



## Expression of Immunogenic S1 Gene of Infectious Bronchitis Virus from Field Outbreak in Eukaryotic Cells

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### ABSTRACT

Infectious bronchitis (IB) is an acute and contagious disease of poultry. The spike glycoprotein (S) of IB virus is a dimer and is cleaved into two glycopolypeptides, S1 and S2 post-translationally. S1 gene defines the serotype and plays a major role in induction of protective immunity. Eukaryotic expression systems are frequently employed for the production of recombinant S1 proteins as it is highly glycosylated protein. In present study the S1 gene amplified from isolated field strain of IBV was cloned into eukaryotic expression vector and express in vero cell line. The pQE-TriSystem vector was used as eukaryotic expression vector to express the corresponding protein. The successful expression was confirmed at 24 and 48 hrs post transfection by Reverse Transcriptase-PCR. These promising observations emphasize the need of expression of S1 gene recombinant protein for the development of effective recombinant DNA vaccine against IB in near future.

**Keywords:** Infectious bronchitis, IBV, Spike protein, S1 gene, Eukaryotic expression

Infectious bronchitis (IB) is highly contagious, acute and constitutes one of the most common disease of poultry caused by avian infectious bronchitis virus (IBV). IBV is highly transmissible virus belongs to group III of the genus Coronavirus of the *Coronaviridae* family (Cavanagh and Naqi, 1997). The various reports of southern Asia showed regular emergence of new strains (Yan *et al.*, 2011; Patel *et al.*, 2015) of this virus indicate its continue threat to poultry industry. Inclusion of IBV in OIE list signifies its economic importance.

Spike protein is a highly glycosylated protein of approximately 1160 amino acids, which cleaved to form S1 and S2 subunits of about 520 and 625 amino acids residues respectively post-translationally. Spike protein forms the club shaped characteristic surface projection in which the S2 protein is anchored to the membrane while S1 is attached to S2 and form its bulbous head. S1 defines the serotype and believed to play a major role in induction of protective immunity (Karaca *et al.*, 1992). It enables

virus entry into the host cell and subsequently viral spread by cell fusion (Soonjeon *et al.*, 2005).

The choice of a suitable expression system depends largely on the biochemical and biological properties of the protein of interest (Geisse *et al.*, 1996). The major advantage of mammalian expression system is that it will post-translationally modify the expressed product with higher degree of fidelity as compared to the other expression system. This may be crucial for producing efficacious antigen (Dertzbaugh, 1998). The recombinant S1 protein previously expressed in different eukaryotic expression system like in Baculovirus system (Dai *et al.*, 2002), plant expression vector (Zhou, 2003), recombinant plasmid transfected into COS - 7 cells (Jiaogongmei *et al.*, 2006) etc.

The present study describes the RT-PCR amplification of S1 gene from field isolate and its subsequent expression in Vero cell line. The PCR amplified 1.5 kb gene was ligated in to the pTZ57R/T vector, this construct was

transformed into *E. coli* DH5 $\alpha$  cells. For expression and characterization of S1 protein in *in-vitro* system and the S1 gene was further subcloned in eukaryotic expression vector pQE-TriSystem. The positive clones were confirmed by the release of identical size fragment on RE analysis and by PCR amplification of S1 gene with specific primers. The vero cell line was transfected with these pQE-TS-S1 plasmids isolated from positive clone. The successful expression was confirmed at 24 and 48 hrs post transfection by RT-PCR. The RT-PCR was performed from total RNA isolated from transfected cells at 24 and 48 hrs post transfection.

## MATERIALS AND METHODS

### RT-PCR amplification of S1 gene from field isolate

IB positive field samples were used to amplify the S1 gene of IBV. Total RNA was isolated from Trachea and Lung tissue of infected bird with the standard Trizol (Sigma, USA) method with some changes. cDNA were synthesized from this RNA with the random hexamer primers. For PCR amplification of S1 gene, self designed gene specific forward (5'-ATGTTGGTAACACCTCTT-3') and reverse (5'-AGACCATATTCACCTTGTA-3') primers were used. S1 gene amplification was then conformed by nested PCR by using internal HVR primers (Table 1). The amplified product was purified from agarose gel elution.

**Table 1: Self-designed Primes list used in present investigation**

Primer Set no.	Primer Name	Primer sequence (5'-3')	Amplicon Size (bp)
1	S1F	ATG TTG GTA ACA CCT CTT	1448
	S1R	AGA CCA TAT TCA CCT TGT A	
2	IBH12F	TGCAGGCTCTTCATCTG	503
	IBH12R	TAGGTGATCCATCACAC	
3	IBH3F1	TTAAGCAGAAGTT-TATAGTC	387
	IBH3R1	GCAAAGGACCTCCATATG	
4	IBH3F2	AATCCTAGTGGTGTTTCAG	257
	IBH3R2	GTTGCTCTACCACTAAAG	

### Cloning of S1 gene into T/A cloning vector

The purified S1 gene then ligated into a pTZ57R/T vector using T/A cloning. The ligated plasmid was transformed into DH5 $\alpha$  *E.coli* host cell strain. Positive clones were selected by blue white screening, colony PCR and double restriction enzyme (RE) analysis. The positive clones were then sequenced by ABI automatic DNA sequencer to confirm their orientation. The clone having S1 gene in correct confirmation was selected to release the inserted gene by *Sac*-I and *Hind*- III restriction enzyme for directional cloning into the expression vector.

### Subcloning of S1 gene into eukaryotic expression vector

Eukaryotic expression vector pQE-TriSystem was isolated from DH5 $\alpha$  clones using miniprep alkaline lysis method of plasmid isolation. This vector then digested with same set of restriction enzyme i.e. *Sac*-I and *Hind*- III to generate corresponding sticky ends. The RE digested vector the ligated with RE released S1 gene to construct pQE-trisystem-S1 recombinant plasmid. The DH5 $\alpha$  competent cells were then transformed with this recombinant plasmid. Positive clones were screened using colony PCR with S1 gene specific primers and double restriction enzyme (RE) analysis with *Sac*-I and *Hind*- III enzymes. The recombinant pQE-trisystem-S1 was isolated from positive *E.coli* colony.

### Transfection of Vero cell line

The Vero cells maintained at 37°C with 5% CO<sub>2</sub> and 80% humidity were used for transfection experiment. Cell were maintained in maintenance media Dulbaco's Modified Eagles Medium (DMEM, HyClone, USA) containing 10% NBCS (HyClone, USA), 50 $\mu$ g/ml gentamicin and 2.5 $\mu$ g/ml Amphotericin-B.

The day before transfection, 1 $\times$ 10<sup>5</sup> cells per well were plated in 12 well plate. These plates were incubated in CO<sub>2</sub> incubator at 37°C and 5% CO<sub>2</sub> for 24 hrs to get 60-80% confluency. SuperFect transfection reagent (Qiagen, Germany) was used for vero cell transfection as per the manufacturer's instruction with some modifications. 1.5 $\mu$ g plasmid DNA was diluted to make 75 $\mu$ l with OptiMEM media (Invitrogen, UK). After mixing it properly, 7.5 $\mu$ l

SuperFect transfection reagent was added to the DNA solution and mixed by pipetting. After incubated for 5-10 min at room temperature, 200µl growth medium containing NBCS and antibiotic was added to the reaction tube containing transfection complex. After mixing by pipetting immediately total volume was transfer in the well. In the similar manner, the mock transfected (vector without insert, i.e. without S1 gene) and the blank were maintained for the comparative study of transfection. The plate was incubated for 6 hrs with the transfection complexes under their normal growth conditions. The medium was remove containing remaining complexes from the wells by gentle aspiration, and washed once with PBS. Again fresh growth medium was added containing NBCS and antibiotic. The expression of transfected gene was analyzed after 24 and 48 hrs post transfection.

#### Assessments of S1 gene expression

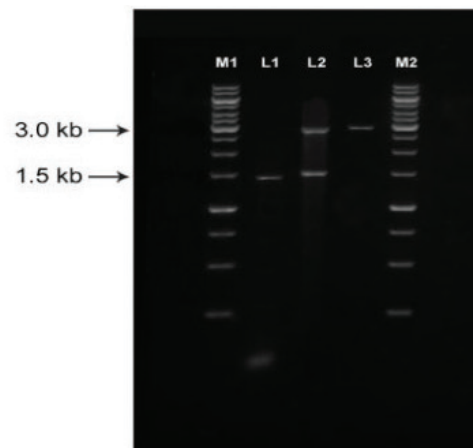
To confirm the expression of S1 gene in transfected cell, RT-PCR was performed from total RNA isolated from transfected cells at 24 and 48 hrs post transfection. Total RNA were isolated from test wells along with Mock transfected and negative control wells. The entire experiment was carried out in the biological triplicates. The S1 gene specific primers were used for RT-PCR amplification of S1 gene for confirming its expression.

#### RESULTS AND DISCUSSION

IB is an acute, rapidly spreading, viral disease of chickens. The disease is prevalent all over the world, with incidence approaching hundred percent in most locations. Reasons behind this great survivability of virus are wide variations in the serotypes, generation of new strains continuously, highly contagious nature, virulence of the strains, the evolution of specific tissue tropism and use of live vaccine. The mutable character of genes of infection related IBV antigens have complicated and increased the cost with the disease outbreaks. Further this has impaired its prevention through immunization (Cavanagh & Naqi, 1997).

The molecular characterization of IBV is based mainly on analysis of the S1 protein gene (Zhou *et al.*, 2004; Kwon *et al.*, 1993). IBV undergo a high frequency of mutation resulting in the emergence of different serotypes (Patel *et al.*, 2015; Yan *et al.*, 2011; Gelb *et al.*, 1991; Cook and

Huggins, 1986). S1 gene sequence comparison is a better tool of serotyping (Ladman *et al.*, 2006). Koch *et al.* (1991) reported that the sequencing results of S1 gene revealed most of the neutralizing epitopes to be located within the regions 40-129 (HVR-I) and 271-378 (HVR-II).



**Fig. 1:** Agarose gel electrophoresis (1%) showing 1.5 kb amplified S1 gene from field isolate and restriction enzyme analysis of InsT/A-S1 positive clone

M1 & M2 – 1kb DNA molecular weight marker

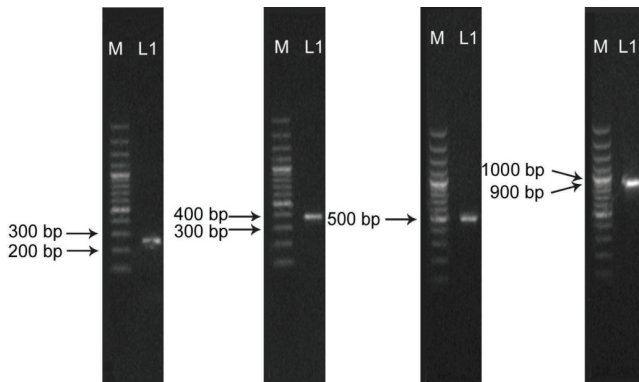
L1 – PCR amplified 1.5 kb S1 gene from field isolate

L2 – Restriction enzyme digested InsT/A-S1 clone showing released 1.5 kb S1 gene

L3 – Linearised InsT/A vector of 2.9 kb

In current study PCR amplification of cDNA were done using S1 specific primers by optimizing the PCR conditions at 48°C annealing temperature. Cavanagh *et al.* (1988) sequenced five vaccine strains of Mass type and reported amino acid substitutions in S1 gene among these strains. These areas were designated as hypervariable region 1 and 2 (HVR1 and HVR2). PCR amplification produced a product of 1448 bp (on agarose gel electrophoresis near to 1.5 kb marker band) from the field sample (Fig. 1). The confirmation was done by nested PCR amplification of hyper variable regions (HVR) of S1 gene of IBV. Nested PCR or multiplex PCR is the tool which is used for confirmation of PCR product. This provides a specific and sensitive approach for identification of gene. For nested PCR by set-1, set-2 and set-3 primers yielded desired PCR product of 503 bp, 387 and 257 bp respectively and a product of 965 bp amplified with forward primer of HVR12

and reverse primer of HVR3 set1 (Fig. 2). Surprisingly the published primer set which has shown reproducibility in amplifying S1 gene from vaccine strain (Sylvester, 2005) could not amplify the gene in present study.



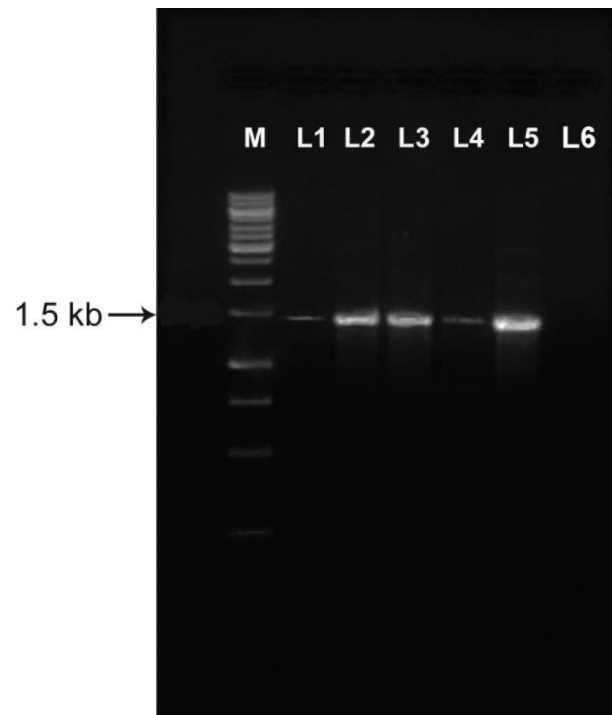
**Fig. 2:** Nested PCR by set-1, set-2 and set-3 primers yielded desired PCR product of 503 bp, 387 and 257 bp respectively and a product of 965 bp amplified with forward primer of HVR12 and reverse primer of HVR3 set1

PCR amplified 1.5 kb S1 gene was ligated in pTZ57R/T cloning. On transformation in *E. coli* DH5 $\alpha$  strain transformed cells characteristically produced blue and white colonies on agar containing X-gal and IPTG. Positive clones were designated as InsT/A-S1. Plasmids from positive white colonies were isolated and these InsT/A-S1 plasmids were digested with *Sac*-I and *Hind*-III restriction enzymes to released 1.5 kb insert (Fig. 1).

InsT/A-S1 plasmid containing S1 gene insert was sequenced using vector specific T7 primer and M13 primer. Around 1500 bp nucleotide sequence was generated. Out of seven positive clones, three were found in desired orientation.

pQE-TriSystem vector was used for IBV S1 gene expression in present study. This vector also has the same RE sites and linearized with the same set of RE i.e. *Sac*-I and *Hind*-III restriction enzyme. Eluted 1.5 Kb gene encoding S1 protein was ligated in linearized pQE Trisystem vector. The construct was designated as pQE-TS-S1 and transformed in *E. coli* DH5 $\alpha$  cells. The developed colonies for positive pQE-TS-S1 clone were confirmed by colony PCR and RE analysis. Release of S1 gene insert on double digestion and PCR amplified S1 gene of 1.5 Kb were obtained in 5 colonies out of 6 colonies screened.

Bacterial based expression system is the most convenient to use to express the heterologous antigen and can express antigen at very high level. Limitation of prokaryotic system is that complex eukaryotic proteins are usually not expressed because; post-translational modifications like glycosylation, phosphorylation, acetylation and amidation which cannot be done in bacterial expression vectors (Billman-Jacobe, 1996; Makrides, 1996). All these issues can be resolved by expressing the gene in eukaryotic cells. For the eukaryotic expression of S1 gene in present study Vero cell line was used. The vero cell line was transfected with the pQE-TS-S1 plasmid isolated from positive clone. The transfection was carried out by using the SuperFect, an active dendrimer, possesses a defined spherical architecture with radiating from a central core and terminating at charged amino group. This provides net positive charge to the SuperFect-DNA complex, which allows them to bind to negatively charged receptors (e.g. sialyated glycoproteins) on the surface of eukaryotic cells.



**Fig. 3:** RT-PCR amplified product of 1.5kb from Vero cell line

M – 1kb DNA molecular weight marker

L1 & L2 – PCR amplified 1.5 kb S1 gene from Vero cell line after 24 hrs.

L3 & L4 – PCR amplified 1.5 kb S1 gene from Vero cell line after 48 hrs.

L5 – Known positive 1.5 kb S1 gene fragment

L6 – Negative Control

The cDNA prepared from 24 hrs and 48 hrs post transfected vero cells was used for RT-PCR amplification. The amplified product of 1.5 kb was obtained in vero cells transfected with the pQE-TS-S1 indicated expression of S1 gene in cell system. The amplification was not seen in mock transfected and negative control cells (Fig. 3). The result showed amplification of 1.5 kb S1 gene in all transfected samples like the findings of Johnson *et al.* (2003) who got the expression of the S1 gene by RT-PCR by 20 hrs post-infection. The results showed that the S1 protein could be expressed in a large amount in Vero cells.

These promising observations emphasize further confirmation and validation of eukaryotic expression of S1 gene recombinant protein for the development of recombinant DNA vaccine against IB. Diverse nature of S1 gene can be exploited for the strain identification present in local area and to improve the diagnostic efficacy.

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