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Vaccination with UV-inactivated nodavirus partly protects European sea bass against infection, while inducing few changes in immunity

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ABSTRACT

Developing viral vaccines through the ultraviolet (UV) inactivation of virus is promising technique since it is straightforward and economically affordable, while the resulting viruses are capable of eliciting an adequate antiviral immune response. Nodavirus (NNV) is a devastating virus that mainly affects European sea bass juveniles and larvae, causing serious economic losses in Mediterranean aquaculture. In this work, a potential vaccine consisting on UV-inactivated NNV (iNNV) was generated and administered to healthy juveniles of European sea bass to elucidate whether it triggers the immune response and improves their survival upon challenge. First, iNNV failed to replicate in cell cultures and its intraperitoneal administration to sea bass juveniles also failed to produce fish mortality and induction of the type I interferon (IFN) pathway, indicating that the NNV was efficiently inactivated. By contrast, iNNV administration induced significant serum non-specific antimicrobial activity as well as a specific antiviral activity and immunoglobulin M (IgM) titres against NNV. Interestingly, few changes were observed at transcriptional level in genes related to either innate or adaptive immunity, suggesting that iNNV could be modulating the immune response at protein or functional level. In addition, the iNNV vaccinated group showed improved survival, reaching a relative survival percentage of 57.9%. Moreover, challenged fish that had been vaccinated presented increased serum antibacterial, antiviral and IgM titres, as well as the higher transcription of *mhc1a*, *ifn*, *isg15* and *cd8a* genes in brain, while in the head-kidney the transcription of *mhc1a*, *mhc2b* and *cd8a* was down-regulated and *mx*, *isg15* and *tcrb* was up-regulated. Although the UV-inactivated vaccine against NNV showed promising results, more effort should be addressed to improving this prophylactic method by increasing our understanding of its action mechanisms, thus enabling the mortality rate of NNV to be further reduced.

1. Introduction

For viral vaccine development purposes, viruses can be inactivated by several chemical or physical methods; formaldehyde, β-propiolactone (BPL) or binary ethylene imine (BEI) being the most widely used chemicals. However, physical inactivation is more practical since the resulting vaccines could be considered chemical-free products. Thus, viral inactivation by irradiation, including ultraviolet (UV), mainly UV-C (200–280 nm), offers a promising tool for vaccine development since it is easy, affordable and fast. UV induces dimer formation between adjacent pyrimidines in RNA, blocking the RNA molecule as a transcription template, but can also produce significant alterations in the coat proteins (Delrue et al., 2014). However, although virus inactivation by UV is obviously feasible, its potential use and efficacy for vaccination is controversial since it is unclear whether these kinds of vaccine induce proper immunity, and any protection greatly depends on the virus isolate and the severity of the UV-exposure. Preliminary studies in mammals indicated that eastern equine

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encephalomyelitis (EEE) and rabies (CVS) virus inactivated by UV were not suitable for vaccinating mice since no protection was conferred (LoGrippo, 1958). More recently, UV-inactivation of the murine leukemic virus (Cas) strain Cas-Br-M was seen to induce a strong cytotoxic T-lymphocyte (CTL) response in mice, protecting them against disease and inhibiting viral replication (Sarzotti et al., 1994). Furthermore, UV-inactivated porcine reproductive and respiratory syndrome virus (PRRSV) induces virus-specific and neutralizing antibody responses (Vanhee et al., 2009). By contrast, UV-inactivated vaccines consisting of foot and mouth disease virus (FMDV) failed to induce correct antibody response (Mahdy et al., 2015), while, in the case of influenza A, the vaccine failed to induce protection or an antibody and CTL response (Furuya et al., 2010). Therefore, although UV is useful for inactivating a virus, its potential use for vaccine development needs to be cautiously evaluated.

Aquaculture is a fast and highly growing industry worldwide. For this sector, diseases triggered by viruses and the lack of effective vaccines against them are bottleneck factors for its success. Among such viruses, nodavirus (NNV) is the causal agent of viral encephalopathy and retinopathy (VER), which mainly alters brain and retina structure and function, causing mortality rates of up to 100% in more than 50 fish species (Munday et al., 2002; OIE, 2013). NNV is a non-enveloped bipartite single stranded RNA virus composed of 2 RNA strands in positive sense, RNA1 coding for viral RNA-dependent RNA polymerase (RdRp), and RNA2 coding for the capsid protein (CP), which composes the virus coat by assembling multiple units of the single protein (Delsert et al., 1997; Munday et al., 2002; Sommerset and Nerland, 2004; Tan et al., 2001). European sea bass (*Dicentrarchus labrax*) is a very susceptible species to this virus which can induce up to 100% mortality, mainly in juveniles and larvae stages (Breuil et al., 1991), affecting negatively the Mediterranean aquaculture. To date, much effort has been put into obtaining a deeper knowledge of European sea bass immunity, cell-mediated cytotoxicity (CMC), antimicrobial peptides (AMPs) and interferon (IFN) responses having been identified as pivotal mechanisms against NNV (Chaves-Pozo et al., 2012; Novel et al., 2013; Scapigliati et al., 2010; Valero et al., 2015a; b; c; Valero et al., 2016a). Despite the great negative impact of NNV in fish farms, all vaccine types tested so far have failed to totally eradicate the mortalities elicited by this virus. Thus, recent studies have reported different types of vaccine against NNV, such as live/inactivated NNV, virus-like particles (VLPs), DNA or recombinant proteins, all of which only produced partial protection in fish (Kai and Chi, 2008; Kai et al., 2014; Lin et al., 2016; Luu et al., 2017; Nishizawa et al., 2012; Núñez-Ortiz et al., 2016; Oh et al., 2013; Sommerset et al., 2003; Valero et al., 2016b; Vimal et al., 2014). Most studies have focused on inactivated vaccines but always using chemicals such as formalin or BEI (Kai and Chi, 2008; Kai et al., 2014; Núñez-Ortiz et al., 2016; Pakingking et al., 2010, 2011), but no study has addressed the efficacy of UV-inactivated vaccines on NNV infection. For practical purposes there is only a single commercial vaccine (ALPHA JECT micro® 1 Noda; PharmaQ), consisting on inactivated NNV, with limited application to sea bass in some Mediterranean countries but its effectiveness is not reported yet.

UV inactivation of aquatic virus has been widely evaluated (Lytle and Sagripanti, 2005). Interestingly, viruses of the family *Rhabdoviridae* (VHSV) are the most susceptible to UV-C radiation, while viruses of the families *Birnaviridae* (IPNV) and *Nodaviridae* (NNV) are the most resistant (Frerichs et al., 2000; Oye and Espen, 2001). In fact, while the World Organization for Animal Health (OIE) recommends the use of 10 mJ/cm^2 of UV to inactivate most aquatic viruses and bacteria the dosage is increased to 125–200 mJ/cm^2 for IPNV and NNV. Unfortunately, even considering its potential application, little effort has been directed towards generating and testing UV inactivation for fish virus vaccines. Only one study has tested a vaccine against infectious Salmon anaemia virus (ISAV) in Atlantic salmon (*Salmo salar*) (Rivas-Aravena et al., 2015). In this case, UV-inactivated ISAV was encapsulated in chitosan and administered orally. Upon challenge, the vaccine elicited a partial relative protection (RPS) of 39%, which increased to 77% when the vaccine contained a DNA adjuvant. Interestingly, when the immunity was evaluated, no antibodies were detected in serum and the expression of immune-related genes suggested that the vaccine is capable of stimulating the innate immune response through IFNα and IFNγ, but not cellular immunity, and regulated by the stimulation of interleukin (IL)-10 and tumour growth factor (TGF)-β (Rivas-Aravena et al., 2015). Given the lack of knowledge about the efficiency of viral UV-inactivated vaccines for fish this work looks at the inactivation of NNV by UV irradiation and studies the immune response triggered in healthy European sea bass juveniles by vaccination and challenge with NNV, and the rates of protection offered.

2. Material and methods

2.1. Animals

European sea bass juveniles (*Dicentrarchus labrax*; 10–12 g body weight) were bred in the facilities of *Instituto Español de Oceanografía* in Mazarrón (COM-IEO, Spain) and transported to the University of Murcia (Spain). Fish were kept in 250 L running seawater (28‰ salinity) aquaria at 24 ± 2 °C, with a 12 h light:12 h dark photoperiod and fed daily with 3% biomass of a commercial pellet diet (Skretting). Before sampling, all specimens were anesthetized with 40 μL of clove oil, completely bled and immediately beheaded and weighed. 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 (Permit Number: A13150104) and the *Instituto Español de Oceanografía* (Permit Number: 2010/02) for the use of laboratory animals.

2.2. Nodavirus (NNV) stocks

Nodavirus (NNV; strain It/411/96, genotype RGNNV) was propagated in the E−11 cell line. Cells were inoculated with NNV and incubated at 25 °C until the cytopathic effect (CPE) was extensive. The supernatant was harvested and centrifuged to eliminate cell debris. Virus stocks were titrated in 96-well plates before use in the experiments (Reed and Müench, 1938).

2.3. Preparation of vaccine

A previous study demonstrated that UV exposure to 254 nm with a dose of 440 μW/cm^2 for 10 min (equivalent to 264 mJ/cm^2) resulted in the complete inhibition of NNV infectivity (Frerichs et al., 2000). Based on this, and to ensure complete NNV inactivation, 100 μL of a NNV batch of 10^{10} $TCID_{50}$/mL were diluted 100-fold with phosphate buffer (PBS) and exposed to UV-C (254 nm; Bio-Link) with a total dose of 800 mJ/cm^2. To verify the NNV infectivity, inactivated NNV (iNNV) was cultured by 2 successive passages on E−11 cultures at 25 °C for 10 days. In addition, cell cultures were processed for RNA isolation and NNV detection by PCR as described below.

2.4. Fish vaccination

European sea bass fish specimens were randomly divided into 4 aquaria (250 L each) forming two experimental groups in duplicate. Fish were gently sedated by 20 μL of clove oil and vaccinated as follows: one group was intraperitoneally (ip) injected with 100 μl per fish of PBS (Control) while the other group received a single ip injection with 10^7 $TCID_{50}$/fish (iNNV). After vaccination, fish (n = 6 fish/group and time) were sampled 1, 15 and 30 days post-vaccination (dpv). Blood was obtained from the caudal peduncles and serum samples by centrifugation at 10,000 g for 10 min at 4 °C and immediately stored at −80 °C until use. Head-kidney was removed by dissection, immediately frozen in TRIzol Reagent (Life Technologies) and stored at −80 °C until use.