoclean gel DNA recovery kit prior to the standard sequencing protocol. Three volumes of ADB buffer were added to each excised gel piece, and incubated at 55ºC for 5 - 10 min. The melted agarose solution was applied into a Zymo-spin column, placed into a collection tube, and centrifuged for 30 sec. A volume of 200 µℓ of wash buffer was added to the column and spun for 30 sec; this step was repeated. The Zymo-spin column was placed into a sterile 1.5 mℓ tube, and 30 µℓ of water was added directly to the column matrix and spun to elute the DNA.

The setup of a standard sequencing reaction was as follows: each reaction contained 2 µℓ terminator mix v3.1, 3 µℓ sequencing buffer, 1 µℓ (3.2pmol) primer, 3-7 µℓ template (DNA), and an appropriate volume nuclease free water was added to the mixture to add up to final volume of 20 µℓ in a micro-

centrifuge tube. The sequencing cycling parameters consisted of an initial denaturing step at 94°C for 1 min, followed by denaturation at 94°C for 10 sec, annealing at 50°C for 5 sec, and elongation at 60°C for 4 min for 25 cycles. Following purification, the sequencing reaction product was purified using the EDTA/sodium acetate/ethanol method of precipitation.

2.2.5.2 Sequence analysis and genotyping

Sequence data from both strands were edited in BioEdit Sequence Alignment Editor (V.7.0.9.0) and aligned using MAFFT Version 6 (https://align.bmr.kyushu-u.ac.jp/mafft/online/server/). The sequences of representative isolates were compared with AdV sequences present in GenBank by using the BLAST-N program (version 2.2.18) to search for the most similar strains available. Below is a table presenting the referenced HAdV strains used for comparison with the strains detected in water samples, respiratory and stools specimens from SA.

Table 2.2: GenBank accession numbers of the adenovirus isolates used as reference strains for phylogenetic analyses

Species/ Type	**Accession number**	**Species/ Type**	**Accession number**
A12	AB330093.1; X73487.1	D26	AB330107.1; EF153474.1
A18	AB330099.1; DQ149610.1	D32	DQ149629.1; AB330113.1
B3	AF542129.1; EF486506.1 DQ099432.4; AY854178.1	D42	AB330123.1; DQ149635.1
		D49	AB330130; DQ393829
B7	AB243118.1; AY601634.1		
C1	EU867492.1; AB330082.1	F40	AB330121.1; L19443.1
C2	EU867472.1; AY819853.1 AF542120.1; AY224391.1	F41	AY819814.1; AY819812.1 AB330122.1; DQ315364.2
C5	EU439587.1; AF542130.1		
C6	AB330087		
D8	AB361058; AB090344	Fowl AdV	Aviadeno; AC000014
D13	AB330094.1; DQ149616.1	Duck AdV	Atadeno; Y09598
D15	AB330096.1; DQ149617.1	Feline AdV	AY512566
D25	AB330106.1; DQ149623.1	Sim Ad7	DQ792570.1

Phylogenetic trees were constructed from the nucleic acid sequence alignments by using the Neighbor-Joining (NJ) method of the MEGA software version 4 (Tamura *et al.*, 2007). For NJ, a distance matrix calculated from the aligned sequences by Kimura two-parameters formula was used, and for determining the reliability of tree topology, bootstrap analysis was carried out on 1000 replicates.

2.3 RESULTS

2.3.1 Prevalence and characterisation of adenoviruses

2.3.1.1 Stool specimens

Of the 20 HAdV positive stool specimens tested, 9 specimens could be sequenced. Pairwise comparison of the nucleotide sequences of a conserved hexon genome fragment of 9/20 (45%) of the stool specimens identified HAdV-F as the predominant species, comprising 7/9 (77.8%) of the identified strains. Species HAdV-C and –A were identified in 1/9 (11%) specimens each (Figure 2.1).

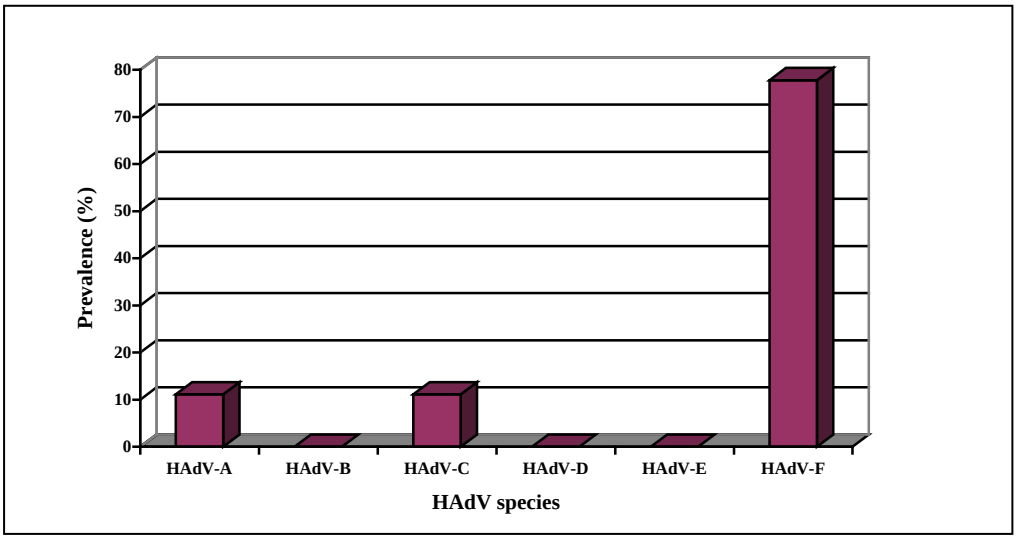

Figure 2.1: Distribution of HAdV species in the diarrhoeal stool specimens

2.3.1.2 Respiratory specimens

Of the 22 HAdV positive respiratory specimens analysed, HAdV strains from 14 specimens could be sequenced. Pairwise comparison of nucleotide sequences of a conserved hexon genome fragment from 14 of the HAdV strains indicated that HAdV-B was the predominant species, being identified in 10/14 (71.4%) strains, while HAdV-C was identified in 4/14 (28.6%) strains (Figure 2.2).

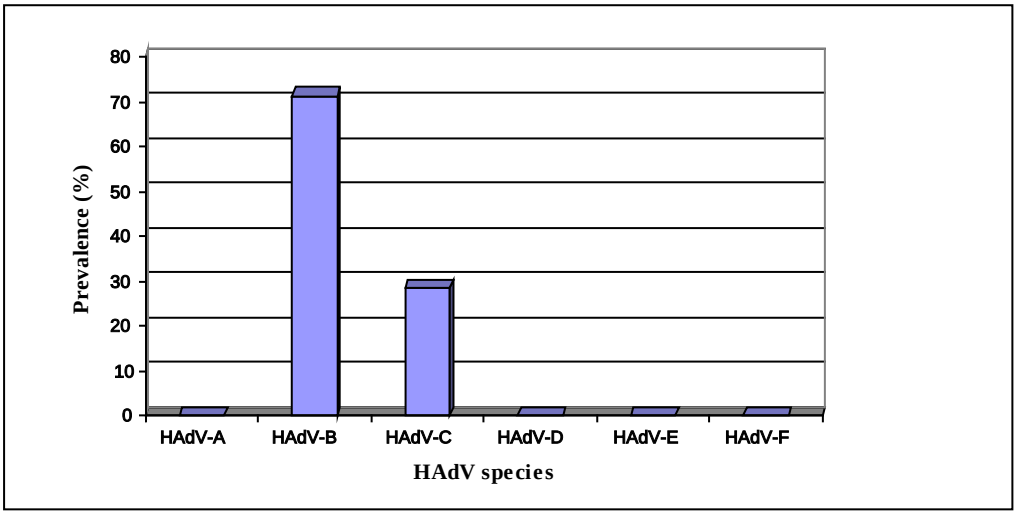

Figure 2.2: Distribution of HAdV species in selected respiratory specimens

2.3.1.3 Water samples

Human adenoviruses were detected in 65/756 (8.6%) of the water samples analysed. In the untreated surface river water (K19) HAdVs were detected in 17% (40/236) of the samples. From Figure 2.3, it is evident that there was no seasonal distribution of HAdVs in these untreated surface water samples, because HAdVs were detected throughout the sampling period. In the treated drinking water drawn at sites A and B, HAdVs were detected in 0.8% (4/520) of the samples.

From Figure 2.4, it is evident that HAdV-F was the predominant species detected in the untreated water sources. From the HAdV-positive water samples, 34 (52%) HAdV isolates (32 [32/34: 94%] from untreated and 2 [2/34: 5.9%] from treated water) were characterised. In the untreated water,

HAdV-F was the dominant species, being identified in 21/32 (66%) isolates with HAdV-D in 7/32 (22%) of isolates. Species HAdV–B was identified in 2/32 (6.3%) and HAdV-A and –C was detected in 1/32 (3.1%) isolates each. From treated water HAdV-D and –F were identified in one (50%) isolate each.

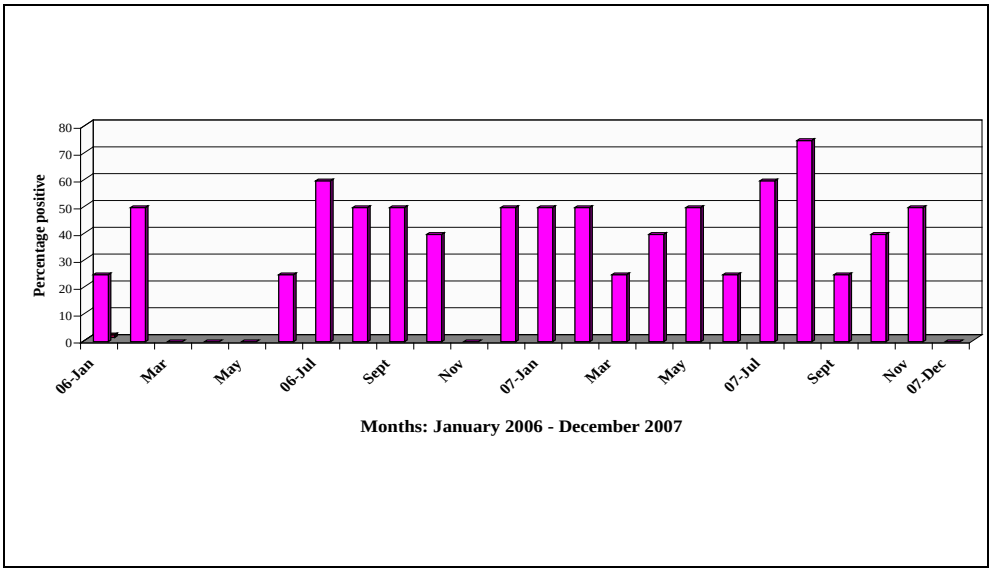

Figure 2.3: Prevalence and seasonal distribution of HAdVs in one of the untreated water sources (K19) for the period January 2006 to December 2007.

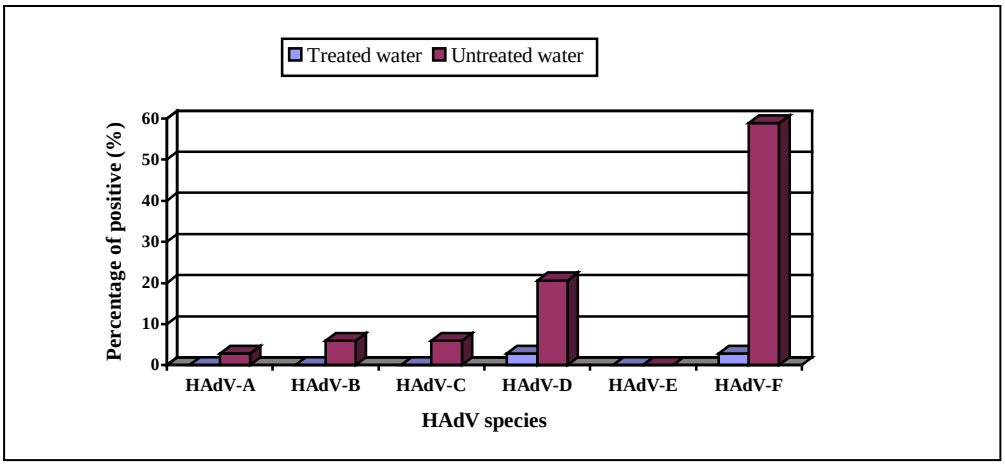

Figure 2.4: Prevalence of HAdV species in untreated and treated water samples.

2.3.1.4 Genotype profiles of adenoviruses

The differences in the genotype profiles of the HAdVs detected in the clinical specimens [stool (A) and respiratory (B)] and water samples (C) is presented in Figure 2.5. A marked difference in the distribution of HAdV species and genotypes in stools, respiratory specimens, and water samples was noted. Species F (HAdV-41) predominated in the stool specimens and water samples,

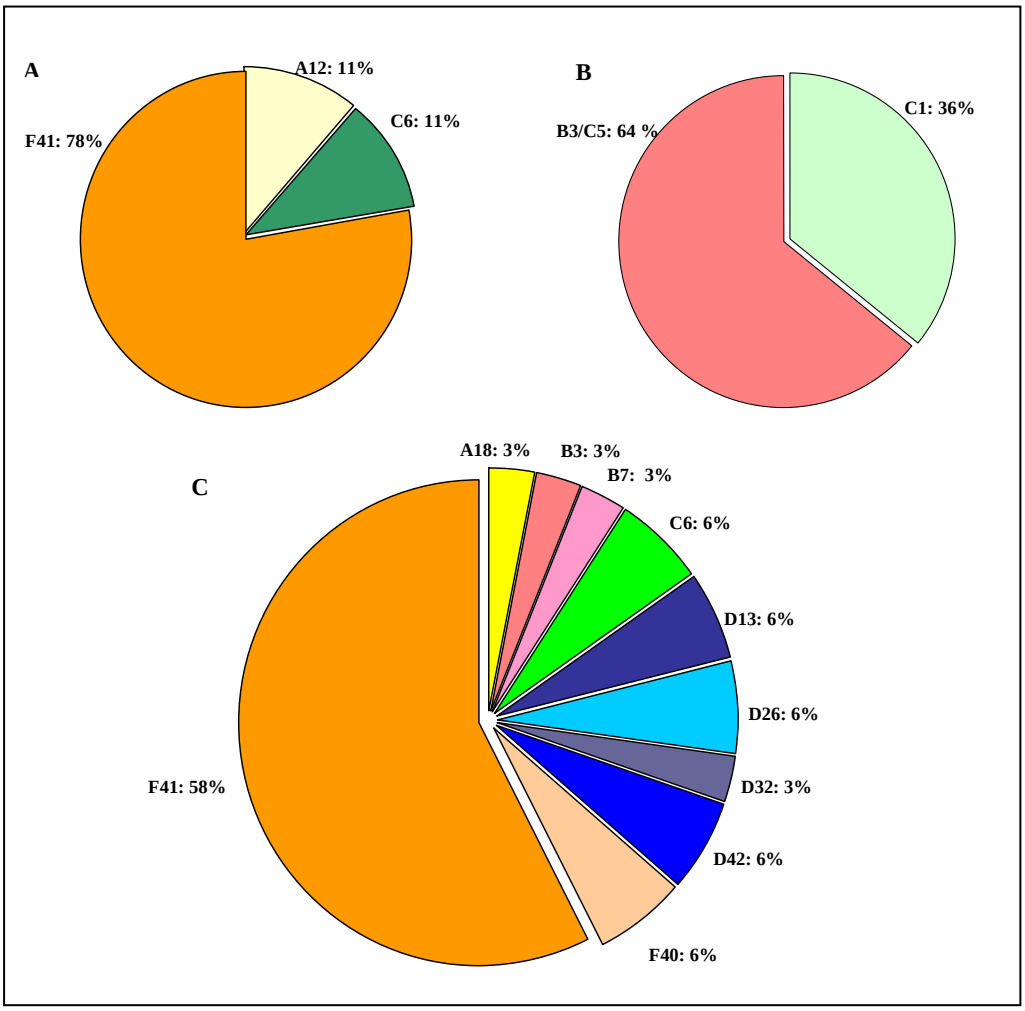

Figure 2.5: Distribution of HAdV genotypes among patients presenting with gastroenteritis (A), patients presenting with respiratory infection (B), and in the combined treated and untreated water samples (C).

comprising 78% and 61% of the identified strains, respectively. In the stool specimens species A (HAdV-12) and species C (HAdV-6) were detected to a lesser extent, each representing 11% of strains identified (Figure 2.5[A]). In the respiratory specimens species B (HAdV-B3/HAdV-C5: 64.3%)

predominated and species C (HAdV-1: 35.7%) was also noted. In contrast, in the water samples the predominance of species F (HAdV-41: 58%; HAdV-40: 6%) was noted, with the detection of other HAdV species (-D [21%], -B [6%], -A [3%], -C [6%]) to a lesser extent. Of note was that only 3% of HAdV-41 among all detected HAdVs in water samples was from the treated drinking water.

2.3.2 Phylogenetic analysis of adenoviruses

The genetic relationship between the HAdV strains from selected untreated and treated water samples and the majority of the stool specimens (ST) is depicted in Figure 2.6. From Figure 2.6, a number of the HAdVs from untreated (n=16) and treated (n=1; B12060612) water samples grouped together with HAdV-F41. Human AdV strains from stool specimens ST13, 14, 19 and water sample K19070226 showed 100% nucleotide sequence identity to each other and 97% identity to the HAdV-F41 reference strain in the region analysed. HAdV strains from the same surface water sample site which were collected almost a year apart, K19060724 and K19070611, showed 100% nucleotide sequence identity to each other and 97% to the reference HAdV-F41. In comparison, HAdVs from the same site collected a week apart, K19060710 and K19060717, showed 99% nucleotide sequence identity to each other and 96% to HAdV-F41. Within genotype F41 four distinct clusters were noted. Three of the HAdV strains from untreated water samples, K19070521, N20060605 and N19060505, resorted separately, each in their own distinct cluster, with 91%, 93% and 87% nucleotide sequence identity to the HAdV-F41 reference strain (AY819814), respectively. The HAdV from treated drinking water sample B12060612 grouped together with HAdVs from surface water samples K19060821, K19070129 and K19070326. Only two HAdV-F40 strains were identified from surface water samples K19070402 and K19080121. These strains showed 93% of nucleotide sequence identity to each other, and 93% and 98% of nucleotide sequence identity to the reference HAdV-F40 strain, respectively.

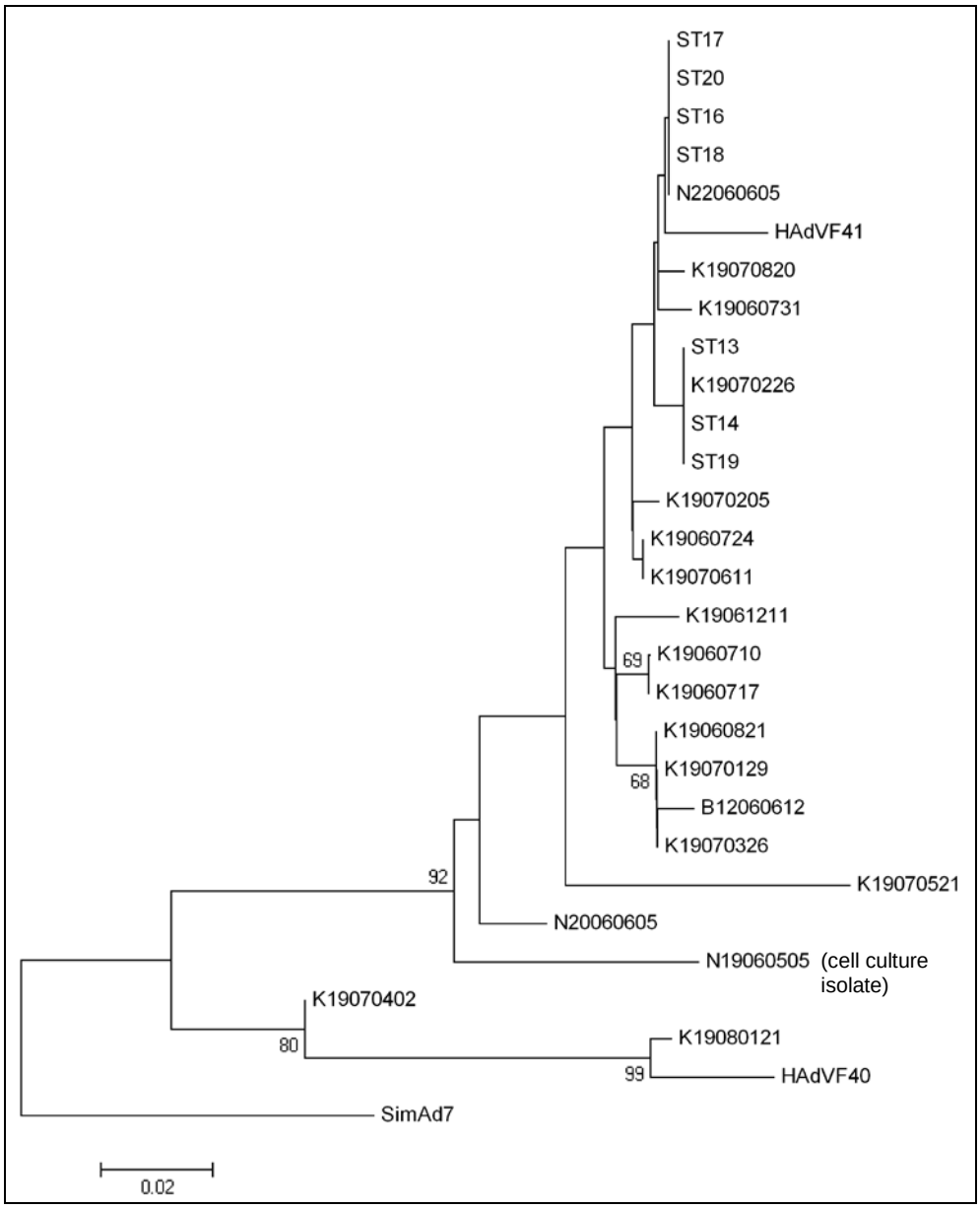

Figure 2.6: Neighbour-Joining phylogenetic tree based upon a 159 base-pair fragment of the hexon genomic region, showing the relationships between species F HAdV strains from stool specimens and water samples and the reference HAdV strains. The bar indicates nucleotide changes per site and bootstrap percentages are indicated.

In Figure 2.7, the relationship between HAdV strains from clinical specimens and water samples and HAdV types -B3, -B7, -C5 and -C6 is presented. In the hexon genome region analysed, there was a 100% nucleotide sequence identity between HAdV-B3 and HAdV-C5. Human AdV strains from respiratory specimens RESP3, 5, 10, 12, 13, 15, 19 and 20 showed 99% to 100% nucleotide sequence identity to each other and 97% to 98% identity to

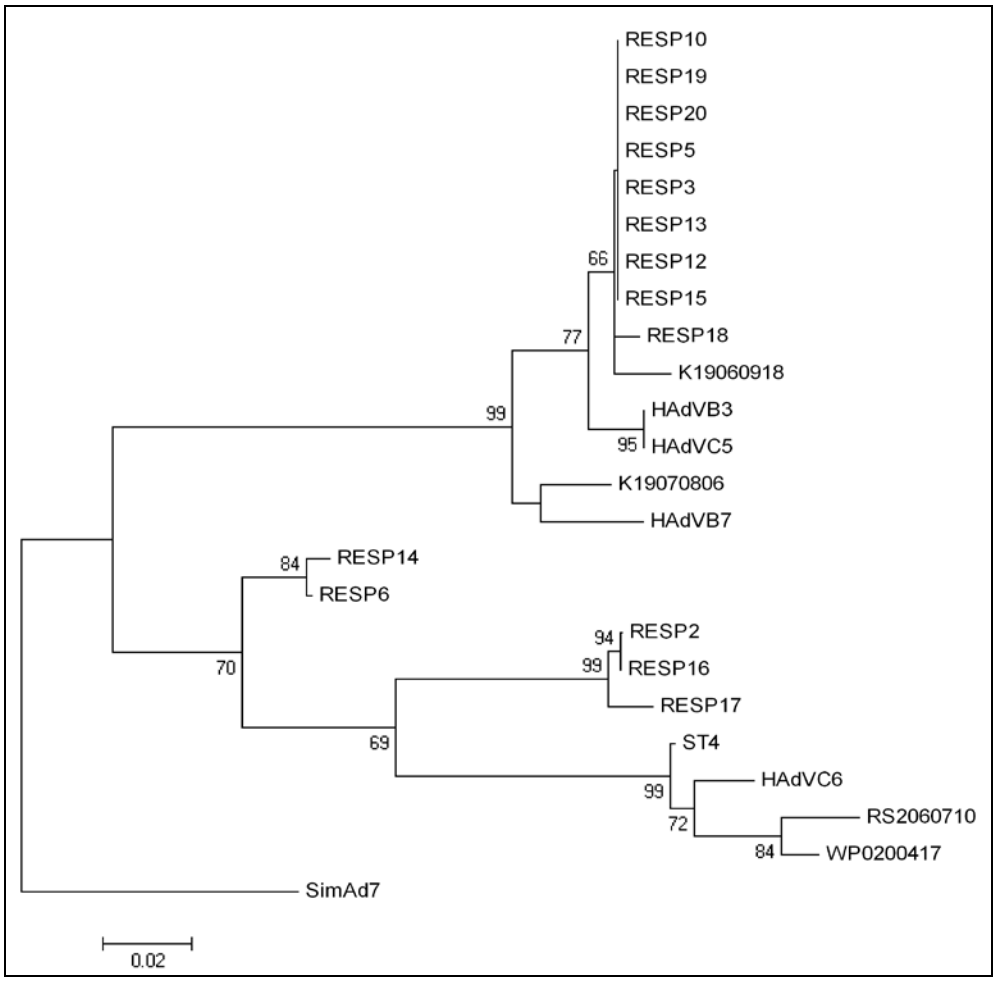

Figure 2.7: Neighbour-Joining phylogenetic tree based upon a 159 base-pair fragment of the hexon genomic region showing the relationships between HAdV-B and -C strains from respiratory specimens and water samples and the reference HAdV strains. The bar indicates nucleotide changes per site and bootstrap percentages are indicated.

HAdV-B3/HAdV-C5 in the region analysed. Figure 2.7, shows that these HAdV strains from respiratory specimens grouped together in the same cluster with HAdV strains from a respiratory specimen (RESP18) and from an untreated surface water sample (K19060918). These strains were sorted in a separate cluster to the reference HAdV-B3 (AF542129) and HAdV-C5 (AF542119) strains. On the basis of pairwise comparison of nucleotide sequence, the HAdV strain from an untreated surface water sample, K19070806, drawn in 2007 could be genotyped as an HAdV-B7 showing a

96% nucleotide sequence identity to the reference HAdV-B7 (AB330088) strain. Two distinct clusters of HAdV-C1 strains from RESP6, 14, 2, 16, 17 were evident. One cluster (RESP6, 14) showed a 89% nucleotide sequence identity and the other (RESP 2, 16, 17) a 96% to 97% nucleotide sequence identity to the reference strain (not shown). A combination of HAdV strains from stool specimens as well as treated water (WP0200417) and untreated water (RS2060710) samples could be characterised as HAdV-C6.

There were three groups of HAdV strains with the HAdVs from the respiratory specimens which sorted into separate clusters from the stool specimen and water samples (Figure 2.7). Human adenovirus strains from respiratory specimens RESP2 and RESP16, and RESP17 showed 87% nucleotide sequence identity to the reference HAdV-C6 (AB330087) strain, while the HAdVs from RESP14 and RESP6 showed 85% to 86% nucleotide sequence identity to the same HAdV-C6 reference strain. The HAdV strains from treated (WP0200417) and untreated water (RS2060710) samples showed 94% nucleotide sequence identity to the reference HAdV-C6 (AB330087) strain, and 95% nucleotide sequence identity to the clinical stool (ST4) specimen.

In Figure 2.8 the relationship between HAdV strains from a treated water sample (A19070402) and untreated surface water (Vierderhof), and a HAdV cell culture isolate from an untreated water sample K19060626 showed 98% to 99% nucleotide sequence identity to each other and 96% to 98% identity to the reference strain of HAdV-D13 in the region analysed. Within species D HAdV strains from untreated water sample MISLKF and treated water destined for a cruise ship, N13060505, showed 99% nucleotide sequence identity to each other and 98% to 99% nucleotide sequence identity to HAdV-D42. Strains from water samples K19070903 (untreated surface water) and N19060505 (treated water destined for use on a cruise ship) showed 89% nucleotide sequence identity to each other, 96% and 87% nucleotide sequence identity to the reference HAdV-D26, respectively. Within species A stool

isolate ST6 showed 96% nucleotide sequence identity to HAdV-A12 while a strain derived from an untreated surface water, K19061002, showed 92% nucleotide sequence identity to HAdV-A18.

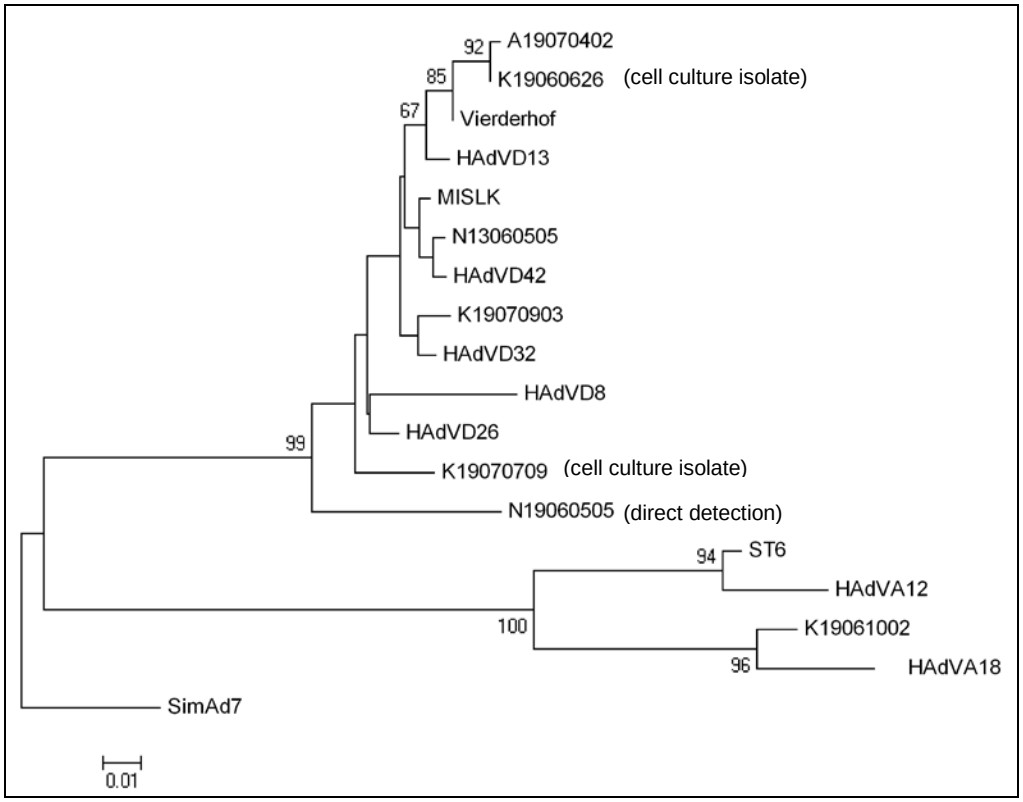

Figure 2.8: Neighbour-Joining phylogenetic tree based upon a 159 base-pair fragment of the hexon genomic region showing the relationships between HAdV-D and -A strains from clinical specimens and water samples and the reference HAdV strains. The bar indicates nucleotide changes per site and bootstrap percentages are indicated.

2.4 DISCUSSION

This section of the dissertation focused on the genetic relatedness between HAdVs genotypes occurring in the water sources (including treated potable and untreated surface river water) used by SA communities for domestic and recreational purposes and clinical (stools and respiratory) specimens from South African patients with gastrointestinal or respiratory symptoms. Gastrointestinal HAdV infection may be a consequence of ingestion and respiratory and eye infections due to inhalation and recreational exposure to

faecally contaminated water (Jiang, 2006). Previous studies from SA only dealt with the occurrence of HAdVs in water samples (van Heerden *et al.*, 2003, 2005a, b) and clinical specimens (Kidd *et al.*, 1986; Marx *et al.*, 1998; Moore *et al.*, 1998) separately and the possible link between HAdVs in water sources and those associated with clinical infections was not investigated.

The detection of HAdV strains associated with gastrointestinal and respiratory infections has also been reported worldwide (Hierholzer, 1992; Echavarría, 2008). In this study an overall HAdV prevalence of 8.3% was recorded for the stool specimens and 5.9% for the respiratory specimens. The overall HAdV prevalence of 8.3% in the stool specimens is similar to the 9.8% reported by Moore *et al.* (1998), also for diarrhoeal samples from the Gauteng region. In this study, a prevalence of 3.1% was noted for HAdV-F40/41 which is lower than the prevalence of 6.5% reported for hospitalised children in Soweto (Kidd *et al.*, 1986) but similar to the 3.7% reported by Marx *et al.* (1998) for the stool samples from the same geographical area, and 3.4% for stool samples from another area within Gauteng (Moore *et al.*, 1998). This HAdV-F40/41 prevalence of 3.1% also correlates with prevalence rates reported for paediatric diarrhoeal stool specimens from other African regions, including 2.6% in Tanzania (Moyo *et al.*, 2007), 3.1% in eastern Tunisia (Fodha *et al.*, 2006) and 2% in Botswana (Basu *et al.*, 2003). The HAdV prevalence of 5.9% in the respiratory specimens recorded in this study is lower than the prevalence of 11% reported by McAnerney *et al.* (1994).

The overall prevalence of HAdVs detected in water samples in this study was 8.6%, of which 8.1% was from untreated waters and 0.53% from treated waters. It is difficult to make direct comparisons regarding the prevalence of HAdVs in untreated surface and treated drinking water sources from other countries, because there are no universal standard procedures for the detection of viruses in environmental samples (Verheyen *et al.*, 2009). Within the SA context, where the same recovery and detection techniques were applied, the prevalence of HAdV in 0.8% treated drinking water recorded in this study was

substantially lower than the 4.4% and 5.3% reported for the same water sources in 2000-2001 and 2002-2003, respectively (van Heerden *et al.,* 2003, 2005a). This lower prevalence in treated drinking water could be ascribed to either the application of an automated nucleic acid extraction method in the current study or an improvement in the water treatment process. In contrast, a higher prevalence of 17% was noted compared to 13% recorded in 2000-2001 (van Heerden *et al.,* 2003) for the same K19 surface water source. However, the prevalence of 17% was lower than the 22% reported by van Heerden *et al*. (2005a) for 2002 to 2003. This variation could possible be due to differing levels of faecal contamination in the sampling periods. The characterisation of the HAdVs detected in the water samples drawn in 2002-2003 indicated that species D predominated in the treated drinking water samples with single strains of HAdV-C2, HAdV-F40 and HAdV-F41 being identified (van Heerden *et al*. 2005a). In the current study, HAdV-D and HAdV-F were also identified in the treated drinking water samples, suggesting that species D and F are more resistant to water treatment processes. In this study, the HAdVs detected in the untreated water sources were identified to be predominately HAdV-F strains. This result is similar to the typing data reported by van Heerden *et al.* (2005a), however, in contrast to the latter study, species A and species B were also identified in the untreated water sources (Figure 2.7 and 2.8). The identification of HAdV-D13 and HAdV-D26 in the harvested cell cultures infected with untreated water samples K19060626 and K19070709, respectively (Figure 2.8) indicates that the HAdVs detected in the water sources are potentially infectious. In one of the water samples, N19060505, HAdV-D26 was detected directly by PCR in the recovered virus extract while HAdV-F41 was detected in the cell culture extract of the same sample. Therefore, the detection of single genotype in a sample does not preclude the possibility that multiple genotypes are present; a similar finding was noted for HAstV (Nadan *et al.,* 2003).

The phylogenetic analysis of HAdV strains demonstrated a close genetic relationship between the clinical (ST and RESP) strains and the strains from

untreated and treated water sources. Within genotype HAdV-F41, the HAdVs from stool specimens clustered in two distinct clusters. In one of the clusters, the strains showed 100% nucleotide sequence identity to a strain from treated drinking water (N22060605) and, in the other cluster, as strain from river water indicating that HAdVs detected in water sources are genetically closely related to those detected in clinical specimens. All stool samples in each cluster presented in Figure 2.6 showed a 100% nucleotide sequence identity to the grouped water samples, N22060605 and K19070226, and a 97% to 98% identity to the reference HAdV-F41. Of note was the nucleotide sequence identity of strains from water samples K19060724 and K19070611. These water samples were collected a year apart, but the HAdV-F41 strains showed a 100% similarity to each other and 97% to the reference HAdV-F41 which demonstrates the persistent circulation of this strain in the water source and surrounding community. This finding that viruses persist in the environment is further highlighted by strains from water samples collected a week apart, i.e. K19060710 and K19060717, which showed 99% nucleotide identity to each other, and 96% to reference HAdV-F41. In comparison to HAdV-F41, the detection of HAdV-F40 showed that this strain type was rarely detected in the water samples and was only identified in two water samples collected at different times. The fact that HAdV-F41 was more frequently detected in the stool specimens and water could either be due to seasonal circulation of the virus or due to the fact that HAdV-F41 causes more severe diarrhoea and the stool samples were all from hospitalised patients. The detection of a high percentage of HAdV-F40/41 strains in untreated water suggests a high level of contamination with human faeces. The HAdV-B3/C5 strain from untreated river water sample K19060918 clustered closely with the majority of the HAdV strains from the respiratory (RESP) specimens and the reference strain (Figure 2.7), which identifies exposure to aerosols of water from this water source as a potential source of HAdV-associated respiratory infection. Other HAdV strains, namely HAdV-B7 and HAdV-C6, which are associated with respiratory infection, were detected in water samples K19070806, RS2060710 and WP0200417 (Figure 2.7). Genotypes HAdV-A18 and HAdV-A12

clustered with K19061002 (surface river water) and ST6 (stool specimen), respectively. This close genetic relatedness between HAdV strains detected in water and clinical strains highlights that water may be a potential source of human infection in communities using the water for domestic and recreational purpose.

CHAPTER 3

MOLECULAR DETECTION AND CHARACTERISATION OF ADENOVIRUSES FROM STOOL SPECIMENS FROM KENYAN CHILDREN

3.1 INTRODUCTION

Diarrhoeal disease is a major cause of morbidity and mortality in children, especially in developing countries (Bern and Glass, 1994). To date, studies on viral diarrhoeal pathogens in Kenya have focussed on HRVs (Kiulia *et al.*, 2006, 2008, 2009) and human astroviruses (HAstVs) (Kiulia *et al.*, 2007) and there are no significant data on the prevalence of HAdVs in children with diarrhoea. In a study on acute gastroenteritis in early childhood in Kenya where AdV infection was determined by seroconversion studies, no AdV infection was noted (Mutanda, 1980). There is only a single study on HAdVs in stool specimens from paediatric patients in Kenya. In this study, HAdVs were detected in 22% (11/49) of the stool specimens using an AdV group-specific latex-agglutination assay and no further characterisation was done (Forbes *et al.*, 2004). The classic enteric HAdVs, species F types 40 and 41, are considered to be the second most important cause of childhood gastrointestinal illness worldwide (Avery *et al.*, 1992; Scott-Taylor and Hammond, 1992), but unlike rotavirus they show no seasonal variation in their incidence (Wood, 1988; de Jong *et al.*, 1993; Ison, 2006). Human adenovirus types A31, F40 and F41 have been associated with severe gastroenteritis in infants and young children (Madisch *et al.*, 2006) and these enteric HAdVs are a common cause of diarrhoea in child care settings (Van *et al.*, 1992; Taylor *et al.*, 1997; Gompf and Oehler, 2005). In patients with AIDS HAdV-associated gastroenteritis is usually due to species D viruses while in other immunocompromised patients gastroenteritis is usually due to types A31, C2, F40 and F41 (Hierholzer, 1992).

The objective of this study was to determine the prevalence of HAdVs in stools from Kenyan infants and children, and to determine the HAdV genotypes circulating in this sub-group of the population. The use of molecular techniques, i.e. group-specific PCR and sequence analysis, is necessary as all the HAdV types will be detected and characterised. This is important to fully understand the epidemiology of faecally shed AdVs, especially among individuals living in closed settings (Bern and Glass, 1994; Kroes *et al.*, 2007).

3.2 MATERIALS AND METHODS

3.2.1 Clinical specimens

3.2.1.1 Ethical approval

Ethical approval for the use of stool specimens was granted by the Kenyatta National Hospital Ethics and Research Committee (KNH-ERC) from Kenya, and the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, South Africa: S27/2008.

3.2.1.2 Study population and specimen collection

Stool specimens from Kenyan children ≤14 years of age were collected as part of an ongoing public health initiative to document rotavirus infection and epidemiology in Kenya. Specimens included 228 diarrhoeal stools from children younger than ten years of age who attended urban clinics (Kibera, Ngong, St Odilias and Embulbul) in and around Nairobi, and 135 diarrhoeal stools from children younger than ten years of age with severe diarrhoea who were either hospitalised or attended the out-patient department of the Maua Methodist Hospital in the Meru North District. The HIV status of the children who attended the urban clinics and rural Maua Methodist Hospital is unknown. In addition, 110 stool specimens (43 diarrhoeal; 67 non-diarrhoeal) were collected from HIV-seropositive children <14 years of age at the Nyumbani Hospice and Nyumbani community-based foster care programme. Of the 473 stool specimens, 278, of which 104 (43 diarrhoea; 61 non-

diarrhoea) were from the urban hospice, 94 from the urban clinics and 80 from the rural setting, were available for adenoviral analysis.

3.2.1.3 Specimen preparation

A 10% stool dilution was prepared by adding a pea-sized portion of the stool specimen to 9 ml distilled water in a 10 ml tube (Sterilin, Barloworld Scientific Ltd, Stone Staffordshire, UK), and mixed by vortexing for 1 min to give a well defined suspension.

3.2.2 Molecular detection of adenoviruses

3.2.2.1 Nucleic acid extraction

Viral nucleic acid was extracted from the faecal suspensions using the method as described Section 2.2.4.1 (page 42).

3.2.2.2 Detection of adenoviruses by PCR

Nested-PCR of the viral DNA was done using the degenerate primer pairs (Table 2.1; page 43) following the methods described in Section 2.2.4.2 (page 43).

3.2.3 Molecular characterisation of adenoviruses

3.2.3.1 Sequencing of PCR amplicons

Amplicons from a randomly selected convenience subset (n=61) of HAdV-positive specimens were characterised. The sequencing reaction for the amplicons from the second round nPCR reaction was undertaken following the detailed methods described in section 2.2.5.1 (pages 44-55), using the nPCR primers listed in Table 2.1.

3.2.3.2 Sequence analysis and genotyping

Sequence data from both strands of the PCR products were aligned with Sequencher 4.7. Multiple sequence alignments were generated using MAFFT Version 6 (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) and edited using BioEdit Version 7.0.9.0 (Hall, 1999). The sequences of representative

isolates were compared with AdV sequences present in GenBank by using the BLAST-N program (version 2.2.18) to search for the most similar strains available. A phylogenetic tree was constructed using the Mega software version 4 (Tamura *et al.,* 2007). The AdV reference strains used for comparative purposes are presented in the Table 3.1:

Table 3.1: GenBank accession numbers of reference adenovirus nucleotide sequence data used for comparative purposes

Species/ Type	**Accession number(s)**	**Species/ Type**	**Accession number(s)**
A12	AB330093	D26	EF153474; AY819873
A18	AB330099	D28	AB330109; DQ149626
A31	X74661.1	D29	DQ149627; AB330110
B3	AF542129	D30	AB330111; DQ149628
B7	AB330088; AB243118.1	D32	DQ149629; AB330113
B21	AB330102	D33	DQ149630
B34	AB052911	D36	AB330117; DQ149631
C1	AB330082	D37	DQ900900
C2	AJ293903	D38	AB330119
C5	AF542119; EU867493.1	D39	AB330120; DQ149634
C6	AB330087	D42	AB330123; DQ149635.1
D8	AB361058; AB090344	D43	AB330124; AY819885
D9	AB245425; X74664	D44	DQ149637
D10	AB330091	D45	AB330126
D13	AB330094	D46	AB330127
D15	AB330096.1; AY819865	D47	AB330128;AB330128.1
D17	AB330098; AF108105	D48	AB330129; HAU20821
D19	DQ149618; AB330100	D49	AB330130; DQ393829
D22	AB330103; DQ149620	F40	AB330121
D23	AB330104; DQ149621	F41	AY819814
D24	AB330105; DQ149622	Sim Ad7	DQ792570
D25	AB330106; DQ149623	Feline AdV	AY512566
Fowl AdV	Aviadeno; AC000014		
Duck AdV	Atadeno; Y09598		

3.2.4 Statistical analysis

Demographic data were summarised using descriptive statistics. The exposed groups from the rural and urban areas were compared with respect to HAdV positivity using the Mantel-Haenszel chi-square test (http://www.openepi.com/TwobyTwo/TwobyTwo.htm).

3.3 RESULTS

3.3.1 Prevalence of adenoviruses

Two-hundred-and-eighty stool specimens were tested for the presence of HAdVs and 104 (37%) were positive. The prevalence of HAdVs in diarrhoeal and non-diarrhoeal stool specimens in relation to children's age and HIV serological status and the geographic setting is summarised in Table 3.2.

Table 3.2: Prevalence of HAdVs in diarrhoeal and non-diarrhoeal stool specimens from urban and rural Kenyan children.

	Urban setting						Rural setting	
	HIV-seropositive				HIV status unknown		HIV status unknown	
Age range	Non-diarrhoeal		Diarrhoeal		Diarrhoeal		Diarrhoeal	
	No.[a]	Pos[b] (%)	No.	Pos (%)	No.	Pos (%)	No.	Pos (%)
0-12 mo	-	-	-	-	33	27.3 %	29	58.6 %
13-24 mo	-	-	-	-	29	24.1 %	17	23.5 %
25-36 mo	-	-	-	-	10	10 %	9	22.2 %
3-5 y	-	-	-	-	16	43.8 %	14	71.4 %
>5 y	-	-	-	-	3	0 %	1	100 %
Unknown	61	16.4 %	43	81.4 %	3	33.3 %	10	0 %
Total	61	16.4 %	43	81.4 %	94	26.6 %	80	42.5 %

a = number of specimens tested

b = percentage of HAdV positive specimens

From Table 3.2, the overall detection of HAdVs in diarrhoeal and non-diarrhoeal stool specimens was 94/217 (43%) and 10/61 (16%), respectively. In the urban hospice setting, 45/104 (43%) of the stool specimens from HIV-seropositive children (average age 6.3 years; range 5 months to 14 years) tested positive for HAdV. In this setting, there was a highly significant association between HAdV and diarrhoea compared to no diarrhoea [35/43 (81%) versus 10/61 (16%); p <0.001]. Twenty-five (26%) of the 94 diarrhoeal stool specimens from children of unknown HIV status (average age 2.1 years; range 2 months to 10 years) attending clinics in the same urban areas as the hospice tested positive for HAdV. The prevalence rates of HAdV infection in children aged 0-12 months and 13-14 months were similar, 27% and 24%, respectively. Although a HAdV prevalence rate of 44% was recorded for the children between three and five years of age, due to the low number of specimens (n=16), this prevalence rate did not differ significantly from that recorded for the younger age groups. In the urban setting, the association between HAdV-positive diarrhoeal stools and the HIV-seropositive status of the children in the hospice environment was highly significant, when compared to HAdV-positive diarrhoeal stools from children of unknown HIV status (35/43 [81%] versus 25/94 [26%]; p <0.001). In the rural setting 34/80 (42%) of the stool specimens from children of unknown HIV status (average age 2 years; range 2 months to 6 years) with diarrhoea were HAdV-positive. For the age groups 0-36 months, the HAdV prevalence rates in stool specimens from the children from the rural settings were similar to, and did not significantly differ from, those recorded for stool specimens from urban children of unknown HIV status. In the 3-5 year old age group, however, a child of unknown HIV status from a rural area with gastroenteritis was more likely to have an HAdV infection compared to a child of unknown HIV status from an urban area with gastroenteritis (10/14 [71%] versus 9/16 [56%]; p <0.40). When comparing the overall HAdV prevalence in diarrhoeal stool specimens from children with unknown HIV status from the urban and rural settings, a significant dis-association was noted between HAdV-positive

diarrhoeal specimens from urban and rural locations of the children (25/94 [26%] versus 34.80 [42%]; p <0.028).

3.3.2 Characterisation of adenoviruses

From 104 HAdV-positive specimens, 61 HAdV strains were sequityped. On the basis of pairwise comparisons of a 475 bp fragment of a conserved genomic region of the hexon protein, 55 HAdV strains could be genotyped while an additional 6 HAdV strains could be speciated (Figure 3.1).

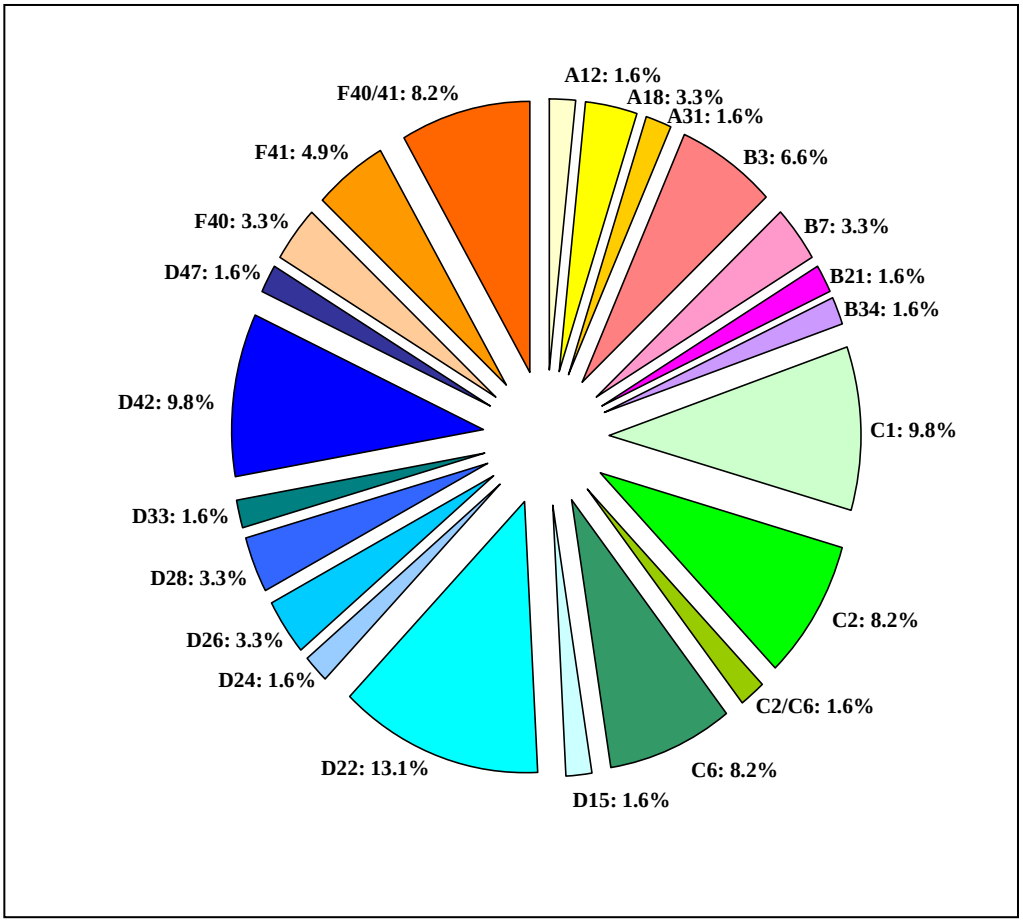

Figure 3.1: The prevalence of HAdV species and genotypes identified in stool specimens from Kenyan children, combined with and without diarrhoea.

The overall detection of HAdVs species and genotypes in the stool specimens was as follows: HAdV-D predominated and was detected in 22/61 (36.1%), HAdV-C 18/61 (29.5%), HAdV-F 10/61 (16.4%), HAdV-B 8/61 (13.1%), and

HAdV-A 4/61 (6.5%). No species E or G were detected among the HAdVs genotyped. The distribution of HAdV species in the urban clinics (Kibera, Ngong, St Odilias, Embulbul), urban hospice (Nyumbani) and rural hospital (Maua) are presented in Figure 3.2.

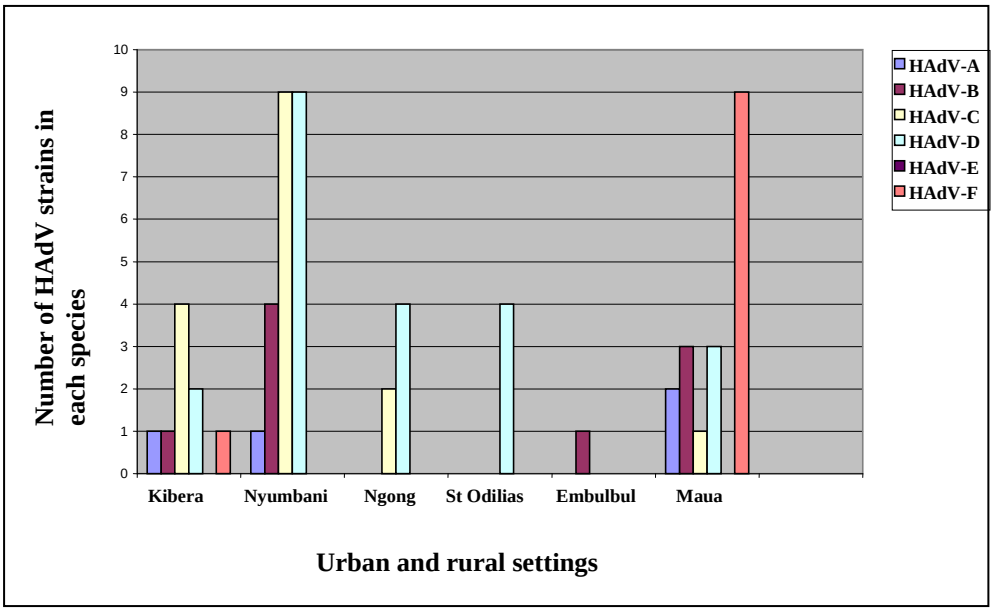

Figure 3.2: Distribution of HAdV species in urban and rural setting in Kenya.

In the urban clinics and urban hospice, HAdV–C and HAdV-D predominated, while, in the rural hospital setting HAdV-F was the most prevalent species identified (Figure 3.2). In addition, a variation of the distribution of HAdV species within the urban setting was noted. A high prevalence of HAdV-D was noted from the Ngong and St Odilias clinics while HAdV-C was the predominant species detected in specimens from the Kibera clinic.

The HAdV genotype distribution in diarrhoeal stool specimens from children in the urban and rural settings, irrespective of their HIV status, is presented in Figure 3.3. In urban children with diarrhoea HAdV species D predominated comprising 47.2% (HAdV-22: 41.2%; HAdV-42: 23.5%) of the typed strains (Figure 3.3[A]). HAdV species F and A, classically associated with gastroenteritis, were detected to a lesser extent each representing 2.8% of

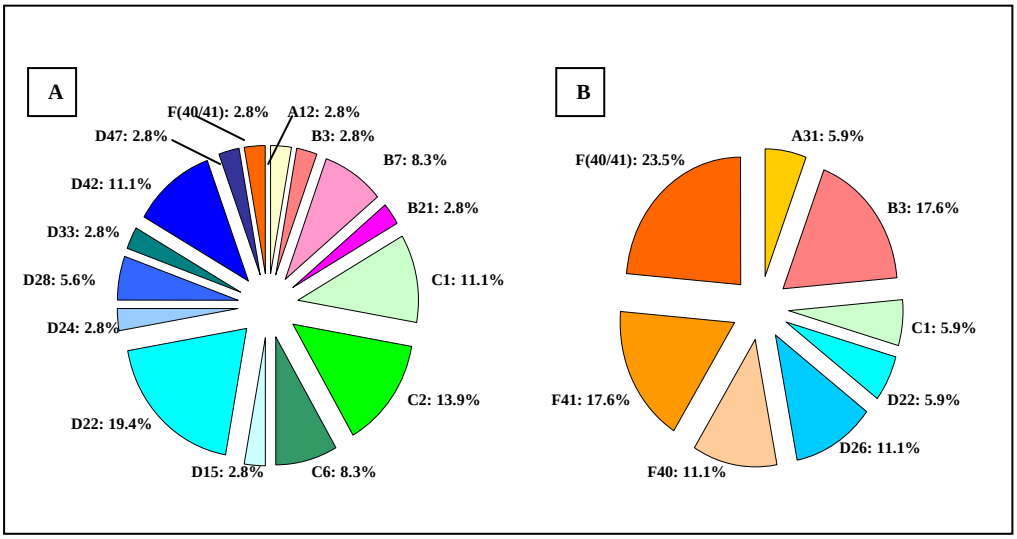

Figure 3.3: Distribution of HAdV species and genotypes presented in the diarrhoeal stool specimens from Kenyan children in urban (A) and rural (B) settings irrespective of their HIV status.

strains identified. In contrast, species F (HAdV-40: 11.8%; HAdV-41: 17.6%; HAdV-40/41: 23.5%) and A (HAdV-31: 5.9%) were detected in the diarrhoeal stools specimen of children from the rural setting Figure 3.3[B]. Major differences were noted in the distribution of species C and B HAdVs between the two groups. Species C (HAdV-2: 13.9%; HAdV-1: 11.1%; HAdV-6: 8.3%) were identified in 33% of the typed strains from the urban setting while in the rural setting species C was only identified in 5.9% of the strains. Variation was also noted in the distribution of species B, namely in the urban setting B7 (8.3%), B3 (2.8%) and B21 (2.8%) were identified while only B3 (17.6%) was identified from the rural setting.

The HAdV genotype distribution in diarrhoeal stool specimens from HIV seropositive children and children of unknown HIV status in urban and rural areas is presented in Figure 3.4. Marked differences were noted in the distribution of HAdV species and genotypes in diarrhoeal stool specimens from urban and rural children in relation to their HIV status. In the urban setting species D predominated in both HIV seropositive children (Figure 3.4 [A]: 43.8% [D-22: 42.9%; D-42: 14.3%]) and children of unknown HIV status

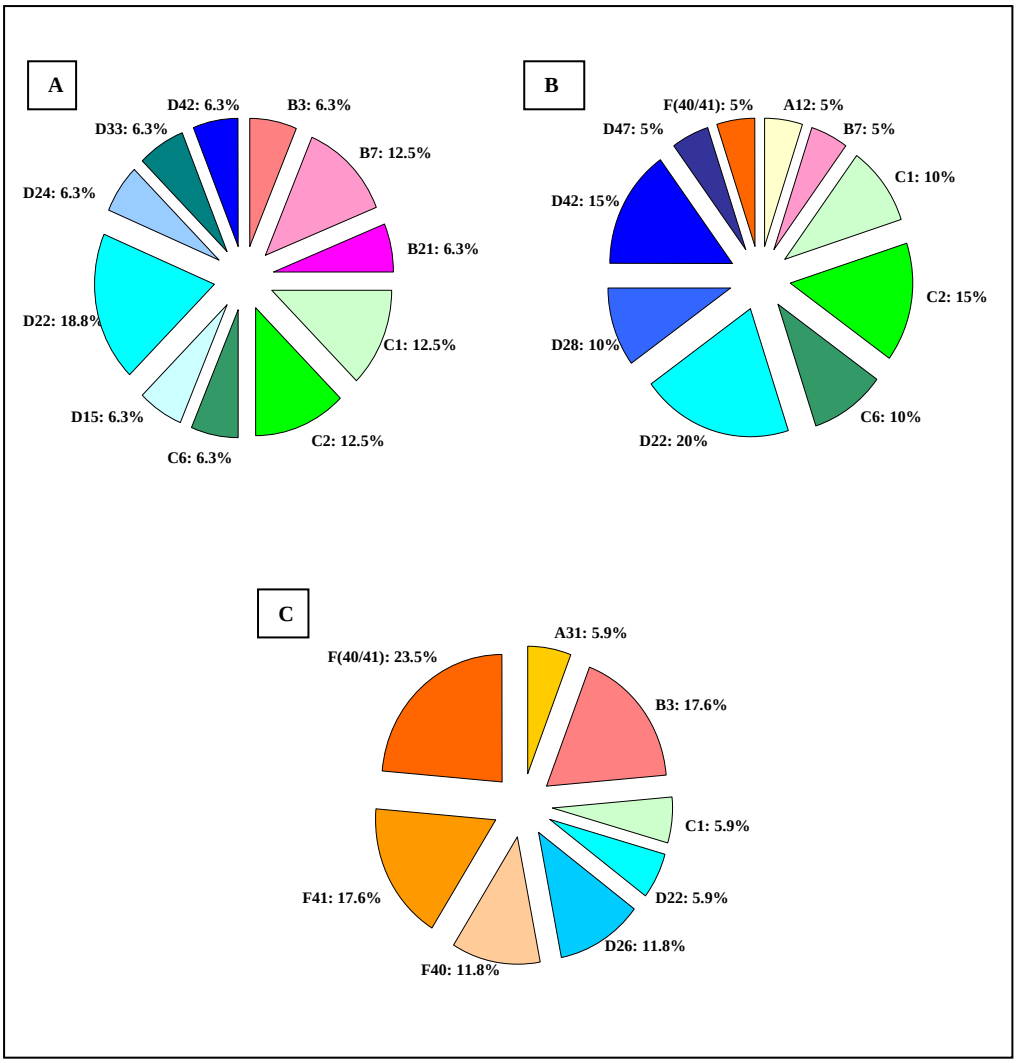

Figure 3.4: Distribution of HAdV species and genotypes in paediatric diarrhoeal stool specimens from: (A) urban HIV-seropositive children; (B) urban children of unknown HIV status; and (C) rural children of unknown HIV status.

(Figure 3.4 [B]: 50% [D-22: 40%; D42: 30%]). HAdV species C and B showed minor differences between these two groups. From both urban (Figure 3.4[B]) and rural (Figure 3.4[C]) children of unknown HIV status, occurrence of HAdV strains varied markedly. Species F was shown to be detected in high prevalence from the rural setting, comprising 52.9% of the identified HAdVs, whereas from the urban settings the detection was 5%. The detection of species A from urban (Figure 3.5B [A-12: 5%]), and rural (Figure 3.4[C] [A-31: 5.9%]) children showed no major differences.

3.3.3 Nucleotide sequence and phylogenetic analysis of Kenyan HAdVs

Within each species the HAdV strains grouped together in a number of clusters with 99-100% nucleotide sequence identity within each cluster. The representative stains for each cluster are presented in Table 3.3. The phylogenetic tree shows the genetic relatedness of Kenyan HAdV strains to reference strains in GenBank (Figure 3.5).

Table 3.3: Summary of the characterised Kenyan HAdV strains from diarrhoeal and non-diarrhoeal paediatric stool specimens, identifying the representative strain included in the phylogenetic analysis.

HAdV genotype	**Representative Kenyan strain**	**Kenyan strains with 99-100% nucleotide identity to the representative strain**
HAdVD42	NK 460	NK212 NK204 NK521
HAdVD22	NK247	NK546 NK649 NK205 NK491 NK197 NK488 NK512
HAdVD28	NK518	NK519
HAdVC1	NK485	NK165 NK544 NK747 NK178
HAdVC2	NK513	NK515 NK506 NK164 NK473 NK161 NK209 NK471 NK211 NK267
HAdVB7	NK510	NK245
HAdVB3	NK201	NK740 NK701 NK647

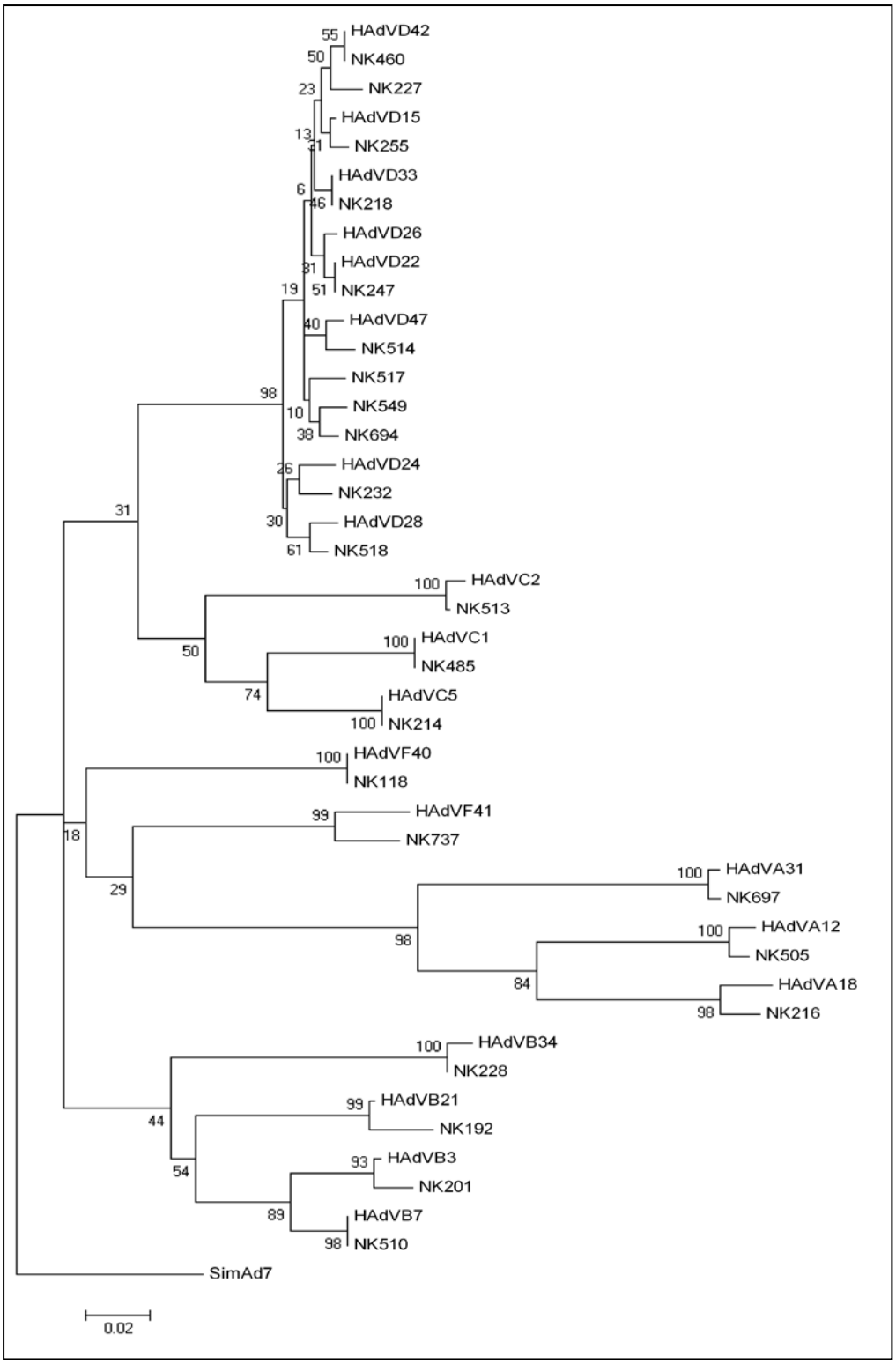

Figure 3.5: Neighbour-Joining phylogenetic tree based upon a 475 base-pair fragment of the hexon genomic region of the representative Kenyan HAdV and reference HAdV strains. The bar indicates nucleotide changes per site and bootstrap percentages are indicated.

Within type HAdV-D42, three distinct clusters with representative strains NK460, NK227 and NK517, all from the urban area, were noted. Strains NK549 and NK694 showed a 98.6% nucleotide sequence identity to the reference HAdV-D26 strain in GenBank but clustered separately to the reference strain.

3.4 DISCUSSION

This study represents the first in depth investigation into the molecular epidemiology of HAdVs in both urban and rural settings in Kenya and highlights the previously unreported diversity of HAdVs shed in stool specimens from African children. In a previous study from Kenya, no data on HAdV genotypes in stool specimens was presented (Forbes *et al.*, 2004).

The analysis of stool specimens for HAdVs by PCR showed a very high prevalence (34.4%) compared to the 22% previously reported for Kenya (Forbes *et al.*, 2004). In almost all studies done in Africa, the prevalence of enteric viruses, including HAdV, has been determined using commercial ELISA or related immunological-based assays specifically HAdV-F40/41 specific assays. In a study in Tanzania, a HAdV prevalence of 2.6% was recorded (Moyo *et al.*, 2007), while in Botswana the prevalence of HAdV was 7.8% (2% HAdV F40/41) (Basu *et al*., 2003). In South Africa, Moore *et al*. (1998) reported a HAdV prevalence of 9.8% (3.4% HAdV F40/41). A recent study from Nigeria, where stool specimens from children were screened for AdVs using a commercial genus-specific EIA (Adenovirus Ridascreen® r-Biopharm, UK) a positivity rate of 22.3% was recorded (Aminu *et al.*, 2007). In other studies in Africa, i.e. in Ghana, where PCR was used to determine the prevalence of HAdVs a similar higher prevalence, i.e, ~28%, to that noted in this study was reported (Reither *et al.*, 2007; Weitzel *et al.*, 2007).

Of note was the observation that the most commonly detected species from diarrhoeal stool specimens of infected infants and young children, HAdV-F

(40/41) and HAdV-A (31), were less prevalent in the diarrhoeal stool specimens from children in the urban setting. The detection of a high number of strains that are not associated with gastroenteritis infection, i.e HAdV-D in 22/61 (36.1%), HAdV-C in 18/61 (29.5%), HAdV-B 8/61 (13.1%), and HAdV-A 4/61 (6.5%), was significant. From Figure 3.1, HAdV-D types -22, -42, -47, previously reported to be detected mostly from HIV/AIDS patients (Hierholzer, 1992; Lord *et al*., 2000) were detected in high numbers in this study. These types have been associated with diarrhoea in HIV/AIDS patients (Hierholzer, 1992), but the aetiological association of these HAdVs with the diarrhoeal episodes reported in this study needs to be elucidated. The observation that in the diarrhoeal stool specimens from children five years and younger of age of unknown HIV status from the urban and rural setting the HAdV-D prevalence was 50% and 17.6%, respectively, suggests that many of the children in this study may have been HIV-positive or had compromised immune systems. The occurrence of HAdV-B types -3 and -7, and HAdV-C types -6, -2 and -1, species associated with respiratory infections was unexpectedly detected in large numbers, although it has been noted that such species can be excreted in stool specimens (Garnett *et al*., 2002).

The findings from this investigation highlight the limitations of the immunological-based assays currently used for the detection of HAdVs in stool specimens as many types could possibly be missed if only HAdV-F40/41 species-specific EIAs are used for the detection or screening of HAdV from stool specimens. The use of molecular techniques for the detection of enteric viruses may not be possible in developing countries due to limitations in resources, but it will improve the accuracy of detection and characterisation of gastroenteritis-associated viruses.

CHAPTER 4

GENETIC HETEROGENEITY OF ADENOVIRUSES FROM SOUTH AFRICA AND KENYA COMPARED TO THOSE FROM THE REST OF THE WORLD

4.1 INTRODUCTION

Adenoviruses are common worldwide and AdV infection patterns depend on their serotype, population group, and type of exposure (Ruuskanen *et al.*, 2009). Clinical outcomes of HAdV infection in humans are usually influenced by their viral type-specificity and coinfection with multiple strains (McCarthy *et al.*, 2009). Most of the known higher numbers of HAdV serotypes -D42 to -G52 were isolated from immunocompromised individuals, and about half of all known HAdV serotypes are recognised to cause illness to human population (Echavarría, 2009). It is, therefore, important to know the geographical distribution of HAdVs types and their virulence patterns, to understand the global burden of HAdV infection (Madisch *et al.*, 2006; Gray *et al.*, 2007).

The typing of HAdV by serum neutralisation or haemagglutination inhibition is no longer considered by many because both methods are labour-intensive and time-consuming (McCarthy *et al.*, 2009). The use of genomic RFLP/restriction enzyme (RE) analysis is also no longer popular, as it requires cultured virus, is labour-intensive and is also reported to have difficulties in interpretation of some of the results (Lu and Erdman, 2006). The introduction of PCR and sequencing has been advantageous, as these methods have the potential to provide rapid results for public health evaluation, treatment of patients and also for response to outbreaks (Gray *et al.*, 2007). One method used the PCR amplification and sequencing of hypervariable regions 1 to 6 (HVR 1-6), while another method was based on sequencing of the hexon

hypervariable region 7 (HVR 1-7) (Sarantis *et al.*, 2004). These methods were both successful in identifying the 52 recognised HAdVs (McCarthy *et al.*, 2009). A third method based on the hexon gene was described by Casas *et al.* (2005). In their study, sequencing and phylogenetic analysis of a moderately conserved region (amino acids 540-662) allowed speciation and serotype identification. Their publication indicated that they had prepared a website (www.greeneidlab-columbia.edu) where laboratories could submit hexon sequences and an automatic report would be generated detailing the date, serotype and location of the most similar isolate in the database (Casas *et al.*, 2005). Madisch *et al.* (2006) used a two-step typing system whereby a generic PCR product closely adjacent to loop 1 of the Є neutralisation determinant was sequenced and for speciation loop 2 of the hexon region was sequenced. An alternative strategy was the use of multiplex PCRs targeting the fiber gene (Xu *et al.*, 2000). This method does not differentiate strains to the serotype level, but was subsequently modified to include sequencing of smaller amplicons generated from the fibre gene to identify the HAdV serotype (McCarthy *et al.*, 2009).

The objective of this aspect of the study was to determine the genetic heterogeneity of the African HAdV strains in relation to strains from other regions of the world using the genotyping system proposed by Casas *et al.* (2005). This typing system was selected as it offered a website for direct comparison of African strains to those from other regions of the world.

4.2 MATERIALS AND METHODS

4.2.1 Adenovirus strains

Representative strains of HAdVs detected from clinical specimens and environmental samples from SA (Table 4.1) and clinical specimens from Kenya (Table 4.2) were selected for phylogenetic analysis. Representative strains of appropriate genotypes from other regions of the world were selected from GenBank (Table 4.3).

Table 4.1: Representative HAdV strains from southern Africa

Sample ID	Country	Detection method	Source	Species/type
K19061211	South Africa	Direct	River water	F41
K19060821	South Africa	Direct	River water	F41
K19070326	South Africa	Direct	River water	F41
K19070129	South Africa	Direct	River water	F41
B12060612	South Africa	Direct	Drinking water	F41
K19060710	South Africa	Direct	River water	F41
K19070205	South Africa	Direct	River water	F41
K19060724	South Africa	Direct	River water	F41
ST13	South Africa	Direct	Stool	F41
K19070626	South Africa	Direct	River water	F41
k19070820	South Africa	Direct	River water	F41
K19060731	South Africa	Direct	River water	F41
ST18	South Africa	Direct	Stool	F41
N22060605	South Africa	Direct	Treated water	F41
K19070521	South Africa	Direct	River water	F41
N19060505	South Africa	Cell culture	Treated water	F41
N20060605	South Africa	Direct	Treated water	F41
K19070402	South Africa	Direct	River water	F40
K19080121	South Africa	Direct	River water	F40
K19060918	South Africa	Direct	River water	B3
RESP18	South Africa	Cell culture	NPA	B3
RESP15	South Africa	Direct	NPA	B3
K19070806	South Africa	Direct	River water	B7
RESP14	South Africa	Direct	NPA	C1
RESP6	South Africa	Direct	NPA	C1
RESP2	South Africa	Direct	NPA	C1
RESP17	South Africa	Cell culture	NPA	C1
RS2060710	South Africa	Direct	Untreated water	C2
WP0200417	Namibia	Direct	Untreated water	C2
ST4	South Africa	Direct	Stool	C2
Vierderhof	South Africa	Direct	Irrigation water	D13
A19070402	South Africa	Direct	Drinking water	D13
K19060626	South Africa	Cell culture	River water	D13
N13060505	South Africa	Direct	Treated water	D42
N19060505	South Africa	Direct	Treated water	D42
ST6	South Africa	Direct	Stool	A12
K19061002	South Africa	Direct	River water	A18
K19070903	South Africa	Direct	River water	D32
MISLK	South Africa	Direct	Irrigation water	D33
K19070709	South Africa	Cell culture	River water	D26

NPA: nasopharyngeal aspirate

Table 4.2: Representative HAdV strains from Kenya

Sample ID	Region	Detection method	Source	Species/type
NK737	Maua	Direct	Stool	F41
NK118	Kibera	Direct	Stool	F40
NK201	Nyumbani	Direct	Stool	B3
NK510	Kibera	Direct	Stool	B7
NK192	Nyumbani	Direct	Stool	B21
NK228	Nyumbani	Direct	Stool	B34
NK214	Nyumbani	Direct	Stool	C1
NK485	Kibera	Direct	Stool	C1
NK513	Ngong	Direct	Stool	C2
NK227	Nyumbani	Direct	Stool	D13
NK460	Ngong	Direct	Stool	D42
NK232	Nyumbani	Direct	Stool	D42
NK514	Ngong	Direct	Stool	D42
NK517	St Odilias	Direct	Stool	D42
NK505	Kibera	Direct	Stool	A12
NK216	Nyumbani	Direct	Stool	A18
NK549	Maua	Direct	Stool	D32
NK694	Maua	Direct	Stool	D26
NK255	Nyumbani	Direct	Stool	D24
NK218	Nyumbani	Direct	Stool	D28
NK247	Nyumbani	Direct	Stool	D28
NK518	St Odilias	Direct	Stool	D28
NK697	Maua	Direct	Stool	A31

Table 4.3: Representative HAdV strains from the rest of the world.

Species/ Type	Accession number & Country	Species/ Type	Accession number & Country
A12	X73487 (USA); AY819855 (Spain)	D22	AB330103 (Japan); DQ149620 (Austria);
A18	AB330099 (Japan); DQ149610 (Austria); AY819856 (Spain)		FJ404771 (USA); AY819869 (Spain)
A31	X74661 (Germany)	D24	AB330105 (Japan); DQ149622 (Austria); AY819871 (Spain)

Table 4.3 continued: Representative HAdV strains from the rest of the world.

Species/ Type	**Accession number & Country**	**Species/ Type**	**Accession number & Country**
B3	AF542129 (Korea); EF564601 (USA); AB330084 (Japan); AY599834 (USA) X76549 (Germany); DQ086466 (Switzerland); AY819911 (Spain)	D26	EF153474 (The Netherlands); AY819873 (Spain)
		D28	AB330109 (Japan); DQ149626 (Austria); FJ824826 (USA); AY819875 (Spain)
B7	U75955 (Sweden); X76551 (Germany); AY594255 (USA); GQ478341 (China) AB330088 (Japan); AF065065 (USA); Z48571 (Sweden); EU078562 (China)	D32	AB330113 (Japan)
		D33	DQ149630 (Austria); AY819879 (Spain)
B21	AB053166 (Japan); AY601633 (USA); AY819908 (Spain)	D42	AB330123 (Japan); DQ149635 (Austria); AY819884 (Spain)
B34	AB052911 (Japan)	D47	AB330128 (Japan); DQ149640 (Austria); AY819889 (Spain)
C1	EU867492 (Germany); AB330082 (Japan); AF534906 (USA)	F40	L19443 (UK); X51782 (UK); AY819809 (Spain); AY819818 (Spain)
C2	AJ293903 (Germany)	C5	AF542129 (Korea)
C6	EU867472 (Germany); AY819853 (Spain)		
D8	AB500121 (Japan); AB448769 (Japan); AY819903 (Spain); X74663 (Germany)	D13	AB330094 (Japan); DQ149616 (Austria); AY819864 (Spain)
		D9	AB245425; X74664
D15	AB330096 (Japan) AY819865 (Spain); DQ149617 (Austria)	F41	AY819814 (Spain); AY819812 (Spain)
			AB330122 (Japan); DQ315364 (USA); X51783 (UK)
Sim Ad7	DQ792570		

USA: United States of America ; UK: United Kingdom

4.2.2 Phylogenetic analysis

Nucleotide sequences were analysed using the BioEdit sequence alignment Editor (V.7.0.9.0) and BLAST-N (version 2.2.18). The sequences were aligned using MAFFT version 6 (https://align.bmr.kyushu-u.ac.jp/mafft/online/server/). To study the genetic heterogeneity among HAdV and their relationships with HAdVs circulating in other countries pairwise comparisons of partial sequences of the hexon genome was done. To confirm the HAdV genotype and species detected, the phylogenetic trees were constructed from the nucleotide sequence alignments of the detected strains and the most closely related nucleotide sequence published in GenBank using the Neighbor-joining (NJ) method of the MEGA software version 4 (Tamura *et al.*, 2007). For determining the reliability of tree topology, bootstrap analysis was carried out on 1,000 replicates.

4.3 RESULTS

The genetic relationships between the species F HAdVs from SA, Kenya and the rest of the world are presented in Figure 4.1. The nucleotide sequences of HAdV-F40 and HAdV-F41 formed well-defined clades with their reference strains. The HAdV-F41 strains showed a greater genetic variability than the HAdV-F40 strains. Within HAdV-F41, a number of genome types could be identified. Within one genetic cluster, strains from the USA (DQ315364), United Kingdom (UK, X51783) and Japan (AB330122) showed a 100% nucleotide sequence identity to each other and ≥95% sequence identity to a number of HAdV-F41 strains from stool specimens and surface and treated drinking water sources from SA. The HAdV-F41 from Kenya (NK737) sorted in a genome type separate from the strains from SA and the rest of the world showing only a 93% nucleotide sequence identity to the reference strains from Spain (AY819814) and strains from the UK and USA. Two distinct genetic clusters were evident within HAdV-F40. The Kenyan stool specimen NK118 and South African surface water K19080121 had a 92% and 98% nucleotide sequence identity to the reference strain HAdV-F40 (AB330121), respectively.

One of the HAdV-F40 strains from a South African water source (K19070402) showed greater genetic variation (92% nucleotide sequence identity) to the reference strain and clustered separately from the other HAdV-F40 strains.

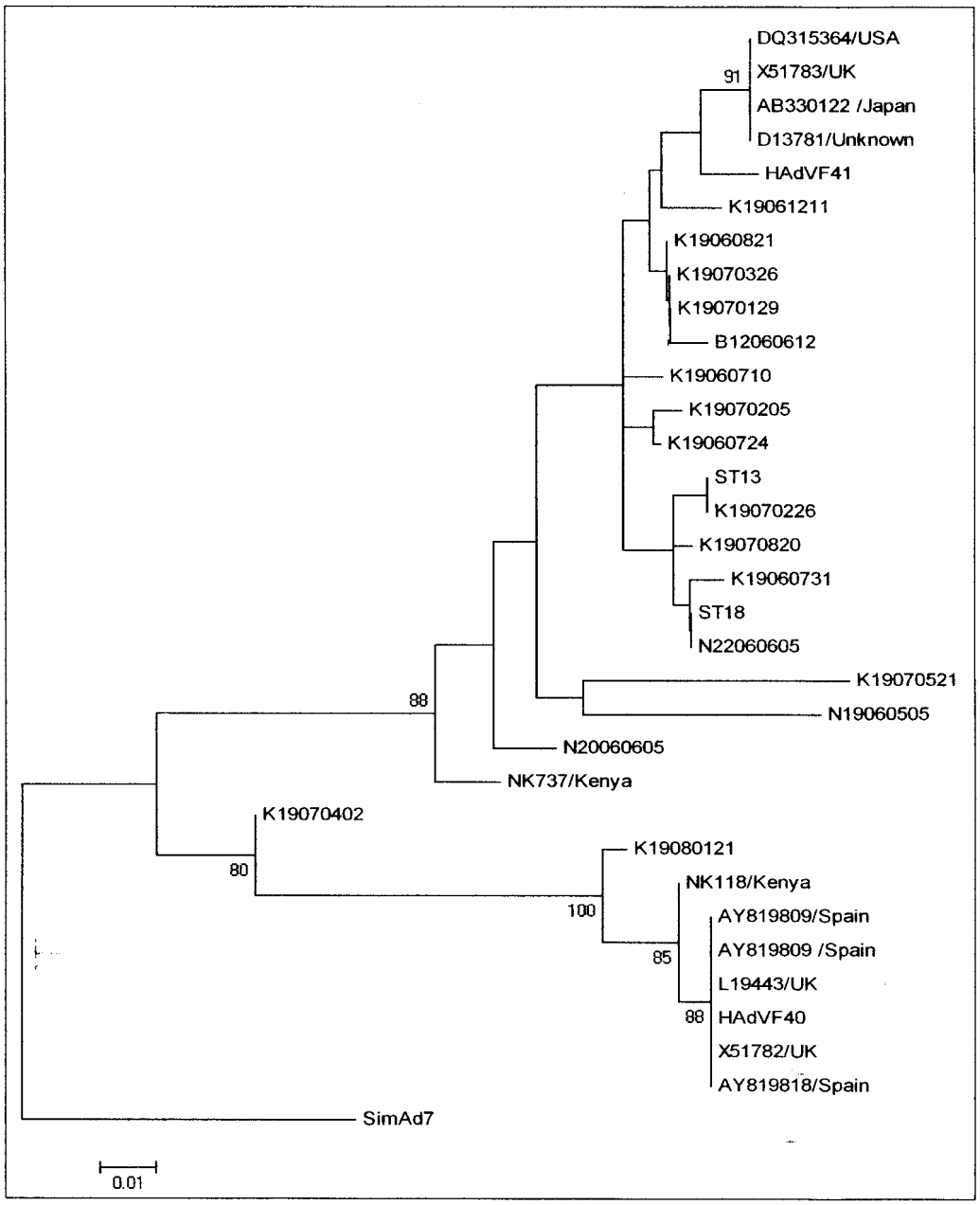

Figure 4.1: Neighbour-Joining phylogenetic tree based upon a 159 base-pair fragment of the hexon genomic region showing the relationships between species F HAdV strains from stool specimens (ST = SA; NK = Kenya), water samples (southern Africa), and strains from the rest of the world as well as the reference HAdV strains. Simian AdV-7 served as an outgroup. The bar indicates nucleotide changes per site and bootstrap percentages ≥75% are indicated.

Most of the species B HAdVs serotypes could be resolved by molecular typing, as noted previously (Chapter 2). No differentiation between serotypes HAdV-B3 and HAdV-C5 could be made in the hexon genome region analysed. From Figure 4.2, the African HAdV-B3/HAdV-C5 strains NK201 from Kenya and RESP18 from SA clustered separately from the reference strains from Korea and strains from the rest of the world. Serotypes B7, B21 and B34 formed separate well defined clades. The Kenyan strains NK192 and NK228 segregated into clades HAdV-B21 and HAdV-B34, each in their own distinct cluster with, 88% and 92% nucleotide sequence identity to the reference strains, respectively. Two distinct sub-clusters were evident with HAdV-B7. In the one sub-cluster HAdV-B7 strains from the rest of the world (Sweden, Germany, USA, China and Japan) showed 100% nucleotide sequence identity to the reference strain while in the other sub-cluster strains from Africa, NK510 from a Kenyan stool specimen and K19070806 from a South African river, showed a 99% nucleotide sequence identity to each other and only 95% nucleotide sequence identity to the strains from the rest of the world.

The species C HAdVs were less clearly defined by molecular analysis (Figure 4.2). Three distinct clusters were evident within HAdV-C1 and strains from Africa clearly clustered separately from the reference strain and strains from Germany, Japan and the USA. Genetic variation was evident as NK485 and RESP17 showed 97% and 96% nucleotide sequence identity to the reference HAdV-C1 strain, EU867492, respectively, while RESP14, RESP6 and NK214 showed only 89.4% nucleotide sequence identity to the reference strain. Little genetic variation was evident in HAdV-C2 as AdVs from Kenyan and South African stool specimens, NK153 and ST4, showed a 99% and 97% nucleotide sequence identity to the reference HAdV-C2 strain, AY819853, respectively. Greater genetic variation was noted within HAdV-C6 whereas the HAdVs from the South African untreated water sample RS2060710 and Namibian untreated water sample WP0200417 showed a 94% nucleotide sequence

identity to the reference HAdV-C6 strain, AY819853, and 97% nucleotide identity to each other.

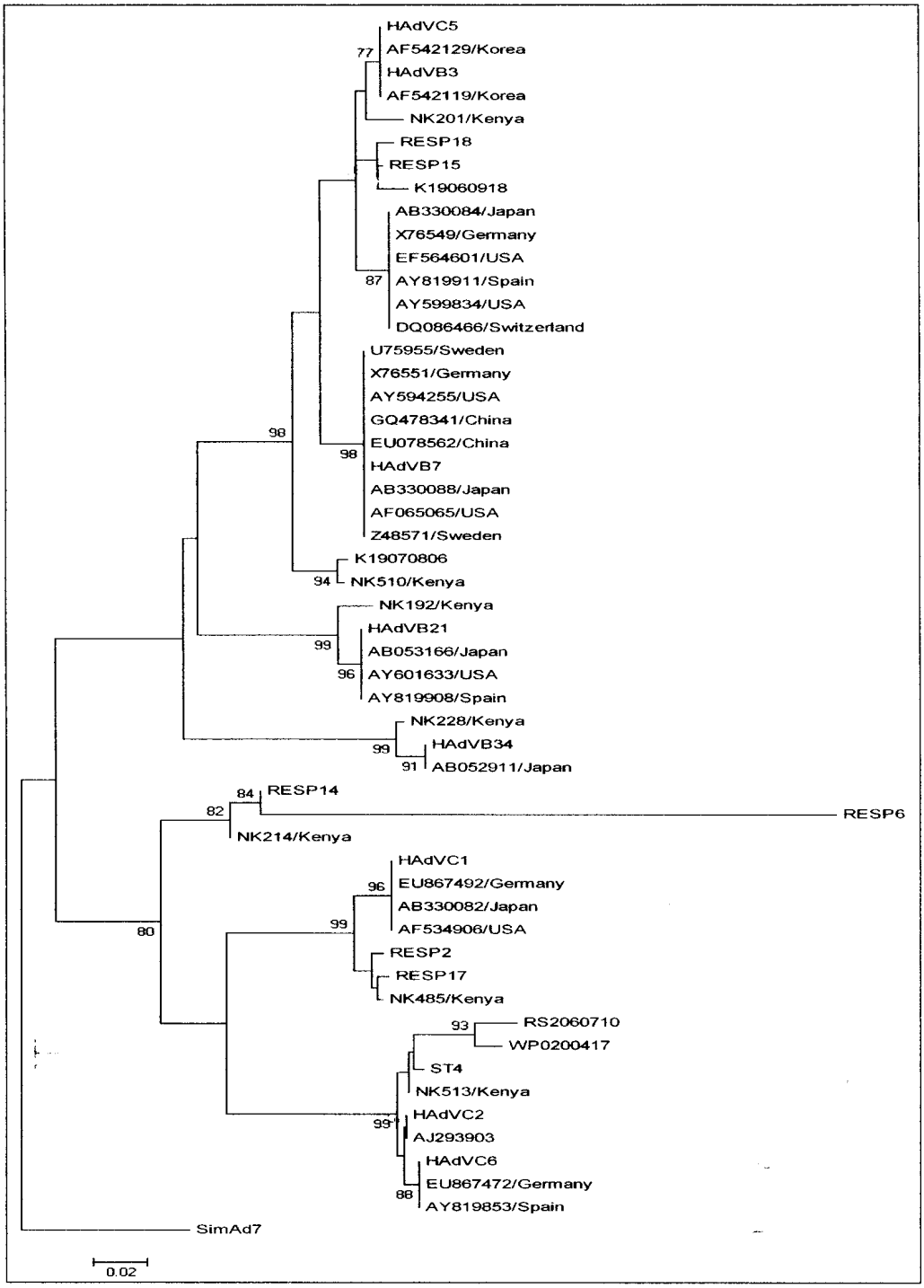

Figure 4.2: Neighbour-Joining phylogenetic tree based upon a 159 base-pair fragment of the hexon genomic region showing the relationships between HAdV-B and -C strains from stool (ST) and respiratory specimens (RESP)(South Africa), stool specimens (NK)(Kenya), water samples (southern Africa), the rest of the world and the reference HAdV strains. Simian AdV-7 served as an outgroup. The bar indicates nucleotide changes per site and bootstrap percentages ≥75% are indicated.

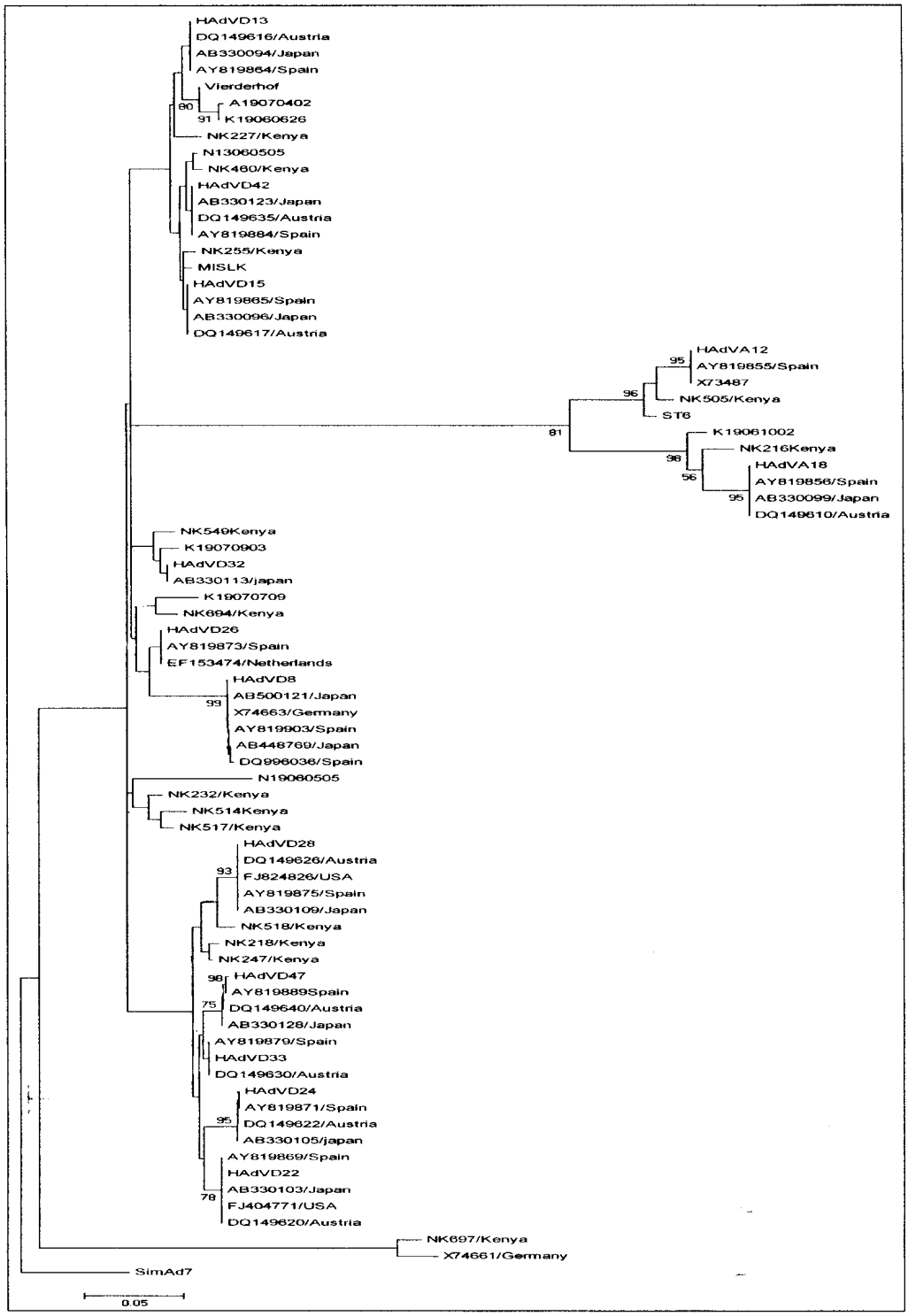

Figure 4.3: Neighbour-Joining phylogenetic tree based upon a 159 base-pair fragment of the hexon genomic region showing the relationships between HAdV-D and -A strains from stool (ST) and respiratory specimens (RESP) (South Africa), stool specimens (NK)(Kenya), water samples (southern Africa), the rest of the world and the reference HAdV strains. Simian AdV-7 served as an outgroup. The bar indicates nucleotide changes per site and bootstrap percentages ≥75% are indicated.

The species A HAdVs were readily distinguished by molecular analysis and the African strains segregated in well-defined clades with the reference strains. Within serotype HAdV-A12 strains NK505 from Kenya and ST8 from SA showed little genetic variation (98% nucleotide sequence identity). There was also very little genetic variation between these strains and the reference strain and strains from the USA (X73487) and Spain (AY819855), as they showed 96% nucleotide sequence identity to HAdV-C2 strains from the rest of the world. Compared to HAdV-A12, strains within serotype HAdV-A18 showed more genetic variation. Strains NK216 and K19061002 from a Kenyan stool specimen and a South African river water sample, respectively, showed 91% nucleotide sequence identity to each other and 92% to 94% nucleotide sequence identity to HAdV-A18 strains from other regions of the world. Of note, Kenyan stool specimen NK697 clustered with the HAdV-A31 from Germany (X74661) showing 96% nucleotide sequence identity.

When the nucleotide sequence from the species D viruses from Africa were compared to those from the rest of the world it was evident that the genetic relatedness of the strains within the types was less clearly defined (Figure 4.3). There appeared to be little genetic variation between some of the HAdV-D42 strains from Africa and those from the rest of the world. Strains N13060605 from South African cruise ship water and NK460 from a Kenyan stool specimen showed a 99% nucleotide identity to each other and 98%-99% nucleotide sequence identity to the reference strain, AB330123, from Japan. However, another African strain, namely NK227, from a Kenyan stool specimen, showed only 95.6% nucleotide sequence identity to the reference strain. South African water sample K19070709 and a Kenyan stool specimen NK694 showed a 97% nucleotide identity to each other and 96%-97% nucleotide identity to the reference HAdV-D26 strain, AB330107, from Japan, and strains from Spain (AY819873) and the Netherlands (EF153474). There appeared to be a little genetic variation within the HAdV-D28 clade, with a Kenyan stool specimen NK518 showing a 97.5% nucleotide sequence identity to the strains from the rest of the world.

4.1 DISCUSSION

The objective of this aspect of the study was to determine the genetic relatedness of HAdVs from SA and Kenya to HAdVs from the rest of the world. To this end, phylogenetic analysis of nucleotide sequences from a conserved fragment of the hexon gene of HAdV, as described by Casas *et al.* (2005), was applied. The nucleotide sequence of a moderately conserved region of African representative HAdV strains detected were compared to HAdV strains from those of the rest of the world. The importance of comparing the HAdV strains from Africa to those of the other regions of the world was to determine if different serotypes and strains were more common on a continent with an increasing immunocompromised population, as intermediate types are more common in this population group (Hierholzer, 1992).

From Figure 4.1, 4.2, and 4.3 it is evident that the majority of the African HAdV strains clustered separately from the strains from other regions of the world, suggesting that some of the African HAdV strains are genetically distinct from those from the rest of the world. Human AdV-F41 strains detected from South African treated and untreated water samples showed a close relationship with South African stool specimens (ST), e.g. ST13 and K19070226, and ST18 and N22060605 (Figure 4.1), but clustered separately from the reference strain and HAdV-F41 strains reported from other regions of the world (Figure 4.1). Whether this was due to antigenic drift or environmental adaptation peculiar to the African continent is not known. Many of the early studies showed HAdV-F40 and HAdV-F41 to occur equally in number (Li *et al.*, 2004), however after 1986, as also noted in Japan, Vietnam and Korea, the prevalence of HAdV-F41 increased. This change was thought to be due to antigenic drift with a concomitant increase in occurrence in susceptible individuals (Li *et al.*, 2004). In this study, HAdV-F41 also predominated. In addition, as described in other studies (Li *et al.*, 2004; Lu and Erdman, 2006), the HAdV-F41 strains showed greater genetic variability

than the other strains (Figure 4.1). It is thought that the HAdV-F41 genome types have a scattered global distribution and that novel genome types are present worldwide. This still needs further investigation. The lack of definition within species D could be due to the presence of recombinant or intermediate strains (Hierholzer, 1992, Echavarría, 2008), which were not differentiated by analysis of the hexon gene fragment. In addition, the possibility of mutations should be considered where differences were noted in HAdV serotypes detected directly from water samples and those detected from cell-cultured strains from water samples as this might be the mechanism of HAdV adaption to survival in the environment or to cell culture (Foy, 1997; He and Jiang 2005; Gerba, 2007).

This study had many limitations. The website www.greeneidlab-columbia.edu (Casas *et al.*, 2005) was found to be non-functional, and only a limited database of sequences in GenBank was available for comparison. In addition the PCR and sequencing-based method applied focused on a limited area of the HAdV hexon genome and new HAdV variants and intermediates could have been missed or mistyped. In addition, a shorter hexon genome fragment (159 bp) was sequenced and analysed than described by Casas *et al.* (2005) who recommended the analysis of 360 bp fragment. Of note, the difficulties encountered in the comparison of HAdV genotypes in this study to those reported by other researchers could have been due to the different hexon region or other protein site, such as fibre protein, being targeted. As highlighted by other groups (Gray *et al.*, 2007; Lebeck *et al.*, 2009) the hexon sequencing approach is useful but needs to be augmented by other molecular methods to determine the genetic relatedness of HAdVs from Africa and the rest of the world.

CHAPTER 5

GENERAL DISCUSSION

Adenoviruses are reported as the causative agent associated with sporadic and epidemic disease that result in significant economic losses and morbidity (Gompf and Oehler, 2005). Infections by HAdVs are known to occur throughout the year, but outbreaks are reported to be more common in late winter, spring, and early summer. Respiratory infections can be both endemic and epidemic, causing outbreaks in closed communities (Desselberger and Gray, 2003), while enteric infections are well known as a common cause of infantile diarrhoea in the day-care setting (Gompf and Oehler, 2005). With the genetic variability in HAdVs and with the description of seven types (HAdV-D42 to -D49) exclusively from patients with AIDS, it is important to be vigilant and to routinely monitor for the emergence of new types and variants, especially in the developing countries with a high percentage of immunocompromised individuals, e.g. South Africa and Africa in general. Therefore, this study was conducted to determine the genetic heterogeneity of AdVs circulating in communities in selected regions of Africa.

Although AdVs have successfully been detected in clinical specimens (Kidd *et al.,* 1986; Marx *et al.* 1998; Moore *et al.* 2000) and in water sources in SA (van Heerden *et al.,* 2003, 2004, 2005b), there has been no comparative genetic analysis on clinical and environmental isolates to determine the clinical relevance of environmental isolates. Characterisation and comparative genetic analysis of AdVs from water sources and clinical specimens was done to ascertain whether or not the same strains have been associated with human infection, and also to provide additional information regarding AdVs circulating in a given SA community. The phylogenetic trees (Figures 2.6, 2.7, and 2.8) constructed based on the hexon genome region, showed the relationship between the strains from the SA clinical (ST and RESP) specimens and water (untreated and treated) samples. Based on the results of

this study, it is evident that water may play a very important role as source of infection in the surrounding communities. The detection of a high percentage of HAdV-F (40/41) strains in untreated surface water suggests a high level of contamination with human faeces.

To establish the type of AdV genotypes circulating in Kenya and their clinical relevance with known HAdV genotypes, a phylogenetic tree (Figure 3.5) was constructed based on the hexon genome region analysed. The introduction of molecular techniques (nPCR and sequencing) showed a high prevalence of HAdV-D, HAdV-C, and HAdV-B which could have been missed if species-specific-EIAs had been used. Although nPCR is prone to false-positive results, the precautions taken to avoid cross-contamination (section 2.2.4.2) and evidence that the nucleotide sequence of the positive control (HAdV-C2) differed to that of the strains detected in this study, indicates that the results of this study are correct. The findings from this investigation highlighted the possible limitations of the immunological-based assays currently used for the detection of HAdVs in stool specimens. These findings highlighted the high number of HAdV-D strains present in stool specimens from HIV-infected individuals with the potential for recombinant. This study was possibly the first to be conducted using molecular method (PCR and sequencing) in Kenya. The findings of this study support the findings of Sarantis *et al.* (2004). The detection of HAdV-C1, -C2, -C5 and –C6 from stool specimens of immunodeficient patients was reported by Sarantis *et al.* (2004), and from this study species C (HAdV-C1, -C2, and –C5) from stool specimens of Kenyan immonucompromised patient (Figure 3.4) was noted.

The importance of the study was to ascertain whether the type of strains detected in Africa, especially SA and Kenya, would be genetically related to those reported from the rest of the world. The difficulty of comparing the results of this study with other existing evidence is due to the genomic region used for identification, and also the short length of the hexon region analysed. In addition, recombinant and intermediate strains, which might also play a role

in the genetic variability, may not have been detected or clearly defined by the genomic region analysed.

In conclusion, the findings of this study showed the benefits of PCR and sequencing techniques for detection and characterisation of HAdVs from different regions of the world, however, the precise genetic variation between the HAdV serotypes was not very good because of the shorter hexon region utilised. A similar phenomenon was raised when species D's node values on a phylogenetic tree were observed (Sarantis *et al.*, 2004). The evidence that HAdV strains detected in both treated and untreated waters had >90% nucleotide sequence identity as compared to those detected in clinical specimens and the known reference HAdV strains, support the evidence that water may be an important reservoir of HAdVs in a given communities. The detection of a number of different HAdV species from stool specimens, especially in Kenyan cohorts, highlighted the importance of using a molecular technique for burden of disease studies, and also for prevention and treatment programmes.

CHAPTER 6

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APPENDIX A

A.1 SOUTH AFRICAN RESPIRATORY SPECIMENS

Laboratory accession number	Specimen type	Specimen ID number	Detection Method	Patient details	
				Age	Sex
GTS0579011	NPA	RESP 1	Direct	1 yr 1 mo	M
GTS0714764	NPA	RESP 2	Direct	11 mo	M
GTS0600250	NPA	RESP 3	Direct	5 mo	F
GTS0884090	NPA	RESP 4	Direct	7 mo	F
GTS0914003	NPA	RESP 5	Direct	2 mo 20 d	M
GTS0584042	NPA	RESP 6	Direct	4 mo	F
GTS0710259	Sputum	RESP 7	Direct	1 yr 6 mo	M
GTS0585858	-	RESP 8	Direct	25 yr	F
GTS0437448	NPA	RESP 9	Direct	1 mo 13 d	M
GTS0891045	NPA	RESP 10	Direct	5 mo	M
GTS0900134	NPA	RESP 11	Direct	3 mo	F
GTS0539892	Luki	RESP 12	Direct	7 mo	M
GTS0419198	NPA	RESP 13	Direct	3 mo	F
GTS0553505	NPA	RESP 14	Direct	5 mo	M
GTS0923959	NPA	RESP 15	Direct	2 yr 5 mo	F
GTS0656735	NPA	RESP 16	Direct	10 mo	M
GTS1245470-1 (Cell culture)		RESP 17	Direct	Unknown	Unknown
GTS1245470-2 (Cell culture)	-	RESP 18	Direct	Unknown	Unknown
GTS1245470-3 (Cell culture)	-	RESP 19	Direct	Unknown	Unknown
GTS1245470-4 (Cell culture)	-	RESP 20	Direct	Unknown	Unknown
GTS0544993	-	RESP 21	Direct	2yr 6 mo	M
GTS0703742	-	RESP 22	Direct	1 yr	M

NPA = nasopharyngeal aspirate; yr = year; mo = month; d = day

A.2 SOUTH AFRICAN STOOL SPECIMENS

Laboratory accession number	Sample type	Specimen ID number	Detection method	Patient details	
				Age	Sex
GTS0765295	Stool	ST 1	Direct	16 d	F
GTS0763743	Stool	ST 2	Direct	4 mo	F
GTS0745180	Stool	ST 3	Direct	61 d	M
GTS0778490	Stool	ST 4	Direct	1 yr 4 mo	M
GTS0773479	Stool	ST 5	Direct	2 yr 7 mo	M
GTS0775429	Stool	ST 6	Direct	7 mo	F
GTS0770678	Stool	ST 7	Direct	12 d	M
GTS0666775	Stool	ST 8	Direct	93 d	M
GTS0785817	Stool	ST 9	Direct	93 d	M
GTS0785090	Stool	ST 10	Direct	9 mo	M
GTS0788115	Stool	ST 11	Direct	12 mo	F
GTS0390156	Stool	ST 12	Direct	1 yr 7 mo	M
GTS0370595	Stool	ST 13	Direct	3 yr	M
GTS0395192	Stool	ST 14	Direct	2 yr	F
GTS0391904	Stool	ST 15	Direct	1 yr 8 mo	M
GTS0447931	Stool	ST 16	Direct	93 d	F
GTS0430974	Stool	ST 17	Direct	7 d	F
GTS0507801	Stool	ST 18	Direct	Unknown	Unknown
GTS0517973	Stool	ST 19	Direct	11 mo	F
GTS0410109	Stool	ST 20	Direct	93 d	M
VGU707/07	Stool	ST 21	Direct	Unknown	Unknown
	Stool	ST 22	Direct	Unknown	Unknown

yr = year; mo = month; d = day

A.3 ADENOVIRUS POSITIVE WATER SAMPLES

Date of collection	Sample identification	Source of collection	Detection method
1/9/2006	K19	River water	Cell
2/6/2006	K19	River water	Direct
2/27/2006	K19	River water	Cell
3/13/2006	K19	River water	Direct
3/27/2006	K19	River water	Cell
5/29/2006	RWS2	Untreated water	Direct
4/6/2006	Viederhof	Irrigating water	Direct
6/5/2006	N13	Cruise ship water	Direct
6/5/2006	N19	Cruise ship water	Direct
6/5/2006	N20	Cruise ship water	Direct
6/5/2006	N22	Cruise ship water	Direct
6/12/2006	B12	Treated drinking water	Direct
6/12/2006	N19	Cruise ship water	Cell
6/19/2006	RWS2	Untreated water	Direct
6/26/2006	K19	River water	Direct
6/26/2006	K19	River water	Cell
7/10/2006	K19	River water	Direct
7/10/2006	RWS2	Untreated water	Direct
7/17/2006	K19	River water	Direct
7/24/2006	K19	River water	Direct
7/31/2006	K19	River water	Direct
8/14/2006	B2	Treated drinking water	Direct
8/14/2006	K19	River water	Direct
8/21/2006	K19	River water	Direct
8/21/2006	RWS2	Untreated water	Direct
9/18/2006	K19	River water	Direct
10/2/2006	K19	River water	Direct
10/30/2006	K19	River water	Direct
11/27/2006	B2	Treated drinking water	Direct
12/11/2006	K19	River water	Direct
1/8/2007	K19	River water	Direct
1/16/2007	WPO020	Untreated water	Direct
1/29/2007	K19	River water	Direct
2/5/2007	K19	River water	Direct

A.3 ADENOVIRUS POSITIVE WATER SAMPLES (continued)

Date of collection	Sample identification	Source of collection	Detection method
1/30/2007	Z.R	Irrigation water	Direct
2/26/2007	K19	River water	Direct
3/26/2007	K19	River water	Direct
4/2/2007	A19	Treated drinking water	Direct
4/2/2007	K19	River water	Direct
4/16/2007	K19	River water	Direct
4/17/2007	WPO020	Untreated water	Direct
5/14/2007	K19	River water	Direct
5/21/2007	K19	River water	Direct
6/11/2007	K19	River water	Direct
7/4/2000	MVFin	Treated water	Direct
7/9/2007	Z-Blend	Treated water	Direct
7/9/2007	Z-Blend	Treated water	Cell
7/9/2007	K19	River water	Cell
7/16/2007	K19	River water	Direct
7/2/2007	N20	Cruise ship water	Direct
7/30/2007	K19	River water	Direct
8/6/2007	K19	River water	Direct
8/13/2007	K19	River water	Direct
7/2/2007	N19	Cruise ship water	Cell
7/2/2007	N20	Cruise ship water	Cell
8/20/2007	K19	River water	Direct
9/3/2007	K19	River water	Direct
10/8/2007	K19	River water	Direct
10/15/2007	K19	River water	Direct
10/29/2007	RWS2	Untreated water	Direct
11/2/2007	MISLK	Irrigating water	Direct
11/5/2007	K19	River water	Direct
11/12/2007	K19	River water	Direct
1/7/2008	K19	River water	Cell
1/21/2008	K19	River water	Cell

A.4. KENYAN ADENOVIRUS POSITIVE STOOL SPECIMENS

Lab No	Age	Hospital /Clinic	Specimen type	STATUS	
				HIV	Diarrhoeal
118	6 mo	Kibera	Stool	Unknown	D
157	-	Nyumbani	Stool	+ve	D
158	-	Nyumbani	Stool	+ve	D
161	-	Nyumbani	Stool	+ve	D
164	-	Nyumbani	Stool	+ve	D
165	-	Nyumbani	Stool	+ve	D
171		Nyumbani	Stool	+ve	ND
174	-	Nyumbani	Stool	+ve	D
176	-	Nyumbani	Stool	+ve	D
178	-	Nyumbani	Stool	+ve	ND
181	-	Nyumbani	Stool	+ve	D
185	-	Nyumbani	Stool	+ve	D
186	-	Nyumbani	Stool	+ve	ND
189	-	Nyumbani	Stool	+ve	D
190	-	Nyumbani	Stool	+ve	ND
192	-	Nyumbani	Stool	+ve	D
197	-	Nyumbani	Stool	+ve	D
200	-	Nyumbani	Stool	+ve	D
201	-	Nyumbani	Stool	+ve	D
204	-	Nyumbani	Stool	+ve	ND
205	-	Nyumbani	Stool	+ve	D
207	-	Nyumbani	Stool	+ve	D
209	-	Nyumbani	Stool	+ve	ND
210	-	Nyumbani	Stool	+ve	D
211	-	Nyumbani	Stool	+ve	D
212	-	Nyumbani	Stool	+ve	D
214		Nyumbani	Stool	+ve	D
216	-	Nyumbani	Stool	+ve	ND
218	-	Nyumbani	Stool	+ve	D
226	-	Nyumbani	Stool	+ve	D
227	-	Nyumbani	Stool	+ve	D
228	-	Nyumbani	Stool	+ve	ND
231	-	Nyumbani	Stool	+ve	D
232	-	Nyumbani	Stool	+ve	D
233	-	Nyumbani	Stool	+ve	D
234	-	Nyumbani	Stool	+ve	D

D = Diarrhoeal; ND = Non-diarrhoeal.

A.4. KENYAN ADENOVIRUS POSITIVE STOOL SPECIMENS (continued)

Lab No	Age	Hospital /Clinic	Specimen type	STATUS HIV	Diarrhoeal
235	-	Nyumbani	Stool	+ve	D
236	-	Nyumbani	Stool	+ve	ND
237	-	Nyumbani	Stool	+ve	D
239	-	Nyumbani	Stool	+ve	D
245	-	Nyumbani	Stool	+ve	D
247	-	Nyumbani	Stool	+ve	D
255	-	Nyumbani	Stool	+ve	D
256	-	Nyumbani	Stool	+ve	D
261	-	Nyumbani	Stool	+ve	D
267	-	Nyumbani	Stool	+ve	ND
460	2 yrs	Ngong	Stool	Unknown	D
471	1 yr 3 mo	Kibera	Stool	Unknown	D
473	4 yrs 1 mo	Kibera	Stool	Unknown	D
485	10 mo	Kibera	Stool	Unknown	D
488	1 yr 6 mo	Kibera	Stool	Unknown	D
491	5 yrs	Ngong	Stool	Unknown	D
505	1 yrs 3 mo	Kibera	Stool	Unknown	D
506	7 mo	Kibera	Stool	Unknown	D
510	3 yrs 1 mo	Kibera	Stool	Unknown	D
512	2 yrs 4 mo	Ngong	Stool	Unknown	D
513	4 yrs	Ngong	Stool	Unknown	D
514	5 yrs	Ngong	Stool	Unknown	D
515	4 yrs	Ngong	Stool	Unknown	D
517	-	St Odilias	Stool	Unknown	D
518	2 yrs	St Odilias	Stool	Unknown	D
519	11 mo	St Odilias	Stool	Unknown	D
521	2 yrs	Kibera	Stool	Unknown	D
531	4 yrs	Kibera	Stool	Unknown	D
537	2 yrs	Kibera	Stool	Unknown	D
540	1 yr	Kibera	Stool	Unknown	D
544	1 yr	Embulbul	Stool	Unknown	D
546	4 yrs	St Odilias	Stool	Unknown	D
549	6 yrs	Maua	Stool	Unknown	D
550	5 yrs	Maua	Stool	Unknown	D
551	9 mo	Maua	Stool	Unknown	D

D = Diarrhoeal; ND = Non-diarrhoeal.

A.4. KENYAN ADENOVIRUS POSITIVE STOOL SPECIMENS (continued)

Lab No	Age	Hospital /Clinic	Specimen type	STATUS	
				HIV	Diarrhoeal
552	2 yrs 6 mo	Maua	Stool	Unknown	D
554	5 yrs	Maua	Stool	Unknown	D
556	1 yr	Maua	Stool	Unknown	D
557	6 mo	Maua	Stool	Unknown	D
558	7 mo	Ngong	Stool	Unknown	D
581	8 mo	Kibera	Stool	Unknown	D
682	4 yrs	Embul-bul	Stool	Unknown	D
733	5 mo	Maua	Stool	Unknown	D
735	2 mo	Maua	Stool	Unknown	D
736	1 yr	Maua	Stool	Unknown	D

D = Diarrhoeal; ND = Non-diarrhoeal.

APPENDIX B

B.1: **Magwalivha M**, Wolfaardt M, Taylor MB. Molecular characterisation of human adenoviruses from treated and untreated drinking water in Gauteng. [Presentation]. Faculty day, Faculty of Health Sciences, University of Pretoria 14-15 August 2007, BMS Building, Prinshof Campus, Pretoria.

Human adenoviruses (HAdVs) are non-enveloped DNA viruses, currently comprising 51 serotypes which are divided into six species (designated A to F). The HAdVs are associated with a number of diseases affecting respiratory, urinary, gastrointestinal tracts and the eye. Human adenoviruses are ubiquitous in the environment resulting in the possible contamination of treated and untreated drinking-water supplies by faecally contaminated water. The presence of adenoviruses (AdV) in water sources are considered important as they are exceptionally resistant to selected water treatment processes allowing for prolonged survival in the environment. Adenoviruses have also shown to be up to 60 times more resistant to UV irradiation than RNA viruses such as enteroviruses and hepatitis A virus. As part of ongoing surveillance of treated and untreated water sources for enteric viruses, 350 water samples from Gauteng region were tested, by a nested polymerase chain reaction (nPCR), for HAdVs. Of these samples, 28 (8%) untreated water and 3 (0.86%) of the treated water samples were positive for HAdVs. Isolates were characterised by amplifying and sequencing a conserved region of the hexon protein. Nucleotide sequences were compared, by pairwise analysis, to sequences of all HAdVs available in the GenBank database. Sequence analysis of 20/31 (64.5%) of the isolates identified HAdV-41 (species F) as the predominant (12/20; 60%) strain detected. This is of great significance as HAdV-F is the most common enteric AdV associated with paediatric gastroenteritis. HAdV-D was detected in 3/20 (15%) of the samples, while species HAdVs -A, -B, and –C were detected in one sample (5%) each. The predominance of HAdV-F in water sources in the Gauteng region confirms previous findings, and highlights the risk to communities using faecally contaminated water for domestic purposes.

B.2: **Magwalivha M**, Wolfaardt M, Taylor MB. Molecular characterisation of human adenoviruses from treated and untreated drinking water in Gauteng. [Poster]. The 2007 MCBG symposium. Department of Medical Virology, University of Pretoria/Molecular and Cell Biology Group of South Africa. 17 October 2007. HW Snyman North, Faculty of Health Sciences, University of Pretoria, Pretoria

Human adenoviruses (HAdVs) are non-enveloped DNA viruses, currently comprising 51 serotypes which are divided into six species (designated A to F). The HAdVs are associated with a number of diseases affecting respiratory, urinary, gastrointestinal tracts and the eye. Human adenoviruses are ubiquitous in the environment resulting in the possible contamination of treated and untreated drinking-water supplies by faecally contaminated water. The presence of adenoviruses (AdV) in water sources are considered important as they are exceptionally resistant to selected water treatment processes allowing for prolonged survival in the environment. Adenoviruses have also shown to be up to 60 times more resistant to UV irradiation than RNA viruses such as enteroviruses and hepatitis A virus.

As part of ongoing surveillance of treated and untreated water sources for enteric viruses, 350 water samples from Gauteng region were tested, by a nested polymerase chain reaction (nPCR), for HAdVs. Of these samples, 28 (8%) untreated water and 3 (0.86%) of the treated water samples were positive for HAdVs. Isolates were characterised by amplifying and sequencing a conserved region of the hexon protein. Nucleotide sequences were compared, by pair wise analysis, to sequences of all HAdVs available in the GenBank database. Sequence analysis of 20/31 (64.5%) of the isolates identified HAdV-41 (species F) as the predominant (12/20; 60%) strain detected. This is of great significance as HAdV-F is the most common enteric AdV associated with paediatric gastroenteritis. HAdV-D was detected in 3/20 (15%) of the samples while species HAdVs -A, -B, -C, and –E were detected in one sample (5%) each. The predominance of HAdV-F in water sources in the Gauteng region confirms previous findings, and highlights the risk to communities using faecally contaminated water for domestic purposes.

B3: Magwalivha M, Wolfaardt M, Kiulia N, van Zyl W, Mwenda J, **Taylor MB**. Molecular characterisation of adenoviruses from Kenyan children with diarrhoea [Presentation]. HIV and AIDS Research Indaba 26-27 February 2009 Moot Court Room, Faculty of Law, University of Pretoria.

Introduction: Human adenoviruses (HAdVs) cause a wide range of clinical syndromes and have increasingly been recognized as important pathogens in immunocompromised individuals. HAdVs are classified in seven species, A to G, comprising 52 serotypes and organotropism and virulence are type related. Precise typing of HAdVs is therefore essential for epidemiological surveillance and the understanding of infection chains. In developing countries diarrhoea is a major cause of morbidity and mortality and after rotaviruses HAdVs are considered to be the second most important cause of viral infantile diarrhoea. There are no data on the HAdVs associated with diarrhoea in paediatric patients in Kenya.
Methods: In this study 280 diarrhoeal stool specimens from children <10 years of age in urban and rural areas in Kenya were screened for HAdVs using a nested polymerase chain reaction and the HAdVs genotyped by sequence analysis of a conserved hexon gene fragment.
Results: HAdVs were detected in 104/280 (37.1%) of the stool specimens tested: 45/104 (43.3%) from HIV-seropositive children from an urban hospice; 25/96 (26%) from children of unknown HIV status from the same urban area as the hospice; and 34/80 (42.5%) from children of unknown HIV status in a rural area. Nucleotide sequence analysis of 58/105 (55.2%) strains identified HAdV-D as the predominant species amongst the HAdV strains from children with diarrhoea in the urban setting. In the rural setting species A and F were identified with species F, commonly associated with gastroenteritis, comprising 60% of the strains.
Conclusion: This study provides valuable new data on the prevalence and distribution of HAdV genotypes in diarrhoeal stool specimens in Africa and highlights the necessity for further investigations, especially in immunocompromised children.

B4: **Magwalivha M**, Wolfaardt M, Kiulia NM, van Zyl WB, Mwenda JM, Taylor MB. High prevalence of species D human adenoviruses in faecal specimens from Kenyan children with diarrhoea [Poster]. Faculty Day, Faculty of Health Sciences, University of Pretoria 18-19 August 2009: HW Snyman Building, Pretoria.

Introduction: Human adenoviruses (HAdVs) cause a wide range of clinical syndromes and have increasingly been recognized as important pathogens in immunocompromised individuals. HAdVs are classified in seven species, A to G, comprising 52 serotypes and organotropism and virulence are type related. Precise typing of HAdVs is therefore essential for epidemiological surveillance and the understanding of infection chains. In developing countries diarrhoea is a major cause of morbidity and mortality and after rotaviruses HAdVs are considered to be the second most important cause of viral infantile diarrhoea. There is very little data on the prevalence and distribution of HAdV serotypes associated with diarrhoea in paediatric patients in Kenya.

Methods: In this study 280 diarrhoeal stool specimens from children <10 years of age in urban and rural areas in Kenya were screened for HAdVs using a nested polymerase chain reaction and the HAdVs genotyped by sequence analysis of a conserved hexon gene fragment.

Results: HAdVs were detected in 104/280 (37.1%) of the stool specimens tested: 45/104 (43.3%) from HIV-seropositive children from an urban hospice; 25/96 (26%) from children of unknown HIV status from the same urban area as the hospice; and 34/80 (42.5%) from children of unknown HIV status in a rural area. Nucleotide sequence analysis of 61/104 (58.7%) strains identified HAdV-D as the predominant species amongst the HAdV strains from children with diarrhoea in the urban setting. In the rural setting species A and F were identified with species F, commonly associated with gastroenteritis, comprising 60% of the strains.

Conclusion: This study provides valuable new data on the prevalence and distribution of HAdV genotypes in diarrhoeal stool specimens in Africa and highlights the necessity for further investigations, especially in immunocompromised children.

APPENDIX C

Journal of Medical Virology 82:77–84 (2010)

High Prevalence of Species D Human Adenoviruses in Fecal Specimens From Urban Kenyan Children With Diarrhea

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Human adenoviruses (HAdVs) cause a wide range of clinical syndromes and are classified in seven species, A–G, comprising 52 serotypes. HAdV-A31, -F40, and -F41 have been associated with diarrhea in infants and young children. In developing countries gastroenteritis is a major cause of morbidity and mortality in children and, in comparison to rotaviruses, there are no data on the HAdVs associated with diarrhea in pediatric patients in Kenya. This study investigates the prevalence and genotypes of HAdVs in 278 stool specimens (211 diarrheal; 67 non-diarrheal) from children $\leq$14 years of age in urban and rural areas in Kenya. Stool specimens were screened for HAdVs using a nested polymerase chain reaction and the HAdVs genotyped by sequence analysis of a conserved hexon gene fragment. HAdVs were detected in 104/278 (37.4%) of the stool specimens: 35/43 (81.4%) of diarrheal and 10/61 (16.4%) of non-diarrheal stool specimens from children in an urban hospice; 25/94 (26.6%) of diarrheal specimens from urban children and 34/80 (42.5%) of diarrheal specimens from children in a rural area. Species D HAdVs were identified as the most prevalent HAdV species in diarrheal stool specimens from urban children comprising 18/37 (48.6%) of the strains identified. In contrast HAdV species F predominated in pediatric diarrheal specimens from the rural area, being identified in 7/16 (43.8%) of the characterized strains. This study provides valuable new data on the prevalence and distribution of HAdV genotypes in diarrheal stool specimens in Kenya and Africa, and highlights the necessity for further investigations. ***J. Med. Virol. 82:77–84, 2010.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: gastroenteritis; rural; HIV-seropositive

INTRODUCTION

Human adenoviruses (HAdVs) are ubiquitous and are recognized as the etiologic agent of a variety of diverse clinical syndromes [Erdman et al., 2002; Rezig et al., 2006]. HAdVs can affect the respiratory, urinary, gastrointestinal tracts, and the eyes [Horwitz, 2001]. HAdVs are endemic in pediatric populations [Hierholzer, 1992] and are frequently isolated from the pharynx and stool of asymptomatic children [Wadell, 1999]. Adenoviruses (AdVs) have been increasingly recognized as a major problem in immunocompromised hosts where infections have the potential to cause fatal disseminated disease [Leen and Rooney, 2004; Kroes et al., 2007]. Exposure to and transmission of HAdVs are possible by a variety of routes. Person-to-person contact plays a major role in the transmission of AdVs and depending on the nature of illness, this can include fecal–oral, oral–oral and hand–eye contact transmission, as well as indirect transfer through contact with contaminated surfaces or shared utensils [Puig et al., 1994; Chaperon et al., 2000; World Health Organization (WHO), 2004; Boone and Gerba, 2007]. AdVs are highly infectious and local outbreaks are common [Van et al., 1992; Horwitz, 1996; Taylor et al., 1997; Rezig et al., 2006].

Grant sponsors: National Research Foundation, South African Co-operation Fund for Scientific Research and Technological Developments (in a collaborative project between: Department of Medical Virology, University of Pretoria, South Africa and the Institute of Primate Research (IPR) & Kenya Medical Research Institute (KEMRI) (Kenya)); Grant sponsor: South African National Health Laboratory Service Research Trust.

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Accepted 2 September 2009
DOI 10.1002/jmv.21673
Published online in Wiley InterScience (www.interscience.wiley.com)

On the basis of neutralization assays 52 serotypes of HAdVs, which are grouped into seven species (A–G), have been distinguished [Benkö et al., 2005; Jones et al., 2007]. Pathogenicity varies according to species and serotype, and organ specificity and disease patterns appear to be serotype dependent [Horwitz, 2001; Madisch et al., 2006; Larrañaga et al., 2007]. Lower respiratory tract infection has been associated with HAdV-B3, -B7, -B21, and -E4, while less severe upper respiratory tract infection is often ascribed to HAdV-C1, -C2, -B3, -C5, and -B7 infection [Madisch et al., 2006; Echavarría, 2008]. Persistent infections of kidneys and hemorrhagic cystitis are caused by HAdV-B11, -B34, and -B35 and are the most common HAdVs isolated from the urine of patients with acquired immunodeficiency syndrome (AIDS) and bone marrow transplant recipients [Wadell, 1999]. In addition to acute disease, the species C AdVs establish persistent infections in immunocompetent hosts characterized by intermittent viral excretion. Although primary infections affect the respiratory tract, HAdV-C display prolonged fecal excretion for months, and even years after virus is no longer detected in nasopharyngeal washings [Garnett et al., 2002]. All of the recently described species D HAdVs (HAdV-43 to HAdV-49) were first isolated from AIDS patients [Lord et al., 2000]. Species D HAdVs, namely HAdV-D20, -D22, -D26, -D28, -D29, -D30, -D32, -D37, -D43 to D46, and untyped species D viruses have been detected in stool specimens from AIDS patients with pneumonia and/or diarrhea [Hierholzer, 1992]. Among HAdV species D, serotypes HAdV-D8, -D19, and -D37 have been reported to be responsible for sporadic cases, as well as outbreaks of severe epidemic keratoconjunctivitis in particular in dry climates or in densely populated areas. These strains are frequently associated with nosocomial infections [Jernigan et al., 1993]. The classic enteric HAdVs, that is, species F type-40 and -41, are considered to be the second most important cause of childhood gastrointestinal illness worldwide [Avery et al., 1992; Scott-Taylor and Hammond, 1992], but unlike rotavirus they show no seasonal variation in their incidence [Wood, 1988; de Jong et al., 1993; Ison, 2006]. HAdV type-A31, -F40, and -F41 have been associated with severe gastroenteritis in infants and young children [Madisch et al., 2006] and these enteric HAdVs are a common cause of diarrhea in child care settings [Van et al., 1992; Taylor et al., 1997; Gompf and Oehler, 2005]. In AIDS patients species D HAdVs have been found in association with gastroenteritis while in other immunocompromised patients HAdV-related gastroenteritis is usually due to type-A31, -C2, -F40, and -F41 [Hierholzer, 1992]. The newly described HAdV-52 (proposed to be species G) was detected in a patient presenting with gastroenteritis [Jones et al., 2007].

Diarrheal disease is a major cause of morbidity and mortality in children, especially in developing countries [Bern and Glass, 1994]. In comparison to rotaviruses [Steele and Ivanoff, 2003], studies on the prevalence of HAdVs in acute pediatric gastroenteritis in Africa are limited. In these studies, HAdVs have been detected in stool specimens using either species F specific AdV type-40 and -41 (AdV40/41) immuno- [Marx et al., 1998; Moore et al., 1998, 2000; Basu et al., 2003; Moyo et al., 2007; Sdiri-Loulizi et al., 2008] and hybridization assays [Kidd et al., 1985] or group specific enzyme immunoassays (EIAs) [Audu et al., 2002; Rossouw, 2004; Fodha et al., 2006] and immunochromatographic assays [Weitzel et al., 2007]. Except for isolated studies [Moore et al., 1998; Rossouw, 2004; Silva et al., 2008] the characterization of strains has predominantly focused on HAdV-F40 and -F41 strains [Kidd et al., 1986, 1996]. There are no significant data on the prevalence of HAdVs in children with diarrhea in Kenya. In a study on acute gastroenteritis in early childhood in Kenya where AdV infection was determined by seroconversion studies, no AdV infection was noted [Mutanda, 1980]. There is only a single study on HAdVs in stool specimens from pediatric patients in Kenya. In this study HAdVs were detected in 22% (11/49) of the stool specimens using an AdV group-specific latex-agglutination assay and no further characterization was done [Forbes et al., 2004]. There is also a dearth of data on the prevalence of human immunodeficiency virus (HIV) infection in infants and young children in Kenya. Children <14 years of age years comprise 42.2% of the total population (~37,953,840) [Central Intelligence Agency, 2009]. In 2001 there were an estimated 110,000 (0.7%) to 160,000 (1%) children <14 years of age living with HIV which increased to an estimated 130,000 (0.8%) – 180,000 (1.1%) children <14 years of age living with HIV in 2007 [UNAIDS, 2008].

The objective of the current study was: (i) to determine the prevalence of HAdVs in stool specimens from infants and children with diarrhea in Kenya, and (ii) to characterize and determine the HAdV genotypes in the study population. Characterization and typing is important in order to establish the epidemiology of virus infections, especially in closed settings where groups of individuals, by virtue of increased exposure or impaired immunity, are at increased risk of gastroenteritis [Bern and Glass, 1994; Kroes et al., 2007]. The African Rotavirus Network and their associates play an important role in elucidating the causes of viral gastroenteritis in Africa. These epidemiological data are required to better understand the etiological agents of viral gastroenteritis in Africa so as to formulate policies for the prevention and control of diarrheal disease.

PATIENTS AND METHODS

Ethical Approval

This study was approved by the Kenyatta National Hospital Ethics and Research Committee (KNH-ERC) and the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, South Africa: Number S27/2008.

Study Population and Specimen Collection

During the period February 1999 to September 2005, as part of an ongoing public health initiative coordinated by the African Rotavirus Network to document rotavirus infection and epidemiology in Kenya, stool specimens were collected from children ≤14 years of age. Stool specimens included 228 diarrheal stool specimens from children younger than 10 years of age who attended urban clinics in and around Nairobi and 135 diarrheal stool specimens from children younger than 10 years of age with severe diarrhea who were either hospitalized or attended the out-patient department of the Maua Methodist Hospital in the Meru North District. The HIV status of the children who attended the urban clinics and rural Maua Methodist Hospital is unknown. In addition, 110 stool specimens (43 diarrheal; 67 non-diarrheal) were collected from HIV seropositive children <14 years of age at the Nyumbani Hospice and Nyumbani community-based foster care program. Of the 473 stool specimens 278 (58.8%), of which 104 (43 diarrhea; 61 non-diarrheal) were from the urban hospice, 94 from the urban clinics and 80 from the rural setting, were available for adenoviral analysis (Table I). These samples had all previously been tested for rotaviruses [Kiulia et al., 2006, 2009] and astroviruses [Kiulia et al., 2007].

Specimen Preparation

A 10% stool suspension was prepared by adding a pea-sized portion of the stool specimen to 9 ml sterile distilled water in a 10 ml polypropylene tube (Sterilin, Barloworld Scientific Ltd, Stone Staffordshire, UK) and vortexed for 60 sec to give a well-defined suspension. The stool suspension was aliquoted and stored at −20°C until further processing.

Nucleic Acid Extraction

Genomic viral nucleic acid was extracted from 200 µl of the stool suspension using the MagNA Pure LC Total Nucleic Acid Isolation kit (Roche Diagnostics GmbH, Mannheim, Germany) in a MagNA Pure LC instrument (Roche), following the manufacturer's instructions. Purified nucleic acid was aliquoted and stored at −70°C until use. A suspension of cell cultured HAdV-2 (ATCC VR-1079 AS/Rab) and ultrapure water were included in all reactions as positive and negative controls, respectively.

Detection of Adenoviruses by PCR

Published primer pairs ADHEX1F and ADHEX2R, and ADHEX2F and ADHEX1R which amplify a conserved region of the AdV hexon gene fragment and which reportedly detect all known serotypes of HAdVs [Casas et al., 2005] were used in a nested polymerase chain reaction (nested PCR), as described by Avellón et al. [2001] and modified by Van Heerden et al. [2004]. To exclude the possibility of cross-contamination, reagents for the PCR were prepared in rooms separate from those used for the initial processing of the specimens and for the analysis of the amplicons. Unless stated to the contrary the reagents were from Promega Corp., Madison, WI. In brief the first round reaction (primary amplification): 10 µl of extracted nucleic acid was added to 40 µl of a reaction mixture containing 5× green Go Taq flexi buffer (5 µl), 25 mM $MgCl_2$ (8 µl), 10 mM dNTPs (2 µl), 20 pmol/µl primer ADHEX1F (0.5 µl), 20 pmol/µl primer ADHEX2R (0.5 µl), 5 U/µl Go Taq DNA polymerase (0.5 µl), and nuclease-free water (22 µl). The PCR cycling parameters consisted of an initial denaturing step at 94°C for 2 min, followed by denaturation at 93°C for 1 min, annealing at 50°C for 1 min, and extension at 70°C for 1 min for 30 cycles. Nested PCR (second amplification): 1 µl of first round amplification product was added to 49 µl of a reaction mixture containing 5× green Go Taq flexi buffer (5 µl), 25 mM $MgCl_2$ (8 µl), 10 mM dNTPs (1.5 µl), 20 pmol/µl primer ADHEX2F (0.5 µl), 20 pmol/µl primer ADHEX1R (0.5 µl), 5 U/µl Go Taq DNA polymerase (0.5 µl), and nuclease-free water (33 µl). The cycling parameters consisted of an initial denaturing step at 94°C for 2 min followed by denaturation at 93°C for 1 min,

TABLE I. HAdV Prevalence by Age in Stool Specimens From Kenyan Children From Urban and Rural Settings

	Urban setting						Rural setting	
	HIV seropositive				HIV status unknown		HIV status unknown	
	Non-diarrheal		Diarrheal		Diarrheal		Diarrheal	
Age range	No.[a]	% Pos[b]	No.	% Pos	No.	% Pos	No.	% Pos
0–12 months	—[c]	—	—	—	33	27.3	29	58.6
13–24 months	—	—	—	—	29	24.1	17	23.5
25–36 months	—	—	—	—	10	10	9	22.2
3–5 years	—	—	—	—	16	43.8	14	71.4
>5 years	—	—	—	—	3	0	1	100
Unknown	61	16.4	43	81.4	3	33.3	10	0
Total	61	16.4	43	81.4	94	26.6	80	42.5

[a]Number of specimens tested.
[b]Percentage of specimens HAdV positive.
[c]—, age groups not specified (average age 6.3 years; range 5 months to 14 years).

annealing at 54°C for 1 min, and extension at 72°C for 1 min for 30 cycles, with a final extension step at 72°C for 7 min. The PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining and UV illumination using a 100 bp marker (O'GeneRuler, Fermentas Life Sciences, Burlington, Ontario) to determine amplicon size.

Sequencing of PCR Amplicons

Amplicons (475 bp) of a randomly selected subset of available specimens (n = 60) of HAdVs were extracted from gel bands and purified using the DNA Clean & Concentrator™-25 kit (Zymo Research, Orange, CA). The amplicons were sequenced in both directions using the ABI PRISM BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and primers ADHEX2F and ADHEX1R.

Sequence Analysis and Genotyping

Sequence data from both strands were edited in BioEdit Sequence Alignment Editor (V.7.0.9.0) and aligned using MAFFT Version 6 (https://align.bmr.kyushu-u.ac.jp/mafft/online/server/). The nucleotide sequences of the HAdV strains were compared with AdV sequences present in GenBank by using the BLAST-N program (version 2.2.18) to search for the most similar strains available. To confirm the genotyping and speciation results phylogenetic trees were constructed from the nucleotide sequence alignments of the Kenyan strains and the most closely related nucleotide sequences published in GenBank using the Neighbor-joining (NJ) method of the MEGA software version 4 [Tamura et al., 2007]. For NJ, a distance matrix calculated from the aligned sequences by Kimura two-parameters formula was used, and for determining the reliability of tree topology, bootstrap analysis was carried out on 1,000 replicates. The accession numbers of the published HAdV nucleotide sequences used in the phylogenetic analysis included: HAdV-1, AB330082; HAdV-2, AJ293904; HAdV-3, AF542129; HAdV-5, EU867493.1; HAdV-6, AB330087; HAdV-7, AB330088; HAdV-12, AB330093; HAdV-18, AB330099; HAdV-21, AB330102; HAdV-22, AB330103; HAdV-27, AB330108; HAdV-31, 330112.1; HAdV-32, AB330113; HAdV-34, AB052911; HAdV-40, AB330121; HAdV-41, A819814; HAdV-42, AB330123.

Statistical Analyses

Demographic data were summarized using descriptive statistics. The exposed groups from the rural and urban areas were compared with respect to HAdV positivity using the Mantel–Haenszel Chi-square test (http://www.openepi.com/TwobyTwo/TwobyTwo.htm).

RESULTS

The HAdV prevalence in the different settings in relation to the children's age distribution is summarized in Table I. Overall HAdVs were detected in 104/278 (37.4%) of the stool specimens (94/217 [43.3%] diarrheal; 10/61 [16.4%] non-diarrheal) tested. In the urban hospice setting 45/104 (43.3%) of the stool specimens from HIV-seropositive Kenyan children (average age 6.3 years; range 5 months to 14 years) tested positive for HAdV. In this setting there was a highly significant association between HAdV and diarrhea compared to no diarrhea (35/43 (81.4%) versus 10/61 (16.4%); $P < 0.0000001$). Twenty-five (26.6%) of the 94 diarrheal stool specimens from children of unknown HIV status (average age 2.1 years; range 2 months to 10 years) attending clinics in the same urban area as the hospice tested positive for HAdV. In this group of children HAdV was detected in diarrheal stool specimens from children in all the age groups tested (Table I). The prevalence rates of HAdV infection in children aged 0–12 months and 13–24 months were similar, 27.3% and 24.1%, respectively. Although a HAdV prevalence rate of 43.8% was recorded for the children between three and 5 years of age, due to the low number of specimens this prevalence rate did not differ significantly to that recorded for the younger age groups. In the urban setting the association between HAdV positive diarrheal stools and the HIV-seropositive status of the children in the hospice environment was highly significant when compared to HAdV positive diarrheal stools from children of unknown HIV status (35/43 [81.4%] vs. 25/94 [26.6%]; $P < 0.0000001$). In the rural setting 34/80 (42.5%) of the stool specimens from children of unknown HIV status (average age 2 years; range 2 months to 6 years) with diarrhea were HAdV positive. For the age groups 0–36 months the HAdV prevalence rates in stool specimens from the children from the rural setting were similar to, and did not differ significantly from, those recorded for stool specimens from urban children of unknown HIV status (Table I). However in the 3–5 year old age group a child of unknown HIV status from a rural area with gastroenteritis was more likely to have an HAdV infection compared to a child of unknown HIV status from an urban area with gastroenteritis (10/14 [71.4%] vs. 7/16 [43.8%]; $P = 0.1335$). When the overall HAdV prevalence in diarrheal stool specimens from children with unknown HIV status from the urban and the rural settings were compared, a significant association was noted between HAdV positive diarrheal specimens and the rural location of the children (25/94 [26.6%] vs. 34/80 [42.5%]; $P < 0.02765$).

On the basis of pairwise comparisons and phylogenetic analysis of a conserved hexon genomic fragment 56 HAdV strains could be genotyped while possible intermediate types were detected in four HAdV strains (Table II; phylogenetic tree not shown). No species E or G were detected among the HAdVs genotyped from either the rural or the urban settings. From Table II it is evident that species D was more prevalent among the HAdV strains from patients with diarrhea in the urban setting, comprising 47.1% of the typed strains from diarrheal stool specimens from the HIV-seropositive children and 50% of the typed strains from diarrheal stool specimens from the children of unknown HIV

TABLE II. Distribution of HAdV Species and Genotypes by Age in HAdV Positive Stool Specimens From Kenyan Children

	Urban setting				Rural setting	
	HIV seropositive		HIV status unknown		HIV status unknown	
Age range	Non-diarrheal (n = 10)	Diarrheal (n = 35)	Diarrheal (n = 25)		Diarrheal (n = 34)	
0–12 months	—[a]	—	1 (C1); 1 (C2); 1 (C5); 1 (D28); 1 (F40)		1 (B3); 1 (C5); 2 (F40); 2 (F41); 2 (F40/41)	
13–24 months	—	—	1 (A12); 1 (C6); 1 (D22); 1 (D28); 2 (D42)		1 (A31); 1 (B3)	
25–36 months	—	—	1 (D22)		1 (F40/41)	
3–5 years	—	—	1 (B:1[7]); 2 (C2); 1 (C6); 2 (D22); 1 (D47)		1 (A18); 1 (B3); 1 (D22); 2 (D26)	
Unknown	1 (A18); 1 (B34); 1 (C2/6); 1 (C5); 2 (C6); 1 (D42)	2 (B:1[7]); 1 (B3); 1 (B21); 2 (C2); 2 (C5); 1 (C6); 1 (D15); 3 (D22); 1 (D24); 1 (D33); 2 (D42)	1 (D42)			
Not typed	3		5		18	

[a]—, age groups not specified (average age 6.3 years; range 5 months to 14 years).

status. In the children of unknown HIV status from the urban clinics species D HAdVs were present in the diarrheal stool specimens from children in all age categories. In the non-diarrheal stool specimens collected in the urban area from HIV-seropositive children species C predominated, comprising 57.1% of the genotyped strains; only one HAdV-D42 strain was identified. In contrast, species A (HAdV-31) and F (HAdV-40 and HAdV-41), were detected among the HAdV strains from patients of unknown HIV status from a rural setting, with species F predominating, comprising 43.8% of the genotyped strains. From Table II it is evident that in the rural setting species A and species F HAdVs were detected more often in diarrheal stool specimens from children in the younger age groups (0–36 months) while the species D viruses were identified from the older children (>3 years).

Genotypes HAdV-D22 and HAdV-D42 were identified in the diarrheal stool specimens from the urban setting, irrespective of the HIV status. The HAdV-D22 strains showed 97.5–99.4% nucleotide sequence identity and 100% amino acid sequence identity to the most closely related strain in GenBank (accession no: AB330103; HAdV-D22 from a nosocomial keratoconjunctivitis epidemic). The nucleotide sequences of two of the HAdV-D42 strains from the urban clinics were 100% identical to each other and to the most closely related strain in GenBank (accession no: AB330123; HAdV-D42 from a nosocomial keratoconjunctivitis epidemic). The remaining HAdV-D42 strains showed 98–99.3% nucleotide sequence identity and 100% amino acid sequence identity to the most closely related strain in GenBank (accession no: AB330123; HAdV-D42).

DISCUSSION

This study represents the first comprehensive investigation into the molecular epidemiology of HAdVs in both urban and rural settings in Kenya and highlights the previously unreported diversity of HAdVs shed in stool specimens from African children.

HAdVs have previously been detected in diarrheal stool specimens from pediatric patients from Africa, predominantly <5 years of age, with prevalence rates varying from 2.6% (HAdV F40/41) in Tanzania [Moyo et al., 2007], 5.5% (3.1% HAdV F40/41) in eastern Tunisia [Fodha et al., 2006], 6.7% in Nigeria [Audu et al., 2002], 7.8% (2% HAdV F40/41) in Botswana [Basu et al., 2003] to 9.8% (3.4% HAdV F40/41) in South Africa [Moore et al., 1998] depending on whether the assays applied were group-specific or species F specific. In a recent study in northern Ghana, which included children up to 12 years of age, an HAdV prevalence of 28% was reported [Reither et al., 2007]. The HIV status of the children in the aforementioned studies was unspecified. In this investigation the HAdV prevalence in diarrheal stool specimens from Kenyan children was much higher than that previously reported from other African regions. In the diarrheal stool specimens from children 5 years of age and younger of unknown HIV

status from the rural and urban setting the HAdV prevalence was 47.8% and 27.3%, respectively, which is significantly greater than in previous studies in Africa. The HAdV prevalence, that is, 26.4%, in the diarrheal stool specimens from children of unknown HIV status in the urban setting (average age 2.1 years; range 2 months to 10 years) was marginally higher than that recorded previously, that is, 22%, for diarrheal stool specimens from children (age range 1 month to 16 years) with acute gastroenteritis presenting at a hospital in the same urban setting [Forbes et al., 2004]. In addition the overall prevalence of HAdVs in the diarrheal stool specimens from children (age range 2 months to 14 years) from different settings in Kenya was 43.3%. This is nearly twice the HAdV prevalence of 22% reported in the only previous study in Kenya for children with acute gastroenteritis presenting at an urban hospital [Forbes et al., 2004]. A similar higher HAdV prevalence rate, that is, 28%, to that reported in this study was noted in acute pediatric diarrhea in northern Ghana [Reither et al., 2007]. In this study and the Ghanaian study the higher HAdV prevalence rate can be ascribed to the more sensitive detection method, namely nested PCR which reportedly detects all described HAdV species, applied in these studies compared to the latex agglutination assay [Forbes et al., 2004] and the group or species specific EIAs [Moore et al., 1998; Audu et al., 2002; Basu et al., 2003; Fodha et al., 2006; Moyo et al., 2007] used in other investigations and the broader age range of the children included in the studies. These data re-affirm the limitations of the immunological-based methods for the detection of enteric HAdVs which were described previously [Moore et al., 2000]. From this study it is evident that the HAdV prevalence data in Africa is dependent on and must be interpreted in accordance with the test method applied and age group of the children investigated.

On the basis of pairwise analysis and phylogenetic analysis of a partial hexon genomic region, it was evident that the predominant species and genotypes of the HAdVs identified from the rural setting were the common enteric HAdV species, namely HAdV-F [types 40/41] and HAdV-A [type 31], which are typically associated with pediatric gastroenteritis [de Jong, 2003; Madisch et al., 2006; Echavarría, 2008]. These enteric HAdVs comprised 72.7% of the strains identified in the diarrheal stool specimens in the children younger than 3 years of age while species D viruses were identified in the older children (>3 years old) (Table II). In contrast, the HAdVs detected in the diarrheal stool specimens from the urban sources were not those typically associated with pediatric gastroenteritis, that is, species A and species F. In the children younger than 2 years of age of unknown HIV status in the urban setting typical enteric HAdVs (species A and F) were only identified in two diarrheal stool samples while species D viruses were detected in all age groups (Table II). In the diarrheal specimens from the HIV-positive children from an urban hospice setting species D HAdVs predominated, comprising 47.1% of the strains identified. Species D HAdVs are reportedly the predominant species in HIV-infected patients [Echavarría, 2008] and gastroenteritis in AIDS patients has been ascribed to species D HAdVs [Hierholzer, 1992]. The high prevalence of species D shedding in the children of unknown HIV status presenting at the urban clinics, that is, 50%, and the identification of species D HAdVs in the children 3 years and older in the rural setting suggests that many of the children may have been HIV-infected. HAdV genotypes 1, 2, 3, and 5, all of which are associated with respiratory infections, were detected in the stool specimens from both the rural and urban areas. HAdV serotypes 1, 2, 3, 5, and 6 have previously been identified in respiratory infections in Nairobi [Hazlett et al., 1988]. Respiratory illness is a common manifestation of HAdV infection in children [de Jong, 2003] and HAdVs that are known to infect respiratory sites can be shed in the feces of an infected person for months [Garnett et al., 2002]. The identification of species B and species C HAdVs, which are often associated with respiratory disease [de Jong, 2003; Echavarría, 2008], in 46% of diarrheal and 71% of non-diarrheal stool specimens from the urban setting (Table II) confirm the fecal shedding of these HAdV species. The possible reason that the non-enteric HAdVs are more prevalent in the stool specimens of HIV seropositive children is that AdVs reportedly set up persistent and generalized infections in hosts with impaired or compromised immune systems [Hierholzer, 1992; Echavarría, 2008].

There are no previous published studies regarding the HAdV prevalence in diarrheal stool specimens from HIV-seropositive children in Africa. The statistically significant high prevalence of HAdVs in diarrheal stool specimens from HIV-seropositive children in the urban hospice setting, that is, 81.4%, and HAdV prevalence rates recorded for children of unknown HIV status in the urban setting, that is, 26.6%, and for children of unknown HIV status in the rural setting, that is, 42.5%, and the high prevalence of species D HAdVs highlights the contribution of HAdVs to the burden of enteric virus infections in Kenyan children younger than 14 years of age. Considering the impact of the HIV pandemic and the fact that children younger than 14 years of age comprise 42.2% of the Kenyan population [Central Intelligence Agency, 2009], the precise typing of HAdV strains in diarrheal specimens is necessitated for the further understanding of persistence, infection chains, and possible therapeutic interventions. Although species D HAdV infection has been associated with diarrhea in AIDS patients [Hierholzer, 1992], the etiological association of the species D HAdVs with the diarrheal episodes reported in this study needs further elucidation as HAdVs are endemic in pediatric populations [Hierholzer, 1992]. The results from this study however suggest that the contribution of HAdVs to the burden of diarrheal disease in Africa may previously have been underestimated. Further investigations into the molecular epidemiology of HAdVs in diarrheal stool

specimens from older children and adults in Africa is therefore warranted.

ACKNOWLEDGMENTS

We wish to thank Dr. S. A. S. Olorunju, Biostatistics Unit, Medical Research Council and Dr. W. D. Cubitt, Gt. Ormond St. Hospital for Children, London for reviewing the manuscript. Mpho Magwalivha acknowledges a post-graduate bursary from the Poliomyelitis Research Foundation.

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