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Expression and Study on Immunogenicity of *P12A-3A* Genes of Type O Foot and Mouth Disease Virus in Recombinant Attenuated Goatpox Virus Strain

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Abstract: The *P12A* gene fragments and *3C* gene fragments of O/China 99 strains of foot and mouth virus were ligated into bilateral tandem Vaccinia virus promoter P7.5 which could initiate expression of *EGFP* gene and the expression box p-EGFP-N1-P7.5-P12A-3C was constructed. The expression box was ligated to the linear vector pUC119-TK after KpnI enzyme digestion by blunt end ligation to obtain the recombinant plasmid pUC119-TK-EGFP-P7.5-P12A-3C. The homologous recombination took place in the BHK-21 cell between the recombinant plasmid and the Capripox virus attenuated strain by deleted *TK* gene. The PCR identification, the Western blot analysis and immunological experiment on mice were carried out. The results showed that the recombinant strain could be subcultured in BHK-21 cell for 10 passages, expressing *P12A-3C* gene which was 2900 bp by sequence analysis, the Western blot analysis demonstrated that the expressed protein of recombinant plasmid pUC119-TK-EGFP-N1-P7.5-P12A-3C in BHK-21 cells infected GTPV AV41 could be specifically recognized by the hyperimmune serum to type O FMDV. Immunity assay showed that the expression protein had immunogenicity in mice. All the results showed that the Goat Pox virus attenuated strain which could express *P12A-3C* gene of type O FMDV was obtained.

Key words: Promotor P7.5, *P12A-3C* gene, live vector vaccine, FMDV, immunogenicity

INTRODUCTION

Foot and Mouth Disease (FMD) is an extremely contagious viral disease of cattle, pigs, sheep, goats and many cloven-hoofed wild animals (Ramanoon *et al*., 2013; Haas *et al*., 2012; Biswal *et al*., 2012; Muroga *et al*., 2012; Valdazo-Gonzalez *et al*., 2011). Capri Pox is also called sheep Smallpox including Sheep Pox and Goat Pox which were acute, feverish and contagious infectious disease of sheep and goat caused by Sheep Pox Virus or Goat Pox Virus (Yan *et al*., 2012; Bowden *et al*., 2009; Venkatesan *et al*., 2012). In FMDV, the *VP1* gene encodes capsid protein.

The capsid proteins precursor P1-2A contains major epitope of FMDV and the mature virion is assembled by means of proteolysis by proteases such as 3C, etc. all that can induce the production of cellular immunity and humoral immunity (Porta *et al*., 2013; Reddy *et al*., 2010; Pan *et al*., 2008; Li *et al*., 2008). In this study, the recombinant vector pUC119-TK-EGFP-N1-P7.5-P1-2A-3C was constructed. The expression protein was used for immunological experiment on mice and protection test against FMDV challenge after screening and purification. The research is to establish foundation for study on non-replicability recombinant FMDV vaccine and Pox Virus recombinant live vector vaccines.

MATERIALS AND METHODS

Strain and vector: GTPV AV41 stains were provided by China Institute of Veterinary Drugs Control.O/China99 of FMDV strain, DH5α and BHK-21 cells were provided by China State Key Laboratory of Veterinary Etiological Biology. The vector p-EGFP-N1-P7.5 containing Pox Virus promoter P7.5 that had divergent promoting activity and pUC119-TK (Sequence analysis showed that there was only one KpnI restriction site in *TK* gene and the site was used to introduce promoter and screening gene) were constructed by China State Key Laboratory of Veterinary Etiological Biology. BALB/c female mice (16-18 g in weight) were purchased from Lanzhou Institute of Biological Products.

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Primer design: Specific primers (F1, F2) of *P12A* gene and specific primers (F3, F4) of *3C* gene were designed, respectively. The (F1, F2) region was about 2256 bp and (F3, F4) region was about 639 bp.

F1: 5'-GTC, AAGCTT, CCACC, ATG, GGC, GCC, GGG, CAA, TCC, AGC-3'
F2: 5'-TATA, GGATCC, CCC, AGG, GTT, GGA, CTC, GAC, GT-3'
F3: 5'-TATA, GGATCC, AGT, GGT, GCT, CCC, CCG, ACT, GAC-3'
F4: 5'-GTTG, GCTAGC, TTA, CTC, GTG, GTG,TGG, TTC, GGG, ATC-3'

The HindIII restriction enzyme site and the Kozak sequences CCACCATG were inserted at 5' end of F1 primer. The BamHI restriction enzyme sites were inserted at 5' end of F2 and F3 primers. The NheI restriction enzyme site and termination codon TTA were inserted at 5' end of F4 primer. Primers were designed by TaKaRa.

Construction and identification of expression cassette p-EGFP-N1-P-7.5-P12A-3C: F1, F4 as primers and pMD18-T-P1-2A-3C constructed as template, P12A-3C was amplified by PCR. The amplification conditions were initial denaturation for 2 min at 94°C, denaturation for 30 sec at 94°C, anneal for 30 sec at 61°C and after 35 cycles elongation at 72°C for 5 min. The plasmid p-EGFP-N1-P7.5 was digested by HindIII and the P12A-3C was digested by NheI. After connection of two digested products p-EGFP-N1-P7.5 and P12A-3C. The product was transformed into competent cell DH5α and cultured at 37°C in LB culture medium. The plasmid was identified by PCR amplification and enzyme digestion after extraction in the extract. The positive plasmids identified were subjected to sequencing by TaKaRa.

Construction and identification of recombinant vector pUC119-TK-EGFP-N1-P7.5-P12A-3C: The digested products were recovered after the recombinant plasmid digested by KpnI and under 37°C water bath for 3 h. These terminus of restriction fragments recovered could be filled into blunt ends and the products were put at ice for reserve. Then, the dephosphorylation of fragments was conducted and the products were put at ice for reserve. The expression cassette p-EGFP-N1-P7.5-P1-2A-3C was digested by NheI and NotI then the restriction fragments were recovered. These terminus of restriction fragments recovered could be filled into blunt ends and the products were put at ice for reserve.

The blunt end ligation of EGFP-N1-P7.5-P12A-3C after filled and recombinant plasmid pUC119-TK after filled and dephosphorylation was conducted in the ligation system: EGFP-P7.5-P12A-3C 4 μL, pUC119-TK 2 μL, Ligation Solution A 48 μL, Ligation Solution B 6 μL, reaction at 16°C for 30 min. The ligation product was transformed into competent cell DH5α and cultured in LB culture medium contained Amp+. Next, the plasmid was identified by PCR amplification and BglII enzyme digestion after extraction in the extract. The positive plasmids identified were subjected to sequencing by TaKaRa.

Cell passage of BHK-21 infected GTPV AV41 strain: The GTPV AV41 strain was inoculated into BHK-21 cells to proliferate and put at 37°C for culture. The CPE occurred, the cell sap would be collected and continue the cell passage until the CPE time was stable. The virus was collected and preserved at -70°C to reverse.

Co-transfection with GTPV AV41 and liposome into BHK-21 cells: The BHK-21 cells were infected by 0.1 mol L^{-1} CPV (2 mL) and cultured at 37°C for 8-12 h then observed by electron microscopic. The recombinant plasmid extracted was transfected into BHK-21 cells infected CPV that was cultured at 37°C for 18-48 h and the level of transfection was detected by fluorescence microscope. When 80% of cells had CPE, the virus was harvested. After washing by 0.01 mol L^{-1} PBS (pH7.4) and digestion with trypsin, the supernatant was removed by centrifugation. Then, the precipitation was lysed and by centrifugation and the supernatant was used to screen and purify the recombinant virus.

Screening of recombinant GTPV: The viral supernatant preserved was diluted in the ratio of 1:100. The BHK-21 cells were infected viral supernatant and cultured at 37°C and 5% CO_2 for 48-72 h in 24 well culture plate. The viral supernatant which had green fluorescent was harvested by digestion with trypsin and the supernatant was removed by centrifugation, next the precipitation was lysed and the supernatant was removed by centrifugation. The 100 μL supernatant was subcultured in BHK-21 cells in 24 well culture plate and cultured 3 times repeated. And also the supernatant was subcultured in 96 well culture plate and screened 10 times continuously. After centrifugation and purification of the 1st to 10th generation cell cultures, the supernatant was preserved to use.

The cell total RNA was extracted in the 6th generation cell cultures and amplified by RT-PCR specific primers of which were *3C* gene, *P12A* gene and *P12A-3C* gene of FMDV. The amplified products were analyzed by electrophoresis (1% agarose gel) and sequence.

Genetic stability test of recombinant virus: The cell total RNA in the 1st, the 6th and the 10th generation cell cultures was extracted, respectively and then amplified by RT-PCR specific primers of which were *3C* gene and *P1-2A* gene of type O FMDV.

Table 1: Vaccine combinations and usage for mice immunization

Groups	Number	Vaccine	Dose
1	6	pUC119-TK-EGFP-N1-P7.5-P12A-3C	5×10^7 PFU
2	6	Foot and Mouth Disease Type O Inactivated Vaccine	0.2 mL
3	6	PBS	0.2 mL

Western blot analysis of expression products: The guinea pig anti hyperimmune serum to type O FMDV was diluted in the ratio of 1:300 and the HRP-rabbit anti guinea pig IgG horseradish peroxidase conjugate was diluted in the ratio of 1:1000. At last, DAB was used to involve in color reaction.

Animal immunization test and antibody test: Eighteen BALB/c female mice were separated into three groups (six mice/group), vaccinated by intramuscular injection two times (28 days interval each time) with different adjuvants (Table 1). Mice sera were collected when 0, 14, 28 and 45 days after second vaccination. The sera were detected their titers with type O foot and mouth disease indirect ELISA at $OD_{492\ nm}$.

Protection against FMDV challenge: Every mouse was injected into vastus medialis by intramuscular injection with 0.2 mL (250 ID_{50}) O/China 99 virulent strain of FMDV on 45th day after immune. The 36th h after injection, four mice sere were collected at random in every group. The sera were diluted to 1:10 with DMEM. Every blood sample was injected into 40 neonatal mice, respectively and the control group was established. Continuously observed attacks and death of neonatal mice for 7 days. After end of test, all mice were put to death according to the Declaration of Helsinki and the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China in 2006). All experiments were conducted in BSL-3 lab.

RESULTS AND DISCUSSION

Identification of expression cassette p-EGFP-N1-P-7.5-P12A-3C: F1, F2; F3, F4; F1, F4 as primers, respectively, P1-2A, 3C and P12A-3C were amplified by PCR from vector p-EGFP-N1-P-7.5-P12A-3C, respectively. After digestion of plasmid p-EGFP-N1-P-7.5-P12A-3C by HindIII and BamHI; BamHI and NheI; HindIII and NheI respectively, P12A and p-EGFP-N1-P7.5-3C, p-EGFP-N1-P7.5-P12A and 3C, P12A-3C and p-EGFP-N1-P7.5 were obtained, respectively. They were all consistent with reference sequences by sequencing (Fig. 1). The results showed that expression cassette p-EGFP-N1-P-7.5-P1-2A-3C was successfully constructed.

Identification of PCR and enzyme of recombinant vector pUC119-TK-EGFP-N1-P7.5-P12A-3C: The plasmid pUC119-TK was digested by KpnI and after digestion by NotI and NheI, the p-EGFP-N1-P7.5-P12A-3C was flushed with T4 DNA polymerase and conducted dephosphorylation. The gene fragments obtained after treatment were about 5.2 and 6.6 kb long. The single band was obtained after digestion of pUC119-TK-EGFP-N1-P7.5-P12A-3C by BglII and P12A, 3C and P12A-3C fragments were obtained by PCR. The sequences were consistent with reference sequences by sequencing. The results showed that transfer vector pUC119-TK-EGFP-N1-P7.5-P12A-3C was successfully constructed (Fig. 2).

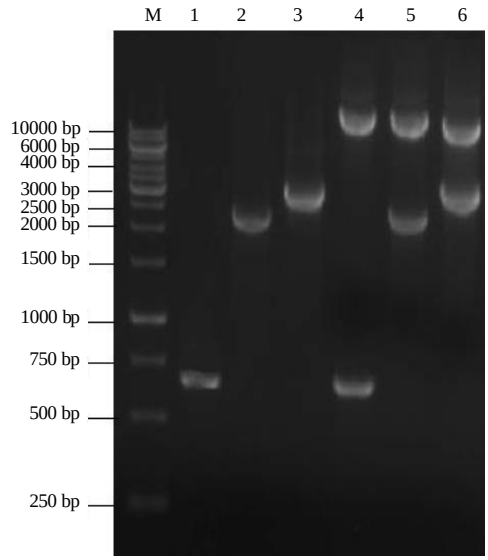

Fig. 1: Identification of PCR and restriction enzyme of expression cassette p-EGFP-N1-P-7.5-P12A-3C. Lane M is GenRuler™ 1 kb DNA Ladder; Lane 1 is product of 3C by PCR, the size of fragment is about 640 bp; Lane 2 is product of P12A by PCR, the size of fragment is about 2260 bp; Lane 3 is product of P12A-3C by PCR, the size of fragment is about 2900 bp; Lane 4 is product of digestion by BamHI and NheI, the size of p-EGFP-N1-P7.5-P12A is about 9990 bp and the size of 3C is about 640 bp; Lane 5 is product of digestion by HindI and BamHI, the size of p-EGFP-N1-P7.5-3C is about 8370 bp and the size of P12A is about 2260 bp. Lane 6 is product of digestion by HindI and NheI, the size of p-EGFP-N1-P7.5 is about 7730 bp and the size of P12A-3C is about 2900 bp

CPE of BHK-21 cells: The obvious CPE appeared after BHK-21 cells infected GTPV AV41 3-4 days later. The cells were infected 24 h later, the cell edge became clear firstly, cell membrane contracted next, separated from surrounding cells (Fig. 3).

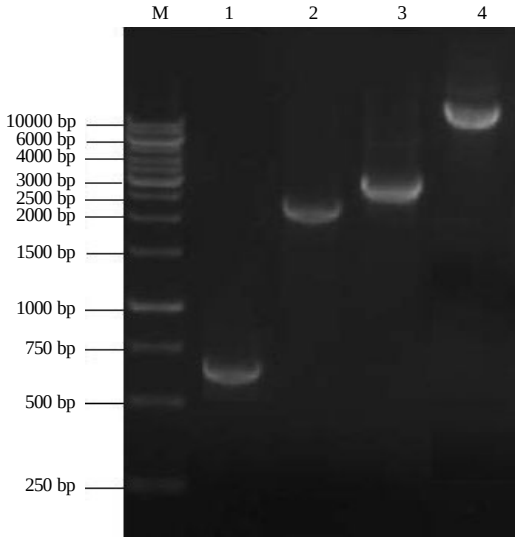

Fig. 2: Enzyme and PCR identification of transfer vector pUC119-TK-EGFP-P7.5-P12A-3C. M: GenRuler™ 1 kb DNA Ladder; Lane 1 is PCR products of FMDV 3C, the size of fragment is about 640 bp; Lane 2 is PCR products of FMDV P12A, the size of fragment is about 2260 bp; Lane 3 is PCR products of FMDV P12A-3C, the size of fragment is about 2900 bp; Lane 4 is enzyme products of pUC119-TK-EGFP-N1-P7.5-P12A-3C by KpnI, the size of fragment is about 11800 bp

Screen of recombinant GTPV: Transfection 16-48 h later, green fluorescent was observed in BHK-21 cells infected parent GTPV after transfection with plasmid pUC119-TK-EGFP-N1-P7.5-P12A-3C. The fluorescent was not observed in normal BHK-21 cells and cells only infected goat pox virus (Fig. 4). After consecutive screening in 24 well cell plate and 96 well cell plate, preliminary prediction was that the recombinant GTPV protein expressing *P1-2A-3C* gene of FMDV was obtained (Fig. 5).

Identification of RT-PCR of BHK-21 cells infected recombinant GTPV: By amplification of the sixth generation viral cell cultures infected recombinant GTPV by RT-PCR, P12A, 3C and P12A-3C were obtained and the objective bands were consistent with expected gene fragments. The sequences were consistent with reference sequences by sequencing. It showed that the target gene had been integrated into genome of recombinant GTPV and accomplished translation in BHK-21 cells (Fig. 6).

Result of genetic stability test: The supernatant purified of 1st generation, 6th and 10th generation pathological

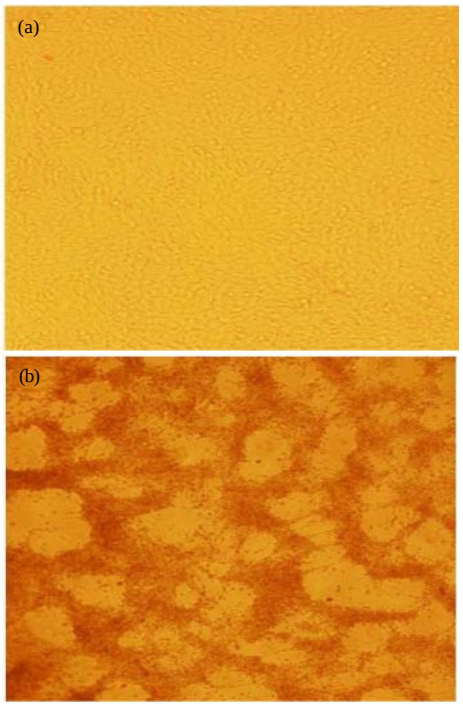

Fig. 3: The CPE of GTPV infected BHK-21 cell (x200). a) Normal BHK-21 cell; b) 24 h post-infection BHK-21 cell

cells were detected by PCR. The products P12A and 3C amplified were consistent with expected sequences. The sequences were consistent with reference sequences by sequencing. The results showed that *P12A-3C* gene of FMDV had been transferred into GTPV stably (Fig. 7).

Western blot analysis: The supernatants after centrifugation of pathological cells of BHK-21 cells infected GTPV AV41 and BHK-21 cells infected GTPV AV41 which was transfected by plasmid pUC119-TK-EGFP-N1-P7.5-P12A-3C both were analyzed by Western blot.The special protein band was only observed in cell lysate and supernatant of BHK-21 cells which were infected GTPV AV41 and transfected by plasmid pUC119-TK-EGFP-P7.5-P12A-3C. Moreover, the band was about 100 kDa long and that was consistent with expected band and recognized by hyperimmune serum to type O FMDV. The results showed that P12A-3C gene of type O FMDV was expressed in BHK-21 cells infected GTPV AV41 and had good antigenicity (Fig. 8).

Result of indirect ELISA: The specific antibody had arisen in the 15th day after immune and antibody titer was higher after the 28th day immune. The antibody titer of Foot and Mouth Disease Type O Inactivated Vaccine

Table 2: FMDV antibody titer by indirect ELISA ($OD_{492\ nm}$)

		Average antibody titers at different immunity-time (IgG) (Days)			
Groups	Vaccine	0	14	28	45
1	pUC119-TK-EGFP-N1-P7.5-P12A-3C	0.312	1.362	1.420	1.468
2	Foot and Mouth Disease Type O Inactivated Vaccine	0.334	1.447	1.493	1.526
3	PBS	0.306	0.343	0.332	0.312

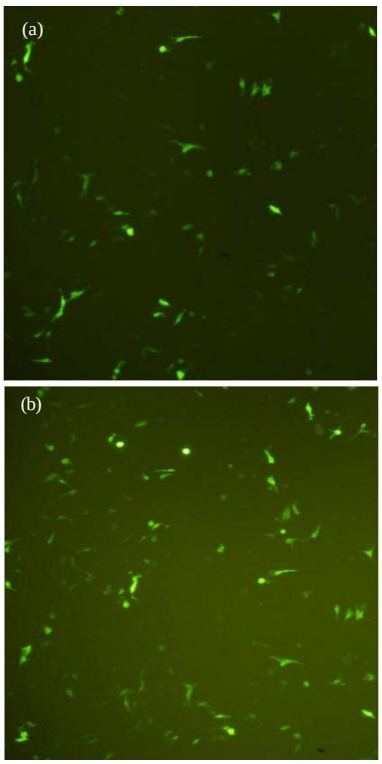

Fig. 4: The pictures of green fluenuence in BHK-21 cells membrane (x200). a) 24 h after transfection; b) 48 h after transfection

group was highest, antibody titer of pUC119-TK-EGFP-N1-P7.5-P12A-3C group was lower and control group had no specific antibody (Table 2).

Result of protection against FMDV challenge: The protection rate against FMDV challenge of two immune groups were 75.0 and 82.5% but the control group was 0 (Table 3). It has been reported that Kozak sequence (GCCACCATGG) can improve translation efficiency of foreign gene in eukaryotic cells (Kozak, 1987, 1986; Shuman and Moss, 1988). The Kozak sequence was inserted in the front of 5'terminal initiation codon ATG of *P1-2A* gene of FMDV. The expression time and expression level of foreign gene in recombinant CPV are related to promoter (Pozzi *et al*., 2009; Yanagida *et al*., 1992). The P7.5×16 and P7.5×20 in Poxvirus of vector pUTAL as early promotors, the foreign genes were

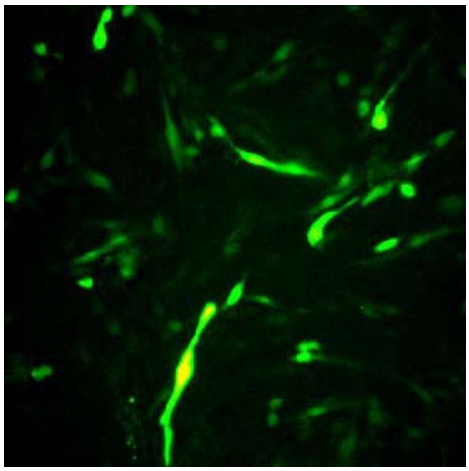

Fig. 5: The screening and purification of the F6 generation BHK-21 cells infected by recombination capripox virus (x300)

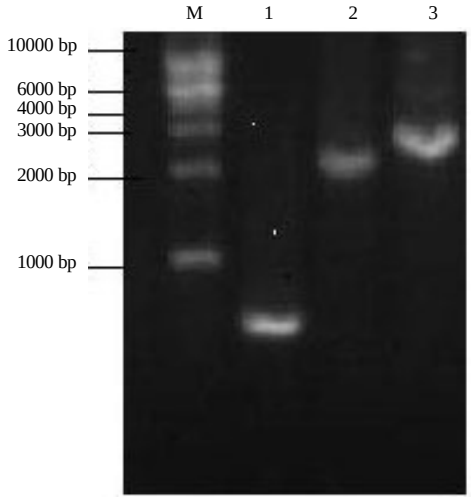

Fig. 6: Identification of the recombinant GTPV by RT-PCR. M: DL10000 DNA marker; Lane 1 is PCR product of FMDV 3C, the size of fragment is about 640 bp; Lane 2 is PCR product of FMDV P12A, the size of fragment is about 2260 bp; Lane 3 is PCR product of FMDV P12A-3C, the size of fragment is about 2900 bp

expressed. P7.5×16 and P7.5×20 are bidirectional tandem promoters and the connection direction needed not

Table 3: Protection against FMDV challenge in sucking mice

Groups	Challenged dose	Challenge dcounts	Survival counts	Death counts	Protect ion rates (%)
pUC119-TK-EGFP-N1-P7.5-P12A-3C	0.2 mL ($250ID_{50}$)	40	30	10	75.0
Foot and Mouth Disease Type O Inactivated Vaccine	0.2 mL ($250ID_{50}$)	40	33	7	82.5
PBS	0.2 mL ($250ID_{50}$)	40	0	40	0.0

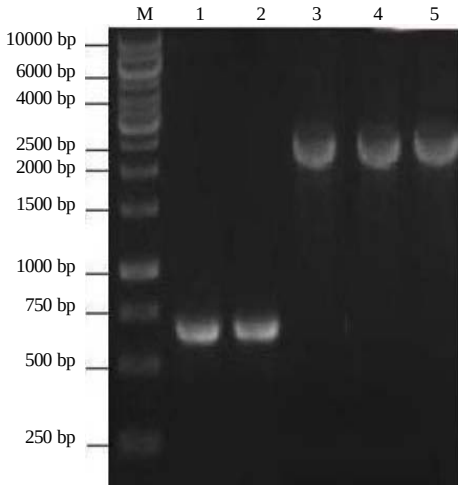

Fig. 7: Stability detection of the recombinant GTPV. M: GenRuler™ 1 kb DNA; Ladder 1, 2: PCR product of FMDV 3C from the 1st and 10th generation recombinant GTPV, the size of fragment is about 640 bp; 3, 4, 5: PCR product of FMDV P12A from the 1st, 6th and 10th generation recombinant GTPV, the size of fragment is about 2260 bp

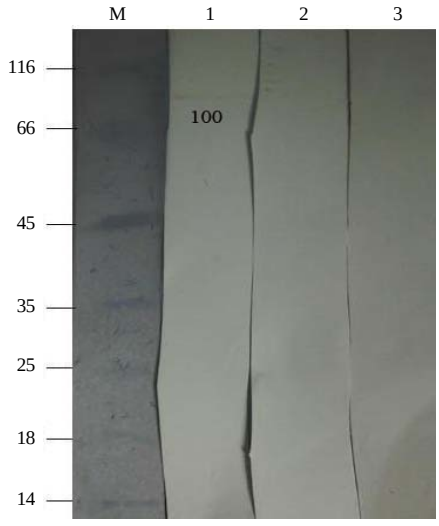

Fig. 8: Western-blot analysis of the expressed protein. M: Protein marker; 1: BHK-21 cells infected GTPV AV41 was transfected by pUC119-TK-EGFP-P7.5-P12A-3C; 2: BHK-21 cells infected GTPV AV41 was transfected by pUC119-TK-EGFP-P7.5; 3: BHK-21 cells infected GTPV AV41

controlled which can largely promote the stable expression of foreign genes. The live vector vaccine pUC119-TK-EGFP-N1-P7.5-P1-2A-3C of recombinant goatpoxvirus low virulent strain was screened successfully. This vector vaccine and Foot and Mouth Disease Type O Inactivated Vaccine were both used to conduct mice immunization test. The results showed that specific antibody titers of both of immune groups were high. The results of protection against FMDV challenge showed that the protection rate of two immune groups were 75.0 and 82.5%, respectively.

The vector vaccine conducted could product mice against virulent FMDV challenge, in addition immune effect and protective rate were ideal. However, mice are different from swine in species, it needs to be further proved that whether immunity and specific antibody will be produced in swine.

CONCLUSION

In this experiment, the *P12A-3C* gene of O/China99 strain of FMDV was inserted into recombinant vector pUC119-TK-EGFP-N1-P7.5. The *TK* gene of non essential region of recombinant sheep pox virus was accomplished homologous arm recombination in parent sheep pox virus attenuated strain. The expression protein had good immunogenicity in mice and specific antibody at high level was produced. It provided preliminary method to study on the candidate live vector vaccine for prevention of FMD.

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