Molecular and Epidemiological of Human Adenovirus and Classic Human Astrovirus in Children With Acute Diarrhea in Shanghai, 2017-2018

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Research Article

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Abstract

Background: In addition to rotavirus and norovirus, human adenovirus (HAdV) and classic human astrovirus (classic HAstV) have been identified as important pathogens of acute diarrhea in infants and young children. Here, we presented the molecular epidemiology of HAdV and classic HAstV in children with acute diarrhea in Shanghai.

Methods: Fecal specimens were collected from 804 outpatient infants and young children diagnosed as acute diarrhea in Shanghai from January 2017 to December 2018. All the samples were screened for the presence of HAdV and classic HAstV. HAdV and Classic HAstV were detected using traditional PCR and reverse-transcription PCR, respectively. All the HAdV and classic HAstV positive samples were genotyped by phylogenetic analysis.

Results: Among these 804 fecal samples, 8.58% (69/804) samples were infected with at least one of HAdV or classic HAstV, and one of them was co-infected with these two viruses. The overall detection rates of HAdV and classic HAstV were 3.47% (28/804) and 5.22% (42/804), respectively. Four subgroups (A, B, C and F) of HAdV were detected and seven different genotypes (HAdV-C1, -C2, -B3, -C5, -A31, -F40 and -F41) were identified. Subgroup F had the highest constituent ratio at 64.29% (18/28) followed by non-enteric HAdV of subgroup C (21.43%, 6/28) and subgroup B 10.71% (3/28). HAdV-F41 (60.71%, 17/28) was the dominant genotype, followed by HAdV-C2 (14.29%, 4/28) and HAdV-B3 (10.71%, 3/28). Two different genotypes of classic HAstV (HAstV-1 and HAstV-5) were identified in 42 samples during the study period. HAstV-1 (95.24%, 40/42) was the predominant genotype and the other two strains were genotyped as HAstV-5.

Conclusions: Those findings indicate that HAdV and classic HAstV play an important role in the pathogenesis of acute diarrhea in children in Shanghai. Systematic and long-term surveillance of HAdV and classic HAstV are needed to monitor their prevalence in children and prevent major outbreak.

Background

Acute diarrhea is one of the major health problems in children under five years. About 1.0 billion children < 5 years of age are infected with diarrheal diseases and 0.45 million deaths occur due to diarrhea each year [1–3]. Diarrhea can be caused by various types of viruses, bacteria and parasites. Viruses have long been considered as the most important pathogens responsible for acute gastroenteritis, with rotavirus group A and norovirus being the most prominent causes of acute diarrhea in children [4–6]. Human adenovirus and classic human astrovirus are also recognized as important causes of sporadic diarrhea and outbreaks of diarrhea in children [7, 8].

Human adenovirus (HAdV) belongs to the genus Mastadenovirus of family Adenoviridae. HAdV are non-enveloped, double-stranded, 26-45kbp linear DNA viruses. This virus possess outer capsid and inner core structural proteins. The outer capsid is comprised of fiber proteins, penton, and hexon. The fiber proteins are attached to the penton base and penton is the second-most abundant component consisting of 12
penton bases. The hexon is the principal component of the capsid. HAdV are categorized into seven species (HAdV-A through HAdV-G) based on genomic sequence analysis, and more than 100 genotypes have been recognized [9–11]. Different genotypes have been identified by multiplex PCR techniques and sequencing targeting fiber genes or hexon genes [12, 13]. HAdV infections lead to pathogenesis of several systems in human, including acute respiratory illness, acute gastroenteritis, conjunctiva, hemorrhagic cystitis, hepatitis, hemorrhagic colitis, pancreatitis, nephritis, or meningoencephalitis [13]. Genotypes 40 and 41 belonging to HAdV-F are the most frequently reported causes of HAdV-associated diarrhea in young children and called enteric HAdV. HAdV-40 and 41 have been found to be responsible for 1–20% of diarrhea cases in both outpatients and hospitalized children worldwide [14–18]. Some acute diarrhea in children have also been reported in association with HAdV-12, -18, and -31 of HAdV-A. Moreover, HAdV-B, HAdV-C, HAdV-D and HAdV-G were also detected in fecal samples from children with acute gastroenteritis [14, 15, 18, 19].

Human astrovirus (HAstV) belongs to the Astroviridae family which is divided into two genera, Mamastrovirus and Avastrovirus based on their ability to infect mammalian and avian species, respectively. HAstV are non-enveloped, positive sense, single-stranded RNA viruses. The genome is 6.8–7.9 kb in length and consists of a 5’untranslated region (UTR), followed by three open reading frames (ORFs) (ORF1a, ORF1b and ORF2), a 3′ UTR and a poly A tail. ORF1a and ORF1b encode nonstructural proteins including the RNA-dependent RNA polymerase (RdRp), while ORF2 encodes the capsid protein precursor [20]. The initial prototype strain of human astrovirus species was originally isolated in 1975, called classic human astrovirus (classic HAstV). With the development of next-generation sequencing technologies, two novel groups of highly divergent HAstV, Melbourne (MLB) and Virginia/Human-Mink-Ovine-like (VA/HMO), emerged and have been identified in human stools of individuals with diarrhea worldwide [7, 21, 22]. The overall detection rate of novel HAstV in stool was much lower than that of classic HAstV which are still the second or third most common viral pathogens responsible for diarrhea in young children. Up to now, eight genotypes of classic HAstV (HAstV-1 to HAstV-8) have been identified [23]. Classic HAstV is responsible for 2 to 18.8% of acute diarrhea cases in children globally. HAstV-1 is the most prevalent genotype detected in children, whereas HAstV-2 ~ HAstV-8 are less prevalent [14, 20, 24–26].

In Shanghai, the majority of studies focused on the molecular and epidemiological of rotavirus and norovirus, fewer studies were conducted for the molecular epidemiology of HAdV and classic HAstV in outpatient [14, 27–29]. Therefore, this study investigated the detection rate, viral co-infection, seasonal distribution, age distribution and genetic diversity of HAdV and classic HAstV infections in children with acute diarrhea in Shanghai during 2017 and 2018.

**Materials And Methods**

**Study design**
During 2017 and 2018, a total of 804 stool specimens were collected from children under five years who were diagnosed as acute diarrhea and admitted to the outpatient of the Children's Hospital of Fudan University, Shanghai, China. All the enrolled specimens were routinely collected and stored at -70 °C before investigation. The definition of acute diarrhea was diarrhea (three or more loose, watery, thin paste texture or the presence of mucous stools within 24 hours), possibly accompanied by vomiting, abdominal pain, fever, and nausea. This definition excluded the presence of pus or blood regardless of the presence of fever [14]. Demographic information and clinical diagnoses were gathered from the children's medical histories. Because the stool specimens were collected during the normal course of patient care, informed consent from each patient was not required. The whole study proposal has been approved by the Institutional Review Board of Children's Hospital of Fudan University. All methods were carried out in accordance with relevant guidelines and regulations.

The viral genomic RNA and DNA were extracted from a 10% fecal suspension supernatant using the TIANamp Virus DNA/RNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. Extracted genetic material was reversly transcribed into cDNA with a random primer using Prime-Script™ II Reverse Transcriptase (Takara, Biotechnology (Dalian) Co., Ltd.) for detection of the Classic HAstV. A conserved region (C4) in the HAdV hexon gene was amplified using the Ad-1 (5′-TTCCCC-ATGCGCAYAACAC-3’) and Ad-2 (5′-CCCTGGTAKC-CRATRTTGTA-3’) primers [30]. The expected size of amplicon was 482 bp. The PCR cycling program was as follows: an initial denaturation at 94 °C for 2 min, followed by 35 cycles of 30 sec at 94 °C, 30 sec at 55 °C, and 1 min at 72 °C with a final extension cycle at 72 °C for 7 min. Classic HAstV detection in fecal specimens were detected using primers Mon269 (5′-CAACTCAGGAAACAGGGTGT-3’) and Mon270 (5′-CTGGCTTAACCCACATTCC-3’) which targeted on the ORF2 region C [31]. The expected size of the PCR product was 449 bp. The PCR amplification was performed under the following conditions: 94 °C for 2 min, 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 1 min followed by 72°C for 7 min. All the PCR products were electrophoresed in a 2% agarose gel with ethidium bromide and a DNA ladder of 100 bp (Takara Bio Co., Dalian, China).

All the amplicons of HAdV and classic HAstV were purified and sequenced for phylogenetic analysis by first-generation sequencing technologies (Sangon Biotech (Shanghai) Co., Ltd.). Phylogenetic trees were constructed using the Neighbor-Joining method and Kimura two parameters substitution model replicons with 1,000 bootstrap by MEGA (v6.0) software. Nucleotide sequences of HAdV and classic HAstV detected in this study were compared with the sequences of corresponding reference virus strains available in the GenBank database, respectively.

The nucleotide sequences of HAdV strains and the accession numbers used were as follows: HAdV-1: AC_000017, AF534906; HAdV-2: J01917, AC_000007; HAdV-3: AY599836; HAdV-4: AY487949; HAdV-5: AY339865; HAdV-8: AB448768; HAdV-9: AJ854486; HAdV-11: AY163756; HAdV-12: X73487; HAdV-14: AY803294; HAdV-16: AY601636; HAdV-17: AF108105; HAdV-21: AY601633; HAdV-22: FJ404771; HAdV-26: EF153474; HAdV-28: FJ824826; HAdV-29: AB562587; HAdV-31: AM749299; HAdV-34: AY737797; HAdV-35: AY128640; HAdV-36: GQ384080; HAdV-37: AB448777; HAdV-40: L19443; HAdV-41: DQ315364; HAdV-46: AY875648; HAdV-48: EF153473; HAdV-49: DQ39829; HAdV-53: AB605240; HAdV-54: NC
012959; HAdV-A: NC_001460; HAdV-B: NC_011203; HAdV-C: NC_001405; HAdV-D: AC_010956; HAdV-E: NC_003266; and HAdV-F: NC_001454. The reference classic HAstV strains and accession numbers used were as follows: HAstV-1: L23513, Z25771; HAstV-2: L13745; HAstV-3: AFl41381, L38505; HAstV-4: AY720891, L38506; HAstV-5: DQ028633, U15136; HAstV-6: L38507, Z46658; HAstV-7: L38508, Y08632; HAstV-8: AF260508, Z66541.

**Statistical analysis**

The differences of HAdV and classic HAstV detection rates between in girls and boys and the year of 2017 and 2018 was compared using a two-sided chi-square test in SPSS Statistics v.20.0 (IBM Corp., Armonk, NY, USA), and a $P$-value less than 0.05 was considered statistically significant.

**Results**

**Clinical characteristics, epidemiological of HAdV and classic HAstV infections**

During the study period, a total of 804 stool samples from children with acute diarrhea were enrolled in our study. Among them, 497 children were boys and 307 were girls. All the children were diagnosed as acute diarrhea when coming to outpatient of the Children's hospital of Fudan University in Shanghai.

Among these 804 fecal samples, 8.58% (69/804) samples were infected with at least one of HAdV and/or classic HAstV, and one of them was co-infected with these two viruses. The overall detection rates of HAdV and classic HAstV were 3.47% (28/804) and 5.22% (42/804). The frequency of HAdV in boys and girls were 3.22% (16/497) and 3.91% (12/307) ($P=0.604$). The prevalence of classic HAstV in boys and girls were 5.84% (29/497) and 4.23% (13/307) ($P=0.275$). For HAdV, the prevalence in 2017 and 2018 were 2.84% (12/423) and 4.20% (16/381) ($P=0.293$), respectively. The annual detection rates of the classic HAstV varied significantly according to the year: 2.84% (12/423) in 2017, 7.87% (30/381) in 2018 ($P=0.001$).

**Seasonal and age distribution of HAdV- and classic HAstV-infected children**

The seasonal distribution of HAdV detected was observed with a peak both in June of 2017 (18.75%, 6/32) and 2018 (15.38%, 4/26). In this study, HAdV was detected in 13 of the total 24 months (Figure. 1). The peak of classic HAstV detection occurred in December of 2017 (11.76%, 4/34) and November of 2018 (33.33%, 10/30). In 10 of the total 24 months considered, classic HAstV was not detected (Fig. 1).

Infections of HAdV and classic HAstV were found in all the age groups. About 82.14% (23/28) of HAdV-infected cases and over half of the classic HAstV infected children (66.67%, 28/42) were found in children less than 36 months, respectively. The group of children between 37 and 48 months old presented the highest prevalence of HAdV infections (13.33%, 6/45) and HAstV infections (5.17%, 3/55) (Fig. 2).

**Genotypes of HAdV and classic HAstV infections**
During the whole study period, a total of 28 and 42 nucleotide sequences of HAdV and classic HAstV were obtained, respectively. The phylogenetic trees of nucleotide sequences of the HAdV and classic HAstV isolates were constructed in comparison with the reference strains.

According to the phylogenetic tree analysis conducted based on a partial genomic region of hexon, four subgroups (A, B, C and F) of HAdV were detected and seven different genotypes (HAdV-A31, -B3, -C1, -C2, -C5, -F40 and -F41) were identified. Subgroup F classified as enteric HAdV had the highest constituent ratio at 64.29% (18/28) followed by non-enteric HAdV of subgroup C (21.43%, 6/28) and subgroup B (10.71%, 3/28). Of the seven genotypes, HAdV-F41 (60.71%, 17/28) was the dominant genotype, followed by HAdV-C2 (14.29%, 4/28) and HAdV-B3 (10.71%, 3/28). HAdV-F41 was the most predominant genotype assigned at 83.33% (10/12) and 43.75% (7/16) in 2017 and 2018, respectively. The second prevalent genotype varied from 2017 to 2018. HAdV-C2 (16.67%, 2/12) was the second prevalent genotypes in 2017 while HAdV-B3 (18.75%, 3/16) was in 2018. Of note, only two different genotypes (HAdV-C2 and HAdV-F41) were identified in 2017 and all the seven different HAdV genotypes were detected in 2018 (Fig. 3).

Based on the ORF2 region C of classic HAstV, two different genotypes of classic HAstV (HAstV-1 and HAstV-5) were identified in 42 samples during the study period. HAstV-1 (95.24%, 40/42) was the predominant genotype in this study and it was the only genotype detected in 2017. Except for HAstV-1 (93.33%, 28/30), HAstV-5 (6.67%, 2/30) was also identified in 2018 (Fig. 4).

**Discussion**

Although HAdV and classic HAstV usually cause a self-limiting short time watery diarrhea, they are frequent causes of acute diarrhea in children under five years of age [3, 20]. The real-time monitoring of HAdV and classic HAstV can help us to monitor the prevalence of those two pathogens in children with acute gastroenteritis, and play a guiding role in the prevention of major epidemics in Shanghai.

In the present study, the overall stool positivity rate for HAdV infection in this study was 3.47%, which is similar to what was reported in Brazil (3.9%), Bangladesh (4.82%), our previous studies (5.2%), but is much lower than in Northwest Ethiopia (32.0%) and Albania (23.2%) [18, 24, 32–34]. According to our continuous monitoring data, the detection rate of HAdV in children with acute diarrheas was relatively stable in Shanghai from 2010 to 2018 [14]. In addition with our previous study from 2010 to 2011 (1.9%), the detection rate of classic HAstV (5.22%) in Shanghai was also lower than the average global positive rate of 11.0% [14, 20]. This frequency was similar to that observed in other studies carried out in Thailand (2.6%), Asian Russia (2.8%), Brazil (3.9%), Lebanon (5.5%), Germany (5.0%) [35–39]. However, the detectable rate of classic HAstV in 2018 (7.87%) was significantly increased compared to the detection rate in 2017 (2.84%) in this study. Long-term monitoring is needed to determine the reason for this increase. Furthermore, gender was not found to play a role in HAdV and classic HAstV infections in our study. This conclusion is consistent with the findings describing data in Tanzania and Northwest Ethiopia.
Moreover, we found that only one child aged 5 years in 2018 was co-infected with HAdV and classical HAstV.

Although a small number of positive samples of HAdV and classic HAstV were reported in this study, description of seasonality of those two viruses infection were also analyzed. HAdV and classic HAstV infections had a tendency to occur in oscillatory fluctuations. The highest rates of HAdV infection was observed in July both in 2017 and 2018, which was similar to Tianjin from 2008 to 2009 and Thailand from 2011 to 2017 [8, 41]. However, in our previous study on inpatients with acute diarrhea from 2006 to 2011, HAdV infection was more frequent during the winter months [27]. Besides, the seasonal pattern of HAdV infection was not observed in our previous study on outpatients from 2012 to 2016 [36]. All those data indicate that seasonal pattern of HAdV infection was not obvious and consistent in Shanghai. A longer time-series analysis is needed to describe the discrepancies in HAdV prevalence drawn from the acquired data of inpatients and outpatients ≤ 5 years of age. The same phenomenon was also found in Thailand and Indian [8, 42]. Similar to several other studies conducted in Germany, Spain, Northern Italy and our previous study, classic HAstV infection was also frequent during the cold-weather period in Shanghai [14, 26, 39, 43].

According to our data, a higher HAdV (82.14%, 23/28) and classic HAstV (66.67%, 28/42) positive component ratios were both identified in children ≤ 3 years which were in line with the findings of other studies [19, 27, 32]. In this study, both of HAdV (13.33%, 6/45) and classic HAstV (6.67%, 3/42) infection were most frequently detected in children of 37 to 48 months old. This finding suggest that herd immunity to HAdV and classic HAstV may be developed gradually in children after 4 years old in Shanghai. However, the neutralizing antibody production and duration of herd immunity and the epidemiological pattern to HAdV and classical HAstV still need to be determined.

The molecular characterization of HAdV through phylogenetic analysis revealed a genetic diversity in the analyzed samples in this study. A total of seven HAdV genotypes including five non-enteric HAdV genotypes were found in children with acute diarrhea from 2017 to 2018. Our survey of HAdV genotypes in children with acute diarrhea indicated that enteric HAdV including HAdV-F40 and -F41 accounted for 64.29% (18/28), being considered as the most remarkable pathogens associated with acute diarrhea in Shanghai. However, HAdV-F40 was found in only one child. This finding coincides with previous reports from our previous studies, Bangladesh and Japan [14, 32, 44]. One reason for the predominance of HAdV-F41 over HAdV-F40 is an antigenic drift of HAdV-F41. Meanwhile, some studies have discovered that the GTC1 and GTC2 subdivisions trigged by the build-up of amino acid mutations in the HVRs (hexon hypervariable regions) of hexon may allow the HAdV-F41 to escape from the host immune response and cause an increased HAdV-F41 infection in the individuals [45–47].

In this study, non-enteric HAdV including HAdV-A31, -B3, -C1, -C2, -C5, might play an important role in causing acute diarrhea in children, although they primarily caused conjunctiva and the upper and lower respiratory tracts infections [13]. Interestingly, non-enteric HAdV-C2 and HAdV-B3 unexpectedly exceeded HAdV-F40 and became the second and third leading genotype in diarrhea children. In addition, different
from our previous studies during 2012 to 2016, the detection rate of HAdV-C2 exceeded HAdV-A31 and became the second prevalent genotype from 2017 to 2018 [39]. All those results suggested that the genotypes of non-enteric HAdV in diarrhea children were in a dynamic change in Shanghai. Our investigation showed that continuous surveillance of HAdV in diarrhea children in Shanghai is very important.

For classic HAstV, HAstV-1 is the most prevalent genotype detected worldwide whereas HAstV-2 to -8 are less [7, 23]. According to the phylogenetic tree analysis of classic HAstV, only two genotypes including HAstV-1 and HAstV-5 were identified in Shanghai from 2017 to 2018. HAstV-1 (95.24%) was the absolute predominant genotype detected in children with diarrhea which is consistent with our previous study from 2008 to 2011 as well as with other reports conducted in Japan, Switzerland, Asian Russia, Korea, German and Brazil [36, 37, 39, 48–51]. HAstV-5 was only detected in two samples in early 2018. To our knowledge, this was the first time to report the appearance of HAstV-5 in Shanghai. Nevertheless, long-term monitoring data on HAstV-5 are needed to derive the epidemic characteristics of this genotype.

**Conclusions**

In conclusion, this investigation clarified the epidemiological role of HAdV and classic HAstV in children under five years with acute diarrhea in Shanghai from 2017 to 2018. HAdV-41 as a significant involvement in the etiology of acute diarrhea in children younger than 5 years has been proved; however, the role of non-enteric HAdV in children cannot be ignored. We found that HAstV-1 was the most predominant genotype in Shanghai. These findings can enhance our knowledge on the significance of HAdV and classic HAstV infections in children. Systematical and long-time surveillance of the genetic variability of HAdV and classic HAstV are important for understanding periodicity of the relative prevalence of different genotypes.

**Abbreviations**

**HAdV**: Human adenovirus **HAstV**: Human astrovirus **ORFs**: Open reading frames **UTR**: untranslated region **RdRp**: RNA-dependent RNA polymerase **MLB**: Melbourne **VA/HMO**: Virginia/Human-Mink-Ovine-like **MEGA**: molecular evolutionary genetics analysis **GTC**: genome-type clusters **HVRs**: hexon hypervariable regions

**Declarations**

**Ethics approval and consent to participate**

This study and informed consent waiver statement was reviewed and approved by the Institutional Review Board of Children's Hospital of Fudan University. Because fecal specimens enrolled in this study were the remaining samples after routine examination, consent was not sought from the parent or legal guardians of the children.
Consent for publication

Not applicable.

Availability of data and material

The datasets used in the current study are available from the link:
http://purl.org/phylo/treebase/phylows/study/TB2:S27343?x-access-code=3821b7362045cab03c705697db437f13&format=html and the accession number is 27343.

Competing interests

The authors have no competing interest.

Funding

None.

Authors' contributions

JX and LJL conceived of and designed the study. LJL performed experiments and wrote the manuscript. HQZ and MHX assisted in analysis all the data and interpretation of data for the work. LYS, LFC and RJ acquired the clinical data and coordinated in the design of the study. All authors have read and approved the final manuscript.

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