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Expression of full length fusion (F) protein gene of Newcastle disease virus in mammalian expression system

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ABSTRACT

The present study was carried out to clone and express fusion protein gene of Newcastle disease virus in mammalian expression system. The Newcastle disease virus (2K3) isolate from pigeon was propagated in 9–11 day-old embryonated chicken eggs and infected allantoic fluid was collected for RNA isolation. The fusion (F) gene of 1.68 Kb was amplified with cDNA as template and was cloned in TOPO cloning vector. The recombinant TOPO colonies were digested with *NcoI* and *XhoI* enzymes, the insert released was further ligated into pTriEx Neo 1.1 expression vector digested with the same enzymes and was transformed in *Escherichia coli*. The recombinant pTriEx colonies confirmed by colony PCR and restriction digestion were induced with 1mM IPTG which showed expressed fusion protein of 55 kDa at 4 h post induction and increased in overnight induced cultures. The recombinant pTriEx plasmid was transfected into vero cells. The cell lysate collected at 48 h and 72 h post transfection showed expressed fusion protein with the molecular weight of 55 kDa in 12% SDS-PAGE. The protein was further confirmed to be NDV specific by its immunoreactivity with NDV antiserum raised in rabbits showing fusion protein of 55 kDa. The immunofluorescence assay of transfected vero cells exhibited a bright cytoplasmic fluorescence confirming the fusion protein expression.

Key words: Fusion gene, Newcastle disease virus, Immunofluorescence assay, Mammalian expression, Prokaryotic expression, SDS PAGE, Sub-cloning pTriEx, TOPO cloning, Western Blot

Newcastle disease (ND) is a highly contagious and economically important disease having a wide host range infecting 27 of the 50 orders of the birds (Alexander 2000).

The disease is caused by Newcastle disease virus (NDV), also known as avian paramyxovirus type–1 (APMV-1). ND viral genome has 6 genes in 3' to 5' orientation i.e NP, P, M, F, HN and L encoding 6 structural proteins i.e nucleoprotein, phosphoprotein, matrix, fusion, haemagglutinin-neuraminidase and large protein (Millar *et al*. 1988, Chambers *et al.* 1986, Wilde *et al.* 1986). The HN and F glycoproteins play an important role in virus infectivity, and the attachment of the virus to the host cells is mediated by HN having both haemagglutitnin and neuraminidase activities. HN has an undefined role in the fusion mediated by the fusion protein (Yusoff and Tan 2001). The fusion protein allows penetration of the virus into host cells via fusion of viral envelope and cell membrane (Romer-oberdorfer 2003). The fusion glycoprotein is translated as precursor F_0 which is cleaved within the cells by host proteolytic enzymes into F_1 and F_2 (Schied and Choppin 1977). The pathogenicity of NDV is related to cleavability of F_0 protein (Nagai and Klenk 1977). The protective efficacy of chimeric vaccines was evaluated generating 3 recombinant viruses based on Lasota strain, chickens immunized with this chimeric vaccine expressing F or F plus HN were efficiently protected against shedding virulent virus efficiently preventing spread of the virus due to F protein (Kim *et al.* 2013). These 2 proteins bear major virus neutralizing epitopes, and antibody to these proteins can neutralize the virus independently. For the presently available commercial ND vaccines serological differentiation of naturally infected birds from vaccinated birds using qualitative assay is impossible (Lee *et al.* 2008). To overcome this disadvantage, in the present study fusion protein was expressed in mammalian expression system, the recombinant fusion (F) protein thus obtained can be used as sub-unit marker vaccine to differentiate naturally infected birds from vaccinated birds and as an antigen in immunodiagnostic assays in birds for future Newcastle disease eradication programme. Thus with the above factors in mind the fusion protein gene was amplified by RT-PCR and the recombinant protein was assessed *in vitro* after expression in mammalian expression system for use in immunodiagnostics in future.

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MATERIALS AND METHODS

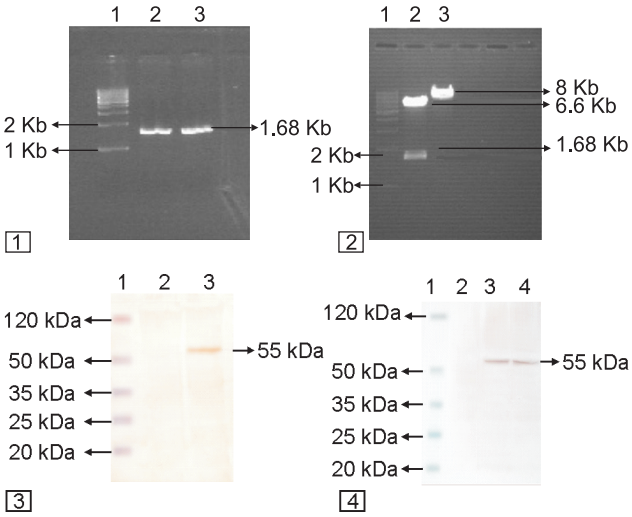
NDV isolate: Newcastle disease virus isolate (2K3) from pigeon available at the Department of Animal

Biotechnology was propagated in 9–11 day-old embryonated chicken eggs through intra-allantoic route of inoculation. The infected amnio allantoic fluid was collected 62 h post infection for RNA isolation and further studies.

RNA Isolation and RT-PCR: Infected AAF was checked by haemagglutination (HA) test for HA activity and used for RNA isolation (Lien *et al.* 2007) using TRIZOL reagent. Reverse transcription was carried out using random hexamers (50 ng/µl), thermoscript RT (15U/µl), 5X RT buffer (250mM Tris-HCl, 250 mM KCl, 20mM $MgCl_2$, 50 mM DTT) by incubating at 25°C for 10 min, 50° C for 50 min, 85° for 5 min for one cycle. Full length fusion gene sequence was amplified from c-DNA using the following primers (forward 5'-GCAGCCATGGGCTCCAGATCTA CC-3' and reverse 5'-CGACTCGAGTATTTTGGTAGC GGCCCT-3'). *Nco*I and *Xho*I restriction sites placed in the forward and reverse primers respectively were used to facilitate cloning in cloning vector. Amplification was carried out in 50 µl reaction using 5 µl c-DNA, 0.2 mM dNTP's, XT-5 polymerase, XT-5 poly buffer A with the following temperature conditions: initial denaturation at 94°C for 2 min followed by split cycle (94°C- 30 sec, 52°-58° C for 30 sec, 72°C- 40 sec for 10 cycles and 94°C- 30 sec, 54.4°C for 40 sec, 72°C for 40 sec for 20 cycles) followed by final extension of 72° C for 7 min.

Cloning of fusion gene in TOPO pCR2.1 and pTriEx Neo1.1 vectors : Amplified fusion gene product was purified by gel extraction kit and used for cloning into TOPO pCR 2.1 as per manufacturer's instructions. Recombinant clone containing fusion gene was selected based on ampicillin selection. Recombinant TOPO plasmid was isolated using plasmid extraction kit and subjected to automated sequencing. The nucleotide sequence obtained was subjected to BLAST analysis which showed 94% homology with 2K3 pigeon isolate (Accession No.F.J 986192.2). The fusion gene was released from recombinant TOPO clone and was ligated to pTriEx vector using T_4 DNA ligase (5U/µl). The recombinant pTriEx clones were selected based on ampicillin selection, and were subjected to automated sequencing to confirm the orientation of the insert.

Expression of recombinant protein in prokaryotic and eukaryotic hosts: BL21DE3 cells were used as prokaryotic host to confirm the expression of the recombinant fusion protein. The confirmed recombinant pTriEx colony was induced with 1 mM IPTG. The protein was confirmed to be NDV specific by western blotting, the proteins were electroblotted onto the nitrocellulose membrane. After blocking overnight in 2% BSA, the membrane was incubated with the diluted monoclonal antiserum raised in rabbits (1:10) for 1 h the membrane was washed 3 times with PBST buffer and then incubated with diluted goat anti-rabbit HRP conjugate (1:1000) for 1 h at 37° C. The membrane was washed and band was visualised following incubation with diaminobenzidine tetrahydrochloride (DAB). Vero cells grown to 70% confluency were used for transfection with recombinant pTriEx plasmid using lipofectamine-2000 as per manufacturer's instructions. Expression of the fusion gene in vero cells was analysed and confirmed to be NDV specific by reacting with antiserum raised in rabbits against NDV in western blotting (Patel *et al.* 2008). Expression of recombinant fusion protein in vero cells was further confirmed by immunofluorescence assay using NDV antiserum raised in rabbits as primary



Figs. 1–4. **1.** Agarose gel (1%) electrophoresis of fusion gene amplicon of 1.68 Kb. Lane 1. 1 Kb DNA ladder; lane 2 and 3.1.68 Kb fusion gene amplicon. **2.** Agarose gel (0.8%) electrophoresis of NcoI and XhoI digested recombinant pTriEx plasmid confirming the presence of 1.68 Kb F gene amplicon. Lane 1. 1 Kb DNA ladder. Lane 2. Recombinant pTriEx plasmid digested with ncoI and XhoI showing the release of 1.68 Kb F gene; Lane 3. Recombinant pTriEx plasmid after single digestion with NcoI. **3.** Western blot showing 55 kDa F protein in BL21DE3. Lane 1, Protein marker; lane 2, cell lysate from uninduced cells, lane 3, cell lysate from induced cells showing 55 kDa F protein. **4.** Western blot analysis showing 55 kDa fusion protein in vero cells. Lane 1, Marker, lane 2, normal vero cells; lane 3, and 4, 48 h and 72 h post transfection.

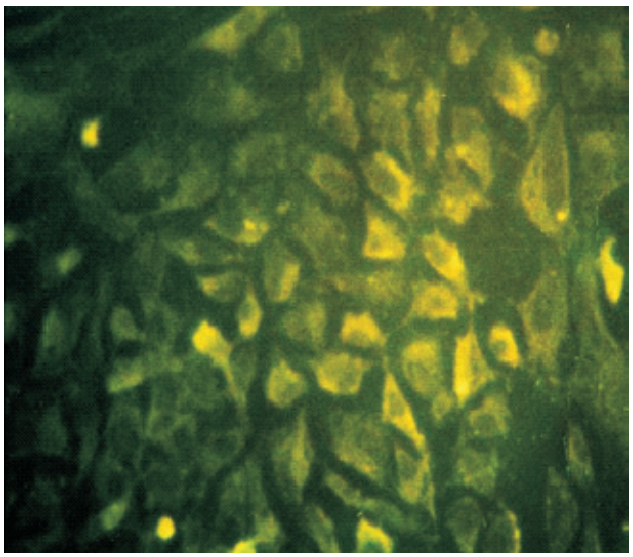


Fig. 5. Expression of recombinant fusion protein on transfected vero cells by immunofluorescence assay (IFA).

antibody and FITC labelled anti-rabbit IgG antibody as secondary antibody (Loke *et al.* 2005).

RESULTS AND DISCUSSION

Newcastle disease is a globally important and highly contagious viral disease of poultry which exhibits epizootic proportions if effective control measures are not implemented. Molecular biology approaches such as RT-PCR, restriction endonucleases analysis of amplification products, heteroduplex mobility assay, real time quantitative RT-PCR were used to detect NDV (Aldous *et al.* 2001, Berinstein *et al.* 2001, Creelan *et al.* 2002, Wise *et al.* 2004). The genomic diversity of NDV increases the possibility of the diagnostic failures, resulting in unidentified infections (Miller *et al.* 2010). The continuous outbreaks of fatal ND in commercial poultry flocks demonstrated that the current vaccination strategies are not fully efficacious and should be improved by new generation vaccines (Liu *et al.* 2010). Moreover, use of whole cell live vaccines is complicating the diagnostic approaches and necessitates the need for a diagnostic assay that 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 gene was amplified using F gene specific primers with cDNA as template under appropriate thermal cyclic conditions and the expected amplicon of 1.68 Kb was obtained (Fig. 1). Ligation of PCR amplified F gene to TOPO pCR2.1 vector and transformation of *E.coli* (DH5α) cells resulted in white colonies. Fusion gene positive colonies were selected by restriction enzyme digestion which released the 1.68 Kb F gene insert. Recombinant TOPO clones were subjected to automated sequencing and the nucleotide sequence obtained was subjected to BLAST analysis. The nucleotide sequence showed 94% homology with Newcastle disease virus isolate 2K3/Chennai/Tamil Nadu, complete genome (Accession No. FJ986192). The fusion gene was further sub-cloned into pTriEx expression vector. The recombinant pTriEx clones were further subjected to restriction digestion with *Nco*I and *Xho*I which released the insert of 1.68 Kb (Fig. 2).

The recombinant pTriEx colonies were induced with 1 mM IPTG. High levels of protein expression were observed in overnight induced cultures. The recombinant fusion protein was immunoreactive with NDV antiserum raised in rabbits in immunoblotting (Fig. 3).

The vero cells transfected with recombinant pTriEx plasmid were also analysed for protein expression. The protein expression was observed in 48 and 72 h post transfection. The expressed fusion protein in vero cells too immunoreacted with NDV antiserum raised in rabbits (Fig.4). Further, the expression of fusion protein of Newcastle disease virus in transfected vero cells was confirmed by immunofluorescence assay. Cells transfected with recombinant pTriEx plasmid containing fusion gene exhibited bright cytoplasmic fluorescence (Fig.5).

The 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 in prokaryotes. Hence in our study the fusion protein was first expressed in prokaryotic expression system and later in mammalian expression system as it offers proper 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). In the present study, transient transfection was done in vero cells using recombinant pTriEx plasmid. Protein expression was observed in cell lysate collected 48 h post-transfection and there was no increase in protein expression in the cell lysate collected 72 h post transfection. Hence 48 h post-transfection was observed as optimum time for protein harvest in this study. Cells transfected with recombinant pTriEx plasmid showed bright cytoplasmic fluorescence indicating that fusion protein was produced in the cytoplasm which was in agreement with earlier findings (Rajawat *et al.* 2008).

In conclusion, we have successfully cloned and expressed fusion protein gene of NDV in mammalian expression system. The recombinant fusion protein thus can be purified to assess its potential as an antigen in the development of diagnostic assays or as recombinant vaccines. It is also concluded that the expression of the recombinant fusion protein can be increased for mass antigen production.

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