The Immungenicity and Cross-Neutralizing Activity of Enterovirus 71 Vaccine Candidate Strains

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Abstract

Objective: This study aimed to evaluate enterovirus 71 (EV-A71) vaccine candidate strains, including their genotypes, immunogenicity and cross-neutralization capacity.

Methods: From clinical samples, EV-A71 strains were separated by using Vero cells. Six strains were chosen for vaccine candidates, and the sequences were analyzed. To detect the immunogenicity of the strains, we used them to immunize NIH mice at 0 and 14 days. Cytopathic effects (CPE) were examined to determine the EV-A71 neutralizing antibody (NTAb) titer 14 d after the first and second inoculations. To evaluate the cross-neutralizing capacity of the EV-A71 vaccine candidate strains, we tested serum immunized mice with ten EV-A71 genotype strains.

Results: Six EV-A71 vaccine candidate strains were identified, all belonging to sub-genotype C4, the prevalent genotype in China. The sequence similarity of the VP1 regions of the six candidate vaccine strains and three approved inactivated vaccines was 97.58%–97.77%, and the VP1 amino acid similarity was 98.65%–99.33%. Experiments were performed to evaluate the immunogenicity and cross-neutralizing activity of the EV-A71 vaccine candidate strains. The strains had good immunogenicity 14 d after two immunizations, inducing an NTAb titer ranging from 1:94 to 1:346. The NTAb seroconversion rates 14 d after one immunization were above 80% (except HB0007), and significantly increased immunogenicity of EV-A71 strains was observed post-inoculation. Furthermore, our candidate vaccine strains had broad cross-neutralizing activity after challenge with ten sub-genotypes of EV-A71. The highest NTAb titer/lowest NTAb titer ratios of sera against EV-A71 sub-genotypes were 8.0 (JS0002), 8.0 (JS0005), 21.3 (HB0005), 21.3 (HB0007), 10.7 (HB0040) and 8.0 (GD0002), respectively.

Conclusions: Our EV-A71 strains had good immunogenicity and cross-neutralization activity, and have the potential to serve as vaccine strains for multivalent hand, foot and mouth disease vaccines.

Key words: enterovirus 71, genotypes, immunogenicity, cross-neutralization, hand, foot and mouth disease (HFMD), vaccine
BACKGROUND

Enterovirus 71 (EV-A71), a single-stranded RNA virus in the Picornaviridae family, has been associated with a range of diseases, including hand, foot and mouth disease (HFMD) and central nervous system complications. EV-A71 mortality rates can be particularly high in children 5 years of age and younger, and it is considered the most severe neurotoxic enterovirus. HFMD has become a major public health problem, and it was listed as a category-C infectious disease in China in 2008.

The genome of EV-A71 contains a single-stranded positive genomic RNA approximately 7,400 bp in length [1]. Although EV-A71 includes only one serotype, 11 sub-genotypes have been identified to date, with genotypes A, B (including sub-genotypes B1–B5) and C (including sub-genotypes C1–C5) [2–4]. The EV-A71 BrCr strain (A sub-genotype) was first isolated from a woman with encephalitis in 1969 in California [5], and was the only A genotype among EV-A71 virus strains. In the 1980s, the B1 and B2 sub-genotypes became epidemics in Europe and America [6–9]. Then the C1 sub-genotype gradually replaced B2 in the late 1980s to early 1990s. In 1997, hundreds of children died from HFMD due to the C2, B3 and B4 sub-genotypes in the Asia-pacific region. In 2004–2005, C4 replaced the B4 sub-genotype as the main genotype in Taiwan [10]. In mainland China, C4 has remained the predominant subgenotype since its first isolation in 1997 [3,11,12]. Vaccination is the most economical and effective method to prevent and control the spread of infectious diseases. Three EV-A71 inactivated vaccines have been approved in China and have been demonstrated to be more than 90% effective in clinical trials [2,13–15]. However, recent epidemiological findings have revealed a sharp increase in cases of HFMD due to other enteroviruses [16–21], and EV-A71 inactivated vaccines cannot protect patients against other enteroviruses. Additionally, because of the lack of preventive and therapeutic measures, the development of safe and effective multivalent HFMD vaccines has become an urgent matter, particularly in China.

In our study, we identified six EV-A71 vaccine candidate strains belonging to sub-genotype C4, which is currently prevalent in China. Experiments were performed to evaluate the immunogenicity and cross-neutralizing activity of the EV-A71 vaccine candidate strains.

METHODS

Cells
Rhabdomyosarcoma (RD, Minhai Biotechnology Co., Ltd.) cells were used for viral culture and titer detection, and neutralizing antibody (NTAb) titer detection.

Green monkey kidney cells (Vero cells, Minhai Biotechnology Co., Ltd.) were used for viral culture and titer detection.

MEM solution (Gibco) containing 10% (v/v) fetal bovine serum (Gibco) and 2 mmol-glutamine (Gibco) was used for cell culture; MEM containing 2% (v/v) newborn bovine serum (Lanzhou Minhai Bioengineering Co., Ltd.) and 2 mmol glutamine was used for viral culture, dilution and NTAb titer detection. The pH of the above solutions was adjusted to 7.4±0.2.

Viruses
The EV-A71 vaccine candidate strains were separated from samples with HFMD from the EV-A71 serum epidemiological investigation of Jiangsu Province and Guangdong Province, the surveillance of the EV-A71 vaccine before the phase III clinical trial and the Hubei Center for Disease Prevention and Control. EV-A71 virus B0, B1, B2, B3, B4, C1, C2, C4JP, C4YA and C5 were kindly made available by the National Institute for Food and Drug Control. EV-A71 vaccine candidate strains were propagated in Vero cells. The 50% tissue culture infectious doses (TCID50) were determined by using Vero cells, and the values were calculated with the Behrens-Karber method. Each sample was tested in three replicates.

Sequence amplification and phylogenetic analyses
Total RNA was extracted with a MagMAXTM-96 Viral RNA Isolation Kit (Ambion). One-step RT-PCR amplifications were conducted with a SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase kit (Invitrogen). The forward primer of EV-A71 VP1 was 5′-ATAATAGCAGTACGCGGCAGCC-3′, and the reverse primer was 5′-CAAGATGTCCGGTACCCACTC-3′. The segments were sequenced by BGI LifeTech Co., Ltd., China. Nucleotides of other EV-A71 VP1 regions were obtained from the Genbank database (https://https.ncbi.nlm.nih.gov/genbank/, Table S1). A phylogenetic tree was constructed on the basis of VP1 alignment with the neighbor-joining method in MEGAX software (http://www.megasoftware.net/), and bootstrap values at the branch points were calculated from 1,000 replicates.

Serological assays
Specific-pathogen-free female NIH mice (18–22 g) were immunized with the EV-A71 vaccine candidate strains, which were diluted to an equivalent EV-A71 viral titer of 6.0 logTCID50/ml. Each virus was inoculated in ten mice. The first dose was delivered by intraperitoneal injection at day 0, and a second dose was given 14 d later. Serum samples were collected 2 weeks and 4 weeks after the first immunization. Blood samples were allowed to stand at room temperature for 1 hour before centrifugation at 4000 rpm at 4°C for 10 min, and then were stored at −60°C.

Microneutralization for detection of NTAb
Blood samples were inactivated at 56°C for 30 min and serially diluted two-fold from 1:8 to 1:16384. A total of 50 µL serially diluted sera and 50 µL viral preparation containing 100TCID50 of EV-A71 (523-07T, Genbank No. EU753398.2) were mixed in 96-well microplates and incubated with RD cells. CPE were observed after
incubation for 7 days. NTAb titers of EV-A71 were confirmed when RD showed 50% inhibition of CPE. Samples were run simultaneously with cell control, positive serum control and viral back titration samples. NTAb titers equivalent to or greater than 1:8 were considered sero-positive, whereas titers less than 1:8 were considered sero-negative. Neutralization data were analyzed in GraphPad Prism 9.0 and SPSS 22.0. Common logarithmic transformation of the NTAb titer raw data was used to calculate the geometric mean titer (GMT). Data were considered significant at $P < 0.05$. A comparison of EV-A71 NTAb positive rates was performed with the $\chi^2$ test. NTAb positive rates were tested with Fisher’s exact test.

**Detection of NTAb titers against different EV-A71 sub-genotypes**

The method used to measure NTAb titers against different EV-A71 sub-genotypes (Table S2) was the same as that in Method 2.5, except for the detection of the virus instead of other EV-A71 sub-genotypes (Method 2.2).

**RESULTS**

**Phylogenetic analysis of the EV-A71 vaccine candidate strains’ VP1 amino acid sequences**

Phylogenetic analysis of the EV-A71 vaccine candidate strains and 55 EV-A71 strains identified from the Genbank database was performed on the basis of the VP1 coding region with the neighbor-joining method. The results revealed that the EV-A71 strains could be divided into three genotypes: A, B (including B1 to B5 sub-genotypes) and C (including C1 to C5 sub-genotypes). The EV-A71 vaccine candidate strains belonged to genotype C4 (C4a) (Fig 1), which remains predominant in mainland China. The sequence similarity of the VP1 regions among the six candidate vaccine strains and the reference strains (three inactivated vaccines approved in China) was 97.58%–97.77%, and the VP1 amino acid similarity was 98.65%–99.33%. The amino acids at sites 146, 220, 226, 237, 241, 283, 289 and 293 differed (Table 1).

**Viral titers and growth curves of the EV-A71 vaccine candidate strains**

The TCID$_{50}$ of the EV-A71 vaccine candidates, determined with Vero cells, were 6.4 (JS0002), 6.1 (JS0005), 6.6 (HB0005), 6.4 (HB0007), 6.7 (HB0040) and 6.0 (GD0002) LgTCID$_{50}$/ml, respectively. HB0040 was the highest titer strain, and GD0002 was the lowest titer strain. The growth curves of six candidate strains in Vero cells were consistent (Fig 2). The viral titers generally increased substantially 2 days after inoculation and then increased slightly thereafter. The highest viral titer reached 6.0–7.0 LgTCID$_{50}$/ml on the 4$^{th}$ day post-infection.

**NTAb responses to the EV-A71 vaccine candidate strains**

The EV-A71 vaccine candidate strain NTAb GMTs were not significantly different at each time point, in the same order (Fig 3), and the NTAb seroconversion rate of mice 28 d after the same immunization was 100% (Table 2). However, the NTAb seroconversion rates at 14 d after
immunization ranged from 50% to 100%, and those of JS0002 and JS0005 were significantly higher than that of HB0007 ($P = 0.03$).

The anti-EV-A71 NTAb GMTs elicited by the EV-A71 vaccine candidate strains at 14 d post-inoculation were 1:28.5 (JS0002), 1:13.5 (JS0005), 1:11.1 (HB0007), 1:13.5 (HB00040) and 1:15.5 (GD0002), and no differences were observed in NTAb GMTs among candidate strains ($P > 0.05$; Fig 3).

The NTAb GMTs were 1:230.9 (JS0002), 1:346.4 (JS0005), 1:123.7 (HB0005), 1:93.5 (HB0007), 1:206.9 (HB0040) and 1:150.5 (GD0002), which were significantly higher than those at 14 d ($P < 0.01$; Fig 3), thus indicating markedly increased immunogenicity of EV-A71 post-inoculation. No substantial differences among candidate strains of NTAb GMTs at 28 d were observed ($P > 0.05$).

Cross-neutralization activity of sera from mice immunized with EV-A71 vaccine candidate strains, tested against different EV-A71 sub-genotypes

To evaluate the cross-neutralizing capacity of the EV-A71 vaccine candidate strains, we tested serum immunized mice with ten different EV-A71 genotype strains. The fold difference in each serum sample was calculated as highest NTAb titer/lowest NTAb titer among the ten EV-A71 sub-genotypes. The commonly used fold difference was used to analyze the cross-neutralizing capacity of sera [22–24]. The highest NTAb titer/lowest NTAb titer ratios of sera against ten EV-A71 sub-genotypes were 8.0 (JS0002), 8.0 (JS0005), 21.3 (HB0005), 21.3 (HB0007), 10.7 (HB0040) and 8.0 (GD0002), respectively (Table 3), thus indicating that EV-A71 vaccine candidate strains had broad neutralization capacity for different EV-A71 sub-genotypes. A comparison of the six candidate strains indicated that the order of the highest NTAb titer/lowest NTAb titer ratios from low to high was as follows: anti-JS0002/anti-JS0005/anti-GD0002 (8.0) < anti-HB00040 (21.3). Thus, our findings suggested that JS0002, JS0005 and GD0002 had better cross-neutralization capacity than HB0005 and HB0007. The NTAb GMT against B3 was lowest (176.0), and that against C4JP was highest (1290.2).

### TABLE 1 | Different sites of VP1 amino acids.

| Strain   | Amino acid position |
|----------|---------------------|
|          | 146  | 220  | 226  | 237  | 241  | 283  | 289  | 293  |
| JS0002   | I    | L    | P    | T    | S    | T    | A    | S    |
| JS0005   | I    | L    | P    | T    | S    | T    | A    | S    |
| HB0005   | V    | L    | P    | T    | T    | A    | S    |
| HB0007   | V    | L    | P    | T    | S    | T    | A    |
| HB0040   | V    | L    | P    | T    | S    | T    | A    |
| GD0002   | V    | L    | P    | T    | S    | T    | A    |
| EUB12515 KM | V  | L    | S    | T    | S    | A    |
| HQ328793 Sinovac | V  | L    | P    | T    | S    | S    |
| JX025561 WH | V  | V    | P    | N    | S    | S    |
|          | T    |

### TABLE 2 | NTAb seropositivity rate (dilution ≥1:8) of mice inoculated with EV-A71 vaccine candidate strains.

| Strain   | Seropositive rate (%) |
|----------|------------------------|
|          | 14 d           | 28 d           |
| JS0002   | 100% (10/10)$^a$ | 100% (10/10)$^a$ |
| JS0005   | 100% (10/10)$^a$ | 100% (9/9)$^a$  |
| HB0005   | 90% (9/10)$^b$  | 100% (10/10)$^a$ |
| HB0007   | 50% (5/10)$^b$  | 100% (9/9)$^a$  |
| HB0040   | 90% (9/10)$^b$  | 100% (10/10)$^a$ |
| GD0002   | 80% (8/10)$^a$  | 100% (10/10)$^a$ |

$a,b$: Groups sharing letters indicate significantly different NTAb seropositivity rates ($P < 0.05$).
DISCUSSION

Immunogenicity and cross-neutralization capacity are two key indexes required for the approval of a new vaccine. Selecting a candidate vaccine strain that can induce strong and broad neutralization effects is key to the development of an inactivated vaccine. To select the best vaccine strain, we examined six candidate vaccine strains from different places and times. We found that our candidate vaccine strains all had the C4 sub-genotype, as did the three inactivated vaccines approved in China. The VP1 similarity of the three inactivated vaccines was higher than 99%, and our strains derived from samples since 2017 showed 97.58%–97.77% identity with them, thus indicating that mutations and recombination commonly occur during outbreaks of EV-A71. The annual rate of evolution in both the B and C genotypes of EV-A71 was estimated to be approximately 1.35 × 10−2 substitutions per nucleotide, and EV-A71 is a genetically diverse, rapidly evolving virus [8]. The VP1 of EV-A71 was found to contain important epitopes that contribute to the neutralization of the virus. The synthetic peptide designated PEP27 (amino acids 142–156) carried the EV-A71 IgM-specific immune-dominant epitope [25], and Liu [26] has confirmed that SP70 (amino acids 208–222) is a neutralizing linear epitope of EV-A71. The VP1 amino acid at position 146 in JS0002 and JS0005 were I, and the vaccine strains were V, respectively; at position 220, one of the vaccine strains had V, and our strains all had L, thus potentially influencing differences in immunogenicity. Sun’s [27] study has shown that the amino acid variation in A293S and S283T in VP1 occurs with high frequency, in accordance with our findings. Other VP1 amino acid sites were not among the key sites reported [28–31].

Immunogenicity was tested with sera from mice immunized with our candidate vaccine strains. Our strains showed good immunogenicity at 14 d after two immunizations, inducing NTAb titers higher than 1:94 and a rate seroconversion of 100%. Post-inoculation, the immunogenicity of EV-A71 strains significantly increased. However, the NTAb seroconversion rates at 14 d after one immunization were not all 100%, and the NTAb titer ranged from 1:11.1 to 1:28.5. Only JS0002 and JS0005 showed 100% NTAb seroconversion, and these values were far better than that of HB0007. Mao [4] has reported NTAb seroconversion rates of mice immunized with the three EV-A71 vaccine strains ranging from 90.0% to 100.0% at 14 d after immunization, values better than those of some of our candidate strains, as well as NTAb GMTs ranging from 1:14.9 to 1:54.5 after 14 d inoculation, values similar to our results. The immunizing dose in Mao’s study was higher than that in our study, and the experimental animals (BALB/c and NIH) differed between studies, thus potentially explaining Mao’s better NTAb seroconversion rates.

Broad cross-neutralizing activity is key for vaccine strains. Our candidate vaccine strains displayed good cross-neutralization capacity. The highest NTAb titer/lowest NTAb titer ratios of sera against EV-A71 sub-genotypes B and C were 8.0 (JS0002), 8.0 (JS0005), 21.3 (HB0005), 21.3 (HB0007), 10.7 (HB0040) and 8.0 (GD0002), respectively. Anti-JS0002, anti-JS0005, anti-GD0002 and anti-HB0040 (highest NTAb titer/lowest NTAb titer ratio = 8.0) were found to have the potential to be better candidate vaccine strains. Moreover, Liu [2] and Mao [24] have demonstrated that C4 EV-A71 vaccine strains can provide infants and children with global protection against HFMD caused by different sub-genotypes. However, we used sera of mice immunized with our candidate vaccine strains; therefore, our

### TABLE 3 | Neutralization activity of serum samples obtained from mice challenged with ten sub-genotypes of EV-A71 individually.a

| Viral sub-genotype | Immune sera | GMTs | Highest NTAb titer/lowest NTAb titer |
|--------------------|-------------|------|-------------------------------------|
|                    | Anti-JS0002 | Anti-JS0005 | Anti-HB0005 | Anti-HB0007 | Anti-HB0040 | Anti-GD0002 |                |
| B0                 | 2048        | 384       | 192         | 768         | 1536        | 256        | 597.7         |
| B1                 | 2048        | 512       | 192         | 1536        | 1536        | 1536        | 948.8         |
| B2                 | 1024        | 96        | 24          | 512         | 384         | 384         | 237.2         |
| B3                 | 256         | 128       | 128         | 96          | 192         | 384         | 176.0         |
| B4                 | 256         | 128       | 192         | 128         | 256         | 512         | 217.4         |
| C1                 | 512         | 768       | 96          | 512         | 512         | 512         | 414.4         |
| C2                 | 384         | 512       | 128         | 768         | 512         | 384         | 395.0         |
| C4JP               | 2048        | 512       | 512         | 2048        | 2048        | 2048        | 1290.2        |
| C4YA               | 1536        | 384       | 384         | 1536        | 2048        | 1536        | 1015.1        |
| C5                 | 1024        | 512       | 384         | 1024        | 1536        | 1024        | 828.9         |
| GMTrs              | 841.6       | 322.7     | 170.0       | 627.1       | 772.0       | 672.1       | /             |
| Highest NTAb titer/lowest NTAb titer | 8.0 | 8.0 | 21.3 | 21.3 | 10.7 | 8.0 | / |

aThe detection samples were a mixture of sera from nine or ten mice inoculated with the EV-A71 vaccine candidate strain.
results might be different from those with an inactivated vaccine, which could be influenced by the entire preparation process. More studies evaluating vaccine strains are needed.

In summary, our six candidate vaccine strains had the C4 sub-genotype and showed good immunogenicity after two immunizations. We observed broad cross-neutralizing activity of different EV-A71 sub-genotypes by using a standardized NTAb assay and examining NTAb responses.

CONCLUSIONS

Six EV-A71 candidate vaccine strains were evaluated in our study. They displayed good immunogenicity and cross-neutralization capability, and have the potential to serve as vaccine strains. However, more studies are necessary, including investigations of animal lethality and protective effects, genetic stability and process adaptability assessment. Therefore, further studies will be conducted in our project.

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COMPETING INTERESTS

The authors declare that they have no conflicts of interest.

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