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Bicistronic DNA vaccine against *Edwardsiella tarda* infection in *Labeo rohita*: Construction and comparative evaluation of its protective efficacy against monocistronic DNA vaccine

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ABSTRACT

DNA-based vaccination or genetic immunization is one of the most promising, effective and prophylactic measures to control aquatic animal diseases. This immunization strategy involves administration of eukaryotic antigen expression vectors (DNA vaccine) into the host that encode for an antigen under the control of a eukaryotic promoter which resulted into elicitation of strong cellular and humoral immune responses. In the present study, a bicistronic DNA vaccine (designated as pGPD + IFN) was constructed which contains an additional immune adjuvant gene (Interferon gamma gene of *Labeo rohita*) along with a regular antigenic gene (glyceraldehyde-3-phosphate dehydrogenase gene of *Edwardsiella tarda*) with the purpose to maximize the protective efficacy of the vaccine against *E. tarda* infection. After construction of the vaccine a pilot study was orchestrated in vitro to ascertain the positive co-expression of the dual genes in vaccine-transfected SSN-1 cell line. Successful co-expression of GAPDH and IFN-γ genes in the transfected cell were confirmed by Western blot and RT-PCR respectively. Further, an in vivo vaccine trial was conducted in which rohu (*L. rohita*) fingerlings were intramuscularly (I/M) injected (initial and booster immunised) with two DNA constructs one group with pGPD + IFN and the other with pGPD (containing GAPDH gene only) and challenged with *E. tarda* (1×10^5 CFU/fish) at 35 day post-initial vaccination. The protective immune responses were determined in terms of relative percentage survival (RPS), specific antibody production, non-specific immune response and expression kinetics of immune-related iNOS gene. Evaluation of RPS analysis revealed that pGPD + IFN group recorded highest RPS of 63.16% while the pGPD vaccinated group showed 47.37% when compared with 63.33% cumulative mortality of control group. The results regarding respiratory burst activity, myeloperoxidase activity as well as antibody titre also showed pGPD + IFN group with highest activities at all the time points. Furthermore, the current study displayed pGPD + IFN group having significant ($p < 0.05$) upregulation of iNOS gene transcript at 24–48 h post-immunization (both initial and booster dose) as well as after challenge. Thus, from this study, we can conclude that the bicistronic vaccine can be an effective immunization strategy in orchestrating a coordinative immune response against *E. tarda* in *L. rohita*.

1. Introduction

The control of bacterial diseases in aquaculture has relied mainly on the use of the antibiotics. Nevertheless, indiscriminate use of antibiotics has raised many issues such as emergence of multidrug resistance and other environmental concerns. Recently, there has been a paradigm shift which is focusing more on prevention rather than control of bacterial disease. With this strategic view, concentrated research efforts have been taking place for developing fish vaccines against various pathogenic diseases. In fish vaccinology study, DNA vaccination is quite a recent strategy which is now being focused on (Heppell et al., 1998; Li et al., 2013; Bedekar et al., 2015).

DNA-based vaccination or genetic immunization has been regarded as an effective vaccination strategy and a promising intervention in fish disease management. DNA vaccines encodes for a specific protein which is antigenic to the host against the specific pathogen, when

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administered to the host resulted in generating systematic immunity in host against the invasion of pathogen (Heppell et al., 1998). Immunization with antigen-encoding plasmid DNA can elicit very strong and long-lasting humoral and cellular immune responses (Donnelly et al., 1997; Cavazzana-Calvo et al., 2000; Jiang et al., 2004). In addition, DNA-based vaccine has several advantages, including no risk of infection and low cost in comparison with traditional vaccines.

Edwardsiella tarda (*E. tarda*) is a gram negative, facultative anaerobic bacterium belonging to the family Enterobacteriaceae, causes *Edwardsiellosis*/putrefactive systemic infection in both marine and freshwater fishes (Sun et al., 2011). The enormous loss caused by this pathogen is being observed in the USA, Japan, Europe and Asian countries; although, no exact figures are available to quantify the loss (Meyer and Bullock, 1973). The disease is characterized with different pathological manifestation like distended abdomen, prolapsed rectum, cutaneous lesions inside the musculature, fibrinous peritonitis, gas pockets in the kidney or musculature and necrosis of the hepatic and renal tissue (Mohanty and Sahoo, 2007). Experimental and natural infections by *E. tarda* in Indian major carps have also been reported (Swain et al., 2002; Swain and Nayak, 2003a, 2003b). In order to prevent fish from *edwardsiellosis* disease, a pDNA vaccine against *E. tarda* has been constructed in our laboratory. Several potential virulence factors of *E. tarda* viz. sialidase NanA, outer membrane proteins (OMP), dermatotoxin, haemolysins, catalase, EseDs and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been reported previously. Among these GAPDH in the outer membrane is selected as the antigenic gene as it is reported to be highly antigenic and is considered to be an effective vaccine candidate to counter both Gram negative and Gram positive bacterial infections (Kawai et al., 2004; Bolton et al., 2004).

Most of the research work on DNA vaccine for fish is based on using a single antigenic gene to generate protective immune response in the host. But in the current study, we incorporated an additional host immune gene (also called immune adjuvants) along with the antigenic gene to enhance the cumulative immune response leading to host survival. In order to maximize the effect of immune adjuvants, Interferon gamma (IFN-γ) is selected as it plays a major role in adaptive cell mediated immune responses being produced by $CD4^+$ T helper 1 (T-H1) lymphocytes and $CD8^+$ cytotoxic T lymphocytes (CTL) in response to MHC-presented antigens (Biron and Sen, 2001).

Thus, the present study deals with the construction of the bicistronic DNA vaccine having an antigenic gene (GAPDH of *E. tarda*) and an immune adjuvant gene (IFN-γ of host fish, *Labeo rohita*). Co-expression of both these genes *in vitro* has been demonstrated to ascertain the viability of the pDNA construct. Further, the constructed bicistronic DNA vaccine was administered to *L. rohita* to evaluate its effect in eliciting immune response in the host as well as its protective immunity by challenge study. In addition, a comparative analysis of two DNA constructs (with or without IFN-γ) has also been evaluated.

2. Material and methods

2.1. Experimental animal

Rohu (*L. rohita*) juveniles (20.0 ± 2.6 g) obtained from a local farm were stocked into 500 L fiber reinforced plastic (FRP) tanks with aerated freshwater in our indoor rearing facility for 3-weeks prior to vaccination trial and were fed twice a day with a standard pelleted diet at 3% of their body weight. Water quality of the tanks was maintained. The water temperature varied from 27 °C to 28 °C and the pH of the water varied from 7.4 to 7.6 during the experiment. To confirm the fishes were *E. tarda*-free, bacterial isolation in SS-agar plate from kidney and liver of ten randomly selected individuals was carried out.

2.2. Bacteria

Edwardsiella tarda used in the study was obtained from ATCC, USA. Pure culture of the bacterium, *Edwardsiella tarda* ATCC® 15947™ was revived in brain heart infusion (BHI) broth from cult loop. The broth was incubated for 18–24 h at 37 °C. The bacterial culture was then streaked on Salmonella-Shigella Agar (SS agar) media and incubated at 28 °C. Single black colony grown on agar plate was inoculated in BHI broth and incubated at 28 °C for 22 h.

2.3. Construction of bicistronic DNA vaccine

2.3.1. Isolation of genomic DNA from E. tarda

The genomic DNA from freshly cultured *E. tarda* was isolated by using GenElute Bacterial Genomic DNA kit (Sigma, USA) according to the manufacturer's protocol. Concentration of DNA obtained was measured using Nanodrop (Thermo Scientific, USA). The resultant DNA was stored at − 20 °C until further use.

2.3.2. Extraction of GAPDH gene from genomic DNA of E. tarda

The genomic DNA prepared from *E. tarda* was amplified with specific primer sets for GAPDH gene (996 bp) designed using Primer Express software from the available NCBI sequences (Gene bank accession no. FJ605131.1) (Table 1) in a 25 μL reaction with Taq DNA polymerase (5 U) (Thermo scientific, USA), with following cyclic condition in a thermal cycler (Applied Biosystems, USA); Initial denaturation at 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min, then a single step of final extension at 72 °C for 10 min. Amplified PCR product was run in 1.2% agarose gel and subsequently purified by gel extraction using Fermentas GeneJET™ Gel Extraction kit.

2.3.3. Extraction of IFN-γ gene from Poly I:C stimulated L. rohita kidney tissue

For induction of IFN-γ gene 8 naive *L. rohita* from same lot were induced by injecting 100 μL of Poly I:C (100 mg/mL in HBSS solution). The kidney tissue was collected from 2 fish at 6 h, 12 h, 24 h and 48 h post-injection and were collected in TRIzol® reagent and used for RNA isolation.

Total RNA from kidney tissues of fish collected at different time-

Table 1
Primers used for amplification of inserts in the DNA vaccine.

Gene (accession no.)	Oligo name	Sequence (5′–3′)	RE site
GAPDH	GPD/pIRES/F	CCC**CTCGAG**ATGACTATCAAAGTAGGTATCA	*Xho* I
(FJ605131.1)	GPD/pIRES/R	CCC**ACGCGT**TTACTTAGAGATGTGTGCGA	*Mlu* I
IFN-γ	IFN/pIRES/F	CCC**GTCGAC**ATGATTGCGCAACAAACAATG	*Sal* I
(HQ667144.1)	IFN/pIRES/R	CCC**GCGGCCGC**TCAAGACTTCTGATTCTTTTTG	*Not* I

Bold in the table signifies the restriction enzymes

Table 2
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	AAGGAAAGCCTCCTAATCGTTGT	62 bp
	iNOS-R	CCATTTCCTGGACAGTCTCCAT	

point was extracted with TRIzol® reagent (Invitrogen, USA) as per the manufacturer's protocol and quantified by Nanodrop (Thermo Scientific, USA). The residual genomic DNA was removed using RNase-free DNase I (Fermentas, USA). Total RNA (1 μg) was reverse-transcribed into first-strand cDNA using First-strand cDNA synthesis kit (Fermentas, USA) using oligo-dT primer and M-MuLV reverse transcriptase in a 20 μL reaction volume. This cDNA was further used as template for extraction of IFN-γ gene.

The IFN-γ gene (552 bp) was amplified with specific primer sets designed using Primer Express software from the available NCBI sequences (Gene bank accession no.HQ667144.1) (Table 1) 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 min). Amplified PCR product was run in 1.2% agarose gel and subsequently purified by gel extraction using Fermentas GeneJET™ Gel Extraction kit.

2.3.4. Cloning of GAPDH gene and IFN-γ gene in pTZ57R/T cloning vector

The gel extracted GAPDH and IFN-γ gene products were cloned into pTZ57R/T vector (Fermentas, USA) and 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 blue-white screening. The recombinant plasmid DNA was isolated using the QIAGEN Plasmid Mini Kit (QIAGEN, Germany) as per the manufacturer's protocol. The concentration of the recombinant plasmids was measured using Nanodrop spectrophotometer (Thermo Scientific, USA) in terms of ng/μL.

2.3.5. Cloning of GAPDH gene and IFN-γ gene in pIRES expression vector

GAPDH gene from pTZ57R/T was released by digestion of recombinant plasmid using restriction enzyme *Xho I* and *Mlu I*. Simultaneously pIRES vector (6.1 kb, Clontech, USA) was digested with same restriction enzyme. Thereafter the GAPDH gene was cloned in pIRES vector in Frame A and designated as pGPD.

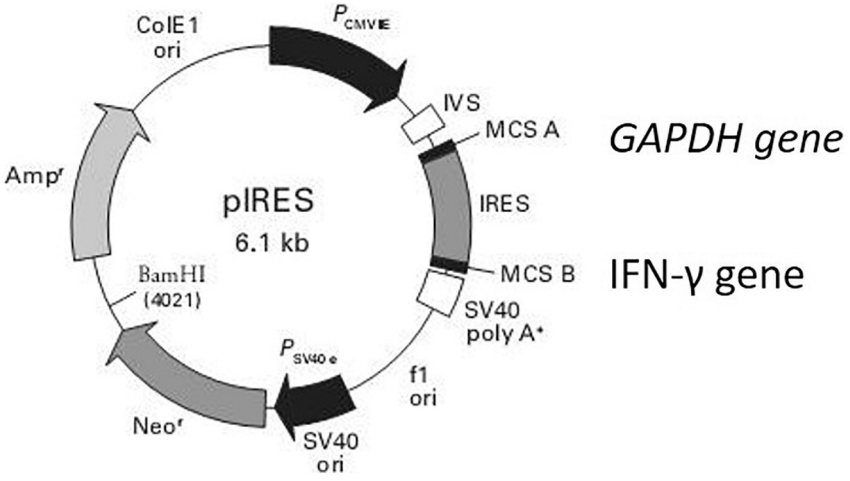

Fig. 1A. Schematic demonstration of the constructed bicistronic DNA vaccine. GAPDH gene and IFN-γ gene cloned in the eukaryotic expression vector, pIRES vector (6.1 kb having CMV promoter) at Frame A and Frame B respectively.

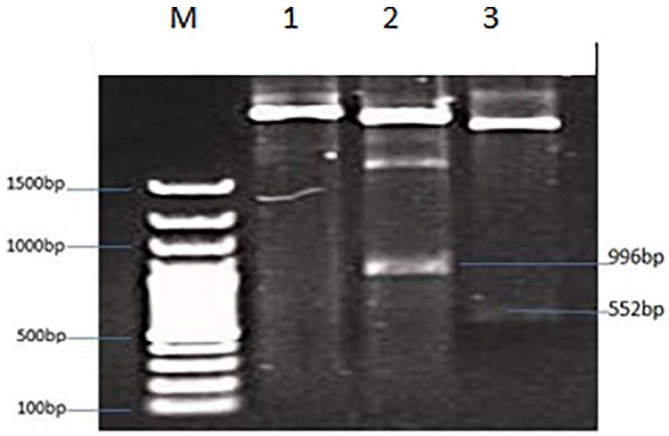

Fig. 1B. Agarose gel electrophoresis (1.2%).showing restriction digestion of DNA construct (in pIRES vector) confirming presence of GAPDH gene and IFN-γ gene inserts; Lane M: 100 bp DNA ladder; Lane 1: naked pIRES vector; Lane 2: release of GAPDH gene (996 bp) after RE digestion of pGPD + IFN construct with *Xho I and Mlu I* restriction enzymes and Lane 4: release of IFN gene (552 bp) after RE digestion of pGPD + IFN construct with *Sal I* and *Not I* restriction enzymes.

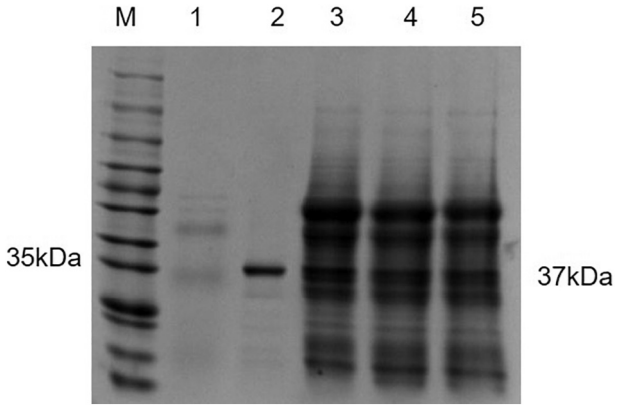

Fig. 2A. Coomassie Blue stained 12% SDS-PAGE showing expression of recombinant 37 kDa protein; Lane M: prestained protein marker (10–245 kDa); Lane 1: negative control (mock pIRES vector transfected SSN-1 cell); Lane 2: purified recombinant 37 kDa GAPDH protein as positive control; Lane 3–5: whole cell extract of SSN-1 cells transfected with pGPD + IFN construct. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Similarly, IFN-γ gene from pTZ57R/T was released by digestion of recombinant plasmid using restriction enzyme *Sal I* and *Not I* and subsequently cloned in pGPD in Frame B after digestion with same

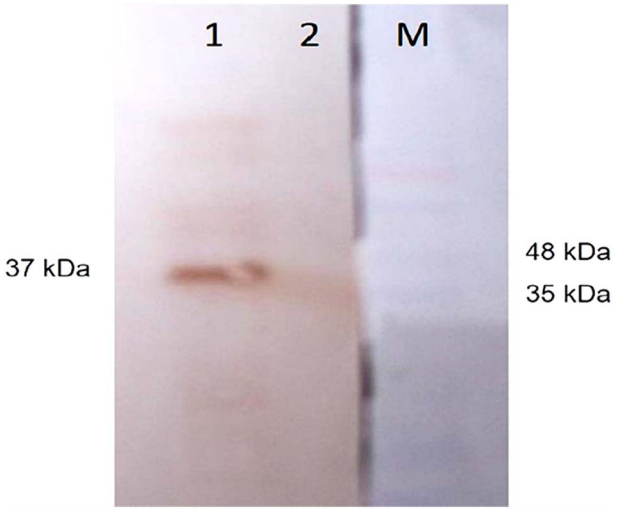

Fig. 2B. Western blot assay of pGPD + IFN transfected SSN-1 cells showing expression of recombinant 37 kDa GAPDH protein specifically reacting with anti-GAPDH MAbs; Lane1: pGPD + IFN transfected SSN-1 cell lysate; Lane 2: negative control (Mock transfected SSN-1 cells); Lane M: prestained protein marker (10–245 kDa).

restriction enzyme. The resultant plasmid vector containing both GAPDH and IFN-γ gene was designated as pGPD + IFN.

2.4. Co-expression of pGPD + IFN in vitro

2.4.1. Transfection of cells using Lipofectamine™ 3000 Reagent

Striped snakehead (SSN-1) cell line (75–80% confluency) was transfected with the purified recombinant plasmid pGPD + IFN using Lipofectamine™ 3000 Reagent, (Thermo Fisher Scientific, USA) as per the manufacturer's protocol. Briefly, the 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 lipoplexes formation. The pDNA-lipid complexes was added to the cells and incubated for 2 to 3 days for the in vitro studies.

2.4.2. Confirmation of expression of GAPDH gene by Western Blot

Cells were collected 48 h post-transfection for confirmation of transgene expression of GAPDH by Western Blot using anti-*E. tarda* GAPDH monoclonal antibodies (MAbs) developed by Banu et al. (2017). Purified recombinant GAPDH protein (Banu et al., 2017) used as positive control.

2.4.3. Confirmation of expression of IFN-γ gene by Immunofluorochemistry

Transfected SSN-1 cells were subjected to Immunofluorochemistry (IFC) 48 h post-transfection for confirmation of trans-gene expression of IFN-γ, using anti-rohu IFN-γ polyclonal antibodies raised in mice

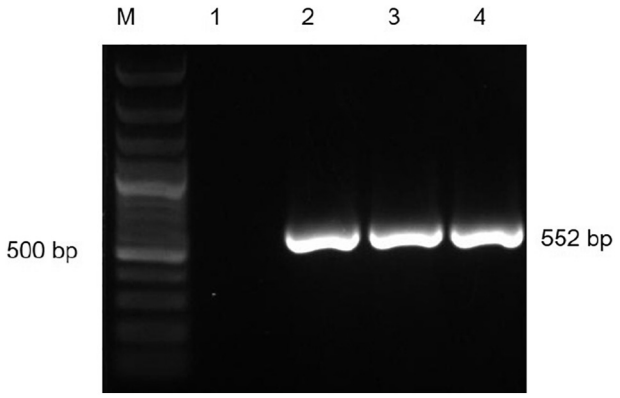

Fig. 3B. Agarose gel electrophoresis (1.2%) of RT-PCR products showing positive expression of recombinant IFN-γ in pGPD + IFN plasmid transfected SSN-1 cells; Lane M: 100 bp DNA marker; Lane 1: negative control (Mock transfected SSN-1 cells), Lane 2–4: IFN-γ gene (552 bp) amplified from pGPD + IFN transfected SSN-1 cells.

(produced in our lab according to the method developed by Belperron et al., 1999) (Supplemental file 1). Briefly, the media was removed and the transfected cells were washed with 1 × PBS prior to the IFC study. The cells were incubated in 3.7% para-formaldehyde for 10 min in 4 °C followed by washing with PBS (twice) and proceeded to permeabilization step. The cells were then incubated with 0.1% Triton X-100 for 5 min at 4 °C. The cells were blocked with 1% BSA in PBS for 30 min at room temperature. The prepared primary antibody of dilution (1:50) in 1% BSA was added and incubated for 2 h in room temperature (antibody: Mouse anti-rohu-IFN-γ polyclonal antibody). The cells were washed three times with washing buffer and subsequently secondary antibody (Goat anti-mouse IgG conjugated FITC, Thermo Fisher Scientific, USA) at a dilution of 1:50 was added and incubated for 45 min in dark at room temperature. The cells were washed thrice with 1 × PBS and cells were mounted with 1, 4- diazobicyclo-2, 2,-octanex (DABCO) in mounting medium. The signals were recorded using Axio-observer A1 Zeiss Fluorescent Inverted Microscope.

2.4.4. Confirmation of expression of IFN-γ gene by RT-PCR

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 residual genomic DNA was removed using DNase I (Fermentas, 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

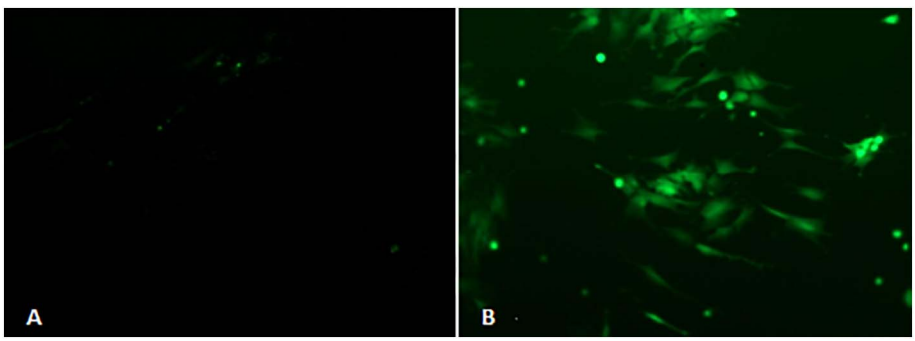

Fig. 3A. Analysis of recombinant IFN-γ expression in SSN-1 cell. Fluorescence observation of (A) mock transfected SSN-1 cells showing no fluorescence after probing with anti IFN-γ polyclonal antibodies and (B) positive fluorescence after probing with anti IFN-γ polyclonal antibodies confirming IFN-γ expression in SSN-1 cells following transfection with pGPD + IFN.

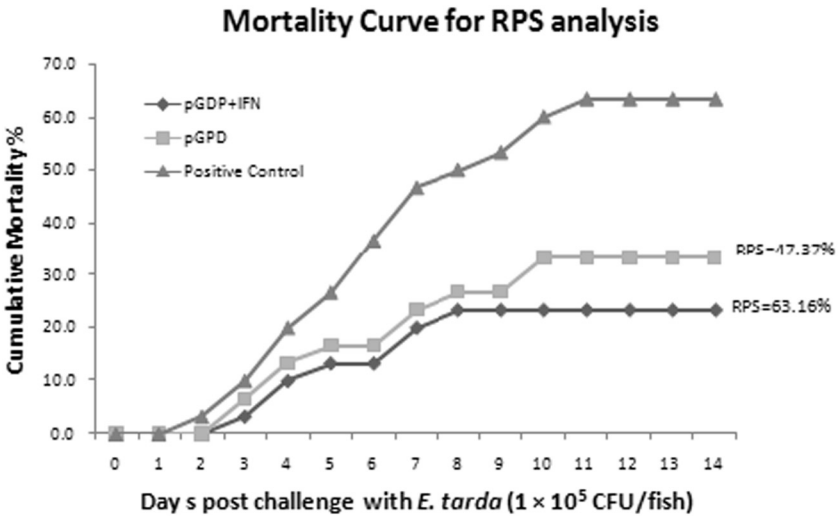

Fig. 4. Cumulative mortality and relative percentage survival (RPS) analysis of different treatment groups (pGPD + IFN, pGPD and positive control) of *L. rohita* (n = 30, 3 tanks with 10 fish/tank for each groups) challenged with *E. tarda* (1×10^5 CFU/fish) at different time intervals.

plasmid construct. The cDNA extracted from SSN-1 cell sample transfected with mock pIRES vector was taken as negative control. The PCR amplification conditions were same as described earlier. The PCR product was run on 1.2% agarose gel.

2.5. In vivo evaluation of vaccine efficacy of pGPD + IFN

2.5.1. Experimental design for immunization trial challenge study

Rohu fingerlings (n = 280, n = number of fish) were randomly distributed into 3 treatment groups with 70 fishes in each group and designated as pGPD, pGPD + IFN, positive control (challenged group) and remaining 70 fishes were kept as naïve group without any treatment. For immunization, the vaccine groups were intramuscularly (I/M) injected with 100 μL of pGPD (10 μg/fish) and pGPD + IFN (10 μg/fish) plasmid constructs respectively while the positive control group was mock injected with 100 μL of PBS. Booster dose was administered at 21 day post-initial immunization. Three fish from each group (treatment groups and positive control group) were randomly selected for sampling at 24 h, 48 h and 96 h post-initial vaccination (0-day) and booster vaccination (21-day) while 3 fish per group were sampled before challenge (35-days post-immunization). Blood, serum and kidney tissue samples were collected at each time point for further analysis of immune parameters.

2.5.2. Challenge study

Viable count of *E. tarda* was determined as colony forming unit (CFU) following 10 fold serial dilutions and plating on nutrient agar. The LD_{50} dose was calculated following the method of Reed and Muench (1938) from same experimental group of fishes (10 fish per dilution). For challenge study, remaining 49 fish from each of the 3 treatment groups viz., pGPD, pGPD + IFN, and positive control groups were intra-peritoneally injected with 100 μL of bacterial suspension (LD_{50} dose of 1×10^5 CFU/fish) 35 day post-initial immunization. For mortality observation, 30 fish per group were divided into 3 separate tanks (n = 10 / tank where n is number of fish). Mortality pattern was observed for 14 days post-infection. The cause of mortality was confirmed by re-isolating the bacteria from the kidney of dead fish. Relative percentage of survival (RPS) was calculated as described by the formulae (Amend, 1981); Relative percentage of survival (RPS) = {1 − (%Mortality in vaccinated group / %Mortality in control group)} × 100. While from the remaining fishes in each group (including naïve group) 3 fish per time-point were randomly sampled for blood, serum and kidney tissue samples at 24 h, 48 h, 96 h and 144 h post-challenge.

2.5.3. Non-specific immune responses

For non-specific immune responses, respiratory burst activity and myeloperoxidase activity were analysed from blood and serum samples respectively collected at different time intervals. Respiratory burst activity was assessed by Nitroblue tetrazolium (NBT) reduction assay according to the method described by Stasiack and Bauman (1996). Briefly, 100 μL of blood was placed into the wells of flat bottom microtitre plates and incubated at 37 °C for 1 h. After 1 h, supernatant was removed from the wells and were washed 3 times with PBS. After washing, 100 μL of 0.2% NBT solution (Sigma, USA) was added and incubated for further 1 h. The reaction was stopped by adding 100% methanol. The wells were washed with 70% methanol, and the reduced formazan was solubilised in 120 μL of 2 N KOH and 140 μL of DMSO. The optical density of the wells (OD) was recorded at 540 nm in ELISA plate reader (BIOTEK, USA). The total myeloperoxidase (MPO) content of the serum samples from different time points was determined as described by Quade and Roth (1997) and partially modified by Sahoo et al. (2005). Briefly, 15 μL of serum was diluted with 135 μL of Hanks balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+} in 96-well microtitre plate to which 25 μL of 20 mM 3, 3, 5, 5-tetramethyl benzidine hydrochloride (TMB) (Genei, India) and 25 μL of 5 mM hydrogen peroxide (H_2O_2) were added. The reaction was stopped by adding 50 μL of 4 M sulphuric acid after 2 min of incubation. Plate was centrifuged at 400 × g for 10 min. 150 μL of supernatant was transferred to a fresh microtitre plate. The optical density was read at 450 nm in an ELISA plate reader (BIOTEK, USA).

2.5.4. Competitive ELISA for specific antibody quantification

Antibody (IgM) quantification was done by competitive enzyme-linked immunosorbent assay (c-ELISA) as described by Swain and Nayak (2003a, 2003b) with slight modification. Briefly, 100 μL of *E. tarda* cells (1×10^5 CFU/mL) diluted in coating buffer (carbonate – bicarbonate buffer, pH 9.6) was coated in 96 well ELISA plates (Nunc, Denmark) and incubated at 4 °C overnight. The plates were washed with wash buffer (PBS-T, 0.05% tween20 in PBS pH 7.4) thrice for 5 min and blocked by 300 μL blocking buffer (3% BSA solution in PBS-T) and incubated further for 1 h at 37 °C. The plates were washed again with washing buffer thrice for 5 min each. Serum from all four groups i.e. pGPD, pGPD + IFN, positive control and naive groups collected in triplicate at different time-points were diluted with 1% BSA in PBS-T @ 1:200. 50 μL of each sample was added in different wells and incubated in an ELISA shaker at room temperature for 30 min. Thereafter 50 μL of diluted (1:200) monoclonal antibody raised against GAPDH protein (MAbs developed by Banu et al., 2017) was added to each well and kept overnight at 4 °C. While in one row of the plate only 50 μL of MAbs was

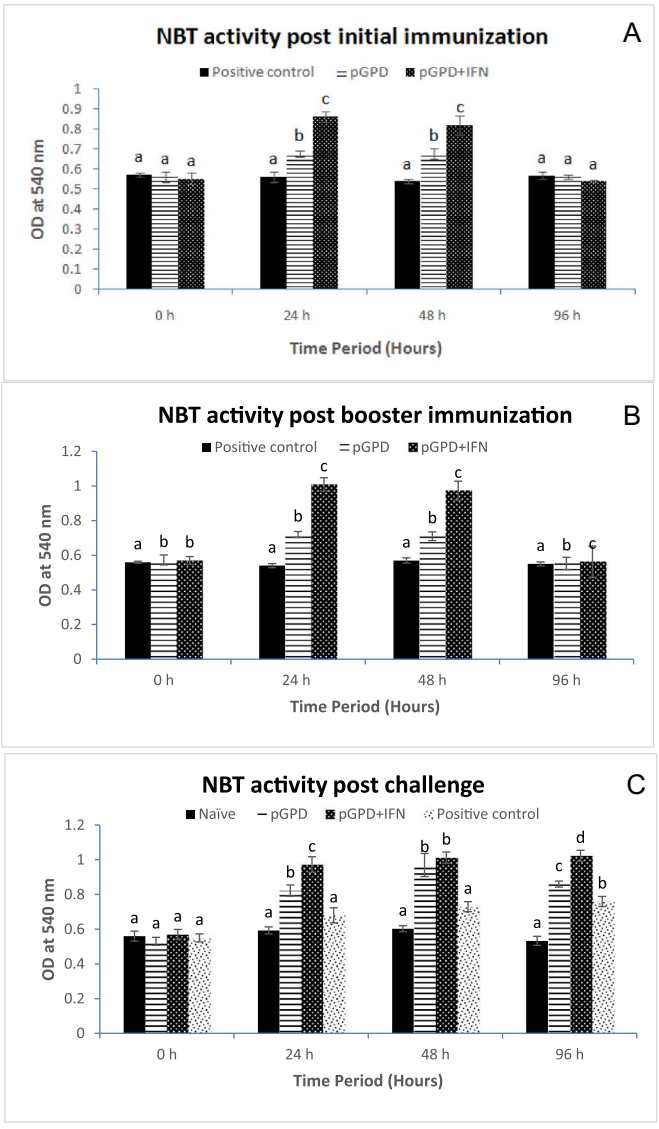

Fig. 5. Respiratory burst activity (NBT) of different experimental group viz. pGPD + IFN, pGPD, Positive control and Naïve group at different time intervals post-initial immunization (A), post-booster immunization (B) and post-challenge with *E. tarda* (1×10^5 CFU/fish). The mean values ($n = 3$) of the NBT activities in the blood cells were plotted with standard error as a function time after immunization/challenge. A one-way ANOVA ($p < 0.05$) and Duncan's multiple range test (homogenous subsets indicated by alphabets) was used to determine statistical significance of the NBT activities at various time points.

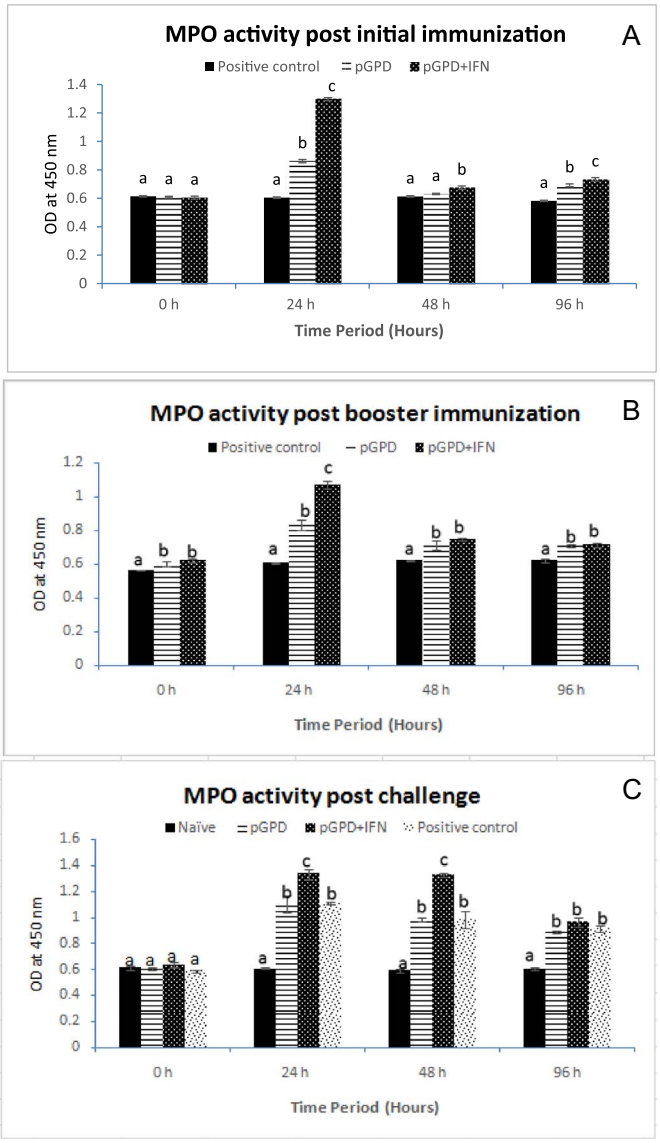

Fig. 6. Myeloperoxidase activity (MPO) of different experimental group viz. pGPD + IFN, pGPD, positive control and naïve group at different time intervals post-initial immunization (A), post-booster immunization (B) and post-challenge with *E. tarda* (1×10^5 CFU/fish). The mean values ($n = 3$) of the MPO activities in the serum were plotted with standard error as a function time after immunization/challenge. A one-way ANOVA ($p < 0.05$) and Duncan's multiple range test (homogenous subsets indicated by alphabets) was used to determine statistical significance of the MPO activities at various time points.

added in each well for MAbs reading. The plates were washed thrice by wash buffer next day and incubated with 100 μL of secondary antibody (Goat anti-mice Alkaline phosphatase conjugate) diluted 1:2000 immediately in PBS-T at 37 °C for 1 h. The plates were thoroughly washed with PBS-T five times and substrate *O*-phenylenediamine tetra hydrochloride (OPD) (100 mg/mL of OPD and 40 μL of H_2O_2 (30% *v*/v) in 5 mL of citrate-phosphate buffer, pH 5) was added to each well at the rate of 100 μL per well. The plates were incubated for 5–10 min at 37 °C in dark chamber. After that the reaction was stopped with 50 μL of 3 M H_2SO_4 and optical density (OD) was recorded at 492 nm in ELISA plate reader (BIOTEK, USA). The result was expressed as percentage inhibition, derived by following formula: PI = 100 − (mean OD_{492} of test serum × 100) / (mean OD_{492} of MAbs).

2.5.5. *iNOS gene expression as an indicator for IFN-γ induction*

The relative expression of Inducible nitric oxide synthase (iNOS) gene was studied as an indicator of induction by IFN-γ of pDNA. For expression kinetic study, kidney tissues collected from different fish-group at different time points were processed for total RNA extraction using TRIzol® reagent (Invitrogen, USA) as per the manufacturer's protocol and quantified by Nanodrop (Thermo Scientific, USA). The residual genomic DNA was removed using RNAse-free DNAse I (Fermentas, USA). Total RNA (1 μg) was reverse-transcribed into first-strand cDNA using First-strand cDNA synthesis kit (Fermentas, USA) using oligo-dT primer and M-MuLV reverse transcriptase in a 20 μL reaction volume.

PCR primers were designed for iNOS and β-actin (internal control) genes using Primer Express software from the available NCBI sequences as mentioned in Table 2. The cDNA prepared from samples were used as template. 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

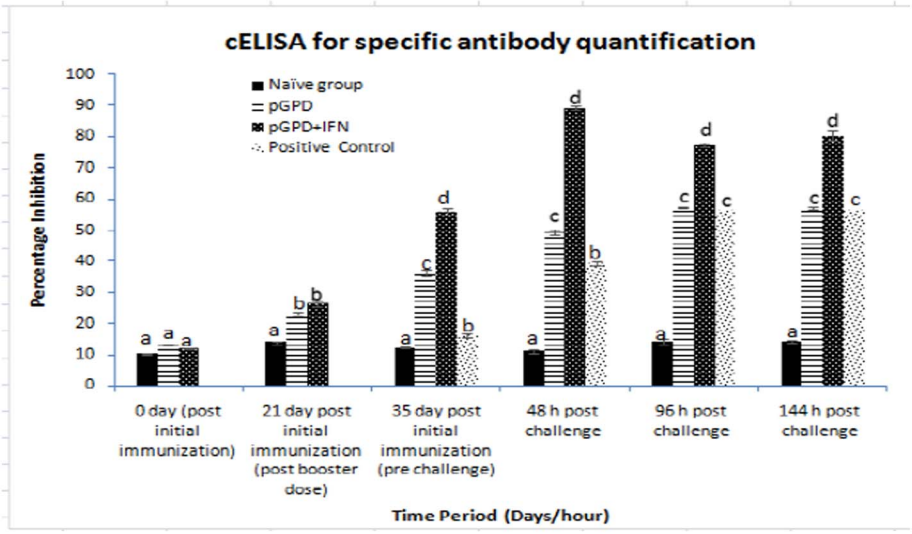

Fig. 7. Percentage inhibitions (PI) of serum-antibody against *E. tarda* were determined by competitive ELISA using anti-GAPDH MAbs. Percentage inhibition of anti-GAPDH antibody binding to *E. tarda*, present in the fish serum of different experimental group viz. pGPD + IFN, pGPD, positive control and naïve group were plotted at different time intervals post-initial immunization (A), post-booster immunization (B) and post-challenge with *E. tarda* (1×10^5 CFU/fish). The mean values ($n = 3$) of the PI activities in the serum were plotted with standard error as a function time after immunization/challenge. A one-way ANOVA ($p < 0.05$) and Duncan's multiple range test (homogenous subsets indicated by alphabets) was used to determine statistical significance of the PI activities at various time points.

each gene-specific primers (iNOS and β-actin) 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, 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 iNOS was normalised by internal control, β-actin gene and comparison of control vs. treatment groups. Fold change of iNOS was calculated using $2^{\Delta\Delta ct}$ method (Livak and Schmittgen, 2001).

2.6. Statistical analysis

The data generated for different gene expression were statistically analysed by statistical package SPSS version 16 (SPSS Inc., USA) in which data were subjected to one-way ANOVA and Duncan's multiple range test was used to determine the significant differences between the means. Comparisons were made at the 5% probability level. *p*-Value below 0.05 was considered statistically significant. The results were expressed as the mean ± S.E.

3. Results and discussion

3.1. Confirmation for positive construction of bicistronic DNA vaccine

In the present study, a bicistronic DNA vaccine was constructed which is illustrated with the help of a schematic diagram (Fig. 1A). The DNA construct consists of pIRES vector (6.1 kb, vector with internal ribosomal entry site), in which GAPDH gene (996 bp antigenic gene from *E. tarda*) and IFN-γ gene (552 bp immune-adjuvant gene from L. *rohita*) were inserted at Frame A and Frame B of the vector respectively. The CMV promoter present in the vector helps the antigenic GAPDH gene as well as IFN-γ gene to express inside a eukaryotic system after administration and thus serves the purpose for constructing this DNA vaccine as a prophylactic measures to combat *edwardsiellosis* in *L. rohita*. The presence of GAPDH and IFN-γ gene in the recombinant plasmid (pGPD + IFN) was confirmed by restriction enzyme digestion. Release of 996 bp GAPDH fragment and 552 bp IFN-γ fragments (Fig. 1B) confirmed that the recombinant plasmid was successfully constructed.

3.2. Co-expression of recombinant GAPDH and IFN-γ gene in vitro

In order to investigate whether GAPDH and IFN-γ gene will express in a eukaryotic system, the recombinant pGPD + IFN was transfected in SSN-1 cell line in vitro. The SDS-PAGE (Fig. 2A) and Western Blot (Fig. 2B) results from the transfected cells showed that the recombinant plasmid expresses GAPDH protein *in vitro*. The resultant band at 37 kDa coincides with the recombinant GAPDH protein band produced by Banu et al. (2017). Thus, it can be ascertained that the GAPDH protein can also be expressed from the recombinant plasmid, when administered in the eukaryotic host i.e. *L. rohita* and helps in inducing protective immune responses in the host.

We have confirmed expression of IFN-γ by IFC as well as RT-PCR. The positive fluorescence signal (Fig. 3A) in the pGPD + IFN construct transfected SSN-1 cells confirmed positive expression of recombinant IFN-γ. In addition, the RT-PCR results (Fig. 3B) displayed that the transfected SSN-1 cells contains mRNA transcript for IFN-γ gene which can be only possible when there is positive expression of recombinant IFN-γ, as DNase I treatment ruled-out the possibility of amplification of IFN-γ from other sources. From these positive expressions of GAPDH and IFN-γ *in vitro*, we can assure that the vaccine will also express in the *L. rohita* and give an enhanced immunogenic effect in the host fish which can counter *E. tarda* infection effectively.

3.3. Challenge study and relative percentage survival (RPS) analysis

After challenged with the bacterium, the moribund fishes across various treatment groups showed typical signs of acute septicaemia with dropsy, external lesions, haemorrhages and congested vent starting at 36–48 h post-challenge (hpc). No gross lesion or mild congestion at ventral parts of body was demonstrated in the survivor fish during peak infection stage which subsides in the later phase of infection. Mortality was observed first time at 2 day post-challenge (dpc) and continued up to 11 dpc after which no mortality was observed. From the challenge study (Fig. 4), it was revealed that, the pGPD + IFN immunised group recorded highest RPS of 63.16% while the pGPD vaccinated group showed 47.37% when compared with the cumulative mortality percent in the positive control group (63.33%). The cause of mortality was confirmed by re-isolating the bacteria from kidney of freshly dead or moribund fish. The RPS data proves the efficacy of the constructed vaccine against *E. tarda*. The results also highlighted how most of the control fish succumbed to pathogen-mediated lethality while fish in the vaccinated groups evade the bacterial infection. In addition, the difference in the RPS value in the pGPD + IFN and pGPD groups showed that there is an obvious effect of IFN-γ in the host

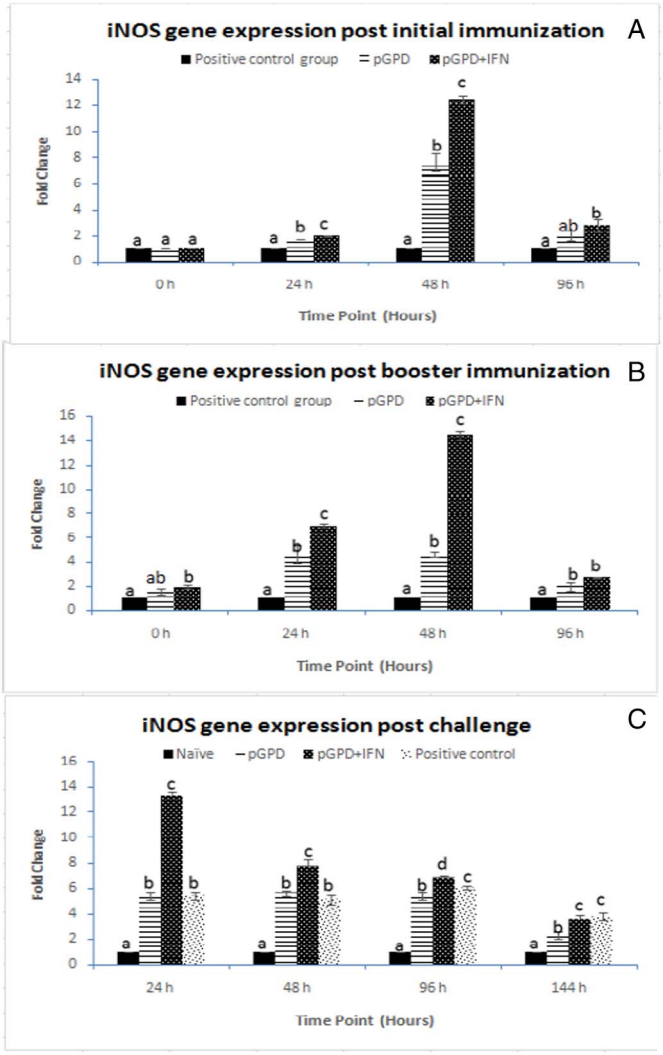

Fig. 8. Relative quantification of iNOS gene (normalised with internal control β-actin) in kidney of *L. rohita* of different experimental group viz. pGPD + IFN, pGPD, positive control and naïve group at different time intervals post-initial immunization (A), post-booster immunization (B) and post-challenge with *E. tarda* (1×10^5 CFU/fish) at different time intervals. The mean values ($n = 3$) of the iNOS transcript in the kidney were plotted with standard error as a function time after infection. A one-way ANOVA ($p < 0.05$) and Duncan's multiple range test (homogenous subsets indicated by alphabets) was used to determine statistical significance of the time-oriented iNOS expression at various time points.

immune response to the bacterial invasion. Similar synergistic effect of IFN-γ was previously recorded where it enhances non-specific immune responses by triggering the cascade of cytokines and other immune molecules (Xiang and Ertl, 1995; Zaidi and Merlino, 2011; Parhi et al., 2015). But the exact effect of IFN-γ as an immune adjuvant needs to be studied in detail.

3.4. Evaluation of non-specific and specific immune responses

Apart from the RPS analysis, specific antibody responses as well as innate immune responses are critical parameters for an effective vaccine. The respiratory burst and myeloperoxidase activity are oxygen-dependent reactions which are widely used to evaluate the defense ability of the host against pathogens (Sharp and Secombes, 1993). In the present study, the immunised group showed significantly ($p < 0.05$) higher NBT activity (Fig. 5A and B) and MPO activity (Fig. 6A and B) at 24 h and 48 h post-vaccination (both initial and booster dose) when compared with the control group. Moreover the pGPD + IFN group showed relatively higher activity than the pGPD group. During the challenge study, both NBT activity (Fig. 5C) and MPO activity (Fig. 6C) increased significantly ($p < 0.05$) after challenge with bacteria irrespective of groups but the pGPD + IFN group displayed highest activities at all the time points. These results indicate possible neutrophils activation in the host after countering foreign antigen (in vaccine) or whole bacterium which ultimately helped in the clearance of the bacteria. Furthermore, the highest activities in the pGPD + IFN group could explain the role of IFN-γ in stimulating different chemokines which in turn helps in attracting various immune cells to the infection sites.

In addition to the non-specific immune assays, a competitive ELISA (Fig. 7) was carried out for the detection of fish-anti-GAPDH antibody. The present results showed significantly ($p < 0.05$) higher immune response in the immunised group than the control group both pre- and post-challenge period. The increased level of antibody titre at 21 day post-immunization (after booster dose) from the minimal level of titre recorded after initial immunization signifies gradual production of IgM in response to the DNA vaccine. The antibody titre during the peak infection phase (post-challenge) can be correlated with the mortality pattern where higher specific antibody level in the vaccinated groups apparently helps to overcome the bacterial pathogenicity. Similar findings of increased antibody titre in response to DNA vaccines were recorded previously (Joosten et al., 1997; Kumar et al., 2008; Tian et al., 2008). However, the reason behind the sudden rise in the antibody level in the pGPD + IFN group after challenge is not well understood suggesting a further detailed study.

Furthermore, we have also studied the expression kinetics of iNOS gene (Fig. 8) as it is an essential effector molecule and a well-known immune-regulatory factor important in the defense against various pathogens (Acosta et al., 2004; Bridle et al., 2006; Kole et al., 2017). In the current study, iNOS mRNA transcripts significantly ($p < 0.05$) increases at 24–48 h post-immunization (both initial and booster dose) as well as after challenge however the expression level declines to minimal level after 96 h. The observed up-regulation in iNOS expression in the immunised group might have helped in the production of reactive nitrogen intermediates (RNIs) which may inhibit the pathogen at the initial phase of infection leading to host survival in contrast with the prolonged mortality in the control group. Also, the difference in the expression level of iNOS gene transcripts in the pGPD + IFN and pGPD group allowed us to predict the role of IFN-γ in stimulating various antimicrobial molecules like iNOS which have a coordinative immune response against *E. tarda* infection.

In conclusion, our study revealed that the constructed bicistronic DNA vaccine having GAPDH as antigenic gene and IFN-γ as immune adjuvant is an effective immunization strategy for protection against *edwardsiellosis* in *L. rohita*. The moderate RPS value (63.16%) obtained in the present experiment shows that there is a scope for further study to improve the efficacy of the vaccine as well as the delivery mechanism. Nonetheless, based on this study we can conclude that the presence of the additional IFN-γ gene increases the potentiality of the DNA vaccine in inducing protective immune responses in the host.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2017.11.052.

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