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Re-engineering of bicistronic plasmid pGPD/IFN to construct fusion gene co-expressing Glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) of *Edwardsiella tarda* and Interferon-gamma (IFN-γ) gene of *Labeo rohita* (Hamilton) and its *in vitro* functional analysis

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Edwardsiella septicemia disease in the cultured Indian major carps is caused by the fish pathogen *Edwardsiella tarda* and it is preventable by DNA vaccination. Here, we tried to develop a bicistronic DNA vaccine pGPD/IFN expressing the Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene of *Edwardsiella tarda* and Interferon-gamma (IFN-γ) gene of *Labeo rohita.* The vaccine showed high protective efficiency in our previous studies; however as a limitation of bicistronic construct the expression of gene cloned in second frame (B) is poor. To overcome this limitation we re-engineered the construct and designed a fusion gene co-expressing the GAPDH and IFN-γ genes as one frame with an aim to get the optimum expression of both the genes. For this purpose, a fusion insert comprising GAPDH and IFN-γ coding sequences was cloned in to pcDNA3.1(+) plasmid vector. The fusion genes' *in vitro* expression was confirmed in the striped snakehead fish cell line (SSN-1). Successful expression of the re-engineered fusion gene DNA vaccine in the cell line was achieved at 48h post-transfection, which was confirmed by amplifying the expression transcripts of GAPDH and IFN-γ genes. Thus, the study concludes that the re-engineered fusion vaccine pcGPD/IFN (pcDNA3.1(+) plasmid having fusion GPD/IFN) is functional and can be effectively utilized to vaccinate rohu (*Labeo rohita*) as it contains the species-specific immune gene (IFN-γ) as an adjuvant.

Keywords: DNA vaccine, Immune adjuvant, pcGPD/IFN, pIRES, Rohu

Edwardsiellosis has been associated with epizootics in cultured Indian major carps and the disease occurs in fishes of all ages causing high mortality rate[1]. The pathogen *Edwardsiella tarda* is a motile, Gram negative, rod-shaped short bacterium of about 3 µm in length and 1 µm in diameter[2]. Moreover, *Edwardsiella tarda* causes severe gastroenteritis, wound infection, meningitis, and urinary tract infection in humans[3]. One of the most virulent factors of *Edwardsiella tarda* is the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the outer membrane (37 KDa)[4]. *Labeo rohita* (Hamilton, 1822), commonly called Rohu, is one of the most cultured fishes in the world contributing 2484.8 MT to the aquaculture production[5]. The interferon-gamma (IFN-γ) of *Labeo rohita* is an adjuvant gene typically involved in the immune response of the fish against invaders of different microbial origins[6].

A bicistronic DNA vaccine for *Labeo rohita* against *Edwardsiella tarda* was constructed in our laboratory by combining the GAPDH and IFN-γ genes in the pIRES vector and designated as pGPD/IFN[7]. pIRES is a bicistronic plasmid vector with an ampicillin selection marker having frames 'A' (cloned GAPDH gene) and 'B' (cloned IFN-ɣ gene) separated by the IRES sequence[7]. Even though the IRES-mediated expression utilizes a cap-dependent single promoter upstream which avoids promoter

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Abbreviations: GAPDH, Glyceraldehyde 3, phosphate dehydrogenase; GFP, Green Fluorescence Protein; IFN-ɣ, Interferon, gamma; IRES, Internal Ribosome Entry Site; GPD/IFN, GAPDH and IFN-ɣ genes; PCR, Polymerase Chain Reaction; RT PCR, Reverse Transcriptase PCR; pIRES, Plasmid having IRES; pGPD/IFN, pIRES having GPD/IFN; pcGPD, pcDNA3.1(+) plasmid having GAPDH gene; pcGPD/IFN , pcDNA3.1(+) plasmid having fusion GPD/IFN; RPS, Relative Percent Survival; RE, Restriction Enzyme; SSN-1, striped snakehead cell line

interference, there is a decreased expression efficiency report of the second gene luciferase [secreted alkaline phosphatase (SEAP) and chloramphenicol acetyltransferase (CAT)] in the system both *in vitro* (CHO cells, HeLa, and L) and *in vivo* (mouse)[8]. The decreased expression of the second gene (20-50% of those of the first gene) is due to dependence on IRES compared to the cap-dependent first gene expression[8]. In the pGPD/IFN vaccine[7], the second gene (IFN-ɣ) of *Labeo rohita* coordinates a diverse array of cellular programs through transcriptional regulation of immunologically relevant genes[9]; however, the vaccine with the limited expression of the IFN-ɣ gene has been proven to be moderately effective in *in vivo* trials with RPS ranging from only 63.16% (naked vaccine) to 64.7% (nano-conjugated vaccine)[10]. Hence, to facilitate the effective expression of the second gene in an IRES independent manner, fusion expression of the vaccine genes is conceptualized.

Also, fusion genes are found to be effective in inducing protective immunity in various findings as in fragment C of tetanus toxin fused with DNA vaccine[11] and another DNA vaccine (hemagglutinin-based influenza vaccine) that was fused to CTLA4 (cytotoxic T-lymphocyte-associated protein 4)[12]. Thus in the present study, we tried to re-engineer pGPD/IFN for avoiding IRES region by genetic fusion of genes and to confirm its functionality by *in vitro* assay.

Materials and Methods

Plasmids, genes, bacterial strain, and cell line

The plasmids used in the study are pGPD/IFN (pIRES, 6.1kb, Clontech, USA), pcDNA3.1(+) (Invitrogen, San Diego, CA), and pmaxGFP (Lonza). The vaccine genes are GAPDH (Gene bank accession no. FJ605131.1) and IFN-ɣ (Gene bank accession no.HQ667144.1), for which fusion and internal amplification primers were designed using Primer Express software (Table 1).

The DH5α *Escherichia coli* strain was used for the cloning purpose in the study, grown in Luria Broth (Difco, USA) at 37°C. Ampicillin (final concentration, 100 µg/mL) was added as per the requirement of the LB media for transformation. The fish cell line (SSN-1) was maintained in L-15 medium with 7.4 pH and supplemented with 15% fetal bovine serum (FBS), 10,000 IU penicillin /mL, 10 mg streptomycin /mL, and amphotericin B (25 µg/mL) as an antifungal agent. The cell line was sub-cultured every 3-5 days[13].

DNA isolation and PCR using fusion primers

The plasmid DNA (pGPD/IFN) was isolated using the plasmid miniprep kit (Thermo Scientific) following the manufacturer's protocol. PCR amplification of the gene GAPDH is carried out in a 25 µL reaction comprising 10X PCR buffer (2.5 µL), 10mM dNTP (0.5 µL), 50mM $MgCl_2$ (0.5 µL), Platinum Taq DNA polymerase (0.1 µL) (Thermo scientific, USA), forward and reverse fusion primers each 1 µL and 100 ng of template plasmid (pGDP/IFN) and the volume is made up by nuclease-free water. The following reaction conditions were followed in a thermal cycler (Applied Biosystems, USA), 95°C for 5 min, 30 cycles of 95°C for 1 min, 56°C for 1 min, 72°C for 1 min, then a final single step extension of 72°C for 10 min.

PCR amplification cyclic conditions of IFN-γ gene (using fusion primer) in a 25 µL reaction was 95°C for 5 min, 30 cycles of 94°C for 30 s, 57.5°C for 40 s, 72°C for 50 s, then a final extension of 72°C for 10 min. The amplified fragments were purified by Geneget gel extraction kit (Thermo Scientific) after electrophoresis (Bio-Rad, USA) in 1% agarose gel.

PCR of internal gene amplification

Using the internal primers, PCR amplification of the gene GAPDH was under the cycling conditions of 95°C for 5 min, 30 cycles of 95°C for 1 min, 58°C for 1 min, 72°C for 30 s and a final extension of 72°C for 10 min. Amplification of the gene IFN-γ (using

Table 1 — List of specific primers used in the study

Gene (Accession No.)	Oligo name	Sequence (5'-3')	Amplicon size
GAPDH (FJ605131.1)	RT GAPDH F	GGCGCTAGCCAGAACATCAT 20mer	200bp
	RT GAPDH R (Internal primer)	TCAGAAGCGGCCTTCATCAC 20mer	
	GAPDH KpnI F	AAAGGTACCATGACTATCAAAGTAGGTATCA 31mer (*Kpn*I)	1014bp
	GAPDH BamHI R (fusion primer)	AAAGGATCCATACTTAGAGATGTGTGCGA 29mer (*BamH*I)	
IFN-γ (HQ667144.1)	RT IFN F	ACAGGATATTCACTCGCATGGA 22mer	200bp
	RT IFN R (Internal primer)	CAGCTCGAACAATGCTTTGC 20mer	
	IFN EcoRI F	AAAGAATTCTATGATTGCGCAACAAACAATG 31mer (*Eco*RI)	574bp
	IFN XhoI R (fusion primer)	AAACTCGAGTCAAGACTTCTGATTCTTTTTG 31mer (*Xho*I)	

internal primers) was done under the cycling conditions of 95°C for 5 min, 30 cycles of 94°C for 30 s, 59°C for 40 s, 72°C for 30 s and final extension of 72°C for 10 min.

Restriction enzyme (RE) digestion and purification

In order to generate fusion genes by cloning, RE digestion using *Kpn*I, *BamH*I, *EcoR*I, and *Xho*I (Fast digest Thermo Scientific) enzymes using two combinations at a time following the supplier's protocol was done. For the GAPDH gene, *Kpn*I and *BamH*I enzymes were used. Similarly, for the IFN-γ gene, *EcoR*I and *Xho*I enzymes were used.

A 20 µL reaction mixture was prepared by adding purified GAPDH DNA (up to 1 µg), 10X FD buffer (2 µL), *Kpn*I (1 µL), *BamH*I (1 µL) and nuclease-free water (to make up to 20 µL). The reaction mixture was incubated (37°C for 60 min and 80°C for 5 min) and subsequently purified. Similarly, RE digestion of the pcDNA 3.1(+) plasmid, IFN-γ gene, and pcGPD plasmid was done using appropriate enzyme combinations at required stages of cloning. The digested DNA (GAPDH and IFN-ɣ) was purified using the Geneget gel extraction kit (Thermo Scientific) as per the suppliers' protocol. The concentration of purified DNA at each step was measured using Nanodrop (Thermo Scientific, USA) and stored at –20°C (Voltas, India) till further use.

For confirmation of each clone, the plasmids at the end of each stage were RE digested releasing the fragments for which the enzyme combinations and the resultant product release are given in Table 2.

Ligation

The RE digested, purified GAPDH insert was ligated into the RE digested and purified pcDNA 3.1(+) vector using the T4 DNA ligase (Fermentas, USA). The ligation reaction was done in a 20 µL total volume by adding 5X Ligation Buffer (4 µL), vector, purified PCR fragment (based on concentration and base pair of PCR product of GAPDH), 5 u/µL T4 DNA Ligase (1 µL), and nuclease-free water (to make up the final volume of 20 µL). The reaction mixture was mixed and incubated (16°C overnight) in a thermal cycler for successful ligation. A similar method was carried out for subsequent IFN-γ insert ligation with the GAPDH cloned pcDNA3.1(+) vector (pcGPD).

Table 2 — Details of enzyme combinations of RE digestion and product release from plasmids

Plasmid type	Enzymes used	Released product
pcDNA3.1(+)	*Kpn*I &*BamH*I	8 bp release
pcGPD	*Kpn*I &*BamH*I	GAPDH gene (1002bp)
	*EcoR*I &*Xho*I	33bp release
pcGPD/IFN	*Kpn*I &*Xho*I	GPD/IFN fusion gene (1588bp)
	*EcoR*I &*Xho*I	IFN-ɣ gene (562bp)

Competent cell preparation and transformation

The transformation protocol of the InstAclone PCR Cloning kit (Fermentas, USA) was carried out as per the procedure supplied by the manufacturer. DH5α strain of *Escherichia coli* was inoculated into 2 mL of C-medium, and the culture was incubated overnight at 37°C. Fresh C-medium (1.5 mL) was taken to which 150 µL of the overnight culture was added and incubated (37°C for 20 min at 200 RPM). The bacterial culture thus obtained was used for transformation.

T-solution was prepared by mixing equal volumes of T-solution (A) with T-solution (B) and was kept on ice. The 1.5 mL of *Escherichia coli* culture was centrifuged and re-suspended in 120 µL of T solution and incubated on ice, and further centrifugation and resuspension were done. The 50 µL of the re-suspended cells were added to the ice stored 2.5 µL ligated plasmid and kept on ice for transformation. The cells were plated on the pre-warmed LB agar (100 µg/mL ampicillin) and incubated (Wadegati Pvt. Ltd., India) overnight at 37°C until the transformed bacterial colonies were visible.

Screening of clone by colony PCR

Colonies were screened for the vaccine genes using colony PCR. The colonies were picked individually using a sterile disposable inoculation loop and suspended in a total volume of 25 µL reaction mixture prepared by adding 12.5 µL of the 2X master mix (Thermoscientific, USA), 10.5 µL of nuclease-free water, 1 µL of each forward and reverse primers (25 picomoles). The PCR amplification was done (using reaction conditions of fusion primers), and the amplicons were checked in a 1% agarose gel.

***In vitro* gene expression analysis**

The SSN-1 cells in culture plates (6 well) were grown until the subconfluent monolayer (80%) with an average of 87.2×10^4 cells/mL. Transfection of fusion gene plasmid (pcGPD/IFN) was done in triplicate using the Lipofectamine3000 reagent according to the manufacturer's protocol (Thermo Fisher Scientific, USA). Control transfection was carried out using the pmaxGFP (Green Fluorescence Protein) plasmid. Detailed transfection procedure is mentioned elsewhere[7]. The cells were used for expression analysis after 48 h incubation at 28°C. The total RNA was isolated using Trizol™ reagent (Invitrogen, USA) from the fusion gene transfected

cell line, and DNase I (Fermentas, USA) treatment was done. RevertAid First-strand cDNA synthesis kit (Thermo Scientific, USA) protocol was followed for cDNA synthesis using the total RNA of 1 µg. Amplification of GAPDH and IFN-γ genes from the cDNA (diluted to the uniform final concentration) was done following the conditions of internal gene amplification as described earlier, and the PCR amplicon was run on the 2% agarose gel. The control plasmid (pmaxGFP) expression was analyzed using filter light after 48 h under an inverted fluorescence microscope (10X).

Fusion gene sequence analysis

The signal peptide prediction of the fusion vaccine sequence was analyzed by SignalP-5.0 (www.cbs.dtu.dk/services/SignalP/).

Results

Re-engineering of bicistronic vaccine pGPD/IFN

In the present study, GAPDH and IFN-γ genes previously cloned (in our laboratory) in the bicistronic DNA vaccine (pGPD/IFN) have been re-engineered. Thus, the two genes were first amplified separately from the bicistronic DNA vaccine (pGPD/IFN) and cloned in another plasmid vector, i.e., pcDNA3.1(+) in a two-step manner. The primer sites in full-length fusion primers and internal primers used are depicted for both GAPDH and IFN-γ genes (Fig. 1). The corresponding PCR picture is represented (Fig. 2). Complete length primers are designed especially for fusion purposes having the specific restriction site of the specific enzymes. At the same time, the internal primers are designed for the PCR amplification and confirmation of the GAPDH and IFN-γ gene products at the *in vitro* stage of the study.

Removal of IRES site and construction of fusion gene vaccine pcGPD/IFN

The GAPDH gene (1002 bp) alone cloned pcDNA3.1(+) vector was designated as pcGPD. The fusion construct contains the IFN-γ gene (562 bp) as an immune adjuvant fusion-partner in the pcGPD plasmid next to the GAPDH gene leaving 23bp vector fusion sequence and designated as pcGPD/IFN. The cloning in the plasmid vector pcDNA3.1(+) was done to effectively remove the IRES site, which was present in between the vaccine genes in the original pIRES plasmid (pGPD/IFN). After successful cloning at each step, followed by colony PCR (Figs 3 and 4), the gene fragment was released from the corresponding plasmid to confirm its size and restriction site (Figs 5 and 6).

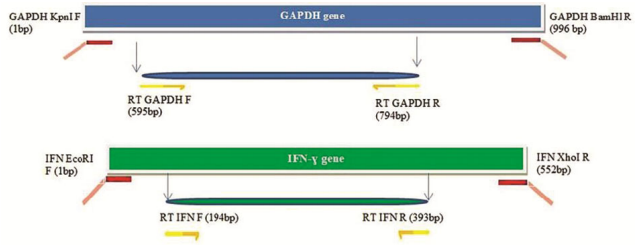

Fig. 1 — 1mPCR primer sites in the GAPDH (996 bp) and IFN-ɣ genes (552 bp).

Codon optimization and signal peptide prediction

To clarify, in the current study, the fusion gene segment was created by changing the stop codon of the GAPDH gene during the PCR process using the changed primers, and the fusion sequence was selected from the pcDNA3.1(+) vector backbone. At the same time, the selected fusion sequence comprises a 23bp vector backbone between the *BamH*I and *EcoR*I RE cutting sites which suggest the possibility

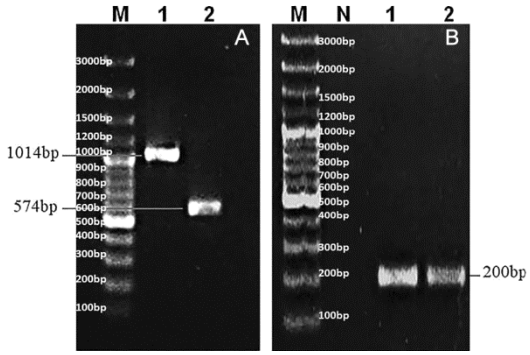

Fig. 2 — Agarose gel electrophoresis (1%) showing the PCR amplification of GAPDH and IFN-ɣ genes using (A) full length; and (B) internal primers. [Lane M: 100 bp plus molecular weight marker (Thermo scientific generuler). (A) Lane 1**:** PCR amplicon of the GAPDH gene (1014 bp); Lane 2: PCR amplicon of the IFN-ɣ gene (574 bp). (B) Lane N: Negative control; Lane 1: Internal amplicon of the GAPDH gene (200 bp); Lane 2: Internal amplicon of IFN-ɣ gene (200 bp)]

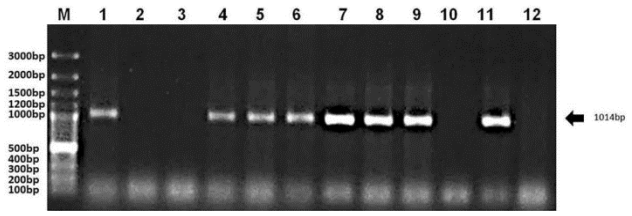

Fig. 3 — Agarose gel electrophoresis (1%) showing cloned GAPDH gene in pcDNA3.1(+) vector by colony PCR. [Lane M: 100 bp plus molecular weight marker (Thermoscientific generuler). Lane 1-12: Colony PCR showing positive amplification of GAPDH gene in lanes 1, 4-9, 11, and the rest were negative]

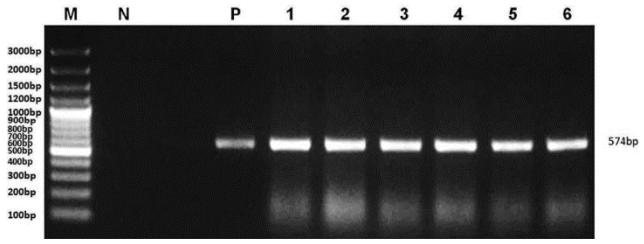

Fig. 4 — Agarose gel electrophoresis (1%) showing the cloned INF-ɣ genes in the pcGPD vector by colony PCR. [Lane M: 100 bp plus molecular weight marker (Thermoscientific generuler); Lane N: Negative control; Lane P: Positive control of IFN-ɣ gene amplified from the pGPD/IFN; Lane 1 - 6: Colony PCR of positive amplification of 574 bp IFN-ɣ gene]

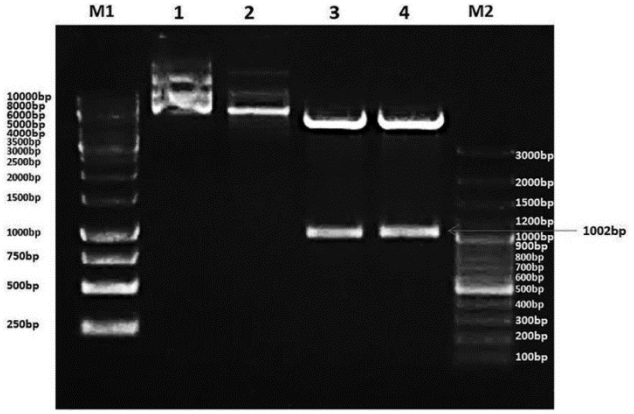

Fig. 5 — Agarose gel electrophoresis (1%) showing RE (*BamH*I & *Kpn*I) digested pcGPD. [Lane 1 & 6: Molecular weight markers of 1 Kb ladder and 100 bp ladder, respectively; Lane 2 & 3: Undigested pcGPD plasmid containing supercoiled and nicked strands (6422 bp)**;** Lane 4 & 5: *Kpn*I & *BamH*I digested pcGPD showing the release of the GAPDH gene (based on restriction site 1002 bp fragment results from plasmid rather than the amplicon size of 1014 bp which results from bp inclusion by extending primer sequence)]

of frame-shift during codon processing in the subsequent translation process of the continuous gene. Consequently, this frame-shift in the continuous IFN-γ gene was corrected during the PCR amplification of the IFN-γ gene by the designed codon-optimized primers. To make it comprehensible, the whole re-engineering process is exemplified (Fig. 7). Further to confirm the fusion sequence of the vaccine genes, any presence of signal peptide was tested using software analysis of the complete fusion gene. The sequence

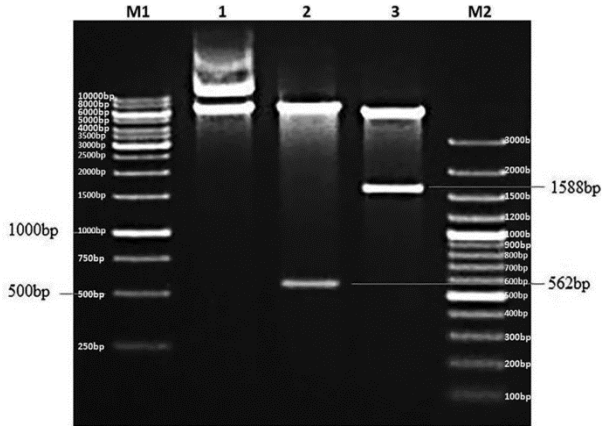

Fig. 6 — Agarose gel electrophoresis (1%) showing RE (*EcoR*I & *Xho*I and *Kpn*I & *Xho*I) digested pcGPD/IFN. [Lane 1: 1 Kb molecular weight marker; Lane 2: Undigested pcDNA-GPD/IFN plasmid containing supercoiled and nicked strands; Lane 3: *EcoR*I & *Xho*I digested plasmid showing the release of the IFN-ɣ gene (based on restriction site 562 bp fragment results from plasmid rather than the amplicon size of 574 bp which results from bp inclusion by extending primer sequence); Lane 4: *Kpn*I & *Xho*I digested plasmid showing the release of the GPD/IFN (based on restriction site 1588 bp fragment results which includes the fusion sequence); Lane 5: 100 bp plus molecular weight marker]

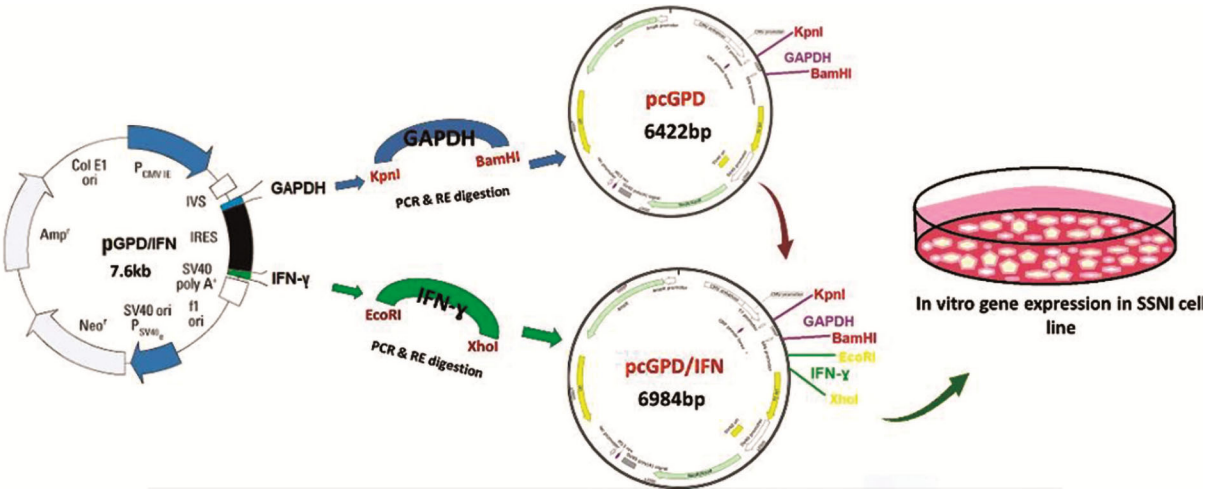

Fig. 7 — Schematic representation of the re-engineering process

analysis showed no presence of signal peptide as the likelihood value is 0.0012 (Fig. 8).

***In vitro* expression of fusion gene vaccine pcGPD/IFN**

The SSN-1 cells were adherent cells initially derived from the snakehead fish and were transfected with the fusion gene-containing plasmid. Positive transfection of the re-engineered plasmid was confirmed by reverse transcriptase PCR of IFN-γ and GAPDH genes. An equal amount of RNA was used for the reverse transcription (1 µg), and an equal amount of cDNA (2µL) was used for the PCR conditions (using internal primers with the same product size of 200bp for both the genes) to estimate the gene-specific amplification of the fusion vaccine without discrimination in amplicon size. Hence, from the resultant amplification regime, the result can be semi-quantitatively taken for both the genes displaying the same level of expression as shown in Fig. 9. The SSN-1 cells treated with the control plasmid (pmaxGFP) showed GFP gene expression at 48 h post-transfection, confirming the successful transfection process (Fig. 10).

Discussion

Bicistronic DNA vaccine against *Edwardsiella tarda*

Labeo rohita cultured worldwide as food fish was affected by *Edwardsiella tarda* infection, and studies on challenge tests are available for this pathogen in *Labeo rohita*[14,15]. Besides vaccinating the fishes against this pathogen, a bicistronic DNA vaccine was constructed with an ampicillin resistance gene as a selective marker[7]. However, the vaccine had an IRES region for the combined expression of GAPDH and IFN-γ genes. Thus in an attempt to remove the IRES sequence, a fusion vaccine concept was established. The IFN-γ is an immune cytokine with secretory property and is reported to have at least 20 signal peptides in exon1 of the gene sequence[16]. For constructing the fusion DNA vaccine, the sequence of

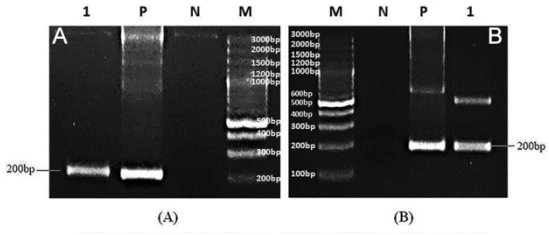

Fig. 9 — Agarose (2%) gel electrophoresis showing the RT PCR amplification of (A) IFN-ɣ; and (B) GAPDH genes. [(A) Lane 1: RT PCR amplified product of the IFN-ɣ gene; Lane 2: Positive control of the IFN-ɣ gene; Lane 3: Negative control; Lane 4: 100 bp plus molecular weight marker. (B) Lane 1: 100 bp plus molecular weight marker; Lane 2: Negative control; Lane 3: Positive control of the GAPDH gene; Lane 3: RT PCR amplified product of the GAPDH gene]

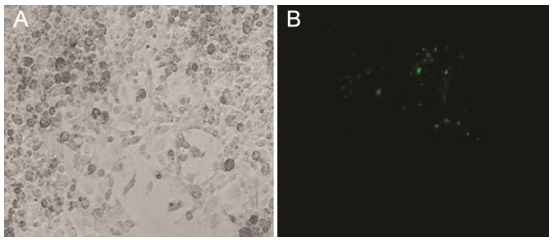

Fig. 10 — pmaxGFP plasmid transfected SSN-1 cells (10X). (A) Cells in white light; and (B) Cells showing green fluorescence in filter light

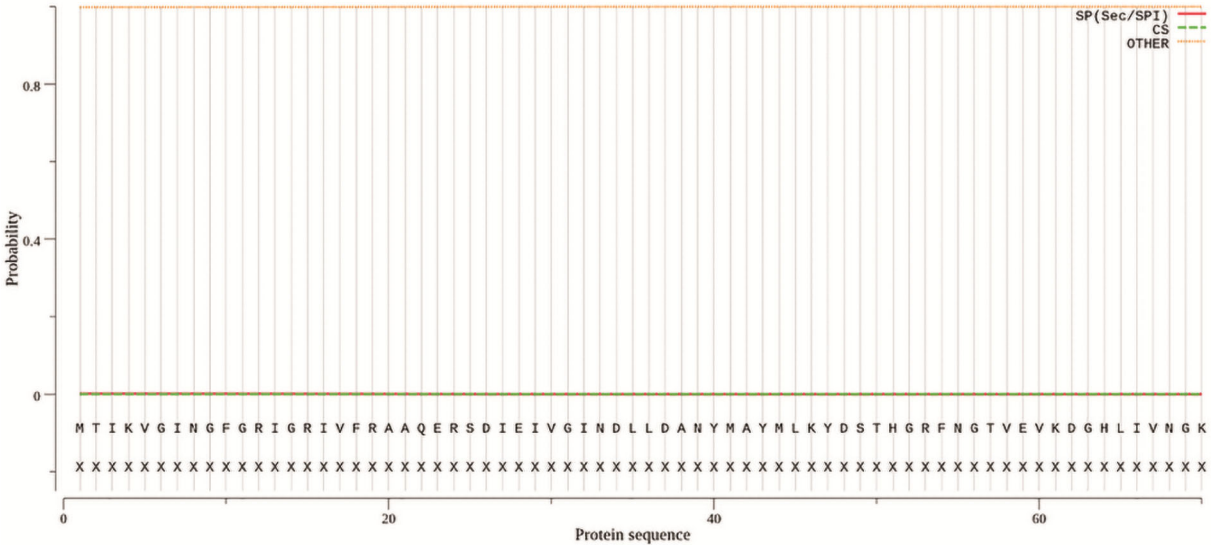

Fig. 8 — Signal peptide prediction graph showing the no presence of signal peptide

the IFN-γ gene devoid of the signal peptide region was chosen (cDNA of mRNA transcripts in the original pGPD/IFN[7]) for cloning; further analysis for signal peptide prediction confirmed this.

Functionality of the re-engineered fusion vaccine

The accomplished aim of this study is to transfer the vaccine inserts to another vector without the IRES region. The crucial factor is that by changing the DNA vaccine without IRES, its immune function should not get altered, and it should accomplish its aim at the final host, i.e., provoking the expected immune response against *Edwardsiella tarda* infection in *Labeo rohita*. This can be checked by the expression functionality of the fusion genes of the vaccines by the *in vitro* method. Likewise, the re-engineered DNA vaccine of this study transfected in the SSN-1 fish cell line showed that the vaccine is compatible with efficient protein expression in fish cells as indicated by mRNA expression. The expression level by RT-PCR semi-quantitatively confirmed the same pattern for both the genes since the single promoter mediates the transcription at the 5' end rather than the suggested IRES dependent differential expression of the second gene of the earlier vaccine (pGPD/IFN).

Studies on transfection using a viral vector with a mammalian expression cassette showed its suitability of protein expression in human glioblastoma cells, and the expression cassette in hepatocellular carcinoma cell lines showed stable protein expression[17,18]. Similarly, the current expression cassette of the fusion gene was found to be functionally stable. The efficient transfection of the primary cultures of rainbow trout gill cells with plasmids encoding viral or reporter proteins was also reported[19]. A study on the *in vivo* expression of chemokines in rainbow trout (*Oncorhynchus mykiss*) in response to a DNA vaccine and interleukin 8 was carried out[20]. The report is one step ahead of the present discussing study of the re-engineered vaccine in which only the *in vitro* expression was confirmed. It is highly desirable to check the re-engineered vaccine expression in the *in vivo* system; however, *in vivo* efficiency has been demonstrated for pGPD/IFN[7]. Efficient expression of both the genes (GAPDH and IFN- ɣ) in the SSN-1 cell line has been confirmed earlier (pGPD/IFN), where 48 h post-transfection using lipofectamine resulted in successful expression[7]. Ortega-Villaizan et al. have given the possibility of using pronephros cells in early primary culture for preliminary screening of fish DNA vaccines[21]. This is a good strategy for screening candidates for the best immune response induced by fish DNA vaccines. Interferon (IFN)-γ responsive stable cell line RTG-3F7 has been developed by transfection with a plasmid construct[22]. This kind of cell line would be an excellent tool for monitoring the presence of IFN-ɣ in biological samples, which may enable the study of intracellular signaling pathways of IFNs.

To confirm and to check the functional expression of the target genes continuous cell lines are ideal[23]. The SSN-1 used in the study is a continuous fish cell line[24], which previously showed successful transfection and transgene expression of the dual genes in the prototype vaccine (pGPD/IFN)[7]. Hence, to re-confirm the functionality of the fusion vaccine the same method was followed using the SSN-1 cell line. Various other studies used SSN-1 cell line for transfection of gene encoding the protein of interest; for example, Nectin-4[25], and Cathepsin H[26].

Plasmids pcDNA3.1(+) and pmaxGFP

The pcDNA3.1(+) was a choice vector for cloning since it had desired RE cutting sites in the preferred order of arrangements such as *Kpn*I, *BamH*I, *EcoR*I, and *Xho*I. These enzymes were selected since they did not cut the genes of interest, i.e., GAPDH and IFN-γ internally. The pcDNA3.1(+) plasmid facilitated the cloning process of the fusion vaccine genes. Many authors have used the pcDNA3.1 vector for various cloning purposes and as a control in transfection studies[27-29]. In the present study, along with the fusion gene transfection (pcGPD/IFN), the control transfection was performed using the pmaxGFP plasmid in the SSN-1 cell line. The expression timing of the GFP gene was observed to occur at 48 hr after transfection. Likewise, a similar expression of the pmaxGFP plasmid used as a control during transfection and co-transfection studies was found to occur at 48 hr by other authors[30,31]. The control pmaxGFP plasmid was reported to contain the transfection efficiency of 7% at 48 hr post-treatment in fish cell line[32].

The efficacy of the prototype (pGPD/IFN) vaccine

Using the prototype (pGPD/IFN) of the re-engineered vaccine of this study, many experimental studies have been conducted in *Labeo rohita* in the Aquatic Animal Health Management laboratory (AAHM) at ICAR-CIFE, of which some were published, including the comparative evaluation of the

protective efficacy of the bicistronic DNA vaccine against the monocistronic DNA vaccine[7] and nano conjugation of DNA vaccine (pGDP/IFN) with chitosan nanoparticles and studying its protective efficacy and immune-modulatory effects in *Labeo rohita*[33]. Also, the vaccine complex containing chitosan/PLGA (poly lactic-co-glycolic acid) nanoparticles was evaluated for its persistence, bio-distribution, and environmental transmission[34]. The environmental transmission study showed the safety of the vaccine with no recorded transmission in the environment. The mucosal immune response of the pGPD/IFN conjugated with chitosan/PLGA nanoparticles was studied in *Labeo rohita* against *Edwardsiella tarda* infection. The high RPS of 64.7% and adaptive mucosal immunity was obtained in the chitosan/PLGA nanoparticles conjugated vaccine treatment than the PLGA nanoparticle alone conjugated vaccine treatment[10]. The DNA vaccine (pGPD/IFN) is also evaluated for the generation of immune response in mucosal-associated lymphoid tissues (MALT) in *Labeo rohita* fingerlings which showed the expression correlation of Polymeric immunoglobulin receptor (pIgR) gene with IgM gene expression[35]. A further study using the vaccine (pGPD/IFN) conjugated with chitosan/PLGA, resulted in augmentation of the total serum protein, globulin concentrations in *Labeo rohita*. The study also showed the increment in the non-specific immune parameters such as myeloperoxidase and lysozyme levels in the vaccinated fishes[36]. Hence, the current study does not include *in vivo* experimentation because of the proven efficacy of the vaccine in various *in vivo* models in our laboratory.

Desirability of DNA vaccines

Other than the current vaccine, Liu et al.[37] have designed a bicistronic vaccine against *Edwardsiella tarda*, co-encoding antigenic (flagellar protein, FlgD) and adjuvant proteins (C5a), which have also been found to be a better vaccine than using the monocistronic vaccine. DNA vaccines have several benefits, including ease of manufacture, cost effectiveness, and the capacity to produce a wide spectrum of immune responses (cellular and humoral responses). Long-lasting immunity is produced via DNA vaccinations[38]. In a study the C57BL/6 mice were vaccinated using a fusion DNA vaccine made of *Mycobacterium tuberculosis* HSP70 (Heat shock protein 70) and Melanoma antigen-encoding gene 3 (MAGE-3) against B16 or B16-MAGE-3 tumour cells suggesting the immune evoking advantage of fusion gene[39]. Similar to our study the pIRES vector was used for the creation of a bivalent DNA vaccine against Newcastle disease (ND), using the hemagglutinin-neuraminidase (HN) and fusion (F) genes of the velogenic (virulent) strain which were amplified and cloned at multiple cloning sites A and B, respectively and the recombinant construct was evaluated against ND[40].

Conclusion

A re-engineered DNA vaccine successfully expressing the immunodominant protein of *Edwardsiella tarda* and immunoadjuvant IFN-γ has been designed and its functionality is checked in fish cell line. Edwardsiellosis being a serious bacterial infection in Indian major carp an effective preventive measure is urgently required. Vaccination is the most suitable method of disease prevention; it also addresses the issue of antimicrobial resistance in fish culture. In this study, the plasmid pcDNA3.1(+) helped with the fusion gene vaccine's development and the expression of the pcGPD/IFN fusion gene in the SSN-1 cell line was demonstrated 48 h after transfection and verified the genes' functionality, which was the ultimate goal of the study. The fusion DNA vaccine has a broad range of applications for immunizing fish to prevent edwardsiellosis in aquaculture compared to any single gene DNA vaccine, ultimately to prevent the economic losses due to this disease.

Ethical statement

The study was undertaken after the approval of the Board of Studies of the Department of Aquatic Animal Health Management, ICAR-CIFE, Mumbai, and was approved by the Institutional Bio-Safety Committee (dated 15/01/2013). The current study does not deal with animal experimentation.

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Conflict of Interest

Authors declare no competing interests.

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