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Adjuvant effects of interleukin-2 co-expression with VP60 in an oral vaccine delivered by attenuated *Salmonella typhimurium* against rabbit hemorrhagic disease

Zhaobin Deng, Yi Geng*, Kaiyu Wang, Zehui Yu, Ping Ou Yang, Zexiao Yang, Changliang He, Chao Huang, Lizi Yin, Min He, Li Tang, Weimin Lai

College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan, 611130, China

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ABSTRACT

Rabbit hemorrhagic disease (RHD) is a highly contagious infection that has caused significant damage to the rabbit industry since 1984. Inactivated vaccines, the currently used prevention measures, are effective in controlling RHD. However, these vaccines are derived from the livers of infected rabbits, which constitutes a major concern in terms of animal welfare and safety. Administration of DNA vaccines in collaboration with appropriate adjuvants, in particular, cytokines, to strengthen the immune response presents a novel optimization strategy to generate more efficient vaccines. In this study, the adjuvant effect of interleukin (IL)-2 co-expression with the VP60 gene in a DNA vaccine was evaluated. In total, four groups of 60 RHD virus (RHDV)-free rabbits (30 days old) were orally or subcutaneously administered recombinant SL7207-pVAX1-IL2-VP60, SL7207-pVAX1-VP60, SL7207-pVAX1 bacteria or the commercial inactive vaccine, and the induced immunity evaluated by challenge with the RHDV(Y8504/China) strain on day 56. The Recombinant SL7207-pVAX1-IL2-VP60 induced a higher level of antibodies than the vaccine SL7207-pVAX1-VP60 and inactivated vaccines to a significant extent. The concentrations of interleukin (IL)-4 were markedly higher than those in groups immunized with the naked or inactive vaccine alone. Furthermore, the fusion gene vaccine provided higher protection (93.33%) after virus challenge relative to immunization with the single gene (SL7207-pVAX1-VP60). The collective results indicate that recombinant SL7207-pVAX1-IL-2-VP60 bacteria exert enhanced protective effects against RHDV and therefore present a strong candidate as a potential vaccine. Moreover, IL-2 enhanced both humoral and cellular responses, highlighting the utility of rabbit IL-2 as an effective adjuvant.

1. Introduction

The first epidemic of rabbit hemorrhagic disease (RHD), a contagious and highly pathogenic disease, occurred in China in 1984, causing the death of millions of rabbits (Liu et al., 1984). In subsequent years, the disease extended to Korea and Italy (Abrantes et al., 2012), followed by other continents (Nowotny et al., 1997). The high lethality of RHD has caused significant damage to the rabbit farming industry and extreme economical losses over the years. Infected rabbits characteristically display high fever (> 40°C), congestion and hemorrhage in spleen, kidney, liver and other solid organs, ring hemorrhages and congestion at the tracheal mucosa (Fuchs and Weissenböck, 1992; Ueda et al., 1992). Death is generally reported within 48–72 h with a ˜90% lethality rate (Park et al., 1995). The etiological agent, rabbit hemorrhagic disease virus (RHDV), is a single-stranded positive-sense RNA virus belonging to the Caliciviridae family (Ohlinger et al., 1993) that consists of 7437 nucleotides encoding two open reading frames (ORF) designated ORF1 and ORF2. The former encodes a polyprotein cleaved by viral proteases into seven non-structural and one major capsid protein, VP60. ORF2 encodes another structural protein, VP10, which is significantly involved in the packaging of genomic RNA. As the main capsid protein and the only protective antigen documented so far, the VP60 gene has been effectively employed for diagnosis and vaccination purposes. Over the past few decades, the VP60 gene has been successfully produced using many heterogenous expression systems and shown to induce immunity against RHDV (Cheng et al., 2013; Qiu et al., 2013; Wang et al., 2012; Yang et al., 2015). However, in terms of costs, protective effects and other aspects, inactivated vaccine is more effective. RHDV does not proliferate in specific cell lines, and therefore, prevention of the disease is mainly achieved through the generation of

* Corresponding author at: Huimin Road No. 211, Wenjiang, Sichuan Province, 611130, China.
E-mail address: gengyisicau@126.com (Y. Geng).

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inactive vaccines produced by livers collected from infected rabbits. Although the currently available vaccine provides effective protection against RHD, serious concerns regarding biological safety and animal welfare have been raised (Pérez-Filgueira et al., 2007).

DNA vaccines are superior to their traditional counterparts in terms of biosecurity since the immune response is induced by partial genes and not the whole virus, preventing the infection caused by occasionally imcompleted inactivation and potential viral spread, in addition to being beneficial for animal welfare (Farnós et al., 2005). Results to date have shown that recombinant VP60 can induce obvious immunogenicity in both prokaryotic and eukaryotic expression systems (Chen et al., 2014; Lópezvidal et al., 2015). However, DNA vaccines remain slightly inferior with regard to protective effects. Several strategies have been developed to improve the immunogenicity of DNA vaccines, among which cytokine adjuvants appear to present an effective approach.

Cytokines are a class of low molecular weight secretory proteins that significantly stimulate humoral and cellular immunity against invasion of bacteria, viruses and other pathogens (Schijns, 2000). IL-2 has been identified as a potential adjuvant for several human and animal vaccines (Baek et al., 2015; Toubaji et al., 2007; Li et al., 2015). IL-2 acts on a wide range of cells and specifically enhances cellular immune responses by induction of cytotoxic T cells, lymphokine-activated killer cells and natural killer cells and promoting the expression of related cytokines produced by T cells (Siegel et al., 1987), for activated B-lymphocytes it also had an obvious stimulation on the secretion of immunoglobulin (Collins and Oldham, 1993). In addition to conventional options, bacterial vectors present a good choice of adjuvant. For example, *Salmonella typhimurium* is not only a type of intestinal bacterium but also an effective adjuvant that contributes to oral delivery of heterologous antigens to the immune system (Qiu et al., 2013).

In the current study, a eukaryotic vector expressing a fusion gene based on IL-2 of rabbits and VP60 of RHDV was constructed, and the recombinant plasmid delivered using attenuated *S. typhimurium* via oral immunization of rabbits. The effects of the vaccine and containing IL-2 as an adjuvant were comprehensively evaluated.

2. Materials and methods

2.1. Bacterial strains, viruses and cells

Attenuated *S. typhimurium aroA* strain SL7207 is maintained in our laboratory (*S. typhimurium* 2337-65 derivative hisG46, DEL407 [*aro*A::Tn10 (Tc^s)]). *Escherichia coli* DH5α cells were purchased from Invitrogen (Carlsbad, CA, USA). Propagated cultures were grown at 37°C on Luria–Bertani plates or liquid medium and 50 μg/mL ampicillin for proliferation of DH5α cells. 293 T cells were cultured in Dulbecco's modified eagle medium (DMEM, Gibco Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), penicillin and streptomycin (100 U/mL). Incubation was performed at 37°C and 5% CO_2. The RHDV strain (Y8504/China) was identified and maintained by our laboratory. Viruses used for were collected from liver homogenates prepared in sterile phosphate-buffered saline (PBS) at a 1:10 (w/v) ratio. Homogenates were stored at −80 °C until use.

2.2. Construction of expression plasmids

Total RNA was extracted from liver of infected rabbits and spleen of healthy rabbits with the RNAiso Plus Kit (Takara, Dalian, China), cDNA synthesis was performed using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China), following the instructions of manufacture and stored at −20°C. To amplify IL-2, VP60 and fusion genes, four specific primer pairs were designed (Table 1) based on the sequences published in GenBank (Accession number: NM_001163180.1 and DQ069282.1) (IL2-L-F and VP60-L-R were the same as IL2-VP60-F and IL2-VP60-R, respectively). The single genes, IL-2 and VP60, were amplified from cDNA, and after gel extraction, products were initially cloned into pMD19-T (Takara, Dalian, China), designated pMD19-T-IL2 and pMD19-T-VP60, respectively. The two recombinant plasmids were used to amplify the fusion gene, IL2-VP60, with the IL2-VP60-F and IL2-VP60-R primer pair. A 6 × His tag of the fusion gene was used to validate expression and the fusion gene cloned into pMD19-T to generate pMD19-T-IL2-VP60. All recombinant plasmids were identified via hydrolysis by restriction endonucleases and DNA sequencing. VP60 and fusion genes were cloned into the eukaryotic expression vector pVAX1 (Invitrogen, Carlsbad, CA, USA) through the *Nhe* I and *Hind* III sites, generating pVAX1-VP60 and pVAX1-IL2-VP60, respectively. The correct sequences were confirmed via hydrolysis by restriction endonucleases and DNA sequencing.

2.3. Transfection into human embryonic kidney 293 T cells in vitro

Endotoxin-free plasmids for transfection were obtained from recombinant bacteria cultured at 37°C for 18 h using the GoldHi EndoFree Plasmid Maxi Kit (CWBIO, Beijing, China), in keeping with the manufacturer's instructions. 293 T cells were cultured at 37°C with 5% CO_2 in six-well plates. pVAX1-VP60 and pVAX1-IL2-VP60 plasmids were mixed with 10 μL transfection reagent (4 μg), respectively, using the pVAX1 plasmid as a negative control. The mixture was diluted in 100 μL preheated Reduced-Serum Medium (Opti-MEM) (Thermo Scientific, Waltham, MA, USA) and incubated at room temperature for 20 min, followed by dropwise addition into 293 T cells with 80% confluence. Next, culturing was performed at 37°C with 5% CO_2 for 6 h without antibiotics, after which growth medium (DMEM supplemented with 10% FBS) replaced Opti-MEM. After 60 h incubation, protein expressed from cells was incubated in lysis buffer, mixed with 6 × loading buffer at a ratio of 5:1 (v/v) and stored at −20°C before use.

2.4. SDS-PAGE and western blot

To assess expression of fusion IL2-VP60 and single VP60 genes and the corresponding translation products, cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot. In brief, fractionated protein was transferred to polyvinylidene fluoride membrane at 150 V for 90 min and blocked in Tris-buffered saline with Tween-20 (TBST) buffer containing 3% bovine serum albumin (BSA) under slow shaking for 2 h. After washing with TBST buffer three times, the membrane was incubated in TBST with 0.5% BSA containing anti-6x His-tagged mouse Immunoglobulin G (IgG) (Abcam, Cambridge, UK) at a ratio of 1:1000 at 37°C with light shaking for 1 h. Following three further washes, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000) diluted in TBST with 0.5% BSA for 2 h at 37°C. After further washing, detection was performed by a chemical chromogenic method with enhanced chemiluminescence and blots developed using a visualizer.

2.5. Construction of the attenuated Salmonella typhimurium vaccine

Attenuated *S. typhimurium* SL7207 was transformed with plasmid DNA by electroporation according to the method of Huang et al. (2014). Competent cells were thawed naturally on ice, transferred into a 0.2-cm cuvette and mixed with 2–5 μg pVAX1, pVAX1-VP60 or pVAX1-IL2-VP60 on ice. To generate recombinant bacteria, the mixture was transferred into SL7207 using a Bio-Rad Gene Pulser (Richmond, CA, USA) at 1800 V, 200 Ω and 25 μF. Bacteria were rapidly removed from the cuvette and added into sterile culture containing 800 μL Lysogeny Broth (LB) with shaking at an appropriate speed for 1 h at 37°C. Recombinant bacteria were collected by centrifugation (1600 × g, 5 min), followed by uniform plating on LB plates. Positive transformants were selected by culturing on LB-agar plates containing 100 μg/mL

Table 1
Primers used for amplification of IL-2, VP60 and IL2-VP60.

Gene Names	Primer names and sequences (5′-3′)	Digestion site	Size/bp
VP60	VP60-F: CGGCTAGC**ATGGAGGGCAAAGCCCGCAC**	*Nhe* I	1760
	VP60-R: CCAAGCTTTCA*ATGATGATGATGATGATG***GACATAAGAAAAGCCATTG**	*Hind* III	
IL-2	IL2-F: CGGCTAGC**ATG**GGT**TACAAAGTACAACTCTTG**	*Nhe* I	482
	IL2-R: CCAAGCTTTTA*ATGATGATGATGATGATG***TGAACTCGATGCTGAGATG**	*Hind* III	
IL2-linker	IL2-L-F: CGGCTAGCATGGGTTACAAAGTACAACTCTTG	*Nhe* I	
	IL2-L-R: CATAGATCCACCTCCACCAGATCCTCCTCCTCCTGAACTCGATGCTGAGATGATGC		
VP60-Linker	VP60-L-F: GATCTGGTGGAGGTGGATCTATGGAGGGCAAAGCCCGCAC		
	VP60-L-R: CCAAGCTTTCA*ATGATGATGATGATGATG*GACATAAGAAAAGCCATTG	*Hind* III	
IL2-VP60	IL2-VP60-F: CGGCTAGCATGGGTTACAAAGTACAACTCTTG	*Nhe* I	2253
	IL2-VP60-R: CCAAGCTTTCA*ATGATGATGATGATGATG*GACATAAGAAAAGCCATTG	*Hind* III	

[a]Histidine coding sequences is in italic and coding sequences of IL-2 and VP60 are in bold.
[b]Sequences "GCTAGC" and "AAGCTT" are restriction sites *Nhe I* and Hind III.

kanamycin for 16 h and further confirmed using Polymerase chain reaction (PCR) analysis followed by restriction digestion with *Nhe* I/*Hind* III. Recombinant strains containing pVAX1, pVAX1-VP60 and pVAX1-IL2-VP60 plasmids were designated SL7207-pVAX1, SL7207-pVAX1-VP60 and SL7207-pVAX1-IL2-VP60, respectively.

2.6. Vaccination and virus challenge

Sixty RHDV-free Chinese white rabbits (30 days old) were purchased from the farm of Sichuan Agriculture University, China, and randomly divided into four groups. The animal lab was fumigated before arrival of the rabbits. A 7-day period of adaptation to the surrounding was allowed prior experimental manipulation. Animals were water-deprived for 8 h while food was supplied normally before vaccination. The first group was subcutaneously injected with 1 mL inactivated vaccine (Nanjing Tianbang Bio-industry Co. Ltd., Nanjing, China), the second group orally immunized with SL7207-pVAX1, adjusting the concentration to 5×10^9 cfu/mL in sterile PBS (1 mL each rabbit), and the third and fourth groups immunized orally with the same concentration of recombinant SL7207-pVAX1-VP60 and SL7207-pVAX1-IL2-VP60 (1 mL each rabbit), respectively. The rabbits in group second-fourth were boost-immunized in a similar manner at 21th and 42th day post-immunization (dpi). All operations were performed in compliance with the guidelines of Institutional Animal Care and Use Committee (IACUC)-approved protocols of Sichuan Agriculture University. Blood samples were drawn from the ear artery at 7, 14, 28, 42 and 56 dpi for detection of antibody levels and cytokine expression.

All rabbits were challenged with the RHDV strain (Y8504/China) at a concentration of 2^8 hemagglutination units (0.5 mL) through intramuscular injection on day 56. Animals were observed daily for clinical symptoms for 14 days. All dead rabbits were submitted to necropsy. The livers were collected and stored at −80 ℃.

2.7. Evaluation of specific antibodies

Antibody levels were determined by indirect ELISA using commercial kits, purchased from Shandong, Binzhou Animal Science & Veterinary Medicine Academy (Binzhou, China). Results were expressed in terms of optical density (OD) at 450 nm of 100-fold diluted serum using a microplate reader. All samples were assayed in triplicate.

2.8. Cytokine assays

The collected serum was used to evaluate the levels of interferon (IFN)-γ and IL-4 belonging to Type 1 and Type 2 helper T cells (Th1-type and Th2-type) cytokine families, which play important roles in humoral and cellular immunity. The assay was performed using commercial ELISA kits for cytokines purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.9. Statistical analysis

Experimental data, including antibody levels, and results of cytokine assays, were compared among the different groups by one-way analysis of variance (ANOVA) in GraphPad Prism version 5.00 (GraphPad Software, La Jolla, CA, USA). All statistical analyses were performed using SPSS software 19.0 (SPSS, Chicago, IL, USA). The differences among the groups were considered significant at P values < 0.05.

3. Results

3.1. Plasmid construction

Both the single gene, VP60, and fusion gene, IL2-VP60 with sizes of 1760 bp and 2253 bp, respectively, were amplified using PCR and the purified products cloned into pMD19-T vector. Plasmids, pMD19-T-VP60, pMD19-T-IL2-VP60 and expression vector pVAX1 were digested with *NheI* and *HindIII* to produce sticky ends for inserting the target genes before ligation. Using T4 ligase, the target genes were cloned into the eukaryotic expression vector, pVAX1, designated pVAX1-IL2-VP60 and pVAX1-VP60, respectively. The correct constructs were confirmed by means of DNA sequencing.

3.2. Expression of VP60 and the fusion gene in 293 T cells

To detect the expression of the recombinant pVAX1-VP60 and pVAX1-IL2-VP60 plasmids, SDS-PAGE and western blot were used. The recombinant pVAX1-VP60 and pVAX1-IL2-VP60, were used to transfect 293 T cells, and the target proteins recognized using anti-6× His tagged mouse IgG, which also provided evidence of pVAX1-VP60 and pVAX1-IL2-VP60 expression. As expected, two bright and single brands with molecular weights of 61 and 85 kDa were detected (Fig. 1A, B).

3.3. Antibody analysis

To evaluate the immunogenicity of the recombinant strains, indirect ELISA was employed and specific IgG induced by the oral vaccine examined (Fig. 2). The total IgG antibody response was observed in all experimental groups at 7th day after the first immunization in addition to the control group, SL7207-pVAX1. At 28 dpi, the IgG response to the fusion vaccine was significantly higher than that to SL7207-VP60 alone ($P < 0.05$). Similarly, the recombinant strain, SL7207-IL2-VP60, induced IgG to a peak level at 42 d after immunization, which was significantly higher than that of the two other experimental groups ($P < 0.05$), while there was no striking difference between the other two experimental groups. Subsequent antibody levels were slightly decreased in all three experimental groups.

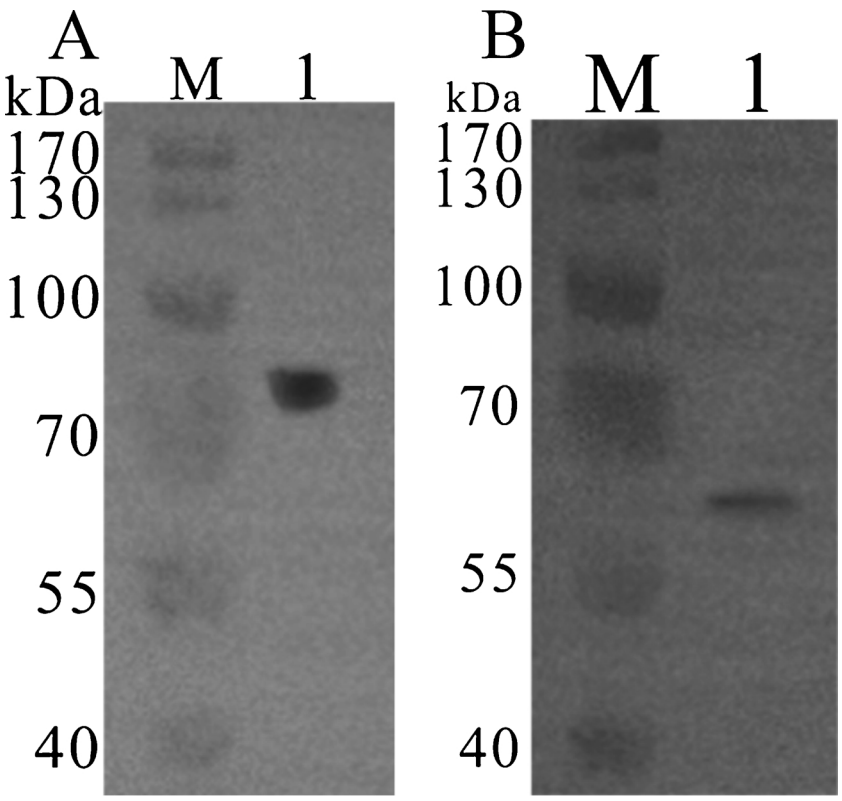

Fig. 1. A, B. Western blot analysis of proteins expressed in 293 T cells, transfected with recombinant plasmids pVAX1-VP60 and pVAX1-IL2-VP60.
A: Western blot analysis of IL2-VP60 protein expression. Lane M, molecular weight marker; Lane 1, pVAX1-IL2-VP60
B: Western blot analysis of VP60 protein expression. Lane M, molecular weight marker; Lane 1, pVAX1-VP60

3.4. Cytokine assay

To evaluate cell-mediated immune responses, ELISA kits were used to detect the concentrations of IL-4 and IFN-γ, primary indicators of Th2 and Th1-type immunoreactions, respectively (Figs. 3 and 4). Mean levels in the control group were stable while the three other groups showed an obvious increase (Fig. 3). In the group immunized with SL7207-pVAX1-IL2-VP60, IL-4 reached peak level at 42 d, which was markedly higher relative to the group inoculated with SL7207-pVAX1-VP60 at 14, 28, 42 and 56 d. There were no significant differences between the fusion and commercial vaccines in terms of IL-4 induction. On the other hand, the IFN-γ concentrations (Fig. 4) were comparable among the three experimental groups, but significantly higher than that in the control group inoculated with SL7207-pVAX1. The IFN-γ concentration in the empty vector group used as a negative control was not significantly changed ($P > 0.05$). Th2 cells are highly involved in controlling humoral immunity because of the production of IL-4, IL-5 and IL-13, furthermore, the presence of IL-2 also plays an important role during the Th2 differentiation (Cote-Sierra et al., 2004). The IL-4 levels induced by fusion SL7207-pVAX1-IL2-VP60 were markedly higher than SL7207-pVAX1-VP60 vaccine, indicating that the SL7207-pVAX1-IL2-VP60 vaccine could promote the Th2 immune response to a significant extent.

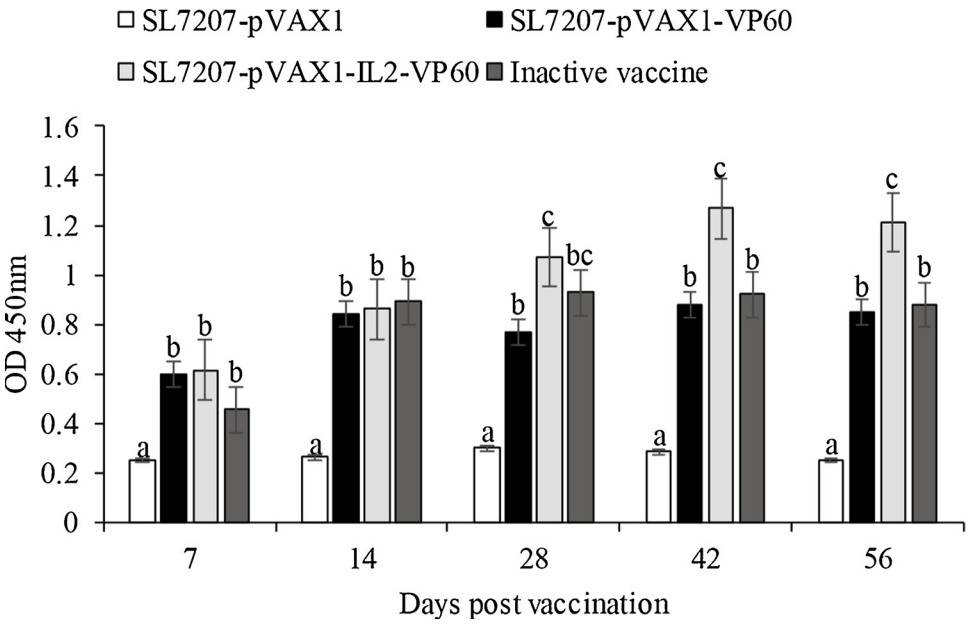

Fig. 2. Kinetic changes of the humoral immune responses in rabbits vaccinated with SL7207-pVAX1, SL7207-pVAX1-VP60, SL7207-pVAX1-IL2-VP60 and commercial vaccine. Serum samples were collected at 7, 14, 28 42 and 56 dpi, and OD values recorded at 450 mm using indirect ELISA. Data are presented as mean ± standard error. Different letters indicate significant differences in antibody levels at the same time ($P < 0.05$).

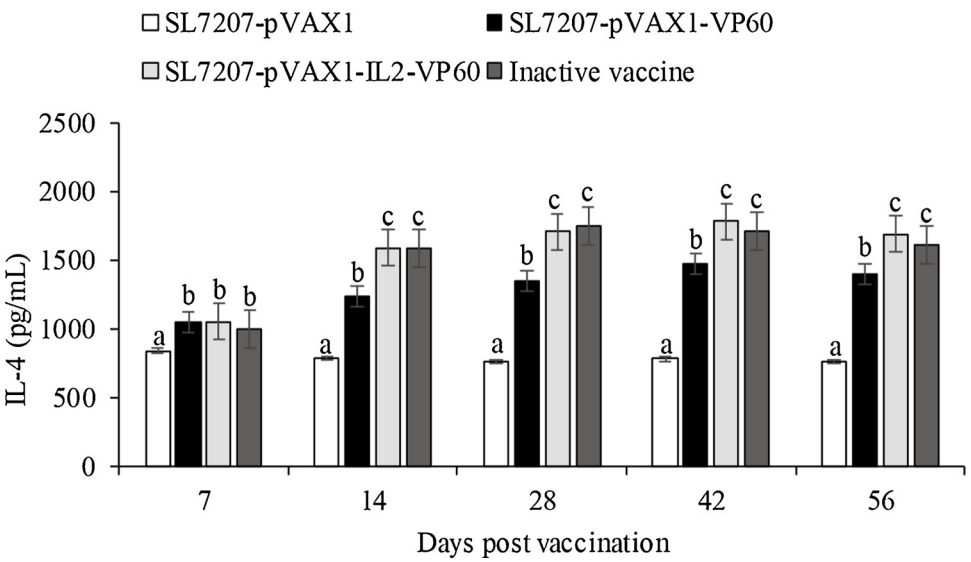

Fig. 3. Analysis of serum levels of the Th2-type cytokine, IL-4. Serum samples were collected at 7, 14, 28, 42 and 56 d, and IL-4 levels examined using a commercially available cytokine ELISA kit (Qiu et al., 2013). Samples were tested in triplicate and significant differences at the same time are indicated by different letters ($P < 0.05$). Data are presented as mean ± standard error.

3.5. Protection against virulent RHDV challenge

To evaluate the protection induced by the vaccine against the RHDV strain (Y8504/China), all rabbits in 4 groups (15 each group) were challenged intramuscularly with RHDV (Y8504/China; 0.5 mL). After 2 weeks, based on anatomopathological examination and RT-PCR findings, dead rabbits were diagnosed with RHDV infection (Fig. 5). Following challenge, all rabbits in the SL7207-pVAX1 group died within 72 h. Dead rabbits exhibited typical clinical symptoms in the liver and lung along with oronasal hemorrhage while no typical clinical signs were observed in surviving rabbits. The protection rate afforded by SL7207-pVAX1-IL2-VP60 was 93.3% higher than that by the single gene, clearly confirming that the fusion gene provides effective protection against RHDV infection.

4. Discussion

RHD, a highly transmittable and lethal disease, has had a devastating impact on both domestic and wild rabbits since the pandemic in 1984 (Cheng et al., 2013). In view of the severity of the disease, prevention and control of RHDV is a critical issue. However, research progression on the development of virus-based vaccines is hindered owing to the lack of a suitable system for propagation *in vitro* (Farnós et al., 2009). The commercially available vaccine effectively prevents RHDV but several concerns remain regarding side-effects, in particular, biological safety and animal welfare (Farnós et al., 2005). To date, no other vaccine candidates have been developed that are superior to the current commercial vaccines. To evoke innate mechanisms with robust and long-lasting immune responses, different types of adjuvants, such as Freund's incomplete/complete adjuvant and aluminous oxide, have been co-immunized with vaccines. However, these are unsuitable for application in humans and animals due to high toxicity and side-effects (Tafalla et al., 2013). Co-expression of cytokine adjuvants with antigen in the same plasmid has been identified as an effective strategy to enhance immunization. Co-delivery of the two ingredients with different vectors has a more efficacious impact than a single gene vaccine (Chen et al., 2010). Significant research has focused on the utility of cytokines as potential adjuvants (Kalams et al., 2012; Tafalla et al., 2013; Xie et al., 2015). The pleiotropic cytokine, IL-2, is activated during both priming for differentiation and maintenance of a differentiated state. The adjuvant effects of IL-2 have been documented in various mammalian models (Baek et al., 2015; Yan et al., 2005). Strikingly, IL-2 not only promotes Th1 differentiation but also plays a key role in the early phase of Th2 differentiation and affects cellular and humoral immunity

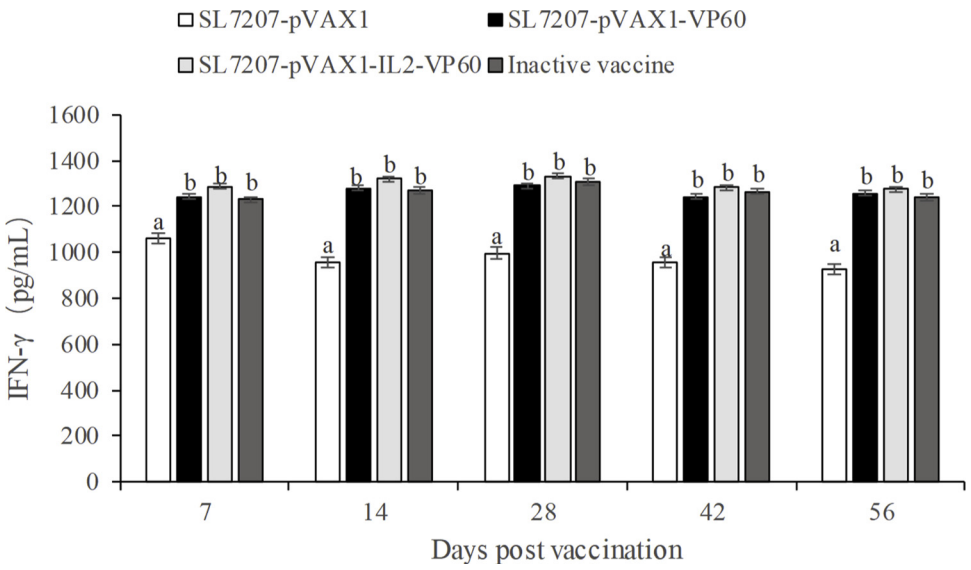

Fig. 4. Production levels of Th1-type cytokine-specific interferon, IFN-γ, in immunized rabbits. Samples were collected at 1, 2, 4, 6 and 8 weeks after the first immunization. Cytokine levels were assessed using a commercially available cytokine ELISA kit (Qiu et al., 2013). Data are presented as mean ± standard error. Different letters indicate significant differences in the IFN-γ levels at the same time ($P < 0.05$).

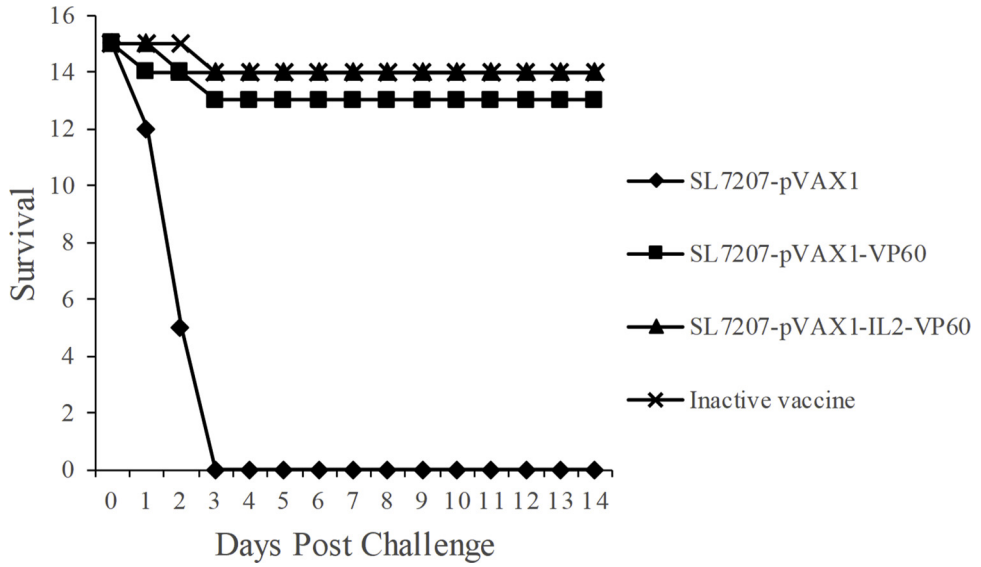

Fig. 5. Challenge results of the RHDV Y8504/China strain. All the rabbits in 4 groups (15 rabbits each group) were challenged with RHDV at 8 weeks after first immunization and survival rates were recorded for 2 weeks after challenge of each group.

by stimulating secretion of IFN-γ and IL-4. However, limited information is available on the potential utility of IL-2 as a cytokine adjuvant in rabbits.

To evaluate the adjuvant effect of rabbit IL-2 for the RHDV antigen, VP60 was co-expressed in the same DNA vaccine. Using the attenuated *S. typhimurium* strain, SL7207, as the antigen carrier, recombinant bacteria with or without IL-2 were generated, and their immunogenicity and protective effects evaluated. Levels of antibody against RHDV, IL-4 and IFN-γ were examined through indirect ELISA. Measurement of the antibody levels before the virus challenge indicated that the humoral immune response against RHDV attack is induced after oral immunization. Notably, antibody levels in rabbits immunized with SL7207-pVAX1-IL2-VP60 were significantly higher than those administered SL7207-pVAX1-VP60 alone and even higher than levels in the group injected with the commercial vaccine. To establish an optimal immune effect, rabbits were boost-immunized with the same dose at 21 and 42 d after the first immunization. The antibody level of the fusion gene vaccine reached a peak, which was significantly higher than that of inactive vaccine, suggesting that co-administration of VP60 and IL-2 effectively stimulates the humoral immune response against RHDV challenge. Relative percentage of survival (RPS), one of the most evident indices to determine the effectiveness of a vaccine, also reflects pathogenic infection (Costa et al., 2011). Our RPS data showed that the SL7207-pVAX1-IL2-VP60 group provides better protection than the SL7207-pVAX1-VP60 group with a 6.67% difference and similar protection to the commercial vaccine.

Antibodies are important for the immune response against RHDV infection but insufficient to evaluate vaccine efficacy. In view of the critical roles of cytokines in the development of cellular immune response and prevention of viral infection, IL-4 and IFN-γ were examined as indicators of Th2-type and Th1-type response. The IL-4 response to SL7207-pVAX1-IL2-VP60 displayed an ascending trend following vaccination and differences between the SL7207-pVAX1-VP60 and SL7207-pVAX1-IL2-VP60-treated groups were significant, consistent with the levels of antibodies induced by both recombinant bacteria. Our observations were similar to earlier findings (Guo et al., 2016). Notably, the recombinant plasmid stimulated higher antibody and similar IL-4 levels relative to inactivated vaccine. Interestingly, however, no obvious changes in IL-4 were documented by in an earlier study by Qiu et al. (2013). The inactivated vaccine is known to stimulate cellular responses to a limited extent but also provides a long-lasting protection for rabbits, suggesting that humoral immunization plays an important role in prevention against RHDV, consistent with our observations. In contrast, we observed no significant changes in IFN-γ that plays a central anti-viral role. Specifically, low upregulation in experimental groups, compared with the control group SL7207-pVAX1, was evident. However, the differences among the experimental groups were not significant. The results suggest that the IL-2 adjuvant activates the cellular response to a limited extent. Our findings are in accordance with earlier studies on IL-2 co-expression with the HA gene of avian influenza virus (AIV) that showed no promotion of the cellular response by the fusion gene vaccine (Hu et al., 2007). According to a report by Lalsiamthara and Lee (2017), the double mutant heat-labile enterotoxin secreted by *Salmonella enteritidis* could stimulate IFN-γ expression. The reasons underlying upregulation of IFN-γ by SL7207 or the fusion gene vaccine are unclear. It is important to note that we performed immunization three times to boost immunity while other reports associated with RHDV VP60 only performed a single immunization step (Guo et al., 2016; Zheng et al., 2016). Further studies are therefore required to confirm these findings and optimize immunization strategies.

Attenuated *S. typhimurium* is widely used as a vector in the development of DNA vaccines, especially for viruses (Kamble et al., 2017; Liu et al., 2018; Zhang et al., 2016). Although *S. typhimurium* is transformed to lose its virulence, the bacterium maintains its invasion ability. After crossing the intestinal mucosal barrier, attenuated *S. typhimurium* is often trapped by antigen presenting cells and delivered to gut-associated lymph tissues, particularly the Peyer's patch, gathering and replicating in macrophages or dendritic cells of gut-associated lymph tissues and gut lymph tissues. However, attenuated *S. typhimurium* dies of nutritional deficiencies and rupture, releasing plasmids, which transfer into host cells to stimulate immune responses.

In conclusion, recombinant SL7207-pVAX1-IL2-VP60 is a potentially effective candidate DNA vaccine for inducing specific antibody and immune responses against RHDV in rabbits. As a potential adjuvant, rabbit IL-2 could strengthen the humoral and cellular responses and provide more potent protection after virus challenge with 93.33% RPS, compared with the naked vaccine. Our results confirm the utility of rabbit IL-2 as a promising adjuvant for vaccine development against RHDV. In view of the limited number of rabbits analyzed, further studies should be implemented to provide detailed evidence on the effects of IL-2.

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References

Abrantes, J., Loo, W.V.D., Pendu, J.L., Esteves, P.J., 2012. Rabbit haemorrhagic disease (RHD) and rabbit haemorrhagic disease virus (RHDV): a review. Vet. Res. 43, 1–19.

Baek, S., Kim, Y.M., Kim, S.B., Kim, C.S., Kwon, S.W., Kim, Y.M., Kim, H.S., Lee, H., 2015. Therapeutic DC vaccination with IL-2 as a consolidation therapy for ovarian cancer patients: a phase I/II trial. Cell. Mol. Immunol. 12, 87–95.

Chen, H.Y., Zhao, L., Wei, Z.Y., Cui, B.A., Wang, Z.Y., Li, X.S., Xia, P.A., Liu, J.P., 2010. Enhancement of the immunogenicity of an infectious laryngotracheitis virus DNA vaccine by a bicistronic plasmid encoding glycoprotein B and interleukin-18. Antiviral Res. 87, 235–241.

Chen, M.M., Song, Y.H., Fan, Z.Y., Jiang, P., Hu, B., Xue, J.B., Wei, H.J., Wang, F., 2014. Immunogenicity of different recombinant rabbit hemorrhagic disease virus-like particles carrying CD8+ T cell epitope from chicken ovalbumin (OVA). Virus Res. 183, 15–22.

Cheng, Y.J., Chen, Z.Y., Li, C.F., Meng, C., Wu, R., Liu, G.Q., 2013. Protective immune responses in rabbits induced by a suicidal DNA vaccine of the VP60 gene of rabbit hemorrhagic disease virus. Antiviral Res. 97, 227–231.

Collins, R.A., Oldham, G., 1993. Recombinant human interleukin 2 induces proliferation and immunoglobulin secretion by bovine B-cells: tissue differences and preferential enhancement of immunoglobulin A. Vet. Immunol. Immunopathol. 36, 31–43.

Costa, A.A., Leef, M.J., Bridle, A.R., Carson, J., Nowak, B.F., 2011. Effect of vaccination against yersiniosis on the relative percent survival, bactericidal and lysozyme response of Atlantic salmon, Salmo salar. Aquaculture 315, 201–206.

Cote-Sierra, J., Foucras, G., Guo, L., Chiodetti, L., Young, H.A., Hu-Li, J., Zhu, J., Paul, W.E., 2004. Interleukin 2 plays a central role in Th2 differentiation. Proc. Natl. Acad. Sci. U. S. A. 101, 3880–3885.

Farnós, O., Boué, O., Parra, F., Martínalonso, J.M., Valdés, O., Joglar, M., Navea, L., Naranjo, P., Lleonart, R., 2005. High-level expression and immunogenic properties of the recombinant rabbit hemorrhagic disease virus VP60 capsid protein obtained in Pichia pastoris. J. Biotechnol. 117, 215–224.

Farnós, O., Fernández, E., Chiong, M., Parra, F., Joglar, M., Méndez, L., Rodríguez, E., Moya, G., Rodríguez, D., Lleonart, R., 2009. Biochemical and structural characterization of RHDV capsid protein variants produced in *Pichia pastoris*: advantages for immunization strategies and vaccine implementation. Antiviral Res. 81, 25–36.

Fuchs, A., Weissenböck, H., 1992. Comparative histopathological study of rabbit haemorrhagic disease (RHD) and European brown hare syndrome (EBHS). J. Comp. Pathol. 107, 103–113.

Guo, H.M., Zhu, J., Tan, Y.G., Li, C.F., Chen, Z.Y., Sun, S.Q., Liu, G.Q., 2016. Self-assembly of virus-like particles of rabbit hemorrhagic disease virus capsid protein expressed in *Escherichia coli* and their immunogenicity in rabbits. Antiviral Res. 131, 85–91.

Hu, H.Q., Pan, Z.M., Zhang, C.Q., Jiao, X.A., Liu, X.F., 2007. The adjuvant effects of chicken IL-2, IL-18, IFN-γ and CpG DNA on HA DNA vaccines against H5 subtype of avian influenza virus delivered by attenuated Salmonella. Chin. J. Microbiol. Immunol. 27, 570–573.

Huang, L.Y., Wang, K.Y., Xiao, D., Chen, D.F., Geng, Y., Wang, J., He, Y., Wang, E.L., Huang, J.L., Xiao, G.Y., 2014. Safety and immunogenicity of an oral DNA vaccine encoding Sip of *Streptococcus agalactiae* from Nile tilapia *Oreochromis niloticus* delivered by live attenuated *Salmonella typhimurium*. Fish Shellfish Immunol. 38, 34–41.

Kalams, S.A., Parker, S., Xia, J., Elizaga, M., Metch, B., Wang, M., Hural, J., Lubeck, M., Eldridge, J., Cardinali, M., 2012. Safety and immunogenicity of an HIV-1 gag DNA vaccine with or without IL-12 and/or IL-15 plasmid cytokine adjuvant in healthy, HIV-1 uninfected Adults. PLoS One 7, e29231.

Kamble, N.M., Hajam, I.A., Lee, J.H., 2017. Orally administered live attenuated *Salmonella typhimurium* protects mice against lethal infection with H1N1 influenza virus. Vet. Microbiol. 201, 1–6.

Lalsiamthara, J., Lee, J.H., 2017. Immunization with *Salmonella Enteritidis* secreting mucosal adjuvant labile toxin confers protection against wild type challenge via augmentation of CD3+CD4+ T-cell proliferation and enhancement of IFN-γ, IL-6 and IL-10 expressions in chicken. Vaccine 35, 767.

Li, Z.R., Tang, X.M., Suo, J.X., Qin, M., Yin, G.W., Liu, X.Y., Suo, X., 2015. Transgenic Eimeria mitis expressing chicken interleukin 2 stimulated higher cellular immune response in chickens compared with the wild-type parasites. Front. Microbiol. 6, 533.

Liu, S.J., Xue, H.P., Pu, B.Q., Qian, N.H., 1984. A new viral disease in rabbits. Anim. Husb. Vet. Med. 16, 253–255.

Liu, S.Y., Jia, R.Y., Qing-Qing, L.I., Feng, D.S., Shen, H.Y., Yang, C., Wang, M.S., Zhu, D.K., Chen, S., Liu, M.F., 2018. Immunogenicity and protective efficacy of DHBV DNA vaccines expressing envelope and capsid fusion proteins in ducks delivered by attenuated Salmonella typhimurium. J. Integr. Agric. 17, 928–939.

López vidal, J., Gómezsebastián, S., Bárcena, J., Nuñez, M.D.C., Martínezalonso, D., Dudognon, B., Guijarro, E., Escribano, J.M., 2015. Improved production efficiency of virus-like particles by the baculovirus expression vector system. PLoS One 10, e0140039.

Nowotny, N., Bascuñana, C.R., Ballagi-Pordány, A., Gavier-Widén, D., Uhlén, M., Belák, S., 1997. Phylogenetic analysis of rabbit haemorrhagic disease and European brown hare syndrome viruses by comparison of sequences from the capsid protein gene. Arch. Virol. 142, 657–673.

Ohlinger, V.F., Haas, B., Thiel, H.J., 1993. Rabbit hemorrhagic disease (RHD): characterization of the causative calicivirus. Vet. Res. 24, 103–116.

Park, J., Lee, Y.-S., Itakura, C., 1995. Pathogenesis of acute necrotic hepatitis in rabbit haemorrhagic disease. Lab. Anim. Sci. 45, 445e449.

Pérez-Filgueira, D.M., Resino-Talaván, P., Cubillos, C., Angulo, I., Barderas, M.G., Barcena, J., Escribano, J.M., 2007. Development of a low-cost, insect larvae-derived recombinant subunit vaccine against RHDV. Virology 364, 422–430.

Qiu, L., Wang, X.L., Hao, H.F., Mu, G.H., Dang, R.Y., Wang, J., Zhang, S.X., Du, E.Q., Yang, Z.Q., 2013. Oral administration of attenuated *Salmonella typhimurium* containing a DNA vaccine against rabbit haemorrhagic disease. J. Virol. Methods 188, 108–113.

Schijns, V.E., 2000. Immunological concepts of vaccine adjuvant activity: commentary. Curr. Opin. Immunol. 12, 456–463.

Siegel, J.P., Sharon, M., Smith, P.L., Leonard, W.J., 1987. The IL-2 receptor beta chain (p70): role in mediating signals for LAK, NK, and proliferative activities. Science 238, 75–78.

Tafalla, C., Bøgwald, J., Dalmo, R.A., 2013. Adjuvants and immunostimulants in fish vaccines: current knowledge and future perspectives. Fish Shellfish Immunol. 35, 1740–1750.

Toubaji, A., Hill, S., Terabe, M., Qian, J.H., Floyd, T., Simpson, R.M., Berzofsky, J.A., Khleif, S.N., 2007. The combination of GM-CSF and IL-2 as local adjuvant shows synergy in enhancing peptide vaccines and provides long term tumor protection. Vaccine 25, 5882–5891.

Ueda, K., Park, J.H., Ochiai, K., Itakura, C., 1992. Disseminated intravascular coagulation (DIC) in rabbit haemorrhagic disease. Jpn. J. Vet. Res. 40, 133.

Wang, X., Qiu, L., Hao, H., Zhang, W., Fu, X., Zhang, H., He, S., Zhang, S., Du, E., Yang, Z., 2012. Adenovirus-based oral vaccine for rabbit hemorrhagic disease. Vet. Immunol. Immunopathol. 145, 277–282.

Xie, X., Wang, L., Yang, W., Yu, R., Li, Q., Pang, X., 2015. Co-administration of antigen with chemokine MCP-3 or MDC/CCL22 enhances DNA vaccine potency. Invest. New Drugs 33, 1–6.

Yan, L., He, Q.G., Yu, X.L., Bei, W.C., Chen, H.C., 2005. The co-administrating of recombinant porcine IL-2 could enhance protective immune responses to PRV inactivated vaccine in pigs. Vaccine 23, 4436–4441.

Yang, D.K., Kim, H.H., Nah, J.J., Song, J.Y., 2015. Rabbit hemorrhagic disease virus variant recombinant VP60 protein induces protective immunogenicity. J. Microbiol. Biotechnol. 25, 1960.

Zhang, D., Huang, X.B., Zhang, X.H., Cao, S.J., Wen, X.T., Wen, Y.P., Wu, R., Liang, E.T., 2016. Construction of an oral vaccine for transmissible gastroenteritis virus based on the TGEV N gene expressed in an attenuated *Salmonella typhimurium* vector. J. Virol. Methods 227, 6–13.

Zheng, X., Wang, S., Zhang, W., Liu, X., Yi, Y., Yang, S., Xia, X., Li, Y., Zhang, Z., 2016. Development of a VLP-based vaccine in silkworm pupae against rabbit hemorrhagic disease virus. Int. Immunopharmacol. 40, 164.