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Genetic diversity of enterovirus 71 isolated from cases of hand, foot and mouth disease in Yokohama City between 1982 and 2000

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Summary. Enterovirus 71 (EV71) is known as one of the major causative agents of hand, foot and mouse disease (HFMD) and is also associated with neurological manifestations such as aseptic meningitis, polio-like paralysis and encephalitis. Recently, large HFMD outbreaks, involving severe neurological complications, have been experienced in Malaysia, Taiwan and some other countries in the Western-Pacific region. To investigate the genetic diversity of EV71 isolates in a single community in Japan, nucleotide sequences of the VP4 region of 52 EV71 isolates in Yokohama City from 1982 to 2000 were determined and the phylogenetic relationship was compared with other referential EV71 strains in Japan and in the world. There were two major genotypes of EV71 in Yokohama City through the 1980's and 1990's. Six EV71 isolates in the early 1980's in Yokohama City were closely related to those from HFMD outbreaks in Japan and from outbreaks of polio-like paralysis in Europe in the 1970's. During recent HFMD outbreaks in 1997 and 2000, two distinct genotypes of EV71 were co-circulating in Yokohama City as in HFMD outbreaks in Malaysia and Taiwan. However, the genetic diversity of EV71 in Yokohama City was not directly correlated with the severity of HFMD. The results confirmed the circulation of two distinct genotypes of EV71 over the past 20 years in Japan.

Introduction

Enterovirus 71 (EV71) is one of the most recently described serotypes of human enterovirus, and was first isolated from patients with neurological manifestations in California between 1969 and 1972 [25]. Although EV71 is known as a common causative agent of hand, foot and mouse disease (HFMD) along with other enteroviruses such as coxsackievirus A10 and A16 (CA16), serious neurological complications due to the EV71 infection were also described. Many EV71 outbreaks with various neurological manifestations have been reported in Europe, America, Australia and East Asia [6, 7, 9–11, 16, 18, 19, 21]. Especially, recent HFMD outbreaks in Malaysia and Taiwan were associated with severe neurological involvement including significant fatalities among children and infants, and a number of EV71 strains was isolated from fatal and nonfatal cases of HFMD or herpangina during the outbreaks [1, 2, 10, 14, 15, 32].

EV71 belongs to the *Human enterovirus A* species of the family *Picornaviridae* [22] and possesses 60 copies of four capsid proteins, VP1, VP2, VP3 and VP4 in the virion. The VP4 protein lies buried in close association with the genomic RNA, and the amino acid sequences of the VP4 region are highly conserved among EV71 isolates. To investigate the genetic lineage of EV71 isolates among HFMD outbreaks in different areas, molecular epidemiological analyses of EV71 have been conducted using the sequence of the VP4 region as well as the 5′-untranslated region (UTR) and VP1 regions [1–3, 17, 26–29, 34]. Most of the recent molecular epidemiological studies are based on the analysis of EV71 isolates from outbreaks with severe neurological complications. However, information on the genetic diversity of EV71 isolated from common HFMD epidemics without severe neurological complications is still limited. Here we analyzed the genetic diversity of the VP4 sequence of 52 EV71 isolates from HFMD cases in a single district, Yokohama City, in Japan from 1982 to 2000, and compared those with the results with those for 9 other Japanese isolates and 34 referential strains from EV71 outbreaks throughout the world.

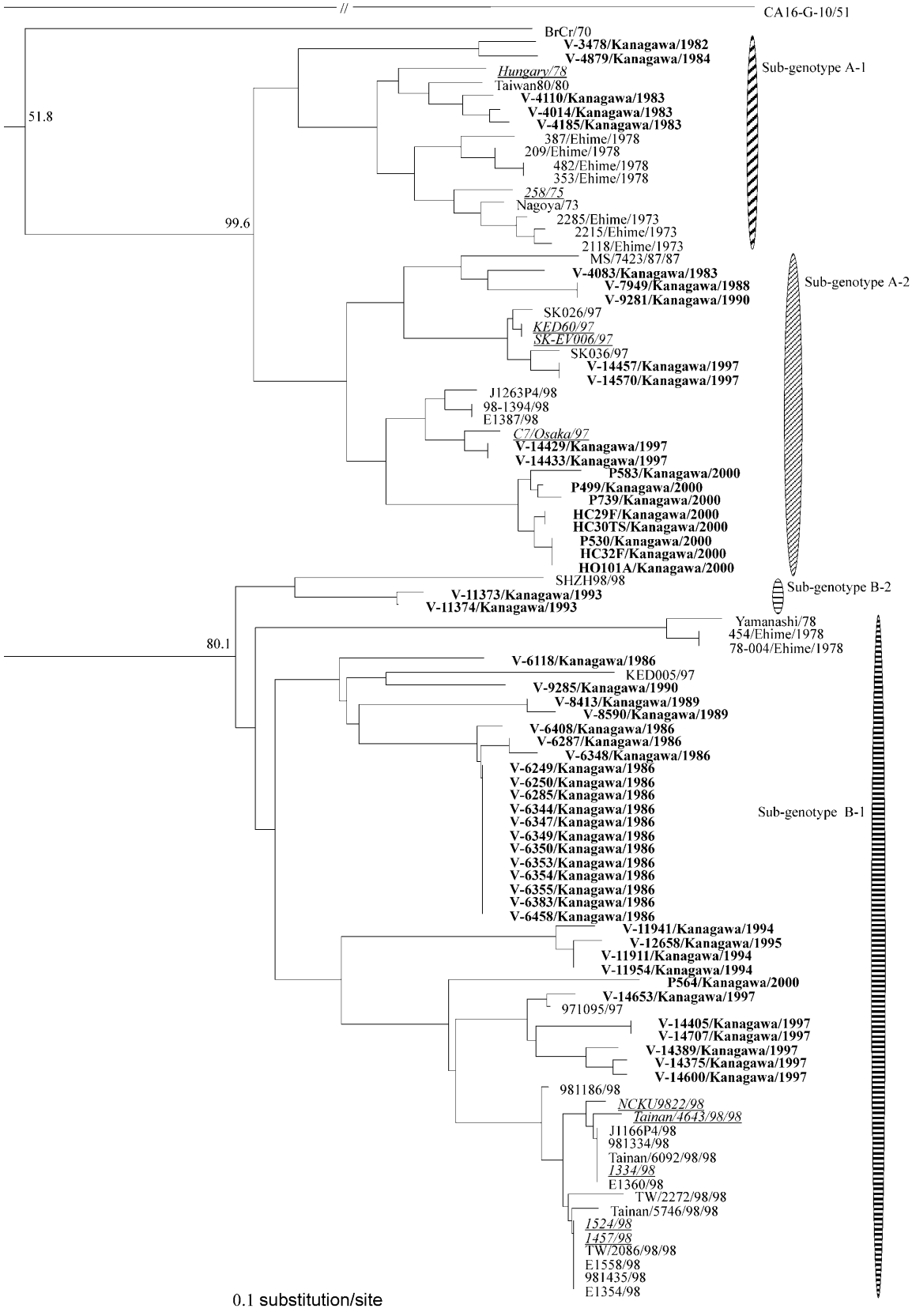
Materials and methods

Virus isolation

Viruses were isolated from throat swab and feces of 52 patients with HFMD, exanthem, herpangina or upper respiratory tract infection in Yokohama City (approximate population of 3.5 million in 2001), from 1982 to 2000. Two cases involved aseptic meningitis. The clinical specimens were inoculated into Vero cells. The inoculated cells were incubated for at least for 2 weeks at 34 °C in an atmosphere of 5% CO_2 in Eagle's minimum essential medium (containing 2% (v/v) fetal calf serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B). The virus isolates were identified by the microneutralization assay with anti-serum against EV71 as previously described [28]. Nine EV71 isolates from cases of large HFMD outbreaks in Ehime prefecture, Japan in the 1970's were also used as referential strains in Japan [12].

Table 1. EV71 isolates in Yokohama, Japan in 1982–2000 and other isolates in the world

Strain	Year	Place	Major Symptom	GenBank accession number	Strain	Year	Place	Major Symptom	GenBank accession number
V-3478/Kanagawa/1982	1982	Kanagawa/Japan	HFMD	AB081342	BrCr	1970	USA	meningitis	U22521
V-4014/Kanagawa/1983	1983	Kanagawa/Japan	HFMD	AB081343	Nagoya	1973	Aichi/Japan	HFMD	AB051301
V-4083/Kanagawa/1983	1983	Kanagawa/Japan	HFMD	AB081344	2118/Ehime/1973	1973	Ehime/Japan	HFMD/meningitis	AB081394
V-4110/Kanagawa/1983	1983	Kanagawa/Japan	HFMD	AB081345	2215/Ehime/1973	1973	Ehime/Japan	HFMD/meningitis	AB081395
V-4185/Kanagawa/1983	1983	Kanagawa/Japan	HFMD	AB081346	2285/Ehime/1973	1973	Ehime/Japan	HFMD/meningitis	AB081396
V-4879/Kanagawa/1984	1984	Kanagawa/Japan	HFMD	AB081347	258	1975	Bulgaria	encephalitis (fetal)	AB051302
V-6118/Kanagawa/1986	1986	Kanagawa/Japan	HFMD	AB081348	Hungary	1978	Hungary	encephalitis (fetal)	AB051303
V-6249/Kanagawa/1986	1986	Kanagawa/Japan	exanthem	AB081349	Yamanashi	1978	Yamanashi/Japan	HFMD	AB051305
V-6250/Kanagawa/1986	1986	Kanagawa/Japan	HFMD	AB081350	209/Ehime/1978	1978	Ehime/Japan	HFMD	AB081397
V-6285/Kanagawa/1986	1986	Kanagawa/Japan	HFMD	AB081351	353/Ehime/1978	1978	Ehime/Japan	HFMD	AB081398
V-6287/Kanagawa/1986	1986	Kanagawa/Japan	HFMD	AB081352	387/Ehime/1978	1978	Ehime/Japan	HFMD	AB081399
V-6344/Kanagawa/1986	1986	Kanagawa/Japan	herpangina	AB081353	454/Ehime/1978	1978	Ehime/Japan	HFMD	AB081400
V-6347/Kanagawa/1986	1986	Kanagawa/Japan	HFMD	AB081354	482/Ehime/1978	1978	Ehime/Japan	HFMD	AB081401
V-6348/Kanagawa/1986	1986	Kanagawa/Japan	HFMD	AB081355	78-004/Ehime/1978	1978	Ehime/Japan	HFMD	AB081402
V-6349/Kanagawa/1986	1986	Kanagawa/Japan	HFMD	AB081356	Taiwan80	1980	Taiwan	HFMD	AB051304
V-6350/Kanagawa/1986	1986	Kanagawa/Japan	HFMD	AB081357	MS/7423/87	1987	USA	paralysis	U22522
V-6353/Kanagawa/1986	1986	Kanagawa/Japan	HFMD	AB081358	SK-EV006	1997	Sarawak/Malaysia	encephalitis (fetal)	AB051331
V-6354/Kanagawa/1986	1986	Kanagawa/Japan	HFMD	AB081359	SK026	1997	Sarawak/Malaysia	HFMD	AB051332
V-6355/Kanagawa/1986	1986	Kanagawa/Japan	HFMD	AB081360	SK036	1997	Sarawak/Malaysia	HFMD	AB051333
V-6383/Kanagawa/1986	1986	Kanagawa/Japan	HFMD	AB081361	KED60	1997	Peninsular/Malaysia	encephalitis (fetal)	AB051335
V-6408/Kanagawa/1986	1986	Kanagawa/Japan	HFMD	AB081362	KED005	1997	Peninsular/Malaysia	HFMD	AB051334
V-6458/Kanagawa/1986	1986	Kanagawa/Japan	respiratory tract inflammation	AB081363	C7/Osaka	1997	Osaka/Japan	encephalitis (fetal)	AB051328
V-7949/Kanagawa/1988	1988	Kanagawa/Japan	HFMD	AB081364	971095	1997	Shiga/Japan	HFMD	AB051330
V-8413/Kanagawa/1989	1989	Kanagawa/Japan	HFMD	AB081365	1334	1998	Taiwan	HFMD/pulmonary edema (fetal)	AB051306
V-8590/Kanagawa/1989	1989	Kanagawa/Japan	respiratory tract inflammation	AB081366	1457	1998	Taiwan	HFMD/pulmonary edema (fetal)	AB051307
V-9281/Kanagawa/1990	1990	Kanagawa/Japan	HFMD	AB081367	1524	1998	Taiwan	HFMD/pulmonary edema (fetal)	AB051308
V-9285/Kanagawa/1990	1990	Kanagawa/Japan	respiratory tract inflammation	AB081368	981186	1998	Taiwan	herpangina	AB051310
V-11373/Kanagawa/1993	1993	Kanagawa/Japan	HFMD	AB081369	981334	1998	Taiwan	HFMD	AB051311
V-11374/Kanagawa/1993	1993	Kanagawa/Japan	HFMD	AB081370	981435	1998	Taiwan	herpangina	AB051312
V-11911/Kanagawa/1994	1994	Kanagawa/Japan	HFMD	AB081371	E1354	1998	Taiwan	HFMD	AB051317
V-11941/Kanagawa/1994	1994	Kanagawa/Japan	HFMD	AB081372	E1360	1998	Taiwan	HFMD/meningitis	AB051318
V-11954/Kanagawa/1994	1994	Kanagawa/Japan	HFMD	AB081373	E1387	1998	Taiwan	HFMD	AB051313
V-12658/Kanagawa/1995	1995	Kanagawa/Japan	respiratory tract inflammation	AB081374	E1558	1998	Taiwan	herpangina	AB051314
V-14375/Kanagawa/1997	1997	Kanagawa/Japan	HFMD	AB081375	98-1394	1998	Taiwan	HFMD	AB051309
V-14389/Kanagawa/1997	1997	Kanagawa/Japan	respiratory tract inflammation	AB081376	TW/2086/98	1998	Taiwan	HFMD	AF119796
V-14405/Kanagawa/1997	1997	Kanagawa/Japan	HFMD	AB081377	TW/2272/98	1998	Taiwan	pulmonary hemorrhage	AF119795
V-14429/Kanagawa/1997	1997	Kanagawa/Japan	HFMD	AB081378	SHZH98	1998	China		AF302996
V-14433/Kanagawa/1997	1997	Kanagawa/Japan	HFMD	AB081379	NCKU9822	1998	Taiwan	HFMD/encephalitis (fetal)	AF136379
V-14457/Kanagawa/1997	1997	Kanagawa/Japan	HFMD	AB081380	Tainan/4643/98	1998	Taiwan	encephalitis (fetal)	AF304458
V-14570/Kanagawa/1997	1997	Kanagawa/Japan	HFMD	AB081381	Tainan/5746/98	1998	Taiwan	HFMD	AF304457
V-14600/Kanagawa/1997	1997	Kanagawa/Japan	HFMD	AB081382	Tainan/6092/98	1998	Taiwan	HFMD	AF304459
V-14653/Kanagawa/1997	1997	Kanagawa/Japan	HFMD	AB081383	J1166P4	1998	Taiwan	Acute Flaccid Paralysis	AB051315
V-14707/Kanagawa/1997	1997	Kanagawa/Japan	HFMD	AB081384	J1263P4	1998	Taiwan	Acute Flaccid Paralysis	AB051316
P-499/Kanagawa/2000	2000	Kanagawa/Japan	HFMD	AB081385					
P-530/Kanagawa/2000	2000	Kanagawa/Japan	HFMD	AB081386	CA16 G-10	1951	South Africa		NC 001612
P-564/Kanagawa/2000	2000	Kanagawa/Japan	herpangina	AB081387					
P-583/Kanagawa/2000	2000	Kanagawa/Japan	HFMD	AB081388					
P-739/Kanagawa/2000	2000	Kanagawa/Japan	HFMD	AB081389					
HC29F/Kanagawa/2000	2000	Kanagawa/Japan	HFMD/meningitis	AB081390					
HC30TS/Kanagawa/2000	2000	Kanagawa/Japan	stomatitis	AB081391					
HC32F/Kanagawa/2000	2000	Kanagawa/Japan	HFMD/meningitis	AB081392					
HO101A/Kanagawa/2000	2000	Kanagawa/Japan	HFMD	AB081393					


//
CA16-G-10/51
BrCr/70
V-3478/Kanagawa/1982
V-4879/Kanagawa/1984
Hungary/78
Taiwan80/80
V-4110/Kanagawa/1983
V-4014/Kanagawa/1983
V-4185/Kanagawa/1983
387/Ehime/1978
209/Ehime/1978
482/Ehime/1978
353/Ehime/1978
258/75
Nagoya/73
2285/Ehime/1973
2215/Ehime/1973
2118/Ehime/1973
MS/7423/87/87
V-4083/Kanagawa/1983
V-7949/Kanagawa/1988
V-9281/Kanagawa/1990
SK026/97
KED60/97
SK-EV006/97
SK036/97
V-14457/Kanagawa/1997
V-14570/Kanagawa/1997
J1263P4/98
98-1394/98
E1387/98
C7/Osaka/97
V-14429/Kanagawa/1997
V-14433/Kanagawa/1997
P583/Kanagawa/2000
P499/Kanagawa/2000
P739/Kanagawa/2000
HC29F/Kanagawa/2000
HC30TS/Kanagawa/2000
P530/Kanagawa/2000
HC32F/Kanagawa/2000
HO101A/Kanagawa/2000
SHZH98/98
V-11373/Kanagawa/1993
V-11374/Kanagawa/1993
Yamanashi/78
454/Ehime/1978
78-004/Ehime/1978
V-6118/Kanagawa/1986
KED005/97
V-9285/Kanagawa/1990
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V-8590/Kanagawa/1989
V-6408/Kanagawa/1986
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V-14707/Kanagawa/1997
V-14389/Kanagawa/1997
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V-14600/Kanagawa/1997
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NCKU9822/98
Tainan/4643/98/98
J1166P4/98
981334/98
Tainan/6092/98/98
1334/98
E1360/98
TW/2272/98/98
Tainan/5746/98/98
1524/98
1457/98
TW/2086/98/98
E1558/98
981435/98
E1354/98
51.8
99.6
80.1
Sub-genotype A-1
Sub-genotype A-2
Sub-genotype B-2
Sub-genotype B-1
0.1 substitution/site

RNA extraction, RT-PCR and sequencing

The VP4 region of 61 EV71 isolates, 52 from Yokohama City and 9 from Ehime, was sequenced. Viral RNA was extracted from 200 μl of viral suspension using High Pure Viral RNA Kit (Boehringer Mannheim GmbH, Germany) and finally suspended with 50 μl of water. To amplify the partial 5′-UTR/VP4/VP2 of the viral genome, reverse transcription-coupled PCR (RT-PCR) was performed according to the manufacture's instructions (Access RT-PCR System, Promega, Madison, WI). The primers 60–80F (5′-AAA TCC TTG T (G/A) C GCC TGT TTT A-3′) and OL68-71R (5′-GGG AAC TTC CAG TAC CA (C/T) CC-3′) were used for the amplification. The following protocol was used; 45 min at 48 °C (reverse transcription), 2 min at 94 °C (denaturing), then 30 cycles of 94 °C for 10 sec, 50 °C for 10 sec, and 65 °C for 1 min and an additional 5 min for elongation at 65 °C in the last cycle. The amplified DNA fragment was confirmed by electrophoresis on 1.5% agarose gel. After purification of the DNA fragments with the QIAquick PCR Purification Kit (QIAGEN GmbH, Germany), the nucleotide sequence was determined with an automated DNA sequencer (ABI 310 DNA sequencer, Applied Biosystems, CA) using the Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The primers 60–80F, OL68-71R, EVP4 (5′-CTA CTT TGG GTG TCC GTG TT-3′) and 640R (5′-GGA TGG CCA ATC CAA TAG CTA TAT GG-3′) were used for sequencing. The nucleotide sequence accession number in this study is shown in Table 1.

Phylogenetic analysis

The VP4 sequence of 34 EV71 isolates and a prototype CA16 strain (CA16/G-10) was obtained from GenBank [24] (Table 1). Phylogenetic analysis was performed as described previously [27]. Briefly, all 96 VP4 sequences (207 bp) were aligned by the CLUSTAL W program [31] and a phylogenetic tree was constructed by the neighbor-joining method using the Kimura two parameter method. The tree was drawn with the TreeView software [23] and CA16/G-10 was used as an outgroup. The reliability of the tree was estimated using 1,000 bootstrap replications.

Results

HFMD epidemics in Yokohama City

In Japan, large HFMD outbreaks mainly caused by EV71 were reported in 1983, 1986, 1990, 1993, 1997 and 2000 [Infectious Agents Surveillance Report (*http://idsc.nih.go.jp/iasr/*)]. Earlier reports showed two other large HFMD outbreaks due to EV71 in 1973 and 1978 [8, 30]. The frequent EV71 isolations in 1986, 1997 and 2000 in Yokohama City might correspond to HFMD outbreaks throughout Japan at that time. Unlike the recent HFMD outbreaks in other countries in the Western-Pacific region, only a few HFMD outbreaks with severe neurological complications have been reported in Japan [12, 13]. Also in

Fig. 1. Dendrogram of 95 EV71 isolates and a prototype CA16 strain, G-10, based on the VP4 region. The bootstrap values (%) are indicated at the node of each branch. The strains isolated in Yokohama City are written in bold letters. The strains isolated from fatal cases are written in italic letters

Yokohama City, most of the EV71 was isolated from uncomplicated HFMD cases (Table 1).

Phylogenetic analysis of EV71 in Yokohama City

All of the EV71 strains, including 52 isolates from Yokohama City, were clustered into two major genotypes A and B except the prototype BrCr strain (Fig. 1). In this study, the terminology of the major VP4 genotypes, A and B, was according to our previous reports [20, 28]. The genotypes A and B were further divided into two sub-genotypes (A-1 and A-2, B-1 and B-2), respectively. The homology of nucleotide sequences within the same sub-genotype was more than 92%. Genotype A viruses showed 15–22% nucleotide diversity with genotype B viruses.

Five EV71 strains isolated in Yokohama City in the early 1980's were grouped into sub-genotype A-1 and were closely related to two historical EV71 strains (258 and Hungary) from Europian EV71 outbreaks in the 1970's. Two EV71 outbreaks, in Bulgaria in 1975 and in Hungary in 1978, were characterized by severe neurological complications including many fatalities but were not associated with HFMD [6, 21]. However, sub-genotype A-1 was a dominant genotype in Japan through the 1970's and early 1980's, and all of the EV71 isolates were obtained from cases of uncomplicated HFMD in Yokohama City (Table 1, Fig. 1). In 1986, a total of 16 EV71 strains were isolated from HFMD patients in Yokohama City, and all were grouped into the sub-genotype B-1. From 1986 to 1997, the genotype B was consistently dominant in Yokohama City and sub-genotype B-1 strains were consecutively isolated from uncomplicated HFMD cases (Fig. 2).

During the recent large HFMD outbreaks in Japan in 1997 and 2000, two distinct genotypes of EV71, sub-genotypes A-2 and B-1, were simultaneously found in Yokohama City. The corresponding two genotypes were also identified from recent HFMD cases in other areas of Japan [28, 33]. The sub-genotypes A-2 and B-1, isolated in Yokohama City in 1997 were closely related to two dominant genotypes from large HFMD outbreaks, Malaysia 1997 and Taiwan 1998, respectively (Fig. 1). Pairwise comparison of the nucleotide sequence in the VP4 region among sub-genotype A-2 viruses in Malaysia 1997 and Yokohama City 1997 showed more than 96% nucleotide homology. Likewise, nucleotide diversity among sub-genotype B-1 viruses in Taiwan 1998 and Yokohama City 1997 was less than 7% (data not shown).

Amino acid alignment

Alignment of the deduced amino acid sequence for the VP4 region (69 amino acids) of the 52 EV71 isolates from Yokohama City indicated only a few differences. Nine out of 52 isolates had a single amino acid difference compared with the prototype BrCr strain and the others no differences (data not shown). Thus, most nucleotide substitutions were synonymous and the amino acid sequence in the VP4 region was highly conserved among EV71 isolates as described previously [5].

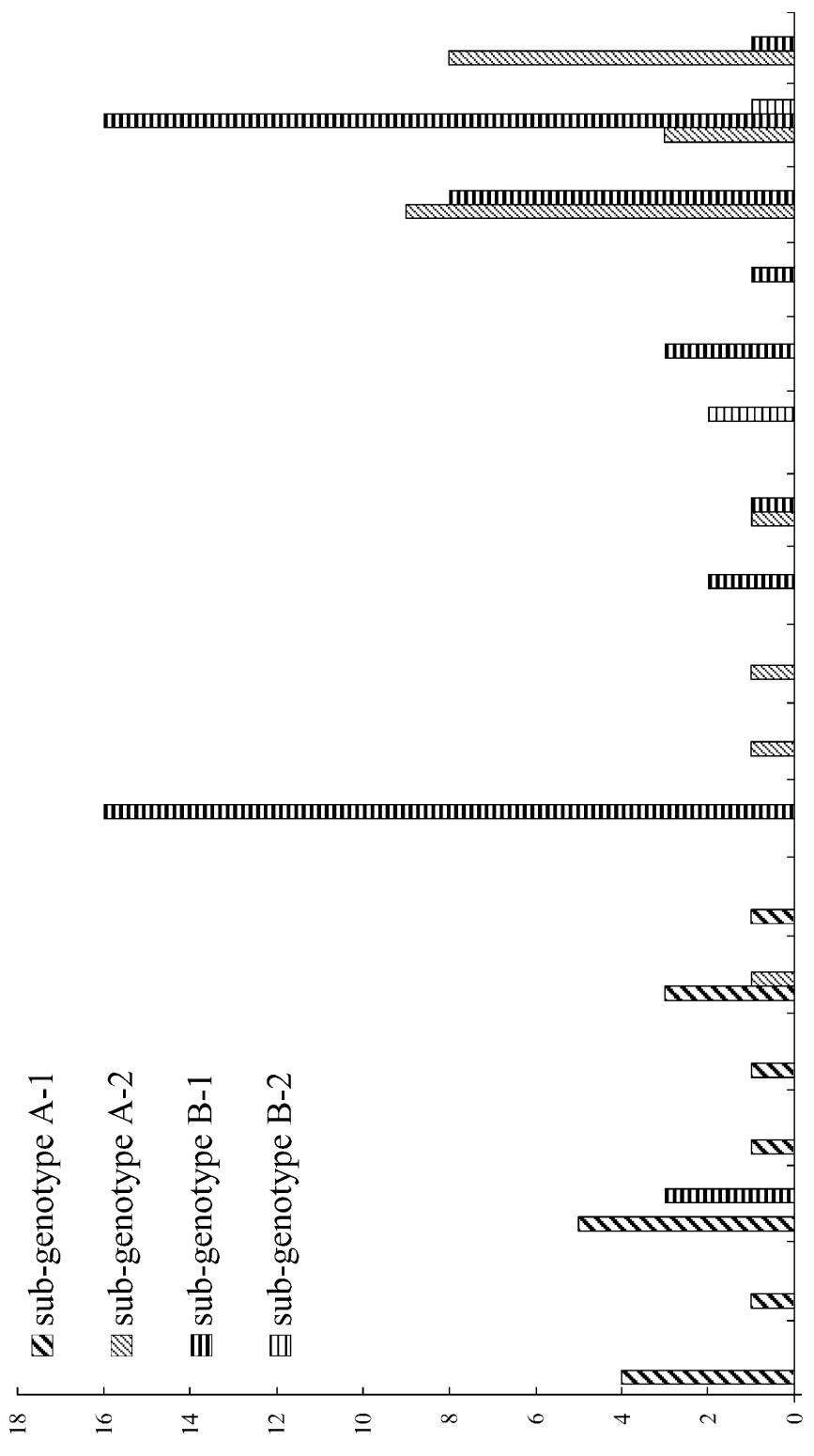

Fig. 2. The VP4 genotype of EV71 isolates. The genotypes were grouped according to the phylogenetic analysis shown in Fig. 1. The horizontal axis is the year of isolation. The vertical axis indicates number of isolates

Discussion

A number of molecular epidemiological studies on recent EV71 isolates obtained from HFMD cases with or without neurological manifestations in the Western-Pacific region have revealed the co-circulation of two major EV71 lineage in this area [3, 5, 17, 20, 26–29, 32, 34]. Although phylogenetic analyses have been performed using several different regions of the genome, 5′-UTR, VP4 and VP1, the two major clusters among recent EV71 strains were identical in the analysis of each region [5, 20]. Consequently, the VP4 genotypes, A and B, in this study might correspond to the VP1 genogroups B and C which were identified by Brown et al. [3]. Phylogenetic analysis of recent EV71 isolates from uncomplicated HFMD cases in Yokohama City indicated that two major lineage of EV71 (VP4 genotypes A and B) were co-circulating in a single community in Japan, neither of which might be aboriginal in Japan. The VP4 sub-genotype A-2 viruses isolated in the 1997 outbreak in Yokohama City were closely related to four Malaysian strains, SK-EV006, SK026, SK036 and KED60, isolated in 1997 (Fig. 1). Other B-1 viruses from Yokohama City 1997 could be grouped into the same B-1 sub-genotype dominant in Taiwan in 1998. The VP4 sub-genotypes, A-2 and B-1, were related to large HFMD outbreaks with severe neurological disease in Malaysia and in Taiwan, but B-1 was isolated from uncomplicated HFMD cases in Yokohama City.

Since 1997, a number of HFMD outbreaks with severe neurological involvement, mainly caused by EV71, have been described in many countries in the Western-Pacific region such as Malaysia, Taiwan and Australia [4, 10, 14, 16, 18, 32]. On the other hand, according to the Infectious Disease Weekly Report (*http://idsc.nih.go.jp/index.html*) and Infectious Agents Surveillance Report (*http://idsc.nih.go.jp/index.html*), the successive circulation of EV71 was described for at least 20 years in Japan before and after the “re-emergence” of EV71 epidemics in this region. In Japan, EV71 has been isolated mainly from HFMD patients and sometimes from patients with aseptic meningitis, but rarely in cases with severe neurological involvement. Due to the large degree of variability in clinical manifestations and the severity of the disease during each EV71 outbreak throughout the world, several possible molecular determinants (genotype or mutation) of the neurovirulence of EV71 have been proposed. However, the present phylogenetic analysis of EV71 revealed that neither VP4 genotype, A or B, in Yokohama City was associated with severe neurological disease. In Taiwan, a recent report by Wang et al. indicated a change in the major genotypes of EV71 between 1998 and 2000, however, both genotypes could be associated with HFMD outbreaks with severe neurological involvement including fatalities [32]. Other molecular epidemiological analyses of EV71 revealed no close relationship between the genotype and severity of the disease [3, 5, 17, 26–29, 32]. In addition, all three genotypes of EV71 (VP4 genotypes A, B and BrCr) caused various neurological manifestations and histopathological lesions of the central nervous tissues in our experimental infection model using cynomolgus monkeys [20]. Even so, some extent of contributions of single or slight mutations determining

the neurovilurence of EV71 remains to be considered regardless of the genotypes. Further studies on viral and host factors are still needed to elucidate the severity of EV71 infection.

In conclusion, two recent lineage of EV71 in the Western-Pacific region have been major causative agents of HFMD epidemics, and are possible to be neurovirulent. Further molecular epidemiological analysis of EV71 strains in Japan using other genome regions, along with the VP4 region, will be needed to track in more detail EV71 lineage in this part of the world.

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