Herpesviruses associated with mortalities among Pacific oyster, *Crassostrea gigas*, in France - Comparative study

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SUMMARY

Sporadic mortalities were reported in june and july of 1992 and 1993 among batches of hatchery-reared larval Pacific oyster, *Crassostrea gigas,* and at the beginning of July and in August of 1993 among five batches of 3-7 month old young spats, *C. gigas.* Observations with transmission electron microscope showed the presence of herpes-like virus particles in infected larvae and young spats. The viruses observed in diseased larvae and affected young spat oysters were very similar. Elevated temperature and crowding may increase susceptibility of oysters to these herpes-like virus infections.

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KEY-WORDS : Herpes-like virus - mortality - Pacific oyster - *Crassostrea gigas* - larvae - spat.

RESUMÉ

Herpesvirus associés à des mortalités chez l'huître japonaise, *Crassostrea gigas*, en France. Etude comparative. Par T. RENAULT, R.M. LE DEUFF, N. COCHENNEC et P. MAFFART.

Des mortalités épisodiques ont été observées au cours des étés 1992 et 1993, parmi des lots de larves d'huître creuse, *Crassostrea gigas,* élevées en écloserie. Par ailleurs, au début du mois de juillet et au mois d'août 1993, des mortalités sporadiques ont également été enregistrées parmi cinq lots de juvéniles d'huître creuse, *C. gigas,* âgés de 3 à 7 mois. Des observations en microscopie électronique à transmission ont permis de révéler la présence de particules virales apparentées aux herpesvirus, chez les larves et les juvéniles moribonds. Les virus observés chez les larves et sur le naissain semblent très proches. Les températures élevées et les conditions d'élevage intensif pourraient augmenter la sensibilité des huîtres à ces infections virales.

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MOTS-CLÉS : Herpès-like virus - mortalités - huître japonaise - *Crassostrea gigas* - larve - naissain.

Introduction

Pacific oyster, *Crassostrea gigas,* are increasingly cultured in a great number of countries, from animals ultimately introduced from Japan. Introduction into France has occured in the late 1970's, after the disappearance of the Portuguese oyster, *C. angulata,* decimed by two iridovirus infections [4, 5].

The discovery of viruses in Pacific oyster, *C. gigas,* is fairly recent. The first detection of virus infection was reported by COMPS *et al.* [6] among *C. gigas* cultured at Arcachon and Marennes-Oléron (France). With respect to its morphology and morphogenesis, this virus closely ressembled to *Iridoviridae.* In 1979, ELSTON [9] reported an other virus infection in larval Pacific oysters and the electron microscope examination revealed the presence of viral particles assumed to belong to the family *Iridoviridae.* Moreover, herpes-like virus associated with mortality among hatchery-reared larvae of Pacific oyster, *C. gigas,* was observed in France during the summer of 1991 [16]. At the same time, HINE *et al.* [14] described a herpes-like virus responsible for larval fatality of hatchery-reared *C. gigas* in New Zealand.

In summer of 1992 and 1993 sporadic high mortalities (90 to 100 %) occured among some batches of *C. gigas* larvae in several french hatcheries. Moreover, during July and August of 1993, abnormal sporadic high mortalities (80 to 90 %) were also reported, among five batches of cultured young spat of *C. gigas* from different French marine locations. Histological and electron microscopy examinations were performed to search for an explanation of these abnormal mortalities. We describe here herpes-like infections of both hatchery-reared larvae and cultured young spats of Pacific oyster, *C. gigas,* from French coasts and we make a comparative study between the two viruses found respectively among larvae and young oysters and other herpesviruses described among bivalve molluscs by different authors.

Material and methods

Samples of moribund hatchery-reared larvae and samples of moribund cultured 3-7 month old oyster spat were fixed in Davidson's fluid for light microscopy examination. Samples were then dehydrated through an ascending ethanol series, cleared in xylene and infiltrated with paraffin on a tissue processor. Following these different steps, they were embedded in paraffin, sectionned at 3 or 4 μm, then stained by Hematoxyline Eosine (H/E) and carefully checked for lesions using a photomicroscope. The nucleal reaction of Feulgen and Rossenbeck was also used on some slides.

Samples of healthy wild-type larvae, samples of healthy or affected by moderate mortality cultured young oysters, and young spat survivors taken one to four months after reported high mortalities were also analysed with the same protocol.

For transmission electron microscopy, larvae and pieces (gill, mantle and digestive gland) of young spat were fixed 1 h in cold 2.5 % glutaraldehyde in cacodylate buffer and post-fixed in 1 % osmium tetroxyde in the same buffer. Tissues embedded in Epon were cut on a LKB ultramicrotome. One μm sections for light microscopy were stained in 0.5 % toluidine blue in 1 % aqueous sodium borate solution. Ultrathin sections were collected on copper grids, stained with uranyl acetate and lead citrate. These sections were then examined with a JEOL JEM 1200 EX transmission electron microscope at 60 kV.

Results

COURSE OF DISEASE AND EPIDEMIOLOGY

Three to four days after the spond, reduction in feeding and swimming larval activity were observed. Significant mortality occured by Day 6, with 100 % mortality by Day 8 to Day 10 in most batches. Moribond larvae showed a less extended velum and parts of this velum were often observed free in the pond water.

For young spat, high mortalities occured in 1993 at the begining of July in four different marine locations and in August for one batch (Table I). 80 to 90 % mortality appeared in few days (less than a week). In these areas, high mortality was not detected during this period among the Pacific oysters cultured around the batches in which fatality of young spat was reported (Table I). One to four months after outbreaks, no mortality was observed among the surviving animals in two locations (Table I).

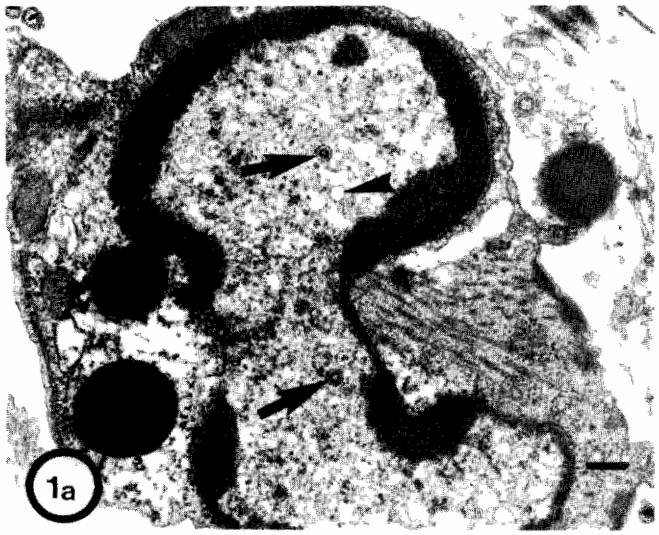
HISTOLOGICAL AND ULTRASTRUCTURAL OBSERVATIONS

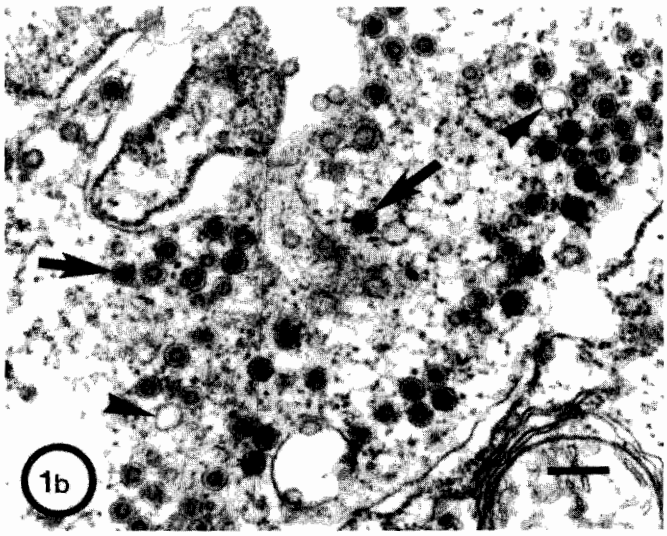
The main histological changes in the diseased larvae and spat consisted essentially of the presence of enlarged nuclei that showed abnormal shape and abnormal chromatin pattern throughout the connective tissues. The inflammatory reaction around infected cells was reduced. For larvae, these lesions were observed into velum and mantle and for young spat these abnormal nuclei were reported into gill and mantle connective tissues. Accumulations of Feulgen positive material were detected into nuclei and cytoplasm of affected cells.

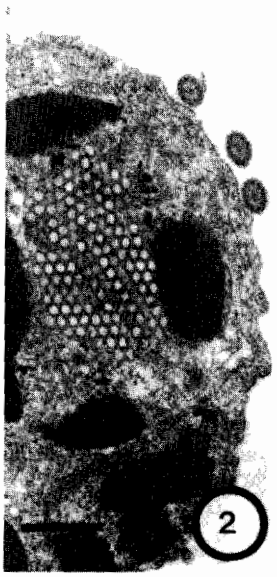
By electron microscopy, infected cells of larvae and young oyster spat exhibited intranuclear and intracytoplasmic virus-like particles. The nuclei contained spherical or polygonal particles, 70-75 nm in diameter (Figs 1a and 1b). Some particles appeared empty and consisted of structures assumed to be capsids ; other contained an electron-dense core or a ovoid annular translucent core and were interpreted as being nucleocapsids (Figs 1a and 1b). Some empty capsids appeared in the nucleus of infected cells with a paracrystalline arrangement (Fig. 2). Naked cytoplasmic nucleocapsids were observed in the cytoplasm of myocytes (Fig. 3). Enveloped virions were detected into cytoplasmic vesicles in other cells (Figs 4a and 4b) among both larvae and young oysters. In cytolytic cells and in extracellular spaces, enveloped viruses were seen too (Figs 5 a and 5b). These particles consisted of a capsid with an electron-dense nucleoid that was in turn surrounded by a unit-membrane like structure (Figs. 5a, 5b and 6). The core was 54 nm in length and 36 nm in diameter when viewed longitudinally. Envelop and capsid were separated by a reduced electron-lucent gap. Fine filaments passed from the toroidal core to the inside of the capsid (Fig. 6). The enveloped particles, about 120 nm in diameter, exhibited spike-like protrusions on the surface (Fig. 5a).

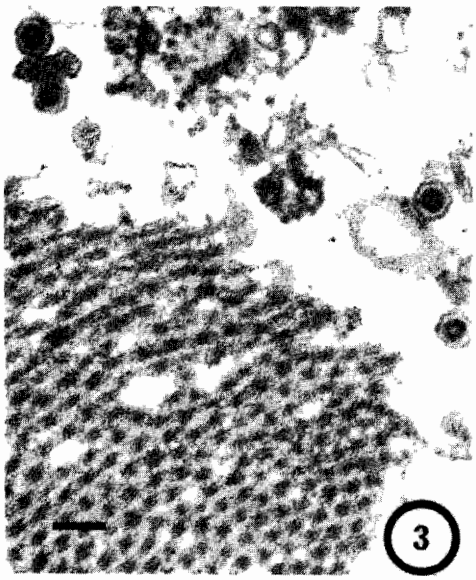
Ultrastructural changes of infected cells were found to be related to the presence of the virus in Japanese oyster larvae and young spat. Abnormal accumulations of granular endoplasmic reticulum associated with large swollen mitochondria (Figs 7 and 8) and condensed nuclei with electron-lucent center (Fig. 9) or electron-lucent areas (Fig. 10) were often observed in connective tissues of diseased animals. Moreover infected cells nuclei of affected larvae showed abnormal chromatin pattern with marginalisation (Fig. 11). Degenerating and lysing infected nuclei were frequently present too. A few large dense granular bodies, lacking a bonding membrane were reported in infected cells. Other than fibroblastic cells, the infected cell types could bot be identified with certainty, but nucleocapsids occured in cells that might have been myocytes and particles were also observed into the cytoplasm of cells assumed to be haemocytes.

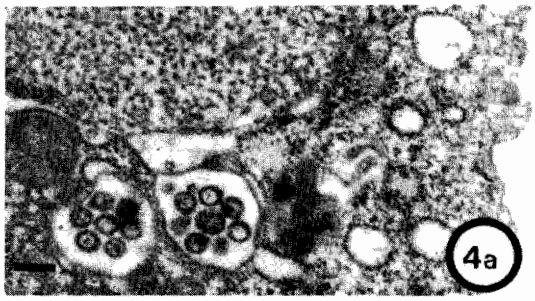
In this study, we found viruses in eight batches of hatchery-reared larvae and in five batches of cultured young oyster spat (Table I). On an other hand, histological and electron microscopy examinations on survivors of two batches of young oyster spat taken one to four months after high mortalities failed to reveal the presence of

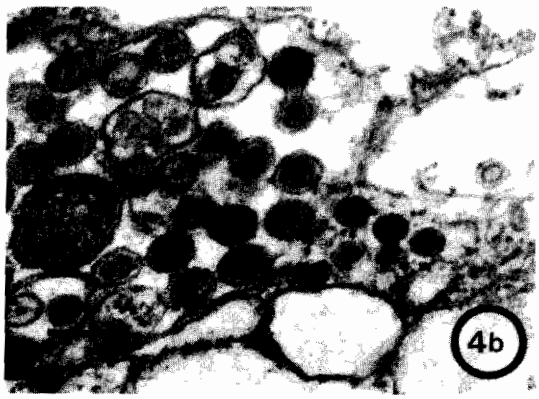


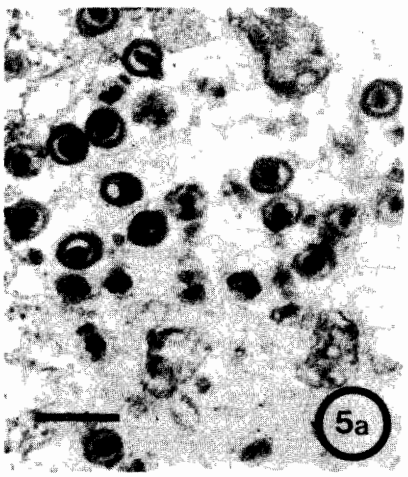


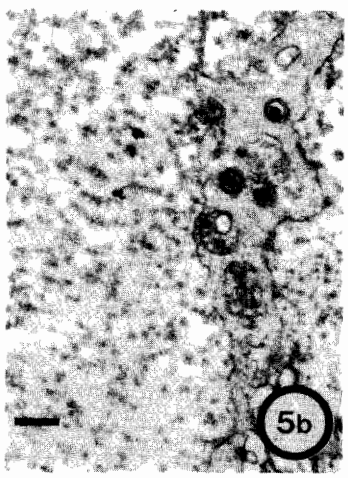












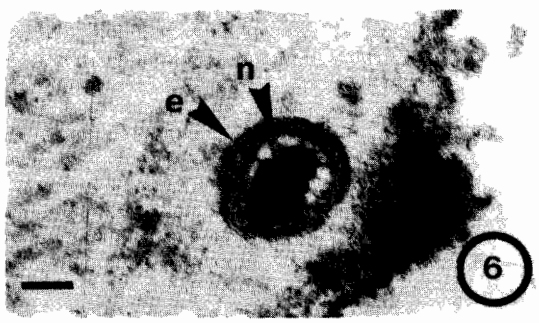


FIGURE 1. — Infected fibroblastic cell showing intranuclear spherical or polygonal particles. Some particles appear empty (arrowheads) and other contained an electron-dense core (arrows). (1a) *C. gigas* larva infected cell. (1b) Viruses observed in infected *C. gigas* oyster spat. EM (bar = 200 nm).

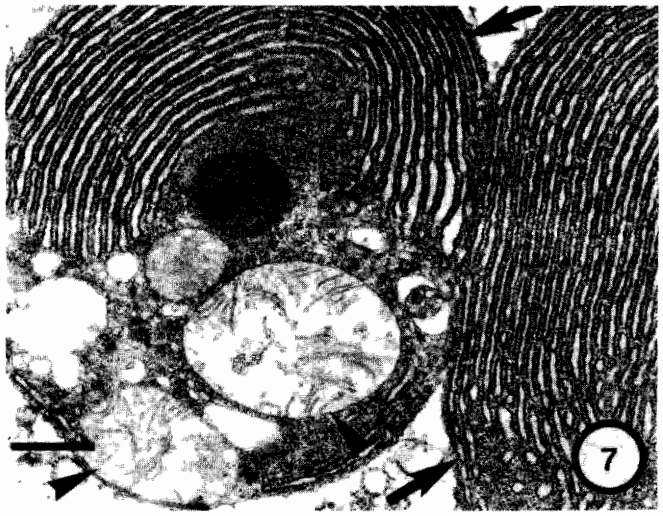
FIGURE 2. — Intranuclear assembly of empty capsids with a paracrystallin arrangement within *C. gigas* larva infected cell. EM (bar = 500 nm).

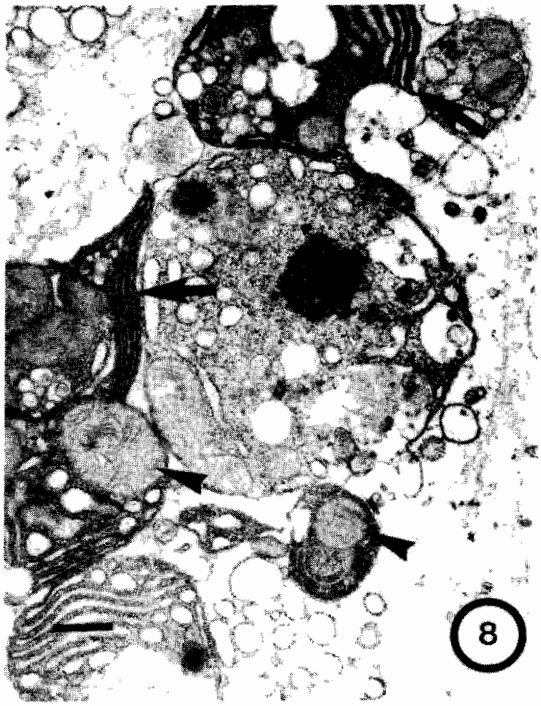
FIGURE 3. — Unenveloped particles in myocyte cell cytoplasm of a *C. gigas* young oyster. EM (bar = 200 nm).

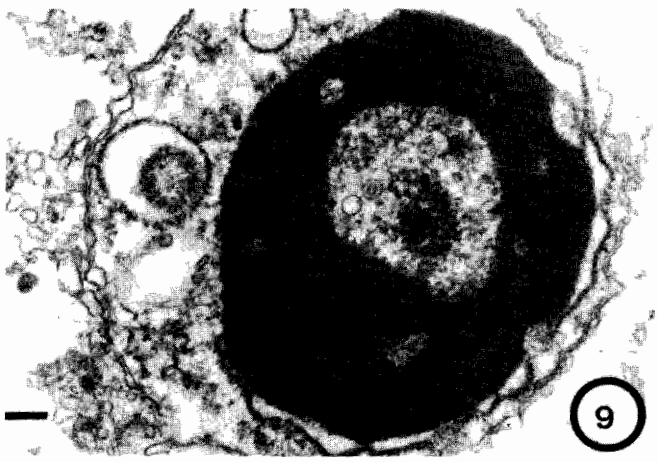
FIGURE 4. — Enveloped viruses (arrows) within cytoplasmic vesicles (4a) in infected cell of a *C. gigas* larva and (4b) in infected cell of a *C. gigas* young spat. EM (bar = 200 nm).

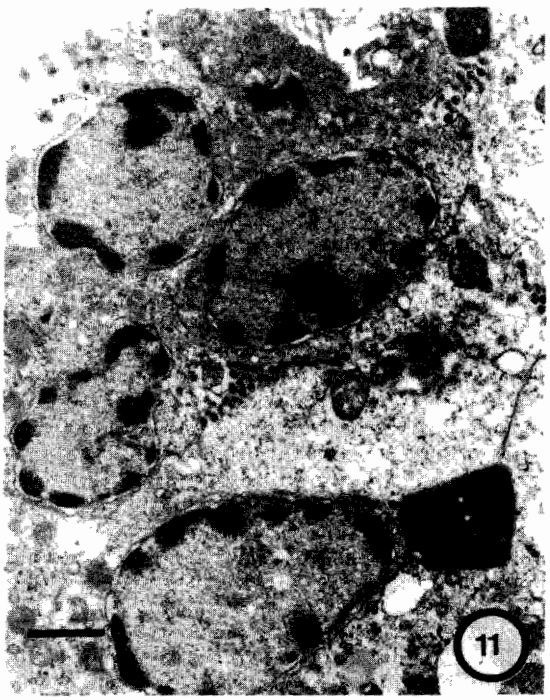
FIGURE 5. — Enveloped particles found in extracellular spaces (5a) in infected *C. gigas* larvae and (5b) in infected *C. gigas* young oysters. EM (bar = 100 nm).

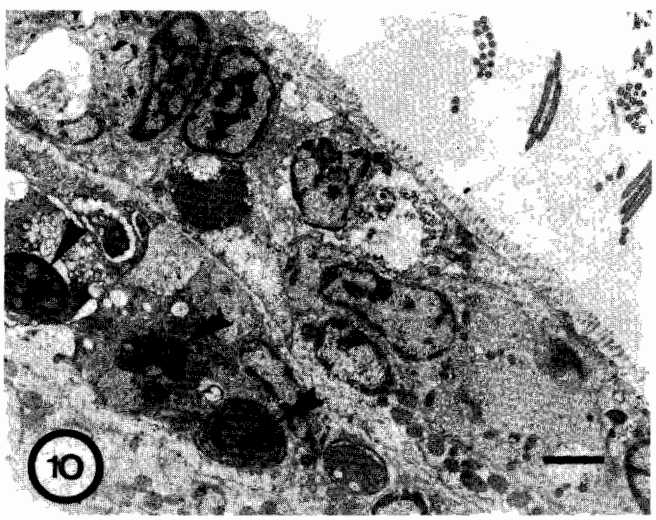
FIGURE 6. — Fine structure of an extracellular enveloped virus found in infected young oyster spat, *C. gigas* : the nucleocapsid (n) is surrounded by an envelope (e). Fine filaments pass the core to inside of the capsid (arrows). EM (bar = 50 nm).











FIGURES 7 and 8. — Ultrastructural changes in infected young oysters *C. gigas* cells : accumulation of granular endoplasmic reticulum (arrows) and large swollen mitochondria (arrowheads). EM (bar = 500 nm).

FIGURE 9. — Infected cell of *C. gigas* young oyster spat shows a condensed nucleus with an electron-lucent center where some particles are visible. EM (bar = 200 nm).

FIGURE 10. — Infected cells of *C. gigas* young oysters show condensed nuclei with electron-lucent areas (arrowheads) in the mantle connective tissue. EM (bar = 2 μm).

FIGURE 11. — Infected cell nuclei of affected *C. gigas* larvae show abnormal chromatin pattern with marginalisation (arrows). EM (bar = 1 μm).

viruses. However some analysed animals exhibited abnormal nulei and condensed chromatin without virus detection (Table I).

No virus was observed among healthy wild-type larvae and healthy or affected by moderate mortality cultured young oysters (Table I).

Discussion

The virions described in this report ressemble hespesviruses in morphological characteristics, in cellular locations and in size range [18, 15, 17]. In addition, the fibrils spanning the space from the core to the inner surface of the capsid are similar to the arrangement in herpesviruses [11, 17].

The herpes-like virus associated with mortalities among hatchery-reared larval Pacific oysters, *C. gigas,* seems to be the same as those reported in *C. gigas* young spat. They have the same structural characteristics, the same cellular locations and comparable sizes (Tables II and III).

The virogenesis for the two viruses begins in the nucleus where capsids and nucleocapsids appear. Then the viral particles pass through the nuclear membranes into the cytoplasm. Enveloped virions are then released at the cell surface or by cytolysis. Extracellular naked nucleocapsids may derive from lysed infected cells.

The presence of Feulgen positive cytoplasmic and nuclear components in the infected cells is in accordance with herpesvirus infections. These oyster viruses ressemble the *Betaherpesvirinae* (Cytomegaloviruses) on the points of the enlargement of infected cells and their nuclei [18], their association with dense bodies in the cytoplasm [20], and the ovoid ring of nuclear granular material similar to that reported in human cytomegalovirus infection [22]. However

Origine of sample	Date of sample	Nature of animals	N	% mortality	Detection of herpes-like virus
Loire-Atlantique (hatchery-reared)	24.06.92	L	30	86	+
"	13.07.92	L	30	100	+
"	15.07.92	L	30	100	+
"	29.07.92	L	30	100	+
Charente-Maritime (wild-type)	08.07.92	L	30	0	-
"	29.07.92	L	30	0	-
"	29.07.92	L	30	0	-
Charente-Maritime (hatchery-reared)	25.06.93	L	30	100	+
"	12.07.93	L	30	100	+
"	14.07.93	L	30	100	+
"	19.07.93	L	30	100	+
Loire-Atlantique	08.07.93	YS	15	90	+
"	25.10.93	YS	15	0	-
Charente-Maritime (Oléron)	08.07.93	YS	18	90	+
"	17.09.93	YS	180	0	-
Charente-Maritime (Oléron)	08.07.93	YS	15	50	-
Charente-Maritime (Boyardville)	23.08.93	YS	20	90, the first week of july	-, but abnormal nuclei were observed in different tissues.
Charente-Martime (Ronce-les-Bains)	26.08.93	YS	30	80-90	+
Bretagne (Cancale)	10.07.93	YS	30	80-90	+
Bretagne (Morlaix)	9.08.93	YS	30	10	-
Bretagne (Etell)	13.08.93	YS	15	30	-
Bretagne (Golfe du Morbihan)	21.09.93	YS	30	58	-
Méditerranée (Etang de Thau)	07.93	YS	30	ND	+

N : number of examined animals per sample. L : larvae, YS : young spat.

TABLE I. — *Crassostrea gigas.* Detection of herpes-like virus among larvae or young spat.

	Capsids	Nucleocapsids	Enveloping virions	Enveloped virions
Size (n = 50)	81 ± 3.0 nm	75 ± 3.0 nm	99 ± 8.0 nm	122 ± 8.0 nm
Nucleus	+	+		
Perinuclear space				+
Cytoplasm	+	+	+	
Dense bodies		+	+	
Cytoplasmic vesicles			+	+
Extracellular		+		+

TABLE II. — Occurrence and size of different stages of replication of oyster herpes-like virus in different cell parts of *Crassostrea gigas* larvae.

	Capsids	Nucleocapsids	Enveloping virions	Enveloped virions
Size (n=50)	81 ± 3.0 nm	75 ± 3.0 nm	100 ± 9.0 nm	121 ± 7.0 nm
Nucleus	+	+		
Perinuclear space				+
Cytoplasm	+	+	+	
Dense bodies		+	+	
Cytoplasmic vesicles			+	+
Exracellular		+		±

TABLE III. — Occurrence and size of different stages of replication of oyster herpes-like virus in different cell parts of young oysters, *Crassostra gigas*.

such similarities are not sufficient on a taxonomic point. Details of replication are variable within subfamilies [8] and vary with cell type infected [8, 21], time after infection [18] and virus strains [3]. Nevertheless, the nucleic acid nature of these viruses should be confirmed by chemical analysis.

Several reports of herpes-like viruses from marine vertebrates or invertebrates are found in the literature [2, 12, 13, 19], including herpes-type viruses in oysters [1, 7, 10, 14, 16]. The morphological characteristics of the nucleocapsid of the viruses described in this study are closed to those of the viruses reported among Pacific oyster, *C. gigas,* larvae in french hatcheries [16] and in New Zealand [14]. The nucleocapsids are similar in dimension to those detected in European flat oyster, *Ostrea edulis,* by COMPS and COCHENNEC [7], in Pacific oyster, *C. gigas,* by NICOLAS *et al.* [15] and in American oyster, *C. virginica,* by FARLEY *et al.* [10], but at 70-75 nm, they are smaller than the 97 nm of nucleocapsids observed by HINE *et al.* [14], among *C. gigas* in New Zealand (Table IV). Moreover, mature virions described in this study are smaller than the 160 to 180 nm of enveloped paraspherical particles observed in *O. edulis* [7] and larger than the 90 nm of enveloped particles reported among French Pacific oyster larvae [16] (Table IV). Mature particles noted into Pacific oyster larvae in New Zealand [14] and in our report are similar in size (Table IV). However, particles sizes are dependent on the technique of specimen preparation and measurement.

Elevated temperature and crowding seem to increase the susceptibility of animals to herpes-like virus infections among Pacific oyster, *C. gigas.* Indeed mortalities associated with herpes-like viruses detection were only observed during summer in this report. Moreover, the detection of herpesviruses in animals held at elevated temperatures among *C. virginica* [10], the occurrence of herpesviruses in summer among *C. gigas* in Australia [1] and the observation of mortalities in mid-summer among the same species reported in New Zealand [14], in France [16] and in this study, suggest ostreids herpesviruses may only develop and induce mortalities at elevated temperatures, particularly under stressed conditions. So high temperatures appear to favor the spread of the infections or activation of viruses from an occult to an overt phase or both.

Host species	Size		References
	Capsids	Enveloped virions	
C.virginica	70 / 90 nm	/	[6]
C. gigas	70 ± 2.0 nm	90 ± 5.0 nm	[12]
C. gigas	97 ± 4.0 nm	131 ± 9.0 nm	[10]
O. edulis	80 nm	160 / 180 nm	[4]
C. gigas (larvae)	75 ± 3.0 nm	122 ± 8.0 nm	In this report
C. gigas (young spats)	75 ± 3.0 nm	121 ± 7.0 nm	In this report

TABLE IV. — Morphological characteristics of herpes-like viruses observed among bivalve molluscs.

Indeed, it seems that the herpes-like viruses detected among bivalve molluscs are not a real danger for animals cultured at low temperatures like the observations performed by FARLEY *et al.* [10] suggest. Thus, since 1970, no catastrophic mortalities associated with herpes-like virus detection were reported among the species *C. virginica.* However it seems necessary to perform regular controls for a survey of the evolution of these virus infections among *C. gigas* oysters.

The evolution of the disease in hatchery-reared larvae with the first signs visible three to four days after the spond seems to point out that contamination of larvae occurs quite early, and may result of a vertical transmission. Among the batches of 3-7 month old young oysters, the mortalities appeared suddenly and the course of the disease was very brief in time, only a week. These reports suggest a high pathogenicity of this herpes-like virus, with a great infectivity in a same batch. But in the case of young spat oysters, the origin of virus contamination is unclear. Moreover, the presence of two similar viruses among *C. gigas* larvae and *C. gigas* young oyster spat may indicate that some larvae, survivors of animals infected by herpes-like viruses could be healthy latent carriers and express subsequently the disease under stressed conditions. In point of fact, four batches of herpes-like virus infected *C. gigas* young oyster spat are originating from French hatcheries and the larvae were produced in 1993. One batch of these infected young oysters came from a hatchery in which mortalities in association with herpes-virus detection were observed among several breedings during the summer of 1993. The three other batches were originating from hatcheries in which no larvae sample was taken and no examination was made during their productions. So it is impossible to know if the larvae were infected or not by the herpes-like virus before their exit of these hatcheries.

Purification and fine characterization seem to be necessary to develop sensitive diagnosis methods for detection of these pathogens described among *C. gigas* larvae and *C. gigas* young oysters and to determine relationship between these viruses.

Acknowledgements

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