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Construction of a recombinant BHV-1 expressing the VP1 gene of foot and mouth disease virus and its immunogenicity in a rabbit model

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Abstract Foot-and-mouth disease (FMD) and infectious bovine rhinotracheitis (IBR) are two important infectious diseases of cattle. Using bovine herpesvirus type 1 (BHV-1) as a gene delivery vector for development of live-viral vaccines has gained widespread interest. In this study, a recombinant BHV-1 was constructed by inserting the synthetic FMDV (O/China/99) VP1 gene in the the gE locus of BHV-1 genome under the control of immediately early gene promoter of human cytomegalovirus (phIE CMV) and bovine growth hormone polyadenylation (BGH polyA) signal. After homologous recombination and plaque purification, a recombinant virus named BHV-1/gE⁻/VP1 was acquired and identified. The immunogenicity was confirmed in a rabbit model by virus neutralization test and enzyme-linked immunosorbent assay (ELISA). The result indicated that the BHV-1/gE⁻/VP1 has the potential for being developed as a bivalent vaccine for FMD and IBR.

Introduction

Foot-and-mouth disease virus (FMDV) is the causative agent of a highly contagious and economically important disease affecting cloven-hoofed animals, particularly, domestic livestock species such as cattle, swine and sheep. FMDV consists of a single-stranded, plus-sense RNA genome of approximately 8,500 bases surrounded by four structural proteins, VP1, VP2, VP3 and VP4. It has been demonstrated that VP1 could induce neutralizing antibodies in the experimental and natural hosts. Two immunogenic sites on the VP1 were located at residues 141–160 amino acid (aa) and 200–213aa. On the residues 145–147aa, the loop contains a conserved triplet: Arg^{145}-Gly^{146}-Asp^{147} (RGD) motif, which is involved in the interaction between virus and cell surface receptor (Mason et al. 2003). FMD inactivated vaccines were shown to induce neutralizing antibodies, protect animals from FMDV and play a key role in controlling campaigns and eradication of FMD. However, inactivated vaccines require growth and inactivation of live virus, which requires containment facilities and has the potential for an escape of live virus from those facilities or from improper vaccine preparation. So,

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development of novel vaccines is necessary to control this disease (Sáiz et al. 2002).

Infectious bovine rhinotracheitis (IBR) is another widespread viral disease of cattle that causes serious economic losses in the cattle industry worldwide. The causative virus is bovine herpesvirus type 1 (BHV-1), commonly known as infectious bovine rhinotracheitis virus, a member of the Herpesviridae family, alpha herpesvirinae subfamily. BHV-1 is one of the most prevalent pathogenic agents in cattle and causes severe respiratory disease, most commonly rhinotracheitis, conjunctivitis, and genital infections (Muylkens et al. 2007). BHV-1 consists of double-stranded linear DNA with an approximate size of 140 kb, and foreign genes can be stably inserted into the genome of BHV-1, which makes the BHV-1 a promising candidate for the development of a live vaccine vector for economically important bovine diseases. Several recombinants expressing immunogenic foreign proteins have been reported as vaccines for other infectious diseases (Kit et al. 1991; Schmitt et al. 1999). In this study, a recombinant BHV-1 expressing VP1 gene of FMDV (O/China/99) was constructed and its immunogenicity was investigated in a rabbit model.

Materials and methods

Viruses and cells

BHV-1/gE$^-$/LacZ$^+$ is a recombinant derived from infectious bovine rhinotracheitis virus (IBRV) Bartha Nu/67, in which gE gene was deleted and *E. coli* β-galactosidase gene (LacZ) expression cassette was inserted under the control of immediately early gene promoter of human cytomegalovirus (phIE CMV) and bovine growth hormone polyadenylation (BGH polyA) signal (unpublished work). Viruses were propagated in Madin-Darby bovine kidney (MDBK) cells. MDBK cells and bovine turbinate (BT) cells were cultured in minimum essential medium (MEM) supplemented with 10% (v/v) fetal calf serum (FCS), 100 U penicillin/ml and 100 µg streptomycin/ml.

Plasmids

The plasmid pUC57-VP1 contained a synthetic gene that encodes FMDV (O/China/99) VP1 starting at the *Hin*dIII site and ending at the *Xba*I site; the plasmid

pGEM-TgILdgELacZ contained the LacZ gene expression cassette under the control of phIE CMV, BGH polyA signal and upstream recombinant sequence of BHV-1 genomic DNA; the plasmid pGEM-TgE$_{SN}$ contained the downstream recombinant sequence of BHV-1 genomic DNA.

Construction of the transfer vector plasmid

The plasmid pUC57-VP1 was digested with *Xba*I and *Hin*dIII, the isolated VP1 fragment was ligated into the *Xba*I/*Hin*dIII-cut plasmid pGEM-TgILdgELacZ, the resulting plasmid was named gILdgEVP1, the LacZ gene was removed and the VP1 gene was inserted under the control of phIE CMV and BGH polyA signal in the plasmid gILdgEVP1; the plasmid pGEM-TgE$_{SN}$ was digested with *Sma*I and *Nsi*I, then the isolated downstream recombinant fragment was ligated into the *Sma*I/*Nsi*I-cut plasmid gILdgEVP1, the resulting plasmid named gILdgEVP1gE$_{SN}$ was the transfer vector plasmid. Then the transfer vector plasmid was linearized with *Nsi*I for cotransfection.

Construction of the recombinant BHV-1 expressing VP1 gene of FMDV

Recombinant BHV-1 expressing FMDV VP1 gene was constructed by homologous recombination between genomic DNA of vector virus (BHV-1/gE$^-$/LacZ$^+$) and the transfer vector plasmid as described by Wang (2007). Monolayers of BT cells were cotransfected with the mixtures of purified BHV-1/gE$^-$/LacZ$^+$ DNA and 2.5 µg linearized transfer vector plasmid mediated by calcium phosphate. The recombinant viruses were acquired by selection for white plaques which failed to express the *E. coli* LacZ gene on the medium containing 150 µg X-gal/ml, and were named BHV-1/gE$^-$/VP1.

Identification of the recombinant virus BHV-1/gE$^-$/VP1 by PCR

The selected recombinants were further propagated in MDBK cells and the insertion of FMDV VP1 gene was identified by PCR. The VP1 specific primers (sense: 5′-ATTAGGATCCATGGGGGTTGACGCT CGCACGCAGAC-3′; antisense: 5′-CGCCAAGCT TATTACGCCACAATCTTTTGTTTGTGT-3′) were applied.

Production of anti-FMDV VP1 specific mouse serum

The complete FMDV (O/China/99) VP1 of gene was cloned into *Bam*HI and *Hin*dIII site of *E. coli* expression vector pET30a (Novagen), generating the pET30a-VP1 expression vector. Recombinant fusion protein was induced by the addition of IPTG to a culture of BL21 cells transformed with the pET30a-VP1 plasmid. The recombinant fusion protein was purified with Ni-NTA Purification System (Invitrogen). The BALB/c mice were immunized with recombinant VP1 protein, the sera from immunized mice were collected and used as anti-VP1 protein antibody to detect VP1 expression in the cells infected with BHV-1/gE$^-$/VP1.

Analysis of the expression of VP1 protein

The expression of VP1 protein in cells infected with BHV-1/gE$^-$/VP1 was determined by Western blotting and indirect immunofluorescence assay. Briefly, MDBK cells or BT cells infected with BHV-1/gE$^-$/LacZ$^+$ or BHV-1/gE$^-$/VP1, were either harvested for Western blotting or fixed and processed for immunofluorescence analysis (Qian et al. 2004). For Western blot analysis, cell lysates were separated by SDS-PAGE, and transferred to a nitrocellulose membrane. The anti-FMDV (O/China/99) VP1 specific mouse serum diluted 1:200 and horseradish peroxidase (HRP)-conjugated anti-mouse IgG diluted 1:5000 produced in sheep (Sigma) were used as the primary and the secondary antibody, the reaction was developed with 4-chloro-1-naphthol. For immunofluorescence analysis, BT cells were fixed by immersion in 70% (v/v) ethanol, mouse anti-FMDV (O/China/99) VP1 specific mouse serum diluted 1:50 and FITC-conjugated anti-mouse IgG diluted 1:100 produced in sheep (Sigma) were used as the primary and the secondary antibody.

Animal immunization

Eight New Zealand white rabbits were randomly divided into two groups, after collection of preimmune sera, the rabbits were inoculated subcutaneously with 1 ml 10^7 50% tissue culture infective dose (TCID$_{50}$) BHV-1/gE$^-$/VP1 virus and 1 ml 10^7 TCID$_{50}$ vector virus respectively. The second inoculation was given

with the same dose at 2 weeks post-inoculation and the blood samples were obtained once a week, and sera were collected to determine the presence of antibodies against IBRV and VP1 protein.

Detection IBRV antibody by virus neutralization (VN) test

Sera were inactivated and diluted two-fold serially in MEM supplemented with 3% (v/v) FCS, then mixed with the same volume of 100 TCID$_{50}$ IBRV and incubated at 37° for 4 h. Then 100 µl mixture was added to each well of 96-well plate with monolayers of MDBK cells, and incubated at 37° for 72–96 h. Virus neutralization titers were calculated as the highest dilution that there was no CPE on MDBK cells.

Detection VP1 antibody by ELISA

96-well ELISA plates were coated with 1 µg purified VP1 protein per well as antigen. Rabbit sera were diluted 1:100 and HRP-conjugated donkey anti-rabbit IgG diluted 1:5000 was used as the primary and the secondary antibody respectively. *o*-Phenylenediamine (OPD) was substrate, the absorbance at 490 nm was determined.

Results

Construction of transfer vector plasmid and recombinant BHV-1

The BHV-1/gE$^-$/VP1 was constructed by inserting FMDV VP1 gene under the control of phCMV in gE locus of the BHV-1 genome. To generate the BHV-1/gE$^-$/VP1, the transfer vector plasmid was linearized with *Nsi*I and cotransfected into BT cells with genomic DNA of BHV-1/gE$^-$/LacZ$^+$ for homologous recombination (Fig. 1a). Because the vector virus (BHV-1/gE$^-$/LacZ$^+$) contains a LacZ gene, β-D-galactosidase encoded by the LacZ gene, could be expressed in the infected MDBK cells. If β-D-galactosidase is produced from the vector virus, a blue plaque will be formed on the medium containing X-gal because of hydrolysis of the chromogenic substrate, X-gal. The virus from blue plaques was not the BHV-1/gE$^-$/VP1 but vector virus. So the white

virus plaques were selected and propagated in MDBK cells for further identification by PCR. There were specific amplifications from the MDBK cells infected with BHV-1/gE⁻/VP1. The PCR result showed that the VP1 gene was successfully inserted into the genome of BHV-1 (Fig. 1b).

Analysis of the expression of VP1 protein

As shown in Fig. 2a, there was a specific immuno-blotting reaction in MDBK cells infected with BHV-1/gE⁻/VP1. And there was no reaction in MDBK cells infected with BHV-1/gE⁻/LacZ⁺. Immunofluorescence was detected in the BT cells infected with BHV-1/gE⁻/VP1, but there was no immunofluorescence in the BT cells infected with BHV-1/gE⁻/LacZ⁺ (Fig. 2b). The result showed that the VP1 gene was expressed in the cells infected with BHV-1/gE⁻/VP1.

Serum neutralization antibody to IBRV

The rabbits were randomly divided into two groups and numbered. After collection of preimmune sera, the rabbits in the group one (number 1–4) were inoculated with 1 ml 10^7 TCID$_{50}$ of BHV-1/gE⁻/VP1, and the rabbits in the group two (number 5–8) were inoculated with 1 ml 10^7 TCID$_{50}$ BHV-1/gE⁻/LacZ⁺. Virus neutralization test was carried out to confirm the infection of BHV-1 in the rabbits. The result showed that no neutralization antibody to IBRV was induced before immunization, and virus neutralization antibodies were induced in all rabbits after the first inoculation. Significant increases in virus neutralization antibody titers were observed after boosting immunization in all rabbits except number 3. The details were shown in Table 1.

The antibody against VP1 protein

The serum samples collected from these rabbits were subjected to ELISA test to measure antibodies against VP1 protein. Anti-VP1 protein antibodies were induced in the rabbits inoculated with BHV-1/gE⁻/VP1. Although BHV-1/gE⁻/VP1 expressed VP1 at low levels, the rise of anti-VP1 protein antibody titers after the second inoculation with BHV-1/gE⁻/VP1 suggested that there was a booster effect after the second inoculation (Fig. 3).

Fig. 1 a Sketch map of construction the transfer vector ▶ plasmid and BHV-1/gE⁻/VP1. First the LacZ gene in the expression cassette (pGEM-TgILdgELacZ) was replaced by the VP1 gene, then the downstream recombinant sequence of BHV-1 DNA (gE$_{SN}$) was ligated to construct the transfer vector plasmid (pGEM-TgILdgEVP1gE$_{SN}$). The transfer vector plasmid containing the VP1 gene expression cassette under the control of phIE CMV, upstream and downstream recombinant sequences was cotransfected BT cells with genomic DNA of BHV-1/gE⁻/LacZ⁺. The recombinant virus BHV-1/gE⁻/VP1 was acquired by selection white plaques on the medium containing X-gal. **b** Identification of BHV-1/gE⁻/VP1 by PCR. Total DNA from virus infected MDBK cells was amplified by PCR with specific primers for FMDV VP1 gene. About 690 bp fragment was amplified in total DNAs from BHV-1/gE⁻/VP1 infected MDBK cells as expected, but no amplification in BHV-1/gE⁻/LacZ⁺ infected MDBK cells. *Lanes 1–4* MDBK cells infected with different virus plaques of BHV-1/gE⁻/VP1; *lane 5* Positive control, pUC57-VP1; *lane 6* MDBK cells infected with BHV-1/gE⁻/LacZ⁺; *M* DNA Marker DL2000 (Takara, Dalian, China)
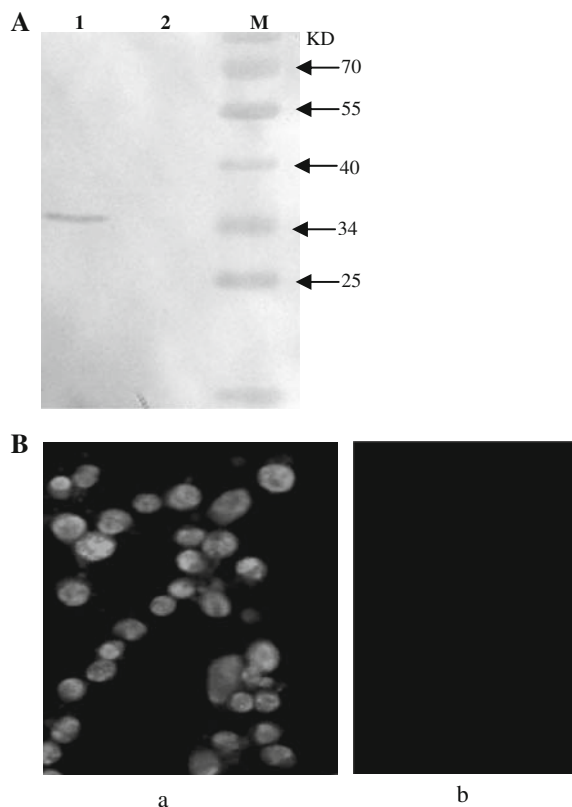
Discussion

Although inactivated virus vaccines effectively prevent FMD, their use is accompanied by dangerous residual potency problems. As a result, alternative approaches are being investigated, including subunit vaccines, synthetic peptides, DNA vaccines and recombinant viruses. Cellular immunity, as humoral immunity, plays an important role in protection against FMDV, especially against FMDV type O. Recombinant viruses can offer significant advantages over the traditional virus inactivated vaccines or recombinant proteins as they can induce cellular immunity and humoral immunity. DNA vaccines can also induce cellular immunity and humoral immunity, but, unlike DNA vaccines, recombinant viruses are more stable, versatile for manufacture, storage, and have no limitation that DNA vaccines remains low relative efficacy, requiring multiple boosts with high doses. Several virus vectors were used as FMDV antigen delivery, such as fowlpox virus, pseudorabies virus, adenovirus (Qian et al. 2004; Zheng et al. 2006; Du et al. 2007). Among the virus vectors, BHV-1 provides many unique advantages. The attenuated strains of BHV-1 are widely available and the basis of their attenuation is well known. Because cattle are routinely and safely immunized against BHV-1, the use of BHV-1 as a vector should not pose a new risk to cattle or humans and would obviate the need for an additional vaccination against BHV-1. Several

A

Recombinant virus BHV-1/gE⁻/VP1

B
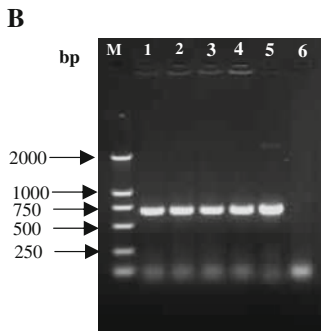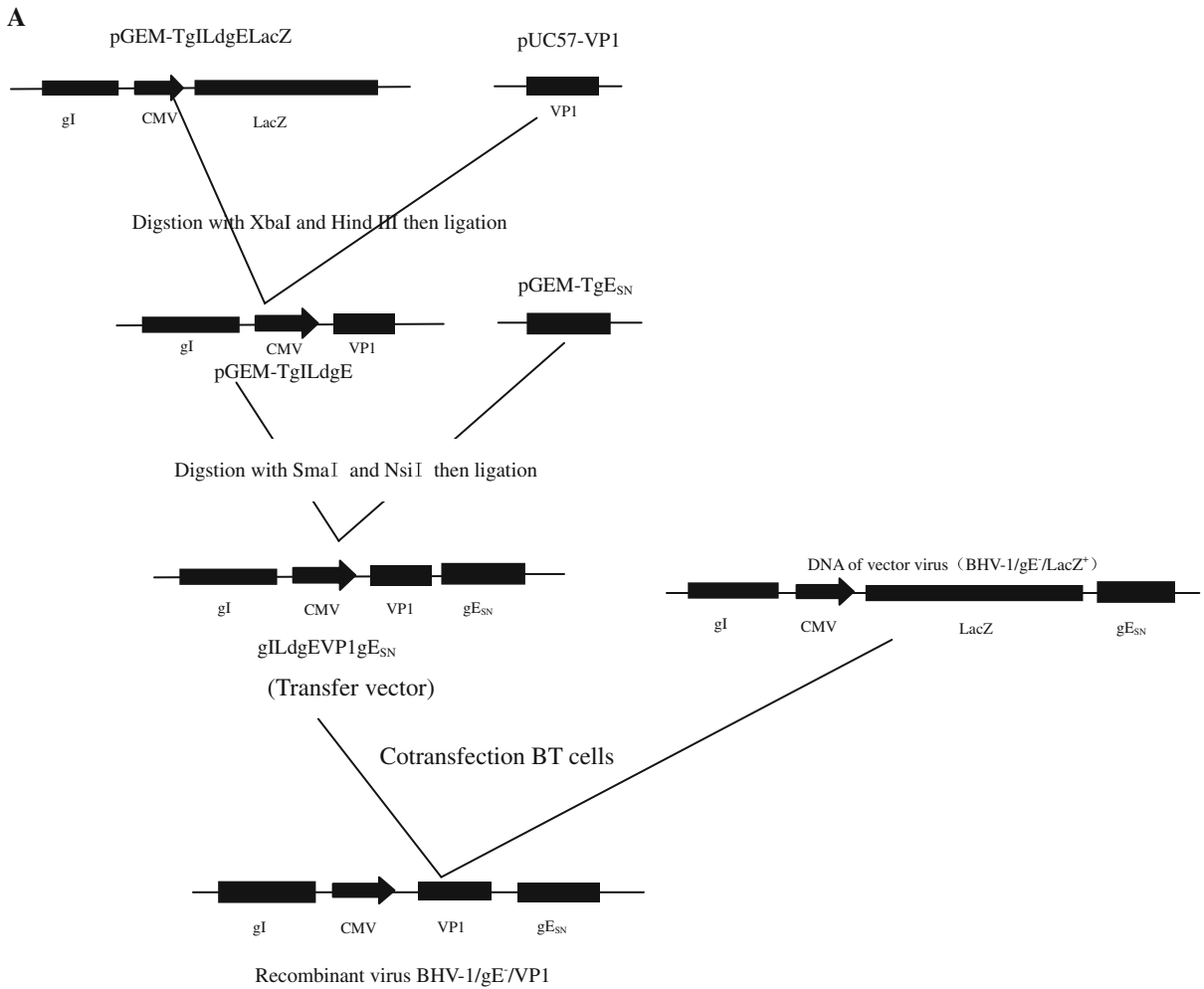
recombinant BHV-1 expressing immunogenic foreign proteins of important bovine pathogens have been reported as vaccines for BHV-1 and other infectious diseases (Schmitt et al. 1999; Wang et al. 2003).

Kit et al. (1991) constructed a recombinant BHV-1 expressing the FMDV VP1 epitopes as fusion protein with BHV-1 gC. Protective levels of anti-FMDV antibodies were induced in calves and protected the calves against virulent BHV-1 challenge after vaccination with the recombinant BHV-1. Qian et al. (2004) reported that a novel recombinant FMD vaccine was constructed by expressing the FMDV VP1 gene using an attenuated pseudorabies virus

A

B

Table 1 Virus neutralization (VN) to IBRV

Rabbit no.	VN to IBRV weeks postinoculation				
	0	1	2[a]	3	4
1	<2	8	8	32	16
2	<2	4	8	16	8
3	<2	4	16	8	4
4	<2	2	8	16	8
5	<2	4	8	16	8
6	<2	2	4	8	4
7	<2	4	8	32	16
8	<2	8	8	32	8

Eight New Zealand white rabbits were randomly divided into two groups and numbered, after collection of preimmune sera, the rabbits in the group one (number 1–4) was inoculated with 1 ml 10^7 $TCID_{50}$ of BHV-1/gE$^-$/VP1, and the rabbits in group two (number 5–8) was inoculated with 1 ml 10^7 $TCID_{50}$ BHV-1/gE$^-$/LacZ$^+$. Booster injection was given with the same dose at 2 weeks post-vaccination. And the blood samples were obtained once a week. Virus neutralization antibodies were induced in all rabbits inoculated with BHV-1/gE$^-$/VP1 (no. 1–4) and BHV-1/gE$^-$/LacZ$^+$ (no. 5–8). Significant increases in virus neutralization antibody titers were observed after boost immunization

[a] All rabbits were boosted at 2 weeks postinoculation with respective virus

Fig. 2 a Western blot analysis of cell lysates infected with BHV-1/gE$^-$/VP1 with anti-VP1 specific mouse serum. MDBK cells infected with BHV-1/gE$^-$/VP1 or BHV-1/gE$^-$/LacZ$^+$ were separated by SDS-PAGE, then transferred to a nitrocellulose membrane for Western blot analysis, there was an obvious immunoblotting reaction in MDBK cells infected with BHV-1/gE$^-$/VP1. And there was no reaction in MDBK cells infected with BHV-1 gE$^-$/LacZ$^+$. *Lane 1* MDBK cell lysates infected with BHV-1/gE$^-$/VP1; *lane 2* MDBK cell lysates infected with BHV-1 gE$^-$/LacZ$^+$; *M* Pre-stained protein marker (MBI). **b** Immunofluorescence analysis of BT cells infected with BHV-1/gE$^-$/VP1 with anti-VP1 specific mouse serum. BT cells infected with BHV-1/gE$^-$/VP1 or BHV-1/gE$^-$/LacZ$^+$ were fixed for immunofluorescence analysis, immunofluorescence was detected in the BT cells infected with BHV-1/gE$^-$/VP1. And there was no immunofluorescence in the BT cells infected with BHV-1/gE$^-$LacZ$^+$. *a* BT cells infected with BHV-1/gE$^-$/VP1 (100 fold); *b* BT cells infected with BHV-1/gE$^-$/LacZ$^+$ (100 fold)
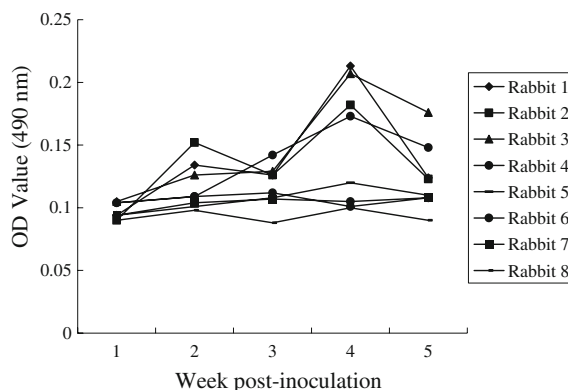
(PRV) vector (TK$^-$/gG$^-$/LacZ$^+$). The VP1 gene was inserted into the *Bam*HI site of PRVgG gene and expressed as a fusion protein. The recombinant PRV-VP1 virus could induce humoral immunity against PRV and FMDV but could not protect swine from challenge with 20 minimal infecting doses of FMDV. The gG promoter is not so strong to express high

Fig. 3 Detection of antibodies against VP1 protein by ELISA. The rabbits were randomly divided into two groups and inoculated with BHV-1/gE$^-$/VP1 and BHV-1/gE$^-$/LacZ$^+$ respectively. Booster injection was given with the same dose at 2 weeks after the first inoculation. The blood samples were obtained once a week. And the antibodies against VP1 protein were measured by ELISA. The antibodies against VP1 protein were induced in the rabbits inoculated with BHV-1/gE$^-$/VP1 (number 1–4). Although BHV-1/gE$^-$/VP1 expressed VP1 at low levels, the rise of anti-VP1 protein antibody titers after the second inoculation with BHV-1/gE$^-$/VP1 suggested that there was a booster effect after the second inoculation

levels of VP1 protein is one of reasons for this. Hooft van Iddekinge et al. (1996) used different promoters to control the transcription of the E1 gene of hog cholera virus and found that the phCMV was better than other promoters. In this study, FMDV VP1 was inserted in the BHV-1 genome under the control of phCMV in order to express high level of VP1 protein. To our knowledge, this is the first description of inserting the entire FMDV VP1 gene in the BHV-1 genome at the gE locus under the control of phCMV.

In order to investigate the immunogenicity of the BHV-1/gE⁻/VP1, the rabbits were chosen as experimental animal model, because BHV-1 can efficiently replicate in the rabbit although BHV-1 has a narrow host range and cannot efficiently infect small experimental animals (the costs were also considered of course) (Valera et al. 2008). The result showed that high level virus neutralization antibodies against IBRV were induced in all rabbits (Table 1). Although low level VP1 antibodies were induced in the rabbits inoculated with BHV-1/gE⁻/VP1, there was obvious increase after the second inoculation. This suggested that there was booster effect after the second inoculation (Fig. 3).

Live marked (gE-negative) attenuated BHV-1 vaccine is one of the most effective vaccines against IBR. Some European countries have initiated control programs aimed at IBR eradication based on the use of marker vaccines (van Oirschot et al. 1996). The immunogenicity of BHV-1/gE⁻/VP1 will be subsequently extended to the cattle, the actual target species, and the BHV-1/gE⁻/VP1 would be hopeful to be developed as a BHV-1 marker vaccine in China and also can elicit protective immune response against FMDV.

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