Ovine interferon-γ gene of indigenous sheep: Cloning, sequencing and expression studies in *Escherichia coli*

T J Rasool*, M Hosamani, A Premraj¹, E Sreekumar¹ and R K Singh

Biotechnology Laboratory, Indian Veterinary Research Institute, Mukteswar 263 138 (Nainital), India
¹Animal Biotechnology Laboratory, Rajiv Gandhi Center for Biotechnology (RGCB), Pujapura, Trivandrum 695 012, India

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Ovine interferon-gamma (IFN-γ) cDNA was isolated from total mRNA of peripheral blood mononuclear cells from indigenous sheep by reverse transcription PCR. The cDNA was successfully cloned, sequenced and expressed in *Escherichia coli* as thioredoxin fusion protein. Sequence comparison of Indian sheep IFN-γ showed high nucleotide homology with published IFN-γ sequences of sheep and other species of ruminants. The cDNA codes for a mature polypeptide with a putative molecular weight of 17 kDa. The recombinant ovine IFN-γ, expressed in BL21 *E. coli* cells as His-tagged protein, was purified using Ni-chelation chromatography. Up to 250 µg of recombinant ovine IFN-γ could be obtained from 50 mL of bacterial culture. The production of this important cytokine has significant implications in therapy of major infectious diseases of small ruminants. It also has potential applications as adjuvant to enhance the protective efficacy of the existing vaccines.

**Keywords**: cDNA, cytokine, expression, interferon gamma, sheep

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**Introduction**

Interferon-gamma (IFN-γ) is a pleiotrophic cytokine that plays central role in regulation of body’s immune responses. It plays the major role in immunity against intracellular pathogens, where cell mediated immunity is predominant. It has antiviral activity against a number of viruses¹ and also antitumor activity². The cytokine is principally secreted by activated lymphocytes and natural killer (NK) cells. In addition, IFN-γ is the most widely tested adjuvant cytokine in many vaccine formulations including DNA vaccines. In view of the multiple roles and potential practical applications attributed to this lymphokine, it has been characterized from many livestock species, including cattle, buffalo, goat and sheep³⁻⁷. In the present study, we have attempted the cloning and expression of IFN-γ from Indian ovines so as to be useful as an adjuvant along with the diverse vaccines being developed/produced for small ruminants, such as peste des petits ruminants, sheep and goat pox, etc.

*Author for correspondence:*
Tel: 91-5942-286346; Fax: 91-5942-286347
E-mail: tjrasool@rediffmail.com

**Materials and Methods**

**Cloning and Sequencing of Ovine IFN-γ**

Sheep peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood employing gradient centrifugation method using histopaque 1.077 (Sigma, St Louis, MO). Cells were suspended in RPMI 1640 medium containing 10% fetal bovine serum (Sigma), 25 mM L-glutamine and concanavalin A at final concentration of 5µg/mL. Cells were incubated for 8 h at 37°C under 5% CO₂ tension. Total RNA was isolated using TRI reagent (Sigma) according to manufacturer’s instructions. RNA was reverse transcribed by MMLV reverse transcriptase using oligo dT primer. From the cDNA, IFN-γ specific cDNA was amplified by PCR amplification using specific primers. Primers were designed based on the published sequence of ovine IFN-γ cDNA and have the following sequence: forward primer: ggtttttctggttcttatgg and reverser primer: ttgcaggcaggagaaccatta. The amplicon (472 bp) was gel purified and ligated into pGEMT Easy vector (Promega, Madison, WI) and transformed into JM109 *Escherichia coli* competent cells. Orientation of the insert in the recombinant plasmid was verified by restriction digestion analysis and...
sequencing. Sequencing was carried out using automated sequencer (ABI 377).

**Sequence Analysis**

Sequencing was carried out in automated DNA sequencer (ABI 370). The deduced amino acid sequences of IFN-γ homologues from different species, available from the NCBI database, were assembled into multiple sequence alignment using clustal W (version 1.81) program and edited manually. Phylogenetic analysis was carried out by MEGA version 2.1 using neighbor joining method with 1000 bootstrap replicates.

**Construction of Expression Plasmid Vector**

For construction of IFN-γ cDNA, another set of primers were employed that amplified the entire open reading frame excluding the signal sequence. The sequence of the primer was as follows. Sense primer 5'-gTTCTTATggCCAgggCCAAT-3'; anti-sense primer 5' TTACgTTgATgCTCTCCggCCTCg 3'. The PCR product (443bp) was cloned into pGEMT-Easy vector and sub cloned into pET32b vector using EcoRI site (Novagen, Madison, WI). The recombinant pET32_oIFN-γ was bidirectionally sequenced to confirm the correct orientation and fidelity of frame. Recombinant plasmid was transformed into BL21DE3pLyss host and the recombinant colonies were grown overnight in the presence of ampicillin. Bacterial culture was diluted with fresh LB broth after pelleting overnight culture and grown for 2 h at 37°C. Expression was induced by growth at 30°C for 4 h with addition of IPTG at final concentration of 1mM.

**SDS-PAGE Analysis**

Bacterial pellet was lysed in equal volume of 2× sample loading buffer and subjected to SDS-PAGE analysis using 12% acrylamide gel. The protein was visualized with comassie brilliant blue stain.

**Western Blotting**

Protein-samples were separated on a 12% SDS-PAGE and transferred onto a nitrocellulose membrane (Amersham) using a Trans-Blot SD Semi dry electrophoretic transfer apparatus (BioRad, Hercules, CA, USA). The membrane was then incubated with anti-bovine IFN-γ monoclonal antibody CC302 TC supernatant (a kind gift from Dr Chris Howard, Institute of Animal Health, Compton, UK), diluted at 1:5 in 3% BSA in TBS buffer, for 2 h at room temperature. Anti-mouse IgG alkaline phosphatase conjugate antibody (Sigma) diluted at 1:5000 in 3% BSA (Sigma) in TBS buffer was used as the secondary antibody and membrane was developed in alkaline peroxidase substrate solution [100 mM Tris-Cl, pH 9.5; 5 mM MgCl₂; 100 mM NaCl; 0.1 mg/mL NBT; 0.05 mg/mL BCIP (Sigma)] to visualize the positive reaction.

**Purification of Recombinant Protein and Protein Estimation**

For bulk production and subsequent purification of roIFN-γ, 50 mL bacterial culture was induced as previously described. The protein was purified from whole bacterial lysate using Ni chelation chromatography (Invitrogen, USA) under denaturation conditions, according to manufacturer’s protocol.

**Results**

cDNA encoding ovine IFN-γ of 472 bp was amplified using primers designed on the published sequence of ovine IFN-γ (X52640) from a nondescript sheep. The amplicon was gel purified and cloned into pGEMT-easy vector. The recombinant plasmid was transformed into BL21DE3pLyss host and the recombinant colonies were grown overnight in the presence of ampicillin. Bacterial culture was diluted with fresh LB broth after pelleting overnight culture and grown for 2 h at 37°C. Expression was induced by growth at 30°C for 4 h with addition of IPTG at final concentration of 1mM.
Expression of the recombinant construct was induced by addition of 1 mM IPTG. SDS-PAGE analysis of induced bacterial lysate showed expressed fusion protein of 34 kDa (Fig. 3). Western blot using anti-bovine IFN-γ monoclonal antibody showed the band of 34 kDa as expected (Fig. 4). Purification of the recombinant protein was carried out under denaturing conditions. Protein was obtained by elution with both 200 and 300 mM concentrations of EDTA, with higher yield being observed with 200 mM EDTA. Protein was free from extraneous proteins as evident from the SDS-PAGE analysis (Fig. 5). Total yield of the protein ranged from 4.0-5.0 mg per L of bacterial culture.

Fig. 1—Multiple sequence alignment of deduced amino acid sequences of mature IFN-γ of sheep (Ovis aries* NCBI Acc.No. DQ028767) with the reported sequences of different domestic animals including sheep (Ovis aries, X52640), goat (Capra hircus, AY603405), Zebu cattle (Bos indicus, AF533686), exotic cattle (Bos taurus, NM_174086), water buffalo (Bubalus bubalis, AF484688) Red Deer (Cervus elaphus, X63079) and camel (Camelus bactrianus, AB107657) using Clustal W (1.81) program. (*) Indicates highly conserved residue, (.) shows fairly conserved, and (:) shows less conserved residue across various species.

Fig. 2—Phylogenetic analysis of IFN-γ nucleotide cDNA of different animals using MEGA 2.1 constructed by neighbor joining method with 1000 bootstrap replicates. The details of the sequences obtained from the database are mentioned in Fig. 1. The scale bar beneath the tree indicates nt substitution per site.

orientation and proper reading frame of the insert. Expression of the recombinant construct was induced by addition of 1 mM IPTG. SDS-PAGE analysis of induced bacterial lysate showed expressed fusion protein of 34 kDa (Fig. 3). Western blot using antibovine IFN-γ monoclonal antibody showed the band of 34 kDa as expected (Fig. 4). Purification of the recombinant protein was carried out under denaturing conditions. Protein was obtained by elution with both 200 and 300 mM concentrations of EDTA, with higher yield being observed with 200 mM EDTA. Protein was free from extraneous proteins as evident from the SDS-PAGE analysis (Fig. 5). Total yield of the protein ranged from 4.0-5.0 mg per L of bacterial culture.
Discussion

IFN-γ plays a central role in immune regulation by augmenting both innate and adaptive immunity. This is the key cytokine in immunity against intracellular pathogens. Levels of IFN-γ are significantly enhanced in active infections involving intracellular pathogens, such as Mycobacterium, Neosporum, Toxoplasma, Corynebacterium and other viral infections. Detection of bovine IFN-γ using monoclonal antibody test is based on this principle.

Sheep IFN-γ has been sequenced and expressed as recombinant GST fusion protein in E. coli. Present study reports the cloning and sequencing of ovine IFN-γ and its phylogenetic relatedness with published sequences available in the database. It also reports the expression of recombinant protein in E. coli and standardization of conditions for optimized expression and its subsequent purification using affinity purification.
Full-length ovine interferon is 166 amino acid long polypeptide having signal peptide spanning 23 amino acids on the N-terminal end. In the present study, cDNA corresponding to mature polypeptide of 432 bp (143 aa) was amplified using primers designed on the published ovine sequence. Results of PCR amplification of cDNA showed that unstimulated PBMC did not produce IFN-γ under normal conditions, while it could be upregulated by stimulation with Con-A. Further, isolation of mRNA at different time intervals showed that maximum yield could be obtained from PBMC following 8 h of incubation with mitogen. Both nucleotide and deduced amino acid sequences of mature interferon gamma of sheep were compared with sequences of different ruminant species obtained from the NCBI database. Sequence analysis reveals high-level conservation of the cytokine among the ruminants. Presence of Q3, R6 and R38 were uniquely observed in cattle and buffalo sequences, while residues P3, K6 and E38 were characteristic of sheep and goat sequences. Occurrence of K94 was consistent in all the species, except ovines in which K94R substitution was observed. Similarly, presence of S19 was consistent in the cattle sequences unlike in other species. Phylogenetic relationship of the IFN-γ of all the domestic ruminant species was examined. Sequences of small ruminants comprising sheep and goat IFN-γ clustered together, while large ruminants including cattle and buffalo sequences formed another cluster. Red Deer and camel sequences showed high divergence, as they did not cluster with any of the above species.

Mature ovine interferon-γ has a predicted mol wt of 17 kDa. In the present study, mature cytokine was expressed as thioredoxin fusion protein with histidine tag at the N-terminal as 34 kDa protein. This strategy was pre-empted by failure of earlier attempts to express the full-length bovine IFN-γ with signal sequence. Bacterial cultures harvested at different intervals showed that maximum level of expression is achieved at 4 h of induction. Purification of the protein under native purification conditions did not show the presence of protein, implying that the protein formed inclusion bodies and was insoluble in nature. Therefore, purification was carried out under denaturation conditions. Further work is in progress to determine the antiviral and adjuvant properties of purified protein. It would be important to use recombinant cytokine as therapy in the treatment of major infectious diseases of sheep or as an adjuvant along with the vaccines, such as PPR and sheep pox.

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References