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# Evolution of EV71 Genogroup in Taiwan From 1998 to 2005: An Emerging of Subgenogroup C4 of EV71

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In Taiwan, enterovirus 71 (EV71) has played an important role in severe enterovirus-related cases every year since the devastating outbreak in 1998. Three genogroups A, B, C occur worldwide; with the B and C genogroups being subdivided into B1–B4 and C1–C4 subgenogroups respectively. To understand the mutation of the EV71 genogroup in Taiwan before and after 1998, a total of 54 worldwide strains were studied including 41 Taiwanese strains obtained in 1986 and 1998–2004. A fragment of 207 bp of the VP4 region was amplified and sequenced. Genetic analysis was performed using MEGA software (version 3.0) for the nucleotide sequence alignment and phylogenetic analysis. In Taiwan, the subgenogroup B1 was predominant before 1998 while subgenogroup C2 was the major etiologic group in 1998 outbreak. A minor etiologic group outbreak in 1998, subgenogroup B4, became predominant during the period from 1999 to 2003. In this study, subgenogroup C4 emerged and became predominant in 2004 in Taiwan. The nucleotide differences between B1 and C2, C2 and B4, B4 and C4 were 20%–26%, 19%–27%, 18%–22%, respectively. Nucleotide sequence alignment revealed 67 substitutions. Most of the substitutions (62/67) were silent mutations. This is the first report about the emergence of EV71 subgenogroup C4 in Taiwan. ***J. Med. Virol. 78:254–262, 2006.*** 

**KEY WORDS:** enterovirus 71; genogroup; subgenogroup

## INTRODUCTION

Human enterovirus 71 (EV71) was first isolated in 1969 [Schmidt et al., 1974]. Outbreaks of EV71 infection have been reported worldwide in countries in Europe, America, and the West Pacific region (WPR) [Brown et al., 1999; Cardosa et al., 1999, 2003; Shimizu et al., 2004]. EV71 and coxsackie virus A16 (CA16) belong to the *Enterovirus* genus of the family *Picornaviridae* and both are common etiologic agents of epidemic hand-foot-and-mouth disease (HFMD). However, EV71 infections have been also associated with aseptic meningitis, encephalitis, and poliomyelitis-like paralysis and even death in addition to febrile rash illness [Melnick, 1984; Alexander et al., 1994].

EV71-associated severe neurological disease was rarely seen in earlier outbreaks in the 1980s in Australia [Gilbert et al., 1988], Asia [Samuda et al., 1987], Taiwan (unpublished data), and the United States [Alexander et al., 1994]. However, it was reported in outbreaks in Bulgaria in 1975, which caused 44 deaths and in Hungary in 1978, which recorded 47 deaths [Chumakov et al., 1979; Nagy et al., 1982]. In the past decade, several EV71 outbreaks have occurred. EV71 outbreaks with several fatal cases were reported in WPR countries after 1997. Death-associated EV71 outbreaks were reported in Sarawak, Malaysia in 1997 where there were 34 deaths [Chan et al., 2000; Herrero et al., 2003]. In Taiwan, a devastating EV71 outbreak occurred in 1998, followed by another two death-associated outbreaks in

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2000 and 2001, which caused 78, 25, and 26 deaths, respectively. Bulbar and brain stem encephalitis and pulmonary edema were the major clinical syndromes found in these fatal cases [Chang et al., 1998; Lam et al., 1998; Ho et al., 1999; Lin et al., 2003].

The virulence of EV71 has raised considerable public concern in many countries in WPR and the phylogenetic relationships of EV71 isolates worldwide have been described [Shimizu et al., 1999, 2004; McMinn et al., 2001; Shih et al., 2000; Wang et al., 2000, 2002; Chu et al., 2001; Cardosa et al., 2003; Chan et al., 2003; Li et al., 2005]. There are three genogroups (A, B, and C) worldwide based on the nomenclature of Brown et al. [1999]. The prototype strain, BrCr is the sole member of genogroup A. Most EV71 isolates belong to either genogroup B or C, which are further divided into four sub-lineages,that is, subgenogroups B1–B4 and C1–C4, respectively [Cardosa et al., 2003; Shimizu et al., 2004]. This indicates that EV71 is a genetically diverse, rapidly evolving virus.

According to previous reports from Taiwan based on VP4 and VP1 genes, isolates obtained in 1980 and 1986 in Taiwan belonged to the B genogroup. However, two distinct genogroups, C (major) and B (minor), co-circulated in Taiwan in the 1998 outbreak while only genogroup B was found in 1999–2003. Intriguingly, only genogroup C was found again in 2004. [Shih et al., 2000; Wang et al., 2000, 2002; Chu et al., 2001, data from CDC Taiwan, 2004]. To have a more comprehensive study on the changing genogroups and subgenogroups and to update the molecular epidemiology data for control measures in Taiwan and around the world, 41 virus strains isolated from 1986 and 1999–2005 were studied, based on the nucleotide sequences of VP4 region. The sequences were analyzed together with the sequences data of isolates from around the world deposited in GenBank. A phylogenetic tree was constructed for a comprehensive study of the molecular epidemiology of EV71 in Taiwan, showing for the first time the emergence of subgenogroup C4 in Taiwan. Our results also confirmed previous research that indicated several subgenogroups of the virus have been circulating in the WPR since 1997[Cardosa et al., 2003].

## MATERIALS AND METHODS

### Viruses

Forty-one strains of EV71 obtained in Taiwan from 1986, 1999 to 2005 were analyzed in this study (Table I). Viruses were inoculated in Vero cells with Eagle's minimal essential medium supplemented with 2% fetal bovine serum, and were harvested when the cytopathic effect was almost 100%. The infected cells were frozen and thawed three times, and then were centrifuged. The viral supernatant was stored at −80°C.

### Viral RNA Extraction

Viral RNA was extracted using QIAamp viral RNA purification kit according to the manufacturer's instructions (Qiagen Chatsworth, CA)

### RT-PCR and Sequencing

The primers OL68-1(5′-GGT AAQ TTC CAC CAC CAY AA-3′,Y is either C or T, 1179–1198) and EVP2 (5′-CCT CCG GCC CCT GAA TGC GGC TAA-3′, 449–473) were used for VP4 amplification of nucleotide 449–1198 relative to BrCr. Reverse transcription and polymerase chain reaction (RT-PCR) were performed as previously described [Kitamura et al., 1997]. Briefly, a mixture of 5 μl of the RNA and 1 μl of 50 μM of each primer (EVP2 and OL68-1) was heated at 95°C for 5 min and was put on ice immediately. Then 43 μl of the reaction mixture composed of 10× PCR reaction buffer, 40 U of RNase inhibitor (Promega, Madison, WI), 200 U of MMLV (Promega, 2.5 U of cloned Pfu DNA polymerase (Protech, Taipei, Taiwan) and 15 mM of dNTP(Promega) was added. The following RT-PCR program was used in all isolates: 37°C for 1 hr, 94°C for 5 min, followed by 40 cycles of 95°C for 1 min, 55°C for 30 sec, 72°C for 1 min, and a final elongation step of 72°C for 5 min. A fragment of 750 base pairs spanning the 5′-noncoding region to one third of the VP2 region including the entire region of VP4 was amplified. Cycle sequencing was performed using the purified PCR products with the ABI Prism Ready Reaction Dideoxy Terminator cycle sequencing kit (Model 3730 version3.4, Applied Biosystems, Foster City, CA).

### Phylogenetic Analysis

Alignment of the 207 base pairs of VP4 gene sequence was undertaken by using the Clustal method of DNASTAR MegAlign. A phylogenetic tree was constructed by the neighbor-joining method with MEGA 3 (Molecular Evolutionary Genetics Analysis, Version 3.0). The reliability of the neighbor-joining tree was estimated by bootstrap analysis with 1,000 pseudoreplicates.

### Accession Numbers of the Nucleotide Sequences

Sequence data from the present study were deposited to the GenBank nucleotide sequence database. Their accession numbers are shown in Table I.

## RESULTS

### Nucleotide Sequences Analysis

A total of 41 strains isolated in Taiwan from 1986 and 1999 to 2004 were analyzed in this study (Table I). Based on 207 bp VP4 nucleotide sequence, a total of 45 worldwide strains of EV71 including the prototype, and some of the Taiwanese strains, were aligned using the Clustal method of MegAlign in DNA STAR program. Three genogroups A, B, and C were revealed by the alignment (Fig. 1). Furthermore four subgenogroups, B1–B4 and C1–C4, were observed in B and C genogroups, respectively. Subgenogroup C4 is a newly emerged subgenogroup in Taiwan. The nucleotide changes found in a total of 64 sites were compared to that of the prototype, BrCr. Ninety-three percent (62/67)

TABLE I. EV71 Isolates in This Study

| Isolate | Year | Origin | Clinical outcome | Accession number | Subgenogroup |
|---|---|---|---|---|---|
| 250/86 | 1986 | Taiwan | HFMD | AY817061 | B1 |
| 251/99 | 1999 | Taiwan | HFMD | AY817062 | B4 |
| 427/99 | 1999 | Taiwan | HFMD | AY817063 | B4 |
| 2002/00 | 2000 | Taiwan | HFMD | AY817068 | B4 |
| 532/00 | 2000 | Taiwan | HFMD | AY817064 | B4 |
| 75/00 | 2000 | Taiwan | HFMD | AY817059 | B4 |
| C1153/00 | 2000 | Taiwan | Herpangina | AY817076 | B4 |
| C1481/00 | 2000 | Taiwan | HFMD | AY817073 | B4 |
| 1043/01 | 2001 | Taiwan | HFMD | AY817066 | B4 |
| 109/01 | 2001 | Taiwan | HFMD | AY817060 | B4 |
| 1104/01 | 2001 | Taiwan | HFMD | AY817067 | B4 |
| C2446/01 | 2001 | Taiwan | HFMD | AY817074 | B4 |
| C2564/01 | 2001 | Taiwan | HFMD | AY817075 | B4 |
| K002/01 | 2001 | Taiwan | HFMD | AY817078 | B4 |
| K0381/01 | 2001 | Taiwan | HFMD | AY817080 | B4 |
| C3346/02 | 2002 | Taiwan | HFMD | AY817077 | B4 |
| C3485/02 | 2002 | Taiwan | Herpangina | AY817079 | B4 |
| N2838/03 | 2003 | Taiwan | HFMD | DQ008993 | B4 |
| S37/03 | 2003 | Taiwan | HFMD | DQ008996 | B4 |
| CDC153/03 | 2003 | Taiwan | Herpangina | DQ008988 | B4 |
| CDC691/03 | 2003 | Taiwan | Herpangina | DQ008989 | B4 |
| CDC695/03 | 2003 | Taiwan | HFMD | DQ008990 | B4 |
| CDC701/03 | 2003 | Taiwan | Herpangina | DQ008998 | B4 |
| VI4705/04 | 2004 | Taiwan | HFMD | DQ008997 | C4 |
| N1416/04 | 2004 | Taiwan | HFMD | DQ008991 | C4 |
| N2472/04 | 2004 | Taiwan | Herpangina | DQ008992 | C4 |
| N2852/04 | 2004 | Taiwan | HFMD | DQ008994 | C4 |
| N2947/04 | 2004 | Taiwan | HFMD, CNS, involvement | DQ008995 | C4 |
| 2644/04 | 2004 | Taiwan | HFMD | AY817069 | C4 |
| 4688/04 | 2004 | Taiwan | HFMD | AY817070 | C4 |
| 4693/04 | 2004 | Taiwan | HFMD | AY817072 | C4 |
| 4694/04 | 2004 | Taiwan | HFMD | AY817071 | C4 |
| T685/04 | 2004 | Taiwan | HFMD | AY817065 | C4 |
| K614/04 | 2004 | Taiwan | HFMD | AY817081 | C4 |
| K650/04 | 2004 | Taiwan | HFMD | AY817084 | C4 |
| K793/04 | 2004 | Taiwan | HFMD | AY817085 | C4 |
| K847/04 | 2004 | Taiwan | HFMD | AY817086 | C4 |
| K848/04 | 2004 | Taiwan | HFMD | AY817087 | C4 |
| 1301/05 | 2005 | Taiwan | Herpangina | DQ100442 | C4 |
| 1386/05 | 2005 | Taiwan | Acute Bronchitis | DQ100444 | C4 |
| 1396/05 | 2005 | Taiwan | Herpangina | DQ100443 | C4 |

of the substitutions occurred in the third codon, and are responsible for causing silent mutation (data not shown). Unique sequence patterns were found in each subgenogroup. Moreover, unique nucleotide base changes were found in specific subgenogroups. G at nucleotide 9 and A at nucleotide 48 were unique to subgenogroup C4. G at nucleotide 66 and 120 were specific to subgenogroup C3. C at nucleotide 60 was unique to subgenogroup C2. G at nucleotide 117 and 123 was unique to subgenogroup B4. T at nucleotide 51 and 75 was unique to subgenogroups B1∼B4.

EV71 isolates from Taiwan were found in subgenogroup B1 (in 1980, 1986), subgenogroup C2 (in 1998), subgenogroup B4 (in 1999–2003), and subgenogroup C4 (in 2004), respectively. The nucleotide differences between subgenogroups B1 and C2, C2 and B4 as well as B4 and C4 were 20%–26%, 19%–27%, and 18%–22%, respectively (Table II). However, the nucleotide difference between C4 and other subgenogroup were 9%–18%. The results suggested that subgenogroup C4 is a newly evolved lineage.

## Phylogenetic Analysis

The genetic relationship among 54 isolates worldwide was inferred by the neighbor-joining method based on the 207 nucleotides (744–950) of the VP4 gene in the GenBank. Three genogroups, A, B, C were deduced in the phylogenetic tree (Fig. 2). The prototype of EV71, BrCr, was the only strain in genogroup A. Genogroup B was divided further into four subgenogroups, B1–B4 (Figs. 2 and 3). Subgenogroup B1 consisted of isolates collected from Taiwan, Japan, Hungary, and Bulgaria in 1973–1986. Subgenogroup B2 included isolates obtained from USA in 1987. Subgenogroup B3 comprised of isolates from Sarawak, Singapore, and Australia in 1997–1999. Subgenogroup B4 included

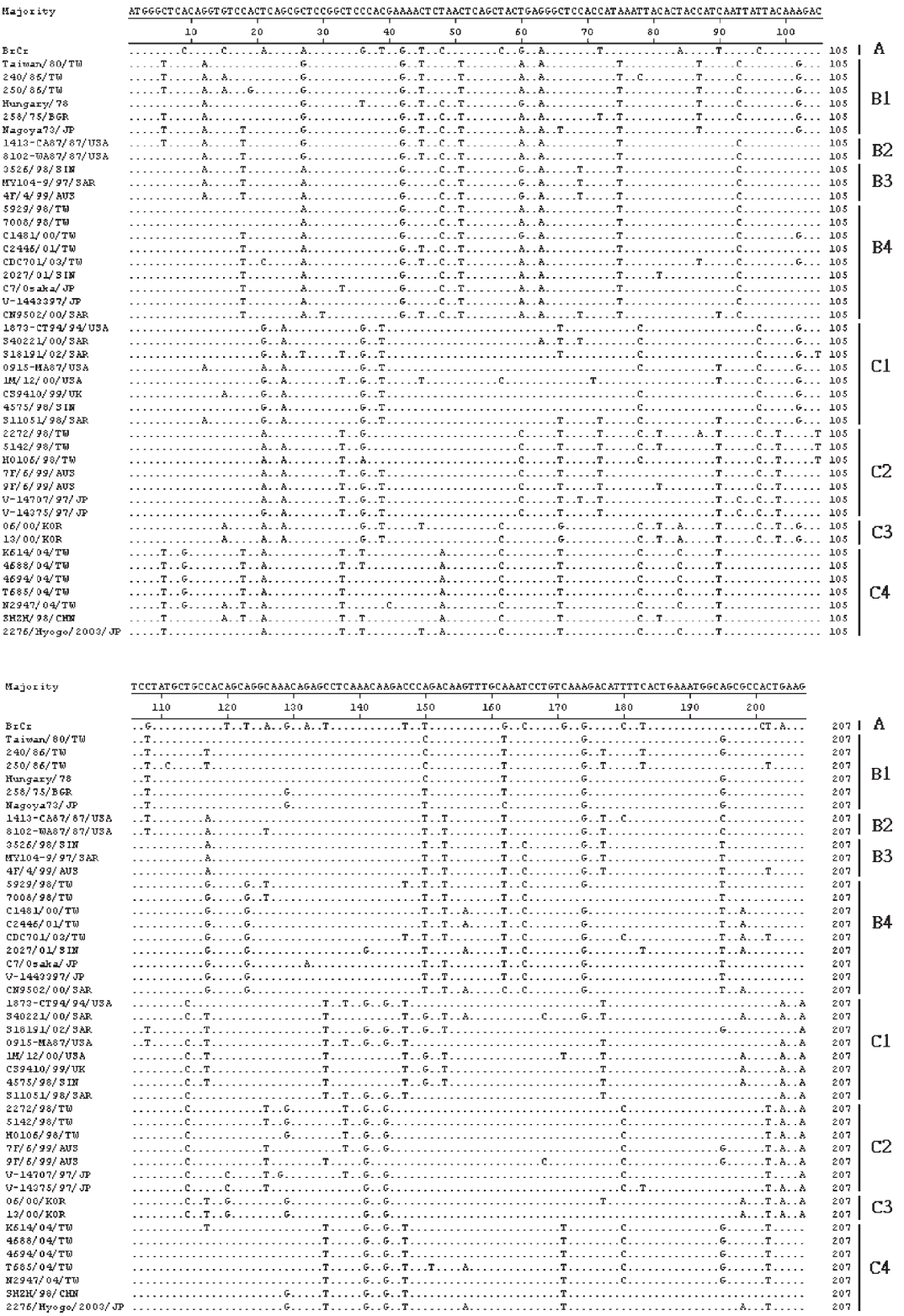


Fig. 1. The alignment of nucleotide sequences of 45 EV71 strains worldwide based on VP4 gene sequences.

strains isolated from Japan, Sarawak, Singapore, and Taiwan in 1997–2003. Genogroup C was also divided into four subgenogroups, C1–C4 (Figs. 2 and 4). Subgenogroup C1 included isolates obtained from USA, Japan, Singapore, and Sarawak in 1987–2002. Subgenogroup C2 consisted of isolates collected from Taiwan in 1998 first major outbreak, Japan in 1997, and Australia in 1999. Subgenogroup C3 comprised

TABLE II. Nucleotide Differences in Different Genogroups and Subgenogroups

| | A | B1 | B2 | B3 | B4 | C1 | C2 | C3 | C4 |
|---|---|---|---|---|---|---|---|---|---|
| A | 0 | | | | | | | | |
| B1 | 22–23 | 3–8 | | | | | | | |
| B2 | 21 | 5–8 | 2 | | | | | | |
| B3 | 20 | 8–11 | 4–5 | 0 | | | | | |
| B4 | 17–20 | 8–14 | 6–11 | 3–7 | 0–6 | | | | |
| C1 | 20–24 | 17–21 | 17–21 | 16–20 | 15–24 | 1–9 | | | |
| C2 | 19–23 | 20–26 | 21–25 | 21–25 | 19–27 | 9–19 | 0–6 | | |
| C3 | 20 | 21–23 | 24–26 | 24–25 | 23–26 | 9–14 | 8–12 | 1 | |
| C4 | 19–20 | 19–23 | 18–21 | 18–20 | 18–22 | 9–18 | 14–17 | 12–17 | 0–6 |

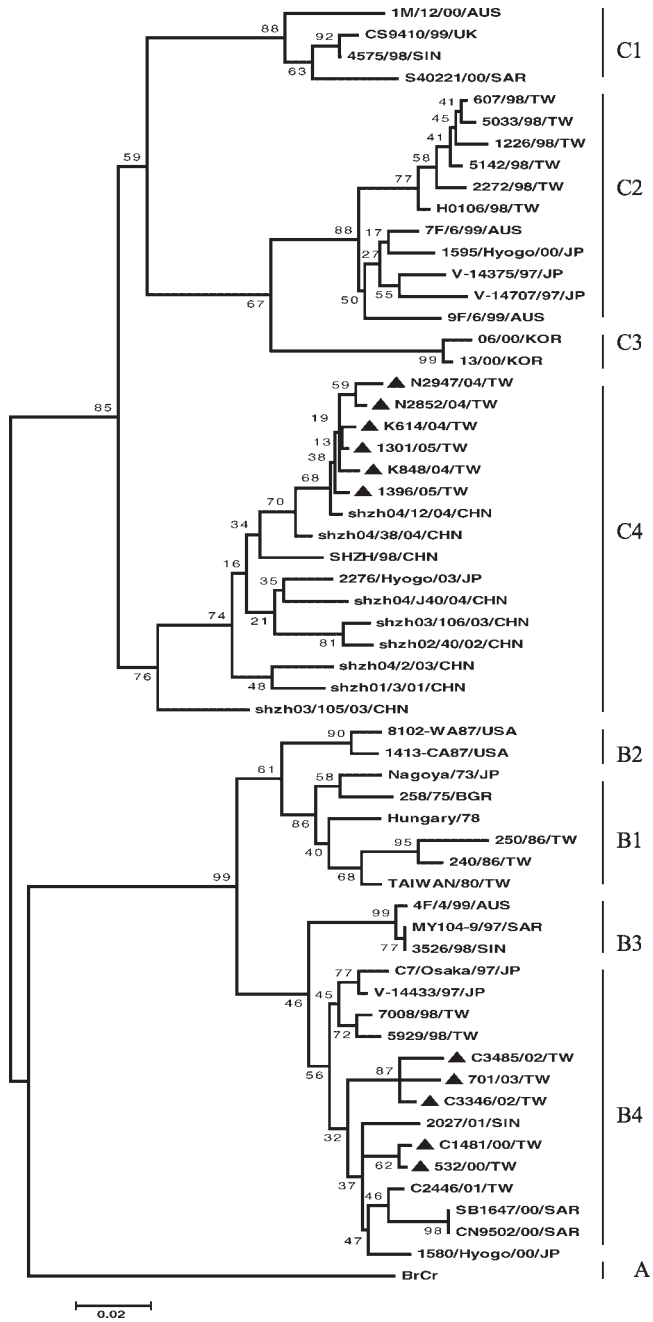


Fig. 2. Phylogenetic analysis of 59 EV71 strains worldwide based on VP4 gene sequence. The VP4 nucleotide sequence of the prototype BrCr strain was used as an outgroup. Bootstrap values (%) in 1,000 pseudoreplicates for major lineages are indicated at the branch nodes.

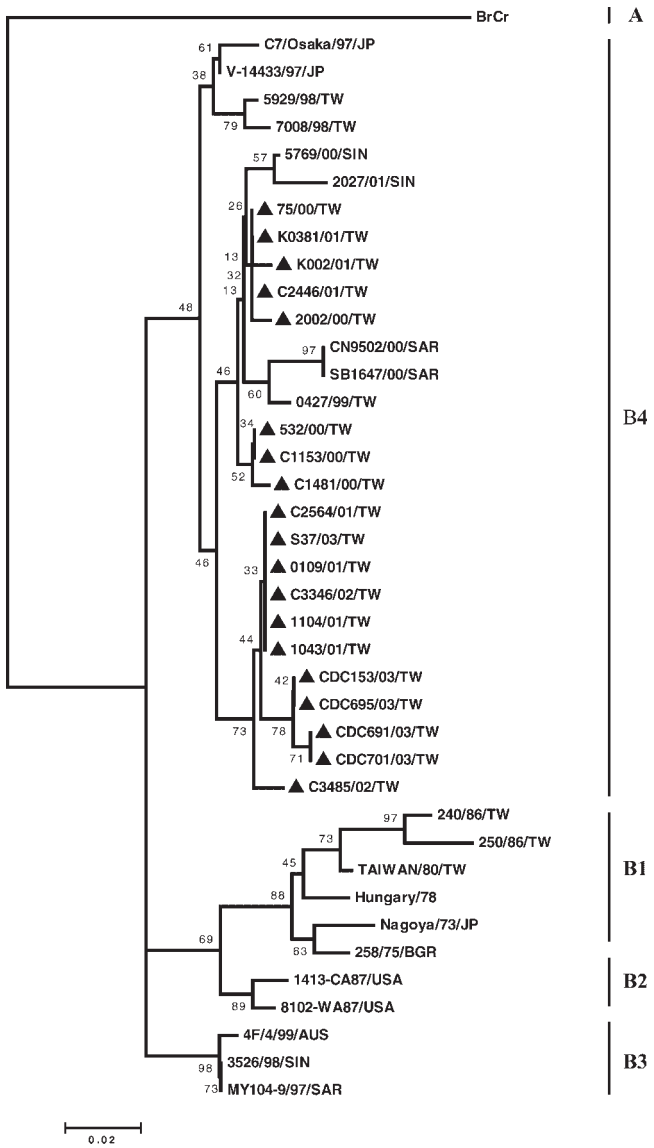


Fig. 3. Phylogenetic analysis of 40 EV71 strains of genogroup B worldwide based on VP4 gene sequence. The VP4 nucleotide sequence of the prototype BrCr strain was used as an outgroup. Bootstrap values (%) in 1,000 pseudoreplicates for major lineages are indicated at the branch nodes.

isolates obtained from Korea in 2000. Subgenogroup C4 consisted of isolates collected in China, Japan, and Taiwan during 1998–2004.

### Genogroup Changes Based on VP4 Gene Sequence Analysis

In Taiwan, HFMD cases caused by EV71 of subgenogroup B1 had been found in 1980 and 1986. In 1998 a severe outbreak of EV71 occurred in Taiwan, the virus detected was subgenogroups C2, which also caused outbreaks in Japan in 1997, and in Australia in 1999 [Cardosa et al., 2003]. Subgenogroup B4, which had co-circulated as a minor group in the 1998 outbreak, provoked a severe outbreak in 2000, 2001. Outbreaks caused by subgenogroup B4 were also reported from Sarawak in 2000 and Singapore in 2000–2002 [Cardosa et al., 2003]. In this study, it was found that subgenogroup of EV71 isolates obtained in Taiwan 2004–2005 had changed from subgenogroup B4 to subgenogroup C4 which had also been found in China and Japan in 1998, 2001–2004, and 2003–2004, respectively (Table III).

## DISCUSSION

Several outbreaks of EV71 have occurred in Taiwan since 1980. The most devastating one was the outbreak in 1998, which caused 78 deaths followed by outbreaks in 2000 and 2001, which caused 25 and 26 deaths, respectively [Wang et al., 2002; Lin et al., 2003]. Many research efforts were carried out in laboratories

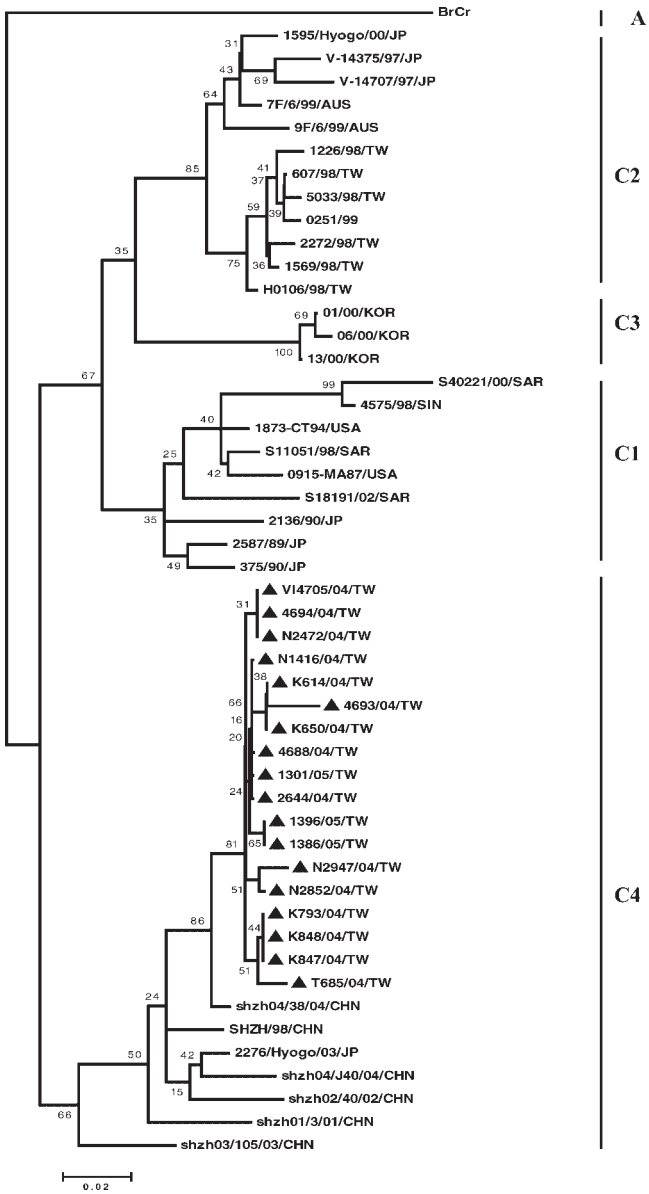


Fig. 4. Phylogenetic analysis of 50 EV71 strains of genogroup C worldwide based on VP4 gene sequence. The VP4 nucleotide sequence of the prototype BrCr strain was used as an outgroup. Bootstrap values (%) in 1,000 pseudoreplicates for major lineages are indicated at the branch nodes.

TABLE III. Chronological Change of EV71 Subgenogroups in West Pacific Region (Modified From Cardosa et al. [2003] With Permission)

| | 1980 | 1986 | 1997 | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Singapore | — | — | B3, B4 | B3,C1 | B3 | **B4** | B4 | C1,B4 | — | — | — |
| Sarawak | — | — | **B3** | C1 | No EV71 | **B4**, C1 | No EV71 | C1 | C1, **B5** | No EV71 | — |
| Thailand | — | — | — | — | — | — | — | C1 | — | — | — |
| Taiwan | **B1** | **B1** | — | **C2**[a],B4 | B4 | **B4** | B4 | B4 | B4 | C4 | C4 |
| Japan | — | — | B3, B4, C2 | — | — | — | — | B4,C2 | C4 | C4 | — |
| China | — | — | C3 | C4 | — | C4 | C4 | C4 | C4 | C4 | — |
| Korea | — | — | — | — | | **C3** | No EV71 | No EV71 | — | — | — |
| Perth | — | — | — | — | **B3**, **C2**[a] | C1 | No EV71 | No EV71 | — | — | — |

—, No data available. No EV71, no EV71 identified despite active surveillance.
Boldface typing indicates major subgenogroups causing large outbreaks.
[a]Severe neurological diseases were found in the outbreaks.

island-wide. The genetic analysis of the EV71 isolates revealed that genogroup B was found in 1980 and 1986 outbreaks, genogroup C was the major genogroup in 1998 outbreak. However, genogroup B coexisted in the outbreak as a minor group (1/10) which in turn became the major genogroup in 2000 and 2001 outbreaks [Shih et al., 2000; Wang et al., 2000, 2002; Chu et al., 2001]. As EV71 became an endemic causative agent that cause severe neurologic disease in addition to HFMD. A surveillance system was organized by the CDC, Taiwan. Similar surveillance systems were also established in Japan Malaysia, Hong Kong, Singapore, and Australia. Through the surveillance system, the change of EV71 genogroups in Taiwan was monitored.

An overview of the molecular epidemiology of EV71 from 1997 to 2002 was reported through phylogenetic analysis of the VP4 and/or VP1 genes of recent EV71 isolates [Cardosa et al., 2003]. Because of the similar clustering of recent EV71 strains in both the VP1 and VP4 gene based phylogenetic trees as well as the simple and convenient screening we chose the VP4 gene as the target region for the phylogenetic analysis of EV71 molecular epidemiology worldwide in this study [Shimizu et al., 2004]. According to the nomenclature proposed by Cardosa and Shimizu, our results of nucleotide alignment and phylogenetic analysis confirmed that the major genogroup of the EV71 in Taiwan had changed chronologically from subgenogroup B1 (in 1980, 1986) to subgenogroup C2 (in 1998), to subgenogroup B4 (in 1999–2003) and to subgenogroup C4, which had emerged in 2004. This is the first description concerning the emergence of subgenogroup C4 in Taiwan.

The EV71 outbreaks in the West Pacific Region (WPR) during recent years resulted in many fatalities due to severe neurological complications not only in Taiwan in 1998, 2000, and 2001 but also in Malaysia in 1997 [Wang et al., 2002; Cardosa et al., 2003; Herrero et al., 2003; Lin et al., 2003]. They were caused by subgenogroups C2 and B4 in Taiwan as well as subgenogroup B2 in Malaysia, respectively. In addition, subgenogroups B3 and C2 were found in the EV71 outbreaks in Australia in 1999 and subgenogroup B4 was found in Singapore EV71 large outbreak in 2000. Subgenogroup C3 was found in EV71 outbreak in Korea (Table III) [Cardosa et al., 2003]. Recently, EV71 infections have also been reported from Japan, Singapore, Australia, Korea although no fatal cases were reported [Shimizu et al., 2004].

Because of the convenience of the global traffic, the EV71 outbreak is not unique to the WPR. It occurred in California in 1969, Bulgaria in 1975, Hungary in 1978, and causing devastating results in Taiwan in 1998 and Malaysia in 1997. Serious CNS involvement was not common in the earlier outbreaks during 1969–1974. However, the disease spectrum of EV71 infection expanded from mild disease to neurological involvement and even death in the later outbreaks during 1975–2001. It has been reported that a large outbreak of EV71 began in February 2003 and 80% of the isolates belonged to subgenogroup C1 at the beginning of the outbreak, which was overtaken by subgenogroup B5 thereafter (personal communication with Dr. Cardosa). Subgenogroup C4 had emerged in 2004 in Taiwan after its appearance in China in 1998, 2000–2004 and in Japan in 2003–2004, respectively. This implies that subgenogroup C4 might be the next candidate that may provoke an EV71 outbreak in Taiwan. Thus, it is important to keep health workers alert on the possibility of the introduction of a new subgenogroup.

Intriguingly both the B and C genogroups evolved intermittently in recent years (Table III). For the activity of genogroup B in Taiwan and WPR based on VP1 and VP4 nucleotide sequences, strains of subgenogroup B4 have been circulating in the WPR at least since 1997, as these strains were isolated sporadically in Japan in 1997 and in Taiwan during 1998 outbreak as a minor group before its large outbreaks in 2000 in Singapore, Sarawak, and Taiwan [Cardosa et al., 2003]. EV71 strains of subgenogroup B4 continued to be isolated in Singapore during 2001–2002 and in Taiwan during 1999–2003. EV71 strains from Taiwan in 1998 and 1999–2003 evolved chronologically and formed distinct clusters in subgenogroup B4. The isolates from the subgenogroup B4 isolates in 1999–2003 were closely related to those from Sarawak and Singapore in 2000 and 2001, respectively while those from 1998 were closely related to those from Japan. In subgenogroup B1, isolates from Taiwan in 1980 and 1986 were closely related to those from Hungary in 1978. However, only HFMD cases were found in 1986 outbreak in southern Taiwan (unpublished data).

For the activity of genogroup C in Taiwan and the WPR based on both the VP1 and VP4 region, the major strains in Taiwan 1998 outbreak belonged to a distinct cluster in the subgenogroup C2, which was also found in Japan in 1997, 2002 and in Australia in 1999. Subgenogroup C3 appeared only in Korea. Subgenogroup C1 caused several outbreaks in Sarawak in 1998, 2000, and 2002–2004. In this study subgenogroup C4 in Taiwan was first reported based on VP4 sequence analysis. Our results showed that Taiwanese isolates in 2004–2005 formed a distinct cluster in subgenogroup C4 and were much closer to those from China in 1998 and 2000–2004 than those from Japan in 2003–2004. Furthermore, it was reported that most EV71 isolates from the 1998 epidemic belonged to genogroup C, while only 1/10 of the isolates were genogroup B [Wang et al., 2002]. And virtually all the isolates in genogroup C belonged to subgenogroup C2 while only one EV71 isolate from the 1998 epidemic belonged to subgenogroup C4 [Li et al., 2005]. This suggests that EV71 strains of subgenogroup C4 currently circulating in Taiwan and even in Japan may derived from Chinese strains. There are two possible explanations for this. First, EV71 ancestors of the subgenogroup C4 was imported into Taiwan in1998 and evolved thereafter. Second, the viruses were imported into Japan and

Taiwan in 2003 and 2004, respectively, after they had evolved into subgenogroup C4.

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## REFERENCES

Alexander JP, Baden L, Pallansch MA, Anderson LJ. 1994. Enterovirus 71 infection and neurologic disease—United States, 1977–1991. J Infect Dis 169:905–908.

Brown BA, Oberste MS, Alexander JP, Jr., Kenneth ML, Pallansch MA. 1999. Molecular epidemiology and evolution of enterovirus 71 strains isolated from 1970 to 1998. J Virol 73:9969–9975.

Cardosa MJ, Krishnan S, Tio PH, Perera D, Wong SC. 1999. Isolation of subgenus B adenovirus during a fatal outbreak of enterovirus 71-associated hand, foot, and mouth disease in Sibu, Sarawak. Lancet 354:987–991.

Cardosa MJ, Perera D, Brown BA, Cheon D, Chan HM, Chan KP, Cho H, McMinn P. 2003. Molecular epidemiology of human enterovirus 71 strains and recent outbreaks in the Asia-Pacific region: Comparative analysis of the VP1 and VP4 genes. Emerg Infect Dis 9:461–468.

Chan LG, Parashar UD, Lye MS, Ong FG, Zaki SR, Alexander JP, Ho KK, Han LL, Pallansch MA, Suleiman AB, Legathesan M, Anderson LJ. 2000. Deaths of children during an outbreak of hand, foot, and mouth disease in Sarawak, Malaysia: Clinical and pathological characteristics of the disease. For the Outbreak Study Group. Clin Infect Dis 31:678–783.

Chan KP, Goh KT, Chong CY, Teo ES, Lau G, Ling AE. 2003. Epidemic hand, foot and mouth disease caused by human enterovirus 71 Singapore. Emerg Infect Dis 9:78–85.

Chang LY, Huang YC, Lin TY. 1998. Fulminant neurogenic pulmonary oedema with hand, foot and mouth disease. Lancet 352:1391.

Chu PY, Lin KH, Hwang KP, Chou LC, Wang CF, Shih SR, Wang JR, Shimada Y, Ishiko H. 2001. Molecular epidemiology of enterovirus 71 in Taiwan. Arch Virol 146:589–600.

Chumakov M, Voroshilova, Shindarov ML, Lavrova I, Gracheva L, Koroleva G, Vasilenko S, Brodvarova I, Nikolova M, Gyurova S, Gacheva M, Mitov G, Ninov N, Tsylka E, Robinson I, Frolova M, Bashkirtsev V, Martiyanova L. Rodin V. 1979. Enterovirus 71 isolated from cases of epidemic poliomyelitis-like disease in Bulgaria. Arch Virol 60:329–340.

Gilbert GI, Dickson KE, Waters MJ, Kennett ML, Land SA, Sneddon M. 1988. Outbreak of enterovirus 71 infection in Victoria, Australia, with a high incidence of neurologic involvement. Pediatr Infec Dis J 7:484–488.

Herrero LJ, Lee CSM, Hurrelbrink RJ, Chua BH, Chua KB, McMinn PC. 2003. Molecular epidemiology of enterovirus 71 in peninsular Malaysia, 1997–2000. Arch Virol 148:1369–1385.

Ho M, Chen ER, Hsu KH, Twu SJ, Chen KT, Tsai SF, Wang JR, Shih SR. 1999. An epidemic of enterovirus 71 infection in Taiwan. Taiwan Enterovirus Epidemic Workimg Group. N Engl J Med 341:929–935.

Kitamura A, Narisawa T, Hayashi A, Ashihara Y, Ishiko H. 1997. Serotype determination of enteroviruses that cause hand-foot-mouth disease: Identification of enterovirus 71 and coxsackie virus A 16 from clinical specimens by using specific probe. J Jpn Assoc Infect Dis 71:715–723.

Li L, He Y, Yang H, Zhu J, Xu X, Dong J, Zhu Y, Jin Q. 2005. Genetic characteristics of human enterovirus 71 and coxsackievirus A16 circulating from 1999 to 2004 in Shenzhen, People's Republic of China. J Clin Microbiol 42:3835–3839.

Lin TY, Twu SJ, Ho MS, Chang LY, Lee CY. 2003. Enterovirus 71 outbreaks, Taiwan: Occurrence and recognition. Emerg Infect Dis 9:291–293.

Lum LC, Wong KY, Lam SK, Chua KB, Goh AY. 1998. Neurogenic pulmonary oedema and enterovirus 71 encephalomyelitis. Lancet 352:1391.

McMinn P, Lindsay k, Perera D, Chan HM, Chan KP, Cardosa MJ. 2001. Phylogenetic analysis of enterovirus 71 strains isolated during linked epidemics in Malaysia, Singapore, and Western Australia. J Virol 75:7732–7738.

Melnick JL. 1984. Enterovirus type 71 infections: A varied clinical pattern sometimes mimicking paralytic poliomyelitis. Rev Infect Dis 6:S387–S390.

Nagy G, Takatsy S, Kukan E, Mihaly I, Domok I. 1982. Virological diagnosis of enterovirus type 71 infections: Experiences gained during an epidemic of acute CNS diseases in Hungary in 1978. Arch Virol 71:217–227.

Samuda GM, Chang WK, Yeung CY, Tang PS. 1987. Monoplegia caused by enterovirus 71: An outbreak in Hong Kong. Pediatr Infect Dis J 6:206–208.

Schmidt NJ, Lennette EH, Ho HH. 1974. An apparently new enterovirus isolated from patients with disease of the central nervous system. J Infect Dis 1 29:304–309.

Shih SR, Ho MS, Lin KH, Wu SL, Chen YT, Wu CN, Lin TY, Chang / SNM> LY, Tsao KC, Ning HC, Chang PY, Jung SM, Hsueh C, Chang /SNM> KS. 2000. Genetic analysis of nterovirus71 isolated from fatal and non-fatal cases of hand, foot and mouth disease during an epidemic in Taiwan, 1998. Virus Res 68:127–136.

Shimizu H, Utama A, Yoshii K, Yoshida H, Yoneyama T, Sinniah M, Yusof MA, Okuno Y, Okabe N, Shih DR, Chen HY, Wang JR, Kao CL, Chang KS, Miyamura T, Hagiwara A. 1999. Enterovirus 71 from fatal and nonfatal cases of hand, foot and mouth disease epidemics in Malaysia, Japan and Taiwan in 1997 1998. Jpn J Infect Dis 52:12–15.

Shimizu H, Utama A, Onnimala N, Li C, Li-Bi Z, Yu-Jie M, Pongsuwanna Y, Miyamura T. 2004. Molecular epidemiology of enterovirus 71 infection in the Western Pacific Region. Pediatics Int 46:231–235.

Wang JR, Tsai HP, Chen PF, Lai YJ, Yan JJ, Kiang D, Lin KH, Liu CC, Su IJ. 2000. An outbreak of enterovirus 71 infection in Taiwan, 1998. II. Laboratory diagnosis and genetic analysis. J Clin Virol 17:91–99.

Wang JR, Tuan YC, Tsai HP, Yan JJ, Liu CC, Su IJ. 2002. Change of major genotype of enterovirus 71 in outbreaks of hand-foot-and-mouth disease in Taiwan between 1998 and 2000. J Clin Microbiol 40:10–15.