Research in Veterinary Science 115 (2017) 332–339

Contents lists available at ScienceDirect

Research in Veterinary Science

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

Orally administered recombinant *Lactobacillus casei* vector vaccine expressing β-toxoid of *Clostridium perfringens* that induced protective immunity responses

Mojtaba Alimolaei[a,b], Mehdi Golchin[a,*], Majid Ezatkhah[c]

[a] Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran
[b] Department of Molecular Microbiology, Kerman branch, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Kerman, Iran
[c] Department of Anaerobic Bacterial Vaccine Research and Production, Kerman branch, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Kerman, Iran

ARTICLE INFO

Keywords:
Clostridium perfringens
Lactobacillus casei
β-toxoid
Vaccine

ABSTRACT

Clostridium perfringens types B and C cause enteritis and enterotoxemia in animals. The conventional vaccine production systems need time-consuming detoxification and difficult quality control steps. In this study, a modified β-toxoid gene was synthesized, cloned into the pT1NX vector, and electroporated into *Lactobacillus casei* competent cells to yield *L. casei*-β recombinant strain. Surface expression of the recombinant β-toxoid was evaluated by ELISA and confirmed by immunofluorescence microscopy. Vaccinated BALB/c mice with *L. casei*-β induced potent humoral and cell-mediated immune responses that were protective against lethal challenges with 100 MLD/mL of the β-toxin. Safety and efficacy of the recombinant clone was evaluated and the presumptive toxicity of *L. casei*-β was studied by toxicity test and histopathological findings, which were the same as negative controls. Our results support the use of *L. casei* as a live oral vector vaccine, and that the recombinant *L. casei*-β is a potential candidate for being used in the control of enterotoxemia diseases caused by *C. perfringens* types B and C.

1. Introduction

Clostridium perfringens (*C. perfringens*) causes numerous gastrointestinal infections in livestock and poultry (Uzal and McClane, 2011). This bacterium is an obligatory anaerobic, rod-shaped bacterium subdivided in 5 toxinotypes (A, B, C, D, and E) according to the production of four major toxins (α, β, ε, and ι). *C. perfringens* types B and C, which both produce β-toxin as the main virulence factor, cause enteritis and enterotoxemia in animals (Fernandez-Miyakawa et al., 2007; Sayeed et al., 2008; Uzal and McClane, 2011). Type B is mainly pathogenic for small ruminants, and causes lamb dysentery, hemorrhagic enteritis, and enterotoxemia. Type C is pathogenic for humans, ruminants, and birds, and causes struck, hemorrhagic enteritis, enterocolitis, and enterotoxemia (Niilo, 1980).

Most pathogens initiate their infections at the mucosal surface such as gastrointestinal system (Marasini et al., 2014). Thus, mucosal vaccination is highly desired as a primary prevention step for these infectious diseases. The most effective method for prevention of enterotoxemia is vaccination (Milach et al., 2012). In current commercial vaccines, virulent strains are grown and used for toxins production. These toxins are being used with optional purification steps for vaccine production. Such vaccine producing systems need the time-consuming detoxification and quality control steps (Nijland et al., 2007b). Therefore, the production of a non-toxic variant of β-toxin, which still retains full immunogenicity, in a safe bacterial vector, could be a beneficial alternative. This would be a good choice to prevent of enterotoxemia or to reduce its severity in initial steps in small ruminants.

The research field of live bacterial vectors (LBVs) has shown significant progress over the last years and various LBVs such as *Lactobacillus casei* (Li et al., 2009; Song et al., 2014), *Salmonella enterica* (Kulkarni et al., 2010; Kulkarni et al., 2008; Zekarias et al., 2008) and *Bacillus subtilis* (Hoang et al., 2008) have proved to be effectual and potent tools for using in livestock health (Silva et al., 2014).

Lactobacillus casei (*L. casei*) has potential immune-modulatory properties and is widely used for expressing several heterologous antigens as vaccines in animal models, with promising results (Adachi et al., 2010; Campos et al., 2008; Ferreira et al., 2009; Grangette et al., 2002; Lee et al., 2006; Maassen et al., 1999; Oliveira et al., 2006; Wen

* Corresponding author at: Post Box: 76169-133, 7616914111 Kerman, Iran.
E-mail address: golchin@uk.ac.ir (M. Golchin).

http://dx.doi.org/10.1016/j.rvsc.2017.06.018
Received 29 January 2017; Received in revised form 5 June 2017; Accepted 26 June 2017

et al., 2012; Yoon et al., 2012). It is a probiotic with GRAS (generally recognized as safe) status and could be a promising host for β toxoid production. *L. casei* has been previously used for gene delivery and expression of protective clostridial antigens, the fragment C of tetanus toxin (Maassen et al., 1999), and the alpha toxin of *C. perfringens* (Li et al., 2009). Moreover, *L. casei* is a Gram-positive organism, which increases the chance of success for production of the β toxoid. This probiotic bacterium applies commonly in ruminants and it is a good vector vaccine to make protective immunogenicity against bacterial antigens, such as clostridial vaccine. This is a good alternative procedure for vaccination against infectious diseases, which can be used as a probiotic at any time in each age. The purpose of this study was to construct and evaluate a safe, non-toxic recombinant vector vaccine for surface expression of a *C. perfringens* β-toxoid.

2. Materials and methods

2.1. Bacterial strains, medium, and growth conditions

L. casei ATCC 393 (obtained from Pasteur Institute of Iran) and recombinant *L. casei* expressing β-toxoid (*L. casei*-β) were anaerobically grown in deMan, Rogosa and Sharpe (MRS) medium (Himedia, India), at 37 °C without shaking. *Lactococcus lactis* subsp. *cremoris* MG1363 carrying pT1NX expression vector, obtained from BCCM/LMBP plasmid collection of University of Ghent, Belgium (http://bccm.belspo.be/about/lmbp.php), was used in this study and grown in M_{17} medium (Micromaster Laboratories, India) containing 0.5% glucose (GM_{17}) at 30 °C without shaking. Plating of bacteria was performed on the respective media with 1.5% agar, and erythromycin (Ery) was utilized at final concentration of 5 μg/mL when it was necessary.

2.2. Gene synthesis

The gene encoding β-toxin of *C. perfringens* (based on gene bank: L13198.1) was modified to construct an inactive form (toxoid) of this toxin. The toxicity of β-toxin was eliminated using three point mutations (D81A, K83A, and C292A), rendering it no longer toxic, but still immunogenic (Nijland et al., 2007a; Segers et al., 1999). Moreover, *Nae*I and *Bam*HI restriction sites and a dendritic cell-targeting peptide sequence (FYPSYHSTPQRP) were added to this modified gene (Mohamadzadeh et al., 2009). This modified β-toxoid gene sequence was synthesized and cloned into pGH plasmid by Generay Biotechnology Company (Shanghai, China).

2.3. DNA procedure and electrotransformation

Nucleic acid manipulation and cloning procedures were performed as previously described (Alimolaei et al., 2016). Briefly, the pGH-β plasmid was digested with *Bam*HI and *Nae*I (Jena Bioscience, Germany) and the target beta gene ligated into the likewise digested pT1NX expression vector containing the lactococcal P1 constitutive promoter to construct pT1NX-β plasmid. The pT1NX vector carries the lactococcal usp45 secretion signal sequence and the sequence encoding the cell wall anchor of *Staphylococcus aureus* protein A (spaX) (Schotte et al., 2000). The encoded protein can be anchored to the cell wall and displayed at the cell surface when the insertion of the coding region is done between unique *Nae*I and *Bam*HI sites.

For the preparation of competent cells, a stationary phase culture (18 h) of *L. casei* ATCC 393 was inoculated (1:50 inoculum) (*v*/v) into 200 mL MRS broth containing 1% glycine and 0.5 M sucrose, and incubated at 37 °C without shaking. The cells were harvested at OD_{600} = 0.3 by centrifugation at 5000 rpm for 10 min at 4 °C and washed thrice with an ice-cold washing solution (10 mM $MgCl_2$) and then bacteria were resuspended 1:100 in 10% glycerol.

For electroporation, 10 μL of ligation mixture or empty pT1NX vector, as the control, was transferred to a volume of 100 μL of competent *L. casei* cells. Electroporation was performed with a single electric pulse (1.75 kV, 200 Ω, 25 μF in 0.2 cm cuvettes) using a Gene Pulser™ apparatus (Bio-Rad Laboratories, Richmond, CA), as described previously (Alimolaei et al., 2016).

Recombinant *L. casei* colonies which contain the pT1NX-β (*L. casei*-β) or carrying the empty vector (*L. casei*-P) were selected on the solid medium by screening the Ery (Erythromycin)-resistant clones. Plasmid was extracted as previously explained (Klaenhammer, 1984) and selected clones were checked for correct ligation by PCR and sequencing, using specific primers: 5′-GCGAATATGCTGAATCATCTA-3′ and 5′-GCAGGAACATTAGTATATCTTC-3′. PCR reaction was performed as previously described (Alimolaei et al., 2014). The expected size of the amplified fragment corresponded to 196 bp.

2.4. Protein isolation, gel electrophoresis, and Western blotting

For the expression analysis of the modified β-toxoid, the recombinant *L. casei*-β and negative controls (*L. casei*-P and wild type *L. casei*) were grown in MRS broth at 37 °C until an OD_{600} = 1.5 was reached. Bacterial cells were collected by centrifugation at 5000 rpm for 10 min at 4 °C. The pellets were suspended in a lysis buffer (50 mM Glucose, 25 mM Tris-HCl, 10 mM EDTA, 20 mg/mL Lysozyme, pH 8.0) and incubated in water bath at 37 °C for 30 min. Then, bacteria were exposed to three consecutive freeze-thaw cycles, and lysates were centrifuged at 13000 rpm for 5 min at 4 °C. The cell protein fractions were processed following Perez et al. (2005). Cell fractions of strains were separated by SDS-PAGE, and either stained with coomassie brilliant blue directly, or transferred to a nitrocellulose membrane and probed with the polyclonal *C. perfringens* beta antitoxin (NIBSC, UK) and horseradish peroxidase (HRP)-conjugated goat anti-Horse IgG antibody.

2.5. Indirect ELISA

The supernatant and cell wall protein fractions were analyzed via indirect ELISA as follows: protein extracts (100 μg/mL) of *L. casei*-β and negative controls (*L. casei*-P and *L. casei*) were coated onto a 96-well ELISA plate (Nunc, Denmark) overnight at 4 °C. International reference preparation of *C. perfringens* beta toxoid (NIBSC, UK) (100 μg/mL) was coated as a positive control. After washing the wells with Phosphate-buffered saline (PBS), pH 7.4 and blocking with bovine serum albumin (3% BSA in PBS), the polyclonal *C. perfringens* beta antitoxin (NIBSC, UK) was diluted (1 mg/mL) in PBS–1% BSA, added to each well and incubated for 1 h at 37 °C. After three-times washing with PBS, 0.05(*v*/v) Tween 20, horseradish peroxidase-conjugated goat anti-Horse IgG antibody (KPL, USA) was added to each well (1:200) and incubated for an additional 1 h at 37 °C. Finally, the plate was washed and developed with TMB substrate, and the absorbance was measured at 450 nm.

2.6. Expression of β-toxoid on the cell surface

Immunofluorescence microscopy was used to confirm the expression of β-toxoid protein on the surface of recombinant *L. casei* cells. Briefly, *L. casei*-β, *L. casei*-P, and wild-type *L. casei* were grown in MRS broth as described. The amount of 500 μL of cultures was collected by centrifugation at 5000 rpm for 5 min, and pellets were washed twice with PBS (pH 7.4). Polyclonal *C. perfringens* beta antitoxin was labeled with fluorescein isothiocyanate (FITC) according to a standard protocol (Harlow and Lane, 1988), diluted (1:200) in PBS, and 20 μL was added to each tube and incubated for 3 h at 37 °C. Samples were washed three-times with PBS, smeared on a microscope slide, and viewed by an immunofluorescence microscope.

2.7. Immunization and challenge

The study was approved by Animal Experimentation Ethics