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Cloning and expression of fusion (F) and haemagglutinin-neuraminidase (HN) genes of Newcastle disease virus in insect host (*Sf*9cells)

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Outbreak of Newcastle disease (ND), one of the major diseases with significant economic importance, causes chaos in poultry industry including subsidiary agriculture field which gains its revenue in terms of feed. The growing demand for acceptance in the global market for poultry products warrants India to declare freedom from OIE notified avian diseases like Newcastle disease and avian influenza (AI) (OIE 2009). Hence, there is a need to develop better vaccines against Newcastle disease.

Newcastle disease virus (NDV) has 2 surface glycoproteins namely, fusion (F) and haemagglutinin-neuraminidase (HN) (Alexander 2000). The fusion gene inserted in herpes virus of turkeys resulted in a vaccine which gave good protection against virulent NDV (Morgan *et al*. 1993). A recombinant baculovirus expressing the F protein of NDV D26 induced a protective effect (Mori *et al*. 1994). Swayne *et al.* (2003) reported recombinant paramyxovirus type 1–avian inûuenza-H7 virus as a vaccine for protection of chickens against inûuenza and Newcastle disease. It was reported that in contrast to the whole virus vaccines, recombinant vaccines provide immunity and eliminate the development of antibodies to many non specific epitopes. Recombinant Newcastle disease virus was found as a vaccine vector (Haung *et al*. 2003). Kamiya *et al.* (1994) reported that immunization with recombinant Newcastle disease fusion (rNDF) or recombinant Newcastle disease haemagglutinin-neuraminidase (rNDHN) proteins of the velogenic Miyadera strain protected the challenged birds. HN protein of Newcastle disease virus was expressed in mouse myeloma cells and its immune potentials were studied (Risinskaya *et al*. 2001). Apart from the immunogenic potentials of the recombinant antigens, these recombinant antigens could also be used as marker antigens for differentiating vaccine viruses and epizootic viruses (Kirubaharan *et al.* 2000, Koppad *et al.* 2009). In this context, the present study described expression of fusion and HN genes of NDV in insect cells. These expressed proteins were used to assess their immunogenicity in experimental birds trial.

Virus propagation: Newcastle disease virus isolate available at the Department of Animal Biotechnology, Madras Veterinary College, Chennai, was inoculated through the allantoic cavity route in 9-day old specific pathogen free (SPF) embryonated chicken eggs (ECE) received from a commercial hatchery. Amnio allantoic fluid (AAF) was harvested from inoculated embryos and presence of the virus was checked by haemagglutination (HA) and haemagglutination inhibition (HI) tests.

RNA isolation and RT-PCR: RNA was isolated from AAF using trizol LS reagent; 10 µg of RNA was reverse transcribed using oligo-dTs primers and reverse transcriptase superscript-III enzyme. Full length amplification of fusion (F) and haemagglutinin-neuraminidase gene was done by PCR using gene specific primers (F-gene primers: Forward-: 5'GCAGCCATGGGCTCCAGATCTACC3', Reverse-: 5'CGACTCGAGTATTTTGGTAGCGGCCCT3' and HN gene primers: Forward: 5'ATGGATCCATGGTCCGTTCC GCATCACCA3', Reverse : 5'ATGAATCTCGAGCCTTG GCATTGCAGAAG 3') and XT-5 long PCR polymerase enzyme with the following cycle conditions (initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 sec, annealing at gradient temperature ranging from 52°C to 58°C with 0.5 degree differences, for 30 sec, extension at 72°C for 40 sec, of 30 cycles, final elongation at 72°C for 7 min). Amplified products were checked by 1% agarose gel electrophoresis and purified by using gel purification kit. Gel purified products were confirmed by sequencing using a commercial kit.

Cloning and expression: PCR amplified products were cloned into pTriEx vector using T_4 DNA ligase enzyme and transformed into *E coli,* BL21, DE3 cells. Recombinant colonies were screened by colony PCR using gene specific primers and insert was released from recombinant clones by restriction enzyme digestion using *Nco I* and *Xho I* enzymes

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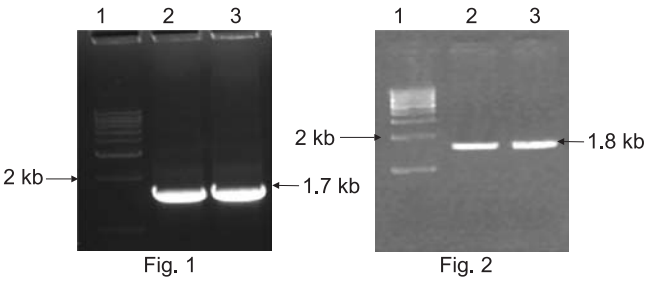
at 37°C for 1 h. Positive colonies were induced with 1mM IPTG overnight. SDS-PAGE and immunoblotting were done with the overnight induced cultures for expression.

*Sf*9 cells were grown in 6 well plates and when the cells reached 80% confluency, cells were transfected with the mixture of recombinant pTriEx containing the gene and baculovirus expression vector. Transfected cells were incubated at 28°C for 80 h for the plaques to develop. After 80 h plaques were stained with 0.8% of neutral red and randomly selected plaques were screened for the presence of transfected gene using gene specific primers. The positive plaques were again transfected into T_{25} flasks containing confluent layer of *Sf*9 cells after 72 h of post transfection, the expression of proteins were confirmed by immunofluorescent assay, SDS-PAGE and immunoblotting.

The objective of this study was to explore the possibility of producing recombinant F and HN proteins of NDV in an insect host system. HA test performed with the harvested AAF indicated a HA titre of 2^8 and a HI titre of 2^7 with known NDV antiserum.

Reverse transcription PCR of the fusion and HN genes resulted in the amplification of 1.7 and 1.8 kb amplicons, respectively, as expected (Figs 1, 2). Both the genes were cloned into pTriEx neo 1.1 expression vectors and transformed into *E coli,* BL21 (DE3) cells. For expression of recombinant fusion and HN proteins in insect cells, recombinant clones obtained were transfected to insect cells using Baculovirus expression vector. After 72 h of post transfection, recombinant virus was isolated from the plaques. The plaques were screened for the presence of F and HN gene inserts by PCR using gene specific primers. The positive plaques (Fig. 3) were used for checking the expression of recombinant fusion and HN proteins.

Expression of recombinant fusion and HN proteins in *Sf*9 cells was confirmed by immunofluorescence assay. Positive expression was confirmed by observing the green fluorescence (Fig. 4). Further, the expressed proteins were also confirmed by SDS-PAGE by running cell lysates of transfected and non-transfected cells. SDS-PAGE gels revealed the presence of 55 kDa and 65 kDa proteins as



Figs 1–2. **1.** Agarose gel (1%) electrophoresis of Fusion gene of 1.7 Kb size. Lane 1: 1Kb DNA ladder, Lane 2 and 3: Fusion gene amplicon of 1.7 Kb. **2.** Agarose gel (1%) electrophoresis of HN gene of 1.8 Kb size. Lane 1: 1Kb DNA ladder, Lane 2 and 3: haemagglutinin neuraminidase gene amplicon of 1.8 Kb.

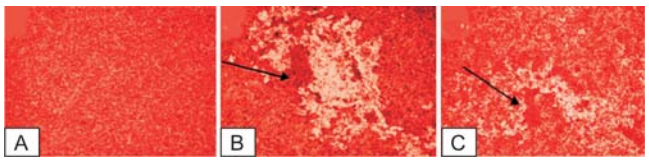


Fig. 3. Plaque assay for the isolation of recombinant baculovirus with F and HN gene in *Sf9* insect cells under 1% agarose overlay, stained with 0.33% neutral red. Plaques are indicated by arrows (100 ×). A, Non transfected *Sf*9 cells; B, recombinant baculovirus plaque with F gene; C, recombinant baculovirus plaque with HN gene.

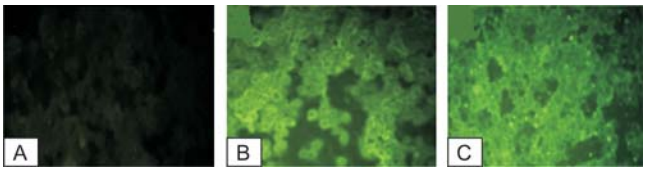
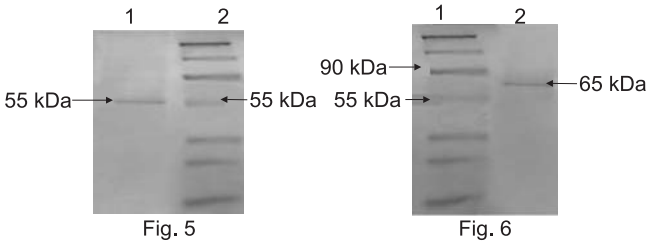


Fig. 4. Indirect immunofluorescence test to detect expression of recombinant F and HN protein in *Sf9* cells. Cells were fixed 72 h post-infection (200 ×). A, Uninfected *Sf*9 cells; B, recombinant F gene transfected cells showing strong positive fluorescence; C, recombinant HN gene transfected cells showing strong positive fluorescence.



Figs 5–6. **5.** SDS- PAGE (12%) analysis of expressed Fusion (F) protein showing 55 kDa in *Sf* 9 cells. Lane 1, 72 h post-transfection; lane 2, prestained protein marker showing different proteins in kDa. **6.** SDS- PAGE (12%) analysis of expressed heamagglutinin-neuraminidase (HN) protein showing 65kDa in *Sf* 9 cells. Lane 1, prestained protein marker showing different proteins in kDa; Lane 2, 72 h post-transfection.

expected (Figs 5, 6). Immunoblotting was done by transferring expressed recombinant proteins from SDS-PAGE into nitrocellulose membranes and their specificities were confirmed using NDV specific serum (Figs 7, 8), which was raised in department by injecting commercial ND vaccine into chicken.

Newcastle disease is a highly contagious, globally important viral disease of poultry which exhibits epizootic proportions if effective control measures are not implemented. The disease is still rampant in several parts of the globe, though some good vaccines are available. Pathotype diversity of NDV increases the possibility of diagnostic failures, resulting in undetected infections (Mebatsion *et al.* 2005, Miller *et al.* 2010). Persistent ND infection in commercial and domestic chicken indicated that

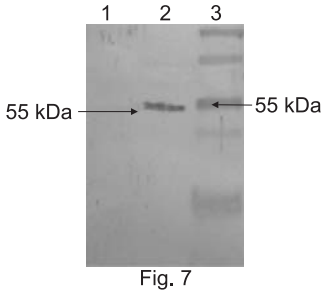


Fig. 7

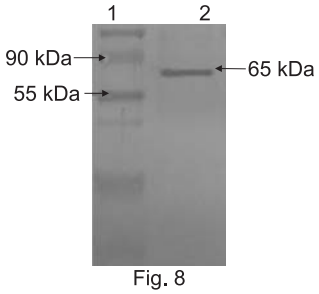


Fig. 8

Figs 7–8. **7.** Western blot analysis showing 55 kDa fusion (F) protein in *Sf* 9 cells. Lane 1, Control *Sf* 9 cells; lane 2, 72 h post-transfection; lane 3, prestained protein marker. **8.** Western blot analysis showing 65 kDa heamagglu-tinin-neuraminidase (HN) protein in *Sf* 9 cells. Lane 1, Prestained protein marker; Lane 2, 72 h post-transfection.

the current vaccination strategies are not fully efficacious and needs to be improved by new generation vaccines (Liu *et al.* 2010). Moreover, use of whole cell live vaccines complicates the diagnostic approaches and necessitates the need for a diagnostic assay which can differentiate vaccine viruses from epizootic viruses isolated from field. Subunit/ recombinant antigen based marker vaccines might be an alternative. In this context, the present study was undertaken to clone and express fusion (F) protein gene of a pigeon isolate of NDV in eukaryotic expression system for its application in developing immunodiagnostics and sub-unit vaccines.

Fusion glycoprotein expressed in prokaryotic expression system will be in the precursor, F_0 form as no cleavage occurs in prokaryotes due to the absence of Golgi membrane and also the protein will not be in its active form due to the absence of post-translational modifications (Römer-Oberdörfer *et al*. 2003). Hence in our study the fusion protein was first expressed in prokaryotic expression system and later in mammalian expression system since it offers post-translational modifications and relevant cellular compartmentalization of the proteins so that the expressed fusion protein can be used for further *in vivo* studies and immunodiagnostics (Loke *et al.* 2005). Transient transfection was done in *Sf*9 cells using recombinant pTriEx plasmid. Protein expression was observed in cell lysate collected 72 h post-transfection and hence 72 h post-transfection was considered as optimum time for protein harvest. Transfected cells showed bright cytoplasmic fluorescence indicating that fusion protein was produced in the cytoplasm as reported earlier (Lee *et al.* 2008, Rajawat *et al.* 2008).

A multifunctional protein, HN is also the major antigenic determinant of the paramyxoviruses. HN is responsible for the adsorption of the virus to sialic acid containing cell surface molecules. In addition, HN mediates enzymatic cleavage of sialic acid (neuraminidase activity) from the surface of virions and the surface of infected cells (Lamb and Park 2007, Chambers *et al.* 1986). The fusion (F) protein mediates virus - cell and cell - cell fusion for all paramyxoviruses (Choppin and Scheid 1980), following the proteolytic generation of a 'fusion peptide' (Nagy *et al.* 1991). A complex between NDV HN and F protein can be detected at the surface of both infected and transfected cells (Berinstein *et al.* 2001, Lamb and Park 2007). Among the 6 NDV proteins, fusion and HN proteins were reported to be immunogenic and hence DNA vaccines using these genes of NDV were exploited by many workers (Heckert *et al.* 2002, Loke *et al.* 2005, Patel *et al.* 2007).

Like the F protein, HN protein expression was also observed in cell lysates collected 72 h post-transfection. HN clone of NDV was transfected in insect cells using baculovirus transfection system. In conclusion, we have successfully cloned and expressed F protein and HN protein genes of NDV in insect host. The recombinant F and HN proteins thus can be purified to assess their immune potentials in birds. The antigens can be used in the development of diagnostic assays or as recombinant vaccines. It is also concluded that the expression of the recombinant fusion protein and haemagglutinin neuraminidase protein can be increased for mass antigens production in insect host for commercial use.

SUMMARY

The lentogenic strain of Newcastle disease virus (NDV) was propagated in 9–11 day-old specific pathogen free (SPF) embryonated chicken eggs. Allantoic fluid was collected after the death of embryo, the obtained allantoic fluid showed virus titre (HA) of 1:256. Viral RNA was extracted from allantoic fluid by Trizol method, complementary RNA was synthesized by reverse transcriptase enzyme (superscript-III) and full length recombinant fusion and recombinant haemagglutinin-neuraminidase gene was amplified by PCR using gene specific primers. Gel purified PCR products were cloned into pTriEx neo 1.1 expression vector and transformed into *E. coli* BL-21 DE3 cells. The presence of insert in recombinant colonies was confirmed by colony PCR, restriction digestion and sequencing of insert released products. Cloned recombinant plasmids of fusion and haemagglutinin neuraminidase genes were expressed in *Sf9* cells using baculovirus expression vector system. After 72 h of transfection plaque assay was done to confirm the presence of recombinant virus and then positive recombinant viruses were transfected to insect cells. The expression of recombinant proteins in *Sf9* cells was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting and immunofluorescent assays (IFA).

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