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).

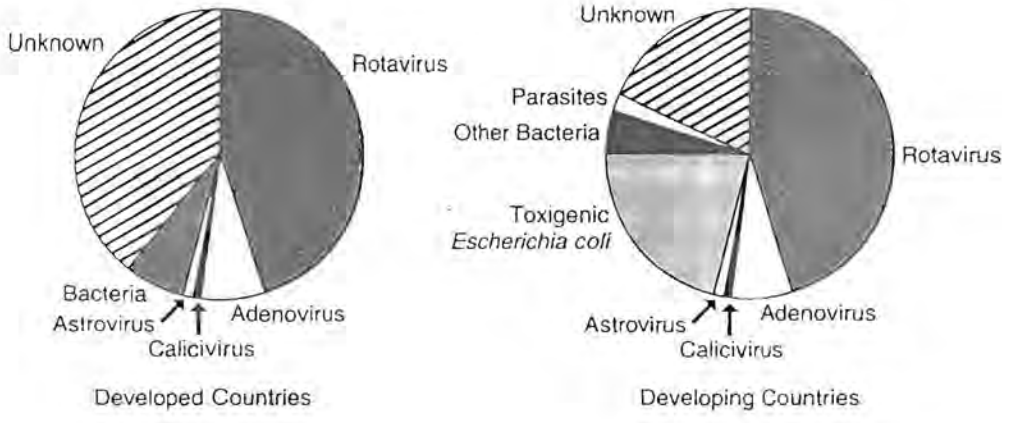


Figure 1: Microbial agents associated with severe infant and childhood diarrhoea in developed and developing countries (Kapikian, 1996).

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).

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).

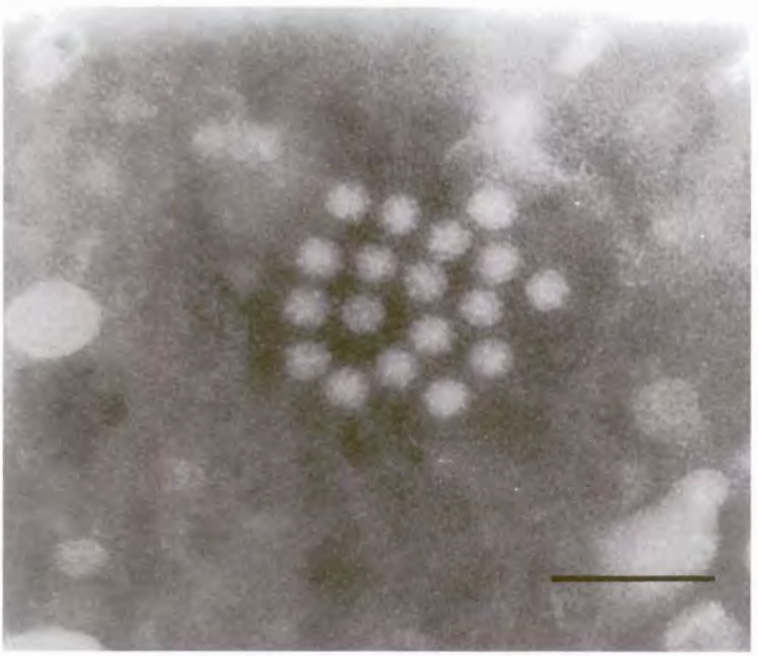
AstV infection in animals has also been documented (Kurtz, 1994; Caul, 1996). AstV-associated diarrhoea has been reported in lambs (Snodgrass and Gray, 1977; Herring *et al.*, 1981), calves (Woode and Bridger, 1978), piglets (Bridger, 1980; Shimizu *et al.*, 1990), cattle (Bridger *et al.*, 1984), cats (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,

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).

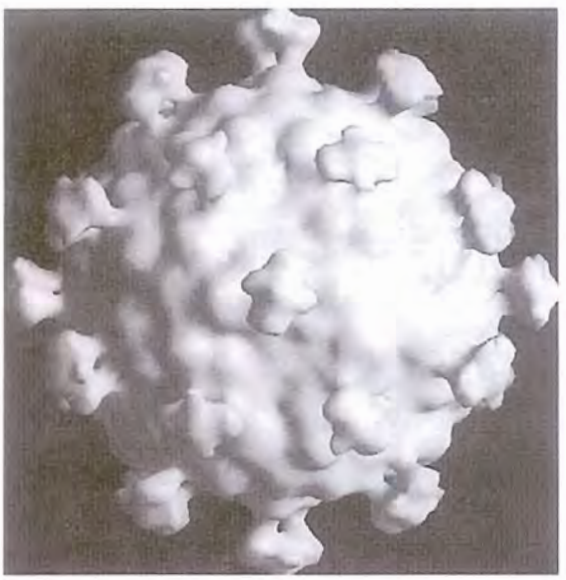


Figure 3: Image processing of negatively stained HAstV-1 viewed along the twofold axis of symmetry (Matsui and Greenberg, 2001)

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).

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).

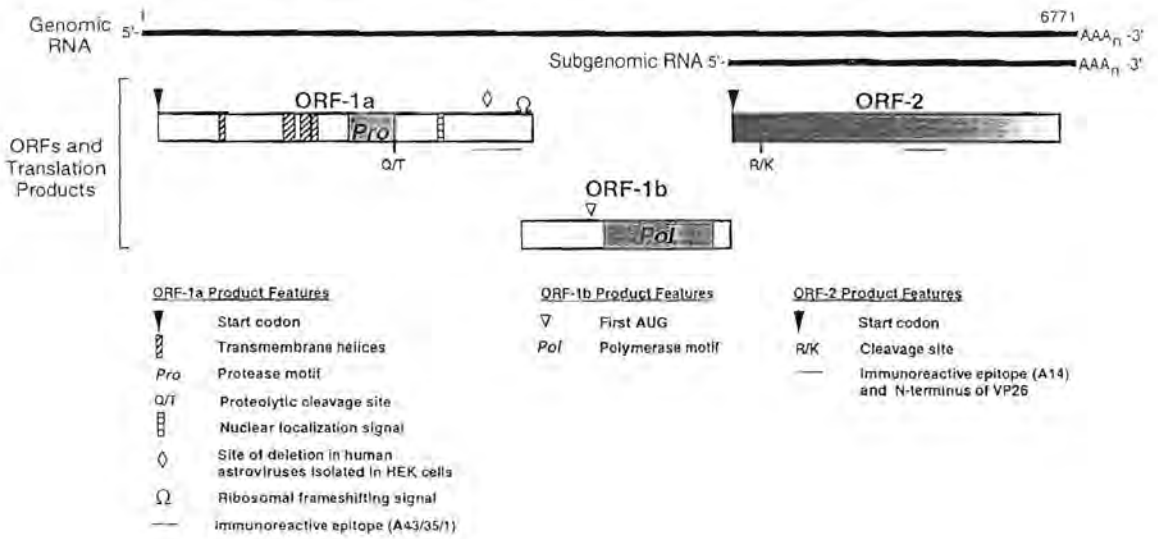


Figure 4: Genomic organisation of HAstV-1. Genomic and subgenomic RNAs are indicated above the ORFs and translation products (Matsui and Greenberg, 2001)

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

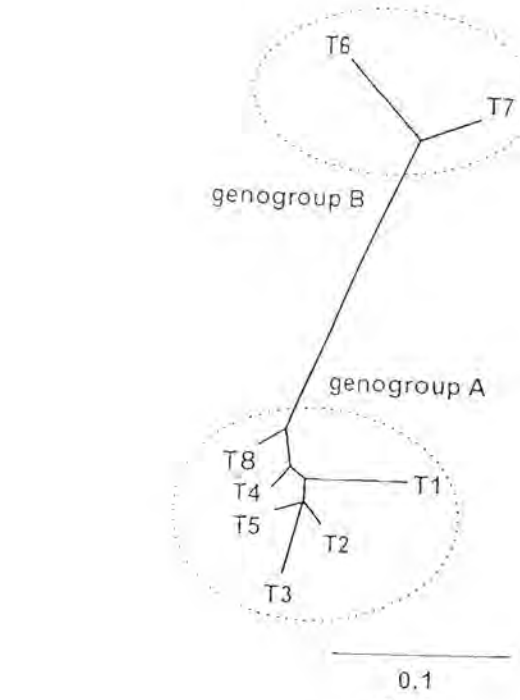
The length of this region varies in each of the reference strains, i.e. 2358 nt [HAstV-1, Newcastle] (Willcocks and Carter, 1993; Carter and Willcocks, 1996), 2362 nt [HAstV-1, Oxford] (Lewis *et al.*, 1994) and 2388 nt [HAstV-2, Oxford] (Jiang *et al.*, 1993). The greatest nucleotide sequence variability 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).

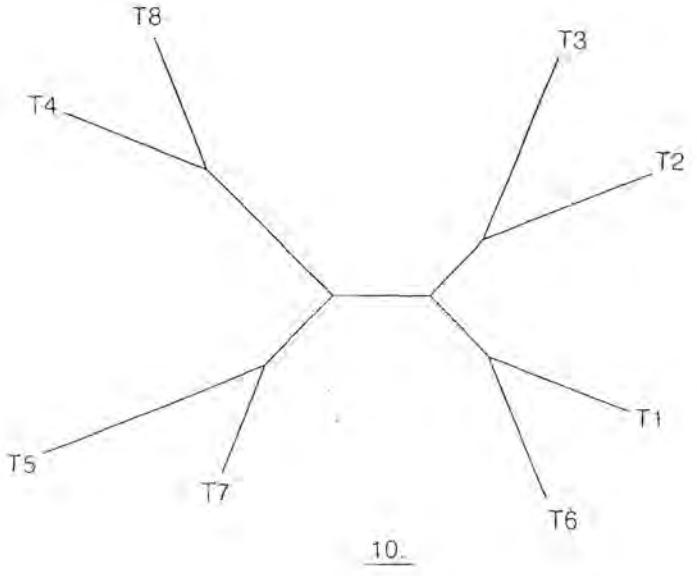
2.5.2 Genotypic properties

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).

ORF1a



A



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

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	~10^{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]	~10^{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.*,

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® (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 v2.1.1 (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.