

EXPERIMENTAL TRANSMISSION OF A HERPES-LIKE VIRUS TO AXENIC LARVAE OF PACIFIC OYSTER, *CRASSOSTREA GIGAS*

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Introduction

A herpes-like virus which was associated with mortalities amongst hatchery-reared larvae of Pacific oysters, *Crassostrea gigas*, was observed for the first time in France during the summer 1991 (Nicolas *et al.*, 1992). Since this first report, we have noted further outbreaks of the disease in the summers of 1992 and 1993 in hatcheries on the western coasts of France in which a similar virus was associated with moribund larvae. Moreover, in the summer 1991, Hine *et al.* (1992) described a herpes-like virus responsible for larval mortality of hatchery reared *C. gigas*, in New Zealand. All these viruses are closely related with respect to their size and morphology and to their apparent cellular tropism for fibroblastic cells. Considering these reports and the economic importance of *C. gigas* to shellfish culture, it seems important to characterise this virus and develop sensitive diagnostic methods in order to study the epidemiology of the disease and prevent its potential spread. Amongst the infectious diseases of bivalve molluscs, viral diseases are not well known. Indeed, in most laboratories interested in molluscan pathology, the basic for examination of suspect samples is still light microscopy. However, with viral disease, this method appears inadequate and needs to be improved by other methods such as electron microscopy. In addition, due to the lack of bivalve cell lines, research into viral cytopathogenic effects in homologous cell culture is impossible.

Here, we report an alternative method to cell culture for diagnosis and production of *C. gigas* herpes-like virus using axenic larvae.

Materials and methods

Axenic larvae: To obtain axenic larvae Langdon's method (1983) was applied as follows. *C. gigas* were well brushed and washed under flowing water, then sterilised externally in a 30 sec. bath of 70% ethanol before opening them, in a sterile area. The shells were then washed for 5 min. with commercial Betadine solution diluted 50% with distilled water. The gonad was then incised with a sterile scalpel and gametes were recovered by pipetting. Fertilisation was performed by mixing spermatozoa and ova in a ratio of 10:1. Axenic larvae were reared in sterile sea water at a density of 20 larvae per ml. Larvae of *O. edulis*, which are laid by parent one week after fertilisation, were recovered by filtering the water and then decontaminated by a 20 min. bath in antibiotics (oxolinic acid, kanamycin and erythromycin, 0.1 mg/ml each; penicillin, 0.75 mg/ml and streptomycin sulphate, 1.50 mg/ml), then reared as described up but to a density of 3 larvae/ml. Axenicity of larvae was confirmed by inoculation of a few drops of sea water in marine broth.

Inoculation: fresh or thawed (-20°C) larvae infected with herpes-like virus were macerated in sea water and filtered to 0.22µm. This virus suspension was inoculated to two day old *C. gigas* larvae, or to seven day old *O. edulis* larvae.

Results and discussion

Two days after inoculation of the virus suspension, *C. gigas* larvae ceased to swim and fell to the bottom of the glass containers. These larvae exhibited velar lesions (Figure 1) but not all were dead. Identical results were obtained when axenic larvae were inoculated with virus suspensions from fresh samples or from infected larvae stored at -20°C for several months.

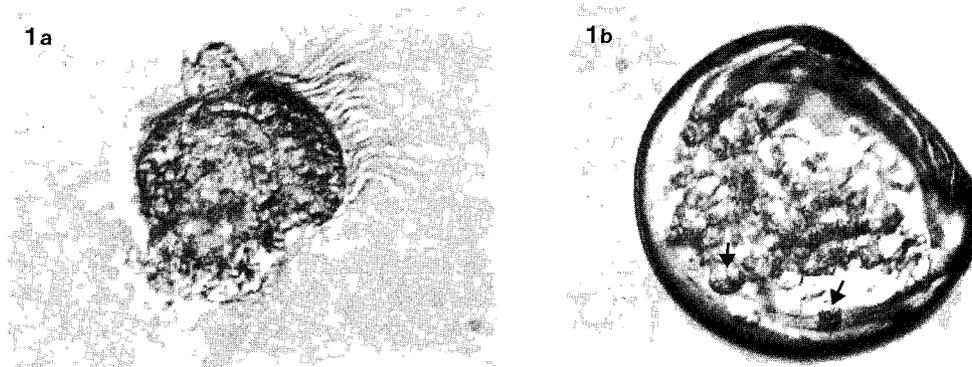


Figure 1 : Macroscopic velar lesions of axenic *C. gigas* larvae, 48h after inoculation of the herpes-like virus. 1a) Portion of velum detached from larva. 1b) hypertrophied cells in larval shell.

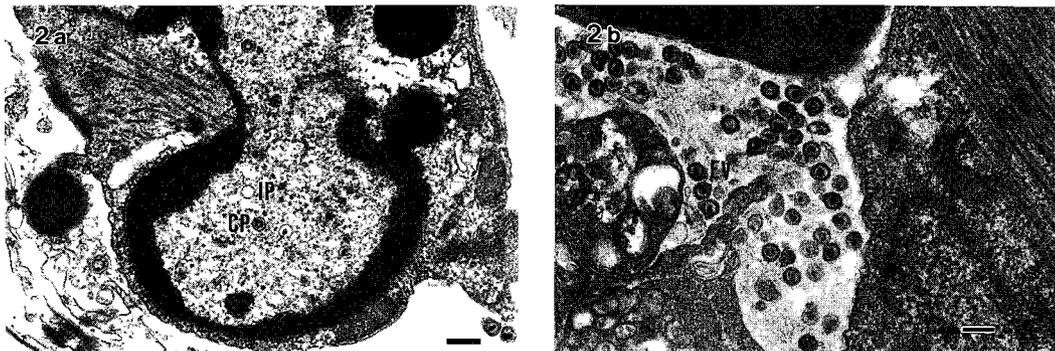


Figure 2 : Electron micrographs of infected tissues of axenic larvae showing herpes-like particles at different stages of maturation. 2a) Infected nucleus with incomplete empty viral particles (IP) and a complete particle with nucleoid (CP). 2b) Extracellular enveloped virions (EV) containing an electron dense parallelepipedal core. Bar = 200nm

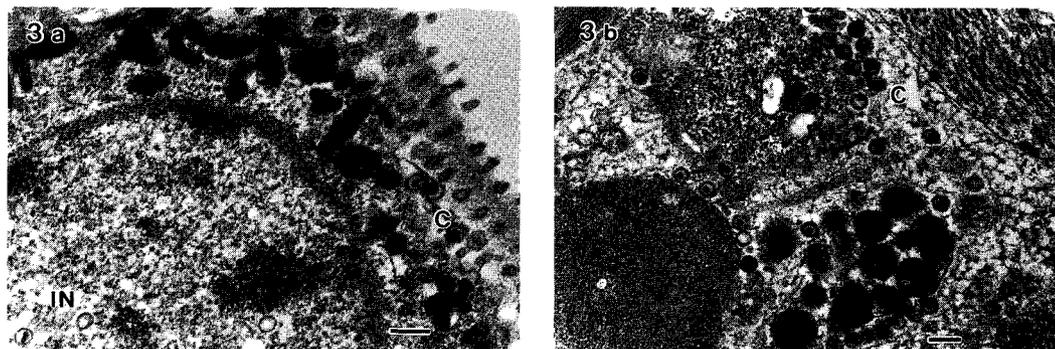


Figure 3 : Electron micrographs of hatchery-reared infected larvae. Intranuclear (IN) and cytoplasmic (C) particles exhibit the same features as viruses observed in experimentally reared axenic larvae. Bar = 200nm.

This disease spread quite quickly because all larvae were moribund within 48 hours post inoculation (p.i.) with the virus. This observation seems to indicate that several viral cycles occurred within this time. Such a short productive cycle is characteristic for the *Alphaherpesvirinae*, indeed, viruses belonging to this subfamily of the *Herpesviridae* are characterised by a productive cycle of less than 24 hours (Matthews, 1982), whilst the cycle of *Betaherpesvirinae* is at least 48 hours long. However, this virus also shows features similar to the *Betaherpesvirinae*, particularly velum cells are hypertrophied and detached from the tissue (Figure 1). Also, very large "cytomegalic" cells could be observed in sea water (not shown). Total cumulated mortalities on the sixth day p.i. reached 100% when larvae were inoculated with viral suspension from either fresh or thawed samples, while only three per cent mortalities occurred in control healthy larvae. In a previous report, Hine (1992) described a similar evolution of the disease in hatchery reared *C. gigas* larvae in New Zealand. Indeed, these naturally infected larvae ceased to swim three or four days after exposure and mortality reached 100% at the seventh day. This seems to suggest that contamination of larvae occurs quite early, and may result of a vertical transmission from parents.

Transmission electron microscopy examination revealed the presence of viral particles in inoculated larvae (Figure 2), whilst in control larvae neither lesion, nor virus could be observed.

Two days p.i., viral particles were observed in nuclei of connective cells of the velum. On the third and fourth days p.i., virions were still present in the nucleus but also in the cytoplasm showing a later stage of the infection. In both cases, the viral particles exhibited the same characteristics (morphology, size of capsid 72-75nm, cellu-

lar localisation, presence of an envelope) as for virions previously described in infected larvae from hatcheries (Figure 3).

A herpes-like virus has also been found in five months old spat of the flat oyster, *Ostrea edulis*, and was suspected to be responsible for the 90% mortality observed in these animals (Comps and Cochenec, 1993). This virus was similar in structure to the herpesvirus previously described from *C. gigas* larvae, with some morphological differences. Therefore, we tried to inoculate the *C. gigas* herpes-like virus into *O. edulis* larvae and approximately 80% of the larvae fell to the bottom of the inoculated flasks at the 10th to 12th day p.i., but in the uninoculated control, approximately 50% larvae were also deposited on the bottom. No velar lesions were noticeable and no viral infection could be detected by transmission electron microscopy.

Nevertheless, herpes-like viruses from *O. edulis* and *C. gigas* should not necessarily be considered to be different or unable to infect other species of oyster. Indeed, the failure to obtain the development of a viral infection in *O. edulis* larvae may be attributed to several other causes. Firstly, the method used to decontaminate *O. edulis* larvae may be inadequate to obtain real axenic conditions, and could result in the failure of viral infection. This last hypothesis is based on the observation that it was difficult to perform the experimental transmission of the virus on non axenic *C. gigas* larvae (data not shown). Furthermore, the biology of *O. edulis* obliged us to inoculate the larvae when they were seven days old, and may be more resistant to viral infection than younger larvae, as with the two day old *C. gigas* larvae. Alternatively, natural herpesvirus infections of *O. edulis* have so far only been observed in young spat, and never in larvae, suggesting that larval stages could be less or in-sensitive to this disease .

Conclusion

This method of inoculation of a herpes-like virus to axenic larvae is, to our knowledge, the first report of a successful experimental transmission of a viral disease affecting bivalve molluscs. Indeed, the lack of available molluscan cell lines leads to obvious difficulties in studying and maintaining viruses from molluscs.

For the purpose of diagnosis of a viral aetiology, the method described here complemented ultrastructural examinations. It has the advantage of taking little time and first results can be expected within four to five days. However, as this method does not substitute for transmission electron microscopy, we consider it only as the first step in our work. Indeed, since we are able to store infectious virus for long periods and to obtain infected larvae, we intend to develop other diagnostic methods based on the use of specific reagents such as DNA probes and antibodies.

Summary

A herpes-like virus associated with mortalities among hatchery reared larvae of Pacific oyster, *Crassostrea gigas*, has been described in France and New Zealand. Considering the economic importance of *C. gigas* to shellfish culture, we have developed a method for diagnosis and production of *C. gigas* herpes-like virus on axenic *C. gigas* larvae. The virus spreads quite quickly: 100% larvae showed lesions within 48h post infection, and cumulative mortalities reached 100% on the sixth day p.i. Ultrastructural examination revealed the presence of the same viral particles as previously described

in infected larvae from hatcheries. Virus stocks were still infectious after being stored at -20°C for several months. We failed to obtain infection of flat oyster, *Ostrea edulis*, larvae. This method is the first report of a successful experimental transmission of a viral disease affecting bivalve molluscs.

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