**Preliminary study of immunogenicity and protection against nodavirus following injection of synthetic peptides from its capsid protein.**

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Jean-Luc COEURDACIER, Florent LAPORTE, Jean-François PEPIN, 

*IFREMER, Route de Maguelone, 34 250 PALAVAS les FLOTS, France*

Cette présentation orale aborde les aspects non traités dans la publication relative aux essais de vaccination contre la Nodavirose par peptides synthétiques (https://w3.ifremer.fr/archimer/doc/00000/10600/7038.pdf) dont évaluation par différentes méthodes de la présence ou des traces d’un contact avec le Nodavirus qu’il soit vivant (culture de cellule), sous forme de fraction protéique (ELISA antigène et PCR) ou qu’il ait laissé des traces sériques (séropositivité par ELISA anticorps) ou sous forme de vacuoles encéphaliques (immunohistochimie).

**VIRUS CULTIVATION AND QUANTIFICATION**

One nodavirus strain, Sea bass 1 (Sb1), was isolated from sea bass collected in the Atlantic Ocean; this strain corresponds to the virus from which the DIEV RNA2 protein was extracted. A second strain was isolated from sea bass collected in the Mediterranean Sea (Sb2) and corresponds to SB-med sequenced by Thiery et al. (1999). These virus strains were purified after cultivation on Striped Snake fish cell lines (SSN-1a slightly modified version of the method describe by Frerichs et al. (1996), as follows: Leibovitz medium was supplemented with L glutamine(1%) streptamicin-penicillin (2%) and foetal calf serum (10%) and the cells were grown at 29°C. Two day old SSN1 lines were infected with the virus for production or tissue samples for quantification, and kept 7 days. After quantification using tissue samples, the supernatant was used to initiate a second culture.

The virus was purified on CsCl2 using the method previously described by Breuil and Romestand (1999) and the viral protein was quantified by reading the optical density at 260 nm and 280 nm respectively, according to the method described by Harlow and Lane (1988).

Virus titre was measured on cell culture by immunofluorescence (FITC). The infected cell was estimated by counting reactive fluorescence cell in regard of dilution and expressed in Fluorescent Cell Unit (FCU/mL). Briefly, after dehydration of the cells, the rabbit anti-nodavirus polyclonal serum was added to the SSN1 cells. FITC-conjugated anti-rabbit IgG polyclonal serum were used to count the infected cells, which appeared as UV fluorescence spots under the microscope.

**CHALLENGE**

Pre-challenges were performed with different concentrations of nodavirus: from 9 X 10^7 FCU to 9 X 10^10 FCU of Sb2 and from 9 X 10^9 FCU to 9 X 10^11 FCU of Sb1 per fish, to find the concentration which doesn’t kill all the animals but at least 60% of them, as the required R.P.S. (Relative Percent Survival, A. E Ellis. 1988).

No challenge performed with Sb1 succeeded and no symptoms of illness were ever observed.
70 days after the first vaccination all the fish were given an intramuscular injection of $9 \times 10^9$ FCU of virulent Sb2 nodavirus in PBS. A group of fish that had not been handled was added at this stage. Dying and dead fish were counted and sampled daily over 40 days. Diagnosis of infection for those animals were based on clinical and histopathological observations, ELISA and cell culture as defined upper.

**RESULTS**

**CHALLENGE AND PROTECTION**

**Mortality** (fig3)

Table 2. Mortality percentage and relative survival of fish Sb2 challenged.

<table>
<thead>
<tr>
<th>Group</th>
<th>C-ter</th>
<th>Lp1</th>
<th>Lp2</th>
<th>HI Sb1</th>
<th>KLH</th>
<th>No stress</th>
<th>N-ter</th>
<th>HI Sb2</th>
<th>KLH</th>
<th>No stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish challenged</td>
<td>31</td>
<td>28</td>
<td>31</td>
<td>34</td>
<td>37</td>
<td>16</td>
<td>29</td>
<td>20</td>
<td>32</td>
<td>40</td>
</tr>
<tr>
<td>Dead fish</td>
<td>19</td>
<td>14</td>
<td>18</td>
<td>11</td>
<td>24</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>Dead fish%</td>
<td>61</td>
<td>50</td>
<td>58</td>
<td>32</td>
<td>65</td>
<td>63</td>
<td>4</td>
<td>5</td>
<td>31</td>
<td>33</td>
</tr>
<tr>
<td>RPS</td>
<td>6</td>
<td>23</td>
<td>11</td>
<td>50</td>
<td>3</td>
<td>87</td>
<td>83</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

Experimentation was performed in two times, C-Ter, Lp1, Lp2 in first and N-Ter latter.

RPS=Relative Per cent Survival=$\left\{1-(\% \text{ vaccine mortality}/\% \text{ control mortality})\right\} \times 100$

(A.E. Ellis 1988)

**Fig. 3.** All injected fish and a group of fish that had not been handled were given an intramuscularly injection of $9 \times 10^9$ FCU of virulent Sb2 in PBS. No difference was observed between the mortality of peptide injected fish and negative controls (31%) but N-Ter injected fish had a significantly lower mortality (4%).

The difference between the control and N-Ter RPS is promising but does not prove there is protection because the control mortality was too low (60% at least required)
Fig. 4. A group of fish that had not been handled (----) was included to investigate the influence of stress on mortality with regard to negative controls ( KLH — — —). HI SB1 was used as a positive control (———); a similar pattern was obtained with HI SB2. After one week the mortality of non-handled fish was similar to the positive controls; then, during the third week it rapidly rose to the levels of the negative controls. Stress only changes the kinetics of mortality but not the final mortality. The fish, which had not been handled, had the same final percentage of mortality as the controls but the kinetics were different, with the control group of fish dying more regularly.

**Presence of virus**

The relation between death and virus injection was investigated by 4 methods. In fact the presence of nodavirus was investigated at different levels: nodavirus protein by antigen ELISA, live virus by SSN1 culture, or signs of vacuolating encephalopathy was observed under the microscope and infected cells detected by immunochimistry microscopy (Thiery et al 1997)

In general, the samples used for microscopy analyses came from different fish to those used in ELISA or in cell culture because the small seize of the brains sampled.

Vacuolating areas could be seen on stained or fluorescent sections of all the brains of dead or dying fish, but not on sections of gonad or spleen. No evidence of encephalopathy was observed in surviving fish.

The results of ELISA and SSN1 culture were slightly different, 38% of brains from dead fish tested had no viable virus and half of them had no viral protein at all. Only 10% of surviving fish had viable virus and 30% viral protein. In all cases the presence of viable virus was consistent with the presence of protein (fig 4 & 5).
Fig. 5. Presence of viral protein was quantified by ELISA (ag +/-) and live virus by growing on SSN1 cells (cc +/-). The brain tissues of both fish that survived and fish that died were tested. No case of viable virus in the absence of protein (ag-cc+) was found. The ag-cc-fish, which died probably destroyed the virus and the protein but still had fatal lesions. The survivors who were ag+cc+ may be considered as healthy carriers.

DISCUSSION

The difference in mortality between two fish batches of the same family from one week to another is a major problem. Moreover, the fish were not individually marked and all the animals were challenged. We don’t known if the surviving fish had antibodies to nodavirus.

So, if the dead fish did not have any antibodies this would suggest a lack of protection rather than a lack of immunogenicity. However, if the surviving animals that did not have antibodies are included in the calculation they increase the RPS although they are protected by another mechanism (natural or non specific defence...)

All dead fish examined showed characteristic lesions but only 64% had living virus and 70% had antigen in the brain. One hypothesis is that some of them eliminated the virus after it induced lethal lesions. In fact, the samples used for microscopy came from different fish than the samples used for ELISA or cell culture and a difference in the samples cannot be totally ruled out. 70% of the surviving fish did not have virus in their brain, which is consistent with results obtained on sevenband grouper (Epinephelus sepmfasciatus) (Tanaka et al., 1998). Nevertheless, this was also true for 18% of dead fish. If technical problems are ruled out, there are two possible explanations : i) the fish died from another cause, but all of them were seropositive and all the fish of the control group were tested negative. ii) the virus is located elsewhere than the brain. This would be consistent with results found in sea bass brood stock (Breuil p.c)

The most promising avenue for vaccine research is the use of recombinant proteins process that produce natural or new proteins. One or more of all these proteins is bound to be able to induce an unexpected reaction and then the production of the recombined protein will have to be stopped by the destruction of all the transfected cells or animals. This is possible in theory but not in practice. Peptides, which are entirely synthetic chemical products, do not have this disadvantage.
6E11b, Biotin conjugated monoclonal antibody against sea bass IgM
Bioenvirotech, Marseille, France
Abαpt Specific antibody activity against synthetic peptide
Abαpt Specific antibody activity against synthetic peptide
CPV Canine Parvovirus
DIPCDI Diisopropylcarbodiimide) in DMF (N,N-dimethylformamide)
DIEV RNA2 Capsid protein of *Dicentrarchus labrax* encephalitis virus (Delsert and Comps 1997)
DMF N,N-dimethylformamide
ELISA Enzyme-linked immuno-sorbent assay
Fmoc Fluorenylmethoxycarbonyle
HOBt 1-hydroxybenzotdazole
IFA Incomplete Freund’s adjuvant
KLH Keyhole Limpet Haemocyanin (SIGMA H-7017)
KLHS+ Anti-KLH sea bass serum used as a standard
NdS+ Anti-nodavirus sea bass serum used as standard (6000 UI mL⁻¹)
PBS Phosphate buffered saline
PBS-SM5% or 05% 5% or 0.5% skimmed milk in PBS (w/v)
PBS-T 0.5% Tween 20 in PBS(v/v).
FCU Fluorescent Cell Unit
PpS+ sea bass anti-peptide standard serum
OPD Orthophenylenediamine
rb abαKLH rabbit antibody serum against KLH (SIGMA)
Sb1 = DIEV=Sb-atl *Dicentrarchus labrax* nodavirus (Delsert and Comps 1997, Thiery et al, 1999)
Sb2 = Sb-med *Dicentrarchus labrax* nodavirus (Thiery et al, 1999)
SSN-1 Striped Snake cell line
TIPS Triisopropylsilane
TFA, Acide trifluoracétique
HI Sb1; HI Sb2 Heat inactivated *nodavirus*