A DNA vaccine encoding the FMDV capsid precursor polypeptide P1 and the enhancing effect of bovine herpesvirus 1 VP22 protein as molecular adjuvant

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Summary. – DNA vaccines containing the capsid precursor polypeptide P1 gene of foot-and-mouth disease virus (FMDV) alone or combined with the VP22 gene of bovine herpesvirus 1 (BVP22) as molecular adjuvant were constructed and used for immunization of BALB/c mice. The latter were challenged with FMDV and their humoral as well as cell-mediated immune responses and virus clearance capacity were assayed. Both DNA vaccines elicited specific immune responses, however, the DNA vaccine with the BVP22 adjuvant showed stronger responses and more efficient virus clearance. A stronger Th1 response was indicated by the IgG2a/IgG1 ratio. These results indicate that (i) a DNA vaccine based on FMDV P1 can stimulate significant immune responses and virus clearance and (ii) BVP22 is a potentially useful molecular adjuvant for such a vaccine.

Keywords: DNA vaccine; foot-and-mouth disease virus; bovine herpesvirus 1

Introduction

Foot-and-mouth disease (FMD) is a highly contagious and economically devastating disease of swine, cattle, and other cloven-hoofed ruminants. Epidemics of FMD are a constant threat to domestic livestock throughout the world (Brown, 2003). The causative agent, FMDV, is an RNA virus and belongs to the genus *Aphthovirus* of the family *Picornaviridae*. The FMDV genome comprises 8.5 kb of single-stranded RNA encapsulated in an icosahedral capsid, which consists of 60 copies each of four structural proteins (VP1–VP4), which are the secondary cleavage products of P1 (McCullough *et al.*, 1992; Li *et al.,* 2001).

Vaccination with chemically inactivated viral vaccines has historically played a key role in the control and eradication of FMDV outbreaks. A major drawback of these vaccines is the potential for incomplete inactivation of the virus and the risks of handling live virus, such as the escape of the virus from production facilities leading to outbreaks of FMD (McCullough *et al.,* 1992; Li *et al.,* 2001). Therefore, a vaccine that is not derived from live FMDV material would be a potentially useful tool in the global control and eradication of FMD.

The precursor polypeptide P1 of FMDV is encoded by the P1 gene and is recognized by most FMDV neutralizing antibodies (Acharya *et al.,* 1989; Belsham, 1993; Mason *et al.,* 2003). Animals immunized with P1 expressed in yeast or with a recombinant adenovirus expressing P1 developed antibodies detectable by ELISA, virus neutralization antibodies, and were protected from FMDV challenge, but those responses were partial (Sanz-Parra *et al.,* 1999; Balamurugan *et al.,* 2003).

DNA immunization is a vaccination strategy, in which plasmid DNA encoding foreign antigens is administered directly to a host to induce specific humoral and cell-mediated immune responses (Liu *et al.,* 1998). DNA vaccines can be relatively safe and allow the long-term expression of encoded antigen in cells. They are able to elicit both cell-mediated and humoral immune responses and are being investigated widely to protect against a large number of infectious disease, including FMD. However, DNA-vaccinated animals are not

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Abbreviations: BHV-1 = bovine herpesvirus 1; BVP22 = VP22 gene of bovine herpesvirus 1; FMD = foot-and-mouth disease; FMDV = FMD virus; P1 = capsid precursor polypeptide; SI = stimulation index

completely protected from FMDV challenge, which suggests the need for improvement (Ward *et al.,* 1997; Chinsangaram *et al.,* 1998).

One major limitation of DNA vaccines is their inability to spread *in vivo*, opposite to some replicating viral vaccine vectors. Therefore, a strategy that facilitates the spread of antigen may significantly enhance the potency of naked DNA vaccines. Recently, studies have shown that alphaherpesvirus VP22 protein, one of the tegument proteins, possesses the property of intercellular transport. VP22 can ferry various genetically fused effector proteins to the bystander cells without the loss of the effector function (Elliott and O´Hare, 1997; Brewis *et al.,* 2000; Bennett *et al.,* 2002). It has been reported that herpes simplex virus 1 (HSV-1) VP22 protein and MDV-1VP22 protein were capable of enhancing the intercellular spread of the linked protein, which led to increased numbers of E7-specific $CD8^+$-T-cell precursors and to the enhancement of the antitumor effect in mice vaccinated with HSV-1 VP22/E7 DNA, compared to mice vaccinated with wild-type E7 DNA (Hung *et al.,* 2001). Recent studies have demonstrated that BVP22 is able to enhance the potential of DNA vaccines and have revealed that the genetic adjuvant properties of BVP22 are much stronger than those of other VP22 proteins (Oliveira *et al.,* 2001; Hung *et al.,* 2002). A DNA construct that encoded BVP22-linked antigen elicited enhanced immune responses in mice compared with a construct encoding antigen alone (Harms *et al.,* 2000).

In order to improve the biosafety and immunogenicity of conventional DNA vaccines against FMD, we investigated the effect of BVP22 as a molecular adjuvant on a DNA vaccine encoding the FMDV P1 polypeptide. The humoral and cellular immune responses and protective efficacy of DNA vaccines encoding FMDV P1 alone or fused with BVPP22 were evaluated in a mouse model.

Materials and Methods

Virus and cells. BHK-21 and HeLa cells were obtained from ATCC, maintained in DMEM (Invitrogen) supplemented with 10% (v/v) FCS, streptomycin 100 μg/ml and penicillin 100 μg/ml at 37°C in 5% CO_2. The FMDV O/ES/2001 strain was propagated and titered in BHK-21 cells and the supernatant of the infected cells was clarified and stored at -70°C.

Construction of DNA vaccines. To generate the expression plasmid pcDP1, a 2.2kb DNA fragment encoding the complete precursor polypeptide (P1) of FMDV strain O/ES/2001 was cut from the plasmid pMD-P1 (Yu *et al.,* 2005) by *Bgl*II and *Xba*I, and subcloned into the *Bam*HI and *Xba*I site of pcDNA3.1 (+) (Invitrogen), resulting in a DNA vaccine encoding FMDV P1. To construct the recombinant DNA vaccine pcDBP1 co-expressing FMDV P1 and the BVP22 gene, the 2.2 kb *Bgl*II–*Xba*I DNA fragment encoding FMDV P1 was subcloned into the *Bam*HI and *Xba*I site of the vector pcDBVP22, in which pcDNA3.1 (+) contains the BVP22 gene, resulting in pcDBP1. The accuracy of these constructs was confirmed by restriction analysis and sequence analysis. The constructed plasmids were prepared for transfection and immunization and subsequently purified using the Wizard Plus Maxipreps Purification System (Promega).

P1 gene expression from DNA vaccine in vitro. DNA transfection and gene expression were performed according to Yu *et al.* (2006). At 48 hrs after transfection, the plates were fixed with 3.7% formaldehyde in PBS (pH 7.4) and incubated with rabbit polyclonal anti-P1 antiserum. The plates were then incubated with goat anti-rabbit IgG-FITC and observed under a fluorescence microscope (Olympus).

Immunization of mice. Immunization of mice was performed with plasmid pcDP1, pcDBP1, and empty vector pcDNA3.1 (+), separately, according to Yu *et al.* (2006). Serum samples were collected from the retro-orbital plexus of the mice at various time points after immunization to detect P1-specific antibodies and neutralizing antibodies against FMDV. The mice were sacrificed and their splenocytes were harvested for the lymphocyte proliferation assay 4 weeks after the secondary immunization.

ELISA of P1-specific antibody. The P1-specific antibody responses were determined by ELISA (Yu *et al.,* 2006). The A_{630} was measured by an ELISA reader (Labsystems MK3).

Assay of neutralizing antibodies. Prior to testing, serum samples from the test animals were inactivated at 56°C for 30 min and two-fold serial dilutions were made. Serum (50 μl) was mixed with 50 μl FMDV O/ES/2001 strain (200 $TCID_{50}$) in a 96-well tissue culture plate and incubated for 1 hr at 37°C in 5% CO_2. Subsequently, 100 μl of BHK-21 cell suspension containing 1×10^6 cells/ml was added to each well and the plates were incubated for 4 days at 37°C in 5% CO_2. The cells were monitored for CPE and the neutralization titers were calculated as the $\log_2$ of the reciprocal value of the highest dilution that resulted in complete neutralization.

Lymphocyte proliferation assay. The spleens were removed from the immunized mice and the splenocytes were plated in 96-well flat-bottomed plates for MTS assay (Yu *et al.,* 2006). Each splenocytes sample was plated in triplicate. The erythrocyte cell suspension was lysed with 0.75% Tris-NH_4Cl (pH 7.4). Subsequently, the medium with or without the recombinant P1 (10 μg/ml) was added and mixed. After 72 hrs of incubation, 20 μl of MTS (Promega) was added to each well and the plates were incubated for a further 4 hrs. A_{492} was measured in an ELISA scanner. The stimulation index (SI) was calculated as the ratio of the average OD value of wells containing antigen-stimulated cells to the average OD of wells containing only cells with medium.

Virus detection. Virus detection in the sera of mice after FMDV challenge was performed as described by Shieh (Shieh *et al.,* 2001). Briefly, 4 weeks after the second immunization, the mice were injected intraperitoneally with virus suspension containing 1×10^6 $TCID_{50}$ of FMDV strain O/ES/2001. Blood was collected from the test mice at 24, 48, and 72 hrs post-challenge. Each blood sample (100 μl) was added to the BHK-21 monolayer culture and incubated with gentle rocking at 37°C for 1 hr. The cell monolayers were

washed twice with medium and incubated with fresh medium supplemented with 3% fetal calf serum for 3 days in the presence of 5% CO_2. The presence of the virus in the sera of test mice was determined by the manifestation of the cytopathic effect of FMDV in BHK-21 cell cultures. Virus clearance was defined as the absence of the virus in the sera of the test mice at 48 hrs post-challenge.

Statistical analysis. All data from the VN tests, lymphocyte proliferation assays and ELISA were analyzed using the *t*-test. Results were expressed as means ± SE. Differences with $P < 0.05$ were considered significant.

Results

Construction of vaccines and expression of the P1 gene in vitro

The P1 cDNA of the FMDV type O/ES/2001 strain was subcloned into pcDNA3.1 (+) and into pcDBVP22, which contained BVP22, to create two cDNA expression plasmids, namely pcDP1 and pcDBP1. In the case of pcDP1, the P1 gene was directly under the transcriptional control of the human cytomegalovrus (HCMV) immediate-early promoter/enhancer. In the second recombinant plasmid, pcDBP1, the P1 gene was downstream of the BVP22 gene, where BVP22 gene was directly under the transcriptional control of the HCMV immediate-early promoter/enhancer (Fig. 1). To test whether the two types of expression plasmid could express authentic P1 protein *in vitro*, the expression analysis was performed by indirect immunofluorescence. As shown in Fig. 2, obvious fluorescence staining was detected in cells transfected with pcDP1 or pcDBP1 (Fig. 2b,c), but not in the cells transfected with the empty vector pcDNA3.1 (+) (Fig. 2a). This indicates that the P1 protein can be correctly expressed in transfected cells *in vitro*. The protein expression from the plasmid pcDBP1 was higher than from the plasmid pcDNA3.1) (Fig. 2c).

Humoral immune responses to DNA vaccines

To determine the ability of different DNA vaccine constructs to induce P1-specific antibody responses, groups of five mice were immunized intramuscularly with 100 μg of each DNA construct. Serum samples were collected at various time points, and P1-specific IgG, IgG1, and IgG2a antibodies were analyzed by ELISA using a standard single

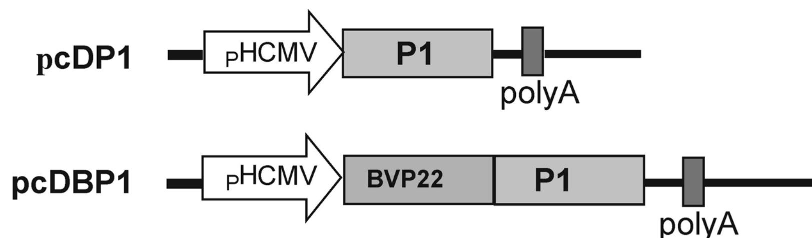

Fig. 1

Scheme of plasmid constructs used as the DNA vaccines

pHCMV = the HCMV promoter/enhancer.

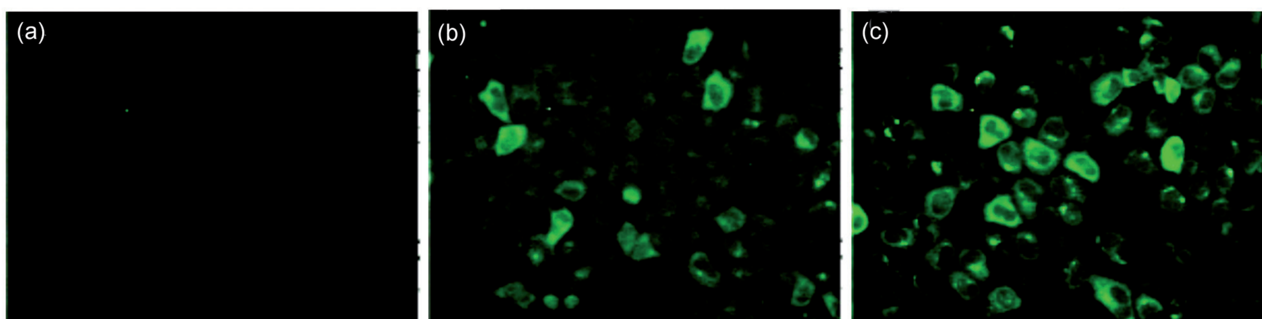

Fig. 2

P1 gene expression from the DNA vaccines *in vitro*

Indirect immunofluorescence assay. Cells transfected with pcDNA3.1(+)(a), pCDP1 (b), pCDBP1 (c).

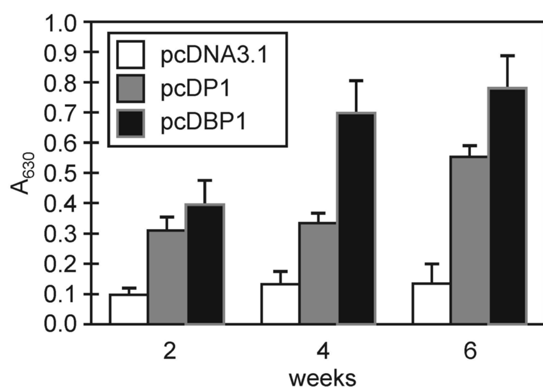

Fig. 3

Induction of P1-specific IgG antibodies by the DNA vaccines in mice

ELISA of P1-specific IgG antibodies in mouse serum 2, 4, and 6 weeks after immunization.

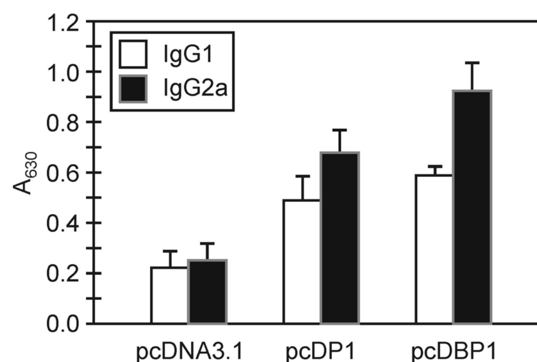

Fig. 4

Induction of P1-specific IgG1 and IgG2a antibodies by the DNA vaccines in mice

ELISA of P1-specific IgG1 and IgG2a antibodies in mouse serum 2, 4, and 6 weeks after immunization.

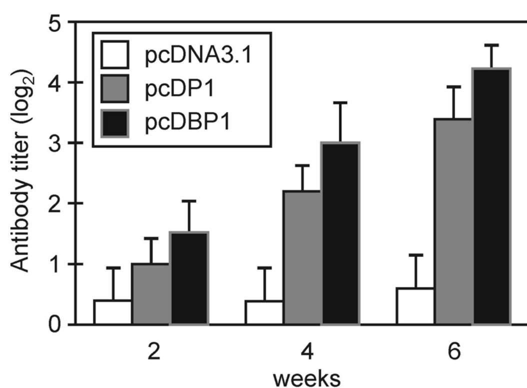

Fig. 5

Induction of virus neutralizing antibodies by the DNA vaccines in mice

Virus neutralizing antibodies assayed 2, 4, and 6 weeks after immunization.

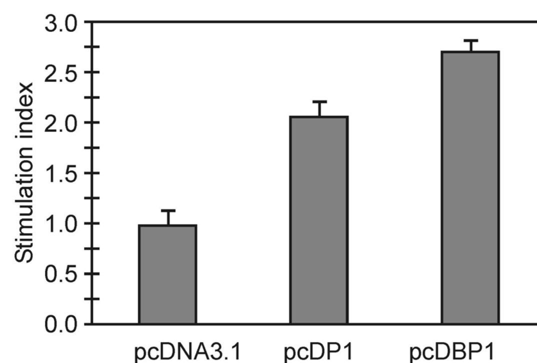

Fig. 6

Lymphocyte proliferative responses of mice to DNA vaccines

The responses were assayed 4 weeks after the second immunization.

dilution (1:40). As shown in Fig. 3, the groups immunized with pcDP1 or pcDBP1 produced P1-specific ELISA antibodies, and no significant difference ($P > 0.05$) was observed between these groups two weeks after the primary immunization. After a booster immunization, the increase in the level of anti-P1 IgG antibody in the sera of mice immunized with pcDBP1 was greater than that in mice immunized with pcDP1 ($P < 0.05$). The same results were obtained with respect to anti-P1 IgG1 and IgG2a at four weeks after the primary immunization (Fig. 4). Moreover, six weeks the after primary immunization, the level of P1-specific antibodies and the ratio of IgG2a/IgG1 were slightly higher in the group immunized with pcDBP1.

Serum samples were further evaluated for their ability to neutralize FMDV *in vitro* using a virus neutralization assay. As shown in Fig. 5, detectable levels of neutralizing antibodies were developed in the sera of mice immunized with both pcDP1 and pcDBP1 after a single immunization. After the booster, higher levels of neutralizing antibodies were observed in the sera of mice immunized with the plasmid pcDBP1, which is similar to the results of ELISA

Cell-mediated immune responses to DNA vaccines

The above results clearly showed that the DNA vaccine pcDBP1 induced higher humoral immune response than DNA vaccine pcDP1. Cell-mediated immune responses are considered important for the immune protection against FMDV (Sanz-Parra *et al.*, 1999). To investigate

Table 1. Virus clearance in mice immunized with DNA vaccines and challenged with FMDV

DNA vaccine	Virus clearance[a]
pcDNA3.1	0/5 (0%)
pcDP1	3/5 (60%)
pCDBP1	4/5 (80%)

[a]The ratio of the number of viremia-free mice to the total of challenged mice.

whether pcDBP1 could also enhance the cell-mediated immune response, mice were sacrificed four weeks after the secondary immunization, and their splenocytes were harvested for use in a lymphocyte proliferation assay. The lymphocyte proliferation assay was performed using recombinant P1 protein for stimulation and ConA as the positive control. As shown in Fig. 6, similar to the humoral immune responses, the P1-specific proliferative response was significantly higher in mice immunized with pcDBP1 than in mice immunized with pcDP1 ($P <0.05$). The mean SI of pcDBP1 and pcDP1 were 2.71 and 2.07, respectively. A low level of lymphocyte proliferative response was observed in the group immunized with empty vectors. These results indicated that pcDBP1 induced an enhanced Th1-type immune response.

Virus clearance in DNA-vaccinated and the FMDV-challenge mice

The above results, which showed enhanced induction of P1-specific antibodies, neutralizing antibodies and lymphocyte proliferative responses following immunization with the pcDBP1 DNA vaccine, encouraged us to investigate whether more efficient immune protection would be developed by immunization with pcDBP1. Mice were immunized twice as described above and challenge was carried out intraperitoneally with 1×10^6 $TCID_{50}$ of the O/ES/2001 strain of FMDV four weeks after the secondary immunization. The apparent vaccine efficacy induced by pcDP1 or pcDBP1 was demonstrated by the rapid clearance of the virus from the sera of test mice after the challenge with live FMDV (Table 1). The absence of the virus in their sera two days after the challenge was used as an indicator of viral clearance. Our results showed that 40% of the mice immunized with pcDP1 still had detectable virus levels in their sera after two days. On the other hand, the sera of mice immunized with pcDBP1 were 80% virus-free at this timepoint. All mice immunized with the empty vector, pcDNA3.1, exhibited virus in their sera after 2 days. These data indicated that the approach of using the DNA vaccine containing the fusion of BVP22 and antigen can effectively induce virus-neutralizing activities and efficient clearance of the virus.

Discussion

A previous study showed that DNA vaccines encoding the FMDV VP1 protein, which included the major antigenic site, did not induce either humoral or cell-mediated responses (Sanz-Parra *et al.*, 1999). However, we found that a DNA vaccine encoding the FMDV P1 protein could induce not only humoral responses but also cell-mediated responses (Yu *et al.,* 2006). At the same time, we demonstrated that vaccination with the DNA vaccine encoding P1 resulted in the clearance of FMDV from the sera of immunized mice challenged by FMDV, while the DNA vaccine encoding the FMDV VP1 protein did not protect animals against the challenge (Yu *et al.,* 2006; Shieh *et al.,* 2001). Although the FMDV VP1 protein carries the main continuous antigenic site recognized by host B-lymphocytes, which produce neutralizing antibodies, other structural proteins (VP2–VP3) have additional antigenic sites that are important in the immune response to FMDV (Mateu, 1995). Thus, the P1 gene of FMDV may be the appropriate candidate for design of DNA vaccines against FMDV.

In this study we compared the DNA vaccine pcDP1, encoding the FMDV P1 protein alone, and the DNA vaccine pcDBP1, encoding the BVP22-P1 fusion protein, because previous studies showed that the BVP22 could enhance the potency of naked DNA vaccines (Harms *et al.,* 2000; Oliveira *et al.,* 2001; Hung *et al.,* 2002). We compared the induction of humoral and cell-mediated immune responses and protective efficacy against virus challenge in a mouse model. The results revealed that pcDBP1 and pcDP1 were expressed in transfected HeLa cells. The cells transfected with pcDBP1 showed more intense fluorescence staining than the cells transfected with pcDP1. This result confirmed that BVP22 might be useful as a genetic adjuvant of a DNA vaccine against P1.

We observed that the treatment of mice with the DNA vaccine containing BVP22 (pcDBP1) increased the ratio of O/ES/2001 specific IgG2a/IgG1, which correlated with the level of neutralizing antibodies. Given that the neutralizing ability of an antibody is determined primarily by its affinity and the antigenic epitope to which it binds (Liang *et al.*, 1985; Mosmann and Coffman, 1989; Raz *et al.*, 1996), the positive relationship between a higher ratio of IgG2a/IgG1 and the neutralization activity perhaps indicates that most of the anti-O/ES/2001 IgG2a either binds to the neutralization epitope(s) or has higher affinity than anti-O/ES/2001 IgG1. The higher elevation of IgG2a/IgG1 by pcDBP1 DNA is consistent with the previous results, which showed an increased elevation of IgG2a/IgG1 induced by DNA priming and peptide boosting (Shieh *et al.,* 2001). The results of presented study imply that BVP22 enhances the level of anti-O/ES/2001 IgG2 directed against the neutralization epitope(s), but whether the increase of IgG2a/IgG1 is attributed to BVP22 remains to be elucidated.

Similarly, the lymphocyte proliferative response in DNA-vaccinated mice was significantly increased by pcDBP1, which also indicates that the immunization with pcDBP1 induced an enhanced cellular immune response. An explanation for the enhanced P1-specific response is that the intramuscular administration of pcDBP1 DNA can introduce DNA directly into professional APCs in the muscle, which allows APCs to present P1 directly through the MHC class I pathway (Raz *et al.*, 1996). Another important reason for this enhancement is that the fusion of BVP22 to P1 may facilitate the spread of antigen from cells expressing pcDBP1 DNA to surrounding APCs, thus increasing the number of APCs that present P1 through the MHC class I pathway.

Another potential explanation for the observed enhancement of the P1-specific T-cell immune response may be the so-called "cross-priming effect" that can occur when APCs process BVP22-P1 protein by phagocytosis of apoptotic bodies from other cells, which enhances cell-mediated immunity (Albert *et al.*, 1998, Akbari *et al.*, 1999). However, other studies have shown that direct priming of T-cells may be a more important mechanism in DNA immunization than the cross-priming mechanism (Albert *et al.*, 1998; Shieh *et al.,* 2001). Thus the extent of the contribution of the cross-priming mechanism to cell-mediated immune responses remains to be elucidated.

In summary, our results demonstrate that DNA plasmids encoding P1 effectively induce significant and specific immune responses in tested mice. Enhancing vaccine potency by the linkage of BVP22 to the antigen increased the immune responses and virus clearance from the sera of immunized mice. Based on our studies in mice reported in this paper, we expect that FMDV DNA vaccines will also provide protection in naturally susceptible animals such as swine and cattle.

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