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An Epidemiological Analysis of Enterovirus 71: Taiwan, 1998-2004

Abstract

Enterovirus 71 (EV71) is one of the major causes of hand, foot, and mouth disease (HFMD). Sometimes a patient infected with EV71 may suffer from serious neurological manifestations including encephalitis and poliomyelitis-like paralysis. Recently isolation of EV71 and small-scale epidemics of disease caused by EV 71 have been continuing to be reported in the Western Pacific Region (WPR). Taiwan had a major outbreak of this virus in the summer of 1998, when a total of 405 suspected cases of EV71 infection with acute complications were reported. Young children and newborns compose the high-risk group of the infection and often the hardest hit victims of such outbreaks. In this study, we took clinical specimens from our stock that had been collected, isolated and identified by the Taiwan CDC and its contract laboratories as EV71 positive, analyzed them by a Reverse Transcription Polymerase Chain Reaction (RT-PCR), followed by a genomic sequencing process by amplifying the VP1 segments of the virus genome, and conducted a phylogenetic tree analysis. Our results concur with the theory that the same B

and C subgenotypes of EV71 have been co-circulating throughout Australia, Malaysia, Singapore, Japan, and Taiwan over the past decade. We also found that the major subgenotype causing the 1998 Taiwan epidemic was C2, while the following five years between 1999 and 2003, the prevailing infections were caused by B4 subgenotype instead. It appears that the trend changed to yet another subgenotype C4 in 2004, which proved to be quite similar to the EV71 strain SHZH03-China which was circulating on Mainland China in the previous year. There is a sequential homologous similarity of 97~99% among the various C4 isolates collected in Taiwan, and their average homologous similarity to SHZH03-China is 96.9%.

Introduction

Enterovirus 71 (EV71) was first discovered in 1969 in California, USA through isolation from a stool sample of an infant suffering from encephalitis. After that, further isolates were detected in other parts of the United States, in Bulgaria, Japan, Hong Kong, Malaysia, Taiwan, and Australia [1-6]; they caused epidemics of various degrees. EV71 and Coxsackie virus (CA16) are very much alike and both cause hand, foot, and mouth disease HFMD. However, EV71 is certainly associated with the further development of acute neurologic manifestations, including poliomyelitis-like paralysis, encephalitis, and aseptic meningitis. Though EV71 epidemics have occurred in the past in North America, Asia, and Australia, few cases developed severe neurologic manifestations, and even in those cases, the mortality rate was not alarming [7]. The major outbreak which occurred in Taiwan in the summer of 1998, however, was exceptional. A total of 405 infants and young children were reported to be HFMD patients, and many of them developed severe neurological symptoms and

other complications, and eventually 78 died. [8-10].

Enterovirus is a plus-sense, single stranded RNA virus, which belongs to the family of *Picornaviridae*. The size of this particular virus is quite small with a diameter of only 20-30nm. It is non-enveloped, and with an icosahedron appearance [11, 12]. The genome of EV71 has a total length of approximately 7.5kb. From its 5' end to the 3' end, it is arranged in the following order: 5'-NCR, P0 (VP4, VP2), VP3, VP1, 2A (protease), 3D (RNA polymerase), and the 3 end poly-A. The 5'-NCR is composed of about 750 nucleotides, and is a highly conservative region in terms of genetic evolution. Segment VP4 is known as closely related to the stability of the viral RNA, while VP1-is somewhat relevant to the combinations of the virus to the host cell receptors as well as to the antibodies. Of the latter, the VP1 is not only the major interacting region responsible for antibody neutralization, but also one of the more variable segments in the entire genetic sequence, in addition it is where we can differentiate the enterovirus from other serotypes [13]. Currently, for the purpose of EV71 detection or its genotype differentiation, people often make use of the 5'-untranslated region (UTR) [14, 15], VP1, and VP4 regions [7, 11, 14,16]. In this study, we employed RT-PCR and gene sequencing technology to analyze the VP1 region of the Taiwan EV71, in order to get a picture of the genetic diversity and identity of the prevalent subgenotypes in Taiwan over the time period from 1998~2004.

Materials and Methods

*Sources of the specimens analyzed in this study

They are either clinical specimens from patients with suspected EV71 infection originally sent directly to the Center of Research & Diagnostics, Taiwan

CDC for routine examination, or isolates of EV71 gathered by 12 contract viral infection laboratories across Taiwan. All 2073 specimens examined in this study were collected between 1998 and 2004.

*Virus culture

200ul of a pretreated specimen (which was derived from an anal swab, a throat swab, or a stool sample) was inoculated into a thriving culture of RD, HEp-2C, MRC5, or Vero cell line. The culture was then retained inside a 36℃ incubator and maintained with 5% CO_2 for 14 days under observation. When obvious cytopathic effect (CPE) occurred in the cell culture, we proceeded with centrifugation at 5000xg to collect the supernatant solution, which was then used for subsequent molecular bioassays and enterovirus genomic sequencing. The leftover cells were fixed on a glass plate and stained for Indirect Immunofluorescence Assay (IFA) to identify the virus type.

*Identification by IFA

Having fixed the cytopathic effect-bearing cells on the glass plate, we treated the plate with a solution of commercially available EV71 mouse monoclonal antibody (CHEMICON Inc, CA, USA; Catalog No. 3321, 3323, 3324). The plate was then incubated, rinsed, and allowed to react with a FITC-labeled anti-mouse monoclonal antibody. After further incubation and more rinsing, the plate was examined under a fluorescence microscope. If infection was present, the cytoplasm of the cell would emit an apple-green fluorescence, which indicates the case to be positive. Otherwise, if negative, the cytoplasm would appear reddish in color.

*RNA extraction

We used a commercial viral nucleic acid purification reagent kit (QIAGEN Inc, CA, USA) to purify our RNA samples. The procedure is as follows: first

140ul of the specimen was measured out and added into 560ul of the AVL buffer provided. The mixture was allowed to react at room temperature for 10min before adding to it 560ul absolute alcohol. After mixing well, the solution was made to pass through a QIAmp spin column, followed by washing of the column twice with the AW buffer. Eventually, the extracted RNA was dissolved out by passing some pure water (RNase free) through the column. The prepared viral RNA samples could then be used in the Reverse Transcription Polymerase Chain Reaction (RT-PCR).

* RT-PCR [11,15]

(1) Reverse Transcription (RT)

5ul viral RNA, was added into an RT reaction mixture, which consisted of 75mM KCl, 50mM Tris-HCl, 3mM $MgCl_2$, 10mM DTT, 0.5mM dNTP mixture, RNasin (38U/ul), and 50pmoles antisense primer162 [15] (see Table 2). After the mixture was placed at 70℃ for 10min, a solution of 100 units of MuLV-reverse transcriptase was added, and that was followed by a 90-min incubation period at 37℃.

(2) Polymerase Chain Reaction (PCR)

PCR proceeded using the cDNA obtained from the aforementioned RT: to the cDNA was added 50mM KCl, 10mM Tris-HCl, 1.5mM $MgCl_2$, 0.1% Triton-X 100, 1mM dNTP mixture, and a primer mixture of 159 and 162 [11, 17, 18], which consists of 50pmoles of each, plus 5 units of Taq polymerase (Promega Inc, WI, USA). First the mixture was maintained at 94℃ for 3 min for denaturation. Following that, the temperature cycle program was set at 94℃ for 1min, 48℃ for 1min, and 72℃ for 2min. The cycle was repeated 35 times, and in the final stage the mixture reacted at 72℃ for 15min before stopping.

*Sequencing analysis

The sequencing experiments were carried out on an ABI 3730 sequencer along with a commercial fluorescence nucleic acid sequencing reagent kit of the same brand, i.e. the ABI PRISM(™) BigDye(™) Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), for labeling the nucleic acid product to be analyzed. Since the purity of the nucleic acid sample decisively affects the quality of the sequencing reaction outcome, it is recommended to use only highly purified ($OD_{260/280}$ >1.8) PCR product as the sequencing template. The appropriate amount of DNA needed for analysis should be somewhere between 200~500ng, or in case the sample is double-stranded (e.g. a plasmid), between 50~100ng if it is single-stranded. However, only 30~90ng is sufficient for most PCR products. The standard procedure was as follows: the appropriate amount of the DNA template was added to a combination of 3ul premix (including a Tris-HCl buffer, pH 9.0, $MgCl_2$, a dNTP mix, labeled A-dye terminator, C-dye terminator, G-dye terminator, T-dye terminator, and AmpliTaq DNA Polymerase FS with thermally stable pyrophosphatase), 3.2~5.0pmole DNA primer pair 159/162, and a suitable amount of water to make the total volume of the reaction solution 10ul. The solution was covered with a layer of waxy oil and placed in the micro centrifuge tube holding the reaction product in the PCR machine already preheated to 94℃. The conditions of each cycle had been programmed at 94℃ for 30sec, 55℃ for 15sec, and 60℃ for 4min, the number of repeats set at 25; the reaction was terminated at 4℃ before proceeding with the electrophoresis process to determine the sequence.

*Phylogenetic tree analysis

The numerical codes of the local isolates employed in this study are: 2004496~20041055 for the year 2004, 2003-0695~2003-6720 for the year 2003, 2002-042~2002-4348 for the year 2002, 2001-336~2001-522 for the year 2001,

99195~99214 for the year 1999, and 1341 and 980200~981321 for the year 1998. Whereas the reference strains we used are N5101-TW-98, TW-1743-982003-SHZH03-china, HK1159, 2381MA, EV71BrCr, Malaysia, EV71MS, 0627-MAA00~0815MAA00, S1971SAR03~S19741SAR03, 0128-MAA97~0889-MAA97, 7629PA87~7631PA87, 1413CA87, 4826CT83, 3984OH82, 4644AR83, 2259CA82, 1011ND79, 2234NY77, 2229NY76~2238NY77, 2604AUS74~2610AUS74, KOR-EV71-02~07, 013-KOR-00, 2132VA95, 2037MD95, 2007CT94, 2006CT94, 0756MAA97, S19691SAR03MA, CN30014SAR03MA, 2261CA91, 2583CAN91, 9323TX89, 0915~0916MA87, 2262CA92, 9718TX89, 1919NM94, 0390TX90, 2641~2644AUS95, 2355OK97, and 2286TX97. The analysis was conducted on a computer installed with software called Molecular Evolutionary Genetics Analysis (MEGA) version 2.1. The adopted calculating methods were “Neighbor-joining” and “Kimura 2 parameter” with a bootstrap of 1,000 repeats to obtain the phylogenetic treeanalysis results.

Note: The reference sequences of EV71 cited in this study are selected from some of the published papers [14-16, 19, 20].

Results

Briefly, what we did in this study was that after a purifying pretreatment we extracted the viral RNA from the specimen. Then we used a specific EV71 primer pair 159/162 to amplify a genomic segment at the VPI region by means of RT-PCR. Following that, using agarose gel electrophoresis and EtBr staining analysis we could witness and isolate a 485bp genomic segment (Figure 1). After further purification, this DNA segment was put on an ABI 3730 sequencer to determine its sequence, which was then compared through BLAST comparison

analysis available at the NCBI website to ascertain whether the sample was indeed EV71 or another, but similar enterovirus strain. Finally, we took all those EV71 genomic sequences obtained in out laboratory, and those of reference strains of various EV71 subgenotypes stored in our Gene Bank at Taiwan CDC, and compared side-by-side a selected length of 414bp in their sequences by means of a MEGA 2.1 version software to perform a phylogenetic tree analysis.

The results indicated that our phylogenetic tree is comprised of two major genotypes, Type B and Type C, which are obviously quite different from the original Type A genotype to which the prototype BrCr belongs. According to Betty A. Brown and others, through VPI nucleic acid sequential analysis, EV71 can be divided into three genotypes, A, B, and C. The BrCr Prototype is a Type A strain; Type B appeared in the 1970s and 1980s, while Type C did not appear till the mid-1980s. The current situation is that all the recently isolated EV71s throughout the world are exclusively Type B and Type C. However, when comparing differences in their genomic sequences and homology of the phylogenetic tree established, one can make further classification by the genotype. The research of B. A. Brown et al [11], Mc Minn et al [20], and Cardosa et al [7], uncovered a few subgenotypes within both genotypes B and C. Namely, Type B can be divided into four subgenotypes, B1, B2, B3, and B4, while Type C can be divided into C1, C2, C3, and C4 as well (see Figure 2). And based on the definition established by B. A. Brown et al [11] in their genomic sequencing and phylogenetic tree research, it is now universally recognized that within the same genotype the total variation between two strains is not supposed to exceed 12%, while strains belonging to two different genotypes may display a variation in the range of 16.5~19.7%. Any two subgenotypes of Type B EV71 may differ from each other by a variation of somewhere within a range of 6~11% in their DNA

compositions, whereas such variation between two Type C viruses may be about 6~10%.

In this study, through comparison of their locations on the phylogenetic tree and nucleic acid variations, not only did we identify those Type B isolates, but also divided them into the four subgenotypes, B1~B4, by definition (Figure 3). One of our findings was that the most prevalent EV71 in 1999~2003 was B4 subgenotype, and in 1999 alone, EV71 isolates formed a small cluster, which is rather close to the Malaysian reference strains isolated in 1999 and 2000 (0627MAA99~0778MAA00). The homology between the two groups is about 96~97%. However, the enterovirus isolates collected in 2002~2003 in Taiwan are quite similar to one another, and together they formed a separate cluster among themselves except one isolate, 2002-119, which is clustered with 0627-MAA99 instead and the two have a homology of 99%. Furthermore, we identified two EV71 isolates, 8623VNR3 and TW/1743/98, as being B1 subgenotype. The former is a 1986 EV71 isolate and identical with a strain called TW/253/86(AF116805) registered by Chang Gung University, and the latter is another EV71 isolated by Chang Gung University in 1998. The similarity between the two turned out to be 94%.

As for the Type C isolates involved, we identified them and also classified them into the four subgenotypes, C1~C4, by definition (Figure 4). During the great 1998 enterovirus epidemic in Taiwan, the most prevalent strain happened to be EV71 C2 subgenotype, but this particular C2 subgenotype is not exactly the same as that isolated earlier in Australia and North America, as they appear in two separate small clusters on the phylogenetic tree. The homology between the two is 96~98%. In that particular year, a small part of the isolates were identified as Type B. For instance, clinical strain 981263 and isolate N5101-TW-98 by

Cheng Kung University are B4 subgenotype. According to the research done by Professors S. R. Shih and J. R. Wang, the major EV71 circulating on Taiwan in 1998 was Type C with just a minor portion being of Type B [14, 15, 21]. However, it is worth noting that among 2004 EV71 isolates, the majority are C4 subgenotype virus, which is quite similar to the one circulating in Mainland China in the previous year. Among the C4 subgenotype virus strains isolated in Taiwan during its prevalence period starting from the end of 2003 through the entire year 2004, the VPI nucleic acid similarity remained at 97~99%.

Pertaining to sequential homologous similarity, from what we have analyzed in this study, the predominant subgenotypes occurring in Taiwan over the past six years are B1, B4, C2, and C4. The homology range percentage among those B1 isolates examined was 92-98%; among the B4 ones it was 95-99%, the C2 ones 98-99%, and the C4 ones 94-99%. Comparatively, the range of homology similarity between Type A, and the said four subgenotypes is only 81~86%. Of the latter four, C2 and C4 are a bit more similar to Type A than the rest, and the average homology similarity between them is 84%. Comparing the two groups of Type B and Type C isolates, we found the VPI sequential variation between the two lay between 15~19%. Yet the homology similarity among the subgenotypes in the Type B group was in the range of 88~96%, and 89-94% in the Type C group. On the other hand, the homology similarity between EV71 C4 and C3 subgenotypes, which were prevalent in Taiwan in the year 2004, were somewhat higher with an average value of 90.9%, and those Type C subgenotypes are about 15~17% different from the Type B subgenotypes isolated in the same year (Table 1).

Discussion

In 1989, the Taiwan Department of Health (Taiwan DOH) initiated the establishment of a sentinel surveillance system for disease outbreaks in Taiwan [9, 10]. Through a joint effort in examination and detection performed by medical specialists, health workers, and clinical laboratory technicians in the established system, many enterovirus infection cases have since been identified. In the year 1998 alone, the total case number of identified HFMD and herpangina patients was 129,106, among which 405 cases were diagnosed as severe enterovirus infections and 78 of them eventually died of the disease [9, 15]. Some experts deduced that there might be two reasons for such a large epidemic outbreak in that year: 1) the mutation of the virus itself, i.e. a certain single mutation or a combination of point mutations resulting in a sudden increase of virulence, or the introduction of some exotic strains. 2) host factors, which simply means an increase in susceptible population and individual genetic susceptibility [9].

In this study we attempted to analyze the problem from the standpoint of viral gene variations and carried out a general survey on all EV71 isolates collected between 1998 and 2004 in Taiwan by Taiwan CDC's own virology laboratory and contract laboratories across the country. Through a complicated phylogenetic tree analysis, we found the prevalent EV71 strains circulating in Taiwan during the time period investigated were classified into the B1, B4, C2, and C4, four subgenotypes. The villain responsible for the great 1998 enterovirus epidemic in Taiwan, that caused many young children to suffer from HFMD, severe acute neurologic symptoms and even death, turned out to be EV71 C2 subgenotype. In addition, we also found a few isolates belonging to B1 and B4 subgenotypes.

In 1997, just one year before the severe epidemic outbreak in Taiwan, a

major enterovirus epidemic occurred in Malaysia, one of our Western Pacific region neighbors. However, the major causes of that particular epidemic turned out to be EV71 subgenotypes B3 and B4, while some C1 and C2 showed up on a much smaller scale; this is likely related to the fact that some young Malaysian children suffered from severe neurologic manifestations and death. Malaysia had another major outbreak in 2000 with a prevalent, gradual shift of EV71 subgenotypes to B4 and C1. In addition, since 1997 the B4 subgenotype EV71 has slowly spread from Malaysia to Singapore, Sarawak, and Taiwan [13]. Initially when certain experts viewed the fact that right after the great enterovirus epidemic in 1997 Malaysia, and Taiwan's own epidemic began in March 1998, they seriously doubted the two might be closely related. In this study we were finally able to clear up these doubts and demonstrated through subgenotype phylogenetic tree analysis that the two epidemics had quite different prevalent strains. Although two isolates of EV71 subgenotype C2, 03907-MAA-97 and 03750-MAA-97, surfaced in the 1997 Malaysia epidemic [13], we have found through phylogenetic analysis that the strains are quite different from those circulating in 1998 in Taiwan, and the VPI gene similarity range between the two groups is 96~97%, which makes them appear in separate small clusters on our phylogenetic tree.

By the same token, based on a 5'-NCR region phylogenetic tree analysis, Professor S. R. Shih and his team at Chang Gung University also concluded that the prevalent EV71 strains in 1998 Taiwan were different in genotype from those circulating in 1997 Malaysia [14]. However, our virology laboratory's findings in recent years indicate that as far as the VPI region genomic sequence is concerned, there are no obvious differences between the EV71 isolated from specimens of enterovirus patients with severe disease and those of cases with non

life-threatening symptoms. Professor Shih's group of Chang Gung University did applied themselves to determine the entire length of two of their EV71 isolates; i.e. TW/2272/98 which came from a patient who died, and TW/2086/98 from a patient with no serious disease manifestations [14]. Comparing the two detailed sequences, they found the nucleic acid sequential similarity stayed in the range of 97-100% in the 5'-NCR, VP1~VP4, 2A, 2B, 2C, 3A, 3B, and 3D regions but with two exceptions, namely the similarity dropped to 90-91% in the 3C and the 3-UTR regions. Another research team of the National Cheng Kung University took a different approach in their study of the same question by establishing an animal model suitable for enterovirus infections [23], in which they induced infection in ICR young mice through oral ingestion of EV71 and thus isolated a more virulent EV71 strain, then compared its nucleic acid sequence with a less virulent strain. What they found were 4 mutated points in the 5'-UTR region, 3 points in the VP2 region, and 8 points in the 2C region. They reasoned that since the 5'-UTR region has a sector called internal ribosome entry site (IRES), whose secondary structure is thought somehow able to regulate the duplicating speed of enterovirus inside cells, it would thus affect the virulence of the virus [23-24].

On the other hand, as regards possible host factors that some researchers turned their attention to, we find two things appearing to be essential, and they are herd immunity and susceptibility. There is evidence that the production of herd protective antibody is very much related to the outbreak of large scale EV71 epidemics. Research done by L. Y. Chang et al [27] indicates that there was a correlation between the pre-epidemic antibody positive seroprevalence rates and the mortality and severity of the disease during the enterovirus epidemic. In a separate study [28], C. Y. Lu et al (under the supervision of Dr. L. M. Huang) at

National Taiwan University Hospital analyzed the EV71 antibody seroprevalence in infants and young children over the past decade, and they found that it was 7~11% in 1994 but gradually dropped to 3~4% during 1994~1997 [9]. We can see from the antibody data and actual records that the epidemic level of EV71 was quite low in 1995~1997, which might have resulted in the lack of antigen stimulation to the herd immunity of infants and young children; this situation was thought to be at least one major cause of increased host susceptibility that made the great 1998 outbreak inevitable. Still other researchers studied the problem from the standpoint of cytokines in the immune system. According to a study done by T. Y. Lin et al [25] at National Cheng Kung University Hospital, whenever a severe EV71 case developed encephalitis and pulmonary edema, it was evident that the patient's blood level of cytokines IL-1β, IL-6, and TNF-α would be much higher than normal. In addition, during the first two days of infection, the IL-6 level in CSF also was apparently elevated. Therefore, if patients produce a combination of CNS and systemic inflammatory reactions, this would in turn induce a EV71-related functional cardio-respiratory failure. Yet another study by S. M. Wang et al [26] at the National Health Institute also noted that the complications of encephalitis and pulmonary edema arising during EV71 infection would result in higher disease mortality. They also showed that the severe acute case would develop leukocytosis and thrombocytosis phenomena, obvious increases in plasma IL-10, IL13, and INF-γ, but the number of lymphocytes $CD4^+$, $CD8^+$, and natural killer cell (NK cells) in the peripheral circulation would drop significantly. Therefore, in order to better understand why we had such a large scale enterovirus epidemic in 1998 along with so many severe cases, besides considering the above-mentioned factors for discussion, we believe more studies from various perspectives and more retrospective

investigations are needed.

Before 1998, Taiwan had no laboratory diagnostic framework specific for enterovirus surveillance. Back in 1994, Taiwan launched a new surveillance system for acute flaccid paralysis (AFP), whose purpose was to determine whether cases with such a symptom were caused by poliovirus; poliovirus happens to be one kind of enterovirus. At that stage, all enterovirus infections proven not to have been caused by poliovirus were not further classified and just lumped together as non-polio enterovirus (NPEV). Because there were almost no statistics or records regarding the state of enterovirus infection in Taiwan before the great 1998 epidemic, the National Institute of Preventive Medicine (NIPM), one of the three organizations that later merged into the current Taiwan CDC, tried to search for the presence of EV71 isolates among the NPEV category of the stock specimens of the AFP surveillance system, but failed to find any. In the meantime, our laboratory also sent the genomic sequencing data to the U.S. CDC for confirmation and further information. The opinion of the U.S. CDC was that the 1998 Taiwan EV71 was a unique type and there was no way to tell from where it originated.

Therefore, its source remained unknown. It might have originated from some local EV71 strain, which through an evolutionary process reached a certain level and took advantage of the lack of protective antibodies in most infants and young children during that time period, resulting in the great outbreak. Or it might have been an exotic virulent strain brought over directly from one of our neighboring countries, such as Mainland China. There was just no relevant data available to make a comparative analysis to prove or disprove the above two possibilities. After the 1998 epidemic, however, the NIPM and then the new Taiwan CDC have gradually joined forces with 12 contract laboratories across

Taiwan in a concerted effort to monitor the local serotype-changing trends in enterovirus, and have established a complete database. That is the reason that we do know the course of the viral variation in the following years; but we still know very little about what influences these gene variations might have on the phenotype such as neutralization antibody effects. More importantly, we have no idea in what direction they are going to evolve in the future, and what level or critical point in such evolution would result in another large-scale epidemic. And it seems that any new predictive patterns, variables, and parameters would only complicate the situation even more and make the problem harder to solve.

From the perspective of EV71 evolution analysis, at the moment there are different schools of experts using VP1, VP4, VP4-VP2 junction, or 5'-UTR region to perform evolution analysis [6, 7, 11, 14, 15]. As you can see, we chose to use the VP1 region in this study for our subgenotype analysis, since VP1 is the genomic section responsible for the most outer layer of enterovirus capsid protein, and where the targets of immune system reactions and antibody neutralization locate. According to a report by B. A. Brown et al published in 1999 [11], through an evolution analysis employed VP1, they managed to divide EV71 into three genotypes A, B, and C, with further division of subgenotypes B1~B2 and C1~C2. They found that the differences between Type B and Type C were in the range of 15.5~18.7%. Such differences within the B1 subgenotype were no more than 9.5%, while the difference between B1 and B2 was in the range of 6.9~11.1%. By the same token, the differences within C1 and C2, were 1~6.3% and 0.7~1.1%, respectively, and those between C1 and C2 were in the range of 6.1~10.1%.

In 2001, P. McMinn et al [20] reported their results of a study on the prevalent EV71 in Malaysia, Singapore, and Western Australia. Through the

study they discovered two new subgenotypes B3 and B4. The homologous similarity within the B3 isolates were 98-99% and that of the B4 isolates were 96-99%. Then a paper by M. J. Cardosa et al was published in 2003 [7] regarding a molecular epidemiology study on EV71 in Asia-Pacific Region. These authors discovered a new subgenotype C3 through examining sequential evolution of some 2000 EV71 isolates (KOR-EV71-05, KOR-EV71-06, etc.) collected in Korea. The next year (2004), H. Shimizu et al published a paper [16] about a molecular epidemiology study on EV71 in the Western Pacific Region. They noted that the prevalent EV71 strains in this region happened to be Type B and Type C. They also surveyed some EV71 isolates collected in the 2000 Shanghai area such as F1-CHN-00, H25-CHN-00, etc. Through the difficult process of sequence comparison and phylogenetic tree analysis, they found another new subgenotype C4. In our laboratory, we performed an analysis of some of the EV71 isolates collected in 2004 Taiwan by comparing their sequences with those available on the NCBI website and found that they were quite similar to a 2003 Mainland China isolate named SHZH03-China. Their homologous similarity was within the range of 96~97%. This particular strain is a C4 according to a phylogenetic analysis and its sequence differs from those of some 2000 Shanghai isolates by 4~6%.

According to our own analysis of all Taiwan local EV71 isolates collected in 1998~2004, we now know that the predominant circulating strains in 1998 belong predominantly to the C2 subgenotype with only a few B1 and B4 exceptions, whereas the most prevalent subgenotype among the isolates collected in 1999~2003 is B4, but it changed suddenly into C4 in the year 2004. In our homologous analysis, we found that the average homologous similarity within each subgenotype of B1, B4, C2, and C4 was 94.9%, 96.7%, 99.1%, and 96.5%,

respectively. Furthermore, the difference between the two subgenotypes B4 and C4 is 15~16% and their homologous similarity averages 84.2%. However, since mainland Chinese researchers identified those EV71 isolates collected in the Shanghai area in 2000 as C4 subgenotype [16], our own record shows there are two C4 isolates (2001-336 and 2001-522) which emerged in Taiwan one year later, in the same small cluster with F1-CHN-00 and H25-CHN-00. Later, again in 2004, some Taiwan enterovirus isolates were discovered to be in a small cluster with the 2003 Mainland China EV71 isolate SHZH03-China.

One thing worth noticing is that a C4-subgenotype isolate with the serial number of 1341 on the phylogenetic tree was collected and isolated in 1998 by our virology laboratory. Since it was found to differ from the then predominant prevalent strains at the gene sequence level, it was named as a C minor genotype. This fact tells us that back in 1998 the C4-subgenotype was already in existence, but we have no means of knowing whether the appearance of this Taiwan C4 is an earlier strain than that of its mainland China counterparts or not. One reason is that we do not have access to information from mainland China EV71 about isolates collected before 2000; so we are left solely with comparison through the BLAST method available on the Internet and phylogenetic tree analysis to appreciate that our strains are closet to the mainland China C4 subgenotype. Furthermore, in order to prevent the EV71 C4 genotype from returning to cause future large scale epidemics and to understand the protective capability of herd immunity against the antibodies of this C4 subgenotype, our laboratory has been taking paired sera from all 2002 and 2003 suspected severe enterovirus cases, and serum samples of 1998 EV71 C2 subgenotype, 2002 EV71 B4 subgenotype, and 2004 C4 subgenotype, and their antibody-neutralizing titers determined. Our results reveal that people with a EV71 infection history possess serum antibodies

capable of protectively neutralizing invading C4-subgenotype EV71 [22]. From the standpoint of herd immunity, it is possible the reason that although the subgenotype of the virus does change from time to time, in most cases it does not create major outbreaks. Other factors which may also contribute to the prevention of future major epidemics are successful enterovirus disease prevention strategies and well promoted public health education on the relevant causes of the disease.

So what is the benefit of this sort of study? The answer is that knowledge gathered in the past concerning EV71 subgenotype evolutional epidemiology analysis will provide essential references for the future development of EV71 vaccines and effective antiviral drugs. It also provides important information for disease prevention policy making and clinical treatment. Furthermore, the Taiwan CDC and the twelve contract laboratories have formed an enterovirus surveillance network, which is capable of and effective in identifying the serotype of locally emerging enterovirus strains as well as the evolutionary course of those subgenotypes within the same serotype.

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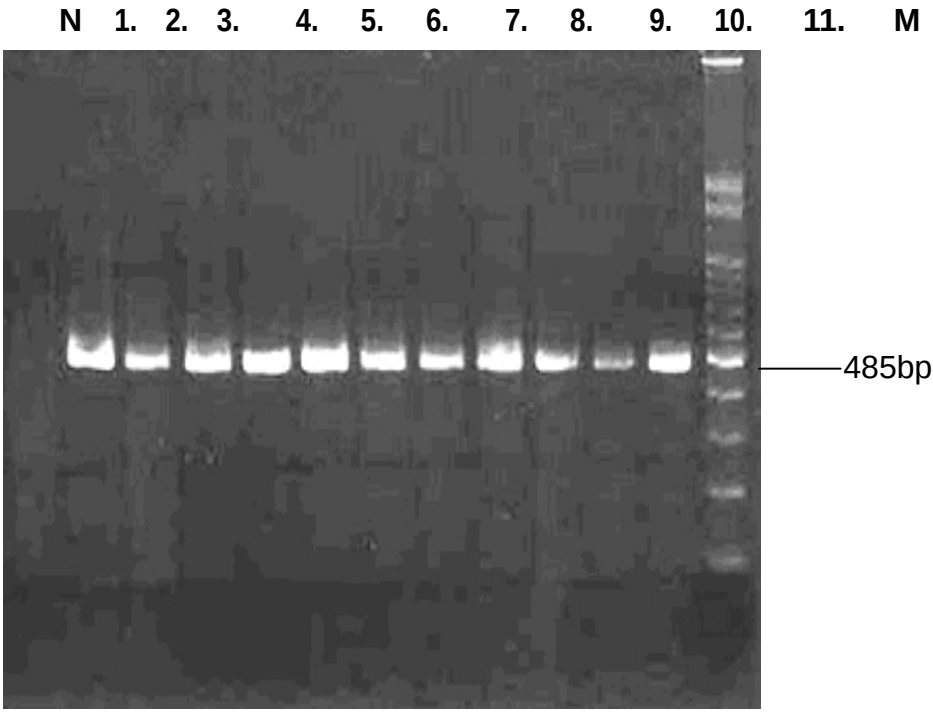
Figure 1. A diagram of agarose gel electrophoretic analysis of the product after applying primer pair 159/162 to amplify the VP1 region



In above diagram, N is a negative control, M is a 100bp ladder marker, 1~11 are clinical EV71 isolates. The size of the amplified VPI segment after applying primer pair 159/162 is about 485bp.

Figure 2. Phylogenetic tree of EV71 subgenotypes under genotypes B and C

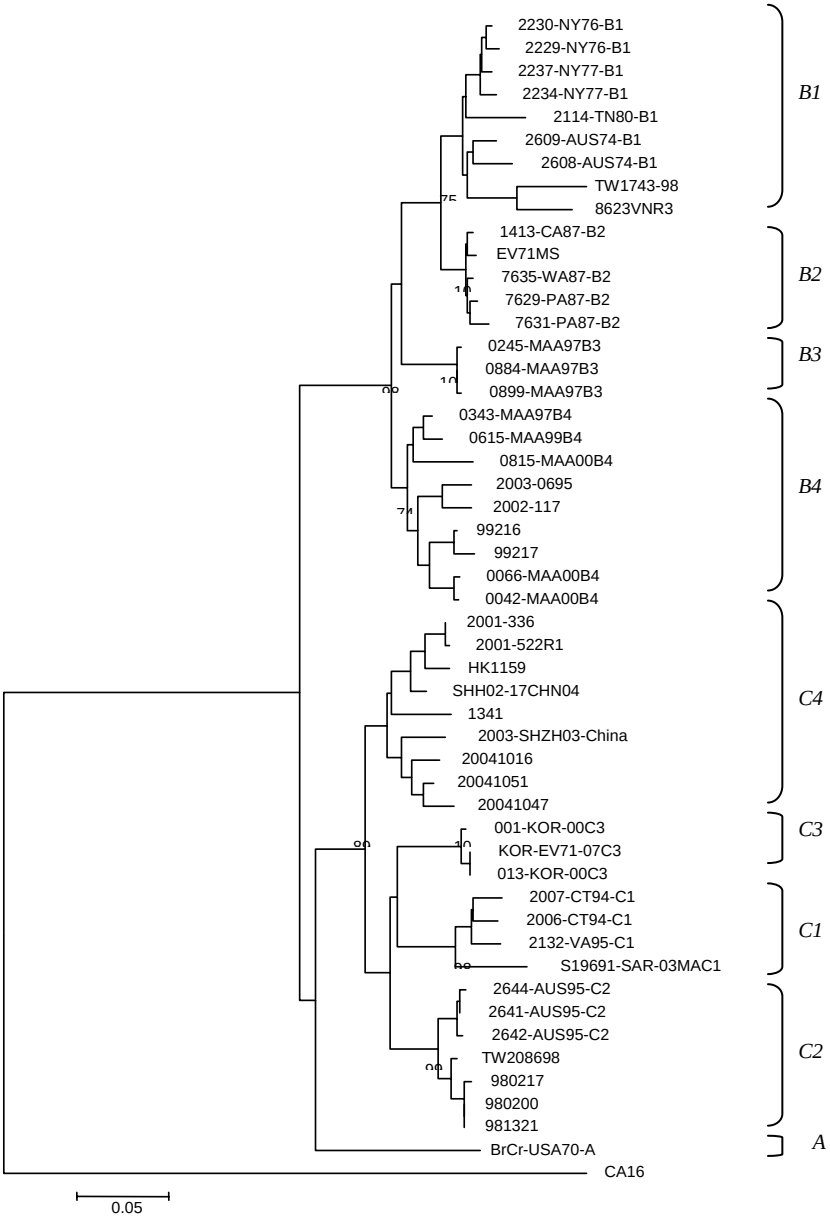


Figure 3. Phylogenetic tree analysis of EV71 subgenotypes B1~B

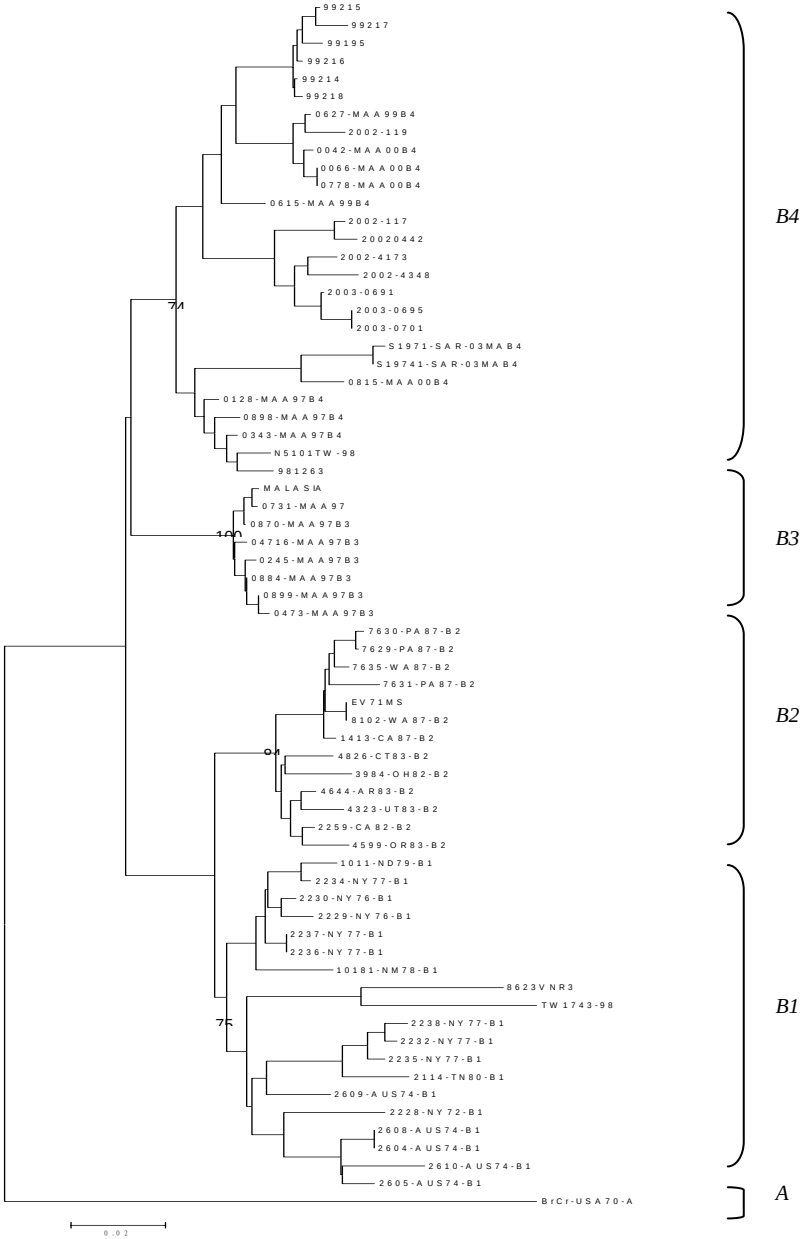


Figure 4. Phylogenetic tree analysis of EV71 subgenotypes C1~C4

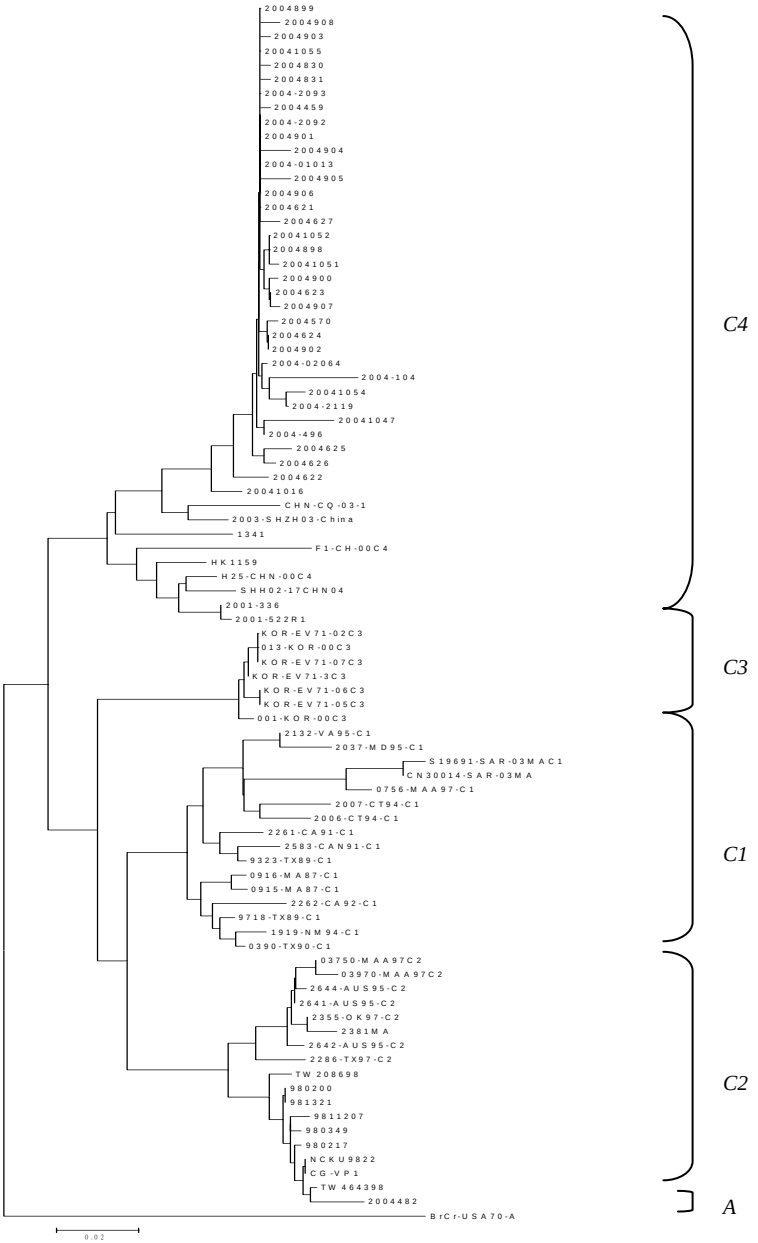


Table 1. Percentage analysis of homology among EV71 subgenotypes

EV71 subgenotypes	Comparison	%Homology range	Mean % homology
Within B1	-	92-98	94.9
Within B4	-	95-99	96.7
Within C2	-	98-99	99.1
Within C4	-	94-99	96.5
B2	B4	91-94	92.2
B2	C2	82-84	82.9
B2	C4	83-85	83.1
B4	C2	83-84	83.3
B4	C4	84-85	84.2
C2	C4	89-91	90.1
B1	A	82-84	83.5
B4	A	81-83	82.1
C2	A	82-86	84.3
C4	A	82-86	84.1
B1	B2	90-96	93.3
B1	B4	88-93	90.4
B1	C2	83-85	83.9
B1	C4	84-85	84.4
B2	B3	93-94	93.6
B4	B3	93-95	93.5
C2	B3	83-84	83.8
C4	B3	84-85	84.2
B2	C3	82-83	82.6
B4	C3	84-85	84.2
C2	C3	91-92	91.8
C4	C3	90-93	90.9
B2	C1	81-83	81.9
B4	C1	83-84	83.6
C2	C1	91-94	92.7
C4	C1	89-93	90.3

Table 2. Primer sequences

Primer	Sequence	Relative location
159	5’-ACYATGAAAYTGTGCAAG G-3’	2385-2403
162	5’-CCRGTAGGKGTRCACGCRAC-3’	2869-2850