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**Supplementary Data**

**Seroprevalence of neutralizing antibodies against HFMD associated enteroviruses among healthy individuals in Shanghai, China, 2022**

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**Materials and methods**

**Sample collection**

Serum samples from 307 healthy participants were collected between September 27th and December 7th in 2022 in three local districts (Jing’an, Changning and Yangpu) of Shanghai (**Supplementary Table S1**). All the serum samples were obtained from the routine hand, foot and mouth disease (HFMD) and Dengue Fever Surveillance Program. Healthy individuals were defined as those without any signs of disease at the time of sample collection. Serum samples were stored at -80 ℃ and inactivated at 56 ℃ for 30 min before use.

**Etiological Surveillance**

Clinical samples including throat swabs and faeces from outpatients who are diagnosed with HFMD were collected at local sentinel hospitals in each district in Shanghai. The criteria for clinical HFMD diagnosis were established per the official National Guidelines for HFMD Control and Prevention (2009 edition). These samples were then sent to microbiology labs at the local CDCs, where EV, EV-A71, CVA16, CVA6, CVA10 can be confirmed using commercial real-time PCR Kits. Those EV-positive but EV-A71, CVA16, CVA6 and CVA10 negative were defined as other EV infected HFMD cases.

**NtAb detection**

EV-A71, CVA16, CVA6, CVA10 and CVA4 clinical isolates (**Supplementary Table S2**) were used to quantify neutralizing antibodies (NtAbs) through a micro-neutralization test. Genotyping analysis (**Supplementary Figure S1**) showed that the five isolates belonged to evolutionary branches C4a, B1b, D3a, C and C2, respectively. These isolates represent the predominant genotypes or subgenotypes circulating in the mainland of China (T. Ji et al., 2018, T. Ji et al., 2019, Z. Han et al., 2020, Y. Song et al., 2020, J. Xiao et al., 2022). Viruses were propagated in human rhabdomyosarcoma (RD) cell line and the viral infectivity titers were determined following the official National Guidelines for HFMD Control and Prevention (2009 edition) (<https://www.gov.cn/gzdt/2009-06/04/content_1332078.htm>).

All the samples were diluted serially two-fold (1:8 to 1:2048) in duplicate and incubated with an equal volume of viruses at 100 CCID50/50 μL. After a two-hour incubation at 37 ℃, the mixtures were then added to RD cells in 96-well cell culture plates (1×105 cells/mL) and incubated at 37 ℃ for 7 days. Cytopathic effect (CPE) was observed under an inverted microscope. Each batch of tests included virus back titration, serum toxicity control, and cell control. The virus back titration should be within the range of 32–320 CCID 50/50 μL. NtAb titers were defined as the highest dilution capable of inhibiting over 50% of CPE of the well.

**Statistical analysis**

NtAb titers from positive serum samples were log-transformed to calculate the geometric mean titer (GMT) values and 95% confidence intervals (CI). NtAb titers below 8 and more than 2048 were assigned as 4 and 4096. Titers of ≥ 8 were considered as the threshold of NtAb-positive. All statistical analyses were performed using Graphpad Prism 9.0. The *χ2* test was used to compare the distribution of seropositive rates of different age and gender groups. Kruskal-Wallis test was used to compare the GMT values of different age and gender groups. A *P*-value < 0.05 was considered statistically significant.

**References**

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**Supplementary Figure 1.** Phylogenetic analysis of enterovirus isolates used in current study based on complete *VP1* sequences. The phylogenetic dendrogram was constructed by the neighbor-joining method and validated with 1000 replicates. Only bootstrap values over 70% are shown. Sequences used in the current study were marked with red dot. **A.** EV-A71. **B.** CVA16. **C.** CVA6. **D.** CVA10. **E.** CVA4.

**Supplementary Table S1** Demographic Profile of participants in the current study (n = 307).

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| Groups | N (%) |
| **Age (Mean ± SD, years)** |  |
| ≤ 1 (0.45 ± 0.30) | 13 (4.23) |
| 1–2 (1.2 ± 0.40) | 16 (5.21) |
| 3–5 (4.04 ± 0.82) | 37 (12.05) |
| 6–10 (7.78 ± 1.39) | 42 (13.68) |
| 11–18 (12.72 ± 1.92) | 47 (15.31) |
| 19–40 (29.42 ± 5.56) | 66 (21.50) |
| 41–59 (50.34 ± 5.31) | 56 (18.24) |
| ≥ 60 (63.73 ± 2.71) | 30 (9.77) |
| Overall |  | 307 |
| **District** |  |  |
| Jing'an |  | 47 (15.31) |
| Changning |  | 210 (68.40) |
| Yangpu |  | 50 (16.29) |
| **Gender** |  |
| Male | 160 (52.12) |
| Female | 147 (47.88) |

**Supplementary Table S2** Information of enterovirus isolates used in micro-neutralization test.

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| --- | --- | --- | --- | --- |
| **Viruses** | **Origin** | **Year** | **GenBank Accession No** | **Genotype** |
| EV-A71 | Chinese CDC | 2008 | EU703812 | C4a |
| CVA16 | Chinese CDC | 2007 | GQ429229 | B1b |
| CVA6 | Shanghai CDC | 2016 | OR271573 | D3a |
| CVA10 | Shanghai CDC | 2018 | MW929287 | C |
| CVA4 | Shanghai CDC | 2019 | OR271574 | C2 |

The EV-A71and CVA16 isolates were kindly provided by National Institute for Viral Disease Control and Prevention, Chinese CDC. The CVA6 and CVA10 isolates were isolated from HFMD patients in 2016 and 2018. The CVA4 isolates were isolated in 2019 from a patient with herpangina.