

*Int. J. Aquat. Biol.* (2016) 4(5): 353-359  
ISSN: 2322-5270; P-ISSN: 2383-0956  
Journal homepage: [www.ij-aquaticbiology.com](http://www.ij-aquaticbiology.com)  
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## Original Article

# Transcription of adaptive-immune genes upon challenge with infectious pancreatic necrosis virus (IPNV) in DNA vaccinated rainbow trout (*Oncorhynchus mykiss*)

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**Abstract:** In the present study, rainbow trout weighting  $3\pm 0.3$  g were vaccinated with an oral DNA vaccine encoding VP2 gene of a prevalent isolate of IPNV in Iranian trout farms encapsulated in sodium alginate microspheres and Chitosan tripolyphosphate (CS/TPP) nanoparticles. The vaccinated fish were then challenged with a virulent isolate of IPNV at 30 days post-vaccination. The transcriptional changes of adaptive-immune genes (IgM and IgT), as well as the VP4 gene of IPNV, as an indicators of viral replication were studied 45 days post-challenge. Analysis of RT-qPCR data showed lower levels of VP4 gene expression in the oral DNA vaccinated trout after IPNV challenge compared with the control one. Moreover, the constructed DNA vaccine did not enhance the expression of IgM and IgT genes above the levels observed in the carrier control group but it showed a mimic of viral activity and contributes to maintaining them at appreciable levels in vaccinated group.

### Article history:

Received 2 July 2016

Accepted 21 September 2016

Available online 25 October 2016

### Keywords:

DNA Vaccine

IPNV

IgT

IgM

VP4 gene

## Introduction

Infectious pancreatic necrosis (IPN) is an important viral disease of both salmonid and non-salmonid fishes caused by infectious pancreatic necrosis Virus (IPNV) belonging to the family *Birnaviridae*, and genus *Aquabirnavirus* (Dobos, 1977; Reno, 1999; Rodriguez Saint-Jean et al., 1991). This virus contains a bisegmented genome (A and B) of double-stranded RNA with segment A being larger (approximately 3.1 kbp) encoding VP2 that the most of viral neutralizing epitopes are mapped to it (Tarrab et al., 1993; Frost et al., 1995; Hill and Way, 1995; Fridholm et al., 2007). IPNV infects both fry and juveniles of Atlantic salmon, brook trout and rainbow trout resulting in high morbidity and mortality (Evensen, 2008). The surviving fish may become asymptomatic carriers shedding virus via urine, feces and reproductive products (Rodriguez Saint-Jean et al., 1991; Johansen and Sommer, 1995).

After many researches still need to an appropriate vaccine against IPNV with a long lasting protection especially at an early age and preventing the carrier formation is crucial. However, different antigen delivery systems, including live, inactivated whole virus, fusion protein, subunit, virus-like particles, and intramuscular DNA vaccines have been investigated (Munang'andu and Evensen, 2015). Oral DNA vaccines are considered as a new strategy and ideal way to immunize large numbers of small fish against IPNV (Hølvold et al., 2014). DNA vaccines are plasmid DNAs encoding specific proteins that can be expressed in cells of an inoculated host, inducing strong and long-lasting humoral and cellular immune responses. Intramuscular injection is an effective common route of DNA vaccination in fishes. Also, some antigen-encapsulation methods have been approved for oral delivery of such vaccines (Bozkir and Saka, 2004; de las Heras et al., 2010). However, the mechanisms

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responsible for protection of this delivery system are not completely clarified.

Iran is one of the leading countries in trout farming which its production remarkably decreased due to viral disease outbreaks such as IPN (Soltani et al., 2015). In the present study, we assessed transcriptional changes of adaptive-immune genes (IgM and IgT), as well as the VP4 gene of IPNV in vaccinated rainbow trout with an oral DNA vaccine encoding VP2 gene of a prevalent isolate of IPNV in Iranian trout farms encapsulated in sodium alginate microspheres and chitosan tripolyphosphate (CS/TPP) nanoparticles. Hence, the trout were challenged with a virulent IPNV at 30 days post-vaccination and then the transcript change of mentioned genes measured in the survived fish at 45 days after challenge.

## Materials and Methods

### ***Construction and preparation of the DNA vaccine:***

The VP2 gene of a prevalent isolate of IPNV in Iranian trout farms, cultured in CHSE-214 cell line, was cloned into a eukaryotic expression vector pcDNA3.1 (Invitrogen, USA) under the control of the immediate-early cytomegalovirus (CMV) promoter, yielding pcDNA3.1-VP2. The pcDNA3.1-VP2 was verified using Hind III and Xho I endonuclease analysis and the recombinant plasmid was then amplified in *Escherichia coli* (TOP10), and the cells were grown in LB broth with ampicillin (Fig. 1). The plasmid DNA was isolated with the Endofree Plasmid Mega purification Kit (Qiagen, USA) according to the manufacturer's instructions. The DNA concentration was measured in a spectrophotometer (NanoDrop 2000, Thermo scientific, Spain) before it was aliquoted, and stored at -20°C until use.

### ***Encapsulation of DNA vaccine (pcDNA3.1-VP2):***

The pcDNA3.1-VP2 and pcDNA3.1 plasmids were encapsulated in sodium alginate microspheres and CS/TPP nanoparticles as described by Bozkir and Saka (2004) and de las Heras et al. (2010), and lyophilized and stored at 4°C. Briefly, an equal volume of heated CS/TPP (prepared by ionic

gelation process; Vimal et al., 2012) and pcDNA3.1-VP2 solutions was quickly mixed together and vortexed at 2500 rpm for 30 s. The pcDNA3.1-VP2 loaded CS/TPP particles were separated by centrifugation at 20,000 rpm for 30 min at 10°C.

For preparation of microspheres, 1.5 mL of pcDNA-VP2 (1 mg ml<sup>-1</sup>) were mixed with 2.5 mL of sodium alginate (3% w/v), then the mixture after stirrer (10 min at 500 rpm) was emulsified (30 min at 900 rpm) with Span 80 (0.5 mL) and paraffin oil (100 mL). A volume of 2.5 ml of 0.15M CaCl<sub>2</sub> was then added to emulsion and stirred (900 rpm for 2 hrs). Microspheres were collected by centrifugation (10 min at 1000 g) and washed with ethanol (70%). The unbound pcDNA-VP2 content in the supernatant was quantified by UV spectrophotometer at 260 nm. The encapsulation efficiency (EE) was calculated using the following equation:  

$$EE = [(Total\ amount\ of\ pcDNA3.1-VP2 - Free\ amount\ of\ pcDNA3.1-VP2\ in\ supernatant) / Total\ amount\ of\ pcDNA3.1-VP2] \times 100$$

***Fish vaccination and IPNV challenge:*** Rainbow trout weighing 3 g were orally immunized with feed pellets at 5% of body weight containing alginate microspheres and CS/TPP nanoparticles loaded with pcDNA3.1-VP2 (named Alg-pcDNAVP2 and CS/TPP-pcDNAVP2, respectively) in separate trial groups (each group 90 fish) at temperature 15°C for 90 days.

Two groups of rainbow trout were immunized with vaccine impregnated feed pellets (containing alginate microspheres loaded with 10 and 25 µg of pcDNA3.1-VP2, and boosted 15 days later with the same amount of the plasmid. In control group, fish orally immunized with alginate microspheres containing 10 µg of the empty-plasmid (pcDNA3.1) and boosted 15 days later with the same amount of empty-plasmid. Furthermore, a same experiment was performed via CS/TPP nanoparticles loaded with pcDNA3.1-VP2 in three separate groups. Unvaccinated fish were used as negative controls. At 30 days post vaccination, two subgroups of 15 fish from each group were challenged by IP injection of 0.2 ml/ fish with IPNV at a concentration of 10<sup>7</sup>

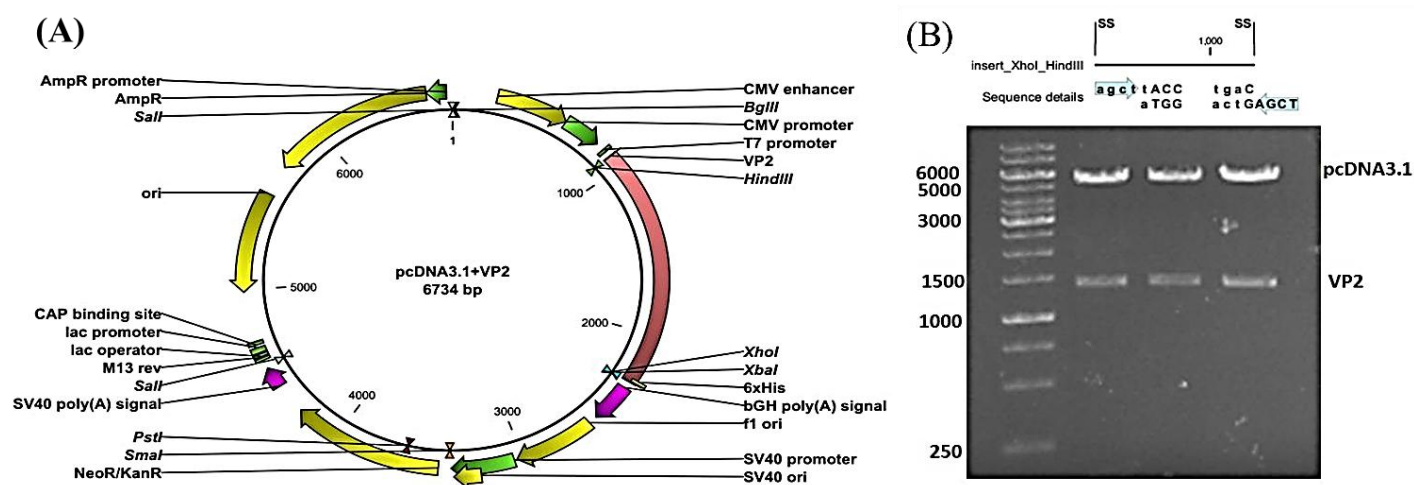


Figure 1. (A) Diagram of the pcDNA3.1 vector encoding VP2 genomic fragment of IPNV, and (B) the agarose gel (1%) of pcDNA-VP2 digested by Hind III and Xho I enzymes.

Table 1. The primers used for the real-time quantitative RT-PCR.

Genes	Acc. No	FW-Primer (5'-3')	Rev-Primer (5'-3')
VP4	M18049.1	AGGAGATGACATGTGCTACACCG	CCAGCGAATATTTTCTCCACCA
IgM	X65263.1	ACCTTAACCAGCCGAAAGGG	TGTCCATTGCTCCAGTCC
IgT	AY870265	AGCACCAGGGTCAAACCA	GCGGTGGGTTTCAGAGTCA
EF-1 $\alpha$	AF498320	GATCCAGAAGGAGGTCACCA	TTACGTTTCGACCTTCCATCC

TCID<sub>50</sub> mL<sup>-1</sup>. At day 45 post-challenge when fish did not exhibit clinical symptoms, three trout from each group were sacrificed by overexposure to clove oil, spleen and head kidney were removed for assessing of transcript change of IgT, IgM and IPNV-VP4 genes.

**Transcriptional changes of IgM, IgT and IPNV-VP4 genes:** RNA was extracted from 20 mg of the homogenized head kidney and spleen samples using Exgene<sup>TM</sup> Viral DNA/RNA kit (GeneAll, Korea), and then cDNA was synthesis was carried out in a total volume of 25  $\mu$ l from 5  $\mu$ l of extracted RNA using HyperScript<sup>TM</sup> First strand Synthesis Kit (GeneAll, Korea) according to the manufacturer instruction.

Relative expression of adaptive-immune genes (IgM and IgT), as well as the VP4 gene of IPNV, as an indicator of viral replication, were quantified in duplicate for each cDNA sample on the Real-Time PCR detection system (Applied Biosystems) using SYBR Green qPCR Master Mix. Each reaction containing 12.5  $\mu$ l 2xSYBR Green PCR Master Mix,

200 nM of each primer 100 ng cDNA template and nuclease-free water up to final volume of 25  $\mu$ l were incubated for 15 min at 95°C (1 cycle), followed by 40 cycles at 95°C for 15 s, 59°C for 1 min.

The melting curve of each amplicon was examined, and the expression of the target genes was corrected based on the endogenous control expression (*EF-1  $\alpha$* ) and calculated as fold change in relative expression according to the 2<sup>- $\Delta\Delta$ Ct</sup> method (Livak and Schmittgen, 2001). Primers are listed in Table 1.

**Statistics analysis:** Data were statistically analyzed by one-way analysis of variance (ANOVA) using SPSS package (SPSS 1998). Differences were considered statistically significant at  $P < 0.05$ .

## Results

The encapsulation efficiency of alginate sodium and CS/TPP to encapsulate pcDNA3.1-VP2 were determined and the results revealed a high encapsulation efficiencies with 87.8% and 84.2% of DNA binding with alginate sodium microspheres

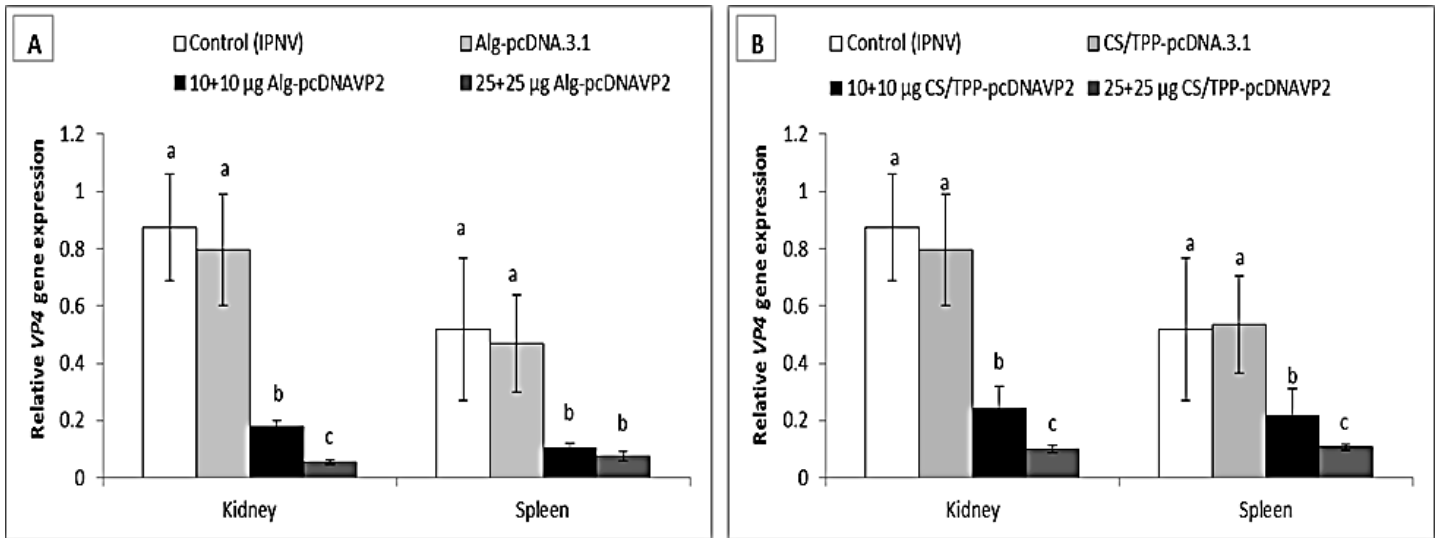


Figure 2. Relative expression of the IPNV-VP4 gene in the kidney and spleen of vaccinated and IPNV infected trout. Rainbow trout (n=90) orally immunized with alginate microspheres (A) and CS-TPP nanoparticles (B) containing 10 and 25  $\mu\text{g}$  of pcDNA3.1-VP2 and boosted 15 days later with the same amount of vaccine. Fish orally immunized with alginate microspheres (A) or CS-TPP nanoparticles (B) containing 10  $\mu\text{g}$  of the empty-plasmid and boosted 15 days later with the same amount of empty-plasmid, and unvaccinated fish were used as negative controls. At 30 days post-vaccination two subgroups of 15 fish from each group were challenged by IP injection 0.2 ml/fish with IPNV at a concentration of  $10^7$  TCID<sub>50</sub> mL<sup>-1</sup>. Transcription of the VP4 gene was recorded in the kidney and spleen of both vaccinated and virus control fish at 45 days post-challenge. Data are represented relative to EF1-a expression ( $2^{-\Delta\Delta\text{CT}}$  method; n=3). Different letters indicate significant differences between groups at  $P < 0.05$ .

and CS/TPP nanoparticles, respectively. Furthermore, distribution of DNA vaccine in different tissues of vaccinated fish was determined by RT-PCR amplification of VP2 gene at 30 days post vaccination (data not shown).

**IPNV-VP4 gene transcription:** The relative expression of VP4 gene was determined to detect the rate of IPNV replication or the viral load (Fig. 2). VP4 expression in the virus control group showed stronger expression in the kidney as approximately 15 and 9 times greater than the infected fish that were vaccinated with 25  $\mu\text{g}$  Alg-pcDNAVP2 and 25  $\mu\text{g}$  CS/TPP-pcDNAVP2, respectively. Moreover, VP4 expression in vaccinated fish with 25  $\mu\text{g}$  of vaccines was around 3 times greater than 10  $\mu\text{g}$  fish treatments. The results showed a significantly lower levels of the virus in the vaccinated fish 45 days post-challenge ( $P < 0.05$ ).

**Adaptive immune-related genes (IgM and IgT) transcription:** In five days post challenging fish with IPNV, the levels of IgM and IgT genes expression were examined in the kidney and spleen of the vaccinated fish (10 and 25  $\mu\text{g}$  dose of pcDNA3.1-VP2) and the results are showed in

Figure 3. Transcript of IgM and IgT genes in the virus control group was greater than vaccinated fish (around 2 times). However, the expression patterns were similar. Hence, the oral DNA vaccine did not increase the transcript of IgT and IgM genes above the levels showed in the virus control, but it showed a mimic of viral activity and was kept them at appreciable levels in all vaccinated groups especially that of IgT gene in vaccinated fish with 25  $\mu\text{g}$  CS/TPP-pcDNAVP2.

### Discussion

IPNV is the causal agent of an acute contagious disease particularly in young salmonids, with high morbidity and mortality, and establishes an asymptomatic carrier state in the survivors (Evensen, 2008). In the present study, the relative expression of IgM and IgT genes, as well as the VP4 gene of IPNV were assessed in orally vaccinated rainbow trout with different dose of encapsulated pcDNA3.1-VP2 DNA vaccine with CS/TPP nanoparticles and alginate sodium microsphere, 45 days post-challenge with IPNV. The results showed that fish of all treatments survived and displayed no clinical

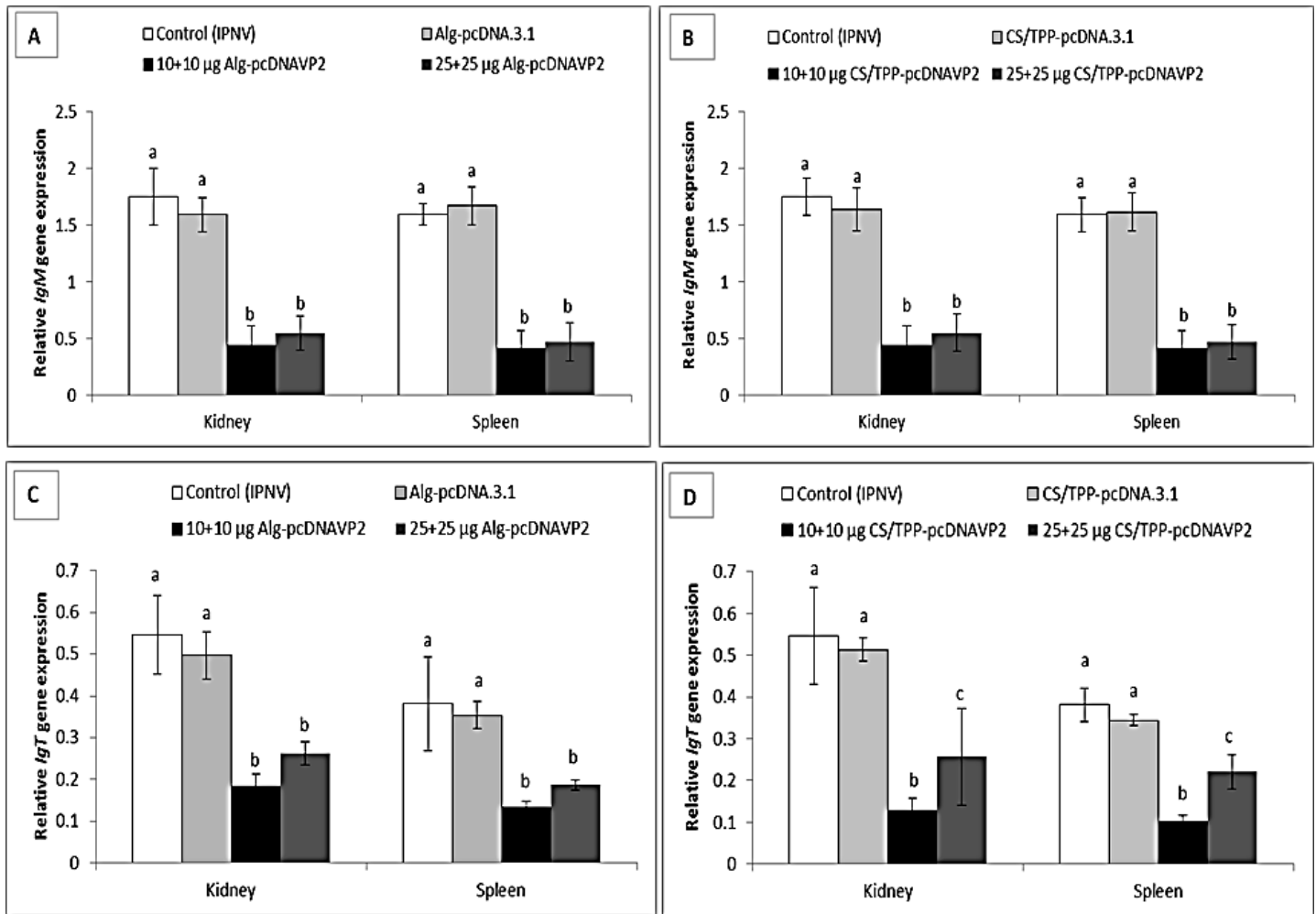


Figure 3. Expression of adaptive immune-related genes of IgM and IgT in the kidney and spleen of rainbow trout orally vaccinated with Alg-pcDNAVP2 (A and C) and CS/TPP-pcDNAVP2 (B and D) after challenging with IPNV as described in legend Figure 2. Different letters indicate significant differences between groups at  $P < 0.05$ .

symptoms of disease. The transcript of IPNV-VP4 gene in the control group was significantly higher than vaccinated fish suggesting that oral vaccination has decreased the viral load. Similarly in the other studies, oral DNA vaccination of fishes has reduced replication of different viral agents such as IPNV and IHNV (Marjara et al., 2011; Ballesteros et al., 2014; Ballesteros et al., 2015), suggesting different immune modulatory effects of the vaccine may result in the inhibition of the viral replication.

The results showed that CS/TPP nanoparticles and alginate sodium microsphere effectively protected the constructed IPN DNA vaccine from degradation in the fish stomach. The results also showed that IgT and IgM genes are express-able in both vaccinated and virus control fish, but with more intensely in the control carrier fish. However,

vaccination did not enhance the expression of IgM and IgT above the levels observed in the control carrier group, but it showed mimicked viral activity and contributes to maintain at the appreciable levels in all vaccinated groups suggesting the presence of Ig secreting cells, and confirming the vaccine activity and the gene response to oral stimulation of vaccine administration. However, it remains unclear what immune activity is being induced by the virus that persists in surviving fish or by the exogenous VP2 gene.

In conclusion, the results showed that CS/TPP nanoparticles and alginate sodium microspheres loaded with pcDNA3.1-VP2 vaccine are able to reduce viral replication in the surviving fish after challenging with IPNV. Moreover, the constructed DNA vaccine did not enhance the expression of IgM

and IgT genes above the levels observed in the virus control carrier group but it shows mimics viral activity and contribute to keep them at appreciable levels in all vaccinated groups. Further works are in progress to assess the correlation between these genes and clinical efficacy of the vaccine in the same fish groups.

### Acknowledgements

This study was funded by the grant of Research Council of the University of Tehran and the Centre of Excellence of Aquatic Animal Health. Authors thank E. Alirahimi and M. Ahmadpoor for their technical assistance.

**Conflict of interest:** Authors declare that they do not have any conflict of interest.

**Authors' Note:** The subject matter of this publication is included in pending patent applications.

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