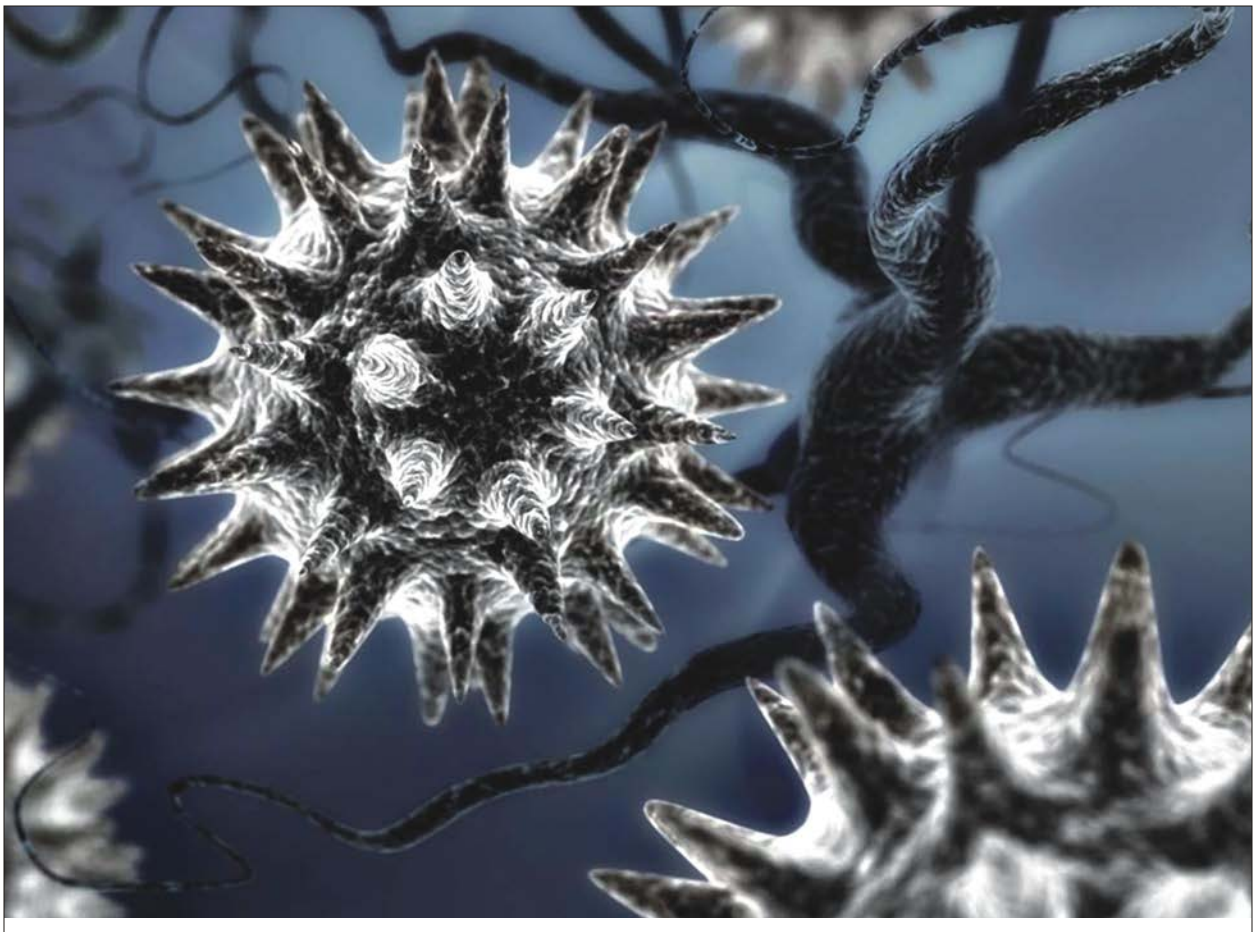

International Journal of Virology

ISSN 1816-4900

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS International Journal of Virology

ISSN 1816-4900
DOI: 10.3923/ijv.2017.1.13

Review Article
DNA Vaccines: Important Criteria Against Avian Viruses

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Abstract

Developing DNA vaccines against avian viruses provides researchers to develop technology that offers new approaches for the prevention of emerging avian viral diseases. The DNA vaccine is ease to construction and manufacture and the potential for world-wide usage even in low-resource settings. The principle behind the DNA vaccine is injection of plasmid DNA encoding a foreign antigen of interest can result in the subsequent expression of the foreign antigen and the induction of an immune response within a host. This is relevant to prophylactic vaccination strategies that subsequently recognizes the infectious agent and fights off the disease. In this review we focused on the detail information on the development of vaccination strategies that include the incorporation of immunostimulatory sequences in the backbone of the plasmid, codon optimization, Kozak sequences, co-expression of stimulatory molecules, appropriate delivery methods for target antigens to increase the potency of the DNA vaccines, in understanding their immunological mechanisms that play a role to generate both cellular and humoral immune responses and in their applications and efficacy in clinical trials so far.

Key words: Avian viruses, DNA vaccine, vaccine vector optimization, delivery methods, immunology of DNA vaccine

Received: September 05, 2016 Accepted: October 29, 2016 Published: December 15, 2016

Citation: Bellam Praveen and Golla Narasimha, 2017. DNA vaccines: Important criteria against avian viruses. Int. J. Virol., 13: 1-13.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The poultry farming is an organized and mechanized when compared to livestock farming, it is the one of the important industry in developing countries. Occurrence of disease outbreaks in poultry industry has led to recognize the importance of health management in poultry farming. However, poultry industry is threatened by progressively more virulent pathogens by exotic and emerging diseases cause losses to this sector. Avian respiratory diseases are mainly caused by mycoplasma, viruses and bacteria. The most avian viral respiratory diseases are caused by Infectious Bronchitis Virus (IBV), Infectious Bursal Disease Virus (IBDV), Newcastle Disease Virus (NDV), Avian Influenza Virus (AIV), Infectious Laryngotracheitis Virus (ILV). Infection with these viruses reduces the performance of broilers and egg production, making a severe economic impact on domestic commercial poultry industry. Therefore, considerable efforts on vaccination for preventing viral diseases have been made for over half a century. Traditional treatments against virus infection usually involve inactivated vaccines and live attenuated vaccines. However, pitfalls have been found in both types of vaccines. The inactivated vaccines can induce high titters of antibody but usually with lower cytotoxic T-cell (CTL) responses. Live vaccines are usually applied by spray and they hardly induce clinical signs, such as mucous discharge[1]. Protection offered by these vaccines is good but limited as the protection is sero-specific and does not offer any protection again new serotypes. Therefore, the situation warrants development of new generation DNA vaccines, which are preventive measures that could be used alone or in tandem with the conventional immunizing agents. The DNA vaccines generated using recombination of a pathogen's immunogenic gene and an optimized bacterial plasmid is a novel approach that could ably support the efforts made to the development of new immune prophylactics for controlling infectious diseases of poultry. These third generation vaccines (DNA vaccines) are having many advantages when compared to the conventional inactivated or live vaccines like superior cellular immunity generation and non-requirement of cold chain. Nevertheless, the doubts regarding the potential of DNA vaccine at times to develop immune response to sufficient levels has been a worrying factor. To overcome this unfilled space, various technological and immunological approaches are being employed to improve the efficacy of DNA vaccines to make their practical implementation, which could provide a novel alternative to the conventional vaccines for the prevention of various infectious as well as emerging diseases of poultry. Several DNA vaccines have been successfully tested against pathogens, such as infectious laryngotracheitis[2,3], avian influenza[4], coccidian[5], infectious bursal disease virus[6], infectious bronchitis virus[7,8] and Newcastle disease virus[9]. Developing vaccines using rDNA technologies requirea thorough understanding of the disease causing agent, particularly immunogenic site. In addition, it is important to know the different antigen-processing mechanisms and immune response of the host. Understanding of host immune responses will ensure that DNA vaccine designed will induce the appropriate immunological reaction or not. This review will provide a platform for improving DNA vaccines for poultry farms to improve immunity against various viral diseases.

AVIAN VIRAL GENES IN PROTECTIVE IMMUNITY

Infectious bronchitis virus: Infectious bronchitis virus is a member of the genus *Gammacoronavirus*, family Coronaviridae and have a non-segmented, positive-sense, single-stranded RNA genome. In IBV the S1 protein plays a crucial role in IBV pathogenesis. The S1 protein subunit carries hemagglutination inhibiting antibodies, serotype specific sequences, neutralization epitopes[10]. The S1 spike protein subunit is necessary and sufficient to induce protective immunity and has been successfully constructed a DNA vaccine against IBV. The N protein combines with the chromosome of IBV located inside the virions and plays an important role in viral replication, assembly and immunity[11-13].

Infectious bursal disease virus: Infectious Bursal Disease Virus (IBDV) belongs to genus *Avibirnavirus* and is a member of the family Birnaviridae with a non-enveloped, double-stranded RNA (dsRNA) genome. The VP2 is one of the major structural protein which forms the viral capsid. The VP2 is consider as protective immunogenic antigen responsible for inducing virus neutralizing antibodies and is closely related to antigenic variations and viral virulence[14,15]. The VP2 is an apoptotic inducer[16].

Newcastle disease virus: Newcastle Disease Virus (NDV) a member of the genus *Rubulavirus* of the family Paramyxoviridae with enveloped single stranded non-segmented negative-stranded RNA genome. In this haemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins play important role in virus-host cell interaction and virulence of the virus. These are primary targets of ND DNA vaccine development. Studies have been

conducted to evaluate the potential of plasmid expressing NDV HN and F glycoproteins to induce anti-NDV immune responses in chickens with variable protection efficacy[17,18].

Chicken anemia virus: Chicken Anemia Virus (CAV) the only member of the genus *Gyrovirus* of the family Circoviridae containing non-enveloped icosahedral single stranded, negative sense, circular DNA genome. The VP1 is the main protective protein inducing neutralizing antibodies[19]. Co-synthesis of the VP1 and VP2 proteins can induce neutralizing antibodies that protect progeny chicks in the efficient induction of antibody response against CAV challenge[20].

Avian influenza virus: Avian Influenza Virus (AIV) is member of the genus *Influenza virus A* and family Orthomyxoviridae consisting of negative-sense ssRNA genome. In AIV HA protein is a glycosylated integral membrane protein. This mediates adsorption and penetration of virus during infection. Another protein NA is an integral membrane glycoprotein, which promotes the release of virus particles from host cell receptors. The haemagglutinin (HA) and neuraminidase (NA) proteins of Avian Influenza (AI) are the most predominant immunogenic proteins in inducing immunity[21].

Marek's disease virus: Marek's Disease Virus (MDV) belongs to the family Herpesviridae and genus *Mardivirus* with dsDNA genome. The VP22 of MDV is tegument protein involved in intercellular transport and movement between cells from the original cell of expression into the neighbouring cells. The studies shown that VP22 protein possesses the ability to improve DNA vaccine potency by facilitating intercellular spreading of the linked protein[22].

DNA vaccine strategy: A conventional DNA vaccine is made by using standard molecular biology techniques. First, the immunogenic gene of interest of a pathogen which is protective to the host is amplified by using Polymerase Chain Reaction (PCR) with a pair of primers, a cDNA template and cloned into a suitable mammalian expression vector. Secondly, the resultant plasmid construct is examined to verify the fidelity of the insert to avoid cloning errors, such as frame shifts through sequencing. Following construct is confirmed by sequence analysis and the expression of correct protein is verified by Western Blotting (WB), ELISA, immunofluorescent test (IFT) and immunoprecipitation test.

Characteristics of DNA vaccine: The DNA vaccines are also called as genetic vaccines, nucleic acid vaccines or naked DNA vaccines, which are composed of simple ring form of double-stranded DNA that generally consists of two components: The first one is the mammalian expression cassette (promoter/enhancer-incite expression in the host cells, antigen encoding double stranded DNA sequence(s) of interest-capable of stimulating immune responses and polyadenylation sequences for the stabilisation of the mRNA transcripts) (Fig. 1) and the second one is a bacterial plasmid backbone (which is derived from bacteria but it totally unable to produce infection) with origin of replication which is necessary for the amplification of the plasmid in a bacterial system, an antibiotic resistance gene for plasmid selection during bacterial culture, also contains some immune stimulatory sequences such as unmethylated CpG motifs which induce unspecific immune activation, acting as adjuvant in DNA immunization[23,24].

APPROACHES TO OPTIMIZE DNA VACCINES

Vector optimization: Selection of an appropriate vector is one of the most important issues in optimizing a DNA vaccine. With regard to the plasmid itself, significant advances have been made in optimizing the genetic sequence of the encoding gene as well as other related components. It is generally believed that the expression level of antigen-encoding genes *in vivo* following DNA vaccination correlates positively with the plasmid DNA-induced immune response[25-27].

Promoters: In order to get maximum protein production from a plasmid transfected into an animal cell, the regulatory elements of the plasmid must be optimized according to the cell type. In plasmid vaccine design promoter strength is considered as the critical factor[28]. The DNA vaccines have traditionally incorporated the viral promoters with broad cell

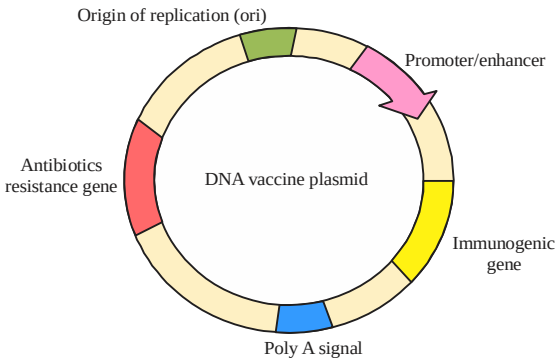

Fig. 1: Features of DNA vaccine plasmid

type specificity such as human cytomegalovirus/ immediateearly gene (CMV/IE) promoter, simian virus 40 (SV40) and Rous Sarcoma Virus (RSV) promoters. These promoters have been the most frequently used vehicles for driving the high constitutive expression levels of the vector-encoded genes. Tissue-specific promoters are also used in the construction of DNA vectors, such as the muscle-creatine kinase (MCK) promoter[29] and muscle-specific desmin[30] are expected to be safer than viral promoters, but they induced low levels of antigenic protein expression and weak immune responses[31]. Intron and poly adenylation signals can also affect the expression levels of the antigen. The addition of an intron, such as the intron A of the CMV/IE gene[32] and poly adenylation signals to the plasmid leads to increased protein expression.

CpG motifs in plasmid DNA vector backbone: In animal models, experimental studies of inactivated vaccines, naked DNA vaccines without adjuvant can induce effective immune responses. Part of this effectiveness is attributable to the plasmid DNA itself, whose backbone specific motif consists of unmethylated cytosine-phosphate-guanosine (termed CpG motifs) dinucleotide with optimal flanking regions composed of two 5' purines and two 3' pyrimidines[33-35]. The CpG motifs in the plasmid vector backbone of DNA vaccines elicited a higher antibody response, more CTLs and greater IFN-γ production than the original vector[36,35]. The Toll Like Receptor (TLR-9), present on effector cells of the immune system can bind and recognize CpG motifs[37] and these motifs-CpG oligo deoxy nucleotides (ODNs) can directly stimulate multiple types of immune cells[38]. Interaction of TLR-9 with CpG motifs activates several signalling pathways and results in an immune stimulatory cascade[39]. Motif-CpG ODNs inducing professional antigen-presenting cells (APCs) which is critical in their striking enhancement of cellular immune responses and results in activation of natural killer cells (NK)[40-42].

Antibiotic free plasmids: In plasmid, antibiotic resistance genes are primarily inserted for selection purpose during cloning. Due to generating microbial resistance, the incorporation of antibiotic resistance genes into DNA vectors for vaccination purposes is not favoured for vaccine producers. Thus novel plasmids without antibiotic resistance genes based on "Operator-repressor titration" are being investigated [43]. In that Minimalistic Immunogenically Defined Gene Expression (MIDGE) vectors are one of the examples for antibiotic resistance gene-free plasmid with linear covalently closed double stranded DNA containing minimal gene expression elements (include a promoter/intron, gene of interest and poly adenylation signal). The elimination of bacterial DNA sequences, antibiotic resistance genes from plasmid DNA[44] make easier for MIDGE vectors to go through the different membranes of the cell and to get integrated into the genome of the host cell[45]. The MIDGE vectors are safe, easy to scale-up and allows chemical modifications, which can increase expression and immune response.

Codon usage: Codon usage is observed in all species and the use of selective codons in genes often correlates with gene expression efficiency[46]. Many pathogens (bacteria, virus) have a very different codon usage and genomic GC content as compared with mammals[47]. In this case of codon usage, the DNA vaccines may result in inefficient translation and low level expression of microbial genes in transfected mammalian cells[48,44]. Thus, to overcome this optimizing the codons in plasmid-encoded genes may become an approach for enhance efficacy in genetic immunization. The introduction of multiple CpG motifs into the plasmid backbone by altering the coding sequence of particular genes of interest to conform the preferred mammalian codons[47]. By increased CpG motifs by codon-optimized gene shows characteristic adjuvant effect[36].

Kozak sequences: One control point that can influence protein synthesis from plasmid vectors is at the stage of translation of mRNA transcripts[49]. A comparison of several hundred mRNA sequences showed that presence of the translational initiating consensus sequence (-6 CCA/GCCAUGG +4), named 'Kozak' consensus sequence, located upstream of the initiator codon. This is necessary for studying the conditions required for initiation of optimal translational efficiency of the mammalian genes[50,51]. An optimal sequence present upstream of the AUG initiation codon within mRNA influences its recognition by eukaryotic ribosomes[52]. Prokaryotic genes and some eukaryotic genes do not contain Kozak sequences, therefore, the incorporation of a Kozak sequence into a plasmid vector backbone may increase the expression level of the transgenes in the context of DNA vaccines[48].

Bidirectional and biocistronic plasmids: The DNA vaccines have the potential to express multiple antigens from one or more pathogens in a single vector[53]. Bidirectional plasmids allowed co-expression of two antigens *in vitro*, which was in accord with increased immune response *in vivo*[54]. The disadvantage of these plasmids was competition for gene expression from the promoters, plasmid instability due to the presence of more than one expression cassette and possible

lower transfection efficacy of such plasmids. Bicistronic plasmids can transcribe from a single promoter and express proteins from a single mRNA[55]. Thus, the bivalent DNA vaccine represents an innovative approach for enhancing immune response[56].

Vaccine delivery: In DNA vaccination, the optimized gene sequence of interest (plasmid) is delivered through different routes including intramuscular (IM), intradermal (ID), intravenous (IV), intranasal (IN), intratracheal (IT), orally, intra peritoneal (IP), subcutaneously (SC), epidermal (by scarification of skin) and *in ovo* to the developing embryo[57,58]. Using the host cellular mechanism, the plasmid enters into the nucleus of transfected local cells, such as skin fibroblasts, keratinocytes and also resident Antigen Presenting Cells (APCs). The most popular method of administering DNA vaccines is needle injection into muscle or skin. It is relatively simple and effective way to inject aqueous solution of DNA plasmid into tissue result in the direct transfection of cells (Fig. 2). Another efficient method for intra dermal delivery is carried out by DNA-covered particle bombardment called "Gene-gun". This consists of gold covering micro particles with recombinant plasmid DNA and shooting them by mechanical force are propelled by helium or CO_2 pressure on to the tissue or skin[59] (Fig. 2). Each one of these methods of delivery introduce vaccine to distinct areas of immune surveillance and therefore, primes the immune system in distinct ways. A few studies have suggested that a combination of routes is more effective than any one single route; however this has not been conclusively proven[58,6,60]. Therefore, because of simplicity and effectiveness IM injection is still the most common route of DNA vaccination in the avian species.

IMMUNOLOGY OF DNA VACCINE

Antigen presenting cells: After entry of the plasmid DNA into the host cells, it is transported into the nucleus, where the encoding viral gene uses host's transcriptional and translational mechanism to produce the desired target antigenic protein product and later processed into small antigenic peptides fragments (8-10 amino acids) by using host proteases. Obscuring its shape, distinctive peptides would be released which are processed endogenously synthesized protein antigens that are initially translated on the ribosomes of the Endoplasmic Reticulum (ER) where the resulting peptides are then presented together with MHC class I molecules in the lumen of the ER with in the antigen presenting cells (APC) (Fig. 3). The peptide-MHC class I complex is transported to the exterior of the cell membrane where it is recognized by the antigen binding T-cell receptor (TCR) expressed on $CD8^+$ T lymphocytes cells (CTLs). The MHC class II pathway, after uptake of viral proteins by antigen presenting cells which are processed extracellular proteins are acquired by endocytosis and are processed in endosomes into small antigenic peptide fragments where it is recognized by the T-cell receptor (TCR) expressed on $CD4^+$ T-cells. The $CD4^+$ T-cell then act as a T-helper lymphocyte and activate antigen through specific surface immunoglobulin of B cells to secrete antibody. The encoded proteins could be delivered by the MHC I or MHC II antigen-processing pathways to induce high levels of $CD4^+$ or $CD8^+$ T-cell activation[61], resulting in enhanced immunogenicity. It has been reported that $CD4^+$ T-cell responses produce directly antiviral cytokines as well as increase the proliferation, maturation and functional activity of $CD8^+$ CTL, while $CD8^+$ CTL plays a critical role in

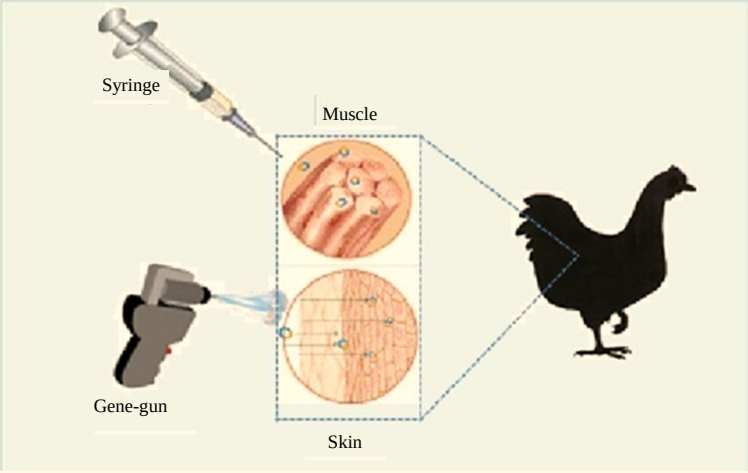

Fig. 2: Mechanism of vaccine delivery

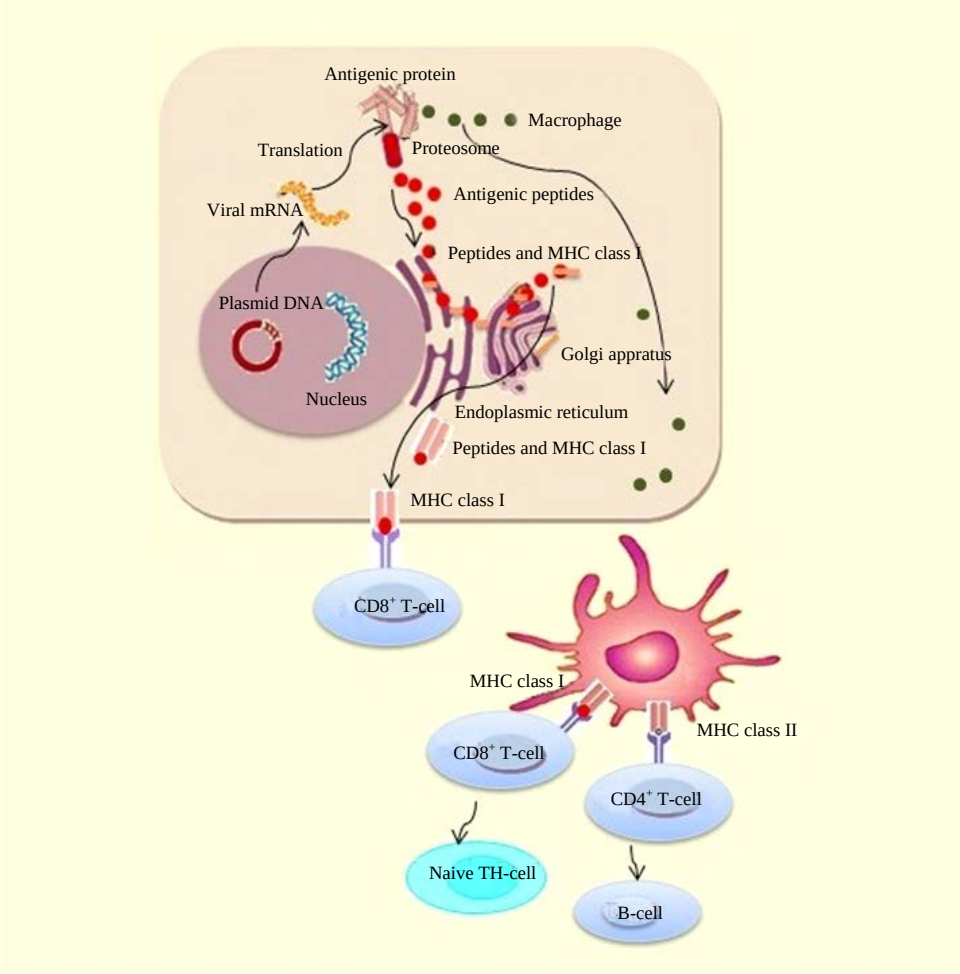

Fig. 3: Mechanism of antigen presentation and processing

controlling IBV infection in poultry[62]. This form of antigen presentation and processing induces both MHC class I and class II-restricted cellular and humoral immune responses.

Role of T-cells, cytokines and co-stimulatory molecules: The $CD4^+$ T-cell population has been subdivided into TH1 and TH2 subsets based on the profile of cytokines secreted activation. Co-stimulatory or cytokine molecules are responsible for promoting B cell survival and antibody production and also provide helper function to $CD8^+$ T-cells. Among the pro-inflammatory cytokines secreted by the TH1 cells are granulocyte-macrophage colony stimulating factor (GM-CSF), Transforming Growth Factor β (TGF-β), TNF-α, β, IFN-γ (cell mediated immunity)[63], IFN-α, β (innate immune defence against virus infections)[64], interleukin-12 (IL-12), interleukin-2 (IL-2) (T-cell growth factor)[65], interleukin-18 (IL-18), Myelomonocytic Growth Factor (MGF)[66] proliferate and activate cell mediated immune responses by stimulating effector CTLs into memory cells, NK cells and increasing the phagocytic activity of monocytes and macrophages (Fig. 4). After few days of viral post infection, the TH2 subtype of $CD4^+$ T-cells are involved in induction of humoral immunity and by producing IL-4 cofactor in the proliferation of resting B-cells[67], IL-5, IL-6, IL-10 and IL-13 may replace TH1-cells. At this stage of viral infection, the chicken immune system may depend on humoral immunity to control viral infection (major role in supplying B-cell help in the generation of a humoral immune response, antibody production)[68]. Helper TH2 type cell recognized by specific epitopes or intact virus through their surface IgM and promote B-cell differentiation into plasma cells producing large amounts of IgG, IgA, IgE antibodies and memory B-cells[69,70].

EXPERIMENTAL EVIDENCE

The delivery of a bicistronic vector containing IBV N gene and IL-2 cytokine as DNA vaccination by injecting intramuscularly in 7-day-old chickens with 150 μg of plasmid

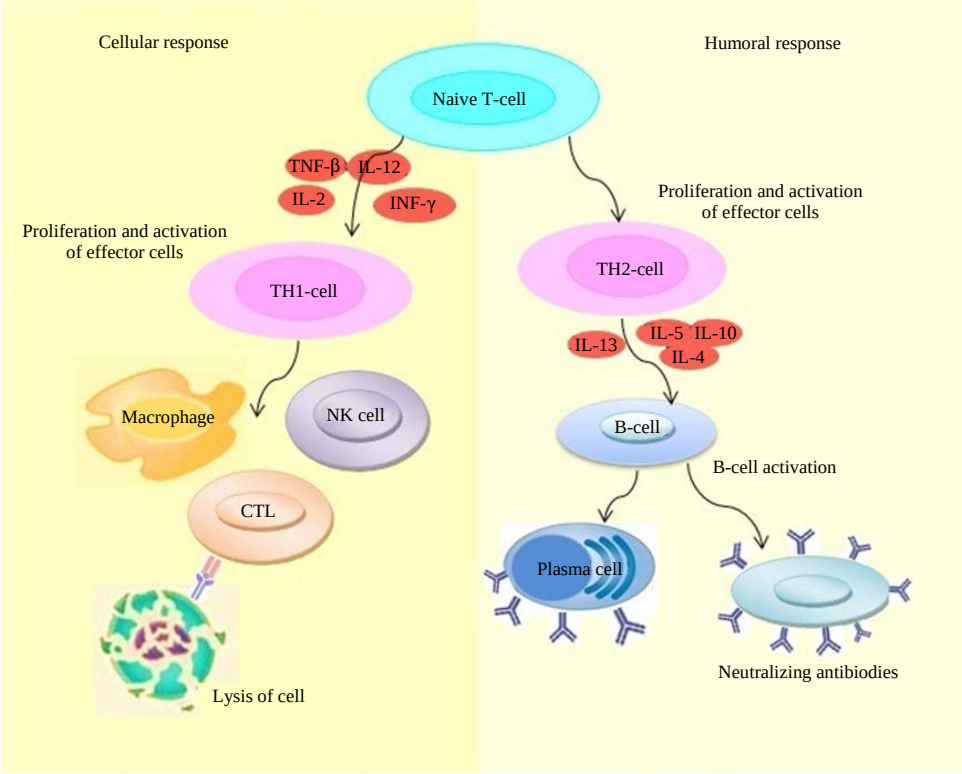

Fig. 4: Mechanism of cellular and humoral response

(N/IL2) accelerated specific antibody induction with an increase in T-cell response[71]. In the same way delivery of DNA vaccines in specific pathogen-free (SPF) chickens immunized intramuscularly with monovalent individual plasmids (S1, M and N)[72], multivalent combination of plasmids (S1/M/N) and also by boosting with an inactivated IBV vaccine before being challenged with virulent IBV with equivalent molar ratio for DNA component containing 100 μg of plasmid vector induced humoral and cellular mediated immune responses[73]. The multi-epitope chimeric DNA vaccine with mini genes of IBV (S1, S2 and N) which contain both B-cell epitopes for protective antibody response and T-cell epitopes that induce CTL response in IBV infection, which were immunized to 7-day-old chickens intramuscularly with 150 μg of plasmid encapsulated by liposome[74]. The poly-CTL-epitope based vaccine strategy provides a safe mechanism to induce a broad spectrum of immunity against most serotypes of IBV. The S1-derived CTL epitopes based DNA vaccines represents a possible strategy to elicit efficient cellular immune response against virus[75]. The IBDV DNA vaccine with IL-18 as an adjuvant were immunized to 14-day-old SPF chickens intramuscularly with 100 μg of the plasmids (VP243, VP243/IL-18) twice at 2 weeks intervals increases the immune responses and protection efficacy against IBDV infection[76]. In Newcastle disease virus immune responses induced by 100 mg μg^{-1} of recombinant plasmids immunized intramuscularly as DNA vaccines separately, in combination (ND.HN.F, ND.F and ND.HN)[77] and 60 μg plasmid vector with interferon-γ and interleukin-4 genes (HN.F, IFN-γ/IL-4) as adjuvants increased NDV specific antibodies as well as TH1 or TH2 Cell Mediated Immune (CMI) response[78]. The immunodominant VP2 fragment was injected intramuscular with 100 mg of plasmid (pVAX-VP2) in immunized chickens as a potential DNA vaccine against IBDV infection in 7-84 days old chickens[79]. It is determining that the adjuvant property of the C-terminal domain of *M. tuberculosis* HSP70 (cHSP70) by genetically linking cHSP70 with the IBDV-VP2 gene (100 g, intramuscularly) and evaluating this fusion gene construct as a vaccine candidate[80]. The IBDV VP243 gene-based DNA fused with AIV HA gene could trigger dual expression of both proteins and induce specific humoral immune responses to both IBDV and AIV by a single plasmid construct (500 μg, intramuscularly)[81]. The immunization with 100 mg μg^{-1} of Avian Influenza Virus (AIV) strain H5 gene and Esat-6 of *Mycobacterium tuberculosis* gene (H5/Esat-6) as a genetic adjuvant improve antibody responses[82]. Ability of adjuvants (aluminum hydroxide, essai microparticle) and phema (nanoparticle)) to showed some

enhancement of an avian influenza (H6N2) HA DNA-induced immune response in chicken[83]. The administration of herpes virus of turkey (HVT) with 5 μg of rChIFN-γ plasmid reduced the incidence of tumour development and suggesting that rChIFN-γ increases the potency of HVT against challenge with a virulent strain of MDV in chickens[84]. As with any vaccination program, the regimen of vaccine delivery and the age of the animal being vaccinated are important. In the case of DNA vaccination in the avian, most protocols have involved a primary immunization in birds less than 1 week of age with at least one or two secondary immunizations. In many reports a good response to the DNA vaccine (either immunity or protection) was seen only after the boost[85,86]. Summary of DNA vaccines for avian infectious viral diseases is summarized in (Table 1).

Abrogating interference from maternal antibodies: The DNA vaccination and maternal antibody interference shows two important features: (1) The neonate's immune system sufficiently mature to elicit a response. (2) The offspring of an immune mother be immunised despite the high levels of passively transferred maternal antibodies to the antigen. The DNA immunisation will be effective in early life in the absence of maternal antibodies. But in commercial chicks, significant titres of maternal antibodies are present up to 35 days of age. Antibody response in neonates decreases when structural protein, such as glycoprotein of HA of influenza virus were immunised in presence of maternal antibodies[91]. On the other hand, vaccination with DNA encoding nucleoprotein induces antibodies in neonates even in the presence of maternal antibodies[92]. In NDV the interference from maternal antibodies the DNA vaccines containing HN and/or F genes fail to protect commercial chicks[77]. But in CAV infection DNA vaccine are protective in the presence of a maternal antibody[93].

Epitope based DNA vaccine: Epitope DNA vaccine which is a newly-developed DNA vaccine with short DNA sequences, encoding well-defined cytotoxic T-lymphocyte (CTL), antibody, helper-T lymphocyte (HTL) specific epitopes are used as immuno genes, which induces protection against large and complex viral pathogens. In influenza virus multi-epitope DNA vaccine injected intra muscularly with three CTL epitopes increases the percentage of $CD4^+$ and $CD8^+$ T-lymphocytes in peripheral blood of immunized chickens[94]. The S1 protein of IBV has serotype-specific and neutralisation-specific epitopes. Fifty two glycoprotein of S1 carries epitopes which induce cross-reactive antibodies[95,96]. These multi-epitope vaccines induce cellular and humoral immunity in chickens.

Table 1: Summary of DNA vaccines for avain infectious viral diseases

					Immune responses				
Pathogen/family	Challenge antigen in vaccine	Types of plasmids	Dosage (μg)/Route	Adjuvant	Humoral immunity	Cell mediated immunity	Age of bird	Protection	References
IBV	S1, N, M	pVAX1	100 μg/IM	Cationic liposomes	Yes	Yes	7 days old	85%	Yang *et al*.[56]
Coronaviridae	S, N		150 μg/IM	Cytokine (IL-2)	Yes	Yes	7 days old	80%	Tang *et al*.[71]
	Nucleocapsid		150 μg/IM	None	ND	Yes	5 days old	Yes	Seo *et al*.[8]
	S1		150 μg/IM	None	Yes	Yes	7 days old	Yes	Tan *et al*.[75]
IBDV	VP243	pCAG, pCI, pCDNA3, pCR3.1	100 μg/IM, O	Cytokine (IL-18)	Yes	Yes	14 days old	93%	Li *et al*.[76]
Birnaviridae	VP2/4/3		200 μg/IM	Liposome	Yes	ND	14 days old	Yes	Li *et al*.[87]
	Large segment gene		400 μg/IM	None	Yes, but low	ND	1 days old	Protection after 2-3 injections	Chang *et al*.[88]
	vp2 and vp2, vp4, vp3		150-800 μg/IM and IP	None	Yes, but low	ND	1 days old, 3 Weeks-old, 4 Weeks-old	Moeini *et al*.[89] vp2-no protection, vp2, vp4, vp3-protection	Fodor *et al*.[6]
NDV	HN and/or F	pVNO2,	60 μg/IM	Cytokine (IFN- γ, IL-4)	Yes	Yes	21 days old	10-40%	Sawant *et al*.[78]
Paramyxoviridae	F protein	PCAG,	100 μg/IM	Cationic lipids	Yes	ND	2 Weeks-old	Partial	Sakaguchi *et al*.[9]
AI	HA (H7)	pCI-neo,	100-200 μg/IM, IV, IN	None	Yes	ND	2 Weeks-old	Partial	Fynan[58]
Orthomyxoviridae	HA	pCMV/H7	100, 300 μg/IM, IV, IT, IB, O	None	Yes	ND	3 Weeks-old	Partial	Fynan[57]
	HA (H5)		10, 50,100, 250 μg/IM	$CaPO_4$, sucrose, cationic lipids, DEAE dextran, polybrene	Yes	ND	1 day old	Yes	Suarez and Schultz-Cherry[86]
CAV	VP1-VP2	pBudCE4.1	150 μg/IM	None	Yes	Yes	2 week-old	Yes	Moeini *et al*.[89]
Circoviridae									
MD	VP22	pCDNA3	2 μg/GG	DNA-coated gold particles	Yes	Yes	6-8 weeks-old females C57BL/6 mice	Yes	Hung *et al*.[90]
Herpesviridae									

IBV: Infectious bronchitis virus, CAV: Infectious chicken anemia virus, AI: Avian influenza, NDV: Newcastle disease virus, IBDV: Infectious bursal disease virus, MD: Marek's disease, IM: Intramuscularly, IV: Intravenously, IN: Intranasally, IT: Intratracheally, O: Ocularly, IB: Intrabursally, GG: Gene-gun, ND: Not done

STRATEGIES TO IMPROVE DNA VACCINE IMMUNOGENICITY

Genetic adjuvants: Cytokines can serve as potent adjuvants in DNA vaccines technology[97,98]. Cytokines are co-expressed with the viral protein to enhance the potency of DNA vaccines against viral infections[42]. Primarily the challenge experiments are to be needed to verify the efficacy of virus immunogenic antigen and virus vaccine bearing membrane-bound immune modulators. Then after the incorporation of virus cytokines, immunogenic vaccine antigen, immune modulators which determine the scope and limitations of vaccine development. Finally the effects of various cytokine adjuvants on T-cell responses need to be evaluated, in this cellular immunity plays an important role in protection during viral infections. The efficacy of chicken IL-18 (chIL-18) as adjuvant enhances both humoral and cellular immune responses against IBDV challenge[76]. The immune-modulatory effect of plasmids encoding chicken IFN-γ and IL-4 genes when co-delivered with HN-F bivalent ND DNA vaccine induces high antibody response[99,100]. Interferon-gamma (IFN-γ) is a type II interferon, is associated with adaptive immune responses and protective antiviral defences against MD[101,102].

Chemical adjuvants: Chemical adjuvants can function as activators of innate immunity provide slow release depots and alter immune cell trafficking[103]. Aluminium salts are the most widely used adjuvant for both human and veterinary vaccines[104]. Liposomes are synthetic spheres possessing lipid layers that can encapsulate antigens and act as both a vaccine delivery vehicle and adjuvant[105]. Nanoparticles have shown promise for enhancing immune responses to plasmid DNA vaccines[106-108]. Adjuvants with IM vaccination, found lipfectin to be better than lipfect amine in producing a protective response[9]. Where gene-gun deliveries of DNA on gold beads have been used in the chicken, good results have been reported[4,109].

ADVANTAGES OF DNA VACCINES

The DNA vaccines are relatively inexpensive and easy to development and produce long term persistence of immunogenicity, immune response focused only on antigen of interest and multiple immunogenic epitopes, evoke protective humoral and cellular immune responses, antigen presentation by both MHC class I and class II molecules, ability to polarize TH-cell toward type 1 or type 2 responses, DNA vaccines are thermo stable, DNA vaccines are safer, more stable and easy to handle.

Disadvantages of DNA vaccines: Inducing antibody production against DNA, may induce immunologic tolerance by antigens expressed inside host body, DNA vaccines may have a relatively low immunogenicity, insertion of foreign DNA into the host genome may cause the cell to become apoptosis.

CONCLUSION

After several hurdles scientific developments in the field of DNA vaccines have resulted in notable improvements in their potency. The key technical challenges going forward to improve proper optimized design of the plasmid vector, suitable route of delivery, potency of low doses of DNA to enhance the immunity towards DNA vaccines, utility of a variety of regulatory elements, codon usage, kozak sequences and produce immune m odulators like cytokines and co-stimulatory molecules induce longer immune response and active immunization against avian infectious diseases. The better understanding of the mechanisms by which DNA vaccination lead to generate strong protective cellular immune responses and role for DC as the principle APC and the importance of both $CD4^+$ and $CD8^+$ T-cell activation through MHC class II and I restricted epitopes as key players in cellular immunity. The current review provides a comprehensive and unbiased analysis of the literature with latest key developments for the developmentof different DNA vaccine strategies and their applications are discussed in detail with respect avian viral diseases. This review will be informative for beginners, who would like to work in the areas of vaccinology with respect to avian diseases. In this way, this review will aid in increasing the current scientific knowledge in the field of avian vaccinnology.

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