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Nanoencapsulation of inactivated-viral vaccine using chitosan nanoparticles: Evaluation of its protective efficacy and immune modulatory effects in olive flounder (*Paralichthys olivaceus*) against viral haemorrhagic septicaemia virus (VHSV) infection

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ABSTRACT

Viral haemorrhagic septicaemia virus (VHSV), a (−) ssRNA virus belonging to the genus *Novirhabdovirus* of rhabdoviridae family, is the aetiological agent of viral haemorrhagic septicaemia (VHS) disease which causes huge economic losses in farmed olive flounder (*Paralichthys olivaceus*) and significant mortalities among several other marine fish species in Korea, Japan, and China. Previously, we developed an inactivated vaccine viz., formalin-inactivated VHSV mixed with squalene as adjuvant which was effective in conferring protective immunity (58–76% relative percentage survival) against VHSV but the mode of administration was intraperitoneal injection which is not feasible for small sized fingerling fish. To overcome this limitation, we presently focused on replacing the injection route of vaccine delivery by oral and immersion routes. In this context, we encapsulated the inactivated VHSV vaccine with chitosan nanoparticles (CNPs-IV) by water-in-oil (W/O) emulsification method. After encapsulation, two sets of *in vivo* vaccination trials were conducted viz., preliminary trial-I and final trial-II. In preliminary trial-I, olive flounder fingerlings (10.5 ± 1.7 g) were vaccinated with CNPs-IV by different delivery strategies involving oral and immersion routes (single/booster dose) followed by challenge with VHSV (1×10^6 $TCID_{50}$ virus/fish) to evaluate an effective method amongst different applied delivery strategies. Subsequently, a final trial-II was conducted to better understand the immune mechanism behind the efficacy of the employed delivery strategy and also to further improvise the delivery mechanism with prime-boost (primary immersion and oral boosting) combination in order to improve the transient anti-VHSV response in the host. Evaluation of RPS analysis in trial-I revealed higher RPS of 46.7% and 53.3% in the CNPs-IV (immersion) and CNPs-IV (immersion/immersion) groups, respectively compared to 0% RPS in the CNPs-IV (oral) group and 20% RPS in the CNPs-IV (oral/oral) group when calculated against 100% cumulative mortality percentage in the NVC (non-vaccinated challenged) control group, whereas, in the trial-II, RPS of 60% and 66.6% were obtained for CNPs-IV (immersion/immersion) and CNPs-IV (immersion/oral) groups, respectively. In addition, specific (anti-VHSV) antibody titre in the fish sera, skin mucus and intestinal mucus of the immunized groups were significantly ($p < 0.05$) enhanced following vaccination. Furthermore, CNPs-IV immunized fish showed significant ($p < 0.05$) upregulation of different immune gene transcripts (IgM, IgT, pIgR, MHC-I, MHC-II, IFN-γ, and Caspase3) compared to control, in both the systemic (kidney) and mucosal (skin and intestine) immune compartments of the host post immunization as well as post challenge. To conclude, mucosal immunization with CNPs-IV vaccine can orchestrate an effective immunization strategy in organizing a co-ordinative immune response against VHSV in olive flounder thereby exhibiting higher protective efficacy to the host with minimum stress.

1. Introduction

Viral diseases are considered to be the major limiting factors in the development and profitability of aquaculture. Among several viral pathogens affecting the industry, viral haemorrhagic septicaemia virus (VHSV), causative agent of viral haemorrhagic septicaemia (VHS)

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disease, is one of the most serious pathogen of both freshwater and marine finfishes worldwide in terms of its wide host-range, pathogenicity, disease course, and mortality rates [1,2]. The virus is an enveloped negative sense single strand RNA virus which is classified under the genus *Novirhabdovirus* of rhabdoviridae family [3]. Among several genotypes of the virus, the genotype IVa has been causing menace to the olive flounder (*Paralichthys olivaceus*) aquaculture industry in Korea [3] with repeated outbreaks during late winter and spring season (water temperature 8–15 °C) every year since its first isolation in 2001 [4,5]. Once the fish gets infected with the virus, it shows pathognomonic signs viz., dark colouration, ascites and haemorrhages on external body surface, congested liver, splenomegaly and hydronephrosis [5] and ultimately succumbs to the virus-mediated lethality in a very short time with a very rare chance of recovery. In view of the constant threat of VHSV to flounder in particular and aquaculture industry as a whole, a number of prophylactic measures have been devised by constant research worldwide in fish vaccinology and allied fields for over three-decades. Among these, DNA-based vaccine has proven to be the most promising vaccination strategy in conferring the substantial protection against VHS [6]. However with the legitimate concerns and legal restriction on the usage of DNA vaccine in food fishes in most of the countries, including Korea, alternative immunization strategy is needed which can induce long lasting protective immunity and should have universal acceptability.

We have previously developed an injection-based vaccination strategy containing formalin-inactivated VHSV mixed with squalene as adjuvant and it proved to be effective in conferring protective immunity to olive flounder (58–76% RPS) [7]. However, the administration route (intraperitoneal injection) of the vaccine was only suitable for relatively large-sized advanced fingerlings and not feasible for delivery in the hatchery reared small-sized fingerlings, due to its stressful and labour-intensive procedure. Hence, a need was felt on replacing the injection route of vaccine delivery by non-stressful mucosal (immersion/oral) route. Although, the mucosal vaccination route seems to be the reliable approach in developing a successful immunization strategy, it carries some practical problems viz., requirement of comparatively higher amount of antigen [8] and degradation of the naked formalin-killed antigen in the hostile *in vivo* (due to enzymatic action in gut via oral route) as well as in aquatic environment (via immersion route), resulting in an unsuccessful transportation of antigen to the target immune organs in the host alongwith its failure in inducing transient and specific antiviral immune response against the virus. Given these issues, nanotechnologies including nanoconjugation and nanoencapsulation, are being sought as suitable alternatives for developing efficacious vaccines that can protect the antigen from degradation as well as permit sustained or pulsed release of conjugated/encapsulated antigen which can minimize the requirements of antigen dosage and repeated administration in ensuring long-term protection [9].

Among various polymers used for nanoconjugation/nanoencapsulation, chitosan ((1,4)-2-amino-2-desoxy- beta-D-glucan), a natural biopolymer derived from deacetylation of chitin (major component of the shells of crustaceans) has received much attention because of its excellent biocompatibility, low immunogenicity, and reduced cytotoxicity compared to other polymers [10–12]. Chitosan and its derivatives are considered to be good candidates for targeted drug delivery; drug transport, protein delivery and gene transfer [13–16]. The unique muco-adhesive cationic property of chitosan assists in stable bonding with therapeutic materials like antigens, which has raised its demand in the pharmaceutical research [17]. Chitosan has been used previously to deliver various reporter genes orally in different animals [18–21]. Fish vaccine research is now-a-days shifting its focus on the use of chitosan as vaccine carrier as is evident from increasing number of studies where it was reported that, chitosan used for nano-encapsulation or nano-conjugation of target antigens facilitates successful vaccine delivery into host fish by mucosal routes as well as induces strong protective immune response against the respective pathogens [22–27]. However, most of these studies investigated suitability of chitosan as DNA vaccine carrier or recombinant protein carrier but till date its use in carrying an inactivated virus antigen in fish has been rarely investigated.

In this context, the present study was undertaken to formulate nanoencapsulated inactivated VHSV vaccine using chitosan nanoparticles (CNPs) followed by its administration to fishes by different delivery strategies involving oral and immersion routes (single/booster dose or single/combine route) in order to evaluate its ability *per se* in carrying the antigen to the target immune cells and thereby induce protective immunity in olive flounder against VHSV. To our knowledge, this is the first report describing chitosan NPs for encapsulation of inactivated whole viral antigen against VHSV.

2. Material and methods

2.1. Experimental animals

Olive flounder (*Paralichthys olivaceus*) fingerlings (8.1 ± 0.7 g) obtained from a local hatchery were disinfected with 50 ppm formalin and acclimatized in our indoor rearing flow-through facility provided with UV-treated aerated seawater and were fed twice a day with a standard pelleted diet at 3% of their body weight for 3-weeks prior to the vaccination trial. The water temperature and pH varied from 19 °C to 20 °C and from 8.0 to 8.2, respectively during rearing time. To confirm the pathogen free status of fishes, bacterial isolation was carried out in BHI (Brain Heart Infusion) agar, TCBS (Thiosulfate-citrate-bile salts-sucrose) agar and SS (Salmonella-Shigella)-agar plates, virus isolation was carried out in CHSE-214 cells and microscopic observation for detection of parasites was carried out from ten randomly selected individuals. Further, diagnosis for the presence of VHSV was done by nested polymerase chain reaction (PCR) using VG1-VD3 and VD5-VD3 primers (VG1: 5′-atggaatggaacactttttc-3′, VD5: 5′-tcccgctatcagtcaccag-3′ and VD3: 5′-tgtgatcatgggtcctggtg-3′) [28].

2.2. Virus culture

VHSV (F1Wa05 strain) was cultured in fathead minnow (FHM) epithelial cell line maintained at 15 °C in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Invitrogen, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, Invitrogen, USA), 100 IU/mL penicillin G, and 100 μg/mL streptomycin (Gibco, Invitrogen, USA). The cells were inoculated with virus at a multiplicity of infection (m.o.i) of 0.01. Cells along with virus were harvested when the cytopathic effects (CPE) were apparent following which the cell culture supernatant was centrifuged at 5000 × *g* for 30 min at 4 °C for removal of cell debris, and aliquots were stored at −80 °C until use. The virus titre was measured by conventional bio-assay method in 96-well tissue culture plate (Nunc, Denmark).

2.3. Preparation of virus antigen – formalin inactivation

VHSV was inactivated as described in our previous study with slight modification [7]. Briefly, 0.3% formalin was added to 410 mL (180 mL initial set of vaccination trial-I and 230 mL for final set of vaccination trial-II; vaccine dose was calculated as 1 mL/fish.) of harvested VHSV having a virus titre of 10^8 $TCID_{50}$/mL and was stirred for 24 h at 4 °C on a magnetic stirrer. Complete inactivation was confirmed by re-inoculation into FHM cells. The inactivated virus, designated as ‘IV’ was concentrated by ultracentrifugation at 30000 × *g* for 2 h at 4 °C using ultracentrifuge (Hitachi, Japan). Aliquots of concentrated IV antigen for various experimental fish groups in 2 different immunization trials were pooled accordingly and suspended in 1 mL of sterile distilled water followed by storage at 4 °C until nanoencapsulation.

2.4. Encapsulation of IV antigen in chitosan nanoparticles (CNPs)

2.4.1. Preparation of chitosan NPs

The chitosan NPs were prepared by following the method as described previously [18]. Briefly, 0.5 g of chitosan (Sigma-Aldrich, USA) was dissolved in 100 mL of sodium acetate buffer (pH 5.4) and kept on magnetic stirrer for 3 h with vigorous stirring. After complete dissolution the solution was filtered using 0.2 μm syringe-filters.

2.4.2. Determination of size of blank CNPs

The blank CNPs were characterized in terms of size and size distribution by dynamic light scattering (DLS) using Zetasizer S-90 Malvern instruments (Malvern, UK). Briefly, 1.3 mL of CNPs sample at a concentration of 0.3 mg/mL was taken in a polystyrene cuvette and the measurement was performed at 25 °C. The viscosity and refraction index were set equal to those specific for water.

2.4.3. Synthesis of chitosan NPs encapsulated IV antigen (CNPs-IV)

Encapsulation of IV antigen in chitosan NPs was carried out using water-in-oil (W/O) emulsification procedure with slight modification [29]. Briefly, aliquot of 10 mL of the prepared chitosan solution (0.05%) in sodium acetate buffer (pH 5.4) was mixed with 1 mL of aqueous solution of pooled IV antigen and homogenized at 2500 rpm for 5 min. The mixture (W) of chitosan and IV antigen was added into 200 mL liquid paraffin oil (O) containing 1.5 mL Span-80 (sorbitan monolaurate, an emulsifying agent), followed by homogenization at 2500 rpm for 10 min to form water-in-oil primary emulsion (W/O). To the primary emulsion, 4 mL of 50% (w/v) sodium polyphosphate (TPP, a gelification solution) was added drop-wise while stirring in mechanical stirrer (2500 rpm) for 1 h. The chitosan-IV nanospheres (CNPs-IV) were separated by centrifugation at 1000 × g for 30 min, washed twice using 70% alcohol, and stored at 4 °C.

2.4.4. Nanospheres characterization

The morphology and size distribution of CNPs-IV nanospheres was evaluated by transmission electron microscopy (TEM). For TEM analysis, a thin formvar film was coated on finely meshed copper grids, in that 5 μL of aqueous solution of CNPs-IV was added followed by addition of 5 μL of PTA (phosphotungstic acid as negative stain). After 10 s the grids were washed with 5 μL of distilled water and air-dried under ambient conditions. The grids were then examined under transmission electron microscope (Hitachi, Japan).

2.5. Vaccine preparation for immunization

The CNPs-IV nanospheres prepared for immersion immunization was suspended in 20 mL of distilled water prior to vaccination. Whereas, for oral vaccination, CNPs-IV nanospheres were mixed with 5% (w/w of feed) aqueous emulsion of hydroxypropyl methylcellulose (HPMC as binder) (Sigma-Aldrich, USA), sprayed gently on the commercial feed pellets and kept at 50 °C for 1 h. An extra outer coating of 2% HPMC (w/w of feed) and 1.6% TEC (triethylcitrate as plasticizer, v/w of feed) (Sigma-Aldrich, USA) was sprayed over the vaccine coated pellet, and kept for 1 h at 37 °C prior to oral immunization to prevent vaccine leaching.

2.6. Experimental design for immunization trial-I

Olive flounder fingerlings (n = 180, n = number of fish) (10.5 ± 1.7 g) were randomly distributed into six experimental groups viz., CNPs-IV (immersion), CNPs-IV (oral), CNPs-IV (immersion/immersion), CNPs-IV (oral/oral), NVC control (non-vaccinated challenged control) and naive control (without treatment and challenge) groups, with 30 fish in each group and reared in 300 L FRP (fibre reinforced plastic) flow-through tanks supplied with UV-treated seawater maintained at 20 °C. For primary immersion immunization, fishes from CNPs-IV (immersion) and CNPs-IV (immersion/immersion) groups were distributed into 2 (1 for each group) plastic aquaria (30 fish/aquaria) and immersed in 1 L of water with 20 mL of CNPs-IV vaccine solution for 2 h with vigorous aeration and transferred back to the original tank following immersion. Similarly, booster dose was applied to the CNPs-IV (immersion/immersion) group after 2-weeks (300 ^{0} days) post primary vaccination. Whereas for oral immunization in CNPs-IV (oral) and CNPs-IV (oral/oral) groups, fishes were fed with the prepared feed containing CNPs-IV nanospheres (as described above) for 2 consecutive days (twice/day) during primary (both groups) and booster (only oral/oral group) immunization at the same time while as normal commercially available feed was given to the other groups. NVC control and naive fish group were kept untreated. After 30 days of primary immunization, all the fishes from each of the 6 experimental groups were transferred into plastic aquaria (2 aquaria per group) containing 25 L of UV-treated seawater, and shifted to cold room challenge facility where temperature was maintained around 15 °C. Fishes from the immunized groups as well as NVC control groups were intra-peritoneally injected with 100 μL of VHSV (10^6 $TCID_{50}$virus/fish) strain homologous to the vaccine strain while as naive group was left unchallenged. Mortality pattern and clinical signs of VHS was observed daily for 20 days post infection in 2 aquaria (n = 15, fish/aquaria) per group (including naive group). Dead fishes were collected and examined for VHSV-specific mortalities. Relative percentage of survival (RPS) was calculated by the formulae [30]; Relative percentage of survival (RPS) = {1 – (%Mortality in vaccinated group/%Mortality in control group)} × 100. (Pictorial designs for trial-I experiment in Supplementary file 1).

2.7. Experimental design for immunization trial-II

Following the efficacy analysis of the pilot immunization trial-I, a second experimental set-up was designed for immunization trial-II (pictorial details of experimental design for trial-II in Supplementary file 1). In the trial-II, olive flounder fingerlings (n = 240, n = number of fish) (12.5 ± 1.5 g) were distributed into three treatment groups with 60 fishes in each group and reared in 500 L FRP flow-through tanks supplied with UV-treated seawater maintained at 20 °C. The groups were designated as CNPs-IV (immersion/immersion), CNPs-IV (immersion/oral), NVC control group (non-vaccinated challenged group) and remaining 60 fishes were kept as naive group (without any treatment or challenge). For initial immunization, fishes from both the CNPs-IV (immersion/immersion), and CNPs-IV (immersion/oral) groups were distributed into 2 (1 for each group) plastic aquaria (60 fish/aquaria) and immersed in 2 L of water with 20 mL of CNPs-IV vaccine solution for 2 h similar to the immersion immunization in trial-I. After 14 days post initial immunization (300 ^{0} days) booster dose was administered to the CNPs-IV (immersion/immersion) group, same as initial immunization, whereas, the CNPs-IV (immersion/oral) group was fed with the prepared feed containing CNPs-IV nanospheres as described in trial-I for 2 consecutive days (twice/day). To mention here, the present immunization trial-II was run in parallel with the set-up of our previously published report regarding the use of PLGA nanoparticles encapsulated IV vaccines (PNPs-IV) against VHSV in olive flounder [31], using the same NVC control and naive control groups; and with identical immunization and challenge schedule.

Five fish from each group (immunized groups, NVC control group and naive group) were randomly selected for sampling at 48 h post initial vaccination (hpiv), 48 h post booster vaccination (hpbv) and before challenge (30 d post initial immunization). Blood serum and head-kidney tissue samples were collected at each time point for further analysis of immune parameters. Skin (portion from caudal peduncle site) and skin mucus samples were collected at same time point from CNPs-IV (immersion/immersion), NVC control and naive groups whileas intestine (pool of representative section from foregut, midgut and hindgut) and intestinal mucus samples were collected from CNPs-IV (immersion/oral), NVC control and naive groups at 48 hpbv and before challenge (30 dpiv).

2.8. Challenge study for immunization trial-II

For challenge study, remaining 45 fish (16.5 ± 0.8 g) from each of the 4 experimental groups viz., CNPs-IV (immersion/immersion), CNPs-IV (immersion/oral), NVC control and naive group were transferred to the cold room challenge facility (15 °C) into plastic aquaria (3 aquaria per group, n = 15/aquaria, where n is number of fish) containing 25 L of UV-treated seawater. Fish from the treatment groups were intraperitoneally injected with 100 μL of VHSV (10^6 $TCID_{50}$virus/fish) strain as described in trial-I while naive group remained unchallenged. Mortality pattern and clinical signs of VHS was observed daily for 20 days post infection in 2 aquaria (n = 15, fish/aquaria) per group (including naive group) and RPS was calculated as described above in section 2.6. Fishes from the remaining aquaria in each group (including naive group) were sampled randomly (5 fish per time-point) for blood serum and head-kidney tissue sample for all groups at 24 h, 48 h and 96 h post challenge (hpc). Skin (portion from caudal peduncle site) samples were collected at same time point from CNPs-IV (immersion/immersion), NVC control and naive groups whileas intestine (pool of representative section from foregut, midgut and hindgut) samples were collected from CNPs-IV (immersion/oral), NVC control and naive groups. Skin mucus and intestinal mucus samples were collected at 48 hpc from CNPs-IV (immersion/immersion) and CNPs-IV (immersion/oral) groups, respectively, as well as from NVC control and naive groups.

2.9. Collection of skin and intestinal mucus

Skin mucus samples from CNPs-IV (immersion/immersion) vaccinated groups as well as NVC control and naive group were collected by placing the fish in a zip-lock plastic bag, containing 200 μL of sterile PBS with 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich, USA), followed by gently rubbing the skin surface of the fish. The collected mucus in the zip-lock bags were then transferred to microcentrifuge tubes, vortexed for 1 min, centrifuged at 2000 × *g* for 15 min at 4 °C.

For collection of gut mucus, fishes were dissected aseptically and whole intestine was excised. Prior to collection of gut mucus, all the gut contents were removed carefully by gently squeezing the intestine and afterwards collecting the mucus to avoid contamination. Gut mucus was collected in microcentrifuge tube, containing 200 μL of sterile PBS with PMSF (1 mM), by gently squeezing the gut of the sampled fish using sterile forceps. The gut mucus samples were then vortexed for 1 min and centrifuged at 2000 × *g* for 15 min at 4 °C. Both the mucus supernatants were stored at −20 °C for further analysis.

2.10. Competitive ELISA for specific antibody quantification in experimental fish

Specific antibody (anti-VHSV Ig) quantification in the fish sera, skin mucus and intestinal mucus of the experimental fish groups was done by 3 different competitive enzyme-linked immunosorbent assays (c-ELISA), respectively, as described according to previously published protocol by Kole et al. [25] with slight modifications. Briefly, 100 μL of VHSV (10^8 $TCID_{50}$/mL) diluted in coating buffer (carbonate – bicarbonate buffer, pH 9.6) was coated in triplicate in 96-well ELISA plates (Nunc, Denmark) and incubated at 4 °C overnight. The plate was washed with wash buffer (PBS-T, 0.05% tween 20 in PBS pH 7.4) thrice for 5 min and blocked by adding 300 μL blocking buffer (3% bovine serum albumin (BSA) solution in PBS-T) and incubated further for 1 h at 25 °C. The plates were washed again with washing buffer thrice for 5 min each. 100 μL of serum (standardised dilution at 1:200 in 1% BSA in PBS-T), skin mucus and intestinal mucus (undiluted supernatants) sampled from all experimental groups at different time points were added in different wells and incubated in an ELISA shaker at room temperature for 1 h. Subsequently, 100 μL of diluted (1:200 in 1% BSA in PBS-T) monoclonal antibody (MAb) raised against VHSV glycoprotein (Enbiogene, Korea) was added to each well and kept at 4 °C overnight. For MAbs reading, only 100 μL of MAb (1:200) was added in 3-wells of each plate. The plates were washed thrice by wash buffer on the following day and incubated with 100 μL of secondary antibody (Goat anti-mice HRP conjugate, Thermo Fisher Scientific, USA), freshly diluted in PBS-T @ 1:2000, at 25 °C for 1 h. The plates were thoroughly washed with PBS-T five times and substrate *O*-phenylenediamine tetra hydrochloride (OPD) solution (100 mg/mL of OPD and 40 μL of H_2O_2 (30% v/v) in 5 mL of citrate (50 mM) – phosphate (100 mM) buffer, pH 5) was added to each well. The plates were incubated for 20 min at 25 °C in dark chamber. Afterwards, the reaction was stopped with 50 μL of 2 N H_2SO_4 and optical density (OD) was recorded at 492 nm using VERSA max microplate reader (PerkinElmer, USA). The result was expressed as percentage inhibition, derived by following formula: PI = 100- (mean OD_{492} of test sample × 100)/(mean OD_{492} of MAbs).

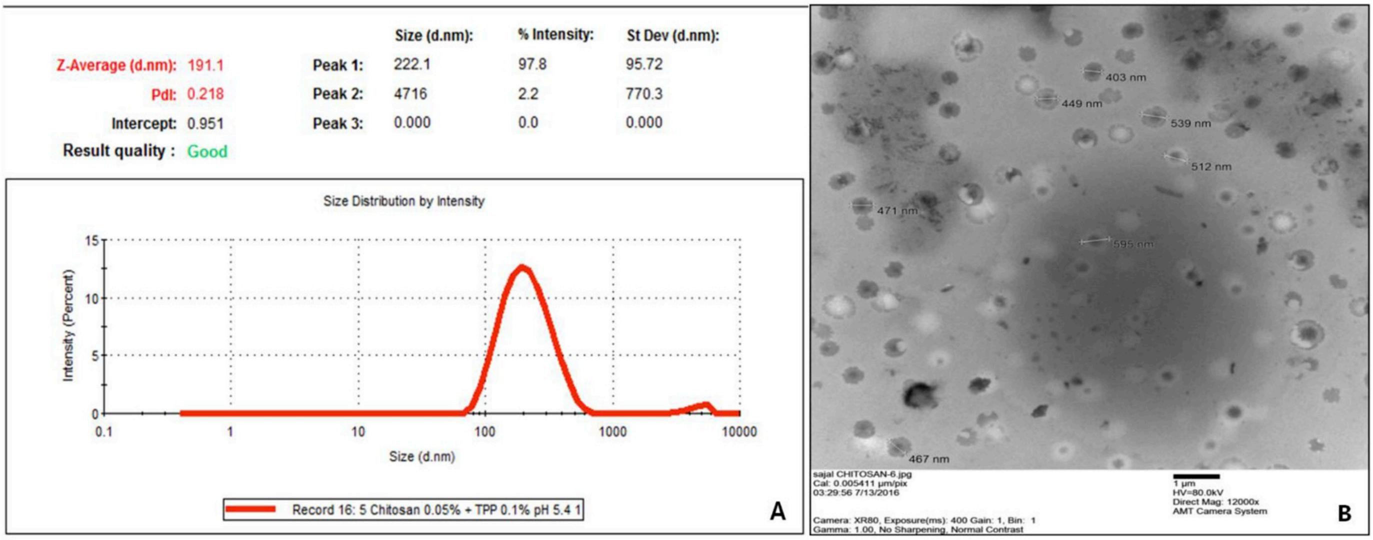
2.11. Immune gene expression

2.11.1. RNA isolation and cDNA synthesis

Total RNA was extracted from the kidney, intestine and skin tissues using RNAiso Plus (Takara Bio Inc, Japan) as per manufacturer's protocols and quantified by NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, USA). The residual genomic DNA was removed using RNase-free DNase I (Takara Bio Inc, Japan). Total RNA (1 μg) was reverse-transcribed into first-strand cDNA using ReverTra Ace® qPCR RT Kit (Toyobo, Japan) using oligo-dT primer and ReverTra Ace reverse transcriptase in a 10 μL reaction volume as per the manufacturer's protocol. The resulting cDNA was stored at −20 °C until use.

Table 1
Primers used for real-time PCR.

Target gene	GenBank acc. no	Product length	Sense primer	Antisense primer	Ta
VHSV N	JF792424	138	ATCTGGAGGCAAAGTGCAAG	CCATGAGGTTGTCGTTGTTG	62
β-actin	HQ386788	131	CCTCTTCCAGCCTTCATTC	TGGTTCCTCCAGATAGCAC	56
IgM	AB052744	115	GCCTCCTTCTTCTGCTCTG	CCTCAGTGGATGTTGTGATT	56
IgT	KX174302	150	TAATTGTTCAGTAACTCATGCCG	GATTGAAGTGTTCCTATGCGTCT	56
pIgR	HM536144	478	AAGGAGGAGGACTCTGGGTG	TGGTGATGGGTCTGGATGG	58
MHC I	AB126921	148	TCTCCCTCCTCTCCAGTCAGC	GCTCATCTGGAAGGTCCCGTCAT	58
MHC II	AY848955	107	GTCGTCAGGCTTCACTCTGT	TCTCTTTGCCAGCTCACTTT	56
IFN-γ	AB435093	126	CTACAAGCGGCGATATGATG	GGAGGTTCTGGATGGTTTTG	64
Caspase3	JQ394697	115	ACATCATGACACGGGTGAAC	TCCTTCGTCAGCATTGACAC	58



			Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm):	191.1	Peak 1:	222.1	97.8	95.72
PdI:	0.218	Peak 2:	4716	2.2	770.3
Intercept:	0.951	Peak 3:	0.000	0.0	0.000
Result quality :	Good				

Fig. 1. Chitosan nanoparticles characterization; (A) Particle size distribution of blank chitosan nanoparticles (CNPs) by dynamic light scattering (DLS) using Zetasizer S-90 Malvern instruments; (B) Transmission electron micrograph of IV-loaded chitosan nanospheres (CNPs-IV) showing spherical nanospheres with mean particle size range of 450–500 nm.

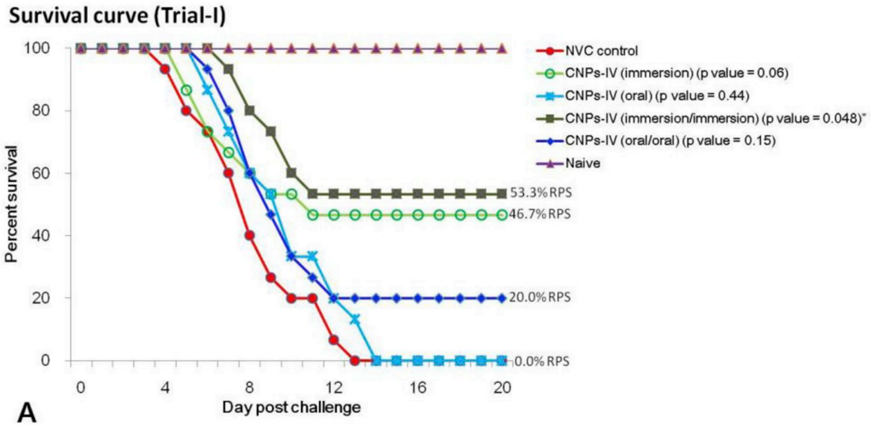


Fig. 2A. Survival curve of immunization trial-I, showing cumulative mortality and relative percentage survival (RPS) analysis of different experimental groups viz., CNPs-IV (immersion), CNPs-IV (oral), CNPs-IV (immersion/immersion), CNPs-IV (oral/oral), and NVC control groups of olive flounder (n = 30, 2 tanks with 15 fish/tank for each groups) challenged with virulent VHSV (10^6 $TCID_{50}$ virus/fish) at different time intervals. Naive group was kept unchallenged. Log-rank test was analyzed between non-vaccinated NVC control group and the vaccinated groups viz., *p*-values of 0.06, 0.44, 0.048 and 0.15 with CNPs-IV (immersion), CNPs-IV (oral), CNPs-IV (immersion/immersion) and CNPs-IV (immersion/oral) groups, respectively. Significant difference (*) was observed only in CNPs-IV (immersion/immersion) fish group with respect to NVC control.

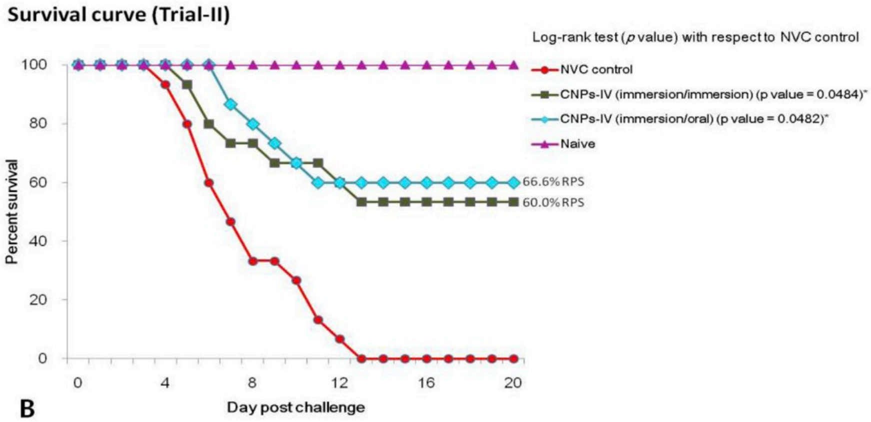


Fig. 2B. Survival curve of immunization trial-II, showing cumulative mortality and relative percentage survival (RPS) analysis of different experimental groups viz., CNPs-IV (immersion/immersion), CNPs-IV (immersion/oral), and NVC control groups of olive flounder (n = 30, 2 tanks with 15 fish/tank for each groups) challenged with virulent VHSV (10^6 $TCID_{50}$ virus/fish) at different time intervals. Naive group was kept unchallenged. Significant difference (*) by log-rank test was noted between non-vaccinated NVC control group and the vaccinated groups viz., *p*-values of 0.0484 and 0.0482 with CNPs-IV (immersion/immersion) and CNPs-IV (immersion/oral) groups, respectively. The mortality values for NVC control and naive control are identical to our previously published paper [31] as both the experiments were run simultaneously with same NVC control and naive control groups.

2.11.2. Quantitative expression analysis of immune genes in experimental samples

Gene-specific primers for immune-related genes were designed using Primer3Plus online software tool based on available sequences from NCBI database. Olive flounder β-actin was selected as a housekeeping gene and all primers used for gene expression are listed in Table 1. Real-time PCR was carried out in an Exicycler™ 96 Real-Time Quantitative Thermal Block (Bioneer, Korea) using SYBR Green AccuPower® PCR PreMix (Bioneer). For relative quantification of each gene, cDNA synthesised from kidney, skin and intestine tissues from the experimental groups as well as from the naive group was used as template. The reaction was performed (in duplicate) in a final reaction volume of 20 µL with a 10 min initial denaturation at 94 °C, followed by 35 cycles of 20 s denaturation (94 °C), annealing (temperature is given

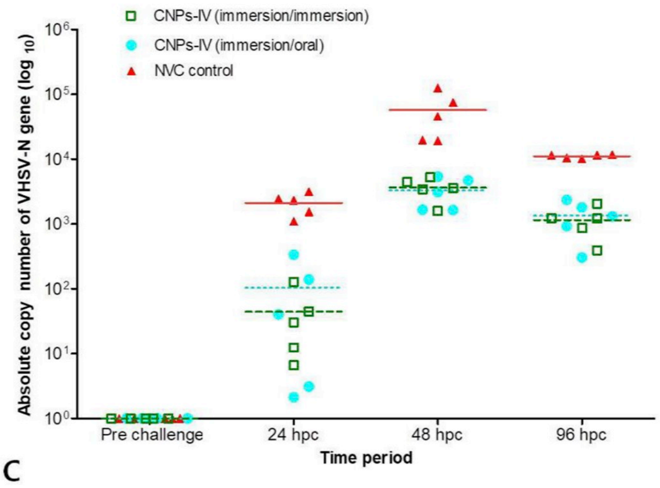


Fig. 2C. Absolute copy number of VHSV in 50 ng total RNA from kidney tissues (n = 5, n is number of fish sampled per time point) of different experimental groups viz., CNPs-IV (immersion/immersion) (□), CNPs-IV (immersion/oral) (●), and NVC control (▲) groups, plotted against time after challenged with virulent VHSV (10^6 $TCID_{50}$ virus/fish) reared at 15 °C. Absolute copy numbers of VHSV for NVC control are identical to our previously published paper [31] as both the experiments were run simultaneously with same NVC control group.

in Table 1) and scanning. The threshold cycle (Ct) value was determined using the automatic setting on the Bioneer Exicycler™ 96 Real-Time PCR system. Relative quantification of immune response was estimated with respect to the expression in the naive control using $2^{-\Delta\Delta Ct}$ method [32] whereas the viral mRNA was quantified as described in our previous study [33].

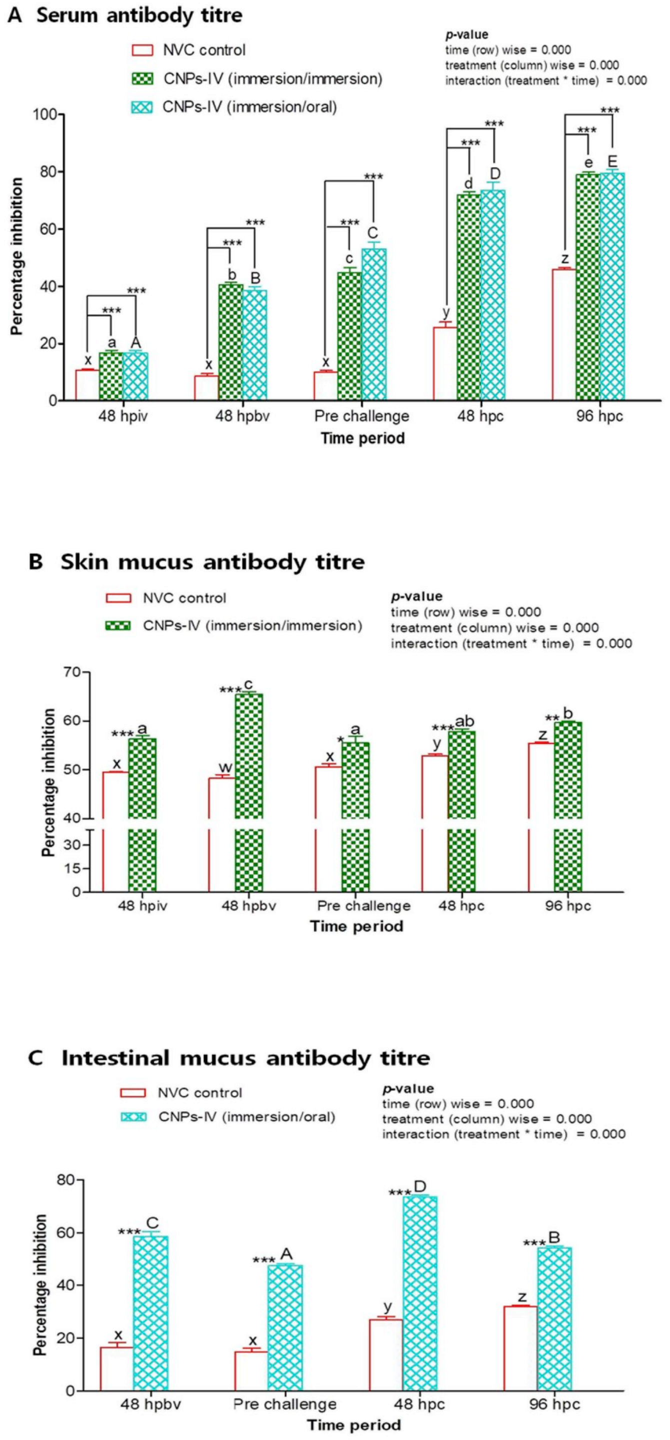
2.12. Statistical analysis

For survival analysis of the immunization trials, Kaplan–Meier curve analysis and log rank test were carried out using GraphPad Prism 5 Software and survival curves were constructed in Microsoft Excel Software. Whereas, the data generated for VHSV-specific antibody titre in serum and mucus samples as well as for gene expression in different tissue samples collected from all experiment groups at different time points were statistically analyzed by using statistical package SPSS version 22 (SPSS Inc., USA). Each data set was subjected to two-way ANOVA in order to determine the statistical significance within the group (time wise), between the groups (group wise) as well as to evaluate the interaction effect. Post-hoc analysis was followed by Duncan's multiple range test and an unpaired *t*-test was used to determine the significant differences in antibody titre and gene expressions at different time points within and between the vaccinated fish groups, respectively. Comparisons were made at the 5% probability level and *p*-value below 0.05 was considered statistically significant. The results were expressed as the mean ± standard error.

3. Results and discussions

3.1. Positive encapsulation of inactivated VHSV antigen (IV) with chitosan NPs

Chitosan NPs based vaccine delivery system has proven to be highly effective in fish, particularly as DNA vaccine and recombinant protein carrier, as it forms electrostatic bonds with the antigenic DNA or protein and provides them the required stability against exogenous degrading factors such as temperature, pH and nucleases activities, thereby helps in successful transport of the target antigen into the immune organs and conferring long lasting protective immune responses in the host [17,22–27,34]. However, studies on its use in encapsulating inactivated whole virus are limited. In the present study, we encapsulated the inactivated VHSV (IV) into the CNPs by modified water-in-oil (W/O) emulsification procedure. Exploratory experiments were carried out for determination of the size distribution and morphology of the CNPs prior and post 'IV' loading by nanoanalyzer and transmission electron microscopy, respectively. As shown in Fig. 1A, the blank CNPs were having uniform distribution with average size of 191.1 nm whereas, the TEM (Fig. 1B) displayed that the nanoparticles obtained after encapsulating 'IV' antigen, have rounded morphology with high electron density in the centre, and their diameters ranged around 450–500 nm. The central electron dense region of the CNPs-IV indicated that the IV antigen was positively encapsulated inside and was



(caption on next page)

Fig. 3. inhibitions (PI) of specific antibody against VHSV were determined by competitive ELISA using anti-VHSV-G MAbs. Percentage inhibition of anti-VHSV antibody binding to VHSV antigen, present (A) in the fish serum of different experimental group viz., CNPs-IV (immersion/immersion), CNPs-IV (immersion/oral), NVC control and naive groups; (B) in the skin mucus of CNPs-IV (immersion/immersion), NVC control and naive groups; and (C) in the intestinal mucus of CNPs-IV (immersion/oral), NVC control and naive groups, were plotted at different time intervals post immunization and post challenge with virulent VHSV (10^6 $TCID_{50}$ virus/fish). The mean values (n = 5) of the PI activities in the serum, skin mucus and intestinal mucus were plotted with standard error as a function of time after immunization/challenge. A two-way ANOVA was performed with each of the data set. The *p*-values within the group (day wise), between the group (treatment wise) and for interaction effect are evaluated to determine statistical significance ($p < 0.05$). Duncan multiple range test (homogenous subsets indicated by lowercase (w–z) for NVC control, lowercase (a–e) for CNPs-IV (immersion/immersion) and uppercase (A–E) for CNPs-IV (immersion/oral) groups) within the groups (time-wise) and an unpaired *t*-test (indicated by asterisks *) between the groups at different time points were also performed to analyze the statistical differences. (hpiv – h post initial vaccination; hpbv – h post booster vaccination). The PI values for NVC control are identical to our previously published paper [31] as both the experiments were run simultaneously with same NVC control and naive control groups.

surrounded by the CNPs, whereas, the size range and uniformity in their size distribution validated the suitability of the prepared CNPs-IV nanospheres for vaccine delivery to fish via oral or immersion routes, as smaller size allows the NPs to easily cross the outer epithelial barrier to reach the target immune sites and ensures a wider tissue distribution [35]. Moreover, this size distribution is also supported by several previous findings where the authors reported that antigen-loaded CNPs in the size range of 100–500 nm, are ideally suited for drug delivery and are biocompatible for effective transfection in fish tissue [25,26,36,37].

3.2. Relative percentage survival (RPS) and viral mRNA copy number analysis

To mention here, prior to the present immunization trial, we tested the efficacy of inactivated viral particles (without encapsulation) delivered by oral and immersion routes which failed to provide any protection (0% RPS) to the fish [data not shown] and this prompted us to conduct this study. In the present study, two sets of immunization trials were conducted viz., preliminary trial-I for evaluation of an effective method (among different delivery strategies) which can successfully deliver the prepared vaccine (CNPs-IV) in olive flounder and elicit protective immunity against VHSV challenge, and final trial-II to better understand the immune mechanism behind the efficacy of that delivery strategy and also to further improvise the delivery mechanism with prime-boost (primary immersion and oral boosting) combination in order to improve the transient anti-VHSV response in the host. The trial-I challenge study (Fig. 2A) revealed that, immersion based delivery of the CNPs-IV was effective in comparison with the oral route, as indicated by the higher RPS of 46.7% and 53.3% in the CNPs-IV (immersion) and CNPs-IV (immersion/immersion) groups, respectively against 0% RPS in the CNPs-IV (oral) group and 20% RPS in the CNPs-IV (oral/oral) group when calculated with 100% cumulative mortality percentage in the NVC control group. From the results, it was apparent that the oral immunization either failed to deliver the vaccine to the target immune organs of the fish or was unable to protect the IV antigen from enzymatic degradation in the gut during the process of transportation. This is in contrast with previous studies involving CNPs based vaccines wherein, they reported oral route elicits higher protective immunity in fish [23–26], however as those studies involve recombinant DNA as antigen, this variation in efficacy of the oral route can be understood as the inactivated virus antigen used in our experiment has altogether different action mechanism compared to recombinant DNA. Further, the trial-II RPS analysis (Fig. 2B) showed that initial immersion followed by oral boosting in the CNPs-IV (immersion/oral) group resulted in an effective antiviral immune induction in the host giving 66.6% RPS which was similar to the only immersion strategy in CNPs-IV (immersion/immersion) group (60% RPS). Thus considering the results of both the immunization trials, it can be inferred that the CNPs-IV vaccine was not degraded by enzymatic activity in the gut, instead, some pre-sensitization such as primary immersion immunization or repeated boosting (as RPS% increase from 0 to 20 by boosting) is needed in case of the oral delivery of CNPs encapsulated inactivated viral antigen to become effective. Nevertheless, the similar RPS data for the CNPs-IV mucosal vaccine (> 60%) in the present study and that of injection (more precise method of targeted vaccine delivery) of squalene adjuvant-IV vaccine (58–73% RPS) in our previous study [7] suggests that the present strategy can be a promising alternative to injection vaccine. In addition, the viral mRNA copy number in the kidney of olive flounder post challenge with VHSV (Fig. 2C) demonstrated that both the immunized fish groups in trial-II were able to combat viral replication and proliferation to a certain extent (10–100 times less viral mRNA copies) compared to the non vaccinated (NVC) control fish at the peak phase of infection which correlates with the survival of the immunized fish and lethality in the NVC control fish.

3.3. Specific antibody quantification

Apart from the RPS analysis, we quantified the anti-VHSV antibody titre in the fish sera, skin mucus and intestinal mucus of the immunized fish in trial-II experiment to know the efficacy of the vaccination strategy in terms of antibody production. Although indirect ELISA is being commonly used for determination of specific antibody titre in efficacy evaluation of fish vaccine, but it seemed to be inappropriate for our present study, as it was giving false positive and high background. Hence, we employed competitive ELISA, to minimize the background signal to get a very specific anti-VHSV antibody titre. The present results demonstrated an increasing trend for antibody titre in serum (Fig. 3A) of both the CNPs-IV vaccinated fish groups, having minimum titre value at 48 h post primary immunization which increased significantly ($p < 0.05$) post booster dose and maintained until 30 days post primary immunization. This was in contrast with the very low titre level in the untreated NVC control fish confirming that the CNPs-IV had successfully activated the specific humoral response in the immunized fish which was accompanied by gradual production of anti-VHSV immunoglobulin (Ig) in response to the inactivated virus antigen. During the challenge period, the anti-VHSV inhibition percentage increased in all the groups including NVC control fishes at 48 hpc and 96 hpc, however the percentage level was significantly ($p < 0.05$) higher (> 80%) in the immunized groups than the NVC control group (~40%). The high antibody titre level is consistent with the RPS% in the vaccinated fish and can be correlated with several previous findings wherein, they reported chitosan NPs as vaccine carrier was efficient in eliciting fish humoral response for production of antibodies against respective antigen of interest [22–26,33]. Further, the similar level of antibody response in both the immunized groups i.e. CNPs-IV (immersion/immersion) and CNPs-IV (immersion/oral) substantiates the fact that oral route is equally efficient in delivery of the constructed CNPs-IV vaccine but needs pre-stimulation or repeated boosting.

Besides, enhancing the serum antibody responses in the systemic immune compartment of the fish, the encapsulated vaccine was able to induce immune response locally at mucosal sites (delivery route specific) viz., skin (Fig. 3B) and gut (Fig. 3C) of CNPs-IV (immersion/immersion) and CNPs-IV (immersion/oral) fish group respectively. The results demonstrated highest increase in antibody titre in the skin mucus of CNPs-IV (immersion/immersion) fish at 48 hpbv unlike serum antibody response where highest titre value recorded post challenge, indicating production of cutaneous antibody by the skin associated lymphoid tissue (SALT) [38] in response to the CNPs-IV post immersion vaccination. Furthermore, the antibody (anti-VHSV) titre of around

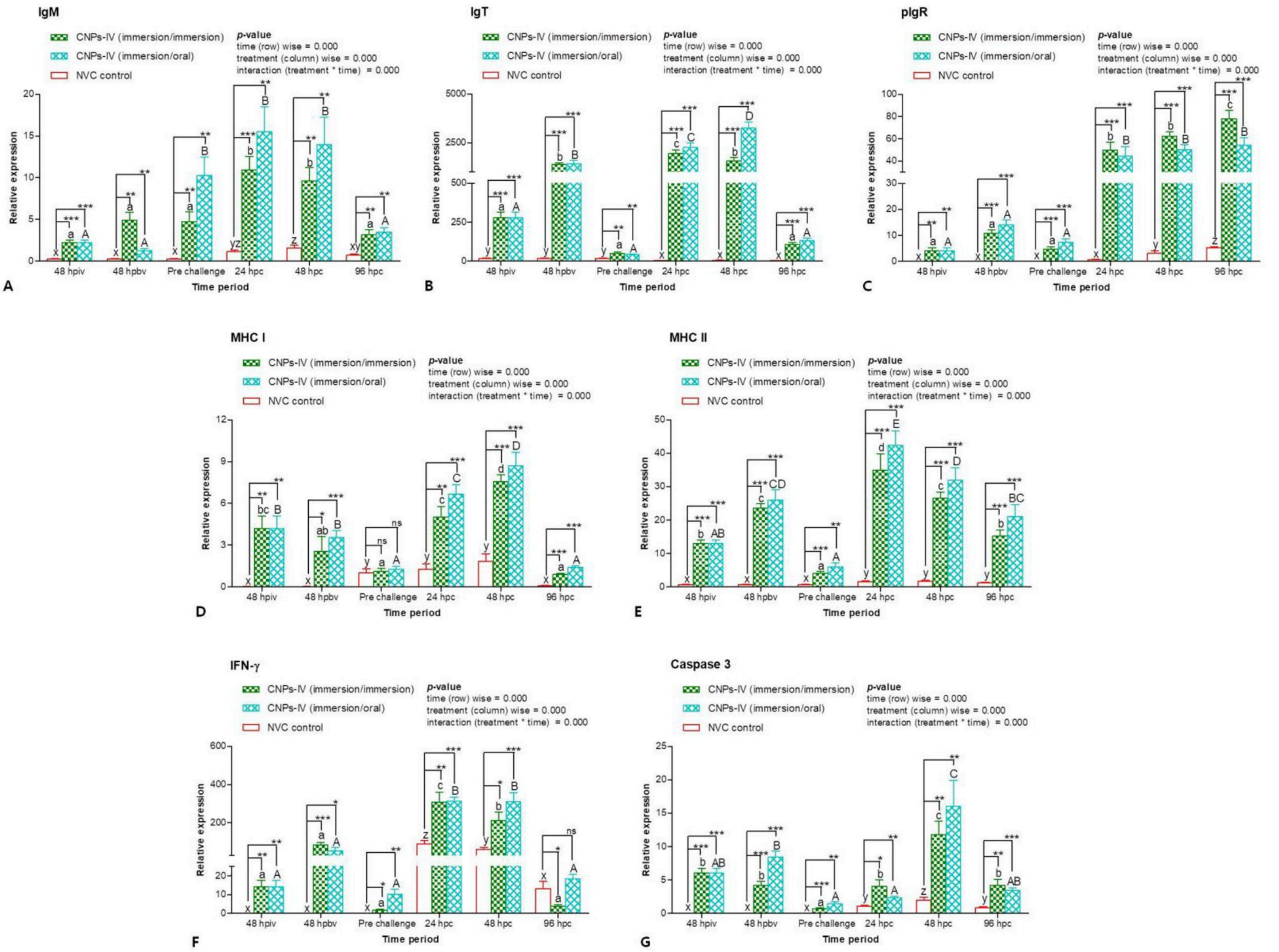


Fig. 4. Relative expression analysis of immune genes in anterior kidney tissue of olive flounder of different experimental group viz., CNPs-IV (immersion/immersion), CNPs-IV (immersion/oral), and NVC control groups. Expression levels of each gene were compared among the experimental groups relative to the naive control and the mean (n = 5) relative expression of IgM (A), IgT (B), pIgR (C), MHC I (D), MHC II (E), IFN-γ (F), and Caspase 3 (G) were plotted with standard error at different time intervals post immunization and post challenge with virulent VHSV (10^6 $TCID_{50}$ virus/fish). A two-way ANOVA was performed with each of the data set. The *p*-values within the group (time wise), between the group (treatment wise) and for interaction effect are evaluated to determine statistical significance ($p < 0.05$). Duncan multiple range test (homogenous subsets indicated by lowercase (x–z) for NVC control, lowercase (a–d) for CNPs-IV (immersion/immersion) and uppercase (A–E) for CNPs-IV (immersion/oral) groups) within the groups (time-wise) and an unpaired *t*-test (indicated by asterisks * for significance level or 'ns' for non significance) between the groups at different time points were also performed to analyze the statistical differences. (hpiv – h post initial vaccination; hpbv – h post booster vaccination). The gene expression values for NVC control are identical to our previously published paper [31] as both the experiments were run simultaneously with same NVC control and naive control groups.

50% PI in the skin mucus of all the experimental fish groups, including non-vaccinated control fish, at all time points stipulates the fact that teleostean skin mucus contain some innate non-specific natural antibodies which provides them with a barrier against viral invasion in normal condition [39,40]. In contrast, the antibody responses in the intestinal mucus derived from the gut associated lymphoid tissue (GALT) in naive untreated fish was ~20% indicating that their activity remains in subdued state normally but can be activated remarkably with oral vaccination as was evident from the stable anti-VHSV antibody titre level (~60%) in the gut mucus of CNPs-IV (immersion/oral) fish group post booster immunization as well as post challenge with VHSV. This higher antibody titre in the CNPs-IV (immersion/oral) group again validates the fact that CNPs-IV vaccine was able to reach the second gut segment without denaturation in the acidic stomach environment as well as cross the mucosal barriers to gain access to antigen presenting cells (APCs) for induction of local and/or systemic responses [41,42]. The present results can be correlated with the previous findings wherein they reported increment of antibody titre in the gut mucosa in Atlantic salmon after oral immunization with alginate NPs encapsulated IPNV antigens [43]. Thus, from the specific antibody response analysis in sera, skin and gut mucus, it can be inferred that unlike injection vaccine where only systemic immune response is elicited for generation of protective immunity in the host fish, administration of the encapsulated vaccine by immersion or oral routes stimulates both the systemic and mucosal immune responses thereby endowing a wider protective armoury to the fish against virus infection.

3.4. Expression kinetics of immune-related genes

Acquired or adaptive immunity is the basis of vaccination as it creates immunological memory after an initial exposure to a specific antigen, in our case the CNPs encapsulated 'IV' antigen, which leads to an enhanced response on subsequent encounters with the same antigen. In fishes, head kidney is regarded as the primary immune organ responsible for the initiation of adaptive immunity as it is the place of origin for both B- and T-lymphocytes as well as maturation of B-cells

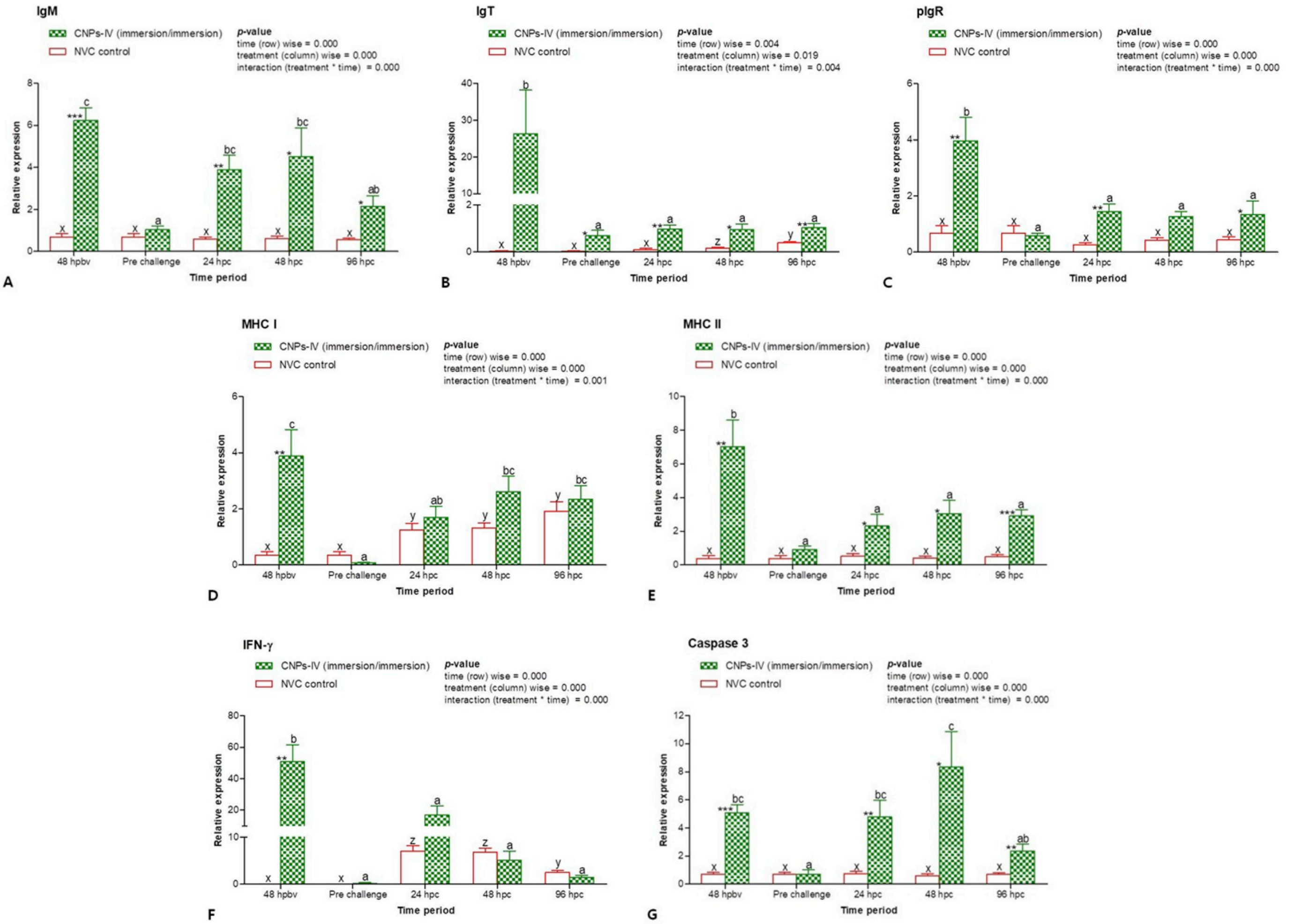


Fig. 5. Relative expression analysis of immune genes in skin tissue of olive flounder of CNPs-IV (immersion/immersion), and NVC control groups. Expression levels of each gene were compared among the experimental groups relative to the naive control and the mean (n = 5) relative expression of IgM (A), IgT (B), pIgR (C), MHC I (D), MHC II (E), IFN-γ (F) and Caspase 3 (G) were plotted with standard error at different time intervals post booster immunization and post challenge with virulent VHSV (10^6 $TCID_{50}$ virus/fish). A two-way ANOVA was performed with each of the data set. The *p*-values within the group (time wise), between the group (treatment wise) and for interaction effect are evaluated to determine statistical significance ($p < 0.05$). Duncan multiple range test (homogenous subsets indicated by lowercase (x–z) for NVC control, and (a–c) for CNPs-IV (immersion/immersion) groups) within the groups (time-wise) and an unpaired *t*-test (indicated by asterisks * for significance level) between the groups at different time points were also performed to analyze the statistical differences. (hpbv – h post booster vaccination). The gene expression values for NVC control are identical to our previously published paper [31] as both the experiments were run simultaneously with same NVC control and naive control groups.

[44,45] whereas skin and gut mucosa forms the main induction sites for antigen uptake and subsequent mucosal immunity [46]. Thus in order to understand the immunological basis of the CNPs-IV vaccine efficacy against VHSV, the present study included a comparative expression kinetics of different classes of immune-related genes in the systemic (head kidney) and mucosal (skin and intestine) compartment of the experimental fish at post immunization and post challenge period.

Immunoglobulins (Ig) expressed either as B-lymphocyte receptor or secreted in plasma and mucus, are the key humoral component of the teleost adaptive immune system [46–48]. Among the three Ig isotypes characterized so far in fishes, IgM is most abundantly found in the systemic circulation [49] whereas IgT has more prominence in the mucosal compartments [50]. Alongside, the polymeric IgR (pIgR), which is reported to be strongly expressed in the skin epithelial cells, gut enterocytes and hepatocytes, binds with the Ig molecules and transcytose them to the immune reaction site [46]. Our present results demonstrated significant upregulation in the gene transcripts of both IgM (Figs. 4A, 5A and 6A), IgT (Figs. 4B, 5B and 6B), and their receptor, pIgR (Figs. 4C, 5C and 6C), post booster dose in head kidney, skin and intestine of the immunized fish. This can be correlated with the efficiency of the CNPs-IV mucosal vaccine in substantiating production of Ig secreting cells in both the systemic and mucosal immune compartment of the recipient fish and triggering the antibody dependent humoral responses. The results are consistent with the production of specific antibodies, as observed in the cELISA results, and are also in accordance with several previous studies wherein, they reported nanoencapsulated mucosal vaccine resulted in high expression of Ig molecules [33,51–53]. It is noteworthy to mention here that comparatively very high expression of IgT (> 2000 folds change) in head kidney at 48 hpbv and 24 and 48 hpc against the IgM expression (< 15 folds change) at all time points, was mainly due to very low basal expression of IgT (Ct value ~37) in the naive control fish. Further, the contrasting expression pattern of these 3 genes in the skin (significantly high expression post booster but lower response post challenge) compared to intestine (significantly higher transcripts level at 48 hpbv and post challenge) signifies that the CNPs-IV vaccine positively evokes immune responses at both the mucosal sites but due to the applied challenge route (intra peritoneal injection), the skin's immune responses minimizes post challenge, as most of the immune reaction gets concentrated at systemic compartment to combat the virulent virus.

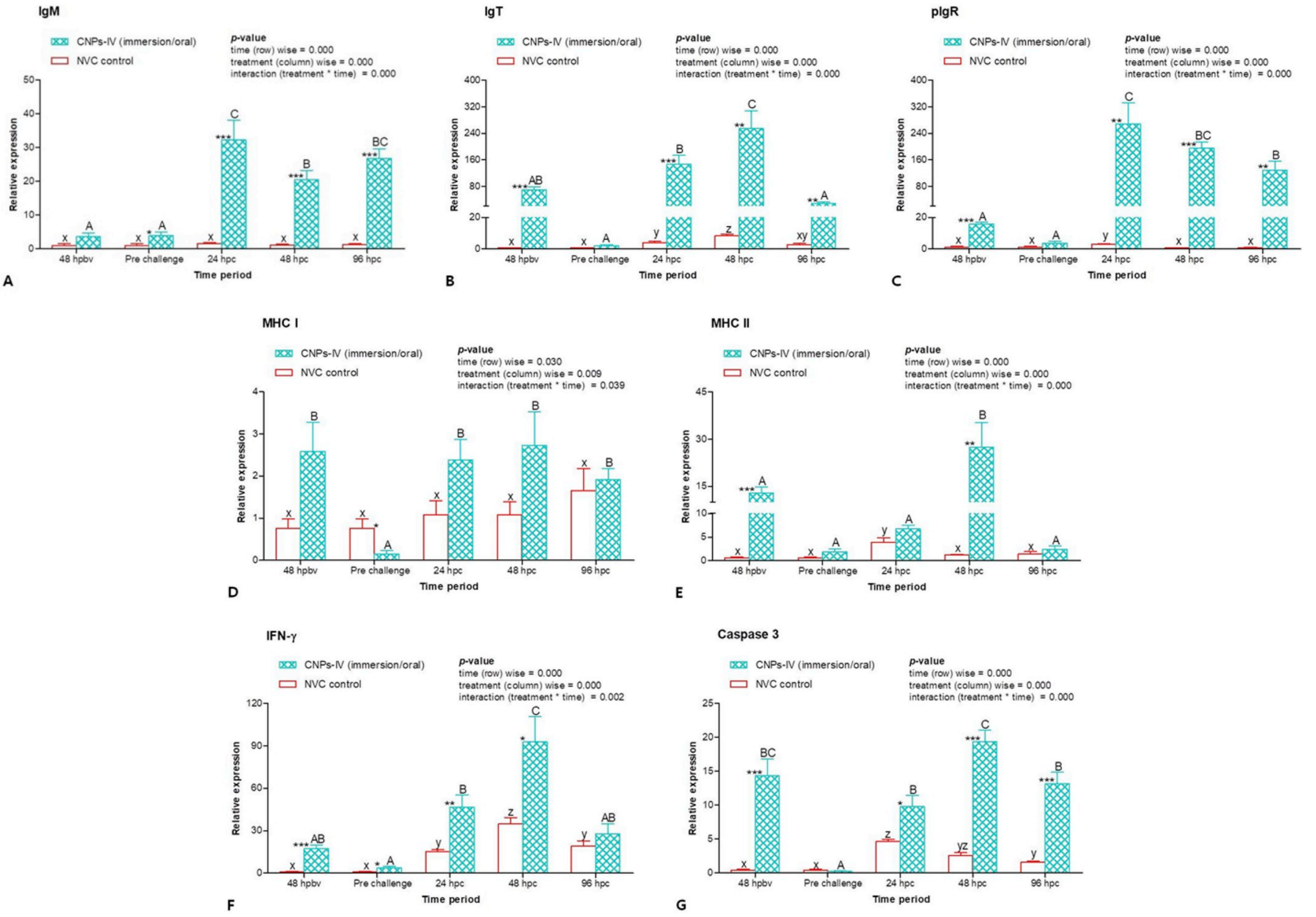


Fig. 6. Relative expression analysis of immune genes in intestine tissue of olive flounder of CNPs-IV (immersion/oral), and NVC control groups. Expression levels of each gene were compared among the experimental groups relative to the naive control and the mean (n = 5) relative expression of IgM (A), IgT (B), pIgR (C), MHC I (D), MHC II (E), IFN-γ (F) and Caspase 3 (G) were plotted with standard error at different time intervals post booster immunization and post challenge with virulent VHSV (10^6 $TCID_{50}$ virus/fish). A two-way ANOVA was performed with each of the data set. The *p*-values within the group (time wise), between the group (treatment wise) and for interaction effect are evaluated to determine statistical significance ($p < 0.05$). Duncan multiple range test (homogenous subsets indicated by lowercase (x–z) for NVC control, and uppercase (A–C) for CNPs-IV (immersion/oral) groups) within the groups (time-wise) and an unpaired *t*-test (indicated by asterisks * for significance level) between the groups at different time points were also performed to analyze the statistical differences. (hpbv – h post booster vaccination). The gene expression values for NVC control are identical to our previously published paper [31] as both the experiments were run simultaneously with same NVC control and naive control groups.

The fish adaptive immune responses being centred around the functional relevance of B- and T-lymphocytes in both the systemic and mucosal immune compartments, which depends on antigen presentation by major histocompatibility complex (MHC) marker present on antigen presenting cells (APC) [46,47,54,55], as such, the expression kinetics of these marker molecules can help us to evaluate the CNPs-IV vaccine efficacy. In our study, there was significant increase in transcripts level of both MHC I (Figs. 4D, 5D and 6D) and MHC II (Figs. 4E, 5E and 6E) in all the 3 tissues (head kidney, skin and intestine) of vaccinated fish, post immunization as well as post challenge compared to the NVC control group. The upregulation of MHC-I and MHC-II in head kidney of both the immunized groups as well as in skin and intestine of respective route-dependent immunized fish groups, revealed that the CNPs-IV vaccine was efficiently processed and presented by MHC receptors, which might ultimately elicited the activation of MHC-II–CD4-B-cell proliferation and MHC-I–CD8-T-cell mediated cytotoxic immune responses in both the systemic and mucosal sites of the immunized fish.

In addition, the present study investigated the expression dynamic of IFN-γ gene, which is an important gene for viral vaccine potency [56–58]. The IFN-γ gene transcript showed significant upregulation post booster immunization with nanovaccines in the analyzed tissues (Figs. 4F, 5F and 6F). Among the two mucosal sites, the skin showed slightly more IFN activity than intestine whereas in the head kidney both the immunized fish groups showed similar expression level of IFN-γ. As expected, the expression level peaked after challenge in the kidney of the vaccinated fish reflecting a quick and transient antiviral mechanism inside the host which ultimately resulted in the host survival among the vaccinated groups. Similar upregulation of IFN-γ have been shown in trout, vaccinated by nanoencapsulated viral (DNA) vaccines [34,56,57], thereby validating the efficiency of the CNPs-IV based mucosal vaccination strategy in inducing host IFN response against VHSV. Interestingly, the IFN-γ responses post challenge in the skin (relatively lower expression) and intestine showed variation which may be due the site of host's antiviral activity against VHSV.

Apart from IFN mediated immune responses, apoptosis constitutes an integral part of antiviral immune defense mechanism. An early and effective apoptotic response can cease viral replication and disease development in the host [59,60]. Apoptosis inducers activate different caspases, and subsequently lead to the activation of caspase 3, the final executioner of apoptosis [61]. In view of this, the present study also analyzed the expression kinetics of caspase 3 gene (Figs. 4G, 5G and

6G). Our results demonstrated high induction of caspase 3 at 48 hpc in all the analyzed tissue of both the vaccinated groups compared to non vaccinated control fish. The caspase 3 expression pattern was similar with the MHC-I expression suggesting a rapid T-cell response causing cytotoxicity in the host which ultimately limits viral proliferation intime leading to its survival.

In conclusion, our study displayed that efficiency of the adopted immunization strategy involving encapsulation of inactivated VHSV antigen in chitosan NPs (CNPs-IV) and administration via mucosal routes in prime-boost method in order to develop protective antiviral immunity in olive flounder against VHSV. The high RPS values observed in the present experimental trial-II along with specific antibody response and positive expression of immune genes in both the systemic and mucosal immune compartments suggests that nanoencapsulation of 'IV' offered protection to the antigen against degradation thereby facilitating its non-stressful but effective administration to the target immune organs and henceforth enhanced the overall efficiency of the constructed vaccine. However, our findings suggest that there is a further scope to improve the efficacy of the vaccine using a suitable adjuvant as an additional supplement to CNPs-IV, in order to ensure better penetration and transport of the vaccine to the targeted immune organs.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.05.017.

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