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Development of a DNA vaccine for chicken infectious anemia and its immunogenicity studies using high mobility group box 1 protein as a novel immunoadjuvant indicated induction of promising protective immune responses

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ABSTRACT

Chicken infectious anaemia (CIA) is an economically important and emerging poultry disease reported worldwide. Current CIA vaccines have limitations like, the inability of the virus to grow to high titres in embryos/cell cultures, possession of residual pathogenicity and a risk of reversion to virulence. In the present study, a DNA vaccine, encoding chicken infectious anaemia virus (CIAV) VP1 and VP2 genes, was developed and co-administered with truncated chicken high mobility group box 1 (HMGB1 ΔC) protein in young chicks for the evaluation of vaccine immune response. CIAV VP1 and VP2 genes were cloned in pTARGET while HMGB1 ΔC in PET32b vector. *In vitro* expression of these gene constructs was evaluated by Western blotting. Further, recombinant HMGB1 ΔC was evaluated for its biological activity. The CIAV DNA vaccine administration in specific pathogen free chicks resulted in moderately protective ELISA antibody titres in the range of 4322.87 ± 359.72 to 8288.19 ± 136.38, increased $CD8^+$ cells, and a higher titre was observed by co-administration of novel adjuvant (HMGB1 ΔC) and booster immunizations. The use of vaccine with adjuvant showed achieving antibody titres nearly 8500, titre considered as highly protective, which indicates that co-immunization of HMGB1 ΔC may have a strong adjuvant activity on CIAV DNA vaccine induced immune responses. The able potential of HMGB1 protein holding strong adjuvant activity could be exploited further with trials with vaccines for other important pathogens for achieving the required protective immune responses.

1. Introduction

Chicken infectious anaemia (CIA) is an emerging and economically important disease of poultry having worldwide distribution [1–7]. It is caused by chicken infectious anaemia virus (CIAV), family *Circoviridae*, genus *Gyrovirus*. CIAV has circular ss-DNA genome (2.3 kb) which encodes three distinct viral proteins: VP1 (52 kDa), major capsid and immunogenic protein; VP2 (28 kDa), non-structural scaffold protein; and VP3 (13 kDa), known as apoptin. CIAV is highly contagious, hardy and ubiquitous in nature, and can be vertically transmitted [1,4].

The conventional vaccines (live/killed) available for CIA have limitations like, the inability of the virus to grow to high titres in embryos/cell cultures, residual pathogenicity and a risk of reversion to virulence. Under such circumstances development of a DNA vaccine for CIAV is of importance as DNA vaccines have been reported to be safe, stable, induce both humoral and cell mediated responses, work even in presence of maternal antibodies and are well tolerated. However, most of the DNA vaccines developed till date proved to be less immunogenic as compared to conventional vaccines, and on account of which only few DNA vaccines are available commercially [8–10]. Currently, research has focused on developing

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novel strategies to enhance immunogenicity of the DNA vaccines, and inclusion of genetic adjuvants to enhance and direct immune responses is one among them [8–16].

Genetic adjuvants are expression vectors encoding biologically active molecules such as cytokines, chemokines, soluble forms of cell ligands, adhesion proteins or other immunoreactive molecules [17–19]. Amongst these, high-mobility group box 1 (HMGB1) protein has been reported to possess potent immune enhancing activities because HMGB1 promotes inflammation and induces innate immune responses, DC maturation and mediate Th1 polarization [20–22]. Recent report shows that co-immunization of HIV-1 DNA and tuberculosis subunit vaccine with HMGB1 drives strong cellular and humoral immunity [23,24]. The present work was designed with an aim to develop a DNA vaccine for CIAV and explore the role of chicken HMGB1 protein as a potential vaccine adjuvant.

2. Materials and methods

2.1. Specific pathogen free chicks

Specific pathogen free (SPF) eggs ($n \equiv 70$) were procured from M/s Venkateshwara Hatcheries Group Limited, Pune, and chicks hatched out in Hatchery Unit of Central Avian Research Institute, Izatnagar were maintained following required recommendations in the Experimental Animal Shed of Avian Disease Section, Indian Veterinary Research Institute, Izatnagar, India. All the experimental procedures on birds were carried out according to the recommendations and approval of the Institute Animal Ethics Committee.

2.2. Cloning and characterization of CIAV VP1 and VP2 gene constructs

The polymerase chain reaction (PCR) amplification of CIAV VP1 and VP2 genes was carried out in an automated thermal cycler (PTC 200, MJ Research, USA) in a 25 μl reaction volume containing 12.5 μl PCR master mix (Fermentas, USA), 10 pmoles of each primer (Table 1) and 20 ng of DNA. Cycling conditions included initial denaturation of 94 °C/4 min followed by 34 cycles of denaturation, annealing and extension at 94 °C/1 min, 60 °C/1 min and 72 °C/2 min, respectively, and final extension at 72 °C/8 min. Amplified genes were ligated with linear pTARGET vector (Promega, USA) using T4 DNA ligase (Promega, USA), transformed into JM109 competent cells, incubated overnight at 37 °C, plasmid DNA was isolated by Miniprep Kit (Fermentas, USA). The constructed clones were characterized by restriction endonuclease (RE) digestion with *Eco*RI and gene sequencing. The primers were designed and restriction sites were predicted by using Lasergene software (DNASTAR, USA).

2.3. In Vitro expression analysis of CIAV VP1 and VP2 gene constructs

The CIAV VP1 and VP2 gene constructs at the concentration of 4 μg/well of 6 well plate was used to transfect Vero cells by polyfect method. For Western blotting, transfected cells were harvested by trypsinisation and washed twice with ice-cold phosphate buffer saline (PBS). Cells were lysed, and then centrifuged at 16,000 × *g* for 5 min. Supernatant containing protein extract were separated. Finally, 30 μg protein from each group was resolved in 15% SDS polyacrylamide gel and electroblotted on to the nitrocellulose membrane using semi-dry blotting apparatus. Membrane was blocked with 2% BSA and probed with appropriate dilutions of CIAV serum, and incubated with rabbit anti-chicken IgY-HRPO conjugate (1:12,000 dilution). The bands were visualized following incubation with diaminobenzidine tetra-hydrochloride.

2.4. Cloning and expression of chicken HMGB1ΔC gene construct

The total RNA was extracted from the unstimulated peripheral blood mononuclear cells (PBMC) of SPF chicks, separated by density-gradient centrifugation using histopaque (1.077 g/ml, Sigma, USA). The reverse transcription was carried out as described by Sawant et al. (2011) [19]. The cDNA synthesized was used to amplify HMGB1ΔC employing PCR using HMGB1 F and R primers, introducing *Eco*RI and *Xho*I restriction sites, under same reaction conditions as described in Section 2.2. The cycling conditions included initial denaturation of 94 °C for 5 min followed by 36 cycles of denaturation, annealing and extension at 94 °C/1 min, 55.6 °C/1 min and 72 °C/1 min, respectively, and final extension at 72 °C/8 min. The amplified HMGB1ΔC gene was digested and ligated to pET32b vector (Novagen, USA) digested with the same restriction enzymes. The constructed plasmid was then transformed into *Escherichia coli* BL21 Poly Lys competent cells, incubated overnight at 37 °C, and the plasmid DNA isolated and characterized by gene sequencing. Further, 50 ml culture of transformed *E. coli* BL21 Poly Lys was induced by IPTG at 37 °C and the samples were taken at 0 h, 2 h, 4 h and 6 h. The expressed protein was confirmed by Western blotting using anti-His HRPO conjugate.

2.5. Functional characterization of rHMGB1ΔC

Monocytes were isolated from PBMCs using plastic adhesion methodology [25]. Assuming that 10% population of the PBMCs (10^7 cells/ml) in the wells (100 μl/well) of cell culture plate will be monocytes, the concentration of the adherent monocytes would be approximately 5×10^6 cells/ml. The monocytes were stimulated with different concentrations of rHMGB1ΔC (1, 10, 100 ng) *in vitro* for 24 h with or without polymyxin B (10 μg/ml) and in the presence of lipopolysaccharide (LPS) (positive control, 1 μg/ml). After that monocytes were harvested, RNA samples (1 μg for each reaction) were DNase treated and reverse transcribed to cDNA as

Table 1
Description of primer sequences used for PCR and real-time PCR.

Sr. no.	Primer	Mers	Primer sequence (5′–3′)	Amplicon size (bp)
1	HMGB1 F	30	GCATTGAATTCAATGGGCAAAGGTGATCCC	569
	HMGB1 R	29	GGCGCCTCGAGCTTCTTCTTCTTGCTCTT	
2	VP1 F	18	ATGGCAAGACGAGCTCGC	1350
	VP1 R	18	TCAGGGCTGCGTCCCCCA	
3	VP2 F	19	ATGCACGGGAACGGCGGAC	651
	VP2 R	20	TCACACTATACGTACCGGGG	
4	IL-6 F	19	CAAGGTGACGGAGGAGGAC	254
	IL-6 R	18	TGGCGAGGAGGGATTTCT	
5	β-actin F	22	GCACCACACTTTCTACAATGAG	184
	β-actin R	19	ACGACCAGAGGCATACAGG	