

Construction of New Genetically Engineered Vaccine Strain for O-type Foot-and-Mouth Disease of Pig

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ABSTRACT

Foot-and-mouth disease is an acute and highly contagious viral infectious disease. Although the foot and mouth disease vaccine has been applied in some parts of the world since the beginning of the 20th century, the current epidemic of foot and mouth disease in the world is still serious and constitutes an obstacle to the trade of animal and animal products in the world. The porcine pseudorabies virus gene deletion strain PRV TK- / gE- / LacZ +, which is constructed by our laboratory, has the advantages of good safety, large capacity and high recombination efficiency. In this study, the artificial O-type foot-and-mouth disease P1 gene as an antigen gene, UbiP1 fused with ubiquitin (Ub) as another antigen gene to enhance cellular immunity, and then shRNA designed for FMD 3B gene and porcine IFN- γ with antiviral and immune-regulatory effects. The two functional genes were constructed in turn to construct a transfer vector with four functional genes. The transfer vector was transfected into TK- / gE- / LacZ+ cells with PRV deletion vector, and the plasmids were purified and identified. The recombinant pseudorabies virus, which contains the six functional genes of P1 gene, UbiP1, shRNA and IFN- γ , was obtained, which laid the foundation for further construction of new genetically engineered vaccine.

KEYWORDS: antigen gene; ubiquitin gene; interferon gene; recombinant virus

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1. Introduction

1.1. The basic characteristics of foot and mouth disease

1.1.1 Basic concept of foot and mouth disease virus

Foot-and-mouth disease (FMD) is an acute and severe infectious disease caused by foot and mouth disease virus (FMDV) infection. The disease is widely distributed in the world. The outbreak often creates huge economic losses. The development of new genetically engineered vaccines has attracted much attention because of the potential danger of inactivation of inactivated inoculum and virulence.

1.1.2 Biological characteristics of foot and mouth disease virus

Foot and mouth disease virus is spherical, positive icosahedral structure, diameter 20-25nm; no capsule, sensitive to acid and alkali, foot and mouth disease virus particles from the capsid and viral nucleic acid composition, the shell by 60 asymmetric subunits, each sub-unit contains one molecule of VP1, VP2, VP3 and VP4, where VP4 is located inside the virus particles. FMDV belongs to the small RNA virus family, and there are seven serotypes, namely, O, A, C, SAT 1, SAT 2, SAT 3 and Asia type (Rodriguez and Grubman, 2009), with no cross-reactivity between serotypes (Lu Chengping, 2005), the virus of this feature, to its quarantine, epidemic prevention has brought great difficulties.

The FMDV genome is an infectious single stranded RNA with a size of about 8.5 kb. There is only one open reading frame (ORF), each with a noncoding region (NCR). The open reading frame is first translated as a polyprotein, cleaved by protease into mature structures and nonstructural proteins as well as some intermediates. FMDV genome P1 region encoding structural protein, P12A precursor in the formation of 3C protease by VP4-VP2, VP3 and VP1 composed of the original body. 5 protoplasts form a pentamer, twelve pentamers assembled into RNA containing pre-viral particles or lack of RNA empty capsid. In the infected cells, FMDV 3C protease gene encoding protein can catalyze the processing

of P12A precursor capsid protein VP0, VP1, VP3, these three proteins can interact to form 5S original body, a virus particle structure of the components, the formation of virus-like particles, such virus-free particles without viral nucleic acids, have the same immunogenicity as the whole virus.

1.1.3 Foot and mouth disease virus hazards

FMDV is mainly infected with cloven-hoofed species such as pigs, cattle, sheep, goats and buffalo. Many wild cloven-like species may also be susceptible to infection, in which camels are proven to be susceptible to foot and mouth disease (Larska et al., 2009). Due to infection of foot and mouth disease and sick animal mouth, tongue, lips, hoof, breast and other parts of the blisters, ulceration and the formation of rotten spots. Foot and mouth disease incidence of almost 100%, the disease once the outbreak, can quickly evolve into biological disasters, directly by the major and even devastating blow to the animal husbandry and its related industries. In order to control the spread of the disease, the general action is to directly destroy a large number of animals, such a measure not only cannot fundamentally solve the epidemic often lead to serious public health problems, causing social panic. Foot and mouth disease outbreak of the destructive power will also spread to other industries, such as foreign exports were forced to close, the epidemic area was tightly blocked, tourism blocked, such as last April in South Korea outbreak of FMD was the national spread of the state, so that the Korean epidemic prevention authorities into a state of emergency. According to the degree of harm of foot and mouth disease, the World Organization for Animal Health (FMO) classified foot and mouth disease as a class A animal infectious disease, and the Ministry of Agriculture classified foot and mouth disease as a class of animal diseases and classified foot and mouth disease virus as a class of animal pathogenic microorganisms.

1.2. The purpose and significance of this experimental study

Pseudorabies and foot and mouth disease is a serious harm to the global pig industry, two major infectious diseases, respectively, caused by pseudorabies virus and foot and mouth disease virus. PRV mainly caused pig reproductive disorders, with the large-scale development of China's pig industry, pig pseudorabies epidemic spread trend, and to the pig industry brought huge losses. Foot and mouth disease is a common animal and animal disease, acute infectious diseases, because of its highly infectious, and the traditional inactivated vaccine in the production process inactivation does not completely cause the virus to escape the factory and the risk of foot and mouth disease, foot and mouth disease genetic engineering vaccine research is highly valued.

In this study, the porcine IFN- γ gene, the shRNA located in the FMDV 3B region and the two FMDV antigen genes P1-UbP1 were inserted into the transfer vector PIECMV, and then the pseudorabies deletion strain PRV TK- / gE- / LacZ+ was co-transfected, and the U gene of the O-type foot-and-mouth disease antigen gene and the Ubi-P1 gene fused with the ubiquitin gene were obtained by the method of plaque screening, and the porcine IFN- [gamma] gene was expressed and the O- 3B segment of the RNAi effect, to together to achieve the ultimate antiviral effect.

2. Experimental materials

2.1. Strains, strains, vectors and plasmids

Pseudorabies attenuated vaccine strain PRVTK- / gE- / LacZ+ is constructed by the State Key Laboratory of Agricultural Microbiology. The mutant strain lacked 205 bp of the PRVEa strain TK gene and inserted into the LacZ expression cassette in the gE coding region, resulting in a deletion of the gE inactivated gene.

The engineering bacteria DH5a and XL-gold were used in this laboratory.

Vector PIECMV by the experimental Ma Rui master graduate students to build.

PUC57pansUbiP12A by the Shanghai Chun Chun company synthesis.

PCAsIFN γ siF by the laboratory Hao Genxi graduate students to build.

PCApansUbiP12AsiF by the laboratory Hao Genxi graduate students to build.

Porcine kidney passage cells PK-15 and African green monkey kidney cells Vero were purchased from China Collections.

2.2. Major drugs and reagents

Newborn bovine serum (NCS) was purchased from Hangzhou Sijiqing Material Co., Ltd., before use inactivated by 56 °C 30min.

The DMEM powder for cell culture is GIBCO products.

Ampicillin (Amp), fetal bovine serum are Invitrogen products.

Various restriction enzymes, ligase, Taq enzyme, DNA Marker are Dalian Bao biological company products.

DNA Recycling Kit is TaKaRa products.

The plasmid is small and a large number of preparation kits are OMEGA products.

Liposome Transfection Kit LIPOFECTIN2000 and serum-free medium OPTI-MEM are products of Invitrogen.

2.3. Main medium and preparation

LB liquid medium: tryptone 10.0g, yeast extract 5.0g, NaCl10.0g, dissolved in ddH₂O, completely dissolved with 5mol / LNaOH adjusted to pH 7.0-7.2, constant volume to 1000.0mL, 15 pounds high pressure steam sterilization under 20min, room temperature preservation.

LB solid culture medium: agar powder was added to the LB liquid medium which had not yet been sterilized to a final concentration of 1.5% and autoclaved for 20 min. The temperature of the medium to be reduced to about 50 °C, add the appropriate antibiotics, paving plate. After the medium was solidified, it was kept at 4°C for storage.

DMEM basal culture medium (for conventional cell culture): 13.5g of DMEM powder (Gibic, USA) was dissolved in 950 mL of DMH, 3.7 g of NaHCO₃ was added, adjusted to pH to 6.8-7.0 with 1 mol /L HCl, ML, 0.22µm filter sterilization, after storage at room temperature to save spare.

DMEM cell growth solution: 10% of the inactivated newborn bovine serum, 100 µg / mL penicillin, 100 µg / mL streptomycin and 4 °C were added to the DMEM basal medium.

DMEM cell maintenance solution: 1% -3% of newborn bovine serum, 100 µg / mL penicillin, 100 µg / mL streptomycin, and 4 °C were added to the DMEM medium.

Cell digestion: NaCl 8.0g, KCl 0.2g, Na₂HPO₄ 1.15g, KH₂PO₄ 0.2g, trypsin 2.5g, followed by dissolved in 900.0mLddH₂O, to be completely dissolved, fixed to 1000.0mL, 0.22µm filter bacteria, sub-installed after -20 °C save reserve.

2.4. Buffer and its preparation

TAE (50 x): 242.0 g Tris base, 57.1 mL glacial acetic acid, 100.0 mL 0.5 mol / LEDTA (pH 8.0), plus ddH₂O volume to 1000.0 ml.

PBS Buffer: NaCl 8g, KCl 0.2g, Na₂HPO₄ 1.42g, KH₂PO₄ 0.27g dissolved in 800.0mLddH₂O, to be completely dissolved, add concentrated HCl to adjust the pH to 7.4, constant volume to 1000.0mL, high temperature autoclave room temperature save.

10% SDS: Weigh 10g of high purity SDS in 80mLddH₂O, dissolve at 68 °C, add concentrated HCl to adjust the pH to 7.2, set to 100.0mL, at room temperature.

0.5 M EDTA: Weigh 186.1g Na₂EDTA. 2H₂O dissolved in 800.0mLddH₂O, stir well, add NaOH to adjust the pH to 8.0, constant volume to 1000.0mL, high temperature autoclave after storage at room temperature.

2.5. Other solutions

TEN: 100.0 mol / L NaOH, 10.0 mol / LTris-Cl, 1.0 mol / LEDTA (pH 8.0)

LCM: 30 mol / L (DMSO) 6.0 mmol / L [beta]-MoracPtoethnael, 0.5% NP-40, 125.0 mol / L KCl

Plaque Screening PCR Sample Cell Lysis Solution: 0.5% SDS, 100.0 mmol / L NaCl, 10.0 mmol / L Tris-CL, 1.0 mmol / LEDTA, 0.25 mg / mL Protease K.

2.6. Oligonucleotide primers

The oligonucleotide primers used in this experiment PCR are shown in Table 1.

Table 1. Oligonucleotide primers used in this study

Number	Sequence	Uses
<i>R primer</i>	5- AGCTTTGCGTGACTTTGTGTTTTTC- 3'	Identification of positive recombinants
<i>F primer</i>	5- GCAGGGGGCTGTTTCATATACTGAT- 3'	

3 Experimental methods

3.1 Construction strategy of transfer vector PIECMVUbiP1P1SiFIFN γ

The construction process of the recombinant transfer vector PIECMVUbiP1P1SiFIFN γ is shown in Fig. 4

Figure 4: Transfer vector PIECMVUbiP1P1SiFIFN γ Construction process

Fig. 4: Building process of PIECMVUbiP1P1SiFIFN γ

3.1.1 Restriction endonuclease digestion

When the digested product was used for detection only, 1 μ L of plasmid DNA was added and the corresponding restriction enzyme reaction buffer was added. After the addition, the restriction enzyme was added and the mixture was homogenized and placed in a 37 °C water bath for 3 hours. If the enzyme is used for the recovery of the product, according to the actual situation can increase the amount of plasmid to expand the digestion reaction system.

3.1.2 Digestion of the product by agarose gel electrophoresis

Preparation of 0.8% agarose gel (gel concentration should be adjusted according to the size of the target band, if the large fragment is low concentration of agarose gel): 20mLddH₂O into the 400 μ L (50 \times) TAE, then add 0.16g agarose, heated to completely dissolved, slightly cooled and added to the amount of EB. Gel into the glue tank, if the bubble with a comb away, plug the comb, waiting for the gel cooling solidification. Pull out the comb and place the gel in an electrophoresis tank with 1 x TAE electrophoresis buffer to ensure that the buffer is drowned. Then 1.5-3 μ L of the enzyme digest was mixed with a trace 6 \times loading buffer and added to the sample wells while taking about 2 μ L of DNA marker as a control. Electrophoresis at 100-120V. When the bromophenol blue electrophoresis to gel 2/3, you can end the electrophoresis. Remove the gel in the gel imaging system to take pictures and save the photos.

3.1.3 Recovery of the digested product

The digested product was digested by agarose gel electrophoresis. After identification, the target bands were cut under long-wave UV lamp. The DNA fragments were recovered by agarose gel DNA recovery kit (see the kit for specific recovery methods). The recycling process is as follows:

(1) The cut the target DNA band into the 1.5mL centrifuge tube. (2) Add 600 μ L of LCI, 50 °C water bath for 10min, turn the centrifuge tube to fully dissolve. (3) The previous step of the solution added to the adsorption column, room temperature. (4) Add 600 μ L PW solution, wait 12000r / min after centrifugation 60min, discard the waste; (5) Add again 600 μ L PW liquid, 12000r / min centrifugal 60sec. (6) 12000r / min centrifugal 2min, open the lid for 5min. (7) discard the collection tube, the adsorption column into the centrifuge tube, hanging drop 30-50 μ L E_B eluent (or ddH₂O), 37 °C incubator for 5min. (8) 12000r / min centrifugation 2min collection of DNA solution, centrifuge tube marker preservation.

3.1.4 Dephosphorylation of the recovered product

In the case of molecular cloning, we need to dephosphorylate the carrier in order to prevent the self-ligating of the carrier prepared by the single enzyme digestion during the ligation. The specific steps: recovery of 34 μ L ddH₂O in 50 μ L system by adding 2 μ L CIAP, 4 μ L Buffer placed in 37 °C reaction 30min after adding 1 μ L CIAP placed at 50 °C reaction 15min, with the kit for liquid recovery dissolved 20 μ L ddH₂O, gel test.

3.1.5 Enzyme-linked reaction of the target gene with the vector

The recovered DNA fragment was ligated with the vector by T4 DNA ligase, and the enzyme system was prepared according to the instructions of T4 DNA ligase provided by Dalian Bao Bioengineering Co., Ltd., as shown in

Table 2.

Table 2. Target gene and vector enzyme system

Reaction system	Volume (μL)
Carrier	1
Target gene	4
T4 DNA Ligase(2~5U / μL)	1
10×T4 DNA Ligase Buffer	2
ddH ₂ O	12
Total	20

Placed at 16 °C or 4 °C overnight.

2.7. Preparation of competent cells, plasmid transformation, plasmid preparation and purification

3.2.1 Preparation of Escherichia coli competent cells (calcium chloride method)

A single colony was picked from the Escherichia coli DH5 α plate and seeded in 2 mL of LB liquid medium at 37 °C for 200 r / min shaking under shaking overnight at 1: 100 and transferred to a 50 ml L₂ saline solution. 3-4h, the bacteria were transferred to a sterile pre-cooled 50 ml polypropylene centrifuge tube under sterile conditions, placed on ice for 30 min, centrifuged at 4 h 4000 r / min for 10 min. Discard the supernatant, the centrifuge tube inverted 1min so that the residual culture flow. Add 10ml ice pre-cooling of 0.1mol/ LCaCl₂ re-suspended precipitation, ice bath for 30min, 4 °C 4000r/min centrifugal 10min. Discard the supernatant and add 2 mL of 0.1 mol/L CaCl₂ pre-cooled with ice to re-suspend. Suspended competent cells can be used immediately for transformation. Such as preservation, you need to add sterile glycerol to the final concentration of 15%, sub-installed 0.1ml/ tube, -80 °C refrigerator to save spare.

3.2.2 Transformation of plasmids

The incubation of the vector with cDNA or DNA gene fragment was mixed with 100 μL of competent cells, the ice bath was 30min; the bath was heated at 42 °C for 90sec; ice bath for 2min; if the plasmid was transformed, 30 ~ 50 μL antibiotic LB plate; if the conversion of the connection, then add 0.4mL 37 °C preheated LB medium, 37 °C shaking culture 45min; 5000r / min centrifugal 3min, discarded 450 μL supernatant, the remaining part of the direct coating Cloth containing the corresponding antibiotic LB plate, 37 °C culture 12 ~ 16h, the emergence of transformation of colonies.

3.2.3 Identification of positive plasmids

The transformed colonies were picked and seeded in 4 mL of LB liquid medium containing the corresponding resistance. Cultured overnight at 37 °C. Plasmid DNA was prepared in small amounts. The recombinant plasmid was identified by digestion with suitable restriction endonucleases, agarose gel electrophoresis, EB staining and UV lamp observation. In addition, positive plasmids can be further identified by sequencing.

Construction of recombinant virus expressing the FMDV antigen gene P1, ubiquitin protein and antigen P1 fusion gene Ubi- P1, RNA interference gene shRNA and interferon gene IFN- γ with PRV as vector

3.3.1 Cell culture, cryopreservation and resuscitation

(1) Cell culture: sterile stage will have grown into a single layer of cell culture flask tilted culture medium, washed with PBS 1-2 times, add 1mL trypsin 37 °C digestion for several minutes, discarded trypsin , add fresh growth liquid blowing scattered, according to 1: 3 or 1: 4 pass.

(2) Cell cryopreservation: sterile stage will grow into a single layer of cells with trypsin digestion, discard the trypsin, with a small amount of cell cryopreservation (100mL bottle frozen 1-2 tube) Cells, about 1.5 mL per tube in the cell cryopreservation tube. Before going to 4 °C for 40min-1h, -80 °C overnight into the liquid nitrogen tank can be stored for several years (or -80 °C can save 1-3 months). (10% of serum for 1 case of serum + 1 part of DMSO) and 70% DMEM + 20% serum + 10% DMSO)

(3) Cell resuscitation: the water bath pre-adjusted to 37 °C, the growth fluid added to the cell bottle and 37 °C preheating, and then from the liquid nitrogen tank (or -80 °C refrigerator) to remove a tube of frozen cells, the cells were quickly melted, and the cell fluid was transferred into the cell bottle in a sterile platform. The incubation was carried out in a 5% CO₂ incubator at 37 °C and the fresh growth fluid (preheated) was continued for 6 hours.)

3.3.2 PRV TK- / gE- / LacZ + Genomic DNA extraction

The PRV attenuated strain TK- / gE- / LacZ + was inoculated into PK-15 cells grown in monolayer, cultured at 37 °C to 80% of cells. The cells were stripped from the wall of the flask by blowing with a straw, 4°C 8000 r/min centrifugation for 15 min to collect cells. The cell pellet was re-suspended in cell lysate LCM to cell lysis, ice bath for 5-10min, after adding trichlorotrifluoroethane extraction, shake 5min, 4 °C 3000r / min centrifugal 10min, absorb the supernatant in another clean Centrifuge tube, then add trichlorotrifluoroethane extraction time, shake 5min, 4 °C 3000r / min centrifugal 10min, take the supernatant, 4 °C 8000r / min centrifugal 10min, take the supernatant, add 5% -45% (10% of the volume of supernatant added), equilibrated on an electronic balance, centrifuged at 26,000 r / min for 2 h, carefully discarded supernatant, the pellet was resuspended in TEN buffer, 10% SDS to the final concentration of 0.5%, room temperature for 30min, add the same amount of saturated phenol, gently shake about 10min, 10000r / min centrifugal 10min, the supernatant in another centrifuge tube, with the same volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) twice, take the supernatant by adding 2 times the volume of absolute ethanol, mixed with -20 °C 3h or overnight, at 4 °C 10000r / min centrifugal 20min, discard the supernatant, 70% Ethanol washed precipitate, dried at room temperature, dissolved in an appropriate amount of RNaseTE, set 37 °C 1h, and finally stored at -20 °C standby.

3.3.3 Liposome-mediated co-transfection

Using liposome-mediated method according to their kit instructions. The cells were cultured in 24-well cell culture plates. The cells were cultured for 4 hours before transfection, and the cells were grown to 40% to 60%. At the time of transfection, two 1.5 mL sterile Eppendorf tubes were administered, 1 to 2 µg of transfer plasmid, 1 to 2 µg of genome and 50 µL of serum-free medium OPTI-MEM, tube B was added to 3 µL of LIPOFECTIN Reagent and 50 µL of serum-free medium OPTI-MEM was incubated at room temperature for 5 min. Subsequently, the reagent in tube B was added to tube A and allowed to run at room temperature for 20 min. During the 20 min course of the mixture, the medium in the 24-well culture plate was discarded and the cells were washed once with serum-free DMEM. The mixture was added to the cell monolayer, incubated in a 5% CO2 incubator at 37 °C for 6 h, and then the liquid on the cell culture was discarded and the culture medium was added to the cell culture.

The co-transfected product with cytopathic effect was repeatedly freeze-thawed three times for 10-fold dilution. The virus solution of 10-4-10-6 dilution was taken 400 µL of the PK-15 cells and incubated at 37 °C for 1 h. The culture broth was washed and washed three times with PBS (pH 7.4). Each well covered 2.5 mL of low melting agarose and incubated at 37 °C in CO2 incubator until blank (About 48h), the extraction of a single plaque in 200 µL maintenance solution, -20 °C repeated freeze-thaw 2 to 3 times, inoculated in PK-15 cells have grown into a single layer of 24-well cell culture plate to be After the cell disease, the IFN-γ gene was amplified by PCR, and the positive recombinants were subjected to the next plaque assay until 100% purified recombinant virus was obtained.

3.3.4 Identification with PCR template treatment

The template process is as follows:

(1) 24-well plate has been infected cells repeatedly freeze-thawed 2 times, each hole to absorb 200µL supernatant virus solution. (2) 10 µL of 10% SDS, 10 µL of 0.5 molLEDTA, 1.3 µL of protease k at 50 °C for 1 h or at 37 °C for 6 h or more (overnight). (3) add the same volume (220µL) phenol: chloroform: isoamyl alcohol extraction time, 12000r / min centrifugal 10min, take supernatant plus 1/10 volume 3MNaAc (PH5.2) and 2 times the volume of anhydrous ethanol, -20 °C precipitation more than 30min. (4) 12000r / min centrifugal 10min, discard the supernatant, precipitate with 500µL 70% ethanol wash once, 12000r / min centrifugal 10min, discard the supernatant, drying, each tube by adding 20µL ddH2O dissolved.

3.3.5 Recombinant virus PCR identification

The PCR products were amplified by PCR with 0.8% agarose gel. After the electrophoresis, the gel samples were observed and prepared by gel imaging system. Screening of positive recombinants. The PCR amplification system conditions for FM-γ gene of foot-and-mouth disease virus are shown in Table 5 and Table 6.

Table 3. reaction system of identification of recombinant virus by PCR

Reaction system	Volume (µL)	Remarks
DNA template	10	10 × PCR buffer Ingredients
10×PCR buffer	5	Tris-HCl(PH8.3)100mmol/ L ,KCl500 mmol/L
dNTP Mixture	2	MgCl215 mmol/L
F primer	2.5	DNTP Mixture Ingredients: 2.5 mmol / L, in the presence of pH 7.2-9.0 Na
R primer	2.5	salt aqueous solution.

RTaq enzyme	0.5	F primer and R primer by Shanghai Bioengineering Company:
ddH ₂ O	27.5	Nmol were: 4.1 and 4.5, respectively.
Total	50	RTaq enzyme: 5U / μ L.

Table 4. Recombinant virus PCR identification reaction conditions

PCR process	Temperature (°C)	Time	Cycle number
Pre-degeneration	94	3min	
Transsexual	94	1min	} A total of 30 cycles
Annealing	55	45s	
Extend	72	45s	
Extended again	72	10min	
End	4	forever	

After the reaction, the PCR product was subjected to 0.8% agarose gel electrophoresis. After gel electrophoresis, gel pictures were observed and prepared by gel imaging system.

3. Results and Analysis

3.1. Construction of transfer plasmid PIECMVUbiP1

The UbiP12A gene was digested by restriction endonuclease BamHI, Sall and ScaI, and the UbiP12A gene was ligated into PIECMV by restriction endonuclease BglII and XhoI. The obtained transfer plasmid PIECMVUbiP1 was consistent with the expected result by BamHI digestion, indicating that the transfer plasmid PIECMVUbiP1 was successfully constructed.

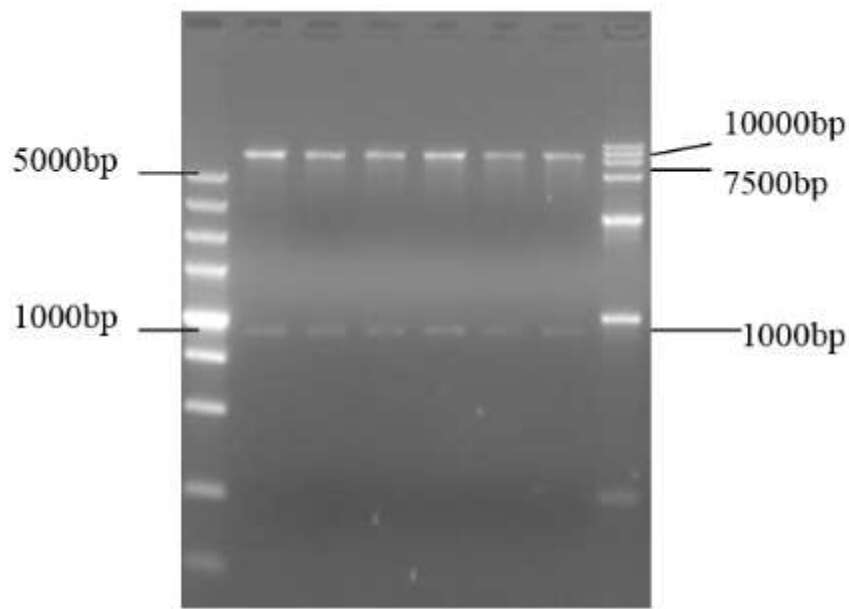


Figure 1. Identification of Recombinant Plasmid PIECMVUbiP1

3.2. Construction of Transferring Plasmid PIECMVUbiP1P1

The P12A gene was digested by restriction endonuclease BglIII, and the plasmid P12A was digested by restriction endonuclease BamH, and the P12A gene was ligated into the vector PIECMVUbiP1. The resulting transfer plasmid PIECMVUbiP1P1 was detected by XhoI and it was judged whether the orientation of the P12A gene was consistent with that of the promoter. The results were consistent with the expectation that the transfer plasmid PIECMVUbiP1P1 was successfully constructed.

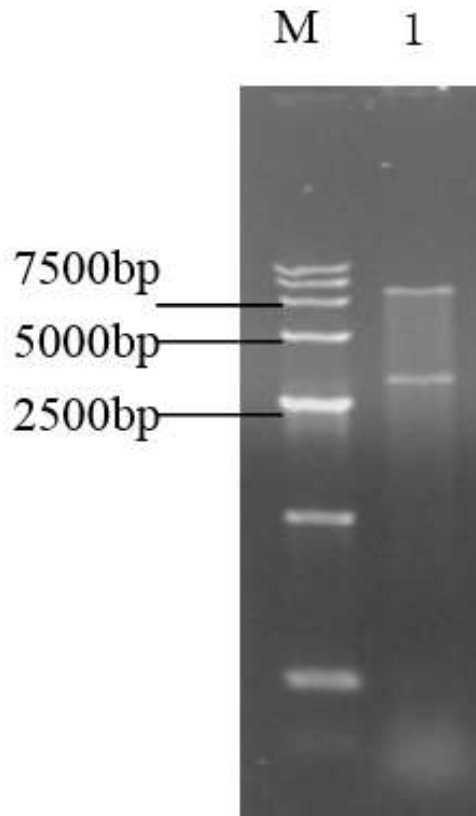


Figure 2. Recombinant plasmid PIECMVUbiP1P1 digestion

3.3. Construction of Transferring Plasmid PIECMVUbiP1P1SiFIFN γ

The sIFNgsiF gene was obtained by digesting the plasmid pCAsIFNgsiF with the restriction enzymes Sall, ScaI and HindIII. The sIFNgsiF gene was ligated into the vector PIECMVUbiP1P1 by restriction endonuclease Sall and HindIII to construct the transfer plasmid PIECMVUbiP1P1SiFIFN γ . The results showed that the UbiP12A gene, P12A gene and sIFNgsiF gene were successfully cloned into the vector ($P < 0.05$). The recombinant plasmid PIECMVUbiP1P1SiFIFN γ was identified by restriction endonuclease digestion. The results showed that the UbiP12A gene, P12A gene and sIFNgsiF gene were successfully cloned into the vector PIECMV, the transfer vector PIECMVUbiP1P1SiFIFN γ was successfully constructed and the results of restriction enzyme digestion were shown in Fig. 8 below:

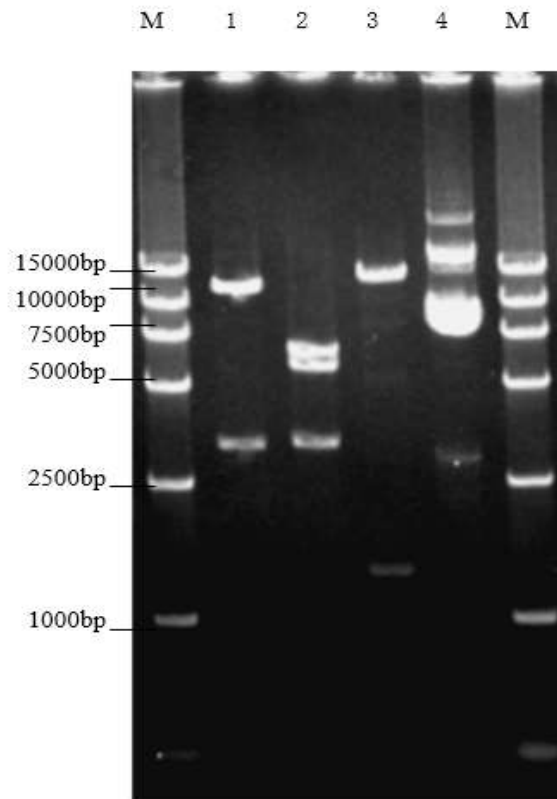


Figure 3. Identification of Recombinant Plasmid PIECMVUbiP1P1SiIFN γ

3.4. Co-transfection products were identified by the third generation PCR

The transfected product was transfected into cells filled with monolayer cells. The cells were harvested for the third generation of the cells. The virus genome was extracted and a pair of primers F primer and R primer were designed according to the nucleotide sequence. The recombinant virus IFN- γ gene was fragmented and the PCR product was electrophoresed by 0.8% agarose gel. The results showed that the gene fragment of 716bp was amplified with the expected size (716bp) (as shown in Fig. 9). Indicating that the successful transfection, there can be stable proliferation of the positive recombinant virus, followed by plaque purification experiments.

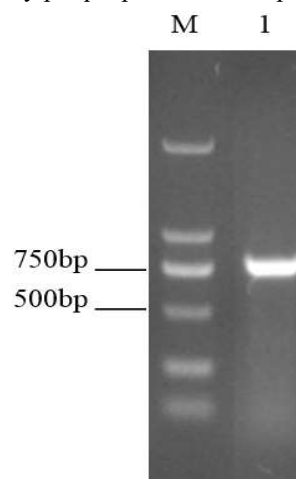


Figure 4. PCR product identification of the third generation genome of the transfected product

3.5. Purification and PCR identification of recombinant virus

3.5.1 Formation of plaques

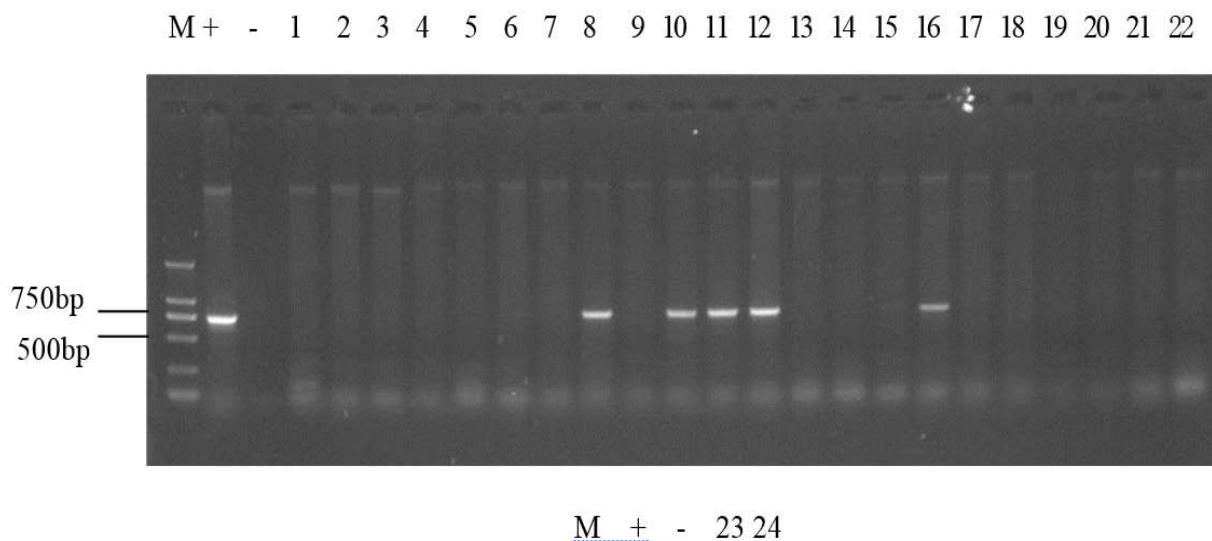
After 48 hours of co-transfection, the cells were infected and the transfected product was harvested. After 10 days of dilution, the virus solution with 10⁻²-10⁻⁷ dilution was inoculated into 6 wells of monolayer PK-15 cells plate, the adsorption of low melting point agarose 37 °C carbon dioxide incubator culture 2d, the visible macroscopic spot, which in the 10⁻⁵ dilution formed the number of plaques suitable for easy selection of plaques (as shown in Figure 10).



Figure 5. Plaque Photo

3.5.2 Screening of the first round of plaques on PK-15 cells

Twenty-four plaques were picked up in the plaques formed at the dilutions of 10⁻⁴, 10⁻⁵ and 10⁻⁶ to carry out recombinant virus amplification in 80% of PK-15 covered with monolayer 24-well plates, poison. The PCR results of the first round of plaque screening of the recombinant virus were shown in Fig. 11. Select the positive recombinant virus, the next round of the blank spot screening.



4. Discussion

4.1. The construction of the carrier

In the construction of the carrier, in order to improve the efficiency of the connection and the accuracy of the connection we can through the following aspects to cleverly design the experiment: (1) with the use of the same enzyme. When the vector and the plasmid containing the sub-cloned fragment do not contain the same restriction site and contain the same coenzyme site that cleaves the complementary cohesive ends, we can use the same enzyme to obtain the fragment and vector containing the complementary sticky end used for connection (2). The dephosphorylation of the carrier. When the carrier used to ligate the subcloned fragment is treated with only one endonuclease, in order to prevent the carrier from self-dephosphorylation, it is necessary to reduce the possibility of carrier self-linking in the ligation reaction, fragment connection efficiency. (3) Identification of the connection direction. When the vector and the target fragment were digested only after the production of a sticky end, we can cleverly select the endonuclease, digestion and identification of the target fragment into the direction of whether and the vector promoter in the same direction (4). The temperature and time when the reaction is connected. Most of the ligation reaction conditions were 16 °C overnight. Since the transfer plasmid was relatively large in this experiment, the difficulty of construction was correspondingly large, especially the number of times of connection failure. Therefore, after adjusting the condition of a series of reactions, Etc. We can also set the connection reaction temperature to 4 °C, the time can be controlled within the range of 8-48 hours.

4.2. Subculture of cells

PK-15 and Vero cells used in this study have good growth and proliferation ability, and can be infinitely passaged, but in order to get the morphology and activity are very good cells, cell passage details is particularly critical.

Like most cell lines, PK-15 and Vero cells were first washed 2-3 times with PBS buffer before subculturing, and the appropriate amount of trypsin was placed in a 37 °C incubator. The cells were incubated with the appropriate amount of cell culture medium. Under the microscope to see 1-3 cells for the dispersion so far, and then the ratio of about 1: 3 sub-packaging culture. But the difference between the two cell passage processes, as most of the different types of cell passage process, is the difference between trypsin digestion processes. When the cell is not well digested, there will be cells adherent growth, uneven distribution, when the cell digestion time is too long easy to aging, poor shape, low activity. And cell digestion is largely dependent on the amount of trypsin and digestion time, when the amount of trypsin added too much, the cells are easy to large pieces from the bottle wall off the lumps, so trypsin cannot evenly distributed on the cell surface, resulting in even if the blow cannot be dispersed into a single cell, in this case, increase the digestion time is necessary, but too long will cause excessive cell membrane digestion, cell activity decreased or even cracked. So the digestion process of trypsin addition and digestion time for different types of cells must have a good grasp in order to cultivate the morphology and activity are very good cells.

4.3. Plaque screening experiment

4.3.1 Selection of plaque screening cells

Because of the genetically engineered vaccine against porcine O-type foot and mouth disease recombinant pseudorabies virus, the most representative of which is the role of pigs, for these reasons, for such recombinant virus plaque screening and a series of experiments is the most commonly used PK-15 cells, a large number of experimental results show that the selection of such cells on the general reorganization of the virus screening is no side effects, so this study was directly transfected positive recombinant virus directly selected with PK-15 cells screening. At the same time, taking into account the construction of recombinant virus containing γ interferon gene, and PK-15 cells containing α , β , γ interferon gene coding region, may regulate the recombinant virus interferon gene, including a series of expression, regulation, immune and other physiological processes, the inhibition of the growth of the virus itself is not conducive to the acquisition of positive recombinant virus, so at the same time use another very commonly used Vero cells for the study of recombinant virus screening.

From the plaque screening experiment and the viral genome PCR amplification results can be seen that interferon gamma did not affect the growth of PK-15 cells, so the use of the cells as a carrier can theoretically be used for the screening of the recombinant virus.

4.3.2 Effects of RNA interference on plaque screening results

Experiments have shown that the addition of exogenous synthetic siRNA molecules to mammalian cells can induce specific RNA interference.

4.3.3 Effects of Interferon - γ on Plaque Screening Results

Studies have shown that cattle γ -interferon (rBoIFN- γ) gene and VP1 gene recombination in foot and mouth disease vaccine, can be integrated in *Pichia pastoris* co-expression, and the expression of a high concentration of the product to the supernatant, can significantly improve the body fluids and cellular immune responses induced in mice (Shi et al., 2006).

This study first demonstrates that the FMDV vaccine, which incorporates the gamma interferon gene, enhances the body's immune response, and secondly suggests that the interferon-gamma gene does not affect yeast and fungi and mammals Normal growth activity, followed by FMDV vaccine in the process of screening the formation of the plaque state is very good, so γ -interferon gene does not interfere with the recombinant genome foot and mouth disease vaccine itself, but the plaque from the PCR results can be seen in the PK15 cells the screening on the Vero cells cannot extract the genome of the cells, so the recombinant virus γ -interferon gene on its host cell growth is affected, but also to be further verified by subsequent experiments.

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