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Sea bass *Dicentrarchus labrax* nervous necrosis virus isolates with distinct pathogenicity to sea bass larvae

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ABSTRACT: Reproduction of nodavirus disease was performed by experimental infection of sea bass eggs during fertilization or at larval stage 4 with 2 genetically distinguishable nodavirus strains (Sb1 and Sb2) isolated from sea bass collected along the Atlantic and Mediterranean French coast. The pathogenicity of the virus strains was assigned after detection of the virus by ELISA and immunohistochemistry (IHC). The Atlantic (Sb1) strain was more pathogenic than the Mediterranean (Sb2) strain during the fertilization step whilst both strains were pathogenic following experimental exposure of 4 d old larvae. Virus lesions developed in the brain 4 to 6 d following experimental exposure. Experimental ELISA proved very sensitive for detecting the nodavirus in Sb1 or Sb2 experimentally infected larvae, as well as in naturally infected sea bass larvae collected in French hatcheries or in barramundi larvae reared in the Pacific area. The development of an ELISA specific for the 2 nodavirus strains isolated from the sea bass should be useful for the detection of the virus, in addition to other techniques recommended by the Office International des Epizooties (OIE).

KEY WORDS: Fish encephalopathy · Nodavirus · ELISA

INTRODUCTION

Fish nodaviruses are the causative agents of viral encephalopathy and retinopathy, which is responsible for mortalities in larvae and juveniles of several fish species (OIE 1995). The causative agents (striped jack nervous necrosis virus [SJNNV] and fish encephalitis virus [FEV]) were isolated and characterized in striped jack *Pseudocaranx dentex* in Japan (Mori et al. 1992) and in the sea bass *Dicentrarchus labrax* in France (Comps et al. 1994). Successful experimental transmission of the disease to juveniles has been performed in several fish species including sea bass *D. labrax* (Peducasse et al. 1999), the redspotted grouper *Epinephelus akaara* (Mori et al. 1991), brown spotter grouper *E. malabaricus* (Boonyaratpalin et al. 1996), seven band grouper *E. septemfasciatus* (Tanaka et al. 1998) and barramundi *Lates calcarifer* (Glazebrook et al. 1990). However, experimental transmission to larvae was only reported in striped jack (Arimoto et al. 1993, Nguyen et al. 1996) with differences in susceptibility between larvae and juveniles (Arimoto et al. 1993). In the present study, the transmission of the disease was performed in sea bass larvae by using 2 genetically distinguishable nodavirus strains (Thiery et al. 1999) isolated in naturally infected sea bass larvae and juveniles, respectively. In addition, an ELISA system was developed to detect the nodavirus in infected larvae, and this technique was used along with the immunohistochemistry method (IHC) or the Fluorescent Antibody Technique (FAT) detection method recommended by the Office International des Epizooties (OIE 1995).

MATERIALS AND METHODS

Virus collection. Two nodavirus strains (Sb1 and Sb2) were isolated from affected sea bass reared in the

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Atlantic or in the Mediterranean Sea, respectively, and purified according to the method previously described (Breuil et al. 1991, Breuil & Romestand 1999). The virus was cultivated on striped snake (SSN-1) fish cell lines (Frerichs et al. 1996) and the cell supernatants were collected, titrated and stored frozen (–20°C) until used in the experiments.

Test fish: All the larvae or eggs used in the test were obtained from seronegative females (Breuil & Romestand 1999) reared in the IFREMER (Institut Français de Recherche et d'exploration de la Mer) station of Palavas. In addition, infected sea bass larvae were collected in 1997 in commercial hatcheries and were used as a positive control. Infected barramundi larvae collected in Tahiti (French Polynesia) were also tested (Renault et al. 1991).

Infection experiments: Exposures to the virus were performed during fertilization by mixing 25 ml of fresh eggs (i.e. 25 000 eggs) with 100 µl sperm diluted with contaminated (Sb1 1.6×10^8 plaque-forming units [PFU] ml^{-1} or Sb2 9×10^8 PFU ml^{-1}) seawater for 10 min. The cell supernatant (SSN-1) containing the associated retrovirus was used as a control. Following experimental exposure, the fertilized eggs were rinsed with seawater and placed in 50 l tanks at a water temperature of 14 to 15°C for 3 d. At the end of the incubation period, the newly hatched larvae (larval stage 1) were collected at the water surface and placed in 12 l tanks at a density of 50 to 100 larvae l^{-1} and a water temperature of 16 to 17°C for 10 d. Exposure of larval stage 4 was performed by bathing some larvae from the control group previously described in contaminated seawater (same virus doses as used for the experimental exposure of the eggs) for 10 min. In all the experiments, the dead larvae were collected daily for quantification of the virus by ELISA and for detection of lesions by IHC or FAT analysis. The water effluent was chlorine disinfected using an automatic injection system (HYDROBOX M1, HYDROCHIM, France).

ELISA analysis. Samples of more than 30 larvae were collected and centrifuged for 1 min at 15 000 × *g* and the supernatant was discarded. The larvae were ground using a hand-held micro potter (Eppendorf) and diluted $^1/_4$ (v/v) with 0.5% skimmed milk in phosphate-buffered saline (PBS) pH 7.4 and then stored frozen at –20°C. Two positive controls were made of naturally infected sea bass (pool of 50 larvae) and barramundi larvae (pool of 30 larvae). Sea bass larvae (pool of 30 larvae) collected in hatcheries with no signs of the disease were used as negative samples. Sea bream *Sparus aurata* and A*rtemia salina* larvae were also tested.

ELISA plates (Nunc maxisorp) were coated overnight with a rabbit IgG raised against the Sb1 virus strain and diluted (2.5 µg ml^{-1}) in carbonate buffer pH 9.4 at 4°C. After washing 3 times with PBS, the plates were blocked by adding 250 µl of 3% (w/v) bovine serum albumin PBS for 1 h at 37°C. Diluted samples (1:500 and 1:5000) or purified virus (standard) diluted in 0.5% (w/v) skimmed milk-PBS were then deposited in each well in duplicates and incubated for 1 h at 37°C. After washing with PBS-0.05% Tween, a biotinylated antinodavirus rabbit IgG (the IgG was biotinylated at a ratio of 1:45 according to the method described by Harlow & Lane 1988) diluted (1.14 µg ml^{-1}) in 0.5% skimmed milk-PBS was added for 1 h at 37°C. The reaction was developed by adding 50 µl of avidin labeled with horse-radish peroxidase (Pierce) diluted 1:2500 in 0.5% (w/v) skimmed milk-PBS for 30 min at 37°C. The enzyme was detected after 15 min incubation with a chromogenic substrate (orthophenylenediamine) and after reading the optical density (OD value) of each well at 492 nm. The immunoassays were carried out in duplicate, and individual results were calculated using labsystem GENESIS windows-based software (Labsystem, Helsinki, Finland). Results were analyzed using the Bienaymé-Tchebitcheff inequality with a significant limit of $p < 0.06$.

IHC analysis. Samples of more than 30 larvae were fixed in Davidson's fixative and brain lesions (vacuolation) were detected by IHC analysis (Peducasse et al. 1999).

RESULTS

ELISA

Sea bass and barramundi larvae with clinical signs of disease (whirling behavior and encephalitis) reacted in the ELISA and gave an OD value of 1.2 at a 1000-fold dilution (Fig. 1). All the controls made with non-diseased sea bass and sea bream larvae or *Artemia salina* cysts were negative (OD < 0.1). Negative larvae (54 different pools of 30 larvae) were tested by ELISA and the cut off limit (arrow) was determined by using the Bienaymé-Tchebicheff inequality for $p = 0.06$ (Table 1). The samples were positive for an OD value higher than the cut off limit of OD = 0.344 (probability for an OD value > mean of the negative control + 4 standard deviation of the negative control). The ELISA was sensitive enough to detect 6 ng ml^{-1} of purified virus. The precision of the ELISA was assayed with 8 positive replicates, and the coefficient of variation (CV) within and between assays ranged from 1.15 to 4.62%, respectively (Table 2).

Both of the purified viruses isolated from the Atlantic (Sb1) and Mediterranean coasts (Sb2) were also detected in the ELISA although significant differences were seen. The Atlantic strain reacted better than the Mediterranean strain (Fig. 2).

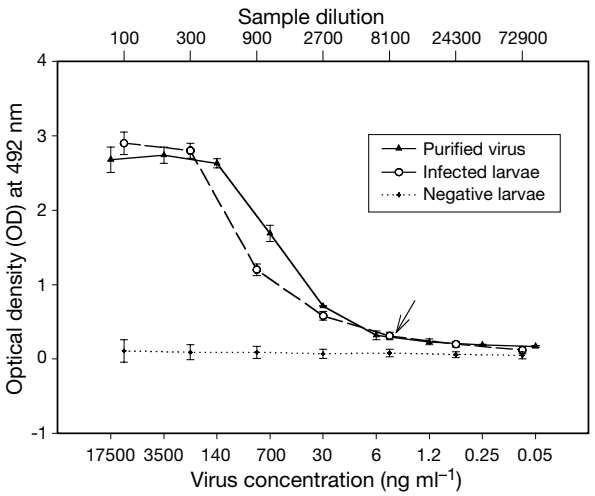

Fig. 1. Virus detection in sea bass larvae extracts. Standard curve for purified nodavirus and dose response curve for extracts of larvae (positive and negative larvae). Each data point represents the mean (+SE) of duplicate wells. Optical density was the OD value of the sample minus the OD value of the non-specific buffer response. Samples were positive (significantly different from the control) when the OD values were higher than 0.344 (arrow)

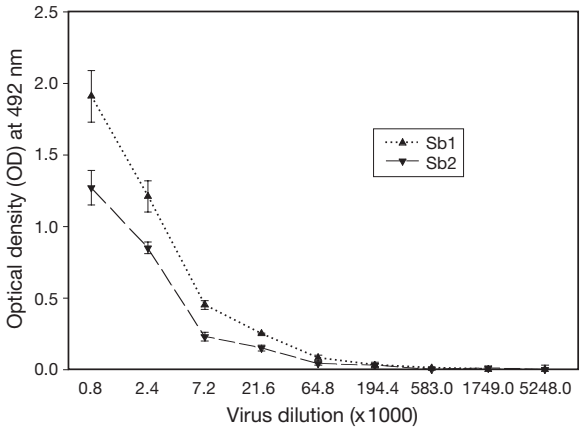

Fig. 2. Dose response curve for Atlantic (Sb1) and Mediterranean (Sb2) nodavirus strains. Each data point represents the mean (+SE) of duplicate wells. Optical density was the OD value of the sample minus the OD value of the non-specific buffer response

Virus detection in larvae from a commercial sea bass hatchery

The Sb2 virus strain was detected at different periods of encephalitis in a Mediterranean sea bass hatchery (Table 3). The disease appeared in 1 single tank from larvae that had been collected from 1 spawning. The nodavirus was first detected by ELISA at Day 36 following hatching and 7 d prior to the detection of encephalitis lesions in the brain by IHC or the observation of abnormal swimming behavior of the larvae (Day 43 after hatching). There was no outbreak of disease in the other larval rearings, and the affected tank was dried and disinfected at Day 51 to prevent contamination of the whole hatchery.

Table 1. Determination of the cut off limit of the ELISA. ELISA optical density (OD) values of different pools of negative sea bass larvae diluted 1:500. According to the Bienaymé-Tchebicheff inequality, the probability for an OD value > $m + k\sigma$ is $1/k^2$. (For $k = 4$ the probability is 0.06.) The samples were significantly (p = 0.06) different from the control when the OD values were higher than 0.344 (cut off limit of the ELISA)

No. of samples	Mean OD values (m)	SE (σ)	Cut off limit ($m + 4\sigma$)
54	0.084	0.065	0.344

Experimental transmission to eggs and larvae

Transmission to eggs

The Sb1 strain was infective for all eggs tested (Fig. 3, Expts 3a–d) while the Sb2 strain was infective only for Expt 3c. The Sb1 virus was detected by ELISA 8 to 11 d following the experimental exposure of the eggs (Fig. 3), corresponding to larval stages 5 and 8, respectively, and the highest antigen concentration was reached at Day 11 (OD > 3) in larvae extract diluted 1:500 (Fig. 3a,c,d). The positive reaction observed 11 d following exposure of the eggs with the Sb2 strain (Fig. 3c) is probably due to the exposure of newly hatched larvae to the virus and not to a true egg exposure as compared to the development of the virus following exposure of 4 d old larvae (Fig. 4a) from the

Table 2. Precision test of the ELISA showing the coefficients of variation (CV) within and between positive (8 replicates) and negative (6 replicates) assays. OD: optical density

	OD value (dilution 1:500)	SE (σ)	CV (%) within assays	CV (%) between assays
Positive sample (8 replicates)	1.25	0.014	1.15	4.62
Negative sample (6 replicates)	0.049	0.001	2.72	3.15

Table 3. Virus detection by ELISA (optical density [OD] at dilution 1:500) and by Immunohistochemistry (IHC) following outbreak of encephalitis in a commercial sea bass hatchery in 1997. Diseased sea bass larvae *Dicentrarchus labrax* collected in an Atlantic hatchery and barramundi larvae *Lates calcarifer* collected in a French Polynesian hatchery were used as positive controls. Samples were positive (significantly different from the control) when the OD value was higher than 0.344 (cut off limit of the ELISA). *Positive result

Day of rearing	ELISA (OD)	Brain lesions (IHC)	Symptoms whirling behavior
15	0.05	Negative	No
25	0.08	Negative	No
33	0.24	Negative	No
36	0.35*	Negative	No
43	0.6*	Positive	Yes
51	1.5*	Positive	Yes
Sea bass larvae	2.2*	Positive	Yes
Barramundi larvae	1.9*	Positive	Yes

same batch of eggs. Transmission of the disease to the larvae was confirmed by IHC analysis of the brain at the end of all the experiments; results (data not shown) were similar to those described in the transmission to 4 d old larvae.

Transmission to 4 d old larvae

As shown in Fig. 4a,b, both virus strains (Sb1 and Sb2) were infective to 4 d old larvae. In both experiments, the Sb1 virus was detected earlier than the Sb2 (Fig. 4) and the ELISA values detected in larvae extract diluted 1:500 at Day 9 following exposures were similar for both viruses. The first ELISA detection was obtained 4 d following contamination with the Sb1 (Fig. 4a) corresponding to the larval stage L8. The positive ELISA results following exposures to Sb1 and Sb2 virus strains were confirmed by IHC analysis (Fig. 5b) of the brain and retina of the larvae. After immunostaining using rabbit anti-nodavirus IgG, staining was observed in nervous cells 4 d following the experimental exposure (Fig. 5a) and no vacuolation was detected at this step. Vacuolation of the nervous cells was observed 9 d after experimental exposure (Fig. 5b).

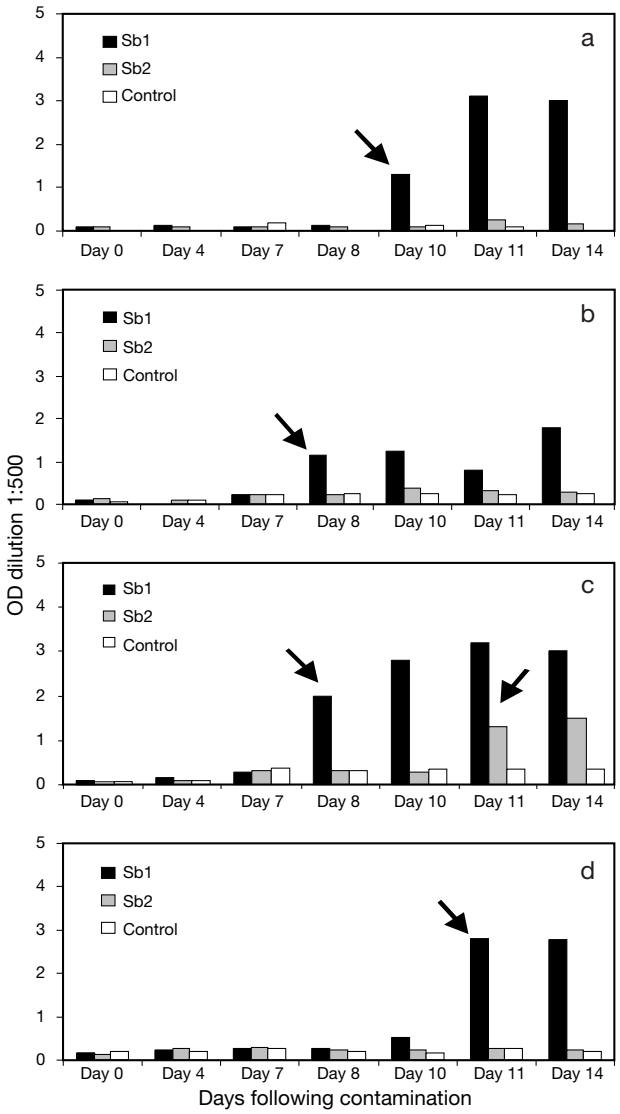

Fig. 3. Dose response curve for sea bass larvae (Expts a, b, c and d) following experimental exposure during fertilization with the Sb1 and Sb2 strains at 15°C. Optical density was the OD value of the sample minus the OD value of the non-specific buffer response. Arrows represent the first larval stage showing positive ELISA results associated with viral lesions in the brain

DISCUSSION

Virus detection by ELISA

The first detection of nodavirus in fish tissues by ELISA was performed in striped jack using a rabbit polyclonal serum raised against the SJNNV (Arimoto et al. 1992). However, this ELISA allowed the detection of 25 ng ml^{-1} of purified virus, which is less sensitive than our method (6 ng ml^{-1}), which uses a biotin-labeled rabbit IgG allowing the avidin-biotin amplification step. The nodavirus isolated from sea bass and barramundi reacted in the ELISA, and these viruses belong to the red-spotted grouper nervous necrosis virus cluster according to phylogenetic analysis of the coat protein gene (Nishizawa et al. 1997). As the nodavirus disease is now spreading worldwide (Munday & Nakai 1997), this ELISA should also be tested with the different genotypes (Nishizawa et al. 1997). In

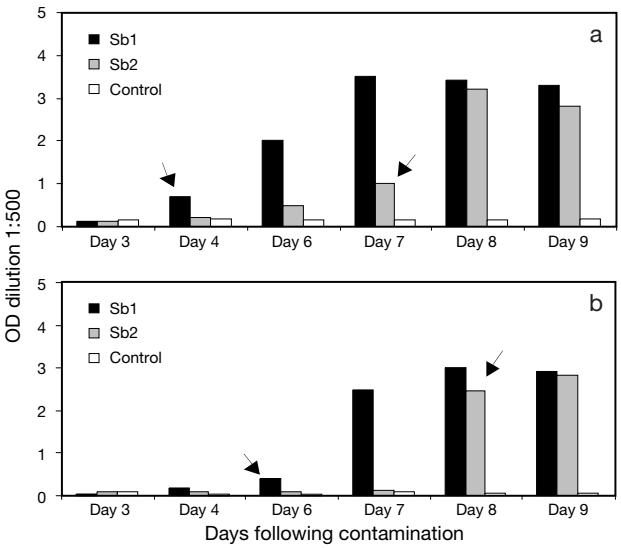

Fig. 4. Dose response curve for sea bass larvae (Expts a and b) following experimental exposure at larval stage 4 with the Sb1 and Sb2 strains at 15°C. Optical density was the OD value of the sample minus the OD value of the non-specific buffer response. Arrows represents the first larval stage showing positive ELISA result associated with viral lesions in the brain

sea bass, the existence of at least 2 distinct genotypes (Atlantic and Mediterranean virus strains) has been reported (Thiery et al. 1999). Although both genotypes reacted in the ELISA, optimal results were obtained with the Atlantic isolate (Sb1) that had also been used for the rabbit immunization. This preliminary result suggests a close but non-identical antigenic relationship between the 2 nodavirus genotypes isolated from sea bass.

According to the OIE manual (OIE 1995), the diagnosis of the disease is based on the detection of lesions in the brain. Our preliminary results on the detection of nodavirus in a commercial hatchery as well as in experimentally contaminated larvae or eggs using both techniques (IHC and ELISA) indicate that an ELISA should also be useful as a rapid and specific diagnostic method for the disease. The ELISA is very useful for screening numerous samples, and positive results can be confirmed by FAT or other techniques such as PCR (Mushiake et al. 1994, Nishizawa et al. 1994, Chi et al. 1997, Thiery et al. 1997) and cell cultures (Frerichs et al. 1996).

Transmission to larvae

Experimental exposures confirm that the 2 nodavirus strains are pathogenic to sea bass larvae, and this is in accordance with the isolation of both strains in sea bass hatcheries. Preliminary infection studies have shown the high pathogenicity of the Sb2 strain to sea bass juveniles (Peducasse et al. 1999), but this is the first report of comparative studies of the 2 virus strains to sea bass larvae. Viral lesions were detected in the brain and the retina as early as 4 d following challenge, and these results are similar to other experiments performed on fish larvae (Munday & Nakai 1997). Sb1

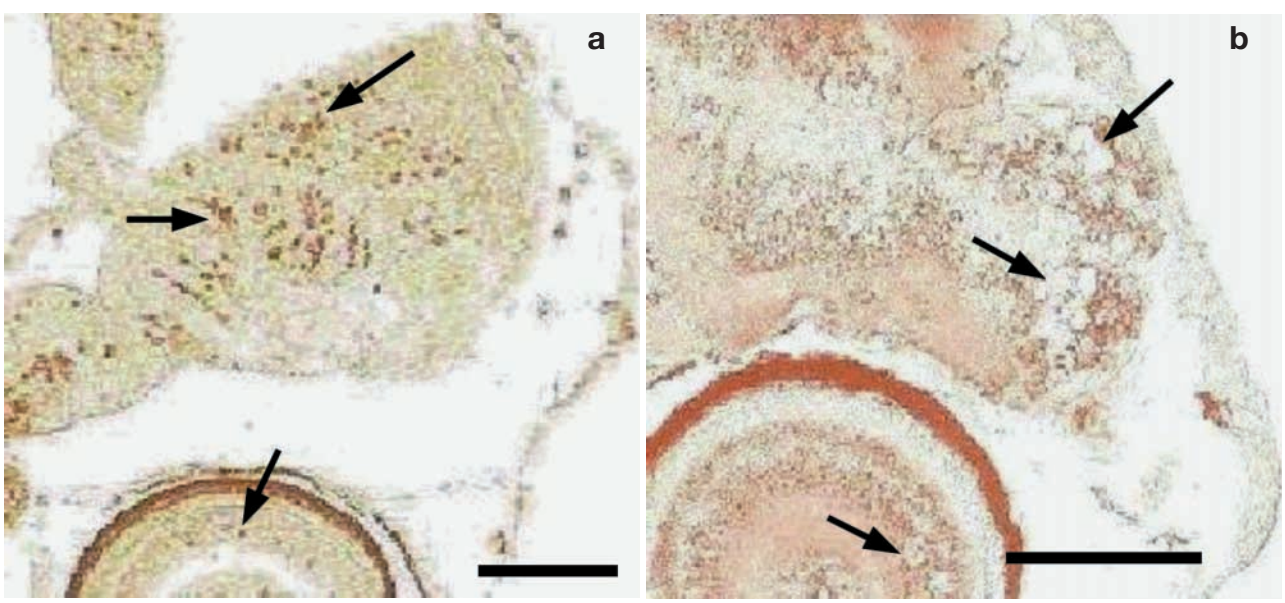

Fig. 5. Histological section through the brain and retina of a sea bass larvae showing (a) immunostaining of the nervous cells by anti-nodavirus antibodies (arrows) 4 d following challenge with the nodavirus and (b) extensive vacuolation (arrows) of the nervous cells 9 d following challenge with the nodavirus. Scale bars = 100 µm

is highly infective during fertilization in comparison to Sb2, but both strains are pathogenic to 4 d old larvae. Such differences in virulence may be related to different factors such as the temperature and age of the larvae. In redspotted grouper *Epinephelus akaara*, rearing water temperature (16 to 28°C) influences development of the disease, causing higher mortalities and allowing earlier detection of lesions (Tanaka et al. 1998). The Sb2 strain is highly pathogenic for sea bass juveniles and adults (Peducasse et al. 1999) and less pathogenic during fertilization (our experiments), and this could be related to the water temperature, as all the experiments with larvae were performed at 14 to 15°C while juvenile experiments were performed at 25°C. The age-dependent susceptibility of sea bass larvae is another interesting finding. When the contamination experiments are performed at the same water temperature (14 to 15°C), Sb1 is highly pathogenic during the larval development (our experiments) and less pathogenic (or non-pathogenic) to juveniles or adults (Coeurdacier pers. comm.), and this is in accordance with preliminary experiments showing that the SJNNV is pathogenic for striped jack larvae and not for juveniles (Arimoto et al. 1993). The transfer of specific maternal immunity from naturally immunized females to the eggs could also affect the development of the virus in the eggs and larvae. An antibody activity has been detected in the eggs of various fish species (Shors & Winston 1989, Kawahara et al. 1993, Lillehaug et al. 1996) including sea bass (Pepin et al. 1997), and this activity is based on monomeric immunoglobulins (Mor & Avtalion 1990). Furthermore nodavirus antibodies have been detected in the serum of adult sea bass and in our broodstock (Breuil et al. 1999). The transfer of nodavirus antibodies to the eggs could explain the apparent resistance of the sea bass eggs to the Sb2 strain, as this virus was first isolated in a French Mediterranean sea bass farm (Thiery et al. 1999). Further experiments using monoclonal antibodies raised against the 2 nodavirus strains Sb1 and Sb2 are needed to confirm this hypothesis.

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