Tropical Journal of Pharmaceutical Research May 2020; 19 (5): 927-932
ISSN: 1596-5996 (print); 1596-9827 (electronic)
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Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v19i5.3

Original Research Article

An oral vaccine against CVA16 (Coxsackievirus A16) was developed by constructing a recombinant *Lactococcus lactis*

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Sent for review: 16 January 2020 *Revised accepted: 30 April 2020*

Abstract

Purpose: *To develop an oral vaccine against CVA16 (Coxsackievirus A16) by constructing a recombinant Lactococcus lactis that expresses VP1 from CVA16.*
Method: *An oral CVA16 vaccine was prepared by expressing CVA16 VP1 protein with Lactococcus lactis. CVA16 VP1 gene was incorporated into a Lactobacillus expression vector, namely, pNZ8148, and then expressed in NZ9000, a food-grade lactic acid bacterium which serves as a carrier for oral vaccines.*
Results: *There was statistically significant difference in CVA16-specific IgG antibody level between NZ9000-pNZ8148-CVA16-VP1 group (0.49 ± 0.05) and control group (0.05 ± 0.00) when the antiserum was diluted 1:10 ($t = 19.84$; $p < 0.05$). Furthermore, the level of CVA16-specific IgA antibody in NZ9000-pNZ8148-CVA16-VP1 group (0.17 ± 0.02) was significantly higher than in control group (0.05 ± 0.00) following antiserum dilution of 1:10 ($t = 12.08$; $p < 0.05$).*
Conclusion: *A CVA16 oral vaccine made from Lactobacillus elicits protective antibodies against CVA16. Thus, it is a potential as oral vaccine against CVA16 but further studies in vivo are required to ascertain its safety and effectiveness.*

Keywords: *Coxsackievirus A16, Hand, foot and mouth disease, Lactococcus lactis, Oral vaccine, Enterovirus 71*

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INTRODUCTION

Enterovirus 71 (EV71) and coxsackievirus A16 (CVA16) are the main pathogens that hand-foot-cause and-mouth disease (HFMD) [1-4]. A small number of children may present severe symptoms such as aseptic meningitis and acute flaccid paralysis [5,6]. With the use of vaccines against EV71, the level of severe HFMD caused by EV71 is decreased in children [7,8]. However, there are still no available clinical vaccines for CVA16. Inactivated vaccine, the most frequently used type of vaccine, has been used against EV71 in the clinics [9]. However, inactivated vaccines are associated with some disadvantages, one of which is that their immune

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effect is not sustainable, thereby necessitating multiple injections. Research has shown that the complex of EV71 and CVA16 VLPs stimulates the production of bivalent antibodies [10]. However, there are reports showing that infants exhibited discomfort due to pain after they were vaccinated through injection [11].

Expectations are high for the development of a vaccine that can exist in the body for a long time, with continuous production of antibodies without any adverse effects. Lactic acid bacteria are used as safe and food-grade bacteria, and as carriers for oral vaccines [12]. The lactic acid bacteria expression system, being an advanced prokaryotic expression system, has some advantages. In the first place, it is safe because lactic acid bacteria are often used as food. Secondly, the level of protein expressed by lactic acid bacteria is low, implying that it is easy to purify the exogenous proteins. Moreover, lactic acid bacteria-based oral vaccine is administered through gastrointestinal mucosa antigen presentation, and can induce the production of an effective immune response [13].

In this study, an oral vaccine against CVA16 was developed by constructing a recombinant *Lactococcus lactis* that expressed VP1 from CVA16. The results suggest that the oral vaccine induced antibodies against CVA16 via intestinal mucosal immunity.

EXPERIMENTAL

Cells, virus, vectors and reagents

Rhabdomyoma (RD) cells purchased from ATCC were cultured in DMEM supplemented with 10 % FBS and antibiotics. The CVA16-GZ08 strain (GenBank accession no. FJ198212) was obtained from Guangzhou Medical University. The CVA16 was titrated to obtain $TCID_{50}$, arising from typical cytopathic effect (CPE) due to viruses. Plasmid pNZ8148, as well as competent cell line MC1061 and *Lactococcus lactis* NZ9000 which expresses exogenous proteins, were obtained from Nanjing Zoonbio Biotechnology Co. Ltd, while MRS broth and MRS solid medium were purchased from Guangdong Huankai Microbial Sci and Tech Co. Ltd. TKARA BIO Inc. was the source of DNA Marker and Taq DNA Polymerase.

Protein marker was bought from TransGen Biotech. The restriction enzymes *NcoI* and *XbaI* were purchased from New England Biolabs, while anti-coxsackievirus A16 antibody was product of Jianglai Biotechnology Co. Ltd.

Construction of the plasmid, PNZ8148-CVA16VP1

The complete gene for CVA16 VP1 (GenBank accession number JF420555.1) was synthesized by Sangon Biotech (Shanghai) Co. Ltd. The sequences of the restriction enzymes *NcoI* and *XbaI* were designed to the 5' ends of the upstream and downstream primers. The initiation codon ATG and the termination codon TTA were designed to be behind the restriction site of the upstream and downstream primers, respectively. The primer sequences are displayed as follows:

CVA16 VP1 forward primer: 5'-CCATGGATGGGGATCCTATTGCAGATATGATTGA-3'

CVA16 VP1 reverse primer: 5'-TCTAGATTACAACGTTGTTATCTTGTCTCTACTA-3'

To obtain CVA16 VP1 that contained the sequences of the restriction enzymes *NcoI* and *XbaI*, CVA16 VP1 forward and reverse primers were used to amplify the fragment of CVA16 VP1 with Bio-Rad PCR instrument (USA). Polymerase chain reaction was carried out at 94°C for 4 min, with 30 cycles of amplification (94 °C for 30 sec, 58 °C for 30 sec, and 72 °C for 60 sec); and 72 °C for 160 sec after the last cycle. The PCR fragment was purified using a DNA fragment purification Kit Ver.4.0 (TAKARA BIO Inc.). After purification, the DNA fragment and the plasmid pNZ8148 were digested overnight at 37°C with the restriction enzymes *NcoI* and *XbaI* (New England Biolabs Ltd). The digested DNA fragment and plasmid were purified using the DNA fragment purification Kit Ver.4.0. Nucleic acid concentration was measured with Nano Drop One/one C (Thermo Scientific Ltd). The digested CVA16 VP1 fragment and plasmid (3:1 molar ratio), and 1 μl of T4 DNA Ligase (NEB) and 1 μl of 10×T4 DNA Ligase Reaction Buffer (NEB) were added. Ultra-pure water was added to bring the reaction volume to 10 μl, and the solution was mixed and incubated at 16°C overnight. Thereafter, the linked products were transferred to *E. coli* MC1061-competent cells for amplification. Positive clones were picked for sequencing. The match sequence plasmid (named pNZ8148-CVA16 VP1) was kept frozen at -80 °C prior to use.

Construction of the expression CVA16 VP1 of NZ9000

To obtain *Lactococcus lactis*-competent state of NZ9000, a single colony was picked, inoculated in 5 ml of GM medium, and incubated at 30 °C

overnight. Then, 5 ml NZ9000 was transferred to 50 ml GSGM17 medium at 30 °C overnight. Next, 5 ml NZ9000 was transferred to 400 ml GSGM17 medium, incubated at 30 °C to an absorbance of 0.2 - 0.3 at a wavelength of 600 nm, and centrifuged at 4 °C and 4000 rpm for 20 min. The cells were collected and washed with 400 ml of solution I. Then, 100 ml of solution II was added, vortexed and allowed to stand for 15 min on ice, before it was centrifuged for 20 min at 4 000 rpm at 20 °C. The cells were collected and washed with 100 ml of solution I, suspended in 4 ml of solution I, dispensed into aliquots of 40 µl per tube, and kept frozen at -80 °C.

The plasmid pNZ8148-CVA16 VP1 and 40 µl of competent cells were mixed, transferred to a 0.2-cm electro-conversion cup in an ice bath, and subjected to electro-transformation at 2000 V, 25 µF, and 200 Ω. Thereafter, 1 ml of GMMC was immediately added to the electro-conversion cup and transferred into a 1.5-ml tube. Then, the samples were placed in an ice bath for 5 min and incubated at 30 °C for 1 - 1.5 h. Conversion bacteria were coated onto the GM culture board containing chloramphenicol. Next, 10 µl, 100 µl, and 900 µl were separately added to the plate and cultured at 30°C for 40 h.

A single colony was introduced into 5 ml of GM medium at 30°C overnight. The plasmid was extracted and identified with PCR using Bio-Rad PCR instrument (USA). The forward primer was 8148-VF1:5'-ACGCGAGCATAATAAACGG-3', while the reverse primer was 8148-VR1:5'-CGAAAGCGAAATCAAACGA-3'. Polymerase chain reaction was carried out at 94 °C for 5 min, with 30 amplification runs, and 72 °C for 10 min after the last run.

Western blot assay for protein expressions

The recombinant *Lactococcus lactis* NZ9000-pNZ8148-CVA16 VP1 cells were cultured in a medium containing 10 µg/ml chloramphenicol at 30 ℃ for 8 – 10 h in the presence of glucose (0.5 %). Dilution was carried out to achieve an absorbance value of 0.4. Then, nisin was separately added to the remaining cultures to a final concentration of 50 ng/ml or 100 ng/ml, and cultured overnight at 30°C for 4 h to induce expression of fusion protein. After induction, the cells were subjected to SDS–polyacrylamide gel electrophoresis. Western blot assay was done as outlined previously to identify the expression of the CVA16 VP1 protein, using monoclonal antibody CVA16 IgG (Jianglai Biotechnology Co. Ltd, 1:1000) as 1^0 antibody, and horse radish peroxidase-linked secondary antibody.

Oral vaccination

In all, ten 6-week-old BALB/c female mice were bought from Guangdong Medical Laboratory Animal Centre and fed in an IVC squirrel cage. They were assigned to 2 groups: pNZ8148-CVA16 VP1 and pNZ8148-vector groups, each with 5 animals. Mice in pNZ8148-CVA16VP1 group and pNZ8148-vector group were subjected to oral vaccination using recombinant *Lactococcus lactis* NZ9000-pNZ8148-CVA16VP1 and *Lactococcus lactis* NZ9000-pNZ8148, respectively, at a dose of 5×10^{11} CFU/ ml in 500 µl 3 times a day. The mice were vaccinated on days 1, 3, 5, 7, 14, 21, 28 and 35. After 35 days, all mice were sacrificed, and serum and nasal lavage samples were obtained.

ELISA

Titres of I*gA* were determined in serum and nasal lavage using enzyme-linked immunosorbent assay (ELISA) [14]. A 96-well enzyme plate was coated with purified CVA16 virus (10^7TCID50/ml), and 50 µl/well was added. Then, the plate was incubated at 4 °C for 12 h. Thereafter, horse radish peroxidase-linked anti-mouse IgG (for serum) or IgA (for nasal lavage) diluted 1: 8000 or 1: 10000, respectfully, was used in mice as 2^0 antibody for determination of the levels of antibodies against CVA16.

***In vitro* test for CVA16-neutralizing antibodies**

Mouse sera were subjected to incubation for ½ h at 56 °C before the test, and were diluted 1:2. Then, 50 ml of each serum was added to 50 mL of 100 $TCID_{50}$ of virus, followed by incubation at 37 °C for 1 h and adsorption onto 96-well microtiter plates pre-seeded with RD cells. The plates were incubated at 37 °C for 48 h, followed by titre reading in terms of the highest dilution that suppressed viral multiplication.

Statistical analysis

Differences between two groups were analysed using Student's *t*-test. Statistical analysis was done using SPSS 16.0 (IBM). Values of $p < 0.05$ were taken as indicative of statistically significant differences.

RESULTS

The CVA16 gene was cloned into the pNZ8148 plasmid. Then, the plasmid pNZ8148-CVA16VP1 was transformed into NZ9000 *Lactococcus lactis* bacteria. The primer pair 8148-VF1 and 8148-VR1 were used to detect positive clones of the *Lactococcus lactis* NZ9000 containing the

plasmid pNZ8148-CVA16VP1. The fragment was a 1200-bp band, as shown in Figure 1 A. Double digestion with the restriction enzymes *NcoI* and *XbaI* produced a small 800-bp band, as shown in Figure 1 B.

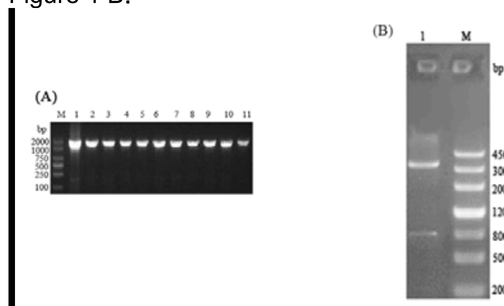

Figure 1: Map of nucleic acid electropherogram. Map of recombinant *Lactobacillus* plasmid PCR nucleic acid electropherogram. M: DNA marker (down to up: 100, 250, 500, 750, 1000 and 2000 bp); 1: plasmid of PNZ8148-CVA16 VP1 as the template; 2 to 11: plasmid of extraction from bacterial fluid; Map of recombinant *Lactobacillus* plasmid enzyme digestion nucleic acid electropherogram. M: DNA marker (down to up: 200, 500, 800, 1200, 2000 and 3000, 4500 bp) 1: plasmid of pNZ8148-CVA16 VP1 which served as the enzyme digestion template

Expression of CVA16 antigen and identification by Western blotting

The positive clones were picked for induction with different concentrations of nisin for 4 h and overnight. Figures 2A and 2B show the SDS-PAGE results of NZ9000-pNZ8148-CVA16VP1 after nisin induction. The results indicate that NZ9000-pNZ8148-CVA16-VP1 was expressed at 35 kDa, the molecular weight of the CAV16-VP1 protein band. The broken supernatant of the positive strain that was not induced was the control. The protein bands were not obvious between induction samples and non-induction samples.

The results of Western blotting are shown in Figures 2 C and 2D. The broken supernatant and sediment that were induced by two concentrations of nisin for 4 h showed immunoreactive protein bands of 35 kDa. Moreover, there were no inductions of target proteins overnight by nisin at doses of 50 ng/ml or 100 ng/ml.

pNZ8148-CVA16VP1 induced CVA16-specific antibody response via oral vaccination

The CVA16-specific IgG antibody was induced in sera of mice orally given NZ9000-pNZ8148-CVA16-VP1 (Figure 3A). In addition, IgA titre in nasal lavage was detected in mice orally fed NZ9000-pNZ8148-CVA16-VP1, as shown in Figure 3B. As expected, IgG in sera and IgA in nasal lavage were absent in the control group mice. There was statistically difference in CVA16-specific IgG antibody level between the NZ9000-pNZ8148-CVA16-VP1 group (0.49 ± 0.05) and control group (0.05 ± 0) when the antiserum was diluted *1:10 (t = 19.84; p < 0.05)*. In addition, there was statistically significant difference in *CVA16-specific IgA antibody* level between the *NZ9000-pNZ8148-CVA16-VP1* group (0.17 ± 0.02) and control group (0.05 ± 0) at antiserum dilution *1:10 (t =12.08; p < 0.05).*

To test the neutralization titre of antisera of oral vaccine against CVA16, mice anti sera raised against CVA16 were subjected to in *in vitro* microneutralization test, with RD cells infected with 100TCID$_{50}$ of CVA16. The RD cells were completely protected from CPE by antiserum from mice subjected to whole-virion immunization at a neutralization titre of 1:16. This result is shown in Figure 3C.

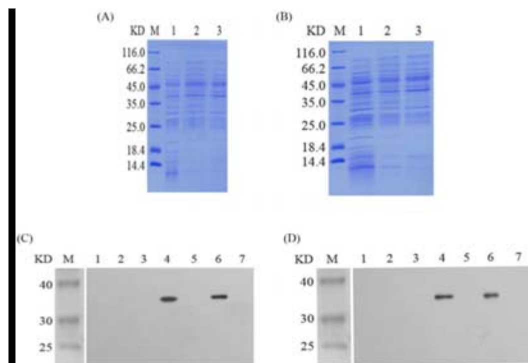

Figure 2: SDS-PAGE result for expression of CVA16 VP1

DISCUSSION

Lactic acid bacteria, as probiotics, settle securely on the intestinal tract surface, which is highly beneficial to the body. Some studies on the expression of alien proteins in *L. lactis* showed that it could be a versatile expression system and delivery vehicle for various proteins. Many oral vaccines have been made with lactic acid bacteria. These include protected rotavirus vaccine [15], porcine epidemic diarrhoea virus oral vaccine [16], and human papillomavirus type 16 vaccine [17]. Foreign proteins expressed by lactic acid bacteria direct stimulation of the intestine to produce immunity. In this study, the VP1 protein of CVA16 was expressed in cells. However, a signal peptide and optimized expression conditions were added. This protein may have higher merit than other secreted expression proteins.

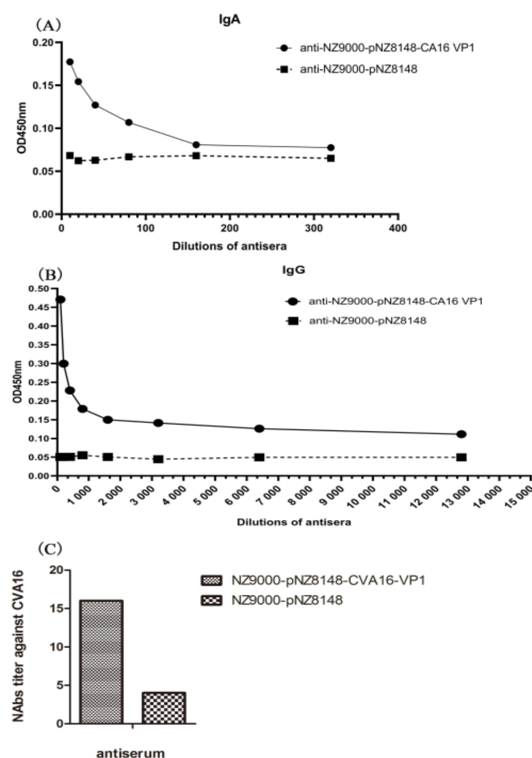

Figure 3: CVA16-VP1-specific antibody responses in mice with oral vaccination of NZ9000-pNZ8148-CVA16-VP1

The lactic acid bacteria that secrete expression proteins need to constantly express proteins to stimulate the intestine. Therefore, the concentration of expression protein may be low. In contrast, foreign protein is constantly expressed intracellularly until the bacteria are degraded.

In addition to inducing the body to produce IgG antibodies, lactic acid bacteria stimulate the production of IgA antibodies. In the anti-HPV field, researchers constructed recombinant strains of *Lactococcus lactis* NZ9000 that express HPV E6 protein which could be used to treat HPV-16 associated with cervical cancer.

However, there are two problems with oral vaccines made by lactic acid bacteria, one of which involves stomach acid. Research has shown that overexpressing the recT gene of lactis NZ9000 enhances tolerance of acid stress. In this study, the lactic acid bacterium NZ9000 was used to express CVA16 VP1. Therefore, theoretically, it could withstand the hydrochloric acid effect. The second problem has to do with drug-resistant genes in recombination-prone lactic acid bacteria. The main factor in this problem originates from lactic acid bacterial expression plasmid. With advances in biotechnology, non-resistant plasmids have been used to express foreign proteins, but it is difficult to screen positive bacteria.

Tetracyclines have some desirable attributes that make them suitable as antibiotics: they are active against gram +ve and gram-ve microorganisms, and they are associated with safety and tolerability by patients. Moreover, tetracyclines are available in intravenous (IV) and oral dosage forms. The pNZ8148 plasmid has an anti-chloramphenicol gene which leads to chloramphenicol resistance. Chloramphenicol has been widely used in the treatment of various sensitive bacterial infections, with serious adverse reactions in the haematopoietic system. Thus, its clinical application has been strictly controlled. Therefore, there is no need to worry about this problem.

CONCLUSION

An oral CVA16 vaccine made from *Lactobacillus* elicited protective antibodies against CVA16. Thus, it has a potential for use as an oral vaccine against CVA16; however, further investigations, including animal trials, are required to ascertain its suitability.

DECLARATIONS

Acknowledgement

This work was supported by the Dongguan Bureau of Science and Technology for the City Key Programmes of Science and Technology (project nos. 2015108101020, 2018507150 1001618, 2016108101029 and 2017507150 100444). The funders played important roles in the study design, data collection and analysis, decision to publish, and preparation of the manuscript.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. Zaixue Jiang performed most experiments and took part in drafting the manuscript. Xingui Tian designed the work, analysed the data and participated in drafting the

manuscript. Xiaomei Lu helped analyse the data. Baimao Zhong designed and supervised the work, and edited the final version of the manuscript. All authors read and approved the final version of the manuscript.

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