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G Senthilnathan
Ph.D. Scholar, Department of Veterinary Microbiology, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India

K Shoba
Professor and Head, Department of Veterinary Microbiology, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India

TV Meenambigai
Professor and Head, Vaccine Research centre- Viral Vaccines, Centre for Animal Health studies, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India

K Anbu Kumar
Assistant Professor, Bioinformatics and ARIS cell, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India

NR Senthil
Associate professor and Head, Department of Veterinary Public health and Epidemiology, Veterinary College and Research Institute, Tirunelveli, Tamil Nadu Veterinary and Animal Sciences University, Tamil Nadu, India

Corresponding Author:
G Senthilnathan
Ph.D. Scholar, Department of Veterinary Microbiology, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India

Multivalent DNA vaccine expressing the glycoproteins and tegument protein of infectious laryngotracheitis virus elicits robust humoral and cellular immune response in chickens

G Senthilnathan, K Shoba, TV Meenambigai, K Anbu Kumar and NR Senthil

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Abstract
Infectious laryngotracheitis poses a severe threat to chickens causing huge economic loss to poultry industry globally. DNA vaccination is a novel approach to induce protective immunity to infectious diseases. The immunogenicity of multivalent DNA vaccine against ILT virus was evaluated in chicken. Three expression plasmids targeting the glycoproteins and tegument protein of ILTV were constructed. Experimental chickens were immunized either with individual plasmids or as combination of all three plasmids (multivalent). A higher level of ILTV specific antibody response was detected in the chickens immunized with combination of three plasmids. The multivalent immunized group of birds showed augmented T cell response than the monovalent immunized groups. The Th1 type cytokines IFNγ and IL-2 were observed to be significantly upregulated in the multivalent immunization group in comparison with monovalent groups. This study showed clear picture of robust humoral and cellular immune response by multivalent DNA expressing the glycoproteins and tegument protein of ILTV.

Keywords: Infectious laryngotracheitis, Multivalent DNA vaccine, glycoproteins, tegument proteins, humoral immune response, cellular immune response

1. Introduction
Over the last few decades poultry farming in India has been transformed into a dynamic entity with more structured and intensified operations. In India, the production of eggs and poultry meat has been unfailingly increasing at the rate of 8 to 10% per annum against the 1.5 to 2% increase in agricultural crops [1]. The occurrence of unanticipated emerging and re-emerging diseases are the current challenges in poultry farming. Infectious laryngotracheitis (ILT) stands top in the ladder among the emerging respiratory pathogens of galliforms primarily infecting the chickens. It poses a severe threat to chickens disturbing the poultry welfare globally, causing huge economic loss since the bloom of intensive modern poultry industry [2]. The infectious laryngotracheitis is caused by Gallid alphaherpes virus 1 (GaHV-1) under genus Ilto virus, subfamily alphaherpesvirinae and family herpesviridae. The disease may occur either as severe acute form or as mild form under natural prevalence [3].
Universally vaccination has proven to be an efficient tool to control ILT in poultry [4]. Further, ILT claims the honour of first avian disease controlled by effective vaccination [5], but still today poses an important threat for poultry industry worldwide. Currently, there are two major types of commercial vaccines available worldwide; the live attenuated vaccines and viral vectored vaccines. The live attenuated vaccines have several limitations like residual virulence, spread from vaccinated to naive birds, reversion of virulence on bird-to-bird passage and establishment of latency [6]. Though the viral vectored vaccines are safer than live attenuated vaccines, thy fail to prevent shedding of challenge virus and have only partial protection [7]. DNA vaccination can be the better alternative to overcome the constraints of various types of ILT vaccines. The first attempt for DNA vaccines in poultry was directed against Avian influenza virus in 1993 [8]. Encouraging results have been declared by many researchers for DNA vaccines against various avian diseases like Infectious Bursal disease [9], Infectious bronchitis [10], Newcastle disease [11] and Mareks disease [12].

ILTV glycoproteins are immunogenic and responsible for stimulating humoral and cell mediated immune responses [13]. Glycoprotein C in herpesviruses has been shown to be a target for cellular and humoral immune responses, capable of inducing neutralizing antibodies and T-cell immune responses [14]. Glycoprotein D were found to be important for ILTV replication and elicits humoral and cell-mediated immune responses in the host [15]. The UL47 gene, which encodes a major tegument protein of ILTV is not essential for replication *in vitro*, but is required for virulence in chickens [16].

DNA vaccines could generate broad immune responses, similar to other live-modified virus platform, without the need for a replicating pathogen [17]. The multivalent DNA vaccine by inducing synergistic response represents an innovative approach for enhancing DNA vaccine potency [18]. In this background, a study was undertaken to develop Multivalent DNA vaccine comprising gC, gD and UL47 genes of Infectious laryngotracheitis virus and its immunogenicity was evaluated in chickens.

2. Materials and Methods

2.1. Birds, Virus and Cell culture system

The Chickens hatched from SPF eggs were housed in experimental poultry shed at Madras Veterinary college, TANUVAS and used for vaccination trial. Institutional Biosafety Committee and Institutional Animal Ethical Committee approval has been received for this trial. The SPF chicken eggs were used for isolation and further propagation of ILT virus. Infectious laryngotracheitis virus (ILTV) was isolated from samples collected from commercial poultry farms in Namakkal region of Tamil Nadu. ILTV was propagated in 10 days old egg by Chorio allantoic membrane route. The Chicken embryo fibroblast (CEF) cells prepared from 9-10 days old egg was cultured in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% foetal bovine serum (Gibco) and maintained at 37˚C with 5% CO_2.

2.2. Construction and screening of recombinant plasmids

The cloning primers were designed with *Hind*III and *Kpn*I restriction sites as overhangs (Table-1) and were used to amplify full-length sequence of gC, gD and UL47 genes. The pVAX1 vector and gC, gD and UL47 genes were digested by *Hind*III-HF and *Kpn*I-HF (New England biolabs) restriction enzymes and purified by QIAquick PCR purification kit (Qiagen). The three genes were cloned individually into pVAX1 vector between *Hind*III and *Kpn*I restriction sites. The pVAX1-gC, pVAX1-gD and pVAX1-UL47 recombinant plasmids were transformed in DH5α cells and recombinant colonies were screened on to LB Agar with Kanamycin (50 µg/ml) plates by colony PCR using vector screening primer. The recombinant colonies confirmed by colony PCR were sequenced and nucleotide sequence was verified. The presence and size of insert gene was also checked by insert release using *Hind*III and *Kpn*I restriction enzyme digestion.

2.3. *In vitro* expression of recombinant plasmids

All the three recombinant plasmids were transfected in CEF monolayer using Lipofectamine® LTX and PLUS™ reagent (Invitrogen) as per the manufacturer's instructions. The transfected cells were harvested after three days of incubation using Laemmli buffer (Biorad) and the expressed protein was resolved in SDS-PAGE. The resolved proteins were transblotted onto a nitrocellulose membrane using turbo trans-blot apparatus (Biorad) at a constant voltage of 25 V for 15 minutes and then the nitrocellulose membrane was subjected to immunoblotting with ILTV polyclonal antiserum to detect ILTV specific proteins.

2.4. Scaling up of recombinant plasmids

The recombinant plasmids pVAX1-gC, pVAX1-gD and pVAX1-UL47 were scaled up in DH5α *E. coli* cells using LB broth with kanamycin antibiotic. The plasmids were extracted from overnight grown *E. coli* cells by modified alkaline lysis method [19] with modifications. Precipitation by 5M $CaCl_2$ and 20% polyethylene glycol was followed for extraction of RNase free plasmid [20, 21].

2.5. Immunization of chickens with DNA vaccines

The 21 days old SPF chickens were divided into 6 groups (Table-2) and immunized as follows by intramuscular injection in chest muscle. Group I and II received 0.5 ml of PBS and 150 µg of empty pVAX1 vector respectively. Groups III to V received 150 µg of pVAX1-gC, pVAX1-gD and pVAX1-UL47 respectively. Group VI received 450 µg combined DNA vaccine containing 150 µg of each of three plasmids. All the chickens were administered with equivalent booster dose two weeks after primary vaccination. Blood samples for evaluation of immunogenicity was collected on 0-day and 3, 4, 5, 6 weeks after primary immunization.

Table 1: List of PCR primers designed and used in this study

Sl.no	Primer Name	Primer sequence	Product size
1.	gC CLON-FP	CAGAAAGCTTATGCAGCATCAGAGTACTGC	1242 bp
	gC CLON-RP	CAGAGGTACCGGACAGACAGTAGTGCACG	
2.	gD CLON-FP	CAGAAAGCTTATGCACCGTTCTCATCTCAG	1302 bp
	gD CLON-RP	CAGAGGTACCGAGGCGTGGCATGTAGG	
3.	UL47 CLON-FP	CAGAAAGCTTATGACCTTGCCCCATCGATT	1872 bp
	UL47 CLON-RP	CAGAGGTACCTCATATTTCCGATTTCCGGCG	

Table 2: Experimental design for vaccination trial

Sl.no	Group	Immunization details	No. of birds
1	Group I	Negative control (PBS)	6
2	Group II	Empty Vector control	6
3	Group III	Monovalent DNA Vaccine (gC gene)	10
4	Group IV	Monovalent DNA Vaccine (gD gene)	10
5	Group V	Monovalent DNA Vaccine (UL47 gene)	10
6	Group VI	Multivalent DNA Vaccine (gC, gD and UL47)	10

2.6. ILTV specific antibody response by Indirect ELISA

The ILTV specific antibody response to experimental vaccines were estimated using commercial indirect ELISA kit (BT Lab). The test sera was diluted as indicated by manufacturer, performed the test and optical density values were measured at 450 nm. As per the manufacturer's instructions the cutoff value has to be calculated by average negative control OD_{450nm} value plus 0.15. The OD_{450nm} value greater than or equal to cutoff value was considered positive and lesser than cutoff value was considered negative to ILTV antibody response.

2.7. Lymphocyte proliferation assay for CMI response

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood sample of each group by density gradient centrifugation using Histopaque® (Sigma). The PBMCs were adjusted to 1×10^7 cells per ml in RPMI 1640 medium (Gibco)

containing 10% FBS and seeded at the rate of 100µl per well in 96 well plate. After an overnight incubation, 50% of wells in each group were stimulated by adding 10 µl of ILTV (10^4 EID_{50}) and the remaining 50% of wells were added with PBS. After 72 hours of incubation, 10 µl of MTT dye (5mg/ml) was added to all wells and incubated for 4 hours at 37˚C. Then 150 µl of DMSO was added to all wells, incubated under dark for 4 hours and OD was measured at 570 nm. The stimulation index was calculated by dividing the mean optical density of stimulated wells by mean optical density of unstimulated cells.

2.8. Total RNA isolation and cDNA synthesis

Total RNA was isolated from the stimulated PBMCs of different vaccinated groups and control group using TRIzol™ reagent (Invitrogen). The RNA extracted was reversely transcribed using PrimeScript™ cDNA synthesis kit (Takara). The cDNA was diluted to 100 ng/µl working concentration for measurement of cytokines by RT-qPCR.

2.9. RT-qPCR and relative quantification of cytokine gene expression

The cytokine gene panel in this study included IFNγ and IL-2. Actin B was used as reference gene to normalize the samples. The RT-qPCR reaction was conducted with a total volume of 10 µl and the reaction mixture included SYBR green qPCR mater mix (Takara), 1 µM of each primer and 2 µl of diluted cDNA (100 ng/µl) [18]. The ΔC_t value of target gene was calculated by subtracting the C_t value of the reference gene from the C_t value of the target gene. The $\Delta\Delta C_t$ was calculated by using the formula $(C_{t\ Target} - C_{t\ Actin})_{Time\ x} - (C_{t\ Target} - C_{t\ Actin})_{Time\ 0}$, where time X is any point of time in treatment groups and time 0 is the expression of target gene normalized to Actin B. The fold change in gene expression between immunized and control group was calculated by using $2^{-\Delta\Delta Ct}$ algorithm [23].

3. Results

3.1. Construction of recombinant plasmids

The full-length gC, gD and UL47 genes of ILTV were successfully amplified by respective cloning primers (Fig-1A, 1B and 1C). These three genes which encode 414, 434 and 623 amino acids were cloned efficiently between HindIII and KpnI restriction sites of pVAX1 vector. The recombinant colonies were screened on LB agar with kanamycin antibiotic (Fig-2A, 2B and 2C). The colony PCR using vector screening primer confirmed the directional cloning of insert genes into vector (Fig-3A, 3B and 3C). The sequence data of recombinant plasmids also confirmed their nucleotide identity.

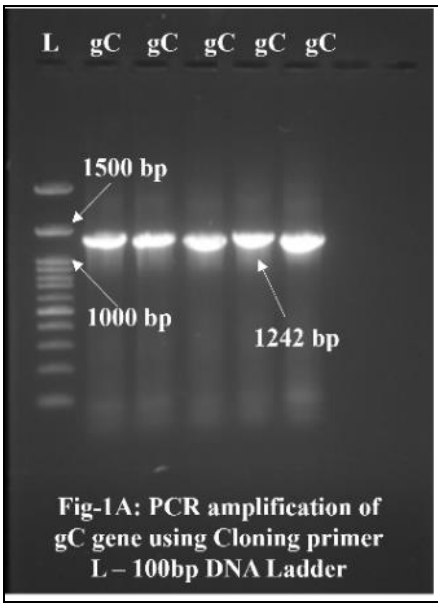

Fig-1A: PCR amplification of gC gene using Cloning primer L – 100bp DNA Ladder

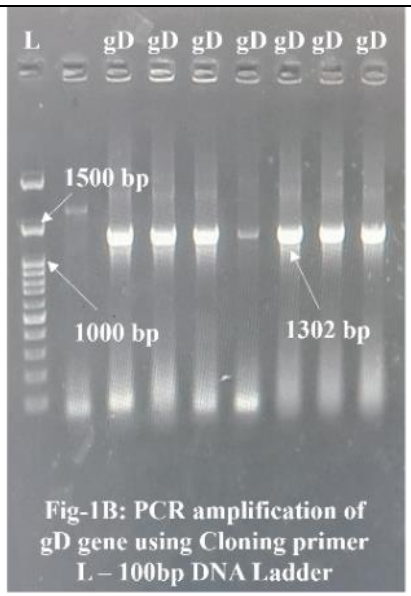

Fig-1B: PCR amplification of gD gene using Cloning primer L – 100bp DNA Ladder

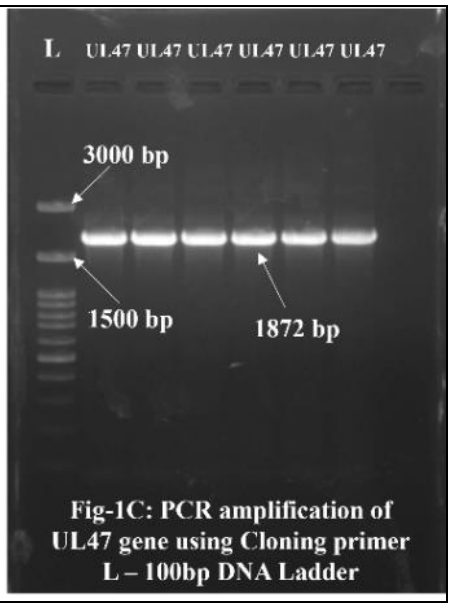

Fig-1C: PCR amplification of UL47 gene using Cloning primer L – 100bp DNA Ladder

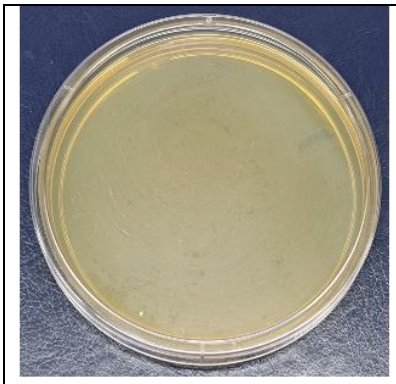

Fig-2A: No colonies on transformation of cut linear vector without insert

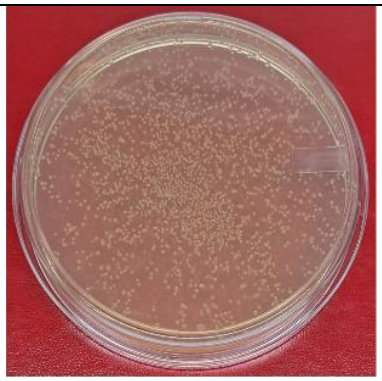

Fig-2B: Numerous colonies on transformation of uncut pVAX1 vector

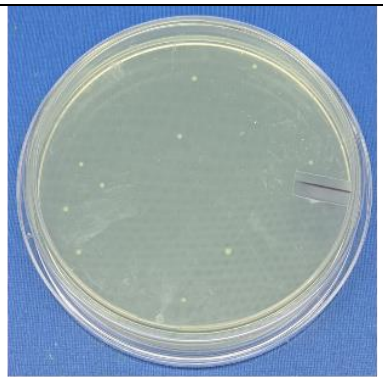

Fig-2C: Few colonies on transformation of recombinant plasmids containing insert gene

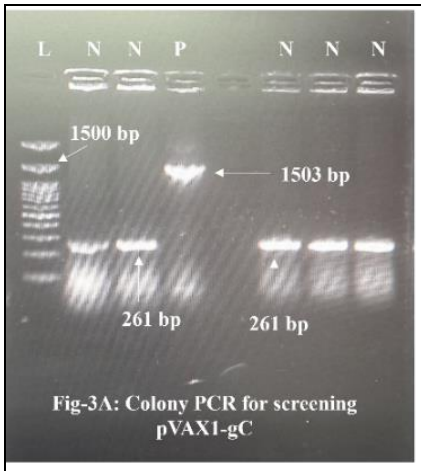

Fig-3A: Colony PCR for screening pVAX1-gC

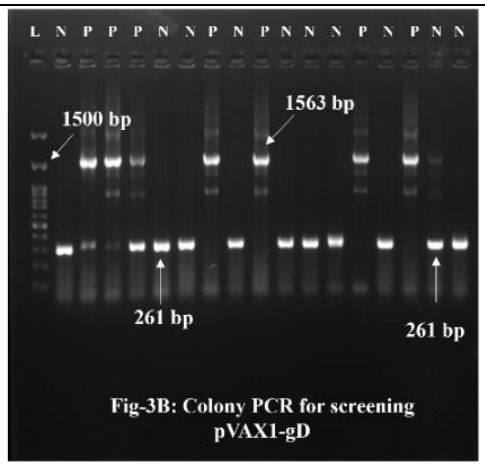

Fig-3B: Colony PCR for screening pVAX1-gD

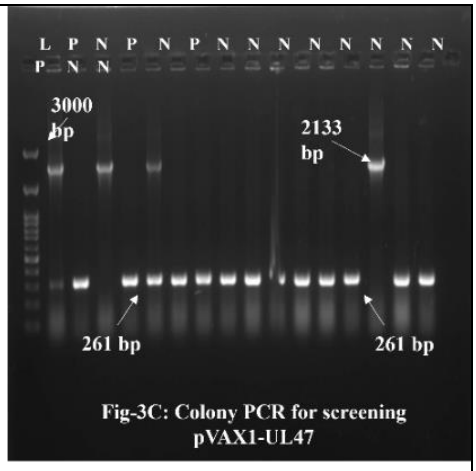

Fig-3C: Colony PCR for screening pVAX1-UL47

L- 100bp DNA ladder, N – Non recombinant colony, P – Recombinant colony

3.2. *In vitro* expression of recombinant plasmids

The recognition of recombinant proteins r-gC, r-gD and r-UL47 by anti-ILTV serum was confirmed by formation of bands relative to the molecular masses of recombinant proteins. In the r-gC, r-gD and r-UL47 lanes, a dominant band around 46 kDa, 48 kDa and 68 kDa was detected respectively which were found to be proportionate to their molecular weight (Fig-4).

3.3. Scaling up of recombinant plasmids

The average yield of plasmid DNA in LB broth was estimated to be 1.5mg/L in this study. The concentration of plasmid DNA was found to be in the range of 800ng/µl to 1.2µg/µl and purity ($260/280_{nm}$) was estimated as 1.9 to 2.1. The recombinant plasmids were again checked for presence of insert gene by single and double enzyme digestion (Fig-5).

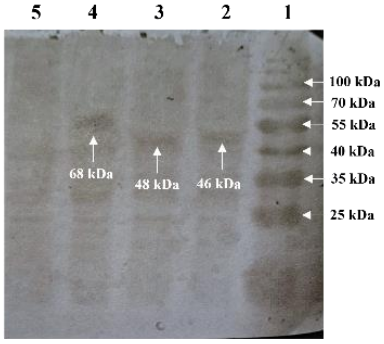

Fig-4: Identification of gC, gD and UL47 proteins by western blot analysis

Lane 1: Protein Ladder, Lane 2: pVAX1-gC, Lane 3: pVAX1-gD, Lane 4: pVAX1- UL47, Lane 5: CEF control

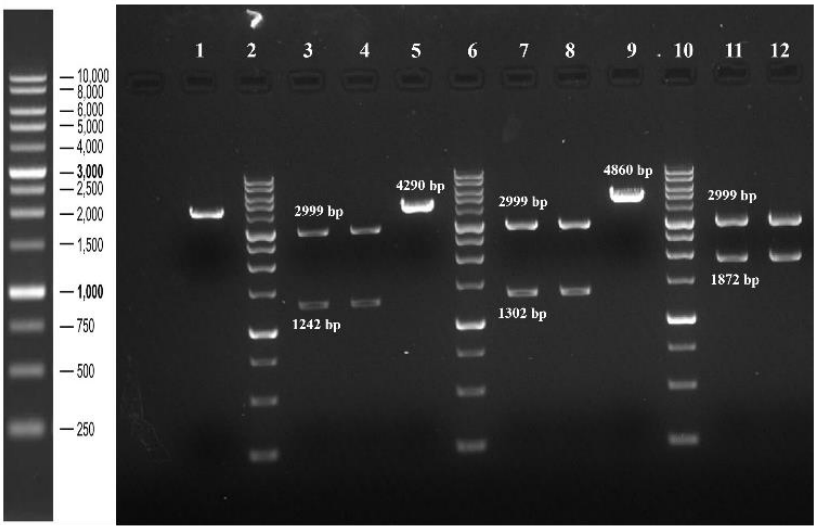

Fig-5: Insert release from recombinant plasmids by restriction endonuclease digestion

Lane 2,6,10: 1 Kb DNA Ladder, Lane 1: pVAX1-gC digested with *Hind* III only, Lane 3&4: pVAX1-gC digested with *Hind* III and *Kpn* I, Lane 5: pVAX1-gD digested with *Hind* III only, Lane 7&8: pVAX1-gD digested with *Hind* III and *Kpn* I, Lane 9: pVAX1-UL47 digested with *Hind* III only, Lane 11&12: pVAX1-UL47 digested with *Hind* III and *Kpn* I

3.4. Assessment of ILTV specific antibody response induced by DNA vaccination

The ILTV specific antibody response was detected in chickens immunized with the monovalent constructs encoding gC, gD and UL47 proteins (Table-3). The pVAX1-gC and pVAX1-gD construct showed higher antibody response than pVAX1-UL47 plasmid. However, the three constructs in combination as multivalent induced higher antibody response than monovalent groups suggesting a greater potency for inducing humoral immune response (Fig-6). In contrast no antibody response was detected in PBS and empty vector control groups.

3.5. Lymphocyte proliferation assay

The *in vitro* proliferative response of lymphocytes upon ILTV specific stimulation was clearly observed in birds immunized with monovalent and multivalent DNA vaccine (Table-4). But the mean stimulation index of multivalent immunized birds was markedly higher than the monovalent groups (Fig-7). This indicated that use of three plasmids as combined vaccine elicited a synergistic and consistent proliferation of lymphocytes. The PBS control and empty vector control group have not shown any stimulation response.

Group name	PBS control	Empty vector	pVAX1-gC	pVAX1-gD	pVAX1-UL47	Multi valent
0 day (Pre.Vac)	0.209	0.216	0.208	0.210	0.216	0.206
3 Weeks (Post.Vac)	0.233	0.219	0.458	0.443	0.424	0.446
4 Weeks (Post.Vac)	0.230	0.216	0.516	0.503	0.446	0.533
5 Weeks (Post.Vac)	0.231	0.227	0.515	0.521	0.463	0.549
6 Weeks (Post.Vac)	0.238	0.243	0.517	0.498	0.426	0.523
P value	NS	0.9997 NS	<0.0001 ****	<0.0001 ****	<0.0001 ****	<0.0001 ****

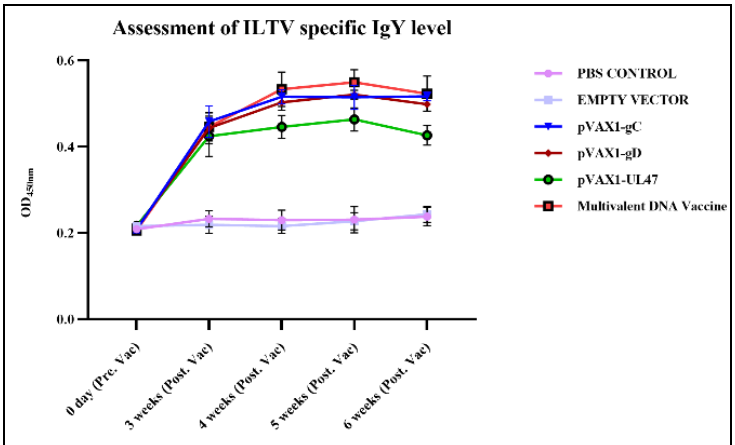

Fig-6: Graph showing ILTV specific antibody response of birds immunized by different DNA vaccines

	PBS control	Empty Vector	pVAX1-gC	pVAX1-gD	pVAX1-UL47	Multi valent
0 day (Pre-Vac)	1.031	1.120	1.052	1.036	1.157	1.076
3 Weeks (Post.Vac)	1.028	1.048	1.762	1.626	1.651	1.880
4 Weeks (Post.Vac)	1.110	1.076	1.778	1.675	1.752	2.043
5 Weeks (Post.Vac)	1.047	1.034	1.760	1.801	1.737	2.094
6 Weeks (Post.Vac)	1.054	1.003	1.631	1.759	1.764	2.168
P value	NS	>0.9999 NS	0.0009 ***	0.0014 **	0.0007 ***	<0.0001 ****

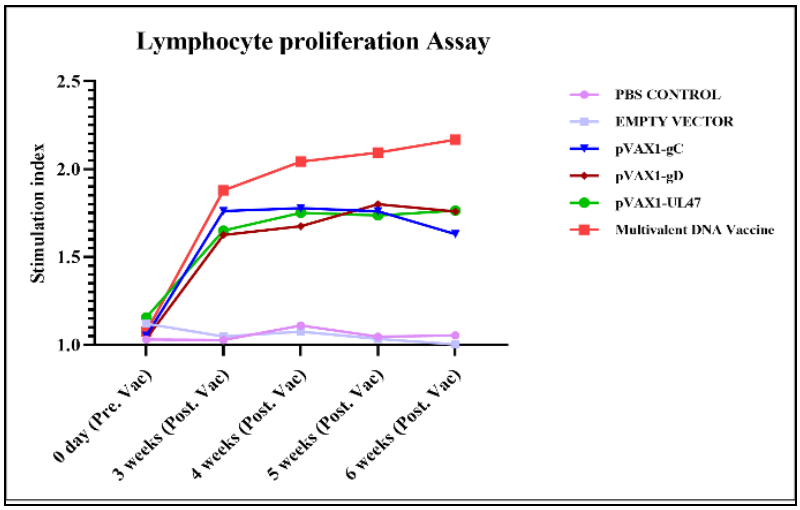

Fig 7: Graph sowing mean stimulation index of birds immunized with different DNA vaccines

3.6. Relative gene expression of IFNγ and IL-2 in immunized birds

The C_t values of two target cytokines and Actin B from RT-qPCR was used to calculate $2^{-\Delta\Delta Ct}$ on different weeks. Both genes IFNγ and IL-2 were found to be upregulated in the vaccinated groups (pVAX1-gC, pVAX1-gD, pVAX1-UL47 and multivalent DNA). The expression of both target genes followed the same pattern in the vaccinated groups. Highest level of expression among the groups was observed in the multivalent DNA vaccine group followed by pVAX1-gD and pVAX1-UL47 monovalent groups whereas pVAX1-gC group was observed to be the least among three (Fig-8 and 9).

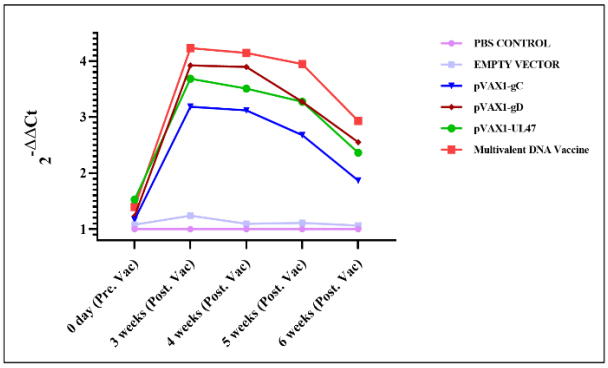

Fig-8: Graph showing IFNγ Cytokine gene expression levels in different group of birds

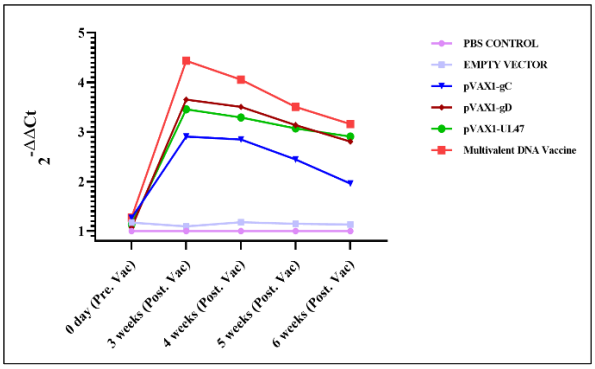

Fig-9: Graph showing IL-2 Cytokine gene expression levels in different group of birds

4. Discussion

In DNA vaccination, the expression of antigens in the target host resembles native pathogen epitopes more closely, and thus preserves the antigenicity than the conventional vaccines. The three plasmids targeting the glycoproteins (pVAX1-gC, pVAX1-gD) and tegument protein (pVAX1-UL47) of ILTV developed in this study were the potential target for development of vaccine [24, 25]. The expression of gC, gD and UL47 proteins were successfully detected in transfected CEF cells. The modest variation in protein size from previous studies [26, 16] may be due to presence of immature form or differently processed protein in cell culture system.

The humoral and CMI response was assessed in chickens immunized with recombinant plasmid DNA either as monovalent or as combined multivalent vaccine regimen. Each of the three ILTV target antigens constructed in this study exhibited an appreciable ILTV specific immune response. The pVAX1-UL47 plasmid elicited lower antibody response than pVAX1-gC and pVAX1-gD as evaluated by ELISA. The glycoprotein gC and gD was reported as major immunogens of ILTV and are capable of producing high level of neutralizing antibodies [27]. The higher antibody response detected from the chickens immunized with combination of all three plasmids (multivalent) confirmed their synergistic effect.

Early studies indicated that CMI response rather than humoral responses were crucial for protection against ILTV following vaccination [28]. All the three monovalent constructs showed almost similar lymphocyte proliferative response but in contrast the multivalent DNA immunized group showed augmented proliferative response and was consistently maintained through the entire study period. This shows clear picture of enhanced T cell response by multivalent combination in accordance to previous studies [18, 29]. The IFNγ and IL-2 are primary activators of macrophages and are important indicators of cell-mediated immune response in chickens [30]. Both the cytokines were found to be significantly upregulated in the multivalent immunization group in comparison with monovalent groups [31]. The challenge study of vaccinated birds with virulent ILTV could not be carried out due to the constraint of BSL III lab facility to perform experimentation.

5. Conclusion

Taken together, the results of this study demonstrated that multivalent DNA vaccine expressing gC, gD and UL47 proteins of ILTV synergistically augmented the humoral and cellular immune response in chickens than individual plasmids. The results from this study may initiate the potential usefulness of multivalent DNA vaccine against ILTV.

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