

Detection of Serum Antibodies Against Nodavirus in Wild and Farmed Adult Sea Bass: Application to the Screening of Broodstock in Sea Bass Hatcheries

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Abstract

Antibodies to nodavirus were detected by ELISA in the serum of adult sea bass. Preliminary epidemiological analysis revealed no significant differences between wild (17 % seropositive fish) and farmed reared (18 % seropositive) adult fish in Palavas-les-Flot, France. A higher proportion of seropositive fish was found in farmed reared females (16%) compared to the males (3%). Furthermore, 30 fish were separated in two groups (seronegative and seropositive) with regard to their serological results following three successive analyses at 2 months intervals. All fish have been individually tested for two years at 2 months intervals. No fish belonging to the seronegative group have developed nodavirus antibodies for 2 years, and all the samples tested, ovarian biopsies, sperm, eggs and larvae have been found virus negative. The seropositive fish have remained seropositive for 2 years and developed a lower serum antibody level during winter. These observations suggest that the screening of the broodstock by repeated ELISA detection of the nodavirus antibodies could be a valuable method for the detection of seronegative fish. For optimal results in the sea bass, the broodstock should be screened during summer prior to the maturation period of the females. This method should also be used in conjunction with the antigen detection in ovarian biopsies.

Introduction

Nodavirus is known for inducing mass mortalities of sea bass (*Dicentrarchus labrax*) reared in cages (Lebreton *et al.*, 1997) and in hatcheries (Breuil *et al.*, 1991). In fish hatcheries the control of the disease can be based on preventing both horizontal and vertical transmission of the virus (Munday and Nakai, 1997) as spawners have been demonstrated as a source of larval infection in the sea bass (Comps *et al.*, 1996) and in the striped jack, *Pseudocarenx dentex* (Arimoto *et al.*, 1992).

The usual methods used for the diagnosis of the disease (OIE Manual 1995) are not useful for the broodstock as they require that the fish are killed. Thus the detection of nodavirus antibodies in the serum of adult fish could be an advantage in that it detects the serological status of an individual without necessitating the killing of the animal. Such methods have been carried out on the striped jack, (Mushiake *et al.*, 1992; Mushiake *et al.*, 1993), and in the sea bass (Breuil and Romestand, 1999).

In this study, a previously described ELISA technique (Breuil & Romestand, 1999) was used to detect the nodavirus antibody in the serum of the sea bass. This ELISA allowed us to study (i) the epidemiology of the disease in captive and wild sea bass, and (ii) the application of this technique for the selection of seronegative fish (broodstock) in sea bass hatcheries.

Material and Methods

Adult sea bass (Broodstock)

210 adult fish (farmed reared sea bass) from both sexes weighing 1 to 6 kg were individually tagged with an implanted electronic transponder (reader HS 5105 L, Destron, South St Paul, MN, USA). Blood samples were collected every two months by heart puncture and allowed to clot at room temperature. The serum were collected after centrifugation and kept frozen -20 °C until ELISA quantification of nodavirus antibody. Serologic examination was also performed on 41 adult wild sea bass from both sexes collected near the Palavas area.

Samples from seronegative fish (ovarian biopsies, eggs, milt) were collected and frozen for cell culture and RT-PCR analysis.

Nodavirus antibody quantification by ELISA.

Quantification of nodavirus antibody in the serum of the sea bass was performed according to the ELISA method previously described (Breuil and Romestand, 1999). Results were expressed as negative, positive and intermediate. Differences between groups (seronegative, seropositive and unclassified groups) were analysed using the sigmastat software using chi 2 test or Friedman repeated measures analysis of variance on ranks.

Nodavirus detection by cell cultures

SSN1 cell line from *Channa striatus* (Frerichs et al, 1996) was used for virological analysis and production. The cells were propagated at 29° C in Leibovitz medium (L15) according to the method previously described (Peducasse *et al.*, 1999). Briefly, samples were thawed and homogenised with sand in a mortar, diluted to 10-1 in PBS containing 200 U.I. ml-1 of penicillin, 0.2 mg.ml-1 of streptomycin and 0.2 mg. ml-1 kanamycin. The homogenate was then centrifuged at 3000xg for 15 min at 4° C and 100 µl of the supernatant diluted 10-1 and 10-2 in PBS were inoculated into 48 hours old cells grown in 24 well plate. The cells were incubated at 24° C and observed for a cytopathic effect (cpe). Seven days later, a second passage was performed onto new cells and the virus was detected by an indirect immunofluorescence technique (IFAT).

Nodavirus detection by RT-PCR

The same samples that were used for virological examination were also processed for nodavirus detection by RT-PCR. Total RNAs from each sample were isolated using RNA NOW (Biogenetex, USA), a reagent based upon a modification of the Guanidinium salt-Phenol-Chloroform method (Chomczynsky & Sacchi, 1987), according to the manufacturer instructions. Three microliters of the total RNA samples were subjected to RT-PCR using MuLV reverse transcriptase (Perkin Elmer, France), and Ampli Taq DNA polymerase (Perkin Elmer, France) essentially according to a previously described protocol (Nishizawa *et al.*, 1994), but using primers (R3, F2) designed to amplify the T4 region of RNA2 from sea bass nodavirus (Thiéry *et al.*, 1999). Fifteen microliters of the PCR products were analysed

	Seropositive Fish (0.37<OD 1/400<3.2)	Seronegative Fish (OD 1/400<0.23)	Unclassified Fish (0.23<OD 1/400<0.37)
Wild Sea Bass n=41	7 (17%)	29 (72%)	5 (11%)
Captive Sea Bass n=210	38 (18%)	126 (60%)	46 (22%)

Table 1. Serology of wild and captive sea bass. No significant differences between groups ($P>0.05$)

by electrophoresis on a 2% agarose gel stained with ethidium bromide.

Selection of broodstock

Adult captive sea bass (110 fish) were tested three times at 2 months intervals for nodavirus antibodies. At the end of the serological analysis, 30 fish were classified in two groups (seropositive and seronegative groups) regarding to the individual serological results and placed in separated tanks receiving UV treated sea water: fish that had been seronegative for the three repeated analysis were placed in SN (seronegative) tank and fish that had been seropositive for the three repeated analysis were isolated in SP (seropositive) tank; two seronegative fish were placed in SP tank for testing an horizontal transmission of the virus from seropositive to seronegative fish. Repeated serological analysis of these 30 captive sea bass were then performed at two month intervals from April 97 to March 99.

Results

Serology of wild and captive sea bass

Serological examination of 41 adult sea bass collected from the sea (wild fish) revealed 17%

seropositive and 72 % seronegative fish (Table 1). No significant differences (χ^2 test) were seen between wild and captive sea bass. The percentages of seropositive fish within these two populations were 17 % (wild fish) and 18 % (farmed reared fish).

Serological differences between males and females

110 adults sea bass (32 males and 78 females) reared in captivity were tested for nodavirus antibodies (Table 2). A higher proportion of seropositive fish ($P<0.05$) was found in the female group (16 %) compared to the male group (3%).

Selection of the broodstock: serological differences related to seasons

22 fish (18 females and 4 males) were classified in the seronegative group and reared in G7 tank, and 16 fish (13 females and 3 males) were classified in the seropositive group and reared in SP tank.

Seronegative fish (SN tank)

The fish were individually screened 15 times from April 97 to May 99. None of them became seropositive during this period and few serologic variations were noted: some seron-

	Seropositive Fish (0.37<OD 1/400<3.2)	Seronegative Fish (OD 1/400<0.23)	Unclassified Fish (0.23<OD 1/400<0.37)
Male Sea Bass n=32	1 (3%)*	26 (81%)*	5 (16%)
Female Sea Bass n=78	13 (16%)*	43 (56%)*	46 (22%)

Table 2. Serological differences between domestic sea bass males and females. *Significant differences between marked groups ($P<0.05$)

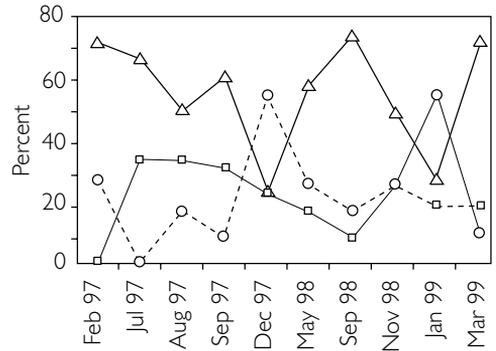
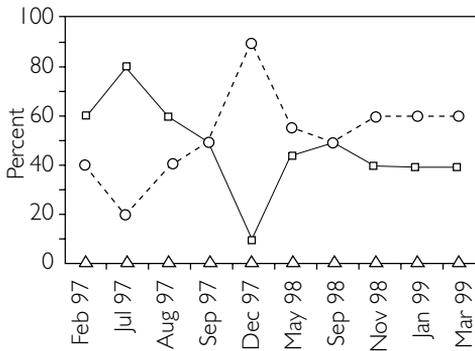


Fig. 1. a) Serological variations related to season for 19 seronegative fish (SN tank). b) Serological variations related to season for 11 seropositive fish (SP tank). In each case, symbols: circles = seronegative; triangles = seropositive; squares = unclassified.

egative fish developed a slight response in the ELISA (intermediate results) for a short period of time during summer 1997 and 1998 (Fig. 1a). Furthermore, all virological controls made on the sexual products of these fish by cell culture or RT-PCR were always negative (16 fish tested).

Seropositive fish (SP tank)

The fish were individually screened 14 times since April 1997 (Fig. 1b). The nodavirus antibody level of each individual was relatively stable during this period (> more than one year). Only one fish (sea bass female F5590) became negative and the two seronegative fish that had been placed in SP tank remained seronegative during the experiment. Repeated serological analysis of the 16 seropositive fish revealed that the level of nodavirus antibodies significantly decreased in winter 1997 (Friedman repeated measures analysis of variance on ranks: a multiple comparison procedure (Dunnett Method) was used to isolate groups that differed from the others). In December 1997 the proportion of seropositive fish (<30%) was significantly ($P > 0.001$) reduced (Dunnett method) and by opposite the

percentage of seronegative fish increased to reach 55%. Few individual serological variations were seen during 1998-1999 and the proportion of seropositive fish decreased (no significant differences) again in January 99 (30%).

Discussion

Epidemiological data

No differences were seen between the proportion of seropositive fish in wild and farmed reared sea bass. This result is in accordance to previous data obtained in the striped jack (Mushiake *et al.* 1992). However, the epidemiological data of these two species are very different. A high proportion of seronegative fish (72%) was found in the sea bass reared in Palavas station compared to the striped jack (28%) reared in Komame station (Mushiake *et al.* 1992). This observation is of great importance for the selection of a sufficient amount of seronegative fish in a fish farm.

The higher proportion of seropositive fish in female sea bass (16%) compared to the males (3%) found in the present study was not noted in the striped jack (Mushiake *et al.*, 1992). In the previous work of Mushiake *et al.*, 1992,

serological data were obtained from wild fish by opposite to our observations on captive fish. Thus, the difference between male and female sea bass was probably due to husbandry techniques (stripping and repeated ovarian biopsies) inducing specific stress of the females. The only fish that died during our experiment were sea bass females (3 seronegatives and 5 seropositives fish). Maturation and spawning may also inhibit the fish humoral response as 17 hydroxy corticosteroid hormones are known to be immunosuppressive in fish (Ezenwa *et al.*, 1986).

Individual serological variations

For each adult individually tagged sea bass (broodstock), the nodavirus antibody level was lower in winter and higher in summer and consequently, the summer season was the period that gave optimal results for the antibody level detection by ELISA. The inhibition of the fish immune system as well as the decreasing of serum IgM and antibody level can be related to cold temperature in various fish species (Bureson and Frizell, 1986; Bly and Clem, 1992). The vitellogenesis period of sea bass females occurs when the water temperature is below 15 °C (Autumn) whilst the fish immune response is optimal at 18 °C (Deschaux *et al.*, 1983). However the decrease in the nodavirus antibody level during winter has not been reported in the striped jack (Mushiake *et al.*, 1993) probably because the vitellogenesis period of the striped jack occurs at a higher water temperature.

Screening of the broodstock

Repeated screening of sea bass broodstock by ELISA detection of nodavirus antibodies allowed the selection of seronegative fish. We

also have evidence that these fish are nodavirus free for the following reasons: Fish classified in the seronegative group never became seropositive. Furthermore, all the samples tested (ovarian biopsies, sperm, eggs and larvae) were found virus negative by RT-PCR and cell culture. In addition, no symptoms (whirling behaviour) nor brain lesions were found in larvae from seronegative fish (5 spawns were placed into incubator tanks and the larvae were reared for 10 days), as well as in adult females (3 fish) that died during the experiment. All these results support the hypothesis that fish placed in the seronegative group are also virus free fish. Preliminary studies in the striped jack gave confusing results because the fish were selected by performing one single serological analysis (Mushiake *et al.*, 1992). A more recent study, although based on a single screening of the broodstock showed that combining serology and PCR analysis of ovarian biopsies represents a good tool for the selection of virus free broodstock (Mori *et al.*, 1998; Mushiake *et al.* 1994).

Repeated ELISA detection of the nodavirus antibodies in the serum of sea bass during summer can also be used in combination with the antigen detection in ovarian biopsies during the maturation period (autumn). Both techniques should be used for the control of the nodavirus in sea bass broodstock.

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The detection of nodavirus in Tunisia occurred several years ago in farmed sea bass and sea bream [4-20]. In this study, the causative agent was detected from feral fish species for the first time. To our best knowledge, this is the first report of VERN infection in these fish species in Tunisia from coastal waters. The exchange of pathogens between farmed and wild fish has been repeatedly observed and investigation results indicated that marine strains are not without significance to the farmed fish industry. The healthy carrier species, although asymptomatic but also migratory, could induce the spread of virus to other fish cage in other locations. In this assay 17% and 18% sera of wild and farmed European sea bass broodstock were positive for nodavirus antibodies, respectively (Breuil et al., 1991). In a similar study, 9% sera of commercial barramundi were positive for antibodies (Huang et al., 2001) by ELISA method. Furthermore, the production of multivalent or recombinant vaccines against VNN virus, the increase of application of some immunostimulant drugs, the eco-epidemiological investigation on the global spreading of VNN in the new regions and new susceptible hosts should be considered in future studies.

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