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A Recombinant Baculovirus Expressing VP2 Protein of Infectious Bursal Disease Virus (IBDV) and Chicken Interleukin-18 (ChIL-18) Protein Protects Against Very Virulent IBDV

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Abstract: Infectious Bursal Disease (IBD) is a highly contagious viral disease of young chickens which is characterized by destruction of the lymphoid cells in the bursal of Fabricius; other lymphoid organs are also affected but to a lesser degree. Vaccination against the disease with inactivated or attenuated live vaccines is widely practiced but less effective due to the emergence of very virulent or antigenic variant strains of IBDV in recent years. VP2 is the major antigen of IBDV and contains major epitopes responsible for protection against IBDV. IL-18 can significantly promote T lymphproliferation response to enhance immunological effect of vaccine. In this study, a genetically engineered vaccine candidate, recombinant coexpression protein encoding the *VP2* gene from the IBDV and the mature chicken interleukin-18 (*mChIL-18*) gene has been shown to have the ability to elicit both $CD4^+$/$CD8^+$T cell proliferations and neutralizing antibodies against IBD in chickens. Furthermore, the results demonstrate that recombinant coexpression protein VP2-IL18 (pVP2-IL18) can significantly protect against very virulent IBDV (vvIBDV) in chickens than recombinant protein VP2 (pVP2) and traditional vaccines. These results indicate that the recombinant protein simultaneouly expressing the VP2 protein of the IBDV and the mChIL-18 protein can be potentially used as a vaccine for protection of chickens from vvIBDV.

Key words: *VP2* gene, mature *ChIL-18* gene, coexpression, genetically engineered vaccine, immunogenicity, China

INTRODUCTION

Infectious Bursal Disease (IBD) causes considerable economic losses to the poultry industry worldwide by causing a high rate of morbidity and mortality in an acute form or as a consequence of severe immunosupression provoked by the destruction of immature B-lymphocytes within the bursa of Fabricius (Van den Berg, 2000). Vaccination against the disease with inactivated or attenuated live vaccines is widely practiced. However, such traditional vaccines have become less effective due to the emergence of very virulent or antigenic variant strains of IBDV in recent years (Majo *et al*., 2002). The defect of traditional vaccines is that it only raises humoral immunity while cellullar immune response is very important in cleaning viral infection. A number of studies in mice have shown that genetically engineered vaccines can induce antibody and cell-mediated responses to a variety of bacterial, viral and parasitic antigens (Scheerlinck *et al*., 2001). Furthermore, genetically engineered vaccines are known to efficiently prime the immune response to generate strong antibody and cell-mediated responses when boosted with either recombinant protein (Rothel *et al*., 1997) or viral vectors encoding a vaccine antigen (Hanke *et al*., 1998; Kent *et al*., 1998). In the previous review, the role of $CD4^+$ and $CD8^+$T cells in the cell-regulation of infection was stressed (Scott and Kaufmann, 1991).

IBDV is a member of the birnavirus genus, family Birnaviridae (Brown, 1986). The genome of IBDV consists of two segments of double-stranded RNA named A and B. The larger segment, A is proteolytically cleaved to form the viral proteins (VP) VP2-VP4 by the viral protease VP4 (Birghan *et al*., 2000). A second open reading frame preceding and partially overlapping the polyprotein gene (Spies *et al*., 1989; Bayliss *et al*., 1990) encodes nonstructural protein VP5. Furthermore, VP2 and VP3 are major capsid proteins whereas VP2 is the major antigen of IBDV and contains major epitopes responsible for protection against IBDV (Bayliss *et al*., 1991).

VP2 can stimulate neutralizing antibodies and accordingly induce humoral immuniny *in vivo*. Therefore, the VP2 protein has been used quite effectively in developing subunit/DNA vaccines with other expression

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system against IBDV (Chang *et al*., 2003; Mahmood *et al*., 2007; Rong *et al*., 2007). Protein immunization opens a new approach to the development of gene vaccines for chickens against infectious diseases. The level of protection afforded by genetically engineered vaccine is often inferior to the efficacy of conventional subunit vaccines (Schrijver *et al*., 1997; Chaplin *et al*., 1999). Fordor testified that the polyprotein of IBDV could generate protective reaction but the effect was inferior to traditional attenuated vaccine (Fodor *et al*., 1999). Recently some study shown that plasmid encoding cytokine gene was able to enhance immunological effect of genetically engineered vaccine. Some of the cytokines have been proven to be effective immunomodulator in animal model or clinical test in many reports.

For example, IL1-IL8, IL12 ifN, colony-stimulating factor and TNF, etc., as the immunomodulator, it has been demonstrated that the cytokine adjuvant can promote the vaccines of bacterium, virus, parasite to induce protective immune response and enhance the vaccines effect of immunoprotection (Noll and Autenrieth, 1996) so, the cytokine adjuvant is widely used lately.

IL-18 is one cytokine that has been studied as a vaccine adjuvant and an immunomodulatory molecule. IL-18 is a member of the IL-1 family of proinflammatory cytokines and it was originally identified for its ability to induce high levels of IFN-γ secretion from both NK and T cells. IL-18 is a pleiotropic cytokine whose multiple biologic activities include IFN-γ Natural Killer (NK) cells, stimulation of Fas ligand-mediated cytotoxicity by NK and T cells, enhancement of the production of Granulocyte-macrophage Colony-stimulating Factor (GM-CSF), IL-2 and anti-CD3-induced T cell proliferation inhibition of IgE synthesis by B cells and anti-tumor effects (Manajit *et al*., 1996). Furthermore, IL-18 can promote lymphproliferation response to enhance immunological effect of vaccine.

It is observed that chicken IL-18 could significantly enhance the proliferation responses of T lymphocytes from spleen and B lymphocytes from bursa. Meanwhile, it can raise the neutralization antibody level and enhance the protection against virulent IBDV induced by genetically engineered vaccine (Yu *et al*., 2008). These results indicate that IL-18 may be used in settings where, it is desirable to promote a strong cellular response such as for certain infectious disease vaccines and for cancer immunotherapies.

In this study, the researchers have constructed recombinant protein that coexpressing *VP2* and *IL-18* genes to boost immunological effect of IBDV genetically engineered vaccine. It is the foundation of developing neotype IBDV vaccine.

MATERIALS AND METHODS

Construction of recombinant bacmid: The full-length *VP2* gene of IBDV 2001 strain isolated from Shandong was amplified by PCR with a pair of primers (VP2-XhoI and VP2-KpnI). The forward primer VP2-XhoI, 5'-CTCTCGAG ATGCACCACCACCACCACCACGCAGCGATGACGA AC-3', contained sequences for a XhoI site before the start codon and six histidines after the start codon of the *VP2* gene and the reverse primer VP2-KpnI, 5'-CTGGTACCTAGCCTTAGGGCCCGGATTA TGT-3' included a KpnI site (Fig. 1). The amplified product was subcloned into the corresponding sites in pFastBac™ Dual plasmid (Invitrogen, Fig. 2a) under the control of the baculovirus p10 promoter (Fig. 2b). The *mChIL-18* gene was amplified by PCR with the forward primer mChIL-18-BamHI, 5'-CGCGGATCCATGCACCAC CACCACCACCACGCCTTTTGTAAG-3'whichcontained sequences for a BamHI site before the start codon and six histidines after the start codon of the *mChIL-18* gene and the reverse primer mChIL-18-HindIII, 5'-CGGAAGCTTTA GTCATAGGT TGTGCCT-3' which included a HindIII site. The amplified product was then subcloned into the Multiple Cloning Sites (MCS) under the control of the baculovirus polyhedrin promoter (Fig. 2c) in plasmid pFastBac™ Dual/VP2 (Fig. 2b).

The purified plasmid DNA was transformed into DH10Bac *E. coli* (Invitrogen) which contained a baculovirus shuttle vector to obtain the recombinant bacmids DNA: Bacmid-VP2 (rBac-VP2), Bacmid-mChIL18 (rBac-IL18) and Bacmid-VP2-mChIL18 (rBac-VP2-IL18).

Cells and viruses: *S. frugiperda* (Sf 9) cells were grown at 27°C in Sf-900 II SFM (Invitrogen). Sf9 cells (9×10^5) were plated in 35 mm wells of a six-well plate covered with Sf-900 II SFM and allowed to attach for at least 1 h before transfection using Cellfectin Reagent II (Invitrogen) to obtain recombinant Bacmid-VP2 virus (rBac VP2), recombinant Bacmid-mChIL18 virus (rBac IL18) and recombinant Bacmid-VP2- mChIL18 virus (rBac VP2-IL18). The subsequent selection, plaque purification, amplification and end-point titration were completed according to the manufacturer's instruction. The identification of recombinant baculovirus was carried out by PCR with a pair of primers. The primer sequences were as follows: M13 forward primer, 5'-GTTTTCCCAGTCAC GAC-3' and M13 reverse primer, 5'- CAGGAAACAGCTA TGAC-3'. Expression of recombinant protein: 6×10^5 Sf9 cells were infected with the recombinant baculovirus (rBac virus) at 0.1 M.O.I. and recombinant VP2 protein (pVP2), mChIL18 protein (pIL18) and VP2-mChIL18 protein (pVP2-IL) were harvested at 72 h postinfection. Infected cells were washed with Sf-900 II SFM and broken into pieces by supersonic spallation meter. Cell lysates and

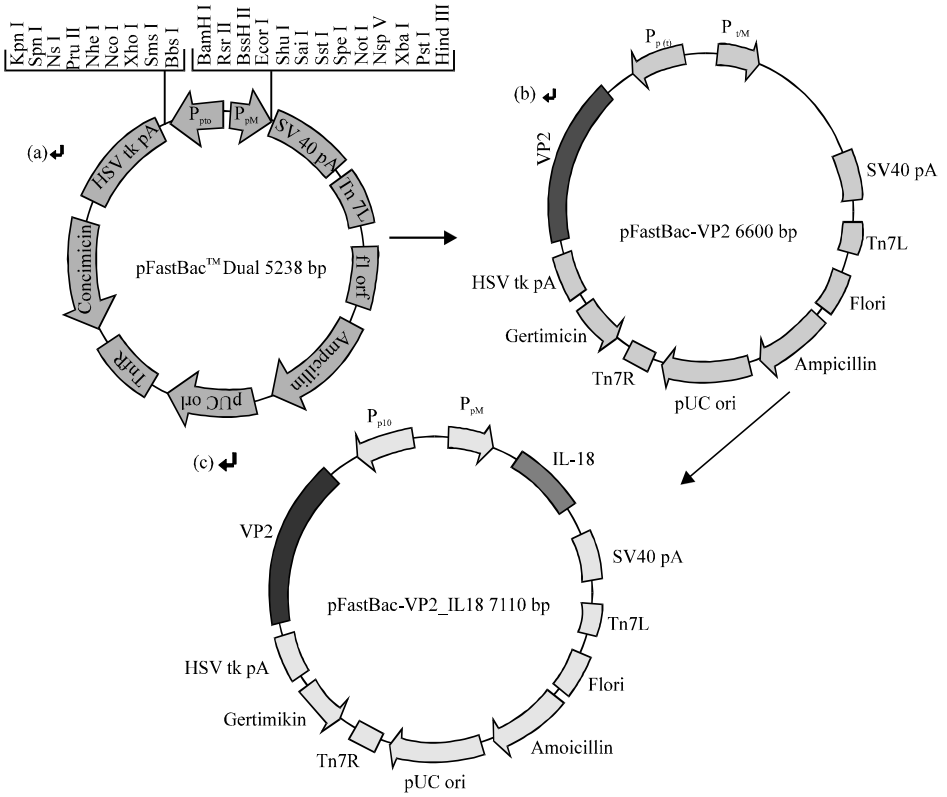

Fig. 1: Gene arrangement on recombinant donor plasmid (pFastBac™ Dual/VP2/IL18). *VP2* gene was subcloned into the corresponding sites in pFastBacDual plasmid under the control of the baculovirus p10 promoter and then *IL-18* gene was subcloned into MCS under the control of the baculovirus polyhedrin promoter in plasmid pFastBac-VP2

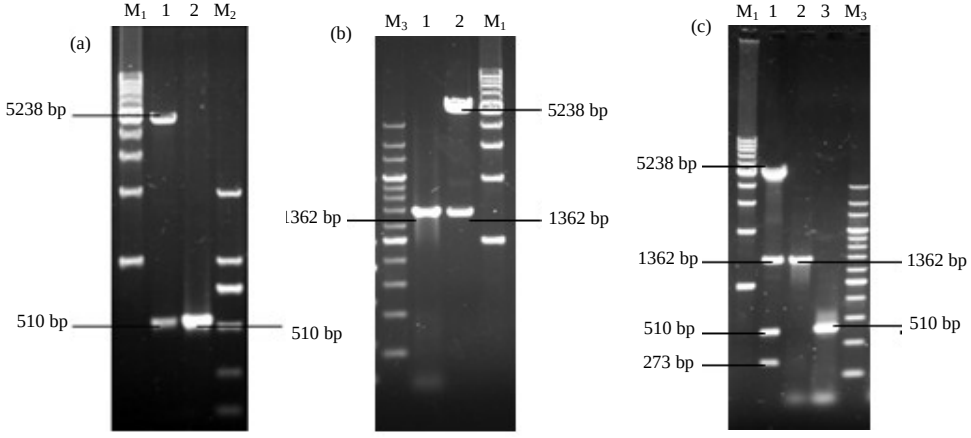

Fig. 2: Agarose gel images of PCR and digested products of recombinant plasmids pFastBac Dual /IL18; a), pFastBac Dual/VP2 and b) pFastBac Dual/IL18/VP2; c). M1: 1 kbp DNA Ladder Marker; M2: DL2,000 DNA Marker; M3: 200 bp DNA Ladder Marker; a) Lane 1: BamHI–HindIII digestion of pFastBac Dual-IL18; Lane 2: PCR products of *IL18* gene; b) Lane 1: PCR products of *VP2* gene; Lane 2: Kpn I–Xho I digestion of pFastBac Dual-VP2; c) Lane 1: BamHI–Hind III and KpnI–XhoI digestion of pFastBac Dual-IL18/ VP2; Lane 2: PCR products of *VP2* gene; Lane 3: PCR products of *IL18* gene

pellets were collected separately after centrifugation and analyzed by the indirect immunofluorescence test. The transfected cells containing recombinant proteins were washed with PBS (pH 7.4, 137 mM NaCl, 2 mM KH_2PO_4, 10 mM $NaHPO_4$ and 2.7 mM KCl) three times and fixed with ice-cold acetone/alcohol (ratio 3:2) for 8 min. After

washing with PBS for three times, the wells were then overlaid with rabbit anti-mChIL18 polyclonal antibody and positive serum of IBDV and then incubated at 37°C for 1 h. The wells were then washed three times with PBS and incubated with TRITC anti-rabbit IgG (Jackson) and FITC anti-chicken IgG (Santa Cruz, 1:200) at 37°C for 1 h. The washing steps were repeated, the wells were covered by 50% (v/v) of glycerine and analyzed by an inverted phase contrast microscope with different fluorescence lights.

Purification of recombinant protein: Ni-NTA-agarose beads (Gibco-BRL) were washed with PBS (pH 7.4, 137 mM NaCl, 2 mM KH_2PO_4, 10 mM $NaHPO_4$ and 2.7 mM KCl) to achieve equality with the baseline. Washed beads were then incubated with the cell disruption supernatant (recombinant protein) for 30 min at 4°C and loaded into 1 mL plastic column. The column was washed with 5 mL of Denaturing binding buffer (pH 6.0, 8M Urea, 20 mM Na_2HPO_4 and 500 mM NaCl) to remove the unbound proteins. A second wash was carried out with 10 column volumes of Denaturing wash buffer (pH 6.0, 8M Urea, 20 mM Na_2HPO_4 and 500 mM NaC_1) to remove the nonspecific proteins bound to the beads. Elution was carried out in Denaturing elution buffer (pH 4.0, 8M Urea, 20 mM Na_2HPO_4 and 500 mM NaCl). The filtrate containing purified recombinant protein was stored in aliquots at -70°C.

Preparation of protein employed immunity: Penicillin and Phytomycin were added into the purified recombinant protein filtrate. The concentration of protein was determined with the Bradford method. Then, the protein was admixed to isochoric Freund's Complete Adjuvant (FCA) for the first immune or Freund's Incomplete Adjuvant (FIA) for the boosted immune.

The proliferative response of T lympholeukocytes: About 2 weeks old Specific-Pathoge n-Free (SPF) chickens were randomly divided into 5 groups (20 chickens per group). Group 1 received 1 μg of chicken IL18 protein and 2 μg of Infectious Bursal Disease vaccine (B78, Strain); group 2 received 2 μg of IL18 and 2 μg of B78; group 3 received 3 μg of IL18 and 2 μg of B78; group 4 received 2 μg of B78 and group 5 served as the normal control. All the chickens were immunized sub-cutaneouly with IL18 protein after B78 at 14th day of age. Six chickens per group were randomly bled via wing veins to prepare anticoagulated blood before immunization at day 14th and weekly after each injection at day 21, 28, 35 and 42th post-immunization. The mouse anti-chicken CD4-FITC Monoclonal Antibody (MoAb) and mouse anti-chicken CD8-RPE MoAb were added into unicell suspension of 1×10^5 cells mL^{-1} which prepared with routine. The data was obtained with flow cytometry then analyzed by SPSS and Microsoft Excel.

Immunogenicity and protective efficacy of the recombinant proteins in chickens: About 2 weeks old, SPF chickens were randomly divided into 7 groups with 20 chickens $group^{-1}$. Group 1 received 200 μg of B78. Group 2 received 200 μg each of pIL18 and B78. Group 3 received 200 μg each of pIL18 and pVP2.

Group 4 received 200 μg of pVP2-IL18. Group 5 received 200 μg B78 in primary immunization and 200 μg pVP2-IL18 in booster immunization. Group 6 received 200 μg of pVP2. Group 7 served as PBS control and the normal control. All the chickens were immunized sub-cutaneouly at 14th day of age. After 2 weeks, chickens were boosted with the protein vaccines by the same route dosage.

Detection of T lymphocyte sub-type: Six chickens per group were randomly selected from each group to detect the change of T lymphocyte sub-types with mouse anti-chicken CD4/FITC (Southern biotech) and mouse anti-chicken CD8a/R-PE (Southern biotech).

The chickens were bled via wing veins to prepare anticoagulated blood before immunization at day 14th and weekly after each injection at day 21, 28, 35, 42 and 49th post-immunization. The following experiment was progressed by the same method. The ivory lympholeukocyte layer was isolated by adding the lympholeukocyte separating medium into prepared anticoagulated blood.

The ivory lympholeukocyte layer was gathered and washed twice with PBS (pH 7.4, 137 mM NaCl, 2 mM KH_2PO_4, 10 mM $NaHPO_4$ and 2.7 mM KCl) and then added the mouse anti-chicken CD4-FITC MoAb and mouse anti-chicken CD8-RPE MoAb. The unicell suspension of 1×10^5 cells mL^{-1} was prepared and detected by FACS.

Detection of anti-IBDV antibody titer by ELISA: Six chickens per group were randomly selected to detect the antibody titer. A commercial antibody-capture ELISA kit for IBD (IDEXX) was used to determine the serum anti-IBDV antibody titer. The chickens were bled via wing veins to prepare serum before immunization at day 14th and weekly after each injection at day 21, 28, 35, 42 and 49th post-immunization. The serum were clarified and kept at -20°C. The relative level antibody titer was determined by calculating the Sample to Positive (S/P) ratio. Endpoints titers were calculated by the equation:

$$\log 10 \text{ titer} = 1.09 (\log 10 S/P) + 3.36 \text{ (IDEXX)}$$

Protective efficacy experiment: At week 2 post-boost, chickens of each group were challenged with 100 LD50/0.2 mL of vvIBDV by the eyedrop route. Protection rate was initially calculated for each challenge group based on gross bursal lesions.

RESULTS AND DISCUSSION

Construction of recombinant plasmids: The constructed recombinant plasmids were confirmed by PCR amplifications using appropriate primer combinations and enzyme digestions (Fig. 2). Sequence analysis of pFastBac™ Dual/IL18, pFastBac™ Dual/VP2 and pFastBac™ Dual/VP2/IL18 recombinants also confirmed the sequence identity and the correct reading frames. Each recombinant construct was sequenced twice from both directions to confirm the ligation of the insert and fusion of the *IL18* and *VP2* genes.

Identification of recombinant baculovirus: Recombinant bacmid DNA is >135 kb in size and the restriction enzyme analysis is difficult to perform. Therefore, the researchers used PCR analysis to verify the presence of the gene of interest in the recombinant bacmid. The bacmid contains pUC/M13 forward and reverse priming sites flanking the mini-attTn7 site within the lacZ-complementation region to facilitate the PCR analysis (Fig. 3a). The size of PCR product was calculated by 2560 bp mini-attTn7 plus size of insert gene on the basis of Bac-to-Bac Baculovirus Expression system user manual (Fig. 3b). The size of amplified fragments for rBac IL18, rBac VP2 and rBac VP2-IL18 was respectively 3070, 3922 and 4432 bp.

Indirect immunofluorescence test of recombinant protein: Indirect Immunofluoresence Antibody Test (IFAT) was performed to further confirm the expression of the recombinant protein. The cells transfected with rBac VP2-IL18 exhibited bright red fluorescence and bright green fluorescence, respectively by rabbit anti-mChIL18 polyclonal antibody linked TRITC anti-rabbit IgG (Fig. 4a) and positive serum of IBDV linked FITC anti-chicken IgG (Fig. 4b) in the same eyesight. It demonstrated that IL18 protein and VP2 protein were simultaneously expressed in sf 9 cells. No fluorescent staining was detected from nontransfected cells and cells transfected with pFastBac™ dual only (Fig. 4c).

The proliferative response of T lympholeukocytes with pIL18: Short-term proliferation assays were carried out to determine the capability of VP2 to induce proliferation of T lympholeukocytes in Peripheral Blood (PB) in chicken with FACS. Different concentrations of VP2 protein were used (0.1 and 10 μg μL^{-1}). All experiments were performed in the absence of IL18 protein. The results demonstrate that VP2 protein was not able to induce significant proliferation of $CD4^+$ and $CD8^+$ T lympholeukocytes in chicken. We were then interested in whether the generation of an inflammatory environment by supplementation different small amounts of IL18 (0.1, 0.2 and 0.3 ng μL^{-1}) could enhance the proliferative response against VP2 protein (1 μg μL^{-1}). In the presence of IL18, proliferation in response to VP2 protein could be strongly enhanced in $CD4^+$ (Fig. 5a) as well as in $CD8^+$ (Fig. 5b) T lympholeukocytes. The dominance was non presentation with high concentration of IL18 while 0.2 ng μL^{-1} was found to be optimal concentration (Fig. 5a, b).

Detection of T lymphocyte sub-types: The number of $CD4^+$ and $CD8^+$T lympholeukocytes in peripheral blood were detected by FACS. As showed in Fig. 6, the number of $CD4^+$ and $CD8^+$T lympholeukocytes in peripheral blood of chickens immunized with genetically engineered vaccine were obviously higher than the control group and only B78 vaccine immunity class which demonstrated satisfactory Cell-mediated Immunity (CMI) induced by genetically engineering vaccine. PVP2-IL group was always higher than pVP2+pIL group and B78 group and

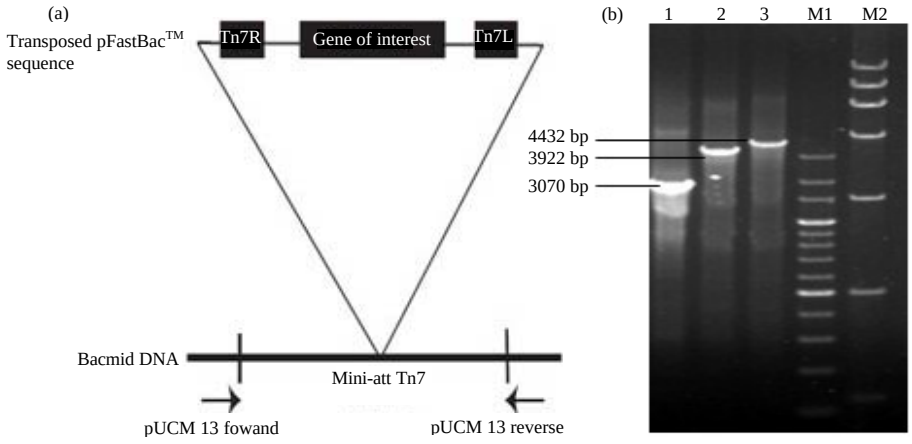

Fig. 3: PCR identification of recombinant baculovirus; a) Map and features of transposed pFastBacTM sequence and b) Agarose gel electrophoresis of rBac. M1: 200 bp DNA Ladder Marker; M2: DL15,000 DNA Marker; Lane 1: PCR products of rBac IL18; Lane 2: PCR products of rBac VP2; Lane :3 PCR products of rBac VP2-IL18

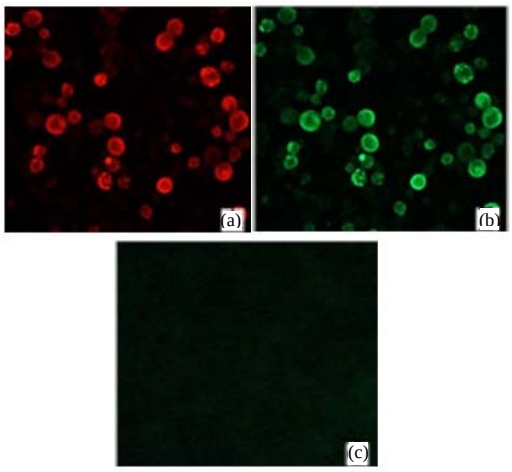

Fig. 4: The expression of VP2 and chicken IL-18 proteins in cells transfected with rBac VP2-IL18 was visualized respectively by different excitation lights in the same eyesight. Expression of pVP2-IL18 was visualized as fluorescent cells in bright field (a and b). Sf9 cells transfected with pFastBac™ dual were used as a negative control (c)

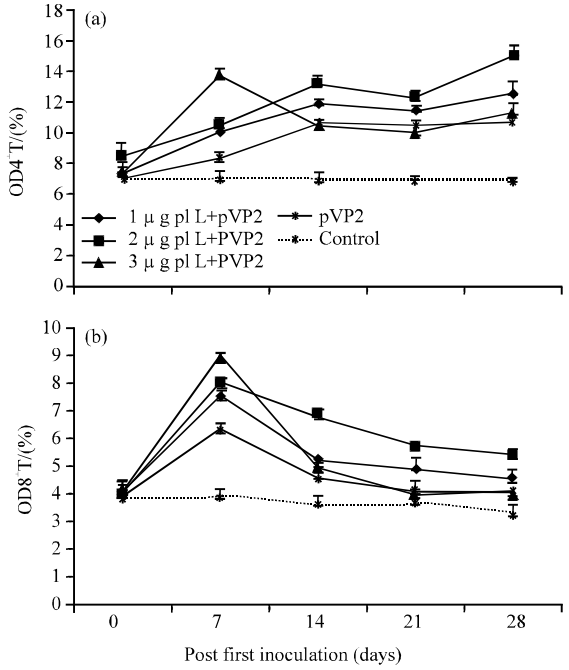

Fig. 5: IL18 at different concentrations enhanced the proliferative response of T lympholeukocytes (n = 6); a) Proliferative response of $CD4^+$T lympholeukocyte and b) Proliferative response of $CD8^+$T lympholeukocyte

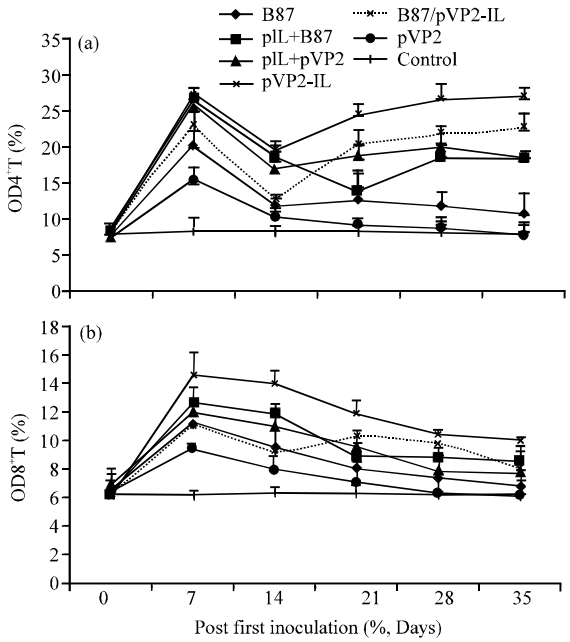

Fig. 6: Different proteins enhanced the proliferative response of T lympholeukocytes (n = 6); a) Proliferative response of $CD4^+$T lympholeukocytes and b) Proliferative response of $CD8^+$T lympholeukocytes

then presented significant difference. The number of $CD4^+$T lympholeukocytes in pVP2-IL immunized group kept increasing tendency and longer persistence comparing with the B78 group after the secondary immunization (Fig. 6a). The number of $CD8^+$T lympholeukocytes of group pVP2-IL kept decreasing tendency with higher percentages comparing with the B78 group after the secondary immunization (Fig. 6b).

Detection of antibody titers of IBDV: Peripheral blood anti-IBDV antibody titers of chickens immunized with different vaccines were detected by ELISA. The control chickens inoculated with PBS control and the normal control showed no evidence of any anti-IBDV antibody response (Table 1). In addition, the antibody titers remained relatively low and no-significant differences among all groups except from the control group in 7 days after the first immunization in most of the chickens. However, anti-IBDV antibody titers of all experiment groups were presented upgrade tendency after secondary immunity and up to maximum in 4 weeks post inoculation. The antibody against IBDV detected in six chickens immunized with pVP2-IL was always higher than other groups at day 7, 14, 21, 28 and 35th post-immunization. It presented significant difference ($p<0.05$) between

Table 1: Peripheral blood anti-IBDV antibody titers in chickens immunized with different vaccines

Groups	Before inoculation (0 day)	Post inoculation 7th day	14th day	21st day	28th day	35th day
B78	362.7±23.6	1834.6±26.8	1944.4±14.2	2276.9±8.90	2585.3±7.20	2151.7±7.00
pIL+B78	303.2±34.7	1834.6±41.3	2013.3±23.3	2712.4±24.5	3282.8±34.1	2925.2±34.5
pIL+pVP2	410.9±26.8	1698.0±31.1	1848.3±30.6	1972.0±17.6	2304.8±37.4	1999.5±29.9
pVP2-IL	362.7±17.6	2068.6±27.3	2500.9±29.4	4066.0±31.1	4639.6±29.7	3890.8±36.6
B78/pVP2-IL	410.9±28.0	1807.2±16.4	1972.0±24.1	3168.0±31.5	4080.6±27.6	3297.2±36.6
pVP2	350.8±30.0	1188.0±17.2	696.0±38.0	978.2±20.0	683.3±35.7	484.0±32.1
Control	315.1±19.3	423.0±19.4	327.0±34.4	386.7±29.9	268.0±34.6	210.1±29.4

Table 2: The mortality and protection rate of different groups challenged by vvIBDV

Groups	No. of death/total	No. of infection/total	Rate of protection (%)
B78	2/20	3/20	75
pIL+B78	1/20	4/20	75
pVP2	4/20	6/20	50
pVP2-IL	0/20	2/20	90
B78/pVP2-IL	1/20	3/20	80
P IL+pVP2	2/20	4/20	70
Control	14/20	20/20	0

group pVP2-IL and group B78 at day 14, 21, 28 and 35th post-immunization. In addition, it also presented significant difference ($p<0.05$) between group pVP2-IL and group pVP2+pIL at day 7, 14, 21, 28 and 35th post-immunization. Comparing with group B78, anti-IBDV ELISA antibody titer of group pVP2 was lower but non-significant difference. The anti-IBDV ELISA specific antibody titer was boosted in the present of pIL+B78 comparing with only B78 while non-specific antibody in the present of only pIL and only PBS control.

Protection efficacy against challenge with IBDV: The groups used in the animal studies were shown in Table 2. Different quantitive chickens of all groups were dead except pVP2-IL group. No dead chicken was showed only in group pVP2-IL.The chickens immunized with pVP2-IL had demonstrated 90% protection against IBDV challenge while chickens first immunized with B78 and booster immunized with pVP2-IL had demonstrated 80% protection. The chickens immunized with B78 and pIL+ B78 also demonstrated 75% protection against IBDV challenge. However, the number of dead chickens showed 2 in group B78 while the number of dead chickens showed 1 in group pIL+ B78. The chickens immunized with pVP2 had merely demonstrated 50% protection against IBDV challenge. All chicken of conteracting toxic sub-stances control group show up typical clinical symptom, pathological change and histology pathological changes. Recombinant vaccines have recently been shown to generate protective immune responses in a number of expression system (Chang *et al*., 2003; Hu *et al*., 2006; Oveissi *et al*., 2010) in the poultry industry. Baculovirus/insect cell system is also used widely for recombinant protein production (Fodor *et al*., 1999; Gao *et al*., 2007). At present, passive protection of chickens against IBDV is achieved by vaccinating breeding hens with conventional attenuated or inactivated IBDV vaccines. Maternal antibodies are transmitted to the progeny through the egg yolk and provide protection to chickens for the 1st few weeks of life. However, some chickens immunized with the live attenuated vaccines also show certain degree of bursal atrophy and are not fully protected against IBDV infection (Giambrone and Closser, 1990; Rong *et al*., 2001). Since, these strains still retain some degree of pathogenicity, young chickens vaccinated with the intermediate live attenuated IBD vaccines which can induce moderate bursal atrophy may have immunosuppression interfering with vaccination against poultry diseases (Giambrone *et al*., 1976).

With so many disadvantages associated with the currently available live attenuated vaccines against IBDV infection, the search for a new approach to improve the vaccine or to produce new vaccines is warranted. The VP2 contains the antigenic regions responsible for neutralizing antibodies and serotype specificity (Fahey *et al*., 1989). During the past decade, successful expression of VP2 protein of IBDV has been reported in several systems (Macreadie *et al*., 1990; Tsukamoto *et al*., 1999; Yu *et al*., 2002). Although, prokaryotic expression system such as *E. coli* is simple, it lacks the modification mechanism of eukaryotic expression. Post-translational modifications of eukaryotic systems such as glycosylation, disulfide bond formation and proteolytic processing can be performed in insect cells. Previous studies demonstrated that virus infected insect cells (Trimble *et al*., 1991) was capable of expressing glycoproteins processed in a manner similar to *P. pastoris*. To provide a source for subunit vaccine or a diagnostic reagent, the VP2 protein of vvIBDV that induces neutralizing antibodies (Fahey *et al*., 1989) was expressed in the recombinant baculovirus expression system. In vaccinal immunization effect test, latency period of genetically engineering vaccine was longer than B87 attenuated vaccine while both of protection effect was equivalency.

Accordingly, IL possessing molecule immunologic adjuvant effect was used in boosting immunization effect of IBDV genetically engineering vaccine (Yu *et al*., 2002).

In most of these studies, IL-18 enhanced the development of Th1-driven antigen-specific T helper and cytolytic immune responses. The combination pPSA/pIL-18 vaccine also induced stronger and more rapid $CD4^+$ and $CD8^+$T cell responses than pPSA alone based on the proliferation and cytolytic assays and the proportions of antigen-specific T cells detected by flow cytometry (Marshall *et al*., 2006). We investigated the immunostimulating activities of recombinant His-tagged chicken IL-18 (rHis-ChIL-18) on immunization-induced not only the proliferation of $CD4^+$T cells but also antigen-specific antibody responses (Degen *et al*., 2005).

The data demonstrated that low amounts of IL18 could strongly enhance in $CD4^+$ and $CD8^+$T lympholeukocyte. In addition, the dominance was non-presentation with high concentration of IL18 while 0.2 ng mL^{-1} was found to be optimal concentration (Fig. 5). IL had double characters of adjusting organismal normal function with suitable dose while causing side effect with bulk (Jiang and Li, 2003). This study clearly demonstrates that pIL18 is a strong adjuvant that increases vaccine potency as measured by its ability to induce the generation of significant numbers of antigen-specific $CD4^+$ and $CD8^+$T cells and boost of anti-IBDV antibody titer when immuned with pVP2-IL genetically engineered vaccine. The data is consistent with the results of other immune response study (Hung *et al*., 2010). The number of $CD4^+$ and $CD8^+$T lympholeukocyte in peripheral blood of genetically engineered vaccine immunity class were obviously higher than control group and only B78 vaccine immunity class which demonstrated satisfactory CMI induced by genetically engineered vaccine (Fig. 6).

Group pVP2-IL was always higher than group pVP2+pIL and group B78 and then presented significant difference (Fig. 6). The data are consistent with the results of other immune response studies (Stephen *et al*., 2002; Wong *et al*., 2002). ELISA detection shown significant difference ($p<0.05$) between group pVP2-IL and group B78 or group pVP2+Pil (Table 1). Group pVP2-IL had demonstrated 90% protection against IBDV challenge while group B78 attenuated vaccine demonstrated 75% protection and group pVP2 merely demonstrated 50% protection (Table 2). In previous studies, a DNA vaccine using P/VP243/STC in SPF chickens provided 80-100% protection against IBD (Chang *et al*., 2001).

It had been shown that vaccination of susceptible chickens with VP2 expressed by many prokaryotic and eukaryotic expression systems could induce virus-neutralizing antibody response and protected chickens against IBD infection (Wu *et al*., 2004). But those productions of subunit vaccine were less biology safe than Baculovirus expression system was employed. So, it could be safe for us to produce the protein vaccine using the new expression system. In addition, the fact of IL-18 appears to enhance all aspects of the cellular and humoral immune response suggests that it is a broadly effective Th1 adjuvant that could be useful in many settings.

CONCLUSION

In this study, the results demonstrated that a recombinant baculovirus expressing the VP2 protein of the IBDV and the mChIL-18 protein could protect against very virulent IBDV in chickens. These results indicate that the recombinant baculovirus expressing the VP2 protein of the IBDV and the mChIL-18 protein can be potentially used as a vaccine for protection of chickens from very virulent IBDV.

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