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Cloning and expression of chicken granulocyte-macrophage colony stimulating factor (GMCSF) gene

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Granulocyte-macrophage colony stimulating factor (GMCSF), a multifunctional cytokine can enhance immune responses when administered along with DNA vaccine. Aim of the present study was to clone and express the chicken GMCSF cytokine for use as 'genetic adjuvant'. Chicken GMCSF gene 435bp was amplified using specific primers in which restriction sites of *Bam*HI and *Hind*III were at forward and reverse primers respectively. The PCR product was cloned into eukaryotic expression vector pcDNA 3.1(+) and clones were confirmed by restriction digestion and nucleotide sequencing. Functional activity of recombinant GMCSF was checked by expression of GMCSF specific mRNA in transfected Vero cells by RT-PCR of total RNA isolated from transfected Vero cells. The recombinant plasmid can be used as genetic adjuvant in chicken.

Keywords: Genetic adjuvant, GMCSF, PCR

Cytokines are essential effector molecules of innate and acquired immunity which initiate and coordinate cellular and humoral responses against pathogens. Proper communication between pathogen and host cells via pattern recognition receptors (PRRs) and pathogen associated molecular patterns (PAMPs) initiates signal transduction pathways which in turn induces the expression of cytokines and costimulatory molecules which direct the appropriate immune response[1].

Cytokines, chemokines and T cells co-stimulatory molecules as part of vaccine itself help in improving the efficacy of vaccines as they work as 'genetic adjuvant'. Effectiveness of recombinant cytokines in various vaccine adjuvant has been well established[2]. These recombinant immunomodulators either increase antigen uptake, their processing and presentation capacities or their functional polarization towards selective stimulation of T cell subsets and B cells[3]. By this strategy, the direction of an immune response can also be modulated towards a cell mediated Th1, or antibody mediated Th2 type responses by differential expression of cytokine patterns.

Co-administration of plasmid cytokine adjuvant can augment DNA vaccine-elicited humoral and cellular immune responses in animal models. GM-CSF is a glycoprotein that regulates proliferation and differentiation of hematopoietic progenitors and has multifunctional role including reversal of neutropenia associated with cytotoxic chemotherapy, bone marrow and haemopoietic stem cell transplantation. It has a potent effect on differentiation and maturation of dendritic cells (DCs) as well as expression of MHC and co-stimulatory molecules[4-6]. The DC system is the initiator and modulator of the immune response.

Adjuvant properties of GM-CSF are due to its ability to attract the antigen presenting cells to the site of inoculation[7] and stimulate maturation of dendritic cells[8]. Research finding revealed that GM-CSF exhibit its adjuvant property without skewing the Th1/Th2 balance. Thus, facilitating generation of both humoral and cell-mediated immunity[9,10]. Its use has been explored in conjunction with a number of experimental vaccines including those for human

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immuno-deficiency virus (HIV)[11,12] hepatitis C[13], leishmaniasis[14], influenza[15], pseudorabies virus[8] and hepatitis C virus[16]. However, till now GMCSF based genetic adjuvant has not been developed for use in chicken. Keeping in view the role of GMCSF in modulating immune response, in the present study we have amplified chicken GMCSF and expressed in eukaryotic system for using it as a genetic adjuvant along with DNA vaccine.

Materials and Methods

Primer designing — Primers were designed for chicken GMCSF using *Lasergene* software (DNASTAR, Inc. Madison, WI, USA), from the sequences available at GenBank. Specificity of primers was checked by NCBI blast programme (http://www.ncbi.nlm.nih.gov/BLAST/). For chicken GMCSF, restriction enzyme (RE) sites (*Bam*HI in forward and *Hind*III in reverse primer) were added to facilitate cloning in the same RE Sites in pcDNA3.1(+) expression vector.

Peripheral blood mononuclear cells (PBMC) culture — Blood collected from Rhod Red Island chicken in heparinized tube was first depleted of thrombocytes by low-speed centrifugation. The buffy coat was then layered on Histopaque (Sigma, USA) and centrifuged for 15 min at 800 g. Cells at interface were collected and washed three times with medium. PBMC were cultured at a density of 5×10^6 cells/well in RPMI-1640 medium. Then, PBMC were placed in 25 cm^2 flask and induced with 10 µg/ml concanavalin A (Con A) (Sigma).

RNA extraction and preparation of cDNA — Total RNA was extracted by using TRIreagent (Sigma, USA) as per the manufacturer's protocol. Briefly, TRIreagent (1 ml) was added to 200 µl of culture fluid, mixed thoroughly and incubated for 10 min at room temperature (RT). 200 µl of chloroform was added, mixed and centrifuged at 10,000 rpm for 10 min at RT. Total RNA present in the aqueous phase was precipitated with 500 µl of iso-propanol and RNA pellet was washed with 70% ethanol, air dried, and resuspended in 20 µl of nuclease free distilled water.

cDNA was synthesized in 25 µl reaction volume using random hexamer primers. For this, 5 µg of total RNA was mixed with 50 ng random hexamer primers and incubated at 70°C for 10 min followed by 5 min incubation at 25°C. Then 5 µl of 5 × RT buffer, 40 U of RNase inhibitor, 1 µl of 10 m*M* dNTPs mix and 200 U of 'RevertAid' reverse transcriptase (MBI Fermentas) was added and incubated at 42°C for 1 h followed by 15 min at 75°C to denature the enzyme. cDNA thus formed was stored at -20°C till used.

Amplification and cloning of GMCSF — GMCSF was amplified using specific primers, forward 5'-GGCCGGATCCATGCTGGCCCAGCTCACTA-3' and reverse 5'-GGCCAAGCTTTTAGATGCAGTC TTTCTCCTCTG-3'. For amplification of GMCSF gene, reaction was performed in 50 µl volume containing 5 µl of 10 × PCR buffer, 1.5 m*M* Mg^{++}, 10 pmol of each forward and reverse primer, 200 µ*M* of dNTPs mix, 2.5 U of Taq DNA polymerase and 5 µl of cDNA. After initial denaturation at 95°C for 2 min the reaction was carried out in a thermal cycler (Biometra, U.K.) using following conditions: 35 cycles each of denaturation (95°C for 30 sec), annealing (48°C for 45 sec) and extension (72°C for 1 min). Final extension was done at 72°C for 5 min. The PCR amplicon (10 µl) was analyzed in 1.5% agarose gel in containing 0.5 µg/ml ethidium bromide at 70 volts for 1 h and visualized on ultraviolet transilluminator.

The PCR product was gel purified using 'MinElute Gel Extraction Kit' (QIAGEN, Germany) as per the protocol given by the manufacturer. DNA eluted with 20 µl of elution buffer was checked again by gel electrophoresis on 1.5% agarose gel. Concentration and purity of DNA was assessed by Nanodrop (Nanodrop 2000c, Thermo Scientific). Then eluted PCR product was digested with *BamHI* and *HindIII* restriction enzymes. Further, this digested amplicon i.e GMCSF was cloned into a eukaryotic expression vector pcDNA3.1 (+) at *BamHI* and *HindIII* restriction sites. Recombinant plasmid was isolated and confirmed by restriction enzyme digestion using *Bam*HI and *Hind*III enzymes.

Nucleotide sequencing — Recombinant positive clone pcDNA.ch.gmcsf was got sequenced at "DNA sequencing facility" Chromous Biotech, Bangalore. Nucleotide sequences were checked using BLAST (NCBI) and compared with the reported sequences from UK and China using Lasergene software (DNASTAR, Inc. USA).

Expression by RT-PCR — For transfection, 1.5 µg plasmid DNA mixed with 12 µl PolyFect

(QIAGEN, Germany) was used for 4×10^5 Vero cells in serum free medium in a six well plate. After 5-10 min incubation at room temperature, 0.6 ml fresh cell growth medium (DMEM with 10% FBS) was added to the mixture and layered over cells grown to 60% confluency in a six well plate and incubated again at 37°C and 5% CO_2 for expression for 72 h. At the end of incubation, cells were harvested, total RNA was extracted and RT-PCR was performed using nested set of primers (forward 5'-CAGCGGCCACAGCA GGTC-3' and reverse 5'-GCGGGGTGGGAGTTAT GTTC-3') as described earlier. PCR product was analyzed by gel electrophoresis on 1.5% agarose gel to confirm the expression.

Results and Discussion

Recombinant cytokines have been used clinically in the treatment of human diseases including cancers and infectious diseases[17-20]. In the present study, chicken GMCSF was amplified and cloned into a eukaryotic expression vector for use as genetic adjuvant. Total RNA was extracted from ConA stimulated PBMC using TRIreagent and GMCSF was amplified using 5 µl of cDNA. Specific single band of approximately 435bp in PCR as seen in ethidium bromide stained 1.5% agarose gel (Fig. 1) confirming the amplification of chicken GMCSF. Amplified PCR product was gel purified and digested with *BamHI* and *HindIII* restriction sites. The digested product was visualized in long-range UV light and the band representing the GMCSF was excised and purified using "Gel Extraction kit" (QIAGEN). The concentration of purified insert was 30 ng/µl. Stimulated macrophages has also been used to extract total RNA for amplification of GMCSF genes[21].

Therefore, GMCSF was cloned into pcDNA 3.1(+), a eukaryotic expression vector at *Bam*HI and *Hind*III sites. Following transformation to *E. coli* DH5α cells, the recombinant colonies were selected and propagated. The recombinant plasmids with GMCSF gene insert (pcDNA.ch.gmcsf) was confirmed following the digestion with *Bam*HI and *Hind*III which released the fragment of desired 435bp (Fig. 2). In the present study, pcDNA3.1 was used to develop genetic adjuvant. It is a high level constitutive expression vector designed for use in a variety of cell lines. The vector has cytomegalovirus (CMV) enhancer promoter, bovine growth hormone, polyadenylation signal and transcription sequence, f1 origin for single-stranded rescue of the sense strand DNA and SV40 origin for episomal replication. Ampicillin resistance gene and ColE1 origin is provided for selection and maintenance of recombinant in *E.Coli*. The studies by many workers revealed that vectors containing the CMV

Fig. 1— Agarose gel electrophoresis showing amplification of chicken GMCSF at induced PBMC [Lane M: 100 bp DNA Ladder; Lane 1-2: amplification of 435bp PCR product for chicken GMCSF gene]

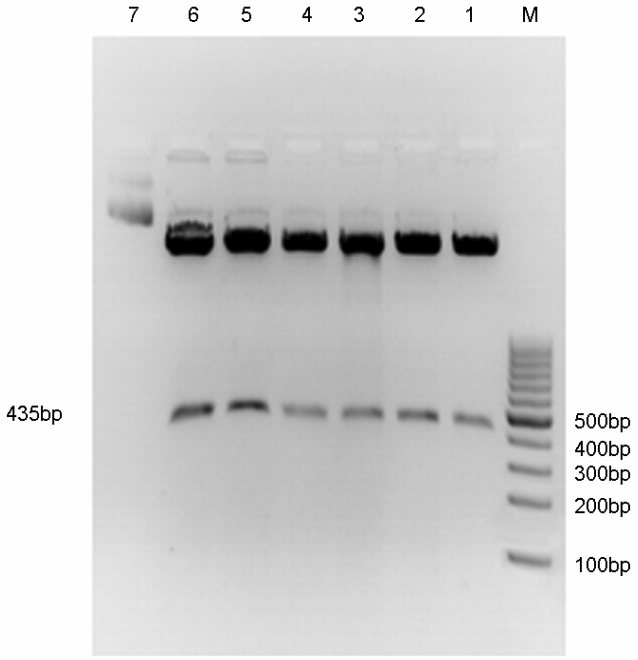

Fig. 2— Agarose gel electrophoresis showing confirmation of GMCSF gene insert in pcDNA3.1 (+) eukaryotic expression vector through restriction enzyme analysis [Lane M: 100bp DNA Ladder; Lane 1-6: recombinant clones releasing 435bp insert on digestion with *BamHI* and *HindIII* restriction enzymes; Lane 7: undigested recombinant clone]

promoter and the bovine growth hormone termination signal, work well in the DNA vaccination[22,23]. There are many reports where pcDNA3.1(+) has been used as a vector for developing DNA vaccine[24,25].

The recombinant plasmid pcDNA.ch.gmcsf was sequenced from the sequencing facility, Chromous Biotech Pvt. Ltd., Bangalore. The sequence data was stored in "EditSeq" programme of 'Lasergene' software (DNASTAR) for sequence analysis. The sequence was submitted to GenBank and is available with accession number was GQ421598. The sequence obtained in this study was compared and analyzed using MegAlign programme of Lasergene software with reported sequences of GMCSF EU939770.1 from China and NM_001007078.1 from UK. Nucleotide alignment showed there was a nucleotide substitutions 28G-T in the GMCSF used in this study and 367A-G in GMCSF reported from birds of china (Fig. 3) (Accession no. EU939770.1) Nucleotide comparison of the GMCSF amplified in this study (GQ421598) with those of reported sequences) demonstrated 99.5% homology with the sequence from China and 99.8% homology with UK. (Fig. 4)

For expression studies, the recombinant pcDNA.ch.gmcsf containing complete GMCSF gene was purified using 'Endofree Maxi columns' (QIAGEN). The purified plasmid DNA was checked by taking $OD_{260/280}$ and ratio of OD_{260}/OD_{280} was found to be 1.8, which indicated that the DNA preparation was pure double standard DNA and free of RNA and protein contaminations.

The purified plasmid DNA at the concentration of 1.5 µg per well of 6 well plates was used to transfect Vero cells by Polyfect^{R} Transfection reagent (QIAGEN). The transfected cell lysate was collected at 72 h post transfection, along with mock and vector transfected control to check the expression by RT-PCR using nested set of primers which yielded specific band of 202 bp (Fig. 5), indicating that GMCSF cloned in this study are functionally active. Many other workers have also used RT-PCR for confirmation of expression *in vitro*[24,25]. As recombinant pcDNA.ch. gmcsf contains functionally

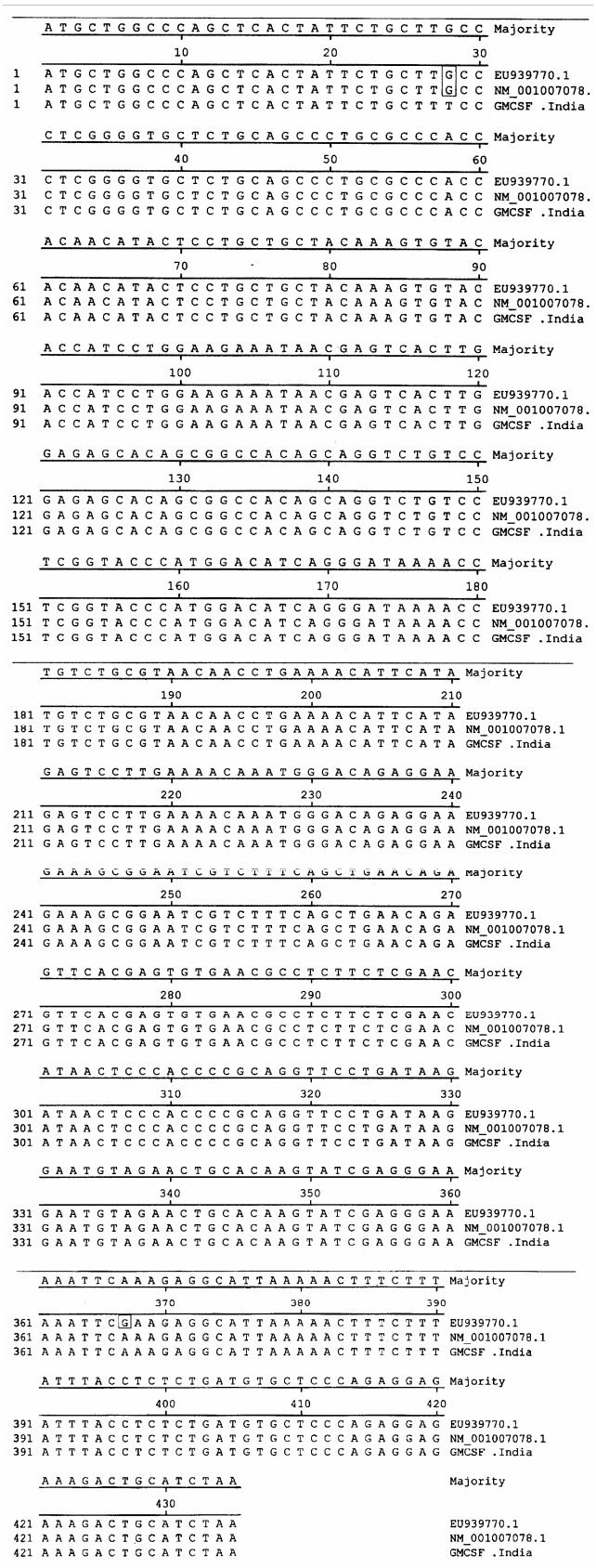

Fig. 3— Sequence alignment of 435bp of GMCSF. Only changes in relation to the consensus sequence are indicated

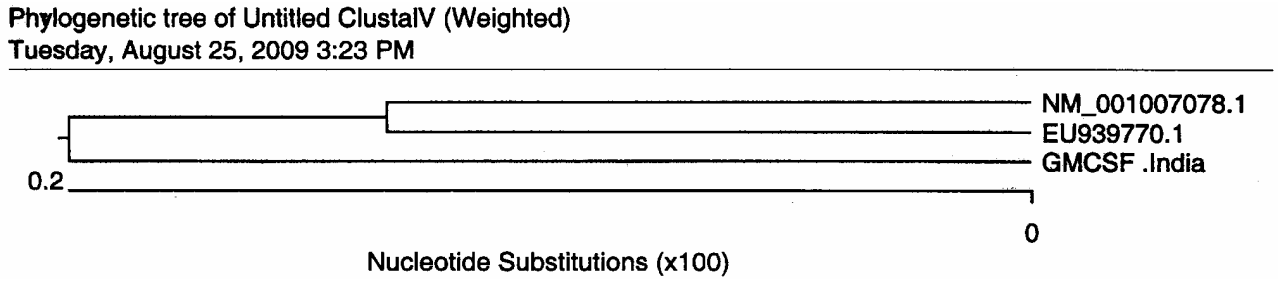

Fig. 4— Rooted phylogenetic tree showing relationship between GMCSF India and others

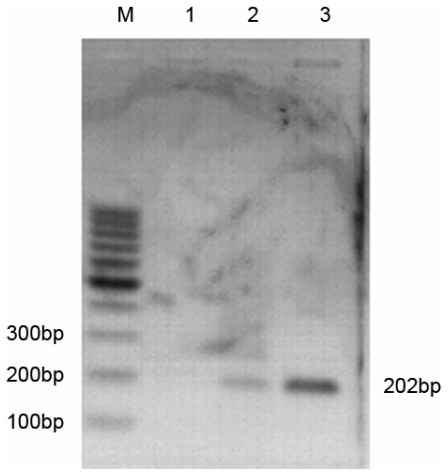

Fig. 5— Characterization of *in vitro* expression of GMCSF by RT-PCR [Lane M: 100bp DNA ladder (Fermentas); Lane 3: transfected cell lysate collected at 72 h post transfection; Lane 2: transfected cell lysate collected at 48 h post transfection; Lane 1: mock control]

active chicken GMCSG, which expresses GMCSF, it can be used as a genetic adjuvant with different vaccine formulations.

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