

## Cloning and high level expression of bovine interferon gamma gene in eukaryotic cells (COS-7)

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### Summary

Interferon gamma (IFN- $\gamma$ ) is one of the key cytokines in defining T helper 1 lymphocyte immune responses. In this study, the bovine IFN- $\gamma$  gene was cloned from spleen tissue RNA using the reverse transcription-polymerase chain reaction (RT-PCR). IFN- $\gamma$  cDNA was sub-cloned and expressed in mammalian expression plasmid (pcDNA3.1(+)) under the control of the human cytomegalovirus (CMV) promoter. The predicted amino acid (aa) sequence of bovine IFN- $\gamma$  compared with corresponding known sequence from bovine (*Bos taurus*) was 100% identity and with ovine, caprine, camel, lama, equine, canine, feline, human, mice and chicken cytokine was 95, 95, 86, 83, 77, 75, 75, 61, 44 and 35%, respectively. *In-vitro* expression of recombinant bovine IFN- $\gamma$  (rBoIFN- $\gamma$ ) and secretion to culture medium was confirmed by ELISA test. Maximum expression of rBoIFN- $\gamma$  occurred at 96 and 144 h after transfection in COS-7 cells. These results showed that pcDNA3.1 expression vector and COS-7 cells transfected by diethylaminoethyl (DEAE)-dextran allowed the high level expression of bovine IFN- $\gamma$  gene and the release of protein in supernatant of cell culture.

**Key words:** Interferon gamma, Bovine, Cloning, COS-7

### Introduction

Interferon-gamma (IFN- $\gamma$ ), a cytokine produced mainly by sensitized T lymphocytes, is known to play an important role in the immune response to many intracellular bacteria via its effect on macrophage activation and induction of MHC class I and class II. This cytokine also regulates T cell proliferation and differentiation, mediates leukocyte trafficking, and enhances antigen presentation (Young and Hardy, 1995; Schroder *et al.*, 2004).

During the early 1980s, the first IFN- $\gamma$  genes were identified and completely cloned. Then its biologically active recombinant human IFN- $\gamma$  proteins were expressed in bacteria. Purified recombinant

IFN- $\gamma$  was subsequently used to perform numerous experimental trials in humans and domestic animals (Tanaka *et al.*, 1983). Recently, in human medicine IFN- $\gamma$  has attracted extensive attention, and scientists have explored its clinical uses and therapeutic potential. IFN- $\gamma$  has applications in the treatment of a number of immunological, viral, and neoplastic diseases. Interest in IFN- $\gamma$  and many therapeutic possibilities remains high, as clinical trials continue to explore its use in the treatment of inflammation (Raghu *et al.*, 2004), cancer (Kleeberg *et al.*, 2004), and infectious diseases (Kuberski *et al.*, 2004).

Bovine interferon gamma (BoIFN- $\gamma$ ) has also been investigated for its therapeutic potential and could be a new product used in the control of bovine diseases. The antiviral

activity of recombinant bovine interferon gamma (rBoIFN- $\gamma$ ) against bovine leukaemia virus (BLV) has been demonstrated by Sentsui *et al.* (2001). They have been also revealed that rBoIFN- $\gamma$  suppresses the replication of BLV *in vitro*, but its biological activity is limited to cells of homologous species (Sentsui *et al.*, 2001).

This study has described the identification of interferon gamma gene in bovine (*Bos taurus*) and also clone bovine interferon gamma gene in a eukaryotic vector, evaluating its expression in cell culture transfected by DEAE-dextran method before using *in vivo*.

## Materials and Methods

### Cloning and sequencing of BoIFN- $\gamma$ gene

Total cellular RNA was isolated from the spleen tissue using RNX<sup>plus</sup> reagent (Cinnagen Co., Tehran, Iran) following the manufacturer's procedure. One ml of RNX<sup>plus</sup> reagent was added to 100 mg of spleen tissue and homogenized at room temperature for 5 min. Chloroform (200  $\mu$ l) was added to the tube and mixed thoroughly. After 5 min, samples were centrifuged at 12,000 rpm for 15 min. The clear aqueous supernatant was transferred to a fresh tube and an equal volume of isopropanol was added, mixed well, and centrifuged at 12,000 rpm for 15 min at 15-20°C. The RNA pellet was washed with 75% ethanol, air-dried and dissolved in diethyl-pyrocabonate-treated (DEPC) (0.1%) water and stored at -70°C.

First-strand cDNA synthesis was performed using a RevertAid<sup>TM</sup> M-MuLV Reverse Transcriptase Kit (Fermentas, GmBH, Germany). BoIFN- $\gamma$  cDNA production was conducted using an BoIFN- $\gamma$  specific primer, 5'-GTGATATCCTGAAGC GCCA-3' (Start from 1097 bp), complementary to the 3' untranslated region (UTR) of cattle IFN- $\gamma$  mRNA (Accession No: NM174086). BoIFN- $\gamma$  cDNA amplification was performed in two steps (nested PCR), using the same 3' primer with addition of a specific polymerase chain reaction (PCR) primer corresponding to the 5' UTR of the BoIFN- $\gamma$  sequence, 5'-CATAACACAGGAGCTACCG-3' (Start

from 40 bp). In the first step, PCR was carried out for 30 cycles; 1 cycle of 94°C for 3 min, 30 cycles of 94°C for 60 sec, 56°C for 60 sec and 72°C for 2 min, with a terminal step of 10 min at 72°C, using Bio Rad thermal cycle. BoIFN- $\gamma$  cDNA open reading frame (ORF) was performed with 5  $\mu$ l of the first-step amplification product after a second round of PCR, utilizing a specific forward primer flanked on its 5' side by a HindIII restriction site and Kozak site, 5'-GAAGCTTACCATGAAATATACAAG CTATTCTTAGCTTTACTGCTCTTGTG GG-3', and a specific reverse primer flanked on its 5' side by a XbaI restriction site, 5'-GGTCTAGATTACGTTGATGCTCTCCG GCCTCG-3', following steps: 1 cycle of 94°C for 3 min, 30 cycles of 94°C for 60 sec, 57°C for 45 sec and 72°C for 2 min, with a terminal step of 10 min at 72°C in a thermal cycler device (Bio Rad, Mexico). In this step a fragment of 516 bp was amplified.

To facilitate sequencing, PCR products were initially cloned into pDrive cloning vector using the Qiagen PCR cloning kit (Qiagen, Milden, Germany). Recombinant plasmids were transformed into *E. coli* DH5 $\alpha$  and cultured in LB medium with ampicillin (100  $\mu$ g.ml<sup>-1</sup>) as control, and screened for BoIFN- $\gamma$  cDNA inserts by T7-M13 primers. Then recombinant plasmids of positive clones were purified using the Plasmid DNA Purification kit (Qiagen, Milden, Germany). Plasmids containing insert were verified by double digestion with 5' HindIII and 3' XbaI enzymes and then followed by agarose gel electrophoresis. Three confirmed clones were sequenced by Macrogen Inc. (Seoul, South Korea) in an ABI 3730 XL automatic DNA sequencer. The BoIFN- $\gamma$  cDNA sequence in this step included 432 bp corresponding to mature protein and 69 bp signal peptide.

### Construction of expression plasmid (pcDNA3.1-IFN- $\gamma$ )

BoIFN- $\gamma$  cDNA ORF portions of the recombinant pDrive plasmid was directly subcloned into the 5' HindIII and 3' XbaI restriction enzyme sites of the pcDNA3.1(+) expression cloning vector (Invitrogen, Carlsbad, CA, USA). Recombinant

pcDNA3.1 plasmid (pcDNA3.1-IFN- $\gamma$ ) was isolated from transformed *E. coli* DH5 $\alpha$  after single selection for ampicillin resistance. Randomly selected colonies were screened for BoIFN- $\gamma$  cDNA inserts utilizing T7-BGH primers and positive clones cultured in LB broth medium. Recombinant plasmid was purified using the Plasmid DNA Purification kit (Qiagen, Milden, Germany). A clone containing plasmid pcDNA3.1-IFN- $\gamma$  was sequenced bidirectionally using Macrogen Inc. (Seoul, South Korea) in an ABI 3730 XL automatic DNA sequencer to verify correct insertion and sequence fidelity.

### Transfection and expression of rBoIFN- $\gamma$

COS-7 cells were routinely grown in DMEM (Sigma Aldrich, MO, USA) containing FCS 10% (Gibco, Linz, Austria), nonessential amino acids 1%, L-glutamine 1%, penicillin 1 U.ml<sup>-1</sup>, and streptomycin 1  $\mu$ g.ml<sup>-1</sup> at 37°C in CO<sub>2</sub> 5% and passaged using standard conditions. Twenty-four h before transfection with pcDNA3.1-IFN- $\gamma$  plasmid, exponentially growing cells were harvested by trypsinization and transferred to 35-mm tissue culture dishes at a density of  $5 \times 10^4$  cells/dish. The culture was incubated for 20-24 h at 37°C in a humidified incubator with an atmosphere of 5% CO<sub>2</sub>. Plasmid DNA (6  $\mu$ g) was mixed with 1 ml DEAE-dextran (1 mg.ml<sup>-1</sup> in PBS) and incubated at room temperature for 30 min. Following one wash with DMEM medium, DNA/DEAE-dextran/PBS mixture was added (150  $\mu$ l per well) and the cultures incubated for an appropriate time (25 min). Then DNA/DEAE-dextran/PBS solution was removed and after washing with PBS, warmed medium (3 ml) supplemented with serum and chloroquine (100  $\mu$ M) was added and incubated for 3-5 h. The transfection medium was removed and cells were washed with PBS for 2 min and the PBS was then replaced with 3 ml of medium. After 24 h growth at 37°C in 5% CO<sub>2</sub>, supernatant was collected and replaced with new DMEM. Collection of supernatant was followed every 48 h for two weeks (Sambrook and Russell, 2001). Transfection was performed in triplicate.

### RT-PCR

For *in vitro* study of rBoIFN- $\gamma$  expression in cell culture and to synthesize cDNA, 1  $\mu$ g of total RNA was isolated (described above, section 1) in the same conditions from transfected and non-transfected COS-7 cells 72 h after transfection and then mixed with 500 ng of random hexamer primer and heated at 70°C for 10 min. The mixture was immediately chilled on ice for 5 min, followed by the addition of 9.5  $\mu$ l of a reverse transcription mixture prepared in a total volume of 20  $\mu$ l containing 5  $\mu$ l of 5X reaction buffer, 2  $\mu$ l of 10 mM dNTP, 2  $\mu$ l DDT water, and 0.5  $\mu$ l RNase inhibitor. The mixture was incubated at 25°C for 5 min and then 1  $\mu$ l RevertAid<sup>TM</sup> M-MuLV Reverse Transcriptase (Fermentas, GmBH, Germany) was added. The mixture was further incubated at 42°C for 50 min. The reverse transcriptase reaction was terminated by the incubation of the reaction tubes sample at 70°C for 10 min. RT-PCR was completed using primers corresponding to nt 64-94 (F: 5'-AGTAGCCCAGATGTAGCTAAGGGT-3') and 472-500 (R: 5'-GACTTCTCTCCGCTTTCTGAGGT-3') of the cloned BoIFN- $\gamma$  cDNA. The PCR program was as follows: 1 cycle of 94°C for 4 min, 30 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 60 s, followed by a cycle of 72°C for 10 min.

### ELISA for rBoIFN- $\gamma$ expression assay

Sandwich ELISA for the detection of rBoIFN- $\gamma$  expression in culture supernatant was performed using a bovine IFN-gamma ELISA kit according to the manufacturer's instruction (Mabtech, Hamburg, Germany). The cells were incubated for 48 hrs and supernatants were collected for 336 hrs. Then the supernatants were stored at -70°C until use. The Maxisorp plate wells (NUNC) were coated overnight at 4°C with 2  $\mu$ g.ml<sup>-1</sup> of the monoclonal antibody BoIFNg-I (Mabtech, Hamburg, Germany) suspended in PBS, and were blocked with 2% (W/V) skim milk prepared in PBS followed by washing with PBS and Tween 20 (0.5%) (PBS-T). The plates were inoculated with 100  $\mu$ l supernatant and rBoIFN- $\gamma$  standards (75, 150, 300, 600, 1200 pg/ml) in corresponding wells and then kept for 2 h at

37°C. After washing, 100 µl of monoclonal antibody PAN-biotin (Mabtech, Hamburg, Germany) at 0.1 µg/ml in 2% skim milk prepared in PBS was added and incubated for 1 h. 100 µl Streptavidin-HRP (Mabtech, Hamburg, Germany), diluted 1:1000 in PBS-T, was added to each well and incubated at 37°C for 1 h before the addition of 100 µl per well of indicator solution containing 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) and incubation at 37°C. After incubation for 20 to 30 min at 37°C, the reaction was stopped by the addition of 100 µl of 2 M H<sub>2</sub>SO<sub>4</sub> (stop solution) to each well and quantification was carried out at 450 nm. The standard curve allowed for the calculation of rBoIFN-γ concentration in supernatant using the relationship  $f(y) = 0.0003 f(x) + 0.0612$  ( $R^2 = 0.9908$ )

where,

f(y): the titer response as A450

f(x): the rBoIFN-γ concentration

### Sequence analysis

BoIFN-γ cDNA and protein sequences were analyzed by BLAST through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) (Altschul *et al.*, 1997). The Sequence alignments were made using Clustal software and also bovine IFN-γ protein prediction was done using the BioEdit version 7.0.5.3 software package (<http://jwbrown.mbio.ncsu.edu/Bioedit/bioedit.html>).

### Data analysis

All data were analyzed using the general linear model (GLM) procedure of SAS software (Duncan, 1955) for one way analysis of variance. Significant differences among treatment means were determined by least squares means of SAS software only when a significant probability value ( $P < 0.01$ ) was detected.

### Results

The ORF of BoIFN-γ cDNA was 501 bp (Fig. 1) and had one nucleotide mutation (G for A, 432n) in sequence that was silent compared with the sequence in the GeneBank database, (Cerretti *et al.*, 1986).

The nucleotide and predicted aa sequences results of bovine IFN-γ obtained in the current study (assigned with Accession No: FJ263670) are illustrated in Figs. 2 and 3.

BoIFN-γ cDNA encoded a putative 166-aa polypeptide with a predicted molecular weight (MW) of 19362.39 Da. The predicted aa sequence of rBoIFN-γ with reference sequences of bovine and other species from GeneBank database (GB), are aligned in Fig. 2.

The predicted aa sequence of rBoIFN-γ was 100% homologous to bovine IFN-γ, 95% homologous to caprine and ovine IFN-γ, 86% homologous to camel and 83% to lama IFN-γ. Homology to non-ruminant species was considerably less, with 77% homology to equine, 75% homology to feline and canine IFN-γ, 61% homology to human IFN-γ, 44% homology to mice IFN-γ and 35% homology to chicken IFN-γ (Fig. 3). Recombinant IFN-γ of all four ruminant species contained 166 aa residues and the greatest aa homology occurs between these sequences.

### Characterization of recombinant BoIFN-γ

The pcDNA3.1-IFN-γ clone contained a signal peptide, simulating naturally secreted bovine IFN-γ. This leaves the first 23 aa with many features of an expected signal sequence for a secreted protein (Cerretti *et al.*, 1986). PCR is performed with T7-M13 and T7-BGH primers and then double digestion with HindIII and XbaI restriction enzymes on pDrive-BoIFN-γ and pcDNA3.1-BoIFN-γ produced fragments with sizes of 711 bp, 598 bp, 510 bp and 510 bp (Figs. 4a, b and c). Sequencing analysis was also performed on the pcDNA3.1-IFN-γ clone for further confirmation of the correct sequence and orientation of insert.

### BoIFN-γ transcription in COS-7 cells

Recombinant plasmid (pcDNA3.1-IFN-γ) clone was expressed in COS-7 cells. RT-PCR analysis of DNase-treated total RNA from COS-7 cells transfected with pcDNA3.1-IFN-γ yielded a predicted band of 437 bp confirming transcription of recombinant plasmid encoded BoIFN-γ (Fig. 5). The corresponding band was not

amplified from RNA extracted from non-transfected COS-7 cells.

### Production of rBoIFN- $\gamma$ in COS-7 cells

Expressed protein was detected by a quantitative BOIFN- $\gamma$  capture ELISA in culture supernatant from COS-7 cells transfected with recombinant plasmid (pcDNA3.1-IFN- $\gamma$ ). The secretion value of rBoIFN- $\gamma$  was monitored for 336 h and maximum secretion was observed at 96 and 144 h after transfection (Table 1), which was continued for 336 h after transfection. The most significant difference ( $P < 0.01$ ) in the rBoIFN- $\gamma$  concentration was observed between different times (Table 1).

### Discussion

The IFN- $\gamma$  gene from bovine was cloned, sequenced and expressed. The nucleotide sequence of BoIFN- $\gamma$  cDNA encodes a 166 aa protein that has one nucleotide substitution. Substitution was observed in nt A<sub>432</sub> to G<sub>432</sub>, but the aa sequence has 100 homology with a BoIFN- $\gamma$  aa sequence from the GB database. Mutation and nucleotide diversity in the BoIFN- $\gamma$  gene sequence have been recorded by Schmidt *et al.* (2002). All mutations have been demonstrated in coding regions of BoIFN- $\gamma$  gene. As they have declared, G-T transversion in the coding region of exon 1 causes a Gly<sub>14</sub> to Val<sub>14</sub> exchange in the BoIFN- $\gamma$  signal peptide of different bovine species and a G-A transition in exon 2 encodes a Ser(19) to Asn(19) change in the mature protein of the Tibetan yak cattle (Schmidt *et al.*, 2002). In our experiment the ORF of BoIFN- $\gamma$  cDNA had one nucleotide mutation (G for A, 432n). It was a silent mutation when compared with the reference sequence in the GeneBank database (Cerretti *et al.*, 1986). The predicted aa sequence of rBoIFN- $\gamma$  was also identical to bovine IFN- $\gamma$ .

BoIFN- $\gamma$  has individual aa differences throughout the sequence in comparison with other ruminants (ovine, caprine and camel). Nevertheless, its high sequence homology might be the best explanation for the preparation of proper anti-IFN- $\gamma$  antibody that is able to react against IFN- $\gamma$  from other

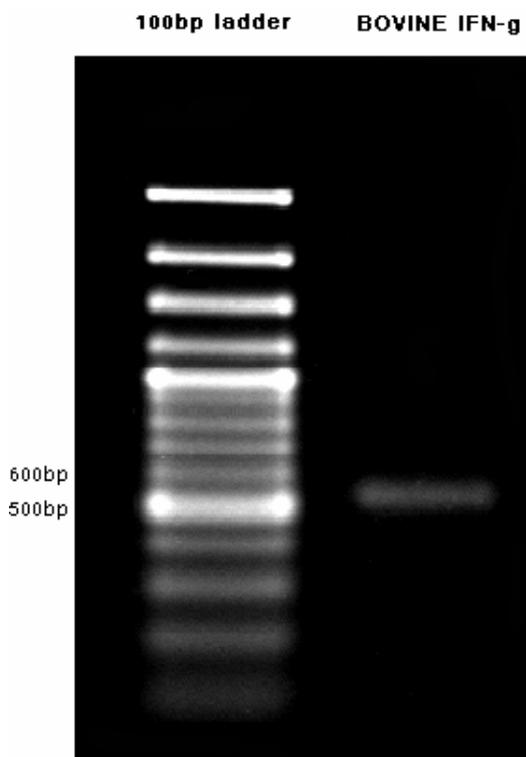
ruminant species. It may also suggest further applications of rBoIFN- $\gamma$  or directly pcDNA3.1-IFN- $\gamma$  for cytokine therapy in different animals.

In this study, ELISA test showed that COS-7 cells successfully transfected with

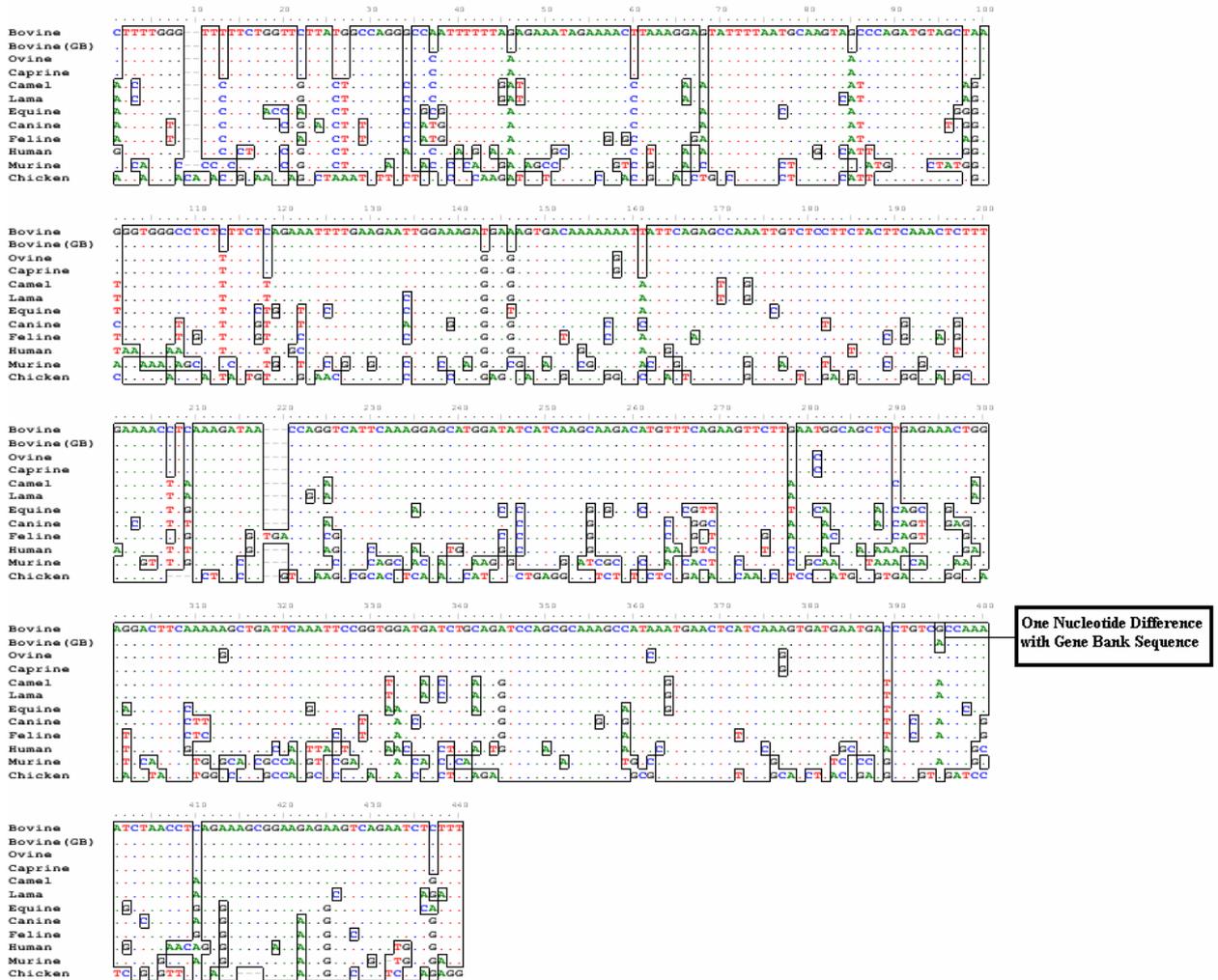
**Table 1: Concentration of rBoIFN- $\gamma$  after transfection (n = 3). Protein expression assay by ELISA test showed the rBoIFN- $\gamma$  expression in cell culture supernatant was started 24 h after transfection and reached the highest level at 96 and 144 h, which was significantly different from other times ( $P < 0.01$ ), and expression continued for 336 h**

Time (h)	Concentration of rBoIFN- $\gamma$	
	Transfected cells	Non-Transfected cells
24	403.61 <sup>e</sup>	96.4
48	9007.38 <sup>d</sup>	106.5
96	9438.98 <sup>a</sup>	119.3
144	9443.35 <sup>a</sup>	90.7
192	9255.206 <sup>c</sup>	112.6
264	9395.98 <sup>b</sup>	109.1
336	9374.93 <sup>b</sup>	107.2
SEM	5.523	6.05
CV (%)	0.912	9.494
Probability	0.0001	0.1

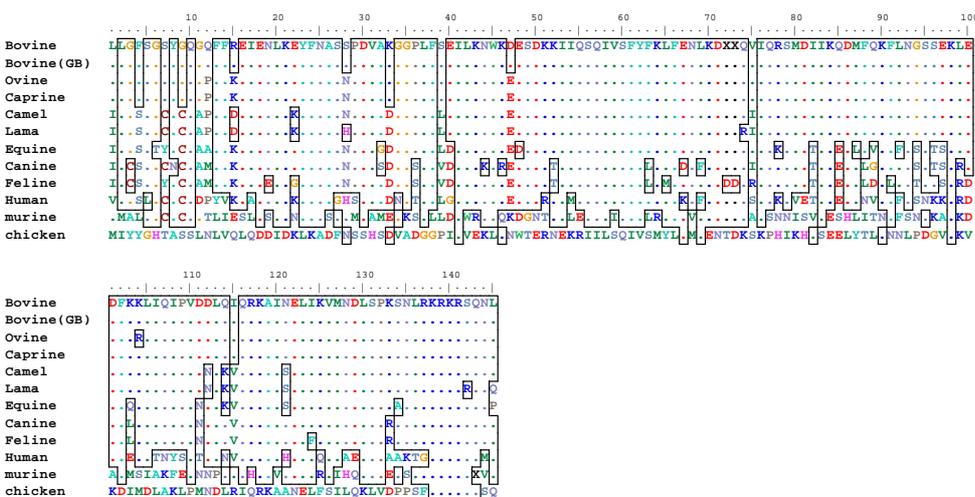
\*a-e: Columns values with the same superscript are not significantly different ( $P < 0.01$ ). SEM: Standard error of mean



**Fig. 1: RT-PCR amplification of BoIFN- $\gamma$  gene isolated from spleen tissue produced fragment with size of 516 bp**



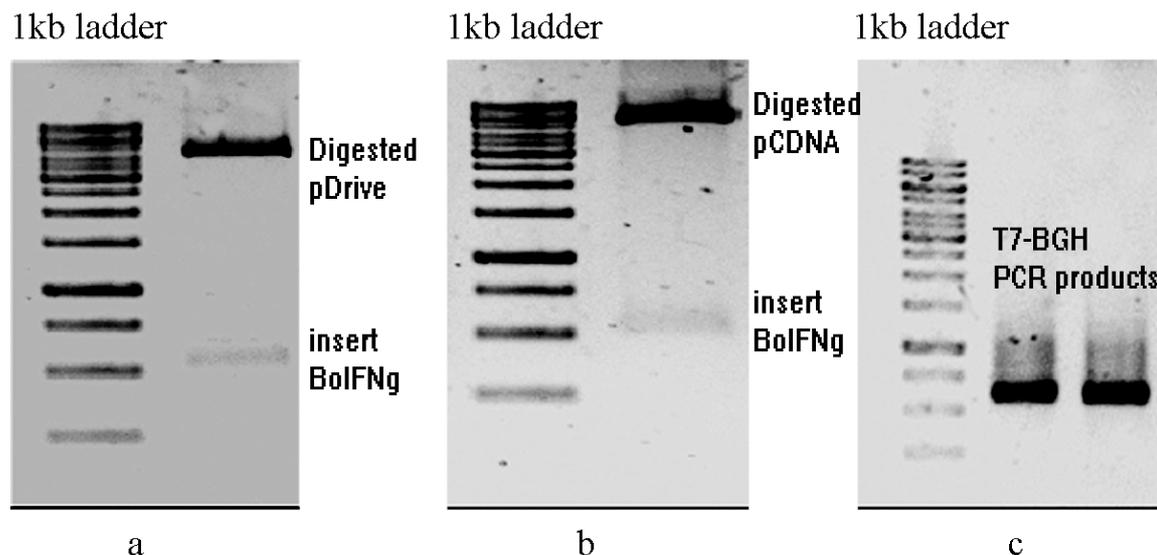
**Fig. 2: Nucleotide sequence of rBoIFN- $\gamma$  and alignment with data base sequences of bovine IFN- $\gamma$  and other species in GeneBank (GB)**



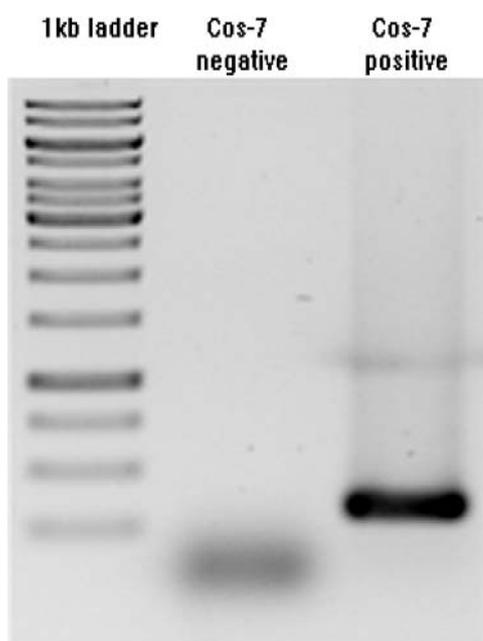
**Fig. 3: Predicted aa sequence of rBoIFN- $\gamma$  and alignment with data base sequences of bovine IFN- $\gamma$  and other species in GeneBank (GB)**

pcDNA3.1-IFN- $\gamma$  containing CMV promoter by using the DEAE transfection method and

secreted rBoIFN- $\gamma$  protein in supernatant with the maximum level at 96 h (9438



**Fig. 4: Double digestion pDrive-IFN- $\gamma$  and pcDNA3.1-IFN- $\gamma$  with HindIII and XbaI restriction enzymes (a, b). PCR performed with T7-BGH primers on two positive clones containing pcDNA3.1-IFN- $\gamma$ , produced fragment with size of 598 bp (c)**



**Fig. 5: RT-PCR analysis of COS-7 cells transfected with pcDNA3.1-IFN- $\gamma$  produced fragment with size of 437 bp**

pg.ml<sup>-1</sup>) and 144 h (9443 pg.ml<sup>-1</sup>) after transfection, which was significantly different from other times and secretion continued for two weeks. Stoeckle *et al.* (1996) have demonstrated that human IFN- $\gamma$  can be secreted *in vitro* by transfected HeLa cells and fibroblasts using a replication defective adenovirus for >4 weeks. To express caprine IFN- $\gamma$  protein in COS-7 cells, Beyer *et al.* (1998) have used vectors

containing CMV and CAEV LTR (caprine arthritis encephalitis virus long terminal repeat) promoters and employed the Lipofectamine transfection method. In their experiment, protein concentration in supernatant had been calculated as 2100 pg.ml<sup>-1</sup> for CMV and 400 pg.ml<sup>-1</sup> for CAEV LTR promoters (Beyer *et al.*, 1998). The results of the current experiment showed that rBoIFN- $\gamma$  was secreted at a higher concentration after 96 h and 144 h (9438-9443 pg.ml<sup>-1</sup>) in comparison with Beyer *et al.* (1998).

Since IFN- $\gamma$  has been shown to possess numerous immunomodulatory capabilities and antiviral activity, the development of the efficient production system described in this report is an important breakthrough to establish IFN- $\gamma$  reagent for bovine diseases. At present, recombinant DNA techniques have made it possible to produce IFN- $\gamma$  on a large scale, which will help make it economically feasible to bring IFN- $\gamma$  into general use for control and treatment of many infectious diseases in animals.

A variety of gene transfer and expression systems are available, but there has been intense research into the development of efficient vector systems and transfection methods. These methods are basically different in their use of chemical, viral, bacterial and synthetic delivery vectors. Although this research has mainly been

concerned with the expression of recombinant IFN- $\gamma$  from bovine on mammalian cell lines that were transfected with synthetic delivery vectors by a chemical method, selecting a system for the production of recombinant proteins cannot always predict how well the protein will be expressed in the selected host. In general, DEAE-dextran DNA transfection is ideal for transfection and is suitable for overexpression of recombinant protein in transient transfections. The major advantages of the technique are its relative simplicity and speed, limited expense, and remarkable transfection efficiency.

Two glycosylation sites are present in the mature IFN- $\gamma$ , in agreement with studies in other species (Kelker *et al.*, 1983). As already noted, when rBoIFN- $\gamma$  is produced through genetically engineered bacteria, posttranslational glycosylation of the protein molecule does not take place; this will influence the circulatory half-life of the molecule (Kelker *et al.*, 1983; Rutenfranz and Kirchner, 1988; Yamaguchi *et al.*, 1991). Furthermore, non-glycosylated rBoIFN- $\gamma$ , when present in relatively high concentrations is more prone to aggregate (Gyorffy *et al.*, 2001). A variety of gene transfer and expression systems are available. Based on these findings, we tried to adapt efficient production system with COS-7 cells for glycosylated rBoIFN- $\gamma$ .

The IFN- $\gamma$  acts as an adjuvant, and enhances the immune response to the tumor cells and viral infections. Because of the anti-viral property of IFN- $\gamma$ , IFN- $\gamma$ -pcDNA3.1 construct could be directly used in cattle for *in vivo* expression of BoIFN- $\gamma$  as a therapeutic agent against viral infections or an adjuvant in bovine vaccination. Furthermore, rBoIFN- $\gamma$  from COS-7 cells could be used to immunize animals for the recovery of antibodies that are specific to the natural bovine IFN- $\gamma$ . In subsequent studies other methods of transfection will serve for better expression of rBoIFN- $\gamma$  in COS-7 cells and as a sensitive technique for evaluation of the biological activity of rBoIFN- $\gamma$ .

We have demonstrated a useful method for transfection of Cos-7 cells and its applicability for overexpression of rBoIFN- $\gamma$  protein in transient transfections. This can be

used as a basis for further studies on DNA vaccine technology in animals.

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