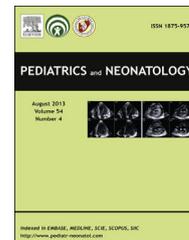


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REVIEW ARTICLE

Epidemiology of Enterovirus 71 Infections in Taiwan

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Enterovirus 71 (EV71) was first described in USA in 1969 but retrospective studies in The Netherlands further detected EV71 in the clinical specimens collected in 1963. EV71 has one single serotype measured by using hyperimmune animal antisera but can be phylogenetically classified into three genogroups (A, B, and C) including 11 genotypes (A, B1–B5, C1–C5). In Taiwan, EV71 caused a large-scale nationwide epidemic in 1998. Retrospective studies further detected EV71 in clinical specimens collected from hand–foot–mouth disease patients in 1980 and 1986. Therefore, EV71 may have circulated in Taiwan prior to 1980. Since 1998, EV71 has cyclically caused nationwide epidemics with different predominant genotypes in 1998 (genotype C2), 2000–2001 (B4), 2005 (C4), 2008 (B5), and 2012 (B5). Phylogenetic analysis revealed that C4 viruses isolated in 2005 were probably from China, B5 viruses isolated in 2008 were probably from South Eastern Asia, and B5 viruses isolated in 2012 were probably from Xiamen, China. Several studies have collected postinfection sera from children to measure cross-reactive neutralizing antibody titers against different EV71 genotypes and found that antigenic differences between genogroup B and C viruses did not have a clear pattern but that genotype A virus was antigenically different from genogroup B and C viruses. In conclusion, EV71 cyclically caused nationwide epidemics through international importations. EV71 surveillance in Taiwan should combine genetic and serological methods.

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1. Introduction

Enteroviruses (EVs) are single-stranded, positive-sense RNA viruses in the Picornaviridae family. They cause various clinical manifestations, including cutaneous, visceral, and neurological diseases.¹ Human EV can be further classified

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into four species including human EV A (20 serotypes such as EV71 and coxsackievirus A), human EV B (59 serotypes), human EV C (21 serotypes), and human EV D (4 serotypes). Overall, human EV has more than 100 serotypes.^{1,2} For many years, polioviruses were the most important EVs because they caused large outbreaks of paralytic disease before poliovirus vaccines were available. EV71 was first described in California, USA, in 1969. Since then, EV71 has been detected worldwide.^{2–5} Globally, two patterns of EV71 epidemic have been reported: small-scale outbreaks with few central nervous system (CNS)-complicated cases and deaths, and large-scale outbreaks with frequent CNS-complicated cases and deaths. The latter pattern occurred in Bulgaria (1975), in Hungary (1978), in Malaysia (1997), in Taiwan (1998), in Singapore (2000), in southern Vietnam (2005, 2007–2009, and 2011), in Brunei (2006), in Korea (2009), and in China (2007–2009).^{2,6} Because EV71 mortality rates are heavily affected by healthcare accessibility and standards, a more relevant clinical definition, such as CNS complication should be also used to quantify disease burden of EV71 infections in prevalent areas.

Although EV71 was first isolated in 1969, a retrospective analysis shows that this virus circulated in The Netherlands as early as 1963.⁵ Molecular evolution studies further predicted that EV71 could have emerged in the human population around 1941.^{5,7} Recently, EV71 repeatedly caused life-threatening outbreaks of hand–foot–mouth disease (HFMD) with neurological complications in Asian children. The neurological manifestations progress very quickly and range from aseptic meningitis to acute flaccid paralysis and brainstem encephalitis.² Due to its tremendous impact on healthcare systems, development of EV71 vaccines is a national priority in Taiwan and other Asian countries. Several vaccine candidates are being evaluated in humans and some will be licensed in the near future. Epidemiological characteristics are critical to the design of vaccine trials and formulation of vaccination policy when vaccines are licensed. Therefore, this review will focus on epidemiology of EV71 infections in Taiwan.

2. Clinical spectrum of EV71

According to previous clinical studies conducted in northern Taiwan, symptomatic EV71 infections progress through four stages: HFMD/herpangina (Stage 1), CNS involvement (Stage 2), cardiopulmonary failure (Stage 3), and convalescence (Stage 4).² This classification was recently recommended in a World Health Organization (WHO) report.⁶ Follow-up studies further demonstrated that EV71 infection can cause long-term sequelae including neurological development and cognitive function.⁸ In a prospective hospital-based case-finding study, 21% of 183 EV71 infections in children aged < 18 months of age developed neurological complications such as meningitis and encephalitis.⁹ Based on national severe EV surveillance and two cross-sectional serological surveys, Lu et al¹⁰ estimated that 130,617 Taiwanese children aged < 3 years were infected with EV71 infections in 1998 and that 273 (0.21%) of these infected children developed neurological complications. Overall, the prospective hospital-based case-finding study would overestimate the proportion of EV71

infections with neurological complications and the national surveillance data would underestimate the proportion of EV71 infections with neurological complications. In a cohort study conducted from 2006 to 2012 in northern Taiwan, about 100 EV71 primary infections were detected serologically in children younger than 5 years and none of them developed neurological complications (Lee MS et al, unpublished data). Therefore, the proportion of EV71 infections with neurological complications would be between 0.21% and 1%.

3. Laboratory diagnosis of recent EV71 infections

EVs include over 100 serotypes and usually cause self-limited infections with nonspecific symptoms in children, with the exceptions of polioviruses and EV 71 which frequently cause neurologic complications. Therefore, early detection and serotyping of EVs are critical in clinical management and disease surveillance. Moreover, herpangina and HFMD are major clinical manifestations for EV71 and other EV species A viruses. Therefore, laboratory diagnosis is critical to differentiate EV71 infections from other EVs. There are several methods for laboratory diagnosis of recent EV71 infections, including viruses isolation, molecular methods, and serology (Table 1). The traditional methods for detection and serotyping of EV infections are virus isolation and immunofluorescence assay, which are time-consuming and labor-intensive.^{11,12} Several clinical studies have documented that molecular diagnosis based on polymerase chain reaction saves time and is more sensitive than virus isolation for the detection of EV infections in hospitalized patients,^{13–16} but few studies have been conducted on outpatients. Moreover, no study has compared molecular tests and virus isolation/immunofluorescence

Table 1 Laboratory diagnosis methods for recent EV71 infections.

Method	Advantages	Disadvantages
Virus isolation/IFA	Provide virus isolates for further study	Low sensitivity Time consuming Requires skillful manpower
Nested RT-PCR	High sensitivity Time-saving	Requires skillful manpower False positivity Multiple primers for different genotypes
CODEHOP RT-PCR	High sensitivity Time saving	Requires skillful manpower Requires sequencing
Serology: neutralizing antibody	High sensitivity High specificity	Requires skillful manpower Requires pair sera
Serology: ELISA IgM	Rapid diagnosis	False positivity

ELISA = enzyme-linked immunosorbent assay; IFA = immunofluorescence assay; RT-PCR = reverse transcription polymerase chain reaction.

assay for serotyping of human EVs using clinical specimens. Although these methods have been used to detect EVs in clinical specimens including throat swabs, stool samples, and cerebrospinal fluid,^{12–14,16–19} reports elucidating the comparison among the diagnostic approaches are limited.

Molecular tests for the detection of human EVs in clinical specimens usually target highly conserved sites in the 5' untranslated region.²⁰ Due to low virus titers in clinical specimens, several reverse transcription-nested or reverse transcription-semi-nested polymerase chain reaction have been developed to further increase its sensitivity and specificity.^{21–23} However, serotyping of EVs based on 5' untranslated region sequences directly amplified from clinical specimens have not been well evaluated. In addition, EV VP1 capsid gene has recently been proposed to be an ideal target for the detection and serotyping of EVs using the consensus degenerate hybrid oligonucleotide primer (CODEHOP).²⁴ A recent study compared virus isolation and the two molecular tests for detection and serotyping of EVs in clinical samples and found that the VP1 CODEHOP test performed well for detection and serotyping of EVs in clinical specimens and that it could reduce unnecessary hospitalization during EV seasons.²⁵ Although molecular methods are much more sensitive than the virus isolation for the detection of EV infections, they require skillful manpower and are not suitable for clinics and community hospitals.

There are two serological methods available for laboratory diagnosis of recent EV71 infections, including neutralization assay and enzyme-linked immunosorbent assay (ELISA) immunoglobulin M (IgM). The neutralization assay is the most reliable method but it requires collection of pair sera, which is not feasible for most situations. Several studies have tried to develop ELISA serum IgM methods for rapid diagnosis of EV71 infections but these serum IgM assays all share the drawback of frequent false positive reactions (>20%) in patients infected with other human EVs such as CVA6 and CVA16 (Lee MS et al. unpublished data).^{26–28} Serum IgM tests with low false positive reactions are desirable to reduce unnecessary hospitalization during EV seasons.

4. EV surveillance in Taiwan

A nationwide EV71 epidemic occurred in Taiwan in 1998, which may have caused the largest number of severe cases and deaths in human history. Since then, a national EV surveillance system has been established by Taiwan Centers for Disease Control. The national EV surveillance system includes three components: (1) a sentinel physician network to collect weekly number of HFMD and herpangina, which was recently replaced by the Real-time Outbreak and Disease Surveillance through the National Health Insurance Database;^{29,30} (2) a laboratory network for virus identification by collecting throat swabs from EV-like patients (herpangina and HFMD); and (3) mandatory notification of EV-like severe cases, which collects throat swab, serum, and contact information through an epidemiological investigation. Based on these surveillance data, nationwide EV71 epidemics have occurred cyclically in Taiwan since 1998 (Table 2). Retrospective studies further found that EV71

Table 2 EV71-related severe and fatal cases in Taiwan, 1998–2012.

Year	Severe case no.*	Death no. (case fatality rate [†])	Predominant genotype
1998	405	78 (19)	C2
1999	6	1 (17)	B4
2000	152	25 (16)	B4
2001	181	27 (15)	B4
2002	58	8 (14)	B4
2003	44	4 (9)	B4
2004	20	5 (25)	C4
2005	82	8 (10)	C4
2006	2	0	C5
2007	7	2 (29)	C5
2008	344	14 (4)	B5
2009	25	2 (8)	B5
2010	12	0	C4
2011	59	3 (5)	C4/B5
2012	144	2 (1)	B5

Data are presented as *n* (%).

Data source: Taiwan Centers for Disease Control.

* Different laboratory methods were used for different years so these data should be interpreted with caution.

[†] Case fatality rate = number of deaths/number of severe cases.

epidemics may have occurred in Taiwan in 1980 and 1986.^{31,32} As shown in Table 2, the first recognized EV71 epidemics occurred in 1998 with 405 severe cases including 78 fatal cases. Nationwide epidemics occurred again in 2000–2001, 2005, 2008, and 2012 with different predominant genotypes. Overall, clinical spectra of these epidemics were similar although different genotypes predominantly circulated in different years.^{32,33} The laboratory diagnosis methods only employed virus isolation in early years but were revised to include serum IgM and molecular methods later. Therefore, it is not feasible to compare the magnitude of EV71 epidemics based on number of EV71 confirmed severe cases. However, case fatality rates decreased significantly in recent years, which may be related to an early warning of EV71 epidemics and stage-based clinical management.^{6,32}

5. Age-specific incidence rates

To design clinical trials of EV71 vaccines, age-specific incidence rates of EV71 infections are required to identify target populations, estimate disease burdens, define end-points of clinical efficacy, and calculate the sample size for efficacy trials. Age-specific incidence rates of EV71-related severe infections during the 1998 epidemic have been estimated to be 27.3/100,000, 37.1/100,000, 30.0/100,000, and 23.1/100,000 for children aged <6 months, 6–11 months, 12–23 months, and 24–35 months, respectively, which are too low to be a suitable endpoint of efficacy trials.³⁴ Alternatively, EV71-related mild illness such as herpangina and HFMD could be suitable clinical endpoints. In an infant prospective cohort study initiated in 2006 in

northern Taiwan and employing serum neutralization assay to detect EV71 infections, the age-specific incidence rates of EV71 infection during the 2008–2009 epidemic were observed to increase from 1.71/100 person–years at age 0–6 months infants to 4.09/100 person–years, 5.74/100 person–years, and 4.97/100 person–years in children aged 7–12 months, 13–24 months, and 25–36 months, respectively. In addition, the cumulative incidence rate was 15% by age 36 months, 39% of EV71 infections developed HFMD/herpangina, and 29% of EV71 infections were asymptomatic in young children.³⁵ A retrospective serosurvey also found that 37% of seropositive children reported to develop HFMD/herpangina during the 1998 nationwide epidemic in Taiwan.³⁴ Overall, development of EV71 vaccines should target children age < 6 months, and EV71-related mild illness such as herpangina and HFMD could be used as the clinical endpoints of efficacy trials.³⁶

6. Seroprevalence of serum EV71 neutralizing antibody

In addition to age-specific incidence rates that require time-consuming prospective cohort studies, seroprevalence studies of neutralizing antibody can be readily conducted to estimate cumulative infection rates. Several seroprevalence studies have been completed in Taiwan using sera collected in 1994, 1997, and 1999 (Table 3). Overall, seropositive rates of EV71 neutralizing antibody in sera collected in 1994 were 39% in 5-year-old children and 57% in 8-year-old children, which indicates that EV71 was prevalent from 1986 to 1994. Seropositive rates of EV71 neutralizing antibody in children aged <3 years in 1997 in Taipei City were about 0% (age 0.5–0.9 years) to 15% (age 3 years), which are much lower than in the same age groups in 1999. In a large-scale cross-sectional serosurvey conducted in 1999 in six areas including two urban (Taipei City and Kaohsiung City) and four rural areas, seropositive rates

were higher in the preschool children in rural areas than in the urban areas but similar in school children in both areas, which indicates more intrafamily transmission affecting preschool children occurred in rural areas.

7. Genetic and antigenic evolutions

According to analysis of VP1 sequences, EV71 was phylogenetically divided into three distinct genogroups: A, B, and C.^{1,2} Genogroups B and C can be further divided into genotypes B1–B5 and C1–C5, respectively, and genotype C4 is further classified into subgenotypes C4a and C4b.³⁶ Recently, genogroups D, E, and F were identified in India.³⁷ Genogroup A includes the EV71 strain (BrCr-CA-70), which was identified in 1970 in the USA but was not detected again until 2008. In an investigation of the HFMD outbreak in Anhui, China in 2008, five EV71 isolates were identified and they were closely related to genotype A based on analysis of VP1 genes.³⁸ In 2009, genotype A viruses were further detected in Beijing, Hubei, and Yunnan (Figure 1). Reasons for the re-emergence of genotype A in China are not clear and should be clarified. By contrast, genotypes B and C continued to circulate around the world after the 1970s and the 1980s, respectively. Recently, retrospective studies have shown that a genotype B0 virus circulated in The Netherlands as early as 1963 and a genotype C0 virus circulated in Japan as early as 1978.^{4,5} Interestingly, genogroup B viruses seemed to evolve through positive selection and only genotype B5 viruses are circulating. By contrast, genogroup C viruses have evolved through neutral selection and multiple genotype C viruses including C2, C4, and C5 are cocirculating globally.^{5,7,36} Moreover, EVs frequently recombine to generate new genotypes and serotypes, which could only be elucidated by complete genome analysis. Therefore, more efforts are required to generate complete genome data especially early EVs isolated before 1980.

Table 3 Age-specific seroprevalence of EV71 neutralizing antibody in Taiwan.

Location ^{ref}	Year	Findings
Taipei city ¹⁰	1994	39% for 5 y, 57% for 8 y, 67% for 10–20 y, 58% for 31–50 y
Taipei city ¹⁰	1997	44% for <6 mo, 0% for 6–11 mo, 5% for 1 y, 16% for 2 y, 15% for 3 y, 38% for 5 y, 47% for 10–20 y
Taipei city ¹⁰	1999	38% for <6 mo, 15% for 6–11 mo, 20% for 1 y, 22% for 2 y, 21% for 3 y, 29% for 4 y, 29% for 5 y, 33% for 6 y, 51% for 7 y, 47% for 8 y, 64% for 9 y, 63% for 10–20 y, 69% for 21–30 y, 69% for 31–50 y, 77% for >50 y
Taipei city ³⁴	1997	36% for <0.5 y, 4% for 0.5–0.9 y, 4% for 1–1.9 y, 22% for 2–2.9 y, 36% for 3–5.9 y, 63% for 6–11 y, 66% for 12–19 y, 57% for 20–29 y
Taipei city ³⁴	1999	7% for <0.5 y, 0% for 0.5–0.9 y, 8% for 1–1.9 y, 11% for 2–2.9 y, 34% for 3–5.9 y, 56% for 6–11 y, 54% for 12–19 y, 60% for 20–29 y, 48% for 30–49
Ilan county ³⁴	1999	8% for <0.5 y, 15% for 0.5–0.9 y, 18% for 1–1.9 y, 15% for 2–2.9 y, 49% for 3–5.9 y, 79% for 6–11 y, 74% for 12–19 y, 78% for 20–29 y, 50% for 30–49 y
Taoyuan county ³⁴	1999	13% for <0.5 y, 15% for 0.5–0.9 y, 30% for 1–1.9 y, 36% for 2–2.9 y, 49% for 3–5.9 y, 58% for 6–11 y, 60% for 12–19 y, 55% for 20–29 y, 47% for 30–49 y
Taichung county ³⁴	1999	12% for <0.5 y, 0% for 0.5–0.9 y, 14% for 1–1.9 y, 30% for 2–2.9 y, 51% for 3–5.9 y, 65% for 6–11 y, 81% for 12–19 y, 73% for 20–29 y, 75% for 30–49 y
Kaohsiung city ³⁴	1999	10% for <0.5 y, 3% for 0.5–0.9 y, 5% for 1–1.9 y, 15% for 2–2.9 y, 26% for 3–5.9 y, 57% for 6–11 y, 56% for 12–19 y, 58% for 20–29 y, 72% for 30–49 y
Kaohsiung county ³⁴	1999	24% for <0.5 y, 9% for 0.5–0.9 y, 12% for 1–1.9 y, 25% for 2–2.9 y, 40% for 3–5.9 y, 61% for 6–11 y, 68% for 12–19 y, 63% for 20–29 y, 67% for 30–49 y

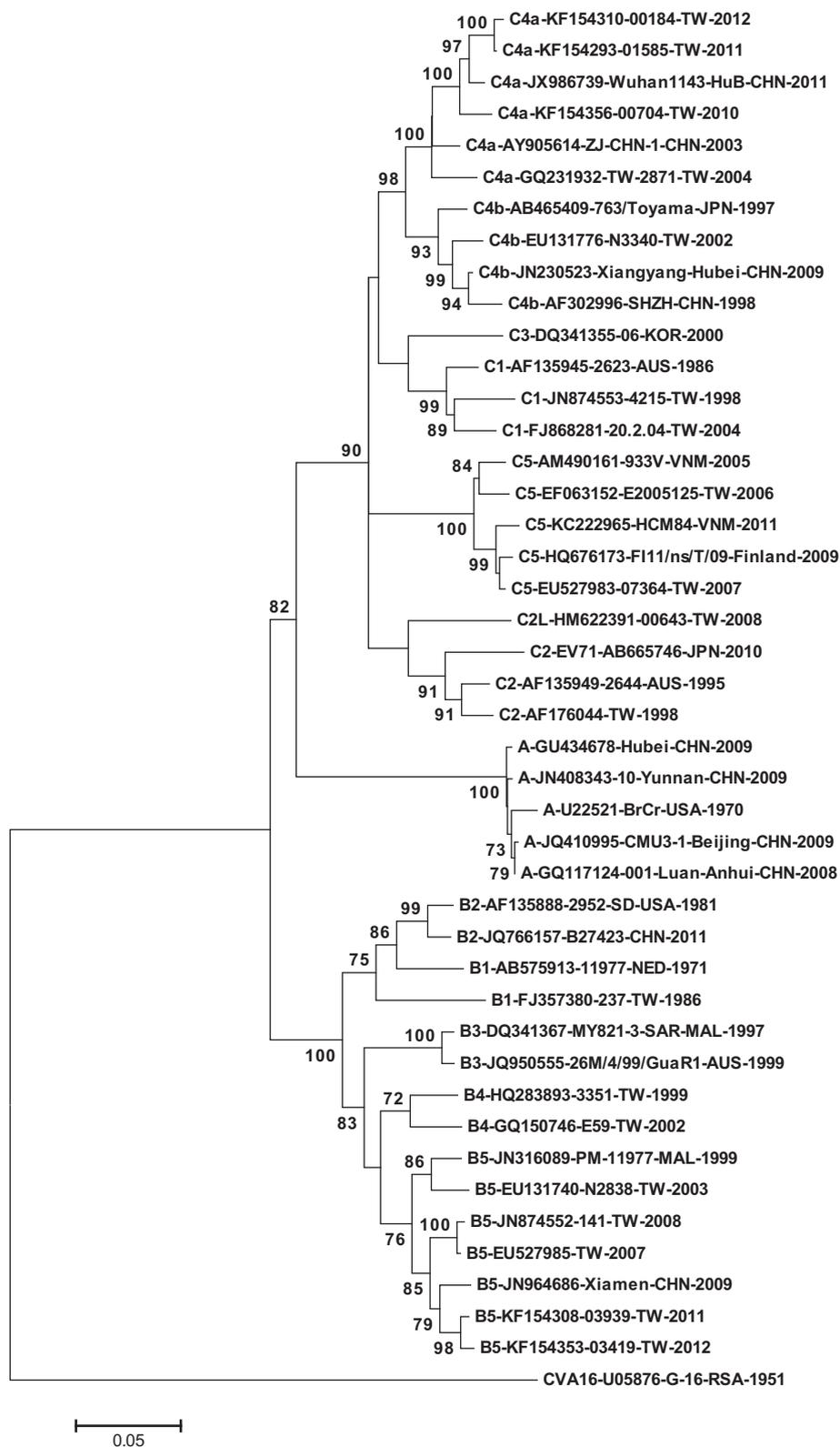


Figure 1 Phylogenetic analysis of VP1 genes of representative EV71 strains. The phylogenetic tree was generated by the neighbor-joining method with 1000 bootstraps based on nucleotide sequence from 2481 to 3278 using genome of EV71 (accession number is U22521) as reference. Only bootstrap values over 70% are shown. Coxsackievirus A16 strain was used as the outlier.

In Taiwan, different predominant genotypes were identified in different epidemics (Table 2; Figure 1). Based on retrospective studies, genotype B1 viruses circulated in Taiwan from 1980 to 1986. In the 1970s, genotype B1 viruses circulated widely in Europe, Japan, and USA. Phylogenetic analysis of VP4 genes found that the genotype B1 viruses isolated in Taiwan in 1980 and 1986 clusters together but could be differentiated from other genotype B1 viruses, which indicates that genotype B1 viruses have been introduced to Taiwan for some time.³¹ Information about circulating genotypes is not available from 1987 to 1997 in Taiwan. In 1998, Taiwan experienced the largest epidemic caused by genotype C2. Genotype C2 viruses were widely detected in the middle 1990s in Australia, Japan, and The Netherlands and they were likely to have emerged through recombination between EV71 and CAV8.³⁹ The genotype C2 viruses were replaced by genotype B4 viruses, which caused nationwide epidemics in 2000–2001 in Taiwan. Genotype B4 viruses were widely detected in the late 1990s and could evolve from genotype B2 viruses. The B4 viruses were replaced by genotype C4a viruses, which caused nationwide epidemics in 2005. Genotype C4 viruses were first detected in Japan and China in 1997 and 1998, respectively and evolved into two subgenotypes (C4b and C4a) in 2003 (Figure 1). Since then, genotype C4a viruses have become predominant and spread to a lot of countries including Australia, Austria, Japan, Taiwan, and Vietnam. Genotype C4a viruses could evolve from recombination between genotype C4b and genogroup B viruses. The genotype C4a viruses were replaced by genotype C5 viruses, which only spread sporadically in 2006–2007 in Taiwan. Genotype C5 viruses were first detected in Vietnam in 2003 and caused a large scale of epidemic in 2005.⁴⁰ Genotype B5 viruses emerged in late 2007 in Taiwan and caused a nationwide epidemic in 2008. Genotype B5 viruses were first detected in South Asia (Singapore or Malaysia) in 1999 and spread to several Asian countries in the early 2000s (Figure 1). In 2003, genotype B5 viruses were detected sporadically in Taiwan. They were not detected again in Taiwan until 2007. After the 2008–2009 epidemic, genotype B5 viruses disappeared and only genotype C4a viruses were detected sporadically in 2010 and 2011 in Taiwan. Genotype B5 viruses re-emerged in mid-2011 in Taiwan and caused nationwide outbreaks in 2012. Interestingly, the re-emerging genotype B5 viruses in 2011–2012 were phylogenetically closer to B5 viruses circulated in Xiamen, China in 2009 than to the B5 viruses isolated in Taiwan in 2008–2009 (Figure 1; Lee MS et al, unpublished data). International spreading of EV71 is common in Asia and should be monitored through international networks.

Because EV71 genetic variants have been widely identified in Taiwan and globally, it is necessary to examine their antigenic variations, which is critical to the selection of vaccine strains. EV71 has one single serotype as measured by using hyperimmune animal antiserum but antigenic variations have been reported recently in human studies. Using sera collected from young children with a primary infection of genotype B5, two studies detected partial antigenic differences between genogroups B and C but not between viruses in the same genogroup (B5 and B4 viruses).^{18,33} Kung et al⁴¹ did not detect significant antigenic differences between genotypes B4 and C4 viruses using acute-phase sera from EV71 inpatients. A serological survey in healthy Japanese children and adults

detected partial antigenic differences between genotype B5 and A viruses but not among different genotypes in genogroups B and C that had previously circulated in Japan.⁴² By constructing an antigenic map, however, Huang et al⁴³ detected antigenic differences between genogroups B and C, and also between B5 and B4 viruses. A recent study found that Taiwanese children infected with genotypes C2, C4, B4, and B5 had lower GMTs (≥ 4 -fold difference) against genotype A than other genotypes but antigenic variations between genogroups B and C did not have a clear pattern.⁴⁴ It is hard to compare different studies that employed different human sera and laboratory procedures, in particular the cell lines used in the neutralization assay. A network to harmonize laboratory procedures including standard sera and viruses is required to make the comparison possible. Moreover, the clinical and epidemiological significance of the observed antigenic variation requires longitudinal serological studies for clarification. In addition, it is not feasible to collect a large amount of serum from children postinfection to measure cross-reactive neutralizing antibody titers against multiple EV71 genotypes. Recently, a rabbit model has been developed to generate antisera for monitoring antigenic variations of EV71, which could be integrated to EV surveillance system (Lee MS, unpublished data).

8. Conclusions

EV71 is highly contagious and causes life-threatening outbreaks in children in Taiwan and several Asian countries. In the past 10 years, EV71 has caused nationwide epidemics every 3–4 years in Taiwan. Different genotypes dominate in different epidemics but the pattern is not predictable in Taiwan. Case-fatality rates of EV71 infections in recent epidemics seem to decrease significantly in Taiwan due to early warning of EV71 epidemics and stage-based clinical management. Overall, EV71 seems to have evolved rapidly and spread widely in Asia in the past 15 years. The Taiwanese experience on control and prevention of EV71 would be valuable to other countries facing EV71 epidemics.

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