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Construction and immunological evaluation of recombinant *Lactobacillus plantarum* expressing HN of Newcastle disease virus and DC- targeting peptide fusion protein

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ABSTRACT

Newcastle disease virus (NDV) has been considered as one of the most severe threats to poultry industry. In this study, we constructed a series of food grade recombinant *Lactobacillus plantarum* (*L. plantarum*) strains (RLP) synthesizing either virus hemagglutinin–neuraminidase protein (HN) alone or HN fused with DC cells targeting peptides (DCpep), named RLP(pSIP409-HN) and RLP (pSIP409-HN-DCpep), respectively. The immune responses and protective efficacy were then evaluated in chickens. Results showed that the presence of DCpep in RLP (pSIP409-HN-DCpep) group significantly increased the production of secretory immunoglobulin A (SIgA) in intestines and the percentages of $CD3^+CD4^+$ T cells in spleen and peripheral blood leukocytes ($P < 0.05$) compared to chickens immunized with RLP (pSIP409-HN). In addition, the similar enhancement effects were also observed with regard to trachea SIgA, T lymphocytes proliferation and survival rates after NDV challenge, even without significant statics differences. The results demonstrated the possibility to take use of DCpep as an immune adjuvant in the design of NDV vaccine.

1. Introduction

Newcastle disease virus (NDV), a member of the genus *Avulavirus* in the family *Paramyxoviridae*, has been considered to be one of the most important chicken diseases, resulting in severe economic losses in the poultry industry worldwide (Ganar et al., 2014). In addition to the traditional live attenuated and inactivated vaccines, there have been a number of studies focusing on new technologies to design more efficient NDV vaccines, such as the reverse genetics generated recombinant vaccine (Wang et al., 2015; Zhao et al., 2014), DNA vaccine (Sawant et al., 2011), virus like particles (Park et al., 2014) and subunit vaccine (Esaki et al., 2013; Kumar et al., 2011). Since the surface envelope of NDV is mainly surrounded by two kinds of glycoproteins, the virus hemagglutinin-neuraminidase protein (HN) and the fusion protein (F), which play critical roles in virus infectivity and pathogenicity. The HN and F proteins have been selected as the main targets for vaccine design and studied extensively in the last several decades (Kim et al., 2013; Kumar et al., 2011; Li et al., 2014).

Dendritic cells (DCs) play a critical role in initiating and regulating immune response due to their effective antigen presenting ability (Banchereau and Steinman, 1998) and have been considered as the bridge between host innate and adaptive immunity (Sehgal et al., 2014). Both foreign and self antigens could be efficiently captured by DCs and presented to T cells, inducing different immune response according to the circumstance (Banchereau and Steinman, 1998). Due to the important role of DCs in immune response, the DCs targeting strategy has received more and more attention in the field of vaccine design. In order to trigger strong immune responses, an efficient approach to deliver specific antigens to DCs should be necessary. To data, there have been plenty of DCs' surface receptors or short peptides involved in the specific DCs targeting approach, such as DEC205 (Hawiger et al., 2001), DC-SIGN (Hesse et al., 2013), Dectin-1 (Ni et al., 2010), DCIR (Klechevsky et al., 2010), Mannose R (He et al., 2004), LOX-1 (Li et al., 2012) and a 12-mer peptides (Curiel et al., 2004; Mohamadzadeh et al., 2009).

Lactic acid bacteria (LAB) have been considered as GRAS (generally regarded as safe) organisms (Garcia-Fruitos, 2012) due to its potential health benefits, such as the improvement of immunity and thus control of intestinal infections (Hosoya et al., 2011;

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Perdigon et al., 2001, 2002), improved nutritional value of food (Gilliland, 1990), control of serum cholesterol levels (Lee do et al., 2009) and some cancers (Hirayama and Rafter, 1999; Rafter, 2002). LAB belong to a clade of Gram-positive, non-sporeforming, acid-tolerant, either rod- or cocci-shaped bacteria. Among the various sources of LAB, two of them including *Lactobacillus* and *Lactococcus*, have been extensively genetic engineered as a cell factory to synthesize heterologous proteins (Chowdhury et al., 2014; Morello et al., 2008; Nouaille et al., 2003; Tauer et al., 2014). Since LAB are naturally associated with mucosal surfaces, particularly the gastrointestinal tract, they have also been considered as promising mucosal delivery vesicles to produce protective antigens. In particular, *Lactobacillus plantarum* (*L. plantarum*) has gained more and more attention due to its better performance in some studies compared to *Lactobacillus casei* or *Lactococcus lactis* (Grangette et al., 2002; Shaw et al., 2000).

In this study, we constructed a recombinant *L. plantarum* (RLP) expressing HN-DCpep fusion protein under an inducible promoter in plasmid pSIP409 (Sorvig et al., 2005), confirmed by immunological analysis in vitro. Oral administration of RLP significantly increased the production of secretory immunoglobulin A (SIgA) and the percentages of $CD3^+CD4^+$ T cells in chickens, providing at least partial protection against NDV challenge.

2. Materials and methods

2.1. Bacteria strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* (*E. coli*) DH5α cells were grown in Luria-Bertani medium at 37 °C with shaking and *L. plantarum* NC8 cells (Thea Aukrust, 1992) were grown aerobically in MRS medium at 30 °C without shaking. Solid media were prepared by adding 1.5% (w/v) agar to the broth. When required, ampicillin was added 200 μg/mL for *E.* coli, erythromycin (erm) was added as follows: 200 μg/mL for *E. coli* and 10 μg/mL for *L. plantarum.*

2.2. DNA manipulation

RNA from NDV LaSota strain was extracted using TRIzol reagent (Life Technologies) and used as template for reverse transcription PCR (RT- PCR). Primer pairs HN-P1/P2 and HN-P1/P3 (Table 1) were used to amplify HN gene and HN gene together with DCpep, respectively. The products of PCR amplification were ligated with pMD18-T vector, followed by double digestion using NcoI/HindIII. The digestions were then ligated with pSIP409 digested with the same enzymes, yielding pSIP409-HN and pSIP409-HN-DCpep, respectively (Fig. 1).

2.3. Immunological analysis

Overnight culture of *L. plantarum* NC8 harboring individual plasmid was inoculated into MRS medium with 10 μg/mL Erm at the ratio of 1:50 and incubated at 30 °C until $OD_{600} = 0.3$. Then inducer pheromone SppIP was added at the final concentration of 50 ng/mL and incubated for further 5 h. The induced cells were harvested by centrifugation and suspended in 50 mM phosphate buffer, followed by sonic disruption. The cell-free extract was analyzed on 12% sodium dodecyl sulfate–polyacryamide gel electrophoresis (SDS-PAGE) and subjected to western blotting analysis using mouse anti-NDV HN monoclonal antibody (lab stock) as the primary antibody followed by HRP-labeled goat anti-mouse IgG antibody (Cell Signaling Technology) incubation. Immunoreactive bands were detected by the addition of ECL substrates (Thermo Scientific Pierce).

2.4. Animal experiments

1-day-old chickens were purchased from Qinguan Animal Technology Co., Ltd. (Handan, China). A total of 80 2–3 week chicken were random divided into 4 groups with 20 birds each group, subjected to either phosphate buffered saline (PBS), RLP(pSIP409), RLP(HN) or RLP(HN-DCpep), respectively. In detail, the chickens were orally immunized with RLP with individual plasmid at the dose of 5.0×10^9 CFU/0.2 mL PBS for continuous 3 weeks with 3 doses per week. After one week interval, the chickens were boosted at week 5 and then 4 birds from each group were necropsied for sample collections and immunological analysis as described below. The rest chickens were then challenged with NDV NA-1 strains at the dose of 10^2 ELD_{50} by nasal drops and the clinical signs, body weight gain (BWG) and survival rates were observed and recorded during the next 10 days. The intestinal samples were also collected for hematoxylin–eosin (HE) staining and histopathologic analysis.

2.5. Determination of hemagglutination inhibition (HI) antibody and SIgA antibody

The presence of HI antibodies were determined according to the protocol described by OIE (2012). Briefly, serum was transferred to 96-well round-bottom plates in duplicate and serially diluted in 2-fold steps in PBS. After that, a volume of 50 μL NDV (LaSota) antigen was added to each well and incubated for 60 min at room temperature. Then 50 μL of a 0.5% chicken erythrocyte suspension in PBS was added to each well and incubated for additional 1 h at room temperature. The titer of HI antibodies was recorded when the highest dilution capable of preventing hemagglutination.

The contents of small intestines and tracheas were collected by washing with 6 mL cold phosphate buffered saline Tween-20 (PBST) containing 1% bovine serum albumin (BSA) (PBST-BSA), followed by centrifugation at 12,000 rpm/min for 10 min. The supernatants were collected and stored at −20 °C for further analysis.

Enzyme-linked immunosorbent assay (ELISA) was performed to determine the SIgA levels in intestines and tracheas as described previously (Li et al., 2008) with minor modifications. In detail, Nunc Immunoplate Maxisorb F96 plates (Nalge Nunc, Rochester, NY) were coated overnight at 4 °C with purified NDV virus at 1 μg/well suspended in sodium carbonate–bicarbonate buffer (pH 9.6). The plates were blocked with PBST-BSA for 1 h at 37 °C and then 100 μL diluted intestinal samples (1:40) or tracheas samples (1:5) were added with triplicate repeats. After 1 h incubation at 37 °C, wells were washed three times with PBST and incubated with goat anti-chicken IgA antibodies conjugated with horseradish peroxidase (HRP) for additional 1 h. After three times washings, 100 μL 3,3′,5,5′ tetramethylbenzidine (TMB) was then added to develop the reaction. Color development (absorbance) was recorded at 450 nm.

2.6. Isolation of lymphocytes and fluorescence activated cell sorting (FACS)

The single cell suspensions were prepared from spleens and peripheral blood leukocytes (PBL) as described previously (Helmby et al., 2000; Nolte et al., 2000) with minor modifications, then T cell proliferation and the percentages of $CD4^+$ T cells were evaluated by FACS.

In detail, spleen samples were washed 3 times with RPMI 1640 medium without fetal bovine serum (FBS). A cell strainer (0.76 μm, Falcon) was used to mash the organs gently with the plunger of a 10 mL syringe and the single cells were resuspended in 20 mL Hanks' balanced salt solution (HBSS). The cell pellets were collected after centrifugation at 1200 × *g* for 5 min at room temperature and treated with 2 mL ammonium–chloride–potassium (ACK) lysing buffer (Life Technologies) for 8 min at 37 °C. After centrifugation,