Molecular Characteristics of VP1 Region of Enterovirus 71 Strains in China

Haiyan Sun
Shaoxing Second Hospital
Min Gao
Huzhou Central Hospital
Dawei Cui (daweicui@zju.edu.cn)
The First Affiliated Hospital, Zhejiang University School of Medicine https://orcid.org/0000-0003-3840-1486

Research

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Abstract

Background Enterovirus 71 (EV71) is the most common causative agent of severe and fetal hand, foot, and mouth disease (HFMD) outbreaks worldwide, and the VP1 protein, a capsid protein of EV71, is responsible for the genotype of EV71, which is important for vaccine selection and effectiveness. We performed an observational study of the genetic characteristics and genotype of EV71 isolates in China.

Methods The VP1 gene sequences of 3712 EV71 virus strains from China, excluding repetitive sequences, and 30 known EV71 genotypes, as reference strains, between 1986 and 2019 were obtained from GenBank. A phylogenetic tree, amino acid homology, genetic variation and genotype analysis of the EV71-VP1 protein were performed with MEGA 6.0 software.

Results The amino acid identity of all Chinese EV71 strains was 88.33%-100%, 93.47%-100% with the vaccine strain H07, and 93.04%-100% with the vaccine strains FY7VP5 and FY-23K-B. Since 2000, the prevalent strains of EV71 were mainly the C4 genotype; the C4a subgenotype was predominant, the C4b subgenotype was the second, and other subgenotypes appeared sporadically between 2005 and 2018 in mainland China. The B4 genotype was the main genotype in Taiwan, and the epidemic strains were constantly changing. Some amino acid variations in VP1 of EV71 occurred with high frequencies: A289T (20.99%), H22Q (16.49%), A293S (15.95%), S283T (15.11%), V249I (7.76%), N31D (7.25%), and E98K (6.65%).

Conclusion The C4 genotype of EV71 in China can match the vaccine, which can effectively control EV71 epidemiology. However, the efficacy of the vaccine strain is partially affected by the continuous change in epidemic strains in Taiwan, China. These studies suggested that the genetic characteristics of the EV71-VP1 region should be continuously monitored, which is critical for epidemic control and vaccine design against EV71 infection in children.

Background

Enterovirus 71 (EV71) is a common pathogen of hand, foot, and mouth disease in children, and it is also the most important risk factor for severe cases and deaths[1–4]. Some children with hand, foot, and mouth disease (HFMD) can have severe neurological diseases, such as aseptic meningitis, encephalitis and acute delayed paralysis, and develop serious brainstem encephalitis, neurogenic pulmonary edema and even death[5–6]. The surviving children with severe neurological diseases often have irreversible sequelae, seriously threatening the health of children[7–8].

EV71 was first discovered in 1969; the virus was distributed mainly in the Americas, Europe and other countries in sporadic form, and there were outbreaks in some European countries in the 1970s and 1980s[9–10]. After 1997, EV71 began to emerge and spread in Asia, and the Asia-Pacific region is the most prevalent place for EV71. There are reports of EV71 outbreaks in China, Singapore, Malaysia and so on[11–12]. Diseases caused by EV71 infection have been widely prevalent in China since 2007[13]. Hand, foot and

mouth disease (HFMD) pandemics in Linyi city, Shandong Province, and Guangdong, Anhui Province, in 2008 resulted in tens of thousands of childhood infections and dozens of child deaths[14–15].

EV71 virus belongs to the enterovirus genus of the RNA virus family. According to the nucleotide differences in the VP1 region, EV71 can be divided into three genotypes, and only one member of genotype A (Brcr), genotype B and genotype C, which can be divided into five genotypes, B1mur5 and C1mur5, among which C4 is subdivided into C4a and C4b[16–18]. In China, the C4 genotype is the main epidemic strain[19]. The C4 genotype persists in mainland China, C4b was the predominant epidemic genotype from 1998 to 2004, and it was mainly the C4a subgenotype after 2004, although Taiwan strains changed from the C2 to B4 to B5 subtypes[20–25]. At present, phase III clinical trials of vaccines from three companies in China have been completed, and the genotypes of their vaccine strains are all C4a subgenotypes[26]. The Vaccine Research and Development Center of National Institutes of Health in Taiwan has also developed an FI-EV71 vaccine based on the B4 subtype (EV71vac), which can cause a strong cross-neutralizing antibody reaction against different EV71 gene subtypes, such as B4, B1, B5 and C4a[26–27].

However, there are many reports on recombination between different genotypes of EV71[28–31], suggesting that EV71 has high variability and recombination ability, which may lead to the production of new pathogenic strains. Therefore, genome monitoring of EV71 epidemic strains is of great significance for the prevention and control of EV71 epidemics and can guide the application of the EV71 vaccine to a certain extent.

Therefore, in this study, the VP1 sequences of all EV71 viruses registered in GenBank in China from 1996 to 2019 were collected, and the genetic variation characteristics of genes were analyzed by bioinformatics software to provide a scientific basis for the prevention and control of HFMD epidemics.

Methods

1.1 Acquisition of the EV71-VP1 gene sequence

The complete VP1 gene sequences of EV71 strains isolated from children with HFMD from 1986 to December 31, 2019 in China with known collection dates and isolation regions, including mainland China, Hong Kong, Macao and Taiwan, were obtained from the GenBank public database on the National Center for Biotechnology Information (NCBI) PubMed website (http://www.ncbi.nlm.nih.gov/genbank/). A total of 8340 EV71 strains from China were collected from GenBank, consisting of 6572 strains from mainland China, 26 strains from Hong Kong, 0 strains from Macao, and 1742 strains from Taiwan. If EV71 strains were isolated the same year and from the same region and had 100% nucleotide homology, only one strain was included. Therefore, a total of 3470 strains from mainland China, 2 strains from Hong Kong, and 1156 strains from Taiwan were removed. Finally, 3712 strains, consisting of 3102 strains from mainland China, 24 strains from Hong Kong, and 586 strains from Taiwan, were retained in this study. The nucleotide sequence of the complete VP1 gene of all virus strains was 891 bp. The time and

geographical distribution of all isolates are shown in Figs. 1 and 2. Moreover, the VP1 gene sequences of Chinese vaccine strains (H07, FY7VP5, and FY-23K-B)[26]and 27 known genotypes of EV71 reference strains that were considered reference sequences that are shown in Table 1.

Table 1
Data of reference strains of EV71 genotypes

No.	GenBank No.	Year of Isolation	Place of Isolation	Genotype
1	HQ328793(H07)	2008	China	C4a
2	JX025561(FY7VP5)	2008	China	C4a
3	EU812515(FY-23K-B)	2008	China	C4a
4	HQ828086	2010	China	C4a
5	EU753365	2007	China	C4a
6	KU936124	2014	China	C4a
7	KU936125	2014	China	C4a
8	KU936130	2014	China	C4a
9	JQ742002	2001	China	C4b
10	JQ742001	2001	China	C4b
11	AF376081	1998	Malaysia	C3
12	AY207625	2000	Malaysia	C3
13	AF304457	1998	Taiwan	C2
14	AF376110	1999	Australia	C2
15	AY125969	2002	Korea	C1
16	AY125976	2002	Korea	C1
17	JN874558	2007	Taiwan	C5
18	KU888174	2008	Vietnam	C5
19	U22521	1970	USA	A
20	GU434678	2009	China	A
21	AF135886	1974	Australia	B1
22	AB059814	1975	Bulgaria	B1
23	AF009540	1988	USA	B2
24	AF009534	1987	USA	B2
25	AF376119	1998	Singapore	B3
26	AF376073	1997	Malaysia	B3
27	AJ586873	1997	Malaysia	B4

No.	GenBank No.	Year of Isolation	Place of Isolation	Genotype
28	AF376084	2000	Malaysia	B4
29	AB177815	2003	Japan	B5
30	AB177816	2003	Japan	B5

1.2 Construction of an EV71-VP1 evolutionary tree and gene sequence analysis

The complete VP1 sequences of EV71 strains were compared by Molecular Evolutionary Genetics Analysis (MEGA) version 6.0. A phylogenetic tree of the EV71-VP1 gene was constructed by the adjacency method (neighbor-joining, N-J) with 1000 bootstrap replications. The homology and variation of the VP1 gene sequences of the EV71 strains were analyzed in a previous study[32].

2. Results

2.1 Phylogeny and homology analysis of the EV71 VP1 region

A phylogenetic tree of the VP1 amino acid sequence was constructed with 3712 EV71 isolates from China and 30 reference strains (Fig. 3). The amino acid identities of all Chinese EV71 strains were 88.33%~100% in comparison with the vaccine strain H07 and 93.04%-100% with the vaccine strains FY7VP5 and FY-23K-B. Among these EV71 strains, the C4 genotype accounted for the predominant strains of EV71, and the C4a subgenotype was the most common. Moreover, B4, C4b and C2 were also important genotypes of EV71, and other subgenotypes appeared sporadically. The amino acid identity of all Chinese EV71 strains was 88.33%~100%, 93.47%-100% with the vaccine strain H07, and 93.04%-100% with the vaccine strains FY7VP5 and FY-23K-B.

2.2 Genotypic distribution of EV71 strains in different years in China

Among 3712 strains, the only 6 strains first isolated from China in 1986 were all B3 genotypes. Next, different genotypes/subgenotypes of EV71 were found in China. From 1986 to 2007, there was a small epidemic peak of EV71 in 1998 with the C2 genotype. Moreover, between 1999 and 2003, the B4 genotype was the predominant strain of EV71. Most EV71 strains between 2004 and 2005 were C3 genotypes. From 2006 to 2018, the C4a subgenotype was the most common epidemic strain, and the B4 and C4b genotypes also accounted for a certain proportion of EV71 strains. These results are shown in Table 2 and Fig. 4.

Table 2
Number of EV71 genotypes for every year in China

Year	Genotypes of EV71												Number
	C4a	C4b	C1	C2	C3	C5	A	B1	B2	B3	B4	B5	
1986										6			6
1996				1									1
1997					1						1		2
1998		3		119		2			1	1	9		135
1999				1					2	6	8		17
2000		3							2	4	35		44
2001		5							1		84		90
2002		4									49	1	54
2003	7	7							1		9		24
2004	1	14		1	11								27
2005	3		1	1	18								23
2006	6	1	2		1								10
2007	56	2	3								2		63
2008	281	8		2	6		4				45		346
2009	341	16					2				6		365
2010	565	15									6		586
2011	313	6	1	4	1		1	2	2	3	14	2	349
2012	276	27		1			1				77		382
2013	231	1		1	6						2		241
2014	415	22		4							1		442
2015	162	12		1							2		177
2016	153	15										1	169
2017	148												148
2018	11												11

Next, the EV71 strains isolated from mainland China, Hong Kong, and Taiwan, China, were further analyzed to explore their genotypic distribution in different years. The results indicated that from 2005 to

2018, the C4a subgenotype was absolutely the predominant strain of EV71, the C4b subgenotype was second, and other subgenotypes appeared sporadically in mainland China. Moreover, among the 24 strains isolated from Hong Kong, there were 10 strains of the C4a subgenotype (1 strain in 2008, 10 strains in 2010, and 1 strain in 2012) and 14 strains of the C4b subgenotype (1 strain in 2008 and 13 strains in 2010). The results are shown in Table 3 and Fig. 5.

Table 3
Number of EV71 genotypes for every year in mainland China

Year	Gene subtype of EV71												Number
	C4a	C4b	C1	C2	C3	C5	A	B1	B2	B3	B4	B5	
1986													0
1996				1									1
1997					1								1
1998		1		1									2
1999													0
2000		3											3
2001		4											4
2002		4											4
2003	7	7							1				15
2004	1	13		1									15
2005	3		1										4
2006	6	1	1										8
2007	56	2											58
2008	280	6		2	1		4						293
2009	341	16					2						359
2010	551	15											566
2011	302	6	1	3	1		1	2	2	3		2	323
2012	273	27					1						301
2013	231	1		1									233
2014	415	22		4									441
2015	159	12		1									172
2016	148	15										1	164
2017	148												148
2018	11												11

However, the epidemic strains of EV71 in Taiwan in recent years are quite different from those in mainland China, and epidemic strains are not predominantly represented by a single subgenotype but are

constantly changing. The first 6 strains isolated from Taiwan, China, in 1986 were all of the B3 genotype. In 1998, C2 was the predominant EV71 genotype in Taiwan. From 1999 to 2003, 2008 to 2009, and 2011 to 2012, the epidemic strains in Taiwan evolved mainly into B4 genotype EV71 strains. Additionally, between 2010 and 2011, C4a was an important genotype (Table 4 and Fig. 6).

Table 4
Number of EV71 genotypes for every year in Taiwan, China

Year	Gene subtype of EV71												Number
	C4a	C4b	C1	C2	C3	C5	A	B1	B2	B3	B4	B5	
1986										6			6
1996													0
1997											1		1
1998		2		118		2			1	1	9		133
1999				1					2	6	8		17
2000									2	4	35		41
2001		1							1		84		86
2002											49	1	50
2003											9		9
2004		1			11								12
2005				1	18								19
2006			1		1								2
2007			3								2		5
2008	1	2			5						45		53
2009											6		6
2010	12										6		18
2011	11			1							14		26
2012	5			1							77		83
2013					6						2		8
2014											1		1
2015	3										2		5
2016	5												5
2017													0
2018													0

2.3 Analysis Of Amino Acid Variation Of Ev71-vp1

The analysis of the common amino acid variation sites in the EV71-VP1 region showed that of the 3712 strains, the major common amino acid site variation was site 289, with a total variation rate of 23.55%, and A289T was the most common variant, accounting for 20.99%. Moreover, 10.24% of strains from mainland China, including Hong Kong, exhibited this variation, and 10.75% of strain from Taiwan also had this variation. There was a total mutation rate of 21.85% at amino acid site 22 in VP1 region. The frequency of the H22Q variation was 16.49%, of which mainland China, including Hong Kong, exhibited 5.50%, and Taiwan exhibited 10.99%; furthermore 23% of the EV71 strains had an H22R mutation. Moreover, the mutation rates of A293S, S283T, V249I, N31D and E98K were 15.95%, 15.11%, 7.76%, 7.76% and 6.65%, respectively. Among the common amino acid variation sites of the EV71-VP1 region, the most common amino acid variation was at site 293, and there were five kinds of amino acid variation, among which the A293S mutation was the most common (Table 5).

Table 5
Analysis of common amino acid variation sites in VP1 of EV71

Common amino acid	**22H**		**31N**		**98E**		**145E**		**249V**	
Mutant amino acids	Q	16.49%	D	7.25%	K	6.65%	G	3.13%	I	7.76%
	R	5.23%	S	0.13%	G	0.08%	Q	3.50%	A	0.05%
	N	0.13%	G	0.03%	R	0.03%	A	0.30%	F	0.03%
			R	0.03%			L	0.05%		
	total	21.85%	total	7.44%	total	6.76%	total	6.98%	total	7.84%
Common amino acid	262I		282N		283S		289A		293A	
Mutant amino acids	V	2.77%	S	1.78%	T	15.11%	T	20.99%	S	15.95%
	Q	0.03%	D	0.32%	A	0.08%	V	2.32%	V	0.13%
			K	0.08%	F	0.05%	D	0.16%	G	0.08%
			T	0.05%			I	0.08%	L	0.03%
									P	0.03%
	total	2.80%	total	2.24%	total	15.25%	total	23.55%	total	16.22%

Note: H: Histidine; Q: Glutamine; R: Arginine; N: Asparagine; D: Aspartic acid; S: Serine; G:Glycine; E: Glutamic acid; K: Lysine; A: Alanine; L: Leucine; V: Valine; I: Isoleucine; F: Phenylalanine; T: Threonine; P: Proline

3. Discussion

It has been reported that the major EV71 strains basically have belonged to the C4 genotype in mainland China since 2004[18, 33]. In this study, it was found that the epidemic strains of EV71 in mainland China have been dominated by the C4 subtype since 2000 and mainly the C4b subtype from 2000 to 2004, and the proportion of the C4a subtype has increased significantly since 2005. From 2005 to 2018, the C4a subtype was dominant, the C4b subtype was secondary, and other subtypes appeared sporadically, which was similar to previous reports at in China and abroad[20–21, 30, 33]. From 2004 to 2018, most of the EV71 isolates in mainland China were genotype C, and most of them were subtype C4 (especially subtype C4a), while other genotypes occurred sporadically (9 strains of genotype B in 2011, 4 strains of genotype A in 2008, 2 strains of genotype A in 2009, 1 strain of genotype A in 2011 and 1 strain of genotype A in 2012). The immune effectiveness of inactivated vaccines depends mostly on the antigenic correlation between epidemic strains and vaccine strains, which are often best for preventing the infection of the same subtype virus but poor against different subtypes[30]. Recent studies have also shown that the EV71 vaccine (especially in children who receive 2 doses of immunization) can effectively prevent and control childhood EV71-associated HFMD but has no protective effect against coxsackievirus (CV) A6 (CVA-6) or CVA16, and there is no explanation for the effectiveness of other subtypes of EV71 (excluding C4a subtypes)[34]. These studies show that the vaccine research and development for EV71 combined with CVA6 and CVA16 and other multivalent vaccines could better prevent EV71 infection.

Interestingly, this study found that the subtypes of EV71 epidemic strains in Taiwan were mainly B4 subtypes, which were different from those in mainland China, and EV71 epidemic strains are also constantly changing, which is consistent with early reports[26]. A previous study indicated that the FI-EV71 vaccine (EV71vac) based on the B4 genotype from Taiwan, China, was safe and induced a high titer of neutralizing antibodies against EV71 in a human phase 1 clinical trial on adults in 2010 and was highly effective against B1, B5, and C4a strains. However, the titers of neutralizing antibodies against C4b and CVA16 were low in 20% of volunteers, and virus-neutralizing antibodies against the C2 genotype were not detected in 90% of the vaccine recipients[26–27]. These studies indicate that it is necessary to strengthen the monitoring of EV71 genotypes; new multivalent and effective vaccines that can cover local strains should be designed and applied according to the genotypes of local predominant EV71 epidemics to ensure the vaccine plays a more accurate role in the control of HFMD epidemics.

Some studies have shown that the H22Q mutation in the VP1 protein of EV71 virus can lead to a decrease in the adsorption capacity of the C4 genotype to host cells[35–37]. The amino acid at position 22 of 78.15% of the 3712 strains isolated in China was H (histidine), which suggested that most of the viruses had strong adsorption capacity to host cells. Furthermore, H22Q was present in 10.99% of all EV71 strains in Taiwan, which was significantly more prevalent than that in mainland China (including Hong Kong) (5.50%), suggesting that the adsorption capacity of some strains in Taiwan to host cells was weak in comparison with that of strains in mainland China.

Studies have shown that the variation A289T in the EV71-VP1 protein is closely related to the occurrence of severe HFMD and that the neurological symptoms caused by EV71 infection are significantly

increased when the amino acid at position 289 of VP1 is A (alanine); in contrast, there is low neurotoxicity when the amino acid at position 289 is T (threonine)[36, 38]. In this study, 76.45% (2838 strains) of the virus strains contained an A (alanine), suggesting that most of the EV71 viruses have high neurotoxicity. Moreover, 10.24% (380 strains) of the strains in mainland China (including Hong Kong) and 10.75% (399 strains) of those in Taiwan contained a T (threonine), suggesting low neurotoxicity. Additionally, whether these new mutations will cause the emergence of severe HFMD remains to be further studied, regarding A289V (valine)/D (aspartic acid)/I (isoleucine) mutations.

EV71 can infect human lymphocytes by binding to its receptor molecule, Pselectin glycoprotein ligand-1 (PSGL-1). When the amino acid at position 145, E (glutamic acid), in VP1 is mutated to G (glycine) or Q (glutamine), to the virus binds PSGL-1 more readily, whereas its PSGL-1-binding ability is weakened or lost if position 145 is E[39]. In this study, the amino acid at position 145 is E in most strains, and the mutation rate of E145G/Q is 6.63%, suggesting that the emergence of this mutation may be result in a virus that is more likely to infect human lymphocytes.

It has been reported that the E98K mutation may increase the hydrophobicity of VP1, making it easy for large compounds to enter and interfere with VP1 receptor binding, suggesting that E98K mutant viruses are sensitive to larger compounds[40]. Other studies have shown that E145G and N31D mutations are associated with increased virulence of EV71 and may increase the risk of neurological complications, while I262V mutations reduce the risk of neurological complications[41–43]. In this study, the mutation rates of E98K, E145G, N31D and I262V were 6.65%, 3.13%, 7.25% and 2.77%, respectively. These findings indicate that these mutations may play an important role in the pathogenicity of mild and severe EV71-associated HFMD.

Humans are the only natural host and source of EV71, as EV71 cannot infect rodents, which is due mainly to the incompatibility between the virus and rodent cells and the different expression of its scavenger receptor in humans and rodents[44–46]. However, some studies have found that the simultaneous substitution of K98E, E145A and L169F in VP1 of EV71 can cause infection in mice[44]. Our study showed that among 3712 strains, the mutation frequencies of K98E, E145A and L169F were 93.24%, 0.30% and 0.03%, respectively, but no strain with three mutations was found. These findings suggested that humans are still the only host of EV71 in China, but the existence of individual mutations does not rule out the emergence of strains that can infect other mammals after a few years. Therefore, it is important to closely monitor the mutation of the key sites of the EV71-VP1 region.

EV71 is the most important pathogen of children with severe HFMD, which can lead to irreversible sequelae or death of infected children, and it is a serious threat to the health of children[4, 7]. At present, there is no specific drug for the treatment of EV71 infection. The development and marketing of an inactivated EV71 vaccine in China is crucial for the prevention of HFMD caused by EV71 infection[26, 47–49]. Phase III clinical trials of the EV71 inactivated vaccine approved in China in 2015 showed that its protective effectiveness against EV71-associated HFMD was more than 90%[47, 50–52]. However, molecular

epidemiological studies of EV71 show that EV71 gene mutations occur frequently, leading to genetic diversity[28–31, 53]. These studies imply that there is still a need for strengthening surveillance of EV71 genotypes and the development of new EV71 vaccines.

This study is a retrospective study, and there are some limitations. First, the EV71-VP1 gene sequences from China analyzed in this study were downloaded from the GenBank database but were not tested by us. Second, the Chinese EV71-VP1 strains registered in GenBank do not cover all provinces of China, and the data in some years are missing, which means that we may have missed some isolates of other genotypes. Third, it is not clear whether some variations in the amino acid sites found in the study are related to the severity of disease or the route of transmission.

4. Conclusion

In summary, the prevalent strains of EV71 belonged to mainly the C4 genotype; the C4a subgenotype was predominant, the C4b subgenotype was the second most prevalent, and other subgenotypes appeared sporadically in mainland China. The B4 genotype is the major genotype in Taiwan, China, and the epidemic strain is constantly changing. Moreover, the variation in amino acids in the key sites of the EV71-VP1 region is very important for the development of severe HFMD. Taken together, these studies indicated that the genetic characteristics of the EV71-VP1 region should be continuously monitored, which is essential for the prevention and control of EV71-associated HFMD in children and EV71 vaccine design.

Declarations

Competing interests

The authors declare that they have no conflicts of interest.

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Authors' contributions

Dawei Cui participated in the design of the study. Dawei Cui, Haiyan Sun and Min Gao performed data collection and statistical analysis, Dawei Cui and Haiyan Sun drafted the manuscript. All authors read and approved the final manuscript. We gratefully acknowledge American Journal Experts (AJE) (**Verification code: 053B-2FE3-F3E3-887C-4651**) for providing English language editing services for our manuscript.

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Availability of data and material

The available data used and/or analyzed during the current study are all included in the manuscript.

Ethics approval and consent to participate

Compliance with ethical standards.

Consent for publication

Not applicable.

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Figures

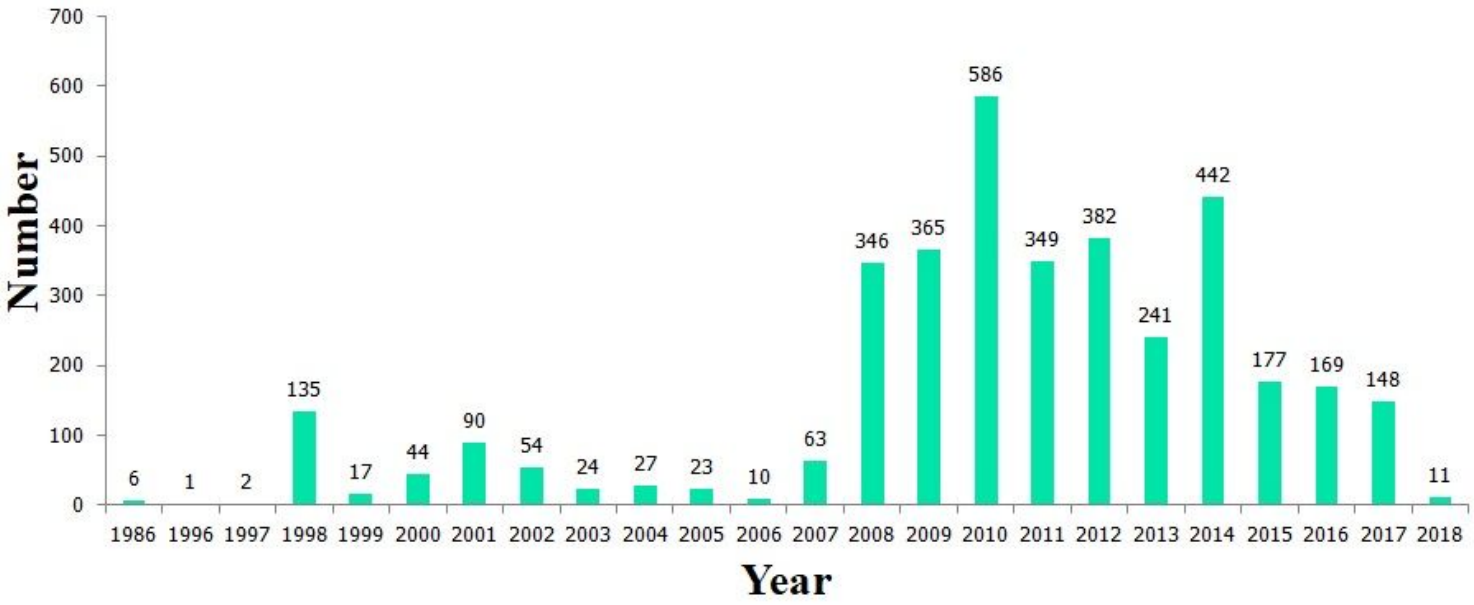

Figure 1

The number of EV71 strains isolated in different years of China. The 3712 EV71 strains with complete VP1 gene sequences collected from China were obtained from the GenBank public database on the National Center for Biotechnology Information (NCBI) PubMed website (http://www.ncbi.nlm.nih.gov/genbank/) between 1986 and December 31, 2019, however, no EV71 strains were collected in 2019 in this study.

Figure 2

The number of EV71 strains isolated in different provinces of China. The 3712 EV71 strains were collected from China including different provinces of mainland China (3102), Hong Kong (24), Macao (0) and Taiwan (586) between 1986 and December 31, 2019. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

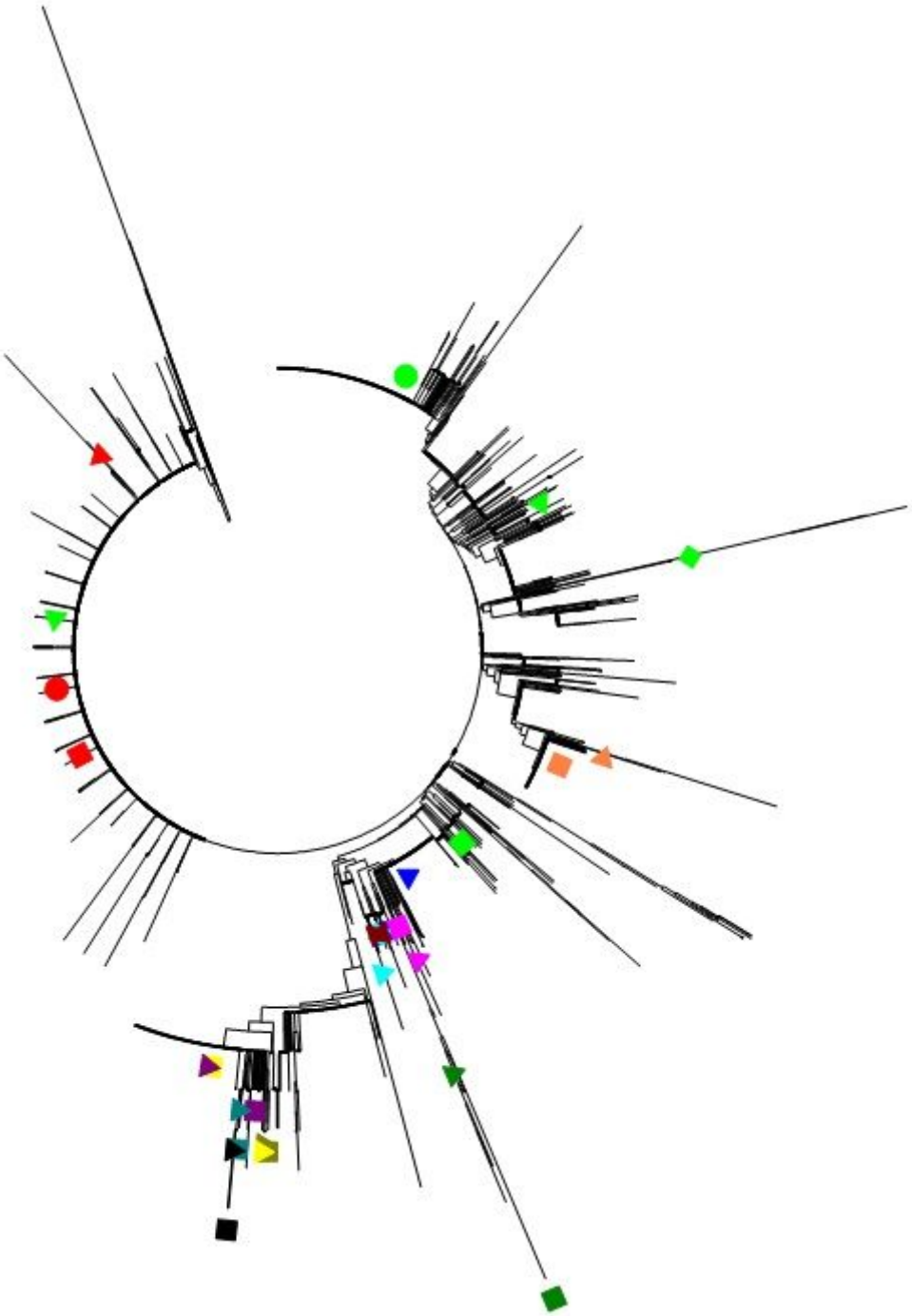

Figure 3

Phylogenetic tree of entire VP1 gene in EV71. The 30 reference strains were indicated in different colored shapes, as shown below: □:HQ328793(H07) ▲:JX025561(FY7VP5) ●:EU812515(FY-23K-B) □:HQ828086(C4a) ▲:EU753365(C4a)●:KU936124(C4a) ▼:KU936125(C4a) □:KU936130(C4a) □:JQ742002(C4b) ▲:JQ742001(C4b) □:AY207625(C3) ▲:AF376081(C3) □:AF304457(C2) ▲:AF376110 (C2) □:AY125969(C1) ▲:AY125976 (C1) ▲:JN874558 (C5) ▼:KU888174(C5) □:U22521(A) ▲:GU434678(A) □:AB059814(B1) ▲:AF135886 (B1) □:AF009534(B2) ▲:AF009540(B2) □:AF376119(B3) ▲:AF376073(B3) □:AF376084(B4) ▲:AJ586873 (B4) □:AB177815 (B5) ▲:AB177816 (B5)

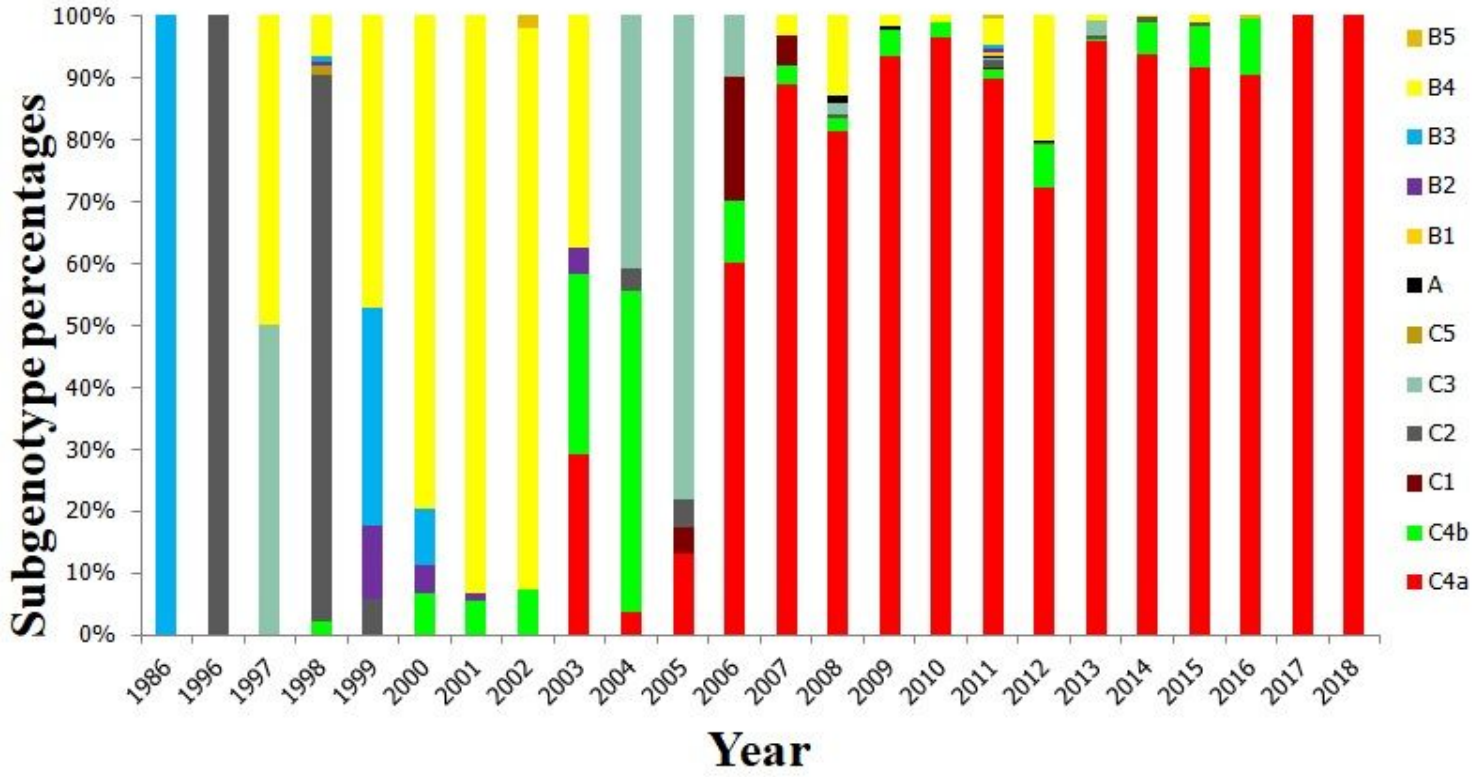

Figure 4

The subgenotype percentages of EV71 for every year in China. Among 3712 strains, 12 genotypes were found in this study. The B4 genotype was the predominant strain between 1999 and 2003, C3 genotypes was main from 2004 to 2005, the C4a subgenotype was the most common epidemic strain from 2006 to 2018.

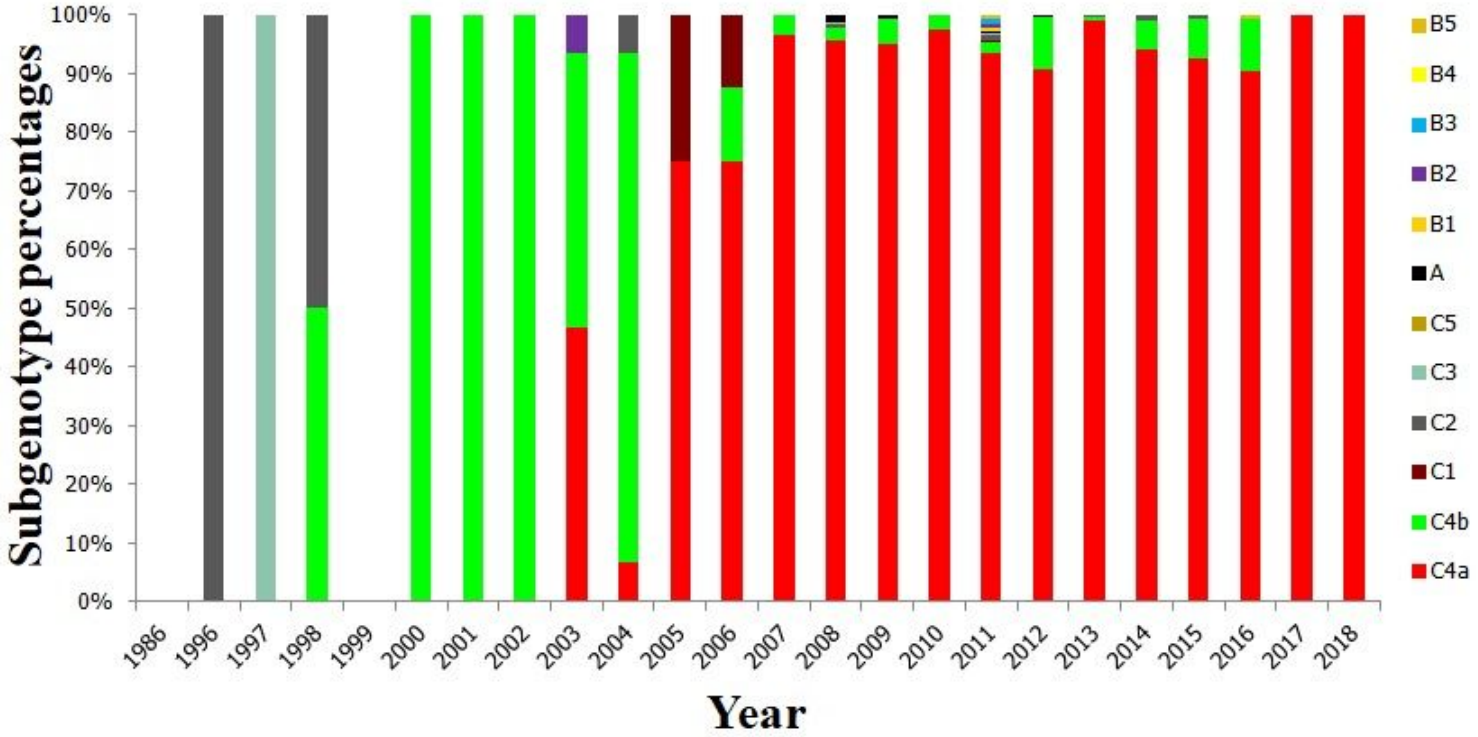

Figure 5

The genotypic distribution of EV71 for every year in mainland China. The C4a subgenotype was absolutely the predominant strain of EV71 from 2005 to 2018, the C4b subgenotype was second, and other subgenotypes appeared sporadically in mainland China.

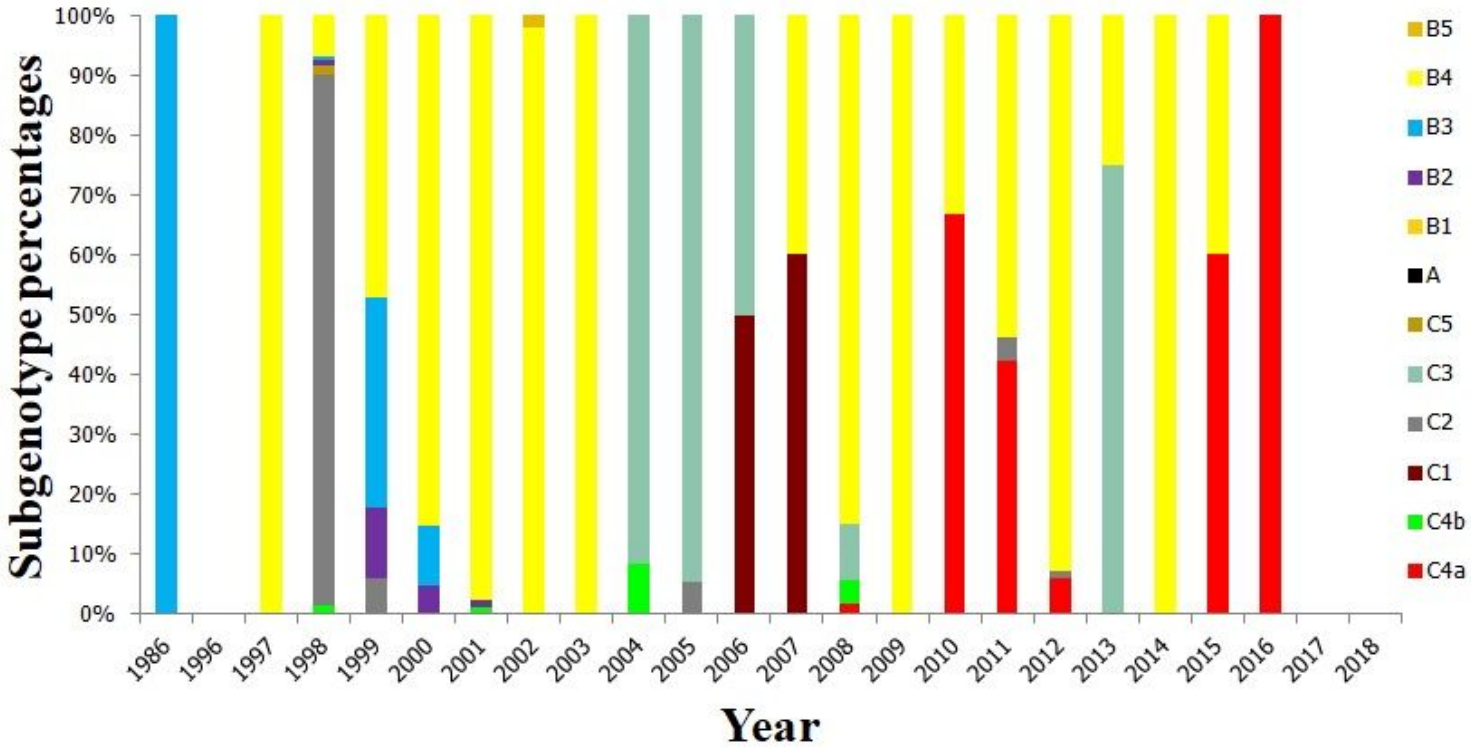

Figure 6

The subgenotype percentages of EV71 for every year in Taiwan, China. The epidemic strains of EV71 in Taiwan in recent years are constantly changing. The B3 genotype was first emerged in 1986. From 1999 to 2003, 2008 to 2009, and 2011 to 2012, the B4 genotype was predominant strains. Moreover, between 2010 and 2011, C4a was a mainly important genotype.