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Production and application of recombinant haemagglutinin neuraminidase of Newcastle disease virus

Selvan Nallaiyan[1*], Ranjit Singh Amirtha Jacob Abbadorai[2], Sukumar Sundaramoorthy[3], Jeyakumar Nelson[4], Suryanarayana Veluvarthy Venkata Sanyasi[5]

[1]*Centre for Biotechnology, Muthayammal College of Arts & Science, Rasipuram – 637 408, Tamil Nadu, India*
[2]*Department of Advanced Zoology, Sri Paramakalyani College, Alwarkuruchi – 627412, Tamil Nadu, India*
[3]*Centralized Instrumentation Laboratory, Madras Veterinary College, Chennai – 600 007, Tamil Nadu, India*
[4]*Unit of Microbiology, Faculty of Medicine , AIMST University, Malaysia*
[5]*Molecular Virology Laboratory, Indian Veterinary Research Institute Campus, Hebbal, Bangalore–560024, Karnataka, India*

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ABSTRACT

Objective: To discuss the possibility of expressing the haemagglutinin–neuraminidase (HN) protein in prokaryotic system such as *Escherichia coli* (*E. coli*) cells by cloning the full length HN gene. **Methods:** The full length HN gene of Newcastle Disease Virus (NDV) of size 1 734 bp was preciously isolated by RT–PCR. The sequence was assessed and submitted to Nucleic Acid Databank (NCBI) and the gene ID was EU215390.1 after cloning and sequencing. Now the assessed HN gene was subcloned into pET 32 a+ expression vector for production the HN protein in *E. coli*, BL21 (DE3) $P^{LYS}S$ cells following standard protocols. The crude lysate protein from the induced positive clone was size assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and their haemagglutination (HA) property against chicken RBC was assessed by standard micro HA test. **Results:** The molecular size of the full HN gene of NDV as assessed by cloning and digesting the positive clone to release the insert was 1.7 kb. The expressed protein in both crude and pure form was assessed to be 63 kDa and 81 kDa, respectively. The HA activity of the crude protein of the positive clone was 1 in 40. **Conclusions:** This finding indicates that the fusion protein retains the biological activity of native protein in the crude form and therefore could be used as a diagnostic reagent for antibody detection and for routine assessment of immune status in commercial layer forms.

1. Introduction

The development of the Indian poultry industry in general, Namakkal layer industry in particular, is remarkably fast and it is growing on leaps and bounds with respect to poultry egg production. But it is surprisingly sad to note that the development in poultry vaccinology is pacing far behind the production achievements. The vaccines used in the poultry industry are as old as the childhood of the industry.

Recombinant vaccines for several diseases of poultry have recently been produced in the laboratories in India and abroad in an effort to eliminate some of the disadvantages of live and killed vaccines. Genetically modified viruses that possess large regions of their genome and lack the genes for the infection associated proteins are of recent interest as they can be used as marker vaccines[1,2]. These modified viruses also serve as viral vectors for inserting genes of other viruses[3]. However, recombinant vaccines could neither totally replace the conventional vaccines now preferred over conventional vaccines by the commercial poultry industry.

NDV is an Office International des Epizooties list A pathogen that causes a serious respiratory and neurological disease in all species of birds and is an economically important infectious agent, causing substantial losses to the poultry industry. Newcastle disease varies in the degree of severity, ranging from an unapparent infection to severe disease causing 100% mortality. In China, the first Newcastle disease outbreak was recorded in 1928, and the standard strain F48E9 was isolated in 1948 for the first time[4].

Unique to paramyxoviruses, the Haemagglutinin–neuraminidase (HN) protein of Newcastle Disease Virus

*Corresponding author: Selvan Nallaiyan, Centre for Biotechnology, Muthayammal College of Arts & Science, Rasipuram – 637 408, Tamil Nadu, India.
Tel: +91–9994200335, +91–4286–285555
E–mail: selvanbt@gmail.com

(NDV) has the activity of haemagglutinating the red blood cells (RBC) and it has been shown that NDV can bind to and haemagglutinate amphibian, reptilian, avian, guinea pig, mouse and human RBCs[5]. The haemagglutination seen with NDV is due to the HN protein's ability to bind to RBCs and to other cells with the proper sialic (neuraminic) acid receptors[6]. Haemagglutination of RBCs has provided a powerful tool in the diagnosis of NDV. Burnet (1942) demonstrated that NDV anti-sera prevented the agglutination of RBCs by NDV. This discovery provided the basis of the haemagglutination-inhibition (HI) test that has been the standard for the diagnosis and measuring the antibody response to natural or vaccinal exposure of birds to NDV. In present days, the whole virus cultivated in embryonated chicken eggs is used as a source of haemagglutinin protein and HI assay is the only method widely followed worldwide.

The serotypes and genotypes of Infectious Bronchitis Virus (IBV) was mainly determined by the Spike (S)1 glycoprotein. A prevalent IBV strain ZY3 was isolated and the highly antigenic region of its S1 gene was amplified and expressed in *Escherichia coli (E. coli)* using the pET-32a (+) vector[7].

The present study aims at cloning the entire HN gene into the available prokaryotic expression vector system and use the expressed HN protein in the cell lysate of the bacteria as the source of haemagglutinin required for the routine HI tests in the laboratory for at least sero-monitoring of vaccination response. If the hypothesis is proved successful, the labour intensive laboratory cultivation of virus in embryonated chicken eggs and the recontamination of the environment through possible laboratory spillage of the virus can greatly be obviated.

2. Materials and methods

Tissue samples from ailing birds supplied by the kind courtesy of Avian Disease Laboratory, Namakkal, Tamil Nadu, India, were used for virus isolation through chicken embryo cultivation. Total RNA from infected eggs was isolated using TRIZOL reagent as per manufacturer's specification which is a single step isolation method[8,9]. The isolated RNA was used as template for amplification of HN gene by RT-PCR.

Primers encompassing full length HN gene were designed from the published sequence. The oligonucleotides were reconstituted to 1 nmol/μL concentration in sterile Tris EDTA (TE) as a stock and stored at -70 ℃. The primers were used at working dilution of 20 pmol/μL in filtered distilled water (FQW). The sequences of the primers were as follows:

Forward 5′-GCG GGA TCC ATG GAC CGC GCC GTT AGC CAA AT-3′

Reverse 5′-GCG AAG GCTT CTA GCC AGA CCT GGC TTC TCT-3′

The position of the primers with this gene is 6412 to 8145 location in locus NC_002617 of NDV B1 complete genome in NCBI nucleotide database.

cDNA for the HN RNA was synthesized in 0.5 mL micro centrifuge tube. Eight μL of total RNA was mixed with 2 μL of HN specific reverse primer (50 pmol) and they were heated in boiling water for 5 minutes, then immediately chilled in ice for 5 minutes. To the RNA primer mixture, 4 μL of 5× RT buffer (250 mM Tris HCl, 25 mM $MgCl_2$, 300 mM KCl), 2 μL of 10 mM each of dNTPs, 30 U of ribonuclease inhibitor, 1 μL (15u/μL) of avian myeloblastosis virus (AMV) reverse transcriptase (Promega, USA) and 3 μL of nuclease free water were added and the reaction mixture was incubated at 42 ℃ for 90 min for cDNA synthesis. Later the reaction was terminated by heating the mixture at 90 ℃ for 3 min. HN gene was amplified by subjecting 2 μL of cDNA to PCR in a reaction mixture contains PCR assay buffer (25 mM Tris HCL, 10 mM KCL, 1.5 mM $MgCl_2$), 0.1 mM dNTP′s, 50 pmol each of forward and reverse primers, 0.5 unit of Pfu polymerase (Promega, USA).

Optimum annealing temperature was determined by employing gradient PCR. Reactions were carried out in a thermal cycler (Techne, USA) with forward and reverse primers using the reaction conditions; initial denaturation for 3 min at 94 ℃, followed by 35 cycles of denaturation at 94 ℃ for 2 min, annealing at 55-60 ℃ (gradient) for 1 min and extension at 72 ℃ for 2 min. Finally, the reaction was held at 72 ℃ for 10 min to complete the synthesis of unfinished products. Specific and optimum amplification of the gene was seen at 58 ℃ of annealing temperature. Subsequently the gene was amplified at 58 ℃ and the amplified PCR product (1.7 kb) was purified from low melting point agarose gel, as per the standard protocol, for further cloning.

The purified PCR fragment was digested with BamHI and HindIII (Promega, USA) as per standard protocol and ligated into BamH1 and HindIII digested pBSKII+ plasmid. Ligation reaction was carried out as per standard protocol using T4 DNA ligase (Promega, USA). Briefly 50 ng of insert DNA was mixed with 25 ng of linearized vector in the presence of 1 ×ligase buffer (supplied by manufacturer). The ligated mixture (1 mL) was transferred into competent *E. coli* DH5 α cells and plated on LB agar containing 50 mg/mL ampicillin, 1 mM IPTG and 0.02% X-gal. The white colonies observed after 16 h of incubation were screened for the presence of the vector with insert by colony lysis and release of the insert by restriction enzyme digestion. One of the positive colonies was designated as pBND-HN.

The insert was released from pBND-HN and ligated into linearized pET 32a+ (Novagen, USA) expression vector. A few pETND-HN plasmid DNA was transferred into electro competent *E. coli* BL21 (DE3) $P^{lys}S$ cells by electroporation and plated in ampicilline-agar plate and incubated 16 hrs at 37 ℃. A few colonies were picked and grown in LB broth overnight in the presence of ampicillin. Cells were collected by centrifugation at 6 000 g at 4 ℃, suspended 5 volumes of fresh LB broth without ampicillin, grown to 0.6 OD at A600 nm and to that IPTG was added to 0.4 mM for protein induction. Protein induction was done at 30 ℃ for 3 hr in a shaking incubator.

The cells were collected by centrifugation, suspended in TE containing 10 mM Phenyl methyl sulphonyl fluoride (PMSF) and lysed by freezing-thawing twice. The crude lysate proteins from the induced clone were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The crude form of protein was checked for the HA activity with 1% washed chicken RBC. The specificity of HA activity was also ascertained by HI test using NDV specific

serum.

3. Results

Upon transformation of the *E. coli* DH5 alpha cells with the ligated mixture and plating of the transformed cells on LB-ampicillin-agar plates, several ampicillin resistant colonies could be seen on the plates. These may represent *E. coli* with pET 32a+plasmid and with or without inserted DNA. Colonies were screened by colony lysis, the plasmid DNA from the colony showing slower mobility was subjected for restriction digestion and analyzed by agarose gel electrophoresis using DNA molecular size markers (Figure 1). The actual RT and PCR product obtained both in the pBKII+ and pET32a+ clones, each in these forms namely clones without insert (HN gene), with insert, and along with released insert. Figure 1 showed well separated DNA fragments could be seen in the gel, the lower fragment of 1.7 kb may correspond to the inserted HN gene and the upper band of above 5.0 kb may correspond to the linearized pET 32+ vector of 5.9. This recombinant plasmid has been designated as pETND-HN.

Well separated protein bands could be seen both in case of induced and un-induced cell lysates which may be of both host and plasmid origin. However, there was an additional thick Coomassiebrilliant Blue (CBB) stained protein band of size corresponding to 81 kDa which may be of insert origin. As per the derived amino acid (aa) sequence the expressed from the cloned insert is expected be of 63 kDa. Along with the fusion tag of 18 kDa from the vector the total size of the fusion protein would be 81 kDa, which was the observed size. The crude protein gave HA at 1 in 40 dilutions (Figure 2). The crude protein expressed by the true positive clone, which gave haemagglutination with chicken RBC up to 1 in 40 dilution was also subjected to neutralization test with equal volume of known NDV positive serum and the results are shown in Figure 3. Formation of clear and complete haemagglutination of RBC was seen in the wells (Row 1, wells 1 to 4, Figure 3).

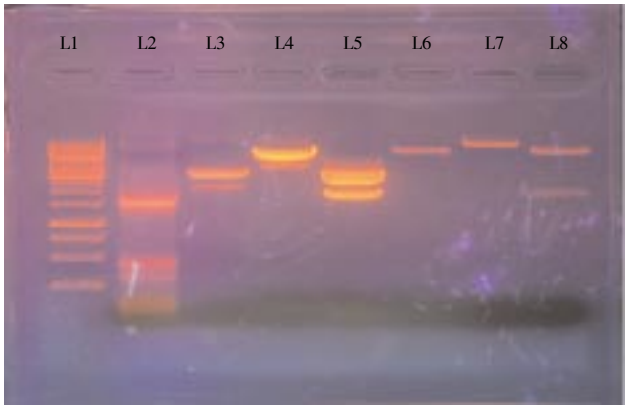

Figure 1. The actual RT and PCR product.
Lane 1: Promega 1 kb ladder, Lane 2: RTPCR product, Lane 3: Digested pBSKII+ vector, Lane 4: Single Digested pBSKII+ insert, Lane 5: Insert release from pBSKII+, Lane 6: Digested pET32a+ vector, Lane 7: Single Digested pET32a+ vector + insert, Lane 8: Insert release from pET32a+.

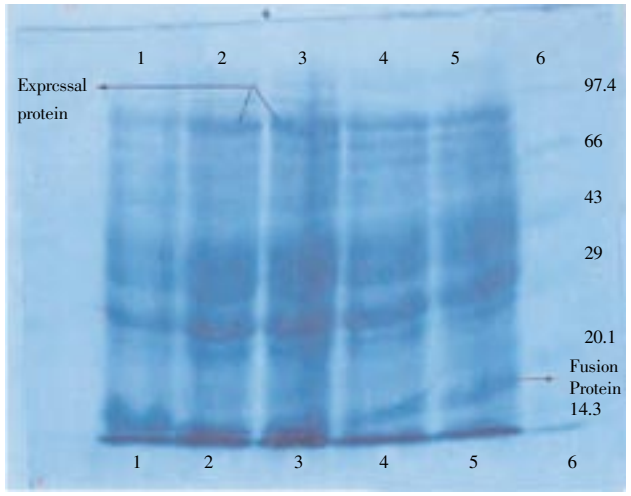

Figure 2. CBB stained polyacrylamide gel electrophoresis of the crude protein expressed by the pET32a clone in BL21(DE3) p^{LYS}'S cells.
Lanes 1 and 4: Protein expressed by uninduced clones, Lanes 2 and 3: Proteins expressed by induced clones yielding about 81 kDa of HN protein (63 kDa) plus **18** kDa of fusion protein, Lane 5: Protein expressed by the vector, Lane 6: Protein molecular weight marker.

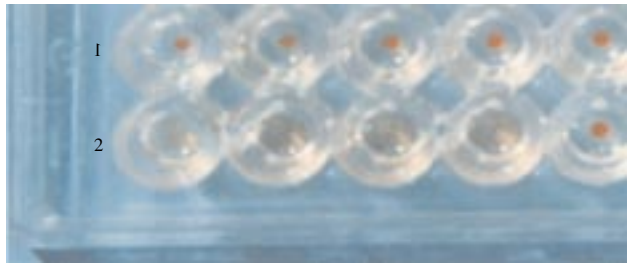

Figure 3. HA activity of the induced protein with chicken RBC.
1: HA activity of crude protein of positive clone-HA in four wells i.e. 1 in 40 dilution;
2: HA activity of crude protein expressed by vector (control)-No HA activity.

4. Discussion

The focus of this study is to explore the possibility of producing the HN protein of NDV (locally isolated B1 strain) and study its activity in haemagglutination for the purpose of using it in serological assay of NDV antibodies. For expression of HN gene in *E. coli*, BL21 (DE3), pET32a+vectors were used which have necessary features for over expression of cloned genes[10,11]. The HN gene of NDV amplified by RT-PCR[9,12] was inserted down stream of strong T7 promoter, ribosome binding site and initiation signal and upstream of strong terminator signal. Presence of thyrodoxin tag makes the protein soluble thereby maintaining the biological activity of the fusion protein. Though the quantity of HN protein expressed by the clone has not been measured, the intensity of the stained band may represent around 10% of the total proteins. Another important benefit of this system is its ability to maintain target genes transcriptionally silent

in the uninduced state[13]. The crude protein was confirmed of their specificity only by size measurement along with known protein molecular weight markers. The expressed crude protein of size 81 kDa inclusive of 63.2 kDa of haemagglutinin protein and 18kDa of fusion protein of the host cell is clearly appreciable. Haemagglutinin protein of HN gene of NDV possesses a specific affinity to bind on receptors of normal chicken RBC and this process make the RBC to become agglutination on mixing. This reaction is termed as haemagglutination and this reaction is being used to assess the antibody of NDV by an assay called Haemagglutination inhibition (HI) assay. Hence the crude protein was checked for its haemagglutination activity with 1% washed chicken RBC using "U" bottom 96 well micro haemagglutination plates and was found to exhibit haemagglutination titre of 1 in 40.

The present study highlighted the possibilities of using the expressed protein as diagnostic reagent for HI-assays being done routinely in avian diagnostic laboratories mainly for monitoring the vaccination efficiency[14], in commercial layers, the HA efficacy of the expressed protein was given importance, rather than its immunologic activities.

Conflict of interest statement

We declare that we have no conflict of interest.

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