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Human Astroviruses

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SUMMARY

Human astroviruses (HAtVs) are positive-sense single-stranded RNA viruses that were discovered in 1975. Astroviruses infecting other species, particularly mammalian and avian, were identified and classified into the genera *Mamastrovirus* and *Avastrovirus*. Through next-generation sequencing, many new astroviruses infecting different species, including humans, have been described, and the *Astroviridae* family shows a high diversity and zoonotic potential. Three divergent groups of HAstVs are recognized: the classic (*MAstV 1*), HAstV-MLB (*MAstV 6*), and HAstV-VA/HMO (*MAstV 8* and *MAstV 9*) groups. Classic HAstVs contain 8 serotypes and account for 2 to 9% of all acute nonbacterial gastroenteritis in children worldwide. Infections are usually self-limiting but can also spread systemically and cause severe infections in immunocompromised patients. The other groups have also been identified in children with gastroenteritis, but extraintestinal pathologies have been suggested for them as well. Classic HAstVs may be grown in cells, allowing the study of their cell cycle, which is similar to that of caliciviruses. The continuous emergence of new astroviruses with a potential zoonotic transmission highlights the need to gain insights on their biology in order to prevent future health threats. This review focuses on the basic virology, pathogenesis, host response, epidemiology, diagnostic assays, and prevention strategies for HAstVs.

INTRODUCTION

Using electron microscopy (EM), Appleton and Higgins reported in 1975 the occurrence of 28- to 30-nm particles in stools of children suffering from vomiting and mild diarrhea (1). The same year, Madeley and Cosgrove used the term astrovirus (AstV) to describe the small round viruses with a characteristic star-like appearance ("astron" means star in Greek) found in the feces of hospitalized infants with gastroenteritis (2). These particles were placed together with other viruses with a smooth entire edge within the category of "small round viruses" (SRVs), which differed from the "small round structured viruses" (SRSVs) that grouped particles with a rough, hairy, or irregular edge, such as caliciviruses (3).

The genomic and subgenomic organization of AstV and its polyprotein processing led to the proposal of the new family *Astroviridae*, separated from the families *Picornaviridae* and *Caliciviridae*, within the positive-sense single-stranded RNA (ssRNA) viruses (4). In 1995, the International Committee for the Taxon-

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TABLE 1 Main characteristics of the *Astroviridae* family

Characteristic
Virion structure
Icosahedral particles, 28–41 nm in diam
Nonenveloped
Distinctive five- or six-pointed star-like shape under the electron microscope in about 10% of the virions
Genome
A single molecule of infectious, positive-sense ssRNA, 6.4–7.7 kb, with a poly(A) tail
Three ORFs: ORF1a and ORF1b at the 5′ end encoding the nonstructural proteins and ORF2 at the 3′ end encoding the structural proteins
VPg protein linked to the 5′ end
A ribosomal frameshifting signal present between ORF1a and ORF1b
Lack of a helicase domain
Replicative cycle
Structural proteins are expressed from an ~2.8-kb subgenomic RNA

omy of Viruses (ICTV) definitively established the *Astroviridae* family in their sixth report (5). Table 1 depicts the main characteristics of the *Astroviridae* family.

Initially, the *Astroviridae* family consisted of a single genus, *Astrovirus*, based on virion morphology (5). However, later on, two genera were established based on their hosts of origin: *Mamastrovirus* (MAstV) and *Avastrovirus* (AAstV), infecting mammalian and avian species, respectively. Although initially detected in children's stool, AstVs have been found in the feces of a wide variety of mammalian species, i.e., cats (6), cattle (7), deer (8), dogs (9), mice (10), rats (11), pigs (12), sheep (13), mink (14), bats (15), cheetahs (16), rabbits (17), and even sea lions and dolphins (18), as well as in avian species, i.e., turkeys (19), chickens (20), ducks (21), pigeons (22), and guinea fowl (23) and other wild aquatic birds (24).

Very recently, the complete picture of the AstV field has dramatically changed with the discovery after metagenomic surveillance studies of a variety of highly divergent novel AstVs able to infect different animal species, including humans, which are unrelated to the previously described 8 serotypes of HAstVs, now termed classic HAstV (25–29). The first novel HAstVs were identified in 2008 in pediatric stool specimens in Melbourne, Australia (26). They were termed HAstV-MLB, and so far several MLB-related strains (named MLB1, MLB2, and MLB3) have been detected in different parts of the world (27, 30, 31). In 2009, a second group of novel HAstVs was described in samples from children with diarrhea in Virginia (VA) (28) and in Nigeria, Pakistan, and Nepal (HMO, referring to human, mink, and ovine-like astroviruses) (29). In this review, the term HAstV-VA/HMO will be used to refer to this group. So far, up to four VA/HMO strains have been described (27, 31). Thus, a single host species may be susceptible to be infected by different AstVs (15, 32). Altogether, the number of AstVs associated with human infections has almost doubled in the last few years.

The present review focuses on HAstVs within the genus *Mamastrovirus*, which according to molecular surveillance data are one of the most important causes of pediatric acute gastroenteritis, after rotaviruses and arguably caliciviruses (33–36).

BASIC VIROLOGY

Virion Structure and Assembly

The AstV virion is an icosahedral nonenveloped particle with a smooth margin and a distinctive five- or six-pointed star identified on the surface of some (around 10%) virions (Fig. 1). Particles are assembled from the VP90 precursor protein (approximately 90 kDa), which is further processed by cellular caspases to generate the VP70 protein, losing an acidic C-terminal domain (37). Particles composed of the 70-kDa protein are immature and require trypsin cleavages, which occur extracellularly through a rather complicated pathway producing highly infectious particles with capsid proteins of 32 to 34, 27 to 29, and 25 to 26 kDa (VP34, VP27/29, and VP25/26) (38). VP34 is derived from the highly conserved N-terminal region of the polyprotein and builds up the capsid shell, while VP27/29 and VP25/26 are both derived from the variable C-terminal domain with a different N terminus and form the dimeric spikes (39, 40) (see below).

Transmission EM observation reveals that viruses shed in stool are 28 to 30 nm in diameter, while viruses produced in cell monolayers are larger, with an external diameter of 41 nm, and distinct protruding spikes but without the characteristic star-like appearance (41). This surface star-like shape could, however, be observed after alkaline treatment of the particles. There is evidence that particle diameter could vary according to the source of the virus and sample processing for the preparation of EM suspensions.

Recent cryo-electron microscopy studies of immature (VP70) and mature (processed protein) particles of cell-adapted HAstV have revealed a number of distinctive features that provide insight on AstV structure and polyprotein processing (42). The cryo-EM reconstructions show a capsid shell of 35 nm organized as $T = 3$

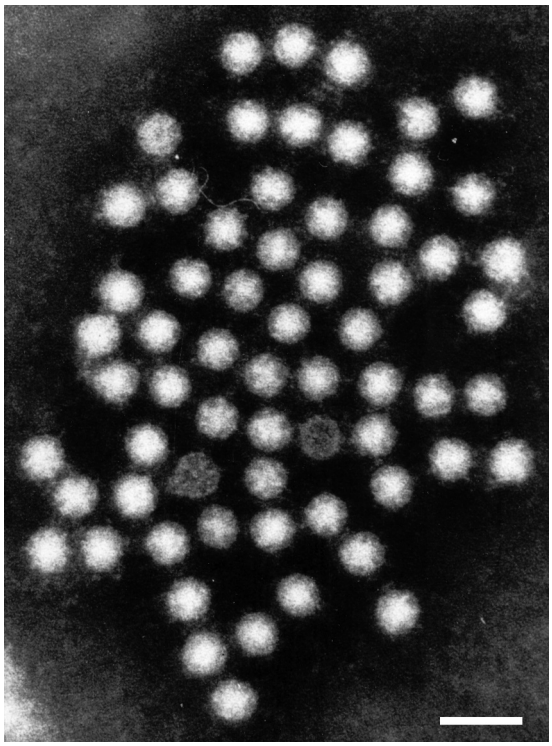


FIG 1 Human astrovirus particles observed by transmission immunoelectron microscopy in feces negatively stained with phosphotungstic acid. Bar, 50 nm.

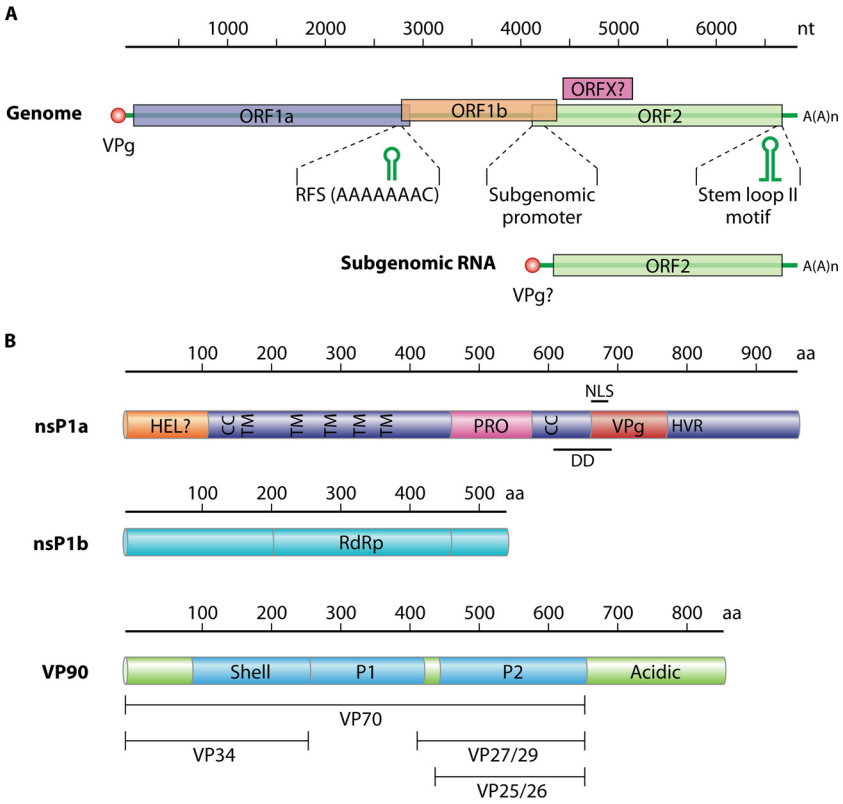


FIG 2 Genome organization and polyprotein products of human astrovirus. (A) Genomic and subgenomic RNA organization, with open reading frames (ORFs) ORF1a, ORF1b, ORF2, and putative ORFX represented as boxes. Nucleotide sequences represent highly conserved sequences located in the ribosomal frameshift (RFS) signal and upstream of the initiation site of subgenomic RNA transcription. Putative RNA secondary structures conserved in the RFS and in the 3′ end of the genome are depicted. (B) Characteristic motifs of the HAstV polyprotein products. HEL, putative helicase domain; TM, transmembrane domain; CC, coiled-coil domain; PRO, protease domain; VPg, coding region for a VPg protein; HVR, hypervariable region; NLS, putative nuclear localization signal; DD, putative death domain; RdRp, RNA dependent-RNA polymerase motif. The ORF2-encoded structural polyprotein (VP90) consists of conserved regions (shell and P1 domains), a variable region containing the P2 domain (capsid spikes), and an acidic C-terminal region which is cleaved by cellular caspases to result in the VP70 precursor. Particles containing VP70 are further cleaved by trypsin to yield the VP34, VP27/29, and VP25/26 proteins.

icosahedral symmetry assembled from 180 protein subunits and decorated with either 30 (mature particles) or 90 (immature particles) globular dimeric spikes, resulting in a total diameter of 44 nm.

The crystal structure of the AstV spike without its C-terminal end has been elucidated (40). Remarkably, the shape, size, and architecture of the AstV spike show a notable similarity with those of hepatitis E virus, the only member of the recently established *Hepeviridae* family (43).

Despite the advances in the determination of virion structure, the AstV protein composition, which is critical to determine virus specific infectivity (38, 44), remains to be clearly defined.

Genome Organization

The AstV genome is a positive-sense ssRNA molecule of around 6.8 (6.2 to 7.8) kb, excluding the polyadenylated tail at the 3′ end (45). A VPg protein is covalently linked to the 5′ end of the genome (46). The genome contains three open reading frames (ORFs), named from the 5′ end to the 3′ end ORF1a, ORF1b, and ORF2. ORF1a and ORF1b encode the nonstructural proteins (nsPs) involved in RNA transcription and replication, while ORF2 encodes the structural proteins, which are expressed from a subgenomic RNA (Fig. 2) (4, 47). In the classic HAstVs and in other mammalian AstVs, a new ORF, termed ORFX, overlapping the 5′ end of ORF2 in the +1 reading frame, has been described (48). It has been suggested that ORFX could be translated through a leaking scanning mechanism. The translation product of this ORFX has not yet been experimentally confirmed.

The AstV RNA is infectious and upon transfection produces infectious virus progeny (49). Infectious particles may also be obtained after *in vitro* transcription of a full-length cDNA clone of the HAstV genome and transfection into permissive cells (49, 50). During the replicative cycle, a positive-sense single-stranded subgenomic RNA corresponding to around 2.8 kb of the 3′ end of genomic RNA is produced in infected cells, coding for the virion structural proteins (51).

Regarding genome organization, two untranslated regions (UTR)s, the 5′ UTR and the 3′ UTR, of 11 to 85 and 80 to 85 bases, respectively, are located at the ends of the AstV genome. No internal ribosome entry site (IRES) has so far been described in AstV. This element is present at the 5′ ends of the genomes of other positive ssRNA viruses belonging to the families *Picornaviridae* (52) and *Flaviviridae* (53) but is absent in the family *Caliciviridae* (54). Besides the poly(A) tail consisting of around 30 adenines, a highly conserved secondary element is present at the 3′ end of the AstV genome. This secondary structure motif, termed the stem-loop II motif, is present in the genomes of classic HAstVs, HAstV-

VA/HMO, and cat, ovine, porcine, and avian AstVs, but it is only partially conserved in the genome of HAstV-MLB (55–57). Interestingly this structure is also found at the 3′ ends of the genomes of some members of coronaviruses, noroviruses, and rhinoviruses (56, 58). This stem-loop II motif appears to be conserved at both the nucleotide sequence and RNA structure levels, indicating a strong evolutionary selection for its conservation. Although its function is yet to be determined, it has been suggested that it could provide stability to the secondary structure of RNA and interact with viral and cellular proteins essential for genome replication (56).

The lengths of ORF1a, ORF1b, and ORF2 vary depending on the AstV strain. This variation is largely dependent on the insertions and deletions present at the 3′ end of ORF1a (59, 60). Likewise, the overlapping regions of the different ORFs also vary in length depending on the AstV strain. In mammalian AstVs the overlapping region of ORF1a and ORF1b ranges from 10 to 148 nucleotides (nt), while the overlap is only 12 to 45 nt long in avian AstVs (61). A ribosomal frameshifting signal (RFS), essential for the translation of the RNA-dependent RNA polymerase (RdRp) encoded in ORF1b, is present precisely in this overlapping region (62, 63). The RFS has been found in all AstVs except in a newly described chicken astrovirus, in which ORF1b has its own start codon (64). The overlap found between ORF1b and ORF2 may also vary in length or be nonexistent, as occurs in duck AstV (65). A highly conserved sequence that could be part of the promoter for subgenomic RNA synthesis is located in this region (Fig. 2) (13).

ORF1a encodes a putative helicase domain (HEL), several transmembrane (TM) and coiled-coil (CC) domains, the protease domain (PRO), a VPg, a hypervariable region (HVR), a nuclear localization signal (NLS), and a putative death domain (DD). ORF1b encodes the RNA dependent-RNA polymerase (RdRp). ORF2 contains the region coding for the shell proteins, a P1 domain of unknown function, a variable region containing the P2 domain which includes the spike proteins, and an additional acidic domain at the C terminus (Fig. 2).

General Overview of the HAstV Replicative Cycle

The following sections dealing with the replicative cycle of HAstVs essentially refers to the so-called classic HAstVs. The HAstV replication cycle shares many common features with the replication cycle of members of the *Caliciviridae* family (Fig. 3). After cell entry and uncoating, the two main nonstructural polyproteins, nsP1a (~102 kDa) and nsP1a1b (~160 kDa), are translated from the VPg-linked genomic RNA. Nonstructural polyprotein nsP1a1b is expressed thanks to the RFS that exists between ORF1a and ORF1b (62, 63). Cleavage of these polyproteins results in the individual nonstructural proteins required for genome replication, which takes place in replication complexes assembled in close association with intracellular membranes. This process results in the formation of both genomic and subgenomic RNAs, which are produced in large quantities to ensure the production of high yields of structural proteins. After encapsidation and maturation, virions are released from the cell. While it is known that both genomic and subgenomic RNAs are encapsidated into virions of animal caliciviruses (66), it is yet to be elucidated whether a similar process occurs in AstVs.

Cell Binding and Entry

The main cellular receptor for HAstVs still remains unknown, but the susceptibilities of different cell lines to HAstV infection differ depending on the serotype (67), suggesting that these viruses could take advantage of having more than one attachment or receptor molecules, some of which may be of a polysaccharide nature and common to all HAstVs (40). Besides revealing a close relationship with hepatitis E virus, determination of the structure of the HAstV dimeric surface spikes has identified a putative receptor binding site that is conserved in all HAstV serotypes and which would recognize a polysaccharide molecule (40). In the same study, it was noted that dextran sulfate, heparin sulfate, and particularly heparin could partially block the infectivity of HAstVs, while sialic acid did not seem to take part in the virus-cell recognition event. Thus, as has been hypothesized for other related viruses such as noroviruses, a carbohydrate molecule may act as cellular receptor or at least as an initial recognition factor on the cell surface. Interestingly, binding of HAstVs to *in vitro*-differentiated intestinal epithelial cell monolayers results in an increase in cell barrier permeability, and this phenomenon occurs independently of viral replication (68). Whether these changes are required for the viruses to reach putative receptors present in the basolateral membrane or whether they are a consequence of the activation of cellular signaling pathways after initial binding is still uncertain.

Subsequent steps of the life cycle have been recently studied on the highly permissive CaCo-2 cells (69), confirming previous observations on HEK293 cells (70) which indicated that HAstVs use a clathrin-mediated endocytosis pathway as the viral entry mechanism. Drugs that inhibit clathrin-mediated endocytosis and actin filament polymerization, as well as those that reduce the presence of cholesterol in the cell membrane, decrease the infectivity of the virus (69). Furthermore, entry also depends on the acidification and maturation of endosomes where membrane permeabilization and RNA uncoating would occur. Overall, it has been estimated that while the half time of virus binding to the cell surface is about 10 min, virus uncoating takes around 130 min (69).

Cellular signaling pathways activated after HAstV initial infection have been analyzed using specific drug inhibitors, and it is known that interaction of HAstVs with cells results in the activation of the extracellular signal-regulated kinase (ERK1/2) and the phosphoinositide 3-kinase (PI3K) pathways, both being required for effective entry and establishment of a productive infection (71, 72). It seems that the PI3K-mediated cascade would act independently or downstream of that mediated by ERK1/2 (72). Finally, it has also been observed that activation of the ERK1/2 pathway does not require binding of infectious viruses and can be achieved after binding of inactivated viruses and/or virus-like particles (71). Although it is clear that ERK1/2 pathway activation is required for a productive HAstV infection and it has been speculated that ERK could phosphorylate the HAstV RdRp or other nonstructural proteins, the function of activated ERK still remains unknown, and it may apply an indirect mechanism.

Translation/Replication

The VPg protein linked to the HAstV genome plays an essential role in infectivity following viral RNA uncoating. Removal of the VPg protein by proteolytic treatment of viral RNA completely abolishes infectivity (46), indicating that the VPg protein may play

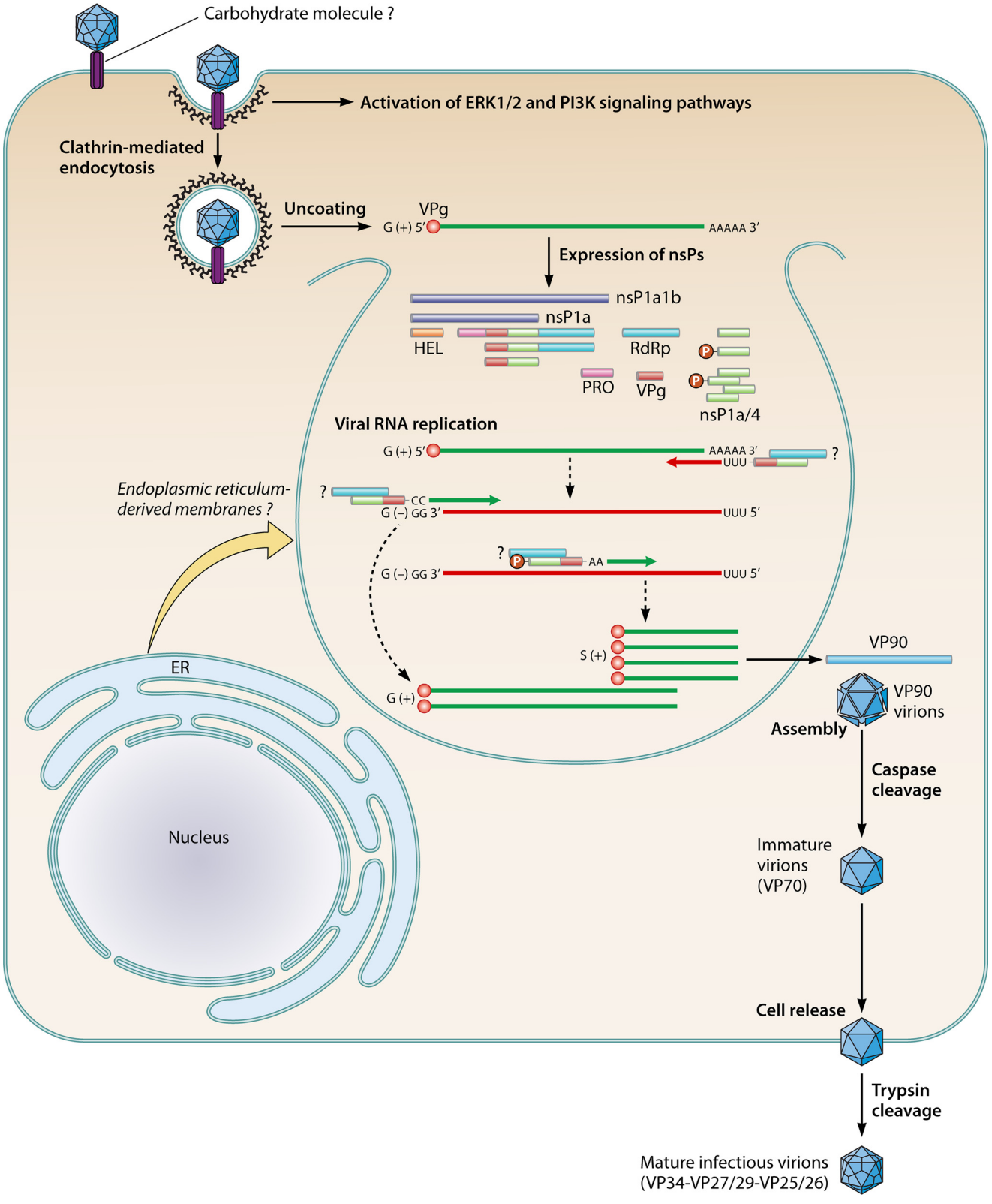


FIG 3 Replication of human astroviruses. After binding to one or more cellular receptors, virus uptake occurs via clathrin-dependent endocytosis. A drop in the pH leads to viral uncoating. Two main nonstructural polyproteins, nsP1a and nsP1a1b, are translated from the VPg-linked genomic RNA and further cleaved by viral and cellular proteases, resulting in mature nonstructural proteins (nsPs), which are required for genome replication. Replication complexes assemble in close association with intracellular membranes. The nsP1a/4 protein or one of its precursors, which would include the VPg domain, may interact with the RdRp protein and contribute to the regulation of the synthesis of negative- and positive-sense RNA strands as well as of subgenomic RNA. The phosphorylation status of the nsP1a/4 protein may contribute to this regulation step. Subgenomic RNAs are produced in large quantities and are used for the expression of capsid proteins. The structural VP90 polyprotein initially assembles into immature virions in association with intracellular membranes. Several cellular caspases further cleave these VP90 polyproteins once they have dissociated from membranes, resulting in VP70 immature viral capsids. Release of VP70 particles into the medium seems to occur without cell lysis, and virions mature extracellularly by the action of trypsin.

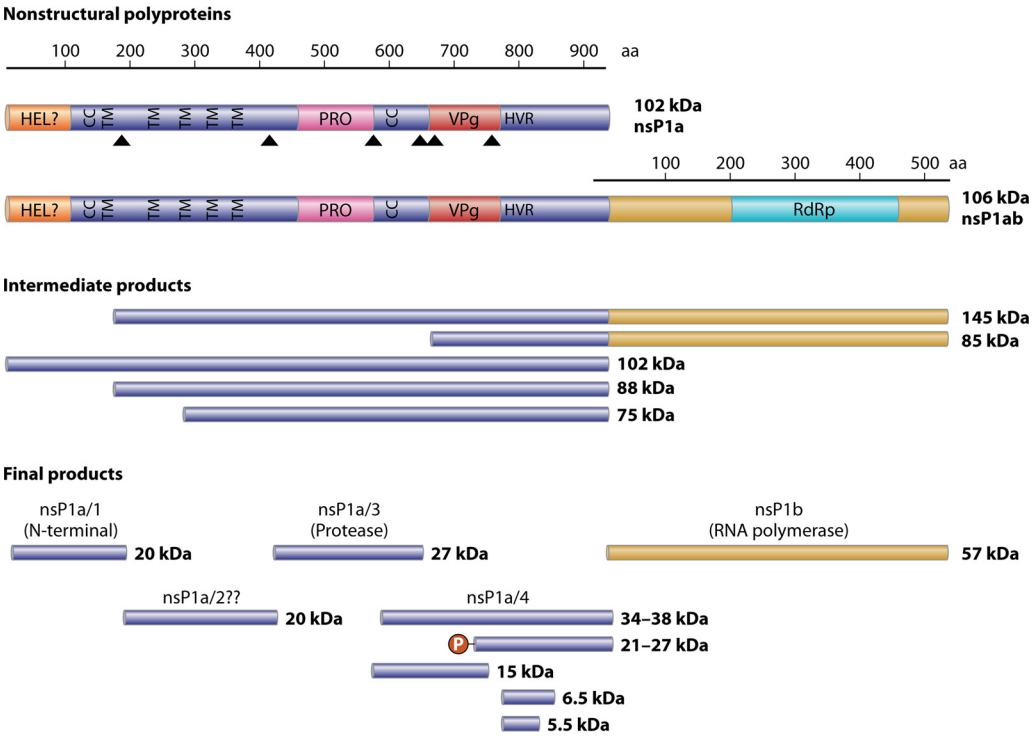


FIG 4 Putative processing of the astrovirus nonstructural proteins expressed from ORF1a and ORF1b. HEL, putative helicase domain; CC, coiled-coil domain; TM, transmembrane domain; PRO, protease domain; VPg, viral protein genome-linked coding region; HVR, hypervariable region; RdRp, RNA-dependent RNA polymerase motif; P, posttranslational phosphorylation. Triangles depict proteolytic cleavage sites described in the literature (75, 77, 78, 80).

a role in the recruitment of cellular translation initiation factors, analogous to what has been described for caliciviruses (73). The genome is then translated, giving rise to nsP1a1b and nsP1a polyproteins, which are then cleaved by the viral serine protease (in nsP1a) as well as some cellular proteases, resulting in the individual nonstructural proteins (Fig. 4). The crystal structure of the viral protease has been resolved, showing properties of trypsin-like enzymes with a catalytic Asp-His-Ser triad typical of the serine proteases (74).

The cleavage process of the virus nonstructural polyproteins is rather unknown. None of the processing products has been confirmed by N-terminal sequencing, and studies performed in different experimental systems with a variety of classic HAstVs reported different intermediate and final products (75–79). It appears, however, that proteolytic processing of polyproteins nsP1a1b (around 160 kDa) produces the nsP1b protein (RdRp) (around 57 kDa) and the nsP1a protein (around 102 kDa), which is subsequently cleaved to yield several mature products (Fig. 4). Several putative transmembrane domains that could help in driving and anchoring the nonstructural replication complexes on cellular membranes have been identified in the nsP1a polyproteins (13, 62). Four potential cleavage sites have been proposed in the nsP1a protein, specifically between residues Ala_{174} and His_{175}, between Val_{409} and Ala_{410}, between Glu_{567} and Thr_{568}, and between Glu_{654} and Ile_{655}. Although discrepancies exist in regard to the exact cleavage sites, it seems clear that a viral protease would be responsible for all proposed proteolytic cleavages except for the one between Ala_{174} and His_{175}, yielding at least four proteins, nsP1a/1 to -4 (75, 77, 78, 80). It has been shown that some of the resulting nonstructural proteins are posttranslationally modified by phosphorylation mechanisms (81, 82), and these modifications may modulate the interaction among them. Whether the proteolytic processing of these nonstructural polyproteins takes place before or after anchoring to intracellular membranes is still unknown, but large membrane rearrangements are observed in HAstV-infected cells both *in vitro* (82, 83) and *in vivo* (84). Since both viral nonstructural proteins and viral RNA have been found colocalizing with the endoplasmic reticulum and an endoplasmic reticulum retention signal has been predicted at the end of nsP1a (82), it is believed that endoplasmic reticulum-derived intracellular membranes would be the cellular site for HAstV genome replication.

The HAstV genome replication process has not been characterized in detail and is inferred from those of other positive-sense RNA viruses. Thus, a full-length negative-sense genomic RNA would be synthesized and used as a template for the production of both positive-sense genomic RNA and positive-sense subgenomic RNA, but the existence of a negative-sense subgenomic RNA template cannot be completely ruled out. Regarding the kinetics of this process, Jang et al. (85) observed that *de novo* synthesis of positive-sense RNAs occurs almost instantly following negative-sense RNA synthesis. They also estimated the total amount of HAstV negative-sense RNA synthesized in cells as nearly 0.7 to 4.0% of that of the HAstV positive-sense RNA. Many other aspects of the HAstV genome replication process, such as the role of the VPg protein as a primer for initiation of RNA strands, have not been addressed yet. Involvement of cellular host proteins in the process and the mechanisms used by the virus to switch between replication and transcription are other issues that remain to be elucidated. The expression of one of the nonstructural proteins

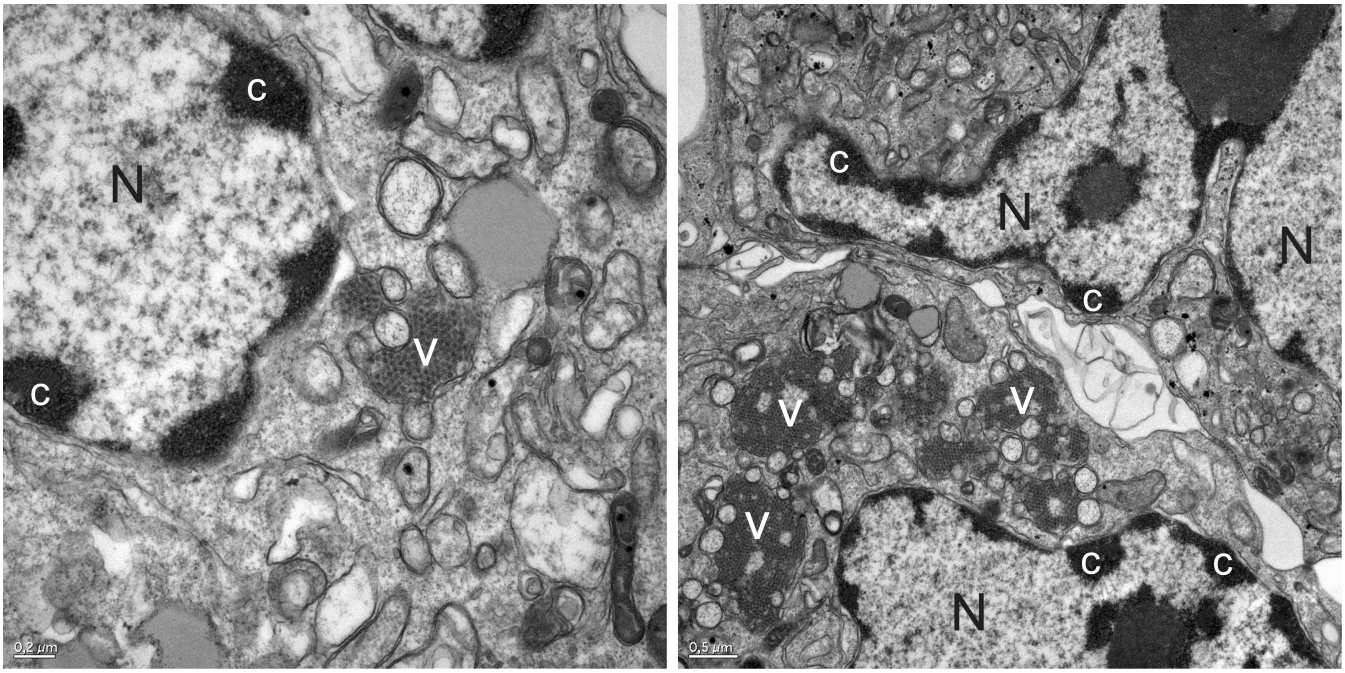


FIG 5 HAstV particles are associated with membranes in infected cells. Ultrastructural analysis of CaCo-2 cells infected with HAstV-4 at 48 h postinfection shows aggregates of virions accumulated in the cytoplasm in close association with double-membrane vesicles (V). Nuclei (N) of infected cells show masses of condensed chromatin dispersed at the periphery (c).

(the nsP1a/4 protein) and the RdRp in the baculovirus expression system has suggested that oligomerization and phosphorylation states of nsP1a/4 protein may regulate its interaction with the RdRp protein (81), and although not confirmed, it is likely that these changes may contribute to the regulation of the synthesis of negative- and positive-sense RNA strands as well as of subgenomic RNA. In addition, variability in the nsP1a/4 gene, which contains a hypervariable region, also affects the virus replication phenotype (60). Percentages of genomic, subgenomic, and antigenomic RNAs in total cell-associated RNAs produced during infection vary depending on the nsP1a/4 gene. While the amount of subgenomic RNA produced by viruses containing certain nsP1a/4 variants represents only between 0.8 and 17% of the total RNA, the proportion of subgenomic RNA produced by other nsP1a/4 mutants can reach up to 85 to 95%, and this also results in significantly higher numbers of infectious progeny (60). Accordingly, the viral load in feces from children with gastroenteritis correlates with nsP1a/4 variability, and this phenotype can be typed based on the hypervariable region contained in the nsP1a/4 gene (86) (see below).

Expression of Structural Proteins, Virion Assembly, and Cell Release

Structural proteins are expressed from the subgenomic RNAs as an ~90 kDa polyprotein (VP90) through a mechanism that it is likely to involve the 5′-end VPg (46). Cell fractionation studies have shown that structural protein synthesis takes place in the same cellular compartments where the replication complexes form (83). The model of HAstV morphogenesis suggests that VP90 polyprotein initially assembles into immature virions in association with intracellular membranes through its C terminus and that several cellular caspases further cleave these VP90 polyproteins close to their C termini once they have dissociated from membranes, resulting in viral capsids composed of 70-kDa polyproteins (VP70) (83, 87). In infected cells, virus aggregates surrounded by a large quantity of double-membrane vacuoles may be observed in the vicinity of the nucleus (Fig. 5) (82, 83). Finally, VP70-containing viral particles would be released from cells and proteolytically processed by trypsin to enhance infectivity. Processing of VP90 to VP70 by caspases is required for viruses to exit the cell, and release of VP70 particles into the medium seems to occur without cell lysis (37). Virus release from cells is more dependent on caspase activity than on cell death itself (37).

PATHOLOGY, PATHOGENESIS, AND HOST RESPONSE

Clinical Disease Correlates

HAstVs, especially classic HAstVs, are considered gastrointestinal pathogens affecting children worldwide, with very few reports of HAstV-mediated disease in normal healthy adults (88, 89). Serological studies indicate that most children are infected with HAstV and develop antibodies to the virus early in life (90, 91), which are thought to provide protection against future infections. Immunocompromised individuals and the elderly also represent high-risk groups. Typically, HAstV infection induces a mild, watery diarrhea that lasts 2 to 3 days, associated with vomiting, fever, anorexia, and abdominal pain. Vomiting is less prevalent in astrovirus infection than in rotavirus or calicivirus infection, and HAstV infections also show a longer incubation period. Based on data from adult volunteer studies and outbreaks of gastroenteritis in a child care center, the mean incubation period of HAstV infections was calculated to be 4.5 days (92). In general, HAstV diarrhea is milder than those caused by rotaviruses or noroviruses, and it resolves spontaneously, although in some cases HAstV in-

fections have required hospitalization. Asymptomatic infections have also been described in children and adults (93–96), although HAstV prevalence as asymptomatic pathogens has yet to be characterized. Studies in immunodeficient patients, including HIV-infected individuals, have associated HAstV infections with symptomatic gastroenteritis (97, 98), but a recent report has also shown that classic HAstV infections can also spread systemically and cause severe disseminated lethal infections in highly immunocompromised children (99). Consistent with this idea, recent work on murine AstVs (MuAstVs) has demonstrated distribution of viruses beyond the gastrointestinal tract (i.e., in the spleen, liver, and kidney) in immunodeficient mice (100). Finally, the relationship of HAstV infection to the etiology of intussusception in children has also been studied. Although with less frequency than rotavirus, norovirus, and adenovirus infection, HAstV infection has also been identified as a potential risk factor for intussusception in infected children (101–103).

Correlations between specific serotypes of classic HAstVs, level and duration of viral load, and severity of disease have not been deeply analyzed. In one study, quantification of shedding levels of serotypes 1, 2, 3, 4, and 8 ascertained viral concentrations ranging from 3.4×10^8 to 1×10^{13} per gram of feces and significantly higher titers in the serotype 3-containing feces. In addition, serotype 3 was associated with a more severe gastroenteritis (104). Later on, differences in viral load in fecal samples were further associated with variability in one of the viral nonstructural proteins, the nsP1a/4 protein (60), suggesting that nonstructural proteins may also influence the pathogenicity of the HAstV strain.

Besides classic HAstVs, infections caused by the other two novel types of nonclassic HAstVs (HAstV-MLB and HAstV-VA/HMO) are generally associated with gastroenteritis as well, but their pathogenic role in human health has not been clearly demonstrated. Both types of viruses were almost simultaneously identified in pediatric stool specimens from children with diarrhea (26–29), but their role as etiological agents of gastroenteritis needs to be further clarified.

HAstV-MLB (MLB1, MLB2, and MLB3) strains have so far been detected in children with gastroenteritis in Australia (26), the United States (30), India (27), Mexico (105), Turkey (106), Egypt (107), Bhutan (108), China (109), and Europe (110). However, a case-control study on HAstV-MLB1 and classic HAstV infections in India showed that while classic HAstVs were significantly associated with diarrhea, HAstV-MLB1 was not (111). Furthermore, MLB1 titers in stool did not differ significantly between symptomatic and asymptomatic individuals. Since HAstV-MLB2 viruses have also been isolated in the plasma and nasopharynx of a child with an upper respiratory infection (112, 113), the authors have hypothesized that HAstV-MLB pathogenicity may affect extraenteric tissues and that its presence in stool may simply be a consequence of its mode of transmission. No other pathogen was detected in these samples, suggesting that HAstV-MLB2 was the cause of the patient's fever. Hence, the tropism of selected strains of HAstV-MLB may not be restricted to the gastrointestinal tract, and the role of these viruses in pediatric infections demands further investigation.

HAstV-VA/HMO (VA1, VA2, and VA3 [also known as HMO-C, HMO-A, and HMO-B, respectively] and VA4) have been detected in pediatric gastroenteritis samples in Nigeria, Nepal, and Pakistan (29), the United States (27), Egypt (107), and China (109), but so far they have never been reported in Europe (114). Serological studies in the United States have confirmed that HAstV-HMO-C (VA1) is a highly prevalent human-infectious agent (115). In a recent study, a strain closely related to HAstV-VA1 was isolated in a patient with new-onset celiac disease (114). Finally, similarly to what has been observed for classic HAstVs, extraintestinal dissemination in immunodeficient individuals has also been observed in HAstV-HMO-C (VA1) virus infections, when the agent was detected in neural tissue from an immunocompromised child with encephalitis (116). Future research efforts should assess the extent and severity of diseases associated with novel HAstVs.

Histopathological Changes

Despite the impact of HAstV on human health, knowledge on astrovirus pathogenesis is still limited (117), mainly due to the lack for many years of a good small-animal model and to the fact that most data gathered so far are restricted to classic HAstVs. For humans, there is only one report describing the histopathological examination of small intestinal biopsy specimens in an immunodeficient child with HAstV infection and persistent diarrhea after a bone marrow transplant (118). The child shed HAstV for more than 60 days. The main findings evidenced villous blunting, irregular superficial epithelial cells, and an increase in lamina propria inflammatory cell density. Viral replication was targeted to surface epithelial cells, mostly at the villus tips in the jejunal and duodenal biopsy specimens. Although the patient was immunocompromised, the study concluded that the intestinal inflammatory response was only mild.

Ultrastructural studies in symptomatic animals have been performed in experimentally infected lambs, calves, and turkeys. Experimentally infected lambs showed villus atrophy in the small intestine (84, 119). Astroviruses infected only mature enterocytes (viruses were seen in paracrystalline arrays, along the microvilli, and within lysosomes and autophagic vacuoles) in the villi of the small intestine and macrophages (viruses were seen in lysosomes) in the lamina propria (84, 119). Infected enterocytes could be observed sloughing into the gut, and by 5 days postinfection, the villi appeared normal. Maximum lesions were observed in the jejunum and ileum, without observation of infected cells in the colon (119).

The bovine virus was found to mostly infect the epithelium covering the dome villi of the jejunal and ileal Peyer's patches (120). Both M cells and absorptive enterocyte cells of the dome epithelium contained virus, and microvilli of infected M cells were often severely stunted.

In turkeys, replication of turkey astroviruses (TAstVs) was detected in the upper sectors of the small intestine, but it was later on detected in the large intestine as well, infecting epithelial cells at the basal borders of the villi (121). Infectious TAstV-2 was isolated from the bursa, thymus, spleen, kidney, skeletal muscle, pancreas, and plasma, confirming that a viremic stage occurs during infection (122). However, despite detection of infectious particles and viral antigen in these extraintestinal tissues, *in situ* hybridization assays indicated that astrovirus replication was confined to the intestines. No clear histological changes were either observed in any of these tissues. Histological analysis of the intestine indicated only minor enteric damage, in the form of mild epithelial necrosis, lamina propria infiltration, minimal villous atrophy, and mild crypt hyperplasia. The lack of major villus atrophy, cell death, and inflammation indicated that astrovirus-induced diarrhea is

caused by a mechanism other than damage of the intestinal epithelium or an inflammatory response. The increased activation of the potent immunosuppressive cytokine transforming growth factor β (TGF-β) observed in infected turkeys may partially explain the lack of inflammation (122).

Finally, the recent isolation and characterization of MuAstVs offers new perspectives for the use of the mouse model for the study of many aspects of viral replication *in vivo* and the histopathological changes induced by the infection (100). Although in the murine model, animals do not suffer diarrhea and different mouse strains show different susceptibilities, MuAstV shedding in wild-type mice for more than 14 days has been observed, and viral RNA has been detected in duodenum, ileum, proximal colon, and mesenteric lymph nodes (100).

Mechanisms of Pathogenesis and Induction of Diarrhea

Since astrovirus-infected intestines show relatively minor histological changes and inflammation, other mechanisms of virus-induced diarrhea have been studied. Among the primary mechanisms described for diarrhea induction by other enteric infections (reviewed in references 123 and 124), major destruction of the intestinal epithelium and inflammatory responses do not seem to play a role in AstV infection. Alternatively, rapid loss of fluids and electrolytes may originate from inhibition of the usual absorptive function of the intestine, activation of secretory processes, or loss of intestinal epithelial barrier permeability.

Studies in the turkey animal model showed that specific maltase activity decreases during infection. Decreased specific maltase activity caused disaccharide maldigestion and malabsorption, followed by osmotic diarrhea affecting intestinal absorption (125). Later on, sodium malabsorption, likely by redistribution of specific sodium transporters, was reported in turkey poults with diarrhea (126). Levels of the sodium hydrogen exchanger NHE2 were found to increase upon infection, and NHE3 was translocated from the membrane to the cytoplasm. In addition, analysis of TAstV-infected enterocytes failed to show evidence of paracellular dilatation, although F-actin rearrangements were identified in the apical regions of cells.

Finally, using HAstV-infected differentiated CaCo-2 cells, F-actin rearrangements have also been observed, together with disruption of the cell barrier and redistribution of occludin (68). Discrepancies with data from AstV infection in turkeys, in which no alterations in tight junctions were observed, could be due to the use of different viruses and/or different cellular models or to differences in the virus infection stage at the time of analysis. Of note, the effect of HAstV infection on epithelial barrier permeability occurs independently of viral replication, suggesting that the capsid protein of HAstVs may act as an enterotoxin (68). Although the study did not report significant amounts of cell death upon HAstV infection at the time of the loss of barrier function and these changes were mainly attributed to the disruption of tight junctions, HAstV-induced enterocyte death may eventually occur at a late stage of the infection. Indeed, other studies using CaCo-2 cells have shown that infection induces cell death by apoptosis (37, 127). In agreement with these hypotheses, the release of virions from infected cells is thought to occur through a nonlytic mechanism (37).

Host Immune Responses and Control of Infection

The observation that AstV infection and gastroenteritis in most species are age dependent led to the assumption that immune protection is acquired after an initial infection. However, the specific immune responses activated upon AstV infection are not yet completely understood (reviewed in reference 128). Epidemiological and clinical studies have demonstrated that the humoral immune response plays a role in restraining infection and disease in humans. Seroprevalence studies show that the vast majority of healthy young adults have antibodies against the most prevalent classic HAstV serotypes (90) and also against some of the novel HAstVs (115) and that the increase in seroprevalence correlates as well with age during childhood (91, 115, 129). Maternal-fetal transmission of antibody occurs, and antibody levels decrease by 6 to 8 months of age (91, 129). Infections induce antibodies in humans of all age groups, which may persist for extended periods of time (90, 129). These findings are in agreement with the observations made during the two clinical studies performed in human volunteers; the severity of infection signs negatively correlated with the presence of anti-HAstV antibodies before the challenge (95, 96). Although seroprevalence studies detected serotype-specific neutralizing antibodies, further studies would be necessary to assess whether cross-reaction and cross-protection may occur between all serotypes. Although mucosal IgA levels in the gastrointestinal tract have been shown to be major immune effector products against gastrointestinal viral infections and IgAs are induced upon AstV infection, it is yet to be elucidated whether they are required for protection (130).

In addition to this indirect evidence that points to a major role of humoral responses, the challenge of small intestinal biopsy specimens from healthy adults in an organ culture system with inactivated HAstVs demonstrated the presence of HAstV-specific $CD4^+$ and $CD8^+$ T cells residing in the normal tissue (131). Thus, it is clear that both humoral and cellular adaptive immune responses are involved in protecting normal healthy adults from reinfections. Finally, besides its role in protection from reinfections, adaptive immunity has also been shown to restrict astroviral replication in primary infections in the murine model (100). Compared to wild-type mice, $Rag1^{-/-}$ mice deficient in B and T cells show significantly higher levels of viral shedding in feces and higher levels of viral genome copies detected in intestinal tissues, mesenteric lymph nodes, spleen, liver, and kidney, showing that dissemination of infection is restricted by B and T cells in wild-type mice.

While it is evident that adaptive immunity is a clear mediator both for protection and for controlling infection and disease, many authors suggest that innate immune responses may also play an important role in controlling and limiting disease in primary infections, especially in young individuals. Indeed, evidence in this regard comes from published *in vitro* data on human viruses (132–136), as well as from *in vivo* studies with infected young turkeys (122, 137) and immunodeficient mice (100). Unpublished studies in our laboratory have also explored the interplay between HAstV replication and the interferon (IFN) response in cultured cells, observing that infection of CaCo-2 cells induces little type I IFN (IFN-I) response and that this occurs at late stages of infection.

Data on human viruses have demonstrated that the capsid protein of HAstV acts as an inhibitor of the complement system,

which constitutes an important innate response against bacterial and viral infections. Similarly to what has been observed with the IFN response, many pathogens have developed ingenious methods by which to evade destruction by the complement system (138). Purified recombinant HAstV capsid protein is able to inhibit complement-mediated hemolysis through direct interaction with two of the complement regulatory molecules, C1 and MBL (133, 134). Both the classical and lectin activation pathways of complement may be inhibited by HAstV capsid protein. The capsid region responsible for the interaction was narrowed down to a 15-residue sequence located in the highly conserved N-terminal domain of the capsid polyprotein (residues 79 to 108), which is highly conserved among classic HAstVs, feline AstVs (FAstVs), and porcine AstVs (PAstVs) but less conserved in the novel HAstVs and mink, ovine, and avian viruses. The biological role of the interaction of the HAstV capsid protein and the complement system remains to be elucidated. Among possible effects, this mechanism of immune evasion would inhibit the process of inflammatory cell migration to infected tissues, although some authors have additionally speculated that HAstV particles may bind C1q (or MBL) and use C1q receptors expressed on the cell surfaces for cellular entry or initial attachment (139). Besides its role on the pathophysiology of HAstV infection, the discovery of this peptide has inspired novel therapeutic options to treat complement-mediated diseases such as ABO-incompatible blood transfusion reactions, which result in acute intravascular hemolytic disease processes (136).

In the turkey model, in which adaptive immune responses do not seem to be critical for viral clearance (137), infection associated with poult enteritis and mortality syndrome (PEMS) induces functional defects in macrophages. In the context of TAstV infection, macrophages showed decreased phagocytosis and intracytoplasmic bacterial killing, and reduced activities of macrophage-mediated proinflammatory cytokines (interleukin-1 [IL-1] and IL-6) were also detected, leading to an increase in the susceptibility of turkeys to secondary infections (140). Infection also compromised the lymphocyte-mediated immune defenses by reducing lymphoproliferative responses (141) and induced increased levels of active TGF-β in serum (122). Finally, the expression of inducible nitric oxide synthase (iNOS) and the subsequent increase in its immune mediator NO have been shown to play a role in controlling TAstV replication both *in vitro* and *in vivo* (137). When stimulated *ex vivo* with lipopolysaccharide, adherent spleen cells isolated from infected animals produced more NO, suggesting that TAstV infection primes macrophages *in vivo* and makes them more susceptible to activation upon stimulation. In addition, pretreatment of viruses with NO donor compounds significantly inhibited replication, while treatment of embryonated turkey eggs with specific iNOS inhibitors enhanced virus replication (137). Although treatment of a macrophage cell line with the virus resulted in increased NO activity, the source of NO *in vivo* was not identified until recently, and it is now known that infected intestinal epithelial cells are the ones responsible for iNOS upregulation upon TAstV infection (142).

In the murine model, although it is clear that adaptive immunity restricts replication, innate immunity contributes to the control of MuAstV replication as well (100). Although infection is asymptomatic, viral replication in intestinal tissues of Stat1$^{-/-}$ mice is significantly higher than that in wild-type mice. Overall, the Jak/Stat1 pathway is required for type I and II IFN signaling, and it has been shown to be critical for resistance to other enteric viruses such as noroviruses (143). Although it has not yet been proved whether iNOS activity is also relevant in controlling the infection in mice as happens in avian species and which cell type would produce it, it has been demonstrated that it plays a role in protecting mice from norovirus infection (143), suggesting that it might play a role against astroviruses as well. On the other hand, however, since NO has also been shown to regulate intestinal mucosal permeability and ion transport, a role of NO as a mediator of diarrhea caused by other viral infections such as rotaviruses has also been demonstrated (144, 145).

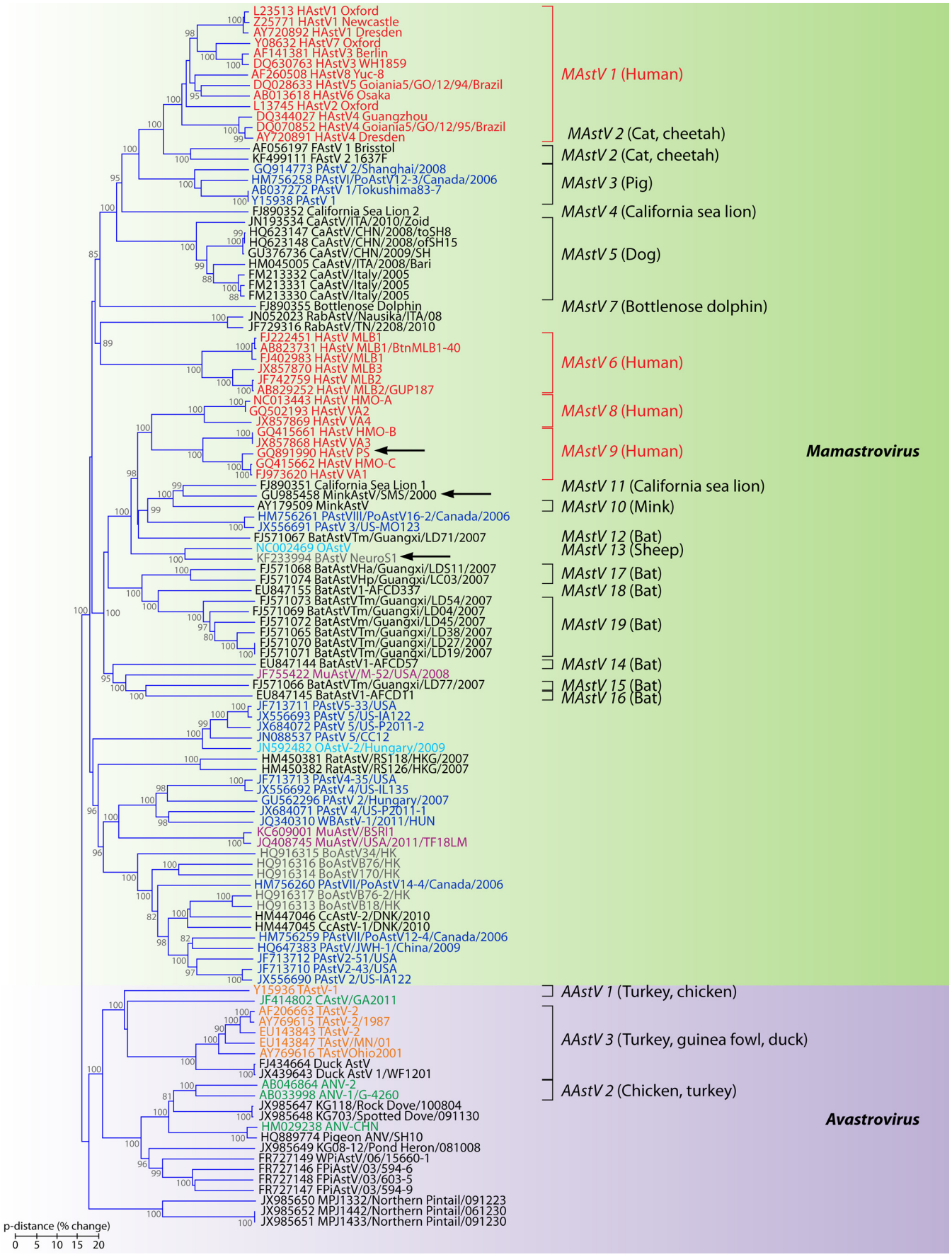
TAXONOMY, EVOLUTION, AND EMERGENCE

The *Astroviridae* family was established by the ICTV in 1995, and, consistent with phylogenetic reconstruction, it is now divided in two genera: genus *Mamastrovirus* (MAstV), including viruses infecting mammals, and genus *Avastrovirus* (AAstV), including viruses infecting avian species. Since 2008, the number of animal hosts that have been found to be infected with AstVs has quadrupled (comprising at least 30 mammal species and 14 avian species), and classification within the family has gained complexity (146). Classification within each genus was based initially only on the species of the host of origin, but at present genera are divided into "viral species" or "genotypes" on the basis of host range but also on genetic differences in the complete capsid sequence (viruses with differences at the capsid protein level that are higher than approximately 0.30 to 0.35 are regarded as different genotype species). On average, the mean amino acid distance (p-dist) between the two genera is 0.83, while within genera, mean distances are 0.72 and 0.64 for MAstV and AAstV, respectively. The official 2012 ICTV release (http://www.ictvonline.org/virusTaxonomy.asp) recognized 19 species within the MAstV genus and 3 species within the AAstV genus (Fig. 6), but following the standardized criteria for classification, at the time of writing of this review, the two genera could be divided into 33 and 11 genotype species, respectively. Thus, the *Astroviridae* family currently constitutes a remarkably genetically diverse emerging family with a long evolutionary history and a broad spectrum of host species, and its nomenclature and taxonomy should be discussed, agreed upon, and updated regularly.

In fact, within the *Astroviridae* family, it is common to find that a single host species, such as humans, is susceptible to infection by divergent astroviruses (Fig. 6). Besides humans, many other animal species, such as pigs (147), bats (15, 148), sea lions (18), sheep (149), cows (150–152), mice (100, 153), mink (154), turkeys (20, 24, 155), chickens (64), ducks (24, 156), and doves (24, 32, 157), can be infected with viruses belonging to different genetic lineages.

For most of the cases, it is still unknown whether these divergent viruses are dispersed globally or whether they are restricted to specific geographic areas. Finally, whether these viruses may show different clinical presentations when infecting the same animal species is yet to be elucidated for most cases. In general, AstV infections are restricted to the gastrointestinal tract, with symptomatic cases occurring predominantly in young individuals, but extraintestinal symptomatic and asymptomatic infections have also been described for both mammal and avian viruses, indicating that the tissue tropism of these viruses may be wide.

Reconstruction of evolutionary events within the *Astroviridae* family indicates that the separation between mammalian and


L23513 HAstV1 Oxford
Z25771 HAstV1 Newcastle
AY720892 HAstV1 Dresden
Y08632 HAstV7 Oxford
AF141381 HAstV3 Berlin
DQ630763 HAstV3 WH1859
AF260508 HAstV8 Yuc-8
DQ028633 HAstV5 Goiania5/GO/12/94/Brazil
AB013618 HAstV6 Osaka
L13745 HAstV2 Oxford
DQ344027 HAstV4 Guangzhou
DQ070852 HAstV4 Goiania5/GO/12/95/Brazil
AY720891 HAstV4 Dresden
AF056197 FAstV 1 Brisstol
KF499111 FAstV 2 1637F
GQ914773 PAstV 2/Shanghai/2008
HM756258 PAstVI/PoAstV12-3/Canada/2006
AB037272 PAstV 1/Tokushima83-7
Y15938 PAstV 1
FJ890352 California Sea Lion 2
JN193534 CaAstV/ITA/2010/Zoid
HQ623147 CaAstV/CHN/2008/toSH8
HQ623148 CaAstV/CHN/2008/ofSH15
GU376736 CaAstV/CHN/2009/SH
HM045005 CaAstV/ITA/2008/Bari
FM213332 CaAstV/Italy/2005
FM213331 CaAstV/Italy/2005
FM213330 CaAstV/Italy/2005
FJ890355 Bottlenose Dolphin
JN052023 RabAstV/Nausika/ITA/08
JF729316 RabAstV/TN/2208/2010
FJ222451 HAstV MLB1
AB823731 HAstV MLB1/BtnMLB1-40
FJ402983 HAstV/MLB1
JX857870 HAstV MLB3
JF742759 HAstV MLB2
AB829252 HAstV MLB2/GUP187
NC013443 HAstV HMO-A
GQ502193 HAstV VA2
JX857869 HAstV VA4
GQ415661 HAstV HMO-B
JX857868 HAstV VA3
GQ891990 HAstV PS
GQ415662 HAstV HMO-C
FJ973620 HAstV VA1
FJ890351 California Sea Lion 1
GU985458 MinkAstV/SMS/2000
AY179509 MinkAstV
HM756261 PAstVIII/PoAstV16-2/Canada/2006
JX556691 PAstV 3/US-MO123
FJ571067 BatAstVTm/Guangxi/LD71/2007
NC002469 OAstV
KF233994 BAstV NeuroS1
FJ571068 BatAstVHa/Guangxi/LDS11/2007
FJ571074 BatAstVHp/Guangxi/LC03/2007
EU847155 BatAstV1-AFCD337
FJ571073 BatAstVTm/Guangxi/LD54/2007
FJ571069 BatAstVTm/Guangxi/LD04/2007
FJ571072 BatAstVm/Guangxi/LD45/2007
FJ571065 BatAstVTm/Guangxi/LD38/2007
FJ571070 BatAstVTm/Guangxi/LD27/2007
FJ571071 BatAstVTm/Guangxi/LD19/2007
EU847144 BatAstV1-AFCD57
JF755422 MuAstV/M-52/USA/2008
FJ571066 BatAstVTm/Guangxi/LD77/2007
EU847145 BatAstV1-AFCD11
JF713711 PAstV5-33/USA
JX556693 PAstV 5/US-IA122
JX684072 PAstV 5/US-P2011-2
JN088537 PAstV 5/CC12
JN592482 OAstV-2/Hungary/2009
HM450381 RatAstV/RS118/HKG/2007
HM450382 RatAstV/RS126/HKG/2007
JF713713 PAstV4-35/USA
JX556692 PAstV 4/US-IL135
GU562296 PAstV 2/Hungary/2007
JX684071 PAstV 4/US-P2011-1
JQ340310 WBAstV-1/2011/HUN
KC609001 MuAstV/BSRI1
JQ408745 MuAstV/USA/2011/TF18LM
HQ916315 BoAstV34/HK
HQ916316 BoAstVB76/HK
HQ916314 BoAstV170/HK
HM756260 PAstVII/PoAstV14-4/Canada/2006
HQ916317 BoAstVB76-2/HK
HQ916313 BoAstVB18/HK
HM447046 CcAstV-2/DNK/2010
HM447045 CcAstV-1/DNK/2010
HM756259 PAstVII/PoAstV12-4/Canada/2006
HQ647383 PAstV/JWH-1/China/2009
JF713712 PAstV2-51/USA
JF713710 PAstV2-43/USA
JX556690 PAstV 2/US-IA122
Y15936 TAstV-1
JF414802 CAstV/GA2011
AF206663 TAstV-2
AY769615 TAstV-2/1987
EU143843 TAstV-2
EU143847 TAstV/MN/01
AY769616 TAstVOhio2001
FJ434664 Duck AstV
JX439643 Duck AstV 1/WF1201
AB046864 ANV-2
AB033998 ANV-1/G-4260
JX985647 KG118/Rock Dove/100804
JX985648 KG703/Spotted Dove/091130
HM029238 ANV-CHN
HQ889774 Pigeon ANV/SH10
JX985649 KG08-12/Pond Heron/081008
FR727149 WPiAstV/06/15660-1
FR727146 FPiAstV/03/594-6
FR727148 FPiAstV/03/603-5
FR727147 FPiAstV/03/594-9
JX985650 MPJ1332/Northern Pintail/091223
JX985652 MPJ1442/Northern Pintail/061230
JX985651 MPJ1433/Northern Pintail/091230
MAstV 1 (Human)
MAstV 2 (Cat, cheetah)
MAstV 2 (Cat, cheetah)
MAstV 3 (Pig)
MAstV 4 (California sea lion)
MAstV 5 (Dog)
MAstV 7 (Bottlenose dolphin)
MAstV 6 (Human)
MAstV 8 (Human)
MAstV 9 (Human)
MAstV 11 (California sea lion)
MAstV 10 (Mink)
MAstV 12 (Bat)
MAstV 13 (Sheep)
MAstV 17 (Bat)
MAstV 18 (Bat)
MAstV 19 (Bat)
MAstV 14 (Bat)
MAstV 15 (Bat)
MAstV 16 (Bat)
Mamastrovirus
AAstV 1 (Turkey, chicken)
AAstV 3 (Turkey, guinea fowl, duck)
AAstV 2 (Chicken, turkey)
Avastrovirus
p-distance (% change)
0 5 10 15 20

avian virus lineages occurred 310 million years ago and was followed by the divergence within the mammalian viruses and the occurrence of several cross-species transmissions (158, 159). Besides observational studies, analysis of the tree topology suggests that interspecies transmission may be plausible for these viruses and highlights their zoonotic potential. Indeed, some of the officially recognized AstV species (genotypes *MAstV 2*, *AAstV 1*, *AAstV 2*, and *AAstV 3*) may infect more than one animal species, indicating that cross-species transmission is frequent, especially in avian viruses. While cross-species infections have been well documented in poultry (160, 161) and have also been suggested in mammals, especially in pigs, cats, and humans (147, 159, 162, 163), it is still unclear whether avian viruses may be able to infect mammal species and vice versa. Interestingly, especially within viruses infecting humans, the distinct evolutionary relationships among the different genotype species that infect humans indicate that several introductions of HAstVs into the human population have occurred (31). As a consequence, the zoonotic potential of these viruses is high, and future nonhuman-to-human transmissions are likely to occur.

Human Astroviruses

Four phylogenetic clades or genotype species of HAstVs have been identified in human stools. Besides the 8 serotypes of classic HAstV (*MAstV 1*), which are widely recognized as a common cause of nonbacterial gastroenteritis in children, human viruses are found in genotypes *MAstV 6*, *MAstV 8*, and *MAstV 9* (Fig. 6, red). While classic HAstV (*MAstV 1*) and HAstV-MLB (*MAstV 6*) form a monophyletic group together with viruses from cats, pigs, dogs, rabbits, California sea lions, and dolphins, the other two genotypes, *MAstV 8* and *MAstV 9*, are genetically related to each other and to viruses from mink, sheep, California sea lions, bats, cattle, pigs, and mice.

Within genotypes, strains can be grouped in serotypes based on their antigenicity. While all eight classic HAstVs clearly correspond to different serotypes, antigenic differences between MLB1, MLB2, and MLB3 and between VA1, VA2, VA3, and VA4 still need to be investigated. While the eight serotypes of *MAstV 1* share 63 to 84% amino acid similarities at the capsid level, similarity among strains within *MAstV 6*, *MAstV 8*, and *MAstV 9* ranges between 74 and 90%, 78 and 79%, and 75 and 77%, respectively, suggesting that they would likely show different antigenic properties. Finally, subtypes or lineages have also been identified within each serotype, with a cutoff 93 to 95% nucleotide homology to a reference strain needed to be considered a new subtype (164–168). In light of this, HAstV-1 has so far been classified into 6 lineages (1a to 1f), HAstV-2 into 4 lineages (2a to 2d), HAstV-3 into 2 lineages (3a and 3b), and HAstV-4 into 3 lineages (4a to 4c) (166, 169–174).

Although strains are usually classified according to ORF2 variability and a high correlation is detected between serotypes and genotypes, classic HAstVs have also been typed based on ORF1a. Phylogenetic analysis of several partial sequences of ORF1a resulted in only two clearly separated groups, which have been called genogroup A (which comprises serotypes HAstV-1 to HAstV-5 and HAstV-8) and genogroup B (serotypes HAstV-6 and HAstV-7) (86, 175). As suggested, this different phylogenetic clustering may have resulted from recombination events between structural and nonstructural coding regions (86, 175).

As viruses with RNA genomes, nucleotide mutations and recombination events are, among other factors, important in their genome evolution (reviewed in reference 176). The HAstV genome mutation rate has been analyzed, and it generally matches the variation rates for single-stranded positive-sense RNA viruses which evolve rapidly, such as picornaviruses (177). According to this study, variation rates are approximately 3.7×10^{-3} nucleotide substitutions per site per year and 2.8×10^{-3} synonymous changes per site per year. A higher genetic variability in ORF2 than in ORF1a and -1b has been confirmed, suggesting the independent evolution of HAstV genes and even gene regions and pointing to different selection pressures and/or evolutionary constraints for specific genomic regions (158, 178). Overall, negative or purifying selection is a dominant force among all HAstV genes, and only few codon sites, such as those coding for residues located within the capsid protein exposed to the immune pressure or which are involved in cellular recognition, show positive selection and molecular adaptation during evolution (155, 178). Sites which are prone to positive selection are concentrated within the C-terminal half of ORF2, but they are not restricted to it. In ORF1a, clusters of such sites are also located in nonconserved regions around amino acid positions 614 to 624, 775 to 777, and 812 to 817, also close to its C terminus (178). Finally, when comparing HAstVs belonging to different genetic lineages or genotypes, a marked constrained evolution has been observed within both ORF1a and ORF1b in classic HAstVs (*MAstV 1*) but not in MLB and VA/HMO viruses (*MAstV6*, *MAstV8*, and *MAstV 9*), suggesting that ORFs for nonstructural genes of classic HAstVs may contain RNA secondary structures or cryptic overlapping ORFs that are absent in the same region of the other human viruses (31).

When comparing evolutionary rates among different AstVs, higher rates and an enhanced accumulation of synonymous substitutions, especially in ORF2, have been observed in porcine, ovine, mink, and turkey AstVs than in viruses infecting humans,

FIG 6 Phylogenetic relationships within the family *Astroviridae*. The predicted amino acid sequences of the entire capsid polyprotein were aligned using Clustal Omega (309). The phylogenetic tree was generated using the neighbor-joining algorithm (310) implemented in the MEGA6 program (311). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) which are higher than 70 are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances (p-dist) used to infer the phylogenetic tree. All positions containing alignment gaps and missing data were removed only in pairwise sequence comparisons (pairwise deletion option). Genotype species officially recognized by the ICTV are indicated, with hosts of origin shown in parentheses. Human viruses are colored in red, porcine viruses are colored in dark blue, mink viruses are colored in dark gray, bovine viruses are colored in brown, ovine viruses are colored in light blue, bat viruses are colored in black, murine viruses are colored in purple, turkey viruses are colored in orange, and chicken viruses are colored in green. Arrows indicate strains which have been isolated from neurologic tissues. HAstV, human astrovirus; FAstV, feline astrovirus; PAstV, porcine astrovirus; CaAstV, canine astrovirus; RabAstV, rabbit astrovirus; OAstV, ovine astrovirus; BoAstV, bovine astrovirus; MuAstV, murine astrovirus; WBAstV, wild boar astrovirus; CcAstV, deer astrovirus; TAstV, turkey astrovirus; CAstV, chicken astrovirus; ANV, avian nephritis virus; WPIAstV, wild pigeon astrovirus.

cats, and chickens (178). The major evolutionary forces acting specifically at synonymous positions are nucleotide composition, translational selection, and codon usage. A study performed in 2007 (159) demonstrated that the codon usage is an operational driving force especially among the expansion of mammalian AstVs after their separation from the avian AstVs and that AstV codon usage points toward the host species codon usage. In addition, this switch in codon usage during diversification of mammalian AstVs was related to their different nucleotide compositions, rather than being caused by translational selection (159).

Finally, recombination events after coinfection of the same host by different viruses are another of the main mechanisms that have played a role in HAstV evolution and in generation of novel strains. Natural recombinants have been commonly found between strains belonging to the same genotype, such as different strains within the same serotype or different serotypes of classic HAstV (*MAstV 1*) (168, 172, 179–182), but it is still unknown whether recombination may also occur between strains belonging to different genotype species even if they are adapted to infect the same host. So far, recombination events involving human strains have been suggested to have occurred between classic HAstV and a California sea lion AstV (18) and between classic HAstV and PAstV (163). Recombinations between different animal AstVs have also been described (152, 155, 162, 183). Although most recombination breakpoints have been identified upstream of the conserved ORF1b/ORF2 junction region (155, 168, 172, 182), they are also frequent within ORF2 (152, 155, 172, 177), and they may also exist within ORF1a (155, 181) and ORF1b (155, 177, 180).

Nonhuman Astroviruses

Besides HAstVs, many other mamastroviruses show a remarkable genetic diversity, and some of them are of special interest because they have also been isolated both in feces and in extraintestinal tissues. These include mink AstVs (154) and bovine AstVs (BoAstVs) (150–152), which have been isolated in neurologic tissues in association with neurologic diseases, porcine AstVs (PAstVs) (184, 185), which have been isolated in blood, and murine AstVs (MuAstVs), which have been isolated in mesenteric lymph nodes, spleens, livers, and kidneys of wild-type or immunodeficient mice (100).

AstVs infecting mink (genotype *MAstV 10*) have traditionally been considered agents of the preweaning diarrhea syndrome (14), but recently a novel virus which could be classified as a different genotype (Fig. 6, dark gray) has been isolated in neural tissue from minks suffering from the so-called shaking mink syndrome (SMS) (154). Similarly, among BoAstVs, which are also associated with gastrointestinal infections and may be grouped in 4 genotypes (Fig. 6, brown), the strain called BoAstV-NeuroS1 has been detected in brain tissue in association with neurologic disease (150). Of note, the 3 MAstV species associated with neurologic symptoms (HAstV-PS, mink AstV-SMS, and BoAstV-NeuroS1) are included in the same genetic cluster of astroviruses (Fig. 6).

Pigs have been extensively studied in different parts of the world because they serve as a reservoir for many zoonotic diseases. To date, the ICTV officially recognizes only one PAstV genotype (*MAstV 3*), but at least five genetic lineages which would eventually correspond to genotypes have been documented (162, 185–188) (Fig. 6, dark blue). PAstVs are widely distributed, and they have been isolated from feces of both diarrheic and healthy pigs in many different parts of the world (187, 189–191). Recently, viremia in healthy pigs infected with lineages 2 and 4 has also been documented, with a viral RNA prevalence in serum of 3.89% (184).

Surprisingly, it has recently been recognized that MuAstVs are commonly found infecting laboratory mice in different countries, including breeding facilities, research institutions, and commercial research mouse facilities (100, 153, 192, 193) (Fig. 6, purple). Although the mice are asymptomatic, the viruses have been isolated in the gastrointestinal tracts and mesenteric lymph nodes of wild-type mice but also in spleens, livers, and kidneys of immunodeficient mice (100, 192).

Among the other MAstVs described to date, large genetic diversity has been described in ovine AstVs (OAstVs) (Fig. 6, light blue) (119, 149) and bat AstVs (BatAstVs) (Fig. 6, black) (15, 148, 194, 195). In most cases, viruses have been mostly isolated from healthy animals, and prevalences are high. The genetic diversity of feline AstVs (FAstVs), canine AstVs (CaAstVs), rabbit AstVs (RabAstVs), rat AstVs, and AstVs infecting sea lions and dolphins has not been studied so extensively, and the clinical impact of their infections seems to be low and has not been well explored yet (11, 16, 17, 196–200).

In contrast, the pathogenicity of *Avastroviruses* is significant, especially in poultry, with high economic costs and affecting the food industry worldwide. These viruses have been associated with avian diseases, including poult enteritis mortality syndrome (PEMS) in turkeys, chickens, and guinea fowl, mild growth loss, nephritis, the runting-and-stunting syndrome (RSS), and gout in chickens, and fatal hepatitis in ducklings (20, 64, 146, 201, 202). The ICTV officially recognizes 3 species within AAstVs: *AAstV 1* (turkey AstV 1), *AAstV 2* (avian nephritis virus [ANV]), and *AAstV 3* (turkey AstV 2 and duck AstV) (Fig. 6). Among them, TAstV and ANV from chickens are the two most widely studied virus strains, and surveys indicate that these viruses are worldwide distributed. Recently, AstVs infecting pigeons have been described (157), and a novel virus related to ANV has also been isolated and characterized in pigeons with gastroenteritis (22, 32), as well as in healthy chickens (22). Finally, viruses similar to previously known viruses infecting poultry have been also isolated in wild aquatic birds, and wild migratory bird populations have been shown to commonly carry novel and diverse AAstVs, likely playing a role in AAstV ecology (24).

EPIDEMIOLOGY

HAstV Transmission

HAstVs are transmitted essentially through the fecal-oral route, as proven in human volunteer studies (95, 96). However, in these studies, few adult recipient individuals developed gastroenteric disease with virus shedding in feces, probably because of their age.

Food and water may act as vehicles for human enteric virus transmission (203, 204). Several large HAstV outbreaks have been associated with consumption of contaminated food (205–207). In addition, fecally contaminated fomites have been implicated in outbreaks declared in institutions such as day care centers, hospitals, nurseries, schools and military facilities (208, 209). Moreover, waterborne transmission of HAstVs has been suggested as a risk of digestive morbidity for the general population (210), and HAstV was recovered in surface water from an area in which an outbreak of gastroenteritis was concomitantly occurring (211). The envi-

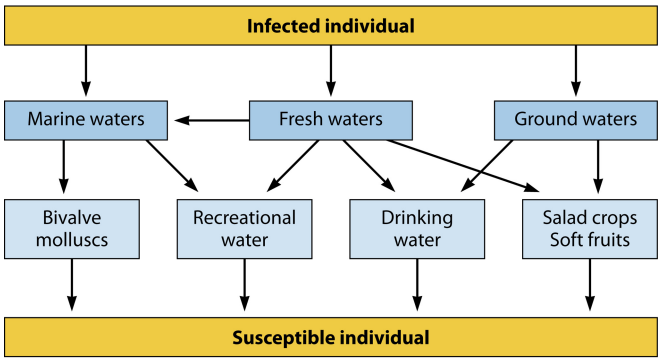


FIG 7 Flowchart of potential routes of environmental transmission of human astroviruses (see the text for details).

ronmental transmission of HAstV is supported by the stability of the virus in drinking water (212), freshwater (213), and marine water (213).

Figure 7 depicts the potential environmental routes of HAstV transmission. HAstV may be shed in very high numbers, reaching up to 10^{13} genome copies per gram, in the stools of infected individuals (104). Since wastewater treatment practices do not ensure the complete removal of viral pathogens, HAstV present in untreated and treated wastewaters are discharged into the environment (214–217) and may become contaminants of marine water, freshwater, and groundwater (211, 218). Groundwater may also receive viral contamination through the injection of reclaimed wastewater (219).

Among foods susceptible to become contaminated with HAstV at the preharvest stage are bivalve mollusks grown in polluted waters (218) and fresh produce irrigated with contaminated water, such as lettuce, green onion, and other greens, as well as soft fruits such as raspberries and strawberries (220). These products usually undergo little if any processing before consumption and hence are susceptible to act as vehicles for human enteric virus transmission (221).

Surface water and groundwater are employed as sources of drinking water for public consumption, and poor water quality is recognized as a major health threat. The WHO estimates that almost 90% of the global burden of gastroenteritis is attributable to unsafe water and poor sanitation and hygiene, with the pediatric population in developing areas being the most vulnerable group (222). As an example of this situation, HAstV genomes have been detected in tap water in Ghana (223).

Although the risk derived from recreational activities in sewage-polluted waters is much lower than that from drinking contaminated water, an outbreak of gastroenteritis occurred among children and adults who bathed in a Helsinki outdoor wading pool that had norovirus and astrovirus contamination (224). Additionally, a seroprevalence study in the United Kingdom showed that 93% of surfers had HAstV antibodies, while only 23% of age-matched controls had these antibodies (225). Chlorine and other disinfectants are routinely employed for drinking water treatment and are effective for the inactivation of HAstV in water (212, 226).

Poor hygiene practices with improper food handling are usually responsible for postharvest contamination of food products, particularly ready-to-eat foods such as salads, sandwiches, and deli meat that are all consumed either uncooked or lightly cooked. HAstVs cause asymptomatic infections in children and adults (61, 93), and asymptomatic food workers are more often implicated in gastroenteritis outbreaks than symptomatic food handlers (227). It is critical to ascertain the significance of asymptomatic as well as presymptomatic and postsymptomatic HAstV shedding in the transmission of food-borne infections.

Fomites or inanimate surfaces play an important role in the institutional spread of HAstV infections (208, 209, 228). Fomites include serving and chopping utensils, and the ability of the virus to persist dried onto these surfaces long enough and in numbers high enough to represent a risk is a major public health issue. Disinfection practices, as well as improved hygiene measures, particularly in high-risk settings such as hospitals, institutions for the elderly, day care centers, or restaurants, are of major importance in preventing the spread of infections. Antiseptics and disinfectants must be employed for the inactivation of pathogens on skin (notably hands) and fomites, respectively.

Although astroviruses were initially considered to be so species specific that it led to their classification based on the host species (45), concern about the potential zoonotic transmission of astroviruses to humans has recently arisen (146). As discussed above, the advent of pyrosequencing enabled the characterization of previously unrecognized HAstVs, termed nonclassic HAstVs (25). Some of these nonclassic HAstVs have been shown to have common ancestors with recently described rat AstV (11), while others are phylogenetically related to mink and ovine AstVs (29).

As mentioned above, there is evidence of recombination events between human and animal AstVs. Recombination in the variable region of ORF2 has been shown to occur between human and porcine strains of AstV (163). These AstV recombinants were described in areas in which human and porcine strains were in close contact. Since the genetic variability of the HAstV strains appeared to be much higher than that of the porcine strains, it was concluded that zoonotic transmission likely took place from humans to pigs instead of vice versa, which had previously been suggested (158, 163).

High genetic variability, together with the occurrence of interspecific infections and recombination events, makes AstVs serious candidates for emerging zoonotic infections.

Age, Geographic, and Temporal Distribution

HAstVs affect predominantly the pediatric population (117, 164, 229, 230), although infections in elderly people and immunocompromised hosts are also reported (231–236). In addition, astrovirus gastroenteritis may also affect healthy adults (88, 89, 207). The age of children infected with classic HAstV is highly variable, ranging from newborns to over 5 years (33, 164, 167, 237–243); however, infection is more common among those younger than 2.

Classic HAstVs are distributed all over the world and are associated with 2 to 9% of cases of acute, nonbacterial diarrhea in children (reviewed in reference 146), although incidences as high as 61% have been reported (94). Table 2 shows some examples of studies on classic HAstV prevalence in stool conducted in different regions and settings. The mean incidence worldwide is 11%, with 7% and 23% incidences in urban and rural areas, respectively (244). HAstV incidence is usually higher in developing countries, although paradoxically, a low incidence of infection is observed in sub-Saharan Africa (245–247).

A tendency toward a decrease in classic HAstV incidence seems to have occurred in the last decades, which is evidenced by com-

TABLE 2 Studies of human astrovirus prevalence in stool

Region and country (setting, period of study)	Age (yr) of study population	Astrovirus prevalence (%)	Detection method	Reference
North Africa				
Egypt (rural, 1995–1998)	<2	26.7	EIA	237
Tunisia (urban and rural 2003–2005)	<5	6.0	EIA	306
Sub-Saharan Africa				
Botswana (urban, 2001–2002)	<5	2.7	EIA	245
Kenya (urban and rural 1999–2005)	<5	5.3	EIA	246
Ghana (urban, 2005–2006)	<12	4.8	RT-PCR	247
Asia				
Thailand (urban, 1985–1987)	<5	8.6	EIA	239
Japan (urban and rural 1982–1992)	Children of all age groups, mostly <10	18	EIA	249
China (urban and rural, 1998–2005)	<5	5.5	EIA plus RT-PCR	307
Vietnam (urban, 2005–2006)	<3	13.9	RT-PCR	241
India (urban, 2004–2008)	<8 years, adults	3.1	RT-PCR	308
Japan (urban 2008–2009)	Children of all age groups	1.7	RT-PCR	250
North America				
Mexico (rural, 1992–1995)	<3	61	EIA and RT-PCR	94
USA (urban, 1993–1994)	<3	20	EIA	266
USA (urban, 1996–1997)	Children of all age groups, mostly <3	6.9	EIA	230
Central America				
Guatemala (rural, 1987–1989)	<3	38.6	EIA	33
South America				
Chile (urban, 1985–1987; 1993–1995)	<3	23.5	EIA	240
Brazil (urban, 1990–1992)	<2	5.8	EIA plus RT-PCR	265
Argentina (urban, 1995–1998)	<3	3.7	EIA plus RT-PCR	267
Colombia and Venezuela (urban, 1997–1999)	<5	5.0	EIA	167
Europe				
France (urban, 1995–1998)	<3	6.0	EIA	242
Spain (urban, 1997–2000)	Children of all age groups, Mostly <5	4.9	RT-PCR	164
Italy (urban, 1999–2000)	<2	3.1	EIA	264
Germany (urban, 2000)	All ages	1.2	EIA	276
France (urban, 2007)	<5	1.8	EIA	248
Oceania				
Australia (urban, 1995)	<5	4.2	Northern (RNA) dot blot	35

paring the data from studies conducted in the same area several years apart, e.g., in France (242, 248) or Japan (249, 250). The mean classic HAstV incidence in the 1980s was 22%, while it was 15% in the 1990s and 5% in the first decade of the new millennium (244). However, there are differences in the methods employed and the study populations that make difficult to adequately compare these studies. It is plausible that the decrease in the incidence of classic HAstV infection may be related to the displacement of classic HAstV infections by those with novel HAstV strains such as MLB and VA/HMO, which could be outcompeting classic HAstV. As a matter of fact, recent reports point to a high prevalence of some of these strains (115). The diminution in the number of astrovirus gastroenteritis cases is not observed with other viral agents such as noroviruses, whose incidence has increased in the same time period (251–255).

The classic HAstV distribution follows a seasonal pattern with a higher incidence of infection in temperate regions in the cold-weather period, and although there is a dramatic decline in the number of cases in the warm-weather period, HAstVs continue to circulate throughout the year, and infections do occur in summer too (61, 164, 243, 249, 256, 257). This seasonality may be the consequence of a better stability of the virus at lower temperatures (208, 212). Other viral gastroenteritis agents such as rotaviruses and noroviruses, but not adenoviruses, show a similar seasonal pattern (258–260). In tropical areas, the maximum incidence of HAstV infections tends to occur in the rainy season (33, 94). Nevertheless, some studies have failed to observe a distinct peak of infection throughout the year (243). In addition, other studies have reported a greater burden of astrovirus diarrhea in children in alternate years (164, 167, 243, 257).

TABLE 3 Prevalence of serotypes (genotypes) of classic human astrovirus

Region and country (setting, period of study)	Prevalence (%) of serotype: 1	2	3	4	5	6	7	8	Reference
North Africa									
Egypt (rural, 1995–1998)	43.3	3.6	12.0	4.8	15.7	7.2	0	12.0	237
Asia									
Vietnam (urban, 2005–2006)	100.0	0	0	0	0	0	0	0	241
India (urban, 2004–2008)	6707	9.7	0	0	6.5[a]	0	0	16.0	308
Japan (urban 2008–2009)	91.0	0	9.0	0	0	0	0	0	250
North America									
USA (urban, 1993–1994)	55.0	17.0	—[b]	—	—	—	—	—	266
South America									
Brazil (urban, 1990–1992)	45.5	27.3	12.1	12.1	0	3.0	0	0	265
Argentina (urban, 1995–1998)	41.0	13.0	13.0	25.0	8.0	0	0	0	267
Europe									
Spain (urban, 1997–2000)	38.0	6.0	19.0	26.0	0	0	0	11.0	164
Oceania									
Australia (urban, 1995)	85.0	0	0	15.0	0	0	0	0	35

[a] ORF1a/ORF2 recombinant strains.
[b] —, there were no data on this genotype in the study.

A different behavior regarding seasonality seems to occur with nonclassic HAstV strains. In one study (109), novel astrovirus (MLB1, MLB2, and VA/HMO) infections were observed in March, April, May, July, and November. However, more studies are required to elucidate whether nonclassic HAstV strains follow a seasonal distribution pattern.

Molecular Epidemiology of HAstV

In addition to the intrinsic variability caused by the error-prone RdRp (176), the occurrence of recombination events between nonstructural and structural coding regions, sometimes from viruses infecting different host species, adds more diversity and complexity to the AstV molecular typing (18, 146, 163, 175, 181).

In 1984, Kurtz and Lee set the methodology, based on the use of reference sera to recognize the different serotypes (five at the time) of HAstVs (261). Since what can be considered the birth of AstV epidemiology, up to eight serotypes of classic HAstV (HAstV-1 to HAstV-8) have been identified based on their antigenicity. Phylogenetic analysis of the capsid region, particularly of the carboxy-terminal hypervariable domain of the ORF2-encoded structural protein, points to a high correlation between serotypes and genotypes, thus allowing typing of the different AstV isolates through sequence analysis (45, 158, 262, 263). As explained above, sequence diversity in the capsid region allows the recognition of subtypes (165, 166, 168) or lineages (164, 167, 175, 264, 265) within the different genotypes.

Moreover, analysis of the well-conserved partial sequence near the protease motif-coding region enables the establishment of two genogroups, A (with serotypes HAstV-1 to HAstV-5 and HAstV-8) and B (with serotypesHAstV-6 and HAstV-7) (175). Sequence analysis of the hypervariable region in the nsP1a/4-coding region in ORF1a enables the additional differentiation of 12 genotypes, nine within genogroup A and three within genogroup B (86). In order to clearly distinguish them from ORF2-derived serotypes, the ORF1a-derived genotypes are named using roman numerals. While in most cases, a correlation exists between ORF1a genotypes and ORF2 serotypes, some genotypes are shared by more than one serotype, and some serotypes may be found in association with more than one genotype, highlighting the occurrence of recombination events between strains. Selected ORF1a genotypes have been associated with higher levels of viral shedding (86).

Table 3 shows the prevalence of classic HAstV serotypes worldwide. Overall, HAstV-1 is the most frequently isolated type (35, 164, 237, 241, 250, 256, 265–268). However, there are exceptions, such as in one study conducted in Mexico (238) in which HAstV-2 was reported to be the most prevalent. It is not well defined which is the second most frequent type, since differences related to the geographical localization are observed (164, 243, 269). Classic HAstV prevalence may be influenced by the lack of heterotypic immunity, since children in a given location may suffer subsequent infections with different serotypes. This was observed in one study (164) in which most infections with HAstV-1 and HAstV-3 occurred in children younger than 2 years old, while HAstV-4 infections were observed in children older than 4 years of age, thus confirming the apparent lack of cross-protection after infection with one serotype before infections with other types or variants.

LABORATORY DIAGNOSTICS

Early Developments: Electron Microscopy and Immunological Assays

Although cell culture propagation of some classic HAstVs is possible with the aid of trypsin (51, 59, 270), virus isolation in cell culture is not applicable for astrovirus diagnosis in fecal specimens. In the past, HAstVs were routinely detected by direct transmission electron microscopy (EM) in negatively stained stool

samples (1, 2, 228). This procedure is laborious, time-consuming, and subjective and involves highly experienced personnel. Further limitations were that, as mentioned above, only around 10% of the particles show the characteristic star-like surface structure and that the sensitivity of EM requires concentrations of around 10^7 particles per gram of stool for visualization of the virus (34). Such levels are usually detected for only 12 to 48 h after the onset of symptoms, with numbers often too low thereafter for EM detection.

The use of antibodies in immune electron microscopy (IEM) or solid-phase immune electron microscopy (SPIEM) enhances the sensitivity of the assay while enabling confirmation of the identity of the viral agent (88, 207, 252, 271). However, the limited availability of antibodies for the different HAstV types hampers the use of these techniques (34).

Enzyme immunoassays (EIAs) were developed for the detection (272, 273) and typing (256, 271, 274) of HAstV. Herrmann and coworkers (273) developed in 1990 an EIA based on the use as capture antibody of a monoclonal antibody (8E7) directed toward the conserved domain of the capsid and a peroxidase-labeled polyclonal antibody as the detection antibody. A further refinement was labeling the detection antibody with biotin (275).

At least two commercial EIAs are available for classic HAstV detection in stool specimens. Although the assays do not enable typing of the isolates, they are useful for rapid detection of the virus when large numbers of samples must be analyzed (167, 276–279). EIA-based techniques have been reported to have the same sensitivity as direct EM observation (228) but obviously are much less time-consuming. In addition, several rapid immunochromatographic tests against astroviruses are commercially available, and some of them have been evaluated (280).

Molecular Assays

The advent of molecular detection techniques, based initially on probe hybridization and later on genome amplification, opened the possibility to develop assays with exquisite sensitivity and specificity for the diagnosis of AstVs in stool. While the sensitivity of probe hybridization has been described to be on the same level as that of EIA (34), reverse transcriptase PCR (RT-PCR) offers thresholds of detection as low as 10 to 100 genome copies per gram of stool (34). Nevertheless, the sensitivity of these molecular assays relies greatly on several variables, such as primer/probe selection, enzymatic amplification reaction conditions, and, last but not least, the efficiency of the target nucleic acid extraction (281). In any case, studies employing EIA and RT-PCR in parallel reported a higher number of positive samples scored by the latter technique as well as a longer period of viral shedding that extended beyond the resolution of symptoms (206, 228).

Conventional RT-PCR methods are still broadly used today in many research laboratories both for screening and for further isolate typing. While some of the most extensively used common-type and type-specific primers for the detection of 8 serotypes of classic HAstV have been previously reviewed (282), primers Mon244/Mon245 (and Mon269/Mon270) described by Noel et al. (274), targeting the conserved 5′ end of ORF2 and allowing typing by sequencing, are some of the most widely used ones. Upon the discovery of novel HAstVs, consensus astrovirus RT-PCR primers targeting ORF1b were also published (15, 29, 30). Although these primers should be able to detect all known human and animal AstVs, so far they have allowed the detection of both classic and novel human AstVs (107, 110). Primers designed to specifically detect HAstV-MLB and HAstV-VA/HMO have also been described (27, 30, 114).

Finally, microarrays have also been shown to be an especially useful option for virus detection and typing. One of the described microarray-based methods is able to distinguish representatives of the eight known serotypes of classic HAstVs (283), and another one is a combimatrix for simultaneous detection of various enteric viral pathogens, including HAstVs (284). Additionally, other, less conventional molecular techniques, such as nucleic acid sequence-based amplification (NASBA), have also been described for HAstVs (285).

A further refinement was the development of quantification techniques such as real-time RT-quantitative PCR (RT-qPCR) assays, which provide high speed, sensitivity, and reproducibility and reduction of contamination risk in the diagnostic of HAstVs, (214, 286–289). Similarly, a real-time reverse transcription loop-mediated isothermal amplification has been developed for rapid and quantitative detection of HAstV-1 (290). Interestingly, some of the published RT-qPCR methods already come in a multiplex format allowing the simultaneous detection of classic HAstVs and other targets such as noroviruses, rotaviruses, sapoviruses, adenoviruses, and/or bocaviruses (291–294). One of these methods even includes up to 19 targets, including bacteria, protozoa, and helminths (295). Interestingly, that assay also includes the detection of two extrinsic controls to monitor extraction and amplification efficiencies (295), which may be highly relevant in surveillance or food virology applications. Commercial monoplex and multiplex real-time RT-qPCR kits are also available, although most of them are especially relevant for the clinical diagnostic setting. Remarkable among them is the FilmArray gastrointestinal panel (BioFire Diagnostics, Inc., USA), which is currently under development and aims to simultaneous detect 23 gastrointestinal pathogens from stool, including HAstVs and 4 other enteric viruses. Regarding novel HAstVs, although not designed to be used in surveillance studies, two RT-qPCR assays targeting HAstV-MLB have been applied for quantification of viral loads in stool and serum (111, 112). If the importance of novel HAstVs in human health is clinically confirmed, molecular methods should be soon updated to incorporate broadly reactive primers/probes to universally detect all HAstVs.

Finally, although classic HAstVs are somewhat fastidious to grow *in vitro* and novel HAstVs have not yet been propagated in any cell line, the combination of molecular techniques with infectivity assays may help to ultimately analyze the importance that positive results in terms of genome copies may have on public health when present in environmental or food samples. The use of CaCo-2 cells combined with pretreatment of virus inoculums with trypsin remains the gold standard for classic HAstV isolation from clinical and environmental samples (59, 67), and several integrated cell culture (ICC) methods combined with RT-PCR detection have been described (296, 297). Whether these methods allow the isolation of novel HAstVs or whether these viruses show significant differences in cell tropism still remains to be elucidated.

Next-generation technologies, including whole-genome sequencing and viral metagenomics, may rapidly generate sequence-based data that will be determinant for optimizing the currently used primers and will also help to identify additional genomic regions for analysis. Furthermore, whole-genome se-

quencing will provide genetic information that may also correlate with virulence factors and may help us to better understand the basis of viral pathogenesis, and metagenomics will be useful to identify new emerging strains.

PREVENTION AND TREATMENT

The prevention of HAstV infections is essentially based on the control of the transmission routes and on the prevention and control of disease at the host level. The control of the transmission routes includes virus detection and inactivation in water and food and disinfection of contaminated fomites. Methods for the detection and quantification of HAstVs are available (see above), but the threshold of acceptable genome copy numbers in water and food matrices has yet to be defined not only for HAstVs but for other gastroenteritis agents as well. Although the survival of classic HAstV in drinking water is high, disinfection treatments of 2 h with1 mg/ml of free chlorine is quite effective (212). However, genogroup B is somewhat more resistant to chlorine disinfection than genogroup A (215), suggesting that differences in environmental persistence may exist between strains. Astrovirus survival and inactivation in food matrices have not been extensively studied, probably because only a few HAstV food-borne outbreaks have been described (207, 298) and because efforts have been dedicated to the application of emerging technologies for the inactivation of other viral agents, such as norovirus and hepatitis A virus (299, 300). Regarding disinfection of contaminated fomites, 90% alcohol has been proved useful (301). Unfortunately, no information on inactivation is yet available for nonclassic HAstVs.

Prevention of disease development in the host includes vaccination and potentiation of the natural defenses such as the gut microbiota. No vaccines have been developed for HAstVs despite several descriptions of virus-like particle (VLP) production in different systems (302, 303). This lack of commercial interest in vaccine production may be due to the low clinical impact of astrovirus infection in healthy patients and to the need for a multivalent vaccine to cover all circulating serotypes and strains or at least the most prevalent ones, since apparently there is not heterologous protection (90). Some authors have speculated that probiotics, which may interfere with the biological cycle of enteric virus at many different steps, may be used as a measure to prevent and/or treat intestinal viral infections (304, 305). Additionally, the antiviral activity of some synthetic flavonoids on astrovirus replication has been described (226).

The usual therapy applied to patients (young children or adults) suffering from serious gastroenteritis consists of oral or intravenous fluid replacement to avoid dehydration. Intravenous treatment with IgG for immunocompromised hosts with severe or persistent diarrhea has also been proposed, although the efficacy of this treatment has yet to be established in large-scale studies (231).

CONCLUSIONS

In the last few years, the global vision of AstV diversity has dramatically changed. Although they were traditionally regarded as agents of acute gastroenteritis in children, it is now known that the viruses may spread systemically, especially in immunocompromised individuals. Viremia has been documented in some human cases, pigs, and turkeys, and AstVs have been isolated in neurologic tissue in association with neurologic disorders in humans, cattle, and mink. The emergence of such a number of different viruses infecting so many different species indicates the possibility of zoonotic transmission of viruses to humans. In this regard, the AstV diversity found in avian species, pigs, and bats is remarkable, with these species frequently being associated with the zoonotic transmission and the emergence of new strains of influenza viruses and coronaviruses. In fact, the novel HAstV-VA/HMO viruses (genotypes *MAstV 8* and *MAstV 9*) are phylogenetically closer to the bat isolates than to the classic HAstVs (*MAstV 1*), and some of these novel viruses have been associated with extraintestinal manifestations. All these concerns highlight the need to improve broadly reactive diagnostic techniques allowing the detection of all HAstVs. A better understanding of the AstV replicative cycle, particularly of the novel viruses, and of their role in clinical disease and their mechanisms of pathogenesis is required to prevent and treat potential new infections.

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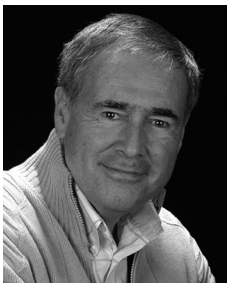

Albert Bosch (Full Professor) has over 35 years of experience in virology and has published over 150 peer-reviewed articles on enteric viruses in scientific journals as well as complete books. He has been member of numerous scientific committees, notably the scientific committee of the Spanish Food Safety Authority and the CEN committee of the EU on standardization of molecular methods for virus detection in foods; he also belongs to the scientific panel on food- and waterborne diseases of the European Centre for Disease Control and Prevention (ECDC). He acted as chairman of the Astrovirus Study Group of the International Committee on Taxonomy of Viruses (ICTV). He is president of the International Society for Food and Environmental Virology and the Spanish Society for Virology. He was the president of the water microbiology section of the Spanish Society for Microbiology and is a member of the steering committee of the Health-Related Water Microbiology Group of the International Water Association.

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Susana Guix graduated from the University of Barcelona in 1997 with a degree in biology. After graduation, she performed her Ph.D. work on human astroviruses at the Department of Microbiology of the University of Barcelona. She performed the first studies on the molecular epidemiology of astroviruses in Spain and characterized the apoptotic response in infected cells. In 2004, she received a Fulbright postdoctoral fellowship and performed her postdoctoral work at Baylor College of Medicine in Houston, TX, working on human noroviruses. For the past 6 years, she has worked as a Lecturer Professor at the Department of Microbiology of the University of Barcelona. She was a member of the Astrovirus Study Group of the International Committee on Taxonomy of Viruses (ICTV) from 2005 to 2012. Her current research interests on astroviruses and noroviruses include their molecular epidemiology, the study of the role of the viral nonstructural proteins, and the characterization of the innate immune responses activated upon infection.