



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.



Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

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

Diqiu Liu^a, Xiangqing Wang^a, Junwei Ge^a, Songmei Liu^{a,b}, Yijing Li^{a,*}

^a Department of Preventive Veterinary Medicine, College of Veterinary Medicine, Northeast Agricultural University, 59 Mucai Street, Harbin 150030, PR China

^b Microbiology Key Laboratory, College of Life Sciences, Heilongjiang University, Harbin 150080, PR China

ARTICLE INFO

Article history:

Received 25 January 2010

Accepted 16 February 2010

Keywords:

Oral immunization

Porcine parvovirus VP2

LTB

Lactobacillus casei

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.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

Porcine parvovirus is one of mainly causative agents responsible for reproductive failure, characterized by still-birth, 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 a 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

* Corresponding author. Tel.: +86 451 55190385;

fax: +86 451 55103336.

E-mail addresses: liudiqiu1980@yahoo.com.cn, yijingli@yahoo.com (Y. Li).

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-labile 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 GM₁ 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-labile toxin B subunit (LTB) was obtained from the plasmid pMD18-T Simple-LTB by *Sall* and *XhoI* digestion. This fragment was inserted the corresponding site in the plasmid pPG611-VP2 or pPG612-VP2 (constructed preserved in our laboratory) previously digested with *XhoI*, 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 ssUP 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 immunoelectron 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 NaH₂PO₄, 0.01 M Na₂HPO₄, pH 6.5) with 1.0% BSA at 4 °C as electron microscopy probes.

2.4. SDS-PAGE, Western blot, immunofluorescence 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

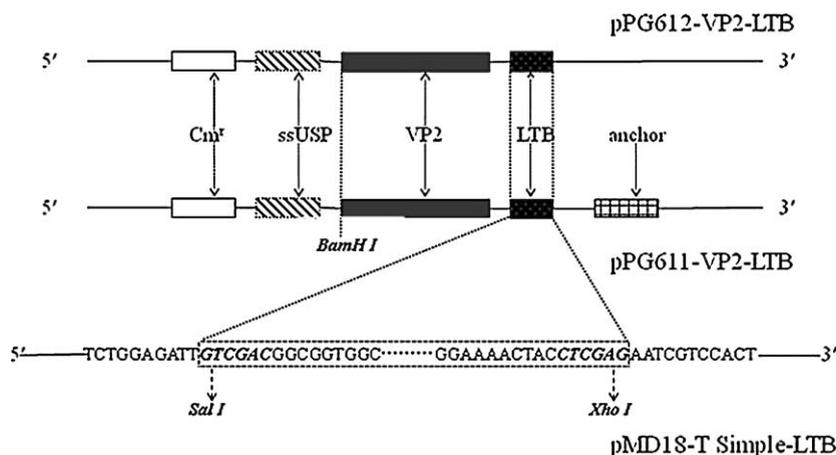


Fig. 1. The scheme of recombinant vectors expressing VP2-LTB protein. The LTB fragment was cleaved with *Bam*HI and *Xho*I restriction endonuclease and inserted into the corresponding sites of pPG611-VP2 and pPG612-VP2, respectively, giving rise to pPG611-VP2-LTB and pPG612-VP2-LTB.

(pH 7.4). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Invitrogen) was utilized as a secondary antibody at a dilution of 1:5000, which was visualized with the Chemiluminescent Substrate reagent (Pierce, Rockford, IL) following the manufacturer's instruction. The reduced rLc:pPG611 or rLc:pPG612 acted as negative control.

For detection of the surface-displayed expressing of rLc:pPG611-VP2-LTB, immunofluorescence was used as described previously [20]. In short, rLc:pPG611-VP2-LTB cells were cultured and induced in MRS overnight at 37°C. The cell pellets were sequentially incubated with mouse anti-LTB serum (1:500), fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Invitrogen) secondary antibodies (1:2500) and analyzed with a confocal microscopy. Furthermore, immunoelectron microscopy was used to reconfirm the location of the fusion protein in rLc:pPG611-VP2-LTB. The cell pellets were prepared as described above, then washed thrice with PB and incubated overnight with mouse anti-VP2 McAb-colloidal gold conjugates at 37°C followed by thrice washing using PB. At last, the complex was analyzed by transmission electron microscope.

The secreted VP2-LTB fusion protein expression by rLc:pPG612-VP2-LTB was examined in the supernatant. Overnight cultures of rLc:pPG612-VP2-LTB in MRS supplemented with xylose, were obtained by centrifugation at 12,000 × *g* for 5 min. The supernatant were concentrated 20 times using a Centrifugal Filter Unit (Millipore, Billerica, MA), and then examined using 12% SDS-PAGE and Western blot as above.

2.5. Immunization and sample collection

Groups of 10 female SPF BALB/c mice (8-week-old) were immunized with equal quantity of live rLc:pPG611-VP2-LTB, rLc:pPG612-VP2-LTB, rLc:pPG611-VP2, rLc:pPG612-VP2 and rLc:pPG611 that were grown overnight in basal MRS medium containing Cm (10 μg/ml) supplemented with xylose at 37°C, respectively. For the oral route, 2 × 10⁹ cells in 0.1 ml PBS (pH 7.4) suspension were administered on 3 consecutive days at days 0–2, 14–16 (first booster),

28–30 (second booster). The mice were handled and maintained under strict ethical conditions according to the international recommendations for animal welfare.

Sera were prepared from the blood samples collected from the tail vein on days 0 (pre-immune), 7, 21, 35, and 49. Feces were directly obtained from the anus of the mice on days 0 (pre-immune), 5, 12, 19, 26, 33, 40, 47, and 54, 200 mg subsequently suspended and lysed in 400 μl of PBS containing 0.01 M EDTA-Na₂ overnight at 4°C, which was followed by centrifuge 12,000 × *g* for 30 min and the supernatant were stored at –20°C. On days 0 (pre-immune), 7, 21, 35, and 49, vaginal and ophthalmic lavage fluids were obtained by washing the respective organs three times with 200 and 50 μl sterile PBS. The cleared extracts of all samples were collected by centrifugation and stored at –20°C for the afterward analysis, in addition, supplemented with protease inhibitors.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) plates were coated overnight at 4°C with the full PPV virus propagated on ST cells and the culture of ST cells used as negative control antigen. After the wells were blocked for 2 h at 37°C with PBS containing 5% skimmed milk, lavage fluid or serum samples were serially diluted in PBS-1% BSA and added in triplicate and incubated for 1 h at 37°C. After the plates were washed three times with PBS-Tween20 0.05%, horseradish peroxidase-conjugated goat anti-mouse IgG or IgA antibody (Invitrogen) was added into each well (1:5000) and incubated for an additional 1 h at 37°C. After another round of washing, colour development was carried out using *o*-phenylenediamine dihydrochloride as substrate, and then absorbance was measured at 490 nm.

2.7. PPV neutralization assays

Sera of mice orally immunized with rLc:pPG612-VP2-LTB, rLc:pPG611-VP2-LTB, rLc:pPG612-VP2, rLc:pPG611-VP2, rLc:pPG611 were evaluated via a plaque reduction assay to determine the neutralizing ability of the elicited

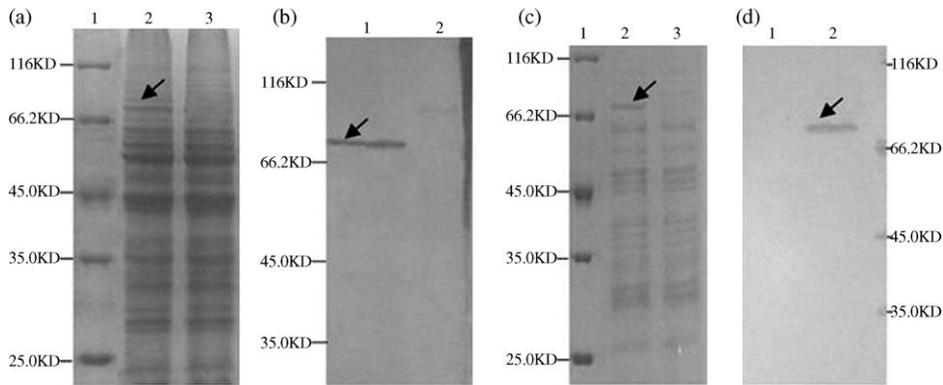


Fig. 2. Secretion expression of VP2-LTB protein in rLc:pPG612-VP2-LTB. (a) Coomassie blue gel staining shows the expression of a 75,000 kDa fusion protein in lysates of rLc:pPG612-VP2-LTB (lane 2), but not in that of rLc:pPG612 (lane 3); lane 1, molecular mass marker. (b) Western blot analysis of VP2-LTB expression in recombinant strain. An immunoreactive band was observed (lane 1) in a similar position as observed in the SDS-PAGE shown in the panel (a), but not in that of rLc:pPG612 (lane 2). (c) 75,000 kDa fusion protein in supernatant of rLc:pPG612-VP2-LTB (lane 2), but not in that of rLc:pPG612 (lane 3); lane 1, molecular mass marker. (d) An immunoreactive band was observed (lane 2) in a similar position as observed in the SDS-PAGE shown in the panel (c), but not in that of rLc:pPG612 (lane 1).

antibodies with slight modifications as described previously [17]. Briefly, 50 μ l of 2-fold serially diluted sera samples were mixed with the equivalent volume of PPV adjusted to 200 TCID₅₀ in microcentrifuge tubes, and then incubated at 37 °C for 2 h. A 24-cell plate with confluent monolayer of swine testicular (ST) cell inoculated with the mixture at 37 °C for another 2 h rocked at 30 min intervals. Subsequently, the inoculum was removed and overlaid with medium, and the plate was incubated in a 5% CO₂ incubator at 37 °C for 5 days. The overlay medium was then discarded followed by thrice washing with sterile PBS, pH 7.4 and stained with 1% crystal violet solution. Differences in the number of plaque formed between treatments were analyzed by Student's *t*-test for the level of significance.

3. Results

3.1. VP2-LTB secretion expression by *L. casei*

Cell lysates and supernatant of overnight cultures of rLc:pPG612-VP2-LTB or rLc:pPG612 in basal MRS medium with additional xylose was obtained by centrifugation, which were subjected to SDS-PAGE and Western blot to examine the target protein expression. The results of SDS-PAGE followed by Coomassie blue gel staining analysis showed a 75 kDa fusion protein in the cell lysates (Fig. 2(a): lane 2) and the supernatant (Fig. 2(c): lane 2) of the rLc:pPG612-VP2-LTB induced by xylose, but not in that of rLc:pPG612 induced by xylose (Fig. 2(a): lane 3, Fig. 1(c): lane 3). An immunoreactive band of 75 kDa was detected in the cell lysates (Fig. 2(b): lane 1) and supernatant (Fig. 2(d): lane 1), whereas there was no band in the negative control lane (Fig. 2(b): lane 2, Fig. 2(d): lane 2). The results demonstrated that VP2-LTB was able to be induced by xylose in *L. casei* ATCC 393.

3.2. VP2-LTB surface-displayed expression by *L. casei*

Overnight cultures of rLc:pPG611-VP2-LTB and rLc:pPG611 induced in basal MRS supplemented with

xylose were pelleted and cell lysates were analyzed by SDS-PAGE and Western blot. Coomassie blue gel staining showed that there was a 80 kDa fusion protein in the cell lysates of rLc:pPG611-VP2-LTB induced by xylose (Fig. 3(a): lane 3), but not in that of rLc:pPG611 (Fig. 3(a): lane 2), which was reconfirmed at the corresponding position by Western blot analysis of rLc:pPG611-VP2-LTB (Fig. 2(b): lane 2) and rLc:pPG611 (Fig. 2(b): lane 1).

The immunofluorescence reaction of rLc:pPG611-VP2-LTB and rLc:pPG611 induced by xylose developed with the mouse anti-VP2 serum and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG also showed that there was only green fluorescence on the cell surface of rLc:pPG611-VP2-LTB (Fig. 4(a)), but not on that of rLc:pPG611 (Fig. 4(b)).

The immunogold electron microscopy analysis demonstrated that the target protein VP2-LTB was only expressed on the surface of rLc:pPG611-VP2-LTB (Fig. 5(a)), but it did not appeared on the surface of rLc:pPG611 (Fig. 5(b)).

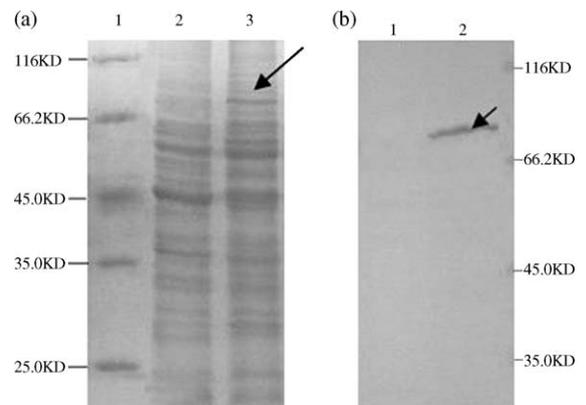


Fig. 3. Expression of VP2-LTB protein in rLc:pPG611-VP2-LTB. (a) SDS-PAGE shows the expression of a 80,000 kDa fusion protein in lysates of rLc:pPG611-VP2-LTB (lane 3), but not in that of rLc:pPG611 (lane 2); lane 1, molecular mass marker. (b) Western blot analysis showed that an immunoreactive band in a similar position as observed in the SDS-PAGE in the panel (a).

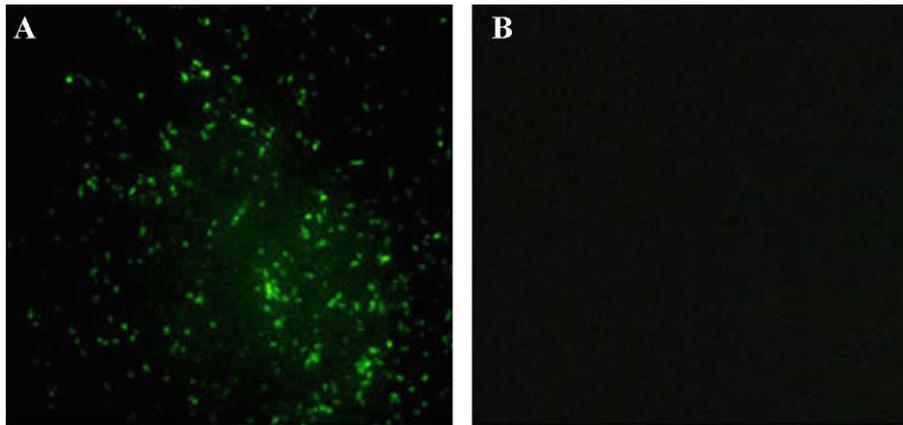


Fig. 4. The immunofluorescence analysis of VP2-LTB surface-displayed expression by *Lactobacillus casei*. The rLc:pPG611-VP2-LTB and rLc:pPG611 was induced in basal MRS supplemented with xylose, the bacteria pellets were incubated with mouse anti-VP2 serum, FITC conjugated goat anti-mouse IgG. (a) There was green fluorescence on the surface of rLc:pPG611-VP2-LTB. (b) There was no immunofluorescence reaction on the cell surface rLc:pPG611.

This result was identical to of immunofluorescence reaction.

3.3. Systemic and mucosal immunogenicity of VP2-LTB expressed by *L. casei*

BALB/c mice were immunized orally with recombinant *L. casei*-expressing VP2-LTB or VP2. As negative controls, mice were immunized with rLc:pPG611. The mucosal response was further investigated by measuring anti-PPV-VP2 sIgA level in the feces, vaginal and ophthalmic lavage fluids post intragastric immunization via ELISA. The level of specific IgG anti-PPV-VP2 in the sera of experimental mice indicated that systemic immune response was induced by the recombinant *L. casei*. The concentration of anti-PPV-VP2 sIgA or IgG antibody was expressed as titres that were determined by expression of the test samples to a standard curve generated by serial dilution of commercially purchased IgA or IgG (Sigma) of

known titre. Since seventh day post first immunization, the higher levels of IgA could be observed in animal group that received rLc:pPG612-VP2-LTB or rLc:pPG611-VP2-LTB in three kinds of samples including feces (Fig. 6(a)), vaginal (Fig. 6(b)) and ophthalmic lavage (Fig. 6(c)) fluids with means significantly different from groups that received rLc:pPG612-VP2 ($P < 0.001$, $P < 0.001$ and $P < 0.05$, respectively) and rLc:pPG611-VP2 ($P < 0.001$, $P < 0.001$ and $P < 0.05$, respectively). Analysis of the sera obtained from the same animals revealed that the animals that displayed the higher levels of anti-PPV-VP2 IgA also displayed the higher level of IgG (Fig. 6(d)), showing correlation in the production of these two classes of antibodies. The levels of IgG or IgA in all animal groups did not show significantly different from each other before the first immunization (pre-immune). By contrast, as might be expected, no significant elicitation of antibodies was observed in control group or used the ST cell culture as negative control antigen (data not shown).

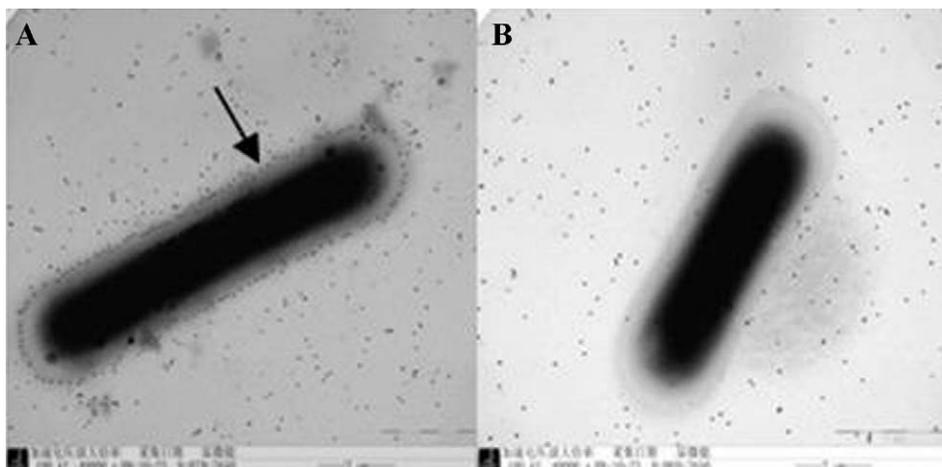


Fig. 5. Identification of the fusion protein on the cell surface of recombinant *L. casei* labeled with McAb-colloid gold conjugates via immunoelectron microscopy (4000 \times). rLc:pPG611-VP2-LTB or rLc:pPG611 was grown in basal MRS supplemented with xylose followed by incubation with McAb-colloid gold conjugates. (a) The gold particles adhered to the cell surface of rLc:pPG611-VP2-LTB; (b) nothing happened to that of rLc:pPG611.

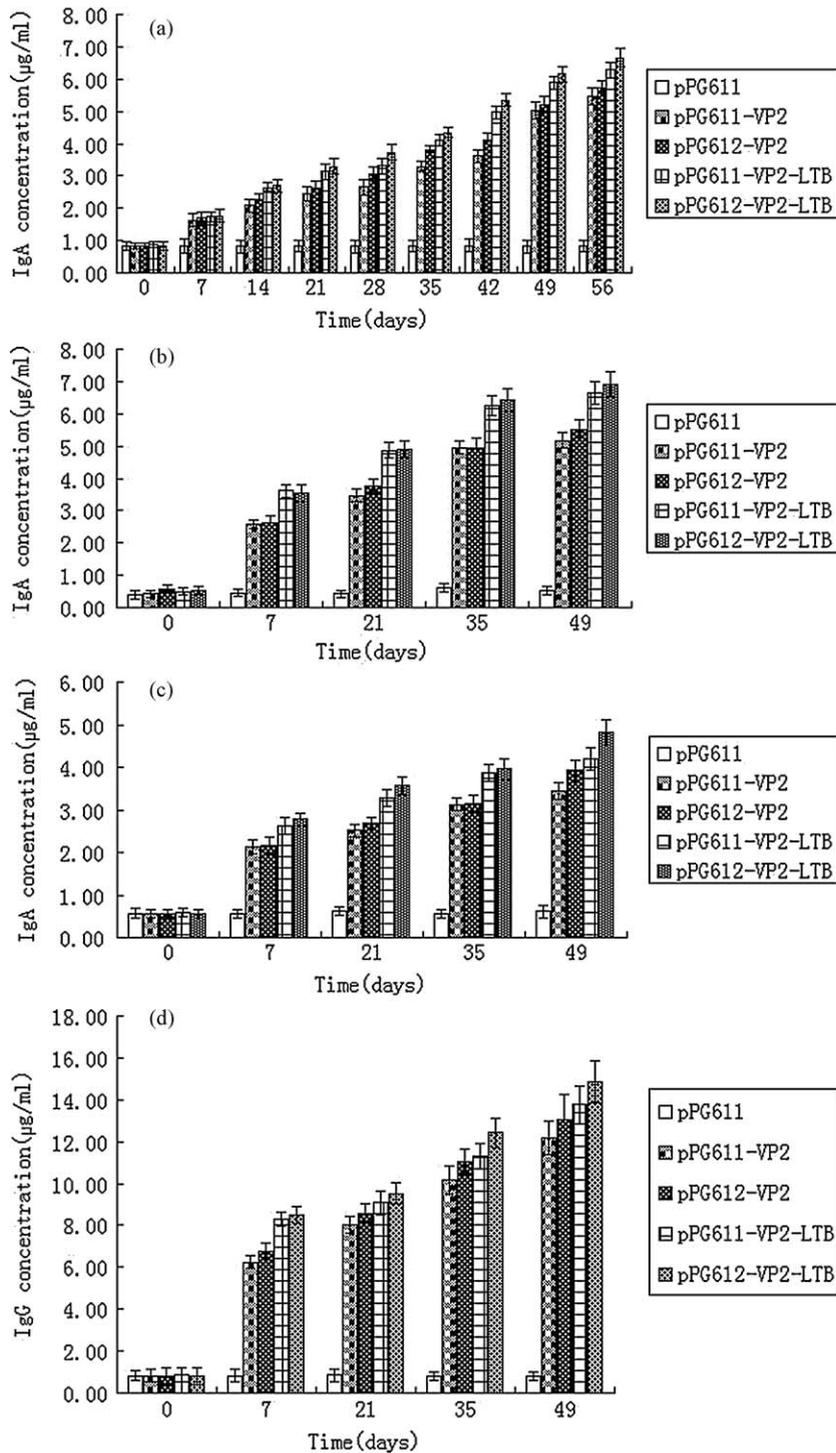


Fig. 6. Titers of mucosal and serum antibody. Feces, vaginal fluids, ophthalmic fluids and sera of mice received recombinant strains expressing VP2-LTB or VP2 and equivalent doses of *L. casei* harboring empty plasmid pPG611 were analyzed by ELISA, using the PPV as the coating antigen. Values are means (\pm S.D.) of three replicates per treatment in one experiment, repeated twice. (a) Feces were collected on 0, 7, 14, 21, 28, 35, 42, 49, 56 days and analyzed for the presence of PPV-VP2 specific IgA. (b) Vaginal fluids were obtained on 0, 7, 21, 35, 49 days for the analysis of specific IgA. (c) Ophthalmic fluids were performed as (b). (d) Sera were analyzed for the presence of PPV-VP2 specific IgG.

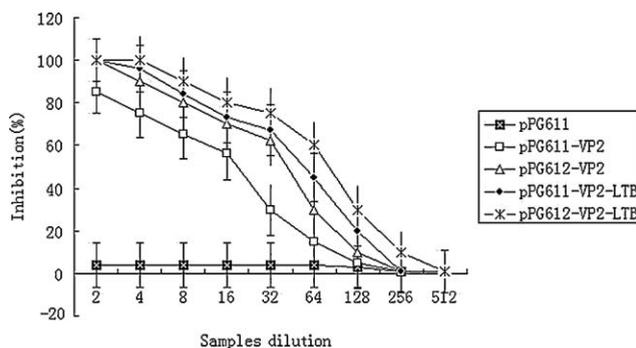


Fig. 7. IgG neutralization activity against PPV *in vitro*. Sera were prepared from mice administered with rLc:pPG612-VP2-LTB, rLc:pPG611-VP2-LTB, rLc:pPG612-VP2, rLc:pPG611-VP2 and rLc:pPG611. Results are mean values and standard errors of triplicates.

3.4. PPV-neutralizing activity *in vitro*

The PPV-neutralizing efficacy of IgG from the sera of mice received the recombinant *L. casei* was detected via plaque reduction assay. Results indicated that the presence of anti-rPPV-VP2 IgG in the culture medium conferred statistically significant neutralizing effects ($P < 0.05$) on PPV infection (Fig. 7). It was consistently observed that IgG antibody response of mice orally immunized with *L. casei*-expressing rPPV-VP2-LTB fusion protein displayed stronger PPV-neutralizing activity than *L. casei*-expressing rPPV-VP2 from 4-fold to 128-fold dilution of sera, which was exhibited by reduction in the number of plaque. The final dilution of antibody obtained from the mice immunized with rLc:pPG612-VP2-LTB or rLc:pPG611-VP2-LTB was 1:128 significantly different from rLc:pPG612-VP2 (1:64) and rLc:pPG611-VP2 (1:32). Not surprisingly, no neutralizing activity was observed in the sera of mice received rLc:pPG611.

4. Discussion

PPV can result in an economically important disease of swine. The initial infection of PPV mainly occurs at the mucosa of intestines [21]; this is a critical step in the infection process. Therefore, mucosal administration is the first choice of vaccination as an effective prophylactic method against PPV invasion, which is taken for granted. Mucosal vaccination is able to induce the effective not only mucosal but also systemic immune response, but parental vaccination can not. As such, the most of researches is currently focused on the development of appropriate mucosal vaccine for oral administration. For example, the recombinant attenuated pseudorabies virus [22] was able to protect mice against viral challenge. Nevertheless, the majority of these live vectors are potentially risk prone.

LAB is utilized as a live carrier delivering model pathogen on mucosal surface to induce systemic and mucosal effective immune responses, which may be preferable in terms of safety control and minimization of side effects. Several LAB-associated model vaccines have been constructed and evaluated in some studies such as LAB expressing tetanus toxin fragment C (TTFC) [23,24], SpaA from *Erysipelothrix rhusiopathiae* [25], glycoprotein S from TGEV [12], the Spike protein segment from SARS-CoV

[26], which mostly achieved the goal but not all. To improve immune protective responses induced by *L. casei*-expressing rPPV-VP2, in this study, we constructed the recombinant *L. casei*-expressing rPPV-VP2-LTB fusion protein for the first time.

L. casei mainly distributed on the mucosal surface of duodenum, jejunum, ileum and colon in mice and was able to persist in about 6 days [27]. The inducible expression systems in this study base on xylose operon regulation. The target protein expression was completely depended on the xylose induction. According to the above statement, the induced recombinant strains were able to stimulate the mucosal immune system of gastrointestinal tract for several days but not a lifetime after oral administration in mice, which did not result in immunotolerance against target antigen. When the induced recombinant bacteria colonized on the mucosal surface, although the xylose was not present, VP2-LTB protein has been still keeping secretory or trans-membrane state in host cell and was recognized, processed and present by relevant lymphocytes in the mucosal immune system, which could induce local immune responses. Theoretically, secretory antigen was more easily uptaken by lymphocytes than membrane-anchored antigen.

We performed the comparison of immunogenicity among four strains expressing rPPV-VP2 protein after inoculation to mice via intragastric route. The administration process consisted of three sets of three successive daily doses of the recombinant *L. casei*, which was required in order to ensure that antibody response to PPV could be induced in all mice orally administered by live bacteria. Oral vaccination is based on antigen delivery to the gastrointestinal tract, the largest mucosal surface and the central site of IgA secretion. Secretory immunoglobulin A (sIgA) plays the most important role in the mucosal immune response against pathogen invasion, which mainly existed in local mucosa fluids. The results of immunization showed that the recombinant strains were capable of inducing systemic immune response producing IgG and mucosal immune response producing sIgA.

LTB is able to facilitate an accumulated uptake of the antigen by binding to GM1 on intestine enterocytes, which has been applied to plant vaccine production and delivery system such as potato tuber, *Nicotiana benthamiana* plants or rice [28–30]. By contrast, the recombinant *L. casei*-

expressing PPV-VP2-LTB fusion protein were able to induce higher antibody level of local mucosal or systemic immune response than that induced by the recombinant *L. casei*-expressing PPV-VP2, in addition, rLc:pPG612-VP2-LTB was greater than rLc:pPG611-VP2-LTB in induction of antibody immune responses, which were conspicuous events in this study. Statistically significant differences were observed since the seventh day post the first immunization. Owing to the above characteristics, we hypothesized that LTb played the crucial role in the induction of higher antibody level as a mucosa-targeting molecule, as well as LTb of the fusion protein on the surface of rLc:pPG611-VP2-LTB was partly covered by cell wall so that it was partly accessible to ligands on the enterocytes resulting in lower level of immune antibodies than that induced by rLc:pPG612-VP2-LTB.

Moreover, in order to confirm the differences in the induced antibody level in inhibiting the virus whether LTb was present or not, we tested sera collected from immunized mice could inhibit the infection of ST cells in a plaque reduction neutralization assay. The results showed that serum obtained from mice immunized with *L. casei*-expressing rPPV-VP2-LTB demonstrated more potent neutralizing activity than *L. casei*-expressing rPPV-VP2. As well, sera obtained from mice immunized with rLc:pPG612-VP2-LTB was more effective than that after immunization of mice with rLc:pPG611-VP2-LTB. For more satisfactory results, it was worth deeply investigating whether the various amount or locus of LTb molecule in a fusion protein was able to affect the immunogenicity of orally live vaccines based on recombinant *L. casei* ATCC 393 expression or delivery system. Testing the efficacy in a porcine vaccination and infection model is a next step in examining the efficacy of this vaccine formulation.

LTb was able to function as a molecular adjuvant to enhance immunogenicity of heterologous antigen expressed by *L. casei* for intragastric immunization. Higher level of systemic or mucosal antibody immune responses was observed after immunization with *L. casei*-expressing rPPV-VP2-LTB, especially rLc:pPG612-VP2-LTB producing the antigen in the external milieu, which made it a more appropriate candidate as orally live bacteria vaccine against PPV infection.

Acknowledgements

This study was supported by Grant 30371074 from the National Natural Sciences Funds of China. The authors wish to thank Jos Seegers for providing the plasmid pPG611, pPG612 and the bacterial strain *L. casei* ATCC 393.

References

- [1] Wilhelm S, Zimmermann P, Selbitz HJ, Truyen U. Real-time PCR protocol for the detection of porcine parvovirus in field samples. *J Virol Methods* 2006;134:257–60.
- [2] Bergeron J, Menezes J, Tijssen J. Genomic organization and mapping of transcription and translation products of the NADL-2 strain of porcine parvovirus. *Virology* 1993;197:86–98.
- [3] Allan GM, Kennedy S, McNeilly F, Foster JC, Ellis JA, Krakowka SJ. Experimental reproduction of severe wasting disease by co-infection of pigs with porcine circovirus and porcine parvovirus. *J Comp Pathol* 1999;121(1):1–11.
- [4] Ellis JA, Bratanich A, Clark EG, Allan G, Meehan B, Haines DM. Coinfection by porcine circoviruses and porcine parvovirus in pigs with naturally acquired postweaning multisystemic wasting syndrome. *J Vet Diagn Invest* 2000;12(1):21–7.
- [5] Parke CR, Burgess GW. An economic assessment of porcine parvovirus vaccination. *Vet J* 1993;70(5):177–80.
- [6] Adriaan FG, Christianne JM, Paloma R. A novel recombinant virus-like particle vaccine for prevention of porcine parvovirus-induced reproductive failure. *Vaccine* 2006;24:5481–90.
- [7] Ginkel FW, Nguyen HH, McGhee JR. Vaccines for mucosal immunity to combat emerging infectious diseases. *Emerg Infect Dis* 2000;6(2):123–32.
- [8] Friedman RS, Frankel FR, Xu Z, Lieberman J. Induction of human immunodeficiency virus (HIV)-specific CD8 T-cell responses by *Listeria monocytogenes* and a hyperattenuated *Listeria* strain engineered to express HIV antigens. *J Virol* 2000;74:9987–93.
- [9] Lee JS, Shin KS, Pan JG, Kim CJ. Surface-displayed viral antigens on *Salmonella* carrier vaccine. *Nat Biotechnol* 2000;18:645–8.
- [10] Shata MT, Hone DM. Vaccination with a *Shigella* DNA vaccine vector induces antigen-specific CD8⁺ T-cells and antiviral protective immunity. *J Virol* 2001;75:9665–70.
- [11] Shata MT, Reitz MS, DeVico AL, Lewis GK, Hone DM. Mucosal and systemic HIV-1 Env-specific CD8⁺ T-cells develop after intragastric vaccination with a *Salmonella* Env DNA vaccine vector. *Vaccine* 2001;20:623–9.
- [12] Ho PS, Wang JK, Lee YK. Intragastric administration of *Lactobacillus casei* expressing transmissible gastroenteritis coronavirus spike glycoprotein induced specific antibody production. *Vaccine* 2005;23:1335–42.
- [13] Simmons CP, Ghaem-Magami M, Petrovska L, Lopes L, Chain BM, Williams NA. Immunomodulation using bacterial enterotoxins. *Scand J Immunol* 2001;53:218–26.
- [14] Millar DG, Hirst TR, Snider DP. *Escherichia coli* heat labile enterotoxin B subunit is a more potent mucosal adjuvant than its closely related homologue, the B subunit of cholera toxin. *Infect Immun* 2001;69:3476–82.
- [15] Lauterslager TG, Florack DE, van der Wal TJ, Molthoff JW, Langeveld JP, Bosch D. Oral immunization of naive and primed animals with transgenic potato tubers expressing LTb. *Vaccine* 2001;19:2749–55.
- [16] Cristian S, Ines M, Juan P, Roy C, Luis E. A continuous epitope from transmissible gastroenteritis virus S protein fused to *E. coli* heat-labile toxin B subunit expressed by attenuated *Salmonella* induces serum and secretory immunity. *Virus Res* 1996;41:1–9.
- [17] Yigang XU, Yijing LI. Construction of recombinant *Lactobacillus casei* efficiently surface displayed and secreted porcine parvovirus VP2 protein and comparison of the immune responses induced by oral immunization. *Immunology* 2008;124(1):68–75.
- [18] Josson K, Scheirlinck T, Michiels F. Characterization of a Gram-positive broad-host-range plasmid isolated from *Lactobacillus hilgardii*. *Plasmid* 1989;21:9–20.
- [19] Mühlpfordt H. The preparation of colloidal gold particles using tannic acid as an additional reducing agent. *Cell Mol Life Sci* 1982;38:1127–8.
- [20] Cortes-Perez NG, Bermúdez-Humarán LG, Le Loir Y, Rodriguez PC. Mice immunization with live lactococci displaying a surface anchored HPV-16 E7 oncoprotein. *FEMS Microbiol Lett* 2003;229:37–42.
- [21] Joo HS, Donaldson-Wood CR, Johnson RH, Campbell RS. Observations of the pathogenesis of porcine parvovirus infection. *Arch Virol* 1976;51:123–9.
- [22] Wu FS, Li WG, Fan SH, Wang Y, Tang GF. Construction of the pseudorabies virus transfer vector to express porcine parvovirus VP2 gene. *Chin Henan Agric Sci* 2006;2:102–4.
- [23] Maassen CBM, Laman JD, Heijne MJ. Instruments for oral disease-intervention strategies: recombinant *Lactobacillus casei* expressing tetanus toxin fragment C for vaccination or myelin proteins for oral tolerance induction in multiple sclerosis. *Vaccine* 1999;17:2117–28.
- [24] Reveneau N, Geoffroy MC, Lochet C. Comparison of the immune responses induced by local immunizations with recombinant *Lactobacillus plantarum* producing tetanus toxin fragment C in different cellular locations. *Vaccine* 2002;20:1769–77.
- [25] Cheun HI, Kawamoto K, Hiramoto M, Tamaoki H. Protective immunity of SpaA-antigen producing *Lactococcus lactis* against *Erysipelothrix rhusiopathiae* infection. *J Appl Microbiol* 2004;6(6):1347–53.
- [26] Jong-Soo L, Haryoung P, Dong PH, Seung-Pyo H. Mucosal immunization with surface-displayed severe acute respiratory syndrome coronavirus spike protein on *Lactobacillus casei* induces neutralizing antibodies in mice. *J Virol* 2006;80(8):4079–87.

- [27] Yigang XU, Lichun CUI, Lijie Tang, Lili ZHAO, Yijing LI. Colonization ability and distribution of *Lactobacillus casei* 393 in mouse gut. China Dairy Ind 2007;35(4):20–2.
- [28] Wagner B, Hufnagl K, Radauer C, Wagner S, Baier K, Scheiner O. Expression of the B subunit of the heat-labile enterotoxin of *Escherichia coli* in tobacco mosaic virus-infected *Nicotiana benthamiana* plants and its characterization as mucosal immunogen and adjuvant. J Immunol Methods 2004;287(1–2):203–15.
- [29] Companjen AR, Florack DE, Slootweg T, Borst JW, Rombout JH. Improved uptake of plant-derived LTB-linked proteins in carp gut and induction of specific humoral immune responses upon infeed delivery. Fish Shellfish Immunol 2006;21(3):251–60.
- [30] Zhang X, Yuan Z, Duan Q, Zhu H, Yu H, Wang Q. Mucosal immunity in mice induced by orally administered transgenic rice. Vaccine 2009;27(10):1596–600.