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Short communication

Novel astrovirus types circulating in Shandong Province (Eastern China) during 2016: A clinical and environmental surveillance

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A B S T R A C T

Background: In recent years, several novel astroviruses have been discovered by molecular assays. Their prevalence in many parts of the world remains unclear.
Objectives: To investigate the existence and genetic diversity of novel astroviruses in China.
Study design: Stool specimens were collected from patients suffering gastroenteritis from two hospitals in Jinan city in 2016, and were screened for novel astroviruses by real-time RT-PCR assay. Positive samples were subject of ORF2-amplification by conventional RT-PCR and Sanger sequencing. In addition, 1 liter of sewage sample was collected monthly in 2016 and concentrated via the membrane adsorption/elution method. Partial ORF2 amplification, cloning and Sanger sequencing were conducted, and phylogenetic analysis was performed for genotyping all the obtained sequences.
Results: 18 of 635 specimens (2.8%) were positive for novel astrovirus detection by real-time RT-PCR assay and were further genotyped by Sanger sequencing as 13 MLB1, 4 MLB2, and 1 VA3. 100% (12/12) of sewage samples were positive for novel astroviruses by conventional RT-PCR. After cloning and sequencing, six known novel astroviruses (MLB1, MLB2, VA1, VA2, VA3, and VA5) were identified. VA2, MLB1, and VA1 were the most common subtypes being detected in 100.0% (12/12), 91.7% (11/12), and 91.7% (11/12) of the samples, respectively. Eighteen sequences that could not be classified into any known subtypes were found.
Conclusions: These results demonstrate the wide circulation and high diversity of novel astroviruses in Jinan, Shandong Province, China, and underline the significance of environmental surveillance combined with population-based surveillance for understanding the molecular epidemiology of enteric viruses.

1. Background

Classic human astroviruses (HAstVs) are well-established causative agents of viral gastroenteritis and are classified into 8 serotypes. Since 2008, the screening of human feces by next-generation sequencing has revealed two novel HAstV groups: MLB and VA/HMO. MLB group consists of three clades of MLB1, MLB2, and MLB3, and they are all assigned to *Mamastrovirus 6* species. VA group contains VA1 to VA5. Of these, VA2 and VA4 belong to *Mamastrovirus 8*, VA1 and VA3 belong to *Mamastrovirus 9* species, whereas VA5 may be classified as a new species [1–4]. Novel HAstVs are phylogenetically distant from the classical HAstVs and little is known in terms of epidemiology and pathogenesis. Their association with diarrhea remains to be determined.

Circulation of novel HAstVs has been confirmed in several parts of the world, including Australia, India, Japan, Brazil, Uruguay, Thailand, Egypt, Nigeria, Kenya, Gambia, etc [1]. In China, novel astrovirus was first discovered from diarrheal specimens in Hong Kong during 2004–2005 [5]. Subsequently, an investigation on fecal specimens of acute gastroenteritis patients during 2010–2011 revealed 3 novel astrovirus strains of MLB1, MLB2 and VA2 with a frequency of detection of 1.5% [6]. Since these are the only descriptions on novel HAstVs in China, the genetic diversity and circulation in human population are yet to be understood.

As enteric virus-infected individuals shed a large number of virions in the feces, we can monitor the transmission of enteric virus by examining environmental specimens supposedly contaminated by human feces. A study on the wastewater in Japan has identified sequences belonging to MLB2, VA1, and VA2 clades [7]. Here, we collected clinical and sewage specimens and analyzed the presence of novel HAstVs, to evaluate their prevalence and genetic diversity in eastern China.

2. Study design

2.1. Clinical and sewage samples, treatment and RNA extraction

The study was conducted in accordance with the Declaration of Helsinki and the ethical approval was given by the Ethics Review Committee of the Shandong Center for Disease Control and Prevention (2015-01). Written informed consents were obtained from the patients or the legal guardians.

Stool specimens were collected from patients with acute gastroenteritis visiting or admitted to Shandong Provincial Hospital and Qilu

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Children's Hospital of Shandong University in Jinan, Shandong Province, China from January to December, 2016. A 10% dilution of stool specimens was prepared, followed by vortexing and centrifugation at 3000 ×*g* for 10 min.

A wastewater treatment plant (WWTP) which gathered sewage from the west half of Jinan and covered a population over 0.8 million inhabitants was selected as the sampling site. Sewage specimens were collected monthly at the inlet canal of the WWTP from January to December in 2016, and were concentrated 100-fold via membrane adsorption / elution method [8]. Briefly, one liter of sewage was centrifuged at 3000 ×*g* for 30 min. $MgCl_2$ and hydrochloric acid was added to the supernatant to a final Mg^{2+} concentration of 0.05 M and pH value of 3.5. Then the solution was filtered through a 10 μm cellulose acetate membrane filter and a 0.45 μm mixed cellulose ester (MCE) membrane filter (ADVANTEC, Tokyo, Japan). Absorbents on the MCE filter were eluted with 10 ml of 3% beef extract solution (pH 8.5) by 3-min ultrasonication. After centrifugation at 3000 ×*g* for 30 min, the supernatant was ready for detection.

Viral RNA was extracted from 140 μl of 10% dilution of stool samples or concentrated sewage samples by using the QIAamp Viral RNA Mini Kit (QIAGEN, Germany).

2.2. Novel HAstV real-time PCR for clinical samples

Primers and probes used for real-time PCR assay were designed in this study (Table 1) on the basis of the nucleotide sequence alignments of 16 MLB strains and 11 VA strains in the GenBank database. For clinical specimens, two real-time PCR reactions detecting MLB and VA clades respectively were performed by using AgPath-ID One-Step RT-PCR Reagents (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. A threshold C_t value of 38 was chosen as the cut-off for detection. To investigate the detection limits of real-time PCR assay, three plasmids containing the synthesized MLB1, VA1 and VA2 target inserts respectively were obtained from Shanghai Sangon Company. Ten-fold serial dilutions of MLB1, VA1 and VA2 plasmids with concentrations ranging from 1×10^0 to 1×10^4 copies per reaction were amplified in triplicate by corresponding MLB and VA real-time PCR assays, respectively, and the assay was repeated in twice. The detection limit was based on the lowest concentration at which positive PCR signal was produced in all reactions.

2.3. Conventional one-step RT-PCR, Sanger sequencing, and phylogenetic analysis

Positive clinical samples for novel HAstV real-time PCR assay and all sewage samples were forwarded to the conventional one-step RT-PCR assay on ORF2 region (encoding the capsid protein) by using the Superscript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, USA). The primers (Table 1) are designed in this study. The length PCR products is 896 bp (nucleotide position 4068–4963 according to MLB1 reference strain NC_011400) for MLB clades, and 696 bp (nucleotide position 4212–4907 according to VA1 reference strain NC_013060) for VA clades. PCR products were gel purified via a QIAquick gel extraction kit (Qiagen, Germany). Purified products from clinical samples were directly forwarded to bi-directional Sanger-sequencing at Shanghai Sangon Company. Purified products from sewage were cloned into pGEMT vector (Promega, USA) and transformed into competent *E. coli* TG1 via the heat shock method. The transformants were incubated at 37 °C on LB (Ampicillin) plate. After blue and white screening, 6–12 white clones for each transform were selected and Sanger-sequenced bi-directionally.

The phylogenetic tree of novel astroviruses were constructed by using Mega 7.0 via the Neighbor-Joining method with Kimura 2-parameter model, and bootstrapping was performed with 1000 duplicates. The tree incorporated all MLB and VA sequences alongside with reference strains of all other *Mamastrovirus* species, and was constructed based on 450-bp capsid coding sequences (nucleotide position 4068–4517 according to MLB1 NC_011400) which represent the common coding region of MLB and VA sequences obtained in this study. Environmental sequences sharing > 99% similarities within a subtype were removed in order to simplify the tree. The capsid gene sequences in this study were deposited into GenBank with the accession numbers MF490481–MF490796.

3. Results

3.1. Novel astrovirus detection in stool specimens from patients suffering gastroenteritis

During January to December, 2016, a total of 635 stool specimens were collected from patients with acute gastroenteritis in Jinan.

Table 1
Primers and probes used in this study.

Assay	Target virus	Primer	Sequence (5′–3′)	Polarity	Location (nt)	Size (bp)
real-time PCR	MLB	qMLB-F	AATMGGAATCGCCGTCGTAGG	+	3987-4004[a]	/
		qMLB-R	AAATTTGATCCAACGGTGCC	–	4084-4065[a]	/
		qMLB-P	FAM-TACAACTGGGCCTAARCCTGCGGTGTC-BHQ1	+	4016-4042[a]	/
	VA[h]	qVA-F	CTTTGGAGGGGMGGWCCAAAG	+	4184-4204[c]	/
		qVA-R1	ACCACTGGTTCCTTCTTCTCCTG	–	4306-4284[c]	/
		qVA-R2	TCCTCCTTGACAACCTCTTTAGC	–	4295-4273[d]	/
		qVA-R34	CGCTCCTGCTTAACTACCTCCTT	–	4298-4276[e]	/
		qVA-R5	TTTTCCTCCTTAACCACCTCCTT	–	4260-4238[g]	/
		qVA-P	FAM-ATGGCTGGTAARCAGCCCCAGCA-BHQ1	+	4212-4234[c]	/
Conventional PCR[i]	MLB	MLB-F	ACCGTTGGATCAAATTTRAGTGATGTTGTTG	+	4068-4098[a]	
	MLB1&2	MLB12R	GAAATTGCTGGTTTATCATCTCT	–	4963-4941[a]	896
	MLB3	MLB3R	CTAGAAATGGCGGGTTTGTCATCTCT	–	4960-4935[b]	893
	VA	VA-F	ATGGCTGGTARRCAGCCCCAGCA	+	4212-4234[c]	
	VA1	VA1R	GTCTCCAGCCCATGCTGCATCCTTGTA	–	4916-4890[c]	705
	VA2	VA2R	CCAGGCTTCATCCTTATAAGTTGA	–	4914-4891[d]	703
	VA3	VA3R	GTCACCGTTCCATGCTGCATCCTTGTA	–	4905-4879[e]	694
	VA4	VA4R	CCATGGTGCATCACGGTAAGTGGA	–	4909-4886[f]	698
	VA5	VA5R	ATCACCTGCCCACGCAGCATCCTTGTA	–	4882-4856[g]	671

[a–g]Corresponding nucleotide position of Astrovirus MLB1 (accession no. NC_011400), MLB3 (accession no. NC_019028), VA1 (accession no. NC_013060), VA2 (accession no. GQ502193), VA3 (accession no. NC_019026), VA4 (accession no. NC_019027), and VA5 (accession no. KJ656124), respectively.
[h]The real-time PCR for VA is performed in one reaction with all these 6 primers and probes mixed together.
[i]For conventional amplification, altogether 7 distinct RT-PCR reactions are performed for each sample with the combination of universal forward primers (MLB-F or VA-F) and the rest of specific reverse primers.

Table 2
Information of novel HAstV in gastroenteritis patients in 2016.

Type	Strain	Co-infection	Patient			
			Sex	Age	Onset	Clinical Presentation
MLB1	HuA169	–	M	35 y	2016/6/11	Fever, diarrhea
MLB1	HuA171	–	F	31 y	2016/9/7	Abdominal pain, diarrhea
MLB1	HuB041	AdV	M	2 m	2016/7/10	Diarrhea
MLB1	HuB045	–	M	3 m	2016/7/15	Diarrhea
MLB1	HuB048	–	F	2 m	2016/7/30	Diarrhea
MLB1	HuB054	–	M	7 y	2016/8/6	Nausea, vomiting, diarrhea
MLB1	HuB057	–	M	5 y	2016/8/9	Nausea, abdominal pain, diarrhea
MLB1	HuB058	–	F	4 y	2016/8/7	Vomiting, diarrhea
MLB1	HuB061	–	M	6 m	2016/8/6	Diarrhea
MLB1	HuB064	–	M	16 m	2016/8/8	Diarrhea
MLB1	HuB092	AdV	F	2 m	2016/8/27	Diarrhea
MLB1	HuB111	–	F	3 m	2016/10/23	Diarrhea
MLB1	HuB112	–	M	8 m	2016/10/21	Diarrhea
MLB2	HuC074	–	F	35 y	2016/6/9	Nausea, abdominal pain, fatigue, diarrhea
MLB2	HuC075	–	M	58 y	2016/8/3	Nausea, vomiting, abdominal pain, diarrhea, cyanosis, dehydration, edema
MLB2	HuC091	–	F	19 y	2016/6/3	Nausea, vomiting, diarrhea
MLB2	HuD039	GII NoV	F	13 m	2016/3/15	Nausea, diarrhea
VA3	HuA037	–	F	42 y	2016/5/20	Nausea, vomiting, diarrhea

Table 3
Detection of novel astroviruses from sewage in 2016.

Month	Subtype[a]								
	MLB1	MLB2	MLB3	VA1	VA2	VA3	VA4	VA5	VA6
January	+ (8)	+ (1)	–	+ (1)	+ (10)	+ (1)	–	–	–
February	+ (6)	+ (8)	–	+ (6)	+ (9)	+ (1)	–	+ (2)	+ (4)
March	+ (10)	–	–	+ (6)	+ (11)	–	–	+ (16)	+ (1)
April	+ (6)	+ (2)	–	+ (8)	+ (11)	–	–	+ (11)	–
May	+ (5)	–	–	+ (17)	+ (11)	–	–	+ (2)	+ (1)
June	+ (6)	+ (2)	–	+ (11)	+ (11)	–	–	+ (2)	–
July	+ (5)	–	–	+ (3)	+ (5)	–	–	+ (6)	–
August	+ (2)	+ (8)	–	+ (5)	+ (8)	+ (1)	–	–	+ (11)
September	+ (6)	–	–	+ (2)	+ (8)	–	–	–	–
October	+ (4)	+ (13)	–	+ (12)	+ (12)	–	–	+ (1)	–
November	+ (2)	+ (1)	–	+ (1)	+ (8)	–	–	–	+ (1)
December	–	–	–	–	+ (8)	–	–	+ (2)	–
Positive sewage samples[b]	11 (60)	7 (35)	0	11 (72)	12 (112)	3 (3)	0	8 (42)	5 (18)

[a] PCR results for the subtypes tested are shown as follows: +, positive; –, negative. The numbers in parentheses indicate the numbers of viral sequences derived from plasmids after cloning mixed sequence amplicons.
[b] The numbers in parentheses indicate the sum of the numbers of sequences.

TaqMan-based real-time PCR assay revealed 18 novel HAstV infections, resulting in a detection rate of 2.8%. The detection limits of the real-time PCR assays were 10, 100 and 10 copies per reaction for MLB1, VA1 and VA2, respectively. Of all novel HAstV positive specimens, 17 were positive with MLB and 1 was positive with VA clade. Subsequent conventional RT-PCR, Sanger sequencing and BLAST analyses based on partial ORF2 region identified these 18 positive specimens as 13 MLB1, 4 MLB2, and 1 VA3, respectively (Table 2).

Mixed viral infections were found in 3 out of the 18 novel HAstV positive specimens (Table 2). A mixture of MLB1 and enteric adenovirus was detected in two specimens, and a mixture of MLB2 and GII norovirus was detected in the other specimen.

3.2. Novel astrovirus detection from sewage

Novel astroviruses were detected in all 12 months of 2016 by conventional RT-PCR reactions. After cloning and Sanger sequencing of the PCR products, a total of 342 sequences of novel astrovirus were obtained. Of these, 324 sequences belonged to 6 known astrovirus subtypes of MLB1 (n = 60), MLB2 (n = 35), VA1 (n = 72), VA2 (n = 112), VA3 (n = 3), and VA5 (n = 42) (Table 3). The remaining 18 unclassified sequences were derived from the PCR reactions using primer pairs VA-F/VA1R and VA-F/VA3R, and they have 98.2–100.0% nucleotide similarities among themselves, and relatively higher genetic diversity with all other known novel astroviruses. BLAST search with these 18 sequences showed that two VA5 reference sequences had 100% coverage, highest score, and lowest E-value with them. Reference strain VA5/human/Gambia/102139/2009 had closest genetic relationship with them with 84.6–85.4% nucleotide identities.

Of all the novel astrovirus clades, VA2, MLB1, and VA1 were the most common subtypes in sewage being detected in 100.0% (12/12), 91.7% (11/12), and 91.7% (11/12) of the samples, respectively, followed by VA5 (8/12, 66.7%), MLB2 (7/12, 58.3%), and VA3 (3/12, 25%). The unclassified clade in this study was detected in 41.7% (5/12) of the samples.

3.3. Subtypes diversity of novel astroviruses

We constructed phylogenetic trees by using Mega 7.0 based on 450-bp partial capsid coding sequences of novel astroviruses. In the tree with all *Mamastrovirus* species (Fig. 1 A), all sequences from sewage segregated with the corresponding reference strains of each subtype of novel astroviruses. Generally, Chinese strains within a clade have multiple lineages in the tree of common MLB and VA clades (Fig. 1B to

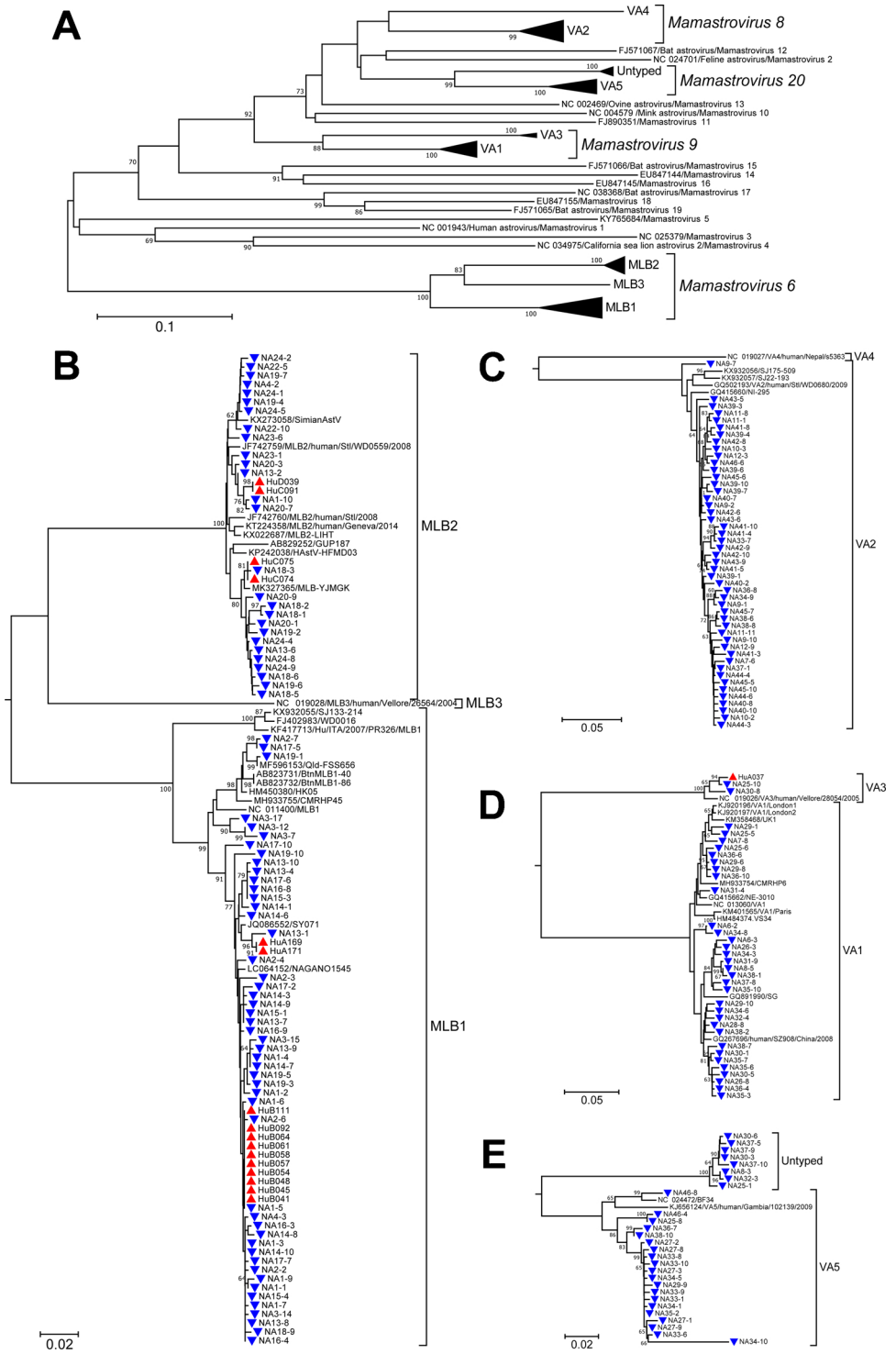

Fig. 1. Phylogenetic tree on partial ORF2 region of novel astroviruses by using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. A, Phylogenetic tree incorporating all MLB and VA sequences alongside with reference strains of *Mamastrovirus 1 to 20* based on 450-bp capsid coding sequences (nucleotide position 4068–4517 according to MLB1 NC_011400). All MLB and VA branches obtained in this study are compressed in the tree. B–E, Expanded subtrees of *Mamastrovirus 6, 8, 9,* and *20,* respectively. Red triangle indicates sequences from diarrheal patients, blue inverted triangle indicates sequences from sewage, and the rest branches with no taxon markers are reference strains. Unclassified sequences are indicated as a cluster of "Untyped" in Fig. 1E. Scale bars indicate branch distance (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

E). Clinical and environmental sequences within a subtype segregated together and no distinct separation was observed between them.

4. Discussion

Generally, novel astroviruses account up to 6.4% of the gastroenteritis cases [2]. This study revealed a detection rate of 2.8% in diarrheal patients in Jinan city, Shandong Province, China in 2016. However, novel HAstV RNA was continuously present in all sewage samples in 2016 with considerably higher number of obtained sequences and subtypes. Phylogenetic analysis revealed close genetic relationships between environmental and clinical sequences within the three subtypes of MLB1, MLB2, and VA3. These results further demonstrate that environmental surveillance is a sensitive and important approach in understanding the local circulation of enteric viruses, especially for those viruses with low incidence in the population. In a previous study, seasonal coincidence of acute viral gastroenteritis outbreaks and monthly peak occurrence of enteric viruses (including HAstVs) in river water was observed [9]. Further combined surveillance on sewage and clinical patients at a much larger scale may provide valuable information on the early warning of outbreaks and the pathogenic role of novel astroviruses.

Our results showed that novel astroviruses were detected in gastroenteritis cases aged from 2 months to 58 years, and adults accounted for 33.3% of total detection, indicating further population-based surveillance should not just focus on children. Besides diarrhea, some novel astroviruses subtypes including MLB1, MLB2, and VA1 are linked to nervous system diseases in humans [1], their detection in sewage from China leads to pay attention at the public health level. Also, humans could be exposed to these viruses in sewage polluted surface waters. Evaluation of technologies for microbial removal should be considered in the city of Jinan.

In this study, we identified 18 sequences that cannot be classified into any known subtypes. VA5 reference strains had highest nt similarities (˜85%) with them. Using current molecular typing methods for group species assignment and identification of astroviruses, a distinct subtype within the same species is defined as sharing < 93–95% of nucleotide identity with a reference strain, or > 0.05 distance by phylogenetic analysis, based on the capsid gene sequence [1,2,10,11]. Hence, these 18 sequences might belong to a new subtype. Further complete genome sequencing of this clade is needed to ascertain whether these sequences can be proposed as a new subtype.

In order to increase the sensitivity and to avoid the interference of predominant strains on minor strains in sewage samples, we used a total of 7 specific primer pairs designed for each novel astroviruses, which means each sewage specimen was examined by 7 individual conventional RT-PCR reactions. This methodology inevitably increased the workload, but it had led to the finding of 6 known clades from sewage samples. It should be noted that the higher number of subtypes in sewage than that from clinical specimens is not likely to be the consequence of the different methods employed for environmental samples and clinical specimens, because by comparing the real-time PCR assay with the conventional PCR by using 184 clinical specimens in 2016, we obtained concordant 4 positive results in either assay (data not shown). The real-time and conventional RT-PCR detection method designed in this study will be useful for future astrovirus detection efforts.

Generally, Chinese strains within a clade have multiple lineages, suggesting that the circulation of these novel astrovirus in local population has been established for years. Previously, MLB1, VA1 and VA2 have been detected in human specimens across the world including Australia, Asia, Africa, North America and Europe [1,6,12–14]. The wide distribution of these 3 novel astroviruses is concordant with their frequent detection in sewage in this study. In opposite, VA5 has only been reported to be identified in two African countries, Gambia and Burkina Faso [3,4]. The frequent detection of VA5 in sewage in this study reflects its wide circulation in China.

There are some limitations in this study that could be addressed in future research. The sensitivity of the real-time PCR assay was performed only for MLB1, VA1 and VA2 genotypes. Also, no data have been provided regarding the efficiencies of sewage concentration method and conventional RT-PCR assay for different subtypes, so percentages of detections from sewage do not necessarily reflect the exact distribution in the sewage. In addition, the unclassified clade was not identified in human specimens but in sewage, which does not necessarily reflect human infection.

In conclusion, this study demonstrates the existence of multiple non-classical astroviruses in China and among these identifies a new clade which is genetically different with any of the current subtypes. Future screening for the presence of these novel astroviruses in diarrhea patients and other human population are needed to gain better insight into the epidemiology and pathogenesis of novel astroviruses.

Conflicts of interests

None declared.

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Author's contributions

AX and HW designed the study. WZ was responsible for clinical aspects and analyzed the results. ZT and WZ performed laboratory determinations and analyzed laboratory data. ZT drafted and revised the manuscript. All authors contributed to the final draft.

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