Effect of difference between EV-A71 virus epidemic strain and “vaccine strain” on neutralizing antibody titer

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ABSTRACT
Hand, foot and mouth disease was mainly caused by EV-A71 virus. The main antigen structure of VP1 region of EV-A71 was easily varied. Here, we investigated the seroprevalence of EV-A71 based on a large group of healthy individuals in Beijing, China, in order to study the effectiveness of EV-A71 vaccine in a real-world setting. BrCr and the clinical strain isolated from the Chinese mainland in 2008 (“vaccine strain”: CMU4232/BJ/CHN/2008), EV-A71 C4 epidemic strains isolated in 2010, 2013, and 2016, were tested for neutralizing antibodies (NtAb) in every year. Phylogenetic tree analysis of the EV-A71 strains above, as well as amino acid composition homologous sequence analysis were applied. The “vaccine strain” has 83.0% homology with FY23, HO7 and FY7VP5. It belongs to the same branch of C4a as 10 C4, 13 C4 and 16 C4, and differs from the amino acid sites 283 and 293 of 16 C4. Compared with “vaccine strains,” there was a significant difference between the 50–59 years old age group when the NtAb titer of 16 C4 strain was 1:512–1:1024. Our results suggest that changes in the functional epitopes of NtAb caused by amino acid 283 and 293 loci in EV-A71 strains may affect the production of neutralizing antibodies.

Introduction
Hand, Foot and Mouth Disease (HFMD) was first reported in New Zealand in 1957. Although HFMD is typically a self-limited disease, severe complications may occur including brain-stem encephalitis, acute flaccid paralysis and aseptic meningitis.1,2 It has been implicated in a series of outbreaks across the Asia-Pacific region since the early 20th century.3–7 The largest Asia-Pacific epidemic occurred in China in 2008. According to data from the Chinese Center for Disease Control and Prevention, 24.64 million cases of HFMD were reported in mainland China among which 3,700 were fatal from 2008 to February 2022.

Enterovirus-A71 (EV-A71) was the dominant pathogen in severe and fatal HFMD cases.8 Human EV-A71 is a group of genetically diverse viruses belonging to the genus Enterovirus species A, family Picornaviridae.9 The emergence of the EV-A71 epidemic in the Asia-Pacific region has been associated with the circulation of different genetic lineages (genotypes B3, B4, C1, C2, and C4) that appear to be undergoing rapid evolutionary changes.10 From March 2008 to June 2009, more than 600,000 cases of HFMD and 126 deaths were reported in Fuyang City, Anhui Province, PRC. Subsequently, it was reported that the epidemic spread rapidly to other areas. This was a new epidemic caused by recombinant EV-A71 C4a.11 Then we assume that the EV-A71 outbreak may be caused by the infection of the epidemic strain and the vaccine are of different genotypes, which are phylogenetically unmatched.

Although there is no effective medicine for EV-A71 treatment at present, EV-A71 inactivated vaccine independently developed in China has been approved to appear on the market in December 2015, which can effectively prevent HFMD caused by EV-A71.12,13 However, it is not known whether the widespread vaccination of EV-A71 inactivated vaccine would cause the serotype change of domestic EV-A71 epidemic strains, or the mutation and recombination would lead to the generation of new pathogenic strains. When humans are infected with EV-A71, protective neutralizing antibodies will be generated. The long-term existence of antibodies in the body can effectively protect the body from re-infection,14 and neutralizing antibodies can effectively evaluate the immune effect of vaccines. In the long-term existence of EV-A71, mutations in amino acid sites may occur, whether there is a change in the protective effect of neutralizing antibodies produced by vaccination is unknown.

Therefore, the difference of amino acids between the three EV-A71 epidemic strains and the “vaccine strain” was analyzed, and the effects of serum neutralizing antibodies in healthy people were monitored as well. It is of great significance for prevention and treatment of EV-A71 epidemic, which plays a certain guiding role for EV-A71 vaccine vaccination.

Methods
Serum samples
In this study, the serum samples were collected from 200 healthy human in 2016, including 80 males and 120 females, categorized into five age groups: 15 for aged 10–19, 60 for aged 20–29, 51 for aged 30–39, 45 for aged 40–49, 29 for aged 50–59. The number of males and females in each group and the
average age of each group are shown in Table 1. The study was approved by the ethics committee of the Sixth Medical Center of PLA General Hospital, Beijing, China (Table 1).

**EV-A71 cells and viruses**

The RD cells (human embryo rhabdomyosarcoma) were cultured in DMEM containing 4.5 g/L glucose, L-glutamine, and sodium pyruvate (Corning, 10-013-CVR, USA), supplemented with 10% FBS (Corning, 35-076-CV, USA), 100 IU of penicillin, and 100 μg of streptomycin per ml. The cells were incubated at 37°C with 5% CO₂. The “vaccine strain”, the three epidemic strains and BrCr that harvested from RD were cultured by freezing and thawing three times and were stored at −80°C. The titers of the virus stocks were tested using a modified plaque-forming assay and determining the CCID₅₀.

The BrCr strain was derived from the American 1969 isolate, “vaccine strain” was a clinical isolate from China in 2008, 10 C4 strain (2010 years), 13 C4 strains (2013 years) and 16 C4 strains (2016 years) were all isolated from Chinese clinical isolates of corresponding years, all above are provided by the Centers for Disease Control (CDC).

**Neutralization assays**

The neutralizing potency of the EV-A71 antibody was manipulated by following standard protocols for microplate neutralization assays with some modifications. Initially, the serum sample was diluted to 1:4, inactivated at 56°C for 30 minutes, and stored in a refrigerator at 4°C overnight. The serum was diluted from 1:4 to 1:1028 at the beginning of the experiment, and 50 μL of virus strain (CCID₅₀) was mixed and incubated at 37°C for 2 hours. Finally, the RD cell suspension (2 x 10⁵ cells/mL) was added to the mixture. The plates were then placed in a 36°C CO₂ incubator for 7 days and the potential cytopathic effect was determined under the microscope. Virus back titrations were established for each experiment. This test is considered valid if the virus back titration shows 32–320 TCID₅₀/well. The lowest dilution observed in >50% of the cytopathic effect was considered to be the anti-titer of the serum sample, and a titer >1:8 was considered an antibody positive cutoff.⁶⁻¹⁸

**Data availability**

Thirty-three VP1 sequences of enterovirus types determined in this study have been deposited in the GenBank database under the accession numbers BrCr, FY23, FY7VP5, H07. The nearly VP1 genome sequences of EV-A71 strains associated with “vaccine strains”, 10 C4 (GS 2010–13 T), 13 C4 (GS 2013–009) and 16 C4 (GS 2016–001).

**Statistical analysis**

Chi-square test and t-test were applied for statistical analysis. The antibody titer of >1:8 was considered to be positive. The geometric mean titers (GMT) of antibody titers and 95% confidence intervals (CI) were calculated. Paired t-tests were performed for comparing cross-reactive neutralizing antibodies (NtAb) titers because we measured the serum neutralizing antibody titers against the five EV-A71 viruses for each people. Statistical analyses were performed using SPSS, version 20.0 (SPSS Inc., Chicago, IL, USA). P-values less than 0.05 were regarded as statistically significant.

**Results**

**Phylogenetic tree analysis and difference of amino acid between five strains**

Representative strains isolated from China and other countries in 1998–2017, including “vaccine strains,” FY23, FY7VP5, and H07, were clustered in the C4a genotype. The VP1 genome sequences of “vaccine strains” from this study showed the closest genetic relationship to those of FY23, FY7VP5 and H07 from mainland China shared 97.9%–99.2% nucleotide sequence. 99.8%-100.0% amino acid sequence identity with the closest strains. “vaccine strains” had great similarity in VP1, VP2 and VP3 (Figure 1). The VP1 of “vaccine strains” sequence was identical to amino acid of the three inactivated vaccine strains (FY23, FY7VP5, H07) in China, except four sites of VP1 (Figure 2). The “vaccine strains” for this research were isolated from the 2008 clinical strain, and were analyzed as the “vaccine strain” in this study.

The phylogenetic tree of 33 EV-A71 strains from China and other countries were constructed, including BrCr (BrCr-USA -1970), and belong to the A genotype, which are consistent with previous research findings. The amino acid homology alignment analysis of VP1 of EV-A71 showed that the prototype strain BrCr (type A) were distinctive from other strains, in which 18 amino acid sites were found their positions has changed. The nucleotide homology and amino acid homology of the five strains were within 76.8–97.8% and 93.8–100.0% respectively (Figures 1 and 3).

The sequence homology difference was 15.0% and the same branch was found of “vaccine strains,” 10 C4, 13 C4 and 16 C4 in this study. The four strains were clustered in the C4a genotype. The nucleotide homology and amino acid homology of the four strains were within 96.2–97.8% and 99.3–100.0%, respectively. The 297 amino acid homology alignment analysis of EV-A71-VP1 showed that “vaccine strains” and three epidemic strains (10 C4, 13 C4, 16
Figure 1. Neighbor-joining phylogenetic trees for EV-A71 complete VP1 sequences (891 bp). The nucleotide substitution model used was the p-distance model. One thousand bootstrap replicates were used for construction of the phylogenetic trees; values >70% are shown. The scale bar represents a genetic distance of 0.02 nucleotide substitutions per site. The symbol “■” indicates 2008 clinical isolates; “●” indicates 2010, 2013, 2016 Chinese clinical isolates and BrCr-USA-1970 strain; “▲” indicates three strains of vaccine produced in China, the reference sequences are labeled with GenBank accession no./country/year.

Figure 2. Variations were found in EV-A71 (VP1) of our study. “·” indicates matching to the FY23, FY7VP5, H07.
C4) have two amino acid positions and 54 nucleotide position mutations, and only nucleotide variations of 847 and 877 sites cause amino acid changes, corresponding to 283 and 293 sites, respectively. Still others are meaningless mutations. For the 16 C4 sequences, a substitution of aspartic acid (S) to asparagine (T) at residue 283 (S283T) and a substitution of aspartic acid (A) to asparagine (S) at residue 293 (A293S) were exhibited (Figures 1 and 4).

**Gender difference of five EV-A71 strains at the same age**

No gender-specific difference was found in male and female neutralizing antibody GMT (p > .05). The GMT of BrCr NtAb was lower than other strains. It shows the feasibility of the experimental method (Figure 5).

**Seroprevalence and positive GMT values of five EV-A71 strains NtAb in each age group**

The seroprevalence of “vaccine strain” NtAb was about 80.0% for all age groups. The highest seroprevalence of “vaccine strain” NtAb was 90.2% for 30–39 years old, The NtAb rates of 40–49 and 50–59 were 73.3% and 75.9%, respectively (Table 2). The positive GMT of the “vaccine strain” was at its highest in age group 10–19 (67.5, 95% CI: 28.5–160.0), and the positive GMT reduced gradually with the increase of age. There are significant differences between 10–19 and 20–29 (56.8, 95% CI: 41.0–78.6, p < .001), 30–39 (50.3, 95% CI: 34.9–72.5, p < .001) and 40–49 (24.9, 95% CI: 16.9–36.9, p = .002). The positive GMT was at its lowest in age group 50–59 (22.6, 95% CI: 13.7–37.3). There was no statistical difference between 50 and 59 years old and other age groups (22.6, 95% CI: 13.7–37.3, p > .05) (Table 2).

10 C4, 13 C4, 16 C4 strains, the seroprevalence of three strains NtAb was about 80.0% in all groups. The lowest positive in age group of 10–19 in the 10 C4 (86.7%), 13 C4 (86.7%), 16 C4 (86.7%), but 20–29 in the BrCr (18.3%). Then, it gradually increased, and peaked (88.2%, 96.1%, 88.2%, 42.2%) in 30–39, and decreased slightly (80.0%, 91.1%, 82.2%, 42.2%) in 40–49, and then gradually increased at the age of 50–59 (82.8% and 93.1%) in 10 C4 and 13 C4, but it is still going down in 16 C4 (72.4%) and BrCr (37.9%), GMT of 10 C4, 13 C4, 16 C4 was at its lowest in age group 50–59. Significant differences were detected by pairwise comparison between age groups of 20–29 and 50–59 (10 C4, p = .000; 13 C4, p = .003; 16 C4, p = .000), 30–39 and 50–59 (13 C4, p = .005; 16 C4, p = .001), 40–49 and 50–59 (10 C4, p = .015; 16 C4, p = .015). However, there was statistically significant difference in BrCr between each group except 10–19 and 50–59 (Table 2).

**Correlation analysis of NtAb GMT between “vaccine strain” and other four strains**

EV-A71 NtAb titers between 10 C4, 13 C4, 16 C4 and “vaccine strain” were strongly and positively correlated (r = 0.6267, p < .0001; r = 0.7060, p < .0001; r = 0.6277, p < .0001). No correlation was found between the titers against the “vaccine strain” and BrCr (r = 0.0120, p = .12) (Figure 6).
**Titer distribution of NtAb in seropositive individuals and age-dependent immunity to EV-A71 infections**

To analyze the immunity level, three NtAb titer ranges were defined: 1:8-1:32 (low), 1:64-1:256 (medium), and 1:512-1:1024 (high). Our analysis showed that the distribution of low and high EV-A71 NtAb titers among the different age groups was inconsistent (Figure 7).

The distributions of NtAb titers in “vaccine strain” are shown in Figure 7(a). For the high NtAb titer, the percentages of three groups (aged 30–39 and 40–49 levels) were <5.0%, while those of the remaining age groups were all >5.0%, and the proportion of the titer 1:512-1:1024 among “vaccine strain”-seroprevalence also decreased with age (p < .05).

The distributions of NtAb titers in BrCr are presented in Figure 7(b). In the age group of 10–19 none of the NtAb showed titers in the range of 1:64-1:256 and 1:512-1:1024. On the contrary, for the higher NtAb titer, the highest percentage (3.45%) was found in the older age group (50–59). The distributions of NtAb titers in 10 C4, 13 C4 and 16 C4 are illustrated in Figure 7(c–e). The younger children presented higher NtAb titers, which is similar to the trend observed in case of “vaccine strain.” However, the NtAb titers in 10 C4, 13 C4 and 16 C4 were in medium and high ranges, mostly in 1:8-1:32 or 1:64-1:256. Furthermore, in age between 50 and 59, none of the NtAb titer was of 1:512-1:1024 (p < .05) in 16 C4.

**Discussion**

The EV-A71 genotype C4 first appeared in 1998 and caused the latest large-scale outbreak in China in 2008. It is estimated that the incidence of HFMD in China is 1--2 per 1,000 people, and the number of deaths reported per year is 350–900, mainly young children. Since then, China has required clinicians and hospitals to report clinical cases of HFMD to the National Legal Infectious Disease Surveillance System (NNIDSS) within 24 hours of diagnosis. From 2008 to 2018, China CDC registered a total of 20,537,199 cases of HFMD. In 2018, the incidence of hand, foot and mouth disease in China occurred 169,4129/100,000, and the mortality rate was 0.0025/100,000. Considering the changing trend of HFMD epidemics, we suggest that vaccine strategies for hand, foot and mouth disease should be closely monitored.

In December 2015, China approved three new inactivated EV-A71 vaccine (FY23, FY7VP5, H07) for HFMD, vaccine measures starting in 2016 have reduced the total number of patients in 2016 and 2017 by 17.0% and 22.0%, respectively. It has been reported that the immunization induced by the inactivated EV-A71 vaccine has good persistence within 5 years after the initial vaccination. Furthermore, a phase III clinical trial of inactivated diploid enterovirus has been reported to demonstrate satisfactory safety. However, between 1986 and 2008, the full-length genomic sequence of Taiwan’s popular EV-A71 was internally and recombinated, and dominant genotype changes from B to C or to B occurred at least three times. Subtypes of EV-A71 subtypes were observed over time, but most people did not know their appearance and extinction at the exact time. Therefore, continuous monitoring of the EV-A71 strain is recommended, including monitoring genetic evolution and antigenic changes, monitoring serum neutralizing antibodies, and contributing to the development of the EV-A71 vaccine.

China, however, has seen persistent predominance of subgenogroup C4 over the past 15 years, and represents more exception than the rule. The introduction of three monovalent C4 vaccines in China may however drive strain replacement and lead to a shift in EV-A71 genogroup or subgenogroup dominance, as seen with other infectious diseases. At present, based on the sequence analysis of the VP1 gene, people have a deep understanding of the evolution and molecular epidemiology of enterovirus circulating strains. Studies have shown that in the Asia-Pacific countries, there has been a shift between enterovirus EV-A71 genotypes B and C. Phylogenetic tree analysis shows that the prototype strain BrCr (genotype A) is the most different strain from others. The “vaccine strain” and the three epidemic strains belong to the C4a genotype, and the “vaccine strain” has a certain homology with the inactivated vaccine strain in mainland China.
Although the identified EV-A71 sequences belonged to a single subgenogroup across our study period, novel amino acid changes are also detected when compared to the sequences reported in previous molecular epidemiological studies. Additionally, we identify amino acid residue variations associated with major neutralization/antigenic epitopes. In this study, site variation, nucleotide variations at positions 847 and 877 caused amino acid changes (corresponding to amino acids 283 and 293, respectively) and other nonsense mutations. In addition, polymorphic loci at S283T and A293S in VP1 region have been previously reported. It has been reported that the VP1 mutation is a major determinant of the immunogenicity of EV-A71, and that single amino acid variation in VP1 can lead to the breadth and efficacy of immune responses against major EV-A71 isolates, as well as the sensitivities of EV-A71 to heterologous neutralization difference. In our research, the NtAb titer ranges of 16 C4 was significantly lower than 13 C4 and BrCr [GMT (95% CI): p = .003 vs. p = .000]. In particular, the EV-A71 vaccine had a high mutation rate and frequent recombination, so it was important that we cannot use the same vaccine candidate invariably.

The GMT range of neutralizing antibody BrCr increased with the increase of age, but “vaccine strain,” contrary to the three strains, was on a downward course. In general, there was no significant difference between male and female among the five strains (p > .05). There was no difference between male and female, which indicated that the neutralization method was accurate to detect neutralizing antibody in serum. The results further highlight that inclusion of EV-A71 in the HFMD vaccine was a suitable strategy in vaccine development.

At present, the main method of serological epidemiology research is microneutralization experiment, which is a cross-sectional study of serum antibody positive rate and neutralization antibody titer of different age groups, as well as a retrospective or prospective study of multi-time sampling. According to the change of serum antibody positive rate and neutralizing antibody titer, epidemic characteristics of disease and population immune status can be scientifically evaluated. The positive rate of BrCr serum was less than 50.0%, because BrCr was isolated in 1970, and the infection in the current population was limited. The seropositive rate was high for those of 50–59 years old, and no significant difference was found among all age groups. The high proportion of samples positive for BrCr in the age group of 50–59 could be explained by two hypotheses. This age group was most probably naturally infected during their childhood and most likely with genotypes A, C, which were circulating during the 1950s to 1970s. The immunity raised by natural infection during childhood of the age group of 50–59 could provide better cross-neutralization against different genotypes than that induced by vaccination, as seen for rotavirus and mumps virus.

But “vaccine strain” was different. Fewer individuals were found to be positive against the “vaccine strains” in the 40–49 and 50–59 age groups than other younger age groups (20–29 and 30–39), and there was a significant difference (p < .05). Studies have shown that the more variable P1 region, coding for the viral capsid proteins was believed to undergo mutations more frequently, particularly at the VP1 region, as interactions with antibodies and host cell receptors require
the virus to evolve quickly in order to evade the host immune system, while maintaining its ability to bind to its host receptors. Mutations occur at high rates in RNA viruses due to the lack of proofreading activity in the RdRp, which leads to approximately $1 \times 10^{-4}$ substitutions per nucleotide copied. Such high mutation rate allows EV-A71 to adapt rapidly to selection pressures, which select for beneficial mutations. It can be inferred that the epitope of the C4 strain functional protein has changed in 16 years, and the epitope on the surface protein may make the immune response anti-infective. The type has a certain degree of cross-neutralization. It is concluded that changes in important functional epitopes of this strain caused changes in the titer of neutralizing antibodies, and epitopes on this surface protein may reduce the immune response against infection.

In accordance with the divergent trend of the major HFMD serotype, the overall EV-A71 seropositive rate exhibited

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**Figure 6.** Scatterplot representation of correlation between neutralizing antibody titers (NT50) against “vaccine strains” and four strains. (a) Scatterplot for samples seroneutralization-positive against both “vaccine strains” and BrCr strain. (b) Scatterplot for samples seroneutralization-positive against both “vaccine strains” and 10 C4 strain. (c) Scatterplot for samples seroneutralization-positive against both “vaccine strains” and 13 C4 strain. (d) Scatterplot for samples seroneutralization-positive against both “vaccine strains” and 16 C4 strain.

**Figure 7.** Age-stratified distribution of NtAb titers against. Antibody titers from 10-19-year, 20-29-year, 30-39-year, 40-49-year and 50-59-year of age are shown. The y-axis represents the percentage of the population with a given antibody concentration; the x-axis represents different ages. (a) vaccin, (b) BrCr, (c) 10 C4, (d) 13 C4 and (e) 16 C4 among seropositive participants. NtAb, neutralizing antibody. GMT, geometric mean titers.
a fluctuating downward trend, which also prompted the higher risk of EV-A71 infection over the next several years. High seroprevalence and percentages of high-level EV-A71 NtAbs confirmed a documented EV-A71 “silent” epidemic in the 2008 post-HFMD epidemic period. Our study revealed that older than younger had higher NtAb seroprevalences against BrCr and “vaccine strain.” For BrCr, seropositivity rates and NtAb titer ranges increased with age; however, these rates and titer ranges were generally low. This finding indicates that very few people were exposed to BrCr. For “vaccine strain,” the seropositivity rates and higher NtAb titer ranges showed a decreasing trend with age, indicating that younger people are being continuously exposed to EV-A71 over time, and they often experience repetitive infections. Younger people may have increased chance of having been exposed. This serological finding suggests that EV-A71 is the major pathogen of HFMD and that its inclusion in vaccines is suitable. For “vaccine strain,” in comparison to other serotypes, higher seropositivity rates and higher NtAb titer ranges were observed in the age group of 50–59. Although EV-A71 causes limited damage in adults, the high level of titer also suggests that these individuals were infected with the virus at a young age, making early vaccination meaningful. In the 16 C4 group, there was no higher NtAb titer in 50–59 group. Thus, it is necessary to supervise the antigenic evolution of EV-A71 and update the vaccine in time like influenza viruses.24–27 Although the relationship between antibody titers and protection against reinfection remains unknown, high NtAb levels are always considered to indicate recent infection. In our study, the highest percentage of high level EV-A71 NtAbs was in the group of 10–19 and 20–29. As such, monitoring surveillance of changes within the VP1 region of EVs may allow for the early identification of epidemiological and clinical changes, as well as the identification of potential novel vaccine targets.28

**Limitations**

In this study, there is an absence of serum in 0–10-year-old children, and therefore studies on EV-A71 vaccine have certain limitations. The five strains were clustered in C4a Genotype, although it had an impact on evaluating the effectiveness of vaccine, the neutralizing antibody titer of C4 genotype could also be evaluated.

**Conclusion**

In conclusion, the VP1 protein plays a major role in the infectivity, replication and virulence of EVs and is the primary target for neutralizing antibodies. Our study revealed that with the passage of time, the amino acid of EV-A71 differed, and this difference was observed in the neutralization experiment of serum in 2016. In summary, if we use the vaccine produced by the isolated strains in 2008, the protective effect of the current vaccine has an impact.

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**Authors’ contributions**

All authors contributed to the study conception and design. Material preparation, manuscript finalization and data analysis were performed by Yiwei Ding. Project supervision was performed by Zhihai Han. All authors read and approved the final manuscript.

**Consent to participate**

Informed consent was obtained from all individual participants included in the study.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Ethics approval**

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the Sixth Medical Center of PLA General Hospital, Beijing [No. 201,907,100].

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**Data Availability statement**

The data sets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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