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High-Yield Expression and Purification of Recombinant σB Protein of Avian Reovirus (ARV) in Prokaryotic System

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A B S T R A C T

The σB protein of avian reovirus (ARV) is diagnostically important protein for development of an ARV-specific enzyme-linked immunosorbent assay (ELISA) and for which production of an antigenic recombinant σB protein in abundance is a prerequisite. In the present study, we successfully employed the prokaryotic system for expression of antigenic σB protein of ARV. The ARV isolated from the cases of viral arthritis and propagated on chicken embryo fibroblast primary culture was used for isolation of viral RNA. Isolated viral RNA was employed as a template for amplification of σB encoding gene. Further, the amplified fragment was cloned and subcloned in TA and pET32a(+) vectors, respectively. The continuity of open reading frame (ORF) was confirmed by sequenceing. The developed pET32a(+)-σB expression construct was transformed and expressed in BL21 (DE3) Rosetta cells. The SDS-PAGE and Western blot results confirmed the abundant expression of 56 kDa σB protein at 4 hr post induction. The expressed protein was further confirmed to be avian reovirus specific by its immunoreactivity with ARV hyperimmune serum. Overall, the results of the current study showed that the prokaryotic system can be exploited for expression of recombinant σB protein from avian reoviruses.

Introduction

Avian reovirus (ARV) infection is a ubiquitous disease of poultry, affecting mostly broiler breeds. Pathogenic effects of ARV such as arthritis and tenosynovitis are attributed to significant economic losses to poultry industry. ARV is classified under genus *Orthoreovirus* of family *Reoviridae* (Van Regenmortel *et al.*, 2000). The genome of ARV encodes at least ten structural proteins (λA, λB, λC, μA, μB, μBC, μBN, σC, σA and σB) and four non-structural proteins (μNS, P10, P17 and σNS) (Benavente and Martínez-Costas, 2007). Among these proteins, S3 gene-encoded σB is a major outer capsid protein of ARV, functioning in the induction of cell fusion and viral pathogenesis (Ni and Ramig, 1993). ARV σB protein is structurally similar to the σB protein of Duck reoviruses and Mammalian reoviruses (Vakharia *et al.*, 1996; Yin *et al.*, 1997) and share functional similarity with them. The σB protein is also known to induce group-specific

neutralizing antibodies against ARV and can be used for detection of ARV-specific antibodies in infected or vaccinated chickens.

Timely and specific diagnosis of ARV outbreak is a prerequisite for the implementation of vaccination programs for efficient control and subsequent prevention of outbreaks.

Various serological diagnostic techniques have been developed for efficient detection of antibodies against ARV, including serum neutralization test (Wickramasinghe *et al.,* 1993), AGID (Meanger *et al.,* 1995), immunoblot assay (Ide and Dewitt, 1979) and ELISA (Liu *et al.,* 2002). Several indirect ELISA diagnostic test based on recombinant σC and σB protein have been developed and validated for detection of antibodies against ARV (Zhang *et al.,* 2007).

Extensive sequence similarity of recombinant σB proteins with isolates prevalent in local outbreaks is a prerequisite for more specific and accurate detection of ARV infection. Previous reports of sequencing and phylogenetic analysis of σB encoding gene of Indian ARV isolate has revealed 89.9% sequence homology with ARV isolates from other countries (Kumari S. *et al.,* 2011). The substantial sequence divergence may contribute to significant reduction in the specificity and accuracy of ELISA-based detection.

Therefore, expression and purification of recombinant σB protein from Indian ARV isolate is favorable for the development of specific and accurate ELISA test for timely detection of ARV outbreaks in India.

In this study, we studied cloning, expression and purification of the σB protein of an Indian ARV isolate in *E. coli* based prokaryotic expression system.

Materials and Methods

Cells and virus

The ARV isolate used in the current study was maintained in the Avian Diseases Section, Division of Pathology, IVRI, Izatnagar (Bareilly, India). The virus was isolated from chicken flocks with the history of viral arthritis and malabsorption syndrome from a poultry farm in India (Kataria *et al.,* 1982). The virus was adapted and subsequently maintained in chicken embryo fibroblast (CEF) cell culture by repeated passaging. The CEF were cultured in Glasgow minimal essential medium (GMEM) (Sigma, USA) containing 10% foetal calf serum (FCS) (Thermo Scientific, USA), 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C with 5% CO_2 level. The supernatant of CEF culture positive for ARV virus was used as viral inoculum to infect a monolayer (70-80% confluency) of CEF cells. After ARV inoculation, the CEF cells were washed and incubated in GMEM containing 2% FCS. After the observation of visible cytopathic effects typical of ARV, CEF culture was harvested by three freeze–thaw cycles and clarified by centrifugation at 10,000 ×g for 10 min in an F2402H rotor (Beckman, USA).

RNA isolation, cDNA preparation and cloning

Total viral RNA was isolated from ARV-infected CEF cells using TRIzol® reagent (Invitrogen, Carlsbad, CA), as per the manufacturers protocol. The extracted RNA was reverse-transcribed to synthesize the first strand cDNA using gene specific primers and Thermoscript™ RT-PCR kit (Invitrogen, Carlsbad, USA). Synthesized cDNA was further used as a template for amplification and TA cloning of σB encoding gene in pTZ57R/T vector. Full length σB gene sequence was amplified from cDNA using the

following primer pairs (forward 5'-CCCGGATCCATGGAGGTACGTGTGCCAAA-3' and reverse 5'-CCCAAGCTTTTACCAACCACACTCCACAAC-3'). *BamHI* and *HindIII* restriction sites placed in the forward and reverse primers, respectively, were used to facilitate cloning. PCR amplification was carried out using the following cycling conditions: 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1.5 min, with a final extension of 72 °C for 10 min. The PCR amplified gene product was cloned into a T/A cloning vector pTZ57R/T (MBI Fermentas, Germany). A band size of 1104bp (~1.1 Kbp) was observed on agar gel electrophoresis and further confirmed by restriction enzyme digestion and sequencing. The complete CDS of σB gene was subcloned in frame into *BamHI* and *HindIII* sites of a 6X His-tagged prokaryotic expression vector, pET 32a (+) (Novagen, Germany). The recombinant plasmid (pET32a-σB) was confirmed by restriction enzyme digestion and DNA sequencing.

Expression and purification of recombinant σB protein

The recombinant plasmid (pET32a-σB) was used to transform *E. coli* BL21 (DE3) Rosetta cells (Novagen, Germany) and protein production was induced with isopropyl-d-thiogalactopyranoside (IPTG; 1 mM final concentration, MBI Fermentas, Germany) for 6 hrs at 37 °C. The bacterial culture pellet containing 6X Histidine tagged σB protein was solubilised using 8 M urea and purified by affinity chromatography by Ni-NTA column (Invitrogen, USA). The recombinant protein was further purified using the Amicon ultra-4 centrifuge filter device (Millipore, USA) with a membrane cut-off of 30 kDa and dialyzed using 10% glycerol in PBS. The concentration of purified protein was determined by Modified Lowry's Protein assay kit according to the manufacturer's protocol (Pierce, USA). The confirmation of the recombinant σB protein was accessed by SDS-PAGE and Western blot using anti-His antibody (Qiagen, Germany) as well as hyperimmune sera raised against the recombinant σB protein.

SDS-PAGE and Western blot

Purified σB protein and protein extracts of the induced recombinant cells were mixed with an equal volume of reducing laemmeli sample buffer, boiled for 3 min, and separated by SDS-PAGE (using 10% gel, in a BioRad, MiniProtean II electrophoresis unit). After electrophoresis, the fusion proteins and protein extracts of the induced recombinant cells were transferred to nitrocellulose membranes by the method described by Towbin *et al.,* (1979). The blots were incubated overnight at room temperature, with 1:200 dilution of chicken anti-ARV hyper-immune serum to probe the recombinant σB protein.

Results and Discussion

Reovirus infection is an economically important and highly contagious viral disease of broiler birds which needs timely diagnosis for implementation of effective control measures. Molecular biotechnology-based approaches *viz*. dot-blot hybridization (Yin and Lee, 1998), PCR combined with RFLP (Hedayati *et al.*, 2013), immunodiffusion and serum neutralization test (Islam and Jones, 1988) has been described for the identification of avian reoviruses in infected chickens. Indirect ELISA kits based on the σB and σC proteins have been developed for the detection of plasma or serum antibodies against ARV infection in chickens (Liu *et al.*, 2002). Therefore, bulk production of a properly folded, antigenic and immunogenic ARV proteins is an absolute neccesity for

developent of ELISA based diagnostic kits. Prokaryote based expression system offers the advantage in terms of its simplicity, robustness, low cost and high yield of recombinant proteins over mammalian and yeast based system, which are cumbersome and costly systems (Chen, 2012).

Chicken embryo fibroblast (CEF) cell culture, the most senstive culture system for propagation of ARV, was used in the current study. The cytopathic effects (CPE) were visualized as the formation of multinucleated cells (syncytia), which appeared as early as 24 hr post inoculation, followed by degeneration and detachment from the monolayer leaving small holes in the cell sheath and dead cells floating in the medium (Fig. 1). These CPE observation were in accordance with the previous study in which ARV-infected CEF cells exhibited shrinkage, rounding, and detachment from the plate, nuclear damage, with chromatin condensation and margination (Labrada *et al.*, 2002).

The culture supernatant of CEF, infected with ARV isolate was used for isolation of RNA template which was further employed for amplification of the σB protein encoding gene using gene specific primers. The expected amplicon of 1.1 Kbp was obtained and run on an agarose gel (Fig. 2A). Further, this amplified product was cloned in pTZ57R/T and the positive colonies were selected by blue-white screening and restriction enzyme digestion that released the 1.1 Kbp σB protein encoding gene insert (Fig. 2B). Recombinant pTZ57R/T-σB clones were subjected to Sanger sequencing and the nucleotide sequence obtained was BLAST analyzed.

Fig.1 Reovirus-induced cytopathic effects observed in chicken embryonic fibroblast cell culture. 1A. Non-infected primary chicken embryo fibroblast cells. 1B. Cytopathic effects observed 48 h post reovirus infection. Multinucleated cells (syncytia) are indicated by arrow. CPE include degeneration and detachment of fibroblast cells from monolayer leaving small holes in the cell sheath and dead cells floating in the medium

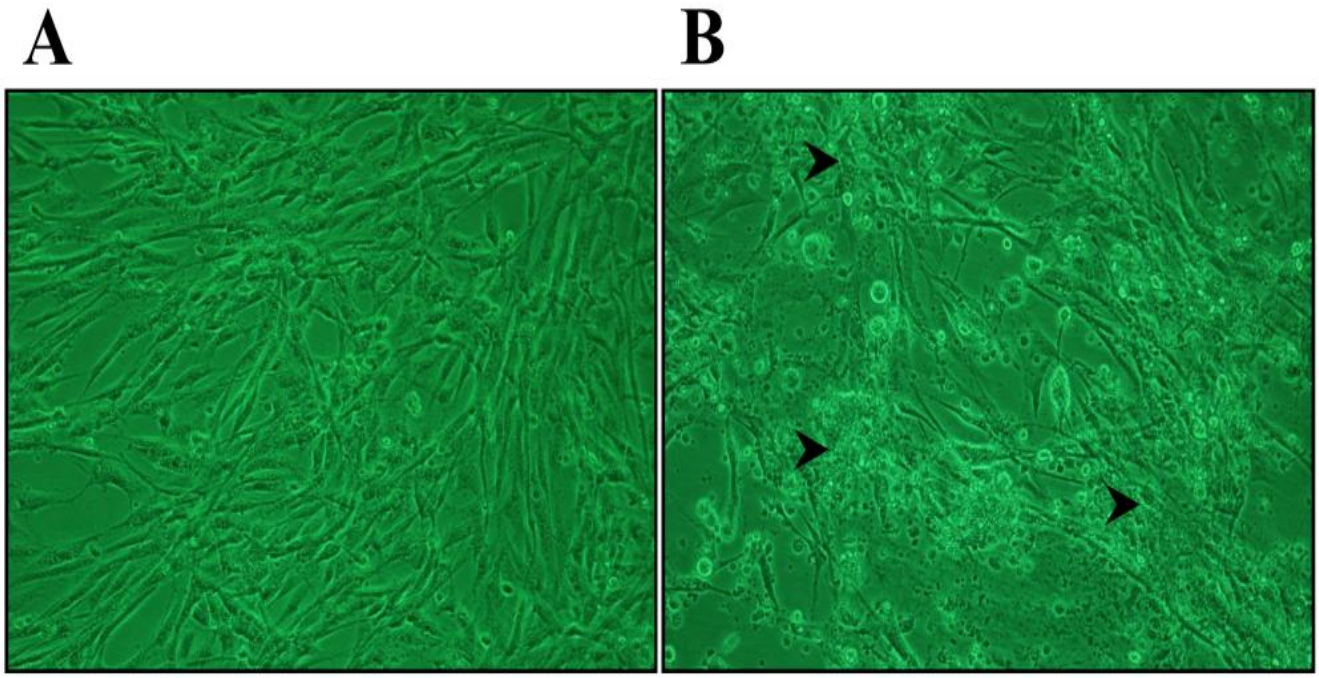


Fig.2 PCR and restriction digestion of σB gene in cloning and expression vector. 2A. Agarose gel electrophoresis of σB gene amplicon of 1.1Kb. Lane 1. 100 bp DNA ladder; lane 2 and 3. 1.1Kb σB gene amplicon. 2B. Agarose gel electrophoresis of *BamHI* and *HindIII* digested recombinant pTZ57R/T plasmid confirming the presence of 1.1Kb σB gene amplicon. Lane 1. 1Kb DNA ladder. Lane 2. Vector control pTZ57R/T plasmid after single digestion with *BamHI*; Lane 3 and 4. Recombinant pTZ57R/T plasmid digested with *BamHI* and *HindIII* showing the release of 1.1Kb σB gene insert.

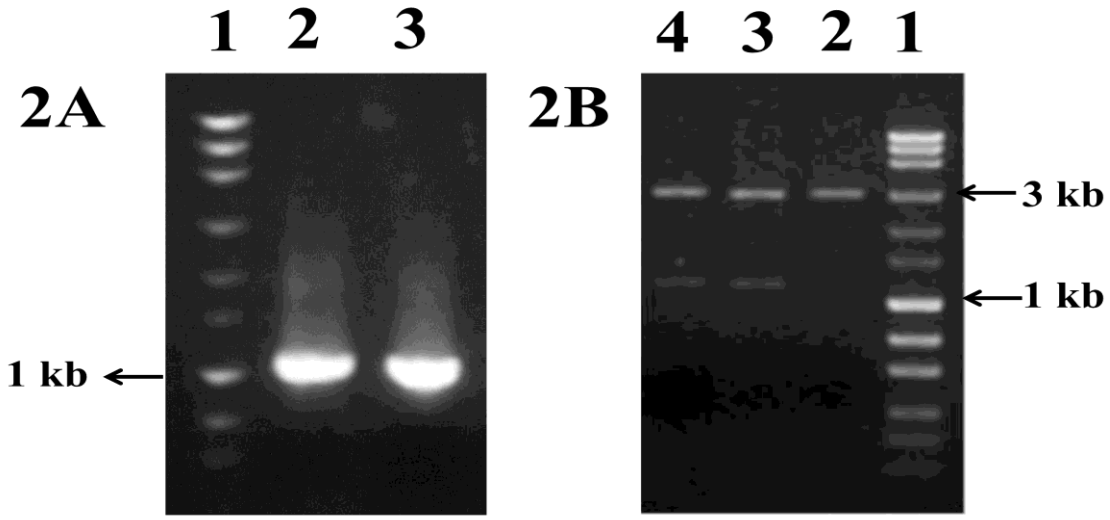
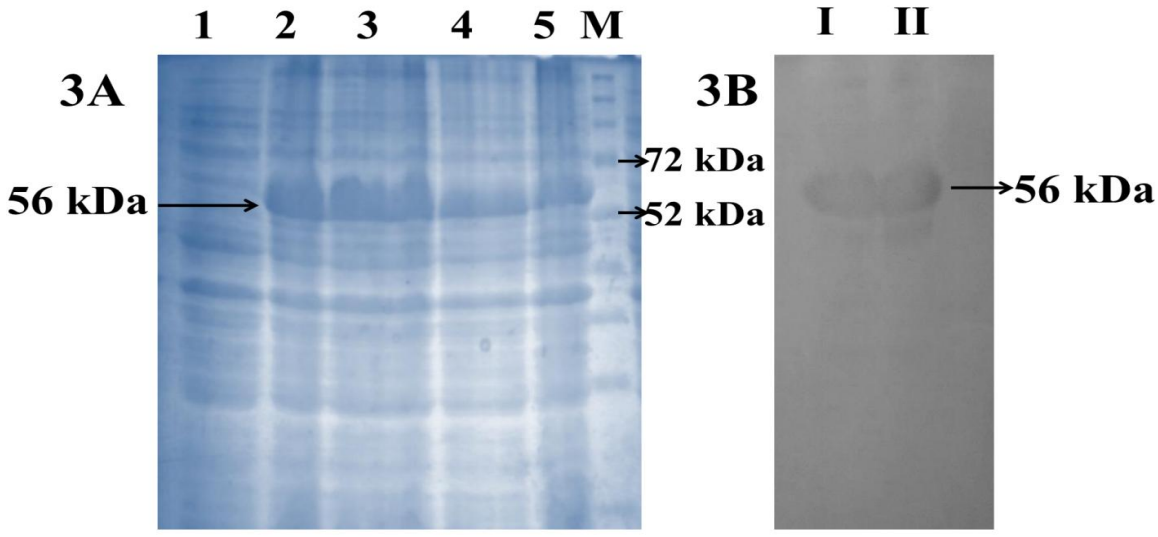


Fig.3 SDS-PAGE and Western blot analysis of σB expression in a prokaryotic expression system. 3A. SDS-PAGE analysis of σB expression kinetics in BL21 cells. Lane M. Pre-stained protein marker; Lane 2. Cell lysate from uninduced cells, lane 2, 3, 4 and 5, Cell lysate from induced cells at 2, 4, 6, and 8 hrs post-induction showing 56 kDa σB protein in BL21 cells. 3B. Western blot analysis showing 56 kDa σB protein in BL21 cells. Lane 1 and 2. Cells lysate from post-induction cells showing 56 kDa σB protein in BL21 cells.



The deduced nucleotide sequence of the σB encoding gene was submitted to GenBank (Acc. No. JQ924284). The nucleotide sequence shared overall 89.9% sequence homology with ARV isolates from other countries. However, it shared only 64.0% homology with Muscovy duck reovirus (strain 89026) and 70.6-71.2% with turkey reovirus (strain NC98 and Tx99, respectively) at nucleotide level (Kumari *et al.,* 2011). This can be attributed to highly variable nature of σB encoding gene as reported earlier (Wickramasinghe *et al.,* 1993).

Further, the amino acid sequence data of σB protein of Indian ARV isolate revealed the presence of two conserved B- cell epitopes as well as variable immunogenic epitopes similar to those found in Tunisian isolates (Hellal Kort *et al.,* 2013). These two conserved minimal determinant of linear B-cell epitopes ^{21}KTPACW^{26} (epitope A) and ^{32}WDTVTFH^{38} (epitope B) were also identified in the σB protein sequence of American chicken ARV (S1133 strain) isolate (Yin *et al.,* 2013). It has been shown that monoclonal antibodies raised against these epitopes are able to protect birds from infection (Yin *at al*., 2013). Due to highly variable nature of the σB protein and its potential role in inducing protective antibodies, there is growing need to develop vaccines and diagnostics according to serotype prevalent in particular area. Therefore, in the present study, expression and purification of σB protein of Indian ARV isolate was carried out for further use in the development of an ELISA assay.

E. coli BL21 expression system has been widely used due to various advantages like relative inexpensive cost, ease of manipulation with rapid growth rate (Mayer and Buchner, 2004). Hence in the present study, *E. coli* BL21 system was choosed for σB protein expression. Following induction with 1 mM IPTG, the recombinant *E. coli* expressed σB protein along with N-terminus 6X His-tag accounting for a total molecular weight of 56 kDa as observed on 10% SDS–PAGE (Fig. 3A). The highest expression level was found at 4 hr post-induction as observed on SDS-PAGE. Further, the σB protein was purified under denaturing condition with dialysis and further subjected to SDS–PAGE analysis, which showed a single band of purified recombinant σB protein at the expected size of 56 kDa.

In another study, the prokaryotic expression of σB fusion protein from S1133 ARV isolate yielded protein with an approximate molecular mass of 59.3 kDa, but the expressed proteins showed varying results when immunoblotted with antiserum from chickens vaccinated with vaccine virus strain S1133 (Liu *et al.,* 2002). Similarly, no antibodies could be detected by ELISA or immunoblot assay in ducks immunized with the recombinant muscovy duck reovirus σB protein and no significant protection was noted after the challenge (Le Gall-Recule *et al.,* 1999). These results has been attributed to the presence of antibodies, specific to different serotypes of ARV prevalent under field conditions, making it unable to get detected by traditional immunoassays based on strain S1133 antigen.

In the present study, the presence of recombinant σB protein was confirmed with Western blot analysis using anti-His antibodies and field hyper-immune serum, indicating validation of immunore activity with antibodies against Indian ARV isolates (Fig. 3B). The concentration of purified σB protein by modified Lowry's protein assay kit was found to be 1.6 mg/100 mL of bacterial culture.

In conclusion, the results from current study validate the use of prokaryotic expression

system for abundant production of immunogenic σB protein of ARV. The produced σB protein can be employed in future for the development of in-house ELISA test for rapid and timely detection of ARV outbreaks in India.

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