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Construction of a Recombinant Fowlpox Virus Expressing the E0/E2 Proteins of Classical Swine Fever Virus and its Evaluation in Experimental Animals

Kang-Kang Guo, Wei-Ming Zhang, Yan-Ming Zhang, Peng-Bo Ning, Jing-Yu Wang and Miao-Tao Zhang

College of Veterinary Medicine, Northwest A and F University, Yangling, Shaanxi 712100, People Republic of China

Corresponding Author: Yan-ming Zhang, College of Veterinary Medicine, Northwest A and F University, Yangling, Shaanxi 712100, People Republic China Tel: +86 29 87092040 Fax: +86 29 87091032

ABSTRACT

Classical Swine Fever (CSF) is a hemorrhagic disease of pigs caused by virulent strains of Classical Swine Fever Virus (CSFV). Vaccination is a useful tool for preventing and controlling CSF. In this study the protective antigens E0 and E2 genes of CSFV were cloned and inserted into a fowlpox virus vector to construct a recombinant plasmid. We transfected this plasmid into chicken embryo cells infected with the fowlpox virus FV282 to package and propagate the recombinant fowlpox virus FV282-E0-E2. Specific anti-CSFV antibodies were detected with enzyme-linked immunosorbent assay in FV282-E0-E2 immunized experimental mice and pigs. Moreover, virus challenge experiments were conducted in experimental pigs immunized with FV282-E0-E2. Immune protection was evaluated in experimental pigs infected with virulent CSFV by observing clinical manifestations and post-mortem examination. The results showed that recombinant fowlpox virus FV282-E0-E2 was obtained after ten cycles of blue plaque selection. The special anti-CSFV antibodies were detected in the experimental mice and pigs. The immunized pigs could resist the attack of the virulent CSFV strain. The recombinant fowlpox virus FV282-E0-E2 represents a potential candidate for the development of a genetic engineered vaccine for the future prevention of CSF.

Key words: Classical swine fever virus, E0/E2 genes, fowlpox virus, live vector vaccine, subunit vaccine

INTRODUCTION

Classical Swine Fever (CSF) is a highly contagious, multi-system hemorrhagic viral infection of pigs which can exhibit acute, subacute, chronic and tardive clinical manifestations. Inapparent infection is the main form of CSF observed in recent years (Moennig *et al.*, 2003). This disease has the potential of severely affecting the international trade of pigs and pig products, causing serious economic losses to the pig industry and is furthermore a public health issue that continues to attract extensive attention worldwide (Edwards *et al.*, 2000). Consequently, CSF is classified by the World Organization for Animal Health (OIE) as a notifiable (previously List A) disease. Vaccine immunization is an effective strategy for preventing and controlling CSF in most developing countries; however, killing CSF diseased and viral infected pigs is also a useful tool to eradicate CSF which has been implemented in the European Union since the early 1990s (Vandeputte and Chappuis, 1999).

Live attenuated vaccines (for example, the Chinese lapinized vaccine, C-strain) have been proven efficient and safe and have been successfully used around the world to control CSF (Van Oirschot, 2003a, b). However, the humoral immune response induced by traditional live attenuated vaccine does not differ from that elicited by infections caused by wild-type viruses; hence, the use of these vaccines has been hampered by their inability of inducing a response differentiable between infected and vaccinated animals (Dong and Chen, 2007). To overcome this drawback, some subunit vaccines have been studied and developed (De Smit *et al.*, 2001a; Dong and Chen, 2006; Lin *et al.*, 2009; Barrera *et al.*, 2010; Toledo *et al.*, 2010). Subunit vaccines are a suitable alternative for the control of classical swine fever. However, such vaccines have as the main drawback the relatively long period of time required to induce a protective response which hampers their use under outbreak conditions.

CSFV is a member of the *Pestivirus* genus within the Flaviviridae family of viruses and has a single-stranded, positive-sense RNA genome that encodes a polyprotein precursor containing 3898 amino acids. This polyprotein precursor can be processed into four mature structural proteins (C, Erns, E1 and E2) and eight mature non-structural proteins (Npro, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B), along with viral protease and host cellular proteases (Meyers *et al.*, 1999; Moormann *et al.*, 2000; Greiser-Wilke *et al.*, 2006). The membrane glycoprotein E2 is a critical immune protein of CSFV and is considered one of the preferred target proteins in vaccine research for the development of a novel engineered or marker vaccine (Dong and Chen, 2007; Dortmans *et al.*, 2008; Lin *et al.*, 2009; Barrera *et al.*, 2010; Toledo *et al.*, 2010). To overcome the issues of immune response delay, repeat vaccination is required to achieve effective immune protection in pigs vaccinated with E2 engineered vaccine (De Smit *et al.*, 2001b). Selection of a better vaccine vector that can stimulate a high immune response or enhance the immune response in vaccinated animals is therefore considered an important factor for novel engineered vaccine development.

Previous research has shown that the anti-CSFV E2 neutralizing antibody was induced in vaccinated pigs which subsequently developed resistance to the lethal virulence of CSFV challenge (Konig *et al.*, 1995; Bouma *et al.*, 1999; Dewulf *et al.*, 2000; De Smit *et al.*, 2001a). The E2 protein was also associated with CSFV adhesion and entry into the host cells during infection, CSFV virulence, induction of protective immune responses and production of neutralizing antibodies (Risatti *et al.*, 2005). The E0 protein can also induce effective neutralizing antibodies and offers resistance against the lethal virulence of CSFV challenge in vaccinated pigs (Hulst *et al.*, 1998). Furthermore, E0 protein possesses RNase activity related to the persistent infection of host cells, can prevent or postpone apoptosis of infected host cells and may also play a significant role in viral replication (Tang *et al.*, 2008).

Increased serological sensitivity was observed when experimental pigs were vaccinated with the chimeric antigen of E0+E2 protein, with anti-CSFV antibody production occurring seven days earlier than in pigs only vaccinated with E2 protein (Lin *et al.*, 2005). This method is therefore a feasible strategy for developing a novel engineered vaccine co-expressing E0/E2 genes with an appropriate vector. Use of a poxvirus vector maybe favorable for live vector vaccine development, such as the fowlpox virus which is a non-replicating vector that has been confirmed not to complete its replication cycle in non-poultry host cells. The fowlpox virus vaccine is also safe and highly efficient for non-poultry animals (Vazquez Blomquist *et al.*, 2002).

In this study (carried out from March 2006 to June 2008), the E0 gene and E2 gene of CSFV were inserted into fowlpox virus FV282 to construct a recombinant fowlpox virus capable of

co-expressing the E0/E2 proteins. We subsequently conducted animal immunization protective experiments in pigs to evaluate the recombinant fowlpox virus co-expressing E2/E0 proteins as a potential candidate vaccine.

MATERIALS AND METHODS

Vectors, virus and bacteria: pMD18-T-E0 and pMD18-T-E2 recombinant plasmids were constructed to contain the full-length of the CSFV virulent Shimen strain, encoding the E0 and E2 genes, respectively. FPV-P11 fowlpox virus expression plasmid, FPV-pSY recombinant fowlpox virus plasmid, pSC11 plasmid with a LacZ reporter gene under P11 promoter and FV282 fowlpox attenuated vaccine strains were available within our laboratory. The competent *E. coli* DH5 α used for cloning were purchased from Tiangen Biotech (China).

Reagents: BamHI, EcoRI, PstI, XbaI, NotI, SmaI, Klenow Fragment enzyme and Polymerase Chain Reaction (PCR) reagents were purchased from Takara (China). Fetal Calf Serum (FCS) was purchased from Hyclone (USA). Lipofectamine™ 2000, Trizol reagent and Dulbecco's Modified Eagle's Medium (DMEM) were obtained from Invitrogen (USA). Wizard Purification Plasmid was purchased from Promega (USA). A CSFV antibody indirect Enzyme-Linked Immunosorbent Assay (ELISA) test kit was obtained from Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. Rabbit anti-CSFV antibody and goat anti-rabbit antibody labeled with fluorescein isothiocyanate were purchased from Beijing Dingguo Changsheng Biotechnology (China).

Target genes cloning: The E0 gene was amplified from the pMD18-T-E0 by PCR with the E0-upstream primer (5'-AAA GAATTCGGAAAATATAACTCAATGGAA-3'; underlined region indicates EcoRI site) and E0-downstream primer (5'-CCCGGATCCGGCATAAG CGCCAAACC AGGT-3'; underlined region indicates BamHI site). The predicted PCR product size was 681 bp. The E2 gene was amplified from the pMD18-T-E2 by PCR with the E2-upstream primer (5'-CTA GGATCC ATGCGACTGGCTTGTAAGGAAGA-3'; underlined region indicates BamHI site) and E2-downstream primer (5'-ATTATCCCGGGACCAGCGCGAGTTGTT-3'; underlined region indicates SmaI site), with a predicted length of 1119 bp. PCR amplification of the E0 and E2 genes was conducted according to the following procedures: denaturation at 95°C for 5 min, followed by 40 cycles at 94°C for 60 sec, 55°C for 60 sec, 72°C for 40 sec and finally, 72°C for 10 min.

Construction of recombinant fowlpox virus plasmid with CSFV E0/E2 genes: The recombinant fowlpox virus containing CSFV E0 and E2 genes was constructed according to the procedure in Fig. 1 and 2. Briefly, the complete E0 and E2 gene PCR amplicons were identified by agarose gel (8 g L⁻¹) electrophoresis and purified using an agarose gel DNA purification kit (Takara). The purified E0 gene was digested with EcoRI/BamHI, inserted into the FPV-P11 vector and digested with the same enzymes (EcoRI/BamHI) to construct the recombinant plasmid FPV-P11-E0. The positive plasmid was verified by restriction enzyme digestion (EcoRI/BamHI) and sequencing. Similarly, the purified E2 gene was digested with BamHI/SmaI and inserted into the FPV-P11 vector which was also digested with the BamHI/SmaI, to construct the FPV-P11-E2 recombinant plasmid. The positive plasmid was verified by enzyme digestion (BamHI/SmaI) and sequencing. The LP2EP2-E2 fragment was obtained from FPV-P11-E2 using NotI digestion and the repair of DNA ends with the Klenow Fragment enzyme. FPV-P11-E0 was digested with SmaI

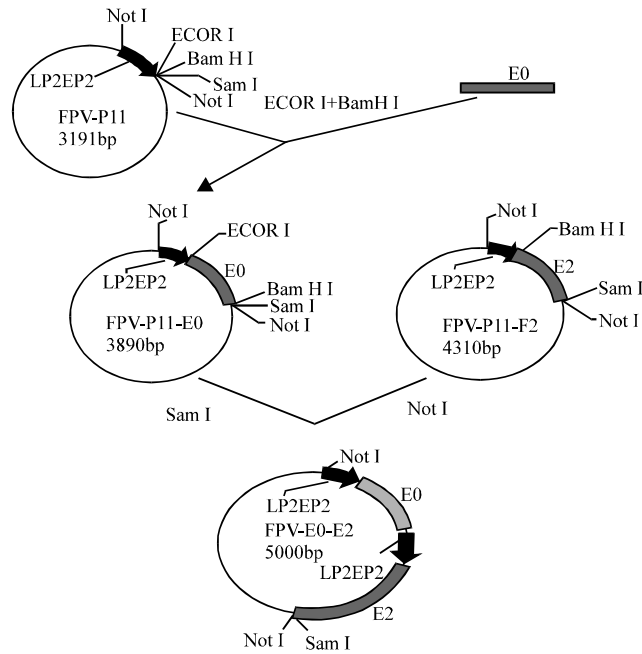


Fig. 1: Construction of the recombinant CSFV E0-E2 genes fowlpox virus vector

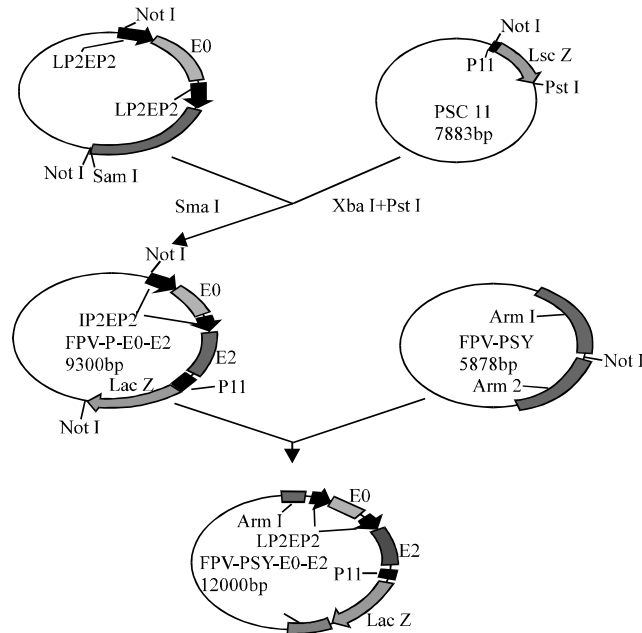


Fig. 2: Construction of the recombinant CSFV E0-E2 genes fowlpox virus migration vector

and 5' dephosphorylated with alkaline phosphatase, before being linked with the LP2EP2-E2 fragment to construct the FPV-E0-E2 recombinant plasmid. The positive plasmid was verified by enzyme digestion (NotI) and sequencing.

P11-Lac Z was obtained from the pSC11 vector using XbaI/PstI digestion, 3' end repair with Klenow fragment enzyme and 5' end-filling with T4 DNA ligase, before being inserted into

FPV-E0-E2 (digested with SmaI) to construct the FPV-P-E0-E2 recombinant plasmid. The positive recombinant plasmid was identified by restriction enzyme digestion and sequencing. The LP2EP2-E0-LP2EP2-E2-P11-LacZ fragment was obtained from FPV-P-E0-E2 digested with NotI and inserted into the recombinant fowlpox virus plasmid FPV-pSY which was digested with the same enzyme (NotI), to construct FPV-pSY-E0-E2 recombinant plasmid. The positive recombinant plasmid was identified and verified by restriction enzyme digestion, PCR and sequencing.

Preparation and purification of recombinant fowlpox virus

Preparation of chicken embryo fibroblasts: Tissues of 9-10 day old, Specific Pathogen Free (SPF) chicken embryos were cut into 2 mm small pieces, being washed with Hank's buffer, transferred into a 50 mL centrifuge tube and treated with trypsin (2.5 g L^{-1}) at 37°C for 20 min. Samples were further centrifuged at low speed to remove the supernatant. The tissue precipitates were washed with Hank's buffer for 2-3 times and centrifuged before the addition of Dulbecco's Modified Eagle's Medium (DMEM). Chicken Embryo Fibroblast (CEF) were obtained by filtering the tissue precipitates with 8 layers of sterile gauze and adjusting the cell concentration to $10^6/\text{mL}$. CEFs were seeded into 15 mm^2 dishes to obtain single-layer cells at 37°C with 5% CO_2 .

Preparation of FV282 fowlpox virus: Confluent CEFs (80%) were washed three times with serum-free DMEM and inoculated with fowlpox virus FV282 (0.25 mL per cm^2) 37°C for 2 h. The cells were washed twice with serum-free DMEM after the surviving virus was removed and then cultured with 5% FCS DMEM at 37°C with 5% CO_2 for 3-4 days. Whole cells and medium were collected when a 100% cytopathic effect (CPE) was observed and then frozen and thawed three times to obtain the virus which was stored at -70°C .

Virus inoculation and transfection of CEFs: The recombinant plasmid FPV-pSY-E0-E2 was purified and the concentration was determined at 260 and 280 nm ultraviolet wavelengths using a spectrophotometer. The purified recombinant plasmid was used for experiments when the optical density (OD) value at 260 nm/280 nm was >1.7 .

CEF were seeded into 6-well plates (Costar, USA) with 5 mL 5% FCS DMEM and cultured at 37°C with 5% CO_2 . The medium was removed and the CEFs were washed three times with serum-free DMEM when they had reached 80% confluence. FV282 virus (1 mL) was inoculated into the CEFs at 37°C for 2 h. Surviving virus was removed and the cells were washed three times with serum-free DMEM. Purified FPV-pSY-E0-E2 plasmid ($10 \mu\text{L}$) was diluted with serum and antibiotic-free DMEM to a final volume of $50 \mu\text{L}$, then mixed gently at room temperature for 5 min. Lipofectamine 2000 ($8 \mu\text{L}$) was also diluted with serum and antibiotic-free DMEM to a final volume of $50 \mu\text{L}$. Equal volumes of diluted FPV-pSY-E0-E2 and lipofectamine 2000 were mixed gently at room temperature for 20 min. The FV282 virus infected CEFs were transfected with the whole mixture (liposome/DNA) drop by drop, shaken gently and inoculated at 37°C with 5% CO_2 for 4-6 h. Transfected CEFs were cultured with 5% DMEM (antibiotic-free) to 80% confluence at 37°C with 5% CO_2 . Recombinant fowlpox virus was harvested by freezing and thawing the whole medium and cells three times.

Identification and purification of recombinant fowlpox virus: The obtained recombinant fowlpox virus was inoculated into 80% confluent CEFs at 37°C for 2 h, before 5% FCS DMEM containing low melting point agarose (10 g L^{-1}) was added to the surface of cells after surviving

recombinant fowlpox virus was removed. The inoculated cells were cultured at 37°C with 5% CO₂ for 72-96 h until the CPE appeared. DMEM agarose with X-gal (250 µg mL⁻¹) was then overlaid onto the cells which further cultured for 12-24 h. Blue plaque cells were selected into 1 mL serum-free DMEM and the recombinant fowlpox virus was extracted by freezing and thawing. The recombinant fowlpox virus was purified by blue plaque selection for 10 rounds as previously described. The obtained recombinant fowlpox virus was identified by PCR and named FV282-E0-E2.

Immunization of mice: Twelve 6-8-week-old BALB/c mice were randomly divided into three groups. Group 1 (FV282-E0-E2 group) and group 2 (FV282 group) mice were immunized with 1×10⁸ plaque forming units (pfu)/100 µL of FV282-E0-E2 or FV282 virus by intraperitoneal injection and boosted twice at 7-day intervals. Group 3 (negative control group) mice were immunized with sterile Phosphate-Buffered Saline (PBS) using the same dose and procedures. Serum samples were collected from the caudal vein of immunized mice every week following the second immunization, for up to five times.

The anti-CSFV antibody titer was detected using ELISA according to kit procedures. Briefly, 50 µL of diluted sera (1:20) were added to the wells of the ELISA plate and incubated at 37°C for 45 min. Each serum sample was tested four times. Wells were washed with washing buffer for four times after the serum was removed, then treated with 50 µL horseradish peroxidase-conjugated goat anti-mouse antibody (1:500 dilution) at 37°C for 45 min. Substrate (50 µL) was added into each well after washing and maintained at 37°C for 15 min. Samples were then treated with 50 µL of Stop Buffer to terminate reactions. Finally, the Optical Density (OD) at 450 nm (OD₄₅₀) of each well was determined.

Positive and negative serum standards were tested according to the same procedures as described above. An absorbance reading (OD₄₅₀) of positive/negative serum of >2.1 was considered a positive test result. Analysis of the R-value for the test results was calculated using: $R = (ODS - ODN) / (ODP - ODN)$ (ODS was the average OD₄₅₀ value of serum sample, ODN was the average OD₄₅₀ value of standard negative serum, ODP was the OD₄₅₀ value of standard positive serum). R < 0.200 was considered negative, R > 0.300 was considered positive and R = 0.200-0.300 was indeterminate.

Immunization and virus challenge of pigs: Twenty 40-day-old CSFV-free and anti-CSFV antibody negative healthy pigs were assigned to three groups. Group 1 pigs (n = 8) were immunized with approximately 1.4×10⁹ pfu/2 mL of recombinant virus FV282-E0-E2, group 2 pigs (n = 6) were immunized with approximately 1.4×10⁹ pfu/2 mL of FV282 virus and group 3 pigs (n = 6) were treated with 2 mL of PBS, by subcutaneous injection into the neck and groin. Booster immunization was carried out 7 and 14 days after the first immunization. Serum samples were collected from the precaval vein of all experimental pigs at the 14, 21, 28, 35 and 42 days after the first immunization. Anti-CSFV antibody titers were detected by ELISA as described above.

All experimental pigs were challenged intramuscularly with 1 mL of blood-stock of the highly virulent CSFV strain. Daily rectal temperatures and clinical signs of disease were recorded. Pigs with obvious clinical signs of CSF, such as fever, apastia, astasia, diarrhea and flushing of the skin, were euthanized and subjected to general pathological examinations. All remaining pigs were euthanized to examine the general pathological changes 14 days after challenge.

Statistical analysis: The statistical analysis performed was achieved by using statistical software SPSS version 13.0. Antibody titers (R value) of the studied groups of mice or pigs were expressed as Means±Standard deviations. The differences in antibody titers were evaluated using Student's t-test. p-values less than 0.05 were considered significant.

RESULTS

Construction and identification of recombinant plasmid: CSFV E0 and E2 genes were successfully amplified by PCR and sequencing revealed that the length of these genes was 681bp and 1119 bp (excluding sequences of enzyme sites), respectively. The E0 gene fragment was amplified from the FPV- pSY-E0-E2 recombinant plasmid with E0 primers (Fig. 3) and the E2 gene fragment was amplified from the FPV-pSY-E0-E2 recombinant plasmid with E2 primers (Fig. 4). E0 and E2 genes were successfully inserted into the vector and the sequencing results of the recombinant plasmid indicated that the insertion sites, reading frame and encoding sequences were correct.

Purification and identification of recombinant fowlpox virus: A recombinant fowlpox virus containing CSFV E0/E2 genes was selected and purified from transfected CEFs via ten cycles of blue plaque selection (Fig. 5) and named FV282-E0-E2. The recombinant virus was identified by PCR with E0 primers to verify the E0 gene and E2 primers to verify the E2 gene which indicated that the E0/E2 genes were contained within the virus. The sequences and open reading frames of E0/E2 were also correct according to the sequencing results.

Detection of anti-CSFV antibody in mice immunized with FV282-E0-E2: Anti-CSFV antibodies were screened for by ELISA for immunized mice at 14, 21, 28, 35 and 42 days after the first immunization. The R values (represents the antibody titer) of group 1 mice were greater than 0.2000, meaning positive for the anti-CSFV antibody on days 21, 28, 35 and 42. The R values of group 2 and 3 were less than 0.2000, meaning negative for the anti-CSFV antibody in entire experiment period. The anti-CSFV antibody of group 1 were significantly different from the group 2 and 3 ($p < 0.05$ at days 21 and 28; $p < 0.01$ at days 35 and 42). These results indicated that the

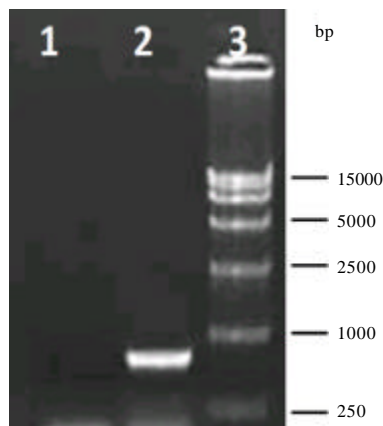


Fig. 3: Identification of CSFV E0 gene form FV282-E0-E2. M: DNA Marker; 1: Amplification of E0 gene in FV282; 2: Amplification of E0 gene in FV282-E0-E2

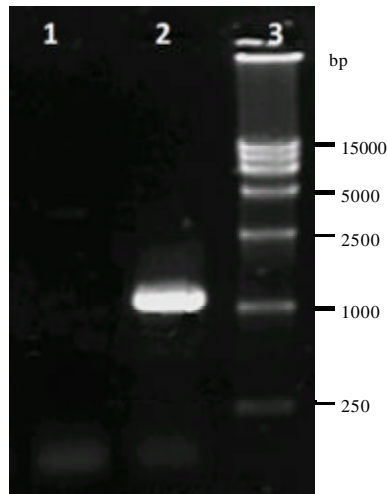


Fig. 4: Identification of CSFV E2 gene form FV282-E0-E2. M: DNA Marker; 1: Amplification of E2 gene in FV282; 2; Amplification of E2 gene in FV282-E0-E2

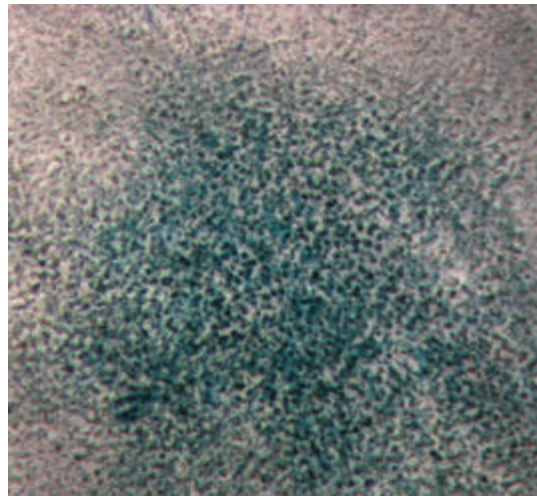


Fig. 5: Blue plaques formed by FV282-E0-E2 in infected CEF cells(x100)

Table 1: Detection of anti-CSFV serum antibodies post vaccination in mice

Groups	Antibody titer (R value)				
	14 days	21 days	28 days	35 days	42 days
FV282-E0-E2	0.1650±0.0026	0.3289±0.0038 ^A	0.3960±0.0011 ^A	0.5670±0.0030 ^B	0.6685±0.0018 ^B
FV282	0.1280±0.0013	0.1375±0.0027	0.1350±0.0034	0.1570±0.0021	0.1620±0.0022
Control	0.1245±0.0014	0.1305±0.0012	0.1568±0.0020	0.1768±0.0012	0.1587±0.0019

A: The significant difference at a 5% level ($p < 0.05$); B: The significant difference at a 1% level ($p < 0.01$)

recombinant fowlpox virus could induce the specific antibody in mice and could further be used for pig immunization (Table 1).

Table 2: Detection of anti-CSFV serum antibodies post vaccination in pigs

Groups	Antibody titer (R value)				
	14 days	21 days	28 days	35 days	42 days
FV282-E0-E2	0.2980±0.0017	0.4150±0.0017 ^A	0.5283±0.0023 ^A	0.6685±0.0019 ^A	0.6906±0.0012 ^A
FV282	0.1438±0.0020	0.1320±0.0022	0.1728±0.0019	0.1655±0.0014	0.1539±0.0018
Control	0.1325±0.0011	0.1425±0.0009	0.1539±0.0011	0.1462±0.0018	0.1521±0.0011

A: The significant difference at a 1% level (p<0.01)

Antibody screening and virus challenge of immunized pigs: The anti-CSFV antibody titers (R value) of group 1 were greater than 0.2000 on days 21, 28, 35 and 42 after the first vaccination. No anti-CSFV antibodies were detected in group 2 and 3 (Table 2). This experiment revealed that the recombinant fowlpox virus FV282-E0-E2 could excite a specific antibody response in experimental pigs.

Clinical manifestations of pigs with virus challenge were as follows:

- Group 1:** (FV282-E0-E2 group) pigs: 4 out of 8 immunized pigs showed clinical signs, such as pyrexia (rectal temperature 40.5-42°C), anorexia and diarrhea on day 5 after virus challenge. Clinical signs were not observed in 2 out of the 4 pigs on day 8, however the remaining 2 pigs exhibited pyrexia (rectal temperature 40.5-42°C); depression, anorexia, respiratory distress, tremors, diarrhea, skin petechiae and ecchymosis of the ears, abdomen, perineum and the inner side of the hmb skin, paralysis of hind legs and nervous symptoms in the later stages of the challenge and finally death on day 10. No clinical signs were observed on the other 4 immunized pigs
- Group 2:** (FV282 group) pigs: experimental pigs (n = 6) showed pyrexia (rectal temperature 40.5-42°C) and diarrhea on day 3 after challenge with virulent CSFV. All of the pigs exhibited anorexia and depression on day 4, while some of the pigs also showed vomiting. On day 6, signs of pyrexia, high-level depression, tremors, mucopurulent secretion in eyes, skin petechiae and ecchymosis of the ears, abdomen, perineum and the inner side of the hmb skin were observed. Three of the six pigs succumbed to disease on day 9 post-challenge, one succumbed to disease on the day 12 post-challenge and the remaining two pigs were euthanized
- Group 3:** (negative control) pigs: experimental pigs (n = 6) presented with similar clinical signs as those in group 2 pigs. All of the six pigs succumbed to disease between days 8 and 12 post-challenge

Post-mortem examination of experimental pigs with virus challenge: Post-mortem examination of deceased pigs revealed pathological lesions typical of virulent CSFV infection, including tonsil necrosis, marginal spleen infarcts, ileocecal valve ulcers, lymph hemorrhages and edemas, urinary bladder hemorrhages and petechiae. Post-mortem examination on six surviving pigs of group 1 showed that the 2/6 pigs did not present obvious lesions beside the hemorrhages in mandibular and mesenteric lymph nodes, two pigs showed hemorrhages on the mandibular, groin and mesenteric lymph nodes and bladder mucosa, while the last two pigs did not show any tissue lesions. The two surviving pigs of group 2 presented with obvious hemorrhages on the mandibular, mesenteric and groin lymph nodes, kidney, spleen, bladder and heart. Pathological changes were shown in Fig. 6a-e.



Fig. 6(a-e): Pathological changes in the tissues of experimentally infected pigs. (a) Pathological changes on spleen of experimental pigs. Marginal spleen infarction in a diseased pig (left) and normal spleen of an immunized surviving pig (right), (b) Hemorrhagia on the bladder mucosal surface in diseased pigs, (c) Hemorrhagia of lymph nodes (left: normal lymph nodes), (d) Hemorrhagia in the mucous membranes of the intestine and (e) Hemorrhagia of the endocardium

DISCUSSION

As well known, vaccination is an efficient strategy to prevent and control CSF, the live attenuated vaccine can provide complete protection for immunized animals. But the limitation of live attenuated vaccine influenced the use in field, especially the antibodies against live attenuated vaccines do not allow differentiating infected from vaccinated animals and the mechanism responsible for attenuation is not known. People studied and developed some novel vaccines, such as, DNA vaccine (Moura *et al.*, 2007; Rawat *et al.*, 2007), immunogenic peptides (Pakzad *et al.*, 2010), viral vectors expressing protein. The E0 and E2 genes of CSFV have also been commonly targeted for novel genetically engineered vaccine development. A new vaccine formulation based on E2 envelope viral glycoprotein produced in the milk of goats (E2his) has been shown to induce a highly protective response in pigs against CSFV infection, the results demonstrated by far the feasibility of using the E2his-based vaccine for preventing and controlling CSF

(Barrera *et al.*, 2010). Another vaccine candidate based on the E2-CSFV antigen co-formulated with recombinant human alpha interferon was produced in goat milk and could confer complete protection by the end of the first week after vaccination (Toledo *et al.*, 2010). E2 gene of CSFV based vaccine development also were carried out by other researchers, all of these study results indicated that it was a feasible strategy of using the E2 gene of CSFV to develop genetically engineered vaccines for preventing and control CSF (Bouma *et al.*, 1999; Dewulf *et al.*, 2000; De Smit *et al.*, 2001b; Solhjo *et al.*, 2007; Dortmans *et al.*, 2008). Glycoprotein E0 also is an important protein of CSFV, it is another target protein for developing subunit vaccine. The vaccinated experimental pigs seroconverted for E0-specific antibody at 2 weeks after vaccination, indicating the possibility of developing vaccine based on E0 protein of CSFV (Moormann *et al.*, 2000). We constructed the recombinant fowlpox virus containing and co-expressing the E0/E2 proteins of CSFV to find the possibility of developing a dual-gene subunit vaccine candidate. Our research data showed that E0 and E2 proteins were expressed in experimental mice and pigs and successfully provoked the immune cells to produce the specific antibodies. Pigs immunized with FV282-E0-E2 plasmid elicited an effective response against CSF post-vaccination. No severe CSF clinical signs were observed in immunized pigs challenged with a highly pathogenic CSFV strain. Our present results were also proven the feasibility of using the E2/E0-based vaccine for preventing and controlling CSF. Furthermore, the results of our study highlight that the recombinant fowlpox virus FV282-E0-E2 represents a potential candidate for the development of a genetic engineered vaccine for preventing and controlling CSF.

CONCLUSION

Present study has shown that a recombinant fowlpox virus containing the E0 and E2 genes of classical swine fever virus successfully invokes the development of a specific antibody response in experimental mice and pigs. Experimental pigs immunized with the recombinant fowlpox virus could partly resist the virulent attack of CSFV. This recombinant vector has the potential to be developed into a novel CSF vaccine in the future.

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