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Environmental Surveillance for Human  
Astrovirus in Shandong Province, China  
in 2013SUBJECT AREAS:  
GASTROENTERITIS  
WATER MICROBIOLOGY  
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Human astroviruses (HAstVs) are one of the leading viral agents of acute gastroenteritis. However, there is limited information on HAstVs in China. Here, we describe the molecular characterization of HAstVs in Shandong, China via sewage surveillance. A total of 23 sewage samples were collected from sewage treatment plants in the cities of Jinan and Linyi in 2013. After concentration via adsorption-elution method, 9 samples (39.1%) were positive by reverse transcription PCR (RT-PCR) for the presence of the 719-nt HAstV nucleotide sequence. Genetic cloning and sequencing were performed on positive PCR products, and 26 HAstV sequences were obtained. Phylogenetic analysis on these sequences revealed 4 genotypes (HAstV-1, -2, -4 and -5), with HAstV-1 and -5 as the most common genotypes in Jinan and Linyi, respectively. Homologous comparison revealed Shandong sequences had relatively less genetic divergence among themselves than with foreign sequences. This study represents the first effort to investigate the genotypes and molecular epidemiology of HAstVs via sewage surveillance in China. The high detection rate in this study reflects that HAstVs circulated at a relatively high frequency in the local population, and demonstrates that environmental surveillance is an effective method in investigating circulating HAstVs.

Human astroviruses (HAstVs) are non-enveloped and positive-sense single-stranded RNA viruses, which belong to the genus *Mamastrovirus*, family *Astroviridae*. Its genome consists of three open-reading frames (ORFs), ORF1a, ORF1b, and ORF2<sup>1</sup>. ORF1a and ORF1b encode the non-structural viral proteins, and ORF2 encodes the capsid protein precursor.

According to the sequence analysis based on 348-bp partial ORF2 coding region<sup>2</sup>, HAstVs can be divided into eight genotypes (HAstV-1 to HAstV-8). Of these, HAstV-1 has been recognized as the most frequent genotype throughout the world<sup>3</sup>, and the second common genotype differs in different regions<sup>4–6</sup>. The prevalence of HAstV infection is seasonal. HAstVs are mostly prevalent in winter in temperate regions, but in the rainy season in tropics<sup>4,7</sup>.

HAstVs are distributed worldwide and considered to be one of the leading viral agents of acute gastroenteritis, especially in young children. HAstV outbreaks have also been reported in many developed and developing countries<sup>8–10</sup>. Generally, the incidence of gastroenteritis induced by HAstVs was reported to be ranged from 2% to 9%<sup>4,11–16</sup>. Nevertheless, a higher rate of 26% was also reported<sup>17</sup>. In Wuhan of China, a study conducted during 2007–2008 revealed a positive rate of 13.6% for HAstV RNA in children with diarrhea<sup>18</sup>.

Raw sewage contains a mass of viruses<sup>19</sup>, mostly belonging to enteric viruses (including human enteroviruses, norovirus, rotavirus, human adenovirus, etc.), which can be shed by individuals and transmitted via the fecal-oral route. Some studies have been successfully conducted to detect these viruses in sewage<sup>20–25</sup>, and a few revealed a positive rate of HAstVs ranged from 6.3% to 89.7%<sup>19,26–28</sup>. Therefore, HAstVs in sewage may contaminate drinking water resources, and cause a high public health risk. Besides, owing to the sufficient information on virus in sewage and the absence of clinical viral diarrheas data in many countries, the surveillance of sewage is able to become an effective approach to reveal the molecular epidemiology of gastroenteritis viruses.

Since 2008, environmental surveillance has been successively conducted in our lab in monitoring polioviruses and other human enteroviruses<sup>25,29–32</sup>. In this study, we investigated the HAstVs in raw sewage in the cities of Jinan and Linyi of Shandong Province in 2013, to obtain the circulation and molecular characterization of HAstVs, and evaluate the possibility of the application of environmental surveillance in monitoring HAstVs.



## Results

**HAsTVs in raw sewage.** By using the virus concentration and RT-PCR, a total of 23 raw sewage samples were collected during the 12-month surveillance period in 2013. Of these, HAsTVs were detected in 9 samples (39.1%, 9/23), 4 (33.3%, 4/12) in Jinan and 5 (45.5%, 5/11) in Linyi, respectively. HAsTV sequences and positive sewage specimens were illustrated in table 1. In Jinan, the positive samples were mostly in winter (October, November, December and January). However, in Linyi, the positive samples did not show an apparent seasonal distribution (detected in April, May, June, October and November).

RT-PCR products were subjected to TA cloning, and positive recombinant clones were selected and sequenced. HAsTV sequences, together with some bacterial sequences from nonspecific amplification, were observed. A total of 26 HAsTV ORF2 sequences were detected, with 6 in Jinan and 20 in Linyi, respectively. After typed by sequence analysis, four genotypes, including HAsTV-1, -2, -4 and -5, were observed. HAsTV-1 was the most common genotype (53.8%, 14/26), followed by HAsTV-5 (38.5%, 10/26). All the 6 HAsTV sequences in Jinan belonged to genotype 1, whereas the 20 sequences in Linyi belonged to 4 different genotypes, including HAsTV-5 (50%, 10/20), HAsTV-1 (40%, 8/20), HAsTV-2 (5%, 1/20), and HAsTV-4 (5%, 1/20).

**Homologous comparison and phylogenetic analysis.** Homologous comparison and phylogenetic analysis were carried out among all 26 environmental sequences and 26 reference sequences of all eight HAsTV genotypes. Table 2 shows the alignment results of nucleotide and amino acid in acquired genotypes. Phylogenetic analysis revealed that Shandong HAsTV-1 sequences in this study can be divided into two clusters comprising 13 and 1 sequences, respectively (Fig 1). Fourteen environmental HAsTV-1 sequences displayed 92.0–100% nucleotide identity to each other. HAsTV-1 sequences in Jinan had 98.3–100% nucleotide identity, and the HAsTV-1 sequences in Linyi had 92.0–100% nucleotide identity among themselves. In the major cluster, the environmental sequences had 97.4–100% nucleotide identity to each other, and 97.9–99.9% identity to the Shanghai HAsTV-1 strain (FJ375759). In the minor cluster, the sequence LY45-2 was closely related to the Oxford strain (L23513) and a Hungarian strain (HQ398856), with 96.8% and 98.1% nucleotide identity, respectively. When compared with strains identified in other regions of China, including Beijing (FJ755403 and FJ755404), the Inner Mongolia (HM1200877) and Liaoning Province (KF211475), all the HAsTV-1 strains in two clusters displayed lower nucleotide identity (91.5 to 95.9%) to them. Moreover, no closer relationship was observed when compared with strains from other countries, such as Germany (AY720892), Korea (JN887820), and Italy (JX087965), with 91.7–92.3%, 92–92.4% and 91.6–92.2% nucleotide identity, respectively.

All HAsTV-5 strains in this study were segregated into one cluster, and the nucleotide identity among themselves was 99.3 to 100%. These sequences showed a close relationship with strains isolated in Liaoning Province of China (JQ403108) and Hungary (KF157967), with 98.3 to 98.7% and 98.1 to 98.3% identity,

respectively. However, great genetic divergence was observed in comparison with HAsTV-5 strains isolated in Beijing (AB037274) and Brazil (DQ028633), with 4.1–4.5% and 4.5–4.9% genetic difference, respectively.

The HAsTV-2 strain isolated in this study had a high similarity of 98% with a strain isolated in Italy (JX087964), and the HAsTV-4 strain displayed 93.7% and 94.4% identities to the reference strains from Brazil (DQ070852) and Germany (AY720891), respectively.

## Discussion

HAsTVs are one of the important viral agents of diarrhea. However, no specialized surveillance system on these viruses has been established in China. In this study, based on the well established enterovirus environmental surveillance system in Shandong Province, we investigated the prevalence and genetic characteristics of HAsTVs in 2013.

As a result, 23 raw sewage samples were collected and examined for HAsTVs, and 26 HAsTV sequences were obtained, with a high HAsTV-positive rate of 39.1%. This positive rate was in accordance with other reported detected frequencies. For example, in a previous report in Hungary, HAsTVs were detected in 43% of raw sewage samples<sup>19</sup>. A detection of enteric viruses in Germany environmental waters, depending on the samples sites, 24–42% of the samples from the sewage plant influent contained HAsTVs<sup>33</sup>. These high positive rates of HAsTVs in sewage reflect the high prevalence and circulation of HAsTVs in population, and the inhabitants are faced with a higher health risk.

Four genotypes were recognized in this study, in which HAsTV-1 accounted for the largest proportion (53.8%), suggesting that HAsTV-1 is the main prevalent genotype in the cities of Jinan and Linyi, consistent with studies in most countries<sup>5,12,34,35</sup>, including China<sup>11</sup>. In Jinan, only HAsTV-1 was identified, however, the absence of other genotypes does not mean that only HAsTV-1 was prevalent in Jinan, because the sample size was limited in this study. In Linyi, HAsTV-1, -2, -4 and -5 were detected, indicating the simultaneous circulation of different HAsTV genotypes in this region and the circulating genotype varies in different geographic location. In addition, the HAsTV-1 and -5 were the most prevalent genotypes in Linyi, and the HAsTV-5 isolates (accounting for 50%) even outnumbered the HAsTV-1 isolates (accounting for 40%), indicating that HAsTV-5 may be more frequent than HAsTV-1 in Linyi. This result is different from many studies mentioned above<sup>5,11,12,34,35</sup>. The small sample set may be a credible reason, however, the dominant genotypes can vary in different geographical areas, and the isolates from a study conducted in Houston and Mexico City were all HAsTV-5 after typed by RT-PCR<sup>36</sup>. Therefore, in spite of HAsTV-1 being the most common genotype worldwide, HAsTV-5 may be the dominant genotype in some areas. Further investigation should be conducted to demonstrate whether HAsTV-5 is the most frequent genotype in Linyi.

HAsTV-2 and -4 were detected at a low rate (both 5%). The emergence of HAsTV-2 and -4 suggests the need to monitor the minor HAsTV genotypes besides the overarching dominant genotypes. HAsTV-6 to -8 are rare all over the world<sup>5</sup>. In this study, HAsTV-3, -6, -7 and -8 were not detected, and it might be explained that these

**Table 1 | Numbers of HAsTV sequences detected in sewage in Jinan and Linyi in 2013, by month and genotype**

Genotype	Jinan												Linyi											
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
HAsTV-1	1									2	1	2					2					3	3	NA*
HAsTV-2																1								
HAsTV-4																							1	
HAsTV-5																	7	3						

\*NA, sewage samples were not available in December of Linyi.



Table 2 | Homologous comparison on ORF2 nucleotide and amino acid sequences

Genotype	Within Shandong sequences		With reference strains	
	Nucleotide (%)	Amino acid (%)	Nucleotide (%)	Amino acid (%)
HAsV-1	92.0–100.0	94.6–100.0	91.3–100.0	94.1–100.0
HAsV-2	/	/	92.2–99.4	95.4–100.0
HAsV-4	/	/	93.7–94.9	96.2–98.3
HAsV-5	99.3–100	98.7–100.0	95.1–100.0	97.9–100.0

genotypes were not prevalent in our sampling sites. However, HAsV-3 and -6 has been identified in the cities of Beijing, Wuhan and Jiangmen of China and other countries<sup>11,37–40</sup>. Furthermore, HAsV-3 was reported to be able to induce more severe gastroenteritis and shed more virus<sup>41</sup>. HAsV-8 also has been examined in Spain, the South Africa and Australia<sup>4,5,27</sup>. Therefore, more attention should be paid to the circulating possibility of these less frequent genotypes.

HAsV prevalence is known to be seasonal, and more HAsV infection was observed in winter and spring in temperate regions<sup>3</sup>. In this study, the seasonal distribution was observed in Jinan with most positive samples detected in winter, and no positive sample was detected in summer, which supports the epidemiology reports<sup>5,7</sup>. This phenomenon may be due to the reason that the viral stability increases in the lowered temperature<sup>42</sup>. However, the HAsVs detected in Linyi did not follow a seasonal pattern. Moreover, a study from Australia has reported that HAsVs could display an unusual biennial winter peak<sup>4</sup>, and the distinct winter peak may be not observed in each year. But, owning the biggest small commodity wholesale market in Northern China, Linyi is a city of frequent population flow. This is quite different from the situation in Jinan. This may partially explain the difference in detection between the two cities. Considering the limit of investigation period in this study, further study should be performed to testify whether the HAsV infection in Linyi follow this seasonal distribution.

According to the homologous comparison and phylogenetic analysis, the environmental HAsV-1 isolates can be divided into 2 clusters. The major viral cluster contain the sequences from Jinan and Linyi, indicating that one HAsV-1 transmission strain circulated in the two cities concurrently and the occurrence of frequent inter-city spread during the survey period. The HAsV-1 strains in Linyi belonged to 2 clusters, suggesting the presence of two HAsV-1 transmission links. Additionally, the strain found in Shanghai of China (FJ375759) had a close relationship (97.9–99.9% identity) with the major viral strain cluster. Therefore, it may be inferred that the strains in the major viral cluster may be transmitted from Shanghai, and these strains, along with the circulating strain in Shanghai should have the same ancestor. Another less transmission strain in Linyi was similar to a Hungarian strain (HQ398856, 98.1% identity). Though HAsV-1 has been identified in some regions in China<sup>37,38,43</sup>, no close relationship was observed when compared with other Chinese reference strains (Beijing, Liaoning and the Inner Mongolia).

The HAsV-5 strains in this study displayed a high identity to each other and were located in one cluster, indicating the circulating of one transmission link. A Hungarian HAsV-5 strain (KF157967) showed a high similarity with these strains, with addition of the relationship of HAsV-1 strain in Linyi and Hungary as is mentioned above, moreover, in 2010, a HAsV-gastroenteritis outbreak occurred in Hungary<sup>44</sup>. On this account, the HAsVs surveillance in Linyi should be strengthened to avoid the outbreak of HAsV-gastroenteritis. In addition, the HAsV-5 isolate in Liaoning Province of China displayed 98.3 to 98.7% identity with our isolates, so the common origin of HAsV-5 strains in Linyi, Liaoning Province and Hungary is probable. No appropriate HAsV-2 and -4 reference strains in China were found. By comparing them with

foreign strains, the two minor genotypes keep close relationship with Italian and Brazilian strain, respectively.

Environmental surveillance has been proved to be an alternate tool to obtain the genetic diversity of enteric viruses in urban population<sup>19–21,27</sup>. Data from sewage can provide an overview on the viral molecular epidemiology. In this study, a one-year environmental surveillance on HAsVs was conducted successfully, and the genetic characteristics of HAsVs in Jinan and Linyi was observed, which offers another convincing evidence on the benefit of environmental surveillance. And long-term surveillance of circulating viruses should be conducted in the inhabitants to control and prevent virus infection. The monitoring time and genotypes obtained are limited in this investigation, and due to the lack of clinical data, we did not compare the environmental isolates with the clinical isolates circulating, however, this study provides the only surveillance data on HAsVs via sewage surveillance.

In conclusion, this study proves the presence of HAsVs in raw sewage in Jinan and Linyi of China. The high detection rate of HAsVs suggests that HAsVs circulated frequently in the population. HAsV-1 and -5 are the most frequent genotypes in Jinan and Linyi, respectively. In the absence of clinical data on gastroenteritis viruses, environmental surveillance can be a sensitive approach to assess the presence of virus and characterize the viral genetic evolutionary relationships.

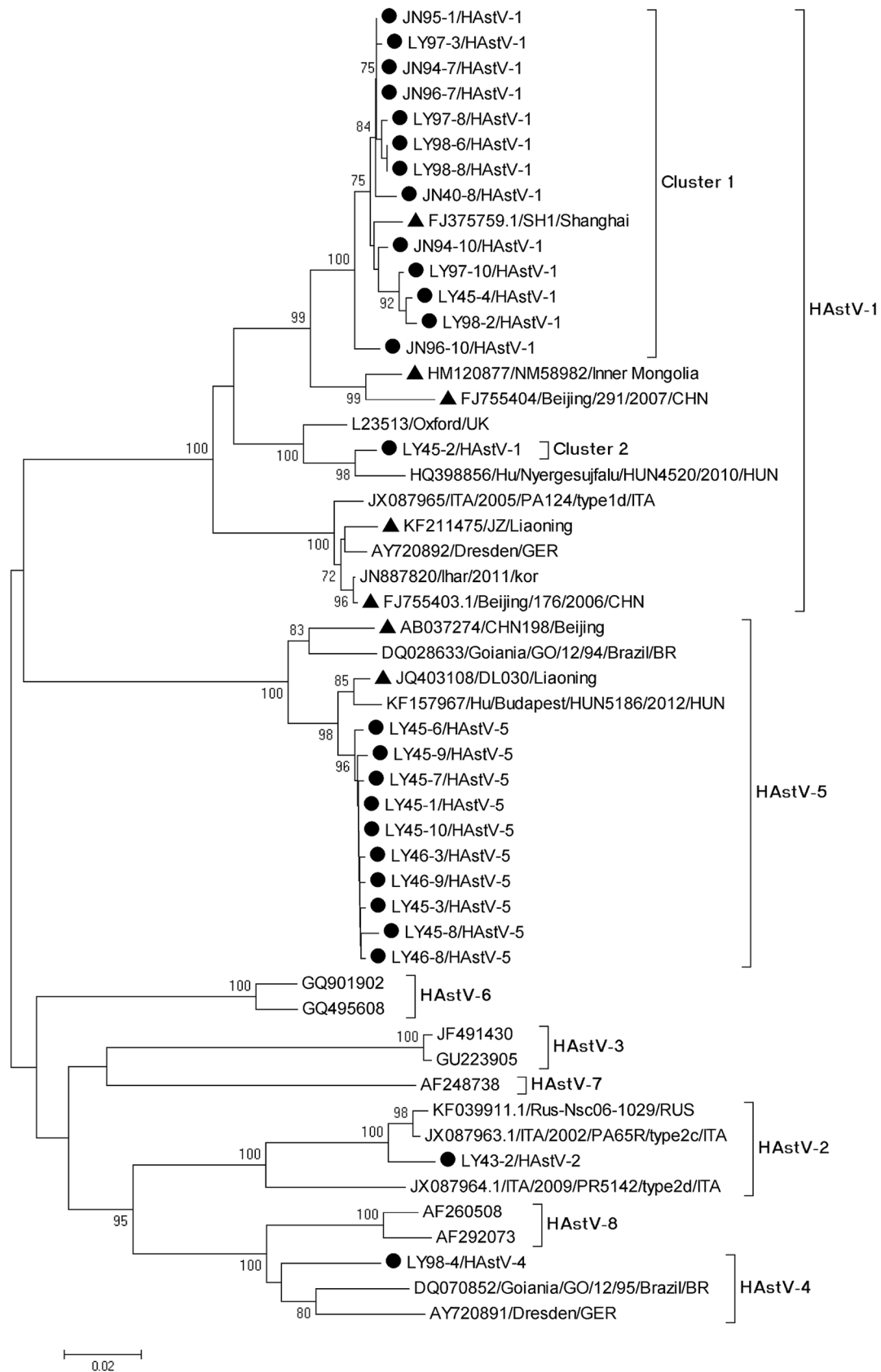
## Methods

**Sampling.** The permission for each sampling location was issued by Shandong Provincial Environmental Protection Department. From January to December in 2013, sewage samples were collected monthly in the inlet collector canal of the municipal sewage treatment plants, Jinan Everbright Water and Linyi Shouchuang Water, by using grab sampling procedure. The samples were transferred to the laboratory, stored at a cold temperature of 4°C, and processed within 24 h.

**Concentration.** Sewage samples were concentrated by adsorption-elution method as described previously<sup>20</sup>. Briefly, the raw sewage was centrifuged at 3000 × g for 30 min at 4°C. MgCl<sub>2</sub> was added to the 800 ml of the supernatant to a final concentration of 0.05 M, and the pH value was adjusted to 3.5 by hydrochloric acid. Then the supernatant was filtered through a mixed cellulose ester membrane filter (pore size of 0.45 μm, ADVANTEC, Tokyo, Japan). The membranes were eluted with 10 ml 3% beef extract solution (pH value, 9.0) by ultrasonication for 5 min. The eluted solution was centrifuged at 3000 × g for 30 min, and the supernatant was filtered through a 0.2 μm filter (PALL, Ann Arbor, USA).

**RNA extracted and reverse transcription-PCR.** Viral genomic RNA was extracted from 140 μl of concentrated sewage using QIAamp viral RNA mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommended procedure. Reverse transcription-PCR (RT-PCR) was carried out to amplify 719-nt HAsV ORF2 sequence (nt position 4235 to 4959 according to Oxford strain) by using Access RT-PCR System (Promega, USA), with primers pairs Precap1 (5' - GGACTGCAAAGCAGCTTCGTG - 3') and 82b (5' - GTGAGCCACCAGCCAT CCCT - 3'), which can amplify all classic serotypes of human astrovirus<sup>45</sup>.

**Cloning and sequencing.** The RT-PCR products were analyzed by electrophoresis with 1% agarose gels. The positive products were gel-purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA, USA). The purified products were ligated into the pGEM®-T Easy vector (Promega, USA) by TA cloning. The ligation products were transformed into competent *Escherichia coli* JM109 cells using the heat shock method. After blue and white screening, ten positive recombinant clones were selected for each transformation. The plasmid was extracted and sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Sequences were analyzed by an ABI 3130 genetic analyzer (Applied Biosystems). Molecular typing was performed by using BLAST.



**Figure 1** | Phylogenetic tree on 719-nt ORF gene of HAstV sequences. ● indicates the environmental isolates obtained in this study. ▲ indicates reference strains identified in China. “JN” and “LY” in the name stand for HAstV sequences from the cities of Jinan and Linyi, respectively.



**Homologous comparison and phylogenetic analysis.** BioEdit 7.0.5.3 software was used to carry out the nucleotide sequence alignments. The phylogenetic tree was constructed by the Mega, version 5.0, using the neighbor-joining method based on 719-nt sequences. The branching confidence was estimated by bootstrapping with 1,000 times. The reference strains are as follows: HAsV-1: L23513, KF211475, JX087965, JN887820, HQ398856, HM120877, AY720892, FJ755404, FJ755403, FJ375759; HAsV-2: KF039911, JX087964, JX087963; HAsV-3: JF491430, GU223905; HAsV-4: DQ070852, AY720891; HAsV-5: JQ403108, KF157967, AB037274, DQ028633; HAsV-6: GQ901902, GQ495608; HAsV-7: AF248738; and HAsV-8, AF292073, AF260508.

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## Author contributions

N.Z. drafted the paper, Z.T. and A.X. conceived the study, X.L. and S.W. gathered and analyzed the data, and H.W. and W.L. helped to interpret results and contributed to the writing. All authors reviewed the manuscript.

## Additional information

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