Vaccine 33 (2015) 6596–6603

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

A new EV71 VP3 epitope in norovirus P particle vector displays neutralizing activity and protection *in vivo* in mice

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ARTICLE INFO

Article history:
Received 31 March 2015
Received in revised form 13 October 2015
Accepted 24 October 2015
Available online 31 October 2015

Keywords:
Enterovirus 71
Norovirus P particle
Neutralizing antibody
Epitope
Antigen presentation

ABSTRACT

Enterovirus 71 (EV71) and Coxsackievirus A16 (CVA16), as the main agents causing hand, foot and mouth disease (HFMD), have become a serious public health concern in the Asia-Pacific region. Recently, various neutralizing B cell epitopes of EV71 were identified as targets for promising vaccine candidates. Structural studies of *Picornaviridae* indicated that potent immunodominant epitopes typically lie in the hypervariable loop of capsid surfaces. However, cross-neutralizing antibodies and cross-protection between EV71 and CVA16 have not been observed. Therefore, we speculated that divergent sequences of the two viruses are key epitopes for inducing protective neutralizing responses. In this study, we selected 10 divergent epitope candidates based on alignment of the EV71 and CVA16 P1 amino acid sequences using the Multalin interface page, and these epitopes are conserved among all subgenotypes of EV71. Simultaneously, by utilizing the norovirus P particle as a novel vaccine delivery carrier, we identified the 71-6 epitope (amino acid 176–190 of VP3) as a conformational neutralizing epitope against EV71 in an *in vitro* microneutralization assay as well as an *in vivo* protection assay in mice. Altogether, these results indicated that the incorporation of the 71-6 epitope into the norovirus P domain can provide a promising candidate for an effective synthetic peptide-based vaccine against EV71.

1. Introduction

Enterovirus 71 (EV71) is the main causative agent of hand, foot and mouth disease (HFMD) and has been associated with severe neurological diseases resulting in high mortality in young children under the age of six [1–3]. EV71 was first isolated in mainland China in 1987 [4]. From 2008 to 2011, HFMD claimed 1893 lives in mainland China, and these deaths were caused mainly by EV71 infection [5–7]. Three candidate vaccines against EV71 have been developed and evaluated in phase III trials in mainland China, demonstrating good safety profiles and abilities to mediate protective effects [8,9], and antiviral drug studies have also been reported [10–15]. However, no vaccine against EV71 is currently available [16].

EV71 is a small, non-enveloped, positive-stranded RNA virus with a genome size of about 7.4 kb, which is encapsidated within an icosahedral capsid, and the four viral structural proteins (VP1, VP2, VP3 and VP4) function as a single structural subunit, the protomer. Five protomers in turn form a pentamer, twelve of which can self-assemble into a naturally native virion [16–19]. VP1, VP2 and VP3 are arranged at the surface of the virion and therefore are exposed to immune pressure, while VP4 is located internally [20,21].

Analysis of the crystal structure of EV71 to 3.8 Å resolution demonstrated that the EV71 virion has a quasi-T = 3 symmetry with β-sandwich "jelly-roll" folds like other picornaviruses. The most variable regions of picornavirus virions are the loops exposed on the virion surface that are also important neutralizing immunogenic sites [22–24]. For example, Foo et al. reported that residues 208–222 (GH loop) of VP1, which is the most surface exposed viral protein, as an important neutralizing epitope of EV71 [25]. Additionally, Xu et al. identified that residues 141–150 of VP2, alongside the VP2 EF loop, as an EV71 neutralization epitope [26]. Therefore, a peptide-based vaccine containing a neutralizing epitope is considered an attractive and promising approach to stimulate an effective

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http://dx.doi.org/10.1016/j.vaccine.2015.10.104
0264-410X/

and specific protective immune response [25]. However, small synthetic peptides containing linear B-cell epitopes are usually poorly immunogenic [27,28]. Virus-like particles (VLPs), such as Hepatitis B core antigen (HBc) VLPs, human papillomavirus (HPV) VLPs and modular VLPs, as well as norovirus P particles [16,29–34], display morphologies similar to natural virions and have proven to be optimal delivery systems for the presentation of epitopes to the immune system. With the ability to efficiently interact with antigen-presenting cells, VLPs can display heterologous epitopes at high density and maximize the immunogenic and protective potential of neutralizing epitopes.

The capsid protein of norovirus, belonging to the *Caliciviridae* family, is composed of a shell (S) domain and protrusion (P) domain [35,36]. When expressed alone, the P domain can assemble spontaneously into P particles with a $T = 1$ octahedral (24-mers) symmetry. As a new platform for displaying foreign antigen epitopes, norovirus P particles offer several advantages. They can be produced in *Escherichia coli* and *Pichia pastoris* (yeast) easily and stably [20,37]. P particles also can enhance immune responses to the inserted foreign antigen [29,36,38,39]. Conceivably, P particles can be applied as a platform for novel vaccine development against various viral and bacterial pathogens.

Enterovirus 71 (EV71) and coxsackievirus A16 (CVA16) both belong to the genus *Enterovirus* within the family *Picornaviridae* [40], but EV71 vaccines have failed to prevent CVA16 infections [41]. By protein alignment of these two viruses, we found differences in amino acids concentrated on particular regions in different loops based on the crystal structure [24,42]. Therefore, we hypothesized that the regions of differential amino acids concentrated on the surface-exposed loops may be immunodominant and antigenic sites. This hypothesis was confirmed by different genotypes of HPV [43,44]. In this study, conserved regions of EV71 were selected based on alignment of EV71 and CVA16 P1 amino acid sequences [7,45,46], as well as the reported major surface-exposed loop domains of EV71 [42]. P particle-EV71 epitope chimeric vaccines were constructed by insertion of selected EV71 epitopes, including the previously identified neutralizing linear epitope SP70 (208–222 of VP1), into loop 2 of the norovirus P particle [25]. We evaluated the potential of the norovirus P particle as a carrier for delivery of EV71 epitopes to achieve enhanced immunogenicity and protection against viral infection in the mouse model. Analysis of sera from immunized mice led to the identification of the neutralizing epitope 71-6 (residues 176–190) within the VP3 protein of EV71. Study of the crystal structure of EV71 have demonstrated that the 71-6 epitope of the GH loop in VP3 could generate potent neutralizing antibodies [24,47]. Immunized mice developed significantly increased immune responses, and 100% of those animals survived after a lethal challenge with the EV71 virus. These results led to the identification of anew cross-neutralizing epitope (residues 176–190) of VP3 utilizing a P particle carrier.

2. Materials and methods

2.1. Cell lines, media and viruses

Human rhabdomyosarcoma (RD) cells, RD-SCARB2 (RDS) and 293-SCARB2 (293S) cell lines were cultured and EV71 C4 strain was propagated as described previously [46,48,49].

2.2. Peptides, inactivated EV71 and VLP vaccines

Keyhole Limpet Hemocyanin (KLH) conjugated synthetic 71-6 peptide (designated here as pep. 71-6) from GL Biochem. Ltd. (Shanghai, China) was dissolved in PBS to 1 mg/ml [49] and stored at −80 °C. Production and purification of inactivated EV71 and VLP vaccines were described previously [46].

Table 1
Peptide sequence selected from different subunits of EV71.

Subunit	Peptide	Amino acid position	Peptide sequence
VP4	71-1	40–54	TAGKQSLKQDPDKF
	71-2	48–62	QDPDKFANPVKDIFT
VP2	71-3	136–150	AGGTGTEDSHPPYKQ
	71-4	219–231	ISPLDYDQGATPV
VP3	71-5	58–69	VPTNATSLMERL
	71-6	176–190	HYRAHARDGVFDYYT
VP1	71-7	7–19	VIESSIGDSVSRA
	71-8	22–34	HALPAPTGQNTQV
	71-9	91–104	GEIDLPLKGTTNPN
	71-10	240–259	TSKSKYPLVVRIYMRMKHVR
	SP70	208–222	YPTFGEHKQEKDLEY

2.3. Selection of epitopes and construction of expression vectors

The expression vector pET28a (+)-P particle containing the P domain of the norovirus Hunter504D strain (genogroup II, cluster 4 [GII.4]) was designed [39]. To obtain chimeric P particles containing EV71 antigens, we inserted the EV71 epitopes into the norovirus P particle as shown in Table 1, Figs. S1 and S2A. The position at which the foreign epitope was molecularly incorporated into the P particle was described previously [29,39].

2.4. Expression and purification of chimeric proteins

The chimeric pET28a (+)-PP-EV71 epitope expression plasmids were transformed into Rosetta competent cells (DE3 strain, Shanghai Tian Jia Biological Technology Co. Ltd.). Protocols for expression and purification are provided in the Supplementary Materials and Methods.

2.5. SDS-PAGE and Western blot analysis

Purified chimeric proteins were analyzed by SDS-PAGE and Western blot. An anti-His monoclonal antibody (mAb) was used for Western blot analysis as described previously [50]. The preparation and immunization of inactivated EV71 and EV71 VLPs were conducted in our previous work and they were also subjected to Western blotting for detecting reactivity with all sera from animals immunized with the PP-EV71 epitope proteins using the same method above [46].

2.6. Transmission electron microscopy (TEM)

TEM was performed to examine the size and shape of the P-particles and chimeric PP-71-6 particles. The samples were analyzed and evaluated using the H-7650 transmission electron microscope (Hitachi, Japan) [51].

2.7. Mouse immunization

Female BALB/c mice (purchased from Changchun Institute of Biological Products) aged 6–8 weeks were randomly divided into 13 groups ($n = 5$ per group). Each mouse was immunized with 25 μg of chimeric protein. PP protein and PBS were used to treat the control groups. The samples were adsorbed on 200 μg of aluminum hydroxide adjuvant and were administered intraperitoneally (i.p.) at week 0, 2 and 4. Serum samples were collected from the tail vein at week 0, 2, 4, 6 and 8. Blood samples were left at 37 °C for 1 h before centrifugation at 3000 × *g*, 4 °C for 30 min and inactivated at 56 °C

for 30 min. The animal protocol was approved by the University Committee on the Use and Care of Animals, Jilin University.

2.8. ELISA

In order to determine the specific IgG responses in immunized mouse serum samples, a modified ELISA was carried out as described previously [26]. The PP protein, pep.71-6 protein, inactivated EV71 and VLPs were used as the coating proteins. Serum samples from animals immunized with PP-EV71 epitopes were 10-fold serially diluted, with the first dilution at 100-fold. The absorbance was determined at 450 nm with an ELISA plate reader. Serum samples and proteins of inactivated EV71 and VLPs were generated in our previous research [46].

2.9. Neutralization assay

The presence of neutralizing antibodies against EV71 was assayed by the traditional cytopathic effects (CPE) method with RD cells [52]. The pseudovirus assay (PVA) was conducted to detect cross-neutralization antibodies in serum samples at 6 week, which presented positive results in both neutralization and passive protection assays below. The pseudovirus construction and detection method have been described previously (Table S4) [7,46,53].

2.10. Passive protection assay

Neonatal mice have been utilized as animal models for EV71 infection [54–57]. In this study, the *in vivo* protective efficacy of the experimental proteins was evaluated by two assays. First, two adult female BALB/c mice were immunized with PBS, PP or several chimeric P domain-EV71 epitope proteins as described above. The PBS and PP groups were used as negative controls in this experiment. After the second immunization, mice were allowed to mate. Ten neonatal mice of each group (age <24 h) were challenged intracerebrally (i.c.) with 1.5×10^6 $TCID_{50}$ of the EV71 C4 strain (KJ508817). Each group was evaluated in two independent experiments. The challenged mice were monitored daily for survival and body weight for 16 days as described previously [58,59].

In another experiment, the serum samples were mixed with a lethal dose of EV71 of about 1.5×10^6 $TCID_{50}$ in equal volumes and then incubated at 37 °C for 1 h 30 min. Ten newborn mice (age <24 h) were challenged by injection i.c. with 30 μl of the serum-virus mixture in each group. All mice were monitored daily as described above, and the data were collected after 16 days [60].

2.11. Protein structure prediction

To analyze the presentation of the 71-6 epitope on the surface of the virion, the crystal structure of human EV71 was downloaded from the PDB Protein Databank (PDB file ID: 3VBS) and rendered using VMD software (version 1.9.2). The software settings recommended by the manual were used to generate the images.

2.12. Statistical analysis

All statistical analyses were performed with GraphPad Prism version 5. Statistically significant differences between groups were determined by using Student's *t*-test and *P* values < 0.05. Data from the experiment were gained with at least three replicates and expressed as the mean ± standard deviation (SD).

3. Results

3.1. Expression and purification of chimeric P particles displaying EV71 epitopes

All purified P particle–EV71 epitope proteins (*i.e.*, norovirus chimeric proteins) were subjected to SDS-PAGE analysis, which showed that their inferred molecular masses (~37 kDa) were slightly higher than that of the unmodified P particles (~35 kDa) (Figure S2B). Concentrations of the chimeric proteins (yields ranging 5–10 mg/L) were determined by using the Odyssey Infrared Imaging System (Fig. S2C), and all proteins reacted with the anti-His mAb in Western blot assays (Fig. S2D).

3.2. Antibody response following PP-EV71 epitope protein immunization

Groups of five mice were immunized intraperitoneally (i.p.) with the chimeric proteins to evaluate their immunogenicity. Western blots were performed to identify the ability of the mouse serum samples at 6 week to recognize inactivated EV71 and VLP antigens. The anti-EV71 VP1 rabbit serum was used as a positive control antibody [46]. Fig. 1 shows the results of Western blot analysis of diluted serum samples from mice immunized with PP-EV71 epitope proteins. The serum samples against PP-71-2, PP-71-4, PP-71-9 and PP-SP70 strongly recognized the inactivated EV71 and VLPs antigens. Meanwhile, the PP-71-3 and PP-71-6 serum samples showed weak reactivity with both types of antigens, and the remaining serum samples presented negative results.

To further investigate specific IgG responses, ELISA was conducted using P particles, inactivated EV71 and VLP proteins as capture antigens to detect specific antibody responses in the serum samples. As expected, we found that all proteins could induce specific antibodies against P particle antigens (Fig. 2). Sera from mice immunized with PP-71-1, PP-71-2, PP-71-3, PP-71-6, PP-71-9 and PP-SP70 could detect the inactivated EV71 and VLP antigens as coating proteins, while other serum samples yielded negative results (Fig. 2). Our findings suggested that some of the norovirus chimeric proteins could induce epitope-specific antibodies in mice.

3.3. Chimeric proteins elicit EV71 neutralizing antibodies in mice

Serum samples from immunized mice were further evaluated for their capacity to neutralize EV71 by the CPE method. Mixtures of serum samples at week 6 from mice immunized with chimeric proteins and EV71 virus (KJ508817) were exposed to RD cells and analyzed by the CPE method. The results are shown in Table S2. The titer of the serum samples against PP-71-6 was 64, slightly higher than those of other samples. Serum samples against PP-71-7, PP-71-8 and PP-71-10 did not show any neutralization effect at the 1:8 dilution (lowest dilution tested), which was identical to the PP group and PBS control group. We concluded that the PP-71-1, PP-71-2, PP-71-4, PP-71-5, PP-71-6, PP-71-9 and PP-SP70 chimeric proteins could elicit specific neutralization antibody responses, and no significant differences were observed between them.

3.4. In vivo *protective efficacy against lethal challenge with EV71*

Serum samples determined to be positive in the neutralization assay were evaluated further for protective efficacy by mixing with the EV71 challenge virus and inoculating one-day-old neonatal mice. The survival and body weight of the mice were monitored daily until 16 days post-challenge. As shown in Fig. 3A and B, serum samples from the PP-71-6 and PP-SP70 immunized groups provided 100% protection against EV71 challenge. Animals of the

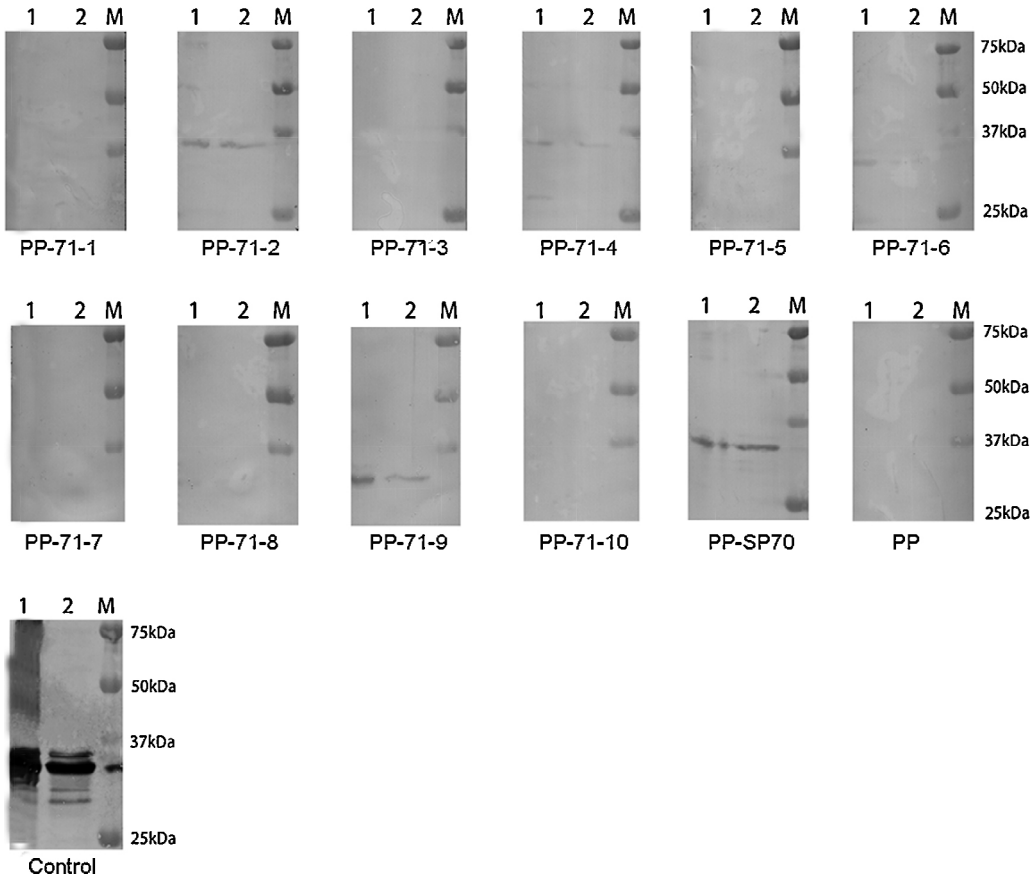

Fig. 1. *Western blot analysis of reactivity of inactivated EV71 and VLP antigens with mouse serum samples (1:300 dilution) raised against the P domain-EV71 epitope chimeric proteins*. Inactivated EV71 (lane 1) and VLP proteins (lane 2) as antigens were analyzed by Western blot for detecting reactivity of serum samples from mice immunized with chimeric P particles (PP-71-1, PP-71-2, PP-71-3. . . PP-71-10, PP-SP70) and P particles. The positive control serum sample was the EV71 VP1 rabbit polyclonal antibody.

negative control group, as well as other groups, manifested significant disease symptoms and died rapidly.

In another assay, two adult female mice from each group were immunized with these proteins and then allowed to mate. Ten newborn mice aged <24 h from each adult female mice were challenged with 1.5×10^6 $TCID_{50}$ EV71 virus, and then survival and body weight were monitored daily until 16 days later. Similar results were also obtained from this experiment as shown in Fig. 3C and D. The PP-71-6 and PP-SP70 groups demonstrated approximately 80% protection. Control mice that were born to the PBS or

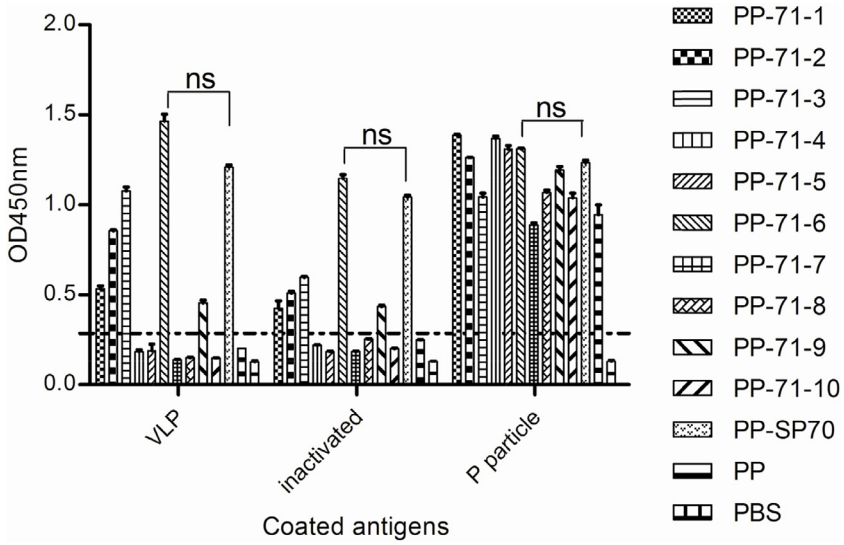

Fig. 2. *Mouse immunogenicity studies of all P domain-EV71 epitope chimeric proteins by ELISA.* EV71 VLPs, inactivated EV71 and P particles were used as ELISA coating antigens to detect specific antibodies in serum samples from mice immunized with different chimeric P particles. Serum samples were diluted 1:100 for the assay. Results are presented as the mean ± SD of three independent experiments. Statistically significant differences in antibody responses were observed between groups. * $P < 0.05$; ns indicates no significant difference.

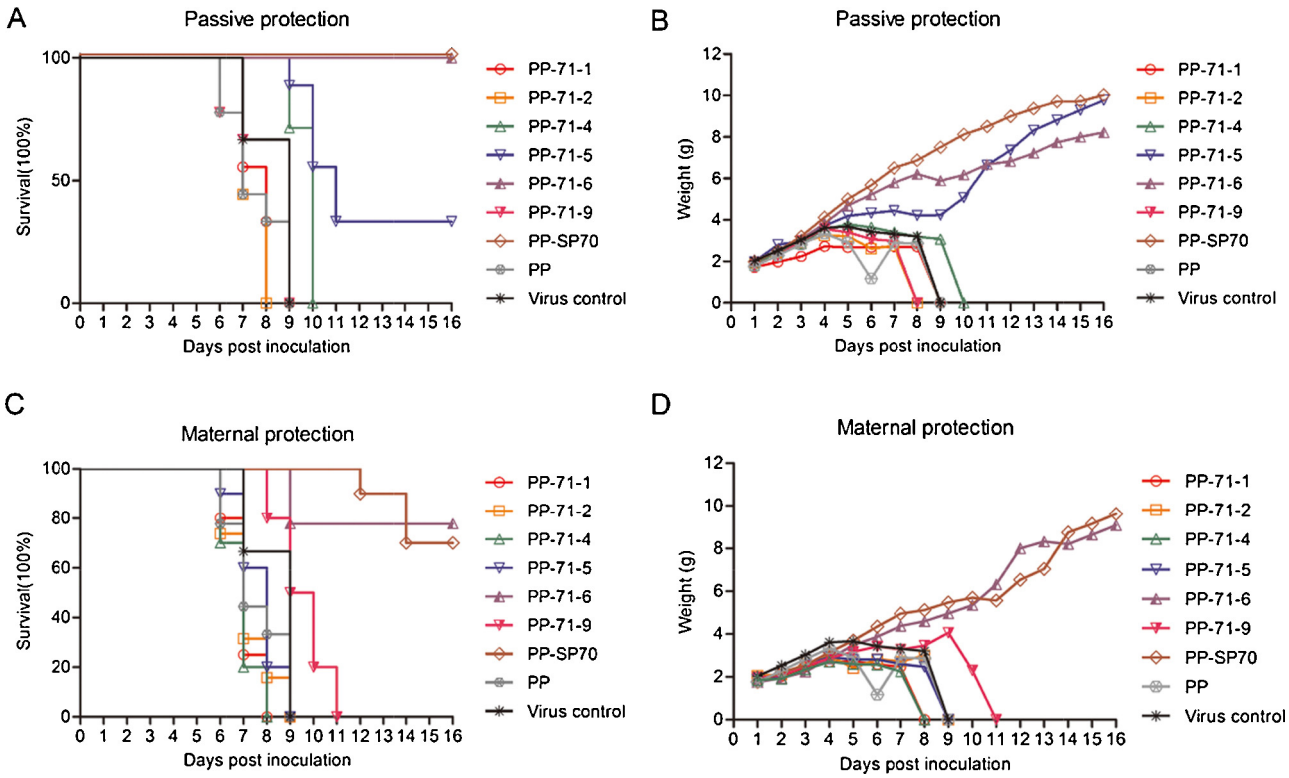

Fig. 3. *Passive protection against EV71 lethal challenge virus in vivo.* Serum from mice immunized with chimeric P domain-EV71 epitope proteins passively protected other mice against lethal EV71 challenge. In one experiment, passive transfer was conducted by co-incubating the serum samples against chimeric proteins with an equal volume of EV71 virus (1.5×10^6 $TCID_{50}$) for 1.5 h at 37 °C. Thereafter, groups of 10 1-day-old mice were i.c. injected with the mixture. The mice were subsequently observed on a daily basis for survival (A) and weight (B) for 16 days. In another experiment, maternal protection was evaluated by i.p. immunized 6-week-old female BALB/c mice at week 0, 2 and 4 with PBS, PP or several chimeric P domain-EV71 epitope proteins. After the second immunization, the mice were allowed to mate. Ten neonatal mice of the immunized dams were i.c. challenged within 24 h of birth with EV71 virus (1.5×10^6 $TCID_{50}$) and subsequently monitored for survival (C) and weight (D) for a period of 16 days, as described above. The Kaplan–Meier plots was used to calculate survival.

PP–treated dams, as well as other groups, had 100% mortality. These results demonstrated that the protein PP-71-6 indeed could induce strong antibody responses and protect newborn mice against EV71 challenge whether in maternal protection or passive transfer.

3.5. TEM of PP-71-6 particles and 3D structure models of EV71 pentamer

Results of Western blot analysis, ELISA, neutralization assay and protection assay were summarized and presented in Table S3. Based on the results above, we focused on the new neutralizing epitope 71-6 (amino acids 176–190 in VP3). To obtain direct evidence of chimeric P particle formation, PP-71-6 and P particle preparations were analyzed by negative staining electron microscopy. In the primary purification of P particles by using a Superdex 200 size exclusion column, three main ultraviolet absorption peaks were formed. The peak profile and all particles with an average diameter of ~20 nm were observed (Fig. S3A and B). The 24-mer P-particles of peak 1 constituted approximately 50% of the mixture and were used for immunization. These data indicated that the PP chimeric proteins self-assembled into VLPs presenting EV71 epitopes.

In order to analyze the presentation of the 71-6 epitope on the virion, the 3D crystal structure of human EV71 (PDB ID, 3VBS) was modified to define the viral surface location of the peptide. The viral pentamer (VP1, VP2, VP3 and VP4) was rendered as an electrostatic surface model (Fig. S4A) and a cartoon (Fig. S4B) with an enlarged view (Fig. S4C). Locations of the 71-6 epitope on the GH loop of VP3 and SP70 on the GH loop of VP1 are displayed. Based on these studies, we can further conclude that the 71-6 epitope is located in the canyon of the viral pentamer.

3.6. Further analysis of PP-71-6 chimeric protein

To determine whether this epitope is a conformational or linear epitope in the neutralization assay, EV71 VLPs, inactivated EV71, PP-71-6 and pep. 71-6 proteins were prepared as ELISA coating antigens for detection by serum samples at 6 week from mice immunized with corresponding proteins (Fig. 4A). All serum samples reacted to corresponding EV71 antigens, while the reactivity of serum samples from mice immunized with the inactivated EV71 proteins to PP-71-6 and pep. 71-6 antigens was low or negative. These results assured that the new neutralizing epitope 71-6 of VP3 was antigenic. We also compared the neutralizing titer *in vitro* (Fig. 4B) and protection *in vivo*, including passive transfer (Fig. 4C) and maternal protection (Fig. 4D), for the PP-71-6 and pep. 71-6 groups. In both the neutralization assay and protection assay, the pep. 71-6 group demonstrated negative results. This finding suggested that the 71-6 epitope may be a conformational neutralizing epitope, and the delivery via the norovirus P particle increased its immunogenicity. Meanwhile, we found the PP-71-6 serum samples failed to cross-react and could not neutralize the CA16 strain (JQ180468.1), nor the challenge virus in our lab (data not shown). To investigate the durability of the effect in serum samples of PP-71-6-immunized animals, blood samples were collected at week 0, 2, 4, 6, 8 and 10. The PP and PBS group were used as negative controls. As revealed in Fig. 5A, the neutralization antibody titer against EV71 virus was undetectable after the first immunization, and it reached the plateau at week 6. Although the titer slightly

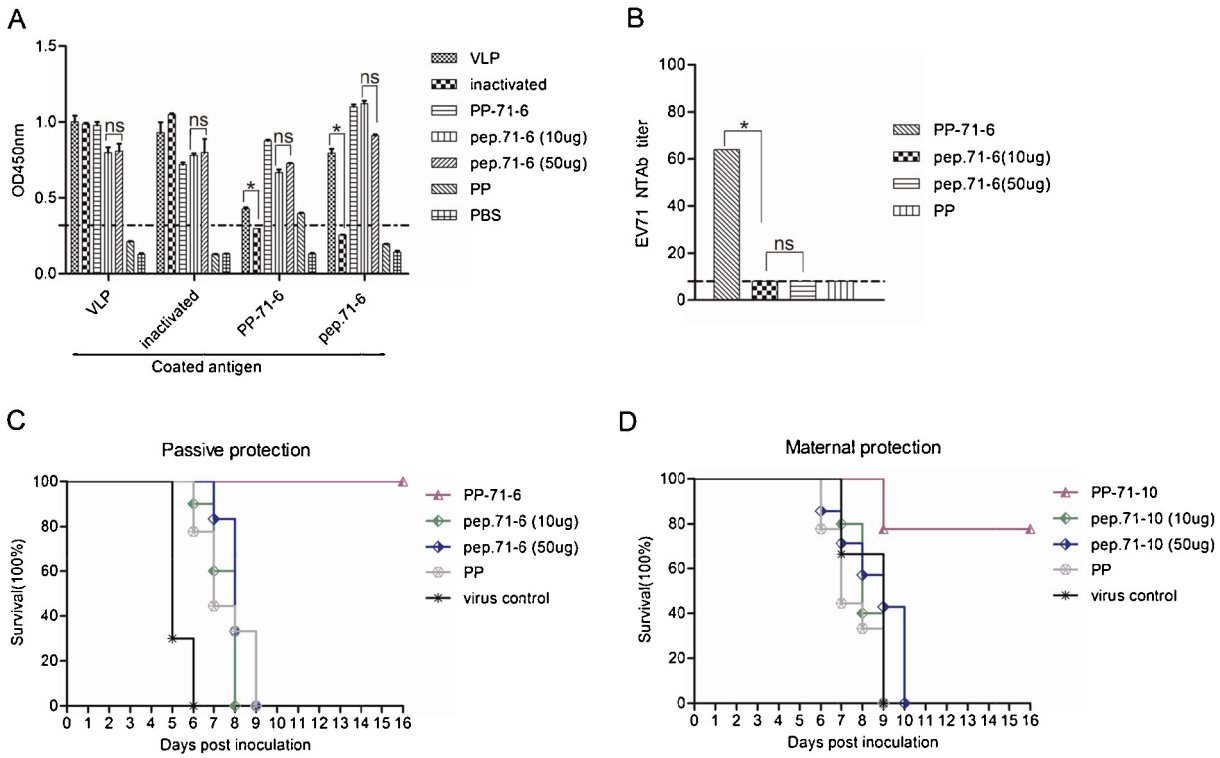

Fig. 4. *Identification and characterization of neutralization epitope 71-6.*Coating antigens of inactivated EV71, VLPs, PP-71-6 and pep. 71-6 were detected by serum samples from mice immunized with corresponding proteins by ELISA (A). Comparison of PP-71-6 and pep. 71-6 groups by neutralization assay *in vitro* (B) and protection assay *in vivo* via passive transfer (C) and maternal protection (D). Statistically significant differences in antibody responses were observed between groups. * $P < 0.05$; ns indicates no significant difference.

decreased thereafter, it still maintained a relatively high level at week 8 and 10.

Subsequently, cross-neutralizing antibodies in the positive serum samples of mice immunized with PP-71-6 were detected by PVA using 10 subgenotypes (B1–B5, C1–C5) of EV71 pseudovirus [7,53]. As demonstrated in Fig. 5B, PP-71-6 could induce cross-neutralizing antibodies from mice in the PVA assay, and a significant difference was observed between the PP-71-6 and PBS groups. Hence, the data confirmed that the 71-6 epitope (residues 176–190) is a cross-neutralizing epitope.

4. Discussion

In previous studies, a cross-protective vaccine against EV71 infections was developed based on the peptide selection method, and two conserved linear neutralizing epitopes (SP70 and SP55) belonging to the VP1 subunit were identified [25,61]. Another VP2 peptide also was demonstrated as a potential neutralization site. However, based on the EV71 crystal structural analysis, the VP3 subunit was thought to have major antigen clusters, but it was not described as having neutralizing antibody epitopes [26]. Furthermore, linear CVA16 virus neutralization epitopes were identified within residues 176–190 of VP3 [62]. Nevertheless, the peptides above did not induce cross-protection against the major pathogens of HFMD (EV71 and CVA16). Therefore, the current study was focused on the non-identical regions in the exposed hypervariable loops of EV71 and CVA16 P1.

VLPs were previously proposed to be an optimal delivery system for presentation and maximizing immunogenic potential of vaccine epitopes. HBc VLPs are extremely immunogenic and have been successfully used as a carrier system for presentation of foreign epitopes without loss of their capsid-forming ability [16,32–34]. Norovirus P particles also are used as a platform for novel vaccine development against other viral and bacterial pathogens [29,36,38]. In our laboratory, P particles have been expressed in the *E. coli* system with high yield and stability [63,64]. Therefore, the norovirus P particle system was chosen to present the EV71 epitopes in our study [29]. We will also attempt to utilize other VLPs carriers, such as HBc VLPs, and compare their immunogenicity with P particles in a later study.

Through immunization experiments and serological tests, the new epitope 71-6 (residues 176–190) within the VP3 protein of EV71 was identified. This epitope, which is well-conserved among different EV71 subgenotypes, yielded a high neutralizing titer and provided 80–100% protection, similar to results with PP-SP70. Simultaneously, by using the crystal structure of human EV71 (PDB file ID:3VBS) and VMD software, residues 176–190 of the VP3 GH loop were identified in the canyon of the viral pentamer and found to be exposed on the whole EV71 virus capsid surface. Pep. 71-6 and chimeric protein PP-71-6 were used to immunize BALB/c mice. Serum samples from mice immunized with the inactivated EV71 proteins showed minimal reactivity to the PP-71-6 and pep. 71-6 antigens. We attributed these results to structural differences between inactivated EV71 and EV71 VLPs. Serum samples from mice immunized with pep.71-6 did not have neutralizing activity, while those from the PP-71-6 group contained significant neutralizing antibody titers. Based on all results above, the 71-6 epitope was identified as a conformational neutralizing epitope. Simultaneously, these results demonstrated the feasibility of using P particles as a platform for presentation and enhancing immune responses to our antigens. In previous studies, P particle-rotavirus VP8* chimera and the P particle-influenza M2e chimeric vaccine induced high antibody responses against both norovirus P particles and the inserted epitope [29,36]. In our study, the 71-6 epitope sequence was also inserted in loop 2, as in the aforementioned

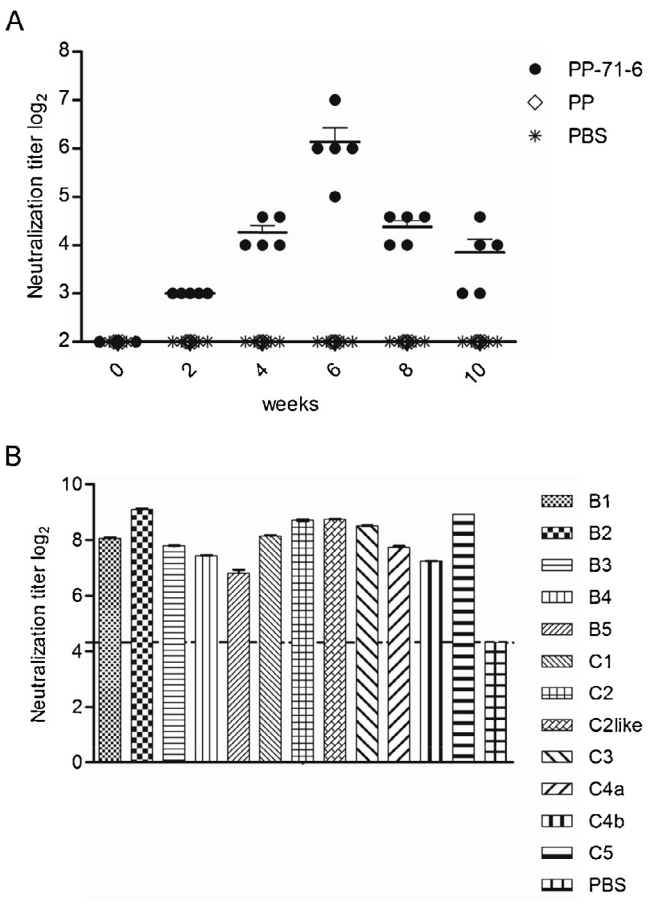

Fig. 5. *Analysis of PP-71-6 specific neutralizing antibodies in vitro.* Groups of five mice were immunized i.p. at week 0, 2 and 4 with PP-71-6 protein, PP protein and PBS as described in Materials and Methods. Serum were collected from each mouse at week 0, 2, 4, 6, 8 and 10. (A) Neutralizing titer profiles of antibody responses determined by CPE are presented after the last immunization with these proteins using serum samples collected at week 0, 2, 4, 6, 8 and 10. Each symbol represents an individual mouse, and the line indicates the geometric mean value of the group. (B) Cross-neutralization titer of PP-71-6 serum at week 6 against EV71 genogroups in PVA (B1–B5, C1–C5).

work, and we speculate that the chimeric protein PP-71-6 may also be a protective candidate vaccine against norovirus infection.

In further experiments, we will conduct another 3D structural protein analysis of PP-71-6 to determine whether the conformation of the 71-6 epitope presented by a norovirus P particle would be similar to that in an authentic EV71 virion. Furthermore, we will examine other selected epitopes spanning the entire P1 protein of EV71 in the same way as for the 71-6 epitope by insertion into the norovirus P particle carrier for further analyses. The purpose of these future studies will be to confirm whether the negative data resulted from the destruction of the native conformational epitopes in EV71 virions due to arbitrarily defined lengths of these peptides.

5. Conclusions

We have successfully identified residues 176–190 within the VP3 capsid protein of EV71 as a conformational neutralizing epitope. Our results suggested that the chimeric protein PP-71-6 might provide an opportunity for the design of a dual vaccine candidate against both EV71 and norovirus. Simultaneously, the discovery of the amino acids 176–190 of VP3 as a conformational neutralizing epitope provides valuable information for the production of mAbs and development of a vaccine for HFMD.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.10.104.

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