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Expression and Immunological Analysis of Capsid Protein Precursor of Swine Vesicular Disease Virus HK/70

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Abstract: VP1, a capsid protein of swine vesicular disease virus, was cloned from the SVDV HK/70 strain and inserted into retroviral vector pBABE puro, and expressed in PK15 cells by an retroviral expression system. The ability of the VP1 protein to induce an immune response was then evaluated in guinea pigs. Western blot and ELISA results indicated that the VP1 protein can be recognized by SVDV positive serum, Furthermore, anti-SVDV specific antibodies and lymphocyte proliferation were elicited and increased by VP1 protein after vaccination. These results encourage further work towards the development of a vaccine against SVDV infection.

Key words: Swine vesicular disease virus; Capsid protein precursor gene (*vp1*); Gene expression; Immune response

Swine vesicular disease (SVD) is the causative agent of highly contagious disease in pigs, characterized by vesicles on the coronary bands, heels of the feet, and occasionally on the lips, tongue, snout, and teats. SVD is caused by a virus belonging to the genus *Enterovirus* within the family *Picornaviridae* and is antigenically closely related to the human pathogen coxsackievirus B5[4]. The disease was first reported in Italy in 1966 and since then several outbreaks have been documented in a number of countries in Europe and Asia[2, 4]. The similarity of clinical symptoms between SVD and Foot and Mouth Disease (FMD) have lead to SVD being classified as a list A disease by the World Organization for Animal Health (OIE) [3].

SVDV particles are composed of 60 copies each of the four capsid proteins VP1–VP4, which enclose a single-stranded, positive-sense RNA genome of about 7400 nt. Proteins VP1, VP2 and VP3 are exposed at the viral surface, whereas VP4 is in close contact with the RNA and thus not accessible from the outer shell surface in the intact virions. It has been shown that both conformation-dependent neutralizing sites and linear epitopes are located mainly in the outer capsid proteins[1, 6, 8, 12, 14]. Furthermore, a recombinant bacterially expressed SVDV polyprotein, P1, is able to

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induce an SVDV-specific cellular and humoral immune response in pigs [7]. For this reason, the *vp1* gene was chosen for this study.

Retrovirus is a class of enveloped viruses containing a single stranded RNA molecule as the genome. Following infection, the viral genome is reverse transcribed into double stranded DNA, which integrates into the host genome & is expressed as proteins. The viral genome is approximately 10kb, containing at least three genes: *gag* (coding for core proteins), *pol* (coding for reverse transcriptase) & *env* (coding for the viral envelope protein). At each end of the genome are long terminal repeats (LTRs) which include promoter/enhancer regions & sequences involved with integration. In addition there are sequences required for packaging the viral DNA (psi) & RNA splice sites in the *env* gene. Retroviral vectors are believed to be advantageous in single gene transfer, which may allow for the simultaneous achievement of more efficient gene delivery and longer-term trans-gene expression[11]. The ability of retrovirus vectors delivering a particular gene to target cells has been applied commonly in both experimental and clinical settings. Under these conditions, we have cloned and expressed *vp1* gene of SVD HK/70[19] in eukaryotic cell, and analyzed the immunological activity in guinea pigs.

MATERIALS AND METHODS

Materials

GP2-293 cells; PK15 cells; IBRS-2; pBABE puro vector，pVSV-G plasmid and SVDV positive serum were preserved at th virology department of Lanzhou Veterinary Research Institute, Gansu Province, P.R. China. Phytohemagglutinin (PHA, Cat No. L9132), 5-diphenyltetre zolium bromide (MTT, Cat No. M5655), 3-(4,5-Dimethylthiazoyl2-yl)-2 and Dimethylsul-foxide (DMSO, Lot No.12H0366) were purchased from Sigma Chemical Co., USA; SVDV Antibody Test Kit was purchased from Cedi-Diagnostics Co., Netherlands. Twenty 2-month-old guinea pigs weighing between 400-500 g were used for the study. All of the animals were kept in an isolation hutch.

Cell culture and SVD virus propagation

GP2-293 cells; PK-15 cells and IBRS-2 were cultured in Dulbecco's Modified Eagle Medium, DMEM (Invitrogen, USA) supplemented with 10% fetal calf serum. SVDV HK/70 (preserved by Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, China[19]) was propagated in IBRS-2 cells cultured in DMEM. After 12 h of incubation at 37℃, when more than 80% of the cells showed cytopathic effect, the cells were subjected to three freeze–thaw cycles. The viral suspension was clarified by centrifugation at 800× *g* for 10 min and stored at -70℃.

Construction of recombinant plasmid

Viral RNA was extracted from the viral suspension using an RNeasy mini kit (Qiagen). A first-strand synthesis reaction was then performed using random hexamers (TaKaRa Bio) to anneal and prime the viral RNA for reverse transcription with avian myeloblastosis virus reverse transcriptase in the presence of RNasin. The cDNA was used in high-fidelity PCR with forward primer *vp*1F: 5'– AA*GAATTC*CACCATGGC ATGATCCAGCTAACGT-3' and reverse primer *vp*1R: 5'-CG*GGATCC*GCTGACTTGCTCGA-3'. The primers were designed according to the sequence of the HK/70 strain (GenBank accession no. AY429470). The forward primer contained an optimal Kozak sequence

CCACC [10] and initiation codon (ATG) for optimal initiation of translation and the reverse primer contained a *Bam*H I restriction site, as indicated by the underlined nucleotides. Following gel purification, the PCR product was cloned into the dephosphorylated *Eco*R I and *Bam*H I site of the pBABE puro vector and the resultant plasmid was named pBABE puro-*vp1*. The fidelity of the recombinant plasmid was confirmed by restriction digestion and sequence analysis. The plasmid was introduced into *E. coli* DH5α and large-scale DNA production runs were performed using a S.N.A.P. Kit (Invitrogen) and concentration was determined by spectrophotometry at 260 and 280 nm.

Production of recombinant virus

The GP2-293 cells were plated at 1:10 (approx. 9×10^5 cells per 100 mm plate) for 24 h before transfection. Then co-transfection of the mixture (recombinant plasmid pBABE puro-*vp1* and pVSV-G) was performed in PK15 cells with lipofectin according to the manufacturer's protocol (Invitrogen). The viruses were collected 72 h after transfection, and the media was replaced with 5 mL of fresh DMEM (complete). Viruses were collected about every 5 h for 2 days and frozen in sterile tubes at －70℃.

Target cells infection and selection

PK15 cells to be infected were split within 24 h before infection and grown to 20% confluency for infection so that they did not overgrow the plate before being cultured in selective media. Medium was replaced with 2 mL of recombinant viral supernatant plus enough growth media to cover the cells for a 60 mm plate, polybrene was added to a concentration of 8 g/mL. The media was changed once a day for the first 2 or 3 days after infection. A positive PK15 cell colony was produced during the following 2 to 4 weeks and named PK15-VP1.

Western blot

Positive cell lysates were electrophoresed in 10% SDS–polyacrylamide gels and transferred to a nitrocellulose membrane (Bio-Rad), incubated for 1 h in blocking solution (5% non-fat dry milk, 0.1% Tween-20 in phosphate buffered saline, PBS) and probed with a SVDV positive serum diluted 1:800 in blocking solution. Normal PK-15 cell lysates were used as negative control. Specific anti-P1 antibodies were detected with a protein A–horseradish peroxidase conjugate (Sigma) and the reaction was developed with luminol.

Immunization procedures

Twenty guinea pigs were randomly divided into four groups of five: Group A, infected by emulsified positive PK15-VP1 cell lysates; Group B, infected by blank vector control; Group C, blank cell control and Group D, PBS. Each group of guinea pigs was inoculated intramuscularly in both hind limbs three times at 3-week intervals with 3.5×10^9 cell of each sample. Serum was collected at weeks 0, 2, 4, 8 and 10 post-immunization (p.i.).

T-lymphocyte proliferation assay

The T-lymphocyte proliferation assay was performed with MTT dye assay. Briefly, the lymphocytes were isolated according to standard protocol by gradient centrifugation and the buoyant cells were washed three times in RPMI 1640 medium with 10% FBS. MTT dye assay was performed with 96-well flat-bottomed plates. T-lymphocyte (2×10^7 cell/mL) mixed with 100 μL PHA (10 μg/mL) were added into wells. The plates were incubated at 37 ℃ in a humid atmosphere with 5% CO_2. After 45 h, 20μL of MTT

solution (5μg/mL) were added to each well and incubated for 3 h. 200μL of a DMSO working solution (192μL DMSO with 8μL 1mol/L HCl) were added to each well for 15min, and then the absorbance was evaluated in an ELISA reader at 570nm with a 630nm reference. The stimulation index (SI) was calculated based on the following formula: SI = the absorbance value for mitogen-cultures divided by the absorbance value for non-stimulated cultures.

Detection of anti-SVDV antibodies of vaccinated animals

Guinea pig sera were prepared at day 0 (pre-bleed), 28, 42 and 56 (after the second and the final booster) and also at the termination of the experiment. SVDV-specific antibodies in pig serum were detected using a commercial competitive sandwich ELISA kit (Ceditest; Ced-Diagnostics B.V.) following the manufacturer's instructions.

Detection of specific neutralizing antibodies

Prior to testing, sera were incubated for 30 min at 56℃ to inactivate complement. Sera taken from all guinea pigs were analyzed for neutralizing antibody titers by using a micro-neutralization assay with monolayer of IB-RS2 cells. Double dilutions of sera were reacted with 200 $TCID_{50}$ of SVDV HK/70 at 37℃ for 1 h. After incubation, 100 microliter IBRS-2 cell suspension containing 10^4 cells was added and plates were incubated for 3 d at 37℃ in 5% CO_2. Thereafter, cells were examined for SVDV-specific cytopathic effect and neutralization titres were calculated as $-\log_{10}$ of the reciprocal of the highest dilution resulting in 50% neutralization.

RESULTS

Expression of *vp1* gene in PK15 cell

In order to demonstrate appropriate expression of the SVDV VP1 proteins, transfected PK15 cells were detected by Western blot and PCR. To detect the activity of expressed VP1 protein in vitro, the medium of the infected cells was harvested and analyzed by Western blot. As seen in Fig.1, the picture revealed a major band of 29 kDa in cells infected with "recombinant retrovirus", whereas no protein band was found in blank cells.

T-lymphocytes proliferation response of guinea pigs was tested after immunization. Compared with the

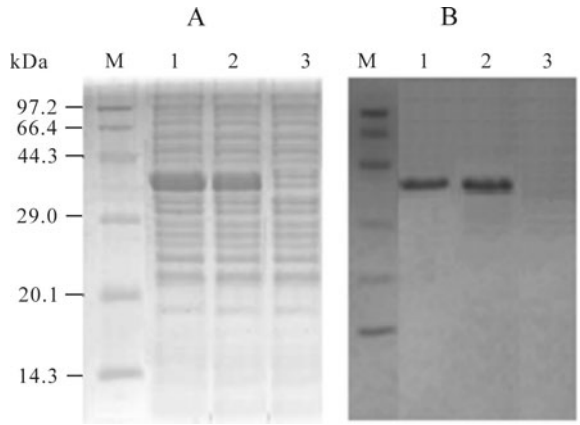

Fig. 1. SDS-PAGE (A) and Western blotting (B) was used to detect the expression and identification of the recombinant *VP1* protein. A: M, Protein markers; Lane 1 and 2, PK15-*VP1* cell lysates; Lane 3. PK15 cell lysates. B: M, Protein markers; Lane 1 and 2, Western blot analysis with PK15-*VP1* cell lysates; Lane 3, Western blot analysis with PK15 cell lysates.

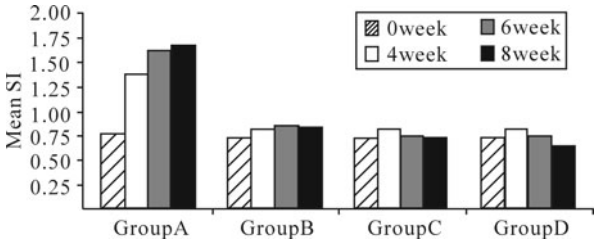

Fig.2. Specific proliferation of lymphocytes in immunized guinea pigs presented as the mean stimulation index (SI). SI was calculated as the ratio of mean absorbance values of wells containing antigen-stimulated cells to mean absorbance values of wells containing only cells with medium.

control group, the vaccinated group showed a significant lymphocyte proliferation response after the first vaccination, which increased after the booster vaccination (Fig.2)

Antibody response

The SVDV specific antibody levels in the sera were measured from 2 weeks after the 1st immunization and the results were shown in Table.1. Antibody positive of group A were of significant level (5/5) compared with groups B–D. But antibody positive titers of group B (0/5) compared with groups C–D were not significant. This finding indicated that PK15-VP1 acted as an antigen and significantly enhanced serum antibody production in guinea pigs.

Neutralizing antibodies study

Serum samples were evaluated further in an SVDV serum neutralization test (SNT). Induction of neutralizing antibody was similar to that observed for the ELISA antibody. Briefly, neutralizing antibody was detected in group A, whilst all of the control animals remained negative (Table.2).

Table 1. Presence of SVDV specific antibodies in immunized guinea pigs` by blocking ELISA test

Group	SVDV-specific antibodies positive (days p.i.)			
	0	28	42	56
A	0/5	4/5	5/5	5/5
B	0/5	0/5	0/5	0/5
C	0/5	0/5	0/5	0/5
D	0/5	0/5	0/5	0/5

Inhibition⩾50 %: SVDV-specific antibodies are present in the sample.

Table 2. Neutralizing antibody titers of guinea pigs

Group	0 week	4 weeks	8 weeks	10 weeks
A	<0.6	1.0±0.2	1.6±0.2	1.9±0.2
B	<0.6	<0.6	<0.6	<0.6
C	<0.6	<0.6	<0.6	<0.6
D	<0.6	<0.6	<0.6	<0.6

Results were measured by SNT and shown as –log titre (mean± SD)

DISCUSSION

In this study, *vp1* gene of the SVDV was engineered successfully into the genome of PK15 cells by use of a retroviral expression system (containing a retroviral expression vector, plasmid pVSV-G and a packaging cell). In this expression system, the genes coding for the viral structural components were stably integrated into the packaging cell line's genome, and the viral particles produced were replication-incompetent; to some extent, these characteristics enhance the organism safety and enlarge the application of this system. To date, there is no effective vaccine available for SVD and the development of effective prophylactic vaccines is of the highest priority for controlling outbreaks of SVDV. Subunit vaccines [16] have been shown to elicit strong cellular and humoral responses and which can overcome many problems associated with conventional vaccination, such as the potential danger associated with the production and distribution of attenuated live vaccines[16]. To this aim we have cloned and expressed the capsid precursor polypeptide (VP1) and analyzed the immunological activity of the expression product in guinea pigs.

The antigenic linear map of Swine vesicular disease virus (SVDV) has been studied using a repertoire of monoclonal antibodies (mAbs) raised against a recombinant SVDV polyprotein P1[9]. Peptidescanning analyses, cross-reactivity studies with homologous and heterologous viruses and predicted location on a computer-generated three-dimensional model of the capsid proteins have allowed the identification of five main linear sites [1, 7]. Two sites, the N terminus of VP3 and amino acids 51-60 on VP1, correspond to internal areas, conserved not only between SVDV

isolates but also in the related enterovirus coxsackievirus B5. In contrast, three other regions, amino acids 142-161 of VP2, 61-70 of VP3 and the C terminus of VP1, are exposed on the external face of the capsid and subjected to antigenic variation, even among different SVDV isolates [12]. Further minor sites that are antigenically conserved were identified on VP1 [15]. For this reason, the *vp1* gene of SVDV HK/70 was chosen in this study.

Over the years, many different vaccine formulations and delivery methods have been developed and tested in an effort to effectively immunize against viral disease [5]. Most studies involving immunization with simple subunit or subunit-in-adjuvant preparations elicited measurable immune responses, and a number of studies have shown that recombinant retroviral vector pBABE puro can be successfully employed as a vector for in vitro delivery of vaccine immunogens[5, 17]. As this vector does not contain any viral proteins, it is not replication competent. Based on those findings, the protein of SVDV VP1 that was cloned into a retrovirus vector and expressed in the mammalian cell line (PK15 cells) was confirmed by Western blot (Fig.1) in our work. Although we have demonstrated appropriate expression of SVDV proteins from the infected cells in this study, whether the *vp1* gene was stably integrated into the PK15 cell genome needs further confirmation. These results at least indicated that *vp1* gene exists in the genomic DNA in 30th generation cells, but not in the control group. Furthermore, the result of Western blot indicated that the expressed VP1 protein can be recognized by SVDV positive serum.

Whether expressed VP1 protein could enhance the cellular immune responses in guinea pigs was evaluated. A significant difference of lymphocyte proliferation in guinea pigs in group A was observed by comparing with the value of control group B, C and D, while there was no significant difference between the groups B, C and D. Taken together, the results indicated that expressed VP1 protein could significantly increase the activation potential of T cells in guinea pigs. The antibody levels in the sera of VP1-immunized guinea pigs are shown in Table 1, which demonstrates that the expressed VP1 protein is likely effective on T cells, as associated sensitively with an enhancement of anti-SVDV specific antibody levels. In our guinea pigs experiments, we found that anti-SVDV antibody titers increased after immunization in the experimental positive group while in the control groups it did not. Summarizing these data, we would predict that this immunity and dose would be adequate for induction of protective immunity.

The approach described in this study is relevant for the potential use of a recombinant VP1 protein as a vaccine and for the possibility of new opportunities for development of a marker vaccine.

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