

**European Commission**

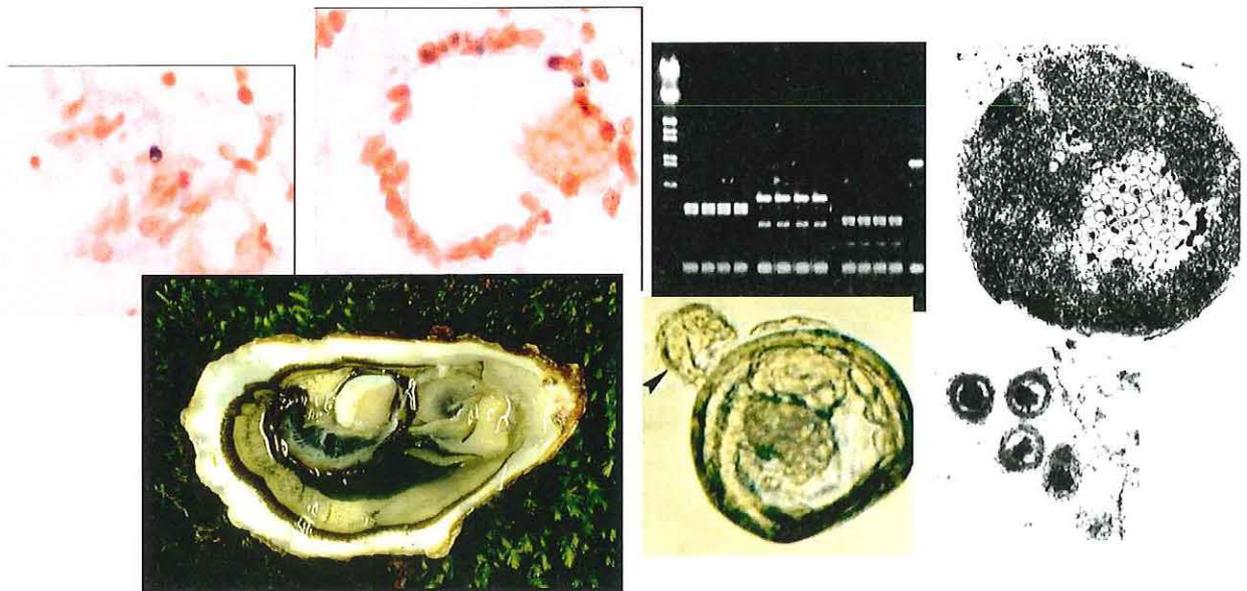
**Contract FAIR-CT98-4334**

**« Diagnosis of oyster herpes-like virus: development and validation of molecular, immunological and cellular tools »**

**« VINO »**

**FINAL REPORT:**

**4<sup>th</sup> January 1999 to 3<sup>rd</sup> January 2002**



- 1. Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER), La Tremblade, France**
- 2. Medical Research Council (MRC), Glasgow, United Kingdom**
- 3. Eurogentec S. A., Seraing, Belgium**
- 4. Université de Bretagne Occidentale (UBO), Brest, France**
- 5. University College Cork, Cork, Ireland**
- 6. Instituto de Investigaciones Marinas (CSIC), Vigo, Spain**
- 7. Centre for Environment, Fisheries & Aquaculture Science (CEFAS), Weymouth, United Kingdom**

**European Commission**

**Contract FAIR-CT98-4334**

**« Diagnosis of oyster herpes-like virus: development and validation of molecular, immunological and cellular tools »**

**« VINO »**

**FINAL REPORT**

## FAIR-CT98-4334

### “Diagnosis of oyster herpes-like virus: development and validation of molecular, immunological and cellular tools”

#### Final Report

**Type of contact:** Shared-cost research project

**Total cost:** 1,284,071 ECU                      **EC contribution:** 649,738 ECU (50.5%)

**Commencement date:** 04-01-99                      **Duration:** 36 months

**Completion date:** 03-01-02

**EC contact:** DG XIV.C.2 (F. Vander Elst: +32 2 299 54 08 and I. Minguez Tudela: +32 2 299 52 56)

**Coordinator:** Dr Tristan Renault (Participant no. 1)  
IFREMER - DRV/RA  
Laboratoire Génétique et Pathologie  
17390 La Tremblade  
France  
Phone: +33 5 46 36 98 36  
Fax: +33 5 46 36 37 51  
Email: [trenault@ifremer.fr](mailto:trenault@ifremer.fr)

**Participant no. 2:** Dr Andrew J. Davison (contractor)  
MRC Virology Unit  
Institute of Virology  
Church Street  
Glasgow G11 5JR  
United Kingdom  
Phone: +44 141 330 6263  
Fax: +44 141 337 2236  
Email: [a.davison@vir.gla.ac.uk](mailto:a.davison@vir.gla.ac.uk)

**Participant no. 3:** Dr Florence Xhonneux (contractor)  
Eurogentec S.A.  
Parc Scientifique du Sart-Tilman  
B-4102 Seraing  
Belgium  
Phone: +32 4 366 61 58  
Fax: +32 4 366 51 03  
Email: [fl.xhonneux@eurogentec.be](mailto:fl.xhonneux@eurogentec.be)

**Participant no. 4 :** Dr Germaine Dorange (contractor)  
Université de Bretagne Occidentale  
Unité de Culture Cellulaire, ISSS, Hôpital Morvan  
5, avenue Foch, 29609 Brest  
France  
Phone : +33 2 98 01 81 16  
Fax. : +33 2 98 01 81 23  
Email: [germaine.dorange@univ-brest.fr](mailto:germaine.dorange@univ-brest.fr)

**Participant no. 5 :** Dr Sarah C. Culloty (contractor)  
University College Cork  
Department of Zoology and Animal Ecology  
National University of Ireland  
Lee Maltings, Prospect Row, Cork  
Ireland  
Phone : +353 21 904187  
Fax. : +353 21 270562  
Email: [s.culloty@ucc.ie](mailto:s.culloty@ucc.ie)

**Participant no. 6 :** Dr Beatriz Novoa (contractor)  
Instituto de Investigaciones Marinas (CSIC)  
Eduardo Cabello, 6  
36208 Vigo  
Spain  
Phone : +34 986 231930  
Fax. : +34 986 292762  
Email: [virus@iim.csic.es](mailto:virus@iim.csic.es)

**Participant no. 7:** Dr Peter Dixon (contractor)  
CEFAS Weymouth Laboratory  
Virology Group  
Weymouth, Dorset, DT4 8UB  
United Kingdom  
Tel : +44 1305 206642  
Fax : +44 1305 20663

## Contents

|  |             |
|--|-------------|
| <b>Executive summary</b>   | <b>p 1</b>  |
| <b>Abstract</b>  | <b>p 1</b>  |
| <b>Project synthesis</b>   | <b>p 3</b>  |
| <i>Obtaining a complete virus genomic library and DNA sequence</i>   | <i>p 3</i>  |
| <i>Development of diagnosis tools</i>  | <i>p 4</i>  |
| <i>Developing molecular tools and techniques</i>   | <i>p 6</i>  |
| <i>Developing immunological tools and techniques</i>   | <i>p 6</i>  |
| <i>Developing and testing cell cultures for virus replication</i>  | <i>p 6</i>  |
| <i>Herpes-like infection surveys</i>   | <i>p 6</i>  |
| <br>   |             |
| <b>Background</b>  | <b>p 7</b>  |
| <br>   |             |
| <b>Obtaining a complete virus genomic library and DNA sequences</b>  | <b>p 8</b>  |
| Genome structure   | p 8         |
| Genetic content  | p 9         |
| Phylogenetic analysis of the oyster virus  | p 10        |
| <br>   |             |
| <b>Developing tools for the diagnosis of herpes-like virus infections</b>  | <b>p 11</b> |
| <b>Obtaining specific primer sequences and probes for diagnosis by PCR and <i>in situ</i> hybridization</b>                                      | <b>p 11</b> |
| <b>Identification of immunogenic viral proteins and preparation of recombinant proteins and antibodies for diagnosis use</b>                     | <b>p 12</b> |
| <i>Identification of immunogenic virus proteins</i>  | <i>p 12</i> |
| <i>Preparation of recombinant proteins</i>   | <i>p 13</i> |
| <i>Preparation of antibodies</i>   | <i>p 13</i> |
| <i>Testing oyster primary cell cultures and vertebrate cell lines, obtention of oyster larval cells and preparation of primary cell cultures</i> | <i>p 15</i> |
| <i>Preparation of oyster primary cultures: embryonic and heart cell cultures</i>   | <i>p 15</i> |
| <i>Cryopreservation of dissociated heart cells and embryoïds</i>   | <i>p 16</i> |
| <i>Cultivation of herpes-like virus in oyster primary cultures</i>   | <i>p 16</i> |
| <i>Cultivation of herpes-like virus in fish cell lines</i>   | <i>p 16</i> |
| <br>   |             |
| <b>Application of DNA probes, immunological reagents and cellular tools for virus detection</b>  | <b>17</b>   |
| Collecting samples   | p 17        |
| Molecular biology workshop   | p 17        |
| Ring trials (PCR and <i>in situ</i> hybridization)   | p 18        |
| <i>Interlaboratory comparison of PCR on frozen samples</i>   | <i>p 18</i> |
| <i>Interlaboratory methodology comparison: ISH on fixed samples</i>  | <i>p 19</i> |
| PCR and <i>in situ</i> hybridisation analysis for virus diagnosis  | p 19        |

|  |             |
|--|-------------|
| <b>Discussion - Conclusion</b>               | <b>p 20</b> |
| <b>Virus genome</b>                          | <b>p 20</b> |
| <b>Molecular diagnosis tools</b>             | <b>p 20</b> |
| <b>Immunological diagnosis tools</b>         | <b>p 20</b> |
| <b>Cellular diagnosis tools</b>              | <b>p 20</b> |
| <b>Validation and use of developed tools</b> | <b>p 21</b> |

|                   |             |
|-------------------|-------------|
| <b>References</b> | <b>p 22</b> |
|-------------------|-------------|

**FAIR-PL98-4334**

**“Diagnosis of oyster herpes-like virus: development and validation of molecular, immunological and cellular tools”**

**Final Report**

**Executive summary**

**Abstract**

Little information is available on viral infections that affect bivalve molluscs. Such a lack of data is due to a certain inadequacy of the diagnosis methods that are employed when massive mortality events occur. Most laboratories involved in mollusc pathology still analyse samples through light microscopy.

World-wide, there is thus currently a lack of information concerning the occurrence of bivalve herpesviruses. This is probably due to the lack of suitable diagnostic tools. The basic method for identification and examination of suspect samples is predominantly histopathology. This enables the identification of any cellular changes, but is not conclusive identification of bivalve herpesviruses. This technique doesn't allow, by itself, to detect viruses unless it is completed by other methods, such as transmission electron microscopy, the study of cytopathogenic effects in cell cultures or the detection through specific reactivities. At present, no bivalve cell-line is available: the detection of cytopathogenic effects in a homologous system is thus impossible. Since invertebrates lack antibody-producing cells, the direct detection of viral agents remains the only possible tool. In these conditions, the use of transmission electron microscopy is a necessity for visual confirmation. However, histology and transmission electron microscopy are time consuming and inadequate for epidemiological studies.

Viral detection in bivalves may be performed on two kinds of biological material. When mortality events occur, moribund animals may be collected in the affected farms. This fresh material may be immediately used for nucleic acid extraction and further analysis. On the other hand, collected infected organisms can be frozen or fixed and remain archived for long periods of time, constituting a bank of reference material.

The aim of the VINO project was therefore the development and validation of molecular, immunological and cellular tools for the diagnosis of, and studies on, the bivalve herpesviruses. The main objective was to develop these 'state of the art' diagnostic techniques. They should be applicable for identification of viruses during disease outbreaks. In addition, these techniques must also be suitable for the detection of subclinical infections and latent virus.

The specific objectives of the programme were:

- 1 - Obtaining the complete oyster herpesvirus (OsHV-1) DNA sequence with determination of the genome structure.
- 2 - Comparing OsHV-1 with viruses belonging to the *Herpesviridae* family on the basis of sequence data and genome structure.
- 3 - Developing molecular tools for OsHV-1 detection.
- 4 - Developing immunological tools for OsHV-1 detection.
- 5 - Developing cellular tools for OsHV-1 detection using oyster primary cell cultures and vertebrate cell lines.
- 6 - Application of developed diagnostic tools for OsHV-1 detection in oyster samples from different geographical locations.

An initial step of the programme involved cloning of virus DNA in cosmids and plasmids. This work provided cloned viral DNA fragments suitable for characterising the virus genome and preparing specific diagnostic probes (PCR primers, labeled DNA probes and specific antibodies). Tests of oyster primary cell cultures and vertebrate cell lines were planned in order to study the ability of the virus to replicate *in vitro*. The development of molecular, immunological and cellular tools for OsHV-1 diagnosis may facilitate virus detection in infected material. Developed reagents have been used by four European laboratories to analyse a wide range of bivalve samples and to confirm the usefulness of the diagnostic tests.

The entire virus genome has been cloned and sequenced. All viral sequences were analysed and the relationship of the OsHV to other members of the *Herpesviridae* family was determined.

To date, polymerase chain reaction (PCR) assays have been developed, which allows the rapid, specific and sensitive diagnosis of herpesviruses in bivalve samples. Another technique that has also been developed is *in situ* hybridisation (ISH). VINO partners have conducted trials using PCR and ISH techniques in order to standardise and further develop the techniques in their respective laboratories.

In addition to continuing the calibration of PCR and ISH, a main target was obtaining viral replication in oyster primary tissue cultures or in fish cell lines. However, all assays failed.

The production of antibodies against bivalve herpesviruses appeared also a necessity for the development of any serological diagnostic/research technique. The development of immunochemistry tests and ELISA (Enzyme Linked Immunosorbent Assay) is now possible because of the availability of antibodies specific for bivalve herpesviruses. Indeed, specific rabbit antisera were obtained and may be used for diagnosis development. The best experimental conditions have been defined for their use in ELISA and Western blot analysis. A good immunization of mice was also obtained with two recombinant viral antigens and clones producing specific monoclonal antibodies have been isolated and characterized by ELISA, Western blot analysis and immunochemistry analysis.

Applied to field samples, this calibration/standardisation step has provided an opportunity to carry out a preliminary epidemiological study. This was currently being achieved by the invaluable provision of bivalve larvae, spat, and adults from private hatcheries and shellfish farms in France, Spain, the United Kingdom and Ireland. Herpesviral infections were confirmed

in France in 1999, 2000 and 2001 and some positive samples were also reported in Spain and in The United Kingdom.

### **Project synthesis**

#### *Obtaining a complete virus genomic library and DNA sequence*

The entire viral genome sequence has been completed and analysed. Virus particles have been purified from fresh infected *Crassostrea gigas* larvae and viral DNA extracted from purified virions. At completion, each nucleotide was determined an average of 10.8 times and 96.1% of the sequence was determined. The overall genome structure is: TR<sub>L</sub> - U<sub>L</sub> - IR<sub>L</sub> - X - IR<sub>S</sub> - U<sub>S</sub> - TR<sub>S</sub> with a 207439 bp total genome size. TR<sub>L</sub> and IR<sub>L</sub> are inverted repeats flanking a unique region (U<sub>L</sub>). TR<sub>S</sub> and IR<sub>S</sub> are inverted repeats flanking a unique region (U<sub>S</sub>), and X is located between IR<sub>L</sub> and IR<sub>S</sub>. A similar genome structure has evolved independently in certain vertebrate herpesviruses (e.g. herpes simplex virus and human cytomegalovirus). The sequences of the genome termini were determined. They are not located uniquely, but a predominant form is apparent for each. The nature of the sequence between IR<sub>L</sub> and IR<sub>S</sub> was also determined. As with the termini, the IR<sub>L</sub> - IR<sub>S</sub> junction is not located uniquely, but the predominant form corresponds to a fusion of the two termini if each possesses two unpaired nucleotides at the 3' end. Unpaired nucleotides are characteristic of herpesvirus genome termini. Southern blot hybridisation experiments using PCR-generated probes from the ends of U<sub>L</sub> and U<sub>S</sub> showed that the two orientations of U<sub>L</sub> and U<sub>S</sub> 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. Both the database and restriction endonuclease digests indicated that a minor proportion (approximately 20-25%) of genomes contain a 4.8 kbp region in U<sub>L</sub> in inverse orientation. These data indicate that the virus contains a mixture of genome forms. In 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 132 unique protein-coding open reading frames (ORFs). Owing to the presence of inverted repeats, 13 ORFs are duplicated, resulting in a total of 145 ORFs. This is an approximation of the gene number, chiefly because of the presence of fragmented genes that might not encode functional proteins. Seven genes encode enzymes (DNA polymerase, deoxyuridine triphosphatase, two subunits of ribonucleotide reductase, helicase, a putative primase and the ATPase subunit of terminase). Seven proteins bear sequence similarities with viral or cellular inhibitors of apoptosis proteins (IAPs). IAPs are also encoded by baculoviruses and entomopoxviruses (both of which have invertebrate hosts) underscores the importance of the apoptotic responses of invertebrates against viral infections. Ten ORFs encode class I membrane proteins. An additional 17 proteins contain a hydrophobic domain indicating a possible association with membranes. A total of 39 proteins share sequence similarities with other proteins encoded by the virus, defining 13 multigene families in addition to the IAPs. An additional notable feature, located between ORFs 50 and 51, is a large palindrome. By analogy with certain vertebrate herpesviruses, this palindrome is a candidate origin of DNA replication.

The sequence data demonstrate that the oyster herpes-like virus type 1 (OsHV-1) is not closely related to herpesviruses with vertebrate hosts (including fish). Amino acid sequence comparisons failed to identify a single protein which has homologues only in other herpesviruses. Several OsHV-1 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 than to homologues in other organisms. However, a genetic indication of a common origin between OsHV-1 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 OsHV-1 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. Moreover, a similar genome structure was observed in certain vertebrate herpesviruses. The presence of several isomers described in the OsHV-1 genome is also a feature reported in vertebrate herpesvirus genomes. 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. 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. Moreover, recent data shown that OsHV-1 can infect several bivalve species. 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.

#### Development of diagnosis tools

To diagnose herpes-like virus infections, the basic method for examination of suspect samples is still light microscopy. This method appears poorly adapted to viral diseases and needs to be improved upon by other techniques such as transmission electron microscopy. Both techniques are time consuming and inadequate for epidemiological surveys. In addition, research into virus cytopathogenic effects in cell cultures is impossible because the lack of bivalve cell lines. A breakthrough was achieved recently in the development of a protocol, based on sucrose gradient centrifugation, for purifying oyster herpes-like virus particles from fresh infected larval *Crassostrea gigas* (Le Deuff and Renault, 1999). This advance has served as an appropriate platform for generating molecular biological reagents to diagnose virus infections. A procedure to detect herpes-like virus in French oysters using the polymerase chain reaction (PCR) was developed (Renault *et al.*, 2000a). PCR offers many advantages for disease diagnosis. With regard to herpes-like viruses from oysters, important advantages include its extreme sensitivity, pathogen specificity, ease of sample processing, and availability of reagents. Another technique that has also been developed is *in situ* hybridisation (ISH) (Lipart and Renault, 2002). In addition to continuing the calibration of PCR and ISH, a main target was the production of antibodies to the virus. The development of immunochemistry and ELISA tests became possible because of the availability of cloned sequences of an oyster herpes-like virus which enables the synthesis of recombinant virus proteins.

#### Developing molecular tools and techniques

A PCR-based procedure for detecting a herpes-like virus that infects the Pacific oyster, *Crassostrea gigas*, in France was developed. Two primer primers (A3/A4 and A5/A6) were designed to provide specific amplification products ranging in size 917 and 1001 bp when performed on oyster herpes-like virus DNA (Renault *et al.*, 2000a). No amplification was observed on oyster genomic DNA nor on the DNA from vertebrate herpesviruses. Crude samples were prepared and submitted to nested PCR, allowing the amplification of DNA fragments of the expected size when performed on infected larval and spat samples. The procedure used to prepare the sample for PCR was found to be critical because of the presence of unidentified substances in

oyster tissues that inhibit the PCR reaction. A quick and convenient sample preparation using ground tissues allowed a sensitive detection of the herpes-like virus infected oysters. The ability of the defined PCR protocol to diagnose herpes-like virus infections in oysters was compared to the transmission electron microscopy technique from 15 *C. gigas* larval batches presenting or not mortalities. PCR amplification is as sensitive diagnosis assay for herpes-like virus as the transmission electron microscopy. However, the nested PCR protocol is more convenient and less time consuming. The relationship between reported mortalities among *C. gigas* oyster spat and herpes-like virus DNA detection by PCR was also investigated. Statistical analysis showed that virus detection and mortalities are correlated (Renault and Arzul, 2001).

A competitive PCR method has also been developed using previously designed primers in order to detect and quantitate herpes-like virus 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 (Arzul *et al.*, 2002). 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 the viral DNA. The method was successfully used to demonstrate the presence of viral DNA in asymptomatic adult oysters indicating that oyster herpes-like virus infects animals presenting no anomalous mortality. Quantitations of herpes-like virus DNA in infected spat and asymptomatic adult oysters were 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.

Two primer pairs were also developed in order to amplify small DNA fragments from OsHV-1 DNA. The first primer pair, called OH1/OH4, yielded 196 bp amplicons when genomic viral DNA was used as template. The size of PCR products obtained with the second primer pair (IAP1/IAP2) was 207 bp. Both primer pairs have been designed in order to obtain PCR amplification when DNA extracted from histological blocks was used as template. Several primer pairs previously designed (Renault *et al.*, 2000, Arzul *et al.*, 2001a, b and c) have already been tested using this type of DNA. They failed producing amplicons. These results could be explained by DNA fragmentation. Both primer pairs OH1/OH4 and IAP1/IAP2 allowed the production of amplicons when DNA extracted from wax blocks was used. A classical technique was chosen for DNA extraction from histological sections using dewaxing in xylene and treatment with proteinase K. Archived material has been used. PCR analysis using DNA extracted from this material showed clear bands presenting expected sizes when both primer pairs were used. This suggested that both primer combinations were reliable tools to detect viral DNA in archived material. Moreover, the primer pairs have been designed in two different areas of the genomic viral DNA enhancing the specificity of the detection. The OH1/OH4 primer pair recognises a gene coding for a protein of unknown function and the IAP1/IAP2 primer combination amplifies a fragment of a gene corresponding to a putative inhibitor of apoptosis (IAP).

*In situ* hybridization of 5 ng/ $\mu$ l probe produced by PCR to paraffin-embedded oyster sections from

infected animals resulted in strong staining of fibroblastic-like cells in connective tissues. *In situ* hybridization appeared thus as a useful technique to detect herpesvirus DNA in histological sections. However, ring trials must be repeated in order to improve results. Indeed, it appears necessary to standardize the method. The study carried out during the programme appears as a first step in a long process of validation. It is important to note that it is the first time that an *in situ* hybridization method was developed to diagnose a viral infection in bivalves and that the technique was used by several European countries on reference material.

#### *Developing immunological tools and techniques*

The immunoscreening with specific anti-OsHV-1 antibodies and the results of sequencing of the virus genome allowed to identify two open reading frames (ORFs) encoding for putative immunogenic viral proteins. The first ORF codes for a protein of 748 amino acids. This protein contains a highly hydrophobic C-terminal domain, potential N-glycosylation sites (Asn-X-Ser/Thr) and a potential signal peptide at the N-terminal end. This ORF could code for a membrane glycoprotein, the typical profile of surface viral antigens. The second identified ORF codes for a protein of which different regions are recognized by anti-OsHV-1 ascitis. This protein of 364 amino acids doesn't present the characteristics of membrane proteins but seems to be a potential immunogenic protein. This ORF codes for a protein presenting homologies with baculovirus, insect and mammal IAPs (Inhibitor of Apoptosis). The two ORFs encoding for putative immunogenic viral proteins have been cloned in baculovirus expression system in order to prepare recombinant proteins and antibodies for diagnosis use. The production of polyclonal and monoclonal antibodies specific for the two selected viral proteins furnished specific immunological reagents.

#### *Developing and testing cell cultures for virus replication*

Several 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. OsHV-1 may be not able to multiply in fish cell lines or under cultivation conditions used viral replication doesn't occur. Tested fish cell lines cannot be used for the herpes-like virus infection diagnosis. Preliminary assays performed in primary cultures of embryonic oyster cells showed the presence of viral DNA in infected cultures using PCR and *in situ* hybridization. However, experiments must be reiterated and other techniques as transmission electron microscopy used in order to demonstrate the presence of the virus in embryonic cells. Although promising results have been observed, primary cultures of embryonic oyster cells are not at this time a reliable tool to detect OsHV-1.

#### *Herpes-like infection surveys*

Periodic losses in bivalve hatcheries are regularly reported in Europe. Current practise in shellfish hatcheries and farms takes account of basic research findings about food provision, rearing and environmental conditions but uncontrolled variables are still damaging the industry, particularly since 1991. Among these uncontrolled variables, herpes-like virus infections seem to play a key role (Renault and Arzul, 2001). The observed association between oyster mortality and herpes-like virus infections provides an imperative to determine the extent to which the virus is involved as a causative agent of massive bivalve mortalities in different European countries. PCR may be used to investigate the presence of herpes-like virus DNA in bivalve samples belonging to different bivalve species from different geographical origins.

The laboratories involved in mollusc epidemiological surveys have collected bivalve samples in 1999, 2000 and 2001 to search viral infections using the developed tools. In 2001, both PCR and *in situ* hybridization were used to diagnose herpes-like virus infections in bivalves. Positive samples were reported in France, in Spain and in the United Kingdom using molecular techniques. These results confirm previous data indicating that herpes-like virus infections may be observed in France in the field and in hatcheries (Nicolas *et al.*, 1992, Comps and Cochenec, 1993 ; Renault *et al.*, 1994a and b ; Renault *et al.*, 2001a and b). Moreover, some PCR positive results were also obtained for bivalve samples originating from Spain and the United Kingdom. Positive samples were observed in four bivalve species: *Crassostrea gigas*, *Ostrea edulis*, *Ruditapes decussatus* and *R. philippinarum*.

## Background

Bivalve cultivation may be endangered by the occurrence of epizootics, especially viral diseases, which are considered one of the putative risks to production. Indeed, mortalities have been described among different species of ostreids and are associated with the presence of viruses belonging to various families. The first description of a virus was reported in adult Eastern oysters, *Crassostrea virginica*, with the detection of particles indicating membership of the *Herpesviridae* (Farley *et al.*, 1972). Mass mortalities of adult Portuguese oysters, *C. angulata*, among French livestock (between 1967 and 1973) were associated with iridovirus infections (Comps *et al.*, 1976; Comps and Bonami, 1977; Comps and Duthoit, 1979). Other viruses described in ostreids are members of the *Iridoviridae*, *Papovaviridae*, *Togaviridae*, *Retroviridae* and *Reoviridae* (Farley, 1976; Farley, 1978; Elston, 1979; Meyers, 1979; Elston and Wilkinson, 1985).

Recently, in 1991, viruses interpreted as belonging to the *Herpesviridae* were associated with high mortality rates of hatchery-reared larval *Crassostrea gigas* in France (Nicolas *et al.*, 1992) and in New Zealand (Hine *et al.*, 1992). Since 1992 sporadic high mortalities of larval *C. gigas* are regularly observed in some private French hatcheries, occurring each year during summer period in association with a herpes-like virus (Renault *et al.*, 1994b). Since 1993, sporadic high mortalities occur also in some batches of Pacific oyster spat cultured in different French locations (Renault *et al.*, 1994a and b). In addition, herpesvirus infections were reported in spat and larvae of the European flat oyster, *Ostrea edulis*, in France (Comps and Cochenec, 1993; Renault *et al.*, 2000b). 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) and in larval *Ruditapes philippinarum* in France (Renault, 1998 ; Renault *et al.*, 2001a and b). Unexplained mortalities were observed in recent years among *C. gigas* larvae in the United Kingdom and Spain, although samples were not examined. High losses were reported among Pacific oyster spat in Ireland in 1994 and 1995. No obvious cause of mortalities was determined (Culloty and Mulcahy, 1995). However, screening using conventional light microscopy yielded little apart from some cell damage most noticeably enlarged cell nuclei and marginated chromatin. Results would now indicate that herpes-like virus is present in at least one site on the south coast of Ireland (Culloty and Mulcahy, unpublished

data). Herpes-like virus infections in bivalves seem to be ubiquitous and are associated with substantial mortalities. The observed association between oyster mortality and herpes-like virus infections provides an imperative to determining the extent to which the virus is involved as a causative agent of massive mortalities. It appeared essential to survey epidemiologically infections in different European countries.

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 the OsHV also showed that it could be transmitted from *O. edulis* larvae to axenic larvae of *C. gigas*. 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 the OsHV to spat of *C. gigas*, 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%. However, when holding conditions were improved, so reducing the stress levels of the animals, no significant mortalities were observed. 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 and b).

## **Obtaining a complete virus genomic library and DNA sequences**

### **Genome structure**

Comparison of the predicted sizes of restriction endonuclease fragments with those determined by digestion of viral DNA indicated that the overall genome structure is: TR<sub>L</sub> - U<sub>L</sub> - IR<sub>L</sub> - X - IR<sub>S</sub> - U<sub>S</sub> - TR<sub>S</sub>. The total genome size is 207439 bp. TR<sub>L</sub> and IR<sub>L</sub> (7584 bp) are inverted repeats flanking a unique region (U<sub>L</sub>, 167843 bp), TR<sub>S</sub> and IR<sub>S</sub> (9774 bp) are inverted repeats flanking a unique region (U<sub>S</sub>, 3370 bp), and X (1510 bp) is located between IR<sub>L</sub> and IR<sub>S</sub>. A somewhat similar genome structure has evolved independently in certain vertebrate herpesviruses (e.g. herpes simplex virus and human cytomegalovirus).

The sequences of the genome termini were determined. The genome termini are not located uniquely, but a predominant form is apparent for each. The nature of the sequences between IR<sub>L</sub> and IR<sub>S</sub> was also determined. PCR products were generated from viral DNA using primers within these elements, close to their boundaries with X (i.e. equivalently close to the genome termini). Two products were obtained. The larger corresponded to the relevant portion of IR<sub>L</sub> - X - IR<sub>S</sub>, as expected. The smaller corresponded to the relevant portion of IR<sub>L</sub> - IR<sub>S</sub> (i.e. X was absent, equivalent to the genome termini joined together). As with the termini, the IR<sub>L</sub> - IR<sub>S</sub> junction is not located uniquely, but the predominant form corresponds to a fusion of the two termini if each possesses two unpaired nucleotides at the 3' end. Unpaired nucleotides are characteristic of herpesvirus genome termini.

Southern blot hybridization experiments were carried out to determine the relative amounts of the two types of junctions (IR<sub>L</sub> - X - IR<sub>S</sub> and IR<sub>L</sub> - IR<sub>S</sub>) present in viral DNA. Only the former was detected, indicating that the latter is present in no more than a small proportion of virion DNA molecules. The hybridization experiments also indicated that a small proportion of molecules

contain an additional X sequence at the left end of the genome. Southern blot hybridization experiments using PCR-generated probes from the ends of  $U_L$  and  $U_S$  showed that the two orientations 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, and results from recombination between inverted repeats during DNA replication.

Both the database and restriction endonuclease digests indicated that a minor proportion (approximately 20-25%) of genomes contain a 4.8 kbp region in  $U_L$  in inverse orientation, with the gain of 1 bp at one end and the loss of 1 bp at the other incidentally keeping the hybrid genes in frame.

These data indicate that the virus contains a mixture of genome forms. In 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. The major form has the basic structure:  $TR_L - U_L - IR_L - X - IR_S - U_S - TR_S$ . By virtue of inversion of  $U_L$  and  $U_S$  (to give  $U_L'$  and  $U_S'$ ), this comprises four isomers in approximately equimolar amounts:

$TR_L - U_L - IR_L - X - IR_S - U_S - TR_S$   
 $TR_L - U_L' - IR_L - X - IR_S - U_S - TR_S$   
 $TR_L - U_L - IR_L - X - IR_S - U_S' - TR_S$   
 $TR_L - U_L' - IR_L - X - IR_S - U_S' - TR_S$ .

Small proportions of molecules either lack the X sequence or contain an additional X sequence at the left terminus. Although the situation might be more complex, this would most simply represent:  $X - TR_L - U_L - IR_L - IR_S - U_S - TR_S$  (as four isomers). Since herpesvirus genomes are packaged into capsids from head-to-tail concatemers (conceptually a circularised form of the parental genome), this minor genome form is explained most readily as resulting from rare cleavage of concatemers at  $X - TR_S$  rather than at  $IR_L - IR_S$ . A minority of genomes also contain a 4.8 kbp region within  $U_L$  that is inverted. Presumably, this form also exhibits the 8 permutations described above.

### **Genetic content**

A detailed analysis of the coding potential of the genome sequence indicated the presence of 132 unique protein-coding open reading frames (ORFs). Owing to the presence of inverted repeats, 13 ORFs are duplicated, resulting in a total of 145 ORFs in the genome. This is an approximation of the gene number, chiefly because of the presence of fragmented genes that might not encode functional proteins.

Amino acid sequence comparisons of the proteins encoded by the 132 ORFs with databases using Blast and FastA yielded the following information about their properties and relationships. Seven genes encode enzymes. These include DNA polymerase (ORF106), deoxyuridine triphosphatase (ORF81), two subunits of ribonucleotide reductase (ORF21 and ORF53), helicase (ORF72), a putative primase (ORF25) and the ATPase subunit of terminase (ORF117). Seven proteins bear sequence similarities with viral or cellular inhibitors of apoptosis proteins (IAPs; ORF43, ORF102, ORF103, ORF114, ORF125, ORF93, ORF105). Of these, the first five listed contain a zinc-binding domain known as a RING finger. One (ORF43) was selected as a potentially

immunogenic protein. The observation that IAPs are also encoded by baculoviruses and entomopoxviruses (both of which have insect hosts) underscores the importance of the apoptotic responses of invertebrates against viral infections. Vertebrate herpesvirus or poxvirus do not encode IAPs, and subvert the battery of host defences by other pathways. Three additional proteins contain RING fingers (ORF10, ORF35, ORF126), and two others possess alternative zinc-binding domains (ORF129, ORF132). One protein is related to a eukaryotic protein of unknown function which is brain-specific in vertebrates (ORF59). Seven ORFs encode class I membrane proteins that traverse the membrane once, and three encode proteins that traverse the membrane more than once. One member of the former class (encoded by ORF94) was also selected as a potentially immunogenic protein. An additional 17 proteins contain a hydrophobic domain indicating a possible association with membranes.

A total of 39 proteins share sequence similarities with other proteins encoded by the virus, defining 13 multigene families in addition to the IAPs. The generally ancient nature of the gene duplication events which have led to this situation is indicated by the fact that relationships are distant and that homologues are generally widely distributed in the genome. Of those ORFs in multigene families, 16 appear to represent eight genes that have become fragmented relatively recently in evolution. It is questionable whether all of these ORFs encode functional proteins.

An additional notable feature, located between ORFs 50 and 51, is a large palindrome. The sequence of this element was particularly difficult to solve. By analogy with certain vertebrate herpesviruses, this palindrome is a candidate origin of DNA replication.

#### **Phylogenetic analysis of the oyster virus**

Even though data indicated that the OsHV-1 capsid is structurally similar to that of other herpesviruses, amino acid sequence comparisons failed to identify a single protein which has homologues only in other herpesviruses. Several OsHV-1 proteins (see above) have homologues that are distributed widely in nature (e.g. DNA polymerase), but these are no more closely related to homologues in other herpesviruses than to homologues in other organisms. This finding is also characteristic of comparisons between herpesviruses which infect fish or amphibians and those that infect mammals or birds, and has been taken to reflect divergence over a very long periods of time.

In this context, detailed phylogenetic analyses are not of great utility in determining whether OsHV-1 and vertebrate herpesviruses have a common origin. The strongest genetic indication of a common origin resides with the ATPase subunit of the terminase (see above), which is involved in packaging DNA into the capsid. Homologous genes are present in all herpesviruses, and the only non-herpesvirus counterparts are specified by T4 and related bacteriophages. The T4 and OsHV-1 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. This scheme is consistent with the generally accepted model of evolution of herpesviruses with their hosts. In the context of this model, 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.

Recent data have shown that OsHV-1 can infect several bivalve species (Arzul *et al.*, 2001a, b and c ; Renault *et al.*, 2000b). 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 intriguing 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.

## **Developing tools for the diagnosis of herpes-like virus infections**

### **Obtaining specific primer sequences and probes for diagnosis by PCR and *in situ* hybridization**

A PCR procedure and an *in situ* hybridisation technique have been developed in 1999. The PCR method allowed the detection of viral DNA in frozen larval and spat samples. The *in situ* hybridisation technique demonstrated the presence of viral DNA in histological sections of infected *Crassostrea gigas* and *Ostrea edulis* spat.

Two sequences of cloned viral DNA were selected in order to design PCR primer pairs: a sequence without significant homology with sequences in data banks and an other one corresponding to a gene encoding for a protein presenting homologies with an Inhibitor Apoptosis Protein (IAP). Twelve primers were designed and eight primer pairs were tested. Two primer pairs, OHV3/OHV114 and OHV1/OHV2, were selected on the basis of systematic detection of low amounts of viral DNA (10 to 20 fg of viral DNA per PCR tube). Reaction parameters were optimised using virus DNA extracted from purified particles. Optimal conditions for PCR and sample preparation were also defined (Arzul *et al.*, 2001a, b and c ; Renault *et al.*, 2000a). Of the different procedures of sample preparation from oyster specimens, boiling of ground tissues was the preferred method (Renault *et al.*, 2000a), because it is simple and consistent.

Two primer pairs have been developed in 2001 in order to amplify small DNA fragments from OsHV-1 DNA. The first primer pair, called OH1/OH4, yielded 196 bp amplicons when genomic viral DNA was used as template. The size of PCR products obtained with the second primer pair (IAP1/IAP2) was 207 bp. Both primer pairs have been designed in order to obtain PCR amplification when DNA extracted from histological blocks was used as template. Several primer pairs (OHV3/OHV4, OHV3/OHV114, A3/A4 and A5/A6) have already been tested using this type of DNA. They failed producing amplicons. These results could be explained by DNA fragmentation. Both primer pairs, OH1/OH4 and IAP1/IAP2, allowed the production of amplicons when DNA extracted from wax blocks was used. A classical technique was chosen for DNA extraction from histological sections using dewaxing in xylene and treatment with proteinase K. Archived material has been used. Five histological blocks prepared in 1995 corresponding to *Crassostrea gigas* spat have firstly been analysed. Animals presenting high mortality rates have been fixed individually in Davidson's fluid during the summer of 1995.

Transmission electron microscopy examination allowed to detect viral particles. PCR analysis using DNA extracted from these blocks showed clear bands presenting expected sizes when both primer pairs were used. This suggested that both primer combinations were reliable tools to detect viral DNA in archived material. Moreover, the primer pairs have been designed in two different areas of the genomic viral DNA enhancing the specificity of the detection. The OH1/OH4 primer pair recognises a gene coding for a protein of unknown function and the IAP1/IAP2 primer combination amplifies a fragment of a gene corresponding to a putative inhibitor of apoptosis (IAP). In 2001, a first validation assay of both primer pairs was carried out using 31 archived histological blocks. Histological blocks corresponding to the material used for preparing reference slides were used for the validation. Each block has already been analysed by histology. Histology examination revealed cellular and nuclear abnormalities suggestive of infection with oyster herpesvirus type 1 (OsHV-1) in 20 individuals. Eleven oysters presented no sign of infection and were interpreted as healthy oysters. DNA was extracted from histological sections using conventional techniques. Both primer pairs OH1/OH2 and IAP1/IAP2 allowed to detect viral DNA from infected oysters. However, some difference may be observed in amplification efficiency. Thus, to enhance viral DNA detection, several primer pairs designed in different genome areas are needed. For routine use, it would be recommendable to run PCR with OH1/OH4 and to confirm negative results with IAP1/IAP2.

*In situ* hybridization protocols have been developed and optimal conditions have been defined (Arzul *et al.*, 2001 ; Lipart and Renault, 2002). *In situ* hybridization of 5 ng/ $\mu$ l labeled probe produced by PCR using the OHV3/OHV114 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. Labeled cells were observed in connective tissues in different organs. The location and the morphology of labeled cells corresponded to the observations made by transmission electron microscopy. No background hybridization to healthy oyster tissues was detected.

#### **Identification of immunogenic viral proteins and preparation of recombinant proteins and antibodies for diagnosis use**

Two OsHV-1 selected ORFs, the ORF43 and ORF94, were expressed via the baculovirus system. The purified recombinant proteins were used for immunization of rabbits and mice in order to select anti-OsHV-1 specific antibodies for diagnosis use. Rabbit antisera and mouse monoclonal antibodies were characterized by ELISA and western-blotting against the OsHV-1 antigens.

#### **Identification of immunogenic virus proteins**

The immunoscreening with specific anti-OsHV-1 antibodies and the results of sequencing of the virus genome have allowed to identify two open reading frames (ORF) encoding for putative immunogenic viral proteins. The first ORF, called ORF94, codes for a protein of 748 amino acids. This protein contains a highly hydrophobic C-terminal domain, potential N-glycosylation sites (Asn-X-Ser/Thr) and a potential signal peptide at the N-terminal end. Thus, this ORF could code for a surface glycoprotein with a transmembrane C-terminal end, the typical profile of surface viral antigen. The second identified ORF, called ORF43, codes for a protein of which different regions are recognized by anti-OHV ascite. This protein of 364 amino acids doesn't present the characteristics of membrane proteins but seems to be a potential immunogenic protein (IAPs). The two ORFs, ORF43 and ORF94, encoding for putative immunogenic viral proteins have been cloned in baculovirus expression system in order to prepare recombinant proteins and

antibodies for diagnosis use. The baculovirus system rather than *Escherichia coli* was chosen as insect cells are more convenient for folding recombinant glycoproteins in a native conformation and with post-translational modifications.

#### Preparation of recombinant proteins

Insect cells (Sf9) were separately infected with the recombinant viruses and harvested two days post infection. Proteins were expressed in the intracellular fraction. Protein expression was also observed in the culture supernatant with the recombinant ORF43s and ORF94. By this way, the secretion of the protein ORF94 without its hydrophobic C-terminal region was demonstrated. The capacity of the insect cells to recognize the natural signal peptide of this protein was confirmed. A kinetic study allowed to assess the best production conditions and the harvest time in roller bottle. Tn5 cells were used since these insect cells are recommended for the secretion of recombinant proteins. Both proteins were detected in the intracellular fraction, by Coomassie blue staining. The ORF43 had an apparent molecular weight of ~50 kDa and was in a doublet form. It was expressed at high yield. The ORF94 presented an apparent molecular weight of 90 kDa and a quite diffused coloration which is the characteristics of glycosylated proteins. Secreted forms were not detected by this way. They were weakly detected by western blotting. The best production conditions were 48h of infection, to avoid important cells mortality.

Concentration of primary antibody and incubation time were optimized to ensure a specific signal and to reduce the background using western blotting. A dilution 1/20000 and an incubation of 1 hour at room temperature or overnight at 4°C were selected. Cells infected with ORF43s and ORF94 baculoviruses were lysed with Triton X-100 and recombinant protein solubilized with NaOH 10mM. Solubilized proteins were submitted to electrophoresis and eluted from gels. The protein concentration of the preparations was ~1.4 mg/ml for ORF43 and ~1.2 mg/ml for ORF94 (2.8 mg of ORF43 and 2.3 mg for ORF94). Both preparations were also tested by western-blotting with an anti-OsHV serum. The ORF43 was thus detected but not the ORF94.

#### Preparation of antibodies

Two rabbits were injected at day 1, 14, 28 and 56 with 100 µg of the recombinant proteins per injection. Bleedings were carried out before all injections (except the second one) and a final bleeding was also carried out after the last injection. Four mice were injected three times at three week intervals (Days 0, 21 and 42) with 50 µg of the recombinant proteins per injection. Bleedings were carried out before all the injections and after the last one. All the sera were tested by ELISA and Western blotting assays in order to determine the mouse to be sacrificed for fusion procedure.

All the sera were tested and characterized by ELISA and Western blotting. ELISA for selection of antisera and monoclonal antibodies was carried out on insect cells rather than on purified antigen. Specific insect cell preparations were obtained for ELISA coating. Tn5 cells were infected with the recombinant baculovirus ORF43 and ORF94 and harvested according to the previous results. Uninfected cells were also prepared as control for antibodies against insect cell epitopes. The antigens used for antibody characterization were non purified antigens. They corresponded to Tn5 insect cells infected by different recombinant baculoviruses: one coding for the ORF43, one coding for the ORF94.

A standard low background was observed with sera collected before injections. Serum titers raised after each injection but reached a maximum level after the fourth one. The specificity of the response was calculated by subtracting the optical densities obtained with the control antigens from the optical densities obtained with the ORF94 and ORF43 antigens. The optimal serum were found to be 500x to 1000x for bleedings after 2 and 3 antigen injections and 25000x to 50000x for the sera collected after 4 and 5 antigen injections. Sera were also tested on western blots. The best experimental conditions were determined as to be an incubation of the western blots with the rabbit sera taken after 4 antigen injections and minimum 1000x diluted.

Selected mice were injected with a fourth dose of antigen (ORF94 and ORF43 respectively) before doing the fusion according to standard protocols. These protocols usually allow the obtention of ~500 hybridoma per spleen. 5-20% of these hybridoma usually secrete antigen-specific antibodies. All the obtained hybridoma were subcultivated in order to maintain them and to produce enough material for their characterization. The hybridoma that secreted anti-ORF94 and anti-ORF43 antibodies were screened by ELISA. Each culture well was incubated with the non purified ORF94 or ORF43 antigen. The specificity of the response (O.D.) was calculated as described above for ELISA by subtracting control optical densities values from the specific ones. A screening was also done in parallel on the basis of the growth of the clones in the appropriate medium.

All the supernatants selected (from clones with good growth) showed a positive O.D. and thus a specific response against injected proteins. After 2 runs, 47 clones still remained among the initial anti-ORF43 hybridoma and only 12 clones among the anti-ORF94 ones. An additional test was performed and 22 anti-ORF43 hybridoma were selected as they showed significant O.D. Only 5 anti-ORF94 hybridoma remained and were selected.

Considering the selection of monoclonal antibodies specific for OsHV-1, an immunohistochemistry protocol was developed. Indeed, it appears necessary to complete results obtained using ELISA and Western-blotting. Histological blocks remain the major material available in laboratories involved with shellfish disease diagnosis. An OsHV-1 infected *Crassostrea gigas* individual previously characterized by transmission electron microscopy, PCR and *in situ* hybridization has been selected to develop the immunochemistry protocol.

In a first step, twenty seven hybridoma supernatants have been tested. Incubation with pure supernatants was carried out one hour at room temperature. No signal was detected with the hybridoma supernatants tested. Then, the hybridoma supernatants were again tested with an overnight incubation. Three hybridoma supernatants specific for ORF43 (LF 1A10, LF 2C11 and LF 3D8) and two hybridoma supernatants specific for ORF 94 (LG 5E5 and LG 6H8) yielded positive results on histological sections. Faint staining was detected in muscle and in connective tissues. The staining appeared stronger with hybridoma supernatants specific for ORF94 than for hybridoma supernatants specific for ORF43. Incubation of hybridoma supernatants overnight at 4°C gave better results than a shorter incubation period (1 hour) at room temperature. Such results have already been observed in the laboratory (T. Renault, personal communication) when immunochemistry techniques were used on fixed material from bivalves. A proteinase K treatment was included in some assays and results obtained with and without this type of treatment have been compared using selected hybridoma supernatants. Five hybridoma supernatants (LF 1A10, LF 2C11, LF 3D8, LG 5E5 and LG 6H8) yielded positive results on

histological sections in absence of proteinase K treatment confirming the first results. Positive results were also observed with the hybridoma supernatant LG 6C8 when a proteinase K treatment was added in the immunochemistry protocol. Moreover, the staining appeared more intense with hybridoma supernatants LG 5E5 and LG 6H8 when proteinase K was used.

Six clones were finally selected on the basis of ELISA and immunochemistry results: 1A10, 2C11 and 3D8 for anti-ORF43 hybridomas and the clones numbers 4A3, 6C8 and 6H8 for anti-ORF 94 hybridomas. Three clones were selected for each antigen to be next subcloned and fully characterized. These were the clones that gave the best response in ELISA and/or immunohistochemistry. The six selected hybridomas at this stage were not pure, some of them contained several clones yet as the technique used for the screening does not allow a real cloning of the hybridoma. The next step was to isolate monoclonal antibody-producing hybridoma cell lines by "limiting dilution cloning". The culture supernatant of each single colony obtained was checked by ELISA as described above against the ORF94 or ORF43 antigen. Positive clones were subcloned again until all the supernatants of the individual next subclones showed an equally positive antibody response. One subclone was then selected for each initial hybridoma except for the clone 3A10 (anti-ORF43) for which two subclones ( 3A10G6 and 3A10H7) were selected. All the subclones were then characterized by ELISA and Western blotting as described below. The isotype of the secreted antibodies was also determined for each of the subclones and all the selected antibodies were immunoglobulins G2A kappa. The subcloned hybridomas were again tested using the immunochemistry protocol including a proteinase K treatment. No signal was detected. Assays have been conducted three times.

#### Testing oyster primary cell cultures and vertebrate cell lines, obtention of oyster larval cells and preparation of primary cell cultures

##### *Preparation of oyster primary cultures: embryonic and heart cell cultures*

Primary cultures of fresh and frozen-thawed *Crassostrea gigas* «embryoïds» obtained by enzymatic treatment of embryos at 2 to 64 stages of cell development were carried out. Some assays were also carried out with oyster heart cells. Such cultures, which can be obtained routinely, may be favorable to the observation of the cytopathogenic effect of virus, because the majority of adherent cells are fibroblastic-like cells and cardiomyocytes. Such cell types appear as target cells for the herpesvirus infecting oysters.

The effect of the incubation temperature on the embryonic and heart cell adherence and cell growth has been studied by measuring the rate of proteins and/or DNA and by using BrdU test, knowing that the optimal temperature for the herpes-like virus may be 26°C (Le Deuff *et al.*, 1996). A similar evolution of embryonic and heart cell cultures at 26°C, 20°C and 15°C, up to 6 days was observed. There was a slight increase of proteins and DNA between 2 and 4 days for embryoïds and between 2 and 8-10 days for heart cells. After BrdU incorporation, some positive dark nuclei were detected by immunocytochemistry, from 2 to 6 days (for embryoïds) or 8 days (for heart cells), showing that there was probably replicative DNA synthesis. At 26°C, culturing cells were contractile earlier and cell networks were established after a shorter time than at 15°C : e.g., in cultures of embryoïds, contractile cells were observed after 1 day at 26°C and only after 3 days at 15°. Cell networks were established after 2 days at 26°C and 6 days at 15°C.

These results enabled to conclude that it was possible to cultivate embryonic and heart cells of *Crassostrea gigas* at 26°C, that appears the optimal temperature for herpes-like virus, during at least 6-10 days . It appeared also possible to try to infected oyster cells by the herpes-like virus, two days after seeding. Indeed, after this time of culture, adherent cells are in a sufficient number to test their sensitivity to the herpes-like virus. The observation of some positively marked nuclei after incorporation of BrdU, suggested that there is in culture a slight mitotic activity. After 2 days, specific membrane receptors, potentially altered by the enzyme used to dissociate hearts and embryos, could be reconstituted. Moreover, it was possible to maintain the cells in culture for a sufficiently long time to be able to verify if the cells could be infected by the virus.

#### *Cryopreservation of dissociated heart cells and embryoïds*

If heart cell cultures may be obtained from fresh cells and from cryopreserved cells using the protocol described by Le Marrec *et al.* (1998, 1999), assays failed to maintain frozen embryonic cells in aggregates : they separated when they were thawed. However, even if the viability percent of thawed cells was at least 80%, cultured cells did not organize in networks as described for embryoïds.

#### *Cultivation of herpes-like virus in oyster primary cultures*

Tools and experimental procedures used to detect virus infections in cells were checked up. These controls concerned the PCR amplification method, the digoxigenin labeling of DNA probes used for *in situ* hybridization, the *in situ* hybridization procedure, using histological sections of *Crassostrea gigas* spat infected by the herpes-like virus, as positive control, the infectivity of infected larval samples using axenic *Crassostrea gigas* D larvae produced by *in vitro* fecondation.

Several assays of herpes-like virus cultivation in oyster primary cultures have been carried out in 1999 and 2000. Virus cultivation experiments were carried out in primary cultures of embryonic oyster cells and oyster heart cells. No obvious cytopathic effect was detected. However, PCR analysis allowed to detect amplicons presenting the expected sizes. PCR carried out 4 and 6 days after inoculation of the herpes-like virus showed positive results for all the conditions of viral infection of embryonic cells in culture, and, in particular, when the herpes-like virus was maintained in contact to the cells during two hours before adding cell culture medium and when PEG was added to the medium. Moreover, *in situ* hybridization assays demonstrated positive labeling at the cellular level. *In situ* hybridization applied to « PCR positive » samples allowed to observed also positive reactions in some cells, in particular around the cells, but nuclei were not positively marked. Experiments must be reiterated and other techniques such as transmission electron microscopy used in order to demonstrate the presence of viral particles in primary cell cultures.

Although promising results were observed, primary cultures of oyster cells are not at this time a reliable tool to detect herpesviruses in bivalves. Indeed, the results obtained in embryonic oyster cells were unclear and need to be reiterated. Molecular techniques showed the presence of viral DNA although no cytopathic effect was detected in infected embryonic oyster cells

#### *Cultivation of herpes-like virus in fish cell lines*

Infected and uninfected (negative control) oyster larvae have been inoculated into seven different fish cell lines (TV1, RTG2, SSN1, TFC, CHSE-214, EPC and BB) at two temperatures in order to detect a cytopathic effect of the herpesvirus. Cells were grown with minimal essential medium

with penicillin and streptomycin and 10% of fetal calf serum (FCS), (MEM 10%). Frozen infected and uninfected larvae were frozen and then, homogenized and filtered through 0,45 µm. Once the cell monolayer was confluent, the medium was withdrawn and samples were inoculated. One hundred µl of the larvae homogenated were inoculated on cell monolayers in 24 well-plates. After 30 minutes, MEM with 2% of FCS was added to the wells and plates were incubated at 15 or 20 °C. Plates were observed daily to detect a cytopathic effect (CPE). Blind passages were conducted although no CPE was detected in order to allow the virus to replicate in these cells. An alteration of the cell monolayer was observed in the first passage of some experiments at 15°C. No definitive cytopathic effect was observed when the second passage to new cells was conducted. No cytopathic effect was detected in any of the passages at 20°C.

## **Application of DNA probes, immunological reagents and cellular tools for virus detection**

### **Collecting samples**

The laboratories involved in epidemiological surveys among bivalves have collected bivalve samples in 1999, 2000 and 2001 in order to carry out analyses to search for herpes-like virus infections using the developed tools.

### **Molecular biology workshop**

A molecular biology workshop has been organised in May 2000 in order to ensure that each participant involved in epidemiological surveys used the same protocols and procedures for molecular diagnosis of OsHV-1 infections. The workshop took place at the IFREMER station in La Tremblade (Charente Maritime, France) during one week (15-19 May 2000).

PCR and *in situ* hybridization protocols have been presented and discussed. Fifteen larval samples were analysed by PCR using the OHV1/OHV2 primer pair. Five participants have analysed by themselves the larval samples. The results obtained by each participant have been compared. All negative controls appeared negative for all participants indicating the absence of contamination. All positive controls appeared positive. This suggests that the PCR procedure is reliable. Five larval samples showed a clear band presenting the expected size in agarose gels in all experiments. Amplicons were detected in three other samples in four of the five PCR assays. These results indicated that no contamination occurred during PCR experiments and that the genomic viral DNA was systematically amplified (positive controls). Moreover, some larval samples (5) appeared systematically positive when PCR analysis was carried out.

Two infected *Crassostrea gigas* juveniles and two non infected *C. gigas* spat have been analysed using *in situ* hybridization. Positive and negative material were used to test two different *in situ* hybridisation protocols. The first protocol was based on an alkaline phosphatase/BCIP-NBT direct detection system and the second protocol on a peroxidase/DAB indirect detection system. Infected oysters showed the presence of labeled nuclei and cells in connective tissues of different organs. No staining was observed in non infected animals.

At the end of the molecular biology workshop, genomic viral DNA and fifteen larval samples in dry ice, and histological sections from two infected and from two non infected oyster spat have been furnished for Ring trials.

#### **Ring trials (PCR and *in situ* hybridization)**

Following the workshop in 2000, reference material has been furnished in order to carry out Ring trials (viral DNA, 15 larval samples and histological slides as positive and negative reference material). Preliminary assays on serially diluted positive control material showed that detection of 10 to 100 fg of OsHV-1 genomic DNA may be routinely achieved using the OHV3/OHV114 primer pair in the different laboratories involved in this test. Moreover, the OHV1/OHV2 primer pair allowed to amplify systematically 10 fg of viral DNA. Fifteen larval samples have also been analysed by PCR. Five samples appeared systematically positive when they were tested in the different laboratories. However, other larval samples appeared positive or negative depending of the laboratories. Positive and negative material (histological sections from fixed spat) were also used to test two different *in situ* hybridization protocols. The protocol based on a alkaline phosphatase/BCIP-NBT direct detection system seemed to give better results than the protocol based on a peroxydase/DAB indirect detection system.

In 2001, more reference material has been furnished in order to carry out other PCR and *in situ* hybridization Ring trials.

- Fifteen *Crassostrea gigas* spat samples collected in several locations in Charente Maritime (France) during the year 2001 were selected and sent as reference material for PCR to participants involved in epidemiological surveys. Fifteen bivalve larval samples collected in 1997, 1998 and 1999 were also selected and sent as reference material for PCR trials.

- Three histological blocks have been selected and sent to participants involved in epidemiological surveys. The selected blocks correspond to *Crassostrea gigas* oyster spat collected in 1994 and 1995 infected by OsHV-1. Moreover, *in situ* hybridization Ring trials were carried on a series of samples collected in the years 1994-1995. Thirty one histological blocks were selected. Each block has already been analyzed by histology. Histology examination revealed cellular and nuclear abnormalities suggestive of infection with OsHV-1 in 20 individuals. Eleven oysters presented no sign of infection and were interpreted as healthy individuals. Four slides of each histological block have been sent in June 2001 to participants involved in epidemiological surveys. Slides were again sent to the same participants in December 2001 in order to repeat *in situ* hybridization Ring trial.

#### Interlaboratory comparison of PCR on frozen samples

Frozen larvae and seed samples analysed in the laboratory A were sent to other European laboratoires (labelled B, C and D). PCR with OHV3/OHV114 (C2/C6) gave consistent results considering laboratories A, B and D. As far as the primer pair OHV1/OHV2 (C5/C13) is concerned, false positives were obtained in laboratories B, C and D. Moreover, several false positive results were observed in the laboratory C with both primer pair. However, results obtained in laboratories A, C and D were very similar using both primer pairs.

Interlaboratory methodology comparison: ISH on fixed samples

*In situ* hybridization analysis was carried on in the laboratory A on a series of samples collected in the years 1994-1995. Additional slides were prepared and sent for analysis to three other European laboratories, labelled B, C and D. The laboratories B and C found 3 false negative results (two of them in common). On the other hand, results from laboratory B indicate that seven false positives were obtained among ten negative samples. Therefore, consistent results were obtained as far as laboratories A and D are considered. However, two false negative results were observed in the laboratory D. *In situ* hybridization is a useful technique to detect herpesvirus DNA in histological sections. However, ring trials must be repeated in order to improve results. Indeed, it appears necessary to standardize the method. The study carried out during the programme appears as a first step in a long process of validation. It is important to note that it is the first time that an *in situ* hybridization method was developed to diagnose a viral infection in bivalves and that the technique was used by several European countries on reference material.

**PCR and *in situ* hybridization analysis for virus diagnosis**

Bivalve samples collected in 1999, 2000 and 2001 were analysed. More than 300 bivalve samples (313) were analysed by PCR in four different laboratories. No positive result was reported among Irish samples (0/70). Three PCR positive results (3/57) were observed in oysters from the United Kingdom. Two samples correspond to *Crassostrea gigas* oysters and the last one to *Ostrea edulis* oysters. A positive result (1/68) was also reported among Spanish samples. Positive PCR batches (27/148) have been observed in France in bivalves collected in 1999, 2000 and 2001 confirming previously reported results.

## **Discussion - Conclusion**

### **Virus genome**

The herpes-like virus infecting oysters is now well identified. The genome structure and the entire sequence have been defined. The virus has been identified as a new member of the *Herpesviridae* family and named Oyster herpesvirus type 1 (OsHV-1). 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. This scheme is consistent with the generally accepted model of evolution of herpesviruses with their hosts. In the context of this model, 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.

### **Molecular diagnosis tools**

To date, a polymerase chain reaction (PCR) assay has been developed, which allows the rapid, specific and sensitive diagnosis of OsHV in bivalve samples. Another technique that has also been developed is *in situ* hybridisation (ISH). ISH is specific, but is relatively time consuming; however it appears to be most suited to the detection of OsHV in low level infections, or in possible latent stages such as occur with other herpesviruses. Trials using PCR and ISH techniques conducted in order to standardise and further develop the techniques showed that assays must be repeated especially for *in situ* hybridization.

### **Immunological diagnosis tools**

Two putative immunogenic viral proteins have been identified by immunoscreening of a lambda library and served to produce two recombinant proteins using the baculovirus system. Polyclonal and monoclonal antibodies specific for the two selected viral proteins have been produced. However, a two month delay in producing monoclonal antibodies was observed. Analyses using these reagents were not possible during the programme. Moreover, a supplementary delay (five months) was also observed in producing subcloned hybridoma supernatants. At the end, it was not possible to reproduce the results using these subcloned hybridoma supernatants. Indeed, several assays have been carried out, but all failed using the subcloned hybridomas.

Polyclonal antibodies production has been completed as anticipated at the beginning of 2001. However, because of the limited amounts of the considered reagents, it has been decided to work only with monoclonal antibodies for diagnosis purpose.

### **Cellular diagnosis tools**

Cellular tools were not available and analysis of oyster samples using such tools as anticipated was not carried out during the programme. Indeed, no cytopathic effect has been observed in 1999 and 2000 in tested fish cell lines. Tested fish cell lines cannot be used for the herpes-like virus infection diagnosis. Assays carried out in 2000 in primary cultures of embryonic oyster cells showed the presence of viral DNA in infected cultures using PCR and *in situ* hybridization. However, these results must be confirmed.

### **Validation and use of developed tools**

Applied to field samples, new developed tools of diagnosis provided an ideal opportunity to perform a preliminary epidemiological study. This was currently being achieved by the invaluable provision of oyster spat and larvae from private hatcheries and shellfish farms in France, in Spain, in the United Kingdom and in Ireland. Molecular methods (PCR and *in situ* hybridisation) were used for herpes-like virus infections in laboratories involved in epidemiological surveys among bivalves. However, most of the PCR analyses of bivalve samples failed to show positive results. Thus, validation of molecular reagents and tools was also carried out using reference material.

## References

- Arzul, I., Renault, T. and Lipart, C. (2001a). Experimental herpes-like viral infections in marine bivalves: demonstration of interspecies transmission. *Dis. Aqua. Org.*, 46: 1-6.
- Arzul I., Nicolas J. L., Davison, A. J. and Renault, T. (2001b). French scallops : a new host for ostreid herpesvirus-1. *Virology*, 290: 342-349.
- Arzul, I., Renault, T., Lipart, C. and Davison, A. J. (2001c). Evidence for inter species transmission of oyster herpesvirus in marines bivalves. *J. Gen. Virol.*, 82: 865-870.
- Comps, M., and Bonami, J.R. (1977) Infection virale associée à des mortalités chez l'huître *Crassostrea angulata* Th. *C. R. Acad. Sc., D*, 285 : 1139-1140.
- Comps, M. and Cochenec, N., (1993) A herpes-like virus from the European oyster *Ostrea edulis* L. *J. Inv. Pathol.*, 62, 201-203.
- Comps, M. and Duthoit, J.L. (1979) Infections virales chez les huîtres *Crassostrea angulata* (Lmk) et *C. gigas* (Th.). *Haliotis* 8 (1977), 301-308.
- Comps, M., Bonami, J.R., Vago, C. and Campillo, A. (1976) Une virose de l'huître portugaise (*Crassostrea angulata* Lmk). *C. R. Hebd. Séanc. Acad. Sc. D* 282, 1991-1993.
- Culloty, S.C. and Mulcahy, M.F. (1995). Mortalities in Pacific oyster *Crassostrea gigas* with particular reference to Ireland. Dept. of Zoology, U.C.C. 27pp.
- Elston, R.A. (1979) Virus-like particles associated with lesions in larval Pacific oysters (*C. gigas*). *J. Invert. Pathol.* 33, 71-74.
- Elston, R. and Wilkinson, M.T. (1985) Pathology, management and diagnosis of oyster velar virus disease (OVVD). *Aquaculture* 48, 189-210.
- Farley, C.A. (1976) Ultrastructural observations on epizootic neoplasia and lytic virus infection in bivalve mollusks. *Prog. Exp. Tumor Res.* 20, 283-294.
- Farley, C.A. (1978) Viruses and virus-like lesions in marine molluscs. *Mar. Fish. Rev.* 40, 18-20.
- Farley, C.A., Banfield, W.G., Kasnic, J.R.G., Foster, W.S. (1972) Oyster herpes-type virus. *Science, Wash.D.C.* 178, 759-760.
- Hine, P.M. (1997) Trends in reserch on diseases of bivalve molluscs. *Bull. Eur. Ass. Fish Pathol.* 17(6), 181-183.
- Hine, P.M., Wesney, B. and Hay B. E. (1992) Herpesvirus associated with mortalities among hatchery-reared larval Pacific oysters, *C. gigas*. *Dis. aquat. Org.* 12(2), 135-142.
- Hine, P.M. and Thorne, T. (1997) Replication of herpes-like viruses in haemocytes of adult flat oysters *Ostrea angasi* (Sowerby, 1871): an ultrastructural study. *Dis. aquat. Org.* 29(3), 197-204.
- Hine, P.M., Wesney, B. and Besant, P. (1998) Replication of herpes-like viruses in larvae of the flat oyster *Tiostrea chilensis* at ambient temperatures. *Dis. aquat. Org.* 32(3), 161-171.
- Le Deuff, R.-M. and Renault, T. (1999) Purification and partial genome characterization of a herpes-like virus infecting the Japanese oyster, *Crassostrea gigas*. *J. Gen. Virol.* 80, 1317-1322
- Le Deuff, R.-M., Renault, T. and Gérard A. (1996). Effects of temperature on herpes-like virus detection among hatchery-reared larval Pacific oyster *Crassostrea gigas*. *Dis. aqua. Org.*, 24: 149-157.
- Le Deuff, R.-M., Nicolas, J.L., Renault, T. and Cochenec, N. (1994) Experimental transmission of herpes-like virus to axenic larvae of Pacific oyster, *Crassostrea gigas*. *Bull. Eur. Ass. Fish Pathol.* 14(2), 69-72.
- Le Marrec-Croq, F., Fritayre, P., Chesne, C., Guillouzo, A. and Dorange, G. (1998). Cryopreservation of *Pecten maximus* heart cells . *Cryobiology*, 37, 200-206.

- Le Marrec-Croq, F., Glaise, D., Guguen-Guillouzo, C., Chesne, C., Guillouzo, V. and Dorange, G. (1999). Primary cultures of heart cells from the scallop *Pecten maximus* (Mollusca-Bivalvia). *In Vitro Cell. Dev. Biol.- Animal*, 35, 289-295.
- Lipart, C. and Renault T. (2002). Herpes-like virus detection in *Crassostrea gigas* spat using DIG-labelled probes. *J. Virol. Meth.* 101: 1-10.
- Meyers, T. R. (1979) A reo-like virus isolated from juvenile American oysters (*Crassostrea virginica*). *J. Gen. Virol.* 46, 203-212.
- Nicolas, J. L., Comps, M. and Cochenec, N. (1992) Herpes-like virus infecting Pacific oyster larvae, *C. gigas*. *Bull. Eur. Ass. Fish Pathol.* 12(1), 11-13.
- Renault, T. (1998). Infections herpétiques chez les invertébrés : détection de virus de type herpes chez les mollusques bivalves marins. *Virologie* 2:401-403.
- Renault, T. and Arzul, I. (2001). Herpes-like virus infections in hatchery-reared bivalve larvae in Europe: specific viral DNA detection by PCR. *J. Fish Dis.*, 24: 161-167.
- Renault, T. and Lipart, C. (1998) Diagnosis of herpes-like virus infections in oysters using molecular techniques. *EAS Special Publication* 26, 235-236.
- Renault, T., Lipart, C. and Arzul, I. (2001a). A herpes-like virus infects a non-ostreid bivalve species: virus replication in *Ruditapes philippinarum* larvae. *Dis. aqua. Org.*, 45: 1-7.
- Renault, T., Lipart, C. and Arzul, I. (2001b). A herpes-like virus infecting *Crassostrea gigas* and *Ruditapes philippinarum* larvae in France. *J. Fish Dis.*, 24: 369-376.
- Renault, T., Cochenec, N., Le Deuff, R.-M. and Chollet, B. (1994a) Herpes-like virus infecting Japanese oyster (*C. gigas*) spat. *Bull. Eur. Ass. Fish Pathol.* 14(2), 64-66.
- Renault, T., Le Deuff, R.-M., Cochenec, N. and Maffart, P. (1994b) Herpesviruses associated with mortalities among Pacific oyster, *C. gigas*, in France - Comparative study. *Rev. Med. Vet.* 145(10), 735-742.
- Renault, T., Le Deuff, R.-M., Lipart C. and Delsert C. (2000a) Development of a PCR procedure for the detection of a herpes-like virus infecting oysters in France. *J. Virol. Meth.* 88, 41-50.
- Renault, T., Le Deuff, R.-M., Chollet B., Cochenec N. and Gérard A (2000b). Concomitant herpes-like virus infections in hatchery-reared larvae and nursery-cultured spat *Crassostrea gigas* and *Ostrea edulis*. *Dis. aqua. Org.* 42(3), 173-183.