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An oral chitosan DNA vaccine against nodavirus improves transcription of cell-mediated cytotoxicity and interferon genes in the European sea bass juveniles gut and survival upon infection☆

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ABSTRACT

Vaccines for fish need to be improved for the aquaculture sector, with DNA vaccines and the oral administration route providing the most promising improvements. In this study, we have created an oral chitosan-encapsulated DNA vaccine (CP-pNNV) for the nodavirus (NNV) in order to protect the very susceptible European sea bass (*Dicentrarchus labrax*). Our data show that the oral CP-pNNV vaccine failed to induce serum circulating or neutralizing specific antibodies (immunoglobulin M) or to up-regulate their gene expression in the posterior gut. However, the vaccine up-regulated the expression of genes related to the cell-mediated cytotoxicity (CMC; *tcrb* and *cd8a*) and the interferon pathway (IFN; *ifn*, *mx* and *ifng*). In addition, 3 months after vaccination, challenged fish showed a retarded onset of fish death and lower cumulative mortality with a relative survival of 45%. Thus, we created a chitosan-encapsulated DNA vaccine against NNV that is partly protective to European sea bass juveniles and up-regulates the transcription of genes related to CMC and IFN. However, further studies are needed to improve the anti-NNV vaccine and to understand its mechanisms.

1. Introduction

Although DNA vaccines are increasingly considered to be a potential method to solve the lack of available treatments to viral diseases in aquaculture (Evensen and Leong, 2013; Kurath, 2008), their administration by injection makes them a limited and inappropriate preventive measure because of its invasiveness and economically unfeasible delivery. Therefore, it remains necessary to develop simple and cost-effective systems to deliver DNA vaccines for mass administration in fish farms. At this point, recent approaches have demonstrated that oral management of encapsulated vaccines improves the survival to different pathogen infections (Rajesh-Kumar et al., 2008; Vimal et al., 2014). In fish vaccination studies, several types of encapsulation substances, including alginate (Ballesteros et al., 2015; de las Heras et al., 2010; Maurice et al., 2004; Tian et al., 2008b), chitosan (Li et al., 2013; Rajesh-Kumar et al., 2008; Tian et al., 2008c) and poly(DL-lactide-*co*-glycolide) (Tian et al., 2008a; Tian and Yu, 2011), have been effectively used to encapsulate bacterial or viral antigens, and the data obtained demonstrate the generation of an effective immune response and a resistance to pathogen challenge. Thus, encapsulated oral vaccines are easy to use for mass intake with minimal fish stress; they are targetable, are easy to produce in large quantities and they store stably. In addition to the capacity of these substances to entrap and protect the antigens in the fish digestive tract, they act by themselves as immunostimulants, offering an additional improvement of the immune response. Thus, the administration of sodium alginate or chitosan is able to increase the fish immune

☆ The genetic nomenclature used in this manuscript follows the guidelines of Zebrafish Nomenclature Committee (ZNC) for fish genes and proteins and the HUGO Gene Nomenclature committee for mammalian genes and proteins.

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response and disease resistance (Abu-Elala et al., 2015; Cheng and Yu, 2013; Fujiki et al., 1994; Lin et al., 2011). Moreover, they are environmentally friendly since they are biodegradable and non-toxic, making the encapsulation with these polymers a possible and ideal route not only for general vaccination, but also for DNA vaccines.

Among fish diseases, viruses present significantly increasing problems in intensive aquaculture as there are no solutions available at preventive and therapeutic levels. One of the most threatening viruses is the nodavirus (*Nodaviridae* family, *Betanodavirus* genus), which is also named the nervous necrosis virus (NNV), which causes the viral encephalopathy and retinopathy (VER) disease that alters brain and retina structure and function (Munday et al., 1992). NNV provokes mortality rates up to 100% in more than 50 marine species (Munday et al., 1992; OIE, 2013). Among them, the European sea bass (*Dicentrarchus labrax*) – a very relevant species in Mediterranean aquaculture – is one of the most susceptible ones, being larvae and juvenile stages those suffering highest mortalities (Breuil et al., 1991; Frerichs et al., 1996). Although some aspects of the fish immune response against NNV are known, very few studies have addressed the generation of an effective vaccine. So far, different studies have demonstrated the increase of the immune response and/or NNV resistance after the administration of live/inactivated NNV or recombinant proteins (Kai and Chi, 2008; Kai et al., 2014; Kim et al., 2000; Nishizawa et al., 2012; Oh et al., 2013; Sommerset et al., 2005). In addition, one study showed that the intramuscular injection of a NNV DNA vaccine failed to protect Atlantic halibut (*Hippoglossus hippoglossus*) (Sommerset et al., 2005); however, more recently, the oral administration of an encapsulated DNA vaccine to Asian sea bass (*Lates calcarifer*) was partly protective against NNV (Vimal et al., 2014). However, further studies are needed to improve their efficiency and applicability in fish farms to control the infections and dissemination of this important virus.

In this study, we orally vaccinated healthy specimens of European sea bass juveniles with a specific DNA vaccine against NNV, which was encapsulated into chitosan nanoparticles. The aim was to study whether the vaccine stimulated the immune response in the gut of the fish and provoked a decrease of the mortality rate after challenge. Our results pointed to the activation of genes related to cell-mediated cytotoxicity (CMC) and the interferon (IFN) pathway in the gut of vaccinated fish, which resulted in improving the survival against an *in vivo* NNV challenge after 3 months of vaccination.

2. Material and methods

2.1. Animals

Juveniles of the marine teleost European sea bass (*D. labrax*) (125 days post-hatching, dph; 6.02 ± 0.70 g body weight) were bred and reared in the *Centro Oceanográfico de Mazarrón, Instituto Español de Oceanografía* (COM-IEO). All animal studies were carried out in accordance with the Guidelines of the European Union Council (2010/63/UE), the Bioethical Committee of the University of Murcia (Spain; Permit Number: A13150104) and the *Instituto Español de Oceanografía* (Spain; Permit Number: 2010/02) for the use of laboratory animals.

2.2. Nodavirus (NNV) stocks

NNV (strain It/411/96, genotype RGNNV; isolated from European sea bass in Italy) was propagated in the E-11 cell line, which is persistently infected with a snakehead retrovirus (Frerichs et al., 1996). The E-11 cells were grown at 25 °C in Leibovitz's L15-medium (Gibco) supplemented with 10% foetal bovine serum (Gibco), 2 mM L-glutamine (Gibco), 100 i.u. ml^{-1} penicillin (Gibco), 100 μg ml^{-1} streptomycin (Gibco) and 2.5 μg ml^{-1} fungizone (Gibco) using Falcon Primaria cell culture flasks (Becton Dickinson). Cells were inoculated with NNV and incubated at 25 °C until the cytopathic effect was extensive. Supernatants were harvested and centrifuged to eliminate cell debris. Virus stocks were titrated in 96-well plates according to a protocol that was previously described (Reed and Müench, 1938) and used in the experiments.

2.3. Plasmid constructs

For the construction of the NNV DNA vaccine (pNNV), the entire open reading frame of the RNA2 gene (genotype RGNNV, strain It/411/96) was amplified by a polymerase chain reaction (PCR) from a cDNA sample obtained from the NNV culture (Table 1), containing both the start and stop codons. The PCR product was cloned into the expression vector pcDNA3.1/V5-His-TOPO according to the manufacturer's instructions (Invitrogen) and used to transform One Shot TOP10 *Escherichia coli* cells (Invitrogen). A clone containing the pNNV was identified by PCR screening, and the proper orientation was verified by sequencing. A religated empty pcDNA3.1/V5-His-TOPO plasmid (pcDNA3.1) was used as a negative control.

2.4. Preparation of vaccine

We synthesized the nanoparticles by complexing high molecular weight (about 390,000 Da) chitosan (Sigma) with DNA plasmid. Chitosan was dissolved in 1% acetic acid with gentle heating and adjusted to pH 5.7. The solution was then sterile-filtered through a 0.45 μm filter and adjusted to pH 5.5. Several concentrations of chitosan (w/v; 0.02%, 0.04%, 0.06% and 0.10%) were prepared and mixed with equal volumes of plasmid (200 μg ml^{-1} in 25 mM of sodium sulphate solution) at 55 °C and rapidly mixed and vortexed at maximum speed for 45 s. The resulting polyplexes were kept at room temperature for 30 min for stabilization. The DNA loading efficiency in the chitosan particles was measured by spinning a sample at 13,000 g for 15 min and measuring the resulting DNA in the supernatant at 260 nm. In addition, the vaccine formulation (CP-pNNV) was studied by transmission electron microscopy to assess the heterogeneity and size of the chitosan-DNA nanoparticles. Briefly, 5 μl of the vaccine formulation were placed onto formvar-carbon-coated 400 mesh copper grids, fixed with 2% glutaraldehyde for 5 min, then washed and negatively stained with 2% phosphotungstic acid, pH 7, for 1 min. Samples were then examined under a Tecnai 12 transmission electron microscope (Phillips).

2.5. Fish vaccination

For the fish vaccination, we selected the optimal chitosan formulations by using 0.04% of chitosan (w/v) and the DNA concentration of 200 μg ml^{-1}. Diets were obtained by spreading the chitosan solutions onto the commercial diet and allowing the pellet to dry. European sea bass juveniles (125 dph of age) were randomly distributed and fed for 2 days with the following formulations into the commercial diet: diet alone (control), chitosan particles (CP), CP containing the empty plasmid (CP-pcDNA3.1) or chitosan particles containing pNNV (CP-pNNV). Fish received the CP-pcDNA3.1 and CP-pNNV diets containing approximately 10 μg of plasmid per fish.

2.6. Sampling

Fish (n = 6 fish/group and time) were sampled at 7, 30 and 90 days after oral vaccination. The posterior region of gut was

Table 1
Sequence of the oligonucleotides used. Underlined are the start and stop codons.

Gene	Symbol	Acc. Number	Sequence (5′3′)		Use
Nodavirus capsid protein	NNV RNA2	D38636	F	ACAATGGTACGCAAAGGTGATAAG	PCR cloning
			R	TTAGTTTTCCGAGTCAACACGG	
			F	CGTGTCAGTCATGTGTCGCT	Conventional PCR
			R	CGAGTCAACACGGGTGAAGA	
Interferon	*ifn*	AM765847	F	GGCTCTACTGGATACGATGGC	Real-time PCR
			R	CTCCCATGATGCAGAGCTGTG	
Interferon gamma	*ifng*	KJ818329	F	TCAAGATGCTGAGGCAACAC	
			R	AGTGCTTTGCTCTGGACGAC	
Interferon-induced GTP-binding protein Mx	*mx*	AM228977, HQ237501, AY424961	F	GTCTGGAGATCGCCTCT	
			R	TCTCCGTGGATCCTGATGGAGA	
MHC class I alpha	*mhc1a*	AM943118	F	GGACAGACCTTCCCTCAGTG	
			R	TCCAGATGAGTGTGGCTTTG	
MHC class II beta	*mhc2b*	AM113466	F	CAGAGACGGACAGGAAG	
			R	CAAGATCAGACCCAGGA	
Immunoglobulin mu heavy chain	*igmh*	FN908858	F	AGGACAGGACTGCTGCTGTT	
			R	CACCTGCTGTCTGCTGTTGT	
Immunoglobulin tau heavy chain	*igth*	FM010886	R	TCACTTGGCAAATTGATGGA	
			F	AGAACAGCGCACTTTGTTGA	
CD8 alpha	*cd8a*	AJ846849	F	CTGTCCTCCGCTCATACTGG	
			R	TTGTAATGATGGGGGCATCT	
T-cell receptor beta chain	*tcrb*	FN687461	F	GACGGACGAAGCTGCCCA	
			R	TGGCAGCCTGTGTGATCTTCA	
Elongation factor 1-alpha	*ef1a*	FM019753	F	CGTTGGCTTCAACATCAAGA	
			R	GAAGTTGTCTGCTCCCTTGG	

removed, then immediately frozen in TRIzol Reagent (Life Technologies) and stored at −80 °C for later RNA isolation to evaluate the transcription of immune-related genes since this part of the gut is the richest in B and T lymphocytes (Ballesteros et al., 2013; Picchietti et al., 2011). The blood was obtained from the caudal peduncle of specimens after 90 days of vaccination, and the serum samples were obtained by centrifugation at 10,000 × g during 1 min at 4 °C, and immediately frozen and stored at −80 °C until they were used to evaluate the immunoglobulin M (IgM) levels and the neutralizing antibodies.

2.7. Total, specific and neutralizing antibody levels

Total serum IgM levels were analysed using the enzyme-linked immunosorbent assay (ELISA) (Cuesta et al., 2004). Thus, 20 μl per well of 1/100 serum diluted in PBS pH 7.4 containing 0.05% Tween 20 (PBS-T; Sigma) were placed in flat-bottomed 96-well plates (Nunc) in triplicate, and the proteins were coated by overnight incubation at 4 °C with 200 μl of carbonate-bicarbonate buffer (35 mM $NaHCO_3$ and 15 mM Na_2CO_3, pH 9.6). After three rinses with PBS-T, the plates were blocked for 2 h at room temperature with the blocking buffer of PBS-T containing 3% bovine serum albumin (Sigma), followed by three rinses with PBS-T. The plates were then incubated for 1 h at room temperature with 100 μl per well of mouse anti-sea bass IgM monoclonal antibody (Aquatic Diagnostics Ltd) at the optimal dilution of 1:100 in the blocking buffer, then washed and incubated with the secondary antibody anti-mouse IgG-HRP (Sigma) at the optimal dilution of 1:1,000 in the blocking buffer. After exhaustive rinsing with PBS-T, the plates were developed using 100 μl of a 0.42 mM TMB solution, and daily prepared in distilled water containing 0.01% H_2O_2. The reaction was allowed to proceed for 10 min and was stopped by the addition of 50 μl of 2 M H_2SO_4. The plates were read at 450 nm. Negative controls consisted of samples without serum or without primary antibody, whose optical density (OD) values were subtracted for each sample value.

Serum specific IgM levels against NNV were analysed using a slightly modified ELISA (Scapigliati et al., 2010). Briefly, 100 μl of purified NNV preparation diluted 1:5 with 50 mM carbonate-bicarbonate buffer pH 9.6 was employed to coat flat-bottomed 96-well plates overnight at 4 °C. After three rinses with PBS-T, the plates were blocked for 2 h at room temperature with the blocking buffer, followed by four rinses with PBS-T. Then, 100 μl of serial dilutions (1:50 to 1:200) of sera (n = 6 fish/group and time) diluted in PBS-T were incubated for 2 h at room temperature, followed by five rinses with PBS-T. The plates were then incubated with mouse anti-sea bass IgM monoclonal antibody and secondary anti-mouse IgG-HRP as above. The absorbance was read at 450 nm. Negative controls consisted of samples without serum or without coating, and positive controls that consisted of sera from *in vivo* infected sea bass with NNV after 15 days were also used in the ELISA (Chaves-Pozo et al., 2012). The OD values of negative controls were subtracted for each sample value.

Neutralizing antibodies were assayed as previously (Pakingking et al., 2009). In brief, two-fold serial dilutions of sera from fish obtained after 90 days of vaccination were incubated with equal volumes of 2×10^3 $TCID_{50}$ ml^{-1} of NNV for 1 h at 25 °C. After incubation, samples were assayed for NNV replication on E-11 cells as above and serum dilution provoking the absence of cytopathic effect was determined. A serum from an infected fish served as positive control.

2.8. Gene expression by real-time polymerase chain reaction

Total RNA was isolated from TRIzol Reagent frozen samples following the manufacturer's instructions. One μg of total RNA from each individual fish was treated with DNAse I to remove genomic DNA, and the first strand of cDNA was synthesized by reverse transcription using the ThermoScript™ Reverse Transcriptase (Invitrogen) with random hexamers (Invitrogen) followed by RNAse H (Invitrogen) treatment.

The transcription of the NNV capsid gene was analysed in the posterior gut of all vaccinated samples by conventional PCR, which was performed with a thermocycler (Mastercycler, Eppendorf). Reaction mixtures were incubated for 2 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at the specific annealing temperature of 58 °C, 1 min at 72 °C and finally 10 min at 72 °C. The primers used are shown in Table 1. For visualizing and comparing the groups, the

PCR products were run on a 1% agarose gel.

Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min 60 °C and 15 s at 95 °C. For each mRNA, gene expression was corrected by the elongation factor 1-alpha (*ef1a*) content in each sample and expressed as $2^{-\Delta Ct}$, where ΔCt is determined by subtracting the *ef1a* Ct value from the target Ct. The primers used are shown in Table 1. Before the experiments, the specificity of each primer pair was studied using positive and negative samples. A melting curve analysis of the amplified products validated the primers for specificity. After these verifications, all amplifications were performed in duplicate cDNAs and repeated once to confirm the results. Negative controls with no template were always included in the reactions.

2.9. NNV challenge

Three months after oral vaccination, 24 fish per group were transported to the University of Murcia aquaria. Fish were randomly divided into two tanks per group, kept in 30 L running seawater (28‰ salinity) aquaria at 24–26 °C, with a 12 h light: 12 h dark photoperiod and acclimatised for 15 days prior to the experiments. The fish were fed daily with a commercial pellet diet (Skretting). Each group received a single intramuscular injection of 100 μl culture medium containing 10^6 $TCID_{50}$ $fish^{-1}$ of the same NNV isolate (n = 24 fish/group) since this route of infection has been proven to be the most effective (Aranguren et al., 2002). Mortality was recorded daily through the challenge, and relative percent survival (RPS) was determined:

RPS = 1 − [(% mortality in vaccinated fish)/(% mortality in control fish)] × 100.

2.10. Statistical analysis

Gene expression data were analysed by one-way ANOVA to denote statistical differences between groups at different sampling times ($P \leq 0.05$). A non-parametric Kruskal–Wallis assay, followed by a multiple comparison test, was used when data did not meet parametric assumptions. Gene expression data are presented as mean ± standard error of the mean (SEM; n = 6 fish/group and time). Cumulative survival was represented for all treatments as mean ± SEM (n = 2 replicates) and analysed by a two-way ANOVA followed by Tukey's post-hoc analysis. All statistical analyses were conducted using the SPSS 20 application.

3. Results

It is worthy to note that the expression of the plasmid coding for NNV detection by PCR analysis in the posterior gut from vaccinated specimens of European sea bass resulted undetected for all the animals (data not shown).

3.1. Characteristics of the chitosan-DNA nanoparticles

The loading efficiency of chitosan nanoparticles with all chitosan concentrations and plasmid DNA amounts that were assayed ranged from 84.71% to 97.47% of the total plasmid DNA (Table 2). The optimal loading efficiency was when 0.04% chitosan concentration and 200 μg ml^{-1} of plasmid DNA were used (Table 2), which was selected for formulating the vaccine. Transmission electron microscopy showed that freshly prepared particles were approximately 0.05–0.2 μm in size and fairly spherical (Fig. 1).

Table 2
Loading efficiency of plasmid DNA (% of total plasmid DNA used to produce the particles) in the chitosan particles produced with different chitosan concentrations.

Plasmid DNA (μg ml^{-1})	Chitosan concentration (%)			
	0.02	0.04	0.06	0.10
85	84.71	87.93	91.03	97.24
200	62.30	97.47	92.18	88.62

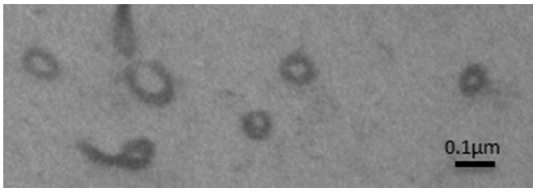

Fig. 1. Appearance and size distribution of chitosan-DNA nanoparticles by transmission electron microscopy.

3.2. Encapsulated DNA vaccine failed to induce the production of specific immunoglobulin M antibodies

Serum IgM levels were analysed at 90 days post-vaccination (Fig. 2) and no significant changes in the total serum IgM levels were detected between groups (Fig. 2A). Moreover, anti-NNV-specific IgM levels were undetected in any experimental group and were only detected in intramuscularly *in vivo* infected specimens at day 15 of infection (Fig. 2B). Similarly, neutralizing antibodies were not detected in any vaccinated fish (data not shown). In addition, we evaluated the *igmh* and *igth* transcription levels at 7, 30 and 90 days post-vaccination. We found significant differences in the CP-pcDNA3.1 and CP-pNNV groups compared to the control and CP groups only after 7 days post-vaccination in the transcription of *igmh* (Fig. 2C). In the case of *igth* transcription, the sea bass specimens receiving the CP-pNNV vaccine significantly increased this gene expression compared to the control group, but it did not significantly increase in the CP-pcDNA3.1 group after 30 and 90 days of vaccination (Fig. 2D).

3.3. Oral DNA vaccine transitorily up-regulated mhc1a

We evaluated the expression of MHC class I and II genes, *mhc1a* and *mhc2b*, respectively (Fig. 3). Interestingly, the CP-pNNV oral vaccine failed to up-regulate their transcription in the posterior gut except for the *mhc1a* gene expression, which was up-regulated at 7 days post-vaccination compared to the control and CP groups (Fig. 3A).

3.4. The oral vaccine stimulated the expression of cell-mediated cytotoxicity-related genes

The vaccine enhanced the expression in the posterior gut of some CMC genes as *tcrb* and *cd8a* (Fig. 4). Interestingly, *tcrb* gene expression was up-regulated in the CP-pNNV group after 7 and 90 of vaccination when compared to the control group; with the CP group at 30 days; and with the CP-pcDNA3.1 group at 7 and 90 days (Fig. 3A). Otherwise, the *cd8a* gene was up-regulated after 30 and 90 days post-vaccination in the CP-pNNV group compared to the control group (Fig. 4B). The CP-pcDNA3.1 group showed stimulation of *tcrb* gene expression after 7 days of vaccination, and *tcrb* and *cd8a* gene expression after 7 and 90 days of vaccination compared to the control group (Fig. 4).

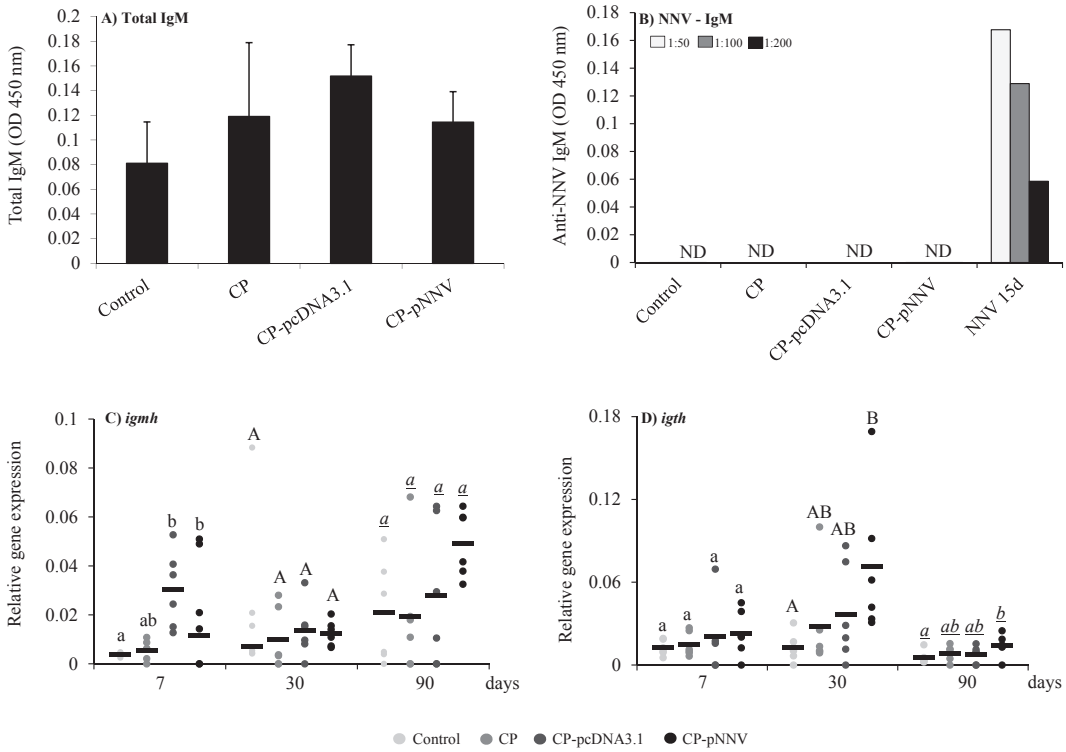

Fig. 2. Total (A) or specific anti-NNV (B) IgM levels in the serum after 90 days and the transcription levels of *igmh* (C) and *igth* (D) genes in the posterior region of the gut of European sea bass after 7, 30 and 90 days of oral vaccination with diet alone (Control) or diet containing chitosan (CP), chitosan plus empty plasmid (CP-pcDNA3.1) or chitosan plus NNV DNA vaccine (CP-pNNV). NNV 15 d represents a positive sample from a sea bass specimen infected with NNV for 15 days. Data represent the mean ± standard error of the mean (SEM; n = 6/group and time). Statistical analysis was performed by ANOVA ($P < 0.05$) followed by Tukey's post-hoc analysis. Different letters denote statistical differences between groups at the same time point (lowercase letters = 7 days; capital letters = 30 days; italic lowercase letters = 90 days).

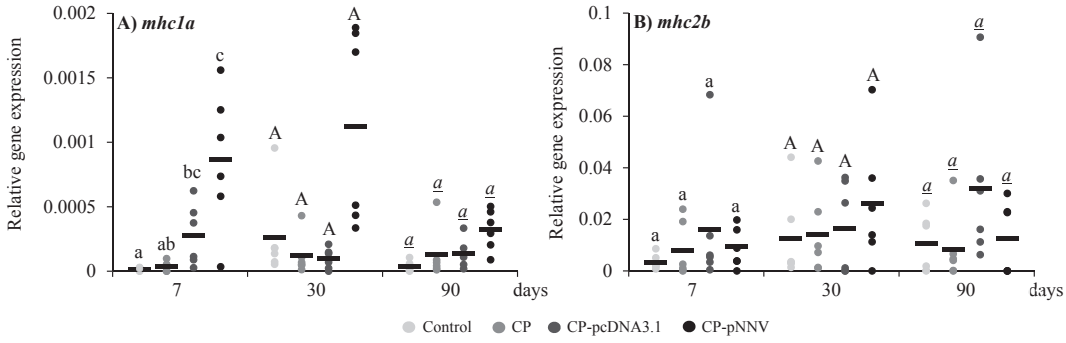

Fig. 3. Expression level of genes related to antigen presentation, *mhc1a* (A) and *mhc2b* (B), in the posterior region of the gut of European sea bass after 7, 30 and 90 days of oral vaccination with diet alone (Control) or diet containing chitosan (CP), chitosan plus empty plasmid (CP-pcDNA3.1) or chitosan plus NNV DNA vaccine (CP-pNNV). Data represent the mean ± SEM (n = 6/group and time). Statistical analysis was performed by ANOVA ($P < 0.05$) followed by Tukey's post-hoc analysis. Different letters denote statistical differences between groups at the same time point (lowercase letters = 7 days; capital letters = 30 days; italic lowercase letters = 90 days).

3.5. Type I and II interferon genes were induced by the oral DNA vaccine

Finally, we analysed the expression of genes related to type I (*ifn* and *mx*) and II (*ifng*) IFN response (Fig. 5). Thus, in the CP-pNNV group, the transcription of *ifn* (Fig. 5A) was significantly up-regulated at day 30 and of *mx* (Fig. 5B) at days 7 and 30. The expression level of the *ifng* gene was significantly up-regulated in the CP-pNNV group after 30 and 90 days of vaccination. Interestingly, the CP-pcDNA3.1 group showed enhanced expression levels when compared with the CP group (Fig. 5C).

3.6. European sea bass resistance against NNV infection was improved by the oral encapsulated DNA vaccine

The infection challenge with NNV was performed in two replicates after 3 months of oral vaccination (Fig. 6). The fish control group and the CP group developed the first symptoms of VER disease 2 or 3 days after the challenge, which manifested as erratic swimming and changes in skin colour (Supplementary data, Videos S1 and S2), which started to dye after 3 or 4 days of challenge in the control group and the CP groups, respectively (Fig. 6). Mortality reached 100% at day 19 in control group and at day 21 in CP group. Interestingly, and although the fish from the CP-pcDNA3.1 group showed a delay on the onset of mortalities that started at day 17

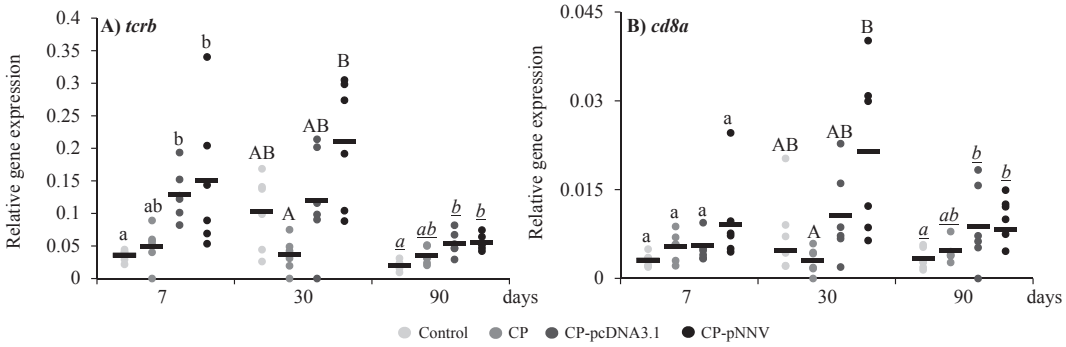

Fig. 4. Expression level of genes related to CMC response, *tcrb* (A) and *cd8a* (B), in the posterior region of the gut of European sea bass after 7, 30 and 90 days of oral vaccination with diet alone (Control) or diet containing chitosan (CP), chitosan plus empty plasmid (CP-pcDNA3.1) or chitosan plus NNV DNA vaccine (CP-pNNV). Data represent the mean ± SEM (n = 6/group and time). Statistical analysis was performed by ANOVA ($P < 0.05$) followed by Tukey's post-hoc analysis. Different letters denote statistical differences between groups at the same time point (lowercase letters = 7 days; capital letters = 30 days; italic lowercase letters = 90 days).

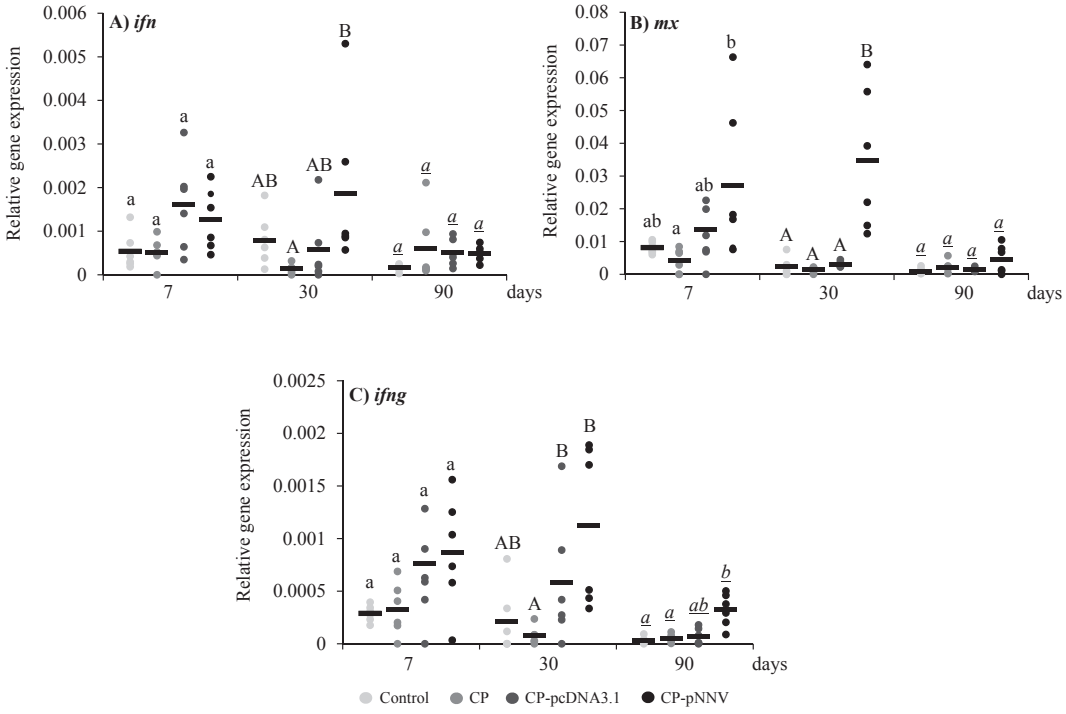

Fig. 5. Expression levels of the type I, *ifn* (A), *mx* (B), and II (*ifng*; C) interferon response genes in the posterior region of the gut of European sea bass after 7, 30 and 90 days of oral vaccination with diet alone (Control) or diet containing chitosan (CP), chitosan plus empty plasmid (CP-pcDNA3.1) or chitosan plus NNV DNA vaccine (CP-pNNV). Data represent the mean ± SEM (n = 6/group and time). Statistical analysis was performed by ANOVA ($P < 0.05$) followed by Tukey's post-hoc analysis. Different letters denote statistical differences between groups at the same time point (lowercase letters = 7 days; capital letters = 30 days; italic lowercase letters = 90 days).

post-challenge, the mortality recorded in this group was 100% at day 22 (Fig. 6). In contrast, and although the fish from the CP-pNNV group started to die at day 21 of infection, they did not show very clear signs of disease (Supplementary data, Video S3) and showed a RPS of 45% at the end of the challenge. Two-way ANOVA found significant differences among the groups ($P = 6.61 \times 10^{-57}$), and the time ($P = 5.3 \times 10^{-62}$) and between the two factors ($P = 2.3 \times 10^{-16}$). Furthermore, Tukey's analysis found significant differences among the groups (Fig. 6 inset). The fish control group and CP group are similar but significantly different to the others. The fish CP-pcDNA3.1 group and CP-pNNV group are also significantly different.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.dci.2016.06.021

4. Discussion

Chitosan nanoparticles seem to be ideal for DNA encapsulation and vaccine administration by oral intake because of their natural characteristics. Thus, chitosan nanoparticles are non-toxic for both animals and humans (Rao and Sharma, 1997). Moreover, their positively charged surface and complex stability in physiological pH conditions allow them to protect the encapsulated DNA plasmid from nuclease degradation in the gut (MacLaughlin et al., 1998; Mao et al., 1996). In addition, sodium alginate or chitosan act by themselves as immunostimulants, offering an additional improvement of the immune response (Abu-Elala et al., 2015; Cheng and Yu, 2013; Fujiki et al., 1994; Lin et al., 2011). However, we did not observe any immune stimulation due to chitosan dietary intake, but we found an up-regulation of the *igmh*, *mhc1a*, *tcrb*, *cd8a* and *ifn*

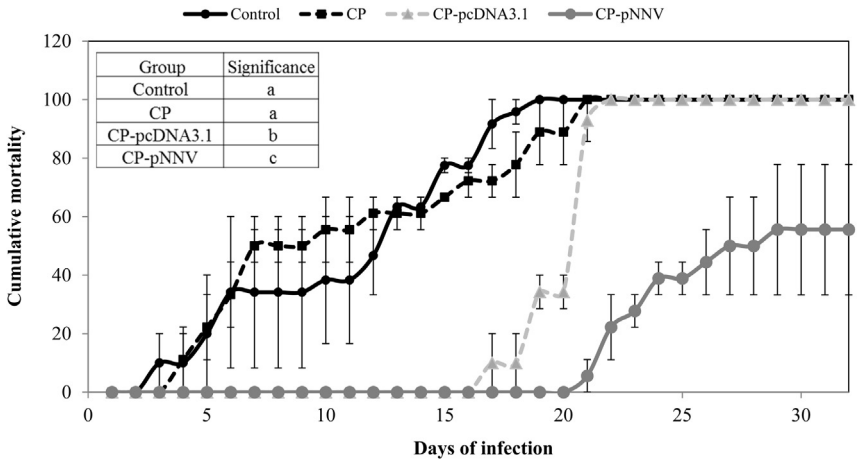

Fig. 6. Cumulative mortality in orally vaccinated European sea bass juveniles after intramuscular injection with 10^6 $TCID_{50}$ NNV per fish 3 months after oral vaccination with diet alone (Control) or diet containing chitosan (CP), chitosan plus empty plasmid (CP-pcDNA3.1) or chitosan plus NNV DNA vaccine (CP-pNNV). Curves show the mean mortality ± SEM (n = 2 replicates). Statistical analysis was performed by two-way ANOVA followed by Tukey's post-hoc analysis. Inset: Different letters indicate significant differences ($P < 0.05$) among groups.

gene expression levels in the CP-pcDNA3.1 group. This was concomitant with a delay in the start of the mortality of the challenged fish, the data of which indicated some protective effect of the empty plasmid, as previously described in other studies dealing with DNA vaccination (Kim et al., 2000; Liang et al., 2010).

We failed to detect any NNV capsid gene transcription in the posterior gut of vaccinated fish from 7 days onwards. This issue could mean that the DNA vaccine shows a very short and transitory expression by the host in the posterior gut while conferring partial protection at the same time. Our post-vaccination results also showed no alterations in total serum IgM levels in any group after 90 days of vaccination. In addition, we failed to detect specific IgM antibodies against NNV or neutralizing antibodies in any vaccinated group. Interestingly, the expression of *mhc2b* and *igmh* genes was unchanged in the posterior gut of vaccinated sea bass at almost all time points except at 7 days post-vaccination, where we found an up-regulation of *igmh* gene expression in the CP-pcDNA3.1 and CP-pNNV groups, which is similar to that of the *igth* transcription after 30 and 90 days post-vaccination. Moreover, many of the studies dealing with DNA vaccines in fish, regardless of the administration route used, demonstrate that this type of vaccine elicits acquired immunity by producing specific antibodies against a virus at different time point that range from 1 month to 6 months post-vaccination (de las Heras et al., 2010; MacLaughlin et al., 1998; Vimal et al., 2014; Zheng et al., 2010). However, in some other trials, specific antibodies were not detected despite good protection being observed (Kurath, 2008; Lorenzen et al., 1998; McLauchlan et al., 2003). Our data are in concordance with these last data that were previously described due to the fact that even when we did not detect specific antibodies against NNV, we observed a clear delay in the beginning of mortalities and a RPS of 45% at the end of the trial in the CP-pNNV vaccinated fish. All these data point to specific cellular antiviral mechanisms involved in fish protection upon vaccination with DNA vaccines. In that sense, and taking into account that the IFN pathway and the CMC are considered as the major immune mechanisms to fight viral infections in fish (Ellis, 2001; Robertsen, 2006), we studied those mechanism at the gene expression levels. Our data show that the orally administered vaccine stimulated the expression of the *tcrb* and *cd8a* genes, suggesting the activation of the CMC status in the gut but further studies need to prove this.

This is the first study in which a prominent role of the CMC-related genes triggered by an encapsulated, orally administered DNA vaccine has been reported in fish, although it had also been demonstrated in mammals and other vertebrates (Jazayeri et al., 2012; Nixon et al., 1996). Interestingly, one study also reported an increase in the CMC antiviral activity after intramuscular DNA vaccination in fish (Utke et al., 2008). Moreover, in previous *in vivo* infections with NNV, we observed that innate CMC activity, as well as some gene transcription (e.g. NCCRP-1) related to this activity, were greatly activated; whereas immune responses played by phagocytes (monocyte-macrophages and granulocytes) such as phagocytosis or respiratory burst activities seem to be less relevant upon NNV infection in the European sea bass (Chaves-Pozo et al., 2012). Furthermore, it has been demonstrated that infected orange-spotted grouper (*Epinephelus coioides*) increased the *cd8a* gene expression, the number of $CD8^+$ lymphocytes and the specific cytotoxic activity against NNV-infected cells, in an MHC I-restricted manner. However, the transcription of CMC-related genes does not appear to be affected by NNV infection in all fish species (Chang et al., 2011; Patel et al., 2008; Scapigliati et al., 2010).

As expected, the protection seem to be also orchestrated by an early phase of non-specific IFN mechanisms due to the up-regulated expression of *ifn* (30 days), *mx* (7 and 30 days), and of the *ifng* (30 and 90 days) genes upon vaccination. In fact, this *ifng* up-regulation might also point to the activation of the CMC since cytotoxic cells are the main producers of IFNγ. These results are in concordance with those found in juvenile specimens of European sea bass upon NNV infection *in vivo*, where different genes involved in the IFN pathway were greatly stimulated in the brain (the target tissue of the virus) and the gonad (Valero et al., 2015), suggesting the same unspecific immune response after vaccination. Similarly to our data, in another DNA vaccination study performed in rainbow trout, early enhancement of IFN mechanisms were reported (Utke et al., 2008).

Independent of the mechanism of action of the oral DNA vaccine used in this study, our data show that after 90 days post-vaccination, the CP-pNNV vaccinated fish showed clear and complete protection against NNV until day 21, when mortality started. Taking into account that mortality in the control group and the CP groups started around day 4 and reached 100% before day 21 post-challenge, and that CP-pNNV vaccinated fish kept the 45% survival proportion at the end of the challenge, we can conclude that the encapsulated DNA vaccine used in this study partly protect the fish

from NNV infection.

5. Conclusion

Our results show that the designed oral DNA vaccine against NNV encapsulated in chitosan nanoparticles up-regulates the transcription of genes related to the CMC and the IFN immune responses in the posterior gut, but fail to trigger the production of specific antibodies after 90 days of vaccination. However, at this time, the infection with a lethal dose of NNV results in a relative survival of 45% of the vaccinated fish. Although this protection is slightly lower than that observed in other studies cited here, most of them evaluate the protection at a few weeks or a month post-vaccination, but not for the length of time shown in our study (3 months). Therefore, further studies are needed to understand the immune response in the gut after oral DNA vaccination and to improve the oral DNA vaccines against NNV in order to ameliorate or abolish the incidence of VER disease and subsequent mortalities.

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