

# Molecular Characterization of Astroviruses by Reverse Transcriptase PCR and Sequence Analysis: Comparison of Clinical and Environmental Isolates from South Africa

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**A comparative analysis was performed with 25 isolates of astroviruses (AstVs) detected in sewage sources and 22 concurrently identified clinical AstV isolates from the Tshwane (Pretoria) Metropolitan Area in South Africa. The samples and specimens were screened for AstVs by using an enzyme immunoassay and/or a reverse transcriptase PCR (RT-PCR) for the highly conserved untranslated region (3' end) of the genome. The RT-PCR results were confirmed by oligonucleotide probe dot blot hybridization. Viable viruses were propagated in cell cultures for amplification when a minimal specimen was available or indeterminate sequences were obtained. AstV strains were characterized by RT-PCR and partial sequence analysis of the capsid region. The presence of multiple human AstV (HAstV) types in a single sewage sample complicated identification of individual strains, and additional type-specific RT-PCR and sequence analyses of the capsid region were required for characterization. Amplification and characterization of one genotype from a sample, therefore, did not preclude the possibility that a sample harbored additional different genotypes. Genotype and sequence information obtained from AstVs in wastewater samples were compared to information obtained from AstV strains from human stools. HAstV type 1 (HAstV-1), as well as HAstV-3, -5, -6, and -8, were identified among the clinical isolates, and HAstV-1, -2, -3, -4, -5, -7, and -8 were identified among the environmental samples. Phylogenetic analysis demonstrated that HAstV-1, -3, -5, and -8, which were present in human stool and sewage samples, clustered together, indicating that these viruses are closely related. The concurrent presence of identical HAstV strains in wastewater samples and in hospitalized patients suggests that AstVs present in the environment pose a potential risk to communities in which fecally contaminated water is used for recreational and domestic purposes.**

Human astroviruses (HAstVs) cause human diarrhea (4) and have been identified as the second most important cause of viral infantile diarrhea in selected areas of South Africa (30, 57) and in other regions of the world (5, 32). HAstV infection has been reported for all age groups, and the young, elderly, and immunocompromised are at the greatest risk (12, 15). Astrovirus (AstV) disease is usually less severe than the disease caused by rotaviruses, and AstVs may also cause asymptomatic infections (8, 28). Transmission of HAstV infections occurs via the fecal-oral route (8, 15). Although contaminated food (47, 63) and water (11) have been associated with outbreaks of HAstV-associated gastroenteritis, the risk attributable to food and water contamination in the transmission of HAstVs has not been fully elucidated yet (15, 16). AstVs also have been associated with scours in young animals, such as calves, lambs, pigs, cats, dogs, and mink (8, 24, 25), as well as with a fatal hepatitis in ducklings (17), with hemorrhagic enteric syndrome in turkeys (23), and with acute intestinal nephritis in chickens (20). To date, eight HAstV serotypes (HAstV type 1 [HAstV-1] to HAstV-8) have been described (33, 37, 61, 65). Certainly two, and possibly three, serotypes of

bovine AstVs (37) and one serotype of porcine AstV (38) have been recognized.

AstVs, which are classified in the distinct family *Astroviridae*, have a single-stranded polyadenylated positive-sense RNA genome that is approximately 6.8 to 7.2 kb long and contains three open reading frames (ORFs), designated ORF1a, ORF1b, and ORF2 (37). ORF2 is located at the 3' end of the genome and encodes the capsid protein precursor (7). This protein has a well-conserved amino terminus (7). Nucleotide sequence analysis of a limited region of ORF2 has facilitated phylogenetic comparisons of HAstVs (45), and there is a good correlation between antigenic and genomic types (3, 45). Partial and complete sequence data are available for a limited number of animal (21, 65) and turkey (23) AstV isolates. Although the capsid proteins of AstVs infecting different hosts are reportedly highly divergent, similarities among HAstV, feline AstV, and porcine AstV capsid sequences suggest that zoonoses involving pigs, cats, and humans could occur (21). However, AstV infection appears to be species specific (32), and to date no interspecies transmission has been documented (21).

Surface waters in both rural and urban areas are affected by fecal contamination from human and animal sources (14, 18). The occurrence of AstVs in water sources (9, 29, 31, 40, 51, 60) and the occurrence of AstVs in sludge biosolids (10) have been reported, but the clinical significance and epidemiological impact of environmental AstV strains is unknown (60). Until

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

Sampling site	Sampling date (mo/day/yr)	Sample	RT-PCR <sup>a</sup>		Genotype(s)
			Polyacrylamide gel electrophoresis	Oligonucleotide probe hybridization	
Sewage treatment plants					
Daspoort (west inflow)	4/19/99	DW1	+ <sup>b</sup>	–	Untypeable
	4/28/99	DW2	+	+	HAstV-1, -3, -4, -7, -2 <sup>c</sup>
	5/24/99	DW3	+	–	HAstV-1, -3, -4 <sup>d</sup>
	10/19/00	DW4	+	+	HAstV-1, -7 <sup>e</sup>
Daspoort (east inflow)	4/19/99	DE1	+	+	Untypeable
	5/24/99	DE2	+	+	HAstV-1, -3, -5, -7 <sup>d</sup>
	7/26/99	DE3	+	+	HAstV-1 <sup>f</sup>
	10/19/00	DE4	+	+	HAstV-7, -2, -8 <sup>g</sup>
Baviaanspoort	4/19/99	B1	+	+	Untypeable
	4/28/99	B2	+	+	HAstV-1, -2 <sup>f</sup>
	10/19/00	B3	+	+	HAstV-1 <sup>h</sup>
Zeekoegat	4/28/99	Z1	+	+	HAstV-2 <sup>h</sup>
	5/24/99	Z2	+	+	HAstV-1 <sup>h</sup>
	9/19/99	Z3	+	+	HAstV-3 <sup>h</sup>
	10/19/00	Z4	+	+	HAstV-1 <sup>h</sup>
Streams					
Pienaars River (downstream to Baviaanspoort)	4/28/99	R1	–	–	
	5/24/99	R2	–	–	
	8/02/99	R3	–	–	
Apies River (downstream to Daspoort)	4/19/99	A1	+	–	Untypeable
	5/24/99	A2	–	–	
	7/26/99	A3	–	–	

<sup>a</sup> RT-PCR was performed with type-common primers Mon2 and Mon67.

<sup>b</sup> +, detected; –, not detected.

<sup>c</sup> The HAstV-1, -3, -4, and -7 genotypes were determined by using amplicons derived directly from the sample by type-specific RT-PCR, and the HAstV-2 genotype was determined by using amplicons derived directly from the sample by group-specific RT-PCR.

<sup>d</sup> The HAstV genotypes were determined by using amplicons derived directly from the sample by type-specific RT-PCR.

<sup>e</sup> The HAstV-1 genotype was determined by using amplicons derived directly from the sample by group-specific RT-PCR, and the HAstV-7 genotype was determined by using amplicons derived directly from the sample by type-specific RT-PCR.

<sup>f</sup> AstV genotypes were determined by using amplicons derived from infected CaCo-2 cell cultures by group-specific RT-PCR.

<sup>g</sup> The HAstV-7 genotype was determined by using amplicons derived directly from the sample by type-specific RT-PCR, and the HAstV-2 and -8 genotypes were determined by using amplicons derived by group-specific RT-PCR from two different types of cell cultures inoculated with the same sample.

<sup>h</sup> The HAstV genotype was determined by using amplicons derived directly from the sample by group-specific RT-PCR.

recently, there have been no reports of antigenic or molecular characterization of environmental AstV isolates, but in a recent publication the authors described using restriction fragment length polymorphism (RFLP) to genotype such isolates (52). The aim of this study was to detect, by using type-common reverse transcriptase PCR (RT-PCR), and to characterize, by partially sequencing the 3' end of the ORF2 capsid gene, AstV strains obtained from water and sewage samples and to compare these strains to AstV strains obtained from the stools of humans in the same geographic region. The comparative data obtained should provide valuable information concerning the possible sources of human infection or concerning sources of fecal contamination of surface waters in communities in which these water sources are used for domestic and recreational purposes.

#### MATERIALS AND METHODS

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

The sewage and water samples were clarified by centrifugation (Beckman GS-6R centrifuge) for 30 min at 3,000 × g. Each resultant pellet was resuspended

in supernatant fluid (10 ml) and clarified by addition of chloroform (10%, vol/vol; Merck, Darmstadt, Germany) and further centrifugation (Beckman GS-6R centrifuge) for 10 min at 3,000 × g. The supernatants from the first and second clarification procedures were pooled, and AstVs were recovered from each supernatant in 10 ml (final volume) of phosphate-buffered saline (pH 7.4) (Sigma Chemical Co., St. Louis, Mo.) by using the polyethylene glycol-sodium chloride precipitation technique described by Minor (34) for the concentration of picornaviruses. The viral suspension was concentrated further to 2 ml by ultrafiltration by using a Biomax-100K NMWL membrane (Ultrafree 15 centrifugal filter device; Millipore Corporation, Bedford, Mass.). The final concentrate was divided into aliquots and stored at -20°C.

**Clinical specimens.** Stool specimens from pediatric patients (age, <5 years) who presented with clinical symptoms of gastroenteritis at two tertiary referral hospitals in the Tshwane Metropolitan Area were submitted for routine diagnosis of gastroenteritis viruses. HAstVs were detected by enzyme immunoassay (EIA) (IDEIA Astrovirus; Dako Ltd., Ely, United Kingdom) in 32 of 1,303 (2.5%) stool specimens referred from January 1998 to October 2000 for analysis. An additional three HAstVs were detected by EIA retrospectively in 1% (3 of 356) of stool samples referred between January 1996 and December 1997 which had been stored at 4°C. Stool specimens and suspensions of stool specimens (10% in phosphate-buffered saline [Sigma]) were stored at 4°C.

**Cell culture amplification.** To enhance detection by RT-PCR or to clarify sequencing data for certain isolates, AstVs in concentrates of selected water samples and stool suspensions were amplified by propagation in cell cultures. Sample concentrates and stool suspensions were treated with penicillin (50 µg/ml), streptomycin (50 µg/ml), and neomycin (100 µg/ml) (100× PSN antibiotic mixture; GIBCO BRL Life Technologies, Paisley, Scotland) and with 100 U of nystatin (GIBCO BRL) per ml and inoculated onto monolayers of human hepatoma cell line PLC/PRF/5 (ATCC CRL 8024) (passages 81 to 85) and

human colonic carcinoma cell line CaCo-2 (ATCC HTB 37) (passages 35 to 58 and 178 to 198). Cells were grown in 25-cm<sup>2</sup> cell culture flasks, as described previously (59), and were incubated for 7 days at 37°C and harvested; an aliquot was blind passaged, and this was followed by incubation for an additional 7 days at 37°C. Cell culture extracts (120 µl) from the initial harvest and after blind passage were assayed for AstV RNA by RT-PCR.

**Detection of AstVs by RT-PCR.** Aliquots of the sludge samples, stool suspensions, and cell culture extracts were pretreated with an equal volume of 1,1,2-trichloro-trifluoroethane (Sigma) prior to extraction of total RNA from 120 µl of each treated sample with TRIZOL reagent (GIBCO BRL) used according to the manufacturer's instructions. The extracted RNA was resuspended in 25 µl (final volume) of sterile nuclease-free water (Promega Corp., Madison, Wis.) and stored at -70°C. For each extraction procedure, nuclease-free water was included as a negative control. The primers and probe were synthesized by Sigma-Genosys Ltd., Pampisford, United Kingdom. RT-PCR was performed by using 5 µl of RNA extract and type-common primers Mon2 and Mon67 (35). The amplicon was confirmed to be an AstV amplicon by performing an oligonucleotide probe hybridization assay as described previously (31, 60). For further characterization, a region at the 3' end of the ORF2 capsid gene (nucleotides [nt] 6513 to 6781, HAstV-1 [accession no. L23513]) of all confirmed AstV-positive samples was amplified by using primers Mon2 and prBEG (54). These primers detect all HAstVs except HAstV-4. The reaction mixture and the conditions for the RT-PCR when these primers were used were essentially the same as those used when the Mon2-Mon67 primer pair was used (60), except that the 1× PCR buffer contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, and 1.5 mM MgCl<sub>2</sub>. Clinical isolates that were undetectable with primers Mon2 and prBEG were subjected to an HAstV-4 type-specific RT-PCR (64) or a type-common RT-PCR with primers Mon348 and Mon340, which amplified a region of ORF1a (3). Environmental isolates that were confirmed to be AstV positive by the RT-PCR-oligonucleotide probe hybridization assay but were undetectable or produced an uninterpretable sequence when primers Mon2 and prBEG were used were subjected to HAstV-1 to HAstV-7 type-specific RT-PCRs as described by Walter et al. (64). Cell culture extracts of HAstV-1 to HAstV-7 Oxford reference strains were used as positive controls.

**Sequencing of RT-PCR amplicons.** DNA amplicons derived from the 3' end of the ORF2 capsid gene or the 289-bp region of ORF1a were sequenced directly by the dideoxy chain termination method (56) by using a Sequenase version 2.0 PCR product sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio) according to the manufacturer's instructions. The sequencing reaction mixtures were electrophoresed on 8% polyacrylamide-6 M urea gels in 1× Tris-borate-EDTA buffer. The gels were vacuum dried and exposed to X-ray film (Hyperfilm-βmax; Amersham) for 12 h at room temperature.

**Sequence analysis and genotyping.** Nucleotide sequences were entered into a database in PC/Gene (version 6.85; IntelliGenetics Inc., Geneva, Switzerland). Basic sequence manipulation and verification were performed by using OMIGA (version 2.0; Accelrys, Madison, Wis.). ClustalX (62) was used to create multiple alignments of the amino acid sequences of selected isolates and reference strains. Nucleic acid sequences were added and aligned with GeneDoc (version 2.3) by using the corresponding amino acid alignment as a template, which resulted in a consensus length of 208 nt for the 3' end of ORF2 (42). Pairwise comparisons of nucleotide sequences of all reference types with the selected isolates were performed by using GeneDoc (version 2.3) for preliminary genotype assignment and for identification of clusters of strains with 99 to 100% homology. Only one representative strain from each cluster was included in the phylogenetic analysis. The sequences of representative isolates were compared with AstV sequences present in GenBank by using the BLAST-N program (version 2.1.1) (1, 2) to search for the most similar strain available. The nucleotide sequence alignment was assessed by likelihood-mapping (43, 58) utilizing TREE-PUZZLE 5.0 (<http://www.tree-puzzle.de>) for tree-likeness of the data and the proper sequence composition for phylogenetic analysis. Phylogenetic trees were constructed from the nucleic acid sequence alignments by using the maximum-likelihood algorithm of the program DNAML of PHYLIP (version 3.52c) running in a UNIX environment (13). We performed the analysis rooted (with HAstV-4 as a root) and unrooted. In the analysis the global rearrangement option was invoked, and the order of the sequence input was randomized 10 times. Phylograms generated in DNAML were visualized by using the TREEVIEW package (version 1.5) (48) and were further edited with Micrografix Designer (version 6.0a).

**Definition of strains.** Although the definitions for strains and subtypes of AstVs are not clear, we arbitrarily considered an isolate with <95% nucleotide homology and a distance of >0.05 as determined by phylogenetic analysis at the 3' end of ORF2 (consensus length, 208 nt) compared to the reference strain a different strain.

**Nucleotide sequence accession numbers.** The accession numbers for the previously published HAstV capsid gene sequences that were used in the pairwise comparisons and phylogenetic analyses and included reference strains with complete capsid sequences are as follows: HAstV-1, L23513; HAstV-2, L13745; HAstV-3, AF117209; HAstV-4, Z33883; HAstV-5, U15136; HAstV-6, Z46658; HAstV-7, AF248738; and HAstV-8, Z66541.

The nucleotide sequence data for the clinical and environmental AstV isolates described here have been deposited in the EMBL/GenBank database under the following accession numbers: T3/SA/DW2\_P3/1999, AY094090; T7/SA/DW2\_P7/1999, AY094091; T4/DW3\_P4/1999, AY094092; T5/SA/DE2\_T5/1999, AY094089; T1/SA/DE3\_C/1999, AY094082; T8/SA/DE4\_P/2000, AY094083; T2/SA/DE4\_S/2000, AY094084; T1/SA/B2\_64/1999, AY094080; T2/SA/B2\_61/1999, AY094079; T1/SA/B3/2000, AY094081; T2/SA/Z1/1999, AY094085; T1/SA/Z2/1999, AY094086; T3/SA/Z3/1999, AY094087; T1/SA/Z4/2000, AY094088; T8/SA/4759/1998, AY093649; T3/SA/5200/1998, AY093650; T5/SA/6899/1998, AY093651; T1/SA/7110/1998, AY093652; T6/SA/126729/1998, AY093653; T1/SA/7052/1999, AY093654; and T1/SA/26025/1999, AY093655.

## RESULTS

**AstV genotypes in water and sewage samples and clinical specimens.** AstVs were detected directly by the HAstV type-common RT-PCR in all of the human stool specimens previously identified by EIA, in all 15 sewage samples, and in one of six (17%) stream water samples. The presence of AstV amplicons was confirmed by an oligonucleotide probe hybridization assay of the RT-PCR products from 100% of the human stool specimens, from 13 of 15 (87%) of the sewage samples, and from none of the stream water samples. Of the 35 AstV isolates from human stool specimens, 22 (63%) could be characterized directly after amplification from the stool specimens by sequencing of the Mon2-prBEG amplicon (296 to 324 nt, depending on the HAstV type) from the 3' end of ORF2. Two additional isolates were confirmed to be HAstV isolates by sequencing of a 246-bp region of ORF1a. After storage at 4°C for an extended period, AstVs could no longer be amplified by RT-PCR from the remaining 11 stool specimens by using type-common primers Mon2 plus prBEG and Mon348 plus Mon340 or type-specific primers. Seven environmental AstV isolates, from seven separate sewage samples, were amplified and characterized directly from the sewage samples by sequencing of the Mon2-prBEG amplicon. An additional five isolates, originating from three sewage samples, could be characterized only after isolation in cell culture. One of the sewage samples (sample B2) yielded two different HAstV types from separate flasks of CaCo-2 cell cultures with different numbers of passages, while another sample (sample DE4) yielded two different HAstV genotypes after amplification with two different cell culture types (i.e., CaCo-2 and PLC/PRF/5) (Table 1). Thirteen isolates, from five of the sewage samples, were typed by sequencing amplicons from amplicons generated by type-specific RT-PCR directly from the sewage samples. Indeterminate sequences were obtained from amplicons derived from sewage samples DW1, DE1, and B1 and their cell culture derivatives.

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

TABLE 2. Summary of the characterized South African HAstV strains from clinical and sewage sources with high levels of identity to the representative isolates included in the phylogenetic analysis

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

<sup>a</sup> Type assignments were based on comparison with the Oxford reference strain.

<sup>b</sup> ND, none detected.

**Sequence analysis of South African AstVs.** The multiple alignment included sequences with a consensus length of 208 nt (after the primer sequences were removed) for all South African isolates. Pairwise comparisons revealed groups of South African isolates with 99 to 100% identity. The groups of isolates and representative strains of each group are summarized in Table 2.

To prepare for phylogenetic analysis, the multiple alignments of the nucleotide sequences were tested by likelihood mapping. The data had a tree-like structure, and all sequences were in the proper sequence composition range. The phylogenetic analysis was performed in two stages. First, all South African strains were included in an unrooted tree. Pairwise analysis and the phylogenetic tree revealed common branch points for the majority of the South African strains within types; therefore, 27 strains with 99 to 100% homology were withheld from the phylogenetic analysis to avoid repeats. Reference strains (HAstV-1 to -8) and representatives of the 21 distinctive South African strains were included in the final phylogenetic analysis. The HAstV-4 strain was less related to the other reference strains and was therefore used as a root for the analysis.

The analysis (Fig. 1) showed that clusters of types with HAstV-1 to -8 reference strains separated with confidence (distances, 0.09 to 0.62;  $P < 0.05$ ). HAstV-3 and -7 and HAstV-5 and -8 were very closely related in this hypervariable region, with 90 and 82% pairwise identity and distances of 0.09 and 0.124, respectively. The HAstV-4 strain was significantly

different from the other HAstV strains. All of the characterized South African isolates could be assigned to a type. The calculated intragenotypic distances suggest that the HAstV-1, -2, -4, -5, and -8 South African isolates represent new strains of the corresponding genotypes (for HAstV-1, 90 to 94% identity and distances of 0.05 to 0.13; for HAstV-2, 88 to 94% identity and distances of 0.10 to 0.13; for HAstV-4, 88% identity and distance of 0.12; for HAstV-5, 91 to 94% identity and distances of 0.05 to 0.10; and for HAstV-8, 94% identity and distance of 0.06). South African strains of HAstV-3, -6, and -7 appear not to be new strains or subtypes (for HAstV-3, 99% identity and distance of 0.01; for HAstV-6, 96% identity and distance of 0.04; and for HAstV-7, 99% identity and distance of 0.005).

HAstV-1, -3, -5, and -8 were detected among the clinical samples and environmental isolates. HAstV-1 isolates comprised 22 (48%) of the 46 isolates characterized by sequence analysis of the 3' end of ORF2. The phylogenetic analysis included eight representative strains, as previously described. The nucleotide identity among the South African HAstV-1 isolates was 88 to 100%, compared to 89 to 94% identity to the prototype strain. Two isolates recovered from wastewater sources formed a distinct subtype (cluster T1a) but were more closely related to the Oxford reference strain (distance, 0.05) than the other South African strains were. Another cluster

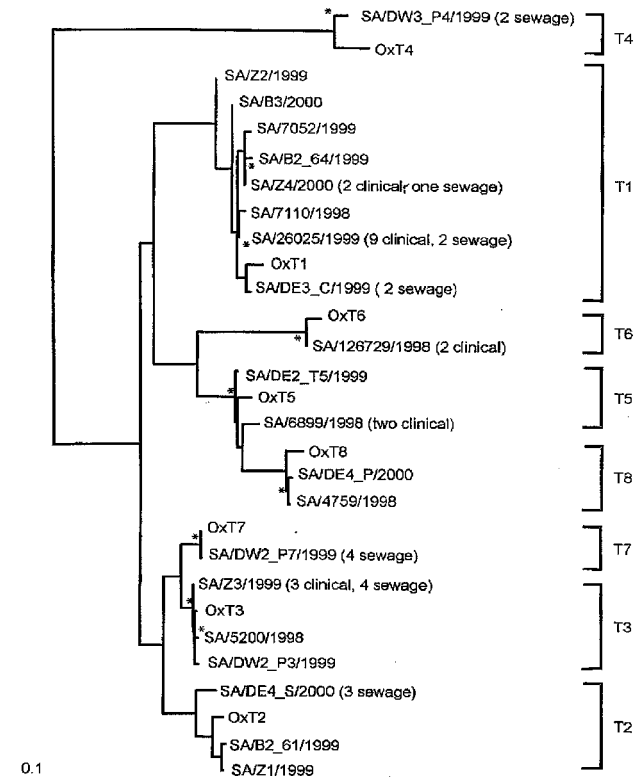


FIG. 1. Maximum-likelihood phylogenetic tree based on a 208-nt region of the 3' end of ORF2, showing the relationships of representatives of the South African environmental and clinical HAstV isolates and the prototypes of HAstV-1 to -8. The branch points of the tree (rooted) had a confidence level of  $P < 0.05$ . Scale bar = 0.1 nucleotide substitution per site. Asterisks indicate nonsignificant branch points ( $P > 0.05$ ).

(cluster T1b), which was distinct from cluster T1a (distance, 0.04 to 0.6), was also observed. Cluster T1b included multiple closely related environmental strains and strains from human stool samples represented by SA/Z4/2000, SA/B2\_64/1999, SA/7110/1998, SA/7052/1999, and SA/26025/1999. The other two strains (SA/B3/2000 and SA/Z2/1999) had unique sequences and were identified only once.

The environmental and clinical HAstV-3 isolates showed high levels of nucleotide sequence identity ( $\geq 98\%$ ) to each other and to the prototype strain and clustered together in a single genotypic cluster, cluster T3 (Table 2; Fig. 1). The HAstV-5 environmental isolate exhibited a higher level of nucleotide identity (95%) to the prototype strain than the clinical isolates exhibited (nucleotide identity, 91%). The HAstV-5 environmental isolate and clinical isolates exhibited only 93% nucleotide identity (distance, 0.07); as a result, we considered them unique strains of HAstV-5 (Fig. 1).

Two HAstV-8 strains, one from a clinical specimen obtained in 1998 and the other from a sewage sample collected in 2000, were analyzed. These two strains are closely related to each other (98% nucleotide sequence identity) but are distinct from the prototype strain (93 to 94% nucleotide sequence identity) (Fig. 1). A nucleotide identity of 100% was recorded for the two clinical HAstV-6 strains, and they exhibited 96% nucleotide identity to the Oxford reference strain. No HAstV-6 strains were detected among the environmental isolates.

HAstV-2, -4, and -7 isolates were detected only among the environmental isolates. The levels of nucleotide identity among the HAstV-2 isolates were 88 to 98%, but when these viruses were compared to the prototype strain, the levels of identity were lower (88 to 94%); this resulted in clustering of the South African isolates separate from the prototype strain within HAstV-2 (Fig. 1). The South African HAstV-2 isolates were members of two unique clusters, represented by SA/B2\_61/1999 and SA/Z1/1999 in one group and by SA/DE4\_S/2000 in the other group (distance, 0.013) (Fig. 1). The HAstV-4 isolates, both from the same sewage works, showed 100% nucleotide sequence homology to each other but 88% nucleotide sequence homology to the prototype strain. The HAstV-7 isolates showed high levels of nucleotide sequence identity ( $\geq 98\%$ ) to each other and to the prototype strain and grouped together in a single cluster (Fig. 1).

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

## DISCUSSION

In this study an RT-PCR–oligonucleotide probe hybridization assay followed by partial sequencing of the C terminus of ORF2 was successfully used to detect and characterize clinical and environmental AstV isolates from the Tshwane Metropolitan Area in South Africa. The RT-PCR–oligonucleotide probe hybridization assay has previously been shown to be a valuable tool for detection of HAstVs in stool specimens (30) and, in conjunction with cell culture, for detection of infectious

HAstVs in water from different sources (31, 60). In this investigation prior amplification in cell culture was also shown to facilitate detection and characterization of multiple HAstV types in a single sample (Table 1). PCR is a procedure that is widely used to detect and genotype viruses (9, 27, 49, 50, 66) and parasites (53) from clinical and environmental sources. Sequence analysis of the 3' region of HAstV ORF2 provides type information, as well as enough diversity to provide additional strain information. There are, however, no data on the ability of RT-PCR amplification of ORF2 to detect and characterize mixed populations of HAstV genotypes. For this study, single HAstV genotypes obtained from the clinical specimens and obtained directly from six of the sewage samples were amplified by RT-PCR by using type-common primers Mon2 and prBEG, and they were characterized by sequencing a 208-nt region of ORF2. However, sequence analysis with type-common primers Mon2 and prBEG for a number of the sewage samples resulted in indeterminate or untypeable sequences (Table 1). We then asked if these strains were truly unique strains or represented a mixed population of strains that underwent the sequencing reaction simultaneously. Subsequent RT-PCR amplification of the same samples with HAstV-1 to -7 type-specific primers, which also amplified the 3' end of ORF2, resulted in identification of multiple genotypes in at least five of the sewage samples (Table 1). In one of the sewage samples, DW4, HAstV-1 was identified by RT-PCR by using the type-common primers Mon2 and prBEG, while HAstV-7 was subsequently detected in the same specimen by using type-specific primers. As has been reported previously for *Cryptosporidium parvum* (53), we showed that amplification and characterization of a single genotype from a clinical specimen or water sample do not preclude the possibility that multiple genotypes are present. A similar finding was reported for human caliciviruses; in this case cloning of PCR products and sequencing of several individual clones resulted in identification of multiple genotypes in a single sewage sample (27).

Among the clinical isolates characterized by sequence analysis of the 3' end of ORF2, HAstV-1 (64%) was the most frequent type identified, and HAstV-3 (14%) and HAstV-5 (9%) were less common. This finding is similar to findings obtained in other regions of the world (19, 22, 26, 39, 44, 45, 46, 49, 55). The occurrence of HAstV-6 and -8 in 9 and 5% of the specimens, respectively, is important as these types reportedly are seldom detected (15, 37). HAstV-8, however, appears to be more common on the African continent (36, 41, 61) and in Barcelona, Spain (19). The absence of HAstV-2 in the South African clinical specimens is noteworthy as this serotype was identified as the predominant type in other parts of the world, including in a periurban community of Mexico City (64). The distribution of HAstV genotypes in the South African environmental isolates is similar to that observed for the clinical isolates in that HAstV-1 was the predominant type identified (36% of the isolates). The difference was the occurrence of HAstV-2, -4, and -7 in the sewage samples, which comprised 16, 8, and 16% of the environmental isolates, respectively, while none of these types were detected in the clinical specimens. Further research is therefore warranted to ascertain whether these types are possibly more resistant to environmental degradation or whether human infection by these types is not as severe as human infection by the other types and thus

does not require medical attention. Finally, is there a difference in the reservoir and/or mode of transmission between HAstV types?

Our results clearly indicate that the HAstVs detected in both clinical and environmental samples are closely related and probably represent identical strains (Table 2; Fig. 1). Further analysis showed that the closely related environmental and clinical isolates were distinct from other HAstV strains detected during the same time period (1997 to 2000) in other geographical locations. This suggests that fecally polluted water could be a reservoir for human infection. In addition, the presence of different strains in the same community indicates that multiple strains and multiple genotypes circulate concurrently. Phylogenetic analysis demonstrated that the South African strains aligned with the corresponding reference strains but were sufficiently different that they represent new strains or subtypes ( $P < 0.05$ ). Two South African HAstV-1 subtypes were detected; one of these subtypes includes both clinical and environmental isolates (distance, 0.05;  $P < 0.01$ ). Two separate subtypes of HAstV-5 and -2 were identified. South African HAstV-8 strains formed a new subtype that included environmental and clinical subtypes. South African HAstV-4 strains also clustered in a distinct subtype. The nucleotide sequences of HAstV-3 and -7 isolates from the South African clinical and environmental sources, as well as from other geographic regions, appeared to be highly conserved, exhibiting 98 to 100% identity. This is similar to what was observed for hepatitis A virus (50).

The AstVs detected by RT-PCR–oligonucleotide probe hybridization in the sewage samples were characterized as HAstVs (namely, HAstV-1, -2, -3, -4, -5, -7, and -8) (Table 1). This suggests that the integrated RT-PCR–oligonucleotide probe hybridization assay used in this and previous studies (31, 60) for the detection of AstVs in water and sewage samples selects for AstVs of human origin. As cross-species infection *in vitro* appears to occur only after prior adaptation of an AstV isolate in a cell culture of the species of origin (6), the amplification of AstVs from water and sewage specimens in cell lines of human origin (namely, PLC/PRF/5 and CaCo-2) should further enhance the selection and detection of viruses of human origin. Additional RT-PCRs performed with primers specific for animal AstVs and/or cell cultures of animal origin would therefore be required to detect AstVs of animal origin in water and sewage samples. The role of zoonotic infection of AstVs is not currently understood; consequently, the possible risk of infection of 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 (52). Therefore, the presence of HAstVs in the environment could pose a potential health risk to persons using contaminated water for domestic or recreational purposes. Although quantitative RT-PCR and larger surface water sample volumes would be required to obtain conclusive evidence, the absence of AstVs in the surface waters downstream of the sewage works in which multiple genotypes of HAstVs were detected suggests that these viruses were removed effectively by the sewage treatment process. This study provides valuable new data on the molecular epidemiology of HAstVs circulating in the communities in the Tshwane Metropolitan Area of South Africa and in southern Africa and provides a feasible alterna-

tive to RFLP analysis (52) for characterization of HAstVs detected in water sources. However RT-PCR with analysis of the sequence in the capsid region provides more information for characterization of environmental isolates than RFLP analysis provides as RFLP analysis is limited by the amount of the RT-PCR product and requires secondary amplification by internal primers.

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