The Journal of Animal & Plant Sciences, 25(4): 2015, Page: 1121-1128
ISSN: 1018-7081

CONSTRUCTION AND EXPRESSION OF RECOMBINANT PLASMIDS CONTAINING NEPHROPATHOGENIC IBV S1 GENE AND LI-KEY SEGMENT OF CHICKEN MAJOR HISTOCOMPATIBILITY COMPLEX II GENE

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ABSTRACT

Infectious bronchitis virus (IBV) produces highly contagious infectious bronchitis disease (IB) of poultry at all ages around the world. In spite of the proper vaccinations, new IBV strains are emerging at poultry farms in China. In this study, a new nephropathogenic infectious bronchitis virus (XDC-2) strain was isolated from a vaccinated flock showing clinical signs of IBV in Jiangsu province of China. The virus was confirmed by SPF chicken embryo lesions, RT-PCR and S1 gene sequencing. The complete spike (S1) glycoprotein and li-key segment of chicken major histocompatibility complex II gene with Flag tagged were amplified (S1-F and Ii-F) with expected band 1684bp and 705bp, respectively. S1 and Ii gene were cloned into pcDNA3.1 eukaryotic expression vector (pcDNA-S1-F and pcDNA-li-F). Recombinant plasmids were confirmed by colony-PCR, enzyme digestion and sequencing. Further, recombinant plasmids were transfected to baby hamster kidney (BHK-21) cells for protein expression in vitro. Cells lysate were used for immune-blot analysis, S1 and Ii genes were successfully expressed with expected band size of protein molecular weight 61KDa and 26KDa, respectively. In conclusion, recombinant plasmids containing IBV complete S1 gene and chicken MHC II li gene expressed *in vitro* could be co-administer to immunize the chickens for the prevention and control of nephropathogenic IBV infection.

Key words: Infectious bronchitis virus; Nephropathogenic, S1 gene, chicken MHC II li gene.

INTRODUCTION

The infectious bronchitis virus (IBV) is an enveloped RNA virus of family coronaviridae. It causes highly contagious disease of respiratory, urinary and reproductive tract of the chicken and produce production losses in poultry industry by high morbidity, mortality, drop in eggs quality and quantity (Cavanagh & Naqi, 2003). IBV is prototype virus and has four structural proteins, Spike protein (S), Envelop protein (E), Membrane protein (M) and Nucleocapsid protein (N). S protein is post-transitionally cleaved into S1 and S2 (Cavanagh, 2007). S1 protein is located at outer membrane of the virus and posses major antigenic determinants that play important role in cell attachment and virus neutralization. IBV S1 gene has high mutation rate, few amino acid differences in S1 subunit decreases the cross protection between serotypes. In recent years, IBV has been a major problem of poultry industry particularly in China (Liu *et al*., 2006b; Yu *et al*., 2001a; Yu *et al*., 2001b; Zeshan *et al*., 2010). Live or attenuated vaccine is used to control the IBV infection, live attenuated vaccine generally induce long lasting protection by humoral and cellular responses but there is always risk of insufficient attenuation that may lead to emergence of new IBV strains or genetic instability (Cook *et al*., 1986; Moore *et al*., 1997; Wang *et al*., 1993; Zhou *et al*., 2002). The live attenuated vaccine is also considered for the spread of live vaccine virus (Cavanagh *et al*., 1984; Kapczynski *et al*., 2003; Meulemans *et al*., 2001; Naqi *et al*., 1993). Inactive vaccine is relatively expensive and induces short time immunity. However, it produces the high antibody titers, low level of cytotoxic T lymphocytes (CTL) and may induce infection in one-day-old chickens (Cavanagh and Cook, 1997; Cavanagh and Naqi, 2003). Therefore, there is need of improved vaccine system that can intractable to nephropathogenic infectious bronchitis strain.

DNA vaccine represents a new and novel approach for the induction of rapid cellular and humoral immune response. DNA recombinant plasmid used as vaccine has an effective vehicle for the delivery of antigens and it is an ideal strategy to induce broad immune responses against viral diseases (Kalinna, 1997; Strugnell *et al*., 1997; Tacket *et al*., 1999). DNA vaccination provides protection by inducing the neutralization antibodies, helper T cells and cytotoxic T cell responses (Gurunathan *et al*., 2000). The construction of the DNA vaccine is based on integral antigen insertion in DNA plasmid that have no risk of integrating gene in

the host cells (Jeon *et al*., 2002; Tian *et al*., 2008). DNA vaccines have been successfully used against several RNA viruses, such as, Togaviruses and Bunyaviuses and Filoviruses (Ulmer *et al*., 2006). Previously, two DNA vaccines have been licensed for the Infectious hematopoietic necrosis virus of the salmon and West Niles Virus (Corbeil *et al*., 2000; Kutzler & Weiner., 2008; Faurez, *et al*., 2010). However, the adjuvants are added to enhance the potency of the DNA vaccine (Calarota and Weiner, 2004; Kent *et al*., 1998; Liu *et al*., 2006a; Scheerlinck, 2001; Stevenson, 2004). The IBV S1 sub-unit gene contains antigenic determinants and is involved for induction of neutralization antibodies (Cavanagh *et al*., 1986; Ignjatovic and Galli, 1994).

Recently, S1 sub-unit gene is targeted for the development of recombinant vaccine (Shi *et al*., 2011). DNA recombinant plasmids encoding IBV S1 have been implied and found with promising results to protect the IBV infection (Kapczynski *et al*., 2003). The entire S1 glycoprotein was expressed in vaccinia virus that induced virus neutralizing antibodies of IBV (Zeshan *et al*., 2010).

It has been documented that immune regulator were shown strong adjuvant effects; such as, interleukins (IL), tissue necrosis factor (TNF), granulocytes macrophage colony stimulating factors (GM-CSF) that enhanced antibodies production, stimulation of the T cells, increased functions of antigens presenting cells and regulate the immune activities of the T cells (Winter *et al*., 2006).

Virus components are presented after the degradation to the antigen presenting cells or MHC class II to the surface of the cells for the immune response. The increase in $CD4^+$ T cell responses by MHCII li is well known (Holst *et al*., 2008). Therefore, MHC II li gene is involved in presentation of degraded virus molecules to present the surface and increasing the activation of the $CD4^+$T cellular response. In the present study, we constructed and expressed the S1 gene of IBV XDC-2 strain and chicken MHC II li fragment by western blot analysis which could be more beneficial for boosting the immune response and protection from nephropathogenic IBV infection.

MATERIALS AND METHODS

Virus, eggs, chicken spleen, plasmid and cells: Infectious bronchitis virus strain XDC-2 strain was isolated from vaccinated broiler flock showing clinical signs and mortality similar to nephropathogenic IBV from Jiangsu province of China. The samples (lungs and kidneys) from infected birds were collected randomly and propagated in 10-days-old specific pathogen free (SPF) embryonated chicken eggs purchased from (Nanjing Tech-Bank Bio-Industry Co. Ltd., Nanjing, China), after three blind passages through SPF eggs and incubated in automatic controlled incubator until the embryo lesions were observed. The chicken embryo died within 2-5 days post inoculation and showing IBV specific lesions were harvested allantoic fluid and 50% chicken infectious dose (EID_{50}) was calculated 10^3 EID_{50} by inoculation of serial 10 fold dilution of virus in 10-day-old SPF embryonated chicken eggs. The presence of virus in the harvested allantoic fluid was confirmed by amplification of complete S1 sub-unit gene by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). White leghorn chicken fresh spleen was used to extract the RNA and amplify chicken li fragment of MHC Class II histocompatibility complex gene. Experiment was conducted under the Guide line Principles for Biomedical Research Involving Animals and approved by the Animal Care and Ethics Committee of Nanjing Agricultural University (permit number IACECNAU20130905).

The DNA cloning vector pcDNA3.1 (Invitrogen, USA) for the construction of recombinant plasmids and baby hamster kidney (BHK-21) cells were used for eukaryotic expression of the protein.

Designing primers: The first pair of primer previously reported by Zeshan *et al*. (2010) was used for the identification of the IBV strain from the samples. Second and third pair of primers were designed by using the Primer Premier Version 5.0 software (Premier Biosoft International, Palo Alto, CA) to amplify the S1 gene according to the published IBV S1 gene sequence in GenBank: accession number: (AY427819) and MHC II Ii GenBank: accession number: (AY508513). S1 and li gene primers were added restriction enzyme, Kozak and Flag sequences, respectively.

First pair

1- P1 5’ GCGCTCGAGATGTTGGGGAAGTCACTG 3'
2- P2 5’ CGCCTCGAGTTACATTTTGGTCATAGAA 3'

Underline represents the *Xho*I enzyme site.

Second pair

S1-F:5’
ATTGGATCCACGACCATGGTGGGGAAGTCACTG 3’
S1-R-Flag:
5'ATACTCGAGTTACTTATCGTCGTCATCCTTGTA ATCCATTTTGGTCATAGAACG 3’
In S1-F and S1-R-Flag primers under line represent the *Bam*HI and *Xho*I enzyme sites respectively.

Third pair

Ii-F:
5’TATGGTACCACGACCATGGCTGAGGAGCAG 3’
Ii-R-Flag:
5’AATGGATCCCTACTTATCGTCGTCATCCTTGTA ATCCTTGGCTT
TCAC3’
Ii-F and Ii-R-Flag primers underline represent the *Kpn*I

and *Bam*HI sites respectively.

Identification of IBV by RT-PCR, amplification of S1 gene and cloning in pMD-18T vector: The allantoic fluid from the dead embryonated eggs showing IBV lesions was collected to identify the virus and to amplify the S1 sub-unit gene. The RNA was extracted using TRIzol (Invitrogen, CA. USA) according to manufacturer's protocol. Briefly, a total of 200µl of allantoic fluid was mixed in Trizol. The mixture was centrifuged at 12,000rpm/5min at 4°C and collected 1ml of the clear supernatant. Trichloromethane 200µl was added with shaking and kept 5min at room temperature. Then mixed fluid was centrifuge at 1000rpm/10min, and the supernatant about 500µl was collected in other Eppendrof tube and gently mixed with equal amount of Isopropanol and stored at -20°C for whole night and centrifuged at 12000rpm/10min. Tube containing pellet was re-suspended in 1ml of 70% Ethanol and three times centrifuged at 7500rpm/5min. Finally, 20µl of Diethyl pyrocarbonate (DEPC) treated water was added to dissolve the RNA pellet and stored at -70ºC. For the RT-PCR, 7µl total RNA of both samples was used by mixing M-MLV Reverse transcriptase (Promaga, USA). The mixture was incubated at 70ºC for 10min then immediately transferred to ice water for 2min, added M-MLV enzyme 0.25µl and kept at 37ºC for 1h.

Synthesis of cDNA and RT-PCR was performed in thermocycler (Eppendorf Mastercycler, USA) using set of primers with total 25µl mixture, 12.5µl Prime taq PCR Mix (TaKaRa, China), 3µl cDNA, 0.5µl of each pair of primers and 8.5µl of *dd*H_2O. The PCR protocol was set as, denaturizing temperature at 94ºC for 5min, followed by 35 cycles at 94ºC for 45sec, annealing at 55ºC for 30sec, extension at 72ºC for 2min and final extension was done at 72ºC for 5min. The amplified S1-F (1650bp) was visualized and photographed under digital Gel Doc-It 3uv Trans-illuminator imaging system (HAMAMATSU, Japan) in 1% agarose gel electrophoresis.

Then S1 gene was T/A ligated into the cloning vector pMD-18T (TaKaRa, China), keeping at 16ºC for 18h in Eppendrof Thermomixer comfort (Eppendrof, New Burnswick,USA). The DH5α *E .coli* cells were transformed with the ligated product. The success of ligation was first screened by restriction enzyme digestion and later was confirmed by DNA sequencing.

Chicken embryo lesions: The 10-day-old SPF chicken embryo eggs were inoculated 200µl allantoic fluid of 10^3 EID_{50}, the eggs were observed for embryo mortality and lesions. The control group eggs were inoculated with the sterile PBS**.**

Amplification of MHC II li gene: The total RNA was extracted from the fresh chicken spleen of the white Leghorn chicken using TRIzol (Invitrogen, USA), according to the manufacturer's instructions described previously. The same protocol of PCR was adopted for li gene amplification as S1, only the annealing temperature and extension time were changed according to the size of gene. The amplified MHCII Ii-F gene (672bp) fragment was visualized in 1% agarose gel electrophoresis.

Cloning of IBV S1 gene and chicken MHC II Ii gene: The IBV complete S1-F gene was successfully amplified from pMD-18T-S1 using as template by PCR. The complete S1-F and li-F gene with Flag were approximate size of 1684bp and 705bp respectively. The S1 and Ii gene were purified and digested with *Bam*HI and *Xho*1 and *Bam*HI and *Kpn*I enzymes and cloned into pcDNA3.1 (Invitrogen, USA) eukaryotic expression vector respectively. The recombinant plasmids were confirmed by colony PCR, enzyme digestion and sequencing.

Confirmation of the recombinant plasmid (pcDNA-S1-F and pcDNA-Ii-F) by colony PCR and Restriction Digestion: The recombinant plasmids were confirmed by PCR amplification of S1 and li gene of equal size by using colony-PCR and restricted digestion with *Bam*HI and *Xho*I, and *Bam*HI and *Kpn*I enzyme, respectively. The bands were visualized in 1% agarose gel with correct size.

***In vitro* eukaryotic expression of recombinant plasmids and western bolt analysis:** BHK-21cells were transfected by using lipofectamine2000 (Invitrogen, CA, USA) according to the manufacturer instruction. BHK-21 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) supplemented with 8% (v/v) fetal bovine serum (FBS, Invitrogen, USA) at 37ºC with 5% carbon dioxide. For transfection of plasmids, BHK-21 cells were grown in 24 wells culture plates before overnight and about at 70-80% cells showing confluence were used. Briefly, cells were washed with DMEM and transfected by using 3µl Lipofectamine2000, they were made complexes with 1µg of each plasmid pcDNA-S1-F, pcDNA- li -F and control group as pcDNA3.1. The cells were collected after 24h, 48h, 72h and 96h of post-transfection by using the 100µl cell lysis buffer (TaKaRa ClonTech, China) per well, collected cells were centrifuged at 4000rpm/5min and supernant was collect, the total protein concentration was measured of each interval, 48h post transfected cells were measured with good concentration (1-2µg/µl) per well using Nanodrop™ 2000 spectrophotometer (Thermo Scientific, USA) at absorbance of 340nm and were used for western blot analysis, 25µl sample was loaded in each well of 12% SDS-page gel for the protein separation as previously described (Calandrella *et al*., 2001; Du *et al*., 2008; Zeshan *et al*., 2011). Further, resolved protein were transferred to nitrocellulose membrane (Pall Corporation, Pensacola, FL, USA) and blocked in 10% skimmed milk for 4h mixed in PBST (Tris-HCL 50mmol/l, 0.1%Tween

20). The membrane was incubated in mouse anti-flag monoclonal antibodies (Abmart, China) with 1/1000µl (v/v) dilution for 2h. After, four times washing in TBST with 5min interval, were subjected to react with secondary antibodies goat-anti-mouse (Boster, China) at 1/10000 dilution. Proteins band were visualized by using chemiluminescence luminal reagents (Chemistar™ High-sig ECL Western Blotting substrate, Tanon, China).

RESULTS

Identification of IBV by chicken lesions: Chicken SPF embryonated eggs were inoculated with the virus. After three consecutive passages, the embryos were shown typical lesions of IBV (early embryos death, retard growth, curled toes and blood traces on the skin) except control group (Fig.1).

Fig.1. The effect of IBV XDC-2 isolates on embryo development. Up, early dead embryo and down control group.

Confirmation of IBV by RT-PCR from allantoic fluid: The harvested allantoic fluid was used to synthesize cDNA and performs RT-PCR. All the harvested samples confirmed the presence of the IBV except the control group (Fig. 2).

Amplification of S1 and MHC II li gene: The IBV S1-F gene and chicken MHC li-F gene were successfully amplified by using designed primers. The result showed S1-F and li-F genes amplified were approximate 1684bp and 705 bp of respective size (Fig. 3).

S1-F and li-F gene cloning and confirmation of recombinant plasmids: The S1-F and li-F was restricted enzyme digested and successfully ligated with pcDNA3.1 cloning vector. The recombinant plasmids (pcDNA3.1-S1-F and pcDNA3.1-li-F) were confirmed by restricted digestion with two bands of equal size visualized, respectively in 1% (w/v) agarose gel electrophoresis, colony-PCR amplification of S1-F and li-F and sequencing (Fig. 4).

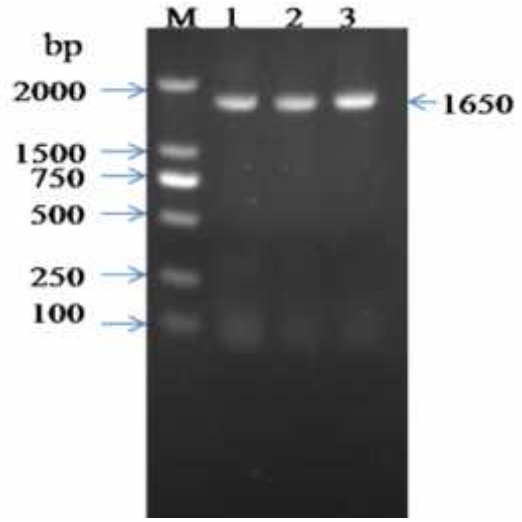

Fig.2. RT-PCR of embryo allantoic fluid, (a) M: DNA marker DL2000bp, lane-1-3 amplified complete S1 gene from allantoic fluid.

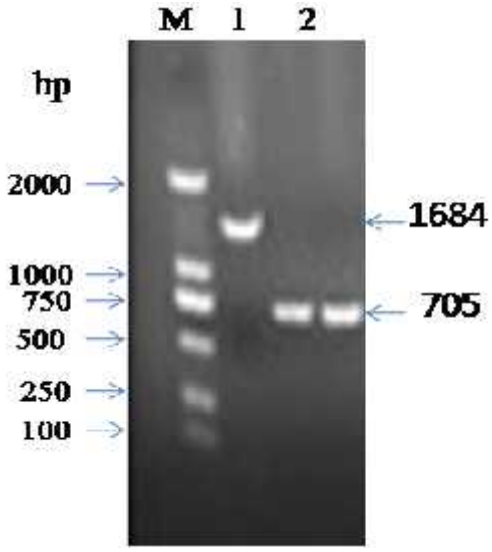

Fig.3. Amplification of the S1-F and Ii-F, Lane1-M-marker DL 2000bp, Lane 2-Amplified IBV XDC-2 S1-F, lane 3-chicken MHC II Ii-F.

***In vitro* expression of recombinant plasmids and Western blot analysis:** For protein expression, the recombinant plasmids pcDNA3.1-S1-F and pcDNA3.1-li-F transfected to BHK-21 cells and collected after 48h post-transfection were used in Western blot analysis. The S1 and Ii genes were successfully expressed with the 61KDa and 26KDa of protein molecular weight, respectively (Fig. 5).

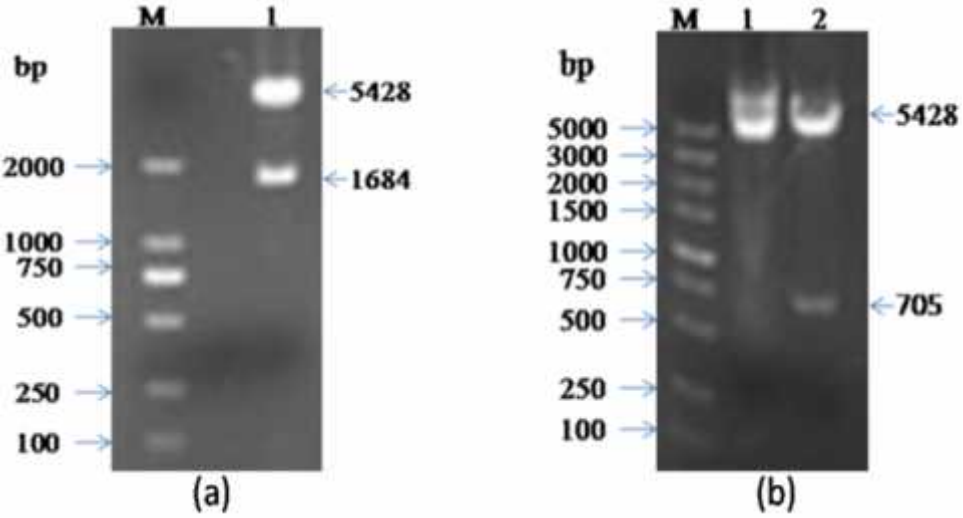

Fig.4. Confirmation of recombinant plasmids (pcDNA3.1-S1-F and pcDNA3.1-li-F by Restricted digestion (a) Lane 1 M-marker 2000bp, lane 2- pcDNA3.1 and S1-F gene (b) Lane 1 M-marker 5000bp, Lane 2 Recombinant plasmid pcDNA-li-F, lane 3 pcDNA3.1 and li-F.

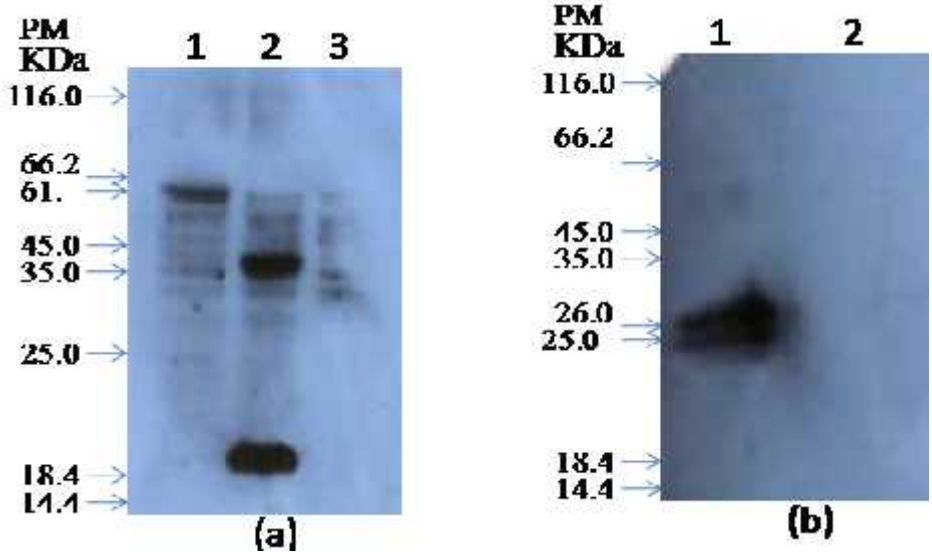

Fig.5. The immune-blotting of recombinant plasmids pcDNA-S1-F and pcDNA-li-F, (a) PM- post-stained protein marker, line 1, expressed S1-F 61KDa, line 2 Flag control positive, lane 3 negative control. (b) PM-post-stained protein marker, lane 1, expressed li-Flag protein, lane 2 negative control.

DISCUSSION

Infectious bronchitis (IB) is highly contagious and pathogenic disease of the chickens and produces severe economic losses to poultry industry (Cavanagh and Naqi, 2003; De Wit, 2000; Ignjatovic *et al*., 2002; Zeshan *et al*., 2010). In the present study, newly identified nephropathogenic IBV XDC-2 strain sequence was submitted to GenBank under accession number (KM213963). The samples of embryo allantoic fluid were positive by RT-PCR. Pathological lesions such as curling, stunting, dwarfing of the embryo, turned neck and less development of the feathers on the body were observed similar to previously observed in IBV nephropathogenic strain. The dead embryos were showing retarded growth in comparison to control group that confirmed pathogenic IBV virus kills the embryo at early stage (Adzhar *et al*., 1996; Cavanagh *et al*., 2005; Cook, *et al*., 1976).

DNA vaccines development is an attractive approach, potentially safe and alternative toll to replace conventional vaccine production methods. The IBV S1 gene play an important role for induction of neutralization antibodies against IBV, it contains antigenic determinants (Cavanagh *et al*., 1986; Ignjatovic and Galli, 1994). Therefore, S1 sub-unit gene is suitable target for the development of recombinant vaccine (Shi *et al*., 2011). DNA recombinant plasmid encoding IBV S1

was shown with promising results to protect the IB (Kapczynski *et al*., 2003).

In the present study, the IBV S1 and li-key segment of chicken major histocompatibility complex II gene were used to construct the recombinant plasmids that the DNA vaccine optimum response could be achieved. MHC II li gene is well known to increase the $CD4^+$ T cells response and vaccine induce prolong protection against viral infection (Holst *et al*., 2008). The pcDNA3.1 plasmid was selected for the *in vitro* protein expression, and recombinant plasmids were constructed and confirmed by colony-PCR, restriction digestion and sequencing. Recombinant plasmids were transfected to BHK-21 cells that showed cytopathic effects (CPE) after the 48h post-transfection. The cells lysate was used to characterize the protein expression by Western blot analysis. The recombinant plasmids (pcDNA-S1-F and pcDNA-Ii-F) expressed protein bands weight were of 61KDa and 26KDa, respectively. The results were similar to the previous study that DNA recombinant plasmid containing S1 gene can be expressed efficiently (Kapczynski *et al*., 2003; Tan *et al*., 2009; Zeshan *et al*., 2011).

The present study supports that recombinant vector pcDNA3.1 containing complete S1 gene and Ii-key segment of chicken major histocompatibility complex II gene could be co-administered effectively for the prevention of nephropathogenic IBV infection in chicken that may prove a suitable candidate for the future vaccine development and to replace the conventional vaccine.

Acknowledgments: This work was mainly supported by the National Natural Science Foundation (31230071), grants from the Ministry of Education, China (20120097110043), and the priority academic program development of Jiangsu higher education institutions (PAPD).

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