

Immune Responses in Pigs Induced by Recombinant Canine Adenovirus 2 Expressing M Protein of Porcine Reproductive and Respiratory Syndrome Virus

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KEY WORDS: : Porcine reproductive and respiratory syndrome virus, M protein, Canine adenovirus vector, Pigs, Recombinant vaccine, Immunization

ABSTRACT

In order to develop a new type vaccine for porcine reproductive and respiratory syndrome (PRRS) prevention using canine adenovirus 2 (CAV-2) as vector, the expression cassette of M protein (MP) derived from plasmid pMD18T-M was cloned into the CAV-2 genome in which E3 region had been partly deleted, and the recombinant

virus CAV-2-M was obtained by transfecting the recombinant CAV-2-M genome into MDCK cells together with Lipofectamine™ 2000. Immunization trials in piglets with the recombinant CAV-2-M showed that CAV-2-M could stimulate a specific immune response to PRRSV. Immune response to the MP and PRRS virus was confirmed by ELISA, western blot analysis, neutralization test and lymphocyte proliferation assays. These results indicated that CAV-2 may serve as a vector for development of PRRSV vaccine in pigs and the CAV-2-M might be a

candidate vaccine to be tested for preventing PRRSV infection.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS), characterized by severe reproductive problems in sows and respiratory distress in piglets and growing pigs, is a kind of communicable disease caused by PRRS virus (PRRSV) infection¹⁻³ and is now one of the most important diseases in the swine breeding industry^{4,5}.

Currently, commercially conventional (inactivated and attenuated) vaccines cannot provide satisfactory immunoprotection against PRRSV⁶⁻⁸ because of the poor immune effect^{9,10} or the intrinsic risk of reversion to virulence under natural condition or residual pathogenicity^{11,12}. Thus, more effective, safer vaccines are urgently needed.

PRRSV is an enveloped, positive-strand RNA virus belonging to the family of Arteriviridae and the genus of Okavirus¹³. The genome of PRRSV is approximately 15 kb in length and contains 7 open reading frames (ORFs)¹⁴. According to the sequence data, the ORF1 encodes functional proteins involved in virus replication. The ORFs 2-7 are postulated to encode structural proteins, in which M protein (MP) is one of the major structural proteins. MP as a candidate antigen has been mentioned in many reports regarding genetic vaccine development of PRRS^{6,15-18}.

Recombinant adenoviruses are one of the preferred viral vectors used in vaccine production. Canine adenovirus 2 (CAV-2) has biological advantages, similar to human adenovirus, including (1) genetic stability, a wide range host, good replication in pigs [19-21]; (2) a good recombinant virus vector. The E3 region, unnecessary for replication, is partially deleted to provide space for the exogenous gene insertion. There will not be pathogenicity after infection, or potential harm to the external environment [18,19]; (3) the low-level replication in human cell lines without packaging new virus particles or recombination with wild type human adenovirus. Therefore, CAV-2 may

be modified to serve as the vector for genetic vaccine development against canine and porcine diseases.

In order to explore the possibility of this recombinant virus as a vaccine against PRRSV, we developed a live recombinant virus vaccine (CAV-2-M) expressing M protein (MP) of PRRSV using CAV-2 as vector, investigated MP expression, and examined its immunological characteristics in piglets.

MATERIALS AND METHODS

Vaccines

The commercial PRRSV KV vaccine was serotype Ch-1R strain (Harbin Weike Biotechnology Development Company, Harbin, China), batch No. 0702001, approval no. (2007) 080011063.

Virus, plasmid and cells

PRRSV-JL (a wild PRRSV strain) was isolated from clinical specimens in our laboratory. Marc-145 and MDCK cells were supplied by Epidemiological Laboratory, Chinese Academy of Military Medical Sciences and cultured in DMEM (Gibco, Inc., Rockville, Maryland, USA) supplemented with 10% new calf serum (NCS, Hyclone, USA), 1% penicillin (100 U/ml), and streptomycin (100 mg/ml). Plasmid pPolyII-CAV-2 containing the whole genome of canine adenovirus 2 (CAV-2) was constructed by Zhang et al.²². It is an infectious plasmid that can produce canine adenovirus particles after being transfected into MDCK cells²². Plasmid pMD18T-M containing the expressing cassette of M protein (MP) was constructed in Epidemiological Laboratory, Chinese Academy of Military Medical Sciences²³. The restriction enzymes, reverse transcriptases and T4 DNA ligases were purchased from NEB, Beijing, China.

Plasmid construction

The 4.8 kb KpnI fragment containing the E3 region from pPolyII-CAV-2 was first cloned into pVAX1 (Invitrogen, Carlsbad, California, USA), forming pVAX-E3. The EcoRV/BamHI double-digested fragment containing MP cDNA from plasmid pMD18T-M was cloned into pEGFP-C1 (Clontech Laborato-

ries, Inc., San Jose, California, USA) at the restriction site of NheI and BamHI, forming pEM-C1. The AseI/MLuI fragment of pEM-C1 containing the MP cDNA expression cassette was filled in and cloned into the SspI/SspI site and Klenow/dNTPs blunted pVAX-E3, forming pVAX-ΔE3-M. The fragment of pVAX-ΔE3-M double digested with Sall and NruI, containing the deleted E3 into which the MP expression cassette was inserted, was cloned into pPolyII-CAV-2 by replacing the fragment between the Sall and NruI sites, forming pPolyII-CAV-2-ΔE3-M. This recombinant genome was prepared for transfection. The cloning steps and plasmid preparation were performed as described elsewhere²⁴, the constructing route was presented in Fig. 1.

Production of the recombinant virus

Recombinant pPolyII-CAV-2-M was transfected into MDCK cells using Lipofectamine 2000TM (Invitrogen) according to the protocol described. Briefly, 4 μg purified recombinant genome and 25 μl Lipofectamine 2000TM were dispersed in 300 μl DMEM respectively, mixed up at room temperature for 20 min, supplemented with 1.4 ml DMEM and then overlaid onto the 80% confluent MDCK cells. After 12 hours of incubation, the medium was replaced by a complete DMEM containing 10% NCS. Transfected cells were cultured at 37 °C and propagated for days until cytopathic effect (CPE) appeared.

Identification of recombinant virus via restriction digestion

Transfected cells were rinsed 2 times by PBS and suspended in 800 μl of fresh lysis solution (0.6% SDS, 10 mmol/l EDTA, 100 μg/ml Proteinase K) at 37 °C for 1h; supplemented with 200 μl of 5 mol/l NaCl, placed on ice for 1h, and then precipitated by centrifugation at 4 °C with 12,000 rpm for 20 min. The supernatant was extracted once by using equivalent phenol and chloroform respectively and precipitated in anhydrous alcohol. The sediments were rinsed by 70% ethanol, dried at room temperature for 5 min, dissolved in aqueous solution contain-

ing 10 μg/ml RNase A, and placed at 37 °C for 20 min. Then they were digested with restriction enzymes and separated on 1% agarose gel electrophoresis. The recombinant virus genome was digested with different restriction enzymes, meanwhile pPolyII-CAV-2 served as the negative control.

Titration of the recombinant virus

The TCID₅₀ of the recombinant canine adenovirus was assayed on 96-well cell culture plate (Nalge Nunc International, Denmark) according to the protocol described elsewhere²⁵.

MP expression from the recombinant virus

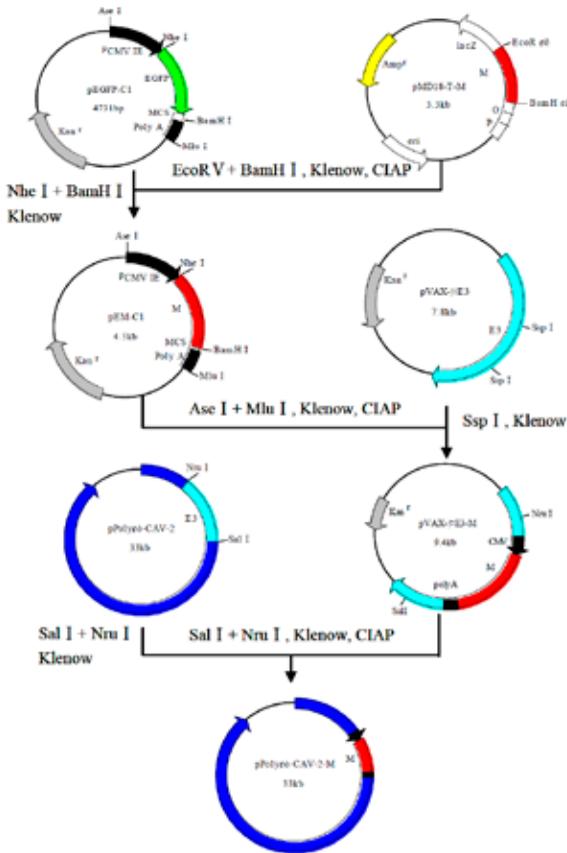
The recombinant virus infected cell lysates were separated on 10% SDS-PAGE, the proteins were blotted onto a nitrocellulose membrane (Pall Corporation) probed with piglet positive serum against PRRSV and detected using horseradish peroxidase-labeled goat anti-pig IgG antibody (Sigma) and chromophore DAB as described elsewhere²⁶.

Immunization of pigs with the recombinant virus

Eighteen piglets, which have not been infected with PRRSV (demonstrated by RT-PCR and indirect ELISA), were purchased from the Changchun Animal Breeding Center for Medicine, Changchun, China, and randomly divided into three groups, six piglets for each group. Groups 1 and 2 were intramuscularly (in the neck) injected only once with 1.5 ml recombinant CAV-2-M (107.8/0.1 ml TCID₅₀) and PRRSV KV vaccine, respectively; group 3 was injected with CAV-2 (negative control) and PBS (blank control) respectively, three piglets for each injection. Blood was collected before inoculation and at an interval of 2 weeks after immunization (p.i.). Sera were separated for detection of specific antibody against PRRSV. The lymphocytes were separated from the spleens of mice for detection of specific cell-mediated immune responses.

Serum antibody against PRRSV via ELISA and western blot analysis

Figure 1. Routes of construct CAV-2 expressing M protein of PRRSV.



MP-specific IgG antibody responses were detected by indirect ELISA based on the purified MP expressed from *E. coli*, with 4µg/ml MP coating the microplate, serum dilution 1:200, HRP-labeled rabbit anti-pig IgG dilution 1:1000, and measuring optical density (OD) at A490.

The new-cultured PRRSV-JL infected Marc145 cell lysates were separated on 10% SDS-PAGE, blotted, and detected as described in the MP expression from the recombinant virus section.

Titration of neutralizing antibodies

Serum neutralizing test to PRRSV-JL was performed as previously described²⁷. Neutralizing activity was expressed as the highest serum dilution that completely prevented the replication of virus in cells.

Lymphocyte proliferation assay

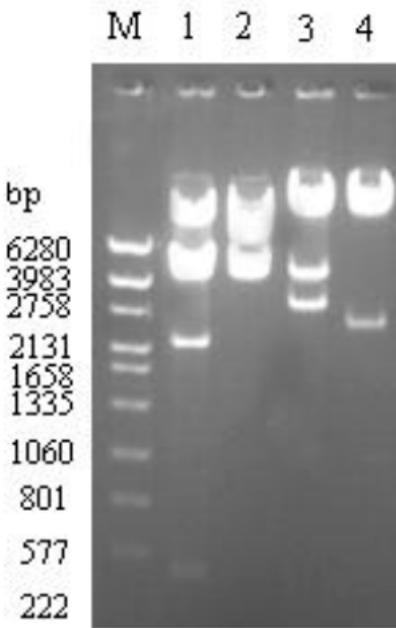
Lymphocyte proliferation assay was performed using peripheral blood mononuclear cells (PBMCs) of immunized piglets. Swine PBMCs were isolated and plated in 96-well flat-bottom plate at 100 µl/well (106 cells/well). Immediately, 100 µl/well of medium with respective 5 µg/ml of CAV-2 and 2 µg/ml of attenuated PRRSV-JL proteins (an extract of PRRSV-JL-infected Marc-145 cells concentrated by ultracentrifugation at 80,000×g for 2 hr) or simple medium (control) was added and mixed. Each PBMCs sample was plated in triplicate. The proliferative activity was measured according to the method described by Bounous et al.²⁸. The stimulation index (SI) was calculated as the ratio of the average A570 value of wells containing antigen-stimulated cells to the average A570 value of wells containing only cells with medium.

RESULTS

Generation of recombinant CAV-2-M

To generate recombinant CAV-2-M (canine adenovirus 2 expressing PRRSV M protein), the M protein (MP) expression cassette, which was driven by the cytomegalovirus (CMV) immediate early (IE) promoter with the SV40 polyadenylation signals²³, was cloned into the plasmid pPolyII-CAV-2. This included the whole CAV-2 genome, producing a recombinant plasmid pPolyII-CAV-2-M. Seven days after transfection with the recombinant pPolyII-CAV-2-M into MDCK cells, typical CPE (grape-cluster-like cells) was observed under a microscope. Adenovirus-like particles were observed under the electron microscope after negative staining of the supernatant of the cell culture with

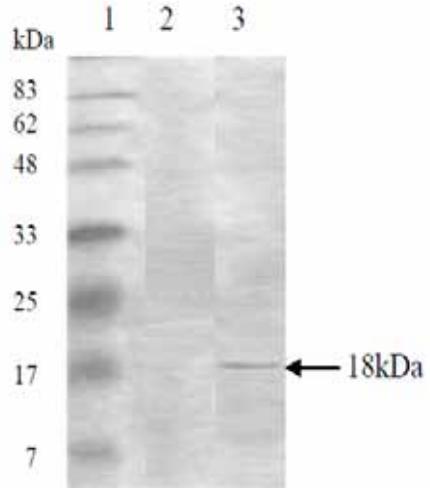
Figure 2. Identification of CAV-2-M by restriction digestion. *pT13 Hind III Marker* (lane M); *CVA-2-M/Xba I* (lane 1); *pPolyII-CAV-2/Xba I* (lane 2); *CAV-2-M/Sal I+Nru I* (lane 3); *pPolyII-CAV-2/Sal I+Nru I* (lane 4).



potassium phosphotungstate. The growth characteristic of the recombinant virus was similar to that of the canine adenovirus vaccine strain YCA18. The recombinant virus was referred to as CAV-2-M.

The identification of the genome from the recombinant virus CAV-2-M by restriction digestion confirmed that the MP cDNA and its expression cassette were included in the recombinant virus (Fig. 1). The XbaI single digestion and the Sall/NruI double digestion obtained six fragments (13288, 5560, 4910, 4730, 2309 and 526 bp) and three fragments (23903, 4606 and 2203 bp) from CAV-2-M, as well as four fragments (23570, 8552, 5560 and 4073 bp) and two fragments (29176 and 3247 bp) from pPolyII-CAV-2 (negative control), respectively (Fig.2). This was consistent with our estimation in advance. The titer of CAV-2-M was stable and the TCID50 based on Kärber

Figure 3. Expression of M protein from virus constructs in MDCK cells via Western blot analysis. Protein size marker (lane 1), CAV-2 (lane 2), CAV-2-M (lane 3).



formula was 107.8/0.1 ml.

MP expression from the recombinant virus

The result of western blot analysis was shown in Fig.3. The molecular weight of the full MP was estimated to be 18,000 Da, which is consistent with the molecule derived from the PRRSV. The results indicated CAV-2-M expressed PRRSV MP that in turn interacted with positive antiserum.

Immune responses in pigs to the recombinant virus

MP-specific antibody responses in piglet sera, which were detected by indirect ELISA based on the purified PRRSV MP derived from E. coli, were measured before immunization and at 4, 6, 8, 10 and 12 weeks p.i. All piglets immunized with CAV-2-M and PRRSV KV vaccine developed ELISA antibodies significantly at 4 weeks p.i. ($P < 0.05$ compared to the control group) and antibodies sustained for 8 weeks until the trials ended, while the control group had no antibodies. The PRRSV KV vaccine could produce higher antibodies titers than that of

Table 1. PRRSV-JL-specific neutralizing antibodies in immunized piglets

| Vaccine | Weeks p.i. | Titers of neutralizing antibodies ^a | | |
|------------------|------------|--|-----|------|
| | | <1:8 | 1:8 | 1:16 |
| CAV-2 | 4 | 3b | 0 | 0 |
| | 6 | 3 | 0 | 0 |
| | 8 | 3 | 0 | 0 |
| | 10 | 3 | 0 | 0 |
| | 12 | 3 | 0 | 0 |
| CAV-2-M | 4 | 6 | 0 | 0 |
| | 6 | 6 | 0 | 0 |
| | 8 | 6 | 0 | 0 |
| | 10 | 6 | 0 | 0 |
| | 12 | 6 | 0 | 0 |
| PRRSV KV vaccine | 4 | 6 | 0 | 0 |
| | 6 | 6 | 0 | 0 |
| | 8 | 3 | 1 | 2 |
| | 10 | 0 | 2 | 4 |
| | 12 | 1 | 2 | 3 |

^a the dilution of antiserum that neutralizes PRRSV-JL to eliminate CPE; b the number of piglets under a certain titer.

the recombinant CAV-2-M (Fig. 4).

In addition, western blot analysis also confirmed that piglets immunized with CAV-2-M developed specific antibodies against the PRRSV MP at 6 weeks p.i. (Fig. 5).

PRRSV-JL-specific neutralizing antibodies were detected at 4, 6, 8, 10 and 12 weeks p.i. As shown in Table 1, piglets inoculated with CAV-2-M developed slightly detectable neutralizing antibodies (the titer is less than 8) at 4 weeks p.i., and these antibodies sustained to 12 weeks until the trials ended. Piglets immunized with PRRSV KV vaccine developed detectable neutralizing antibodies with a high titer (1:8) at 8 weeks, the titer reached the peak (1:16) at 10 weeks. Piglets immunized with CAV-2 (negative control) did not show neutralizing antibodies against PRRSV-JL.

The lymphocyte proliferative responses were analyzed by *in vitro* stimulating the isolated PBMCs of immunized piglets at 4, 6 and 10 weeks p.i. As shown in Fig. 6, vaccination with recombinant CAV-2-M produced significantly higher specific lym-

phocyte proliferative responses than that of the control group ($P < 0.05$), and higher than that of piglets inoculated with PRRSV KV vaccine ($P > 0.05$).

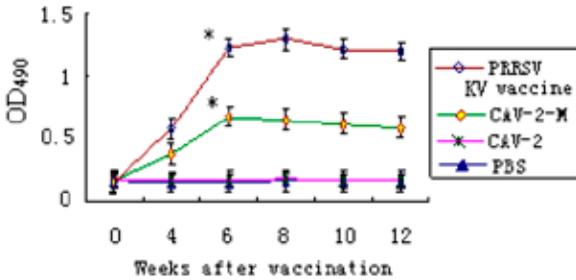
DISCUSSION

Choices of CAV-2 vector and interest gene Adenovirus is one of the major vectors currently used in the research of genetic engineering live vector vaccine. Canine adenovirus infects canines and felids spontaneously, and can express the exogenous gene efficiently as a vector. CAV-2 as a vector has successfully expressed rabies virus glycoprotein gene, foot-and-mouth disease VP1 gene and other viral antigen genes, and achieved satisfactory outcomes²⁰.

M protein (MP), as a non-glycosylated membrane matrix protein, is encoded by PRRSV ORF6. MP is one of the most conservative structural proteins in North American and European PRRSV strains, has strong immunogenicity, can induce the body to produce certain neutralizing antibodies and specific cell-mediated immune response²⁹, and may be associated with the clearance of

Figure 4. Serum MP antibodies in immunized piglets via ELISA. The ordinate: A490. The abscissa: weeks p.i. * $P < 0.05$ compared to the control (injected with CAV-2 and PBS) group.

Fig.4



PRRSV that infects into pigs.

In this study, we adopted the strategy of CAV-2 E3 area partial deletion and inserted MP gene expression cassette into the deletion area to construct recombinant virus CAV-2-M. The recombinant CAV-2-M could stably transcript and express PRRSV MP gene, showing good reactivity.

Construction of recombinant CAV-2-M

The construction of recombinant adenovirus contains two main methods, namely, the in vivo homologous recombination and the in vitro ligation. When conducting the conventional homologous recombination to construct CAV, a gene fragment of E3 area (non-essential area) is first cloned, then subcloned into a plasmid, deleted partially while retaining flank sequences, and then the exogenous gene is inserted into the deletion site of E3 area at the same transcription direction as the E3 promoter. Then, the plasmid, with viral genome together, co-transfects eukaryotic cells and undergoes the homologous recombination in the cells. Finally, the plaque selection and purification are performed to obtain the recombinant adenoviruses. Because of the extremely low recombinant efficiency in eukaryotic cells and because purification is very difficult, it is difficult to obtain the purified recombinant adenovirus using this method.

This study used the in vitro ligation

method. At the beginning, several cloning steps were performed as mentioned in the Materials and methods section. Then, the exogenous gene expression cassette was inserted into the partial deletion site of the E3 area of whole-virus-genome framework plasmid pPolyII CAV-2. The plasmid transfected MDCK cells that appeared pathologically typical and the recombinant adenovirus was successfully obtained. The construction steps are complicated, but the transfection-mediated virus generation is easy without purification. Therefore,

the construction strategy used in this study is convenient and time-saving with high recombination efficiency. Using this method, we have successfully won many strains of recombinant CAV-2 viruses that contain and express the exogenous genes.

Cell transfection is also the key step of recombinant virus. According to the existing experience, the transfection is affected by

Figure 5. Serum MP antibodies (6 weeks p.i.) via Western blot analysis. Protein size marker (lane 1), CAV-2-M (lane 2), CAV-2 (lane 3).

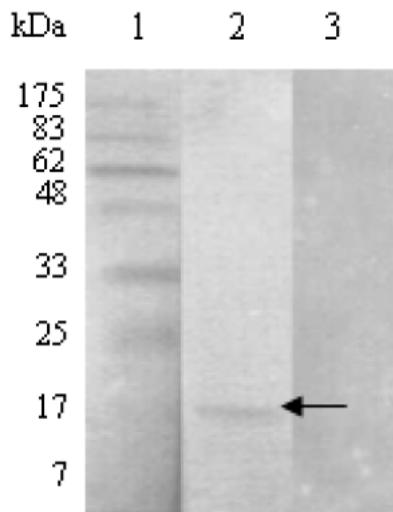
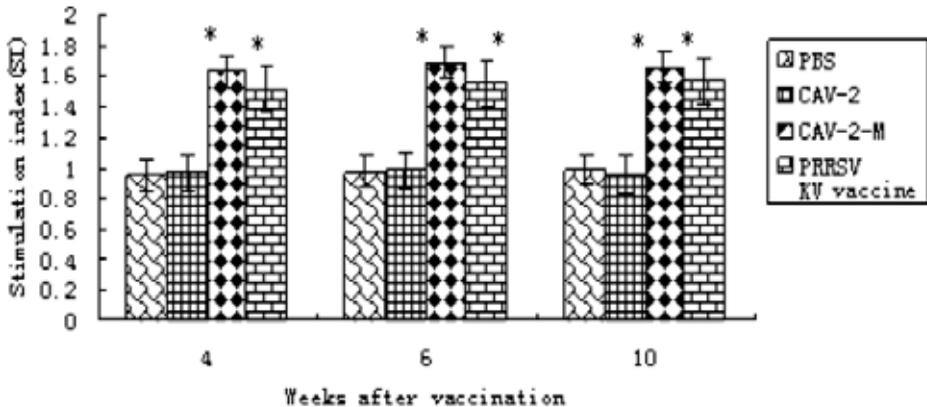


Figure 6. Lymphocyte proliferative responses in immunized piglets. The ordinate: SI(A570). The abscissa: weeks p.i.. * $P < 0.05$ compared to the control group.



many factors, including the cell growth and density, the liposomes and DNA concentrations, the serum in medium, and the gene fragment or plasmid size and purity. Through many trials, we found a satisfactory method: the endotoxin-free supercoiled recombinant framework plasmid and the linearized framework plasmid by double flank restriction digestion co-transfected cells for both the first time and the second time. Both times, co-transfections are completed within 24 h. As results, after three transfections, we successfully obtained multi-strain recombinant canine adenoviruses containing the exogenous genes, including the recombinant CAV-2-M generated in this study. This method improved the achievement ratio of transfection with good reliability and repeatability.

PRRSV infection is now one of the thorniest problems in swine breeding industry and causes tremendous economic losses^{4,5}. Secondary infection, mixed infection, and immunosuppression propose the severe challenges to the current immunization strategy and the vaccine development method. Currently, the commercial vaccines for the protection against PRRS are mainly attenuated and inactivated vaccines. Although these two vaccines play a positive role in the control of PRRS, they are still unsatisfactory. Therefore, many researchers have constructed DNA vaccines, subunit

vaccines and recombinant live virus vector vaccines based on the primary protective antigen GP5 of PRRSV in order to develop new vaccine with enhanced safety and effectiveness. In the research of live equine arteritis virus (EAV) vector vaccines, Balasuriya et al.¹⁷ found the recombinant virus vaccine co-expressing ORF5 and ORF6 could trigger strong humoral immune and cellular immune responses and fight against virulent PRRSV.

This study constructed the recombinant virus CAV-2-M. The immunization test in piglets showed that CAV-2-M could stimulate piglets to produce the specific antibodies against MP, but the neutralizing activities of these antibodies were weak. One possible reason that CAV-2-M induced a low-level neutralizing antibody titer might be that the MP was not correctly folded and the neutralizing epitopes of MP were not completely exposed. A series of modified MP cDNA with epitopes exposed at the surface of a small vector protein is under design and cloning into the CAV-2 vector in our laboratory to investigate an explanation.

In addition, cell-mediated immunity plays an important role in protective immunity against PRRSV³⁰. In the present study, specific lymphocyte proliferative responses were also observed in piglets immunized with recombinant adenovirus CAV-2-M. This finding possibly indicates that CAV-

2-M can serve as a vaccine to provide protection against PRRVS, although the neutralizing activities of the MP antiserum are weak. The mechanism deserves further study.

Post-immunization assessment

CAV-2-M serves as a new kind of genetic vaccine against PRRSV. It is necessary to further evaluate the immunogenicity of the recombinant adenoviruses and determine their ability to protect against infection with virulent PRRSV. In addition, CAV-2-M expressing a single gene can not provide completely efficient protection; therefore, it may be necessary to construct more recombinant virus vaccines containing multiple genes, or conduct co-immunization with recombinant virus vaccines expressing GP5 and GP3, which would be a good subject for future research.

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