Neutralizing antibody responses to enterovirus and adenovirus in healthy adults in China

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Hand, foot and mouth disease (HFMD) is an important public health problem that has emerged over the past several years. HFMD predominantly infects children under seven years old and occasionally causes severe disease in adults. Among the enteroviruses, enterovirus 71 (EV71) and coxsackievirus 16 (CA16) are the major causative agents of HFMD. In addition, adenovirus cocirculates with enterovirus and has become a possible additional pathogenic factor for HFMD in some cases. Here, we have investigated the neutralizing antibody responses to both enterovirus and adenovirus in adults, with the aim of exploring the prevalence trends of these viruses.

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Keywords: AdC7; AdHu5; CA16; EV71; hand, foot and mouth disease; seroprevalence; neutralizing antibody
(AdC7), in the serum of healthy adults. AdHu5 is a subgroup C adenovirus and is one of the most common adenoviruses circulating in both children and adults. AdC7 originated in chimpanzees and is thought to be a rare serotype in humans, although little is known about its prevalence in the human population. Chimpanzee adenoviruses have been considered ideal carriers for the development of vaccines against a broad range of pathogens because their neutralizing antibodies are rare in humans, and this low antibody prevalence would circumvent the negative effects of pre-existing immunity to common human serotypes of adenovirus.17,18 Evaluating the prevalence of neutralizing antibodies to AdC7, compared with AdHu5, may provide evidence supporting the potential use of AdC7 as an additional or alternative vaccine carrier.

MATERIALS AND METHODS

Human serum samples

Serum samples from 391 healthy adult humans were collected at random times throughout 2012 by the Suzhou Industrial Park Centers for Disease Control and Prevention. Two hundred and seven study participants were from Jiangsu province, while the remaining participants were from the other 20 provinces and cities in China. All serum samples were heat inactivated at 56 °C for 30 min prior to testing. Informed consent was obtained from each study participant, and the collection and use of serum samples for this study were approved by the Human Ethics Committee of the Suzhou Industrial Park Centers for Disease Control and Prevention.

Cells and viruses

Human rhabdomyosarcoma (RD) and human embryonic kidney (HEK293) cells were maintained in complete Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA), 2% penicillin and streptomycin (HyClone, Logan, UT, USA). EV71 strain G082 (genogroup C4) and CA16 strain SZ05 (GenBank accession NO EU262658), kindly provided by Dr Zhong Huang (Institute Pasteur of Shanghai, Shanghai, China), were amplified in RD cells and purified by sucrose gradient centrifugation. Recombinant replication-defective adenoviruses AdHu5 and AdC7 expressing green fluorescent protein were constructed in our laboratory using published protocols.17 The recombinant adenoviruses were amplified in HEK293 cells to generate high-titer viral stocks and then purified by cesium chloride density gradient centrifugation. All four viruses were dissolved in phosphate buffered saline with 10% glycerol and stored at -80 °C for use in all tests in this study.

EV71 and CA16 neutralization assays

The titers of the EV71 and CA16 viruses were determined using the Reed and Muench method to analyze the cytopathic effects observed in infected RD cells and were expressed as 50% tissue culture infectious doses (TCID50). The neutralization antibody tests for EV71 and CA16 were performed as previously described.19,20 Viruses were diluted to 2 TCID50/μL. Fifty microliters of diluted viruses was mixed with 50 μL of two-fold serially diluted serum, at dilutions of 1:10–1:1280, in 96-well plates and incubated at 37 °C for 1 h. RD cells (1×104) were added to each well after incubation. Seventy-two hours later, the 96-well plates were examined by fluorescence microscopy. Dulbecco’s modified Eagle’s medium without serum was used as the negative control. The neutralizing antibody titer was expressed as the reciprocal of dilutions for which the proportion of green fluorescent protein-expressing cells was reduced to approximately 50% of that for the negative control. A titer ≥ 20 was regarded as positive for the serotype-specific neutralizing antibodies.

Data analysis

Seroprevalence rates were compared between viruses using the Chi-square test. Analysis of variance23 was performed to compare the means of neutralizing antibody titers across different regions, genders and ages. The correlations between neutralizing antibody titers of the viruses studied were calculated using Spearman’s method.24 All statistical analysis was performed using SPSS (Statistical Package for the Social Sciences, Chicago, IL, USA) software (version 16). A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Study participants and characteristics

This study enrolled 391 healthy adults from 21 provinces and cities (Table 1). Participants were aged 18–71 (mean: 30.7 ± 8.9) years and included 226 males and 165 females. Participants were divided into five age groups. The study sample was representative of the geographical distribution of the population of China, with 239 of participants from coastal (Jiangsu, Zhejiang, Shanghai and Shandong) regions and 152 from inland areas (Henan, Shanxi, Hunan, Hubei and other provinces).

| Table 1 Demographics of all serum sample donors (n=391) |
|---------------------------------------------------------|
| **Group** | **n (%)** |
| **Age (years)** | | |
| ≤ 20 | 18 (4.6%) |
| 21–30 | 212 (54.2%) |
| 31–40 | 102 (26.1%) |
| 41–50 | 48 (12.3%) |
| ≥ 50 | 11 (2.8%) |
| **Gender** | | |
| Male | 226 (57.8%) |
| Female | 165 (42.2%) |
| **Areas** | | |
| Coastal | 239 (61.1%) |
| Inland | 152 (38.9%) |

Adenovirus neutralization assays

An adenovirus neutralization assay was performed based on previously described methods.21 Before testing, 10-fold serial dilutions of the recombinant adenoviruses were prepared, and 50 μL of each dilution was added to 96-well plates, followed by 50 μL of Dulbecco’s modified Eagle’s medium with 5% fetal bovine serum. One hundred microliters of HEK293 cell suspension (2.5×10^5 cells/mL) was added to wells in the same 96-well plate. Twenty-four hours later, green fluorescent protein levels were examined by fluorescence microscopy to determine a suitable virus concentration to use in the neutralization test. Viruses were then diluted to this concentration, mixed with two-fold serially diluted (1:10–1:1280) human serum in 96-well plates and incubated at 37 °C for 1 h. After incubation, 100 μL of an HEK293 cell suspension (2.5×10^5 cells/mL) was added to each well and the plates were then incubated at 37 °C in a 5% CO2 atmosphere. Twenty-four hours later, the 96-well plates were examined by fluorescence microscopy. Dulbecco’s modified Eagle’s medium without serum was used as the negative control. The neutralizing antibody titer was expressed as the reciprocal of dilutions for which the proportion of green fluorescent protein-expressing cells was reduced to approximately 50% of that for the negative control. A titer ≥ 20 was regarded as positive for the serotype-specific neutralizing antibodies.22
Seroprevalence and distribution of neutralizing antibodies to four viruses

The seroprevalence rates were as follows: EV71, 85.7% (335/391, 95% confidence interval (CI): 82.2%–89.2%); CA16, 58.8% (230/391, 95% CI: 53.9%–63.7%); AdHu5, 74.2% (290/391, 95% CI: 69.8%–78.5%); AdC7, 11.8% (46/391, 95% CI: 8.6%–15.0%). Seropositivity for anti-EV71 neutralizing antibody was greater than that for anti-CA16 (P < 0.0001), and the seroprevalence of anti-AdHu5 neutralizing antibodies was much higher than that of anti-AdC7 neutralizing antibodies (P < 0.0001). Because EV71, CA16 and AdHu5 have high seroprevalences, the prevalence of neutralizing antibodies to all three of these viruses was calculated and found to be 39.4% (154/391, male/female ratio: 69: 85), with median neutralizing antibody titers of 80, 40 and 640 for EV71, CA16 and AdHu5, respectively.

The distribution of titers for the four viruses is shown in Figure 1. Positive titers were primarily concentrated in the ranges of 10–160 for EV71 and CA16, 160–1280 for AdHu5 and 20–40 for AdC7. Neutralizing antibody titers for AdHu5 in these participants were very high, while the titers for AdC7 were very low, with only one titer reaching 320.

Virus seroprevalence in different geographical regions

Of the 391 healthy adults whose serum samples were collected, 61.1% were from coastal regions and 38.9% were from inland regions. Viral seroprevalence rates in adults from coastal regions were higher than those from inland regions, except for AdC7. However, only the difference in AdHu5 seroprevalence rates between coastal and inland areas reached statistical significance (P=0.001; Table 2). The median neutralizing antibody titers to EV71, CA16, AdHu5 and AdC7 in seropositive individuals were 80, 40, 640 and 40, respectively, in coastal regions, and 60, 40, 320 and 40, respectively, inland. However, there were no statistically significant differences in the neutralizing antibody titers for any of the four viruses between coastal and inland regions (Figure 2).

Virus seroprevalence by gender and age group

The seroprevalence rates for all four viruses were higher in females (Table 3) but only reached statistical significance for CA16 and AdHu5. A significant difference in neutralizing antibody titer was only observed between males and females for CA16 (P=0.006) (Figure 3). Comparing the seroprevalence rates among different age groups, no significant difference was found for EV71, CA16 or AdC7. The seropositivity rate for AdHu5 in the 21–30 years old age group was significantly lower than that in the 41–50 years old age group (P=0.011), but there was no other significant difference between other age groups for AdHu5 (Table 4). There were no significant differences in titer values of neutralizing antibodies between age groups for all four viruses (Figure 4).

Table 2 Seroprevalence of EV71, CA16, AdHu5 and AdC7 in different regions

| Area       | EV71          | CA16          | AdHu5\(^a\) | AdC7          |
|------------|---------------|---------------|-------------|---------------|
| Coastal    | 87.8% (83.6%–92.0%) | 62.2% (56.0%–68.4%) | 80.3% (75.2%–85.4%) | 11.8% (7.6%–15.9%) |
| Inland     | 82.4% (76.2%–88.5%) | 53.6% (45.6%–61.6%) | 64.7% (57.1%–72.4%) | 11.8% (6.6%–16.9%) |

\(^a\) P=0.001; 95% confidence interval shown in brackets.
Correlation between neutralizing antibody titers for different viruses

To analyze the relations between infections with different viruses, a correlation analysis was performed and showed that neutralizing antibody titers for EV71 were associated with those for CA16 ($r=0.145$, $P=0.004$) and AdHu5 ($r=0.126$, $P=0.013$). A significant correlation between neutralizing antibody titers for other viruses was not observed.

**DISCUSSION**

HFMD predominantly occurs in children and can manifest severe symptoms, especially in those younger than three years old. For this reason, most seroprevalence studies of EV71 and CA16 have focused on the pediatric population.$^{6,9,11}$ Adults can also develop HFMD and neutralizing antibodies when exposed to EV71 and CA16. Previous reports showed that humoral immunity mediated by neutralizing antibodies plays a critical role in protection against EV71 and CA16.$^{27,28}$ Although cell-mediated immunity also contributes to protection against EV71 and CA16,$^{27,28}$ Adult infection with these two viruses is mostly latent and asymptomatic, but the mechanisms underlying this response in adults have not been clarified.

A study in Shanghai demonstrated that subclinically EV71-infected children served as a source of continual spread of EV71 in the population.$^9$ Thus, adults who carry EV71 and CA16 asymptotically may pose a significant threat to children. Children are likely to become infected with EV71 and CA16 from adults, although further research is needed in this area. Protection from HFMD should address both children and adults. A seroprevalence study of pre-existing immunity in adults is indispensable, as this immunity may be associated with less clinical severity in adults, and such a study can shed light on the spread of HFMD in children and the general population.

A survey conducted using serum samples from patients tested for other viruses in Germany revealed that CA16 seroprevalence rates were 70%–85% in adults aged 20–59 years, whereas EV71 seroprevalence rates were 40%–48% in adults in the same age range.$^7$ However, another study in Germany found that 75% of healthy adults aged 20–40 years old had neutralizing antibodies to EV71.$^{25}$ In our study, the EV71 seroprevalence rate was 85.7% in healthy adults, higher than that in Germany, while the seroprevalence rate for CA16 (58.8%) was much lower, which may be because HFMD in Germany tends to be associated with CA16 infections and is less commonly due to EV71.$^7$ Yang et al.$^{28}$ reported that 83.3% (10 of 12 samples) of healthy adults were antibody-positive to EV71 in 2008 in Fuyang City, China, where the national HFMD outbreak originated in that year. Another study on neutralizing antibodies in pregnant women in Jiangsu, China found the seropositivity rate for EV71 to be 85.3%, which was similar to our result, while the CA16 seropositivity rate was 89.1%, which was much higher than we found.$^{15}$ We showed that seroprevalence rates for EV71 and CA16 in adults did not vary significantly across age groups and were comparable (EV71: ≥80%, CA16: 54%) to those in children between 5 and 15 years of age, as reported previously,$^6$ suggesting that infection rates for these viruses may be saturated in children older than 5 years.

Enterovirus outbreaks primarily occur during summer and fall in tropical areas, so infection rates may vary across geographic regions. However, no significant differences in seroprevalence between coastal and inland areas were observed in our study. Higher EV71 and CA16 seroprevalence and neutralizing antibody titer values were found in females, but only those for CA16 were statistically significantly different. We speculate that Chinese women are involved in more household cleaning and care of the sick, so their likelihood of getting infected is higher.

Children are susceptible to adenovirus infection, which accounts for 10% of febrile illnesses in children.$^{29}$ A previous study indicated that neutralizing antibody levels for adenovirus, together with those for EV71 and CA16, approached adult levels after seven years of age.$^{1,10}$ Thus, adenovirus may cocirculate with enterovirus in humans. AdHu5 is one of the most common serotypes to infect both children and adults. AdHu5 seroprevalence rates in adults vary from 60% to 70% in Europe$^{31,32}$ and the USA$^{33,34}$ and up to 98% in warmer African and Asian tropical countries.$^{19,35–37}$ Several studies have shown that AdHu5 seropositivity rates in some areas of China ranged from 72% to 77.34% in adults,$^{22,36,39}$ consistent with our results (74.2%). We found AdHu5 seropositivity to be significantly higher in coastal regions than in inland regions, which could be due to the mild and humid climate in coastal areas; it is possible that the coastal climate contributes to the spread of adenovirus infections. We also found a gender difference in AdHu5 seroprevalence, which was not consistent with previous

**Table 3 Seroprevalence of EV71, CA16, AdHu5 and AdC7 by gender**

| Gender | EV71 | CA16$^a$ | AdHu5$^b$ | AdC7 |
|--------|------|---------|------------|------|
| Male   | 82.7% (77.8%–87.7%) | 53.5% (47.0%–60.0%) | 69.9% (63.9%–75.9%) | 11.1% (6.9%–15.2%) |
| Female | 89.7% (85.0%–94.4%) | 66.1% (58.8%–73.4%) | 80.0% (73.8%–86.2%) | 12.1% (7.1%–17.2%) |

*P*=$0.017$; 95% confidence interval shown in brackets.

*P*=$0.026$; 95% confidence interval shown in brackets.

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**Figure 3** Neutralizing antibody titers against EV71, CA16, AdHu5 and AdC7 of seropositive individuals, male and female. Significant difference in neutralizing antibody titers was only observed between males and females for CA16 ($P=0.006$). The box plot shows the minimum, first quartile, median, third quartile and maximum titer levels.
However, AdHu5 and CA16 have similar gender distributions of seroprevalence. In our study, AdHu5 seroprevalence increased with age (21–50 years), with an older population (41–50 years old) having the highest seropositivity (87.5%), in agreement with a previous report.22

Because the prevalence of AdHu5 is so high in humans and because pre-existing immunity to AdHu5 dampens the immune response induced by AdHu5-based vaccines, rare adenovirus serotypes from other species, such as chimpanzees, have been developed and tested as vectors for vaccine development. AdC7 represents a distinct serotype related to human adenovirus and has already been used as a vaccine carrier in many studies, with promising results.40–42 However, the seroprevalence status of AdC7 in humans has not been investigated extensively. In our study, we showed that the AdC7 seropositivity rate in adults is low (11.8%) and similar to that for AdC68 (12.7%) in Chinese adults,22 and that the AdC7 seroprevalence rate does not vary significantly among regions, genders or age groups. Like AdC7, AdC68 is another rare serotype, related to human adenovirus, from chimpanzees.17 The neutralizing antibody titer for EV71 correlated with those of CA16 and AdHu5, which implies these three viruses cocirculate in adults and that adenovirus infection also should be taken into consideration in the prevention and treatment of HFMD.

Here, we have demonstrated that EV71, CA16 and AdHu5 were highly epidemic among healthy adults in China. The correlation between neutralization titers for EV71, CA16 and AdHu5 further confirms that enterovirus cocirculates with human adenovirus in humans. AdC7 rarely circulates in adults, suggesting that it could be a good choice as a vaccine carrier for vaccine development.

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Table 4  Seroprevalence of EV71, CA16, AdHu5 and AdC7 among different age groups

| Age group | n  | EV71          | CA16          | AdHu5          | AdC7          |
|-----------|----|---------------|---------------|---------------|---------------|
| ≤20       | 18 | 61.1% (36.2%–86.1%) | 72.2% (49.3%–95.1%) | 5.6% (0%–17.3%) |
| 21–30     | 212| 85.4% (80.6%–90.2%) | 58.5% (51.8%–65.2%) | 69.3% (63.1%–75.6%)* | 10.4% (6.2%–14.5%) |
| 31–40     | 102| 85.3% (78.3%–92.3%) | 60.8% (51.2%–70.4%) | 79.4% (71.4%–87.4%) | 14.7% (7.7%–21.7%) |
| 41–50     | 48 | 81.3% (69.8%–92.7%) | 56.3% (41.7%–70.8%) | 87.5% (77.8%–97.2%)* | 10.4% (1.5%–19.4%) |
| ≥51       | 11 | 90.9% (70.7%–100%) | 54.6% (19.5%–89.6%) | 63.6% (29.7%–97.5%) | 18.2% (0%–45.4%) |

*P<0.011; 95% confidence interval shown in brackets.

Figure 4  Neutralizing antibody titers against EV71, CA16, AdHu5 and AdC7 of seropositive individuals among different age groups. No significant differences were found in titer values of neutralizing antibodies between age groups for all four viruses. The box plot shows the minimum, first quartile, median, third quartile and maximum titer levels.
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