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Molecular epidemiology and evolution of worldwide enterovirus 71 strains isolated from 1970 to 2004

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Human enterovirus 71 (EV71) is one of the major etiological agents of the hand-foot-and-mouth disease (HFMD) that often causes severe neurological complications. Recently, its outbreaks mainly take place in the torrid zone of the Asia-Pacific region. To study the evolution and genetic variability, we collected 532 EV71 strains with almost complete or complete VP1 sequences (891 nt) isolated worldwide from 1970 to 2004. The pairwise homologies and genetic distances were analyzed. Most strains belong to previously identified genotype B and C. However, a unique strain R13223-IND-01 appears not to fall into current three genotypes (A, B and C), and probably represents a new genotype D. Some orphan strains were observed in the genotypes B and C, and their significance in the EV71 evolution was discussed. Moreover, there is a significant co-variance of 6 discrete positions on VP1 (amino acid 43, 58, 164, 184, 240 and 249). This high co-variability is tightly related with the subgenotypes.

enterovirus 71 (EV71), VP1, epidemiology, co-variability

Enterovirus 71 (EV71) belongs to human Enterovirus A species of the *Enterovirus* genus, *Picornaviridae* family[1], and closely related with coxsackievirus A16 (CA16). EV71 infection manifests most frequently as the hand-foot-and-mouth disease (HFMD), especially in children. Unlike CA16, EV71 may cause severe neurological complications during acute infection[2,3]. EV71 was first isolated in California in 1969[4] and now its spread is worldwide. The major epidemic area nowadays is the Asia-Pacific region: Japan[5], Malaysia[6,7], Taiwan of China[8−10], Singapore[11], Australia[12] and Mainland of China[13,14]. The 1998 and 2000 large outbreaks in China's Taiwan Province separately caused 129106 reported cases (78 deaths) and 80677 reported cases (41 deaths)[10], and resulted in a panic in the public. So it is necessary to keep surveillance on the molecular epidemiology and genetic variability of EV71.

In previous studies, 3 genotypes, which were A, B and C[15], and 8 subgenotypes, which were B1−B4 and C1-C4 were identified[13,15−17]. However, a complete evaluation of the evolution and genetic variability of EV71 is still lacking so far (Table 1). Because of various methodologies and criteria, it is hard to have a comprehensive insight into the phylogenetic relationships between all the EV71 strains from the results of different investigators. Compared with other enterovirus members, capsid protein VP1 of EV71 is more variable than other regions in the genome[18]. Besides, the specific epitopes responsible for serotypic specificity are clustered mainly on VP1[11]. It was also found that VP1 sequence had better correlation with serotypes than 5′ UTR or the VP4-VP2 junction[26]. Therefore, in this study, 532 EV71 strains with almost complete (≥841 nt) or complete VP1 sequences (891 nt) were collected from GenBank and analyzed with MEGA 3.0.

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Table 1 Recent phylogeny studies of enterovirus 71 (EV71)

Gene	Nucleotides	Strains			Reference
		numbers	country [a]	year	
VP1	891	113	USA, AUS, MAL, COL, CAN, CHN	1970－1998	[18]
	341	75	CHN, MAL, SIN	1974－1998	[11]
	439	23	KOR, USA, MAL, CHN, AUS, SIN, GER	2000	[19]
	891	66	MAL, AUS, SIN	1997－2001	[17]
	841	28	CHN	1970－2000	[10]
	891	73	MAL, USA, AUS, CHN, SIN, KOR, HUN, BGR	1974－2001	[7]
	891 & 840	99	MAL, USA, AUS, SIN, CHN, KOR, HUN, BGR	1974－2001	[16]
	891	57	USA, CHN, KOR, AUS, MAL, SIN	1974－2003	[20]
	891	70	JPN, MAL, CHN, USA, AUS, SIN, KOR	1974－2003	[21]
	891	42	CHN, SIN, USA, AUS, KOR, MAL	1974－2004	[13]
	891	27	USA, AUS, MAL, CHN, JPN, COL, HUG, BGR	1973－2000	[22]
	891	83	AUS, SIN, MAL, USA, CHN, KOR	1983－2001	[23]
VP4	207	44	CHN, JNP, USA	1973－1998	[24]
	207	134	CHN, MAL, USA, JPN, UK, KOR, SIN, AUS, HGR, BGR	1972－2002	[16]
	207	30	CHN, USA, SIN, KOR, AUS, MAL	1977－2004	[13]
	207	87	CHN, JPN, MAL, SIN, AUS, KOR, USA, HUG, BRG	1973－2005	[25]
VP4/VP2	420	29	MAL, CHN, JPN, USA, HUG, BRG	1973－1998	[5]
5′UTR	440	13	MAL	1997－1999	[6]
	681	36	CHN	1998	[9]
	648	48	CHN	1970－2000	[10]

a) AUS, Australia; BGR, Bulgaria; CAN, Canada; CHN, China (including Mainland of China and Taiwan of China); COL, Columbia; HUN, Hungary; JPN, Japan; KOR, Korea; MAL, Malaysia; SIN, Singapore; UK, United Kingdom; USA, United States.

1 Material and method

1.1 EV71 sequence data

All 532 EV71 strains included in this study were from GenBank. They were isolated worldwide during 1970－2004. Among these virus strains, 143 were isolated in Malaysia, 112 in China (81 from Taiwan and 31 from the Mainland), 99 in USA, 81 in Australia, 49 in Japan, 29 in Singapore, 11 in Korea, and 1 in Brazil, Bulgaria, Canada, Colombia, Germany, Hungary, India and Thailand respectively. Among these 532 EV71 strains, 28 lack 50 nt (GenBank Accession Nos. AY055174－AY055201) and one lacks 36 nt in the 3′ end (GenBank Accession No. AY179600), which does not disturb the reconstruction of the phylogenetic trees. Isolates used in the analysis are listed in Supplementary Figure S1 (http://www.SpringerLink.com), arranged by the order of GenBank Accession Numbers.

1.2 Phylogenetic analysis

The analysis program used in this study was MEGA, version 3.0. The VP1 nucleotide sequences of all 532 strains were aligned using the Clustal method[27]. Phylogenetic trees were constructed through neighbor-joining using the Kimura two-parameter method[28,29]. The VP1 sequence of the CA16 prototype strain G-10 (GenBank Accession No. U05876), was included as an outgroup. The reliability of the neighbor-joining trees was estimated by bootstrap analysis using 1000 pseudoreplicate data sets.

1.3 Nucleotide sequence analysis

Genetic distances were calculated by pairwise comparison with the Kimura two-parameter distance method, MEGA program, version 3.0. The mean similarity values within or between genotypes were calculated by computing intra-group and inter-group means method. The maximum and minimum values between every two comparison groups were picked out to estimate the similarity range.

1.4 Amino-acid co-variance analysis

It was found that 6 discrete positions of VP1 protein (43, 58, 164, 184, 240 and 249) exhibited similar property in the amino-acid variability, with 2 dominant residues showing nearly the same abundance (about 50%) on each position. If these residues on 6 positions were considered together, the observed abundance of the amino-acid patterns could be compared with the expected value under the null hypothesis H0. H0, the amino acids on these 6 positions are independent of each other. The consistent degree between the observed and expected values is analyzed by χ^2-test (P=0.005).

2 Results and discussion

2.1 Phylogenetic analysis of EV71

This study is a comprehensive analysis based on VP1. To ensure the high reliability and accuracy, short sequences were not included. According to previous studies, we set up criteria that to make up a genotype or a subgenotype, values of genetic similarity between strains should be more than 85% and 91%, respectively. Among 532 EV71 strains, 140 strains have not been classified in previous studies. An unrooted cladogram was constructed with the 532 EV71 isolates (Figure 1). Almost all strains fell under a certain genotype identified previously. BrCr (GenBank Accession No. U22521) is still the only member of genotype A. 259 strains (48.7%) and 271 strains (50.9%) belong to genotype B and C respectively. However, an outlier strain, R13223-IND-01 (AY179600), could not be classified as genotype A, B or C. The genetic similarity within or between genotypes is represented by the percentage of nucleotide sequence similarity (Table 2). The value of genetic similarity between R13223-IND-01 and genotype A is 78.5%. The maximum and minimum similarity values between R13223-IND-01 and genotype B is 83.6% and 79.0%, and those between R13223-IND-01 and genotype C are 83.2% and 79.9%. These values are close to inter-genotype similarity among genotypes A, B and C, but much lower than intra-genotype similarities of them. So this strain isolated in India probably represents a new genotype D.

Most strains in genotypes B and C can be further divided into 8 subgenotypes (B1－B4 and C1－C4). When genetic similarity was under investigation (Table 2), orphan strains that cannot be classified into certain genotype were not included in the inter- or intra-genotype analysis while orphan strains that cannot be classified into certain subgenotype were not included in the inter- or intra-subgenotype analysis. Diversity degree of genotype C and genotype B is similar. Subgenotypes B3 and C3 have the greatest intra-group similarity (>99%). It is noticeable that the mean similarity value between B1 and B2 is 94.3%, very close to the intra-group mean similarity value of B1 (95.3%). This suggests that the previously named B1 and B2[15] can also merge into one subgenotype.

Our analysis also revealed some mistakes in previous reports. Strain 15M/AUS/8/99 (AF376092) and 14F/AUS/9/99 (AF376091) were respectively identified as B3 and C2 strain based on the complete VP1 gene sequences[16,17]; however, the accurate identification is just the opposite: 15M/AUS/8/99 is a C2 strain, but 14F/AUS/9/99 is a B3 strain. Moreover, 6 strains (AB177815－AB177816, AB213647－AB213650) isolated in Yamagata Japan were previously thought to form a new subgenotype B5[21]. In our study, these 6 strains, together with one isolate from Singapore (AF376121) and 16 isolates from Malaysia (AY207633, AY258303－AY258311, AY258313, AY905545－AY905550), should be identified as a lineage of subgenotype B4 (Figure 1(b)), because these 24 strains share at least 95.5% similarity with each other and 91.2%－96.7% similarity with other B4 strains. In Singh's study based on 341 bp VP1 genes, 25 strains (AF135908－AF135930, AF251358) appeared to form a new genotype D[11], but according to the phylogenetic analysis in our study, they all belong to genotype B. This

Table 2 VP1 nucleotide sequence similarity within or between (sub) genotypes

(Sub) genogroup	Comparison	Homology (%)	Mean homology (%)
(Ⅰ)			
Within B	-	85.4－100	93.7
Within C	-	87.1－100	92.6
B	A	76.5－80.8	79.1
B	C	76.0－84.9	80.4
C	A	77.7－82.2	80.2
(Ⅱ)			
Within B1	-	90.4－99.9	95.3
Within B2	-	93.1－99.9	97.7
Within B3	-	96.5－100	99.0
Within B4	-	91.2－100	96.3
B1	B2	89.0－97.2	94.3
B1	B3	87.5－93.3	91.0
B1	B4	85.4－93.0	89.7
B2	B3	89.0－93.9	92.6
B2	B4	88.4－93.6	91.3
B3	B4	90.2－95.6	93.4
(Ⅲ)			
Within C1	-	91.1－100	96.1
Within C2	-	93.2－100	97.6
Within C3	-	98.9－100	99.4
Within C4	-	93.0－100	96.1
C1	C2	87.1－94.2	91.1
C1	C3	89.3－93.7	91.6
C1	C4	87.1－93.8	90.1
C2	C3	89.4－92.4	91.1
C2	C4	87.2－91.9	89.9
C3	C4	88.4－91.6	89.4

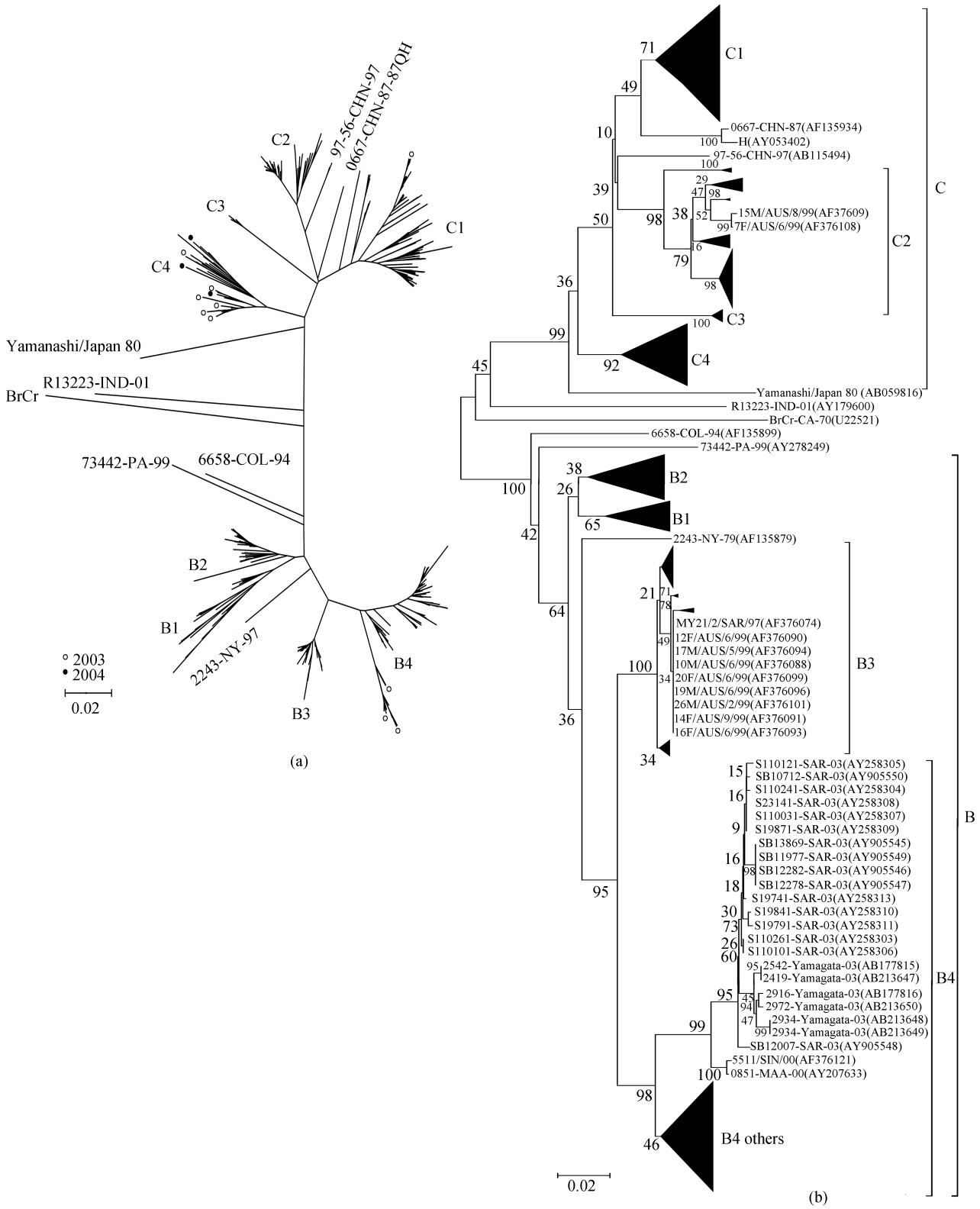

Figure 1 Overview of the genetic relationships of 532 EV71 strains isolated from 1970 to 2004, based on an alignment of their VP1 gene by the neighbor-joining method with the program MEGA 3.0. (a) Black circles (●) and blank circles (○) separately represent the strains that emerged in year 2004 or 2003. (b) Genetic relationships of 532 EV71 strains isolated from 1970 to 2004. To emphasize special strains such as orphan strains and previously falsely demonstrated ones, information of other strains were omitted. Necessary bootstrap values were marked.

indicates that short nucleotides sequence may greatly decrease the reliability of the phylogenetic analysis.

2.2 Orphan strains

Compared with most of B- and C-genotype strains, seven strange strains are observed in the phylogenetic trees (Figure 1(a)): 2243-NY-79 (AF135879), 6658-COL-94 (AF135899), 73442-PA-99 (AY278249), Yamanashi/Japan 80 (AB059816), 0667-CHN-87 (AF135934), H (AY053402), and 97-56-CHN-97 (AB115494). These strains belong to genotype B or C separately, but seem hard to fall under current subgenotypes (B1－B4 or C1－C4) (Table 3). So we call them 'orphan strains'.

The American strain 2243-NY-79 was isolated in New York in 1979. At the same period (1970－1988), EV71 strains reported in USA and Australia were all B1 and B2 strains, and the B3 strains were believed to emerge first in Asia in 1995. It is interesting that 2243-NY-79 has very similar genetic similarity to that of B1, B2 and B3 (Table 3). This indicates that the ancestor of B3 probably existed in USA by the end of the 1970s. Orphan strain 73442-PA-99 isolated in Brazil and 6658-COL-94 isolated in Colombia are much closer to genotype B than genotypes A and C, while their average genetic homologies with B1, B2, B3 and B4 (<93%) are much lower than intra-subgenotype similarity (Table 2), suggesting that circulating strains in these two countries in the 1990s might belong to new subgenotypes (B5 or B6) of genotype B. Yamanashi/Japan 80 was the first reported strain of genotype C. Three Chinese orphan strains, 0667-CHN-87, H and 97-56-CHN-97, isolated during 1980-2000, also belong to genotype C. So genotype C probably originated in East Asia in the early 1980s and has circulated since then.

Orphan strains provided some hints for the origin and evolution of EV71 and genetic variability in various areas; however, the incomplete surveillance gives rise to a great limitation. EV71 had not been widely investigated until the 1990s, especially in developing countries, such as China, Brazil and Colombia, so the emergence time of EV71 in many countries of Asia-Pacific regions was very likely to be much earlier than that was first reported. Moreover, some orphan strains such as R13223-IND-01 (India), 0667-CHN-87(China) and H (China), were probably not occasional reported cases, but represented a panel of strains. Since EV71 is a frequent cause of HFMD epidemics associated with severe neurological complications, it is necessary to include it in the national surveillance system for diseases, especially in Asia-Pacific regions.

2.3 Evolution and genetic variability of EV71

The relation between subgenotypes and isolated area or time is also observed. Before 1996, EV71 virus mostly circulated in the United States and Australia, but widely spread in Asia ever since. The first reported EV71 strain was BrCr isolated in California in 1969[4], which was the only member of genotype A. During 1972－1980, only B1 strains were isolated, while B2 strains were dominant strains during 1981－1988, especially in USA (Figure 2(a)). This indicated that the ancestor of B-genotype EV71 virus probably co-existed in USA with genotype A strain during the late 1960s, and the oldest B1 strains occurred much earlier than B2 strains. Since the 1990s, B1 or B2 strains have scarcely been reported except for a B2 strain, Germany/FS/98 (GenBank Accession No. AY079098). C1 strains were first isolated in Australia in 1986, and gradually became the dominant strains from 1986 to 1993 (Figure 2(a)). C2 strains occurred much later than C1 strains, and the isolated cases were reported several times after 1995. The circulating situation was much complicated during 1996－2006. Four new subgenotypes (B3, B4, C3 and C4) emerged and co-circulated with subgenotype C1 and C2 worldwide (Figure 2). Subgenotypes C3 and C4 show marked area-limited circulation: C3 strains were only isolated

Table 3 VP1 nucleotide sequence similarity of orphan strains and other virus strains

Orphan strain	Mean homology (%)										
	A	B	C	B1	B2	B3	B4	C1	C2	C3	C4
2243-NY-79	80.2	92.2	79.6	93.7	93.1	93.8	90.6				
6658-COL-94	80.3	88.2	81.0	88.6	90.6	88.1	87.3				
73442-PA-99	81.5	89.5	81.5	90.0	92.6	89.6	88.3				
Yamanashi/Japan80	80.1	79.5	88.4					89.4	87.7	86.3	87.8
0667-CHN-87	79.3	80.5	92.9					93.6	92.4	91.5	92.7
H	79.6	80.2	92.5					93.2	91.9	91.1	92.3
97-56-CHN-97	81.8	81.3	91.8					91.6	92.9	92.2	90.6

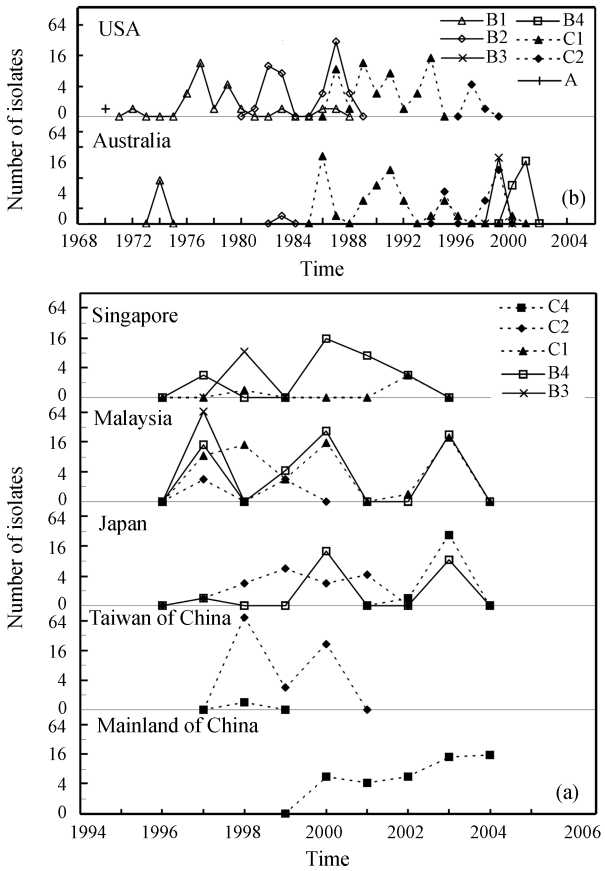

Figure 2 Worldwide circulating situation of EV71. (a) In United States and Australia, from 1970 to 2004. (b) In Asia, from 1994 to 2004.

in Korea, and C4 strains were only isolated in China and Japan.

The origin of genotype C was once considered in the eastern and southeast Asia[15]. However, in Brown's study, a Chinese isolate 0667-CHN-87 (AF135934) was mistaken for 0667-CHN-85. Actually, C1 strains were first reported in Australia in 1986, and then in USA and China in 1987. Subsequently, C1 strains became the predominant subgenotype instead of B1 and B2 strains in USA and Australia. Because of lack of early epidemic data of EV71 in Asia, it is hard to draw an accurate conclusion on the origin of genotype C. Since a Japanese strain Yamanashi/Japan 80 (AB059816) isolated in 1980 was the first known C strain, it is likely that genotype C originated in East Asia in the early 1980s.

2.4 Co-variability and genotype-related sites

Previously, some genotype-related amino-acid sites on VP1 were found in 113 strains isolated in USA[15]. In this study, these sites are further studied with the 532 strains isolated worldwide. A remarkable co-variability existed among 6 discrete positions of VP1 protein (43, 58, 164, 184, 240 and 249) (Figure 3). On each position, two kinds of dominant amino acids were observed and have nearly the same abundance (about 50%). Supposing that the amino acids on these six positions were independent of each other, the expected frequency of certain patterns, such as ETETSV or KADSTI, would be nearly $(1/2)^6$, that is, 1.6%. However, the observed frequencies of these two patterns are 38.0% and 40.2% respectively. Under the null hypothesis H0, χ^2-test indicates that the observed values are markedly different from the expected values (P=0.005). Thus, a tight linkage among the residues exists on these 6 positions. Based on the amino-acid pattern on these 6 sites, 532 strains can be divided into two clusters: one cluster includes strains showing pattern ETETSV or its mutant patterns; the other cluster includes strains showing pattern KADSTI or its mutant patterns. Interestingly, strains in the first cluster are all B-genotype strains, and those in the second cluster belong to genotypes C, A (strain BrCr), or D (strain R13223-IND-01). Because of the mutation (Figure 3), the dominant residue on one position in a certain genotype is not enough for us to identify the genotype of a new isolate. In contrast, amino acid pattern on these 6 positions can provide more accurate standards for identifying genotypes. In previous studies, the relation with the genotype was also observed in positions 262, 289 and 292[7,15]. However, the predominant residues in these 3 positions seem to be related with some certain subgenotypes (B1—B4, C1—C4) rather than genotypes (A, B or C) (Figure 4). Besides, position 124 is actually a highly conserved site position

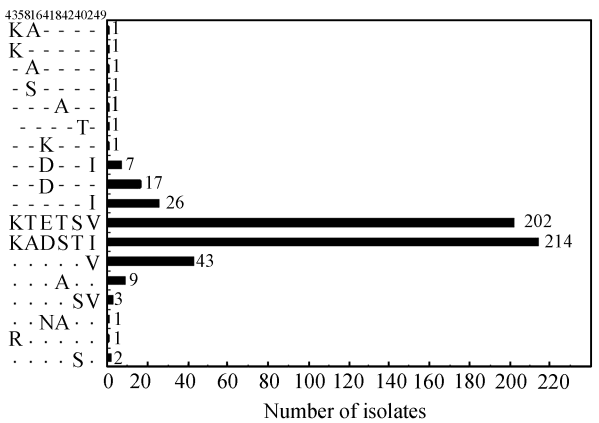

Figure 3 Co-variability in 6 discrete positions on VP1 (from left to right in order: amino acid 43, 58, 164, 184, 240 and 249). ETETSV (35.3%) and KADSTI (37.4%) are two most abundant patterns. Other patterns are respectively similar to them. '-' and '.' separately stand for the same amino acid compared with ETETSV or KADSTI.

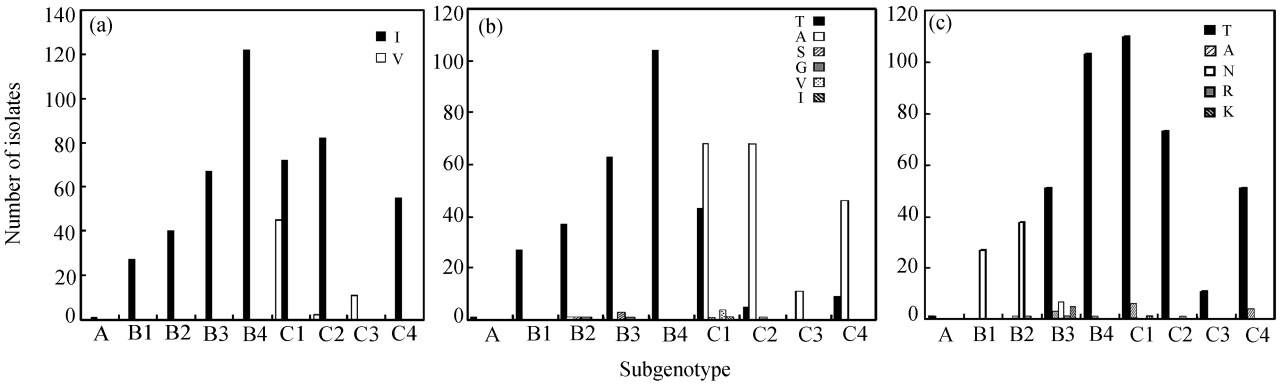

Figure 4 Variability of amino acids on positions 262(a), 289 (b) and 292 (c), and the relationship with subgenotypes.

and almost all strains show the Glutamic residue, so the genotype-relation of this site mentioned in the previous study[15] may be caused by mistaking position 164 for 124.

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