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Recombinant virus-like particle presenting a newly identified coxsackievirus A10 neutralization epitope induces protective immunity in mice

Wenlong Dai[a,1], Pei Xiong[a,1], Xueyang Zhang[a], Zhi Liu[b], Jinhuan Chen[c], Yu Zhou[a], Xiaohua Ye[a], Chao Zhang[a,d,*]

[a] Vaccine Research Center, CAS Key Laboratory of Molecular Virology & Immunology, Institut Pasteur of Shanghai, Chinese Academy of Sciences, University of Chinese Academy of Sciences, Shanghai, China
[b] Biological Imaging and Instrumental Analysis Center, Institut Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai, China
[c] National Center for Protein Science Shanghai, State Key Laboratory of Molecular Biology, CAS Center for Excellence in Molecular Cell Science, Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, University of Chinese Academy of Sciences, Shanghai, China
[d] Joint Center for Infection and Immunity, Guangzhou Institute of Pediatrics, Department of Gastroenterology, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, China

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ABSTRACT

Coxsackievirus A10 (CVA10) has emerged as one of the major pathogens of hand, foot, and mouth disease in recent years. However, there are no approved vaccines or effective drugs against CVA10. Several experimental CVA10 vaccines have been shown to elicit neutralizing antibodies that could confer protection against viral infection. However, neutralizing antigenic sites on CVA10 capsid have not been well characterized. Here, we report the characterization of linear neutralization epitopes of CVA10 and the development of a CVA10 vaccine based on the identified epitopes. We showed that peptide VP2-P28, corresponding to residues 136 to 150 of VP2, were recognized by anti-inactivated CVA10 sera and effectively inhibited anti-CVA10 sera-mediated neutralization, suggesting that this peptide contains neutralizing epitopes. Insertion of VP2-P28 into hepatitis B core antigen (HBc) resulted in a chimeric virus-like particle (VLP; designated HBc-P28) with the CVA10 epitope exposed on the particle surface. HBc-P28 VLP elicited strong antibody responses against VP2-P28 in mice. Anti-HBc-P28 sera could neutralize both CVA10 clinical isolates and prototype strain, consistent with the fact that the VP2-P28 sequence is highly conserved among CVA10 strains. In addition, anti-HBc-P28 sera failed to cross-neutralize other HFMD-causing enteroviruses, indicating that neutralizing antibodies elicited by HBc-P28 VLP were CVA10-specific. Importantly, anti-HBc-P28 sera were able to provide efficient protection against lethal CVA10 infection in recipient mice. Collectively, these data show that peptide VP2-P28 represents a CVA10-specific linear neutralizing antigenic site and chimeric VLP displaying this peptide is a promising epitope-based CVA10 vaccine candidate.

1. Introduction

Hand, foot, and mouth disease (HFMD) is a common pediatric infectious disease that lead to significant morbidity and mortality worldwide, especially in the Asia-Pacific region (Repass et al., 2014; Ventarola et al., 2015). HFMD could be caused by a variety of enteroviruses, including enterovirus 71 (EV71) and coxsackieviruses A16 (CVA16), A6 (CVA6), and A10 (CVA10) (Bian et al., 2015; Lu et al., 2012; Mao et al., 2014; Solomon et al., 2010; Wong et al., 2010). In particular, in recent years numerous HFMD cases and outbreaks have been reported to be associated with CVA10 infection (Chen et al., 2017; Ji et al., 2018; Munivenkatappa et al., 2018; Yang et al., 2015), thus indicating that CVA10 has emerged as one of the major causative agents of HFMD. Moreover, CVA10 co-circulated with CVA6, CVA16, and/or EV71 in several HFMD outbreaks (Blomqvist et al., 2010; Mirand et al., 2012; Wu et al., 2010), possibly resulting in viral co-infections and genetic recombination. CVA10 infections are generally mild and self-limiting, but severe life-threatening complications and even death can occur (Fuschino et al., 2012; Lu et al., 2012).

CVA10 belongs to the A species of the *Enterovirus* genus within the

* Corresponding author. Vaccine Research Center, CAS Key Laboratory of Molecular Virology & Immunology, Institut Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai, China.
E-mail address: chaozhang@ips.ac.cn (C. Zhang).
[1] These authors contributed equally: Wenlong Dai, Pei Xiong.

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Picornaviridae family. CVA10 capsid possesses an icosahedral structure and consists of 60 protomers, each composed of four proteins (VP1–VP4). VP4 is located inside the capsid shell, whereas VP1, VP2, and VP3 are situated at the outer surface of the shell (Fields et al., 2007; Zhu et al., 2018b) and are responsible for induction of immune responses *in vivo*. Similar to other enteroviruses, CVA10 has a deep surface depression (called the canyon) encircling the star-shaped peak at the fivefold axis (Chen et al., 2019; Zhu et al., 2018a, 2018b). The canyon region is thought to be involved in receptor binding.

Currently, there are no licensed vaccines or effective drugs against CVA10. Several experimental CVA10 vaccines, including inactivated whole virus and virus-like particle (VLP) vaccines, have been developed (Li et al., 2017; Liu et al., 2016; Shen et al., 2016; Zhang et al., 2017, 2018; Zhou et al., 2018). These vaccine candidates could elicit neutralizing antibodies that conferred protection against lethal CVA10 challenges in mice (Shen et al., 2016; Zhang et al., 2017, 2018; Zhou et al., 2018). However, antigenic sites responsible for induction of CVA10-specific neutralizing antibodies have not been characterized. In the present study, we identified immunodominant linear neutralization epitopes on the capsid proteins of CVA10 by screening a peptide library covering the whole VP1, VP2 and VP3 sequences with anti-CVA10 neutralizing sera and further examining neutralization-inhibitory effects of these peptides. Our results showed that VP2-derived peptide #28 (VP2-P28; residues 136 to 150 of VP2) contained potential neutralizing epitopes.

To enhance immunogenicity of peptide VP2-P28 and develop an epitope-based CVA10 vaccine, particulate carrier molecules, such as hepatitis B virus core (HBc) particles, are required for delivery of VP2-P28. HBc antigen, when expressed in heterologous systems, can self-assemble into two sizes of VLPs (T = 3 and T = 4), which contain 180 or 240 subunits, respectively (Crowther et al., 1994). Each HBc subunit contains an antiparallel α-helical hairpin and two subunits form a dimer. The dimers form spikes on the particle surface (Bottcher et al., 1997). The major immunodominant region (residues 74 to 89) is located at the tips of the spikes and allows insertion of foreign epitopes (Pumpens and Grens, 1999; Wynne et al., 1999). In this study, we used HBc particles as a carrier to present VP2-P28 and evaluated the immunogenicity and protective efficacy of this chimeric VLP in mice. Our results showed that HBc VLP displaying VP2-P28 peptide could be readily produced in *Escherichia coli* (*E. coli*) and, more importantly, induce CVA10-specific neutralizing/protective antibodies, thus representing a promising CVA10 vaccine candidate.

2. Materials and methods

2.1. Cells and viruses

Human rhabdomyosarcoma (RD) cells were cultured as described previously (Ku et al., 2012). CVA10 strains used in this study include two clinical isolates, CVA10/S0148b and CVA10/S0273b, and the prototype strain CVA10/Kowalik (Shen et al., 2016). EV71 clinical strain EV71/G082 and CVA16 clinical strain CVA16/SZ05 have also been described previously (Cai et al., 2013; Ku et al., 2012). 50% tissue culture infectious dose ($TCID_{50}$) for each virus was determined according to the Reed-Muench method (Reed and Muench, 1938).

2.2. Synthetic peptides, inactivated CVA10 and antibodies

58, 49 and 46 peptides spanning the whole sequences of the VP1, VP2 and VP3 proteins of CVA10/S0148b were synthesized by GL Biochem (Shanghai, China), respectively. Synthetic peptides were 15 amino acid residues long and overlapped by 10 residues. Additionally, VP2 peptide #28 (VP2-P28; sequence: GSNTKPNEAPHPGFT) was linked to keyhole limpet hemocyanin (KLH) by GL Biochem. A polyclonal antibody against VP2-P28 was then generated in our laboratory from BALB/c mice immunized with KLH-linked VP2-P28 plus Freund's adjuvant (Sigma, USA). β-propiolactone-inactivated CVA10/S0148b and mouse polyclonal antibodies against CVA10/S0148b were prepared in a previous study (Shen et al., 2016). Anti-HBc polyclonal antibody was produced by immunizing a New Zealand white rabbit with *Escherichia coli* (*E. coli*)-derived His-tagged HBc protein (Ye et al., 2014).

2.3. Peptide ELISA

Linear B-cell epitopes of CVA10 were identified by peptide ELISA. Briefly, ELISA plates (Nunc, USA) were coated with 1 μg/well of individual peptide in PBS and incubated at 37 °C for 2 h. Purified inactivated CVA10/S0148b virus was used as positive control. After blocking in 5% milk in PBS-Tween-20 (PBST), pooled anti-CVA10/S0148b mouse sera was added at a dilution of 1:100 and incubated at 37 °C for 2 h. After washes with PBST, the plates were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (diluted 1:5,000; Sigma). After color development, absorbance for each well was determined at 450 nm using a microplate reader.

2.4. Neutralization and neutralization-inhibition assays

Neutralizing activities of mouse antisera against CVA10 were measured by cytopathic effect (CPE)-based neutralization assay that was described previously (Shen et al., 2016). Neutralizing titer was defined as the highest serum dilution that fully inhibited appearance of CPE.

Effect of peptide treatment on neutralizing activity of anti-CVA10 sera was determined by neutralization-inhibition assay. Briefly, 25 μL (50 μg/mL) of the selected CVA10 peptides were mixed with equal volume of 1:500 diluted anti-CVA10/S0148b mouse sera and incubated at 37 °C for 1 h. The peptide/sera mixtures were mixed with 50 μL (100 $TCID_{50}$) of CVA10/S0148b and incubated at 37 °C for 1 h. The mixtures (100 μL/well) were added to confluent RD cell monolayers grown in 96-well plates. After three days of incubation at 37 °C, cell viability was evaluated by the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay as described previously (Shi et al., 2013). Percent cell viability was calculated as follows: 100 × (OD490 of the given sample - OD490 of the virus-only sample)/(OD490 of the cell-only sample - OD490 of the virus-only sample).

To determine whether peptide treatment affects viral infection, 100 $TCID_{50}$ (50 μL) of CVA10/S0148b was mixed with 50 μg/mL (25 μL) of peptides and 25 μL of DMEM medium and incubated at 37 °C for 1 h. The peptide/virus mixtures were inoculated into confluent RD cells in 96-well plates. After three days of incubation at 37 °C, cell viability was measured using CellTiter-Glo 2.0 assay kit (Promega, USA) according to the manufacturer's instructions. Percent cell viability was calculated as follows: 100 × (luminescence of the given sample - luminescence of the virus-only sample)/(luminescence of the cell-only sample - luminescence of the virus-only sample).

To determine the effects of different doses of the selected peptides on neutralizing activity of anti-CVA10 sera, the peptides (25 μL) in serial 2-fold dilutions were subjected to neutralization-inhibition assay as described above.

2.5. Sequence alignment

Enterovirus strains used for sequence alignment include CVA10/S0148b (GenBank ID: KX094564), CVA10/S0273b (KX094565), CVA10/Kowalik (AY421767), CVA10/FJ-01 (KY012321), CVA10/FJ-01 (KY012321), CVA10/M10MJ20 (JN639886), CVA10/2014-XMCDC-301-CA10 (KX768155), CVA10/VNM/5.1/2014 (KX430803), CVA10/C125/CHW/AUS/2017 (MH111059), CVA10/P1005/2013/China (KP289394), CVA10/Weihai/SD/CHN/2014 (KU578127), CVA10/FY07/AH/CHN/2013 (KP009580), CVA10/Shenzhen18/CHN/2014 (KX595288), CVA10/USA/TN/2016-OB2038 (KY271944), CVA10/MAD-9802-11 (LT719058), CVA10/08M50227 (JX009031), CVA10/61217-2276 (MF422532), EV71/G082 (Ku et al., 2012), CVA16/SZ05

(EU262658), and CVA6/Gdula (AY421764). Alignment of VP2 protein sequences was conducted by BioEdit.

2.6. Vector construction

To construct a new vector for expression of HBc without a hexahistidine (His) tag, HBc gene was amplified by PCR from the plasmid pET28-HBc-His-tag (Ye et al., 2014) with primers (forward, 5′-GTCCCATGGACATTGACCCTTACAAAGA-3'; reverse, 5′-GTCCTCGAG**TTA**ACATTGAGATTCCCTAGATT-3′), digested with *Nco*I and *Xho*l, and then inserted into the expression vector pET28b, resulting in plasmid pET28-HBc. To construct the plasmid expressing HBc-P28 fusion protein, DNA fragment encoding VP2-P28 peptide of CVA10/S0148b was synthesized and subsequently cloned into the *XbaI/Bgl*II site of pIBT-HBc (Ye et al., 2014) using ClonExpress II One Step Cloning Kit (Vazyme, China), yielding an intermediate plasmid pIBT-HBc-P28. The HBc-P28 fragment was amplified by PCR from pIBT-HBc-P28 and then cloned into pET28b as described above, to generate plasmid pET28-HBc-P28.

2.7. Expression and purification of recombinant proteins

To express HBc and HBc-P28 proteins, *E. coli* BL21 (DE3) cells were separately transformed with the plasmids pET28-HBc and pET28-HBc-P28. A single transformed colony for each construct was cultured overnight at 37 °C in LB medium containing 50 μg/mL kanamycin, diluted 1:100 in fresh LB medium and grown to a OD600 of 1.0. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to 0.05 mM to induce protein expression and the cultures were shaken at 16 °C for 24 h. The induced *E. coli* cells were pelleted, resuspended in 0.15 M PBS buffer and then lysed using a high-pressure cell disruption system (JNBIO, China) at 1000 Bar. The resultant lysates were purified by polyethylene glycol (PEG) precipitation and 10–50% sucrose gradient ultracentrifugation according to a detailed protocol previously described by our laboratory (Zhang et al., 2015). Total protein concentrations of the purified VLP samples were quantified by Bradford assay.

2.8. Western blotting and ELISA assays

Western blotting and ELISA analyses of sucrose gradient fractions were carried out as described previously (Dai et al., 2018; Ku et al., 2013) with minor modifications. Anti-HBc and anti-VP2-P28 polyclonal antibodies were diluted 1:1000 and 1:100, respectively, and then used for detection.

2.9. Electron microscopy

Purified HBc and HBc-P28 VLP samples were separately adsorbed onto glow-discharged carbon-coated copper grids and negatively stained with 0.5% aqueous uranyl acetate. The grids were viewed on a Tecnai G2 Spirit transmission electron microscope (FEI, USA) at 200 kV.

2.10. Mouse immunization

All animal studies were approved by the Institutional Animal Care and Use Committee at the Institut Pasteur of Shanghai.

Purified HBc and HBc-P28 VLPs (100 μg/dose) were separately mixed with Alhydrogel® adjuvant (500 μg/dose; Invivogen, USA) by vortexing to generate experimental vaccines. These vaccines were injected into the peritoneal cavity of female BALB/c mice (6–8 weeks old) at weeks 0, 3 and 5. Antiserum samples were harvested from each mouse at week 7 and then heated to 56 °C for 30 min to destroy complement prior to use.

2.11. Serum antibody measurement

Antigen-specific IgG antibodies in antiserum samples were measured by indirect ELISA. Briefly, ELISA plates were coated with 50 ng/well of HBc or 500 ng/well of VP2-P28 peptide overnight at 4 °C and then blocked with 5% milk in PBST. Antisera were added at a 1:100 dilution and incubated at 37 °C for 2 h. After three washes, the plates were incubated with HRP-conjugated anti-mouse IgG (Sigma). After color development, absorbance at 450 nm was determined.

2.12. In vivo protection assay

Protective efficacy of the HBc-P28 vaccine was tested using passive immunization/challenge assay. Groups of six-day-old ICR mice were injected intraperitoneally (i.p.) with pooled anti-HBc or anti-HBc-P28 sera (100 μL/mouse). One day later, the mice was i. p. infected with CVA10/S0148b (7.9×10^4 $TCID_{50}$/mouse) and then checked daily for survival and clinical score for 15 days. Clinical scores were graded as follows: 0, healthy; 1, reduced mobility; 2, limb weakness; 3, paralysis; 4, death.

2.13. Statistical analysis

All statistical analyses were performed using GraphPad Prism version 5. Cell viability was analyzed by a two-tailed Student's *t*-test. Survival rates were analyzed by Log-rank (Mantel-Cox) test.

3. Results

3.1. Identification of linear B-cell epitopes in the capsid proteins of CVA10

Previous studies have demonstrated that inactivated CVA10 could potently induce the production of neutralizing antibodies in mice (Shen et al., 2016; Zhu et al., 2018b). To identify CVA10 linear B-cell epitopes, a library of synthetic peptides spanning the whole VP1, VP2 and VP3 sequences was screened with sera pooled from inactivated CVA10-immunized mice (Shen et al., 2016) by ELISA. As shown in Fig. 1, anti-CVA10 sera reacted strongly with VP1-derived peptides #1, #2, #6, #7, and #8, as well as VP2-derived peptides #28 and #29, but had no reactivity against other peptides. These results suggest that the seven ELISA-positive peptides contain B-cell epitopes recognized by anti-CVA10 antibodies. Therefore, the seven peptides were chosen for further analysis.

3.2. Identification of the potential neutralizing epitopes of CVA10

To test whether the selected peptides contain CVA10 neutralizing epitopes, neutralization-inhibition assay was performed. In this assay, the seven high-binding peptides were allowed to bind to anti-CVA10 neutralizing polyclonal antibodies for 1 h prior to neutralization assay. As shown in Fig. 2A, anti-CVA10 sera (diluted 1:500) effectively protected cells against virus infection (see column 3, sera + virus) with cell viability similar to that of cells without treatment (see column 1, cell only). Preincubation of anti-CVA10 sera with VP2-derived peptide #28 (designated VP2-P28) significantly reduced neutralizing activity of anti-CVA10 antibodies. VP2-P29 had only a weak neutralization-inhibitory ability, but the effect is not statistically significant. By contrast, treatment with the other five VP1 peptides did not have any neutralization-inhibitory effect (Fig. 2A). These results indicate that of the seven high-binding peptides, only one peptide (VP2-P28) might contain potential neutralizing epitopes.

Note that there could be one other possible interpretation of the current result (Fig. 2A). If VP2-P28 has the ability to bind to the CVA10 receptors on cell surface, a competition of VP2-P28 peptide with CVA10 authentic virions could also inhibit viral infection, independent of involvement of anti-CVA10 neutralizing antibodies. To test this possibility, CVA10 virus was mixed with peptides only and inoculated into cells. Three days later, cell viability was measured. As shown in Fig. 2B, treatment of CVA10 virus with VP2-P28 or control peptide VP1-P2 did

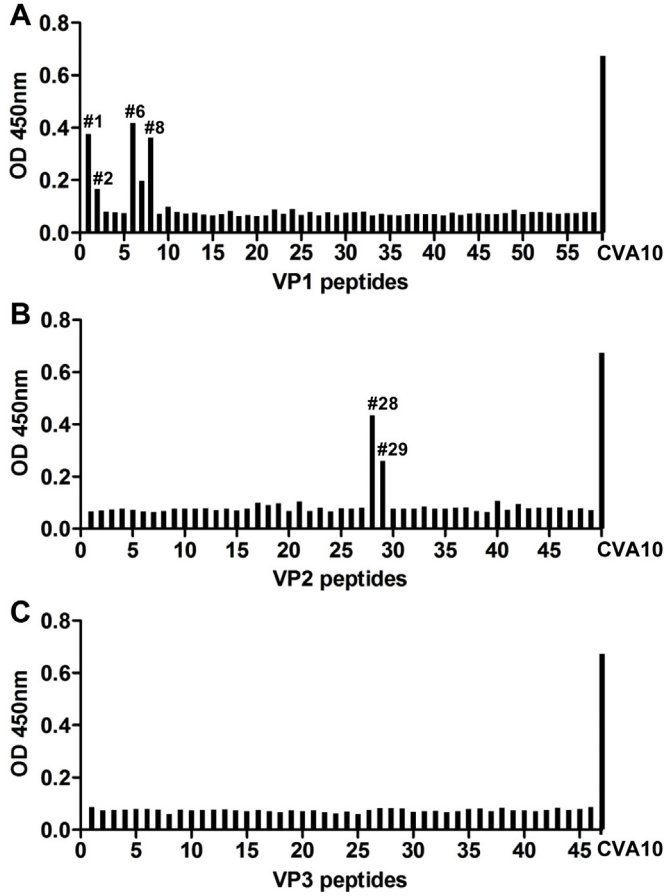

Fig. 1. Identification of linear B-cell epitopes within the capsid proteins of CVA10. A panel of 153 overlapping peptides spanning the whole sequences of **(A)** VP1 (58 peptides), **(B)** VP2 (49 peptides), and **(C)** VP3 (46 peptides) of CVA10 was tested for reactivity with anti-CVA10 immune sera by peptide ELISA. Inactivated CVA10 served as positive control. Results shown are representative of two independent experiments.

not affect viral infection, indicating that VP2-P28 peptide does not compete with CVA10 virions for cellular receptor binding.

The effect of different doses of VP2-P28 on neutralizing activity of anti-CVA10 sera was determined by neutralization-inhibition assay. As shown in Fig. 2C, VP2-P28 showed neutralization-inhibitory activity in a dose-dependent manner with an IC50 (half maximal inhibitory concentration) of 32.95 μg/mL. By contrast, control peptide VP1-P2 did not exhibit any inhibitory effect regardless of the peptide dose.

3.3. Sequence analysis and location of VP2-P28

Sequence alignment of VP2 proteins from 16 representative strains of CVA10 showed that the VP2-P28 region (residues 136 to 150 of VP2) was highly conserved among various CVA10 isolates (Fig. 3A). Moreover, by blast analysis (data not shown), a total of 93 sequences containing VP2-P28 region were retrieved from the NCBI CVA10 database. VP2-P28 epitope is fully conserved in 73 CVA10 strains and relatively conserved among 20 other strains, 15 of which had single amino acid residue substitutions in this epitope. However, comparison with other HFMD-causing enteroviruses (EV71, CVA16, and CVA6) revealed significant differences in amino acid sequences between CVA10 and other enterovirus VP2-P28 regions (Fig. 3A).

According to the cryo-EM structure of mature CVA10 (PDB: 6IIJ) (Chen et al., 2019), the VP2-P28 region is highly exposed on the virion surface and located on the southern rim of the canyon (Fig. 3B). Moreover, the VP2-P28 region forms part of the EF loop or puff (between beta strands E and F of VP2) that is the largest surface loop of VP2 (Fig. 3C). Superposition of CVA10 (PDB: 6IIJ), EV71 (PDB: 3VBS), and CVA16 (PDB: 5C4W) VP2 structures revealed obvious structural differences in the VP2-P28 region (EF loop) between CVA10 and the other viruses (Fig. 3C). Taken together, these results indicate that VP2-P28 represents a CVA10-specific surface-exposed yet highly conserved neutralizing epitope. Therefore, VP2-P28 might be a potentially desirable candidate for further development as an epitope-based CVA10 vaccine.

3.4. Expression and characterization of chimeric VLP displaying VP2-P28

To test the potential of VP2-P28 as a candidate vaccine, we used HBc particles as a carrier to present this CVA10 epitope. To achieve optimal immunogenicity, the VP2-P28 epitope was inserted into the tips of the HBc surface spikes by replacing residues 77 to 83 of HBc (Fig. 4A). Two constructs, pET28-HBc and pET28-HBc-P28, were made for expression of native HBc VLP and chimeric HBc VLP displaying VP2-P28 in *E. coli*, respectively. To eliminate adverse effects of His-tag, a stop codon was added right after the HBc coding sequence (before the 6× histidine) to avoid translation of His-tag. The two plasmids were

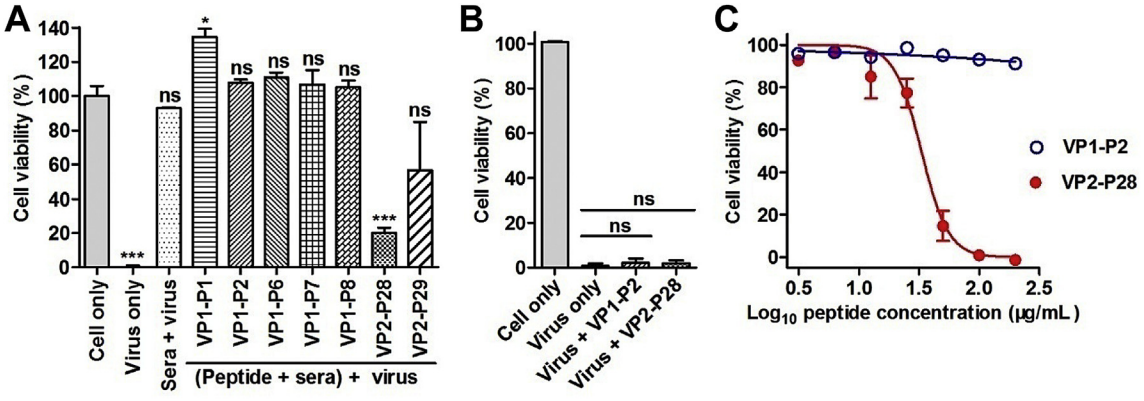

Fig. 2. VP2-P28 significantly reduced neutralizing activity of anti-CVA10 antibodies. (A) Inhibitory effect of the selected peptides on neutralizing activity of anti-CVA10 sera. Anti-CVA10 sera were pre-incubated with the indicated peptides for 1 h before the sera were subjected to neutralization assay. After three days, cell viability was measured by the MTT assay. **(B)** VP2-P28 does not affect CVA10 viral infection. CVA10 virus was mixed with VP2-P28 or control peptide VP1-P2 and inoculated into RD cells. Three days later, cell viability was measured using CellTiter-Glo 2.0 assay. **(C)** Dose-response curve. Anti-CVA10 sera was incubated with different doses of peptides VP2-P28 or VP1-P2 for 1 h prior to neutralization assay. After three days, cell viability was measured using CellTiter-Glo 2.0 assay. Results were expressed as percentage cell viability relative to untreated cell control. Data are mean ± SEM of at least triplicate wells. Statistical significance is indicated as follows: ns, no significant difference ($P \geq 0.05$); *, $P < 0.05$; ***, $P < 0.001$.

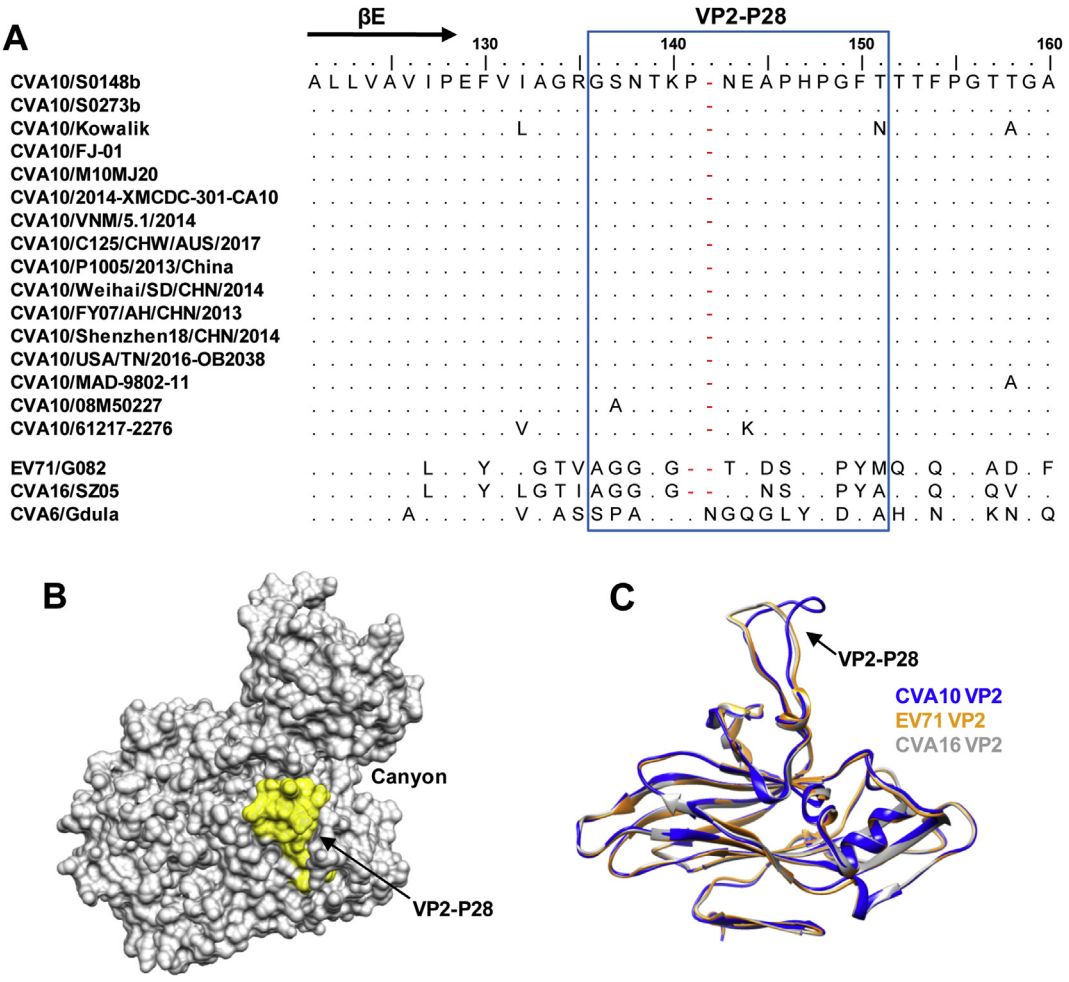

Fig. 3. Sequence analysis and location of CVA10 epitope VP2-P28. (A) Alignment of amino acid sequence of VP2 EF loop of different enteroviruses. Beta strand E of CVA10 is labeled at the top. The VP2-P28 region is boxed with a blue line. Dots represent residues identical to those of CVA10/S0148b and red dashes are gaps. **(B)** Location of CVA10 epitope VP2-P28 on the viral protomer surface. VP2-P28 is colored in yellow. **(C)** Superposition of VP2 of CVA10 (PDB: 6IIJ; blue), EV71 (PDB: 3VBS; orange), and CVA16 (PDB: 5C4W; light gray) mature virus particles. Figures **(B) and (C)** were generated with UCSF Chimera.

separately transformed into *E. coli* strain BL21 and the resulting transformants were subjected to low temperature induction for soluble expression of recombinant proteins. Lysates of induced *E. coli* cells were sedimented through sucrose gradients, and the resultant fractions were analyzed for protein expression and VLP assembly by western blotting and ELISA. Western blotting analysis with HBc-specific antibody showed that strong bands with expected molecular mass of 21–23 kDa were detected in factions #9 and #10 of lysates from *E. coli* cells transformed with pET28-HBc and pET28-HBc-P28, respectively (Fig. 4B). Moreover, fraction #9 of *E. coli* clone expressing HBc-P28 showed strong reactivity with the antibody against peptide VP2-P28, whereas none of factions of *E. coli* clone expressing HBc did (Fig. 4C). These results demonstrate the successful expression of HBc and HBc-P28 proteins and also suggest that the two proteins could separately assemble into particles. Similarly, ELISA analysis revealed that faction #9 of both *E. coli* clones reacted strongly with anti-HBc antibody, but anti-VP2-P28 antibody only reacted with factions (numbers #9 and #10) of *E. coli* clone expressing HBc-P28 but not the clone expressing HBc (Fig. 4D and E). The data (Fig. 4E) suggest that the VP2-P28 epitope is exposed at the outer surface of particles, thus allowing for efficient antibody binding.

Finally, the purified protein samples were subjected to transmission electron microscopy analysis. As shown in Fig. 4Fand G, numerous spherical particles of two different sizes (~30–35 nm diameter) were observed for both HBc and HBc-P28 proteins. Moreover, the surfaces of both the large and small particles were not very "smooth" and protruding spikes could be found on the particle surfaces, which is consistent with the previous research (Crowther et al., 1994). These results convincingly demonstrate the successful assembly of HBc and HBc-P28 VLPs in *E. coli*. In addition, SDS-PAGE analysis of purified proteins showed only one prominent band for each protein and as expected, HBc-P28 protein migrated slower than HBc in the gel (Fig. 4H). Purified HBc-P28 protein could be recognized by both anti-HBc and anti-VP2-P28 antibodies in western blotting analysis, whereas purified HBc only reacted with anti-HBc antibody (Fig. 4H).

3.5. HBc-P28 VLP induced CVA10-specific neutralizing antibodies in mice

To study the immunogenicity, purified HBc-P28 VLP was adsorbed on aluminum hydroxide adjuvant and used to immunize adult BALB/c mice. Control mice were immunized with the same dose of HBc VLP formulated with alum. After three rounds of immunization, antisera were collected and detected for antigen-specific IgG antibodies by ELISA using purified HBc VLP or peptide VP2-P28 as coating antigens. As shown in Fig. 5A and B, HBc-specific antibodies were present in sera of all mice vaccinated with HBc or HBc-P28 VLPs, while anti-VP2-P28 antibodies were detected only in HBc-P28 VLP-vaccinated mice.

Individual antisera were further tested for their ability to neutralize live CVA10 (the S0148b strain) *in vitro*. As shown in Fig. 5C, HBc VLP-immunized mouse sera (control sera) did not show any CVA10-

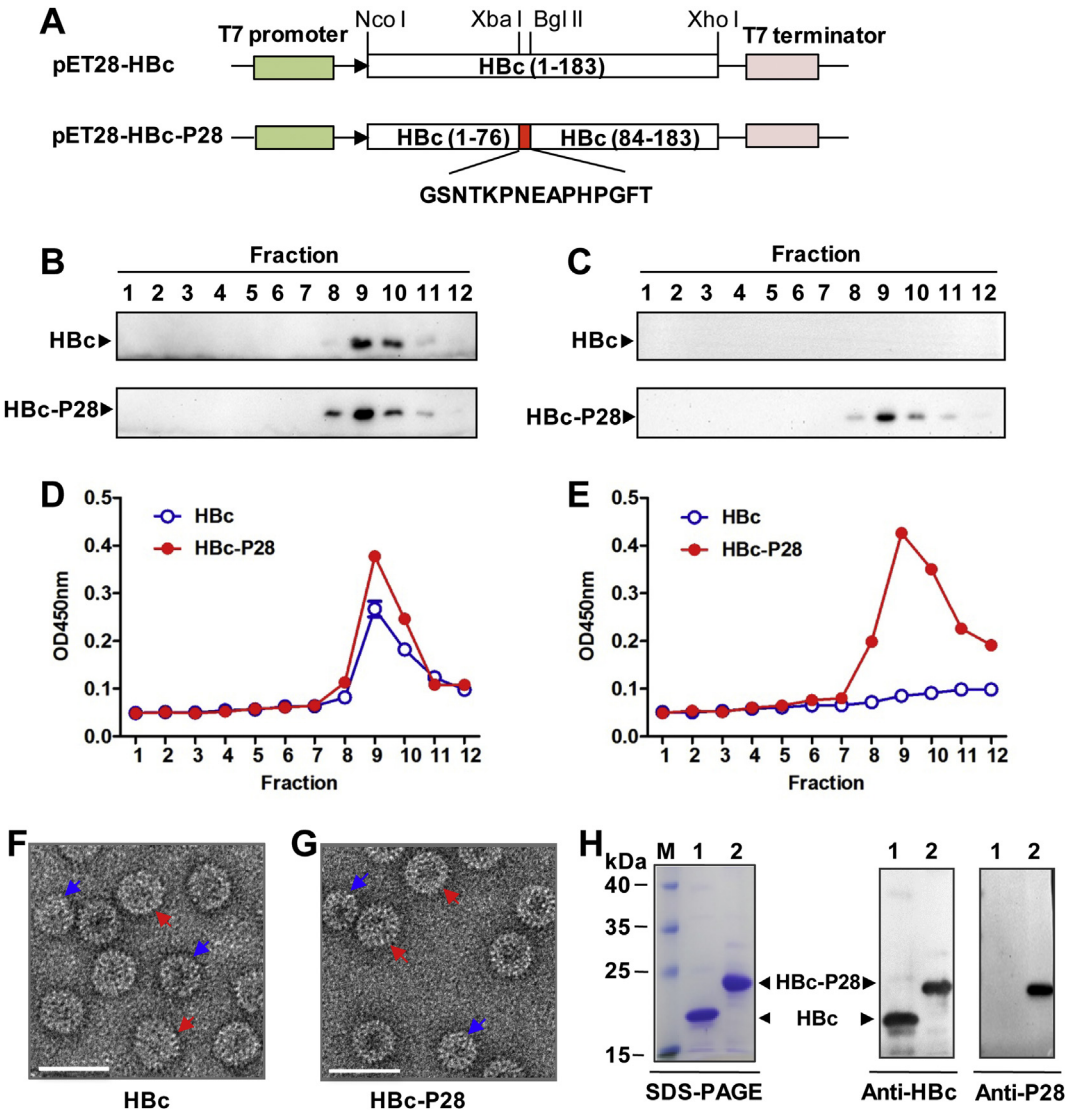

Fig. 4. Expression and characterization of HBc-P28 fusion protein in *E. coli*. **(A)** Schematic diagrams of the constructs used in this study. P28, CVA10 epitope VP2-P28. **(B–E)** Sucrose gradient analyses. Lysates from *E. coli*. transformed with pET28-HBc or pET28-HBc-P28 were subjected to ultracentrifugation through 10–50% sucrose gradients. Twelve fractions were taken from the top and then analyzed by **(B–C)** western blotting and **(D–E)** ELISA with **(B and D)** anti-HBc and **(C and E)** anti-VP2-P28 polyclonal antibodies, respectively. **(F–G)** Electron microscopy micrographs of **(F)** HBc and **(G)** HBc-P28 VLPs. The large and small particles are indicated by red and blue arrows, respectively. Bar = 50 nm. **(H)** SDS-PAGE and western blotting analysis of purified HBc and HBc-P28 VLPs. Lane M, protein marker; lane 1, HBc VLPs; lane 2, HBc-P28 VLPs.

neutralizing activity at the lowest serum dilution tested (1:8). By contrast, all sera from the HBc-P28 VLP group neutralized CVA10/S0148b with titers ranging from 8 to 32.

To determine the breadth of neutralization, pooled antisera for each group were further detected for the cross-neutralization capacity against a panel of CVA10, EV71, and CVA16 strains. As shown in Table 1, pooled anti-HBc VLP sera did not display any neutralization effect against all tested viruses at the lowest dilution tested (1:16). HBc-

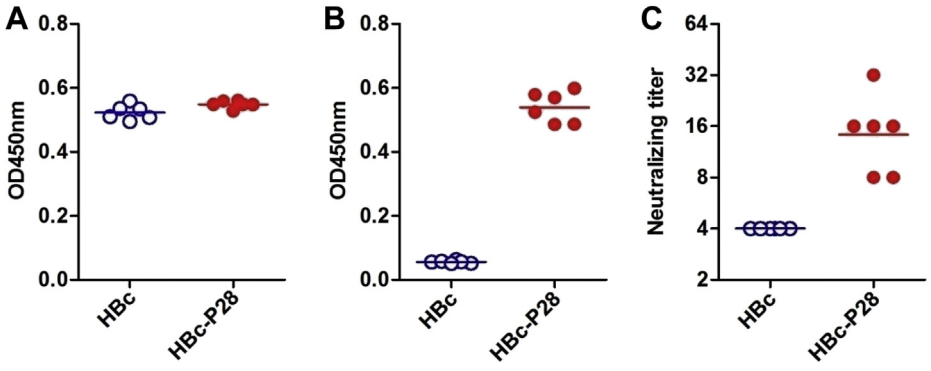

Fig. 5. Humoral immune responses induced by immunization with VLPs. Groups of six mice were immunized three times with purified HBc or HBc-P28 VLPs, and antisera were collected two weeks after the final immunization. The serum samples were diluted 1:100 and tested for reaction with **(A)** HBc VLPs and **(B)** VP2-P28 peptide by ELISA. **(C)** Neutralizing titers of the antisera against CVA10/S0148b. For geometric mean titer (GMT) computation, a titer of 4 was assigned to serum samples that did not show any neutralization effect at 1:8 dilution (the lowest dilution tested). Each symbol represents a mouse and the solid line indicates the geometric mean value of each group.

Table 1
Neutralization activity of pooled antisera against a panel of enteroviruses.

Pooled antisera against	Neutralization titer against				
	CVA10/ S0148b	CVA10/ S0273b	CVA10/ Kowalik	EV71/ G082	CVA16/ SZ05
HBC	< 16	< 16	< 16	< 16	< 16
HBC-P28	32	32	16	< 16	< 16

The lowest serum dilution tested is 1:16.

P28 immune sera could neutralize CVA10 clinical isolate CVA10/S0273b and prototype strain CVA10/Kowalik, but failed to cross-neutralize other HFMD-causing enteroviruses, including EV71/G082 and CVA16/SZ05 (Table 1). These results indicate that HBc-P28 VLP can elicit CVA10-specific neutralizing antibody responses in mice.

3.6. Passively transferred anti-HBc-P28 sera protected neonatal mice from lethal CVA10 challenge

In vivo protective efficacy of HBc-P28 VLP vaccine was assessed by passive immunization with immune sera in a mouse model of CVA10 infection (Shen et al., 2016). Groups of naive ICR mice (six-day-old) were prophylactically administered pooled anti-HBc-P28 or control (anti-HBc) sera and were challenged with CA10/S0148b one day later. After infection, mice that received control sera gradually developed clinical signs, including limb weakness and paralysis, and 56.2% of these mice ultimately died (Fig. 6). In contrast, 92% (12/13) of mice treated with anti-HBc-P28 sera remained healthy and survived (Fig. 6). These results demonstrate that the HBc-P28 VLP vaccine could offer efficient protection to mice against lethal CVA10 infection.

4. Discussion

So far, there have been no reports regarding linear neutralization sites of CVA10. The primary goal of this study was to identify linear neutralization epitopes on the capsid proteins VP1, VP2 and VP3 of CVA10. Results of peptide ELISA and neutralization-inhibition assay showed that only one CVA10 peptide, VP2-P28, could not only be readily recognized by neutralizing polyclonal antibodies against inactivated CVA10 (Fig. 1), but also effectively inhibit neutralization effect of anti-CVA10 sera (Fig. 2), suggesting that this peptide contains potential neutralizing epitopes. In addition, VP1-derived peptides #1, #2, #6, #7, and #8 corresponding to N-terminal region (residues 1 to 50) of VP1 reacted with anti-CVA10 sera (Fig. 1A), indicating that the VP1 N-terminal region contains multiple B-cell epitopes. Hence, these findings would be helpful to CVA10 vaccine design and diagnostic reagent development.

In the present study, we developed a VP2-P28-based CVA10 vaccine by utilizing HBc particles to display this peptide. HBc-P28 protein could assemble into particles in *E. coli* (Fig. 4). Importantly, VP2-P28 was exposed on the surface of HBc particles (Fig. 4E). Moreover, expression levels of HBc-P28 and HBc VLPs in *E. coli* were comparable. Final yields per liter of bacterial culture were 12.8 mg for HBc-P28 VLP and 10.9 mg for HBc. Additionally, *E. coli* expression system has multiple desirable traits, such as high scalability, simpleness in operation and low cost (Baeshen et al., 2015; Baneyx, 1999), thus *E. coli*-expressed HBc-P28 VLP had the potential for future industrial production.

The current study showed that HBc-P28 VLP could induce potent antibody responses against VP2-P28 in mice (Fig. 5B). Sera raised against HBc-P28 could neutralize both CVA10 clinical isolates and prototype strain (Fig. 5C and Table 1). Amino acid sequence in the VP2-P28 region was highly conserved among CVA10 strains (Fig. 3A), which could well explain the broad-spectrum neutralizing activity of HBc-P28-immune sera against CVA10. In addition, anti-HBc-P28 sera failed to cross-neutralize other enteroviruses, including EV71 and CVA16

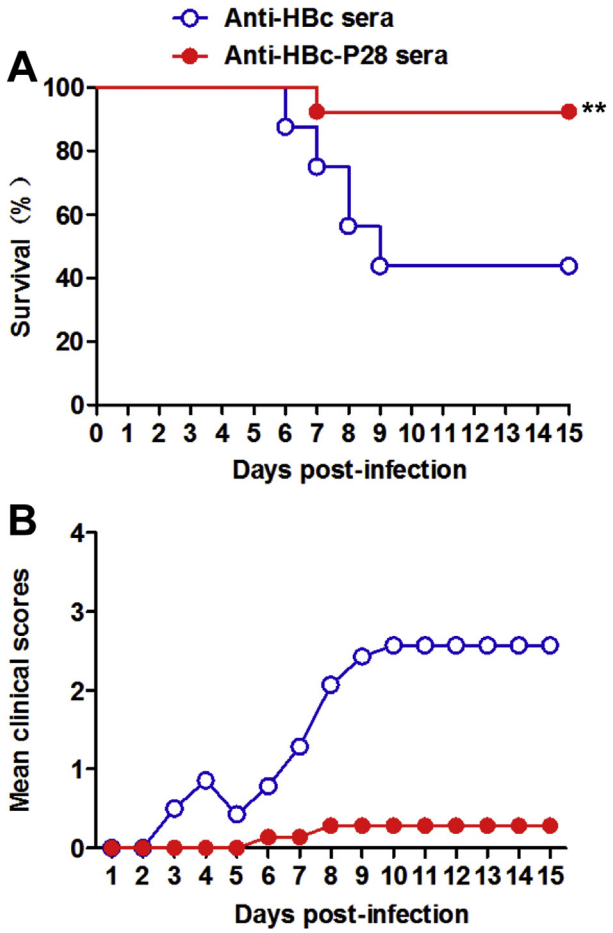

Fig. 6. *In vivo* protective efficacy of anti-HBc-P28 sera against lethal CVA10 challenge. Groups of six-day-old ICR mice (n = 13–16 mice/group) were i. p. administered pooled anti-HBc (control) or anti-HBc-P28 sera. One day later, these mice were infected with CVA10/S0148b. After infection, each mouse was monitored daily for **(A)** survival and **(B)** clinical score for 15 days. Clinical scores were graded as follows: 0, healthy; 1, reduced mobility; 2, limb weakness; 3, paralysis; 4, death. Statistical significance was determined by Log-rank (Mantel-Cox) test; **p < 0.01 vs. control group.

(Table 1), probably due to low level (~30%) of amino acid sequence identity and obvious structural differences in the VP2-P28 region between CVA10 and the other viruses (Fig. 3). It is worth noting that a high degree of sequence homology (79%) exists between EV71 and CVA16 VP2-P28 regions (Fig. 3A).

In this study, *in vivo* protective efficacy of the HBc-P28 VLP vaccine was evaluated by passive immunization/challenge experiment. Passively transferred anti-HBc-P28 sera effectively protected the recipient mice from CVA10 infection, whereas treatment with anti-HBc sera did not improve clinical symptoms and survival rate of CVA10-infected mice (Fig. 6). These data not only demonstrated the efficacy of the HBc-P28 VLP vaccine, but also indicated that anti-VP2-P28 antibodies but not anti-HBc antibodies played role in protection.

In summary, our study demonstrates that VP2-P28 is a CVA10-specific linear neutralizing antigenic site, and *E. coli*-derived HBc-P28 VLP could elicit broadly neutralizing/protective antibody responses against CVA10, thus representing a promising CVA10 vaccine candidate worthy of further development.

Conflicts of interest

The authors declare no conflict of interests.

Acknowledgements

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