

Original Article

Codon optimization of the rabbit hemorrhagic disease virus (RHDV) capsid gene leads to increased gene expression in *Spodoptera frugiperda* 9 (Sf9) cells

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Rabbit hemorrhagic disease (RHD) is contagious and highly lethal. Commercial vaccines against RHD are produced from the livers of experimentally infected rabbits. Although several groups have reported that recombinant subunit vaccines against rabbit hemorrhagic disease virus (RHDV) are promising, application of the vaccines has been restricted due to high production costs or low yield. In the present study, we performed codon optimization of the capsid gene to increase the number of preference codons and eliminate rare codons in *Spodoptera frugiperda* 9 (Sf9) cells. The capsid gene was then subcloned into the pFastBac plasmid, and the recombinant baculoviruses were identified with a plaque assay. As expected, expression of the optimized capsid protein was markedly increased in the Sf9 cells, and the recombinant capsid proteins self-assembled into virus-like particles (VLPs) that were released into the cell supernatant. Rabbits inoculated with the supernatant and the purified VLPs were protected against RHDV challenge. A rapid, specific antibody response against RHDV was detected by an ELISA in all of the experimental groups. In conclusion, this strategy of producing a recombinant subunit vaccine antigen can be used to develop a low-cost, insect cell-derived recombinant subunit vaccine against RHDV.

Keywords: capsid protein, codon optimization, rabbit hemorrhagic disease, *Spodoptera frugiperda* 9 cells

Introduction

Rabbit hemorrhagic disease (RHD) is a highly contagious and lethal infection that affects both wild and domestic rabbits. Its etiological agent, the rabbit hemorrhagic disease virus (RHDV), is considered to be the single most economically important disease of rabbits

worldwide. The disease was first recognised in China and was subsequently identified in other areas of Asia, different European countries, and Mexico [13,17,24]. The etiological agent was classified as a calicivirus, a positive-sense, single-stranded RNA virus that is antigenically related to the European brown hare syndrome virus [23,28]. The first complete genome of the virus was obtained for the German isolate [12]. Subsequently, many whole genomes of RHDV isolates from different countries were sequenced [2,10]. The RHDV genome is about 7.4 kb in length and composed of two narrowly overlapping ORFs: ORF1 and ORF2. ORF1 encodes a polyprotein that is cleaved by a virus-encoded trypsin-like cysteine protease as well as the major structural protein for the capsid (VP60) along with non-structural proteins p16, p23, helicase, p29, VPg, protease, and RdRp. ORF2 encodes a minor structural protein, VP10. Subgenomic mRNA encoding both the structural proteins VP60 and VP10 can also be found in the viral particles. The coat protein has an apparent molecular weight of 60 kDa. A total of 180 copies of this protein are assembled to produce native virus capsids [1,11,19-21].

The lack of a suitable cell culture system for RHDV has hindered large-scale production of the virus as a source of vaccine antigens. Commercially available vaccines are therefore still produced from tissues collected from experimentally infected rabbits. However, this strategy raises serious concerns about biological safety, contaminating residues, and animal welfare issues. During the past 20 years, the capsid (VP60) gene was successfully expressed in several heterologous systems [3-5,8,9] and has been shown to confer full protection against lethal challenge with RHDV in rabbits. For example, Fernández *et al.* [8]

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constructed a single-dose adenovirus vector vaccine against RHDV that induced a potent and long-lasting immune response against RHDV after parenteral or mucosal administration. An insect larvae-derived recombinant subunit vaccine against RHDV was also developed by Pérez-Filgueira *et al.* [25]. The vaccine possessed high levels of antigenicity and immunogenicity, and provided full protection for experimental rabbits. Several recombinant VP60 proteins have been produced in insect cell lines or *Pichia (P.) pastoris*. Nevertheless, the scaling up of insect cell lines and *P. pastoris* in fermentors as well as antigen enrichment are both difficult and expensive. In this paper, we describe an attractive method that significantly improves the expression level of the capsid gene in insect cells by optimizing the VP60 protein codons. The resulting supernatant can be directly used as vaccine antigens without the need for concentration or purification.

Materials and Methods

Optimization of the capsid gene

According to the codon usage frequency of *Spodoptera (S.) frugiperda* cells, the amino acid sequence of the RHDV capsid was optimized online (<http://www.kazusa.or.jp/codon/>). The basic principle was to not change the amino acid sequence of the capsid. A total of 158 bases were changed (Fig. 1). Most of these represented silent mutations, and only two amino acids were changed (D₃₃₄→E and A₅₇₂→T). The opti-Cap gene was generated and synthesized by Shanghai Generay Biotech (China).

Construction of recombinant baculovirus

Recombinant baculoviral DNA harbouring the RHDV VP60 gene (Cap) or optimized VP60 gene (opti-Cap) was prepared using the baculovirus expression vector pFastBac1 (Gibco BRL, USA). The coding regions of

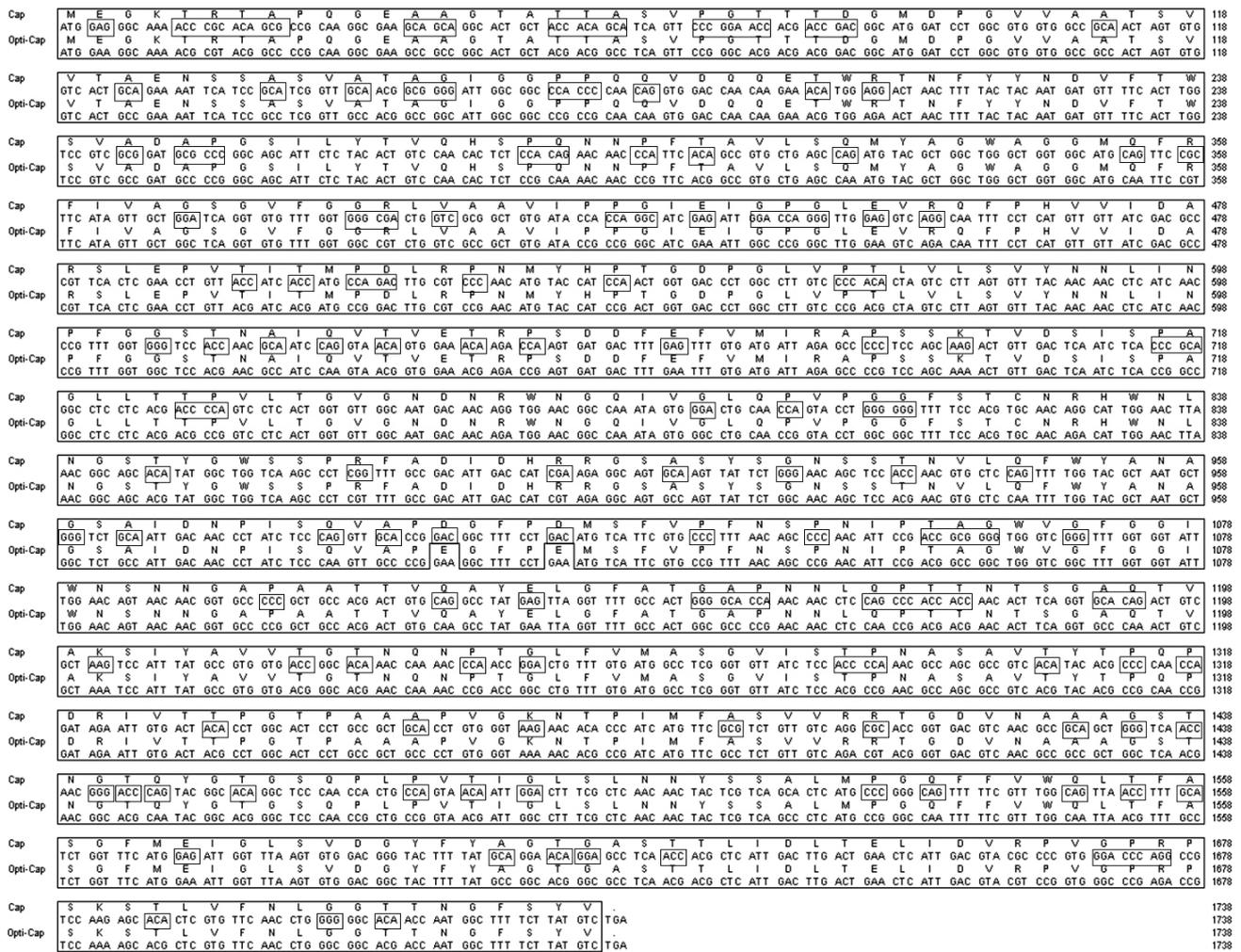


Fig. 1. Sequences of the original and codon-optimized capsid genes. Rare codons of the *Spodoptera frugiperda* 9 (Sf9) cells are marked with boxes.

RHDV Cap or opti-Cap were amplified from the recombinant plasmid pBL-RHDV, which contains the full-length genome of the RHDV strain CHA [16]. The sense primer used to amplify the VP60 gene was Cap-F (5-*GTCGAC*_{SalI} ATGGAGGGCAAACCCG-3) and the antisense primer was Cap-R (5-*AAGCTT*_{HindIII} TTAGACA TAAGAAAAGC-3). The PCR amplification was carried out in a 50 μ L reaction mixture containing 0.2 μ M dNTP, 0.5 mM MgCl₂, 0.2 μ M each primers, 1 \times PCR buffer and 1 U of Platinum Taq polymerase (Invitrogen, USA). PCR thermal profile was 94°C for 3 min followed by 30 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 100 sec, then a final extension step at 72°C for 5 min.

The amplified fragments were digested in reactions at 37°C for 3 h with restriction enzymes *SalI* and *HindIII* (Takara, China) and purified using the TakaRa PCR Cleaning Kit (Takara, China) then inserted into corresponding regions of pFastBac1 (Invitrogen). Identity of the resulting constructs, pFast-Cap and pFast-opti-Cap, were verified by DNA sequencing. The recombinant plasmids were sequenced in both directions using universal pFASTBAC-F and pFASTBAC-R primers. Sequencing was conducted by Sanggong Biotechnology (Sanggong, China). Recombinant baculoviruses (rBv-Cap and rBv-opti-Cap) were generated using a Bac-to-Bac baculovirus expression system (Invitrogen) according to the manufacturer's protocol. Briefly Sf9 insect cells (ATCC, CRL-1711) were maintained in SF900-II serum free medium (Invitrogen) at 28°C and used for transfection with recombinant Bacmid BV DNAs and production of VLPs. A transfection mixture was prepared that contained 250 ng recombinant BV DNA, 6 μ L of Cellfectin (Invitrogen), and 2 mL unsupplemented Grace's Medium (Invitrogen). Following incubation at room temperature for 45 min, the mixture was added to Sf9 cells seeded at 8×10^5 cells per well in a 6-well plate. The transfection mixture and cells were incubated for 5 h at 28°C, after which the transfection mixture was removed and replaced with 2 mL complete growth medium.

Immunofluorescence assay (IFA)

An indirect IFA was used to detect target protein expression in Sf9 cells. Briefly, the cells were fixed in cold methanol: acetone (1 : 1, v : v) at -20°C for 30 min. After three times washed with PBS, cells were incubated at 37°C for 1 h with mouse antiserum specific for VP60 (prepared from Balb/c mouse vaccinated three times with the recombinant RHDV VP60) diluted 1 : 500 in PBS. After washing with PBS three times, cells were incubated at 37°C for 1 h with FITC-conjugated goat anti-mouse IgG antibody (1 : 100 dilution with 0.1% Evans blue solution; ZSbio, China).

Finally, the stained cells washed three times with PBS were observed under a fluorescence microscope equipped with a video documentation system (Nikon, Japan).

SDS-PAGE and Western blot analysis

The recombinant baculovirus infected Sf9 cells were collected and lysed in RIPA lysis buffer (Beyotime Biotechnology, China) for 30 min at 4°C. The lysates were centrifuged for 5 min at 12,000 g at 4°C. The lysates were further denatured by incubation for 5 min at 95°C in sample buffer (2% SDS, 10% glycerol, 60 mM Tris (pH 6.8), 5% β -mercaptoethanol, 0.01% bromophenol blue). The samples were then subjected to 12% SDS-PAGE and stained with coomassie blue or were transferred to nitrocellulose (NC) membranes (Amersham, UK) for western blot analysis. To eliminate possible non-specific binding, the membranes were blocked overnight with 10% skim milk (Sigma-Aldrich, USA) in 0.5% Tween 20 in PBS (PBST) for 1h at room temperature and then incubated with mouse anti-VP60 antiserum (prepared from Balb/c mouse vaccinated three times with the recombinant RHDV VP60) diluted 1 : 500 in PBS. Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (ZSbio, China) diluted 1 : 5,000 in PBS was used as the secondary antibody reacted for 1 h at room temperature. Antibody binding was visualised by 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich) staining. Quantification of protein blots was performed using the Quantity One 1-D software (version 4.4.0; Bio-Rad, USA) on images acquired from an EU-88 image scanner (Seiko Epson, Japan).

Electron microscopy

VP60 VLPs were recovered from the supernatant of infected Sf9 cells, purified by sucrose gradient centrifugation, and diluted in Tris and MgCl₂ (TM) buffer (20 mM Tris, 50 mM MgCl₂, pH 8.0). The VLP samples were applied to glow-discharged carbon-coated grids (TED, UK) for 2 min and negatively stained with 2% (w/v) aqueous uranyl acetate (SPI, USA) at room temperature for 1 min. Images were recorded with a JEM-1230 electron microscope (Philips, The Netherlands).

Immunization experiments and viral challenge

The VLP purification was performed as described with minor modifications [14]. Briefly, the infected cells were lysed by two rounds of freeze and thaw, followed by the addition of NP-40 (Sigma-Aldrich) to a final concentration of 1% and 30 min incubation. The cell debris were removed by centrifugation at $10,000 \times g$ for 30 min, and the supernatant containing the VLPs were purified by sucrose gradient (60 ~ 10%) centrifugation at $80,000 \times g$ for 1.5 h at 4°C (SW 41 Ti ROTOR; Beckman Coulter, USA). The protein concentrations of VLPs was quantified by Bio-Rad protein assay kits (Bio-Rad, USA).

Twelve 10-week-old 2.5 kg New Zealand male rabbits (obtained from the Experimental Animal Center of Shanghai) that were seronegative for RHDV and were randomly divided into three groups of four animals each.

Care and maintenance of all rabbits were in accordance with the animal study protocol 11-08 of the Institutional Animal Care and Use Committee (IACUS) guidelines set by Shanghai Veterinary Research Institute, the Chinese Academy of Agricultural Sciences (CAAS). Rabbits in groups 1 and 2 were injected with 1 ml VLPs suspension each of containing 100 µg native or optimized capsid protein, respectively. Rabbits in groups 3 were injected with PBS only. All of the groups were immunized with same dose on day 0 and boosted on day 14. Blood samples were collected from the marginal ear vein before each round of immunization and 2 weeks after the last injection. The level and specificity of the antibody response against RHDV was subsequently analyzed. All of the rabbits were then challenged intramuscularly with 1,000 LD₅₀ of RHDV (prepared from liver homogenate of rabbits infected with RHDV) 15 days after immunization. The rabbits were examined daily for 7 days after the challenge.

Assessment of anti-RHDV antibody production

To measure antibody titres against RHDV, serological responses were evaluated by an ELISA 0, 7, and 14 days after immunisation. Briefly, the ELISA plate (Corning, USA) well were coated with 50 µL purified RHDV (liver homogenate of infected rabbits diluted 1 : 10 in PBS) overnight at 4°C. The plate was blocked by the assay buffer (5% bovine serum albumin in PBS) and then washed by the wash buffer (0.05% Tween 20 in PBS). After wash, 100 µL collected rabbit serum diluted in 1 : 100 in PBS were added to the wells and incubated for 2 h at 37°C. After 3 more washes, HRP-conjugated anti-rabbit IgG antibody (Kangwei, China) diluted in 1 : 8,000 in PBS was added for incubation for 1 h at 37°C. After color development, absorbance at 450 nm was measured with a Titertek Multiskan spectrophotometer (Titertek-Berthold, USA).

Results

The codon-optimized RHDV capsid gene was cloned into pFastBac1

Based on the codon bias of *S. frugiperda*, we optimized

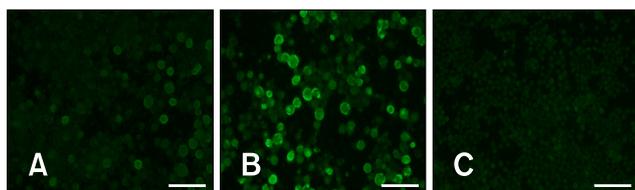


Fig. 2. Detection of RHDV capsid proteins expressed in Sf9 cells by IFA. The expression of capsid (A) and opti-capsid (B) proteins was detected in recombinant rBv-Cap- and rBv-opti-Cap-infected as well as uninfected Sf9 cells (C) with anti-VP60 antiserum as the primary antibody. Scale bars = 100 µm (A ~ C).

158 amino acids by mutating 158 nucleotides (Fig. 1). Most of these mutations were silent but two amino acids were changed (D₃₃₄→E and A₅₇₂→T). Compared to the original VP60 gene, the synthesized opti-Cap gene had a protein sequence identity of 99.5%. This construct was cloned into the pFastBac1 baculovirus expression vector that contains a baculovirus-specific promoter suitable for the expression of proteins in insect cells.

The opti-Cap protein is highly expressed in baculovirus

The expression of capsid and optimized capsid proteins in recombinant baculovirus-infected Sf9 cells was detected with an IFA. As shown in Fig. 2, native capsid and opti-capsid proteins were expressed in Sf9 cells (Fig. 2). The expression level of opti-capsid appeared to be higher (Fig. 2B).

Expression of the recombinant capsid and opti-capsid in Sf9 cells infected with recombinant baculovirus rBv-Cap or rBv-opti-Cap was measured with SDS-PAGE and Western blot analyses. After Coomassie blue staining, lanes corresponding to the rBv-Cap- and rBv-opti-Cap-infected cells contained a major protein band similar in size to the viral VP60 protein from purified RHDV. This major band was not observed in the lane corresponding to the uninfected Sf9 cells (data not shown).

To confirm the identity of this major protein band, cell extracts separated by SDS-PAGE were electrophoretically transferred to NC membranes and incubated with anti-RHDV rabbit serum (Fig. 3). The results demonstrated that the opti-Cap present in the infected Sf9 extracts was

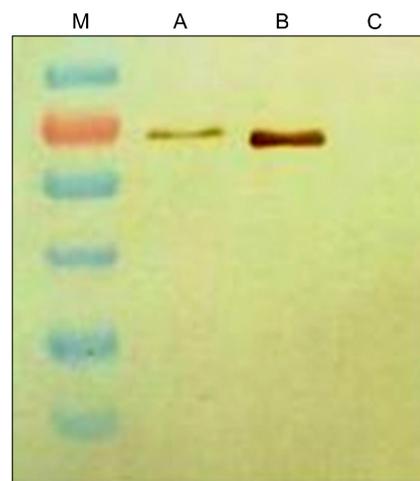


Fig. 3. Western blot analysis of extracts from Sf9 cell expressing the capsid protein using a polyclonal anti-RHDV rabbit serum. Lanes A: baculovirus rBv-Cap-infected Sf9 cells, Lane B: baculovirus rBv-opti-Cap-infected Sf9 cells, Lane C: uninfected Sf9 cells, Lane M: molecular size markers (90, 66, 45, 35, 27, and 15 kDa).

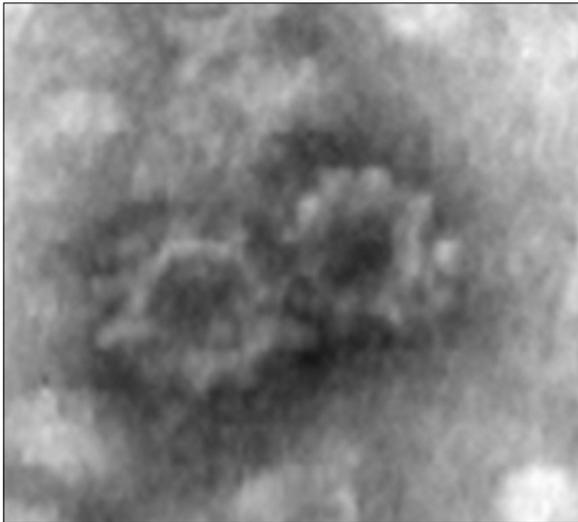


Fig. 4. Electron microscopy evaluation of RHDV-VLP. The sucrose gradient fraction was applied to carbon-coated grids, negatively stained with 2% uranyl acetate, and observed at a magnification of 4,000 \times with electron microscopy.

antigenically similar to RHDV capsid protein (Fig. 3, Lane A and B). However, the opti-Cap band was darker than that of the normal recombinant VP60 protein, indicating that the expression level of this protein in rBv-opti-Cap (Fig. 3, Lane B) was higher than that in rBv-Cap (Fig. 3, Lane A) as expected.

The opti-capsid forms VLPs

Because the optimized capsids were released into the medium, we purified the potential VLPs from both the cell lysates and supernatants with sucrose gradient centrifugation. After centrifugation, a visible band was observed in the middle of the sucrose gradients. Western blot analysis of the sucrose gradient fractions indicated that the visible band contained recombinant capsids composed of 60 kDa polypeptides. These polypeptides reacted with serum from rabbits hyperimmune to RHDV (data not shown), confirming that the optimized capsids were antigenically similar to the native virus. Calicivirus-like particles were observed with Transmission electron microscope TEM in this fraction (Fig. 4).

The opti-capsid induces robust antibody responses to RHDV

Rabbits were immunized with optimized capsid protein released into the cell supernatants and anti-RHDV antibody responses were measured with an ELISA. All of the animals in groups 1 and 2 that were vaccinated with the recombinant capsid protein showed specific antibodies against RHDV 14 days after the first immunization (Table 1). The antibody levels were slightly higher in animals

Table 1. Endpoint titre of specific anti-VP60 antibodies

Animal	Group	Pre-immune	Immune 1 (14 dpi)	Immune 2 (28 dpi)
1		0	1.97	2.49
2	1*	0	1.95	2.58
3		0	1.86	2.50
4		0	1.71	2.68
5		0	1.81	2.30
6	2 [†]	0	1.92	2.15
7		0	1.91	2.10
8		0	1.95	2.13
9		0	0	0
10	3 [‡]	0	0	0
11		0	0	0
12		0	0	0

*Rabbits injected with purified native capsid protein. [†]Rabbits injected with the optimized capsid protein. [‡]Rabbits injected with placebo.

immunized with purified native VP60. Specific anti-VP60 antibodies were not detected in the sera of naive rabbits (group 3). Specificity of the anti-VP60 antibodies was also evaluated with a Western blot analysis (data not shown) just before the viral challenge. All of the rabbits immunized with native VP60 or optimized VP60 were fully protected and survived RHDV challenge. In contrast, the control rabbits died 36~72 h after being challenged with virulent RHDV and developed characteristic signs (clinical manifestations were anorexia and dyspnea, autopsy were liver necrosis and a massive disseminated intravascular coagulopathy in all organs and tissues) of the disease.

Discussion

RHD is contagious and highly lethal in rabbits. Therefore, it is important to prevent and control the disease with effective vaccines. Unfortunately, the lack of an efficient *in vitro* system for propagating RHDV has hindered production of the virus as a source of vaccine antigens [7]. Currently, commercially available vaccines are produced from tissues collected from experimentally infected rabbits. It has been reported that the capsid protein (VP60) of RHDV has good immunogenicity, and rabbits inoculated with enough recombinant VP60 can be fully protected from wild-type RHDV [8,26]. The capsid protein gene has been subsequently expressed in a number of heterologous hosts to obtain a recombinant vaccine against RHDV [5,8,9,18,25]. However, none of these new types of vaccines against RHDV are available for field application. We believe that the main reasons for this are

low expression levels of VP60 and the high cost of antigen purification. Thus, the most important considerations for developing a new type of vaccine are improving antigen expression levels and reducing the production cost.

Codon usage bias has been reported for numerous organisms from viruses to eukaryotes. If a gene contains codons that are rarely used in the host, the corresponding expression level will not be maximized. Codon optimization involves altering the rare codons in a target gene so they more closely reflect the codon usage of the host. This is performed without modifying the amino acid sequence of the encoded protein [6,15,22,27,29].

In the present study, the VP60 codons were optimized according to the codon usage frequency of highly expressed genes in insect cells. Expression of the codon-optimized capsid protein was highly increased in the insect cells while the capability of self-assembly into virus-like particles was preserved. Moreover, we produced promising results in our rabbit immunization experiments by inducing the production of RHDV-specific antibodies after vaccination using the codon-optimized VP60 gene. All of the vaccinated rabbits were fully protected and survived RDHV challenge, indicating that insect cell cultures could be directly used as a candidate vaccine without the need for concentration or purification.

Acknowledgments

This work were supported by the Chinese Natural Sciences Foundation (31270194, 31101848, 31300141), the Fundamental Research Funds for the Central Institutes program (2011JB13, 2013JB07), special fund for Agro-scientific Research in the Public Interest (201303046), and the National Advanced Technology Research and Development Program of China (863 Program) (2011AA10A200).

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