



Original Research Article

Determination of immune potentials of recombinant fusion and recombinant haemagglutinin-neuraminidase antigens of Newcastle Disease virus (NDV)

Maheswarappa Gowrakkal, K.Vijayarani*, K.Kumanan and A.Uttarkumar

Department of Animal Biotechnology, Madras Veterinary College,
TANUVAS, Vepery, Chennai-07, India

*Corresponding author

ABSTRACT

Chicken are the most abundant birds in the world with a population of more than 25 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 (PBMCs) by recombinant antigens were measured using tetrazolium dye (MTT assay). Expression of cytokines such as interferon alfa and interferon gamma in PBMCs 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 immune response 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,
PBMCs,
Lympho-proliferation assay and 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 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 (Ananth *et al.*, 2008) and even in resistant birds like ducks (Stalin, 2008). 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 (Kamaraj *et al.*, 1998), Japanese quail (Kumanan *et al.*, 1990), house crows (Roy *et al.*, 1998), ducks (Roy *et al.*, 2000) and emu (Kumanan *et al.*, 2003). 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.

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 (Alexander and Senne, 2008). Currently available live attenuated and inactivated vaccines were not enough to 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 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. Fifty 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 A, B, C, D groups of 5 birds each and group E (Control) contain 10 birds. Further, group A was divided in to 3 subgroups (A1: Only Fusion antigen (10 µg/birds) with adjuvant, A2: Only HN antigen (10 µg/birds) with adjuvant and A3: Both F and HN antigen (5µg 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. 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.

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 $10^{8.2}$ EID₅₀/0.01ml ND 2K3 virus isolate available in the department, on 42nd day. All the challenged birds were monitored for 14 days and recorded the mortality. On 56th 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 primary dose, 14 and 28 day of 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 μ l NDV antigens (5 μ g/ml of fusion and 5 μ g/ml of HN antigen) in coating buffer and incubated at 4 $^{\circ}$ C overnight. After washing with wash buffer, the plate was blocked with blocking buffer and incubated at 37 $^{\circ}$ 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 $^{\circ}$ 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 $^{\circ}$ 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% H₂SO₄. Optical density values were measured at 492 nm in an automatic ELISA reader.

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

In a 96 well ELISA plate, 2 X 10⁵ PBMCs in 200 μ l of RPMI-1640 medium were seeded and incubated at 37 $^{\circ}$ C overnight. Rec-F and rec-HN antigens were added at 2

μ g/ml concentration to different rows separately. For a separate row, both the antigens were added at half of the concentrations of initial rows (1 μ g/ml of rec-F + 1 μ g/ml of rec-HN) and another separate row was treated with commercial R₂B vaccine at the concentration of 50 μ l/ml. A separate row was maintained for positive control (2 μ g/ml of Con-A) and another separate row was maintained as untreated negative control.

Plate was incubated at 37 $^{\circ}$ C for 3 days, and then media was removed and added 200 μ l of tetrazolium dye (1 mg/ml) was added to all wells, incubated at room temperature for 1 hr in dark. 100 μ l 1N HCl-isopropanol was added into each well to dissolved 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 (Reynolds and Maraqa, 2000). The response was reported as stimulation index (SI) and was calculated by the following formula:

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

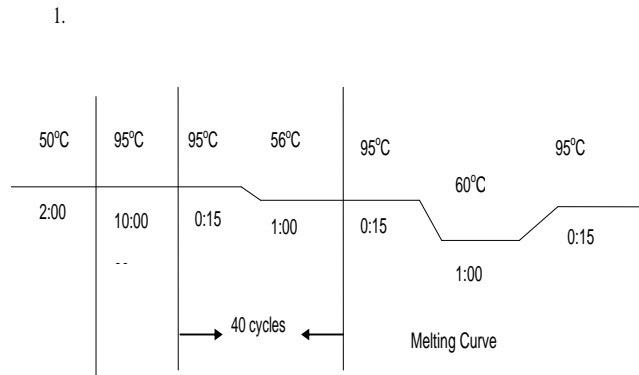
Gene Expression Study

1x10⁶ PBMCs were stimulated with recombinant fusion protein, recombinant HN protein at the concentration of 2 μ g/ml. The 50 μ l/ml of R₂B 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.

Quantitative PCR

Quantitative PCR (qPCR) was carried out using SYBR[®] Green JumpStart[™] Taq Ready Mix[™] (Sigma, USA).

1. Quantitative PCR was carried out using gene specific primers and cDNA as template.
2. For amplification and relative quantification of cellular genes (β 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 Li *et al.* (2007) with an initial heat denaturing step at 94^o C for 3 min followed by the sequence cycle, final extension and melting curve as follows :



3. An end-point single fluorescence was measured after each extension step. The melting curves for PCR products were analyzed from 70^o C to 95^o 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.

1. Using MS-Excel programme, Mean of the Ct values, delta Ct values, delta-delta Ct values and fold change values were estimated.
2. The delta Ct values were obtained by subtracting mean of the β actin values in mean of the INF-alpha and INF-gamma values.
3. 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 (β actin) in each treatment groups.
4. 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.

Result 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 0.01 ml of virulent ND virus of $10^{8.2}$ EID₅₀ 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 (Choppin and Scheid, 1980). A complex between NDV HN and F protein can be detected at the surface of both infected and transfected cells (Lamb and Park, 2007).

In a similar study, Arora *et al.* (2010) had used crude 75 kDa (HN) and 56 kDa (F) proteins purified from R₂B 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 (Kamiya *et al.*, 1994; Lee *et al.*, 2008). 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 (Hanson, 1975). Kumar *et al.*, (1988) 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 (Log₂) values of 1.505 ± 0.252 , 2.709 ± 0.571 and 3.412 ± 0.842 on 28th, 42nd and 56th day, respectively. Commercial live vaccine showed antibody titres as mean HI titres (Log₂) values of 2.107 ± 0.349 ,

2.989 ± 0.954 and 3.812 ± 0.0285 and commercial inactivated vaccines showed antibody titres mean HI titres (Log₂) values of 1.806 ± 0.915, 2.709 ± 0.614 and 3.391 ± 0.474 on 28th, 42nd and 56th 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 (Log₂ values) of 0.301 ± 0.492, 0.220 ± 0.322 and 0.220 ± 0.322 and recombinant HN protein showed mean HI titres of 1.204 ± 0.105, 2.412 ± 0.732 and 3.010 ± 0.910 on 28th, 42nd and 56th 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 (Choppin and Scheid, 1980). Lack of this synergistic interaction in individual antigen groups led to less titre values and high mortality.

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 (Log₁₀) for mixed recombinant antigens, live vaccine and inactivated vaccine were 0.784 ± 0.82, 0.842 ± 0.89 and 0.698 ± 0.53 on 24th day, 2.889 ± 0.91, 4.781 ± 2.14 and 2.701 ± 1.85 on 42nd day and 3.257 ± 0.54, 6.577 ± 1.33 and 3.192 ± 2.04 on 56th 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 (Choppin and Scheid, 1980). It indicated that both fusion and HN proteins play a major role in protecting the birds challenged with virulent NDV.

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 immune response occurs early infection of NDV and is detectable as early as 2-3 hrs after the immunization with live vaccine strains (Timid *et al.*, 1975). Cell mediated immunoresponse elicited by vaccine has major role in curbing the disease out break 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 (R₂B) 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 (Choppin and Scheid, 1980).

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 (R₂B) showed the highest stimulation index value. This indicated that R₂B 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- α and IFN- γ were studied in comparison with house keeping gene like β -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- α)

The expression of IFN- α 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- α 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 three fold 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- α was continuously down regulated till 150 hrs. The down regulation in IFN- α can be attributed to the increasing concentration of V protein, an interferon antagonist as suggested by Jang *et al.*, 2010. However, in a similar study Li *et al.* (2007), a constant expression of the IFN- α gene upto 120 hrs post infection was observed with infectious bursal disease virus. A five fold increase in expression ratio at 72 hrs post infection was also reported. Increased expression of IFN- α gene in cells infected with NDV has also been reported by earlier workers (Munir *et al.*, 2005).

The expression ratio of IFN- α 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 three fold 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- α 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- α 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.

Expression of Interferon gamma (IFN- γ)

Expression of mRNA of IFN- γ 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- γ 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- γ gene was down regulated. The expression ratio of INF- γ 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- γ gene induced by fusion protein was more or less similar to commercial ND vaccine but the expression ratio of IFN- γ gene induced by recombinant HN protein at 72 hrs of post infection was nearly five fold of

the level of expression of the same at 24 hrs. After 72 hrs post infection the expression level of IFN- γ 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- γ gene was noticed from 30 hrs and peak was reached at 42 hrs in PBMC (Ahmed *et al.*, 2007). However, in the present study the peak upregulation of IFN- α 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- γ was reported from 24 hrs post infection. Upregulation of this gene is an indication of induction of immune response (Rue *et al.*, 2011).

Vaccination is an important measure used successfully for prevention and control of Newcastle disease for longer (Gilsson and Kleven, 1993). Koppad *et al.* (2010) stated that inactivated Newcastle disease viral particles coupled with calcium phosphate nanoparticle as a adjuvant exhibited prolonged and improved humoral and cell mediated immune response in comparison with commercial live ND vaccines. Robin *et al.* (1992) 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.

In this study 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.

Inference

Vaccination study results obtained after challenging 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 R₂B 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.

Figure.1 HI antibody titres of immunized birds

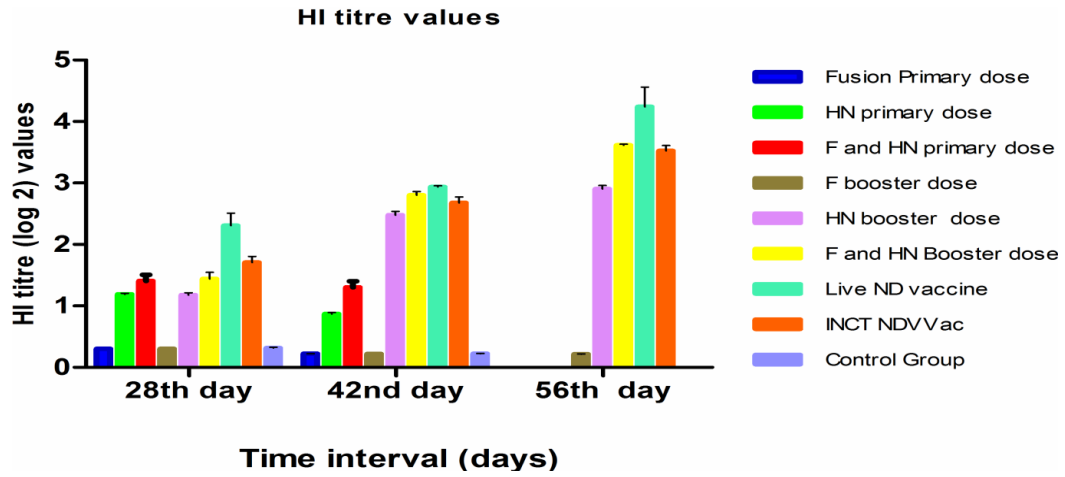


Figure.2 Serum antibody titres of immunized birds measured by ELISA

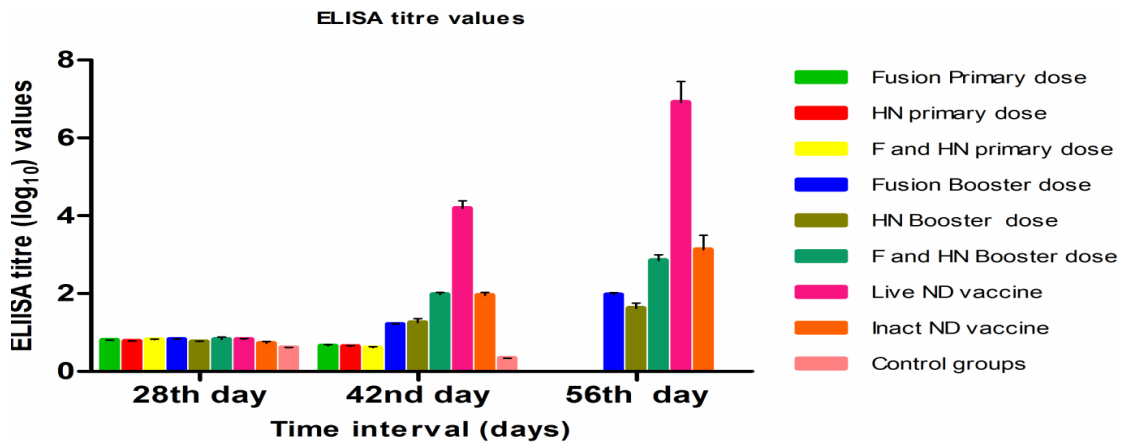


Figure.3 Lymphoproliferation assay results of recombinant antigens of NDV

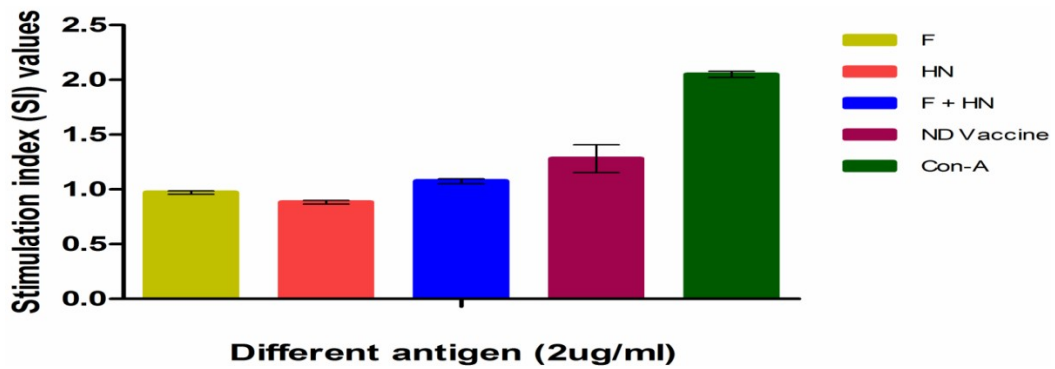


Figure.4 Expression of IFN-alpha in PBMCs stimulated by ND vaccine, Fusion and HN antigens of NDV

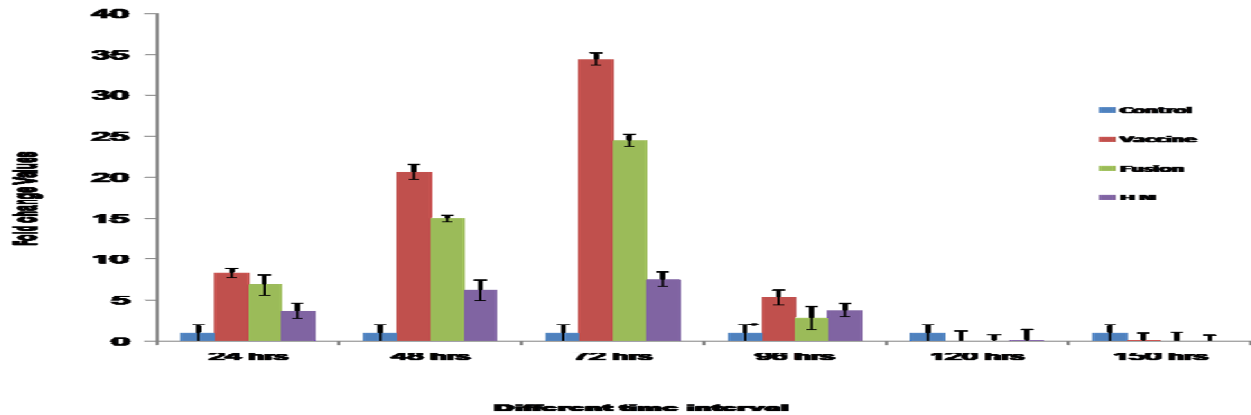
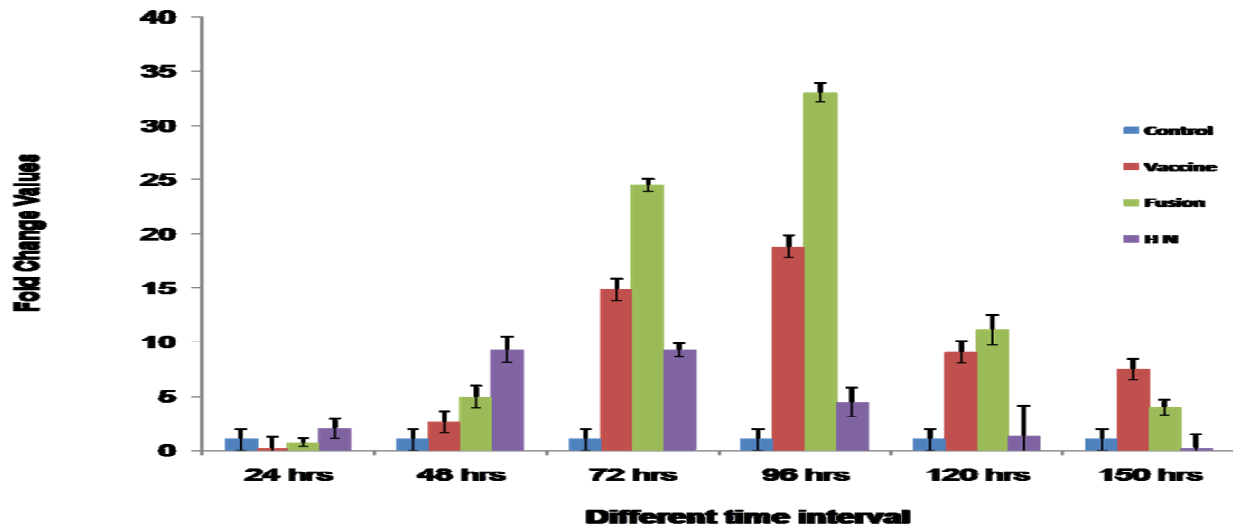


Figure.5 Expression of IFN-gamma in PBMCs stimulated by ND vaccine, Fusion and HN antigens of NDV



Acknowledgement:

The authors thank the Dean, Faculty of Basic Sciences and The Dean, Madras Veterinary College for the facilities provided. The authors also thank Indian Council for Agricultural Sciences, Government of India, New Delhi and National Institute of Animal Nutrition and Physiology, Bangaluru, for funding part of this work through the ICAR Niche area of

Excellence in Animal Biotechnology project.

References

Abbas, A. K., Murphy, K. M. and Sher, A. 1997. Functional diversity of helper T lymphocytes. *Nature*. 383: 787-793.
 Allan, W.H. and Gough, R.E. 1988. A standard haemagglutination

- inhibition test for Newcastle disease. Comparison of macro and micro methods. *Vet. Rec.* 9: 120-123.
- Al- Shahery, M.N., Al-Zubedy, A.Z. and Al- Baroodi, S.Y. 2008. Evaluation of Cell mediated immune response in chickens vaccinated with Newcastle disease virus. *Iraqi J. Vet. Sci.* 22: 21-24.
- Al-Zubeedy, A.Z. 2009. Evaluation and efficacy of two different vaccination programs against Newcastle disease I Nineveh Province. *J. Anim and Vet Advan.*, 8: 2228-2231.
- Andrade, O. G., Rubio, E. L., Flores, T. O., Bone, T. F. and Lim, M. A. G. 2006. Expression of the Newcastle disease virus fusion protein in transgenic maize and immunological studies. *Transgenic Res.* 15: 455-463.
- Andrea, M. M., Krell, P. J. and Nagy, E. 1999. Antibody detection based differential ELISA for NDV-infected versus NDV HN- subunit vaccinated chickens. *Vet. Microbiol.* 66: 209-222.
- Antonio, C.S., Lima, F.S., Denadai, J., Nishizawa, M. and Doretto, L. 2008. Newcastle disease vaccination in guinea fowls: Clinical and Immunological parameters. *Intl. J.Poult.Sci.* 7: 1013-1014.
- Arora, P., Lakhchana, B.D. and Garg, S.K. 2010. Evaluation of immunogenic potentials of 75 kDa and 56 kDa proteins of Newcastle disease virus. *Ind. J. Exp. Biol.* 48: 889-895.
- Bankowski, R. A. 1957. A modified live Newcastle disease virus vaccine. *Proc. Soc. Exp. Biol. Med.*, 96: 114-118.
- Bhaskar, S., Mayank, P., Gaurava, K., Rai, A., Meeta, S., Barkha, R., Mona C, Brijesh, S. Yadav., Arnab, Sen. and Bimelendu, M. 2012. Isolation of Newcastle disease virus from a non-avian host (sheep) and its implications. *Arch. Virol.* 157: 1565-1567.
- Boven, M. V., Bouma, A., Fabri, H.F.T., Katsma, E., Hartog, L. and Koch, G. 2008. Herd immunity to Newcastle disease virus in poultry by vaccination. *Avian Pathol.* 37: 1-5.
- Chandrashekhar, S., Padmanaban, V.D., Masilamony, P.R. and Venkatesan, R.A. 1992. Nature of protective immunity response in chickens against Ranikhet disease. *Ind. Vet. J.* 66: 801-806.
- Cxifra, G., Meszoros, J. and Engstrom, B. 1998. Detection of NDV antibodies and level of protection provided by single vaccination in young chicks. *Avian Pathol.* 27: 562-565
- Dashab, G., Sadeghi, G. and Mehri, M. 2007. Performance and humoral immune response to Newcastle disease in two strains of broiler chickens. *J. Anim. Vet. Adv.* 6: 451-453.
- Deng, R., Mirza, A. M., Mahon, P. J. and Iorio, R. M. 1997. Functional chimeric HN glycoproteins derived from Newcastle disease virus and human parainfluenza virus-3. *Arch. Virol.* 13: 115-130.
- Garib AISO, Giekens, E. A., Gruys, B. and Koch, G. 2003. Review of Newcastle disease virus with particular references to immunity and vaccination. *World's Poul. Sci.* 59: 185-198.
- Hassanzadeh, M. and Bozorgmeri Fard, M. H. 2004. A serological study of Newcastle disease in pre and post vaccinated village chickens in Iran. *Int. J. Poult Sci.* 3: 658-661.
- Heckert, R.A and Nagy, E, 1999. Evaluation of the haemagglutination- inhibition assay

- using a Baculo virus- expressed haemagglutinin- neuraminidase protein for detection of Newcastle disease virus antibodies. *J. Vet.Diagn. Invest.* 11: 99-102.
- Huang, Z., Elankumaran, S., Panda, A. and Samal, S. K. 2003. Recombinant Newcastle disease virus as a vaccine vector. *Poult. Sci.* 6: 899-906
- Huang, Z., Krishnamurthy, S., Panda, A. and Samal, S.K. 2001. High level expression of a foreign gene from the most 3' – proximal locus of a recombinant Newcastle disease virus. *J. Gen. Virol.* 82: 1729-1736.
- Janke, M., Peeters, B. and de Leeuw, O. 2007. Recombinant Newcastle disease virus (NDV) with inserted gene coding for GM-CSF as a new vector for cancer immunogene therapy. *Gene Therapy.* 14: 1639–1649.
- Jayawardane, G. W. and Spradbrow, P. B. 2000. Oral vaccination of chickens against Newcastle disease with V4 vaccine delivered on processed rice grains.” *Australian Vet. J.* 67: 364-369.
- Jinying Ge, Guohua Deng, Zhiyuan Wen, Zhigao Bu, and Hualan Chen. 2007. Newcastle disease virus-based live attenuated vaccine completely protects chickens and mice from lethal challenge of homologous and heterologous H5N1 Avian Influenza Viruses. *J Virol.* 81: 150–158.
- Kirubaharan, J. J., Velmurgan, R., Kumanan, K. and Albert, A. 2000. Inactivated vaccines against Newcastle disease virus and humoral immune response. *Indian. Vet.J.* 77:563
- Koppad, S., Dhinakar Raj, G., Gopinath, V.P., John Kirubaharan, J., Thangavelu, A and Thiagarajan, D. 2010. Calcium phosphate coupled newcastle disease vaccine elicited humoral and cell mediated immune response in chickens. *Res. Vet.Sci.* 68: 22-35.
- Kumar, K.U., Swamy, S. K. and Reddy. T.V. 1988. Humoral and cell mediated immune response in chicks vaccinated against Newcastle disease. *Kerala. J.Vet.Sci.* 19: 116-121.
- Kylasamaier. 1931. A study on madras fowl past. *Ind. Vet. j.* 8: 346-352.
- Madhvan, C., Roy, S., Rai, A. and Kataria, J.M. 2006. Antibody detection based differential ELISA for Newcastle disease virus. *J. Virol. Met.* 138: 117-122.
- Makkay A. M., P. J. Crelll and E. Nagy, 1999. Antibody detection – based on differential ELISA for NDV-infected or Vaccinated versus NDV HN- subunit vaccinated chickens. *Vet. Microbiol.* 66: 209-222.
- Rivetz, B., Weinsman, Y. and Herzberg, Y. 1985. Evaluation of novel rapid kit for visual detection of NDV antibodies. *Avian Dis.* 29: 929-942.
- Robin, W.M., Gelb, J.J., Schreurs, C.S. and Lutticken, D. 1992. Protection of chicken from Newcastle and Mareks disease with a recombinant herpesvirus of turkeys vaccine expressing the Newcastle disease virus fusion and HN proteins. *Avian.Dis.* 28: 65-69.
- Roy, P., Venugopalan, A. T. and Dhillon, A. S. 2003. Efficacy of two commercial Newcastle disease virus lentogenic vaccines against virulent Asiatic type Newcastle disease viruses. *J. Appl.Poult.Res.* 12: 169-173.
- Sakaguchi, M., Nakamura, H., Sonoda, K., Hamada, F. and Hirai, K. 1996. Protection of chickens from

- Newcastle disease by vaccination with a linear plasmid DNA expressing the F protein of Newcastle disease virus. *Vaccine*. 14: 747-752.
- Selvan N., Ranjit Singh., Amirtha J., Sukumar, A. S., Jeyakumar, N. and Suryanarayana, V. V. S. 2010. Production and application of recombinant haemagglutinin neuraminidase of Newcastle disease virus. *Asian Pacific J. Trop. Med.* 3: 629-632.
- Vegad, G.L., Bhindwale, S., Mishra, B. and Sharma, R. 2008. LaSota vaccine for Newcastle disease: observations from India on its adverse effects due to complicating pathogens. *World Poultry Science Association*. 64: 401-409
- Warzecha, H. and Mason, S.H. 2003. Benefits and risks of antibody and vaccines production in transgenic plants. *J.Plant Physiol.* 160: 755-764
- Yang, Z.Q., Liu, Q.Q., Pan, Z.M., Yu, H.X. and Jiao, X.A. 2007. Expression of the fusion glycoproteins of Newcastle disease virus in transgenic rice and its immunogenicity in mice. *Vaccine*. 25: 591-598.
- Young, J.K., Li, D. and Morrison, T. G. 1999. Interaction of peptides with sequences from the Newcastle disease virus fusion protein heptad repeat region. *J. Virol.*73: 5945-5956.
- Yushoff, K., Millar, N. S., Tey, B. T., Chambers, P. and Emmerson, P.T. 1997. Determination of the 3' terminal sequence of the HN gene of Newcastle disease virus isolates by direct nucleotide sequencing. *Asia-Pacific J Mol. Bio. Biotech.* 5: 48-50.
- Zhoa, H., Janke, M., Fournier, P and Schirmacher, V. 2008. Recombinant Newcastle disease virus expressing human interleukin-2 serves as a potential candidate for tumor therapy. *Virus Res.* 136: 75-80.
- Zhuhui, H., Krishnamurthy, S., Aruna, P. and Samal, S.K. 2004. Newcastle disease virus V protein is associated with viral pathogenesis and functions as an alpha interferon antagonist. *J. Virol.* 77: 8676-85.