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Isolation and amplification of fin gene from Newcastle disease virus strain for the production of recombinant protein

S. Ponmani

Assistant professor in Biotechnology, Padmavani Arts and Science College for women, Salem – 636 011.
*Corresponding author: *biotechponmani@gmail.com*

Abstract

Control of poultry diseases, especially of viruses among commercial layer poultry is by regular vaccination. Presently, during the full term of layer life of 72 weeks, all the birds are protected against at least six different viral diseases, totally about 14 vaccination with repetitions in some. Newcastle disease (also called Ranikhet Disease) one among the six, still remains a major and chronic diseases prevalent worldwide. Repeated vaccinations are always done by prior monitoring of antibody levels. Haemagglutination - inhibition (HI) assay is the popular and easy test followed universally. This test involves the use of live and whole virus, cultivated in embroyonated chicken eggs. In addition to the laborious cultivation process, laboratory spillage of the virus may pose danger to the birds because the virus is infective to the birds also. The actual tests for assessment of these activities were done and discussed.

Keywords: Newcastle disease, HI, Recombinant protein.

Introduction

The development of the Indian poultry Industry in general, Namakkal layer industry in particular, is remarkably fast on leaps and bounds with respect to poultry egg production, including even 305 eggs per year per bird with daily feed intake of 100g as against the world breeder record of 300 eggs per year per bird with daily feed intake of 105g (Verma and Pillai, 1989). But it is surprisingly sad to note that the poultry vaccinology is pacing for behind the production achievements. The vaccines used in the poultry industry are as old as the childhood of the industry. For Ranikhet disease, the good old egg embryo adopted lentogenic and mesogenic strains of Ranikhet Disease virus are being used as vaccine, on the principle of immunity by creating mild disease. The lentogenic strain vaccine is meant for chicks and the mesogenic vaccine is meant for adult. Mesogenic strain is contra indicated to chicks because it may induce the disease in chicks. The mesogenic strain may even induce mild side effects in 2 to 3 percent of the adult also in general. These same egg embryo adapted live viruses are also used in the laboratory diagnosis of Ranikhet antibodies. Antibody estimation for Ranikhet disease is also routinely and regularly undertaken for sero monitoring of the vaccinated poultry. So, any spillover of the virus from laboratory may become a recontamination of the healthy stock.

Live vaccine can elicit a strong Immunoglobulin A (IgA) response, local immunity, when administrated in the drinking water as eye drop or by spray. But their potential of reversion of virulence may cause the very disease in birds that do not receive any vaccine or only a partial dose. The disadvantages of killed vaccines are that they do not elicit local immunity at mucous membranes and that each bird must be handled to administer the killed vaccine product. And in general killed vaccines are comparatively costlier than live

vaccines. The majority of commercial layer poultry today are vaccinated several times against Ranikhet Disease either with live virus or with killed virus. As such, the present day poultry vaccines popularly used are only whole virus vaccines which cannot obviate or address the disadvantages enlisted above.

Recombinant poultry vaccines have recently been produced in the laboratory in an effort to eliminate some of the disadvantages of live and killed vaccines. Recombinant vaccines are live viruses that possess large regions of their genome that do not code for protein or code for non essential proteins (Jack Wood, 1999). These types of virus serve as recombinant viral vectors that can carry foreign genes of other viruses (King, 1999).

This present work provides a detailed study of locally (Namakkal, Tamil Nadu, India) isolated strain from commercial layer poultry with respect to its HN gene in an effort to clone and express the gene in prokaryotic vector with an ultimate idea of using the source as reagent for serological use in the laboratory.
Newcastle disease (ND), a highly contagious infection of poultry, reported at Newcastle (hence, termed as Newcastle Disease), United Kingdom in 1926 by Doyle with the subsequent outbreaks also in UK, at Somerset and Stafford shire, produced mortalities up to 100 percent. This disease with relatively mild respiratory symptoms was reported in California in mid 1930s with less than 15% mortality. The first outbreak recorded in India was at Ranikhet in 1927, (Edwards, 1928), hence is also termed as Ranikhet Disease (RD).

The ultimate idea of the present study is to clone the entire HN gene into the available prokaryotic expression vector system so that the expressed HN protein by mere culturing of the bacterial cells as the source of haemagglutinin required for the routine HI tests in the laboratory for at least sero - monitoring of vaccination response. If this idea is proved successful, the labour intensive laboratory cultivation of virus in embryonated chicken embryos and the recontamination of the environment through possible laboratory spillage of the virus can greatly be obviated.

A brief design of the work to be followed in the present study for the successful achievement of the objective can be listed as here under:

- Selection of a suitable NDV strain has to be selected preferably from locally (Namakkal, Tamil Nadu, India) available infected birds as a means of local isolation.
- Isolation of the entire FIN gene in its full frame by RT-PCR using specific primers flanked by known restriction enzyme sequences.
- Purification and partial end sequencing of the isolated FIN gene assessing its suitability for cloning.
- Ligation of the pure and full length HN gene into cloning vector pBKSII+ for identification of the gene and assessment of its orientation by nucleotide sequencing.
- Sub cloning of the cloned HN gene in to prokaryotic expression vector pET 32a+ and its transformation into E.coli. BL 21(DE3)P^{LYS}S cells.
- Assessment of the indused protein either as crude or as pure for molecular size by SDS - PAGE. For serological activity by HA using chicken RBC and for biological activity by HI assay of the antibody induced by the protein in birds.

2. Objective of the study

To explore the possibility of cloning the full length haemagglutinin - Neuraminidase (1-IN) gene of New Castle Disease virus (NDV - Syn: Ranikhet Disease Virus) of local origin in prokaryotic vector system to achieve the invitro expression of the 1-IN gene product in the form of protein, so that the same can be used for serological assays of NDV diagnosis. This aim involves the following biotechnological processes:

- Viral mRNA isolation and purification
- IIN gene isolation by Reverse Transcription (RT) - Polymerase Chain Reaction (PCR) using specifically designed primers for this purpose.
- Cloning of the gene in bacterial vector and transforming the vector into a bacterial cell.
- Analysis of the cloned gene by complete sequencing.
- Recloning of the analyzed gene into an expression vector and transformation of the vector into E-coli.
- Induction of the transformed E-coli for protein expression
- Isolation and purification of the protein.
- Analysis of the protein for its anticipated biological activity

3. Review of literature

Classification:

NDV is a member of the Order Mononegavivales, family Paramyxoviridae, subfamily Paramyxoviridae and genus Avulavirus (Mayo, 2002 & Murphy, 1995). NDV is the only member of the genus Avulavirus (Mayo, 2002). Other important members of the family paramyxoviridae are the mumps virus, SV5 and parainfluenza Virus type 2.

Virion:

The NDV virus particles are pleomorphic in nature and range from 150 - 4090 nm in size. The virions contain a long helical nucleo capsid structure which is 1,000 nm long and 17-18 nm in diameter. The envelope is layered with spike glycoproteins which are 8-12 nm in diameter. The genome of NDV is a single strand of RNA of negative sense, and has a molecular weight of 5.2 to 5.7 x 106 daltons (Alexander, 1997).

The genomic RNA consists of 15,186 nucleotides (Deleeuw et. al. 1999; Krishnamurthy and Samal 1998 and Phillips et. al. 1998).

The helical nucleo capsid, rather than the free genome RNA, is the template for all RNA synthesis. The nucleo capsid protein (NP) and genome RNA together form a core structure, to which the phosphoprotein (P) and the large polymerase protein (L) are attached (Lamb and Kolakofsky 1996).

This core forms the RNP or the transcriptive replicative complex and serves as the minimum infectious unit. The envelope of NDV contains two surface glycoproteins: the hemagglutinin - neuraminidase (HN) protein responsible for attachment of the virus to host cells and the Fusion (F) protein required for fusion of the virus into the host cell membranes (Homma and Obucci 1997).

The F and HN proteins are also the main targets of the immune response of NDV (Meulemans et. al. 1986 and Morgan el. al. 1992). Internal to the envelop is the matrix (M) protein, which is thought to be important in viral assembly (Peeples, 1991).

Genome Organization:

The NDV genome consists of six genes (32' NP-P-M-F-HN-1.5) encoding at least eight proteins (Peeples, 1988 and Steward et. al. 1993).

The genomic RNA contains a 3’ extracistronic region of 55 nucleotides, known as the leader, and a 5' extracistronic region of 144 nucleotides, known as the trailer (Krishnamurthy and Samal 1998).

These regions are essential for replication of the genome, and they flank the six genes. At the beginning and end of each genes are conserved transcriptional control sequences, known as the gene start and gene end sequences, respectively. Between the gene boundaries are intergenic regions, which vary in length from 1-47 nucleotides (Chambers et. al. 1986a; Chamber et. al. 1986b and Krishnamurthy and Samal 1998).

Viral proteins:

The six genes of NDV code for eight proteins: NP, P. M, F, ITN, L, V and W. The V and W proteins are the two additional proteins formed by non - templated nucleotide addition through the RNA editing process during P-gene transcription (Steward el. al. 1993).

Nucleocapsid and its associated proteins:

The nucleocapsid protein (NP) and genome RNA together form a core structure, to which the phosphoprotein (F) and the large polymerage protein (L) are attached (Lamb and Kolakofsky 1996). These three proteins form the transcriptive - replicative complex, which is the minimum infectious unit of NDV.

NP Protein:

The NP protein serves several functions in viral replication, including encapsidation of the genome RNA into a nucleocapsid (the template for the RNA synthesis), association with the P-L polymerase during virus assembly. The intracellular concentration of unassembled NP is also considered to be a major factor controlling the relative rates of transcription and replication from genome templates (Blumberg and Kolakofsky 1981 and Blumberg et. al.1981).

The NP gene of NDV consists of 1747 nucleotides with a coding region of 489 residues. The molecular weight of the protein is predicted to be 54 kilo daltons (KD) (Krishnamurthy and Samal 1998).

P protein:

The P gene of NDV is 1451 nt long. The P gene ORF encodes an unedited version of rnRNA, which results

in formation of the P protein. RNA editing with the addition of one G nucleotide at the editing site (near the center of the OR-F) produces an rnRNA which encodes the V protein, whereas addition of two G nucleotides produces an mRNA that encodes the W protein.(Lamb el. al. 1996 and Steward et. al. 1993).
The P protein is essential for viral RNA synthesis. This protein is highly phosphorylated in nature. It is an essential lamponent of the viral RNA polymerase and the nascent chain assembly complex formed during viral RNA synthesis. The P protein associates with the L- protein forming the viral polymerase (P-L) and thus, functions as a transcriptive and replicative factor. It also associates with the un assembled NP (NP") firming the P - NI"' complex (Hamaguchi et. al. 1983). This property of P protein has been suggested to prevent NP' from assembling RNA non - specifically (Masters et al. 1988). The predicted molecular mass of the polypeptide is 53 KID (Daskalakis el. al. 1992).

L Protein:

The L protein is the least abundant of the structural proteins (about 50 copies per virion). The L - gene is the most promoter - distal in the transcription map and thus the last to be transcribed. Its low abundance, its large size and its localization to transcriptionally active viral cores suggested that it might be the viral polymerase. The P and L proteins form a complex, and both proteins are required for polymerase activity with NP: RNA templates (Curran et. al. 1994: Hamaguchi et. al. 1983 and Marx et. al. 1978).

The L protein is also responsible for capping and polyadenylation of the mRNAs. Polyadenylation is thought to result from polymerase stuttering on a short stretch of U residues. The L gene is 6704 nt long. The predicted molecular mass of the polypeptide is 242 KID (Yasoff et. al. 1987).

Matrix Protein:

M protein is the most abundant protein in the virion. The M gene of NDV is 1241 nt long. Its predicted molecular mass is 40 KID (Chambers et. al. 1986c).
The M protein interacts with the nucleocapsid and the envelope proteins of the virion. This protein is considered to be the control organizer of viral morphogenesis, interacting with the cytoplasmic tails of the integral membrane proteins, the lipid bilayer, and the nucloecapsids. The self - association of M and its contact with the nucleocapsid may be the driving force in forming a budding virus particle (Peeples, 1991).

Envelope glycoproteins:

NDV possesses two integral membrane glycoproteins namely the hemagglutinin - neuraminidase (IN) glycoprotein which is involved in cell attachment and the Fusion (F) glycoprotein which mediates pH - independent fusion of the viral envelope with the plasma membrane of the host cell.

HN protein:

The HN glycoprotein of NDV is a multifunctional protein and a major antigenic determinant of the virus. It is responsible for the adsorption of the virus to sialic acid - containing receptors. In addition, HN mediates enzymatic cleavage of sialic acid (neuraminidase activity) from the surface of virions and the surface of infected cells. In addition to the heamagglutinating and neuraminidase activities, HN also has a fusion promoting activity, through interacting with the fusion glycoprotien of NDV (Lamb and Kolakofsky 1996).
Previous research has indicated that for fusion to occur, a type - specific interaction between the F and HN protein is required. It is proposed that HN undergoes a conformational change on attachment to its ligand and thereby triggers a conformational change in the F protein to release the Fusion peptide (Lamb, 1993).

Studies have shown that a point mutation in the globular domain of HN that abolishes its receptor recognition, neuraminidase and fusion activities, also interferes with its ability to interact with F (Deng et. al. 1999).

Research on the role of the HN protein in the mechanism of cell membrane fusion indicates that the binding of the HN protein to the receptor induces conformational changes of residues near the hydrophobic surface of the protein and this change in turn, triggers the activation of the F protein, which initiates membrane fusion (Takimoto et. al. 2002).
The HN gene is 1998 nt long with a coding region having 577 amino acid residues. The FIN of some strains of NDV is synthesized as a biologically inactive precursor (HNO) and 90 residues from the C-terminal are removed to activate the molecule (Millar et. al. 1988; Miller et. al. 1986 and Nagai et. al. 1976).
The HN proteins are type II integral membrane proteins that span the membrane once. The N - terminus of the HN - protein consists of the cytoplasmic domain, followed by the transmernbrane region and the stalk region. The protein contains a single hydrophobic domain, located near the N-

terminus, that acts as a combined signal and anchorage domain targeting the nascent chain as it emerges from the ribosome to the membrane of the endoplasmic reticulum and ensuring the translocation of the polypeptide chain across the membrane, bringing about the stable anchoring of the protein in the lipid bilayer (Lamb et. al. 1996).

The c-terminus end of the protein is composed of the globular head or the ectodomain. This region of the uN protein is the main site of attachment of the virus to host cells. The HN glycoprotein of NT)V contains Six N - linked carbohydrate chains (McGinnes and Morrison, 1995).

These carbohydrate chains are added to the protein in the rough endoplasmic reticulum as it undergoes modifications in various cell compartments during its formation (Kornfeld and Kornfeld 1995). All but one of the six glycosylation sites on the HN protein, are located in the c-terminal external domain. The secondary structure prediction shows that the c-terminal external domain is mostly arranged in beta - sheets, while alpha - helices are predominantly located in the N - terminal domain of the protein (Sagrera et. al.2001).

The predicted molecular weight of the HN protein is 74 KD (Chambers et. al. 1986a). The HN protein forms an oligomer consisting of di-sulfide linked homodimers that form a non-covalently linked tetramer (Markwall and Fox 1980).

There has been debate for many years as to whether the FIN molecules contain combined or separate active sites for hemagglutinating and neuraminidase activities. Recent studies and analysis of the crystal structure of HN of NDV have predicted that there resides a single site in the HN protein that binds sialic acid tightly (hemagglutimation activity), but hydrolyses the molecule slowly (neuraminidase activity) (Crennell et. al. 2000).

F protein:

The F protein of NDV mediates viral penetration by fusion between the virion envelope and the host cell plasma membrane, in a pH - independent manner. After fusion, the nucleocapsid is delivered to the cytoplasm. Later in infection, the F protein expressed at the plasma membrane of infected cells can mediate fusion with neighboring cells to form syncytia (giant cells). Syncytia formation is an hallmark of NDV infection in host cells. It is a typical cytopathic effect caused by the virus and can lead to tissue necrosis and might also be the mechanism of virus spread. The F protein is a type I integral membrane protein and is synthesized as an inactive precursor (FO) that is cleaved by a host-cell protease. This cleavage releases a new N-terminus of Fl, thus forming the biologically active protein, consisting of disulfide - linked chains Fl and F2 (Scheid and Choppin, 1974).

The cleavage of F0 is a key determinant for pathogenicity of paramyxoviruses . Viruses that have multiple basic residues in the cleavage site of the F protein have proteolytic cleaveage of the F 0 molecule intracellularly by subtilisin - like proteases such as, Furin, during transport of the protein through the trans Golgi network. Paramyxoviruses that have single basic proteins in the F-cleavage site cannot be cleaved intracellularly and require exogenous proteases for cleavage activation (Ortmaim et. al. 1994 and Scheid and Choppin, 1974).
The F gene is 1792 nt long. F 0 has a predicted molecular mass of 66 KD. F 1 is 55 KD and F2 is 12.5 KD (Chambers et. al. 1986b). The Fusion peptides are thought to intercalate into target membranes, starting the fusion process.
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4. Methods

The Startup virus

Virus Strain Selection

The tissue samples from ailing birds supplied by the kind courtesy of Avian Disease Laboratory, Namakkal were proventriculaus, brain and liver showing characteristic lesion for viral isolation.

Tissue sample preparation

Tissue sample was prepared as 15% suspensions by grinding in a mortar and pestle with PBS containing the antibiotics, penicillin (10,000 lU/mi and streptomycin sulfate (2.0 mg/ml) at the pH of 7.0 to 7.4.

These tissue suspensions were centrifuged at 1500 x g for 20 minutes in a refrigerated centrifuge (4° C) to sediment tissue debris and most bacteria. The supernatant was then be aseptically removed and placed in an 1.5 ml sized vial for egg inoculation and storage. Tissue specimens were combined with antibiotics and kept at room temperature for 2 hours before egg inoculation.

This aided in reducing problems with bacteria. Specimens heavily contaminated with bacteria that cannot be removed by centrifugation (or) controlled by antibiotics were filtered through a 450 nm Millipore filter. Filtering was used only as a last resort, because aggregates of virus could be retained by the filter and significantly reduces the titre.

Egg Inoculation

The four most common routes for the inoculation of embroyonating eggs are via the allantoic sac, yolk sac, chorio allantoic membrane and amniotic sac. Here, allantoic sac route of inoculation was used.

Allantoic sac inoculations:

Initially four embryos were used for specimen.
Before inoculation, all embroys were candled for viability, and the site of inoculation was disinfected with a solution of 70% ethyl alcohol containing 3.5% iodine and 1.5% sodium iodide. The disinfection was applied with a cotton swab. A hole was prepared in the shell using a vibrating engraving tool of rotating drill equipped with a pointed tip. The drill tip was disinfected with the alcohol-iodine disinfectant before each use to avoid contaminating the eggs shell.

Candled 8 to 11 days embroynating eggs, making made certain that all air cells were in the normal position. Placed eggs on an eggs stand, air cell up and disinfected the area directly at the top of the egg. Drilled a small hole through the eggs shell along the centre axis at the top of the egg.

Using a syringe with 25 - gauge 5/8-inch (16mm) needle, inoculated 0.1 to 0.3 ml inoculum per egg by inserting the needle vertically and about 45°C inclined angle through the hole, the entire length of the needle and injecting the desired amount. Avoided moving the syringe sideways once the needle was inserted to prevent tearing of the CAM, which could cause bleeding and death of the embryo.

Sealed the hole with melted paraffin and returned the eggs to the incubator. Eggs inoculated by the allantoic route were normally incubated for 3-7 days post inoculation.

Collection of specimens from embroynating eggs:

Inoculated eggs were candled at least once a day to remove eggs with dead embryos. Continued incubation of eggs with dead embryos might result in decreased virus titre due to the thermal inactivation and could cause changes in the embryonic tissue making it difficult to interpret lesions in the embryos or on the supporting membranes. Embryos that died within the first 24 hours were discarded as non specific deaths were caused by injury, toxicity or bacterial contamination. If the inoculated eggs had live embryos following the specified incubation period, eggs would be refrigerated for a minimum of 4 hours to kill the embryo and allowed blood to clot before harvesting egg content. The presence of erythrocytes in the allantoic fluids can significantly reduced the titre of some viruses, such as paramyxoviruses and orthomyxovivuses, which agglutinate erythrocytes.
All eggs from which specimens were to be collected were surface disinfected with 70% ethyl alcohol. The disinfected was allowed to dry before harvesting specimens.

Cracked the egg shell over the air cell by tapping the egg shell with the blunt end of sterile forceps. Removed the eggs shell which covers the air cell, being careful not to rupture the under lying membranes and discarded pieces of the egg shell in disinfectant. Discarded forceps in a beaker of disinfectant.

Used different forceps to tear the egg shell membrane, the CAM and the amniotic membrane to release the allantoic fluid. Depressed the membranes over the yolk sac with the forceps and allowed the allantoic fluid to collect and pool above the forceps. Using a 5 ml pipette aspirated the fluid and places it into a sterile 12 x 75 mm snap-cap tube. Clarified the allantoic fluid by centrifugation at 1500 x g for 10 minutes and tested the allantoic fluid for evidence of virus infection using hemagglutination. Stored at - 20°C for further use.

5. Results and Discussion

Commercial layer poultry industry is under strict vaccination control of a major and a chronic disease called Newcastle disease (ND) because the ND is not eradicated in India. Hence, control of the diseases is by regular and periodical vaccination of all the age group of layers. Regular and periodical vaccination of the same birds during different periods requires a continuous and vigil sero-monitoring of the antibody levels of individual birds in general and of the population in particular. Presently such sero monitoring worldwide is being carried out by Haemagglutination Inhibition assay. This test requires the need of live virus cultivated in the laboratory using embryonated chicken eggs. Since the virus used for the laboratory test is also infective to the birds,

laboratory cultivation of virus posses an obvious danger of spillage and spread of the diseases.

An alternative approach, to replace the use of dangerous whole virus for this serological reaction was attempted in this study.

The possibility of using the FIN gene of NDV responsible for expressing the surface glycoprotein involved in the specific haemagglutination property used in the HI assay was explored and the success of the attempt was presented with all possible illustration.

Being an RNA virus, the full length 1.7 Kb sized RN gene of NDV was precisely isolated by RT-PCR through manually designed flanking primers. The purified gene was cloned into pBKSII + cloning vector for the purpose of checking the gene at the level of nucleotide sequences and its orientation to be directed during the sub-cloning of the gene into pET32a + expression vector so that expression of the anticipated specific protein in prokaryotic vector system is achieved.

6. Conclusion

Though the expressed protein in the pure form did not elicit the invitro HA activity with chicken RBC, the crude protein, before purification, gave agglutination with chicken RBC up to I in 40 dilution of the protein. The specificity of the agglutination was also confirmed by specific neutralization using NDV specific serum. The crude protein was also tested for its sero conversion ability on inoculation to susceptible conversion birds. The inoculation trial were done in ten birds and was found that the mean antibody titre was elevated from 41.6 to 227.6 which is fairly above the value 128, which is considered to be the population mean for protective immune status. The ultimate aim of this study is to produce an edible vaccine by transferring the gene in a plant.

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