Genetic Diversity of Coxsackievirus A16 Associated with Hand, Foot, and Mouth Disease Epidemics in Japan from 1983 to 2003

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Abstract

To clarify the chronologic genetic diversity of coxsackievirus A16 (CV-A16) strains associated with hand, foot, and mouth disease (HFMD) epidemics in a restricted area and their genetic relation with those isolated in other areas, we investigated the genetic diversity of the 129 CV-A16 strains associated with HFMD epidemics in Fukushima, Japan, from 1983 to 2003, and compared their genetic relation with the 49 CV-A16 strains isolated in other areas of Japan and in China using phylogenetic analysis based on the VP4 sequences. Phylogenetic reconstruction of the CV-A16 strains isolated in Fukushima from 1983 to 2003 demonstrated three distinct genetically divergent clusters relating to HFMD epidemics that occurred from 1984 to 1994 (including the 1985 and 1991 outbreaks), those from 1987 to 1998 (including the 1988 and 1998 outbreaks), and those from 1995 to 2003 (including the 1995 and 2002 outbreaks). CV-A16 strains isolated during each period in Fukushima formed a single cluster with those isolated during essentially the same time period in other areas of Japan and in China. Our results demonstrated that prevalent CV-A16 strains causing HFMD in Fukushima, Japan, genetically changed twice during 21 epidemics and changes were also observed in the CV-A16 strains causing HFMD epidemics in other areas. We concluded that repeated outbreaks of CV-A16-related HFMD in Japan were caused, in part, by the introduction

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of genetically changed CV-A16 strains, which might be transmitted overseas.
Introduction

Coxsackievirus A16 (CV-A16) and human enterovirus 71 (HEV71) are both major etiologic agents of hand, foot, and mouth disease (HFMD). The surveillance data indicate that CV-A16 and HEV71 infections independently cause large outbreaks and then become quiescent for a period of a few years [6].

HEV71 illness is more severe with a significantly greater frequency of serious complications and fatality than illness caused by CV-A16 [2]. In 1997, deaths associated with epidemics of HEV71-associated HFMD in Sarawak, Malaysia, followed by outbreaks with high mortality in Taiwan in 1998 and 2000, raised considerable public concern about the virulence of this virus. Since then, several groups have attempted to describe the molecular epidemiology of HEV71 in the Asia-Pacific region, and have reported the relationships of HEV71 epidemics with the genetic diversity of HEV71 strains [1, 5, 10]. The results indicate that HEV71 strains causing HFMD outbreaks were genetically changed.

On the other hand, the molecular epidemiology of CV-A16 associated with HFMD epidemics has not been fully described [9]. In the present study, we evaluated the relationship between the chronologic CV-A16 epidemics in a restricted area, i.e., Fukushima Prefecture, Japan, and the genetic diversity of the CV-A16 strains. We also
examined the geographic genetic relationship between the CV-A16 strains isolated in Fukushima and those isolated in other areas of Japan and China, using phylogenetic analyses constructed using the neighbor-joining method on the basis of the VP4 and VP1 sequences.

Materials and Methods

Virus strains. Pharyngeal swab samples were collected from patients with HFMD in the Fukushima Prefecture for virus surveillance and transferred to the Fukushima Institute for Public Health for virus isolation. HEp-2, Vero, and RD-18 cells were used for the isolation of enteroviruses. Confluent cell cultures were seeded in microplate wells and inoculated with 100 μL of maintenance medium and 50 μL of pharyngeal swab samples. The cell cultures were then incubated at 34°C in 5% CO₂/95% air and observed for 7 days to check for cytopathic effects. A blind passage was performed once if no cytopathic effect was observed by the end of the observation period. Virus isolates were identified by a neutralization test using anti-CV-A16 polyclonal antibodies provided from the National Institute of Infectious Diseases in Japan. A total of 322 CV-A16 strains were isolated and identified from 1983 to 2003. Those isolates were stored at -80°C.
**PCR and sequence determination of VP4 gene.** Randomly selected isolates (63/241 strains) from 1983 to 1999 and all isolates (69 strains) from 2000 to 2003 were used for further genetic analysis. A total of 132 strains were isolated from patients with HFMD. The methods of molecular diagnosis of enteroviruses by nested reverse-transcription (RT) - polymerase chain reaction (PCR) and phylogeny-based classification using the VP4 sequences are described elsewhere [7]. Briefly, viral RNA was directly extracted from 100 µL of the stock virus samples using the Smitest R kit (Genome Science Laboratories), according to the manufacturer’s instructions. The RNA was dissolved with 10 µL of RNase-free distilled water containing 40 U of ribonuclease inhibitor (RNasink; Promega) and 50 pmol of a reverse primer, OL68-1 [nt 1178-1197, 5’-GGTAA(C/T)TTCCACCACCA(A/G/C/T)CC-3’]. The positions of the primers for RT-PCR were numbered according to the complete nucleotide sequence of the attenuated poliovirus Sabin 1 strain [11]. The RNA was subjected to heat denaturation for 15 s at 100°C followed by snap-cooling in an ice-water bath. Reaction mixture [10 µl; 200 U of Moloney murine leukemia virus reverse transcriptase (Life Technology), 2.5 mM dNTPs, and 40 U of RNasin (Promega) ] was added to each RNA sample. cDNA synthesis was performed for 1 h at 37°C. In total, 5 µL of the cDNA reaction mixture was added to 45 µL of 1x Taq buffer containing 12.5 pmol of a forward primer,
MD91 (nt 444-468, 5′-CCTCCGGCCCCTGAATGCGGCTAAT-3′) and 2.5 U of Taq DNA polymerase (Roche Diagnostic Systems). Semi-nested PCR was performed using 5 μL of the PCR product with a pair of primers, EVP4 (nt 541-560, 5′-CTACTTTGGGTGTCCGTGTT-3′) and OL68-1. After initial denaturation at 94°C for 5 min, 40 cycles of amplification were performed using the GeneAmp PCR System 9600 (PE-Applied Biosystems). Each cycle consisted of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, and an extension reaction at 72°C for 1 min. The 40 amplification cycles were followed by a final extension at 72°C for 7 min. The first PCR primer pair allowed for amplification of 753 bp and the semi-nested PCR primer pair allowed for amplification of 656 bp. The final PCR products encoded the 3′ one-third of the 5′ non-translated region, the entire VP4, and the 5′ one-third of VP2 of each CV-A16 strain. The PCR products, including the entire VP4 sequences, were separated in 1% agarose gels and purified with a QIA quick-gel extraction kit (Qiagen). The nucleotide sequence was determined using a 373A DNA auto-sequencer (PE-Applied Biosystems) with fluorescent dideoxy chain terminations (PE-Applied Biosystems) and EVP4 and OL68-1 primers.

**Phylogenetic analysis based on the VP4 gene.** The entire VP4 nucleotide sequences of the 129 strains isolated in Fukushima were determined and used for phylogeny-based
analysis along with those of 64 prototype enterovirus strains. We estimated the evolutionary distances using the Kimura two-parameter method [8] and constructed unrooted phylogenetic trees with the neighbor-joining method [14]. Bootstrap analysis was performed by resampling the data sets 1000 times. Bootstrap values greater than 70% were considered to be statistically significant for the grouping. The VP4 sequences of representative 54 CV-A16 strains isolated in Fukushima were also compared with those of 20 strains isolated in other areas of Japan, 29 strains in China, and 1 strain in the United Kingdom taken from international databases (GenBank) using phylogenetic analysis.

**PCR and phylogenetic analysis based on the VP1 gene.** To confirm the relevance of genogrouping based on the VP4 region, we used the phylogeny-based classification methods using the VP1 sequences that were reported by Oberste et al [12]. Twenty-five CV-A16 strains isolated in Fukushima were randomly selected and their VP1 regions were amplified using two sets of primer pairs, i.e., 055/VP3-011/2A and 055/VP3-009/2A. The VP1 sequences were determined and compared using phylogenetic analysis with those of 28 strains isolated in China and 1 Taiwan strain taken from international databases.
Results

**Chronologic genetic diversity.** Enterovirus surveillance data in the Fukushima Prefecture for the period from 1983 to 2003 indicate that peaks of CV-A16 isolations from HFMD occurred in the years 1985, 1988, 1991, 1995, 1998, and 2002. This epidemic pattern is very similar to that observed in Japanese national surveillance data (Fig. 1) [6]. A total of 322 CV-A16 strains were isolated and identified during this period in the Fukushima Prefecture. A nested RT-PCR assay was performed for the detection of enteroviral genome sequences in 63 randomly selected samples collected from 1983 to 1999 and the 69 samples collected from 2000 to 2003. A positive PCR result was obtained in 129 of 132 samples and all detected enteroviruses were identified as CV-A16 using PCR-based analysis. Phylogenetic analysis of the VP4 sequences of 129 CV-A16 strains isolated in Fukushima demonstrated the existence of at least three genetically distinct groups (Bootstrap value > 70%) relating to the epidemics that occurred from 1984 to 1994, from 1987 to 1998, and from 1995 to 2003 (Fig. 2). Those three groups were designated as genogroup A, B, and C, respectively. The outbreaks in 1985 and 1991 were due to genogroup A, those in 1988 and 1998 genogroup B, and those in 1995 and 2002 genogroup C (Fig. 1). All isolates in Fukushima since 2000 were of genogroup C. CV-A16 strains within a genogroup were slightly genetically
divergent in several epidemics.

**Geographic genetic relationship.** VP4 sequences of representative 54 CV-A16 strains isolated in Fukushima were compared with those of 50 strains taken from international databases (GenBank), which included 20 strains isolated in Japan, 29 strains in China, and 1 UK strain. Genogroup A strains included 11 CV-A16 strains isolated in Fukushima from 1984 to 1994 and 1 strain isolated in another part of Japan in 1986. Genogroup B strains included 30 CV-A16 strains isolated in Fukushima from 1987 to 1998, 6 strains isolated in other parts of Japan from 1979 to 1998, and 3 strains isolated in Asia from 1999 to 2000. Genogroup C strains included 88 CV-A16 strains isolated in Fukushima from 1995 to 2003, 11 strains isolated in other parts of Japan from 1998 to 2002, 26 strains isolated in Asia from 1998 to 2003, and 1 UK strain in 1999. In general, each genogroup relating to the epidemics from 1984 to 1994, from 1987 to 1998, or from 1995 to 2003 in Fukushima formed a single cluster with CV-A16 strains isolated during almost the same time period in other areas of Japan and in China (Fig. 3). Although the clustering seemed to be more closely related to the period of isolation rather than the area of isolation, the VP4 sequences of strains isolated in Japan were not identical to those isolated in China during the same time period. The GenBank accession numbers of the nucleotide sequences of 54 representative CV-A16 isolates in
Fukushima are AB266393 to AB266446. Those are indicated in parenthesis in Fig. 3, as well as 50 additional strains taken from GenBank.

To clarify whether the genetic diversity occurred as clusters in the VP4 gene or scattered across the gene, the entire VP4 nucleotide sequences of all strains were aligned (Fig. 4). Common genetic diversities were observed in each genogroup. They did not form clusters, but appeared randomly throughout the VP4 gene.

**Phylogenetic analysis based on the VP1 gene.** To confirm the relevance of genogrouping based on the VP4 region, bootstrap analysis was performed using VP1 sequences of 25 CV-A16 strains isolated in Fukushima and 29 strains isolated in other countries. The analysis based on the VP1 gene revealed three genetically distinct groups, and the grouping was completely identical with the results based on the VP4 gene (data not shown). Bootstrap values for the grouping of A, B, and C based on the VP1 gene were 97%, 81%, and 100%, respectively, and were higher than those based on the VP4 gene (90%, 39%, and 71%, respectively).

**Discussion**

CV-A16 causes large outbreaks of HFMD worldwide. Enterovirus surveillance data in the Fukushima Prefecture for the period from 1983 to 2003 indicate that the annual
proportion of CV-A16 isolates relative to total enterovirus isolates fluctuated widely, from 0% in 1983, 1986, and 1999 to 35.3% in 2002. Peaks of CV-A16 isolations from HFMD occurred in the years 1985, 1988, 1991, 1995, 1998, and 2002 [6]. This epidemic pattern is very similar to that observed in the Japanese national surveillance data (Fig. 1) [6]. Those observations indicate that CV-A16 follows an epidemic mode of transmission, causing large outbreaks and then becoming quiescent for a period of a few years. Similar quiescence between outbreaks is observed in the meningitis epidemics caused by echovirus type 30. The quiescence is probably due to the development of population immunity that occurs during a high-infection-rate epidemic. The virus might cause only sporadic cases until a large cohort of non-immune individuals has developed, often over a period of several years, setting the stage for another large epidemic [12].

Phylogeny-based classification using the VP4 sequence is useful for the identification of human enteroviruses [3, 4]. The method takes advantage of the detection of the divergence in VP4 sequences both between and within serotypes, and thus is also of use for global epidemiologic studies of enteroviruses [1, 5, 7]. We investigated the genetic diversity of CV-A16 strains associated with HFMD epidemics in the Fukushima Prefecture, Japan from 1983 to 2003 and compared their genetic relation with those isolated in other areas of Japan and in China using the same method. CV-A16 strains
isolated in Fukushima, from 1983 to 2003, made at least three distinct clusters on the phylogenetic tree. The three clusters were designated as genogroups A, B, and C, and were associated with HFMD epidemics from 1984 to 1994 (including the 1985 and 1991 outbreaks), those from 1987 to 1998 (including the 1988 and 1998 outbreaks), and those from 1995 to 2003 (including the 1995 and 2002 outbreaks), respectively. The predominant genogroup was replaced with a new genogroup. CV-A16 strains within a genogroup gradually became genetically divergent in several epidemics. Those results demonstrated that the introduction of a new genogroup in addition to the genetic divergence within a genogroup resulted in repeated HFMD outbreaks in Fukushima, Japan.

Each genogroup formed the same cluster with CV-A16 strains isolated during essentially the same time period in other areas of Japan and in China. The clustering seemed to be more closely related to the date of isolation rather than the geographic location. Genetic diversities appeared randomly throughout the VP4 gene and were common to each genogroup. Those indicated that a new CV-A16 genogroup might derive from other regions of the world, be predominant for several years with genetic divergence, and then disappear.

The protein encoded by the VP1 gene is the most exposed and immunodominant of the
capsid proteins. Serotypic identification and classification rely on antigenic methods, and VP1 gene sequence data is likely to give the most useful information in molecular epidemiologic studies [13]. Shimizu et al analyzed the phylogeny of HEV71 isolates, which is the other major etiologic agent of HFMD, based on the nucleotide sequence alignment of both the VP1 and VP4 regions [15]. Phylogenetic trees based on VP4 sequences revealed that the HEV71 strains isolated from the Western Pacific Region formed two major genogroups, B and C, and were similar to those based on the VP1 sequences. Cardosa et al reported that the VP1 and VP4 gene sequences both provide similar phylogenetic information, but the higher bootstrap values in the VP1 dendrograms provide greater confidence, particularly when elucidating new genotypes. Thus, the use of the shorter VP4 gene might be helpful for HEV71 surveillance, but the VP1 gene should be used for confirming data obtained with VP4-based analysis [1].

Therefore, we confirmed the data obtained from the analysis based on VP4 genes of CV-A16 isolates using VP1-based analysis. Phylogenetic analysis based on the VP1 gene demonstrated the existence of three genetically distinct groups, and the grouping was completely identical with the result obtained with VP4-based analysis. Bootstrap values for the grouping of A, B, and C based on the VP1 gene were higher than 80%, while those for the grouping of A and C based on the VP4 gene were higher than 70%.
Although bootstrap values are slightly higher in the VP1 dendrograms than in the VP4 dendrograms, the VP4 gene as well as the VP1 gene seems to be appropriate for the epidemiologic study of CV-A16. In conclusion, phylogenetic analysis based on the VP1 gene confirmed that CV-A16 strains isolated in Fukushima formed three genogroups and each genogroup formed the same cluster with CV-A16 strains isolated during essentially the same time period in China.

In summary, we describe the evident genetic diversity and changes in the VP4 protein region of CV-A16 strains that were isolated in a restricted region through more than 20 successive epidemics. Our results indicate that CV-A16 strains causing HFMD had genetically changed twice during the period and those CV-A16 strains might have been transmitted overseas. We conclude that the repeated outbreaks of CV-A16-related HFMD might be caused, in part, by the worldwide transmission of the genetically changed CV-A16 strains, as well as a large cohort of non-immune individuals. To test this hypothesis, a worldwide surveillance system for HFMD and genetic analysis of isolated CV-A16 strains is necessary.
Figure Legends

Figure 1

Numbers of CV-A16 isolates in Fukushima Prefecture and in Japan between 1983 and 2003

Numbers of CV-A16 isolates in Fukushima (closed circle and solid line) and in Japan (open circle and broken line) are expressed, as reported to the Infectious Disease Surveillance Center in Japan by prefectural and municipal public health institutes through the Japanese infectious agents surveillance program. A, B, and C mean genogroups designated from phylogenetic analysis of the VP4 sequences of CV-A16 strains. Solid bars and broken bars mean HFMD outbreaks and epidemic periods, respectively, due to corresponding genogroups.

Figure 2

Phylogram depicting the phylogenetic relationships on the basis of the VP4 sequence among 129 CV-A16 strains isolated in Fukushima from 1983 to 2003.

Bootstrap analysis was performed by resampling the data sets 1000 times. Bootstrap values greater than 70% were considered to be statistically significant for the grouping
and were denoted in the figure. Isolated place, strain name, and isolated year were indicated. GenBank accession number of one isolate to represent isolates with identical sequence was also indicated in parenthesis. CA-A16/G-10/51 is prototype CV-A16 strain. The VP4 nucleotide sequence of prototype HEV71/BrCr/71 was used as an outgroup in the analysis.

Figure 3

Phylogram depicting the phylogenetic relationships on the basis of the VP4 sequence among representative 54 CV-A16 strains isolated in Fukushima and 50 CV-A16 strains isolated in Japan, China, and UK taken from international databases (GenBank).

Bootstrap analysis was performed by resampling the data sets 1000 times. Bootstrap values greater than 70% were considered to be statistically significant for the grouping and were denoted in the figure. Isolated place, strain name, and isolated year were indicated. GenBank accession numbers of CV-A16 strains isolated in Fukushima and the strains taken from international databases were indicated in parenthesis. CA-A16/G-10/51 is prototype CV-A16 strain. The VP4 nucleotide sequence of prototype HEV71/BrCr/71 was used as an outgroup in the analysis.
Figure 4

Alignment of the entire VP4 sequences.

Common genetic diversities were observed in each genogroup. Nucleotide difference from the prototype CV-A16/G-10/51 strain observed in genogroup A, genogroup A and B, or genogroup A and C was indicated in halftone and that in genogroup B, genogroup B and C, or genogroup C was indicated in box.
References


Figure 1

Isolation number in Fukushima (●)

Isolation number in Japan (○)

A

B

C

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Figure 3

Strains isolated in Fukushima
Strains taken from GenBank
CV-A16 VP4 Gene Alignment (1-70 bp)

Figure 4-1
CV-A16 VP4 Gene Alignment (71-140 bp)

**Figure 4-2**
## CV-A16 VP4 Gene Alignment (141-207 bp)

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**Fu:** Fukushima  **Ka:** Kanagawa  **To:** Tochigi  **C:** China

*Figure 4-3*