Epidemiological Report

Prevalence of Antibodies against Enterovirus 71 in Children from Lu’an City in Central China

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SUMMARY: We performed preepidemic and postepidemic serological surveys to elucidate the rate of enterovirus 71 (EV71) infection in Lu’an City, Anhui Province, Central China. For the preepidemic study, a total of 472 healthy infants and children (age range, neonates to 15 years) were randomly selected before the 2008 outbreak of EV71 in the region. Blood samples were collected and tested for neutralizing antibodies (NAbs) against EV71 by performing a microneutralization assay. The results of preepidemic serological survey showed that 43.2% (204/472) of the tested samples yielded positive results for NAbs against EV71. The seropositivity rates were 29.6% (93/314) in children who were 0–7 years of age and 74.6% (59/79) in children who were 12–15 years of age. The overall geometric mean titer was 18.1, and the highest antibody titers were detected in children who were 5–7 years of age; this suggests that this group was frequently exposed to EV71 infection. For the postepidemic study, 83 serum samples were collected from healthy children ≤15 years of age in 2010. The seropositivity rate of EV71 NAbs increased in this young population after the 2008 outbreak, especially in 2- to 11-year-old children. This report shows that EV71 was spreading in Lu’an City before the 2008 outbreak, and children under 7 years of age were the main susceptible population.

INTRODUCTION

Human enterovirus 71 (EV71) is a nonenveloped, positive-strand RNA virus that belongs to the family Picornaviridae, genus Enterovirus, and species human enterovirus A (1). On the basis of molecular typing, EV71 strains can be divided into three genogroups, A, B, and C; the B and C genogroups are further subdivided into B0–B5 and C1–C5, respectively (2,3). EV71 is transmitted via the fecal-oral route or via direct contact with throat discharge or the fluid from blisters. EV71 infections manifest most frequently as a mild exanthema known as hand, foot, and mouth disease (HFMD); however, a small number of EV71 infections can be associated with severe neurological and cardiopulmonary complications, such as aseptic meningitis, pulmonary edema, and acute flaccid paralysis.

EV71 was first isolated in California, USA, in 1969 (4). Since then, EV71 has been found in many countries worldwide and has been shown to cause life-threatening outbreaks in young children, mostly in the Asia-Pacific regions (5). Recent outbreaks of infection have been reported in Malaysia in 1997 (6); Australia, 1999 (7); Singapore, 2000 (8); Japan, 1997 and 2000 (9,10); Taiwan, 1998 (11); and mainland China, 1998, 2004, and 2008 (12–14). EV71 was initially isolated in China from clinical specimens collected from HFMD patients in Wuhan City in 1987 (15); soon after, HFMD had reportedly spread to most provinces in China. Since the 1987 epidemic spread rapidly, EV71-associated HFMD received considerable attention from both physicians and the local governments, and HFMD was classified as a category C notifiable infectious disease in China, along with influenza, mumps, and rubella. In China, a total of 39 notifiable infectious diseases are classified into three categories (A, B, and C) on the basis of their epidemic situation, degree of infection, etc. Category C diseases are generally considered less harmful than those in categories A and B. Subsequently, EV71 infection became an endemic disease and posed a significant threat to public health in China.

Between late March and August 2008, an epidemic of HFMD attributable to EV71 occurred nationwide in China, with more than 176,000 reported human cases and at least 40 casualties (16). Most of the victims were children aged 6 years or younger, but no studies were conducted to investigate the seroepidemiology of EV71 infection during and shortly after the epidemic.

Few studies have investigated the seroprevalence of EV71 in Central China. Therefore, we conducted preepidemic and postepidemic serological surveys in children from Lu’an City. The results indicated that infection was common before the 2008 epidemic, especial-
ly in young children aged 5–7 years. This age range coincides with the age at which children begin educational programs (such as kindergarten and preschool), which have recently become very popular in China.

**MATERIALS AND METHODS**

**Cell culture:** Rhabdomyosarcoma (RD) cells were cultured in a humidified incubator at 37°C with 5% CO₂. These cells were grown in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum, 2 mm L-glutamine, and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). All cell culture reagents were purchased from Gibco Life Technologies (Gaithersburg, Md., USA).

**Virus:** EV71 strain 1401-Luan (CHN)-08 (genogroup A of EV71; GenBank accession no. GQ117125) was used for the microneutralization assay. This strain had antibodies against EV71, with low GMT values of 44.1%, 4.5%, 31.0%, 40.5%, 60.9%, and 70.0%, respectively. Analysis of sera sampled 1 year later in 2007 revealed seropositivity rates of 36.4%, 5.4%, 26.0%, 50.0%, 72.7%, and 79.5% in the 6 age groups (Fig. 1). Differences between the seropositivity rates in 2006 and 2007 for each age group were not statistically significant (P > 0.05).

The overall seroprevalence of EV71 among the 472 sera samples collected before the 2008 outbreak was 43.2%, with a GMT of 18.1. Antibody titers <1:8, 1:8–1:64, 1:128, and ≥1:256 were observed in 50.6%, 39.4%, 5.6%, and 4.4% of samples, respectively. In terms of gender, we observed a small difference in the seroprevalence of EV71 between male (53.9%) and female (44.9%) participants.

**Microneutralization assay:** The serum samples were heat-inactivated at 56°C for 30 min and stored at -70°C. Neutralizing antibodies (NAb) against EV71 were detected using a microneutralization test, as described previously (18). Briefly, 50 μL of twofold serially diluted serum (1:8 to 1:2,048) were mixed with 50 μL of EV71 strain 1401 containing 100 TCID₅₀ in each well of a 96-well plate. The plates were incubated at 37°C for 2 h, and then 100 μL of RD cell suspension (1 x 10⁵ cells/mL) was added. Every serum sample was tested in duplicate, with cell-only, serum-only, and virus-only controls in each plate. Virus backdrops were also included in each assay. An assay was considered successful if the result of backdrops was 32–320 TCID₅₀/well. The plates were incubated at 35°C for 7 days, and CPE was observed daily under a microscope. NAb titer was defined as the highest serum dilution that could result in a 50% inhibition in the development of CPE. After 7 days, the cells were fixed using 5% glutaraldehyde and stained with 0.1% crystal violet. The sample was considered to be positive for NAb against EV71 if the neutralizing titer was equal to or greater than 1:8. Undetectable titers (negative sera) were assigned a level of 2 for calculation of the geometric mean titer (GMT) (19). The GMT and 95% confidence interval were calculated, and statistical analysis was performed using Student t test. A P-value < 0.05 was considered statistically significant.

**RESULTS**

Analysis of the serum samples collected in 2006 for the preepidemic serological study showed that the 6 age groups (<1, 1, 2–4, 5–7, 8–11, and 12–15 years) had seropositivity rates of 44.1%, 4.5%, 31.0%, 40.5%, 60.9%, and 70.0%, respectively. Analysis of sera sampled 1 year later in 2007 revealed seropositivity rates of 36.4%, 5.4%, 26.0%, 50.0%, 72.7%, and 79.5% in the 6 age groups (Fig. 1). Differences between the seropositivity rates in 2006 and 2007 for each age group were not statistically significant (P > 0.05).

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The seroprevalence of EV71 among children increased with age after the children reached 1 year of age (Fig. 1). Our results showed that 40.0% of the neonates had antibodies against EV71, with low GMT values of 1:8–1:64; these antibodies could have been derived from their mothers. After 1 year of age, seroprevalence declined to 5.1%, suggesting that maternal immunity was waned in majority of the children. In children between 2 and 4 years of age, the percentage of EV71-positive sera increased to 28.3%; it further increased to 45.6% in children who were 5–7 years of age (Fig. 1). These data suggest that the seropositivity rate might increase at an average rate of 17% annually in children between 2 and 7 years of age. In samples from 8–11 year-old children, age-specific seroprevalence reached 65.4%. Seropositivity further increased to 74.6% in children who were 12–15 years of age (Table 1). The seroprevalence rate was 29.6% (93/314) in children who were 0–7 years of age. Thus, 70.4% of the preschool children (age, ≤7 years) had no NAb against EV71 infection, while majority of children who were 15 years of age had NAb against EV71. Therefore, children under 7 years of age represent the main population (70.4% of all cases) susceptible to EV71 infection.

To analyze the immunity levels in each age group, 4 groups were defined according to the NAb titers detected as follows: <1:8 (negative titer level), 1:8–1:64 (low titer level), 1:128 (medium titer level), and ≥1:256 (high titer level). We observed that serum containing high lev-
Fig. 1. Seroprevalence of neutralizing antibodies against EV71 in 208 serum samples collected in 2006 and 264 serum samples collected in 2007 from Lu’an City in Central China before the EV71 outbreak in 2008.

### Table 1. Seropositive number and GMT value of antibody against EV71 in different populations

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<td>&lt;1</td>
<td>1</td>
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<tr>
<td>No. samples</td>
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<td>78</td>
</tr>
<tr>
<td>No. positives</td>
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<tr>
<td>Positive rate (%)</td>
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<tr>
<td>GMT (1:X)</td>
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<td>3.6</td>
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Fig. 2. The neutralizing antibody levels against EV71 in Lu’an City in Central China before the EV71 outbreak in 2008.

el (≥ 1:256) of EV71 NAbs was mostly observed in 5–7 year age group, followed by 8–11 year age group. Serum containing medium levels (1:128) of NAbs was also most frequently observed in the 5–7 year age group, while low titer levels (1:8–1:64) were generally found in the 12–15 year age group (Fig. 2).

The 6 age groups (<1, 1, 2–4, 5–7, 8–11, and 12–15 years) showed GMT values of 7.8, 3.6, 18.2, 30.3, 24.6, and 20.2, respectively. On the basis of these results, the GMT seems to increase with age, reaching a peak when the children were 5–7 year age group and then declining slightly in children who were >7 years of age (Table 1). There was a statistically significant difference between the GMT values in preschool children (0–7 years of age, with a GMT value of 11.2) and those in older children (8–15 years of age, with a GMT value of 22.3) (*t* test = 2.97, *P* < 0.05).

Additional postepidemic serological studies were per-
formed using samples collected after the 2008 outbreak. EV71 NAbs were detected in 5.6%, 66.6%, 71.4%, 72.7%, and 77.7% of the healthy children who were 1, 2–4, 5–7, 8–11, and 12–15 years of age, respectively. The GMT levels were similar in each group before and after 2008 outbreak (Table 1).

**DISCUSSION**

A few previous studies have investigated the seroprevalence of anti-EV71 antibodies in some countries such as Japan in 1979 (23) and 2009 (21); Brazil, 2002 (24); Singapore, 2002 (25); Germany, 2009 (26); and Taiwan, 2002 (27) and 2010 (22).

In Japan, Hagiwara et al. (23) analyzed 137 samples from healthy children who were 0 to 10 years of age, living in the city of Kawasaki; they detected EV71 NAbs in 6.6% of the samples. Mizuta et al. (21) analyzed NAb titers against 7 subgenogroups of EV71 in 83 residents of Yamagata Prefecture and observed that NAbs from the different subgenogroups were cross-reactive. The seropositivity rates were 24.1% (7/29) in children under the age of 6 years, 44% (11/25) in children between the age of 6 and 14 years, and 51.7% (15/29) in children above the age of 14 years. In Brazil, patients with fever and exanthema were studied (24), and EV71 seroprevalence was 40.8% (97/238) in children under 15 years of age. In Singapore, age-specific seroprevalence ranged from 0.8% in children under 2 years of age to approximately 50% in children over 5 years of age. Moreover, the GMT of EV71 antibodies was higher in preschool children than in school-age children (25). In Taiwan, preepidemic age-specific seropositivity rates for EV71 NAbs in the children were 48% at birth, 1% between 6 and 12 months of age, and 11% at 24 months of age (22). A different study in Taiwan indicated that postepidemic EV71 seropositivity rates in the most susceptible children (less than 3 years of age) were 25% in rural areas and 11% in metropolitan cities (27). However, in Germany, NAbs against EV71 were detected in 60.3% of the tested samples from individuals who were 10 months to 75 years of age (26). The seropositivities of the individuals in the 0–3 year, 3–6 year, 6–10 year, and 10–15 year age groups were 27.3%, 45.6%, 56.4%, and 67.2%, respectively. In older participants, seropositivity reached 75% and remained stable.

Our results show that the seroprevalence of EV71 in Central China was higher than that reported in Japan in 1979, lower than that reported in Germany, and similar to those reported in Singapore, Taiwan, and Japan in 2009.

EV71 seroprevalence was recently investigated by another research group in China (28). They analyzed the presence of antibodies against EV71 and coxsackievirus A16 in 900 serum samples from children who were ≤5 years of age in 2005. In the study, 32.0% of the samples yielded positive results for EV71; this was similar to our results (seropositivity rate of 29.6% among children 0–7 years of age). Zhu et al. (28) reported a GMT value of 1:8.5, which is similar to the GMT value observed in our study (1:8.9 in children 0–4 years of age). Furthermore, we observed that the GMT peaked at 1:30.3 for children who were 5–7 years of age, before gradually decreasing in the older groups (Table 1).

Our data showed that up to 70.4% of the preschool children (age, ≤7 years) had undetectable EV71 NAbs, which may explain why most infections occur in this age group. In the older age groups, more than 50% of the children were seropositive. For example, majority of the individuals who were 15 years of age had EV71 NAb. Our observations revealed that EV71 had spread over a large area in Lu’an City before the 2008 outbreak of HFMD, and the virus represented a serious risk to the health of the children.

In addition, the GMT analysis indicated that the infection was common in children who were 2–15 years of age. This finding was supported by the following observations. (i) Very few children <2 years of age had EV71 NAbs. The seropositivity rate was 22.4% (35/156) in children <2 years of age and 56.6% (179/316) in children who were 2–15 years of age (Table 1). (ii) The proportion of seropositive children peaked after 5 years of age. For example, the seropositivity rates were 45.6% (36/79) in 5–7-year-olds, 65.4% (52/79) in 8–11-year-olds, and 74.6% (59/79) in 12–15-year-olds. (iii) The GMT of anti-EV71 antibodies increased until the children were 5–7 years of age and then declined, suggesting that the endemic spread of EV71 infection mostly occurs in children under 7 years of age. Therefore, substantial levels of transmission of EV71 infections may have occurred in places where preschool-age children congregate, such as day-care centers and kindergartens.

From April to December 2008, an outbreak of HFMD occurred in Lu’an City in Central China. There were 1,311 reported HFMD cases, and the age of the patients ranged from 20 days to 15 years. The age-specific distribution rates among the 1,311 patients were as follows: 0.01%, under 1 year of age; 67.7%, between 1 and 5 years of age; 29.2%, between 6 and 10 years of age; and 2.9%, above 10 years of age (29). Most patients had mild infection, and no deaths were reported. Since poor immunity against EV71 was observed in the younger age groups in our study, we suggest that children under 7 years of age are mainly susceptible to EV71 infection (accounting for 70.4% of all cases).

On the basis of the serological data collected after the HFMD outbreak in 2008, the positive rate of anti-EV71 antibody increased significantly in healthy children who were 2–11 years of age (Table 1).

This study is limited in scope to children in Lu’an City in Central China. A serological study of EV71 NAbs in preschool teachers and parents may prove useful in developing a better understanding of the transmission of EV71 infections in the region.

In conclusion, EV71 infection occurs frequently in children from the central region of China, and mostly preschool-age children are susceptible to this infection. Further control strategies against HFMD, such as immunizing preschool children, should be implemented when the EV71 vaccine is available in China.

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**Conflict of interest** None to declare.
REFERENCES