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Lack of *in vivo* cross-protection of two different betanodavirus species RGNNV and SJNNV in European sea bass *Dicentrachus labrax*

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ABSTRACT

Viral encephalopathy and retinopathy (VER) is a severe infective disease characterized by neuropathological changes in several fish species associated with high mortality. The etiological agent is a virus belonging to the *Nodaviridae* family, genus *Betanodavirus*. To date, four different betanodavirus species have been officially recognized by International Committee on Taxonomy of Viruses (ICTV), namely the red-spotted grouper- (RGNNV), the striped jack- (SJNNV), the barfin flounder- (BFNNV) and the tiger puffer nervous necrosis virus (TPNNV). Moreover, two reassortants RGNNV/SJNNV and SJNNV/RGNNV have been described.

Betanodaviruses can be classified into three different serotypes (A, B and C) that are antigenically different, so none (between serotype A and C) or partial (between serotype B and C) cross-immunoreactivity has been detected *in vitro*.

In this study we investigated the *in vivo* cross-protection of the two main betanodavirus species (RGNNV and SJNNV), which belong to distinct serotype, by immunizing intraperitoneally (IP) juvenile sea bass with formalin inactivated RGNNV and SJNNV vaccines, followed by a challenge with RGNNV.

Fish IP vaccinated with inactivated RGNNV showed a high protection value (85%). Serological analyses highlighted a great specific anti-NNV immunoglobulin M (IgM) production against the homologous virus, while a good seroconversion with low neutralization property was highlighted against the heterologous virus. In fish IP vaccinated with inactivated SJNNV the protection recorded was equal to 25%, significantly lower respect to the one provided by RGNNV IP vaccine. ELISA test detected good IgM production against the homologous virus, and a lower, but still detectable IgM production against the heterologous one. By contrast, serum neutralization test highlighted a poorly detectable antibody production unable to neutralize either the homologous or the heterologous virus.

These results confirm that the two serotypes are not cross-protective *in vivo*. According to these findings, the production of multivalent formulation, or at least the provision of different types of vaccines based on both fish and virus species requirement, should be recommended in order to broaden the range of protection.

1. Introduction

Viral encephalopathy and retinopathy (VER), also known as viral nervous necrosis (VNN), is a severe infective disease characterized by neuropathological changes associated with high mortality in several fish species. The etiological agent is a virus belonging to the *Nodaviridae* family, genus *Betanodavirus*, that is a naked RNA virus characterized by an extremely high resistance to chemical and physical agents [1].

Betanodavirus is one of the most significant viral pathogens of finfish, and it is a bottleneck for mariculture development in several countries [2–4]. *Betanodavirus* is a highly pathogenic virus able to evade the host protective systems and can either replicate and transmit progeny to other cells or remain in a latent condition in nervous tissues [5]. Its viral genome is composed by two genetic segments containing three open reading frames (ORFs). The RNA1 gene of approximately 3.1 kb encodes the viral replicase, while the RNA2 segment of ca.1.4 kb encodes the capsid protein. A third transcript, known as RNA3 (0.4 kb), is cleaved from the RNA1 terminus during viral replication and encodes the B2 non-structural protein, an inhibitor of cell RNA silencing [6,7]. To date, four different betanodavirus species have been officially

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recognized by the International Committee on Taxonomy of Viruses (ICTV) namely, the red-spotted grouper- (RGNNV), the striped jack- (SJNNV), the barfin flounder- (BFNNV) and the tiger puffer nervous necrosis virus (TPNNV) [8]. Due to the segmented nature of their genome, fish nodaviruses can undergo genetic shift resulting in the generation of reassortant viruses. To date, the reassortants RGNNV/SJNNV and SJNNV/RGNNV have been described [1,9,10], whose genotype name designation refers to the donor genotype of the polymerase/capsid protein genes (RNA1/RNA2, respectively). A recent study, conducted on 120 field isolates from as the exact same number of outbreaks in the Mediterranean Sea, highlighted that almost 20% of the circulating betanodavirus species are reassortant RGNNV/SJNNV [10].

Betanodaviruses can be classified into three different serotypes only partially cross-reactive: serotype A comprising SJNNV and RGNNV/SJNNV viruses, serotype B consisting of the BFNNV and the TPNNV genotypes and serotype C encompassing RGNNV and SJNNV/RGNNV strains [11]. These serotypes are antigenically different, so none (between serotype A and C) or partial (between serotype B and C) cross-immunoreactivity has been detected *in vitro* [11].

Several experimental vaccines against VNN have been tested so far (for review, see Ref. [16]), most of them on grouper (*Epinephelus* spp.), which is probably the most important and valuable fish amongst the VER susceptible species [12,13]. An inactivated RGNNV vaccine against VER of sevenband grouper (*E. septemfasciatus*) has been produced and marketed in Japan only.

Sea bass (*Dicentrarchus labrax*) is the second most farmed species in the Mediterranean Sea (FEAP 2005–2014) and it is also highly susceptible to VER. In this species, mortality can vary depending on the fish age and water temperature: outbreaks in hatcheries can be devastating, with a mortality rate reaching 80–100%, but also elder fish may be seriously affected [13–15]. A formalin-inactivated RGNNV experimental vaccine was recently tested on sea bass giving high protection by IP injection but none by bath immunization [16]. In this study the vaccinated sea bass were challenged with the homologous virus only, but due to the circulation of different NNV species in the Mediterranean Basin, there is still the risk that fish may be simultaneously infected by more than one serotype. In this study, we investigated the *in vivo* cross-protection of the two principal betanodavirus species (RGNNV and SJNNV), which belong to a distinct serotype, by immunizing intraperitoneally juvenile sea bass with formalin inactivated SJNNV vaccine, followed by a challenge with RGNNV.

2. Materials and methods

2.1. Fish

Three hundred (300) juvenile European sea bass (average weight of 6.10 ± 0.52 g) were transferred from a VER-free commercial farm to the experimental aquarium at the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe, Legnaro-PD, Italy). On arrival, forty fish were sampled to be checked for the most common pathogens (parasites, bacteria and betanodavirus). Preliminary analyses confirmed that all the fish were free from pathogens. The fish were then equally distributed in 4 different close system tanks holding 65 fish each, filled with 300 L of artificial salt water at 25‰ of salinity, at a temperature of 22 ± 1 °C, oxygen 6 ± 0.5 ppm and artificial photoperiod of 8 h of light and 16 of darkness.

2.2. Virus

Two isolates of *Betanodavirus* with diverse genomes (283.2009 RGNNV and 484.2.2009 SJNNV) and belonging to different serological groups (C and A respectively) were used for the experimental challenge of sea bass juveniles [11,17]. Both virus came from two outbreaks occurred in the Mediterranean Sea in 2009: the RGNNV 283.2009 had been isolated from a severe mass mortality in sea bass, while SJNNV 484.2.2009 from affected Senegalese sole (*Solea senegalensis*).

The isolates were propagated on E−11 cells [18], a clone of SSN-1 cell line [19], both originated from striped snakehead fish whole fry, in 150 cm^2 tissue culture flasks using with L-15 medium (Leibovitz) (Sigma-Aldrich) without fetal calf serum. The collected virus was subjected to titration by endpoint dilutions assays. Titres were calculated according to the Spearman-Karber formula [20] and expressed as TCID50 ml^{-1}.

2.3. Vaccine preparation

The E−11 cell media containing VERv (both initial titre $10^{7,80}$ TCID50 ml^{-1}) were centrifuged at 2000 g for 10 min to remove cell debris, and then inactivated by adding buffered formalin (Carlo Erba, Italy) at a final concentration of 10 μl/mL and left at room temperature (22–25 °C) for 1 week. All vaccine preparations were checked after treatment using virological analyses according to a standard procedure [21], with a third additional blind passage to ensure the complete inactivation of the pathogen [16].

2.4. Vaccination and challenge

After a period of acclimation of 10 days, fish were sedated with 30 ppm Tricaine Pharmaq (Pharmaq, Norway) and then vaccinated by IP of 0,1 ml/fish of the two different vaccine preparations described above (one per tank). Additionally, fish from another tank were mock-vaccinated by IP using PBS 0.01 M to act as “virus positive control”. One tank was left untreated to act as negative control. Thirty days post vaccination (dpv), blood and brain samples were collected from fish (n = 10) from all groups.

After 30 dpv all groups, except for the negative control tank, were challenged with RGNNV 283.2009 by intramuscular injection of 0.1 ml of virus solution (approximately $10^{6.80}$ $TCID_{50}$ $fish^{-1}$) after administration of anesthesia as described above. The fish were then transferred back to the original tanks and kept at a temperature of 25 ± 1 °C, oxygen 6 ± 0.5 ppm and artificial photoperiod of 8 h of light and 16 of darkness. The “negative control” group was mock infected by injection of 0.1 MEM-10 (Sigma) rather than with the virus solution administered to the intramuscularly infected group. Considering that SJNNV is only slightly or not at all pathogenic for sea bass [22–24] the challenge was performed only with RGNNV.

Fish were checked twice a day and the dead ones were removed. Brain samples were collected from each dead fish and stored at −80 °C. The experiment ended on day 30 and the relative percent survival (RPS) calculated. All the remaining fish were euthanized by an overdose of Tricaine Pharmaq, sampled and stored at −80 °C until analyses.

2.5. Immunological analyses

The blood from vaccinated juvenile sea bass (30 dpv) was collected from the caudal vein of lethally anaesthetized fish (10 mg/l of Tricaine Pharmaq). Sera were obtained by centrifugation at 1000*g* for 5 min. Due to the small fish size, sera were pooled in group of 3 or 4 in order to obtain a sufficient amount of sera to be tested with different immunological assays.

Indirect ELISA: VERv-specific IgM detection was performed using a previously developed Indirect ELISA [25,26]. Data resulting from the ELISA assays are presented as the mean absorbance ± SD. Each sample was measured in duplicate wells and optical density values (OD 450 nm) of control wells were automatically subtracted from samples values. All sera were tested against both antigens RGNNV and SJNNV.

Serum neutralization tests (SN): serial 2-fold dilutions (1:40 to 1:2560) of heat inactivated serum were prepared in a 96 well plate (Corning) with MEM Leibovitz medium without FBS. Diluted sera were incubated with a defined amount of infectious virus (100 $TCID_{50}$/50μl), 4 wells were used for each sample. After incubation over-night at

+4 °C, the virus-serum mixture was added to a confluent E−11 cell line and incubated for 10 days at 25 °C. Plates were observed every 3 days for appearance of cytopathic effects (CPE). The neutralization value of a virus is defined as the reciprocal of the highest dilution of serum that completely inhibits CPE.

Immunofluorescence assay (IF): E−11 cells seeded on 96 wells plates were infected with betanodavirus strain 283.2009 (RGNNV) and 484.2.2009 (SJNNV). Immediately after the appearance of the cytopathic effect about 3 days post incubation at 25 °C, the cell monolayer was fixed with 80% acetone for 1 h and then washed with phosphate-buffered saline solution (PBS). Sea bass serum was added at different dilutions (1:10–1:50–1:100) and incubated at 37 °C for 90 min. After washing, a secondary MAb anti-sea bass IgM (Aquatic Diagnostic Ltd) diluted 1:10 was added and incubated at 37 °C for 90 min. After PBS washing, a fluorescein-conjugated MAb against mouse IgG (Sigma-Aldricht) diluted 1:100 was added and incubated for further 90 min. Finally, plates were washed with PBS and immediately read under a fluorescent microscope (Zeiss Axioskop equipped with Axiocam MRC 5) at 20X-40X.

2.6. Virological analyses

Virus isolation in cell culture: samples (cell culture supernatant and fish brains) were examined by standard virological techniques [21].

Molecular analyses: Real Time RT-PCR analyses for detection of betanodavirus RNA2 were performed using a slightly modified version of the published protocol [27]. Briefly, total RNA was extracted from 100 µl of the sample using the NucleoSpin® RNA kit (Macherey-Nagel) and according to the manufacturer's instructions. Real-time PCR was conducted with RotorGene Q (Qiagen), Rotor Gene 6000 (Corbett, Australia) using the OneStep RT-PCR Kit (Qiagen) following the manufacturer's recommendations, adding Random primers (Applied Biosystems) and RNase Inhibitor (Promega). Primers and probe were used at a final concentration of 0.9 µM and 0.75 µM, respectively, random primers at concentration 1X, RNase Inhibitor at final concentration of 8U µl-1 and thermal cycling was performed at 50 °C for 30 min and denaturation at 95 °C for 10 min followed by 45 cycles of 95 °C for 10 s, 54 °C for 35 s and 72 °C for 1 s, with a final incubation at 40 °C for 30 s. Data analyses were performed with the Rotor Gene 6000/RotorGene Q series software.

2.7. Statistical analysis

The number of fish per experimental group was calculated according to the expected mortality rate (35%) and 0.05 as α error (one-side) and power 80% (1-β = 0.80).

Each fish in the study was followed over time and the event “death” was recorded and verified by Real Time RT-PCR.

The Kaplan–Meier method was used to estimate the survival function from lifetime data, which allowed to draw the survival curve for each group as a step curve and to measure the length of time the fish would survive the infection. In the graph, the y-axis plot indicates the cumulative probability of the surviving fish at each time (Van Belle et al., 2004). To compare the different survival curves, the non-parametric Wilcoxon–Breslow–Gehan test was used for equality of survivor functions.

To compare the distributions of the quantitative variable indirect ELISA among two independent groups, two-sample Wilcoxon (Mann-Whitney) rank-sum test was used, after having evaluated the homogeneity of variances through non parametric robust Levene test.

Chi square test was used to compare cumulative mortality between independent groups.

All statistical analyses were performed using the STATA 12.1 (Stata Corp LLC, USA) software. For all tests, a p-value < 0.05 was considered as significant.

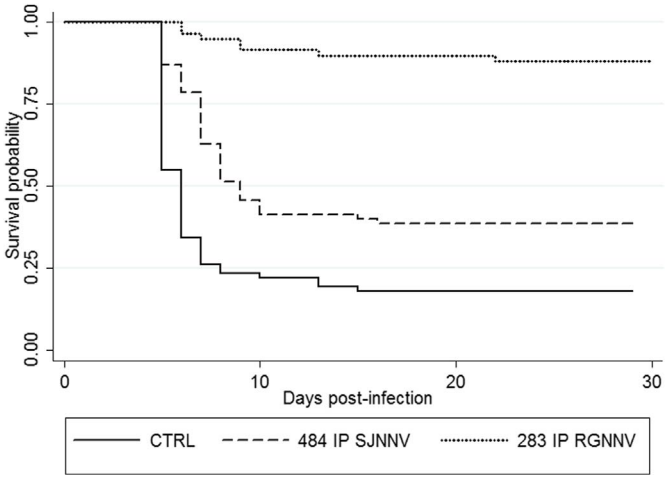

Fig. 1. Cumulative survival rate of sea bass infected with RGNNV after vaccination.

2.8. Ethic statement

The Istituto Zooprofilattico Sperimentale delle Venezie is a public health body belonging to the National Health System, authorized as user by the Italian Ministry of Health (Decree n. 78/2012-A of 13/03/2012). The animal care and use protocol adopted adheres to the Directive 2010/63/EU of the European Parliament and of the Council, implemented at a national level through Law Decree n. 26 of 4 March 2014. The experimental protocol was approved by the internal animal-welfare body (Opinion n° 02/2013 of 4th January 2013) and authorized by the Italian Ministry of Health (Law Decree n°126/2013 of the 24th May 2013).

3. Results

3.1. Challenge and virological analyses

Cumulative mortality was of 82.2% in the positive control, 61.4% in the SJNNV vaccinated group and 11.9% in the RGNNV vaccinated one, with significant differences among groups (Chi-square test: $p < 0.001$; Fig. 1). The RPS of the RGNNV vaccine was 85.6, while for the SJNNV vaccine it was 25.3.

All brain samples from dead fish tested by rRT-PCR resulted positive (ct value range 7.76–26.98). Additionally VERv was re-isolated from all the tested dead fish (sample titration range $1 \times 10^{8.05}$ - > $1 \times 10^{8.80}$).

3.2. Immunological analyses

Anti-VERv specific IgM were detected by Indirect ELISA in sera samples from all the vaccinated groups, both against RGNNV and SJNNV antigens. Fish vaccinated with inactivated RGNNV 283.2009 showed the highest IgM titre (OD 450 nm Median = 0.497; Iqr = 0.059 against the homologous antigen RGNNV and Median = 0.457; Iqr = 0.0085 against the heterologous SJNNV), while fish vaccinated with inactivated SJNNV 484.2.2009 lower titre in both cases (OD 450 nm Median = 0.312; Iqr = 0.040 against RGNNV and Median = 0.233; Iqr = 0.140 against SJNNV) (Wilcoxon test: $p < 0.001$; Fig. 2). The presence of anti-VERv specific antibodies was confirmed in all the vaccinated groups by IF against both RGNNV and SJNNV antigens.

The serum neutralization test showed the presence of neutralizing antibodies against RGNNV 283.2009 only in the homologous vaccinated group (average titre 1:320), whereas all the other groups showed negative results. Regarding the same sera analyzed against SJNNV 484.2.2009, once again only fish immunized with inactivated RGNNV showed low levels of neutralizing antibodies (average titre 1:80), while

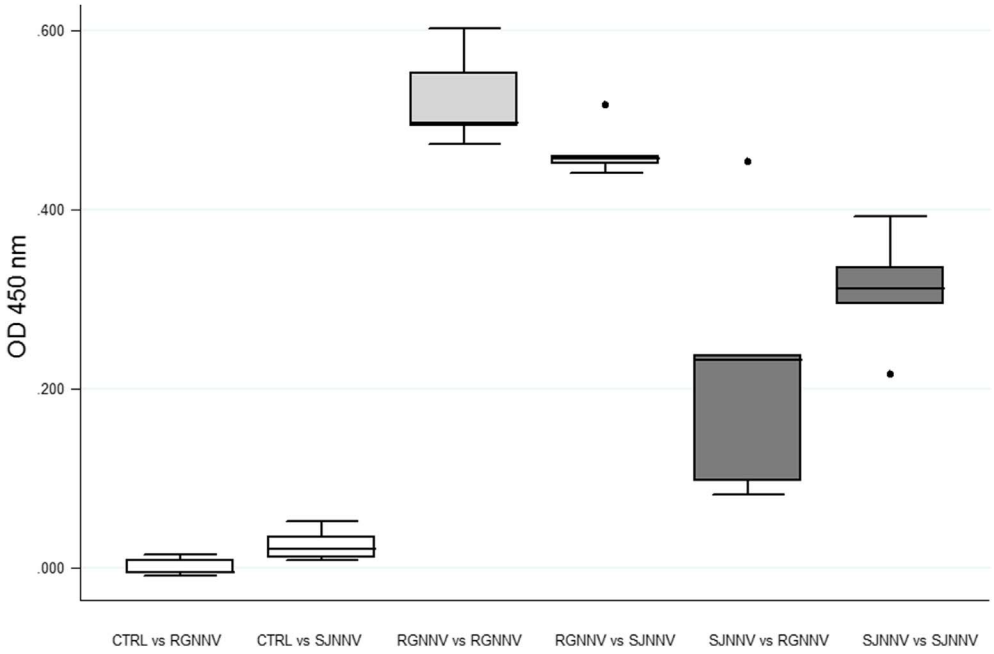

Fig. 2. VERv-specific IgM detected by ELISA 30 days after intraperitoneal immunization with formalin inactivated RGNNV and SJNNV.

all the other groups recorded negative results.

4. Discussion

Viral encephalopathy and retinopathy (VER) is one of the most devastating viral diseases for marine aquaculture with a wide host range [1,2]. To date, in the Mediterranean basin, four species have been detected: RGNNV, SJNNV, BFNNV and TPNNV, plus the two reassortant: RGNNV/SJNNV and SJNNV/RGNNV [10,28–33].

In the past VER had caused several economic losses to the Mediterranean farmers, also because no commercial vaccines were available in Europe [4,34–39]. Recently an autologous vaccine for sea bass against VER has been commercialized, at present available only in Spain and Greece.

Betanodaviruses can be classified into three different serotypes (A, B and C) that are antigenically different, so only very slight or no cross-immunoreactivity at all has been detected *in vitro*, probably because their immunoreactive residues are located at different positions of the coat protein sequence [11].

The aim of this study was to verify *in vivo* the Betanodavirus inability to induce a cross-protection in sea bass against the two principal viral species in the Mediterranean, RGNNV and SJNNV. To reach this goal RGNNV and SJNNV IP immunized sea bass juveniles were challenged with a pathogenic RGNNV strain (283.2009). Cumulative mortality recorded in the virus positive control group was quite high (> 82%) and in line with previous experiments [16], thus validating the infection procedure carried out in this trial. Molecular analyses results confirmed the presence of the virus in each brain sample tested, while virus re-isolation and titration from both dead and survivors fish proved that replication occurred in the fish central nervous system (CNS). All the results, matched with the appearance of the typical clinical signs five days post infection, suggest that the experimental infection was successful.

Fish IP vaccinated with inactivated RGNNV showed a high protection value, equal to 85%. Such results were in accordance to those found by other authors in sea bass [16] (RPS = 81.9%) and in seven-band grouper [40] (RPS = 67%–100%) in trials using a single injection of formalin-inactivated RGNNV followed by homologous challenge. In 2009, Pakingking and colleagues [41] reported strong immune responses in Asian sea bass (*Lates calcarifer*) after a single injection of the formalin-inactivated RGNNV, which induced a protection in vaccinated groups equal to 100%. Our results further confirmed that a single dose of formalin inactivated RGNNV was sufficient to protect sea bass against the homologous virus. Serological analyses highlighted a great IgM production against the homologous virus, reflecting results obtained during the challenge. Particularly, all tests (ELISA, IF and SN) were concordant, showing a good seroconversion level. Concerning serological analyses of RGNNV vaccinated fish against the SJNNV antigen, the ELISA assay reported a high antibody production against the heterologous virus, whose specificity was confirmed also by IF. On the other hand, the SN test reported low antibodies neutralization property. These results may suggests that, although the production of specific antibodies was high, these are not protective against the heterologous virus. Unfortunately, since SJNNV is not pathogenic for sea bass [23,24,42], this hypothesis has not been confirmed *in vivo*.

In the IP group vaccinated with inactivated SJNNV the protection recorded was equal to 25%, therefore significantly lower respect to the one provided by RGNNV IP vaccine. Since this is the first study which uses a formalin-inactivated SJNNV vaccine for the immunization of sea bass, a comparison with other *in vivo* studies results has not been possible. Anyway, considering the serological analyses, the ELISA test detected a good IgM production against the homologous virus, and a lower, but still detectable IgM production against the heterologous. In both cases, the immunofluorescence assay confirmed the specificity of the antibodies detected by ELISA. Such results suggested that a specific seroconversion had occurred. On the other hand, the SN test highlighted a poorly detectable antibody production unable to neutralize either the homologous or the heterologous virus, not even at the lowest dilution. Noteworthy, results from SN are in accordance to those from the challenge, where a scarce protection was recorded, thus suggesting that the specific antibody production detected by ELISA and IF is not always linked to the virus neutralization capacity.

These results are in accordance with those already obtained *in vitro* by several authors [11,43–46], where a little or no cross-immunoreactivity between the two viral species (RGNNV and SJNNV) was found, therefore confirming that the two serotypes are not cross-protective also *in vivo*.

Even though sea bass are not or little affected by SJNNV, it is true that this viral species is highly pathogenic for *Solea senegalensis* [23,24,42], another common species farmed in Mediterranean basin. Indeed sea bass can be infected by the reassortant strain RGNNV/SJNNV which share the same antigenic properties of SJNNV [10,11].

Moreover, the same reassortant strain caused several outbreaks in sea bream hatcheries in the last years, producing great losses [37]. According to these findings, the production of a multivalent formulation or at least the provision of different types of vaccines based on both fish and virus species should be recommended in order to broaden the range of protection.

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