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Construction and immunogenicity of the recombinant *Lactobacillus acidophilus* pMG36e-E0-LA-5 of bovine viral diarrhea virus

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A B S T R A C T

Bovine viral diarrhea/mucosal disease (BVD/MD) is an infectious disease of cattle with a worldwide distribution, creating a substantial economic impact. It is caused by bovine viral diarrhea virus (BVDV). This research was conducted to construct the recombinant *Lactobacillus acidophilus* (*L. acidophilus*) pMG36e-E0-LA-5 of BVDV *E0* gene and to test its immunogenicity and protective efficacy against BVDV infection in the mice model. The BVDV *E0* gene was sub-cloned into the expression vector and then transformed into the *L. acidophilus* LA-5 strain by electroporation. The recombinant *L. acidophilus* pMG36e-E0-LA-5 was confirmed by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The mice were immunized orally with the recombinant *L. acidophilus* pMG36e-E0-LA-5. The serum IgG antibody and fecal sIgA antibody responses, expression levels of interleukin (IL)-12 (IL-12) and interferon gamma (IFN-γ) were detected respectively. On the 7th day after the last-immunization, the mice were inoculated with BVDV to evaluate the protective efficiency of the recombinant *L. acidophilus* pMG36e-E0-LA-5.

The results showed that the expressed products protein E0 in the *L. acidophilus* LA-5 resulted in single band of 27 kDa by SDS-PAGE and its strong reactivity with BVDV antibody was confirmed by Western blotting. The IgG and sIgA antibodies responses, IL-12 and IFN-γ expression levels in the vaccinated mice with recombinant *L. acidophilus* pMG36e-E0-LA-5 were significantly higher than those in the control mice. The protective rate of the vaccinated mice against BVDV increased significantly, and a 90.00% protection rate in virulent challenge was observed. These results indicated that the recombinant *L. acidophilus* pMG36e-E0-LA-5 strain was successfully constructed and it could effectively improve the immune response in mice and might provide protection against BVDV.

1. Introduction

Bovine viral diarrhea/mucosal disease (BVD/MD) is an infectious disease of cattle with a worldwide distribution, creating a substantial economic impact on both the beef and dairy industries (Tajima et al., 2001; Cowley et al., 2012). It is caused by bovine viral diarrhea virus (BVDV) (Evermann and Ridpath, 2002; Wang et al., 2014). BVDV infects a wide range of mammalian hosts including cattle, sheep, goat, deer, camel and other ruminants (Intisar et al., 2010; Passler et al., 2007). Broad range of clinical manifestations are attributed to the BVDV infection in susceptible animals including depression, inappetence, oral erosions and ulcerations, diarrhea, respiratory disorders, nervous system affections, abortion and premature birth as well as immunosuppression, especially in young stocks (Kelling et al., 2007; Ridpath, 2010). Therefore, the control of the BVDV infection is economically important to the cattle industry (Ficken et al., 2006; Chase et al., 2004).

E0 gene is a major protective antigen gene of BVDV, and its product is envelope glycoprotein responsible for the elicitation of neutralizing antibodies. The envelope glycoprotein E0 contains major antigen epitopes of BVDV, which can induce protective immune response. And its gene sequence is highly conserved, which make it a suitable candidate antigen of BVDV gene engineering dependent vaccine and immunodiagnosis (Gao et al., 2011; Zimmerman et al., 2006).

Lactobacillus acidophilus (*L. acidophilus*) is a very important probiotics in human and animal (Sanders and Klaenhammer, 2001; Altermann et al., 2005), play an important role in health benefits by its adhesion and colonization in the intestinal mucosa (Buck et al., 2005; Campbell et al., 1999), shows marked antitumor activities, lowering cholesterol, suppression of harmful bacteria, regulating micro flora, maintaining micro-ecological equilibrium in gut and an ability to modify immune responses (Anderson and Gilliland, 1999; Medellin-Peña and Griffiths, 2009). In addition,

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the *L. acidophilus* can produce some of the metabolites, which has nonspecific antibacterial and anti-diarrhea effects. The effects of the *L. acidophilus* have been manifested by the marked increase of the cytokines, chemokines and inflammatory mediator. The *L. acidophilus* as probiotics is widely used in food and feed processing industry, medical and other fields. The *L. acidophilus* as immune vaccine carrier has potential application value (Peterson et al., 2007; Seegers, 2002). This research is conducted to construct the recombinant *L. acidophilus* pMG36e-E0-LA-5 of BVDV *E0* gene and to test its immunogenicity and protective efficacy against BVDV infection in the mice model.

2. Materials and methods

2.1. Strain, plasmid and reagents

The *L. acidophilus* LA-5 strain, plasmid pMG36e and plasmid pMD19-T-E0 of BVDV E0 gene encoding structural protein antigen were provided by the Health inspection and quarantine Laboratory of the College of Veterinary Medicine, Agricultural University of Hebei, China (Jiang et al., 2014). Taq DNA polymerase, T4 DNA Ligase, restriction endonuclease *Sac* I and *Hin*d III, were provided by TaKaRa (Dalian, China). BVDV positive serum was purchased from China Institute of Veterinary Drug Control (Beijing, China). The mini plasmid extraction kit and DNA gel Extraction kit were from Huashun (Shanghai, China). Horseradish peroxidase-conjugated rabbit anti-bovine secondary antibody was purchased from Solai BaoTechnology (Beijing, China). The ELISA Kit for the Quantitative Analysis of Mouse IFN-γ was purchased from Beijing BLKW Biotechnology *Co., Ltd*. The ELISA Kit for the Quantitative Analysis of Mouse IL-12 was purchased from eBioscience *Co., Ltd.*

2.2. Construction and identification of the recombinant plasmid pMG36e-E0

The recombinant plasmids pMD19-T-E0 of BVDV *E0* gene encoding structural protein antigen and pMG36e vector were digested by the *Sac* I and *Hin*d III at 37 °C for 2 h respectively. The digested products were electrophoresed on 1% agarose Gel and recycled by DNA gel Extraction kit. The purified *E0* gene fragments were ligated to pMG36e vector by T4 DNA ligase at 37 °C for 2 h and transformed into competent *Escherichia coli* DH5. The transformed bacteria were plated on MRS culture medium (containing 300 μg/mL erythromycin) at 37 °C overnight. The recombinant plasmids pMG36e-E0 were extracted by the alkaline lysis method and identified by the *Sac* I and *Hin*d III enzyme digestion and PCR using specific primers p1/p2 (forward primer p1: 5′-CCATGGAAAACATAACACAGTGG-3 and reverse primer p2:5′-TAAGCATATGCTCCAAACCACGT-3′). The primer set (P1/P2) was designed by Primer 6.0 software based on the published *E0* sequence of HB strain of BVDV (Jiang et al., 2013) and multiple cloning sites (MCS) of the expression vectors pMG36e. The reaction mixture consisted of 10 × Ex Taq Buffer (Mg^{2+} plus) (2.0 μL), plasmid pMG36e-E0 template (2.0 μL), dNTP Mixture (2.0 μL, each 2.5 mmol/μL), forward primer p1 (1.0 μL, 2.0 μmol/μL), reverse primer p2 (1.0 μL, 2.0 μmol/μL), Ex Taq DNA polymerase (0.1 μL, 5 U/μL) and DEPC-H_2O (up to 20 μL). PCR was initially denatured at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 1 min. The reaction was subjected for final extension at 72 °C for 10 min. Products were electrophoresed on 1% agarose gel, stained with ethidium bromide and visualized under UV light.

2.3. Transformation of the L. acidophilus LA-5 strain

The recombinant plasmid pMG36e-E0 was transformed into the *L. acidophilus strains* LA-5 by electroporation and then inoculated into 950 μL SMRS medium (containing 0.5 mol/L sucrose) at 37 °C for 3 h. Transformed products diluted 10-fold concentration 100 μL were spread on the MRS plates (containing 300 μg/L erythromycin) and incubated at 30 °C for 36–48 h, until colonies appeared. Single colonies of the transformants were inoculated into 35 mL MRS medium (containing 300 μg/L erythromycin) and incubated at 37 °C overnight. After centrifugation the supernatant was discarded, and the collection of cell was split by supersonic. The supernatant was obtained after centrifugation and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting by electrochemiluminescence (ECL).

2.4. Preparation of the recombinant L. acidophilus pMG36e-E0-LA-5

Identified recombinant strain *L. acidophilus* pMG36e-E0-LA-5 was inoculated into 35 mL MRS medium (containing 300 μg/L erythromycin) and incubated at 37 °C continuously. At OD_{600} = 1.0, the cells were harvested by centrifugation at 5000 rpm for 5 min. After washing with 0.01 mol/L PBS, the cells were resuspended in the normal saline at a concentration of 10^{10} CFU/mL.

2.5. Immunization of the recombinant pMG36e-E0-LA-5

The 8-week-old BALB/c mice, male, 18–20 g, healthy, were purchased from Laboratory Animal Research Center of Hebei Medical University, China, and housed in a temperature-controlled, light-cycled room hygienically controlled environment, and provided with feed and water without any antibiotic. All animal studies complied with Guidelines for Ethical Conduct in the Care and Use of Experimental Animals, Peoples' Republic of China.

The mice were randomly divided into 3 groups (20 mice each group), a recombinant pMG36e-E0-LA-5 group (group 1), a pMG36e-LA-5 group (group 2), and a normal saline control group (group 3). The mice in group 1 were immunized orally with the recombinant *L. acidophilus* pMG36e-E0-LA-5 (10^{10} CFU/mL) at a dose of 0.2 mL/mouse for 3 days, and a booster vaccination was given at the same dosage two week later for 2 times. The mice in group 2 and group 3 were immunized orally with the mock-vehicle *L. acidophilus* pMG36e-LA-5 and normal saline respectively, at same dose as the same method. The serum IgG antibody and fecal sIgA antibody responses, expression levels of the IL-12 and IFN-γ were detected before and after immunization.

2.6. Detection of antibodies

On the 0 day, 10th day, 17th day, 26th day, 33rd day, 42nd day after the first immunization, serum IgG antibody responses and fecal sIgA antibody responses were measured by the indirect ELISA method. The OD at 490 nm was measured by a Model 550 ELISA Microplate Reader (BIO-RAD).

2.7. Detection of cytokines

On the 0 day, 10th day, 17th day, 26th day, 33rd day, 42nd day after the first immunization, the plasma levels of the IL-12 and IFN-γ were measured by ELISA. The plasma level of the IL-12 was measured by indirect ELISA following the manufacturer's instructions. The OD at 492 nm was measured by the ELISA Microplate Reader. The plasma level of the IFN-γ was measured by double-sandwich ELISA following the manufacturer's instructions. The OD at 450 nm was measured by the ELISA Microplate Reader.