Developmental and Comparative Immunology 65 (2016) 64–72

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

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☆

Yulema Valero [a], Elham Awad [b], Francesco Buonocore [c], Marta Arizcun [a], M. Ángeles Esteban [d], José Meseguer [d], Elena Chaves-Pozo [a], Alberto Cuesta [d, *]

[a] Centro Oceanográfico de Murcia, Instituto Español de Oceanografía (IEO), Carretera de la Azohía s/n, Puerto de Mazarrón, Murcia, Spain
[b] Department of Hydrobiology, National Research Center, Giza, Egypt
[c] Dipartimento per l'Innovazione nei Sistemi Biologici Agroalimentari e Forestali, Università della Tuscia, Italy
[d] Fish Innate Immune System Group, Department of Cell Biology and Histology, Faculty of Biology, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, Murcia, Spain

ARTICLE INFO

Article history:
Received 6 April 2016
Received in revised form
26 June 2016
Accepted 26 June 2016
Available online 28 June 2016

Keywords:
Oral vaccine
Chitosan
Nodavirus
European sea bass
Cell-mediated cytotoxicity
Interferon

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.

* Corresponding author. Department of Cell Biology and Histology, Faculty of Biology, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, 30100, Murcia, Spain.
E-mail address: alcuesta@um.es (A. Cuesta).

http://dx.doi.org/10.1016/j.dci.2016.06.021
0145-305X/

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