An Investigation of Epidemic Enterovirus 71 Infection in Taiwan, 2008

Clinical, Virologic, and Serologic Features

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Background: Enterovirus 71 (EV71) is causing life-threatening hand-foot-mouth disease in Asia. In Taiwan, EV71 epidemics with different predominant genotypes occurred in 1998 (C2), 2000–2001 (B4), and 2004–2005 (C4). This genotype replacement may have important implications for vaccine development and prediction of epidemics. A nationwide EV71 outbreak occurred again in 2008, which provided a unique opportunity to characterize clinical, virologic, and serologic features of this epidemic.

Methods: We analyzed clinical and virologic data of 111 EV71 patients hospitalized in 2008 and prospectively conducted follow-ups of healthy children from June 2006 to December 2008.

Results: Among the 111 EV71 inpatients, 21 (19%) developed complications. Among the 21 complicated cases, 15 had central nervous system complication only, 2 had acute heart failure, and 4 had central nervous system and pulmonary complications. In the prospective study, 11 symptomatic infections and 4 asymptomatic infections were detected. Twenty-two EV71 isolates were genotyped, and 21 of them belong to genotype B5, which is phylogenetically close to B5 viruses circulating in Southeast Asia. Serologic tests show that children infected with B5 viruses have lower geometric mean titers of neutralizing antibody against genotype C4 than those against genotype B5 ($P = 0.004$, t test).

Conclusions: The 2008 nationwide EV71 epidemic was caused by genotype B5 that was likely introduced to Taiwan from Southeast Asia. Clinical features of the 2008 epidemic were not different from those observed before in Taiwan. Potential antigenic variations between genotype C4 and B5 viruses could be detected and its long-term epidemiologic significance needs further investigation to clarify.

Key Words: enterovirus 71, seroepidemiology, cohort study, molecular epidemiology

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Human enteroviruses include over 90 serotypes and can be divided into 4 species using molecular typing. With the exceptions of polioviruses and enterovirus 71 (EV71), which frequently cause neurologic complications, human enteroviruses usually cause self-limited infections in children.[1] EV71 was first isolated in California, USA, in 1969.[2] Since then, EV71 has been isolated globally. The clinical spectrum of EV71 infection ranges from asymptomatic infection, mild hand-foot-mouth disease (HFMD) to severe cases with central nervous system (CNS) and cardiopulmonary involvements.[1] Recent studies further demonstrated that CNS-complicated EV71 infections could cause long-term cognitive and motor deficits.[3,4] Globally, 2 patterns of EV71 outbreaks have been reported: small-scale outbreaks with low mortality and large-scale outbreaks with high mortality.[1] The latter pattern occurred in Bulgaria with 44 deaths in 1975,[5] in Hungary with 45 deaths in 1978,[6] in Malaysia with 29 deaths in 1997,[7] in Taiwan with 78 deaths in 1998,[8] in Singapore with 5 deaths in 2000,[9] and recently in China with >22 deaths in 2007 and 2008.[10–12] Since the 1998 epidemic, EV71 has been recognized as an endemic disease in Taiwan; it caused nationwide epidemics again in 2000–2001, 2004–2005, and 2008.[12–19] In the 2008 epidemic, 347 severe complicated cases including 14 fatal cases were reported.[12]

In response to the considerable public health concern worldwide because of the virulence of EV71, there have been intensified studies of the phylogenetic relationships between EV71 isolates. Several regions of the EV71 genome have been used for phylogenetic analysis, the capsid protein VP1 is considered most robust for evolutionary study because of a high degree of diversity and lack of involvement in recombination. Using this region for analysis, EV71 viruses are classified into 3 genogroups (A, B, and C).[1] Genogroup A has disappeared; but genogroups B and C are widely circulating in Asia. Genogroups B and C can be further divided into genotypes B1–B5 and C1–C5, respectively.[1,19,20] Interestingly, genogroup replacements have been well documented in highly epidemic countries such as Malaysia and Taiwan. In Sarawak, Malaysia, the predominant genogroups were genogroup B in 1997, genogroup C in 1998, genogroup B in 2000, genogroup C in early 2003, and genogroup B in late 2003 and 2005–2006.[1,21–23] In Taiwan, the predominant genogroups were genogroup C in 1998, genogroup B in 1999–2003, and genogroup C in 2004–2006.[18–20] In 2008, a nationwide epidemic occurred again in Taiwan, which provided us with a

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unique opportunity to characterize clinical, virologic, and serologic features of this epidemic.

METHODS

Study Populations

Two populations were available: hospitalized patients in Chang Gung Memorial Hospital (CGMH) and healthy children who were prospectively followed up starting from birth in a cohort study at the same hospital. We chose CGMH as a study site because it has large obstetric and pediatric populations and serves residents from rural and urban areas in northern Taiwan.[14,24] In the cohort study, we started to enroll in June 2006. Pregnant women having prenatal examinations in CGMH were invited to participate in the study. Sera were obtained from participating pregnant women and their children for measuring EV71 neutralizing antibody titers in the following schedule: pregnant women right before delivery; neonates at birth (umbilical cord blood); and infants at 6, 12, 24, 36, and 48 months of age. If the participating children developed suspected enterovirus illnesses (herpangina or HFMD), throat swabs were collected from these participating children for virus isolation. Institutional review board approvals were obtained from CGMH and the National Health Research Institutes following the Helsinki Declaration; and informed consent was obtained from all mothers of participating infants.

Clinical and Laboratory Definitions

Evidence of herpangina included oral ulcerations on anterior tonsillar pillars, soft palate, buccal mucosa, or uvula. Evidence of HFMD included oral ulcers on the tongue and buccal mucosa and a vesicular rash on the hands, feet, knees, or buttocks. Nonspecific febrile illness was defined as a rectal temperature greater than 38°C without other symptoms. In complicated cases, aseptic meningitis was defined as a clinically compatible illness with cerebrospinal fluid pleocytosis (>5 leukocytes/mm^3 in patients >1 month or >25 leukocytes/mm^3 in neonates) and negative bacterial cultures. Encephalitis was characterized by an altered level of consciousness accompanied by cerebrospinal fluid pleocytosis. Evidence of a poliomyelitis-like syndrome included acute limb weakness with diminished reflexes and muscular strength. A diagnosis of encephalomyelitis was made when there was evidence of encephalitis and poliomyelitis-like syndrome. Evidence of pulmonary complications include pulmonary edema, hemorrhage or diffuse ground–glass infiltrations on roentgenography with decreased PaO_2/FiO_2 <300.[14] Acute heart failure was characterized by evidence of decreased contractility on echocardiography, arrhythmia, an enlarged heart, and elevations in cardiac enzymes that are markers for cardiac damage.[8] Laboratory evidence of EV71 infection was defined as the isolation of EV71 from a throat swab, a rectal swab, or a stool sample, or a $\geq$4-fold rise or seroconversion in EV71 neutralizing antibody titers in paired sera samples.

Virologic Analysis

In CGMH, clinical samples (throat swabs, rectal swabs, or stool samples) are routinely collected for virus isolation from hospitalized pediatric patients with suspected enterovirus infections (herpangina, HFMD, or nonspecific febrile illness). Samples were inoculated into human embryonic fibroblast, LLC-MK2, HEp-2, and rhabdomyosarcoma cell cultures. When enteroviral cytopathic effect involved $>50\%$ of the cell monolayer, cells were scraped and subjected to indirect fluorescent antibody staining with enteroviruses monoclonal antibodies.[25] VP1 genes of isolated EV71 viruses were sequenced and genotyped by phylogenetic analysis using the Neighbor-joining method in MEGA 4 software as described previously.[18,26] Backgrounds of reference virus sequences used in the phylogenetic analysis were listed in Table, Supplemental Digital Content 1, http://links.lww.com/INF/A503.

Serologic Assays

Laboratory methods for measuring EV71 serum neutralizing antibody titers followed standard protocols.[14,27] Two-fold serially diluted sera (1:8–1:512) and virus working solution containing 100 $TCID_{50}$ of EV71 strain E59-TW-2002 (B4 genotype) were mixed on 96-well microplates and incubated with rhabdomyosarcoma cells. A cytopathic effect was observed in a monitor linked with an inverted microscope after an incubation period of 4 to 5 days. The neutralization titers were read as the highest dilution that could result in a 50% reduction in the cytopathic effect. Each test sample was run simultaneously with cell control, serum control, and virus back titration. The starting dilution was 1:8; the cutoff level of seropositivity was set at 8. Undetectable titer was assigned a level of 2 for the calculation of geometric mean titer (GMT). For deciding serostatus (positive or negative), sera were tested only at 1:8. Postinfection sera from seroconverted individuals were also measured neutralizing antibody titers against genotype B5 (NHRI141-TW-08) and C4 (70516-TW-08) viruses.

Statistical Analysis

Neutralization antibody titers were log transformed to calculate the GMT and 95% confidence interval (95% CI). The statistical association between 2 nominal or ordinal variables was tested by the χ^2 test, Fisher exact test, or the Mantel-Haenszel χ^2 test for trend as appropriate. All statistical analyses were performed using Microsoft Excel (Microsoft, Redmond, WA) or SAS (SAS Institutes, Cary, NC).

RESULTS

Clinical Features

In 2008, there were 588 hospitalized patients with enteroviruses isolated, and 40 of them (6.8%) developed complications. Most of the 588 inpatients were infected with coxsackievirus A2 (CA2, 202 cases) or EV71 (111 cases) (Table 1). EV71 inpatients were first detected in January and peaked in June. A total of 19% (21/111) of the virus culture-confirmed EV71 cases had compli-

TABLE 1. Clinical Symptoms of Hospitalized Children With Enterovirus Isolation in Chang Gung Children's Hospital, 2008

Clinical Symptoms	EV71 (N = 111) n (%)	CA2 (N = 202) n (%)	Other (N = 275) n (%)*
Uncomplicated cases	90 (81)	200 (99)	258 (94)
Herpangina	18	164	117
HFMD	69	11	26
Viral exanthema, febrile illness, or URI	3	25	115
Complicated cases	21 (19)	2 (1)	17 (6)
Aseptic meningitis	0	0	0
HFMD plus aseptic meningitis	1	0	14
Herpangina plus encephalitis	5	1	0
HFMD plus encephalitis	6	0	0
HFMD plus polio-like syndrome	3	0	0
HFMD plus acute heart failure	2	0	0
HFMD plus encephalitis and pulmonary hemorrhage	4	0	0
Other	0	1	3

*Other enteroviruses, including CA4, CA5, CA6, CA9, CA10, CA16, Coxsackievirus B1 (CB1), CB4, CB5, echovirus types 4, 9, and 30, and untypable enteroviruses.
CA2 indicates Coxsackievirus A2; URI, upper respiratory infection.

cations, which is significantly higher than those of CA2 (1%, $P < 0.01$, Fisher exact test) and other enteroviruses (6%, $P < 0.01$, χ^2 test) (Table 1). Among the 90 uncomplicated EV71 cases, 69 (77%) had HFMD, 18 (20%) had herpangina, and 3 had nonspecific illness. In contrast, herpangina were more frequently observed in CA2 (82%) and other enterovirus infections (45%) (Table 1). Among the 21 complicated EV71 cases, 15 had CNS complication only, 2 had acute heart failure, and 4 had CNS and pulmonary complications (Table 1). Except for the 4 cases with CNS and pulmonary complications, the remaining 17 complicated cases recovered without physical sequelae. Among the 4 cases with CNS and pulmonary complications, 1 recovered with left arm and bilateral toes amputated after treatment with extracorporeal membrane oxygenation for twice, 1 remains ventilator dependent during 3 months of follow-up, 1 had sequelae with left hand and right foot weakness, and 1 died with pulmonary and renal failures after hospitalization for 27 days. Three of the 4 cases with sequelae (75%) and 7 of the 17 complicated cases without sequelae (41%) were under 24 months old; but the difference did not have statistical significance because of small sample size ($P = 0.31$, Fisher exact test).

In the children cohort study, we recruited 693 neonates from June 2006 through June 2008. Among them, 494, 347, and 64 children completed follow-up and serum collections at 6, 12, and 24 months of age, respectively, by December 2008. In 2007, there were 25 clinically suspected cases (7 HFMD and 18 herpangina cases); but none of them was EV71 infection based on virus culture and serology tests. In 2008, there were 86 clinically suspected cases (9 HFMD, 60 herpangina, and 17 nonspecific illnesses); none of them developed complications. Among them, 5 had EV71 isolated, 7 had CA2 isolated, and 9 had other enteroviruses isolated (Table 2). All 5 culture-confirmed EV71 cases also seroconverted with EV71 neutralizing antibody tests. One febrile case was virus-culture negative but developed seroconversion. One HFMD case and 4 cases with nonspecific illness did not provide throat swabs for virus culture but were confirmed to have EV71 infections based on serologic tests. In addition, 4 children with serology-confirmed EV71 infections did not report apparent symptoms (herpangina, HFMD, or febrile illness) in 2008 and could have asymptomatic infections. Overall, we detected 15 children with EV71 infections in 2008, including 11 symptomatic and 4 asymptomatic infections (Table, Supplemental Digital Content 2, http://links.lww.com/INF/A504).

Virologic Features

We sequenced VP1 genes of 17 virus isolates from the hospitalized EV71 cases and 5 isolates from the children cohort for phylogenetic analysis. Based on the phylogenetic analysis (Fig., Supplemental Digital Content 3, http://links.lww.com/INF/A505), 21 of the 22 isolates belong to genotype B5, with the remaining 1 isolate belonging to genotype C4 (virus ID 70516-TW-08) (Table, Supplemental Digital Content 1, http://links.lww.com/INF/A503). Nucleotide sequences of these 21 B5 viruses and another Taiwan B5 virus isolated in 2007 were highly similar (98% to 99.7%); and these B5 viruses are phylogenetically related to other B5 viruses isolated in Malaysia in 2006 (FM201323-MY-06, FM201327-MY-06) and can be separated from the B5 viruses isolated in 2006 in Brunei and in 2003 globally (Fig., Supplemental Digital Content 3, http://links.lww.com/INF/A505). The only C4 virus isolated in our study is genetically more related to the C4 viruses isolated in China in 2007–2008 (EU913467-China-08) than the C4 viruses isolated in Taiwan in 2004–2005 (Fig., Supplemental Digital Content 3, http://links.lww.com/INF/A505).

Serologic Features

Seropositive rates of EV71 neutralizing antibody in the participating mother was 61%, and GMT of EV71 neutralizing antibody in the seropositive mother was 20 (95% CI 17–22). Age-specific seropositive rates of EV71 neutralizing antibody in the participating children were 48% at birth, 1% at 6 months of age, 1% at 12 months of age, and 11% at 24 months of age (Fig. 1). Among the 6 children who were seropositive at 6 months of age, 4 had higher neutralizing antibody titers at birth than at 6 months of age, which indicates residual of maternal antibodies. Overall, 15 children developed seroconversion in 2008 (Table, Supplemental Digital Content 2, http://links.lww.com/INF/A504). GMT of EV71 neutralizing antibody in the 15 seropositive children was 213 (95% CI 136–333), which is significantly higher than that in the seropositive mothers. Sera collected from these 15 children have also been measured neutralizing antibody titers against genotypes B5 and C4 viruses, and their GMT against genotype B5 and C4 were 308 (95% CI 202–470) and 122 (95% CI 80–186), respectively (Table, Supplemental Digital Content 2, http://links.lww.com/INF/A504).

DISCUSSION

EV71 viruses are widely circulating in Asian countries and genogroup replacements have been well documented in epidemic countries such as Malaysia and Taiwan. In Taiwan, the predominant EV71 genotypes were C2 in 1998, B4 in 2000–2003, and C4 in 2004–2005, but only 4 C5 isolates were detected in 2006.[19] We found that the predominant EV71 genotype in our study populations in 2008 was B5. Enterovirus surveillance data from the Taiwan Centers for Disease Control indicates that the B5 viruses started to circulate in southern Taiwan in late 2007 and then spread to northern Taiwan in early 2008,[19,28] findings consistent with our study. Phylogenetically, the B5 viruses isolated in our study and the Taiwan CDC surveillance system were very close; they could also be traced back to the B5 viruses isolated in Brunei and Malaysia in 2006.[26] Although 1 B5 virus (N2838TW-03) was

TABLE 2. Virus Isolation in Clinically Suspected Cases in a Cohort Study in Northern Taiwan, 2008

Clinical Symptoms	No. Cases	No. Specimens	EV71	CA2	CA10	Other Enteroviruses†	Other Viruses‡
Herpangina	60	47	2	7	3	6	13
HFMD	9	7	3	0	0	0	2
Other*	17	13	0	0	0	0	3
Total	86	67	5	7	3	6	18

*Including febrile illness, upper respiratory illness, and viral exanthema.

†Including CA5 (1 case), CA6 (1 case), CA16 (1 case), CB4 (1 case), and CB5 (1 case) and untypable enterovirus (1 case).

‡Including cytomegalovirus (7 cases), adenovirus (1 case), herpes simplex virus type I (6 cases), parainfluenza virus types I & III (3 cases), and influenza A virus (1 case).

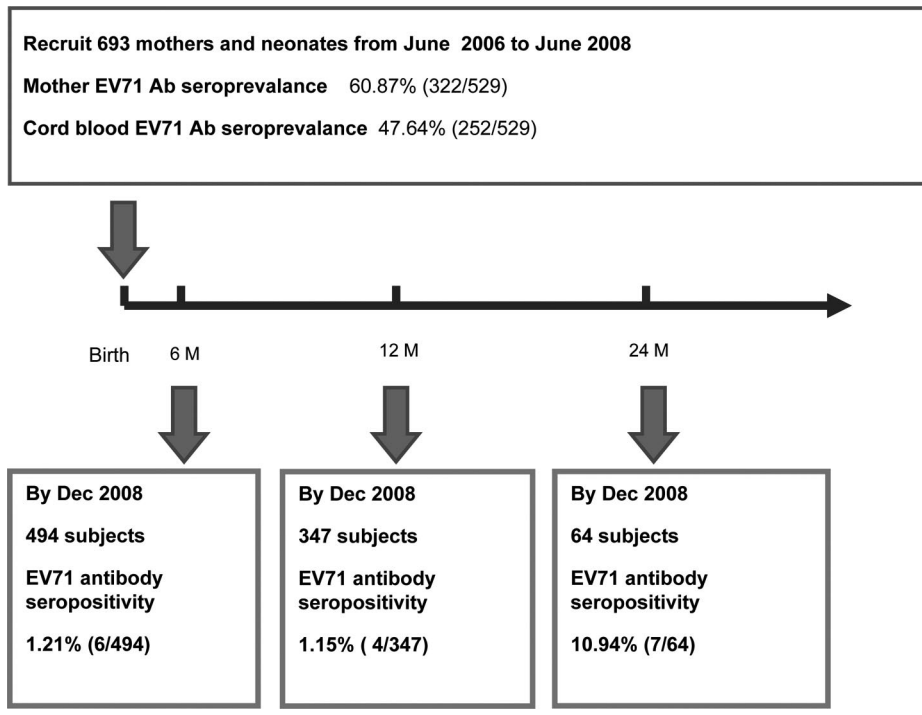

FIGURE 1. Follow-up schedule in a cohort study in northern Taiwan, 2006–2008. Sera were obtained from participating pregnant women and their children for measuring EV71 neutralizing antibody titers in the following schedule: pregnant women right before delivery; neonates at birth (umbilical cord blood); and infants at 6, 12, 24, 36, and 48 months of age.

isolated in southern Taiwan in 2003 when the B4 viruses predominantly circulated nationwide, no B5 virus was detected during 2004–2006 in Taiwan.[18,19] This 2003 B5 strain was genetically close to the B5 viruses isolated in Singapore, Japan, and Malaysia in 2000–2003 and could be genetically separated from the recent B5 viruses isolated in Brunei, Malaysia, and Taiwan during 2006–2008 (Fig., Supplemental Digital Content 3, http://links.lww.com/INF/A505). Globally, the B5 genotype was first detected in Singapore in 2000 and continued to circulate and evolve in Southeast Asia. Occasionally, the B5 viruses spread to neighboring regions such as Brunei, Japan, and Taiwan.[29–31]

The mechanism of genogroup replacement of predominant EV71 viruses in Taiwan and Malaysia is not clear. Serologic data in our study showed that children infected with B5 viruses have similar neutralizing antibody titers against the B5 and B4 viruses but have lower neutralizing antibody titers against the C4 virus. This finding may explain why the B5 virus did not spread in 2003 because B4 viruses had widely circulated in Taiwan during 2000–2002 and population immunity against B4 and B5 viruses would be high in 2003. In addition, nationwide epidemics occurred again in 2004 and 2005 caused by the C4 genotype. C5 viruses emerged in 2006 and 2007 in Taiwan but did not cause nationwide outbreaks.[19] Comprehensive and chronologic cross-reactive serologic data would be required to further clarify the clinical and epidemiologic significances of genogroup replacement, which have critical implications on development of EV71 vaccines.

Based on serum neutralization tests using hyperimmune animal antisera, EV71 is classified as a single serogroup. However, EV71 has tended to evolve more quickly in the last 10 years and more genotypes are now spreading globally.[32,33] In sera collected from monkeys immunized with a live-attenuated EV71 vaccine (genotype A), significant antigenic variations (>10-fold) were detected between homologous (genotype A) and heterologous (genotypes B4 and C2) viruses.[34] In addition, a serosurvey in healthy children and adults in Japan detected partial antigenic variations between genotype B5 and A viruses (GMT: 42 versus 15, $P < 0.05$).[35] Based on antigenic cartography of EV71 using postinfection human sera, Huang et al[36] found that B5 genotype was antigenically distinct from B4 genotype and C genogroup. However, Kung et al[16] did not detect potential antigenic variations between B4 and C4 genotypes using 13 acute-phase sera from hospitalized EV71 patients. It is hard to compare these human serologic studies with our study because of different study designs for collecting sera and different assay protocols. Harmonization of serologic methods plays a critical role to global influenza surveillance and control and should be applied to EV71 surveillance system.

Two studies conducted in Taiwan and Malaysia have identified that younger age is a risk factor of severe EV71 infections.[14,23] Our study also found that EV71 cases with CNS and pulmonary complications occurred mainly in children younger than 2 years (75%). Surprisingly, the Malaysia study suggested that genotype B5 and C1 viruses were more likely related to EV71 infections with CNS complications than B4 viruses. However, this study and previous studies conducted in the CGCH showed that the C2, B4, and B5 viruses caused similar clinical features but that after the stage-based management was developed in 2000 fatality rates in the EV71 cases with cardiopulmonary complications decreased significantly from 83% (15/18) in 1998–1999 to 33% (12/36) in 2000–2002 and 25% (1/4) in 2008.[14,37] The Malaysian study frequently observed severe non-CNS HFMD (34/277 = 12%), which was less observed in our study (2/111 = 1.8%). It is hard to compare these 2 studies because the study design and clinical classification were different. A universal clinical classifi-

cation for EV71 infections is desirable to make international comparisons possible.

Most enterovirus infections are self-limited and do not require hospitalization, but EV71 infections in young children frequently cause complications and can progress quickly. Therefore, Taiwanese parents frequently request hospitalization care if their children develop enterovirus symptoms (HFMD, herpangina, and febrile illness) during EV71 seasons. In our study, 18.9% of enterovirus inpatients were related to EV71 infections during the 2008 EV71 season. Rapid diagnosis kits differentiating EV71 infections from other enterovirus infections are urgently needed to reduce unnecessary hospitalization cares.

In conclusion, the predominant virus in the 2008 epidemic in Taiwan belongs to genotype B5, which had not widely circulated in Taiwan before and is phylogenetically close to the recent B5 viruses circulating in Southeast Asia. Potential antigenic variations between B5 and C4 viruses were detected and may explain the genogroup replacement occurring in the 2008 epidemic. Disease surveillance and development of EV71 vaccines should consider the potential antigenic variations.

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