Nanostructured recombinant protein particles raise specific antibodies against the nodavirus NNV coat protein in sole

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Running title: Nanoparticles induce NNV specific antibodies in sole

Abstract

Nervous necrosis virus (NNV) reassortant strains RGNNV/SJNNV have emerged as a potent threat to the Mediterranean marine aquaculture industry, causing viral encephalopathy and retinopathy (VER) in Senegalese sole (*Solea senegalensis*). In this study, a cheap and practical vaccine strategy using bacterial inclusion bodies made of the coat protein of a virulent reassortant strain of this betanodavirus was devised. The nanostructured recombinant protein nanoparticles, VNNV-C^{NP}, were administered without adjuvant to two groups of juvenile sole, one by intraperitoneal injection and the other by oral intubation. Specific antibodies were raised *in vivo* against the NNV coat protein via both routes, with a substantial specific antibody expansion in the injected group 30 days post homologous prime boost. Expression levels of five adaptive immune-related genes, *cd8a, cd4, igm, igt* and *arg2,* were also quantified in intestine, spleen and head kidney. Results showed *cd4* and *igm* were upregulated in the head kidney of injected fish, indicating activation of an adaptive systemic response, while intubated fish exhibited a mucosal response in the intestine. Neither route showed significant differential expression of *cd8a*. The specific antibody response elicited *in vivo* and the lack of any signs of toxicity over the 6-week study period in young fish (n=100), evidences the potential of the nanoparticle as a vaccine candidate.

Keywords: NNV, protein nanoparticles, vaccine, oral gavage, antibodies, mucosal immunity, viral antigens

1. Introduction

Viral encephalopathy and retinopathy (VER), also known as viral nervous necrosis (VNN), is a serious infectious disease with a high economic impact on the aquaculture of several fish species such as Senegalese sole (*Solea senegalensis*), European sea bass (*Dicentrarchus labrax*), Asian sea bass (*Lates calcarifer*) and groupers (*Ephinephelus spp.*) [1, 2]. The causative agent, viral nervous necrosis virus (NNV) belonging to the family *Betanodaviridae*, is a non-enveloped, small (~30 nm diameter), icosahedral virus with a bi-segmented single-stranded (+) RNA genome. This virus has tropism for the nervous tissue, producing necrotic lesions and vacuolation in the brain, retina and spinal cord of infected fish [3, 4a]. Such damage leads to the typical neurological signs of the disease, such as abnormal swimming, spasms, darkness, anorexia and lethargy [4]. Mortality is particularly high in larvae and juveniles and the virus can be transmitted vertically through broodstock [5] and horizontally through the water body from infected fish and asymptomatic carriers or via live prey used as feed for marine fish larvae (reviewed in [1, 2]). Husbandry practices such as feeding small captured wild fish and squid to farmed species whose biological cycle is not fully controlled could also contribute to horizontal transmission [6].

Structurally, NNV contains two single-stranded, positive-sense RNA segments encoding the polymerase (RNA1) and the capsid protein (RNA2). RNA1 is also involved in determining temperature sensitivity for viral replication [7]. From the 3'end of RNA1, sub-genomic RNA3 is transcribed during viral replication in infected cells, encoding proteins B1 and B2, the latter being an RNA silencing-suppression protein that facilitates accumulation of intracellular viral RNA1 [8]. RNA2 encodes the capsid, or coat protein and contains a variable region which was used to classify four different genotypes [9]: red-spotted grouper nervous necrosis virus (RGNNV), striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV) and barfin flounder nervous necrosis virus (BFNNV). It is the coat protein which plays a key role in infectivity [10].

Recently NNV reassortant strains referred to as RGNNV/SJNNV have emerged in Mediterranean aquaculture, with RNA1 being RGNNV type and RNA2, SJNNV. In a phylogenetic study of 120 betanodavirus isolates from various farmed and wild fish species in Southern Europe, Panzarin et al. (2012) reported 80% were RGNNV genotype, 19.2% were RGNNV/SJNNV, whereas only 0.08% were SJNNV [13]. These NNV reassortants are particularly virulent in Senegalese sole [11, 12], although they can also cause mortality and clinical signs in farmed European sea bass. However in sea bass, the disease is not as severe, nor as widespread as that caused by the RGNNV strain [12, 13]. Genetic reassortment seems to be facilitated by factors related to fish farm housing conditions such as population density,

stress and the incorporation of new fish batches. The reassortant strains analysed have amino acid substitutions in the SJNNV capsid protein (encoded by RNA2), which likely enhance colonization and virulence in new host species [11, 14, 14a]. Worryingly, in 2014-16 hatcheries in Southern Europe started reporting mass mortalities in gilthead sea bream (*Sparus aurata*) larvae which tested positive for RGNNV/SJNNV [15]. Until that time sea bream had been considered resistant to NNV, though they could be asymptomatic carriers. In addition, turbot (*Scophthalmus maximus*) juveniles have been shown to be susceptible to the RGNNV/SJNNV reassortant (SpSs-IAusc160.03) isolated from diseased Senegalese sole [16]. Considering the farming of Senegalese sole is in close proximity to turbot in the Iberian Peninsula and to sea bream in Southern Europe, the interspecies transmission of betanodavirus is currently a major threat for aquaculture [17].

The first commercial vaccine against RGNNV in Europe was released by Pharmaq, ALPHA JECT micro®1Noda. It is a formaldehyde-inactivated culture of RGNNV strain ALV1107 in a liquid paraffin adjuvant for intraperitoneal (i.p.) injection to European sea bass of minimum weight 12 g, with duration of immunity for at least 1 year. This has been recently followed by another inactivated RGNNV vaccine produced by HIPRA using strain 1103, ICTHIOVAC®VNN. This vaccine is recommended to be i.p. injected in a Montanide-based adjuvant to European sea bass of minimum weight 15 g. While these vaccines are suitable for broodstock to avoid vertical transmission, infection otherwise occurs primarily at larval and young stages where i.p. vaccination is not feasible [4]. As a compromise solution, fish may be held in the hatchery until large enough to vaccinate. Both vaccines are appropriate for *D. labrax* and currently, we do not have any data about the protective effects against reassortant RGNNV/SJNNV strains, nor the application of the vaccines in any other species. Cross-reaction of polyclonal antibodies between NNV genotypes has been evidenced but not cross-neutralization, except between RGNNV and BFNNV strains [1,18].

As regards other vaccine developments, apart from inactivated virus, different strategies such as viral like particles (VLPs), DNA vaccines, and recombinant capsid proteins injected i.p. or to muscle (i.m.), have been explored. Using these strategies, protection against a RGNNV or a SJNNV challenge and the raising of neutralizing antibodies has been, for the most part, successfully demonstrated in several species of farmed fish affected by VER in Europe (sea bass and turbot) or in Asia and Oceania (barramundi and groupers) [1]. Recent work in biomaterials science has led us to an alternative approach. Bacteria inclusion bodies (IBs) are biologically active, non-toxic protein nanoparticles which have a propensity to cross cell membranes and can serve as protein releasing agents [19]. They are stable *in vivo* without encapsulation and are cheap and easy to produce as recombinant protein nanoparticles [20]. In addition, they carry immunostimulants for fish in the form of remnants of bacterial lipopolysaccharide (LPS),

peptidoglycans and nucleic acids [21]. We have already demonstrated that IBs made of the RGNNV/SJNNV coat protein, induce a strong, innate anti-viral immune response *in vitro* in zebrafish liver cell line (ZFL), as well as being readily taken up *in vivo* through the zebrafish intestine after oral administration [22]. In this study, we produced these IBs or "nanopellets" (NPs) in *Escherichia coli* using the coat protein of the betanodavirus reassortant strain (SpSs-IAusc160.03) [11]. We tested these IBs, called VNNV-C^{NP}, in juvenile Senegalese sole to determine their capacity to elicit specific antibodies against the NNV coat protein as a surrogate of protection. Finally, we compared the differences between i.p. injection and the oral route using a novel oral gavage (intubation) method to sole that permits delivery of precise amounts of nanoparticle to young fish. Overall, the results presented in this study represent a new, smart vaccine preparation approach against the reassortant RGNNV/SJNNV strain with view to providing a practical tool to combat VER in Senegalese sole, European sea bass, gilthead sea bream and turbot.

2. Materials and Methods

2.1 NNV coat protein production

2.1.1. VNNV-C^{NP} nanoparticle

The protein nanoparticle VNNV-C^{NP} was designed based on the NNV coat protein from the Iberian betanodavirus isolate (strain SpSs-IAusc160.03), NCBI GenBank, accession no: NC_024493. This betanodavirus is a reassortant RGNNV/SJNNV strain with a modified SJNNV capsid amino acid sequence [11, 14a]. Molecular cloning of the targeted coat protein and IB production in *E. coli* were as previously described in [22].

2.1.2 Soluble NNV for ELISA

The clone containing the coat protein gene used to prepare the IBs (VNNV-C^{NP}) was used to produce soluble NNV coat protein-His6 by lowering the culturing temperature to favour recombinant soluble production. Briefly, *E. coli* was cultured at 37ºC in Luria Bertani (LB) with ampicillin (100 µg/mL; Sigma) until OD_{550} nm reached 0.5-0.8. Then protein expression was induced by adding 1 mM IPTG (Panreac), culturing at 16ºC overnight. To confirm production of soluble NNV coat protein-His6, an equivalent number of cells from a sample of the cultures (before adding IPTG and after overnight culture) were collected by centrifugation at 5,000 x *g* for 15 min at 4 ºC and resuspended in 1 mL PBS with protease inhibitor following manufacturer's instructions (cOmplete-EDTA free, Roche); then cells were sonicated on ice and centrifuged at 15,000 x *g* for 15 min at 4 ºC to collect the soluble protein fraction (supernatant). Aliquots were run on a western blot and the protein was detected using an anti-His-tag antibody

(GenScript A00186-100) (**Supplementary Fig. S1 A)**. For use in ELISAs, soluble protein was purified by Ni affinity chromatography on FPLC AKTA Purifier 10 system (GE Healthcare) via the C terminal His-tag. Purification was performed using 1 mL HiTrap Chelating HP column (GE Healthcare), eluting with an imidazole gradient (500 mM imidazole, 500 mM NaCl, 20 mM Tris HCl, pH 7.4). Fractions were tested for the presence of NNV coat protein-His6 via Western blot (**Supplementary Fig. S1, B and C**). Eluates from the peaks were pooled when the western blot was positive for the targeted coat protein. The collected fractions were dialyzed at 4 ºC with stirring overnight using a cellulose membrane (Spectra, 6-8 kD MWCO) against 20 mM Tris-HCl, 5% Dextrose buffer pH 7.4. Traces of precipitate were removed by centrifugation at 15,000 x *g* for 30 min at 4 ºC. Soluble protein concentration in the supernatant was determined by a Bradford assay and aliquots were filtered and stored at -80ºC until use.

2.2 Fish and experimental design

Senegalese sole juveniles were supplied by Cupimar (Cadiz, Spain) and maintained at IFAPA, El Toruño (Instituto de Investigación y Formación Agraria y Pesquera), El Puerto de Santa María, Cádiz, in a flow-through system of filtered sea water. A total of 100 specimens with average weight 15.21 ± 1.93 g were selected for the trial. All procedures were authorized by the Bioethics and Animal Welfare Committee of IFAPA and were given registration number 10-06-2016-102 by the National authorities for regulation of animal care and experimentation.

To carry out the trial and minimize environmental effects, animals were tagged using a two-colour code by subcutaneously injecting a thin line of visible implant elastomer (VIE) (Northwest Marine Tech) in the non-pigmented side as previously described [23, 24]: half of the specimens were tagged in green at the cranial part of body to identify the intraperitoneally (i.p.) injected group and the other half in red at the caudal end for the orally intubated group (**Supplementary Fig. S2**). Before handling, all fish were anesthetized using 99% 2-phenoxyethanol (Panreac) 0.4 mL L^{-1} (sedation 0.1 mL^{-1}, euthanasia 0.6 mL^{-1}). After tagging, fish were distributed into two tanks for 3 days until commencing the trial. In this period, no mortality or infections were registered. Throughout the experiment they were given commercial fish feed daily (1.5 mm diameter, 2% of biomass), except for 24 h prior to VNNV-C^{NP} treatments or samplings.

For the trial two administration routes were used: i.p. injection as a reference method to monitor the action of the nanoparticles as a potential immune stimulus, and oral intubation as a proxy for uptake of the nanoparticle in feeds. For the latter route we established a novel approach to precisely administer the experimental doses. In brief, the anaesthetized fish, with the non-ocular side exposed, was held lightly around the jaw to open the mouth and a fine and semirigid 1.0 mm diameter veterinary cat catheter (Henry Schein Inc.) was inserted softly down the digestive tract (approx. 35 mm depth). Once the volume was delivered (80 µL), the catheter was

withdrawn slowly to avoid regurgitation. Previous tests established the feasibility of the method and the appropriate administration volume using a green dye to confirm the volume was sufficient to reach the intestine (**Fig 1**).

For each administration route (green i.p., and red oral**; Fig. 1 and Fig. S2**), a total of 44 fish were used: 14 were PBS as a control, 14 with 50 µg of VNNV-C^{NP} (dose 1) and 16 with 500 µg of VNNV-C^{NP} (dose 2). The remaining 6 animals of each tag colour were used as untreated controls. For i.p. administration, the volume injected was 100 µL using a 25G needle. For oral intubation, the syringe was shaken between each administration to ensure the particles were in a homogeneous suspension. After the administration, treated fish were distributed in triplicate tanks (n=28,28,32) by treatment (PBS, dose 1 and dose 2) to avoid environmental effects between administration routes. The untreated controls (n=12) were maintained in a separate tank. All tanks were cylindrical, 0.8 m^2 surface in an open flow-through circuit and water renewal each 2 h. Dissolved oxygen ranged from 7.3 to 8.3 mg/L and temperature from 18.4 ± 1.4 ºC, with a natural night/day light regime. On day 14 after starting the experiment, a booster was carried out using the same doses and delivery route as previously (oral intubation or i.p. injection). Survival and welfare were monitored daily throughout the 6 weeks that spanned the experiment.

2.3 Tissue and blood collection

Animals (n=4) were sampled at 3 days post-booster (dpb). Fish were anesthetized as indicated above, weighed (average 16.6 ± 2.4 g) and blood was taken by puncturing the caudal vein. Then animals were immediately euthanized followed by a quick incision in the spinal cord, and tissue samples for intestine (~110 mg), spleen (~30 mg) and head kidney (~ 60 mg) (wet weight) were taken, immersed in RNAlater (Sigma) and stored at -80 ºC until use. The sex of each animal was also recorded (ratio males:females = 1:3) and animals were checked for signs of any alterations to organs or lesions. Blood was left to coagulate 1.5-2 h on ice and then centrifuged at 3,000 x *g* for 20 min at 4 ºC. Sera was recovered and stored at -80 ºC. The remaining fish were mantained for four weeks in the tanks and sampled at 30 dpb using the same procedure as above: four fish per treatment were anaethesized, weighed (26.4 ± 4.3 g) and blood was taken. Then, animals were euthanized, sexed (ratio males:females = 1:2) and tissues dissected and conserved in RNAlater at -80 ºC. The experimental pipeline, described in sections 2.2 and 2.3, is summarised in **Fig 2**.

2.4 Antibody response (ELISA)

To determine if fish treated with VNNV-C^{NP} raised specific antibodies against the NNV coat protein, indirect ELISAs were performed using serum samples at 3 and 30 dpb. Briefly, Maxisorp 96 microwell plates Nunc) were coated with 1 µg $well^{-1}$ of purified soluble NNV coat

protein-His6 (see section 2.1.2) in 50 µL well^{-1} carbonate buffer (100 mM $NaHCO_3$, pH 9.2) overnight at 4 ºC. All further steps were carried out at room temperature (RT). Washes were performed in triplicate with TTN buffer (50 mM Tris, 0.05% Tween 20, 0.15 M NaCl, pH 7.4), blocking with TTN + 3% BSA (Sigma) for 1.5 h, and serum dilutions were prepared with PBS, 0.05% v/v Tween 20 + 0.5% BSA. Each serum dilution was added in duplicate at 100 µL well^{-1} and incubated for 2 h. The primary antibody was a polyclonal anti-Senegalese sole Ig produced in rabbit on order from Sigma-Aldrich. Secondary antibody was an HRP conjugated mouse monoclonal anti-rabbit IgG, γ-chain specific (Sigma A1949). Detection was via 3,3'5,5' tetramethylbenzine (TMB) substrate reagent set (BD Biosciences). Absorbance was measured at 450 nm on a spectrophotometer (Victor 3, PerkinElmer). The specific antibody titre was defined as the inverse of the greatest dilution which still gave a positive result. Controls were sera from fish injected or intubated with PBS. Other controls were wells coated with purified *S. senegalensis* Ig as a positive technical control for the polyclonal antibody, a reference serum extracted from adult Senegalese sole with high levels of IgM and a negative control without serum.

2.5 RNA extraction and RT-qPCR

RNA was extracted from intestine, spleen and head kidney taken 3 days post booster (section 2.3). Tissue was removed from RNAlater, blotted and placed in 200 µL of chilled 1-Thioglycerol Homogenization solution (Promega Maxwell^R RSC simply RNA Tissue kit). Tissue was homogenized in a QIAGEN TissueLyser for 2 rounds of 6 min at 50 Hz using 5 mm stainless steel balls and stored on ice for immediate extraction or at -80 ºC for longer term. Following the Promega Maxwell kit instructions, 200 µL of lysis buffer was added, the mixture was vortexed well and RNA was extracted in an automated nucleic acid purification system including DNAse treatment (Maxwell RSC Instrument, Promega). RNA was eluted in 45 µL of nuclease free water. RNA yield and quality were determined on nanodrop ND-1000 (Thermo Fisher Scientific) and integrity was assessed on the Agilent 2100 Bioanalyzer using the RNA 6000 Nano Lab-Chip kit (Agilent Technologies), achieving RINs of 8.5-10. Then, cDNA was synthesized from 1 µg high quality total RNA using the iScript cDNA systhesis kit (Bio-Rad) according to manufacturer's instructions.

To evaluate the response to nanoparticles a set of five genes involved in the adaptive response were selected: cluster of differentiation 8a (*cd8a*), cluster of differentiation 4 (*cd4*), immunoglobulin M (*igm*), immunoglobulin T (*igt*) and arginase 2 (*arg2*). Sequences were retrieved from SoleaDB [25]. Identities were checked in NCBI gene bank and primers were designed using NCBI Primer 3 and Oligoanalyzer 3.1 (Integrated DNA Technologies). The primer sequences and unigene name are listed in **Supplementary Table S1**. Primers for the

reference gene *eef1a* were previously published [26]. Quantitative real-time PCR (qPCR) was performed at 60°C annealing temperature using iTaq Universal SYBR Green Supermix (Bio-Rad) with 250 nM of primers and 2.5 µl of cDNA previously diluted to 1:5 for the target and 1:50 or 1:200 for the reference gene, *eef1a*. All the samples (n=4 per treatment) were run in triplicate, and data were analysed for individual replicates using the comparative Ct method with the PBS control as the calibrator group [27]. Primer efficiencies are depicted in **Supplementary Fig. S3**.

2.6 Statistical analysis

Prior to statistical analyses all data were checked for normality using the Shapiro-Wilk test. When normality was not accomplished, a non-parametric test was used (Wilcoxon Rank Sum Test). Statistical analyses were performed using Prism 8.1 software (GraphPad). Data are shown as mean ± standard deviation (SD). Comparisons of means for each experimental group versus control, or between treatments were performed using a one-way unpaired t-test with Welch's correction for unequal variances. $P < 0.05$ was considered statistically significant in all analyses.

3. Results

3.1 Fish survival and welfare

Fish did not show any signs of disease or toxicity after nanoparticle administration. There was just one animal death in the PBS control group during the first 24 h after the initial i.p. injection, which we assume was due to the handling procedure. There were no deaths of intubated fish. When blood and tissue samples were collected at 3 and 30 dpb the animals were checked for abnormalities. No signs of morphological alterations were observed such as softening of liver or enlarged spleen. The eyes of the fish were bright and skin healthy.

3.2 Specific antibody response

Fish immunized with VNNV-C^{NP} raised specific antibodies against the NNV coat protein in both routes although to a different extent (**Fig. 3**). As we have measured serum antibodies and IgM is the prevailing isotype in fish sera [28], we refer to the antibodies detected here as anti-NNV coat protein IgMs. In injected fish (Fig. 3A and C), specific anti-NNV coat protein IgMs were significantly higher ($P<0.05$) than the control at 3 dpb. Both low and high VNNV-C^{NP} doses evoked a similar amount of specific antibody with titer 2500. Later at 30 dpb, antibody amounts were greater in animals supplied the high (dose 2) compared to the low (dose 1) group (titer for both groups ≥ 12500. Note **Fig 3A ii**). Interestingly, the titration curve using the pooled adult serum sample indicated the presence of some antibodies recognizing the NNV coat protein

compared to untreated control, but the levels are far less than those found in VNNV-C^{NP} vaccinated juveniles (P<0.05).

In orally intubated fish, the specific antibody response was present but low (**Fig 3B and C**). Nevertheless, fish supplied the high dose at 3 dpb and those supplied both doses at 30 dpb had higher levels than the control. **Fig. 3C** plots all treatment groups at 1:100 serum dilution, clearly showing the systemic IgM response after injection or orally supplying the nanoparticles.

3.3 Gene Expression (qPCR)

To gain more insight into the antibody production, the transcript levels of five adaptive immune-related genes at 3 dpb was quantified. **Fig. 4** upper panel depicts the relative expression levels in fish administrated 500 µg of VNNV-C^{NP} (dose 2) via injection (A) or intubation (B) with respect to the PBS control in spleen, intestine and head kidney. At this dose, the *igm* and *igt* mRNAs were up-regulated in the intestine in both administration routes, though *igm* amounts were particularly high in injected fish, 9.1-fold compared to 2.4-fold in intubated fish, while *igt* appeared more prominently activated in intubated fish, 7.0-fold compared to 4.2-fold in injected fish. The *arg2* mRNAs were also significantly up-regulated in the intestine of the intubated group (1.9-fold). In the spleen, a down-regulation of *igt* in injected fish and *cd8a* and *arg2* in intubated fish was observed. In the head kidney, *cd4* and *igm* transcripts were up-regulated in the injected fish, 2.2- and 2.6-fold, respectively. No significant changes in the intubated fish were detected. In all scenarios the only significant response for *cd8* was a decrease of mRNA levels (2.4-fold) in the spleen of intubated fish.

The expression of *igm*, *igt* and *arg2* were further analysed in fish treated with low and high VNNV-C^{NP} doses in all the tissues sampled. **Fig. 4** lower panel shows the gene expression results according to administration route and nanoparticle dose in intestine. In this tissue (**Fig. 4C**), there was a dose-dependent up-regulation of *igm* (6.0- and 9.1-fold) and *igt* (3.4- and 4.2-fold) transcripts in injected fish, and of *igt* (2.5- and 7.7-fold) in intubated fish. While the up-regulation of *igt* in the intestine was expected, the up-regulation of *igm* was intriguing although *igm*+ B cells have been reported in the intestine of rainbow trout and carp; see [29] and references therein. The *arg2* mRNAs were down-regulated (2.6-fold), though not significantly, in fish injected with a low dose of nanoparticles and significantly up-regulated (1.9-fold) in fish intubated with a high dose. The gene expression dose response data for spleen and headkidney are presented in Figure 4S of the supplementary material. In spleen (**Fig. S4A**), the *igm* mRNAs were up-regulated (2.7-fold) and *arg2* transcripts down-regulated (-2.4-fold) in fish i.p. injected with a low dose of nanoparticles. In intubated fish, the only significant change was the down-regulation of *arg2* expression (1.9-fold) with a high dose of nanoparticles. In head kidney (**Fig.**

S4B) the only significant changes were the up-regulation of *igm* transcript amounts in injected fish (6.1- and 2.6-fold for low and high dose, respectively).

Discussion

This study presents a novel vaccination strategy using VNNV-C^{NP}. We demonstrate that these nanoparticles can enhance the production of specific antibodies against the VNNV capsid protein *in vivo*. This is a key surrogate of protection [30] and it has been achieved in a primary target group for vaccination: juvenile fish of a highly susceptible farmed species, the flatfish Senegalese sole [12]. Further, there was a considerable increase in antibody production after i.p. homologous prime boost indicating that immune memory was triggered. In gene expression studies, *cd4* and *igm* were up-regulated in the head kidney of i.p. vaccinated fish (**Fig. 4A**), indicative of the activation of an adaptive systemic response. The immunogenic potential of this protein nanoparticle in *S. senegalensis* is therefore substantial and was evidenced without adding any adjuvant.

Our data indicate that the response appears to be a humoral rather than a cellular response, as *cd8a* was not significantly differentially expressed in any of the conditions tested. Recombinant protein antigens, being non-replicative vaccines, are delivered into the cell via the exogenous pathway and a CD8+ T-cell response is only thought possible via cross presentation [31]. Indeed, the majority of commercial aquaculture vaccines against viral diseases are non-replicative, using inactivated whole virus (IWV) and entering via the exogenous route [31]. Good protection is generally achieved using IWV vaccines when injected with adjuvant to enhance responses [32].

Apart from the injection route, we also tested VNNV-C^{NP} via oral administration. This practical route is applicable to fish of any size, is cheap for farmers and non-stressful for fish [33]. It is therefore highly desirable. In this study, a feasible oral intubation method was set up for sole. Oral intubation methods have been published for zebrafish [34] and trout [35] and are applicable for testing many compounds such as toxins and immunostimulants, as well as oral vaccine candidates. The method optimized for sole is fast and easy to set up, not difficult to perform and fish showed no subsequent signs of malaise. Success of the method is shown by the gene expression results for intestine (**Fig. 4C**), since *igt* and *arg2* were up-regulated in a dose dependent fashion in intubated fish. Also, systemic antibodies were raised to a different extent depending on oral dose (**Fig. 3B and C**). The method therefore can deliver precise doses to the gut without injuring the fish.

Concerning the antibodies raised and gene expression in orally intubated fish, a dose dependent mucosal immune response was observed; *igt* was up-regulated in intestine (**Fig. 4C**), but the systemic antibody response was low (**Fig. 3B and C**), especially when considering the potential immunogenicity demonstrated in the injected group. In addition, using oral intubation with the high dose, antibodies did not increase after the homologous booster, rather there was a slight drop (**Fig. 3C**). This could be a sign of antibody suppression or deletion (anergy) which is a form of tolerance (hyporesponsiveness) reported to occur with high doses of orally delivered antigen in mammals [36]. In this vein, a study in salmon reported a reduction in serum antibodies after two oral administrations 7 weeks apart using alginate encapsulated inactivated IPNV [37]. In contrast, other authors showed antibodies increased with time when an oral vaccine was delivered 3 days/week for two months. But the same vaccine induced tolerance when given 5 days per month [38]. The tuning of dose and administration regime is therefore critical to achieve a systemic response. Indeed, regulatory T-cell (Treg) induction is the form of tolerance associated with low antigen doses and tolerance is the "default immune pathway" in the intestine [36]. In our case, the low dose was more favourable than the high, but still it only weakly induced systemic antibody production.

In order to optimise a response via the oral route there are various points to consider. First, the protection of nanoparticles to resist the pH and enzymatic degradation in the gut and reach satisfactorily the immune related cells in the lamina propia. The lamina propia along with intraepithelial lymphocyte compartments (IEL) are the main effector sites in the teleost gut-associated lymphoid tissue (GALT) [29]. With flow cytometry and analysis of tissue sections, our group has previously demonstrated that the nanostructured proteins are taken up by intestinal cells and reach the lamina propia, using a fluorescently labelled TNFα inclusion body orally administered to trout [39] and zebrafish [34]. We have also demonstrated the nanoparticles used in the present study, VNNV-C^{NP}, are taken up by zebrafish intestinal cells when orally intubated [22]. The average size of the particle, 422 ± 87 nm measured by field emission scanning electron microscopy (FESEM) [22], is similar to the size range of antigen loaded PLGA nanoparticles (450-500 nm) which induced protective immunity against viral haemorrhagic septicaemia virus (VHSV) in juveniles of the flatfish olive flounder [40].

Another consideration is the administration route regimen. While protection elicited by oral vaccines alone has been variable [33], oral administration as a heterologous booster can be effective. Heterologous boosting is known to raise a stronger response than homologous boosting since it is more likely to induce both humoral and cell-mediated immune (CMI) responses [41, 42]. Kole et al., [40] used 2 administration scenarios to olive flounder, a flatfish closely related to Senegalese sole: a primary immersion immunization with inactivated-VHSV-loaded PLGA nanoparticles, followed two weeks later by either 1) a homologous booster by

immersion or 2) a heterologous oral booster, introducing the loaded nanoparticle into the feed for 2 consecutive days. Results in terms of relative percentage survival (RPS) after VHSV challenge were higher for the immersion/oral group than immersion/immersion, 73.3 vs 60% respectively. In another pilot study using an oral/oral administration strategy, only 23.3% RPS was obtained [40]. This exemplifies the need to try different modes and combinations of delivery to optimize the response.

Interestingly, a recent publication has shown that orally vaccinated fish can attain higher levels of specific antibodies post-challenge compared to i.p. injected fish. Whole bacteria producing the recombinant NNV coat protein were used as a vaccine strategy for European sea bass against RGNNV infection. Recombinant *E. coli* (oral rNNV) was delivered in the feed or by injecting lysed bacteria (i.p. rNNV). Both routes produced specific anti-NNV IgM, with the i.p. route achieving higher levels than the oral. However, after challenge, the orally vaccinated fish had greater levels of anti-NNV IgM [43]. For our work, additional trials will be necessary to demonstrate and quantify the functional protection raised by our particles. It is a notable result that we did see specific antibodies raised by VNNV-C^{NP} via the oral route, though at suboptimal levels.

Very promising results on oral vaccination against VER have been reported using VLPs. In convict grouper (*Epinephelus septemfasciatus*), Wi et al. [46] demonstrated 100% survival with i.p. injection of RGNNV VLPs produced in yeast (*Saccharomyces cerevisiae*) against an RGNNV challenge and 57% survival when the VLPs were orally administered [46]. In orange spotted grouper (*Epinephelus coioides*), Chien et al. [47] tested three different routes obtaining the highest RPS with immersion (81.9%), followed by injection (61.4%) and oral administration (52.3%). Concerning advantages of our nanoparticles over VLPs, the greatest difference is in the ease and low production cost of our biomaterial, which is purified by enzymatic and mechanical disruption [39]. This is a far more straightforward and less costly approach than required to produce VLPs [48]. In addition, our nanoparticles can be lyophilized, retaining biological activity [39]. Therefore, transport and storage would not require the cold chain. Another attractive feature we have already mentioned is that impurities from the production process are immunostimulants for fish, namely LPS, peptidoglycan and nucleic acids. To further enhance antigen uptake and presentation, additional adjuvant solutions for mucosal vaccines such as *Vibrio cholerae* toxin or *E. coli* heat-labile enterotoxin are being tested in mammals, reviewed in [49]. The aim is to boost both cellular and humoral responses non-specifically and could be considered for application in fish.

In summary, we have demonstrated the immunogenicity of VNNV-C^{NP} when i.p. or orally supplied to Senegalese sole. The highest response was obtained via i.p. injection observing

enhanced production of specific anti-VNNV coat protein (capsid) antibodies that increased substantially post booster. This is a major step revealing the potential of the particles to activate the adaptive immune response. The particles were also able to raise specific anti-VNNV capsid antibodies when administered orally, but to a lesser extent. The protein nanoparticles were innocuous to the fish, should not accumulate in the environment and bulk production is cheap and feasible. We are therefore very interested to continue development of this nanoparticle as a potential vaccine candidate, with particular relevance for sole, sea bass, sea bream and turbot.

Authorship

RT, NR and MM designed the study. RT, MCB and MA performed the experiments. DT helped produce the nanoparticle and MP the soluble recombinant protein. JJB provided the polyclonal antibody. RT did the data analysis and wrote the manuscript. NR and MM devised and supervised the research. All authors contributed to the preparation of the final manuscript.

Conflict of interest: The authors declare no conflict of interest

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FIGURE LEGENDS

FIGURE 1. Demonstration of oral intubation (gavage) method to juvenile sole. A. *S. senegalensis*, approx. 15 g weight, were anaethetized & placed with ventral side exposed. A fine, 1.00 mm diameter veterinary cat catheter, attached to a 1 mL syringe, was inserted down

the digestive tract to approximately 35 mm depth. Post administration of liquid, the catheter was withdrawn slowly to avoid regurgitation. **B.** Dissected fish showing successful administration to the intestine of up to 80 µL without regurgitation.

FIGURE 2. Experimental pipeline: *S. senegalensis* response to VNNV-C^{NP} administration. Timeline summarizing the experimental steps performed. d= days, i.p.=intraperitoneal

FIGURE 3. Titration curves for anti-VNNV coat protein IgM in *S. senegalensis* sera from juveniles vaccinated with VNNV-C^{NP}, determined by ELISA. Fish (~15g) were administered 50 µg (■) or 500 µg (▼) of nanoparticle in PBS on days 0 & 14 (booster) by **A. i.p. injection** or **B. oral gavage**. Blood was sampled (n=4) at **i) 3 days post booster** & **ii) 30 days post booster**. Controls were PBS (●) administered same routes & times, & pooled adult serum high in total IgM (♦) as reference serum. Data are mean ± SD. **C. Comparison of anti-VNNV-coat protein IgM in *S. senegalensis* sera at a 1:100 dilution in 2A and 2B.** Broken horizontal line is the background cut off (2 x absorbance without serum). Data are mean ± SD. Differences between each treatment mean & control, & between treatments of the same administration route were analyzed by unpaired one-sided t-tests with Welch's correction for unequal variances. Significance levels *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$, ns = not significant.

FIGURE 4. Gene expression stimulated by VNNV-C^{NP} comparing 5 genes grouped by tissue and route at high dose (upper panel) and 3 genes comparing low and high dose by both routes in intestine (lower panel) Fish (~15 g) were administered VNNV-C^{NP} nanopellet (NP) in PBS on days 0 and 14 (booster) by **A. i.p. injection** or **B. oral intubation** at 500 µg/fish and **C. by both routes at 2 doses,** 50 and 500 µg/fish. Tissues sampled from 4 fish/ treatment for RNA extraction 3 days post booster: Int = intestine, Sp = spleen, Hk = head kidney. Gene expression determined by qPCR with *eef1a* as reference gene and PBS control as the calibrator group using the Livak method. Data are mean ± SD (n = 4). Differences between each treatment mean & control analysed by unpaired one-sided t-tests with Welch's correction for unequal variances. Significance levels *$p < 0.05$