CHAPTER 4

RESULTS

4.1 SPECIMENS AND SAMPLES

4.1.1 Human stool specimens

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

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

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

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

4.1.2 Animal stool specimens

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

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

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

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

4.1.3 Water and sewage samples

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

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

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

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

4.1.4 AstV isolates from river, dam and sewage water samples

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

4.2 OPTIMISATION OF RT-PCR FOR THE DETECTION OF AstVS

4.2.1 Optimisation of HAstV type-common RT-PCRs

4.2.1.1 Mon2/Mon67 primer pair

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

4.2.1.2 Mon348/Mon340 primer pair

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

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

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

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

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

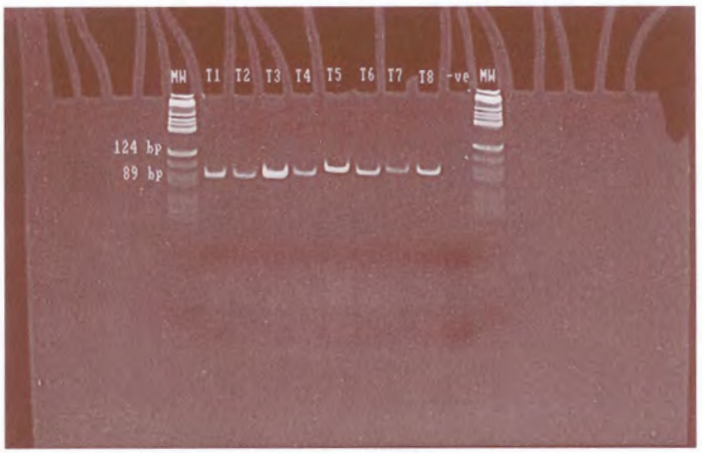

Figure 6: Analysis by ethidium bromide staining and UV illumination of RT-PCR products of HAstV-1 to 7 cell-culture-adapted reference strains (T1-T7) and an HAstV-8 positive stool specimen (AS20) (T8) with type-common primers Mon2/Mon67 using the optimised buffer and RT-PCR reaction mix. Products (89bp)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

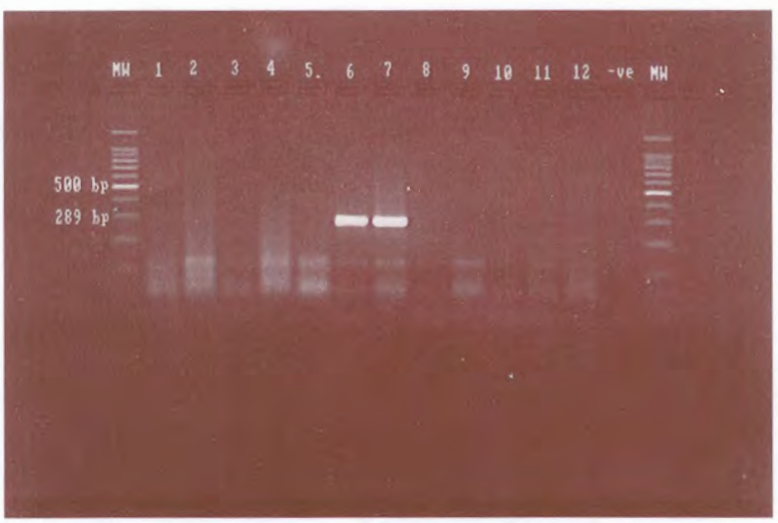

Figure 7: Agarose gel analysis of 289 bp RT-PCR products amplified from *HAstV-6 RNA using buffers 1 to 12 (lanes 1-12)* from the Opti-Prime™ PCR Optimization Kit (Stratagene). Ultrapure water served as negative control (lane "-ve"). Bands were observed in lanes 6 and 7 with 10X buffer containing 100mM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

4.2.1.3 Mon2/prBEG primer pair

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

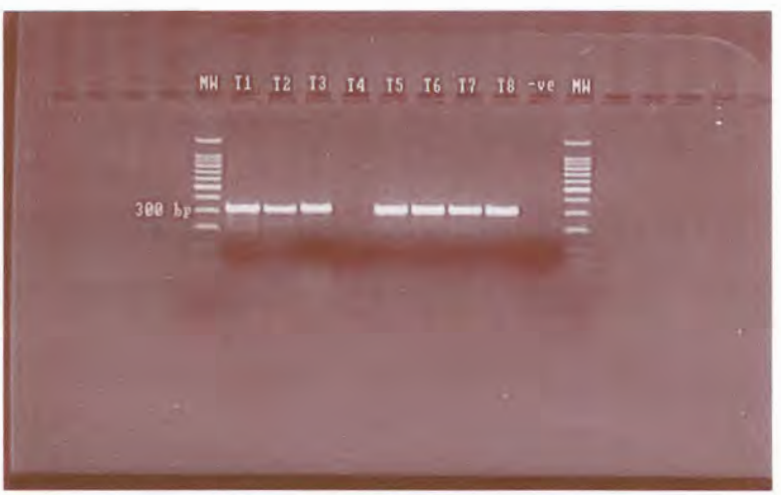

Figure 8: Analysis of 319 bp amplicons derived from HAstV-1 to 3 and HAstV-5 to 7 reference strains (lanes T1-T7), and the HAstV-8 positive stool specimen (AS20) (lane T8) using the optimised RT-PCR reaction mix and primers Mon2/prBEG. Products were resolved by agarose gel electrophoresis. Ultrapure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

4.2.2 Optimisation of HAstV type-specific RT-PCRs

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

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

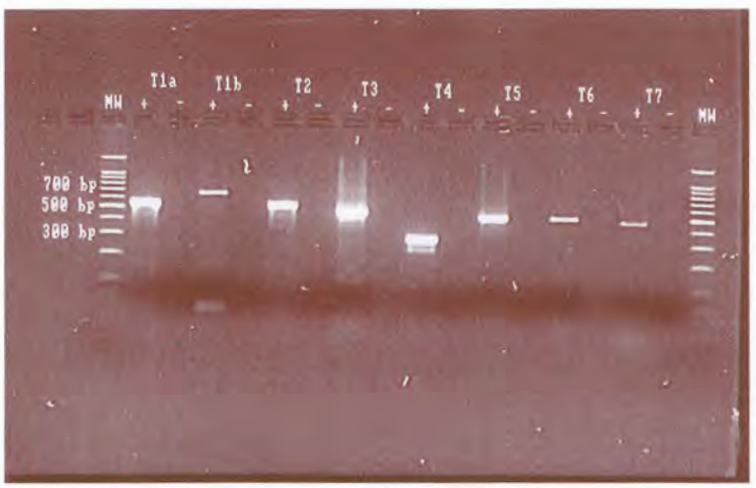

Figure 9: Agarose gel analysis of RT-PCR products derived from HAstV-1 to 7 reference strains (lanes T1a-T7) using type-specific primers in the optimised type-specific RT-PCR reaction mix (lane "+"). T1a and T1b represent primer pairs Mon2/AV3 and Mon2/pr6151, respectively. Ultrapure water served as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

4.3 ASSESSMENT OF CELL CULTURES FOR THE ISOLATION OF HAstVS

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

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

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

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

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

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

4.4 DETECTION AND CHARACTERISATION OF AstVs

4.4.1 Human stool specimens

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

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

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

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

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

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

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

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

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

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

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

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

* Primer pair used for type-specific RT-PCR
1: RT-PCR using this type-specific primer pair not done on isolate
2: untypable by typing RT-PCR or inconclusive sequityping

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

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

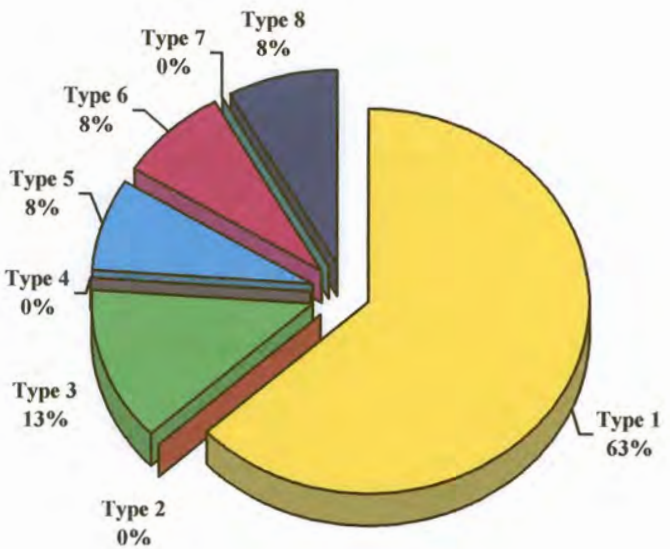

Figure 10: Distribution of HAstV genotypes identified between January 1996 and October 2000 in human diarrhoeal stool specimens, taken from hospitalised patients in the Tshwane Metropolitan Area.

4.4.2 Animal stool specimens

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

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

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

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

4.4.3 Water and sewage samples

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

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

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

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

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

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

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

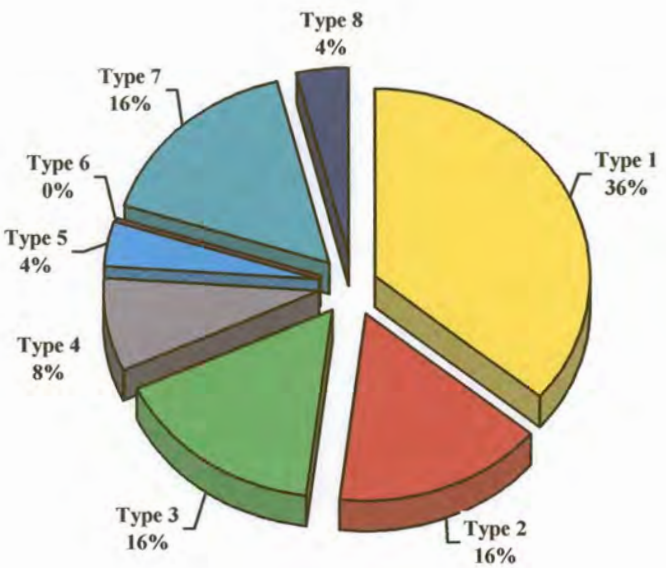

Figure 11: Distribution of HAstV genotypes detected between April 1999 and October 2000 from sewage samples collected from sewage works in the Tshwane Metropolitan Area.

4.4.4 AstV isolates from river, dam and sewage samples

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

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

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

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

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

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

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

4.5 NUCLEOTIDE SEQUENCE AND PHYLOGENETIC ANALYSIS OF SOUTH AFRICAN HAstVs

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

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

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

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

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

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

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

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

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

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

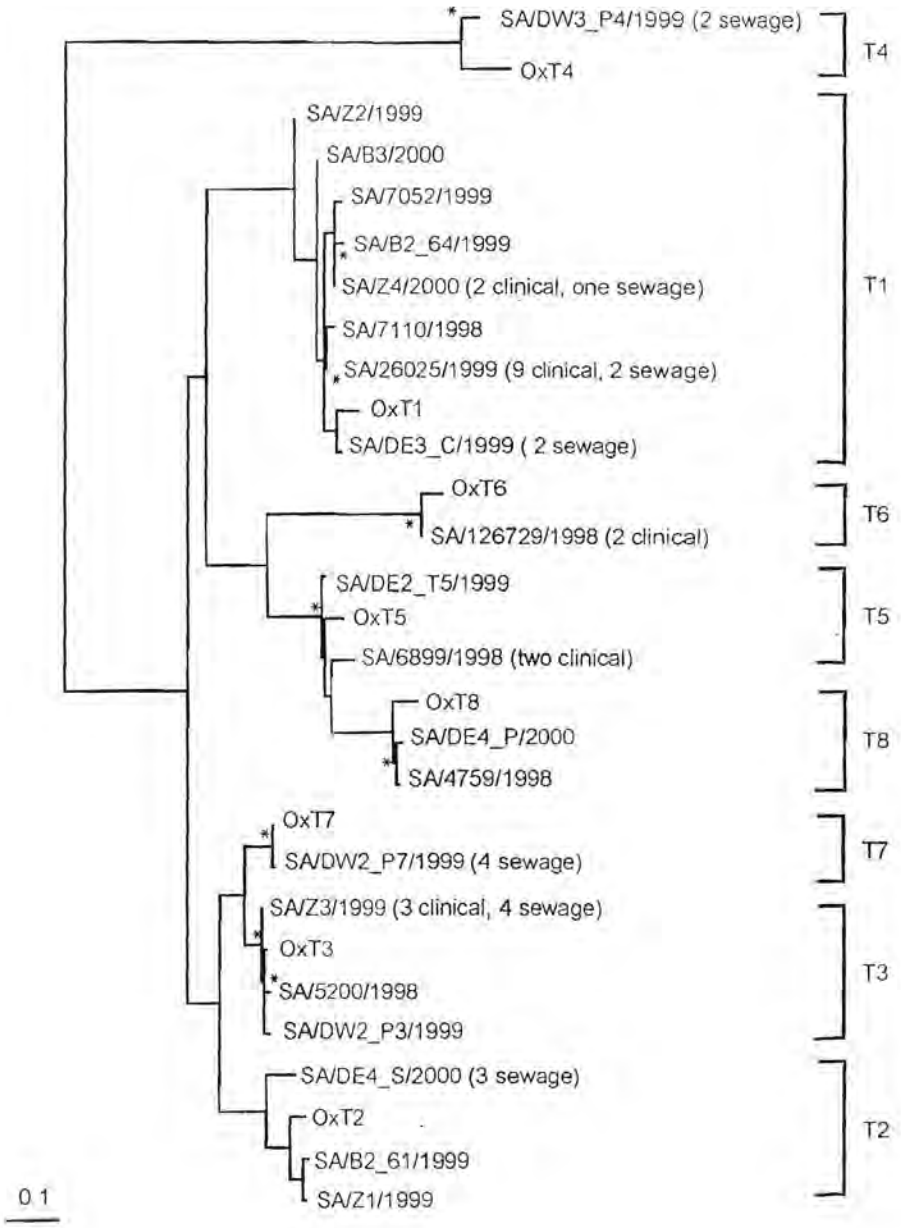

Figure 13: Maximum-likelihood phylogenetic tree based upon a 208 nt region of the 3' end of ORF2 showing the relationships between representatives of the South African environmental and clinical human astrovirus (HAstV) isolates and the prototypes of HAstV types 1 to 8. Branch points of the resulting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

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

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

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

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

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

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

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

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

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

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

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

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

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

CHAPTER 5

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

CHAPTER 6

CONCLUSIONS

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

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

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

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

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