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Cloning and expression of fusion (F) protein gene of Newcastle disease virus (NDV)

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ABSTRACT

The present study was undertaken to amplify, clone and express Fusion (F) protein gene of Newcastle disease virus. The freeze-dried virus of pigeon origin was propagated in embryonated chicken eggs. RNA was isolated from the infected allantoic fluid and cDNA was synthesized by reverse transcriptase polymerase chain reaction (RT-PCR). Primers specific to F gene incorporated with restriction enzyme sites, viz. *Nde*I and *Xho*I were used to amplify the F gene using cDNA as a template by polymerase chain reaction (PCR). The 1680 bp F gene amplicons were gel-purified and first cloned into TOPO vector. Positive clone was confirmed by colony PCR and restriction enzyme digestion. TOPO released insert was ligated with *Nde*I and *Xho*I digested pET22b expression vector using T_4 DNA ligase and transformed into *E.coli*. Recombinant clones were selected by colony PCR and restriction enzyme digestion and induced with 0.5 mM final concentration of Isopropyl-1-thio-b-D-galactosidase (IPTG) for the expression of the recombinant F protein. The expressed protein of 55 kDa was obtained during the 4 h post-induction. The obtained recombinant protein reacted with rabbit anti serum in Western blot confirming it to be NDV specific.

Key words: Fusion protein gene, Newcastle disease virus, Polymerase chain reaction, Prokaryotic expression vector, SDS PAGE, TOPO cloning, Western blot

Newcastle disease (ND), a poultry disease that causes severe outbreaks resulting in huge economic losses, is caused by *Newcastle disease virus* (NDV) (Alexander 2003). The virus belongs to the genus *Avulavirus*, order *Mononegavirales* from the family *Paramyxoviridae* (Mayo 2002). The disease in pigeons occurs due to the spread from diseased chicken flocks, and from domesticated or feral pigeons to poultry (Alexander *et al*. 1985). India is endemic to ND and different pathotypes of NDV were isolated and characterized from several avian hosts (Nanthakumar *et al*. 2000, Roy *et al*. 2000 and Kumanan *et al*. 2003). The nonsegmented, negative-sense, single stranded RNA genome of NDV contains 6 genes in the order 3′-NP-P-M-F-HN-L-5′, which encode the 6 major structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin-neuraminidase (HN) and the large (L) RNA-dependent RNA polymerase (Lamb and Kolakofsky 2001). Fusion protein plays an important role in virulence determination of NDV (Römer-Oberdörfer *et al.* 2003). The NDV F protein is synthesized as a precursor, F_0, activation of which requires proteolytic cleavage into the disulphide-linked polypeptides F_1 and F_2. The inactive precursor F_0 contains 553 amino acids with calculated molecular weight of ~55 kDa (Salih *et al.* 2000). The precursor is proteolytically cleaved at the peptide bond of residues 116 and 117, to generate 2 disulfide- linked polypeptides, F_1 (48–54 kDa) and F_2 (10–16 kDa) by specific cellular proteases (Ogasawara *et al*. 1992). The present study describes cloning and expression of NDV fusion protein (F), in a prokaryotic expression system which could be assessed for its antigenicity and immunogenicity.

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MATERIALS AND METHODS

Virus propagation and cDNA synthesis: Embryonated chicken eggs were used for propagation of freeze-dried NDV 2 k3 available at the Department of Animal Biotechnolgy, Madras Veterinary College, using standard procedures (OIE 2008). RNA was isolated from the infected amnio-allantoic fluid using TRIZOL LS employing manufacturer's protocol. cDNA was synthesized from RNA by RT-PCR using ThermoScript cDNA synthesis kit.

Cloning of fusion protein gene: Forward and reverse primers were designed (FP 5′ GCC ATA TGG GCT CCA GAC CTT TTA CC 3′; RP 5′ GCC TCG AGT CAC ATT TTT GTA GTG GC 3′) for amplifying F protein gene, incorporating *Nde* I enzyme site in the forward primer and *Xho* I site in the reverse primer to suit cloning of F gene in the expression vector, pET22b. F gene was amplified using the cDNA and 5 Kb ampli kit as per the manufacturer's

instructions. Polymerase chain reaction was carried out with and initial denaturation at 94°C for 30 sec, followed by 10 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min 30 sec. This was followed by 20 cycles of 94°C for 30 sec, 50°C for 40 sec, 72°C for 1 min 30 sec. The amplified product was checked, gel purified using columns and used for further study. TOPO pCR2.1 vector was used for initial cloning of the purified fusion gene as per the manufacturer's protocol. Recombinant clones were selected using antibiotic resistance, colony PCR and restriction enzyme digestion (*Nde* I and *Xho* I). Restriction enzyme digested fusion gene was obtained from recombinant TOPO clones and used for ligation with *Nde* I and *Xho* I digested pET22b vector. Recombinant clones were selected based on ampicillin resistance, colony PCR and restriction enzyme digestion.

Expression of recombinant F protein: The plasmid DNA construct with the F gene was transformed into *E. coli* BL21 (DE3 pLysE) cells. The transformed *E. coli* cells were plated onto LB agar plate supplemented with ampicillin (100 µg/ml). A single colony was inoculated into 10 ml of LB broth with ampicillin (100µg/ml), and cultured at 37°C overnight with shaking. The culture was diluted 1:100 in the LB broth containing ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml) and grown at 37 °C with shaking. The *E. coli* BL21 cells containing recombinant pET22b were induced with 0.5 mM IPTG. The cell lysate from the fourth hour culture was electrophoresed in a 12% SDS-PAGE gel at constant voltage of 50 V. After the electrophoresis, the gel was stained with Coomassie brilliant blue and analysed for expression.

RESULTS AND DISCUSSION

NDV 2 k3 was propagated in 9–11 days old embryonated chicken eggs and the amnioallantoic fluid (AAF) collected from the dead embryos had a haemagglutination titre of 2^9 while the HI titre was found to be 2^8. RNA was isolated from infected AAF and the 1.68 Kb F gene was amplified by RT-PCR using gene specific primers having restriction enzyme sites *Nde* I and *Xho* I (Fig. 1).

The F gene amplicon was gel purified and cloned into TOPO vector which has a good cloning efficiency. TOPO cloning resulted in 7 clones positive for F gene as evidenced by digestion with *Nde* I and *Xho* I. Digestion of the recombinant plasmid released an insert of 1.68 Kb size. This result confirmed that the RT-PCR amplified fusion gene of the NDV was successfully cloned into TOPO pCR2.1 vector. To further confirm this, the recombinant TOPO pCR2.1 vector containing the fusion protein gene was subjected to automated sequencing with cloning primers and the sequence showed 94% homology with NDV 2K3 isolate F gene (Accession no FJ986192) available in the GenBank. It was evident from the sequencing results that the fusion gene was in the right orientation in the vector.

Prokaryotic expression vector pET series has been used

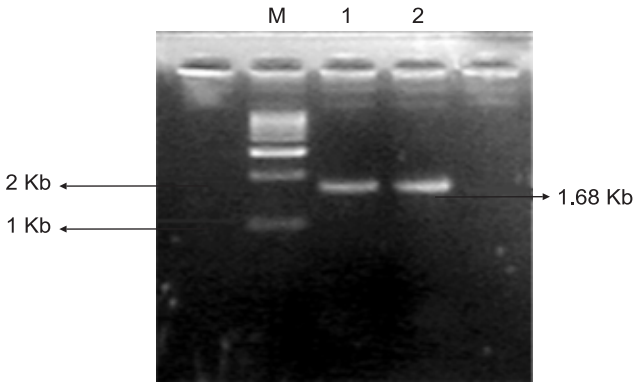

Fig. 1. Agarose gel (1%) electrophoresis of fusion gene showing a 1.68 Kb amplicon. Lane M, 1 Kb DNA ladder; Lane 1 and 2, 1.68 Kb F gene amplicon.

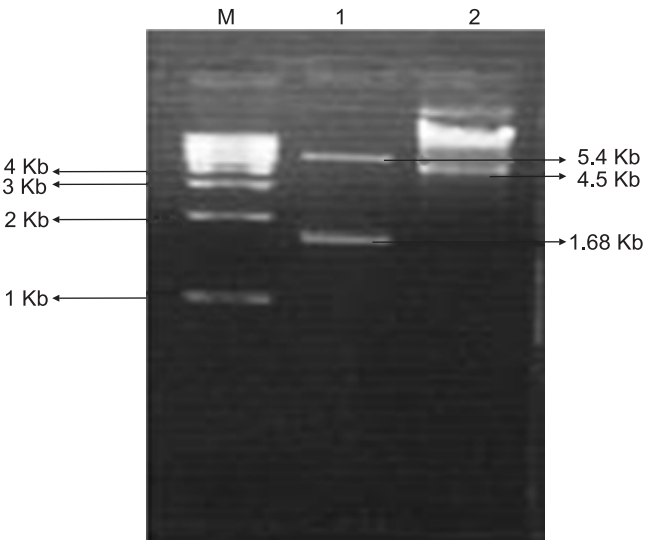

Fig. 2. Agarose gel (0.8%) electrophoresis of restriction enzyme digested recombinant pET22b plasmid confirming the presence of 1.68 Kb F gene. Lane M, 1 Kb DNA ladder; Lane 1, Recombinant pET22b plasmid cut with Nde I and Xho I showing 5.4 Kb pET22b plasmid and 1.68 Kb F gene; Lane 2, Uncut pET22b (3.5 Kb).

by many workers for cloning and expression of viral and bacterial proteins because of its high efficiency and universal application (Xia *et al*. 2009). Hence, in the present study, pET22b was used for cloning and expression of F gene of NDV. Recombinant plasmids were screened by colony PCR and by restriction enzyme digestion. Colony PCR and restriction enzyme digestion with *Nde* I and *Xho* I resulted in 1.68 Kb products as expected (Fig. 2). These results indicated that the F gene was successfully cloned into the prokaryotic expression vector.

Fusion protein of NDV is reported to be of 55 kDa protein and in our study, the recombinant pET22b clone showed expression of 55 kDa protein under the T7 promoter of the vector when induced with 0.5 mM IPTG (Fig. 3). The expressed protein reacted with rabbit antiserum against NDV in Western blot confirming it to be NDV specific. NDV F

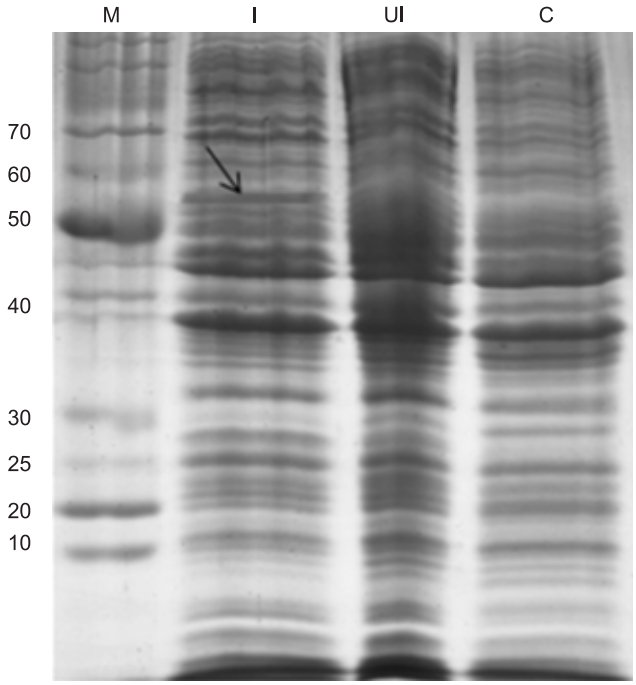

Fig. 3. SDS-PAGE showing expression of recombinant fusion protein in a 12% gel. M, Marker; I, four hour induced culture showing expressed protein (arrow); UI, four hour un induced; and C, induced control pET22b plasmid.

protein is synthesized as a precursor. F_0, the activation of which requires proteolytic cleavage into disulphide-linked polypeptides F_1 and F_2. The inactive precursor F_0 contains 553 amino acids with calculated molecular weight ~55 kDa (Salih *et al*. 2000). Cleavage and post-translational modifications of fusion protein occur in trans-golgi membrane (Morrison *et al*. 1985). Being a prokaryotic expression system, the fusion protein obtained will be in F_0 form as no cleavage is possible in prokaryotes due to absence of golgi membrane. Fusion protein obtained in this study may also be in the precursor F_0 form.

The expressed fusion protein's potential as a candidate antigen in NDV vaccine cocktails can be studied. Moreover, it is well documented that the fusion protein cleavage site sequence is one of the molecular markers for assessing the pathotypes of NDV isolates (Aldous and Alexander 2001). Under these circumstances, the recombinant F protein can be used in developing specific diagnostic assays for differentiating epizootic and vaccine viruses. It is concluded that the F protein of a pigeon isolate of NDV has been successfully cloned and expressed in a prokaryotic system. Further studies can be initiated to assess its potential as an antigen in diagnostic assays or in recombinant cocktail vaccines.

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