

## Determination of Immune Potentials of Recombinant Fusion and Recombinant Haemagglutinin-Neuraminidase Antigens of Newcastle Disease Virus (NDV)

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### Abstract

Chicken are the most abundant birds in the world with a population of more than 24 billion. They are mainly kept for egg and meat production. Important economic loss for the industry can occur, due to viral diseases such as Avian influenza and Newcastle disease. Vaccination is the only available tool to curb the outbreak of epidemics in poultry. Till today only few good vaccines are available to prevent the epidemic diseases. Recombinant DNA technology led to the development of recombinant vaccines and subunit vaccines but immunopotency of recombinant vaccines and subunit vaccines are not well understood in Newcastle disease vaccines. Hence, in this study, we cloned and expressed the fusion and haemagglutinin-neuraminidase genes of NDV in insect cells. Immunological study was carried out to assess the immunopotency of rec-F and rec-HN antigens in commercial broilers. Humoral and cell mediated immune response were evaluated. The immunogenic potentials of recombinant antigens along with commercial live and inactivated vaccine in stimulating humoral antibody response was recorded by haemagglutination inhibition (HI) test and ELISA. Cell mediated immune responses in stimulating the peripheral blood mononuclear cells by recombinant antigens were measured using tetrazolium dye (MTT assay). Expression of cytokines such as interferon alfa and interferon gamma, stimulated by recombinant F and HN antigens and commercial vaccines were studied by Real-time polymerase chain reaction. Our results revealed that recombinant antigens had similar immuneresponse as commercial inactivated vaccines but less efficient in protecting the immunised birds than commercial live vaccine. Fusion protein has shown better protection than HN protein and combination of both antigens revealed more potency than individual antigen.

**Keywords:** Recombinant antigens; Fusion protein; HN protein; ELISA; Lymphoproliferation assay; Cytokines expression

### Introduction

Poultry sector is one of the major components of Indian animal husbandry and provides continuous and consistent income around the year to the poultry farmers. Further, animal products like milk, meat and egg also bring foreign revenue into the country. 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 (ND) and Avian influenza (AI). In India, ND is endemic with regular outbreaks with reports of presence of virulent virus in normal chicken and even in resistant birds like ducks. NDV had high adaptation properties which help it to circulate among different feral birds leading to continued presence of virus as it was isolated in desi birds, Japanese quail [1], house crows, ducks [2] and emu. Hence, the risk at any outbreak remains large to poultry industry which intern cause chaos in subsidiary agriculture. This is the prevailing scenario in the country in spite of the availability of live lentogenic, live mesogenic and inactivated vaccines to control ND along with regular sero prevalence and sero-monitoring (Figure 1).

Newcastle disease (ND) is an Office international des epizooties (OIE) listed disease caused by avian paramyxovirus type-1 (APMV-1) strains, with a wide avian host range resulting in severe economic losses in the poultry sector. Newcastle disease virus (NDV) has a negative sense, single stranded RNA genome which codes for six proteins namely, nucleoprotein, phosphoprotein, matrix protein, fusion protein, haemagglutinin-neuraminidase protein and large polymerase protein. The susceptibility of a wide variety of avian species coupled with frequently mobile wild bird reservoirs has contributed to the vast genomic diversity of this virus as well as diagnostic failures. Currently available live attenuated and inactivated vaccines were not enough to

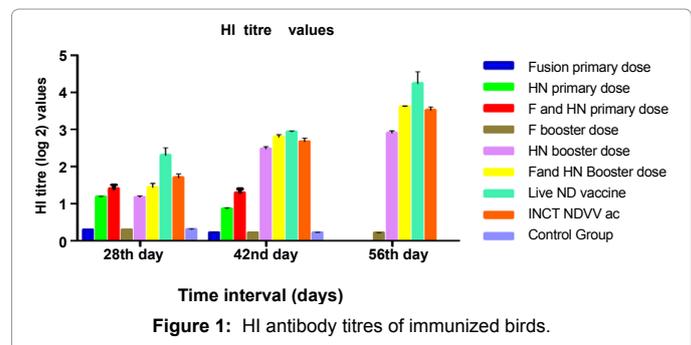


Figure 1: HI antibody titres of immunized birds.

trust full protection from virulent NDV infection. Live ND vaccines have many draw backs such as cold chain maintenance, reversion to virulence, spreading of live virus, local area reactions, etc. Inactivated vaccines have drawbacks such improper inactivation or over inactivation and strain specificity etc. All these hindrances encourages

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in the development of most potent recombinant antigen vaccine against NDV infection.

## Materials and Methods

To determine the immunogenicity of recombinant antigens bird trial was conducted. Hundred numbers of a day old chicks received from Venkateswara hatcheries limited (VHL), Hosur, Tamil Nadu, India, Immunogenicity study was conducted in two week old chicks in order to avoid the effect of maternal antibody against the challenged antigens. All the birds were divided into five groups namely A, B, C, D and E (Control). Further, group A was divided in to 3 subgroups (A1: Only Fusion antigen (10 ug/birds) with adjuvant, A2: Only HN antigen (10 ug/birds) with adjuvant and A3: Both F and HN antigen (5 ug of each antigen/ birds) with adjuvant), similarly group B was divided into B1, B2 and B3 (treatment was similar as group A). Group C was treated with live ND vaccine and group D treated with inactivated ND vaccine. Each treatment group contain 10 birds and group E (Control) contain 20 birds. Immunization was conducted on 14 days in all groups and the booster dose of antigen was administered in group B, C and D only, on 28 days of age (Figure 2).

Blood samples were collected from each group separately on 28, 42 and 56 day of experiment. Serum was separated by centrifugation and stored at -80°C till further use. All the birds were challenged with virulent ND virus of 10<sup>9.2</sup>·EID<sub>50</sub>/0.01 ml ND 2K3 virus isolate available in the department, on 42<sup>nd</sup> day. All the challenged birds were monitored for 14 days and recorded the mortality. On 56<sup>th</sup> day of trial, serum was collected from all the survived birds.

### Haemagglutination Inhibition (HI) test

HI test was performed in a plastic V-bottomed 96 well microtiter plate. HI titre was determined for serum samples collected from all birds on 14 day of post primary dose, 14 and 28 day of post booster.

### Indirect ELISA

The required dilutions of test antigens (Fusion and HN protein), primary antibody and conjugate for the test system were determined by checker board titration. The microplate was adsorbed with 100 µl NDV antigens (5 µg/ml of fusion and 5 µg/ml of HN antigen) in coating buffer and incubated at 4°C overnight. After washing with wash buffer, the plate was blocked with blocking buffer and incubated at 37°C for 60 min. After another washing, 1: 100 dilution of experimental serum samples were added in triplicate and the plate was incubated at 37°C for 45 min. After another washing, anti- chicken peroxidase conjugate at 1: 1000 dilution in blocking buffer was added to plate and was incubated at 37°C for 30 min. The enzyme substrate solution ortho-phenylene diamine dihydrochloride (OPD) was added and incubated for 10 min in the dark. The reaction was stopped by adding 1% H<sub>2</sub>SO<sub>4</sub>. Optical density values were measured at 492 nm in an automatic ELISA reader (Figure 3).

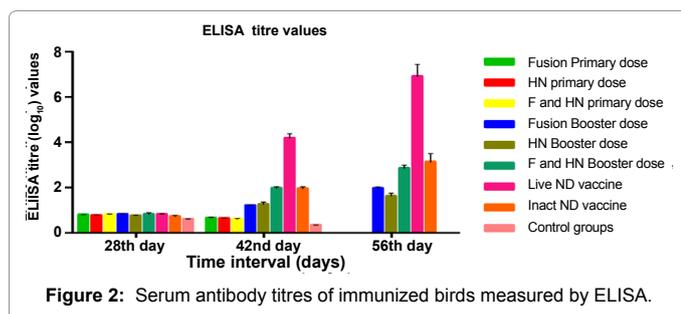


Figure 2: Serum antibody titres of immunized birds measured by ELISA.

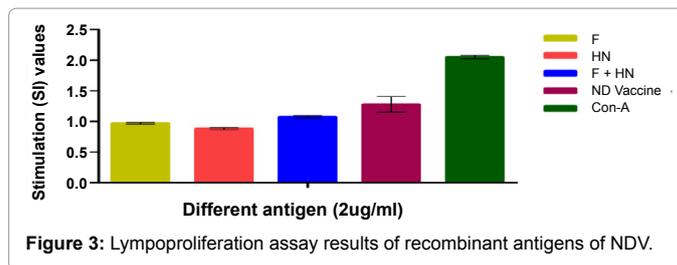


Figure 3: Lymphoproliferation assay results of recombinant antigens of NDV.

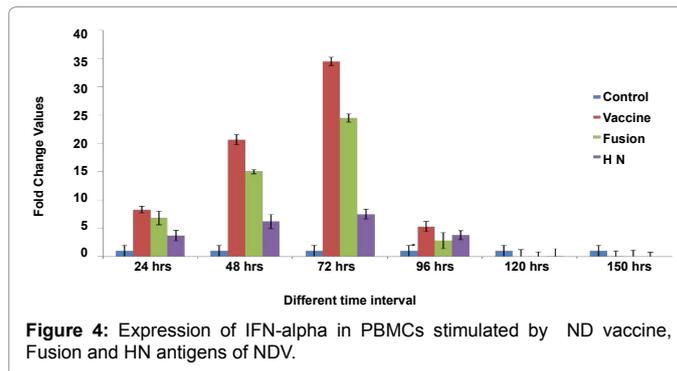


Figure 4: Expression of IFN-alpha in PBMCs stimulated by ND vaccine, Fusion and HN antigens of NDV.

### Lymphoproliferation assay (MTT assay) for rec-F and rec-HN antigens

In a 96 well ELISA plate, 2×10<sup>5</sup> PBMCs in 200 µl of RPMI-1640 medium were seeded and incubated at 37°C overnight. Rec-F and rec-HN antigens were added at 2 µg/ml concentration to different rows separately. For a separate row, both the antigens were added at half of the concentrations of initial rows (1 µg/ml of rec-F + 1 µg/ml of rec-HN) and another separate row was treated with commercial R<sub>2</sub>B vaccine at the concentration of 50 µl/ml. A separate row was maintained for positive control (2 µg/ml of Con-A) and another separate row was maintained as untreated negative control. Plate was incubated at 37°C for 3 days, and then media was removed and added 200 µl of tetrazolium dye (1 mg/ml) was added to all wells, incubated at room temperature for 1 hr in dark. 100 µl 1N HCl-isopropanol was added into each well to dissolve the formazan crystals. The plate was shaken for 20 min to resuspend the lymphocytes and absorbance of each well was measured by a microplate spectrophotometer at a wavelength of 550 nm. The response was reported as stimulation index (SI) and was calculated by the following formula:

$$\text{Stimulation Index (SI)} = \frac{\text{Mean absorbance of stimulated cultures}}{\text{Mean absorbance of unstimulated cultures}}$$

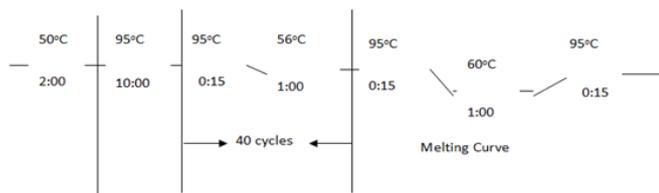
### Gene Expression Study

1×10<sup>6</sup> PBMCs were stimulated with recombinant fusion protein, recombinant HN protein at the concentration of 2 µg/ml. The 50 µl/ml of R<sub>2</sub>B vaccine was also used to stimulate PBMCs as the positive control and untreated PBMCs were maintained for each sample. All the treated cells were harvested separately at different time intervals, RNA was extracted and cDNA was synthesized as per the manufacturer's instructions (Figure 4).

### Quantitative PCR

Quantitative PCR (qPCR) was carried out using SYBR<sup>®</sup> Green JumpStart<sup>™</sup> Taq Ready Mix<sup>™</sup> (Sigma, USA).

- Quantative PCR was carried out using gene specific primers and cDNA as template.
- For amplification and relative quantification of cellular genes ( $\beta$  actin, INF alpha and INF gamma), real time PCR was carried out in Real time Thermal cycle (Master cycler®, Applied Biosystems, USA) as described by Yang et al. [3] with an initial heat denaturing step at 94°C for 3 min followed by the sequence cycle, final extension and melting curve as follows :



- An end-point single fluorescence was measured after each extension step. The melting curves for PCR products were analyzed from 70°C to 95°C to determine the specificity of all amplifications.

### Gene expression data analysis

The relative fold change method was used to analyse the expression of cytokine genes relative to reference (or endogenous control) gene in response to NDV antigen treatment. The following formulae were applied to find out the expression of genes.

- Using MS-Excel programme, Mean of the Ct values, delta Ct values, delta-delta Ct values and fold change values were estimated.
- The delta Ct values were obtained by subtracting mean of the  $\beta$  actin values in mean of the INF-alpha and INF-gamma values.
- Then the delta-delta values were obtained from subtracting the control delta values in treatment delta values. Sample values were normalized with the endogenous gene control ( $\beta$  actin) in each treatment groups.
- The fold change values were obtained by raising the 2 logarithm values to the delta-delta values of each treatment. Graph was plotted on MS Excel sheet using the fold change values versus time interval to confirm the expression of cellular gene. 1.00 was kept as the control and the fold change values which were above the control values were considered as significant (Figure 5).

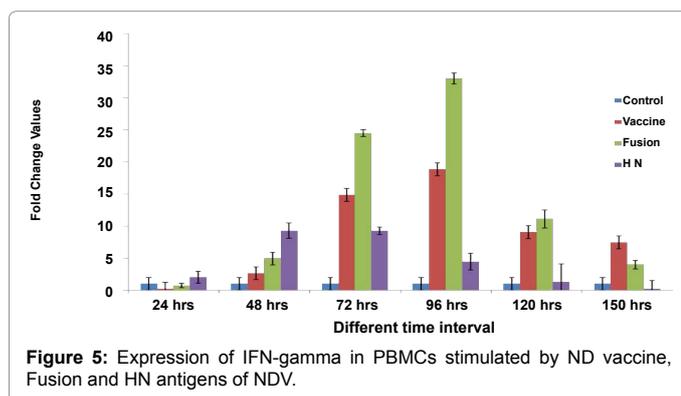


Figure 5: Expression of IFN-gamma in PBMCs stimulated by ND vaccine, Fusion and HN antigens of NDV.

### Results and Discussion

Immunization was conducted on 14 days in all groups and the booster dose of antigen was administered in group B, C and D only, on 28 days of age. Serum samples were collected from all the experimental birds at the age of 28, 42 and 56 days. The efficacy of the antigens in eliciting immunological response in the birds was further assessed by Haemagglutination inhibition (HI) and ELISA for humoral and lymphoproliferation assay and real time RT-PCR for cell mediated immune response, respectively.

The birds of all groups were challenged with 0.001 ml of virulent ND virus of  $10^{9.2}$  EID<sub>50</sub> on 14 day after the booster injection (42 day age) and further monitored for next 14 days. In group A 100% mortality was observed in 48 hrs to 72 hrs. In group B, which received booster dose of antigen F, HN and both recorded the mortality of 40% (B1), 80% (B2) and 20% (B3), respectively. Live ND vaccine was found to be most reliable in imparting protection and no mortality but commercial ND inactivated vaccine group had recorded 20% mortality till 14 days of challenge. Group E, which was maintained as control with no treatment had shown 100% mortality within 48 hrs post challenge. The higher protection compared to individual antigen and only 20% mortality in mixed antigens group could be due to interaction between fusion and HN antigen which in turn had synergistic effect in stimulation of immunity and it is true with all viruses of *Paramyxoviridae* family. A complex between NDV HN and F protein can be detected at the surface of both infected and transfected cells.

In a similar study, Arora et al. [4] had used crude 75 kDa (HN) and 56 kDa (F) proteins purified from R<sub>2</sub>B live vaccine virus. A challenge study was conducted with virulent virus and mortality of 26.6%, 80% and 20% was observed with F, HN and mixed (F and HN) antigen groups, respectively. Though the live vaccines have better immunogenic properties and provide 100% protection, the recombinant vaccines are much safer and more reliable because live ND vaccine have major drawbacks such as reversion to virulence, spreading of virus, vaccination failure due to improper cold chain maintenance and production of severe vaccinal reactions.

Chicken immunized with rNDF and rNDHN glycoproteins of the velogenic Miyadera strain were completely protected from a challenge and glycoproteins derived from velogenic and lentogenic NDV strains have been evaluated [5]. They found that the recombinant glycoproteins from velogenic strain produced complete protection after the second immunization than glycoproteins from lentogenic strains and also found that combined F and HN glycoprotein had synergistic effects against ND. The current study also supports the findings, where in, presence of both F and HN proteins provided complete immunity when given booster immunization against challenged virulent NDV. However, in this study recombinant glycoproteins were derived from lentogenic strain of NDV.

### Humoral immune responses

Haemagglutination is one of the important biological properties of NDV and its ability to agglutinate chicken erythrocyte is the principle of HA and HI tests. Kumar et al. [6] concluded that humoral immune response plays a very vital role in protection against Newcastle disease. In this study serum samples were collected on 28, 42 and 56 days of immunological trials. Haemagglutination Inhibition (HI) test was carried out to determine the antibody titre in the collected serum samples. Birds administered with recombinant antigens in combination (55 kDa of F + 65 kDa of HN) showed serum antibody titers with mean

HI titres ( $\text{Log}_2$ ) values of  $1.505 \pm 0.252$ ,  $2.709 \pm 0.571$  and  $3.412 \pm 0.842$  on 28<sup>th</sup>, 42<sup>nd</sup> and 56<sup>th</sup> day, respectively. Commercial live vaccine showed antibody titres as mean HI titres ( $\text{Log}_2$ ) values of  $2.107 \pm 0.349$ ,  $2.989 \pm 0.954$  and  $3.812 \pm 0.285$  and commercial inactivated vaccines showed antibody titres mean HI titres ( $\text{Log}_2$ ) values of  $1.806 \pm 0.915$ ,  $2.709 \pm 0.614$  and  $3.391 \pm 0.474$  on 28<sup>th</sup>, 42<sup>nd</sup> and 56<sup>th</sup> day, respectively. All these HI titre values were statistically significant at 95% level of significance ( $p < 0.05$ ). This highest post booster antibody titre could be due to the response of memory cells and antigenic polarization exhibited by injected proteins along with Freund's complete adjuvant.

The recombinant fusion protein of booster group showed mean HI titres ( $\text{Log}_2$  values) of  $0.301 \pm 0.492$ ,  $0.220 \pm 0.322$  and  $0.220 \pm 0.322$  and recombinant HN protein showed mean HI titres of  $1.204 \pm 0.105$ ,  $2.412 \pm 0.732$  and  $3.010 \pm 0.910$  on 28<sup>th</sup>, 42<sup>nd</sup> and 56<sup>th</sup> day, respectively. Fusion protein groups (booster) not showed increased in HI titres since fusion protein antibody cannot possess haemagglutination inhibition property but 28 day serum HI titre may be due to maternal antibody. Among the birds receiving F and HN protein individually, fusion protein exhibited better immunity in comparison to HN protein. The birds which received only primary dose of antigen showed lower HI titre values in comparison with booster dosed birds. This lower antibody titre produced by primary dosing was insufficient to protect birds against virulent NDV. This could be due to lack of booster stimulation to memory cells in primary dosed birds. The HI titres of mixed antigens and inactivated vaccine were similar and protection rate was also found to be the same. It could be due to interaction of fusion and HN proteins which is required in synergistic effect. Lack of this synergistic interaction in individual antigen groups led to less titre values and high mortality.

In a similar study, Arora et al. [4] found that combination of F and HN proteins imparts better protection than individual antigen. The fusion protein has developed higher antibody titre than HN protein and it was also found that combination of recombinant fusion and HN protein had equal immunopotency as commercial inactivated whole virus vaccine.

In the present study, ELISA titres indicated that combination of recombinant fusion and HN protein induced higher antibody titer than individual antigens. The ELISA titre values of commercial live ND vaccine were more than commercial inactivated ND vaccine, while recombinant fusion and HN combination showed similar titre values as that of inactivated vaccine. The ELISA titre values ( $\text{Log}_{10}$ ) for mixed recombinant antigens, live vaccine and inactivated vaccine were  $0.784 \pm 0.82$ ,  $0.842 \pm 0.89$  and  $0.698 \pm 0.53$  on 24<sup>th</sup> day,  $2.889 \pm 0.91$ ,  $4.781 \pm 2.14$  and  $2.701 \pm 1.85$  on 42<sup>nd</sup> day and  $3.257 \pm 0.54$ ,  $6.577 \pm 1.33$  and  $3.192 \pm 2.04$  on 56<sup>th</sup> day, respectively. All these ELISA titre values were statistically significant at 95% level of significance ( $p < 0.05$ ). Antibody titres were reported to be not significant in primary dose groups and were insufficient to protect birds challenged with virulent NDV. Recombinant fusion protein had shown higher titres values than HN protein. However, combined injection of F and HN protein elicited significant by higher antibody titre than either of the proteins injected individually and it could be due to interaction between fusion and HN proteins which is required in synergistic effect. It indicated that both fusion and HN proteins play a major role in protecting the birds challenged with virulent NDV.

In a similar study, Arora et al. [4] suggested that ELISA titre values were higher in fusion protein injected birds than HN protein injected one and the combination of both recombinant proteins had shown significantly higher titre than individual and were equal to commercial

killed vaccine. The present study also had similar findings that, inactivated ND vaccine and mixed recombinant antigens had equal immune protection and equal potency of development of antibody but live vaccine was highly significant in the development of antibody titre ( $p < 0.01$ ) than inactivated ND vaccine.

Humoral response of recombinant antigens was determined to be significant when combination of recombinant fusion and HN antigen were used. Fusion protein had shown higher titres ELISA than HN protein which indicated that fusion protein plays a greater role in protection against the virulent virus challenge. The humoral immune response to live ND vaccine was highly significant than that of inactivated ND vaccine. Mixed recombinant antigen (F and HN) had similar potency and efficacy as inactivated ND vaccine.

### Cell mediated immunity

Cell mediated immunity (CMI) plays an important role in defense against viral infections as revealed by Allan et al, [7]. Cell mediated immune response occurs early infection of NDV and is detectable as early as 2-3 hrs after the immunization with live vaccine strains. Cell mediated immunoresponse elicited by vaccine has major role in curbing the disease outbreak and eradication of infectious agent of Newcastle disease.

### Lymphoproliferation assay (MTT assay)

In this study, lymphocytes were stimulated with recombinant fusion protein, HN protein of NDV as well as with commercial ND vaccine ( $R_2B$ ) for 72 hrs. The stimulation indices (SI) of the lymphocytes were recorded. The PBMCs stimulated by mixed (F and HN) antigens have shown higher stimulation index value in comparison with individual antigen and it could be due to synergistic interaction between two glycoproteins. These values were statistically significant at 95% of level of significance ( $p < 0.05$ ). Fusion protein had higher proliferation stimulation than HN protein. Commercial ND vaccine ( $R_2B$ ) showed the highest stimulation index value. This indicated that  $R_2B$  vaccine had significant immune stimulation activity than recombinant antigens in vaccinated birds.

### Expression of cytokine genes

In the present study, expression of cytokine genes like IFN-  $\alpha$  and IFN-  $\gamma$  were studied in comparison with housekeeping gene like  $\beta$ -actin in peripheral blood mononuclear cells. Their transcripts were measured to find out their up/down regulation or time of their expression in terms of hours post infection.

### Expression of Interferon alpha (IFN- $\alpha$ )

The expression of IFN- $\alpha$  gene was measured by real time PCR and expression levels were presented as the fold change values. These fold change values were statistically significant at 95% of level of significance ( $p < 0.05$ ). The expression ratio of mRNA of IFN- $\alpha$  gene induced by commercial ND vaccine in PBMCs was higher than eight fold of the level of expression of the same on day one. It further increased to higher than threefold of that of 24 hrs at 48 hrs. At 72 hrs of infection the expression ratio was increased to four fold of the same gene at 24 hrs. After 72 hrs of expression the level of IFN- $\alpha$  was continuously down regulated till 150 hrs. The down regulation in IFN- $\alpha$  can be attributed to the increasing concentration of V protein, an interferon antagonist as suggested by Selvan et al. [8]. However, in a similar study Young et al. [9], a constant expression of the IFN- $\alpha$  gene upto 120 hrs post infection was observed with infectious bursal disease virus. A fivefold increase in expression ratio at 72 hrs post infection was also reported. Increased

expression of IFN- $\alpha$  gene in cells infected with NDV has also been reported by earlier workers.

The expression ratio of IFN- $\alpha$  gene induced by the recombinant fusion protein in PBMCs was more than six fold of the level of expression of the same, on day one. At 72 hrs of post infection, peak expression ratio was achieved which was four fold more than that of 24 hrs expression. After 72 hrs of post infection, the expression was found to get down regulated and at 150 hrs its expression ratio became non-significant. The same expression ratio was recorded for HN protein which exhibited more than threefold of the level of expression of the same on day one. At 72 hrs only two fold increases in the expression level in comparison to expression ratio on day one was noticed. This expression ratio by fusion and HN protein indicated that fusion protein was more potent in eliciting the cell mediated immune response than HN protein. The expression level of IFN- $\alpha$  gene in PBMCs stimulated by commercial ND vaccine and recombinant fusion protein were more or less similar. This could be due to fusion protein being followed the antigenic polarization in stimulating the T cells, which was lacking in HN protein treated PBMCs. Moreover, there can be down regulation in expression level of IFN- $\alpha$  due to activation viral V protein in commercial ND vaccine treated cells which is absent in recombinant fusion protein treated cells. The down regulation in of cytokine gene expression in fusion and HN protein treated PBMCs could be due to lack of booster stimulation.

In a similar study with virulent Texas GB strain of NDV, the virus was reported to upregulate the IFN- $\alpha$  gene from 36 hrs post infection and no change was observed upto 24 hrs post infection. In another study Selvan et al. [8] found that over expression of V protein, a product of P gene had been reported to enhance the production kinetics of NDV replication in chicken embryo cells. Huang et al. [10] reported that, the P gene of NDV has interferon antagonist property and expression of this protein in cells has been observed to facilitate the replication of virus.

### Expression of Interferon gamma (IFN- $\gamma$ )

IFN- $\gamma$  has been reported to be involved in macrophages and T cells activation and inhibition of virus replication. A protective role of IFN- $\gamma$  against NDV infection has also been demonstrated. In addition, the level of IFN- $\gamma$  mRNA expression has been correlated with the level of IFN- $\gamma$  secretion in chicken. In many economically important poultry diseases a primary role of IFN- $\gamma$  has been suggested [11].

In the present study, the expression of mRNA of IFN- $\gamma$  gene was measured by real time PCR and expression levels were reported as the fold change values. These fold change values were statistically significant at 95% of level of significance ( $p < 0.05$ ). The expression ratio of mRNA of IFN- $\gamma$  gene in induced PBMCs by commercial ND vaccine at 96 hrs of infection was more than 100 fold of its expression level at 24 hrs. However, up to 72 hrs post infection, the expression ratio of this gene was significant and it increased up to 96 hrs. After 96 hrs of infection the expression ratio of INF- $\gamma$  gene was down regulated. The expression ratio of INF- $\gamma$  gene induced by the recombinant fusion protein at 96 hrs of infection was more than the 60 fold of the level of expression of the same at 24 hrs. The expression ratio of IFN- $\gamma$  gene induced by fusion protein was more or less similar to commercial ND vaccine but the expression ratio of IFN- $\gamma$  gene induced by recombinant HN protein at 72 hrs of post infection was nearly fivefold of the level of expression of the same at 24 hrs. After 72 hrs post infection the expression level of IFN- $\gamma$  gene induced by HN protein was down regulated and expression ratio was not significant when compared to fusion protein. This could

be due to HN protein being more potent in humoral immune response than cell mediated immune response. These expression ratios clearly indicated that antigen polarization was followed by both fusion and HN glycoprotein of lentogenic NDV in inducing the interferon gene expression.

In another study, carried out to find the immune response to virulent NDV in chicken lines divergently selected for cutaneous hypersensitivity, upregulation of IFN- $\gamma$  gene was noticed from 30 hrs and peak was reached at 42 hrs in PBMC. However, in the present study the peak upregulation of IFN- $\alpha$  was noticed only at 72 hrs and IFN gamma was at 96 hrs of post infection. The probable reasons could be vaccine used was not virulent and belonged to mesogenic group. Moreover, recombinant antigens used were from lentogenic isolate of NDV. This hypothesis can be further justified by a latest work on host gene response to virulent NDV using DNA microarray, wherein upregulation of IFN- $\gamma$  was reported from 24 hrs post infection. Upregulation of this gene is an indication of induction of immune response.

In a similar study, Chandrashekhar et al. [12] reported that vaccination protected birds by producing humoral and cell mediated immune response which is essential for complete protection against infection. Vaccination is an important measure used successfully for prevention and control of Newcastle disease for longer. Koppad et al. [13] stated that inactivated Newcastle disease viral particles coupled with calcium phosphate nanoparticle as an adjuvant exhibited prolonged and improved humoral and cell mediated immune response in comparison with commercial live ND vaccines.

Robin et al. [14] constructed recombinant HTV clones, which expressed the fusion and HN proteins of NDV and when chickens were inoculated at 1 day age with recombinant HVT expressing the NDV fusion protein, had an immunological response and were protected (>90%) against lethal intramuscular challenge at 28 days of age with the neurotropic velogenic NDV strain Texas GB. Recombinant HVT expressing the NDV haemagglutinin-neuraminidase provided partial protection (47%) against the same challenge. This finding supported our results that recombinant fusion protein was more immunogenic and more potent in eliciting the humoral and cell mediated immune response compared to recombinant HN protein.

This study found that recombinant fusion and HN proteins were more immunogenic as inactivated and live ND vaccine in cytokines expression studies. Interferon expression revealed that recombinant antigen vaccine has maximum peak response than live ND vaccine and prolongation of expression was similar in both fusion protein induced and live ND vaccine induced cells. However, we did not compare the cytokines expression profiles with inactivated ND vaccine but obtained results indicated that recombinant fusion protein vaccine had significant immune response than live ND vaccine in cytokine production.

### Conclusions

Vaccination study was conducted out in experimental birds to assess the immunogenicity of the recombinant fusion and HN proteins as well as commercial ND vaccines. The results obtained after challenge studies indicated that commercial live ND vaccine was highly potent followed by commercial inactivated ND vaccine and mixed fusion and HN proteins. The immune potency of fusion and HN protein as mixed proteins were greater than the individual proteins. This result also suggested that commercial inactivated ND vaccines could be replaced with mixed recombinant protein vaccine in near future. Since protection recorded in these two groups was similar.

Immunogenic potential of recombinant proteins in eliciting the humoral antibody response has been determined by HI and ELISA test. The mean titre values of HI and ELISA test indicated that commercial inactivated ND vaccine and mixed recombinant proteins had similar immune potency. But recombinant subunits have shown slight improved and prolonged ELISA titre values in comparison with inactivated ND vaccine. Even though, the titre values for live ND vaccine were high, many drawbacks of this vaccine not encouraging their utilization in control of NDV infection. The titre value of recombinant fusion protein was found to be higher than HN protein but mixed recombinant proteins were recorded as significant than individual ( $p < 0.01$  and  $0.05$ ).

Immunogenic potential of recombinant proteins in eliciting cell mediated immune response has been determined by lymphoproliferation assay and cytokine genes expression study. Stimulation index values indicated that commercial R<sub>2</sub>B vaccine was more superior to mixed recombinant proteins. The recombinant fusion protein has shown higher stimulation index than the recombinant HN protein. However, stimulation index value of combination of fusion and HN protein has been recorded more significant than individual proteins.

Similarly, cytokine genes expression in lymphocytes stimulated by recombinant live ND vaccine, recombinant fusion protein and recombinant HN protein has been determined. The obtained results indicated that IFN alpha expression was peaked at 72 hrs of post infection and then down regulated to non-significant level. The expression of IFN gamma has reached its peak at 96 hrs of post infection and after 96 hrs started to down regulate. But fusion glycoprotein exhibited higher fold change values than commercial live ND vaccine in expression of IFN gamma and had similar prolongation curve till 150 hrs of post infection. This cytokines gene expression study also revealed that commercial ND vaccine and recombinant fusion protein has similar potency but recombinant HN protein was recorded to be less potent in stimulation of CMI response.

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#### References

1. Kumanan K (1989) Studies on immunogenic proteins of Newcastle disease virus Ph.D., thesis. Tamil Nadu Veterinary and Animal Sciences University, Chennai.
2. Roy P, Venugopalan AT, Dhillon AS (2003) Efficacy of two commercial Newcastle disease virus lentogenic vaccines against virulent Asiatic type Newcastle disease viruses. *Journal of Applied Poultry Research* 12: 169-173.
3. Yang ZQ, Liu QQ, Pan ZM, Yu HX, Jiao XA (2007) Expression of the fusion glycoprotein of Newcastle disease virus in transgenic rice and its immunogenicity in mice. *Vaccine* 25: 591-598.
4. Arora P, Lakhchaura BD, Garg SK (2010) Evaluation of immunogenic potential of 75kDa and 56kDa proteins of newcastle disease virus (NDV). *Indian J Exp Biol* 48: 889-895.
5. Janke M, Peeters B, de Leeuw O, Moorman R, Arnold A, et al. (2007) Recombinant Newcastle disease virus (NDV) with inserted gene coding for GM-CSF as a new vector for cancer immunogene therapy. *Gene Ther* 14: 1639-1649.
6. Kumar KU, Swamy SK, Reddy TV (1988) Humoral and cell mediated immune response in chicks vaccinated against Newcastle disease. *Kerala Journal of Veterinary Science* 19: 116-121.
7. Allan WH, Lancaster JE, Toth B (1974) The production and use of Newcastle disease vaccines. *FAO Animal Production Series No. 10*. FAO, Rome.
8. Selvan N, Ranjit S, Amirtha J, Sukumar AS, Jeyakumar N, et al. (2010) Production and application of recombinant haemagglutinin neuraminidase of Newcastle disease virus. *Asian Pacific Journal Tropical Medicine* 3: 629-632.
9. Young JK, Li D, Abramowitz MC, Morrison TG (1999) Interaction of peptides with sequences from the Newcastle disease virus fusion protein heptad repeat regions. *J Virol* 73: 5945-5956.
10. Huang Z, Elankumaran S, Panda A, Samal SK (2003) Recombinant Newcastle disease virus as a vaccine vector. *Poult Sci* 82: 899-906.
11. Zhuhui H, Krishnamurthy S, Aruna P, Samal SK (2003) Newcastle disease virus V protein is associated with viral pathogenesis and functions as an alpha interferon antagonist. *Journal of Virology* 77: 76-85.
12. Chandrashekhar S, Padmanaban VD, Masilamony PR, Venkatesan RA (1992) Nature of protective immunity response in chickens against Ranikhet disease. *Indian Veterinary Journal* 66: 801-806.
13. Koppad S, Raj GD, Gopinath VP, Kirubakaran JJ, Thangavelu A, et al. (2011) Calcium phosphate coupled Newcastle disease vaccine elicits humoral and cell mediated immune responses in chickens. *Res Vet Sci* 91: 384-390.
14. Morgan RW, Gelb J Jr, Schreurs CS, Lütticken D, Rosenberger JK, et al. (1992) Protection of chickens from Newcastle and Marek's diseases with a recombinant herpesvirus of turkeys vaccine expressing the Newcastle disease virus fusion protein. *Avian Dis* 36: 858-870.