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Original Article

An Outbreak of Coxsackievirus A16 Infection: Comparison With Other Enteroviruses in a Preschool in Taipei

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BACKGROUND/PURPOSE: The transmission rate of enteroviruses in young children remains unclear. Therefore, we carried out active surveillance in preschool children to investigate the transmission rate and clinical manifestation of enteroviruses.

METHODS: From September 2006 to December 2008, we monitored infectious diseases in children (2–3 years of age) in a preschool in Taipei. If any child had a febrile illness or symptoms/signs of enteroviral infection [e.g. herpangina or hand-foot-and-mouth disease (HFMD)], we performed viral isolation and enterovirus polymerase chain reaction. VP1 sequencing was performed to define their serotypes. We also collected clinical data and analyzed transmission rates.

RESULTS: There were eight episodes of enterovirus infection during the study period. The serotypes included coxsackievirus A4 (CA4), CA2 and CA16. The transmission rates of CA4 and CA2 among children in same class were 26% and 35%, respectively. Between November 28 and December 12, 2008, 13/21 (61.9%) children contracted herpangina and/or HFMD. The average age was 2.82 (range, 2.43–3.39) years. CA16 was detected in 10/13 (76.9%) of the throat swabs by polymerase chain reaction VP1 genotyping. Compared with previous CA2 and CA4 outbreaks, CA16 had a significantly higher transmission rate ($p=0.035$) and resulted in more cases of HFMD ($p<0.001$). The transmission duration of coxsackie A viruses within the same class ranged from 12 to 40 days.

CONCLUSION: Compared with CA2 and CA4, CA16 infections resulted in more cases of HFMD and had significantly higher transmission rates in preschoolers.

KEYWORDS: coxsackievirus A16, enterovirus, transmission rate

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Introduction

According to the statistics of the Department of Health, Executive Yuen, Taiwan, infectious diseases, which may result in some kind of severe morbidity or even mortality, account for one-third of all hospitalizations and outpatients' medical care in children younger than 5 years of age.¹ The proportion of infection-related outpatient clinic visits and hospitalizations in preschool children is also much higher than in school children.¹ Due to the increase of infectious sources, preschool attendance may be the most important environmental factor affecting infections in preschool children. Surveillance of these children may help to elucidate the transmission rate, risk factors and outcome of these infectious diseases, which will provide guidance for better child care and disease prevention.

Enterovirus (EV) infections are very common in young children and lead to a wide spectrum of clinical presentations. Most cases are asymptomatic or mild, and usually recover without any special medication. In 1998, an EV epidemic occurred in Taiwan, including more than 120,000 cases of hand-foot-and-mouth disease (HFMD) and herpangina, with 405 severe cases and 78 deaths, which raised wide public concern.² Most of the fatal EV cases were in preschool children who suffered from the sudden lethal complication of cardiopulmonary failure. After the 1998 epidemic, severe EV cases were still identified at a rate of 9–393 per year, and more than 60% of the patients were children under 3 years of age.³ Although it is the main pathogen in children in Taiwan during the summer, there is no antiviral therapy and an EV71 vaccine is still under development.⁴

Due to its high mortality and morbidity, most EV research focuses on EV71 and clinical data for other EVs are limited. The aim of this 2-year prospective cohort surveillance study was to elucidate the transmission and epidemiologic characteristics of enteroviral infections among young children in a preschool.

Methods

Children and follow-up

After obtaining written informed consent, we enrolled all 2–3-year-old preschool children attending a public preschool in Taipei. The study period was from September

2006 to December 2008. Demographic data and medical histories were collected and the children's daily symptoms, medication, hospitalization and school absences were recorded by the school nurse.

Regular health check-ups for the children were performed every week, or every other week, by the study pediatricians and nurses. If there was any febrile illness or symptomatic illness including herpangina, HFMD, pharyngitis, upper respiratory tract infection, or acute gastroenterocolitis, throat swabs were collected for etiology work-up via viral culture and reverse transcription-polymerase chain reaction (RT-PCR). Febrile illness means a rectal temperature $>38^{\circ}\text{C}$ without other symptoms. HFMD was defined as oral ulcers on the tongue and buccal mucosa with vesicular rash on the hands, the feet, the knees or the buttocks. Otherwise, herpangina was defined as oral ulceration on the anterior tonsillar pillars, soft palate, buccal mucosa or the uvula.

The transmission rate was defined as the number of symptomatic children divided by the total number of preschoolers enrolled in the same class. We defined the index case to be the first case of each episode and the other cases of the same episode were regarded as secondary. The transmission period was defined as the onset of illness between the first and last cases of each episode. The transmission, complication and hospitalization rates, and school absences of the preschoolers were all analyzed. An outbreak was defined as two or more cases of EV infection with onset of illness occurring within 10 days in the same institution.⁵ During the study period, there were eight episodes of EV infection, and three fulfilled the criteria of an outbreak. The symptoms of the infected children were fever, herpangina and HFMD. Significantly more HFMD cases were noted during the outbreak in November 2008. Therefore, we compared the clinical course, transmission rate and outcome between the different EV serotypes.

Virus isolation and serotyping

Throat swabs, rectal swabs or stool samples were collected for virus isolation. Clinical specimens were inoculated into human embryonic lung MK2, HEp-2, MDCK and rhabdomyosarcoma cell cultures after pretreatment (decontamination). If there was a cytopathic effect, the cells were scraped and subjected to indirect fluorescent antibody staining with specific antibodies against the suspected viruses.

Molecular diagnosis of EVs

Nucleic acid extraction

RNA and DNA were extracted from clinical samples using an isolation kit according to the manufacturer's instructions (RNA and DNA extraction kit, Qiagen, Hilden, Germany).

Reverse transcription

Reverse transcription (RT) was carried out with the first strand cDNA Synthesis Kit for RT-PCR (Invitrogen, Carlsbad, CA, USA). The previously extracted RNA (8.5 µL) was mixed with 10 mM deoxyribonucleotides and random hexamers, and then incubated at 65°C for 5 minutes. The samples were then mixed with buffer, 25 mM MgCl₂, 0.1 M dithiothreitol, RNase inhibitor, and reverse transcriptase to the final volume of 20 µL, briefly vortexed, and incubated at 25°C for 10 minutes, followed by 50°C for 50 minutes. Finally, the product was incubated at 85°C for 5 minutes and then allowed to cool to 4°C.

Real-time PCR for pan-EVs

The primers and probes for pan-EVs were designed according to the highly conserved regions on in the 5' untranslated region of the EV genome sequences obtained from GenBank (Table 1). cDNA (5 µL) was mixed with DNA MasterPLUS Mix, 25 mM MgCl₂, 10 µM primers and 1 µM probes to a final volume of 10 µL for amplification. The amplification cycle was as follows: denaturation at 95°C for 10 minutes, followed by 55 cycles at 95°C for 10 seconds, annealing at 62°C for 10 seconds, and then extension at 72°C for 5 seconds followed by cooling to

40°C. If the real-time PCR result was positive, the cDNA was sent for subsequent semi-nested PCR.

Molecular typing of the circulating EVs

A semi-nested RT-PCR was used for detecting and amplifying the cDNA sequences. In order to identify the serotype of EVs, we chose the previously described VP1-specific primers (Table 2).⁶ PCR amplicons with three sets of VP1-specific primers were purified using the Gel/PCR DNA fragment extraction kit (Geneaid, Sijhih City, Taiwan) prior to sequencing, and direct sequencing was performed with the previous genogroup-specific primers on an 377 PE/ABI automatic sequencer (Perkin-Elmer Cetus, Norwalk, CA, USA) using the ABI Prism BigDye Termination Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Perkin-Elmer). The serotype of the EVs was inferred by comparison of the partial VP1 sequences with those in the public gene database containing VP1 sequences for the strains of all the 67 human enterovirus serotypes.

Data analysis

The difference between categorical data and continuous variables was analyzed using the χ^2 test with Yates's correction and one-way analysis of variance. A *p* value of <0.05 was considered statistically significant.

Table 1. Primers for enterovirus real-time polymerase chain reaction

Virus	Primer	Sequence
Pan-enterovirus	Forward	5'-TCCTCCGGCCCCCTG AATG-3'
	Reverse-2	5'-AATTGTCACCATAAGCA GCCA-3'
	Probe	5'-6FAM-AACCGACTAC TTTGGGTGTCCGTGTTT CXT-PH-3'

Table 2. Primer sequences for enterovirus genotyping (nested reverse transcription-polymerase chain reaction for the VP1 region)

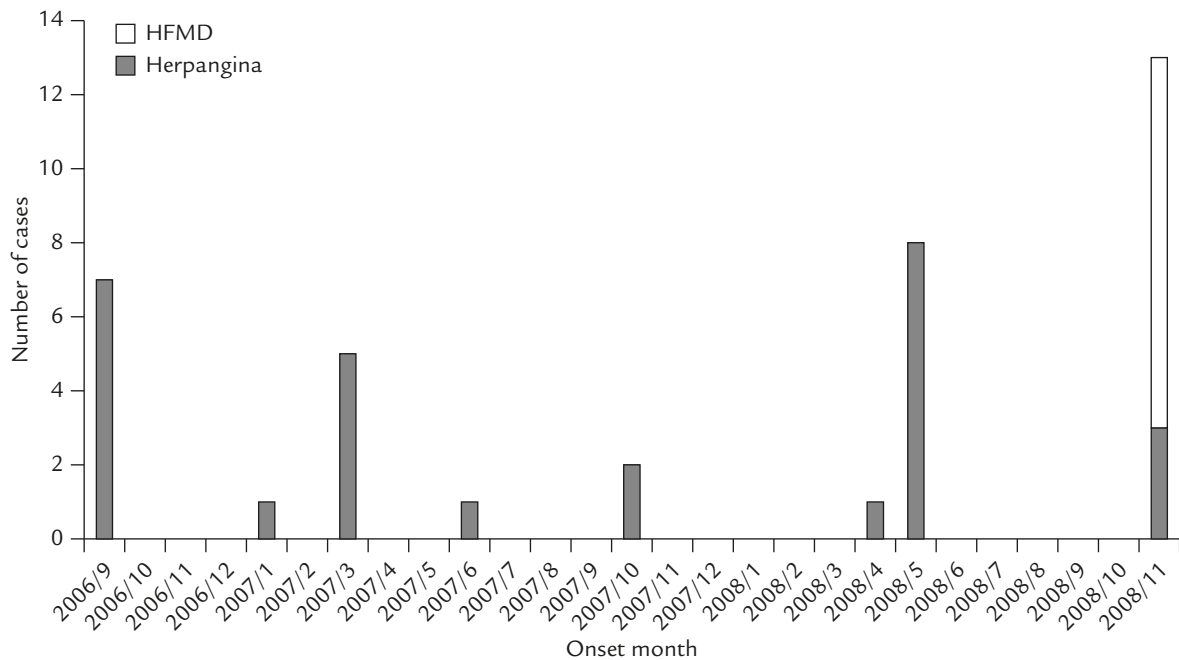
Gene	Sequence
EntA	
Forward	5'-TNCARGCWGCNGARACNGG-3'
Reverse outer	5'-ANGGRTTNGTNGMWGTYTGCCA-3'
Reverse inner	5'-GGNGGNACRWACATRTAYTG-3'
EntB	
Forward	5'-GCNGYNGARACNGGNCACAC-3'
Reverse outer	5'-CTNGGRTTNGTNGANGWYTGCC-3'
Reverse inner	5'-CCNCCNGGBGGNAYRTACAT-3'
EntC	
Forward	5'-TNACNGCNGTNGANACHGG-3'
Reverse outer	5'-TGCCANGTRTANTCRTCCC-3'
Reverse inner	5'-GCNCCWGGDGGNAYRTACAT-3'

A=Adenine; B=GTC; C=cytosine; D=GAT; G=guanine; H=ACT; K=GT; M=AC; N=AGCT; R=GA; S=GC; T=thymine, V=GAC; W=AT; Y=TC.

Table 3. The demography of the 2- to 3-year-old preschoolers (2006–2008)^a

	2006 (n=27)	2007 (n=23)	2008 (n=21)	p
Age (yr)	2.45±0.28	2.52±0.30	2.52±0.23	0.58
Sex, male	10 (37.0)	10 (43.5)	11 (52.4)	0.57
Sibling number	0.67±0.54	0.62±0.72 ^b	0.71±0.63	0.90
Underlying diseases	6 (22.2) ^c	3 (13.0) ^{b,d}	3 (14.3) ^e	0.70

^aData presented as mean±standard deviation or n (%); ^bdata (sibling number and underlying diseases) of two children were missing, making a total of 21 children; ^cunderlying diseases were asthma (3 children), congenital heart disease (1 child), developmental delay (1 child) and atopic dermatitis (1 child); ^dunderlying diseases were developmental delay (2 children) and glucose-6-phosphate dehydrogenase deficiency (1 child); ^eunderlying diseases were asthma (1 child), developmental delay (1 child) and atopic dermatitis (1 child).

**Figure 1.** Monthly distribution of eight episodes of enterovirus cases. HFMD=hand-foot-and-mouth disease.

Results

Enterovirus outbreaks

From September 2006 to December 2008, we continuously monitored all infectious diseases among 2–3-year-old children (range, 21–27 children in each semester) in a preschool. Demographic data of the classes within the study period is listed in Table 3.

During the study period, there were a total of eight episodes of EV infection (Figure 1), but the serotype was only identified in four of these due to low viral loads. Moreover, only three of the four EV infections fulfilled the definition of an outbreak. Most of the cases were detected between April and July and between September and November;

which are the major and minor peaks of EV activity in Taiwan.³ The main clinical symptoms were fever, herpangina and HFMD. Poor activity and dehydration were the main reasons for hospitalization. There was no EV-associated mortality reported during the surveillance period.

The index case of the outbreak in November 2008 was a 2-year-old girl with an initial presentation of herpangina and fever. Afterwards, 13/21 (61.9%) children consecutively suffered from herpangina or HFMD, within 12 days (Figure 2). Ten of them were shown to have coxsackievirus A16 (CA16) by VP1 RT-PCR and direct sequencing of their throat swabs. On the aspect of clinical symptoms, 77% of the infected cases presented with HFMD, while 23% had herpangina. Only 23% suffered from fever and 8% needed hospitalization.

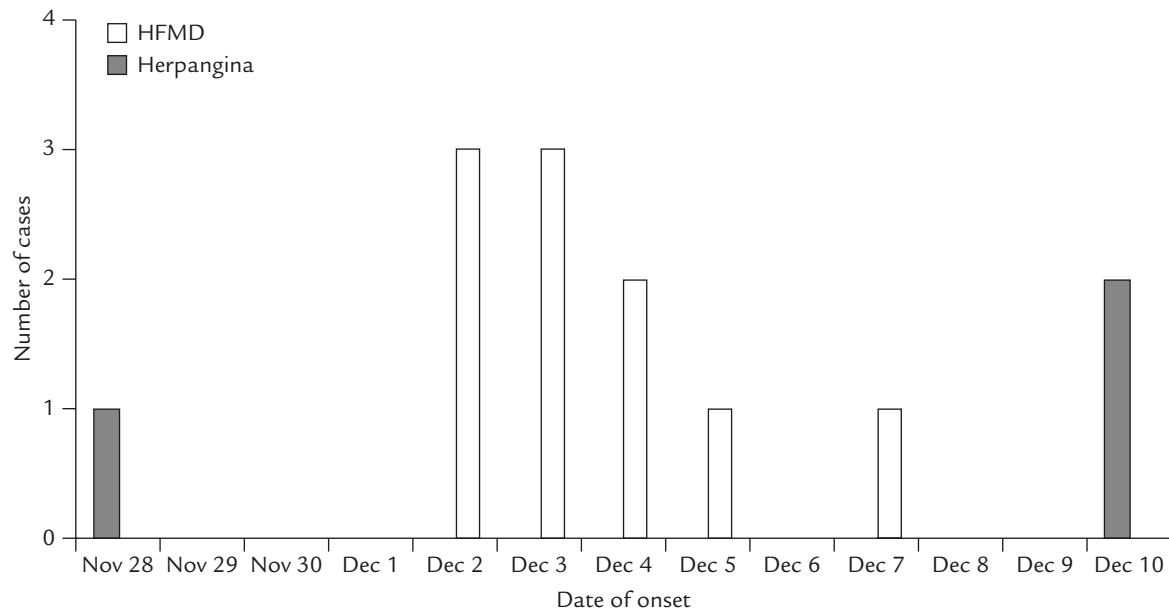


Figure 2. Summary of the coxsackievirus A16 outbreak in November 2008. HFMD=hand-foot-and-mouth disease.

Table 4. Comparison of the different Coxsackie A virus outbreaks^{a,b}

	Sep 2006 (<i>n</i> =7)	May 2008 (<i>n</i> =8)	Nov 2008 (<i>n</i> =13)	<i>p</i>
Sex, male	4 (57.1)	5 (62.5)	9 (69.2)	0.860
Age of symptomatic children (yr)	2.34±0.63	2.92±0.19	2.82±0.27	0.020
Serotyping ^c	CA4	CA2	CA16	
Viral detection				0.020
Culture	6 (85.7)	2 (25.0)	0 (0)	
Real-time PCR	5 (71.4)	1 (12.5)	12 (92.3)	
VP1 PCR followed by direct sequencing	1 (14.3)	0 (0)	10 (76.9)	
Symptoms/signs				
Febrile illness	5 (71.4)	6 (75.0)	3 (23.1)	0.030
Herpangina	7 (100)	8 (100)	3 (23.1)	<0.001
HFMD	0 (0)	0 (0)	10 (76.9)	<0.001
Hospitalization rate	1 (14.3)	2 (25.0)	1 (7.7)	0.550
Transmission period (d) ^d	28	40	12	-
Transmission rate ^e	7/27 (25.9)	8/23 (34.8)	13/21 (61.9)	0.040

^aData presented as mean ± standard deviation or *n* (%); ^b*n* represents the number of the infected cases during each outbreak. % means the percentage of positive viral detection, symptoms and signs, and hospitalization among the infected cases; ^cetiology was confirmed by virus culture or VP1 sequencing; ^dTransmission period was defined as the onset of illness between the first and last cases; ^eTransmission rate was defined as the number of the infected cases divided by the total number of the preschoolers in the same class. CA=coxsackie A virus; PCR=polymerase chain reaction; HFMD=hand-foot-and-mouth disease.

Rates of virus detection

Viral infection was defined as a positive culture result, positive real-time PCR, or positive VP1 RT-PCR followed by direct sequencing from throat swabs. The yield rate

for viral culture in these EV outbreaks was 43%, 25% and 0% (Table 4). However, real-time PCR had significantly higher yield rates, and VP1 RT-PCR followed by direct sequencing was also significantly more sensitive than viral

culture, detecting 77% of the viruses during the CA16 outbreak. The overall yield rates for viral culture, real-time PCR, and VP1 RT-PCR followed by direct sequencing were 28.6% (8/28), 64.3% (18/28), and 39.3% (11/28), respectively ($p=0.02$). Therefore, real-time PCR yielded a higher positive rate of 35% compared with conventional viral culture. Based on the EV viral load measured by real-time PCR, viral culture might give a positive result if the viral load was over 100,000 copies, but VP1 PCR followed by direct sequencing might give a positive result if the EV viral load was over 1,000 copies. During the CA16 outbreak, identical VP1 sequence data suggested that all the infections during this outbreak resulted from the same virus.

Comparison of different coxsackie A virus infections

The clinical comparison of all coxsackie A virus infections is shown in Table 4. The serotypes of the three EV epidemics were CA4, CA2 and CA16. Compared with CA4 and CA2 infections, there were significantly more cases of HFMD with CA16 infection ($p<0.001$). The transmission rates of the three outbreaks were 26%, 35% and 62%, respectively ($p=0.04$). Clearly, there was a particularly high transmission rate of CA16, and most infected cases occurred within a short period (Table 4 and Figure 2). The duration of EV transmission ranged from 12 to 40 days (Table 4).

Discussion

After the severe EV71 outbreak in Taiwan in 1998 with high mortality and morbidity, there was extensive interest in this EV. Most studies focused on EV71 because of its severe complications, high fatality and high transmission rates. However, the transmission rates and transmission duration of other EVs were not clear. This is the first prospective observational study of circulating enteroviruses in a preschool. During the 2-year study, CA2, CA4 and CA16, which were the top five circulating EVs during the last 3 years,⁷ were detected during the eight episodes of EV infection. In this study, compared with other coxsackieviruses, CA16 infection resulted in a significantly higher transmission rate and more HFMD cases. Although there was a similar clinical picture of HFMD, the complication and mortality rate of patients with CA16 infections was significantly lower than those with EV71 infection.⁸

To date, there have been no reports on the transmission rate and duration of transmission for enteroviruses among preschoolers. This study provides important data about the duration and rate of transmission of different enteroviruses in 2–3-year-old preschoolers. We found that the duration of EV transmission within the same class could be as long as 40 days (Table 3). This might be related to prolonged stool viral shedding, poor hand hygiene, or close contact between these children. The duration of EV excretion may range from 3 to 11 weeks, and fecal-oral route is considered to be the most important route of transmission.⁹ Hand-washing, mask wearing and suspending a class if two index cases were noted within 1 week are some of the measures that have been taken in Taiwan. However, it is difficult to remind susceptible young children about personal hygiene, since the care givers and parents have to pay more attention to the isolation of infected children. In addition, the study children were residing in an open community, so there may have been more opportunities to contact other infected persons, at home or in the park for example. The factors affecting the transmission duration are complex and multiple. However, we still consider that the outbreaks of CA16 derived from the same source because their VP1 sequences were identical.

The transmission rates of the different coxsackie A viruses were significantly different. The transmission rate of CA16 was the highest (up to 62%), that of CA4 was 26%, and that of CA2 was 35%. The reason for the significantly higher transmission rate of CA16 needs further investigation.

The rapid diagnosis of EV infections would avoid unnecessary antibiotic use and decrease the clinical cost. Although being the gold standard of pathogen identification, viral culture is a labor-intensive and time-consuming work for clinical researchers. The low sensitivity of viral culture might result from low viral titers, inappropriate transport or storage, and poor growth of the virus.^{10,11} Therefore, accurate and rapid molecular diagnostic tools, e.g. real-time RT-PCR, play an important role in the identification of the pathogen. Compared with conventional cell culture, PCR appears to yield higher positive rates in different specimens (e.g. throat swab, rectal swab and cerebrospinal fluid), ranging from 29% to 68%.¹² In this study, real-time PCR yielded a higher positive rate (35%) compared with conventional viral culture. Also, VP1 RT-PCR followed by direct sequencing may help to identify

“untypeable” EVs that do not react with existing neutralizing anti-sera or in immunofluorescence tests. For example, we were able to obtain positive VP1 RT-PCR results, and then define serotypes, in some cases with negative viral isolation.

This is a community-acquired survey rather than a hospital-based study, and there are some limitations. The sample size was small, and may be not representative of the transmission rates of all infectious diseases. Furthermore, even though some cases had positive real-time PCR results, further VP-1 sequencing and serotyping failed due to low viral titers.

In conclusion, the transmission of CA16 infections is significantly higher than that of CA2 and CA4 in preschool children, but the actual mechanism underlying the high transmission rate still needs to be investigated. Compared with CA2 and CA4, CA16 infection leads to significantly more cases of HFMD. The transmission duration of coxsackie A viruses ranged from 12 to 40 days.

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