UNIVERSITAT DE BARCELONA

Characterization of emerging novel human astrovirus: form bedside to bench

Diem-Lan Vu Cantero

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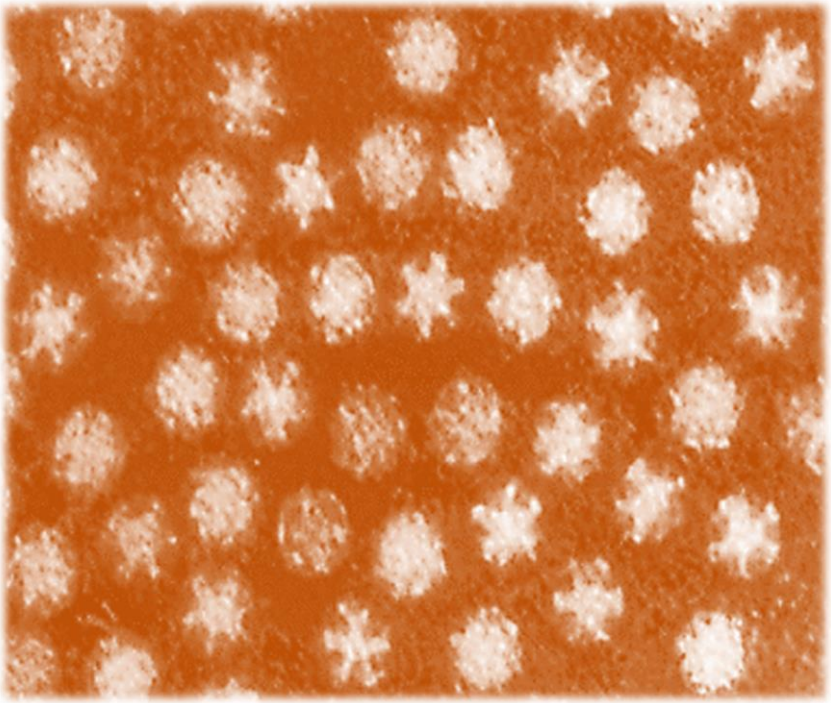

Doctoral Thesis

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Characterization of emerging novel human astrovirus: from bedside to bench

Memòria presentada per Diem-Lan Vu Cantero per a optar al títol de

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ABSTRACT

Novel human astrovirus (HAstV) are enteric virus belonging to the *Astroviridae* family and were discovered 10 years ago by high-throughput sequencing. They are divided in two different clades, the HAstV-MLB including 3 genotypes (MLB1-3) and HAstV-VA including 5 genotypes (VA1-5). While their role during gastroenteritis is debated, they have been reported as the sole agent identified in many cases of severe central nervous system infection, mainly in immunocompromised patients. This suggests that these emerging and highly divergent viruses can be associated with serious clinical manifestations, requiring additional basic and epidemiological studies to better understand their pathogenesis, prevalence and clinical correlation.

We implemented several cell culture systems allowing the propagation of two distinct genotypes of novel HAstV from clinical stool samples, namely HAstV-MLB1 and HAstV-MLB2. Both strains could efficiently replicate in human HuH-7 hepatoma and A549 respiratory cell lines. In addition, both strains could establish a persistent infection over several cell passages in both cell lines, and HAstV-MLB1 could also establish a persistent infection in HuH-7.5 cells. In the latter, electron microscopy revealed a high production of capsid arrays and significant intracellular reorganization. Immunofluorescence assays revealed only a low proportion (5-10%) of infected cells. We explored the innate immune response to HAstV-MLB infection and observed that IFN expression was either abolished or delayed and diminished, depending on the cell line, during acute infection. During persistent infection, IFN expression was abolished in all cases, while when co-stimulated with poly I:C, IFN expression remained inhibited in a cell and genotype-dependent manner. Addition of exogenous IFN led to the cure (IFN-β) and relative inhibition (IFN-λ)

of HAstV-MLB infection in HuH-7 cell line, while there was no effect in A549 infected cell line.

At the epidemiological level, using a sensitive and specific real-time RT-PCR assay, we found that novel HAstVs could be identified in 6-10% of cases of undiagnosed gastroenteritis in Spanish pediatric children of < 5 years old. Together with a Japanese study, our prevalence is the highest observed to date. Children under 2 years old had a higher prevalence rate, compared to older ones. HAstV-MLB1 and HAstV-VA1 accounted for 31% and 26% of all novel HAstV observed in our cohort, while no HAstV-MLB3 were detected. Nevertheless, we found a high rate of co-infection with other enteric viruses (66%), precluding to assess a firm association between the presence of novel HAstV and the occurrence of gastroenteritis in such cases. We could not identify differences in the mean Cq values between mono- and co-infection episodes.

We then performed a case-control study to assess the role of novel HAstV in gastroenteritis. We found a prevalence of 6.3% and 4% in symptomatic and asymptomatic children, respectively, without statistical difference between groups. However, we found that asymptomatic children had statistically significant higher HAstV-MLB viral loads (median 6.52 $\log_{10}$ genomes/ml, IQR 4.52-6.84) compared to symptomatic children (median 2.35 $\log_{10}$ genomes/ml, IQR 2.13-3.76). Similarly, in symptomatic patients, we observed a higher viral load when novel HAstVs were found in coproculture-positive (median 5.19 $\log_{10}$ genomes/ml, IQR 4.24-6.22) compared to coproculture-negative (median 2.31 $\log_{10}$ genomes/ml, IQR 2.11-3.32) stool samples. These findings suggest that novel HAstV are not associated with gastroenteritis, but could modulate the gut

microbiome and may confer protection to invading pathogens, although the mechanism remains to be elucidated.

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List of abbreviations and glossary

aa:	aminoacid
AAstV:	Avastrovirus
AFP:	acute flaccid paralysis
Allo-HSCT:	allogeneic stem cell transplantation
A549:	adenocarcinoma human alveolar basal epithelial cells
BoAstV:	bovine astrovirus
CaCo-2:	heterogeneous human epithelial colorectal adenocarcinoma
CAstV:	chicken astrovirus
Cq:	quantitation cycle
CSF:	cerebrospinal fluid
DAstV:	duck astrovirus
ELISA:	enzyme-linked immunosorbent assay
EM:	electron microscopy
ER:	endoplasmic reticulum
FMDV:	foot-and-mouth disease virus
HAstV:	human astrovirus
HBGA:	histo-blood group antigen

HFMD: human foot-and-mouth-disease

HMO: human-mink-ovine

HNP-1: human neutrophil defensin-1

Hpi: hour post infection

HTS: high-throughput sequencing

HuH: human hepatoma

HVR: hypervariable region

ICTV: International Committee on Taxonomy of Viruses

iNOS: inducible nitric oxide synthase

IFN: interferon

IRF-3: IFN regulatory factor-3

IQR: interquartile range

kb: kylobase

MAstV: Mammastrovirus

MBL: mannose-binding lectin

MuAstV: murine astrovirus

MuNoV: murine norovirus

Nb: number

nm: nanometer

NPS: nasopharyngeal swab

nsP: non-structural protein

nt: nucleotide

ORF: open reading frame

OvAstV: ovine astrovirus

PoAstV: porcine astrovirus

Poly(A): polyadenylated

Poly I:C: polyinosinic-polycytidylic acid

qRT-PCR: real-time reverse transcription polymerase chain reaction

RdRp: RNA-dependent RNA polymerase

Ref: reference

RIG-I: retinoic-acid inducible gene I

RNA : ribonucleic acid

RT-PCR: reverse-transcription polymerase chain reaction

RFS: ribosomal frameshift

TAstV: turkey astrovirus

UK: United Kingdom

URTI: upper respiratory tract infection

US: United States

UTR: untranslated region

VPg: viral protein genome-linked

VP: viral protein

Introduction

1. Astrovirus

Astrovirus is a small (28-30 nm) non-enveloped icosahedral single-stranded RNA-positive virus (Baltimore classification group IV). The first description of the human astrovirus (HAstV) was made in 1975 during an outbreak of gastroenteritis in a maternity ward (2, 3). Since then, HAstV was recognized as the 3rd cause of viral acute gastroenteritis in young children, and predominantly infects infants, young children, immunocompromised hosts and the elderly. Up to 8 serotypes have been described and they are nowadays named "classic HAstV". Nevertheless, with the advent of highly sensitive molecular diagnostic tools, namely high-throughput sequencing (HTS), novel astrovirus variants have been identified in humans and animals throughout the last decade, considerably increasing the number of genotype species belonging to the *Astoviridae* family. These new variants, generally named "novel HAstV", have been considered as emerging viruses which demonstrate distinct clinical characteristics that request many basic and clinical studies. Importantly, several novel HAstV have been associated with extra-intestinal infections, including fatal meningitis and encephalitis, especially in immunocompromised individuals. Unless indicated, the information described in Section 1 of the Introduction mostly refers to classic HAstV, and a deeper description of novel HAstV is presented in Section 2.

Classic HAstV has been identified worldwide with a variable prevalence ranging from 0-20% (4). When only considering studies using real-time RT-PCR assays, mean positivity rate was 9.5% among the outpatient pediatric community compared to 4,2% in hospitalized children (5). One Japanese study found a prevalence up to 23% among asymptomatic control children during multiple outbreaks of gastroenteritis in a day care center (6). Co-infection is frequent particularly with rotavirus and norovirus (7-9), and the

seasonality is controversial (10-13). Among the 8 classic HAstV serotypes, HAstV-1 is the most prevalent, serotypes 1-3 seems to affect younger children and serotypes 4 and 8 older children (8).

Novel HAstVs have also been identified worldwide, without significant differences between industrialized and developing countries (see section 2). Some reports indicate that they may show a lower positivity rate in stool, but the number of studies is still limited, and numbers contradict data from seroprevalence studies, which suggest an abundant circulation of the viruses in the population. Thus, the development and optimization of sensitive diagnostic methods and the more systematic screening for novel HAstVs are mandated to provide a valuable estimate of their prevalence.

Overall, the *Astroviridae* family includes today viruses which infect more than 40 animal species, including mammals and birds. The International Committee on Taxonomy of Viruses (ICTV) officially recognizes 19 mammalian and 3 avian genotype species, respectively (14), but future classification updates will be soon required as the *Astroviridae* family is greatly expanding according to the regular identification of novel strains in various animal species by HTS.

1.1 Genetic diversity and classification

Astroviruses are classified in 2 genera: the *Mammastrovirus* (MAstV) and *Avastrovirus* (AAstV), infecting mammals, including humans, and avian species, respectively. Within each genera, the ICTV defines a distinct genotype species if presenting a genetic amino acid p-distance of at least 0.05 in the 3'-end of the capsid precursor protein, or a genetic nucleotide homology of less than 93-95% in the full capsid-coding sequence of a reference strain (15, 16). To date, there are 19 genotype species recognized within the MAstV and

3 genotype species within the AAstV genera. Nevertheless, novel variants identified are missing and should increase the number of officially recognized genotype species both in MAstV and AAstV in the future, once the official ICTV classification will be updated (17-23).

Classic HAstV serotypes 1-8 belong to the MAstV-1 human genotype species and novel HAstV belong to 3 different genotype species: MAstV-6 (HAstV-MLB1-3), MAstV-8 (HAstV-VA2, 4) and MAstV-9 (HAstV-VA1, 3) (see Section 2 for novel HAstV). HAstV-VA5 is currently still unassigned.

1.2 Astrovirus host range and associated clinical disease

1.2.1 Human astrovirus

Classic HAstV is the second to third most common cause of viral gastroenteritis among young children, accounting for 2-9% of non-bacterial cases. After an incubation period of about 4.5 days, symptoms consist in mild diarrhoea that is self-resolving after 2-3 days, sometimes associated with vomiting, moderate abdominal pain anorexia and fever (15). According to the biphasic age distribution of susceptible individuals, it is supposed that the effective protection conferred by antibodies during adulthood leads to pauci-/asymptomatic infections during this period of age. Nevertheless, asymptomatic carriage was also reported in children (24, 25), and although large case-control studies reveal an association between HAstV infection and diarrhoeal disease (26-30), some others do not (31, 32). In case of immunocompromised conditions, infection can be persistent (33) and/or disseminated (34). With a seroprevalence that can reach 94% at 6-9 year old (35), HAstV-1 is the most observed serotype, while HAstV-6 and HAstV-7 are rarely identified (seroprevalence of 16% and 10%, respectively (36)). HAstV-3 seems to be more frequently

associated with chronic and more severe infections (37). Of note, classic HAstV has occasionally been identified in respiratory samples or coincided with respiratory symptoms (38-42), but a clear association between HAstV and respiratory disease is still missing.

1.2.2 Animal astrovirus

1.2.2.1 Mammalian species

Astrovirus can infect more than 30 distinct mammalian species, including cattle, sheeps, pigs, bats, minks, domestic animals (dogs and cats), but also marine mammals such as California sea lions, bottlenose dolphins, and steller sea lions.

Animal MAstV are not necessarily associated with gastro-intestinal disease and clinical presentation can range from asymptomatic infection to severe disease. Porcine astrovirus (PoAstV) is one of the most diverse among MAstV, belonging to 7 distinct genotype species; despite a prevalence of up to 80-90%, PoAstV infections are frequently asymptomatic, suggesting a potential persistent infection and rendering pigs a good reservoir for astrovirus transmission (43, 44). Nevertheless, one recent study identified a neuroinvasive PoAstV strain (Ni PoAstV-3) as the cause of neurological syndrome and death in newly weaned paraplegic piglets; interestingly Ni PoAstV-3 genome was not identified in faeces of affected pigs (45). There are also hypothesis for PoAstV role in congenital tremor (46) and polio-encephalomyelitis in piglets (47). PoAstV was also detected in respiratory samples (45, 48), as well as in various other organs outside from the gastro-intestinal tracts in a high proportion of asymptomatic and symptomatic pigs; this suggests the absence of a clear association with clinical disease, and a wide tissue tropism (49, 50).

Bovine astroviruses (BoAstV) were formerly classified among 4 distinct genotype species and are not significantly associated with diarrhoeal disease (51). Recently, divergent strains of BoAstV, namely BoAstV-CH13/Neuro S1 and BoAstV-CH15/-BH89/14 were detected in brain samples of cattle presenting non-suppurative encephalitis (52-55). These variants are very distant from other BoAstV and cluster together with human, mink and ovine strains, forming together the human-mink-ovine (HMO) clade. Further retrospective investigations revealed the presence of BoAstV-CH13/Neuro S1 in up to one-third of brain biopsies of cattle with undiagnosed encephalitis disease, some of which cases dating from 1950 (56). Of note, a study identified the presence of astrovirus in cattle with the bovine respiratory disease, although measure of association with the disease was negative (57).

Ovine astrovirus (OvAstV) was the first description of animal astrovirus in 1977 (58) and a second genotype was isolated from a healthy sheep in 2009 (59). While the association between OvAstV and diarrhoeal disease is not evident, cases of non-suppurative meningoencephalitis affecting sheep were associated with a specific neurotropic OvAstV-CH16 genotype species (60-62).

Mink astrovirus is associated with two distinct syndromes, the pre-weaning diarrhoea syndrome (63) and the so-called shaking mink syndrome (64).

As for other viruses, bats represent a great reservoir for astrovirus diversity and their characteristic of being asymptomatically infected by diverse astrovirus variants make them at risk for recombination events and emerging strains with potential cross-species transmission (65).

Of note, the recent identification of new variants has further expanded the range of astrovirus host mammals, notably dromedaries (66), foxes (67), marmots (68), rats (69), mice (70-72), rabbits (73, 74) , wild boar (75) , roe deer (76) and yaks (77).

1.2.2.2. Avian species

Avian astrovirus can infect more than 15 avian species. Many AAstV cause severe systemic disease leading to significant economic loss in poultry farming.

Duck astrovirus (DAstV) was the first AAstV described and cause fatal hepatitis in ducks (78). In turkeys, astrovirus can cause outbreaks of enteritic disease, as well as a severe syndrome called the poult enteritis mortality syndrome consisting in a growth retardation, decreased thymus size, and enteric infection; this syndrome is associated with a systemic infection and a high mortality rate (79-81). Avian nephritis virus can cause nephritis and growth retardation in chicken (82). Chicken astrovirus is also associated with the runting-stunting syndrome characterized by poor weight gain and high mortality (83), as well as with the “white chicks” disease consisting in a high mortality rate among embryos and chicks, weakness and a white plumage (84). Recently, a novel goose astrovirus has been identified and associated with fatal gout disease in goslings (85, 86). **Table 1** shows some cases of extra-intestinal identification for animal and human astroviruses.

Animal/Human	Tissue(s)	Method(s) of Detection
Turkey	Bursa, Thymus, Spleen, Kidney, Liver, Skeletal Muscle, Bone Marrow, Pancreas, Plasma	RT-PCR, immunofluorescence, infectious virus isolation
Duck	Liver	RT-PCR
Cow	Brain	RT-PCR, Sequencing
Mink	Brain	Sequencing
Pig	Blood	RT-PCR, Sequencing
Human	Blood	RT-PCR, Sequencing
Human	Cerebrospinal Fluid	Sequencing
Human	Urine	Sequencing
Human	Brain	Sequencing, Immunohistochemistry
Human	Nasopharyngeal swab	RT-PCR, Sequencing
Human	Pharyngeal swab	RT-PCR, Sequencing

Table 1: Astrovirus localization in extra-intestinal tissues. RT-PCR: reverse-transcription polymerase chain reaction (87)

1.3 Astrovirus diversity and potential for zoonotic transmission and emergence of new strains

According to astrovirus resistance in the environment, fomites, contaminated food (15, 88) and water from treated wastewater (89-93) are all sources of infection with astrovirus from potential diverse host species, which could favour inter-species transmission, mixed infections and recombination events.

The recent MOxAstV-CH18 identified in brain muskox (94) is closely related to OvAstV-1 and BoAstV-CH15 is also closely related to the neurotropic OvAstV-CH16. Some OvAstV, BoAstV and PoAstV clustered together within the same genotype species (95) and the HMO clade includes strains coming from very divergent hosts including humans, pigs, minks, bovines and ovines. These elements argue in favour for inter-species transmission that may have occurred long time ago and could be favoured by ecotones (16). Similarly, novel HAstV-MLB are most closely related to rat astrovirus than to the classic HAstV (69).

Other studies suggests the potential for inter-species transmission - HAstV genome was identified in faeces of non-human primates and canine astrovirus was identified in human stool samples (96, 97) - and even inter-genera transmission (97-99), although the presence of astrovirus nucleic acids in stools alone is not sufficient to prove any active replicative infection. Interestingly, one study identified the presence of seroconversion for turkey astrovirus (TAstV) at higher frequency in humans with exposure to turkeys compared to control population (100).

Alternatively to inter-species transmission, recombination is a well-characterized event in *Astroviridae* family (95, 101-105). Apart from recombination between 2 strains of the same genotype species infecting the same host, host species that are permissive to several highly divergent astroviruses such as pigs could be the reservoir for emerging inter-species recombinant strains (43, 49, 106, 107). Recombination between human and animal astroviruses has also been suspected, such as between HAstV and California sea lion strain (108), HAstV and PoAStV (107), or HAstV and non-human primate astrovirus (97). These data suggest the potential emergence of novel recombinant astroviruses able to infect humans and potentially more virulent than the classic HAstV, which is one of the hypothesis explaining the origins of the HAstV-VA and HAstV-MLB clades.

1.4 Biological characteristics

1.4.1 Genome organization

Astrovirus positive-sense single-stranded RNA genome is 6.2 to 7.8 kb in length and contains 3 open reading frames (ORF) **(Figure 1a)**. ORF1a and ORF1b encode the non-structural proteins (nsP), while ORF2 encodes the structural proteins of the capsid **(Figure 1b)**. In some MAstV, including HAstV, a fourth ORF named ORFX has been described, and

could be translated by a leaky scanning mechanism, but to date its function is still unknown (109). The genome contains a VPg protein covalently-linked at the 5' end and a polyadenylated (poli (A)) tail at the 3' end. The VPg protein was first described in 2012 and was shown to be essential for virus infectivity, potentially through the recruitment of cellular translation initiation factors (110). Genome extremities include 5'UTR and 3'UTR. Besides the poly(A) tail, a highly conserved region called the stem-loop II motif has been described in many MAstVs and AAstVs. Its role is not fully elucidated, but it could provide stability to the secondary structure of the RNA and contribute to efficient genome replication (111).

ORF1a encodes the nsP1a polyprotein and ORF1ab encodes the nsP1ab polyprotein through a ribosomal frameshift (RFS) mechanism at the AAAAAAAC RFS signal (112) **(Figure 1a)**. Both nsP1a and nsP1ab polyproteins need to be cleaved by viral and cellular proteases. Once processed, nsP1a gives rise to a several transmembrane helices, a putative helicase structure, the VPg protein, coiled-coil structures, the serine protease, and a nuclear localization signal antigen. By interacting with the RNA-dependent RNA polymerase (RdRp), the C-terminal region of nsP1a, namely nsP1a/4, has been identified as a regulator of the genome replication. This region contains the VPg protein and the hypervariable region (HVR) and influences the ratio of genomic, subgenomic and antigenomic RNA production and further the amount of infectious progeny (113). After several cleavage steps, nsP1ab encodes the RdRP **(Figure 2a)**.

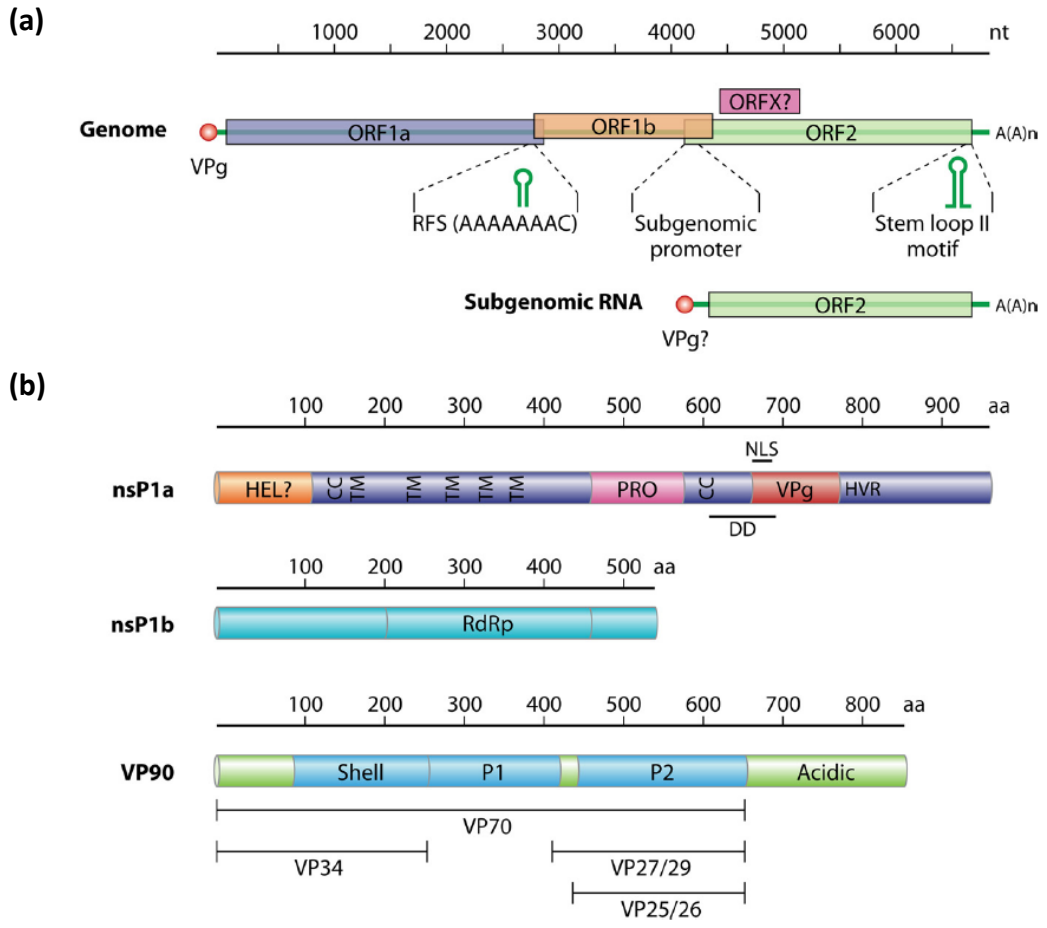
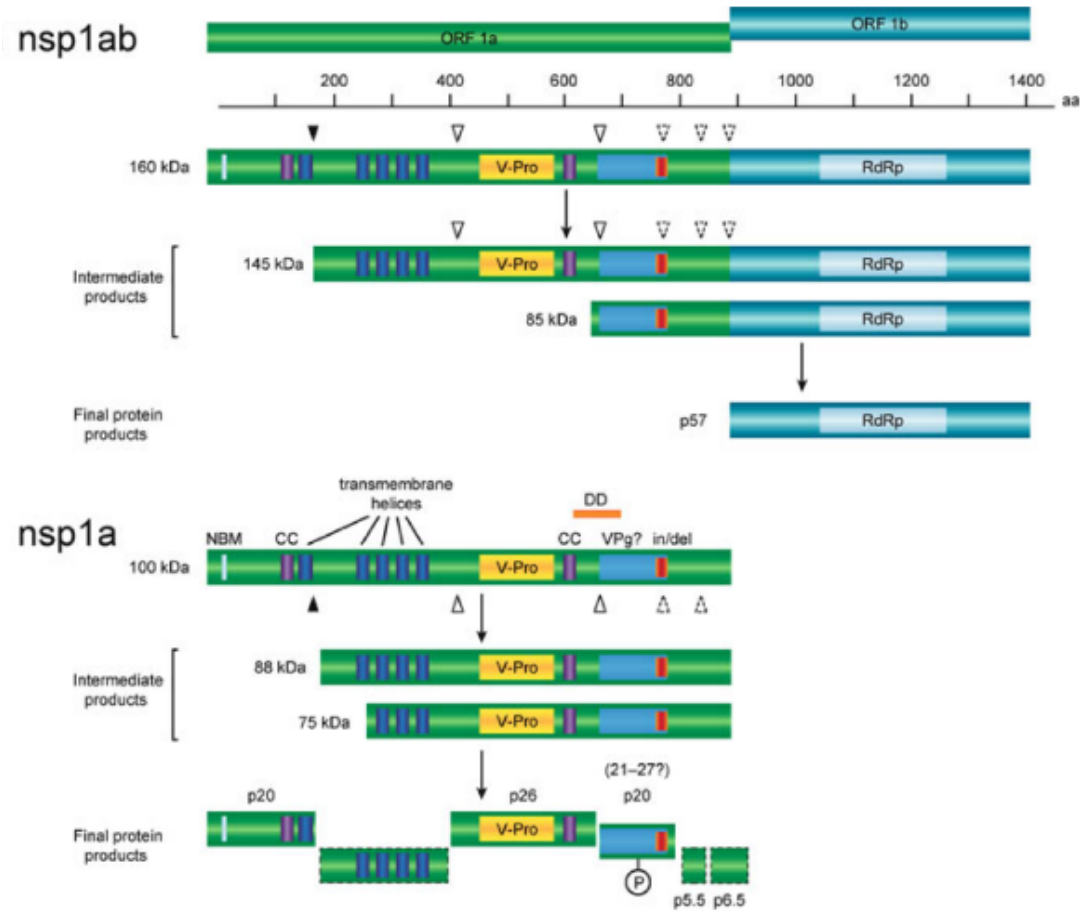


Figure 1: General organization of the astrovirus genome (a) and polyproteins translated from each open reading frame (ORF) (b). VPg: Viral protein genome-linked; RFS: ribosomal frameshifting; HEL: helicase; CC: coiled coil; TM: transmembrane; PRO: protease; DD: death domain; NLS: nuclear localization signal; HVR: hypervariable region; RdRp: RNA dependent RNA polymerase; VP: viral protein (15)

The capsid precursor VP90 is translated from the subgenomic positive-sense RNA genome of ORF2, corresponding to about 2.8 kb of the 3' end of the genomic RNA. A highly conserved region that could correspond to the subgenomic promoter is situated in front of ORF2. The N-terminal region of VP90 is highly conserved and corresponds to the capsid core, while the C-terminal region, highly variable even within a genotype species, forms the spikes of the virion (114). VP90 needs to be cleaved at the C-terminal domain by cellular caspases before cell exit, although the exact mechanism by which virions go out

from the cell is not fully understood. Outside from the cell, the capsid precursor VP70 needs further cleavage by host trypsin in order to obtain infectious mature virions with capsid proteins VP32-34 (capsid core), VP27-29 and VP24-26 (spikes) (115). Both core and spike domains are target for neutralizing antibodies, although spike domain induces a 5-10-fold higher antibodies reactivity (115-117) **(Figure 2b)**.

(a)



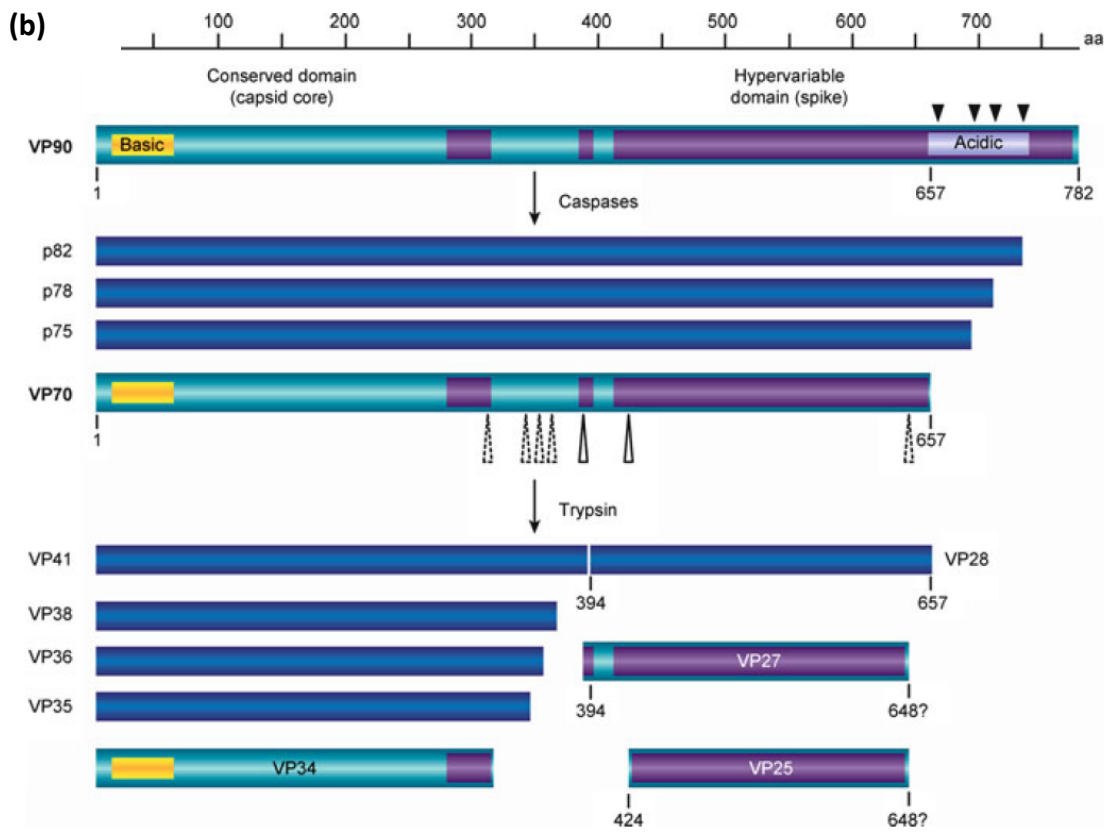


Figure 2: Details on (a) non-structural astrovirus proteins. Close and open triangles represents cellular and viral proteases, respectively, **(b) structural proteins.** Closed arrow heads represent cleavages carried out by caspases, important for virus maturation and cell egress ; open long arrow heads , cleavages by trypsin NBM: nucleotide binding motif; CC: coiled-coil; TH: transmembrane helices; v-Pro: viral protease; VPg: viral protein attached to the genome; in / del: insertion/ deletion; DD: putative death domain; RdRp: RNA-dependent RNA polymerase. (112).

1.4.2 Replicative cycle

Figure 3 summarizes the principal steps of astrovirus replication cycle. Astrovirus cell receptor is currently unknown, but according to a certain serotype-specific tropism for multiple cell lines *in vitro* (118) and multiple organ involvement *in vivo*, it is thought that astrovirus could use more than one specific receptor or a common receptor present on several cell types for cell entry. A putative binding site with amino acids arrangement typical for glycoprotein receptor recognition was previously described (119). After cell attachment, astrovirus enters the cell through a clathrin-mediated endocytosis

mechanism (120). The vesicle is then transported possibly through actin filaments in late endosomes through the cytoplasm to the proximity of the endoplasmic reticulum (ER) (121). Soon after virus entry, there is an activation of the cellular ERK1/2 pathway, which is required for productive virus replication, although the exact mechanism is still unknown (122, 123).

The acidification of late vesicles allows the release of RNA genome into the cytoplasm (121). VPg protein is essential for translation initiation (110), potentially through recruitment of eIF4E protein as described for caliciviruses (124). After translation of proteins from the genomic RNA, a viral replicase complex composed at least of the RdRp and nsP1a proteins, allows the synthesis of negative-sense RNA genome within 6-12 hours post-infection (hpi). The negative-sense RNA strain then serves for the transcription of novel genomic and subgenomic RNA starting since 8 hpi. At 12 hpi, a 10-fold higher amount of subgenomic RNA over genomic RNA can be observed (125).

Astrovirus genome replication occurs in replication complex that are closely related to cell membranes issued from the ER, the Golgi apparatus or lysosomal and endosomal vesicles (126, 127). At 48 hpi, capsid arrays can be observed in the perinuclear space, associated with double membrane vesicles that are otherwise not visible in non-infected cells (127, 128). A recent study identified that, by acting on genomic and subgenomic RNA production, the ubiquitin-proteasome system was required for efficient HAstV replication (129).

Detailed mechanism on the virus egress from the cell is lacking, but it is known that the C-terminal region of VP90 capsid protein needs to be cleaved by cellular caspases at its acidic region site (130). In addition, capsid assembly initiation may also occur in the absence of

the viral genome (131). Despite caspase activation and the identification of a putative death domain (DD) on nsP1a that could trigger caspase activation and cell death, apoptosis after astrovirus infection is still debated (132, 133). In vitro studies show that viral titre begins to increase from 8 hours, and reach peak level at 12 hours after infection **(Figure 4)**

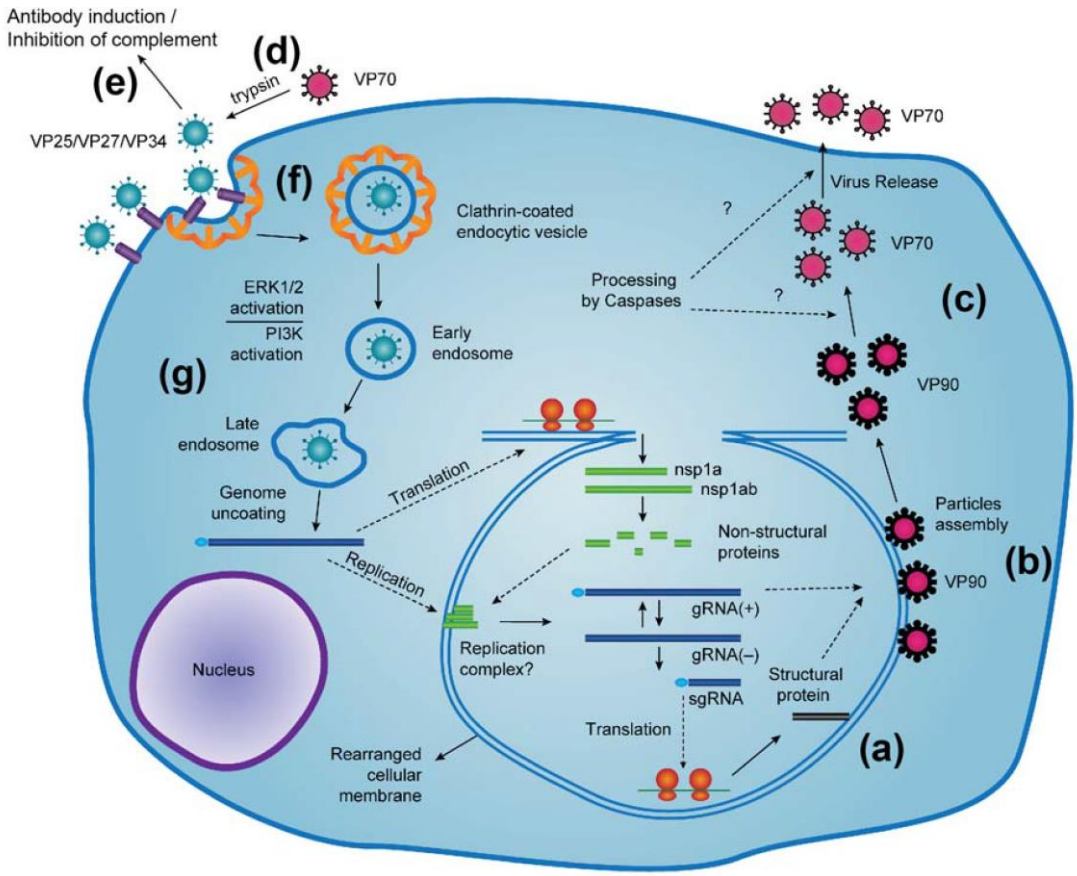


Figure 3: Astrovirus replicative life cycle ERK 1/2: extracellular signal-regulated kinase; PI3K: phosphoinositide 3-kinase; gRNA: genomic RNA; sgRNA; subgenomic RNA (134)**.**

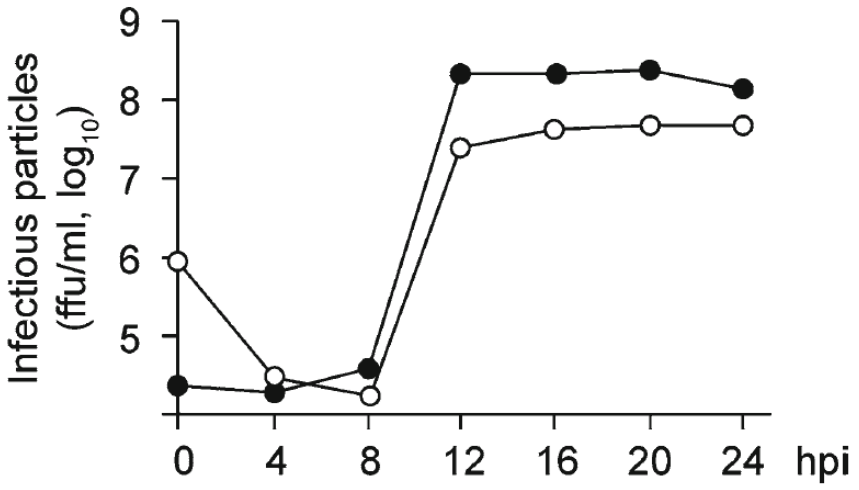


Figure 4: Multistep growth curve of HAstV-8 propagation in CaCo-2 cell line. ffu/ml: fluorescent forming units per ml. Empty circles represent number of virus particles in the supernatant, full circles represent number of cell-associated virus particles. (133).

1.5 Pathogenesis and immune response

Astrovirus pathogenesis remains largely unknown. While *in vitro* studies have demonstrated cell death induced by astrovirus infection (130, 132), this was not observed in histological descriptions of infected humans or animal models, where few morphological changes and inflammation are described: in humans, macroscopic examination of intestinal tissues of a 4 year old boy suffering from HAstV gastroenteritis after allogeneic hematopoietic cell transplantation showed villus blunting and irregularity of surface epithelial cells, without local signs of inflammation. Virus particles were observed by immunostaining and electron microscopy in the luminal surface and the cytoplasm of the surface epithelial cells at the tips of the villi and infection predominated in the upper small intestine (duodenum and jejunum) (135). In turkeys, experimental TAstV-2 infection, which causes severe diarrhoea, growth suppression, and reduction in thymic mass, only induces mild epithelial necrosis, minimal lamina propria mononuclear

infiltrates, minimal villus atrophy, and mild crypt hyperplasia (81, 136). Infectious TAstV can be detected in other organs, including the thymus, the bursa, spleen, liver, kidney, pancreas, skeletal muscle, plasma and bone marrow, suggesting systemic dissemination, but replication seems to be restricted to the intestine (81, 136).

The mechanism of diarrhoea induced by astrovirus is thus not associated with tissue damage. In an *in vitro* study, it has been demonstrated that HAstV infection increases the intestinal epithelial barrier permeability in a dose-dependent manner, independently from virus replication and without cell death, by disruption of the tight junction (137). Increased intestinal epithelium barrier permeability could also be a mechanism developed by astrovirus for systemic dissemination, as seen in turkey models and severe infections in immunocompromised hosts. Disruption of tight junctions can also expose the basolateral surface and potential receptors for additional productive cycles of infection, as described for coxsackie virus and CAR receptor (138). In an additional study, the same group confirmed the role of TAstV-2 capsid protein acting as an enterotoxin in an experimental turkey model: diarrhoea was induced by ingestion of the capsid protein alone in a dose-dependent manner and was associated with increased intestinal barrier permeability and relocalization of two intracellular sodium transporters (NHE3 and SGLT-1), in the absence of cellular damage and inflammation (139).

Previous studies also demonstrated the potential role of maltase decreased activity resulting in disaccharide maldigestion, malabsorption and osmotic diarrhoea (140).

1.5.1 Innate immune response

The absence of inflammation and cell death observed in humans and animal models is supposed to be associated with a mechanism of immune evasion. In addition to its

structural and toxicogenic role, the astrovirus capsid protein contributes to the immune evasion by inhibiting the complement activation. The complement system is composed of three pathways, namely the classical pathway, the lectin pathway and the alternative pathway. Activation of the complement system cascade contributes to pathogen elimination and regulation of the inflammatory and adaptive immune response. By binding directly to C1q and mannose-binding lectin (MBL) of the classical and lectin pathways, respectively, HAstV capsid protein inhibits C1 and C4 activation and prevents further downstream activation, including C3 and C5 which are key components for initiating pathogen phagocytosis, pro-inflammatory signalling, opsonisation, infected cell lysis and intracellular pathogen recognition (141-143). This occurs through HAstV capsid residues 79-139, which demonstrates limited sequence homology to human neutrophil defensin-1 (HNP-1), a known inhibitor of C1q and MBL (144).

The important role of type I interferon (IFN) in astrovirus infection control was reported by two independent studies. The first observed a delayed (peak at 48h) and mild increase in IFN-β expression after infection of CaCo-2 cells with infectious HAstV-4, once viral production had already taken place, and with less than 10% of infected cells showing translocation of the IFN regulatory factor-3 to the nucleus. Inhibition of IFN-β by BX795 inhibitor increased virus infectivity and cellular pre-treatment with exogenous IFN-β reduced the number of infected cells, demonstrating viral sensitivity to exogenous IFN. Of note, HAstV was not able to disrupt the cellular IFN-β expression when cell was concurrently challenged by poly I:C. In conclusion, HAstV is sensitive to IFN-β, but IFN-β cellular expression is delayed and attenuated, enhancing virus replication (145).

Using a different serotype (HAstV-1), the second group studied observed that HAstV induced a significant IFN-β expression since 24 hpi but at a much (almost 10-fold) lower level than the positive control influenza virus. Cell pre-treatment with exogenous IFN-β also reduced the number of infected cells up to 90%. Contrary to Guix *et al,* it was demonstrated here that IFN-β can prevent increased epithelial barrier permeability during infection. IFN-β effects on virus replication and epithelial barrier permeability were further confirmed in a mouse model (146).

Among other mediators of the innate immune response, nitric oxide plays a role in TAstV-2 infection, limiting viral replication (147). Yet, inducible nitric oxid synthase (iNOS) is not only produced by macrophages and splenocytes infected by TAstV-2, but seems also to be secreted by intestinal epithelial cells in the absence of innate or adaptive immune cell response (148).

In an experimental model, macrophages demonstrated lower viability when exposed to TAstV-2, and up to 30% reduction in phagocytosis of *Escherichia coli* as well as a significant reduction in the intracytoplasmic killing of the latter, leading to a 25-88% greater survival of *Escherichia coli* in TAstV-2 exposed macrophages compared to controls. In addition, measurement of the pro-inflammatory IL-1 and IL-6 cytokines revealed that TAstV-2 exposed macrophages significantly secreted less of both cytokines compared to controls (79). This study provides interesting data concerning a potential of astrovirus for immune modulation through macrophages viability and function, which could increase the host‘s susceptibility for other pathogens.

Finally, TGF-beta, a potent anti-inflammatory cytokine, has been identified at a significantly high level in serum during TAstV-2 infection, potentially explaining the low inflammatory response observed (81).

1.5.2 Adaptive immune response

In a study where HAstV was inoculated to healthy volunteers, those with detectable pre-existing HAstV antibodies had asymptomatic or pauci-symptomatic illness, compared to more severe infection in those without previous antibodies (149). Knowing that about 70% of adult patients, who rarely develop symptomatic astrovirus infection, have HAstV antibodies and according to the biphasic age distribution of symptomatic HAstV infection, as well as the severe clinical diseases observed in immunocompromised hosts (see chapter on novel HAstV), it has thus largely been accepted that adaptive humoral and cellular response to HAstV play a critical role in infection control.

This assumption was supported by animal models where mice lacking mature B and T cells demonstrated higher viral titre of murine astrovirus (MuAstV) in the intestinal tract and faeces, and virus dissemination to other organs (72). There are few human data on cellular adaptive response during astrovirus infection, but specific CD4+ T cell for HAstV can be detected in human small intestine of individuals with an asymptomatic infection (150), and there are some descriptions of chronic HAstV infection in patients with T cell deficiencies (151).

A recent experimental vaccine candidate for mink astrovirus demonstrated high efficacy, confirming the protective role of adaptive immunity. After two injections, immunized mice demonstrated a significant increase in specific antibodies and the increase was higher when the N-terminal truncated capsid protein was used for immunization,

compared to the whole capsid or the C-terminal truncated capsid protein. N-terminal truncated capsid protein immunization also allowed a better splenocytes proliferation after re-stimuation with mink astrovirus capsid protein, associated with higher level of Th1 (IL-2 and IFN-γ) and Th2 (IL-5) and Th2/T-reg (IL-10) cytokine secretion. Finally, the authors observed that, when challenged with a mink astrovirus strain causing the shaking mink syndrome, litters with maternal passive immunization had milder signs and symptoms of astrovirus infection compared to the non-vaccinated group. Virus shedding was also significantly attenuated in the vaccinated group (152).

In humans, unfortunately there is still no vaccine available, and it is argued that the immunosuppressing function of the capsid protein discussed above (142, 144) might limit its effectiveness. Yet, while most of vaccine tested were based on the whole capsid protein, the N-terminal truncated capsid protein used for mink astrovirus vaccine does not contain the peptide region responsible for complement inhibition, which can explain its higher immunogenicity. In addition, results from the mink astrovirus vaccine may also be promising for humans, since the virulent HAstV-VA strains belong to the same cluster as mink astrovirus. However, escape variants that can avoid antibody binding with only one single amino acid mutation have been described (153, 154), suggesting that viral evolution may generate vaccine-resistant strains.

2. Novel Human Astrovirus

2.1 Historical perspectives

Novel HAstV were identified a decade ago with the advance of HTS. HAstV-MLB1 was the first non-canonical HAstV described and was found by HTS in the faeces of a 3 year old boy in Melbourne presenting diarrhoea in 1999 (155, 156). HAstV-MLB2 and HAstV-MLB3

were then identified as distinct genotypes and firstly described in a cohort of children in India using the pan-astrovirus RT-PCR assay (157). As for the HAstV-VA/HMO clade, HAstV-VA1/HMO-C was found during a pediatric outbreak of gastroenteritis in Virginia by HTS (158). Strains belonging to this clade were concomitantly identified by two distinct groups, leading to their dual name: HAstV-HMO-A ,-HMO-B and -HMO-C were described in 2009 and were found in faeces of Pakistani and Nigerian children suffering from acute flaccid non-polio paralysis (159) and at the same time, HAstV-VA2 and VA3 were identified in faeces of diarrhoeal children in India (157); nucleotide sequence comparison showed that HAstV-VA1 and HAstV-HMO-C, HAstV-VA2 and HAstV-HMO-A, HAstV-VA3 and HAstV-HMO-B were the same genotypes, respectively. Distinct HAstV-VA4 and HAstV-VA5 genotypes were further described (26, 160, 161).

2.2 Epidemiology and phylogenetic characteristics

After the first descriptions of novel HAstV, many other studies reported the presence of novel HAstV worldwide. **Table 2** is adapted from Vu e*t al* (162) and summarizes the studies where screening for novel HAstV was performed. Since 2016, additional studies revealed the presence of novel HAstV in Germany (163), in Thailand (164), in Tanzania (165), in the US (33), in the Netherlands (166), and in China (167). Of note, in the latter, environmental screening in sewage revealed 18 sequences with only 85% nucleotide similarity with HAstV-VA5 reference strain in the partial capsid region, potentially associated to a novel genotype (HAstV-VA6).

Globally, the prevalence was low (1.5%), except in Japan, where up to 10% of the screened population was positive for a novel HAstV (168).

	Geographical localisation	Specimen	Total screened	Nb positive	Patient	Clinical presentation	Year	Prevalence	Method	Ref
HAstV-MLB1	Australia (Melbourne)	Faeces	9	1	Child	Diarrhoea	1999	-	HTS	(155)
	India	Faeces	416	7	Children	Diarrhoea	2002-7	1.7%	RT-PCR	(157)
	Hong Kong	Faeces	622	1	unknown	Diarrhoea	2004-5	0.2%	RT-PCR	(69)
	Egypt	Faeces	364	5	Children	Diarrhoea	2006-7	1.4%	RT-PCR	(169)
	Nigeria	Faeces	95	4	Children	Non polio AFP	2006-8	4.2%	RT-PCR	(159)
	US	Faeces	254	4	Children	Diarrhoea	2008	1.6%	RT-PCR	(170)
	Kenya	Faeces	362	12	Children	Diarrhoea/no-	2008-9	3.3%	RT-PCR	(26)
	Gambia	Faeces	587	4	Children	Diarrhoea/no-	2008-9	0.7%	RT-PCR	(26)
	Italy	Faeces	4	2	Children	Diarrhoea	2007-10	-	RT-PCR	(171)
	US	Faeces	419	5	Children	Oncologic	2008/ 2010-11	1.2%	qRT-PCR	(33)
	China	Faeces	723	7	Children	Diarrhoea	2010-11	1%	RT-PCR	(172)
	Bhutan	Faeces	unknown	2	Children	Diarrhoea	unknown	-	RT-PCR	(173)
	Brazil	Faeces	200	2	Children	Acute Diarrhoea	2011	1%	RT-PCR	(174)
	Japan	Faeces	330	32	Children	Diarrhoea	2012-13	9.7%	RT-PCR	(168)
	Thailand	Faeces	29	1(pool)	Children	HFMD	2012	-	HTS	(175)
	Uruguay	Sewage	20	4	-	-	2012-13	-	RT-PCR	(90)
	Germany	Faeces	2877	11	Adult. Children	Acute gastroenteritis	2010-15	0.4%	RT-PCR	(163)
	Thailand	Faeces	2034	9	Children	Acute gastroenteritis	2011-16	0.4%	RT-PCR	(164)
	Switzerland	Faeces	548	3	Adult, children	Diarrhoea/no-	2014-15	0.5%	qRT-PCR	(176)
	Japan	CSF, serum, throat, faeces, urine	1	1	Child	Encephalitis	2015	-	HTS	(177)
	Netherland	Faeces	458	1	Adult. Children	Gastroenteritis	2016	0.2%	qRT-PCR	(166)
	China	Faeces	635	13	Adult. Children	Acute gastroenteritis	2016	2.1%	qRT-PCR	(167)
	China	Sewage	12	11	-	-	2016	-	RT-PCR	(167)
	Tanzania	Serum	816	4	Children	Fever	2014-16	0.5%	HTS	In preparation
	Switzerland	NPS	156	1	Children	Respiratory symptoms	2016-17	0.6%	qRT-PCR	(42)

Table 2: Overview of studies with positive screening for novel HAstV. The total screened usually refer to number of patients or samples in case of environmental studies. The year corresponds to the period of samples screening. Nb: number; RT-PCR: reverse transcription polymerase chain reaction; qRT-PCR: real-time reverse transcription polymerase chain reaction; HTS: high throughput sequencing; AFP: acute flaccid paralysis; CSF: cerebrospinal fluid; NPS: nasopharyngeal swab; URTI: upper respiratory infection; HFMD: human foot-and-mouth disease; allo-HSCT: allogeneic hematopoietic stem cell transplantation: UK: United Kingdom: US: United States

	Geographical localisation	Specimen	Total screened	Nb positive	Patient	Clinical presentation	Year	Prevalence	Method	Ref
HAstV-MLB2	India	Faeces	10	2	Children	Diarrhoea	2002-7	-	RT-PCR	(157)
	US	Faeces	4	3	Children	Unknown	2008-9	-	RT-PCR	(157)
	Turkey	Faeces	150	1	Child	Diarrhoea	2005	0.7%	RT-PCR	(178)
	US	Plasma, NPS	176	1	Child	Rash. URTI, febrile neutropenia	unknown	0.6%	HTS	(179, 180)
	Japan	Wastewater	24	13	-	-	2007-8	-	RT-PCR	(89)
	Kenya	Faeces	362	1	Child	No symptoms	2008-9	0.3%	RT-PCR	(26)
	Gambia	Faeces	587	9	Children	Diarrhoea/no-	2008-9	1.5%	RT-PCR	(26)
	China	Faeces	723	2	Children	Diarrhoea	2010-11	0.3%	RT-PCR	(172)
	Japan	Faeces	330	3	Children	Diarrhoea	2012-13	0.9%	RT-PCR	(181)
	Thailand	Faeces	29	1 (pool)	Children	HFMD	2012	-	HTS	(175)
	Switzerland	CSF, plasma	404	2	Adult	Meningitis	2014	0.5%	HTS, qRT-PCR	(182)
	Switzerland	Faeces	615	6	Adult. children	Diarrhoea/no-	2014-15	0.1%	qRT-PCR	(182)
	Switzerland	Faeces	548	4	Adult, children	Diarrhoea/no-	2014-15	0.7%	qRT-PCR	(176)
	Germany	Faeces	2877	5	Adult. Children	Acute gastroenteritis	2010-15	0.2%	RT-PCR	(163)
	Thailand	Faeces	2034	2	Children	Acute gastroenteritis	2011-16	0.1%	RT-PCR	(164)
	Netherland	Faeces	458	1	Adult. Children	Gastroenteritis	2016	0.2%	qRT-PCR	(166)
	China	Faeces	635	4	Adult. Children	Acute gastroenteritis	2016	0.6%	qRT-PCR	(167)
	China	Sewage	12	7	-	-	2016	-	RT-PCR	(167)
	Tanzania	Serum	816	2	Children	Fever	2014-16	0.3%	HTS	In preparation
	Switzerland	NPS	156	1	Children	Respiratory symptoms	2016-17	0.6%	qRT-PCR	(42)
	China	Faeces	27	2	Adult	Allo-HSCT	2016-18	7.4%	HTS, RT-PCR	(183)
HAstV-MLB3	India	Faeces	800	5	children	Diarrhoea/no symptoms	2002-7	0.6%	RT-PCR	(160)
	Kenya	Faeces	362	7	Children	Diarrhoea/no-	2008-9	1.9%	RT-PCR	(26)
	Gambia	Faeces	587	18	Children	Diarrhoea/no-	2008-9	3.1%	RT-PCR	(26)

Table 2 (Continued)

	Geographical localisation	Specimen	Total screened	Nb positive	Patient	Clinical presentation	Year	Prevalence	Method	Ref
HAstV-VA1/ HMO-C	US (Virginia)	Faeces	6	3-6	Children	Diarrhoea (outbreak)	2008	-	HTS, RT-PCR	(158)
	Nepal	Faeces	190	3	Adult	Diarrhoea/healthy	2006-8	1.6%	RT-PCR	(159)
	Japan	Wastewater	24	3	-	-	2007-8	-	RT-PCR	(184)
	US	Brain	1	1	Teenager	Encephalitis	2007	-	HTS	(185)
	Tanzania	NPS	30	1	Children	URTI	2008	-	HTS	(186)
	Gambia	Faeces	587	1	Child	No symptoms	2008-9	0.2%	RT-PCR	(26)
	UK	CSF, brain, serum, stool	1	1	Child	Encephalitis	2013	-	HTS	(187)
	UK	Faeces	726	2	Children	Diarrhoea /unknown	2013-14	0.3%	RT-PCR	(187)
	France	Brain	1	1	Teenager	Encephalopathy. seizure	2013	-	HTS	(188)
	UK	Brain	1	1	Adult	Encephalitis	2013	-	HTS	(189)
	Germany	Faeces	2877	5	Adult. Children	Acute gastroenteritis	2010-15	0.2%	RT-PCR	(163)
	UK	Brain	1	1	Child	Encephalitis	2015	-	HTS	(190)
	China	Sewage	12	11	-	-	2016	-	RT-PCR	(167)
	Tanzania	Serum	816	5	Children	Fever	2014-16	0.6%	HTS	In preparation

Table 2 (Continued)

	Geographical localisation	Specimen	Total screened	Nb positive	Patient	Clinical presentation	Year	Prevalence	Method	Ref
HAstV-VA2/ HMO-A	India	Faeces	10	2	Children	Diarrhoea	2002-7	-	RT-PCR	(157)
	US	Faeces	356	1	Children	Diarrhoea/ healthy controls	2003-5	0.3%	RT-PCR	(31)
	Egypt	Faeces	364	2	Children	Diarrhoea	2006-7	0.5%	RT-PCR	(169)
	Nigeria	Faeces	95	2	Children	Non polio AFP	2006-8	2.1%	RT-PCR	(159)
	Pakistan	Faeces	43	1	Child	Non polio AFP	2006-8	2.3%	RT-PCR	(159)
	Japan	Wastewater	24	3	-	-	2007-8	-	RT-PCR	(89)
	US	Faeces	4	1	Child	Unknown	2008-9	-	RT-PCR	(157)
	Kenya	Faeces	362	4	Children	Diarrhoea/no-	2008-9	1.1%	RT-PCR	(26)
	Gambia	Faeces	587	10	Children	Diarrhoea/no-	2008-9	1.7%	RT-PCR	(26)
	US	Faeces	419	8	Children	Oncologic	2008/ 2010-11	1.9%	qRT-PCR	(33)
	China	Faeces	723	2	Children	Diarrhoea	2010-11	0.3%	RT-PCR	(172)
	Japan	Faeces	330	2	Children	Diarrhoea	2012-13	0.6%	RT-PCR	(181)
	Nigeria	Faeces	103	1	Children	Diarrhoea	2012-13	1%	RT-PCR	(96)
	Germany	Faeces	2877	2	Adult. Children	Acute gastroenteritis	2010-15	0.07%	RT-PCR	(163)
	Switzerland	Faeces	548	3	Adult, children	Diarrhoea/no-	2014-15	0.5%	qRT-PCR	(176)
	Thailand	Faeces	2034	2	Children	Acute gastroenteritis	2011-16	0.1%	RT-PCR	(164)
	China	Sewage	12	12	-	-	2016	-	RT-PCR	(167)

Table 2 (Continued)

	Geographical localisation	Specimen	Total screened	Nb positive	Patient	Clinical presentation	Year	Prevalence	Method	Ref
HAstV-VA3/ HMO-B	India	Faeces	10	1	Child	Diarrhoea	2002-7	-	RT-PCR	(157)
	India	Faeces	800	1	Child	unknown	2002-7	0.1%	RT-PCR	(160)
	Nigeria	Faeces	95	1	Child	Non polio AFP	2006-8	1.1%	RT-PCR	(159)
	Pakistan	Faeces	43	1	Child	Non polio AFP	2006-8	2.3 %	RT-PCR	(159)
	Spain	Serum	pools	3	Unknown	Acute hepatitis /healthy controls	2011	-	HTS	(191)
	Nigeria	Faeces	103	1	Children	Diarrhoea	2012-13	1%	RT-PCR	(96)
	Germany	Faeces	2877	1	Adult. Children	Acute gastroenteritis	2010-15	0.03%	RT-PCR	(163)
	China	Faeces	635	1	Adult. Children	Acute gastroenteritis	2016	0.2%	qRT-PCR	(167)
	China	Sewage	12	3	-	-	2016	-	RT-PCR	(167)
	Tanzania	Serum	816	6	Children	Fever	2014-16	0.7%	HTS	In preparation
HAstV-VA4	Nepal	Faeces	196	2	Children	Diarrhoea	unknown	1%	RT-PCR	(160)
HAstV-VA5	Gambia	Faeces	587	1	Child	No symptoms	2008-9	0.2%	RT-PCR	(26)
	Burkina Faso	Faeces	48	1	Child	Diarrhoea	unknown	2.1%	HTS	(161)
	China	Sewage	12	8	-	-	2016	-	RT-PCR	(167)
HAstV-VA6 ?	China	Sewage	12	5	-	-	2016	-	RT-PCR	(167)

Table 2 (Continued)

Nevertheless, two seroprevalence studies reported that up to 65% of adult patients was positive for HAstV-VA1 (169) and that HAstV-MLB1 seroprevalence increases with age, reaching 100% in the 7-17 year old group (170). This suggests that novel HAstV are circulating in the population worldwide without causing overt significant disease, or that the associated disease is still unrecognized or misdiagnosed. According to the high rate of undiagnosed gastroenteritis for example, novel HAstV could have been overlooked in these situations.

Novel HAstV are genetically very divergent from the classic HAstV. **Table 3** shows the amino acid sequence identity between classic HAstV, HAstV-MLB and HAstV-VA clades.

Mamastrovirus Species	Classic	MLB	VA2-VA4	VA1-VA3	VA5
	Mamastrovirus 1	*Mamastrovirus 6*	*Mamastrovirus 8*	*Mamastrovirus 9*	Unassigned
Serotypes/Clades	HAstV-1 to 8	MLB1, MLB2 and MLB3	VA2 (HMO-A) and VA4	VA1 (HMO-C) and VA3 (HMO-B)	VA5
ORF1a (protease and other nonstructural proteins)					
Classic	100	–	–	–	–
MLB	32.8	100	–	–	–
VA2-VA4	24.1	29.1	100	–	–
VA1-VA3	24.2	28.9	67.4	100	–
VA5	23.9	28.2	61.5	59.6	100
ORF1b (RNA dependent RNA polymerase)					
Classic	100	–	–	–	–
MLB	54.5	100	–	–	–
VA2-VA4	51.8	49.4	100	–	–
VA1-VA3	53.0	49.3	73.7	100	–
VA5	50.2	50.7	74.0	71.5	100
ORF2 (capsid proteins)					
Classic	100	–	–	–	–
MLB	27.5	100	–	–	–
VA2-VA4	24.0	21.9	100	–	–
VA1-VA3	23.0	22.1	51.9	100	–
VA5	23.8	20.6	58.9	53.1	100

Table 3: Amino acid sequence identity between classic HAstVs, HAstV-MLB and HAstV-VA, for the 3 ORFs (4)

HAstV-MLB are phylogenetically closer to rat astroviruses (69), while HAstV-VA clade belongs to the HMO cluster, including **H**uman, **M**ink and **O**vine astrovirus **(Figure 5)**. This close relationship to animal viruses suggests a cross-species transmission or recombination between animal and human strains that could have occurred hundreds or thousands of years ago (171).

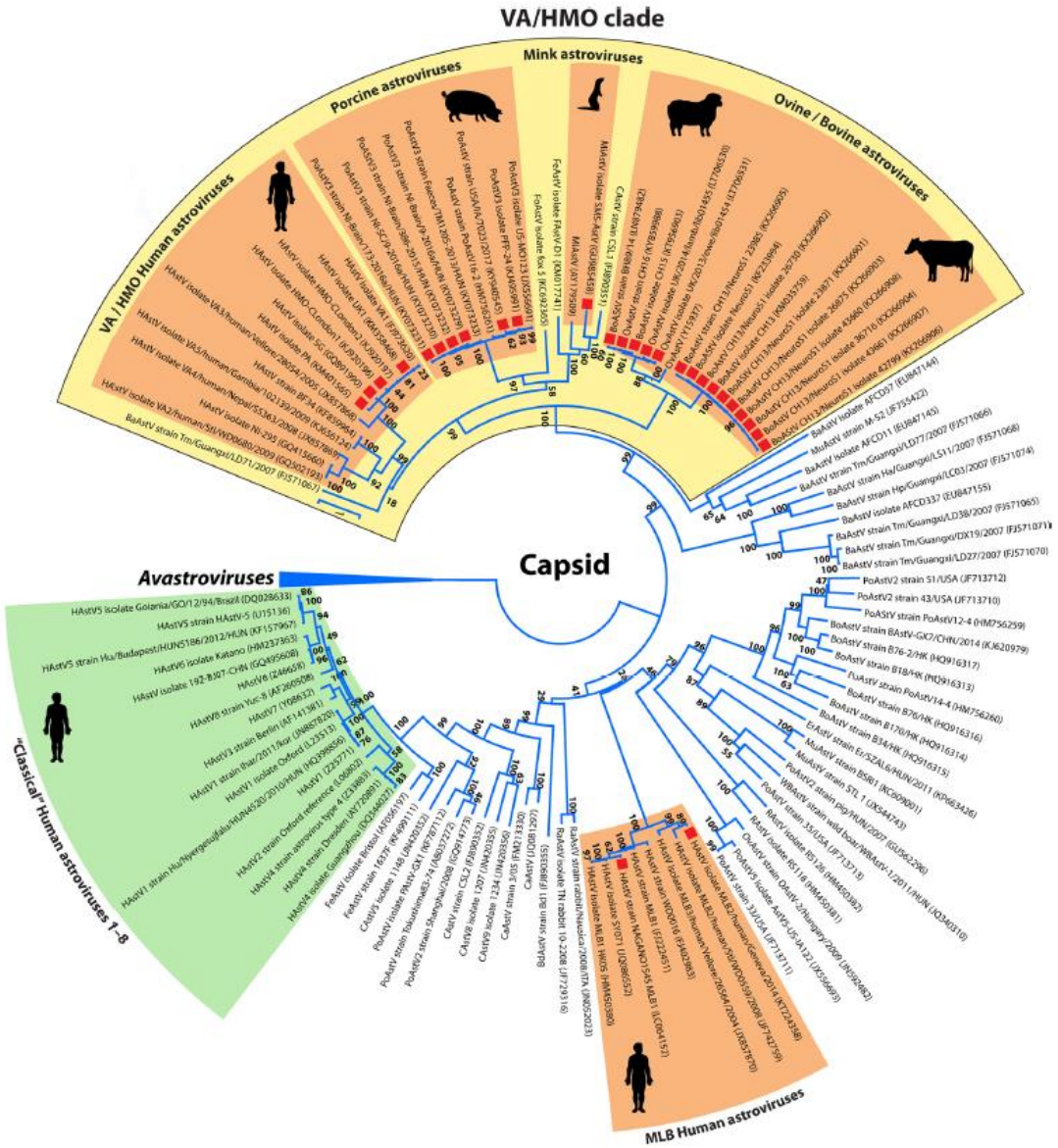


Figure 5: Phylogenetic relationship of animal and human astrovirus based on the full-length capsid sequences. Bootstrap values are indicated on the phylogenetic tree nodes and were determined for 1000 replicates. Neurotropic astroviruses are indicated in orange and classic HAstV are indicated in green. Red squares indicate the neurotropic strains with full-length sequences available. BaAstV: bat astrovirus; BdAstV: bottlenose dolphin astrovirus; BoAstV: bovine astrovirus; CAstV: California sea lion astrovirus; CaAstV: canine astrovirus; ErAstV: European roller astrovirus; FeAstV: feline astrovirus; FoAstV: fox astrovirus; HAstV: human astrovirus; MiAstV: mink astrovirus; MuAstV: murine astrovirus; OvAstV: ovine astrovirus; PoAstV: porcine astrovirus; RaAstV: rabbit astrovirus; RAstV: rat astrovirus; WBAStV: wild boar astrovirus. (1)

Of note, a particular separate strain, named Bastrovirus was identified in 2016 in 8.7% of a human population (HIV-positive and HIV-negative) in the Netherlands (172). This novel

strain shared 45% amino acid identity with the astrovirus HAstV-MLB2 capsid protein and 28% amino acid identity with a non-structural protein of members of the *Hepeviridae* family. Later on, Bastrovirus sequences isolated in swine, rat and bat samples from Vietnam were submitted to Genbank, and the same variant was identified in 31% of screened pigs in the US in 2019 (173).

Recombination event between novel HAstV was also described for HAstV-MLB3 with HAstV-MLB1 and HAstV-MLB2, detected in environmental samples (174).

According to the high rate of co-infection with other enteric pathogens observed with novel HAstV, their pathogenic role in case of symptomatic disease remains to be determined (26, 163, 164).

2.3 Clinical disease spectrum

Two case-control studies have evaluated the pathogenic role of novel HAstV in gastroenteritis, but their results are conflicting. While the first found a higher rate of HAstV-MLB1 in symptomatic patients and higher rate of HAstV-MLB3 in control subjects (26), the second found a higher HAstV-MLB1 prevalence in asymptomatic patients (27). The question as to whether novel HAstV can be real enteric pathogen thus remains non-elucidated. In contrast, nine case reports identified novel HAstVs (mainly HAstV-VA1 genotype), in cerebral tissue samples or cerebrospinal fluid (CSF) of undiagnosed cases of severe meningoencephalitis infections (reviewed in (4)). An additional similar case report had earlier been described for a classic HAstV (serotype 4) infection (34). Most patients **(Table 4)** were immunocompromised and had fatal outcome (34, 175-181). Neurohistopathology described microgliosis and astrogliosis, axonal swelling and neuronal loss; CD3+/CD8+ T cell infiltrate was described in all but one cases (175-179).

According to these case descriptions, three distinct groups performed a large screening of CSF to try to identify additional cases of HAstV undiagnosed central nervous system infections, but none additional positive sample was identified (166, 176, 182). This suggests that although there are growing evidence for a pathogenic role of novel HAstV in meningoencephalitis in immunocompromised hosts, this remains of rare occurrence. Nevertheless, in some available tissue samples of human encephalitis cases, HAstV-VA1 capsid protein was found in astrocytes and in cells morphologically similar to neurons (175-177). A recent *in vitro* study confirmed the permissiveness of primary astrocytes as well as immortalized SK-N-SH cells derived from neuroblastoma for a full replication cycle and production of infectious HAstV-VA1 (183). The authors used a strain derived from a clinical stool sample (158) that share 96.8-99% amino acid homology with the neurological strains, suggesting that HAstV-VA1 strains identified in cases of gastroenteritis and in central nervous system infections are similar. In addition, after HAstV-VA1 infection, authors observed an increase in CXCL10, a chemoattractant leading to the recruitment of immune cells (183).

Patient (country)	Underlying disease and conditions	Clinical diagnosis	Symptoms	Outcome	Astrovirus type (method used for pathogen identification), strain name, available GenBank accession no.	Potential source	Reference
15-yr-old boy (USA)	X-linked agammaglobulinemia (immunocompromised)	Encephalitis	Suicidal and homicidal ideation, headache, memory loss, ataxia, progressive cognitive decline	Fatal (within 71 days)	VA1/HMO-C (NGS), HuAstV-PS (Puget Sound) or HuAstV-SG, GQ891990	Exposure to mink?	9
3-mo-old boy (Switzerland)	Severe combined immunodeficiency, hematopoietic stem cell transplantation, chemotherapy (immunocompromised)	Meningoencephalitis	Multiorgan dysfunction (hepatic and respiratory)	Fatal (within 17 days posttransplantation)	HuAstV4 (NGS), HQ396880–HQ396890	Nosocomial	34
18-mo-old boy (UK)	Cartilage hair hypoplasia with hereditary immunodeficiency, hematopoietic stem cell transplantation, chemotherapy (immunocompromised)	Encephalopathy, encephalitis	Irritability, dystonia, reduced consciousness	Fatal (within 9 mo)	VA1/HMO-C (NGS), VA1/HMO-C UK1(a) London1, KJ920196	Community acquired?	28
42-yr-old man (UK)	Chronic lymphocytic leukemia (CLL); hematopoietic stem cell transplantation, chemotherapy (immunocompromised)	Progressive encephalitis, bilateral hearing loss	Tinnitus, sensorineural deafness, central dyspnea, hypotension, nausea, irritability, agitation	Fatal (within 7.5 mo)	VA1/HMO-C (NGS), VA1/HMO-C-UK1, KM358468	Community acquired?	29
14-yr-old boy (France)	X-linked agammaglobulinemia (immunocompromised)	Progressive encephalitis	Progressive cognitive decline, recurring seizures, ataxia, erratic myoclones, dysarthria	Alive at time of publication	VA1/HMO-C (NGS), VA1/HMO-C-PA, KM401565	Data not available	30
4-yr-old boy (Japan)	Congenital aplastic anemia, hematopoietic stem cell transplantation, chemotherapy (immunocompromised)	Encephalopathy	Cluster of convulsion, fever, diarrhea	Recovered	MLB1 (NGS), MLB1-NAGANO-1545, LC064152	Unknown	32
21-yr-old women (Switzerland)	Healthy	Acute meningitis	Severe headache, fever, neck stiffness	Recovered	MLB2 (NGS), MLB2/human/Geneva/2014, KT224358	Unknown (contact with children?)	33
37-year-old (Switzerland)	Acute myeloid leukemia, relapse hematopoietic stem cell transplantation, chemotherapy (immunocompromised)	Meningitis	Headache, meningeal involvement, vertigo, limb weakness, lightheadedness	Fatal (within 9 mo)	MLB2 (RT-PCR)	Unknown (contact with children?)	33
8-mo-old girl (UK)	Acute myeloid leukemia, hematopoietic stem cell transplantation, chemotherapy (immunocompromised)	Encephalitis	Encephalopathy, uncontrolled dystonic movement, poor respiratory effort	Fatal	VA1/HMO-C (NGS)	Unknown, no history of exposure to animals	31

Table 4: Nine cases of HAstV-associated central nervous system infection identified in humans. NGS: next-generation sequencing (1)

Host	Age (country)	Clinical signs	Natural outcome	Detection source	Method(s) of detection	Ni-AstV type	Abundance	Reference
Mink (*Mustela vison*)	3 to 4 mo (Denmark, Sweden, Finland)	Shaking mink syndrome (SMS), tremor/seizure, paraplegia	Mortality (27–28%)	Brain	NGS	MiAstV/MAstV10	0.2–0.8% morbidity	12, 16
Bovine (*Bos taurus*)	3 yr (USA)	Seizure, paraplegia, circling, and blindness	Not known	Brainstem, cerebellum, and/or spinal cord	NGS, ISH	BoAstV NeuroS1	3/32 animals (9.4%)	13
	1.5 to 7 yr (Switzerland)	Abnormal gait (or recumbency), behavioral changes, and hyperreactivity or depression	Not known	Brain tissue/medulla oblongata	NGS, ISH, RT-PCR	BoAstV-CH13 and/or BoAstV-CH15	6/22 affected animals, 0/33 healthy animals	17, 43, 45
	4 yr (Switzerland)	Unspecified CNS symptoms	Not known	Medulla oblongata, cerebellar cortex, midbrain, cerebral cortex	NGS, RT-PCR	BoAstV-CH15	Single case	43
	1.5 to 10 yr (Switzerland)	Encephalitis	Deceased	Brain, medulla oblongata	NGS, RT-PCR	BoAstV-CH13/BoAstV NeuroS1	2/16 affected animals, 0/50 healthy animals	44
	1 to >10 yr (Switzerland)	Encephalitis, meningoencephalitis	Not known	Brain, brainstem, cerebellum, cerebrum, hippocampus	ISH, RT-PCR	BoAstV-CH13/BoAstV NeuroS1	12/14 affected animals	18
	15 mo (Germany)	Central blindness, circling, inappetence, somnolence	Deceased	Brain, spinal cord, spleen, liver, pancreas	NGS, RT-PCR, ISH	BoAstV-BH89/14	Single case	47
	7 mo to 2 yr (Canada)	Encephalitis, seizures	Mortality (100%)	Brain	RT-PCR, ISH	BoAstV-CH13/BoAstV NeuroS1	2 single cases; 4/9 affected animals	48, 49
	1 to 11 yr (Switzerland)	Encephalitis, gait abnormalities, behavioral changes	Deceased	Gray matter of the CNS (brainstem), cerebellum, cerebrum, hippocampus	ISH, IHC	BoAstV-CH13/BoAstV NeuroS1	33/97 affected animals, 0/52 healthy animals	8
Ovine (*Ovis aries*)	4 yr or 10 days (Scotland)	Trembling, whole-body tremor, circling, recumbency	Not known	Cerebrum, cerebellum, obex, spinal cord, tonsil	NGS, RT-PCR	MAstV-13	Single cases	14
	7 yr (Switzerland)	Clinical signs of nonsuppurative encephalitis	Deceased	Medulla oblongata, cerebellum, thalamus, hippocampus, cortex, caudate nucleus	RT-PCR, IHC	MAstV13-CH16	1/48 affected animals	51
Swine (*Sus scrofa domestica*)	1–6 days (Sweden)	Congenital tremor type AII	Not known	Cerebrum, brainstem, cerebellum	RT-PCR	PoAstV-2 and PoAstV-5	3/3 affected animals, 3/3 healthy animals	52
	3–5 wk (Hungarian pigs with presumed postweaning stress)	Paraplegia, pitching, flaccid paralysis, loss of consciousness	Mortality (100%)	Brainstem, cerebellum, spinal cord, tonsils, serum, lung, nasal mucosa	RT-PCR, ISH	PoAstV-3	1.5–4% morbidity	15
	5 wk (pigs and sows, USA)	Hind-limb weakness, quadriplegia, convulsions	Mortality (75–100%)	Cerebrum, cerebellum, spinal cord	NGS, RT-PCR	PoAstV-3	3/4 affected sows, 1/2 affected pigs	53

Table 5: Cases of animal astrovirus-associated central nervous system infection identified. NGS: next-generation sequencing; ISH: *in situ* hybridization; IHC : immunohistochemistry; RT-PCR: reverse transcription polymerase chain reaction (1)

Novel HAstV genome has also been retrieved in body compartment other than the digestive and central nervous system. HAstV-MLB was identified in the serum of febrile children in two separate studies (165, 184). In respiratory samples, two HAstV-MLB2, one HAstV-MLB1 and one HAstV-VA1 were each identified in febrile children with or without respiratory symptoms (42, 185, 186). Finally, a HAstV-VA3 was identified in the serum of 3 pools of patients with acute hepatitis of unknown origin (187).

In conclusion, novel HAstV were recognized 10 years ago and share genetic similarities with animal astrovirus suggesting cross-species transmission or recombination events long time ago. They have been detected in every continent and seroprevalence in the adult population is high. Although associated clinical manifestations are still not clear, there are evidence that novel HAstV can cause severe central nervous system infection in immunocompromised hosts. This neurotropism is commonly shared among the astrovirus strains of the HMO clade, resulting in neurologic disease in humans, mink, porcine, bovine and ovine mammals **(Tables 4 and 5)**.

2.4 Tools for detection, virus propagation and virus-host interaction studies

2.4.1 Diagnostic assays

Electron microscopy (EM) was historically the method of choice for detecting astrovirus in clinical samples. Astrovirus appeared as 28-30 nm particles with the characteristic star-like appearance which gives astrovirus its name (3, 188, 189). Although this technique is the gold standard for diagnosing viral infections, its laborious use is limited in routine diagnostics. In addition, EM requires a quantity of about 10^6-10^7 virus particles per gram of stools, leading to poor sensitivity (3). Also, only 10% of astrovirus particles demonstrate the typical star-like shape, and misdiagnosis with other enteric virus is thus probable.

Immuno-enzymatic methods have greatly improved the diagnosis of astrovirus, decreasing the limit of detection up to 10^5-10^6 virus particles per gram of stools, with reported sensitivity of 100% and specificity of 98% (190). Firstly developed in 1990 (191), the enzyme-linked immunosorbent assay (ELISA) method using an antibody that could detect all 8 serotypes of HAstV, the MAb8E7, is the one that was the most used in epidemiological studies, through a commercial kit available (IDEIA^{TM} Astrovirus, DAKO Diagnostics, Cambridgeshire, United Kingdom) (192-194). MAb8E7 can also be used for indirect immunofluorescence detection of HAstV capsid after cell culture propagation (110, 195), providing information on percentage of infected cells and intracellular localization of capsid protein.

An immune-chromatographic test was reported to be highly sensitive (100%) and specific (91.2%), compared to RT-PCR assay, and allowed rapid diagnostics in 15-20 minutes (196).

Reverse-transcription polymerase chain reaction (RT-PCR) assays have surpassed the sensitivity of ELISA and EM and is nowadays the method of reference for HAstV detection. **Table 6** presents all the primers targeting distinct regions of classic HAstV genome and providing either generic RT-PCR assays targeting all classic HAstV serotypes ("common-type"), or specific RT-PCR assays for distinct serotypes ("type-specific"). Conventional RT-PCR assays have a limit of detection as low as 10-100 genome copies per gram of stools (15, 197).

Primers	Genomic region	Length (bp)	References
Common-type			
Mon340/Mon348	ORF1a (pro)	289	Belliot *et al.* 1997
A1/A2	ORF1a (C-terminal nsP1a)	192–237	Guix *et al.* 2002; Willcocks *et al.* 1994
Mon343/Mon344	ORF1b (RNA pol)	316	Belliot *et al.* 1997
Mon244/Mon245	ORF2 (N-terminal)	413	Noel *et al.* 1995
Mon269/Mon270	ORF2 (N-terminal)	449	Noel *et al.* 1995
prBEG/Mon2	ORF2 (C-terminal) + 3′-UTR	296–324	Saito *et al.* 1995
DM4/Mon2	ORF2 (C-terminal) + 3′-UTR	1200	Walter *et al.* 2001
Mon2/Mon69	3′-UTR	89	Mitchell *et al.* 1995
Type-specific			
AST-S1 to AST-S8, FOR, END	ORF2 (C-terminal)	118–599	Matsui *et al.* 1998; Sakamoto *et al.* 2000
PR6151, PR6257, DM12, JWT4, AST-S5, DM11, Mon2	ORF2 (C-terminal) + 3′-UTR	321–666	Saito *et al.* 1995; Mitchell *et al.* 1999; Walter *et al.* 2001

Table 6: Primers used for HAstV detection by conventional RT-PCR assay (198)

A pan-astrovirus RT-PCR assay using SF0073 (5′-GATTGGACTCGATTTGATGG-3′) and SF0076 (5′-CTGGCTTAACCCACATTCC-3′) primers was developed in 2009 to commonly detect classic HAstV 1-8, novel HAstV-MLB and HAstV-VA by targeting a conserved region of the RNA-dependent RNA polymerase (199). The use of these primers pair has enabled the detection of novel HAstV in the most important epidemiological studies screening for novel HAstV (26, 90, 157, 160, 199-202). Other sets of primers were also designed and allowed the concomitant detection of novel HAstV by distinct groups (159).

Real-time (q)RT-PCR assay was developed for the rapid and even more sensitive screening of classical HAstV and also allows to obtain quantitative results. Sensitivity was improved x10,000-fold compared to conventional RT-PCR (203). Finally, multiplex qRT-PCR assays were developed for the concomitant detection of multiple enteric viruses including astrovirus, norovirus and adenovirus, or norovirus, astrovirus and enterovirus, which reveal to be as sensitive as the monoplex assays (204, 205). A commercial kit allowing detection of up to 22 enteric pathogens including classic HAstV is currently available (206). Unfortunately, according to the high genetic variability between the three clades of HAstV, there is currently no unique qRT-PCR assay allowing the detection of all HAstV

(166). Thus, the most efficient assays for detecting all three clades of HAstV are the pan-astrovirus RT-PCR assay, HTS or at least 7 distinct qRT-PCR assays (182).

2.4.2 Cellular and small animal models

The classic HAstV also can be propagated in several continuous cell lines, but no small animal model has been described for them yet. The most permissive cell line for all classic HAstV serotypes is the human colonic CaCo-2 cell line, which allows the propagation of many strains directly from original stool samples. Human colon carcinoma T84 and PLC/PRF/5 derived from human liver hepatoma also support the propagation of many wild-type strains. Laboratory-adapted HAstV serotypes 1, 2, 4, 5 and 6 can usually replicate in most of cell lines tested, while HAstV 3 and 7 are more difficult to propagate (118). A HAstV serotype 8 strain named Yuc-8 could be propagated on CaCo2 cells, but there is no report of other cell lines tested (207-209).

Turkey animal models allowed the study of pathogenesis and immune response to TAstV, notably the immune modulation of macrophages during the poult enteritis mortality syndrome (79), and the role of the capsid protein in diarrhoeal disease (139). Nevertheless, the relative phylogenetical distance between avian and mammal astrovirus might limit the applicability of such results in humans.

Murine astrovirus was discovered by chance in laboratory mice in 2012. It was shown that MuAstV is shed in mice faeces at high titre and during several days, is highly infectious, and can disseminate outside from the digestive tract (72). Mice deficient in innate and adaptive immunity demonstrated higher viral load in all organs, arguing for some infection control by the innate and adaptive immune systems. Nevertheless, MuAstV infection is asymptomatic in immunocompetent mice and do not induce intestinal damage, raising

important questions on the role of MuAstV as part of the mouse gut virome. These observations have also led to some groups to propose the use of MuAstV as a useful model for studying asymptomatic human infections (210), and interestingly, using an immunodeficient mouse model, MuAstVs have been recently shown to be elements of the virome, which can protect mice against murine norovirus and rotavirus infections (211).

Objectives

The main objectives of the thesis were to better define the epidemiology of novel astrovirus in the pediatric population in Spain and to establish a cell culture system allowing the propagation of novel HAstV *in vitro.*

Specifically, the thesis was divided in four distinct objectives:

1. To establish a cell culture system allowing the propagation of HAstV-MLB *in vitro.*
2. To determine the prevalence of circulating novel HAstV within a Spanish pediatric population with symptoms of gastroenteritis.
3. To assess the role of novel HAstV during gastroenteritis by means of a case-control study.
4. To assess potential risk factors for being asymptomatically infected by novel HAstV within the control population.

Report of articles

1. Report of published articles and their impact factors

The articles that are part of the report of the Doctoral Thesis presented by Diem-Lan Vu Cantero have been published or have been submitted in International scientific journals in the field of Virology or Infectious Diseases and indexed in the Journal Citation Reports.

Article I:

Diem-Lan Vu, Albert Bosch, Rosa M. Pintó, Enric Ribes, Susana Guix. **« Human astrovirus MLB replication in vitro: persistence in extraintestinal cell lines »** J Virol. 2019 Jun 14; 93 (13). doi: 10.1128/JVI.00557-19.

The article was published in Journal of Virology in June 2019. 2018 Impact factor: 4.3, 1st quartile in the field of Virology.

Article II:

Diem-Lan Vu, Aurora Sabrià, Nuria Aregall, Kristina Michl, Virginia Rodriguez Garrido, Lidia Goterris, Albert Bosch, Rosa Maria Pintó and Susana Guix. **« Novel human astroviruses: prevalence and association with common enteric viruses in undiagnosed gastroenteritis cases in Spain »** Viruses. 2019 Jun 27; 11 (7). doi: 10.3390/v11070585. The article was published in Viruses in June 2019. 2018 Impact factor: 3.8, 1st quartile in the field of Virology.

Article III:

Diem-Lan Vu, Aurora Sabrià, Nuria Aregall, Kristina Michl, Jaume Sabrià, Virginia Rodriguez Garrido, Lidia Goterris, Albert Bosch, Rosa Maria Pintó, Susana Guix. **« Lack of association between novel astrovirus and diarrheic children and higher viral titre among asymptomatic controls»** The article was submitted to Scientific reports in September 2019. 2018 Impact factor: 4.0, 1st quartile in the Multidisciplinary section.

Director

Dr Susana Guix Arnau

Director and tutor

Dr Albert Bosch Navarro

Barcelona, October 2019

2. Co-authored report of published articles

Article I:

Diem-Lan Vu, Albert Bosch, Rosa M. Pintó, Enric Ribes, Susana Guix. **« Human astrovirus MLB replication in vitro: persistence in extraintestinal cell lines »** J Virol. 2019 Jun 14; 93 (13). doi: 10.1128/JVI.00557-19.

In study I, the PhD student has carried out all the experimental part as well as the analysis, statistics and interpretation of the results, under the supervision of the directors of thesis. She wrote the manuscript and the revisions and performed some of the additional experiments requested by the reviewers. None of the co-authors of the articles have used the data to prepare their own doctoral thesis.

Article II:

Diem-Lan Vu, Aurora Sabrià, Nuria Aregall, Kristina Michl, Virginia Rodriguez Garrido, Lidia Goterris, Albert Bosch, Rosa Maria Pintó and Susana Guix. **« Novel human astroviruses: prevalence and association with common enteric viruses in undiagnosed gastroenteritis cases in Spain »** Viruses. 2019 Jun 27; 11(7). doi: 10.3390/v11070585.

Article III:

Diem-Lan Vu, Aurora Sabrià, Nuria Aregall, Kristina Michl, Jaume Sabrià, Virginia Rodriguez Garrido, Lidia Goterris, Albert Bosch, Rosa Maria Pintó, Susana Guix. **« Lack of**

association between novel astrovirus and diarrheic children and higher viral titre among asymptomatic controls» Submitted in September 2019.

In studies II and III, the PhD student has implemented the RT-PCR duplex assay and participated to the stool suspension, extraction and RT-PCR screening of the samples. She participated to the supervision and teaching of 2 students for the sample screening. She performed the results analysis, statistics and interpretation of the results, under the supervision of the directors of thesis. She wrote the protocol, the manuscript and the revisions. None of the co-authors of the articles have used the data to prepare their own doctoral thesis.

Director

Dr Susana Guix Arnau

Director and tutor

Dr Albert Bosch Navarro

Barcelona, October 2019

Publications

Article I: Human astrovirus MLB replication in vitro: persistence in extraintestinal cell lines

Authors: **Diem-Lan Vu**, Albert Bosch, Rosa M. Pintó, Enric Ribes, Susana Guix.

While all serotypes of classic HAstV can efficiently be propagated in several cell lines and particularly in CaCo-2 cells (118), until recently, there was no studies investigating a cell culture system to support the novel HAstV propagation *in vitro*. In 2017 and 2019 respectively, Janowski *et al* described the first cell culture systems allowing the propagation of HAstV-VA1 (183, 212).

The aim of this study was to implement a cell culture system allowing the propagation of HAstV-MLB, by using two clinical strains: one HAstV-MLB1 positive stool sample from a child with symptoms of gastroenteritis and one stool sample from an immunocompromised patient with persistent systemic HAstV-MLB2 infection and possibly HAstV-MLB2 central nervous system infection (180).

While CaCo-2 cells were not permissive to none of the HAstV-MLB strain, we succeeded in propagating both strains on a HuH-7.5 cell line. We observed an increase of HAstV-MLB2 titre in the supernatant of more than 5 $\log_{10}$ genome copies/well, but only 3 $\log_{10}$ genome copies/well for HAstV-MLB1; the intracellular increase after 7 days of infection was similar for both strains. Nonetheless, we could maintain the viral titre after several passages and confirm the production of virions of 32-33 nm of diameter by negative staining of the supernatant. Regarding the lowest excretion of HAstV-MLB1, we hypothesized that this could reflect an underlying persistent infection. To test this

hypothesis, we infected HuH-7.5 cells with HAstV-MLB1 and then performed sequential cell passages without adding a viral inoculum. This model allowed the detection of HAstV-MLB1 genome for up to 20 cell passages at 10 log_{10} genome copies/ml of supernatant, confirming our hypothesis. We confirmed the presence of infectious virions by electron microscopy of infected cells. By using the same model for HAstV-MLB2, HuH-7.5 cells failed to regenerate after few passages and HAstV-MLB2 genome detection was lost.

According to the defect in the RIG-I pathway of HuH-7.5 cells, we used another clone of HuH-7 cells, the HuH-7AI cells, and the A549 cell line (respiratory epithelial cells) to test the model of persistent infection: both HAstV-MLB1 and HAstV-MLB2 could establish persistent infection in these cell lines, with a viral titre ranging between 7-9 log_{10} genome copies/ml quantified in the supernatant. Immunofluorescence assays were used to confirm the RT-PCR results and showed a low percentage of infected cells (5-10%).

Of note, we observed that contrary to the classic HAstV, HAstV-MLB1 and HAstV-MLB2 did not require addition of trypsin for infectivity.

We then explored the innate immune response during acute and persistent HAstV-MLB infection. We first measured the expression of type I and III IFN RNA within infected cells and observed that HAstV-MLB can inhibit the IFN-λ expression in both cell lines; while IFN-β expression in HuH-7AI cells is also abolished, in A549 cells it is attenuated and delayed during acute infection. During persistent infection, HAstV-MLB could inhibit the IFN-β/λ expression in both HuH-7AI and A549 cells. In addition, HAstV-MLB could prevent the expression of IFN-β/λ in A549 cell line despite stimulation of the IFN pathway with poly I:C.

Finally, we tested the effect of IFN treatment of infected cells on the viral titre. During acute infection, exogenous IFN-β and IFN-λ could inhibit HAstV-MLB replication in both HuH-7AI and A549 cell lines. However, during persistent infection, the effect of exogenous IFN was more heterogeneous. In HuH-7AI cells, IFN-β could cure HuH-7AI cell line from HAstV-MLB persistent infection after 7 cell passages, but IFN-λ had only a relative effect on HAstV-MLB replication. This effect was more pronounced for HAstV-MLB2 than HAstV-MLB1 infection. In A549 cell lines, there was no effect of exogenous IFN-β/λ on any HAstV-MLB.

In conclusion, we implemented several cell culture systems for the propagation of HAstV-MLB, allowing the production of a viral stock that will serve for future basic virological studies. We also demonstrated the capacity of HAstV-MLB to establish a persistent carrier-state infection *in vitro* on extra-intestinal human cell lines, which could be of importance for clinical correlation. Finally, we gave some clues to elucidate the mechanisms of immune evasion by HAstV-MLB.

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VIRUS-CELL INTERACTIONS

Human Astrovirus MLB Replication *In Vitro*: Persistence in Extraintestinal Cell Lines

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ABSTRACT MLB astroviruses were identified 10 years ago in feces from children with gastroenteritis of unknown etiology and have been unexpectedly detected in severe cases of meningitis/encephalitis, febrile illness of unknown etiology, and respiratory syndromes. The aim of this study was to establish a cell culture system supporting MLB astrovirus replication. We used two clinical strains to infect several cell lines, an MLB1 strain from a gastroenteritis case, and an MLB2 strain associated with a neurologic infection. Efforts to propagate the viruses in the Caco-2 cell line were unsuccessful. In contrast, we identified two human nonintestinal cell lines, Huh-7 and A549, permissive for both genotypes. After serial passages in the Huh-7.5 cell line, the adapted strains were able to establish persistent infections in the Huh-7.5, Huh-7AI, and A549 cell lines, with high viral loads (up to 10 $\log_{10}$ genome copies/ml) detected by quantitative reverse transcription-PCR (RT-qPCR) in the culture supernatant. Immunofluorescence assays demonstrated infection in about 10% of cells in persistently infected cultures. Electron microscopy revealed particles of 32 to 33 nm in diameter after negative staining of cell supernatants and capsid arrays in ultrathin sections with a particularly high production in Huh-7.5 cells. Interferon (IFN) expression by infected cells and effect of exogenous IFN varied depending on the type of infection and the cell line. The availability of a cell culture system to propagate MLB astroviruses represents a key step to better understand their replicative cycle, as well as a source of viruses to conduct a wide variety of basic virologic studies.

IMPORTANCE MLB astroviruses are emerging viruses infecting humans. More studies are required to determine their exact epidemiology, but several reports have already identified them as the cause of unexpected clinical diseases, including severe neurologic diseases. Our study provides the first description of a cell culture system for the propagation of MLB astroviruses, enabling the study of their replicative cycle. Moreover, we demonstrated the unknown capacity of MLB astrovirus to establish persistent infections in cell culture. Whether these persistent infections are also established *in vivo* remains unknown, but the clinical consequences would be of high interest if persistence was confirmed *in vivo*. Finally, our analysis of IFN expression provides some trails to understand the mechanism by which MLB astroviruses can cause persistent infections in the assayed cultures.

KEYWORDS interferon, novel astrovirus, persistence

First identified in 1975 in stool samples of children with diarrhea (1), human astroviruses (HAstVs) cause viral gastroenteritis worldwide (2), being the third most common cause in the pediatric population after rotavirus and norovirus. Besides children, HAstV gastroenteritis also frequently occurs in the elderly (3) and in immunocompromised individuals (4–6). HAstVs are small (28 to 41 nm in diameter) nonen-

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veloped single-stranded positive-sense RNA viruses. To date, the family *Astroviridae* is divided into two genera, *Mamastrovirus* and *Avastrovirus*, including viruses infecting mammals and birds, respectively. Their genome codes for three open reading frames (ORFs), with ORF1a and ORF1b encoding the protease and polymerase proteins, respectively, and ORF2 encoding the capsid proteins.

With the advent of next-generation sequencing technologies, two novel groups of highly divergent HAstVs (named MLB and VA/HMO) which are more closely related to certain animal astroviruses than to the classic HAstVs, have been identified in human stools of individuals with diarrhea (7–13) but also in asymptomatic healthy controls (14, 15). To date, no definitive association between novel astroviruses and gastroenteritis has yet been established, but further epidemiologic studies have confirmed the presence of novel HAstVs worldwide (14, 16–21). In addition, novel HAstVs have been recently identified as the cause of unexpected central nervous system infections in (mostly immunocompromised) humans (22–28). Specifically, MLB astroviruses have been involved in one case of acute meningitis in a healthy young adult (28) and in two cases of neurologic infections in immunocompromised patients (27, 28). Both groups of novel HAstVs have been further divided into several genotypes, MLB1 to -3 for MLB astroviruses and VA1 to -5 for VA astroviruses (2, 29).

Novel HAstVs are part of the neurovirulent astroviruses, which also include animal astroviruses (30). Other unexpected clinical manifestations recently associated with human and animal astroviruses include respiratory tract infections (31–37), fever of unknown etiology (38, 39), hepatitis (40, 41), and severe gout in geese (42). Altogether, these findings suggest that there are probably other still unrecognized divergent astroviruses with clinical implications beyond gastroenteritis in humans and animals. The potential for cross-species transmission is high (43, 44), and the increasing number of descriptions of nonenteric severe clinical manifestations in animals, especially neurologic involvement, should prompt us to validate appropriate systems to study the pathogenicity of astroviruses. Among the novel human astroviruses, a cell culture system has been recently described for VA1 (45). The present study aims at describing a cell culture system for the propagation of MLB1 and MLB2 astroviruses, the two MLB genotypes most frequently identified, and providing some clues for understanding their pathogenicity.

RESULTS

MLB astroviruses can be propagated in Huh-7.5 hepatoma cells. Several MLB clinical specimens were used to infect different cell lines and perform serial viral passages (V-P), following a protocol for an acute infection (see Materials and Methods). Three MLB1 strains were recovered from stool samples of children under 5 years old with symptoms of acute gastroenteritis, and three MLB2 strains were recovered from stool samples and included a neuroinvasive strain identified in an immunocompromised adult patient (28). Among these, only two strains were able to replicate in cell culture (Fig. 1), with one MLB1 strain recovered from a 1-year-old child and the MLB2 neuroinvasive strain recovered from the 37-year-old immunocompromised patient. Attempts to propagate these strains in a Caco-2 cell line resulted in a loss of genome detection after 2 passages (Fig. 2A). Using Huh-7.5 cells, we observed sustained viral genome detection in the culture supernatant (SN) for up to 8 to 9 passages, and some viral passages were also successful in A549 cells (Fig. 1A and 2A). Electron microscopy of the supernatant of acutely infected Huh-7.5 cells confirmed the presence of viral capsids of both genotypes, with a mean size of 33 $\pm$ 3 nm for MLB1 and 32 $\pm$ 2 nm for MLB2 (Fig. 2B and C).

Multistep growth curves were performed to define infection kinetics (Fig. 3A and B). While an increase in viral RNA in the cellular fraction showed similar kinetics for the two viruses, with a major increase during the first 2 days after inoculation and a total $\log_{10}$ fold increase from 1 h postinfection (hpi) to 7 days postinfection (dpi) of 3.41 $\pm$ 0.37 for MLB1 and 3.22 $\pm$ 0.49 for MLB2 (Fig. 3A), the increase in viral RNA in the supernatant fraction was much higher for MLB2 than for MLB1 (5.14 $\pm$ 0.03 versus 2.99 $\pm$ 0.20,

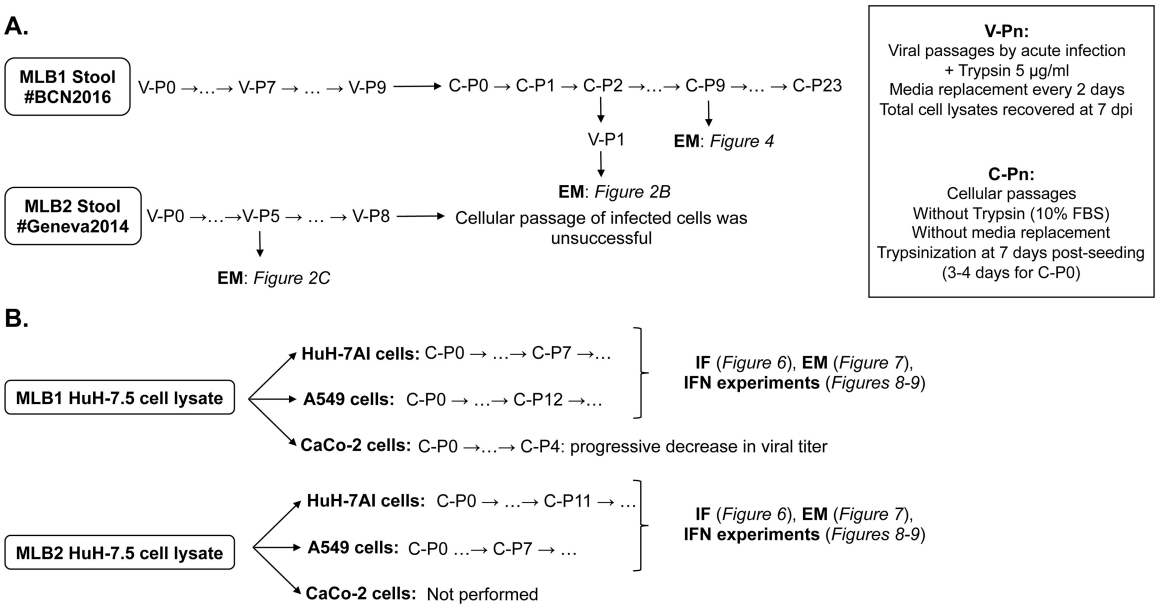

FIG 1 Description of the viral and cell passages performed with HAstV MLB1 and MLB2 on selected cell lines. (A) Passage history in Huh-7.5 cells. Initially, cells were infected using clinical stool samples as an inoculum. After 7 to 8 viral passages of HAstV MLB1 and MLB2, respectively, infected cells were subcultured. (B) Huh-7Al and A549 cell passages using Huh-7.5 cell lysates as an inoculum to establish persistent infections. V-Pn, viral passages; C-Pn, cellular passages; EM, electron microscopy; IF, immunofluorescence; IFN, interferon.

$P < 0.05$) (Fig. 3B), resulting also in overall higher viral production for MLB2. We also confirmed the occurrence of infectious viruses in the inoculum by treating it for 5 min at 99°C and confirming the lack of viral RNA increase in the supernatant of infected cultures (data not shown).

A trypsin treatment was initially included, but no significant differences were observed in the efficiency of MLB replication in the presence or absence of trypsin (5 μg/ml) in the postinfection medium (Fig. 3C).

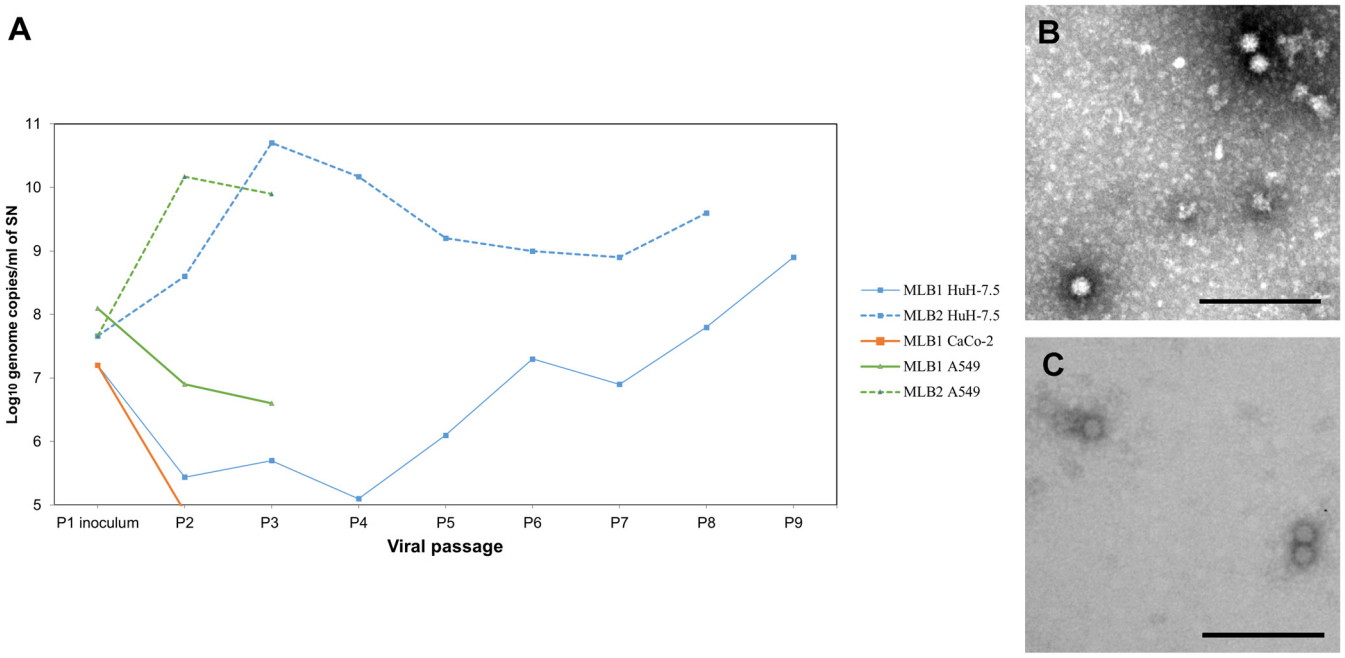

FIG 2 Infection of MLB1 and MLB2 in acutely infected cells. (A) Viral genome titers detected in the culture supernatant (SN) by RT-qPCR assays. (B and C) Electron microscopy of SN from Huh-7.5 cells infected with HAstV MLB1, using SN of a persistently infected cell line as inoculum (B) and MLB2 (C). Bars = 200 nm.

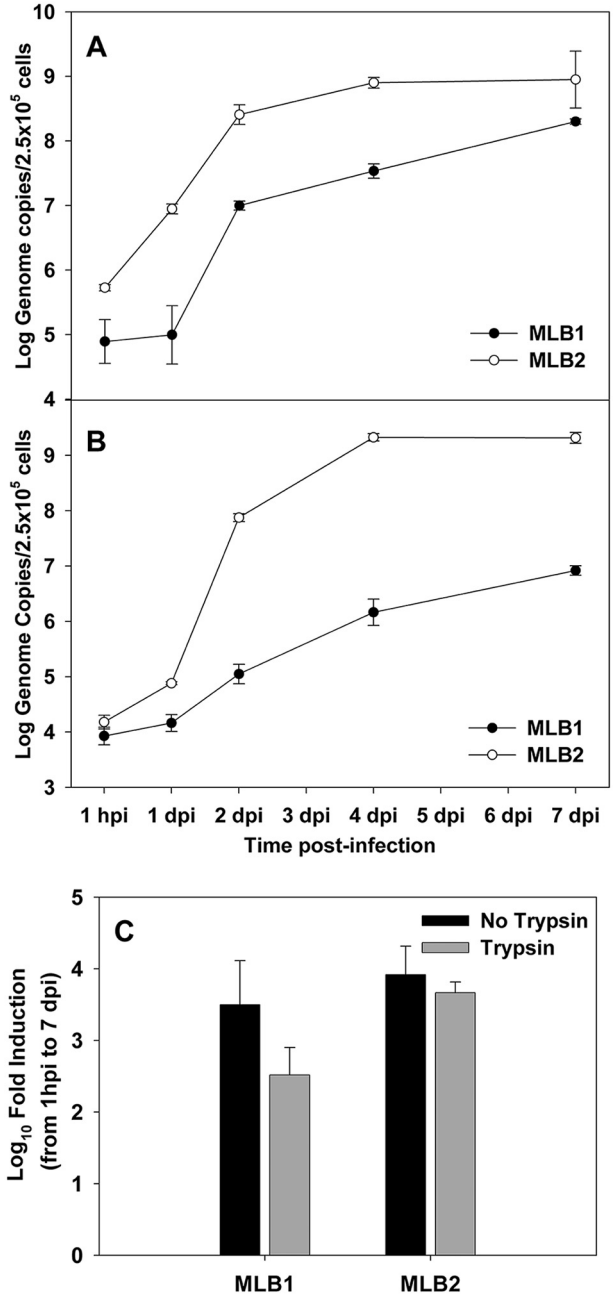

FIG 3 Multistep growth curves of MLB1 and MLB2 on Huh-7.5 cells. Cells were infected using a multiplicity of infection of 20 genome copies/cell, and viral RNA was measured from the cellular (A) and supernatant (B) fractions. Plot shows average values, and error bars indicate one standard deviation from triplicates. (C) Viral replication with or without trypsin for MLB1 and MLB2 on Huh-7.5 cells. Viral replication is expressed as the fold induction of viral genome titers in the SN of infected cells from 1 hpi to 7 dpi. *P* values comparing fold inductions with and without trypsin for each genotype were not significant (by Mann-Whitney test). Plot shows average values, and error bars indicate one standard deviation. Samples were quantified in duplicate from a single experiment.

MLB astroviruses can persistently infect cell cultures. According to the high intracellular viral titer fraction observed for MLB1, we hypothesized that this could reflect a persistent infection. To ascertain whether infected cultures were able to regrow after infection, we used the MLB1 V-P7 cell lysate to establish a persistent infection in the Huh-7.5 cell line. Infected cells were trypsinized at 4 dpi and could be further maintained for up to at least 20 cell passages (C-P) (Fig. 1A). The presence of

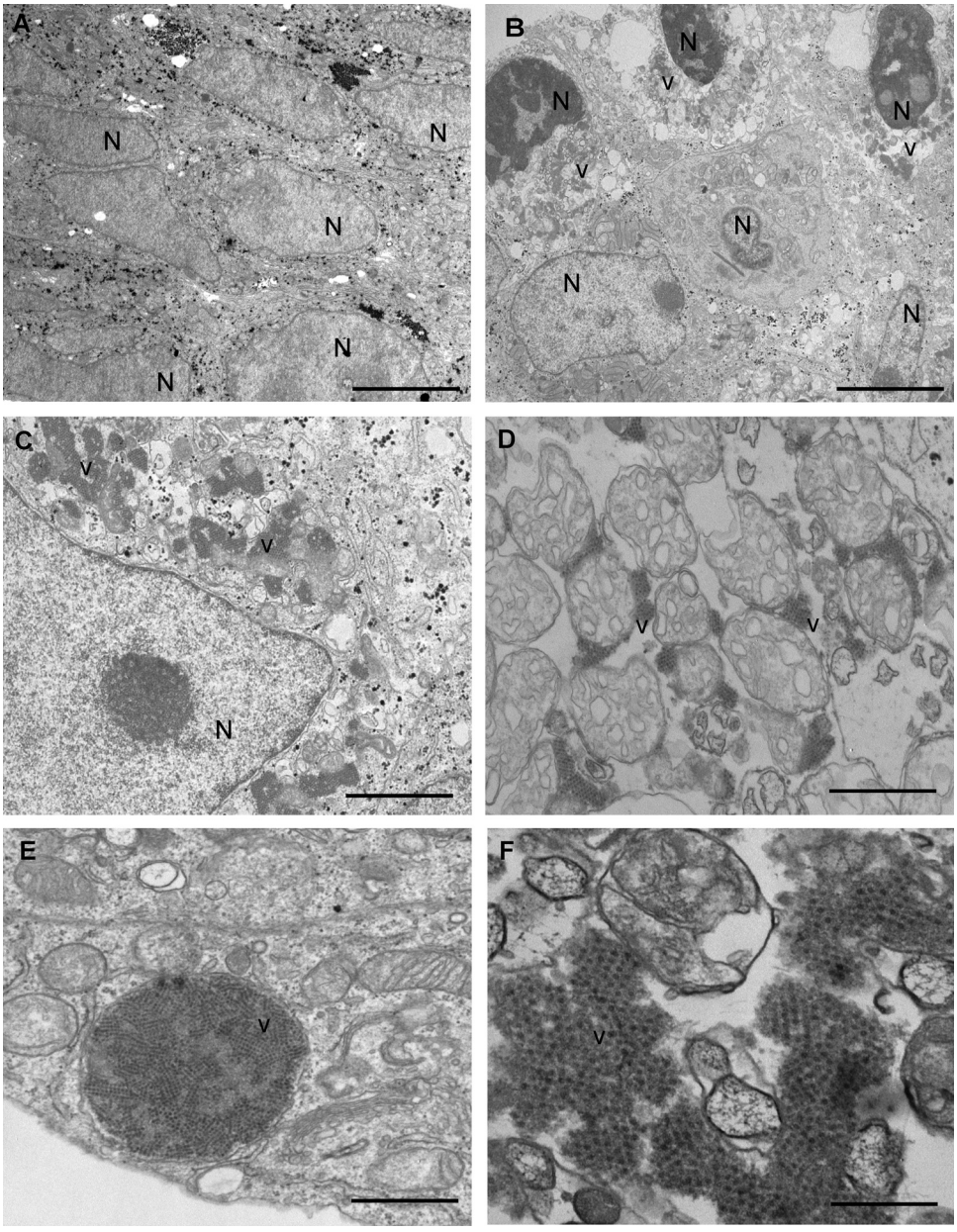

FIG 4 Electron microscopy analysis of the persistent infections on Huh-7.5 cells. (A to F) Noninfected Huh-7.5 cells (A) and persistently infected Huh-7.5 cells (B to F) showing intracellular capsid arrays of HAstV MLB1 at 4 days postseeding. Aggregates of astrovirus particles (v) accumulated in the cytoplasm of infected cells around the nuclei (N). Bars = 5 μm in panels A and B, 2 μm in panel C, 1 μm in panels D and E, and 200 nm in panel F.

numerous capsid arrays in persistently infected Huh-7.5 cells, mostly associated with cell membrane vesicles, was observed (Fig. 4). Cells containing capsid arrays showed remarkable cell structure reorganization.

To elucidate if this was due to the described defect in the interferon pathway of the Huh-7.5 cell line (46, 47), we similarly initiated a persistent infection on Huh-7AI cells with both HAstV MLB1 and MLB2 strains recovered from cell lysates, and also on A549 cells, according to the supposed respiratory tropism of novel astroviruses (Fig. 1B). The titers of viral genomes for both strains detected in the supernatant of the two cell lines during passages of persistently infected cultures are shown in Fig. 5A. The mean viral titer for MLB1 was significantly higher than that for MLB2 in the Huh-7AI and A549 cell lines ($P < 0.002$; Fig. 5B). The MLB1 mean viral titer was also significantly higher in

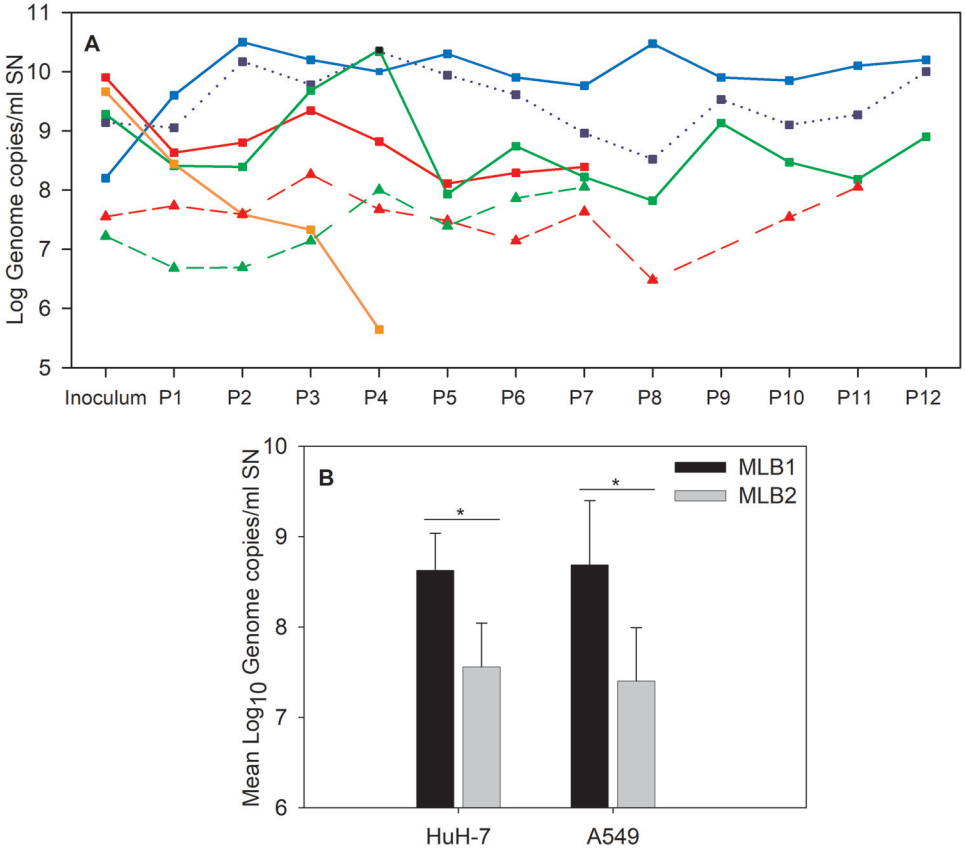

FIG 5 Viral genome titers detected in the culture supernatant (SN) by RT-qPCR assays of MLB1 and MLB2 strains in persistently infected cell lines. (A) Blue line refers to the Huh-7.5 cell line, red lines refer to the Huh-7AI cell line, green lines refer to the A549 cell line, and the orange line refers to the Caco-2 cell line. Continuous lines with squares refer to HAstV MLB1 strain, and dotted lines with triangles refer to HAstV MLB2 strain. The dotted blue line with squares corresponds to the MLB1 persistently infected Huh-7.5 cell line, using the original stool sample as an inoculum. (B) Mean viral genome titers of HAstV MLB1 and MLB2 detected by RT-qPCR assays in the SN of persistently infected Huh-7AI and A549 cell lines. The average was calculated based on 7 to 12 numbers, corresponding to the viral genome titers at each cell passage. Error bars indicate one standard deviation. The mean viral titers were significantly different between HAstV MLB1 and HAstV MLB2 in a comparison of the same infected cell line (*, $P = 0.0009$ in Huh-7AI cells, $P = 0.0018$ in A549 cells, Mann-Whitney test).

Huh-7.5 cells than in Huh-7AI cells, which could confirm our initial hypothesis that the Huh-7.5 interferon pathway deficiency could promote MLB1 replication ($P < 0.001$; Fig. 5A).

Attempts to establish a MLB2 persistent infection on the Huh-7.5 cell line, however, were unsuccessful, and we decided to pursue the rest of the experiments on Huh-7AI and A549 cells only, in order to be able to compare the results between the two genotypes. Except at 4 days after C-P0, persistently infected cultures did not show cytopathic effect, and cells were morphologically identical to noninfected cells. Of note, attempts to establish a persistent infection on Caco-2 cells showed a progressive decline in the viral titer from one cell passage to another, which is a reason why we did not pursue with this cell culture system either (Fig. 5A).

Indirect immunofluorescence assays confirmed the presence of viral capsid proteins in infected cells. Figure 6A shows capsid protein formation in each cell line persistently infected by MLB1 and MLB2 strains. While the fluorescent intensity in each cell line reflects a high production of capsid protein in infected cells, the proportion of cells showing capsid proteins ranged from 1 to 18% (median, 11% and 4.8% for Huh-7AI and A549 persistently infected with MLB1, respectively, and 8.8% for both cell lines persistently infected with MLB2) (Fig. 6B). Overall, for HAstV MLB1, the percentage of capsid-expressing cells was significantly higher in Huh-7AI cells than in A549 cells. No

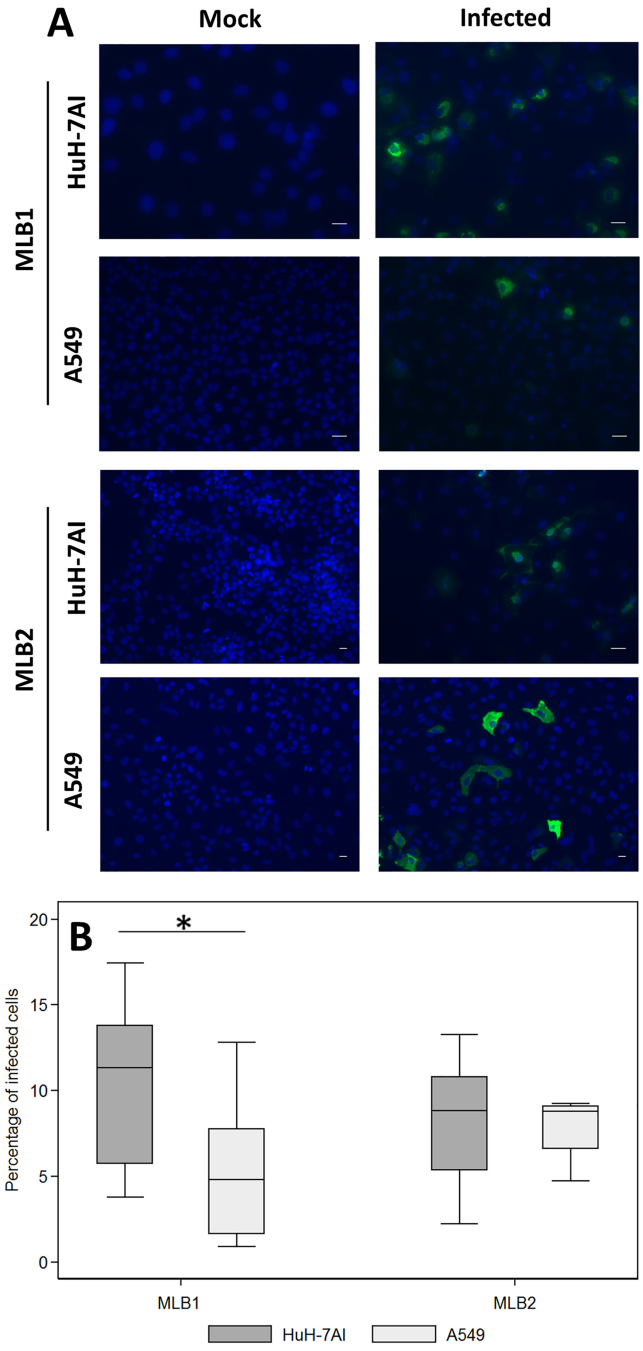

FIG 6 Immunofluorescence assay of MLB1 and MLB2 persistently infected cell lines. (A) Infected and noninfected (mock) cultures were fixed at confluence and were incubated with primary (anti-MLB1 and anti-MLB2 antibodies, respectively) and secondary antibodies. Green corresponds to the viral capsid proteins, and blue corresponds to the nuclei. All samples were fixed at 3 to 5 days postseeding (magnification, ×10 to 20). (B) Estimated proportion of persistently infected cells visualized by the immunofluorescence assay. Central line of each box plot represents the median. Each box plot includes data from 7 to 12 fields from 2 different cell passages. For HAstV MLB1, the proportion of Huh-7AI-infected cells was significantly higher than the proportion of A549 cells (*, $P = 0.0267$, Mann-Whitney test). Bars = 25 μm.

differences were observed for HAstV MLB2. Electron microscopy also confirmed the presence of viral capsid arrays within Huh-7AI- and A549-infected cells (Fig. 7). Table 1 summarizes the results of HAstV MLB1 and MLB2 propagation on selected cell lines.

Complete genome sequences, using primers detailed in Table 2, were obtained for both strains to analyze whether mutations were occurring during replication compared

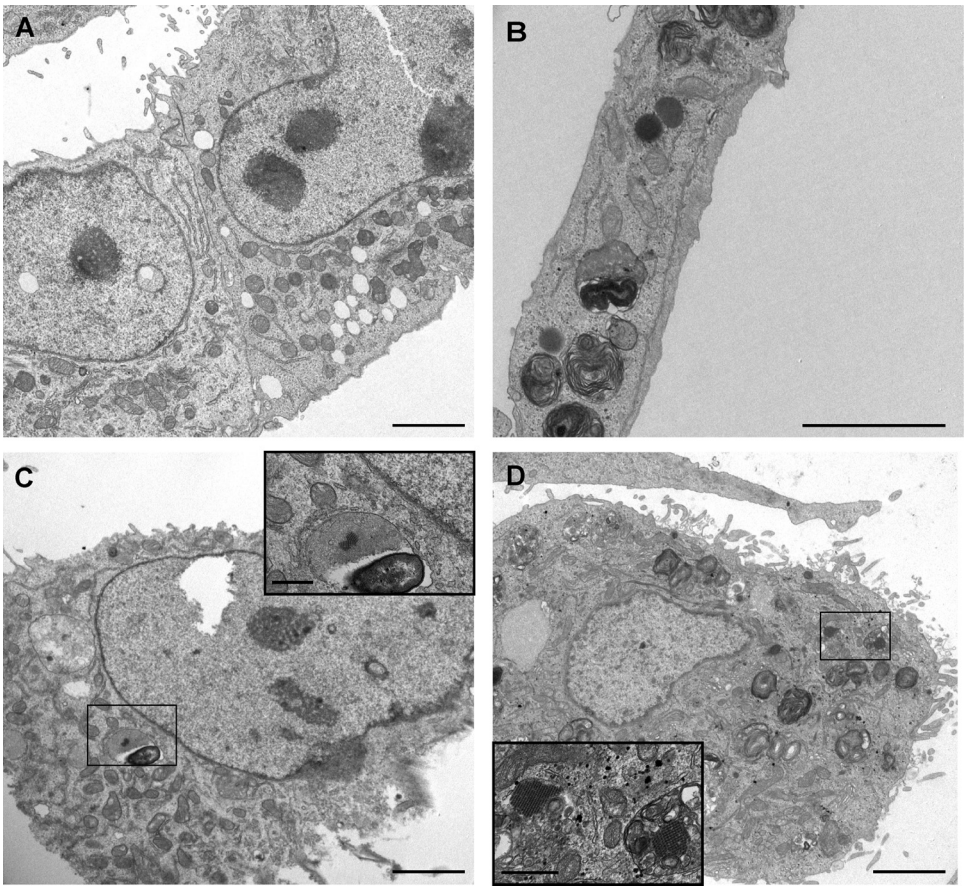

FIG 7 Visualization of capsid arrays by electron microscopy of persistently infected Huh-7AI and A549 cells. (A) Noninfected Huh-7AI cells. (B) Noninfected A549 cells. (C) Huh-7AI cells persistently infected with HAstV MLB1. (D) A549 cells persistently infected with HAstV MLB2. Bars = 2 μm in main images and 200 nm in enlargements.

to viral sequences present in the clinical specimens. The nucleotide sequences of wild-type strains recovered from clinical samples are available at GenBank (accession numbers MK089434 and MK089435). For MLB1, sequences were obtained at V-P6 and C-P2 of acute and persistent Huh-7.5 cell infections, respectively. For MLB2, sequences were obtained at V-P2 on A549 cells and V-P3 in Huh-7.5 cells. No nucleotide changes were detected on the whole genomes throughout the analyzed passages. Nevertheless, we could observe an A-to-C polymorphism at position 1313 and a C-to-T polymorphism at position 5477 of MLB2 at V-P2 in A549 cells, probably reflecting the presence of virus quasispecies. While the latter mutation would be synonymous (and was also present in the wild-type strain), the mutation at position 1313 would result in a substitution of a lysine (K) residue by an arginine (N) in ORF1a. Interestingly, HAstV MLB1 strain directly recovered from the stool sample was also able to establish a persistent infection in the Huh-7.5 cell line, with viral titers comparable to those in the persistent infection with the HAstV MLB1-adapted strain (Fig. 5A), confirming that the ability to establish persistent infections in cell cultures is not dependent on any specific adaptive mutation.

Lack of a strong type I interferon response in MLB-infected cultures. We measured the expression of beta interferon (IFN-β) and IFN-λ1, both known to be implicated in the antiviral innate response. Poly(I·C) transfection was used as a positive control of IFN induction, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were used for normalization. During the acute infection, we could not detect any expression of IFN-λ1 and only a low expression of IFN-β mRNA from 4 to 7 dpi in A549 cells infected with MLB1 and MLB2, compared to those with the poly(I·C) control

TABLE 1 Summary of results of HAstV MLB1 and MLB2 propagation on selected cell lines[a]

Characteristic	HAstV MLB1	HAstV MLB2
Origin of stool sample	1-yr-old child; Barcelona, Spain	Adult allogeneic stem cell transplant recipient for acute myeloid leukemia; Geneva, Switzerland
Patient's symptoms	Gastroenteritis	Meningoencephalitis; leukemia relapse
Viral titer of initial inoculum (genome copies/ml)	7.9×10^7	4.6×10^7
Acute infections		
Infected cell lines		
Caco-2	−	ND
Huh-7.5	+	+
Huh-7AI	+	+
A549	+	+
Mean viral titer (genome copies/ml of SN)		
Huh-7.5	4.5×10^6	2.8×10^9
Persistent infections		
Infected cell lines		
Caco-2	−	ND
Huh-7.5	+	−
Huh-7AI	+	+
A549	+	+
Mean viral titer (genome copies/ml of SN)		
Huh-7.5	5.6×10^9	−
Huh-7AI	4.3×10^8	3.6×10^7
A549	4.9×10^8	2.5×10^7
Percentage of infected cells (median)		
Huh-7AI	11	8.8
A549	4.8	8.8

[a]Mean viral titers for Huh-7AI and A549 cell lines acute infections are not provided due to the few number of assays performed. ND, not done; +, successful replication; −, failure to replicate.

(Fig. 8A). In order to confirm that the absence of IFN expression in Huh-7AI was not due to the fact that only a small proportion of these cells were infected, we infected them with the highest multiplicity of infection (MOI) possible according to the viral titer of the stocks (MOI, 25,000 genome copies/cell for MLB1 and 680 genome copies/cell for MLB2), which resulted in the infection of >80% of Huh-7AI cells (as visualized by immunofluorescence; Fig. 8B and C), and IFN mRNA remained undetectable. During persistent infections, there was basically no expression of IFN-β nor IFN-λ1 mRNAs in any of the two cell lines infected with MLB1 or MLB2 (Fig. 8D).

To understand whether MLB1 and MLB2 replication could block type I IFN expression induced by double-stranded RNA (dsRNA), we analyzed IFN-β and IFN-λ1 mRNA expression after transfecting poly(I·C) in persistently infected cultures. We could observe that IFN-β and IFN-λ1 mRNA expression was almost undetectable in A549 cells for both genotypes compared to the positive control, while in the Huh-7AI cell line, IFN-β and IFN-λ1 induction was only blocked by MLB2. The MLB1 genotype slightly inhibited the expression of IFN-λ1 but not of IFN-β (Fig. 8D). The possibility that A549 persistently infected cultures were refractory to transfection was ruled out by confirming that cells could be efficiently transfected using a green fluorescent protein (GFP)-encoding plasmid (data not shown). Altogether, these results suggest that MLB replication is able to disrupt the innate immune-sensing pathway induced by poly(I·C), although this behavior is cell and genotype (or strain) dependent.

Exogenous IFN inhibits viral replication in a cell-dependent manner. Finally, we tested if the addition of exogenous IFN-β1a and IFN-λ1 at 1,000 U/ml could inhibit viral replication when acutely infected and cure the persistently infected cell lines. During acute infection, pretreatment of cells with both IFN-β1a and IFN-λ1 resulted in a statistically significant reduction in the viral titer compared to the mock-treated controls ($P < 0.005$ and $P < 0.01$ for IFN-β1a and IFN-λ1, respectively, during MLB1 infection of both cell lines; $P < 0.001$ and $P < 0.005$ for IFN-β1a and IFN-λ1, respectively, during MLB2 infection of both cell lines; Fig. 9A and B). The inhibitory effects of both

TABLE 2 Nucleotide sequences of primer pairs used for sequencing of whole genomes of HAstV MLB1 and HAstV MLB2 strains recovered from infected cells

Segment	Primer[a]	Sequence for strain: MLB1	MLB2
1	1 F	CCAAGAGTGGTGGTATGGC	CCAAGAGTGGTGGTATGGC
	1 R	CAGTGCTGTAGACATCCAGAAA	AGCACAAACAACTGATGTAACT
2	2 F	AGAGACCCTGTGTTGCAATAAT	AAAGACCATGTGTTGCCATAAT
	2 R	CTAACTTTGGCTTGAGCAACATAA	CTAACTTTAGCCTGGGCCACATAA
3	3 F	CATAGTTACCGCCGCACAT	TATAGTGACAGCAGCACAT
	3 R	TTCCCTAGTCAGTCCCTTATCC	TTCTCTGGTTAGGCCTTTATCC
4	4 F	CTGACAGAAGAGGAGTACCAAG	TGGCGCACGTCATAGAA
	4 R	CCCATACAGTGGGACCAAA	CCCATACAGTGGGACCAAA
5	5 F	GTACCTTTAGATAGGCCAGTGTATG	GTACCTTTAGATAGGCCAGTGTATG
	5 R	CATCAACAAGGTTGGTGGTATTG	CACCCATAAGCGAGAACCGTAAT
6	6 F	GTTGCGCTCCAAAGGTAATAAA	TCCCTTCTTTGGAGGCTTTG
	6 R	AGTGAAGCGCCTTGGTAAG	AGTGAAGCGCCTTGGTAAG
7	7 F	CCAGTTGTTGATGGCAAATGA	CCAGTTGTTGATGGCAAATGA
	7 R	CCACTCACTAGACGCTGTTT	TTCACAAGGGCCTGAAAC
8	8 F	CTCAACTCATGGTCTGGTCTTG	TTGAATTCATGGTCGGGTC
	8 R	CATGTGCCTTGCTGGAAATTG	GGTGGGCAGTACTAGAAATTG
9	9 F	CAGCGGATGTCTATCGTGTTTA	CAGCTGATGTTTACAGAGTTTACAC
	9 R	TCCTTAGGTATAGCTGGGTATGT	AATGACCCTGTATGCTGGTATG
10	10 F	GGTCATCAGCACCAGCTAATA	GTTCATCTGCAACATCTGAGA
	10 R	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT

[a]F, forward; R, reverse.

IFNs were higher in Huh-7AI cells than in A549 cells ($P = 0.02$). For both genotypes and in both cell lines, the effect of IFN-β1a was approximately 2-fold higher than that of IFN-λ1 (average $\log_{10}$ reductions, 2.91 ± 0.11 versus 1.59 ± 0.11 for MLB1 in Huh-7AI, 1.92 ± 0.52 versus 0.94 ± 0.11 for MLB1 in A549, 4.35 ± 0.03 versus 2.30 ± 0.25 for MLB2 in Huh-7AI, and 2.64 ± 0.20 versus 1.33 ± 0.02 for MLB2 in A549).

During persistent infection, the addition of IFN-β1a to the culture medium succeeded in curing the Huh-7AI cell culture from both MLB1 and MLB2 persistent infection after several cell passages. The addition of IFN-λ1 did not eliminate viral replication completely but reduced viral titer 0.96 ± 0.21 and 2.37 ± 0.09 $\log_{10}$ for MLB1 and MLB2, respectively, after 7 passages (Fig. 9C and D). In contrast, in the A549 persistently infected cell cultures, the addition of none of the tested IFNs produced an inhibitory effect on viral replication (Fig. 9E and F). Increase of IFN concentration up to 5,000 U/ml for two additional passages had no effect either (data not shown).

DISCUSSION

We provide the first description of several cell culture systems permissive for the novel HAstV MLB replication. Both Huh-7 and A549 cell lines were infected by viruses present in clinical specimens and sustained viral replication during serial cell passages without the addition of viral inoculum. Expressed viral capsid protein was detected by immunofluorescence within persistently infected cultures, and viral capsids were visualized by electron microscopy both within membrane vesicles and in the cell cytosol. The production of infectious virions released into the supernatant of persistently infected cultures was confirmed by their ability to acutely infect naive cells when used as an inoculum and generate virions that could be observed in the cell culture media after negative staining. In addition, we provide data on the replication of two distinct genotypes, allowing a comparison of the behaviors of HAstV MLB1 and MLB2. Never-

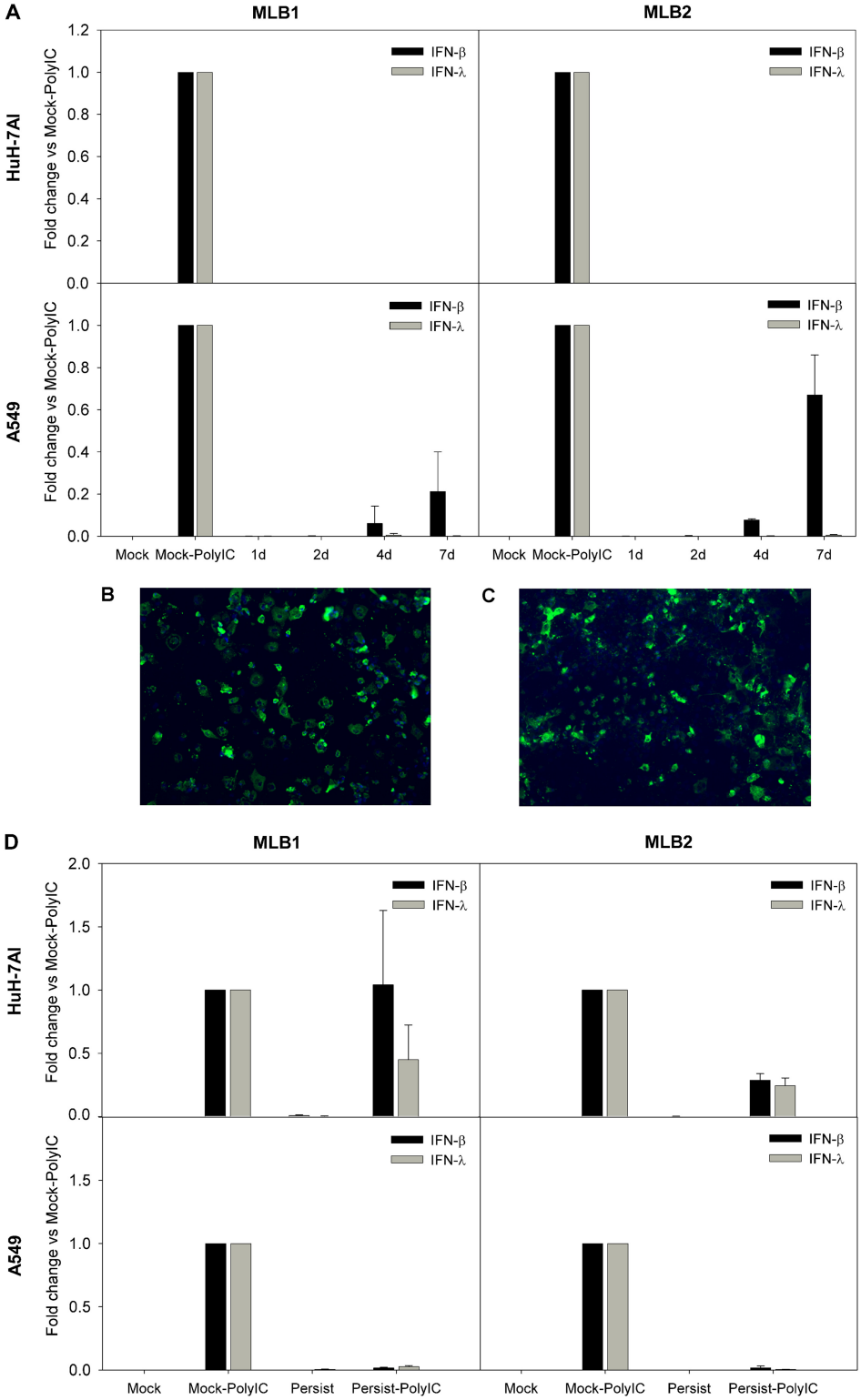

FIG 8 IFN-β and IFN-λ1 expression in infected cell lines. (A) Temporal analysis of IFN-β and IFN-λ1 mRNA expression during acute infection in Huh-7 and A549 cells. Cells were infected with an MOI of 1,000 and 20 genome copies/cell for MLB1 and MLB2, respectively, and poly(I·C)-transfected cells were used as controls. (B and C) Immunofluorescence images correspond to an acute infection using the highest MOI possible (25,000 genome copies/cell for MLB1 and 680 genome copies/cell for MLB2). (D) Analyses of IFN-β and IFN-λ1 expression in persistently infected cultures. Mock, noninfected cells; Mock-PolyIC, noninfected cells transfected with poly(I·C) (positive control); persistent, persistently infected cells; persistent-PolyIC, persistently infected cells that were additionally transfected with poly(I·C); 1d, 2d, 4d, and 7d are the days postinfection (dpi) where IFN expression was measured during acute infections. All samples were quantified at least in duplicate from two distinct experiments.

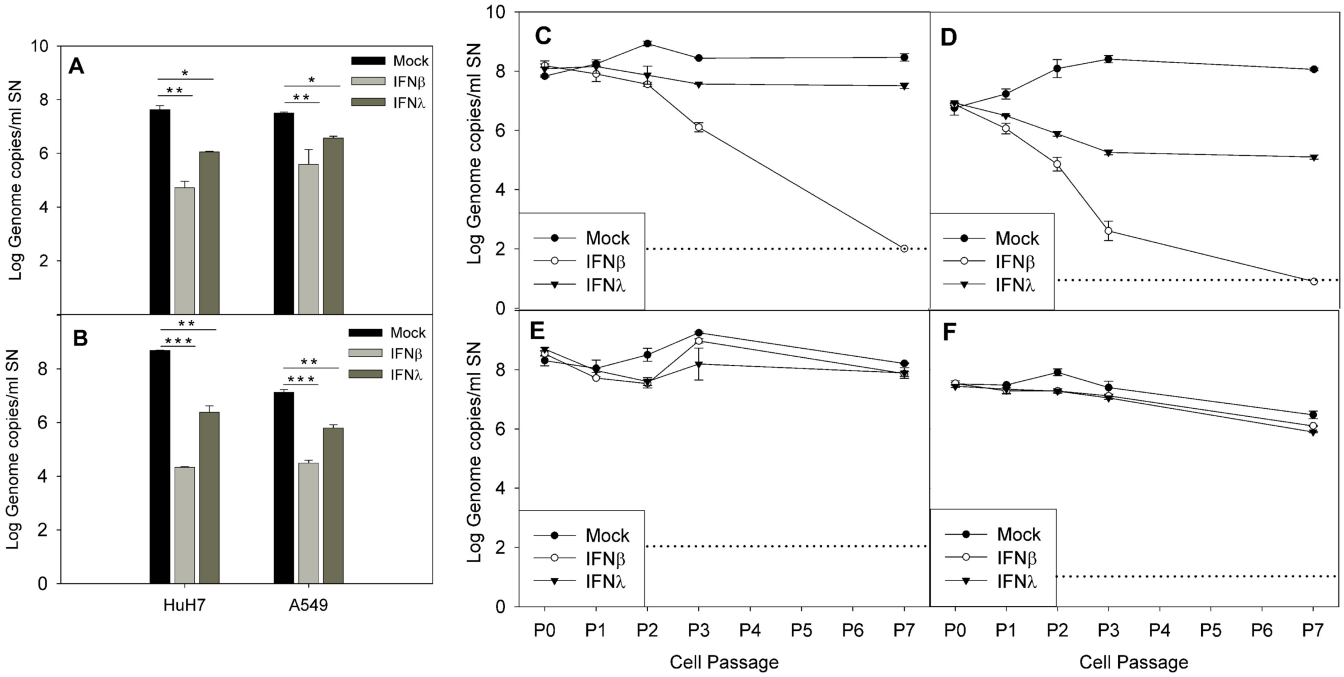

FIG 9 Effect of exogenous IFN during acute infection (A and B) and in persistently infected cultures (C to F). (A and B) Effect of exogenous IFN-β1a and IFN-λ1 on acutely infected Huh-7AI and A549 cells by MLB1 (A) and MLB2 (B). The graphic illustrates the mean viral titer measured in the supernatant at 4 dpi, and error bars show one standard deviation. There was a statistically significant difference in the viral titer between no IFN and IFN-β1a and between no IFN and IFN-λ1 (*, $P < 0.01$; **, $P < 0.005$; ***, $P < 0.001$, ANOVA and Scheffe tests). (C to F) Effect of exogenous IFN-β1a and IFN-λ1 on Huh-7AI cultures persistently infected with MLB1 (C) or MLB2 (D) and on A549 cultures persistently infected with MLB1 (E) and MLB2 (F). Data represent the mean $\pm$ standard deviation titer of viral RNA in the supernatant of each passage measured at 4 to 6 days postseeding in the presence or absence of exogenous IFN-β1a or IFN-λ1. All passages were performed in duplicate. P0 corresponds to the viral titer at 1 day after seeding of the first passage with exogenous IFN. Dotted line indicates the limit of detection. P, passage; SN, supernatant.

theless, we cannot exclude that the results could be strain dependent, as we only succeeded in propagating one strain of each genotype.

We found that the addition of exogenous trypsin is not required for efficient replication of MLB strains, as previously described for VA1 strains by Janowski et al. (45). The efficient propagation of HAstV MLB in extraintestinal cell lines, hepatic and respiratory, reinforces this information, as these tissues do not secrete as much trypsin as the intestinal tract (48). The capacity of novel HAstVs to infect tissues without the need for capsid activation by trypsin opens the door to a potential wider tissue tropism *in vivo*, which could explain the diverse clinical manifestations that have been recently described with divergent astrovirus strains, as follows: acute hepatitis, respiratory illnesses, gout, or encephalitis. Nevertheless, our attempts to infect Caco-2 cells were unsuccessful (or at least not as efficient as with Huh-7 and A549 cells, according to the persistent infection assays), which distinguish our results from those of Janowski et al. (45) and those on classical astroviruses. Whether this difference is strain dependent cannot be formally ruled out, but the hypothesis that divergent astroviruses could show an exclusively extraintestinal tropism has also been advanced by other groups (49), who found porcine astrovirus genome in the central nervous system, respiratory tract, and circulatory system of pigs affected by a neurologic syndrome but not in stool samples. Their work suggests that the respiratory tract could be the primary site of astrovirus infection before spreading to the central nervous system. Of note, classical and VA1 astroviruses are also able to infect A549 cells (45, 50).

Apart from providing a cell culture system for HAstV MLB propagation, we have identified the capacity of this clade to establish persistent infection in the studied continuous cell lines. To the best of our knowledge, this is the first description of an experimentally proven persistent infection for astroviruses. RNA viruses make use of several mechanisms for persistence, including the innate immune system evasion, and

most persistent infections are asymptomatic (51, 52). Thereby, although continuous cell lines are not the best model to infer issues related to pathology occurring *in vivo*, it would be very interesting to further study if there is any role for persistent infection in astrovirus diseases, and if so, which are the determinants for the virus to switch from persistence to virulence. Our immunofluorescence assay and structural changes observed by electron microscopy indicate a carrier-state infection, characterized by a small proportion of capsid-expressing cells, associated with a high degree of cell damage and a high production of virus progeny (53) that can infect surrounding noninfected cells. This model of infection is well described for group B coxsackieviruses (54, 55). An alternate hypothesis is that most of the cells in the culture may be resistant to a full cycle of viral replication (precluding capsid visualization by immunofluorescence) and continue to divide. In light of the impossibility of establishing a MLB2 persistent infection in Huh-7.5 cells, it seems that the high rate of replication of MLB2 genotype during the acute infection prevents the survival and regrowth of the infected cells after subculture.

The results of our IFN experiments provide clues to understand the mechanism for HAstV MLB persistence, suggesting that MLB infection does not induce early strong IFN expression, as it has already been described for the classical HAstVs (56). This would avoid a complete clearance of infection by cells and enable persistence. The fact that no IFN expression was observed in any acutely infected cell line before 4 dpi, the time point when infected cultures were subcultivated from C-P0 to study persistence, supports this idea, permitting the virus to continue replicating in permissive cells before the intervention of the innate immune response. Only a certain level of IFN-β mRNA expression was detected in A549 cells during the late course of acute infection. The inhibition of HAstV MLB replication with exogenous IFN during acute infection also reinforces these data; if there was no shutdown of IFN expression, efficient production of IFN by infected cells would inhibit viral replication and thus possibly prevent persistent infections. Of note, sensitivity of astroviruses to IFN when cells are pretreated before infection has also been demonstrated for classical HAstV (56, 57) and VA1 astroviruses (45).

Exogenous IFNs, especially IFN-β1a, were also able to inhibit and even eliminate MLB viruses from persistently infected cultures, but this was only observed for Huh-7AI cells and not for A549 cells. While this difference based on cell type was unexpected, it suggests that persistence may be maintained in both cell lines by dissimilar mechanisms. Indeed, the inhibitory effects of both IFNs on A549 cells were also significantly milder than on Huh-7AI when cells were acutely infected. It is also noteworthy that the effect of MLB replication on IFN mRNA expression induced by poly(I·C) transfection was different in both cell lines. While it could be efficiently blocked on A549 cells, this effect was only partial in Huh-7AI. Our hypothesis is that while MLBs cannot inhibit IFN expression induced by poly(I·C), they may still avoid activation of IFN response by an unknown mechanism, allowing their persistence in the culture unless IFN is added exogenously. On A549 cells, however, MLBs may find the mechanisms to inhibit both arms of the IFN response (induction and action), allowing them to persist in the culture. The fact that efficient counteracting IFN action on A549 cells is only observed when cells are already infected and not when they are acutely infected suggests that a factor expressed at late stages of the replication cycle may be required.

Whether our inability to establish a persistent infection on the Huh-7.5 cell line with the MLB2 strain was due to the activation of other cellular innate responses that would induce the expression of antiviral genes in noninfected neighboring cells or whether it was due to technical factors such as the schedule of culture passaging or to the MOI remains to be elucidated. In addition, we did not measure other types of IFN, such as IFN-α, which could also play a role in the course of HAstV infection. Altogether, we can see a distinct IFN expression and response to exogenous IFN between the persistent versus acute infection, between MLB1 and MLB2, and between different cell lines. These results suggest that there is an actual coevolution between a virus and its host and that many factors (virus strain, cell type, and model of infection) may uniquely influence the

course of the infection. Interestingly, Nice et al. recently demonstrated that IFN-λ was able to reduce and to cure persistent infection of murine norovirus in the absence of an adaptive immune response (58, 59) and that the interaction between host IFN-λ response and some viral nonstructural proteins determined viral tropism (59). In addition, these results also suggest that, if persistence was confirmed *in vivo*, in the case of coinfections, certain MLB HAstVs could potentially promote the replication of other viruses by inhibiting the IFN response.

In summary, we provide a cell culture system for the propagation of the novel HAstV MLB. We have demonstrated that these viruses can establish a carrier-state infection *in vitro* on extraintestinal human cell lines. IFN expression may be altered by HAstV MLB infections although may vary depending on the strain, the cell line, and the model of infection. Finally, HAstV MLB sensitivity to IFN also depends on the type of infection, the genotype, the cell line, and the type of IFN.

MATERIALS AND METHODS

Cell lines. Human epithelial colorectal adenocarcinoma (Caco-2 cells; ECACC 86010202), human hepatocyte-derived cellular carcinoma (Huh-7AI cells [60] and Huh-7.5 cells [61]), and adenocarcinoma human alveolar basal epithelial (A549 cells; ATCC CCL-185) cell lines were grown at 37°C with 5% CO_2 on minimal essential medium (MEM) with L-glutamine supplemented with 10% fetal bovine serum (FBS; Gibco) and 100 units/ml penicillin and streptomycin (Gibco).

Clinical specimens. Stool samples positive for MLB1 were collected during a screening of stool samples from children under 5 years old with acute gastroenteritis in Barcelona, Spain. Stool samples positive for MLB2 were identified in a previous study (28). Three samples positive for MLB1 and three samples positive for MLB2 were used for infection, with a viral titer ranging from 1.5×10^6 to 1.2×10^8 genome copies/ml of inoculum for MLB1 and from 2.8×10^4 to 4.6×10^7 genome copies/ml of inoculum for MLB2.

Acute infections. Stool suspensions of 0.1 g of sample diluted in 900 μl of phosphate-buffered saline (PBS) were filtered through a 0.45-μm filter (Millipore) and then diluted 1:2 with MEM– 0% FBS and were used as the initial inoculum. The inoculum was pretreated with trypsin (Gibco), at a final concentration of 10 μg/ml, at 37°C for 30 min. Cells were grown on a 24-well plate to 80 to 100% confluence and washed twice with MEM– 0% FBS before infection with 200 μl of the stool inoculum diluted 1:2 after trypsin pretreatment. Cells were incubated for 1 h at 37°C, and the inoculum was then removed and replaced by 500 μl of MEM– 0% FBS supplemented with 0.03% kanamycin, penicillin-streptomycin, and 5 μg/ml trypsin. Cells were maintained at 37°C and 5% CO_2 for 7 days, and the medium was changed every other day. After 7 days, cells were freeze-thawed three times, and 100 μl of the cell lysate was used for the next viral passage. Subsequent viral passages (V-P) as acute infections were performed without trypsin pretreatment and with the addition of MEM supplemented with 10% FBS without trypsin in the postinfection medium.

Persistent infections. A cell lysate of the viral passage 7 (V-P7) of the acute viral passages on Huh-7.5 cells was used to persistently infect the Huh-7AI, A549, and Caco-2 cell lines with MLB1 and MLB2, respectively (Fig. 1B). The viral titer in these selected cell lysates was determined as viral genome copies per milliliter by quantitative reverse transcription-PCR (RT-qPCR) assay and as infectious viruses per milliliter by a 50% tissue culture infective dose ($TCID_{50}$) assay in Huh-7.5 cells. Briefly, for the $TCID_{50}$ assay, cells were infected with 10-fold serial dilutions of each sample in quadruplicate, as described above for acute infections. After 7 days, nucleic acids were extracted from 50 μl of supernatant from each well, and an RT-qPCR assay was performed for the detection of viral genomes (see below). The $TCID_{50}$ was calculated using the Spearman-Karber method, with any detection of viral genome in a well being considered infected. The $TCID_{50}$ corresponded to approximately 1.4×10^4 genomes for MLB1 and to 1.5×10^3 genomes for MLB2. A multiplicity of infection (MOI) of approximately 1,000 genome copies per cell (0.07 infectious viruses per cell) and 20 genome copies per cell (0.01 infectious viruses per cell) was used for MLB1 and MLB2, respectively. For the first passage, cells were grown on 24-well plates to 80 to 100% confluence and washed twice with MEM– 0% FBS. Cell lysates were diluted in MEM– 0% FBS to a final volume of 200 μl at the desired MOI and were inoculated to the cells. After a 1-h incubation at 37°C, the inoculum was removed and replaced by 500 μl of MEM–10% FBS. There was no pretreatment or addition of trypsin for the persistent infections. Cells were incubated at 37°C for 4 days before being subcultivated by trypsinization at a split ratio of 1:3 for a subsequent passage. Cells were then subcultivated by trypsinization for serial passages without the addition of viral inoculum. Persistently infected cells were maintained in T75 flasks and subcultivated every 7 to 10 days at a split ratio of 1:3 to 1:6. An aliquot of the supernatant before each subculture was collected to monitor viral titer.

Viral RNA extraction and quantitative reverse transcription-PCR assay. RNA was extracted from the cell culture supernatants using the NucleoSpin RNA virus kit (Macherey-Nagel), following the manufacturer's instructions, and RT-qPCR-specific assays for MLB astroviruses were performed as previously published (19). Briefly, the following primers and probe for MLB1 were used: forward primer 4320, 5′-GGTCTTGGAGCYCGAATTC-3′; reverse primer 4387, 5′-CGCTGTTTAATGCGCCAAA-3′; and hydrolysis probe 4349, 5′-[FAM]-TAGRGTTGGTTCAAATCT-[MGBNFQ]-3′ (FAM, 6-carboxyfluorescein). The primers and probe used for MLB2 were as follows: forward primer 3762, 5′-CCGAGCTCTTAGTGATGCTAGCT-3′; reverse primer 3832, 5′-CACCCCTCCAAATGTACTCCAA-3′; and hydrolysis probe 3793, 5′-[VIC]-CGCTTCA

CTCGGAGAC-[MGBNFQ]-3′. Plasmids containing a 125-bp fragment of the MLB1 (spanning nucleotides 4292 to 4416 from the virus with GenBank accession no. FJ222451) and MLB2 (spanning nucleotides 3724 to 3848 from the virus with GenBank accession no. KT224358) genomes were used as controls for quantification, and RT-qPCR was performed using the Kapa Probe Fast universal one-step RT-qPCR master mix (Kapa Biosystems), following the manufacturer's instructions, on a CFX96 Touch real-time PCR detection system (Bio-Rad). Fifteen microliters of the RT-qPCR master mix was mixed with 5 μl of extracted RNA. The reaction conditions were as follows: 42°C for 15 min, 95°C for 5 min, and then 40 cycles of 95°C for 3 s, 55°C for 20 s, and 72°C for 10 s. Standard curves were constructed based on 10-fold serial dilutions of the corresponding MLB plasmid analyzed in duplicate.

Multistep growth curve. The multistep growth curve was created using an MOI of 20 genome copies/cell for both MLB1 and MLB2. Infection was performed on 24-well plates according to the protocol used for acute infection (without the use of trypsin nor the change of medium every other day). At each indicated time point, 50 μl of the supernatant as well as the total cells were collected. RNA extraction of the supernatant was performed using the NucleoSpin RNA virus kit (Macherey-Nagel), following the manufacturer's instructions, and RNA extraction from cells was performed using the GenElute mammalian total RNA miniprep kit (Sigma-Aldrich), as indicated by the manufacturer's instructions. The RT-qPCR assay was performed as described above. Samples were quantified in triplicate from a single experiment.

Immunofluorescence. Indirect immunofluorescence assays were performed using rabbit polyclonal MLB1 capsid peptide (DW60) and MLB2 capsid peptide (DW58) antibodies (kindly provided by David Wang, Washington University School of Medicine) (62) as primary antibodies and secondary antibodies labeled with Alexa 488. DAPI (4′,6-diamidino-2-phenylindole) staining was used to detect nuclei. Briefly, cells were rinsed twice with PBS and fixed with 3% paraformaldehyde in PBS for 15 min at room temperature (RT). Permeabilization was performed for 10 min at RT with 0.5% Triton X-100 in 20 mM glycine-PBS. Cells were then blocked for 60 min at RT in 20 mM glycine-PBS containing 10% bovine serum albumin, followed by incubation with primary antibodies at a 1:1,000 dilution for 60 min at 37°C, and then with the secondary antibodies at a 1:500 dilution for another 60 min at 37°C. Incubation with 1 μg/ml DAPI staining was finally performed for 15 min at RT. Cells were washed twice after each step described, except between the blocking step and the incubation with primary antibodies, and were kept in PBS at 4°C until visualization. Negative controls included cells incubated with preimmune serum, primary or secondary antibodies alone, and fixed cells alone (sample autoimmunofluorescence). Nuclei and viral capsids were visualized under a Leica DMIRB/MZFLIII fluorescence microscope.

Electron microscopy. Cell culture supernatants were analyzed by transmission electron microscopy after negative staining. A 10-μl sample was applied to a carbon-coated 400-mesh copper grid and was stained with 2% phosphotungstic acid at pH 6.4. The grids were examined under a JEOL 1200 electron microscope.

For ultrathin sections, persistently infected cells and noninfected controls were seeded on a 90-mm sterile dish for cell culture until reaching 80 to 90% confluence. After removing the medium, cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) during 60 min. Cells were then scrapped in 1.5 ml of PB and collected. After 10 min of centrifugation at 1,000 $\times$ *g*, the pellet was suspended in PB and washed in agitation at 4°C for 10 min ($\times$4). Cells were then postfixed with 1% osmium tetroxide and 0.8% potassium hexacyanoferrate in 0.1 M PB for 1 to 2 h at 4°C. After extensive washing with Milli-Q water, sample dehydration was performed with a graded series of acetone (50% to 100%) and blocks were prepared in an Eponate 12 kit. Sections of 55 nm were cut with a Leica UC6 ultramicrotome (Leica Microsystems, Inc.). Observation was performed under a JEOL 1200 electron microscope.

Sequence analysis. Primer pairs were designed to amplify 10 overlapping amplicons covering the complete genomes (Table 2). Reverse transcription was performed using the SuperScript IV reverse transcriptase (Invitrogen), and cDNA amplification was performed using the Pwo DNA polymerase (Roche), under the following reaction conditions: 70°C for 7 min, 50°C for 25 min, and 80°C for 10 min; 94°C for 4 min; and then 40 cycles of 94°C for 30 s, 50 to 55°C for 40 s, 72°C for 2.15 min, and 72°C for 10 min. Amplicons were purified by gel electrophoresis and Sanger sequenced with the ABI Prism BigDye Terminator cycle sequencing ready reaction kit v3.1 on an ABI Prism 3700 automatic sequencer (Applied Biosystems).

IFN expression analysis. Intracellular RNA was extracted using the GenElute mammalian total RNA miniprep kit (Sigma-Aldrich), as indicated by the manufacturer's instructions. The resulting eluate was treated with RQ1 RNase-free DNase (Promega) to remove any trace of genomic DNA. Quantitative reverse transcription-PCR (RT-qPCR) was performed using the manufacturer's instructions for a KiCqStart one-step probe RT-qPCR assay targeting mRNA of GAPDH, IFN-β, and IFN-λ1 on a CFX96 Touch real-time PCR detection system (Bio-Rad). The primer and probe sequences were as follows: GAPDH forward primer, 5′-GAAGGAAATGAATGGGCAGC-3′; GAPDH reverse primer, 5′-TCTAGGAAAAGCATCACCCG-3′; GAPDH probe, 5′-[6FAM]ACTAACCCTGCGCTCCTGCCTCGAT[OQA]-3′; IFN-β forward primer, 5′-CCTCCGAAACTG AAGATC-3′; IFN-β reverse primer, 5′-GCAGTACATTAGCCATCA-3′; IFN-β probe, 5′-[FAM]TAGCCTGTGCCT CTGGGACT[BHQ]-3′; IFN-λ1 forward primer, 5′-CCACCACAACTGGGAAGG-3′; IFN-λ1 reverse primer, 5′-T TGAGTGACTCTTCCAAGGC-3′; and IFN-λ1 probe, 5′-[FAM]AGCGAGCTTCAAGAAGGCCAGGGAC[OQA]-3′. Fifteen microliters of the RT-qPCR master mix was mixed with 5 μl of extracted RNA. The reaction conditions included 50°C for 20 min, 95°C for 1 min, and then 40 cycles of 95°C for 5 s and 60°C for 35 s. GAPDH mRNA titers were used as an endogenous control to normalize all samples versus the number of cells. All samples were quantified at least in duplicate from two distinct experiments. Positive controls were determined by the transfection of the synthetic analog of double-stranded RNA (dsRNA) polyinosine-poly(C) [poly(I·C)] (InvivoGen) at 1 mg/ml on each cell line, using the X-tremeGENE HD transfection reagent (Roche) and Opti-MEM medium (Gibco). Determination by a RT-qPCR assay of the

IFN expression 24 h after transfection was then performed as described above. To define a standard curve, 10-fold serial dilutions of the GAPDH, IFN-β, and IFN-λ1 RNA were analyzed for each cell line transfected with poly(I·C).

Inhibition of MLB HAstV replication with the addition of exogenous IFN. We used human IFN-β1a and IFN-λ1 (PBL Assay Science) to measure inhibition of MLB HAstV replication. For the acute infection, cells were grown on a 24-well plate and were pretreated with IFN-β1a or IFN-λ1 at a concentration of 1,000 U/ml for 24 h before infection. Cells were inoculated with MLB1 and MLB2 at an MOI of 20 genome copies/cell, as described before (without the use of trypsin). IFN-β1a or IFN-λ1 was added in the postinfection medium at a concentration of 1,000 U/ml. Fifty-microliter aliquots were collected from the supernatant at 4 dpi for RNA extraction and RT-qPCR (see above).

For persistent infection, persistently infected cell cultures were subcultured as described before. IFN-β1a or IFN-λ1 was added in the medium postseeding at a concentration of 1,000 U/ml. Fifty microliters of supernatant was collected before the next subculture, between 4 and 6 days postseeding, and viral RNA was extracted and analyzed by RT-qPCR, as described before. All passages were performed in duplicate.

Statistical analyses. The Mann-Whitney test and analysis of variance (ANOVA), with additional test of Scheffe when appropriate, were used to compare continuous variables. A P value of <0.05 was considered statistically significant. Statistics were performed by Stata/IC 13.1 (StataCorp, College Station, TX, USA).

Data availability. Complete genome sequences for the MLB1 strain obtained from the stool specimens were deposited in GenBank (accession numbers MK089434 and MK089435).

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Article II: Novel human astroviruses: prevalence and association with common enteric viruses in undiagnosed gastroenteritis cases in Spain

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Novel HAstVs were first identified by HTS in cohorts of patients with acute gastroenteritis or control patients in Australia, United States, India and Africa. Despite the development of specific and sensitive RT-PCR assays targeting novel HAstVs, there are only few reports of the systematic screening for novel HAstV in Europe (163, 176, 182). Such data were still missing in Spain.

The aim of this study was to describe the prevalence of novel HAstV in an outpatient pediatric population with symptoms of gastroenteritis by means of a sensitive and specific real-time RT-PCR assay covering all the HAstV-MLB genotypes (MLB1, MLB2 and MLB3) and most of HAstV-VA genotypes (VA1-VA4). We performed a systematic screening of stool samples of children ≤ 5 years old for novel HAstV as well as other viruses commonly involved in gastroenteritis, namely rotavirus, norovirus GI and GII, adenovirus, sapovirus and classic HAstV. We covered two consecutive winter seasons, from January to April 2016 and 2017, respectively. Samples with a microbiological diagnostic by routine diagnostics procedures were excluded.

We included 384 undiagnosed stool samples in the study. Overall, using molecular diagnostics, one or more viruses were found in 57% of undiagnosed cases. Co-infections

were frequently observed in 21% of samples analysed. Adenovirus and rotavirus were the most prevalent (27% and 13%, respectively). Novel HAstVs were identified in 10.2% (39/384) of samples analysed, and HAstV-MLB1 and HAstV-VA1 were the most frequently observed (corresponding to 31% and 26.2% of novel HAstV identified, respectively). In comparison, classic HAstV was observed in 11.2% (43/384) of samples analysed.

Seventy-seven percent of novel HAstV-positive samples were collected from children under 2 years old and 66% of them were found in co-infection with another virus.

The mean Cq value was 30.7±6.7 for HAstV-MLB and 31.7±6.4 for HAstV-VA. There was no significant difference in the Cq value observed during mono-infection, compared to co-infection, for any screened virus, except for the classic HAstV ($p=0.042$).

In conclusion, this study demonstrates that novel HAstVs are circulating in the pediatric Spanish population with the same prevalence as classic HAstV, at least during the studied period. As expected, young children were at highest risk for novel HAstV infection and we observed a particularly high rate of co-infection with other enteric viruses, raising the question of the causality role of novel HAstV during episodes of gastroenteritis.

viruses

MDPI

Article

Novel Human Astroviruses: Prevalence and Association with Common Enteric Viruses in Undiagnosed Gastroenteritis Cases in Spain

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Abstract: A remarkable percentage of acute gastroenteritis cases remain etiologically undiagnosed. The aim of the study was to determine the prevalence of common and emerging enteric viruses, such as novel human astroviruses, among undiagnosed samples from children with acute gastroenteritis. Epidemiological studies for novel human astroviruses are still scarce. Stool samples collected over two consecutive winter seasons (2016–2017) from children with gastroenteritis in Spain, which were negative for bacteria, rotavirus, and adenovirus by routine diagnostics were screened by real-time RT-PCR assays for the presence of classical and novel astrovirus, rotavirus, norovirus GI and GII, sapovirus, and adenovirus. Overall, 220/384 stool samples (57.3%) were positive for at least one virus. Co-infections were identified in 21% of cases. Among a total of 315 viruses identified, adenovirus was the most prevalent (n = 103), followed by rotavirus (n = 51), sapovirus (n = 50), classical astrovirus (n = 43), novel astroviruses (n = 42), and norovirus (n = 26). Novel astroviruses were present in 13.3% of virus-positive cases. Most novel astroviruses were found in children <2-year-old (30/39 children, 77%, p = 0.01) and were found in co-infection (66%). Only classical astroviruses demonstrated significant differences in the Cq values during mono-infections compared to co-infections. In conclusion, common enteric viruses may be frequently found in children with undiagnosed gastroenteritis, indicating the need to implement more sensitive diagnostic methods. Novel astroviruses circulate in the community and could be the cause of gastroenteritis among young children.

Keywords: gastroenteritis; children; novel human astrovirus; classic astrovirus; norovirus; sapovirus; rotavirus; adenovirus; epidemiology; real time RT-PCR

1. Introduction

Despite the large number of pathogens associated with acute gastroenteritis in children, a remarkable percentage of cases remain etiologically undiagnosed, even in developed countries. Among viral etiologies of sporadic acute gastroenteritis in children, rotavirus is historically the leading pathogen, followed by norovirus and astrovirus, although implementation of the rotavirus vaccination has decreased the incidence of rotavirus gastroenteritis in concerned countries [1]. The classical human

astroviruses (HAstVs), first identified in 1975 during an outbreak of gastroenteritis in a maternity ward [2], have been identified worldwide [3] since then. Several epidemiological studies have tried to determine the exact prevalence of classical HAstVs [4], but there is a high heterogeneity among studies depending on the studied population, the diagnostic method used, the timing of sampling, and the geographic area; yet, there are currently few studies using real-time PCR assays. Overall, the prevalence of classical HAstVs in children with acute gastroenteritis ranges between 0% and 30% [4].

Next-generation sequencing technologies have allowed us to identify two novel groups of highly divergent HAstVs named MLB and VA/HMO. Sequences of novel HAstVs have been found in human stools of individuals with diarrhea [5–8], but also in asymptomatic healthy controls [9,10]. Both groups of novel HAstVs have been further divided into several genotypes: MLB1-3 for the MLB astroviruses and VA1-5 for the VA astroviruses [3,4]. To date, no definitive association between novel astroviruses and gastroenteritis has yet been established, but further epidemiologic studies have confirmed the presence of novel HAstVs worldwide [9,11–16]. Interestingly, novel HAstVs have been associated with unexpected central nervous system infections in—mostly immunocompromised—humans [17–23]. While novel astroviruses have been identified in every continent, data in Europe are scarce, and so far only three countries have reported a systematic screening [12,14,18].

The aim of the study was to narrow down the diagnostic gap of acute gastroenteritis in children by determining the prevalence of novel astroviruses in a pediatric population with undiagnosed gastroenteritis, and to compare their prevalence with those of common enteric viruses, using sensitive molecular diagnostic tools.

2. Materials and Methods

2.1. Patient and Sample Population

The study population was children ≤ 5-years-old presenting at outpatient clinics for symptoms of gastroenteritis of unknown etiology. Stool samples had tested negative for enteropathogenic bacteria (*Salmonella* spp., *Shigella* spp., *Yersinia* spp., *Aeromonas* spp., and *Campylobacter* spp.), rotavirus or adenovirus by routine diagnostics, including immunochromatographic tests for viruses, at the Laboratory of Microbiology of Hospital de Vall d'Hebron, which is one of the main hospitals covering the Metropolitan Area of Barcelona (Spain). No clinical data were available except the patients' age and the date of stool collection. A total of 384 stool samples were randomly selected between January and April 2016 and between January and April 2017. This period was chosen to focus on the winter season and according to the higher incidence of viral gastroenteritis in the studied population. The study was conducted in accordance with the Declaration of Helsinki, and was approved by the Clinical Research Ethics Committee of the Hospital Universitari Vall d'Hebron (26 Feb 2016; PR(AG)32/2016).

2.2. Viral RNA Extraction and Real Time Polymerase- or Reverse-Transcription Polymerase Chain Reaction (Real-Time PCR or Real-Time RT-PCR) Assays for Novel Astroviruses and other Enteric Viruses

RNA was extracted from 200 µL of a 30% stool suspension using the Viasure RNA-DNA Extraction Kit (Certest Biotec, Zaragoza, Spain) following the manufacturer's instruction. Real-time RT-PCR specific assays for HAstV-MLB and HAstV-VA were performed using primers and probes previously published [14]. The standard curves and positive controls for MLB1, MLB2, MLB2-3, VA1, VA2, VA3, VA4 real-time RT-PCR assays were obtained using plasmid constructs kindly provided by Dr. S. Cordey from the Geneva University Hospitals. Standard curves were constructed based on 10-fold serial dilutions of the corresponding plasmid and analyzed in duplicate, for monoplex and duplex assays (MLB1-MLB2/3, VA1-VA2, and VA3-VA4). Table 1 was adapted from Reference [14] and provides the results of the monoplex and duplex validation assays. Real-time RT-PCR was performed using the Kapa Probe Fast Universal One-Step real-time RT-PCR Master Mix (Kapa Biosystems, Wilmington, DE, US) following the manufacturer's instructions, on a Stratagene Mx3000P (Thermofischer, Waltham, MA, US) and a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, US). Fifteen µL of

the real-time RT-PCR master mix were mixed with 5 μL of extracted RNA. The reaction conditions were as follows: 42 °C for 15 min, 95 °C for 5 min, then 40 cycles of 95 °C for 3 s, 55 °C for 20 s, 72 °C for 10 s. Samples positive by MLB2-3 real-time RT-PCR assay were further screened with the specific MLB2 real-time RT-PCR assay to confirm the genotype [14].

Table 1. Nucleotide sequences of the primers and probes used and standard curves parameters of the novel HAstV real-time RT-PCR assays.

							Standard Curve (10-Fold Serial Dilutions)					
							Monoplex			Duplex		
Assay	**Viruses Detected**	**Target Region (ORF)/Amplicon size (nt)**	**Fwd primer (5′-3′) [1]**	**Probe (P) (5′-3′) [1]**	**Rev Primer (5′-3′) [1]**	**Final [uM] Fwd/Rev/P**	**Slope**	**Intercept**	**R^2**	**Slope**	**Intercept**	**R^2**
MLB1	MLB1	ORF2/68	GGTCTTGGAGCYCGAATTC	FAM—TAGRGTTGGTTCAAATCT—MGBNFQ	CGCTGTTTAATGCGCCAAA	0.6/0.6/0.25	−4.02	43.06	0.99	−3.5	40.10	0.97
MLB2–3	MLB2-MLB3	ORF1b/71	CCGAGCTCTTAGTGATGCTAGCT	FAM—CGCTTCACTCGGAGAC—MGBNFQ	CACCCCTCCAAATGTACTCCAA	0.6/0.6/0.2	−3.34	44.29	0.99	−3.19	44.69	0.99
VA1	VA1/HMO-C/SG/PS/UK1	ORF2/66	CCATCAGCAGTTACYGGGTCTGT	FAM—TTTCCGCATATCCC—MGBNFQ	CGTGGCTCCAGGTGAYTGT	0.6/0.6/0.2	−2.72	35.24	0.96	−2.83	36.03	0.97
VA2	VA2/HMO-A	ORF2/67	CAGGGCCTGAATTACAAATTTCA	FAM—CATTTATGCATCCTGCTTT—MGBNFQ	GTGCCATCATTTGGCTCTTTC	0.9/0.9/0.25	−3.15	41.24	0.99	−2.71	37.96	0.98
VA3	VA3/HMO-B	ORF1b/67	TTCCAGGCATTTGAGTTTGCT	FAM—TTGAATCCGGATAAAAC—MGBNFQ	CCCATCCTTCTCTCAGTTCATCA	0.6/0.6/0.25	−3.16	39.71	0.98	−3.25	40.39	0.97
VA4	VA4	ORF2/62	GATCCATGTATCGTGCATCGTT	FAM—AACCTTACACAGTCCCCGG—MGBNFQ	GCCCCCCCAAGATGTTG	0.9/0.9/0.25	−3.17	37.94	0.98	−2.5	35.83	0.95

[1] Primers and probes were previously published [14].

We also tested the presence of other enteric viruses in stool samples by real-time RT-PCR assays targeting the classical HAstVs, sapovirus, rotavirus, adenovirus, and noroviruses GI and GII, which were available for the 2017 study period only, using available commercial kits (VIASURE Astrovirus Real Time PCR Detection Kit, VIASURE Sapovirus Real Time PCR Detection Kit, VIASURE Adenovirus Real Time PCR Detection Kit, VIASURE Norovirus GI Real Time PCR Detection Kit, VIASURE Norovirus GII Real Time PCR Detection Kit, and VIASURE Rotavirus Real Time PCR Detection Kit from Certest Biotec; and RIDA®GENE Viral Stool Panel II from R-Biopharm).

2.3. Statistical Analyses

The Kruskal-Wallis test was used to compare continuous variables and Fischer exact test was used to compare categorical variables. The non-parametric equality-of-medians test (Pearson chi2 test with continuity correction) was used to compare the proportion of Cq value above and under the median, respectively, according to mono- or poly-infection status. $p < 0.05$ was considered statistically significant. Statistics were performed by Stata /IC 13.1 (StataCorp, College Station, TX, USA).

3. Results

3.1. More than Half of Samples Undiagnosed by Routine Screening are Positive by Real-Time PCR- or RT-PCR Assays

During the whole study period, 384 undiagnosed stool samples (n = 197 in 2016 and n = 187 in 2017) were analyzed. This selection of samples represents 24.2% of the total amount of samples from outpatient children ≤5-years-old with gastroenteritis symptoms, and to 33.1% of undiagnosed specimens. The overall proportion of undiagnosed samples during 2016 and 2017 was 77.7% (73.0% during the studied period). The mean patients' age was 1.6 years old. Of all analyzed samples, 101/197 (51%) and 119/187 (64%) samples were identified positive for at least one virus screened by the real-time PCR or real-time RT-PCR assays. The proportion of samples that contained more than one viral target was 13% and 28%, for 2016 and 2017, respectively (average of 21% for the total number of tested samples). This led to a total of 133 and 182 distinct viruses identified in 2016 and 2017, respectively. In 2016, adenovirus was the most prevalent (47/197, 23.9%), followed by rotavirus and sapovirus (23/197 each, 11.7%), novel HAstVs (22/197, 11.2%), and classical HAstVs (15/197, 7.6%). In 2017, adenovirus remained the most prevalent (56/187, 29.9%), followed by rotavirus and classical HAstVs (28/187 each, 15%), sapovirus (27/187, 14.4%), norovirus GII (22/187, 11.8%), and novel HAstVs (17/187, 9.1%). Norovirus GI was found in only 4/187 samples (2.1%).

3.2. Diverse Novel HAstVs are Circulating in the Pa]ediatric Population, often in Children under 2 Years Old and in Co-Infection with other Enteric Viruses

Prevalences of novel HAstVs were 11.2% (5.6% of HAstV-MLB and 5.6% of HAstV-VA) and 9.1% (3.2% of HAstV-MLB and 5.9% of HAstV-VA) in 2016 and 2017, respectively. Among HAstV-MLB and HAstV-VA, we found 10/3 MLB1 and 1/3 MLB2, 5/6 VA1, 3/2 VA2, 5/3 VA3, and 1/0 VA4 in 2016/2017, respectively. Of note, two samples were positive for several HAstVs-VA in 2016 (one double co-infection VA1-VA2 and one triple co-infection VA1-VA2-VA3), leading to a total number of 25 novel HAstVs identified in 2016; nevertheless, these samples were considered as mono-infected in the rest of the study.

According to patients' age, 84% (184/220) positive samples for any virus were identified among children 0–3 years old (Figure 1A). Novel HAstV prevalence was highest in children under 2 years old (30/39 positive samples, 77%; $p = 0.01$) (Figure 1B). For all viral targets except for NoV GI, for which the number of positive samples was low, the age group with a higher positivity rate was the group of one-year old children. For rotavirus, classic HAstV, and novel HAstV, this group was followed by children younger than one year of age. For adenovirus, sapovirus, and norovirus GII, the second age

group most affected was children 2 years-old. These same viral targets were also the ones occasionally isolated in children 5 years of age.

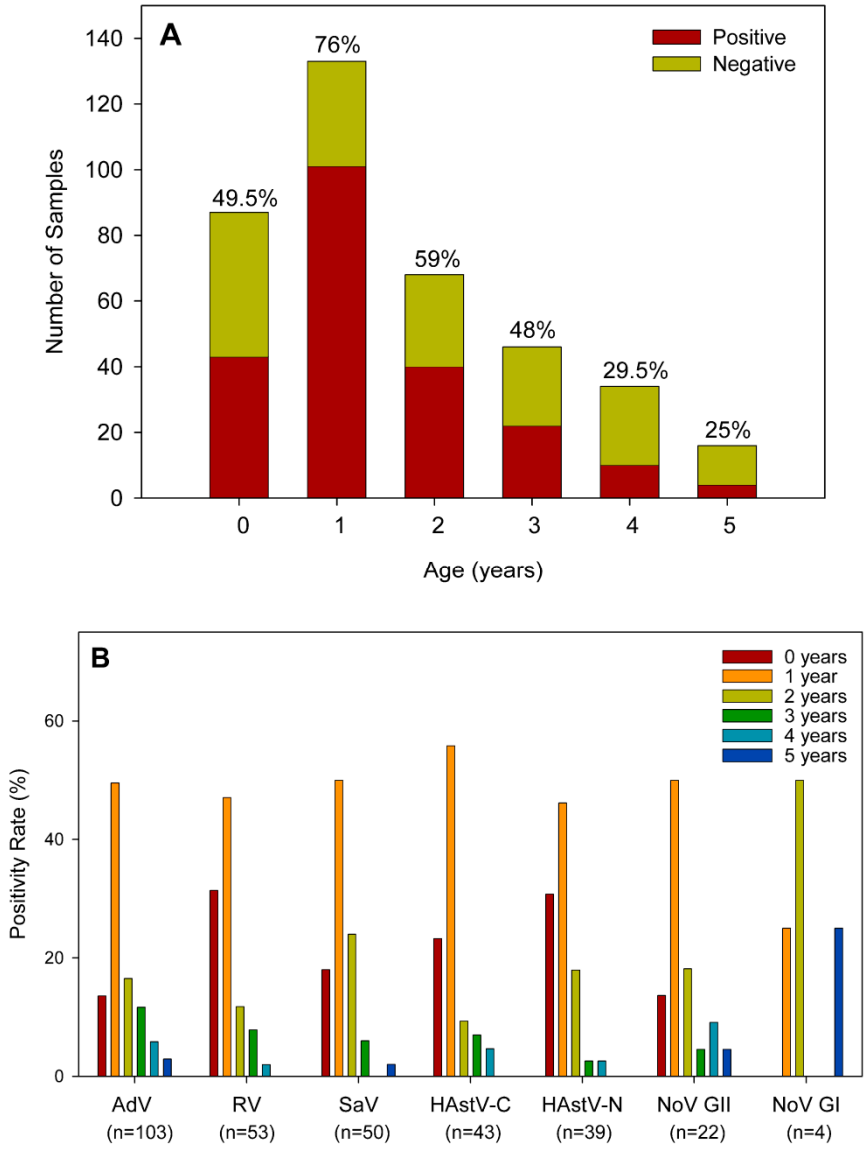

Figure 1. (**A**) Distribution of positive samples for any viral target by age groups. Percentages above each bar indicate the positivity rate in each group category. (**B**) Percentage distribution of positive samples by age group for each viral target. The number in brackets indicates the total number of positive samples for each virus. AdV: adenovirus; RV: rotavirus; SaV: sapovirus; HAstV-C: classic human astrovirus; HAstV-N: novel human astrovirus; NoV: norovirus: GI: genogroup I; GII: genogroup II.

As for most viruses, novel HAstVs were frequently found in co-infection: 76.4% of HAstV-MLB and 54.5% of HAstV-VA positive samples (i.e., 66% of novel HAstV) were also positive for other enteric viruses. Globally, we identified a higher rate of co-infection during 2017 than during the 2016 study period (62.6% vs 42.3%, respectively). In comparison, co-infection was found in 48.5% of adenovirus, 49% of rotavirus, 56% of sapovirus, 58% of classical HAstVs, 63.6% of norovirus GII, and 50% of norovirus GI cases. Figure 2 shows the number of samples and proportion of mono- and co-infections identified per virus for each year analyzed. Overall, HAstV-MLB was identified in co-infection with adenovirus (n = 8), rotavirus (n = 5), sapovirus (n = 4), and classical HAstV (n = 1). HAstV-VA was identified in co-infection with classical HAstVs (n = 6), adenovirus (n = 5), norovirus GII (n = 2), and rotavirus (n = 1). HAstV-MLB and HAstV-VA were found in triple co-infection in three and two

samples, respectively, and HAstV-MLB was also found in one quadruple co-infection (Table S1). There was no case of co-infections between HAstV-MLB and HAstV-VA genotypes.

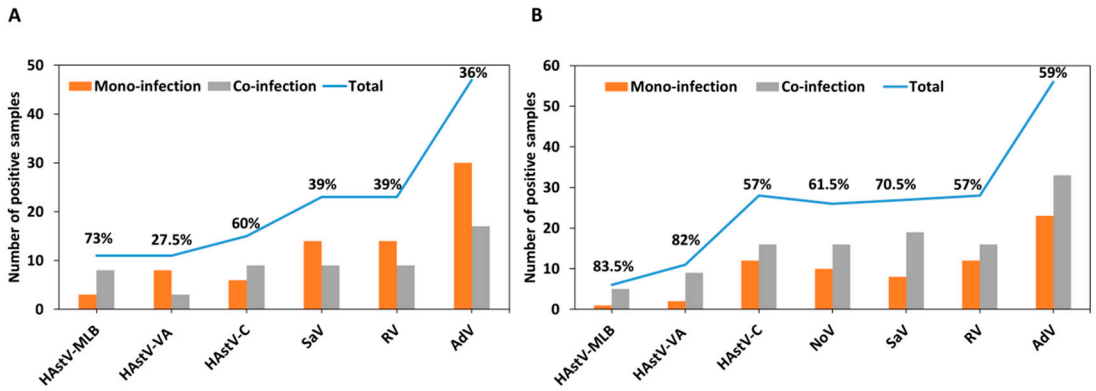

Figure 2. Number of cases identified in mono- and co-infections, respectively, for each virus in 2016 (**A**) and 2017 (**B**). Percentages indicate the proportion of co-infections. HAstV-MLB: MLB human astrovirus; HAstV-VA: VA human astrovirus HAstV-C: classic human astrovirus; NoV: norovirus: GI: genogroup I; GII: genogroup II; SaV: sapovirus; RV: rotavirus; AdV: adenovirus.

Mean global Cq values for novel HAstVs were 30.7 ± 6.7 for HAstV-MLB and 31.7 ± 6.4 for HAstV-VA. Figure 3 shows the median Cq values and confidence intervals for novel astroviruses (Figure 3A) and other enteric viruses (Figure 3B) during mono- and co-infections, respectively. There was no significant statistical difference in the Cq value during co-infection compared to mono-infection for any virus, except for classical HAstVs ($p = 0.042$ by Pearson Chi2 test with continuity correction).

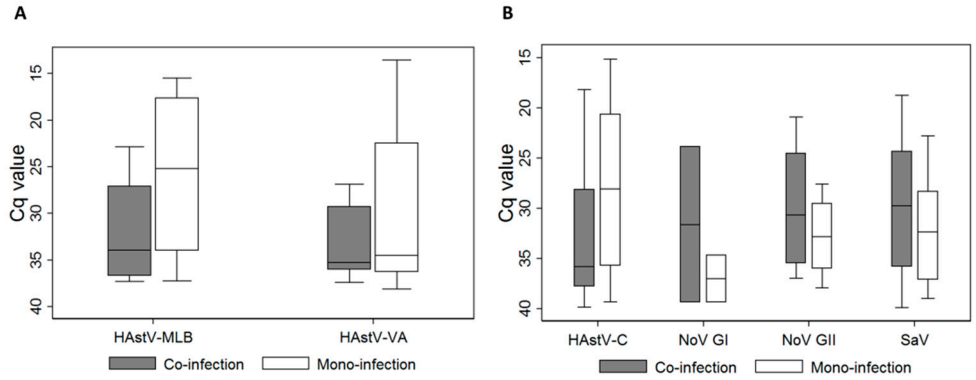

Figure 3. Median Cq values and confidence intervals for novel astroviruses (**A**) and other enteric viruses (**B**) according to mono- or co-infection status. Rotavirus and adenovirus were excluded from the analysis, as samples positive for these viruses by immunochromatography were excluded from the study. There was a statistical difference when comparing the Cq value during co-infection and mono-infection for classical HAstVs ($p = 0.042$ by Pearson chi2 test with continuity correction). HAstV-MLB: MLB human astrovirus; HAstV-VA: VA human astrovirus HAstV-C: classic human astrovirus; NoV: norovirus: GI: genogroup I; GII: genogroup II; SaV: sapovirus.

4. Discussion

In the present study we demonstrated that viral sequences belonging to one or several viruses could be detected in 50%–65% of cases of gastroenteritis of unknown etiology from an outpatient pediatric population, when screened by sensitive molecular assays. These results suggest that the wide use of molecular assays on a routine basis might reduce the proportion of undiagnosed specimens from children with gastroenteritis, which is currently ~75%, down to ~30%.

While most epidemiological studies on enteric viruses in children identified rotavirus as the first viral cause of acute sporadic gastroenteritis [24–28], we found a higher positivity rate for adenovirus, which could be biased on the fact that positive samples for rotavirus and adenovirus by immunochromatography were excluded from the analyses. Yet, two other recent studies also identified higher prevalence of adenovirus than rotavirus infection among a cohort of children with gastroenteritis using real-time PCR assays [29,30], including one study that focused on the post rotavirus vaccine era [30]. In addition, the possibility that the real-time PCR kit used for adenoviruses detects as well other serotypes different from 40 and 41 with a lower efficiency cannot be completely ruled out. As for rotavirus, despite that the prevalence reported in this study (11.7% in 2016 and 15% in 2017) does not include samples that were positive by immunochromatographic tests, it is still remarkable, and could be partially explained by the fact that the rotavirus vaccination coverage in Spain varies between 17%–50%, depending on the year and the area studied [31].

We included the screening for norovirus only during the 2017 study period. We found that norovirus accounted for 14% of the virus-positive cases; similar to what was recently described by Martinez Ascona et al. [26]. Norovirus screening contributed to an increase of 5% (10/187) of samples with a microbiological diagnosis. The proportion of samples that remained undiagnosed decreased from 48.7% in 2016 down to 36.4% in 2017. Genogroup II was predominant, accounting for 85% of identified norovirus, as frequently described in sporadic cases of gastroenteritis [24,25,32].

While most epidemiological studies on classical HAstVs found a 2%–10% positivity rate during acute gastroenteritis [4,13,33], we found an overall prevalence of 11.5%. An 18% positivity rate was also identified by real-time RT-PCR methods in an Italian study [34], and up to 35% in a large cohort of children among three continents [35]. In accordance with the fluctuation for classic HAstV infections according to the year of sampling reported by several studies [3], the observed prevalence for classic HAstV in our study varied between 7.6% in 2016 and 15% in 2017.

Finally, in addition to the most common well-characterized enteric viruses implicated in viral gastroenteritis, we identified for the first time in Spain, a significant positivity rate for novel astroviruses, with a prevalence of 11.2% in 2016 and 9.1% in 2017. Our overall 10% prevalence is by far one of the highest described to date. While other studies found a positivity rate between 0%–6% [4,11–14], a rate similar to ours was found only in Japan [15]. Like in other recent studies [11,12,15], we found HAstV-MLB1 to be the most prevalent one. We detected no HAstV-MLB3 genotypes; interestingly, this genotype was strongly associated with asymptomatic infections in a previous study [9]. Our overall high positivity can be explained by the highly sensitive real-time RT-PCR assay we employed [36], and also by the strict sample selection criteria: absence of a bacterial cause, symptomatic children ≤5 years old, outpatients and winter season. While the winter season is usually associated with peaks of acute viral gastroenteritis [25,26,37], we cannot exclude that novel HAstV may have a different (or no) seasonal pattern. Yet, in Germany, two peaks of HAstV detection were observed, between February and May and between September and December [12], while in Thailand, the pattern of seasonal distribution varied from one year to another [11].

For all viral targets included in the study, the proportion of samples presenting co-infections was high, ranging between 27.5% and 83.5%. A similar high rate of co-infections during viral gastroenteritis was also found in other studies using real-time RT-PCR techniques [34], including classical HAstVs (77%–80%) [34,35]. Khamrin et al. also found overall 46% of co-infections among novel HAstV positive cases [15]. This highlights that viral detection should be interpreted with caution in the era of highly-sensitive molecular diagnostic tests (which detect viral sequences and not antigens). When assessing whether viral load, estimated by the Cq value, could correlate with occurrence of mono- or co-infections, we only found a statistical difference in the mean Cq value for the classical HAstVs. This difference was not statistically significant for the MLB or VA HAstVs, but it is possible that the sample size of the novel HAstVs was too small. Yet, even in the presence of a true viral co-infection, the question is not as simple as to determine which pathogen is responsible for the disease, but what is the respective contribution of each pathogen identified; specific interactions during viral

co-infections should be further investigated by basic virological studies. Of note, for instance, using an immunodeficient mouse model, astroviruses have been recently shown to be elements of the virome, which can protect mice against murine norovirus and rotavirus infections [38].

In conclusion, our study provides additional information on novel HAstVs epidemiology in Europe. As in Germany, UK, and Switzerland, novel HAstVs are circulating in Spain among children with symptoms of gastroenteritis. In light of the high rate of co-infections, it remains to be determined whether novel HAstVs are actual etiologic agents of acute gastroenteritis or only bystanders and/or helpers versus controllers of other pathogens. Yet, our findings demonstrating the presence of one or more viral agents in more than half of undiagnosed gastroenteritis by routine diagnostics in children not only have clinical implications, such as the possible unnecessary use of antibiotics, but also give clues for future research studies on viral interactions and their respective contribution to gastroenteritis symptoms.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4915/11/7/585/s1, Table S1: Number of co- infections identified for novel HAstVs.

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Article III: Lack of association between novel astrovirus and diarrheic children and higher viral titre among asymptomatic controls

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Although the circulation of novel HAstV has been described on each continent and several countries, the causality role of novel HAstV during episodes of gastroenteritis remains non-elucidated. Yet, two case-control studies have contradictory results (26, 27).

The aim of the present study was to define the causality role of novel HAstV during episodes of gastroenteritis in children and, in the case of asymptomatic infections, assess potential risk factors for infection.

We included 363 stool samples of children ≤ 5 years old with symptoms of gastroenteritis (case study population) and 199 stool samples of children ≤ 5 years old visiting a primary health care center for their routine control visits (control study population). The study period covered a one-year period from June 2017 to May 2018. Clinical information included the date of stool collection and patient's age. In the control population, additional clinical and demographic data, corresponding to potential risk factors for being asymptomatically infected were recorded. All samples were tested for the presence of novel HAstV by specific real-time RT-PCR assays. Stool samples of the control population were additionally screened for the presence of adenovirus, norovirus GI and GII, rotavirus, sapovirus and classic HAstV by real-time (RT-) PCR assays commercial kits.

Among the case study population, 23/363 stool samples were positive for a novel HAstV (6.3%), compared to 8/199 stool samples of the control study population (4%). HAstV-MLB1 was the most frequently observed (20/31, 64.5%). The median log_{10} viral RNA copy number/ml of fecal suspension was 2.35 (IQR 2.13-3.76) for HAstV-MLB and 3.23 (IQR 2.87-8.86) for HAstV-VA in the case population, compared to 6.52 (IQR 4.52-6.84) for HAstV-MLB and 2.70 (IQR 1.73-9.05) for VA HAstV in the control population. Overall, there was no statistically significant difference in the prevalence of novel HAstVs between the case and the control population (OR 1.78, 95% CI 0.68-5.45; p=0.30). When analyzing HAstV-MLB and HAstV-VA separately, the difference remained non-significant (HAstV-MLB: OR 2.46, 95% CI 0.80-9.98; p=0.12. HAstV-VA: OR 0.90, 95% CI 0.13-10.07; p=1.00), as well as when only HAstV-MLB1, the most prevalent, was considered (OR 2.47, 95% CI 0.69-13.42; p=0.20). Nevertheless, there was a statistically significant difference in the HAstV-MLB median log_{10} viral RNA copy number/ml of fecal suspension between the case (2.35, IQR 2.13-3.76) and the control population (6.52, IQR 4.52-6.84) even after adjusting for potential confounding factors such as the patient's age and the date of stool collection (p=0.008 by multivariable analysis). Interestingly, the control population had a higher median HAstV-MLB viral titre than the case population.

Within the case population, we included 9.6% of stools with a positive coproculture. We observed that there was no difference in the prevalence of novel HAstV between the coproculture-positive and coproculture-negative patients (p=0.48), but the median log_{10} viral RNA copy number/ml of fecal suspension was statistically higher in coproculture-positive patients (5.19, IQR 4.24-6.22) than in coproculture-negative patients (2.31, IQR 2.11-3.32) (p=0.02). We observed a seasonality with a higher positivity rate during the winter seasons (January-April, p=0.015), but there was no difference in the positivity rate

according to age (p=0.66). Within the control population, 26.1% of samples were positive for adenovirus, 11% for norovirus (including 77.3% of GII), 8.5% for rotavirus, 5% for sapovirus and 3.5% for the classic HAstV. Co-infection with another enteric virus was found in 5 out of 8 novel HAstV-positive samples (62.5%).

In the control population, we did not find any factor predicting the risk for novel HAstV infection, including month of stool collection (p=0.95), patient's age (p=0.06) or sex (p=0.75), the presence of a chronic pathology (p=1.0), breastfeeding (p=1.0), rotavirus complete vaccination (p=0.14), antibiotics (p=0.14) or probiotics (p=1.0) use in the preceding month, the presence of fever or respiratory symptoms (p=0.72) in the preceding 2 weeks, the presence of siblings (p=0.27), school or daycare center attendance (p=1.0), exposition to domestic pets (p=1.0), or the presence of any other enteric virus (p=0.73).

In conclusion, our results show that there is no difference in the positivity rate of novel HAstV between symptomatic and asymptomatic children, suggesting that novel HAstV are not necessarily involved in gastroenteritis pathogenesis. Surprisingly, novel HAstV viral titres were higher in asymptomatic compared to symptomatic children and in coproculture-positive symptomatic children compared to coproculture-negative symptomatic children. All in all, these results should raise our attention on the potential role of novel HAstV as element of the gut virome and as potential modulator of the intestinal homeostasis.

Lack of association between novel astrovirus and diarrheic children and higher viral titre among asymptomatic controls

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Abstract

Novel human astroviruses (HAstV) were discovered 10 years ago and have been associated with fatal cases of central nervous system infections. Their role in gastroenteritis is controversial, as they have been identified in both symptomatic and asymptomatic patients. The aim of the study was to investigate novel HAstV in a gastroenteritis case-control study including a pediatric population in Spain over a one-year period. We included stool samples from patients with gastroenteritis and negative results for viruses screened by routine diagnostics, as well as stool samples of control patients who sought for a routine medical consultation in the first years of age. All samples were screened by real-time RT-PCR assays for the presence of novel HAstV. An additional screening for rotavirus, norovirus GI, GII, sapovirus, classic HAstV and adenovirus was performed for the control group as well. Overall, 23/363 stool samples from case patients (6.3%) and 8/199 stool samples from control patients (4%) were positive for ≥ 1 novel HAstV. MLB1 was predominant accounting for 64.5% of positive cases. A seasonality was observed for the case group (p=0.015), but not the control group (p=0.95). There was no statistically significant differences in the prevalence of novel HAstV between the case and control study groups (OR 1.78, 95% CI 0.68-5.45; p=0.30). Nevertheless, MLB genome copy number/ml of fecal suspension was significantly higher in the control group than in the case group (p=0.008 by multivariable analysis). In conclusion, our study identified a lack of association between novel HAstV and gastroenteritis in children, which could indicate a potential role of reservoir for children, especially given the higher viral load observed in the asymptomatic group.

Introduction

Human astrovirus (HAstV) classically cause acute mild to moderate diarrhoeal illness in young children and the elderly (15). In immunocompromised hosts, they can cause chronic infections (33, 37) and disseminated disease with severe complications (34). In the last years, several cases of central nervous system infections have been associated with HAstV, mainly the novel clades HMO-VA and MLB (175-181), which were identified ten years ago (157). The emergence of these novel clades, which are genetically very distant from the classical HAstV (4) and are associated with unexpected clinical presentation in humans and also various animal species (53, 60, 64), raises the question of their origin and the physiopathology of central nervous system infections. Interestingly, a recent study reported the retrospective identification of an astrovirus strain belonging to the HMO-VA clade on a brain biopsy sample of a muskox who died of suppurative encephalitis in 1982 (94). Another study suggested a common ancestor between the MLB clade and astrovirus identified in rats (69). These data suggest a cross-species transmission (61, 97), which could have occurred long time ago. Sequences of novel HAstVs have been found in human stools of individuals with diarrhoea (157, 161, 213, 214), but also in asymptomatic healthy controls (26, 27), raising the question of their causality role in gastroenteritis. Unfortunately, the two latter reports have contradictory results in their case-control study results, especially for MLB1, which so far is the most common.

The aims of the study were to compare the prevalence of novel HAstV in a pediatric population with and without symptoms of gastroenteritis in Spain and to determine the risk factors for being asymptomatically infected.

Material and Methods

Patient and sample population

The case study group consisted of children ≤ 5 years old attending outpatient clinics for symptoms of gastroenteritis. Stool samples were analyzed for routine diagnostics at the Microbiology Laboratory of Hospital de Vall d'Hebron, Barcelona (Spain). No clinical data were available except the patients' age and the date of stool collection. Stool samples positive for parasites, classic HAstV, rotavirus or adenovirus by routine diagnostics, including immunochromatographic tests for viruses, were excluded. We decided not to exclude bacteria, according to evidence for transkingdom interactions between astrovirus and intestinal bacteria (215). A total of 363 stool samples were randomly selected between August 2017 and May 2018. The study was conducted in accordance with the Declaration of Helsinki, and was approved by the Clinical Research Ethic Committee of the Hospital Universitari Vall d'Hebron (PR(AG)32/2016; February 2016).

The control study group consisted in children of ≤ 5 years old visiting the primary health care center El Serral, Sant Vicenç dels Horts, Barcelona (Spain) for their routine control visits during the first years of age. Patients were included in the study if they did not show symptoms of gastroenteritis such as diarrhoea or vomiting during the preceding 4 weeks before inclusion. The following additional clinical and demographic data were collected: age, sex, date of sample collection, any chronic pathology, breastfeeding history, rotavirus vaccination status, antibiotic or probiotic use in the preceding month, fever and/or respiratory symptoms in the preceding 2 weeks, siblings, school or daycare center attendance, and domestic pet exposition. Parents or legal representatives of all patients signed an informed consent before being included in the present study and the study was

approved by the Clinical Research Ethic Committee of the Institut Universitari d'Investigació en Atenció Primària Jordi Gol (P17/095; May 2017).

Viral RNA extraction and real-time reverse-transcription polymerase chain reaction (real-time RT-PCR) assays for novel HAstV and other enteric viruses

All samples were screened for the presence of novel HAstVs. RNA was extracted from 200 µl of a 30% stool suspension using the Viasure RNA-DNA extraction kit (Certest Biotec) following the manufacturer's instructions. Real-time RT-PCR specific assays for MLB HAstV and VA HAstV were performed in duplex as previously described (182, 216), using the Kapa Probe Fast Universal One-Step real-time RT-PCR Master Mix (Kapa Biosystems) and following the manufacturer's instructions, on a Stratagene Mx3000P (Thermofischer) and a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). The standard curves for quantification were constructed based on 10-fold serial dilutions of plasmids containing a fragment of the corresponding genome strains (MLB1: nucleotides 4292-4416 from FJ222451; MLB2: nucleotides 3724-3848 from KT224358; VA1: nucleotides 4629-4706 from FJ973620.1; VA2: nucleotides 4480-4558 from GQ502193.2; VA3: nucleotides 3678-3756 from JX857868.1; VA4: nucleotides 4646-4721 from JX857869.1).

The presence of other enteric viruses in stool samples of the control population were also assayed by real-time RT-PCR assays for classical HAstVs, sapovirus, rotavirus, adenovirus, and noroviruses GI and GII, using commercial kits (VIASURE Astrovirus Real Time PCR Detection Kit, VIASURE Sapovirus Real Time PCR Detection Kit, VIASURE Adenovirus Real Time PCR Detection Kit, VIASURE Norovirus GI Real Time PCR Detection Kit, VIASURE Norovirus GII Real Time PCR Detection Kit, and VIASURE Rotavirus Real Time PCR Detection Kit from Certest Biotec).

Statistical analyses

The Mann-Whitney and Kruskal Wallis tests were used to compare continuous variables and Fischer exact test or Chi2 test were used to compare categorical variables. Linear and logistic regression analyses were performed to adjust for confounding variables. P<0.05 was considered statistically significant. Statistics were performed by Stata /IC 13.1 (StataCorp, College Station, TX, USA).

Results

Case patients

Among the 363 stool samples analyzed from children with acute gastroenteritis between August 2017 and May 2018, 23 were positive for a novel HAstV (6.3%), including 21 MLB (16 MLB1, 5 MLB2-3) and 4 VA (1 VA1, 1 VA2, 2 VA3). Two patients had a double infection (MLB2+VA1 and MLB1+VA3). Median $\log_{10}$ RNA copy number/ml of fecal suspension for MLB HAstV was 2.35 (IQR 2.13-3.76) and for VA HAstV was 3.23 (IQR 2.87-8.86). Thirty-five out of 363 (9.6%) patients had a concomitant positive coproculture. There was no statistically significant difference in the prevalence of novel HAstV between patients with and without a positive coproculture (p=0.48), but the median $\log_{10}$ RNA copy number/ml of fecal suspension was statistically higher in patients with a positive coproculture (5.19, IQR 4.24-6.22) than in those with a negative coproculture (2.31, IQR 2.11-3.32) (p=0.02). The prevalence of novel HAstV differed significantly between month (p=0.015) (Figure 1A), while there was no difference according to age (p=0.92), or age group categorized as ≤1 year old versus > 1 year old (p=0.66) (Figure 1B).

Control patients

A total of 199 children were included between June 2017 and May 2018. Eight were positive for a novel HAstV (4%), including 5 MLB (4 MLB1, 1 MLB2-3) and 3 VA (1 VA1, 1 VA2, 1 VA3). There were no co-infections observed with several novel HAstV. Median log_{10} RNA copy number/ml of fecal suspension for MLB HAstV was 6.52 (IQR 4.52-6.84) and for VA HAstV was 2.70 (IQR 1.73-9.05). The prevalence of novel HAstV did not significantly differ between month (p=0.95) (Figure 1C), nor between age (p=0.06) (Figure 1D), sex (p=0.75), the presence of a chronic pathology (p=1.0), breastfeeding (p=1.0), rotavirus complete vaccination (p=0.14), antibiotics (p=0.14) or probiotics (p=1.0) use in the preceding month, the presence of fever or respiratory symptoms (p=0.72) in the preceding 2 weeks, the presence of siblings (p=0.27), school or daycare center attendance (p=1.0), exposition to domestic pets (p=1.0), or the presence of any other enteric virus (p=0.73) (Table 1).

Control children were also screened for other common enteric viruses using qualitative commercial assays and viral titres were assessed by using the Cq values. We found 52 adenovirus (26.1%; median Cq value 35.5, IQR 32.5-39.2), 22 norovirus (11%; median Cq value 35, IQR 30.5-38.1), including 5 GI and 17 GII, 17 rotavirus (8.5%; median Cq value 32.7, IQR 30.1-37.3), 10 sapovirus (5%; median Cq value 30.7, IQR 26.3-34.1), and 7 classic HAstV (3.5%; median Cq value 37.2, IQR 31.6-38.2) (Figure 1C). Co-infections were encountered in 5 out of 8 novel HAstV-positive patients with the following viruses: norovirus GI (n=1), classic HAstV (n=1), rotavirus (n=1), adenovirus (n=3). One of these patients had co-infection with 3 viruses (VA3, norovirus GI and adenovirus). There was no statistically significant difference of Cq values between virus groups (p=0.07). The

prevalence of other enteric viruses did not significantly differ between month (adenovirus p=0.14; norovirus p=0.15; rotavirus p=0.59; sapovirus p=0.10; classic HAstV p=0.68). According to other potential risk factors analyzed, we found that breastfeeding was protective for adenovirus infection (p=0.004) and rotavirus vaccination was a risk factor for norovirus infection (OR 5.19, CI 1.69-18.89; p=0.002). Children older than one year old and school attendance were found to be protective for rotavirus infection, but after logistic regression, only age remained statistically significant (p=0.02) (Table 1).

Comparison between case and control groups

Globally there was no statistically significant difference in the prevalence of novel HAstV between the case and the control population (OR 1.78, 95% CI 0.68-5.45; p=0.30) (Table 2). By analyzing MLB and VA clade separately, we also found no differences between case and control patients (MLB: OR 2.46, 95% CI 0.80-9.98; p=0.12. VA: OR 0.90, 95% CI 0.13-10.07; p=1.00), and the same was true when only MLB1, the most frequently detected, was considered (OR 2.47, 95% CI 0.69-13.42; p=0.20). Nevertheless, while the $\log_{10}$ RNA copy number/ml of fecal suspension did not significantly differ between case and control patients (p=0.07), it was the case for MLB when analyzing both clades separately (p=0.008) (Table 1). Unexpectedly, $\log_{10}$ RNA copy number/ml of fecal suspension were higher in the control subjects than the case subjects. Using logistic and linear regression to adjust novel HAstV positivity rate and MLB $\log_{10}$ RNA copy number/ml of fecal suspension obtained in case versus control population for confounding factors such as astrovirus clade, the month of sample collection and patient's age (Table 1), the difference in the positivity rate remained non-significant (p=0.14), while the MLB $\log_{10}$ RNA copy number difference remained statistically significant (p=0.008).

Discussion

In the present study, we demonstrated that novel HAstVs belonging to MLB and VA clades may equally be identified in symptomatic and asymptomatic children. This important finding argues in favor of the potential for children to act as reservoir for transmission of novel HAstV, which is of most public health concern, regarding central nervous system complications that can occur, mostly in immunocompromised patients (4).

We found that 6.3% of case patients were positive for one or several novel HAstV. This is slightly lower than our previous study, which investigated a similar Spanish pediatric population, where 10% of symptomatic patients were positive for any novel HAstV (216). However, this difference can be explained by the study period: our first study focused on two consecutive winter seasons from January-April 2016 and January-April 2017, respectively, while the present one extended over a 10-months period (August 2017-May 2018). Yet, we could demonstrate that there was a statistically significant difference in novel HAstV prevalence according to the month of sample collection, with January-April months being the ones with the highest detection rate. This seasonality of novel HAstV is in agreement with the study of Jacobsen *et al.* (163), although the latter focused on both novel and classic HAstV.

The novel HAstV positivity rate among control patients was 4%. This is comparable to the Indian cohort of Holtz *et al.* (27) and the Gambian cohort of Meyer *et al.* (26). We could not identify factors predicting the asymptomatic carriage of novel HAstV in children. Nevertheless, we demonstrated that asymptomatic children can harbor a high variety of eukaryotic viruses with adenovirus and norovirus being the most frequently identified. In a case-control study screening the presence of several enteric pathogens by conventional

PCR assays, the global positivity rates for norovirus, rotavirus A and sapovirus in control patients were 16%, 14%, and 2%, respectively (217), which is in accordance with our results. Rotavirus is classically associated with severe gastroenteritis in infants (218), but asymptomatic infections can occur (219). Detection of rotavirus in asymptomatic children can reflect an infection during the period of maternal antibodies protection or reinfection with a previously mounted immune response allowing control of the reinfection (219). Additionally, there could be a lower susceptibility for rotavirus infection associated to the genetic variability of the histo-blood group antigen (HBGA) cell receptor (219). Surprisingly, we found no association between rotavirus asymptomatic infection and vaccination status, but this could be due to the small number of rotavirus events. After logistic regression, only children between 0 and 1 year old were significantly at increased risk for rotavirus infection, which is consistent with the lack of immunity in this population group (218). As for norovirus, asymptomatic shedding in children has been reported to be in the range from <1% to >30% in different European and non-European countries (220-224). The higher prevalence of norovirus in patients vaccinated for rotavirus could be explained by a shift in the type of viral infection as has been observed in symptomatic patients in certain countries where rotavirus vaccination has been implemented (225). We found that breastfeeding was only protective for adenovirus infections, which could also be due to the small number of infections by other viruses.

No difference in the prevalence of novel HAstV between the case and the control patients could be observed. While Holtz *et al*. did not find any association between MLB1 infection and diarrhoea (27), Meyer *et al*. reported an association between MLB1 and diarrhoea

and between MLB3 and asymptomatic disease, and no significant association of VA viruses with any of the groups (26, 27). The results of the present study are also in agreement with the high rate of co-infections observed in our previous study: MLB and VA were identified in co-infection with ≥ 1 other enteric virus in 78% and 54.8% of cases (all symptomatic), respectively, raising the question of their contribution to the digestive symptoms (216). In this previous study, we could not observe a difference in the novel HAstV mean RNA copy number during mono- and co-infection. In the present study, the RNA copy number/ml of fecal suspension significantly differed between both groups, particularly for the MLB clade, and was unexpectedly higher in asymptomatic infections than during episodes of gastroenteritis. This finding is interesting and points out to a potential mechanism of protection towards other pathogenic enteric viral infections: the presence of MLB HAstV at higher viral load in asymptomatic patients could infer a protection due to viral interference (226, 227). Our hypothesis is supported by a recent study by Ingle *et al*., who demonstrated that a specific strain of murine HAstV was able to protect immunodeficient mice from infection by murine norovirus and rotavirus through high level of IFNλ 2/3, without inducing intestinal inflammation (211). In addition, novel astroviruses were identified at higher viral load in presence of a pathogenic bacteria, which is in favor of the possibility that some transkingdom interactions may be occurring, as suggested by others (215).

Our study has some limitations: we could not perform a full one-year screening of the case patients, which could have influenced our analysis of seasonality. In the control group, sample collection varied from one month to another probably due to the criteria of exclusion (gastroenteritis, age), the randomness of the pediatric healthy controls and holiday periods.

In conclusion, our study provides additional information on novel HAstV epidemiology among asymptomatic children and reinforces the hypothesis that these emerging viruses are not necessarily associated with gastrointestinal syndromes. The unexpected finding that viral load is higher among asymptomatic than symptomatic children and in presence of pathogenic bacteria should raise our attention on the potential role of novel HAstV in the intestinal homeostasis and drive further investigations to understand the mechanisms underlying severe complications.

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Authors' contribution

Conceptualization: S.G., D-L.V., A.B. and R.M.P; Data curation: D-L.V., A.S., K.M. and S.G.; Formal analysis: S.G. and D-L.V.; Funding acquisition: A.B., R.M.P., S.G. and D-L.V.; Investigation: D-L.V., A.S., N.A., K.M., J.S., V.R.G., L.G. and S.G.; Methodology: S.G. and D-L.V. Project administration: S.G. and D-L.V.; Resources: S.G., J.S., V.R.G. and L.G;

Supervision: S.G.; Validation: S.G.; Visualization: D-L.V., S.G., A.B. and R.M.P.; Writing original draft: D-L.V.; Writing review and editing: S.G., A.B. and R.M.P.

Conflicts of Interest:

None of the authors have any conflict of interest to declare. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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Table 1. Risk factors for asymptomatic infection by novel HAstVs and other enteric viruses in children (2017-2018)

Risk Factor	Virus Prevalence (%)	OR	95% CI	P value
Novel HAstV				
Age (0-1 year/2-6 years)	6.4 / 2.7	2.5	0.47-16.6	0.28
Gender (Boy/Girl)	4.7 / 3.4	1.37	0.26-9.08	0.73
Breastfeeding (Yes/No)	4.7 / 8.7	0.53	0.008-10.98	1.00
Chronic Pathology (Yes/No)	0.0 / 4.6	-	-	1.00
RV Vaccination (Yes/No)	6.3/ 1.9	3.55	0.56-37.94	0.14
Antibiotic Treatment (Yes/No)	12.5 / 3.4	4.02	0.36-25.12	0.14
Resp infection/Fever (Yes/No)	5.1 / 3.5	1.5	0.27-8.30	0.72
Siblings (Any/None)	4.5 / 3.4	1.33	0.23-13.90	1.00
School Attendance (Yes/No)	4.4 / 3.7	1.20	0.23-7.97	1.00
Domestic Animals (Yes/No)	3.9 / 4.3	0.92	0.16-5.08	1.00
Adenovirus				
Age (0-1 year/2-6 years)	19.2 / 30.4	0.54	0.25-1.14	0.09
Gender (Boy/Girl)	25.5 / 27.6	0.89	0.45-1.8	0.74
Breastfeeding (Yes/No)	0 / 34.8	-	-	**0.004**
Chronic Pathology (Yes/No)	25 / 27.2	0.89	0.15-3.79	1.00
RV Vaccination (Yes/No)	24.1 / 28.3	0.80	0.39-1.64	0.61
Antibiotic Treatment (Yes/No)	18.8 / 27	0.62	0.11-2.42	0.56
Resp infection/Fever (Yes/No)	24.7 / 27.8	0.85	0.41-1.72	0.74
Siblings (Any/None)	25.6 / 27.1	0.92	0.44-1.98	0.86
School Attendance (Yes/No)	30.4 / 19.8	1.77	0.86-3.75	0.13
Domestic Animals (Yes/No)	26.7 / 25	1.09	0.55-2.2	0.87
Norovirus				
Age (0-1 year/2-6 years)	13.2 / 10	1.36	0.49-3.76	0.64
Gender (Boy/Girl)	10.5 / 13.1	0.77	0.29-2.10	0.65
Breastfeeding (Yes/No)	9.5 / 19.1	0.45	0.04-3.65	0.66
Chronic Pathology (Yes/No)	25 / 9.5	3.16	0.49-14.37	0.12
RV Vaccination (Yes/No)	20.8 / 4.8	5.19	1.69-18.89	**0.002**
Antibiotic Treatment (Yes/No)	25 / 10	3	0.63-11.34	0.08
Resp infection/Fever (Yes/No)	14.5 / 9.8	1.55	0.57-4.19	0.36
Siblings (Any/None)	9.9 / 15.8	0.59	0.22-1.67	0.32
School Attendance (Yes/No)	11.7 / 11.4	1.03	0.38-2.89	1.00
Domestic Animals (Yes/No)	10.2 / 13.2	0.74	0.27-2.00	0.65
Rotavirus				
Age (0-1 year/2-6 years)	19.2 / 1.8	13.2	2.89-121.3	**<0.001**
Gender (Boy/Girl)	7.5 / 10.3	0.7	0.22-2.15	0.61
Breastfeeding (Yes/No)	38.1 / 17.4	2.92	0.61-15.8	0.18
Chronic Pathology (Yes/No)	8.3 / 9.2	0.89	0.02-6.9	1.00
RV Vaccination (Yes/No)	10.1 / 7.5	1.39	0.43-4.48	0.6
Antibiotic Treatment (Yes/No)	0 / 9.7	-	-	0.37
Resp infection/Fever (Yes/No)	3.8 / 12.2	0.29	0.05-1.09	0.07
Siblings (Any/None)	6.7 / 10.2	0.64	0.19-2.29	0.39

School Attendance (Yes/No)	2.7 / 17.3	0.13	0.02-0.5	**0.001**
Domestic Animals (Yes/No)	5.9 / 11.8	0.47	0.14-1.47	0.2
Sapovirus				
Age (0-1 year/2-6 years)	7.7 / 2.7	3.05	0.63-19.36	0.16
Gender (Boy/Girl)	5.6 / 3.4	1.66	0.34-10.6	0.7
Breastfeeding (Yes/No)	4.7 / 8.7	0.53	0.008-11.0	1.00
Chronic Pathology (Yes/No)	8.3 / 3.5	2.53	0.05-23.84	0.38
RV Vaccination (Yes/No)	6.3 / 3.7	1.74	0.36-9.05	0.49
Antibiotic Treatment (Yes/No)	0 / 5.1	-	-	1.00
Resp infection/Fever (Yes/No)	7.7 / 1.7	4.7	0.81-48.54	0.06
Siblings (Any/None)	4.5 / 5.1	0.88	0.18-5.6	1.00
School Attendance (Yes/No)	6.2 / 2.5	2.61	0.48-26.3	0.3
Domestic Animals (Yes/No)	3.9 / 5.4	0.73	0.14-3.49	0.7
Classic HAstV				
Age (0-1 year/2-6 years)	6.4 / 0.9	7.6	0.82-362	0.04
Gender (Boy/Girl)	4.7 / 1.2	4.2	0.45-199	0.2
Breastfeeding (Yes/No)	0 / 13.0	-	-	0.23
Chronic Pathology (Yes/No)	0 / 2.9	-	-	1.00
RV Vaccination (Yes/No)	6.4 / 0.9	7.3	0.78-346	0.08
Antibiotic Treatment (Yes/No)	6.3 / 2.3	2.83	0.05-30.9	0.4
Resp infection/Fever (Yes/No)	3.8 / 2.6	1.48	0.19-11.32	0.7
Siblings (Any/None)	3.0 / 1.7	1.8	0.17-90.1	1.00
School Attendance (Yes/No)	4.4 / 1.3	3.53	0.38-169	0.4
Domestic Animals (Yes/No)	3 / 3.2	0.93	0.12-7.12	1.00

Only complete vaccinated individuals were included in Yes for rotavirus vaccination.
Breastfeeding Yes includes partial and total breastfeeding of children under 1 year-old.
OR: odds ratio; CI: confidence interval; RV: rotavirus

Table 2. Characteristics of the case and control populations.

	Case (n=363)	Control (n=199)	P Value	OR (95% CI)
Sex (male), n (%)	210 (58)	107 (55.2)	0.5	
• **Missing value**	1	5		
Age, mean (SD)	1.37 (1.16)	2.21 (1.68)	**<0.001**	
• **Missing value**	1	8		
Number of samples collected according to month, n (%)			**<0.001**	
• **Jan-Feb**	86 (23.7)	36 (18.1)		
• **Mar-Apr**	80 (22.0)	37 (18.6)		
• **May-Jun**	40 (11.0)	50 (25.1)		
• **Jul-Aug**	39 (10.7)	20 (10.1)		
• **Sept-Oct**	61 (16.8)	15 (7.5)		
• **Nov-Dec**	57 (15.7)	31 (15.6)		
• **Missing value**	0 (0)	10 (5.0)		
Positive novel HAstV, n (%)*	23 (6.3)	6 (3.7)	0.30	1.78 (0.68-5.45)
• **Log_{10} RNA copies/ml, median (IQR)**	2.54 (2.18-3.76)	6.22 (2.81-7.66)	0.07	
Positive for MLB, n (%)*	21 (5.8)	4 (2.4)	0.12	2.45 (0.81-9.98)
• **Log_{10} RNA copies/ml, median (IQR)**	2.35 (2.13-3.76)	6.52 (4.52-6.84)	**0.008**	
Positive for VA, n (%)*	4 (1.1)	2 (1.2)	1.00	0.90 (0.13-10.07)
• **Log_{10} RNA copies/ml, median (IQR)**	3.23 (2.87-8.86)	2.70 (1.73-9.05)	0.47	

P value for comparison between case and control patients. * The prevalence calculated here included the period between August-May in order to appropriately compare case and control patients. RNA copies correspond to RNA copy number/ml of fecal suspension. OR: odds ratio; SD: standard deviation; IQR: interquartile range; Jan: January; Feb: February; Mar: March; Apr: April; Jun: June; Aug: August; Sept: September; Oct: October; Nov: November; Dec: December; HAstV: Human astrovirus.

Figure

Total number of samples collected per month and per study population

	Jun	Jul	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May
Case	0	0	39	38	23	11	46	46	40	40	40	40
Control	17	18	2	9	6	21	10	15	21	16	21	33

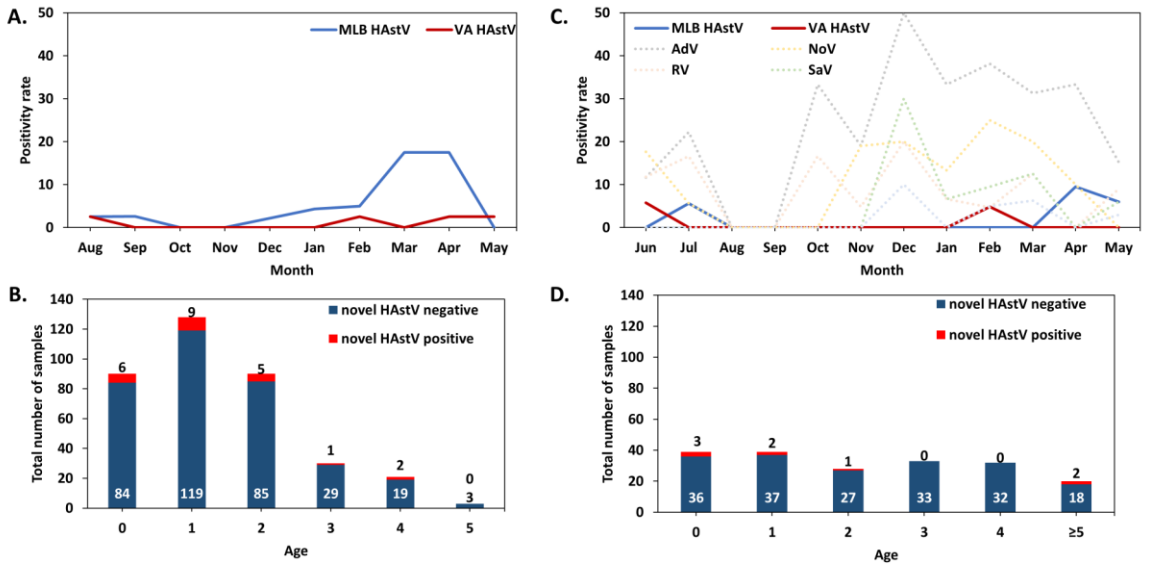

Figure 1. Prevalence of novel astrovirus and other viruses according to month of sampling and age groups.

Prevalence in the case population according to month of sampling **(A)** and age groups **(B)**. The table indicates the absolute number of samples collected per month and per study population. Prevalence in the control population according to month of sampling **(C)** and age groups **(D)**. Numbers above the bar correspond to the absolute number of positive samples for novel HAstVs, numbers at the bottom of the bar correspond to the absolute number of negative samples for novel HAstVs. Two patients in the control population had 6 years old, the screening for novel HAstVs was positive in one and negative in the other. HAstV: Human astrovirus; AdV: Adenovirus; NoV: Norovirus; RV: Rotavirus; SaV: Sapovirus.

Discussion

High throughput sequencing has revolutionized the field of clinical virology by identifying about 9000 novel viral sequences in less than 10 years (228). This expands our knowledge on virus genetics and variants circulating in humans, animals and plants, but also introduce doubts concerning the clinical significance of such identification: while a viral infection has traditionally been associated with a specific disease, highly-sensitive molecular diagnostic tools nowadays force us to redefine the dimensions of a viral infection and introduce the concept of commensal viruses (229). Nevertheless, a virus is considered as commensal until proven otherwise. Novel HAstV are emerging viruses that were identified by HTS a decade ago. Together with the Merkel polyomavirus (230), novel HAstV are one of those few emerging viruses that are associated with a clinical disease (228). Novel HAstV were identified in several cases of severe meningoencephalitis in immunocompromised patients. Although its causality role could not be firmly demonstrated so far by the classical Koch's postulates, many data point to a real neurotropism and pathogenicity for novel HAstV in meningoencephalitis (162). Nevertheless, our understanding of the origin, epidemiology, pathogenesis and clinical aspects of novel HAstV is still in its infancy.

In the first study, we developed a cell culture system allowing the propagation of HAstV-MLB1 and HAstV-MLB2, which represents an indispensable tool for increasing our knowledge about novel HAstV pathogenesis and their interaction profile with the host.

First, we demonstrated that the HAstV-MLB1 identified in stool sample of a diarrhoeal child (216) and the HAstV-MLB2 issued from the stool sample of an immunocompromised patients with possible central nervous system involvement (180) are both infectious. Yet, in the era of molecular diagnostics, the identification of virus genetic materials in

biological samples is sometimes difficult to interpret, contributing to the difficulty of disease association. By isolating both HAstV-MLB on cell culture, we demonstrated that these patients had a true infection. In addition, stool sample of the HAstV-MLB1 positive diarrhoeal child was also screened for other common enteric viruses (namely HAstV-VA, classic HAstV, rotavirus, sapovirus and adenovirus) and no co-infection was identified (216). In view of this and owing to the high viral titre detected, we can speculate that HAstV-MLB1 was responsible for the diarrhoeal disease. In the immunocompromised patient, HAstV-MLB2 was identified in several patient's plasma and CSF samples collected over one and three months, respectively (180). The patient had meningitis that was attributed to meningeal leukemic infiltration and was treated by intrathecal chemotherapy and cranial irradiation. He further developed novel neurological symptoms that were attributed to residual leukemic infiltration and died of disease relapse few months later. It is here difficult to infer an association between HAstV-MLB2 and the patient's neurologic disease, as there was no exacerbation of the neurologic symptoms during the intrathecal chemotherapy, which would be expected in case of infection. Nevertheless, due to the retrospective nature of the investigation, precise clinical evolution is missing.

We provided an *in vitro* cell culture system and HAstV-MLB stocks that are essential to initiate basic research to understand novel HAstV pathogenesis and biology. So, we completed the already available systems for the classic HAstV (118) and HAstV-VA1 (212) with a system for HAstV-MLB. Contrary to the two first, CaCo-2 cells were surprisingly not permissive for efficient HAstV-MLB propagation. We cannot exclude that some abortive replication cycles may have occurred, but according to the discrepant increase in viral titre observed on other infected cell lines, CaCo-2 cells were the worst cell line tested for

HAstV-MLB replication. A previous report showed that HuH-7.5.1 infection with HAstV-8 leads to the same yield of infectious virus production as CaCo-2 cells, although the increase in virus production in the supernatant is slightly delayed, compared to a similar increase in the intracellular fraction (231). The HuH-7.5 defect in the RIG-I pathway probably explains its high permissiveness for HAstV-MLB replication *in vitro* (232, 233). Except one study where HAstV-VA3 was identified in the serum of patients with acute undiagnosed hepatitis, there is no report of HAstV infection and liver disease. Yet, in this study, HAstV-VA3 was not searched in liver tissue (187). In addition to HuH-7 cell lines, A549 respiratory epithelial cells were also permissive for HAstV-MLB propagation, as was already described for the classic HAstV serotypes 1, 2 and 4 (118) and HAstV-VA1 (212). The clinical correlation of these findings is not clear, but it should be emphasized that classic HAstV, HAstV-MLB and HAstV-VA1 have been identified in pediatric respiratory samples of symptomatic children (38, 42, 185, 186). Thus, the evidence suggesting a respiratory tropism for all clades of HAstV keeps growing, and further systematic screening should be performed to address the role of HAstV in respiratory tract infection, if any.

Similar to the work of Janowski *et al.* on HAstV-VA1 (212), we found that HAstV-MLB does not require trypsin for initiating its replication cycle, as is observed for the classic HAstV (234): trypsin requirement for classic HAstV infectivity is related to conformational changes in the capsid proteins - VP70 needs to be cleaved in VP32-34 (capsid core), VP27-29 and VP24-26 (spikes) - allowing the configuration needed for virus infectivity probably through cell attachment and/or entry (115). Hence, it is tempting to speculate that novel HAstV requirements for cell binding/entry are distinct from those of classic HAstV. This could refer to a distinct cell receptor or to the capsid proteins, and additional

crystallography studies could elucidate the question. Knowing that the capsid protein plays several roles in the pathogenesis of astrovirus infection and host's immune response, notably in the mechanism of diarrhoea (137, 139), complement system evasion (141, 142) and neutralizing antibodies recognition (116, 117, 154), understanding its composition will be essential for assessing clinical disease and pathogenesis of novel HAstV.

Despite the use of a high MOI throughout our study, we only found a few proportion of cells infected after inoculation (5-10%). Interestingly, the recent work of Janowski *et al.*, where HAstV-VA1 was propagated in CaCo-2, astrocytes primary cells and the immortalized neuronal SK-N-SH cell line, also showed only 1.1% of CaCo-2 cells and <1% of primary astrocytes or SK-N-SH cells infected using a MOI of 3 (183). In opposite, Guix *et al*. found up to 27% of apoptotic cells after CaCo-2 cell infection with classic HAstV using a MOI of 5 (132). The few infected cells observed in our study is also discordant with the high virus titre detected both intracellularly and in the culture supernatant. A similar observation is described in cases of a *carrier-state* infection, which is characterized by a cytolytic infection that releases a high number of virus progeny, which in turn infect only a small proportion of surrounding cells at any time, maintaining the infection persistently in the culture (235-237). Increasing the concentration of antiviral antibody can normally cure such persistent infections. This description is in accordance with our results where we demonstrated the ability of HAstV-MLB to persist in a cell culture system that can be propagated for several passages, together with the release of high virus titre by a small fraction of infected cells that show significant cellular reorganization (as seen by electron microscopy). In our case, the addition of exogenous IFN could indeed totally or partially cure the persistent infection in HuH-7 cells. Disctinct receptor use (238) and formation of

defective virus particles (239) have been identified as mechanisms influencing persistency. It remains to be determined if some are applicable to HAstV-MLB. Of note, for Coxsackievirus B, it was demonstrated that distinct types of infection occur depending on the virus variant and the cell type, ranging from no infection to lytic and persistent infection (238). In our study, persistent infection was more efficient with HAstV-MLB1 compared to HAstV-MLB2, as measured by the higher mean viral titres observed on both cell lines and the incapacity of HAstV-MLB2 to establish a persistent infection in HuH-7.5. Accordingly, HAstV-MLB2 acute infection was also more efficient than HAstV-MLB1, suggesting that HAstV-MLB2 may induce a more potent host response to infection which could prevent the establishment of persistency. Yet, during acute infection, IFN-β mRNA was detected at higher level during HAstV-MLB2 compared to HAstV-MLB1 infection in A549 cells, which could support this hypothesis; but as no IFN mRNA was detected in HuH-7AI cells neither during HAstV-MLB1 nor during HAstV-MLB2 acute infection, the conditions required for initiating persistence may probably be strain and cell dependant. Once persistent infection is established, no IFN mRNA could be detected in any cell line and with any HAstV-MLB genotype, suggesting that IFN is a key factor required for persistence. Whatever the mechanism, if confirmed by further *in vivo* models, and according to the close relationship between asymptomatic and persistent infections (240, 241), this discovery could increase our knowledge on the pathogenesis of HAstV-MLB infection, where a persistent asymptomatic infection in any body compartment (digestive or respiratory tract, liver or central nervous system for instance), could “reactivate” in certain conditions and cause serious infections, as discussed. A recent MuAstV model also supported astrovirus persistence *in vivo:* by infecting wild-type mice with 2 distinct strains, one issued from a mice deficient in the type I IFN pathway (IFNAR-/-) and the second from

a mice lacking appropriate T cell response (CD1 nude), the authors observed distinct duration of infection, up to 70 days compared to 21 days, respectively. The authors concluded that “differences in host immune pressure could ultimately drive differences in the virus populations”. Of note, the infected mice were totally asymptomatic and no signs of inflammatory response were observed. More important, there was no mounted adaptive immune response after these persistent infections, as reinfection occurred few weeks after viral clearance (210).

Works on persistent murine norovirus (MuNoV) showed that gut bacteria enhance MuNoV persistence by limiting IFN-λ signalling (242), and that IFN-λ can prevent and cure an established MuNoV persistent infection without contribution of the adaptive immune response (243). A recent study using a MuAstV model showed that in turn, some MuAstV strains are associated with a higher level of IFN-λ 2/3 and a high expression of IFN-stimulated genes in the gut, without associated inflammation. Using a deficient mouse model lacking both innate and adaptive immune response (Rag-/-IL2rg-/-), authors demonstrated that acute infection with the MuAstV strain could also prevent MuNoV persistent infection and that this protective effect was associated with IFN-λ 2/3 (211). Our results demonstrate that persistent HAstV-MLB infection *in vitro* does NOT induce a strong type I and III IFN response, which could contribute to the establishment of HAstV-MLB persistence. Yet, a significant number of *in vitro* studies have shown that viruses have developed mechanisms of disruption of the IFN pathway to enhance their replication (244). More strikingly, despite the few infected cells, our IFN assays showed that this was sufficient for inhibiting IFN expression by the whole cell culture even after poly I:C transfection in A549 cells. This potent IFN inhibition had not been observed in classic HAstV-infected CaCo-2 cells (145).

If our results are confirmed *in vivo*, this suggests that HAstV-MLB persistent infection could help or enhance other viral infection by reducing the IFN response, which is the first-line response in any viral infections. Our experiment using exogenous IFN to cure the established persistent cell lines demonstrated that inhibition of IFN-β production could be a mechanism developed by HAstV-MLB to establish persistent infection in HuH-7 cells, as the latest was completely cured by IFN-β, but this may not be the case in A549 cells. Yet, exogenous IFN had no effect on HAstV-MLB replication in this cell line, suggesting that IFN inhibition is not the way by which HAstV-MLB establish persistence in A549 cells, unless the inhibition acts downstream of IFN induction. All in all, inhibition of IFN pathway could reveal to be strongest in A549 cells, as demonstrated by inhibition of IFN mRNA expression despite poy I:C stimulation and absence of effect of exogenous IFN on viral replication. As it was demonstrated that HAstV replication is dependent on the ubiquitin-proteasome system (129), and that ubiquitination regulates the innate immune response (245, 246), it would be interesting to investigate if the ubiquitin-proteasome system increases HAstV replication through inhibition of the innate immune response and whether this mechanism also enhances virus persistence.

Our epidemiological study showed that novel HAstV are quite prevalent in the pediatric Spanish population, with prevalences of 6-10% depending on the study period (articles II and III). We found the highest prevalence described to date, together with a Japanese study (168). Of note, we also performed a screening of 140 CSF from patients with undiagnosed meningoencephalitis, but none was positive for novel HAstV (data not shown). This confirms the relative rarity of the syndrome or points to an incorrect sampling used to identify HAstV central nervous system infection. Yet, as shown in Table

2, HAstV was rarely identified in CSF sample, and brain biopsy seems to have a better yield.

The frequent identification of co-infection between novel HAstV and other enteric viruses, but also between enteric viruses different from novel HAstV, raises the question of the causative pathogen in gastroenteritis. Yet, our average 21% of mixed viral infections slightly exceeds what was observed in other studies (range 5.7-17%), which could be explained by the fact that conventional RT-PCR assays were used in these latter (6, 12, 247-250).

The use of molecular diagnostics in our study also reduced the diagnostic gap from 70% down to 30%, which is also lower than in pre-cited studies where 40-60% of gastroenteritis cases remained undiagnosed (6, 12, 247, 249, 250). Thus, on the one hand molecular diagnostic of gastroenteritis is useful to increase the microbiological diagnostic rate, but on the other hand, in one fifth of cases the causative agent could remain unclear despite microbiological identification, due to detection of mixed infections. Using the Vesikari score, Pang *et al.*, observed a higher diagnostic gap during mild gastroenteritis episodes compared to moderate-severe episodes (54% versus 15%, respectively), which could suggest that undiagnosed gastroenteritis could be non-infectious (12).

In particular, we found one double and one triple co-infection between different HAstV-VA genotypes, and 7 cases (and one additional in the case-control study) of mixed infections between novel and classic HAstV, which could favour recombination events. Except for the classic HAstV, we did not find a difference in the Cq values of any enteric virus when identified in mono- compared to co-infection. These results would be in accordance with other studies which did not find a correlation between clinical

presentations with single or multiple infections (249), but complicate our capacity to analyse the significance of mixed infections. In fact, mixed infections are identified in up to 10% of day care centre outbreaks of gastroenteritis, with the causative virus remaining unknown (251). Yet, mixed infections should be interpreted beyond the frame of the classical Koch's postulates and rather in the scope of the *biocomplexity*, where infection is influenced not only by one pathogen, but also by many other factors including host genetic susceptibility, host health status, host immune imprint, microbial community and transkingdom interactions (229, 252, 253).

In our study, the observed rate of co-infection for novel HAstV was particularly high (mean 66%). An association between *Campylobacter jejuni* and HAstV-MLB1 was previously described in diarrhoeal children (26); yet, in large case-control studies, the association between HAstV-MLB1 or *Campylobacter jejuni,* respectively*,* and diarrhoea is controversial (26, 27, 30, 254-256). In addition, some studies suggest an association between diarrhoea and pathogen excess or mixed infections (254, 256). It could be that in certain circumstances, two (or more) pathogens that are not (always) virulent *per se*, could synergistically lead to diarrhoeal disease when mixed infection occurs (256, 257). It would thus be interesting to further investigate in which conditions novel HAstV are pathogenic. Similarly, it was reported in an experimental animal model, that calves infected with BoAstV alone did not develop symptoms, but did so during mixed infection between BoAstV and rotavirus or Breda virus (258).

Severe manifestations in animals predominate in young animals such as calves, chicks, pre-weaning minks, newly weaned pigs, goslings. This probably reflects their immature immune system allowing uncontrolled infection. Our higher prevalence found in children

<2 years old confirm that younger children are more at risk for acquiring novel HAstV. Among the 9 cases of severe central nervous system infections associated with HAstV, 4 occurred in children < 5 years old and 8 in immunocompromised patients. It could be that, as for classic HAstV, immunity during adulthood confer protection for reinfection and that symptomatic disease develops in immunocompromised patients and young children, which is supported by serological studies (169, 170).

Assuming that children are at higher risk for novel HAstV infection, we investigated their potential role as a reservoir in the case-control study (article III). We found a lower prevalence than in the first epidemiological study, which was probably associated with a seasonality during the winter-spring seasons (corresponding to the period targeted in the first study) observed in the diarrhoeal patients group. In our cohort of asymptomatic children, we found at least one virus in 87/199 (43.7%) of screened stool samples, which is in accordance to a previous study where more than half of control children had ≥ 1 enteropathogen and 46% ≥ 1 virus detected (32). Yet, the prevalence of novel HAstV was similar between symptomatic (6.3%) and asymptomatic (4%) patients. As we found a high proportion of HAstV-MLB1 (68%), our results could parallel those of Holtz *et al.,* who found an association between diarrhoeal patients and classic HAstV, but not with HAstV-MLB1 (27). It is interesting to note that within the same analysed cohort of children, the association between classic HAstV and diarrhoea could differ depending on the diagnostic assay used (29, 31). This supports the idea that as highly sensitive molecular diagnostics are improved, additional criteria to a positive screening are necessary for assessing the role of a pathogen in gastroenteritis.

We found a difference in the HAstV-MLB viral titre between symptomatic and asymptomatic patients, and surprisingly, the latter had almost three times higher median viral loads. This unexpected finding could suggest a protective role of HAstV-MLB in diarrhoeal disease. Yet, among the 5 HAstV-MLB observed in asymptomatic children, one HAstV-MLB1 occurred in mixed infection with rotavirus that was found at a Cq value of 30. This supports a previous study where authors determined a rotavirus Cq value between 24-27 corresponding to the cut-off predictive of symptomatic disease versus asymptomatic carriage (259). Whether HAstV-MLB1 co-infection would have contributed to the low rotavirus titre and by which mechanisms remain to be determined. In 3 other asymptomatic cases, HAstV-MLB1 was the only pathogen identified. A mechanism called viral interference refers to an IFN-associated antiviral state induced by a first viral infection and which limits the ability of other viruses to superinfect the same tissue (227). If HAstV-MLB1 protection against invading viral pathogen occurs and if the underlying mechanism is viral interference, then it would be in contradiction with our *in vitro* results showing that HAstV-MLB infection does not induce a strong IFN response. Yet, cell culture systems may not be representative of what occurs *in vivo* and our results are thus limited: a recent study performed on human intestinal enteroids isolated from biopsy-derived intestinal crypts demonstrated a significant upregulation of the expression of type I and III IFN as well as interferon stimulated genes in response to HAstV-VA1 infection, while such response was missing on CaCo-2 cells. Using our HAstV-MLB1 adapted strain, the authors observed that it was sensitive to endogenous IFN response in human intestinal enteroids (260). Nevertheless, as mentioned before, mixed infections and associated diarrhoeal disease probably result from a more complex equation than simply one or two pathogens responsible of the disease.

Another interesting finding was that median novel HAstV viral load in patients with concomitant positive coproculture was twice higher than in those who were coproculture-negative. This unexpected result could be paralleled with the capacity of HAstV capsid protein to inhibit the complement lectin pathway (141, 142), which can enhance bacterial infections (143), and with animal model studies demonstrating that TAstV induces a decreased capacity of macrophage for *Escherichia coli* phagocytosis and intracytoplasmic killing, and subsequent increase in *Escherichia coli* survival (79). Similarly, bat astrovirus can induce a gut dysbiosis leading to a pathobiont-like shift and potentially enhancing pathogenic co-infections (261). In addition, astrovirus decreased the gut microbiome α and β diversity, namely the diversity of bacterial species within and among hosts, respectively, in young bats, while the opposite was true for adult bats, and this difference was supposed to be associated to the immune system status (261). Thus even if novel human astroviruses were asymptomatic *per se*, some data suggest that they may influence the surrounding microbiome and indirectly induce disease.

As was reported for norovirus (220), we could not identify associated risk factors for asymptomatic novel HAstV infection. Co-infection with other enteric viruses occurred in 5/8 cases of asymptomatic novel HAstV infection (62.5%), which is similar to what we found in symptomatic children in the first epidemiological study (global 66% of mixed infection); thus co-infection cannot be predictive for symptoms although we did not precisely analyse if the number and the type of co-infected viruses were decisive. Among risk factors evaluated, neither antibiotic consumption (262), siblings, day-care attendance, breastfeeding nor a chronic digestive pathology (263), which had previously been associated with risk of acute gastroenteritis infection, were found to be associated with novel HAstV asymptomatic carriage. Different other factors may have also been

described to explain the asymptomatic carriage of a digestive pathogen, including prolonged excretion after a previous diarrhoeal illness, strain-dependent differences in pathogenicity, the detection during the incubation period, host factor susceptibility and a pathogen burden insufficient to cause overt disease (257). Although our cohort may be too small to detect a significant risk factor among those analysed, it is plausible that the detection of novel HAstV in stool sample of asymptomatic children does not rely on isolated risk factors and requires additional longitudinal and basic virological studies to fully understand its causes and consequences.

Overall, our work has provided indispensable tools for the study and the progress of our understanding of novel HAstV biological and clinical significance. We provided a cell culture system and viral stocks issued from clinical samples, including one case with cerebral involvement, which will allow to continue basic virological studies to fully understand the replicative cycle and host-immune interaction of the HAstV-MLB clade. We also completed the epidemiological understanding of novel HAstV by identifying for the first time their circulation among a Spanish pediatric population and highlighted the lack of obligate association with gastroenteritis. Our data also point out to possible interactions between novel HAstV and other enteric pathogens that may influence the course of a digestive infection.

Conclusions

- We provide the first description of several cell culture systems permissive for the novel HAstV-MLB replication.
- Extra-intestinal cell lines (HuH-7 and A549) may efficiently support HAstV-MLB propagation, and, contrary to the classic HAstVs, trypsin is not required for virus infectivity.
- HAstV-MLB can also persistently infect cell lines, suggesting a carrier-state infection. HAstV-MLB1 can establish a persistent infection more efficiently than HAstV-MLB2.
- HAstV-MLB does not induce a strong type I and III IFN response which could be a mechanism developed for persistence. IFN inhibition during HAstV-MLB persistent infection could subsist despite additional IFN pathway stimulation by synthetic double-stranded RNA.
- HAstV-MLB is sensitive to exogenous type I and III IFN, but sensitivity is dependent on the infected cell type.
- Novel HAstVs are circulating in 6-10% of the diarrhoeal pediatric Spanish population with undiagnosed gastroenteritis.
- According to the high rate of co-infection observed, we cannot assess the pathogenic potential of novel HAstV in diarrhoeal disease, and additional basic virological studies are required to assess the role of novel HAstV in gastroenteritis, if any.
- The prevalence of novel HAstVs in symptomatic and asymptomatic subjects in the studied pediatric Spanish population is similar, suggesting that young asymptomatic children are a potential reservoir for novel HAstV transmission.

- A higher viral titre may occur in asymptomatic children as compared to children with gastroenteritis, as well as in coproculture-positive stool samples of symptomatic children positive for novel HAstVs. This argues in favour of potential interaction between the gut microbiome and novel HAstV that needs to be further explored.

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