PhD Dissertation

entitled

Genetic characterization of astroviruses associated with diarrhea among children in a periurban community of Mexico City

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ABBREVIATIONS

Aa	amino acid
AdV	adenovirus
ANV	avian nephritis virus
AstV	astrovirus
BLAST	Basic Local Alignment Search Tool
CCC	child care center
CDC	Centers for Disease Control and Prevention
CV	calicivirus
E	Expect value
EAdV	enteric adenovirus
EIA	enzyme immunoassay
EM	electron microscopy
F81	Felsenstein (model of sequence evolution)
FAstV	Feline astrovirus
HAstV	human astrovirus
HKY85	Hasegawa, Kishino and Yano
HLA	human leukucyte antigen
ICTV	International Committee on Taxonomy of Viruses
IGS	indirect immunogold staining
IRE	immunoreative epitope
JC	Jukes-Cantor (model of sequence evolution)
K2P	Kimura 2 parameter (model of sequence evolution)
LLCMK2	rhesus monkey kidney epithelial cells
M3363	Mexican AstV isolate
NA	neutralization assay
NCBI	National Center for Biotechnology Information
NLS	nuclear localization signal
nt	nucleotide
ORF	open reading frame
OAstV	Ovine astrovirus
PAstV	Porcine astrovirus
PEMS	Poult Enteritis and Mortality Syndrome
POL/Pol	RNA-dependent RNA polymerase
REV	general reversible model (model of sequence evolution)
RNA	ribonucleic acid
RT-PCR	reverse-transcription polymerase chain reaction
RV	rotavirus
TAstV	Turkey astrovirus
TM	transmembrane helices
Th1	helper T-cell subtype 1
T_m	melting temperature
UK	United Kingdom
UPGMA	unweighted pair group method with arithmetic means
USA	United States of America
UTR	untranslated region
VP	viral protein
VPg	genome-linked viral protein
+ssRNA	positive-sense single stranded RNA

Chapter 1
Introduction

Viral infections of the gastrointestinal tract cause approximately 2 billion cases of diarrhea in children per year worldwide resulting in approximately 18 million hospitalizations.[173] Diarrhea is the third leading cause of mortality worldwide behind lower respiratory tract disease and tuberculosis. As many as 3 million pediatric deaths per year result from viral gastroenteritis and dehydration.[63]

The major pathogens of viral gastroenteritis are rotavirus (RV), calicivirus (CV), enteric adenovirus (EAdV) and astrovirus (AstV).

The importance of RV has been well established by the high prevalence and economic burden of the disease and justified research for vaccine development. The first licensed rotavirus vaccine (RotaShield, Wyeth-Lederle, USA) was licensed in the US, but withdrawn due to a possible association with increased incidence of 'intussusception', the slipping of a length of intestine into an adjacent portion, usually producing obstruction. Several new rotavirus vaccines are under trial and soon will be marketed around the world.

Enteric adenoviruses, espacially human AdV subgroup F, types 40 and 41 are major cause of infant diarrhea for which there is no vaccine available.

Human CV and AstV research focus on improving the genetic and antigenic detection methods, assessing prevalence and seroprevalence of these viruses and protection against new infections. This information will provide preliminary data to assess the economic burden of the disease and may justify development of prevention strategies including vaccination.

Following my introductory first year as a PhD student in molecular pathology in the Department of Clinical Chemistry at Faculty of Medicine, Pécs University (mentors: Drs. Eszter Nagy

and Tamás Magyarlaki), I became a member of Dr. Douglas K. Mitchell's research team focusing on astroviruses. In my work I have been involved with the genetic and antigenic characterization of astroviruses at the Center for Pediatric Research, Eastern Virginia Medical School, Norfolk, VA, USA.

In this proposal I summarize my first project to characterize the genetic diversity of AstVs among children in Mexico City. In this project I also aimed to complete a panel of type-specific primer for typing of AstVs. Since this first project I have been involved with the characterization of genetic diversity of AstVs in the United States, Egypt, South Africa and Hungary. I also took an active role in the development of antibody detection methods and studies characterizing the clinical spectrum of astrovirus infections and the economic burden of the disease. Dr. Mitchell's research is supported by the National Institutes of Health (RO1 grant); therefore we had the opportunity to submit a grant with Dr. György Szűcs for a Fogarty International Research Collaboration Award, entitled 'Hospital-Based Assessment of Human Astrovirus Infection in Hungary'. This proposed project would enhance astrovirus research in Hungary.

In this thesis I describe the genetic characterization of HAstVs in a well-defined prospective surveillance study among children in a periurban community of Mexico City. The genetic characterization was performed by reverse transcription-polymerase chain reaction (RT-PCR) and RNA sequence analysis. The RT-PCR data were compared to enzyme immunoassay (EIA), which was the traditional detection method for large community studies. The publications that describe our results from this study are enclosed in Appendix A. My additional publications are attached in Appendix B.

Chapter 2
Hypotheses and specific aims

The objective of this proposal was to define the molecular epidemiology of HAstVs among a cohort of children in a periurban community in Mexico City. The **hypotheses** of this study were that RT-PCR was a more sensitive method than EIA for detection and typing of AstVs (1), multiple HAstV genetic types and strains may be present simultaneously in a community (2), and by the utilization of RT-PCR novel AstVs can be detected which were unrecognized by traditional detection methods (3).

These hypotheses will be tested by the following **specific aims**:

Specific aim 1 To compare RT-PCR, EIA and sequence analysis for detection and typing of astroviruses;

Specific aim 2 To characterize the genetic diversity of HAstVs detected in a prospective community–based surveillance study among children in Mexico City.

Chapter 3
Background and Significance

3.1 General Viral infections of the gastrointestinal tract cause approximately 2 billion cases of diarrhea in children per year worldwide resulting in approximately 18 million hospitalizations.[173] Children in the U.S. have approximately 200,000 hospitalizations due to diarrhea at a cost exceeding $1 billion.[62] Diarrhea is the third leading cause of mortality worldwide behind lower respiratory tract disease and tuberculosis. As many as 3 million pediatric deaths worldwide and 200 deaths in the U.S. per year result from viral gastroenteritis.[63] These numbers translate into 2 to 3 diarrheal

illnesses per child-year in U.S. children younger than 3 years of age; rates in children attending child care centers (CCCs) are significantly higher.[26, 47, 54, 64, 153, 154]

The most common viral enteric pathogens are rotavirus, calicivirus, astrovirus and enteric adenovirus.

3.2 History The term astrovirus was coined by Madeley and Cosgrove in 1975, a year after rotavirus was identified, when the starlike morphology of astrovirus (astron=star in Greek) was seen by electron microscopy (EM) in stool samples from a diarrhea outbreak amongst infants.[104,105](Figure 1) A few months prior to the naming of astroviruses, Appleton and Higgins reported a gastroenteritis outbreak among infants in a maternity ward, where viruses were found in large numbers of virus differing in size and morphology from the previously identified Norwalk virus and rotavirus were shed in the stool.[5] Years later, when specific immunologic reagents became available these viruses were identified as astroviruses.[23,172] Characterization of HAstV molecular epidemiology has proceeded more slowly than rotavirus, the most common cause of severe diarrhea in children. The epidemiology of HAstV associated diarrhea has changed due to recent utilization of reverse transcriptase-polymerase chain reaction (RT-PCR) and enzyme immunoassay (EIA) for virus detection. Human astrovirus is now found to be a common cause of viral diarrhea among young children in hospitals and in the community.[43, 61,106]

Subsequently, viral particles with similar size and characteristic starlike surface features were identified in association with gastroenteritis in a wide variety of young mammals and birds including lambs,[169] calves,[180,197] deer,[181] piglets,[21,22,168] kittens,[65,110] mice,[78] dogs,[109] mink kits[36], and turkey poults.[113,158] Ducklings infected with astrovirus-like particles developed a rapidly fatal hepatitis and viral particles were found in the liver and the feces.[51]

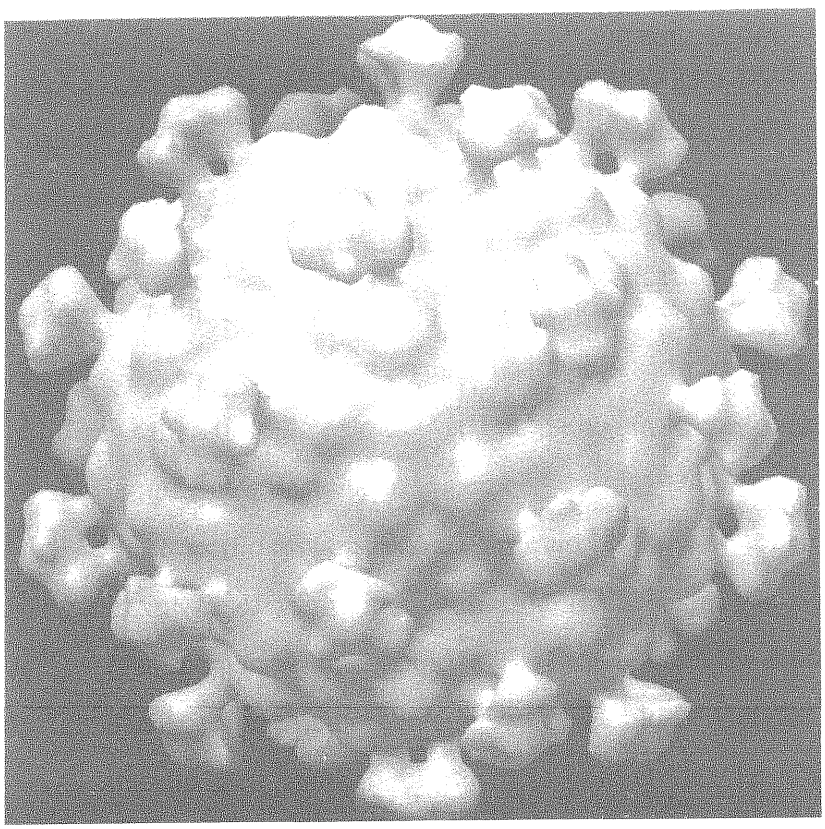


Figure 1 Three-dimensional reconstruction of HAstV serotype 1 imaged by cryoelectron microscopy from negative stain (Yeager et al. 2000)[199]

Although, immune electron microscopy studies demonstrated the presence of antibody in a human serum to cat astrovirus, other studies hypothesized that astroviruses cause infection in a species-specific manner.[110,88] At present there is no animal model to study human astrovirus infection.

3.3 The role of HAstV in disease Preliminary observations of Madeley and co-workers[104,105] established an association between astroviruses and mild gastroenteritis in human. It was difficult to ascertain the role of astrovirus in disease, because astroviruses may be found with other potential gastroenteritis agents in diarrheic feces.[13, 20, 44, 107, 156] Volunteer- and hospital-based clinical studies confirmed the etiologic role of AstV as pathogen in gastroenteritis by fulfilling Koch's Postulates:

1. The specific organism (AstV) should be shown to be present in all cases of humans suffering from a specific disease (gastroenteritis) but should not be found in healthy animals.
2. The specific microorganism (AstV) should be isolated from the diseased human and grown in pure culture on artificial laboratory media.
3. This freshly isolated microorganism, when inoculated into a healthy human, should cause the same disease seen in the original human.
4. The microorganism should be re-isolated in pure culture from the experimental infection.

Ad 1. In several clinical studies HAstV was isolated from children with gastroenteritis.[9,10,13,29,33,38,61,83,86,91,96,99,122,123,125,142,145,147,152,167,171,182,183] The clinical features of gastroenteritis associated with astrovirus are not specific to differentiate it from other viral pathogens (rotavirus, calicivirus). Hermann et al. conducted a study among over 3000 infants in Thailand, where HAstV was associated with 8.6% of the children with gastroenteritis and 2% of the children without gastroenteritis.[61] The finding that HAstVs can be

detected in apparently healthy humans might be due to asymptomatic infections. Not all volunteers challenged with astrovirus, developed symptoms of gastroenteritis but did respond with seroconversion to the infecting type, which is a good indicator for recent infection.[89]

Ad 2. AstV can be isolated from stool specimens and propagated in tissue culture. For volunteer studies virus was filtered from the fecal extract of an infected child and administered to adult volunteers to prove transmission of the disease.[89,118]

Ad 3. Freshly isolated astrovirus, when inoculated into a healthy previously unexposed human, caused gastroenteritis as seen in the original infected human.[89,118]

Ad 4. The volunteers were shedding AstV in their stool after the infection.[89,118]

3.4 Animal hosts of AstVs Astrovirus had been identified in association with disease in several animal species. Known animal hosts of AstVs include both mammals and birds. Astrovirus was associated with mild diarrhea in mammals, including calves, lambs, pigs, cats, dogs, and mice and mink.[8,36,58,88, 109,162,194,198] In most of these cases astroviruses were separated from other viruses and shown to cause illness. Avian AstV infections may result in fatal disease, including acute interstitial nephritis and growth retardation in chickens (avian nephritis virus - ANV), hepatitis in ducklings,[51] and a severe multisystem disease called Poult Enteritis and Mortality Syndrome (PEMS) in turkeys that was featured by diarrhea, stunted growth, immunosuppression, and high mortality rates (25-96%).[80,159,160,165,157,177,200] This latter disease caused millions of dollar loss by the poultry industry in the 1990s in Texas and Virginia, USA.

3.5 Spectrum of clinical features of gastroenteritis in humans The incubation period was calculated to be short ranging from two

to four days based on adult human volunteer studies and outbreak of gastroenteritis in kindergarten.[83, 89, 118]

Several studies highlighted frequent co-infections of HAstV, rotavirus, and calicivirus. For example, astroviruses were detected in 16 to 42% of rotavirus-associated diarrhea episodes.[20,43,56] These co-infections make the complete evaluation of clinical symptoms due to astrovirus alone more difficult.[156](Table 1)

Astrovirus-associated diarrhea has been characterized by a median duration of 3 days (range, 1 to 21), median of 4 stools (range, 1 to 10) during the first 24 hours, vomiting (20-62%), and fever (7-33%).

Astrovirus-associated diarrhea was less severe, as measured by the number of stools and duration of the diarrhea, than RV-associated diarrhea. Fever and duration of vomiting and were significantly more frequent during isolated rotavirus infections than in isolated astrovirus infections.[20,56,151] Studies in developed countries (Finland and France) showed that only 0-3% cases of astrovirus gastroenteritis resulted in dehydration, no repeat symptomatic infections were observed, and very few astrovirus infected children were hospitalized (3%).[56,151] However, a recent population-based study of Egyptian children (younger than 3 years of age) demonstrated that clinical characteristics of the astrovirus diarrheal episodes included some degree of dehydration in 48% and severe dehydration in 17% of the cases based on the WHO criteria. There was no significant difference when episodes with or without co-pathogens were analyzed.[134](Table 1) It is of interest, that severe dehydration was observed in 19% of the first episodes of HAstV diarrhea, whereas 6% in the second or third episodes.[134]

Overall, the clinical severity of astrovirus gastroenteritis was less than that of rotavirus gastroenteritis.[56] In other words, number of children affected provides evidence of the importance of this

Clinical symptoms	Astrovirus	Rotavirus	References
Diarrhea	72%-100%	93%	[151][20][56]
Duration of diarrhea (days)*	0-21 days (average 2-3)	0-12 (average 4)	[151][56]
Maximum number of stools/24 h*	0-10 (average 4)	0-30 (average 6)	[151][56]
Bloody diarrhea	0%	1%	[20]
Abdominal pain	50%	19%	[20]
Vomiting	20-62%	69%	[151][20][56]
Duration of vomiting*	0-4 days (average 1)	0-8 (average 2)	[151]
Maximum no. of vomiting/24 h*	0-10 (average 1)	0-20 (average 3)	[151]
Fever*	25-33%	67%	[20][134]
Maximum fever (°C)	37.5-41.3 (average 37.8)	37.5-40.5 (average 38.9)	[151]
Dehydration (mild)	48%	53%	[134]
Dehydration (severe)	0-17%	17-24%	[151][56][134]
Severity score (1-20)*[a]	2-12 (average 5)	3-19 (average 11)	[151][56]

* $p<=0.01$

[a] Ruuska and Vesikari 20-point scoring system used

Ruuska T, Vesikari T (1990) "Rotavirus disease in Finnish children: use of numerical scores for clinical severity of ırrhoeal episodes." Scand J Infect Dis 22(3): 259-67.

Table 1 Clinical characteristics of HAstV infections compared to RV infections

enteropathogen and the severity may vary from developed to developing countries.

3.6 Immunity The immunity to astrovirus infections is not well understood. Symptomatic infections are primarily found in two age groups: children[122,174] and elderly, institutionalized patients.[99] Volunteers with detectable serum antibody to astrovirus did not develop disease.[89] Therefore, we hypothesize that antibodies acquired early in life provides immunity but the protection wanes late in life.

On the contrary, HAstV infection was reported among adults in outbreaks among recruits in the military in France,[16] staff in kindergarten,[83] and in junior high school in Japan[147,183]; and in Marin Country, California, when an outbreak occurred among elderly patients in a convalescent hospital.[118] Of interest, the outbreak among the military was caused by HAstV-3, the outbreak in the junior high school was caused by HAstV-6, and the Marin County agent was proven to be HAstV-5. Seroprevalence studies indicate that both of these types are less common than HAstV-1 (16-60%).[85,119] Low seroprevalence against HAstV-5 was observed during childhood and increased in early adulthood suggesting that the epidemiology of HAstV-5 is different from that of astrovirus serotypes 1 to 4.[85] These outbreaks might have occurred among the adult population due to the lack of homotypic antibody (anti-HAstV-3, anti-HAstV-6, anti-HAstV-5, respectively); the low protective role of the existing heterotypic antibodies (e.g. HAstV-1), and probably due to the special settings including crowding, poor hygiene or exposure to an unusually high titer of the virus shedding.

The role of homotypic and heterotypic immunity in protection against symptomatic disease is not fully elucidated. Multiple infections with HAstVs in the same person were documented only in a few studies. Mitchell et al. studied outbreaks among children in

CCCs and noted that a single infecting type caused each outbreak, but 25% of the CCCs experienced a second outbreak with a different astrovirus type in the same year (e.g. HAstV-1 followed by HAstV-2).[121] Study of a birth cohort in Egypt observed that children infected by HAstV-1 did not have any subsequent episodes of HAstV-1 diarrhea, but a few of them were infected a second time with other viruses including HAstV-3 and HAstV-5.[134] By these studies the role of homotypic, but not heterotypic immunity in protection is supported. It was noted, that subsequent astrovirus infections were observed to be milder in severity.[134]

Humoral immunity can be easily assessed, but T cells also are proven to play an important role in protecting individuals from repeated infections.[126] T cells residing in normal, duodenal mucosa of adult subjects recognize astrovirus in a human leukocyte antigen (HLA)-restricted manner (HLA-DR). These HAstV specific CD4+ cells showed a helper T-cell subtype 1(Th1)-like cytokine profile (interferon gamma and tumor necrosis factor) when restimulated with astrovirus. The integrity of other T cell lines might be important in modulating the infection. Cubitt et al. observed that the production of CD16+ and CD8+ T cells in patients, who shed virus for a long period of time, was correlated with decreased level of viral shedding (detectable by RT-PCR only).[30]

3.7 Immunocompromised host Defense against HAstV infections largely involves the immune system, therefore it is suggested that novel enteric viruses, such as astrovirus, may be more important etiologic agents of diarrhea in HIV-infected patients than previously recognized and may be more common than either bacterial or parasitic enteropathogens.[53] Immunocompromised hosts infected with HAstV have a prolonged and more complicated course of illness. HAstV-1 outbreak was reported in a pediatric bone marrow transplant unit, where the virus was shed in symptomatic patients for many months.[30] Astrovirus

was identified among 5% of immunosupressed patients with acute or persistent diarrhea in Chile.[179] The most commonly infected patients were those with haematologic disorders and premature infants. This study suggested that in patients with organ transplants an accurate diagnosis about diarrhea etiology is advisable in order to give a specific antimicrobial therapy, or to avoid a misdiagnosis of rejection.[179]

Several reports describe chronic astrovirus diarrhea (up to 31 days) that developed among bone marrow transplant recipients[28,88] or patients with T cell deficiency[196] or receiving fludarabine treatment, which is known to deplete the T cells.[17,19,27] Some of these patients benefited from administered intravenous and oral gammaglobulin, that probably contained high titer of anti-HAstV antibodies (e.g.: 1:128,000 IgG against HAstV-1).[19,30,201] The role of immunoglobulin in treating immunocompromised patients requires further study.

3.8 Detection – Development of laboratory methods Methods used for detection of HAstV significantly affect the estimation of the frequency of HAstV infections.

3.8.1 Electronmicroscopy (EM) EM led to the discovery of AstVs in the United Kingdom, where morphologically distinct viruses with star-like structur were detected in the stool of infants with diarrhea.[5,104] Caul et al. compared the features of enteric viruses (enterovirus, parvovirus, and calicivirus) and proposed an interim classification scheme, based primarily on the morphological appearance of the particles and supported by estimations of size and buoyant density.[23] Later this classification was modified and broadened by Kapikian and included HAstVs.[73] The morphological classification is reliable when viruses display their characteristic morphology, however in many preparations degradation of the particles made the classification difficult. Initial studies of the incidence of HAstV infection were based upon virus detection by

EM, and detected the virus in 1-2% of viral diarrhea cases.[48,97,128,178]

The sensitivity of EM has been estimated to be 10^6 to 10^7 virus particles per gram of stool. In those patients who may be excreting fewer viral particles immuno-electron microscopic (IEM) techniques were useful.[18]

3.8.2 Enzyme immunoassay (EIA) An important milestone was achieved, when Lee and Kurtz successfully propagated the virus in primary human embryonic cells.[93] Successful cultivation of the virus enabled antisera to be raised in rabbits and these sera were used to demonstrate that there were five antigenic types of astroviruses in 1984.[87] At present, there are eight known serotypes of HAstVs (see definition of serotype and antigenic type below) as determined by both hyperimmune sera and typing by sequence analysis.[95,115,175] Production of a group-specific monoclonal antibody (8E7) and its use in combination with rabbit polyclonal antisera enabled development of EIAs for detection of all eight HAstV serotypes, and is now commercially available in Europe (IDEIA™ Astrovirus, DAKO, Cambridgeshire, United Kingdom).[48, 59] Compared to immune electron microscopy used as the standard test, the sensitivity of the astrovirus EIA was 91% and the specificity was 96%. At that time this EIA seemed to provide a sensitive and specific means for the diagnosis of astrovirus gastroenteritis.[60]

The antigen detection EIA facilitated epidemiologic studies demonstrating that HAstVs are a significant cause of diarrhea in many regions of the world, with infection rates significantly higher than determined in surveys utilizing EM as the detection method (2,5-10%).[29,4861,98,183] The sensitivity of EIA is estimated to be 10^5 to 10^6 virus particles per gram of stool.[60,125]

3.8.3 Antigenic typing There are eight known HAstV antigenic

types. This means that polyclonal antibody raised by exposure to a specific antigenic type will react to HAstVs of the same type and not to others. These eight types of astrovirus were tentatively considered serotypes, but it was unclear whether the serotype designation was used properly. Viruses are considered unique serotypes if homotypic antibodies are exclusive and specific in neutralizing growth in plaque assays or neutralization assays. Hudson and Herrmann demonstrated that HAstV-1, 2, and 5 were serotypes.[66] To test human sera for the presence of neutralizing antibodies and to type field strains, neutralization tests were developed using CaCo2 tissue-culture-adapted astrovirus strains and the corresponding rabbit reference sera. Koopmans et al further extended to all other seven types in these experiments.[85] In rabbits, neutralizing antibodies were predominantly serotype-specific, with the exception of low-level cross-reactivity in astrovirus serotype 4 reference serum with astrovirus serotype 1 virus. Similarly, in humans, no evidence of cross-reactivity was found for the serotype combinations tested (all except the combination 1 and 7 and the combination 6 and 7). HAstV-8 was demonstrated to react with the HAstV-8 reference serum exclusively and not sera against serotypes 1-7.[175]

Other methods for serotyping the virus include indirect immunogold staining (IGS), electron microscopy technique, using specific rabbit antisera against astrovirus types as primary antibodies and goat anti-rabbit IgG gold conjugate as secondary antibody.[77]

Polyclonal antisera to HAstV-1 through 8 raised in rabbits by Kurtz and Lee[87] have served as reference reagents. However, stocks of the original sera now are almost exhausted and there is a need to produce more material to be compared with the original antisera and subsequently used for further testing. In addition, many of the rabbit antisera showed cross-reactivity in some assays. Reagents

with greater type-specificity are needed. Typing by neutralization assay was concordant with typing by EIA and genotyping.[15, 85] Therefore one solution for further studies may be genotyping by RT-PCR.

3.8.4 Reverse transcription-polymerase chain reaction RT-PCR detection of HAstV in stool samples among children in closed populations, such as CCCs or hemato-oncologic wards, was an more sensitive method of detection than EIA.[30,122] Marx and coworkers detected HAstV in environmental water samples by RT-PCR, thereby providing a sensitive method to further define possible reservoirs of infection.[111]

Our recent studies among children with gastroenteritis in Egypt and in the USA showed low sensitivity of the commercial EIA compared to RT-PCR. The sensitivity and specificity were only 42% and 60%, respectively.[124] During the investigation of an outbreak in a pediatric hemato-oncologic ward the use of RT-PCR revealed that several patients were infected earlier and shed virus for longer than by using EM or EIA.[30]

RT-PCR has not been applied for screening samples from large studies, because it is expensive and tedious to perform for vast sample sets. Gaggero et al. concluded that EIA is a simple method for screening fecal specimens for HAstV, but ambiguous results should be confirmed by a different technique such as RT-PCR.[43] The study described here is the first example when RT-PCR has been applied for screening in a large community-based surveillance for detection of HAstVs. The sensitivity of RT-PCR is estimated to be 10-100 particles per gram of stool.[161]

3.8.5 Genotyping The complete genome of the prototype Oxford strain (type 1) was sequenced in 1993.[191] Currently, the complete genome of HAstV-1 to 3 and of 8 and capsid sequences for all eight HAstV serotypes are available. Sequence information also is available for two genotypes of turkey AstVs, avian nephritis

AstVs, and one serotype each of feline, porcine and ovine astroviruses.

Belliot compared sequence information from ORF1a, ORF1b, and the 5' end of ORF2 (nts 1182-1470, 3724-4039, and 4574-5022 in HAstV-1, respectively) and found sufficient sequence variability for type differentiation.[15,145] In contrary, our hypothesis is that only the capsid region of HAstVs may be used for genotype assignment defined by phylogenetic analysis because other coding regions may assign different genotype for the isolate (due to recombination events).

3.9 Epidemiology

3.9.1 Epidemiology in general HAstV infections occur worldwide. HAstV was found to be a common cause of viral diarrhea among young children seeking medical care in the outpatient settings, or being hospitalized. HAstVs were shown to be responsible for hospital admission and also as a nosocomial agent during hospitalization.[20,29,43,106,150,151,156,166,167,182]

The incidence rates of HAstV diarrhea range from 2% of children seeking medical care in Baltimore to 17% of children with persistent (>14 days) diarrhea in Bangladesh.[9,10,13,29,33,8,61,83,86,91,96,99,122,123,125,142,145,147,152,167,171,182,183] Although HAstV was detected less frequently than rotavirus or calicivirus in surveillance studies, astrovirus still requires close attention from a public-health viewpoint due to large outbreaks in adults at school,[147] in the military,[16] geriatric care facilities,[52,99] frequent outbreaks in child care centers,[43,121,174] and among immunocompromised patients.[30,45] The prevalence of HAstV infection varied by age groups and indicated that most children were infected during their first two years of life.[79,106,170]

The seasonality of astrovirus infections is controversial and seems to vary by geographical region. In Egypt a definite late-spring early summer peak was noticed,[134] in Mexico no specific

seasonality was observed,[56] in the United States among hospitalized children HAstV infections showed two peaks in the winter simultaneously with the RV and the CV peak. (unpublished data)

3.9.2 Epidemiology of infections by serotype Two distinct serotypes (1 and 2) of HAstVs were first distinguished by an immunofluorescence test in 1982.[94] The importance of HAstV-1 to 5 was described and soon HAstV-6 and 7 were identified.[87,95] The eighth serotype (HAstV-8) was characterized in 2000.[115,175] Initially, HAstV-8 was thought to be rare, but subsequent work shows it to be more common than expected.[134] The clinical significance of HAstV-8 was established by its association with gastroenteritis in hospitalized children.[49, 68]

Early surveys revealed that HAstV-1 was the prevalent strain worldwide accounting for over 65% of the episodes of diarrhea: in Europe [95], North America [31], South America [114], Asia [6] and Australia.[150] With newer assays, HAstV-2 to 8 have been detected more frequently than before.[193 6, 132, 144, 150]

Data obtained by seroprevalence studies provide similar data to the frequency of detection of different HAstV types. Seroprevalence of type-specific HAstV antibodies in children from different locations (USA, Japan, Netherlands) were HAstV-1 (91%-100%), HAstV-2 (31-100%), HAstV-3 (42-69%), HAstV-4 (56%), HAstV-5 (36%) HAstV-6 (16%), HAstV-7 (10%) by 3 to 9 years of age indicating frequent exposure to these enteric viruses in infancy and early childhood. [79, 85, 120]

Some studies noted alternating seasons where HAstV-1 was predominant in one season and other types were predominant the next season.[57,95,132]

A hospital-based study in France observed that HAstV-1, HAstV-2, and HAstV-3 primarily affected children younger than 3 years of age, while HAstV-4 and HAstV-8 had greater impact in older children.[57]

3.10 Source of infection Based on the human volunteer studies, it is known that astrovirus can be transmitted by the fecal-oral route.[89, 118]

Despite scarce information, astrovirus appears to be an important cause of food-borne disease.[41] In June 1991, a large outbreak of HAstV-6 gastroenteritis occurred among students and teachers in Japan. The outbreak affected more than 4700 persons, lasted 5 days, and probably was linked to contaminated food from a common supplier.[147] In another study, astrovirus was detected by RT-PCR in oysters collected in Chiba City Bay, Japan, from 1997 to 1998. HAstV was detected more frequently (13%) than hepatitis A virus (1.8%) or adenovirus (0%) and was comparable to the small round structured viruses (25%). This result indicated that astrovirus was detected in naturally grown oysters during the winter season.[76] Astrovirus infection was reported in a 27-year-old bone-marrow transplant recipient with a history of consuming "sashimi" while visiting Japan before the bone-marrow transplantation.[201]

HAstV detection in water was recently studied extensively. In England, RT-PCR was used to search for HAstV RNA in concentrates of the samples of sewage, seawater, river water and sand and silt collected from a sewage works.[35] No evidence of astrovirus was found in any sample, which suggests that contamination with this virus was not a problem in that region during the summer holiday season or they used inadequate methods. This is in contrast to the findings of Chapron et al. [24, 25] Human astroviruses were detected in all but one sample of sludge biosolids by integrated cell culture-reverse transcriptase-polymerase chain reaction (ICC-RT-PCR) coupled with nested PCR. Pinto et al. also found astroviruses in wastewater samples from sewage treatment plants of Spain and France.[155] Our collaborator, Dr Maureen Taylor in South Africa could detect HAstV-1, 2, 3, 4, 5, 7 and 8 in sewage samples from the

metropolitan area of Pretoria [personal communication]. As indirect evidence of infection in the United Kingdom, the seroprevalence of HAstV-4 among surfers was much higher (93%) compared to aged matched controls (22%).[133]

3.11. Prevention and treatment Control measures for outbreaks of viral gastroenteritis should focus on the removal of an ongoing common source of infection (e.g. an ill food handler or the contamination of a water supply) and on the interruption of person-to-person transmission that can perpetuate an outbreak in a population after the common source has been removed.

Astrovirus was quite resistant to ethanol disinfection, methanol and chlorinated water were shown to be more effective in reducing AstV infectivity.[1,90]

Gastroenteritis caused by astrovirus is generally a mild, self-limiting illness that may disrupt an individual's activity for a few days but does not require specific therapy. In the young child or elderly who becomes dehydrated, oral or intravenous fluid may be necessary. Complicated course of illness may develop in patients with underlying gastrointestinal disease, poor nutritional status, immunodeficiency or severe mixed infections.[29] Intravenous immunoglobulins may be useful for severely immunocompromized patients who fail to respond to conservative measures.[19, 201]

Because improvements in environmental hygiene may not be accompanied by reductions of endemic diarrhea caused by viruses, immunization may play an important role in future control. Vaccine trials for rotavirus are in progress, already. A better understanding of the fundamentals of immunity to astrovirus and estimates of the disease burden are required before vaccine development can be initiated. As astroviruses infect mainly children less than a year old, so a basic requirement of any vaccine candidate would be the ability to induce immunity in very young children.[134]

3.12 Molecular Virology of AstVs

3.12.1 Genomic organization of AstVs AstVs contain a single-stranded, positive-sense RNA (+ssRNA) genome of approximately 6,800 nucleotides (nt; Figure 2). The genome includes three open reading frames (ORFs): ORF1a, ORF1b, and ORF2. ORF1a and 1b encode non-structural proteins, including a serine protease and RNA-dependent RNA polymerase (POL), respectively, and these regions contain sequences highly conserved among AstVs. Functionally important motifs of ORF1a include the catalytic triad of serine protease, proteolytic cleavage sites (Q/T) and a nuclear localization signal (NLS). Of interest is that ORF1a does not include a helicase and encodes an immunoreactive epitope in the 3' end.

The overlap region between ORF1a and ORF1b is highly conserved among serotypes and displays a heptameric shift sequence (AAAAAAC) and a potential for the formation of a downstream stem-loop structure with a possible pseudoknot. It is strongly suggested that a (-1) ribosomal frameshifting mechanism is involved in ORF-1b translation.[69,101,102,190] Even in avian astroviruses the shifty heptamer and the stem-loop motif of human astroviruses were identified.[67, 81]

ORF2 is found in both the genomic and the subgenomic RNA. It has been suggested that the role of the subgenomic RNA is the production of viral structural proteins.[129,130] The N terminal half of ORF2 is highly conserved in contrast to the majority of the C terminal half, which is quite variable among HAstV strains and serotypes.

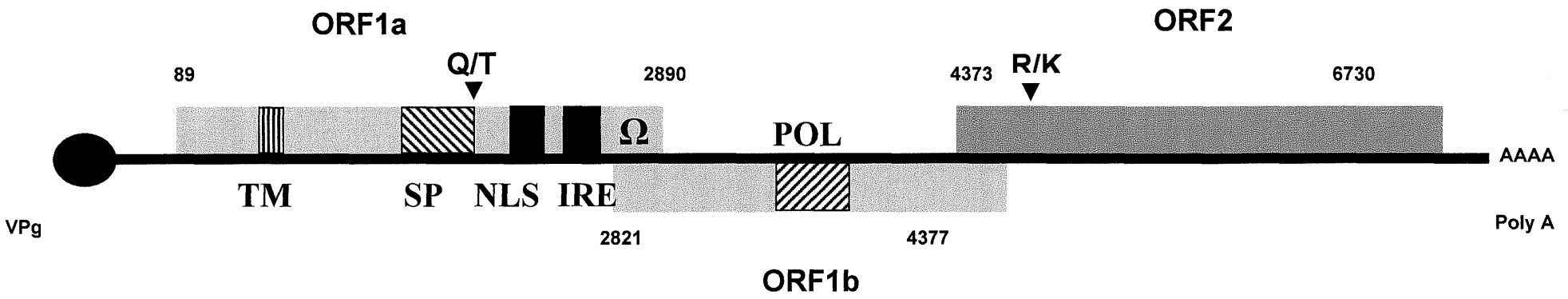


Figure 2 HAstV genome organization (HAstV type 1, 6813 nucleotides)[74] Motif and epitopes of ORF1a include: TM, predicted transmembrane helices; SP, serine protease (1362-1707); Q/T: putative protease dependent cleavage site; NLS, nuclear localization signal (1986-2067); and IRE, immunoreactive epitope (2271-2697). VPg is viral protein bound to the genome, probably encoded in ORF1a. **Ω:** *ribosomal frameshift signal. ORF1b has a motif (POL) common to RNA-dependent RNA polymerases. ORF2 encodes the capsid protein that appears to be cleaved at amino acid 70/71 (RK) before assembly.*

3.12.2 Non-structural proteins The ORF1a/1b translation product appears to be 160 kdal in size.[189] A cleavage occurs near the boundary between the ORF1a and ORF1b resulting in a 101 kdal protein (ORF1a) ad an 59kdal protein (ORF1b). The ORF1a product will further be cleaved into an N terminal (p64) and a C terminal (p38) cleavage product. The catalytic triad of the viral 3C-like serine protease (Ser-551, Asp-489, His-461) proved to have an important role of the processing of the ORF1a product.[74]

It has been suggested that astrovirus encodes a VPg, based on the absence of methyltransferase-encoding region and the similarity of its RNA-dependent RNA polymerase with primarily VPg containing viruses. A convincing VPg domain has not been identified. But it may be located upstream from the putative serine-protease region.

The sequence of the polymerase encoded in the ORF1b region can be aligned with other polymerases of the Koonin's supergroupI.[84]

3.12.3 Structural proteins The astrovirus capsid proteins are derived from a single precursor of approximately 86kdal product (P86) encoded in ORF2.[11] Although description of human capsid proteins have been somewhat variable, there is general agreement that the capsid consists of at least three major components with estimated molecular masses ranging from 20-38 kdal.[11,14] In HAstV-2, ORF2 encodes three proteins: VP35, VP29, and VP26. The more conserved 5' end encodes VP35 whereas the more variable 3' end encodes VP29 and VP26. Partial N-terminal sequencing of VP29 and VP26 polypeptides demonstrated that they originated by alternative processing of P86 after residues 361 and 394, respectively[163] VP29 may be important in viral neutralization, heterotypic immunity, and virus attachment to target cells.[12]

Herman et al. developed A monoclonal antibody (8E7) was developed that detects all human astrovirus types.[60] A

commercially available EIA kit (IDEIA™ Astrovirus, DAKO, Cambridgeshire, United Kingdom) utilizes this antibody for capture. The binding site of the antibody was recently mapped to be in the 5' end, more conservative region of ORF2.[146]

3.13 Taxonomy of astroviruses The family *Astroviridae* was established within the +ssRNA subphylum after a series of observations:

In 1991 it was noted that proteins and RNAs synthesized in astrovirus-infected cells with those of the recognized families of non-enveloped single-stranded RNA animal viruses suggests that astroviruses should not be classified as members of either *Caliciviridae* or *Picornaviridae.*[130] In 1993 the subgenomic RNA sequence of human astrovirus was determined that supported the classification of *Astroviridae* as a new family of RNA viruses.[129] In 1993 the RNA sequence of HAstV-1 was published, which revealed the distinctive genomic organization HAstVs and identified a putative retrovirus-like ribosomal frameshift signal that directs the viral replicase.[69] In 1994 the viral RNA-dependent RNA-polymerase motif was identified and viral structural protein was expressed[100]. Based upon these observations the ICTV classified astrovirus into a new family *Astroviridae* in 1995.

In summary, the characteristics that clearly distinguished astroviruses from members of the same virus subphylum (+ssRNA) are distinct genomic organization, the presence of the serine protease motif and the ribosomal frameshift mechanism.

Recently our research group performed phylogenetic analysis on the complete ORF2 region utilizing maximum likelihood algorithm to compare and estimate the evolutionary relationship among human and animal reference strains. Analysis of the capsid gene revealed two distinct clusters: mammalian astroviruses (including all known human, feline, porcine, and ovine species) and avian astroviruses (including turkey and avian nephritis virus).

(Figure 3) The two clusters also differ in the host of origin with mammalian astroviruses in one cluster and avian astroviruses in another cluster and by the target organ for infection: mammalian astroviruses primarily infect the gastrointestinal tract and cause a self-limiting gastroenteritis, whereas avian astroviruses have been reported to cause extraintestinal disease involving the liver, kidney, and immune system. Utilizing the more conserved regions (ORF1a and ORF1b), Lukashov observed the same two major groups.[103] The phylogenetic distances were comparable to distances between genera of *Caliciviridae* or *Picornaviridae*. Based upon our results we proposed to the Astrovirus Study Group of the ICTV that the family *Astroviridae* be subdivided into two genera, *Mamastrovirus* and *Avastrovirus*, on the basis of phylogenetic clustering of non-structural genes and capsid sequence, host of origin and target organs. *Mamastrovirus* would include astroviruses that infect mammals and primarily cause gastroenteritis. *Avastrovirus* would include astroviruses that infect avian species and may cause intestinal as well as extraintestinal illness.

Other groups performed phylogenetic analyses in different regions of the genome. Belliot stated that phylogenetic grouping and consequent type-assignment in any part of the genome would be consistent.[15] Similar observations were reported by Lukashov analyzing sequences along the whole length of the genome.[103]

3.14 Significance of this study Astroviruses are significant gastrointestinal tract pathogens, but the reservoir, mode of transmission, distribution of antigenic types, and factors associated with immune protection are poorly understood. Through this large prospective study of diarrhea in children in Mexico different molecular diagnostic techniques were compared and information are obtained to describe genetic diversity of HAstV. More sensitive techniques may allow the detection and characterization of previously unknown strains or types. This will lead to the better

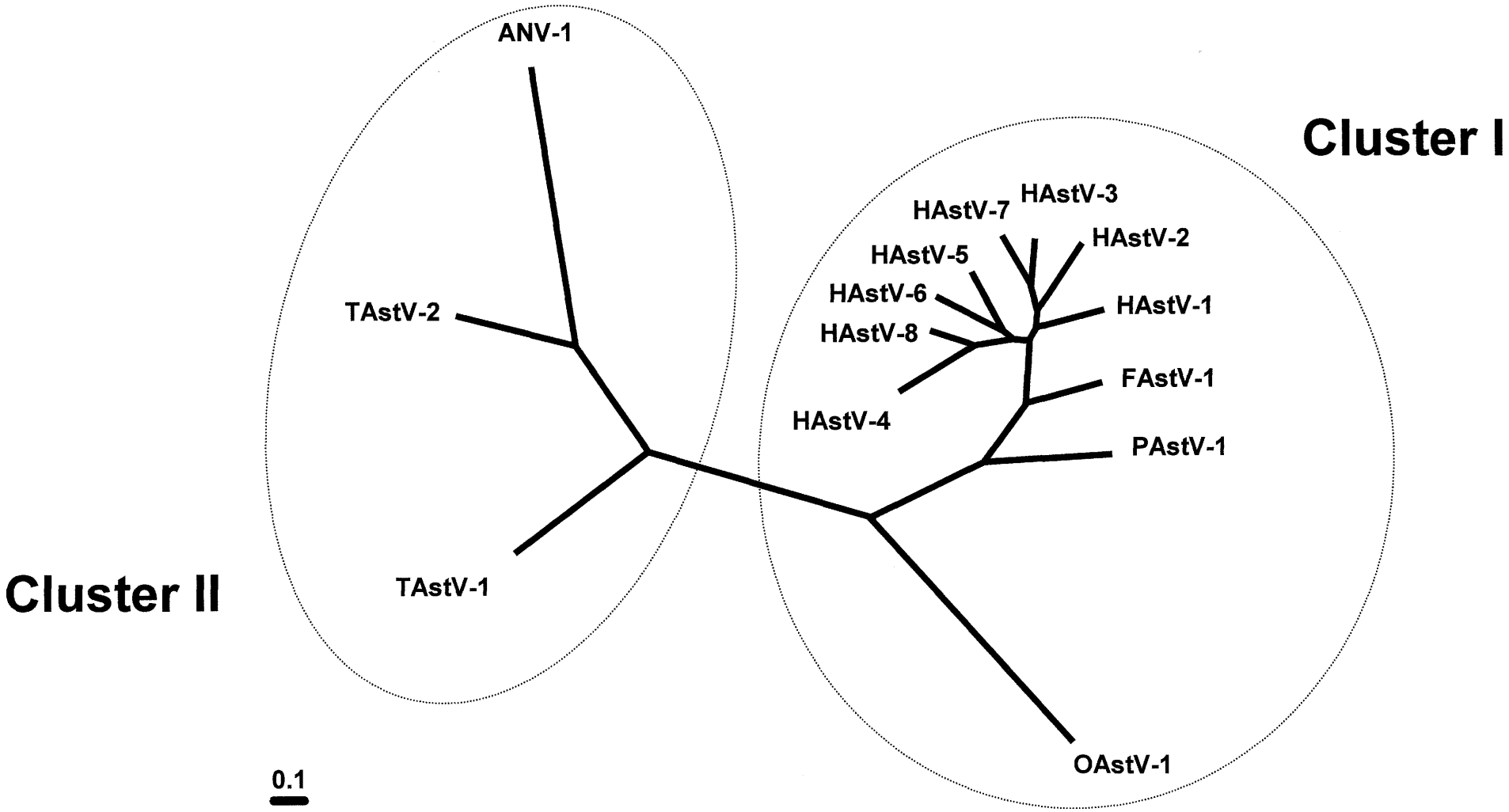


Figure 3 *Maximum likeihood phylogram (global rearrangement, randomized 50 times) of ORF2, including HAstV serotypes 1 to 8: HAstV-1 [Acc#L23513], HAstV-2 [Acc#NC_002470], HAstV-3 [Acc#AF141381], HAstV-4 [Acc#Z33883], HAstV-5 [Acc#U15136], HAstV-6 [Acc#Z46658], HAstV-7 [Acc#AF248738], HAstV-8 [Acc#NC_002499] and animal AstVs: feline AstV (FAstV) [Acc#AF056197], porcine AstV (PAstV) [Acc#Y15938], ovine AstV (OAstV) [Acc#NC_002469], turkey AstVs: TAstV-1 and 2 [Acc#AF206663; NC_002470], and avian nephritis virus serotype 1 (ANV-1) [Acc#AB033998]. Analysis showed that all reference strains appeared statistically distinct ($p<0.01$) and two distinct clusters were evident: Cluster I included all HAstV strains, FastV, PastV and OAstVs and Cluster II included TAstVs and ANV-1.*

understanding of significance of HAstV in gastrointestinal infections in children. These additional data are needed to determine if the prevention strategy of vaccine development is warranted.

Figure 4 illustrates the guiding theme for our research group regarding the analysis of enteric viral pathogens. Each step leads to additional laboratory methods to support further development. The aims of this study address the top two levels of this development scheme.

Chapter 4
Methods

4.1 Study population From November 1988 through December 1991, 214 children in a periurban community of Mexico City were monitored prospectively for diarrhea from birth to 18 months of age. Stool samples obtained during the diarrhea episodes were tested for HAstV by EIA as previously described.[56] Previous studies describe the geographic and socioeconomic characteristics of this community.[131, 185, 186]

4.2 Specimens Stool samples were collected weekly regardless of symptoms and during each episode of diarrhea and stored at -70 °C until tested.[56] HAstVs detected by EIA or RT-PCR were cultured in CaCo-2 as previously described.[145, 192] The extended testing period was defined as the diarrhea episode seven days before and after the episode.

4.3 Definition of diarrhea A diarrhea episode was defined as the presence for at least one day of: (1) three or more watery stools in 24 hours; (2) at least two bowel movements in addition to the usual daily pattern, with a decrease in stool consistency; or (3) at least one bloody stool with a decreased consistency.[56] An extended

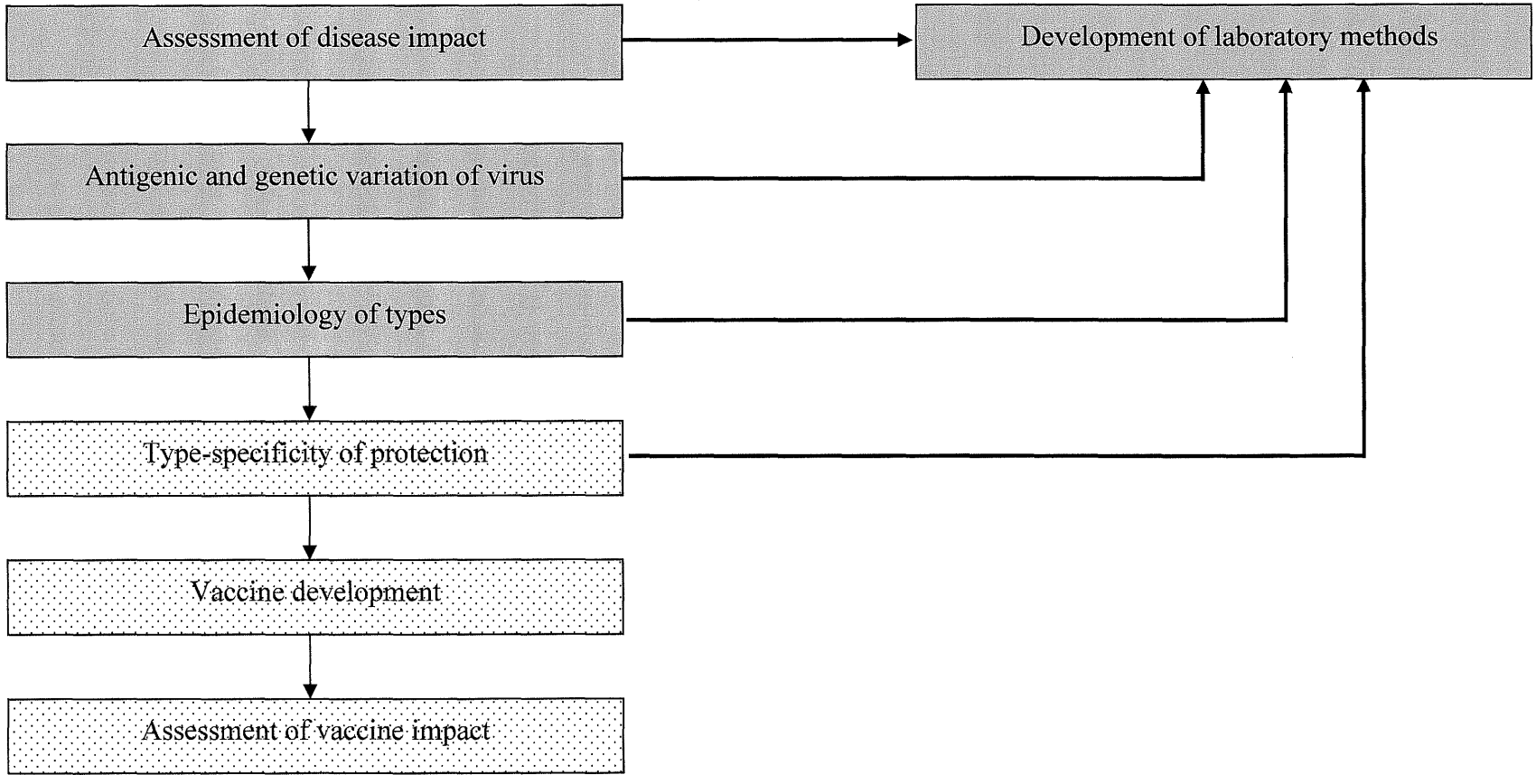


Figure 4 Progression of knowledge needed for a successful immunization program

diarrhea episode was defined as the period of diarrhea plus seven days before and after the diarrhea.

4.4 Detection of HAstVs by EM Virus was concentrated, partially purified on sucrose and examined by electron microscopy. Specimens were placed on a formvar-carbon supported film grid and stained with ammonium moblidate for 30 sec. Micrographs were taken with a transmission electron microscope (Jeol 100CX100) operated at 60 kV. Primary magnification was x29,000-36,000.

4.5 Astrovirus detection and typing by EIA EIA and typing EIA were previously described by Guerrero et al.[56] Typing EIA was performed on tissue cultured HAstV strains. Reagents for the EIA were provided by their collaborations in the Centers for Disease Control and Prevention, Atlanta, GA. Typing EIA was available for HAstV-1 to 5.

4.6 Astrovirus detection and typing by RT-PCR

4.6.1 Extraction of viral RNA RNA was extracted from 300 μL of stool suspension (10-50% in water) or tissue culture samples with an equal volume of trichloro-trifluoroethane (Genetron™, Fischer Scientific, Pittsburgh, PA). After centrifugation the supernatant was further treated with TRIzol™ (Life Technologies, Rockville, MD) and chloroform (Sigma, St. Louis, MO) and the supernatant precipitated with an equal volume of 100% isopropanol (Sigma, St. Louis, MO) to obtain viral RNA. Following removal of the isopropanol the RNA pellet was resuspended in 20 μL RNAse free water.

4.6.2 Primers Type-common RT- PCR utilized the published primer pair Mon348/Mon340, specific for the highly conserved serine protease region of ORF1a (nt 1182-1470, HAstV-1) and selected for detection of all HAstV antigenic types.[15,127] For genotyping a panel of type-specific primers was developed (see Results).

New primers were designed for further genomic characterization of novel isolates. A simple set of rules for primer sequence design is as follows:

1. Primers should be 17-28 nt in length;
2. Nucleotide composition should be 50-60% (G+C);
3. Primers should end (3') in a G or C, or CG or GC: this prevents "breathing" of ends and increases efficiency of priming;
4. Melting temperatures (T_m) between 55-80 °C are preferred;
5. Runs of three or more Cs or Gs at the 3'-ends of primers may promote mispriming at G or C-rich sequences (because of stability of annealing), and should be avoided;
6. 3'-ends of primers should not be complementary (i.e. base pair), as otherwise primer dimers will be synthesized preferentially to any other product;
7. Primer self-complementarity (ability to form secondary structures such as hairpins) should be avoided
8. High sequence identity to the template on the 3'end is more important than on the 5'end.

4.6.3 Type-common and type-specific RT-PCR The reverse transcription reaction mixture (50 μL) contained 3μL RNA and final concentrations: 0.1% bovine serum albumin (BSA), reaction buffer (10mM TRIS, 75 mM KCl, 3.5 mM $MgCl_2$, pH: 8.4), 0.2 mM of all four deoxynucleotide triphosphates, 10U avian myeloblastosis virus reverse transcriptase (AMV-Promega, Madison, WI), 4U RNAsine (Promega, Madison, WI), 0.05 μM dithiothreitol (DTT, Amersham Life Sciences, Cleveland, OH), and 0.15 μM (Mon348) negative strand primer (Sigma-Genosys Ltd., The Woodlands, TX).[15] Samples were incubated at 42°C for 1 hour.

For the PCR reaction we added to the 50μL RT reaction 50μL PCR reaction mixture containing 5% DMSO, reaction buffer (10mM TRIS, 75 mM KCl, 3.5 mM $MgCl_2$, pH: 8.4), Taq

polymerase and 0.15μM (Mon340) positive strand primer (Sigma-Genosys Ltd., The Woodlands, TX).[15] The amplification cycle included denaturation for 2 minutes at 94°C, 30 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute at 50 °C and extension for 1 min at 72 °C. Final extension was performed for 15 minutes at 72°C.[15] The RT and PCR reaction conditions were the same in the type-common and type-specific RT-PCR, except the annealing temperature being 50°C and 40°C, respectively. Amplicons were visualized by agarose gel (1%) electrophoresis in TBE buffer containing ethidium bromide.

4. 7 Typing by sequence analysis

4.7.1 Sequencing RT-PCR amplicons were sequenced to confirm the RT-PCR genotyping. Amplicons were cloned into pGEM-T Vector (Promega, Madison, WI) and sequenced by automated sequencing (AlfWin, Amersham-Pharmacia, Uppsala, Sweden) using fluorescent-labeled primers and SequiTherm Excel™ II Long Read DNA Sequencing Kit-Alf™ (Epicentre Technologies, Madison, WI) according to the manufacturer's recommendations. Newly acquired sequences were submitted to the GenBank.

4.7.2 Sequence verification and multiple alignment Previously published (GenBank) prototype sequences for each antigenic type available for the phylogenetic analysis included: HAstV-1 (Acc#: L23513), HAstV-2 (Acc#: L13745), HAstV-3 (Acc#: AF117209), HAstV-4 (Acc#: Z33883), HAstV-5 (Acc#: U15136), HAstV-6 (Acc#: Z46658), HAstV-8 (Acc#: Z66541) and porcine astrovirus capsid sequence (Acc#: Z66541). HAstV-7 reference strain capsid gene was sequenced in our laboratory for phylogenetic analysis (Acc#: AF248738).

Basic sequence manipulation and verification was performed using PC/Gene (v6.85; IntelliGenetics Inc., Geneva, Switzerland) or OMIGA (v2.0, Genetics Computer Group,

Madison, WI). PC/GENE is DOS-based and was implemented in the early phase of our studies. Later we upgraded to OMIGA that provides a more user-friendly computer environment. Sequences were imported into these programs and ambiguities were identified and resolved by aligning sequences obtained by multiple sequencing reactions. The final verification steps included the identification of a continuous open reading frame and characteristic astrovirus motifs after the translation of the nucleic acid sequences to amino acid sequences. ClustalX was used to create multiple alignments of the amino acid sequences of the selected isolates.[176] The nucleic acid sequences were added and aligned by GeneDoc v2.3 using the corresponding amino acid sequences as template.[143]

4.7.3 Sequence analysis by pairwise comparison Similarity of sequences by pairwise comparison of amino acid and nucleotide sequences provide preliminary data on relatedness and potential type-assignment. Pairwise identities of sequences were calculated form the multiple alignments using GeneDoc v2.3.[143] Pairwise comparison of sequences can be also visualized by dot plot method, when the sequences are on the x and y-axes and a sliding window of sequences are compared. Regions with pairwise identity above a set threshold will be represented by dots. Dot plot diagrams were created in OMIGA (v2.0, Genetics Computer Group, Madison, WI).

4.7.4 Sequence analysis by molecular phylogeny The task of molecular phylogenetics is to convert information in sequences into an evolutionary tree for those sequences. A great number of methods have been described for doing this, which raises the inevitable question of how to select the method that will recover the actual evolutionary relationship among the sequences to be analyzed.

The methods available can be categorized by how they handle the data (1) or the approach taken when building the trees (2).[149](Figure 5)

1. Kinds of data: distance methods versus discrete methods.
    a. The distance methods are based on the idea that if we knew the actual evolutionary distance between all the members of a set of sequences, than we could easily reconstruct the evolutionary history of those sequences. Distance methods first convert the aligned sequences into a pairwise distance matrix based on a selected model of sequence evolution, and then input that matrix into a tree building method. Models of sequence evolution include the Jukes-Cantor (JC), Kimura 2 parameter (K2P), Felsenstein (F81), Hasegawa-Kishino -Yano (HKY85) and general reversible model (REV). The tree building methods are, for example, the neighbor joining method and the unweighted pair group method with arithmetic means (UPGMA).
    b. In contrary, the discrete methods operate directly on the sequences, rather than on pairwise distances. Two major discrete methods are maximum parsimony and maximum likelihood. Maximum parsimony chooses the tree that requires the fewest evolutionary changes. Maximum likelihood chooses the tree that is most likely to have produced the observed data.
2. Approach for constructing a tree: cluster methods vs. search methods:
    a. Cluster methods follow an algorithm and arrive at a tree.
    b. Search method use the optimality criteria to choose among the set of all possible trees.

Size and composition of the dataset and the strength of the available computational background influence the selection of the method for the dataset. Rapid methods of tree assembly, such as neighbor

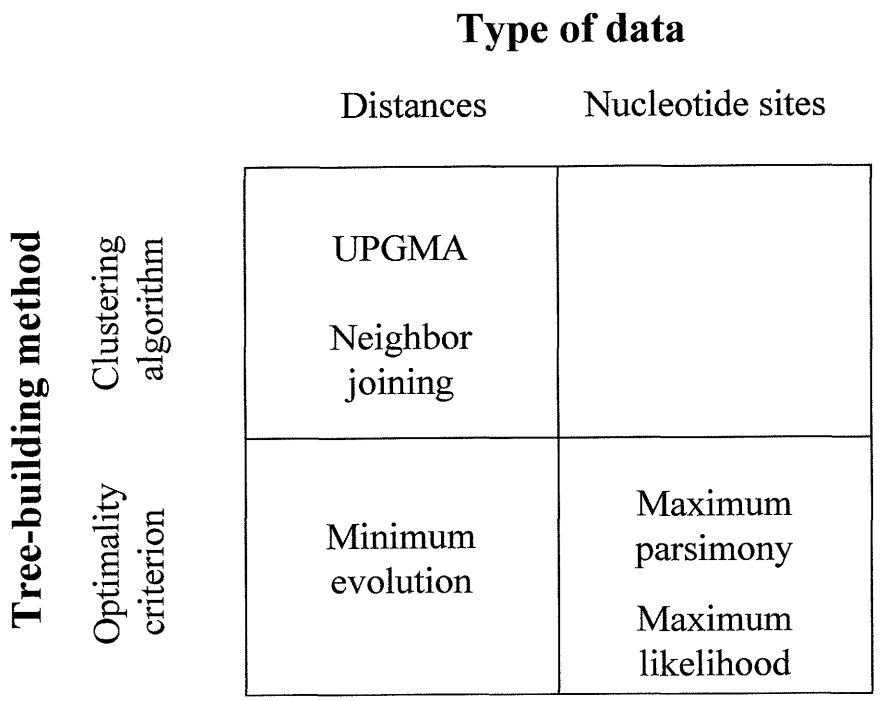


Figure 5 Common phylogenetic methods classified by the method used to build the tree, and by the type of data[149]

joining, come to fore in the analysis of large datasets. The advantage of distance methods is that they are fast and less computationally intensive. The major objections to distance methods are, that summarizing a set of sequences by a pairwise distance matrix they lose information; and branch length estimated by some distance methods may not be evolutionally interpretable.[149] On the other hand maximum likelihood is an appealing method as it can incorporate explicit models of sequence evolution, and also permits the statistical test of evolutionary hypotheses. This method is computationally very expensive; therefore it is used on relatively small datasets.

Not only the method used, but also the data itself are important for a good estimate of phylogeny among viruses. The tree will highly depend on the characters or regions that have been sampled. As a consequence, estimates of phylogeny based on samples will be accompanied by sampling error. The estimation for sampling error is called bootstrapping, when we generate pseudoreplicates from sequence data. These pseudoreplicates will resemble the original dataset in that it only contains sites found in that dataset, but it will differ in the frequencies of different sites. From this pseudoreplicate we build a tree using any of the methods above. We can repeat it a large number of times (100-1000 fold), resulting in a set of bootstrap trees. These bootstrap trees can be assembled into a bootstrap consensus tree, where each node labeled with its frequency of occurrence among the bootstrap trees. It is suggested that only nodes with bootstrap values above 95% should be accepted and well supported.

4.7.5 Molecular phylogeny in this study In this study phylogenetic trees were constructed from the nucleic acid sequence alignment using the PHYLIP package (v3.52c) within UNIX environment.[40]

Neighbor-joining trees were constructed for large datasets using the K2P evolutionary model (program NEIGHBOR in Phylip package) and 1000 fold bootstrapping.

When maximum likelihood algorithm (program DNAML in Phylip package) was applied, the global rearrangement option was invoked and the order of the sequence input was randomized ten to fifty times, depending on the region analyzed. To obtain the best phylogram the analysis was performed both rooted and unrooted using the animal in the rooted analysis as an outgroup. Other menu options were left as default.

4.8 Statistical analysis Statistical association between EIA versus RT-PCR and typing EIA versus RT-PCR was determined by χ^2-test using Epi Info v5.01 (CDC, Atlanta, GA). $P<0.05$ was considered to be significant.

4.9 Definition of subtype and clade Although the definition of strains and subtypes are not clearly defined in the literature we arbitrarily considered an isolate a subtype with <95% homology to the reference strain and >0.05 distance by phylogenetic analysis. Clade is a generic term for a branch on the phylogenetic tree, where closely related isolates cluster together.[146]

4.10 Detection of other pathogens Astrovirus-positive samples were previously tested for RV (67% positive), Shigella and *Giardia lamblia*.[55,131,185] In addition, they were tested for calicivirus by EIA and RT-PCR (19% positive).[39, 71]

4.11 Human subjects This study was approved by the Institutional Review Boards of Eastern Virginia Medical School, Norfolk, Virginia, USA and the Instituto Nacional de la Nutrición, Mexico City, Mexico. This study enrolled children younger than 18 months of age, because infants and young children are at greatest risk for morbidity and mortality due to diarrhea. Informed consent was obtained at enrollment.

4.12 Data management All data were recorded in electronic

databases using PC-based Microsoft Access software (Microsoft Corp.). The clinical, demographic, and laboratory data for the study are maintained in the same relational database.

Chapter 5
Results and Conclusions

5.1 Specific aim 1 To compare RT-PCR, EIA and sequencing in detection and typing of astroviruses in this study

5.1.1 Comparison of EIA vs. RT-PCR Of 698 episodes of diarrhea occurring in 214 children, 510 (73%) episodes were previously tested by EIA. Stool samples (N=396) from 366 (72%) of the EIA-tested episodes were available for further testing by RT-PCR. The availability of a limited number of samples might have resulted in a selection bias that we could not control or predict. HAstV was detected in stool samples from 24 (6.6%) of the 366 extended testing periods and within 17 (7%) of 232 diarrhea episodes. (Figure 6)

Of the 24 HAstV RT-PCR positive stool samples from extended testing periods, only 18 were positive by EIA. Two samples were indeterminate for the presence of HAstV by EIA and were confirmed positive after tissue culturing in CaCo-2 cells. Therefore, EIA was 76% sensitive and 100% and specific compared with RT-PCR for the detection of HAstV in stool samples during diarrhea episodes and extended testing periods. These results were the same if we compared detection from episodes (N=366) or from individual stool samples (N=396). The positive predictive value of EIA was 100% and the negative predictive value was 98.8% for EIA compared to RT-PCR as the gold standard. (Table 2)

In conclusion, the 7% incidence of HAstV diarrhea in this community was similar to that observed in other developing countries by EIA and was increased by RT-PCR testing (35%).[106,

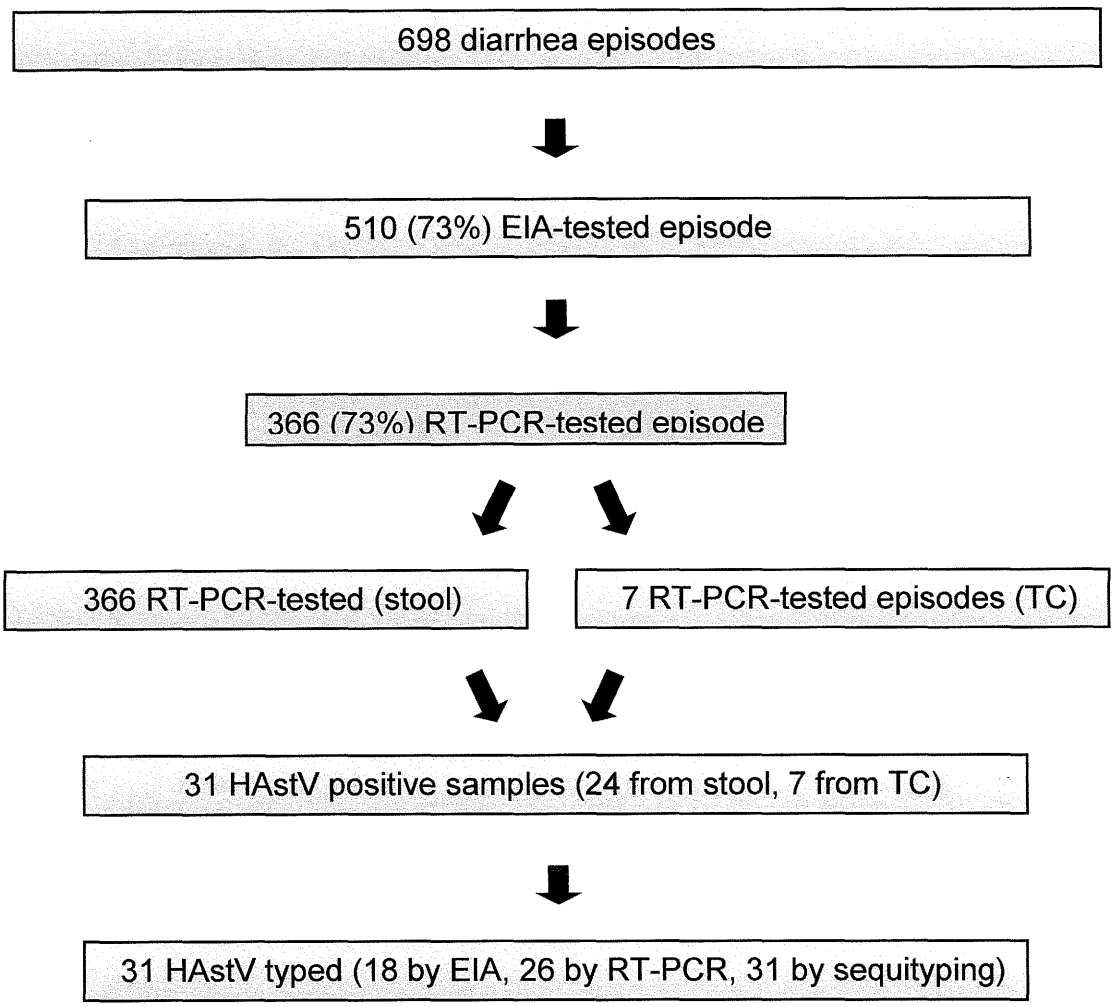


Figure 6 Flowchart of samples available for testing and result of the testing (TC: Tissue culture)

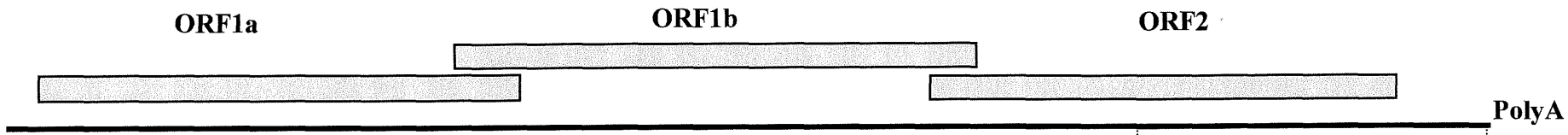
	RT-PCR+	RT-PCR-	Total
EIA+	18	0	18
EIA-	6	342	348
Total	24	342	366

Table 2 Sensitivity and specificity of EIA vs. RT-PCR for detection of HAstV in stool samples during the extended testing period.

[134,182] RT-PCR was more sensitive and as specific as EIA. This agrees with two reports of increased RT-PCR sensitivity when samples from outbreaks were evaluated.[30,122] Recently, we tested two collections of stool samples from children with diarrhea seeking medical care. A subset of samples (N=205) from children (2 weeks to 5 years of age) admitted to three US hospitals and a population-based sample set (N=466) from children in Egypt. In the US subset EIA was 42% sensitive and 100% specific relative to RT-PCR. In the Egypt population study EIA had a sensitivity and specificity relative to RT-PCR of 60% and 98.6% and the positive predictive value of EIA was 78% and negative predictive value was 97%.[124] We conclude that RT-PCR is the most sensitive assay for large community studies.

5.1.2 Panel of type-specific primers for RT-PCR We developed a panel of type-specific primer pairs for rapid type assignment by RT-PCR.(Figure 7) Mon2, a well-established type-common primer at the 3' UTR, served as a negative strand primer in all reactions. For the design of positive strand primers an alignment was created of the 3' end the ORF2 region with HAstV strains available from GenBank and our collection. Regions were identified with great intertype but low intratype differences. We tried to identify potential primer sites with different length from Mon2 for each type-specific positive strand primer. Once the regions were identified selection was made to meet the set of rules described in section 4.6.2. Primers were tested on RNA extracted from tissue culture samples. As Mon2 has a low T_m (54°C) the annealing temperature was set bellow the recommended range (40°C).

HAstV types were determined by use of the following primers pair: HAstV-1 was detected by Mon2/PR6151, HAstV-3 by Mon2/DM12, HAstV-5 by Mon2/AST-S5 and HAstV-7 by Mon2/DM11. HAstV-2 was determined by detection of the amplicons with correct size by both Mon2/PR6257 (541nt) and



Primers	Sequence (5' - 3')	T1	T2	T3	T4	T5	T6	T7	T8	Reference
Mon2(-)	gct tct gat taa atc aat ttt									[122]
6151(+)	atc tat tgt tga tgg ggc ta	666	-	-	-	-	-	-	-	[121]
6257(+)	aca ttg ccc aga att tc	-	541	-	511	-	-	-	-	[121]
DM12(+)	cta gtg agg aac ctg aca ccc atg	-	-	512	-	650	-	-	-	[188]
JWT4(+)	gca gag agc ttg tta tta ac	-	-	-	332	-	-	-	-	[188]
Ast-S5(+)	tag taa ctt atg ata gcc	-	-	-	-	471	-	-	-	[112]
DM11(+)	ggc aga tgt gtt gga act tcc c	-	-	-	-	-	-	430	-	[188]
PRBEG(+)	acc gtg taa ccc tcc tct c	319	321	323	-	324	296	321	324	[161]
DM4 (+)	cta cag ttc act caa atg aa	1241	1273	1260	1193	1237	1215	1252	1231	[188]

Figure 7 Description of the panel of type-specific primers and the size of RT-PCR products amplified from the HAstV genome on the left. Schematic localization of the primer binding sites is depicted on the right.

Mon2/PRBEG (321nt) primer pairs. HAstV-4 was determined by detection of the appropriate sized amplicons by both Mon2/PR6257 (511nt) and Mon2/JWT4 (332nt). Mon2/DM4 (1193-1273nt) primer pair was used for isolates that were untypable by the available type-specific primers to obtain an amplicon for sequence analysis. DM4 is type-common primer having a binding site that is conserved among human strains.

5.1.3 Comparison of typing EIA versus typing RT-PCR and sequence analysis Both typing EIA and RT-PCR were performed on 31 HAstV positive specimens (24 from stool samples and 7 from tissue culture). EIA detected HAstV in 28 of the specimens, whereas type-common RT-PCR for HAstV detected 31 samples (Table 3). Type-specific EIA (for HAstV types 1-5) yielded a type assignment for 18 (56%) of the 28 EIA-positive samples; and type-specific RT-PCR typed 26 (84%) of the 31 RT-PCR-positive samples. The additional five samples were typed by sequence analysis. RT-PCR confirmed the type assignment for the 18 samples typed by EIA. Type-specific RT-PCR yielded significantly more type assignment than was possible by typing EIA alone ($p<0.05$). The difference remained significant even when detection of HAstV-1 to 5 were compared.

In conclusion, RT-PCR typing proved to be consistent with typing EIA results and was confirmed by sequencing. This method is a great alternative for type assignment in laboratories where the resources of typing EIA are not available. The primers are inexpensive; the assay is rapid and sensitive.

5.2 Specific aim 2 To characterize genetic diversity of HAstVs detected in a prospective community–based surveillance study among children in Mexico City.

5.2.1. Genetic diversity of HAstVs based on sequences in the capsid region 31 HAstV isolates were sequenced at the 3' end of

	Number of samples with HAstV Type							
Typing Method	N	T1	T2	T3	T4	T5	T7	UT[a]
EIA	31	1	9	3	4	1	0	13
RT-PCR	31	3	10	3	6	2	2	5
Sequityping	31	3	13	4	7	2	2	0

Table 3 Comparison of HAstV type determination by typing EIA, typing RT-PCR and sequencing; NOTE. [a] UT, untypeable samples that gave either indeterminate or negative results

the genome. The length of the sequences obtained depended on the primer pair used for amplification. (345-1153nt) (Figure 7)

Pairwise comparison of nucleotide sequences of the study isolates (N=31) to reference strains confirmed the typing RT-PCR results and permitted type assignment for five additional samples. (Table 3) Isolates with 98-100% sequence identity were grouped together and a representative strain was identified for each group. The sequences of these isolates were submitted to GenBank (Accession# AF257221-9).

Multiple alignment of the amino acid sequences was created. The nucleic acid sequences were added and aligned using the corresponding amino acid sequences as template and resulted in a consensus length of 363nt of the 3'end of ORF2.

Phylogenetic analysis was performed by maximum likelihood method (randomized 50 times) in two stages. First all Mexican isolates were included in rooted and unrooted trees. Because pairwise analysis and the trees showed common branch points for a majority of Mexican strains within types, 22 strains with 98-100% homology were withheld from phylogenetic analysis to avoid repeats. Reference strains (HAstV-1 to 8) and single representatives of the nine distinctive Mexican strains were included for the final phylogenetic analysis. HAstV-4 was less related to the other reference HAstV strains than porcine astrovirus; therefore the final tree is unrooted.

The analysis (Figure 8) demonstrated that all reference strains appeared statistically distinct (distances 0.11-1.4 and $p<0.01$). HAstV-3 and 7 and HAstV-5 and 8 were very closely related in this hypervariable region. HAstV-4 was significantly different from the other HAstV strains, and less related to other HAstV strains than to two animal astroviruses analyzed (feline and porcine) (data not shown). All Mexican HAstV isolates could be assigned a type, but were significantly different from the corresponding reference

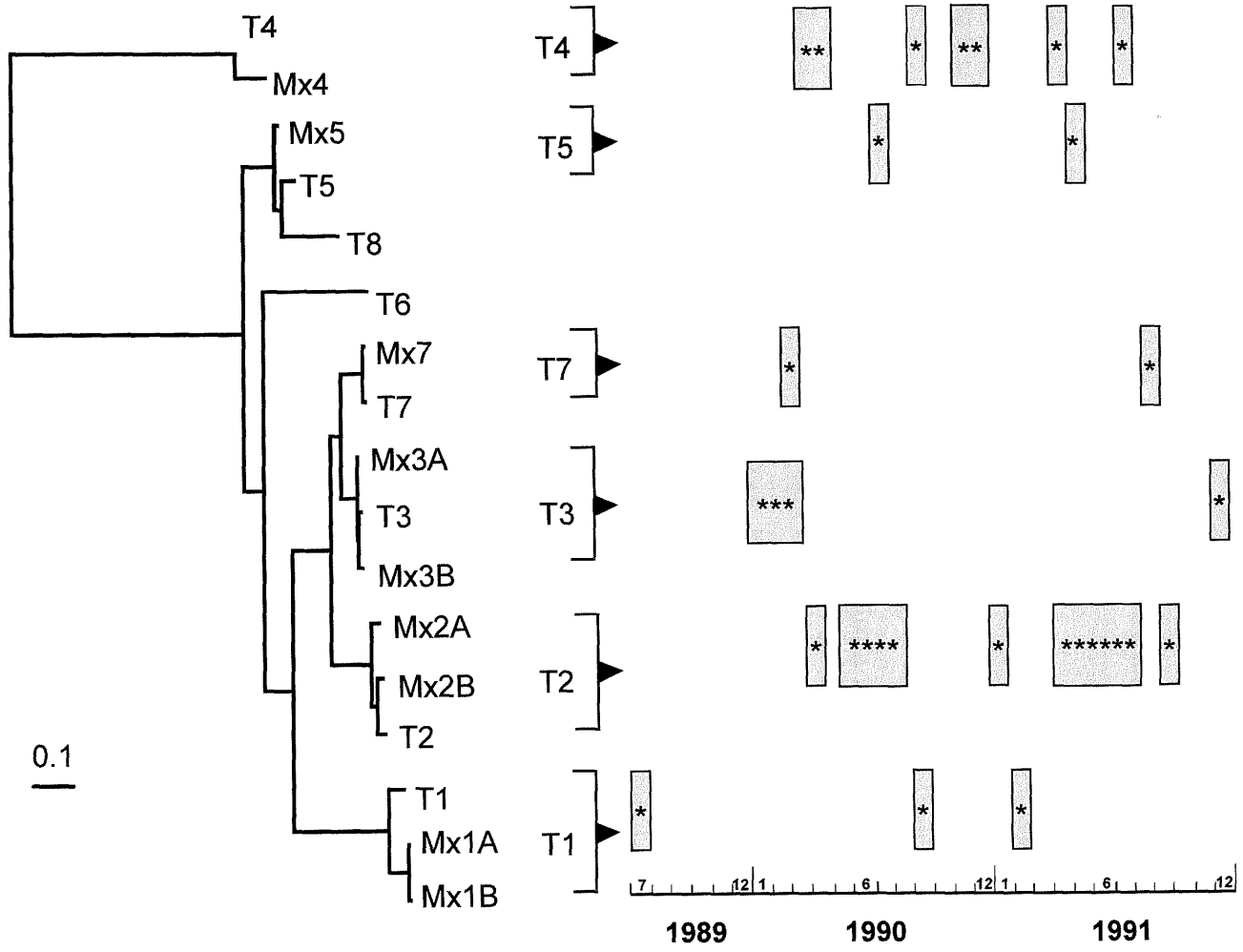


Figure 8 Maximum likelihood phylogram representing the phylogenetic relationship of Mexican HAstV isolates and HAstV references strains (T1-8) (on the left) with distribution of HAstV types in the three year surveillance period (on the right). Partial capsid gene sequences (363 nt) were used for phylogenetic analysis. Branch points of the resulting tree had a confidence level of $P<0.05$. This is an unrooted tree. Distances can be estimated using the scale bar. Timeline of the six detected HAstV types during 3 years of surveillance in Mexico City is represented on the right. The number of Mexican isolates detected are represented by asterics in gray boxes.

strains (p<0.05). Although the definition of strains is not clearly defined in the literature, we arbitrarily considered an isolate a new strain or subtype with <95% homology to the reference strain and >0.05 distance at the 3'end of ORF2 (363nt). Calculated intra-genotypic distances suggest that the Mexican isolates represent new subtypes of the corresponding genotype except in the instance of the HAstV-7 isolate (99% pairwise identity with HAstV-7, dist: 0.009), recovered from 2 children, which fell in the same clade as the prototype.

In summary, multiple types co-circulated in the community of Mexico City during a three-year period. There was no change in the predominant type over the study period. Sequence analysis revealed strains distinct from the prototype for five antigenic types circulating in the community. The predominant type was HAstV-2 (42%) followed by HAstV-4 (23%). This observation was in contrast to previous studies that detected HAstV-1 as a predominant strain including Europe[95], North America[31], South America[114], Asia[6] and Australia.[150]

5.2.2. Unique astrovirus isolate I

5.2.2.1 Detection of a unique HAstV strain We detected two HAstV-5 isolates in our Mexican cohort. Parallel to our study in Mexico, Joseph Briggs from our research group detected two HAstV-5 infections in children attending CCC in Houston, TX. Preliminary sequence analysis showed that the type assignment for these isolates were not consistent based upon sequence information from different genomic regions of the isolates. These isolates were 100% identical to the HAstV-5 isolates from Mexico in the genomic regions sequenced. These observations prompted further investigation of the two isolates from Mexico and two isolates from Houston, TX. These preliminary data supported the occurrence of recombination, which had not been previously reported for astroviruses.

5.2.2.2 Origin of the specimens Two astrovirus strains (H2002, H2067) were detected in diarrhea stool samples collected from two children during a HAstV outbreak in May 1990 at a child care center in Houston, Texas. The other two strains (M240, M370) were detected in this Mexican cohort in diarrheal stool samples in June 1990 and April 1991 from two different children. All four HAstV isolates were detected by type-common EIA.[56] These HAstV strains were detectable by RT-PCR by Mon2/PRBEG (3' terminus of ORF2), RT-PCR by Mon348/Mon340 in ORF1a, Mon344/Mon343 in ORF1b, and Mon2/DM4 in ORF2.[15] (Table 4)

5.2.2.3 Antigenic and genetic typing in the capsid region. Typing EIA identified M370 as HAstV-5 and M240 was untypeable.[56] EIA typing was not available for H2002 and H2067. The inability to EIA type M240 was attributed to a low titer of virus in the stool specimen, which was further supported by the weak amplicon signal resulting from RT-PCR amplification. The isolates from Houston were not typed by EIA. All four isolates were genotyped as HAstV-5 using type-specific primers, Mon2/AST-S5, targeting the 3' end of ORF2. The type assignments were confirmed by sequence analysis in the 3' terminal capsid region (sequence analysis data not shown).[188](Table 4)

5.2.2.4 Extending the information on genome Further data from other genomic regions were needed for these unique AstV isolates. Primer pair Mon2/DM4 was used to amplify a 1.2kb segment of the 3'end of the genome. Primer pair Mon270/Mon344 was used to amplify a 1.2kb segment of the transition region overlapping ORF1b and ORF2.[15,145] There were no available sequences for all reference types in these two genomic regions, therefore we had to design new primers, amplify and sequence several reference strains. Internal primers DM17 (5' GGT TTT GGT CCT GTG ACA CC 3'; location 4563-4544nt in HAstV-1 [L23513]) and DM15 (5' GAT AGG CTC TCC ACT ACA CC 3', location 3901-3920nt in

ID	Symptom	Date of Isolation	Location	Screening EIA[a]	Typing EIA	Screening RT-PCR	Typing RT-PCR
H2002	diarrhea	May-90	Houston, TX	+	n/a[b]	+	T5
H2067	diarrhea	May-90	Houston, TX	+	n/a[b]	+	T5
M240	diarrhea	Jun-90	Mexico City	+	untypeable	+	T5
M370	diarrhea	Apr-91	Mexico City	+	serotype 5	+	T5

Table 4 Epidemiological data for the four HAstV isolates.

NOTE [a] Type-common EIA, [b] Specimen not available for typing EIA, T5: HAstV-5 genotype

HAstV-2 [L13745]) were designed and used to amplify a ~600bp product from the ORF1b-ORF2 transition region of reference HAstV strains. Sequences obtained were submitted in GenBank: 1.2kb amplicon of the 3' end of the genome of H2067 (Accession# AF292080); 1.2kb amplicons from the junction region between ORF1b and ORF2 for HAstV-3 to 7 Oxford strains (Accession# AF292074-8); 1.2kb amplicon from the junction region between ORF1b and ORF2 for HAstV-8 South African strain (As20) (Accession# AF292073) used as a type 8 reference sequence and 1.2kb amplicon from the junction region between ORF1b and ORF2 for H2067 (unique isolate) (Accession# AF292072).

5.2.2.5 Sequence analysis by pairwise comparison The four isolates were 97-100% identical in the ORF1a region (246nt) and 98-100% identical at the 3'end of ORF2 (365nt). Amplicons (1255nt) were obtained for two of the four isolates (H2067 and M370) in the ORF1b/ORF2 transition region and were 99% identical to each other. Pairwise sequence comparison of 1128nt at the 3'end of ORF2 (Mon2/DM4) of H2067 confirmed that it was most similar to HAstV-5 (94%).

Sequence analysis of the transition region of ORF1b-ORF2 (1264nt consensus length) is shown in Figure 9. The overlapping clone was divided into two regions (5' end 576nt, 3' end 637nt) by a highly identical 52nt region. These two regions represented two different ORFs: ORF1b was represented in the 5' end (576nt) and ORF2 was represented in the 3'end (637nt) of this clone.

Pairwise comparison of nucleotide and amino acid sequences of all reference types with H2067 were calculated. In ORF1b (576nt), H2067 was closer to HAstV-3 (92%nt, 98%aa) than to HAstV-5 (88%nt, 96%aa). In the 5'end of ORF2 (637nt), H2067 is closer to HAstV-5 (96%nt, 99%aa) than to HAstV-3 (81%nt, 93%). No the other HAstV types had such high percentage of identity to H2067

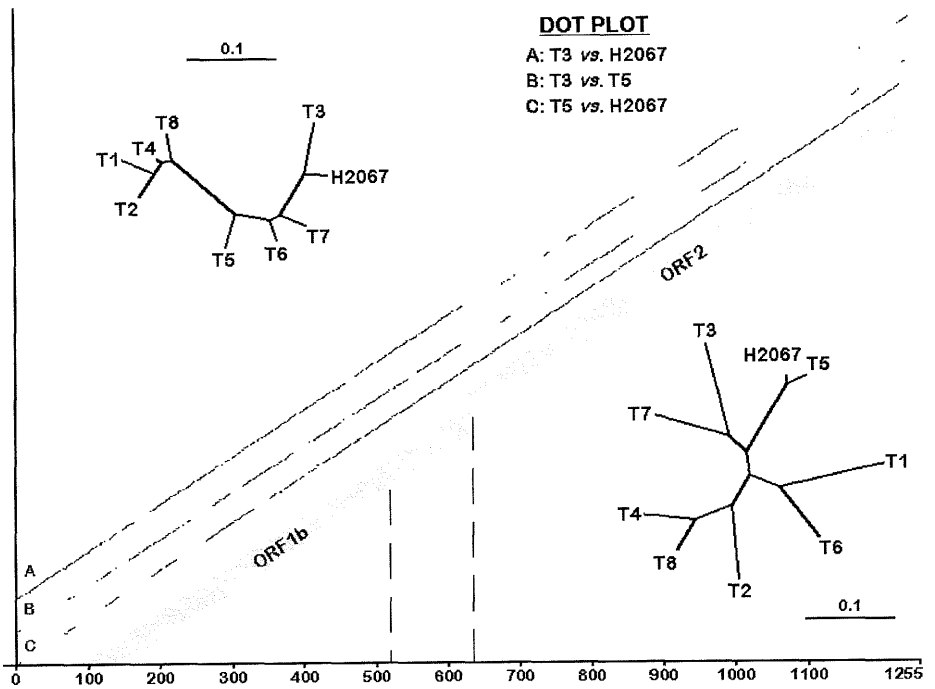


Figure 9 Comparative sequence analysis of the reference human astrovirus type 3 and 5 and H2067 [AF292080] in the transition region of ORF1b-ORF2 (1255nt, x axis). The transition region was divided into two regions (5' end 576nt, 3' end 637nt), excluding a 52nt highly identical region in the middle. Dotted lines indicate the hypothesized 120nt-long recombination site. Maximum likelihood phylograms of 576nt of ORF1b and 637nt of ORF2 5' end are in the left upper and right lower corners, respectively. Phylogenetic distances are given as number of substitutions per site. Identities between selected strains (T5, T3, and H2067) are presented on the diagonal created by dot plot analysis. A dark continuous line visualizes a high rate of identity.

in the ORF1b (84-90%nt, 94-97%aa) and ORF2 (80-83%nt, 89-92%aa) regions. (Table 5)

Pairwise identities between selected strains (T5, T3, and H2067) are presented by dot plot, in which a higher rate of identity is represented by continuous dots (Figure 9 diagonal). The dot plot results demonstrated greater identity between H2067 and HAstV-3 in ORF1b and greater identity between H2067 and HAstV-5 at the 5' end of ORF2. Comparison of HAstV-3 and 5 in this transition region provided the baseline for analysis.

5.2.2.6 Phylogenetic analyses in the ORF1b-ORF2 junction region A maximum likelihood phylogram for the 576nt region of ORF1b of eight HAstV antigenic types and one representative of these strains (H2067) is in the left upper corner of Figure 9. The phylogram shows that H2067 was closest to HAstV-3 and distinct from HAstV-5. The phylogram of the 637nt portion of ORF2 shows that H2067 was closest to HAstV-5 and distinct from HAstV-3 (Figure 9, lower right tree).

5.2.2.7 Evaluation of RNA sequence for putative hotspots for recombination The nucleic acid sequence of the junction region between ORF1b and ORF2 was further analyzed to characterize the possible recombination site: the length, extent of identity and AU-GC content among all human reference strains and one animal strain (porcine [AB037272]) were evaluated. Secondary RNA structure was computed using Zuker's RNA secondary structure predicting program (http://www.ibc.wustl.edu/~zuker/). This structure analysis tool was utilized to predict local secondary structure elements that may represent recombination hotspots.

A 120nt region at the junction of ORF1b and ORF2 was selected for further sequence analysis to characterize the putative recombination site. The consensus nucleotide sequence of the region is shown in Figure 10. All human strains are 93-98% identical in this region. This 120nt region incorporates a 52nt

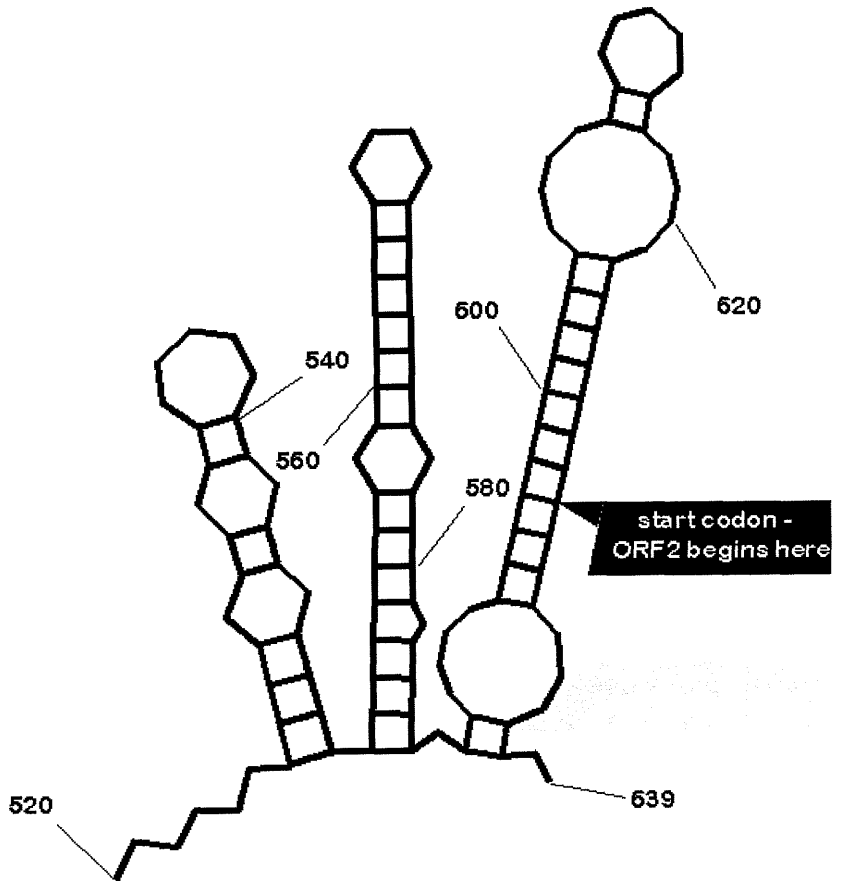
A

aa\nt	H2067	T1	T2	T3	T4	T5	T6	T7	T8
H2067		83	84	92	85	88	90	89	84
T1	94		93	82	94	85	84	84	91
T2	95	97		83	94	85	85	84	92
T3	98	93	94		82	87	86	88	82
T4	95	99	98	94		86	86	85	95
T5	96	94	94	95	94		90	89	85
T6	96	95	95	95	96	96		93	84
T7	97	95	94	96	95	96	98		83
T8	94	96	96	93	96	93	94	93	

B

aa\nt	H2067	T1	T2	T3	T4	T5	T6	T7	T8
H2067		81	80	81	80	96	81	83	81
T1	91		81	80	80	81	83	79	79
T2	89	89		81	84	79	83	82	86
T3	91	91	90		80	80	81	83	80
T4	89	88	93	89		80	83	80	90
T5	99	90	89	91	88		80	82	81
T6	91	91	92	91	93	91		84	83
T7	92	88	90	91	90	91	91		80
T8	90	88	95	89	95	89	93	90	

Table 5 Pairwise nucleotide (nt) and amino acid (aa) identities (%) of H2067 and all HAstV reference types in the junction region of ORF1b (576nt) ***(A)*** *and ORF2 (637nt)* ***(B)****. Comparison of T3 and T5 to H2067 are highlighted. T1-8: HAstV reference antigenic types*




```
520                    540
  GAaCACUGCCU.UCaCGGACUGC.AAGCAGCUUCGUGA.U
560                    580
  CUGG.CU.CC.GCCAG.CUCACAGAAGAGCAACUCCAUCG
600                    620
  CAUuUGGAGGGGAGGACCAAAGAAgUGuGAUGGC   cAA
```

Figure 10 RNA secondary structure of the hypothesized 120nt recombination site in H2067 (520nt-639nt). Nucleotides are numbered according to their position in the 1255nt RT-PCR product [AF292080], including the junction of ORF1b and ORF2. In the hairpin structure, the location for the start codon of ORF2 and the stop codon for ORF1b are indicated. At the bottom of the figure the consensus sequence of the corresponding regions of all reference human strains is described. Uppercase letters indicate 100% and lower case letters indicate ≥89% identity. Dots represent divergence in the nucleotide sequence of the eight reference types. The highly conserved 52nt region is underlined. In the consensus sequence, the start codon for ORF2 and the stop codon for ORF1b are highlighted.

region where sequence identity is 99-100% among human strains. Figure 10 demonstrates the predicted RNA secondary structure for H2067 in the 120nt junction region between ORF1b and ORF2. The predicted structure has a triple-hairpin conformation. The predicted RNA secondary structure was identical among the analyzed human strains. Although the porcine astrovirus has only 80-85% nt identity in this 120nt region, secondary structure analysis predicted the same triple-hairpin conformation (data not shown). The triple-hairpin structure includes the start codon for the capsid gene, as well as the stop codon for the *Pol* gene.

5.2.2.8 Conclusions RT-PCR enabled not only increased detection of HAstVs but also the identification of HAstV recombinants. This human astrovirus strain apparently derived from naturally occurring recombination, and was detected in two different geographic locations and defined by EIA, RT-PCR, and phylogenetic methods.

Based upon our analysis it is highly probable that the recombination occurred at the ORF1b-ORF2 junction region. Analyzing the RNA sequence at the suggested recombination site we observed the following factors that increase the possibility of recombination: high identity among all HAstV antigenic types (120nt 93-98% including a 52nt 99-100% region) and high identity with porcine AstV sequence (80-85%) in the 120 nt region. RNA secondary structure analysis predicted similar triple stable hairpin structures for all human and porcine strains. In summary, this proposed recombination site includes a long homologous region with stable hairpin structure, which supports the classification of our isolate as a homologous or similarity-assisted recombinant.

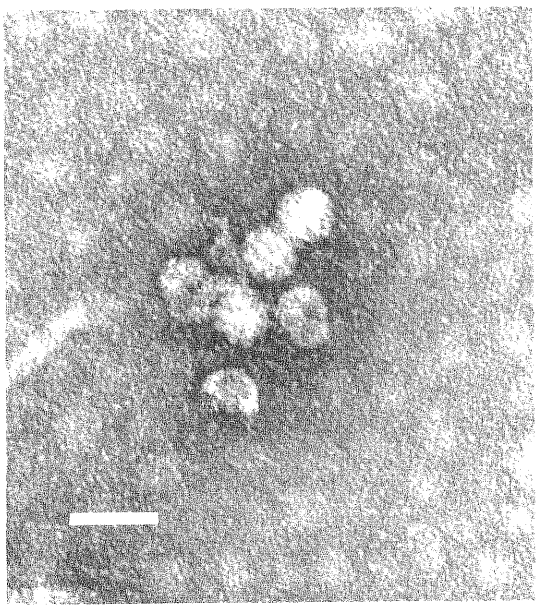
5.2.3. Unique astrovirus isolate II

5.2.3.1 Detection of a novel astrovirus: M3363 The virus (M3363) was discovered in the diarrhea stool of an 8-month-old Mexican infant by screening RT-PCR using primer pair Mon348/Mon340 from ORF1a designed for detection of all serotypes of HAstVs. The initial amplicon was larger (438nt) than that of reference human strains (289nt) and therefore was interpreted as negative. Out of mere curiosity and by serendipity, we sequenced the amplicon and found the virus to be related to astroviruses by using the Basic Local Alignment Search Tool (BLAST), a heuristic search program of National Center for Biotechnology Information (NCBI).[3] M3363 was undetectable by two different EIAs for HAstVs: including a commercially available kit (IDEIA™ Astrovirus, DAKO, Cambridgeshire, United Kingdom) and the EIA developed by the CDC.[56,145] M3363 was concentrated, partially purified from 10%(w/v) stool suspension (6mL) and examined by EM (Figure 11). EM showed typical astrovirus star-like morphology in 50% of the 30-35 nm diameter particles. The size of the virus is slightly bigger than the size of known HAstVs (29-30 nm). There were 30-40 virus particles in clumps at 72,000-fold magnification.

The IDEIA™ Astrovirus kit is an indirect double antibody assay with a monoclonal antibody used as capture antibody and a rabbit antiserum as detector antibody.[60] The antiserum is prepared against astrovirus type 2 but is group reactive by EIA.[59] There are several potential reasons why the kit could not detect the virus:

1. Insufficient amount of the virus present in the test samples. In our case the virus was detectable by EM, which has a higher threshold of viral titer required in viral detection than EIA. The titer of the virus in the original stool samples was calculated to be above 10^5 per gram of stool. The sensitivity of EIA is estimated to be 10^5 to 10^6 virus particles per gram of stool.[60,125]

A



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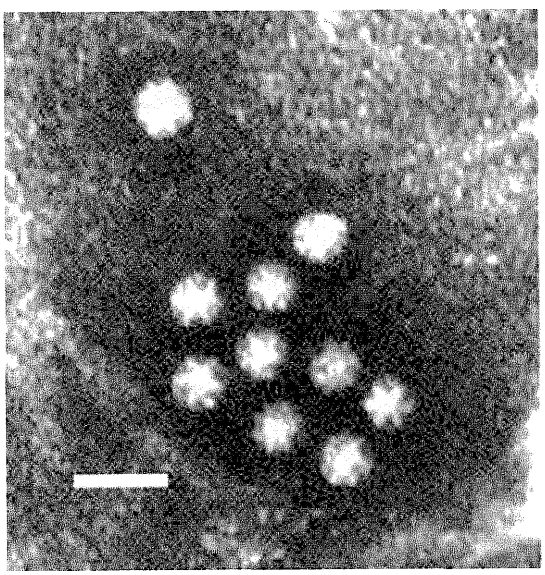

Figure 11 M3363 visualized by EM, stained with ammonium-mobildate. Viral particles appear in clumps with 30-35nm in diameter. 50% of the particles had characteristic surface projections forming star like morphology, typical of astroviruses.(A) HAstVs with starlike structure by EM by F.P. Williams, U.S. Environmental Protection Agency (www.epa.gov/nerlcwww/astro.htm) The bar represents 50 nm.(B)

2. Monoclonal antibody (8E7) did not capture the virus. This monoclonal antibody was raised against HAstV-2 and proved to be a type-common antibody targeting a highly conserved region.
3. Polyclonal anti-HAstV sera did not detect the virus. This polyclonal is proven to be type-common for all HAstVs.

Based on these we hypothesize that the virus does not have the epitopes that would react with either the capture or the detector antibody.

5.2.3.2 Epidemiological description of the host M3363 was isolated from a child residing in San Pedro Martir, Mexico City during a gastroenteritis episode with diarrhea in March 1991. The child was born July 1990 and breastfed until December 1991. The child had had frequent episodes of viral and bacterial diarrhea. In Figure 12 details of the diarrhea episodes are summarized, including the initiation date, length of the diarrhea episodes and the agents identified in the episode. The detection of M3363 was associated with a three-day long diarrhea period. In the same diarrhea episode *Enteropathogenic E. coli* was identified. This diarrhea episode was followed by a new episode associated with HAstV-2 infection. Other diarrhea episodes were associated with CV, RV and Shigella infections. Astrovirus was not detected in any of these other gastroenteritis episodes.

This child lived in a periurban area with frequent animal exposure; including in the home (dogs, cats, chicken).

5.2.3.3 Extending the information on the genome of M3363 Viral RNA was extracted from the stool specimen. Based on the original sequence that was obtained by the screening primer pair Mon348/Mon340 a new internal primer was designed: M3363F (5' CGT GTG CGT GGA GAT TGG C 3'). This primer with the combination of oligo-T primer was utilized to amplify a larger region in ORF1a. OligoT/M3363F successfully amplified a region

	1990	1991								
Initiation date	Sep-7	Jan-22	Mar-7	Mar-27	May-13	May-27	Jun-3	Jun-24	Jul-13	Aug-12
Length (days)	18	6	3	21	3	2	7	7	21	22
Agents identified	?	?	**M3363** EPEC	HAstV-2	CV	EPEC	EPEC	?	?	S

Figure 12 Diarrhea episodes of the host during the study period. Details include the initiation date of the diarrhea episode, the length of the diarrhea episodes and the agents identified in the episode. NOTE. ETEC: Enteropathogenic E. coli; HAstV: human astrovirus; CV: calicivirus; S: Shigella; In case of rotavirus IgG sera-conversion occurred between November 1990 and March 1991.

959nt in length. In ORF2 another primer, DM25 (5' AAA GGT GGA TGG TGG TTT GT 3'), was designed as a type-common primer for all AstVs. OligoT/DM25 successfully amplified a 1337nt region in the 3' end of the genome.

In total approximately 2300nt (34% of the genome) was amplified by RT-PCR. The amplicons from putative ORF1a (959nt)(SeqA_nt) and ORF2 (1337nt)(SeqB_nt) were cloned into pGEM-T vector and sequenced. Nucleotide sequences obtained were translated into amino acid sequences. Both translations resulted in a continuous open reading frame: translation of SeqA_nt resulted in a sequence of 319aa (SeqA_aa) and SeqB_nt resulted in a sequence of 425aa (SeqB_aa). In case of SeqB a non-translated region of 61nt was identified. Submission of these sequences to GenBank is in process.

5.2.3.4 Sequence analysis by PSI-BLAST Amino acid sequences (SeqA_aa and SeqB_aa) were submitted for comparison with known proteins to the NCBI to search the non-redundant GenBank of 1,013,360 sequences (July 22, 2002) using Position-Specific Iterated BLAST (PSI-BLAST).[4] This search program runs at approximately the same speed per iteration as gapped BLAST, but in many cases is much more sensitive to weak but biologically relevant sequence similarities. Simultaneously, a linked program, the NCBI Conserved Domain Search, identifies putative conserved domains searching the Conserved Domain Database of NCBI.

In the PSI-BLAST sequences with the highest identity to the query sequence are scored and an Expect value (E) is assigned. The E value is a parameter that describes the number of hits one can "expect" to see just by chance when searching a database of a particular size. It decreases exponentially with the Score (S) that is assigned to a match between two sequences. Essentially, the E value describes the random background noise that exists for matches between sequences. The E value is used as a convenient

way to create a significance threshold for reporting results. When the E value is increased from the default value of 10, a larger list with more low-scoring hits can be reported. In PSI-BLAST the E value is also used instead of the P value (probability) to report the significance of matches. For example, an E value of 1 assigned to a hit can be interpreted as meaning that in a database of the current size one might expect to see 1 match with a similar score simply by chance.

In SeqA_aa (319aa) a putative conserved domain of peptidase family C4 was identified (aa86-242). After the first iteration in PSI-BLAST, sequences with E values better than the threshold were HAstV-1, 3, and 8 ($E=4e^{-53}$ to $4e^{-52}$), ovine AstV ($E=4e^{-43}$), ANV ($E=2e^{-13}$) and turkey AstV ($E=9e^{-8}$). After the 2nd and 3nd iteration the ovine AstV sequence had the best E value (e^{-119} and e^{-104}) but was very close to the E-value of HAstV-1 (e^{-116} and e^{-100}). No other viruses were identified to match significantly to the sequence of M3363. Of note is that in this region sequence information only from HAstVs and OAstV available among mammalian AstVs.

In SeqB_aa (425aa) putative conserved domain of astrovirus capsid precursor protein were identified in the 5'end (aa1-97) and the 3'end (aa351-421) of the sequences. After the first iteration in PSI-BLAST, sequences with E-values better than the threshold were HAstV-1, 8, 5 and 3 ($E=1e^{-5}$ to 0.003). Sequential iterations identified more AstV sequences to match and longer regions were aligned with better E value above the threshold. After the 4th iteration HAstV-1 Acc#[NC_001943] and HAstV-3 Acc#[AF117209] capsid sequences had the best E-value (e^{-151}), E-value of other HAstV ranged from e^{-145} to e^{-121}. Of the animal strains feline AstV (e^{-123}) and porcine AstV (e^{-112}) sequences gave significant E values. No other viruses were identified to match significantly to the sequence of M3363.

In summary: based upon the conserved domain search, SeqA is derived from a protease-coding region of M3363 and SeqB is from a region coding the capsid gene. This is consistent with the regions for which the primers were designed. The PSI-BLAST search in an international, highly recognized sequence database indicated that M3363 is closest to astroviruses. The search suggests closer relatedness to human and mammalian than to avian AstVs.

5.2.3.5 Pairwise comparison to human and animal reference strains Multiple alignment of aa sequences with the homologous regions of human and animal AstVs was created in ClustalX. First multiple alignment of human astroviruses were aligned with OAstV utilizing the profile alignment option. Sequentially avian AstVs were aligned to each other. Eventually the two alignments were aligned utilizing the profile alignment option. Motifs in the separate alignments of mammalian and avian sequences were also identified in the new alignment. Furthermore, the alignment was compared to the pairwise alignments obtained during the PSI-BLAST search. Pairwise comparison of nucleotide and amino acid sequences of all reference types with SeqA and SeqB were calculated.

In ORF1a (SeqA, 959nt), M3363 was closest to HAstVs (43-44%nt, 37-38%aa) and OAstV (40%nt, 30%aa). (Table 6) For comparison in this region pairwise identity among HAstV1-3 and 8 ranged from 90-92%(nt) and 95-97%(aa). Since this alignment included only four HAstV reference strains, an internal region of 258nt/86aa were selected where sequences from all eight HAstV reference stains were available. Analysis including all eight HAstV reference strains demonstrated M3363 to be closest to HAstVs (36-38%nt, 31-32%aa) and OAstVs (42%nt, 27%aa). (Table 7) In this internal region nt pairwise identity among HAstVs ranged from 76 to 95% and aa pairwise identity ranged from 88-100%.

In the 3'end of ORF2 (SeqB, 1275nt), M3363 was the closest to HAstVs (29-32 %nt, 17-21%aa) followed by PAstV (28%nt,

aa/nt

	M3363	HAstV-1	HAstV-2	HAstV-3	HAstV-8	OAstV	TAstV-1	TAstV-2	ANV-1
M3363		43	44	43	44	40	32	29	30
HAstV-1	38		91	91	90	41	33	31	30
HAstV-2	37	96		92	91	40	34	31	30
HAstV-3	37	97	97		91	40	33	32	30
HAstV-8	37	96	95	97		40	33	31	31
OAstV	30	30	30	30	31		30	28	29
TAstV-1	15	18	18	17	18	15		47	41
TAstV-2	14	15	15	15	15	13	41		37
ANV-1	16	16	16	16	16	15	30	26	

Table 6 Pairwise nucleotide (nt) and amino acid (aa) identities (%) of M3363 and all human and animal AstV reference types in region of ORF1a (959nt). Values above the diagonal represent comparison of nucleotide sequences, and amino acid sequences are compared below the diagonal. NOTE. T1-8: HAstV reference antigenic types

aa/nt	HAstV-1	HAstV-2	HAstV-3	HAstV-4	HAstV-5	HAstV-6	HAstV-7	HAstV-8	M3363	OAstV	ANV	TAstV-1	TAstV-2
HAstV-1		92	90	92	91	76	77	90	38	42	30	35	31
HAstV-2	100		94	95	96	77	79	93	37	40	30	36	30
HAstV-3	100	100		92	94	77	79	91	36	39	31	35	32
HAstV-4	98	98	98		95	79	79	95	37	40	31	36	31
HAstV-5	100	100	100	98		78	79	93	37	40	32	36	31
HAstV-6	90	90	90	88	90		90	80	37	41	32	37	32
HAstV-7	92	92	92	91	92	97		81	38	44	32	35	32
HAstV-8	100	100	100	98	100	90	92		36	40	32	35	31
M3363	32	32	32	32	32	31	32	32		42	30	33	32
OAstV	36	36	36	36	36	37	36	37	27		36	33	33
ANV	16	16	16	16	16	16	16	16	19	18		44	42
TAstV-1	18	18	18	18	18	16	16	18	15	20	36		51
TAstV-2	13	13	13	13	13	13	13	13	15	18	32	45	

Table 7 Pairwise nucleotide (nt) and amino acid (aa) identities (%) of M3363 and all human and animal AstV reference types in region of ORF1a (258nt). Values above the diagonal represent comparison of nucleotide sequences, and amino acid sequences are compared below the diagonal.

18%aa) and FAstV (27%nt, 16%aa). None of the avian AstV types had such high percentage of identity to M3363 in the ORF2 region (19%nt, 23%aa). (Table 8)

5.2.3.6 Motifs in the ORF1a region of M3363 The sequences of ORF1a (serine protease) and ORF2 (capsid) amplicons aligned with the homologous regions of other AstVs. Eight ORF1a amino acid motifs characteristic of human and animal AstVs were identified, including the serine protease, proteolytic cleavage sites and the nuclear localization signal. (Figure 13) These sites are highly conserved among all human and animal AstVs and were located in this unique strain.

5.2.3.7 Phylogenetic analysis of sequences of M3363 The pairwise identities were very low, therefore saturation of the sequences in the less conserved region (outside the "motifs") was suspected. Therefore, the multiple alignment of nucleotide sequences including the motifs was submitted for phylogenetic analysis (consensus length of 501nt). Nucleotide distances were analyzed using the neighbor joining algorithm as implemented in PHYLIP (NEIGHBOR), based on the K2P sequence distance estimation method. Bootstrap replication was performed for the analysis (1000 replications). Phylogenetic trees from both regions had a tree-like structure, and the nodes had significant bootstrap values. Analysis in ORF1a placed M3363 in the mammalian AstV group, distinct from the HAstV cluster and OAstV (bootstrap values 100), however the distance of it from the two clusters was not estimated with confidence (bootstrap value at the node of separation was 74). Analysis in ORF2 placed M3363 in the mammalian AstV group, distinct from the HAstV cluster and in between FAstV and PAstV (bootstrap values of 84 and 93, respectively), however the distance of it from the two clusters was not estimated with confidence (bootstrap value at the node of separation was 75). In summary, phylogenetic analyses in both

aa/nt	M3363	HAstV-1	HAstV-2	HAstV-3	HAstV-4	HAstV-5	HAstV-6	HAstV-7	HAstV-8	FAstV	PAstV	OAstV	TAstV-1	TAstV-2	ANV
M3363		29	29	30	31	30	30	31	32	27	28	22	23	19	19
HAstV-1	17		57	63	50	57	59	61	61	45	39	29	25	23	23
HAstV-2	16	53		61	48	55	55	60	55	45	40	29	26	23	22
HAstV-3	18	62	58		49	59	59	73	60	47	41	29	27	23	22
HAstV-4	15	42	41	43		51	49	50	54	40	35	26	26	23	23
HAstV-5	16	51	48	53	43		62	60	64	47	40	27	27	24	23
HAstV-6	16	53	48	55	42	59		58	59	43	38	28	25	24	23
HAstV-7	17	60	55	77	40	55	55		60	46	43	28	27	24	22
HAstV-8	20	55	50	56	47	59	55	54		45	40	27	26	24	23
FAstV	13	36	36	37	30	37	34	35	36		43	27	26	21	22
PAstV	13	26	28	28	19	26	24	27	25	33		31	27	23	22
OAstV	6	12	10	11	10	11	11	12	12	13	14		25	21	21
TAstV-1	8	9	10	10	9	10	9	10	10	10	11	10		35	29
TAstV-2	8	8	7	9	8	8	8	10	9	8	8	8	24		46
ANV	5	9	8	10	10	10	9	9	10	8	6	7	16	42	

Table 8 Pairwise nucleotide (nt) and amino acid (aa) identities (%) of M3363 and all human and animal AstV reference types in a partial region of ORF2 (consensus length of 1575nt). Values above the diagonal represent comparison of nucleotide sequences, and amino acid sequences are compared below the diagonal.

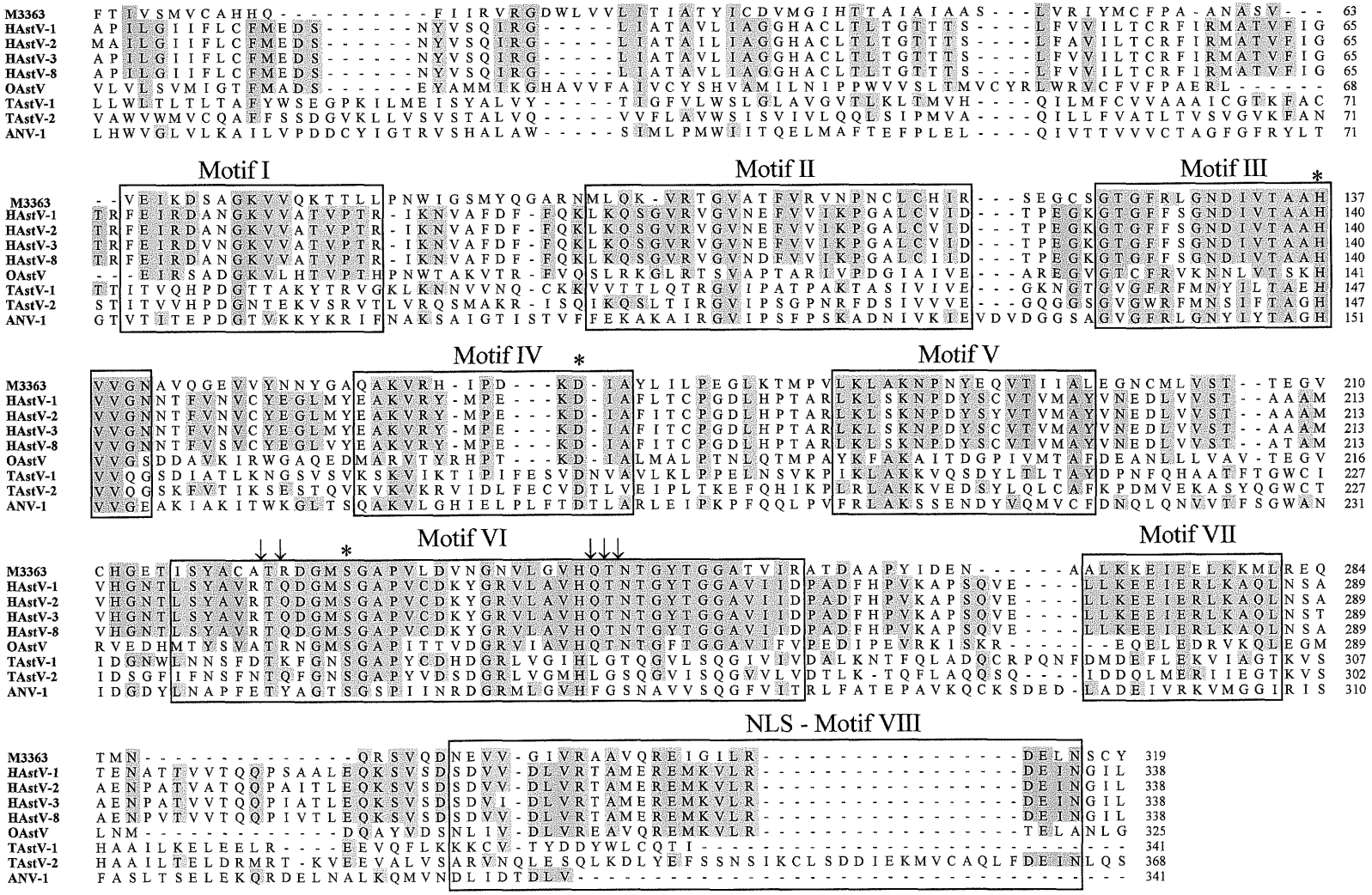

Figure 13 Multiple alignment of aa region in ORF1a, including M3363 and available AstV strains: HAstV-1 [Acc#L23513], HAstV-2 [Acc#NC_002470], HAstV-3 [Acc#AF141381], HAstV-8 [Acc#NC_002499], OAstV [Acc#NC_002469], TAstV-1 and 2 [Acc#AF206663; NC_002470], and ANV-1[Acc#AB033998]. Eight motif regions are highlighted and conserved sites including the catalytic triad of the serine protease: H,D,S (*); putative protease dependent cleavage sites (↓) and NLS, nuclear localization signal .

regions (ORF1a and ORF2) placed M3363 in the mammalian AstV group, distinct from the HAstV, but its time of separation during evolution is not well-defined. (Figures 14 and 15)

5.2.3.8 Cultivation of the virus in tissue culture Attempts were made to cultivate M3363 in LLCMK2 cells,[59] CaCo2 cells[93] and Vero cells. In all cases conditions described for HAstVs did not permit growth of the virus.

5.2.3.9 Conclusions In conclusion, we isolated a virus from a symptomatic child with typical star like AstV morphology by EM. The virus had distinct antigenic features including that it did not react with monoclonal antibody against human AstVs in EIA. It also had distinct genetic features including the presence of common astrovirus motifs and the catalytic triad of serine proteases in ORF1a, but showed low sequence identity to human and known animal AstVs. Pairwise comparison and phylogenetic analysis of the sequences suggest that M3363 is an astrovirus and is more closely related to mammalian than avian astroviruses. M3363 is an outlier to the HAstV group; therefore the possibility of non-human origin is raised.

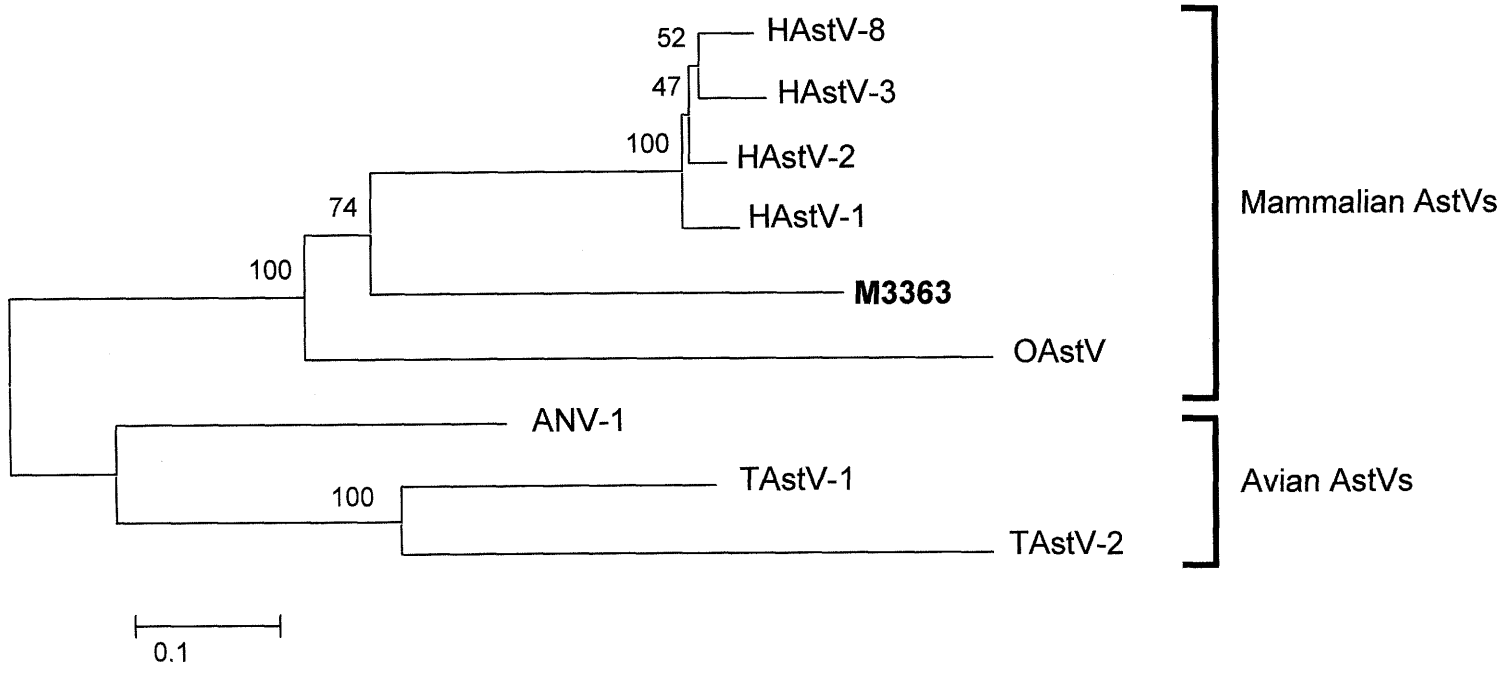

Figure 14 Neighbor joining phylogram (K2P model, bootstrapped 1000 times) of 495 nt region in ORF1a, including M3363 and available AstV strains: HAstV serotype 1,2,3 and 8: HAstV-1 [Acc#L23513], HAstV-2 [Acc#NC_002470], HAstV-3 [Acc#AF141381], HAstV-8 [Acc#NC_002499] and animal AstVs: ovine AstV (OAstV) [Acc#NC_002469], turkey AstV: TAstV-1 and 2 [Acc#AF206663; NC_002470], and avian nephritis virus serotype 1 (ANV-1) [Acc#AB033998]. Numbers at branch points indicate number of replicate trees with that branching.

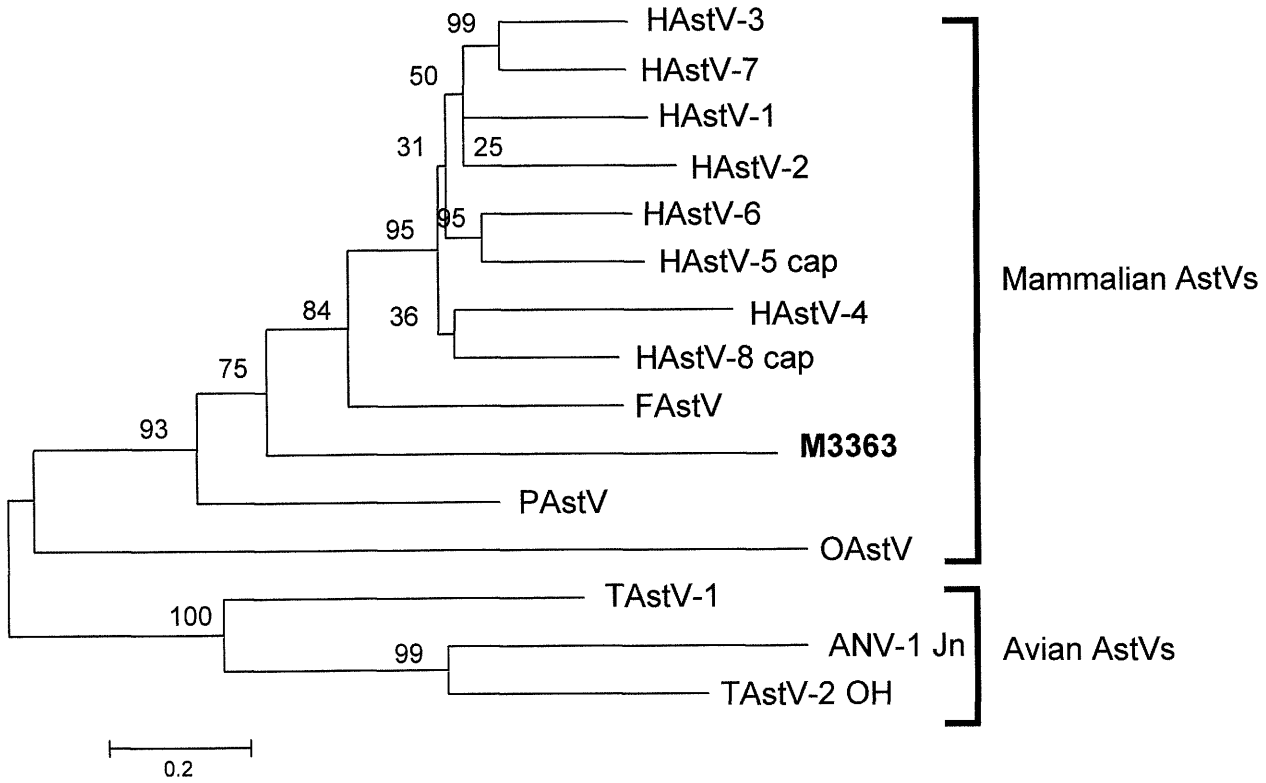

Figure 15 Neighbor joining phylogram (K2P model, bootstrapped 1000 times) of 843 nt region in ORF2, including M3363, HAstV serotypes 1 to 8: HAstV-1 [Acc#L23513], HAstV-2 [Acc#NC_002470], HAstV-3 [Acc#AF141381], HAstV-4 [Acc#Z33883], HAstV-5 [Acc#U15136], HAstV-6 [Acc#Z46658], HAstV-7 [Acc#AF248738], HAstV-8 [Acc#NC_002499] and animal AstVs: feline AstV (FAstV) [Acc#AF056197], porcine AstV (PAstV) [Acc#Y15938], ovine AstV (OAstV) [Acc#NC_002469], turkey AstVs: TAstV-1 and 2 [Acc#AF206663; NC_002470], and avian nephritis virus serotype 1 (ANV-1) [Acc#AB033998]. This region is derived from the original alignment by deleting gapped regions. Numbers at branch points indicate number of replicate trees with that branching.

Chapter 6
Discussion

6.1. Comparison of RT-PCR, EIA and sequencing in detection and typing of astroviruses.

6.1.1 Large community studies: RT-PCR vs. EIA? The 7% incidence of HAstV diarrhea in this community was similar to that observed in other developing countries by EIA[106,134,182]. This is one of the first studies, where RT-PCR was applied to testing large sample sets. In this study, all previously EIA positive samples were detected by RT-PCR and RT-PCR increased the number of positives by 35%. This finding is similar to the findings of Mitchell's current two studies: RT-PCR detected HAstV in stool samples more frequently than EIA.[124] As a result, EIA underestimated the prevalence of HAstV in large population studies. We propose that RT-PCR detection with confirmation should be the standard for future epidemiological studies.

The reason why EIA is less sensitive might be due to the titer of the virus detected or the evolution of the viral epitopes, so the detector monoclonal antibody or the capture polyclonal sera will not react with them. We conducted three different studies, where type-common EIA and RT-PCR were performed and the isolates were typed. Based on these studies we see a trend that EIA tends to detect more HAstV-1 and HAstV-2 than the other types. One explanation of these differences might be that the detector antibody in the EIA was originally prepared against HAstV-2.[59] Even if this sera was claimed to be group reactive by EIA, we speculate that there is a bias towards the detection of HAstV-1 and 2 in this assay.[59]

6.1.2 Typing RT-PCR: a new alternative tool. A typing EIA that uses serotype-specific reference antisera for capture and group-reactive monoclonal antibody as a detector has been developed in

the CDC to assess the antigenic types of astroviruses found in clinical samples.[145] Even if typing can be accomplished by typing EIA, the reagents might not be available for all laboratories. Alternatively, molecular methods for genotyping clinical isolates by RT-PCR with primers from the more conserved N and C terminal regions of ORF2 may be used.[112,145] Beside our study only a few laboratories advanced in developing and testing a full panel of typing primers. Matsui et al published and proved in field samples the use of their panel in 1998.[112] We even adopted a few of their primers into our panel (T5, T6). None of the original sets included typing primers specific to HAstV-8, as this types was thought to be rare. Recently, however, several epidemiological studies proved that HAstV-8 is a more frequent type.[115, 132, 134] Ferenc Jakab, a member of our study group, designed a T8 specific-primer to make the panel complete.(unpublished)

In this study we successfully tested our typing panel. High concordance between genotyping and serotyping was observed and typing RT-PCR permitted the typing of additional virus isolates undetected or untyped by EIA.

Typing EIA could not type 13 isolates. Four of these isolates were not detected by EIA. Nine isolates were not reacting with the typing EIA: two HAstV-1, three HAstV-2, one HAstV-4, one HAstV-5 and two HAstV-7. Since the typing EIA was designed for the typing of HAstV-1 to 5, HAstV-7 could not be detected. The reason for the failure of the typing EIA in the other seven cases might be due to low titer of the virus or changes in the epitopes recognized by the typing antibodies. For example, the untyped HAstV-5 isolate had a low viral titer, as even RT-PCR was amplifying a product with very low yield.

There were five isolates that were not detected by type-specific primers. Sequence information from the isolates untypable by RT-PCR enabled us to analyze the variability of the typing primer

binding sites. Two HAstV-2 isolates, non-detectable by RT-PCR typing were identical to two RT-PCR typable isolates and had 80% identity with primer PR6257 within the sequence of the primer. The sequence of the possible primer binding site for each untypable HAstV-3 and HAstV-4 isolates was 100% identical to the original primer sequence (DM12 and JWT4, respectively). These findings suggest that the failure of RT-PCR could have been because of mismatch (type 2), inhibitors, or low RNA yield (types 3 and 4).

In summary, typing RT-PCR it is a great alternative tool to typing EIA, since it is inexpensive, rapid and sensitive. The amplicons obtained by the RT-PCR can be sequenced and therefore the typing results can be confirmed. Besides, this sequence data can support further characterization of the viruses by phylogenetic analyses.

6.2 Genetic diversity of HAstVs detected in a prospective community–based surveillance study among children in Mexico City.

6.2.1 Multiple astroviruses co-circulate in the community. A new phenomenon? Astrovirus serotype 1 was the most common type found by classical serotyping method.[43,87,144,150] The community acquired astroviruses were monitored in the 1970s and 1980s in different location and HAstV-1 was found to be 65-72% of the astroviruses encountered.[88,95,195] By contrast, our longitudinal study of diarrhea among Mexican children found that AstV serotype 2 was the most common (42%) and serotypes 1, 5 and 7 were the least common (6-10%).

Of interest is that a biennial peak of serotype 1 has been observed in surveillances in the UK, South Africa, Australia and Spain.[57, 95, 132, 170] In the Australian surveillance the biennial winter peak correlated with a greater incidence of serotype 1 virus and an increased rate of hospitalization of children aged 6 to 12 months.[132] Giux from Barcelona also noticed an biennial season of HAstV–1

infections. However, this season was alternating with a season of non-HAstV-1 infections characterized by the presence of multiple types: HAstV-2, 3, 4 and 8. We had similar observation in our recent study among children hospitalized in the US, where multiple HAstV types (predominantly HAstV-2 and HAstV-4) were present in the community from late January to June 1998. The type predominance shifted to HAstV-1 in the following diarrhea season.[187]

Of interest that studies in developing countries seems to identify AstVs with higher type diversity than studies in the developed world.[134]

Studies utilizing RT-PCR detection and typing for AstVs also describe a great diversity of AstVs [161,132]. In our study we noticed the presence of multiple types. It is intriguing that the presence of a predominant type over time was not type during the study period, but all of the types were simultaneously present, co-circulated, in the community.

In summary, distribution of infecting AstV types might be greatly affected by the time period examined (alternating season), by geographical location (developing vs. developed world) or the method used. This study contributes to the better understanding of the epidemiology of HAstVs and may emphasize the epidemiological importance of HAstVs other than type 1.

6.2.2 How could recombination occur in AstV? Recombination is a common event among RNA viruses and is suspected when co-infections with different types occur in one child. Natural, homologous recombinants have been demonstrated among other +ssRNA viruses, including *Picornaviridae*[42] and *Caliciviridae.*[70] In addition, RNA viruses have a tendency toward recombination due to the nature of their *Pol* which naturally shift frame at the ORF1a/ORF1b junction.[7, 69, 138, 139]

The preferred mechanism of recombination among +ssRNA viruses is a copy-choice mechanism where recombination occurs while the *Pol* enzyme switches from copying one RNA molecule (donor template) to another (acceptor template) without releasing the nascent strand.[75] RNA virus recombination events are classified as homologous, aberrant homologous, and non-homologous types depending on the location of the recombination site within the viral genome, and the precision of the event.[92] However, Nagy and Simon suggested a revision of the classification to sequence similarity-essential, similarity-assisted, and similarity-nonessential, based upon the mechanism of the generation and the nature of the recombinant product.[138]

Studies with brome mosaic virus (BMV), a +ssRNA plant virus which is the most frequently used model for homologous RNA virus recombination, revealed several factors that are important in RNA recombination.[135] Those factors include the length of the identical region of the sequence, degree of sequence identity, the presence of AU-rich segments in the 3' vs. relative GC-rich segments in the 5' end, and stable hairpin structures.[137]

Koev et al. showed that the RNA secondary structure at the recombination site plays a unique role in the promoter recognition by the viral *Pol* in vivo in a recombination model of barley yellow dwarf virus.[82]. In the case of *Luteoviridae*, Koev et al. proposed that recombination has occurred during luteovirus evolution, based on sequence analyses of genetic regions of the two major genera, *Luteovirus and Polerovirus*.[82] In *Caliciviridae* and *Luteoviridae* the recombination events were localized at the *Pol*/capsid junction, which is a transition between homologous (non-structural) and divergent (structural) regions. Both of these viruses have subgenomic RNA encoding the capsid protein. In *Luteoviridae* it is proposed that recombination has occurred by *Pol* switching strand

at the subgenomic promoter.[82] Neither natural nor artificially induced recombination has been detected among AstVs.

During the copy-choice mechanism of recombination the short AU rich sequence and stable hairpin structures of the donor template halt the synthesis on the nascent strand, thus providing an opportunity for the *Pol* to interact with the acceptor strand. These AU rich sequences also promote *Pol* slippage.[136] The stable hairpin structures act as replication enhancer in recruiting the *Pol*. Long (or even short, depending on the virus) homologous sequences between the nascent RNA and the acceptor RNA facilitate the association of the nascent strand with the acceptor strand and function as a primer for the template switching and extension.[135]

RNA sequence at the suggested recombination site we observed the following: high identity among all HAstV antigenic types (120nt 93-98% including a 52nt 99-100% region) and high identity with porcine AstV sequence (80-85%) in the 120 nt region. We did not locate definite AU rich segments in the 3' vs. GC rich (or moderately AU-rich) segments in the 5' end of this region. RNA secondary structure analysis predicted similar triple stable hairpin structures for all human and porcine strains. In summary, this proposed recombination site includes a long homologous region with stable hairpin structure, which supports the classification of our isolate as a homologous or similarity-assisted recombinant.

HAstV co-infections with different antigenic types have been reported in epidemiological studies.[112] Co-infection by two different serotypes in one child provides an opportunity for recombination to occur.

The recombination site in our case was in the junction of *Pol* and capsid region similar to other viruses (*Caliciviridae* and *Luteoviridae*).[70] Astroviruses transcribe a subgenomic RNA whose start point maps within the region of the suggested recombination junction, therefore we propose that polymerase recognition of the

subgenomic RNA promoter may be the reason for the recombination event similar to *Luteoviridae*.

We conclude, that phylogenetic analysis of different genomic regions of HAstV can provide contradictory genotyping unlike a previous observation.[15] In our case, the HAstV3-5 recombinant was widespread, apparently stable and pathogenic. Therefore molecular typing methods for AstVs should utilize sequence in the capsid region (ORF2) to correlate with antigenic typing methods.

RNA recombination may contribute to evolutionarily significant RNA rearrangements in astroviruses. Nucleic acid sequence homology and similar RNA secondary structure of HAstV and animal AstVs (porcine) in this transition region of ORF1b and ORF2 may provide a possibility of recombination among AstVs from different species should co-infections occur.

6.2.3 Novel astrovirus from a symptomatic child: infection with a new human type or zoonosis? The infectious agent (1), the host (2) and the environment (3) that allows the host to be exposed to the infectious agent influences the characteristics of an illness. Close examination of all three components is required to finally address the question: did infection occur and if so, was it the cause of the symptoms?

1. The infectious agent, M3363 resembles astroviruses more than any other viruses by electron microscopic features, and genetic characteristics, including the presence of a conserved serine-protease domain, cleavage sites, nuclear localization signal in ORF1a and motifs in the capsid region.

M3363 may be of non-human origin, because the viral particles are slightly bigger that HAstVs, it was undetectable by a type-common EIA, pairwise identities are very low to known HAstVs sequences and the virus was not cultivable by methods described for HAstVs. Finally, phylogenetic analysis showed separation of M3353 from the human clade, but still clustered with the

mammalian group in both regions analyzed. We speculate that M3363 of mammalian but non-human origin.

Based on the amount of viral particles detected in EM, the amount of viral particles is calculated by extrapolation to be at least 10^5/g stool. It is highly improbable that the inoculum contained such vast amount of viruses, so the virus probably replicated in the child thus infection occurred.

2. The infection occurred in a child that was 8 month-old and had a history of multiple diarrhea episodes by bacterial and viral agents, including HAstV-2. The episode with HAstV-2 infection was 21-day-long. This is longer than the average length of HAstV infections (3-4 days) and is considered to be an episode of chronic gastroenteritis. Based on the literature, chronic diarrhea with astroviruses may be due to malnutrition or chronic underlying conditions including immunodeficiency.[28, 88, 179] Since the HAstV-2 infection occurred within a month of infection of M3363, we assume that infection with M3363 did not have much protective role against repeated HAstV infection.

3. The environment: This child with diarrhea lives in a periurban area with frequent animal exposure including dogs, cats and poultry. According to the hygiene history the home of the child had water kept in open container, not exposed to the outside, but no inside faucet in the home. The bathroom was observed as "dirty" and no inside receptacle of garbage was available. Hens and turkeys were in the house and outside. The child was fed both breast milk and water. These circumstances prone for exposure to viruses from both human and animal hosts a young infant that starts to explore the environment and get feeding other than breast milk.

During the episode when M3363 was collected the child had diarrhea but no vomiting and fever. Did M3363 cause diarrhea? Since EPEC was also present in the diarrhea episode it is not clear if diarrhea was attributable to EPEC or M3363. Of interest is that

the child had three sequential episodes of EPEC infections. This is indicative of continuous fecal-oral contamination and poor hygiene. A recent paper has indicated that prior EPEC infection can reduce disease severity upon homologous challenge but not heterologous.[34] The main clinical features of EPEC infected children hospitalized were age below 12 months; low birth weight; precocious weaning and severe protein-calorie malnutrition.[148] Probably EPEC infection alone could cause the symptom and repeated EPEC infection may also indicate an underlying chronic condition. M3363 might have a synergic effect in producing the symptoms of diarrhea. M3363 was not detected any other stool samples collected from this child before and after this diarrhea episode.

The question is not fully answered, whether M3363 is a new human type or mammalian AstV. We speculate that M3363 is of non-human but mammalian origin. This would raise the possibility of interspecies transmission of M3363. Interspecies transmission among AstVs has been examined by sequence analysis of two study group. Jonassen suggest interspecies transmissions involving humans, cats and pigs relatively recently in the evolutionary history of astroviruses based upon high similarities between the HAstV, FAstV and PAstV capsid amino acid sequences.[72] In contrary, Lukashov detected strong selection against non-synonymous substitutions (or amino acid changes) in AstV evolution. Therefore he concludes, that amino acid similarity does not necessarily reflect a recent separation between lineages. Since the synonymous substitutions among mammalian AstVs were saturated, the hypothesis that cross-species transmissions are recent evolutionary event was not supported. Lukashov concluded that a single cross-species transmission introduced astroviruses into the human population, finding no evidence for ongoing animal-to-human transmission.[103] Even if these two reports have conflicting conclusions about the time of interspecies transmission, both agrees

on the fact that interspecies transmission had to occur. These analyses support our observation: the presence of a putative non-human AstV in a human host.

If this interspecies transmission causes disease in the human host, it is considered a zoonosis. Zoonoses can be acquired via insect vectors, food, and direct or indirect contact with animals. More than 250 zoonoses have been described, and they are caused by a wide variety of pathogens, including viruses, rickettsia, bacteria, fungi, and parasites. Humans have very close interactions with working, food-producing, and companion animals. In the developing world close contact with household animals is not uncommon. In the past few years, we have become aware that several enteric pathogens that were thought to be mostly restricted to animals are a major cause of human disease. [37] Examples of such pathogens include bacterial (*Helicobacter pilori*[50]and *heilmannii*[32], *Campylobacter jejuni* and *coli*[108], *Salmonella spp.*[2]); parasitic (*Toxoplasma gondii*, *Giardia ssp.*, *Cryptosporidium ssp.*[164]); and viral agents (rotavirus[140, 141], Norwalk-like virus[184] and hepatitis E virus[116, 117]). Because many pet- and wildlife-related zoonoses are acquired via fecal-oral or direct contact routes, the inquisitive nature of children puts them at a higher risk for infection than adolescents and adults.[46] A study in a periurban shanty town in Lima, Peru was conducted to measure the frequency with which toddler-aged children were contaminated by poultry feces in homes. The study showed that by having in average 5 poultries around the house, the toddlers' hand contact with poultry feces occurred over 5 times/day. Besides 8 feces-to-mouth episodes per household/day occurred both by direct hand-to-mouth contamination and indirectly by handling soiled objects, which were then placed in the mouth. Yet, a survey of 108 families demonstrated that free-roaming poultry were often not thought of as a health risk for children. [108]

Zoonosis might have occurred in our case, because this virus was isolated from a sick young infant with symptoms of diarrhea. The poor hygienic circumstances, including domestic animals in the house created a permissive environment for the infection to occur. No other viral and bacterial agents were detected in the episode except EPEC. The role of M3363 beside EPEC in causing the symptoms is not fully understood.

In the future we need an assay that detects the response of the host to the pathogen, which includes production of pathogen-specific antibodies to further investigate interspecies transmission and zoonosis in this case. The advantage of the antibody detection assay is that large number of serum samples can be screened at low cost in large epidemiologic studies in the future. This antibody detection assay would further characterize this pathogenic virus and may open a new field in the understanding of astrovirus infections among humans. Zoonotic infections must be understood to further develop prevention and immunization strategies.

This large serosurvey for the presence of anti-M3363 antibodies in children and animals would not only investigate the significance of the virus between human and animals, but potentially identify the original host and provide a new source for isolation of the virus. This protein could also serve as an antigen in polyclonal and potentially monoclonal antibody production that could serve as detecting antibody of the virus in human and animal stool specimens. These all would enhance the further characterization of this unique isolate.

The original stool specimen from which the virus was isolated is exhausted. We need to establish ample amount of the virus for further characterization. Previous attempts to culture the virus in well-established human tissue culture (CaCo-2 cell line) were not successful. In the future we plan to culture M3363 in chicken eggs.

Chapter 7
Significance

By successfully utilizing RT-PCR for this large surveillance we propose this method for further epidemiological studies. Furthermore, a new panel of type-specific primers were designed and tested.

The prospective manner of the study with the utilization of RT-PCR and sequence analysis revealed multiple HAstVs co-circulating in the community, AstVs with distinct genetic entities including recombination and interspecies transmission. This is the first evidence that recombination occurs naturally among human astroviruses and the first case report of a novel astovirus type of putative non-human origin isolated from a symptomatic child.

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Selected Abstracts

1. Walter JE, Mitchell DK, Guerrero ML, Berke T, Matson DO, Monroe SS, Pickering LK, Ruiz-Palacios G. Community-Based Assessment of Human Astrovirus-

Associated Gastroenteritis in Children from a Periurban Area in Mexico City by RT-PCR. Poster, Pediatric Academic Society Annual Meeting; May 1-4, 1999, San Francisco, CA, USA

2. Walter JE, Briggs J, Guerrero ML, Matson DO, Pickering LK, Ruiz-Palacios G, Berke T, Mitchell DK. Sequence Analysis of Human Astrovirus ORF1a and ORF2 Genomic Regions Revealed Novel Strains Associated with Gastroenteritis in Children. Platform Presentation, American Society of Virology Annual Meeting; July 10-14, 1999, Amherst, MA, USA
3. Walter JE, Mitchell DK, Berke T, Jiang X, Matson DO, Ruiz-Palacios G, Pickering LK Characterization of a Novel Astrovirus Isolated from a Symptomatic Child. Platform Presentation. American Society of Virology Annual Meeting; July 8-12, 2000, Fort Collins, CO, USA
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5. Jakab F, Walter JE, Parada Ricart E, Berke T, Matson DO, Staat M, Azimi P, Mitchell DK. Hospital-Based Assessment Of Serious Human Astrovirus infections in the United States. Platform presentation. Fifth Annual Meeting of the European Society for Clinical Virology, Sept 2-5, 2001, Lahti, Finland
6. Walter JE, Matson DO, Berke T, Mitchell DK. Reclassification of the family Astroviridae based on Comparative Phylogenetic Analysis of Capsid Genes. Platform presentation. Seventh European Workshop on

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7. Jakab F, Walter JE, Berke T, Matson DO, Szucs Gy, Mitchell DK. Characterization and molecular sequence analysis of human astroviruses among hospitalized children in Hungary. Platform presentation. Annual Meeting of the Hungarian Society of Microbiology, October 10-12, 2001, Balatonfured, Hungary
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13. Parada E, Walter JE, Jakab F, Afflerbach C, Jiang X, Staat M,

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APPENDIX A

Molecular Epidemiology of Human Astrovirus Diarrhea among Children from a Periurban Community of Mexico City

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Human astroviruses (HAstVs) were detected in 23 stool samples from 365 diarrhea episodes among 214 children (<18 months old) prospectively monitored for diarrhea in Mexico City. Stool samples were tested by EIA and reverse transcriptase–polymerase chain reaction (RT-PCR) analysis. EIA was less sensitive (74%) and equally specific, compared with RT-PCR analysis using type-common primers for HAstV detection. Of 31 HAstV isolates, EIA typed 18 (69%) of 26 EIA-positive samples, and RT-PCR analysis typed 26 (84%) of 31 RT-PCR–positive samples. Phylogenetic analysis of the 3′ end of the capsid region (363 nucleotides) confirmed the type assignment by EIA and RT-PCR analysis and determined the type for 5 previously untyped samples. Six HAstV antigenic types cocirculated in the community: HAstV-2 (42%), HAstV-4 (23%), HAstV-3 (13%), HAstV-1 (10%), HAstV-5 (6%), and HAstV-7 (6%). RT-PCR and sequence analysis provided more detailed epidemiology of HAstV in the community than did antigenic detection methods.

Astroviruses were first found in humans in 1975 by use of electron microscopy (EM) to examine specimens from a diarrhea outbreak among infants [1]. Human astroviruses (HAstVs) are a common cause of sporadic cases and outbreaks of viral diarrhea among young children [2, 3]. HAstV is also a common agent in persistent diarrhea, which is a significant public health problem in developing countries [4].

Our understanding of the epidemiology of HAstV-associated diarrhea has changed significantly because of the use of recently developed reverse transcriptase (RT)–polymerase chain reaction (PCR) and EIA detection methods. Previous EM findings suggested a minor role (1%) for HAstVs in gastroenteritis. The prevalence of HAstV infection has been reported to be 2%–16% among children hospitalized with diarrhea and 5%–17% in community studies that used either EIA or RT-PCR analysis [2, 4–10]. Seroprevalence studies indicate that most children are infected during the first 2 years of life [2, 11, 12].

Astroviruses contain a single-stranded, positive-sense RNA genome that is ~6800 nucleotides (nts) in length. The organization of the genome includes 3 open-reading frames (ORFs): ORF1a, ORF1b, and ORF2 [13, 14]. ORF1a and ORF1b encode nonstructural proteins, and these regions contain highly conserved sequences, which is in contrast to ORF2, which encodes the capsid protein and is highly variable at the 3′ end of the genome [15, 16]. Primers specific to the conserved serine protease region in ORF1a or to the untranslated 3′ end of the genome are suitable candidates for RT-PCR detection of all antigenic types [13, 17, 18]. RT-PCR analysis detected HAstV in stool samples with greater sensitivity than did EIA in child care center outbreaks and in a bone marrow transplant unit [18, 19], although these findings are not adequate to predict the superiority of RT-PCR analysis over EIA in evaluating non-outbreak, community-based studies.

HAstV types also have been identified by EIA [20], but type-specific polyclonal or monoclonal antibodies needed for this assay generally are not available, and EIA cannot type a significant portion of isolates [21].

Using type-specific primers, RT-PCR analysis can also be used to type HAstVs. Type-specific primers derived from the 3′ end of the HAstV capsid region were reported for HAstV types 1–7 [3, 22]. The distribution of HAstV antigenic types associated with gastroenteritis varies by region. HAstV-1 was identified more frequently than other types in several countries [5, 7, 8, 10, 22].

Previous phylogenetic analyses of partial sequences in ORF1a, ORF1b, and ORF2 suggested that type information could be determined by sequence analysis of one of several regions of the genome [17, 23, 24]. In this study, we performed phylogenetic analysis on sequences from the 3′ end of the capsid

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Informed consent was obtained from the parents, in accordance with US Department of Health and Human Services guidelines. This study was approved by the institutional review boards at Instituto Nacional de la Nutrición and the University of Texas Medical School of Houston.

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region, to confirm the typing results and to determine the genetic diversity of circulating strains within types.

The aims of this study were to determine the frequency of HAstV infection by RT-PCR analysis, to compare the sensitivity and specificity of RT-PCR analysis with those of EIA during the diarrhea period and in the 7 days before or after diarrhea occurred in an episode of symptomatic infection, and to determine HAstV antigenic types by typing RT-PCR analysis and phylogenetic analysis of the 3′ end of the HAstV capsid gene.

Methods

Study population. From November 1988 through December 1991, 214 children in a periurban community of Mexico City were monitored prospectively for diarrhea from birth to 18 months of age. Stool samples obtained during the diarrhea episodes were tested for HAstV by EIA, as described elsewhere [21]. Previous reports described the geographic and socioeconomic characteristics of this community [25–27].

Stool samples. Stool samples were collected weekly, regardless of symptoms, and also during each episode of diarrhea and were stored at −70°C until tested [21]. HAstVs detected by EIA or RT-PCR analysis were cultured in CaCo-2 cells, as described elsewhere [21, 28]. Sensitivity and specificity of EIA versus RT-PCR analysis were determined by detection of HAstV exclusively from stool samples. Typing EIA was performed on tissue-cultured HAstV strains, whereas typing RT-PCR analysis was performed on RNA extracted directly from stool samples ($n = 23$) and/or from tissue culture ($n = 8$) [21].

Definition of diarrhea. A diarrhea episode was defined as the presence of ≥1 day of ≥3 watery stools in 24 h; ≥2 bowel movements more than the usual daily pattern, with a decrease in stool consistency; or ≥1 bloody stool with a decreased consistency. This definition was validated [21]. An extended-testing period was defined as the diarrhea episode plus 7 days before and after the diarrhea.

Astrovirus detection primers. To detect all HAstV antigenic types, we used RT-PCR analysis with type-common primers (Mon348/Mon340) specific for the serine protease region of ORF1a (nt 1182–1470 of HAstV-1; GenBank accession no. L23513), which permits detection of all 8 serotypes [17].

RNA extraction. RNA was extracted from 300 μL of stool suspension (10%–50% in water) with an equal volume of trichlorotrifluoroethane (Genetron; Fischer Scientific). After centrifugation, the supernatant was extracted with TRIZOL (Life Technologies) and chloroform, and the RNA was precipitated with an equal volume of 100% isopropanol. The isopropanol was decanted, and the RNA pellet was resuspended in 20 μL of RNase-free water.

RT. The RT reaction mixture (50 μL) contained 3 μL of RNA suspension and 0.1% bovine serum albumin, 0.4 mmol each dNTP, 2 U avian myeloblastosis virus RT (Promega), 4 U RNase inhibitor (Promega), 0.05 μmol dithiothreitol (Amersham Life Sciences), and 0.1 μmol negative-strand primer (Mon348; Sigma-Genosys) in reaction buffer (10 m*M* Tris, 50 m*M* KCl, and 3 m*M* $MgCl_2$ [pH, 8.3]) [17]. The samples were incubated at 42°C for 1 h.

PCR. The 100-μL PCR mixture included 50 μL of RT reaction mixture and 50 μL of a mixture containing 10% dimethyl sulfoxide, Taq polymerase (produced in our laboratory for noncommercial use), and 0.1 μ*M* positive-strand primer (Mon340; Sigma-Genosys) in reaction buffer [17]. The amplification cycle included denaturation for 2 min at 94°C, 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 50°C, and extension for 1 min at 72°C. Final extension was done for 15 min at 72°C [17]. Amplicons were visualized by agarose gel (1%) electrophoresis in Tris–boric acid–EDTA buffer containing ethidium bromide.

Type-specific RT-PCR analysis. Astrovirus-positive samples were typed, using the following type-specific primers: PR6151 [3], PR6257 [3], DM12 (5′ CTA GTG AGG AAC CTG ACA CCC ATG, 6306–6329 nt, HAstV-3; GenBank accession no. AF141381), JWT4 (5′ GCA GAG AGC TTG TTA TTA AC 3′, 2084–2103 nt, HAstV-4; GenBank accession no. Z33883), AST-S5 [22], and DM11 (5′ GGC AGA TGT GTT GGA ACT TCC C 3′, 2007–2028 nt HAstV-7; GenBank accession no. AF248738).

The RT and PCR reaction conditions were the same as for the type-common RT-PCR analysis. Mon2 (Sigma-Genosys) served as a negative-strand primer for each reaction.

HAstV types 1, 3, 5, and 7 were assigned by identification of an amplicon of the appropriate size from a single primer pair, as follows: Mon2 with PR6151 (666 nt), DM12 (512 nt), AST-S5 (471 nt) [22], and DM11 (430 nt), respectively. HAstV-2 assignment was determined by detection of the correct size amplicons generated by Mon2/PR6257 (541 nt) and Mon2/PRBEG (321 nt) [29]. HAstV-4 assignment was determined by detection of the appropriate size amplicons generated by Mon2/PR6257 (511 nt) and Mon2/JWT4 (332 nt). The Mon2/DM4 (DM4: 5′ CTA CAG TTC ACT CAA ATG AA 3′, 5525–5544 nt, HAstV-2; GenBank accession no. L13745) primer pair was used to obtain a 1.2-kb amplicon for sequence analysis for isolates that were untypeable using type-specific primers.

Sequence analysis. Typing RT-PCR amplicons were sequenced, to confirm the RT-PCR genotyping. Amplicons were cloned into pGEM-T (Promega) and were sequenced by automated sequencing (AlfWin; Amersham-Pharmacia), using fluorescence-labeled primers and a sequencing kit (SequiTherm EXCEL II Long-Read DNA Sequencing Kit-ALF; Epicentre Technologies), according to the manufacturer's recommendations.

Published (GenBank) HAstV capsid gene sequences used in the phylogenetic analyses included (followed by their accession nos.) HAstV-1 (L23513), HAstV-2 (L13745), HAstV-3 (AF117209), HAstV-4 (Z33883), HAstV-5 (U15136), HAstV-6 (Z46658), HAstV-8 (no. Z66541), and porcine astrovirus (Z66541). The capsid gene of an HAstV-7 Oxford reference strain was completely sequenced in our laboratory (AF248738). Sequences of the Mexican HAstV isolates described here also were submitted to GenBank (AF257221-9).

Basic sequence manipulation and verification were performed using PC/Gene (version 6.85; IntelliGenetics). ClustalX [30] was used to create multiple alignments of the amino acid sequences of the selected isolates. The nucleic acid sequences were added and aligned by GeneDoc (version 2.3) [31], using the corresponding amino acid sequences as template, which resulted in a 363-nt consensus length terminating at the 3′ end of ORF2.

A phylogenetic tree was constructed from the nucleic acid sequence alignment, using the maximum likelihood algorithm in the program DNAML of PHYLIP (version 3.52c) running in a UNIX environment [32]. The global rearrangement option was invoked, and the order of the sequence input was randomized 50 times. The

Table 1. Sensitivity and specificity of EIA vs. reverse transcriptase–polymerase chain reaction (RT-PCR) analysis for the detection of human astrovirus in stool samples during the extended testing period.

EIA	RT-PCR positive	RT-PCR negative	Total
Positive	17	0	17
Negative	6	342	348
Total	23	342	365

NOTE. Data are no. of extended-testing periods. For the EIA vs. RT-PCR, sensitivity was 74%, specificity was 100%, and positive and negative predictive values were 100% and 98.8%, respectively.

analysis was performed both rooted and unrooted, using the porcine astrovirus sequence as an outgroup in the rooted analysis.

Definition of subtype. Although strains and subtypes are not clearly defined in the literature, we arbitrarily considered an isolate to be a subtype with <95% homology to the reference strain and with >0.05 distance by phylogenetic analysis at the 3′ end of ORF2 (363 nt).

Detection of other pathogens associated with diarrhea. Astrovirus-positive samples were previously tested for rotavirus, enteropathogenic *Escherichia coli,* and *Shigella, Salmonella,* and *Campylobacter* species, as described elsewhere [21, 25, 26]. In addition, they were tested for calicivirus by RT-PCR analysis [33].

Statistical analysis. Sensitivity, specificity, and positive and negative predictive values of EIA versus RT-PCR analysis were determined. Concordance between EIA and RT-PCR typing was determined by χ^2 test, using Epi Info (version 5.01; Centers for Disease Control and Prevention [CDC]). Statistical association between the length of the diarrhea episodes and the causative HAstV type was determined by the Kruskal-Wallis test, using Epi Info (version 5.01). Diarrhea episodes associated with infections by multiple pathogens were excluded from this analysis. $P < .05$ was considered to be significant.

Results

EIA versus RT-PCR analysis for HAstV detection. Of 698 episodes of diarrhea occurring in 214 children, 510 (73%) extended-testing episodes were previously tested by EIA for HAstV [21]. Stool samples ($n = 396$) from 365 (72%) of the EIA-tested episodes were available for further testing by RT-PCR analysis. The availability of a limited number of samples might have resulted in a selection bias that we could not control or predict. HAstV was detected in stool samples from 23 (6.3%) of the 365 extended-testing periods (table 1) and within 16 (6.8%) of 231 diarrhea episodes. No children had a repeated HAstV infection.

In 2 of the 16 HAstV diarrhea episodes, additional stool samples from the same episode were tested. In 1 case, HAstV was identified in the first week of the diarrhea episode but was undetectable by both EIA and RT-PCR analysis from a diarrhea stool sample collected a week later. In the second case, HAstV was detected from a stool sample taken on the last day of the diarrhea episode but was undetectable from a diarrhea sample taken a week before. The other 14 HAstV-positive episodes did not have additional stool samples available for testing in the extended-testing period.

We identified 7 HAstV isolates detected within the extended-testing period but outside the diarrhea episode. A stool sample was available from the corresponding diarrhea episode in only 1 of these 7 cases. In this 1 case, HAstV-2 was detected in a sample 1 week before the start of diarrhea, and 2 consecutive stool samples taken during the diarrhea episode and 1 taken after the diarrhea episode were negative for HAstV by both RT-PCR analysis and EIA.

We tested 8 HAstV-positive samples cultured on CaCo-2 cells that were previously positive by EIA. These samples were not available from stool samples for RT-PCR testing. These samples were not included in the comparison of EIA versus RT-PCR detection from stool samples but were included in the analysis of typing EIA versus typing RT-PCR analysis ($n = 31$).

Of the 23 HAstV RT-PCR–positive stool samples from extended-testing periods, only 17 were positive by EIA. Two samples were indeterminate for the presence of HAstV by EIA and were confirmed to be positive after tissue culturing in CaCo-2 cells. Therefore, EIA was 74% sensitive and 100% specific, compared with RT-PCR analysis, for the detection of HAstV in stool samples during the diarrhea episodes and extended-testing periods (table 1). These results were the same when we compared detection from extended episodes ($n = 365$), diarrhea episodes ($n = 231$), or individual stool samples ($n = 395$). The positive predictive value of EIA was 100%, and the negative predictive value was 98.8% for EIA, compared with RT-PCR analysis as the reference standard.

Typing EIA versus typing RT-PCR analysis. Both typing EIA and RT-PCR analysis were performed on 31 HAstV-positive specimens (23 from stool samples and 8 from tissue culture). HAstV was detected in 26 of the specimens by EIA, whereas all 31 samples were positive for HAstV, as determined by type-common RT-PCR analysis (table 2). Type-specific EIA (for HAstV types 1–5) yielded a type assignment for 18 (69%) of the 26 EIA-positive samples, and type-specific RT-PCR analysis typed 26 (84%) of the 31 RT-PCR–positive samples. RT-PCR analysis confirmed the type assignment for the 18 samples typed by EIA. Type-specific RT-PCR analysis yielded significantly more type assignment than was possible by typing EIA alone ($P < .05$).

Sequence analysis. Pairwise identity comparison of nt se-

Table 2. Comparison of human astrovirus (HAstV) type determination by reverse transcriptase–polymerase chain reaction (RT-PCR) analysis, sequence analysis, and typing EIA.

Typing method	No. of samples with HAstV type							
	N	T1	T2	T3	T4	T5	T7	UT[a]
EIA	31	1	9	3	4	1	0	13
RT-PCR	31	3	10	3	6	2	2	5
Sequence analysis	31	3	13	4	7	2	2	0

[a] Untypeable samples that gave either indeterminate or negative results.

quences of the study isolates ($n = 31$) with reference strains confirmed the typing RT-PCR analysis results and permitted type assignment for additional samples (table 2).

Phylogenetic analysis was performed in 2 stages. First, all Mexican isolates were included in rooted and unrooted trees. Because pairwise analysis and the trees showed common branch points for most Mexican strains within types, 22 strains with 98%–100% homology were withheld from phylogenetic analysis, to avoid repeats. Reference strains (HAstV-1 to 8) and single representatives of the 9 distinctive Mexican strains were included for the final phylogenetic analysis. HAstV-4 was less related to the other reference HAstV strains than was porcine astrovirus; therefore, the final tree is unrooted.

The analysis (figure 1) showed that all reference strains appeared to be statistically distinct (distances, 0.11–1.4; $P < .01$). HAstV-3 and 7 and HAstV-5 and 8 were very closely related in this hypervariable region (90% and 85% pairwise identity and distance, 0.11 and 0.17, respectively). HAstV-4 was significantly different from the other HAstV strains and was less related to other HAstV strains than were 2 animal astroviruses analyzed (feline and porcine; data not shown). All Mexican HAstV isolates could be assigned a type but were significantly different from the corresponding reference strains ($P < .05$). Although there is no clear definition of strains in the literature, we arbitrarily considered an isolate to be a new strain or subtype with <95% homology to the reference strain and >0.05 distance at the 3′ end of ORF2 (363 nt). Calculated intragenotypical distances suggest that the Mexican isolates represent new subtypes of the corresponding genotype, except in the instance of the HAstV-7 isolate (99% pairwise identity with HAstV-7 and distance, 0.009), recovered from 2 children, which fell in the same clade as the prototype.

In summary, 6 HAstV antigenic types (1–5 and 7) cocirculated in the community: HAstV-2 (42%) and HAstV-4 (23%) were the most common HAstV types. There was no change in the predominant type over the study period (figure 1).

Variability at the typing primer binding site(s). Sequence information from the isolates that were untypeable by RT-PCR analysis enabled us to analyze the variability of the typing primer binding sites. Two HAstV-2 isolates that were undetectable by RT-PCR typing were identical to 2 RT-PCR–typeable isolates and had 80% identity with primer PR6257 within the sequence

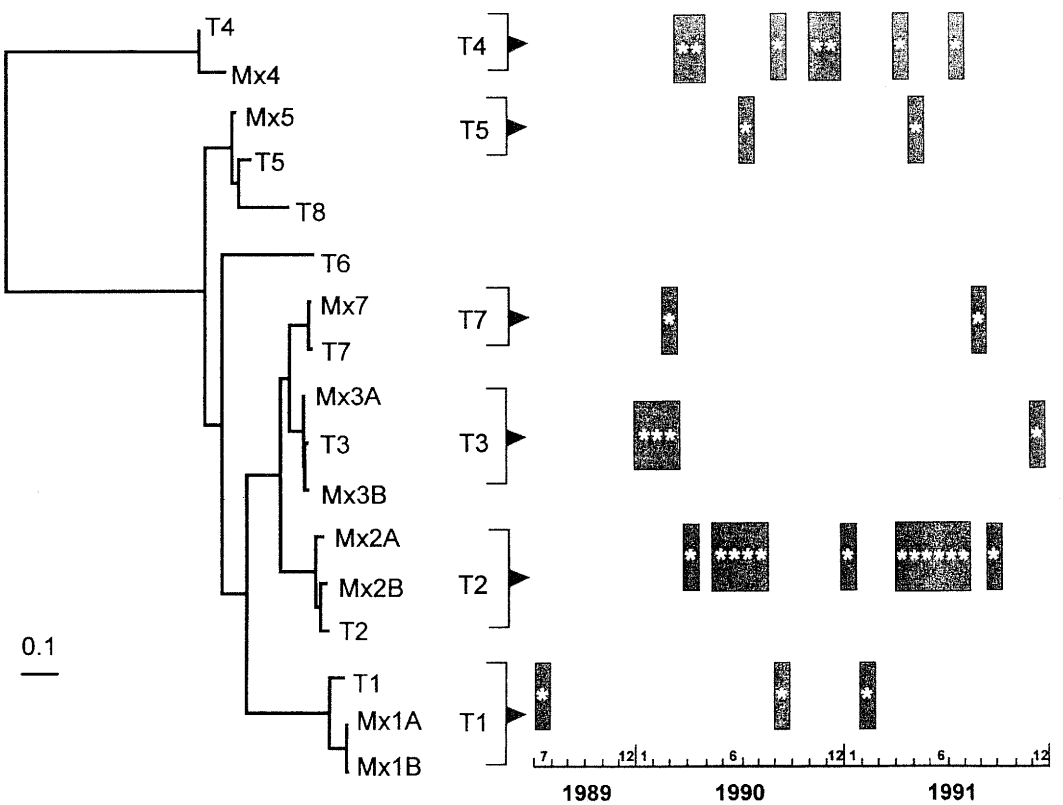

Figure 1. Maximum likelihood phylogram representing the phylogenetic relationship of Mexican human astrovirus (HAstV) isolates and HAstV reference strains (T1–T8; *left*) with the distribution of HAstV types in the 3-year surveillance period *(right)*. Partial capsid gene sequences (363 nucleotides) were used for phylogenetic analysis. Branch points of the resulting tree had a confidence level of $P < .05$. This is an unrooted tree. Distances can be estimated by using the scale bar. Time line of the 6 detected HAstV types during 3 years of surveillance in Mexico City is represented on the right. No. of Mexican isolates detected are represented by asterisks in gray boxes.

of the primer. The sequence of the possible primer binding site for each untypeable HAstV-3 and HAstV-4 isolates was 100% identical to the original primer sequence (DM12 and JWT4, respectively). These findings suggest that the failure of RT-PCR typing could have been due to mismatch (type 2), inhibitors, or low RNA yield (types 3 and 4).

Coinfections and duration of diarrhea. Coinfections with HAstV and other enteropathogens occurred in 6 children: HAstV-3 and calicivirus (1 child), HAstV-7 and rotavirus (1 child), HAstV-2 and *Shigella* species (2 children), HAstV-2 and *Salmonella* species (1 child), and HAstV-3 and enteropathogenic *Escherichia coli* (1 child). *Campylobacter* species were not detected in the 31 HAstV-positive episodes.

The length of the diarrhea episode by HAstV serotypes was analyzed after children with coinfections were excluded. HAstV-2 was associated with significantly longer diarrhea episodes (9.2 ± 6.8 days) than was HAstV-4 (2.7 ± 1.6 days; $P < .04$). We observed 2 groups: HAstV types 1–3 (group 1) and HAstV types 4, 5, and 7 (group 2). Group 1 was associated with longer periods of diarrhea (9.6 ± 7.9 days) than was group 2 (3.3 ± 2.4 days; $P < .023$ days). Similar statistical evaluation could not be performed on the other serotypes because of the small number of infections.

Discussion

Six HAstV antigenic types occurred among children in a periurban community of Mexico City during a 3-year period. Typing RT-PCR analysis was a rapid and effective tool for type determination. Sequence analysis revealed strains distinct from the prototype for 5 antigenic types circulating in the community. Surprisingly, we found that HAstV-2 causes longer diarrhea episodes than does HAstV-4.

The 7% incidence of HAstV diarrhea in this community is similar to that observed (by EIA typing) in other developing countries [2, 4] and was increased by RT-PCR testing (35%). RT-PCR analysis was more sensitive and as specific as EIA. This agrees with 2 reports of increased RT-PCR analysis sensitivity, when samples from within outbreaks were evaluated [18, 19]. We conclude that RT-PCR analysis is the most sensitive assay for large community studies.

We observed that HAstV detection may vary even during the diarrhea period, and additional HAstV-positive samples can be detected during the 7-day incubation and prolonged-excretion periods. These data are not sufficient to evaluate the importance of detection of HAstV before and after diarrhea episodes, but we speculate that it is worth testing for HAstV 7 days before and after the diarrhea period.

Type-specific RT-PCR analysis combined with sequence analysis permitted type determination for all 31 HAstV isolates. Type-specific RT-PCR analysis provided significantly more information than was possible by typing EIA. It is of interest that only 1 of the 3 HAstV-1 isolates was detected by EIA; this suggests that the hypervariability of the epitopes of this HAstV strain may contribute to the low sensitivity of the typing EIA method. RT-PCR genotyping did not detect some strains, presumably because of conditions unrelated to sequence variation.

RT-PCR genotyping and sequence analysis yielded a picture of multiple cocirculating HAstV types with no pattern of changing predominance of HAstV, similar to that revealed by EIA typing. Sequence analysis identified new subtypes of HAstV types 1–5.

Monceyron et al. [23] also analyzed HAstV sequences from the 3′ end of the genome. However, their conclusion was slightly different, which suggests a group including HAstV types 2, 3, and 7. Our phylogram suggests a group that includes HAstV types 1–3 and 7. The difference in the phylogenetic trees obtained is due to the fact that our analysis included 363 nt at the 3′ end of the capsid region, and the analysis of Monceyron et al. [23] included 321 nt at the 3′ end of the genome. There is a 244-nt overlap and 100-nt difference of the 2 consensus regions.

Belliot et al. [17] performed further phylogenetic analysis of partial sequences from the 5′ end of the capsid gene of HAstV strains [17]. This phylogenetic arrangement of HAstV strains was different than the arrangement obtained from the 3′ end of the capsid gene in our study and in the analysis by Monceyron et al. [23]. This can be explained by the different location of the 2 regions analyzed.

Our phylogenetic analysis confirmed that the presently used typing EIA (CDC) [21] can distinguish phylogenetically distinct groups, such as those described by Noel et al. [24], in the 5′ end of ORF2 for the same EIA. Phylogenetic analysis of sequences at the 3′ end of ORF2 could statistically differentiate the reference strains. Phylogeny demonstrated that the Mexican isolates align with the corresponding reference strains but were sufficiently different to represent new subtypes ($P < .01$). Two Mexican HAstV-2 subtypes were detected in different periods of the surveillance (distance, 0.048; $P < .01$). Previous phylograms obtained from different regions of HAstV genome differentiated among the 8 antigenic types but were variable in terms of the relatedness of the types [17, 23, 24]. For future studies, the location and length of a consensus region should be determined for the most reliable characterization of all HAstV genotypes. We propose that this would be at the hypervariable 3′ end of the capsid region.

Previous studies demonstrated that HAstV-1 and HAstV-2 were dominant in communities [10]. In our study, we could demonstrate the coexistence of different strains. However, we also observed that HAstV-2 was associated with a significantly longer period of diarrhea than HAstV-4. We can speculate that the detection of HAstV-2 with less sensitive methods is more probable than the detection of HAstV-4, which results in the bias of observing HAstV-2 as a dominant type.

In summary, the prevalence of HAstV diarrhea was 7% in this community of Mexico City. RT-PCR analysis, using the type-common primer pair in ORF1a, was significantly more

sensitive and equally specific, compared with EIA, for the detection of HAstV infection. RT-PCR genotyping in the capsid protein region is an excellent tool for rapid type determination. Phylogenetic analysis of 363 nts at the 3′ end of the capsid region confirmed type assignment and characterized new strains in Mexico City. Six HAstV types were present simultaneously in a community of Mexico City.

Acknowledgments

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Molecular characterization of a novel recombinant strain of human astrovirus associated with gastroenteritis in children

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Summary. We report a naturally occurring human astrovirus (HAstV) strain detected in two different geographic locations. We identified two isolates of this strain in a diarrhea outbreak at a child care center in Houston, Texas; and two isolates in diarrhea stool samples from two children in Mexico City. All four isolates were detected in stool samples by enzyme immunoassay (EIA). One of the Mexican isolates was typed by EIA and all four isolates were HAstV-5 by typing RT-PCR. The four isolates were >97% nucleotide-identical in two different genomic regions: ORF1a (246nt), and the 3′ end of the genome (471nt). One isolate from each geographic location was further sequenced in the transition region from ORF1b to ORF2 (1255nt) and this region of the two isolates showed ≥ 99% nt identity. Phylogenetic analyses of sequences of eight HAstV antigenic types and the novel strain in the transition region demonstrated the new strain being closely related to HAstV-3 in ORF1b, but closest to HAstV-5 in ORF2. These results and high sequence identity among all HAstV antigenic types in the transition region and RNA structural predictions supported a potential recombination site at the ORF1b/ORF2 junction. This is the first evidence that recombination occurs among human astroviruses.

Introduction

Astroviruses (AstVs) contain a single-stranded, positive-sense RNA (+ssRNA) genome approximately 6.800 nucleotides (nt) long. The genome includes three open reading frames: ORF1a, ORF1b, and ORF2 (Fig. 1) [6, 13]. ORF1a and 1b encode non-structural proteins, including a serine protease (*Pro*) and RNA-dependent RNA polymerase (*Pol*), respectively, and these regions contain

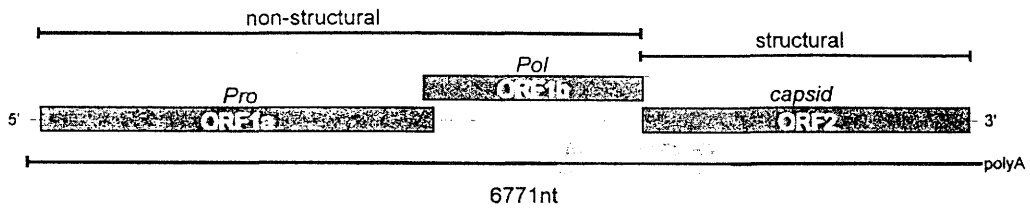


Fig. 1. Schematic diagram of the genome organization of a human astrovirus. Shading indicates the 1200nt junction region between ORF1b and ORF2

sequences highly conserved among AstVs. ORF2 encodes the capsid protein and is highly variable at the 3′ end.

The family *Astroviridae* is distinguished from other virus families that are not enveloped and have ssRNA genomes, such as *Picornaviridae* and *Caliciviridae*, by the presence of ribosomal frameshifting between ORF1a and ORF1b and the lack of the helicase domain [6]. However, AstVs contain eight characteristic amino acid motifs in the *Pol* region (e.g. YGDD) typical of +ssRNA virus polymerases, indicating that astrovirus *Pol* belongs to supergroup I polymerases, which includes the *Pol* enzyme of picornaviruses, caliciviruses, potyviruses and several other plant viruses [6]. +ssRNA plant viruses also share similarities with human astroviruses (HAstVs), such as the substitution of the catalytic cysteine with serine and the genomic organization resembles that of *Luteoviridae* [24].

Belliot et al. characterized three different regions of the astrovirus genome (ORF1a, ORF1b and 5′ end of ORF2) of 64 HAstV isolates from seven countries [2]. Phylogenetic analyses of these partial ORF sequences suggested that genotypes could be assigned equally well by analysis of any of these three regions because of high correlation among the regions. Their findings would suggest that AstV recombinants did not occur among their characterized strains.

The aims of this study were 1) to characterize four HAstV isolates whose genotyping, sequityping and antigenic typing results for the polymerase (ORF1b) and capsid (ORF2) genes resulted in conflicting classification and 2) to determine their genetic and antigenic relationships to known HAstV serotypes.

Materials and methods

Stool specimens

HAstVs were detected in two separate studies. In the first study, stool specimens were collected from 1989 to 1992 during diarrhea outbreaks among children attending childcare centers in Houston, Texas [12]. In the second study, 214 children in a periurban community of Mexico City were monitored prospectively for diarrhea from birth to 18 months of age from 1988 to 1991 [5]. All collected stool specimens were stored at −70 °C until HAstV testing.

Primers

Primer pair Mon2 [12] /DM4 (5′ CTA CAG TTC ACT CAA ATG AA 3′, 5531-5550nt in HAstV-1 [Acc# L23513]) was used to amplify a 1.2 kb segment of the 3′ end of the genome.

Primer pair Mon270/Mon344 [2, 21] was used to amplify a 1.2 kb segment of the transition region overlapping ORF1b and ORF2. Internal primers DM17 (5′ GGT TTT GGT CCT GTG ACA CC 3′; location 4563-4544nt in HAstV-1 [Acc# L23513]) and DM15 (5′ GAT AGG CTC TCC ACT ACA CC 3′, location 3901-3920nt in HAstV-2 [Acc# L13745]) were used to amplify a ~600 bp product from the ORF1b-ORF2 transition region of reference HAstV strains. The novel astrovirus was also genotyped by RT-PCR using Mon2/AST-S5 [11].

Detection of HAstVs

Stool samples from Houston and Mexico obtained during diarrhea episodes were tested for HAstV by EIA [5] and RT-PCR. For RT-PCR, viral RNA was extracted from 300 μL of stool suspension (10–50% in water) by a Genetron™-TRIZOL™ method as previously described [23]. Amplicons were generated by RT-PCR utilizing a type-common primer pair, Mon348/Mon340, that targets a region of ORF1a upstream from the *Pro* motif and that allows detection of all eight serotypes [2].

Typing of HAstVs

HAstV-positive specimens from Mexico were typed by EIA [5] and genotyped by RT-PCR [23]. All typing RT-PCR reagents and conditions were identical to those for type-common detection except the annealing temperature was 40 °C (Mon2/AST-S5).

RT-PCR in the Orf1b-ORF2 region

All RT-PCR reagents and conditions were identical to those for type-common detection except the annealing temperature was 40 °C (Mon270/Mon344, DM17/DM15).

Sequence analysis

RT-PCR amplicons of the four strains were cloned into pGEM-T (Promega, Madison, WI) and sequenced in the ALFwin automated sequencer system (Amersham Pharmacia Biotech, Uppsala, Sweden) using fluorescence-labeled primers and SequiTherm Excel™ II Long-Read DNA Sequencing Kit-ALF™ (Epicentre Technologies, Madison, WI), according to the manufacturer's recommendations.

Published HAstV capsid gene sequences used in the phylogenetic analyses included HAstV-1 [L23513], HAstV-2 [L13745], HAstV-3 [AF117209], HAstV-4 [Z33883], HAstV-5 [U15136], HAstV-6 [Z46658]. The capsid gene of a HAstV-7 Oxford reference strain was sequenced in our laboratory [AF248738]. The 1.2 kb amplicon of the 3′ end of the genome of H2067 was sequenced and submitted to GenBank [AF292080]. Amplicons (1.2 kb) from the junction region between ORF1b and ORF2 for HastV-3 to 7 Oxford strains [AF292074-8], HAstV-8 South African strain (As20) [AF292073], and isolate H2067 [AF292072] were sequenced and submitted to GenBank.

Sequence verification and analysis was performed using OMIGA (v2.0, Genetics Computer Group, Madison, WI). ClustalX was used to create multiple alignments of the amino acid sequences of selected isolates [22]. 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 1264nt in the junction region between ORF1b and ORF2 [20].

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 [3]. The global rearrangement option was invoked and the order of the sequence input was randomized one hundred times.

Pairwise identity was calculated for reference types and H2067 in the transition region of ORF1b-ORF2 based on the original alignment. Identities between selected reference types

and H2067 were visualized by dot plot comparison. The parameters for the dot plot were the following: window size: 25; jump size: 1; hash value: 1; and stringency: 0.85.

The nucleic acid sequence of the junction region between ORF1b and ORF2 was further analyzed to characterize the possible recombination site: the length, extent of identity and AU-GC content among all human reference strains and one animal strain (porcine [AB037272]) were evaluated. Secondary RNA structure was computed using Zuker's RNA secondary structure predicting program (http://www.ibc.wustl.edu/~zuker/). This structure analysis was utilized to predict local secondary structure elements that may represent recombination hotspots.

Results

Detection of viral antigen by EIA and RT-PCR

Two astrovirus strains (H2002, H2067) were detected in diarrhea stool samples collected from two children during a HAstV outbreak in May 1990 at a childcare center in Houston, Texas. The other two strains (M240, M370) were detected in stool samples of diarrhea episodes in June 1990 and April 1991 from two children residing in Mexico City. All four HAstV isolates were detected by type-common EIA [5].

Antigenic and genomic typing

Typing EIA identified M370 as HAstV-5 and M240 was untypeable [5]. EIA typing was not available for H2002 and H2067. The inability to EIA type M240 was attributed to a low titer of virus in the stool specimen, which was further suggested by the weak amplicon signal after RT-PCR amplification. The isolates from Houston were not typed by EIA. All four isolates were genotyped as HAstV-5 using Mon2/AST-S5, type-specific primers targeting the 3′ end of ORF2, and the type assignments were confirmed by sequence analysis (sequence analysis data not shown) [11]. A longer amplicon of H2067 (1128bp) using primers Mon2/DM4 was sequenced.

Sequence comparison

The four isolates were 97–100% identical in the ORF1a region (246nt) and 98–100% identical at the 3′ end of ORF2 (365nt). Amplicons (1255nt) were obtained for two of the four isolates (H2067 and M370) in the ORF1b/ORF2 transition region and were 99% identical to each other. Pairwise sequence comparison of 1128nt at the 3′ end of ORF2 (Mon2/DM4) of H2067 confirmed it's being closest (94%) to HAstV-5.

Sequence analysis of the transition region of ORF1b-ORF2 (1264 nt consensus length) is shown in Fig. 2. The overlapping clone was divided into two regions (5′ end 576nt, 3′ end 637nt) by a highly identical 52nt region. These two regions represented two different ORFs: ORF1b was represented in the 5′ end (576 nt) and ORF2 was represented in the 3′ end (637nt) of this clone.

Pairwise comparison of nucleotide and amino acid sequences of all reference types with H2067 is shown in Table 1. In ORF1b (576nt), H2067 is closer to HAstV-3 (92%nt, 98%aa) than to HAstV-5 (88%nt, 96%aa). In the 5′ end of

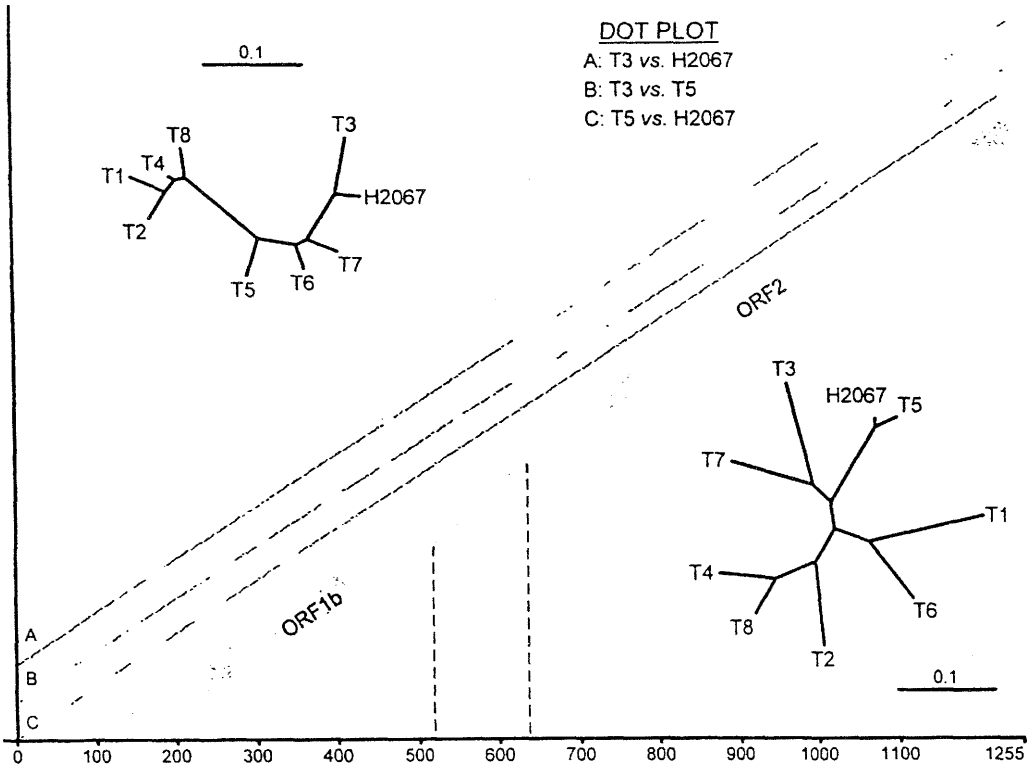


Fig. 2. Comparative sequence analysis of the reference human astrovirus type 3 and 5 and H2067 [AF292080] in the transition region of ORF1b-ORF2 (1255nt, x axis). The transition region was divided into two regions (5′ end 576nt, 3′ end 637nt), excluding a 52nt highly identical region in the middle. Dotted lines indicate the hypothesized 120nt-long recombination site. Maximum likelihood phylograms of 576nt of ORF1b and 637nt of ORF2 5′ end are in the left upper and right lower corners, respectively. Phylogenetic distances are given as number of substitutions per site. Identities between selected strains (T5, T3, and H2067) are presented on the diagonal created by dot plot analysis. A high rate of identity is visualized by a dark continuous line

ORF2 (637nt), H2067 is closer to HAstV-5 (96%nt, 99%aa) than to HAstV-3 (81%nt, 93%). Other HAstV types were less identical to H2067 in the ORF1b (84–90%nt, 94–97%aa) and ORF2 (80–83%nt, 89–92%aa) regions.

Pairwise identities between selected strains (T5, T3, and H2067) are presented by dot plot, in which a higher rate of identity is represented by continuous dots (Fig. 2, diagonal). The dot plot results demonstrated greater identity between H2067 and HAstV-3 in ORF1b and greater identity between H2067 and HAstV-5 at the 5′ end of ORF2. Comparison of HAstV-3 and 5 in this transition region provided the baseline for analysis.

Phylogenetic analysis

A maximum likelihood phylogram for the 576nt region of ORF1b is in the left upper corner of Fig. 2 and shows that H2067 was closest to HAstV-3 and distinct

Table 1. Pairwise nucleotide (nt) and amino acid (aa) identities (%) of H2067 and all HAstV reference types in the junction region of ORF1b (576nt) (**A**) and ORF2 (637nt) (**B**). Comparison of T3 and T5 to H2067 are highlighted

aa\nt	H2067	T1	T2	T3	T4	T5	T6	T7	T8
A									
H2067		83	84	**92**	85	**88**	90	89	84
T1	94		93	82	94	85	84	84	91
T2	95	97		83	94	85	85	84	92
T3	**98**	93	94		82	87	86	88	82
T4	95	99	98	94		86	86	85	95
T5	**96**	94	94	95	94		90	89	85
T6	96	95	95	95	96	96		93	84
T7	97	95	94	96	95	96	98		83
T8	94	96	96	93	96	93	94	93	
B									
H2067		81	80	**81**	80	**96**	81	83	81
T1	91		81	80	80	81	83	79	79
T2	89	89		81	84	79	83	82	86
T3	**91**	91	90		80	80	81	83	80
T4	89	88	93	89		80	83	80	90
T5	**99**	90	89	91	88		80	82	81
T6	91	91	92	91	93	91		84	83
T7	92	88	90	91	90	91	91		80
T8	90	88	95	89	95	89	93	90	

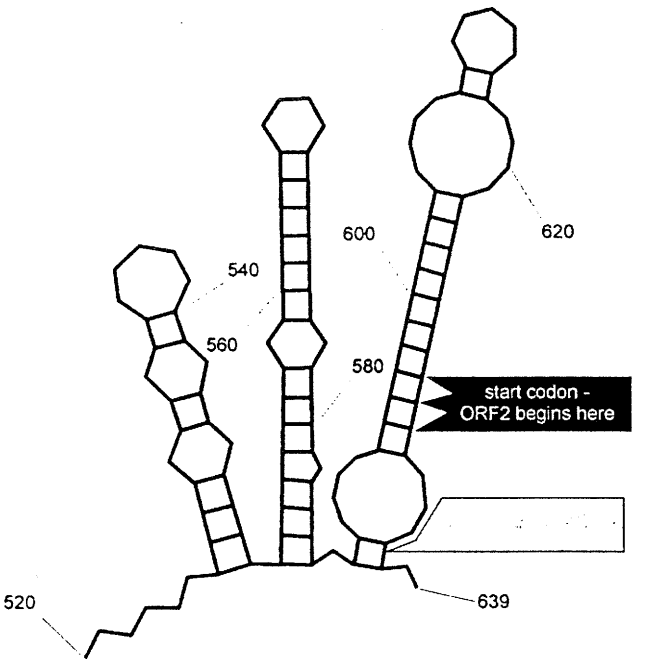
T1-8 HAstV reference antigenic types

from HAstV-5. The phylogram of the 637nt portion of ORF2 shows that H2067 was closest to HAstV-5 and distinct from HAstV-3 (Fig. 2, lower right tree).

RNA structure analysis

A 120nt region at the junction of ORF1b and ORF2 was selected for further sequence analysis to characterize the putative recombination site. The consensus nucleotide sequence of the region is shown in Fig. 3. All human strains are 93–98% identical in this region. This 120nt region incorporates a 52nt region where sequence identity is 99–100% among human strains.

Figure 3 demonstrates the predicted RNA secondary structure for H2067 in the 120nt junction region between ORF1b and ORF2. The predicted structure has a triple-hairpin conformation. The predicted RNA secondary structure was identical among the analyzed human strains. Although the porcine astrovirus has only 80–85% nt identity in this 120nt region, secondary structure analysis predicted the same triple-hairpin conformation (data not shown). The triple-hairpin structure includes the start codon for the capsid gene, as well as the stop codon for the *Pol* gene.




```
520                    540
  GAaCACUGCCU.UCaCGGACUGC.AAGCAGCUUCGUGA.U
560                    580
  CUGG.CU.CC.GCCAG.CUCACAGAAGAGCAACUCCAUCG
600                    620
  CAUuUGGAGGGGAGGACCAAAGAAgUGuGAUGGC   cAA
```

Fig. 3. RNA secondary structure of the hypothesized 120nt recombination site in H2067 (520nt–639nt). Nucleotides are numbered according to their position in the 1255nt RT-PCR product [AF292080], including the junction of ORF1b and ORF2. In the hairpin structure, the location for the start codon of ORF2 and the stop codon for ORF1b are indicated. At the bottom of the figure the consensus sequence of the corresponding regions of all reference human strains is described. Uppercase letters indicate 100% and lower case letters indicate ≥89% identity. Dots represent divergence in the nucleotide sequence of the eight reference types. The highly conserved 52nt region is underlined. In the consensus sequence, the start codon for ORF2 and the stop codon for ORF1b are highlighted

Discussion

In this study we characterize a human astrovirus strain apparently derived from naturally occurring recombination, detected in two different geographic locations and defined by EIA, RT-PCR, and phylogenetic methods.

The cDNA used to obtain sequence in the transition region of ORF1b and ORF2 was generated from a single RT-PCR generating a 1.2 kb product spanning the region and was sequenced for two different isolates (one Houston and one Mexican). High identity between these sequences, the obvious change in their

relationship to the reference HAstV-3 and HAstV-5 types in ORF1b and ORF2, and the lack of similar findings for 64 other HAstV strains [2] indicate that all four isolates are naturally occurring recombinants, probably derived from the same recombinant parent strain. Further, two different researchers characterized the Houston and Mexican isolates one year apart, which minimizes the chance that similarities were due to laboratory contamination.

HAstV co-infections with different antigenic types have been reported in epidemiological studies [11]. Co-infection by two different serotypes in one child provides an opportunity for recombination to occur. In addition, RNA viruses have a tendency toward recombination due to the nature of their *Pol* which naturally shift frame at the ORF1a/ORF1b junction [1, 6, 18, 19].

The preferred mechanism of recombination among +ssRNA viruses is a copy-choice mechanism where recombination occurs while the *Pol* enzyme switches from copying one RNA molecule (donor template) to another (acceptor template) without releasing the nascent strand [8]. RNA virus recombination events are classified as homologous, aberrant homologous, and non-homologous types depending on the location of the recombination site within the viral genome, and the precision of the event [10]. However, Nagy and Simon suggested a revision of the classification to sequence similarity-essential, similarity-assisted, and similarity-nonessential, based upon the mechanism of the generation and the nature of the recombinant product [18].

Studies with brome mosaic virus (BMV), a +ssRNA plant virus which is the most frequently used model for homologous RNA virus recombination, revealed several factors that are important in RNA recombination [14]. Those factors include the length of the identical region of the sequence, degree of sequence identity, the presence of AU-rich segments in the 3′ vs. relative GC-rich segments in the 5′ end, and stable hairpin structures [16, 17]. In the BMV model it was suggested that homologous recombination does not require similar secondary structures between the two recombining sites of the crossover. However, for the effective extension of the 3′-end, the *Pol* requires a hairpin structure in the acceptor RNA region [14, 19]. Koev et al. showed that the RNA secondary structure at the recombination site plays a unique role in the promoter recognition by the viral *Pol* in vivo in a recombination model of barley yellow dwarf virus [9].

Natural, homologous recombinants have been demonstrated among other +ssRNA viruses, including *Picornaviridae* [4] and *Caliciviridae* [7]. In the case of *Picornaviridae*, poliovirus with natural recombinant genome was isolated from vaccine-associated paralytic poliomyelitis in France, detected by double restriction fragment length polymorphism assay and confirmed by partial sequence analysis [4]. In the case of *Caliciviridae*, calicivirus with natural recombinant genome (Arg320) was identified from a diarrhea stool sample from Argentina and confirmed by sequence analysis [7]. In the case of *Luteoviridae*, Koev et al. proposed that recombination has occurred during luteovirus evolution, based on sequence analyses of genetic regions of the two major genera, *Luteovirus and Polerovirus* [9]. In *Caliciviridae* and *Luteoviridae* the recombination events were localized at the *Pol*/capsid junction, which is a transition between homologous

(non-structural) and divergent (structural) regions. Both of these viruses have subgenomic RNA encoding the capsid protein. In *Luteoviridae* it is proposed that recombination has occurred by *Pol* switching strand at the subgenomic promoter [9]. Neither natural nor artificially induced recombination has been detected among AstVs.

During the copy-choice mechanism of recombination the short AU rich sequence and stable hairpin structures of the donor template halt the synthesis on the nascent strand, thus providing an opportunity for the *Pol* to interact with the acceptor strand. These AU rich sequences also promote *Pol* slippage [15]. The stable hairpin structures act as replication enhancer in recruiting the *Pol* [17]. Long (or even short, depending on the virus) homologous sequences between the nascent RNA and the acceptor RNA facilitate the association of the nascent strand with the acceptor strand and function as a primer for the template switching and extension [14].

RNA sequence at the suggested recombination site we observed the following: high identity among all HAstV antigenic types (120nt 93–98% including a 52nt 99–100% region) and high identity with porcine AstV sequence (80–85%) in the 120 nt region. We did not locate definite AU rich segments in the 3′ vs. GC rich (or moderately AU-rich) segments in the 5′ end of this region. RNA secondary structure analysis predicted similar triple stable hairpin structures for all human and porcine strains. In summary, this proposed recombination site includes a long homologous region with stable hairpin structure, which supports the classification of our isolate as a homologous or similarity-assisted recombinant.

The recombination site in our case was in the junction of *Pol* and capsid region similar to other viruses (*Caliciviridae* and *Luteoviridae*) [7, 9]. Astroviruses transcribe a subgenomic RNA whose start point maps within the region of the suggested recombination junction, therefore we propose that polymerase recognition of the subgenomic RNA promoter may be the reason for the recombination event similar to *Luteoviridae*.

We conclude that phylogenetic analysis of different genomic regions of HAstV can provide contradictory genotyping unlike a previous observation [2]. In our case, the HAstV3-5 recombinant was widespread, apparently stable and pathogenic. Therefore molecular typing methods for AstVs should utilize sequence in the capsid region (ORF2) to correlate with antigenic typing methods.

RNA recombination may contribute to evolutionarily significant RNA rearrangements in astroviruses. Nucleic acid sequence homology and similar RNA secondary structure of HAstV and animal AstVs (porcine) in this transition region of ORF1b and ORF2 may provide a possibility of recombination among AstVs from different species should co-infections occur.

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APPENDIX B

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PREDICTIVE MORPHOLOGICAL FINDINGS IN "ZERO-HOUR" BIOPSIES OF RENAL ALLOGRAFTS

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"Zero-hour" biopsies of 65 donors have been performed since 1994. Donor kidneys were categorized into five groups based on the morphological findings in "zero-hour" biopsies. No morphological abnormalities were found in 38% of the cases (group 1). Arteriosclerosis was present in 31% of donor kidneys (group 2). Specific morphological alterations, i. e. acute tubular necrosis [21.5%], tubulointerstitial nephritis [6.2%] or glomerulonephritis [3.1%] were detectable in the cases remained (group 3-5). During an average of 336 posttransplant days clinical and histological follow up was performed (50 rebiopsies). Statistical data of mismatch (1.4–2.0), average of donor/recipient age (35–42 years), cold and warm ischaemic time (1290 and 66 min) were comparable in all groups. According to our observations: 1. higher creatinin was found in grafts with arteriosclerosis (group 2) ($p < 0.05$). 2. there were more non-viabile grafts and longer period of delayed graft function in acute tubular necrosis (group 3), 3. higher creatinin, rejections with the need of rehemodialysis were observed in four cases of tubulointerstitial nephritis (TIN-group 4). Glomerulonephritis (GN-group 5) grafts had only delayed graft function, however these groups were few for statistical evaluation. Biopsy complication in 1/115 cases was found (rebiopsy induced kidney haemorrhage). In conclusion, "zero-hour" biopsies can be useful and safe tools to predict early graft function. Besides "zero-hour" biopsies help the histological interpretation of consecutive graft rebiopsies.

Introduction

Early graft biopsies (called as "zero-hour" or "one-hour" biopsies) have been performed since 1968 as they seemed to serve as early reliable indicator of the status of the transplanted kidney [4]. Controversial results are published about the possible predictive value of these biopsies. In one hand its predictive role is questioned in early rejection [4], early and late graft function [1]. On the other hand it is suggested for prediction of graft loss [2]. Consensus has bee created that these early graft biopsies are useful tools for subsequent biopsy interpretation1 [1, 3, 4]. The aim of this study is to introduce our observations on "zero-hour" biopsies and to reevaluate its role in clinical transplantation.

Materials and Methods

During the period of 1994–1996, 67 pretransplant "zero-hour" biopsies (one from a nonheart-beating donor, 66 from cadaver donors) were studied. 65 out of 67 grafts were transplanted (one with severe arteriosclerosis involving also the renal artery and one because of renal cell cancer with otherwise normal renal histology were excluded).

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"Zero-hour" biopsies ("wedge-biopsies" of 3x5 mm size from the upper pole of the grafts) were performed at the end of cold ischaemic time. No "zero-hour" biopsies complications were observed. 50 consecutive graft rebiopsies were performed in 33/65 transplanted patients to follow their graft complications during 336 days in average. Indications of graft rebiopsies included: 1. Delayed graft function for more than 7 days, 2. Steroid resistant acute rejection, 3. No adequate response to anti-rejection therapy, 4. Increasing serum creatinin without signs of rejection. Immunosuppression protocol included: 1. Sandimmun or Neoral (10 mg/kg/die) monitored by serum cyclosporin A from day 8.; 2. Azathioprin (1–2 mg/kg/die); 3. Methylprednisolon (1 mg/kg/die, sequentially reduced to 0.15 mg/kg/die from day 61.); 4. Prophylactic anti-lymphocyte globulin (ALG) or anti-thymocyte globulin (ATG). Acute rejection therapy: 3–5x500 mg methylprednisolon, rebiopsy was performed if there was no response, ALG or ATG if rebiopsy proved grade II–III rejection. Morphological analysis was done by light microscopy, immunofluorescence (IgG, IgA, IgM, C3 and MAC [membrane attack complex]) and transmission electron microscopy (TEM) using standard techniques1. Acute and/or chronic rejections, cyclosporin nephropathy, acute tubular necrosis were diagnosed on the basis of graft biopsies according to the Banff criteria [5]. Graft thrombosis was suspected by biopsy findings of renal infarction, proved by Doppler-ultrasonography and macroscopic examination of the kidneys after nephrectomy. Clinical follow up was performed by the members of the transplantation team checking of the following parameters regularly: 1. Full laboratory status, 2. Physical examination, 3. Blood pressure, 4. Throat/urine bacteriology, 5. Chest X-ray, 6. ECG, 7. Abdominal ultrasonography. Clinical information was available about donor/recipient age and sex, HLA–B, DR mismatch, pretransplant kidney disease and hemodialysis time of the recipient, blood group of the donor/recipient, periods of handling time, warm/cold ischaemia and non-graft-related clinical complications, treatments and lethal complications.

Definitions: delayed graft function (DGF) was defined as an initial need of hemodialysis. "Good" renal function was classified if the patient had not returned to hemodialysis or died with good renal function during the follow up period.

Results

Early graft function was monitored during the first two posttransplant months in recipients with renal allografts of different "zero-biopsy" morphology. Higher serum creatinin (266 [100–530] μM/L) was observed in recipients with arteriosclerosis (group 1) ($p < 0.05$) and tubulointerstitial nephritis grafts (group 4) (277 [133–600] μM/L) (statistically not evaluatable). Higher number of non-viabile grafts (21.4%) ($p < 0.05$) was present in acute tubular necrosis (ATN – group 3). There were no differences in the frequency of delayed graft function (DGF) (28–30%), but a significantly longer DGF time (18.5 days) ($p < 0.05$) was found in ATN – group 3. All groups were compared to group 1 with normal morphological findings.

Late graft function was monitored by clinical follow up and rebiopsies of viabile grafts during an average of 336 posttransplant days. There was no significant difference in graft survival between statistically evaluable groups (normal:78%, AS:70%). This means increased graft loss in AS-group compared to its early period (0% vs 30%). The graft loss is due to higher frequency of rejections (40%) and a need for rehemodialysis (15%) in arteriosclerosis graft. The worsening function was also reflected in high serum creatinin (253 [100–442] μM/L) levels of these recipients. Rare morphological forms of "zero-hour" biopsies (TIN, GN) were statistically non evaluable and carried either a bad (TIN) or a good (GN) long term graft survival. There was no significant difference in mismatch, age, sex, blood group, handling time, warm/cold ischaemia time and in clinical complications or therapy between the groups.

Postoperative mortality rate was 4,7% (3/64), overall mortality was 17,2% (11/64) with 10,95% (7/64) extrarenal causes.

Discussion

"Zero-hour" biopsies of 65 donors have been performed since 1994. Only 38% of the cases showed no morphological abnormalities which motivated us to study and reevaluate the significance of "zero-hour" biopsies.

Based on our findings we support the the literature that "zero-hour" biopsies do not predict late graft function and complications [1, 3, 4]. In addition we can conclude that there are potentially predictable morphological parameters of the graft at early stages. Renal allografts with arteriosclerosis had worse early function in their recipients reflected in higher serum creatinin levels compared to grafts with normal morphological findings. Acute tubular necrosis of renal allografts in our material statistically reduced graft viability, prolonged the delayed graft function time period. A similar value of tubular damage predicting graft loss was reported in renal allograft rebiopsies [2].

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"Zero-hour" biopsies of 65 donors have been performed since 1994. Donor kidneys were categorized into five groups based on the morphological findings in "zero-hour" biopsies. No morphological abnormalities were found in 38% of the cases (group 1). Arteriosclerosis was present in 31% of donor kidneys (group 2). Specific morphological alterations, i. e. acute tubular necrosis [21.5%], tubulointerstitial nephritis [6.2%] or glomerulonephritis [3.1%] were detectable in the cases remained (group 3-5). During an average of 336 posttransplant days clinical and histological follow up was performed (50 rebiopsies). Statistical data of mismatch (1.4–2.0), average of donor/recipient age (35–42 years), cold and warm ischaemic time (1290 and 66 min) were comparable in all groups. According to our observations: 1. higher creatinin was found in grafts with arteriosclerosis (group 2) ($p < 0.05$). 2. there were more non-viabile grafts and longer period of delayed graft function in acute tubular necrosis (group 3), 3. higher creatinin, rejections with the need of rehemodialysis were observed in four cases of tubulointerstitial nephritis (TIN-group 4). Glomerulonephritis (GN-group 5) grafts had only delayed graft function, however these groups were few for statistical evaluation. Biopsy complication in 1/115 cases was found (rebiopsy induced kidney haemorrhage). In conclusion, "zero-hour" biopsies can be useful and safe tools to predict early graft function. Besides "zero-hour" biopsies help the histological interpretation of consecutive graft rebiopsies.

Introduction

Early graft biopsies (called as "zero-hour" or "one-hour" biopsies) have been performed since 1968 as they seemed to serve as early reliable indicator of the status of the transplanted kidney [4]. Controversial results are published about the possible predictive value of these biopsies. In one hand its predictive role is questioned in early rejection [4], early and late graft function [1]. On the other hand it is suggested for prediction of graft loss [2]. Consensus has bee created that these early graft biopsies are useful tools for subsequent biopsy interpretation1 [1, 3, 4]. The aim of this study is to introduce our observations on "zero-hour" biopsies and to reevaluate its role in clinical transplantation.

Materials and Methods

During the period of 1994–1996, 67 pretransplant "zero-hour" biopsies (one from a nonheart-beating donor, 66 from cadaver donors) were studied. 65 out of 67 grafts were transplanted (one with severe arteriosclerosis involving also the renal artery and one because of renal cell cancer with otherwise normal renal histology were excluded).

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"Zero-hour" biopsies ("wedge-biopsies" of 3x5 mm size from the upper pole of the grafts) were performed at the end of cold ischaemic time. No "zero-hour" biopsies complications were observed. 50 consecutive graft rebiopsies were performed in 33/65 transplanted patients to follow their graft complications during 336 days in average. Indications of graft rebiopsies included: 1. Delayed graft function for more than 7 days, 2. Steroid resistant acute rejection, 3. No adequate response to anti-rejection therapy, 4. Increasing serum creatinin without signs of rejection. Immunosuppression protocol included: 1. Sandimmun or Neoral (10 mg/kg/die) monitored by serum cyclosporin A from day 8.; 2. Azathioprin (1–2 mg/kg/die); 3. Methylprednisolon (1 mg/kg/die, sequentially reduced to 0.15 mg/kg/die from day 61.); 4. Prophylactic anti-lymphocyte globulin (ALG) or anti-thymocyte globulin (ATG). Acute rejection therapy: 3–5x500 mg methylprednisolon, rebiopsy was performed if there was no response, ALG or ATG if rebiopsy proved grade II–III rejection. Morphological analysis was done by light microscopy, immunofluorescence (IgG, IgA, IgM, C3 and MAC [membrane attack complex]) and transmission electron microscopy (TEM) using standard techniques1. Acute and/or chronic rejections, cyclosporin nephropathy, acute tubular necrosis were diagnosed on the basis of graft biopsies according to the Banff criteria [5]. Graft thrombosis was suspected by biopsy findings of renal infarction, proved by Doppler-ultrasonography and macroscopic examination of the kidneys after nephrectomy. Clinical follow up was performed by the members of the transplantation team checking of the following parameters regularly: 1. Full laboratory status, 2. Physical examination, 3. Blood pressure, 4. Throat/urine bacteriology, 5. Chest X-ray, 6. ECG, 7. Abdominal ultrasonography. Clinical information was available about donor/recipient age and sex, HLA–B, DR mismatch, pretransplant kidney disease and hemodialysis time of the recipient, blood group of the donor/recipient, periods of handling time, warm/cold ischaemia and non-graft-related clinical complications, treatments and lethal complications.

Definitions: delayed graft function (DGF) was defined as an initial need of hemodialysis. "Good" renal function was classified if the patient had not returned to hemodialysis or died with good renal function during the follow up period.

Results

Early graft function was monitored during the first two posttransplant months in recipients with renal allografts of different "zero-biopsy" morphology. Higher serum creatinin (266 [100–530] μM/L) was observed in recipients with arteriosclerosis (group 1) ($p < 0.05$) and tubulointerstitial nephritis grafts (group 4) (277 [133–600] μM/L) (statistically not evaluatable). Higher number of non-viabile grafts (21.4%) ($p < 0.05$) was present in acute tubular necrosis (ATN – group 3). There were no differences in the frequency of delayed graft function (DGF) (28–30%), but a significantly longer DGF time (18.5 days) ($p < 0.05$) was found in ATN – group 3. All groups were compared to group 1 with normal morphological findings.

Late graft function was monitored by clinical follow up and rebiopsies of viabile grafts during an average of 336 posttransplant days. There was no significant difference in graft survival between statistically evaluable groups (normal:78%, AS:70%). This means increased graft loss in AS-group compared to its early period (0% vs 30%). The graft loss is due to higher frequency of rejections (40%) and a need for rehemodialysis (15%) in arteriosclerosis graft. The worsening function was also reflected in high serum creatinin (253 [100–442] μM/L) levels of these recipients. Rare morphological forms of "zero-hour" biopsies (TIN, GN) were statistically non evaluable and carried either a bad (TIN) or a good (GN) long term graft survival. There was no significant difference in mismatch, age, sex, blood group, handling time, warm/cold ischaemia time and in clinical complications or therapy between the groups.

Postoperative mortality rate was 4,7% (3/64), overall mortality was 17,2% (11/64) with 10,95% (7/64) extrarenal causes.

Discussion

"Zero-hour" biopsies of 65 donors have been performed since 1994. Only 38% of the cases showed no morphological abnormalities which motivated us to study and reevaluate the significance of "zero-hour" biopsies.

Based on our findings we support the the literature that "zero-hour" biopsies do not predict late graft function and complications [1, 3, 4]. In addition we can conclude that there are potentially predictable morphological parameters of the graft at early stages. Renal allografts with arteriosclerosis had worse early function in their recipients reflected in higher serum creatinin levels compared to grafts with normal morphological findings. Acute tubular necrosis of renal allografts in our material statistically reduced graft viability, prolonged the delayed graft function time period. A similar value of tubular damage predicting graft loss was reported in renal allograft rebiopsies [2].

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Role of astroviruses in childhood diarrhea

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Human astrovirus (HAstV) is a significant cause of acute diarrhea among children, resulting in outbreaks of diarrhea and occasionally in hospitalization. Improved detection methods for eight antigenic types of HAstV and studies assessing the frequency and severity of HAstV diarrhea have further defined the impact of HAstV infections in children. These studies have shown that HAstV infections are clinically milder (diarrhea, vomiting, fever) than rotavirus infections. However, frequent coinfection of HAstV with rotavirus and caliciviruses in childhood diarrhea complicates the epidemiology. Seroprevalence studies have provided evidence that the majority of children are infected by HAstV by 6 years of age. The route of transmission is probably fecal-oral from food or water sources. Recent and planned studies will help to define the epidemiology and in the future lead to prevention strategies, which could include vaccination. Curr Opin Pediatr 2000, 12:275–279 © 2000 Lippincott Williams & Wilkins, Inc.

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Abbreviations

EIA	enzyme immunosorbent assay
HAstV	human astrovirus
RT-PCR	reverse transcriptase polymerase chain reaction

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In 1975, a year after rotavirus was identified, the starlike morphology of human astrovirus (HAstV) (*astro* means "star") was seen by electronmicroscopy in stool samples from a diarrhea outbreak among infants [1]. The characterization of HAstV molecular epidemiology has proceeded more slowly than that of rotavirus, the most common cause of severe diarrhea in children. The epidemiology of HAstV-associated diarrhea has changed by recent utilization of reverse transcriptase polymerase chain reaction (RT-PCR) and enzyme immunoassay (EIA) for virus detection. Human astrovirus is now found to be a common cause of viral diarrhea among young children in hospitals and in the community [2••,3]. Human astrovirus infections have been reported in 2% to 16% of children hospitalized with diarrhea and 5% to 17% of the children with diarrhea in community studies [2••,3–5,6•,7–9,10••,11••]. Although HAstV was detected less frequently than rotavirus or calicivirus in surveillance studies, astrovirus still requires close attention from a public health viewpoint because of large outbreaks in adults [12,13], frequent outbreaks in child care centers [2••,14••,15], and occurrence among immunocompromised patients [16••,17•]. The prevalence of HAstV infection varied by age groups and indicated that most children were infected during their first 2 years of life [3,18•,19]. The seroprevalences of type-specific HAstV antibodies in children from different locations (United States, Japan, Netherlands) were HAstV-1 (91% to 100%), HAstV-2 (31% to 100%), HAstV-3 (42% to 69%), HAstV-4 (56%), HAstV-5 (36%), HAstV-6 (16%), and HAstV-7 (10%) by 3 to 9 years of age, indicating frequent exposure to these enteric viruses in infancy and early childhood [18•,20•,21].

This review focuses on recent papers on the epidemiology and clinical characteristics of HAstV serotypes as infectious agents in diarrhea among hospitalized children, in children from the community, and in outbreaks. Improved detection methods and the seroprevalence of antibodies against astroviruses are discussed to support the importance of this emerging pathogen. Control measures are discussed, and, in the future, we may consider immunization strategies.

Epidemiology of human astrovirus infections

Several recent studies investigated viral causes for diarrhea among children who were hospitalized or in the community in different parts of the world (China, Chile, France, Finland) [2••,6•,10••,11••]. These studies defined the roles of four viral agents

(rotavirus, calicivirus, astrovirus, enteric adenovirus) in childhood diarrhea and assessed the severity of the symptoms caused by these infectious agents. Two of these studies also were designed to investigate the rotavirus vaccine.

The study in China [6•] was conducted between November 1994 and August 1996 among children 5 years of age or less hospitalized for acute diarrhea in Beijing. Stool samples from patients with diarrhea were investigated for the presence of rotavirus (RT-PCR), calicivirus (RT-PCR), astrovirus (electronmicroscopy), and adenovirus (electronmicroscopy). Astrovirus was the second most common viral agent after rotavirus and was detected in 8% of the hospitalized children. This frequency may be an underestimate as electronmicroscopy is less sensitive than EIA or RT-PCR.

In Chile [2••], the frequency of astrovirus infection was determined by EIA, RT-PCR, and cell culture in 292 children with diarrhea seen in an emergency department and among children hospitalized for acute diarrhea. Astrovirus was detected in 15% of rotavirus-negative and 6% of rotavirus-positive samples obtained from patients seen in emergency departments (median patient age, 5.5 months) and in 20% of rotavirus-negative and 8% of rotavirus-positive samples in hospitalized children (median age, 3 months). In Finland [10••], astrovirus was detected by RT-PCR in 144 (9%) of 1528 episodes of acute gastroenteritis from 102 children.

Nosocomial HAstV infections also are a concern. A study in Stanford, CA, demonstrated that 10% of inpatient children with diarrhea had HAstV infection and half of these infections were nosocomial [7].

Infections in child care centers

Child care settings are associated with an increased frequency and severity of infections including diarrheal diseases [22]. HAstV infections among children attending child care centers in the United States were previously reported in 7% to 36% of diarrhea outbreaks and also in sporadic cases of diarrhea. There also was a report of a child care center outbreak in South Africa [14••,15,23,24]. Investigators in Santiago, Chile detected HAstV in 11% of diarrhea episodes in eight child care centers between 1994 and 1995 [2••].

To further define the epidemiology, the distribution of HAstV antigenic types was assessed in child care center outbreaks and sporadic cases of diarrhea among children attending child care centers [14••]. Astrovirus antigenic types were determined in stool samples by RT-PCR and DNA sequence analysis. Eight of the 14 diarrhea outbreaks were associated with astrovirus in six child care centers, including five outbreaks associated with HAstV-1 and three outbreaks with HAstV-2. The two child care centers with a second outbreak in the same year both experienced an outbreak of a different type, indicating that two antigenic types were present in the community during one diarrhea season. This suggests that type-specific immunity resulting from a previous outbreak could allow a subsequent outbreak by a different type [14••].

Infection among immunocompromised patients

Grohmann *et al.* [25] suggested that the contribution of enteric viruses to the morbidity of AIDS patients may be more important than bacterial and parasitic enteropathogens. Two recent studies showed the association of HAstV with immunocompromised patients. Giordano *et al.* [17•] collected 244 stool samples from HIV-infected and uninfected patients with or without diarrhea and tested them for rotavirus, picobirnavirus, HAstV, and adenovirus. Astrovirus was detected in 4% of the HIV-infected patients with diarrhea and in 5.3% without diarrhea and therefore could not be associated with diarrhea in HIV-infected patients [17•].

Cubitt *et al.* [16••] reported an extensive outbreak of astrovirus in a hospital unit where pediatric patients were treated for leukemia or inherited immune deficiency disorders. Prolonged shedding of astrovirus for many months following infection was demonstrated in three patients who had undergone bone marrow transplantation. RT-PCR revealed that several patients were infected earlier and shed virus for longer than detected by either electronmicroscopy or EIA.

Source of infection

Despite scarce information, astrovirus appears to be an important cause of food-borne disease [26]. In June 1991, a large outbreak of HAstV-6 gastroenteritis occurred among students and teachers in Japan. The outbreak affected more than 4700 persons, lasted 5 days, and was believed to have been linked to contaminated food from a common supplier [12]. In another study, astrovirus was detected by RT-PCR in oysters collected in Chiba City Bay, Japan, from 1997 to 1998. Astrovirus was detected in 15 of 112 samples and the highest incidence was observed during January 1998. Human astrovirus was detected more frequently (13%) than hepatitis A virus (1.8%) or adenovirus (0%) and was comparable to the small round structured viruses (25%). This result indicates that astrovirus was detected in naturally grown oysters during the winter season [27]. Astrovirus infection was reported in a 27-year-old bone marrow transplant recipient with a history of consuming "sashimi" while visiting Japan before the bone marrow transplantation [28].

Detection of HAstV in water is not well studied. In England, RT-PCR was used to search for HAstV RNA

in concentrates of the samples of sewage, sea water, river water, sand, and silt collected from a sewage works [29]. No evidence of astrovirus was found in any sample, which suggests that contamination with this virus was not a problem in that region during the summer holiday season.

Seasonality

The astrovirus peak season is controversial. According to some studies, the peak of HAstV infection is in winter [6•,10••]. However, one prospective study from Mexico demonstrated no HAstV seasonality [30].

Type distribution

There are eight reported HAstV antigenic types. Neutralizing antibodies have been identified for HAstV types 1 to 7 [21,31]; therefore HAstV 1 to 7 can be considered different serotypes. Human astrovirus-1 was reported previously to be the most common and dominant serotype in HAstV infections [2••,7,10••,31]. However, five antigenic types were detected in Mexico City during a prospective surveillance, with HAstV-2 the most common (35%), followed by HAStV-4 (15%), HAstV-3 (11%), HAstV-1 (4%), and HAstV-5 (4%) [30]. A study in Japan using a typing RT-PCR method confirmed that multiple HAstV serotypes cocirculated in that community between 1981 and 1997 with continuous presence of HAstV-1, HAstV-4, and HAstV-3 and sporadic cases of HAstV-2, HAStV-5, and HAstV-6 [32•]. In addition, there is evidence for coinfection of different serotypes of HAstV at the same time [10••,33].

Clinical symptoms and severity of infections

Several studies highlighted frequent coinfections of HAstV, rotavirus, and calicivirus. For example, astroviruses were detected in 16% to 42% of rotavirus-associated diarrhea episodes [2••,11••,30]. These coinfections make the complete evaluation of clinical symptoms caused by astrovirus alone more difficult [6•] (Table 1).

Astrovirus-associated diarrhea has been characterized by a median duration of 3 days (range, 1 to 21), median of four stools (range, one to 10) during the first 24 hours, vomiting (20% to 62%), and fever (7% to 25%). Astrovirus-associated diarrhea was less severe than rotavirus, as measured by the number of stools and duration of the diarrhea. Fever and duration of vomiting and diarrhea were significantly more frequent during isolated rotavirus infections than in isolated astrovirus infections [10••,11••,30]. Only 0% to 3% of cases of astrovirus gastroenteritis resulted in dehydration, and no repeat symptomatic infections were observed. Very few astrovirus-infected children (3%) were hospitalized [10••,30]. In addition, bloody diarrhea and vomiting are statistically less common in viral than in bacterial gastroenteritis [11••] (Table 1).

Overall, the clinical severity of astrovirus gastroenteritis was much less than that of rotavirus gastroenteritis [30,34]. Thus the importance of this enteropathogen is evidenced by the number of children affected and not by the severity of disease.

Viral detection methods

Comparison of detection methods for HAstV showed RT-PCR to be the most sensitive, followed by EIA and electronmicroscopy [16••,24].

Enzyme immunosorbent assay

Enzyme immunosorbent assay incorporating monoclonal antibodies has become the most widely used screening method for detection of HAstV in research laboratories

Table 1. Clinical symptoms of human astrovirus infection versus rotavirus infection in childhood diarrhea

Studies	Clinical symptoms	Astrovirus	Rotavirus
Pang and Vesikari [10••], Bon *et al.* [11••], Guerrero *et al.* [30]	Diarrhea, %	72–100%	93%
Pang and Vesikari [10••], Guerrero *et al.* [30]	Duration of diarrhea, *d**	0–21 (mean 2–3)	0–12 (mean 4)
Pang and Vesikari [10••], Guerrero *et al.* [30]	Maximum stools/24 h*, *n*	0–10 (mean 4)	0–30 (mean 6)
Bon *et al.* [11••]	Bloody diarrhea, %	0	1
Bon *et al.* [11••]	Abdominal pain, %	50	19
Pang and Vesikari [10••], Bon *et al.* [11••], Guerrero *et al.* [30]	Vomiting, %	20–62	69
Pang and Vesikari [10••]	Duration of vomiting, *d*	0–4 (mean 1)	0–8 (mean 2)
Pang and Vesikari [10••]	Maximum vomiting/24 h*, *n*	0–10 (mean 1)	0–20 (mean 3)
Bon *et al.* [11••]	Fever, %	25	67
Pang and Vesikari [10••]	Maximum fever, C°	37.5–41.3 (37.8)	37.5–40.5 (38.9)
Pang and Vesikari [10••], Guerrero *et al.* [30]	Dehydration, %	0–5	17
Pang and Vesikari [10••], Guerrero *et al.* [30]	Severity score (1–20)*†	2–12 (mean 5)	3–19 (mean 11)

*$P < 0.01$. †Ruuska and Vesikari 20-point scoring system used [34].

[35]. EIA also was developed for differentiation of HAstV antigenic types [36]. However, the only commercial EIA for HAstV is not approved in the United States (IDEIA Astrovirus, Dako Diagnostics, Ltd., Ely, UK).

Reverse transcriptase polymerase reaction

Reverse transcriptase polymerase chain reaction is an alternative method of detection for HAstV. The single-stranded, positive-sense RNA genome encodes nonstructural proteins (viral serine protease and RNA-dependent RNA polymerase) and the capsid protein [37,38]. Oligonucleotide primers specific for the conserved serine protease region or for the 3′ end of the genome are suitable candidates for RT-PCR detection of all antigenic types [24,37]. RT-PCR detected the genome of HAstV in stool samples with greater sensitivity than EIA in child care center outbreaks, community studies, and a pediatric bone marrow transplant unit outbreak [3,10••,16••,24]. The sensitivity of virus detection by RT-PCR was further increased by culturing the virus in tissue culture [39]. Type-specific primers have been used to further define the epidemiology of human astroviruses [14••,33].

Seroprevalence

All eight types of astrovirus have been distinguished by EIA or immunoelectronmicroscopy. The presence of neutralizing antibodies to HAstV types 1 to 7 was detected, so HAstV types 1 to 7 are considered serotypes [21,31]. In all studies, the seroprevalence of any HAstV type in infants and children decreased at 6 to 8 months of age, consistent with loss of maternal antibodies.

Children from Norfolk, VA, developed antibody to HAstV-1 (94%) and to HAstV-3 (42%) by 6 to 9 years of age [20•]. Japanese children developed antibodies to HAstV-1 (100%) and HAstV-2 (100%) by 3 to 5 years of age [18•]. The seroprevalences of neutralizing antibodies in an age-stratified sample of the population in the Netherlands were HAstV-1 (91%), HAstV-3 (69%), HAstV-4 (56%), HAstV-5 (36%), HAstV-2 (31%), HAstV-6 (16%), and HAstV-7 (10%), indicating frequent exposure to these enteric viruses in infancy and early childhood. It is not known whether exposure to a serotype will provide homotypic protection in repeated infections with the same serotype. The relationship between different antigenic types and heterotypic protection is not completely understood either.

Prevention

Control measures for enteric infection outbreaks should focus on removal of the common source (ill child, contaminated water or food supply) and interruption of person-to-person transmission. However, improvement of environmental hygiene may not result in reduction of endemic diarrhea caused by viruses, so immunization may play an important role in the future.

Conclusions

As more sensitive assays have become available for astrovirus detection, the relative importance of HAstV in childhood diarrhea compared with that of the other common viruses has been reevaluated. Four studies in different parts of the world demonstrated the importance of HAstV in hospitalized children or among children with diarrhea in the community. Human astrovirus is a frequent pathogen in diarrhea outbreaks in child care settings and among immunocompromised patients. The route of infection is presumed to be fecal-oral from food or water sources. Isolated HAstV infections are clinically milder (diarrhea, vomiting, fever) than rotavirus infections. However, frequent coinfection of HAstV with rotavirus and caliciviruses in childhood diarrhea indicates that HAstV-associated diarrhea is not a single entity and may develop during infections with several pathogens. Seroprevalence studies provide evidence that children are frequently exposed to HAstV. Recent and future studies will help to define the epidemiology, leading to prevention strategies that could include vaccination in the future.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • Of special interest
- •• Of outstanding interest

1 Madeley CR, Cosgrove BP: 28nm particles in faeces in infantile gastroenteritis. Lancet 1975, ii:451–452.

2 •• Gaggero A, O'Ryan M, Noel JS, Glass RI, Monroe SS, Mamani N, et al.: Prevalence of astrovirus infection among Chilean children with acute gastroenteritis. J Clin Microbiol 1998, 36:3691–3693.

This study compared the frequency and clinical symptoms of several enteropathogens (including HAstV) among hospitalized children, children visiting emergency departments, and in child care settings. This study was well designed. Several sensitive methods were utilized for the detection of the pathogens and provided a picture of the broad range of pathogens involved with childhood diarrhea. Human astrovirus was proven to play a significant role in diarrhea of all three target groups.

3 Maldonado Y, Cantwell M, Old M, Hill D, Sanchez ML, Logan L, et al.: Population-based prevalence of symptomatic and asymptomatic astrovirus infection in rural Mayan infants. J Infect Dis 1998, 178:334–339.

4 Palombo EA, Bishop RF: Annual incidence, serotype distribution, and genetic diversity of human astrovirus isolates from hospitalized children in Melbourne, Australia. J Clin Microbiol 1996, 34:1750–1753.

5 Shetty M, Brown TA, Kotian M, Shivananda PG: Viral diarrhoea in a rural coastal region of Karnataka India. J Trop Pediatr 1995, 41:301–303.

6 • Qiao H, Nilsson M, Abreu ER, Hedlund KO, Johansen K, Zaori G, Svensson L: Viral diarrhea in children in Beijing, China. J Med Virol 1999, 57:390–396.

This is the first study to investigate the role of enteric viruses in childhood diarrhea in China. Human astrovirus-associated diarrhea was detected only by electronmicroscopy and not by the more sensitive EIA or RT-PCR methods. The astroviruses were not further characterized by type determination, nor was the severity of symptoms associated with HAstV infection evaluated.

7 Shastri S, Doane AM, Gonzales J, Upadhyayula U, Bass DM: Prevalence of astroviruses in a children's hospital. J Clin Microbiol 1998, 36:2571–2574.

8 Unicomb LE, Banu NN, Azim T, Islam A, Bardhan PK, Faruque AS, et al.: Astrovirus infection in association with acute, persistent and nosocomial diarrhea in Bangladesh. Pediatr Infect Dis J 1998, 17:611–614.

9 Cruz JR, Bartlett AV, Herrmann JE, Caceres P, Blacklow NR, Cano F: Astrovirus-associated diarrhea among Guatemalan ambulatory rural children. J Clin Microbiol 1992, 30:1140–1144.

10 •• Pang XL, Vesikari T: Human astrovirus-associated gastroenteritis in children under 2 years of age followed prospectively during a rotavirus vaccine trial. Acta Paediatr 1999, 88:532–536.

This was a well-designed, *prospective* study of enteric viruses in childhood diarrhea. The clinical characteristics of HAstV-associated diarrhea were compared with those of diarrhea caused by rotavirus. The effect of rhesus rotavirus vaccine–tetravalent vaccination on HAstV infections was addressed and no difference was shown in the clinical symptoms of the HAstV-infected children with or without rhesus rotavirus vaccine–tetravalent vaccination.

11 •• Bon F, Fascia P, Dauvergne M, Tenenbaum D, Planson H, Petion AM, et al.: Prevalence of group A rotavirus, human calicivirus, astrovirus, and adenovirus type 40 and 41 infections among children with acute gastroenteritis in Dijon, France. J Clin Microbiol 1999, 37:3055–3058.

A detailed study of the four major enteric viruses (rotavirus, calicivirus, HAstV, and adenovirus). Clinical characteristics of HAstV infections were described and compared with those of rotavirus infections. Diarrhea with multiple viruses is assessed.

12 Oishi I, Yamazaki K, Kimoto T, Minekawa Y, Nishimura H, Kitaura T: A large outbreak of acute gastroenteritis associated with astrovirus among students and teachers in Osaka Japan. J Infect Dis 1994, 170:439–443.

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14 •• Mitchell DK, Matson DO, Jiang X, Berke T, Monroe SS, Carter MJ, et al.: Molecular epidemiology of childhood astrovirus infection in child care centers. J Infect Dis 1999, 180:514–517.

This prospective study provides evidence of HAstV infections with *different* serotypes at the same time in the same community. The typing RT-PCR method for HAstV-1 and HAStV-2 was utilized.

15 Taylor MB, Marx FE, Grabow WO: *Rotavirus, astrovirus* and adenovirus associated with an outbreak of gastroenteritis in a South African child care centre. Epidemiol Infect 1997, 119:227–230.

16 •• Cubitt WD, Mitchell DK, Carter MJ, Willcocks MM, Holzel H: Application of electronmicroscopy, enzyme immunoassay, and RT-PCR to monitor an outbreak of astrovirus type 1 in a paediatric bone marrow transplant unit. J Med Virol 1999, 57:313–321.

This report of HAstV among immunocompromised patients emphasized the importance of utilizing sensitive methods for detection of HAstV. Human astrovirus infection was revealed earlier and could be followed longer by RT-PCR than by electronmicroscopy or EIA. Immunocompromised individuals excreted astroviruses for long periods.

17 • Giordano MO, Martinez LC, Rinaldi D, Espul C, Martinez N, Isa MB, et al.: Diarrhea and enteric emerging viruses in HIV-infected patients. AIDS Res Hum Retroviruses 1999, 15:1427–1432.

This study showed that diarrhea in AIDS patients was frequently associated with *enteric viruses*. Human astrovirus did occur in these patients but did not show a significant association with diarrhea.

18 • Kobayashi S, Kobayashi M, Araki K, Shinozaki T, Yanagawa Y: Antibody prevalence against astrovirus according to age groups. Kansenshogaku Zasshi 1999, 73:578–583.

This study from Japan provided evidence of frequent exposure to HAstV in childhood. Antiastrovirus antibodies against HAstV-1 and HAstV-2 were detected in nearly 100% of children by 3 years of age.

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Characterisation of a South African Human Astrovirus as Type 8 by Antigenic and Genetic Analyses

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Human astroviruses (HAstV) can, on the basis of immunoassays using type-specific rabbit antisera, be classified into eight serotypes that correlate with genotypes. Very few isolates of HAstV type 8 have been described and there is a paucity of data available with regard to the antigenic and genetic relationships between HAstV type 8 (HAstV-8) and HAstV types 1 (HAstV-1) to 7 (HAstV-7). A wild-type HAstV from a South African paediatric patient with diarrhoea was analysed antigenically, by immune electron microscopy and enzyme immunoassay, and genetically in selected regions of the ORF1a, ORF1b and ORF2 and characterised as a HAstV-8. This HAstV-8 strain exhibited greatest homology with HAstV-4 in the 5′ end of the capsid gene and ORF1a and 1b, and greatest homology with HAstV-5 in the 3′ end of the capsid region. This study confirms, by both antigenic and genetic analyses, that HAstV-8 represents a distinct antigenic and genotype and is the first report of a HAstV-8 from a hospitalised paediatric patient with diarrhoea in southern Africa. ***J. Med. Virol. 64:256–261, 2001.*** © 2001 Wiley-Liss, Inc.

KEY WORDS: astrovirus type 8; RT-PCR; serotype; genotype; homology; phylogeny

INTRODUCTION

Astroviruses (AstVs), one of the viruses associated aetiologically with human gastroenteritis [Kurtz, 1994], were first detected, by electron microscopy, in stool specimens from an outbreak of diarrhoea among babies in a maternity unit [Appleton and Higgins, 1975]. Although human astrovirus (HAstV)-associated diarrhoea occurs more often in children, HAstV infection has been reported in all age groups with the very young, the elderly and the immunocompromised at greatest risk [Glass et al., 1996]. The clinical relevance of HAstV infection has come to the fore with reports that, next to rotavirus, HAstVs are the second most common viral cause of gastroenteritis in children in Thailand and Guatemala [Matsui and Greenberg, 1996], France [Bon et al., 1999], and in selected settings in South Africa [Marx et al., 1998; Steele et al., 1998]. Immunofluorescence (IF) studies using reference antisera raised in rabbits identified seven distinct serotypes of HAstVs [Lee and Kurtz, 1982, 1994; Kurtz and Lee, 1984], with an additional and not yet fully characterised serotype, serotype 8 (1995: GenBank accession number Z66541) [Monroe, 1999; Monroe et al., 2000]. AstVs have also been associated with scours in young sheep and pigs and hepatitis in ducklings [Kurtz, 1994]. AstV infection has also been reported in calves, cats, dogs and turkeys [Kurtz, 1994; Matsui and Greenberg, 1996; Koci et al., 2000], with two distinct serotypes of bovine AstVs described [Monroe et al., 1995].

The AstV genome is a positive-sense, single-stranded RNA molecule approximately 6.8 kb in size [Carter and Willcocks, 1996] and has a 5′ non-coding region (NCR), three sequential open reading frames (ORFs) designated 1a, 1b, and 2, an eighty nucleotide (nt) 3 NCR and a poly-A tail (approximately 30 nt) at the 3′-end [Carter and Willcocks, 1996; Monceyron et al., 1997]. ORF1a and ORF1b encode non-structural proteins including the predicted protease and RNA-dependent RNA polymerase motifs respectively [Monroe, 1999]. The capsid protein precursor is encoded by ORF2 [Matsui and Greenberg, 1996], with the C terminus of the capsid region being more variable than the N terminus region [Carter and Willcocks, 1996; Hachiya et al., 1999]. The

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development of reverse transcriptase-polymerase chain reaction (RT-PCR) assays for regions within ORF1a, ORF1b and ORF2 has facilitated the sensitive detection, genotyping and phylogenetic analysis of HAstV isolates [Noel et al., 1995; Belliot et al., 1997; Cubitt et al., 1999; Mitchell et al., 1999; Mustafa et al., 2000], with a good correlation between serotypes and genotypes [Noel et al., 1995].

Phylogenetic analysis of molecular sequences is a novel and powerful tool for assessing genetic relatedness. This technique was used to confirm the close relationship of a South African isolate, AS20, to the reference HAstV type 8 (HAstV-8) [Z66541] strain by comparison of sequence alignment information from two different regions of the capsid gene. The relationship of AS20 to other known HAstV strains in two additional regions of the genome, ORF1a and ORF1b, is also described. The specific aim of this study was to determine the antigenic and genetic characteristics of AS20 and define it as a representative HAstV-8 serotype.

METHODS AND MATERIALS

Virus Isolate

A single stool specimen (415824), from a hospitalised South African paediatric (31 months of age) patient with gastroenteritis, was the source of the wild-type (WT) HAstV isolate, designated AS20, characterised in this investigation. The isolate was originally detected in the stool specimen by direct electron microscopy (EM) using the method outlined by Taylor et al. [1993] and shown to be cultivable in the PLC/PRF/5 human hepatoma cell line [Taylor et al., 1997]. The isolate was subsequently found to be detectable in a commercial astrovirus enzyme immunoassay (EIA)(IDEIA^{TM} Astrovirus: Dako Ltd, Ely, UK).

Antigenic Typing

Immune electron microscopy. The agar-diffusion method, as described by Cubitt et al. [1979] and Taylor et al. [1993], was used for the immune electron microscopy (IEM) studies. Reference rabbit antisera against HAstV serotypes 1 to 8 (HAstV-1:T59; HAstV-2:T57 HAstV-3:T61; HAstV-4:T62; HAstV-5:T63 HAstV-6:T64; HAstV-7:T66; HAstV-8:T67) were kindly supplied by Mr. T.W. Lee. Reference sera for each of HAstV-1 to 8 were diluted 1:100, 1:200, 1:400, 1:800 and 1:1,000 in sterile phosphate buffered saline (PBS) pH 7.4 and mixed with an equal volume of stool suspension (50% in water). Depending on the amount of antibody coating seen on the viral particles, on a scale from 0 (free particles) to 4+ (aggregates where the surface structure of the virus was masked), the isolate was considered to be reactive with that specific dilution of antisera.

Enzyme immunoassay. A typing enzyme immunoassay (EIA), adapted from the method described Noel et al. [1995], was used. The type-specific rabbit antisera were used as capture antibody and the HAstV group-specific monoclonal antibody (Mab), 8E7 [Herrman et al., 1988], as detector. The reaction was visualised using horse radish-peroxidase conjugated sheep anti-mouse IgG (Amersham International, Amersham, UK) and 3, 3′, 5, 5′ tetramethyl benzidine dihydrochloride (Kirkegaard and Perry Laboratories, Gaithersburg, MD) as substrate. The reaction to a specific serotype was considered positive if the absorbance was at least twice the absorbance to any of the other serotypes.

Oligonucleotide Primers

Published sets of primers were used for the amplification of different regions of the genome. Primers Mon2/PR6151, with an expected product size of 656 bp [Mitchell et al., 1999], were used for the amplification of the 3′ end of ORF2 region; primers Mon269/Mon270, with an expected product size of 449 nt (nt 4529–4977; HAstV-1 L23513), for the 5′ end of ORF2 [Noel et al., 1995]; primers Mon348/Mon340 for a 289 nt (nt 1182–1470; HAstV-1 L23513) region within ORF1a [Belliot et al., 1997]; and primers Mon343/Mon344, with an expected product size of 316 bp, for a region within ORF1b (nt 3679–3997; HAstV-1 L23513) [Belliot et al., 1997]. The primers were synthesised by Sigma-Genosys Ltd. (The Woodlands, TX).

Reverse Transcriptase-Polymerase Chain Reaction

Unless specified to the contrary, all reagents used were from Promega Corp. (Madison, WI). Viral RNA was extracted from a suspension (±10% in distilled water) of the stool using Trizol^{TM} reagent (Gibco BRL-Life Technologies, Rockville, MD) according to manufacturer's instructions. For all sets of primers reverse transcription was performed in a 50 µl reaction mixture containing 1× PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 3 mM $MgCl_2$), 1.25 mM each of four dNTPs, 4 U RNasin, 0.1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO), 1 µM reverse primer and 10 U avian myeloblastosis virus reverse transcriptase for 1 hr at 42°C. After the completion of the reverse transcription reaction, 50 µl PCR reaction mix, containing 1× PCR buffer, 5% DMSO (Sigma), 1 µM forward primer and 2.5 U *Taq* polymerase, was added to the reverse transcription reaction mix. The PCR reaction conditions for primer pairs Mon2/PR6151, Mon343/Mon344 and Mon 270/Mon269 were: 30 cycles of 1 min at 90°C, 2 min at 40°C and 1 min at 72°C with final extension for 7 min at 72°C. For primer pair Mon348/Mon340 the same program with an annealing temperature of 50°C was used. The PCR reaction products were analysed by agarose gel electrophoresis and visualised by ethidium bromide staining and UV illumination.

DNA Sequencing and Phylogenetic Analysis

RT-PCR products were cloned using the pGEM-T (Promega Corp.) vector and the cDNA was sequenced by fluorescent automatic sequencer (ALFexpress,

Amersham Pharmacia Biotech Inc., Uppsala, Sweden) using forward and reverse primers flanking the cloning cassette (SequiTherm Excel™ II Long Read DNA Sequence Kit; Epicentre Technologies, Madison, WI). The sequences of the amplified cDNA products were compared with available sequences for HAstV types 1 to 8. Previously published (EMBL/GenBank) reference sequences for each antigenic type available for the phylogenetic analysis included HAstV-1 (L23513), HAstV-2 (L13745), HAstV-3 (AF117209), HAstV-4 (Z33883 and L23510), HAstV-5 (U15136 and L23512), HAstV-6 (Z46658), HAstV-7 (L38508) and HAstV-8 (Z66541). Additional animal AstV sequences included feline AstV capsid sequence (AF056197) and turkey AstV complete genome sequence (AF206663). The complete capsid gene of HAstV-7 Oxford reference strain (AF248738), 246 nt (nt 1182–1470 of the HAstV-1 genome L23513) of HAstV-3 to HAstV-7 Oxford strains and AS20 in the ORF1a region (AF290504-09), 1255–64 nt (nt 3700–4957 of HAstV-1 L23513) of HAstV-3 to HAstV-7 Oxford strains (AF292074-78) and AS20 (AF29073) in the transition region of ORF1b and ORF2, and 568 nt (coding region) of the 3′ end of ORF2 (AS20: AF292079) were sequenced, deposited in GenBank, and available for comparative purposes.

Sequence manipulation and verification was performed using PC/Gene (v6.85; IntelliGenetics Inc., Geneva, Switzerland). ClustalX [Thompson et al., 1997] was used to create multiple alignments of the amino acid sequences of AS20 and the reference human and animal AstVs. The nucleic acid sequences were added and aligned by GeneDoc v2.3 [Nicholas et al., 1997] using the corresponding amino acid sequences as template. A phylogenetic tree was constructed from the nucleic acid sequence alignment using the program DNAML of the PHYLIP package (v3.52c), utilising the maximum likelihood algorithm, running in UNIX operating environment [Felsenstein, 1993]. The global rearrangement option was invoked and the order of the sequence input was randomised fifty times. To obtain rooted phylograms for the different genomic regions, animal and avian AstVs sequences were included in analyses as outgroups. In the highly conserved regions of ORF1a and ORF1b, the corresponding sequence from the genome of the reference turkey AstV served as an outgroup. Phylogenetic distances were compared for each tree (data not shown). Pairwise identity of nucleotide and amino acid sequences were calculated using the original alignment in GenDoc. The EIA and IEM typing results and the amino acid sequence identity of the 3′ end of the ORF2 capsid gene were compared among AS20 and all HAstV reference types.

RESULTS

Antigenic Typing

The results of the typing EIA studies using HAstV-1 to HAstV-8 type-specific antisera, shown in Table I, indicate that AS20 was antigenically most closely related to serotype 8, but no type 8 reference strain was available for comparison. The results of the EIA were confirmed by the IEM studies. Single particles and clusters of particles were seen on the control grid (virus + PBS) and preparations with dilutions of HAstV-1 to HAstV-7 reference antisera, but there was no evidence of antibody binding to the virus even at serum dilutions of 1:100. In contrast there were clusters of particles clearly showing binding of antibody and masking of surface structure in preparations of virus and HAstV-8 reference serum at dilutions of 1:100 to 1:400, but not 1:800.

TABLE I. Genetic Relationship (Percentage Amino Acid [aa] Identity in the 3′ End of the ORF2 Region [225 aa]) of the South African Isolate AS20 to the Prototypes of Human Astrovirus Type 1 (HAstV-1) to HAstV-8 Compared to Antigenic Typing of AS20, by Immune Electron Microscopy and Enzyme Immunoassay, Using HAstV-1 to 8 Type-Specific Antisera

Prototype viruses[a]	% Amino acid identity with AS20	Antigenic typing	
		EIA (OD)	IEM
HAstV-1	47%	0.39	<100[b]
HAstV-2	46%	0.27	<100
HAstV-3	53%	0.26	<100
HAstV-4	34%	0.26	<100
HAstV-5	64%	0.20	<100
HAstV-6	52%	0.44	<100
HAstV-7	48%	0.16	<100
HAstV-8	89%	0.88	400

EIA, enzyme immunoassay; OD, absorbance at 450nm; IEM, immune electron microscopy.
[a]GenBank references for the prototype strains were 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).
[b]Dilution endpoint: reciprocal of the highest dilution of antisera where antibody binding to the virus was evident.

Reverse Transcriptase-Polymerase Chain Reaction

RT-PCR with AS20 RNA resulted in amplicons of the expected size, 289 nt in ORF1a using primers Mon348/340, 319 nt in ORF1b using primers Mon343/344 and 449 nt in the 5′ end of ORF2 using primers Mon269/Mon270. An amplicon of 1255 nt, whose sequence conformed with the two previous sequences, was produced using primer pair Mon270/Mon344 but was not included for further phylogenetic analyses. In addition, AS20 reacted with the HAstV-1-specific primer pair Mon2/PR6151 in the 3′ end of the genome resulting in a product 656 nt in size.

Phylogenetic Analysis

Alignment of ORF1a, ORF1b, ORF2 5′ end and ORF2 3′ end sequences resulted in consensus lengths of 246, 282, 409 and 675 nucleotides, respectively, for Mon348/Mon340, Mon343/Mon344, Mon270/Mon269, Mon2/PR6151 products. Satisfactory alignment with the turkey sequence was obtained only in the ORF1b region of the genome. Feline AstV sequence was included for the analysis in the two regions of ORF2,

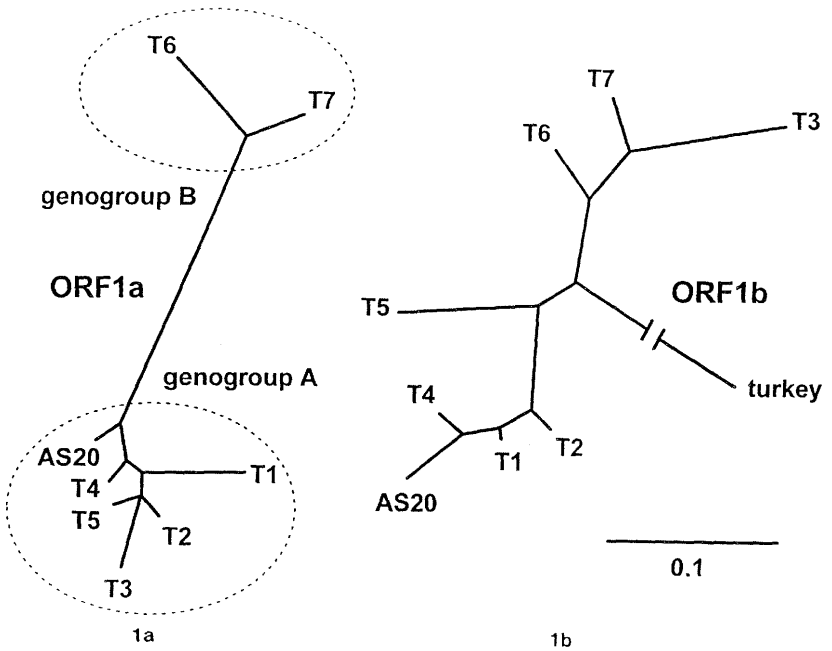

Fig. 1. Maximum likelihood phylogram representing the phylogenetic relationship of AS20 to prototypes of HAstV-1 to HAstV-7. **A:** 246 nt sequences from ORF1a were used for phylogenetic analysis. Branch points of the resulting tree (unrooted) had a confidence level of $P < 0.05$. **B:** 282 nt sequences from ORF 1b were used for phylogenetic analysis. Branch points of the resulting tree (rooted) had a confidence level of $P < 0.05$. Reference turkey AstV served as an outgroup. Scale bars = number of nucleotide substitutions per site.

but the trees could not be rooted because it has high similarity to the HAstV reference strains.

Phylogenetic analysis was performed on nucleotide sequences from these four regions of the AS20 genome and available sequences of the representative reference human, animal and avian AstV strains. Analysis of 246 nt consensus region in ORF1a exhibited that AS20 grouped with HAstV-1 to HAstV-5 in genogroup A as defined by Belliot et al. [1997]. AS20 clustered closest to HAstV-4 but the distance between the two was comparable to differences between other types within the genogroup (Fig. 1A). Pairwise comparisons reflected a similar relationship: HAstV-4 vs AS20 (95% nt, 98% aa identity) and intertype differences of 77–96% in nt and 88–100% in aa. Nucleotide sequence data for the reference HAstV-8 strain were not available for this region.

Analysis of a 282 nt consensus region in ORF1b exhibited that AS20 again grouped closest to HAstV-4 but was significantly different from any of HAstV-1 to HAstV-7. Corresponding sequence from the RNA-dependent RNA polymerase region of turkey AstV genome served as an outgroup (Fig. 1B). Pairwise comparisons reflected a similar relationship: HAstV-4 vs AS20 (93% nt, 95% aa) and intertype differences of 80–96% in nt and 91–97% in aa. There was no available sequence for reference or other HAstV-8 isolates in this region.

Analysis of a 409 nt consensus region of the 5′ end of the ORF2 exhibited that AS20 grouped closest to HAstV-8, with the pairwise similarity being higher (96% nt, 92% aa) than for any other type differences (78–88% nt, 86–96% aa). When compared with HAstV-1 to 7, the reference HAstV-8 and AS20 clustered closest to HAstV-4 (Fig. 2A). Analysis of 675 nucleotides of the 3′ end of ORF2 exhibited that all reference human and feline AstVs were separated with confidence. The feline sequence appeared closer (nucleotide identity 41–49%) to HAstV reference strains 1–3 and 5–8 than to HAstV-4. Therefore, it could not provide rooting for the tree. AS20 again was closest to the reference HAstV-8 strain (94% nt, 89% aa). In comparison to the other HAstV types, AS20 and the reference HAstV-8 strains were closest to HAstV-5 and clearly distinct from HAstV-4 (Fig. 2B). Correlation was noted between the genetic typing of AS20, using the percentage sequence identity of AS20 to the HAstV reference strains in the 3′ end of the ORF2 (225 aa), and antigenic typing, as determined by EIA and IEM using HAstV type specific sera (Table I).

DISCUSSION

HAstV-1 has been reported to be the most prevalent serotype detected in the United Kingdom [Lee and Kurtz, 1994; Noel and Cubitt, 1994], Norway [Kjeldsberg, 1994], United States of America, Korea [Noel et al., 1995], Australia [Palombo and Bishop, 1996; Mustafa et al., 2000] and Japan [Sakamoto et al., 2000]. HAstV-2 to HAstV-5 seem to be less common and HAstV types 6 and 8 seldom detected [Glass et al., 1996; Monroe, 1999]. The occurrence of single isolates of HAstV-8 have been reported from Ghaza and Uganda [Monceyron et al., 1997], Australia (1997) [Mustafa

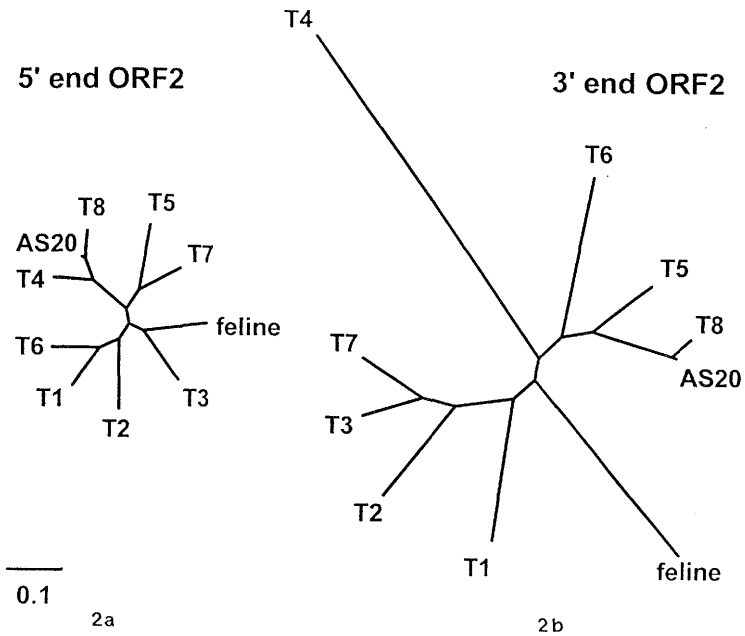

Fig. 2. Maximum likelihood phylogram representing the phylogenetic relationship of AS20 and HAstV-1 to HAstV-8 prototypes. **A:** Partial capsid gene sequences (409 nt) from the 5′-end of the ORF2 were used for phylogenetic analysis. The overall tree pattern is significant ($P < 0.01$). This is an unrooted tree. **B:** Partial capsid gene sequences (675 nt) of the 3′-end of the ORF was used for phylogenetic analysis. The overall tree pattern is significant ($P < 0.05$). This is an unrooted tree. Scale bars = number of nucleotide substitutions per site.

et al., 2000], Pakistan (1993) [Sakamoto et al., 2000] as well as an unspecified source (1995)[GenBank accession number Z66541]. Recently Monroe et al. [2000] reported ten HAstV-8s among 76 HAstV African (Egypt and Malawi) isolates identified by molecular typing. There is, however, very little, data available with regard to the antigenic and genetic relationships between HAstV-8 and HAstV-1 to HAstV-7.

Complete nucleotide sequences are available for HAstV-1 [Willcocks et al., 1994], HAstV-2 [Jiang et al., 1993], HAstV-3 Berlin strain (AF141381) and capsid protein sequences are available for HAstV-1 to HAstV-7 [Carter and Willcocks, 1996]. The complete capsid sequence from ORF2 is available for the prototype HAstV-8 (GenBank accession number Z66541). These sequence data have facilitated confirmation of the finding that HAstVs could be grouped genetically and that these groups correlated with the serotypes [Noel et al., 1995; Belliot et al., 1997]. Phylogenetic analysis of nucleotide sequences from two different regions of the capsid gene of AS20 confirmed its close relationship to the capsid gene of the reference HAstV-8 strain. Therefore, the South African isolate AS20 can be classified as HAstV-8 genetic type. This corresponds with the antigenic characterisation of AS20, by EIA and IEM, as HAstV-8. The high background optical densities observed in the typing EIA (Table I) could be ascribed to the high degree of cross-reactivity occurring with type-specific antisera due to the presence of a group antigen [Herrmann et al., 1988; Noel et al., 1995]. Intertypic relationships between the reference and South African HAstV-8 strains and the other reference HAstV types exhibited that HAstV-8 exhibited greatest homology with HAstV-4 in the 5′ end (Fig. 2A) and to HAstV-5 in the 3′ end of the capsid gene (Fig. 2B). The differences in relationships among HAstV-8 and HAstV-1 to HAstV-7 can be explained by the different degree of variability between the 3′ and 5′ ends of the capsid gene. This is well represented by the different resolution of the two phylograms obtained from these regions (Fig. 2A,B). A similar relationship to that shown between HAstV-8 and HAstV-4 in the 5′ end of ORF2 was illustrated for a 348 nt region of ORF2 [Monroe, 1999].

Nucleotide sequence data from conserved areas within ORF1a from HAstV-1 to HAstV-7 indicate that HAstVs fall into two distinct genogroups, but no HAstV-8 sequence data were included [Belliot et al., 1997]. Phylogenetic analysis of the genome of AS20 in ORF1a and ORF1b exhibited that distances between AS20 and available sequences of reference strains of HAstV-1 to HAstV-7 were comparable to those among other HAstV types. AS20 exhibited the greatest identity with HAstV-4 in both regions. These results suggest that AS20, and consequently HAstV-8, falls within HAstV genogroup A as defined by Belliot and colleagues [Belliot et al., 1997].

In conclusion, the sequence data of AS20 in the ORF1a and ORF1b regions provide valuable new information on the genetic relationships of HAstV-8 with HAstV-1 to HAstV-7. This is the first study that confirms, by both antigenic and genetic analyses, that HAstV-8 represents a distinct antigenic and genotype, sorting within HAstV genogroup A in ORF 1a. In

addition, this is the first report of a HAstV-8 from a hospitalised paediatric patient with diarrhoea in southern Africa.

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A human astrovirusok jelentősége a gyermekkori gastroenteritisekben

Jakab Ferenc,** Walter Jolán, dr.*** Szűcs György, dr.**

Összefoglalás

A rota- és calicivirusok felfedezése után, 1975-ben egy újabb vírust írtak le hasmenéses esetek elektronmikroszkópos vizsgálata során. A jellegzetes csillagszerű morphologiai sajátosság alapján, a kicsiny, kerek vírusokat astrovirusoknak nevezték el (astro=csillag). Az astrovirusok világszerte elterjedtek, és mind szórványos, mind járványos gastroenteritist okoznak, főleg kórházban ápolt csecsemőkben, bölcsődés gyermekek körében és bizonyos esetekben felnőttekben, idősekben és immuncompromittált betegekben is. Az eddigi tanulmányok alapján az összes human viralis eredetű gastroenteritis 4-20%-át okozzák astrovirusok. A fertőzés a rotavíruséhoz hasonló, de jóval enyhébb tünetekkel jár. Magyarországon 1995-ótа folyik a vírus diagnosztikai kimutatása és kutatása. Ez idő alatt járványos astrovirus fertőzést nem sikerült kimutatni, de szórványos astrovirus fertőzések gyakran előfordultak.

Történeti áttekintés

1975-ben Madeley és Cosgrove egy angliai — csecsemőket érintő — gastroenteritis járványból gyűjtött széklet-mintákban egy új gastroenteralis vírust figyelt meg és írt le. Az elektronmikroszkópos képen látható csillagszerű structurájuk alapján e kis kerek vírusokat astrovirusoknak nevezték el (astro=csillag).[25,26] Ezzel a felfedezéssel kezdődött a közel negyed-százados kutatás, ami alapján a human astrovirust (HAstV) ma a négy legfontosabb viralis hasmenést okozó ágens között említhetjük (a rotavirus, calicivirus és enteralis adenovirus mellett). A vírus megismerésében jelentős lépés volt a 80-as évek elején a vírusok sikeres tenyésztése sejtkultúrában, amely egyértelműen megkülönböztette őket a Norwalk és egyéb human calicivirusoktól, melyek *in vitro* még ma sem tenyészthetők.[21] Szintén ezekben az években Herrmann és munkatársai sikeresen adaptálták az ELISA (enzyme-linked immunosorbent assay) vizsgálatot a vírus gyors kimutatására.[9,10] A viralis nucleinsav teljes sequentiájának ismeretével nagymértékben fejlődött a vírus molekuláris szerkezetének analysise is,[41] és a genetikai elemzések alapján Jiang és munkatársai bebizonyították, hogy az astrovirusok önálló családot (*Astroviridae*) alkotnak.[15] Jonassen és munkatársai pedig kifejlesztettek 1995-ben egy revers transcriptio-polymerase láncreakción (RT-PCR) alapuló módszert, amivel hatékonyan tudták kimutatni az astrovirusok különböző serotypusait.[16]

A kimutatás érzékenységének növekedésével az astrovirusok jelentősége a gastroenteritisekben egyre egyértelműbbé vált. 1991-től, gyermekeket érintő megbetegítő szerepének igazolásával e vírusok orvosi jelentősége tovább nőtt.[11] Újabb fordulatot hozott az astrovirus fertőzések epidemiológiájában, amikor 1991-ben Japánban több mint 4700 személyt, iskolásokat és tanáraikat egyaránt érintő hasmenéses járványban human astrovirust tudtak kimutatni,[33] 1997-ben pedig francia kiskatonák közötti hasmenéses járványban bizonyították a szerepét.[1] Ez alátámasztotta azt a feltételezést, hogy nemcsak gyermekeket fertőzhet meg az astrovirus. Ezért napjainkban ennek a vírusnak a vizsgálata is indokolt mind a sporadikus, mind az epidemiás gastroenteritisekben.

A human astrovirusok általános jellemzői

A human astrovirusok hozzávetőlegesen 28-30 nm átmérőjű, ikozahedrális szimmetriájú, gömb alakú, burok nélküli particulumok, öt- vagy hatágú csillaghoz hasonló felszíni structurával. E legjellemzőbb morphologiai tulajdonságuk csupán a vírusok kb. 10%-ában figyelhető meg (*1. ábra*).

Az astrovirusok mind emberben, mind pedig fiatal emlősökben (sertés, borjú, bárány, macska, kutya) enyhe gastroenteritist okoznak. Madarakban súlyosabb, más szervrendszert is érintő betegséggel járhat az astrovirus (AstV) fertőzés; például kiskacsákban fatalis hepatitist okozhatnak, és létezik egy madár nephritist okozó astrovirus is.[12] A human és állati AstV-okon belül számos

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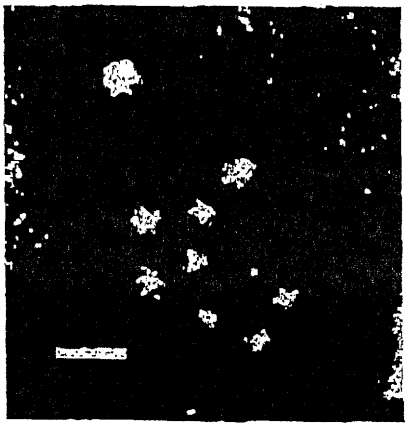
Rövidítések: AstV — astrovirus; HAstV — human astrovirus; ELISA — enzyme-linked immunosorbent assay; RT-PCR — revers transcriptio-polymerase láncreakció; ORF — open reading frame; Pro — protease; Pol — polymerase.

1. ábra. Human astrovirusok elektronmikroszkópos képe jól felismerhető 5-6 ágú csillagszerű struktúrával (F.P. Williams, United States Environmental Protection Agency)



serotypus létezik. Jelenleg nyolc különböző HAstV antigéntípust ismerünk, és neutralisatiós tesztben 7 serotypust azonosítottak.[28]

A vírusgenom körülbelül 6800 nucleotid hosszúságú, egyfonalú, pozitív-szálú RNS, mely egy poly-A farokkal rendelkezik a genom 3' végén (*2.ábra*). A genomban három fehérjét kódoló regiót (open reading frame: ORF) különböztetünk meg. Az 5' véghez közelebb álló, ORF1a és ORF1b a vírus szaporodásához nélkülözhetetlen nem structuralis fehérjéket: proteaset (Pro) és polymerasét (Pol), míg a 3' végnél lévő ORF2 regio a virion capsidját felépítő három fő structuralis fehérjét[23,32,40] kódolja. Ezek egyenként körülbelül 33, 24 és 29 kDa molekulatömegűek és a capsid praecursor-fehérje hasítása során keletkeznek.[14] Található még egy, genomon kívüli (subgenom) RNS is, ami körülbelül 2400 nucleotid hosszúságú és a genom capsid-regiójával azonos. Fertőzéskor a sejtben mind a teljes genom, mind pedig a subgenom RNS replicálódik.[27,32]

Az astrovirusok taxonómiai helye a teljes genom analysisével tisztázódott. 1993-ban Jiang és munkatársai a genom szerkezeti jellegzetességei és az általuk kódolt fehérjék alapján a vírust új családba sorolták *Astroviridae* néven.[15]

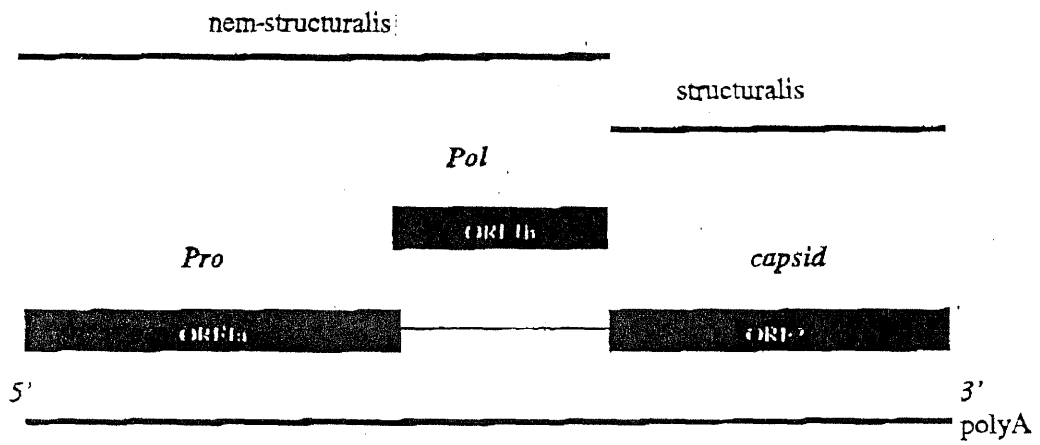
Az astrovirus fizikai tulajdonságai közül kiemelkedő, hogy aktivitását még 60 C°-on is 5-10 percig megőrzi, és nagyon alacsony hőmérsékleten (–70, –85 C°-on) akár 6-10 évig is fertőzőképes maradhat.[42] A vírus ezen tulajdonsága lehetővé teszi, hogy szobahőmérsékleten is könnyen terjedjen. A vírus stabilitását még rendkívül savas környezetben (pH 3,0) is megőrzi; emellett chloroform, különböző detergensek és lipid-oldószerek hatásával szemben is meglehetősen ellenálló.[20]

Epidemiológia

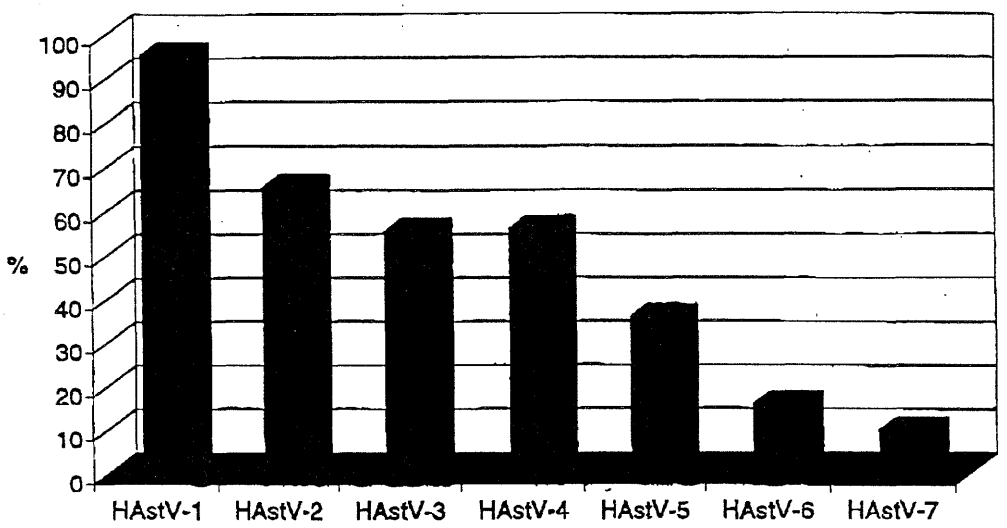
Az astrovirusok világszerte elterjedtek, és szórványos, valamint járványos gastroenteritist egyaránt okoznak. Főleg kórházban ápolt csecsemőkben, bölcsődés gyermekek körében és bizonyos esetekben felnőttekben, idősekben és immuncompromittált betegekben váltanak ki klinikai tüneteket. A jelenleg ismert 8 HAstV serotypus közül eddig az 1-es típus dominanciáját észlelték. Három távolálló földrajzi regio adatai alapján (Amerikai Egyesült Államok, Japán, Hollandia) is egyértelműnek látszik, hogy a fejlett országokban a leggyakoribb, dominans serotypus az 1-es, ami ellen már a két éves korosztálynak is közel 100%-a ellenanyagokkal rendelkezik.[17,19,31] A HAstV fertőzés direkt bizonyítékát jelentő székletvizsgálatok alapján tudjuk, hogy fejlődő országokban a HAstV-1 kevésbé dominans és több serotypus is jelen lehet egyszerre a populációban.[38] Emellett különböző HAstV serotypusok egyidejű fertőzése is előfordulhat.[17,19,29,31,34] Mindez lehetőséget ad a vírusok genetikai állományának rekombinálódására.[39]

A világ különböző országaiban végzett vizsgálatok alapján a HAstV-ok a sporadikus viralis eredetű hasmenéseknek 4-20%-át okozzák. Kínában a vírusos hasmenéses esetek 8%-ában volt kimutatható — a második leg-

2. ábra. A human astrovirus-genom szerkezete.



3. ábra. Három különböző földrajzi területről gyűjtött serummintákban a típusspecifikus ellenanyagok előfordulása alapján a human astrovirus serotypusok megoszlása (Amerikai Egyesült Államok, Japán, Hollandia). [17,19,31]



gyakoribb ágensként a rotavirus után.[35] Hasonló százalékban volt jelen Thaiföldön, ahol a megbetegedések 8,6%-át,[11] Guatemalában 7,6%-át,[11] Finnországban 9%-át,[34] és Franciaországban 6%-át okozta.[2] Igen magas százalékban volt kimutatható a vírus Chilében, ahol a gastroenteritis súlyos következményei miatt sürgősségi ellátásban részesült gyermekek székletmintáinak 15%-ában, a kórházi ellátásra szorultak székleteinek pedig mintegy 20%-ában volt human astrovirus.[6] Az Amerikai Egyesült Államokban végzett tanulmány szerint, az óvodások körében előforduló járványok 4-6%-áért az astrovirus volt felelős.[22,30]

Magas kockázatú csoportot alkotnak a kisgyermekek mellett az immuncompromittáltak és idősek is. Human immundeficientia vírussal (HIV) fertőzött felnőttekben a viralis hasmenéses esetek nagy részéért a HAstV-t teszik felelőssé.[7] HIV fertőzött gyermekek között a HAstV gyakrabban fordult elő, mint a nem fertőzöttekben, de a HAstV növekvő jelenléte nem eredményezett több, vagy súlyosabb hasmenést.[24] Csontvelő-transzplantáción átesett betegekben az enteralis vírusok mellett, az astrovirusok is okozhatnak súlyos hasmenést az esetek 13%-ában.[4] Angliában csontvelő-transzplantáción átesett gyermekek között a HAstV hónapokig tartó járványt okozott.[5] Több esetben írtak már le egyidejű fertőzést human calicivirusokkal és rotavirusokkal is. A HAstV fertőzés szezonalítása még vitatott. Néhány tanulmány szerint a téli hónapokban magasabb a fertőzöttség aránya, mint az év többi részében.[34,35]

Hazánkban 1995 óta folyik a vírus diagnosztikai kimutatása és kutatása az Állami Népegészségügyi és Tisztiorvosi Szolgálat Baranya Megyei Intézetének Regionális Virológiai Laboratóriumában, Pécsen. A vírus kimutatására mind az ELISA, mind az RT-PCR módszert alkalmazzuk. Számos törzset sikerült szövettenyészetben is isolálnunk, és több serotypus kimutatása is sikerrel járt.[13] Az elmúlt években bizonyítható, járványos astrovirus fertőzést nem találtunk, azonban szórványos esetek gyakran előfordultak, főleg az öt év alatti korosztály körében.[36]

Pathogenesis

Az astrovirus fertőzés pathogenesise még nem teljesen ismert. Az eddigi eredmények alapján tudjuk, hogy a vírusok a vékonyból epithel sejtjeiben szaporodnak villus atrophiát okozva. Szaporodásukat a villusok alsó részének epithel sejtjeiben tudták leginkább kimutatni.

Laboratóriumi körülmények között a vírus szaporodásához nélkülözhetetlen a trypsin. (Rotavirus esetén a trypsin *in vivo* és *in vitro* szerepe az, hogy az egyik felszíni fehérjét [VP4] hasítja, ami elősegíti a vírus penetrációját.[18]) Az astrovirus szaporodásában a trypsin szerepe viszont még nem ismert.

Átvitel

Hasonlóan a többi gastroenteralis vírushoz, a human astrovirusok is leggyakrabban faecalis-oralis úton terjednek, de előfordulhat fertőzés közvetlen ember-ember kontaktus útján is. Ez lehet az oka a kórházi vagy családon belüli megbetegedéseknek. A fertőzésekben átvivő közegként szerepelhet astrovirussal szennyezett víz,[3] illetve étel is (pl. osztriga).

Klinikai sajátosságok

A human astrovirusok a rotavirus fertőzéshez hasonló, de jóval enyhébb tüneteket okoznak (*1. táblázat*). Leggyakrabban heveny hasmenéssel társul a fertőzés (72-100%), mely általában két-három napig tart, de akár 21 napig is elhúzódhat, naponta átlagosan négyszeri (1-

10) székletürítéssel. További jellemző tünetek még az émelygés, hányinger, hányás, mely az esetek 20-62%-ában van jelen, 1-4 napig tart, naponta átlagosan 1-2 alkalommal ismétlődve. A fertőzések 7-25%-a jár együtt kb. 37-41 C°-os lázzal. Mindezen jellemző tünetek mellett felléphet még hasi fájdalom (50%), fejfájás és a hasmenés okozta dehydratio (0-5%).[2,3,34]

1. táblázat. Az astro- és rotavirus fertőzések klinikai tüneteinek összehasonlító táblázata[27]

Klinikai jellemzők	Astrovirus	Rotavirus
Hasmenés (%)	72-100	93
Hasmenés időtartama (nap)	0-21	0-12
Hasmenés gyakorisága / 24 óra	0-10	0-30
Véres hasmenés (%)	0	1
Hasi fájdalom (%)	50	19
Hányás (%)	20-62	69
Hányás időtartama (nap)	0-4	0-8
Hányás gyakorisága / 24 óra	0-10	0-20
Láz (%)	25	67
Maximum láz (°C)	37,5-41,3 (37,8)	37,5-40,5 (38,9)
Dehydratio	2-12	3-19

Egy-négy napos inkubációs idő után jelentkezhetnek a klinikai tünetek. Bizonyos esetekben azonban a fertőzés tünetmentesen is lefolyhat.

Immunitás

A gyermekkori fertőzéssel hosszú távú védettség alakul ki. Az idősek között előforduló esetek azonban arra utalnak, hogy a szervezet ellenanyagszintje és immunválasz adó képessége e vírusra is az öregedéssel csökken.[28]

Laboratóriumi diagnosztikai lehetőségek

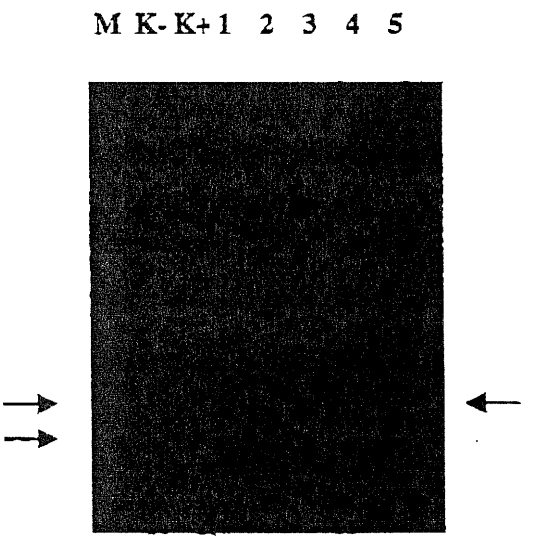
A vírusok diagnosztizálásának legelső és legrégibb módja a székletminták direkt elektronmikroszkópos (EM) vizsgálata. Az EM vizsgálat jó hatásfokkal detektálja az astrovirust, ha a vírusparticulumok igen nagy számban vannak jelen a mintákban (10^8-10^{10}/ml).[20] Napjainkban már e módszer kevésbé alkalmazható rutin diagnosztikai vizsgálatok elvégzésére, mivel nagyszámú minta tesztelését nem teszi lehetővé. Az elektronmikroszkópos vizsgálat másik hátránya, hogy nem ad mindig egyértelmű eredményt, hiszen a vírusok mindössze 10%-án látható a jellegzetesen csillag alakú structura.

Sokkal inkább elterjedt a vírus kimutatására szolgáló immunológiai módszer, az ELISA, mely már kereskedelemben is kapható (DAKO, Dánia). Lényege, a vírus mono- és polyclonalis savókkal történő kimutatása az úgynevezett szendvics-ELISA rendszerben. A módszer érzékenysége 91%-os, specifitása pedig 98%-os értéket mutat. Előnye, hogy lehetővé teszi nagyszámú minta gyors, és megbízható vizsgálatát.

A hagyományos virológiai módszernek számító tenyésztés rutin diagnosztikai célokra nem használható. Ennek fő oka egyrészt, hogy a vírusok nehezen szaporíthatók szövetkultúrában, másrészt, hogy a mikroszkópos morphologiai kép alapján egyértelműen nem lehet következtetni a vírus szaporodására. Ennek oka a magas trypsin tartalmú közeg hatása, mely a vírus cytopathogen hatásához hasonló elváltozásokat okoz a sejteken. Az eddigi tanulmányok azt bizonyítják, hogy a vírus leginkább human colon carcinoma (CaCo2) sejtvonalon tenyészthető trypsint tartalmazó közegben. Hazánkban is több ízben sikerült már astrovirust isolálni ezen a sejtkultúrán (Szűcs, Jakab).

Napjainkban a legérzékenyebb módszer a viralis RNS-nek specifikus RT-PCR módszerrel való kimutatása, ami megfelelően felszerelt laboratóriumot és felkészültséget igényel. E módszerrel mind a szövetkultúrában szaporított, mind pedig a székletmintákban található astrovirusból kivont RNS jól detektálható. A *4. ábrán* szövetkultúrában szaporított human astrovirusok specifikus primerekkel elvégzett amplificatiójának eredménye látható ethidium bromiddal történt festést követő UV megvilágítás alatt.

4. ábra. Szövetkultúrában szaporított HAstV-ok RT-PCR vizsgálatának eredménye a 89 bp nagyságú ampliconok kimutatásával. (M – marker, 60 és 100 bp jelölve; K- - negatív kontroll; K+ - pozitív kontroll /HAstV típus-1/; 1-5 – klinikai HAstV isolatumok)



Terápia és megelőzés

A betegség legtöbbször enyhe lefolyású; ha szükséges, folyadék- és electrolyt pótlással jól kezelhető.

A megelőzésnek kulcsszerepe van az astrovirus okozta gastroenteritisekben is. A megfelelő higiénés követelmények betartásával (kézmosás, tiszta ivóvíz, stb.) a fertőzés veszélye csökkenthető. Gyakran azonban a higiénés

intézkedések önmagukban nem csökkentik eléggé a HAstV okozta hasmenések előfordulását. Végleges megoldást egy oltóanyag jelentene, de jelenleg még várni kell egy olcsó és hatékony vaccina kidolgozására.

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Jakab, F., Walter, J., Szűcs, Gy.: **Human astroviruses in children with gastroenteritis**

Summary

In 1975, after the first detection of rotaviruses and caliciviruses, a new virus was observed and described. These small, star-shaped round viruses were named astrovirus based on their characteristic star-like structure (astro=star). Astroviruses were detected worldwide in sporadic and epidemic gastroenteritis, mainly among hospitalized infants, children attending day care centers and in certain cases, among adults, elderly and immunosuppressed patients. Human astrovirus is the causative agent of 4-20% of the sporadic cases of viral gastroenteritis. Astrovirus infection is similar to rotavirus infection but with milder symptoms. Routine detection of astrovirus has been available in Hungary since 1995. Sporadic cases were frequently revealed but astrovirus epidemic has not yet been identified in the country.

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Comparative Analysis of Infection Caused by Human Astrovirus Types Among Children Hospitalized in the United States

Jolan E Walter [1], Ferenc Jakab [1], Ester Parada Ricart [1], Carol Afflerbach [1], Mary A Staat [2], Parvin Azimi [3], Tamas Berke [1], David O Matson [1] and Douglas K. Mitchell [1]. [1]Center for Pediatric Research, Children's Hospital of The King's Daughters, Eastern Virginia Medical School, Norfolk, VA; [2]Division of Infectious Disease, Children's Hospital Medical Center, Cincinnati, OH; and [3]Infectious Disease Section, Children's Hospital Oakland, Oakland, CA.

Background:
Objective: To evaluate the demographics, clinical characteristics, and type-specific differences of human astrovirus (HAstV) infections among hospitalized children with gastrointestinal symptoms at three geographic sites in the US.
Design/Methods: Children hospitalized between November 1997 and October 1999, 15 d- 5 yr of age, with at least one symptom of diarrhea, vomiting, or fever were evaluated. Demographic information and stool samples were collected. Viral RNA was extracted from stool specimens by TRIZOL method and HAstV was detected by type-common reverse transcriptase–polymerase chain reaction (RT-PCR). Presumptively positive samples with a amplicon of the correct size (289nt) were further analyzed by additional RT-PCR in the capsid region for confirmation and genotyping.
Results: 2413 children were enrolled and 1842 stool samples were tested. HAstV was confirmed in 46 (2.5%) of hospitalized children. Co-infections with rotavirus (N=9, 20%) and human calicivirus (N=1, 2%) were observed. Multiple HAstV types (predominantly HAstV-2 and HAstV-4) were present in the community from late January to June 1998. The type predominance shifted to HAstV-1 in the following diarrhea season ($p<.02$). Clusters of

hospitalization among children infected with an individual type (HAstV-1, 2 or 4) were observed. Children less than 6 months of age were significantly more likely to be infected with HAstV-1 than other HAstV types ($p<.008$). Children hospitalized for HAstV infection were less likely to have an admission diagnosis for gastroenteritis than other children in our study with RV infections ($p<.002$).

Conclusions: Multiple HAstV types are present simultaneously in the community and the predominant type changes over time. Clusters of the same type suggest hospitalization due to community outbreaks. Young infants are more likely to be infected by HAstV-1 than other by HAstV types. Infections by HAstV as a sole enteric agent are more likely to result in hospitalization for non-gastroenteritis diagnosis than RV, even if symptoms of gastroentiritis are present.

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