Aquaculture International (2018) 26:1401–1413
https://doi.org/10.1007/s10499-018-0292-9

Evaluation of interferon gamma (IFN-γ) of *Labeo rohita* as an immunomodulator: in vitro expression model

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Received: 7 January 2018 / Accepted: 31 July 2018 / Published online: 10 August 2018

Abstract

Interferon gamma (IFN-γ) or type II interferon is a cytokine that is critical for innate and adaptive immunity against viral and some bacterial and protozoal infections. The importance of IFN-γ in the immune system lies in its ability to inhibit viral replication directly and most importantly from its immunomodulatory effects. Previously, we successfully co-administered IFN-γ along with GAPDH gene of *Edwardsiella tarda* as bicistronic DNA vaccine in *Labeo rohita*. In order to ascertain the individual role of IFN-γ, the present study involves cloning and expression of 552-bp IFN-γ open-reading frame (ORF) of *L. rohita* in striped snakehead (SSN-1) cell line using eukaryotic expression vector system (pQE-TriSystem) followed by transfection in peripheral blood lymphocytes (PBMCs) to evaluate its immunomodulatory ability in comparison to polyinosinic-polycytidylic acid (Poly I:C)-treated PBMCs. The 18.7-kDa protein, expressed in the pQE-IFNγ-transfected SSN-1 cells, reacted with anti-His antibody in Western blot confirming it to be recombinant IFN-γ, whereas the relative expression of IFN-γ, iNOS, Mx, and IL-1β genes in PBMCs was quantified at 24 h and 48 h post treatment by qPCR. The comparative kinetics of all four genes showed significantly ($p < 0.05$) high upregulation pattern in both pQE-IFNγ-transfected cell group and Poly I:C-treated cell group demonstrating recombinant IFN-γ as an equally efficient inducer like Poly I:C. Thus, our in vitro experiment results highlight the immunomodulatory potential of recombinant IFN-γ as an analogue to synthetic Poly I:C which warranted future studies to further explore the potential of recombinant IFN-γ as an effective vaccine adjuvant against different microbial invasion.

Keywords IFN-γ · *Labeo rohita* · Peripheral blood mononuclear cells · Poly I:C

Highlights
- The 552-bp ORF of IFN-γ of *L. rohita* cloned in pQE-TriSystem expression vector.
- The recombinant IFN-γ positively expresses in SSN-1 cells and PBMCs.
- IFN-γ, iNOS, Mx, and IL-1β genes significantly upregulated in pQE-IFNγ-transfected cells.
- The recombinant IFN-γ was observed to be an equally efficient inducer like Poly I:C.
- Recombinant IFN-γ can be used as an immunomodulator in therapeutic treatment in fish.

Introduction

Interferon gamma (IFN-γ) is a pleiotropic pro-inflammatory dimerized soluble cytokine that is produced by many different cell types viz. activated Th1 T helper cells (Mosmann and Coffman 1989), cytotoxic T lymphocytes of the TC1 phenotype (Sad et al. 1995), and natural killer (NK) cells (Perussia 1991) in response to viruses. Unlike type 1 interferon (IFN-α and IFN-β) which are responsible for arresting viral infection allowing sufficient time for the generation of adaptive immune response, the main biological activity of type II interferon (IFN-γ) appears to be immunomodulatory (Caipang et al. 2005; Zhou et al. 2007) thereby regulating the production of an array of cytokines that induce an antiviral state in host cells. In addition to its antiviral role, IFN-γ is demonstrated to be a central participant in host immune response to many intracellular bacteria via its effect on macrophage activation and induction of MHC class I and class II (Digby and Lowenthal 1995; Zou et al. 2005; Swain et al. 2015).

IFN-γ shares low homology among different species such as 50.3% between human and murine and 43% between human and chicken (Digby and Lowenthal 1995). Similar modest homology was observed in teleosts also, indicating strict species specific functions of IFN-γ. To date, IFN-γ has been identified in several fish species including zebrafish (Igawa et al. 2006), Japanese pufferfish (Zou et al. 2004), trout (Zou et al. 2005), Atlantic salmon (Robertsen 2006), catfish (Milev-Milovanovic et al. 2006), common carps (Stolte et al. 2008), goldfish (Grayfer and Belosevic, 2009), Atlantic cod (Furnes et al. 2009), and grass carp (Chen et al. 2010) with some teleosts having multiple isoforms of the gene.

From functional analysis, teleost IFN-γ have several properties in common with mammalian IFN-γ including the ability to enhance respiratory burst activity, nitric oxide production, and phagocytosis of bacteria in macrophages (Zou et al. 2005; Parhi et al. 2015; Swain et al. 2015), along with induction of IFN-γ inducible protein 10 (IP-10), MHC class II β-chain, and STAT1 (Zou et al. 2005; Arts et al. 2010; Skjesol et al. 2010). IFN-γ has also shown to induce the antiviral gene GBP (guanylate-binding protein) in trout (Robertsen et al. 2006).

Owing to this functional importance in Th1 subset of immune response, recently, IFN-γ has been tested as immune adjuvant in many DNA vaccine trials conducted in higher vertebrates. Co-administration of the IFN-γ gene with DNA antigen of woodchuck hepatitis virus (Wang et al. 2007), mycobacterium paratuberculosis (Kadam et al. 2009), porcine reproductive and respiratory syndrome virus (Du et al. 2012), and infectious salmon anemia virus (Chang et al. 2015; Robertsen et al. 2016), demonstrated to enhance the specific cellular immune response and improved the protective efficacy of vaccine constructs. These results prompted us to investigate the potentiality of IFN-γ as an immune adjuvant in developing fish vaccine also. Accordingly, our lab has characterized IFN-γ gene in *Labeo rohita* (Parhi et al. 2015) and subsequently co-administered IFN-γ gene as vaccine adjuvant with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene of *Edwardsiella tarda* in *Labeo rohita* in order to check the vaccine efficacy against virulent *E. tarda* challenge in vivo (Kumari et al. 2018; Kole et al. 2018). Now, the current research work was conducted to evaluate the synergistic effect of IFN-γ over the DNA antigen as an immunomodulator by studying the in vitro induction profile of some important genes in peripheral blood mononuclear cells (PBMCs) after transfection with recombinant IFN-γ individually. In addition, the induction profile was compared with polyinosinic-polycytidylic acid (Poly I:C)-transfected PBMCs to reaffirm our previous in vivo findings that IFN-γ can truly act as an efficient vaccine adjuvant.

Materials and methods

Experimental animal

Adult Rohu (*L. rohita*) (400–500 g) obtained from a local farm were stocked into 500-L fiber-reinforced plastic (FRP) tanks provided with UV-treated aerated freshwater in our indoor rearing facility and were fed twice a day with a standard pelleted diet at 3% of their body weight before collecting blood. Water quality of the tanks was maintained by exchanging 30% of the tank water daily prior to the experiment. The water temperature varied from 27 to 28 °C and the pH of the water varied from 7.4 to 7.6 during rearing time. To confirm the fishes were free of bacterial diseases, bacterial isolation in brain heart infusion (BHI) agar and *Salmonella-Shigella* (SS) agar plate from kidney of three randomly selected individuals was carried out. Morphological examination was done to detect presence of any parasites.

Peripheral blood mononuclear cell culture

Peripheral blood mononuclear cells (PBMCs) from *L. rohita* were collected by using Histopaque®-1077 (Sigma, USA) as per manufacturer's protocol. Briefly, 5 mL of blood was collected aseptically from *L. rohita* and diluted with PBS in the ratio of 1:1. The diluted blood was layered over an equal volume of Histopaque solution and carefully centrifuged at 1000 rpm for 10 min. After centrifugation, upper aqueous layer was discarded and lymphocytes from middle layer were collected. The collected lymphocytes were then transferred in a T-25 flask (Nunc, Denmark) at the seeding density of 3×10^5 cells/flask (counted by using Neubauer hemocytometer) and incubated overnight at 28 °C. The flask was kept for shaking in incubator at minimum speed. RPMI-1640 (Sigma, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, USA), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Gibco, Invitrogen, USA) was used as the cell culture media. After overnight incubation, 2×10^5 cells/well was seeded in a 24-well cell culture plate (Nunc, Denmark) and cultured using same RPMI growth media for the transfection experiment.

PCR amplification of IFN-γ gene of *L. rohita*

Poly I:C induction of kidney cells from *L. rohita* was carried out as previously described (Parhi et al. 2015). Total RNA was extracted from the cultured kidney cells induced with Poly I:C using TRIzol® reagent following the manufacturer's protocol (Invitrogen, USA). Complementary DNA was synthesized from the total RNA using oligo-dT primers using the first strand cDNA synthesis RevertAid™ kit (Fermentas, USA) following the manufacturer's protocol and was used immediately for PCR amplification of IFN-γ gene. Gene-specific forward primer IFNγ-F (5′ CC GGATCC ATG ATT GCG CAA CAA ACA ATG 3′) containing *BamHI* restriction site and reverse primer IFNγ-R (5′ AAGCTTT CAA GAC TTC TGA TTC TTT TTG 3′) containing *HindIII* restriction site were designed based on our submitted sequences of IFN-γ gene of *L. rohita* to NCBI database (Gene bank accession no. HQ667144.1) using Primer express software. The IFN-γ gene was amplified using the designed primer in a 25-μL reaction with Taq DNA polymerase (5 U) (Thermo scientific, USA). The cyclic condition for amplification of IFN-γ was 30 cycles of denaturation (94 °C, 30 s), annealing (65 °C, 40 s), and extension (72 °C, 1 min) with a further final extension (72 °C, 10 mins).

Cloning of IFN-γ gene in pQE-TriSystem vector

Double restriction digestion of pQE-TriSystem vector (Qiagen, Germany) and PCR-amplified IFN-γ gene product was done by *BamHI* and *HindIII* restriction enzymes for 4 h at 37 °C separately. After restriction digestion, IFN-γ was ligated in pQE-TriSystem vector using T_4 DNA ligase, for 1 h at 22 °C. Cloned IFN-γ gene in pQE-TriSystem vector (pQE-IFNγ) was subsequently transformed into *Escherichia coli* DH5-α strain maintained in our laboratory. Recombinant clones grown on LB-ampicillin (100 mg/mL) agar were identified by antibiotic selection in presence of ampicillin. The recombinant plasmid DNA was isolated using the QIAGEN Plasmid Mini Kit (QIAGEN, Germany) as per the manufacturer's protocol and cleaved with restriction enzymes for confirmation of the positive clones.

Confirmation of expression of IFN-γ gene in eukaryotic cells

Transfection of recombinant plasmid pQE-IFNγ in SSN-1 cell line

Striped snakehead (SSN-1) cell line was procured from the European Collection of Authenticated Cell Cultures (ECACC 96082808, England). The cells were maintained at 28 °C in Leibovitz's L-15 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1× antibiotic antimycotic solution. After propagation (75–80% confluency), the cells were transfected with the purified recombinant plasmid pQE-IFNγ using Lipofectamine™ 3000 Reagent, (Thermo Fisher Scientific, USA) as per the manufacturer's protocol. Briefly, Lipofectamine was diluted in a tube with Opti-MEM and in another tube master mix was prepared with diluted pDNA in Opti-MEM-containing diluted p3000 reagent. Diluted pDNA was then added to the tube of diluted Lipofectamine and incubated for 5 min for the lipoplex formation. The pDNA-lipid complexes was added to the cells and incubated for 24 to 48 h for trans-gene expression analysis.

Confirmation of expression of IFN-γ gene by Western blot

Cells were collected 48 h post transfection for confirmation of trans-gene expression of IFN-γ by Western blot using a Western blot kit (Amresco, USA). Penta·His antibody (Qiagen, Germany) was used as the primary antibody for Western blot. Anti-His antibody coupled with horseradish peroxidase was used for chromogenic detection using BCIP/NBT solution (Sigma, USA).

Confirmation of expression of IFN-γ gene by RT-PCR and sequence analysis

The cells were collected after 48 h post transfection for the extraction of RNA. RNA extraction was done by Trizol™ reagent (Invitrogen, USA). Briefly, the cells were rinsed once with prechilled PBS and 1 mL of Trizol reagent was added to the cells and scrapped using cell scraper and transferred to a 1.5-mL microcentrifuge tube. RNA extraction was done by Trizol™ reagent (Invitrogen, USA) as per the manufacturer's protocol and quantified by Nanodrop (Thermo Scientific, USA). The RNA was reverse transcribed by oligo-dT primer and M-MuLV reverse transcriptase in a 20-μL reaction volume using RevertAid First strand cDNA synthesis kit (Thermo Scientific, USA) as per the manufacturer's protocol. Amplification of IFN-γ gene from the cDNA prepared was done for confirmation of expression of IFN-γ gene from the plasmid construct pQE-IFNγ. The cDNA extracted from SSN-1 cell

sample transfected with mock pQE-TriSystem vector was taken as negative control. The PCR amplification conditions were same as described earlier. Amplified PCR product was run in 1.2% agarose gel and subsequently purified by gel extraction using Fermentas GeneJET™ Gel Extraction kit. The gel extracted product was cloned into pTZ57R/T vector and sequenced by ABI BigDYE terminator method (Bioserve Biotechnologies Pvt. Ltd., India).

Comparative analysis of immune modulation effect of pQE-IFNγ with Poly I:C

Transfection of pQE-IFNγ and Poly I:C into PBMCs

The 24-well plate containing PBMCs (2×10^5 cells/well) as described earlier was divided into four groups viz. untreated cell well, mock-transfected well (cells transfected with empty pQE-TriSystem vector), recombinant pQE-IFNγ-transfected well, and Poly I:C-induced cell well. For transfection of recombinant pQE-IFNγ, 1 μg of pQE-IFNγ plasmid diluted in 100 μL of serum and antibiotic-free Opti® MEM media was added to 2 μL of Lipofectamine®2000 reagent and incubated for 15 min at room temperature. The mixture was subsequently added to each well of recombinant pQE-IFNγ-transfected group and incubated at 28 °C with intermittent shaking. Similarly, mock-transfected group was transfected with 1 μg of empty plasmid vector. In Poly I:C-induced group, 10 μg/mL Poly I:C was added to each well. Cells from each group were harvested after 24 h and 48 h of incubation and processed for RNA extraction and cDNA synthesis as described earlier. The synthesized cDNA was used as template for gene expression analysis. The mock-transfected cell group was taken as control to the pQE-IFNγ-transfected cell group whereas for Poly I:C-treated cell group, untreated cell group was considered as control for gene expression analysis. The whole experiment was conducted in triplicates.

Gene expression study

Quantitative real-time PCR was conducted for analysis of four immediate responsive immune genes viz. interleukin-1β (IL-1β), interferon gamma (IFN-γ), inducible nitric oxide synthase (iNOS), and Mx gene with respect to housekeeping gene, β-actin. Real-time PCR primers for each using Primer Express software from the available NCBI sequences as mentioned in Table 1. Real-time PCR amplification was carried out in ABI 7500 Real-Time PCR detection system (Applied Biosystems, USA). The 25-μL reaction mix volume contained 12.5 μL of Takara SYBR® Premix Ex Taq™ II Master Mix (Takara, Japan), 0.5 μL of ROX-dye, 0.5 μL of 25 pmol

Table 1 Primers used for immune gene expression and size of PCR amplicons

Gene (accession no.)	Oligo name	Sequence (5′–3′)	Amplicon size
β-actin (GU338376.1)	β-actin-F	CCATGTTGCACACTTGATGGA	56 bp
	β-actin-R	GCACTGCCTGCACAAAGAAC	
iNOS (AM932526.1)	iNOS-F	ACCAGCACCTTTGGCAATG	148 bp
	iNOS-R	GGGCGAATGCACAGAAGTGT	
IFN-γ (HQ667144.1)	IFN-γ-F	TTCACTCGCATGGAAAATGA	165 bp
	IFN-γ-R	GACCGGATCATCAGCCTTTA	
Mx (KR349112.1)	Mx-F	CCTCTGACCGACTGGCTAAC	204 bp
	Mx-R	GTTCTTTGCCAGCACTCCTC	
IL-1β (AM932525.1)	IL-1β-F	TTGAAGGCCGTGACACTGACT	114 bp
	IL-1β-R	GATTCCCAGGCACACAGGTT	

each gene-specific primers, and 2 μL of template. The reactions were carried out in a 96-well reaction plate (Applied Biosystems, USA) with two replicates of 10 μL for each sample. The default thermal profile was used for PCR amplification consisting of initial denaturation of 95 °C for 10 min, followed by 40 cycles of each having denaturation step at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Melt curve analysis of the amplification products were performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. The threshold cycle (Ct) was recorded for each samples. The amount of mRNA transcripts of IL-1β, IFN-γ, iNOS, and Mx was normalized by internal control, β-actin gene. Fold change of each gene was calculated using $2^{\Delta\Delta ct}$ method (Livak and Schmittgen 2001).

Statistical analysis

The data generated for different gene expression were statistically analyzed by statistical package SPSS version 16 (SPSS Inc., USA) in which data were subjected to one-way ANOVA to determine the significant differences between the means. Comparisons were made at the 5% probability level. A p value below 0.05 was considered statistically significant. The results were expressed as the mean ± S.E.

Results

Confirmation of positive cloning of IFN-γ gene in pQE-TriSystem vector

Release of IFN-γ gene (552 bp) from double digestion of the plasmid construct pQE-IFNγ with *BamHI* and *HindIII* restriction enzymes confirms positive cloning of IFN-γ gene in pQE-TriSystem vector (Fig. 1).

Confirmation of expression of IFN-γ gene in SSN-1 cells

In order to investigate whether IFN-γ gene will express in a eukaryotic system, the recombinant pQE-IFNγ was transfected in SSN-1 cell line in vitro. The presence of 18.7 kDa band in Western blot (Fig. 2a) on reacting with the Anti-His antibody showed that the recombinant plasmid

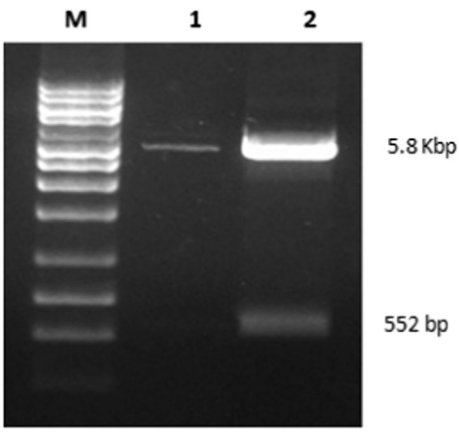

Fig. 1 Agarose gel electrophoresis (1.2%) showing restriction digestion of DNA construct (in pQE-TriSystem vector) confirming presence of IFN-γ gene inserts; Lane M: 1 Kbp DNA ladder. Lane 1: naked pQE-TriSystem vector. Lane 2: release of IFN-γ gene (552 bp) after RE digestion of pQE-IFNγ construct with *BamHI* and *HindIII* restriction enzymes

expresses IFN-γ protein in vitro. In addition, the RT-PCR result (Fig. 2b) followed by sequence analysis of the deduced amplicon (Fig. 2c) displayed that the transfected SSN-1 cells contains mRNA transcript for IFN-γ gene which confirmed positive expression of recombinant IFN-γ.

Comparative analysis of immune modulation effect of pQE-IFNγ with Poly I:C

To evaluate the effect of recombinant IFN-γ as an immunomodulator, a comparative gene expression analysis from pQE-IFNγ-transfected PBMC cell group and Poly I:C-treated PBMC

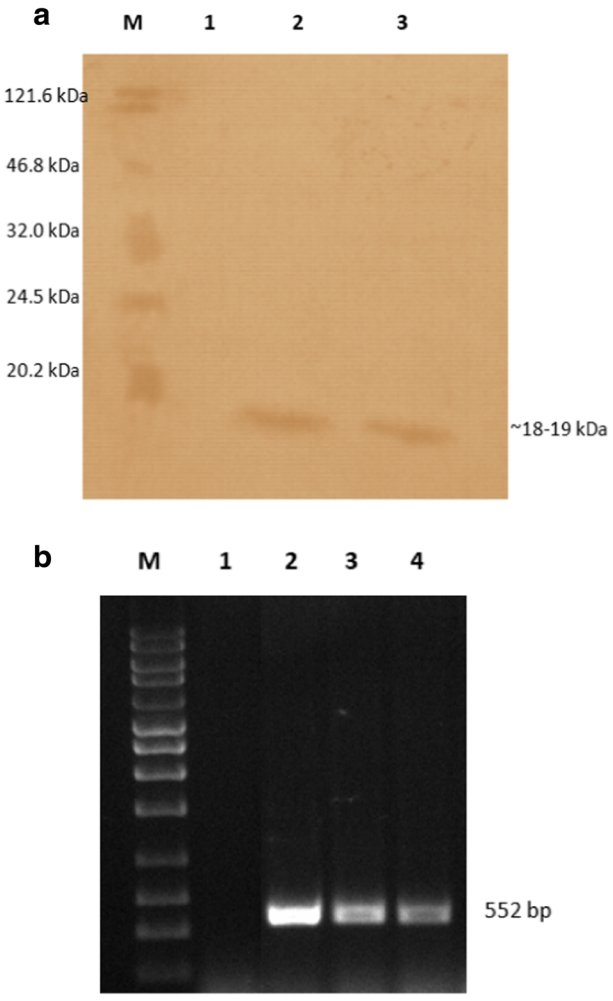

Fig. 2 **a** Western blot assay of pQE-IFNγ-transfected SSN-1 cells showing expression of recombinant IFN-γ protein (~ 18–19 kDa) specifically reacting with anti-His antibody. Lane M: pre-stained molecular mass marker (Thermo fisher, USA). Lane 1: negative control (mock-transfected SSN-1 cells). Lane 2–3: pQE-IFNγ-transfected SSN-1 cell lysate. **b** Agarose gel electrophoresis (1.2%) of RT-PCR products showing positive expression of recombinant IFN-γ in pQE-IFNγ plasmid-transfected SSN-1 cells. Lane M: 1 Kbp DNA marker. Lane 1: negative control (mock-transfected SSN-1 cells). Lane 2–4: IFN-γ gene (552 bp) amplified from pQE-IFNγ-transfected SSN-1 cells. **c** Genomic sequence of the deduced amplicon product from RT-PCR exactly align with the 552-bp ORF of IFN-γ of *L. rohita* confirming positive expression of recombinant IFN-γ in pQE-IFNγ plasmid-transfected SSN-1 cells

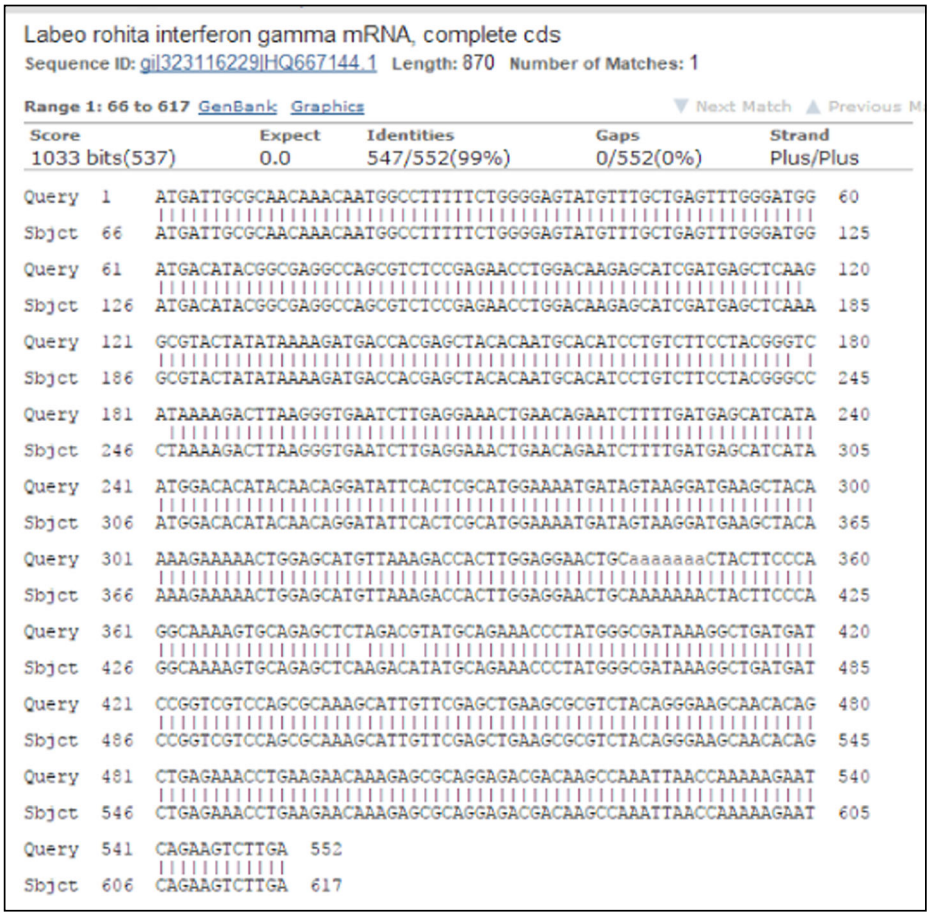

Fig. 2 (continued)

cell group was carried out. In the pQE-IFNγ-transfected cell group (Fig. 3), expression of all the four immediate responsive genes (IL-1β, IFN-γ, iNOS, and Mx) was significantly (< 0.05) high compared to mock-transfected group at 24 h and 48 h post transfection. INF-γ gene showed ~ 55.3-fold (with respect to internal control, β-actin) increase at 24 h post transfection which again increases to ~ 66-folds (with respect to β-actin) at 24 h post transfection. Similar expression pattern was noticed for Mx (~ 18- and ~ 58-fold increase against β-actin at 24 h and 48 h post transfection respectively) and IL-1β (~ 6- and ~ 18-fold increase against β-actin at 24 h and 48 h post transfection respectively) genes whereas the expression of iNOS gene remains elevated (~ 95-folds with respect to β-actin) at both time point (24 h and 48 h) post transfection.

As expected, the expression of all four genes (IL-1β, IFN-γ, iNOS, and Mx) in the Poly I:C-treated PBMC cell group (Fig. 4) was significantly upregulated in comparison with the untreated cell group. Expression of IFN-γ increased to ~ 65-folds and ~ 73-folds with respect to β-actin at 24 h and 48 h post transfection respectively. Mx mRNA transcript was upregulated to ~ 55-folds at 24 h which again increased to ~ 66-folds after 48 h post transfection. In the case of iNOS gene, ~ 66-fold and ~ 95-fold elevations were recorded at 24 h and 48 h post

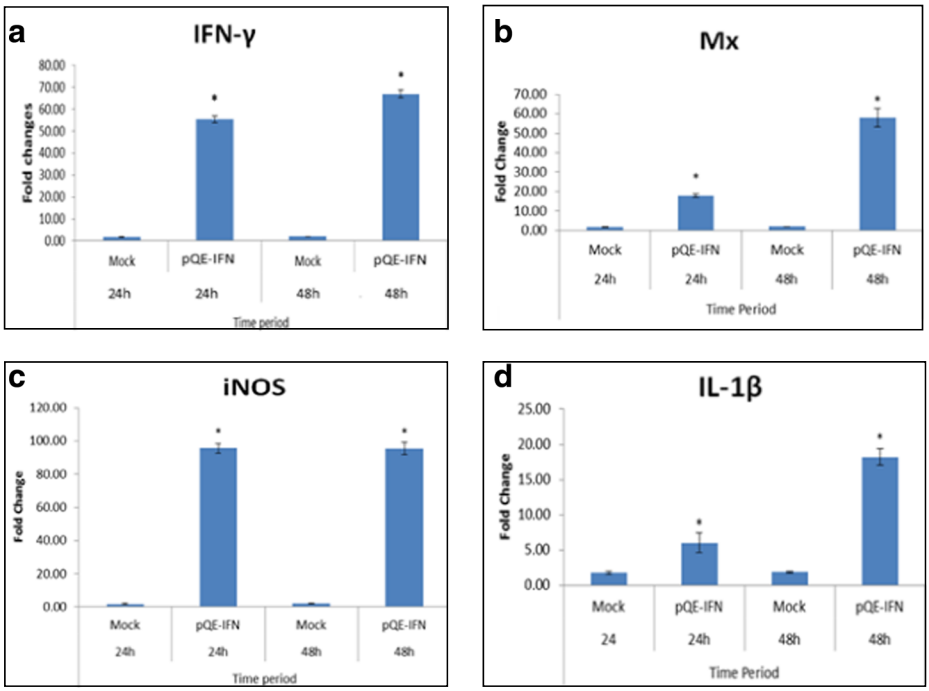

Fig. 3 Relative quantification of IFN-γ (**a**), Mx (**b**), iNOS (**c**), and IL-1β (**d**) genes (normalized with internal control β-actin) in recombinant pQE-IFNγ-transfected PBMCs of *L. rohita* at 24 h and 48 h post transfection. The mean values of the fold change in expression ($n = 9$) of each gene compared to mock-transfected control from three independent experiments were plotted with standard error as a function of time after transfection (shown as bar). A one-way ANOVA ($p < 0.05$) was used to determine statistical significance (denoted by asterisks*) of the difference in expression of individual gene between the two groups at each time interval

transfection respectively. Expressions of IL-1β were relatively low (~ 6-folds to ~ 11-folds with respect to β-actin) at both time points.

Discussion

IFN-γ- and IFN-γ-related genes of *L. rohita* were previously characterized by Parhi et al. (2015) and Swain et al. (2015) respectively. The complete sequences of IFN-γ gene of *L. rohita* consist of 552-bp ORF, 65-bp 5′ UTR, and 253-bp 3′ UTR (Parhi et al. 2015). In the present study, we designed primers for amplification of the 552-bp ORF. The primers contain sites for directional cloning. With these primer set, we were able to amplify the ORF of IFN-γ gene and positively cloned in eukaryotic expression vector pQE-TriSystem. Teleost IFN-γ precursors contain 180–189 amino acids (Robertsen 2006). The theoretical molecular weight of the rohu IFN-γ was recorded to be 18.7 kDa and the protein produced from rohu IFN-γ contains 157 amino acids (Parhi et al. 2015). Thus, our resultant protein band in Western blot around 18–19 kDa from pQE-IFNγ-transfected SSN-1 confirmed the positive expression of the IFN-γ ORF. Also, the RT-PCR amplicon of 552 bp followed by the sequence analysis of the amplicon deduced the exact ORF sequence which we were targeting to use as recombinant clone.

In order to develop efficacious vaccines against various viral and bacterial fish pathogens, the potential of interferon and other cytokines as potent vaccine adjuvant have been

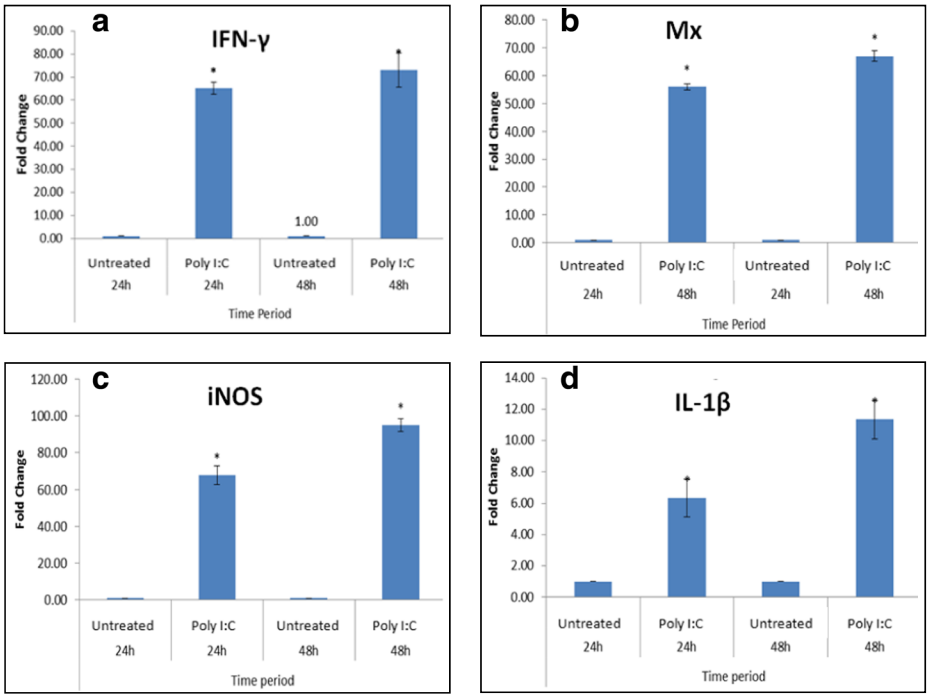

Fig. 4 Relative quantification of IFN-γ (**a**), Mx (**b**), iNOS (**c**), and IL-1β (**d**) genes (normalized with internal control β-actin) in Poly I:C (10 μg/mL) of treated PBMCs of *L. rohita* at 24 h and 48 h post transfection. The mean values of the fold change in expression ($n = 9$) of each gene compared to untreated control from three independent experiments were plotted with standard error as a function of time after transfection (shown as bar). A one-way ANOVA ($p < 0.05$) was used to determine statistical significance (denoted by asterisks*) of the difference in expression of individual gene between the two groups at each time interval

explored by several authors (Caipang et al. 2005; Jimenez et al. 2006; Robertsen et al. 2016; Lazarte et al. 2017). In this context, our previous study has also demonstrated the efficacy of IFN-γ as a suitable adjuvant in bicistronic DNA immunization in *L. rohita* against *E. tarda* (Kumari et al. 2018). To further ascertain the role of IFN-γ as a suitable candidate for antiviral therapeutic treatment or vaccine adjuvant in fish, we compared its effect with commercial ligand Poly I:C-transfected PBMC cell. The kinetics of IFN-γ, Mx, iNOS, and IL-1β showed significantly ($p < 0.05$) high upregulation pattern in both pQE-IFNγ-transfected cell group and Poly I:C-treated cell group. As Poly I:C is a proven immune inducer, its effect on the immune genes was expected; however, the ability of recombinant IFN-γ in simulating the action of Poly I:C as a potent immunomodulator is an interesting finding from our study which in turn substantiates its positive immunostimulatory effect on host immune system.

Coming to the expression of individual gene in the pQE-IFNγ-transfected cell group, it was seen that IFN-γ gene transcript increases from 24 h post transfection to 48 h post transfection. This could be due to self-activation of transfected PBMCs (lymphocytes) by recombinant IFN-γ secreting IFN-γ, which in turn induced the neighboring cells resulting into more production of IFN-γ at 48 h post transfection. This phenomenon was explained by several authors where they reported B cells and professional antigen-presenting cells (APCs) like monocytes/macrophage, dendritic cells secreting IFN-γ which can boost the

immune responses by activation of nearby cells to produce more IFN-γ (Frucht et al. 2001; Harris et al. 2000; Flaishon et al. 2000). The expression of Mx gene showed differential upregulation in the pQE-IFNγ-transfected cell group (increases from 18-folds at 24 h to 58-folds 48 h) when compared with the Poly I:C-treated group (very high expression from early time point). The abrupt rise in expression at 24 h post treatment with Poly I:C may be due to some special mechanisms of the ligand influencing Mx expression immediately but from our results, it was clear that, although stimulation of Mx gene takes some extra time compared to Poly I:C, the recombinant IFN-γ is able to induce Mx gene to the same extent as Poly I:C. Similar upregulation of Mx gene by interferon induction was recorded in salmon where the Mx transcript level was low at 24 h post stimulation but increased remarkably at 48 h and 96 h post stimulation (Zou et al. 2005; Sun et al. 2011). Nonetheless, this high induction of Mx gene can be a potent antiviral response in the host. Besides the genes from interferon driven pathway, our results elicited immediate and strong induction (~ 95-folds) of inducible nitric oxide synthases (iNOS) by recombinant IFN-γ. Unlike the pQE-IFNγ-transfected cell group where there was constantly high expression of iNOS at both time points, the Poly I:C-treated group showed accelerating pattern of expression (~ 66-folds at 24 h and ~ 95-folds at 48 h post transfection). From this expression kinetics, it can be assumed that iNOS expression is directly influenced by IFN-γ which in turn will help in the production of reactive nitrogen intermediates (RNIs) that may damage the pathogen at the earliest phase of infection. Similar upregulation of iNOS gene was also observed in recombinant IFN-γ-treated gold fish monocytes and macrophages (Grayfer and Belosevic 2009). In addition, the present experiment also involved the expression of pro-inflammatory cytokine, IL-1β. This cytokine is an important mediator of the inflammatory response to microbial invasion and tissue injury and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis (Secombes 1996). Our findings showed similar kinetics of IL-1β gene transcript in both pQE-IFNγ-transfected and Poly I:C-treated cell group which can be correlated with the upregulation pattern in the recombinant IFN-γ-induced gold fish monocytes (Grayfer and Belosevic 2009). The induction of IL-1β by the recombinant IFN-γ can thereby stimulate immune responses by activating lymphocytes or by inducing the release of other cytokines capable of triggering macrophages, natural killer (NK) cells, and lymphocytes during pathogen invasion.

In conclusion, the present study demonstrated positive expression of recombinant IFN-γ in a eukaryotic expression system. In addition, we compared the immune modulatory ability of recombinant IFN-γ with Poly I:C and its efficacy in triggering various immune gene mediated pathways for combating bacterial and viral pathogen. The recombinant IFN-γ was observed to be an equally efficient inducer like Poly I:C. Thus, based on this study, we reaffirmed that recombinant IFN-γ of *L. rohita* is a potential immunomodulator which can be effectively incorporated as an immune adjuvant in therapeutic treatments in fish.

Acknowledgements The authors thank the present Director Dr. Gopal Krishna and Ex-Director Dr. W S Lakra, ICAR-Central Institute of Fisheries Education, Mumbai, India, for providing necessary facilities and support for this study.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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