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Selecting the Best Combination of Adjuvant and the Delivery Vehicle for Optimizing the Th1 and Th2 Biased Responses of NDV Live Vaccine in Indigenous Aseel Chicken

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ABSTRACT

Background: This study aimed at the evaluation of the efficacy of TLR ligands (*viz*. Poly I:C, Lipopolysaccharide and CpG:ODN) as vaccine adjuvants in combination with a suitable carrier (oil-in-water emulsion, normal saline solution, or alum) by studying the expression profile of cytokines belonging to Th1 and Th2 biased immune systems.

Methods: Whole blood was collected from 108 birds that were divided into 9 different experimental groups: 3 TLR ligands each with 3 delivery vehicles. Th1 (IFN-γ) and Th2 (IL4) biased cytokines were detected by qPCR followed by ELISA. Both the results showed the highest level of IL4 (Th1 biased) with LPS and the highest level of IFNγ (Th1 biased) with CpG-ODN. Uneven TLR expression was detected from day 3 onwards till day 21 but ligands and delivery vehicles administered along with NDV vaccine did not show significant influence on the immune response.

Result: It is evident that the TLR-3, 4 and 9 expressions increase following exposure to the respective ligands. Hence, TLR ligands can be used as reliable adjuvants in NDV vaccine. The expression of the Th2 biased cytokine IL4 mRNA and Th1 biased cytokine Interferon-gamma was found to be the highest on day 3 in the LPS+NSS group and on day 21 in CpG-ODN+Alum group, respectively. The findings of the real-time PCR were supported to some by the ELISA.

Key words: Cytokines, Ligands, NDV, TLR, Vaccine.

INTRODUCTION

The poultry industry constitutes a major sector of world agriculture. However, several infectious diseases (*viz*. Newcastle disease (ND), infectious bursal disease (IBD), *etc*.) shake the poultry industry with huge economic losses. Hence, such infectious diseases must be handled with immediate concern. Many viral diseases, such as Newcastle disease, require a strong cell-mediated immune response apart from the humoral response (Khalifeh *et al* 2009). To combat infectious diseases systemic response through initial inflammatory and antigen-specific immune responses and Toll-like receptors are very critical (Beutler *et al* 2007).

Vaccination against a viral disease is known to elicit an immunological response against the virus in such a way that it does not cause the disease. The simplest way to do this is to take the virus, kill it by physical or chemical treatment and then inject it into the bird as an inactivated vaccine. A formulation that is designed to enhance the efficacy of a vaccine is usually referred to as an “adjuvant”, after the Latin verb “*adjuvare*” - which means ‘to help’ (Alexander 1997). Many compounds have been described as adjuvants and the term has become a catch-all for a gamut of immune potentiating activities that range from acting as a concentrating depot of a vaccine in the host to a compound that specifically affects the quality and quantity of the immune response by directly acting upon cells of the immune system (Elston *et al* 1998). The most prevalent adjuvants found in licensed veterinary vaccines are aluminum salts and oil emulsions (oil in water emulsions, or water in oil emulsions). All of these formulations are primarily considered to act as depots for injected vaccines. The inactivated vaccines which are based on emulsions formed with mineral oil can cause a serious localized reaction. These usually require incision and washing. In Asian countries, mesogenic strains, for example, Mukteswar of the ND virus are used and can be administered by injection only. This vaccine should be used in birds over eight weeks of age and following primary vaccination with a lentogenic strain such as F strain (Giambrone 1985).

The pathogen-derived molecules (*viz*. PAMPs or DAMPs) bind to different immune sensors in hosts, *i.e*. various pathogen recognition receptors (PRRs). Toll-like receptors (TLR) and nucleotide-binding oligomerization domain-like receptors (NLR) are the important PRRs that activate innate immune pathways and provide us with potential targets for novel adjuvant development as well as enlighten us on an understanding of how current vaccines and adjuvants work. TLR recognize unique patterns of

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individual pathogen-associated molecular patterns (PAMP) and signal to induce the production of pro-inflammatory cytokines, thereby resulting in the activation of innate and adaptive immunity (Takeda *et al* 2003). It is well known that TLRs are crucial in triggering both innate and adaptive immunity and their involvement in immunostimulatory activities defines their ligands as powerful vaccine adjuvants.

Aseel is a popular indigenous breed of game-bird of South-Eastern Asia. However, very limited research has been directed towards exploring the systems biology associated with disease resistance of the Aseel breed. Besides, the use of TLR-ligands as vaccine adjuvants has not been studied in indigenous chicken breeds. The current work aims at identifying the best combination of 'TLR-ligand as vaccine adjuvant in combination with the 'suitable delivery vehicle' to elicit enhanced Th1 and/or Th2 biased immune response against Newcastle disease virus in indigenous Aseel chicken. Besides, the study also attempts to measure the impact of the alum-based as well as emulsion-based delivery systems to be used with the TLR ligands on immune response against/concerning Newcastle disease vaccination.

MATERIALS AND METHODS

Design of experiment

108 day-old non-vaccinated chicks (Aseel breed, representing both sexes) were procured from Poultry Farm, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU) from the period of April to May. The chicks were randomly divided into 9 groups (Table 1): 3 TLR-ligands as vaccine adjuvants (Poly I:C, LPS and CpG-ODN) each with 3 delivery vehicles (alum, oil-in-water and normal saline solution). Intramuscular (i/m) NDV vaccine of *LaSota* strain was given to all the birds (Sub-Groups- I to IX) on day 1. along with their ligands (Poly I:C, LPS and CPG:ODN of TLR-3, TLR-4 and TLR-9, respectively) (Table 1).

Blood collection

Peripheral blood sample (0.25-0.5 ml) was collected using a tuberculin syringe (Dispovan) from wing veins of chicks on day-0 (before vaccination), day-3, -7, -14, -21 and -35 (post-vaccination). The blood was immediately subdivided into two tubes with anticoagulant (0.5M EDTA; for total RNA extraction) and without anticoagulant (for serum preparation for ELISA).

RNA extraction and cDNA synthesis

RNA isolation was done from freshly collected samples with trizol reagent (Ambion), after the required modification of the protocol given by Sambrook and Russell (2001). The optical density of the extracted RNA was measured in a Nanodrop spectrophotometer (Thermo, USA) at wavelengths of 260 nm and 280 nm. Total RNA (1500 ng) extracted was reverse transcribed into cDNA using High Capacity Reverse Transcriptase Kit (Applied Biosystem) (Table 2). These components were mixed properly, briefly centrifuged and the mixture was set in PCR for reverse transcribing mRNA into cDNA as described in Table 3. The cDNA synthesis reaction cycle consisted of one step each of Incubation (25°C × 10 minutes), Reverse Transcription (37°C × 120 minutes) and Stopping the reaction (85°C × 5 minutes).

Real-time PCR

The mRNA expression of Th1 cytokines (IFN-gamma), Th2 cytokines (IL4) and TLR-genes (TLR-3, -4 and -9) was quantified across 9 experimental groups keeping beta-actin as an endogenous control. The real-time PCR was performed using SYBR Green chemistry, with 2 biological replicates and 2 technical replicates for each group under study. The detail of the SYBR-Green primer pairs has been tabulated in Table 3.

ELISA of serum-Cytokines

Chicken serum samples were collected from all the birds from day0 (before vaccination) and day-3, -7, -14, -21 and -35 (post-vaccination) and maintained at -20°C. The amount of IFN-γ and IL4 in each serum sample was measured using IFN-γ and IL4 ELISA kit reagents (IFN-γ and IL4 Chicken Antibody Kit, Cusabio Biotech Co. Ltd.) according to the manufacturer's instructions (Su *et al.,* 2011).

HA-HI test

The HI antibody was measured in the microfilter plates as described by Su *et al*. (2011). Briefly, 25ul of a series two-fold diluted serum was mixed with an equal volume of NDV (LaSota strain) that contained eight hemagglutinating units. The mixtures were incubated at 37°C for 1 hour. A volume of 25 ul of 0.5% chicken red blood cells was added to each well. The mixture was incubated at 25°C for an additional 45 min. The HI antibody titers were reciprocal to the serum dilution that was sufficient to inhibit NDV-induced hemagglutination. The HI titers were expressed as log base 2.

Table 1: Detail of experimental groups, number of experimental birds and description of each group.

Groups	A			B			C		
	Poly I:C (36 Birds)			LPS (36 Birds)			CpG-ODN (36 Birds)		
Sub-groups	I	II	III	IV	V	VI	VII	VIII	IX
Delivery Vehicles	TLR3+ Alum-Based	TLR3+ Oil in water-based	TLR3+ NSS	TLR4+ Alum-Based	TLR4+ Oil in water-based	TLR4+ NSS	TLR9+ Alum-Based	TLR9+ Oil in water-based	TLR9+ NSS

Statistical analysis

The qPCR analysis of the available data from all the blood samples was done using the ΔΔCt method (Livak and Schmittgen 2001). The target Ct values were normalized against the endogenous control Ct and ΔΔCt was calibrated by taking the NSS group (Control) as calibrator. Later, fold change was determined from the "2^Mean ΔΔCt" value, along with their respective Standard error ($SE_{\Delta\Delta Ct}$). Graphs were plotted to study the relative expression of the Gene/ cytokine to the days and groups interaction.

RESULTS AND DISCUSSION

Proteins or peptides that play a major role in immune as well as inflammatory responses *via* activation and regulation of other cells and tissues are known as cytokines. In comparison to other species, avian cytokines have been poorly defined, both in terms of structure and function. However, in recent years advances in avian immunology and genetics have lead to the discovery of a range of cytokines mainly in the chicken, but also the turkey and other avian species. The following results were obtained from this study:

Expression profiling of Th1 and Th2 biased genes

IL4 is a Th2 biased cytokine that is mainly secreted during humoral immune response. The expression of IL4 at mRNA level (detected by real-time PCR) was found to be the highest on day 3 in group LPS+NSS (P<0.05) as compared to that of other experimental groups (Fig 1). The serum (ELISA) could detect the highest level of IL4 protein on day 7 in group LPS+NSS and a non-significant (P>0.05) increase on day 14 in group Poly I:C+Alum, LPS+Alum and LPS+Oil in water as compared to 0 days (Fig 2). Upon activation by IL4, Th2 cells subsequently produce additional IL4 in a positive feedback loop. IL4 is also a characteristic TH2 biased cytokine that induces differentiation of naïve helper T-cells (Th0 cells) to Th2 cells. The cell that initially produces IL-4, thus inducing Th0 differentiation, has not been identified, but recent studies suggest that basophils may be effector cells (Sokol *et al* 2008). The fold-change values of the IL4 gene were also less as compared to the Th1 biased cytokine genes. In one of the studies, the main findings were that cytokine gene expression was significantly lower in the spleen of chick embryos compared with post-hatch chicks, particularly D7 chicks. Significant expression of cytokine genes in the spleen of D7 chickens may be an indicator of the acquisition of the functional ability of the spleen as a secondary lymphoid organ (Abdul-Careem 2007).

IFNγ, a Th1 biased cytokine released from dendritic cells, leads to the elicitation of CMI response. The expression of IFNγ at mRNA was found to be the highest on day 21 in group CpG-ODN+Alum but no significant difference (P>0.05) was seen in other days and other groups. There was a similar expression of all the three adjuvants with different delivery vehicles (Fig 3). Comparatively, the ELISA results indiacted that day 21 elicited high (if not the highest of all the experimental groups) in all the subgroups (considering the TLR-ligand adjuvants and the delivery vehicles). Here, the treatment groups did not show much variation in expression of IFNγ cytokine and ELISA could detect the highest level of IFNg on day 3 in CpG-ODN+NSS and a slight increase on day-7 (in Poly I:C with all the combinations and in CpG-ODN in oil in water) and on day-14 (in CpG-ODN with all the

Table 2: cDNA synthesis reaction.

Components	Volume
10X RT buffer	2 µl
dNTP mix	0.8 µl
RT primers	2 µl
RT enzyme	1 µl
Nuclease-free water	4.2 µl
Sub-total	10 µl
RNA mix	10 µl
Total	20 µl

Table 3: Detail of the primer-pairs used for real-time PCR relative quantification using SYBR-Green chemistry.

Oligo Name	Primer sequence	Length	Template seq*	Amplicon-size	Tm	GC%
IFNγ-F	AAGTCAAAGCCGCACATCAAAC	22	X99774.1	132	60.54	45.45
IFNγ-R	CTGGATTCTCAAGTCGTTCATCG	23			59.45	47.83
IL-4-F	AATGACATCCAGGGAGAGGTTTC	23	AJ621249.1	100	60.05	47.83
IL-4-R	AGGCTTTGCATAAGAGCTCAGTTT	24			61.05	41.67
TLR-3-F	AGACACAGCAATTCAGAAC	19	NM_001011691	198	53.20	42.11
TLR-3-R	TTAATGATGTTATTATCCTCCAAG	24			52.38	29.17
TLR-4-F	CCATCCACTCAGACAACCTTTCC	23	AY064697.1	219	61.12	52.17
TLR-4-R	AGTAAACGCAGCAGCACAAG	20			59.41	50.00
TLR-9-F	AGCTGGAGCTGTTGGACCTA	20	NM 001030558	148	60.55	55.00
TLR-9-R	TTCACGTGCCATAGCATCTC	20			58.06	50.00
Ch_ActB-F	TTTGGCGCTTGACTCAGGAT	20	L08165.1	202	59.96	50.00
Ch_ActB-R	GCCTTCACAGAGGCGAGTAA	20			59.75	55.00

*NCBI Nucleotide accession numbers of the coding sequences of the target genes from which the primers have been designed using NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

combinations) as compared to day-0 (Fig 4). IFNγ is secreted by T helper cells, cytotoxic T cells (T_C cells), macrophages, mucosal epithelial cells and NK cells. IFNγ has antiviral, immunoregulatory and anti-tumor properties. Hsieh *et al* (1993) predicted that the involvement of IL12 in converting naïve T helper cells into Th1 biased cells and IL12 inhibiting causes Th1 suppression. Further, incorporation of IL-18 into attenuated vaccine strains such as the 9R vaccine (Smith 1956) could cause an increased Th1-type response in turn leading to the production of IFN-γ and macrophage activation, with subsequent clearance of *Salmonella* from macrophages. A possible problem with such an approach

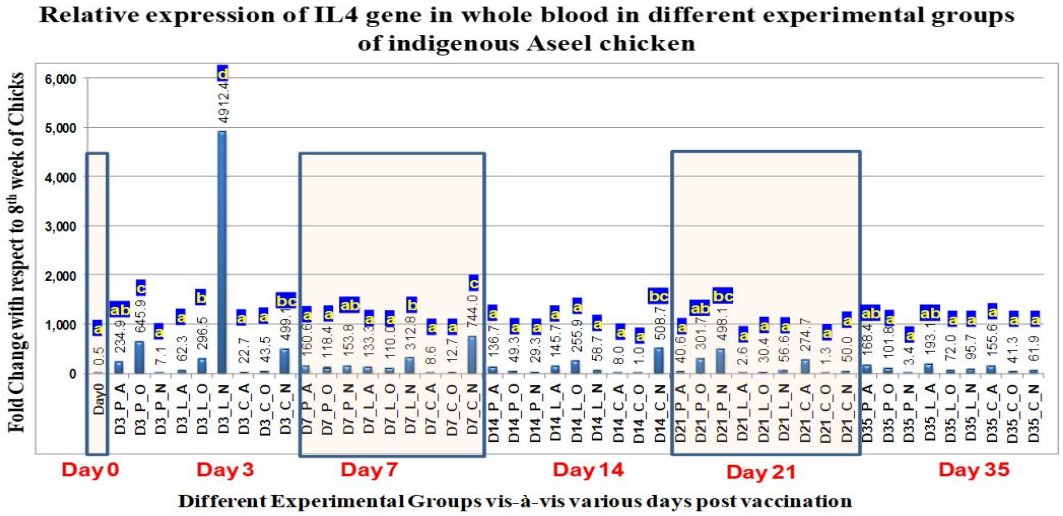

Fig 1: Relative expression of IL4 cytokine gene in whole blood of indigenous chicken over a period of 35 days. Significant difference among fold change is shown with different symbols, determined by post-hoc analysis of the dCt values of IL4 gene under study. Each bar indicates respective standard error (SE).

a,b,c, No common superscript indicates significant differences (P<0.05) between the fold changes of the experimental groups.

- D3, D7, D14, D21 and D35 stands for 3, 7, 14, 21 and 35 days post-vaccination.
- P, L and C indicates for Poly I:C (TLR3-ligand), Lipopolysaccharide (TLR4-ligand) and CpG-ODN (TLR9 ligand).
- A, O and N denotes Alum, Oil-in-water and normal saline solution delivery vehicles of NDV vaccine.

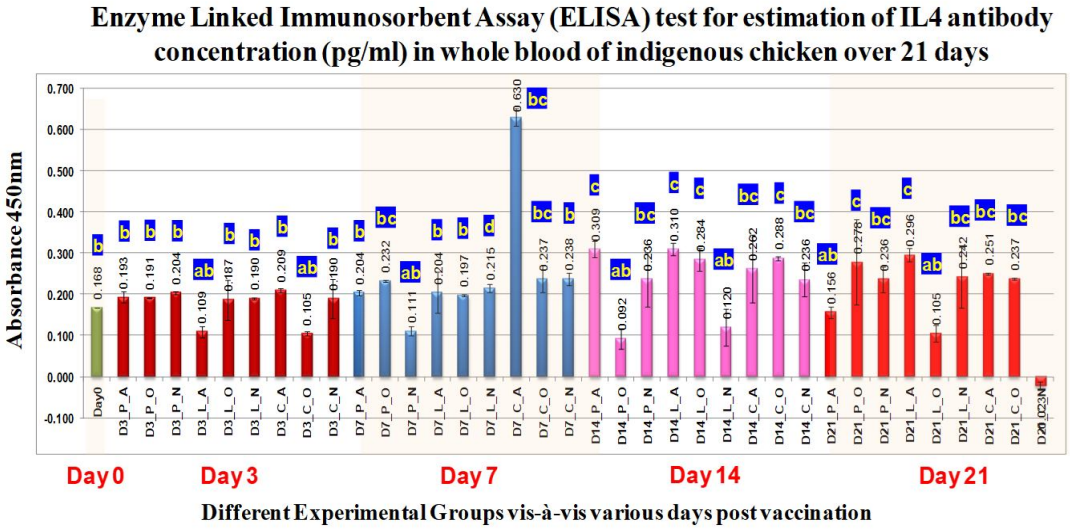

Fig 2: Enzyme Linked Immunosorbent Assay (ELISA) test was done for estimation of IL4 antibody concentration (pg/ml) in whole blood of indigenous chicken over 21 days. Each bar represents respective standard error (SE).

a,b,c, No common superscript indicates significant differences (P<0.05) between the fold changes of the experimental groups.

- D3, D7, D14 and D21 stands for 3, 7, 14 and 21 days post-vaccination.
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- A, O and N denotes Alum, Oil-in-water and normal saline solution delivery vehicles of NDV vaccine.

is that interference from a protective antibody response may occur, reducing the long-term efficacy of the vaccine (Sundick and Gill-Dixon 1997).

TLR expression pattern

The mRNA expression of the TLR3 gene was quantified across 9 different groups keeping beta-actin as endogenous control and across 6 different days (day-0, -3, -7, -14, -21 and -35). There was a significant (P<0.05) increase in TLR3 expression in the groups that were injected with oil in water emulsion was given. Poly I:C recognition has been very well studied in TLR3 expression (Alexopoulou *et al* 2001). The significant (P<0.05) increase in TLR3 gene expression was only observed in the groups that were injected with oil in water emulsion. The expression

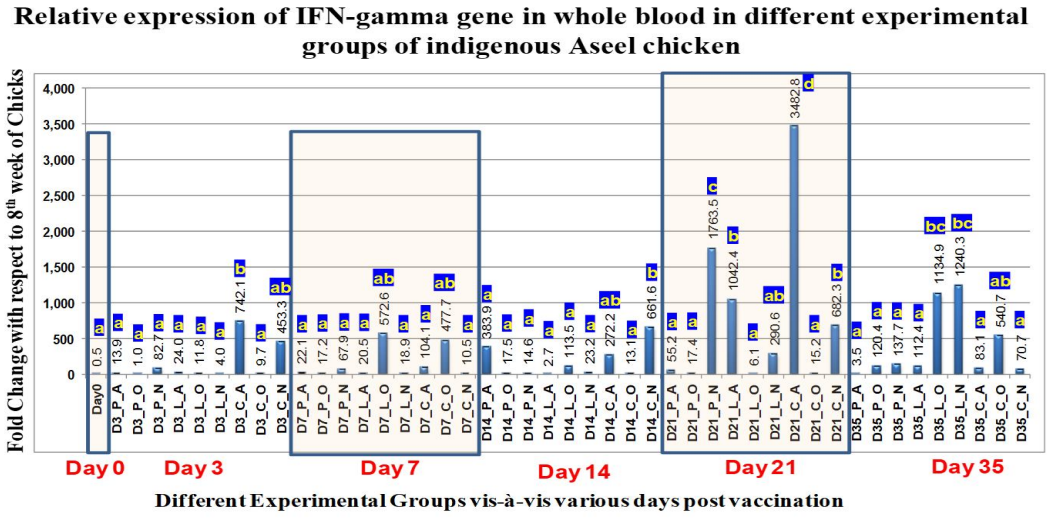

Fig 3: Relative expression of IFN-γ cytokine gene in whole blood of indigenous chicken over a period of 35 days. Significant difference among fold change is shown with different symbols, determined by post-hoc analysis of the dCt values of IFN-γ gene under study. Each bar indicates respective standard error (SE).

a,b,c, No common superscript indicates significant differences (P<0.05) between the fold changes of the experimental groups.

- D3, D7, D14, D21 and D35 stands for 3, 7, 14, 21 and 35 days post-vaccination.
- P, L and C indicates for Poly I:C (TLR3-ligand), Lipopolysaccharide (TLR4-ligand) and CpG-ODN (TLR9 ligand).
- A, O and N denotes Alum, Oil-in-water and normal saline solution delivery vehicles of NDV vaccine.

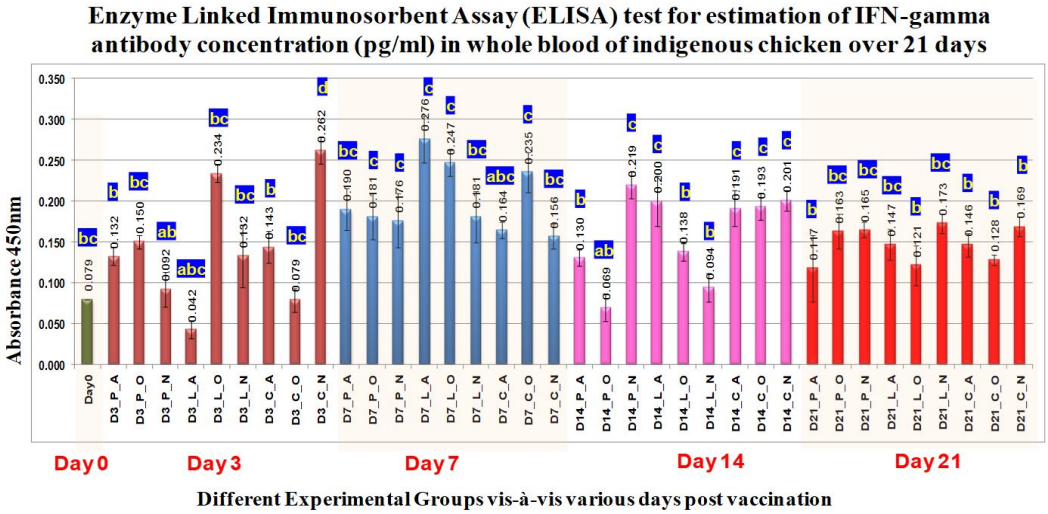

Fig 4: Enzyme Linked Immunosorbent Assay (ELISA) test was done for estimation of IFN-γ antibody concentration (pg/ml) in whole blood of indigenous chicken over 21 days. Each bar represents respective standard error (SE).

a,b,c, No common superscript indicates significant differences (P<0.05) between the fold changes of the experimental groups.

- D3, D7, D14 and D21 stands for 3, 7, 14 and 21 days post-vaccination.
- P, L and C indicates for Poly I:C (TLR3-ligand), Lipopolysaccharide (TLR4-ligand) and CpG-ODN (TLR9 ligand).
- A, O and N denotes Alum, Oil-in-water and normal saline solution delivery vehicles of NDV vaccine.

pattern observed in (Fig 5) shows an increase in gene expression on day 7 with oil in water emulsion in combination with CpG-ODN. The fold change later decreased rapidly after day 14 till day 35, as TLR3 is an innate immune response element and its expression is observed relatively early as shown in (Fig 5).

The expression of TLR4 was also checked at the mRNA level. A significant increase in TLR4 gene expression was observed in CpG-ODN in combination with oil in water emulsion. The expression pattern observed in (Fig 6) shows an uneven pattern of expression from day 0 till day 21 in the group in which delivery vehicles were given. The increased expression of TLR4 was observed in oil in water emulsion irrespective of delivery vehicles. TLR ligands like flagellin have been used as a recombinant fusion protein with the protective antigenic protein against AI as well as in DNA

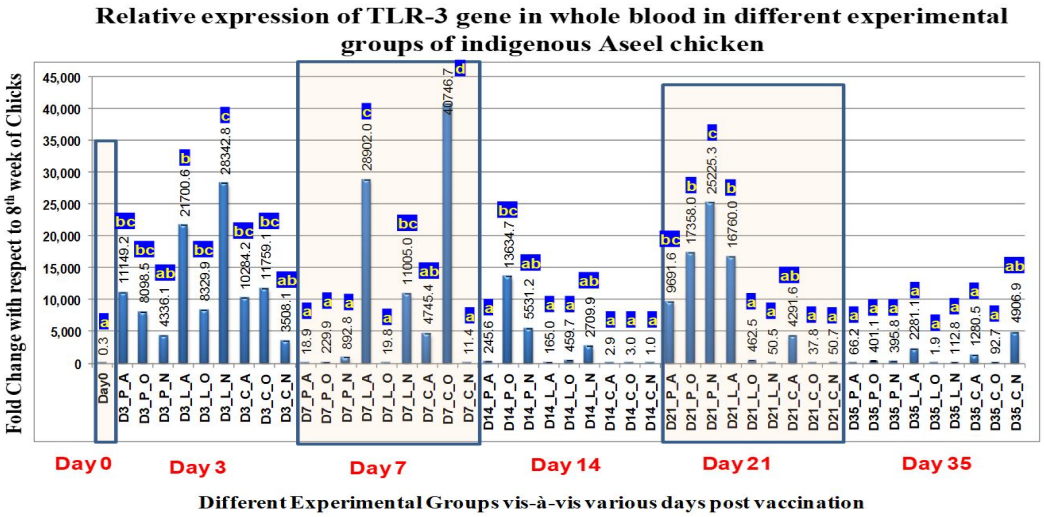

Fig 5: Relative expression of TLR3 gene in whole blood of indigenous chicken over a period of 35 days. Significant difference among fold change is shown with different symbols, determined by post-hoc analysis of the dCt values of TLR3 gene under study. Each bar indicates respective standard error (SE).

- D3, D7, D14, D21 and D35 stands for 3, 7, 14, 21 and 35 days post-vaccination.
- P, L and C indicates for Poly I:C (TLR3-ligand), Lipopolysaccharide (TLR4-ligand) and CpG-ODN (TLR9 ligand).
- A, O and N denotes Alum, Oil-in-water and normal saline solution delivery vehicles of NDV vaccine.

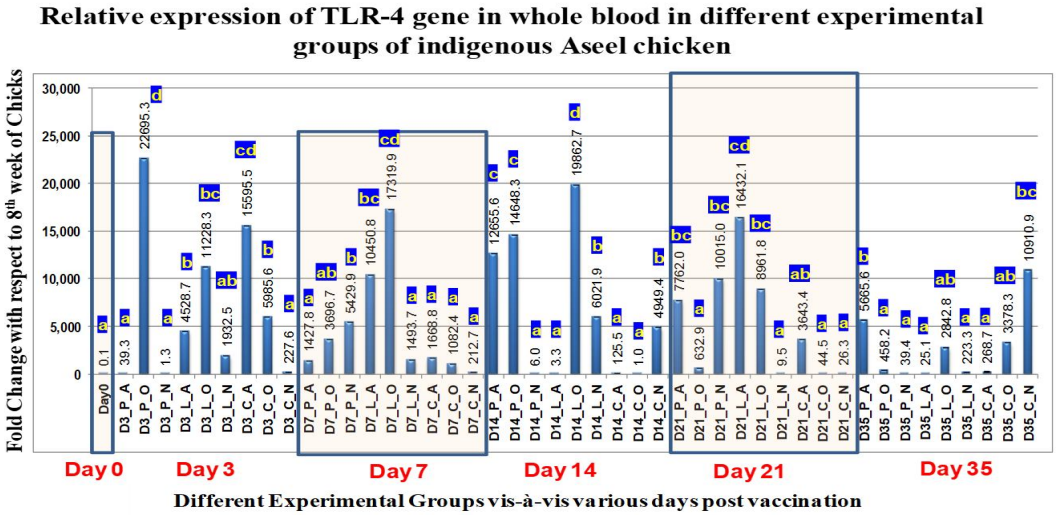

Fig 6: Relative expression of TLR4 gene in whole blood of indigenous chicken over a period of 35 days. Significant difference among fold change is shown with different symbols, determined by post-hoc analysis of the dCt values of TLR4 gene under study. Each bar indicates respective standard error (SE).

a,b,c, No common superscript indicates significant differences (P<0.05) between the fold changes of the experimental groups.

- D3, D7, D14, D21 and D35 stands for 3, 7, 14, 21 and 35 days post-vaccination.
- P, L and C indicates for Poly I:C (TLR3-ligand), Lipopolysaccharide (TLR4-ligand) and CpG-ODN (TLR9 ligand).
- A, O and N denotes Alum, Oil-in-water and normal saline solution delivery vehicles of NDV vaccine.

vaccination against AI and IBD. With the advent of virulent strains, present vaccines for the MD are becoming less effective and in such a scenario, a TLR3 ligand, poly I:C-based HVT vaccines have shown vast potential and will be of great interest in more such future investigations (Ranjith-Kumar *et al* 2008).

TLR4 (LPS), when administered with different delivery vehicles in combination with the Newcastle disease vaccine, provides an early as well as efficient immune response not only against the Newcastle disease but also against gram-negative bacteria as it induces significant TLR4 expression. Thus, LPS in combination with the Newcastle disease vaccine provides combinatorial protection against both Newcastle disease virus and also to other gram-negative bacterial infections. A recent study conducted (Abu-Baker and Masoud 2016) predicting the importance of LPS based vaccines in providing high cross-protection against various strains of the pathogen in mice.

The best TLR9 expression is characterized by CpG-ODN and therefore its expression is also checked at mRNA level. CpG- motifs recognition has been very well studied in TLR9 expression (Han *et al* 2014). There was nonsignificant variation in the TLR9 expression, but a slight increase in expression was seen during day 3 (in group LPS+NSS), day 7 (in group Poly I:C+NSS and CpG-ODN+NSS) till day 14 (LPS+Alum) (Fig 7).

Affirmation of Th1 and Th2 biased genes by ELISA

The expression of IL4 (Th2 biased) at mRNA level (Real-time PCR) was found to be highest on day 3 in group LPS+NSS but no significant difference was seen in other days and other groups. The detection by serum (ELISA) could detect the highest level of IL4 protein on day 7 in group LPS+NSS and a slight increase on day 14 in group Poly I:C+Alum, LPS+Alum and LPS+Oil in water as compared to day-0s. The expression of IFNγ (Th1 biased) at mRNA level (Real-time PCR) was found to be highest on day 21 in group CpG-ODN+Alum but no significant difference was seen in other days and other groups.

There was a similar expression of all the three adjuvants with different delivery vehicles. Treatment groups did not show much expression of IFNγ cytokine and ELISA could detect the highest level of IFNg on day 3 in CpG-ODN+NSS and slight increase on day 7 (in Poly I:C with all the combinations and in CpG-ODN in oil in water) and on day 14 (in CpG-ODN with all the combinations) as compared to day 0. ELISA and qPCR showed the highest level of IL4 (Th2 biased) with LPS and the highest level of IFNγ (Th1 biased) with CpG-ODN.

Haemagglutination inhibition test (HI)

The haemagglutination inhibition test is a gold standard technique to find out the antibody titer for all the viral disease and it also provides an edge over the other techniques to differentiate between the vaccinated from non-vaccinated samples. The hemagglutination-inhibition (HI) test is used to detect and quantitate serotype-specific antibodies to avian Newcastle disease (NDV) in serum, plasma, or yolk following infection or vaccination. The basis of the HI test is that hemagglutination (HA) occurs when hemagglutinins on the virus envelope interact with receptors on the surface of erythrocytes and inhibition of HA occurs in the presence of serotype-specific antibodies in serum, plasma, or yolk.

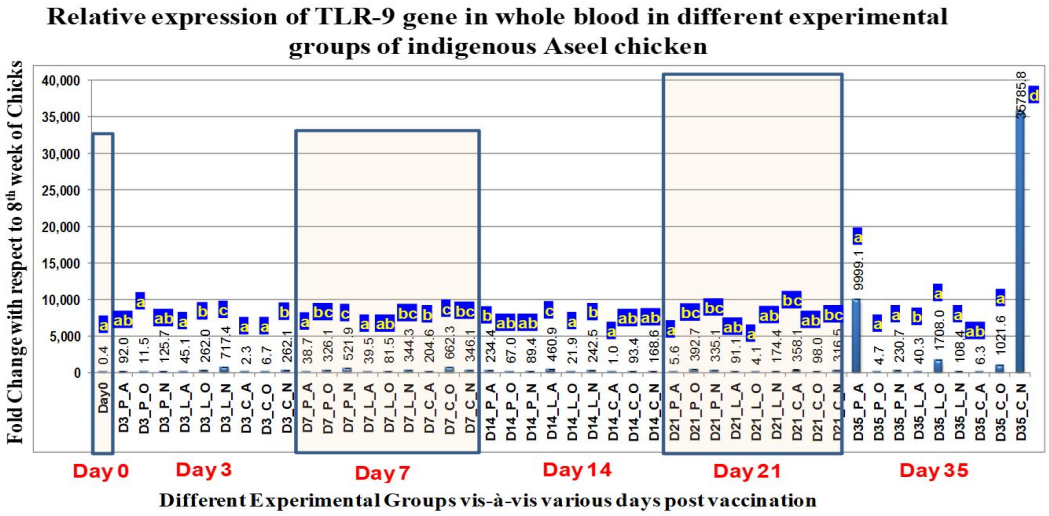

Fig 7: Relative expression of TLR9 gene in whole blood of indigenous chicken over a period of 35 days. Significant difference among fold change is shown with different symbols, determined by post-hoc analysis of the dCt values of TLR9 gene under study. Each bar indicates respective standard error (SE).

a,b,c, No common superscript indicates significant differences (P<0.05) between the fold changes of the experimental groups.

- D3, D7, D14, D21 and D35 stands for 3, 7, 14, 21 and 35 days post-vaccination.
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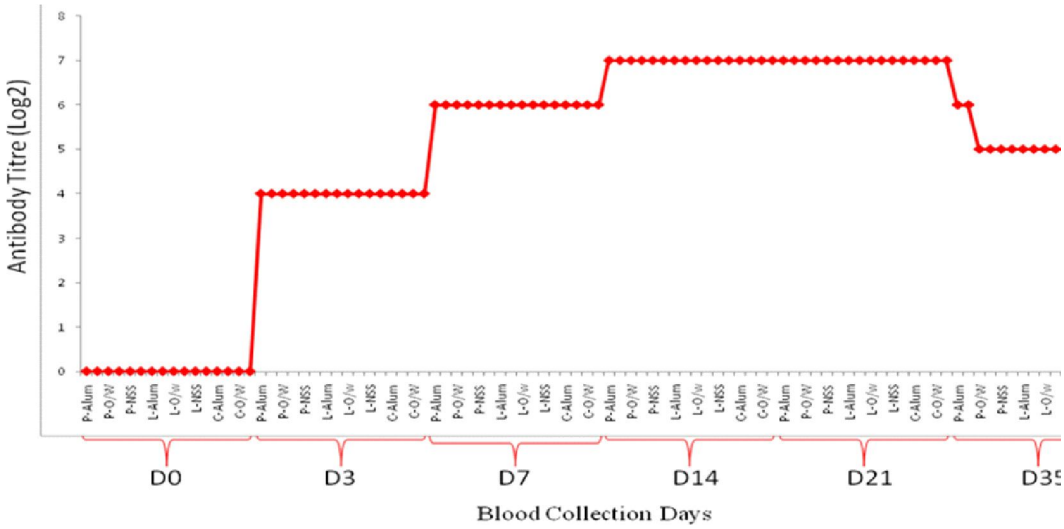

Fig 8: Haemagglutination inhibition test indicating the antibody titer (log_2 scale) for the experimental groups at different interval of days post blood collection.

➢ D0, D3, D7, D14, D21 and D35 stands for 0th day (pre-vaccination) and 3, 7, 14, 21 and 35 days post-vaccination.

The log-transformed antibody titer values were plotted against blood collection days for HI results (Fig 8). The graph showed that immune response against NDV was detected from day 3 onwards and the peak was at day 14 and day 21, but ligands and delivery vehicles administered along with NDV vaccine did not show significant influence on immune response against NDV.

CONCLUSION

The results obtained suggest that serum and plasma both can be used in serodiagnostic haemagglutination inhibition assays although serum titers were generally higher than corresponding plasma titers (Defang *et al* 2012). The various molecular techniques were compared for the best detection of infectious bursal disease virus (IBDV). The conventional agarose gel-based PCR and ELISA were compared with the SYBR Green real-time PCR. Though the results for IBDV detection favored the SYBR Green real-time PCR technique, it was redundant for the detection of RNA viruses such as NDV (Aini *et al.,* 2008). Therefore, in this study, the NDV specific antibody titer in serum was detected by the conventional HI test. Another report predicts the importance of haemagglutination inhibition test titer over the ELISA by showing better titer values with the survivability of mice infected with influenza virus as compared to that of IgG neutralization antibody (Lin *et al.,* 2013).

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