Virus-Like Particles Produced in *Pichia Pastoris* Induce Protective Immune Responses Against Coxsackievirus A16 in Mice

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**Background:** Coxsackievirus A16 (CA16) is one of the main causative agents of hand, foot, and mouth disease (HFMD), and the development of a safe and effective vaccine has been a top priority among CA16 researchers.

**Material/Methods:** In this study, we developed a *Pichia pastoris* yeast system for secretory expression of the virus-like particles (VLPs) for CA16 by co-expression of the P1 and 3CD proteins of CA16. SDS-PAGE, Western blot, and transmission electron microscopy (TEM) were performed to identify the formation of VLPs. Immunogenicity and vaccine efficacy of the CA16 VLPs were assessed in BABL/c mouse models.

**Results:** Biochemical and biophysical analysis showed that the yeast-expressed CA16 VLPs were composed of VP0, VP1, and VP3 capsid subunit proteins, and present spherical particles with a diameter of 30 nm, similar to the parental infectious CA16 virus. Furthermore, CA16 VLPs elicited potent humoral and cellular immune responses, and VLPs-immunized sera conferred efficient protection to neonatal mice against lethal CA16 challenge.

**Conclusions:** Our results demonstrate that VLPs produced in *Pichia pastoris* represent a safe and effective vaccine strategy for CA16.

**MeSH Keywords:** Coxsackievirus Infections • Immunogenetics • Pichia • Vaccines, Virus-Like Particle

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Background

Over the past several years, hand, foot, and mouth disease (HFMD) has been prevalent in the Asia-Pacific region, causing significant morbidity and mortality, particularly in infants and young children [1–3]. However, there are still no available antiviral drugs against HFMD. Development of a safe and effective vaccine has been of high priority in HFMD research. Coxackievirus A16 (CA16) and human enterovirus 71 (EV71) are the two major etiological agents responsible for HFMD, which circulated alternatively or together causing numerous outbreaks of HFMD in the epidemic area [4–6]. EV71 infections were often associated with severe neurological complications and significant mortality, and therefore have been the priority focus of HFMD vaccine development [7,8]. Studies on EV71 vaccines have progressed rapidly in recent years [9,10], and one of the inactivated whole-virus EV71 vaccine candidates has been approved in China since 2015 [11]. However, vaccine development against CA16 still lags far behind. Accumulating reports suggest that CA16 infections can be associated with serious complications like myocarditis, irreversible shock, aseptic meningitis, and even death [12–14]. Furthermore, co-infection with CA16 and EV71 and recombination between these two viruses present the greater challenge for the control and prevention of epidemic HFMD [15–18]. Taken together, these findings suggest that development of CA16 or combined CA16/EV71 vaccines should be targeted to ensure broad and effective protection against HFMD.

Virus-like particles (VLPs), composed of viral structural proteins, are morphologically and immunologically similar to the native virions, but lack viral genetic material and therefore mitigate the potential side effects [19]. VLPs can elicit broad, strong, and long-lasting humoral and cellular immune responses thanks to the surface-displayed, structured, and densely repeated viral antigens and epitopes [20]. VLPs have emerged as a safe and effective strategy for vaccine development against viral diseases. To date, it has been shown that a wide variety of VLPs from important viruses have been approved for use as vaccines or gene therapy tools [21–23]. VLPs can be produced in different expression systems, such as Bacterial systems (Escherichia coli cells) [24], insect systems (baculovirus/insect cells) [25,26], mammalian cell systems [27], and yeast systems (Saccharomyces cerevisiae and Pichia pastoris cells) [28,29].

The yeast expression system P. pastoris has been widely used in vaccine production with the advantages of easy manipulation, high production levels, and low cost [29–31]. In the present study, we demonstrated the production of CA16 VLPs in a P. pastoris yeast system (pGAPZαA-SMD1168) by co-expression of the P1 and 3CD proteins of CA16, and investigated its immunogenicity and protective efficacy as a potential vaccine candidate against CA16 infection.

Material and Methods

Cell lines and virus strains

RD cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS), penicillin 100 U/mL, and streptomycin 100 μg/mL. Three local CA16 strains, JB141030268 (B1a, GenBank No. KC866864), JB141030026 (B1a, GenBank No. KC866856), and JB141030230 (B1b, GenBank No. KC866863), were isolated from stool specimens of HFMD patients in Shenzhen in 2010. All viral stocks were propagated and titrated for the 50% tissue culture infectious dose (TCID₅₀) in RD cells. CA16 strain JB141030268 was used as a candidate vaccine strain, and JB141030026 and JB141030230 were used as virulent strains to challenge the neonatal mice.

Constructions of vector and recombinant plasmids

Viral RNA was extracted from the JB141030268-infected RD cells and subjected to reverse transcription PCR to obtain the CA16-specific cDNA sequences. The resultant cDNA was used as a template for the amplification of the gene fragments coding for P1 and 3CD of CA16, respectively. The P1 fragment was amplified with primers (forward 5'-GGATCTTCCAGAGATTCCGTGATGGGTCACAGTC TCCAC-3' and reverse 5'-CTGCCGTTCGACGATTCCGCGCT CTAATGTTGTATCTTG TTCTTAC-3'), digested with Pml I and Not I. The 3CD fragment was amplified with primers (forward 5'-GAATTCAGGGGACGCTGCTGCTGCTGCCTG CTCAATGTTGTATCTTG TTCTTAC-3' and reverse 5'-TCTAGACTAAAAATATCCTGACGCAATTCTTCTT-3'), digested with EcoRI I and XbaI I. The resultant digestion products were purified and then inserted into the yeast expression vector pGAPZαA (Invitrogen) at the same sites, designated as pGAPZαA-P1 and pGAPZαA-3CD, respectively. This vector was labeled with the α-factor secreting signal sequence and the Zeocin™ resistance marker. The resultant recombinant plasmids were maintained in E. coli JM109, and identified by Zeocin™ resistance, PCR, and double restriction enzyme digestion. The proper recombinant plasmids were sequenced using the pGAP forward and the 3′AOX1 primer sequences to confirm that the target genes were in frame with the α-factor of pGAPZαA vector.

Expression of virus-like particles

The strategy of yeast transformation was performed according to the Pichia Expression Manual (Invitrogen). Briefly, the above-constructed plasmid pGAPZαA-P1 or pGAPZαA-3CD was linearized by BspHI (NEB) and electrooporated into SMD1168 competent cells to make a genome-integrate transformant, designated as pGAPZαA-P1/SMD1168 or pGAPZαA-3CD/SMD1168, respectively.YPD (yeast extract peptone dextrose) medium plates containing Zeocin™ 100 μg/mL (Invitrogen) were used...
for selection of desired transformants. Subsequently, the proper pGAPZαA-3CD/SMD1168 yeast strain was transformed by electroporation with the linearized construct pGAPZαA-P1 (BspHII) and was plated on YPD medium containing gradient increasing Zeocin™ (100, 300, 500, and 1000 μg/mL) for selection of double transformants. DNA sequencing using the CA16-specific primer was performed for further confirming the positive Zeocin™-resistant transformants at each step of electroporation. The positive transformants containing both the P1 and 3CD genes were designated as pGAPZαA-VLPs/SMD1168 for producing VLPs. A negative control containing the empty pGAPZαA vector was constructed in parallel.

For expression of VLPs, a single colony of pGAPZαA-VLPs/SMD1168 yeast transformant was picked and inoculated into YPD liquid medium containing Zeocin™ 100 μg/mL with shaking at 300 rpm at 30°C. After 96 h, the supernatant of the yeast culture was collected by centrifugation at 5000 rpm for 10 min and stored at –80°C until ready to assay.

Sucrose gradient analysis

The above-collected yeast culture supernatant was concentrated 10-fold with 100 KDa Shut off Ultrafilter (Millipore) and then loaded onto 15–60% (w/v) noncontinuous sucrose gradients, followed by ultracentrifugation in a P65ST swing rotor (Himac CP80 WX; Hitachi) at 34,000 rpm for 4 h at 4°C. On basis of pre-experiments, the VLPs-rich fractions between 30% and 45% sucrose were collected carefully, diluted in precooled PBS buffer, and ultracentrifuged in a P65NT angle rotor (Himac CP80 WX; Hitachi) at 28,000 rpm for 2 h at 4°C to remove the residual sucrose. The resultant pellet containing purified VLPs was resuspended in PBS buffer and subjected to Western blot and transmission electron microscopy (TEM). The protein concentration was quantified using the BCA Protein Assay Kit (Pierce) according to the manufacturer’s instructions.

SDS-PAGE and Western blot

The purified VLPs samples were mixed with SDS gel-loading buffer, boiled for 5 min, and separated on 10% SDS-PAGE gels. Proteins were visualized by Coomassie blue R-250 staining or transferred onto PVDF membranes for Western blot analysis. The rabbit anti-CA16 antiserum diluted at 1: 1500 was used as the primary antibody and the secondary antibody was goat anti-rabbit IgG (H+L) HRP-conjugated antibody (Thermo) diluted at 1: 3000. Positive signals were visualized by chemiluminescence using the SuperSignal West Pico Kit (Thermo), recorded by ImageQuant LAS4000 (GE Healthcare). The anti-CA16 monoclonal antibody used in this study was generated in house from a rabbit immunized with inactivated CA16.

Transmission electron microscopy

The purified VLPs samples were absorbed onto carbon-Formvar-coated 200-mesh copper grids and subjected to negative staining with 2.5% phosphotungstic acid for 5 min. The grids were dried overnight and then examined using a Hitachi H-7650 electron microscope at 200 KV.

Mouse immunization and serum samples collection

All of the mice used in this study were obtained from Guangdong Medical Laboratory Animal Center. All animal experimental procedures were carried out in strict accordance with and approved by the Institutional Animal Care and Use Committee at CDC of Shenzhen. Female BABL/c mice aged 6–8 weeks were randomly divided into 8 groups (8 animals per group). Prior to immunization, one part of purified CA16 VLPs antigens (see above, this section) was diluted to 50 μg/mL with PBS, and the other part was mixed with complete (for primary injection) or incomplete (for booster injection) Freund’s adjuvant (50%, v/v) (Sigma) under the same concentration. The heat-inactivated CA16 virus (JB141030268) was prepared in an identical way and served as the positive control antigen. The similarly purified empty vector pGAPZαA and pGAPZαA-P1-transformed yeast antigens, together with PBS and Freund’s adjuvant, were used as negative control antigens for immunization. Each mouse was intraperitoneally (i.p.) injected with 0.2 mL of antigens (10 μg/mouse) at week 0 and boosted with the same dose of antigens at week 2. Blood samples were collected from all mice before immunization and at weeks 0, 1, 2, 3, 4, 5, and 6 after the primary immunization. All mice were euthanized by cervical dislocation under anesthesia. The serum samples were inactivated at 56°C for 30 min and stored at –80°C until the following assay.

Analysis of total IgG and antibody subclasses against CA16

The total anti-CA16 IgG titers and its subtypes in the sera were detected by enzyme-linked immunosorbent assay (ELISA). To measure the total anti-CA16 IgG titers, each well in the 96-well plate was pre-coated overnight with 100 μL of purified heat-inactivated CA16 virus (10 μg/mL in PBS, pH 7.4) at 4°C. After 3 washes with PBST buffer (0.05% Tween 20 in PBS), the wells were blocked with 5% bovine serum albumin (BSA) in 300 μL of PBST at 37°C for 2 h. After another wash, 100 μL of two-fold serial serum dilutions starting at 1: 100 or PBS control was added for incubation at 37°C for 1 h, followed by the addition of 100 μL of horseradish-peroxidase (HRP)-conjugated goat anti-mouse IgG (1: 5000 dilution) and incubation at 37°C for 40 min. The color development was initiated with 100 μL/well of 3, 3’, 5, 5’-tetramethylbenzidine (TMB) substrate (Sigma) in the dark at 37°C for 10 min, and followed
by addition of 50 μL/well of H₂SO₄ (2M) stop solution. The optical density (OD) was read at 450 nm by a microplate reader (Bio-Rad). The mean absorbance value in duplicate wells was recorded, and the anti-CA16 IgG titers were defined as the highest dilutions at which the absorbance was 2.0 times that of control mouse sera.

The subtype IgG titers were measured by commercially available Mouse Monoclonal Antibody Isotyping Kits (Sigma) with the following procedures: the 96-well plates were coated with 100 μL of IgG1, IgG2a, IgG2b, and IgG3 capture antibodies (1:1000 dilution with PBS) at 37°C for 1 h. After 3 more washes with PBST, the immune or control sera were added to the wells and incubated at room temperature for 2 h. The HRP-conjugated goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 antibodies (1:600 dilution with the assay buffer) were added into the appropriate wells as the secondary detection antibody. After 30 min of incubation and another wash, the color was developed with 100 μL/well of TMB substrate in the dark at 37°C, and terminated 20 min later by adding 50 μL/well of NaOH (3N). The positive well was colored brown, and the OD₄₅₀ was read as described above. The mean absorbance value in triplicate wells was used to express the subtype IgG antibody level in serum. 

Neutralization assay

The titers of neutralizing antibodies against CA16 were determined by the TCID₅₀ reduction assay in RD cells. Briefly, RD cells were seeded to 96-well plates overnight until 70% confluence. Fifty microliters of two-fold serially diluted serum samples (2⁻⁴ to 2⁻¹⁰ dilutions) were mixed with equal volumes of 100 TCID₅₀ CA16/JB141030268 virus stock and incubated onto the new 96-well plates at 37°C for 1 h. Subsequently, 2×10⁴ RD cells cultured in 100 μL of DMEM containing 10% FBS were added into each well, and the cytopathic effect (CPE) was observed after 3 days culture. Cells and virus control were carried out simultaneously. The neutralization titers were defined as the highest serum dilutions that could result in a 50% reduction in CPE.

To assess the cross-reactivity, the sera from VLPs-immunized mice were collected at 2 weeks after the booster injection, as described in this section. CA16/JB141030026 (B1a genotype) and CA16/JB141030230 (B1b genotype), along with EV71 (C4 genotype), CA4, CA6, CA10, and Echovirus 30 (ECHO 30) isolated from throat swabs or stool specimens during the HFMD epidemic in Shenzhen in 2010, were used as the challenge viruses, respectively. Procedures were performed as described above.

Splenocyte proliferation and cytokine analysis

The splenocyte proliferation was investigated using the commercially available Cell Counting Kit-8 (CCK-8, Dojindo). Briefly, the spleens were aseptically removed from immunized mice (n=3 for each group) at two weeks after the booster injection as described in this section. The splenocytes were isolated and cultured in 96-well plates at a density of 5×10⁴ cells per well in 100 μL of RPMI 1640-10% FBS for 24 h. Then, the splenocytes were stimulated with 100 μL of purified VLPs (5 μg/mL, experimental groups), ConA (5 μg/mL, Sigma, positive control), or RPMI 1640 medium (negative control) for 48 h, followed by the incubation of 10 μL of CCK-8 solution for 4 h. The OD₄₅₀ was read by a microplate reader. The entire experiment was carried out at 37°C with 5% CO₂; each well was done in sextuplicate, and the mean absorbance value was recorded. The splenocyte proliferation was assessed by the stimulation index (SI) calculated according the formula: SI = (experimental or ConA OD₄₅₀—negative control OD₄₅₀)/negative control OD₄₅₀.

For cytokine analysis, splenocytes were harvested and cultured as described above. After 48 h of stimulation, the supernatants were collected and assayed in duplicate wells for IL-2, IL-4, IL-10, and INF-γ using the commercially available ELISA kits (Life).

Lethal virus challenge and in vivo protection assays

In vivo protective efficacy of the VLPs antisera against CA16 challenge was evaluated in an established neonatal mice model, and the mouse-adapted virulent strains JB141030026 (CA16/B1a) and JB141030230 (CA16/B1b) were used to challenge newborn mice.

In vivo protective efficacy of the maternal VLPs antisera was evaluated by lethal challenge of the neonatal mice born to the VLPs-immunized dams by following steps: groups of adult female BABL/c mice received primary immunization as described above and were allowed to mate two weeks later, followed by booster injections one week after pregnancy. The neonatal mice born to the immunized dams were i.p. challenged with 100 LD₅₀ of CA16/JB14103026 or CA16/JB141030230 within 24 h after birth, respectively. All mice were monitored daily for clinical symptoms and death until all mice in the control groups died. Clinical scores were graded as follows: 0, healthy; 1, reduced mobility; 2, limb weakness; 3, paralysis; 4, death.

To evaluate the passive protective efficacy in vivo, groups of one-day-old BABL/c mice (n=10 for each group) were i.p. inoculated with 20 μL of heat-inactivated antisera from VLPs-immunized or empty pGARPz/A vector antigen-immunized mice at week 4 as described above. One day later, the mice were challenged i.p. with either JB141030026 (CA16/B1a, 100 LD₅₀) or JB141030230 (CA16/B1b, 100 LD₅₀). The survival and clinical scores were recorded daily for up to 14 days post-infection as described above.
Statistics

The statistical significance was determined the Student's two-tailed t test or nonparametric one-way analysis of variance (ANOVA), and \( p < 0.05 \) was considered statistically significant. The Kaplan-Meier survival curves were compared by the log-rank test.

Results

Co-expression of P1 and 3CD in yeast

The P1 and 3CD genes were cloned from the local endemic genotype CA16 B1a strain (JB141030268), which was also the predominant genotype currently circulating in mainland China. The full length of P1 was 2586 bp, which encoded 862 amino acids. The full length of 3CD was 1935 bp, which encoded 645 amino acids. The P1 and 3CD genes were inserted into the yeast expression vector pGAPZ\(a\)A, and the resultant recombinant plasmids pGAPZ\(a\)A-3CD and pGAPZ\(a\)A-P1 were electro-porated successively into the SMD1168 yeast strain to make the double transformants pGAPZ\(a\)A-VLPs/SMD1168. CA16 VLPs were secreted in culture supernatant of positive transformants and purified by ultracentrifugation for SDS-PAGE and Western blot analysis. As shown in Figure 1, the yeast strain SMD1168 and empty vector pGAPZ\(a\)A transformant did not express P1 (lane 1 and lane 2), whereas the pGAPZ\(a\)A-P1/SMD1168 expressed an obvious protein band, approximately 97 kDa (lane 4). Lane 5 (VLPs harvested at 96 h) and lane 6 (concentrated VLPs) show three protein bands ranging from 25 to 40 KDa, and further analysis of Western blot with rabbit anti-CA16 antiserum confirmed that the purified VLPs contained three major subunit proteins, with molecular weight corresponding to VP0 (39 KDa), VP1 (36 KDa), and VP3 (28 KDa), respectively. The TEM observation further unraveled that VLPs presented as 27~30 nm spherical particles consistent with the native CA16 virions (Figure 2). All these results indicated that co-expression of P1 and 3CD in the transformed \( P. \) pastoris successfully led to the cleavage of P1 polyprotein into VP0, VP1, and VP3 by functional 3CD protease and subsequent self-assembly into the icosahedral VLPs. The yield of VLPs was approximately 2.45 mg/L, and the purity was greater than 83%.

Humoral immune response of VLPs-immunized mice.

Humoral immune response elicited by VLPs immunization was evaluated by levels of anti-CA16 IgG and neutralizing antibodies. Groups of female BALB/c mice were i.p. immunized twice at two-week intervals with PBS, Freund's adjuvant (FA), empty pGAPZ\(a\)A vector antigen (pGAPZ\(a\)A), P1 antigen (P1), purified VLPs (VLPs) or purified VLPs with Freund's adjuvant (VLPs+FA), purified heat-inactivated CA16 virus (CA16), and purified CA16 virus with FA (CA16+FA), respectively. The serum samples were collected and pooled at different time points after priming and boosting. Levels of specific IgG against CA16 in sera were measured by ELISA as described in the Material and Methods section. Figure 3 shows that the FA, pGAPZ\(a\)A,
and P1 groups induced negligible anti-CA16 IgG titers, flattening against the background level induced by negative control PBS. Conversely, the VLPs or VLPs+FA triggered significantly higher anti-CA16 IgG antibodies than those provoked by negative controls since week 1. The titers gradually increased and peaked at 2020 or 7132 at week 2 after booster immunization (p<0.05), respectively. Despite the slight drop, the anti-CA16 IgG antibodies elicited by the VLPs or VLPs+FA were sustained at high levels at week 4 after booster immunization (the last time point tested). Compared with the VLPs or VLPs+FA, the inactivated CA16 or CA16+FA induced statistically similar IgG antibodies with the same changing trends (p>0.05). Furthermore,

Figure 2. Characterization of purified CA16 VLPs. The purified VLPs by sucrose gradient ultracentrifugation were characterized by SDS-PAGE (A), Western blot (B), and TEM (C, D) as described in the Material and Methods section. The PAGE gel was stained by Coomassie blue R-250. The primary antibody used in the Western blot was rabbit anti-CA16 antiserum diluted at 1: 1500. Lane M: 10–170 KD protein marker; Lane 1, 5: purified empty pGAPZα vector culture supernatant; Lane 2: purified P1 protein; Lane 3, 4: purified VLPs. Panels C and D were magnified 8000 and 12,000 times, respectively. Bar=100 nm.
the profiles of specific IgG subclasses were measured in the sera harvested from VLPs-immunized and inactivated CA16-immunized mice at week 2 after booster immunization. The results showed that IgG1 and IgG2b were dominantly induced by the VLPs or inactivated CA16, followed by IgG2a and IgG3; the ratio of IgG1 to IgG2a was 1.54 in VLPs and 1.16 in inactivated CA16 (Table 1).

Neutralizing antibody titers against CA16 were measured in RD cells by the TCID₅₀ reduction assay. As delineated in Figure 3, no CA16-specific neutralizing antibodies were detected in sera of negative control groups even at the lowest dilution of 1: 16 before or after immunization as expected. By contrast, the VLPs or VLPs+FA induced significantly higher neutralizing antibodies against the homologous CA16, with the titers approaching up to 815 or 1192 at week 2 after booster immunization (p<0.05), respectively. Moreover, the VLPs showed slightly stronger ability to elicit anti-CA16 neutralizing antibodies compared with the inactivated CA16 under the same sample treatment (p>0.05). To evaluate the cross-neutralization, the sera from VLPs-immunized mice at two weeks after the booster injection were collected and subjected to neutralization assays against a virus panel. As shown in Table 2, the anti-VLPs sera exhibited potent neutralization against the heterologous CA16 virus with titers up to 456, but failed to cross-react with EV71, CA4, CA6, CA10, or ECHO 30 strains.

The above results suggested that immunization with VLPs in mice potently induced the CA16-specific IgG and long-lasting neutralization antibodies.

### Table 1. Subtype IgG profile in the VLP or CA16 immune mice sera.

| Antigen      | IgG1 (log2) | IgG2a (log2) | IgG2b (log2) | IgG3 (log2) |
|--------------|-------------|--------------|--------------|-------------|
| VLP          | 0.43 (0.02) | 0.28 (0.03)  | 0.41 (0.07)  | 0.62 (0.04) |
| VLP+FA       | 0.64 (0.08) | 0.41 (0.02)  | 0.62 (0.05)  | 0.80 (0.06) |
| CA16         | 0.36 (0.03) | 0.31 (0.03)  | 0.32 (0.02)  | 0.52 (0.05) |
| CA16+FA      | 0.60 (0.04) | 0.49 (0.06)  | 0.56 (0.03)  | 0.73 (0.02) |

**Figure 3.** Titer profiles of total IgG and neutralizing antibody. (A) Titers of total anti-CA16 IgG. (B) Neutralization titers. Eight groups of adult female BABL/c mice received priming and boosting injection at two-week intervals with PBS, FA, pGAPZαA, P1, VLPs, VLPs+FA, CA16, and CA16+FA, respectively. The serum samples were collected at the indicated times and measured by ELISA as described in the Material and Methods section. The data represent the mean ± standard deviation (S.D.) of two independent immunization experiments (n=8 for each group). Statistical significance was indicated as follows: n.s. p>0.05; * p<0.05; and ** p<0.01 comparing between each vaccine group and the PBS control group.
The lowest serum dilution tested 1:16.

Table 2. Cross-neutralization of VLPs antisera.

| Antisera       | Neutralization titers against |
|---------------|--------------------------------|
| Anti-VLPs     | JB141030026  | JB141030030  | EV71 | CA4 | CA6 | CA10 | ECHO 30 |
|               | 815          | 456          | <16  | <16 | <16 | <16  | <16      |

VLPs+FA-immunized mice could be significantly induced to proliferate by VLPs compared with spleenocytes of negative control groups (p<0.05). Compared to the inactivated CA16 group, the SI values for the VLPs group were statistically higher with ConA or VLPs stimulation (p<0.05). Profiles of cytokines secreted by VLPs-stimulated spleenocytes were evaluated by measuring the levels of INF-γ and IL-2 (Th1 cytokines) and IL-4, IL-6, and IL-10 (Th2 cytokines). As shown in Figure 5, spleenocytes of VLPs-immunized or VLPs+FA-immunized mice produced significantly higher levels of INF-γ, IL-2, IL-4, IL-6, and IL-10 compared to those from the negative controls (p<0.05). Under the same sample treatment, cytokine profiles of inactivated CA16-immunized mice were similar to those of the VLPs group, except for lower levels of IL-4 (p<0.05). The results suggested that VLPs immunization induced both Th1-type and Th2-type cellular immune response.

In vivo protection against lethal CA16 challenge in neonatal mice

The in vivo protective efficacy of the VLPs was evaluated by maternal immunization and passive protection assays. For the maternal challenge/protection experiment, the neonatal mice born to the immunized dams were i.p. challenged with a lethal dose of CA16 within 2-4 h after birth. As shown in Figure 6, mice from control groups began to get sick at 3 days post-infection (dpi) (Figure 6A), gradually developed limb paralysis at 5 dpi (Figure 6B), and eventually died at 7 dpi. Mice born to the VLPs-immunized and VLPs+FA-immunized dams were partially protected from the homologous CA16 challenge, with a final survival rate of 60% and 87.5% at 14 dpi, respectively (Figure 6C, 6D). When challenged with the heterologous CA16, the survival rate increased to 62.5% and 87.5% at 14 dpi for the VLPs and VLPs+FA groups (Figure 6E, 6F). Maternal VLPs antisera conferred higher protection against homologous and heterologous CA16 challenge to the neonatal mice than that of inactivated CA16 at the same sample treatment, with lower mean clinical scores. For the passive protection assay, groups of one-day-old BABL/c mice were i.p. inoculated with antisera from VLPs-immunized or empty pGAPZαA vector antigen-immunized mice, followed by i.p. challenge with a lethal dose of CA16 at 24 h post-inoculation. As shown in Figure 7, the anti-VLPs serum conferred full protection against homologous and heterologous CA16 challenge for the neonatal mice. In contrast, neonatal mice that received antisera from pGAPZαA-immunized mice died by 7 dpi.

Discussion

CA16 is a major agent responsible for HFMD, and the development of a safe and effective vaccine has been a focal point in CA16 research. When compared with EV71, the vaccine development against CA16 still lags far behind, and the inactivated whole-virus vaccine is the preferred vaccine type [32,33]. However, the inactivated whole-virus vaccine raises high safety concerns. Past and ongoing vaccine research indicates the efficiency of VLPs in developing vaccine for CA16. The majority of CA16 VLPs are produced using the baculovirus/insect cell system [22] and the Saccharomyces cerevisiae yeast system [34]. However, the lower yield and contamination with baculovirus particles [35] in the former system and over-glycosylation, methanol residue, and unnecessary inclusion bodies [36] in the latter system still need to be solved. Past and ongoing vaccine research indicates the efficiency of VLPs in developing vaccine for CA16. The majority of CA16 VLPs are
produced using the baculovirus/insect cell system [22] and the yeast system [37,38]. CA16 VLPs were produced in insect cells by co-expression of P1 and 3CD proteins of CA16 using recombinant baculoviruses and induced passive protection against lethal CA16 challenge in neonate mice. It was also reported that CA16 VLPs yield reached around 8 mg per liter of Sf9 insect cell culture [22]. In addition, Zhao et al. produced CA16 VLPs in the *Saccharomyces cerevisiae* yeast system, and immunization with CA16 VLPs elicited full protection in neonatal mice against lethal CA16 infection [39]. Recently, Zhang et al. demonstrated that CA16 VLPs could be produced in the *Pichia pastoris* yeast system at slightly higher levels, around 8.3 mg per liter of yeast culture [37]. However, the high cost and contamination with baculovirus particles [40] in the former system and over-glycosylation, methanol residue, and unnecessary inclusion bodies [41] in the latter two systems still need to be solved.

The primary aim of this study was thus to develop a highly efficient, safe, and low-cost expression system of VLPs and...
Figure 6. Maternal antibodies protected neonatal mice from lethal CA16 virus challenge. Adult female BABL/c mice were immunized with VLPs or other control antigen as indicated. (A) A representative picture of wasting caused by CA16/JB141030026 at 3 dpi is shown (indicated by arrows). The mouse on the left-hand side was an age-matched control from the PBS-immunized group. (B) A representative picture of right-hind-limb paralysis caused by CA16/JB141030026 (indicated by arrows). The neonatal mice born to the immunized dams were i.p. challenged with CA16/JB141030026 (B1a) or CA16/JB141030230 (B1b) within 24 h after birth and monitored daily for clinical scores (C, E) and survival (D, F) for a period of 14 days. Clinical scores were graded as follows: 0, healthy; 1, reduced mobility; 2, limb weakness; 3, paralysis; 4, death. The logrank test was used to compare the survival curves between each vaccine group and the PBS control group. Statistical significance was indicated as follows: n.s. \( p > 0.05 \); * \( p < 0.05 \); and ** \( p < 0.01 \).
evaluate its vaccine potential against CA16. In this study, we established a P. pastoris yeast system (pGAPZαA-SMD1168) to produce VLPs. The pGAPZαA vector uses the GAP promoter and α-factor secretion signal sequence for constitutive secretory expression of recombinant proteins in P. pastoris, and its Zeocin™ resistance marker makes it easier to select the positive transformants. SMD1168 is a protease-deficient P. pastoris yeast strain in which the secretory proteins are protected from being degraded. Our results demonstrated the formation of VLPs by co-expression of P1 and 3CD proteins of CA16 in P. pastoris. The VLPs exhibited similarity in both protein composition and morphology to native CA16 virions. In this yeast expression system, VLPs products are secreted into the culture supernatant with no need for cell lysis or galactose or methanol induction, making it more convenient, economical, and safe for future industrial production of VLPs vaccine. The production yield of VLPs by ultracentrifugation was 2.45mg/L, which is lower than that in other expression systems [22,29,34]. The large-scale production may be improved by use of the yeast fermentation tanks and optimization of expression and purification conditions and is underway.

In this study, we evaluated the potential of VLPs as a vaccine candidate against CA16 infection. The CA16-specific IgG and neutralizing antibodies could be detected since week 1 after primary injection, and increased following the booster injection, peaking at week 2 after booster immunization in VLPs-immunized BABL/c mice. More importantly, the titers of both IgG and neutralizing antibodies were sustained at a higher level until 4 weeks after booster immunization. The anti-VLPs antisera exhibited potent neutralization with cross-reactivity and conferred protection to the neonatal mice against lethal challenge with CA16 homologous and heterologous strains, implying a potential humoral mechanism of protection via neutralizing antibodies. It is worth noting that the anti-VLPs antisera exhibited no cross-reactivity against other HFMD-causing strains, including EV71, CA4, CA6, CA10, and ECHO 30, thereby suggesting the urgent need for bivalent (EV71/CA16) or multivalent vaccines for effective HFMD prevention [42,43].

In addition to the humoral responses, VLPs immunization also efficiently stimulated splenocyte proliferation and induced high levels of INF-γ and IL-2 (Th1 profile), and IL-4, IL-6, and IL-10 (Th2 profile). The IgG subclass isotyping revealed that IgG1 and IgG2b were dominantly induced by the VLPs. Taken together, the VLPs induced a mixed Th1/Th2 immune response to potentiate both the activation of effector T cells and antibody production. Immunization with VLPs mixed with Freund’s adjuvant elicited higher levels of antibody titers and T cell responses than VLPs alone, which indicated that the presence of adjuvant could enhance VLPs-specific immune responses in mice [44]. Compared with VLPs, the heat-inactivated CA16 elicited relatively lower levels of IgG and neutralizing antibodies, and thus conferred poorer protection for neonatal mice against lethal CA16 virus challenge. The same results were reported in other previous research [45], and again confirmed the importance of preserving conformation-dependent epitopes.

In the present study, the maternal-transferred antibody immunized with VLPs provided 60% and 62.5% protection to neonatal mice from the lethal challenge with CA16 homologous and heterologous strains, but the rates were both 100% for passive transferred anti-VLPs antisera. This may be explained by the lower mouse age (one day old) at the time of virus inoculation.
in the maternal immunization experiment compared with that (two days old) in the passive protection experiment.

To assess the immunogenicity of P1 polyprotein, the pGAPZaA-P1/SMD1168 yeast transformant experiment was conducted in an identical way. SDS-PAGE analysis showed a protein band of approximately 97 kDa in the culture supernatant of pGAPZaA-P1/SMD1168, but no protein band was detected when probed with rabbit anti-CA16 antiserum in Western blot. Moreover, the single P1 protein did not show any immunogenicity in mouse immunization experiments. These collective results implied that a single P1 polyprotein could be expressed in P. pastoris, but with no immunogenicity although containing all the major structural proteins. The correct cleavage of P1 by protease 3CD is critical for VLPs self-assembly and vaccine potential.

Conclusions

In conclusion, we successfully established the Pichia pastoris yeast platform for production of CA16 VLPs. Immunization with VLPs induced strong and long-lasting humoral and cellular immune responses, and VLPs-immunized antisera conferred efficient protection to neonatal BALB/c mice against lethal CA16 challenge. These data imply that P. pastoris yeast is a convenient and efficient expression system and that the yeast-expressed VLPs are a potential vaccine candidate against CA16 infection.

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Competing interest statement

No conflicts.

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