Molecular Immunology 94 (2018) 61–67

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

VP2 (PTA motif) encoding DNA vaccine confers protection against lethal challenge with infectious pancreatic necrosis virus (IPNV) in trout

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ARTICLE INFO

Keywords:
IPNV
Trout
DNA vaccine
Intramuscular
VP2
PTA motif

ABSTRACT

IPNV in Atlantic salmon is represented by various strains with different virulence and immunogenicity linked to various motifs of the VP2 capsid. IPNV variant with P_{217}, T_{221}, A_{247} (PTA) motif is found to be avirulent in Atlantic salmon, but virulent in rainbow trout, and other salmonid species. This study describes a DNA vaccine delivered intramuscularly encoding the VP2 protein of infectious pancreatic necrosis virus (IPNV) with PTA motif that confers high protection in rainbow trout (*Oncorhynchus mykiss*). Intramuscular injection of 2, 5 and 10 μg of DNA (pcDNA3.1-VP2) in rainbow trout fry (4–5 g), confers relative protection of 75–83% in the different vaccine groups at 30 days post vaccination (450° days). The VP2 gene is expressed in spleen, kidney, muscle and liver at day 30 post-vaccination (RT-PCR), and IFN-1 and Mx-1 mRNA are upregulated at early time post vaccination, and so also for IgM, IgT, CD4 and CD8 in the head kidney of vaccinated fish compared to controls, 15 and 30 days post vaccination. Significant increase of serum anti-IPNV antibodies was found 30–90 days post-vaccination that was correlated with protection levels. Mortality corresponded with viral VP4 gene expression were significantly decreased in vaccinated and challenged fish. This shows for the first time that a VP2-encoding DNA vaccine delivered intramuscularly elicits a high level of protection alongside with high levels of circulating antibodies in rainbow trout and a lowered viral replication.

1. Introduction

Infectious pancreatic necrosis virus (IPNV) belongs to *Birnaviridae* family and is the type strain of the genus Aquabirnavirus. It is one of the most widely distributed viruses in aquaculture and in the wild, affecting more than 63 marine aquatic animal species, including fish, mollusks and crustaceans causing high morbidity and mortality in fry and juveniles of farmed fish with asymptomatic adult carriers surviving the disease (Rodriguez Saint-Jean et al., 1991, 2003; Evensen and Santi, 2008).

IPNV is a non-enveloped virus with a bi-segmented (A and B) dsRNA genome (~5 kbp) (Dobos, 1976) where segment A encodes VP2 (Capsid protein) containing most of the neutralizing epitopes of the virus (Frost et al., 1995; Fridholm et al., 2007), VP3 (Structural protein), and the protease, VP4 (Dobos, 1976). It also encodes VP5, a non-structural protein of unknown function (Santi et al., 2005). Segment B only encodes an RNA-dependent RNA polymerase (VP1; Dobos, 1977).

So far IPNV isolates have been grouped into 7 genogroups based on the VP2 sequences, which correlated with serotypes (A1–A9 and B) and geographical distribution (Hill and Way, 1995; Nishizawa et al., 2005).

Depending upon the host species, viral strain and environmental conditions, IPN outbreak may result in mortality rates ranging from 5 to 100% (Evensen and Santi, 2008).

VP2 and amino acid residues 217, 221 and 247 are the main molecular determinants of IPNV virulence and pathogenicity for Atlantic salmon (*Salmo salar* L.) (Santi et al., 2004; Song et al., 2005) and also correlate with immunogenicity (Munang'andu et al., 2013a). Highly virulent isolates from Atlantic salmon possess threonine and alanine at residues 217 and 221, respectively, while, Ala-to-Thr substitution at position 221 is indicative of a non-virulent or low virulent nature for

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https://doi.org/10.1016/j.molimm.2017.12.015
Received 15 October 2017; Received in revised form 12 December 2017; Accepted 18 December 2017
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this fish species (Santi et al., 2004).

Despite the avirulent nature for Atlantic salmon, IPNV variant with P_{217}, T_{221}, A_{247} (PTA) motif is found to be virulent in rainbow trout, and other salmonid species. This variant (PTA) is associated with high mortality in rainbow trout in Norway (Evensen, personal communication), and trout hatcheries in Iran (Raissy et al., 2010; Dadar et al., 2013; Ahmadivand et al., 2016), as well as in Finnish fish farms (Eriksson-Kallio et al., 2016), and in wild and farmed fish in Scotland (Bain et al., 2008).

Prevention against mortality through vaccination is an important control strategy to avoid of IPN related losses. However, the current commercial IPN vaccines (Inactivated and subunit vaccines) induce an immune response biased towards humoral immunity and vaccinated fish may become carriers post infection (Bootland et al., 1995; Munang'andu et al., 2013c).

DNA vaccines consist of antigen-encoding plasmid DNA delivered traditionally through the intramuscular(i.m) route in fish, and for some virus infections a strong and long-lasting immunity has been obtained under experimental (McLauchlan et al., 2003) and commercial use (Alonso and Leong, 2013).

Recently, experimental VP2 encoding DNA vaccines delivered orally have been shown a lower virus infection level with a high protection against mortality in trout (Ballesteros et al., 2014; Ahmadivand et al., 2017).

However, most plasmid-based IPN vaccines have been tested by intramuscular delivery, and those tested in laboratory trials the vaccines were not tested against real challenge since low mortality was obtained in non-vaccinated control groups (< 40%) and with disappointing levels of efficacy for VP2-encoding plasmid vaccines (Mikalsen et al., 2004). Other studies of VP2 plasmid vaccines testing by intramuscular delivery have not included lethal challenge post vaccination (de las Heras et al., 2009) and thus real efficacy cannot be decided.

In this study, we have shown that an intramuscularly delivered DNA vaccine encoding the VP2 gene of a IPNV variant with PTA motif elicits a protective immune response in rainbow trout fry where the level of protection correlates with the level of circulating antibodies and the ability to limit virus replication in the internal organs post challenge.

2. Materials and methods

2.1. Ethics statement

All applicable guidelines for the care and use of animals were followed according to the instructions given by the University of Tehran Ethics Committee for Animal Experimentation.

2.2. Virus and cell culture

The vaccine strain (IPNV with PTA motif, genotype 5, serotype Sp) was used for immunoassays and challenge studies. The strain was selected based on epidemiological studies of the disease outbreaks in Iranian trout farms during 2009–2016 (GenBank Acc No: KX665156, KX665157, KX665158, KX665159, GU338037, KF279643, KC489465). The virus was propagated in CHSE-214 cell line (Fryer et al., 1965) and titrated according to Reed and Muench producer (1938).

2.3. Vaccine construction

The DNA vaccine (pcDNA3.1-VP2) was prepared as previously described by Ahmadivand et al. (2017). Briefly, the VP2 gene of IPNV was inserted in pcDNA3.1 (Invitrogen, USA) under the control of the CMV promoter, verified using HindIII and XhoI endonuclease analysis and then amplified in *Escherichia coli* (TOP10). The constructed plasmid DNA was then isolated with the Endofree Plasmid Mega Purification Kit (Qiagen, USA) according to the manufacturer's instructions. The DNA concentration was measured using a spectrophotometer (NanoDrop 2000, Thermo scientific, Spain), and stored at −20 °C until use.

2.4. Vaccination and challenge

Rainbow trout weighing 4–5 g were vaccinated by intramuscular (i.m.) injection using three doses, 2, 5 and 10 μg/fish of pcDNA3.1-VP2 in the left epaxial musculature in separate trials, each contains 90 fish. Two control groups (each group contains 90 fish) were also injected with 10 μg/fish empty plasmid (pcDNA3.1) and PBS. The vaccinated fish were then kept at 15 °C for 90 days.

On day 30 post vaccination (450° days), 30 fish from each trail (15 fish per replicate) were challenged by IP injection of 0.2 ml/fish with IPNV at a concentration of 10^7 $TCID_{50}$ mL^{-1}.

The relative percent survival (RPS) value of each experimental group was calculated by the formula [1 − (Cumulative percent mortality in vaccinated fish/cumulative percent mortality in non-vaccinated fish)] × 100.

To demonstrate the viral load in the survivals the expression of IPNV-VP4 gene in the head kidney and spleen of fish (n = 5) were analyzed by RT-qPCR on day 45 post-challenge as described previously (Ballesteros et al., 2014; Ahmadivand et al., 2017).

2.5. Expression of VP2 gene from DNA vaccine

On day 30 post-vaccination (day of challenge) three fish from each trial were sacrificed via overexposure to clove oil, and RNA was extracted from 20 mg of each homogenized tissues of spleen, muscle, head kidney and liver using the RiboEx SL Total RNA extraction kit (GeneAll, Korea). To eliminate genomic or plasmid DNA contamination, RNA samples were treated with RNase-free DNase (Promega), and then cDNA synthesis was carried out in a total volume of 25 μl from 5 μl of extracted RNA using HyperScript™ First Strand Synthesis Kit (GeneAll, Korea) according to the manufacturer's recommendations. RT-PCR amplification of VP2 gene with an expected size of 405 bp was performed using SVP2-F and SVP2-R primer pairs according to Ahmadivand et al. (2017). The amplification products were resolved by electrophoresis using a 1% agarose under UV light.

2.6. Immune genes transcription (RT-qPCR)

The relative expression of IFN-1 and Mx-1 mRNA was assessed on days 3, 7 and 15 post-vaccination. Also, IgM, IgT, CD4 and CD8 genes related to adaptive immune responses were quantified on days 15 and 30 post-vaccination. The head-kidney tissues of 5 fish in each trail were processed for gene expressions using Real-Time PCR (Applied Biosystems) and SYBR Green qPCR Master Mix as described previously (Ballesteros et al., 2014; Ahmadivand et al., 2017). 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 α*) calculated relative to empty plasmid according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.7. Antibody titer by ELISA

On days 15, 30, 45, 60 and 90 post-vaccination, five non-challenged fish from each vaccine group were anesthetized by clove oil and blood samples were collected from the caudal vein, clotted and sera were separated by centrifuging at 500*g* for 10 min. Level of antibodies was assessed by ELISA, as described previously by Ahmadivand et al. (2017). Briefly, 96-well ELISA plates were coated by with 100 μl (10^7) $well^{-1}$ IPNV and incubated overnight at 4 °C. After washing with PBS-T (phosphate buffered saline containing 0.05% Tween 20), the wells were blocked for 2 h at 22 °C with 3% dried skimmed milk in PBS (300 μl/well). Following a wash with PBS-T, 100 μl of each fish serum sample (serially diluted with PBST-5% BSA) was added in triplicate and

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