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Immunogenicity Evaluation of Virus-like Particles of Enterovirus 71 in *Hansenula polymorpha*

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ABSTRACT

Objective The virus-like particles（VLPs）of enterovirus 71（EV71）expressed in Hansenula polymorpha, EV71 vaccine was prepared by different test design and the immunogenicity was evaluated. **Methods** Vaccine stock solution with recombinant was obtained from fermented product processed through series of purification operations. EV71 vaccine was prepared with VLPs and the serum neutralizing antibodies（NTAbs）was determinated to evaluate the immunogenicity of different lines of mice, different aluminum adjuvants and different immune doses. **Results** EV71 vaccine was prepared with aluminum hydroxide and immunized SPF NIH mice were more sensitive to determinate NTAbs. Different immune doses of EV71 vaccine all have good immunogenicity, and the second immunization can enhance the immune effect significantly, and the NTAbs increases gradually with the increase of immune dose. The results showed that the immunogenicity in mice was dose-effect relationship with the immune dose. **Conclusion** EV71 VLPs vaccines prepared in this study have good immunogenicity, which provide reference data for the subsequent formulation and the development of polyvalent vaccine.

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Key Words: Enterovirus 71; Virus-like particles; Immunogenicity.

Abbreviation: EV71, Enterovirus 71; VLPs, Virus-like Particles; NTAbs, Neutralizing Antibodies; HFMD, Hand Foot and Mouth Disease.

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Introduction

Hand, foot and mouth disease (HFMD) is a common disease in young children have emerged across the Asia-Pacific regions and have been mainly caused by enterovirus, which is characterized by fever, rash or herpes on the hands, feet, and mouth and so on. Most patients with mild symptoms, few could be complicated with aseptic meningitis, encephalitis, acute flaccid paralysis, respiratory infection and myocarditis, etc. Some severe children show up rapid progression, which is easy to lead to nervous system development retardation and cognitive decline and high disability rate and mortality[1]. A large-scale outbreak of HFMD occurred in Fuyang of China in 2008, the same year HFMD was included in the "Law of the People's Republic of China on Prevention and Control of Infectious Diseases" as a Class C infectious disease by the Ministry of Health[2].

There are more than 20 types of enterovirus causing HFMD, mainly are EV71 and CA16 type, and most severe patients were infected by EV71. In recent years, the number of cases caused by CA6 and CA10 has a tendency to increase [3,4], and surpassed EV71 and CA16 to become the main pathogens even. There is no effective treatment drug of EV71 infection clinical, so vaccination is the best strategy to prevent and control the disease. Lots of vaccine research work was carried out by several institutions and companies for prevent the disease, including inactivated vaccine, attenuated vaccine, subunit vaccine, virus-like particles, etc., and there are three inactivated vaccines on the market already and all had a good protective effect against HFMD caused by EV71, but had no obvious cross-protective effect against other types of enterovirus infection. EV71 vaccine was prepared with VLPs expressed in *Hansenula polymorpha*, and the NTAbs were determinated to evaluate the immunogenicity of different lines of mice (BALB/c\ KM\NIH), different aluminum adjuvants (aluminum hydroxide\aluminum phosphate) and different immune doses, so as to provide experimental basis for the subsequent ormulation and the development of polyvalent vaccine.

Materials and Methods

1. Materials

1.1 Stock solution and Adjuvant: EV71 vaccine stock solution was prepared by the laboratory[5,6]. Aluminum hydroxide and aluminum phosphate adjuvant are provided by production department. Import Aluminum hydroxide adjuvant, purchased on the market.

1.2 Virus and Cell: EV71/523-07T strain (C4, EV71 standard detection strain); Rhab--domyosarcoma cell (RD cell), used for NTAb detection and stored in the lab.

1.3 Animals: BALB/c/NIH/KM mice, SPF, female, 18-22g, purchased from Chinese Experimental Animal Resources Research

Institute for Food and Drug Control or Beijing Vital River Laboratory Animal Technology Co., Ltd.

1.4 Instruments and Equipments: Biosafety Cabinet, LABCONCO/A2; Magnetic agitator, IKA/RCT Basic; Acidity meter, METTLER TOLEDO/FE28, etc.

2. Methods

2.1Vaccine preparation: EV71 stock solution diluted in advance was mixed with adjuvant in a certain proportion, stirred, and adsorpted for 10-30min. EV71 vaccine were prepared according to different test designs follow different adjuvants and immune doses. The final aluminum content of vaccine was 0.5mg/mL, and the prepared vaccines were stored at 2~8℃.

2.2 Immunogenicity Evaluation: Mice were randomly grouped according to experimental design, 10 mice/group and each of which was injected intraperitoneal and immunized once on day 0 and 14, 0.5ml dose per mouse, and blood was collected on 14 day and 28 day after injection. Serum samples were incubated at 37℃ for 1h, placed at 4℃ for 2h, and then centrifuged at low temperature. The serum should be stored below -60℃ for use. The mice were euthanized after the last blood drawing.

2.2.1 Immunogenicity test of different lines of mice: EV71 vaccine was prepared with two dose groups of 0.5μg/0.5mL and 5.0μg/0.5mL dose each mouse by aluminum hydroxide. 6 experimental groups of SPF BALB/c mice, NIH mice and KM mice and 3 blank controls (BALB/c mice, NIH mice and KM mice) were immunized, respectively. Other operations were the same as 2.6 immunogenicity evaluation and the differences in different lines of mice immunized with EV71 vaccine were analysed statistically.

2.2.2 Immunogenicity test of different adjuvants: EV71 vaccine was prepared by using aluminum hydroxide(homemade), aluminum phosphate (homemade) and aluminum hydroxide (imported), the dose of EV71 vaccine was 5.0μg/0.5mL. 60 SPF NIH mice were selected and divided into 6 groups randomly. 3 experimental groups and 3 blank control groups were immunized, respectively. Other operations were the same as 2.6 immunogenicity evaluation and the differences of NTAbs in different adjuvants were analysed statistically.

2.2.3 Immunogenicity test of different immune dose: 70 SPF NIH mice were selected and divided into 7 groups randomly. The EV71 vaccine was prepared by using aluminum hydroxide (homemade) and the dose of EV71 vaccine was 0.25μg/0.5ml、0.50μg/0.5ml、1.0μg/0.5ml、2.0μg/0.5ml、4.0μg/0.5ml、8.0μg/0.5ml. 6 experimental groups and 1 blank control groups were immunized, respectively. Other operations were the same as 2.6 immunogenicity evaluation and the differences of NTAbs in different immune dose were analysed statistically.

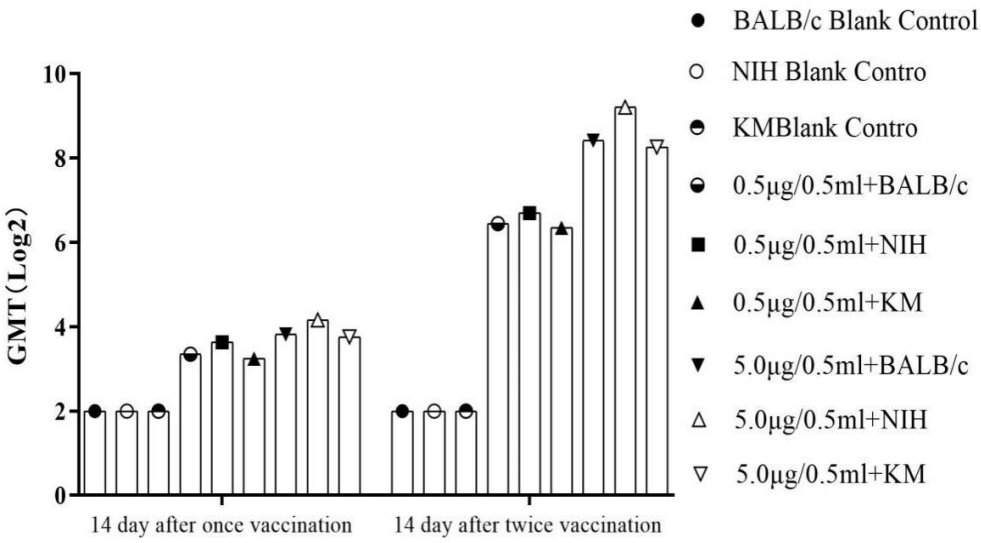
2.4 Determination of NTAbs: Blood samples

were inactivated at 56℃ for 30 min, and serially diluted two-fold from 1:8 to 1:16 384. A total of 50μL serially diluted sera and 50μL virus preparation containing 100$CCID_{50}$ of EV71 (523-07T) were mixed in 96-well microplates and incubated with RD cell. CPE were observed after incubation for seven days. NTAbs of EV71 were confirmed when RD showed 50% inhibition of CPE. Samples were run simultaneously with wells of cell control, positive serum control, and virus back titration. NTAb titers were equivalent to or more than 1:8 assigned as sero-positive, while those less than 1:8 were considered sero-negative[7, 8]. Neutrali--sation data were analysed by GraphPad Prism 9.0 and SPSS 22.0.

Results and Conclusion

1. Immunogenicity test of different lines of mice

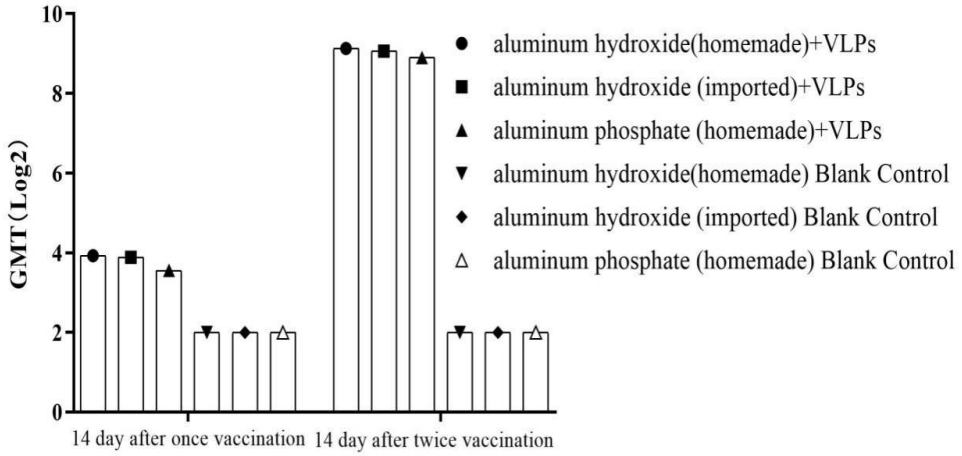
The immunogenicity study result of different lines of mice was shown in figure 1. In the 0.5 μ g and 5.0 μ g immunization dose group, the NTAbs 14 days after immunization in NIH mice were slightly higher than those in BALB/c mice and KM mice at one dose and two doses, but there was no significant difference in statistical analysis. In addition, the seropositive conversion rate of all the three kinds of mice reached 100% 14 days after immunization, indicating that the EV71 vaccines had good immunogenicity in all the three lines of mice.



Figrue 1: Immunogenicity test of different lines of mice

2. Immunogenicity test of different adjuvants

The immunogenicity test results of vaccines prepared with different adjuvants was shown in figure 2. The NTAbs of the three adjuvants, aluminum hydroxide adjuvant (homemade), aluminum phosphate adjuvant (homemade) and aluminum hydroxide adjuvant (imported), were consistent 14 days after immunization at one dose and two doses, with no statistical



Figrue 2: Immunogenicity test of different adjuvants

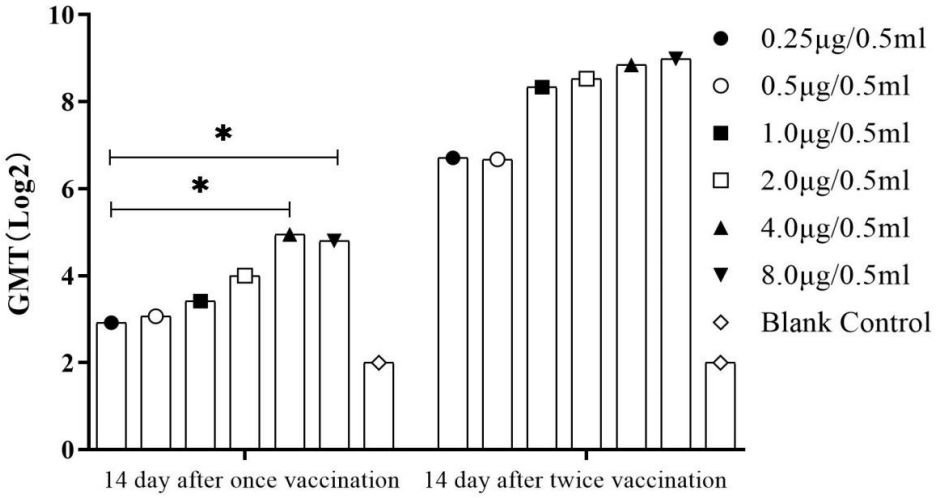
difference. The results indicated that the EV71 vaccine prepared with three aluminum adjuvants in this study had no significant difference in immunogenicity and had well immune effect.

3.Immunogenicity test of different immune dose

The results of immunogenicity test of different immune doses of vaccine were shown in figure 3. The NTAbs in each dose group showed an increasing trend, and the NTAbs of 0.25μg dose group was lower than the 4.0μg and 8.0μg dose group 14 days after immunization significantly ($P<0.05$). There were no significant difference between 0.5μg ~2.0μg dose groups and others.

The results showed that there was a dose-effect relationship between immuno--genicity and immune dose 14 days after once immunization. The NTAbs of the 0.25μg and 0.50μg groups were lower than that of the 1.0μg -8.0μg groups 14 days after the second immunization slightly, and the difference was not statistically significant, indicating that the dose-effect relationship between immunogenicity and immune dose was weakened after the second immunization.

The NTAbs of twice immunization vaccine were higher than once immunization in all groups significantly, indicating that the second immunization vaccine can enhance the immune effect significantly.



Figrue 3: Immunogenicity test of different immune dose

Discussion

The Enteroviruses that caused HFMD belong to two different species and have exist at least 23 serotypes in the past 50 years, including EV71, CA10, CA6, CA16, CA12, CA4, CA14 and CB5, in which EV71 and CA16 serotype are relatively common. The first pathogen of CA16 was isolated from HFMD in 1958, followed by EV71 in the United States in 1969. HFMD was reported firstly in Shanghai in 1981, subsequently it was reported in Tianjin, Wuhan, Shandong, Anhui and other provinces. HFMD has been prevalent in China since then and showed a certain trend of spread about the diseases' outbreak. So far there is no effective antiviral drug for the treatment of the disease still. Therefore, vaccination is particularly important and urgent in prevention of the prevalence of the disease. The genotype of EV71 of HFMD outbreak in China is mainly C4 subtype, and all the three inactivated vaccine on the market are C4a subtype. So we selected the precursor protein (P1) of C4a subtype as the initial sequence of VLP in this study[9].

The VLPs of EV71 expressed in *Hansenula polymorpha* were femented with high density and purified and identified, and the structure of VLPs was similar to natural virus particles. Compared with the traditional inactivated vaccine, it was safe, non-toxic and had good immunogenicity. In this study, it was preliminarily shown that the immunogenicity in mice was dose-effect relationship with the immune dose, and the level of NTAbs increased significantly after

the second immunization, which provided some reference data for the immune program in future. Meanwhile, the selection of mouse line also had a certain effect on evaluation of the immunogenicity of EV71 vaccine accurately. And the NTAbs in NIH mice was slightly higher than in BALB/c mice and KM mice. In addition, the three aluminum adjuvants selected showed no difference in this test, but adjuvants are important components of adjuvant vaccine, and the formulation, preparation process, buffer system, pH and particle size distribution have some impact on the immune effect of the preparation process [10]. There is lots of work to be done to research the preparation process and immunogenicity for the development of novel vaccines or polyvalent vaccines, yet.

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Conflicts of Interest: The authors declare no conflict of interest.

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