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ORIGINAL ARTICLE

# Seroprevalence of enterovirus 71 and no evidence of crossprotection of enterovirus 71 antibody against the other enteroviruses in kindergarten children in Taipei city

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**KEYWORDS**
Crossprotection;
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*Background/Purpose:* Enterovirus 71 (EV71) infection may cause severe neurological and cardiopulmonary complications, especially in preschool children. This study is to investigate the seroprevalence and seroconversion of EV71, and the crossprotection of EV71 antibody against other enteroviruses among kindergarteners.
*Methods:* Overall 228 children in a public kindergarten were enrolled during two academic years, 2006 and 2007, in Taipei, Taiwan and we measured their EV71 neutralizing antibody. When the participants had herpangina; hand, foot and mouth disease (HFMD); febrile illness or respiratory symptoms, throat swabs were sampled and processed for viral culture and enterovirus real-time reverse transcriptase polymerase chain reaction (RT-PCR). Questionnaires, completed by the participants' guardians, surveyed the history of allergy and annual incidence of symptoms related to enterovirus infection.
*Results:* Seropositive rates of EV71 were 20% (32/163) in 2006 and 6% (4/65) in 2007. The rate of EV71 seropositivity increased with age ($p < 0.01$) in 2006 but it did not differ between genders ($p = 0.14$). No seroconversion was observed from 2006 to 2007. Herpangina

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occurred in 64% of children with EV71 seropositivity and 48% of those without EV71 antibodies ($p = 0.12$). Non-71 enterovirus infection, confirmed by viral study, occurred in 53% (19/36) of the EV71-seropositive children and in 53% (102/192) of EV71-seronegative children ($p = 0.89$). No participants had EV71 infection during the study period.
*Conclusion:* EV71 did not frequently circulate in Taipei City from September 2006 to June 2008. Presence of EV71 neutralizing antibody was not associated with lower incidence of enterovirus infection caused by non-71 serotypes.
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## Introduction

Enterovirus 71 (EV71), discovered in the USA in 1969, belongs to the family of *Picornaviridae*.[1] Compared with the other serotypes of enteroviruses, EV71 is well known for its neurotropic characteristics of invading the nervous system and it may cause acute flaccid syndrome, encephalitis and meningitis.[2] In the past 30 years, outbreaks of EV71 infection have brought about great casualties and socioeconomic burdens in the endemic countries, such as Bulgaria (1975), Hungary (1977) and Malaysia (1997).[3–5] The most devastating outbreak caused by EV71 occurred in Taiwan in 1998, resulting in 78 deaths and 405 children with severe neurological complications. The major causes of mortality and morbidity are cardiopulmonary failure and pulmonary edema/hemorrhage followed by brainstem encephalitis, which more commonly affect young children.[6–8]

Transmission in kindergartens and among family members was considered an important pathway of disease spread during the EV71 epidemic in Taiwan in 1998.[9,10] Preschool children who attended kindergarten and day-care centers had elevated rate of EV71 seropositivity.[10] In Singapore, Ooi et al. reported higher geometric mean titer (46.8) of EV71 antibody in children aged 2–5 years compared with mean titer (28.8) in children aged 6–12 years.[11] This suggested that preschool children were more frequently infected with EV71 than older children. Moreover, the surveillance in Taiwan indicated EV71 seropositivity increased with age.[10] Annual seroconversion rate, an indication of latest infection in previously uninfected cohort, was 7–11% during 1989–1993 but the annual seroconversion rate markedly decreased to 4% in 1994 and to 3% in 1997.[10,12] Low seroconversion rate is therefore speculated as a possible factor related to the EV71 epidemic in 1998.

Because enterovirus infection commonly occurs among children, it has been a puzzle for decades that the severity is diverse among individuals after infection. Research on the foot-and-mouth disease virus revealed that a member of *Picornaviridae* causing hand, foot and mouth disease (HFMD)-like symptoms in cloven-hoofed animals, serum IgM, acquired after foot-and-mouth disease virus infection, neutralized the virus of other serotypes.[13] Crossreactivity has also been observed among human enteroviruses, for example, after infection of coxsackievirus A9 the monoclonal antibody recognized coxsackievirus A21 and all three serotypes of poliovirus but did not affect their infectivity.[14] Therefore, the relation between crossreactivity and crossprotection is still unclear. Clinical investigation is therefore required to verify crossprotection among different serotypes of enteroviruses.

Seroepidemiology provides information of great importance in the surveillance of EV71 activity. We thus conducted this study to analyze the seropositivity and seroconversion of EV71 in preschool children and to delineate the possibility of crossprotection of EV71 antibody against other serotypes of enteroviruses.

## Methods

A. Case enrollment and study design

1. Agreement from the institutional review board of National Taiwan University Hospital, and from the education bureaus of Taipei city

The institutional review board of National Taiwan University Hospital approved this study and the education bureaus of Taipei city also agreed to this study after reviewing the project.

2. Enrollment of children and kindergartens

One public kindergarten located in Taipei City agreed to this study. After informed consent was obtained from their parents, we enrolled 228 preschool children (123 boys and 105 girls) in a kindergarten in Taipei City in the academic year of 2006 (September 2006–June 2007) and academic year of 2007 (September 2007–June 2008). Among the 228 children, 163 children (91 boys and 72 girls) were enrolled in year 2006, and 65 new participants (33 boys and 32 girls) and 75 participants from year 2006 enrolled in the following academic year. These children were aged between 2 years 7 months and 6 years 7 months. Their growth and neurological development were within normal limits. A flow chart of the study design is shown in Fig. 1.

3. Demography, family history and past history

All the guardians of the enrolled preschool children received a questionnaire to record their children's age, gender, medical history, allergy history, vaccine history and history of developing herpangina and HFMD during each academic year. Allergy history included atopic dermatitis, allergic rhinitis, hyperactive airway and asthma. The age of a participant was defined as the age of the child at the beginning of the academic year (September 1$^{st}$ of each academic year).

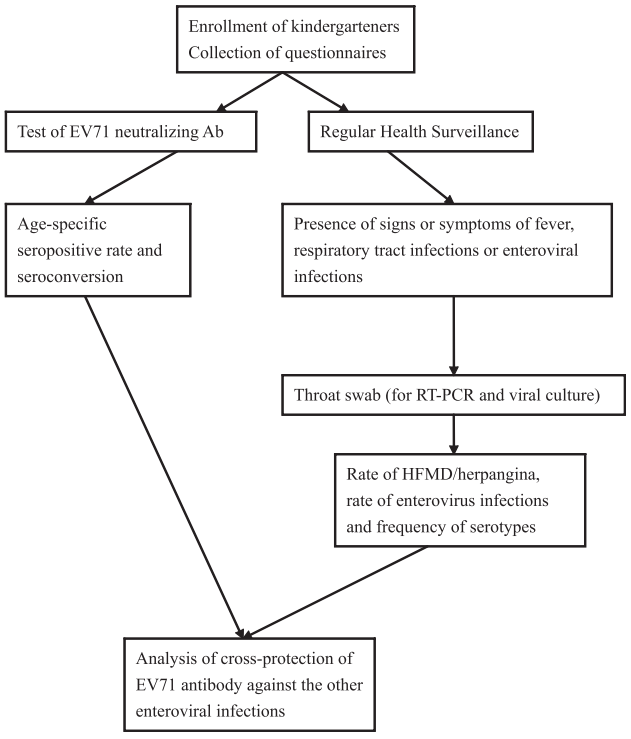


**Figure 1.** Flow chart of study design. After retrieving the questionnaires, all participants received blood tests for enterovirus 71 (EV71) neutralizing antibodies (Ab). If the children presented symptoms of respiratory tract infection, then throat swabs were collected and sent for real-time reverse transcriptase polymerase chain reaction (RT-PCR) and viral cultures.

4. Routine evaluation

The kindergarten nurse recorded the daily medication and the causes of receiving medication of every kindergartener. If the children had fever or symptoms of enterovirus infection, for example herpangina or HFMD, throat swabs would be collected by study nurses and sent for viral isolation and real-time reverse transcriptase polymerase chain reaction (RT-PCR).

Regular physical examinations of the participants were performed by several senior pediatricians every other week. If the participants had symptoms of respiratory infections or enterovirus infection (e.g., herpangina or HFMD), the abnormal physical signs and clinical diagnoses would be recorded and throat swabs would also be sampled and sent for viral culture and RT-PCR.

B. Laboratory methods

1. Virus isolation and serotyping

Throat swabs were submitted for virus isolation. Samples were inoculated into human embryonic fibroblast, LLC-MK2, HEp-2 and rhabdomyosarcoma (RD) cell cultures. When cytopathic effect involved more than 50% of the cell monolayer, cells were scraped and subjected to indirect fluorescent antibody staining with specific antibodies (Chemicon International Inc., Temecula, CA, USA) or typed by VP1 sequencing.

2. Molecular diagnosis for enteroviruses by real-time reverse transcriptase polymerase chain reaction

Total nucleic acid extraction from throat swabs was performed using an Isolation Kit (RNA and DNA extraction kit, Qiagen) according to the manufacturer's guidelines (Roche, MagNA Pure LC 2.0). Reverse transcription was performed with first strand cDNA Synthesis Kit for RT-PCR according to the manufacturer's guidelines (Invitrogen, Carlsbad, CA, USA). Real-time PCR for pan-enterovirus was performed with primers and probes based on the highly conserved region in the 5′-untranslated region of the enterovirus genome. The sequences of the primers (Genomics BioSci & Tech, Taiwan) and probes (TIB MOLBIOL, Germany) are listed as follows: forward primer, 5′-TCCTCCGGCCCCTGAATG-3′; reverse primer, 5′-AATTGTCACCATAAGCAGCCA-3′; PanEV probe (TaqMan), 6FAM-AACCGACTACTTTGGGTGTCCGTGTTTCXT-PH.

3. Molecular typing of the circulating enteroviruses during the surveillance

If there were positive results of enteroviral cytopathic effect or enteroviral RT-PCR, further molecular typing would be performed. Polymerase chain reaction (PCR) followed by direct sequencing was performed. Three genogroup-specific degenerate oligonucleotide primers flanking VP1 region were made including EntAF TNCARGC WGCNGARACNGG, EntAR outer ANGGRTTNGTNGMWGTY TGCCA, EntAR inner GGNGGNACRWACATRTAYTG, EntBF GCNGYNGARACNGGNCACAC, EntBR outer CTNGGRTTNGTN GANGWYTGCC, EntBR inner CCNCCNGGBGGNAYRTACAT, EntCF TNACNGCNGTNGANACHGG, EntCR outer TGCCANGTR TANTCRTCCC and EntCR inner GCNCCWGGDGGNAYRTACAT. PCR products were purified using the Gel/PCR DNA fragments extraction kit (Geneaid) before sequencing and direct sequencing was performed with the previous genogroup-specific primers on 377 PE/ABI automatic sequencer (Perkin-Elmer Cetus, Norwalk, Connecticut, USA) with ABI Prism BigDye Termination Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Perkin-Elmer). The serotype of enteroviruses was inferred by comparing the partial VP1 sequence with VP1 sequences for all strains of the 67 human enterovirus serotypes held in the public gene database.

4. Blood sampling and measurement of EV71 neutralizing antibodies

The participants were encouraged to donate a blood sample annually after written consent was obtained from their guardians. If the children did not have EV71 neutralizing antibody in academic year 2006, then a second blood sample would be taken to investigate the EV71 seroconversion rate in the next academic year.

The measurement of EV71 neutralizing antibody followed the standard protocol for the neutralizing test on microtiter plates. A mixture of serum and 50 μL EV71 solution, which contained 100 50% tissue culture-infective

doses of EV71 strain TW/2272/98 (GenBank accession number AF119795), was incubated with RD cells at 35°C in a 5% carbon dioxide incubator. After an incubation period of 2 to 7 days, the neutralizing antibody titer was determined at the time when cytopathic effect was observed in 1 50% tissue culture-infective dose of the virus back titration under an inverted microscope. The neutralizing antibody titer is defined as the highest dilution of serum that prevents the occurrence of cytopathic effect. Seropositivity is defined as EV71 neutralizing titer $\geq$8, and seroconversion of EV71 is defined as the change of EV71 from seronegative to seropositive or a fourfold rise in the titer of EV71 neutralizing antibodies.

C. Statistics

We compared the clinical course and outcome among different serotypes of enteroviruses and compared the serotypes in different years. We analyzed the rate of enterovirus infections and the rate of herpangina/HFMD between EV71-seropositive kindergarteners and EV71-seronegative kindergarteners. The methods of statistical analysis include the student *t* test and analysis of variance for continuous variables, and appropriate $\chi^2$ tests for categorical data. A *p* value less than 0.05 was considered to be significant. The data were analyzed with SAS 9.2 statistical software (SAS Institute, Inc, Cary, NC).

## Results

### Demography

Overall 228 preschool children (123 boys, 105 girls) were recruited in a kindergarten in Taipei City between September 2006 and June 2008. The recruitment numbers and EV71 seropositivity in each academic year are charted in Fig. 2. In academic year 2006, 91 boys and 72 girls participated, their mean age was 4.1 $\pm$ 1.0 years and the median age was 3.9 years. In academic year 2007, total participants were 140 children, which included 75 participants who were enrolled in 2006 (16 seropositive and 59 seronegative) and 65 new participants, 33 boys and 32 girls. The mean age of the new participants was 3.6 $\pm$ 1.0 years and the median age was 3.5 years.

Of the questionnaires 92% were retrieved. More than half of the participants (51%) reported allergy history and 37% had more than two allergic illnesses. Allergic rhinitis (47%) was the most common allergic illness, followed by hyperreactive airway (26%), atopic dermatitis (23%), asthma (10%) and allergic conjunctivitis (9%).

### EV71 seropositive rates among genders and different age groups

Seropositive rate of EV71 was 20% (32/163) in academic year 2006 and 6% (4/65) for the 65 new participants in academic year 2007. Although boys seemed to have a higher incidence of EV71 seropositivity in both two years, no significant gender difference was found (boy: 20%, girl: 11%; $p = 0.14$). Age-specific EV71 seropositive rates in academic year 2006 were 10% (2/20) for 2-year-old children, 8% (5/60) for 3-year-old children, 33% (16/49) for 4-year-old children and 26% (9/34) for 5-year-old children ($p < 0.01$). The result in 2006 indicated seropositive rate increased with age. In academic year 2007, only 4 children among the 65 new participants (6%) were seropositive (3 boys and 1 girl at the age of 3 years).

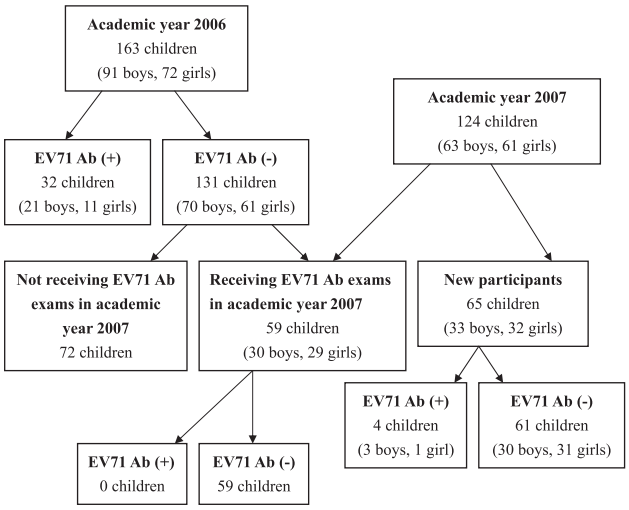


**Figure 2.** Distribution and enterovirus 71 seropositivity of participants in two academic years. In academic year 2006, 163 children were recruited. Among these participants, only those without enterovirus 71 (EV71) antibodies (Ab) would receive another blood test for serum Ab in academic year 2007 to determine the seroconversion rate. Among 32 seropositive children, half of them withdrew from the study in 2007. Eight children attended elementary school and the rest transferred to other schools; none of them had seroconversion. As for 131 seronegative children, 72 dropped out from the test in 2007 because 31 of them had transferred to other schools, 25 attended elementary school and 16 withdrew for personal reasons. Overall 59 children with EV71 seronegative received Ab testing in 2007. In academic year 2007, 65 children were newly recruited and 4 cases were seropositive.

### Seroconversion rate

Among the cohort of 59 children with seronegative status in the academic year of 2006, no seroconversion of EV71 antibody was found in the academic year of 2007 as Fig. 2 shows.

### Rates of herpangina and HFMD during the surveillance of the two academic years

None of the participants had HFMD during the study period. As for the incidence of herpangina, 55% (89/163) of the participants in academic year 2006 and 34% (47/140) in academic year 2007 had herpangina.

### Infection rate of enteroviruses and serotypes

Confirmed by PCR and/or viral culture, the incidence of enterovirus infection was 72% (118/163) in academic year

2006 and the serotypes were identified in 27% (32/118) of samples. The incidence of enterovirus infection in academic year 2007 was 26% (36/140) and the serotypes among 77% (28/36) of the samples were identifiable. The serotypes of enteroviruses are presented in Table 1. In 2006, coxsackievirus A4 (44%) and A2 (16%) were the leading serotypes. Later in 2007, the incidence of coxsackievirus A2 infection increased (39%), which was followed by EV68 (32%). In both academic years, coxsackieviruses A2 and A6 were isolated; however, neither EV71 nor coxsackievirus A16 was isolated during the study period.

## The relation between presence of EV71 antibody and infections of other enteroviruses

During this period, 64% of the participants (23/36) with EV71 antibodies had herpangina whereas 48% (92/192) of the EV71 seronegative group had a history of herpangina ($p = 0.12$).

Furthermore, Table 2 shows the rate of other enterovirus infections was 53% (19/36) in EV71 seropositive group and 53% (102/192) in EV71 seronegative group ($p=0.89$) indicating that the participants with or without prior EV71 neutralizing antibody showed a similar incidence of non-71 enterovirus infection.

## Discussion

The EV71 seropositive rate was relatively low (20%) in preschool children in Taipei City between September 2006 and August 2008 in comparison to the pre-endemic (22–36%) and post-endemic seroprevalence (24–42%) in children 2–5 years of age in the whole country before and after the 1998 outbreak.[10] Low seropositivity and absence of EV71 seroconversion in our study might indicate the infrequency of EV71 circulation in Taipei City during the period of investigation. This result also suggests the accumulation of susceptible hosts of EV71. Moreover, we found that whether children had EV71 antibodies or not, they had a similar incidence of acquiring non-EV71 enterovirus infection. Herpangina occurred more frequently in children with EV71 antibodies than in the seronegative group but the difference was insignificant. This showed that previous EV71 infection or the presence of EV71 neutralizing antibody did not have a strong impact on decreasing the incidence of EV infection caused by other serotypes.

In our study, the incidence of EV71 seropositivity increased with age, which might indicate transmission was more frequent among elder children in kindergarten because of increased interpersonal activities. This finding was also observed in the studies before the 1998 EV71 endemics in Taiwan in much larger populations,[10,12] which presented as an epidemiologic feature among preschool children. Furthermore, we noted that the presence of EV71 antibodies was not associated with lower risks of non-71 EV infection, especially the infection of class A non-polio enteroviruses (e.g., coxsackievirus A2, A4, A6 and A10), which shared more RNA homology with EV71. In a recent murine study, passive immunization of avirulent EV71 or coxsackievirus A16 induced antiEV71 antibodies with neutralizing activity. This study, nevertheless, did not observe the crossprotection toward coxsackievirus B3 or poliovirus.[15] The prior pre-endemic research reported that a higher seroprevalence rate of EV71 antibodies might be associated with minor clinical symptoms of EV71 infection among elder children and adolescents, which suggested greater humoral protection.[9,10] In our viral surveillance, however, there was no infection related to EV71 or coxsackievirus A16. Due to the absence of circulating EV71 and coxsackievirus A16 in our research period, the relation between seroprevalence and crossprotection of EV71 antibodies was undetermined. Thus, our finding could be a lead for further investigations to clarify the circumstantial influences by expanding the population and extending the study periods.

In our investigation, coxsackievirus A4 was the most common serotype in academic year 2006 and coxsackievirus A2 had the highest incidence in academic year 2007. In both years, none of our participants had virological-confirmed infections of EV71 or coxsackievirus A16. According to the analysis from the Center of Disease Control (CDC) in Taiwan, the number of cases of severe EV infection were significantly higher than usual in 2000 (291 cases), 2001 (393 cases) and 2005 (142 cases) but the number dramatically declined in 2006 (11 cases) and in 2007 (12 cases). Coxsackievirus A16 and EV71 were the two prevailing serotypes between 1998 and 2005, followed by coxsackievirus B3, echovirus 4, coxsackievirus B4.[16] The most common serotypes in the following years were coxsackievirus A4 in 2006,

**Table 1** Case percentage of different serotypes of enteroviral infections

| Serotypes | Year | |
|---|---|---|
| | Academic year 2006 (%) | Academic year 2007 (%) |
| CA1 | 0 (0) | 1 (4) |
| CA2 | 5 (16) | 11 (39) |
| CA4 | 14 (44) | 0 (0) |
| CA6 | 2 (6) | 1 (4) |
| CA9 | 1 (3) | 0 (0) |
| CA10 | 0 (0) | 2 (7) |
| CB2 | 3 (9) | 0 (0) |
| CB4 | 0 (0) | 4 (14) |
| Echo 4 | 3 (9) | 0 (0) |
| Echo 6 | 4 (13) | 0 (0) |
| EV68 | 0 (0) | 9 (32) |
| Total | 32 (100) | 28 (100) |

CA: coxsackie A virus; CB: coxsackie B virus; EV: enterovirus.

**Table 2** EV71 serostatus versus non-71 enterovirus infection/herpangina

| | EV71 seropositive (N=36) | EV71 seronegative (N=192) | *p* |
|---|---|---|---|
| Non-71 EV infection | 19 (53%) | 102 (53%) | 0.89 |
| Herpangina | 23 (64%) | 92 (48%) | 0.12 |

EV: enterovirus.

coxsackievirus A16 in 2007 and coxsackievirus A2 in 2008. EV71 accounted for 12% (330/2728) of confirmed EV infection in Taiwan in 2005 and its incidence markedly dropped to 0.05% (1/1995) and 0.6% (16/2471) in 2006 and 2007 respectively. Coxsackievirus A16 presented similar findings. In 2005, the incidence of coxsackievirus A16 infection was 26% (696/2728). It decreased to 3% (60/1995) in 2006 but rebounded to 23% (578/2471) in 2007. These findings were further evidence that since 2005 the circulation of EV71 has become less constant, which was related to the absence of seroconversion in our study population.

At the time our project ended, unfortunately, an EV epidemic recurred in the summer of 2008. In the annual report of CDC in Taiwan, by December 2008, 3677 cases of EV infection were confirmed, 369 cases presented with severe complications (227 males and 142 females) and 90% were under 5 years of age. Fourteen cases died during this endemic. Coxsackievirus A2 (33%, 1199/3677) and EV71 (26%, 966/3677) are the two leading serotypes, followed by coxsackievirus B4 (10%, 382/3677) and undifferentiated non-polio enteroviruses (9%, 338/3677).[17] Therefore, low seropositivity and seroconversion of EV71 in high risk group, indicating large number of susceptible hosts, may be considered an indicator of EV71 endemic as Lu et al. previously suggested.[12]

We performed the study in Taipei City, where there were fewer cases of EV71 compared with other areas in Taiwan. EV infection more frequently occurs in tropical climates with higher humidity. Further investigations of seroprevalence in other regions are necessary to unfold the effect of environmental varieties. Also, an extension of the study period may elucidate the mystery that contributes to the EV71 outbreak.

We found the presence of EV71 neutralizing antibody did not lower the risks of enterovirus infection by other serotypes and further monitoring of EV activity is needed to control its spread.

## Acknowledgments

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