Serological Study of Neutralizing Antibodies Against Enterovirus D68 Among Healthy Population in Xiamen, China

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Abstract

**Background**: Enterovirus D68 (EV-D68) infections poses a serious public health threat, which caused outbreaks of severe respiratory illness and has been closely associated with "polio-like" central nervous system disease. The patterns of immune responses to EV-D68 have not been fully understood. This study was performed to investigate the level of neutralizing antibody against EV-D68 among the healthy population in Xiamen City.

**Methods**: A total of 515 serum samples from healthy people were selected by stratified random sampling in Xiamen City in 2016, and the titer of neutralizing antibody against EV-D68 was detected by an efficient micro-neutralization assay.

**Results**: The overall seroprevalence of neutralizing antibody against EV-D68 was 81.9%, and the overall geometric mean titers (GMT) was 248.0. The positive rates were 42.2%, 49.3%, 71.9%, 94.2% and 98.5%, respectively, in groups of <1, 1-3, 4-6, 7-19 and 20-39 years old, and were up to 100% in the 40-59 and ≥ 60 age groups. The GMTs in these seven groups were 1:39.8, 1:80.6, 1:133.2, 1:283.7, 1:253.3, 1:308.1 and 1:405.0, respectively. The seroprevalence and GMT of EV-D68 showed an increased trend with age, while gender and geographic area didn’t show significant impact.

**Conclusions**: EV-D68 infection was prevalent in Xiamen City. Children are the high-risk groups for EV-D68-related illnesses, especially those aged ≤ 3 years. It is necessary to establish preventive measures and strengthened the surveillance on EV-D68 to prevent disease outbreaks.

Background

Enterovirus D68 (EV-D68), a member of human enterovirus D ([family Picornavirus, genus Enterovirus](#)), is a pathogen that can cause acute respiratory diseases[1]. Unlike most enteroviruses, EV-D68 is acid-sensitive and prefers to be grown at a lower temperature, which is similar to human rhinoviruses (HRVs) [2]. EV-D68 replicates in the human respiratory tract, that results in a wide range of clinical symptoms from mild cold-like illness, such as fever, cough and sore throat, to severe lower respiratory tract infections (LRTIs) and central nervous system diseases, including bronchitis, pneumonia, paralysis, acute flaccid myelitis (AFM), or even death[3–5]. Notably, children with a history of asthma or wheezing and adults with underlying diseases are more likely to develop severe EV-D68 infections[6, 7]. EV-D68 was first isolated from children with LRTIs in California in 1962, while the infection cases were rarely reported until an upsurge of cases has occurred during the last decade worldwide[1, 8–12]. Particularly, an outbreak in the United States in 2014 accounted for more than 1000 cases and 14 deaths of children[13]. EV-D68 infection was first identified in Beijing, China, in 2006[14]. Since then, more and more EV-D68-related illnesses had been reported in other regions of China, such as Shandong[15], Zhejiang[16], Shanghai[17], Jiangsu[18], Hong Kong[19] and Taiwan[20]. However, no vaccine or specific therapy is yet available.

Due to the lack of a universal EV-D68 clinical detection and report management system, the current low incidence cannot accurately reflect the prevalence of the virus in China. Therefore, understanding the
status of neutralizing antibodies (nAbs) against EV-D68 in population is important to assess the prevalence of previous infection and transmission potential of EV-D68. Unfortunately, only a few serological investigations have been conducted in a small part of China (including Beijing[21, 22], Jiangsu[18], Chongqing[23] and Taiwan[24]), as well as in the United States[25], the United Kingdom[26] and the Netherlands[27], the pattern of immune responses have not been well studied for EV-D68. Xiamen is a major seaport and tourist city along the southeast coast of China with an increased floating population. The spread risk of EV-D68 importation into Xiamen exists. In order to further understand the sero-epidemiology of EV-D68, we carried out a serological survey of EV-D68 in a large sample of children and adults in Xiamen City.

**Methods**

**Study subjects and serum samples**

A total of 515 participants aged between 5 months and 83 years, who were previously enrolled in an immune status surveillance of infectious diseases at Xiamen City Center for Disease Control and Prevention (CDC), China, in 2016[28]. All healthy participants were recruited through a stratified random sampling method by districts and age groups, and then divided into seven age groups (< 1, 1-3, 4-6, 7-19, 20-39, 40-59 and ≥ 60 years). The serum samples were stored at -20 °C after centrifugation and inactivated at 56°C for 30 min before testing.

**Measurement of EV-D68 neutralizing antibodies**

EV-D68 neutralizing antibodies in serum samples was measured with an efficient micro-neutralization assay based on enzyme linked immunospot (ELISPOT) assay as previously described[29], with some modifications. Serum samples were serially diluted two-fold from 1:16 to 1:2048 and incubated in 1:1 volume ratio with EV-D68 strain STL-2014–12 (GenBank accession no. KM881710)[30] containing 2×10⁴ TCID₅₀/well at 33°C for 1 h. The mixtures of samples and viruses were added into pre-seeded rhabdomyosarcoma (RD) cells and incubated in a CO₂ incubator at 33 °C for 16 h. After incubation, cells were detected by the ELISPOT assay. The positive and negative control wells were set for each plate. The neutralizing titer was defined as the highest dilution to achieve 50% inhibition of the spots. A titer of ≥ 16 was considered as the cutoff for a positive antibody response.

**Statistical analysis**

All statistical analyses were performed with Microsoft Excel 2010, SPSS 19.0 and GraphPad Prism 7. The titers > 2048 were assigned as 2048. A chi-square test was used to compare the differences in the seroprevalence among different groups, while the log-transformed neutralizing antibody titers of positive serum samples were compared using an unpaired Student’s t test. P< 0.05 was considered statistically significant.
Results

Overall seroprevalence and GMT of EV-D68

In total, 515 participants aged between 5 months and 83 years (male to female ratio was 0.98:1) were tested for nAb against EV-D68. Groups of participants aged < 1, 1-3, 4-6, 7-19, 20-39, 40-59 and ≥ 60 years, included 45, 67, 96, 86, 67, 86 and 68 samples, respectively. The overall seroprevalence of EV-D68 was high, with 422 (81.9%; 95% CI: 78.4-85.0) seropositive samples among 515 participants, and the overall GMT in seropositive samples was 248.0 (95% CI: 215.3-285.7).

The seroprevalence and GMT of EV-D68 in age groups

The analysis of the different age group indicated that EV-D68 seroprevalence trended to increase with age ($z = 131.2, P < 0.0001$), ranging from 42.2% (95% CI: 29.0-56.7) in infants to 49.3% (95% CI: 37.7–60.9) in toddlers to 71.9% (95% CI: 62.2–79.9) in preschoolers, and reached over 90% in groups of participants aged ≥ 7 years, especially as high as 100% in the 40–59 and ≥ 60 age groups (Fig. 1). Highly statistically significant differences in seroprevalence were observed among the 1–3, 4–6 and 7–19 age groups ($\chi^2 = 39.2, P < 0.0001$). Moreover, the GMT of nAb against EV-D68 showed an increased trend with age, except for a small dip at the 20–39 age group (Fig. 1). Children under 1 year of age presented the lowest GMT value (39.8, 95% CI: 21.2–74.8), and the elderly older than 60 years of age had the highest one (405.0, 95% CI: 323.8-506.6). A significant increase in GMT was observed between the 4–6 age group (133.2, 95% CI: 97.5-182.2) and the 7–19 age group (283.7, 95% CI: 203.5-395.6) ($t = 3.262, P = 0.0014$). To further analyze the immune responses, four nAb antibody titer ranges were defined: no (< 1:16), low (1:16 – 1:1:32), moderate (1:64 – 1:256) and high (1:512-1:2048). The analysis indicated that most participants aged ≥ 7 years presented with moderate and high nAb titer levels, whereas children aged ≤ 6 years with low and moderate ones (Fig. 2).

The seroprevalence and GMT of EV-D68 in gender and district groups.

The overall seroprevalence against EV-D68 were 82.8% (95% CI:77.64–86.9) and 81.2% (95% CI:76.0-85.4) in males and females, respectively. As shown in Table 1, EV-D68 seroprevalence trended to increase with age, from 45–100% of males and from 40–100% of females, but no significant gender-specific difference in seroprevalence of EV-D68 was observed ($\chi^2 = 0.2203, P = 0.6388$). In addition, the overall GMT in seropositive samples was 206.8 (95% CI:171.0-250.1) and 234.3 (95% CI:195.0-281.5) in males and females, respectively, and gender also didn't show significant impact on GMT value ($t = 0.9309, P = 0.3524$). Moreover, serum samples were collected from 6 districts of Xiamen City (Table 2). There was no significant difference in seroprevalence of neutralizing antibody against EV-D68 between downtown and suburbs ($\chi^2 = 2.727, P = 0.099$), although slightly lower GMT value for EV-D68 was found in the city center when compared to the suburb group ($t = 2.715, P = 0.0069$). These data indicated that gender and geographic area are not the main factors affecting the EV-D68 epidemic trend in Xiamen City.
### Table 1
Seroprevalence and GMT of nAb against EV-D68 by gender

| Age groups (year) | Gender | Seroprevalence% (95%CI) | GMT (95%CI) |
|-------------------|--------|--------------------------|-------------|
|                   | Male   |                          |             |
| < 1               |        | 45.0 (25.8–65.8)         | 34.6 (12.1–98.5) |
| 1–3               |        | 51.4 (35.6–67.0)         | 83.8 (39.6–177.2) |
| 4–6               |        | 75.5 (61.9–85.4)         | 132.9 (87.2–202.6) |
| 7–19              |        | 93.3 (82.1–97.7)         | 217.1 (133.2–353.7) |
| 20–39             |        | 96.7 (83.3–99.4)         | 201.6 (125.5–323.9) |
| 40–59             |        | 100.0 (91.8–100.0)       | 315.7 (223.1–446.6) |
| ≥ 60              |        | 100.0 (89.6–100.0)       | 501.4 (379.6–662.1) |
|                   | Female |                          |             |
| < 1               |        | 40.0 (23.4–59.3)         | 45.3 (17.6–116.1) |
| 1–3               |        | 46.7 (30.9–63.6)         | 77.0 (41.3–143.6) |
| 4–6               |        | 68.1 (53.8–79.6)         | 133.7 (81.8–218.5) |
| 7–19              |        | 95.1 (83.9–98.7)         | 378.5 (240.9–594.6) |
| 20–39             |        | 100.0 (90.6–100.0)       | 303.0 (197.1–466.0) |
| 40–59             |        | 100.0 (91.8–100.0)       | 300.8 (214.4–422.0) |
| ≥ 60              |        | 100.0 (90.1–100.0)       | 331.2 (234.1–468.5) |

### Table 2
Seroprevalence and GMT of nAb against EV-D68 in geographic regions

| Regions | Seroprevalence% (95%CI) | GMT (95%CI) |
|---------|--------------------------|-------------|
| Downtown| 78.1 (71.5–83.5)         | 170.1 (137.0–211.1) |
| SM      | 68.7 (58.1–77.6)         | 134.4 (95.3–189.5) |
| HL      | 86.3 (78.0–91.8)         | 200.3 (151.7–264.7) |
| Suburb  | 84.0 (79.7–87.5)         | 249.8 (212.0–294.4) |
| HC      | 81.0 (71.3–87.9)         | 344.0 (245.0–483.2) |
| JM      | 87.8 (79.0–93.2)         | 217.4 (155.3–304.3) |
| TA      | 82.0 (72.8–88.6)         | 217.8 (156.6–303.5) |
| XA      | 85.4 (76.2–91.4)         | 243.6 (177.4–334.7) |

### Discussion
Recently, EV-D68 has emerged as one of the major pathogens causing respiratory infection illnesses worldwide. Our study conducted a cross-sectional analysis of the seroprevalence and GMT of neutralizing antibodies against EV-D68 of healthy people of all ages, which can comprehensively and objectively reflect the distribution profiles of EV-D68 nAb among healthy population in Xiamen City. Our data showed that the overall seroprevalence and GMT were high, at 81.9% and 248.0, respectively, which indicating that a high percentage of people have been exposed to EV-D68.

In this study, we found that the seroprevalence of EV-D68 was increased with age. More than 90% of people aged ≥ 7 years and 100% of people aged ≥ 40 years were positive for nAb against EV-D68 in Xiamen in 2016, which were similar to those in Taiwan in 2017[24]. A nearly 100% seroprevalence was also found for the adults in the Beijing of China[22, 31], the United States[25], the United Kingdom[26] and the Netherlands[27]. In addition, our results showed that the GMT of nAb against EV-D68 trended to increase with age, from 39.8 in infants to 405.0 in the elderly. Moreover, a retrospective study of serum samples collected between 2007 and 2011 revealed that the anti-EV-D68 nAb levels increased over time in adults[31]. Together, these data suggest that not only infants and children can get infected by EV-D68, but also adults, and most adults have been infected with EV-D68 in their lifetime.

In the general population, EV-D68 infection is usually mild or asymptomatic, and most young people aged ≥ 7 years and adults present with moderate and high anti-EV-D68 nAb titer levels after infection. Therefore, there is no report of EV-D68 outbreak in China so far, which may be mainly attributed to the herd immunity against it. However, our survey showed that more than 50% of young children were negative of nAb against EV-D68, and the anti-EV-D68 nAb levels of positive subjects was low. Significantly, the seroprevalence and GMT value of EV-D68 increased with age among children aged ≤ 6 years. These findings indicated that children were the high-risk group for EV-D68-related illness, especially children aged ≤ 3 years, which is closely associated with their poor immunity. Moreover, children of this age group have insufficient self-protection awareness, and their maternal transferred antibodies decrease with time after birth. Therefore, it is very necessary to strengthen the joint prevention and control of the epidemic of EV-D68 infection in families, communities, nurseries and kindergartens.

In previous studies, we investigated the seroprevalence and GMT of nAb against eight major human enteroviruses, including A (Enterovirus 71, Coxsackievirus A16, Coxsackievirus A6 and Cosackievirus A10) and B (Cosackievirus B3, Cosackievirus B5, Echovirus 25 and Echovirus 30) species, among healthy population in Xiamen City in 2016[28]. We found that the seroprevalence of most of these eight enteroviruses, as well as EV-D68, increased with age and subsequently reached a plateau, but the positive rates of them (ranged from 14.4–42.7%) were obviously lower than EV-D68 (81.9%). For GMT value, children and adolescents showed higher nAb titers than others in these eight enteroviruses, whereas the anti-EV-D68 nAb levels showed an increased trend with age. These serological characteristics indicated that, in contrast, all populations are susceptible to EV-D68, and the virus can be spreading efficiently, which is closely related to its mainly route of transmission by air.

Conclusions
In conclusion, this study demonstrated that EV-D68 has spread rapidly in Xiamen of China in recent years. Because the lack of specific immunity to EV-D68 in susceptible population, it is necessary to strengthen the surveillance on EV-D68 and improve the development of the vaccine and therapy for EV-D68-related illness prevention and control.

**Abbreviations**

EV-D68: Enterovirus D68  
GMT: Geometric mean titers  
HRV: Human rhinovirus  
LRTI: Lower respiratory tract infection  
AFM: Acute flaccid myelitis  
NAb: Neutralizing antibody  
CDC: Center for Disease Control and Prevention  
ELISPOT: enzyme linked immunospot  
RD: rhabdomyosarcoma

**Declarations**

**Ethics approval and consent to participate**

This study was approved by the ethics committee of Xiamen City CDC and Xiamen University. Written informed consents from participants and/or their parents or legal guardians were obtained.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

No conflict of interest to declare by any of the authors.

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Authors' contributions

Y.L, R.Z, L.X, T.C, S.H, N.X, contributed to the experimental design. Y.L, R.Z, L.X, Q.Z, Y.Q, C.L, contributed to the manuscript preparation. Y.L, Y.W, Z.Y, H.Y, contributed to the virus preparation. S.H, contributed to serum samples collection. Y.L, Y.W, Z.Y, H.Y, performed the micro-neutralization assay. Y.L, R.Z, L.X, Q.Z, Y.Q, C.L, contributed to statistical analysis. All authors approved the final version. All authors discussed the results and commented on the manuscript.

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**Figures**
Figure 1

Seroprevalence and GMT of EV-D68 neutralizing antibodies in different age groups among healthy population.
Figure 2

Distribution of neutralizing antibody titers of EV-D68-seropositive samples by age.