CHAPTER 7

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APPENDIX A

A.1 GLASS-WOOL ADSORPTION-ELUTION PROCEDURE

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

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

A.2 PEG/NaCl CONCENTRATION METHOD

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

APPENDIX B

B.1 NUCLEIC ACID SEQUENCING REACTIONS

a) Reagents

Gel Stock

40% Acrylamide (19:1)	200 ml
10 X Tris-Borate-EDTA (TBE)	100 ml
Urea	420 g

Make up to 1L with distilled H_2O

For X1 Gel:

Stock solution	75 ml
10% AMPS	300 µl
TEMED	23 µl

b) Sequencing reactions

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

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

c) Sequencing gel

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

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

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

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

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

APPENDIX C

C.1 Summary of astrovirus detection from animal stool specimens

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

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

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

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

CODE

C : calf

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

BB : cattle – Bronkhorstspruit farm

BV : Pig – Bronkhorstspruit farm

HB : cattle – Kameeldrift: plot

HK : calf – Kameeldrift: plot

HV : pig – Kameeldrift: plot

PB : cattle – UP Research Farm

PP : pig – UP Research Farm

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

DB : cattle – Pretoria Zoo

DV : pig – Pretoria Zoo

DE : duck – Pretoria Zoo

DMG : mountain goat – Pretoria Zoo

DT : Turkey – Pretoria Zoo

KT : kitten – local vet

DG : dog – local vet

APPENDIX D

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

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

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

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

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

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

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

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

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

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

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

APPENDIX E

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

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Running title: Genetic characterization of South African astroviruses

ABSTRACT

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

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

INTRODUCTION

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

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

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

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

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

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

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

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

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

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

RESULTS

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

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

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

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

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

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

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

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

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

Footnote to Table 1

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

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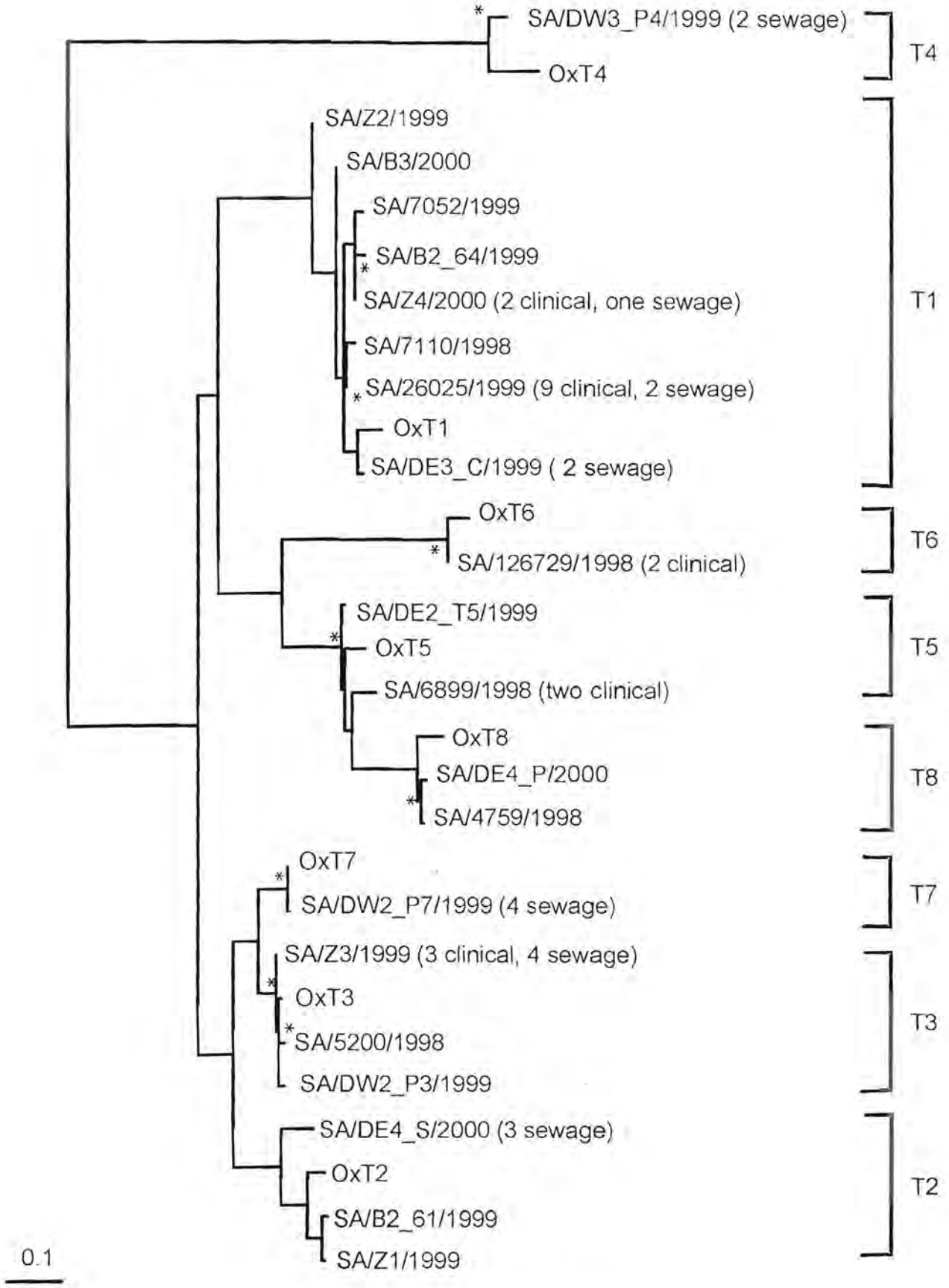

FIG.2. Maximum likelihood phylogenetic tree based on a 208 nt region of the 3' end of ORF2 showing the relationships between representatives of the South African environmental and clinical human astrovirus (HAstV) isolates and the prototypes of HAstV types 1 to 8. Branch points of the resulting tree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

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

Representative SA strains (n=21)	SA strains with 99-100% nucleotide identity to the representative isolate (n=25)
T3[a]/SA/Z3/1999	T3/SA/7169/1996; T3/SA/113768/1998; T3/SA/DE2_T3/1999; T3/SA/DW2_P3/1999
T3/SA/5200/1998	nd[b]
T3/SA/DW3_P3/1999	nd
T7/SA/DW2_P7/1999	T7/SA/DE2_T7/1999; T7/SA/DE4_T7/2000 T7/SA/DW4_T7/2000
T6/SA/126729/1998	T6/SA/5236/1998
T8/SA/4759/1998	nd
T8/SA/DE4_P/2000	nd
T5/SA/DE2_T5/1999	nd
T5/SA/6899/1998	T5/SA/126585/1998
T1/SA/DE3_C/1999	T1/SA/DE2 _T1/1999
T1/SA/26025/1999	T1/SA/3144/1997; T1/SA/124893/1998; T1/SA/4642/1999; T1/SA/6802/1999; T1/SA/9559/1999; T1/SA/22320/1999; T1/SA/25786/1999; T1/SA/5114/2000; T1/SA/DW3_P1/1999; T1/SA/DW4/2000;
T1/SA/Z4/2000	T1/SA/29903/1999; T1/SA/3621/2000
T1/SA/7110/1998	nd
T1/SA/B3/2000	nd
T1/SA/Z2/1999	nd
T1/SA/7052/1999	nd
T1/SA/B2_64/1999	nd
T2/SA/Z1/1999	nd
T2/SA/B2_61/1999	nd
T2/SA/DE4_S/2000	T2/SA/DW2_S/1999, T2/SA/DW2_P/1999
T4/SA/DW3_P4/1999	T4/SA/DW2_P4/1999

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

b: nd = none detected

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

FIG.1.

FIG. 1. Relationship of selected South African human astrovirus (HAstVs) sequences (nucleotide 6481 to 6688; HAstV-1 [123513] of ORF2, to the Oxford (Ox) reference strains. The numbers show the percentage nucleotide identity, by pairwise analysis, of the aligned nucleotide sequences.