Intercity Spread of Echovirus 6 in Shandong Province, China: Application of Environmental Surveillance in Tracing Circulating Enteroviruses

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Environmental surveillance is an effective approach in investigating circulating enteroviruses and had been conducted in the cities of Jinan and Linyi since February 2008 and April 2010, respectively. This study analyzed 46 sewage samples collected in the two cities in 2011 and found that echovirus 6 (E6) was the predominant serotype, with 134 isolates (65 in Jinan and 69 in Linyi) from 23 (50%) samples. This differs from the 2010 data that found 29 E6 isolates in Jinan and only 3 in Linyi. Phylogenetic analysis of the VP1 coding region showed that all environmental E6 samples from 2008 to 2011 ($n = 167$) segregated into two lineages and revealed an increase in VP1 gene diversity in 2011, suggesting that the increased number of E6 detections reflects a real epidemic in the two cities. Most Linyi isolates ($n = 61$, or 88%) in 2011 segregated into sublineage 1a, together with 18 Jinan isolates in 2011. Interestingly, the ancestral VP1 sequence of sublineage 1a inferred using the maximum-likelihood method had 100% identity with the sequence of one environmental isolate from Jinan in August 2010, suggesting an intercity spread from Jinan to Linyi. By Bayesian phylodynamic methods, the most recent common ancestor of Linyi isolates in sublineage 1a dated back to 24 December 2010, revealing that this sublineage was likely imported into Linyi from August to December in 2010. This study demonstrates that environmental surveillance is a sensitive method in tracing transmission pathways of circulating enteroviruses among different regions and reveals that E6-associated aseptic meningitis is an emerging concern in China.

Human enteroviruses (HEVs) comprise more than 100 serotypes, including polioviruses (PVs), coxsackieviruses (CVs) A and B, echoviruses, and newer enteroviruses. HEVs are important human pathogens. They are frequently associated with some severe diseases in children, such as aseptic meningitis (AM), acute myocarditis, acute flaccid paralysis (AFP), and hand, foot, and mouth disease (HFMD), and most are emerging concerns in many part of the world (11, 17).

In China, HEV surveillance based on human specimens is very limited and mainly includes testing of specimens collected through AFP surveillance and HFMD surveillance. However, their application in understanding HEV circulation in a given period is limited because of the low incidence of AFP and the limited pathogen spectrum of HFMD. Environmental surveillance is recommended by WHO as a supplemental method to AFP surveillance for global poliomyelitis eradication (26). It has been revealed to be a sensitive method to monitor the circulation of PVs or nonpolio enteroviruses (NPEVs) (7, 28, 29). In China, continuous environmental surveillance is conducted at two member laboratories of the Chinese poliovirus laboratory network, and one of these is the Shandong Provincial Poliovirus Laboratory.

Environmental surveillance has been conducted in many parts of the world, and in most regions it has served primarily as part of PV surveillance (6, 18). However, gradually more studies have been published on the circulation and phylogenetic characterization of environmental NPEVs, such as the research work in the United States, Japan, Finland, Georgia, Greece, Iran, China, etc.

(1, 7, 9, 10, 12, 19, 21, 25, 27). A high degree of genetic diversity and multiple genetic lineages of environmental NPEVs were also found by studies conducted in Georgia and Shandong (10, 25, 27), respectively, which probably resulted from the evolution of endemic viruses over a long period or from the importation of viruses from other regions.

HEVs possess the ability to spread over large geographical areas. So, if environmental surveillance can be conducted in different regions, when an epidemic of associated disease occurs, the HEV transmission pathways among different regions can be determined via VP1 sequence analysis of environmental isolates. The sensitivity and continuity of surveillance are prerequisites in such circumstances. However, to the best of our knowledge, no such studies of NPEVs have been published yet.

Shandong is a coastal province with a large population (~96 million) and major ports that could potentially serve as portals for

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FIG 1 Locations of Shandong Province and the two cities where environmental surveillance was conducted. Maps were created using Mapinfo software; data are from the National Fundamental Geographic Information System (NFGIS) website (<http://ngcc.sbsm.gov.cn/>).

importation of exogenous viruses. So, environmental surveillance is of great importance in the early warning of related diseases. Surveillance has been conducted in the cities of Jinan and Linyi since 2008 and 2010, respectively. Previous surveillance data had revealed high echovirus 6 (E6) activity in Jinan in 2010 (25). In the present study, the surveillance in 2011 suggested high E6 activities in both cities. Furthermore, all VP1 sequences of environmental E6 isolates obtained since 2008 were phylogenetically analyzed, and a transmission pathway from Jinan to Linyi in 2010 to 2011 was identified. These findings underscore the value of continuous environmental surveillance and genetic analysis to trace HEV spread in different populations.

MATERIALS AND METHODS

Sampling sites. Shandong Province is located in the eastern part of China, with an area of 156,700 km² and a population of 95.79 million (2010 census data) (Fig. 1). Jinan is the capital city, and Linyi is the largest city in Shandong, with total populations of 6.8 million and 10.0 million, respectively. The sewer networks had been established in the metropolitan areas of the two cities, and the inlets to the sewage treatment plants, namely, Jinan Everbright Water (JNEW) and Linyi Shouchuang (LYSC), were the sampling sites.

Sampling, concentration, and virus isolation. Sewage samples were collected monthly in Jinan from January to December and semimonthly in Linyi from January to November in 2011. All samples were collected from the inlet collector canal by the grab sampling method in the afternoon between 1400 and 1500. Approximately 1 liter of flowing water was collected as a sewage sample. Two samples were collected in Jinan on each sampling day, and one was collected in Linyi. The temperature was maintained at approximately 4°C during sample transport to the laboratory, storage (<24 h), and processing. Sewage samples were concentrated for virus isolation using the anion filter membrane absorption method as

described previously (24, 27). Briefly, 800 ml of the sewage was centrifuged at $3,000 \times g$ for 30 min at 4°C, and 2.5 M MgCl₂ was added to the supernatant to a final concentration of 0.05 M. The pH value was adjusted to 3.5 by 0.5 M hydrochloric acid. Then the solution was filtered through a 0.45-μm-pore-size, mixed cellulose ester membrane filter (A045A142C; Advantec, Tokyo, Japan). Absorbents on the filter were then eluted with 10 ml of 3% beef extract solution by ultrasonication two times (1 min each time). After centrifugation at $12,000 \times g$ for 30 min, the supernatant was filtered through a 0.22-μm-pore-size filter and was ready for cell inoculation. So, each sewage specimen was concentrated from an initial 800 ml to two samples of 10 ml (40-fold). L20B, RD, and Hep-2 cell lines were used for virus isolation. For each cell line and each concentrated solution, nine parallel cell vials with standard monolayer cell culture were inoculated with 200 μl of concentrated solution for each vial.

E6 isolation from AM cases. There is no AM surveillance system for HEV in China. The AMES (acute meningitis and encephalitis syndrome) Project has been conducted via serological IgM examination on related viruses (Japanese B encephalitis virus [JEV], mumps virus [MuV], enterovirus, and herpes simplex virus [HSV]) from AMES cases in five sentinel hospitals in Jinan since 2007. So, the remaining cerebrospinal fluid (CSF) specimens ($n = 226$) from this project were used for HEV isolation in our lab. RD and Hep-2 cell lines were used for enterovirus isolation.

Extraction, VP1 amplification, and sequencing. Viral RNA was extracted from the infected cell cultures using a QIAamp viral RNA mini kit (Qiagen, Valencia, CA). Reverse transcription-PCR (RT-PCR) was performed using an Access one-step RT-PCR system (Promega). Primer pair 187/011 (16) that corresponds to the 3' end of VP1 and 5' end of the 2A protease was used for amplification of a 796-nucleotide (nt) sequence. In order to prevent cross-contamination, an RT-PCR using the RNA extracted from normal RD cells served as a blank control, and a negative control containing all the components of the reaction mixture except for the template was also included. PCR products were purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA), and the amplicons

FIG 2 Numbers of sewage samples and numbers of PV, E6, and other NPEV isolates in the cities of Jinan and Linyi, 2010 to 2011.

were bidirectionally sequenced using an ABI 3130 genetic analyzer (Applied Biosystems, Hitachi, Japan).

Homologous comparison, phylogenetic analysis, and ancestral sequence reconstruction. Nucleotide sequence alignments were carried out by BioEdit, version 7.0.5.3, software (4). The nucleotide sequence diversities of different lineages or sublineages on 684 nt (positions 2629 to 3312 on strain D'Amori) of the VP1 region were visualized by using an interactive and hierarchical multiple-logo visualization tool, Phylo-mLogo (22), based on nucleotide composition for grouping. Phylogenetic trees were constructed by Mega, version 5.0, using the maximum-likelihood (ML) method based on 684-nt partial VP1 sequences of environmental E6 isolates (3, 23). The ancestral state of the node (i.e., ancestral sequence) for sublineage 1a was reconstructed via the ML method using Mega, version 5.0, based on the 684-nt partial VP1 sequences of all environmental E6 isolates.

Bayesian evolutionary analysis. We inferred the time scale and tempo of E6 evolution using a Bayesian statistical approach implemented in BEAST, version 1.6.1 (2). The evolution rate (number of substitutions/site/day) and the time of most recent common ancestor (t_{MRC}) by day, with 95% highest posterior density (HPD) of environmental E6 isolates, were estimated based on the data of the sampling day recorded. We used the Hasegawa-Kishino-Yano (HKY) model of nucleotide substitution with gamma-distributed rate variation among sites and an uncorrelated log-normal distribution (UCLD) relaxed molecular clock model. A Markov chain Monte Carlo (MCMC) chain was run for 30 million steps and diagnosed by using Tracer (<http://beast.bio.ed.ac.uk/Tracer>). The evolutionary history was summarized in the form of a maximum clade credibility tree by using TreeAnnotator (<http://beast.bio.ed.ac.uk/TreeAnnotator>) and visualized by using FigTree (<http://tree.bio.ed.ac.uk/software/figtree>).

Nucleotide sequence accession numbers. The VP1 nucleotide sequences of E6 isolates described in this study were deposited in the GenBank database under the accession numbers JX138356 to JX138496.

RESULTS

Virus isolation. In this study, a total of 46 sewage samples, i.e., 24 in Jinan and 22 in Linyi, were collected in 2011. Seventeen (37.0%) samples were PV positive, yielding 39 PV isolates. The numbers of isolates for PV1, PV2, and PV3 were 9, 17, and 13, respectively. All were Sabin strains with no wild virus or vaccine-derived poliovirus (VDPV). PV isolation was distributed randomly throughout the year with no peaks. Thirty-four (73.9%) samples were NPEV positive, with 446 isolates. E6 was the most frequently isolated serotype. Twenty-three samples were E6 positive, with 134 isolates, or 30.4% of the total number (65 isolates from Jinan and 69 from Linyi).

Compared with 2010 surveillance data in Jinan, the number of samples increased by 100% in 2011 (Fig. 2). Accordingly, the numbers of isolates of PV, NPEV, and E6 increased by 180%,

168%, and 124%, respectively. The frequent detection of E6 from 2010 to 2011 revealed high local E6 activity in this period. In contrast, only three E6 isolates were detected in the 2010 surveillance in Linyi, unlike the frequent isolation in 2011, which revealed that E6 was active in Linyi in 2011. In evaluation of the RD and HEP-2 cell lines used to isolate the 167 E6 viruses detected from 2008 to 2012, 52.7% ($n = 88$) of viruses were isolated in RD cells, and 47.3% ($n = 79$) were isolated in HEP-2 cells.

The tempo of distribution of E6 and other NPEVs in 2008 to 2011 is illustrated in Fig. 3. In Jinan, NPEV isolation peaked in summer and autumn months. Frequent E6 isolation occurred in 2010 to 2011, and a similar seasonal pattern was also revealed. In other seasons, E6 and other NPEVs were detected at low frequencies. Similarly, in Linyi in 2011, isolation of E6 and other NPEVs peaked from July to November. In 2010, however, there were two periods when NPEVs were frequently isolated, from July to August and from November to December, and E6 was not a predominant serotype in Linyi in 2010.

E6 from AM cases. Altogether four E6 viruses were isolated from clinical CSF specimens of AMES surveillance in Jinan from 2007 to 2011, with two in 2007, one in 2010, and one in 2011. In 2010, another two E6 viruses (strains 2010LY059 and 2010D0010005) were also isolated from JEV surveillance (24).

Phylogenetic analysis and homologous comparison. To investigate the genetic relationships of environmental E6 viruses in 2011 to those isolated in 2008 to 2010, the 684-nt partial VP1 coding regions of all 134 environmental E6 isolates in 2011 were sequenced and phylogenetically analyzed with 33 previously isolated environmental viruses (E6) (see Table S1 in the supplemental material). As shown in Fig. 4, all environmental E6 isolates in 2008 to 2011 were segregated into two lineages. A total of 151 isolates segregated into a major lineage (lineage 1), while the other 16 belonged to a minor one (lineage 2) (Table 1). Members of lineage 1 were further divided into two major sublineages, 1a and 1b.

Homologous analysis revealed 79.8 to 82.4% nucleotide identity between the two lineages, with 92.5 to 100% within lineage 1 and 91.9 to 100% within lineage 2. The complete VP1 coding regions of six isolates from aseptic meningitis cases were sequenced and compared. Two isolates from 2010 and 2011 AMES surveillance and one isolate from JEV surveillance (2010LY059) belonged to lineage 1. They had 96.3 to 97.4% VP1 nucleotide identity with each other and 93.4 to 99.5% identity with environmental E6 in lineage 1; the closest (highest identity) relationship was with strain JNEW100811.2. The other one isolate from the JEV surveillance (2010D0010005) belonged to lineage 2. It had 92.8 to 97.6% VP1 nucleotide identity with environmental E6 isolates in lineage 2, with the closest relationship to strain JNEW100913.7. The two remaining AMES isolates in 2007 belonged to neither of these two lineages. They had 99.8% identity with each other and 79.3 to 84.8% identity with other AM isolates.

Ancestral reconstruction. In the phylogenetic tree based on VP1 sequences of all environmental E6 isolates, a close relationship was observed between strain JNEW100811.24 and the ancestral node of sublineage 1a (Fig. 4). Hence, the ancestor sequence of this node was inferred using Mega, version 5.0, via the ML method. Homologous comparison was performed between the inferred sequence and strain JNEW100811.24, and 100% identity was revealed, suggesting that JNEW100811.24 was the ancestor of sublineage 1a, which mainly consisted of Linyi E6 viruses from

FIG 3 Monthly distribution of isolates of two E6 lineages and other NPEVs in Jinan, 2008 to 2011 (A) (25) and in Linyi, 2010 to 2011 (B). (Adapted from reference 25.)

2011. The pathway of E6 transmission is illustrated in Fig. 5. Strain JNEW100811.24 from Jinan in 2010 served as the ancestor of a majority of isolates in Linyi in 2011 and of a considerable number of strains in Jinan in 2011.

The VP1 pairwise contrast of environmental E6 was visualized in Fig. 6. In the data set comprised of 79, 142, and 167 sequences, respectively, of sublineage 1a, lineage 1, and lineage 1 plus lineage 2 ([lineages 1 plus 2] all environmental E6 isolates), the frequency of each base at each position was determined. Nucleotide changes were identified in 27 positions in the VP1 regions of sublineage 1a when the range of the substitution per site ratio was set within 0.03 to 0.97 (Fig. 6). The nucleotide with the highest frequency at each position was identical with the correlating nucleotide of strain JNEW100811.24. There was an increasing trend in the number of nucleotide changes. That is in sublineage 1a, only a small number of nucleotide changes were found; more changes occurred in lineage 1, and the largest number of nucleotide changes was found in lineages 1 plus 2.

Origin, evolutionary rate, and molecular clock phylogeny of lineage 1. The 684-nt VP1 sequences of environmental E6 isolates of lineage 1 ($n = 151$) were analyzed for divergence time and evolutionary rate using the Bayesian MCMC method (Fig. 7). The collection date of the samples served as the time information of environmental isolates. The mean rate of lineage 1 is estimated as 3.024×10^{-5} substitutions per site per day (95% HPD, 2.289×10^{-5} to 3.716×10^{-5}). The t_{MRCA} of all Linyi members of sublineage 1a was traced back to 24 December 2010 (95% HPD). So,

under the assumption that it is an ancestor of a lineage—not of all the members of the lineage itself—that is spread between the two cities, the ancestor of this sublineage was likely to be imported into Linyi from between August and December 2010. The t_{MRCA} estimate for lineage 1 was dated to 22 June 2007 (Fig. 7).

DISCUSSION

In addition to contributing to the polio eradication program, environmental surveillance has shown its value in monitoring the circulating NPEVs. It is a useful approach to trace prevalent and minor circulating enteroviruses in human populations (7). Moreover, several studies had revealed a close relationship among environmental and clinical isolates, reflecting the potential power of environmental surveillance to herald epidemics in the context of the low morbidity/infection ratio of HEV infection (19). In regions where there is no specialized HEV surveillance system, such as the situation in China, environmental surveillance is of more significance in understanding HEV circulation.

The titers of HEVs in environmental specimens are lower than those in fecal specimens of infected humans. So, concentration is a necessary step before inoculation. The filter absorption method was used in this study, and this procedure is relatively more convenient and simpler than the two-phase separation method and polyethylene glycol (PEG) precipitation method (26). Although no systematic comparative studies on sensitivity have been published (6), the high HEV-positive rate and the large number of isolates detected in this study demonstrated the high efficiency of

FIG 4 Phylogenetic relationships among all E6 isolates from the environmental surveillance in the two cities from 2008 to 2011. The phylogenetic tree was constructed using Mega, version 5.0, using the ML method based on 684-nt (nt 2629 to 3312 of strain D’Amori) partial VP1 sequences. Branches in red and blue indicate E6 isolates from Linyi and Jinan, respectively. An arrow indicates the location of the isolate JNEW100811.24. For clarity, the name of each isolate is not shown in this figure; instead isolates are represented and distinguished by various colors for different sampling times.

the method used. In the cell culture approach, the abundance of predominant serotypes or lineages in the sewage might have the potential to interfere with the minor circulating serotypes or lineages present in the same samples. So, increasing the number of parallel vials inoculated will improve sample sensitivity (6). In this study, 18 parallel cell vials with standard monolayer cultures were used for each cell line, and along with the abundant isolation of predominant lineage 1 of E6, a minor circulating E6 lineage and other serotypes were also obtained. Another benefit from the increased number of inoculated vials is that more VP1 sequence data

will be achieved, especially for predominant serotypes or lineages. VP1 sequence is correlated with HEV evolution and widely used for molecular epidemiology investigations (7, 15). Hence, VP1 sequences of environmental isolates with records of collection

TABLE 1 E6 isolation from sewage in Shandong Province, 2008 to 2011

Sampling site	Year	No. of samples	No. of E6 isolates		
			Lineage 1	Lineage 2	Total
Jinan	2008	6	1	0	1
	2009	11	0	0	0
	2010	12	22	7	29
	2011	24	59	6	65
Linyi	2010	18	2	1	3
	2011	22	67	2	69
Total		93	151	16	167

FIG 5 The distribution of the two lineages of E6 in the two cities in 2010 and 2011. An E6 virus in Jinan in 2010 (JNEW100811.24) served as an ancestor of a majority of isolates in Linyi in 2011, suggesting possible intercity transmission.

FIG 6 Sequence logo showing nucleotide diversity in the 684-nt VP1 region among the environmental E6 isolates in the two cities in 2008 to 2011. More mutated positions occurred in lineages 1 and 2 combined (all environmental E6 isolates) than in lineage 1 and sublineage 1a. The sequence logo was constructed using Phylo-mLogo. The ranges of the substitutions per site ratios are set as 0.03 to 0.97, 0.1 to 0.9, and 0.15 to 0.85 for sublineage 1a, lineage 1, and lineages 1 plus 2, respectively.

dates in the context of long-term surveillance will provide valuable information for comprehensively exploring viruses in local circulation.

HEV infection typically peaks in the summer months in temperate climates (17, 20). In this study, the monthly fluctuation of the numbers of isolates reflected similar seasonal patterns (Fig. 3). Low yield or negative results occurred in winter and spring, and peaks occurred in summer and autumn for both E6 and total NPEVs, demonstrating that environmental surveillance is useful for establishing temporal patterns of circulation. However, in interpreting the surveillance results, two factors should be considered. First, the solids in the sewage were removed in the procedure

of concentration. This will result in the loss of solid-associated viruses. Second, some serotypes, such as some coxsackie A viruses of HEV-C species, cannot produce visible cytopathic effects (CPE) in these cell lines. So, these viruses cannot be recovered in the cell culture method. Considering these two factors, the actual HEV distribution in the sewage might not be completely identical with that reflected by this study.

In the environmental surveillance in 2011, the frequent E6 isolation revealed a large number of infected individuals in the two cities. According to the results of serological examination of serum specimens from AMES cases in Jinan in 2010 to 2011, HEV was demonstrated to be the predominant pathogen (positive rate,

FIG 7 Origin of lineage 1 of E6 in the two cities. The 684-nt VP1 sequences of environmental E6 isolates of lineage 1 ($n = 151$) were analyzed for divergence time and evolutionary rate using the Bayesian MCMC method. (A) MCMC tree of lineage 1 viruses visualized in FigTree. The two major sublineages are highlighted. An arrow indicates the position of isolate JNEW100811.24. (B) The date of the MRCA of lineage 1 of E6 was estimated to be 22 Jun 22 2007 using a relaxed molecular clock model implemented in BEAST and visualized in Tracer. The numbers on the horizontal axis indicate the days before 25 November 2011, when the last batch of E6 viruses of lineage 1 was isolated.

37.9%), followed by HSV and MuV (13). Also, 10 enteroviruses were isolated from limited CSF specimens, including the two E6 strains which were genetically closely related to environmental viruses. Considering the high E6 activity reflected by environmental surveillance and the intimate genetic relationship between environmental and AM isolates, it is reasonable to assume that E6 was responsible for a considerable proportion of AM cases in these 2 years.

Several studies had assessed the sensitivity of PV environmental surveillance. Hovi et al. (5) reported that by analyzing a single 400-ml specimen, PV circulation could be detected if about 100 individuals were infected with PV in a population of 700,000 people. Lodder et al. (14) found that it is possible to detect 100 PV-infected individuals in a population of tens of thousands uninfected individuals. In our situation, both the JNEW and LYSC plants treated 200,000 tons of sewage daily. So, based on our procedure of concentration and inoculation and assuming (i) that the virus excreted into stools is 10^7 infectious doses/day/person, (ii) that the viruses are dispersed uniformly in the sewage, and (iii) that the recovery rate of the filter method is 50%, it can be calculated that one isolate from 0.8 liters of sewage corresponds to 140 infected individuals. Following this inference, the number of infected persons of a certain serotype can be deduced. For example, the 15 E6 isolates detected in Linyi in August 2011 suggested 2,100 infected individuals at that time. The inference is theoretical, but it might explain why a high degree of genetic diversity of NPEVs can be observed (10), even from a single sewage specimen (25, 27). The viruses from sewage are from a population, not a single person. So, when viruses with the same serotypes are simultaneously isolated from a sewage specimen, they probably derive from a population of several thousands and represent respective evolution positions in the transmission chain. Hence, the high diversity among the isolates is observed.

Environmental surveillance was initially conducted in Jinan. Linyi was selected as another surveillance site because of frequent NPEV-associated AM outbreaks in recent years in an effort to evaluate the prevalence and to monitor the circulation of HEVs. Our study showed that the predominant transmission lineage of E6 was active in Jinan in 2010 to 2011 and in Linyi in 2011. Furthermore, the results of VP1 sequence analysis revealed that the VP1 sequence of strain JNEW100811.24—an environmental isolate from Jinan in 2010—was identical with the reconstructed ancestor sequence of sublineage 1a, which mainly consisted of Linyi E6 viruses isolated in 2011, suggesting the existence of a transmission pathway from Jinan to Linyi of this lineage. It is worth noting that this is the first time that environmental surveillance has been applied to trace NPEV transmission within two human populations. Nevertheless, because environmental surveillance cannot provide information on the infected person, the transmission chains existing in the populations cannot be determined. Molecular clocks for other enteroviruses had been inferred previously (8). In this study, the estimated higher evolution rate of lineage 1 than that of global E6 (25) suggested that lineage 1 evolved faster than other E6 members.

In conclusion, this study demonstrates high E6 activities in the two cities and revealed a transmission pathway from Jinan to Linyi, demonstrating that environmental surveillance can be applied to trace HEV transmission among different regions.

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