

Cloning, expression and identification of truncated Spike gene of IBV (S_{f200}) and chicken granulocyte- macrophage colony stimulating factor (GM-CSF)

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Abstract

The aim of this study was to clone, express and identify the truncated S1 gene of nephrotropic infectious bronchitis virus (IBV) and granulocyte-monocyte colony stimulating factor (GM-CSF) of chicken. Firstly, two genes were amplified by polymerase chain reaction (PCR) and cloned into pMD18-T vector. The truncated S1 gene designated as S_{f200} (containing five antigenic sites of S1 glycoprotein on amino acid residues (aa) 24–61, (aa) 291–398 and (aa) 497–543 and GM-CSF were then amplified from the respective recombinant pMD18-T plasmids and cloned into pET-32a (+) vector resulting pET-S_{f200}, pET-GM which were identified by restriction enzyme digestion and sequencing analysis. The in vitro expression of truncated S_{f200} and GM-CSF constructs were later expressed in *E. coli* BL21 with a molecular mass of approximately 38kDa and 29kDa respectively as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. Polyclonal antibodies were developed by injecting *E. coli* expressed S_{f200} and GM-CSF into the SPF mice and were used to identify the recombinant proteins by Western blot analysis. These findings indicated that the polyclonal antibodies produced in mice could be used to detect the recombinant truncated S_{f200} and GM-CSF and vice versa.

Keywords: IBV virus, cloning, expression, Truncated S1, GM-CSF

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Introduction

Avian infectious bronchitis is an acute highly contagious and economically important disease of poultry caused by infectious bronchitis virus (IBV), which is a member of the family *Coronaviridae*. Infectious bronchitis virus has been a major cause of respiratory infection and poor egg-laying performance in chickens. Later, it became apparent that the same virus could also infect the kidneys and the oviduct. In many reports, nephropathic lesions were observed in IB-vaccinated flocks, suggesting that the currently used IB vaccination may not be providing complete protection (Cavanagh and Naqi, 1997). Nephropathogenic IBV has been a major problem in the poultry industry particularly in China recent years (Liu et al., 2006; Yu et al., 2001). Several strategies have been introduced to increase the potency of the vaccines including antigen targeting to endoplasmic reticulum for rapid intracellular degradation (Hung et al., 2001), directing antigens to antigen-presenting cells (APCs) by their fusion to APC receptor ligands (Cusi et al., 2004) and co-injecting cytokines/co-stimulatory molecules as molecular adjuvants (Calarota and Weiner, 2004). Cytokines are produced by activated T cells, macrophages, endothelial cells and stromal fibroblasts, and act on bone marrow cells to increase inflammatory leukocyte populations including granulocytes and macrophages (Huleihel et al., 1993). Recombinant granulocyte-monocyte colony stimulating factor (GM-CSF) is increasingly under clinical study for the treatment of various diseases including cancer, infectious diseases and hematopoietic diseases, and has become one of the most clinically successful applications of any biological therapeutic agent (Jones, 1999; Mellstedt et al., 1999). In recent years, GM-CSF has been proposed as a potent adjuvant for stronger induction of immune response (Anandh et al., 2000). GM-CSF has been reported to initiate the proliferation, differentiation and activation of macrophages, neutrophils, various antigen presentation cells and, to some degree, T cells, in addition to several direct immune-stimulatory functions. Clearly, these properties make GM-CSF a potent adjuvant (Du et al., 2007; Shi et al., 2006; Wang et al., 2009).

The IBV encodes four major structural proteins, known as the spike (S) protein, nucleocapsid (N) protein, membrane (M) protein and envelope (E) protein. The spikes of IBV are synthesized as S precursor glycoprotein which is post translationally cleaved to form the two subunits, S1 and S2 (Stern and Sefton, 1982). The S1 subunit carries the neutralizing epitopes and plays an important role in the attachment of the virus to the host cell membrane, induction of antibodies for virus neutralization and haemagglutination-inhibition and induction of IBV-specific cytotoxic T lymphocytes (Kant et al., 1992). The S1 region carries the receptor binding domain that defines tissue and host tropisms. The S1 region is responsible for serotype variability among IBV isolates particularly within hypervariable portions of the S1 region. Although the S1 subunit is highly variable (Cavanagh et al., 1988), previous studies have shown that it was an excellent candidate for developing

recombinant vaccine against IB and the corresponding monoclonal antibodies (mAbs) and polyclonal were main tools for identifying functional fragments and for developing the diagnostic reagents (Johnson, 2003; Wang et al., 2002). As the S1 of IBV was very difficult to obtain the full-length S1 expressed in *E. coli*; consequently, studies on engineering vaccine against IB and immunological and biostructural analyses of IBV have been developed slowly since 1990s. To date, the focus is on mapping antigenic regions in the S1 subunit (Cavanagh et al., 1992; Kant et al., 1992; Koch et al., 1990) and the in vitro high-level expression of the S1 subunit.

In this study, we described molecular cloning, expression and identification of the truncated S1 gene of nephrotropic infectious bronchitis virus (IBV) and granulocyte-monocyte colony stimulating factor (GM-CSF) of chicken. This research will open the study of protective efficiency against challenge and the development of a new approach to vaccines against this IBV disease.

Materials and Methods

Strategies for protein expression in *E. coli*: *E. coli* is a convenient host for heterologous protein expression. Its advantages include high levels of heterologous gene expression and scalability of experiments, low cost, fast growth, a lack of post-translational modification and an ability to express labeled (isotope or selenomethionine) proteins.

Virus and mice: The nephropathogenic IBV XDC-2 strain was propagated in the allantoic cavities of 10-day-old SPF embryonated chicken eggs and then harvested allantoic fluid 36 h post inoculation. Four-week-old specific pathogen free (SPF) BALB/c mice were purchased from the Laboratory Animal Center, National institute of hygiene and epidemiology, Hanoi, Vietnam. All animal experiments were approved by the Institutional Animal Care and Use Committee of Bac Giang Agriculture and Forestry University (IACUC No.19-03) and were performed based on the ethical rules and laws of the University.

RNA extraction and reverse transcription: Total viral RNA was extracted from IBV propagated allantoic fluid using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, after adding 500µl TRIzol reagent, the samples were shaken vigorously and incubated at room temperature for five minutes. 200µl chloroform was added followed by vigorous shaking for 15 seconds. Then, the samples were incubated at room temperature for 2-3 mins and centrifuged at 12,000 rpm for 15 minutes. The aqueous supernatant was carefully aspirated into new tubes and 700µl isopropyl alcohol was added. The tubes were incubated at room temperature for about 2 to 3 minutes and centrifuged at 12,000 rpm for 10 minutes. The supernatant was discarded and the pellet was washed with 1 ml of 75 % ethanol (diluted in diethyl pyrocarbonate (DEPC) water). Finally, the tubes were centrifuged at 7500 rpm for five minutes. The supernatant was discarded and the pellet was air dried for 5 to 10 minutes. The pellet

was dissolved in 15 µl of DEPC water then total RNA was reverse transcribed to first strand cDNA.

Amplification of truncated fragments of S1 (*S_{f200}*) and GM-CSF: For amplification of three fragments of the S1 gene, reverse transcription (RT) PCR primers (Table 1) were designed based on S1 sequence published in GenBank (accession no. AY427819). Meanwhile the mature peptide of (GM-CSF 282 bp) was amplified from pCl-neo-GM-CSF using forward and reverse primers GM-P1 and GM-P2 (Table 1). The amplification was performed in a 50 µl reaction mixture containing 1.5 mM MgCl₂, 1×PCR buffer, 0.2 mM of dNTP, 20 pM of each forward and reverse primer, 1.5 U of Taq DNA polymerase (Promega, Madison, USA) and 2 µl of cDNA. The PCR reaction was run in a thermocycler (PTC-150) with the following program: Initial denaturation at 94°C for 5 mins; then 30 cycles as follows, denaturation at 94°C

for 40 secs, annealing at 58°C for 45 secs, extension at 72°C for 1-2 mins, and the final extension at 72°C for 10 mins was performed.

For amplification of *S_{f200}*, an antigenic epitope prediction within the TCoV-S1 gene was accomplished using antigenicity scale (Kolaskar and Tongaonkar, 1990), hydrophilicity prediction (Parker *et al.*, 1986) and linear epitope prediction (Larsen *et al.*, 2006). The resulting epitope predictions were used collectively to select a *S_{f200}* gene fragment. The selected region carrying a number of putative B cell epitopes was located and this region generated a gene fragment 650 bp approx in length encoding 200 amino acids of the S1 protein; this truncated S1 protein fragment was designated *S_{f200}*. Five antigenic sites of S1 on amino acid residues (aa) 24–61 (S1D), (aa) 291–398 (S1CAB) and (aa) 497–543 (S1F) (Kant *et al.*, 1992; Koch *et al.*, 1990) were amplified by overlap PCR from pMD18T-S1 vector using four pairs of primers (Table 1).

Table 1 Primer sequences for amplification of the *S_{f200}* genes of nephropathogenic IBV XDC- 2 strain and chicken GM-CSF

S No.	Primer ID	Sequence	Enzyme site	Gene (Size)
1	S1 F	5' GCGCTCGAGATGTTGGGGAAGTCACTG 3'	<i>XhoI</i>	S1 (1650bp)
2	S1 R	5' CGCCTCGAGTTACATTTTGGTCATAGAA 3'	<i>XhoI</i>	
3	Sense 1	5' GCGGGATCCATGGATAGTTATGTTT 3'	<i>BamHI</i>	S1CAB (351bp)
4	Anti 1	5' CACCACCTTTATTGCCTGCATTATT 3'	No site	
5	Sense 2	5' CAGGCAATAAAGGTGGTGTGATAC 3'	No site	S1D (150bp)
6	Anti 2	5' CCTCACAAGGCTGCGTCAATTCACC 3'	No site	
7	Sense 3	5' TGACGCAGCCTTGTGAGGATGTTAA 3'	No site	S1F (162bp)
8	Anti 3	5' TATCTCGAGTTACCTGGAACGACG 3'	<i>XhoI</i>	
9	GM-P1	5' GAGGGATCCATGCCCATGGACATCAGGGAT 3'	<i>BamHI</i>	GM-CSF (282bp)
10	GM-P2	5' GACCTCGAGTTAGATGCAGTCTT 3'	<i>XhoI</i>	

Construction of prokaryotic expression vectors: PCR products were electrophoresed on 1% agarose gel and before purification, and subcloned into a prokaryotic expression pET-32a (+) vector (Novagen, Madison, WI, USA) at *BamHI* and *XhoI* cloning sites as shown on the vector map. The recombinant expression plasmid designated as pET-32a-*S_{f200}* and pET-32a-GM was then constructed and identified by PCR.

The purification of PCR products of *S_{f200}*, GM-CSF, and pET-32a (+) expression vector from agarose gel as follows by DNA rapid purification kit AccuPower PCR PreMix (BIONEER, Korea). The purified PCR products thus were stored at -20°C for further use.

The compatibility of two restriction enzymes was checked and it was decided which digestion buffer to use (TaKaRa, bio catalog Japan). The gel purified PCR products of *S_{f200}* and GM-CSF were separately digested with *BamHI* and *XhoI*. The reaction mixture was as follows; The mixture was incubated at 37°C for 2 hours. The empty pET-32a (+) vector was also digested using the same enzymes and the reaction mixture. The digested PCR products of *S_{f200}*, GM-CSF and empty pET-32a (+) vector were purified again using DNA gel purification kit as described previously.

After digestion of the PCR products of *S_{f200}*, GM-CSF and empty vector (pET-32a (+)), ligation of the individual product with the vector was performed

using following mixture. The ligation mixture was incubated in a flask at 16°C overnight.

Transformation protocol: 3 mL fresh Luria Bertani (LB) broth was inoculated with DH5α strain of *E coli* followed by incubation overnight. 60-100 µl (depending on turbidity of broth) of culture was inoculated to LB broth and incubated at 37°C for 2-3 hours in a shaker. One tube containing 3mL *E. coli* culture was transferred into two 1.5 ml sterile tubes and centrifuged at 12,000 rpm for 30 seconds. Supernatant was discarded and the pellet was resuspended in 1 mL of 0.1M (ice cold) CaCl₂. And then centrifugation was performed at 12000 rpm/30s. The supernatant was discarded completely and the pellet was resuspended in 100 µl cold CaCl₂.

For cloning, DH5α cells were used, mixed well and kept in ice for 45min (>30min required); Heat shock at 42°C (at water bath) for 90s. Then transformed cells transferred into 1ml LB in growth tube with a loose cap; Incubated at 37°C for 1 hr (40-60min required) with shaking. Transferred into microtube and spun down at 4000 rpm for 1 min. 800 mL of supernatant removed and cells pellets resuspended. Cells were spread on LB/AMP plate by putting in several autoclaved glass beads and gently shaken; the beads were removed and the plate sat on a bench for 5 mins to soak the cells into the surface of the medium.

Cells were grown overnight by putting the plate upside down at 37°C still incubator.

Plasmid extraction (Mini prep method): The plasmid was extracted from the transformed clones using plasmid extraction kit (Omega biotech) according to the manufacturer's instructions.

E. coli BL21 containing the truncated S1 gene pET-32a-S_{f200} was propagated in LB broth overnight at 37°C. One milliliter of overnight culture was inoculated into 100 ml of fresh LB. The culture was induced by adding isopropyl β-d-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Bacteria were harvested by centrifugation at 6,000 rpm for 15 mins at 4°C (Beckman, Coulter, J2-MC centrifuge, Mississauga, ON, Canada). The supernatant was decanted and the pellet was re-suspended in 5 ml of phosphate-buffered saline (PBS) and subsequently analysed by SDS-PAGE and Western blot. The purified protein in Buffer D and Buffer E was detected by SDS-PAGE prior to protein quantification by Bradford method (Kruger, 1994).

Generation of polyclonal antisera in mice: The purified recombinant pET-32a-S_{f200} and pET-32a-GM proteins were inoculated subcutaneously into two groups of BALB/c mice (5/group) to obtain polyclonal antiserum. The purified S_{f200} and GM-CSF antigens were emulsified with Freund's complete adjuvant and were inoculated subcutaneously into BALB/c mice on day 1 and reinoculated on day 21 at 50µg per mouse. The antigen emulsified with Freund's incomplete adjuvant was inoculated intraperitoneally on day 40. Two weeks after the final immunization the serum samples were collected.

SDS-PAGE and Western blot: Estimation of molecular weight and binding properties of S_{f200} fragment of S1 glycoprotein was determined by immunoblotting with minor modifications in the method described by Calandrella et al, 2001 (Calandrella et al., 2001). The protocol of SDS-PAGE and Western blot is as follows; the Recombinant protein was separated by 12% SDS-PAGE and transferred onto nitrocellulose transfer-membrane (Millipore, USA). After being blocked with 5% skimmed milk powder in TBS-Tween 20 (TBST), the membranes were incubated with the primary antibodies (mouse-antisera against recombinant S_{f200} and GM-CSF protein) for 1h at 37°C (dilution 1:100). Then HRP-conjugated Anti-mouse IgG (Sigma, USA) was added and incubated for 1h at 37°C (1:5000 dilution). Finally, the bound antibody was detected using 3,3'-diaminobenzidine tetra hydrochloride (DAB) kit (Boster Bio-technology) according to the manufacturer's instructions.

Results

Antigenicity Index of S_{f200} and GM-CSF: In this study, S_{f200} fragments of XDC-2 strain of IBV and chicken GM-CSF proteins were expressed in *E. coli* BL21 strain at a higher level using a prokaryotic pET32a (+) vector system. Furthermore, polyclonal anisera was raised against these proteins in mice and was used to identify the proteins using immunoblotting.

The two proteins were analyzed for different helices, coils and turns, hydrophilicity, surface probability and specially their antigenicity prediction using Protean software in the DNASTar package. The antigenicity index (Jameson-Wolf) is shown in Fig. 1. Truncated S1 gene designated as S_{f200} (containing five antigenic sites of S1 glycoprotein on amino acid residues (aa) 24–61, (aa) 291–398 and (aa) 497–543).

Cloning of S_{f200} and GM-CSF genes: After isolation and propagation of the virus both S_{f200} and GM-CSF were amplified using primers as described in table 1. The three fragments of the S1 gene with sizes of 150bp, 160bp, and 350bp were joined together by overlap PCR resulting in S_{f200} having the size of 660bp (Fig. 2).

Identification of recombinant pET-32a-S_{f200} and pET-32a-GM: After amplification the PCR products were ligated to pET-32a (+) vector which is a prokaryotic expression vector with a size of 5900bp and has several enzymes but only those enzymes were selected that could not cut the sequence of our inserted gene.

Recombinant pET-32a-S_{f200} and pET-32a-GM were confirmed by restriction enzyme analysis. After plasmid pET-32a-S_{f200} was digested by *Bam*HI and *Xho*I enzymes, the result showed that an anticipated band of 652 bp (approx) was generated in the digested product. This indicated that the three overlapped fragments had been amplified and inserted into the expression vector pET-32a correctly (Fig 3). The recombinant pET32a-S_{f200} produced a fragment of approximately 282 bp which is equal to a molecular mass of S_{f200} gene and a PCR product of about 282 bp was obtained. The results showed that S_{f200} was successfully ligated into pET32a vector.

SDS-PAGE and immunoblotting: After induction by 1 mM IPTG at 37°C for 4 h, the S_{f200} and GM-CSF proteins were found in the inclusion bodies in the cell lysate of *E. coli* BL21 (DE3) with a molecular weight of around 38 and 29kDa, respectively (Fig. 4 A and B). The observed molecular mass of the expressed fusion proteins was within the expected range. There were additional amino acids for the Histidine tag in the C-terminal of the expressed fusion protein. These extra amino acids increased the molecular mass of expressed target protein (Fig. 5).

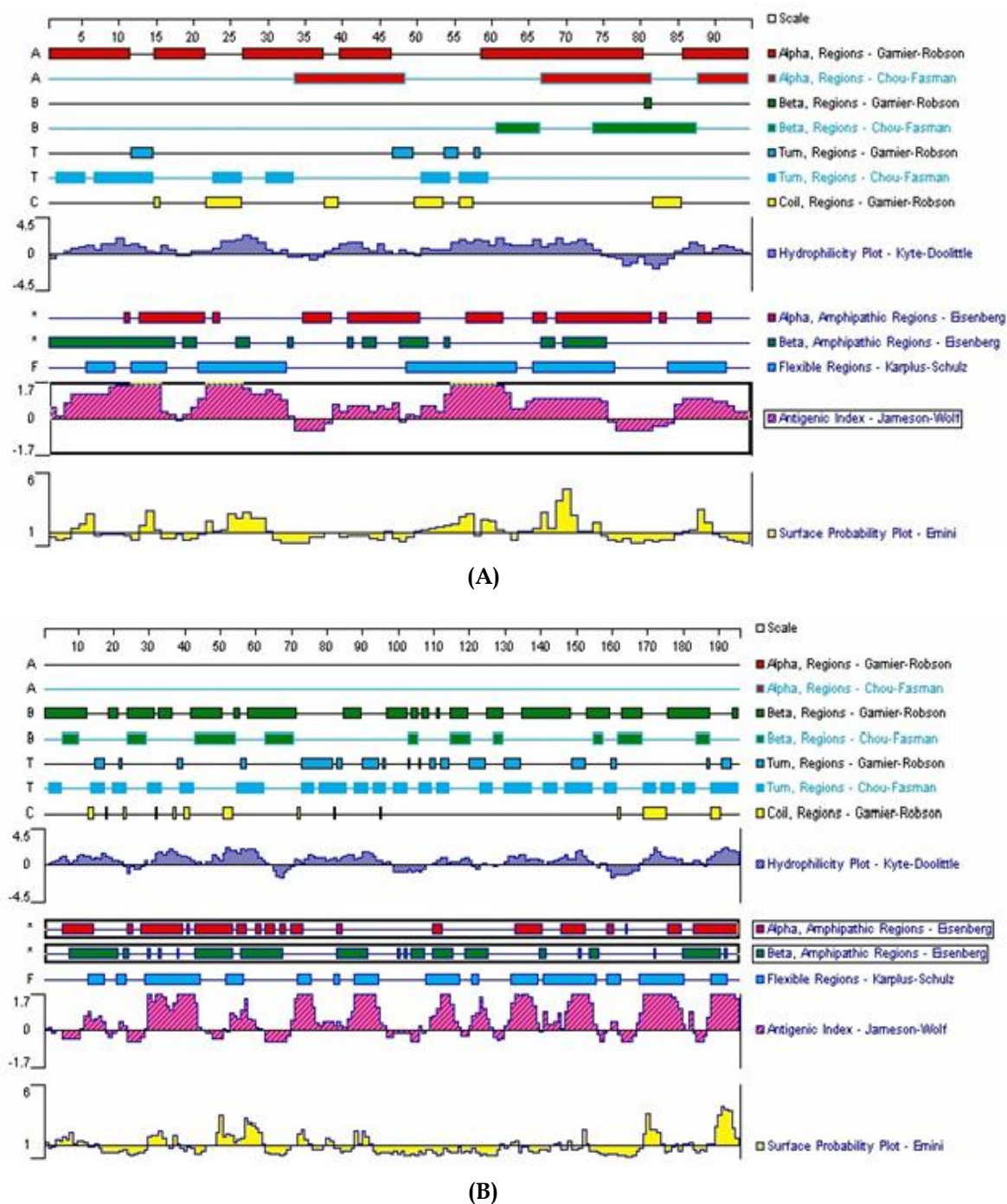


Figure 1 Predictive antigenic analysis of the GM-CSF (A) and S_{F200} (B) proteins was performed using DNASTAR package.

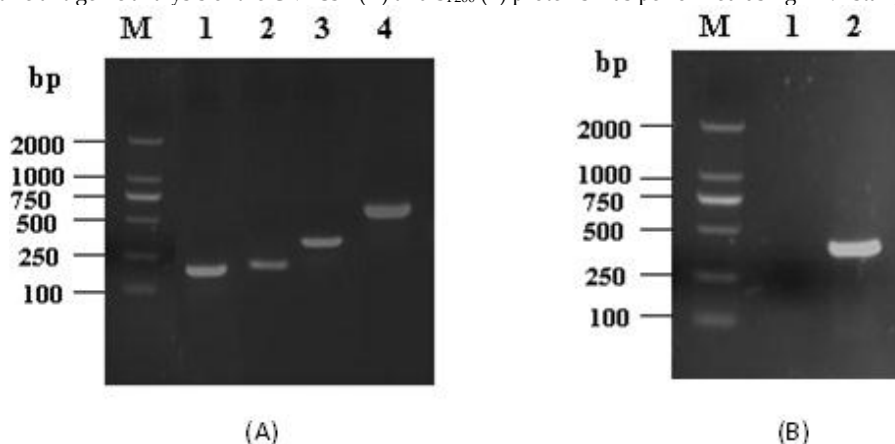


Figure 2 A) Three fragments of S1 with a size of 150bp (Lane 1), 160bp (Lane 2), and 350bp (Lane 3) and S_{F200} (Lane 4), arrow indicate DL2000 DNA marker. (B) Amplification of GM-CSF with a size of 282 bp (right), while the marker is on the left side.

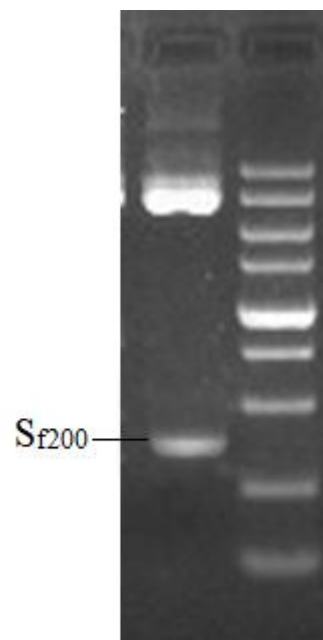


Figure 3 Restriction enzyme analysis of pET-32a-Sf₂₀₀ after digestion with *Bam*H I and *Xho* I. DNA marker is on the other side.

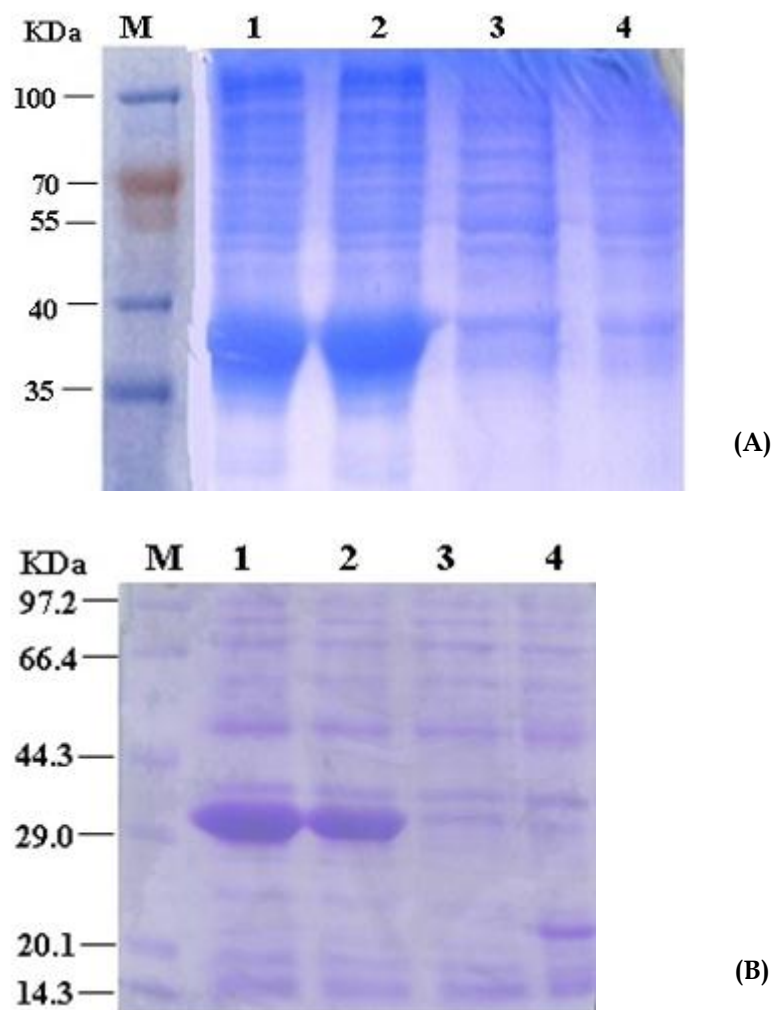


Figure 4 **A)** SDS-PAGE of the cell lysate of *E. coli* harbouring the recombinant plasmid pET-32a-Sf₂₀₀ after induction with IPTG containing *E. coli* BL21 (lane 1), *E. coli* Rosetta srian (lane 2), before induction with IPTG (lane 3), pET-32a (+) vector control (lane 4). **(B)** SDS-PAGE of the cell lysate of *E. coli* harbouring the recombinant plasmid after induction with IPTG containing *E. coli* BL21 (lane 1), *E. coli* Rosetta srian (lane 2), pET-32a-GM-CSF before induction with IPTG (lane 3), pET-32a (+) vector control (lane 4), while the proteins standards are shown on the right side of the panel.

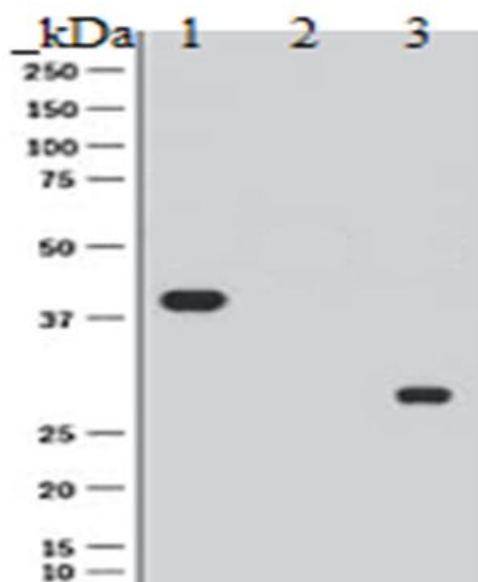


Figure 5 Western blot analysis of the prokaryotic expressed Sf200 in BL21 *E. coli* after probing with polyclonal antibodies raised in mouse model (lane 1), pET-32a (+) vector control (lane 2), BL21 *E. coli* expressed GM-CSF after probing with mouse polyclonal antibodies (lane 3), while the proteins standards are shown on the left side of the panel.

Discussion

In this study, keeping in view the antigenic mapping regions of S1 gene and chicken GM-CSF, we expressed both the protein antigens in *E. coli* designated as S_{f200} [(truncated S1 containing five antigenic sites of S1 glycoprotein on amino acid residues (aa) 24–61 (S1D), (aa) 291–398 (S1CAB) and (aa) 497–543 (S1F) (Kant *et al.*, 1992; Koch *et al.*, 1990) and GM-CSF by pET-32a (+) vector and produced polyAbs against both the proteins in mice and lastly identified the antibodies produced in mice.

The results of DNA sequence analysis of the recombinant plasmids indicated that the reading frame was correct and there was no mutation in the encoding region. Sequences were BLAST in NCBI Gene Bank with showed 100% similarity with the parent sequences. PCR identification and restriction enzyme digestion analysis indicated that the S_{f200} segments and GM-CSF gene were successfully inserted into the pET-32a (+) vector, respectively. Their sequences were identical to the published ones.

The expressed proteins were purified, and their corresponding yields in bacteria culture were calculated to be determined. Additionally, we also expressed the full-length of S1 protein or fragments of S1 protein in *E. coli*. Unfortunately, this protein can be expressed at very low levels or cannot be expressed at all. Poor growth of the host, inclusion body (IB) formation, protein inactivity and even not obtaining any protein at all are some of the problems often found down the pipeline (Dethardt *et al.*, 2006). Limitations in prokaryotic expression systems for viral glycoprotein production can be found with slow destination of product such as maximum and average values for specific growth rates, specific product formation (secretion) rates and space time yields (volumetric productivities). This data enables us to generalize the understanding of the biological limitations of protein synthesis and secretion, thus obviating the major

potential for the optimization of currently available expression systems.

The polyclonal antisera reacted specifically with the homologous GM-CSF and S_{f200} proteins expressed in *E. coli* BL21 (DE3), while no reaction with cell lysate of *E. coli* BL21 (DE3) harbouring the blank pET-32a (+) vector was found in Western blot (Fig. 5). This indicated that the recombinant S_{f200} and GM-CSF proteins are potentially valuable antigens to detect antibody. Five epitopes (A–E) and six epitopes (S1-A to S1-F) within the S1 subunit of IBV have been located by competitive antibody binding assay respectively (Koch *et al.*, 1990). Unfortunately, we cannot verify whether the antigenic epitopes confirmed in our experiment were the same as those identified by Koch *et al.* (1990), because of the lack of mAbs to S1 gene of IBV.

In future study, the polyclonal anisera which was raised against S_{f200} and GM-CSF will be used to identify the recombinant adenoviruses expressing S1 and GM-CSF-S1 and GM-CSF.

In conclusion, The truncated Spike gene of IBV (S_{f200}) and chicken granulocyte- macrophage colony stimulating factor (GM-CSF) were successfully cloned and expressed for further studies.

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Conflict of interest: All authors confirm that, we have no conflict of interest.

References

- Anandh U, Bastani B, Ballal S 2000. Granulocyte-Macrophage Colony Stimulating Factor as an adjuvant to Hepatitis B vaccination in maintenance hemodialysis patients. *Am. J. Nephrol.* 20: 53-56.

- Calandrella M, Matteucci D, Mazzetti P, Poli A 2001. Densitometric analysis of western blot assays for feline immunodeficiency virus antibodies. *Vet. Immunol Immunopathol.*79: 261-271.
- Calarota SA and Weiner DB. 2004. Enhancement of human immunodeficiency virus type 1-DNA vaccine potency through incorporation of T-helper 1 molecular adjuvant. *Immunol. Rev.* 199: 84-99.
- Cavanagh D, Davis PJ, Cook JK, Li D, Kant A, Koch G 1992. Location of the amino acid differences in the S1 spike glycoprotein subunit of closely related serotypes of infectious bronchitis virus. *Avian Pathol.* 21: 33-43.
- Cavanagh D, Davis PJ, Mockett AP. 1988. Amino acids within hypervariable region 1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with neutralization epitopes. *Virus Res.*11: 141-150.
- Cavanagh N 1997. Infectious bronchitis virus, in: Calnek, B.W., Barnes, H., Beard, C.W., R., M.L., Saif, Y.M. (Eds.), *Disease of Poultry*, 9 ed. Iowa State University Press, Ames, pp. 511-526.
- Cusi MG, Terrosi C, Savellini GG, Di Genova G, Zurbriggen R, Correale P 2004. Efficient delivery of DNA to dendritic cells mediated by influenza virosomes. *Vaccine.*22: 735-739.
- Dethardt M, Karl B, Diethard M 2006. Potentials and limitations of prokaryotic and eukaryotic expression systems for recombinant protein production - a comparative view. *Microbial Cell Factories.*5: p61.
- Du Y, Jiang P, Li Y, He H, Jiang W, Wang X, Hong W 2007. Immune responses of two recombinant adenoviruses expressing VP1 antigens of FMDV fused with porcine granulocyte macrophage colony-stimulating factor. *Vaccine.*25: 8209-8219.
- Huleihel M, Douvdevani A, Segal S, Apte RN. 1993. Different regulatory levels are involved in the generation of hemopoietic cytokines (CSFs and IL-6) in fibroblasts stimulated by inflammatory products. *Cytokine.*5: 47-56.
- Hung CF, Cheng WF, Chai CY, Hsu KF, He L, Ling M 2001. Improving vaccine potency through intercellular spreading and enhanced MHC class I presentation of antigen. *J. Immunol.*166: 5733-5740.
- Johnson M 2003. A recombinant fowl adenovirus expressing the S1 gene of infectious bronchitis virus protects against challenge with infectious bronchitis virus. *Vaccine.*21: 2730-2736.
- Jones TC. 1999. Use of granulocyte-macrophage colony stimulating factor (GM-CSF) in prevention and treatment of fungal infections. *Eur. J. Cancer.*35: S8-10.
- Kant A, Koch G, van Roozelaar DJ, Kusters JG, Poelwijk FAJ, van der Zeijst BAM. 1992. Location of antigenic sites defined by neutralizing monoclonal antibodies on the S1 avian infectious bronchitis virus glycopolyptide. *J. Gen. Virol.*73: 591-596.
- Koch G, Hartog L, Kant A, van Roozelaar DJ. 1990. Antigenic domains on the peplomer protein of avian infectious bronchitis virus: correlation with biological functions. *J. Gen. Virol.*71: 1929-1935.
- Kolaskar AS, Tongaonkar PC. 1990. A semi-empirical method for prediction of antigenic determinants on protein antigens. *FEBS Lett.*276: 172-174.
- Kruger NJ. 1994. The Bradford method for protein quantitation, in: Walker, J.M. (Ed.), *Methods in Molecular Biology*. Humana Press, Totowa, pp. 9-15.
- Larsen JE, Lund PO, Nielsen M 2006. Improved method for predicting linear B-cell epitopes. *Immun. Res.*2: 1-7.
- Liu S, Zhang Q, Chen J, Han Z, Liu X, Feng L, Shao Y, Kong X, Rong J, Tong G 2006. Genetic diversity of avian infectious bronchitis coronavirus strains isolated in China between 1995 and 2004. *Arch. Virol.*151: 1133-1148.
- Mellstedt H, Fagerberg J, Osterborg A 1999. Local lowdose of soluble GM-CSF significantly augments an immune response against tumour antigens in man. *Eur. J. Cancer.*35: S29-32.
- Parker JM, Guo D, Hodges RS. 1986. New hydrophilicity scale derived from high-performance liquid chromatography peptide retention data: correlation of predicted surface residues with antigenicity and X-ray-derived accessible sites. *Biochemistry.*25: 5425-5432.
- Shi Y, Liu CH, Roberts AI, Das J, Xu G, Ren G, Zhang Y, Zhang L, Yuan ZR, Tan HSW, Das G, Devadas S 2006. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and T-cell responses: what we do and don't know. *Cell Research.*16: 126-133.
- Stern DF, Sefton BM. 1982. Coronavirus protein: biogenesis of avian infectious bronchitis virus virion proteins. *J Virol.*44: 794-803.
- Wang CH, Hong CC, Seak JC. 2002. An ELISA for antibodies against infectious bronchitis virus using an S1 spike polypeptide. *Vet. Microbiol.*85: 333-342.
- Wang X, Li J, Jiang P, Li Y, Zeshan B, Cao J, Wang X 2009. GM-CSF fused with GP3 and GP5 of porcine reproductive and respiratory syndrome virus increased the immune responses and protective efficacy against virulent PRRSV challenge. *Virus Research.* 143: 24-32.
- Yu L, Liu W, Schnitzlein WM, Tripathy DN, Kwang J 2001. Study of protection by recombinant fowl poxvirus expressing C-terminal nucleocapsid protein of infectious bronchitis virus against challenge. *Avian Dis.*45: 340-348.