other than PI kinases as targets for anti-PV activity and that the role of GBF1 in the inhibitory effect of enviroxime-like compounds remains to be further elucidated.

We tested the specificity of the effects of enviroxime-like compounds on PV and HCV replication because PI4KB has been suggested as a host factor of HCV replication by experiments using PIK93 and siRNA (13, 30). However, Berger et al. showed that PI4KA, but not PI4KB, is required for HCV replication by providing biological evidence (colocalization of PI4KA with the double-stranded RNA [dsRNA] and NS5A and cofractionation with NS5A) and using siRNA (10). An inhibitory effect of PIK93 on both PV and HCV replication was observed, but the effect on HCV replication was weaker than that on PV replication (Fig. 5 and Table 1), and T-00127-HEV1 did not show any anti-HCV activity. Surprisingly, AN-12-H5 showed the highest anti-HCV activity among the compounds. Our data support that PI4KB activity is not essential for HCV replication, consistent with the report by Berger et al. (10), and that PI4KB is an enterovirus-specific host factor.

Enviroxime-like compounds have been identified with a high hit rate, i.e., approximately 0.1% of analyzed compounds, in small-scale screenings (4, 5). Identification of the cellular protein kinase inhibitors GW5074 and Flt3 inhibitor II as enviroxime-like compounds, both of which were suggested to interact with ATP-binding sites of kinases (32, 35), suggested that a structural similarity to ATP is essential for the enviroxime-like activity. This common structural requirement might have contributed to the high hit rate for enviroxime-like compounds. Another factor contributing to the high hit rate would be the apparent low cytotoxicity of enviroxime-like compounds. This might be attributable to a relatively high specificity of enviroxime-like compounds for limited sets of cellular protein kinases and PI kinases (6, 32) (see the supplemental material). In our current screening, four out of five identified candidate compounds were enviroxime-like compounds (data not shown). We also identified 10 noncytotoxic compounds that target the early stage of infection, possibly capsid-binding inhibitors, in the current screening (data not shown). This suggested that enviroxime-like compounds and capsid-binding inhibitors are predominant groups of anti-PV compounds with low cytotoxicity.

In summary, we identified a novel enviroxime-like compound, T-00127-HEV1, that is a highly specific inhibitor of PI4KB. T-00127-HEV1 did not inhibit HCV replication, in contrast to PV replication. Another enviroxime-like compound, AN-12-H5, did not show any inhibitory effect on PI4KB activity, and its anti-PV activity was not affected by knockdown of PI4KB. These results suggested that PI4KB is an enterovirus-specific host factor required for the replication process and targeted by some enviroxime-like compounds (T-00127-HEV1 and GW5074) and that enviroxime-like compounds may have targets other than PI kinases for their antiviral effect.

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Original Article

An Enterovirus 71 Epidemic in Guangdong Province of China, 2008: Epidemiological, Clinical, and Virogenic Manifestations

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SUMMARY: Enterovirus 71 (EV71) is shown to be a major causative agent in outbreaks of hand, foot, and mouth disease (HFMD) reported in Guangdong (GD) Province of China in 2008. A total of 48,876 HFMD cases (131 severe and 21 fatal) were reported to the GD HFMD web-based surveillance system, which covers 871 clinics. The main causes of death included central nervous system damage, heart failure, and pulmonary edema. The incidence rate was 52 per 100,000, and the epidemic peak appeared in May and June. EV71 was found in 59% and coxsackievirus A16 in 26% of 936 laboratory-confirmed cases. Other viruses are likely to be responsible for the remaining 15% of cases. Of the 185 EV71 cases collected, 62% were mild, 27% were severe, and the remaining 11% were fatal. A total of 17 EV71 isolates were subjected to nucleotide sequencing of the entire VP1 gene. Phylogenetic analysis showed that the GD EV71 strains belonged to the C4 subgenotype and that EV71 circulates at a national rather than a regional level. A comparison with the VP1 gene from a different clinical case showed that there was no obvious virulence determinant in this locus. Furthermore, this study found that most deaths occurred in rural areas, thereby indicating that delayed diagnosis and incorrect treatment may play an important role.

INTRODUCTION

Hand, foot, and mouth disease (HFMD) is a common childhood infection that is characterized by fever, rash on the hands and feet, and mouth ulcers. HFMD usually resolves spontaneously, although it can be life-threatening if the virus causes inflammation of the brain stem, which can progress to heart failure and pulmonary edema. It is most commonly associated with coxsackievirus A and enterovirus 71 (EV71). Indeed, EV71-related HFMD has been linked to major outbreaks involving infections with severe neurological complications and death (1-5).

Since the first report of EV71 in California, USA in 1969 (6), there have been sporadic outbreaks in Australia, Sweden, the USA, Bulgaria, and Hungary. The EV71 outbreak in Bulgaria in the 1970s resulted 750 infections, 149 cases of paralysis, and 44 deaths (7). The prevalence of EV71 infection in the Asia-Pacific region has increased greatly since 1997. The outbreaks in Malaysia (8), Singapore (9), and Taiwan (10) caused 129,106 cases in 1998 (10), including 405 cases of severe neurological disease and 78 deaths. These deaths were due primarily to the development of brainstem encephalitis and neurogenic pulmonary edema.

The first cases of HFMD on mainland China were reported in Shanghai in 1981, with Beijing (11), Jiangsu (12), Zhejiang (13), Shandong (14), Guangdong (15,16,18), Jilin (17), and Fujian (18) provinces having reported cases since then. However, few epidemiological and laboratory data were collected due to the absence of a surveillance network.

An epidemic outbreak of EV71-related HFMD in Anhui Province, China in 2008 resulted in the deaths of many infected children. As EV71 infection is life-threatening for children, HFMD was listed as one of the category "C" notifiable diseases in China in May 2008. In the same year, several epidemic HFMD outbreaks with EV71 as the major causative agent occurred in Guangdong (GD) Province. Thus, a total of 48,876 HFMD cases, including 131 severe cases, 21 deaths, and 551 confirmed EV71 infections, were reported, with an incidence rate of 52 per 100,000.

GD, which is located in the south of China, is a subtropical region that neighbors Jiangxi, Hunan, and Guangxi provinces as well as Hong Kong and Macao. It covers an area of some 179,800 km^2 and has a population of around 94,490,000, with a population density of 526 persons/km^2. GD has 21 cities, 9 of which are located on the Pearl River delta, with the remaining 12 grouped into the eastern, western, and mountainous regions.

Several cities in GD reported HFMD outbreak in the period 1999 to 2004. However, due to the absence of a

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surveillance system before 2008, very little is known about these epidemics. This study was therefore conducted to try to gain an understanding of the epidemiology of EV71 in GD. All of the laboratory-confirmed cases obtained from HFMD surveillance data in GD in 2008 were analyzed retrospectively. Information regarding age, region and time distribution, and clinical and etiological features was collected. Furthermore, the complete VP1 gene from 17 typical isolates was sequenced and investigated to determine their genetic diversity. This study therefore provides meaningful information regarding the epidemiology of EV71 in GD which could contribute to subsequent surveillance and treatment methods in this region.

MATERIALS AND METHODS

Clinical case definition: HFMD was defined as fever, accompanied by oral ulcers and a maculopapular or vesicular rash on the hands and feet, with or without buttock involvement. This definition for diagnosis and notification of HFMD was provided in a guidebook that is available to all surveillance sites and their practitioners.

Severe HFMD was defined as a patient with or without a clinical manifestation of HFMD, who had signs or symptoms indicating a more serious illness. These included high fever, myoclonus, encephalitis, acute flaccid paralysis, pulmonary edema, or heart failure.

An HFMD case was considered confirmed with a positive laboratory result, such as enterovirus isolated from a throat swab, rectal swab, feces, cerebrospinal fluid (CSF), or other specimen, a positive IgM test, or acute and convalescent IgG 4-fold lever up, or PCR positive.

Surveillance: The GD web-based infectious disease surveillance system was established in 2004, and a total of 2,789 clinics have since joined this system. HFMD was defined as a category C notifiable disease in May 2008, thus meaning that all clinical and laboratory-diagnosed cases must be reported via this disease surveillance and information management system within 24 h of diagnosis. Standards for the clinical and laboratory diagnosis of HFMD cases can be viewed on the Ministry of Health (MOH) website. In 2008, 871 clinics in GD reported at least one HFMD case, and a total of 1,259 cases were sampled.

For the purpose of this study, the local center for disease control and prevention (CDC) in 21 cities and 121 counties reviewed the records of all HFMD cases notified to the MOH by medical practitioners and conducted follow-up investigations, particularly for cluster cases, severe cases, and those that resulted in death. The data collected including demographic information, epidemiological data, clinical visits, other clinical features, as well as laboratory data regarding any enteroviruses detected.

Each month, the local CDC submitted all case-investigation records, along with standard survey questionnaires, to the provincial department for disease control and prevention (GDCDC).

To ensure quality control, the GDCDC held training workshops on HFMD surveillance techniques for local practitioners and epidemiological and laboratory staff. Standard operating procedures for surveillance and laboratory testing were also provided. All reports were reviewed and analyzed by the GDCDC.

Enterovirus isolation and identification: Laboratory notifications of enteroviruses identified in stool samples, throat and rectal swabs, and swabs from vesicular fluid and oral ulcers collected from outpatient and inpatient cases of HFMD, and CSF from some severe cases and deaths. Human rhabdomyosarcoma (RD) cells were used to isolate viruses from these specimens. Thus, cultures that exhibited a characteristic enterovirus cytopathic effect were evaluated by RT-PCR and sequencing, then viral RNA was extracted from the clinical specimens using a QIAamp Mini Viral RNA Extraction Kit (Qiagen, Valencia, Calif., USA). The entire VP1 gene of the EV71 strains isolated was amplified by RT-PCR using in-house primers that flank the VP1 gene, namely EV71-VP1-S 5′-GCAGCCCAAAAGAA CTTCAC-3′ (nucleotides 2372–2392 from strain EV71/BrCr) and EV71-VP1-A 5′-AAGTCGCGAGA GCTGTCTTC-3′ (nucleotides 3434–3454 from strain HEV71/BrCr) (19). The RT-PCR reactions were performed using a QIAGEN One-Step RT-PCR Kit. The PCR products were subsequently purified using a QIAquick Gel Extraction Kit (Qiagen) and the amplicons bidirectionally sequenced using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Hitachi, Japan). The entire VP1 nucleotide sequences of the EV71 isolates were aligned using the Clustal W software. A phylogenetic tree was constructed using the neighbor-joining method in the MEGA program (Sudhir Kumar, Arizona State University, Ariz., USA) (20).

Statistical analysis: All HFMD data were managed and compiled into a database using EpiData (The EpiData Association, Odense, Denmark). Excel (Microsoft, Redmond, Wash., USA) was used to analyze descriptive variables, including age, gender, disease duration, clinical level of first diagnosis, symptoms at first diagnosis, clinical symptoms, and cause of death.

Nucleotide sequence accession numbers: The entire VP1 nucleotide sequences of the 17 GD EV71 strains isolated in this study were deposited in the GenBank database under the accession numbers HM037792–HM037808 (see Table 4).

RESULTS

Distribution of patients: According to the provincial surveillance system, GD reported a total of 48,876 HFMD cases in 2008 including 131 severe cases and 21 fatalities. The overall incidence rate was 52 per 100,000. The HFMD epidemic in GD lasted throughout the year, with two periods of high prevalence in weeks 17–19 (25,908 reported cases) and weeks 42–45 (5,665 reported cases) (Fig. 1). The majority of cases occurred in young children and were geographically distributed in the Pearl River delta. Indeed, 71% of cases (34,511/48,876) were reported in children younger than 5 years of age and 70% (34,407/48,876) in children younger than 3 years of age. The male-to-female ratio was 1.84:1. EV71 was detected in 59% of the 936 laboratory-confirmed cases, with coxsackievirus A16 being responsible for a further 26% and other enteroviruses for the remaining

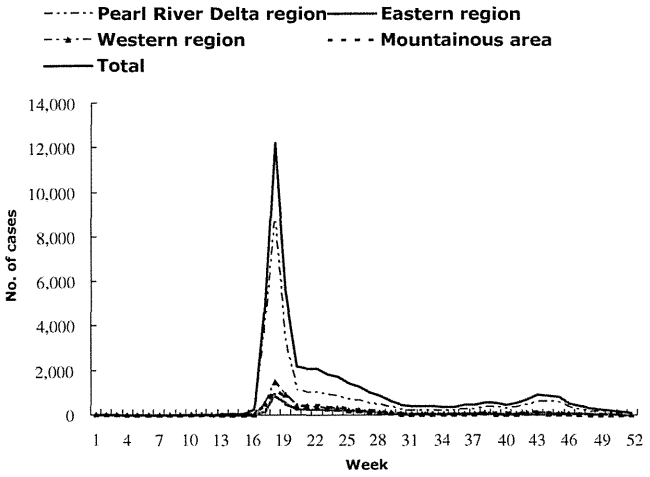

Fig. 1. Residence distribution of hand, foot and mouth disease (HFMD) cases in Guangdong Province, China in 2008.

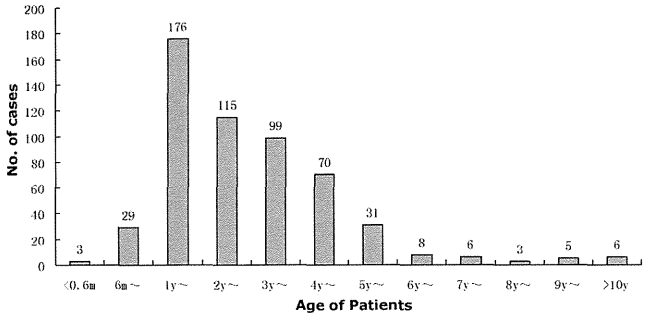

Fig. 2. Age distribution of patients with enterovirus 71 infection in Guangdong Province, China in 2008.

Table 1. General clinical manifestation of 185 enterovirus 71 (EV71) infected patients

Symptom/sign	No. of patients (%)
Skin rash	176 (95)
Hand	172 (93)
Foot	163 (88)
Buttock	47 (25)
Oral ulcer	66 (36)
Fever (°C)	117 (63)
37-	12 (6)
38-	45 (24)
>39	60 (32)
Cough	37 (20)
Vomiting	30 (16)
Sore throat	29 (16)
Runny	17 (9)
Headache	6 (3)
Nausea	8 (4)
Abdominal pain	7 (4)
Diarrhea	5 (3)
Emotional disturbance	6 (3)
Lethargy	8 (4)
Conscious disturbance	6 (3)
Coma	3 (2)
Convulsion	3 (2)
Arrhythmia	8 (4)
Stiffneck	7 (4)
Babinski sign	7 (4)
Kernig sign	2 (1)
Brudzinski's sign	3 (2)
Abnormal tendon reflexes	7 (4)
Abnormal muscular tension	10 (5)

15%.

Patient description: The male-to-female ratio in the 551 laboratory-confirmed EV71 cases was 1.76:1. The age distribution of these patients are shown in Fig. 2. The median age was 2 years (range, 2 months to 25 years), with 422 cases being reported in children younger than 3 years of age, 523 cases in those younger than 5 years of age, and 3 cases in infants aged less than 6 months. Most EV71 cases occurred between weeks 18 and 20.

Clinical presentations and outcome of the patients:

(i) Disease categories: Complete data were available for 185 of the 551 confirmed EV71 cases (34%), 62% of which were mild cases, 27% were severe, and 11% resulted in death. Some 82% of cases required admission to hospital, with a median duration from onset to admission of 1 day (range, 0-10 days). Complete medical records were available for 62 hospitalized cases, and the average course of the disease was 6 days (range, 1-14 days).

(ii) General clinical presentations: The most common symptom was rash (95%), most often on the hands (93%), feet (88%), mouth (36%), and buttocks (25%). Some patients also presented fever (63%), cough (20%), and vomiting (16%), and a small number had neurological symptoms (Table 1).

(iii) Complications: Of the 185 EV71 cases investigated, 11 had neurological symptoms (7 encephalitis, 1 paralysis) and 9 had respiratory symptoms (5 neurogenic pulmonary edema, 4 pneumonia).

(iv) Death: Death occurred in 21 cases, with more male than females being affected. Around 86% of deaths occurred in patients aged between 1 and 2 years. The average course of the disease was 4 days (range, 1-9 days), with 71% lasting for between 3 and 7 days. A total of 76% of these death cases visited a county-level clinic or below initially, with 67% being diagnosed with HFMD and 33% with upper respiratory tract infection, stomatitis, pneumonia, and eczema. Around 95% of death cases presented with fever, which was moderate for 67%. All cases presented a rash, mainly on the hands and feet, which in some cases was accompanied by vomiting (48%), arrhythmia (29%), consciousness disturbance (24%), lethargy (19%), emotional disturbance (19%), coma (14%), and convulsions (14%). Encephalitis was the cause of death in 44% of cases, with respiratory failure as a result of neurogenic pulmonary edema and viral pneumonia accounting for 28% of cases, multiple organ failure as a result of septic shock 17% of cases, and circulatory failure 11% of cases (Table 2).

Virogenic presentations and patient outcomes: The 17 viruses studied herein were isolated from samples submitted from all over GD, including the Pearl River delta and the eastern, western, and northern regions. The age of the patients from which they were isolated ranged from a few months to 5 years, and the severity of the resulting disease could be divided into mild, severe,

Table 2. Characteristics and symptoms of the 21 fatal patients with confirmed EV71

Characteristic		Value
Male sex	no./total no. (%)	12/21 (57)
Age		
Median (range)		1 y 9 mo (9 mo–5 y)
Age group	no./total no. (%)	
<1 y		1/21 (5)
1 y-		12/21 (57)
2 y-		6/21 (29)
3 y-		0 (0)
4 y-		1/21 (5)
≥5 y		1/21 (5)
Course of disease		
Median (range)		4 days (1–9 days)
1–2 days		5/21 (24)
3–4 days		8/21 (38)
5–6 days		7/21 (33)
7–9 days		1/21 (5)
First diagnosis clinic level		
Village		10/21 (48)
County		6/21 (29)
City		5/21 (24)
First diagnosis		
HFMD		14/21 (67)
Upper respiratory infection		4/21 (19)
Stomatitis		1/21 (5)
Pneumonia		1/21 (5)
Eczema		1/21 (5)
Clinical symptom		
Fever (°C)		20/21 (95)
38–		14/21 (67)
≥39		6/21 (29)
Rash		21/21 (100)
Vomiting		10/21 (48)
Arrhythmia		6/21 (29)
Consciousness disturbance		5/21 (24)
Emotional disturbance		4/21 (19)
Lethargy		4/21 (19)
Coma		3/21 (14)
Convulsion		3/21 (14)
Diagnosis of the cause of death		
Encephalitis		8/18 (44)
Respiratory failure		5/18 (28)
Septic shock		3/18 (17)
Circulatory failure		2/18 (11)

and fatal (Table 3). These isolates are thus likely to represent the EV71s circulating throughout GD. To determine the molecular epidemiology of the GD EV71 strains, a phylogenetic tree was constructed from these 17 strains, together with 5 strains from 5 provinces in mainland China that circulated in the period 2003–2008, and 10 international EV71 strains representing all 11 known subgenotypes (A, B1–B5, and C1–C5) (Fig. 3, Table 4). Similar to the cases with the EV71 sequences from mainland China isolated during 2008, the GD EV71 strains clearly belong to subgenotype C4. When compared with the Beijing, Anhui, and Zhejiang isolates from 2008, the percentage VP1 identity of these GD isolates was 95.1–97.3%, 93.8–99.1%, and 94.2–98.4%, respectively. The identity of VP1 sequences among mild, severe, and fatal cases was greater than 95.0%.

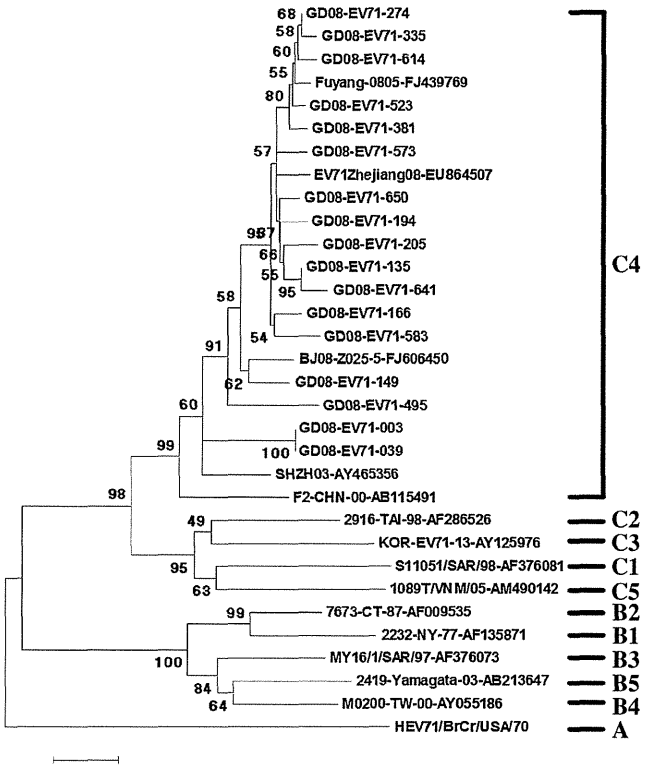

Fig. 3. Phylogenetic analysis of 17 Guangdong (GD) EV71 strains with other found worldwide based on partial VP1 gene sequence. The phylogenetic tree was constructed by the neighbor-joining method with MEGA version 4 software, and the reliabilities indicated at the branch nodes were evaluated using 1,000 bootstrap replications. The prototype a type BrCr strain was used as an out-group.

DISCUSSION

HFMD was epidemic in GD in 2008, with EV71 being the main causative agent. Since May 2008, HFMD has been a category C notifiable disease in China, which means that all cases must be notified to the MOH within 24 h of diagnosis. There are currently 871 clinics in the GD HFMD web-based surveillance network. The highest incidence in the outbreak studied herein occurred in May and June. Most cases involved young children between 2 and 4 years of age, the majority of whom lived in the Pearl River delta. The population density in this area (863 per km^2) is higher than average, and at the time there were 9.5 million susceptible children under 14 years of age living in the Pearl River delta (50% of those in the provinces as a whole).

The EV71 cases reported in GD presented typical HFMD symptoms, with the most severe cases also presenting encephalitis, neurogenic pulmonary edema, and/or septic shock. Eighty-six percent of the patients who died came from rural areas, where poor health awareness among parents often means that they fail to take affected children to the clinic in good time. Furthermore many clinics in villages or counties do not have the appropriate facilities and/or equipment to identify cases. About 33% of deaths were diagnosed as being due to upper respiratory tract infection or other

Table 3. Epidemiological information of 17 EV71 strains isolated in Guangdong in 2008

Strain	Gender	Age	Place	Onset date (y/m/d)	Case	Specimen
GD08-EV71-003	Male	1 y 8 mo	Foshan	2008-4-23	Fatal	Feces
GD08-EV71-039	Male	1 y 4 mo	Foshan	2008-4-28	Mild	Feces
GD08-EV71-135	Female	1 y 8 mo	Qingyuan	2008-5-5	Severe	Feces
GD08-EV71-149	Female	3 y 8 mo	Zhanjiang	2008-5-3	Severe	Rectal swabs
GD08-EV71-166	Male	6 mo	Foshan	2008-5-5	Severe	Feces
GD08-EV71-194	Male	3 y 3 mo	Foshan	2008-5-5	Mild	Feces
GD08-EV71-205	Female	1 y 7 mo	Meizhou	2008-5-9	Mild	Vesicle fluid
GD08-EV71-274	/	5 y	Donguang	2008-5-11	Mild	Rectal swabs
GD08-EV71-335	Male	1 y 6 mo	Yunfu	2008-5-12	Fatal	Feces
GD08-EV71-381	Female	4 mo	Meizhou	2008-5-8	Severe	Throat swabs
GD08-EV71-495	Female	2 y 7 mo	Meizhou	2008-5-22	Mild	Feces
GD08-EV71-523	Female	3 y	Shenzhen	2008-5-23	Severe	Rectal swabs
GD08-EV71-573	Male	1 y	Zhuhai	2008-5-2	Severe	Feces
GD08-EV71-583	Male	5 y	Dongguan	2008-6-17	Severe	Throat swabs
GD08-EV71-614	Male	4 y	Qingyuan	2008-6-21	Mild	Feces
GD08-EV71-641	Female	5 y	Yunfu	/	Fatal	Feces
GD08-EV71-650	Male	4 y 6 mo	Huizhou	2008-8-12	Fatal	Throat swabs

Table 4. Entire VP1 gene nucleotide sequences of the EV71 strains used to generate the EV71 phylogenetic tree

Strain	Source	GenBank no.	Strain	Source	GenBank no.
EV71Zhejiang08	GenBank	EU864507	GD08-EV71-039	This study	HM037793
BJ08-Z025-5	GenBank	FJ606450	GD08-EV71-135	This study	HM037794
SHZH03	GenBank	AY465356	GD08-EV71-149	This study	HM037795
Fuyang-0805	GenBank	FJ439769	GD08-EV71-166	This study	HM037796
2027/SIN/01	GenBank	AF376111	GD08-EV71-194	This study	HM037797
HEV71/BrCr/USA/70	GenBank	U22521	GD08-EV71-205	This study	HM037798
2419-Yamagata-03	GenBank	AB213647	GD08-EV71-274	This study	HM037799
7673-CT-87	GenBank	AF009535	GD08-EV71-335	This study	HM037800
2232-NY-77	GenBank	AF135871	GD08-EV71-381	This study	HM037801
2916-TAI-98	GenBank	AF286526	GD08-EV71-495	This study	HM037802
MY16/1/SAR/97	GenBank	AF376073	GD08-EV71-523	This study	HM037803
S11051/SAR/98	GenBank	AF376081	GD08-EV71-573	This study	HM037804
1089T/VNM/05	GenBank	AM490142	GD08-EV71-583	This study	HM037805
KOR-EV71-13	GenBank	AY125976	GD08-EV71-614	This study	HM037806
F2-CHN-00	GenBank	AB115491	GD08-EV71-641	This study	HM037807
GD08-EV71-003	This study	HM037792	GD08-EV71-650	This study	HM037808

related causes. It is therefore important to improve the facilities and equipments available to these clinics.

Analysis of the entire VP1 gene sequence is considered to be the most rigorous method for determining the EV71 genotype. A key study of the molecular epidemiology of EV71 by Brown et al. (21) identified three EV71 genotypes (A, B, and C). The EV71 prototype strain (BrCr strain) is the sole member of the A genotype, whereas the B genotype is currently known to contain 5 subgenotypes (B1-B5), and the C genotype a further 5 subgenotypes (C1-C5). The phylogenetic analysis of the VP1 gene sequence from the 17 isolates studied herein, which was supported by a high bootstrap, indicated that the GD EV71 strains belong to subgenotype C4, which has been circulating continuously in China since the first reported occurrence in Shenzhen City in 1998. The C4 subtype can, however, be divided into 2 lineages (C4a and C4b) in mainland China. The C4b strain mainly circulated in the period 1998-2004, whereas C4a became the predominant strain after 2004. In accordance with previous studies, the results of our phylogenetic analysis suggest that GD EV71 strains from 2008 and the Shenzhen strain from 2003 were in different clusters, thus indicating that EV71 infections circulate at a national rather than a regional level.

The genetic determinants of EV71 virulence remain unknown. Therefore, as VP1 is one of the structural proteins of EV71 surrounding the virion, we decided to determine whether the virulence determinant of EV71 is present in VP1. During the 2008 EV71 epidemic in GD, viruses were isolated from all of the identified cases divided into 3 classes, namely mild, severe, and fatal. Four of the 17 selected isolates were from fatal cases, 7 from severe cases, and 6 from mild cases. These viruses showed 95-99% nucleotide sequence identity with one another. We also compared the VP1-related amino acid sequence for these viruses and found it to be highly conserved, with identities of close to 100%. Any differences in the clinical outcome of infection must therefore be determined by other factors, such as attenuating muta-

tions within other regions of the viral genome or different host susceptibility factors.

The HFMD surveillance system in GD worked for a little over a year, and more data will be required to establish its epidemiologic usefulness. However, given the nature of passive surveillance, it is highly likely that some infected patients did not visit a clinic and others were misdiagnosed, thus escaping the surveillance system. In addition, clinical specimens were only obtained from cluster cases, severe cases, and deaths, thereby limiting the interpretation of the results obtained.

Most EV71 cases (99.7%) in GD were mild, with only 0.3% being classified severe or resulting in death. This observation may be a result of EV71 strain virulence, individual risk factors, and social factors. However, as EV71 can cause death in children, further, more detailed studies regarding the molecular biology, genetics, and epidemiology of EV71 will be required to determine the mechanism by which such severe cases or death cases.

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Conflict of interest None to declare.

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Short communication

A single-tube multiplex PCR for rapid detection in feces of 10 viruses causing diarrhea

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A novel multiplex polymerase chain reaction assay was developed to identify 10 viruses in a single tube. The assay was targeted to detect group A and C rotaviruses, adenovirus, norovirus GI, norovirus GII, sapovirus, astrovirus, Aichi virus, parechovirus, and enterovirus. A total of 235 stool samples were collected from infants and children with acute gastroenteritis in Kyoto, Japan, from 2008 to 2009, then tested by this novel multiplex PCR and compared with a multiplex PCR described previously, which used 3 primer sets. The novel multiplex PCR could detect the targeted viruses in 111 of the 235 (47.2%) stool samples. Of these, 9 out of 10 types of viruses were identified, including group A rotavirus, norovirus GII, enterovirus, sapovirus, adenovirus, parechovirus, group C rotavirus, astrovirus, and norovirus GI. In contrast, the multiplex PCR that used 3 sets of primers could detect the targeted viruses in 109 of the 235 (46.4%) stool samples. Among these, 8 types of viruses were identified, including group A rotavirus, norovirus GII, enterovirus, adenovirus, parechovirus, group C rotavirus, sapovirus, and astrovirus. The results suggested that the new multiplex PCR is useful as a rapid and cost effective diagnostic tool for the detection of major pathogenic viruses causing diarrhea.

Acute gastroenteritis is one of the most common illnesses in humans worldwide. An estimated 25–30% of all deaths among children younger than 5 years of age are caused by virus infections. Different types of viruses such as rotavirus, norovirus, sapovirus, astrovirus, and adenovirus have been known to associate with these diseases (Clark and McKendrick, 2004). In recent years, several novel viruses have been discovered, mostly by advanced molecular screening methods (Tang and Chiu, 2010; Svraka et al., 2010). Recently, Aichi virus, parechovirus, enterovirus, and human bocavirus have been considered as agents associated with diarrhea in humans (Stanway et al., 2000; Phan et al., 2005; Pham et al., 2007, 2010; Reuter et al., 2009; Chow et al., 2010). The standard laboratory methods for diagnosing viral infections are based mainly on viral isolation in cell culture. However, in those cases, some viruses cannot be isolated by the cell culture system. For epidemiological study, application of reverse transcription-polymerase chain reaction (RT-PCR) and sequencing techniques have become the standard methods for the detection and characterization of those viral pathogens (Yan et al., 2003, 2004).

RT-multiplex PCR methods had been developed previously for the detection of 8 viruses causing diarrhea using 2 sets of specific primers, set A and B (Yan et al., 2003, 2004). Set A was used to identify group A, B, and C rotaviruses and adenovirus, while set B could detect norovirus (genogroup GI and GII), sapovirus, and astrovirus. Most recently, another RT-multiplex PCR for the detection of 4 additional viruses (Aichi virus, parechovirus, enterovirus, and bocavirus) has been reported and a new set (set C) of specific primers was described (Pham et al., 2010). Although these 3 sets of primers showed good results for detecting several types of viruses causing diarrhea, each one had to be used in a separate reaction. In order to develop a simple, rapid, and cost-effective diagnostic tool for screening clinical specimens, a novel multiplex PCR for simultaneous detection of 10 viruses causing diarrhea (group A and C rotaviruses, adenovirus, norovirus GI, norovirus GII, sapovirus, astrovirus, Aichi virus, parechovirus, and enterovirus) in a single reaction tube has been developed.

A total of 235 stool samples were collected from infants and children with acute gastroenteritis in Kyoto, Japan, from 2008 to 2009. Only patients with clinical diagnosis of acute gastroenteritis with watery diarrhea were included in this study. The ages of the

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Table 1
Oligonucleotide primers used for the amplification of 10 viruses causing diarrhea.

Virus and primer	Sequence 5′–3′	Sense	Amplicon size (bp)	Reference
Astrovirus				
PreCAP1	GGA CTG CAA AGC AGC TTC GTG	+	719	Yan et al. (2003)
82b	GTG AGC CAC CAG CCA TCC CT	–		Yan et al. (2003)
Group A rotavirus				
VP7 1′(F)	AAA GGA TGG CCA ACA GGA TCA GT	+	569	Yan et al. (2004)
End9(s)	GTA TAR AAH ACT TGC CAC CAT	–		This study
Adenovirus				
Ad1	TTC CCC ATG GCI CAY AAC AC	+	482	Yan et al. (2004)
Ad2	CCC TGG TAK CCR ATR TTG TA	–		Yan et al. (2004)
Enterovirus				
F1	CAA GCA CTT CTG TTT CCC CGG	+	440	Zoll et al. (1992)
R1	ATT GTC ACC ATA AGC AGC CA	–		Zoll et al. (1992)
Norovirus GII				
COG2F	CAR GAR BCN ATG TTY AGR TGG ATG AG	+	387	Yan et al. (2003)
G2SKR	CCR CCN GCA TRH CCR TTR TAC AT	–		Yan et al. (2003)
Norovirus GI				
G1SKF	CTG CCC GAA TTY GTA AAT GA	+	330	Yan et al. (2003)
G1SKR	CCA ACC CAR CCA TTR TAC A	–		Yan et al. (2003)
Parechovirus				
Ev22(+)	CYC ACA CAG CCA TCC TC	+	270	Joki-Korpela and Hyypia (1998)
Ev22(-)	TRC GGG TAC CTT CTG GG	–		Joki-Korpela and Hyypia (1998)
Group C rotavirus				
GCMP-F	CAA ATG ATT CAG AAT CTA TTG	+	205	This study
G8NA2	GTT TCT GTA CTA GCT GGT GAA	–		Yan et al. (2004)
Aichi virus				
C94b	GACTTCCCCGGAGTCGTCGTCT	+	158	Yamashita et al. (2000)
AiMP-R	GCR GAG AAT CCR CTC GTR CC	–		This study
Sapovirus				
SLV5317	CTC GCC ACC TAC RAW GCB TGG TT	+	100	Yan et al. (2003)
SMP-R	CMW WCC CCT CCA TYT CAA ACA C	–		This study

patients ranged from neonate to 5 years old. The study was conducted with approval from the ethical committee in human rights related to human experimentation, Aino University.

The viral genomes were extracted from a supernatant of 10% fecal suspension using a QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany). The reverse transcription (RT) was performed using random primer (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan). The RT was carried out at 50 °C for 1 h, followed by 95 °C for 5 min and then rapidly cooled on ice.

For the conventional multiplex PCR, the presence of group A, B, and C rotaviruses and the adenovirus were detected by RT multiplex PCR using the primer set A as a protocol, described previously by Yan et al. (2004). Primers Beg9 and VP7-1′, ADG9-1F and ADG9-1R, G8NS1 and G8NA2, and Ad1 and Ad2 were used for the amplification of group A, B, C rotaviruses and the adenovirus, respectively. For the detection of norovirus GI, norovirus GII, sapovirus, and astrovirus, primer set B; G1SKF and G1SKR, COG2F and G2SKR, SLV5317 and SLV5749, 82b and PreCAP1 was used to amplify these viruses, respectively (Yan et al., 2003). For set C, primers 6261 and 6779, EV22 (+) and EV22 (−), 188F and 542R, F1 and F2 were used to detect Aichi virus, parechovirus, bocavirus, and enterovirus, respectively (Pham et al., 2010; Yamashita et al., 2000; Joki-Korpela and Hyypia, 1998; Zoll et al., 1992).

The cDNA was amplified further for detection of the targeted viruses. The polymerase chain reaction (PCR) components contained 10.9 μl of MilliQ water, 5.0 μl of 5× Colorless GoTaq PCR buffer containing $MgCl_2$ at a final concentration of 1.5 mM in the 1× reaction (Promega, Madison, WI), 2.0 μl of 2.5 mM dNTP Mix (Roche, Germany), 0.5 μl of each 20 pmol/μl primer pairs of mixed-primers (for set A, B, or C), 0.1 μl of 5 units/μl GoTaq DNA polymerase (Promega, Madison, WI), and 3.0 μl of cDNA template. The total PCR mixture was 25.0 μl. The amplification was performed for 30 cycles under the following thermal cycling conditions: 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 10 min. The PCR products were detected by electrophoresis through 1.5% agarose gel. Positive results of targeted viruses were assigned based on the expected size of PCR products corresponding to reference viruses. These PCR results were used for comparison with a novel multiplex PCR.

For the novel multiplex PCR, 10 pairs of specific primers for the detection of group A and C rotaviruses, adenovirus, norovirus GI, norovirus GII, sapovirus, astrovirus, Aichi virus, parechovirus, and enterovirus were mixed in a single reaction (Table 1). The PCR amplification components contained 10.9 μl of MilliQ water, 5.0 μl of 5× Colorless GoTaq PCR buffer (containing $MgCl_2$ at a final concentration of 1.5 mM in the 1× reaction) (Promega, Madison, WI, USA), 2.0 μl of 2.5 mM dNTP Mix (Roche, Mannheim,Germany), 0.2 μl of each 20 pmol/μl of 10 primer pairs, 0.1 μl of 5 units/μl GoTaq DNA polymerase (Promega, Madison, WI, USA), and 3.0 μl of cDNA template. Then, the amplification was performed for 35 cycles under the following thermal cycling conditions: 94 °C for 1 min, 48 °C for 1 min, 72 °C 1 min 15 s and a final extension at 72 °C for 10 min. The PCR product sizes were determined by electrophoresis through 2.5% agarose gel. The gel was stained with SYBR Safe (Invitrogen, CA, USA) and then visualized under ultraviolet light source. All 10 reference targeted viruses which were identified previously in the laboratory were used for standardizing the specificity of this novel multiplex PCR. The primer sequences and expected PCR product sizes are shown in Table 1. When samples from the PCR results were not concordant by conventional and novel methods, monoplex PCR was performed again as a confirmation test.

A total of 235 stool samples were screened by the conventional multiplex PCR method using 3 sets of primers, and it was observed that 109 (46.4%) of them were positive for 8 types of the target viruses. Group A rotavirus was seen to be the most prevalent virus detected in this study (28.5%, 31 out of 109), followed by norovirus GII (22.9%, 25 out of 109), enterovirus (12.9%, 14 out of 109), adenovirus (7.3%, 8 out of 109), parechovirus (6.4%, 7 out of 109), group C rotavirus (4.6%, 5 out of 109), sapovirus (2.8%, 3 out of 109), and astrovirus (1.8%, 2 out of 109). In addition, mixed-infection among 2 or 3 viruses were observed as well (12.8%, 14 out of 109). The

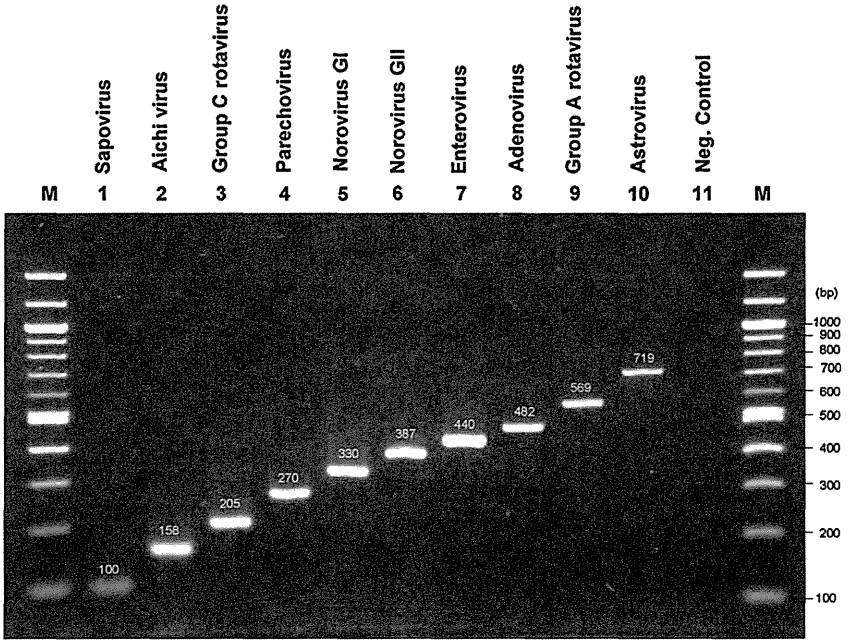

Fig. 1. Agarose gel electrophoresis demonstrating the expected PCR product sizes of 10 viruses detected from clinical samples by the novel multiplex PCR method. Lanes 1–10 represent the specific viruses detected: sapovirus (100 bp), Aichi virus (158 bp), group C rotavirus (205 bp), parechovirus (270 bp), norovirus GI (330 bp), norovirus GII (387 bp), enterovirus (440 bp), adenovirus (482 bp), group A rotavirus (569 bp), and astrovirus (719 bp). Lane 10 is the negative control and M represents the standard 100 bp DNA ladder marker.

patterns of mixed-infections were a wide variety. Triple infection among group A rotavirus/norovirus GII/enterovirus was found in 1 sample. Mixed-infection of 2 viruses between enterovirus and norovirus GII and between enterovirus and group A rotavirus were detected in 3 and 2 samples, respectively. Each mixed-infection of sapovirus with astrovirus or enterovirus or parechovirus, also was observed. In addition, mixed-infections of parechovirus with group A rotavirus or norovirus GII or adenovirus or enterovirus were found in each sample. One other sample was found to have a mixed-infection between adenovirus and astrovirus. In this panel of specimens tested, no norovirus GI, group B rotavirus, Aichi virus, or bocavirus were detected.

To evaluate the efficiency of new multiplex PCR method, 10 known cDNA of the targeted viruses were used also as the template for the novel multiplex PCR method. As shown in Fig. 1, the expected PCR product sizes for the targeted viruses were observed in agarose gel at a 100 bp for sapovirus, 158 bp for Aichi virus, 205 bp for group C rotavirus, 270 bp for parechovirus, 330 bp for norovirus GI, 387 bp for norovirus GII, 440 bp for enterovirus, 482 bp for adenovirus, 569 bp for group A rotavirus, and 719 bp for astrovirus. For testing with clinical samples, a panel of 235 stool samples, which were tested by conventional multiplex PCR, was tested also by this novel multiplex PCR. The results were compared, and 111 (47.2%) of the 235 samples were found to be positive. Of these, 9 types of viruses were identified. The majority of the samples were positive for group A rotavirus (19.8%, 22 out of 111) and norovirus GII (18.9%, 21 out of 111), followed by enterovirus (15.4%, 17 out of 111), sapovirus (9.9%, 11 out of 111), adenovirus (7.2%, 8 out of 111), parechovirus (5.4%, 6 out of 111), group C rotavirus (5.4%, 6 out of 111), astrovirus (3.6%, 4 out of 111), and norovirus GI (0.9%, 1 out of 111). Fifteen samples were identified by the novel multiplex PCR method as mixed-infection between 2 and 3 viruses. Triple infection among group A rotavirus/astrovirus/enterovirus was found in 1 sample. Each of two mixed-infections between enterovirus and group A rotavirus or norovirus GII or sapovirus or parechovirus was detected in 2 samples. Mixed-infection of group C rotavirus and parechovirus was observed in 2 samples. Mixed-infection of norovirus GII with group C rotavirus or adenovirus or parechovirus was found in each sample. Another sample had mixed-infection between adenovirus and parechovirus. It was, however, observed that the results with 51 samples were not concordant between the conventional multiplex PCR and novel multiplex PCR. In this case, a monoplex PCR was carried out as the confirmatory test, and it was interesting to note that the results with 33 samples by the novel multiplex PCR and 18 samples by the conventional multiplex PCR were in agreement with the monoplex PCR.

Viral gastroenteritis is one of the most common illnesses in humans worldwide, and different viral agents have been described to associate with the disease. The clinical presentation of patients with acute gastroenteritis symptoms is not generally indicative of a specific pathogen. Therefore, a rapid, sensitive, and specific diagnostic test would be helpful in the administration of appropriate treatment for the patients. For this reason, a new multiplex PCR method was developed for the detection of 10 pathogenic viruses that are currently known to be associated with diarrheal disease in humans (group A and C rotaviruses, adenovirus, norovirus GI, norovirus GII, sapovirus, astrovirus, Aichi virus, parechovirus, and enterovirus). The advantage of a novel multiplex PCR is that 10 targeted viruses can be detected simultaneously in a single tube. The method could differentiate multiple types of viruses on the basis of different amplicon sizes by combining the primers from conventional multiplex PCR methods (set A, B, and C). However, in a novel multiplex PCR, the detection of group B rotavirus and bocavirus was omitted, because the prevalence rates of these 2 viruses were much lower than those of other viral pathogens. It was observed clearly that the newly designed specific primers for the detection of group A and C rotaviruses, Aichi virus, and sapovirus could be used in combination with the primers reported previously for the detection of these viruses in clinical samples. In comparison between conventional and novel multiplex PCR methods, the overall detection rates for viruses causing diarrhea were not different (46.4% versus 47.2%). However, a novel multiplex PCR requires a shorter time to perform the test. The non-concordant results of the clinical samples between these 2 multiplex PCR methods were confirmed by monoplex PCR, and it was shown clearly that the novel multiplex PCR was in better agreement with mono-

plex PCR results when compared to the conventional multiplex PCR method. It might be possible that newly designed primers incorporated in this novel multiplex PCR were designed based on virus sequences circulating recently and, therefore, could detect recent strains circulating in patients. A problem encountered frequently with the multiplex PCR assay is a reduction in sensitivity, due to competition for reagents when multiple templates are amplified in a single reaction. Therefore, the sensitivity and specificity of this assay should be validated and evaluated further on a larger scale of clinical specimens and compared with the conventional monoplex PCR. The preliminary results showed the potential application of the novel multiplex PCR method as a rapid and cost-effective diagnostic tool for detecting a variety of viruses causing diarrhea.

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Cocirculation of Two Transmission Lineages of Echovirus 6 in Jinan, China, as Revealed by Environmental Surveillance and Sequence Analysis▽

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Enterovirus environmental surveillance on sewage from the city of Jinan, Shandong Province, China, was initiated in 2008. Thirty echovirus 6 (E6) strains—1 in 2008 and 29 in 2010—were isolated and identified. Most E6 isolates (n = 21) came from the sewage collected on August 2010, revealing high local E6 activity at that time. Interestingly, the VP1 sequences of most isolates, even from the same sewage, were not identical. Phylogenetic analysis of VP1 sequences revealed two lineages for these isolates, with 78.0 to 80.0% nucleotide identities with one another, 94.8 to 100.0% identity within the major lineage, and 92.7 to 98.5% identity within the minor one. The VP1 sequences of environmental isolates, clinical isolates from 1998 to 2010, and global E6 were subjected to evolutionary analysis using Bayesian phylodynamic methods. The inferred E6 VP1 ancestral sequence dated back to 1901 (range, 1873 to 1928) and evolved with 7.047×10^{-3} substitutions per site per year. Shandong E6 segregated into three clusters, and the two environmental lineages belonged to clusters A and C, which originated in 2003 and 1992, respectively. The antigenicity analysis via neutralization assay confirmed great antigenic differences between Shandong isolates and a prototype strain. These findings underscore the value of continuous environmental surveillance and genetic analysis to monitor circulating enteroviruses in the population and give further insight into E6 evolution.

Human enteroviruses (HEVs) belong to the genus *Enterovirus*, family *Picornaviridae*. Their infection is known to be generally asymptomatic, but they sometimes may cause illnesses such as summer colds, aseptic meningitis (AM), acute myocarditis, acute flaccid paralysis (AFP), acute hemorrhagic conjunctivitis (AHC), hand, foot, and mouth disease (HFMD), etc.

Enteroviruses were traditionally typed by their antigenic properties via a neutralization test (NT), and the introduction of the molecular typing method, which suggested that enteroviruses should be classified into the same serotype if they have >75% nucleotide similarity in the VP1 coding sequence (>85% amino acid sequence similarity) and into different serotypes if they have <70% nucleotide similarity (<85% amino acid similarity) (18), has been broadly used for typing enteroviruses and for molecular epidemiology investigations (19, 20, 22). HEV comprises more than 90 serotypes, which are classified into four species, HEV-A to HEV-D (6, 28).

Echovirus 6 (E6) is a member of HEV-B. Children less than 1 year old were the most commonly affected by E6 in the period from 1970 to 2005 in the United States (13). For children with E6 infection requiring hospitalization, AM was the most common manifestation, followed by meningismus, upper respiratory tract infection, pneumonia, and herpangina (15). Previously, many outbreaks of AM caused by E6 had been reported throughout the world, including an outbreak that occurred in Anhui Province, China, in 2005 (1, 7, 16, 17). However, there is limited information about other E6 isolates from other regions of China. Whether the genetic characterization of other Chinese E6 isolates is similar to that of Anhui isolates needs further investigation.

There has been to date no specialized enterovirus surveillance system in China. AFP surveillance, developed for a polio eradication program, can obtain non-polio enterovirus (NPEV) isolates as a side benefit and produce baseline data of local NPEV distribution and a genetic overview (3). But the NPEVs isolated from AFP surveillance cannot reflect the current prevalent virus in circulation in a given region due to the low incidence of AFP. Nevertheless, environmental surveillance has been demonstrated to be a sensitive method to detect silently circulating viruses (24, 25). Therefore, the results from environmental surveillance not only reflect the periodic trend of NPEV distribution and variation but also possess a unique

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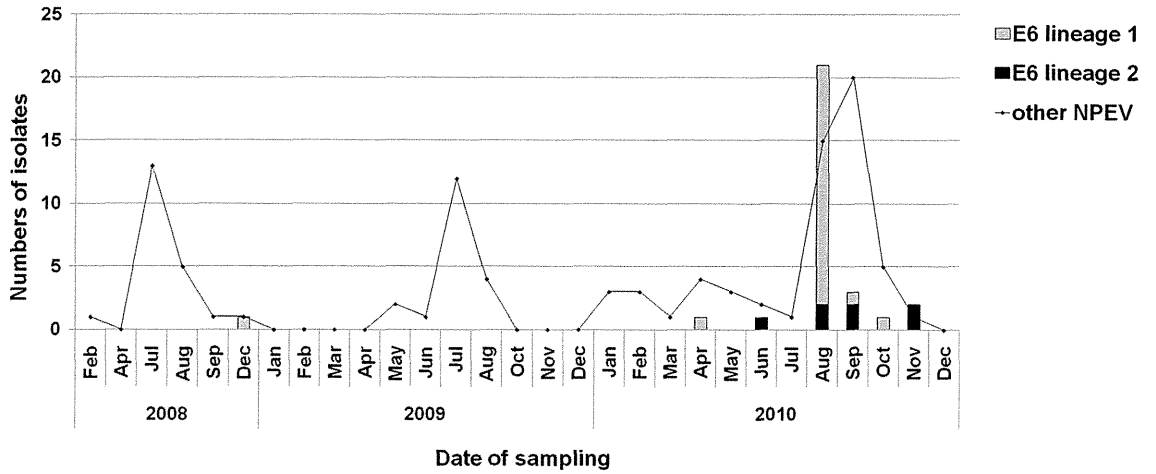

FIG. 1. Numbers of E6 isolates and other NPEVs from sewage collected in different months from 2008 to 2010 in Jinan city, China.

advantage for investigating silently circulating viruses in the corresponding area.

Surveillance for enteroviruses in environmental samples has been conducted in the city of Jinan, Shandong Province, China, since 2008 in order to monitor poliovirus (30) and NPEV circulation in the population of the city. Here, we investigated the genetic characterization of E6 isolated from sewage collected from February 2008 to December 2010 and phylogenetically analyzed the Shandong E6 isolates from both the environmental surveillance samples and clinical cases to assess the capability of environmental surveillance to monitor the prevalent enteroviruses in the population and to present a genetic overview of E6 strains isolated in Shandong Province.

MATERIALS AND METHODS

Shandong Province and Jinan City. Shandong Province is located in the eastern part of China, with an area of 156,700 km^2 and a population of 94.7 million. Jinan is the capital city of Shandong Province. Its metropolitan area and population are 296 km^2 and 2.6 million, respectively. There are two sewage treatment plants located in the metropolitan area of Jinan city, and the number 2 plant treats the domestic sewage from the western part of the city, with a daily sewage treatment capacity of 200,000 tons.

Sampling. Sewage samples were collected monthly from the number 2 sewage treatment plant in Jinan from February 2008 to December 2010. The samples were collected from the inlet collector canal by the grab sampling method in the afternoon between 1400 and 1500 h. A stainless plastic bucket was lowered into the flowing water to collect approximately 0.5 to 1 liter of sewage sample. A cold temperature (approximately 4°C) was maintained during sample transport to the laboratory, storage (<24 h), and processing.

Clinical E6 isolates. The specimens from AFP cases from 1998 to 2010 were collected and processed according to standard protocols recommended by the WHO (31). Cerebrospinal fluid specimens from two aseptic meningitis patients in Shandong Province in 2010 were inoculated onto RD and HEp-2 cell lines directly.

Concentration of sewage. Sewage samples were concentrated by the method described by Iwai et al. (12). Briefly, the sewage samples were centrifuged at 3,000 × *g* for 30 min at 4°C; 2.5 M $MgCl_2$ 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) by positive pressure pump. Absorbents on the filter were then eluted with 10 ml of 3% beef extract solution by ultrasonication three times (1 min each time), and the solution was centrifuged at 12,000 × *g* for 30 min. Subsequently, the supernatant was filtered through a 0.22-μm-pore-size filter and was ready for cell inoculation.

Virus isolation and serotyping. L20B, RD, and HEp-2 cell lines were used for virus isolation. A total of 200 μl of treated solution was added to each of the cell culture tubes (18 tubes of each cell line for one sewage sample). After absorption at 36°C for 2 h, 1 ml of Eagle's minimal essential medium with 2% fetal calf serum was added, and the tubes were kept in a 36°C incubator for 7 days and examined every day. After 7 days, the tubes were frozen and thawed and repassaged in L20B, RD, and HEp-2 cell lines, and another 7-day examination was performed. If a complete cytopathic effect (CPE) was obtained in the RD or HEp-2 cell line, the cells in the tube were frozen and thawed and inoculated into L20B cells to rule out the possibility of poliovirus.

According to standard protocols recommended by the WHO (31), microneutralization assays were carried out in 96-well tissue culture plates using an antibody pool for enterovirus (National Institute for Public Health and the Environment, [RIVM], Netherlands). The antiserum-virus mixtures were incubated for 1 h at 36°C. Subsequently, a suspension fluid of RD or HEp-2 cells was added to the plate, which was subsequently examined daily for the presence of CPE. The antiserum that inhibited the development of CPE was evaluated according to the manufacturer's instructions. Isolates identified as E6 were used for further investigation.

Nucleotide sequencing and molecular typing. Total RNA was extracted from 140 μl of the infected RD and HEp-2 cell culture using a QIAamp viral RNA minikit (Qiagen, Valencia, CA) according to the manufacturer's recommended procedure. Reverse transcription-PCR (RT-PCR) was performed using an Access RT-PCR System (Promega). Approximately 730 bp, including the 3′ end of VP3 and 480 bp of the 5′ end of a partial VP1 gene, was amplified using the primer pair 490/492 (21). Primer pair 491/493 that corresponded to the 3′ end of VP1 and 5′ end of the 2A was used to amplify a 760-bp sequence. The combination of the two sequences yielded the entire VP1 coding region.

The products were analyzed by agarose gel electrophoresis, and positive products were purified and sequenced directly with a BigDye Terminator, version 3.0, Cycle Sequencing kit (Applied Biosystems, Foster City, CA); sequences were analyzed by an ABI 3130 genetic analyzer (Applied Biosystems). The PCR products were sequenced in both directions to avoid possible ambiguous nucleotides. The VP1 sequence was compared with sequences available in GenBank using BLAST, obtained online from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/). Isolates showing >75% nucleotide sequence identities with the E6 D'Amori prototype strain were designated relative serotypes (18).

Homologous comparison and phylogenetic analysis. Nucleotide sequence alignments were carried out by BioEdit software, version 7.0.5.3 (11). Phylogenetic trees were constructed by using Mega, version 4.0 (29), using the neighbor-joining method after estimation of genetic distance using the Kimura two-parameter method (14). A bootstrapping test was performed with 1,000 duplicates, and the transition/transversion rate was set at 2.0.

Evolutionary analysis based on the Bayesian MCMC method. The evolution rate and molecular clock phylogeny of global E6 isolates were inferred using the Bayesian Markov Chain Monte Carlo (MCMC) method in BEAST, version 1.6.1 (9), and the time of the most recent common ancestor (t_{MRCA}) with 95% highest posterior density (HPD) of global E6 and Shandong clusters was estimated. In

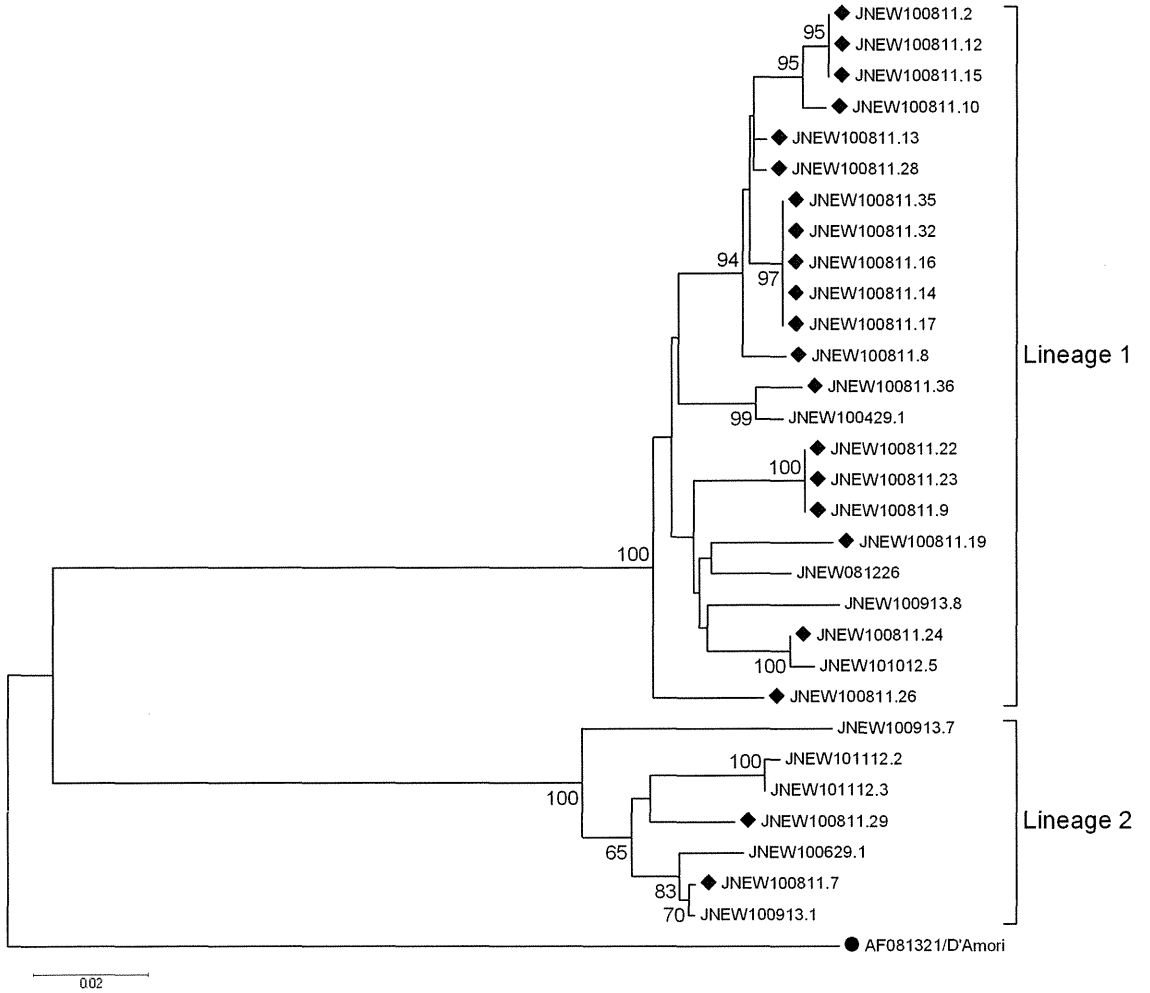

FIG. 2. Phylogenetic tree based on 470-nt 5′ partial VP1 sequences of all environmental E6 isolates in Shandong Province. A circle indicates the prototype strain D'Amori, and diamonds indicate the E6 isolates from the sewage collected on 11 August 2010. The remaining nine E6 isolates were collected at other times. The isolates are identified by a code that consists of JNEW, followed by the sample date (presented as YYMMDD [i.e., year, month, day]), and the tube number.

order to reduce the computation load, sequences with high homogeneity and identical isolation years were deleted. The data were analyzed under both the Hasegawa-Kishino-Yano (HKY) and the general time reversible (GTR) nucleotide substitution models with gamma distribution of among-site rate variation. Two different models of rate variation among branches were implemented in our analysis: the strict clock and the uncorrelated log-normal distributed (UCLD) relaxed molecular clock. Both constant and exponential growth (EG) population sizes were used as tree priors. For each model, the MCMC chain was run for 30,000,000 steps and sampled every 1,000 steps. The first 3,000,000 steps of each run were discarded as burn-in.

Neutralization assay. To examine the antigenic properties of Shandong E6 isolates, a microneutralization assay was carried out in 96-well tissue culture plates using polyclonal antiserum for E6 (Denka, Japan). Briefly, 25 μl of the E6 antiserum, with a 4-fold serial dilution from 1:4 to 1:1,024, was added into the 96-well plates. Virus with 100 50% tissue culture infective doses ($TCID_{50}$)/25 μl was added to them and incubated for 1 to 2 h at 36°C. Subsequently, RD cells were added to the plate, which was subsequently examined daily within 7 days postinfection. The number of wells with CPE was counted, and the neutralization antibody titers were calculated. All assays were performed in triplicate, and back titration controls were performed for each assay.

Nucleotide sequence accession numbers. The VP1 nucleotide sequences of E6 isolates described in this study were deposited in the GenBank database under accession numbers GU272016, GQ329778 to GQ329785, HQ399470 to HQ399495, and HQ829944 to HQ829961.

RESULTS

Virus isolation. During the environmental surveillance from February 2008 to December 2010, a total of 29 sewage samples were collected, and 128 NPEVs and 18 polioviruses (3, 9, and 6 strains for poliovirus types 1, 2, and 3, respectively) were isolated. As shown in Fig. 1, the numbers of enterovirus isolates reached a peak from July to September. Thirty E6 isolates (23.4% of total NPEVs) were detected from seven sewage samples, with 1 isolate in December 2008, none in 2009, and 29 isolates from six samples in 2010. Most environmental E6 strains (21 isolates) were isolated in August 2010. In other seasons, E6 was detected at a low frequency.

The RD and HEp-2 cell lines were evaluated in isolating environmental E6, and 63.3% (19/30) of E6 viruses were isolated on the RD cell line, reflecting relatively higher sensitivity to E6 than HEp-2 cell line.

Genetic relationship of environmental E6. To investigate the genetic relationship among all environmental E6 isolates, especially from the samples collected in August 2010, the 470-

TABLE 1. Information of E6 isolates from AFP and AM cases

Isolate	Prefecture	Year	Patient data		
			Age	Sex	Clinical symptom
98186	Linyi	1998	1	F	AFP
98215	Jinan	1998	1	M	GBS[a]
00059	Dezhou	2000	10	M	AFP
01229	Linyi	2001	1	F	Myelitis
01444	Weifang	2001	8	F	Myelitis
02203	Linyi	2002	3	M	GBS
02295	Jinan	2002	1	M	AFP
05397	Heze	2005	6	F	AFP
06415	Dezhou	2006	1	M	AFP
08346	Binzhou	2008	3	M	AFP
08351	Zibo	2008	1	F	AFP
09086	Weifang	2009	3	M	AFP
10154	Liaocheng	2010	1	F	AFP
10165	Heze	2010	3	F	AFP
10180	Taian	2010	6	M	AFP
10193	Jining	2010	0.5	M	AFP
10208	Linyi	2010	0.5	M	AFP
2010LY059	Linyi	2010	1	M	AM
2010D0010005	Heze	2010	1	M	AM

[a] Guillain-Barré syndrome.

nucleotide (nt) partial VP1 coding regions (position 2447 to 2916 on prototype strain D'Amori) were sequenced and analyzed. Phylogenetic analysis revealed two lineages of environmental E6 isolates (Fig. 2), indicating two transmission links of E6 in Jinan. For the 21 isolates from August 2010, 19 isolates segregated into a major lineage (lineage 1), while the other 2 isolates belonged to a minor lineage (lineage 2). For the nine environmental E6 viruses isolated from the other six sewage samples, four were grouped into lineage 1, and the remaining five were assigned to lineage 2. Homologous analysis revealed 78.0 to 80.0% nucleotide identities between the two lineages, 94.8 to 100.0% identity within lineage 1, and 92.7 to 98.5% identity within lineage 2.

Origin, evolutionary rate, and molecular clock phylogeny. The entire VP1 coding regions of representative E6 isolates from environmental surveillance ($n = 16$), AFP cases ($n = 17$), and AM patients ($n = 2$) in Shandong Province (Table 1) and from global sequences (43 out of 113) were selected for divergence time and substitution rate estimation with the Bayesian MCMC method. Different models were used for data analysis, and it was found that UCLD with EG fit our data best, while the HKY and GTR nucleotide substitution models had no significant impact on the analysis (Table 2). The coefficient of variation of the evolutionary rates among branches was 0.335 (95% HPD, 0.209 to 0.465) estimated by the HKY model, indicating the rate heterogeneity that existed among different branches.

In the molecular clock phylogenetic tree (Fig. 3), Shandong clinical E6 isolates segregated into three clusters, designated A, B, and C, composed of isolates from 2008 to 2010, 2001 to 2002, and 1998 to 2010, respectively. Lineage 1 and 2 environmental E6 isolates belonged to clusters A and C, respectively. The Anhui isolates from an aseptic meningitis outbreak in Anhui Province, China, in 2005 did not fall into any of the three clusters. High intracluster similarities were observed among VP1 sequences of environmental and clinical isolates. In cluster A, environmental E6 had 95.3 to 97.2% identity with the isolate 2010LY059 from an AM patient and 91.8 to 98.5% identities with AFP isolates. Similarly, isolate JNEW100913.7 in cluster C had only 2.1% nucleotide divergence with the isolate 2010D0010005 from an AM case.

Different evolution rates for different branches were observed, and the mean evolution rate was 7.047×10^{-3} substitutions per site per year (HKY). The most recent common ancestor of global E6 can be traced back to 1901 (range, 1873 to 1928). The t_{MRCA} estimates for Shandong clusters were dated to 2003 (A), 1996 (B), and 1992 (C), and the t_{MRCA} estimates for environmental lineages 1 and 2 were dated to 2005.2 and 2003.7, respectively.

Antigenic property. We tested the antigenicity of nine Shandong E6 isolates from clusters A, B, and C (three for each cluster) with E6-specific antiserum. The neutralization to prototype D'Amori served as a control. It was found that all isolates were neutralized with E6-specific antiserum, but the degree of neutralization of these viruses was dramatically less than the result of the prototype strain and varied slightly between isolates (Fig. 4). The variation in the degree of neutralization with E6 antiserum did not depend on the cluster.

DISCUSSION

As a supplemental method to AFP surveillance for global poliomyelitis eradication, environmental surveillance is of great importance in investigating the circulation of wild-type poliovirus or vaccine-derived polioviruses (VDPV) (4, 10, 27, 32). In addition, environmental surveillance constitutes a sensitive method for monitoring NPEV and estimating the extent and duration of enterovirus circulation in a population (23, 24, 26).

Enterovirus environmental surveillance in China was first conducted in the Shandong Provincial Poliovirus Laboratory in

TABLE 2. Origin and evolution rate inferred with Bayesian MCMC method on VP1 coding region

Parameter	Mean value of the parameter (95% HPD) as determined by:	
	HKY+UCLD+EG	GTR+UCLD+EG
t_{MRCA} cluster A	2,003.3 (2,001.2–2,005.2)	2,003.3 (2,001.3–2,005.3)
t_{MRCA} cluster B	1,996.9 (1,994.0–1,999.6)	1,996.8 (1,992.6–1,999.2)
t_{MRCA} cluster C	1,992.1 (1,989.1–1,994.7)	1,992.2 (1,989.4–1,994.9)
Root ht (t_{MRCA} global E6)	1,901.7 (1,873.6–1,928.1)	1,901.2 (1,873.5–1,928.1)
Mean evolutionary rate (10^{-3} substitutions/site/yr)[a]	7.047 (5.507–8.803)	7.351 (5.677–9.110)
Coefficient of variation	0.335 (0.209–0.465)	0.323 (0.192–0.455)

[a] Rate of molecular evolution given as numbers of nucleotide substitutions per site per year.

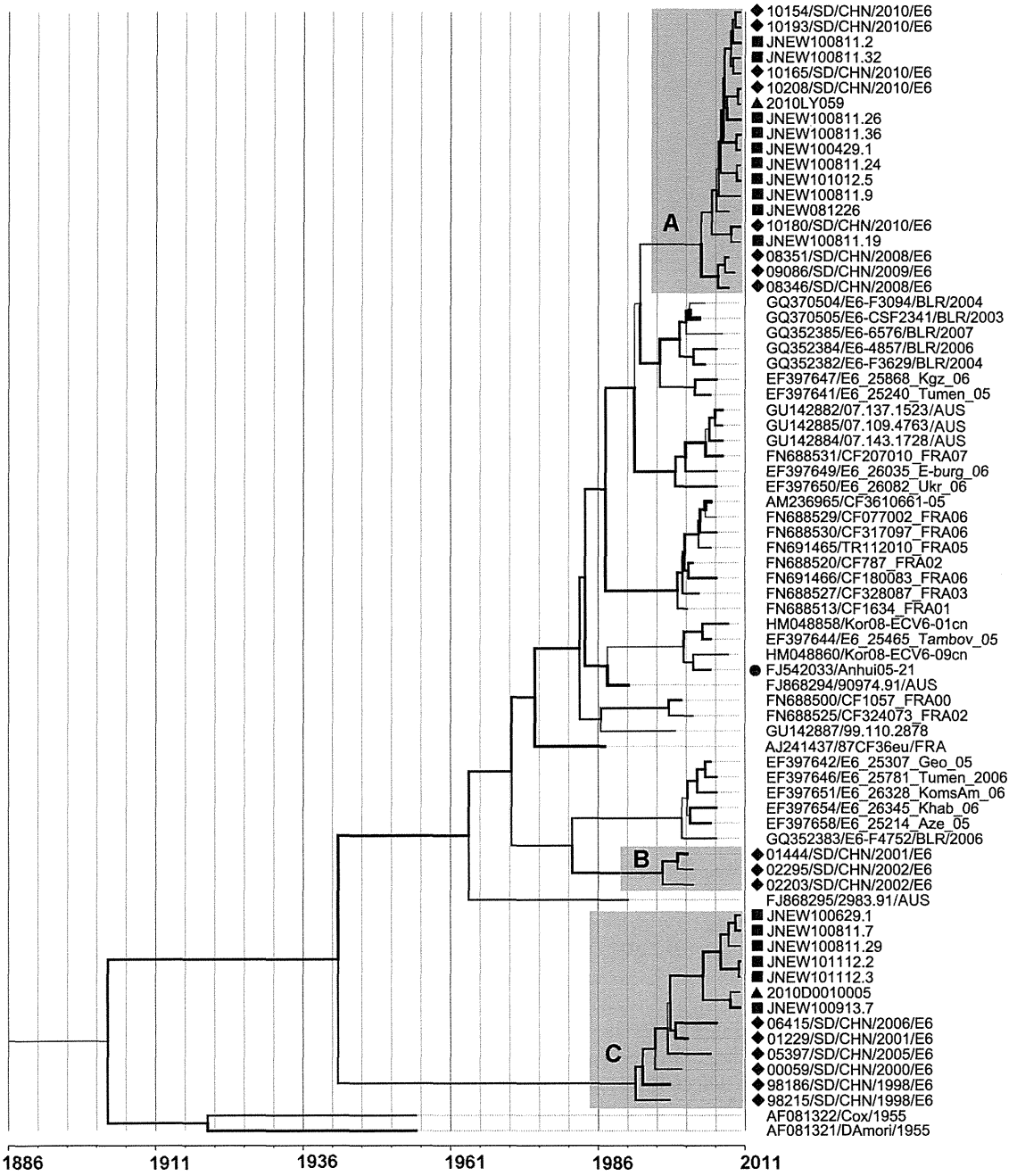

FIG. 3. MCMC tree of the VP1 sequences of E6 isolates throughout the world visualized in FigTree. The width of a branch reflects the evolution rate of individual sequences and their reconstructed ancestors. E6 strains from Shandong segregated into three clusters (A, B, and C). A circle indicates an isolate from aseptic meningitis outbreak in the Anhui Province of China, squares indicate Shandong environmental isolates, diamonds indicate isolates from AFP surveillance in Shandong Province, and triangles indicate isolates from aseptic meningitis cases. Names of environmental isolates (JNEW prefix) are shown as described in the legend of Fig. 2. AUS, Australia; AZE, Azerbaijan; BLR, Belarus; CHN, China; FRA, France; GEO, Georgia; KEO, South Korea; KGZ, Kyrgyzstan; RUS, Russia; UKR, Ukraine; USA, the United States of America.

February 2008. The sewage examined in this study was obtained monthly from treatment plant 2, which services the west half of Jinan City. By the end of 2010, E6 was isolated in sewage collected in 2008 and 2010. No E6 was present in sewage in 2009. There was just one isolate detected in each sample until an abrupt increase in the E6 isolates occurred for the sewage collected in August 2010. Taking into account the similar sewage concentrations and inoculation methods in these years, it is reasonable to assume that the increase in E6 isolates is a reflection of high local E6 activity at that time.

Moreover, the VP1 sequences and phylogenetic analysis provided more information on environmental E6 circulating in Jinan City. Normally, the divergence among enteroviruses isolated in an outbreak of EV-associated diseases, such as AM,

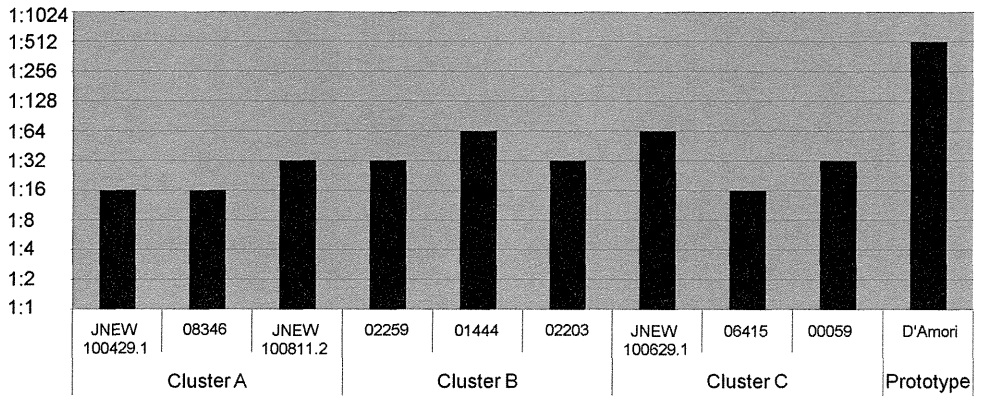

FIG. 4. Results of the neutralization assay of random selected Shandong E6 isolates (three isolates for each cluster) with E6-specific antisera. The E6 neutralization antibody titers in the presence of 100 $TCID_{50}$/25 μl of the viruses are shown.

was no more than 2% (5, 16). The two lineages in environmental E6 and the relatively high intralineage genetic divergence (up to 7%) revealed by phylogenetic analysis indicated that the E6 isolates from the sewage samples in August 2010 were not simply the result of current transmission activity. By Bayesian MCMC evolutionary analysis, the most recent common ancestors for environmental lineages 1 and 2 were dated to 2005.2 and 2003.7, respectively, indicating that the two lineages have circulated for 5 to ~7 years.

Enteroviruses were known to be active in summer and early autumn (24). So, the E6 circulation in recent years might be enlarged in the summer of 2010, resulting in the peak detection via environmental surveillance in Jinan in August. The two lineages of environmental E6 probably represented the local prevalent and minor circulating E6 strains at that time. Furthermore, the cocirculation of two E6 transmission lineages in the same region and period might lead to the occurrence of intratypic recombination, which might affect E6 evolution or even result in the antigenicity and virulence alterations.

E6 can cause serious illnesses such as aseptic meningitis. However, no specialized enterovirus surveillance system for associated diseases has been established in China. Therefore, we cannot obtain sufficient clinical isolates for comparison. Considering the high rate of inapparent infection for enteroviruses, including E6, the prevalent and minor circulating E6 strains detected via environmental surveillance in this study offered unique information of E6 strains currently circulating in Jinan. Two E6 isolates from AM patients in Shandong Province, initially reported as Japanese encephalitis (JE) clinical cases, were grouped into the two clusters of environmental viruses, with high intracluster identities (Fig. 3), indicating that the environmental viruses might also be causative for AM. So, it is reasonable to conclude an E6-associated AM epidemic might occur in Jinan City in the summer of 2010.

The EV genome evolved at a rate of 1% to ~2% mutation per year (8), and the previously estimated substitution rate of the E6 VP1 gene was 8.597×10^{-3} substitutions per site per year (2), slightly different from the results of this study (7.047×10^{-3} substitution/site/year). Moreover, global E6 strains investigated in this study shared a recent common ancestor that originated in 1901, 16 years earlier than the t_{MRCA} estimated previously (2). The differentiation in estimated origin year and evolution rate was thought to come from the joining of Shandong E6 sequences, and analysis of more sequences with time information will yield more precise data to determine the origin and evolution of E6 strains. Shandong E6 isolates were grouped into three clusters. Isolates from cluster B were from 2001 to 2002, and 8 years has passed since the last isolation, indicating that cluster B viruses may have been eliminated from Shandong Province. Cluster A consisted of isolates from 2008 to 2010, suggesting that it may have been introduced into the Shandong area recently. The isolation years of cluster C spanned from 1998 to 2010, revealing the long-time circulation of this cluster in Shandong Province.

Another Chinese isolate (Anhui05-21) from Anhui Province was not grouped into any of the Shandong clusters, demonstrating that the E6 circulating in China consisted of different transmission lineages with high genetic divergence among them. However, the information on Chinese E6 isolates was limited, and more sequences are needed to determine domestic E6 transmission and evolution. The results of phylogenetic analysis also suggested that E6 isolates from the same cluster might come from different regions. As for the three clusters of E6 in Shandong Province, no geographic accumulation of isolates was found, and they were all distributed throughout the province.

Prototype strains of enterovirus serotypes were mostly isolated decades ago, and many have become extinct. The antigenicity of current circulating enteroviruses has changed a lot in comparison with that of prototype strains. This constituted a reason for the frequent failure in serotyping via neutralization testing using combined sera directed at the prototype enteroviruses. Likewise, the present study revealed a considerable differentiation in antigenicity between Shandong E6 isolates and the prototype D'Amori. These isolates could be serotyped by RIVM combined sera in a neutralization test, but a prolonged observation time could result in the failure of serotyping. Hence, a single antiserum directed at currently circulating viruses should be developed as a substitution.

In conclusion, the cocirculation of two E6 transmission lineages in Jinan City, China, was revealed via environmental surveillance and VP1 sequence analysis. The evolutionary genetics of global E6 were investigated, and three clusters of Shandong E6 were revealed. This is the first report of the

application of environmental surveillance in monitoring circulating enterovirus in the population in China.

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The anti-apoptotic protein L* of Theiler's murine encephalomyelitis virus (TMEV) contains a mitochondrial targeting signal

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ABSTRACT

L* protein of TMEV is out-of-frame with the viral polyprotein from an alternative initiation codon AUG, 13 nucleotides downstream from the authentic polyprotein AUG. Anti-apoptotic activity of L* was demonstrated by both 'loss of function' and 'gain of function' experiments. However, the precise mechanism(s) of anti-apoptotic activity of L* remains to be clarified. In this study, L* was demonstrated to be localized to mitochondria. It was also shown by the GFP fusion protein that N-terminal sequence of L* may contain a mitochondrial targeting signal (MTS). Surprisingly, L*$_{(5-70)}$–GFP and L*$_{(41-70)}$–GFP were localized to mitochondria although L*$_{(1-70)}$–GFP was distributed in the cytosol, suggesting L* has an MTS between amino acid (AA) positions 41 and 70, and that L*$_{(1-4)}$ inhibits its mitochondrial targeting. Furthermore, L*$_{(1-70)}$–GFP was localized to the mitochondria by co-expression of L*$_{(65-156)}$, indicating that L*$_{(65-156)}$ suppresses the inhibition of mitochondrial targeting by L*$_{(1-4)}$. These results suggest that the intra- or inter-molecular interaction of L* regulates its mitochondrial localization. It is also suggested that L* may inhibit the intrinsic apoptosis through the localization to mitochondria.

1. Introduction

Theiler's murine encephalomyelitis virus (TMEV) belongs to the genus *Cardiovirus* of the family *Picornaviridae* and is divided into two subgroups on the basis of their different biological activities (Oleszak et al., 2004; Roos, 2010; Takano-Maruyama et al., 2006). GDVII subgroup strains cause acute and fatal encephalomyelitis in mice without virus persistence or demyelination (Oleszak et al., 2004; Roos, 2010; Takano-Maruyama et al., 2006). In contrast, TO subgroup including DA and some (other) viral strains induces an early, non-fatal polioencephalomyelitis of weanling mice, followed by virus persistence and chronic demyelination in the spinal cords (Oleszak et al., 2004; Roos, 2010; Takano-Maruyama et al., 2006). This late demyelinating disease serves as an experimental model for the human demyelinating disease, multiple sclerosis (MS) (Oleszak et al., 2004; Roos, 2010; Takano-Maruyama et al., 2006). Viral persistence is essential for bystander demyelination (Drescher et al., 1997; Lipton et al., 2005; Monteyne et al., 1997). However, the precise mechanism(s) of virus persistence and demyelination remains to be elucidated.

A small 17–18 kDa protein designated L* is out-of-frame with the viral polyprotein from an alternative initiation codon AUG, 13 nucleotides downstream from the authentic polyprotein AUG (Kong and Roos, 1991). L* protein is synthesized in TO subgroup strains, but not GDVII subgroup strains (Michiels et al., 1995; Roos, 2010; Takano-Maruyama et al., 2006). It is shown to be essential for virus growth in J774.1 macrophage cells, the major site of virus persistence, by both 'loss of function' and 'gain of function' experiments (Himeda et al., 2005a; Obuchi et al., 1999; Takata et al., 1998).

Anti-apoptotic activity of L* protein was also demonstrated by both 'loss of function' and 'gain of function' experiments (Ghadge et al., 1998; Himeda et al., 2005b). However, precise mechanism(s) of inhibition of apoptosis by L* protein remains to be clarified. We previously reported that L* is immunocytologically co-localized with α/β-tubulin (Obuchi et al., 2001). Thereafter, it was reported that tubulin is an inherent component of mitochondrial membranes that interacts with the voltage-dependent anion channel (VDAC), which is the major channel of mitochondrial outer membrane (MOM) (Carre et al., 2002; Rostovtseva et al., 2008). Regulation of MOM permeability is important in apoptosis by release of apoptogenic factors into the cytosol. Therefore, it is of great interest to investigate the relationship between L* and mitochondria.

Interestingly, a PSORTII analysis of the amino acid (AA) sequence of L* protein predicted a cleavage site of mitochondrial targeting signal (MTS) between residues 64 and 65 that matched R-3 motif (Schneider et al., 1998) (Fig. 1A). In addition, it was reported that 3×FLAGL*, N-terminus of L* was tagged with 3×FLAG, could not rescue the growth of DAL*-1 in J774 cells (Himeda et al., 2005a), suggesting that N-terminus of L* may play an important role for its activity. Therefore, we studied the distribution of L* in the L*-

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