Indian Journal of Animal Sciences **83** (4): 398–401, April 2013/Article

Cloning, expression and phylogenetic analysis of IFN-γ gene in chicken

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Received: 23 June 2012; Accepted: 10 November 2012

ABSTRACT

In the present study, chicken interferon gamma (Ch.IFN-γ) gene was amplified using specific primers by RT-PCR and cloned into pTZ 57R/T cloning vector. The clone was confirmed by restriction digestion analysis using *Kpn*I and *Apa*I restriction enzymes and orientation was confirmed by colony PCR using T7 promoter primer and gene specific forward primers. Further, the gene fragment was sub-cloned in eukaryotic expression vector pcDNA 3.1(+) and confirmed by restriction digestion. The recombinant pcDNA 3.1 having IFN gene (pcDNA.Ch-IFN-γ) was checked for its ability to express IFN-γ *in vitro* in cell culture and expression was confirmed by RT-PCR, indicating the gene is functionally active. The study to evaluate ability of pcDNA.Ch-IFN-γ to combat infection and enhance efficacy of DNA vaccine is under way.

Key words: Genetic adjuvant, IFN γ, RT-PCR

Cytokines are essential effector molecules of innate and acquired immunity that initiate and coordinate cellular and humoral responses aimed for eradicating pathogens. The cytokines have been successfully used in humans for the treatment of immune-deficiencies. There are various methods of cytokine administration that are currently being developed by several groups which include delivery via live vectors (viral and bacterial), DNA injection and injection of protein. The genes for chicken myelomonocytic growth factor (cMGF) (Hilton *et al.* 2002), stem cell factor (SCF) (Zhou *et al.* 1993), interleukin-8 (IL-8) (Leutz *et al.* 1984), type 1 interferon (Ch.IFN-γ) (Sekellick *et al.* 1994) and type 2 interferon (Ch.IFN-γ) (Digby and Lowenthal 1995) were cloned and recombinant protein was expressed and characterized.

IFN-γ is a member of the interferon family of cytokines that share the property to modulate the immune response and inhibit viral replication and has anti-tumor properties. IFN-γ stimulates immunoglobulin production (Samuel 2001) and specific cytotoxicity of T cells (MacDonald *et al.* 2002). IFN-γ, secreted by Th-1 cells, Tc cells, dendritic cells and NK cells, is also known as immune interferon. Ch.IFN-γ is one cytokine that was assessed for its ability to enhance antibody response. When co-administered with antigen, recombinant Ch.IFN-γ produces a prolonged secondary antibody response that persists at higher levels and for longer periods compared to antigen injected alone (Lowenthal *et al.* 1998). Hence, it is candidate 'Genetic adjuvants' for humoral responses to soluble protein antigens (Schijns *et al.* 2000). The ability of Ch-IFN-γ to combat and enhance vaccine efficacy makes it an excellent candidate as a therapeutic agent and adjuvant. Therefore, in the present study, chicken IFN-γ was amplified and cloned in mammalian expression vector for use as 'Genetic Adjuvant' along with DNA vaccine.

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MATERIALS AND METHODS

Pooled heparinized blood (2 ml) collected from 3 birds was first depleted of thrombocytes by low-speed centrifugation. The buffy coat was then layered on histopaque and centrifuged for 15 min at 800 ×g. Cells at interface were collected and washed 3 times with PBS. Viability of these cells was assessed by dye exclusion test using trypan blue stain. The cells were counted in a countess, the concentration of cells adjusted to 2×10^6 cells / ml in RPMI-1640 medium, plated in a 6 well plate and induced with 1 µg of LPS (lipopolysacchride)/ml of medium. The induced PBMC were

harvested by low-speed centrifugation at 2 h, 4 h and 6 h post induction. Total RNA was extracted from harvested cells using RNAgents-total RNA isolation system according to the manufacture's instruction. Possible traces of genomic DNA were removed by treating RNA samples with RNase free DNase. The total RNA sample was reverse-transcribed using random hexamer primers and the cDNA synthesis kit according to the manufacturer's instruction. The cDNA conversion and intergrity of RNA was tested by amplification of ß-actin gene fragment of 273 bp using specific primers (forward 5'-GAGAAGCTGTGCTACGTCGC-3' and reverse 5'-CCAGACAGCACTGTGTTGGC-3'). PCR was then performed on the cDNA using primers designed from the 5' and 3' untranslated regions of the IFN-γ gene in a thermal cycler. The amplification was carried out in a 25 µl reaction volume, containing 10 pmol each of forward 5'-GAGCCATCACCAAGAAGA-3' and reverse primer 5'-GAGCACAGGAGGTCATAAGA-3', 2 µl cDNA, 1X Taq DNA polymerase buffer containing 2.5 mM $MgCl_2$, 200µM dNTPs mix and 1 U Taq DNA polymerase. After initial denaturation at 94° C for 3 min, the amplification was carried out for 35 cycles each consisting of 40 sec at 94°C, 45 sec at 52°C and 1 min at 72°C with a final extension of 10 min at 72°C. The resultant product of 495 bp was checked on 1% agarose gel (Fig. 1a) and cloned into InsT/A pTZ57R/T as per the manufacturer's instruction. The insert was confirmed by restriction enzyme digestion using *Kpn*I and *Apa*I (Fig. 1b). This recombinant plasmid containing IFN-γ gene insert was digested with *Kpn*I and *Apa*I and the Ch.IFN-γ was gel purified using gel extraction kit and subcloned in to pcDNA 3.1(+) eukaryotic expression vector at *Kpn*I and *Apa*I restriction enzyme sites (Fig. 1c). The recombinant pcDNA.ch.IFN-γ plasmid DNA was prepared by endo-free plasmid maxi kit for use in transfection experiments. The expression of IFN-γ was evaluated *in vero* cells grown to 70% confluency in a 6 well plate and pcDNA.ch.IFN-γ after transfecting pcDNA.ch.IFN-γ using the transfection reagent as per the manufacture's protocol. Briefly, 1.5µg recombinant plasmid DNA mixed with 12 µl polyfect was used to transfect approximately 4×10^5 cells in serum free medium. The mixture was incubated for 5–10 min at room temperature for complex formation then 0.6 ml growth medium was added and layered over the cell monolayer and incubated at 37°C under 5% CO_2 tension. The expression of IFN-γ was confirmed by RT-PCR. Total RNA was extracted from transfected cells at 48 h and 72 h post transfection. The isolated RNA treated with RNAse free DNAse was used in RT-PCR to confirm the expression of IFN-γ using nested set of primers (forward 5'-GCCGCACATCAAACACATATCTG-3' and reverse 5'-GCGCTGGATTCTCAAGTCGTTC-3') to amplify 119bp fragment. This proved the expression of IFN-γ in pcDNA.ch.IFN-γ transfected vero cells. Suitable controls (mock and vector transfected control) were also used in the experiment. This reaction was performed in 50 µl reaction volume containing 5µl of 10× PCR buffer, 1.5 mM Mg^{++}, 10 pmol of each forward and reverse primer, 200µM of dNTPs mix, 2.5U of Taq DNA polymerase and 5µl of cDNA. After initial denaturation at 95°C for 2 min, the reaction was carried out in a thermal cycler for 35 cycles each of denaturation (95°C for 30 sec), annealing (52°C for 45 sec) and extension (72°C for 1 min) with final extension at 72°C for 5 min.

The IFN-γ gene was sequenced at a commercial company, from the recombinant plasmid pcDNA.ch.IFN-γ. The sequence was submitted to GenBank and is available with accession number of GQ421600. Neighbor-joining, maximum parsimony and UPGMA (unpaired group mean average) methods were used for phylogenetic analysis (MEGA 4.0) (Tamura *et al.* 2007).

RESULTS AND DISCUSSION

The nucleotide sequence of IFN-γ was aligned with the available reference sequences (AY163160.1, AJ634956.1, AF424744.1, DQ474071.1 and AY501004.1) using DNASTAR (Lasergene). Upon comparison of the nucleotide sequence GQ421600 with those of reference sequences, it was found that there was 99.4% identity with AF424744.1, 100% identity with AJ634956.1, 96.8% identity with AY163160.1, 99.6% identity with AY501004.1 and 99.4% identity with DQ470471.1. In case of clustering (rooted phylogenetic tree), the isolate of the study was found to be closer to the sequence of AF424744.1 and AJ634956.1. Also,

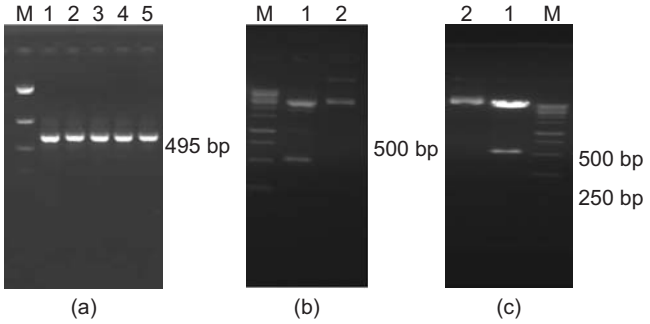

Fig. 1a-c**. A**. Agarose gel electrophoresis showing amplification of Ch IFN-γ at induced PBMC were harvested by low –speed centrifugation at regular time intervals after induction (2, 4 and 6 h). Lane M: 1 Kb DNA Ladder (NEB).Lane 1 to 5: Amplification of 495bp PCR product for chicken IFN-γ gene.**b.** Agarose gel electrophoresis showing confirmation of IFN-γ gene insert in ptz57R/T cloning vector through restriction enzyme analysis. Lane M, 1 Kb DNA ladder. Lane 1, recombinant clone releasing 495 bp insert on digestion with *Apa* I and *Kpn* I restriction enzymes. Lane 2, undigested recombinant clone. **c.** Agarose gel electrophoresis showing confirmation of IFN-γ gene insert in pcDNA3.1 (+) eukaryotic expression vector through restriction enzyme analysis.Lane M: 1 Kb DNA Ladder.Lane 1: Recombinant clones releasing 495 bp insert on digestion with *Apa* I and *Kpn* I restriction enzymes.Lane 2: Undigested recombinant clone.

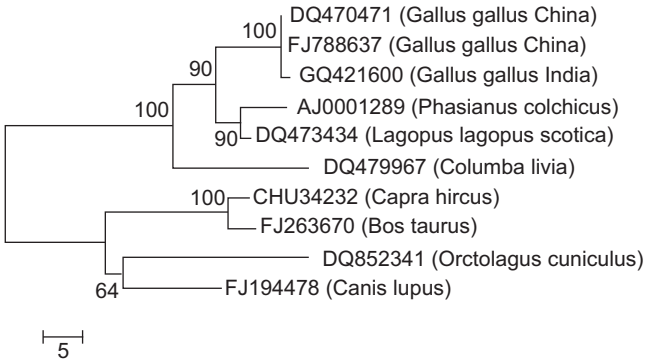

Fig. 2. Neighbour- joining tree obtained from IFN-γ gene sequence data of different species. Bootstrap values and distances are indicated.

the sequence in the present study was subjected to nucleotide blast (Altschul *et al.* 1997) and from the top 50 hits, representative sequences were chosen for phylogenetic analysis.

Both the neighbor joining and the maximum parsimony trees were consistent in generating trees with similar topologies. The neighbour joining tree with relatively higher bootstrap values provided clear resolution of all the nodes. The sequence in the present study clustered along with other avian sequences to a common node. This node was closer to the ruminants and farthest to the rabbit and dog sequences (Fig. 2).

Many cloned cytokine are available as recombinant genes or proteins derived from *in vitro* expression in eukaryotic or prokaryotic systems respectively. Previous studies showed the potential of cytokines, such as IFN-γ, as therapeutic agent and adjuvant (Lawmann *et al.* 1990, Heath and Playfair 1992, Hilton *et al.* 2002). IFN-γ was found to be an effective anti-bacterial (Kagaya *et al.* 1989), anti-parasitic and anti-viral agent in a variety of animal species (Heath and Playfair 1992). Other studies have demonstrated that T cell responses such as delayed type hypersensitivity and T helper cell for antibody production were enhanced following the co-administration of IFN-γ with a vaccine. An *in vivo* challenge study of bicistronic DNA vaccine expressing IBDV-VP2 and chicken IL-2 showed effective protection against a lethal IBD infection in chickens (Sachin *et al.* 2009). Boretti *et al.* (2000) observed increased antibody response when ChIL-1ß was administered *in vivo* as a recombinant protein compared to antigen alone. Combined administration of Ch.IFN-α, Ch.IFN-γ and ChIL-1ß increased antibody responses to tetanus toxoid in an additive manner (Lowenthal *et al.* 1998). This supports the possibility that cytokines in synergy could be effective adjuvants and immune responses may be better when used in combination. IFN-γ activates antigen presenting cells (APCs) by stimulating the expression of class I and class II MHC and co-stimulatory molecules, as well as it increases their microbicidal activity through formation of nitric oxide and reactive oxygen intermediates (ROI). Keeping in view the advantages of using cytokine gene in immunity, IFN-γ gene is cloned into eukaryotic expression vector to be used as an adjuvant with DNA vaccine in poultry.

The vectors containing the CMV promoter and the bovine growth hormone termination signal, work well in the DNA vaccination (Jouanguy *et al.* 1999, Eckmann and Kagnoff 2001). Therefore pcDNA3.1 (+) containing cytomegalovirus (CMV) enhance promoter, bovine growth hormone, polyadenylation signal and transcription sequence, f1 origin for single stranded rescue of the sense DNA and SV40 origin for episomal replication was a vector of choice in the present study.

Despite the suggested potential of IFN-γ, the number of studies addressing *in-vivo* IFN-γ adjuvanticity has been limited. When co-administered to birds with antigen, recombinant rCh.IFN-γ produced a prolonged secondary antibody response that persisted for extended period compared to antigen injected alone (Lowenthal *et al.* 1998). However, the study to evaluate pcDNA.Ch.IFN-γ as genetic adjuvants and enhance efficacy of DNA vaccine is under way.

ACKNOWLEDGEMENNT

The authors are grateful to the Department of Biotechnology, Government of India for financial assistance (project code: BT/PR6473/AAQ/01/242/ 2005).

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