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Original Article

Manifestations of enterovirus D68 and high seroconversion among children attending a kindergarten

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KEYWORDS
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Abstract *Background/purpose(s):* Enterovirus D68(EV-D68) is an emerging disease that affects mostly children. There have been few relevant investigations to clarify transmission and seroprevalence within daycares and kindergartens.
Methods: This prospective cohort study investigated respiratory viral transmission among preschool children in a public kindergarten in Taipei City of Taiwan between September 2006 and June 2008. After children were enrolled, daily monitoring of illness and regular biweekly physical examinations were performed. We performed viral isolation to detect acute EV-D68 infection and neutralization tests to detect specific EV-D68 antibodies and to measure the seroprevalence and seroconversion rates.
Results: Among 190 kindergarten attendees aged between two to five years old, nine children had acute EV-D68 infection in September 2007. The clinical manifestations included pharyngitis, cough and other unspecified upper respiratory tract infection. None of the infected children had acute flaccid paralysis or severe respiratory illness. The phylogenetic tree of partial viral protein 3 and viral protein 1 was clustered in clade A1. The EV-D68 seropositive rate increased from 19% (25/130) at the beginning to 67% (83/124) at the end of the study. The seroconversion rate of 49 children with initial seronegative and paired sera was 73% (36/49).
Conclusions: A high seroconversion rate (73%) for EV-D68 was found among kindergarten attendees, which indicates preschool-aged children are highly susceptible to EV-D68 infection and that the disease burden may be extremely underestimated. Once EV-D 68 circulates, preventive measures may be advocated, especially within kindergartens or daycares, to reduce transmission and subsequent development of severe cases.

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Introduction

Preschool aged children face many kinds of infectious diseases, of which viral infections account for the majority. Enterovirus D68 (EV-D68) is the emerging serotype that has attracted much attention in recent years. EV-D68 was first identified in 1962 and caused an outbreak starting in North America that then spread worldwide in 2014.[1,2] Most of the infected patients were children. The clinical manifestations varied from mild to severe respiratory symptoms and were even complicated with acute flaccid myelitis. According to the Taiwan Centers for Disease Control surveillance data, there have been sporadic cases of EV-D68 during recent years.[3] Most of the patients were younger than five years old. In total, 51 cases were confirmed by viral isolation or reverse transcription polymerase chain reaction (RT-PCR) from June 2015 to December 2017, and 10 patients had neurological complications.[3]

Previous studies of EV-D68 focused only on severe infections or inpatients. A study performed in Japan placed an emphasis on pediatric outpatient cases and found that the hospitalization rate was 9% (six of 69 children).[4] Many EV-D68 cases presented with mild clinical symptoms and might thus be undiagnosed. Viral culture and subsequent genetic sequence analysis to identify the serotype are time-consuming. Therefore, it is very likely that the disease burden of EV-D68 is underestimated. One study performed in Finland revealed that the mean antibody levels against EV-D68 decreased during 1983, 1993 and 2002, suggesting antigenic drift among circulating EV-D68 strains.[5] However, many countries, including Taiwan, lack EV-D68 surveillance or seroprevalence data.

Our team conducted a prospective study to investigate respiratory viral infectious diseases among kindergarten attendees between September 2006 and June 2008,[6] and a cluster of EV-D68 infections occurred in September 2007. Blood sampling was collected for neutralization tests to detect specific viral antibodies before and after September 2007 so that the seroconversion rate could be measured. We reported the clinical manifestations, the seroprevalence and seroconversion of EV-D68 among preschool-aged children at a single kindergarten.

Methods

We enrolled children from one public kindergarten in Taipei city. The kindergarten had six classes grouped by age, and each class included 20 to 35 preschool-aged children (Classes A & B: four-year-olds, Class C: five-year-olds, Classes D & E: three-year-olds, Class F: two-year-olds). There were 193 kindergarten attendees during the 2006 academic year (from September 2006 to June 2007) and 202 kindergarten attendees during the 2007 academic year (from September 2007 to June 2008).

Ethical approval

The institutional review board of National Taiwan University Hospital approved this study. When the academic year began, pediatricians and study nurses met with parents/guardians of the kindergarten attendees and explained the purpose, methods, and potential benefits of this study and the discomfort of sampling. Parents/guardians of the kindergarten attendees gave their written informed consent after the meeting. We gave the results of the viral workup to the parents/guardians of the kindergarten attendees but did not provide any treatment for children found infected by viruses.

Data collection

After written informed consent was obtained from parents or guardians, questionnaires about each child's age, sex, vaccination history and past medical history, including allergic condition, were completed by the parents/guardians. Study nurses would check the questionnaire later, verify the contents and perform a telephone interview to complete it if essential data were missing. Blood samples were collected for baseline EV-D68 neutralization tests in the beginning of the study between March and June 2007 if the parent or guardian agreed. A full-time nurse was responsible for the measurement of the participating children's daily body temperatures by infrared tympanic thermometers and examination for signs of oral ulcers and/or viral exanthems. Study nurses recorded daily health conditions via phone contact or interview. For the participants, pediatricians would perform physical examinations every other week.

If any participant presented with signs and symptoms of infection, we would perform throat and rectal swabs for viral isolation and real-time RT-PCR for specific viruses such as enteroviruses, adenoviruses and influenza viruses. We also followed up the clinical features of each participant and monitored the transmission conditions. At the end of the study, we repeated blood sampling for EV-D68 neutralization tests for participants leaving or graduating from the kindergarten between March and May 2008. Finally, we evaluated the risk factors for and severity of EV-D68 infection.

Laboratory methods

Throat and rectal swabs were submitted to the virology laboratory of National Taiwan University Hospital for virus isolation. Molecular diagnosis and typing were performed at

the molecular viral laboratory of National Taiwan University Hospital. Viral RNA and DNA extraction from viral isolates, throat and rectal swabs were performed by using an Isolation Kit (RNA and DNA extraction kit, Qiagen, Hilden, Germany), and reverse transcription (RT) was performed with 1st strand cDNA.

A Synthesis Kit for RT-PCR (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's instructions. For enterovirus serotyping, semi nested RT-PCR was performed with primers according to a previous report, and the PCR products were purified.[7,8] Then, auto sequencing with the forward primer was performed. The serotypes of the enteroviruses were inferred by comparison of the partial VP1 (viral protein 1) sequence to those in the public gene database containing VP1 sequences for the strains of the human enterovirus serotypes. The detection sensitivity of this enteroviral serotyping was approximately 1000 copies of RNA.

Neutralization test (NT)

The neutralizing antibody test of EV-D68 followed the standard protocol of a plaque reduction neutralization test. Serum samples were heat-treated for 30 min at 56 °C, serially diluted and mixed with 100 50% tissue culture-infective doses of EV-D68, and then incubated for 2 h at 33 °C in microtiter plates seeded with rhabdomyosarcoma cells. Each plate included a cell control, serum control and virus back-titration. The cytopathic effect was monitored from two to seven days after incubation, and the serotiter was determined when the cytopathic effect was observed in one 50% tissue culture-infective dose of the virus back-titration. Cells were fixed with 5% glutaraldehyde and stained with 0.1% crystal violet. Seropositivity was defined as the serotiter ≥ 8.

Phylogenetic study

We constructed a phylogenetic tree based on the partial nucleotide sequences between the VP3 and the VP1 genes of EV-D68. These included nine different strains in the study as well as strains previously isolated in Taiwan and in other countries. GenBank accession numbers for the partial nucleotide sequences of the genes generated in this study are MH780655.1, MH780656.1, MH780657.1, MH780658.1, MH780659.1, MH780660.1, MH780661.1, MH780662.1 and MH780663.1. The phylogenetic tree was built using the neighbor-joining method by the MEGA program 7.0 (Sudhir Kumar, Arizona State University).[9,10] The reliability of the tree was assessed by 1000 bootstrap replicates.

Statistical analysis

We used Student's t-tests and Mann—Whitney tests to examine continuous variables and analyzed categorical variables such as age and sex by the chi-square test. All statistical analysis performed via SPSS version 22 was two-tailed, and $p < 0.05$ was considered statistically significant.

Results

Clinical manifestations of EV-D68

After written informed consent was obtained from the parents/guardians, 180 (93%) of the 193 children were enrolled in the study in the 2006 academic year (between September 2006 and June 2007) and 190 (94%) of the 202 children enrolled in the study in the 2007 academic year (between September 2007 and June 2008). Among them, 130 children underwent baseline blood sampling for neutralization tests between March and June 2007, and 124 children underwent blood sampling when leaving or graduating from the public kindergarten in the end of study between March and May 2008. In total, 59 children underwent blood sampling both at the beginning and at the end of the study.

Soon after the new semester started in the 2007 academic year, 69 children had respiratory tract infections from the second week of September 2007 (Fig. 1). There were 15, 14, 23, seven, six and four infected children in Classes A, B, C, D and F, respectively, among which Class C children accounted for the majority (33%, 23/69). The most common symptom was sore throat or pharyngitis (39%, 27/69), followed by cough (26%, 18/69) and unspecified respiratory symptoms (16%, 11/69). Among them, 7% (5/69) of the infected children had wheezing and sputum production, accounting for 4% (3/69).

Among all cases with throat swabs for viral isolation, nine children were proven to have an EV-D68 infection in September 2007 (Fig. 1). The basic data and clinical manifestations are shown in Table 1. The mean age of the nine EV-D68-infected children was 4.9 years old. The male to female ratio was 2:1. Most children were infected in the second week of September 2007. Two children had an allergic history, but they did not have wheezing during the disease course. None of the nine EV-D68-infected children had oral ulcers or skin rashes. Severe complications, including acute respiratory distress syndrome and acute flaccid myelitis, did not occur during the study period.

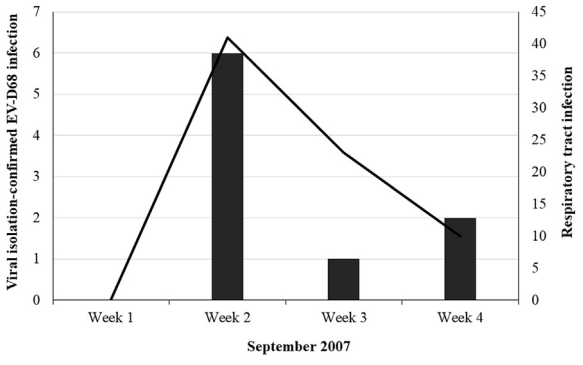

Fig. 1. Time distribution of cases with confirmed EV-D68 infection and respiratory tract infection. A total of nine children had positive viral isolations of EV-D68, and 74 children had respiratory tract infections in September 2007. The bar shows the number of positive viral isolations of EV-D68, and the line shows the number of respiratory tract infections.

Table 1 Basic data and clinical manifestations of viral isolation-confirmed EV-D68 cases in September 2007.

Case	Class	Age (years)	Sex	Onset week	Clinical manifestation	Baseline NT titer[a]	End NT titer[b]
1	A	4.4	Boy	Second	Pharyngitis	<4	16
2	B	4.9	Boy	Second	Pharyngitis	<4	8
3	B	4.6	Girl	Second	Pharyngitis	<4	32
4	C	5.2	Boy	Second	Pharyngitis	<4	16
5	C	5.7	Boy	Second	Upper respiratory tract infection	<4	8
6	D	3.7	Girl	Second	Upper respiratory tract infection	Not checked	Not checked
7	C	5.7	Girl	Third	Cough	Not checked	32
8	A	4.8	Boy	Fourth	Pharyngitis	Not checked	Not checked
9	C	5.8	Boy	Fourth	Upper respiratory tract infection	<4	8

[a] Baseline EV-D68 neutralization test (NT) titer was checked between March and June 2007.
[b] End EV-D68 neutralization test titer was checked between March and May 2008.

Six of the nine viral-isolation-confirmed EV-D68-infected children underwent blood sampling for neutralization tests both in the beginning and end of the study. The results all turned from seronegative at the beginning to seropositive at the end of the study (Table 1).

Seroconversion rate among participants who were seronegative in the beginning

In total, 59 participants underwent blood sampling both at the beginning and at the end of this study. Among them, 49 participants were seronegative for EV-D68 in the beginning and 36 (73%) of these 49 participants became seropositive by the end of study, which provided definite evidence of EV-D68 seroconversion.

Seropositive rates

For all the participants with blood sampling, the seropositive rate in the beginning of study was 19% (25/130). After the EV-D68 cluster infection occurred in September 2007, the seropositive rate increased to 67% (83/124) by the end of study. There was no sex predilection (girls: 67%, 41/61; boys: 67%, 42/63). The seropositive rates and geometric mean titers of EV-D68 neutralization tests among different age groups at the beginning and at the end of study are shown in Fig. 2. Both the seropositive rate and geometric mean titers of EV-D68 NT increased markedly among all age groups by the end of study. There was no statistically significant difference in the EV-D68 NT titers among the different age and sex groups.

According to the phylogenetic tree, all the nine EV-D68 strains identified in our study belonged to clade A1 (Fig. 3). The most recent outbreak strains that caused acute flaccid myelitis worldwide were classified as clades B1, B2 or B3.[11]

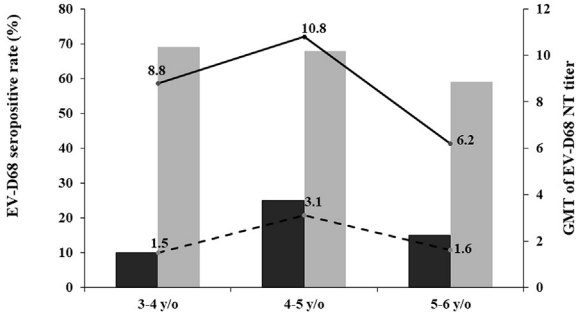

Fig. 2. Change in seropositive rates and EV-D68 NT titers among different age groups. The black bar shows the seropositive rate at the beginning of the study and the gray bar shows the seropositive rate at the end of the study. The dotted line shows the geometric mean titer of EV-D68 NT at the beginning of the study and the solid line shows the geometric mean titer of EV-D68 NT at the end of the study. GMT denotes geometric mean titer.

Discussion

This study revealed that the clinical manifestations of EV-D68 were usually mild and preschool-aged children were highly susceptible. EV-D68 is a unique serotype that differs from most enteroviruses and shares more physiochemical characteristics with human rhinovirus. Human rhinovirus 87 was reclassified as e EV-D68 in 2002.[12] EV-D68 differs from other enteroviruses in terms of its temperature sensitivity, its ability to grow in cell culture at 33 °C (the temperature of the nose) rather than at 37 °C (the temperature of the throat),[13] and its isolation mainly from respiratory specimens but very rarely from stool. There is a limitation in our study, which is that we performed throat and rectal swabs rather than nasopharyngeal swabs when participants had infections. Therefore, the positive isolation rate was low and may have led to underestimation of EV-D68 incidence by culture results.

There are few published data about the seroepidemiological data of EV-D68. One study investigated the seroepidemiology of EV-D68 in infants and children in Jiangsu, China.[14] A total of 319 participants between 6 and 35 months old were included. The seropositive rate of EV-D68 at the beginning of study (January 2012) was 49.2% and increased to 60.5% two years later in January 2014. Since EV-D68 infection causes mostly mild symptoms, it is worthwhile to place emphasis on seroepidemiological data to understand the actually public disease burden.

In the present study, a high seroconversion rate (73%, 36/49) was found in children who were initially seronegative. The seropositive rate was 19% (25/130) at the beginning and 67% (83/124) at the end of study. The

Fig. 3. Phylogenetic dendrogram based on the partial nucleotide sequences of the VP3 and VP1 genes. The sequence of J67071 EV-D70 JPN 2007 was used as the outgroup. These EV-D68 sequences included different strains from those in this study (marked with ▲), from Taiwan in different years, and from various countries in different years. The strain name, country, and year are shown in each sequence and the GenBank accession numbers are indicated in parentheses. TWN denotes Taiwan; USA, United States of America; JPN, Japan; CHN, China; SEN, Senegal; and ITA, Italy. The numbers at the nodes are percentages indicating the levels of bootstrap support based on a neighbor-joining analysis of 1000 replicates.

seroepidemiological change indicated that EV-D68 infection among preschool-aged children did occur during this study period and it might take place in this public kindergarten, households or community. EV-D68 has marked seasonal variability with a late summer to fall predominance,[15] unlike other enteroviruses that typically occur in the summer. EV-D68 was observed to circulate for about one month in September 2007 in our study. Outbreaks in the US in 2014 and many other countries also started in the fall (mostly in September) in the Northern Hemisphere.[1,16–18] Another explanation for EV-D68 circulation in September 2007 is that kindergarten attendees have increased exposure to respiratory viruses after the start of the new school year or after the beginning of kindergarten.[6]

EV-D68 can be further classified into three clades, A, B, and C, based on VP1 region. Clades A and B can be further divided into subgroups, subclades A1 and A2 and subclades B1, B2 and B3.[19] The EV-D68 cultured in our study in 2007 belonged to clade A1. No participant had acute respiratory distress syndrome or acute flaccid myelitis. According to the Taiwan Centers for Disease Control monitoring data, a total of 92 strains of EV-D68 were collected between 2007 and 2016[20]; subclades A1, A2 and B1 were the prevalent genotypes from 2007 to 2013.[21] However, the predominant genotype shifted to clade B3 in 2014.[20,21] Similar findings were mentioned in another Chinese study which reported that strains of clade A were predominant (90.9%) from August 2006 to August 2011, and clade B strains emerged in October 2011.[22] In addition, severe infected cases isolated from Taiwan and China in 2014 belonged to clade B3.[19,23,24] VP1 is mostly involved in viral binding to host receptors as well as in host immune recognition. Both sequence and structural analyses indicate that viral adaptation to the local host environment and/or viral evasion from host immune recognition most likely contributed to the spread of Clade B EV-D68 strains in the US.[25] We believe that different clades of EV-D68 might have different viral virulence and may contribute to variant disease severity and clinical manifestations,[19] which requires further investigation in the future. In addition, various genetic lineages of circulating EV-D68 strains might have contributed to a rise in serious infections.[26]

Among the nine children having positive viral isolation of EV-D68, the male to female ratio was 2:1. This finding is similar to those of previous studies in which boys had higher severities of most infectious diseases than did girls.[27,28] One study performed in the Netherlands found that the male to female ratio among EV-D68 infected patients was 1.5.[16] However, the seropositive rate at the end of our study was the same between males and females. Neutralization antibody titers also showed no statistically significant differences between the sexes. Another study in China did not find significant differences in EV-D68 seroepidemiology between the sexes, either.[29] This suggests that the incidence of EV-D68 infection is similar between the sexes but that males may have a higher proportion of symptoms or severe disease.

The predominant patient group of enterovirus infection is children. Most of the EV-D68 infected cases in the 2014 worldwide outbreak were children.[1] Moreover, children younger than five years old are more vulnerable to enterovirus infection and have higher mortality rates.[14] Both seroconversion rates and neutralization test titers of EV-D68 showed no statistically significant differences among different age groups in our study. Preschool aged children are just starting to learn hygiene practices including handwashing hygiene and proper cough and sneeze etiquette. Viral transmission is easy because of inadequate personal health habits, close contact when playing together and environmental contamination of potentially infected surfaces and objects.[30] This study revealed that preschool-aged children were highly susceptible to EV-D68 infection. We believe that our results reflect a more accurate estimation of the disease burden of EV-D68 because our study participants belonged to a general population rather than an inpatient group. We also provide some information about the seroepidemiology of EV-D68 in Taiwan and the impact of EV-D68 on the health of kindergarten children. Cautious monitoring and preventive measures are mandatory during the circulation period.

There are some limitations in this study. First, this cohort study was performed between 2006 and 2008, approximately ten years ago. There have been changes in social behaviors, public health care and prevention that

might result in different transmission rates at present. Second, we only monitored the cohort in one kindergarten in Taipei city. Different countries, settings and socioeconomic statuses may affect the transmission of EV-D68; therefore, our results might not reflect the transmission rate in other conditions. Third, although we performed daily monitoring for illness among the kindergarten attendees on weekdays, we did not monitor nor did we perform viral isolation during the weekends, and the sensitivity of viral isolation might not be high. We might have missed many cases of acute EV-D68 infection; therefore, only nine children had positive viral isolations of EV-D68. However, we tested their EV-D68 neutralization antibodies at the beginning and at the end of this cohort study to minimize this limitation.

In conclusion, EV-D68 circulated for about one month in a public kindergarten in September 2007 and most of the infected children had mild upper respiratory symptoms. The high seroconversion rate among kindergarten attendees in our study indicates that EV-D68 is highly communicable. The disease burden of EV-D68 may be extremely underestimated if meticulous monitoring is not performed. Once EV-D68 circulates, preventive measures may be implemented, especially within kindergartens or daycares, to reduce transmission and subsequent severe cases.

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