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Oral immunization of mice against *Clostridium perfringens* epsilon toxin with a *Lactobacillus casei* vector vaccine expressing epsilon toxoid

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ABSTRACT

Clostridium perfringens type D infects ruminants and causes the enterotoxemia disease by ε-toxin. A mutated ε-toxin gene lacking toxicity was designed, synthesized, and cloned into the pT1NX vector and electroporated into *Lactobacillus casei* competent cells to yield LC-pT1NX-ε recombinant strain. BALB/c mice, immunized orally with this strain, highly induced mucosal, humoral, and cell-mediated immune responses and developed a protection against 200 MLD/ml of the activated ε-toxin. This study showed that the LC-pT1NX-ε could be a promising vaccine candidate against the enterotoxemia disease.

1. Introduction

Clostridium perfringens (*C. perfringens*) causes numerous gastrointestinal infections in most animals. Enterotoxemia caused by *C. perfringens* type D (overeating or pulpy kidney disease) has great economic and sanitary importance for farming small ruminants worldwide. Enterotoxaemia is a condition caused by the absorption of large amounts of toxins from the intestines into the general circulation (Uzal and Songer, 2008). The most virulent toxin produced by *C. perfringens* type D is epsilon toxin (Niilo, 1980).

The best effective method to control enterotoxaemia is vaccination (Milach et al., 2012). Development and production of current conventional clostridial vaccines have some problems such as time consuming production processes, detoxification, purification, antigen concentration, quality control steps, etc. (Nijland et al., 2007; Salvarani et al., 2013). Thus, the production of these vaccines, despite their efficacy, has some limitations.

A genetically engineered live vaccine (GELV) system is an effective method for vaccine delivery (Qin et al., 2014). Recombinant toxoid vaccines, as GELV, are considered alternative candidates for the conventional clostridial vaccines. Non-toxicity, more stability, and high-yielding process with superior biosafety characteristics are among the advantages of these vaccines (Salvarani et al., 2013). For the GELV production, non-pathogenic bacteria like probiotics have been recommended to be used as a vaccine carrier, since they enhance the safety of vaccines (Qin et al., 2014).

Among probiotics, lactic acid bacteria (LAB) have been generally recognized safe with intrinsic immunogenicity and offer several advantages over current systemic vaccinations. They have a significant potential for oral and mucosal immunity. Expression of bioactive compounds on the cell surface of LAB can stimulate appropriate immune responses (Bermúdez-Humarán et al., 2008; Poo et al., 2006; Wells and Mercenier, 2008; Song et al., 2014; Douglas and Klaenhammer, 2011). Stimulation of the immune system by *Lactobacillus casei* (*L. casei*) has been extensively studied (Galdeano and Perdigon, 2004; Perdigon et al., 2002; Bonet et al., 2006; Galdeano and Perdigon, 2006; Galdeano et al., 2007). This bacterium has been previously used for gene delivery and expression of protective antigens against different microorganism pathogens (Wei et al., 2010; Liu et al., 2011; Campos et al., 2008) such as clostridial antigens, fragment C of the tetanus toxin (Maassen et al., 1999), and alpha toxin of *C. perfringens* (Li et al., 2009).

The aim of the present study was, first, to make a safe and non-toxic enterotoxemia vector vaccine for expressing ε-toxoid on the cell surface of *L. casei* and, second, to evaluate its protective capacity in a mouse model.

2. Materials and methods

2.1. Bacterial strains and growth conditions

L. casei ATCC 393 (purchased from Pasteur Institute of Iran) and recombinant *L. casei* expressing ε-toxoid (LC-pT1NX-ε) were anaerobically

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grown in deMan, Rogosa, and Sharpe (MRS) medium (Himedia, India) at 37 °C without shaking. *Lactococcus lactis subsp. Cremoris* MG1363 was grown in M_{17} medium (Micromaster Laboratories, India) containing 0.5% glucose (GM_{17}) at 30 °C without shaking. Plating of bacteria were performed on the respective media with 1.5% agar. MRS and GM_{17} media were supplemented with 5 μg/ml erythromycin (Ery) when necessary.

2.2. Plasmids and gene synthesis

The pT1NX vector was obtained from BCCM/LMBP plasmid collection of University of Ghent, Belgium (http://bccm.belspo.be/about/lmbp.php). This expression vector was designed for the expression of cloned genes in Gram-positives, particularly LAB species, under the control of the lactococcal P1 constitutive promoter (Schotte et al., 2000). The pT1NX vector carries the lactococcal usp45 secretion signal sequence and the sequence encoding the cell wall anchor of *Staphylococcus aureus* protein A (spaX). The encoded protein can be anchored to the cell wall and displayed at the cell surface by the insertion of the coding region between the unique *Nae*I and *Bam*HI sites.

The gene encoding epsilon toxoid of *C. perfringens* (based on gene bank: AY858558.1) was modified to eliminate the toxicity by the substitution of $proline_{106}$ for $histidine_{106}$ ($H_{106} \rightarrow P_{106}$) using site-directed mutagenesis (Oyston et al., 1998). Moreover, *Nae*I and *Bam*HI restriction sites and a dendritic cell-targeting peptide sequence were added to this modified gene (Mohamadzadeh et al., 2009). This gene was synthesized and cloned in pGH plasmid by Generay Biotechnology Company (Shanghai, China).

2.3. Cloning and recombinant procedures

Nucleic acid manipulation and general cloning procedures were performed according to the laboratory manuals (Sambrook et al., 1989). Briefly, the pGH-epsilon and pT1NX vectors were digested with *Bam*HI and *Nae*I (Jena Bioscience, Germany) and, then, a gel-purified epsilon toxoid gene was ligated into pT1NX and electroporated into *L.casei* ATCC 393.

For the preparation of competent *L.casei*, an overnight culture was inoculated 1:50 in MRS broth containing 1% glycine and 0.5 M sucrose and incubated at 37 °C until the OD_{600} of 0.3 was obtained. The culture was maintained on the ice for 15 min and the bacteria were collected by centrifugation at 4000 *g* for 10 min at 4 °C. The pellet was washed three times in a washing solution (10 mM $MgCl_2$). The bacteria were resuspended 1:100 in 10% glycerol and the volume of 100 μL was used for electroporation.

Electroporation was carried out as previously described (Posno et al., 1991). Ten microliters of ligation mixture or empty pT1NX vector (approximately, 150 ng), as the control, was added to the competent cells. The competent bacteria were electroporated at 1.75 kV, 200 Ω, 25 μF in 0.2 cm cuvettes using a Gene Pulser™ apparatus (Bio-Rad Laboratories, Richmond, CA). Then, the electroporated bacteria were resuspended in MRS broth containing 0.3 M sucrose, 20 mM $MgCl_2$ and 2 mM $CaCl_2$ and incubated anaerobically at 37 °C for 2.5 h. Afterwards, the bacteria were plated in MRS containing 0.3 M sucrose supplemented with Ery and incubated at 37 °C for 3 days under anaerobic conditions.

Recombinant *L. casei* strains were obtained by screening the Ery-resistant clones. Plasmid extraction was performed same as Klaenhammer protocol (Klaenhammer, 1984). These clones were investigated for the presence of the insert of interest by PCR and sequencing, using specific primers: Forward 5′ TGGGAACTTCGATA CAAGCA 3′ and Reverse 5′ CCACTTACTTGTCCTACTAAC 3′. PCR reaction was performed on the extracted plasmid as previously described (Alimolaei et al., 2014). The expected size of the amplified fragment corresponded to 238 bp. Positive clones were frozen and stored in MRS broth containing glycerol (final concentration 25%) at −80 °C.

2.4. Protein expression

For the expression analysis of the modified ε-toxoid, the recombinant LC-pT1NX-ε and *L. casei* as the negative control were grown in MRS medium at 37 °C until $OD_{600} = 1.5$ was achieved. Bacterial cells were collected by centrifugation at 4000 g 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 exposed to three consecutive freeze–thaw cycles. The protein fractions were processed as previously described (de Lúcia Hernani et al., 2011; Perez et al., 2005).

The cell protein extracts of LC-pT1NX-ε and *L. casei* were fractionated by a 12% SDS-PAGE gel. After the electrophoresis, the proteins were transferred to a nitrocellulose membrane and probed with the polyclonal *C. perfringens* epsilon antitoxin (NIBSC, UK) and horseradish peroxidase (HRP)-conjugated goat anti-Horse IgG antibody (KPL, USA). Colorimetric detection was performed using the 4-chloro-1-naphthol substrate. The exposition of ε-toxoid on LC-pT1NX-ε surface was confirmed with FITC- *C. perfringens* epsilon antitoxin using immunofluorescence.

2.5. Immunization

Female BALB/c mice, 6–8 weeks old, and 20–25 g in weight (10 mice per group), were used according to the local guidelines for the animal care. The study was approved by Animal Experimentation Ethics Committee of Kerman University of Medical Sciences. The mice in the vaccinated group orally received the recombinant LC-pT1NX-ε and the mice from two control groups received *L. casei* carrying the empty vector (LC-pT1NX) or PBS. The bacteria grown overnight ($OD_{600} \geq 2$) were collected by centrifugation (2500 g, 10 min at room temperature), washed twice with PBS and resuspended in PBS until 1×10^9 CFU/ml. The amount of 200 μl of the cell suspensions (suspension in PBS alone) was inoculated at three doses on three consecutive days via gastric gavage with each cycle being started on days 0, 16, and 37.

2.6. Cytokine profile

Interleukin-2 (IL-2), IL-4, IL-10, and interferon-γ (IFNγ) concentrations were measured in sera from the vaccinated mice with LC-pT1NX-ε and controls (LC-pT1NX and PBS groups) using a mouse Th1/Th2 ELISA Ready-SET-Go Kit (eBioscience, San Diego, CA) according to the manufacturer's instructions.

2.7. Challenge

The immunized mice were challenged subcutaneously by the injection of activated ε-toxin (with trypsin) from the overnight culture of *C. perfringens* type D reference strain (CN409, RVSRI, Islamic Republic of Iran) after 17 days of the last booster. Minimum lethal dose (MLD) was calculated according to the standard operating procedure (ANB.0024.SOP, Razi Vaccine and Serum Research Institute, Iran). The challenge was performed with 1 MLD, 2 MLD and 10 MLD of the activated toxin.

2.8. Detection of anti-specific ε-toxin antibody

The mice were bled on days -1, 14, 30, 51, and 63 through tail vein. Intestinal washes were collected from sacrificed mice seven days post-challenge. Anti-epsilon IgG, IgA, and sIgA (secretory IgA) antibodies were detected from sera and intestinal washes by indirect ELISA using the international reference preparation of *C. perfringens* epsilon toxoid (NIBSC, UK) as the coating antigen (100 μg/ml) and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:1000) (Serotec, UK) or anti-mouse IgA (1:2500) (KPL, USA). The final absorbance was read at 450 nm.