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# Comparison of the immune responses induced by oral immunization of mice with *Lactobacillus casei*-expressing porcine parvovirus VP2 and VP2 fused to *Escherichia coli* heat-labile enterotoxin B subunit protein

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ABSTRACT

The major structural protein VP2 of porcine parvovirus (PPV) was used as the model parvovirus antigen, which has been expressed in *Lactobacillus casei* fusing with *Escherichia coli* heat-labile enterotoxin B subunit (LTB) as mucosal adjuvant. The VP2-LTB DNA fragment was cloned into vector pPG611 or pPG612 to generated inducible surface-displayed and secretion expression systems based on xylose promoter, designated as rLc:pPG611-VP2-LTB (recombinant *L. casei*) and rLc:pPG612-VP2-LTB, respectively. Expression of the fusion protein was verified by SDS-PAGE, Western blot immunofluorescence and electron microscopy. It was observed that the level of IgG or sIgA from mice orally immunized with VP2-LTB was higher than that from mice received VP2 and negative control, which demonstrated significantly statistically different. Especially, the titer of IgG or sIgA in mice immunized with rLc:pPG612-VP2-LTB is the highest in this study. In summary, LTB as mucosal adjuvant was able to effectively facilitate induction of mucosal and systemic immunity by *L. casei*-expressing VP2 fusion protein.



## 1. Introduction

Porcine parvovirus is one of mainly causative agents responsible for reproductive failure, characterized by stillbirth, mummification, embryonic death and infertility, which is an autonomous parvovirus belonging to the genus *Parvovirus*, subfamily Parvovirinae, family Parvoviridae [1]. Its genome has a single-stranded DNA with negative orientation, containing three open reading frames (ORFS) located on the complementary strand, ORF1, ORF2 and ORF3 [2]. Only ORF2 codes the structural protein VP1, VP2 and VP3 to generate an non-enveloped icosahedral particle encapsidating genome, of which VP2 possesses a significantly spacial position in the structure as model antigen of pathogenic porcine parvovirus.

The main characteristics of PPV existence in swine exhibit the extremely durable and highly infection, besides its role in post-weaning multi-systemic wasting syndrome, and porcine respiratory disease complex [3,4]. PPV is capable of transplacental infection during gestation and spreading between fetuses, which resulted in the reproductive failure so as to reduction of litters in size or serious economical losses in swine industry [5]. It is firmly believed that the acquired immunity originating from the humoral immune response is able to provide lifelong protection for the herds against PPV infection [3,4,6].

In addition, mucosal surfaces are prominent in the gastrointestinal, urogenital, and respiratory tracts and provide portals of entry for pathogens. The mucosal immune system composed of organized lymphoid structures gives

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immunity to pathogens that impinge upon mucosal surfaces by the synthesis of secretory immunoglobulin A (sIgA) antibodies, which created the critical first line of defense against invasion of deeper tissue by the pathogens [7]. Thus, novel vaccine formulation should aim at effective induction of systemic and mucosal immune responses. In view of the prominent role of the mucosa in pathogenic agents transmission and infection, direct mucosal vaccination could be an effective strategy for prophylaxis by induction of systemic and mucosal immune responses.

For mucosal immunization, *Lactobacillus casei* ATCC 393 was selected as live bacterial carrier more attractive than other live vaccine vectors such as *Shigella*, *Salmonella*, and *Listeria* [8–11] because lactobacilli are considered "generally regarded as safe" and able to survive or colonize at the intestinal tract. Furthermore, lactobacilli exhibit adjuvant properties and weak immunogenicity [12]. More importantly, the recombinant *L. casei*-expressing VP2 should effectively stimulate mucosal immune system in gut associated lymphoid tissue (GALT).

As the mucosal application of antigen alone mostly shows low efficacy, co-administration of certain mucosal adjuvant is necessary. Two well-characterized bacterial proteins with an outstanding potential to function as mucosal adjuvant are the heat-labile enterotoxin (LT) of *Escherichia coli* and the cholera toxin (CT) of *Vibrio cholerae*. Their non-toxic mucosal binding B subunits (LTB/CTB) have already been tested for their immunogenicity and their ability to act as mucosal adjuvant [13]. LTB has been found to be a more potent adjuvant than CTB [14]. Therefore, VP2 has been expressed as a fusion protein with *E. coli* heat-liable toxin B subunit (LTB) in the *L. casei*. LTB is a gut adhesion molecule and helpful to antigen uptake from and binding to gut [15]. It was reported that the expression of heterologous antigen fused with LTB in attenuated *Salmonella* has been described an optimized strategy for the induction of mucosal and systemic antibodies responses [16]. LTB is responsible for heterologous protein exposure to mucosal immune system via binding to $GM_1$ ganglioside anchored on the surface of intestinal epithelial cells so that it can function also as mucosal adjuvant producing higher titer serum and secretory antibodies in mice orally administered with *L. casei*-expressing VP2-LTB.

In this study, the recombinant *L. casei* was constructed with the shuttle plasmid pPG611-VP2-LTB or pPG612-VP2-LTB based on xylose promoter. The data showed that compared to rLc:pPG611-VP2 or rLc:pPG612-VP2 (previously constructed in our laboratory) [17], the new recombinant lactobacilli can elicit more potent systemic and mucosal immune response according to higher titers of serum IgG and secretory IgA after oral administration in mice, which is just promising result utilizing LTB as mucosal adjuvant.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

*L. casei* ATCC 393 was a free-plasmid strain grown in MRS medium (Sigma), at 37 °C, without shaking. Chloramphenicol (Sigma) was utilized at a concentration of 10 μg/ml. For the cloning of plasmids, *E. coli* JM109 was used in this study and grown in LB medium containing 100 μg/ml of ampicillin. The plasmids pPG611 and pPG612 was kindly provided by Dr. Jos Seegers (NIZO Institute, Netherlands).

### 2.2. Plasmids, DNA procedures and eletrotransformation

A 375 bp fragment encoding *E. coli* heat-liable toxin B subunit (LTB) was obtained from the plasmid pMD18-T Simple-LTB by *Sal*I and *Xho*I digestion. This fragment was inserted the corresponding site in the plasmid pPG611-VP2 or pPG612-VP2 (constructed preserved in our laboratory) previously digested with *Xho*I, which generate the new plasmid by fusion of LTB to the 3′ end of VP2 with Gly-Gly-Gly linker.

The resulting fragment VP2-LTB was behind the ssUSP secretion signal peptide sequence. There is an anchor peptide sequence behind the target fragment in pPG611.1 as a type of surface-displayed expressing. Taken together, two novel plasmids have been constructed and designated pPG611-VP2-LTB and pPG612-VP2-LTB (Fig. 1), respectively. Preparation and electrotransformation of competent *L. casei* were performed as described previously [12,18].

### 2.3. Monoclonal antibody-colloidal gold conjugate

Colloidal gold particles, as immunoeletron microscopy markers, were prepared as described previously [19]. In short, 2 ml of sodium citrate solution (0.01%, w/v) together with 0.45 ml of freshly prepared tannic acid solution (0.01%, w/v) as reducing agents were added into 100 ml freshly working solution of 0.01% (w/v) HAuCl4 (Sigma) at the boiling point. At last, the gold particles with an average diameter of 5.7 nm were produced.

One anti-VP2 McAb $IgG_{2a}$ (previously prepared in our laboratory) was applied for preparation of colloidal gold conjugate at final concentrations 80 μg/ml at pH 6.5. The antibody-colloidal gold complexes have been stored in 0.01 M PB (phosphate buffer containing 0.01 M $NaH_2PO_4$, 0.01 M $Na_2HPO_4$, pH 6.5) with 1.0% BSA at 4 °C as electron microscopy probes.

### 2.4. SDS-PAGE, Western blot, immunofulorescence and immunoelectron microscopy

For analysis of the expression of VP2-LTB fusion protein in the rLc:pPG611-VP2-LTB or rLc:pPG612-VP2-LTB, transformant bacteria were grown overnight in basal MRS medium containing Cm (10 μg/ml) supplemented with xylose at 37 °C. Bacterial cells were collected by centrifugation at 3000 × *g* for 15 min. The pellets were washed twice with sterile phosphate-buffer saline (PBS, pH 7.4) and lysed in a Bead-Beater (Biospec, Bartlesville, OK) by vigorous shaking. The cell debris was centrifugation at 3000 × *g* for 10 min and the supernatant were analyzed via 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blot was carried out as follow: protein extractions were electrotransferred on a nitrocellulose membrane and the bolts were developed using mouse anti-LTB serum at a dilution of 1:800 with PBS