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PLGA encapsulated inactivated-viral vaccine: Formulation and evaluation of its protective efficacy against viral haemorrhagic septicaemia virus (VHSV) infection in olive flounder (*Paralichthys olivaceus*) vaccinated by mucosal delivery routes

Sajal Kole [a], Syed Shariq Nazir Qadiri [a], Su-Mi Shin [a], Wi-Sik Kim [a], Jehee Lee [b], Sung-Ju Jung [a,*]

[a] Department of Aqualife Medicine, Chonnam National University, Republic of Korea
[b] Department of Marine Life Science, Jeju National University, Republic of Korea

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ABSTRACT

Viral haemorrhagic septicaemia virus (VHSV), an OIE listed viral pathogen, is the etiological agent of a contagious disease, causing huge economic losses in farmed olive flounder (*Paralichthys olivaceus*) and significant mortalities among several other marine fish species in Korea, Japan, and China. In continuation with our previous work, where injection vaccination with inactivated VHSV mixed with squalene (as adjuvant) conferred higher protective immunity to olive flounder, the present study focused on replacing the injection route of vaccine delivery by immersion/oral route to overcome the limitations of the parenteral immunization method. Here, we encapsulated the inactivated VHSV vaccine with PLGA (poly lactic-co-glycolic acid) nanoparticles (PNPs-IV) and evaluated its ability to induce protective immunity in olive flounder (12.5 ± 1.5 g) by initially immunizing the fishes by immersion route followed by a booster with the same dose two weeks later with half of the fish through immersion route and other half through oral route (incorporated into fish feed). Cumulative mortalities post-challenge (1×10^6 $TCID_{50}$ virus/fish) with virulent VHSV-isolate, were lower in vaccinated fish and RPS of 60% and 73.3% were obtained for PNPs-IV (immersion/immersion) and PNPs-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, PNPs-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 controls, in both the systemic (kidney) and mucosal (skin and intestine) immune compartment of the host post immunization as well as post challenge. Thus it can be inferred that the adopted immunization strategy efficiently protected and transported the inactivated viral antigen to target immune organs and positively stimulated the protective immune response against VHSV in olive flounder.

1. Introduction

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 affects more than 70 species of both freshwater and marine fishes worldwide [1]. In Korea, the olive flounder aquaculture industry has been severely affected by VHS since 2001, inflicting huge economic losses in the flounder farms [2]. The disease mainly occurs in late winter and spring season when the water temperature revolves around 8–15 °C causing 50–70% mortalities in all age-groups of flounder [2,3]. The infected fish frequently exhibits dark colouration, ascites and haemorrhages on external body surface, along with internal pathognomonic signs viz., congested liver and enlargement of spleen and kidney during the disease outbreaks [3]. Purposively, various immunization strategies have been adopted for more than three decades, including killed vaccines, attenuated live vaccines, recombinant vaccine in prokaryotic and eukaryotic expression systems, and DNA-based vaccines amongst which DNA vaccines has proven to confer good protection against VHS [4]. However, with the legal restriction to the use of DNA vaccine in food fishes in most of the countries

* Corresponding author at: Department of Aqualife Medicine, Chonnam National University, 50, Daehak-ro, Yeosu 59626, Republic of Korea.
E-mail address: sungju@chonnam.ac.kr (S.-J. Jung).

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including Korea, development of vaccine based on empirical formulation is essential, which can elicit strong and long-lasting immune response as well as be suitable for farmed fishes.

Previously we developed an injection based inactivated vaccine viz., formalin-inactivated VHSV mixed with squalene as adjuvant which was effective in eliciting protective immunity (58–76% RPS) against VHSV in advanced fingerling of olive flounder [5]. However, this method of immunization is stressful, labour-intensive and strategically not suitable for small-sized fingerlings. Vaccine delivery by immersion/oral route can serve as a better alternative to overcome these limitations, as both the routes are non-stressful to small-sized fish, but it does require comparatively higher amount of antigen [6]. Moreover, naked formalin inactivated antigen either degrades in the *in vivo* environment when delivered through oral route or fails to reach the immune cells when administered through immersion route, which results in an unsuccessful immune induction in the host fish. Nanoencapsulation is a suitable intervention to address the issues regarding efficient vaccine administration as it permits a sustained or pulsed release of encapsulated antigen thereby minimizing the requirements of antigen dosage and repeated administration in ensuring long-term protection [7].

Among various polymers used for nanoencapsulation, PLGA (Poly (D,L-lactic-co-glycolic)-acid), a synthetic polymer, is widely used for vaccine delivery in mammals and other biomedical research due to its excellent tissue compatibility, biodegradability, non-toxic nature and its approval by the Food and Drug Administration (FDA) for safe use in human [8,9]. In the recent past, most of the studies have investigated suitability of PLGA as DNA vaccine carrier or recombinant protein carrier [7,10–14] but their use in carrying an inactivated virus antigen in fish is limited so far except one study by Munang'andu et al. [15] where they encapsulated inactivated infectious pancreatic necrosis virus (IPNV) vaccine for administration in Atlantic salmon. To this cause, the present study was undertaken to formulate nanoencapsulated inactivated vaccine against VHSV using PLGA nanoparticles and administered to the fishes initially by immersion route followed by a booster with the same dose two weeks later with half of the fish immunized by immersion route and other half through oral route (incorporated into fish feed) in order to evaluate its ability *per se* in carrying the antigen to the host immune cells and induce protective immunity in olive flounder against VHSV.

2. Material and methods

2.1. Experimental animals

Olive flounder (*Paralichthys olivaceus*) fingerlings (12.5 ± 1.5 g) procured from a local hatchery were disinfected with 50 ppm formalin and acclimatized in our indoor rearing 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. Water quality of the tanks was maintained by continuous flow-through system. The water temperature and pH were maintained at 19–20 °C and 8–8.5, respectively. Ten randomly selected fish were sacrificed for confirmation of their pathogen-free status (detailed in Supplementary file 1). Further, absence of VHSV was confirmed by nested polymerase chain reaction (PCR) [16].

2.2. Virus culture and preparation of virus antigen – formalin inactivation

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 concentration of 0.01 multiplicity of infection (m.o.i). Cells with virus were harvested when the cytopathic effects (CPE) were apparent. 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). 230 mL of harvested virus having titre value of 10^8 $TCID_{50}$/mL was inactivated by adding 0.3% formalin followed by stirring for 24 h at 4 °C on a magnetic stirrer. Complete inactivation was confirmed by re-inoculation on FHM cells. The inactivated virus, designated as 'IV' was concentrated by ultracentrifugation at 30000 rpm for 2 h at 4 °C using ultracentrifuge (Hitachi, Japan). Aliquots of concentrated IV antigen for initial and booster immunization for two vaccinated fish groups were pooled accordingly and suspended in 1 mL of sterile distilled water followed by storage at 4 °C until nanoencapsulation.

2.3. Encapsulation of IV antigen in PLGA nanoparticles (NPs)

PLGA NPs containing IV antigen were formulated using a double emulsion-solvent evaporation technique as described by Delgado et al. [17] with slight modification. Briefly, aliquots of 1 mL of aqueous solution of pooled IV antigen (W_1) and 500 mg of PLGA (L:G = 50:50, M_W 30–60 kDa) (Sigma-Aldrich, USA) in 10 mL dichloromethane (Sigma-Aldrich, USA) organic solvent (5% w/v) was emulsified (100 μL of Span-80 was used as emulsifier) by homogenization for 2 min followed by gentle vortexing to form water-in-oil primary emulsion (W_1/O). The resultant primary emulsion (W_1/O) was further emulsified in 50 mL of aqueous PVA (poly vinyl alcohol) solution (5% w/v) to form water-in-oil-in-water (W_1/O/W_2) emulsion. Nanospheres were prepared by homogenization of the W_1/O/W_2 emulsion for 5 min. After homogenization, the emulsion was kept overnight on magnetic stirrer at 25 °C to allow evaporation of the organic solvent. The PLGA encapsulated IV nanospheres (PNPs-IV) were recovered by centrifugation at 5000*g* for 30 min at 4 °C followed by three times washing (centrifugation at 5000*g* for 10 min at 4 °C) with distilled water. The isolated final product was stored at 4 °C.

2.4. Determination of size of blank PLGA NPs

The blank PLGA NPs (without IV antigen) was characterized in terms of size and size distribution by dynamic light scattering (DLS) using Zetasizer S-90 Malvern instruments (Malvern, UK).

2.5. Nanospheres characterization

The morphology and size distribution of PNPs-IV nanospheres was evaluated by transmission electron microscopy (TEM) (Hitachi, Japan). (Detailed procedure is attached in Supplementary file 1).

2.6. Vaccine preparation for immunization

The PNPs-IV nanospheres prepared for primary and booster immersion immunization were suspended in 20 mL of distilled water prior to vaccination. Whereas, for oral booster vaccination, PNPs-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. To minimize leaching of vaccine in water, 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. The detailed vaccine dose information is attached in Supplementary file 1.

2.7. Experimental design for immunization trial

Olive flounder fingerlings (n = 240, n = number of fish) (12.5 ± 1.5 g) were randomly distributed into three treatment groups with 60 fish in each group and reared in 500 L FRP (fibre reinforced plastic) flow-through tanks supplied with UV-treated seawater maintained at 20 °C. The groups were designated as PNPs-IV (immersion/immersion), PNPs-IV (immersion/oral), NVC control group (non-vaccinated challenged group) and remaining 60 fish were kept as naive group (without any treatment or challenge). For initial immunization (pictorial details of experimental design in Supplementary file 2), fishes from both the PNPs-IV (immersion/immersion), and PNPs-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 PNPs-IV vaccine solution for 2 h with vigorous aeration and transferred back to the original tank after immersion. After 14 days post initial immunization (300° days), booster dose was administered to the PNPs-IV (immersion/immersion) group, same as initial immunization, whereas, the PNPs-IV (immersion/oral) group was fed with the prepared feed containing PNPs-IV nanospheres (as described above) for 2 consecutive days (twice/day) at the same time while as normal commercialized feed was given to the other groups.

2.8. Challenge study

For challenge study, 45 fish (16.5 ± 0.8 g) from each of the 4 experimental groups viz., PNPs-IV (immersion/immersion), PNPs-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 intra-peritoneally injected with 100 μL of VHSV (10^6 - $TCID_{50}$ virus/fish) strain homologous to the vaccine strain while 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 [18]; Relative percentage of survival (RPS) = {1 − (% Mortality in vaccinated group/%Mortality in control group)} × 100.

2.9. Sampling

Post vaccination, five fish from each group (immunized groups, NVC control group and naive group) were randomly selected for sampling at 48 h post initial vaccination and 48 h post booster vaccination and before challenge (30 d post initial immunization). Blood serum and anterior kidney samples were collected at each time point for further analysis of immune parameters. Skin (portion from caudal peduncle site) and skin mucus were collected at same time point from PNPs-IV (immersion/immersion), NVC control and naive groups while intestine (representative section from foregut, midgut and hindgut) and intestinal mucus were collected from PNPs-IV (immersion/oral), NVC control and naive groups at 48 h post booster vaccination and before challenge (30 d post initial immunization). While after challenge, from the remaining aquaria (apart from RPS analysis) in each group (including naive group) 5 fish per time-point were randomly sampled for blood serum and anterior kidney sample for all groups at 24 h, 48 h and 96 h post challenge (hpc). Skin (portion from caudal peduncle site) was collected at same time point from PNPs-IV (immersion/immersion), NVC control and naive groups and intestine (representative section from foregut, midgut and hindgut) were collected from PNPs-IV (immersion/oral), NVC control and naive groups. Skin mucus and intestinal mucus were collected at 48 hpc from PNPs-IV (immersion/immersion) and PNPs-IV (immersion/oral) groups respectively as well as from NVC control group.

2.10. Collection of skin and intestinal mucus

Skin mucus samples 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. For collection of gut mucus, fishes were dissected aseptically and the whole intestine was excised. Although it is practically impossible to isolate gut mucus totally free of faecal matter, however, care was taken to remove the gut contents by gently squeezing the intestine and afterwards collecting the mucus to avoid contamination of the samples. Gut mucus was collected in microcentrifuge tube, containing 200 μL of sterile PBS with PMSF (1 mM), by gently squeezing the empty gut of the sampled fish using sterile forceps. Both the mucus samples were then vortexed for 1 min, centrifuged at 2000*g* for 15 min at 4 °C followed by storing the supernatants at −20 °C for further analysis.

2.11. 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 competitive enzyme-linked immunosorbent assays (c-ELISA), as described according to previously published protocol by Kole et al. [19] with required modifications. VHSV (10^8 $TCID_{50}$/mL) diluted in coating buffer (carbonate – bicarbonate buffer, pH 9.6) was used as antigen. Serum samples (dilution at 1:200 in 1% BSA in PBS-T), skin mucus and intestinal mucus (undiluted supernatants as mentioned above) were used as test samples whereas, commercially available monoclonal antibody (MAb) raised in mice against glycoprotein (G) of VHSV (Enbiogene, Korea) (1:200 dilution in 1% BSA in PBS-T) was used as competitive antibody (detailed procedure is attached in Supplementary file 1).

2.12. Immune gene expression

2.12.1. RNA isolation and cDNA synthesis

Total RNA was extracted from the collected anterior kidney, intestine and skin samples 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.

2.12.2. Quantitative expression analysis of immune genes in experimental samples

Gene-specific primers for immune-related genes were designed using Primer3Plus based on available sequences from NCBI database are listed in Supplementary file 1. For gene expression analysis, real-time PCR was carried out in an Exicycler™ 96 Real-Time Quantitative Thermal Block (Bioneer, Korea) using SYBR Green AccuPower® PCR PreMix (Bioneer). Relative quantification of

immune gene was estimated using $2^{-\Delta\Delta Ct}$ method [20] whereas the viral mRNA was quantified as described in our previous study [21]. Detailed real-time PCR method is attached in Supplementary file 1.

2.13. Statistical analysis

Kaplan–Meier curves and log rank test were carried out using GraphPad Prism5 Software for survival analysis of the immunization trial. 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 analysed 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, carried-out by Duncan's multiple range tests, and an unpaired *t*-test were 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 PLGA NPs

PLGA NPs has gained importance in targeted drug delivery and drug transport, and has proven to be very successful in providing effective protection to the antigen against degradation as well as eliciting long lasting immunity in the host fish [7,10–13]. The most frequently applied method for antigen loading, in particular for DNA vaccine or subunit proteins, is the double-emulsion solvent-evaporation technique, yielding smaller size spherical particles, which are ideally suited for vaccine delivery in fishes via immersion or oral routes [22]. In the current study, we attempted to employ similar emulsification technique in order to encapsulate whole inactivated VHSV (IV) into the PLGA NPs. The present TEM analysis result of the PNPs-IV nanospheres (Fig. 1B) confirmed that there was positive encapsulation of 'IV' in PLGA NPs, which yielded smooth and rounded nanospheres of regular and uniform dimension having electron dense centre. The central electron dense region of the PNPs-IV indicates that the IV antigen was positively encapsulated inside PNPs which could help the IV antigen to remain stable in hostile environmental conditions. It is to be mentioned here that dichloromethane (DCM) used in the encapsulation process may alter the envelope or antigenic structure of the viral particle but nonetheless the results obtained are conclusive that viral particle was intact enough to elicit protective immune response in the host.

Along with positive encapsulation, the physiological parameters especially the size of the NPs, the zeta potential or the charge of the particle and the complexation efficiency with antigen are very important attributes for efficient gene delivery [23]. For better control over the encapsulated antigen dose, its releasing behaviour and biocompatibility with cells and tissues of the host, the size of NPs is critically important [24]. Although NPs are considered to be in nanodimensions (diameters <100 nm size), the size ranging between 100 nm and 500 nm is accepted in the field of drug delivery wherein sufficient amount of drug loading is needed [25,26]. In the present study, the mean particle size of blank PNPs was 206.3 nm (Fig. 1A) whereas the mean nanospheres (PNPs-IV) size was approximately 450–500 nm as measured from the TEM image (Fig. 1B). Previous studies by Kanchan et al. [27] and Rauta et al. [28] reported that antigen loaded PLGA NPs ranging between 200 and 600 nm were efficiently taken up by macrophages initiating the downstream immune responses. The present NPs size falls within the reported size range which could possibly assist in easy delivery of the PNPs encapsulated viral antigen to the host immune cells.

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

In continuation to our previous study [5], we tested the efficacy of inactivated viral particles delivered by oral and immersion routes which failed to provide any protection (0% RPS) to the fish, and this prompted us to nanoencapsulate the IV vaccine to check its protective efficacy. Thereafter, we tried different delivery strategies to evaluate the protective efficacy of the PNPs-IV vaccine viz., primary immersion (46.7% RPS), primary oral (13.3% RPS), prime-

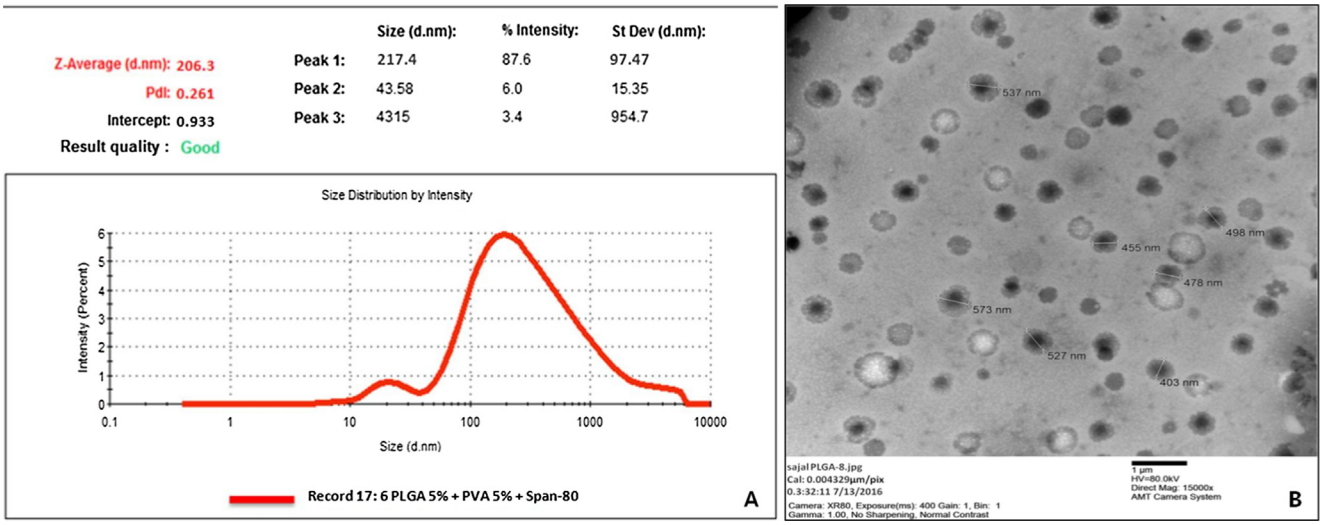

Fig. 1. PLGA nanoparticles characterization; (A) Particle size distribution of blank PLGA nanoparticles (PNPs) by dynamic light scattering (DLS) using Zetasizer S-90 Malvern instruments; (B) Transmission electron micrograph of IV-loaded PLGA nanospheres (PNPs-IV) showing smooth and spherical nanospheres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

boost immersion/immersion (56.7% RPS) and prime-boost oral/oral (23.3% RPS) [data not shown] but they did not provide sufficient protection in terms of RPS (>50%) except prime-boost immersion/immersion strategy. Taking cue from positive results obtained by immersion vaccination in the aforementioned pilot study, the further work was focused on immersion vaccination as primary immunization route followed by booster with both immersion (prime-boost immersion/immersion) and oral (prime-boost immersion/oral) routes in order to investigate the protective efficacy of both the immunization strategies. Interestingly, the modified strategy proved to be effective as is supported by the results from the challenge study (Fig. 2A), which showed PNPs-IV (immersion/oral) group yielding higher RPS of 73.3% (although not statistically significant) than the PNPs-IV (immersion/immersion) group (60% RPS), when compared with 100% cumulative mortality percentage in the NVC control group. The RPS data for the nanoencapsulated vaccines was similar to our previous study with injection of squalene adjuvant-IV vaccine (58–73% RPS) [5], which is quite encouraging as injection route of immunization is known to be more precise method to deliver antigen for immune induction in the host suggesting that PNPs encapsulated IV vaccine can be delivered successfully by mucosal route and can be a promising alternative for injection vaccine. In addition, the viral mRNA copy number in the kidney of olive flounder post challenge with VHSV (Fig. 2B) demonstrated that both the immunized fish groups 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.

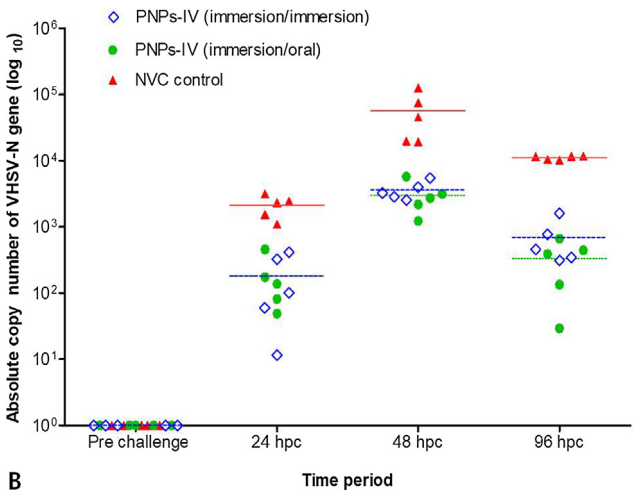

Fig. 2B. Absolute copy number of VHSV in 50 ng total RNA from anterior kidney tissues (n = 5, n is number of fish sampled per time point) of different experimental groups viz., PNPs-IV (immersion/immersion) (◇), PNPs-IV (immersion/oral) (●), and NVC control (▲) groups, plotted against time after challenged with virulent VHSV (10^6 $TCID_{50}$ virus/fish) reared at 15 °C.

3.3. Specific antibody quantification

Alongside the RPS analysis, determination of specific antibody titre constitutes an important aspect for evaluation of fish vaccine efficacy. We employed competitive ELISA to minimize background and get specific OD values for anti-VHSV antibody titre. Present results for antibody titre in serum (Fig. 3A) demonstrated an increasing trend in both the PNPs-IV vaccinated fish groups, starting from minimum titre value after primary immunization, and afterwards significant ($p < 0.05$) increase in titre was observed following booster dose in comparison to very low response in the non-vaccinated control fish. This can be correlated with gradual production of anti-VHSV immunoglobulin (Ig) in the immunized fish in response to the inactivated virus antigen, activating the components of humoral immune response. 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 although the exact functional aspects of the produced antibodies in preventing establishment of pathological VHSV disease is not clear which needs a detailed study. Nevertheless, the serum antibody response are in accordance with previous findings, wherein, they reported PLGA encapsulated vaccines was successful in producing antibodies against respective antigen of interest [7,10,12,14,15]. Further the results also displayed no significant difference between the two vaccinated groups indicating that both the mucosal route of vaccine delivery were equally capable in inducing the systemic immune responses.

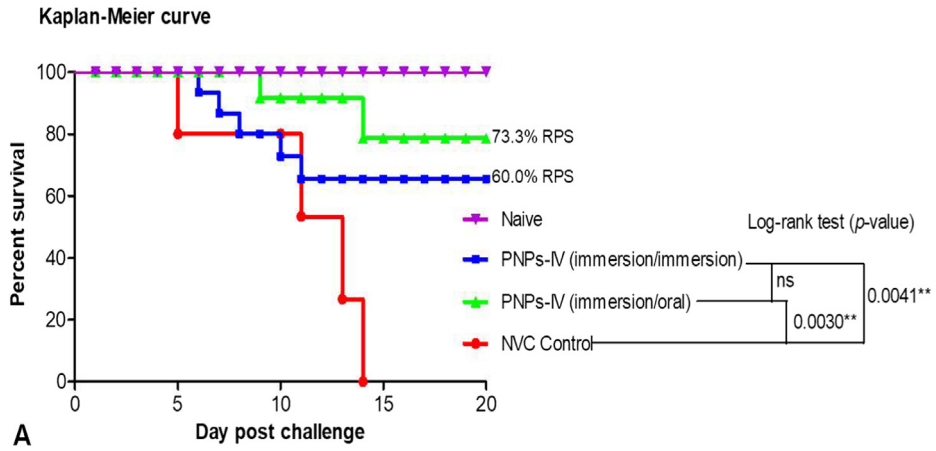

Fig. 2A. Kaplan-Meier curve showing cumulative mortality and relative percentage survival (RPS) analysis of different experimental groups viz., PNPs-IV (immersion/immersion), PNPs-IV (immersion/oral), and NVC control groups of olive flounder (n = 30, 2 tanks with 15 fish/tank for each groups) challenged 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 0.0041 and 0.0030 with PNPs-IV (immersion/immersion) and PNPs-IV (immersion/oral) groups, respectively. No significant difference (ns) was observed between the two vaccinated fish groups.

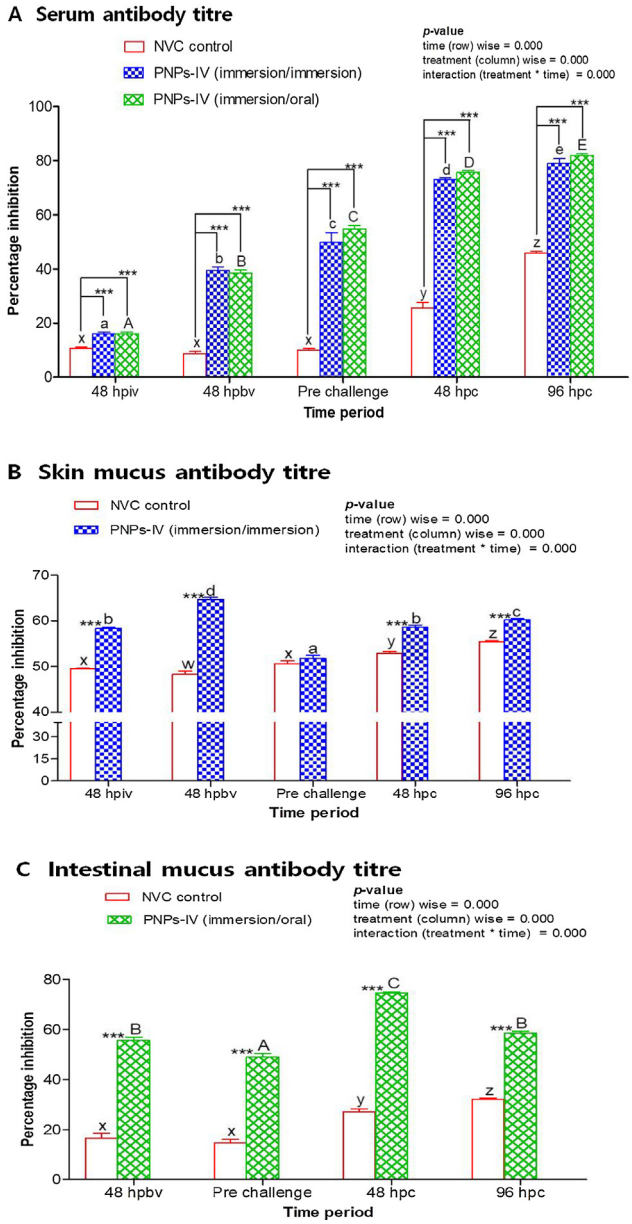

Fig. 3. Percentage inhibitions (PI) of specific antibody against VHSV were determined by competitive ELISA using anti-VHSV-G MAbs. Percentage inhibition of anti-VHSV antibody binding to VHSV antigen, present (A) in the fish serum of different experimental group viz., PNPs-IV (immersion/immersion), PNPs-IV (immersion/oral) and NVC control groups; (B) in the skin mucus of PNPs-IV (immersion/immersion) and NVC control groups; and (C) in the intestinal mucus of PNPs-IV (immersion/oral) and NVC control 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 PNPs-IV (immersion/immersion) and uppercase (A-E) for PNPs-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).

Apart from inducing systemic (serum antibody) immune response, 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 PNPs-IV (immersion/immersion) and PNPs-IV (immersion/oral) fish group respectively. The results displayed highest increase in antibody activity in the skin mucus of PNPs-IV (immersion/immersion) fish at 48 h post booster immersion unlike serum antibody response where highest titre value recorded post challenge. This can be explained by the fact that immersion vaccination activates skin associated lymphoid tissue (SALT) which in turn produces cutaneous antibody [29] resulting in increment of antibody titre in skin mucus post vaccination. Moreover, the higher antibody titre (~50% PI) in the skin mucus in all the experimental fish groups including non-vaccinated control fish at all time points indicates non-specific viral neutralising property of fish skin mucus that might help in evading viral attachment and entry in normal circumstances [30]. However, a further detailed study in this regard is warranted to explore the antiviral humoral activity in the skin mucus. Likewise, boosting with oral nanovaccine in the PNPs-IV (immersion/oral) group induced the gut associated lymphoid tissue (GALT) which is evident from the present result as more than 40% increase in the anti-VHSV antibody titre level over and above the titre value of naive fish was observed. This higher antibody titre in the gut of experimentally immunized fish compared to non-vaccinated control fishes further validates the fact that PNPs encapsulation provided stability to the inactivated antigen against enzymatic degradation in the gut, and we presume that it ultimately helps in transport and presentation of the antigen to the macrophage and lymphoid cell population, present in the intestinal lamina propria, and subsequent establishment of immunological memory for antibody production against the viral antigen as has been reported previously [31]. The antibody responses against VHSV in the immunized fish can be correlated with a recent study by Yun et al. [32] wherein they reported protective immune response in both the systemic and mucosal compartment after administration of PLGA microparticles-encapsulated formalin killed *Aeromonas hydrophila* in *Cyprinus carpio*.

3.4. Expression kinetics of immune-related genes

The expression kinetics of different classes of immune-related genes were studied in anterior kidney, skin and intestine of experimental fish at post immunization and post challenge period in order to get a better picture of host immune dynamics in stimulation of systemic and mucosal immune pathways after vaccination. Consistent with the production of specific antibodies during the immunization process, the immunoglobulins (Ig) viz., IgM (Fig. 4A) and IgT (Fig. 4B) and their receptor polymeric IgR (pIgR) (Fig. 4C) gene transcripts were upregulated significantly ($p < 0.05$) in anterior kidney of the immunized fish after booster immunization irrespective of vaccine delivery route. The Ig molecules are regarded as indicator of specific humoral immune responses which are key parameters for evaluation of vaccine potency in instigating adaptive immunity against viral pathogens in fish [33,34]. To mention here, relatively very high expression of IgT (>2000 folds change) at 48 h post booster and 24 and 48 hpc in compared to the IgM (<10 folds change) at all time points, was mainly due to very low basal expression of IgT (Ct value ~37) in the naive control fish. It is presumed that the differential expression kinetics of the Ig molecules and their receptor, between the vaccinated and non vaccinated fish groups, possibly initiates the humoral responses in immunized fish. The finding is well supported by previous studies wherein oral/immersion delivery of nanovaccine in fish resulted in high expression of Ig molecules [35–38]. Prior to the Ig response, fish adaptive immune response consists of activation of B- and T-lymphocytes which again depends on antigen presentation by major histocompatibility complex (MHC) marker present on antigen presenting cells (APC) [39,40]. Our results exhibits significant increase in tran-

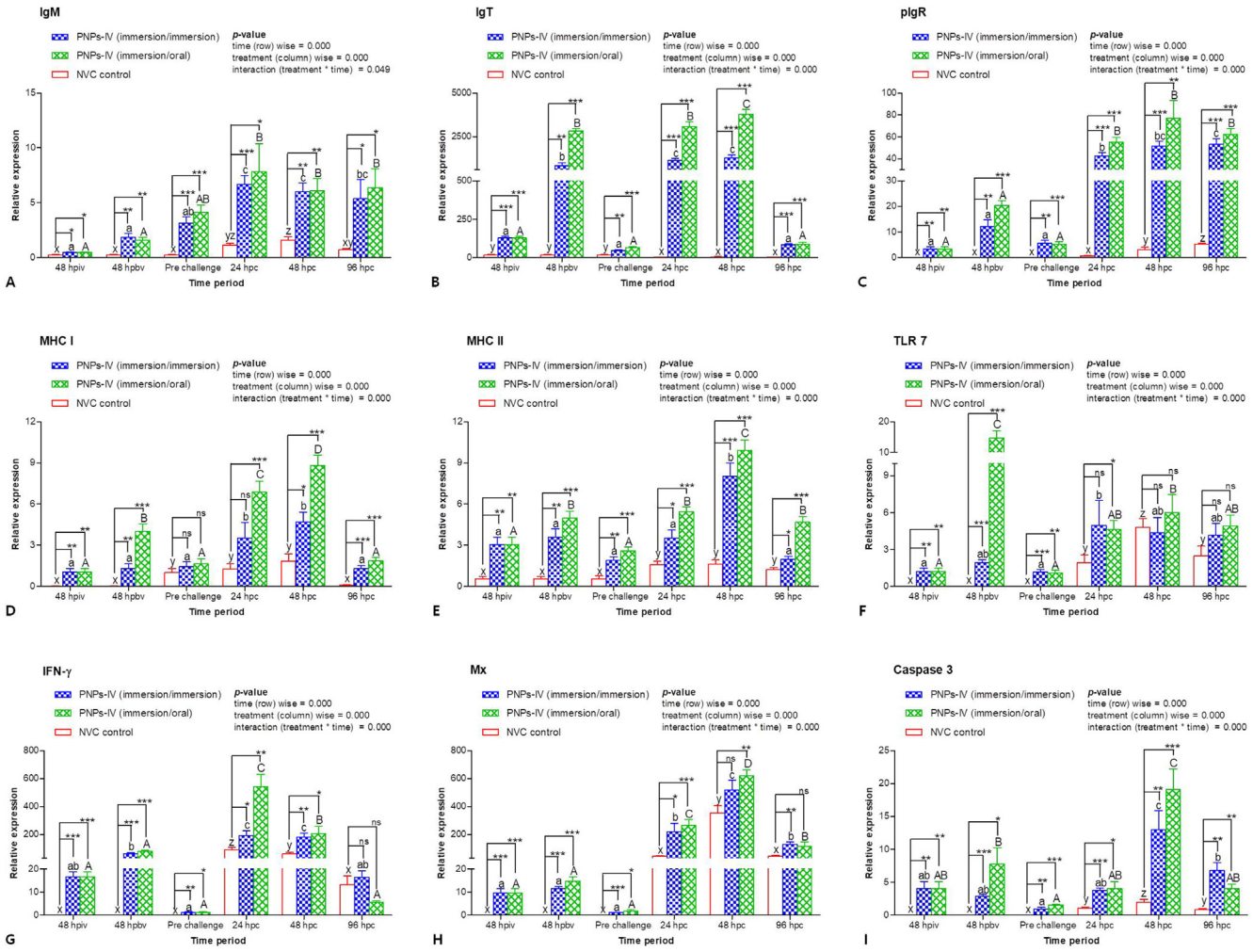

Fig. 4. Relative expression analysis of immune genes in anterior kidney tissue of olive flounder of different experimental group viz., PNPs-IV (immersion/immersion), PNPs-IV (immersion/oral), and NVC control groups. Expression levels of each gene were compared among the experimental groups relative to the naive control and the mean (n = 5) relative expression of IgM (A), IgT (B), pIgR (C), MHC I (D), MHC II (E), TLR 7 (F), IFN-γ (G), Mx (H) and Caspase 3 (I) 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-c) for PNPs-IV (immersion/immersion) and uppercase (A-D) for PNPs-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).

scripts level of both MHC I (Fig. 4D) and MHC II (Fig. 4E) post immunization as well as post challenge compared to the NVC control and it seems that PNPs-IV vaccine was efficiently processed and epitopes presented by MHC receptors, which ultimately elicited the activation of MHC-II–CD4-B-cell proliferation and MHC-I–CD8-T-cell mediated cytotoxic immune responses. However, clarification to this assumption warrants further study. In addition, the present study investigated the expression profiles of IFN-γ and Mx genes, which are key genes with respect to antiviral mechanism [41–43]. Both IFN-γ (Fig. 4G) and Mx (Fig. 4H) genes showed significant upregulation post immunization with the nanovaccine. The expression level of both the genes peaked after challenge in the vaccinated fish reflecting a quick and transient antiviral mechanism leading to host survival among the vaccinated groups. Apart from IFN-mediated immune responses, PRR-mediated host defenses are also important for early antigen recognition and induction of different downstream antiviral pathways [21,44]. Among different PRRs, TLR7 (of TLR family) is responsible for viral-derived ssRNA antigen recognition. Our results showed significantly high TLR7 (Fig. 4F) transcript level in the vaccinated fish group compared to NVC control fish post booster vaccination thereby underlining successful recognition and subsequent binding of the encapsulated antigen by the receptor. It is pertinent to mention here that both Mx and TLR7 genes are part of the innate immune response unlike Ig and MHC molecules of adaptive immunity, as evident by their similar expression levels in both the vaccinated and non-vaccinated fish post VHSV challenge. Further, the study also included the expression kinetics of caspase3 gene, the final executioner of apoptosis [45]. The high induction of caspase 3 (Fig. 4I) at 48 hpc in both the vaccinated groups compared to non vaccinated control fish can be correlated with MHC-I expression in the vaccinated fish indicating a rapid T-cell mediated cytotoxic reaction upon encountering the virus ultimately limiting viral proliferation.

Like the systemic immune response, the PNPs-IV immunization strategy was found to be effective in stimulation of mucosal immune responses as was evident from the significant upregulation of most of the aforementioned immune gene transcripts in the skin (Fig. 5) and intestine (Fig. 6) of PNPs-IV (immersion/immersion) and PNPs-IV (immersion/oral) fish groups respectively. Among the two different mucosal surfaces, the oral route seems to be more efficient in ensuring positive uptake of the antigen and

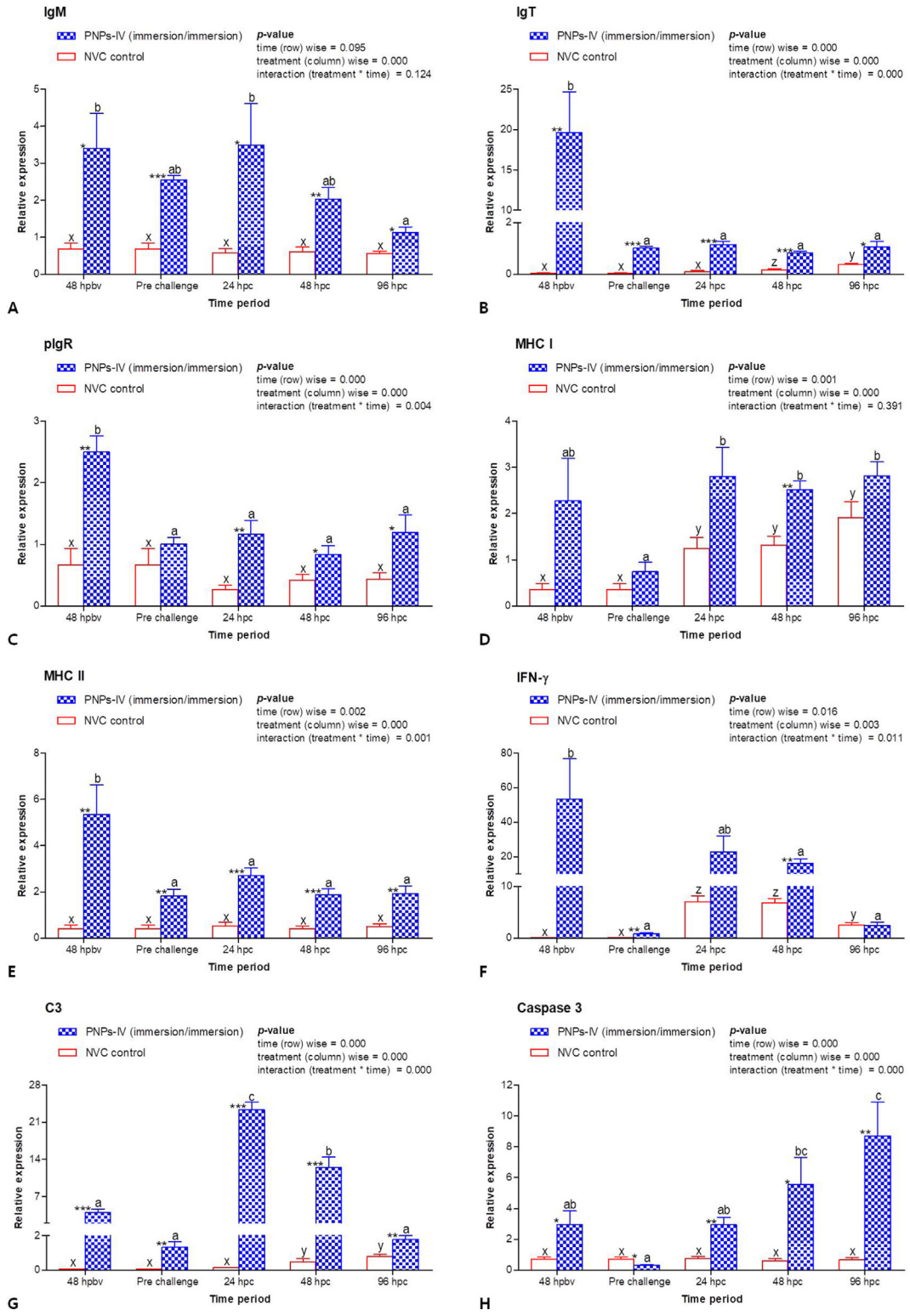

Fig. 5. Relative expression analysis of immune genes in skin tissue of olive flounder of PNPs-IV (immersion/immersion), and NVC control groups. Expression levels of each gene were compared among the experimental groups relative to the naive control and the mean (n = 5) relative expression of IgM (A), IgT (B), pIgR (C), MHC I (D), MHC II (E), IFN-γ (F), C3 (G) and Caspase 3 (H) 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 PNPs-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).

subsequent stimulation of the mucosa associated lymphoid tissue (MALT) than the immersion route. Previous findings have also reported similar observations wherein oral vaccination of nanoencapsulated viral antigen positively activates the MALT along with systemic immune organs [32,35,36]. Furthermore, the contrasting expression pattern of the immune genes viz., IgM, IgT, pIgR,

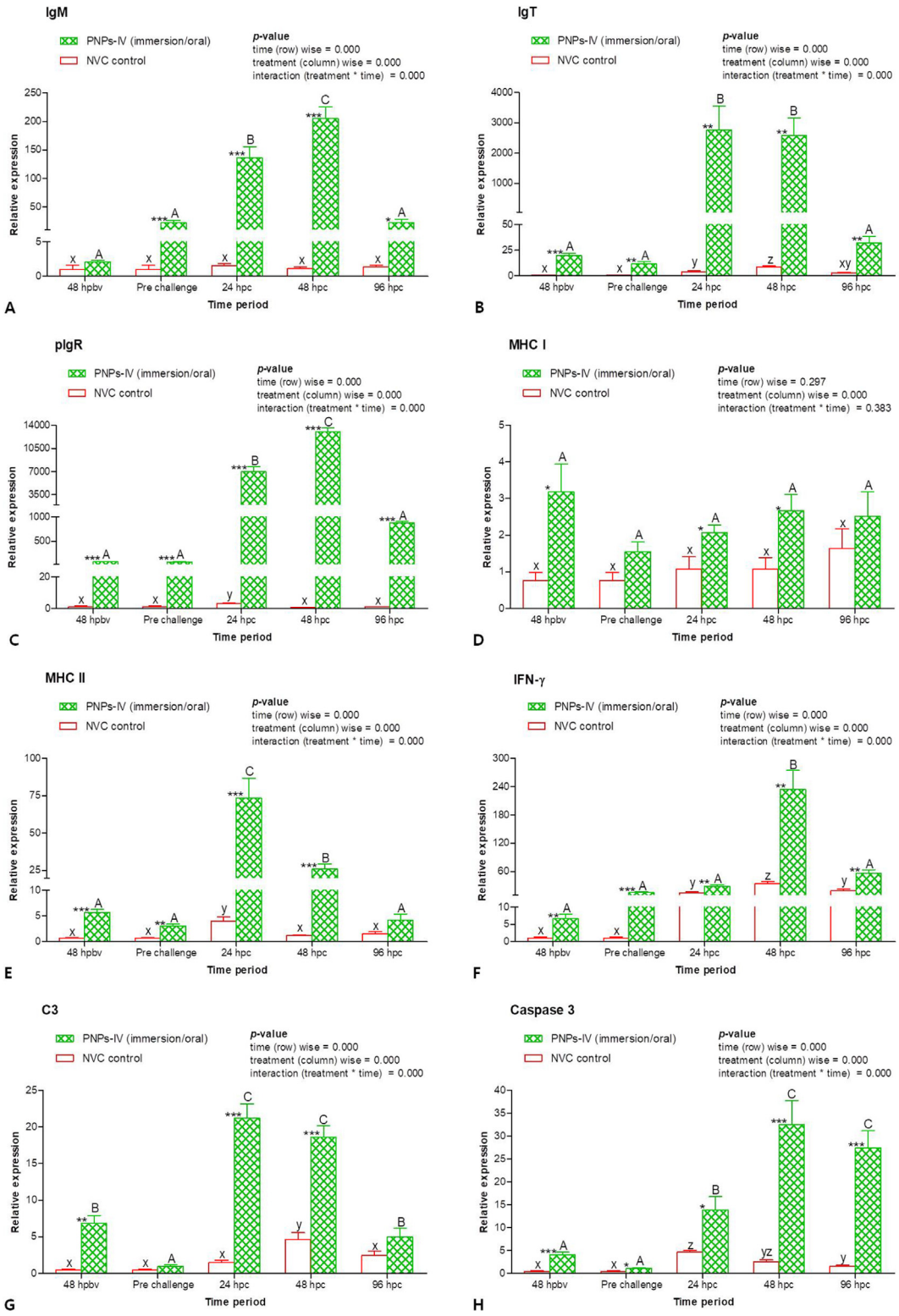

Fig. 6. Relative expression analysis of immune genes in intestine tissue of olive flounder of PNPs-IV (immersion/oral), and NVC control groups. Expression levels of each gene were compared among the experimental groups relative to the naive control and the mean (n = 5) relative expression of IgM (A), IgT (B), pIgR (C), MHC I (D), MHC II (E), IFN-γ (F), C3 (G) and Caspase 3 (H) 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 PNPs-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).

MHC-II, IFN-γ, in the skin compared with intestine, as reflected by significantly high expression of immune genes in skin at 48 hpbv and lower response post challenge, but significantly higher transcripts level of immune genes in intestine at 48 hpbv and post challenge, signifies that the PNPs-IV vaccine positively evokes immune responses at both the mucosal sites. As the applied chal-

lenge route was intra peritoneal injection, therefore most of the immune reaction gets concentrated at systemic compartment to combat the virulent virus, which might have minimized the post-challenge immune response in the skin. Thus, the differential gene expression kinetics in the systemic and mucosal compartments of the immunized and non-immunized control fish suggests that the host's self immune machinery was primed due to vaccination and was well prepared to control viral proliferation in-time leading to its survival, whereas, the NVC control fish failed to combat the virus and ultimately succumbed to death due to non priming and absence of any immunological memory.

In conclusion, our study revealed that PLGA encapsulated inactivated VHSV antigen (PNPs-IV) delivered by mucosal routes is an effective immunisation strategy for protection against VHS disease in olive flounder. Nanoencapsulation of 'IV' protected the antigen against degradation and facilitated its non-stressful administration unlike the injection route and henceforth enhanced the overall vaccine efficiency. The present study provides insights and baseline information about the protective efficacy of PNPs-IV vaccine and our findings suggest that there is a further scope to improve the efficacy of the vaccine using an adjuvant-based approach in order to ensure better penetration and transport of the vaccine to the targeted immune organs. Our study is unique and of significance in the sense that it shows positive correlation between vaccine administration and mucosal immune responses thereby paving the way for future research in the area of fish mucosal viral vaccines. Taken together, it can be inferred that the adopted immunization strategy efficiently protects and transports the inactivated viral antigen to target immune organs and confers a broader protection to the fish against virus invasion, colonization and establishment of infection.

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Declarations of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2018.12.063.

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