

DIAGNOSIS OF HERPES-LIKE VIRUS INFECTIONS IN OYSTERS USING MOLECULAR TECHNIQUES

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Introduction

Several herpes-like virus infections have been reported since 1991 among different bivalve species around the world (Nicolas et al., 1992; Comps and Cochenne, 1993; Renault et al., 1994; Hine and Thorne, 1997; Hine 1997). To date, detection of these infections has been strongly correlated with severe losses of oyster larvae and spat. The virus infections are currently diagnosed by laborious histological and transmission electron microscopical examinations. These techniques, however, facilitate neither rapid diagnosis nor the ability to analyse large sample numbers. As a result of the economic importance of oyster species to mariculture, the development of sensitive and rapid diagnosis methods for virus infections was absolutely necessary in order to monitor and control animal health. A breakthrough was achieved in the development of a protocol for purifying a herpes-like virus from infected *Crassostrea gigas* larvae (Le Deuff, 1995). Using extracted virus DNA as first material, different molecular techniques as PCR and *in situ* hybridization have been developed.

Material and Methods

Oyster (*Crassostrea gigas* and *Ostrea edulis*) larvae and spat obtained from hatcheries and shellfish farmers were investigated by PCR or by *in situ* hybridization retrospectively. Samples were collected and stocked frozen or fixed in Davidson's fluid. For PCR analysis, larvae and spat after grounding in distilled water were denatured in boiling water for 10min and centrifuged at 10 000rpm for 5min. Supernatants were 10 fold diluted in double distilled water and frozen at -20°C. The primer-pair OHV3/OHV4 derived from a M13 cloned restriction virus DNA fragment sequence was used. Thermal cycling was performed using a microprocessor controlled heating block thermal cycler Crocodile III (Appligene Oncor). Each 50µl reaction contained appropriate reaction buffer (Eurogentec), 0.05 mM of each dNTP, 100 ng of each primer, 2.5 mM MgCl₂, 2.5 units of DNA polymerase (Eurogentec) and 1 µl of sample. After heating tubes for 2min. at 94°C, 35 cycles were performed followed by a final elongation step of 5min. at 72°C. Each of the 35 cycles consisted of a DNA melting step at 94°C for 1min, a primer annealing step for 1min at 50°C and a primer elongation step at 72°C for 1min. PCR products were analysed on 1% agarose gels. For *in situ* hybridization, probes were produced by PCR using virus DNA as template, the primer-pair OHV3/OHV4 and digoxigenin-11-dUTP (Boehringer Mannheim). Oyster samples preserved in Davidson's fixative were embedded in paraffin. Sections were cut 7µm thick and placed on silane-prepTM slides (Sigma Aldrich). After dewaxing and rehydration, tissues were treated with proteinase K (100µg/ml in distilled water) at 37°C for 30min. After dehydration, the tissue sections were prehybridized in 500µl of 4x SSC, 50% formamide, 1x Denhardt's solution, 0.25mg/ml yeast tRNA and 10% dextran for 30min at 42°C. The solution was replaced with prehybridation buffer containing 5ng

of digoxigenin-labelled probes. After denaturation of target DNA and probes (95°C, 5min) sections allowed to hybridize overnight. The sections were washed for 10min in 1x SSC at 42°C. Then, they were blocked with phosphate buffer containing 6% of powdered milk for 1h. Sections were incubated for 1h at room temperature with an antidigoxigenin-mono-clonal antibody (Boehringer Mannheim, 1:300 in phosphate buffer). Unbound antibodies were removed with six 5min washes. Anti-mouse-peroxydase conjugate (Sanofi Diagnostics Pasteur) was diluted 1:400 in phosphate buffer and slides were incubated at room temperature for 1h. After six 5min washes, tissue sections were incubated in color development solution (diaminobenzidine/H₂O₂, phosphate buffer).

Results and Discussion

The PCR protocol defined in this study permit to observe amplification products of the expected size (896bp) on agarose gels from different clinical samples presenting high mortality rates. Of the different procedures of sample preparation from oyster specimens, boiling of ground tissues was the preferred method, because it was simple and it was the most consistent in our hands. The presence of compounds in oyster that inhibit DNA polymerase is a potential problem in using ground tissues in PCR. In order to minimize inhibitory effects, a 10 fold dilution is performed after preparation of ground tissues. 0.1 mg of oyster tissues is added in each PCR tube permitting the detection of virus DNA, but avoiding inhibition of PCR amplification. *In situ* hybridization of 5ng/μl probe produced by PCR using OHV3/OHV4 primer-pair to paraffin-embedded oyster (*Crassostrea gigas* and *Ostrea edulis*) sections infected with a herpes-like virus yielded strong hybridization of the probe to infected cells in connective tissues of different organs. The location and the morphology of labelled cells correspond to the observations made by transmission electron microscopy. No background hybridization to healthy oyster tissues was observed. This nucleic probe should be very useful as a diagnostic tool for herpes-like virus infections. Indeed, the *in situ* hybridization technique can be used to confirm the suspicion of the presence of viruses obtained using histological examinations.

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