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Analysis of Immune Gene Expression in Seabass (*Lates calcarifer*) Immunized with Inactivated Vaccine against Similar Damselfish Virus

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ABSTRACT

Background: Control of viral disease outbreaks in aquaculture and minimizing the loss of production can be achieved by development of effective vaccines. Efficacy of these vaccines can be improved by using adjuvants, immunostimulants or vaccine carriers. In this study, inactivated similar damselfish virus (SRDV) vaccine was prepared and expression profiles of immune related genes against virus challenge of the vaccine were investigated in seabass (*Lates calcarifer*).

Methods: Formalin-inactivated virus vaccine was developed to assess its immune responses to SRDV challenge in fish. The immune response was induced by intra-peritoneal injection with inactivated viral vaccine added Quil-A® adjuvant. The transcriptional levels of immune genes IRF-7 and IL-10 were evaluated in the spleen and kidney of seabass from different groups by quantitative real-time RT-PCR assays.

Result: Expression profiles of both genes (IRF-7 and IL-10) in the kidney and spleen of seabass immunized with vaccine added adjuvant were up-regulated at 48 hpi of the virus. In comparison, spleen of seabass immunized with vaccine added adjuvant showed highest expression profiles than kidney. The current study provides evidence for the presence of expression profiles of immune-related genes during the SRDV infection. The study also strongly suggests that Quil-A® adjuvant enhances the immune response of the vaccine candidates.

Key words: Formalin-inactivated vaccine, Immune genes expression, Seabass, SRDV.

INTRODUCTION

Asian seabass (*Lates calcarifer*) is a popular cultivable food species also commonly known as barramundi. Seabass has considerable production potential as the hatchery technology and farming practices have already been standardised for this species. However, most of the production losses in seabass aquaculture are caused by infectious pathogens, particularly virus. The viral disease is very difficult to be controlled once established within the culture system. Avoidance is suggested as one of the best control measures of viral infections. Recently, iridovirus disease has become one of the major constraints in Asian seabass culture (INFOFISH, 2019, Grisha *et al*., 2020). Reduction of viral disease outbreaks and minimizing the loss of production can be achieved by development of effective vaccines. In general, the fish viral vaccines fall under three major categories, namely, killed whole cell vaccine, live-attenuated vaccine and recombinant DNA-based vaccines (Hegde and Sin, 2006). Efficacy of these vaccines can be improved by using adjuvants, immunostimulants or vaccine carriers. Several inactivated vaccines have been developed against diseases caused by various viral pathogens that include Red seabream iridovirus (RSIV) (Nakajima *et al*. 1999; Caipang *et al*. 2006), Nervous necrosis virus (NNV) (Yamashita *et al*. 2005), Cyprinid herpes virus-3 (CyHV-3) (Michel *et al*. 2010), Infectious spleen and kidney necrosis virus (ISKNV) (Dong *et al*. 2013), Lymphocystis disease virus (LCDV) (Nakajima *et al*. 1997, 1999; Yoshimizu and Iwamoto, 2001) and Singapore grouper iridovirus (SGIV) (Ou-yang *et al*. 2012). Administration of vaccines and immunostimulants can be done in three ways in aquatic animals such as injection, immersion and oral uptake. Injection of immunostimulants can produce a strong non-specific response, but it is time-consuming and labour intensive. It has been reported that injection of immunostimulants offer protection against a wide range of pathogens (Labh and Shakya, 2014). The intra-peritoneal route of delivery is the most commonly used administration route for injection of vaccines for fish

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(Sommerset *et al*. 2005). Generally, the intra-muscular route is rarely used for administration of fish vaccines because there is a possibility of creating unsightly scar as well as leakage of the inoculum after injection (Evensen and Leong, 2013).

Spleen, kidney and thymus are considered as the major lymphoid organs in fish (Rombout *et al*. 2005) and represent suitable sources for immune cells which respond to viral infection (Rakus *et al*. 2012, Ouyang *et al*. 2013). The innate immune system is the first line of defence against infection caused by the pathogens. Interferons (IFNs) are secreted mediators that play a fundamental role in the innate immune response against viruses in vertebrates (Adamek *et al*. 2012). Interferon regulatory factors (IRFs) are a family of transcription mediators involved in the transcriptional regulation of type 1 IFN and IFNs stimulating genes (ISGs) (Mamane *et al*. 1999). IRF-7 is known as the master regulator of type I interferon response in vertebrates and it plays a vital role in the innate antiviral immunity (Hu *et al*. 2011). IL-10, initially known as a cytokine synthesis inhibitory factor, is a multifunctional cytokine and demonstrates immuno suppressive function. The main function of IL-10 seems to be regulation of immunity and the inflammatory response, thereby minimizing damage to the host induced by the response to a pathogen or by the self-immune system. The expression of the IL-10 gene was observed in the head kidney, spleen, intestine and gill tissues of normal (healthy) carp (Savan *et al*. 2003). Modulation of IL-10 has been observed in fish fed with various nutritional supplements. Interleukin 10 (IL-10) transcript was found to be up-regulated in seabass (*Dicentrarchus labrax*) fed supplemented with L-arginine at 1% compared to control fish. In contrast, IL-10 and IL-20 transcripts decreased in fish fed supplemented with L-arginine at 2% compared to control diet (Azeredo *et al*., 2015). More recently, we reported a strong evidence for the expression of the immune genes, IFN and IL-10 in juvenile koi carp immunized with the inactivated vaccine of similar damselfish virus (SRDV) and also that the Quil-A® adjuvant was effective in enhancing the immune response of the vaccine candidates (Sivasankar *et al*. 2017). Quil A® adjuvant contains the water-extractable fraction of saponins from the South-American tree, *Quillaja saponaria* Molina. The saponins induce a strong adjuvant response to T-dependent as well as T-independent antigens (Petrovsky and Aguilar, 2004). Quil-A® adjuvant can activate both the cell-mediated and the antibody-mediated immune responses to a broad range of viral, bacterial, parasitic and tumor antigens (Sun *et al*., 2009). In the present work, we report the expression of the immune genes, IRF-7 and IL-10 in seabass immunized with an inactivated vaccine against similar damselfish virus (SRDV).

MATERIALS AND METHODS

The study was conducted in the period of 2016-17 to 2017-18 at the Department of Fish Pathology and Health Management of Fisheries College and Research Institute, Thoothukudi, Tamil Nadu.

Cell line

Virus propagation for vaccine development was carried out on Epithelioma papulosum cyprini (EPC) cell line. Prior to inoculation of the virus, cells were maintained in cell culture plastic flasks (25 cm^2) (Thermo, Korea) in Leibovitz-15 (L-15) (Gibco, USA) medium supplemented with 10% foetal bovine serum (Gibco, USA) and 1x antibiotic-antimycotic solution (Gibco, USA) at 27°C.

Virus

SRDV previously isolated from similar damselfish (*Pomacentrus similis*) and characterized in our laboratory was used for the present study (John and George, 2011; Sivasankar *et al*. 2017). Stock preparation of SRDV was prepared in EPC cell line. On completion of cytopathic effect (CPE), the infected culture supernatant was harvested and clarified by centrifugation at 3000 x g for 10 min. Aliquots of the cell culture supernatant containing SRDV were distributed in cryotubes and stored at -80°C until use. One aliquot was thawed and used for estimating Median Tissue Culture Infectious Dose ($TCID_{50}$)/ml of the virus by inoculating 10-fold serial dilutions in 96-well microplates, seeded with newly sub-cultured EPC cell suspension. The plate was incubated at 27°C and development of CPE was noted for 10 days for calculating the viral titre.

Fish and rearing condition

Healthy seabass (*Lates calcarifer*) juveniles weighing about 10.14 g were obtained from a government hatchery and maintained in the laboratory until non-specific mortalities had stopped and acclimatization achieved to the tank conditions. The fish were conformed negative to similar damselfish virus, koi ranavirus and nervous necrosis virus infection by PCR technique before starting of the experiment. Fish were held in 100 L glass aquarium tanks maintained with ~ 60 L seawater at a temperature of 31± 1°C, salinity 33-35 ppt and pH 8 ± 0.3 with a closed re-circulatory system with carbon filters for acclimatisation but no additional aeration was given. Fish were fed with commercial pelleted diet twice daily. The bottom of the tanks was cleaned daily by siphoning out waste material along with a partial exchange (~10%) of water.

Vaccine preparation

Formalin-inactivated vaccine was prepared according to the protocol given by Ito and Maeno (2015) with slight modification. To prepare the vaccine, supernatants of SRDV infected EPC cell cultures were centrifuged at 1500 x g for 10 min at 4°C. Formalin was added to the culture supernatants (having a virus titre of $10^{6.0}$ $TCID_{50}$ ml^{-1}) to a final concentration of 0.1% (V/V) and incubated at 4°C for 2 days. The presence of infectious virus in inactivated samples and sequential aliquots was measured by cell culture assay by passaging of treated virus samples on EPC cell monolayers for a period of up to 10 days (Fig 1).

Vaccination

Seabass fish were randomly divided into 4 groups of 12 fish

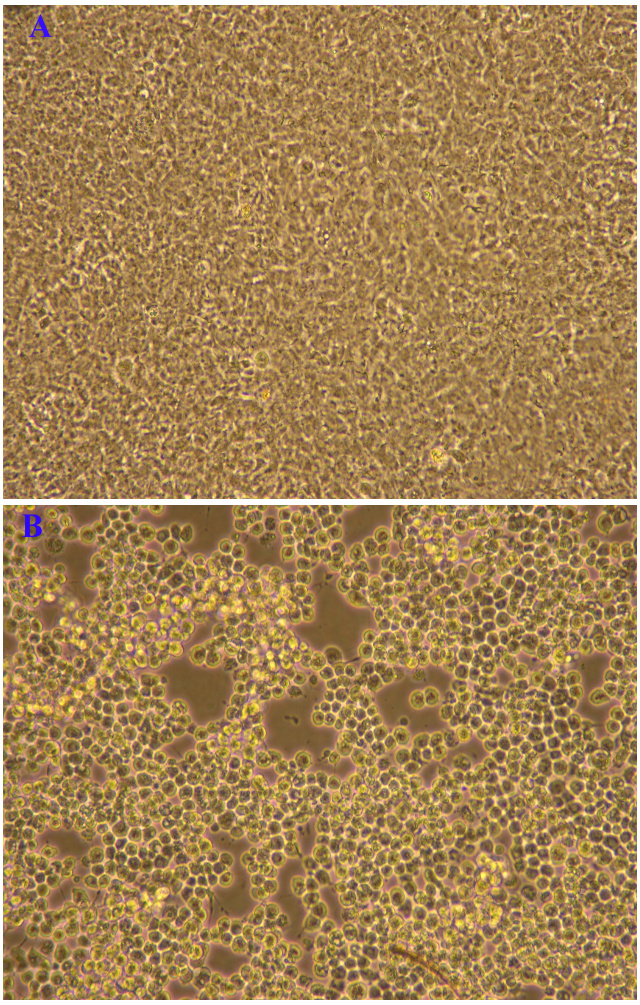

Fig 1: Cytopathic effect induced by SRDV in EPC cell line used in the study. (A) Control EPC cell line (Multiplication × 200). (B) SRDV infected EPC cell line (Multiplication × 200).

each in duplicate namely: experimental control-A (2 tanks), virus control-B (2 tanks), vaccine alone (2 tanks) and vaccine + adjuvant (Qil-A®, InvivoGen) (2 tanks) (Fig 2). The concentration of adjuvant (Qil-A®) was adjusted to 5 µg / fish with sterilized double distilled water. Fish were starved for 24 h prior to vaccination and anaesthetized by immersion in benzocaine (Himedia) solution at the concentration of 40 ppm. Vaccine preparations were then injected intra-peritoneally at 50 µl / fish. Control group of fish received the same volume of PBS. After vaccination, each group was kept in separate aquaria glass tank and was monitored continuously.

Challenge experiment

On the 28th day post-vaccination, fish from each group were challenged by intra-peritoneal injection with SRDV. With the exception of the control-A group, all three groups of fish were challenged with 50 µl of the SRDV stock ($10^{7.17}$ $TCID_{50}$ / volume inoculated). Control-A group of fish received the same volume of PBS.

Transcription of immune genes post-vaccination

The transcriptional levels of immune genes IRF-7 and IL-10 were evaluated in the fish from different groups by quantitative real-time RT-PCR assays. Spleen and kidney were obtained for analysing the expression profiles of immune genes of vaccinated fish at 12, 24 and 48 h following challenge with SRDV. Total RNA was isolated from the both tissues taken from three fish in each treatment group at all the time points by using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. The RNA was dissolved in 100 µl of DEPC treated RNase-free water.

Reverse transcription of total RNA

The RNA concentration was adjusted to 1 µg for reverse transcription to cDNA synthesis. The concentration of RNA was measured by Colibri Microvolume Spectrometer

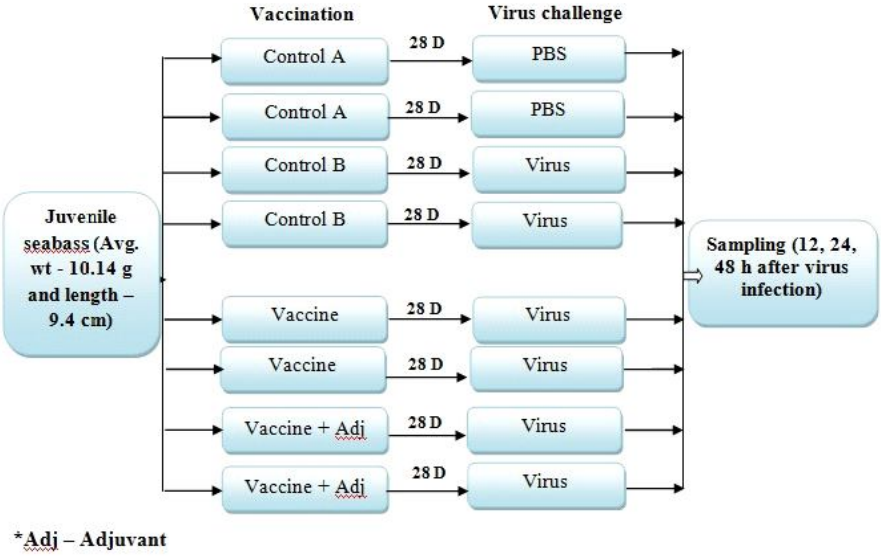

Fig 2: Experimental design of seabass following SRDV challenge.

(Titertech-Berthold, Germany). cDNA was synthesized from all the RNA samples extracted from selected tissues using the specific reverse primers for IRF-7, IL-10 and β actin with cDNA Synthesis Kit (Thermo Fisher Scientific, USA) as per the manufacturer's instructions. The reaction mixture contained extracted RNA (10 µl) with 10 µl of cDNA synthesis mixture containing 2 µl (50 pmol) of specific reverse gene specific primer, 2 µl of 10x RT buffer, 0.8 µl of 25x dNTP, 1 µl of multi scribe, 1 µl of RNase inhibitor and 3.2 µl of nuclease-free water. The reaction mixture was incubated at 25°C for 10 min, 37°C for 160 min and 85°C for 5 min. The cDNA thus synthesized was used for gene expression studies using quantitative real-time RT-PCR.

Quantitative real-time reverse transcriptase-PCR (qRT-PCR)

An aliquot (2 µl) of the cDNA was analyzed by real-time PCR in the reaction mixture of 25 µl containing 2 µl (50 pmol) each of forward and reverse primer, 12.5 µl of 2x power SYBR green (Thermo Fisher Scientific, USA) master mix and 6.5 µl of deionized water. The amplification was carried out in Step-One Plus Real-Time PCR Detection system (ABI, Invitrogen, Life sciences, USA). The primers used for qRT-PCR analysis of IRF-7, IL-10 and β-actin gene and cycling conditions are given in Table 1. The expression level of the immune genes in the samples was analyzed in triplicate using the comparative threshold cycle (Ct) method ($2^{-\Delta\Delta CT}$) with β-actin gene as an internal control. With this method, the Step-One Plus software measures amplification of the target and of the endogenous control (β-actin) in samples and in a reference samples. Measurements are normalized using the endogenous control. The software determines the relative quantity of target in each sample by comparing normalized target quantity in each sample to normalized target quantity in the reference sample. Thus quantification by comparative Ct method was carried out. The Ct values were converted into expression values normalized against the reference gene, β-actin, using the comparative Ct method. Each sample was analyzed in triplicate and the data were calculated as the mean ± standard deviation (SD) and standard error (SE) of relative mRNA expression of IRF-7 and IL-10 in spleen and kidney.

RESULTS AND DISCUSSION

Immune gene expression

Ranaviruses are associated with numerous disease outbreaks and high mortality among the natural and cultured population of freshwater and marine fish. The chemicals and/or antimicrobial agents used for treating fish diseases are associated with several disadvantages. In particular, they can lead to the development of disease resistance in the microorganisms and pose negative impacts to the aquatic environments (Ou-yang *et al*. 2012). Vaccination is a powerful tool for health management in aquaculture and different types of vaccines have been successfully developed against several viral and bacterial diseases in aquaculture. Ou-yang *et al*. (2012) have reported that formalin inactivation of the virus is an effective method to develop safe and efficacious human and veterinary vaccines. Adjuvants are compounds that enhance the specific immune response against co-inoculated antigens (Petrovsky and Aguilar, 2004) and hence used in most of the vaccination studies. The protective potential of DNA vaccine with QCDC (Quil A/cholesterol/DDA/Carbopol) adjuvant and its effects on the innate and adaptive immune responses in Chinese perch has been examined by Fu *et al*. (2014).

IRF-7 is a master regulator of type-I interferon-dependent immune responses and is essential for the induction of IFN-a/b genes (Fu *et al*. 2014; Honda *et al*. 2005; Zou and Secombes, 2011). IL-10 is the most important immunoregulatory cytokine produced by various types of cells (Nam *et al*. 2014). IRF7 has been shown to be involved in the host immune response to infection by a variety of viruses (Shin *et al*. 2013). Xiang *et al*. (2010) have found that IRF7 expression in zebrafish infected with ISKNV increased 10-fold at 8 days of post infection. Infected orange-spotted grouper was also showed a positive regulation of IRF7 (Cui *et al*. 2011). Changes in gene expression of IRF-7 and IL-10 in spleen and kidney tissues obtained from seabass at 12 h, 24h and 48 h after SRDV infection was analysed. Expression profiles of IRF-7 gene and IL-10 were investigated in seabass with SRDV infection alone, SRDV

Table 1: Primers used for the qRT-PCR analysis of IRF-7 and IL-10 gene expression following SRDV challenge.

Gene	Primers sequence 5′-3′	Accession number	Size (bp)	Cycling condition	Reference
IRF-7 Fw2	ATTCACCAACCGCATCCTTA	KP861885	80	95°C 10 min	
IRF-7 Rv2	GCCTCCAGGCATAGATACCA			95°C 30 sec	Valero *et al*., 2015
				58°C 1 min	
				72°C 1 min	
IL-10 Fw3	ACCCCGTTCGCTTGCCA	AM268529	164	95°C 10 min	
IL-10 Fv3	CATCTGGTGACATCACTC			95°C 1 sec	Buonocore *et al*., 2007
				53°C 30 sec	
				72°C 1 sec	
β-actin Fw	TACCACCGGTATCGTCATGGA	GU188683	150	95°C 10 min	
β-actin Rv	CCACGCTCTGTCAGGATCTTC			95°C 30 sec	Parira *et al*., 2016
				60°C 1 min	
				72°C 1 min	

infected fish vaccinated with formalin-inactivated SRDV vaccine and SRDV infected fish vaccinated with formalin-inactivated SRDV vaccine + Quil-A® adjuvant. PBS injected fish was used as a control group of the experiment.

Expression of IL-10 in spleen after virus infection

IL-8 was constitutively expressed in spleen, head kidney and liver of rainbow trout (*Oncorhynchus mykiss*) and its transcription was also induced in response to VHSV (Tafalla *et al*. 2005). Similarly, highest IL-8 mRNA expressions have also been reported in kidney and spleen tissues of golden mandarin fish, as high as 8- to 10-fold difference in mRNA expression levels at 10 dpi (Shin *et al*. 2013). The present experiment revealed that the expression profiles of IL-10 in the spleen of unvaccinated, vaccinated and vaccine plus Quil-A® adjuvant group of fish challenged with SRDV injection were up-regulated as 1.23 fold, 1.39 fold and 2.00 fold at 12 hpi. Unvaccinated and vaccinated group of fish challenged with SRDV infection were showed down-regulation at 48 hpi (Fig 3). Significant up-regulation of IL-10 in the spleen was noted during all phase of CyHV-3 infection compared with mock-infected common carp (Sunarto and McColl, 2015). In our observation, seabass immunized with vaccine added Quil-A® adjuvant was found up-regulation of IL-10 in the spleen 1.87 fold at 24 hpi and it was further up-regulated (4.81 fold) at 48 hpi with SRDV. In the spleen, the IL-10 expression has been strongly induced in response to the virus at days 1 and 2 post-infection. Recently, Xiao *et al*. (2019) have studied the expressions of the IL-8 and IL-10 in various tissues including liver, heart, spleen, kidney, intestine, brain, gill and muscle of yellow catfish. The study indicates that both genes are most abundantly expressed in the spleen.

Expression of IRF-7 in spleen after virus infection

Strong up-regulation of IRF-7 mRNA expression has been found in the spleen of koi immunized with vaccine plus Quil-A® adjuvant at 24 hpi with ranavirus infection (Sivasankar *et al*. 2017). In the present study, IRF-7 expression in spleen of vaccinated group was down regulated while the adjuvant

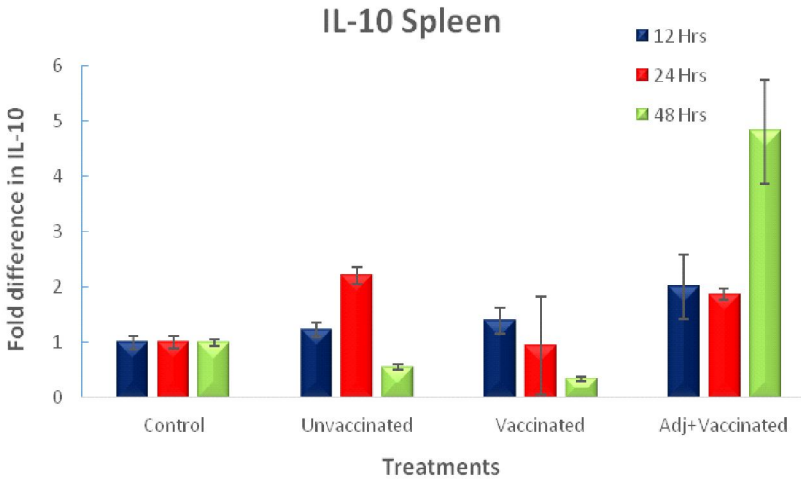

Fig 3: Relative folds induction ($2^{-\Delta\Delta CT}$) of IL-10 gene in spleen of seabass challenged with SRDV at 12 h, 24 h and 48 h post-infection**.**

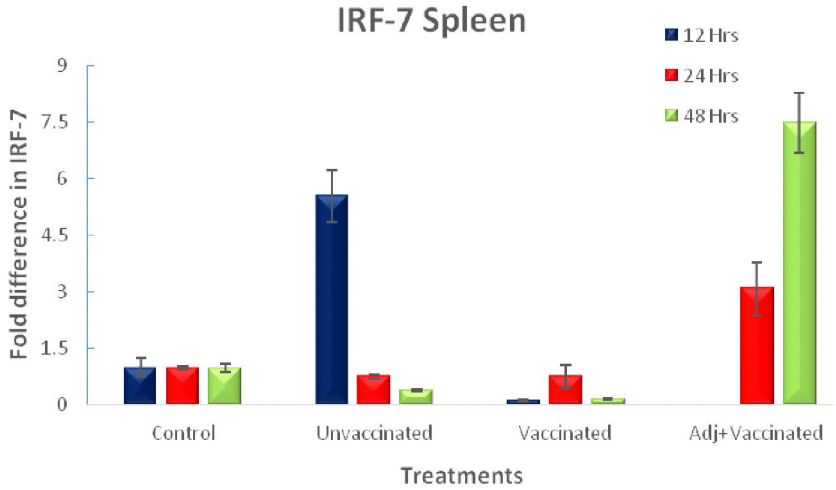

Fig 4: Relative folds induction ($2^{-\Delta\Delta CT}$) of IRF-7 gene in spleen of seabass challenged with SRDV at 12 h, 24 h and 48 h post-infection.

plus vaccinated groups were highly up-regulated (3 fold) at 24 hpi, which further increased to 7.4 fold at 48 hpi (Fig 4). Previously, Fu *et al*. (2014) found that IRF-7 expression in spleen of QCDC + pcMCP administered Chinese perch reached a peak at 48 h post-vaccination, then decreased but increased again at day 21. Spleen IRF-7 transcript expression increased in response to formalin killed *Aeromonas salmonicida* injection, although the increase was only at 6 hpi (2.28 fold) and 24 hpi (1.60 fold) (Inkpen *et al*., 2015). In contrast, the level of IRF-7 gene expression in the spleen was also found down regulated at 12 h in vaccinated and Quil-A® adjuvant plus vaccinated group of fish challenged with SRDV injection. The expression level of IRF-3/IRF-7 in seabass was found to be significantly up-regulated post NNV infection compared to control in all the examined tissues including kidney, spleen, hindgut, liver, heart, muscle, brain and gills (Krishnan *et al*., 2019). They also noticed that the fold change of IRF-3 expression was higher in spleen and brain tissues in the initial stages and up regulated in heart and liver as the infection progressed.

Expression of IL-10 in kidney after virus infection

Transcription of immune-related genes in the kidney of rainbow trout was found increasing when induced by the DNA vaccine against IPNV (Ballesteros *et al*. 2014). The highest expression of IL-10 was observed in kidney and liver of fugu (Zou *et al*., 2003) and head kidney of carp (Savan *et al*., 2003). At 12 hpi, the expression of IL-10 in kidney was up-regulated (2.67 fold) in vaccinated group following virus challenge. The expression level of IL-10 was increased (6.81 fold) at 24 hpi but its level was slightly decreased (2.58 fold) at 48 hpi (Fig 5). In head kidney of seabass, the anti-inflammatory cytokine IL-10 had a 3-fold increase after infection and an expression increase (10 fold and 20 fold) at 1 day and 10 days after boosting with encephalopathy and retinopathy virus vaccine (Scapigliati *et al*. 2010). In the present experiment on seabass, the expression of IL-10 in the kidney was up-regulated (2.11 and 2.23 fold) in Quil-A® adjuvant plus vaccine administered group following virus challenge at 12 and 48 hpi when compared to control group. Holopainen *et al*., (2012) found that IL-10 was up-regulated only at the early time points (1, 6 and 12 hpi) in the ranavirus-infected EPC cells. After 24 hpi, IL-10 expression was lower in all ranavirus treatment groups than in the negative control. Similarly, significant mRNA expression of IL-10 in the kidney of olive flounder has also been observed at 1 hpi and the highest levels of up-regulation at 6 hpi (3 fold) following VHSV challenge (Nam *et al*. 2014).

Expression of IRF-7 in kidney after virus infection

The immune genes such as IRF1, IRF2 and IRF7 showed significant changes in mRNA expression at 24 hpi in the kidney of golden mandarin fish challenged with infectious spleen and kidney necrosis virus (ISKNV) -like agent (Shin *et al*. 2013). Our study also indicated that the mRNA expression of IRF-7 is up-regulated (7.2 fold) in vaccinated seabass at 24 hpi. But the level of expression (4.4 fold) was decreased in vaccinated groups at 48 hpi. IRF-7 expression in infectious salmon anaemia virus (ISAV) infected salmon cells displayed heightened fold change at 72 h post infection (Kileng and Bergan, 2009), but in Asian seabass, high transcript abundance was observed at 24 h post infection in brain, heart, kidney and spleen (Krishnan *et al*., 2019). Interestingly, the expression of IRF-7 in the kidney of unvaccinated seabass group showed up-regulation of 25.9 fold at 12 hpi but its expression level was decreased to 1.74 fold at 24 hpi. A significant induction of IRF3 and IRF7 has been reported in fish vaccinated with DNA vaccine plus ODN C7 compared to vaccine alone at 7 d post-viral challenge (Zhou *et al*. 2014). In fish vaccinated with Quil-A® adjuvant, down-regulation was noticed at 12 hpi but its expression level was up-regulated (4.8 fold) at 24 hpi. Like spleen, the IRF-7 expression level in the kidney of vaccinated and Quil-A® adjuvant plus vaccinated were also up-regulated (7.23 fold) at 48 hpi (Fig 6). Similar to this experiment, the highest level of up-regulation has been recorded at 96 hpi of ranavirus in koi vaccinated with adjuvant (Sivasankar *et al*.

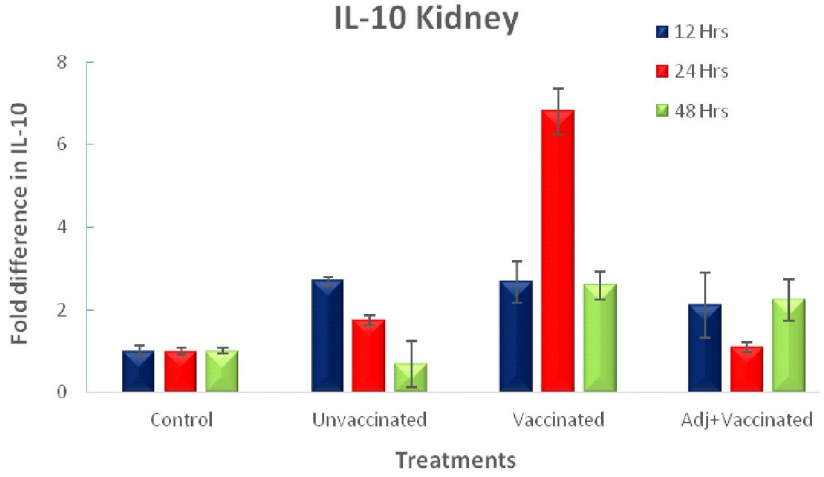

Fig 5: Relative folds induction ($2^{-\Delta\Delta CT}$) of IL-10 gene in kidney of seabass challenged with SRDV at 12 h, 24 h and 48 h post-infection.

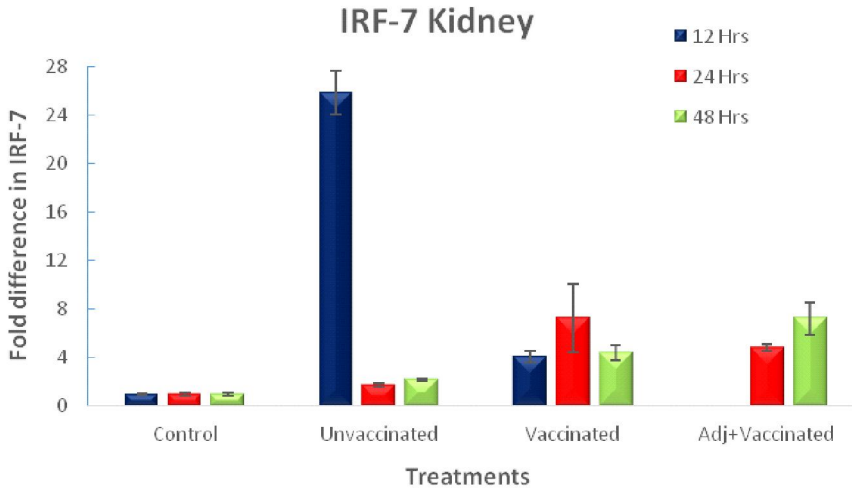

Fig 6: Relative folds induction ($2^{-\Delta\Delta CT}$) of IRF-7 gene in kidney of seabass challenged with SRDV at 12 h, 24 h and 48 h post-infection.

2017). Adamek *et al*. (2014) have also found a strong mRNA expression by up-regulating both IRFs -3 and -7 in the skin and head kidney of two genetic lines of common carp (*Cyprinus carpio*) following CyHV-3 infection.

CONCLUSION

In conclusion, we have demonstrated that formalin inactivated SRDV vaccine could trigger the expression profiles of immune genes (IRF-7 and IL-10) following virus challenge. The study provides an evidence for the induction of the mRNA expression profiles of immune-related genes during the ranavirus (SRDV) infection. In addition, the current study strongly suggests that Quil-A® adjuvant enhances the immune response of seabass when co-administered with inactivated viral vaccine.

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