Construction of recombinant *Lactobacillus casei* efficiently surface displayed and secreted porcine parvovirus VP2 protein and comparison of the immune responses induced by oral immunization

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Summary

Lactobacillus casei ATCC 393 was selected as a bacterial carrier for the development of mucosal vaccine against porcine parvovirus (PPV) infection. The PPV major structural polypeptide VP2 was used as the model parvovirus antigen. Two inducible expression systems, namely pPG611.1 of the cell-surface expression system and pPG612.1 of the secretion expression system based on the xylose operon promoter were used to express the VP2 protein. The immunogenicity of recombinant strains producing VP2 protein in two cellular locations, cell-surface exposed and secreted, was compared to each other by immunizing mice through the intragastric administration. The two types of constructs were able to induce strong specific immune responses against VP2 via intragastric administration and maximum titres of IgA and IgG were attained on days 46 post oral immunization, while the highest antibody levels were obtained with the strain producing the VP2 protein in extracellular milieu. The induced antibodies demonstrated neutralizing effects on PPV infection.

Keywords: porcine parvovirus; VP2 protein; *Lactobacillus casei*; mucosal vaccine; antigen location

Introduction

It is now clear that the gastrointestinal mucosas are the primary sites of natural virus transmission and also serve as a reservoir of virus infection and persistence. Although parenteral vaccination is usually effective in eliciting a protective immune response, the response is not desired as the virus enters the body via the mucosal surfaces. This mucosa-centric nature of virus infection provides a strong rationale for development of mucosal vaccines to induce sufficient mucosal response to prevent the virus from establishing the mucosa as a site of continued replication and dissemination to other tissues.[1–3] Furthermore, these studies suggest that a vaccine that induces local mucosal immunity, in particular the induction of local $CD8^+$ cytotoxic T lymphocyte (CTL) may control viral replication within local tissues prior to systemic dissemination.[4] These results also provide strong support for development of new generation vaccines that induce mucosal secretory immunoglobulin A (IgA) against virus.[5,6]

Porcine parvovirus (PPV), an autonomous parvovirus, is a major cause of reproductive failure in swine resulting in early embryonic death, fetal death, stillbirths, and delayed return to oestrus.[7–10] The viral genome consists of a linear, minus-polarity single-stranded 5 kb DNA,[11] and is constructed of three structural polypeptides with MW of 84 000, 64 000 and 60 000, respectively.[12] The VP2 structural polypeptide encompasses the major antigenic domains and could induce neutralizing antibodies for neutralization of PPV infection.[13,14] Like many other pathogens, the PPV initiates its infectious cycle at the mucosal surfaces,[7,15] especially intestinal mucosal epithelial surface. Efficient protection against mucosal infections requires the development of new vaccines that induce protective immune responses not only at systemic level, but also mucosal level in order to prevent or reduce dissemination of virus at the mucosa. This can best be achieved by mucosal vaccination, a route which offers several advantages over the traditional parenteral vaccines.[16–18] However, as the local administration of pure antigens or subunit vaccines is usually rather inefficient, several strategies have been developed to deliver the antigen in an immunogenic and protected form at the targeted mucosa. One promising approach relies on the use of live bacterial vehicles.[19–21]

The potential development of lactic acid bacteria (LAB) to deliver heterologous antigens to the mucosal immune

system has been proposed;[22–27] these have many properties that make them attractive candidates as antigen delivery carriers for the presentation to the mucosa of compounds with pharmaceutical interest, in particular vaccines. For example, LAB have long been used in industrial food fermentation and preservation and are known for the beneficial effects on the health of humans and animals, and considered 'generally regarded as safe, GRAS' micro-organisms. In addition, many strains of LAB are able to survive and colonize in the intestinal tract.[28,29] and induce a non-specific immunoadjuvant effect;[30] this has provoked serial studies aimed at determining the capability and feasibility of the application of LAB as safe oral vaccines.

Therefore, developing efficient gene expression and protein secretion systems in nonpathogenic gram-positive LAB is a new strategy for parvovirus vaccination. In this study, we chose to develop *Lactobacillus casei* ATCC393 as an antigen-delivery carrier for oral vaccine of PPV, and constructed recombinant strains producing the parvovirus model antigen VP2 structural polypeptide in two cellular locations: the external milieu or anchored to the cell surface using plasmids pPG611.1 and pPG612.1, which differ only by targeting. The immunogenicity of the two recombinant strains was analysed after intragastric administration of live bacteria to mice. Our data have indicated that intragastric intubation of two recombinant strains could induce the specific mucosal and systemic immune response against PPV. The higher anti-VP2 specific immune responses were obtained with the recombinant strain producing the VP2 in the extracellular milieu.

Materials and methods

Bacterial strain and growth conditions

Lactobacillus casei ATCC 393 kindly supplied by Jos Seegers (NIZO, Holland), was grown in Mann Rogosa Sharpe (MRS) medium (Sigma, St Louis, MO), at 37° anaerobically, without shaking. For the analysis of expression of VP2 protein, recombinant strains were grown in basal MRS medium supplemented with 2% xylose. Antibiotic concentration used for the selection of transformants was 10 μg/ml of chloromycetin, Cm (Sigma).

Plasmids, DNA manipulation and transformation

The expression plasmid pPG612.1, a type of secretion expression vector containing ssUsp secretion signal peptide sequence and the expression plasmid pPG611.1, a type of cell-surface expression plasmid containing the structures of ssUsp secretion signal sequence and cell wall anchor domain, were kindly supplied by Jos Seegers (NIZO, Holland). Nucleic acid manipulation and cloning procedures were performed according to standard procedures.[31] A gene fragment of about 1·74 kb encoding the VP2 structural polypeptide of PPV was obtained from the genome of PPV strain LJL12 by polymerase chain reaction (PCR) amplification with the primers 5′-CGAGGATCC TATGGTTCACTGGTTCGACGACCGCGAG-3′ (forward) containing a *Bam*HI site (underlined) and 5′-AGCTT CTCGAGCCATGCTACCTGATTAACCGAGTAACTG-3′ (reverse) containing an *Xho*I site (underlined). PCR amplification conditions were as follows: 95°, 5 min; 30 cycles of 94°, 1 min; 55°, 1 min; 72°, 1·2 min; 72°, 10 min for the final extension. The PCR product of VP2 gene was cleaved with *Bam*HI and *Xho*I restriction endonuclease (MBI) and inserted into the corresponding sites of pPG611.1 and pPG612.1 digested by *Bam*HI and *Xho*I respectively, giving rise to pPG611.1-VP2 and pPG612.1-VP2 (Fig. 1).

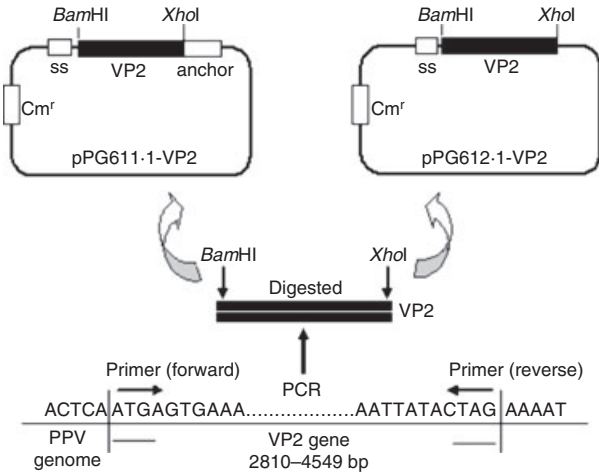

Figure 1. The construct of recombinant vectors expressing VP2 protein. The gene fragment encoding VP2 structural polypeptide of PPV was amplified by PCR with the primers. The PCR product was cleaved with *Bam*HI and *Xho*I restriction endonuclease and inserted into the corresponding sites of pPG611.1 and pPG612.1, respectively, giving rise to pPG611.1-VP2 and pPG612.1-VP2.

Electroporation of *L. casei* was carried out as previously described[32] with some modifications. In brief, recombinant plasmid DNA (10 μl) was added to 150 μl of *L. casei* 393, gently mixed at 4° for 5 min and subjected to a single electric pulse (25 μF of 2·5 kV/cm). The mixer was incubated in MRS medium without Cm at 37° anaerobically for 2 hr. Recombinant strains were selected on MRS-agar medium containing 10 μg/ml of Cm. The presence and integrity of the constructions carried by the *L. casei* 393 transformants were checked by extraction of recombinant plasmid DNA following by restriction analysis and sequencing.

Protein expression and Western blot analysis

To analyse the expression of the VP2 fusion protein by xylose-induced rLc393:pPG611.1-VP2, overnight cultures

grown in basal MRS medium supplemented with xylose were collected by centrifugation at 12 000 ***g*** for 10 min. The pellets were washed twice with sterile 50 mM Tris-Cl, pH 8·0 and lysed in a Bead-Beater (Biospec, Bartlesville, OK) by vigorous shaking. The lysates were centrifuged at 15 000 ***g*** for 10 min and the supernate were examined using 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Western blot analysis: Proteins were electrotransferred onto a nitrocellulose membrane and the immunoblots were developed using mouse anti-VP2 serum at a dilution of 1 : 1000 with phosphate-buffered saline (PBS). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma) diluted at 1 : 2000 was used and visualization of immunolabelled bands were then carried out using the Chemiluminescent Substrate reagent (Pierce, Rockford, IL) according to the manufacturer's instruction.

To assay the localization of the VP2 fusion protein, the same cells were treated with 10 mg/ml lysozyme at 37° for 60 min, centrifuged and the supernate were maintained at −20° for further analysis. In a parallel experiment, the same cells were treated with 10 mg/ml of protease (Sigma) at 37° for 1 hr, washed thrice to remove the protease and then treated with lysozyme. The bacterial protein extracts and the supernate were analysed using Western blot (as previously described). Immunofluorescence was also used to analyse the surface expression of the VP2 protein by the rLc393:pPG611.1-VP2 carried out as previously described.[33] In brief, 2 ml induced cultures were harvested at OD_{600} = 0·5–0·6 and then resuspended in 1 ml sterile PBS–3% bovine serum albumin (BSA) containing anti-VP2 antibodies, incubated overnight at 37°. The cells were pellets, washed thrice with sterile PBS-Tween-20 0·05% to remove non-combination antibodies. The cell–antibody complex was incubated for 6 hr at 37° (avoiding light exposure) with fluoreoscein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma) containing 1% Evans blue. Cells were washed thrice with PBS–Tween-20 0·05%, laid on a glass slide, air-dried. Analysis was performed on a confocal microscopy. The non-induced recombinant strain used as negative control.

To analyse VP2 secretion expression, overnight cultures of rLc393:pPG612.1-VP2 grown in basal MRS medium supplemented with xylose, were collected by centrifugation at 12 000 ***g*** for 10 min. The centrifuged supernate were concentrated 10 times using a Centrifugal Filter Unit (Millipore, Billerica, MA), and then the concentrated supernate were examined using 10% SDS–PAGE and Western blot (as previously described).

Immunization

Seven-week-old BALB/c mice were used as animal model, which had free access to a standard mouse diet and water. RLc393:pPG611.1-VP2 or rLc393:pPG612.1-VP2 was cultured and centrifuged. Cell pellets were washed once with sterile PBS, resuspended in PBS (pH 7·4) to a concentration of 10^{10} colony-forming units (c.f.u.)/ml. The vaccine groups of 30 mice received dose of 10^9 cells of two recombinant strains, respectively. The groups of 30 mice immunized with equivalent dose of *L. casei* 393, *L. casei* 393 containing empty plasmid or received PBS were used as control. All the mice were immunized via oral route. The immune protocol was administered on three consecutive days at days 0, 1and 2. A booster immunization was given at days 14, 15 and 16 and a second booster was given at days 28, 29 and 30.[34]

Enzyme-linked immunosorbent assay (ELISA) analysis

Sera of mice were collected on days 18, 32, 46, 58 after the first immunization and stored at −20° until required. The intestinal mucus of mice was extracted as described previously.[35,36] ELISA analysis: Polystyrene microtitre plates were coated overnight at 4° with the full PPV virus propagated on ST cells and the culture of ST cells used as negative control antigen. The ELISA plates were washed thrice in PBS–Tween-20 1% then saturated with PBS containing 5% skimmed milk for 2 hr. Serum or intestinal lavage was serially diluted in PBS–1% BSA using as primary antibodies. After incubation at 37° for 1 hr, the plates were washed thrice with PBS–Tween-20 1%. Bound antibodies were detected using HRP-conjugated goat anti-mouse IgA (Sigma) or IgG (Sigma) diluted at 1 : 2000, followed by colour development using *o*-phenylene diamine dihydrochloride (Sigma) as substrate, and then absorbance was measured at 490 nm.

Neutralization ability of the induced antibodies

Intestinal fluids and serum samples from mice immunized with two kinds of recombinant strains expressing rPPV-VP2 were evaluated using a plaque reduction assay to determine the neutralization ability of the induced antibodies according to the method described by PS Ho *et al.*[27] In brief, lavages and sera from non-expressor strains or PBS fed mice were used as negative control. Fifty microlitres of samples in twofold serial dilutions were prepared in microcentrifuge tubes. PPV adjusted to 200 $TCID_{50}$ in 50 μl virus diluent was added to the tube containing serially diluted serum or intestinal lavage. The antibody and virus mixture was mixed, pulsed, centrifuged and then incubated at 37° for 1 hr. A 24-cell plate with confluent monolayer of swine testicular (ST) cell (used for virus infection) inoculated with antibody–virus mixture at 37° for another 1 hr rocked at 20 min intervals. Then, the inoculum was removed and overlayed with medium (equal volume of concentrated cell culture medium) and the plate was incubated in a 5% CO_2 incubator at 37° for 5 days. The overlay medium was then

discarded, after which the wells were washed thrice with sterile PBS, pH 7·4 and stained with 1% crystal violet solution. Differences in the number of plaques formed between treatments were examined for the level of significance by Student's *t*-test after analysis of variance.

Results

VP2 protein cell-surface expression

Overnight cultures of rLc393:pPG611.1-VP2 grown in basal MRS medium supplemented with either xylose or glucose were collected and cell lysates were analysed by SDS–PAGE and Western blot. Coomassie blue gel staining showed the expression of a 74 000 MW fusion protein in lysates of rLc393:pPG611.1-VP2 induced by xylose (Fig. 2a, lanes 2, 5), but not the same cells grown in glucose (Fig. 2a, lane 4) or wild-type *L. casei* 393 grown in xylose (Fig. 2a, lane 3). Western blot analysis showed that an immunoreactive band was detected ((Fig. 2b, lane 3) in a similar position as observed in the SDS–PAGE shown in Fig. 2(a), but not when the recombinant strain was grown in MRS medium containing glucose (Fig. 2b, lane 2) or when wild type *L. casei* 393 was grown in MRS medium containing xylose (Fig. 2b, lane 1). The results show that the xylose promoter from *L. casei* can be used to efficiently induce the expression of PPV heterologous protein.

The localization analysis of the VP2 protein by xylose-induced rLc393:pPG611.1-VP2 via Western blot showed that the induced rLc393:pPG611.1-VP2 was treated with lysozyme and a 74 000 MW band was released to the supernate (Fig. 3. lane 2) in a similar position as observed when the same cells were lysed by Bead-Beater (Fig. 3. lane 1), which could not be detected in supernate of cells only

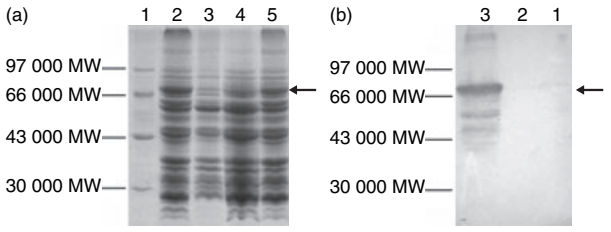

Figure 2. Expression of VP2 protein in rLc393:pPG611.1-VP2. Total cell lysates were analysed by SDS–PAGE and Western blot with specific antiserum. (a) Coomassie blue gel staining shows the expression of a 74 000 MW fusion protein in lysates of rLc393:pPG611.1-VP2 induced by xylose (lanes 2, 5), but not in glucose (lane 4) or wild-type *L. casei* 393 grown in xylose (lane 3); Lane 1. MW, molecular mass marker. (b) Western blot analysis of VP2 expression in recombinant strain. An immunoreactive band was observed (lane 3) in a similar position as observed in the SDS–PAGE shown in the panel A, however, there were no immunoblots in the same cell lysates induced by glucose (lane 2) or in wild-type *L. casei* 393 (lane 1).

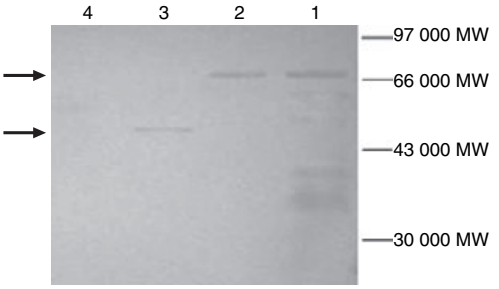

Figure 3. Localization analysis of VP2 expression from the rLc393:pPG611.1-VP2 via Western blot. lane1, total lysates of xylose-inducible cells expressing VP2 fusion lysed by Bead-Beater; lane 2, supernatant of the same cells treated with lysozyme; lane3, supernatant of cells treated with protease followed by lysozyme; lane 4, supernatant from untreated cells.

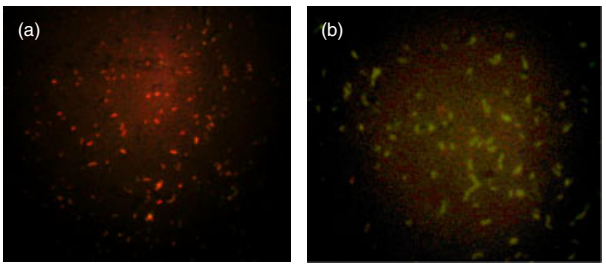

Figure 4. The immunofluorescence of VP2 protein on the cell surface of rLc393:pPG611.1-VP2. (a) The rLc393:pPG611.1-VP2 was grown in MRS medium containing glucose, the bacteria pellets were incubated with mouse anti-VP2 serum, FITC conjugated goat anti-mouse IgG containing 1% Evans blue. There were no immunofluorescence reaction on the cell surface, the bacteria were red, stained by Evans blue. (b) rLc393:pPG611.1-VP2 was induced by xylose, there was green–yellow fluorescence on the surface of the bacteria.

treated with protease (data not shown). Treatment with protease previous to lysozyme gives a single band about 50 000 MW (Fig. 3. lane 3). None of bands was observed in supernate from untreated cells (Fig. 3. lane 4). The immunofluorescence reaction of rLc393: pPG611.1-VP2 induced by xylose developed with the mouse anti-VP2 serum and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG also showed that there was green–yellow fluorescence on the cell surface of rLc393:pPG611.1-VP2 grown in xylose (Fig. 4b), but not in glucose; the recombinant strain was red dyed by Evans blue (Fig. 4a). These results suggested that the VP2 protein could be displayed on the cell surface, while a fragment of VP2 protein appeared to be embedded in the cell wall.

VP2 protein secretion expression by recombinant *L. casei*

Overnight cultures of rLc393:pPG612.1-VP2 grown in basal MRS medium supplemented with either xylose or

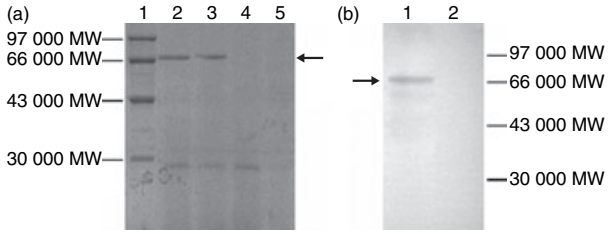

Figure 5. Secretion expression of VP2 protein in rLc393:pPG612.1-VP2. (a) SDS–PAGE analysis of concentrated supernate. lane 1. MW, molecular mass marker; lanes 2, 3. (a) 70 000 MW fusion protein in the supernate of rLc393:pPG612.1-VP2 induced by xylose; but not in same cells induced by glucose (lanes 4, 5). (b) Western blot analysis of VP2 expression. An immunoreactive band was observed (lane 1) in a similar position as observed in the SDS–PAGE shown in (a), however, there were no immunoblots in the supernate grown in glucose (lane 2).

glucose, were collected by centrifugation. The supernate were concentrated 10 times, and analysed by SDS–PAGE and Western blot. SDS-PAGE analysis of the supernate of rLc393:pPG612.1-VP2 induced by xylose showed a 70 000 MW fusion protein in the supernate (Fig. 5a: lanes 2, 3), but not in glucose (Fig. 5a: lanes 4,5). An immunoreactive band of 70 000 MW was detected in the supernate of rLc393:pPG612.1-VP2 induced by xylose (Fig. 5b: lane 1), whereas there was no band in the control lane of the supernate of rLc393:pPG612.1-VP2 grown in glucose (Fig. 5b: lane 2).

Immune responses of recombinant strains induced by intragastric immunization

The immunogenicity of the two recombinant strains was evaluated by intragastric administration to mice. The local immune response was studied by measuring the anti-PPV-VP2 sIgA level in intestinal lavage post intragastric immunization. The concentration of anti-PPV-VP2 mucosal sIgA 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 (Sigma) of known titre. As the results showing, there was no substantial difference in mucosal sIgA level between experimental groups and control groups prior to intragastric immunization, while oral immunization of recombinant strains expressing VP2 elicited an antigen specific mucosal sIgA response, after the second boost, high levels of anti-VP2 sIgA were obtained in intestinal lavages (Fig. 6) by oral administration of rLc393:pPG611.1-VP2 or rLc393:pPG612.1-VP2, whereas no anti-VP2 antibody was observed in control groups of mice or used the ST cell culture as negative control antigen (data not shown). The mucosal sIgA level elicited by rLc393: pPG612.1-VP2 was higher than that elicited by

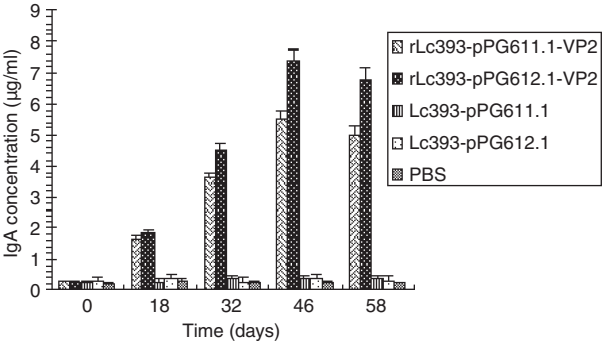

Figure 6. Anti-PPV-VP2 specific sIgA response in intestinal lavage after intragastric immunization. The mice received three consecutive doses of 10^9 rLc393:pPG611.1-VP2 or rLc393:pPG612.1-VP2, three times at 2-week intervals. Control mice received 10^9 Lc393 or Lc393 containing empty plasmid, while the negative group received PBS. Intestinal lavages that were collected on days18, 32, 46 and 58 after the first immunization, were analysed via ELISA using PPV as the coating antigen. Bars represent the IgA titre ± SEM in each group.

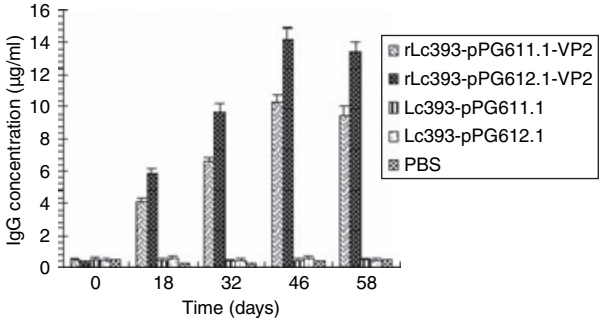

Figure 7. Anti-PPV-VP2 serum IgG response induced after intragastric immunization with recombinant strains. Sera from groups of mice orally immunized with 10^9 recombinant strains expressing VP2 and equivalent doses of Lc393, Lc393 containing empty plasmid and negative control sera from mice received with PBS were analysed for the presence of PPV-VP2 specific IgG by ELISA, using the PPV as the coating antigen. Bars represent the IgG titre ± SEM in each group.

rLc393:pPG611.1-VP2. Statistically significant difference was observed on days 46 and 58.

The anti-VP2 specific IgG level in sera of immunized mice was determined (Fig. 7). The mice orally administrated with rLc393:pPG611.1-VP2 or rLc393:pPG612.1-VP2 after the second boost elicited a prompter and stronger anti-VP2 IgG level in comparison to the mucosal IgA induced in the intestine. No significant elicitation of anti-VP2 antibodies was observed in the control groups of mice or used the ST cell culture as negative control antigen (data not shown). The serum IgG level elicited by rLc393:pPG612.1-VP2 was higher than that elicited by rLc393:pPG611.1-VP2. A statistically significant difference ($P < 0{\cdot}05$) was observed on days 32, 46 and 58.

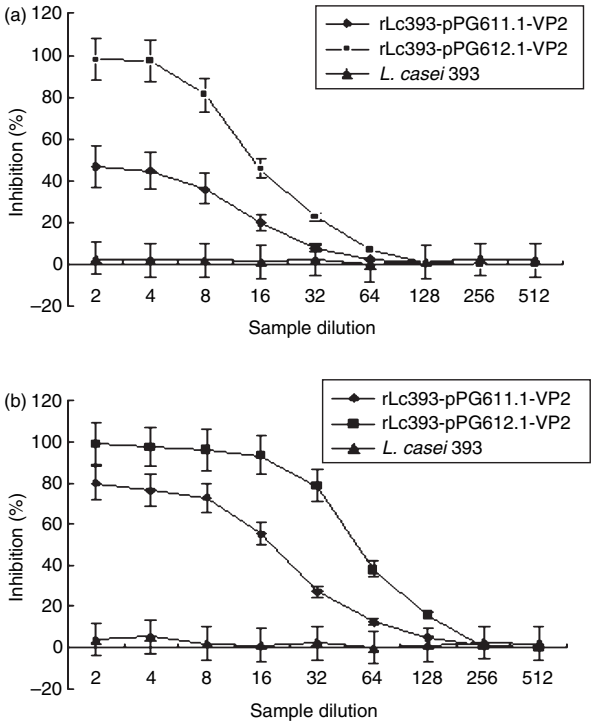

Figure 8. Inhibition of viral plaque formation by (a) intestinal lavages and (b) sera prepared from mice immunized with rLc393:pPG612.1-VP2 and rLc393:pPG612.1-VP2. Results are mean values and standard errors of triplicates.

Plaque reduction assay

Plaque reduction assays were performed to further detect whether the antibody responses were against PPV. Results demonstrated that the presence of anti-rPPV-VP2 sIgA or IgG in the culture medium conferred statistically significant neutralizing effects ($P < 0{\cdot}05$) on PPV infection (Fig. 8). A near 98·0% reduction in the number of plaques was consistently observed when plaque reduction assays were carried out using two- to fourfold diluted intestinal lavages or two-to 16-fold diluted sera from mice immunized with rLc393:pPG612.1-VP2. The significant differences of the final significant dilution of antibody obtained from the mice immunized with rLc393:pPG612.1-VP2 for Fig. 8(a) was 1 : 32 and for Fig. 8(b) is 1 : 64, while the rLc393:pPG611.1-VP2 was 1 : 16 and 1 : 32, respectively. The inhibitory effect decreased gradually on further dilutions of intestinal lavages and sera and reached to the level similar to that of the control, which the rLc393:pPG612.1-VP2 is 1:64 for Fig. 8(a) and 1 : 128 in Fig. 8(b), the rLc393:pPG611.1-VP2 is 1:32 for Fig. 8(a) and 1 : 64 for Fig. 8(b). The neutralizing efficacy of anti-VP2 IgA or IgG from mice immunized with rLc393:pPG611.1-VP2 was lower than antibodies obtained from mice immunized with rLc393:pPG612.1-VP2 (Fig. 8).

Discussion

Infection with PPV is of economic concern to pig breeders worldwide. The initial infection of PPV mainly occurs at the mucosa of intestines; this is a critical step in the infection process.[7] Vaccination is the main prophylatic method of preventing PPV infection. Parenteral vaccination is usually effective in eliciting systemic immune responses; however, the responses obtained can not prevent the virus from entering the body via the mucosal surfaces. Therefore, the elicitation of efficient immune response, not only at the systemic, but also at the mucosal level is highly desirable after vaccination. This goal can be only achieved when the vaccine formulation is administered by the mucosal route. As such, a great deal of research is currently focused on the development of adequate mucosal vaccines, with various vaccine delivery systems being explored for oral application. Attenuated pseudorabies virus carrying the VP2 protein of PPV was able to protect mice against viral challenge.[37] Other live vectors such as vaccinia virus expressing PPV proteins stimulated protective immune responses. However, the majority of these live vectors are potentially risk prone.

LAB are of increasing interest for the expression of heterologous molecules.[38] The absence of an outer membrane in these Gram-positive micro-organisms makes them particularly attractive for the display or secretion of biologically active molecules. To date, series of recombinant lactobacilli expressing heterologous pathogen antigens have been constructed, such as *Streptococcus pneumoniae* antigens PsaA and PspA,[26] transmissible gastroentritis coronavirus S glycoprotein,[27] tetanus toxin fragment C.[23,24] and cholera toxin B subunit.[39] In this study, we engineered for the first time, using *L. casei* 393 to express the PPV main immune protective antigen VP2 protein with two xylose-induced expression systems pPG611.1, pPG612.1 to explore the potential value as oral vaccine. The VP2 protein was expressed in the external milieu and on cell surface, respectively, whose own good antigenic properties could be recognized by antiserum.

We conducted a controlled comparison of the immunogenicity of two recombinant strains producing VP2 protein of PPV in the external milieu or on the cell surface after administration to mice by intragastric route. The oral administration regime was consisted of three sets of three successive daily doses of the recombinant strain as the experimental vaccine. Three successive daily doses of recombinant bacteria were required in order to ensure that systemic antibody response to PPV could be elicited in all mice received recombinant strains intragastrically.

The principal antibody type involved in mucosal immunity is secretory immunoglobulin A (sIgA), the majority of which is released into the gastrointestinal fluid.[40] The results of immunization indicated that the recombinant strains constructed in this study were able to

elicit mucosal immune response producing sIgA antibody and a systemic immune response producing IgG. In contrast, rLc393:pPG612.1-VP2 expressing VP2 in the external milieu induced much higher antibody level of local mucosal or systemic immune response in mice than that elicited by rLc393:pPG611.1-VP2 displaying VP2 on cell surface. Statistically significant differences were observed on days 32, 46 and 58. As estimated by quantitative immunoblot analysis, this difference could be related to varying levels of VP2 production depending on the cellular location of the antigen; therefore, the strong immunogenicity most probably results from the high level of external milieu VP2 product in rLc393:pPG612.1-VP2, which could effectively reacted with intestine mucosal tissues. We hypothesize that the VP2 protein expressed by rLc393:pPG611.1-VP2 may be embedded in the cell wall and only partly accessible to cell surface, which could not fully associate with gut lymphoid tissue resulting in the lower level of immune antibodies than that induced by rLc393:pPG612.1-VP2.

Furthermore, in order to confirm the efficacy of the induced antibodies in inhibiting the virus, we tested whether intestinal lavages and sera collected from immunized mice could inhibit the infection of ST cells in a plaque reduction neutralization assay. The results showed that serum and intestinal samples collected from mice immunized with rLc393:pPG612.1-VP2 demonstrated statistically significant inhibition. Although the pPG611.1 recombinant vector could also induce the neutralizing antibodies, the neutralization by intestinal lavage antibodies obtained from mice immunized with pPG612.1 recombinant vector was more effective than that after immunization of mice with the pPG611.1 recombinant vector.

On the other hand, the immunization results with the cell surface expression antigen by rLc393:pPG611.1-VP2 contrast with those published by Shaw *et al.*[41] who found that cell surface presentation of heterologous antigen did not induce detectable immune responses by the intragastric route. Several factors may explain these differences. Successful mucosal immunization requires the delivery of a minimal threshold load of antigen. Therefore, it is possible that the recombinant strain described by Shaw *et al.* produces insufficient antigen quantities at the cell surface. Alternatively, intrinsic immunostimulatory properties of *L. casei* ATCC 393, yet to be determined, may differ from those of *L. plantarum* 256, used by Shaw *et al.* The strain used in this study was able to adhere and colonize the intestinal tracts and tolerate bile (data not shown), which would render it more appropriate an oral vaccine carrier with the probiotic effects and the harmless nature of *L. casei* 393.

In summary, using the methods described here, better results (i.e. high serum VP2-specific IgG and local IgA responses) were obtained with the strain rLc393:pPG612.1-VP2 producing the antigen in the external milieu, which has a better immunogenicity and was able to elicit high specific mucosal and systemic immune responses against the antigen. This would make it a more appropriate oral vaccine against PPV infection. The mechanism of how the secreted antigen was also able to transude into the intravascular compartment to induce systemic immune responses will be further investigated.

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