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Viruses infecting bivalve molluscs

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Abstract – Bivalve molluscs are filter feeders and as a consequence they may bioaccumulate in their tissues viruses that infect humans and higher vertebrates. However, there have also been described mortalities of bivalve molluscs associated with viruses belonging to different families. Mass mortalities of adult Portuguese oysters, *Crassostrea angulata*, among French livestocks (between 1967 and 1973) were associated with irido-like virus infections. Herpesviruses were reported in the eastern oyster, Pacific oyster and European flat oyster and lately in scallops and clams. Disseminated neoplasia, a proliferative cell disorder of the circulatory system of bivalves, although of uncertain aetiology, has been suggested to be caused by retroviral infections. Other viruses described in bivalves are interpreted as members of the Papovaviridae, Togaviridae, Reoviridae, Birnaviridae and Picornaviridae. However, the lack of bivalve cell lines renders difficult virus isolation from molluscs although some viruses can be isolated in fish cell lines.

Key words: Bivalves / Molluscs / Virus / Infection

Résumé – Infections virales affectant les coquillages. Les mollusques bivalves sont des filteurs et de ce fait peuvent accumuler dans leurs tissus des virus d'origine anthropique. Par ailleurs, des mortalités ont été constatées chez des bivalves en association à la détection de virus apparentés à différentes familles. Ainsi, les mortalités massives d'huîtres portugaises, *Crassostrea angulata*, observées en France, de 1967 à 1973, ont été associées à la présence de virus apparentés aux Iridoviridae. Des virus de type herpès ont été détectés chez l'huître américaine, l'huître creuse, l'huître plate, et plus récemment chez la coquille Saint-Jacques et la palourde japonaise. Des néoplasies touchant les cellules circulantes chez les bivalves sont encore aujourd'hui d'étiologie incertaine. Cependant, l'implication de retrovirus dans le processus a été suspectée. Chez les coquillages, d'autres virus ont été décrits et interprétés comme appartenant aux Papovaviridae, Togaviridae, Reoviridae, Birnaviridae et aux Picornaviridae. Cependant, l'absence de lignées cellulaires de bivalves rend difficile l'isolement de virus à partir d'échantillons de ces mêmes bivalves, alors que des virus peuvent être isolés sur lignées cellullaires de poissons.

Abbreviations used: GNV Gill necrosis virus, OVVD Oyster velar virus disease, HIV Hemocyte infection virus, OsHV Ostreid herpes virus, BIRP Baculovirus inhibitor of apoptosis repeat protein, MABV Marine birnavirus.

1 Introduction

The potential of aquaculture to meet the challenge of food security and to generate employment and foreign exchange is clearly demonstrated by the rapid expansion of this sector, which has grown at an average annual rate of almost 10% since 1984 compared with 3% for livestock meat and 1.6% for capture fisheries production. According to FAO data (1996), aquatic production (including plants) has steadily increased since 1984, and in 1996 total world production of finfish and shellfish from capture fisheries and aquaculture reached 120.3 million tons. A significant proportion of this increased production was of cultured origin. The annual contribution of cultured species to total finfish and shellfish production rose from 13% in 1990 to 22% in 1996. Global production of molluscs, the greatest proportion of which are bivalves, was estimated at 4 388 967 tons in 1994 (FAO 1996). However, high density culture conditions mean that many aquacultured animals are under stress conditions and are generally more susceptible to infections. Major disease outbreaks result in loss of income and sometimes the complete shutdown of operations. Besides causing massive mortalities in cultured species, viral pathogens are often highly infectious and easily transmissible. Indeed, several viral diseases have seriously affected the aquaculture industry. The control of viral diseases is a priority for sustainable aquaculture and attempts must be made to develop new approaches and technologies suited to health improvement of farmed species that are not harmful for the environment.

Bivalves are filter feedeers. As a consequence they may bioaccumulate viruses from humans and other vertebrates (Meyers 1984). Molluscs may act as a transient reservoir for

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vertebrate hosts upon ingestion of the bivalve (Lees 2000; Potasman et al. 2002; Nishida et al. 2003). There is considerable literature on the human health implications which is not the subject of this review. The main objective of the authors is to give updated information on viruses that infect bivalves and replicate in their tissues. Indeed, bivalve cultivation may be endangered by the occurrence of epizootics including viral diseases which are considered one of the major risk to production. Mortalities have been described among different bivalve species associated with the presence of viruses belonging to various families. Mass mortalities of adult Portuguese oysters, *Crassostrea angulata*, among French livestocks between 1967 and 1973 were associated with irido-like virus infections (Comps et al. 1976; Comps and Bonami 1977; Comps and Duthoit 1979). Other viruses described in bivalves are interpreted as members of the Herpesviridae, Papovaviridae, Togaviridae, Retroviridae, Reoviridae, Birnaviridae and Picornaviridae (Farley et al. 1972; Farley 1976; Farley 1978; Oprandy et al. 1981; Meyers 1979; Rasmussen 1986; Bower 2001). However due to the lack of mollusc cell lines and limited application of molecular tools, virology of bivalves is still a relatively primitive science based largely on morphological studies and a few in vivo experimental studies.

2 Irido-like viruses

2.1 Hosts and locations

Several irido-like viruses have been reported in bivalves in different countries around the world. Two distinctive pathological infections have been described in *C. angulata* adult oysters in France (Comps et al. 1976; Comps 1978; Comps and Duthoit 1979; Comps 1980). The first one called "Maladie des branchies" (gill necrosis) was caused by gill necrosis virus (GNV) and was interpreted as the cause of recurrent mass mortalities in Portuguese oysters, *C. angulata*, from 1966 to 1968 along French coasts. In 1970, epizootic mortalities were again reported in French *C. angulata* oysters and associated with the detection of an irido-like virus named hemocyte infection virus (HIV). A possible identical GNV was also associated with disease in *C. gigas* oyster populations (Comps and Bonami 1977). Finally, oyster velar virus disease (OVVD) was reported from hatchery-reared larval Pacific oysters on the west coast of North America (Washington state) (Elston 1979; Elston and Wilkinson 1985). The virus is associated with occasional mortality of oyster pediveliger larvae.

2.2 Disease manifestations and epizootiology

GNV disease is regarded as the main factor of mass mortalities occurring in the late 1960s among Portuguese oysters on the Atlantic coast of France (Comps 1970; Comps and Duthoit 1979). During certain years, the disease affected 70% of the oyster populations from important culture areas including the Marennes Oleron region and the Arcachon area. Maximum losses occurred in 1967 and losses subsequently declined. In 1968, survivors of previous outbreaks recovered from the disease presenting healing of old lesions. The first gross sign of the disease was the appearence of one or several yellow spots on gills and labial palps. The spots increased in size, and the tissues at the centre of the lesion died, became brown and eventually left a perforation in the tissue. This lesion sometimes enlarged to cause a deep indentation and, in advanced stages of the infection, total destruction of affected gill filaments. Yellow or green pustules also developed on the adductor muscle and on the mantle. Mantle perforation could occur as on the gills (Alderman and Gras 1969; Comps 1969 and 1970). The infection of branchial epithelial cells was progressive and lytic.

Gill disease also affected the Pacific oyster, *C. gigas*, although to a lesser extent. The gill lesions were always reduced, suggesting partial resistance in this species. Pacific oysters tranplanted into French waters from Japan in 1968, exhibited gill lesions in 27% of individuals, while 56% of a lot obtained from Korea showed lesions. At the same period, the incidence of gill lesions in *C. gigas* imported from British Columbia was only 14% (Comps 1970; Marteil 1976). Pacific oysters cultivated in France appeared subsequently to have acquired complete resistance to gill disease, as no gill-diseased individuals of this species were found between 1969 and 1971, although mortalities among *C. angulata* reached 70% (Comps 1972).

In 1970, mortality outbreaks of French *C. angulata* were again associated with the detection of an irido-like virus (HIV) infecting hemocytes. The disease affected adult oysters from 1970 to 1973, but no distinctive signs were reported (no gill lesions). Mortality was first observed in the estuary of Marennes Oléron on the French Altantic coast and was paralleled by a similar event at Etel in Brittany (France). By 1971, the disease had also reached the Arcachon region. High mortality rates led to the almost total extinction of *C. angulata* in French Atlantic waters in 1973. Histological observations included an acute inflammatory reaction consisting of atypical virus-infected hemocytes. Pacific oysters seemed to be resistant to the virus (HIV) and, after the disappearence of *C. angulata*, replaced the latter species in France (Marteil 1976; Bonami 1977). However, a similar disease associated with a morphologically similar virus was reported from Pacific oysters in France during an episode of summer mortality of that species in the Bay of Arcachon in 1977 (Comps and Bonami 1977). In 1977 a 15% mortality was noticed in oysters kept in a purification plant in Arcachon. As was the case for *C. angulata*, affected Pacific oysters exhibited virtually no external signs of disease, except for a greyish discoloration of the visceral mass in some cases. Histological examination, however, revealed considerable degeneration of connective tissues and the presence of atypical cells interpreted as infected hemocytes.

Pacific oyster larvae exibiting clinical lesions of OVVD are less active than normal animals. This results from a tendency to keep the visceral mass retracted within valves. Infected larvae are unable to execute normal active movements when the velum is extended. The disease results in the sloughing of ciliated velar epithelial cells forming the characteristice "blisters" (Elston and Wilkinson 1985). Infected velar epithelial cells which are in the process of detaching from the velum appear as blebs along the periphery of the velum. Other cells lose cilia and the larvae become unable to move normally. Samples from the tank bottom appear to have a much more higher proportion of animals with advanced lesions. Elston and Wilkinson (1985)

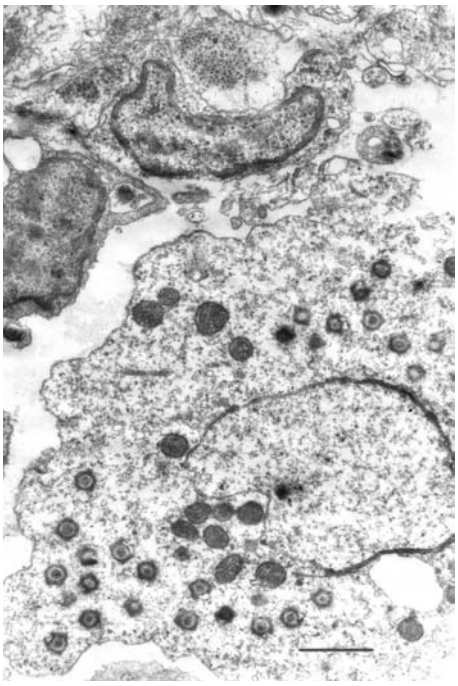


Fig. 1. A hypertrophied *Crassostrea angulata* cell containing intracytoplasmic viral particles. Scale bar = 1 μm.

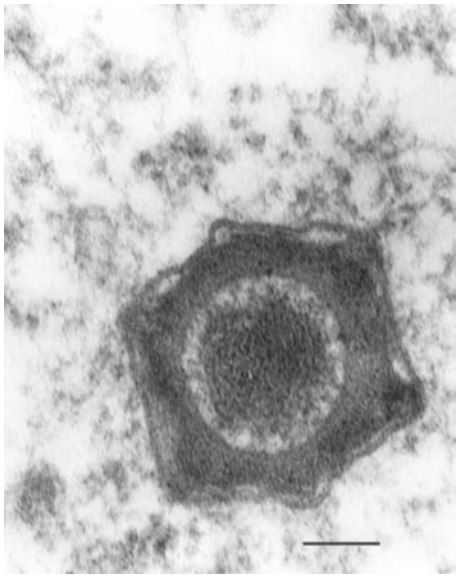


Fig. 2. An irido-like virus particle from *Crassostrea angulata*. The virion is icosahedral in shape with a central electron-dense core, surrounded by an electron-lucent zone followed by another dense layer. Two unit membranes separated by a clear zone enclose the particle. Scale bar = 100 nm.

reported that velar inclusions occurred in 38% of larvae collected from the tank bottom but in only 28% of larvae collected from the water column. Mortality of larvae greater than 170 μm in shell length typically begins at about 10 days of age when cultured at 25−30 °C. Typically, the infection occurs in larvae in which the shell height is between 175 μm and 190 μm. This indicates some size specificity of the disease. Detailed hatchery records from 1976 through 1984 indicate that a disease consistent with the clinical lesions and behavior described above occurred each year more often from mid-March through mid-June (Elston and Wilkinson 1985). Over the 8-year period and during the April to May period, losses of up to 50% of hatchery production were presumptively attributed to OVVD. Even under intensive husbandry, larvae are usually cultured for only about 6 months of each year. This suggests that the virus may have some reservoir host (adult oysters or other species) which allows it to reinfect larvae each year. OVVD seasonality also suggests that environmental conditions are involved in the expression of the disease.

2.3 Descriptive histopathology

OVVD lesions have been reported in clinically affected individuals but not in non-affected larvae. The disease is manifested histologically by the presence of intracytoplasmic inclusion bodies and separation and detachment of infected cells from surrounding tissues. The intracytoplasmic inclusions bodies, 1.2−4 μm in diameter, are located most commonly in ciliated velar epithelium. Lesions are observed in velar, oral and distal esophageal epithelia (Elston and Wilkinson 1985). Rarely, inclusion bodies are observed in mantle epithelium. Initially, the inclusion bodies are spherical but become irregular and less basophilic as the virions form. Utilizing hematoxylin and eosin staining, these inclusions appear basophilic but also contain diffusely distributed eosinophilic components. The inclusion bodies exhibited a deep red, positive reaction when stained by the Feulgen and Rosenbeck reaction. Utilizing ribonuclease controls the acridine orange reaction yields a green staining reaction indicating DNA presence in inclusion bodies (Elston and Wilkinson 1985). Some epithelial cells containing inclusions appear demarcated from surrounding cells. Detachment of epithelial cells from their supporting matrix can occur prior to the formation of viral particles. Nuclear and mitochondrial swelling accompany the development of the infection.

2.4 Virus characteristics

Electron microscopy demonstrates that the globular cells reported in *C. angulata* oysters with gill necrosis are hypertrophied host cells containing intracytoplasmic viral entities (Fig. 1) (Comps et al. 1976; Comps 1978; Comps and Duthoit 1979). Virions are icosahedral in shape and have a diameter of approximately 300 nm. A central electron-dense core, 190 nm in diameter, is surrounded by an electron-lucent zone followed by another dense layer 45 nm in thickness. Two unit membranes separated by a clear zone enclose the particle (Fig. 2). There is no evidence of nuclear involvment. The same type of virus has also been reported in association with hemocyte disease in French *C. angulata* oysters. The virus appears to be icosahedral, 350 nm in diameter with an electron-dense core 190 nm in diameter, Viral particles are observed within the cytoplasm of hemocytes in connective tissues (Comps et al. 1976; Comps 1978). A third virus is also observed in *C. gigas* oysters in Arcachon (Atlantic coast, France)

(Comps and Bonami 1977). The virus exhibits an icosahedral morphology with viral particles 350 nm in diameter. The electron-dense core measures 250 nm in diameter.

In the case of OVVD, electron microscope examinations of all tissues of diseased larval oysters demonstrate viroplastic inclusion bodies in velar, oral and epithelial tissues (Elston 1979; Elston and Wilkinson 1985). The viroplasms are typically granular and electron dense and correspond to intracytoplasmic inclusions visible at the light microscopy level. On the periphery of some viroplasms the initiation of viron formation is observed (Elston 1979; Elston and Wilkinson 1985). Empty capsids detached from the viroplasm may be interpreted as incomplete particles. Complete particles are typically separated from the viroplastic stroma. Particles are predominantly hexagonal in profile and averaged 228 ± 7 nm in diameter. Viral particles are limited by two bilayered membranous structures 8.5 nm wide and separated by a space of approximately 3.6 nm. An electron-lucent space separates the limiting membranes from the adjacent central zone of moderate electron density. Within this central zone, an electron-opaque core is observed with circular and pararectangular profiles. The circular profile measures 160 nm in diameter, and the pararectangular profile measures 106 nm in the shortest dimension. Electron microscopy analysis of serial sections show that cells containing aggregates of particles correspond to cells exhibiting degenerative changes at the light microscopy level (Elston and Wilkinson 1985). Cells containing particles are characterized by poorly defined plasma membranes and loss of the normal homogenous texture of the cytoplasm in the vicinity of the particles.

The various properties of the different viruses described above, as well as their assemblage within the cytoplasm, characterise them as members of the Iridoviridae. However, identity of the four viruses described here cannot be made at this point. Molecular characterization of irido-like viruses infecting bivalves is nedeed. Different findings provide a presumptive link between the detection of these viruses in different bivalve species and the diseases reported. Although the presumptive link is substantial, definitive demonstration of viral etiology of the diseases must be accomplished by experimental transmission.

3 Herpesviruses

3.1 Hosts and locations

The first description of a virus from a bivalve indicating membership of the Herpesviridae was reported in adult Eastern oyters, *C. virginica* (Farley et al. 1972). In 1991, viruses interpreted as belonging to the Herpesviridae were associated with high mortality rates of hatchery-reared larval *C. gigas* in France (Nicolas et al. 1992) and in New Zealand (Hine et al. 1992). Since 1992 sporadic high mortalities of larval *C. gigas* have been regularly observed in some commercial European hatcheries, occurring each year during summer period in association with a herpes-like virus (Renault et al. 1994b; Renault and Arzul 2001). Since 1993, sporadic high mortalities have also occurred in some batches of Pacific oyster spat cultured in different French locations (Renault et al. 1994a,b).

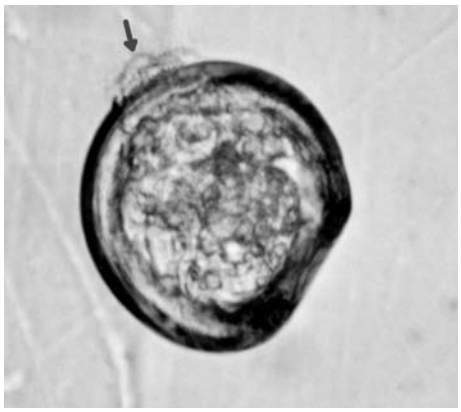


Fig. 3. Velar lesions (arrow) of a *Crassostrea gigas* larva infected with OsHV-1.

Viral infections were also reported in Pacific oyster seed suffering losses in Tomales Bay (California, USA) (ICES 2004) and along the northwestern Pacific coast of Baja California (Mexico) (Renault unpubl. data). In addition, herpesvirus infections were reported in spat and larvae of the European flat oyster, *Ostrea edulis*, in France (Comps and Cochennec 1993; Renault et al. 2001b). Concomitant mortalities were observed among larvae and spat of *C. gigas* and *O. edulis*, in 1994 and 1995, with the detection of herpes-like virus particles by transmission electron microscopy (Renault et al. 2000b). Replication of herpes-like viruses was also described in *O. angasi* adults in Australia (Hine and Thorne 1997), in larval *Tiostrea chilensis* in New Zealand (Hine 1997; Hine et al. 1998), in larval *Ruditapes philippinarum* and larval *Pecten maximus* in France (Renault 1998; Arzul et al. 2001b; Renault et al. 2001a,b; Arzul and Renault 2002).

3.2 Disease manifestations and epizootiology

Infected larvae show a reduction in feeding and swimming activities (Hine et al. 1992; Nicolas et al. 1992). Moribund larvae swim weakly in circles with their velum noticeably less extended than healthy larvae (Fig. 3). They exhibit velar and mantle lesions. Free pieces of velum may be observed in tank water. Shortly before death infected larvae settle at the bottom of the tanks. For spat, sudden and high mortalities are observed in a short period (less than one week) often during summer time (Renault et al. 1994a,b). Histologically, lesions are confined to connective tissues in infected larvae (Hine et al. 1992; Nicolas et al. 1992; Renault et al. 2001a,b). Fibroblastic-like cells exhibit an abnormal cytoplasmic basophilia and enlarged nuclei with marginated chromatin (Fig. 4). Other cells, interpreted as hemocytes, show extensive chromatin condensation (Fig. 4). The main histological change in spat is the presence of abnormal nuclei throughout connective tissues, especially in mantle, labial palps, gills and digestive gland (Renault et al. 1994a,b; Renault et al. 2000b). Abnormalities consist of enlarged nuclei associated with marginated chromatin in fibroblastic-like cells and highly condensed nuclei in ovoid cells interpreted as hemocytes. Some nuclei show peculiar patterns of chromatin with a ring-shape or crescent-shape

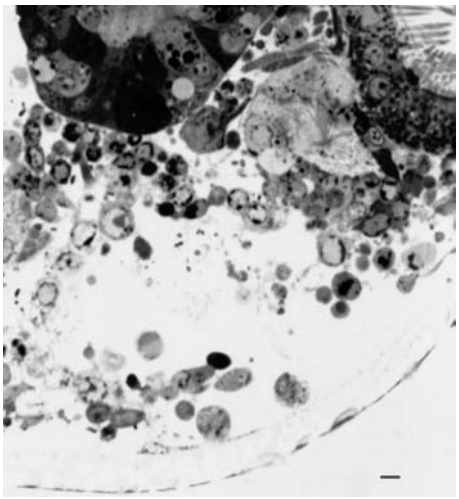
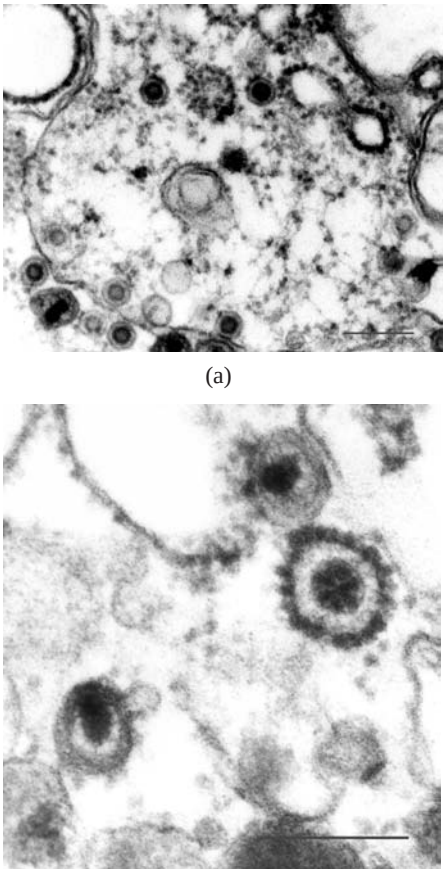


Fig. 4. Toluidine blue-stained semithin section of a *Crassosstrea gigas* larva infeceted with OsHV-1. Condensed hyperbasophilic nuclei and enlarged nuclei presenting chromatin marginalisation are observed. Scale bar = 2 μm.

characteristic of apoptosis. Cellular abnormalities are not associated with a massive inflammatory reaction.

Adult bivalves appear less sensitive to herpesvirus infections compared to young stages. The same observation has been noticed concerning vertebrate herpesviruses, such as fish herpesviruses. Severe diseases and high mortality rates essentially affect fingerlings. The Channel Catfish Virus (CCV) provokes a fatal disease among young fish *Ictalurus punctatus* (Plumb 1973), and CCV was also detected in asymptomatic adults (Wise et al. 1988). The *Oncorhynchus masou* virus (OMV) has proved lethal for salmonid fry, whereas it was isolated from ovarian fluid of normal appearing adults (Kimura et al. 1981). Although no abnormal mortality occurred among *C. gigas* adults in France, the question arises as to whether herpesviruses are able to persist in normal appearing adults. The presence of a herpesvirus was thus demonstrated in asymptomatic *C. gigas* adults using molecular and immunological techniques (Arzul et al. 2002). Viral DNA and proteins were detected in most of the asymptomatic adult oysters analysed (Arzul et al. 2002). Like other herpesviruses, the herpesvirus infecting *C. gigas* seems to be able to persist in its hosts. Adult oysters may play the role of carriers and reservoirs of the virus, promoting virus transmission from adults to larvae (Le Deuff et al. 1996; Arzul et al. 2002). In a recent study, three successive generations of *C. gigas* were screened for herpesvirus by PCR (Barbosa-Solomieu et al. 2004). Viral DNA was detected in two-day old larvae, indicating that infection may take place at early stages. Although the results strengthen the hypothesis of a vertical transmission, it was not possible to predict the outcome of a particular type of cross. The detection of viral DNA in parental oysters did not systematically correspond to a productive infection or result in a successful transmission to the progeny (Barbosa-Solomieu et al. 2004). However, the infective status of the parents appeared to have an influence on both the infection and the survival rates of the progeny (Barbosa-Solomieu et al. 2004). Crosses involving an infected male and a non-infected female resulted in hatching and larval



(a)

(b)

Fig. 5. Transmission electron micrographs of OsHV-1 infected cells from Pacific oyster larvae. Scale bar = 200 nm. (a) Intranuclear spherical or polygonal viral particles are observed; Some particles appear empty and other contained an electron-dense core. (b) Extracellular enveloped particles.

survival rates statistically lower than those observed in the other types of cross (Barbosa-Solomieu et al. 2004). These results suggest that infected females may transmit to their offspring some kind of protection or resistance against viral infection.

3.3 Virus ultrastructure

The basophilic fibroblastic-like cells in infected oysters (larvae and spat) show hypertrophied nuclei and marginated chromatin. Cell shrinkage is associated with large amounts of condensed chromatin (crescent shape) in hemocytes and highly condensed electron-dense cytoplasm. Abnormal fibroblastic-like cells contain intranuclear virus-like particles. Nuclear particles are circular or polygonal in shape, 70 to 80 nm in diameter (Hine et al. 1992; Nicolas et al. 1992; Renault et al. 2001a,b). Some particles appear empty and are interpreted as being capsids, others contain an electron-dense toroidal or brick-shaped core and are presumed to be nucleocapsids (Fig. 5a). Capsids and nucleocapsids are scattered throughout the nucleus in infected cells. Enveloped single

virions or groups of viruses are observed in cytoplasmic vesicles. These intracytoplasmic particles possess an envelope formed by a trilaminar unit-membrane. Core and capsid are separated by an electron-lucent gap of approximately 5 nm with fine fibrils spanning the lucent-space from core to capsid. Naked capsids are also observed free in the cytoplasm of infected cells. Extracellular viruses are usually enveloped and measure 100 to 180 nm in diameter (Fig. 5b) (Hine et al. 1992; Nicolas et al. 1992; Renault et al. 2001a,b). No, or a reduced tegument is observed between the outer membrane and the capsid shell of enveloped particles. Nucleocapsids also occur in myocytes. Abnormal accumulations of granular endoplasmic reticulum are often observed in affected tissues. The occasional occurrence of nuclear tubular structures 45 to 55 nm in diameter with a helical substructure has been reported (Renault et al. 2001a,b). Transverse sections of tubular structures show a tube within a tube arrangement similar in appearance to capsids with lucent cores (Renault et al. 2001a,b).

Oyster herpesvirus capsids (Davison et al., pers. comm.) are similar in overall appearance to those of other herpesviruses that have been studied to date (Booy et al. 1996; Trus et al. 1999, 2001; Cheng et al. 2002). The diameter estimated from cryo-electron microscopic images (approximately 116 nm) is slightly less than that of herpes simplex virus type 1 (HSV-1) capsids (125 nm). This is somewhat surprising in view of the larger genome size (207 kbp compared with 152 kbp), but the DNA could be accommodated if packaged to the same density as in human cytomegalovirus (HCMV) (Bhella et al. 2000). The reconstruction of the oyster herpesvirus capsid reveals an icosahedral structure with a triangulation number of $T = 16$ (Davison et al., pers. comm.). To date, this surface lattice geometry has been observed only with herpesviruses. Other features of the oyster herpesvirus capsid are also distinctively herpesvirus-like. These include prominent external protrusions at the hexon sites, rising above the continuous floor region, and the relatively flat and featureless appearance of the inner surface (Davison et al., pers. comm.).

3.4 Oyster herpesvirus genome

Virus particles have been purified from fresh infected *C. gigas* larvae and viral DNA extracted from purified virions (Le Deuff and Renault 1999). The genome sequence has been completed and analysed (Davison et al., pers. comm.). The overall genome structure is: TR_L - U_L - IR_L - X - IR_S - U_S - TR_S with a 207 439 bp total genome size. TR_L and IR_L (7584 bp) are inverted repeats flanking a unique region (U_L, 167 843 bp). TR_S and IR_S (9774 bp) are inverted repeats flanking a unique region (U_S, 3370 bp), and X (1510 bp) is located between IR_L and IR_S. A similar genome structure has evolved independently in certain vertebrate herpesviruses (e.g. herpes simplex virus and human cytomegalovirus). Southern blot hybridisation experiments using PCR-generated probes from the ends of U_L and U_S showed that the two orientiations of U_L and U_S are present in approximately equimolar amounts in viral DNA, giving rise to four genome isomers. This is also a feature of the vertebrate herpesvirus genomes with similar structures. A minor proportion (approximately 20−25%) of genomes contain a 4.8 kbp region in U_L in inverse orientiation. These data indicate that the virus contains a mixture of genome forms. In the light of the fact that the virion DNA that was sequenced originated from a virus that had not been clonally purified, this was not unexpected.

A detailed analysis of the coding potential of the genome sequence indicated the presence of 124 unique protein-coding genes. Owing to the presence of inverted repeats, 12 open reading frames (ORFs) are duplicated, resulting in a total of 136 genes in the viral genome. These numbers include several fragmented genes, each of which was counted as a single ORF. No indication of splicing emerged from sequence analysis. One striking property of the gene complement is the presence of 38 genes in 12 families of related genes. These include two families whose products are predicted to be secreted, three families predicted to encode membrane-associated proteins, one family whose products contain motifs of helicases, one family whose products are related to inhibitors of apoptosis, one family derived from a deoxyuridine triphosphatase gene, two families of RING-finger genes and two other families. The ancient nature of the duplication events that have resulted in these families is illustrated by the fact that relationships are distant and that family members are generally widely distributed in the genome. Gene families are also present in all sequenced vertebrate herpesviruses. The Baculovirus Inhibitor of apoptosis Repeat (BIR) family has four members that encode products belonging to a family of viral and cellular proteins known as baculovirus inhibitor of apoptosis repeat proteins (BIRPs). Several of the previously identified BIRPs have been shown to have anti-apoptotic activity. BIRP-regulated pathways are evidently important mediators of defence against viral infection in invertebrates, since BIR genes are found not only in oyster herpesvirus but also in other large DNA viruses with invertebrate (insect) hosts, including members of the Baculoviridae (Crook et al. 1993), Ascoviridae (Stasiak et al. 2000), Poxviridae (Afonso et al. 1999), Iridoviridae (Jakob et al. 2001) and Asfarviridae (Yanez et al. 1995). In contrast, large DNA viruses of vertebrates do not encode BIRPs, and subvert host apoptotic defences by other means. Database searches also provide functional information on 25 genes that are not members of families. These include genes encoding a tentative primase, two subunits of ribonucleotide reductase, a helicase, the catalytic subunit of DNA polymerase, the ATPase subunit of terminase (Davison 2002) and two RING-like proteins. The ORF30 protein is related in an N-terminal cysteine-rich domain to a protein of unknown function in two subfamilies (Beta- and Gammaherpesvirinae) of the higher vertebrate herpesviruses. A total of 15 genes encode proteins which have predicted signal or transmembrane sequences and therefore may be associated with membranes, one specifying a putative ion channel.

The sequence data demonstrate that oyster herpesvirus is not closely related to herpesviruses with vertebrate hosts (including fish). Several oyster herpesvirus proteins have homologues that are distributed widely in nature (e.g. DNA polymerase), but these are no more closely related to homologues in other herpesviruses that to homologues in other organisms. However, a genetic indication of a common origin between oyster herpesvirus and vertebrate herpesviruses resides with the ATPase subunit of the terminase. Homologous genes are present in all herpesviruses, and the only non-herpesvirus

counterparts are specified by T4 and related bacteriophages. The T4 and oyster herpesvirus genes are unspliced, whereas those in herpesviruses of mammals and birds contains one intron and those in herpesviruses of fish and amphibians contains two introns. The available data support the view that herpesviruses of mammals and birds, herpesviruses of fish and amphibians and herpesviruses of invertebrates form three major lineages of the herpesviruses (Davison 2002). They also indicate that the oyster herpesvirus is the first identified member of the third major domain of the Herpesviridae. The herpesvirus infecting *C. gigas* oyster larvae was named Ostreid Herpesvirus 1 (OsHV-1). OsHV-1 would have established a separate lineage about a billion years ago, and the fish viruses about 400 million years ago. OsHV-1 is currently the single representative of what may be a large number of invertebrate herpesviruses. Morover, recent data shown that OsHV-1 can infect several bivalve species (Renault et al. 2000b; Arzul et al. 2001a,b,c; Renault et al. 2001b). This contrasts with vertebrate herpesviruses, which are generally confined to a single species in nature. Consequently, the true host of OsHV-1 is unknown. The apparent loss of several gene functions in OsHV-1 prompts the speculation that this may have promoted interspecies transmission in the context of introduction of non-native bivalve species and use of modern aquaculture techniques. It is possible that the parental virus still resides in its natural host.

3.5 Experimental demonstration of pathogenicity

The pathogenicity of the virus for larval stages of *C. gigas* was demonstrated by experimental transmission to axenic larvae (Le Deuff et al. 1994; Le Deuff et al. 1996). Experimental studies on OsHV-1 also showed that it could be transmitted from *O. edulis* larvae to axenic larvae of *C. gigas*. Furthermore, the demonstration that the herpesvirus can be transmitted from infected larvae of the manila clam *Ruditapes philippinarum* to axenic larvae of *C. gigas* has been reported (Arzul et al. 2001a,c). To date, attempts to reproduce symptoms experimentally in spat and adult oysters have been inconclusive. The first experimental data indicated that it was possible to transmit OsHV-1 to *C. gigas* spat, in cohabitation experiments using live infected larvae. A 40% mortality rate of challenged spat was only observed when the spat were kept in stressful conditions. In those experiments, control mock-challenged spat presented a mortality rate of 20% (Renault, unpubl. data). However, when holding conditions were improved, so reducing the stress levels of the animals, no significant mortalities were observed.

3.6 Diagnosis

A breakthrough was achieved in the development of a protocol, based on sucrose gradient centrifugation, for purifying oyster herpes-like virus particles from fresh infected larval *C. gigas* (Le Deuff and Renault 1999). This advance has served as an appropriate platform for generating molecular biological reagents to diagnose virus infections (Renault and Lipart 1998; Renault et al. 2000a; Lipart and Renault 2002).

Procedures to detect herpesvirus in French oysters using the polymerase chain reaction (PCR) (Saiki et al. 1985; Mullis et al. 1986) were developed (Renault et al. 2000a; Arzul et al. 2001a,b,c). PCR offers many advantages for disease diagnosis (Henson and French 1993; Jones and Bej 1994; Martin 1994). With regard to herpesviruses from oysters, important advantages include its extreme sensitivity, pathogen specificity, ease of sample processing, and availability of reagents. A quick and convenient sample preparation using ground tissues allowed a sensitive detection of infected spat and larvae. PCR was used to investigate the relationship between reported mortalities among *C. gigas* oyster spat and herpesvirus DNA detection demonstrating a significant correlation (Renault and Arzul 2001). A competitive PCR method has also been developed using previously designed primers (Arzul et al. 2001b; Renault et al. 2004) in order to detect and quantitate herpesvirus DNA. The method is based on the use of oyster herpesvirus specific primer pairs and an internal standard competitor that differs from the target DNA by a deletion of 76 base pairs. The internal standard DNA molecule was generated by PCR and then co-amplified with the target DNA. The resulting PCR products which were different in size were separated on agarose gels. The assay was found to be specific and sensitive, allowing the detection of 1 fg of viral DNA among 0.5 mg of oyster tissues. The method was used to demonstrate the absence of PCR inhibitors in oyster spat ground tissues. PCR inhibition was observed in adult oyster samples when the same tissue preparation procedure was used. On the contrary, classical phenol/chloroform DNA extraction from adult oyster tissues allowed amplification of the internal standard competitor and viral DNA (Arzul et al. 2001b; Renault et al. 2004). The method was successfully used to demonstrate the presence of viral DNA in asymptomatic adult oysters. Quantitation of herpesvirus DNA in infected spat and asymptomatic adult oysters was also carried out. Although between 1.5 pg and 325 pg of viral DNA per 0.5 mg of oyster tissues were detected in adults, amounts of viral DNA in infected oyster spat varied from 750 pg to 35 ng per 0.5 mg of ground tissues (Renault et al. 2004). Another technique that has also been developed is in situ hybridisation (ISH) (Renault and Lipart 1998; Lipart and Renault 2002). In addition to continuing the calibration of PCR and ISH, the main target is the production of antibodies to the virus. The development of immunochemistry and ELISA tests will be possible because of the availability of cloned sequences of OsHV-1 which enables the synthesis of recombinant virus proteins. Assays of herpes-like virus cultivation in oyster primary cultures and fish cell lines have been carried out. No cytopathic effect has been observed in tested fish cell lines. Preliminary assays carried out in primary cultures of embryonic oyster cells showed the presence of viral DNA in infected cultures using PCR and in situ hybridisation. However, experiments must be repeated and other techniques as transmission electron microscopy used in order to demonstrate the presence of the virus in embryonic cells.

3.7 Herpesvirus infection surveys

The observed association between oyster mortality and herpesvirus infections provides an imperative to determine the extent to which the virus is involved as a causative agent of massive mortalities in different countries around the world. PCR was use to investigate the presence of herpes-like virus

DNA in bivalve samples belonging to different bivalve species from different geographical origins in Europe (Renault and Arzul 2001). Seventeen larval samples of the 81 analysed appeared positive for the detection of herpesvirus DNA by PCR (Renault and Arzul 2001). These results confirm previous data indicating that herpesvirus infections may be observed in commercial French hatcheries. Morover, some PCR positive results were also obtained for bivalve larval samples originating from Spain and United Kingdom. The number of virus DNA positive samples depended on the primer pair used (Arzul et al. 2001a,b,c). PCR positive samples were observed in four bivalve species: *C. gigas*, *O. edulis*, *R. decussatus* and *R. philippinarum*. Samples presenting viral DNA detection by PCR correspond mostly to larval batches presenting mortalities. Herpesviruses may be proposed as one of the major causative agents of anomalous mortalities observed in bivalve hatcheries.

Most of the studies focused on detection of viral infections of economically important species. However, the persistence of bivalve herpesviruses in the marine environment is poorly documented. A recent study focused on the role of seawater parameters in OsHV-1 detection by PCR (Vigneron et al., in press). Viral DNA extracted from purified particles or virions present in infected oyster larvae were detected by PCR after storage in different media at different temperatures. The lowest detection threshold was found using distilled water or Tris EDTA buffer. In seawater, the threshold was higher. The use of sterile media permitted detection of viral DNA stored over a longer period. Storage temperature also had a significant influence on detection, with lower temperatures promoting DNA detection over a longer period. In summary, water parameters such as temperature influenced detection of OsHV-1 DNA by PCR. However, the PCR technique may be successfully applied to samples in natural seawater. The PCR technique permitted detection of naked viral DNA at 100 ng L^{-1} in seawater in bioassays (Vigneron et al., in press).

4 Picornavirus-like and other small virus-like particles

There have been many reports of small virus-like particles in different bivalve species. Rasmussen (1986) reported virus-like particles of 27 nm in diameter in the mussel, *Mytilus edulis*, from Denmark. Virus-like particles were enclosed in vesicles, arranged singly or in paracrystaline arrays, and were associated with chronic inflammatory conditions interpreted as granulocytomas. Granuloytomas were localized in haemolymph spaces of the digestive gland and mantle and involved areas of vesicular connective tissue. Previously, Lowe and Moore (1978) had reported similar granulocytomas from mussels in Britain although no ultrastructural studies were performed in order to investigate a viral aetiology. Virus-like particles were also isolated from farmed seed and adult green-lip mussel, *Perna canaliculus*, suffering mortalities in New Zeland during the summer and autumn of 1994 (Jones et al. 1996). Histological examination showed extensive haemocytosis and multifocal liquefaction necrosis of interstitial cells, basal cells and digestive tubule epithelial cells. Ultrastructural examination showed a highly modified rough endoplasmic reticulum associated with electron-dense uncoated virus-like particles varing from 25 to 45 nm in diameter (Jones et al. 1996). Identical cell damage and virus-like particles were subsequently found in *M. galloprovincialis* from the same area. One year later, Hine and Wesney (1997) reported apparent replication of small DNA-negative virus-like particles from digestive and secretory cells of scallops, *Pecten novaezelandiae*, and toheroa, *Paphies ventricosum*, that had been sampled during an outbreak of mass mortalities. Virus-like particles varing from 22 to 30 nm in diameter were seen in an orderly array on the surfaces of the outer nuclear membrane and along the endoplasmic reticulum (Hine and Wesney 1997). A morphologically similar non-enveloped virus, 25 to 33 nm in diameter, was reported in Japanese pearl oysters, *Pinctada fucata martensii*, in association with necosis and degeneration of muscle fibers (Miyazaki et al. 1999). The authors proposed that the virus, called "akoya-virus", was responsible for mass mortalities which had occurred in western regions of Japan since 1994 among Japanese pearl oysters. Virus-like particles were also detected in granulomas associated with focal necrosis within the adductor muscle of the pearl oyster, *P. margaritifera*, from French Polynesia (Comps et al. 1999). The lesions found in the muscle of *P. margaritifera* displayed some similarities to granulocytomas which developed in the mussel *M. edulis* infected by a picorna-like virus (Rasmussen 1986). Paraspherical or polygonal shaped VLPs (virus-like particles), with a diameter of 40 nm, consisted of a membrane-like envelope coating a central electron dense core which measured 35 nm in diameter (Comps et al. 1999). The first description of a virus affecting the carpet-shell clam, *Ruditapes decussatus*, was reported by Novoa and Figueras (2000). The authors reported virus-like particles in the cytoplasm of connective tissue cells from cultured carpet-shell clams suffering mortalities in Galicia (NW Spain). The virus-like particles appeared free in the cytoplasm or associated with endoplasmic reticulum membranes and cytoplasmic vesicles. They were non-enveloped, icosahedrical-spherical in shape, varing from 27 to 35 nm in diameter. Due to their size, morphology and replication in the cytoplasm in association with endoplasmic reticulum and cytoplasmic vesicles, they were suggested to belong to the Picornaviridae. Histological studies of the clams showed the presence of several species of protozoan parasites (haplosporidian, probably *Haplosporidium tapetis*, and an unidentified coccidian) but with low prevalence and intensities. This fact suggested that the virus-like particles might be the cause of the high mortality rate recorded. More recently, Carballal et al. (2003) detected virus-like particles associated with large foci of heavy hemocytic infiltration in cockles, *Cerastoderma edule*, from Galicia (NW Spain). By electron microscopy non-enveloped virus-like particles were seen in paracrystaline arrays in the cell cytoplam. The virus-like particles had an icosahedral capsid and measured 19 to 21 nm in length. They were determined to be RNA viruses since histological sections of hemocyte foci stained with methyl green pyronin showed red deposits. Virus-like particles were similar to those described in mussels from Denmark (Rasmussen 1986) and due to their size, morphology and the formation of paracristalline arrays they were supposed to belong to the Picornaviridae.

5 Disseminated neoplasia, papovavirus-like particles and other reported virus

Disorders of the circulatory system of bivalve mollucs, termed disseminated neoplasia, occur worldwide in at least 20 bivalve species and are of unconfirmed but possible viral aetiology (Elston et al. 1992). The disease has been determined to be transmissible through water-borne exposure. In the soft-shell clam, *Mya arenaria*, affected by disseminated neoplasia, Oprandy et al. (1981) reported the physical isolation of virus-like particles 120 nm in diameter that due to their ultraviolet absorbance and density characteristics were suggested to belong to the Retroviridae. This hypothesis was later confirmed by inducing viral replication and circulating neoplasia in the soft shell clam with 5-bromodeoxyuridine within 4 days of exposure (Oprandy and Chang 1983). This compound can induce the expression of retroviral particles in some mammalian tissue culture systems.

Papova-like viruses have been reported from the gonadal epithelia of *C. virginica* in Canada (McGladdery and Stephenson 1994) and the USA (Farley 1976; Meyers 1981; Farley 1985; Winstead et al. 1998; Winstead and Courtney 2003), as well as from other bivalve species (Elston 1997). Farley (1976) reported for the first time a papova-like virus in hypertrophied gametocytes of eastern oysters although no clinical effects were recognized in infected individuals. Ultrastructural studies revelead the presence of intranuclear, non-enveloped virus-like particles varing from 50 to 55 nm in diameter with an icosahedral symetry. Norton et al. (1993) also reported a papova-like virus infection in the golden-lip pearl oyster, *Pinctada maxima*, from Australia. Epithelial cells in labial palps were hypertrophied and their nuclei contained eosinophilic inclusion bodies. By electron microscopy, virus-like particles 60 nm in diameter resembling Papovaviridae were detected in those inclusions. During a recent health survey of cultured Pacific oyster, *C. gigas*, from the Tongyoung area on the southern coast of Korea, a papova-like virus was detected in gonadal tissues (Choi et al. 2004). Electron microscopy observation revealed non-enveloped, icosahedral particles 40 to 45 nm in diameter (Choi et al. 2004). Histological examination of *C. gigas* oysters in France revealed also several cases of abnormally large basophilic cells in gonadal tissues that resembled cells characteristic of ovacystis disease (Viral Gametic Hypertrophy; Garcia, unpubl. data) previously reported in *C. virginica* oysters from Maine and Long Island in the USA (Winstead and Courtney 2003). Transmission electron microscopy examination of Feulgen-positive inclusions, observed in both males and females, revealed the presence of non-enveloped free viral particles ranging around 48 nm in diameter. Although similar abnormal cells have been observed occasionally before in *C. gigas* in France, viral particles were described for the first time in 2003 in French oysters (Garcia, unpubl. data)

Reports described the in vitro isolation of virus-like particles assigned to belong to the Reoviridae and Birnaviridae from bivalve molluscs. A reo-like virus was isolated from juvenile eastern oysters, *C. virginica* (Meyers 1979; Meyers and Hirai 1980). Virus particles were isolated using the bluegill fry fish cell line (BF-2). By negative staining, purified virus-like particles with a mean diameter of 79 nm were observed. Some particles had hexagonal profiles and an inner capsid layer. However, most of the particles appeared slightly oval with clear spike-like projections on the outer capsid and a distinct inner core (Bower 2001). Birnaviruses have been found in bivalves in Europe (Hill 1976) and Taiwan (Lo et al. 1988). During a high mortality episode, Suzuki et al. (1998a) isolated a virus from oysters cultured in the Uma Sea (Japan) and tentatively named it “Marine birnavirus”. Marine birnaviruses (MABV) have been defined as a group within the genus *Aquabirnavirus*. Although MABV and infectious pancreatic necrosis virus (IPNV) resemble each other, genogrouping based on the nucleotide sequence of the VP2/NS junction region (A segment) separates them (Hosono et al. 1996). MABV comprise the yellowtail ascites virus (YAV) first isolated from *Seriola quinqueradiata* affected by serious mortality in Japan (Sorimachi and Hara 1985) and other similar viruses isolated from several marine fish (Bonami et al. 1983; Schultz et al. 1984; Hedrick et al. 1986; Novoa et al. 1993; Novoa and Figueras 1996). MABV have also been associated with serious mortality in shellfish (Lo et al. 1988). Although the pathogenicity of certain strains appears to be weak, physiological stressors such as spawning, heavy metals and changes in temperature can increase host susceptibility and result in mortality. This was observed in clam, *Meretrix lusoria* (Chou et al. 1994, 1998), Agemaki (jack knife clam), *Sinovacura constricta* (Suzuki et al. 1998b) and Japanese pearl oyster, *P. fucata* (Suzuki et al. 1997a and 1998a). MABV may thus be opportunistic pathogens which persistently infect marine organisms and become pathogenic under stressful conditions (Suzuki et al. 1998c; Suzuki and Nojima 1999; Kitamura et al. 2000). IPNV-like virus particles were also isolated from *Tellina tenuis* and *O. edulis* from the coast of Britain and east coast of Canada (Hill 1976; Bower 2001). Virus-like particles were isolated using BF-2 cell line. Infected bivalves showed a mild haemocytic infiltration as well as a necrosis on the connective tissue of the digestive gland. Icosahedrical, non-enveloped virus-like particles with a mean diameter of 55 nm were observed. Hill (1982) reported that MABV isolated from shellfish appear to be pathogenic to trout. Moreover, based on serological and genomic properties, strains isolated from shellfish and fish seem similar (Suzuki et al. 1997b, 1998b). This may indicate that the host range of MABV may be broad (Suzuki and Nojima 1999). Furthermore the MABV isolation from a variety of apparently healthy marine shellfish (Hill 1982; Suzuki and Nojima 1999) and from environmental samples (Rivas et al. 1993) suggests that shellfish species can act as reservoirs. Different infection experiments performed with the reovirus-like and birnavirus-like particles isolated on the fish cell line BF-2, showed inconsistent results and a firm conclusion on the significance of these viruses for shellfish is still unknown (Bower 2001).

6 Discussion

The discovery of viruses in marine bivalves is a fairly recent event. Although several viruses are detectable only in molluscs that are suffering from another disease or from environmental stress such as pollution, several massive mortality outbreaks have been correlated to viral infections. Irido-like virus infections led to the almost total extermination of

the Portuguese oyster, *C. angulata*, in French and European Atlantic waters in 1973. Viruses morphologically similar to members of the Herpesviridae have been identified in various marine bivalve species around the world as pathogens in larvae and spat.

Selective breeding of bivalve stocks appears suitable for aquaculture development. Only shellfish hatcheries are able to supply such animals. There may be a substantial international trade in bivalve gametes and larvae, allowing for the distribution of seedstocks improved through selective breeding. Although hatchery technology is constantly being improved, significant production problems including infectious diseases must be solved before hatcheries become a major supplier of juveniles for the industry. Current practice in the commercial shellfish hatcheries takes account of basic research findings about food provision and avoidance of *Vibrio* infection but uncontrolled variables are still damaging the industry. Among these uncontrolled variables, virus infections seem to play a key role. The development of viral diseases in aquaculture partly comes from high density production systems including commercial hatcheries and nurseries. Bivalves in intensive culture are continuously affected by environmental fluctuations and management practices which can impose considerable stress, rendering them susceptible to a wide variety of infectious diseases including viral diseases. Viral diseases in aquacultured invertebrates are of serious concern since no specific chemotherapies and vaccines are available. Knowledge of viruses in cultured bivalves is needed in order to develop new tools for the control of viral diseases. This knowledge may give new insights into the management and control of infectious diseases in aquaculture. The basic method to diagnose virus infections has long been light microscopy, although this procedure appears to be poorly adapted to viral diseases and additional techniques, such as transmission electron microscopy, have to be undertaken to complete the diagnosis. Despite being time consuming and somehow inadequate for epidemiological surveys, both techniques were extensively employed through lack of suitable alternative diagnostic procedures. Indeed, the research into virus cytopathogenic effects in cell cultures is not practical owing to the absence of mollusc cell lines, whereas the development of serological methods is impeded by the absence of immunoglobulin production in molluscs. Within this context, the development of protocols for purification of viral particles from infected bivalves appears as a way out from both methodological deadlocks. For an example, extraction and sequencing of OsHV-1 DNA from purified herpesvirus particles infecting *C. gigas* larvae rendered the development of molecular tools possible.

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