

Original Research Article

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Immune Gene Expression against Similar Damsel Fish Virus (SRDV) Induced by Immunization with Inactivated Vaccine in Juvenile Koi Carp

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ABSTRACT

Vaccination is one of the best preventive measures against viral diseases in aquaculture. In this study, experimental inactivated similar damselfish virus (SRDV) vaccine was prepared and immunogenicity and protection against virus infection of the vaccine was investigated in koi carp (*Cyprinus carpio*). Formalin-inactivated virus at 4°C for 2 days was exhibited no typical clinical symptoms of SRDV infection and no mortality following challenge at 28-day post-vaccination. The immune response was induced by intraperitoneal injection of koi with formalin-inactivated vaccine added Quil-A[®] adjuvant. The expression levels of genes including IRF-7 and IL-10 in spleen of koi immunized with vaccine added Quil-A[®] adjuvant was highly up-regulated (52.2 fold) at 24 h post infection of the virus but in kidney was showed down-regulation (0.4 fold). Interestingly, the highest level of up-regulation (16 fold) was recorded at 96 h post infection of SRDV. Like IRF-7, highest up-regulation of IL-10 gene was observed in spleen of koi immunized with vaccine added Quil-A[®] adjuvant at 24 post infection of the virus while kidney was showed down-regulation. The study provides strong evidence for the existence of expression profiles of immune related genes (IRF-7 and IL-10) during the ranavirus infection. It also suggests that Quil-A[®] adjuvant enhances the immune response of the vaccine candidates.

Keywords

SRDV, Formalin-inactivated vaccine, Immune genes, Expression profiles.

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Introduction

Viral diseases are one of the major challenges that are threatening a sustainable growth of fish farming and wild populations globally. Fish viral diseases account for large-scale mortality in farmed fish and are very difficult to treat directly. In the last decade, ranaviruses have been identified as responsible agents for causing disease outbreaks mainly in hatchery reared larvae and juveniles of a wide variety of fishes throughout the world (Whittington *et al.*,

2010). Ranaviruses are associated with numerous disease outbreaks in natural and cultured populations of fish, amphibians and reptiles (Chinchar, 2002; Chinchar *et al.*, 2009). Several ranavirus isolates have been recovered from different host species in Australia, the Americas, Europe and Asia (Williams *et al.*, 2005). Ranaviruses belong to one of the five genera of the family Iridoviridae (Jancovich *et al.*, 2011). They are large icosahedral, double-stranded DNA

(dsDNA) viruses usually 120-200 nm in diameter in size and with a genome size of 105 and 140 kbp that can infect a broad range of lower vertebrates (Williams *et al.*, 2005; Holopainen, 2012). The core of the virion consists of a nucleoprotein filament surrounded by a lipid membrane containing transmembrane proteins and a capsid composed of identical capsomers (Jancovich *et al.*, 2011). We have isolated and characterised a ranavirus from similar damselfish, which was found to be pathogenic to seabass and similar damselfish (John and George, 2011; Sivasankar, 2014).

Vaccination of farmed fish plays an important role in commercial fish farming in order to mitigate diseases caused by many pathogens. There are several diseases which could be controlled by vaccination (Schnick *et al.*, 1997). Inactivated vaccines generally refer to preparations of killed pathogens when referring to viral vaccines. The first generation of inactivated vaccine was developed by Louis Pasteur during 1880 (Munang'abdu *et al.*, 2014). The inactivation methods consist into two broad categories, namely physical and chemical. Physical inactivation methods for microorganisms can be done by ultraviolet (UV) light, heat and sonication. There are different chemicals are used for preparing inactivated vaccines such as β -propiolactone (BPL) and binary ethylenimine (BEL), formalin and chloroform (Ou-yang *et al.*, 2012; Munang'abdu *et al.*, 2014; Ito and Maeno, 2015). Formalin is the most commonly used inactivators and demonstrated to be fairly effective in inactivating virus infectivity (Jiang *et al.*, 2008). β -propiolactone (BPL) is another widely used chemicals to inactivate viruses for human as well as veterinary vaccine development (Lawrence, 2000).

Killed vaccines have been developed for some pathogenic fish viruses such as

infectious pancreatic necrosis virus (IPNV), infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV) and spring viremia of carp virus (SVCV) (Hegde and Sin, 2006). Red sea bream immunized with inactivated iridoviral antigens was found to be significantly showing better protection than non-vaccinated fish after experimental infection (Nakajima *et al.*, 1997). Fish immunized with formalin-inactivated vaccines have showed good protective effects against lymphocystis disease virus (LCDV) (Nakajima *et al.*, 1997, Nakajima *et al.*, 1999; Yoshimizu and Iwamoto, 2001). Successful use of killed VHSV in rainbow trout has also been recorded. Formalin-inactivated IHNV was found to protect rainbow trout against lethal IHNV when immunized at high virus concentration (Hegde and Sin, 2006). Immunogenicity and protective effects of inactivated Singapore grouper iridovirus (SGIV) vaccines have been investigated in orange-spotted grouper, *Epinephelus coioides* (Ou-yang *et al.*, 2012). Efficacy of a formalin-killed vaccine against infectious spleen and kidney necrosis virus (ISKNV) and the major immunogenic proteins of ISKNV were found out by Dong *et al.*, (2013).

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 a transcriptional regulation of type 1 IFNs and IFNs stimulating genes (ISGs). IRF 7 is known as the master regulator of type 1 immune response in vertebrates and it plays a vital role in the innate antiviral immunity (Hu *et al.*, 2011). IL-10, initially known as cytokine synthesis inhibitory factor, is a multifunctional cytokine and demonstrates immunosuppressive 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 in head kidney, spleen, intestine and gill tissues obtained from normal (healthy) carp (Savan *et al.*, 2003). However, a more pronounced expression of carp IL-10 was observed in head kidney and intestine tissues. In the present work, we report expression of the immune genes, IFN and IL10 in juvenile koi carp immunized with inactivated vaccine of similar damselfish virus (SRDV).

Materials and Methods

Cell line

Epithelioma papulosum cyprini (EPC) cell line was used for virus propagation and vaccine development. The cells were grown in cell culture plastic flasks (25 cm²) (Thermo, Korea). The cell lines were maintained 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

Similar damselfish virus (SRDV) isolated from similar damselfish (*Pomacentrus similis*) and characterized in our laboratory was used for the present study (John and George, 2011; Sivasankar, 2014). Stock preparation of SRDV was prepared in EPC cell line. On completion of 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 placed in cryotubes and stored at -80 °C until use. One aliquot was thawed and used for estimating TCID₅₀/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 noticed for 10 days for calculating the viral titre.

Fish and rearing condition

Healthy koi (*Cyprinus carpio*) were obtained (average weight 9.5 g) from the local ornamental fish farm and maintained in the laboratory until non-specific mortalities had stopped and acclimatization achieved to the tank conditions. Fish were held in 100 l glass aquarium tanks maintained with 60 l freshwater at a temperature of 31± 1 °C with a carbon re-circulating water system. 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

To prepare 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 10⁷ TCID₅₀ 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 periods of up to 10 days.

Vaccination and challenge

Koi juveniles were randomly divided into 6 groups of 17 fish each in duplicate namely: control (4 tanks), vaccine alone (4 tanks) and vaccine + adjuvant (saponin Qil-A[®]) (InvivoGen), (4 tanks). The concentration of adjuvant (saponine Qil-A[®]) was adjusted to 5 µg / fish with sterilized double distilled water. Fish were starved for 24 h prior to vaccination

and anesthetized by immersion in benzocaine (Himedia) solution at the concentration of 40 ppm. Vaccine preparations were then injected intra-peritoneally at 50 µl / fish. Control 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 intraperitoneal injection with SRDV. Two groups of fish from each group were challenged with 50 µl of the SRDV stock ($10^{5.17}$ TCID₅₀ /50 µl). Control fish received the same volume of PBS and fish were monitored for 50 days.

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. Total RNA was isolated from the spleen and kidney tissues taken from three fish in each treatment group at 24h, 48h and 96h after vaccination by using TRIzol reagent (Life Technologies) as per the manufacturer's instructions. The RNA was dissolved in 100 µl of DEPC treated RNase-free water.

cDNA synthesis

The RNA concentration was adjusted to 1 µg for reverse transcription to cDNA synthesis. The concentration of RNA was measured by means of a UV spectrophotometer (Hitachi High-Technologies Corporation, Tokyo Japan) at OD₂₆₀ nm. cDNA was synthesized from all RNA samples extracted from selected tissues using the specific reverse primers for IRF-7, IL-10 and β actin with cDNA Synthesis Kit (Thermo, USA)

according to the manufacturer's instructions. The reaction mixture contained extracted RNA (10 µl) with 10 µl of cDNA synthesis mixture containing 2 µl 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 nucleus-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, USA) master mix and 6.5 µl of deionised water. The amplification was carried out in Step-One Plus Real-Time PCR Detection system (ABI, Invitrogen, Life sciences, USA). The expression level of the immune genes in the samples was analyzed in triplicate using the comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with β-actin gene as an internal control. The primers used for qRT-PCR immune genes and cycling conditions have been listed in the table 1.

Results and Discussion

Protection of inactivated SRDV vaccination

On the 28th day post-vaccination, fish from each group were challenged with SRDV and fishes were maintained for 50 days after viral challenge along with control. In challenge trials, the fish showed no typical clinical symptoms of SRDV infection and no mortality was recorded from all groups during the study period (Fig. 1).

Immune gene expression

Spleen, kidney and thymus are considered 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). Expression of IRF-7 gene and IL-10 were tested in koi with SRDV infection alone, SRDV infected fish vaccinated with formalin-inactivated SRDV vaccine and SRDV infected fish vaccinated with formalin-inactivated SRDV vaccine + Qil-A[®] adjuvant.

Expression of IRF-7 in spleen after virus infection

As shown in figure 2, IRF-7 expression in spleen of koi immunized with vaccine plus Qil-A[®] adjuvant was highly up-regulated (52.2 fold) at 24 h post infection with SRDV. When the fish are immunized with inactivated SRDV vaccine alone and inactivated SRDV vaccine with Qil-A[®] adjuvant were challenged by virus administration, it showed 17 and 5 fold induction respectively after 48h. When the fish are infected with SRDV alone, it showed 11 fold inductions. No expression was noticed after 96h.

Expression of IRF-7 gene in kidney after virus infection

In contrast, the expression of IRF-7 in kidney koi immunized with vaccine added Qil-A[®] adjuvant did not show up-regulation at 24 h post infection (Fig. 3). The highest level of up-regulation (16 fold) was recorded at 96 h post infection of SRDV in fish vaccinated with adjuvant. The expression of IRF-7 in kidney was up-regulated in fish infected with virus alone at all three-time points (2.2, 9.3 and 2.7 fold) respectively when compared to control group. Overall the results show that IRF-7 is up-regulated in both spleen and kidney of koi in responses to SRDV infection.

Expression of IL-10 gene in spleen after virus infection

Changes in gene expression of IL-10 in spleen and kidney tissues obtained from koi at 24 h, 48h and 96 h after SRDV infection was analysed. The levels of IL-10 expression in spleen of vaccine + adjuvant and vaccine alone groups showed high fold induction of 331 and 224 fold respectively when compared to those in non-vaccinated fish at 24 h post-vaccination (Fig. 4). The level of IL-10 gene expression in spleen was found decreased later and stood slightly up-regulated (1.8 fold) in virus infected group while the vaccinated group at 96 h post infection with SRDV was up-regulated at 8 fold.

Expression of IL-10 gene in kidney after virus infection

Unlike to the expression of IL-10 in spleen, its expression in kidney of koi immunized with vaccine alone and unvaccinated group showed up-regulation at 28.9 and 29.6 fold at 24h post viral challenge. The expression of IL-10 continued up-regulated at 48h in both the above groups. In fish vaccinated with adjuvant slight up-regulation was noticed (4.1 fold) at 96 h (Fig. 5). Overall the results show that IL-10 is up-regulated in both spleen and kidney of koi in responses to SRDV infection.

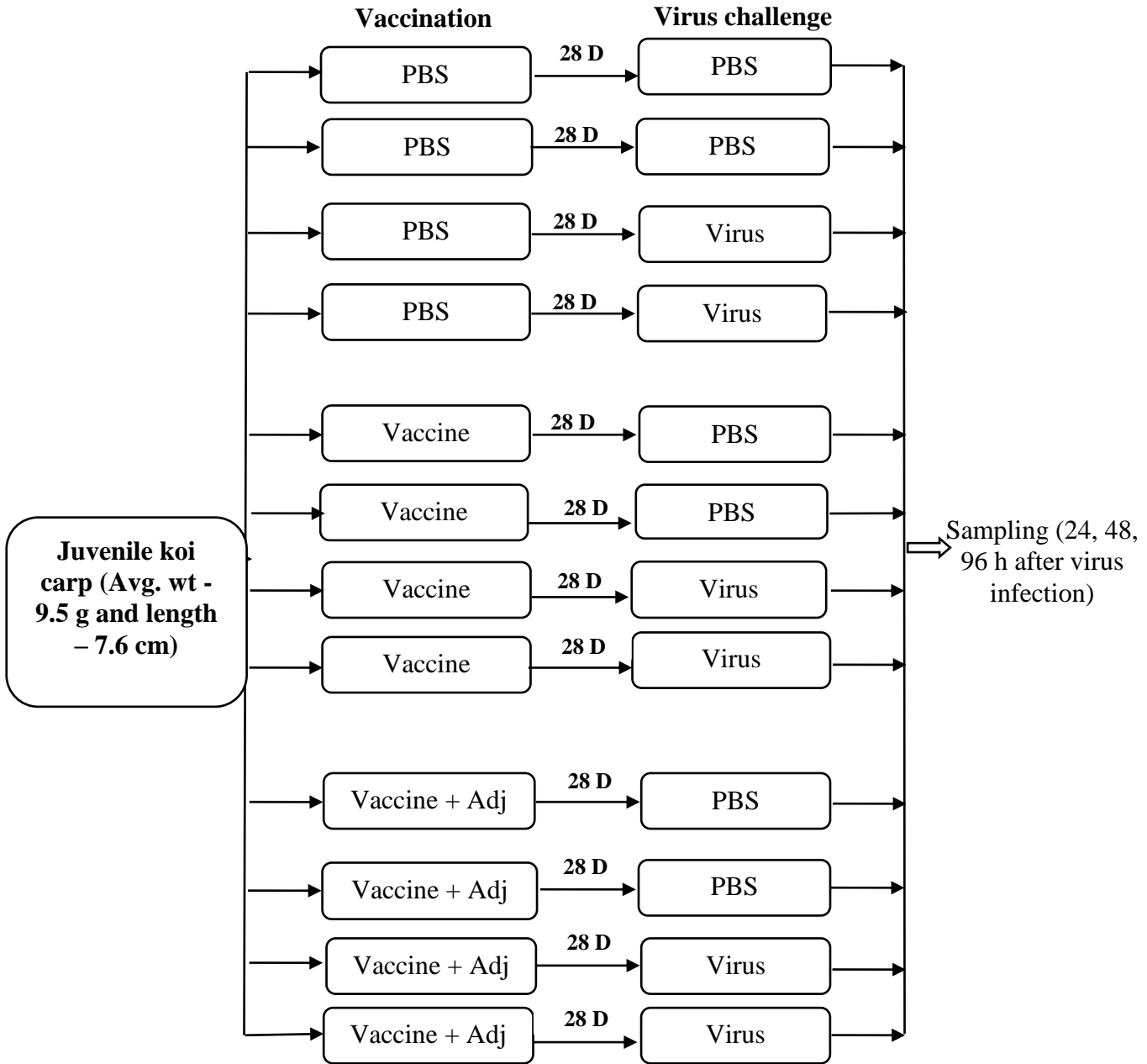
Iridovirus induced mortality remains as an important constraint in the sustainable aquaculture of cultivable and ornamental fish.

The genus *Rana virus* of the family of *Iridoviridae* includes several major pathogens of economically and ecologically important fishes, which are associated with numerous disease outbreaks in the natural and cultured population of fish. Ranavirus disease outbreaks also have high mortality among freshwater and marine ornamental fish farms. The chemicals and/or antimicrobial

treatments are being discouraged because of leading to the development of resistance in the microorganisms and posing negative impacts to the aquatic environments (Ou-yang *et al.*, 2012). Vaccination has become a

powerful tool for health management in aquaculture in term of prevention of diseases and several vaccines against viral and bacterial diseases have been successfully developed (Fu *et al.*, 2014).

Fig.1 Experimental design of juvenile koi fish following similar damselfish virus (SRDV) infection



Adj- Adjuvant

Fig.2 Relative folds induction ($2^{-\Delta\Delta CT}$) of IRF-7 gene in spleen of koi fish challenged with SRDV at 24 h, 48 h and 96 h

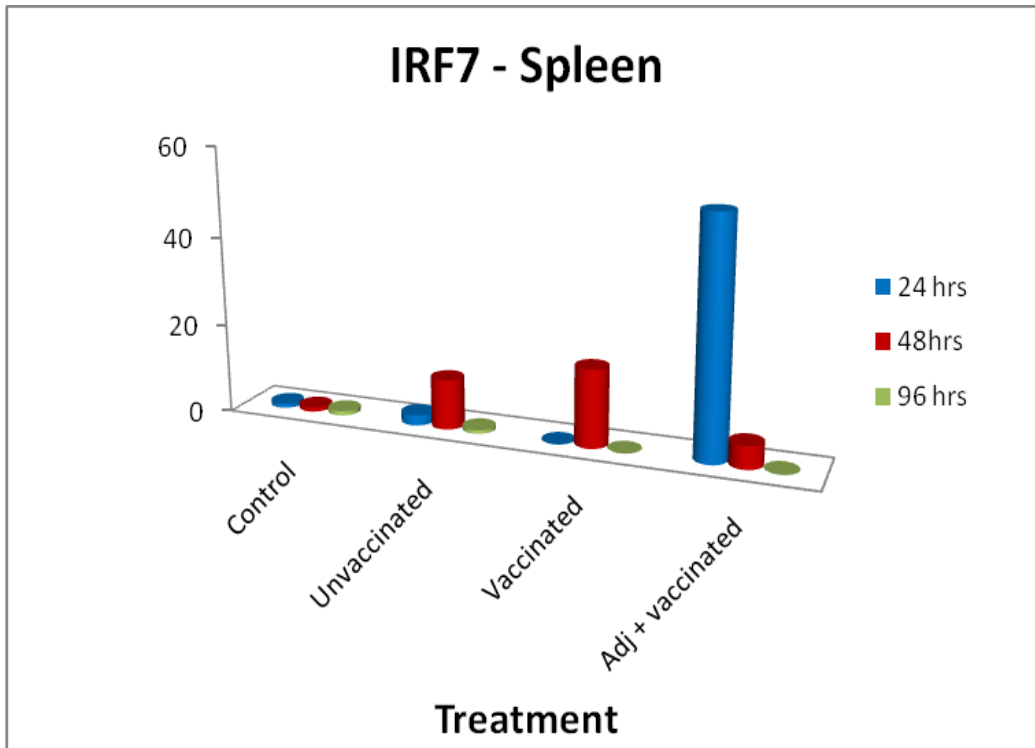


Fig.3 Relative folds induction ($2^{-\Delta\Delta CT}$) of IRF-7 gene in Kidney of koi fish challenged with SRDV at 24 h, 48 h and 96 h

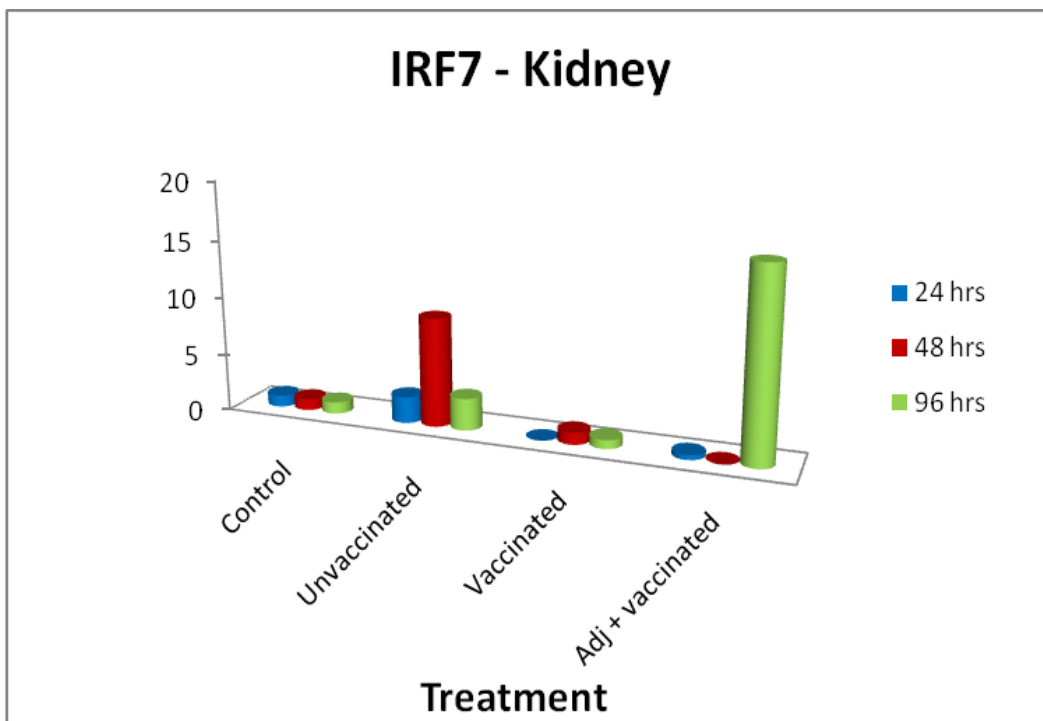


Fig.4 Relative folds induction ($2^{-\Delta\Delta CT}$) of IL-10 gene in spleen of koi fish challenged with SRDV at 24 h, 48 h and 96 h

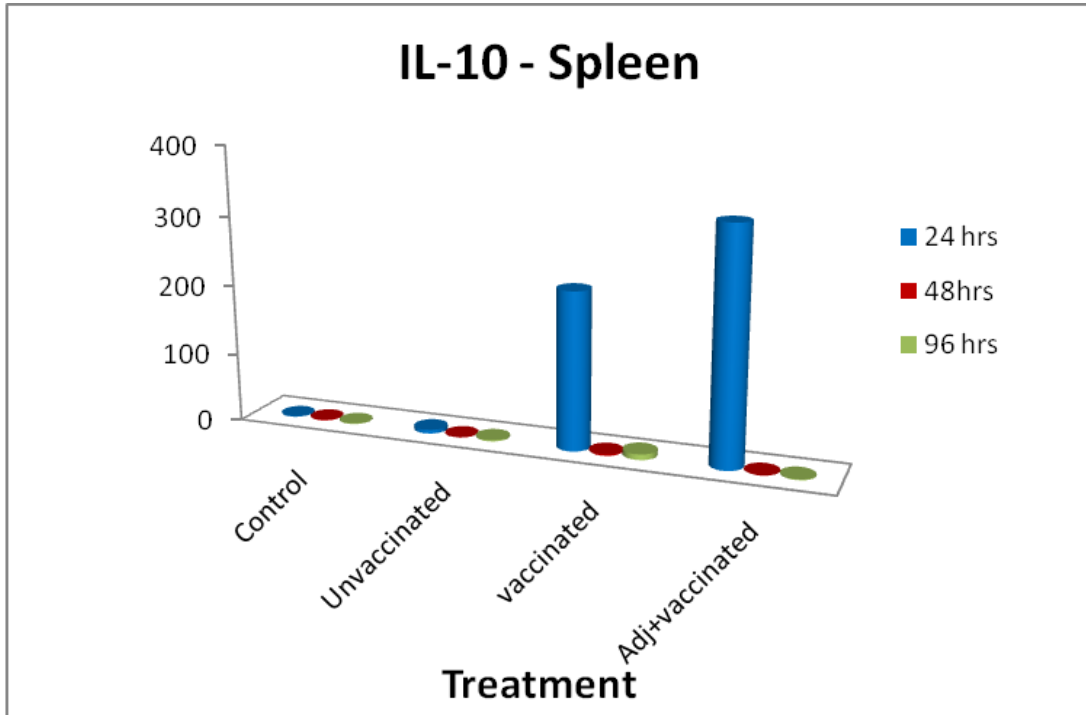


Fig.5 Relative folds induction ($2^{-\Delta\Delta CT}$) of IL-10 gene in Kidney spleen of koi fish challenged with SRDV at 24 h, 48 h and 96 h

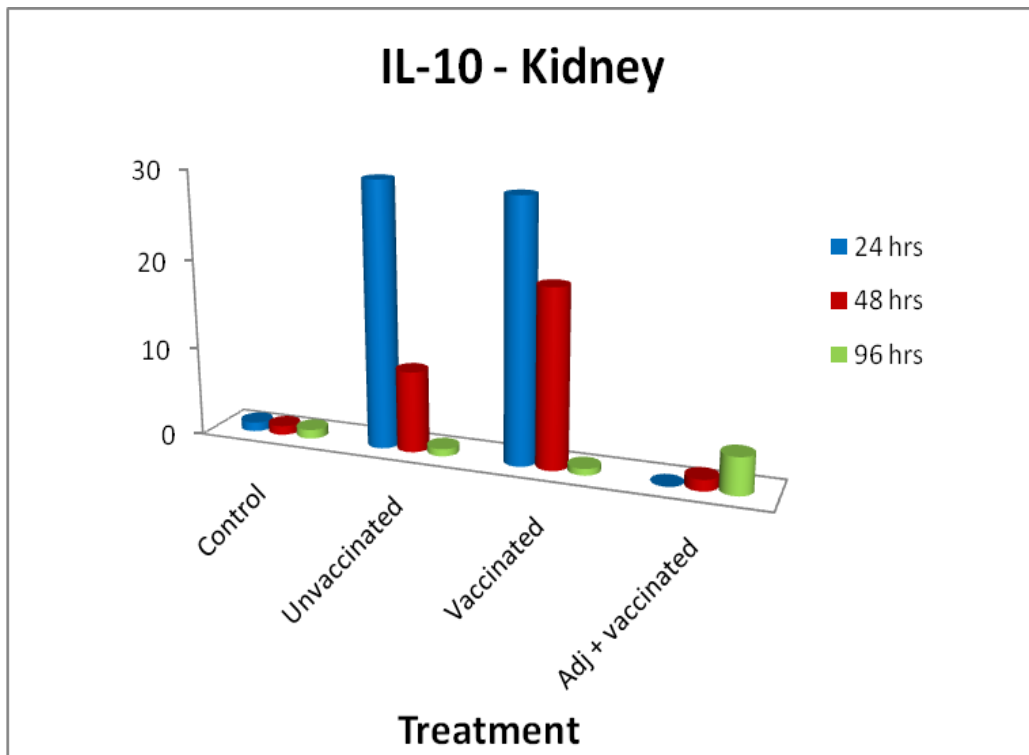


Table.1 Primers used for the qRT PCR analysis of IRF7 and IL10 gene expression following SRDV challenge

Gene	Primers sequence 5'-3'	Accession number	Size (bp)	Cycling condition
IRF-7 Fw2	TCCACTGAGGGTCTGATTGA	JQ698666	148	95 °C 10 min
IRF-7 Rv2	CGCTGGTGCTGACGAAGA			95 °C 15 sec
IL-10 Fw3	TGATGATTTGGAACCATTATTGAA	KX622694	284	55 °C 1 min
IL-10 Fv3	CACCTTTTTTCCTTCATCTTTTCAT			72 °C 1 min
β-actin Fw	TCACCACCACAGCCGAGAG	M24113	110	95 °C 10 min
β-actin Rv	CAGGGAGGAGGAGGAAGCAG			95 °C 15 sec
				54 °C 1 min
				72 °C 1 min

Formalin inactivation of the virus has been effectively used to develop safe and efficacious human and veterinary vaccines (Ou-yang *et al.*, 2012). RT-PCR assays are usually carried out to measure the expression of immune genes in vaccination experiments to assess the efficacy of vaccine preparations and delivery of vaccine candidates. IRF-7 is a master regulator of type-I interferon-dependent immune responses and is essential for the induction of IFN- α/β genes (Fu *et al.*, 2014). IL-10 is the most important immunoregulatory cytokine produced by various types of cells (Nam *et al.*, 2014).

The present study describes the expression of two important immune genes that has specific roles in antiviral activity upon SRDV infection in juvenile koi carp following immunization with inactivated vaccine with and without an adjuvant. In our study, expression of IRF-7 up-regulated in the spleen of koi immunized with vaccine added Quil-A[®] adjuvant (52.2 fold) at 24 h post infection with SRDV then decreased at 48 and 96 h. Similar increase first at 48h followed by decreasing trend across a 21 day period was also reported in Chinese perch post vaccination with QCDC + pcMCP (Fu *et al.*,

2014). Similar observation was noticed in the group of fish immunized with inactivated vaccine where IRF expression increased to 17 fold at 48h and declined by 96 h. In kidney of koi, IRF-7 was up-regulated 16 fold at 96 h post infection of SRDV in the group administered with vaccine plus adjuvant. Expression was subdued in vaccinated fish without adjuvant during all the time points. Expression of IRF7 was however noticed in challenged fish of unvaccinated group. In the kidney of golden mandarin fish, the immune genes such as IRF2, IRF7, Mx and IRF1 showed significant changes in mRNA expression levels at 1 dpi with infectious spleen and kidney necrosis virus (ISKNV) - like agent (Shin *et al.*, 2013). In skin and head kidney of two genetic lines of common carp (*Cyprinus carpio*), CyHV-3 induced a strong mRNA expression by up-regulating both IRFs 3 and 7 at 72 and 120 hpi, and at 24, 72 and 120 hpi, respectively (Adamek *et al.*, 2014).

IL10 expression was very high in the spleen of koi in the current study after 24h of challenge in both groups of vaccinated fishes. Up-regulation of IL10 in fish in response to bacterial and viral infection has been reported (Nam *et al.*, 2014). Formalin and BPL

inactivated SGIV vaccine administered grouper fish induced the expression of IL-8 in the spleen with a peak of 2.0-fold at day 7 and 2.2-fold at day 1 respectively (Ou-yang *et al.*, 2012). In our study, the levels of IL-10 gene expression was up-regulated at 224 fold in vaccinated group while the gene expression was 331 fold in vaccine plus adjuvant group after the virus challenge. Unvaccinated controls did not show any induction of IL10. IL-8 demonstrated a peak in mRNA expression at 10 dpi in spleen of golden mandarin fish following ISKNV-like agent challenge (Shin *et al.*, 2013). Significant up-regulation of carp IL-10 in the spleen was noted during the acute phase of CyHV-3 infection, but not during the persistent and reactivation phases (Sunarto and McColl, 2015). They also observed that the IL-10 host gene and the CyHV-3v IL-10 gene (khvIL-10) were expressed at significantly higher levels during all phases of CyHV-3 infection compared with mock-infected common carp. In the kidney of golden mandarin fish, IL-8 demonstrated a peak in mRNA expression at 4 dpi and then returned to basal level or decreased slightly with ISKNV-like agent challenge (Shin *et al.*, 2013). Significant mRNA expression of IL-10 in the kidney of olive flounder was observed at 1 h post infection and the highest level of upregulation was noted at 6 h (3 fold) following VHSV challenge (Nam *et al.*, 2014). In the present study, IL-10 gene was highly upregulated at 24h post viral challenge in the kidney in both unvaccinated and vaccinated (without adjuvant) fish (29.6 and 28.9 fold). The expression was reduced at 48h in both the cases. IL-10 gene upregulation (4.1 fold) was observed at 96h in the kidney of koi immunized with vaccine added Quil-A[®] adjuvant. The expression of IL-10 mRNA in kidney tissue of olive flounder fish was highly up-regulated (2.7 fold) at 24 h post infection with *Edwardsiella tarda* (Nam *et al.*, 2014). Overall the results show that IL-10 is up-

regulated in both spleen and kidney of koi in responses to SRDV infection at 24 h.

Protection against various pathogens is a major challenge faced by aquaculturists and researchers because of the development of serious diseases associated with large-scale aquaculture. Our results demonstrate that inactivated SRDV prepared from supernatants of SRDV infected EPC cells by formalin at 4°C for 2 days could trigger immune gene expression following SRDV challenge, which is an indication of the immunity development in fish. The effective vaccination is capable of inducing immune-related genes involving the immune system of fish. The study provides strong evidence for the upward modulation of expression profiles of immune-related genes (IRF-7 and IL-10) during the ranavirus infection. The study also suggests that Quil-A[®] adjuvant enhances the immune response of the vaccine candidates.

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