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

X. U. Yigang and L. I. Yijing

*Veterinary Department, Northeast Agricultural University, Harbin, 150030, PR China*  

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
Development of oral vaccine for PPV

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 BamHI and Xhol 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.

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 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. 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 TATGTTCTAGTTGAGACGGGAG-3′ (forward) containing a BamHI site (underlined) and 5′-AGCTT CTCAAGGCTATGCTACCATTGAAGGTAAG-3′ (reverse) containing an Xhol 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 BamHI and Xhol restriction endonuclease (MBI) and inserted into the corresponding sites of pPG611.1 and pPG612.1 digested by BamHI and Xhol respectively, giving rise to pPG611.1-VP2 and pPG612.1-VP2 (Fig. 1).

Electroporation of L. casei was carried out as previously described 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°C for 60 min, centrifuged and the supernate were maintained at −20°C for further analysis. In a parallel experiment, the same cells were treated with 10 mg/ml of protease (Sigma) at 37°C 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 by the rLC393:pPG611.1-VP2 and the 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, 1 and 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°C until required. The intestinal mucus of mice was extracted as described previously.\(^{35,36}\) ELISA analysis: Polystyrene microtitre 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. 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°C 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°C 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°C 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°C 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 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 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 (Fig. 4a). rLc393:pPG611.1-VP2 was induced by xylose, there was green–yellow fluorescence on the surface of the bacteria.

![Figure 2](image)

**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](image)

**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](image)

**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.

**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...
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 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).

**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 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 administered 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 \( (P < 0.05) \) was observed on days 32, 46 and 58.
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.\textsuperscript{7} Vaccination is the main prophylactic 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.\textsuperscript{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.\textsuperscript{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 \textit{Streptococcus pneumoniae} antigens PsaA and PspA,\textsuperscript{26} transmissible gastroenteritis coronavirus S glycoprotein,\textsuperscript{27} tetanus toxin fragment C,\textsuperscript{23,24} and cholera toxin B subunit.\textsuperscript{39} In this study, we engineered for the first time, using \textit{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.\textsuperscript{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.

Acknowledgements
This work was supported by Grant 30371074 from the National Natural Sciences Funds of China. The authors wish to thank Jos Seegers for providing the plasmid pPG611.1, pPG612.1 and the bacterial strain L. casei ATCC 393.

References
1 Belyakov IM, Hel Z, Kelsall B et al. Mucosal AIDS vaccine reduces disease and viral load in gut reservoir and blood after mucosal infection of macaques. Nat Med 2001; 7:1320–6.
2 Belyakov IM, Berzofsky JA. Immunobiology of mucosal HIV infection and the basis for development of a new generation of mucosal AIDS vaccines. Immunity 2004; 20:247–53.
3 Baba TW, Liska V, Hofmann-Lehmann R et al. Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. Nat Med 2000; 6:200–6.
4 Belyakov IM, Kuznetsoy VA, Kelsall B et al. Impact of vaccine-induced mucosal high-avidity CD8+CTLs in delay of AIDS viral dissemination from mucosa. Blood 2006; 107:3258–64.
5 Lamm ME. Interaction of antigens and antibodies at mucosal surfaces. Annu Rev Microbiol 1997; 51:311–40.
6 Michalek SM, Eldridge JH, Curtis R III, Rosenthal KL. Antigen delivery systems: new approaches to mucosal immunization. In: Ogra PL, Mestecky MD, Lamm ME, Strober W, McGhee JR, Bienenstock J, eds. Handbook of Mucosal Immunology. San Diego: Academic Press, 1994:373–90.
7 Joo HS, Donaldson-Wood CR, Johnson RH, Campbell RS. Observations of the pathogenesis of porcine parvovirus infection. Arch Virol 1976; 51:123–9.
8 Carwright SF, Huck RA. Viruses isolated in association with herd infertility, abortions and stillbirths in pigs. Vet Rec 1967; 81:196–7.
9 Mengeling WL, Cutlip RC. Pathogenesis of in utero infection: experimental infection of five-week-old porcine fetuses with porcine parvovirus. Am J Vet Res 1975; 36:1173–7.
10 Joo HS, Johnson RH. Porcine parvovirus: a review. Vet Bull 1976; 46:653–60.
11 Molitor TW, Joo HS, Collett MS. Porcine parvovirus DNA: characterization of genomic and replicative form DNA of two virus isolates. Virology 1984; 137:241–54.
12 Molitor TW, Joo HS, Collett MS. Porcine parvovirus: virus purification and structural and antigenic properties of virion polypeptides. J Virol 1983; 45:842–54.
13 Molitor TW, Joo HS. Clinical and pathological features of porcine parvovirus related disease and its diagnosis. In: Tijssepn, P.
14 Ridpath JF, Mengeling WL. Uptake of porcine parvovirus into host and nonhost cells suggests host specificity is determined by intracellular factors. Virus Res 1988; 10:17–28.

15 Bican J, Svoboda M, Drabek J. Porcine parvovirus infection in boars in the Czech Republic. Acta Vet Brno 2002; 71:45–9.

16 Mestecky J. The common mucosal immune system and current strategies for induction of immune responses in external secretions. J Clin Immunol 1987; 7:265–76.

17 Mc Ghee JR, Mestecky J, Dertzbaugh MT, Eldridge JH, Hirasawa M, Kiyono H. The mucosal immune system: from fundamental concepts to vaccine development. Vaccine 1992; 10:75–88.

18 Staats HF, Jackson RJ, Marinaro M, Takahashi I, Kiyono H, Mestecky J. The mucosal immune system: from fundamental concepts to vaccine development. Curr Opin Immunol 1994; 6:572–83.

19 Medina E, Guzman CA. Use of live bacterial vaccine vectors for antigen delivery: potential and limitations. Vaccine 2001; 19:1573–80.

20 Guzman CA, Weiss S, Chakraborty T. Listeria monocytogenes a promising vaccine carrier to evoke cellular immune responses. In: Wells JM, Pozzi G, eds. Gram-Positive Bacteria as Vaccine Vehicle for Mucosal Immunization. Berlin: Springer, 1997:145–73.

21 Sirard JC, Niedergang F, Kraehenbuhl JP. Live attenuated Salmonella: a paradigm of mucosal vaccines. Immunol Rev 1999; 171:5–26.

22 Pouwels PH, Leer RJ, Boersma WJ. The potential of Lactobacillus as a carrier for oral immunization: development and preliminary characterization of vector systems for targeted delivery of antigen. J Biotechnol 1996; 44:183–92.

23 Maassen CBM, Laman JD, Heijne MJ et al. 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, Locht C et al. 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 Scheppler L, Vogel M, Zuercher A et al. Recombinant Lactobacillus johnsonii as a mucosal vaccine delivery vehicle. Vaccine 2002; 20:2913–31.

26 Oliveria MLS, Monedero V, Miyaji EN, Leite LCC, Lee Ho P, Perez-Martinez G Expression of Streptococcus pneumoniae antigens, PsaA and PspA by Lactobacillus casei. FEMS Microbiol Lett 2003; 227:25–31.

27 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.

28 Alander M, Satokari R, Korpela R, Saxelin M, Vilpponen-Salmenla T, Mattila-Sandholm T. Persistence of colonization of human colonicmucosa by a probiotic strain, Lactobacillus rhamnosus GG, after oral consumption. Appl Environ Microbiol 1999; 65:351–4.

29 Lee YK, Ho PS, Low CS, Arvilommi H, Salminen S. Permanent colonization by Lactobacillus casei is hindered by the low rate of cell division in mouse gut. Appl Environ Microbiol 2004; 70:670–4.

30 Ogawa T, Asai Y, Yasuda K et al. Oral immunoadjuvant activity of a new symbiotic Lactobacillus casei subsp casei in conjunction with dextran in BALB/c mice. Nutrition Research 2005; 25:295–304.

31 Sambrook J, Fritisch EF, Maniatis T. Molecular Cloning: A Laboratory Manual, 3rd Edn. New York: Cold Spring Harbor Laboratory, 2001.

32 Shifang J, Iinyu W, Xinhu G, Liandong H. The factors affected transformation efficiency of Lactobacillus by electroporation. Chin J Biotechnol 1998; 14:429–33.

33 Cortes-Perez NG, Bermúdez-Humarán LG, Le Loir Y, Rodriguez-Padilla C, Gruss A, Saucedo-Cárdenas O, Langella P, Montes-de-Oca-Luna R. Mice immunization with live lactococci displaying a surface anchored HPV-16 E7 oncoprotein. FEMS Microbiol Lett 2003; 229:37–42.

34 Challacombe SJ. Salivary antibodies and systemic tolerance in mice after oral immunization with bacterial antigens. Ann NY Acad Sci 1983; 409:177–93.

35 Elson CO, Ealding W, Lefkowitz J. A lavage technique allowing repeated measurement of IgA antibody in mouse intestinal secretion. J Immunol Methods 1984; 75:101–8.

36 Robinson K, Chamberlain LM, Schofield KM, Wells JM. Oral vaccination of mice against tetanus with recombinant lactococcus lactis. Nat Biotechnol 1997; 15:653–7.

37 Wu FS, Li WG, Fan SH, Wang Y, Tang GF. Construction of the pseudorabies virus transfer vector to express porcine parvovirus VP2 gene. Chinese Henan Agricultural Sciences 2006; 2:102–4.

38 Servig E, Grönqvist S, Naterstad K, Mathiesen G, Elsímk VGH, Axelsson L. Construction of vectors for inducible gene expression in Lactobacillus sakei and L. plantarum. FEMS Microbiol Lett 2003; 229:119–26.

39 Sloo P, Dutot P, Reymund J et al. Production of cholera toxin B subunit in lactobacillus. FEMS Microbiol Lett 1998; 169:29–36.

40 Brandtzaeg P. Distribution and characteristics of mucosal immunoglobulin-producing cells. In: OL Pearay, H Metecky, eds. Laboratory Manual, 3rd Edn. New York: Cold Spring Harbor Laboratory, 2001.

41 Shaw DM, Gaerthe B, Leer RJ et al. Engineering the microflora to vaccinate the mucosa: serum immunoglobulin G responses and activated draining cervical lymph nodes following mucosal application of tetanus toxin fragment C-expressing lactobacilli. Immunology 2000; 100:510–8.