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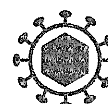
VIROLOGY JOURNAL

RESEARCH Open Access

Phylogenetic and molecular characterization of coxsackievirus A24 variant isolates from a 2010 acute hemorrhagic conjunctivitis outbreak in Guangdong, China

Wu De[1], Zheng Huanying[1*], Li Hui[1], Monagin Corina[2], Guo Xue[1], Liu Leng[1], Zeng Hanri[1], Fang Ling[1], Mo Yanling[1], Zhou Huiqiong[1], Zhang Huan[1], Kou Jing[1], Long Caiyun[1], Hiromu Yoshida[3] and Ke Changwen[1]

Abstract

Background: Acute hemorrhagic conjunctivitis is a common disease in China. As a notifiable disease, cases are registered by ophthalmologists on the AHC surveillance system. An AHC outbreak caused by CA24v was observed in Guangdong Province in 2007 by the National Disease Supervision Information Management System. Three years later, a larger outbreak occurred in Guangdong during the August-October period (2010). To characterize the outbreak and compare the genetic diversity of CA24v, which was determined to be the cause of the outbreak, the epidemiology and the molecular characterization of CA24v were analyzed in this study.

Results: A total of 69,635 cases were reported in the outbreak. 73.5% of index cases originated from students, children in kindergarten and factory workers, with the ≦ 9 age group at the highest risk. The male to female ratio was 1.84:1 among 0-19 years. 56 conjunctival swabs were collected to identify the causative agent from five cities with the AHC outbreak. 30 virus strains were isolated, and two of the genomes had the highest identity values (95.8%) with CA24v genomes. Four CA24v genotypes were identified by phylogenetic analysis for the VP1 and 3C regions. CA24v which caused the outbreak belonged to genotype IV. Furthermore, full nucleotide sequences for four representative isolates in 2010 and 2007 were determined and compared. 20 aa mutations, two nt insertions and one nt deletion were observed in the open reading frame, with 5'- and 3'- UTR respectively between them.

Conclusions: CA24v was determined to be the pathogen causing the outbreak and belongs to genotype IV. VP1 is more informative than $3C^{Pro}$ for describing molecular epidemiology and we hypothesize that accumulative mutations may have promoted the outbreak.

Keywords: CA24v, AHC, Molecular epidemiology, Phylogenetic analysis, Guangdong

Background

Acute hemorrhagic conjunctivitis (AHC) is a highly contagious infection, characterized by an abrupt onset of ocular pain, swelling of the eyelids, a foreign body sensation or irritation, epiphora, eye discharge and photophobia [1,2]. CA24v, EV70 and some additional adenovirus serotypes are the major etiological agents of AHC [3]. Outbreaks of AHC caused by CA24v were first described in Ghana in 1969 [4], with the first isolation of CA24v causing AHC reported during an outbreak in Singapore in 1970 [5]. In the past several decades, CA24v was recognized as the major causative agents of AHC outbreaks [6-8]. AHC spread to Mainland China in 1971 [9], and CA24v was identified in Hong Kong in 1975 [10].

In a previous study, nucleotide sequence variations of the $3C^{Pro}$ regions of the CA24v genome were compared by using isolates from various regions of the world. Phylogenetic analysis revealed that CA24v appeared at one

* Correspondence: zhenghy.gdcdc@gmail.com
[1]Institute of Microbiology, Center for Disease Control and Prevention of Guangdong, No.176, Xingang Road W, Guangzhou, Guangdong, 510300, People's Republic of China
Full list of author information is available at the end of the article

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focal point in Asia around 1963 [11]. Numerous AHC epidemics have occurred since 1969. A high degree of infectivity of CA24v to the human conjunctiva was conferred by mutations accumulating in the RNA viral genome causing expanded epidemics [12]. Four genotypes of the CA24v were described and identified by phylogenetic analysis of the $3C^{Pro}$ and VP1 regions of the genome [13]. Recently, a large epidemic by CA24v was documented in China in 2007. Phylogenetic analyses revealed these isolates were located in the same cluster, and have the closest relationship to the 2005 Singapore isolates [8,14-16].

Another larger AHC outbreak caused by CA24v was observed in 2010 in Guangdong, China. In this report, we briefly describe the epidemiology of the outbreak. In addition, in order to characterize the viral genome of the CA24v isolates in 2010, the $3C^{Pro}$ and VP1 region of the viral genome, as well as complete nucleotide sequences, were determined and phylogenetically analyzed for isolates collected from AHC patients.

Results

The outbreak

As AHC is a notifiable infectious disease in China, all cases diagnosed by physicians were registered in the NDSIMS. Surveillance data showed the number of AHC cases noticeably began to increase from the 36th week and reached a peak of over 20,000 cases in the 38th week in 2010 in Guangdong province. The number of AHC cases returned to a baseline of around 200 cases in the 43rd week. Although in a previous report [8], the peak of the outbreak was identified to be during the 35th-37th weeks, the peak of the 2010 outbreak was in fact between the 37-39th weeks (Figure 1). A total of 72,181 cases were reported in 2010 in Guangdong. In this outbreak, 69,635 AHC cases (74.9 cases/100,000 population) were reported between Sep 1 and Oct 31, 2010. The number of AHC cases during 32~44 weeks was on average 195-fold (69,759/358 cases) and 2.5-fold (69,759/28,381 cases) higher than 2009 and the outbreak of 2007 respectively. Among these cases, 56% percent were male. The male to female ratio was 1.84:1 (1.62:1,2007) among 0 ~ 19 years, and 1.33:1 (1.44:1, 2007) among 0 ~ 49 years, but the ratio was 0.85:1 (0.79:1, 2007) among the ≧ 50 age groups.

Although cases were reported throughout the province, cases were most frequently reported in 5 cities; Heyuan, Guagnzhou, Zhaoqing, Foshan and Jianmen. 23.9% of cases were students, followed in frequency by factory workers (22.8%) and children in kindergartens (16.8%). Case-patients occurred in all age groups, and most of them (81.1%) were < 40 years of age, similar to the 2007 outbreak (87.8%). Two age peaks occurred in the ≦ 9 and the 30 ~ 39 age group. The population at highest risk was ≦ 9 years of age (27.3%) (Figure 2). Only one age peak was observed in the 20~29 age group in the 2007 outbreak (Figure 2).

Samples collection and virus isolation

A total of 56 conjunctival swabs were collected from 56 AHC patients aged 2 months -56 years during the acute phase (1-5 days after onset of symptoms). These cases were comprised of 27 males and 29 females, mainly from students at kindergartens and schools, and factory workers in 5 cities of the Guangdong region. These samples were collected from Guangzhou (10 samples), Heyuan (16 samples), Jiangmen (8 samples), Zhaoqing (10 samples) and Foshan (12 samples) on the 14th and 30th of September. Virus isolation was performed in HEp-2 cell line. CPE were clearly observed on the HEp-2 cell line for 30 of the 56 samples.

RT-PCR tests

The 3, 4, 3, 3 and 3 representatives from Guangzhou, Heyuan, Jiangmen, Zhaoqing and Foshan (respectively) were subjected to one step RT-PCR with 3C-1, 3C-2 and CVA24v-S, and CVA24v-A specific primers encoding the $3C^{Pro}$ and VP1 region on the CA24v genome. 32

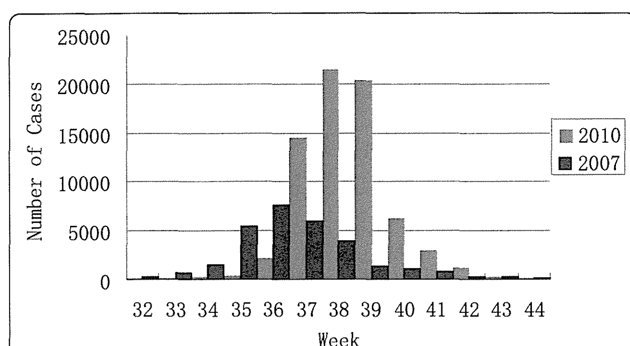

Figure 1 Weekly case numbers of acute hemorrhagic conjunctivitis reported by the disease surveillance network in Guangdong between July and October of 2007 and 2010.

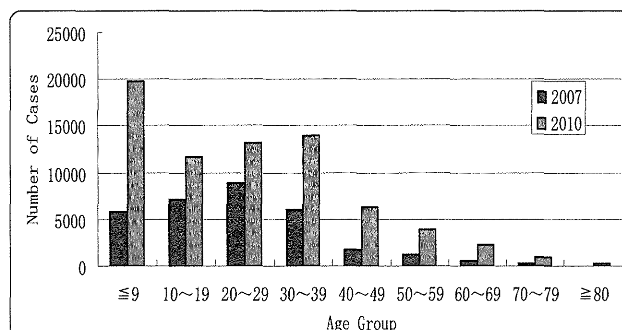

Figure 2 Age distribution of acute hemorrhagic conjunctivitis cases confirmed by ophthalmologists in 2007 and 2010.

special RT-PCR products from 16 isolates were observed clearly in 1.0% agarose gels. In addition, we used the four representatives isolated from Guagnzhou and Heyuan in 2010 and 2007 and performed one-step RT-PCR with eight other special primers. 32 special bands from four isolates were observed in 1.0% agarose gels.

Sequence comparison and phylogenetic analysis of the 3C^{pro} and VP1 regions

As there were few CA24v full genome sequences available in GenBank, and our main objective was to not only describe the epidemiology of the 2010 outbreak, but also to compare the Guangdong sequences with those from other AHC outbreaks, we carried out a phylogenetic analysis in the 3C^{Pro} and VP1 regions which were available from previous studies.

16 isolates from 2010 with a 549 bp size fragment on the 3C^{Pro} region were used for analysis with 27 CA24v strains from each genotype that had caused epidemics of AHC. The 3C^{Pro} nucleotide sequence identity values among 43 strains had a range of 85.4-100% (data not shown), corresponding to a 95.1-100% of amino acid identity values on the 3C^{Pro} protein. Four genotypes were chronologically clustered in the tree (Figure 3). Genotype I (G I) contained early isolates from Singapore, Hong Kong and the CA24v prototype obtained in 1970-1971. 98.5-100% identity values in the nucleotide sequence were observed. Genotype II (G II) was composed of isolates from Singapore and Thailand obtained in 1975, with 98.7% identity values. Eight isolates from 1988 to 1994 were from South-East Asia and the Americas, and were included in genotype III (G III), with 88.5-99.8% identity values in their nucleotide sequences. The other isolates from 2002 to 2010 were located in genotype IV (G IV), with 90.3-100% identity values. Guangdong strains isolated in the 2007 and 2010 outbreaks belonged to G-IV, but were located in different clusters of the Singapore strains in 2005 and South Korea strains in 2007.

To elucidate the nucleotide variation in the VP1 region of strains isolated in Guangdong in 2010, 16 virus strains from 2010, three from 2007 (JF742580-JF742595, JF742578, JF742579, EU391644) and 12 strains from each genotype and different geographical locations were selected for the analysis of 915 bp of the VP1 region. Percent of nucleotide identity values ranged from 85.6 to 100% on the VP1 region, corresponding to a 97.4-100% of amino acid identity values on the VP1 protein. Phylogenetic analysis of the CA24v strains showed 4 distinct genotypes: group I, consisting of one prototype strain EH24/70 (D90457); group II, consisting of BRA87-10629 (EF015038) and JAM87-10628 (EF015037) strains from 1987, with 100% identity values; group III, consisting of USA-Fl98 10631 (EF015040) and DOR93-10630 (EF015039) strains from 1993 to 1998, with 98% identity values; and group IV, consisting of more diverse strains and including some AHC isolates from 2003 to 2010, with 94-100% identity values (Figure 4). From the phylogenetic analysis, the Guangdong 2010 and 2007 isolates were included into group IV, with CA24v isolates associated with AHC epidemics isolated in China, Korea, Singapore, Spain and Australia since the year 2000, which had a close relationship with singapore/DSO-26/2005 (DQ443002).

Genome sequence comparison and analysis

Four complete genome sequences of CA24v, which were isolated from two large outbreaks in 2007 and 2010, were obtained from the overlapping amplicons using 10 primer sets total, which were also isolated from two outbreaks in 2007 and 2010 respectively.

Their genome sizes had lengths of 7,463 bp with 750 nt at the 5'-UTR, and a short, 69-nt sequence at the 3'-UTR, with G + C contents of 45.2% (2010) and 45.6% (2007) respectively. The polyprotein precursor is encoded by a long open reading frame of 6642 nt, corresponding to 2214 aa. Phylogenetic analysis was performed using the complete genome of four strains from this study and 10 worldwide strains from different AHC outbreak events and regions in GenBank. The genome sequence identity values among 14 strains were 80.3-99.3% (data not shown). Two genomes from 2010 had a range of 95.7-95.8% identity values with two 2007 genomes, and 95.6% identity values with Singapore/DSO-2/2005 (DQ443001) at the nucleotide level. Phylogenetic analysis showed the four genome isolates were located in a large cluster, and were separated into two sub small branch clusters (Figure 5).

Twenty common amino acid residue changes, two nucleotides insertion and one nucleotide deletion were observed in polyprotein precursors, 5'-UTR and 3'-UTR among the two isolates from 2010 against 2007's respectively. Two inserted nts were observed at the 98th and the 120th site in 5'-UTR respectively, and one nt was deleted at the 14th site in 3'-UTR in two 2010 genomes. Mutation aa residues were distributed mainly in VP2 (43, D → N; 153, K → R; 165, A → T; 172,R → K), VP3 (156, D → E;182,Y → N), VP1 (301,N → D), 2A (71,Y → H;75,T → S;104,F → Y;137,V → I), 2B (62,S → T), 2C (256,I → V), 3C^{Pro} (68,V → T;150,T → V) and 3D (21,S → R;42,A → V,139,K → R;147,T → A;371,V → I) regions. Comparing the mutation of CA24v nucleotides and amino acids, we found roughly 90% nucleotide mutations were synonymous mutations.

Discussion

AHC is a common disease in China. As CVA24v and influenza A virus share the same receptor (sialic acid)

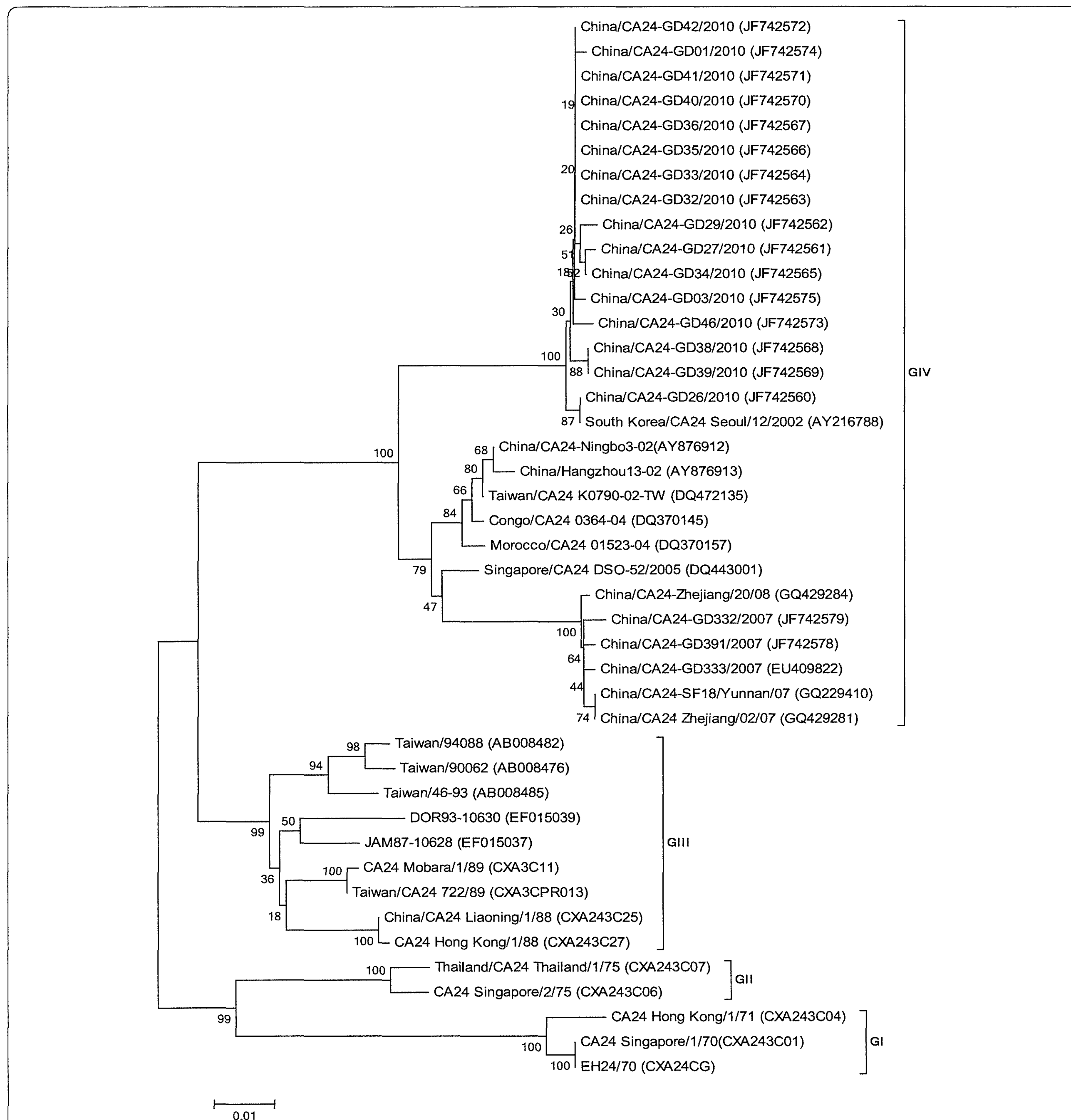

Figure 3 Phylogenetic analysis based on the partial 3C3C^{Pro} (549 nucleotides). The nucleotide sequences were analyzed using a MEGA5.0 package. Four topologies were inferred by Neighbor-joining cluster analysis with the bootstrap option. The numbers at the branching points are bootstrap values estimated with 1,000 pseudo-replicate data. Four genotypes (G I-IV) are indicated. The last numbers of each strain name indicate the year of isolation. The locations are shown to the right of the strain name.

[17], CVA24v is frequently associated with respiratory disease [18]. Causative agents are likely spread among people through conjunctival secretions and direct contact, but respiratory transmission may also occur [19] and may even explain the rapid and extensive spread of AHC during outbreaks [19]. The past 10 years is associated with rapid urbanization in China, with more of the population living in urban areas. High population density and poor sanitation permit these causative agents to spread quickly. Schools, particularly kindergartens and factories become key places for AHC outbreaks to develop. Several large AHC outbreaks were

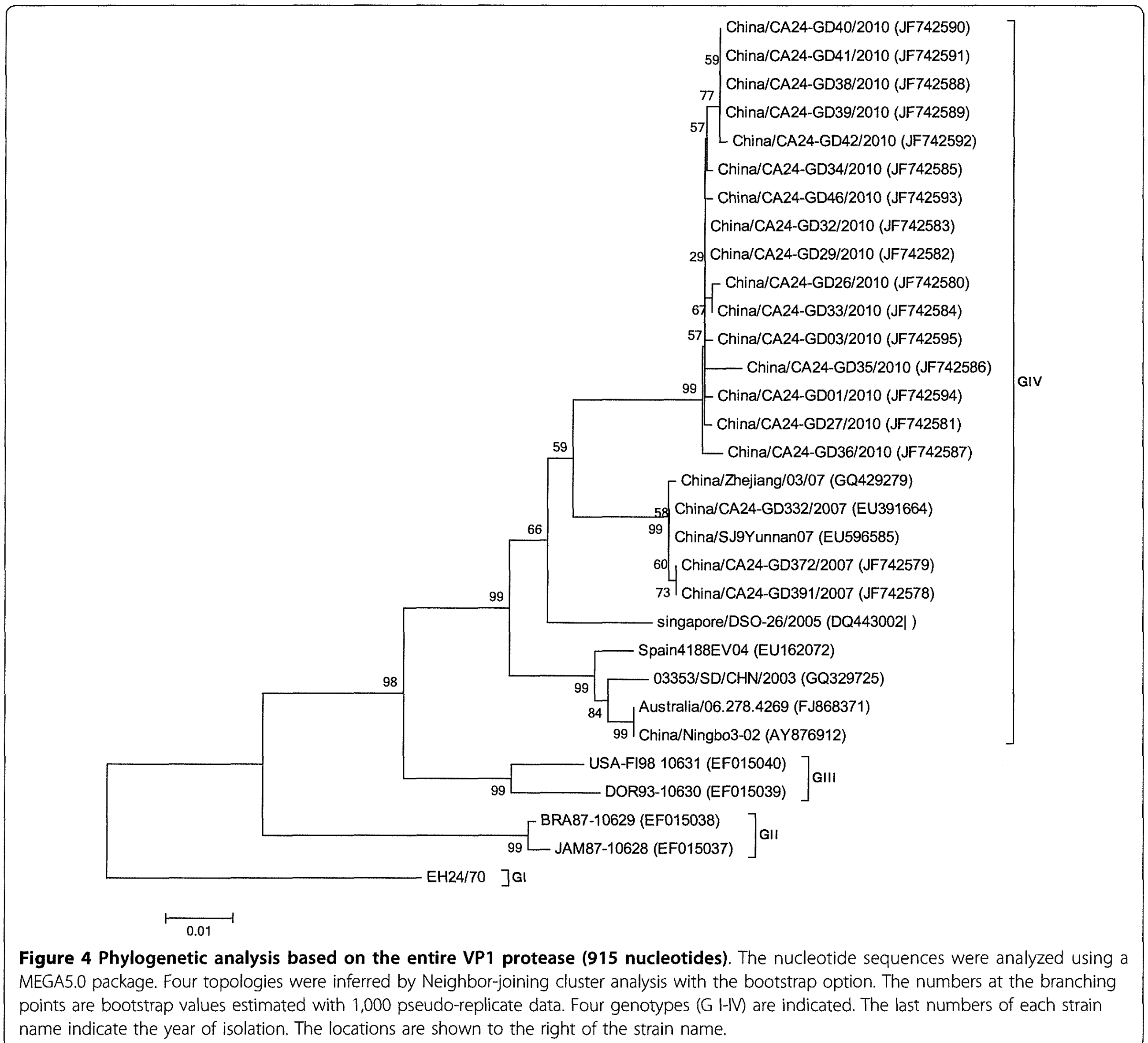

Figure 4 Phylogenetic analysis based on the entire VP1 protease (915 nucleotides). The nucleotide sequences were analyzed using a MEGA5.0 package. Four topologies were inferred by Neighbor-joining cluster analysis with the bootstrap option. The numbers at the branching points are bootstrap values estimated with 1,000 pseudo-replicate data. Four genotypes (G I-IV) are indicated. The last numbers of each strain name indicate the year of isolation. The locations are shown to the right of the strain name.

documented in China in 1971, 1988, 1994, 2002 and 2007 [20]. Periodic outbreaks are the result of considerable infection immunity decline after 7 years. This loss of herd immunity to the virus may have permitted the widespread transmission and periodic outbreaks [21]. Analyzing data from the AHC surveillance system, we find that most AHC outbreaks in Guangdong occur during the period from August to October in the autumn season. Mild climate and school openings in the autumn provide adaptive conditions for CA24v outbreaks.

Sex and age distribution were described in this study. In previous studies, $\leqq$ 9 and $\geqq$ 45 age groups were of lower risk, with children being the notably affected age group since 2007. Interestingly, the male to female ratio is higher in the $\leqq$ 49 than in $\geqq$ 50 age group in the two recent outbreaks. The highest ratio (1.86:1) occurred in $\leqq$ 9 age group, and the ratio decreases with the increase of age. A similar ratio (1.9:1) was described in hand-foot-mouth disease caused by enterovirus 71 and coxsackievirus 16 [22]. This predominance in children has been observed in other enteroviral infections, in which the male-to-female ratio ranged from 1.5:1 to 2.5:1 [23,24]. These results cannot be reasonably explained at present, but may suggest susceptibility at the host genetic and immune level.

In order to identify the phylogenetic relationship of the representative strains from the large Guangdong outbreak of 2010, we amplified and sequenced the $3C^{Pro}$ and VP1 of EV to screen genetic material and type positive EV specimens. In this study, nucleotide sequences

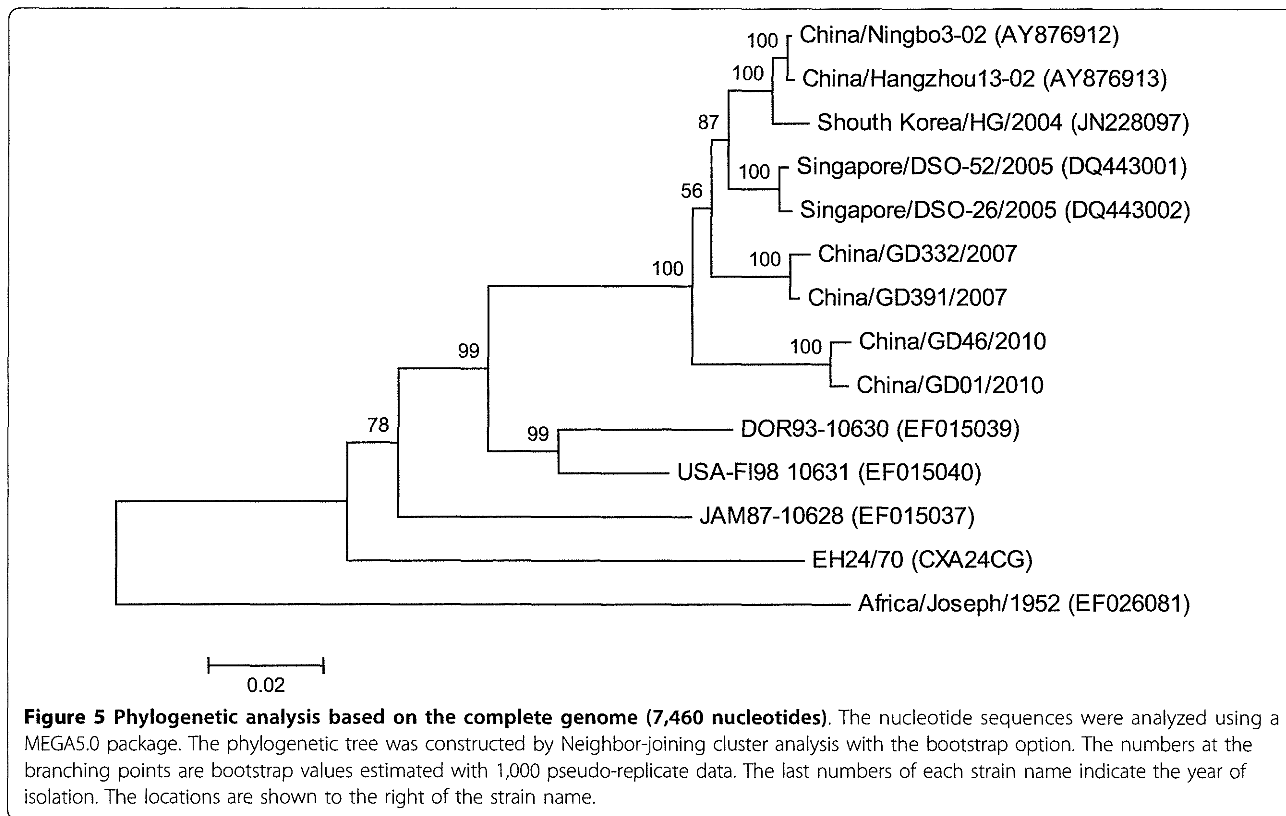

Figure 5 Phylogenetic analysis based on the complete genome (7,460 nucleotides). The nucleotide sequences were analyzed using a MEGA5.0 package. The phylogenetic tree was constructed by Neighbor-joining cluster analysis with the bootstrap option. The numbers at the branching points are bootstrap values estimated with 1,000 pseudo-replicate data. The last numbers of each strain name indicate the year of isolation. The locations are shown to the right of the strain name.

of the complete VP1 and partial $3C^{Pro}$ regions were compared, together with the prototype strain (Singapore) in 1970 and other worldwide strains. The results show the phylogenetic trees of CA24v were similar with that previously reported [13]. Due to the high recombination rates in $3C^{Pro}$ region and the major neutralization sites in VP1 region, both are generally used for evolution analysis, but some propose that the VP1 region is demonstrably more informative for molecular epidemiological studies than the $3C^{Pro}$ region. Comparing phylogenetic trees of VP1 with $3C^{Pro}$'s and the genome tree, we found both the VP1 and $3C^{Pro}$ trees could correctly determine four genotypes, but the VP1 tree was superior than the $3C^{Pro}$'s chronologically. This indicated that the VP1 sequence was more informative than the $3C^{Pro}$ sequence regarding genetic trees. In addition, the phylogenetic trees show that isolates from five cities shared one cluster and had a close relationship among them. The nucleotide distance observed within them suggests a common source.

In this study, the two outbreaks, 2007 and 2010, were compared. The scale of the 2010 outbreak is 2.28 times larger than the 2007 outbreak in population. Obviously, the 2010 outbreak curve shapes are different from 2007's. One notable difference is that one large peak was observed in the ≦ 9 age group, which had been the highest risk population in the 2010 outbreak. The absence of protective antibodies against enterovirus in young children may result in higher risk, which can be observed in other enterovirus diseases as well [22].

The 5'-and 3'-UTR of the enterovirus genome contribute to the replication of the virus RNA [25]. The 5'-UTR of the enterovirus RNA contains an *ori*L (nt 1-90) and an IRES (nt 91-745), which directs the initiation of translation in a cap-independent manner [26]. In this study, we found that two inserted nucleotides resided at the 98th and 120th sites of the 5'-UTR of two 2010 genomes. Curiously, the insertion is not found in other VA24v genomes. As the two inserted nucleotides were located in the IRES of CA24v, we infer the two insertions may have an effect on the initiation of translation of CA24v. Coincidentally, one nt deletion occurred at the 14th of 3'-UTR, and the deletion is only found in two 2010 genomes. The 3'-UTR of the enterovirus genome contains virus oriR [25]. Since 3'-UTR is a strictly *cis*-acting element that needs to make contact with the 5'-oriL [25], we propose the deletion may work in concert with the insertion of the 5'-URT.

Immunologic barriers against CA24v are usually formed among the infected population after a large outbreak or pandemic, which will hamper the viruses' ability to quickly spread among population. To escape from neutralization, like influenza A, CVA24v will normally rapidly change its' antigen epitope, which results in a

CA24v outbreak in a short time in the same region. The high evolutionary rate has been estimated to be 3.0-3.7 $\times 10^{-3}$ substitutions per site per year, 30 years after the emergence of the CVA24v [27], which is similar to influenza A (2.4-3.4 $\times 10^{-3}$) [28]. To further elucidate the CA24v antigen epitope variation, genome amino acid sequences from the 2010 outbreak strains were compared with 2007's. One, two and four amino acid changes were found in VP1, VP3 and VP2 respectively. In enterovirus, VP1, VP2, and VP3 proteins are located at the surface of the viral capsid and are exposed to immune pressure [29]. Major neutralization sites reside in the VP1, VP2 and VP3 proteins, but VP1 contains most of the neutralizing epitopes [30]. Previous studies show most antigen sites induced neutralizing antibodies reside in N-terminal [31], few sites were found at C terminus of VP1. But the C-terminal region (293-302 C-terminal) of the VP1 protein has also been shown to be highly antigenic by using peptide scanning techniques in CA9 [32]. An aa variation at the C terminus of VP1 was observed (301,N $\rightarrow$ D) in two 2010 outbreak strains. Aspartic acid (D) and asparagine (N) have a similar space structure, but different hydrophilicity. Two hydrophilicity and one hydrophobicity aa change were observed on VP2 (43 D $\rightarrow$ N; 165 A $\rightarrow$ T) and the VP3 (182 Y $\rightarrow$ N) regions as well. Hydrophilic aa are usually exposed to the surface of the capsid protein and form antigen epitope sites. In contrast, hydrophobic aa resides inside of the capsid protein. This indicated that these aa substitution might have an effect on capsid protein secondary structure and antigen epitopes, eventually caused escape mutation, promoting an AHC outbreak in a relatively short time span.

Conclusions

We described the 2010 Guangdong outbreak of AHC, and identified the likely etiological agent to be CA24v. We characterized the full genome of 4 CA24v strains from 2010 and 2007. Sequence comparison, phylogenetic analyses, and evolutionary studies reveal that CA24v throughout the world has been divided into 4 genotypes. The CA24v causing the 2010 Guangdong outbreak belongs to genotype IV, and VP1 is more informative than $3C^{Pro}$ for describing molecular epidemiology. This study also confirms significant variations of multiple aa and nt in the 2010 genome, which helps to infer that accelerative virus change may have promoted the AHC outbreak in 2010.

Materials and methods

Sample collection and virus isolation

A total of 56 conjunctival swabs collected from 56 AHC patients were processed immediately by using 4 ml Hanks media containing penicillin (1,000 u/ml) and streptomycin (1000 μg/ml) for 4 h. They were then cultured in fresh monolayers of the HEp-2 cells [8]. The cell line was maintained in the medium supplemented with 10% FBS. When the cells in monolayer presented 70% of confluence, the medium was discarded and 0.2 ml of conjunctival sample was added to a 24-well culture plate. Specimens were allowed to adsorb for 1 h at 36°C before adding 1 ml of fresh MM plus 2% FBS to each well. The cultures were incubated at 36°C and observed daily for CPE for 7 days, with the medium replaced every 4 days. Two blind passages were performed when no CPE was observed. To compare the genome of CA24v, 2 isolates from 2007 were used.

Ethics Statement

Use of conjunctival swab, which was collected for this study, was approved by the Ethical Committee for Centers for Disease Control and Prevention, and written informed consent was obtained from the study participants.

RNA extraction and RT-PCR

RNA was extracted from virus culture supernatant with a QIAamp MinElute Virus Spin kit (QIAGEN Inc., Valencia, Calif)) according to the manufacture's instruction manual.

Eight-pair primers were designed according to CA24v strain sequences (DQ443002) from Singapore. A one-step RT-PCR amplification reaction was performed by using the SuperScript TM III OneStep RT-PCR with Platinum Taq (Invitrogen USA). The reaction system consisted of 2 × Reaction Mix (a buffer containing 0.4 mM of each dNTP, and 2.4 mM MgSO4), 0.5 μl SuperScript III RT/Platinum Taq High Fidelity Enzyme Mix, and 0.4 μM specific primers CVA24v-S and CVA24v-A for VP1, specific primers 3C-1 and 3C-2 for 3C region or 0.5 μM for other specific primers (Table 1) for other regions of the genome. 5 μl of extracted RNA was added to a final volume of 25 μl. The cycling conditions for the 10 RT-PCRs for the genome were: an initial cycle at 45°C 10 min, 50°C for 20 min and 94°C for 2 min; followed by 35 cycles at 94°C for 30 s, 45°C 30 s (increase in increments of 0.3°C for 1 s each up to 55°C) and 68°C for 1 min; and a final incubation at 68°C for 10 min. The band of PCR amplicons visualized after electrophoresis were subsequently excised from 1% agarose gel, and purified by use of a QIAGEN gel extraction kit (QIAGEN, Germany).

Sequencing and genetic analysis

Nucleotide sequencing reactions were performed with a BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems). They were subjected to the initial denaturation at 96°C for 2 min and 30 cycles consisting of 96°C

Table 1 Primer sequences used in this study

Primers	Regions	Nucleotide sequences (5'→3')	Nucleotide position	Amplicons (bp)	References
CA24-1F	5'-UTR	GAAATTAAAACAGCTCTGGGGTTGTTCC	1~820	820	This study
CA24-1R	VP4	TGGATCCGCCAGTTGCCACATTAG			
CA24-2F	5'-UTR	GGCAACACTATTACAATGGGTGCC	736~1620	885	This study
CA24-2R	VP2	GGCCATACAGTCTATTGCTAGGGAG			
CA24-3F	vp2	CGAATAATTCGGCAACTCTTGTGCTG	1558~2523	965	This study
CA24-3R	vp1	AGTAATAACGGTGTCAATGGTCTCC			
CVA24v-S	VP3	GTGAGTGCTTGCCCAGATTT	2407~3438	1032	Ding et al. 2009
CVA24v-A	2a	ATACACCGCCATGTTCTGGT			
CA24-5F	vp1	TAAGGTGTTGGTGTCCTAGACCG	3292-4197	905	This study
CA24-5R	2c	CCCTTTGGCAGCATTACAGGC			
CA24-6F	2b	GCGACTGTTCACCCTGGC	4072-4853	781	This study
CA24-6R	2c	TCGCTGTGTGAGACTGTTGGAG			
CA24-7F	2c	GCAACGACATGAAGCTGTTCTGTC	4696-5322	626	This study
CA24-7R	3a	CAAAACAGTCATGGCCCTGTTCAG			
3C-1	3a	AAAGGGATGGATCGTCAAGC	5250~6181	932	Wu et al.2008
3C-2	3d	TAGCCTCTTCAAAGTCTGTC			
CA24-9F	3d	GAGCCCAGTGTCTTCCATTGTG	6084-6748	664	This study
CA24-9R	3d	CGAACCATGCAGGACTGAGTGA			
CA24-10F	3d	CCAGGCGTAGTGACAGGATCAG	6612-7461	849	This study
CA24-10R	3'-UTR	CCGAATTAAAGAAAAATTTACCCCTACAAC			

for 10 s, 50°C for 5 s, and 60°C for 4 min in a Gene Amp PCR system 2700 (Applied Biosystems). The products labeled by fluorescence were purified by use of the illustra Autoseq G-50 kit (Amersham Biosciences, UK[Q10]) and applied to ABI 3100 Genetic Analyzer (Applied Biosystems).

For molecular typing, nucleotide sequences for PCR amplicons by both CVA24v-S and CVA24v-A covering the entire VP1 region and 3C-1 and 3C-2 for 3C region, were used and compared with the sequence of EH24/70 strain.

To identify respective divergence and infer the genetic relationship among the isolates, the nucleotide sequences were analyzed by using MEGA software version 5.0. Phylogenetic trees were constructed by a neighbor-joining method after 1000 bootstrapping [33].

Nucleotide sequence accession numbers

The VP1 and 3C sequences from this study are available in GenBank with the following accession numbers: JF742580-JF742595, and JF742560-JF742575 We also sequenced the genome of CA24v isolates from four isolations. GenBank accession numbers of these sequences are JF742576-JF742579.

Abbreviations

AHC: Acute hemorrhagic conjunctivitis; CA24v: Variant of coxsackievirus A24; EV70: Enterovirus 70; CPE: Cytopathic effect; FBS: Fetal bovine serum AA Amino acid; NDSIMS: National disease supervision information management system; UTR: Untranslated region; IRES: Internal ribosome entry sites; Nt: Nucleotide.

Acknowledgements

We thank all the people involved in sample collection and in the follow-up investigations of the patients and their close contacts. This study was supported by the Science and Technology Council of Guangdong (2007B031507002). Global Viral Forecasting is graciously supported by the U.S. Department of Defense Global Emerging Infections, Surveillance and Response Systems (DoD GEIS) and the Defense Threat Reduction Agency (DTRA), Cooperative Biological Engagement Program (CBEP), http://Google.org, the Skoll Foundation, and the U.S. Agency for International Development (USAID) Emerging Pandemic Threats Program, PREDICT project, under the terms of Cooperative Agreement Number GHN-A-OO-09-00010-00. The contents are the responsibility of authors and do not necessarily reflect the views of USAID or the United States Government.

Author details

[1]Institute of Microbiology, Center for Disease Control and Prevention of Guangdong, No.176, Xingang Road W, Guangzhou, Guangdong, 510300, People's Republic of China. [2]Global Viral Forecasting, San Francisco, CA, 94104, USA. [3]National Institute of Infectious Diseases Gakuen, Tokyo, 2080011, Japan.

Authors' contributions

WD carried out genetic analysis, drafted the manuscript. ZH_Y performed whole-genome sequencing, participated in the study design. GX carried out viral isolation. LL carried out viral isolation. MY participated in sample collection. ZH_R participated in sample collection. ZH participated in sample collection. ZH_Q performed the sequence alignment. FL performed the sequence alignment. LC participated in whole-genome sequencing. LH helped to draft the manuscript. KC participated in the design of the study. MC helped editing of the manuscript. HY helped editing. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 4 August 2011 Accepted: 15 February 2012
Published: 15 February 2012

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doi:10.1186/1743-422X-9-41
Cite this article as: De *et al.*: Phylogenetic and molecular characterization of coxsackievirus A24 variant isolates from a 2010 acute hemorrhagic conjunctivitis outbreak in Guangdong, China. *Virology Journal* 2012 9:41.

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Virus Genes
DOI 10.1007/s11262-012-0719-8

Evaluating the prevalence and molecular epidemiology of echovirus 11 isolated from sewage in Shandong Province, China in 2010

Jing Yang · Ning Cui · Haiyan Wang · Zexin Tao · Yao Liu · Heng Zhang · Hiromu Yoshida · Yanyan Song · Yong Zhang · Lizhi Song · Yan Li · Xiaojuan Lin · Shengxiang Ji · Wenbo Xu · Aiqiang Xu

Received: 29 November 2011 / Accepted: 18 January 2012
© Springer Science+Business Media, LLC 2012

Abstract Echovirus 11 (E11) is an important human pathogen, but its genetic information in China is in scarce. In this study, 12 sewage samples from Jinan city and 18 from Linyi city were collected in Shandong Province, China in 2010, and E11 was the predominant serotype with 54 isolates from 16 samples. Numbers of E11 isolates reached peaks in August in both Jinan and Linyi city, while another peak occurred in December in Linyi. The complete VP1 genes of all these isolates were sequenced and phylogenetically compared with clinical isolates from Shandong in 1994–2010 ($n = 29$) and global E11. Shandong isolates segregated into five clusters, four in genogroup A and one in genogroup C. Environmental isolates belonged to two clusters of genogroup A, with high inter-cluster genetic divergence (18.5–20.9%). No local clinical E11 was isolated in the two cities in 2010, revealing the value of environmental surveillance in investigating circulating viruses. These findings underscored the significance of environmental VP1 sequence divergences in comprehending the local enterovirus circulation, and updated the global molecular epidemiology of E11.

Keywords Echovirus 11 · Environmental surveillance · VP1 · Phylogenetic analysis · Molecular epidemiology

Introduction

Enteroviruses (EVs) are important human pathogens and circulate worldwide. Echovirus type 11 (E11) is a member of

Jing Yang, Ning Cui, and Haiyan Wang contributed equally to this study and are the first authors.

J. Yang · Y. Song (✉) · A. Xu
Department of Epidemiology, School of Public Health, Shandong University, 44 Wenhuaxi Road, Lixia District, Jinan 250012, People's Republic of China
e-mail: yysong@sdu.edu.cn

N. Cui
Department of Preventive Medicine, College of Basic Medical Sciences, Shandong University of Traditional Chinese Medicine, Jinan, People's Republic of China

H. Wang · Z. Tao · Y. Liu · L. Song · Y. Li · X. Lin · A. Xu
Shandong Provincial Key Laboratory of Infectious Disease Control and Prevention, Shandong Center for Disease Control and Prevention, Jinan, People's Republic of China

H. Zhang
Jinan Environmental Monitoring Center, Jinan, People's Republic of China

H. Yoshida
Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan

Y. Zhang · W. Xu
WHO WPRO Regional Polio Reference Laboratory and State Key Laboratory for Molecular Virology and Genetic Engineering, Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, People's Republic of China

S. Ji
Linyi Center for Disease Control and Prevention, Linyi, People's Republic of China

A. Xu (✉)
Shandong Center for Disease Control and Prevention, No. 16992 Jingshi Road, Jinan 250014, People's Republic of China
e-mail: aqxuepi@163.com

Published online: 07 February 2012

Species B human EV (HEV). It is among the most frequently isolated serotypes in many countries [1–3]. Besides asymptomatic or mild infections, it is also responsible for more serious disorders, such as aseptic meningitis (AM), multisystem hemorrhagic disease of newborn, and uveitis [4, 5]. More seriously, E11 was frequently associated with nosocomial infections [6]. In China, no indigenous wild-type polioviruses have been detected since 1995. However, importation of wild-type polioviruses has been reported in 2011. And also, outbreaks associated with non-polio EVs (NPEVs) have been sequentially reported in recent years [7, 8].

Molecular methods based on VP1 sequences have been broadly used for typing of EV infection and molecular epidemiology investigations [9, 10]. Previous study revealed that at least four monophyletic genogroups of E11 strains circulated worldwide during 1953–2001 based on sequence analysis of complete VP1 sequences from 16 countries [1]. And in another study, by molecular epidemiological research on 257-nt partial VP1 sequences of E11 from 1993 to 2007, designated genogroup D strains were found to have a striking prevalence in Finland [3]. However, except for six partial VP1 sequences of E11 from Yunnan province, no further genetic information on E11 in China was available [11].

The environmental surveillance, designed as a supplementary method for polio eradication program, offered an effective approach in investigating circulating HEV [12, 13]. Combination of virus isolation from environmental sources and phylogenetic analysis is demonstrated to be an effective approach to trace prevalent circulating EVs in the human population [14]. Because the VP1 gene of EVs plays an important role in viral pathogenesis and virulence [15–17], understanding the tempo and mode of evolution of VP1 coding region can provide new insights into the epidemiological dynamics of E11 that may be useful in predicting the genetic basis and periodicity of future E11 epidemics [18].

In this study, the circulation of E11 was revealed and evaluated via environmental surveillance in the two cities of Shandong, China in 2010, and the phylogenetic comparison on complete VP1 sequences between environmental E11 and clinical isolates was performed, and different transmission chains of E11 were identified to co-circulate in the two cities.

Materials and methods

Sewage sampling sites

Shandong Province, with an area of 150,000 km^2 and a population of 91 million, is located in the eastern part of China. Jinan is the capital city of Shandong Province with a population of 2.6 million. Jinan Everbright Water (JNEW) sewage treatment plant treated the domestic sewage from the western part of the city and was a sampling site. Linyi, the largest city (in area) in Shandong Province, is located 217 km south of Jinan city with a population of 1.8 million. Linyi Shouchuang (LYSC) sewage treatment plant was another sampling site.

Sampling and concentration

Sewage samples were collected monthly in Jinan from January to December 2010 and twice per month in Linyi since April. All these samples were collected from the inlet collector canal using grab sampling method in the afternoon during 14:00–15:00. Approximately, 1 l of flowing water was collected by a stainless plastic bucket as a sewage sample. The samples were transported to the laboratory within 1 h, and the temperature was maintained approximately 4°C during transportation. Samples were concentrated for virus isolation immediately (<24 h) as described before [14, 19, 20]. In brief, 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 mixed cellulose ester membrane filter (ADVANTEC, A045A142C, Tokyo, Japan). Absorbents on the filter were then eluted with 10 ml 3% beef extract solution by ultrasonication for three times (1 min for each time), and the solution was centrifuged at 12,000×*g* for 30 min. Subsequently, the supernatant was filtered through a 0.22-μm filter and was ready for cell inoculation.

Virus isolation and VP1 sequence analysis

L20B, RD, and HEp-2 cell lines were used for virus isolation [21]. The microneutralization assays were carried out in 96-well tissue culture plates according to standard protocols recommended by the WHO, using pools (Pools A–G, Coxsackie B, and Polio) of horse antiserum for typing EV (RIVM, the Netherlands). Viral RNA extraction was performed using QIAamp viral RNA mini kit (Qiagen, Valencia, CA, USA), and RT-PCR was performed using Access One-step RT-PCR System (Promega, USA). Primer pairs 008-013 and 187-011 designed by Oberste et al. [9, 22] were used for obtaining the entire VP1 sequence.

PCR products were purified and sequenced directly with the BigDye Terminator v3.0 Cycle Sequencing kit (Applied Biosystems, Foster City, CA), and sequences were analyzed by ABI 3130 genetic analyzer (Applied Biosystems). Nucleotide sequence alignments were carried out by BioEdit 7.0.5.3 software. Phylogenetic trees were constructed by Mega 4.0 using neighbor-joining method after estimation of genetic distance using the Kimura

two-parameter method [23]. A bootstrapping test was performed with 1,000 duplicates, and the transition/transversion rate was set at 2.0.

Nucleotide sequence accession numbers

The VP1 nucleotide sequences of E11 isolates described in this study were deposited in the GenBank database under the accession numbers DQ220784–DQ220786, GQ329790–GQ329795, HM446206–HM446215, HQ286038–HQ286044, and JF969406–JF969462.

Results

Virus isolation

In environmental surveillance conducted in Shandong Province in 2010, 87 and 64 NPEVs were isolated in Jinan and Linyi, respectively. E11 was the most frequently isolated serotype with 22 isolates from 67% (8/12) sewage samples in Jinan and 32 isolates from 44% (8/18) samples in Linyi. Most of these E11 viruses (50/54) were isolated on RD cell line, with higher sensitivity than HEp-2 cell line (4/54).

The monthly results of E11 and other NPEVs in Jinan and Linyi cities were shown in Fig. 1. Numbers of E11 isolates reached peaks in August in both Jinan ($n = 10$) and Linyi ($n = 13$), and another peak occurred in December in Linyi ($n = 12$). The increase of environmental E11 in winter (Fig. 1b, from Nov 30 to Dec 31) in Linyi was not coincident with other NPEVs. In other months, E11 was detected at a low frequency.

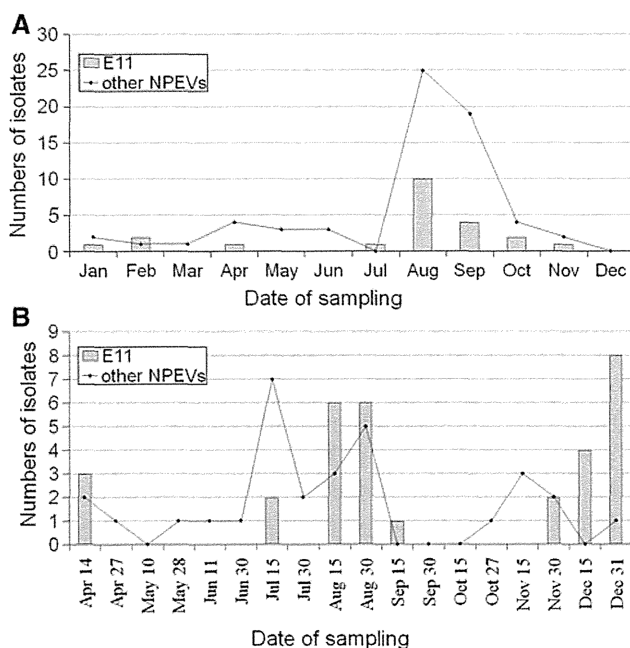

Fig. 1 Monthly distribution of echovirus 11 and other NPEV isolates from sewage collected in Jinan (**a**) and Linyi (**b**) sewage treatment plants in 2010

VP1 sequence analysis

Homologous comparison and phylogenetic analysis were performed on complete VP1 sequences of 54 environmental E11 isolates, 29 isolates from acute flaccid paralysis (AFP) patients in Shandong Province in 1994–2010 (Table 1), and 21 global reference strains which are representatives of different genogroups. Shandong E11 segregated into five clusters—four in genogroup A and one in genogroup C—and genogroup A was predominant with 78 isolates (Fig. 2). Means of genetic distances within and between the five clusters of Shandong E11 were estimated by kimura two-parameter method. The overall mean distance of all analyzed E11 was 0.198. The within-cluster mean distances were below 0.1, and between-cluster mean distances were 0.139–0.275 (Table 2).

Most Shandong E11 isolates belonged to genogroup A. Five Shandong isolates segregated in genogroup C, and they were isolated from AFP cases in 1994–1997, suggesting that they might be extinct in Shandong Province. In genogroup A, cluster 3 consisted of only two isolates in 1994–1996, implying they might also be extinct. Cluster 2 was completely composed of clinical isolates. Cluster 1 was the major one with 54 members from AFP and environmental surveillance. Cluster 4 was composed entirely of environmental isolates from Linyi city.

Environmental isolates segregated into two clusters in genogroup A. All 22 environmental E11 isolates from Jinan were grouped into cluster 1. For 32 Linyi strains, 24 were grouped into cluster 1 and the other 8 belonged to cluster 4 (Fig. 2). Homologous analysis revealed 79.1–81.5% nucleotide identities between the two clusters, 91.3–100.0% within cluster 1, and 99.5% within cluster 4. Interestingly, in cluster 1, an intimate phylogenetic relationship was observed between Jinan isolates JNEW100811.4/27 and some Linyi isolates (nt similarities, 98.5–99.0%), revealing the long distance transmission between the two cities.

Discussion

In environmental surveillance conducted in Shandong Province in 2010, E11 was the most frequently isolated serotype. A negative finding in sewage may not be meaningful unless long-term follow-up results become available [24]. So, the frequent detection of E11 in 2010 suggests the existence of continuous circulation in the two cities. EV is known to be active in summer season, and in this study, as

Table 1 Shandong Echovirus 11 isolates used in this study

Isolates	Year	Sources	GenBank Ac. No.
JNEW100125.2	2010	Sewage	JF969406
JNEW100223.1	2010	Sewage	JF969407
JNEW100223.2	2010	Sewage	JF969408
JNEW100429.5	2010	Sewage	JF969409
JNEW100730.1	2010	Sewage	JF969410
JNEW100811.4	2010	Sewage	JF969411
JNEW100811.11	2010	Sewage	JF969412
JNEW100811.18	2010	Sewage	JF969413
JNEW100811.21	2010	Sewage	JF969414
JNEW100811.25	2010	Sewage	JF969415
JNEW100811.27	2010	Sewage	JF969416
JNEW100811.30	2010	Sewage	JF969417
JNEW100811.31	2010	Sewage	JF969418
JNEW100811.33	2010	Sewage	JF969419
JNEW100811.34	2010	Sewage	JF969420
JNEW100913.2	2010	Sewage	JF969421
JNEW100913.10	2010	Sewage	JF969422
JNEW100913.11	2010	Sewage	JF969423
JNEW100913.20	2010	Sewage	JF969424
JNEW101012.1	2010	Sewage	JF969425
JNEW101012.2	2010	Sewage	JF969426
JNEW101112.1	2010	Sewage	JF969427
LYSC100414.1	2010	Sewage	JF969428
LYSC100414.2	2010	Sewage	JF969429
LYSC100414.4	2010	Sewage	JF969430
LYSC100715.1	2010	Sewage	JF969431
LYSC100715.2	2010	Sewage	JF969432
LYSC100815.1	2010	Sewage	JF969433
LYSC100815.2	2010	Sewage	JF969434
LYSC100815.5	2010	Sewage	JF969435
LYSC100815.6	2010	Sewage	JF969436
LYSC100815.7	2010	Sewage	JF969437
LYSC100815.9	2010	Sewage	JF969438
LYSC100830.2	2010	Sewage	JF969439
LYSC100830.5	2010	Sewage	JF969440
LYSC100830.6	2010	Sewage	JF969441
LYSC100830.7	2010	Sewage	JF969442
LYSC100830.9	2010	Sewage	JF969443
LYSC100830.11	2010	Sewage	JF969444
LYSC100915.1	2010	Sewage	JF969445
LYSC101130.1	2010	Sewage	JF969446
LYSC101130.2	2010	Sewage	JF969447
LYSC101215.1	2010	Sewage	JF969448
LYSC101215.2	2010	Sewage	JF969449
LYSC101215.4	2010	Sewage	JF969450
LYSC101215.5	2010	Sewage	JF969451
LYSC101231.1	2010	Sewage	JF969452
LYSC101231.2	2010	Sewage	JF969453
LYSC101231.3	2010	Sewage	JF969454
LYSC101231.4	2010	Sewage	JF969455
LYSC101231.5	2010	Sewage	JF969456
LYSC101231.6	2010	Sewage	JF969457
LYSC101231.7	2010	Sewage	JF969458
LYSC101231.8	2010	Sewage	JF969459
94321	1994	AFP	HQ286040
94301	1994	AFP	HQ286041
95353	1995	AFP	HQ286043
96339	1996	AFP	JF969460
96387	1996	AFP	GQ329795
97219	1997	AFP	GQ329796
97174	1997	AFP	HQ286042
99350	1999	AFP	HQ286044
00352	2000	AFP	HM446206
00293	2000	AFP	HM446207
00348	2000	AFP	HM446208
00338	2000	AFP	HM446209
00334	2000	AFP	GQ329791
01602	2001	AFP	HM446210
03471	2003	AFP	GQ329792
SD-E11-03-1	2003	AFP	DQ220784
SD-E11-03-2	2003	AFP	DQ220785
SD-E11-03-4	2003	AFP	DQ220786
06359	2006	AFP	GQ329793
06375	2006	AFP	GQ329794
07060	2007	AFP	HQ286038
07300	2007	AFP	HQ286039
08238	2008	AFP	HM446211
08286	2008	AFP	HM446212
08294	2008	AFP	HM446213
09073	2009	AFP	HM446214
09263	2009	AFP	HM446215
10278	2010	AFP	JF969461
10324	2010	AFP	JF969462

shown in Fig. 1, numbers of E11 isolates reached peaks in August in both cities, reflecting the high local activity at that time. However, another peak occurred in December in Linyi, and the increase of environmental E11 in winter was not coincident with other NPEVs, revealing a short-term accumulation of E11 infected population. E11 can cause serious illnesses such as AM [14]. However, there is no specific case-based EV surveillance system in China. So, the environmental surveillance provides the unique information for the local circulating EVs.

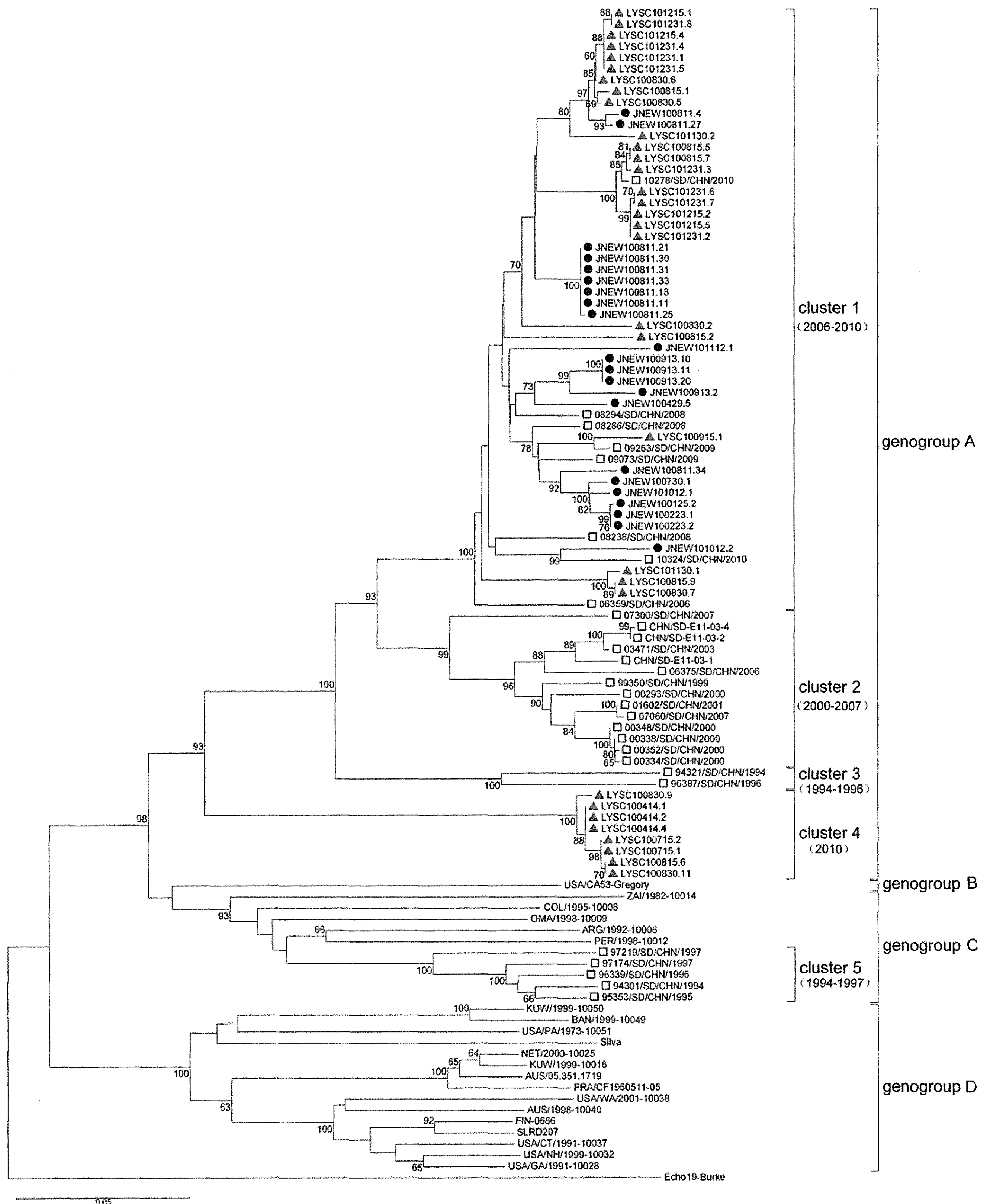

Fig. 2 Phylogenetic tree based on the alignment of the entire VP1 coding regions of global E11 isolates. *Circle* and *triangle* indicated environmental isolates from Jinan and Linyi, respectively, and *square* indicated isolates from AFP surveillance in Shandong Province. The prototype E19-Burke strain was used as an outgroup. The environmental isolates are identified by a code that consists of JNEW (from Jinan) and LYSC (from Linyi), followed by the sample date presented as YYMMDD and the tube number

Table 2 Means of genetic distances estimated by kimura two-parameter method within and between the five clusters of Shandong Echovirus 11

Cluster	Nucleotide distance				
	1	2	3	4	5
1	0.061				
2	0.139	0.052			
3	0.174	0.175	0.092		
4	0.234	0.228	0.257	0.007	
5	0.266	0.262	0.275	0.264	0.062

Phylogenetic analysis on global E11 revealed Shandong E11 belonged to genogroups A and C. In previous molecular epidemiology study on global E11, genogroup D was revealed to be predominant in American and European countries, and the numbers of isolates in genogroups A, B, and C were small [1, 3]. However, in this study, no Shandong E11 belonged to genogroup D, and a great number of genogroup A strains were identified, suggesting E11 circulating in China mainly consisted of isolates of this genogroup. Although five 1994–1997 Shandong isolates segregated in genogroup C, this five-member cluster appears extinct.

Cluster 1 is a major group for Shandong E11. Most environmental isolates in the two cities, together with E11 from AFP surveillance in 2006–2010, belong to this cluster, suggesting this cluster is the predominant lineage in Shandong Province in recent years. Cluster 4 is composed entirely of environmental isolates from Linyi city. This cluster has long genetic distances with the other three clusters in genogroup A. Interestingly, this cluster had not been found in recent 20-year AFP surveillance until the surveillance on domestic sewage in 2010. Due to the low incidence of AFP cases, the NPEVs isolated from AFP surveillance could not be sensitive enough to reveal the local EV circulation promptly. Hence, it is reasonable to conclude that this cluster might be imported into Linyi city recently and is a new virus for the region.

More interestingly, two types of VP1 divergence from the same sewage were identified from E11. First commonly, isolates from a same sewage possessed identical (or almost identical, less than 1%) sequences, such as the E11 of high identities seen in cluster 1 (Fig. 2). Considering the evolution rate of 4.8×10^{-3} (substitutions per site per year) for E11 VP1 region [25], this indicated an occurrence of short-term transmission in corresponding area. Nevertheless, it could not be ruled out that these isolates were derived from a single person, although the possibility is very low for metropolitan sewage specimens. Second strikingly, the isolates from a same sewage consist of VP1 sequences with considerable differences, such as the sewage in December in Linyi contained isolates from several lineages. This phenomenon is usually accompanied with the increase in isolation number. This type of VP1 divergence reflects local co-circulation of different lineages of the serotype, whether they derived from an importation of a common ancestor and evolved for several years, or several importations of different ancestors.

The summarized information of VP1 divergence of environmental isolates from continually collected sewage samples contributes to the understanding of the annual circulation of the virus. As for E11 in the two cities, the high local activity was revealed, and the co-circulation of different lineages of environmental isolates with high genetic divergence was observed, leading to the assumption that E11 had circulated in the two cities for a long time. The co-circulation can result in intra-typic recombination, which might affect the E11 evolution or even cause antigenicity and virulence alterations [25], and is an important issue in further surveillance.

In conclusion, the global E11 molecular epidemiology was updated, and the significance of VP1 sequence analysis in environmental surveillance was highlighted.

Acknowledgments This study was supported by a grant for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor and Welfare of Japan, and two grants from Health Department of Shandong Province (2011QZ013, 2011HZ058). The authors express sincere thanks to the colleagues of Division of EPI, Shandong CDC.

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

Zexin Tao,[a] Yanyan Song,[b] Haiyan Wang,[a] Yong Zhang,[c] Hiromu Yoshida,[d] Shengxiang Ji,[e] Aiqiang Xu,[a,b] Lizhi Song,[a] Yao Liu,[a] Ning Cui,[f] Feng Ji,[a] Yan Li,[a] Peng Chen,[b] and Wenbo Xu[c]

Shandong Provincial Key Laboratory of Infectious Disease Control and Prevention; Shandong Center for Disease Control and Prevention, Jinan, People's Republic of China[a]; School of Public Health, Shandong University, Jinan, People's Republic of China[b]; WHO WPRO Regional Polio Reference Laboratory and State Key Laboratory for Molecular Virology and Genetic Engineering, Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, People's Republic of China[c]; Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan[d]; Linyi Center for Disease Control and Prevention, Linyi, People's Republic of China[e]; and Department of Preventive Medicine, College of Basic Medical Sciences, Shandong University of Traditional Chinese Medicine, Jinan, People's Republic of China[f]

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

Received 8 June 2012 Accepted 16 July 2012
Published ahead of print 27 July 2012
Address correspondence to Aiqiang Xu, aqxuepi@163.com, or Wenbo Xu, wenbo_xu1@yahoo.com.cn.
Z.T. and Y.S. contributed equally to this article.
Supplemental material for this article may be found at http://aem.asm.org/.
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doi:10.1128/AEM.01861-12

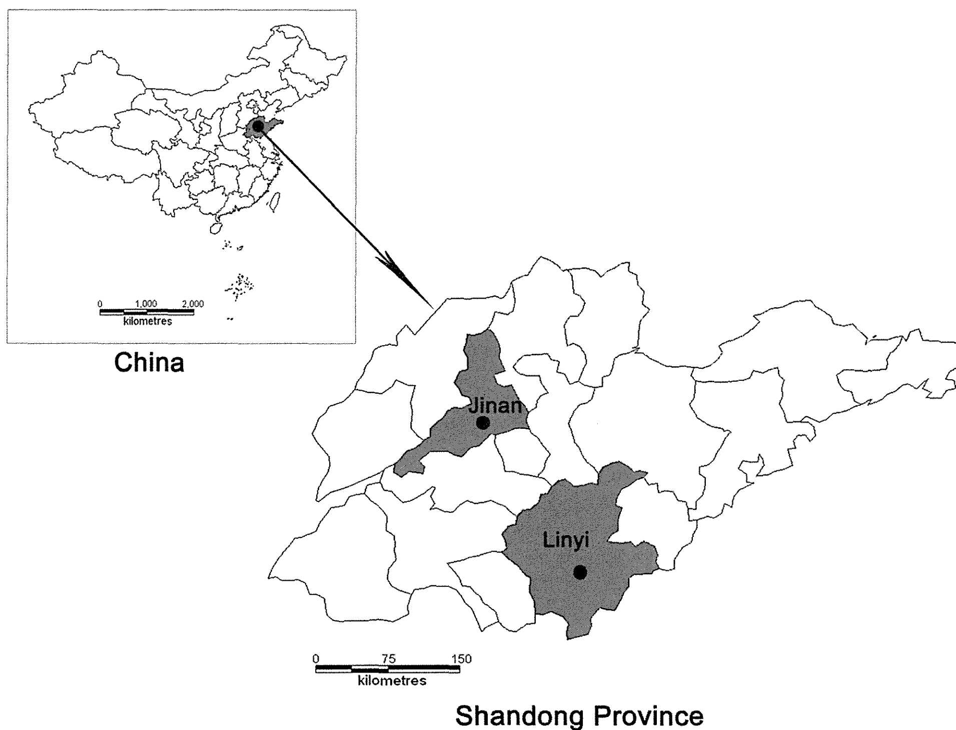

FIG 1 Locations of Shandong Province and the two cities where environmental surveillance was conducted. Maps were created using Mapinfo software; data are from the National Fundamental Geographic Information System (NFGIS) website (http://ngcc.sbsm.gov.cn/).

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 km^2 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 × g for 30 min at 4°C, and 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). 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 × 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

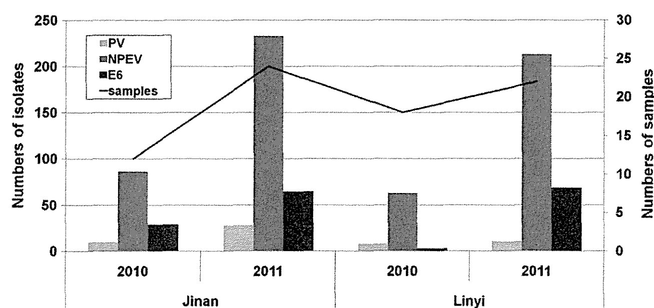

FIG 2 Numbers of sewage samples and numbers of PV, E6, and other NPEV isolates in the cities of Jinan and Linyi, 2010 to 2011.

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_{MRCA}) 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