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Immunogenicity of *Lactobacillus*-expressing VP2 and VP3 of the infectious pancreatic necrosis virus (IPNV) in rainbow trout

Liu Min [a,1], Zhao Li-Li [a,1], Ge Jun-Wei [a], Qiao Xin-Yuan [a], Li Yi-Jing [a,*], Liu Di-Qiu [b,*]

[a] *Veterinary Microbiology Department, Veterinary Medicine College, Northeast Agricultural University, Harbin 150030, PR China*
[b] *Key Laboratory of Animal Models and Human Diesease Mechanisms, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223, PR China*

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ABSTRACT

Infectious pancreatic necrosis virus (IPNV) infects salmonid fish with high mortality and causes serious economic losses to salmonid aquaculture. *Lactobacillus* strains have a number of properties that make them attractive candidates as delivery vehicles for the presentation to the mucosa of compounds with pharmaceutical interest, in particular vaccines. Here, *Lactobacilli*/*Escherichia coli* shuttle vector pPG1 (surface-displayed) or pPG2 (secretory) with the capsid VP2 gene inserted was transformed into *Lactobacillus casei* to yield two recombinant strains: Lc:PG1-VP2 and Lc:PG2-VP2, respectively. Rainbow trout immunized respectively with Lc:PG1-VP2, Lc:PG2-VP2, Lc:PG1-VP3 and Lc:PG2-VP3 elicited anti-IPNV immune responses (serum IgM) via oral route. Statistical results of serum IgM titer with neutralizing activity showed that immunogenicity of Lc:PG2-VP2 was more powerful than that of Lc:PG1-VP2 ($P < 0.001$), Lc:PG1-VP3 ($P < 0.001$) and Lc:PG2-VP3 ($P < 0.001$), which was confirmed by viral loads reduction analyzed by real-time RT-PCR in orally immunized rainbow trout after virus challenge. Comparing with negative control, rainbow trout orally dosed with Lc:PG2-VP2 resulted in ~46-fold reduction in virus load on days 10 post viral challenge as well as Lc:PG1-VP2(~20-fold), Lc:PG2-VP3(~6-fold) and Lc:PG1-VP3(~3-fold). Taken together, Lc:PG2-VP2 exhibited a more appropriate candidate as live bacteria vaccine against IPNV infection in rainbow trout.

1. Introduction

Infectious pancreatic necrosis, caused by the infectious pancreatic necrosis virus (IPNV), is an important viral disease to salmonids, especially juvenile fish [1,2]. It is up in the mortality as 70% in young salmonid fish and establishes an asymptomatic carrier state in survivors, both in different species of salmonids and in other species of farmed fish such as turbot and Atlantic cod [3–5]. IPNV is a member of the family *Birnaviridae*, a group of viruses that are non-enveloped, ~60 nm in diameter, and contain two segments of dsRNA as genetic components, Segments A and B [6–8]. Segment B encodes a non-structural protein that has helicase, protease, and RNA dependent-RNA-polymerase (RdRp) domains. Segment A is the larger segment of about 3.1 kbp and it encodes VP2 and VP3, the two major structural proteins of the virus. Following capsid assembly, the pVP2 protein is cleaved further to produce VP2 [9] as protective antigen that induces neutralizing antibodies which protect susceptible fish from IPNV infection [10,11]. Although VP3 is believed to be a more internal capsid protein than VP2, some neutralizing epitopes have been mapped to it [12,13].

In the last few years, *Lactobacillus casei* have been utilized as vector to deliver exogenous antigen on the mucosal surface to induce local mucosal and systemic immune responses in rodents and swine [14–17]. Lactic acid bacteria (LAB) has long been used in industrial food fermentation and preservation and was known for the beneficial effects on the health of humans and animals and considered 'generally regarded as safe, GRAS' micro-organism [18,19]. Most strains of LAB are able to survive and colonize on the intestinal tract. They are preferable in terms of safety control, minimization of side effects and non-specific immunoadjuvant effect [20,21]. The above-mentioned facts provoke advanced studies aimed at determining the capability and feasibility of LAB as a novel mucosal vaccine formulation.

An ideal vaccine against IPNV must induce long lasting protection at an early age and prevent carrier formation. Injection cannot be used for small fish. Therefore, either oral delivery or immersion

* Corresponding authors. Jiaochang East Road 32, Kunming Institute of Zoology–Chinese Academy of Sciences, Kunming, Yunnan Province 650223, China. Tel.: +86 871 5199045; fax: +86 871 5123798.
E-mail addresses: yijingli@yahoo.com (L. Yi-Jing), liudiqiu@mail.kiz.ac.cn (L. Di-Qiu).
[1] Both authors equally contributed to this study.

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is more preferred routes for early vaccination. Although there were several researches involved in evaluating immunogenicity of IPNV VP2 expressed in DNA vaccine, yeast and subviral particles (SVPs) [22–25], these vaccine formulation were not suitable for oral administration. Based on above reasons, we selected *Lactobacillus* as vector expressing and delivering IPNV VP2/VP3 capsid protein via oral route to induce protective immune responses in younger fish.

Here, we report that IPNV VP2/VP3 capsid protein could be expressed successfully in *Lactobacillus* with natural antigenicity and capable of inducing antibody against IPNV. After rainbow trouts immunized with *Lactobacillus*-derived VP2/VP3 were challenged with IPNV, there was a significant reduction in viral loads in vaccinated fish comparing to the sham-injected ones. All in all, developing oral vaccine based on *Lactobacillus* against IPNV infection was a promising method. In this way, exploring multivalent vaccines against viral diseases in fish using *Lactobacillus*-expressing platform is more possible.

2. Materials and methods

2.1. Bacterium, virus and cell line

L. casei ATCC 393(*Lc*) is a free-plasmid strain grown in MRS medium (Sigma), at 37 °C, without shaking. Chloramphenicol (Sigma) is utilized at final concentration of 10 μg/ml. For the cloning of plasmids, *Escherichia coli* competent cell JM109 (DE3) was used in this study and grown in LB medium containing 100 μg/ml of ampicillin. The Sp strain of IPNV, obtained from American Type Culture Collection (ATCC VR-1318), was used for this study. Viral titer was determined by assaying the dose that infected 50% the cell culture ($TCID_{50}$ ml^{-1}) [26]. An IPNV specific rabbit antiserum was prepared from New Zealand rabbits immunized with purified virus following the protocols of Hill et al. [27]. Chinook salmon embryo cells (CHSE-214) were grown as monolayers at 17 °C, 5.0% CO2 in Eagle minimal essential medium (MEM) (Gibco) supplemented with 100 μg/ml streptomycin, 60 μg/ml penicillin, 2 mM L-glutamine, 1% nonessential amino acids, and 10% fetal bovine serum (FBS) (Invitrogen). Prior to infection experiments, cells were grown to 80% confluence.

2.2. Adhesion on mucosal surface

Bacterium was labeled with fluorescence probe as described previously [28]. In brief, Log-phase culture of *Lc* was harvested, washed twice with sterile phosphate-buffered saline (PBS) and adjusted to a concentration of 10^{10} CFU ml^{-1} prior to labeling with 50 μM five-(and 6-) carboxyfluorescein diacetate succinimidyl ester (cFDA-SE) at 37 °C for 20 min. Fluorescent labeling was terminated by pelleting the bacteria, washing twice with PBS to remove excess cFDA-SE, and resuspending the pellet in PBS.

A group of 15 trout were orally dosed with approximately 10^9 cFDA-SE-labeled *Lactobacilli* by orogastric intubation while the control trout had been orally fed with sterile PBS. The trout were anaesthetized by immersion in 50 mg/mL buffered tricaine methane sulfonate (MS-222, Sigma, Madrid, Spain) prior to handling. Groups of three trout each were sacrificed on days 1, 3, 5 and 7, which the intestine was extracted from each trout and cut longitudinally. Visible residual food particles or fecal material were first removed from the intestine before being examined for the presence of adhering cFDA-SE-labeled *Lc*. This was performed by adding 150 μl of PBS to every 1.0 cm of tissue and dislodging microbes from the mucosal surface of the tissues with the aid of a plunger from a syringe (1.0 ml; Terumo, Tokyo, Japan). Cell extracts were fixed with formaldehyde (0.75%, v/v) prior to flow cytometry analysis.

2.3. Recombinant bacteria procedure

Total RNA from IPNV infected CHSE-214 cells was extracted using the GeneJET™RNA Purification Kit (Fermentas, Shenzhen, China) according to the manufacturer's instructions, and the concentration and purity of the RNA obtained were measured spectrophotometrically (Eppendorf biophotometer, Hamburg, Germany). All used primers were designed on IPNV segment A pVP2-VP4-VP3 gene for polyprotein (Genebank accession number: AJ622822.1). First strand cDNA was transcribed with RevertAid™ M-MuLV reverse transcriptase (Fermentas) and briefly, RNA (2 μg) was incubated with 1 μL of a universal reverse primer U2 for VP2 (Table 1) for 5 min at 65 °C. Subsequently, 4 μL of 5 × Reaction buffer, 1 μL of RNase Inhibitor (20u/μL), 2 μL of dNTP mix and 1 μL of RevertAid™ M-MuLV reverse transcriptase were added and mix gently and centrifuge followed by incubating at 42 °C for 5 min. The reaction was terminated by heating at 70 °C for 5 min and the cDNA template was used in PCR reaction performed over 30 cycles at 95 °C (1 min), 49 °C (1 min),72 °C (1 min), followed by 72 °C for 10 min. The forward and reverse primers used were U1 and U2 respectively. The PCR product was visualized on an ethidium bromide stained 1.2% agarose gel, and it was recovered and purified with TIANgel Midi Purification Kit (Tiangen, Beijing, China). The purified product as template performed on the next round of PCR reaction with S1 and S2 over 30 cycles at 95 °C (1 min), 49 °C (1 min), 72 °C (1 min), followed by 72 °C for 10 min. The target PCR product was purified as above and was sequenced prior to and after cloning into the *Lactobacillus/E. coli* shuttle vector pPG1 and pPG2 under the control of a xylose promoter, respectively. There is an anchoring matrix-encoding *pgsA* gene derived from *Bacillus subtilis* behind the target gene in the pPG1 as a type of surface-displayed expressing. The pPG1 and pPG2 has the ssUSP secretion signal before the target gene to ensure the target protein outside the *Lc*. This construct was designated as pPG1-VP2 or pPG2-VP2 and standard recombinant techniques were used to amplify the plasmid in *E. coli* (JM109).

Preparation and electrotransformation of competent *L. casei* were performed as described previously [28]. Briefly, a stationary phase (16–18 h) culture of recipient *L. casei* was inoculated (1/50 inoculum) (v/v) into 100 ml MRS broth and incubated at 37 °C without shaking. The cells were harvested at OD_{600} 0.3–0.4 by centrifugation at 3000 × g for 10 min at 4 °C and washed twice with an equal culture volume of ice-cold sucrose–magnesium chloride electroporation buffer (SMEB) (250 mM sucrose, 1 mM $MgCl_2$, 5 mM sodium phosphate, pH 7.4). The cells were concentrated 100-fold of original culture volume in ice-cold SMEB buffer.

Table 1
Primers used in this study for standard and RT-PCR.

Gene	Type	Name	Primers	Product size (bp)	Cycles	Cycling conditions
VP2	Universal	U1	F:5′AGCCCTTTCTAACAAACAAAC 3′	1500	30	95 °C 1 min, 49 °C 1 min, 72 °C 1 min
		U2	R:5′ GCTGAGTTGGTCTTGGTGAG 3′			
	Specific	S1	F:5′GGATCCAGAGAGACACATCTTAAAACAAGAGAC 3′	1095	1	95°C 5 min
		S2	R:5′CTCGAGTTCGGGGTCGTACTTGCCATAG 3′		30	95 °C 1 min, 63°C1 min,72 °C 1 min