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CHARACTERISATION OF ASTROVIRUSES FROM SELECTED CLINICAL AND ENVIRONMENTAL SETTINGS

by

SANDRAMA NADAN

submitted in partial fulfilment of the
requirements for the degree

MAGISTER SCIENTIAE
MSc (Medical Virology)

in the

Faculty of Health Sciences
University of Pretoria
Pretoria, South Africa

April 2002

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ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to the following:

Prof WOK Grabow, Head of the Department of Medical Virology, for the opportunity to do this research in his department and for his Interest and guidance throughout this investigation;

My supervisor, Prof MB Taylor, for teaching me what science really is, Thank you, and co-supervisor, Prof DO Matson, for his keen interest and advice;

Profs JE Walter and DK Mitchell, Center for Pediatric Research, Eastern Virginia Medical School, Norfolk, Virginia, USA, for their interest, provision of valuable control material and phylogenetic analyses on the South African isolates;

Dr DW Cubitt, Great Ormond Street Hospital for Sick Children, London, for supplying reference isolates;

Personnel from the Diagnostic Services of the Department of Medical Virology, University of Pretoria for assisting with the collection of clinical specimens and for performing selected assays;

Prof EJ van Rensburg and the research students and staff, Department of Human Genetics, University of Pretoria, for their invaluable advice; and

The Poliomyelitis Research Foundation and National Research Foundation for post-graduate bursaries;

This study was supported, in part, by grants from the Water Research Commission and the National Research Foundation of South Africa.

This dissertation is dedicated to my parents and family for their continued encouragement and support.

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SANDRAMA NADAN

SUPERVISOR : PROF MB TAYLOR
CO-SUPERVISOR : PROF DO MATSON
DEPARTMENT : MEDICAL VIROLOGY
DEGREE : MAGISTER SCIENTIAE (MEDICAL VIROLOGY)

SUMMARY

Astroviruses (AstVs), classified within the family ***Astroviridae***, include both animal and human pathogens. Human AstVs (HAstVs) include eight serotypes and after rotaviruses, are the most common pathogen in childhood viral diarrhoea. With the impending licensure of a rotavirus vaccine, the significance of HAstVs in diarrhoeal disease needs to be reassessed. HAstV infection has been reported in all age groups, with the young, elderly and immunocompromised at greatest risk. Transmission occurs via the faecal-oral route and the occurrence of AstVs in water sources has been documented. The significance to humans of AstVs in environmental sources has not been quantified.

The aim of this study was to optimise and apply molecular techniques for the detection and characterisation of AstVs in human stool specimens and water samples. The HAstV serotypes primarily responsible for gastroenteritis in this region of South Africa (SA) could then be established. Animal stools would also be screened for AstVs to obtain a SA reference strain for further characterisation and comparative studies. Nucleotide sequences

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of the clinical and environmental isolates could be compared with each other and to AstVs found outside SA. These data would also provide information on the role of water as a source of human infection and of the source of faecal contamination of surface waters.

Human stool specimens, water concentrates and cell culture derivatives of these, were screened for HAstVs by enzyme immunoassay and type-common reverse transcriptase polymerase chain reactions (RT-PCR). AstV isolates were characterised by nucleotide sequence analysis of RT-PCR amplicons, generated in the 5' and 3' ends of the genome, using type-common and type-specific primer pairs.

Of a total of 35 clinical isolates, 22 AstV strains were characterised and compared to 25 environmental strains obtained from 15 surface water and wastewater samples. Cell culture amplification of selected specimens enabled the amplification of isolates present in low titres as well as the isolation of multiple AstVs serotypes from single sewage samples. All AstVs from the stool specimens and water samples were identified as HAstVs. All eight HAstV serotypes were represented in the combined study samples. Phylogenetic analyses of the nucleotide sequences of each of the HAstV isolates and comparisons with isolates from the rest of the world showed that some SA strains formed unique genetic clusters, as has been observed in other studies at other sites. AstVs in some clinical and environmental samples were identical. The existence of HAstVs in water samples highlights the potential health risk posed by these waters used for recreational and domestic purposes. This study also presents new baseline data on the molecular epidemiology of HAstVs in SA.

KEYWORDS: Human astroviruses, sewage, multitypes, reverse transcriptase-polymerase chain reaction, molecular characterisation, sequencing, cell culture amplification

KARAKTERISERING VAN ASTROVIRUSSE VAN GESELEKTEERDE KLINIESE EN OMGEWINGS BRONNE

deur

SANDRAMA NADAN

PROMOTOR : PROF MB TAYLOR

MEDE-PROMOTOR : PROF DO MATSON

DEPARTEMENT : GENEESKUNDIGE VIROLOGIE

GRAAD : MAGISTER SCIENTIAE (GENEESKUNDIGE VIROLOGIE)

OPSOMMING

Astrovirusse (AstVs), geklassifiseer in die familie *Astroviridae*, bevat beide dierlike en menslike patogene. Daar is agt serotipes van mens astrovirusse (HAstVs) en is na rotavirusse die mees algemene patogeen van virale diarree in kinders. Met die naderende lisensiëring van 'n rotavirus entstof, moet die belangrikheid van HAstVs met betrekking tot diarree herevalueer word. Alhoewel HAstV infeksie in alle ouderdomsgroepe gerapporteer word, is kinders, bejaardes en immuungekompromitteerde individue meer vatbaar. Oordrag is deur die fekaal-orale roete en die voorkoms van AstVs in gekomtamineerde waterbronne is al beskryf. Die kliniese betekenis van omgewings AstVs tot mense is onbekend.

Die doel van hierdie studie was om molekulêre tegnieke te optimiseer en toe te pas vir die opsporing en karakterisering van AstVs in mens-stoelgang- en water-monsters. Die HAstV-serotipes wat primêr verantwoordelik was vir gastroenteritis kan dan vasgestel word. Stoelgang monsters van diere sou ook ondersoek word vir AstVs om 'n SA bron van dierlike AstV te verkry vir verder karaktiseering en vergelykende studies. Nukleotied basispaar volgorde-bepaling van kliniese en omgewings isolate kan dan

vergelyk word om vas te stel wat die verhouding van SA AstVs tot stamme in die res van die wêreld is. Hierdie data sal ook inligting verskaf tot die moontlik rol van water in menslike infeksie en die bron van fekale kontaminasie van oppervlak water.

Mens stoelgang monsters, water konsentrate en hul selkultuur ekstrakte is ondersoek vir HAstVs deur middel van ensiem immuunbepaling en tipe-algemeen tru-transkripsie polimerase ketting reaksie (TT-PKR). AstV isolate is deur nukleotied basispaar volgorde-bepaling, van die 3' en 5'-kant van die genoom, deur tipe-algemene en tipe-spesifieke voorvoerdes gekarakteriseer.

Uit 'n totaal van 35 kliniese isolate, kon 22 AstV stamme gekarakteriseer en vergelyk word met 25 omgewings stamme geïsoleer uit 15 oppervlak en rioolwater monsters. Deur middel van selkultuur vermeerdering kon isolate teenwoodig in lae titers in geselekteerde monsters geamplifiseer word. Selkultuur amplifikasie het ook die isolasie van veelsoortige AstVs van enkele rioolmonsters bevorder. Alle AstVs vanuit stoelgang en water monsters was geïdentifiseer as HAstVs. Ten minste een van elk van die agt HAstV serotipes is geïdentifiseer uit die totale aantal studie monsters. Filogenetiese analise van die nukleotied basispaar volgorde-bepaling van elk van die HAstV isolate, asook vergelyking met isolate uit die res van die wêreld, het getoon dat geselekteerde SA stamme hul eie unieke groepe vorm, soortgelyk aan patrone gerapporteer in ander studies. Sekere AstV-stamme vanaf kliniese en omgewingsbronne was identies. Die identifisering van HAstVs in water monsters beklemtoon die potensiële gesondheidsrisiko vir individue wat hierdie waterbronne vir huishoudelike en ontspanningsdoeleindes gebruik. Hierdie studie verteenwoordig nuwe inligting oor die molekulêre epidemiologie van HAstVs in SA.

SLEUTELWOORDE : Mens astrovirusse, riool, veelsoortige tipes, tru-transkripsie polimerase kettingreaksie, molekulêre karakterisering, nukleotied basispaar volgorde-bepaling, selkultuur vermeerdering

PUBLICATIONS AND PRESENTATIONS

Publications

Nadan S, Walter JE, Grabow WOK, Mitchell DK, Taylor MB. Molecular characterization of astroviruses: comparison between clinical and environmental isolates from South Africa. Applied and Environmental Microbiology *(submitted)*

Presentations

Nadan S, Grabow WOK, Taylor MB. The molecular detection and characterisation of astroviruses from human stool specimens and sewage [Poster/Presentation]. Faculty Day, Faculty of Health Sciences, University of Pretoria 21-22 August 2001: Pretoria.

Taylor MB, **Nadan S**, Grabow WOK, Walter JE. Molecular epidemiology of human astroviruses from the Tshwane area (Pretoria), Gauteng [Presentation]. Joint Congress of the Infectious Diseases & Sexually Transmitted Diseases Societies of Southern Africa. 2-7 December 2001: Spier Estate, Stellenbosch, South Africa.

Nadan S, JE Walter, Grabow WOK, Taylor MB. The molecular detection and characterisation of astroviruses from human stool specimens and sewage [Presentation]. "Microbial Diversity" 12th Biennial Congress of the South African Society for Microbiology, Faculty of Health Sciences, University of the Free State. 2-5 April 2002: Bloemfontein.

WB van Zyl, **S Nadan**, JC Vivier, JME Venter, K Riley, EKM Tlale, LR Seautlueng,WOK Grabow, MB Taylor. The prevalence of enteric viruses in patients with gastroenteritis in the Pretoria and Kalafong Academic Hospitals, South Africa [Poster]. "Microbial Diversity" 12th Biennial Congress of the South African Society for Microbiology, Faculty of Health Sciences, University of the Free State. 2-5 April 2002: Bloemfontein.

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D.3 **ABSTRACT: Nadan S**, JE Walter, Grabow WOK, Taylor MB. The molecular detection and characterisation of astroviruses from human stool specimens and sewage.

D.4 **ABSTRACT**: WB van Zyl, **S Nadan**, JC Vivier, JME Venter, K Riley, EKM Tlale, LR Seautlueng,WOK Grabow, MB Taylor. The prevalence of enteric viruses in patients with gastroenteritis in the Pretoria and Kalafong Academic Hospitals, South Africa.

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ABBREVIATIONS

Å	:	angstrom
aa	:	amino acid
Ab	:	antibody
AMPS	:	ammonium persulfate
AMV	:	avian myeloblastosis virus
Ag	:	antigen
ANV	:	avian nephritis virus
ARC	:	Agricultural Research Council
AstV	:	astrovirus
ATCC	:	American Type Culture Collection
BAstV	:	bovine astrovirus
BK	:	bovine kidney cell line
bp	:	base pair
(C)	:	carboxy terminal
CCC	:	child care centre
CaCo-2	:	colonic carcinoma cell line
cpe	:	cytopathic effect
CsCl	:	caesium chloride
CV	:	caliciviruses
DAstV	:	duck astrovirus
DTT	:	dithiothreitol
EIA	:	enzyme immunoassay
ELISA	:	enzyme-linked immunosorbent assay
EM	:	electron microscopy
E-MEM	:	Eagle's Minimum Essential Medium
EtBr	:	ethidium bromide
FAstV	:	feline astrovirus
FCS	:	foetal calf serum
FEA	:	feline embryo cells
GBEB	:	glycine-beef-extract buffer
g/cm^3	:	gram per cubic centimeter
g/ml	:	gram per millilitre
HAdV-40/41	:	human adenovirus 40/41
HAstV	:	human astrovirus
HAstV-1	:	human astrovirus serotype 1
HAstV-2	:	human astrovirus serotype 2
HAstV-3	:	human astrovirus serotype 3
HAstV-4	:	human astrovirus serotype 4
HAstV-5	:	human astrovirus serotype 5
HAstV-6	:	human astrovirus serotype 6
HAstV-7	:	human astrovirus serotype 7
HAstV-8	:	human astrovirus serotype 8

HIV	:	Human Immunodeficiency Virus
HEK	:	human embryonic kidney cell line
HEL	:	human embryonic lung fibroblasts
HRP	:	horseradish peroxidase
HRV	:	human rotavirus
HS	:	horse serum
h	:	hour
IEM	:	immune electron microscopy
IF	:	immunofluorescence
ISEM	:	immunosorbent electron microscopy
kb	:	kilobase
kDa	:	kilodalton
L	:	litres
LLCMK2	:	rhesus monkey kidney cell line
MAb	:	monoclonal antibody
MDBK	:	Madin-Darby bovine kidney cell line
min	:	minute
ml	:	millilitre
MW	:	molecular weight
(N)	:	amino terminal
NaCl	:	sodium chloride
NCR	:	non-coding region
NBK	:	neonatal bovine kidney cell line
NIV	:	National Institute for Virology
nm	:	nanometre
nt	:	nucleotide
NV	:	Norwalk virus
OAstV	:	ovine astrovirus
ORF	:	open reading frame
PEG	:	polyethylene glycol
PAGE	:	polyacrylamide gel electrophoresis
PAstV	:	porcine astrovirus
PBK	:	primary bovine kidney cell line
PBS	:	phosphate buffered saline
PCR	:	polymerase chain reaction
RFLP	:	restriction fragment length polymorphism
p.i.	:	post infection
PLC/PRF/5	:	primary hepatoma cell line
RIA	:	radio immunoassay
RNA	:	ribonucleic acid
rRNA	:	ribosomal ribonucleic acid
RT	:	reverse transcription
RT-PCR	:	reverse transcriptase-polymerase chain reaction
SA	:	South Africa

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SDS-PAGE	:	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SPIEM	:	solid phase immune electron microscopy
SRV	:	small round virus
SRSV	:	small round structured virus
ss	:	single stranded
TEMED	:	N,N,N',N'-tetramethyl-ethylenediamine
tRNA	:	transfer ribonucleic acid
TYPE-EIA	:	typing enzyme immunoassay
TAstV	:	turkey astrovirus
TAstV-1	:	turkey astrovirus serotype 1
TAstV-2	:	turkey astrovirus serotype 2
TMB	:	3,3',5,5'-tetramethylbenzidine
UK	:	United Kingdom
UNICEF	:	United Nations International Children's Fund
US	:	United States of America
UTR	:	untranslated region
UV	:	ultraviolet
WT	:	wild type

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CHAPTER 1

GENERAL INTRODUCTION

Acute diarrhoeal disease in humans has been documented since ancient times as a leading cause of morbidity and mortality throughout the world (Farthing and Keusch, 1989; Bern and Glass, 1994; Kapikian, 1996). In developing countries, diarrhoeal disease is one of the leading causes of death among infants and children, accounting for 25 – 30% of deaths in children five years of age and younger (Farthing and Keusch, 1989; Bern and Glass, 1994). An adaptation of a United Nations International Children's Fund (UNICEF) poster, for The World Summit for Children, New York in 1990, highlighted the importance of diarrhoea, by stating that during the two-day summit 22 000 children would die of diarrhoea, whereas 12 000 would die of pneumonia in the same time period (Kapikian, 1996). The occurrence and significance of acute diarrhoeal disease in older age groups is not as well documented as in infants and young children (Bern and Glass, 1994). In developed countries, however, diarrhoeal-related deaths in the elderly are more common than in young children, with concomitant acute and chronic diseases being contributing factors (Bern and Glass, 1994). Major risk factors for diarrhoeal disease include increased susceptibility, such as compromised immune status, increased exposure associated with low economic conditions and the grouping together of susceptibles such as in care centers (Bern and Glass, 1994). Immunocompromised persons have been shown to

be at greater risk of infection for diarrhoeal diseases (Grohmann *et al.*, 1993; Bern and Glass, 1994; Pollok, 2001). In South Africa (SA), poor nutrition, unsuitable sanitation and an inadequate health education policy have been identified as major risk factors for the development and spread of diarrhoeal disease (Westaway and Chabalala, 1998). The economic burden and impact of diarrhoeal disease in SA, resulting from increased medical costs, absenteeism, loss of the revenue, and the losses suffered by the tourism industry, are unknown (Keddy, 1998).

Since the early 1970s, the list of aetiologic agents of acute diarrhoea has been lengthened by the discovery of a number of different agents, including previously unrecognised bacteria, parasites, viruses and toxins (Fig.1) (Bern and Glass, 1994; Kapikian, 1996; Glass *et al.*, 2001).

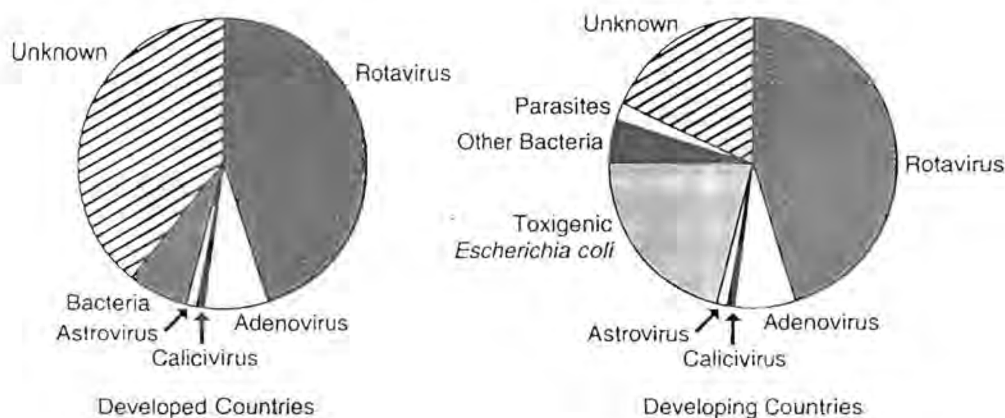

Figure 1: Microbial agents associated with severe infant and childhood diarrhoea in developed and developing countries (Kapikian, 1996).

In developed and developing countries, a diverse range of viruses play an important role in non-bacterial gastrointestinal disease (Bern

and Glass, 1994; Glass *et al.*, 2001). To date, the range of diarrhoeal pathogens in SA has not been fully elucidated (Keddy, 1998). Continual research and active surveillance, therefore, are necessary for determining a disease burden and effective interventions for viral diarrhoeal disease (Keddy, 1998; Glass *et al.*, 2001). Interventions such as water treatment dramatically reduce the spread of bacterial and parasitic diarrhoeal disease (Keddy, 1998) but not of viral diarrhoea pathogens (Centers for Disease Control and Prevention, 1999). Thus, vaccines have been considered to be the most likely approach to succeed in preventing viral gastroenteritis (Glass *et al.*, 2001). With the development of a licensed rotavirus (HRV) vaccine, human astrovirus (HAstV) may become the next target for vaccine development (Keddy, 1998).

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CHAPTER 2

LITERATURE REVIEW

2.1 HISTORY

Astroviruses (AstVs) were first identified as a causative agent of infantile diarrhoea during an investigation of an outbreak of mild diarrhoea and vomiting among infants in a maternity ward (Appleton and Higgins, 1975). Examination of the stool specimens by electron microscopy (EM) revealed viral particles that were different from Norwalk virus (NV) and HRV, commonly associated with childhood diarrhoeal disease. A five-to-six-pointed star-like surface structure was the most distinct feature of the particles. The name "astrovirus", based on the Greek word "astron" for star, was used to describe these newly identified viral particles (Madeley and Cosgrove, 1975). The aetiological association between AstVs and gastroenteritis was subsequently confirmed by challenge studies in volunteers (Kurtz *et al.*, 1979). AstVs have also been detected in the stools of apparently healthy individuals (Madeley and Cosgrove, 1975; Caul, 1996).

In many regions of the world, AstVs are reportedly the second most important childhood viral diarrhoeal pathogen after HRVs (Matsui and Greenberg, 1996; Marx *et al.*, 1998a; Steele *et al.*, 1998; Bon *et al.*, 1999; Dennehy *et al.*, 2001). However, all age groups are susceptible to AstV infection, with the elderly (Gray *et*

al., 1987) and immunocompromised being at increased risk (Glass *et al.*, 1996; Cubitt *et al.*, 1999; Grohmann *et al.*, 1993; Matsui and Greenberg, 2001).

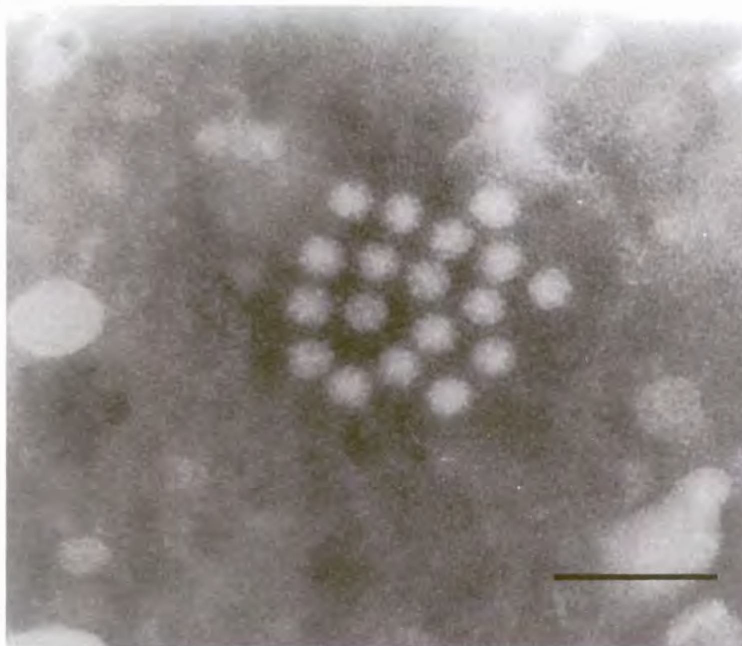
AstV infection in animals has also been documented (Kurtz, 1994; Caul, 1996). AstV-associated diarrhoea has been reported in lambs (Snodgrass and Gray, 1977; Herring *et al.*, 1981), calves (Woode and Bridger, 1978), piglets (Bridger, 1980; Shimizu *et al.*, 1990), cattle (Bridger *et al.*, 1984), cats (Hoshino *et al.*, 1981), dogs (Williams, 1980), red deer (Tzipori *et al.*, 1981), turkey (McNulty *et al.*, 1980) and mice (Kjeldsberg and Hem, 1985). AstVs have also been associated with a fatal hepatitis in ducklings (Gough *et al.*, 1984), haemorrhagic enteric syndrome in turkeys (Koci *et al.*, 2000) and acute nephritis in chickens (Imada *et al.*, 2000). It appears that the association of AstVs with fatal hepatitis in ducklings long preceded the association between AstVs and gastroenteritis in humans and animals (Kurtz and Lee, 1987).

AstV infection appears to be species-specific (Monroe *et al.*, 2000a; Matsui and Greenberg, 2001) and to date cross-infection between humans and animals has not been documented (Kurtz and Lee, 1987; Jonassen *et al.*, 2001; Matsui and Greenberg, 2001). However, similarities between the capsid sequences of HAstV, feline AstV (FAstV) and porcine AstV (PAstV) suggest that zoonoses involving pigs, cats and humans could have occurred relatively recently (Jonassen *et al.*, 2001).

2.2 CLASSIFICATION AND MORPHOLOGY

2.2.1 Morphology

AstVs are a family of small round viruses (SRVs) that display a unique surface structure (Matsui and Greenberg, 2001). They are identifiable by being non-enveloped particles with icosahedral symmetry, being approximately 28 nanometers (nm) in diameter, having with a smooth outer edge and showing a characteristic five-to-six-pointed star apparent on the surface of approximately 10% of particles (Fig. 2) (Madeley, 1979; Kurtz and Lee, 1987; Matsui and Greenberg, 1996, 2001; Monroe, 1999).



(Bar = 100nm)

Figure 2: Electron micrograph of negatively stained HAstV in a faecal specimen. Courtesy: Prof MB Taylor

Immune electron microscopy (IEM) results in antibody coating of the virions, which obscures the star-like features of the virus (Ashley *et al.*, 1978; Kurtz and Lee, 1987). Variation in the diameter of the virus also may be observed, depending upon the source of the virus and preparation technique (Matsui and Greenberg, 2001). Bovine AstV (BAstV) isolated from primary neonatal bovine kidney (NBK) cells has a diameter of 34 nm (range 30-37 nm) (Aroonprasert *et al.*, 1989), whereas similarly stained and fixed sections of BAstV from infected calf ileum produce virions with a diameter of 30 nm (range 27-35 nm) (Woode *et al.*, 1984). The length of passage in cell culture may also influence the size of the AstV particle (Willcocks *et al.*, 1990). Structures have been observed by EM that bridge neighbouring viral particles and that extend between the apices of the points of the star configurations (Snodgrass and Gray, 1977, Hoshino *et al.*, 1981; Kurtz and Lee, 1987; Monroe, 1999). The star-like shape is not readily apparent in AstVs propagated in cell culture, but could be induced by alkaline treatment (Risco *et al.*, 1995). Electron micrographs of purified viral preparations that were not alkaline-treated have showed round particles with spikes on the surface and an external diameter of 41 nm (Risco *et al.*, 1995).

More information regarding the unique surface of the AstV was obtained using cryo-electron microscopy (Matsui *et al.*, 2001; Matsui and Greenberg, 2001). AstV particles were purified from a preparation of cell culture-adapted HAstV type 1 (HAstV-1), frozen-hydrated and stained with uranyl acetate. The image-processing technique confirmed spherical particles of uniform size with clearly visible surface spikes. The three-dimensional reconstruction of these cryo-electron microscopy images revealed a smoothly rippled,

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solid capsid shell with a diameter of 330 angstrom (Å). Thirty dimeric spikes extended about 50 Å from the surface and were centred at the twofold axis of symmetry (Fig. 3) (Matsui and Greenberg, 2001).

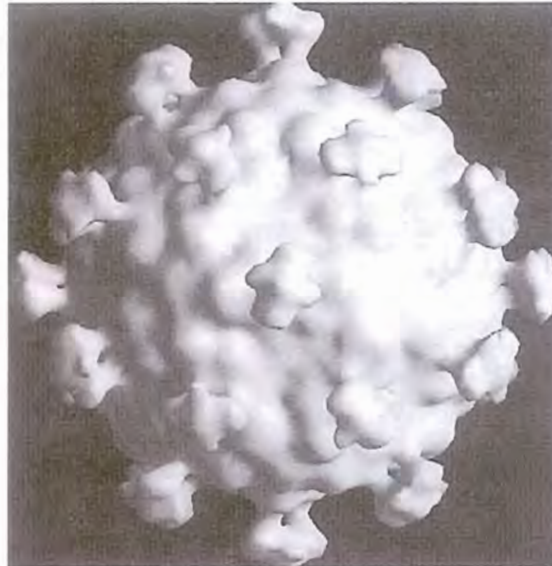

Figure 3: Image processing of negatively stained HAstV-1 viewed along the twofold axis of symmetry (Matsui and Greenberg, 2001)

2.2.2 Taxonomy and classification

AstVs are positive-sense, single-stranded (ss) RNA viruses classified in the *Astroviridae*. The family comprises a single genus, *Astrovirus*, with HAstV-1 as the prototype strain. Virus species are identified by the host species of origin namely, human (HAstV), bovine (BAstV) etc., with serotypes being denoted and identified by a number, e.g. HAstV-1 (Monroe, 1999).

AstVs represent a third family of positive-sense, non-enveloped ss RNA viruses (Matsui and Greenberg, 2001), however particle construction, genomic organisation and replication strategy differ from those of the other two families, *Picornaviridae* and *Caliciviridae* (Matsui and Greenberg, 2001). A feature unique to AstVs is the five-to-six-pointed star motif on the surface of viral particles (Kurtz and Lee, 1987; Doane, 1994). In comparison, picornaviruses have small, round and featureless icosahedral virions and caliciviruses (CVs) have a structured surface with a "Star of David" formed by cup shaped depressions at each of the icosahedral fivefold and threefold axes (Green *et al.*, 2000). In the NV-like CV genus, the surface arches are blunted, yielding a ragged surface (Prasad *et al.*, 1994).

The organisation of the AstV genome resembles that of CVs, however, several features distinguish AstVs from CVs, namely; size, number and processing of structural proteins, lack of a helicase motif in AstVs and presence of a ribosomal frame-shifting during translation of the AstV ribonucleic acid (RNA)-dependent RNA polymerase (Jiang *et al.*, 1993; Matsui and Greenberg, 2001). The distinctions between picornaviruses and AstVs include differences in genomic organisation, AstVs lack of a helicase domain, the presence in AstVs of ribosomal frame-shift and AstV production of a subgenomic RNA (Jiang *et al.*, 1993; Monroe *et al*, 1993; Matsui and Greenberg, 2001).

HAstVs have been cultivated in the presence of trypsin in at least two cell lines (Willcocks *et al.*, 1990; Taylor *et al.*, 1997a; Brinker *et al.*, 2000), which clearly differentiates them from NV-like viruses and Sapporo-like viruses, which have not been propagated *in vitro*

(Clarke and Lambden, 2001). A tentative phylogenetic tree to define the phylogenetic position of AstVs based upon sequence comparison and genomic organisation showed that these viruses had no close relatives (Jiang *et al.*, 1993). AstVs incorporated some features of each of different virus families without resembling one particular family in all features (Matsui and Greenberg, 2001). This justified the assignment of AstVs to a new family (Jiang *et al.*, 1993; Monroe, 1999; Matsui and Greenberg, 2001).

2.3 BIOCHEMICAL AND BIOPHYSICAL CHARACTERISTICS

2.3.1 Buoyant density and sedimentation value

HAstV virions migrate in sucrose gradients with a sedimentation value ($s_{20,w}$) of approximately 160S and have a buoyant density in caesium chloride (CsCl) gradients of 1,36-1,39 g/cm^3 (Monroe, 1999; Monroe *et al.*, 2000a). AstV particles lacking RNA band at a lower density (1,33 g/cm^3) than intact particles containing RNA (1,37 g/cm^3) (Willcocks *et al.*, 1992). HAstVs and ovine AstVs (OAstVs) formed two distinct bands in CsCl density gradients implying differences in AstV density that might be related to strain type (Herring *et al.*, 1981). HAstVs band at a buoyant density of 1,32 g/cm^3 in potassium tartrate-glycerol gradients (Ashley and Caul, 1982), however this method is not optimal for separation of AstVs from NV-like viruses because the process disrupts AstV particles (Ashley and Caul, 1982).

2.3.2 Stability

HAstVs can survive at pH 3 (Kurtz and Lee, 1987). Virus integrity and viability is maintained for 1 h at 50°C and for 5 min at 60°C (Ashley and Caul, 1982). AstVs can withstand the action of chloroform, other lipid solvents and non-ionic, anionic and zwitterionic detergents (Kurtz and Lee, 1987). They are stable when stored for six to ten years at ultralow temperatures (-70°C to -85°C) but the integrity of the viral particle may be disrupted by repeated freezing and thawing (Williams, 1989). HAstV infectivity decreases up to 3 log units after 120 min in the presence of 1 mg of free chlorine/litre of water (Abad *et al.*, 1997). AstVs can survive in seawater at low temperatures and are more rapidly inactivated in marine water than in fresh water (Bosch *et al.*, 1997).

The AstV that causes poult enteric mortality syndrome in turkeys is resistant to inactivation by heat, acidification, detergent treatment and treatment with phenoloic, quaternary ammonia, and benzalkonium chloride-based products. Only treatment with formaldehyde, ß-propriolactone, or the peroxymonosulfate–based product Virkon S® (Antec International Ltd.) completely inactivated AstV in an embryonated egg model (Shultz-Cherry *et al.*, 2001). At a concentration of 90%, ethanol reduced the titre of AstV by $4\log_{10}$, while 90% methanol reduced the virus count to below the sensitivity of detection (Kurtz *et al.*, 1980).

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2.3.3 Nucleic acid composition

The genome of AstVs is a single molecule of infectious RNA, 6,8 – 7,9 kilobase (kb) in length, excluding a poly (A) tract at the 3′ end (Matsui and Greenberg, 1996; Koci *et al.*, 2000; Monroe *et al.*, 2000a). A subgenomic RNA of 2,4 kb is produced in infected cells together with the full-length genome (Monroe *et al.*, 1991). A cap or VPg structure at the 5′ end of the genome is unknown (Monroe *et al.*, 1995; 2000a).

2.3.4 Polypeptide composition

The polypeptide composition of precursor polypeptides, capsid proteins and polypeptides during all of replication in HAstV isolates comprises at least two, possibly three, major proteins 29-39 kDa in size. Several isolates also contain smaller proteins of 13-36 kDa (Monroe *et al.*, 1995). The HAstV precursor polypeptide is specified by the subgenomic RNA synthesized during infection of susceptible cells (Willcocks *et al.*, 1992). The polypeptide composition of virions isolated during the replication cycle in humans, pigs and experimentally infected lambs, have also been investigated (Table 1). For the animal AstV extracts, proteins in the range of 13,0-39,0 kDa were identified, while AstVs isolated from human volunteers and cell cultures collectively, were made up of proteins sized from 5,2 to 36,5 kDa.

Depending on the host species of origin, and method of purification, different numbers and sizes of capsid proteins have been reported (Monroe, 1999). HAstVs have three capsid proteins, namely VP32 or P1 (33 kilodalton [kDa]), VP29 or P2 (32 kDa) and

VP26 or P3 (for HAstV-2)(25 kDa), which are cleaved from a viral structural protein precursor of 90 kDa (Willcocks *et al.*, 1992; Matsui and Greenberg, 1996; Monroe, 1999). Two capsid proteins have been described for OAstVs (Monroe, 1999).

Table 1: Polypeptide composition of selected astroviruses during all of replication

Source	Polypeptide Composition	Comments	Reference
Ovine - *CsCl purified virus* - *"crude" virus*	2 polypeptides that migrated at 33 kDa[1]		Matsui and Greenberg, 1996 Herring *et al.*, 1981
Porcine	5 proteins 13 – 39 kDa		Matsui and Greenberg, 1996; Shimizu *et al*, 1990
HAstV[2]-4 infected cell cultures - *SDS-PAGE*[3]	4 proteins 36.5 kDa 34.0 kDa 33.0 kDa 32.0 kDa	36.5 kDa band thought to represent a precursor protein that undergoes subsequent processing to form one of the smaller proteins which are more abundant	Kurtz and Lee, 1987
HAstV-1 infected cell cultures	2 more proteins than HAstV-4: 24.0 kDa 5.2 kDa		Kurtz and Lee, 1987
HAstV-2 infected cell cultures	3 AstV specific proteins: 33.5 kDa 31.5 kDa 24.0 kDa	Proteolytic digestion of the larger proteins resulted in the 24 kDa polypeptide	Willcocks *et al*, 1990
HAstV –5 isolated from human volunteer	Single protein 30 kDa	Determined by specific immuno-precipitation	Midthun *et al*, 1993

1: kilodalton; 2: Human astrovirus; 3: sodium dodecyl sulphate – polyacrylamide gel electrophoresis

2.4 ANTIGENIC PROPERTIES AND SEROTYPES

2.4.1 HAstV serotypes

On the basis of immune electron microscopy (IEM), neutralisation and immunofluorescence (IF) techniques, HAstVs were classified into eight antigenic or serotypes (Monroe 1999; Matsui and Greenberg, 2001). All eight HAstV serotypes are recognized by a group-specific monoclonal antibody (MAb) (8E7) (Hermann *et al.* 1988), that is directed at a viral structural protein (Matsui and Greenberg, 2001). Neutralisation epitopes have been mapped to the VP26 and V29 proteins of HAstV-1 and HAstV-2, respectively (Monroe *et al.*, 2000a).

The Oxford prototype HAstV 1 to 5 strains were adapted to growth in cell culture (Lee and Kurtz, 1981) and serotyped by IEM, IF and neutralisation (Lee and Kurtz, 1982; Kurtz and Lee, 1984; Hudson *et al.*, 1989). One strain of HAstV-5, also referred to as the Marin County agent, induced a broadly cross-reactive antibody (Ab) response to HAstV-1 to 5 (Midthun *et al.*, 1993). This could possibly be attributed to an anamnestic response rather than to multiple antigenic epitopes on the surface of the Marin County agent (Midthun *et al.*, 1993).

Serotypes 6 and 7, recovered from infants in the Oxfordshire area with gastroenteritis in 1989 and 1991, respectively, were identified by EM (Lee and Kurtz, 1994). Despite having the same structural features as serotypes 1 to 5, these viral particles did not cross react with each other or types 1 to 5 by IF or IEM (Lee and Kurtz, 1994). Further these viruses could be cultured in human colonic carcinoma cell line (CaCo-2) in the presence of trypsin and reacted

with a MAb that was known to react with all the other documented serotypes of AstVs (Lee and Kurtz, 1994). An eighth type was characterised by IF (Belliot *et al.*, 1999), IEM and enzyme immunoassay (EIA) (Taylor *et al.*, 2001a).

2.4.2 Distribution of HAstV serotypes

The distribution of HAstV serotypes differs by year and location (Glass *et al.*, 1996). In general, HAstV-1 is the most commonly identified serotype in the United Kingdom (UK) (Lee and Kurtz, 1994, Noel and Cubitt, 1994), the United States of America (US) (Noel *et al.*, 1995), Australia (Palombo and Bishop, 1996; Mustafa *et al.*, 2000), Norway (Kjeldsberg, 1994), Colombo and Venezuela (Medina *et al.*, 2000), Japan (Sakamoto *et al.*, 2000), Egypt (Naficy *et al.*, 2000), Germany (Oh and Schreier, 2001) and Spain (Guix *et al.*, 2002). Biennial peaks of serotype 1 infection have been reported in UK (Lee and Kurtz, 1994), Australia (Mustafa *et al.*, 2000) and other regions of the world (Matsui and Greenberg, 2001). HAstV-1 has been associated with outbreaks of gastroenteritis in a paediatric bone marrow transplant unit (Cubitt *et al.*, 1999) and from six child care centers (CCCs) (Mitchell *et al.*, 1999a).

HAstV-2 to HAstV-5 seem to be less common and HAstV-6, 7 and 8 are rarely detected (Glass *et al.*, 1996; Monroe, 1999). HAstV-2 was, however, the most frequently identified serotype among a cohort of children in Mexico (Walter *et al.*, 2001a). An outbreak of gastroenteritis in a military camp was attributed to HAstV-3 (Belliot *et al*, 1997a). Single isolates of HAstV-8 have been reported from Ghaza and Uganda (Monceyron *et al.*, 1997), Australia (Mustafa *et al.*, 2000), Pakistan (Sakamoto *et al.*, 2000), South Africa (Taylor

et al., 2001a), Mexico (Mendez-Toss *et al.*, 2000), and HAstV-8 appears to be more common on the African continent (Monroe *et al.*, 2000b) and Spain (Guix *et al.*, 2002) than elsewhere.

Co-infections with different HAstV antigenic types have been reported (Matsui *et al.*, 1998). Consecutive infection by different types of HAstV, namely first HAstV-3 and subsequently HAstV-1, suggests a lack of heterotypic immunity between the different antigenic types (Guix *et al.*, 2002). HAstV-1, 2 and 3 were reportedly more frequently associated with infection in children younger than three years of age while HAstV-4 and 8 were detected in older children (Guix *et al.*, 2002)

2.4.3 Animal AstV serotypes

To date two, and possibly three, BAstV serotypes have been identified (Woode *et al.*, 1985; Monroe, 1999; Matsui and Greenberg, 2001). The two recognized US BAstV serotypes, US1 and US2, exhibit no cross-reaction with each other and the reaction of the third potential serotype, UK, to antisera to US1 and US2, has yet to be analysed (Woode *et al.*, 1985; Matsui and Greenberg, 2001). One serotype each of PAstV, OAstV, duck AstV (DAstV) and FAstV has been described (Monroe, 1999). Two serotypes of turkey AstV (TAstV), TAstV-1 and TAstV-2, are known (Monroe, 1999; Imada *et al.* 2000; Koci *et al.*, 2000; Jonassen *et al.*, 2001).

AstVs from humans, lambs, piglets, red deer, kittens and calves have been shown to be antigenically distinct (Herrmann *et al.*, 1990; Lee and Kurtz, 1982; Tzipori *et al.*, 1981; Woode *et al.*, 1984; Matsui and Greenberg, 2001).

2.5 MOLECULAR BIOLOGY

2.5.1 Genomic organisation and expression

The AstV genome is organised into three sequential open reading frames (ORFs), namely, ORF1a, ORF1b and ORF2 (Fig. 4) (Monroe *et al.*, 1991; Willcocks *et al.*, 1994; Matsui and Greenberg, 2001).

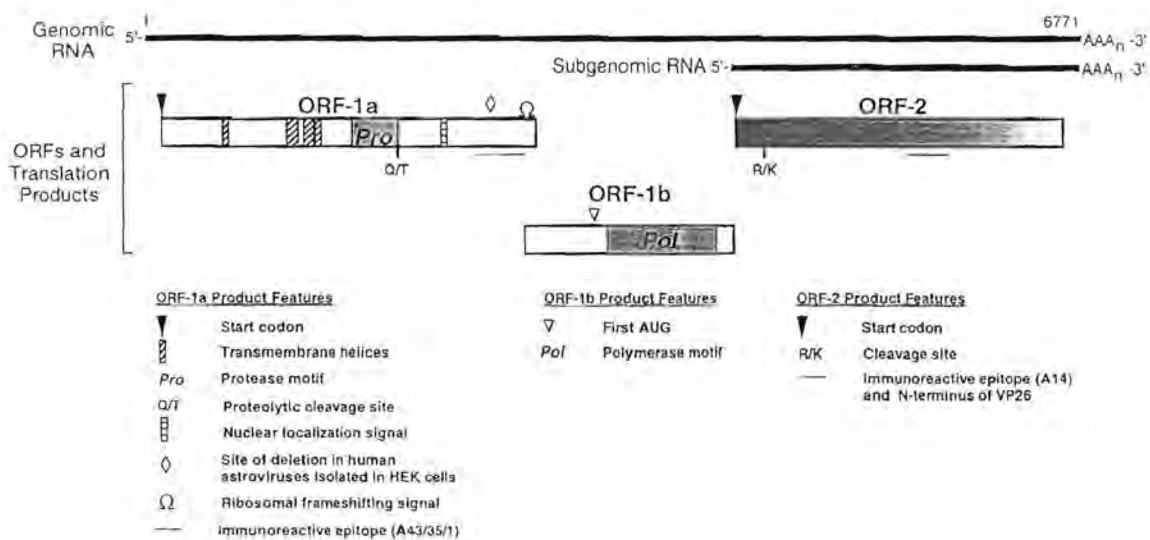


Figure 4: Genomic organisation of HAstV-1. Genomic and subgenomic RNAs are indicated above the ORFs and translation products (Matsui and Greenberg, 2001)

ORF1a begins at the 5′ end of the genome and is followed by ORF1b; they encode motifs for non-structural proteins, including a chymotrypsin-like protease (Pro) and a RNA-dependent RNA polymerase (Pol), that are recognized by distinct amino acid motifs (Jiang *et al.*, 1993; Lewis *et al.*, 1994, Monroe, 1999; Matsui and Greenberg, 2001). A 61 to 73 nucleotide (nt) (depending on serotype) overlap region between ORF1a and ORF1b is highly conserved among HAstV yet characterised (Marczinke *et al.*, 1994;

Monroe 1999; Matsui *et al.*, 2001; Matsui and Greenberg, 2001). ORF2 follows ORF1b and encodes the virion capsid proteins. The overlap between ORF2 and ORF1b is 8 nt for HAstV-1 to 3 and HAstV-8 (Willcocks *et al.*, 1994; Monroe *et al.*, 1995; Carter and Willcocks, 1996; Matsui and Greenberg, 2001; Wang *et al.*, 2001). Subgenomic RNA includes ORF2 only, beginning 2484 nt upstream from the first AUG of ORF2 (Monroe *et al.*, 2000a; Matsui and Greenberg, 2001). At the 3′ end of the genome, an 80 to 85 nt (depending on serotype) untranslated region is found between ORF2 and the poly-A tail (Matsui and Greenberg, 2001).

The three distinctive features of AstV genomic organisation are that:

- the protease and polymerase are encoded in separate reading frames, but are believed to be translated as a polyprotein;
- during infection (proven yet only in cell culture), both full-length genomic and subgenomic RNAs are produced;
- AstVs lack an identifiable RNA helicase domain (Matsui *et al.*, 2001).

Investigation of the location of the structural and non-structural proteins in infected cells by IF (Willcocks *et al.*, 1999) revealed that products from the ORF1a region were concentrated in the nucleus of baculovirus-infected insect cells and of CaCo-2 cells. Products of ORF1b remain predominantly cytoplasmic (Willcocks *et al.*, 1999). An unusual motif for RNA viruses, namely a nuclear addressing signal, contained in the ORF1a product (serine protease) directs the protein into the cell nucleus (Willcocks *et al.*, 1999).

A ribosomal frame-shift mechanism is required for the full expression of the AstV RNA polymerase and is implied by the location of two potential 'slippery' sequences, a shifty heptamer (AAAAAAC), adjacent to a potential stem-loop structure (Jiang *et al.*, 1993; Willcocks *et al.*, 1994; Lewis and Matsui, 1996; Matsui *et al.*, 2001). The genomes of PAstV, OAstV, TAstV-1 and each of the eight HAstV serotypes have a common stem loop structure of 35 nt at the 3' end of the genome (Jonassen *et al.*, 2001). TAstV-2 does not share this structure (Jonassen *et al.*, 2001). The existence of a highly conserved stem-loop structure in HAstVs, PAstV and OAstVs (Jonassen *et al.*, 2001) and of the same virion morphology of animal and HAstVs implies that some features of AstV capsid proteins are conserved (Jonassen *et al.*, 2001). The presence of motifs conserved at the amino acid (aa) level suggests an essential function and thus possibly a role in the assembly and function of the virions for each type of AstV (Jonassen *et al.*, 2001).

2.5.1.1 Open reading frame 1a

This region is 2760 nt in length for Oxford reference HAstV-1 and 2 and 45 nt longer in the Newcastle reference HAstV-1 (Matsui and Greenberg, 1996). The polypeptide encoded by ORF1a contains a serine protease motif. This protease bears strong resemblance to the protease of CVs, except for the substitution of a single amino acid substitution. A bipartite nuclear localization signal is encoded downstream of this viral protease motif, which directs ORF1a encoded proteins to the nucleus (Willcocks *et al.*, 1999). Sequence analysis of cell culture-adapted strains suggests that amino acids encoded by the 3' end of ORF1a determine the host cell range

(Carter and Willcocks, 1996); this region also encodes an immuno-reactive epitope (Matsui and Greenberg, 2001).

2.5.1.2 Open reading frame 1b

This region has 1157 nt in all strains sequenced thus far. It encodes a polypeptide that contains motifs similar to that for a RNA-dependent RNA polymerase (Jiang *et al.*, 1993). This motif is conserved among all HAstVs (Belliot *et* al, 1997b; Lewis *et al.*, 1994; Matsui and Greenberg, 2001). HAstV-1 and 2 appear to be the most closely related and HAstV-4 and 5 are the least related in this region. The aa sequence YGDD, which is presumed at the active site of the RNA-dependent RNA-polymerase, is encoded at position 3940 of ORF1b (Carter and Willcocks, 1996). It has been suggested, on the basis of nucleotide sequence analysis, that the expression of ORF1b is mediated through a ribosomal frameshifting mechanism (Marczinke *et al.,* 1994).

2.5.1.3 Open reading frame 2

The length of this region varies in each of the reference strains, i.e. 2358 nt [HAstV-1, Newcastle] (Willcocks and Carter, 1993; Carter and Willcocks, 1996), 2362 nt [HAstV-1, Oxford] (Lewis *et al.*, 1994) and 2388 nt [HAstV-2, Oxford] (Jiang *et al.*, 1993). The greatest nucleotide sequence variability is found in this region of the genome (Monroe, 1999; Matsui and Greenberg, 2001). Alignments of capsid polypeptide sequences indicate that, among the human serotypes, amino acids are highly conserved at the 5'-terminal (Carter and Willcocks, 1996; Matsui and Greenberg, 2001). In this region, the aa sequence for feline, porcine and sheep strains share certain conserved stretches of aa residues with human strains. Outside of this region there is considerable

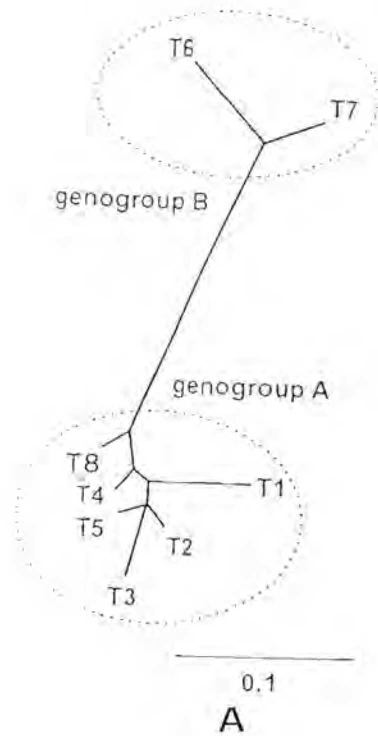
variability in sequence between serotypes, which can only be optimally aligned by the introduction of gaps (Matsui and Greenberg, 2001). The eight aa at the 3'-end of ORF2 are highly conserved, particularly among human strains (Matsui and Greenberg, 2001).

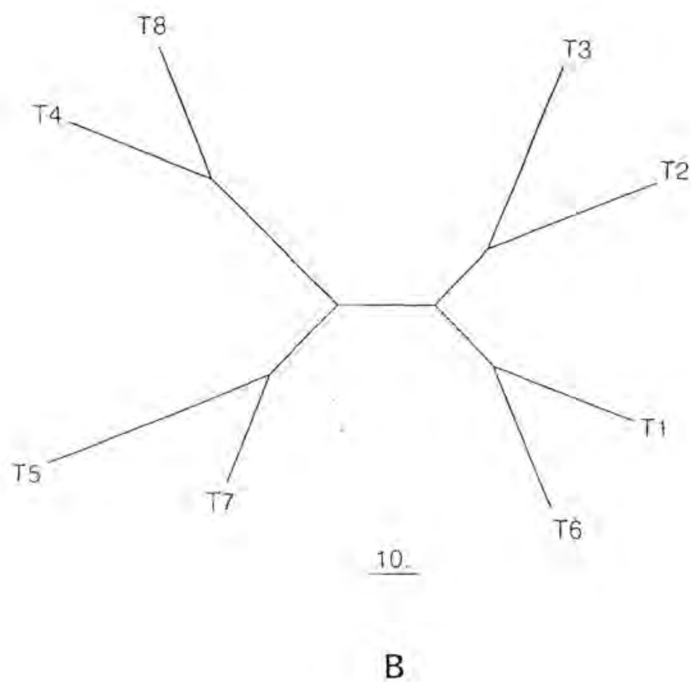
2.5.2 Genotypic properties

The development of reverse transcriptase-polymerase chain reaction (RT-PCR) assays for regions within ORF1a, ORF1b and ORF2 has enabled the sensitive detection, genotyping and phylogenetic analysis of HAstV isolates (Noel *et al.*, 1995; Belliot *et al.*, 1997b; Cubitt *et al.*, 1999; Mitchell *et al.*, 1999a; Mustafa *et al.*, 2000; Taylor *et al.*, 2001a; Walter *et al.*, 2001a; Guix *et al.*, 2002). Concordance between serotypes and genotypes is evident (Noel *et al.*, 1995; Taylor *et al.*, 2001a). An unusual feature of HAstVs is the high level of sequence similarity as a function of the coding region analysed (Monroe, 1999) (Fig. 5). The pairwise nucleotide sequence distances between HAstV-1 to 5, calculated from the 3' end of ORF2, are greater than the range exhibited by sequences of HAstV-1 to 5 from a region within ORF1a (Monroe, 1999). Sequence analysis of ORF1a shows that HAstV-1 to 5 and HAstV-8 cluster in one distinct group, genogoup A, while HAstV-6 and 7 cluster in genogroup B (Belliot *et al.*, 1997b; Monroe, 1999, Taylor *et al.*, 2001a). However, the variability of the 3' end of ORF2 enables better differentiation of strains than do comparisons of the more conserved regions of ORF2, ORF1a or ORF1b (Mitchell *et al.*, 1999a).

i16095133
b15465417

ORF1a





B

Figure 5: Phylogenetic analysis of human astrovirus nucleotide sequences. Unrooted trees generated by maximum parsimony analysis using a 244 nucleotide region of ORF1a (adapted from Taylor *et al.*, 2001a) (A), or a 348 nucleotide region of ORF2 (Monroe, 1999) (B). Scale bars indicate the number of nucleotide changes.

2.6 PROPAGATION OF ASTROVIRUSES

2.6.1 Propagation of human astroviruses

The propagation of HAstVs in cell cultures was first demonstrated in 1977 (Lee and Kurtz, 1977). The HAstVs did not display an observable cytopathic effect (CPE) in infected cells but viral antigen (Ag) was detected in the cytoplasm by IF 24–48 h post infection (p.i.) (Lee and Kurtz, 1977; Kurtz and Lee, 1987). The serial passage of HAstVs in primary human embryonic kidney (HEK) cells in the presence of trypsin was subsequently demonstrated (Lee and Kurtz, 1981). The addition of trypsin to the infected cells resulted in the increase in the yield of viruses released (Lee and Kurtz, 1981). The optimal trypsin concentration of 10 µg/ml growth media resulted in viral titres of 10^4 to 10^7/ml at approximately 48 h p.i. (Lee and Kurtz, 1981). Following six passages and adaptation to growth in HEK cells, HAstVs were successfully passaged into the LLCMK2 rhesus monkey kidney cell line and primary baboon kidney (PBK) cell cultures in the presence of trypsin (Matsui and Greenberg, 1996; Kurtz, 1994). Under an agar overlay containing 10 µg/ml trypsin, HAstV-1, 2 and 5 formed well-defined plaques in LLCMK2 cell culture (Hudson *et al.*, 1989). This cell culture adaptation in the HEK cells was accompanied by a 15 aa deletion in the ORF 1a region. Although the significance of the deletion is not well understood, it is thought to assist in the adaptation of the virus to growth in the cell line (Matsui and Greenberg, 1996).

An important development was the isolation of HAstVs directly from stool specimens on the CaCo-2 human colonic carcinoma cell line without prior adaptation to cell culture (Willcocks *et al.*, 1990).

CPE was observed in the infected cell cultures two days p.i. and was extensive after three to four days. In concert, the viral particles from the earlier passages appeared larger and displayed less distinct features whereas the particles observed after the fifth passage were identical to the particles of the original stool inoculum (Willcocks *et al.*, 1990). Mustafa *et al.* (1998) demonstrated that prior culture of stool samples in the CaCo-2 cell line improved the sensitivity of detection by RT-PCR by 52-100%. With improvements in cell culture techniques it is reportedly possible to culture virus from more than 80% of HAstV antigen (Ag)-positive specimens (Willcocks *et al.*, 1990). The human hepatoma cell line, PLC/PRF/5, has also been shown to support the productive growth of HAstVs in the presence of trypsin (Marx *et al.*, 1995; Taylor *et al.*, 1997a). The human intestinal cell lines T84 and HT-29, and the foetal African green monkey cell line MA104 have also been shown to support the growth of the cell culture-adapted reference strains of HAstV-1 to 7 (Brinker *et al.*, 2000). The prototype HAstV isolates, except HAstV-7, grew exceptionally well in the VERO African green monkey cell line and the 293 human embryonic kidney cell line supported the growth of all HAstVs except HAstV-3 (Brinker *et al.*, 2000). The T84 cell line showed potential for the direct propagation of HAstVs from stool specimens (Brinker *et al.*, 2000). Cross species infection of cell cultures was demonstrated only after the wild type (WT) HAstVs were adapted to growth on cell lines of original host species (Brinker *et al.*, 2000).

The PLC/PRF/5 cell line was found to be more efficient than the CaCo-2 cell line for the detection of HAstVs from environmental water samples (Marx *et al.*, 1998b; Taylor *et al.*, 2001b) and

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selected stool specimens (Taylor *et al.*, 1997a). The results demonstrated that the PLC/PRF/5 cell line supported not only nucleic acid amplification but the production of viral proteins as indicated by the detection of viral Ag by enzyme immunoassay (EIA) (Marx *et al.*, 1998b). As the RT-PCR-oligonucleotide probe hybridisation assay was found to be more sensitive than EIA for detection of HAstVs from the cell culture extracts (Marx *et al.*, 1998b), it was suggested that replication of the environmental HAstVs in cell cultures was restricted to nucleic acid amplification with the production of minimal quantities of capsid proteins (Marx *et al.*, 1995, 1998b).

2.6.2 Propagation of animal astroviruses

Attempts to propagate AstVs from various animals, including bovine (Woode *et al.*, 1985; Aroonprasert *et al.*, 1989), dogs (Williams, 1980; Marshall *et al.*, 1984), ducks (Gough *et al.*, 1984), pigs (Shimizu *et al.*, 1990) and cats (Hoshino *et al.*, 1981), in cell culture has been reported, but only porcine viruses have been adapted to growth in cell culture (Monroe *et al.*, 2000a).

FAstVs have been successfully passaged in feline embryo cells (FEA), with the addition of trypsin (Harbour *et al.*, 1987). BAstVs were passaged in NBK cells in media containing trypsin (Aroonprasert *et al.*, 1989) and PAstVs were propagated in embryonic swine kidney cells with trypsin (Shimizu *et al.*, 1990). Embryonated chicken eggs support the growth of DAstV-1 following blind passage in the amniotic sac. Infected embryos die within 7 days and appear stunted and have greenish, necrotic livers

in which AstV-like particles have been identified (Monroe *et al.* 2000a).

Two factors appear to be important for the propagation of animal AstVs: i) the use of host cells from the animal species from which the AstV was detected, and ii) the incorporation of trypsin into the growth medium (Matsui and Greenberg, 2001). In general, infection of animals and cell culture is species specific (Matsui and Greenberg, 2001).

2.7 VIRAL DETECTION AND CHARACTERISATION

2.7.1 Virus/Antigen detection and characterisation assays

2.7.1.1 Electron microscopy

EM is the only technique available which can detect the full range of gastroenteritis viruses (Lew *et al.*, 1990; Willcocks *et al.*, 1992). AstVs were first identified by EM (Appleton and Higgins, 1975) and for the next 15 years EM remained the only method to detect theses viruses in clinical specimens (Glass *et al.*, 1996). Patients with HAstV-associated gastroenteritis usually shed large quantities of viral particles ($\sim 1X10^{10}$/ml, or 10^8 viable particles/ml) (Kurtz and Lee, 1987), which is within the detection limit of EM, i.e. 10^6-10^7 particles virus per gram stool (Table 2) (Glass *et al.*, 1996; Matsui and Greenberg, 2001). The detection of AstVs by EM is however complicated by the occurrence in stool specimens of other SRVs in the 26-30nm size range, and the AstV star-like surface structure being present on only 10% of the viral particles (Madeley and Cosgrove, 1975; Willcocks *et al.*, 1992; Glass *et al.*, 1996; Matsui

and Greenberg, 2001). EM has also been the technique of choice for the demonstration of AstVs and AstV-like particles from animal stools (Woode *et al.*, 1984; Snodgrass and Gray, 1977; Woode and Bridger, 1978), animal intestinal tissues (Gray *et al.*, 1980; Kjeldsberg and Hem, 1985), turkey faeces (McNulty *et al.*, 1980) and cat faeces (Hoshino *et al.*, 1981).

Table 2: Sensitivity of the different techniques for the detection of HAstVs (adapted from Glass *et al.*, 1996)

Method	**Sensitivity (virus/gram stool**	**Reference**
Electron microscopy	10^{6-7}	Madeley and Cosgrove,1975
Culture	$\sim10^{2}$	Willcocks *et al.*, 1990
Enzyme immunoassay	10^{5-6}	Moe *et al.*, 1991
RNA probes	10^{5-6}	Willcocks *et al.*, 1990
DNA probes	?	
RT-PCR[1]	$\sim10^{2}$	Jonassen *et al.*, 1993

1: Reverse transcriptase-polymerase chain reaction

2.7.1.2 Immune electron microscopy

The sensitivity and specificity of EM for the detection of AstVs can be increased by the use of solid phase IEM (SPIEM), where grids are pre-coated with antisera to increase the adsorption of the virus (Kjeldsberg, 1977; Konno *et al.*, 1982; Oliver and Phillips, 1988; Willcocks *et al.*, 1991; Oishi et al., 1994; Matsui and Greenberg, 2001). Using rabbit antisera to the Oxford reference strains, IEM can also be applied to the serotyping of HAstV isolates (Kurtz and Lee, 1984; Moe *et al.*, 1991; Taylor *et al.*, 2001a). This technique was instrumental in identifying the Marin County agent as HAstV-5 (Herrmann *et al.*, 1990; Midthun *et al.*, 1993).

IEM was used for the investigation of Ab response in rabbits following the oral administration of HAstVs (Kjeldsberg and Mortensson-Egnund, 1983). The quantity of Ab was rated on a 0-4+ scale, with a 1+ change in Ab rating considered to be significant. No Ab response was recorded in rabbits after infection with AstVs (Kjeldsberg and Mortensson-Egnund, 1983).

2.7.1.3 Immunofluorescence and Immunoperoxidase assays

HAstV-Ag detection by IF, using fluorescein-labelled polyclonal antisera, proved to be a valuable technique for the initial detection, and subsequent serotyping, of the prototype HAstVs in infected cell cultures (Lee and Kurtz, 1981; Lee and Kurtz, 1982; Kurtz and Lee, 1987; Lee and Kurtz, 1994; Belliot *et al.*, 1999). IF has also been used for the detection of WT HAstV infection in cell culture (Taylor *et al.*, 1997a). IF has also been applied to determine the localization of astrovirus-specific proteins within infected CaCo-2 cell cultures. An immunoperoxidase assay, where the Ag-Ab reaction is visualized by a colour reaction, was found to be less sensitive than IF for the detection of HAstV replication in PLC/PRF/5 cells (Taylor *et al.*, 1997a).

In animal studies IF was applied for the detection of AstVs in cell cultures, infected with filtrates of intestinal contents, from experimentally infected lambs (Snodgrass and Gray, 1977). It was also used to observe the histopathological effects of an AstV containing stool extract to a calf (Woode *et al.*, 1985), and for detecting the presence of AstVs in the intestinal cells of the calf (Woode *et al.*, 1985). Field isolates from animal stool specimens have been identified as AstVs, by IF, with AstV antiserum derived from gnotobiotic calves (Woode *et al.*, 1985). Two distinct

serotypes of BAstV and possibly a third, were proposed by determining that the antiserum to each of the three BAstVs did not cross-react, as exhibited by IF (Woode *et al.*, 1985).

2.7.1.4 Enzyme immunoassays

The production of a group-specific MAb (8E7) enabled the development of a HAstV-1 to 5-specific EIA for the screening of large numbers of samples (Herrmann *et al.*, 1988). The EIA was found to be 10-100 times more sensitive than EM (Table 2) for the detection of HAstVs in stool specimens (Glass *et al.*, 1996). A more refined EIA with a biotinylated polyclonal detector Ab was developed by Moe *et al.* (1991). Both EIAs had comparable sensitivity (91%) and specificity (98%) to IEM (Matsui and Greenberg, 2001). The sensitivity of EIA is estimated to be 10^5 – 10^6 viral particles per gram stool (Matsui and Greenberg, 2001). A commercial EIA (IDEIA™ Astrovirus, DAKO Diagnostics, UK), with a sensitivity of 100% and specificity of 98,6% (McIver *et al.,* 2000) is now available for the routine detection of HAstVs (Matsui and Greenberg, 2001). The kit is specific for the detection of HAstVs and utilizes a combination of genus-specific monoclonal and polyclonal Abs that will react with and detect known strains of HAstVs (Grant *et al.*, 1996) and will not cross-react with other viral and bacterial enteric pathogens. EIA is the method of choice when large numbers of specimens need to be screened (Glass *et al.*, 1996; Matsui and Greenberg, 2001) and has increased our understanding of HAstV epidemiology (Glass *et al.*, 1996).

The serotyping of HAstVs from stool specimens was demonstrated by the development of a typing-EIA (TYPE-EIA) (Noel *et al.*, 1995), in which rabbit antisera to HAstV-1 to 7 was used as the capture

Ab and, MAb 8E7 the detector. A high degree of cross-reactivity between HAstV-3 and HAstV-1 antisera was observed due of the presence of a group antigen (Noel *et al.*, 1995).

2.7.2 Molecular assays for the detection and characterisation of HAstVs

The cloning and sequencing of the genomes from a number of HAstV genomes lead to the development of very sensitive and specific probes and RT-PCR for the detection and characterisation of HAstVs (Moe *et al.*, 1991; Jiang *et al.*, 1993; Jonassen *et al.*, 1993; Willcocks *et al.*, 1994; Matsui and Greenberg, 2001).

2.7.2.1 Hybridisation assays

A mixture of two cDNA probes was used for the detection of HAstV-1 to 5 in stool specimens (Willcocks *et al.*, 1991). The probes represented a sequence of ~1000 bp from the 3' end and ~900 bp from an internal region of the HAstV genome. The probes did not cross-hybridise with each other and did not react with echovirus 25-infected CaCo-2 cells (Willcocks *et al.*, 1991). These probes used in a nucleic acid dot-blot hybridisation assay were not as sensitive as EM for the detection of low numbers of well-preserved viral particles (Willcocks *et al.*, 1991). However this hybridisation technique was more effective than EM in identifying indistinct AstVs particles from stool specimens, and overall, detected more isolates from stool specimens than EM (Willcocks *et al.*, 1991).

A RNA probe dot-blot hybridisation assay was developed as an alternative diagnostic assay to EIA for the detection of HAstVs

from stool specimens (Moe *et al.*, 1991). The RNA probes displayed greater absolute sensitivity and could detect higher dilutions of virus in tissue culture supernatant and stool samples, but the overall sensitivity was the same as EIA, i.e. 10^{5-6} viruses per gram stool (Moe *et al.*, 1991; Glass *et al.*, 1996).

A dot-blot hybridisation assay using a digoxigenin-11-dUTP-labelled cDNA probe was developed for the detection of infectious HAstVs recovered from sewage polluted water samples (Pintó *et al.*, 1996). The detection limit of the probe, after three passages of HAstV-4 prototype strain on CaCo-2 cells was determined to be 10^2 viral particles per ml. From calculations based on these results, the assumption that WT replication in cell culture would be the same as that of the prototype strain and that 50% recovery was achieved by the MK filters, it was deduced that the hybridisation signal represented a minimum of 20 AstV particles per liter (Pintó *et al.*, 1996).

An oligonucleotide probe (30bp), designed to be homologous to nucleotide sequences in a 59 bp region amplified by two separate primer pairs at the 3′ end of the AstV genome, and used in a dot-blot hybridisation assay provided a sensitive and specific assay for the confirmation of HAstV RT-PCR amplicons (Marx *et al.*, 1998b). This oligonucleotide probe has been applied for the confirmation of HAstV RT-PCR amplicons generated from water samples (Marx *et al.*, 1998b; Taylor *et al.*, 2001b), stool specimens (Marx *et al.*, 1998a) and shellfish (Le Guyader *et al.*, 2000). A nucleotide sequence homology of ~89% was observed between PAstV and HAstV-1 in the ORF2 region amplified by the primer pair of choice (Taylor *et al.*, 2001b), but only 80% homology between the probe

and PAstV. Thus the amplification of animal AstVs using that specific primer pair could not be ruled out, but the subsequent use of the oligonucleotide probe and hybridisation to confirm the identity of the amplicons decreased the likelihood of detection of animal AstVs (Taylor *et al.*, 2001b).

A newly described liquid hybrisation assay, using broadly reactive genogroup-specific probes, enables the preliminary characterisation of HAstVs (Belliot *et al.*, 2001). No cross reactivity was detected between the two probes that were used to characterise WT HAstV strains directly from stool specimens (Belliot *et al.*, 2001). The comparative sensitivity between traditional dot-blot hybridisation assay and the liquid hybridisation was assessed. The newer method was able to detect 0,1 nanogram (ng) of target DNA as compared with only 0,01 ng by the traditional dot-blot hybridisation (Belliot *et al.*, 2001).

2.7.2.2 Reverse transcriptase-polymerase chain reaction

RT-PCR provides an accurate and specific method for detection of HAstVs from stool specimens (Jonassen *et al.*, 1993, 1995; Mitchell *et al.*, 1995; Noel *et al.*, 1995; Saito *et al.*, 1995, Marx *et al.*, 1998a; Guix *et al.*, 2002), surface water (Marx *et al.*, 1995, 1997, 1998b; Taylor *et al.*, 2001b) and wastewater (Chapron *et al.*, 2000; Egglestone *et al.*, 1999; Pintó *et al.*, 2001) samples. The estimated sensitivity of RT-PCR for the detection of HAstVs is ~ 10^2 virus particles per gram of stool specimen (Saito *et al.*, 1995; Glass *et al.*, 1996). This method is more sensitive than EIA and has been used for the confirmation of results where specimens gave ambiguous results by other assays (Grohmann *et al.*, 1993; Mitchell *et al.*, 1995; Glass *et al.*, 1996). It has also improved the

level and extent of diagnosis that was initially indicated by EIA (Mitchell *et al.*, 1995). RT-PCR requires intact nucleic acid for amplification, and thus cannot efficiently distinguish between viable or non-viable virions.

Sequences at the 3' end of the genome encoding the capsid protein exhibits a high degree of variation between serotypes, while sections in the center of ORF2 are almost totally different between serotypes, and the 3' and 5' termini are well conserved (Willcocks *et al.*, 1995). Numerous sets of type-common and type-specific primers, designed from sequences at the 3 'end of ORF2, have been used for the detection of HAstVs (Jonassen *et al.*, 1993; Mitchell *et al.*, 1995; Saito *et al.*, 1995; Yue and Ushijima, 1996; Matsui *et al.*, 1998; Walter *et al.*, 2000, 2001a). A combination of type-common primers designed from the ORF1b/ORF2 translational region (Noel *et al.*, 1995; Belliot *et al.*, 1997b), has enabled the amplification of the HAstV-1 to 7 reference strains in this ORF1b/ORF2 of the genome (Walter *et al.*, 2001b). Nucleotide sequences from a WT HAstV-8 isolate from SA have also been amplified using this set of primers (Taylor *et al.*, 2001a). ORF1a is more highly conserved than ORF2 which has fascilitated the design of a single primer pair which is able to detect all strains of HAstVs (Belliot *et al.*, 1997b).

The genetic characterisation of all eight genotypes of HAstVs has been demonstrated by type-specific RT-PCRs (Walter *et al.*, 2001a), and the sequencing of amplicons generated by selected type-common primer pairs (Saito *et al.*, 1998). The 3' end of ORF2 has been used extensively for the design of primers that enable genotyping by RT-PCR (Noel *et al.*, 1995; Matsui *et al.*, 1998;

Mitchell *et al.*, 1999a; Monroe, 1999; Medina *et al.*, 2000; Naficy *et al.*, 2000; Sakomoto *et al.*, 2000; Sakon *et al.*, 2000; Walter *et al.*, 2001a). The analysis of nucleotide sequences of amplicons generated from the ORF1b region showed no distinct groupings of the reference strains (Belliot *et al.*, 1997b), but could be used to assign a type to a WT HAstV (Taylor *et al.*, 2001a). Sequence analysis of amplicons, generated from ORF1a, grouped the HAstV-1 to 7 reference strains into two genogroups, namely genogroup A and genogroup B (Belliot *et al.*, 1997b). Using the same region for amplification nucleotide sequence analysis, a WT HAstV-8 isolate was characterised into genogroup A (Taylor *et al.*, 2001a). RT-PCR was used for the detection and subsequent analyses of a novel recombinant HAstV, of which four strains were recovered from Houston, Texas and Mexico (Walter *et al.*, 2001b). Phylogenetic analyses demonstrated that this strain as closely related to HAstV-3 in ORF1b but closest to HAstV-5 in ORF2. Nucleotide sequence analysis at the ORF1b/ORF2 junction indicated that this region of the genome could support a potential recombination site (Walter *et al.*, 2001b). The failure to determine genotypes by RT-PCR can be attributed to inhibitors in the sample, the occurrence of multiple serotypes of AstV or the variability of the 3' terminus of the genome (Mitchell *et al.*, 1999a).

From comparative studies of the sequences of the capsid region of human and animal AstVs it was reported that FAstV and PAstV shared closest nucleotide sequence identity to HAstV (Taylor *et al.*, 2001b; Wang *et al.*, 2001). Although stem-loop motifs, similar to HAstVs, were observed in the 3'-end of OAstV and TAstV, the flanking regions of these animal AstVs genomes showed little sequence similarity (Jonassen *et al.*, 1998).

Restriction fragment length polymorphism (RFLP) patterns have also been examined as a means of genotyping AstV isolates from water samples (Pintó *et al.*, 2001). RT-PCR products, generated from ORF1a and ORF2, were subjected to restriction enzyme digestion and the frequency and location of the different enzymatic sites was considered for the differentiation between strains. The different RFLP patterns, A to F, were each assigned into genogroups representing the eight serotypes of HAstVs (Pintó *et al.*, 2001).

2.8 CLINICAL ASPECTS

HAstV infection primarily manifests as gastroenteritis and affects young children worldwide. However all age groups are susceptible with the elderly and individuals of compromised immune status, at greatest risk.

2.8.1 Clinical manifestations

Children <7 years of age are principally affected (Centers for Disease Control, 1990). An incubation period of 3-4 days is most common (Kurtz *et al.*, 1979) but longer and shorter periods have been reported (Centers for Disease Control, 1990; Midthun *et al.*, 1993). The disease is characterised by watery diarrhoea lasting 2-3 days (Greenberg and Matsui, 1992) but diarrhoea, with virus shedding, may be prolonged (7-14 days) (Kurtz and Lee, 1987). The clinical symptoms include fever, headache, malaise, nausea, vomiting and abdominal discomfort which usually lasts four days (Kurtz and Lee, 1987; Greenberg and Matsui, 1992). Although

AstV infection in children is clinically milder than that of HRV (Kurtz and Lee, 1987), the co-infection of AstVs with other viruses such as HRV and HuCV, often causes confusion when assessing the epidemiology of the infection (Walter and Mitchell, 2000). AstV infection has been identified in a dual infection with verotoxin-producing *E. coli* O128: H2 (Bettelheim *et al.*, 2001).

During an outbreak of AstV-diarrhoea at a school in Japan the following features of the illness was reported: the infection was mild, abrupt and characterised by acute watery diarrhoea (72%), abdominal pain (90%) and prodromal fever (13%) (Oishi *et al.*, 1994). These symptoms persisted for 1-4 days and there was no difference observed in the frequency of the symptoms by age or sex (Oishi *et al.*, 1994). Volunteer studies on a adult who ingested a filtrate from a 0,1% stool suspension of the Marin County outbreak developed a gastrointestinal illness characterised by nausea, vomiting, diarrhoea and malaise. The illness started after 5 days of infection and lasted 36 – 48 h (Midthun *et al.*, 1993).

Persistent HAstV infections have recently been recognized in immunocompromised human immunodeficiency virus (HIV)-infected patients (Grohmann *et al.*, 1993) and bone marrow transplant recipients (Cox *et al.*, 1994; Cubitt *et al.*, 1999). Chronic HAstV diarrhoea has also been detected in a child with combined immunodeficiency who had undergone a bone marrow transplant, with the diarrhoea and virus shedding persisting for more than four months, i.e., until the child's death (Kurtz and Lee, 1987). In developing countries, children may have repeated episodes of gastroenteritis in their first few years of life, leading to an estimated 2,4 to 2,8 million deaths each year (Glass *et al.*, 2001).

The mortality rate of HAstV infection is extremely low, but deaths have been reported in young children and the elderly (Monroe, 1999).

2.8.2 Pathogenesis

A duodenal biopsy, during HAstV infection, has shown that the virus is present in the cytoplasm of columnar epithelial cells of the lower third of the villi (Phillips *et al.*, 1982; Kurtz and Lee, 1987; Kurtz, 1994; Monroe *et al.*, 2000a). AstV particles in the biopsy specimen were detected in the epithelial cells in the lower regions off the villus in a patient with sucrase isomaltase deficiency, and in the surface epithelium of a second patient who had severe enteropathy by sensitivity to cow's milk formulae (Phillips *et al.*, 1982).

Examination of the small intestine of gnotobiotic calves experimentally infected with AstVs revealed necrosis of specialised M cells overlying the Peyer's patches (Monroe *et al.*, 2000a), karyorrhexis and an increased number of intra-epithelial mononuclear cells (Woode *et al.*, 1985). In lambs, studies have shown OAstV replicating in mature enterocytes in the villi. Infected cells degenerated and were sloughed off, causing transient villous atrophy. Immature cuboidal epithelial cells from the crypts replaced the lost cells (Kurtz, 1994). A fatal hepatitis is described in ducks (Gough *et al.,* 1984). Avian nephritis virus (ANV) causes a highly pathogenic multisystem disease (Imada *et al.*, 2000).

2.8.3 Assays for the routine diagnosis of HAstV infection

2.8.3.1 Viral detection

For 15 years after the first description of AstVs (Appleton and Higgins, 1975) EM remained the only method for the routine diagnosis of HAstV infection (Glass *et al.*, 1996). The awareness of the importance of AstVs as a cause of infantile diarrhoea has changed as more sensitive detection methods have been developed in recent years (Kurtz, 1994; Monroe *et al.*, 2001). A commercial EIA kit is now available and is the method of choice for diagnostic purposes. It is more sensitive than EM, and enables a quick but effective method of screening large numbers of samples (Matsui and Greenberg, 2001).

2.8.3.2 Serology

Immunosorbent EM (ISEM) (Kurtz and Lee, 1978; Konno *et al.*, 1982), radio immunoassay (RIA) (Wilson and Cubitt, 1988; Midthun *et al.*, 1993), IF (Kurtz and Lee, 1978; Kriston *et al.*, 1996) and type-specific EIAs (Mitchell *et al.*, 1999b) assays have been developed for the serodiagnosis of HAstV infection (Glass *et al.*, 1996). These techniques are, however, used for seroepidemiological research rather than for the routine diagnosis of HAstV infection (Kurtz, 1994).

2.8.3.3 Viral isolation

Although HAstVs have successfully been propagated in a number of cell culture systems (Willcocks *et al.*, 1990; Taylor *et al.*,1997a; Brinker *et al.*, 2000; Mustafa *et al.*, 1998) these techniques are

time-consuming for the routine diagnosis of HAstV infection (Glass *et al.*, 1996).

2.8.4 Treatment

HAstV gastroenteritis is usually mild, self-limiting and may disrupt a person's activities for a few days but does not require specific therapy. Oral or intravenous fluid resuscitation may rarely be required. Dehydration may develop in patients with underlying gastrointestinal disease (Kurtz and Lee, 1987; Cruz *et al.*, 1992; Matsui and Greenberg, 2001).

2.9 EPIDEMIOLOGY

HAstV infections occur throughout the world, typically in the winter months in temperate climates (Kurtz, 1994), and in the rainy season in more tropical climates (Matsui and Greenberg, 2001). HAstVs are associated with 2 to 8% of severe diarrhoeal episodes in children (Mitchell *et al.*, 1999a). Infection is usually in young children with diarrhoea (Appleton and Higgins, 1975; Madeley and Cosgrove, 1975; Ashley *et al.*, 1978; Konno *et al.*, 1982; Spence, 1983; Singh *et al.*, 1989; Herrmann *et al.*, 1991; Lew *et al.*, 1991; Moe *et al.*, 1991; Mitchell *et al.*, 1993; 1999a; Glass *et al.*, 1996; Steele *et al.*, 1998; Unicomb *et al.*, 1998; Mustafa *et al.*, 2000; Naficy *et al.*, 2000), with the elderly (Gray *et al.*, 1987) and immunocompromised individuals at high risk of infection (Grohmann *et al.*, 1993; Cox *et al.*, 1994; Shastri *et al.*, 1998; Yuen *et al.*, 1998; Cubitt *et al.*, 1999; Coppo *et al.*, 2000; Trevino *et al.*, 2001).

Infection is often reported in children younger than 5 years of age (Walter and Mitchell, 2000; Qiao *et al.*, 1999). Co-infection with HRV and HuCV has been detected with AstV infection (Taylor *et al.*, 1997b; McIver *et al.*, 2000; Walter and Mitchell, 2000). HAstVs are responsible for more than 7% of diarrhoeal outbreaks in CCCs (Lew *et al.*, 1991; Mitchell *et* al., 1995; 1999a) as well as in institutions such as hospitals for the elderly and military units (Gray *et al.*, 1987; Palombo and Bishop, 1996; Belliot *et al.*, 1997a).

Seroprevalence studies indicate that most children are infected during the first two years of life (Glass *et al.*, 1996; Maldonado *et al.*, 1998; Mitchell *et al.*, 1999b; Walter *et al.*, 2001a). An antibody prevalence study has shown that 64% of 3-4 yr olds and 87% of 5-10 yr olds had been affected (Kurtz and Lee, 1978; Mitchell *et al.*, 1999b). A seroprevalence of 40% in a small group of adults was reported (Midthun *et al.*, 1993). Studies of selected populations it was reported that 93% of surfers in the UK showed serologic evidence of increased exposure to HAstV-4 (Myint *et al.*, 1994). In an age stratified sample in the Netherlands the seroprevalence of neutralization antibodies was reported to be the highest for HAstV-1 and the lowest for HAstV-7 (Koopmans *et al.*, 1998). A lack of heterotypic immunity between the different types of HAstVs was implied when a patient had a second infection of a different type of HAstV, nine months following the initial infection (Guix *et al.*, 2002).

2.9.1 Routes of transmission

Transmission is typically via the faecal-oral route and is both community acquired (Madeley *et al.*, 1977; Jonassen *et al.*, 1993;

Taylor *et al.*, 1997b) and nosocomial (Appleton and Higgins, 1975; Madeley and Cosgrove, 1975; Lee and Kurtz, 1977; Esahli *et al.*, 1991; Bennet *et al.*, 1995; Shastri *et al.*, 1998). Person-to-person spread is seen in nurseries, paediatric wards, families (Kurtz, 1994), homes for the elderly (Oshiro *et al.*, 1981; Herrmann *et al.*, 1990; Midthun *et al.*, 1993), and in a military camp (Belliot *et al.*, 1997a). Outbreaks have been associated with the consumption of faecally-contaminated shellfish (Kurtz and Lee, 1987) and drinking water (Cubitt, 1991). AstVs are able to survive on inert surfaces long enough to suggest that formites may play a relevant role in the secondary transmission of AstV diarrhoea (Abad *et al.*, 2001).

2.9.1.1 Role of food and water in the transmission of HAstVs

Although the transmission of HAstVs via food and water is considered to be a rare event (Kurtz, 1994), a foodborne outbreak, in Osaka Japan, represented the largest outbreak of AstV-associated gastroenteritis ever reported (Oishi *et al.*, 1994). More than 4 700 persons in ten primary and four junior high schools in Kantano City were affected. The outbreak was spread by contaminated food from kitchens catering for the school lunch system. HAstV-6 was later identified as the causative agent (Sakon *et al.*, 2000).

The survival of AstVs in aquatic environments has been demonstrated by the detection of infectious AstVs in environmental water (Abad *et al.*, 1997) and at sites of sewage-polluted environmental waters (Pintó *et al.*, 1996; Marx *et al.*, 1998b), suggesting that water could be a source of HAstV infection. AstVs have also been detected in seawater (Pintó *et al.*, 1996), sludge biosolids (Chapron *et al.*, 2000) and wastewater (Pintó *et al.*,

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2001). AstVs have been detected in water that was thought to have passed all acceptable bacteriological standard tests (Pinto *et al.*, 1995). The survival rates of HAstVs in water relative to the addition of chlorine have been demonstrated (Abad *et al.*, 1994). It has been suggested that AstVs detected in water environments may be an accurate indicator of the serotypes of AstVs circulating in the community (Pintó *et al.*, 2001). It has been suggested that the absence of HAstV RNA in sewage and environmental samples as reported by Egglestone *et al.* (1999) was due to lack of prior amplification of the virus in cell culture (Chapron *et al.*, 2000).

2.9.2 Prevention and control

The interruption of transmission is an essential element, if not the key factor in preventing AstV infection (Matsui and Greenberg, 2001). Efficient hygienic procedures must be enforced in settings where close person-to-person contact occurs such as hospitals, CCCs and other institutions (Matsui and Greenberg, 2001). Chemical agents such as methanol can be effectively used as a disinfectant (Kurtz *et al.*, 1980). Food handlers must be educated about the dangers existing with their personal incidences of diarrhoeal diseases and need to be aware that the shedding of virus particles in stool specimens may last longer than the symptoms of diarrhoea (Coppo *et al.*, 2000). Foods such as shellfish, which has been associated with outbreaks of AstV-gastroenteritis should be carefully selected and prepared (Matsui and Greenberg, 2001).

2.10 ECONOMIC IMPACT

Although not a debilitating disease, the economic implication of absence from work as the result of a diarrhoeal condition, and hospitalisation for some individuals is important. In the industrialized world, people of all ages have about one episode of gastroenteritis each year, resulting in many hospitalisations (Glass *et al.*, 2001). The annual public and private direct health care costs in SA incurred due to diarrhoea are at least R3,0 billion, and the total social cost of diarrhoeal disease is at least R3,4 billion in SA (Pegram *et al.*, 1998). The contribution of HAstV to the overall burden of gastrointestinal disease and economic impact thereof has yet to be established.

2.11 OCCURRENCE OF AstVs IN SOUTH AFRICA

The occurrence of HAstV infection in SA was first documented in 1983 (Spence, 1983). Recent studies have recorded a prevalence of 5-7% of infection among hospitalised patients in the Tshwane (Pretoria) Metropolitan Area (Steele *et al.*, 1998; Marx *et al.*, 1998a). Seroprevalence studies demonstrated the presence of Abs in a Zulu population from SA, with a similar Ab acquisition pattern to that of the UK (Kurtz and Lee, 1987). As has been found in other regions of the world, HAstVs are the second most important viral diarrhoeal pathogen, surpassed only by HRV (Marx *et al.*, 1998a). HAstVs were also identified as one of a number of viral pathogens associated with an outbreak of gastroenteritis in a Pretoria CCC (Taylor *et al.*, 1997b). Although a HAstV-8 was recovered from a 31 month old patient with gastroenteritis (Taylor

et al., 2001a) and HAstV-6 was isolated from an elderly patient with gastroenteritis, there is no data on the distribution of serotypes in SA.

AstVs have been detected in surface water samples (Marx *et al.*, 1995; 1998b; Taylor *et al.*, 2001b), hospital wastewater (Marx *et al.*, 1998b) by an integrated cell culture RT-PCR-oligonucleotide probe hybridisation assay. There was however no indication with regard to the host source of these environmental AstV strains or what their contribution was to the overall burden of HAstV infection. The potential risk of AstV infection in communities using these water sources for domestic and/or recreational purposes is therefore uncertain.

AIMS OF THIS INVESTIGATION

The hypothesis for this study was that AstVs found in surface waters are distinct from those recovered from clinical specimens in the same geographic region and at the same time.

To confirm or disprove this hypothesis, the individual objectives of the study were:

1. To establish which HAstV serotypes are primarily responsible for gastroenteritis in selected clinical settings;

2. To screen faecal specimens from animals with scours for AstVs to obtain reference material for further characterisation and comparative studies;

3. To characterise AstVs from water sources used for domestic and recreational purposes and compare them to HAstVs from stool specimens to ascertain whether or not the same strains and/or serotypes were associated with human infection.

To achieve these goals there was a need to assess cell culture techniques and optimise RT-PCR for the detection and characterisation of AstVs.

CHAPTER 3

MATERIALS AND METHODS

3.1 STUDY SAMPLE

Samples included in the study were human stool specimens from hospitalised patients with gastroenteritis, stools from animals with and without scours, and sewage and surface water samples. AstV isolates from dam and river water referred for routine viral analysis were also included.

3.1.1 Human stool specimens

HAstV-positive human stool specimens, detected among specimens referred from two tertiary hospitals in the Tshwane (Pretoria) Metropolitan Area, Gauteng, to the Department of Medical Virology, University of Pretoria for routine diagnostic analysis for gastroenteritis viruses, namely HRV, adenovirus 40/41 (HAdV-40/41) and HAstV, served as the source of clinical AstV isolates. After analysis, the stools were stored at 4°C. A 10 % stool suspension, in phosphate-buffered saline (PBS) (Sigma Chemical Co., St Louis, MO) was prepared from the stored stool specimens for further investigation. Where insufficient sample was available for analysis, selected specimens were propagated on cell culture.

3.1.2 Animal stool specimens

Animal stool specimens were collected from a variety of domestic and farm animals and birds in the Gauteng region. Specimens from healthy animals as well as from animals with scours were included. Stools were collected from areas where they could have been potential water contaminants. After collection the stools were stored at 4°C. A 10 % stool suspension, in PBS (Sigma) was prepared from the stored stool specimens for further investigation.

3.1.3 Water and sewage samples

Water and sewage samples were collected from sewage works serving the Tshwane Metropolitan Area, and surface flows downstream of the sewage works. Samples from various stages of the sewage treatment process were collected. The Zeekoegat wastewater treatment plant discharges directly upstream of the Roodeplaat Dam, which is a water-collection area. Where indeterminate AstV detection and characterisation results were obtained, AstVs in selected water and sewage samples were amplified by propagation in cell culture.

3.1.4 AstV isolates from river, dam and sewage water samples

AstV isolates from water samples from the Gauteng region, referred to the Department of Medical Virology for routine virological analysis, were included in the study. The water samples included dam water (A18) which was used for domestic and recreational purposes as well as being the source for a water

purification works. The river water sample (K19) is used by lower socio-economic communities in informal settlements for domestic purposes. Additional AstV isolates, detected in drinking and wastewater from other geographic regions of SA, namely Bloemfontein (BL; S; FS), Windhoek (WH), Natal (U; UW) and Eastern Cape (FH), Venda (V), Gauteng (R; B), Western Cape (K; C) were included for analysis. HAstV positive cell cultures were retested for the presence of HAstV-RNA and if necessary re-inoculated onto fresh cell cultures to further amplify the HAstV RNA.

3.2 VIRAL RECOVERY AND CONCENTRATION

3.2.1 Glass wool adsorption-elution procedure

Viruses were recovered from water samples (>10L) using a glass wool adsorption-elution technique (Grabow *et al.*, 1993; Wolfaardt *et al.*, 1995). Essentially the sample was filtered through the glass wool at a flow rate of 10L/h. Negatively charged viruses adsorbed to the positively charged glass wool. Viruses were then eluted from the glass wool by an alkaline glycine-beef-extract-buffer (GBEB) (pH 9,5), which reverses the ionic charge on the virus. The pH of the eluate was then adjusted to pH7,0 using 1N HCl (Appendix A.1).

3.2.2 Polyethylene glycol/sodium chloride concentration technique

Viruses were recovered from sewage samples or reconcentrated from the glass wool eluate by precipitation with polyethylene glycol

(PEG) (PEG 6000, Merck, Darmstadt, Germany) in the presence of sodium chloride (NaCl) (Merck) as described by Minor (1985) for the concentration of picornaviruses (Appendix A.2).

3.2.3 Ultrafitration

Following PEG/NaCl viral concentration viruses were recovered from the 10 ml concentrate in a final volume of 1 ml by ultrafiltration using a Biomax-100K NMWL membrane (Ultrafree® 15 Centrifugal Filter Device, Millipore Corporation, Bedford, MA), as per manufacturer's instructions. The membrane retains a specified sized particle while allowing smaller particles and buffer to flow through. The final viral concentrate was stored at -70°C.

3.3 CELL CULTURE PROCEDURES

3.3.1 Cell cultures

AstVs from different species reportedly can only be isolated in cell culture of the species of origin (Brinker *at al.*, 2000). Therefore cell cultures of both human and bovine origin were used in this investigation. Cell cultures of ovine, porcine or avian origin were not available and therefore not used in this investigation.

3.3.1.1 Human colonic carcinoma cell line

The continuous colonic carcinoma (CaCo-2) cell line (ATCC HTB 37), has been shown to support the isolation and propagation of HAstVs (Willcocks *et al.*, 1990; Mustafa *et al.*, 1998) as well as

other enteric viruses (Pintó *et al.*, 1994). Cell cultures were used at low (35 - 58) and high (178 to 198) passages.

3.3.1.2 Human hepatoma cell line

The primary liver carcinoma cell line, PLC/PRF/5 (ATCC 8024), which supports the growth of HAstVs in the presence of trypsin (Marx *et al.*, 1995; Taylor *et al.*, 1997a) was used between passages 81 to 85 and 100 to 112.

3.3.1.3 Madin-Darby Bovine Kidney cell line

The Madin-Darby Bovine Kidney (MDBK) cell line (ATCC CRL 22), derived from the kidney of an adult steer, was used between passages 117 and 126. Bovine kidney (BK) cells have been previously shown to support the growth of BAstVs (Aroonprasert *et al.*, 1989).

3.3.2 Media and reagents

Each cell line has its own unique requirements with regard to growth and maintenance media (Grist *et al.*, 1979; Ham and McKeenan, 1979).

3.3.2.1 Serum

a) Foetal Calf Serum: The foetal calf serum (FCS) (Delta Bioproducts, Kempton Park, RSA) was decomplemented for 30 min at 56°C, filter-sterilised and stored at -20°C. The FCS was screened for mycoplasmas and bovine viruses and contained less than 2 EU/ml endotoxins.

b) Horse Serum: Aliquots of horse serum (HS) (Bio Whittaker, Walkersville, MD) were filter-sterilised and stored at –20°C.

3.3.2.2 Growth media

Eagle's Minimum Essential Medium with Earle's salts (E-MEM), L-glutamine, non-essential amino acids, penicillin (100 U/ml) and streptomycin (100 µg/ml) (National Institute for Virology [NIV], Sandringham, RSA) supplemented with serum was used for propagation of all cell cultures. The E-MEM was supplemented with 10 % HS for MDBK cell cultures whereas both PLC/PRF/5 and CaCo-2 cells used E-MEM supplemented with 8% and 15% FCS respectively.

3.3.2.3 Maintenance media

E-MEM supplemented by 5% FCS was used for the maintenance of the PLC/PRF/5 cell cultures, 10% FCS for the CaCo-2 cell cultures and 2 % HS for MDBK cell cultures.

3.3.2.4 Cryopreservation medium

The media used for the cryopreservation of cells was prepared using double strength E-MEM with Earle's salts, with the addition of 40% serum, 10% glycerol (Sigma), penicillin (100 U/ml) and streptomycin (100µg/ml) (GIBCOBRL Life Technologies Ltd., Paisley, Scotland).

3.3.2.5 Starvation medium

Serum-free E-MEM was used as starvation medium prior to the infection of CaCo-2, PLC/PRF/5 and MDBK cells cultures.

3.3.2.6 Trypsin-EDTA

The trypsin-EDTA solution used for the dispersion of the cells contained 0,25% trypsin and 0,05% EDTA diluted in Ca^{2+}-free

Dulbecco buffer supplemented with 200 U/ml penicillin and 100 μg/ml streptomycin (NIV).

3.3.3 Subculturing of cell cultures

The cells were detached from the surface of a confluent flask by the addition of trypsin-EDTA and incubation for 1-3 min at 37°C. The trypsin-EDTA was inactivated by the addition of an equal volume of growth medium and the cells were then gently centrifuged, for 2 min at 2000 rpm (Rota Uni II, BHG, Germany), to form a loose pellet. The pellet was resuspended in fresh growth medium at a concentration of 10^5 cells/ml, and the resultant suspension of cells was distributed evenly among the required number of cell culture flasks. The cells were incubated at 37°C in a humidified 5% CO_2 atmosphere. Fresh growth media was added to the cells in their logarithmic phase to ensure a healthy confluent monolayer.

3.3.4 Maintainence of cell cultures

Maintenance medium, with reduced concentrations of FCS and HS, was used for the maintenance of confluent monolayers of cells.

3.3.5 Cryopreservation of cells

All cells in excess of those immediately required were frozen and stored at -70°C. Monolayers of cells were detached from the flask using trypsin-EDTA, the trypsin neutralised by the addition of an equal volume of growth medium. The detached cells were pelleted by gentle centrifugation for 2 min at 2000 rpm (Rota Uni II). All

traces of growth medium were removed from the pellet and the cells were then resuspended in 1 ml cryopreservation medium. Cells were frozen down gradually in cryovials. This process entailed the vials being held at 4°C for 2 h, then at −20°C for 2 h and finally stored at −70°C.

3.3.6 Revival of cryopreserved cells

Frozen cell cultures were thawed rapidly at 37°C. Pre-warmed growth medium was used to gently reconstitute the thawed cells and the suspension was gently centrifuged to form a loose pellet of cells. The cells were resuspended in 2 ml growth medium and seeded into 25 cm^2 cell culture flasks containing a thin coating of serum. The flasks were incubated undisturbed for 48 h at 37°C. After adsorption of the cells the medium was withdrawn and fresh growth medium was then added to the flask.

3.3.7 Infection of cell cultures

3.3.7.1 Sample preparation

Stool suspensions (10% wet mass in PBS) were treated with penicillin (50µg/ml), streptomycin (50µg/ml) and neomycin (100 µg/ml)(PSN antibiotic mixture [100X]: GIBCOBRL) and 100 units/ml nystatin (Nystatin [100X]: GIBCOBRL), for 1 h at 37°C or overnight at 4°C, prior to infection of cells. Solid material in the treated specimens was precipitated by centrifugation for 5 min at 14 000 rpm (Eppendorf 5413 microcentrifuge) and the supernatant retained for infection procedures. Concentrates of water and sewage samples from the viral recovery procedures were subjected to the same treatment prior to infection of the cell cultures.

3.3.7.2 Infection procedure

For infection of the cells, 25cm^2 flasks were seeded with 5 ml 10^5 cells/ml and incubated at 37°C overnight. The propagation medium was withdrawn from the semi-confluent monolayers, which were washed once with PBS. The cells were starved for 1 h at 37°C in starvation media. The prepared cells were infected with 250 μl of treated stool, water or cell culture suspension and allowed to adsorb for 1 h at 37°C, with gentle rotation at 15 min intervals. The cells were rinsed to remove unattached virus and then 5ml serum-free E-MEM containing 10μg/ml trypsin (Porcine Pancreas Type II, Sigma) was added to each flask. The infected cells were incubated at 37 °C and harvested after 7 days p.i. For subsequent blind passages of the virus, the same procedure was followed using 0,5 ml of infected cell culture material.

3.3.8 Harvesting of infected cell cultures

Cells were harvested 7 days p.i. by vigorous aspiration of the cell culture suspension using a pasteur pipette. This served to create a uniform suspension of infected cells. An aliquot of the suspension was used for AstV detection and the surplus stored at -20°C.

3.3.9 Reference HAstV strains

Cell culture suspensions of the Oxford reference strains of HAstV-1 to HAstV-7 were kindly supplied by Dr DW Cubitt, Great Ormond street Hospital for Sick Children, London, and Prof JE Walter, Center for Pediatric Research, Eastern Virginia Medical School, Norfolk, Virginia. These viruses were further propagated in CaCo-2

cell culture and stored at –70°C. For HAstV-8, a stool suspension of a SA strain (AS20) was used (Taylor *et al.*, 2001a).

3.3.10 Assessment of cell cultures for the isolation of AstVs

Cells cultures of human origin, namely CaCo-2 and PLC/PRF/5, and bovine origin, namely MDBK were assessed for their susceptibility to HAstV infection. No WT or reference animal AstVs were available for cell culture analysis

Cell culture suspensions of HAstV cell culture-adapted prototype viruses, namely HAstV-1, HAstV-2 and HAstV-3; and stool specimens AS21, AS20 G912, G927, G1821 and MRC1086, containing WT strains of AstVs not adapted to growth in cell cultures were tested. Two flasks of the three cell types were infected with 250 μl each of the reference cell culture suspensions and stool suspensions (WT HAstVs). The infected cell cultures were incubated at 37°C and harvested seven days p.i. For selected viruses an aliquot of each of the harvested cell extracts was passaged onto MDBK cells and incubated for a further seven days.

Aliquots of the cell suspensions were tested for the presence of AstV by EIA and RT-PCR using the type-common primer pairs Mon2/Mon67 and Mon348/Mon340. Oligonucleotide probe hybridisation was performed to confirm the RT-PCR results obtained using primer set Mon2/Mon67. Separate aliquots of the suspensions were stored at -20°C.

3.4 VIRAL DETECTION

Because of their surface stars AstVs are clearly distinguishable by EM. This is, however, not a method without shortcomings, as only about 10% of viral particles exhibit the surface star (Matsui and Greenberg, 2001). Sample preparation can also affect the EM appearance of the viral particle (Woode *et al.*, 1985).

3.4.1 Electron microscopy

Faecal samples were prepared by mixing a drop of stool sample with 20 - 50µl 2% phosphotungstic acid (pH 6.8). The stained stool suspension was placed on a 400-mesh Formvar carbon-coated grid. After removing the excess fluid with filter paper, the grid was air dried and examined in a Philips 300 electron microscope (Taylor *et al.,* 1993).

3.5 ANTIGEN DETECTION

Antigenic epitopes located on the surface of the AstV particles provide a means for detection of AstVs in stool specimens, water sample concentrates and cell culture extracts by immunological methods. All stool samples and water and sewage sample concentrates were screened for the presence of AstVs by EIA.

3.5.1 Enzyme immunoassay

A commercial EIA method (IDEIA™ Astrovirus, DAKO) was used for the detection of AstV Ag in stool specimens, water concentrates

and cell culture suspensions. This kit is a qualitative EIA designed for the detection of HAstVs from human stools.

Briefly the principle of the test is as follows:
Murine monoclonal antibodies (MAbs), that recognize group Ags on the surface of HAstV-1 to 8, are coated onto the base of the wells of an EIA plate (capture Ab). During incubation of the sample in the well of the EIA plate, viral Ag present in the specimen binds to the immobilized MAb in the well. A polyclonal rabbit Ab directed against the viral Ag was used as the detector antibody. Excess unbound rabbit polyclonal Ab was rinsed away and anti-rabbit IgG-horse radish peroxidase (HRP) conjugate was added. After incubation and a washing step, a chromogen, 3,3',5,5'-tetramethylbenzidine (TMB) was added to the rinsed wells. The enzymatic reaction between the HRP from the bound conjugate and the chromogen produced a blue colour. If no conjugate had bound there would be no colour reaction and this was observed as a clear well. The reaction was stopped by the addition of H_2SO_4.

Specimens and samples with an absorbance value less than the cut-off were considered negative and an absorbance value greater than the cut-off, positive. Guidelines of the kit recommend that for any results within 0,010 absorbance units of the cut-off value, the test should be repeated or sample retaken.

3.6 MOLECULAR DETECTION

Molecular-based techniques such as RT-PCR are now commonly used to detect low titres of HAstVs in clinical specimens (Mitchell

et al., 1995; Guiz ***et al.***, 2002), water and sewage samples (Egglestone ***et al.***, 1999; Pintó ***et al.***, 2001; Taylor ***et al.***, 2001b) and shellfish (Le Guyader ***et al.***, 2000). Intact viable and non-viable viral particles, containing undegraded RNA, are detected by these assays. All stool samples, water and sewage sample concentrates and cell culture suspensions of AstV isolates, were tested for the presence of AstVs by RT-PCR using the type-common primer pair Mom2/Mon67. The amplicons were confirmed as HAstV by oligonucleotide probe hybridisation assay.

3.6.1 RNA extraction

Total RNA was extracted from pretreated stool specimens, water samples, sewage concentrates and infected cell culture material using the method most suitable for the type of specimen being processed. Three different RNA extraction methods were used. A negative extraction control (ultrapure water) was included with each extraction to ensure that there was no contamination during the procedure.

3.6.1.1 Sample preparation

a) ***Stool specimens***: An equal volume of Freon or Genetron[R] (1,1,2-trichloro-trifluoro-ethane; Sigma) was added to the appropriate volume of stool suspension (10 % in PBS) and mixed thoroughly by vortexing followed by centrifugation at 14 000 rpm (Eppendorf 5402 microcentrifuge) for 5 min. RNA was extracted from the clarified supernatant.

b) ***Infected cell cultures***: Harvested cell culture suspensions (1 ml) were concentrated by centrifugation at 45 000 rpm (Beckman TL–100 Ultracentrifuge) for 1 h at 4 °C. The supernatant was

discarded and the concentrated cell pellet resuspended in the volume of nuclease-free water or lysis buffer.

3.6.1.2 QIAamp Viral RNA Mini Kit

Viral RNA was extracted from cell free material, namely treated stool specimens and infected cell culture supernatants using spin column technology (QIAamp® Viral RNA Mini Kit; QIAGEN GmbH, Hilden, Germany), according to the manufacturer's protocol.

The principle of the RNA extraction using this kit was as follows: The viral particles in the treated sample were lysed in a denaturing buffer containing chaotropic salt which inactivated RNases and preserved intact viral RNA. The lysate was then applied to the membrane. Carrier RNA, contained in the buffer enhanced the binding of the viral RNA to the silica-gel based membrane. Buffering conditions of the sample lysate were adjusted such that the isolated viral RNA bound optimally to the membrane. The salt concentrations and pH values of the lysate ensured that protein and other contaminants were not retained on the membrane but rinsed away during the two washing steps by buffers. The RNA was eluted in 60 µl nuclease-free water (Promega Corp., Madison, WI) or AVL buffer (QIAGEN).

3.6.1.3 TRIZOL® RNA extraction

TRIZOL® reagent (GIBCOBRL) was a mono-phasic solution of phenol and guanidine isothiocyanate, which facilitates rapid total RNA extraction from treated stool specimens, concentrated water samples and infected cell culture material. The TRIZOL® reagent disperses starting material while maintaining the integrity of the RNA. Cells were disrupted and cell components were dissolved.

Chloroform was added and after centrifugation the solution separated into an organic phase and aqueous phase. RNA was precipitated from the upper aqueous phase with isopropyl alcohol. As a modification to the method recommended by the manufacturer, 3M sodium acetate (pH 8.3) (seeDNA™ co-precipitant; Amersham International plc, Little Chalfont, England) was used in the final precipitation step. The pellet comprising the protein-free RNA was dissolved in 30µl nuclease-free water (Promega).

3.6.1.4 RNeasy Mini Kit

The principle of the RNeasy Mini Kit was based on the combination of microspin technology and the selective binding properties of a silica-gel based membrane.

Harvested cell culture suspensions were disrupted in the presence of a lysating buffer and homogenised in a QIAshredder (QIAGEN). Total RNA was extracted from the homogenate using the spin columns provided, as per manufacturer's instructions. DNA was removed using RNase-free DNase (RNase-free DNase Set; QIAGEN) as recommended by the manufacturer.

A specialized high-salt buffer system in which the homogenised lysate was diluted allows RNA longer than 200 bases to bind to the silica-gel based membrane. Smaller RNA molecules such as ribosomal RNA (rRNA) and transfer RNA (tRNA) were able to pass through the column while viral RNA remains bound. Washing steps effectively rinse away these unwanted RNAs leaving high quality RNA in the spin column that is eluted in 30 µl nuclease-free water (Promega).

3.6.2 Oligonucleotide Primers

HAstVs can be detected using type-common primers which amplify the variable region of the capsid protein, i.e. the 3′ end of ORF2 (Mitchell *et al.,* 1995; Saito *et al.,* 1995, Walter *et al*., 2001a) or a 289 bp region of ORF1a (Belliot *et al,* 1997b). Specific HAstV serotypes can be detected using type-specific primers which also amplify regions at the 3′ end of ORF2 (Walter *et al.,* 2001a). The primers used in this study were synthesised and purified by Sigma-Genosys Ltd., Pampisford, UK.

3.6.2.1 Type-common primers

a) *Mon2/Mon67*: A published primer pair (Mon2 reverse primer: 5′-GCT TCT GAT TAA ATC AAT TTT-3′; Mon67 forward primer: 5′-CGA GTA GGA TCG AGG GTA-3′; Mitchell *et al*., 1995) which amplify a 89 bp region of the capsid gene (nt 6909-6797; HAstV-2 [L13745.1]) was used for the screening of specimens and samples for HAstVs.

b) *Mon348/Mon340*: This set of primers (Mon348 reverse primer: 5′-ACA TGT GCT GCT GTT ACT ATG-3′; Mon340 forward primer: 5′-CGT CAT TAT TTG TTG TCA TAC-3′; Belliot *et al.,* 1997b) amplifes a 289 bp within ORF1a region (nt 1470-1182; HAstV-2 [L137451]) was used for the detection of HAstV RNA in HAstV Ag positive samples and specimens found to be HAstV RNA negative using the Mon2/Mon67 primer pair. This primer pair has previously been shown to be specific for the amplification of HAstVs (Belliot *et al*., 1997b).

c) *Mon2/prBEG*: this primer pair (Mon2 reverse primer: prBEG forward primer: 5′-ACC GTG TAA CCC TCC TCT C-3′; Saito *et al*., 1995) amplifies a 319 bp region at the 3′ end of ORF2 (nt 6453-

6771; HAstV-1 [L23513.1]) of all HAstVs except type 4. This primer pair has been shown previously to be specific for HAstVs (Saito *et al.*, 1995).

3.6.2.2 Type-specific primers

a) HAstV-1: Mon2/AV3: (Mon2 reverse primer: AV3 forward primer: 5′-ATG CCT TTG CCT GAG TCC AC-3′; Jonassen *et al.*, 1993) amplifies a 563 bp region at the 3′ end of ORF2 (maps to nt 6209-6771 in HAstV-1 [L23513.1]).

Mon2/pr6151: (Mon2 reverse primer: pr6151 primer: 5′-ATC TAT TGT TGA TGG GGC TA-3′; Mitchell *et al.*, 1999a) amplifies a 666 bp region at the 3′ end of ORF2 (nt 6106-6771, HAstV-1 [L23513.1]).

b) HAstV-2: Mon 2/pr6257: (Mon2 reverse primer: pr6257 forward primer: 5′-ACA TTG CCC AGA ATT TC-3′; Mitchell *et al.*, 1999a) amplifies a 541 bp region at the 3′ end of ORF2 (nt 6257-6797, HAstV-2 [L13745.1]).

c) HAstV-3: Mon2/DM12: (Mon2 reverse primer: DM12 forward primer: 5′-CTA GTG AGG AAC CTG ACA CCC ATG-3′ (nt 6306-6329, HAstV-3 [AF141381]); Walter *et al.*, 2001a) amplifies a 540 bp region at the 3′ end of ORF2.

d) HAstV-4: Mon2/JWT4: (Mon2 reverse primer: JWT4 forward primer: 5′-GCA GAG AGC TTG TTA TTA AC-3′; (nt 2084-2103, HAstV-4 [Z33883]), amplifies a 332 bp region at the 3′ end of ORF2 (Walter *et al.*, 2001a).

e) HAstV-5: Mon2/AstS5: (Mon2 reverse primer: AstS5 forward primer: 5′-TAG TAA CTT ATG ATA GCC-3′, amplifies a 480 bp region at the 3′ end of ORF2 (Matsui *et al.*, 1998).

f) *HAstV-6:* *Mon2/AstS6*: (Mon2 reverse primer: AstS6 forward primer: 5'-TGG CCA CCC TTG TTC CTC AGA-3', amplifies a 500 bp region at the 3' end of ORF2 (Matsui *et al.*, 1998).

g) *HAstV-7:* *Mon2/DM11*: (Mon2 reverse primer: DM11 forward primer: 5'-GGC AGA TGT GTT GGA ACT TCC C-3'; nt 2007-2028, HAstV-7 [AF248738] amplifies a ~500 bp region at the 3' end of ORF2 (Walter *et al.*, 2001a).

3.6.3 Optimisation of RT-PCR

The RT-PCR reactions used for the detection and characterisation of AstV isolates were optimised using the Opti-Prime™ PCR Optimization Kit (Stratagene) as per manufacturer instructions. This kit identifies the buffer system that will provide the highest specificity and yield for a PCR. In addition other commercially available PCR reaction buffers, with modified $MgCl_2$ concentrations, were also assessed. The composition of the final buffers used for the optimisation are given in table 3.

RNA of the Oxford reference type strains of HAstV-1 to 7 was used for optimising type-specific RT-PCR. Stool samples containing WT strains of HAstV-1 (99022320) and HAstV-8 (AS20) were used as RNA source for the optimisation type-common RT-PCRs, with primer pairs Mon2/prBEG and Mon348/Mon340, respectively.

3.6.4 Amplification by RT-PCR

To exclude the possibility of cross-contamination, reagents for the RT-PCR were prepared in a laminar flow cabinet. The RT-PCR reactions were carried out in rooms separately from those used for

Table 3: Composition of different PCR buffers tested in the RT-PCR optimisation reactions.

Buffer (10X)	Tris-HCl (mM)	pH	KCl (mM)	$MgCl_2$ (mM)
Roche[1]	100	8.3	500	15
Promega[2]	100	9.0	500	0
Opti-Prime[3] 1	100	8.3	250	15
Opti-Prime 2	100	8.3	750	15
Roche	100	8.3	500	30
Promega	100	9.0	500	20
Promega	100	9.0	500	30
Opti-Prime 1+2	100	8.3	500	30

1: PCR buffer (Roche Molecular Biochemicals, Mannheim, Germany)
2: PCR buffer (Promega Corp., Madison, WI)
3: Opti-Prime™ PCR Optimization Kit (Stratagene, La Jolla, CA)

the initial processing of the water and stool specimens and the analysis of the amplicons. A positive control (cell-cultured HAstV-1 to 8), negative control (ultrapure water) and negative extraction control were included in all RT-PCR reactions. Unless stated to the contrary, RT-PCR reagents were from Promega Corp.

3.6.4.1 Mon2/Mon67

Reverse transcription (RT) was performed in a 48 µl reaction cocktail. The cocktail contained 5 µl extracted RNA, 5 U of avian myeloblastosis virus (AMV) reverse transcriptase, 1 X PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$) (1:1 Opti-Prime™ 10X PCR buffers 1 and 2, Stratagene), 1 mM dithiothreitol (DTT), 20 pmol reverse primer and 4 U ribonuclease inhibitor (rRNasin®) and 0.5 mM dNTPs (PCR grade: Roche Molecular Biochemicals, Mannheim, Germany). The reaction mix was

incubated for 1 h at 42°C. After completion of the RT reaction, 20 pmol of the forward primer and 2,5U *Taq* DNA polymerase was added and the reaction mix covered with 3 drops nuclease-free mineral oil (Sigma). The PCR cycles included: denaturation for 1 min at 95°C, annealing for 1 min at 45°C, elongation for 1 min at 72°C and final extension for 10 min at 72°C.

3.6.4.2 Mon348/Mon340

Reverse transcription was performed in a 48 µl reaction cocktail. The cocktail contained 5 µl extracted RNA, 5 U AMV reverse transcriptase, 1 X PCR buffer (10 mM Tris-HCl [pH 8.8], 25 mM KCl, 3.5 mM $MgCl_2$) (Opti-Prime™ 10X PCR buffer 7, Stratagene), 1 mM DTT, 20 pmol reverse primer and 4 U rRNasin® and 0.5 mM dNTPs (Roche). The reaction mix was incubated for 1 h at 42°C. After completion of the RT reaction, 20 pmol of the forward primer and 2,5 U *Taq* DNA polymerase was added and the reaction mix covered with 3 drops nuclease-free mineral oil (Sigma). The PCR cycles included: denaturation for 1 min at 95°C annealing for 1 min at 50°C, elongation for 1 min at 72°C and final extension for 10 min at 72°C.

3.6.4.3 Mon2/prBEG

Nucleic acid amplification was essentially the same as for Mon2/Mon67 primer pair except for 1X PCR buffer (10mM Tris-HCl [pH 9.0], 50 mM KCl, 0.1 % Triton® X-100, 1,5 mM $MgCl_2$). The steps of the RT-PCR were the same as for Mon2/Mon67 primer pair.

3.6.4.4 Type-specific RT-PCR

The reaction mix and reaction conditions for the type-specific primers were essentially the same as for primer pair Mon2/prBEG.

3.6.5 Detection of RT-PCR amplicons

3.6.5.1 Polyacrylamide gel electrophoresis

For the RT-PCR using the Mon2/Mon 67 primer pair, 20µl of the PCR products were analysed by polyacrylamide gel electrophoresis (PAGE) and visualized by ethidium bromide (EtBr)(Sigma) staining and ultraviolet (UV) illumination. A molecular weight (MW) marker (DNA Molecular Weight Marker V, Roche) was included on each gel to confirm the size of the amplicon. PCR products from samples that co-migrated with that of a known positive control were considered positive for the presence of HAstV.

3.6.5.2 Agarose gel electrophoresis

PCR products (20 µl) from all other primer pairs were analysed on a 2% agarose gel (SeaKem® LE Agarose, FMC® BioProducts, Rockland, ME) with the addition of 5 µl of 10 mg/ml EtBr (Sigma) per 100ml gel. The PCR products were visualized by UV illumination. A MW marker (100 bp DNA Molecular Weight Marker, Promega) was included on each gel to confirm the size of the amplimer. Those amplicons that co-migrated with a known HAstV reference control (positive control) were considered positive for the presence of HAstV or a specific HAstV type.

3.6.5.3 Oligonucleotide probe hybridisation assay

An oligonucleotide probe hybridisation was performed to detect and confirm the specificity of the amplicon derived by RT-PCR using the

primer pair Mon2/Mon67. A 30 mer oligonucleotide probe (5'-ATC ACC ATT TAA AAT TGA TTT AAT CAG AAG-3') for HAstV (Marx *et al.*, 1998b) homologous to highly conserved regions within the 3' end of ORF2 of all HAstVs was used. The probe was non-radioactively labelled with digoxigenin (DIG)-dUTP using the DIG oligonucleotide 3'-end labeling kit (Roche). Five microlitres (10%) of the total RT-PCR product was denatured at 99°C for 10 min and maintained on ice before application to a nylon membrane (Roche) by dot-blot microfiltration (Bio-Dot Apparatus, Bio-Rad Laboratories, Hercules, CA). The membrane was air-dried and the DNA amplimers fixed to the membrane by UV cross-linking for 5 min on each side, and baking in an 80°C oven for 15 min. The pre-hybridisation and hybridisation solutions and conditions, as described by Marx *et al* (1998b), were used. Chemiluminescent detection of the PCR amplicon-oligonucleotide probe hybrids was then done using the DIG nucleic acid detection kit (Roche) and DIG wash and block buffer set (Roche) according to the manufacturer's recommendations. Positive results were seen as discrete dots on the developed chemiluminescent film (Roche). RT-PCR products where the specificity could not be confirmed by dot-blot hybridisation analysis were considered to be negative for HAstVs.

3.7 MOLECULAR CHARACTERISATION

RT-PCR products from the ORF2 region of the AstV isolates were investigated for sequence identity by direct PCR product sequencing. RT-PCR products derived from the 3' end of the HAstV genome by amplification with type-common primer pair Mon2/prBEG and/or type-specific primer pairs were sequenced.

Where no PCR product was amplified by these primers or untypable characterisation results were obtained, a 289 bp PCR product, generated by primer pair Mon348/Mon340, from the ORF1a region of the genome was analysed.

3.7.1 PCR product sequencing

The sequencing of RT-PCR amplicons was carried out by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977), using the Sequenase Version 2.0 PCR Product Sequencing Kit (USB Corp., Cleveland, OH) according to the manufacturer's instructions.

The sequencing reactions were resolved on an 8% polyacrylamide-6 M urea gel in 1 X Tris-Borate-EDTA buffer. Gels were vacuum-dried and exposed to X-ray film (Hyperfilm™-ßmax: Amersham) for 12 hours at room temperature. The details of the reagents, sequencing reactions and gels are given in Appendix B.

3.7.2 Phlyogenetic analysis

Nucleotide sequences from all characterised AstV strains from the clinical specimens, and water and sewage samples collected in the Tshwane Metropolitan Area were compared to each other and with available sequences for HAstV reference strains type 1 to 8. The published HAstV capsid gene sequences of reference strains included: HAstV-1 [L23513], HAstV-2 [L13745], HAstV-3 [AF117209], HAstV-4 [Z33883], HAstV-5 [U15136], HAstV-6 [Z46658], HAstV-7 [AF248738], HAstV-8 [Z66541]. In addition, nucleotide sequences of the SA clinical and sewage specimens

were compared by pairwise analysis, to HAstV strains from different geographic locations for the same time period.

All nucleotide sequences were entered into a database in PC/Gene (v6.85; IntelliGenetics Inc, Geneva, Switzerland). Basic sequence manipulation and verification (continuous open-reading frame and motifs characteristic of HAstVs) were performed using OMIGA (v2.0, Accelrys, Madison, WI). ClustalX (Thompson *et al.*, 1997) was used to create multiple alignments of the aa sequences of selected isolates and reference strains. Pairwise comparison of nucleotide sequences of all reference types with the isolates were calculated in GeneDoc v2.3 for preliminary genotype assignment. Strains with 99 - 100% nucleotide homology were identified. Representative isolates from each cluster were compared with AstV sequences present in GenBank using the BLAST-N program v2.I.1 (Altschul *et al.*, 1990;1997) to search for the closest strain available. The nucleotide sequence alignment was assessed for tree-likeliness of the data and the proper sequence composition required for multiple alignments by likelihood-mapping (Nieselt-Struwe and von Haeseler, 2001; Strimmer and von Haeseler, 1997) utilizing TREE-PUZZLE 5.0 (http://www.tree-puzzle.de). Phylogenetic trees were constructed from the nucleic acid sequence alignments using the maximum-likelihood algorithm of the program DNAML of PHYLIP (v 3.52c) running in UNIX environment (Felsenstein 1993). A rooted analysis, with HAstV-4 as a root, was performed. In the analysis the global rearrangement option was invoked and the order of the sequence input was randomized ten times. Phylograms generated in DNAML were visualized by TREEVIEW package v1.5 (Page, 1996) and further edited in Micrografx Designer Version 6.0a.

The nucleotide sequence data of the clinical and environmental AstV strains reported here were registered with the EMBL/GenBank database. The viral nomenclature used is similar to that proposed for HuCV (Wang *et al.,* 1994), namely HAstV type/abbreviation of country of origin/strain designation/year of isolation. The SA strains have been assigned the following accession numbers: DW2_P3 (T3/SA/DW2_P3/1999 [AY094090]), DW2_P7 (T7/SA/DW2_P7/1999 [AY094091]), DW3_P4 (T4/DW3_P4/1999 [AY094092]), DE2_T5 (T5/SA/DE2_T5/1999 [AY094089]), DE3_C (T1/SA/DE3_C/1999 [AY094082]), DE4_P (T8/SA/DE4_P/2000 [AY094083]), DE4_S (T2/SA/DE4_S/2000 [AY094084]), B2_64 (T1/SA/B2_64/1999 [AY094080]), B2_61 (T2/SA/B2_61/1999 [AY094079]), B3 (T1/SA/B3/2000 [AY094081]), Z1 (T2/SA/Z1/1999 [AY094085]), Z2 (T1/SA/Z2/1999 [AY094086]), Z3 (T3/SA/Z3/1999 [AY094087]), Z4 (T1/SA/Z4/2000 [AY094088]), 98004759 (T8/SA/4759/1998 [AY093649]), 98005200 (T3/SA/5200/1998 [AY093650]), 98006899 (T5/SA/6899/1998 [AY093651]), 98007110 (T1/SA/7110/1998 [AY093652]), 980126729 (T6/SA/126729/1998 [AY093653]), 99007052 (T1/SA/7052/1999 [AY093654]), 990026025 (T1/SA/26025/1999 [AY093655]).

3.8 STATISTICAL ANALYSIS

Differences of continuous variables between groups were assessed by Student's t test or ANOVA. When Bartlett's test indicated differences in non-normality of distribution of continuous variables, the nonparametic Kruskal-Wallis H test was applied. Differences in distribution by categories were assessed by Yate's corrected χ^2 or

Fisher's exact test, depending upon cell size. When several categories existed in an ordered set, differences in distributions were assessed by Mantel's χ^2 of the trend.

CHAPTER 4

RESULTS

4.1 SPECIMENS AND SAMPLES

4.1.1 Human stool specimens

A total of 35 HAstV-positive human stool specimens, detected between January 1996 and June 2000, were available for further analysis (Table 4). All specimens were from children <5 years of age. From Table 4 it is evident that HRV was the most common viral diarrhoeal pathogen demonstrated in these patients. The prevalence of HAstVs ranged from 0,3% in 1997 to 5% in 2000, with the prevalence of HAdV-40/41 being lower in four of five years. Statistical analyses of virus detection results showed HRV (χ^2 trend = 13,3; p<0,001) and HAstVs (χ^2 trend = 8,3; p = 0,004) were detected more frequently in later years, but not HAdVs (χ^2 trend = 2,7; p = 0,10).

Table 4: Year-to-year detection of HAstV, HRV, and HAdV-40/41 in human stool specimens from tertiary hospitals

Year	Total no.[1] of stool specimens analysed	Number of positive stools (%)		
		HAstV	HRV	HAdV-40/41
1996	143[2]	2 (1%)	30 (21%)	0 (0)%)
1997	286[2]	1 (0,3%)	66 (23%)	4 (1%)
1998	527	17 (3%)	97 (18%)	7 (1%)
1999	503	12 (2%)	132 (26%)	3 (1%)
2000	273	14 (5%)	92 (34%)	8 (3%)
Total	**1732**	**46 (2,7%)**	**417 (24,1%)**	**22 (1,3%)**

1: No. = number; 2: No. of stools referred in 1996 and 1997 that were available for analysis

4.1.2 Animal stool specimens

Seventy-nine animal and bird stool specimens, collected on six days between January 1999 to October 2000, from eight host species and eight sites, were analysed for AstVs (Table 5). Eighteen (22,8%) of the specimens were from animals with scours or loose stools and 61 (77,2%) from healthy animals. On one collection day (2000-01-25), ill and healthy cattle were sampled at three sites. Otherwise, temporal and geographical relationships by host, health status, and site were scattered.

Table 5: Animal host, collection site and consistency of animal and bird stool specimens analysed.

Animal	Site	Date of collection	Stool		No.[1] of stool specimens
			Well Formed[2]	Loose[3]	
Cattle	Pretoria Zoo	2000-01-25	2	2	4
	UP[4] Research Farm	2000-01-25	3	1	4
	Bronkhorstspruit: Farm	2000-01-25	0	3	3
	Kameeldrift: Plot	2000-01-31	4	0	4
	Feedlot: Delmas	2000-02-01	19	5	24
Calves	Alberton: Dairy Farm	1999-06-28	10	3	13
	Kameeldrift: Plot	2000-01-31	2	0	2
Pig	Pretoria Zoo	2000-01-25	4	1	5
	Bronkhorstspruit: Farm	2000-01-25	3	0	3
	Kameeldrift plot	2000-01-31	3	0	3
	ARC[5]	2000-03-23	1	1	2
	UP Research Farm	2000-03-23	1	0	1
Goat	Pretoria Zoo	2000-01-31	6	0	6
Duck	Pretoria Zoo	2000-01-31	2	0	2
Turkey	Pretoria Zoo	2000-01-31	1	0	1
Cat	Pretoria Veterinarian	2000-06-09	0	1	1
Dog	Pretoria Veterinarian	2000-06-09	0	1	1
TOTAL			**61**	**18**	**79**

1: No. = Number; 2: Stool specimen from healthy animal; 3: Stool specimen from animals with scours or loose stools; 4: University of Pretoria Research Farm; 5: Agricultural Research Council: Animal Improvement Institute, Irene, Pretoria.

4.1.3 Water and sewage samples

Twenty-two water and sewage samples were collected on seven days, between April 1999 and January 2000, from sewage works and surface flows downstream of the sewage works (Table 6). Samples from various stages of the sewage treatment process, namely effluent, treated sewage and settled sewage, were collected at irregular times. Samples came from seven sites, of which four handled sewage and three were water sites.

Table 6: Volume and type of water and sewage samples screened for astroviruses (AstVs)

Sample source	Sample type	Sample Code	Collection Date	Vol[1] (L)
Apies River	Urban stream	A1 A2 A3	1999-04-19 1999-05-24 1999-07-26	1,0 2,0 2,0
Baviaanspoort Sewage Works	Sewage effluent[2]	B1 B2 B3	1999-04-19 1999-04-28 2000-01-19	1,0 1,0 2,0
Daspoort Sewage Works (East inflow)	Treated sewage[3]	DE1 DE2 DE3 DE4	1999-04-19 1999-05-24 1999-07-26 2000-01-19	1,0 1,0 2,0 2,0
Daspoort Sewage Works (West inflow)	Settled sewage[4]	DW1 DW2 DW3 DW4	1999-04-19 1999-04-28 1999-05-24 2000-01-19	1,0 2,0 2,0 2,0
Informal settlement (Mamelodi)	Run-off from dam[5]	S1	1999-07-08	1,0
Pienaars River	Treated water	R1 R2 R3	1999-04-28 1999-05-24 1999-08-02	1,0 1,0 1,0
Zeekoegat Sewage Works	Sewage effluent	Z1 Z2 Z3 Z4	1999-04-19 1999-04-28 1999-05-24 2000-01-19	1,0 4,0 2,0 2,0
TOTAL			**22**	

1: Vol. = Sample volume in litres (L); 2: Sewage effluent – treated organic sewage prior to the chlorination step; 3: Treated sewage - end product in process of sewage treatment; 4: Settled sewage - solid organic matter from raw sewage that undergoes anaerobic digestion prior to becoming treated sewage; 5: Surface water used by the community for domestic purposes.

4.1.4 AstV isolates from river, dam and sewage water samples

Eighteen AstV isolates, recovered from 204 river, 204 dam and 816 drinking water samples, referred between 1998 and 2001, from the Gauteng Province, SA, for routine virological analysis, were included in the study. An additional 24 AstV isolates, from drinking and wastewater from other geographic areas in southern Africa, were analysed (Table 7).

4.2 OPTIMISATION OF RT-PCR FOR THE DETECTION OF AstVS

4.2.1 Optimisation of HAstV type-common RT-PCRs

4.2.1.1 Mon2/Mon67 primer pair

Of the Opti-prime™ buffers tested, using an equal combination of buffers 1 and 2 resulted in the most distinct bands from the HAstV cell culture reference strains (Fig. 6) and HAstV EIA-positive stool specimens and water samples. Single amplicons with no additional bands were observed on the gel (Fig. 6).

4.2.1.2 Mon348/Mon340 primer pair

The type-common RT-PCR with primer pair Mon348/Mon340 was optimised using RNA from a HAstV-6-positive stool specimen (AS21). The following 10X buffer resulted in optimal RNA amplification: 100 mM Tris-HCl [pH 8.8], 35 mM $MgCl_2$, 250 mM KCl, (Fig. 7, lane 7). Results of the optimisation analysis are presented in Fig. 7. Similar results were obtained for reference

Table 7: Astrovirus (AstV) positive water and sewage samples from different geographic regions in southern Africa.

Sample site	Sample site	Sample code	Collection date	Cell line in which AstV isolated	Vol.[1] (L)
Vaal Dam	Dam water	A18P	1998-12-14	CaCo-2[2]	130
		A18P	1999-07-19	Direct[3]	150
		A18W	1999-09-27	PLC/PRF/5	180
		A18W	2000-03-20	CaCo-2	?
		A18W	2000-04-17	PLC/PRF/5	?
Klip River	River water	K19K	1998-05-18	CaCo-2	22
		K19K	1998-06-01	CaCo-2	22
		K19K	1999-02-21	PLC/PRF/5	24
		K19K	1999-03-08	CaCo-2	23
		K19K	1999-07-19	Direct	25
		K19W	1999-09-06	Direct	25
		K19K	1999-09-20	CaCo-2	25
		KI9K	1999-09-29	PLC/PRF/5	24
		K19W	1999-10-18	PLC/PRF/5	25
		K19K	2000-02-21	PLC/PRF/5	24
		K19K	2001-07-09	CaCo-2	25
	Drink water	B10	1999-05-10	CaCo-2	10
	Drink water	R1	2000-07-17	PLC/PRF/5	10
Bloemfontein	River	S4	2000-08-14	CaCo-2	20
	Stream	FS3	2000-07-31	PLC/PRF/5	20
	River	BL5	2001-05-14	PLC/PRF/5	20
	Borehole	BL2	2001-06-11	PLC/PRF/5	20
	River	BL3	2001-06-11	CaCo-2	20
	River	BL3	2001-09-10	PLC/PRF/5	20
Windhoek	Drink water	WH8	2001-01-15	CaCo-2	10
	Sewage	WH10	2001-03-19	PLC/PRF/5	10
	Sewage	WH12	2001-03-19	PLC/PRF/5	10
	Sewage	WH5	2001-03-26	CaCo-2	10
	Drink water	WH9	2001-04-02	CaCo-2	10
	Sewage	WH4	2001-05-14	PLC/PRF/5	10
	Sewage	WH5	2001-06-04	CaCo-2	10
	Sewage	WH6	2001-06-04	CaCo-2	10
	Sewage	WH5	2001-08-13	PLC/PRF/5	10
	Sewage	WH11	2001-09-03	CaCo-2	10
Kwa-Zulu Natal	Estuary	UW2	2000-04-05	CaCo-2	10
	Drink water	U6	2001-05-21	CaCo-2	10
	Sewage	U4	2000-07-31	CaCo-2	10
	Drink water	U1	2001-09-17	CaCo-2	10
Eastern Cape	Drink water	FH3	2001-05-28	PLC/PRF/5	10
Western Cape	Drink water	C	2000-05-03	CaCo-2	10
	Drink water	K2	2000-08-28	PLC/PRF/5	10
Venda	Drink water	V1	2000-08-06	CaCo-2	10
TOTAL			**42**		

1: Vol. = Sample volume in litres (L); 2: Cell culture: AstV isolated in specified cell culture type; 3: Direct: AstV detected directly in concentrated water sample

isolates of HAstV-1 to 5 and HAstV-7 and a clinical strain of HAstV-8 (AS20).

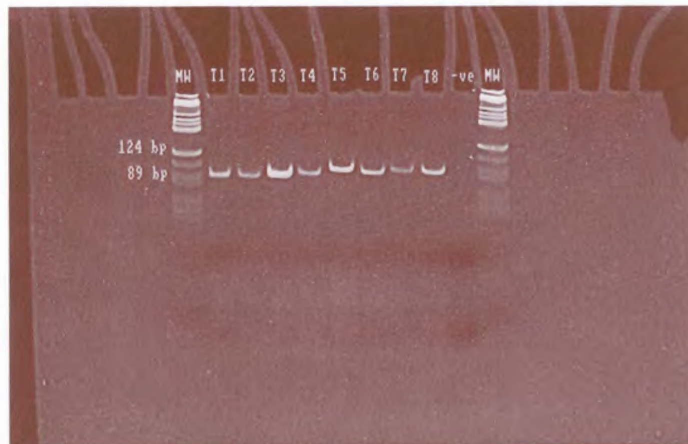

Figure 6: Analysis by ethidium bromide staining and UV illumination of RT-PCR products of HAstV-1 to 7 cell-culture-adapted reference strains (T1-T7) and an HAstV-8 positive stool specimen (AS20) (T8) with type-common primers Mon2/Mon67 using the optimised buffer and RT-PCR reaction mix. Products (89bp) were resolved by polyacrylamide gel electrophoresis. Ultrapure water served as negative control (lane "-ve"). MW = Molecular weight marker V (Roche).

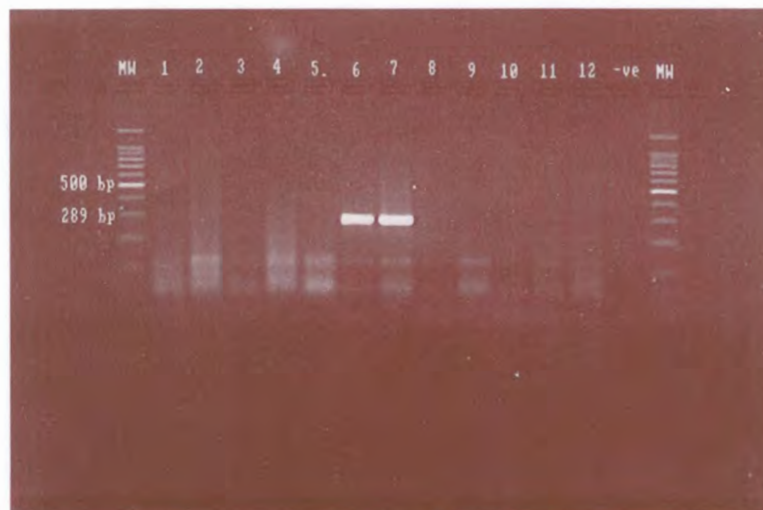

Figure 7: Agarose gel analysis of 289 bp RT-PCR products amplified from *HAstV-6 RNA using buffers 1 to 12 (lanes 1-12)* from the Opti-Prime™ PCR Optimization Kit (Stratagene). Ultrapure water served as negative control (lane "-ve"). Bands were observed in lanes 6 and 7 with 10X buffer containing 100mM Tris-HCl [pH 8,8], 15 mM $MgCl_2$, and 250 mM and 750 mM KCl, respectively. The amplicons were visualised by ethidium bromide staining and UV illumination. MW = molecular weight marker (100 bp DNA ladder: Promega).

4.2.1.3 Mon2/prBEG primer pair

Nucleic acid from reference cell cultures of HAstV-1 to 7 and a clinical HAstV-8 stool specimen was used in the optimisation of type-common RT-PCR primer pair Mon2/prBEG. The buffer system that provided optimal conditions for RT-PCR amplification for this primer pair was: 10X PCR buffer (100 mM Tris-HCl [pH 9.0], 15 mM $MgCl_2$, 500 mM KCl). Single bands could be amplified from each of the RNA samples, except HAstV-4 (Fig. 8). No band was observed for HAstV-4 (lane T4) AS Mon2/prBEG does not amplify this HAstV type. No additional non-specific bands were evident (Fig. 8).

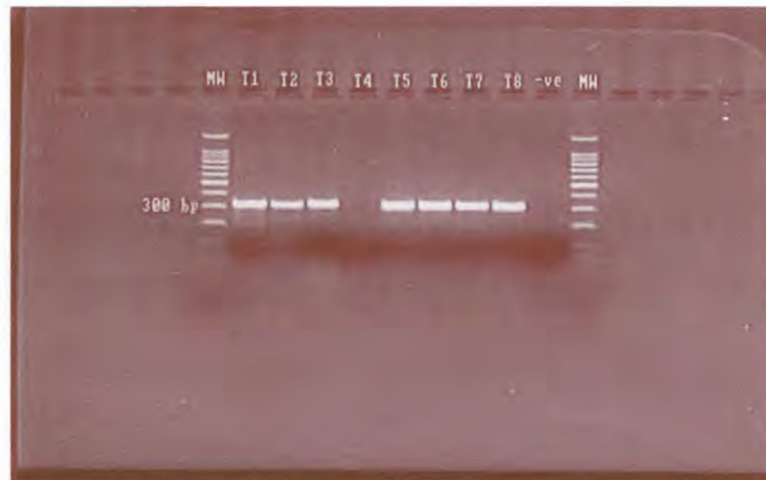

Figure 8: Analysis of 319 bp amplicons derived from HAstV-1 to 3 and HAstV-5 to 7 reference strains (lanes T1-T7), and the HAstV-8 positive stool specimen (AS20) (lane T8) using the optimised RT-PCR reaction mix and primers Mon2/prBEG. Products were resolved by agarose gel electrophoresis. Ultrapure water served as negative control (Lane "-ve"). The amplicons were visualised by ethidium bromide staining and UV illumination. MW = Molecular weight marker (100 bp DNA ladder: Promega).

4.2.2 Optimisation of HAstV type-specific RT-PCRs

Separate optimisation reactions for each type-specific primer pair indicated that a pair's optimal sensitivity and specificity occurred

with a distinct PCR buffer (results not shown). An overall analysis of all results from the type-specific RT-PCR optmisation reactions identified a single 10X PCR buffer, i.e. 100mM Tris-HCl, pH 9.0, 500 mM KCl, 1.0% Triton® X100, 15 mM $MgCl_2$, as an alternate buffer that could be used in a universal reaction mix for all type-specific primer pairs (Fig. 9).

Figure 9: Agarose gel analysis of RT-PCR products derived from HAstV-1 to 7 reference strains (lanes T1a-T7) using type-specific primers in the optimised type-specific RT-PCR reaction mix (lane "+"). T1a and T1b represent primer pairs Mon2/AV3 and Mon2/pr6151, respectively. Ultrapure water served as a negative control for each primer pair (lane "-"). The amplicons were visualised by ethidium bromide staining and UV illumination. MW = Molecular weight marker (100 bp DNA ladder: Promega).

4.3 ASSESSMENT OF CELL CULTURES FOR THE ISOLATION OF HAstVS

We evaluated whether cell culture-adapted and WT HAstV strains preferentially replicated in cell cultures from the host of origin (Table 8). Ag detection by EIA was uniformly unsuccessful and less sensitive than RT-PCR with type-common primers Mon2/Mon67 for the detection of viral presence. From the results

of the different cell cultures to infection with the different virus isolates. The cell culture-adapted HAstV strains replicated more frequently in cell cultures of human origin than in the bovine kidney cell line, whether virus was detected by PAGE ($p=0{,}04$, Fisher's exact test, one-sided) or by probe ($p= 0{,}028$, Fisher's exact test, one-sided).

The primer pair Mon2/Mon67 amplified sequences in five of the MDBK cell cultures, including the uninfected control cultures (result not shown). The specificity of the amplicons could not be confirmed as HAstVs by subsequent oligonucleotide probe hybridisation assay. In addition, further analysis of the MDBK cell culture extracts by RT-PCR using the type-common primer pair Mon348/Mon340 was negative. Therefore *in vitro* cross-species infection could not be demonstrated with the cell culture-adapted or WT HAstV strains and the MDBK bovine kidney cell line used in this study.

Table 8: Assessment of the sensitivity of cell cultures for the isolation and propagation of human astroviruses (HAstVs)

Specimen and cell culture type	Antigen detection[1]	RT-PCR[2] (Mon2/Mon67)		RT-PCR (Mon348/Mon340)
		PAGE[3]	PROBE	
HAstV-1[4]				
CaCo-2	-	+	+	nd
PLC/PRF/5	-	+	+	nd
MDBK	-	+	-	-
HAstV-2[4]				
CaCo-2	nd[5]	+	+	nd
PLC/PRF/5	nd	+	+	nd
MDBK	-	+	-	-
HAstV-3[4]				
CaCo-2	nd	+	+	nd
PLC/PRF/5	nd	+	+	nd
MDBK	-	-	-	-
Stool[6]				
AS21				
CaCo-2	-	+	+	nd
PLC/PRF/5	-	+	+	nd
MDBK	-	+	-	-
AS20				
CaCo-2	-	+	+	nd
PLC/PRF/5	-	+	+	nd
MDBK	-	+	-	-
G912				
CaCo-2	-	+	+	nd
PLC/PRF/5	-	+	+	nd
MDBK	-	+	-	-
G927				
CaCo-2	-	+	-	nd
PLC/PRF/5	-	+	-	nd
MDBK	-	-	-	-
G1821				
CaCo-2	-	+	-	nd
PLC/PRF/5	-	+	-	nd
MDBK	-	-	-	-
MRC1086				
CaCo-2	nd	+	+	nd
PLC/PRF/5	nd	+	+	nd
MDBK	-	-	-	-

1: Antigen detection by enzyme immunoassay; 2: Reverse transcriptase-polymerase chain reaction; 3: Polyacrylamide gel electrophoresis 4: Cell culture-adapted Oxford reference strains; 5: not done; 6: HAstV-positive human stool specimens.

4.4 DETECTION AND CHARACTERISATION OF AstVs

4.4.1 Human stool specimens

AstVs were detected by RT-PCR with HAstV type-common primer pair Mon2/Mon67 in all 35 (100%) of the human stool specimens previously identified as HAstV-positive by EIA. The AstV amplicons were confirmed in all specimens by oligonucleotide probe hybridisation assay of the RT-PCR products (Table 9).

Of the 35 AstVs strains from human stools, 22 (63%) could be assigned a type by nucleotide sequence analysis of a Mon2/prBEG amplicon derived directly from the stool specimen (Table 10). Each stool specimen contained a single HAstV strain identified by nucleotide sequence analysis of the 296 - 324 nt (depending on HAstV type) amplicon from the 3' end of ORF2. Two (8%) additional HAstV strains were confirmed in specimens 96004419 (type 1) and 980128705 (type 8) by nucleotide sequence analysis of the Mon348/Mon340 (246 bp) amplicon from ORF1a (Table 10).

HAstV strains from 14 stool specimens were genotyped by typing RT-PCR (Table 10). For these 14 strains, the type assignment was concordant by typing RT-PCRs and by sequityping of the 3' end of ORF2 (Table 10). Amplicons were not detected by typing RT-PCR in 10 other stool specimens from which the HAstV strains could be assigned by sequityping of the 3' end of ORF2 or a 246 bp region within ORF1a. Two stool specimens (96007169 and 2003621) yielded typing RT-PCR amplicons with type 1 (Mon2/AV3) and type 3 (Mon2/DM12) primer pairs. Sequityping of the Mon2/prBEG amplicon typed a HAstV-3 strain in each specimen.

Table 9: The HAstV detection results from human stool specimens and cell culture derivatives thereof.

Specimen ID number	Collection Date	Stool/Cell culture	Antigen[1] detection	RT-PCR[2]	
				PAGE[3]	Probe
1996					
96007169	96-05-16	Stool CaCo-2 PLC/PRF/5	+ nd[4] nd	+ + +	+ + +
96004419	96-03-21	Stool	+	+	+
1997					
97003144	97-02-17	Stool	-	+	+
1998					
98004759	98-03-04	Stool	+	+	+
98005200	98-03-10	Stool	+	+	+
98005236	98-03-11	Stool	+	+	+
98006400	98-03-25	Stool CaCo-2 PLC/PRF/5	+ nd nd	+ - -	+ - -
98006532	98-03-26	Stool	+	+	+
98006899	98-03-31	Stool	+	+	+
98007004	98-03-31	Stool CaCo-2 PLC/PRF/5	+ nd nd	+ - -	+ - -
98007110	98-04-01	Stool CaCo-2 PLC/PRF/5	+ nd nd	+ - -	+ - -
98007661	98-04-08	Stool CaCo-2 PLC/PRF/5	+ nd nd	+ - -	+ - -
980113786	98-06-14	Stool	+	+	+
980115200	98-07-02	Stool CaCo-2 PLC/PRF/5	+ nd nd	+ - -	+ - -
980116363	98-07-24	Stool CaCo-2 PLC/PRF/5	+ nd nd	+ - -	+ - -
980124893	98-11-04	Stool	+	+	+

Table 9 *continued* : The HAstV detection results from human stool specimens and cell culture derivatives thereof.

Specimen ID number	Collection Date	Stool/Cell culture	Antigen[1] detection	RT-PCR[2]	
				PAGE[3]	Probe
980125359	98-11-08	Stool	+	+	+
		CaCo-2	nd[4]	-	-
		PLC/PRF/5	nd	-	-
980125503	98-11-10	Stool	+	+	+
		CaCo-2	nd	-	-
		PLC/PRF/5	nd	-	-
980126585	98-11-23	Stool	+	+	+
		CaCo-2	nd	-	-
		PLC/PRF/5	nd	-	-
980126729	98-11-24	Stool	+	+	+
980128705	98-12-26	Stool	+	+	+
1999					
99002256	99-01-29	Stool	+	+	+
99002393	99-02-01	Stool	+	+	+
99004642	99-02-23	Stool	+	+	+
99006802	99-03-17	Stool	+	+	+
99007052	99-03-19	Stool	+	+	+
99009559	99-04-22	Stool	+	+	+
990022320	99-09-19	Stool	+	+	+
990025786	99-10-31	Stool	+	+	+
990026025	99-11-02	Stool	+	+	+
990028945	99-12-06	Stool	+	+	+
990029903	99-12-21	Stool	+	+	+
2000					
2003621	00-02-09	Stool	+	+	+
2003622	00-02-10	Stool	+	+	+
2005114	00-02-27	Stool	+	+	+

1: HAstV antigen detection by enzyme immunoassay; 2: Reverse transcriptase-polymerase chain reaction; 3: Polyacrylamide gel electrophoresis; 4: not done

Table 10: Summary of characterisation of astrovirus isolates from human stool specimens.

Specimen number	Type-specific RT-PCR								Sequencing	
	Type 1 2/AV3*	Type 1 2/6151	Type 2 2/6257	Type 3 2/DM12	Type 4 2/JW4	Type 5 2/AstS5	Type 6 2/AstS6	Type 7 2/DM11	Primers	Genotype
96007169	-	-	-	+	-	-	-	-	Mon2/prBEG	3
96004419	-	-	-	-	-	-	-	-	Mon348/340	1
97003144	-	-	-	-	-	-	-	-	Mon2/prBEG	1
98004759	-	-	-	-	-	-	-	-	Mon2/prBEG	8
98005200	-	-	-	+	-	-	-	-	Mon2/prBEG	3
98005236	-	-	-	-	-	-	-	-	Mon2/prBEG	6
98006400	-	-	-	-	-	-	-	-	Mon2/prBEG	untypable[2]
98006532	-	-	-	-	-	-	-	-	Mon2/prBEG	untypable
98006899	-	-	-	-	-	+	-	-	Mon2/prBEG	5
98007004	-	-	-	-	-	-	-	-	-	-
98007110	-	-	-	-	-	-	-	-	Mon2/prBEG	1
98007661	-	-	-	-	-	-	-	-	-	-
980113786	-	-	-	+	nd[1]	nd	nd	nd	Mon2/prBEG	3
980115200	-	-	-	-	-	-	-	-	-	-
980116363	-	-	-	-	-	-	-	-	-	-
980124893	-	-	-	-	-	-	-	-	Mon2/prBEG	1
980125503	-	-	-	-	-	-	-	-	-	-
980125359	-	-	-	-	-	-	-	-	Mon2/prBEG	untypable
980126585	-	-	-	-	-	-	-	-	Mon2/prBEG	5

Table 10 *continued* : Summary of characterisation of astrovirus isolates from human stool specimens.

Specimen number	Type-specific RT-PCR								Sequencing	
	Type 1 2/AV3*	Type 1 2/6151	Type 2 2/6257	Type 3 2/DM12	Type 4 2/JW4	Type 5 2AstS5	Type 6 2/AstS6	Type 7 2/DM11	Primers	Genotype
980126729	-	-	-	-	-	-	+	-	Mon2/prBEG	6
980128705	-	-	-	-	-	-	-	-	Mon348/340	1
99002256	-	-	-	-	-	-	-	-	Mon2/prBEG	untypable
99002392	-	-	-	-	-	-	-	-	Mon2/prBEG	untypable
99004642	+	-	-	-	nd	-	-	-	Mon2/prBEG	1
99006802	+	nd	-	-	nd	-	-	-	Mon2/prBEG	1
99007052	+	nd[1]	-	-	nd	-	-	-	Mon2/prBEG	1
99009559	+	nd	-	-	nd	-	-	-	Mon2/prBEG	1
990022320	+	+	-	-	nd	-	nd	nd	Mon2/prBEG	1
990025786	+	w+	-	-	nd	-	-	-	Mon2/prBEG	1
990026025	+	w+	-	-	nd	nd	nd	nd	Mon2/prBEG	1
990028945	+	-	-	-	-	-	nd	nd	Mon2/prBEG	untypable
990029903	+	+	-	-	nd	-	nd	nd	Mon2/prBEG	1
2003621	+	nd	nd	-	nd	-	-	-	Mon2/prBEG	3
2003622	+	nd	nd	-	nd	-	-	-	Mon2/prBEG	1
2005114	+	+	-	-	nd	-	-	-	Mon2/prBEG	1

* Primer pair used for type-specific RT-PCR

1: RT-PCR using this type-specific primer pair not done on isolate

2: untypable by typing RT-PCR or inconclusive sequityping

HAstV strains from 11 stool specimens could not be typed by typing RT-PCRs or sequityping despite yielding amplicons when utilizing type-common primer pairs Mon2/prBEG and/or Mon348/Mon340.

The distribution of genotypes for the 24 characterised HAstVs from clinical specimens was: 15 (63%) HAstV-1, 3 (13%) HAstV-3, 2 (8%) HAstV-5, 2 (8%) HAstV-6 and 2 (8%) HAstV-8 (Fig. 10). No HAstV-2, HAstV-4 or HAstV-7 strains were identified among the clinical specimens.

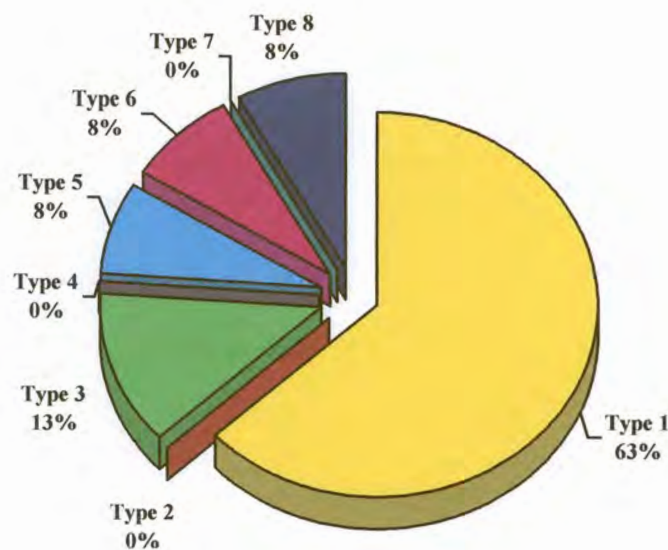

Figure 10: Distribution of HAstV genotypes identified between January 1996 and October 2000 in human diarrhoeal stool specimens, taken from hospitalised patients in the Tshwane Metropolitan Area.

4.4.2 Animal stool specimens

AstVs were not detected in 60 (76%) of the 79 animal stool specimens screened by HAstV-specific EIA and HAstV type-common RT-PCR using primer pair Mon2/Mon67 (Appendix C). However query positive results were obtained by PAGE, in 17 (22%) of the 79 specimens, but none was confirmed by EM or oligonucleotide probe hybridisation assay (Table 11). Because AstVs were not detected among the animal stool specimens, no animal AstVs from SA were available for characterisation. Reference animal AstVs prototype strains were also not available for characterisation and comparative analysis.

Table 11: Summary of virus detection results on animal stool specimens where screening results were query positive.

Specimen ID number	Collection date	Electron microscopy	Antigen[1] detection	RT-PCR[2]	
				PAGE[3]	Probe
C1[4]	28-06-1999	-	-	w+[5]?	-
C2	28-06-1999	-	-	w+?	-
C3	28-06-1999	-	-	w+?	-
C4	28-06-1999	-	-	w+?	-
C5	28-06-1999	-	-	w+?	-
C6	20-08-1999	-	-	w+?	-
C7	20-08-1999	-	-	w+?	-
C9	20-08-1999	-	-	w+?	-
C11	20-08-1999	-	-	w+?	-
C12	20-08-1999	-	-	w+?	-
F5(2)	01-02-2000	-	-	+?	-
F5(3)	01-02-2000	-	-	+?	-
F36(3)	01-02-2000	-	w+?	-	-
HB4	31-01-2000	-	+?	-	-
DB3	25-01-2000	-	-	+?	-
DV4	31-01-2000	-	-	+?	-
DV5	31-01-2000	-	-	+?	-
DE2	25-01-2000	-	-	+?	-
DMG2	31-01-2000	-	-	+?	-

1: Antigen detection by enzyme immunoassay; 2: Reverse transcriptase-polymerase chain reaction; 3: Polyacrylamide gel electrophoresis; 4: C: calf; F: cattle Delmas Feedlot; HB: cattle- Kameeldrift plot; DB : cattle – Pretoria Zoo; DV: pig – Pretoria Zoo; DE: duck – Pretoria Zoo; DMG : mountain goat – Pretoria Zoo; 5: weak positive

4.4.3 Water and sewage samples

HAstVs presence was confirmed by HAstV type-common RT-PCR-oligonucleotide probe hybridisation assay and/or type specific RT-PCR in all 15 sewage samples (Table 12). AstVs were not detected in the water samples drawn downstream of the sewage treatment works (Table 12). A total of 24 AstV strains were detected in the 15 sewage samples

Seven AstV strains in sewage (DW2_P, DW4, B3, Z1, Z2, Z3, Z4), from separate samples, were amplified by Mon2/prBEG primers directly from the sewage samples and characterised by nucleotide sequence analysis (Table 12). Five strains (DE3_C, DE4_P, DE4_S, B2_64, B2_61), from three sewage samples, were characterised only after isolation in cell culture. One sample (B2) yielded two HAstV genotypes from separate flasks of CaCo-2 cells cultures with differing passage numbers. Sample DE4 yielded two HAstV serotypes after separate amplification in CaCo-2 and PLC/PRF/5 cells (Table 12). Thirteen environmental HAstVs (DW2_T1, DW2_P3, DW2_P4, DW2_P7, DW3_P1, DW3_P3, DW3_P4, DW4_T7, DE2_T1, DE2_T3, DE2_T5, DE2_T7, and DE4_T7), from five sewage samples (DW2, DW3, DW4, DE2, DE4), were sequityped from amplicons generated by RT-PCR using type-specific primers. Amplicons derived from three of the sewage samples (DW1, DE1, B1) gave inconclusive sequences i.e. the sequence could not be resolved.

Table 12: Detection and characterisation of astroviruses (AstVs) in sewage and water samples collected, from April 1999 to October 2000 in the Tshwane Metropolitan Area

Sampling Site	Date	Sample code	EIA	RT-PCR[1]		HAstV[2] Genotype
				PAGE[3]	PROBE[4]	
Sewage Works						
Daspoort	19-04-99	DW1	+	+	-[5]	untypable
(west inflow)	28-04-99	DW2	+	+	+	1, 3, 4, 7[6] 2[7]
	24-05-99	DW3	+	+	-	1, 3, 4[6]
	19-10-99	DW4	+	+	+	1[7], 7[6]
Daspoort						
(east inflow)	19-04-99	DE1	+	+	+	untypable
	24-05-99	DE2	+	+	+	1, 3, 5, 7[6]
	26-07-99	DE3	+	+	+	1[8]
	19-10-00	DE4	+	+	+	2[9], 7[6], 8[9]
Baviaanspoort	19-04-99	B1	+	+	+	untypable
	28-04-99	B2	+	+	+	1,2[8]
	19-10-00	B3	+	+	+	1[7]
Zeekoegat	28-04-99	Z1	+	+	+	2[7]
	24-05-99	Z2	+	+	+	1[7]
	19-09-99	Z3	+	+	+	3[7,9]
	19-10-00	Z4	+	+	+	1[7]
Surface water/Streams						
Pienaars river						
(downstream	28-04-99	R1	-	-	-	-
to	24-05-99	R2	-	-	-	-
Baviaanspoort)	02-08-99	R3	-	-	-	-
Apies river						
(downstream	19-04-99	A1	-	+	-	untypable
to Daspoort)	24-05-99	A2	-	-	-	-
	26-07-99	A3	-	-	-	-

1: Reverse transcriptase-polymerase chain reaction with type-common primers, Mon2/Mon67; 2: Human astroviruses; 3: PAGE - polyacrylamide gel electrophoresis; 4: Oligonucleotide probe hybridisation; 5: not detected; 6: AstVs genotyped from amplicons derived directly from the sample by type-specific RT-PCRs; 7: AstVs genotyped from amplicons derived directly from the sample by group-specific RT-PCR; 8: AstVs genotyped from amplicons derived from infected CaCo-2 cell cultures by group-specific RT-PCRs; 9: AstVs genotyped from amplicons, derived by group-specific RT-PCRs, from two different cell culture types (CaCo-2 and PLC/PRF/5) inoculated with the same sample.

From the characterisation data of cell culture isolates it appeared that different cell culture types favoured the growth of selected genotypes of HAstVs. HAstV-1 and 2 were detected more frequently in CaCo-2 cells, HAstV-8 in PLC/PRF/5 cells and HAstV-3 could be isolated from both CaCo-2 and PLC/PRF/5 cell cultures (results not shown).

Seven of the eight HAstV genotypes (HAstV-1 to 5 and HAstV-7 to 8), were detected in the wastewater samples. Multiple HAstV serotypes were identified in six (40%) samples (Table 12). The genotypes of the 24 characterised HAstVs from environmental wastewater samples were: 9 (36%) HAstV-1, 4 (16%) HAstV-2, 4 (16%) (HAstV-3, 2 (8%) HAstV-4, 1 (4%) HAstV-5, 4 (16%) HAstV-7 and 1 (4%) HAstV-8 (Fig. 11).

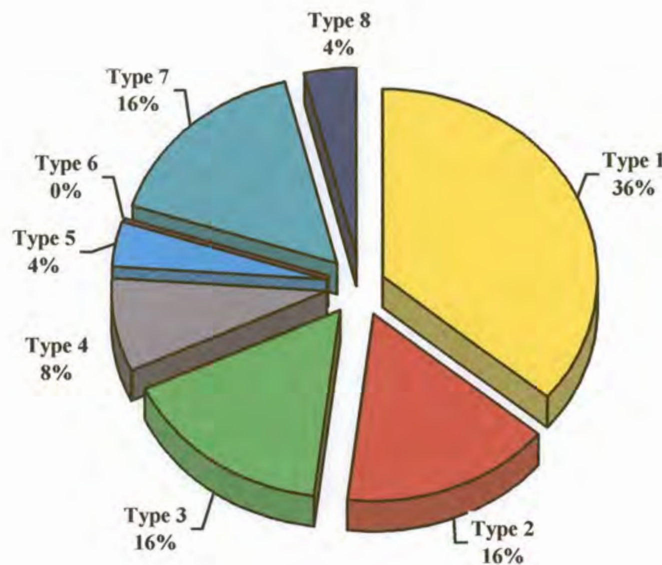

Figure 11: Distribution of HAstV genotypes detected between April 1999 and October 2000 from sewage samples collected from sewage works in the Tshwane Metropolitan Area.

4.4.4 AstV isolates from river, dam and sewage samples

Forty-two extracts of cell cultures infected with river, dam and sewage water samples referred for routine virological analysis, and found to be AstV-positive by type-common RT-PCR-oligonucleotide hybridisation assay (Table 6), were retested for the presence of AstV using the same assay. AstVs could only be detected in 15 (36%) of these cell culture suspensions (Table 13). AstV detection could not be enhanced by further blind passaging of either CaCo-2 or PLC/PRF/5 cell cultures.

Four (27%) of the 15 HAstV isolates could be sequityped from a 217 nt amplicon at the 3′ end of ORF2 (Table 13). HAstV-1 was detected in three (20%) extracts (BL5, BL3 and WH4) and a single isolate (7%) of HAstV-3 (WH5) was identified. The eleven other isolates yielded inconclusive nucleotide sequence profiles.

Table 13: Detection and characterisation of astrovirus isolates from water and sewage samples referred for routine virological analysis.

Sample code	Sampling date	Cell culture	RT-PCR[1]		HAstV[2] Genotype
			PAGE[3]	PROBE[4]	
Gauteng					
K19K	08-03-99	CaCo-2[5]	-	-	nd[6]
(River		P[1] PLC/PRF/5	-	-	nd
water)		P[1] CaCo-2	+	-	untypable
	20-09-99	CaCo-2[5]	-	-	nd
		P[1] PLC/PRF/5	-	-	nd
		P[1] CaCo-2	+	+	untypable
	29-09-99	PLC/PRF/5[5]	-	+	untypable
		P[1] PLC/PRF/5	-	-	nd
		P[1] CaCo-2	-	-	nd
	21-02-00	PLC/PRF/5[5]	-	-	nd
		P[1] PLC/PRF/5	+	-	nd
		P[1] CaCo-2	+	+	untypable
	09-07-01	CaCo-2[5]	+	+	untypable
A18W	27-09-99	PLC/PRF/5[5]	-	-	nd
(Dam		P[1] PLC/PRF/5	-	-	nd
water)		P[1] CaCo-2	-	+	untypable
	20-03-00	CaCo-2[5]	+	-	untypable
		P[1] PLC/PRF/5	-	-	nd
		P[1] CaCo-2	-	-	nd
	17-04-01	PLC/PRF/5[5]	+	-	untypable
		P[1] PL/PRF/5	-	-	nd
		P[1] CaCo-2	-	-	nd
B10	10-05-99	CaCo-2[5]	-	+	untypable
(drinking		P[1] PL/PRF/5	-	+	untypable
water)		P[1] CaCo-2	-	+	untypable

Table 13 *continued*: Detection and characterisation of astrovirus isolates from water and sewage samples referred for routine virological analysis.

Sample code	Sampling date	Cell culture	RT-PCR[1]		HAstV[2] Genotype
			PAGE[3]	PROBE[4]	
Bloemfontein					
(surface BL5	14-05-01	CaCo-2[5]	+	+	1
water) BL3	11-06-01	CaCo-2[5]	+	+	1
Eastern Cape					
(borehole water)	28-05-01	CaCo-2[5]	+	+	untypable
Windhoek					
(wastewater)					
WH4	14-05-01	CaCo-2[5]	+	+	1
WH5	04-06-01	CaCo-2[5]	+	+	3
WH6	04-06-01	CaCo-2[5]	+	+	untypable

*: Original cell culture type in which AstV was detected; 1: Reverse transciptase polymerase chain reaction; 2: Human astroviruses; 3: Analysis of amplicons by polyacrylamide gel electrophoresis; 4: Oligonucleotide probe hybridisation assay; 5: Original cell culture in which AstV was isolated; 6: not done

4.5 NUCLEOTIDE SEQUENCE AND PHYLOGENETIC ANALYSIS OF SOUTH AFRICAN HAstVs

All the AstV strains characterised from human stool specimens and water and sewage samples from the Tshwane Metropolitan Area could be assigned a HAstV genotype by pairwise comparison of a 208 base region at the 3' terminus of ORF2 with those of a consensus region in the Oxford reference strains (Table 14). The SA strains grouped together in a number of clusters with 99-100% nucleotide sequence identity within each clusters. The groups of

Table 14: Summary of the characterised South African (SA) human astrovirus (HAstV) strains from clinical and sewage sources identifying the representative strain included in the phylogenetic analysis.

HAstV Genotype[1]	Representative SA strains	SA strains with 99-100% nucleotide identity to the representative isolate
Type 1	DE3_C	DE2_T1
	Z4	990029903; 2003621
	98007110	nd[2]
	B3	nd
	Z2	nd
	99007052	nd
	B2_64	nd
	990026025	97003144; 980124893; 99006802; 99009559; 990022320; 2005114; DW3_P1; DW4
Type 2	Z1	nd
	B2_61	nd
	DE4_S	DW2_S
Type 3	98005200	nd
	DW3_P3	nd
	Z3	96007169; 980113786; DE2_T3; DW2_P3
Type 4	DW3_P4	DW2_P4
Type 5	DE2_P5	nd
	98006899	980126585
Type 6	980126729	98005236
Type 7	DW2_P7	DE2_P7; DE4_T7; DW4_T7
Type 8	98004759	nd
	DE4_P	nd

1: Type assignment based upon comparison with the Oxford reference strain; 2: none detected

isolates and the representative strains for each group are shown in Table 14. The nucleotide sequence pairwise similarity scores strains in a 208 nt consensus region within ORF2 of the representative of each group and of reference are summarised in Fig. 12.

In preparation for phylogenetic analysis, the multiple alignments of the nucleotide sequences was tested by likelihood-mapping: the data had a tree-like structure, all sequences were in the range of proper sequence composition (data not shown). Pairwise analysis and an unrooted phylogenetic tree demonstrated common branch points for the majority of SA strains within types. Twenty-seven strains with 99-100% sequence homology were therefore withheld from further phylogenetic analysis to simplify the tree. For the final phylogenetic analysis, reference strains HAstV-1 to 8 and representatives of the 21 distinctive SA strains were aligned. HAstV-4 was less related to the other reference strains and was therefore used as a root for the analysis.

The final rooted phylogenetic tree is presented in Fig. 13. The analysis showed that clusters of types with reference strains HAstV-1 to HAstV-8 separated with confidence (distances 0,09-0,62; $p<0,05$). HAstV-3 and 7, and HAstV-5 and 8 showed a close relationship in this hypervariable region, with 90% and 82% pairwise identity and distances of 0,09 and 0,124 respectively. HAstV-4 was distinct from the other HAstV strains.

In this phylogenetic analysis, an isolate was considered a subtype if the nucleotide homology to the reference stain was <95% and the

HAstV strains	T6	126729	T8	4759	T5	DE2	6899	T1	DE3	26025	Z4	Z2	T2	Z1	T3	Z3	T7	DW2	T4	DW3
OxT6 (Z46658)		***96***	57	57	55	58	57	52	54	54	52	54	58	58	56	57	57	58	41	43
T6/126729/1998			58	57	58	61	59	54	56	56	54	56	58	59	57	58	57	57	41	43
OxT8 (Z66541)				***94***	82	83	82	67	67	66	66	70	67	66	70	70	67	67	40	42
T8/4759/1998					85	85	84	67	66	65	65	69	68	67	71	71	68	68	42	43
OxT5 (U15136)						***95***	***91***	65	66	65	65	69	67	67	69	70	70	70	43	41
T5/DE2_T5/1999							***93***	66	66	65	64	70	67	68	72	73	72	72	42	43
T5/6899/1998								64	65	65	65	68	68	66	71	71	69	69	41	42
OxT1 (L23513)									***94***	***92***	***91***	***90***	66	69	69	69	67	66	45	45
T1/DE3_C/1999										***96***	***94***	***90***	66	69	69	69	67	66	45	44
T1/26025/1999											***97***	***94***	69	71	67	68	67	67	44	43
T1/Z4/2000												***92***	69	68	66	66	66	65	44	43
T1/Z2/1999													71	73	73	73	72	71	43	43
0xT2 (L13745)														***94***	80	81	82	81	42	42
T2/Z1/1999															80	81	82	81	43	43
OxT3 (AF117209)																***99***	90	90	44	44
T3/Z3/1999																	91	91	44	44
OxT7 (AF248738)																		***99***	43	43
T7/DW2_P7/1999																			43	44
0xT4 (Z33883)																				**88**
T4/DW3_P4/1999																				

Figure 12. Relationship of selected South African human astrovirus (HAstVs) sequences (nucleotides 6481 to 6688; HAstV-1 [I23513] of ORF2, to the Oxford (Ox) reference strains. The numbers show the percentage nucleotide identity, by pairwise analysis, of the aligned nucleotide sequences.

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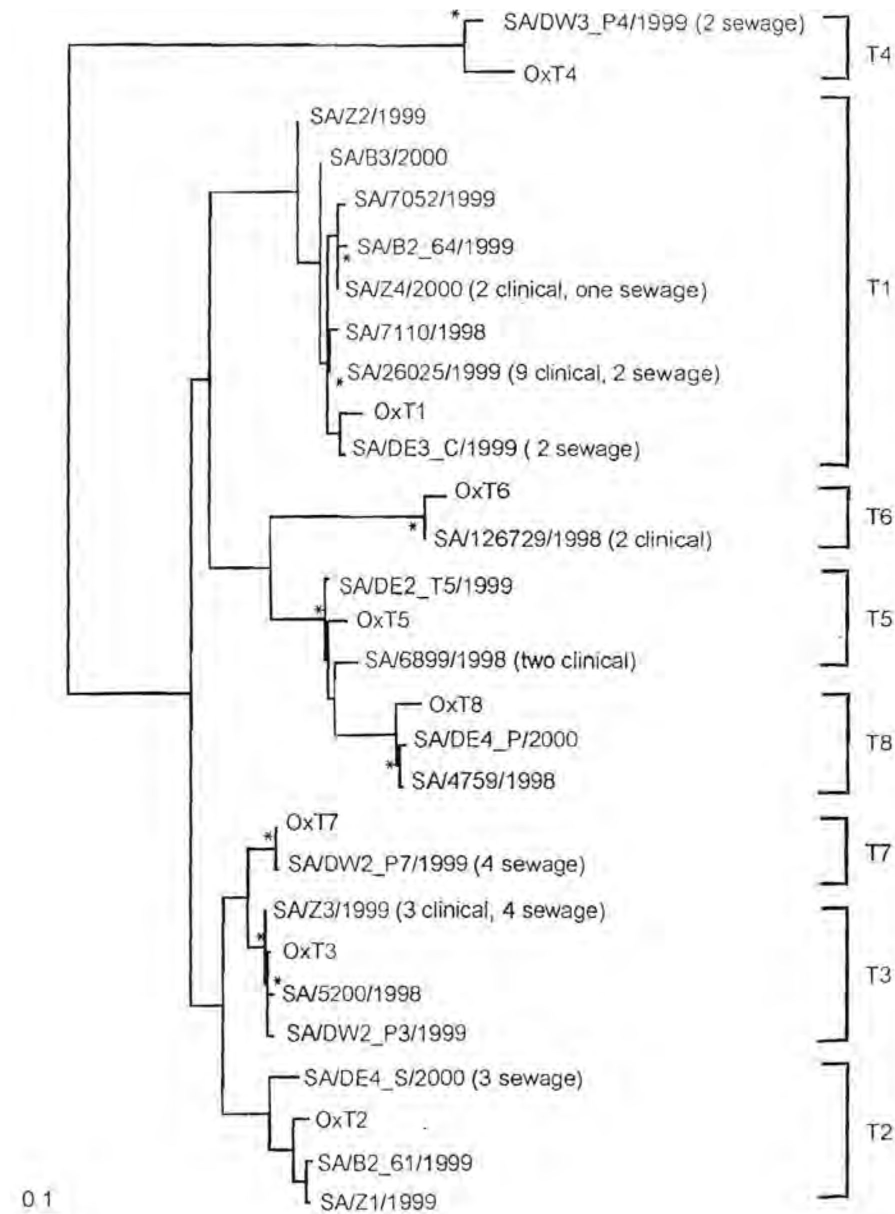

Figure 13: Maximum-likelihood phylogenetic tree based upon a 208 nt region of the 3' end of ORF2 showing the relationships between representatives of the South African environmental and clinical human astrovirus (HAstV) isolates and the prototypes of HAstV types 1 to 8. Branch points of the resulting tree (rooted) had a confidence level of $p<0.05$, scale bar = number of nucleotide substitutions per site except where indicated by an asterisk "*" (Courtesy Prof JE Walter)

distance at the 3' end of ORF2 (208nt) >0,05. Intra-genotypical distances were calculated and data suggested that SA HAstV-1, 2, 4, 5 and 8 strains from the Tshwane Area identified in this study represented new subtypes of the corresponding genotypes with the following percentage similarities and branching distances: HAstV-1 (90-94%, distance 0,05-0,13); HAstV-2 (88-94%, distance 0,10-0,13); HAstV-4 (88%, distance 0,12); HAstV-5 (91-94%, distance 0,05-0,10) and HAstV-8 (94%, distance 0,06). The HAstV-3, 6 and 7 strains appear not to be new subtypes: HAstV-3 (99%, distance 0,01); HAstV-6 (96%, distance 0,04) and HAstV-7 (99%, distance 0,005).

HAstV-1, HAstV-3, HAstV-5 and HAstV-8 were detected among clinical samples and environmental isolates, with HAstV-1 being most common. This serotype comprised 22 (48%) of the 46 isolates characterised by sequityping of the 3' end of ORF2. The SA HAstV-1 strains exhibited a nucleotide identity of 88-100% to each other and 89-94% to the prototype strain. Two strains detected in wastewater sources formed a distinct subtype (T1a) but were more closely related to the reference strain (distance 0,05) than to any of the other SA strains. A second cluster T1b, distinct from T1a (distance 0,04-0,6) was observed. Cluster T1b comprised multiple closely related environmental and clinical strains represented by: Z4, B2_64, 98007110, 99007052 and 990026025. Two other HAstV-1 strains, B3 and Z2, were unique.

Type 3 isolates from both clinical and environmental sources, displayed a high nucleotide sequence similarity (≥98%) to each other and to the prototype strain. All of these isolates clustered into a single genotypic cluster, T3 (Fig. 13). A 95% nucleotide

sequence identity was demonstrated between the SA HAstV-5 environmental isolate and the prototype strain, whereas a 91% nucleotide identity was noted between the clinical isolate and the reference strain. As only a 93% nucleotide identity (distance 0,07) was demonstrated for the SA clinical and environmental HAstV-5 strains, they were placed in unique subtypes within genotype 5 (Fig. 13).

Two HAstV-8 strains were identified and analysed: 98004759 from a stool specimen and DE4_P isolated from a sewage sample in PLC/PRF/5 cell culture. These closely related isolates had a nucleotide identity of 98% to each other, compared with 93-94% nucleotide sequence identity to the reference strain. A 100% nucleotide sequence identity was recorded for the two HAstV-6 strains from stool specimens, with 96% nucleotide identity to the reference strain. No type 6 strains were detected in environmental samples.

HAstV-2, 4 and 7 were only identified in wastewater samples. The nucleotide identity was 88-98% among the HAstV-2 isolates, but, when compared to the prototype strain, the identity was lower (88-94%), resulting in the SA isolates clustering separately from the prototype strain (Fig.13). Two unique subtypes of HAstV-2 were identified among the SA isolates. These are represented by B2_61 and Z1 in one subtype, and DE4_S in the other subtype (Fig 13). The two HAstV-4 isolates showed 100% identity to each other and 88% identity to the reference strain. The type 7 isolates grouped closely together with the reference strain ($\geq$98% nucleotide sequence identity) and comprised a single subtype in HAstV-7.

The results from the comparison of the representative strains from the Tshwane region with AstV sequences present in GenBank, using the BLAST-N program v2.1.1., are given in Table 15.

Table 15: Summary of pairwise sequence comparison between South African (SA) astrovirus (AstV) strains and the AstV isolates in GenBank.

HAstV[1] Genotype	Representative isolate	Closest HAstV isolate in GenBank (% nt[2] identity to representative SA isolate)	
T1	DE3_C	T1/UK[3]/1993	(99%)
	990026025	T1/Bangladesh/55/1996	(99%)
	Z4	T1/Bangladesh/55/1996	(98%)
	Z2	T1/Bangladesh/55/1996	(94%)
T2	Z1	T2/MX[4]/358/1991	(99%)
T3	Z3	T3/MX/352/1991	(99%)
T5	98006899	T5/CH[5]/198/1996	(95%)
T6	980126729	Oxford T6 prototype	(96%)
T8	98004759	T8/SA/AS20/1994	(96%)

1: Human astrovirus; 2: nucleotide; 3: United Kingdom; 4: Mexico; 5: Norway

Three of the SA representative type 1 isolates showed a high percentage (94-99%) nucleotide identity to HAstV-1 isolates from Bangladesh while the other isolate showed 99% nucleotide identity to isolate T1/UK/1993 from the UK. A single subtype of HAstV-2 was identified, with the SA strain sharing 99% nucleotide sequence identity with a HAstV-2 isolate detected in Mexico in 1991. The sequences of HAstV-3 isolates from the SA clinical and environmental sources, as well as from other geographical regions,

appeared to be highly conserved, showing 99% nucleotide sequence identity. The SA strains of types 5 and 6 had nucleotide identity to single isolates of HAstVs of 95% and 96%, respectively. The type 8 HAstV isolated in this study was matched the closest by nucleotide identity to a type 8 HAstV from SA isolated in a previous study (Taylor *et al.*, 2001a), T8/SA/AS20/1994 (96%).

Comparisons of the representative SA strains with AstVs detected in sewage and water samples referred for routine virological analysis from other geographical locations in southern Africa were not carried out. This was on account of the low number of isolates available for nucleotide identity comparison.

CHAPTER 5

DISCUSSION

In this investigation, RT-PCR using type-common and type-specific primer pairs and direct PCR product sequencing, were applied for the detection and characterisation of AstV isolates from clinical and environmental sources. Sequence analysis of the 3' region of HAstV ORF2 provided type information as well as enough diversity to provide additional strain information. There were, however, no data available to this study on the ability of RT-PCR amplification of ORF2 for detecting and characterising mixed populations of HAstV genotypes.

RT-PCR, using published primer pairs for the detection and amplification of HAstVs, was reassessed and the RT-PCR buffering conditions optimised for the most efficient amplification of AstV RNA. The specificity for HAstV RNA of the type-common primer pair Mon2/Mon67 has been previously demonstrated (Grohmann *et al.*, 1993; Oishi *et al.*, 1994; Mitchell *et al.*, 1995; Marx *et al.*, 1997; 1998b; Taylor *et al.*, 2001b). This primer pair was designed from conserved sequences at the 3' terminus of the HAstV-1, HAstV-2 and HAstV-4 genomes (Mitchell *et al.*, 1995) and can be applied for the detection of all HAstV genotypes (Mitchell *et al.*, 1995). However, a 35 nt, stem-loop motif in this highly conserved region (Jonassen *et al.*, 2001), common to HAstV, OAstV, PAstV and TAstV-1, raises the question whether the Mon2/Mon67 primer

pair amplifies only HAstVs. Nucleotide sequence analysis of the 3' end region of ORF2 from different species also suggests that this primer pair may amplify the animal AstVs, PAstV and FAstV (Taylor *et al.*, 2001b). Use of oligonucleotide probe hybridisation as a confirmatory step (Marx *et al.*, 1998b) to demonstrate that Mon2/Mon67 amplicons derive from HAstVs (Taylor *et al.*, 2001b), was sensitive. The detection limit for the oligonucleotide probe for the Mon2/Mon67 primer pair was reported to be 0,7 ng of amplicon (Marx *et al.*, 1998b). Thus, Mon2/Mon67 amplicons generated in this study from animal stool specimens could be confirmed as being HAstVs by subsequent oligonucleotide hybridisation assay. Although RT-PCR has been shown to be more sensitive than EIA for the detection of AstVs (Glass *et al.*, 1996), in this study sewage samples that were concentrated with AstVs were detected with equal success by EIA and RT-PCR using type-common primer pair Mon2/Mon67.

The specificity of Mon2/prBEG (Saito *et al.*, 1995) and Mon348/Mon340 (Belliot *et al.*, 1997b) primer pairs were also reevaluated. Discordant amplification results were obtained for selected isolates from sewage samples, DW2, DW3, DW4, and DE4, and clinical specimens, 96004419, 97003144, 98004759, 98005236 98007110, 90124893, 980126585 and 980128705 (Tables 10 and 12), indicating that variations in WT HAstV genomic sequences influenced primer pair sensitivity and specificity. The typing primer pairs were specific for HAstVs (Matsui *et al.*, 1998; Walter *et al.*, 2001a). This specificity was further confirmed in this investigation as all strains amplified by these type-common and type-specific primer pairs were characterised to be HAstVs. This implies that the RT-PCR

techniques used in this study for the detection of AstVs in human and animal stool specimens, and the water and wastewater samples, were specific for HAstVs. Primers are needed that are animal AstV-specific to detect animal AstVs in animal stool specimens and water samples.

The isolation of AstVs from cell cultures is reportedly dependent upon two factors, i.e. use of cell cultures derived from the host species from which the AstV was recovered and the presence of trypsin in the cell culture medium (Monroe, 1999; Brinker *et al.*, 2000; Matsui and Greenberg, 2001). CaCo-2 and PLC/PRF/5 cells are effective cell lines for the isolation and propagation of HAstVs from stool specimens (Willcocks *et al.*, 1990; Taylor *et al.*, 1997a; Mustafa *et al.*, 1998; Brinker *et al.*, 2000) and from water samples (Marx *et al.*, 1995; Taylor *et al.*, 2001b). Results from this investigation indicate that HAstV infection of bovine cell culture requires adaptation in cells derived from humans. Similar findings have been reported by others (Brinker *et al.*, 2000). Cell cultures of non-human origin would be required for the primary isolation and propagation of animal AstVs from animal stool specimens or environmental water sources. In this study AstVs detected in water samples after primary amplification in CaCo-2 and PLC/PRF/5 cell lines were HAstVs as confirmed by sequencing reactions. Our investigations of a cell culture of animal origin, i.e. MDBK cells, were complicated by the detection of an amplicon generated by Mon2/Mon67 primer pair, in the cell line.

We reconfirmed that use of cell culture prior to RT-PCR increased the sensitivity of HAstV-specific RT-PCR (Marx *et al.*, 1998b; Mustafa *et al.*, 1998; Chapron *et al.*, 2000; Taylor *et al.*, 2001b),

with the added benefit of removing inhibitory agents present in the specimens (Taylor *et al.*, 1997a; Marx *et al.*, 1998b; Mustafa *et al.*, 1998). In this study, selected stains from sewage samples, namely DE3, DE4 and B2 (Table 12), could only be detected and characterised after prior amplification in cell culture.

Of note is the apparent selection by the cell culture types for the propagation of specific HAstV serotypes, i.e. HAstV-1 and 2 were detected more frequently in the CaCo-2 cell line and HAstV-8 in the PLC/PRF/5 cell line, and HAstV-3 was detected in both cell lines (Table 12). In cell cultures infected with sewage water sample DE4, HAstV-2 and HAstV-8 were detected in CaCo-2 and PLC/PRF/5 cell cultures, respectively. A previous example of PLC/PRF/5 propagation of HAstV-8 is given by SA isolate AS20, an isolate from a stool specimen (Taylor *et al.*, 2001a). During cell culture investigations in this study, AS20 was detected by RT-PCR using Mon2/Mon67 from PLC/PRF/5 cells and the amplicon confirmed by oligonucleotide probe, whereas the amplicon from the CaCo-2 cells could not be confirmed (Table 8). Multiple types were detected in sewage sample B2, HAstV-1 and 2, from low and high passages of CaCo-2 cell cultures, respectively (Table 12).

Although animal AstVs do occur and strains have been found in all locations where they have been searched for, their detection is governed by the extent of the search for these viruses (Monroe, 1999). No animal AstVs were detected in this investigation (Table 11), which could be attributed to the small number of samples investigated and the observation that the RT-PCR, EIA and cell culture techniques used in this study selected for the detection of HAstVs. The only non-species-specific technique applied was EM,

as animal AstVs share the surface star-like structure with HAstVs (Monroe *et al.,* 2000a; Matsui and Greenberg 2001). Animal AstVs have been reported in SA with a single isolate among 423 porcine stool specimens examined by EM (Sebata, 1996), confirming that in this study a larger number of stools needed to be screened for the possible detection of animal AstVs.

Type assignment of all SA AstV isolates, by sequencing of PCR products amplified by type-common primer pairs, showed good correlation with characterisation by type-specific primer pairs (Tables 10 and 12). Type assignment by type-specific primer pairs enabled the identification of multiple types of HAstVs from a single sewage sample, e.g. sewage sample DW2 where four serotypes were identified by type-specific RT-PCR. With sewage sample DE4, one serotype (HAstV-7) was detected by type-specific RT-PCR (DE4_T7), and another two, HAstV-2 (DE4_S_BEG) and HAstV-8 (DE4_P_BEG), following amplification in CaCo-2 and PLC/PRF/5 cell lines respectively.

Apparent cross- or non-specific reactions were observed with the HAstV-1 type-specific primer pair Mon2/AV3. For example, using this primer pair, RT-PCR of sewage sample DE4 produced an amplicon of desired size, but no interpretable nucleotide sequence. A single stool specimen, 990028945, was positive by Mon2/AV3 and could not be amplified by any other type-common or type-specific primer sets (Table 10). Attempts at sequencing this Mon2/AV3 amplicon to confirm HAstV-1 identity also yielded uninterpretable nucleotide sequence although the specificity of the methods implied that 990028945 belonged to HAstV-1 genotype. The cross-reactivity of the HAstV-1 Mon2/AV3 primer pair,

therefore, calls for more detailed investigation of the AstV strain from stool specimen 980028945. To this end, amplification of different regions of the genome, in all ORFs, and sequencing of the PCR products is necessary to determine single or multiple type identity. These methods have been used previously for the confirmation that HAstV-8 (AS20), from SA, belongs to genogroup A (Taylor *et al.*, 2001a) and for providing the first evidence that recombination between HAstV RNAs can occur (Walter *et al.*, 2001b).

Two stool specimens, 980128705 (HAstV-8) and 96004419 (HAstV-1), could not be characterised using the type-common primer pair Mon2/prBEG or type-specific primer pairs, but were amplified and subsequently characterised by the Mon348/Mon340 type-common primer pair. Sequencing of RT-PCR products generated by Mon348/Mon340 is thus an effective alternate for the genotyping of AstVs (Belliot *et al.*, 1997b).

For this study, single HAstV genotypes from the clinical specimens and directly from six of the sewage samples were amplified by RT-PCR using type-common primers Mon2/prBEG, and characterised by sequencing of a 208 nt region of ORF2. However, sequence analysis using type-common primers Mon2/prBEG from a number of the sewage samples, directly from the sample concentrate and with no prior cell culture amplification, resulted in indeterminate or untypable sequences. Subsequent RT-PCR amplification of the same sample using HAstV-1 to HAstV-7 type-specific primers, which also amplified the 3'end of ORF2, resulted in the identification of multiple genotypes in at least five of the sewage samples. In one of the sewage samples, DW4, HAstV-1 was

identified by RT-PCR using the type-common primers Mon2/prBEG while HAstV-7 was subsequently confirmed in the same specimen using type-specific primers. As has been reported for *Cryptosporidium parvum* (Reed *et al.*, 2002), we have shown that amplification and characterisation of a single genotype from a clinical specimen or water sample does not preclude the possibility that multiple genotypes may be present. A similar finding was reported for human CVs where, cloning of PCR products and sequencing of several individual clones identified multiple genotypes in a single sewage sample (Lodder *et al.*, 1999).

Of the clinical isolates characterised by sequence analysis of the 3′ end of ORF2, HAstV-1 was the most frequent (64%) type identified, with HAstV-3 (14%) and HAstV-5 (9%), being less common. This is similar to reports in other regions of the world (Kjeldsberg, 1994; Lee and Kurtz, 1994; Noel and Cubitt, 1994; Noel *et al.*, 1995; Palombo and Bishop, 1996; Mustapha *et al*, 2000; Sakamoto *et al.*, 2000; Oh and Schreier, 2001; Guix *et al.*, 2002). The occurrence of HAstV-6 and HAstV-8 in 9% and 5% of the specimens respectively is important as these types are reportedly seldom detected (Glass *et al.*, 1996; Monroe, 1999). HAstV-8 however, appears to be more common on the African continent (Monroe *et al.*, 2000b; Taylor *et al.*, 2001a) and in Barcelona, Spain (Guix *et al.*, 2002) than in others. The absence of HAstV-2 in the SA clinical specimens is noteworthy as this serotype was identified as the predominant type in other parts of the world, as in a peri-urban community of Mexico City (Walter *et al.*, 2001a). The distribution of HAstV genotypes in the environmental isolates, from SA and surrounding countries, is similar to that observed for the clinical isolates, in that HAstV-1

was the predominant type identified, i.e. in 36% of isolates. The difference was the occurrence of HAstV-2, HAstV-4 and HAstV-7 in the sewage samples, comprising 16%, 8% and 16% of the environmental isolates, respectively, whereas none were detected in the clinical specimens. Further research is therefore warranted to ascertain whether these types are possibly more resilient to environmental degradation or whether human infection with these types (HAstV-2, 4 and 7), is not as severe as that of the other types thus not requiring medical attention. Infection with HAstV-1 may be more severe than that of the other serotypes and is thus more frequently identified from clinical and environmental sources.

The results clearly indicate that HAstVs detected from both clinical and environmental sources are closely related and probably represent identical strains. Further analysis showed that the closely related SA environmental and clinical strains were distinct from other HAstV strains detected during the same time period (1997-2000) in other geographical locations. This suggests that faecally contaminated water could be a potential reservoir for human infection. In addition, the different strains present in the same community indicate that multiple strains and multiple genotypes circulate concurrently. Phylogenetic analysis of HAstVs from the Tshwane Metropolitan Area demonstrated that the SA strains aligned with the corresponding reference strains, but were sufficiently different to represent new subtypes ($p<0.05$). Two SA HAstV-1 subtypes were detected, one of them includes both clinical and environmental isolates (distance 0.05; $p<0.01$). The SA HAstV-1 isolates grouped into five clusters with a high level of genetic variability, which is expected when a large number of samples is examined (Guix *et al.*, 2002). Two separate subtypes of

HAstV-5 and HAstV-2 were identified. SA HAstV-8 formed a new subtype including environmental and clinical subtypes. SA HAstV-4 also clustered in a distinct subtype. The sequences of HAstV-3 and HAstV-7 isolates from the SA clinical and environmental sources as well as from other geographic regions appeared to be highly conserved, showing 98-100% nucleotide sequence identity. No phylogenetic analyses were done on AstV isolates from the other geographic regions of SA as the number of characterised isolates was small (n = 4).

No AstVs were detected in any of the six surface water samples collected downstream of the sewage treatment works from which multiple genotypes of HAstVs were detected. This suggests that the sewage treatment process effectively removes or inactivates the AstVs to titres below the level of detection.

The role of zoonotic infection of AstVs is not currently understood; consequently, the possible risk of infection to humans by animal AstVs in water sources needs further clarification. The type of HAstVs found in sewage is a reflection of the clinical epidemiology of HAstVs (Pintó *et al.*, 2001). Therefore, the presence of HAstVs in the environment could pose a potential health risk to persons using contaminated water for domestic or recreational purposes.

This is the first study in which AstV isolates from water sources, identified as HAstVs, have been characterised by sequityping. This provides a feasible alternate assay to RFLP analysis (Pintó *et al.*, 2001) for the characterisation of AstVs from environmental water samples. This study provides new data on the molecular epidemiology of HAstVs circulating in the communities in the

Tshwane Metropolitan Area of SA and other geographic regions. An infrastructure has now been established for the identification of AstVs in epidemic and sporadic outbreaks. The detection of multiple types of AstVs from a single sample implies that a different typing strategy needs to be considered when sequence data are inconclusive.

CHAPTER 6

CONCLUSIONS

This study provides valuable new data on the distribution of HAstV genotypes in SA. Although there are many similarities in the HAstV epidemiology in SA to that reported for the rest of the world, the SA isolates show unique genotypic groupings.

From this study it is evident that AstVs can only be propagated in cells of other species origin after adaptation to growth on cells of species of origin. The techniques and methods applied in this study have proved selective for the detection and characterisation of HAstVs. Of the eight serotypes of HAstVs that infect humans, isolates detected from the SA human stool specimens represented all but HAstV-2 and 7. Not only was HAstV-1 most frequently identified, but a common HAstV-1 strain circulating among many of the patients stools collected in 1999, was identified. The absence of HAstV-2 and 7 from human stool specimens could possibly be ascribed to the fact that all specimens were from patients 5 years and younger. A differential age distribution of HAstV serotypes has been reported in Spain (Guix *et al.*, 2002). In comparing the genotypic data obtained from stool specimens and water samples it was obseved that there was a close relationship between the HAstVs from both sources. It is thus implied that the source of environmental faecal contamination in the SA studies (Marx *et al.*, 1998b; Taylor *et al.*, 2001b) is of human origin.

Analysis of animal stool specimens did not yield any identifiable or typable AstV isolates. This has been attributed to the use of human-specific primer pairs and cell culture types. Further research, using animal-specific primers and cell cultures of animal origin, is therefore required for the detection and characterisation of animal AstVs in stool and water samples.

This study has demonstrated the occurrence of HAstVs in sewage and environmental water samples. In addition, the ability to propagate these viral isolates on cell cultures implies that they are potentially infectious HAstVs. This is the first report of multiple types of HAstVs being present in a single sewage sample. What was initially thought to be novel nucleotide sequences isolated from AstVs, was confirmed by cell culture propagation and type-specific RT-PCR and sequencing, to be the overlapping of multiple nucleotide sequences of HAstVs. The methods applied in this study present an alternative to RFLP analysis (Pintó *et al.*, 2001) for the characterisation of AstV isolates from wastewater samples.

With the development of a rotavirus vaccine AstVs are emerging as an important aetiological agent of viral diarrhoea in infants. Future research should focus on methods for the detection and characterisation of animal AstVs in stool and water samples, and possible cross-species infection. With the possibility of future HAstV vaccine development (Keddy, 1998) the potential recombination between strains of human and animal origin also needs to be investigated.

CHAPTER 7

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APPENDIX A

A.1 GLASS-WOOL ADSORPTION-ELUTION PROCEDURE

Glass wool columns are prepared by the compression of 10 g of glass wool (Saint Grobian, Isover-Orgel, France) into a perspex column (26 cm x 3,0 cm) such that a final density of 0,5 g/cm is reached (glass wool dry weight/volume basis). The column was cleaned by filtering through one volume of 1M HCl (40 ml), two and a half volumes of distilled water and one volume of 1 M NaOH. The column was rinsed with distilled water until the pH of this rinsing water was neutral.

Four grams of dechlorination granules (Wallace and Tiernana, Germany) were placed into the column for the neutralisation of chlorine residuals in the water sample. The water sample was filtered through the prepared perspex column by negative pressure system. After filtration, viruses were eluted from the glass wool with 100 ml glycine-beef-extract buffer (GBEB) (0,05 M glycin, 0,5 % beef extract, pH 9). The 100 ml eluate was further concentrated for viruses using the PEG/NaCl viral concentration method.

A.2 PEG/NaCl CONCENTRATION METHOD

Viruses were concentrated by adding 2,22 g of NaCl (Merck) and 7,0 g of PEG-6000 (Merck) per 100 ml of sample. The sample was maintained at for a minimum of 2 h at 4 °C with constant stirring. The resultant solution was centrifuged at 7000 rpm for 30 min. The resulting pellet was resuspended in 10 ml PBS and supernatant discarded. The PBS solution was sonicated on ice for 5 min and centrifuged for 30 min at 7000 rpm. The supernatant was retained for direct RNA extraction or for further volume-reduction techniques. The pellet was discarded (Minor, 1985; Vilaginès *et al.*, 1997).

APPENDIX B

B.1 NUCLEIC ACID SEQUENCING REACTIONS

a) Reagents

Gel Stock	
40% Acrylamide (19:1)	200 ml
10 X Tris-Borate-EDTA (TBE)	100 ml
Urea	420 g
Make up to 1L with distilled H_2O	

For X1 Gel:	
Stock solution	75 ml
10% AMPS	300 µl
TEMED	23 µl

b) Sequencing reactions

The PCR product was first treated enzymatically. Exonuclease 1 removed residual single stranded primers and extraneous single stranded DNA produced by the PCR. Shrimp alkaline phosphatase removed the remaining dNTPs from the PCR mixture, which would interfere with the labeling step of the sequencing process. Both forward and reverse primer amplification strands were sequenced. The primer annealed to the template and the reaction proceeded by incorporating radioactively labeled bases into the synthesised nucleic acid. The time of the T-7 polymerase activity was

determined by the size of the PCR amplicon being sequenced. Placing the reaction onto ice stopped the polymerase activity. A termination reaction with each of the 4 DNA bases took place separately. An analogue for dGTP, 7-deaza-dGTP was used. The 7-deaza-dGTP formed weaker secondary structures enabling more linear DNA to be formed. This eliminated some compression of the nucleic acid and resulted in a better separation pattern. The addition of a stopping solution halted the reaction. The samples were heated briefly before loading onto gel to separate double strands.

c) Sequencing gel

An 8% polyacrylamide (BioRad, Hercules. CA)-6 M urea (Merck, Darmstadt, Germany) gel was used for separation of the sequencing products. The gel was poured between 2 glass plates of dimensions (34.5 cm x 45 cm x 0.5 cm). The plates were pretreated and cleaned by wiping with acetone [$(CH_3)_2CO$] and methanol [CH_3OH] for the removal of contaminating residues. The surface of the slotted plate was treated with Gel Slick solution (Bioproducts, Rockland, USA). The slicking solution prevents the gel from adhering to the plate.

The plates were separated by two spacers and assembled into a compact unit with clamps holding the sides together. For the pouring of the gel, a 1,5 ml aliquot was made up to seal the open bottom end. The larger volume of gel mixture was then gently poured into the gel space. The sequencing gel was allowed to set overnight. Before loading the sequencing products the gel was pre-heated at 60 V for 1 h. For this the glass plates containing the

cast gel was placed upright into the sequencing apparatus (Hybaid) and connected to a power source (Consort E734). One times TBE buffer (pH 8.3) (Amresco, Solon, OH) was used.

The samples were denatured at 75°C for 2 min to separate double strands before loading onto the gel. Three microlitres of sample was pipetted into the allocated well. All samples were loaded in the order of bases G-A-T-C. Electrophoresis was allowed for a period of time as determined by the size of the initial PCR product.

The gel was separated from the glass plate pretreated with the Slick, adsorbed onto a sheet of blotting paper (3 mm Chr, Whatman, Chromatography paper, Cat. No. 303917, Whatman International Ltd. Maidstone England) and fixed by rinsing with an alcohol reagent. Together with the paper sheet the gel was rolled off the glass plate. The paper containing the intact gel was then dried in a gel drier (Drygel Sr. Slab Gel Dryer. Model SE 1160. Hoefer Scientific Instruments, San Francisco) for 1 h at 80°C. A condensation system (Refrigerated Condensation trap RT400) operated together with a high vacuum pump (VP 190 Two Stage, Savant Instruments Inc.), to combine the gel and Whatman paper into a single membrane. The membrane was exposed to an X-ray film overnight and visualised by development of the film. The membrane was analysed by reading the bands exposed on the film in the ascending order of G-A-T-C from the lower end. The profiles generated by long and short periods of electrophoresis were consolidated to provide a single sequence and analysed. All material in contact with radioactivity, including the membrane was discarded in designated radioactive waste containers for removal and appropriate disposal.

APPENDIX C

C.1 Summary of astrovirus detection from animal stool specimens

Specimen ID number	Date of collection	Antigen[1] detection	RT-PCR[2] PAGE[3]	Probe
C1	28-06-1999	-	w+[4]?	-
C2	28-06-1999	-	w+?	-
C3	28-06-1999	-	w+?	-
C4	28-06-1999	-	w+?	-
C5	28-06-1999	-	w+?	-
C6	20-08-1999	-	w+?	-
C7	20-08-1999	-	w+?	-
C8	20-08-1999	-	-	-
C9	20-08-1999	-	w+?	-
C10	20-08-1999	-	+?	-
C11	20-08-1999	-	w+?	-
C12	20-08-1999	-	w+?	-
C13	20-08-1999	-	-	-
F1(1)	01-02-2000	-	+?	-
F1(2)	01-02-2000	-	+?	-
F1(3)	01-02-2000	-	-	-
F1(4)	01-02-2000	-	-	-
F2(1)	01-02-2000	-	-	-
F2(2)	01-02-2000	-	-	-
F2(3)	01-02-2000	-	-	-
F3(1)	01-02-2000	-	-	-
F3(2)	01-02-2000	-	-	-
F3(3)	01-02-2000	-	-	-
F3(4)	01-02-2000	-	-	-
F4(1)	01-02-2000	-	-	-
F4(2)	01-02-2000	-	-	-
F4(3)	01-02-2000	-	-	-
F5(1)	01-02-2000	-	-	-
F5(2)	01-02-2000	-	+?	-
F5(3)	01-02-2000	-	+?	-
F5(4)	01-02-2000	-	-	-
F5(5)	01-02-2000	-	-	-
F36(1)	01-02-2000	-	-	-
F36(2)	01-02-2000	-	-	-
F36(3)	01-02-2000	w+?	-	-
F37(1)	01-02-2000	-	-	-
F37(2)	01-02-2000	-	-	-

C.1 *continued*: Summary of astrovirus detection from animal stool specimens

Specimen ID number	Date of collection	Antigen[1] detection	RT-PCR[2]	
			PAGE	Probe
BB1	25-01-2000	-	-	-
BB2	25-01-2000	-	-	-
BB3	25-01-2000	-	-	-
BV1	25-01-2000	-	-	-
BV2	25-01-2000	-	-	-
BV3	25-01-2000	-	-	-
HB1	31-01-2000	-	-	-
HB2	31-01-2000	-	-	-
HB3	31-01-2000	-	-	-
HB4	31-01-2000	+?	-	-
HK1	31-01-2000	-	-	-
HK2	31-01-2000	-	-	-
HV1	31-01-2000	-	-	-
HV2	31-01-2000	-	-	-
HV3	31-01-2000	-	-	-
PB1	25-01-2000	-	-	-
PB2	25-01-2000	-	-	-
PB3	25-01-2000	-	-	-
PB4	25-01-2000	-	-	-
PP1	23-03-2000	-	-	-
ARC-P1	23-03-2000	-	-	-
ARC-P2	23-03-2000	-	-	-
DB1	25-01-2000	-	-	-
DB2	25-01-2000	-	-	-
DB3	25-01-2000	-	+?	-
DB4	25-01-2000	-	-	-
DV1	25-01-2000	-	-	-
DV2	25-01-2000	-	-	-
DV3	31-01-2000	-	-	-
DV4	31-01-2000	-	+?	-
DV5	31-01-2000	-	+?	-
DE1	25-01-2000	-	-	-
DE2	25-01-2000	-	+?	-
DMG1	25-01-2000	-	-	-
DMG2	31-01-2000	-	+?	-
DT1	25-01-2000	-	-	-
DA1	25-01-2000	-	-	-
DA2	25-01-2000	-	-	-
DA3	25-01-2000	-	-	-
DA4	25-01-2000	-	-	-
KT1	12-08-2000	-	-	-
DG1	12-08-2000	-	-	-

Footnote to Appendix C tables:
1: Antigen detection by enzyme immunoassay
2: Reverse transcriptase-polymerase chain reaction
3: Polyacrylamide gel electrophoresis
4: Weak positive

CODE

C : calf

F1(1) : Delmas: Feedlot No. 1, cattle No. 1

BB : cattle – Bronkhorstspruit farm

BV : Pig – Bronkhorstspruit farm

HB : cattle – Kameeldrift: plot

HK : calf – Kameeldrift: plot

HV : pig – Kameeldrift: plot

PB : cattle – UP Research Farm

PP : pig – UP Research Farm

ARC-P : pig –Agricultural Research Council: Animal Improvement Institute, Irene, Pretoria

DB : cattle – Pretoria Zoo

DV : pig – Pretoria Zoo

DE : duck – Pretoria Zoo

DMG : mountain goat – Pretoria Zoo

DT : Turkey – Pretoria Zoo

KT : kitten – local vet

DG : dog – local vet

APPENDIX D

D.1 **Nadan S**, Grabow WOK, Taylor MB. The molecular detection and characterisation of astroviruses from human stool specimens and sewage [Poster/Presentation]. Faculty Day, Faculty of Health Sciences, University of Pretoria 21-22 August 2001: Pretoria

ABSTRACT: Astroviruses (AstVs), one of the enteric viruses, are able to persist in the environment and their transmission by food and water has been documented. Astroviral infection is reported to be species-specific and specific AstVs have been associated with diarrhoeal disease in humans and young animals such as calves, piglets, lambs and domestic cats. There are 8 serotypes of human AstVs (HAstVs) and to date 8 serotypes of animal AstVs have been identified. AstVs have been detected, by molecular techniques, in a number of food and water sources but the virus isolates were not characterised to confirm their specificity. The aim of this study was to characterise and compare AstV isolates from human stools and sewage from the same geographical region using type-specific reverse transcriptase-polymerase chain reaction (RT-PCR) and /or sequencing.

Human stool specimens, sewage samples and associated treated effluent were screened for AstVs using a commercial enzyme immunoassay (EIA) kit and a group-specific RT-PCR-oligonucleotide probe assay. Where insufficient specimen was available cell cultures of human origin were infected for virus amplification. Fifteen HAstV isolates from stool and 15 isolates from sewage were subsequently characterised by type-specific RT-PCR and/or sequencing. From the results obtained it is evident that the majority of AstVs detected in sewage samples were of human origin while no AstVs were detected in water samples collected downstream of the sewage works. This is the first study addressing the occurrence and characterisation of AstVs in raw and treated water.

D.2 Taylor MB, **Nadan S**, Grabow WOK, Walter JE. Molecular epidemiology of human astroviruses from the Tshwane area (Pretoria), Gauteng [Presentation]. Joint Congress of the Infectious Diseases & Sexually Transmitted Diseases Societies of Southern Africa. 2 - 7 December 2001: Spier Estate, Stellenbosch, South Africa.

ABSTRACT: Human astroviruses (HAstVs) are an important cause of gastroenteritis worldwide with the young, the elderly and the immunocompromised at greatest risk. To date 8 distinct serotypes of HAstVs, which correlate with genotypes, have been identified. HAstV-1 is the most prevalent serotype detected in many region of the world while HAstV-2 to -5 seem to be less common. HAstV-6 to -8 are seldom detected. In South African children, next to rotavirus, HAstVs have been shown to be the second most common cause of viral gastroenteritis with a prevalence between 5,1 and 7%. There is however little data on the HAstV sero- or genotypes circulating in the South African community. The aim of this study was to characterise HAstV isolates from children with gastroenteritis in the Tshwane area, South Africa (SA), to determine which genotypes were present between 1996 and 2000. To this end a combination of HAstV type-specific reverse transcriptase-polymerase chain reactions and nucleotide sequencing of a limited region of ORF2 were used. To date 20 of 39 HAstV isolates, detected in diarrhoeal stool specimens by enzyme immunoassay, have been characterised. HAstV-1 was the most commonly detected strain, compromising 65% of the typed isolates. HAstV-3, compromising 20% of the isolates typed, was second most common type identified, while only single isolates of HAstV-5, -6 and –8 were detected. No HAstV-2, -4 or –7 strains were identified. This distribution of strains is similar to that reported for other regions of the world. This study provides new and valuable baseline data for the characterisation of isolates from other geographical areas of SA or from outbreaks to determine and identify the source of virus.

D.3 **Nadan S**, JE Walter, Grabow WOK, Taylor MB. The molecular detection and characterisation of astroviruses from human stool specimens and sewage [Presentation]. "Microbial Diversity" 12th Biennial Congress of the South African Society for Microbiology, Faculty of Health Sciences, University of the Free State 2-5 April 2002: Bloemfontein

ABSTRACT: Human astroviruses (HAstVs) HAstVs are an important cause of gastroenteritis worldwide. HAstVs affect all age groups with the very young, the elderly and immunocompromised at greatest risk. Astrovirus (AstV) infection has also been reported in calves, cats, dogs, piglets and lambs but infection is reportedly species-specific and to date no interspecies transmission has been documented. AstVs are able to persist in the environment and their transmission by water and food has been documented. Wastewater and other water sources are therefore a good indicator of which AstVs are circulating in a specific community. There are 8 distinct serotypes of HAstVs with HAstV-1 being the most prevalent serotype. HAstV-2 to HAstV-5 are less common and HAstV-6 to HAstV-8 rarely detected. A number of animal AstV serotypes have also been identified. Genetic analysis of AstV strains can provide valuable information with respect to the source of virus in both sporadic and epidemic human infection. The aim of this study was to isolate and identify AstVs from sewage and water sources and to compare them with AstV isolates from hospitalised patients in the same geographical region. Human stool specimens (n=35), collected between January 1996 and October 2000, sewage samples (n=15) and associated downstream water (n=6), were screened for AstVs using a commercial enzyme immunoassay (EIA) kit and/or a HAstV type-common reverse transcription-polymerase chain reaction (RT-PCR)-oligonucleotide probe assay. Cell cultures were used for the amplification of multiple AstV strains from a single sample. Twenty-two HAstV isolates from human stool specimens and 13 isolates from sewage

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samples were characterised by type-specific RT-PCRs and/or sequencing. HAstV-1 was the most commonly identified serotype in both human specimens (59%) and sewage samples (62%). HAstV-2 was only detected in sewage samples suggesting that either this type is more resistant to environmental inactivation or that the HAstV-2 infection on patients was less severe and therefore did not require hospitalisation. HAstV-3, -5, -6, and -8 were detected less frequently in the stool samples and sewage samples. From the results obtained it was evident that all the AstV isolates from sewage and water sources tested to date were of human origin. The results imply that the cell cultures and techniques used for the isolation and detection of AstVs from sewage and water sources in this and previous studies targeted HAstVs. There is therefore a need to develop specific techniques for the isolation and detection of animal AstVs from water and other sources. This study provides valuable new data on the occurrence and distribution of AstV serotypes in South Africa.

D. 4 WB van Zyl, **S Nadan**, JC Vivier, JME Venter, K Riley, EKM Tlale, LR Seautlueng,WOK Grabow, MB Taylor. The prevalence of enteric viruses in patients with gastroenteritis in the Pretoria and Kalafong Academic Hospitals, South Africa [Poster]. "Microbial Diversity" 12th Biennial Congress of the South African Society for Microbiology, Faculty of Health Sciences, University of the Free State 2-5 April 2002: Bloemfontein.

ABSTRACT: Enteric viruses are important causitive agents of waterborne diseases, such as gastroenteritis, hepatitis A and E and respiratory diseases, which are a major cause of morbidity and mortality worldwide. Although unable to multiply in water, viruses have a low infectious dose of one to ten viral particles. Several studies have addressed the prevalence of gastroenteritis, hepatitis and enteroviruses in South Africa (SA). However, no single study addresses the overall presence of enteric viruses in stool specimens in one cohort study in SA. The aim of the study was to determine the prevalence of enteric viruses in patients with gastroenteritis presenting at the Pretoria and Kalafong Academic Hospitals over a one-year period from January to December 2001. Stool specimens referred to the Dept Medical Virology Diagnostic laboratory for routine analysis for gastroenteritis viruses, were used to determine the presence of the following enteric viruses: adeno (40/41), human astro (HAstV), human calici (HuCV), entero, hepatitis A (HAV) and rotaviruses. Adeno (40/41), HAstV and rotaviruses were routinely detected using commercially available enzyme immunoassays. RNA was isolated from 10% stool suspensions and virus-specific reverse transcriptase-polymerase chain reactions (RT-PCRs) were used to detect HuCV, HAV and enteroviruses. The sensitivity of detection of HAV and enteroviruses was enhance by probe hybridization and nested PCR respectively. Results obtained for 300 stool specimens analysed from January to September 2001 were as follows: entero (54.3%), rota (18%), adeno 40/41 (2.9%); astro (2.2%); calici (1.4%) and HAV (0.01%). From the results to date it is clear that enteroviruses show the highest prevalence of all viruses investigated with rotavirus being the second most prevalent

virus. This is ascribed to the fact that 92% of the stool specimens were obtained from paediatric patients where excretion of poliovirus vaccine strains is common and rotavirus-associate gastroenteritis is the main cause of viral diarrhoea in this age group. As paediatric HuCV infection is usually mild and self-limiting individuals infected with HuCV are seldom hospitalized, explaining the low prevalence recorded in this study. In some studies HAstV, while in others enteric adenovirus has been found to be the second most important cause of acute virus gastroenteritis in infants and young patients children. In this study however similar prevalences for HAstV and enteric adenovirus were noted. The low prevalance of HAV detected in this study is surprising as hepatitis A is endemic in South Africa with subclinical infections commonly found in children. These results provide valuable new data on enteric viruses circulating in a select community with important implications for infection control procedures in paediatric wards.

APPENDIX E

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Molecular characterization of astroviruses: comparison between clinical and environmental isolates from South Africa

S. Nadan[1], JE Walter[2], WOK Grabow[1], DK Mitchell[2], MB Taylor[1*]

1. Department of Medical Virology, University of Pretoria, PO Box 2034, Pretoria 0001

2. Center for Pediatric Research, Children's Hospital of The King's Daughters, 855 W Brambleton Ave, Norfolk, Virginia, USA 23510-1001

Address all correspondence to:

Prof MB Taylor

Dept of Medical Virology

Faculty of Health Sciences

University of Pretoria

P O Box 2034

Pretoria

0001 South Africa

Tel: (+2712) 319-2358 Fax: (+2712) 325-5550

E-mail: mtaylor@medic.up.ac.za

Running title: Genetic characterization of South African astroviruses

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ABSTRACT

Comparative analysis was performed on 25 strains of astroviruses (AstVs) detected in sewage sources and 22 concurrently identified clinical AstV isolates from the Tshwane (Pretoria) Metropolitan Area, South Africa. The samples and specimens were screened for AstVs using enzyme immunoassay and/or type-common reverse transcriptase-polymerase chain reaction (RT-PCR) in the highly conserved untranslated region (3' end) of the genome. The RT-PCR results were confirmed by oligonucleotide probe dot blot hybridization. Viable viruses were propagated on cell cultures for amplification when minimal specimen was available or indeterminate sequences were obtained. AstV strains were characterized by type-common RT-PCR in the capsid region, and sequencing analysis. Selected environmental strains could only be typed by type-specific RT-PCR and sequencing of the same capsid region, facilitating the identification of multiple HAstVs types in a single sewage sample. Amplification of a single genotype from a sample therefore does not preclude the possibility of the sample containing additional different genotypes. Genotype and sequence information obtained from AstVs in wastewater samples were compared to AstV strains from human stools. HAstV-1, 3, 5, 6 and 8 were identified among the clinical strains, and HAstV-1, 2, 3, 4, 5, 7 and 8 among the environmental samples. Phylogenetic analysis demonstrated that HAstV-1, 3, 5 and 8 strains, simultaneously present in human stool and sewage samples, clustered together indicating close relatedness. The concurrent presence of identical HAstV strains in wastewater samples and among hospitalized patients suggests that AstVs present in the environment pose a potential risk to communities using fecally contaminated water for recreational and domestic purposes.

Key words: Astroviruses, sewage, cell culture, RT-PCR, sequencing

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INTRODUCTION

Human astroviruses (HAstVs) cause human diarrhea (4), and have been identified as the second most important cause of viral infantile diarrhea in selected areas of South Africa (SA) (30, 57) and in other regions of the world (5, 32). HAstV infection has been reported in all age groups, with the young, elderly and immunocompromised at greatest risk (12, 15). Astroviruses (AstVs) also cause asymptomatic infections (8, 28). Transmission of HAstV infection is via the faecal-oral route (8, 15). Although contaminated food (46, 63) and water (11) have been associated with outbreaks of HAstV-associated gastroenteritis, the attributable risk of food and water contamination in the transmission of HAstVs has not yet been fully elucidated (15, 16). AstVs also have been associated with scours in young animals such as calves, lambs, pigs cats, dogs and mink (8, 24) as well as a fatal hepatitis in ducklings (17), hemorrhagic enteric syndrome in turkeys (23) and acute intestinal nephritis in chickens (20). To date, eight HAstV serotypes (HAstV types 1-8) have been described (33, 37, 61, 65). Certainly two, and possibly three, serotypes of bovine AstVs (BAstVs) (37) and one of porcine AstV (PAstV)(38) have been recognized.

AstVs have a single-stranded polyadenylated positive-sense RNA genome, approximately 6.8-7.2 kb in size, that contains three open reading frames (ORFs) designated ORF1a, ORF1b, and ORF2 (37). ORF2 is located at the 3' end of the genome and encodes the capsid protein precursor (7). This protein has a well-conserved amino terminus (7). Nucleotide sequence analysis of a limited region of ORF2 has facilitated phylogenetic comparisons of HAstVs (44) with good correlation between antigenic and genomic types (3, 44). Partial and complete sequence data are available for a limited number of animal (21, 65) and turkey (23) AstV isolates. Although the capsid proteins of AstVs infecting different hosts are reportedly highly divergent, similarities between HAstV,

feline AstV (FAstV) and PAstV capsid sequences suggest that zoonoses involving pigs, cats, and humans could be occurring (21). However, AstV infection appears to be species-specific (32) and to date no interspecies transmission has been documented (21).

Surface waters, in both rural and urban areas, are affected by faecal contamination from human and animal sources (14, 18). The occurrence of AstVs in water sources (9, 29, 31, 40, 50, 60) and sludge biosolids (10) have been reported, but the clinical significance and epidemiological impact of environmental AstV strains is unknown (60). Until recently, there have been no reports on the antigenic or molecular characterization of environmental AstV isolates, but a recent publication describes the use of restriction fragment length polymorphism (RFLP) to genotype these isolates (51). The aim of this study was to detect, by type-common reverse transcriptase-polymerase chain reaction (RT-PCR), and to characterize, by partial sequencing of the 3' end of ORF2 capsid gene, AstVs from water and sewage samples and to compare these strains to AstV strains from the stools of humans in the same geographic region. These comparative data will provide valuable information as to the possible source of human infection or source of faecal contamination of surface waters in communities using these water sources for domestic and recreational purposes.

MATERIALS AND METHODS

Sewage and water samples. Three to four sewage samples (1 - 2 L each) were collected from April 1999 to October 2000 at three sewage treatment plants serving residential areas of the Tshwane (Pretoria) Metropolitan Area, Gauteng, SA (Table 1). Three concurrent surface water samples (1 L each) were collected from surface flows downstream from two of the sewage treatment plants, namely Daspoort and Baviaanspoort sewage works.

The sewage and water samples were clarified by centrifugation (Beckman GS-6R centrifuge) for 30 min at 3000g. The resultant pellet was resuspended in supernatant fluid (10 mL) and clarified by the addition of chloroform (10%v/v)(Merck, Darmstadt, Germany) with further centrifugation (Beckman GS-6R centrifuge) for 10 min at 3000g. The supernatants from the first and second clarification procedures were pooled and AstVs were recovered from the supernatant in a final volume of 10 mL phosphate-buffered saline, pH 7.4 (PBS)(Sigma Chemical Co., St.Louis, MO) using the polyethylene-glycol/sodium chloride (PEG/NaCl) precipitation technique as described by Minor (34) for the concentration of picornaviruses. The viral suspension was concentrated further to 2 mL by ultrafiltration using a Biomax-100K NMWL membrane (Ultrafree® 15 Centrifugal Filter Device; Millipore Corporation, Bedford, MA). The final concentrate was aliquoted and stored at -20°C.

Clinical specimens. Stool specimens from pediatric (<5 years of age) patients who presented with gastroenteritis at two tertiary referral hospitals in the Tshwane Metropolitan Area were submitted for the routine diagnosis of gastroenteritis viruses. HAstVs were detected, by EIA (IDEIA™ Astrovirus: Dako Ltd., Ely, UK), in 32 of 1303 (2.5%) stool specimens referred from January 1998 to October 2000 for analysis. An additional three

HAstVs were detected, by EIA, retrospectively in 1% (3/356) of stool samples referred between January 1996 and December 1997 which had been stored at 4°C. Stool specimens and suspension (10% in PBS [Sigma]) thereof were stored at 4°C.

Cell culture amplification. To enhance detection by RT-PCR or to clarify sequencing data from certain isolates, AstVs in concentrates of selected water samples and stool suspensions were amplified by propagation in cell culture. Sample concentrates and stool suspensions were treated with penicillin (50 μg/ml), streptomycin (50 μg/ml) and neomycin (100 μg/ml)(PSN antibiotic mixture [100X]: GIBCOBRL Life Technologies, Paisley, Scotland) and 100 units/ml nystatin (Nystatin [100X]: GIBCOBRL) and inoculated onto monolayers of the human hepatoma cell line, PLC/PRF/5 (ATCC CRL 8024) passages 81 to 85, and the human colonic carcinoma cell line, CaCo-2 (ATCC HTB 37) passages 35 to 58 and 178 to 198. Cells were grown in 25 cm^2 cell-culture flasks, as described previously (59), and incubated for seven days at 37°C. After the appropriate incubation period, the infected cells were harvested and an aliquot blind-passaged, followed by further incubation for seven days at 37°C. Cell culture extracts (120 μl) from the initial harvest and after blind passage were assayed for AstV RNA by RT-PCR.

Detection of astroviruses by RT-PCR. To avoid the possibility of cross-contamination, viral recovery and sample processing procedures, RNA extraction, and analysis of amplicons were performed in separate rooms. Reagents for the RT-PCR were also prepared in a laminar flow cabinet. The primers and probe were synthesised by Sigma-Genosys Ltd., Pampisford, UK.

Aliquots of the sludge samples, stool suspensions, and cell culture extracts were pre-treated with an equal volume of 1,1,2-trichloro-trifluoroethane (Sigma) prior to the extraction of total RNA from 120 μl of treated sample using TRIZOL® reagent (GIBCOBRL)

according to manufacturer's instructions. The extracted RNA was resuspended in a final volume of 25 µl of sterile nuclease-free water (Promega Corp., Madison, WI) and stored at -70°C. For each extraction procedure, nuclease-free water was included as a negative control. RT-PCR was performed using 5 µl of RNA extract and type-common primer pair Mon2/Mon67 (35). The amplicon was confirmed as AstV by an oligonucleotide probe hybridization assay as described previously (31, 60). For further characterization, a region at the 3' end of the ORF2 capsid gene (nucleotides [nt] 6513 - 6781; HAstV-1 [L23513]) of all confirmed AstV positive samples was amplified using the primer pair Mon2/prBEG (53). These primers detect all HAstVs except type 4. The reaction mix details and conditions for the RT-PCR using these primers were essentially the same as for the Mon2/Mon67 primer pair (60), except for the 1 X PCR buffer being 10mM Tris-HCl [pH9.0], 50 mM KCL, 0.1% Triton® X-100, 1.5mM $MgCl_2$. Clinical isolates that were undetectable using the Mon2/prBEG primers were subjected to a HAstV-4 type-specific RT-PCR (64) or a type-common RT-PCR using primer pair Mon348/Mon340 amplifying a region of ORF1a (3). Environmental isolates confirmed to be AstV-positive by the RT-PCR-oligonucleotide probe hybridization assay but were undetectable or resulted in uninterpretable sequence using the Mon2/prBEG primers were subjected to HAstV-1 to HAstV-7 type-specific RT-PCRs as described by Walter et al. (64). Cell culture extracts of HAstV-1 to HAstV-7 Oxford reference strains were used as positive controls.

Sequencing of RT-PCR amplicons. DNA amplicons derived from the 3' end of the ORF2 capsid gene or the 289bp region of ORF1a were sequenced directly by the dideoxy chain-termination method (55) using the Sequenase Version 2.0 PCR Product Sequencing Kit (USB Corp., Cleveland, OH) according to the manufacturer's instructions. The sequencing reactions were run on an 8% polyacrylamide-6 M urea gel in 1 X Tris-

Borate-EDTA buffer. Gels were vacuum-dried and exposed to X-ray film (Hyperfilm™-ßmax: Amersham) for 12 hours at room temperature.

Sequence analysis and genotyping. Nucleotide sequences were entered into a database in PC/Gene (v6.85; IntelliGenetics Inc, Geneva, Switzerland). Basic sequence manipulation and verification (continuous open-reading frame and motifs characteristic of HAstVs) were performed using OMIGA (v2.0, Accelrys, Madison, WI). ClustalX (62) was used to create multiple alignments of the amino acid sequences of selected isolates and reference strains. Nucleic acid sequences were added and aligned in GeneDoc v2.3 using the corresponding amino acid alignment as template, resulting in a consensus length of 208 nt in the 3' end of ORF2 (41). Pairwise comparison of nucleotide sequences of all reference types with the selected isolates were calculated in GeneDoc v2.3 for preliminary genotype assignment and for identification of clusters of strains with 99-100% homology. Only one representative strain from each cluster was included in the phylogenetic analysis. The representative isolates were compared with AstV sequences present in GenBank using the BLAST-N program v2.1.1 (1, 2) to search for the closest strain available. The nucleotide sequence alignment was assessed for tree-likeliness of the data and the proper sequence composition required for multiple alignments by likelihood-mapping (42, 58) utilizing TREE- PUZZLE 5.0 (http://www.tree-puzzle.de). Phylogenetic trees were constructed from the nucleic acid sequence alignments using the maximum-likelihood algorithm of the program DNAML of PHYLIP (v 3.52c) running in UNIX environment (13). We performed the analysis rooted (with HAstV-4 as a root) and unrooted. In the analysis the global rearrangement option was invoked and the order of the sequence input was randomized ten times. Phylograms generated in DNAML were visualized by TREEVIEW package v1.5 (47) and further edited in Micrografx Designer Version 6.0a.

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Nucleotide sequence accession numbers. Published HAstV capsid gene sequences used in the pairwise comparisons and phylogenetic analyses included reference strains with complete capsid sequence: HAstV-1 [L23513], HAstV-2 [L13745], HAstV-3 [AF117209], HAstV-4 [Z33883], HAstV-5 [U15136], HAstV-6 [Z46658], HAstV-7 [AF248738], HAstV-8 [Z66541].

The nucleotide sequence data of the clinical and environmental AstV isolates reported here have been registered with the EMBL/GenBank database and assigned the following accession numbers: T3/SA/DW2_P3/1999 [AY094090], T7/SA/DW2_P7/1999 [AY094091], T4/DW3_P4/1999 [AY094092], T5/SA/DE2_T5/1999 [AY094089], T1/SA/DE3_C/1999 [AY094082], T8/SA/DE4_P/2000 [AY094083], T2/SA/DE4_S/2000 [AY094084], T1/SA/B2_64/1999 [AY094080], T2/SA/B2_61/1999 [AY094079], T1/SA/B3/2000 [AY094081], T2/SA/Z1/1999 [AY094085], T1/SA/Z2/1999 [AY094086], T3/SA/Z3/1999 [AY094087], T1/SA/Z4/2000 [AY094088], T8/SA/4759/1998 [AY093649], T3/SA/5200/1998 [AY093650], T5/SA/6899/1998 [AY093651], T1/SA/7110/1998 [AY093652], T6/SA/126729/1998 [AY093653], T1/SA/7052/1999 [AY093654], T1/SA/26025/1999 [AY093655].

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RESULTS

AstV genotypes in water/sewage samples and clinical specimens. AstVs were detected directly by the HAstV type-common RT-PCR in all of the human stool specimens previously identified by EIA, in 15/15 (100%) of the sewage samples, and in 1/6 (17%) stream water samples. AstV amplicons could be confirmed, by oligonucleotide probe hybridization assay, in the RT-PCR products from 100% of the human stool specimens, 13/15 (87%) of the sewage samples and none of the stream water samples. Of the 35 AstV strains from human stool specimens, 22/35 (63%) could be characterized directly after amplification from the stool specimen by sequencing of the Mon2/prBEG amplicon (296-324 nt , depending on the HAstV type) from the 3' end of ORF2. Two additional strains (T1/SA/4419/1996; T8/SA/128705/1998) were confirmed as HAstV by sequencing of a 246 bp region of ORF1a. After storage at 4°C for an extended period AstVs could no longer be amplified by RT-PCR from the remaining 11 stool specimens using type-common primers Mon2/prBEG and Mon348/Mon340 or type-specific primers. Seven environmental AstV strains (T2/SA/DW2_P/1999, T1/SA/DW4/2000, T1/SA/B3/2000, T2/SA/Z1/1999, T1/SA/Z2/1999, T3/SA/Z3/1999, T1/SA/Z4/2000), from 7 separate sewage samples, were amplified and characterized directly from the sewage samples, by sequencing of the Mon2/prBEG amplicon. An additional five strains (T1/SA/DE3_C/1999, T8/SA/DE4_P/2000, T2/SA/DE4_S/2000, T1/SA/B2_64/1999, T2/SA/B2_61/1999), originating from 3 sewage samples, could only be characterized after isolation in cell culture. One of the sewage samples (B2) yielded two different HAstV types from separate flasks of CaCo-2 cell cultures with differing passage numbers, while another sample (DE4) yielded two different HAstV types after amplification on two different cell culture types, i.e. CaCo-2 and PLC/PRF/5 (Table 1). Thirteen strains (T1/SA/DW2_T1/1999,

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T3/SA/DW2_P3/1999, T4/SA/DW2_P4/1999, T7/SA/DW2_P7/1999, T1/DW3_P1/1999, T3/DW3_P3/1999, T4/DW3_P4/1999, T7/SA/DW4_T7/2000, T1/SA/DE2_T1/1999, T3/SA/DE2_T3/1999, T5/SA/DE2_T5/1999, T7/SA/DE2_T7/1999, T7/SA/DE4_T7/2000), from five of the sewage samples, were sequityped from amplicons generated by type-specific RT-PCR directly from the sewage sample. Indeterminate sequences were obtained from amplicons derived from sewage samples DW1, DE1, and B1 and their cell culture derivatives.

The genotypes of the 24 characterized HAstVs from clinical specimens were: HAstV-1 (63%), HAstV-3 (13%), HAstV-5 (8%), HAstV-6 (8%), and HAstV-8 (8%). The 24 AstV isolates from the sewage samples were: HAstV-1 (36%), HAstV-2 (16%), and HAstV-3 (16%), HAstV-4 (8%), HAstV-5 (4%), HAstV-7 (16%) and HAstV-8 (4%) (Table 1). A seasonal prevalence was not apparent in this small sample set.

Sequence analysis of SA AstVs. Multiple alignment included sequences with a consensus length of 208 nt (after deduction of the primer sequences), from all SA strains. Pairwise comparison revealed groups of SA strains with 99-100% identity. The genetic relationships between the SA strains representing each group, and the reference strains in the 208 nt consensus region, are shown by nucleotide pairwise similarity scores (Fig. 1). The groups of isolates and representative strains for each group are summarized in Table 2.

In preparation for phylogenetic analysis the multiple alignments of the nucleotide sequences was tested by likelihood-mapping: the data had a tree-like structure, all sequences were in the range of proper sequence composition. Phylogenetic analysis was performed in two stages. First, all SA strains were included in an unrooted tree. Pairwise analysis and the phylogenetic tree demonstrated common branch points for the majority of SA strains within types, therefore 27 strains with 99-100% homology were withheld from

phylogenetic analysis to avoid repeats. Reference strains (HAstV-1 to 8) and representatives of the 21 distinctive SA strains were included for the final phylogenetic analysis. HAstV-4 was less related to the other reference strains and was therefore used as root for analysis.

The analysis (Fig. 2) showed that cluster of types with reference strains HAstV-1 to 8 separated with confidence (distances 0.09–0.62; $p < 0.05$). HAstV-3 and 7 and HAstV-5 and 8 were very closely related in this hypervariable region, with 90% and 82% pairwise identity and distances 0.09 and 0.124, respectively. HAstV-4 was significantly different from the other HAstV strains. All characterized SA strains could be assigned a type. Although the definition of strains is not clearly defined in the literature we arbitrarily considered an isolate a subtype if the nucleotide homology to the reference strain was <95% and the distance at the 3'end of ORF2 (208 nt) >0.05. Calculated intra-genotypical distances suggest that the HAstV-1, 2, 4, 5 and 8 SA strains represent new subtypes of the corresponding genotype (HAstV-1 90-94% distance 0.05-0.13; HAstV-2 88-94% 0.10-0.13; HAstV-4 88%, distance 0.12; HAstV-5 91-94%, distance 0.05-0.10; HAstV-8 94%, distance 0.06). SA strains of HAstV-3, 6 and 7 appear not to be new strains or subtypes (HAstV-3 99%, distance 0.01; HAstV-6 (96%, distance 0.04 and HAstV-7 99%, distance 0.005).

HAstV-1, HAstV-3, HAstV-5 and HAstV-8 were detected among clinical samples and environmental isolates. HAstV-1 comprised 22 (48%) of the 46 isolates characterized by sequityping of the 3' end of ORF2. The phylogenetic analysis included 8 representative strains as previously described. The nucleotide identity was 88-100% among the SA HAstV-1 isolates, compared to 89-94% to the prototype strain. Two strains recovered from wastewater sources formed a distinct subtype (T1a) but were more closely related to

the Oxford reference strain (distance 0.05) than the other SA strains. Another cluster (T1b), distinct from T1a (distance 0.04-0.6), was observed. Cluster T1b included multiple closely related environmental strains and strains from human stool samples represented by SA/Z4/2000, SA/B2_64/1999, SA/7110/1998, SA/7052/1999 and SA/26025/1999. The other two strains (SA/B3/2000 and SA/Z2/1999) were unique in sequence and only identified one time.

The environmental and clinical HAstV-3 isolates showed a high percentage ($\geq 98\%$) nucleotide sequence identity to each other and to the prototype strain, and clustered together in a single genotypic cluster, T3 (Table 2; Fig. 2). The HAstV-5 environmental isolate showed a higher percentage (95%) nucleotide identity to the prototype strain than did the clinical isolates with 91% nucleotide identity. The HAstV-5 environmental isolate and clinical isolates showed only 93% nucleotide identity (distance 0.07), as a result we considered them as unique subtypes genotype 5 (Fig. 2).

Two HAstV-8 strains, one from a clinical specimen in 1998 and the other from a sewage specimen collected in 2000, were analyzed. These two strains are closely related to each other (98% nucleotide sequence identity), but distinct from the prototype strain 93-94%)(Fig. 2). A 100% nucleotide identity was recorded between the two clinical HAstV-6 strains, with 96% nucleotide identity to the Oxford reference strain. No HAstV-6 strains were detected among the environmental isolates.

HAstV-2, HAstV-4 and HAstV-7 were detected only among environmental isolates. The nucleotide identity was 88-98% among the HAstV-2 isolates but when compared to the prototype strain the identity was lower (88-94%) resulting in the SA isolates clustering separately from the prototype strain within genotype 2 (Fig. 2). The SA HAstV-2 isolates represented two unique subtypes, represented by SA/B2_61/1999 and

SA/Z1/1999 in one group and SA/DE4_S/2000 in the other group (distance 0.013) (Fig. 2). The HAstV-4 isolates, both from the same sewage works, showed 100% nucleotide sequence homology to each other but with 88% nucleotide sequence homology to the prototype strain. The HAstV-7 isolates showed a high percentage ($\geq$98%) nucleotide sequence identity to each other and to the prototype strain, and grouped together in a single cluster (Fig. 2).

The SA HAstV strains, from clinical and sewage specimens, were compared to HAstV strains from different geographic locations for the same time period (data not shown). The analysis showed that the SA environmental and clinical strains in T1b and T8 cluster together and are distinct from strains isolated at the same time from different geographical locations.

DISCUSSION

In this study a RT-PCR-oligonucleotide probe hybridization assay followed by partial sequencing of the N-terminus of ORF2 was successfully applied to the detection and characterization of clinical and environmental AstV strains from the Tshwane Metropolitan Area, SA. The RT-PCR-oligonucleotide probe hybridization assay has previously been shown to be a valuable tool for the detection of HAstVs in stool specimens (30), and in conjunction with cell culture, for infectious HAstVs in water from different sources (10, 31, 60). In this investigation prior amplification in cell culture was also shown to facilitate the detection and characterization of multiple HAstV types from a single sample (Table 1).

PCR is a widely used means to detect and genotype viruses (9, 27, 35, 47, 49, 66) and parasites (52) from clinical and environmental sources. Sequence analysis of the 3' region of HAstV ORF2 provides type information as well as enough diversity to provide additional strain information. There are, however, no data on the ability of RT-PCR amplification of ORF2 to detect and characterize mixed populations of HAstV genotypes. For this study, single HAstV genotypes from the clinical specimens and directly from six of the sewage samples were amplified by RT-PCR using type-common primers Mon2/prBEG, and characterized by sequencing of a 208 nt region of ORF2. However, sequence analysis using type-common primers Mon2/prBEG from a number of the sewage samples resulted in indeterminate or untypable sequences (Table 1). We then questioned if these were truly unique strains or represented a mixed population of strains that underwent the sequencing reaction simultaneously. Subsequent RT-PCR amplification of the same sample using HAstV-1 to HAstV-7 type-specific primers, which also amplified the 3'end of ORF2, resulted in the identification of multiple genotypes in at least five of the sewage samples

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(Table 1). In one of the sewage samples, DW4, HAstV-1 was identified by RT-PCR using the type-common primers Mon2/prBEG while HAstV-7 was subsequently detected in the same specimen using type-specific primers. As has been reported for *Cryptosporidium parvum* (52), we have shown that amplification and characterization of a single genotype from a clinical specimen or water sample does not preclude the possibility that multiple genotypes may be present. A similar finding was reported for human caliciviruses where, cloning of PCR products and sequencing of several individual clones identified multiple genotypes in a single sewage sample (27).

Of the clinical isolates characterized by sequence analysis of the 3' end of ORF2, HAstV-1 was the most frequent (64%) type identified, with HAstV-3 (14%) and HAstV-5 (9%), being less common. This is similar to reports in other regions of the world (19, 22, 26, 39, 43, 44, 45, 48, 54). The occurrence of HAstV-6 and HAstV-8 in 9% and 5% of the specimens respectively is important as these types are reportedly seldom detected (15, 37). HAstV-8 however, appears to be more common on the African continent (35, 38, 61) and in Barcelona, Spain (19). The absence of HAstV-2 in the SA clinical specimens is noteworthy as this serotype was identified as the predominant type in other parts of the world, like in a peri-urban community of Mexico City (64). The distribution of HAstV genotypes in the SA environmental isolates is similar to that observed for the clinical isolates in that HAstV-1 was the predominant type identified, i.e. in 36% of isolates. The difference was the occurrence of HAstV-2, HAstV-4 and HAstV-7 in the sewage samples, comprising 16%, 8% and 16% of the environmental isolates respectively, while none were detected in the clinical specimens. Further research is therefore warranted to ascertain whether these types are possibly more resistant to environmental degradation or human infection is not as severe as that of the other types thus not requiring medical attention.

Lastly, is there a difference in the reservoir and/or mode of transmission between HAstV serotypes?

Our results clearly indicate that HAstVs detected from both clinical and environmental samples are closely related and probably represent identical strains (Table 2; Fig 2). Further analysis showed that the closely related environmental and clinical samples were distinct from other HAstV strains detected during the same time period (1997-2000) in other geographical locations. This suggests that fecally polluted water could be a potential reservoir for human infection. In addition, the different strains present in the same community indicate that multiple strains and multiple genotypes circulate concurrently. Phylogenetic analysis demonstrated that the SA strains aligned with the corresponding reference strains, but were sufficiently different to represent new subtypes ($p<0.05$). Two SA HAstV-1 subtypes were detected, one of them includes both clinical and environmental isolates (distance 0.05; $p<0.01$). Two separate subtypes of HAstV-5 and HAstV-2 were identified. SA HAstV-8 formed a new subtype including environmental and clinical subtypes. SA HAstV-4 also clustered in a distinct subtype. The sequences of HAstV-3 and HAstV-7 isolates from the SA clinical and environmental sources as well as from other geographic regions appeared to be highly conserved, showing 98-100% nucleotide sequence identity. This is similar to what was observed for hepatitis A virus (49).

The AstVs detected by RT-PCR-oligonucleotide probe hybridization, in the sewage samples were characterized as HAstVs, namely HAstV-1, HAstV-2, HAstV-3, HAstV-4, HAstV-5, HAstV-7 and HAstV-8 (Table 1). This suggests that the integrated RT-PCR-oligonucleotide probe hybridization assay used in this and previous studies (31, 60) for the detection of AstVs in the water and sewage samples selects for AstVs of human origin. As cross-species infection *in vitro* appears only to occur after prior adaptation of the AstV

isolate in cell culture of the species of origin (6), the amplification of AstVs from water and sewage specimens in cell lines of human origin, namely PLC/PRF/5 and CaCo-2, would serve to further enhance the selection and detection of viruses of human origin. Additional RT-PCRs using primers specific for animal AstVs and/or cell cultures of animal origin would therefore be required to detect AstVs of animal origin in water and sewage samples. The role of zoonotic infection of AstVs is not currently understood; consequently the possible risk of infection to humans by animal AstVs in water sources needs further clarification. The type of HAstVs found in sewage is a reflection of the clinical epidemiology of HAstVs (51). Therefore the presence of HAstVs in the environment could pose a potential health risk to persons using contaminated water for domestic or recreational purposes. The absence of AstVs in the surface water downstream to the sewage works from which multiple genotypes of HAstVs were detected indicates that these viruses are removed effectively by sewage treatment process. This study provides valuable new data on the molecular epidemiology of HAstVs circulating in the communities in the Tshwane Metropolitan area of SA and in southern Africa, and provides a feasible alternate assay to RFLP analysis (51) for the characterization of HAstVs detected in water sources.

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ACKNOWLEDGEMENTS

This work was supported by grants from the Water Research Commission and the National Research Foundation, South Africa. Ms. S. Nadan was supported by Grant No. 99/19 from the Poliomyelitis Research Foundation, South Africa, and a Grant-holder linked bursary from the National Research Foundation. Supported in part by NIAD RO1 AI45872-01.

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Table 1. Detection and characterization of astroviruses (AstVs) in sewage samples from three sewage treatment plants and two urban streams downstream of the sewage treatment plants in the Tshwane (Pretoria) Metropolitan Area, South Africa between April 1999 and October 2000.

Sampling site	Sampling date	Sample code	RT-PCR[a] PAGE[b]	RT-PCR[a] Probe[c]	Genotype
Sewage Treatment Plants					
Daspoort	19/04/99	DW1	+	-[d]	untypable
(*West inflow*)	28/04/99	DW2	+	+	HAstV[e]-1, -3, -4, -7[f] HAstV-2[g]
	24/05/99	DW3	+	-	HAstV-1, -3, -4[f]
	19/10/00	DW4	+	+	HAstV-1[g],-7[f]
Daspoort	19/04/99	DE1	+	+	untypable
(East inflow)	24/05/99	DE2	+	+	HAstV-1, -3, -5, -7[f]
	26/07/99	DE3	+	+	HAstV-1[h]
	19/10/00	DE4	+	+	HAstV-7[f],-2[i],-8[i]
Baviaanspoort	19/04/99	B1	+	+	untypable
	28/04/99	B2	+	+	HAstV-1, -2[h]
	19/10/00	B3	+	+	HAstV-1[g]
Zeekoegat	28/04/99	Z1	+	+	HAstV-2[g]
	24/05/99	Z2	+	+	HAstV-1[g]
	19/09/99	Z3	+	+	HAstV-3[g]
	19/10/00	Z4	+	+	HAstV-1[g]
Streams					
Pienaars river	28/04/99	R1	-	-	-
(*downstream*	24/05/99	R2	-	-	-
to Baviaanspoort)	02/08/99	R3	-	-	-
Apies river	19/04/99	A1	+	-	untypable
(*downstream*	24/05/99	A2	-	-	-
to Daspoort)	26/07/99	A3	-	-	-

Footnote to Table 1

[a] RT-PCR, reverse transcriptase-polymerase chain reaction with type-common primers, Mon2/Mon67;[b] PAGE, polyacrylamide gel electrophoresis; [c] Probe, oligonucleotide probe hybridization;[d] -, not detected; [e] human AstVs; [f] AstVs genotyped from amplicons derived directly from the sample by type-specific RT-PCRs; [g] AstVs genotyped from amplicons derived directly from the sample by group-specific RT-PCR; [h]AstVs genotyped from amplicons derived from infected CaCo-2 cell cultures by group-specific RT-PCRs; [i] AstVs genotyped from amplicons, derived by group-specific RT-PCRs, from two different cell culture types inoculated with the same sample.

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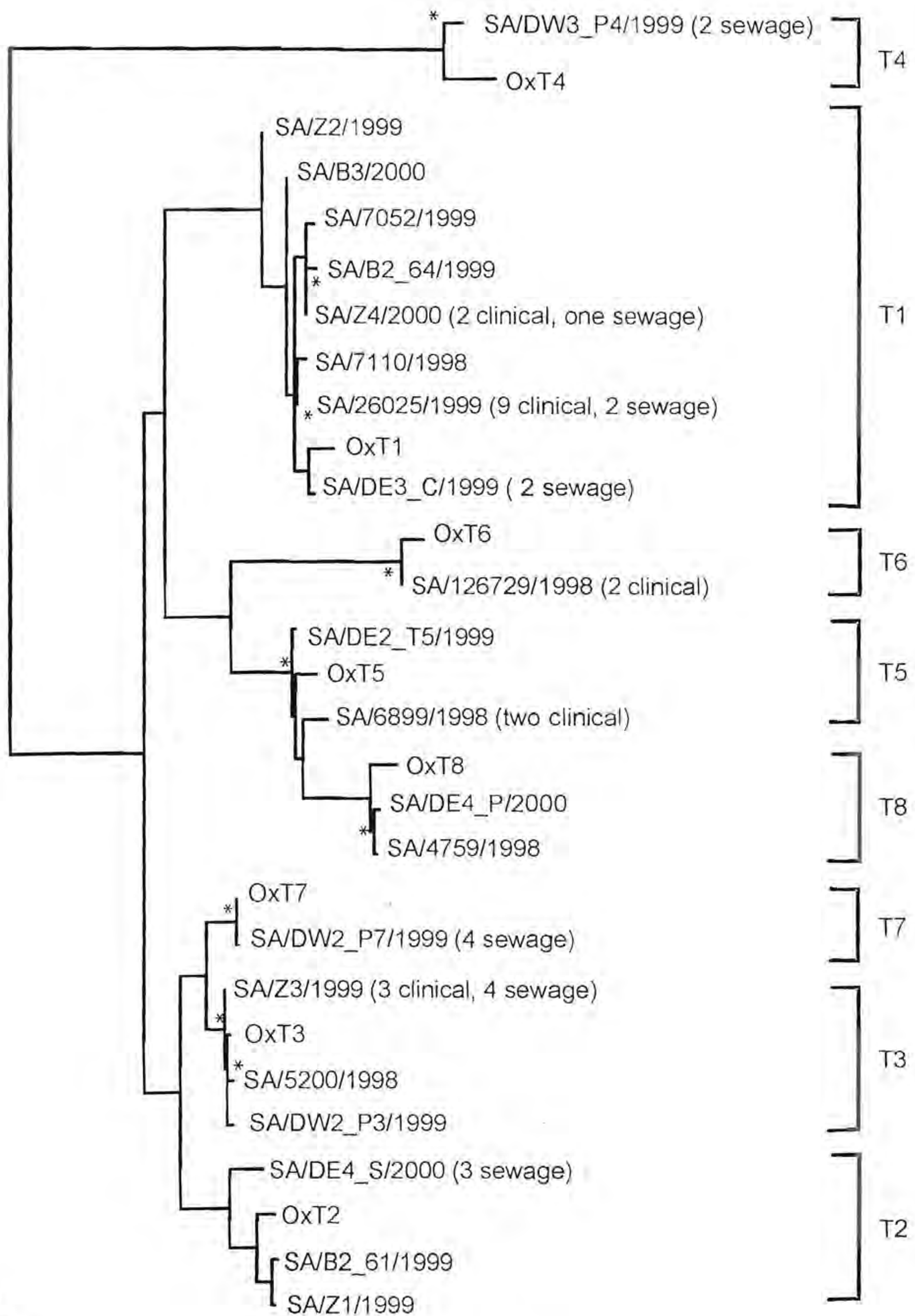

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FIG.2. Maximum likelihood phylogenetic tree based on a 208 nt region of the 3' end of ORF2 showing the relationships between representatives of the South African environmental and clinical human astrovirus (HAstV) isolates and the prototypes of HAstV types 1 to 8. Branch points of the resulting tree (rooted) had a confidence level of $p < 0.05$. Scale bar = number of nucleotide substitutions per site. * represents non-significant branching points (> 0.05).

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Table 2. Summary of the characterized South African (SA) human astrovirus strains from clinical and sewage sources identifying the representative strain included in the phylogenetic analysis.

Representative SA strains (n=21)	SA strains with 99-100% nucleotide identity to the representative isolate (n=25)
T3[a]/SA/Z3/1999	T3/SA/7169/1996; T3/SA/113768/1998; T3/SA/DE2_T3/1999; T3/SA/DW2_P3/1999
T3/SA/5200/1998	nd[b]
T3/SA/DW3_P3/1999	nd
T7/SA/DW2_P7/1999	T7/SA/DE2_T7/1999; T7/SA/DE4_T7/2000 T7/SA/DW4_T7/2000
T6/SA/126729/1998	T6/SA/5236/1998
T8/SA/4759/1998	nd
T8/SA/DE4_P/2000	nd
T5/SA/DE2_T5/1999	nd
T5/SA/6899/1998	T5/SA/126585/1998
T1/SA/DE3_C/1999	T1/SA/DE2_T1/1999
T1/SA/26025/1999	T1/SA/3144/1997; T1/SA/124893/1998; T1/SA/4642/1999; T1/SA/6802/1999; T1/SA/9559/1999; T1/SA/22320/1999; T1/SA/25786/1999; T1/SA/5114/2000; T1/SA/DW3_P1/1999; T1/SA/DW4/2000;
T1/SA/Z4/2000	T1/SA/29903/1999; T1/SA/3621/2000
T1/SA/7110/1998	nd
T1/SA/B3/2000	nd
T1/SA/Z2/1999	nd
T1/SA/7052/1999	nd
T1/SA/B2_64/1999	nd
T2/SA/Z1/1999	nd
T2/SA/B2_61/1999	nd
T2/SA/DE4_S/2000	T2/SA/DW2_S/1999, T2/SA/DW2_P/1999
T4/SA/DW3_P4/1999	T4/SA/DW2_P4/1999

a: Type assignment based upon comparison with the Oxford reference strain

b: nd = none detected

	T6	126729	T8	4759	T5	DE2	6899	T1	DE3	26025	Z4	Z2	T2	Z1	T3	Z3	T7	DW2	T4	DW3
OxT6 (Z46658)		***96***	57	57	55	58	57	52	54	54	52	54	58	58	56	57	57	58	41	43
T6/SA/126729/1998			58	57	58	61	59	54	56	56	54	56	58	59	57	58	57	57	41	43
OxT8 (Z66541)				***94***	82	83	82	67	67	66	66	70	67	66	70	70	67	67	40	42
T8/SA/4759/1998					85	85	84	67	66	65	65	69	68	67	71	71	68	68	42	43
OxT5 (U15136)						***95***	***91***	65	66	65	65	69	67	67	69	70	70	70	43	41
T5/SA/DE2_T5/1999							***93***	66	66	65	64	70	67	68	72	73	72	72	42	43
T5/SA/6899/1998								64	65	65	65	68	68	66	71	71	69	69	41	42
OxT1 (L23513)									***94***	***92***	***91***	***90***	66	69	69	69	67	66	45	45
T1/SA/DE3_C/1999										***96***	***94***	***90***	66	69	69	69	67	66	45	44
T1/SA/26025/1999											***97***	***94***	69	71	67	68	67	67	44	43
T1/SA/Z4/2000												***92***	69	68	66	66	66	65	44	43
T1/SA/Z2/1999													71	73	73	73	72	71	43	43
0xT2 (L13745)														***94***	80	81	82	81	42	42
T2/SA/Z1/1999															80	81	82	81	43	43
OxT3 (AF117209)																***99***	90	90	44	44
T3/SA/Z3/1999																	91	91	44	44
OxT7 (AF248738)																		***99***	43	43
T7/SA/DW2_P7/1999																			43	44
0xT4 (Z33883)																				**88**
T4/SA/DW3_P4/1999																				

FIG. 1.

FIG. 1. Relationship of selected South African human astrovirus (HAstVs) sequences (nucleotide 6481 to 6688; HAstV-1 [l23513] of ORF2, to the Oxford (Ox) reference strains. The numbers show the percentage nucleotide identity, by pairwise analysis, of the aligned nucleotide sequences.