

European Commission

Contract FAIR-CT98-4334

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

« VINO »

**FIRST PERIODIC PROGRESS REPORT
4th January 1999 to 3rd January 2000**

Contactors :

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- 2. Medical Research Council (MRC), Weymouth, 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**

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Abstract

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Abstract of the Progress Report for the period

from **04-01-99** to **03-01-00**

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

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

Abstract of the Progress Report for the period

from 04-01-99 to 03-01-00

I. OBJECTIVES

The aim of the programme is to develop specific tools for diagnosing herpes-like virus infections in bivalves and to validate these reagents by using them in different European laboratories involved in shellfish epidemiological surveys. This will be done using techniques developed by the partners to characterise viruses by studying their genomes and immunologically reactive proteins, to cultivate oyster cells and vertebrate cell lines and to perform epidemiological surveys among bivalves. The programme objectives are :

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

II. DESCRIPTION OF WORK

Preparation of substantial amounts of virus DNA extracted from purified particles and production of specific polyclonal antibodies have been already done by the coordinator of the programme (Participant 1) and serve as an appropriate platform for generating specific reagents for oyster herpes-like virus detection.

An initial step involving cloning extracted virus DNA in cosmids and plasmids has been carried out. This provides cloned viral DNA fragments suitable for characterising the virus genome and preparing specific diagnostic probes. Identification of immunogenic viral proteins provides information that facilitates production of recombinant proteins and antibodies for the development of immunological diagnostic assays. A work concerning tests of oyster primary cell cultures and vertebrate cell lines is planned in order to study the ability of the virus to replicate *in vitro*. Successful culture *in vitro* will provide a means of obtaining virus particles easily and tools to perform diagnostic assays. The development of molecular tools for oyster herpes-like virus diagnosis will facilitate virus detection in infected material. For this purpose,

some PCR primers and labelled probes specific for the oyster herpes-like virus will be obtained. These reagents will be used by four European laboratories to test their usefulness in analysing a wide range of bivalve samples.

III. STATE OF PROGRESS

Viral genomic DNA

Restriction endonuclease analysis of viral DNA showed that the genome contains 29 *Bam*HI fragments. Summation of the fragment sizes indicates a total genome size of 206 kbp. Several libraries of viral DNA fragments cloned into *Escherichia coli* vectors (lambda clones, cosmids and plasmids) were prepared and characterised. All cloned materials are available to other Partners for further studies. Random fragments 600-1000 bp in length produced from viral DNA were used for sequence determination. Sequences were assembled into a database. The database currently takes the form of three large pieces of assembled sequence. Detailed analysis of the genetic content of the virus is not worthwhile until the database is finished and all errors are removed. Nevertheless, certain local analyses have been carried out in order to characterise genes of potential diagnostic interest. Genes encoding the following proteins were identified: DNA polymerase, deoxyuridine triphosphatase, ribonucleotide reductase (large subunit), an inhibitor of apoptosis (IAP), a terminase and a potential surface glycoprotein. The presence of a terminase gene is the only indication that the agent is a herpesvirus. These data raise the possibility that the oyster virus is not a herpesvirus. To tackle this question, viral capsids were isolated from infected larvae and their structure was analysed by reconstruction of electron cryomicroscopic images. The capsid morphology is characteristic of a herpesvirus. The data demonstrate that the oyster herpes-like virus is not closely related to herpesviruses with vertebrate hosts (including fish), consistent with it being a herpesvirus that has evolved with an invertebrate rather than a contaminant acquired by filter feeding. They also indicate that the oyster herpesvirus may be the first identified member of a third major domain of the herpesvirus family, in addition to the mammalian/avian/reptilian and fish/amphibian domains.

Identification of immunogenic proteins

One of the immunologically reactive clones from the screened lambda library has a region that codes for amino acids with high hydrophobic index. This sequence also contains potential N-glycosylation sites (Asn-X-Ser/Thr) and probably corresponds to a surface glycoprotein with a transmembrane anchor near the C-terminus. The sequences of two other reactive clones map to another ORF and overlap each other. The encoded protein is related to the Inhibitor of Apoptosis Protein 2 from the Norway rat and the human Inhibitor of Apoptosis Protein 1. This seems to be a potential immunogenic protein. Indeed, this protein may be produced in high amounts during herpesviral infections. Analyses of other reactive clones showed that they are probably not expressing parts of a real gene.

PCR and in situ hybridisation for virus diagnosis

The PCR protocol defined in this study produces amplification products of the expected sizes from different clinical samples presenting high mortality rates. Of the different procedures for preparing samples from oyster specimens, boiling of ground tissues was the preferred method, because it is simple and gives the most consistent results in our hands. The presence of compounds in oysters that inhibit PCR reactions is a potential problem in using ground tissues. In order to minimise inhibitory effects, a 10-fold dilution is performed after preparation of ground tissues. The primer pairs OHV3/OHV4 and OHV3/OHV114 have been chosen from

eight different primer pairs for further studies because they consistently allow detection of low amounts of viral DNA (10 fg per PCR tube). *In situ* hybridisation of 5ng/μl probe produced by PCR using the OHV3/OHV4 primer-pair to paraffin-embedded oyster (*Crassostrea gigas* and *Ostrea edulis*) sections infected with a herpes-like virus resulted in strong hybridisation to infected cells in connective tissues of different organs. The location and morphology of labelled cells correspond to the observations made by transmission electron microscopy. No background hybridisation to healthy oyster tissues was observed.

Testing culture cells for virus replication

Several assays of cultivation of herpes-like virus in oyster primary cultures have been done. A negative effect of virus on cell cultures was observed 48 hours after inoculation in heart and embryonic cell cultures. The question remains : is this observed effect cytotoxic or cytopathogenic ? In order to answer this question, samples were fixed and prepared for electron microscope to verify if the virus was introduced into the cells. The results of this observation are pending. Infected and uninfected (negative control) oyster larvae have been inoculated into two fish cell lines: one of marine origin TV-1 (derived from turbot (*Scophthalmus maximus*)) and another from a freshwater fish: EPC (from carp epithelioma), in order to detect a cytopathic effect of the herpesvirus. Blind passages were also conducted although no CPE was detected in order to allow the virus to replicate in these cells. No cytopathic effect was detected in any of the passages conducted in these two fish cell lines. In the first passage alteration of the cell monolayer were observed in wells inoculated with infected larvae but in the second passage the morphology of the cells did not change in 30 days of incubation at 15°C.

Collecting samples in 1999

The laboratories involved in epidemiological surveys among bivalves (Participants 1, 5, 6 and 7) have taken bivalve samples during 1999 in order to perform analyses to search for herpes-like virus infections using the tools developed.

IV. ACHIEVEMENTS

Cloning of viral DNA in bacterial vectors (Sub-task 1.1) has been completed by Participant 2. All cloned materials are available to other Partners. Indeed, they served Participant 1 for PCR primer and DNA probe design and Participant 3 as the lambda library for immunoscreening and identification of immunogenic viral proteins.

Obtaining specific primer pairs and probes for viral infection diagnosis by PCR and *in situ* hybridisation (Sub-task 2. 1) has been completed by Participant 1. The PCR and *in situ* hybridisation protocols defined will be used in the four laboratories (Participants 1, 5, 6 and 7) involved in epidemiological surveys among bivalves in 2000 and 2001.

V. FUTURE ACTIONS

Detailed analyses of the genetic content of the virus will be completed after the database has been completed and all errors removed. Global analyses will be carried out in order to characterise viral genes. All the amino acid sequences deduced in Sub-task 1.2 will be compared with protein databases in order to detect similarities to other organisms.

Clones identified by immunoscreening of the lambda library will be used to produce recombinant antigens. These viral fusion proteins will be analysed by SDS-PAGE and immunological techniques. After purification by chromatography, they will serve for immunisation of laboratory animals. Monoclonal antibodies will be produced using immunised mice. Polyclonal and monoclonal antibodies will be characterised by ELISA and Western blotting.

Tests on oyster primary cell cultures will be pursued in 2000 in order to investigate viral replication *in vitro*.

Molecular (PCR and *in situ* hybridisation) and immunological (antigen detection) methods will be used to diagnose herpes-like virus infection in bivalves by laboratories involved in epidemiological surveys (Participants 1, 5, 6 and 7). Bivalve samples taken in 1999 and 2000 will be used. A meeting will be scheduled in February or March 2000 at the IFREMER laboratory in La Tremblade (France) with Participants 1, 5, 6 and 7, in order to ensure common protocols are used for PCR and *in situ* hybridisation. Moreover, Participant 1 will furnish reference material (viral DNA and infected oyster histological slides as positive reference material).

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SCIENTIFIC SYNTHESIS

Intoduction

The aim of the programme is to develop specific tools for diagnosing herpes-like virus infections in bivalves and to validate these reagents by using them in different European laboratories involved in mollusc epidemiological surveys. The programme objectives are :

- 1 - Obtaining the complete oyster herpes-like virus DNA sequence with determination of the genome structure.
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- 6 - Application of developed diagnostic tools for herpes-like virus detection in oyster samples from different geographical locations.

Preparation of substantial amounts of virus DNA extracted from purified particles and production of specific polyclonal antibodies have been already done by the coordinator of the programme (Participant 1) and serve as an appropriate platform for generating specific reagents for oyster herpes-like virus detection.

An initial step involves cloning extracted virus DNA in cosmids and plasmids. This work must provide cloned viral DNA fragments suitable for characterising the virus genome and preparing specific diagnostic probes. Identification of immunogenic viral proteins will make it possible to prepare recombinant proteins and antibodies for the development of immunological diagnostic assays. Work concerning tests of oyster primary cell cultures and vertebrate cell lines is planned in order to study the ability of the virus to replicate *in vitro*. Successful culture *in vitro* will provide a means of obtaining virus particles easily and tools to perform diagnostic assays. The development of molecular tools for oyster herpes-like virus diagnosis will facilitate virus detection in infected material. For this purpose, some PCR primers and labelled probes specific for the oyster herpes-like virus will be obtained. These reagents will be used by four European laboratories to analyse a wide range of bivalve samples and to confirm the usefulness of the diagnostic tests.

Results

Viral genomic DNA

Several libraries of viral DNA fragments cloned into *Escherichia coli* vectors (lambda clones, cosmids and plasmids) were prepared and characterised. All cloned materials are available to other Partners for further studies. Random fragments 600-1000 bp in length produced from viral DNA by sonication were used for sequence determination. Sequences were assembled into a database using Staden's Pregap and Gap4 programs. The database currently takes the form of three large pieces of assembled sequence. It appears probable from the sequence data

that the viral genome contains substantial inverted repeats. Detailed analyses of the genetic content of the virus is not worthwhile until the database is finished and all errors are removed. Nevertheless, certain local analyses have been carried out in order to characterise individual genes. Genes encoding the following proteins were identified: DNA polymerase, deoxyuridine triphosphatase, ribonucleotide reductase (large subunit), an inhibitor of apoptosis (IAP), a terminase and a potential surface glycoprotein. The presence of a terminase gene is the only indication that the agent is a herpesvirus. No other herpesvirus-specific genes were identified. These data raise the possibility that the oyster virus is not a herpesvirus. To tackle this question, the structure of isolated viral capsids was analysed by reconstruction of electron cryomicroscopic images. The capsid morphology is characteristic of a herpesvirus.

Identification of immunogenic proteins

One of the immunologically reactive clones from the screened lambda library contains a region that codes for amino acids with high hydrophobic index. This sequence also contains potential N-glycosylation sites (Asn-X-Ser/Thr) and could so probably correspond to a surface glycoprotein with a transmembrane region near the C-terminus. The sequences of two other reactive clones are different from each other but from an ORF encoding a protein which is related to the Inhibitor of Apoptosis Protein 2 from the Norway rat and the human Inhibitor of Apoptosis Protein 1. This protein may be produced in high amounts during herpes-like viral infections in bivalves. The analysis of other reactive clones showed that they are probably not expressing parts of real genes.

PCR and in situ hybridisation for virus diagnosis

The defined PCR protocol and the designed primer pairs permit amplification products of expected sizes on agarose gels from viral DNA extracted from purified particles and from different clinical samples presenting high mortality rates. Of the different procedures for preparing samples from oyster specimens, boiling of ground tissues is preferred, because it is simple and it is the most consistent in our hands. The OHV3/OHV4 and OHV3/OHV114 primer pairs were selected for further studies because they allow systematic detection of low amounts of viral DNA (10 fg per PCR tube). *In situ* hybridisation of 5ng/ μ l probe produced by PCR using the OHV3/OHV4 primer-pair to paraffin-embedded oyster (*Crassostrea gigas* and *Ostrea edulis*) sections infected with a herpes-like virus yielded strong hybridisation of the probe to infected cells in connective tissues of different organs.

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Several assays of cultivation of herpes-like virus in oyster primary cultures have been done. A negative effect of virus on cell cultures was observed 48 hours after inoculation in heart and embryonic cell cultures. The question remains : is this observed effect cytotoxic or cytopathogenic ? In order to answer this question, samples were fixed and prepared for electron microscope to verify if the virus was introduced into the cells. The results of this observation are pending. Infected and uninfected (negative control) oyster larvae have been inoculated into two fish cell lines: one of marine origin TV-1 (derived from turbot (*Scophthalmus maximus*) and another from a freshwater fish: EPC (from carp epithelioma), in order to detect a cytopathic effect of the herpesvirus. Blind passages were also conducted although no CPE was detected in order to allow the virus to replicate in these cells. No cytopathic effect was detected in any of the passages conducted in these two fish cell lines. In the first passage alteration of the cell monolayer were observed in wells inoculated with infected larvae but in the second passage the morphology of the cells did not change in 30 days of incubation at 15°C.

Collecting samples in 1999

The laboratories involved in mollusc epidemiological surveys (Participants 1, 5, 6 and 7) have taken bivalve samples during 1999 in order to perform analyses to search herpes-like virus infections using the developed tools.

Discussion-Conclusion

The data demonstrate that the oyster herpes-like virus is not closely related to herpesviruses with vertebrate hosts (including fish), consistent with it being a herpesvirus that has evolved with an invertebrate rather than a contaminant acquired by filter feeding. They also indicate that the oyster herpes-like virus may be the first identified member of a third major domain of the herpesvirus family, in addition to the mammalian/avian/reptilian and fish/amphibian domains.

Cloning of viral DNA in bacterial vectors (Sub-task 1.1) has been completed by Participant 2. All cloned materials are available to other Partners. Indeed, they served during 1999 Participant 1 for PCR primer and DNA probe design and Participant 3 as the lambda library for immunoscreening and identification of immunogenic viral proteins.

Obtaining specific primer pairs and probes for viral infection diagnosis by PCR and *in situ* hybridisation (Sub-task 2. 1) has been completed by Participant 1. The PCR and *in situ* hybridisation protocols defined will be used in the four laboratories (Participants 1, 5, 6 and 7) involved in epidemiological surveys among bivalves in 2000 and 2001.

Future actions

Detailed analyses of the genetic content of the virus will be completed after the database has been completed and all errors removed. Global analyses will be carried out in order to characterise viral genes. All the amino acid sequences obtained in Sub-task 1.2 after translation in the six possible reading frames using a computer will be compared with protein databases in order to detect similarities to other organisms.

Clones selected from the lambda library by immunoscreening will be used to produce recombinant antigens. These viral fusion proteins will be analysed by SDS-PAGE and immunotechniques. After a step of purification by chromatography, they will serve for immunisation of laboratory animals. Monoclonal antibodies will be produced using immunised mice. Polyclonal and monoclonal antibodies will be characterised by ELISA and Western blotting.

Tests on oyster primary cell cultures will be pursued in 2000 in order to investigate whether viral replication occurs *in vitro*.

Molecular (PCR and *in situ* hybridisation) and immunological (use of antibodies prepared by Participant 3) methods will be used for herpes-like virus infections in laboratories involved in epidemiological surveys among bivalves (Participants 1, 5, 6 and 7). Bivalves sampled in 1999 and 2000 will be used. A meeting will be scheduled in February or March 2000 at the IFREMER laboratory in La Tremblade (France) with Participants 1, 5, 6 and 7, in order to ensure the use of common protocols and techniques for PCR and *in situ* hybridisation. Moreover, Participant 1 will furnish reference material (viral DNA and infected oyster histological slides) as positive reference material.

METHODOLOGY AND RESEARCH TASKS

Task 1 - Obtaining a complete virus genomic library and DNA sequence

Participant : 2

Duration : 24

Current status : 10 months to completion

Total estimated man-month : 20

N° of man-month devoted already to the task : 12

Objectives :

- ◆ Obtaining the complete DNA sequence of the viral genome
- ◆ Preparing a lambda expression library in order to facilitate identification of immunogenic proteins
- ◆ Determining the virus genome structure and restriction enzyme maps
- ◆ Studying the relationships of the oyster herpes-like virus with other herpesviruses
- ◆ Facilitating further studies of the genetic material in a form independent of the virus

Overview of the methodology :

Three types of clone library were produced. For the first, viral *Bam*HI fragments were inserted into a multipurpose plasmid cloning vector (pCU18) by standard methods. For the second, large quasi-random fragments generated from the viral genome were inserted into a cosmid vector derived from Supercos 1 (Stratagene). For the third, quasi-random viral DNA fragments were inserted into a lambda expression vector (Stratagene Lambda ZAP II), in order to facilitate identification of immunogenic proteins in Sub-task 2. 2.

For DNA sequencing, random fragments of viral DNA derived by sonication were inserted into a standard M13-based sequencing vector (M13mp19). The sequences of several thousands of clones were derived by standard automated methods using an Applied Biosystems 377 sequencer. The sequences were assembled into a database using Staden's software, and will finally yielding the finished sequence. The sequence will be analysed for its genetic content and relationships to other herpesviruses using appropriate software.

Deliverables :

Month 13 : First periodic progress report

- ◆ Cloning of viral DNA in plasmid, cosmid and lambda vectors
- ◆ Preparation of M13 clones and sequencing
- ◆ Analysis of the first sequences of interest

Links with other tasks :

Concomitant derivation of the complete DNA sequence will provide information and materials for use in Sub-task 2. 2.

Sub-task 1. 1. : Cloning of viral DNA in bacterial vectors

Participant : 2

Duration : 6

Current status : completed

Total estimated man-month : 6

N° of man-month devoted already to the task : 6

Objective :

Preparation of cosmid, plasmid and lambda libraries

Methodology :

- Viral DNA was digested with *Bam*HI and ligated into pCU18 digested with *Bam*HI and phosphatased. Ligated DNA was transfected into competent *Escherichia coli* DH5 α (Life Technologies), and ampicillin-resistant colonies isolated. Plasmid DNA was isolated by the alkaline lysis protocol and digested with *Bam*HI. The products were electrophoresed on agarose gels alongside marker DNAs. The gels were stained with ethidium bromide and fragments visualised under short wave UV irradiation. A library of clones representing the majority of the genome was established from the clones analysed.
- Viral DNA was digested partially with *Bam*HI or *Sau*3AI under conditions optimal for production of fragments in the 32-45 kbp size range. The fragments were dephosphorylated and ligated into a cosmid vector (a derivative of Stratagene's Supercos 1) digested with *Xba*I and *Bam*HI. Ligated DNA was packaged into phage lambda particles using a Stratagene III XL kit. Packaged particles were used to infect *E. coli* XL-1 Blue MR, and ampicillin-resistant clones isolated. Cosmid DNA from individual colonies were analysed by agarose gel electrophoresis.
- Information about the genome structure and restriction maps has been and will be produced from restriction enzyme cleavage data and Southern blot hybridization experiments obtained using the plasmid and cosmid libraries.
- Viral DNA was digested partially with *Sau*3AI to produce fragments up to 10 kbp in size. The fragments was ligated into the lambda expression vector (Lambda ZAP II) digested with *Bam*HI and dephosphorylated. Ligated DNA was packaged into phage lambda particles using a Stratagene III Gold kit. Packaged particles were used to infect *E. coli* XL-1 Blue MRF', and an expression phage library was established from the progeny.

Sub-task 1. 2. : Sequencing the virus genome

Participant : 2

Duration : 12

Current status : 3 months to completion

Total estimated man-month : 8

N° of man-month devoted already to the task : 5

Objective :

Obtaining the complete virus DNA sequence

Methodology :

- Random fragments 600-1000 bp in length were produced from viral DNA by sonication. The ends of the fragments were repaired using T4 DNA polymerase. The fragments were then be ligated to *Sma*I-cleaved and dephosphorylated M13mp19, a standard sequencing vector in very widespread use. The ligation was transfected into competent *Escherichia coli* DH5 α F' (Life Technologies) and plated onto a lawn of *E. coli* XL-1 Blue. Recombinant phages were produced in 96-well trays from the resulting clear plaques, and template DNAs prepared by treatment with 4M sodium iodide followed by ethanol precipitation. Approximately 4900 M13 clones were processed into templates for sequencing.
- The templates were sequenced using an ABI PRISM 377 DNA sequencer operating on high throughput. About 2.5×10^6 nucleotides of data were obtained, giving a redundancy of over 10 in sequencing the 2×10^5 bp genome.
- The resulting random sequences were assembled into a database using Staden's Gap4 software, and will be edited in the final stages to remove ambiguities. Residual problems will be resolved by specific experiments involving PCR of viral or cloned DNA and resequencing of problematic regions. This will lead to the completed DNA sequence.

Sub-task 1. 3. : Phylogenetic analysis of the oyster virus

Participant : 2

Duration : 6

Current status : 10 months to completion

Total estimated man-month : 6

N° of man-month devoted already to the task : 1

Objectives :

- ♦ Determination of the genetic content of the oyster herpes-like virus genome
- ♦ Phylogenetic comparison with vertebrate herpesviruses

Methodology :

- The completed DNA sequence will be analysed for genetic content using the standard suite of programs available in the Genetics Computer Group (GCG) package. These analyses will centre on the use of programs that allow identification of open reading frames potentially encoding viral proteins (e.g. Frames).
- Genes of potential diagnostic interest - for example those encoding surface glycoproteins - will be identified from their sequence properties using appropriate programs (e.g. Peplot).
- Global comparisons of predicted protein sequences with proteins encoded by other herpesviruses and other organisms will be carried out using database-searching software available in the GCG package (e.g. Fasta, Blast).
- In order to take a view on the taxonomic position of oyster herpes-like virus (as the first example of a herpesvirus that infects an invertebrate), phylogenetic comparisons will be carried out with vertebrate herpesviruses using appropriate software available from the GCG suite (e.g. Pileup) and other sources.

Task 2 - Developing tools for the diagnosis of herpes-like virus infections

Participants : 1, 3, 4 and 6

Duration : 36

Current status : 16 months to completion

Total estimated man-month : 63 (10, 34, 15 and 4)

N° of man-month devoted already to the task : 29 (5.5, 13.75, 5.5 and 4.25)

Objectives :

- ♦ Obtaining molecular tools for oyster herpes-like virus detection
- ♦ Obtaining immunological tools for oyster herpes-like virus detection
- ♦ Obtaining cellular tools for oyster herpes-like virus detection

Overview of the methodology :

A PCR method was developed and applied for virus detection in oyster samples. The design of primers will be done using virus DNA sequence data obtained in the Task 1 and a Web site (<http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi>). An *In situ* hybridization method was developed using labelled DNA probes obtained by incorporation of digoxigenin-dUTP during PCR. They were applied for virus detection on histological sections of oyster tissues fixed in Carson's and Davidson's fluids.

Immunoscreening of the expressed recombinant proteins was performed according to classical techniques. The recombinant lambda plaques were transferred onto nitrocellulose filters after induction and lysis. Proteins were screened with mouse anti-virus antibodies that are already available (Participant 1). Fixed antibodies were revealed with an appropriate peroxidase conjugated antisera. Immunization of laboratory animals, production and quality control of polyclonal antibodies will be performed following conventional methods. Monoclonal antibodies will be obtained after immunising mice by the principle of hybridoma production. Immunological methods as an immunohistochemical technique will be developed and applied for virus detection in oyster samples.

Larvae are obtained using conventional techniques of *in vitro* fecondation. Adult oysters are provided by shellfish producers. Isolation of oyster embryonic, larval cells and oyster heart cells are performed following a method described for scallop heart and gills and oyster embryos. Freezing and thawing assays of larval isolated cells were carried out according to the method adapted to marine cells. Cells are cultivated in a sea water-based medium. Infected and non infected oyster tissues are inoculated in primary oyster cell cultures and several fish cell lines following routine procedures. For transmission electron microscopy analysis, conventional techniques are used. Antibodies and DNA probes specific for herpes-like virus will be applied to cultured cells cytocentrifuged on slides.

Deliverables :

Month 13 : First periodic progress report

- ♦ Screening of the lambda expression library
- ♦ Obtaining specific primers and probes for diagnosis by PCR and *in situ* hybridisation

Links with other tasks :

Deliverables obtained in task 2 will provide information and techniques used in the task 3.

Sub-task 2. 1. : Obtaining specific primer sequences and probes for diagnosis by PCR and in situ hybridisation

Participant : 1

Duration : 6

Current status : completed

Total estimated man-month : 6

N° of man-month devoted already to the task : 5.25

Objective :

Development of PCR and *in situ* hybridisation techniques for herpes-like virus detection in oysters

Methodology :

- Design of primer pairs from the sequences of viral DNA fragments using a Web site (<http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi>) and primer selection : using sequences of cloned viral DNA fragments (Task 1), primers were designed. The primer selection are performed using virus DNA extracted from purified virions as template. PCR products were analysed using 1% agarose gel electrophoresis. Gels were supplemented with ethidium bromide (0.5 µg/ml). Ten µl of each PCR product were loaded per lane and DNA was visualised using a 302nm UV transilluminator. Sizes of the DNA products were determined relative to those of size markers.
- Determination of optimal conditions for amplification and control of specificity : PCR parameters were optimised using virus DNA extracted from purified particles as template (10fg to 100ng). Different magnesium chloride concentrations (1 to 5mM), different primer concentrations (0.1 to 2µM), different annealing temperatures and different cycle numbers (25, 30, 35 and 40 cycles) were tested. The specificity of the PCR was tested using DNA extracted from healthy oysters and different vertebrate herpesvirus DNAs as template.
- Establishment of protocols for oyster sample processing before amplification : the use of Chelex-100, proteinase K digestion associated with phenol/chloroform extraction and DNA precipitation in ethanol, and denaturation of ground oyster tissues in boiling water were tested as different techniques for sample preparation before the PCR step.
- *In situ* hybridisation protocol development : oyster samples were fixed in Carson's and in Davidson's fluids. Sections were cut 7µm thick and placed on slides pretreated with 3-aminopropyltriethoxysilane. Digoxigenin-labeled DNA probes were obtained by incorporation of digoxigenin-dUTP during a PCR step using oyster herpes-like virus DNA extracted from purified particles as template. After proteolysis with proteinase K treatment and denaturation of the target DNA and probes at 94°C, the tissue sections will be hybridised in buffer containing 1 to 5ng/µl of digoxigenin labelled probes overnight at 42°C. Immunological detection will be done using an anti-digoxigenin monoclonal antibody, a peroxidase conjugate and diaminobenzidine.

Sub-task 2. 2. : Identification of immunogenic viral proteins and preparation of recombinant proteins (3 maximum) and antibodies for diagnostic use

Participant : 3

Duration : 24

Current status : 16 months to completion

Total estimated man-month : 34

N° of man-month devoted already to the task : 13.75

Objectives :

- ♦ Identification of immunogenic virus proteins
- ♦ Preparation of recombinant proteins
- ♦ Preparation of antibodies specific for the recombinant proteins

Methodology :

The lambda library prepared in task 1 was screened using mammalian antibodies generated against virus particles in order to identify immunogenic viral proteins. Mouse polyclonal antibodies are available and were provided by Participant 1. Appropriate proteins will then be expressed via a plasmid in *Escherichia coli* in preparation for development of diagnostic methods. Production of selected recombinant proteins will provide material for positive controls included in immunological diagnostic assays. Moreover, these proteins will be used to prepare specific antigens and antibodies for diagnostic purposes.

- Immunological screening of the recombinant proteins expressed by the lambda library using polyclonal antibodies : bacteria colonies were lysed and transferred from plates to nitrocellulose membranes in appropriate buffer conditions to fix proteins. Membranes were then incubated with specific sera followed by appropriate antibodies conjugated with horse radish peroxidase. Specific antigens were revealed by colorimetric enzymatic reaction and clones expressing these antigens were selected to be amplified and stored.
- Sequencing of viral DNA fragments : plasmids were extracted from immunologically reactive clones. Sequencing reactions were performed first with primers chosen on the cloning vector. After first sequencing results analysis, new specific primers were selected to sequence all the insert. The sequencing work was performed on an ABI377-based fluorescent system using methods including cycle sequencing and engineered thermophilic polymerases specifically dedicated for high accuracy DNA sequencing. In a second step, specific oligonucleotides will be chosen at both extremities of the coding sequence, synthesized and used to amplify in a PCR reaction the DNA fragment to be cloned. Restriction sites will be included at the extremities of the chosen oligonucleotides to make the cloning easier. Amplified fragments will be digested with chosen restriction enzymes and ligated with T4 DNA ligase in appropriate expression vector. This will be chosen in function of sequencing results and protein characteristics by comparison with other well known antigen. Ligation mix will be used to transform competent *E. coli* cells by electroporation. Cells will be plated on appropriate selection medium. Selection will be made by addition of antibiotic (ampicillin usually) corresponding to the resistance gene coded by the vector (Beta-lactamase for ampicillin). Plasmids will be extracted and purified from several clones and analysed by restrictive digestion. One clone for each construct will be selected on the basis of the DNA fragments size observed after electrophoresis on agarose gel. Sequencing of the inserts will be performed to verify the absence of mutations due to PCR amplification as explained above.
- Induction of the expression of a range of recombinant viral proteins : one clone without mutation will be selected and used to be grown in Erlen Meyer following our standard

conditions to produce recombinant antigens. Induction method will depend on the promoter controlling the transcription of the antigen gene cloned in the vector. Recombinant protein molecular weights will be estimated after electrophoresis on polyacrylamide gel in denaturing conditions. Their antigenicity will be studied with specific antibodies. Proteins will be first produced in fermentor according to our standard conditions and several samples will be taken during the process to study the kinetics of production. Several fermentation parameters (nutrients, pH, oxygen,...) will be then tested to attempt to increase the yield and quality (solubility, ...) of the recombinant proteins. Production and harvesting of the different antigens will be performed according to the optimized conditions as determined before. Cells will be disrupted with a Press French or glass beads (Dynomill or Biopilot): lysate will be then centrifuged or tangential filtrated to separate soluble and insoluble fractions. If recombinant antigens are insoluble in inclusion bodies, protein denaturation must be performed with chaotropic agents (guanidin chloride, urea) before optimising refolding conditions. After solubilization if necessary, antigens will be purified by hydrophobic, size exclusion, ions exchange or affinity chromatography depending on the recombinant protein characteristics.

- Antibody production : rabbits will be injected at day 1, 14, 28 and 56 with the recombinant proteins in order to obtain antiserum. Blood sampling will be collected at day 0 (pre-immun serum), day 38 and day 66 to follow the antibody titer. Final bleeding will be achieved at day 80. Mice (minimum 5) will be injected 4 times at three weeks interval in order to develop specific monoclonal antibodies. The best responding mouse will be selected 10 days after the last injection by analysing the specific antibody titers by ELISA. The murine splenic lymphocytes of the best responding mouse will be isolated and fused with the cell line SP2/0-Ag-14 using PEG and the resulting hybridomas will be selected on HAT medium. The hybridomas will be seeded on 96-well plates in HAT medium. and supernatants will be screened against the target antigen for the presence of specific antibodies by ELISA or Western blotting. The positive colonies will be expanded and tested again for specific antibody production. Selected specific hybridomas will be cloned by limit dilution. The production of specific antibodies in clone supernatants will be checked during the procedure. Subsequently, the subclass of every monoclonal antibody will be determined by ELISA testing. In order to obtain large quantities of monoclonal antibodies, hybridomas will be expanded in T flask and the culture supernatants will be collected. Alternatively, the culture will be performed in bio-reactor using the technomouse technology which allows the continuous collection of supernatant containing the monoclonal antibody.

- Characterisation of antisera and monoclonal antibodies : the specificity of antisera and monoclonal antibodies will be analysed by ELISA or Western blotting. In ELISA, the purified antigens will be coated on 96-well microplates, the plates will then be incubated successively with various dilutions of the antibody solutions or antisera and with a peroxidase- or phosphatase alkaline-labelled antibody specific of the bound antibodies. The presence of labelled antibodies will be revealed by a colorimetric enzymatic reaction and quantified by spectrometry. A washing step is performed between each steps of the procedure. In western blotting, the antigens are separated by SDS-PAGE and transferred to nylon or nitrocellulose membranes. The membranes will then be incubated successively with the antibody solutions or antisera and with a peroxidase-labelled antibody specific of the bound antibodies. The presence of labelled antibodies will be revealed by enzymatic reaction using the 4-chloro-Naphtol as peroxidase substrate (BioRad). A washing step is performed between each steps of the procedure.

Sub-task 2. 3. : Testing oyster primary cell cultures and vertebrate cell lines

Participants : 1, 4 and 6

Duration : 21

Current status : 12 months to completion

Total estimated man-month : 23 (4, 15 and 4)

N° of man-month devoted already to the task : 10 (0.25, 5.5 and 4.25)

Objectives :

- ♦ Preparation of oyster primary cell cultures
- ♦ Cultivation of herpes-like virus in oyster primary cell cultures
- ♦ Cultivation of herpes-like virus in vertebrate cell lines

Methodology :

Techniques for culturing oyster cells are used in order to grow *in vitro* different cell types originating from *Crassostrea gigas* larvae and heart from adult Japanese oysters. The resulting primary cell cultures are used to test the ability of the virus to multiply *in vitro*. Vertebrate cell lines (fish cell lines specially) were tested.

- Obtaining gametes and *in vitro* fertilisation : for each assay, 15 to 30 oysters are well brushed and washed under flowing water, then sterilised externally before opening them. The gonad is incised with a sterile scalpel and gametes are recovered using a sterile syringe. Fertilisation is performed by mixing spermatozoa and ova in sterile sea water supplemented with antibiotics.
- Cells isolation : larvae reared in sterile sea water supplemented with antibiotics are isolated by filtration on meshes and are enzymatically treated during 12 hours at 4°C for cell dissociation. Larvae at different development stages are used in order to obtain dissociated cells.
- Cryopreservation of isolated oyster cells : different tests are performed on dissociated larval cells using quick-freezing and slow-freezing techniques and several cryoprotectors.
- Parameters for cell cultures and tests of transport conditions : the primary cell cultures are performed at 26°C using sea water supplemented with amino-acids, sugars, growth factors and antibiotics. Larval cell culture were sent to the Participant 1 using express mail to test their ability to survive transport.
- Techniques for preparing larval samples : frozen oyster larvae are used to try to infect primary cell cultures. Larvae are ground in sterile culture medium or in sterile sea water and filtered to 0.45 or 0.22µm. The virus suspensions are inoculated on cell cultures and incubated from 30 minutes to 2 hours before adding new culture medium.
- Detection of cytopathic effects and virus by transmission electron microscopy : cell incubation is done at 26°C. Primary cell cultures are observed daily during for several weeks in order to detect eventual cytopathic effects. For transmission electron microscopy, cultured cells are fixed in 3 % glutaraldehyde in 0.2 M cacodylate buffer and postfixed in 1 % cacodylate-buffered osmium tetroxide, dehydrated in ethanol and propylene oxide and embedded in Epon araldite mixture. Thin sections are double stained with saturated aqueous uranyl acetate and lead citrate and examined in a transmission electron microscope.
- Detection of cytopathic effects (fish cell lines) and virus by transmission electron microscopy: infected oyster tissues are inoculated in a variety of established fish cell lines (EPC, RTG-2, TV-1, etc) following routine procedures. For transmission electron microscopy, cultured cells are fixed in 4 % glutaraldehyde in 0.2 M cacodylate buffer

and postfixed in 1 % cacodylate-buffered osmium tetroxide, dehydrated in ethanol and propylene oxide and embedded in Epon araldite mixture. Thin sections are double stained with saturated aqueous uranyl acetate and lead citrate and examined in a transmission electron microscope.

Task 3 - Application of DNA probes, immunological reagents and cellular tools for virus detection (i. e. validation)

Participants : 1, 5, 6 and 7

Duration : 36

Current status : 24 months to completion

Total estimated man-month : 62.6 (17, 19, 17 and 9.6)

N° of man-month devoted already to the task : (0.5, 1.25, and 2.5)

Objectives :

- ♦ Detection of herpes-like virus in oysters using molecular tools
- ♦ Detection of herpes-like virus in oysters using immunological tools
- ♦ Detection of herpes-like virus in oysters using cellular tools

The molecular and immunological reagents can facilitate the development of diagnostic techniques based on molecular and immunological detection of virus. Availability of oyster primary cell cultures and of susceptible vertebrate cell lines can allow virus cultivation *in vitro* to be tested. The resulting methods will be transferred to four different laboratories and used in diagnosis of shellfish diseases to perform epidemiological surveys of oyster beds in Europe.

Overview of the methodology :

Different geographical sites are available for sampling where oysters are grown and have suffered losses. Sites that have not suffered losses are also sampled. The cooperation of shellfish farmers in the areas to be sampled is essential for operation of this project. Farmers are identify stocks that are suffering losses so that samples can be taken as soon as they occur. Samples are taken during the crucial period of June/July/August when mortalities have previously occurred, as well as November, February and May of each of the three years in France, Ireland, Spain and the United Kingdom.

In areas where both *Crassostrea gigas* and *Ostrea edulis* are cultured samples of both species are taken. 30 oysters are taken on each occasion from each site. Time restrictions may prevent examination of all oysters from all sites, but extensive sampling will ensure that, even if not looked at immediately that samples will be available for future reference. Samples are divided into two : one for histology and electron microscopy sections for use in routine diagnosis, immunological labelling and *in situ* hybridisation; the other will be stored frozen for PCR, blot hybridisation and cell culture examination.

Deliverables :

Month 13 : First periodic progress report

- ◆ Preparation of larval oyster cell primary cultures (Participant 4)
- ◆ Tests of larval oyster cells and vertebrates cell lines for herpes-like virus cultivation (Participants 1, 4 and 6)

Links with other tasks : the task 3 needs the development of specific diagnosis tools (Tasks 1 and 2).

Sub-task 3. 1. : Application of molecular methods to the diagnosis of oyster herpes-like virus

Participants : 1, 5, 6 and 7

Duration : 27

Current status : 24 months to completion

Total estimated man-month : 32.3 (8, 10.5, 8 and 5.8)

N° of man-month devoted already to the task : (0.25, 1.25, and 1.5)

Objective :

Detection of herpes-like virus in oysters using molecular tools

Methodology :

- Collecting oyster samples : for each participant, 10 batches of *oysters* (*Crassostrea gigas* and/or *Ostrea edulis*) presenting abnormal mortalities and 10 batches of healthy oysters (*Crassostrea gigas* and/or *Ostrea edulis*) are taken each year during the course of the project. For each batch, 30 individuals are sampled. The oysters are removed from the shell, sagittally sectioned, then half is frozen at - 80°C. The other half is again sagittally sectioned and one part is fixed in Davidson's fluid and the other one in Carson's fluid. Analyses using PCR and *in situ* hybridization will be performed on no more than 3 batches presenting mortalities and 3 batches of healthy oysters for each year.
- Testing of molecular tools (PCR and *in situ* hybridization) for diagnosis on samples of virus-infected and healthy oysters with different geographical provenances and at different stages of development : PCR analysis will be performed on frozen oyster samples using the primer pair OHV3/OHV114 and the PCR protocol defined in Sub-task 2.1. The products of PCR will be analysed on 1% agarose gels. The oyster samples will be fixed in Davidson's fluids prior to embedding in paraffin and cutting into 7µm sections. Tissue sections will be placed on slides pretreated with 3-aminopropyltriethoxysilane. Digoxigenin-labelled DNA obtained by incorporation of digoxigenin-dUTP during a PCR step using the OVH3/OHV4 primer pair will be used as specific probes. The immunological detection will be done using an anti-digoxigenin monoclonal antibody and diaminobenzidine.
- Comparison of PCR and *in situ* hybridization with histological and/or transmission electron microscopy for detection of oyster herpes-like virus infections : samples fixed in Davidson's fixative will be used for histological examination. Samples will be embedded in paraffin, cut at 3 µm thickness and stained with hematoxylin and eosin. Samples placed in Carson's fixative will be held for transmission electron microscopy examination. After several rinses in cacodylate buffer, pieces of tissues placed in Carson's fluid will be fixed in 3 % glutaraldehyde in 0.2 M cacodylate buffer and postfixed in 1 % cacodylate-buffered osmium tetroxide, dehydrated in ethanol and propylene oxide and embedded in Epon araldite mixture. Thin sections will be double

stained with saturated aqueous uranyl acetate and lead citrate and examined in a transmission electron microscope.

- Sequencing PCR products from positive samples in order to study possible virus diversity (Participant 6): some PCR products corresponding to positive samples from different geographical origins will be sequenced using an ABI PRISM 377 DNA sequencer operating on high throughput. PCR products will be furnished by Participant 1, Participant 5, Participant 6 and Participant 7.

Sub-task 3. 2. : Application of immunological methods to the diagnosis of oyster herpes-like virus

Participants : 1, 5, 6 and 7

Duration : 27

Current status : 24 months to completion

Total estimated man-month : 24.3 (6, 8.5, 6 and 3.8)

N° of man-month devoted already to the task : 0 (0, 0, 0 and 0)

Objective :

Detection of herpes-like virus in oysters using immunological tools

Methodology :

- Collecting oyster samples (Sub-task 3. 1)
- Validation of immunological methods in virus detection using different samples of virus-infected and healthy oysters with different geographical origins and at different stages of development : oyster samples fixed in Carson's or in Davidson's fluids will be used to prepare paraffin-embedded sections (7µm). Tissue sections will be placed on slides pretreated with poly-D-lysine. Polyclonal and monoclonal antibodies specific for viral recombinant proteins will be used as immunological probes. The detection will be done using a peroxidase conjugate and diaminobenzidine.
- Comparison of immunological detection of virus with DNA probe methods : the results obtained by PCR, in situ hybridisation and immunological labelling will be compared for the same individuals.

Sub-task 3. 3. : Application of primary oyster cell cultures and vertebrate cell lines to the diagnosis of oyster herpes-like virus

Participants : 1 and 6

Duration : 6

Current status : 12 months to completion

Total estimated man-month : 6 (3 and 3)

N° of man-month devoted already to the task : 1.25 (0.25 and 1)

Objective :

Detection of herpes-like virus in oysters using cellular tools

Methodology :

- Collecting oyster samples (Sub-task 3. 1)
- Testing of primary oyster cell cultures and some vertebrate cell lines for diagnosis on samples of virus-infected and healthy oysters with different geographical origins and at different stages of development : frozen oyster samples will be used to infect primary cell cultures. Oyster tissues will be ground in sterile culture medium or in sterile sea

water and filtered to 0.45 or 0.22µm. Cell cultures will be inoculated with virus suspensions and incubated from 30 minutes to 2 hours before adding new culture medium. Primary cell cultures will be observed daily during several days in order to detect eventual cytopathic effects.

- Comparison of virus detection on cell cultures with molecular and immunological tools : the results obtained by PCR, *in situ* hybridisation, immunological labelling and cell cultures will be compared for the same individuals.

Task 4 - General organisation of the programme and synthesis of results

Participants : : 1, 2, 3, 4, 5, 6 and 7

Duration : 36

Current status : 24 months to completion

Total estimated man-month : : 8 (2, 1,1,1,1,1 and 1)

N° of man-month devoted already to the task : 2.3 (0.5, 0.3, 0.3, 0.3, 0.3, 0.3 and 0.3)

Objectives :

- ♦ General organisation of the programme
- ♦ Preparation and discussion of protocols
- ♦ Coordination of tasks between the different participants
- ♦ Presentation and analysis of results of each participant
- ♦ Production of intermediate and final reports
- ♦ Publishing in international scientific journals and participation in national and international meetings for proper dissemination of information
- ♦ Financial management of the project

Overview of the methodology :

A meeting of all participants was scheduled during the first month (January 1999) after the contract was signed in order to adjust the calendar and the content of the different tasks in the light of new scientific or technical developments arising after the proposal of the project. Additionally, three annual meetings are scheduled to take place at the end of each year for the two first years and two months before the end of the contract for the last one (final evaluation of the project). The last meeting of the project will allow the participants to communicate final information and to draw general conclusions to be included in the final report..

Links with other tasks :

Link with tasks 1, 2 and 3 between all participants

MILESTONES

Success of the project is based on availability of one pool of virus DNA extracted from different batches of infected larval *Crassostrea gigas* (Participant 1). There is no cell culture for oyster herpes-like viruses, but progress can nonetheless be made using virus DNA obtained from infected larvae.

1. Cloning of oyster herpes-like virus DNA in bacterial vectors : the results gave rise to information on virus DNA sequences. Generation of a lambda library permitted the identification of immunogenic proteins in order to carry out Sub-task 2. 2.
2. Sequencing of cloned virus DNA and determination of first sequences : the results permitted to determine the sequences of interest for herpes-like virus diagnosis using molecular tools (Sub-task 2. 1).
3. Developing useful molecular techniques (PCR, *in situ* hybridisation) for oyster herpes-like virus detection : the methodology is now fully developed. The development of sensitive and specific techniques has been obtained and is necessary to perform Sub-task 3. 1.
4. Obtaining recombinant proteins and specific antibodies : these materials are necessary to perform Sub-task 3. 2. Immunologically reactive clones are now identified in the lambda library
5. Obtaining oyster herpes-like virus multiplication on oyster primary cell cultures and/or on vertebrate cell lines : considering the general host cell specificity of herpesviruses, it may be that the virus can be cultured only on oyster cells. The results obtained concerning Sub-task 2. 3. will determine the feasibility of Sub-task 3. 3.
6. Collecting oyster samples : application of molecular probes, immunological reagents and cellular tools for oyster herpes-like virus detection will be possible only if mortalities occur among bivalves during the course of the programme. Indeed, if no mortality is observed, validation of the diagnostic reagents will be difficult. However, massive mortalities have been reported among oysters for several years in different geographical locations, and collection of samples over three years will minimise the risk. Moreover positive samples are available in the laboratory from La Tremblade (France, Participant 1) and can serve as reference materials for other laboratories and as material for comparative studies.

DELIVERABLES

Deliverables :

First periodic progress report (Month 13, January 2000)

Task 1 - Obtaining a complete virus genomic library and DNA sequences (Participant 2)

- ◆ Cloning of viral DNA in plasmid, cosmid and lambda vectors
- ◆ Preparation of M13 clones and sequencing
- ◆ Analysis of the first sequences of interest

Task 2 - Developing tools for the diagnosis of herpes-like virus infection (Participant 3)

- ◆ Screening of the lambda expression library
- ◆ Obtaining specific primers and probes for diagnosis by PCR and *in situ* hybridisation

Task 3 - Application of DNA probes, immunological reagents and cellular tools for virus detection

- ◆ Preparation of larval oyster cell primary cultures (Participant 4)
- ◆ Tests of larval oyster cells and vertebrates cell lines for herpes-like virus cultivation (Participants 1, 4 and 6)

Second annual progress report

This report will describe results concerning :

- ◆ Expression of recombinant proteins after the screening of the lambda expression library and subcloning of selected virus DNA sequences
- ◆ Production of recombinant proteins and specific antibodies and development of immunological tests
- ◆ Phylogenetic analysis of the herpes-like oyster virus.

Third annual report (final report)

This report will describe results concerning :

- ◆ Use of molecular (PCR and *in situ* hybridisation), immunological and cellular techniques in diagnosing herpes-like virus infections
- ◆ Comparison of results obtained by histology, PCR, *in situ* hybridisation, immunological tests and cellular assay.

DISSEMINATION OF RESEARCH RESULTS

IFREMER, MRC, PHAROS S. A., UBO, University College of Cork, CSIC and CEFAS reserve the right to protect and commercially exploit any innovation generated by their researches, but no restriction will be placed on the diffusion of the scientific results aside from those proposed by the EU.

Cultures of scallop, heart and other tissue (originating from marine invertebrates) cells, adapted culture media and protocols for cryopreservation of marine invertebrate cells are protected by a patent : FR n° 95 06921, entitled « Procédés de culture de cellules d'invertébrés marins et cultures obtenues ». Results generated by the present research programme will be protected for commercial exploitation by the existing patent.

Publishing in international scientific journals and participation in national and international meetings will be used to ensure proper dissemination of information as it is generated. In addition, participation in scientific and coordinating meetings in the EU and the submission of work reports every six months will ensure that all member states are aware of the results obtained. Publication in professional journals will ensure that information reaches shellfish farmers.

In 1999, two oral presentations concerning the development of PCR primers and procedure have been performed at the 9th European Association of Fish Pathologists International Conference by Dr. I. Arzul (Participant 1) in Rhodes (19-24 September 1999). Publications concerning herpes-like virus genome, PCR and *in situ* hybridisation procedures for virus detection and obtention of larval cell cultures will be made as soon as possible.

European Commission

Contract FAIR-CT98-4334

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

« VINO »

Individual Progress Report

**Reporting Period :
4th January 1999 to 3rd January 2000**

Participant no. 1 (coordinator)

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European Commission

Contract FAIR-CT98-4334

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

« VINO »

Individual Progress Report

**Reporting Period :
4th January 1999 to 3rd January 2000**

Participant no. 1 (coordinator)

Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER)

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FAIR-CT98-4334

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

Individual Progress Report for the period

from 04-01-99 to 03-01-00

Type of contact: Shared-cost research project

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

Participant no. 1 total cost: 233,380 ECU *EC contribution to partner no. 1:* 76,780 ECU (33%)

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

Completion date: 03-01-02

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INDIVIDUAL PROGRESS REPORT

Participant no. 1 : Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER), Laboratoire de Génétique et Pathologie (La Tremblade), France

Scientific team : Dr. Tristan RENAULT
Dr. Isabelle ARZUL (PhD student, Doctor in veterinary medicine)

OBJECTIVES

- ♦ Furnishing oyster herpes-like virus DNA extracted from purified particles and specific polyclonal antibodies
- ♦ Obtaining molecular tools for oyster herpes-like virus detection
- ♦ Obtaining cellular tools for oyster herpes-like virus detection
- ♦ Use of diagnosis tools for herpes-like virus detection in oyster samples

ACTIONS IN THE PROJECT

Task 2 - Developing tools for the diagnosis of herpes-like virus infections

Sub-task 2. 1. : Obtaining tools for PCR and in situ hybridization diagnosis

Sub-task 2. 3. : Testing oyster primary cell cultures and vertebrate cell lines, carrying out infection tests on oyster primary cell cultures and controls (co-ordination of this sub-task by Participant 1, collaboration with Participant 4 and Participant 6)

Task 3 - Application of DNA probes, immunological reagents and cellular tools for virus detection (i. e. validation)

Sub-task 3. 1. : Application of molecular methods to the diagnosis of oyster herpes-like virus, carrying out analysis of oyster samples originating from France

Sub-task 3. 2. : Application of immunological methods to the diagnosis of oyster herpes-like virus, carrying out analysis of oyster samples originating from France

Sub-task 3. 3. : Application of primary oyster cell cultures and vertebrate cell lines to the diagnosis of oyster herpes-like virus, carrying out analysis of oyster samples originating from France

Task 4 - General organisation of the programme and synthesis of results

Coordination of this task. Organisation and participation in annual meetings. Participation and synthesis in writing intermediate and final reports

PLANNED RESEARCH ACTIVITIES

Task	Year 1	Year 2	Year 3
2. 1	Technique development/ P. R. ^a	Technique development and application/P. R.	F. R. ^b
2. 3		Technique development and application/P. R.	F. R.
3. 1	1 st sample collection/P. R.	2 nd sample collection/Laboratory analysis/P. R.	3 rd sample collection/Laboratory analysis /F. R.
3. 2	1 st sample collection/P. R.	2 nd sample collection/Laboratory analysis/P. R.	3 rd sample collection/Laboratory analysis /F. R.
3. 3	1 st sample collection/P. R.	2 nd sample collection/Laboratory analysis/P. R.	3 rd sample collection/Laboratory analysis /F. R.
4	Coordination 2 meetings with all participants	Coordination 1 meeting with all participants	Coordination 1 meeting with all participants

^aP. R. : Periodic Progress Report; ^bF. R. : Final Report

RESEARCH ACTIVITIES DURING THE REPORTING PERIOD

Task 2 - Developing tools for the diagnosis of herpes-like virus infections

Sub-task 2. 1 - Obtaining tools for PCR and in situ hybridisation diagnosis

Two sequences of cloned viral DNA provided by Partner 2 were chosen for the design of PCR primer pairs : a sequence without significant homology with sequences in data banks and an other one corresponding to a gene coding for a protein presenting homologies with an Inhibitor Apoptosis Protein (IAP).

- a) Obtaining primer pairs and PCR conditions. Primer pairs were designed using the Web site <http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi>. They were tested on viral DNA extracted from virions as template. Primers were supplied by Eurogentec (Belgium). Melting temperature of the primers were calculated using the nearest neighbor method. Thermal cycling was performed using a Crocodile III thermal cycler (Appligene Oncor, France). Fifty µl PCR reactions were performed using the Goldstar Polymerase according to manufacturer's (Eurogentec, Belgium) recommendations, each containing the appropriate reaction buffer provided by the DNA polymerase manufacturer, 0.05 mM of each dNTP, 100 ng of each primer, 2.5 mM MgCl₂, and 2.5 units of DNA polymerase. Various amounts of template DNA were added under a volume of 1 µl. After heating the samples for 2 min at 94°C, 35 cycles were performed followed by a final elongation step of 5 min at 72°C. Each of the 35 cycles consisted of a DNA melting step at 94°C for 1 min, a primer annealing step for 1 min at 50°C and a primer elongation step at 72°C for 1

min. Ten μl of each PCR products was size-selected on 1% agarose gels, stained with ethidium bromide (0.5 $\mu\text{g}/\text{ml}$) and visualized using a 302 nm UV transilluminator. The gel was photographed. Sizes of the DNA products were determined relative to those of size markers (Eurogentec, Marker 1 or Marker 2). Relative amounts of amplification products within the same gel were made by comparing bands intensities on photographs. Due to the high sensitivity of PCR, precautions were adopted. All steps, including sample and reaction preparation and PCR amplification were carried out in separate rooms. To limit contamination, all pipetting procedures were performed using filter tips.

Twelve primers were designed and tested (OHV1, OHV2, OHV3, OHV4, OHV5, OHV6, OHV113, OHV114, IAP1, IAP2, IAP3 and IAP4). OHV1 to OHV114 derived from the sequence presenting no obvious homology in data banks and IAP1 to IAP4 were obtained from the sequence corresponding to the gene coding for a protein presenting homologies with an IAP. Eight primer pairs were analysed on viral DNA: OHV1/OHV2, OHV3/OHV4, OHV5/OHV6, OHV113/OHV114, OHV3/OHV114, OHV113/OHV4, IAP1/IAP2 and IAP3/IAP4. All the PCR primers tested gave the expected bands (Annex, Figure 1).

The OHV3/OHV4 and OHV3/OHV114 primer pairs were selected for further studies by Participant 1 and for PCR analyses in the laboratories of Participants 1, 5, 6 and 7 during 2000 because they allow a systematic detection of low amounts of viral DNA (10 to 20 fg per PCR tube, Annex, Figure 2).

- b) Optimal conditions for PCR and specificity controls. Meanwhile no amplification was observed for all primer pairs tested when genomic DNA of *Crassostrea gigas* was used as template or when virus DNA or DNA polymerase was omitted indicating that the amplicons produced were the results of template-dependent DNA amplification and that PCR reagents were not contaminated with virus DNA. In addition, PCR reactions performed on the DNA from various herpesviruses infecting human or lower vertebrates did not amplify DNA fragments indicating that the nested PCR reaction is specific of the herpes-like virus infecting *C. gigas*.

Reaction parameters were optimised using virus DNA extracted from purified particles (10 fg to 10 ng). Magnesium chloride concentrations appeared to be critical, depending on the amount of viral DNA used in the PCR reaction. A 2.5 mM concentration of MgCl_2 allowed production of visible amounts of amplicons when 1 pg of virus DNA was used. Primer concentrations appeared also to be critical, depending on the amount of viral DNA used in PCR reactions. PCR reactions provided visible amplicons on agarose gel for 50, 100, 200 and 300 ng of primers when 1 pg of virus DNA was used as template, but the reaction failed to produce visible amplicons for 400 and 500 ng of primers. Although thermal cycling was routinely performed using 50°C as annealing temperature in PCR reactions, 45°C and 55°C also yielded specific products. Amplicon yield increased as the DNA polymerase concentration increased from 0.01 to 0.05 unit/ μl . Increasing the DNA polymerase concentration above 0.05 unit/ μl resulted in inhibitory effects. The yield of PCR products was partly dependent on the amount of template DNA in the reaction. Using DNA extracted from purified virus particles, the minimum quantity of template needed to produce sufficient amounts of DNA for visualization was 0.1 to 1 pg for the second PCR reaction. The yield of the fragment of the expected size increased when increasing the amount of template and it reached a maximum when 100 pg of virus DNA was used. When more than 1 ng of template virus DNA was used, visible non-specific

amplification products were detected. Cycle number did not appear to be critical. All combinations (30, 35 and 40 cycles) produced visible amounts of PCR products.

The effect of inhibitors contained in animal tissues on the PCR reaction was investigated in mixing experiments. PCR reactions were performed using different amounts of virus DNA extracted from purified particles (1 ng to 10 fg) as template, mixed with increasing amounts of non-infected spat tissues (0.01, 0.05, 0.1, 0.5, 1, 2 and 6 mg per tube). Spat tissues were prepared by grinding oysters controlled as negative by TEM for virus detection (6g of tissues in 1ml of double distilled water) in an Eppendorf tube with a single-use tissue homogeniser. Virus DNA yielded a detectable amplification product of the expected size when PCR was performed on 1 ng to 10 fg in absence of oyster tissues. Increasing the amount of non-infected spat tissues gradually inhibited the PCR reaction ; this inhibitory effect depended on the amount of virus DNA introduced in PCR tubes. The specific reaction product was observed when PCR reactions were performed in the presence of 6 or 2 mg of non-infected spat sample on 1 ng of virus DNA but not on 10 pg of virus DNA. Thus, the inhibitory effect can be partially overcome by increasing the amount of virus DNA introduced in PCR tubes. Noninfected spat tissues (6, 2, 1, 0.5, 0.1, 0.05 and 0.01 mg), in absence of viral DNA, failed to produce amplicons. Thus, in order to optimize the reaction conditions, we routinely ten-fold diluted the oyster tissues (1 g/ml in distilled water) immediately after grinding, so that the amount of tissue added to each PCR reaction was 0.1 mg. This dilution procedure gave more positive results (66,7% versus 40%, n=15) than weighing a lesser amount (0.1g) of tissue without further dilution.

The procedure used to prepare each sample prior to PCR is known to be critical for the success of the amplification reaction. We tested alternative techniques such as the use of Chelex-100, a resin used to prepare crude samples for PCR, and the combination of Proteinase K digestion, phenol/chloroform extraction and ethanol precipitation. Both techniques yielded false negative results. In our system the protocol that constantly provided the best detection, for larvae and as well as for spat, was to grind animals in double distilled water (1 g/ml), and to denature the samples in a boiling water bath for 10 minutes, followed by a quick chill on ice. Samples were then centrifuged at 10,000 rpm for 5 minutes and the supernatant was recovered and immediately diluted ten fold in double distilled water and kept frozen at -20°C for extended periods of time. This protocol allowed detection of amplification from different clinical samples presenting high mortality rates.

- c) Oyster sample processing for PCR. Oyster (*Crassostrea gigas* and *Ostrea edulis*) larvae and spat obtained from hatcheries and shellfish farmers were investigated by PCR. Samples were collected and stocked frozen (-20°C). 50 mg of frozen larvae were weighed out in an Eppendorf microtube and ground in 50 µl of distilled water using a single-use tissue homogeniser. 30 individuals (six pools of five animals) were analyzed for each batch. Animals were removed from the shell and dried on paper. The technique used to grind spat samples depended on the animal size. For spat up to 12 mm in shell size, groups of five animals were weighed out in 1.5 ml Eppendorf microtubes and double distilled water was added at 1 ml/g of tissue. Animals were ground using a single-use tissue homogeniser. For spat greater than 12 mm in shell size, five individuals were ground with a rubber mallet in plastic bags without adding double distilled water. After cutting a corner of the bag, 0.5 g of ground tissue was recovered into a 1.5 ml Eppendorf tube and 0.5 ml of double distilled water was added. Ground larval and spat samples were

vortexed and denatured in a boiling water bath for ten minutes, followed by quick chilling in ice. Samples were mixed again and centrifuged at 10 000 rpm for five minutes. Supernatants were recovered and immediately diluted 10 fold in double distilled water and frozen at -20°C.

- d) *In situ* hybridisation assays. For *in situ* hybridisation, probes were produced by PCR using virus DNA as template, the primer-pair OHV3/OHV4 and digoxigenin-11-dUTP (Boehringer Mannheim). Oyster samples preserved in Davidson's fixative were embedded in paraffin. Sections were cut 7µm thick and placed on silane-prepTM slides (Sigma Aldrich). After dewaxing and rehydration, tissues were treated with proteinase K (100µg/ml in distilled water) at 37°C for 30 minutes. After dehydration, the tissue sections were prehybridized in 500µl of 4x SSC, 50% formamide, 1x Denhardt's solution, 0.25mg/ml yeast tRNA and 10% dextran for 30min at 42°C. The solution was replaced with prehybridation buffer containing 5ng of digoxignin-labelled probes. After denaturation of target DNA and probes (95°C, 5min.) sections allowed to hybridise overnight. The sections were washed for 10min in 1x SSC at 42°C. Then, they were blocked with phosphate buffer containing 6% of powdered milk for 1hour. Sections were incubated for 1hour at room temperature with a antidigoxigenin-monoclonal antibody (Boehringer Mannheim, 1:300 in phosphate buffer). Unbound antibodies were removed with six 5 minute washes. Anti-mouse-peroxydase conjugate (Sanofi Diagnostics Pasteur) was diluted 1:400 in phosphate buffer and slides were incubated at room temperature for 1hour. After six 5 minute washes, tissue sections were incubated in color development solution (diaminobenzidine/H₂O₂. phosphate buffer). *In situ* hybridisation of 5ng/µl probe produced by PCR using OHV3/OHV4 primer-pair to paraffin-embedded oyster (*Crassostrea gigas* and *Ostrea edulis*) sections infected with a herpes-like virus yielded strong hybridisation of the probe to infected cells in connective tissues of different organs (Annex, Figures 3 and 4). The location and the morphology of labelled cells correspond to the observations made by transmission electron microscopy. No background hybridisation to healthy oyster tissues was observed.

Sub-task 2.1 has been completed. The progress of this sub-task is in advance of that anticipated. The work was carried out by Drs. T. Renault and I. Arzul.

The PCR protocol defined in this study permit to observe amplification products of the expected sizes on agarose gels from different clinical samples presenting high mortality rates. Of the different procedures of sample preparation from oyster specimens, boiling of ground tissues was the preferred method, because it was simple and it was the most consistent in our hands. The presence of compounds in oyster that inhibit PCR reactions is a potential problem in using ground tissues. In order to minimise inhibitory effects, a 10 fold dilution is performed after preparation of ground tissues. 0.1 mg of oyster tissues is added in each PCR tube permitting the detection of virus DNA, but avoiding inhibition of PCR amplification. *In situ* hybridisation of 5ng/µl probe produced by PCR using OHV3/OHV4 primer-pair to paraffin-embedded oyster (*Crassostrea gigas* and *Ostrea edulis*) sections infected with a herpes-like virus yielded strong hybridisation of the probe to infected cells in connective tissues of different organs. This nucleic probe should be very useful as a diagnostic tool for herpes-like virus infections. Indeed, the *in situ* hybridisation technique can be used to confirm the suspicion of the presence of viruses obtained using histological examinations.

Sub-task 2.3 - Testing oyster primary cell cultures and vertebrates cell lines

Carrying out infection tests on oyster primary cell cultures and controls

We have made two assays of cell contamination by herpes-like virus using heart cells sent by Participant 4. We have studied the cytopathogenesis of herpes-like virus (inverted microscope). Samples were also fixed for electron microscopy and cytocentrifugated for *in situ* hybridisation assays (they have to be analysed in 2000). We have observed a negative effect of virus on cell cultures for lower dilutions.

The progress of this sub-task is as anticipated. The work was carried out by Dr. T. Renault and Mr. S. Deniau (non appointed student).

Task 3 - Application of DNA probes, immunological reagents and cellular tools for virus detection (i. e. validation)

- a) Sites and bivalve species. The aim of this task is to validate the technology to detect herpes-like virus in oyster larvae, spat and adults. Moreover, since mortalities were detected in France in different bivalve species in some hatcheries at the larval stage (Annex, Tables 1 and 2), we decided to sample larvae to identify the possible cause of the mortalities trying to determine if the herpes-like virus was present in the diseased bivalves. 26 larval batches have been taken in two different hatcheries. Moreover, one batch of *Crassostrea gigas* spat (six-month old) presenting high mortality rates and one batch of healthy animals of the same species at the same age were taken at the IFREMER nursery in La Tremblade.
- b) Oyster samples. The individuals of each spat batch were removed from the shell, sagittally sectioned, then half of the animal was frozen at -20°C and the other half was again sectioned and fixed in Davidson and in Carson. Larval batches were divided in two parts: one part was frozen for molecular studies, the other part was fixed directly in glutaraldehyde for electron microscopy.
- c) PCR analyses. Samples were taken and frozen for their PCR analysis. PCR analysis permitted the detection of viral DNA on several batches of oyster larvae (Annex, Table 3). Moreover, two batches (*Crassostrea gigas*) were analysed individually. 25 animals were found infected on 30 oysters for the batch presenting high mortality rates. No positive result was observed for the batch of healthy spat. *In situ* hybridisation is now being performed on these animals.

The progress of this sub-task is in advance of that anticipated because some comparative analyses are now being performed on the spat samples (PCR and *in situ* hybridisation). The work was carried out by Drs. T. Renault and I. Arzul.

Task 4 - General organisation of the programme and synthesis of results

Organisation and participation in annual meetings, participation in writing intermediate and final reports

Dr. T. Renault organised two meetings in 1999 (January and December) at the IFREMER building in Issy les Moulineaux near Paris. Drs. T. Renault and I. Arzul attended and presented results at two annual meetings held at the IFREMER building in Issy les Moulineaux near Paris. Dr. T. Renault also wrote this intermediate report.

SIGNIFICANT DIFFICULTIES OR DELAYS

None have occurred so far.

ANNEX

Table 1 : PCR analysis results for larval samples (*Crassostrea gigas* and *Ruditapes decussatus*) originating from Hatchery 1 - 1999

Batch	Species	Spawning date	Mortality	Sampling age	PCR results (OVH3/OHV4)
99-1b	<i>C. gigas</i>		No	10 days	-
99-4 G	<i>C. gigas</i>	16/02/99	No	10 days	-
99-5 G	<i>C. gigas</i>	01/03/99	No	10 days	-
99-6 G	<i>C. gigas</i>	16/03/99	No	10 days	-
99-7 G	<i>C. gigas</i>	30/03/99	No	10 days	-
99-8 G	<i>C. gigas</i>	13/04/99	No	10 days	-
99-10 G	<i>C. gigas</i>	27/04/99	No	10 days	-
99-11 G	<i>C. gigas</i>	11/05/99	No	10 days	-
99-12 G 180T	<i>C. gigas</i>	25/05/99	No	10 days	-
99-12 G 180 HT	<i>C. gigas</i>	25/05/99	No	10 days	-
99-12 G 180	<i>C. gigas</i>	25/05/99	No	10 days	-
99-12 G 180 H	<i>C. gigas</i>	25/05/99	No	10 days	-
99-13 G	<i>C. gigas</i>	08/06/99	No	10 days	-
99-15 G	<i>C. gigas</i>	10/08/99	Mortality at Day 3	8 days	+
99-1 PE	<i>R. decussatus</i>	22/02/99		9 days	-
99-3 PE	<i>R. decussatus</i>	07/06/99		8 days	-

Table 2 : PCR analysis results for larval oysters (*Crassostrea gigas* and *Ostrea edulis*) samples originating from Hatchery 2 - 1999

Batch	Species	Spawning date	Mortality	Sampling age Comments	PCR results (OVH3/OHV4)	PCR results (OHV3/OHV114)
P1	<i>C. gigas</i>	15/01/99		10 days / 60 µm / Swimming larvae	+	+
P1	<i>C. gigas</i>	15/01/99		10 days / Bottom	+	+
P2	<i>C. gigas</i>	19/01/99		6 days / 40 µm / Swimming larvae	-	-
P2	<i>C. gigas</i>	19/01/99		6 days / 40 µm / Bottom	-	-
P2	<i>C. gigas</i>	19/01/99		7 days / 60 µm / Swimming larvae	-	-
P3	<i>C. gigas</i>	?		Bottom	-	+
P13	<i>C. gigas</i>	23/06/99		5 days / 60 µm / Swimming larvae	ND	+
P13	<i>C. gigas</i>	23/06/99	Mortality 80 %	5 days / 60 µm / Bottom	ND	-
	<i>O. edulis</i>	27/06/99	No		ND	+
	<i>O. edulis</i>	27/06/99	No		ND	+

Table 3 : PCR analysis results - 1999

1999	Number of sample tubes	PCR results	
		Positive	Negative
<i>Crassostrea gigas</i>	22	5	17
<i>Ostrea edulis</i>	2	2	0
<i>Ruditapes decussatus</i>	2	0	2

Figure 1 : primer pair assays (OHV1/OHV2, OHV3/OHV4 and OHV5/OHV6) on viral DNA extracted from purified particles. Lanes 1,2 and 3 : 10 ng of viral DNA per PCR tube. Lanes 4,5 and 6 : 500 pg of viral DNA per PCR tube. OHV1/OHV2 : lanes 1 and 4, OHV3/OHV4 : lanes 2 and 5 and OHV5/OHV6 : lanes 3 and 6.

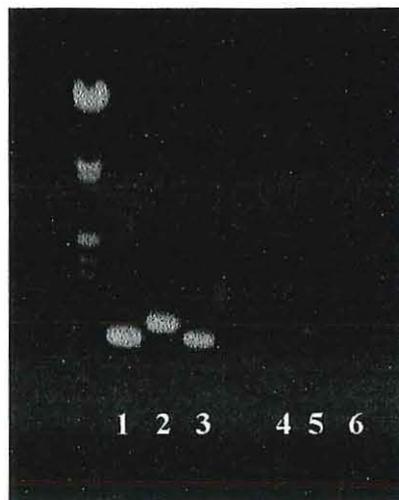


Figure 2 : detection of different amounts of viral DNA using the primer pair OHV3/OHV4. Lanes 1 to 5 : 1 fg, lanes 6 to 10 : 5 fg, lanes 11 to 15 : 10 fg, lanes 16 to 20 : 20 fg, lanes 21 to 25 : 100 fg, lanes 26 and 27 : 500 fg and lane 27 : 10 ng.

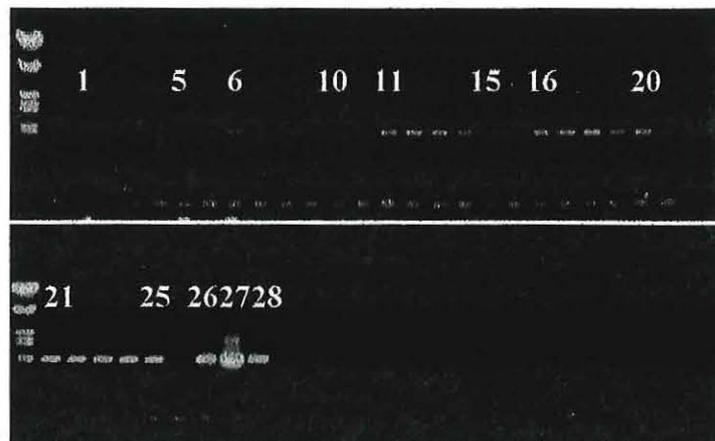


Figure 3 : *Crassostrea gigas* spat infected by the herpes-like virus. *In situ* hybridisation on a histological section of the digestive gland. Labelled cells are observed in the connective tissue of the organ (arrow). Counterstaining with Unna blue.

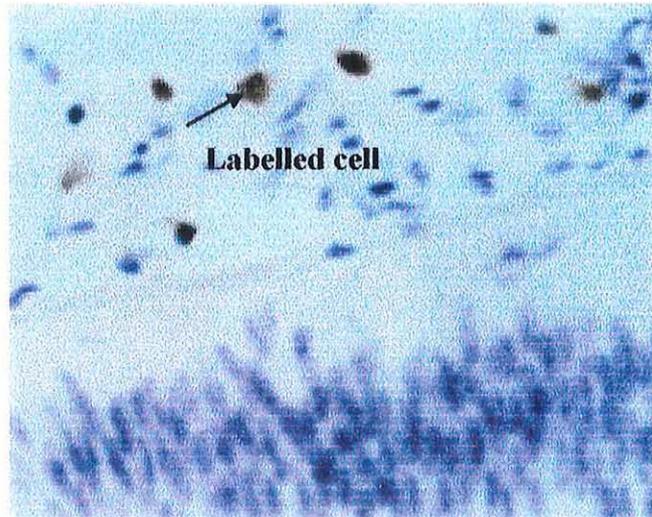
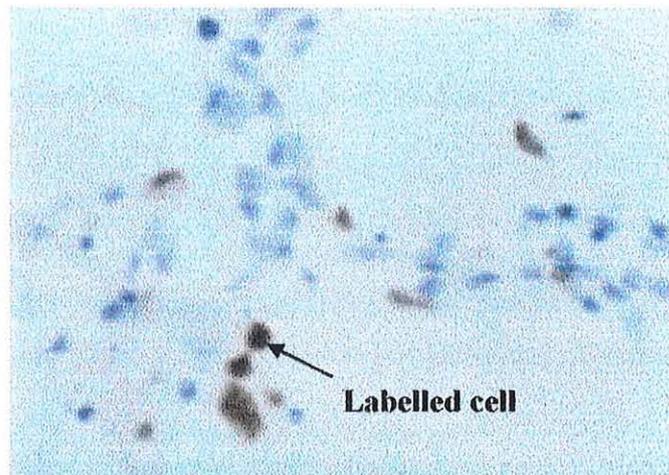


Figure 4 : *Crassostrea gigas* spat infected by the herpes-like virus. *In situ* hybridisation on a histological section of the mantle. Labelled cells are observed in the connective tissue of the organ (arrow). Counterstaining with Unna blue.



European Commission

Contract FAIR-CT98-4334

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

« VINO »

Individual Progress Report

**Reporting Period :
4th January 1999 to 3rd January 2000**

Participant no. 2

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FAIR-CT98-4334

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

Individual Progress Report for the period

from **04-01-99** to **03-01-00**

Type of contact: Shared-cost research project

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

Participant no. 2 total cost: 151,560 ECU **EC contribution to partner no. 2:** 75,780 ECU (50%)

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

Completion date: 03-01-02

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INDIVIDUAL PROGRESS REPORT

Participant no. 2: Medical Research Council Virology Unit, United Kingdom

Scientific team: Dr. Andrew J. DAVISON
Ms. Moira S. WATSON
Mr. Charles CUNNINGHAM

OBJECTIVES

- ♦ Obtaining the complete virus DNA sequence
- ♦ Preparation of a lambda library in order to facilitate identification of immunogenic proteins
- ♦ Determination of the virus genome structure and complete restriction enzyme maps
- ♦ Studying virus taxonomy
- ♦ Facilitation of further studies of the genetic material in a form independent of the virus

ACTIONS IN THE PROJECT

Task 1 - Obtaining a complete virus genomic library and DNA sequence

Sub-task 1.1 - Cloning of viral DNA in bacterial vectors

Sub-task 1.2 - Sequencing the virus genome

Sub-task 1.3 - Phylogenetic analysis of the oyster virus

Task 4 - General organisation of the programme and synthesis of results

Participation in annual meetings, participation in writing intermediate and final reports

PLANNED RESEARCH ACTIVITIES

Task	Year 1	Year 2	Year 3
1.1	Laboratory analysis/PR ^a		FR ^b
1.2	Laboratory analysis/PR	Laboratory analysis/PR	FR
1.3		Laboratory analysis/PR	FR
4	2 meetings with participants	1 meeting with participants	1 meeting with participants

^a PR : periodic progress report; ^b FR : final report

RESEARCH ACTIVITIES DURING THE REPORTING PERIOD

Task 1 - Obtaining a complete virus genomic library and DNA sequence

Sub-task 1.1 - Cloning of viral DNA in bacterial vectors

Restriction endonuclease analysis of viral DNA provided by Partner 1 showed that the genome contains 29 *Bam*HI fragments (Annex, Table 1 : columns 1 and 2). Summation of the fragment sizes indicates a total genome size of 206 kbp. Several libraries of viral DNA fragments cloned into *Escherichia coli* vectors were prepared and characterised.

- a) Lambda clones. Viral DNA was partially digested with *Sau*3AI to produce fragments up to 10 kbp in size. The DNA was ligated into the *Bam*HI site of Stratagene's Lambda ZAP II and packaged into bacteriophage lambda particles using a Stratagene III Gold kit. *E. coli* XL-1 Blue MRF' was infected with the particles to generate an expression library representing approximately 5×10^5 initial recombinants. This library was provided to Partner 3 for immunoscreening. A separate lambda library containing viral *Bam*HI fragments was prepared for generating plasmids by excision (see below).
- b) Cosmids. Viral DNA was partially digested with *Bam*HI or *Sau*3AI under conditions optimal for production of fragments in the 35-45 kbp range. The DNA was cloned into the *Bam*HI site of a derivative of Stratagene's Supercos 1 vector and packaged into bacteriophage lambda particles using a Stratagene III XL kit. *E. coli* XL-1 Blue MR was infected with the particles and ampicillin-resistant clones were isolated. Cosmid DNA from 320 individual colonies was analysed by agarose gel electrophoresis of *Bam*HI digests. Six fragments (U, H, V, I, S and P) were totally absent from the cosmid library, and only parts of four others (L, R, E and A) were represented (Annex 1, column 4). Two of the latter were presumed to originate from the genome termini, which were not expected to be present in the library. The data obtained allowed a partial *Bam*HI map for the genome to be constructed, but derivation of a complete map was precluded.
- c) Plasmids. Viral DNA was completely digested with *Bam*HI and ligated into the *Bam*HI site of the plasmid vector pUC18. *E. coli* DH5 α or TOP10 was transfected and ampicillin-resistant clones were isolated. Plasmid DNA from individual colonies was analysed by agarose gel electrophoresis of *Bam*HI digests. In other experiments, individual viral *Bam*HI fragments were isolated and inserted specifically into pUC18. Some clones of smaller fragments were also produced by excision from the *Bam*HI lambda library (see above). The combined plasmid libraries represent all but five of the viral *Bam*HI fragments (Annex 1, column 5). Two of the missing fragments (on various criteria, L and A) were presumed to originate from the genome termini, which were not expected to be present in the library. Two of the other three (I,R and E) correspond to fragments that were also not represented in their entirety in the cosmid library, and probably represent fragments that cannot be cloned in *E. coli*. The termini of *Bam*HI inserts in the final library were sequenced in order to be able to locate them unambiguously on the genome when the DNA sequence is completed.

All cloned materials are available to other Partners for further studies. Three regions of the genome (in *Bam*HI I, R and E) could not be cloned in *E. coli* vectors. Further efforts to clone them will not be made as they are unlikely to be successful.

Sub-task 1.1 has been completed. The work was carried out by Dr. A.. Davison and Ms. M. Watson with laboratory support from Mr. C. Cunningham.

Sub-task 1.2 - Sequencing the virus genome

Random fragments 600-1000 bp in length were produced from viral DNA provided by Partner 1 by sonication and selective precipitation using polyethylene glycol. The ends of the fragments were repaired using bacteriophage T4 DNA polymerase. The fragments were ligated to *Sma*I-cleaved, dephosphorylated M13mp19. The ligation was transfected into *E. coli* XL-1 Blue, and template DNA was produced from individual recombinant phage plaques.

In an initial experiment, 400 templates were sequenced using autoradiographic techniques. The combined sequence data sampled about one third of the genome, and were used for preliminary phylogenetic analysis (sub-task 1.3).

A further 4500 templates were prepared and sequenced using an ABI PRISM 377 DNA sequencer, and the sequences were assembled into a database using Staden's Pregap and Gap4 programs. The database currently takes the form of three large pieces of assembled sequence. The *Bam*HI map predicted from one arrangement of these pieces is consistent with the *Bam*HI profile of viral DNA and with the partial maps deduced from analysis of cosmids. The fragments are listed in order along the genome in Annex 1, column 1. It is interesting to note that two of the three sequence gaps correspond to regions that could not be cloned as cosmids or plasmids (Annex, Table 1, column 6). They are separated by the six fragments that were absent from cosmids. The combined size of these fragments (29.7 kbp) is less than the minimum size of a cosmid insert (35 kbp), and this, plus the inability to clone *Bam*HI I, explains their absence from cosmids. The sizes of *Bam*HI fragments deduced from the unfinished database sum to 207 kbp (Annex 1, column 3); eventual inclusion of sequences in the gaps is likely to bring this to 208-209 kbp.

Detailed analysis of the genetic content of the virus is not worthwhile until the database is finished and all errors are removed. Nevertheless, certain local analyses have been carried out in order to characterise genes of potential diagnostic interest. Two such genes have been identified by Partner 3, one encoding a potential surface glycoprotein and the other an inhibitor of apoptosis (IAP).

Looking ahead, specific regions in the database where information is absent or of poor quality will be dealt with by PCR amplification of viral DNA using custom primers. PCR products will either be cloned into bacterial plasmids and sequenced, or sequenced directly. It will also be necessary to generate clones from the genomic termini in order to be sure of their location. This will be done by digesting viral DNA with *Pst*I or another restriction endonuclease, "tailing" with dG residues using terminal transferase, and annealing to pUC18 which has been digested with *Sph*I and "tailed" with dC residues. This strategy reconstitutes the *Sph*I sites, and allows terminal fragments to be cloned as well as internal fragments. *E. coli* DH5 α or TOP10 will be transformed with the annealed DNA, and ampicillin-resistant colonies will be analysed by restriction endonuclease digestion. Clones containing the genome termini will be sequenced as appropriate.

It appears probable from the sequence data that the viral genome contains substantial inverted repeats. The genome structure will be investigated by specific restriction endonuclease and DNA hybridisation experiments when the sequence is completed.

The progress of this sub-task is as anticipated. The work was carried out by Ms. M. Watson and Dr. A. Davison with laboratory support from Mr. C. Cunningham.

Sub-task 1.3 - Phylogenetic analysis of the oyster virus

The 400 sequences obtained in sub-task 1.2 were translated in the six possible reading frames using a computer, and the amino acid sequences were compared with protein databases in order to detect similarities to other organisms. Genes encoding the following proteins were identified: DNA polymerase, deoxyuridine triphosphatase, ribonucleotide reductase (large subunit), an inhibitor of apoptosis (IAP) and a terminase. The presence of a terminase gene is the only indication that the agent is a herpesvirus. No other herpesvirus-specific genes were identified. Limited analyses carried out subsequently on various regions of the genome as they are represented in the database support these conclusions.

These data raise the possibility that the oyster virus is not a herpesvirus. To tackle this question, viral capsids were isolated from infected larvae and their structure was analysed by Drs. Alasdair Steven and Benes Trus at the National Institutes of Health (U.S.A.) by reconstruction of electron cryomicroscopic images. The capsid morphology is characteristic of a herpesvirus.

The data demonstrate that the oyster herpes-like virus is not closely related to herpesviruses with vertebrate hosts (including fish), consistent with it being a herpesvirus that has evolved with an invertebrate rather than a contaminant acquired by filter feeding. They also indicate that the oyster herpesvirus may be the first identified member of a third major domain of the herpesvirus family, in addition to the mammalian/avian/reptilian and fish/amphibian domains.

The progress of this sub-task is in advance of that anticipated. The work was carried out by Dr. A. Davison.

Task 4 - General organisation of the programme and synthesis of results

Participation in annual meetings, participation in writing intermediate and final reports

Dr. A. Davison attended and presented results at two annual meetings held at the IFREMER building in Paris. He also wrote this intermediate report.

SIGNIFICANT DIFFICULTIES OR DELAYS

None have occurred so far.

ANNEX

Table 1 : summary of data on the sizes of *Bam*HI fragments (listed in order along the genome), and their representation in clone libraries and the sequence database

<i>Bam</i> HI Fragment	Apparent size (kbp) ^a	Actual size (kbp) ^b	Presence in cosmids ^c	Presence in plasmids ^d	Gaps in the sequence ^e
L	7700	7447			
J	9000	8809			
N	5300	5370			
c	100	123			
M	7100	7029			
Y	760	771			
Z	400	403			
Q	4100	4123			
a	150	241			
W	1750	1728			
K	8500	8335			
D	14000	13550			
X	1650	1635			
C	16000	(15255)			
F	12000	12303			
B	23000	23408			
T	2250	2277			
R	3900	(3812)			
U	2200	2241			
H	9500	9250			
V	2200	2221			
I	9000	8886			
S	2300	2338			
P	4500	4488			
E	13000	(12850)			
G	10500	10151			
O	5200	5255			
b	150	229			
A	30000	32800			
Total	206210	207328			

^a Determined by agarose gel electrophoresis

^b Determined from the unfinished sequence; sizes in parentheses assume no gap

^c Fragments present in their entirety are heavily shaded; those present in part are lightly shaded

^d Fragments present are shaded

^e Fragments containing sequence gaps are unshaded

European Commission

Contract FAIR-CT98-4334

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

« VINO »

Individual Progress Report

**Reporting Period :
4th January 1999 to 3rd January 2000**

Participant no. 3

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European Commission

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Individual Progress Report

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FAIR-CT98-4334

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

Individual Progress Report for the period

from **04-01-99** to **03-01-00**

Type of contact: Shared-cost research project

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

Participant no. 3 total cost: 399,760 ECU **EC contribution to partner no. 3:** 199,880 ECU (50%)

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

Completion date: 03-01-02

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INDIVIDUAL PROGRESS REPORT

Participant no. 3: Eurogentec S.A, Research and Development Department, Parc Scientifique du Sart-Tilman, B-4102 Seraing, Belgium

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Danièle VANDENBERGH
Doriano CINGOLANI
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Corinne BOULLE
Nathalie ROPPE
Yves LECOCQ

OBJECTIVES

- ♦ Identification of immunogenic virus proteins
- ♦ Preparation of recombinant proteins
- ♦ Preparation of antibodies specific to recombinant proteins

ACTIONS IN THE PROJECT

Task 2 - Developing tools for the diagnosis of herpes-like virus infections

Sub-task 2. 2. : Identification of immunogenic viral proteins and preparation of recombinant proteins and antibodies for diagnosis use, carrying out of the total sub-task

Task 4 - General organisation of the programme and synthesis of results

Participation in annual meetings, participation in writing intermediate and final reports.

TIMETABLE OF PLANNED RESEARCH ACTIVITIES

Task	Year 1	Year 2	Year 3
2. 2	Laboratory analysis/P. R. ^a	Laboratory analysis/P. R.	F. R. ^b
4	2 meetings with all participants	1 meeting with all participants	1 meeting with all participants

^aP. R. : Periodic Progress Report; ^bF. R. : Final Report

RESEARCH ACTIVITIES DURING THE REPORTING PERIOD

Task 2 - Developing tools for the diagnosis of herpes-like virus infections

Sub-task 2. 2. : Identification of immunogenic viral proteins and preparation of recombinant proteins and antibodies for diagnosis use

The lambda library we used for the immunoscreening was received from Participant 2. Fragments of the viral genome were cloned into BamHI site of the polylinker of the ZAP Express vector (Stratagene). A lacZ promoter and the sequence coding for the -NH₂ extremity of the β-galactosidase are located upstream this polylinker and were used to drive expression of β-Gal-virus fusion proteins in *Escherichia coli*. This expression could be screened with specific anti-virus antibodies received from Participant 1. The viral DNA inserts can be excised out of the phages in the form of the kanamycin-resistant pBK-CMV phagemid vector (4518pb) to make their characterization easier. The polylinker of the pBK-CMV phagemid is flanked by T3 and T7 promoters sequences that were used as primers for OHV fragments sequencing.

- a) Immunological screening. The library was first titered in the *E. coli* strain XL-1 Blue MRF'. The result in our lab experimental conditions was $7.6 \cdot 10^7$ pfu/ml. Participant 2 obtained $8 \cdot 10^7$ pfu/ml in his lab. This result is thus correct. We determined also the ratio of recombinants to nonrecombinants within this library by complementation and color selection in the presence of IPTG and X-gal. When viral genomic DNA is present in the polylinker, expression from the lacZ gene is disrupted and white plaques are produced. In contrast, without insert in the polylinker, the domain of the β-galactosidase is expressed, the enzyme is thus active since the M15 lacZ domain of the enzyme is coded by the F' episome of the strain XL-1 Blue MRF', and nonrecombinants can be scored visually by the presence of blue plaque. We analysed ~200 clones and observed 94.6% of recombinants. After this first characterization of the library, we performed several successive immunological screenings in order to isolate potential interesting clones, expressing fusion proteins recognized by anti-virus antibodies.

XL1-Blue MRF' bacteria were cultivated in an appropriate medium and infected in exponential growth phase with a known number of phages from library, and plated them. Nitrocellulose membrane were pretreated with IPTG 10mM and deposited on plates 4h at 37°C in order to derepress the lacZ promoter and induce fusion proteins synthesis. After washings, membranes with trapped proteins were saturated with non fat dry milk as blocking agent, incubated with anti-herpes-like virus specific mouse ascites and revealed with a peroxydase conjugated rabbit anti-mouse antibody according to the ECL protocol from Amersham. Several successive screenings were necessary to purify selected plaques, i.e. to isolate clones. For that purpose, each selected plaque at the first step of the screening had to be titered before being used for a following infection and immunological screening. If plaques selected at the second step of the screening were not pure, a third step similar to the previous ones was necessary.

We first worked with ascite n°3 from Participant 1 as this is the ascite that gave the less background in assays performed in immunohistochemistry. We tested several dilutions in

parallel on different membranes and decided to work with the ascite 1000 fold diluted. The signal obtained with a 2000 fold dilution was too weak and using the ascite after 500 fold dilution would not be possible as we had a little quantity of ascite. We also tested ascites n°1 and n°2 at different dilutions but background was too high to use them. It was very difficult to distinguish specific response from non specific one on negative controls. We used non infected cells or cells infected with non recombinant phages as negative controls. This result was in accordance with results of immunochimistry in the lab of the participant 1. As we were limited in the ascite n°3 quantity, assays were performed to use the same antibody preparation for several successive screenings, but no response could be obtained using it for the second time. Ascite seemed to become empty of specific antibodies. So, we had to prepare freshly new ascite dilution for each screening step. A first screening was performed on $4 \cdot 10^5$ pfu of the library. From the first step of the screening, we isolated 14 plaques corresponding to the following numbers : 1, 2, 5, 7, 8, 9,10, 11, 16, 17,19, 20, 21, 23. The second step of the screening showed that plaques n° 16, 21, 23 seemed not to be specific, i.e. not expressing proteins recognized specifically by the ascite n°3. At the third step of this screening, we isolated about a hundred plaques, some of them issued from the same starting plaque, e.g. 1-4 and 1-7 are issued from the plaque n°1 isolated at the first step. A second screening was performed on new $4 \cdot 10^5$ pfu of the library. 13 plaques were selected. A third screening was performed again on new $4 \cdot 10^5$ pfu of the library. No plaques could be specifically selected. All selected plaques, with probably different degree of purity, were conserved in specific buffer at 4°C.

- b) Characterisation of the selected plaques. The pBK-CMV phagemid vectors were excised from the selected purified plaques in order to easy the characterization of the inserts. The *E. coli* strain XL1-Blue MRF' was co-infected with selected plaques and ExAssist helper phage. After heat treatment, excised phagemid vectors were in culture supernatant packed as filamentous phage particules. This stock was used to infect the *E. coli* strain XL0LR before plating on LB medium containing kanamycin. Colonies appearing on the plate contained the PBK-CMV double-stranded phagemid vector with the cloned DNA insert. Helper phage doesn't grown, since helper phage contains an amber mutation that prevents replication of the helper phage genome in a nonsuppressing *E. coli* strain such as XL0LR cells. Helper phage couldn't grow also because it doesn't contain kanamycin-resistance gene. XL0LR cells, that doesn't contain the gene coding for the F' pili formation necessary for filamentous phage infection, are also resistant to lambda phage infection, thus preventing lambda phage contamination. So, only excised phagemids can replicate in this host and colonies can be characterised such as typical *E. coli* cells containing plasmids.

Plasmid DNA was prepared from colonies culture by the well described alkaline lysis and anion exchange chromatography. DNA preparations were digested with different restrictive enzymes, submitted to an electrophoresis on agarose gel in the presence of a DNA molecular weight marker and the restriction pattern was revealed with ethidium bromide. T3 and T7 primers were synthetized in order to perform PCR screening on DNA preparations. As the inserts are flanked by these primers sequences, the Taq polymerase amplified DNA fragments corresponding to the inserts. Their length could be so determined by electrophoresis on agarose gel. Inserts were also partially sequenced from the plasmid preparations. Sequencing was performed on an ABI377-based fluorescent system using methods including cycle sequencing and thermophilic polymerases.

Excision was performed on 33 plaques from the about hundred plaques selected. For each one, we analysed between 4 and 6 XL0LR colonies. For most of them, the restriction

pattern and the PCR fragments length were the same and the plaque used for the excision was considered as pure. But for several, results were different from one colony to another. In these cases, the plaque contained several different phages having integrated different OHV genome fragments, but this didn't interfere in the analysis as each bacterial colony is pure, containing only one sort of insert in phagemid. Size of inserts was comprised between 200 and 4000 pb although the lambda ZAP Express vector could theoretically incorporate until 12 kb. Some restriction sites could be displayed and used to compare and sort out clones. Thirteen clones, with different patterns, were chosen and sequenced with T7 and T3 primers. The sequencing results were analysed in looking for ²²open reading frames (ORF). Then, we compared them to the sequencing results of the Participant 2. One of the sequenced clone (issued from plaque n°7) has a region that codes for amino acids with high hydrophobic index. This sequence contains also potential N-glycosylation sites (Asn-X-Ser/Thr) and could so corresponds to a surface glycoprotein with a transmembrane C-terminal end, the typical profile of surface viral antigen. The sequences of two other clones (issued from plaques n°11-4 and 7-4) are different but both in the same ORF. This result is also very interesting because they seem to code for a protein of which different regions are recognized by anti-virus ascite. This protein present homologies with the Inhibitor of Apoptosis Protein 2 from the Norway rat and the human Inhibitor of Apoptosis Protein 1. This seems to be a potential immunogenic protein. Indeed, this protein must be produced in high amounts during expressed herpesviral infections. The analysis of other sequences showed that they are probably not part of a real gene.

The progress in this sub-task is as anticipated. The work was carried out by Dr. A. M. Lauricella with scientific support from Dr. F. Xhonneux.

We will amplify the two complete selected ORF from cosmids of Participant 2. Indeed, as the complete sequence is already identified and cloned, it would be faster to work from this material than to screen the library for obtaining the other fragments of the genes. These complete ORF will have to clone in appropriate expression system. The potential membrane glycoprotein will possibly expressed in baculovirus system rather in *E. coli* to allow post-traductional modifications of the protein and increase the possibilities to have a recombinant protein with a conformation close to the native one. The other protein will probably expressed in *E. coli*. On the other hand, plaques that were not yet characterized, will be done in ordre to isolate other candidates.

SIGNIFICANT DIFFICULTIES OR DELAYS

The immunological screening was difficult because the ascites we had were not highly specific. Indeed, we observed rather high background particularly with ascites n°1 and 2. The problem was that we had no positive controls such as virus it-self, so it was sometimes difficult to decide if one signal is specific or not. We often had to test several times the same plaques and select only those that are recognized by the ascite at each time. This analysis was also limited by the available quantity of ascite.

European Commission

Contract FAIR-CT98-4334

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

« VINO »

Individual Progress Report

Reporting Period :

4th January 1999 to 3rd January 2000

Participant no. 4

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European Commission

Contract FAIR-CT98-4334

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

« VINO »

Individual Progress Report

**Reporting Period :
4th January 1999 to 3rd January 2000**

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FAIR-CT98-4334

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

Individual Progress report for the period

from **04-01-99** to **03-01-00**

Type of contract: Shared-cost research project

Total cost: 1,283,805.5 ECU **EC contribution:** 649,738 ECU (50.5%)

Participant no. 4 total cost: 72,700 ECU **EC contribution to partner no. 4:** 72,700 ECU (100%)

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

Completion date: 03-01-02

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INDIVIDUAL PROGRESS REPORT

Participant 4 : Université de Bretagne Occidentale, Unité de Culture Cellulaire, Brest, France

Scientific team : Dr. Germaine DORANGE
Dr. Françoise LE MARREC - CROQ
Christine DELBARD
Jean-Paul GUIAVARC'H

OBJECTIVES

- ♦ Preparation of oyster primary cell cultures

ACTIONS IN THE PROJECT

Task 2 - Developing tools for the diagnosis of herpes-like virus infections

Sub-task 2. 3. : Testing oyster primary cell cultures and vertebrate cell lines, obtention of oyster larval cells and preparation of primary cell cultures (collaboration with Participant 1 for this sub-task)

Task 4 - General organization of the programme and synthesis of results

Participation in annual meetings, participation in writing intermediate and final reports.

PLANNED RESEARCH ACTIVITIES

Task	Year 1	Year 2	Year 3
2. 3	Obtention of oyster larval cell and preparation of cell cultures/P. R. ^a	Obtention of oyster larval cell and preparation of cell cultures/P. R.	F. R. ^b
4	2 meetings with all participants	1 meeting with all participants	1 meeting with all participants

^aP. R. : Periodic Progress Report; ^bF. R. : Final Report

RESEARCH ACTIVITIES DURING THE REPORTING PERIOD

Task 2 - Developing tools for the diagnosis of herpes-like virus infections

Sub-task 2. 3. : Testing oyster primary cell cultures and vertebrate cell lines, obtention of oyster larval cells and preparation of primary cell cultures (collaboration with Participant 1 for this sub-task)

- a) Preparation of oyster primary cultures. According to our objectives, we have worked on the establishment of primary cell cultures from fresh and frozen-thawed cells isolated from *Crassostrea gigas* embryos and larvae and also (to compare the results) from oyster heart.

Embryonic primary cell cultures

Obtaining gametes and embryos

For each assay, 30 oysters were used (from the hatchery « Les Ferranges » - Beauvoir sur mer - Vendée - FRANCE or from the bay of Brest - Bretagne – France) during the sexual maturation period. Ripe male and female gametes were stripped from adults and fertilisation was performed as in hatcheries. We have tried to minimise the risks of microbial contaminations in cell cultures taking many precautions at the different steps of this protocol :

- before experiments, during the stabulation period of animals in laboratory, using sterile sea water supplemented with antibiotics,
- before gamete stripping, by decontaminating gonads with antiseptic,
- during *in vitro* fertilisation and embryogenesis, using sterile sea water complemented with antibiotics.

The time of embryogenesis was varied for example :

- after 3 hours, 2 to 24 stages of cell development
- after 16 hours, the trocophore larvae
- after around 24 hours, the D larvae.

Isolation of embryonic and larval cells

Embryos and larvae were isolated by filtration on meshes (50µm). All these developmental stages were exposed to pronase during 16 hours at 4°C to obtain cell aggregates - that we described by the term of embryoïds - and isolated cells that we seeded in culture (Annex, Figure 1).

Embryonic and larval cell cultures

Such embryoïds attach quickly to the plastic of sterile dishes, generally after one day and the cells continue their differentiation *in vitro* as we can see with the ciliated cells on the Annex 4. Some cells probably divide but we have to prove a mitotic activity by establishment of the growth curve, the study of DNA synthesis. After 3 or 4 days, contractile cells appear, connecting different embryoïd structures (Annex, Figures 2 and 4). Concerning D larvae, we have observed that some cells, that stay insides the shell after enzymatic treatment, seem to proliferate to form sometimes network between different shells. This has only been attempted once and so we have to remake such experiments.

Heart primary cell culture

The protocol (Annex, Figure 5) uses pronase at 0.025% during 12 hours at 4°C for heart cell dissociation, allowing us to systematically obtain adherent cells in culture. After one day, round cells, cell aggregates and some fibroblastic cells are attached to the plastic. The number of fibroblastic cells increases in the proximity of cell aggregates after 4 or 5 days (Annex, Figure 7). These cells contract spontaneously *in vitro*. We have proved by patch clamp technique that these contractile cells are cardiomyocytes. Such fibroblastic cell morphology is best for test the pathogenic effect of virus.

- b) Cryopreservation of isolated oyster cells. Embryonic and heart cell cultures may be obtained using fresh cells but also from cryopreserved cells using the protocol described in Figure 3 (Annex). The cryoprotective agent used is Dimethyl sulfoxide (DMSO) at 12% as for *Pecten maximus* hear. This protocol should still be optimized term in of viability percent.
- c) Tests of different parameters for cell cultures and tests of transport conditions. The used culture medium is a simple medium based in sterile sea water supplemented in aminoacids, sugars..., foetal calf serum and antibiotics, as described in Le Marrec *and al.* (1999) for *Pecten maximus* heart cell cultures. This year we don't have tried to optimize this medium by adding growth factors. We have only test incubation temperature of cell cultures (15°C and 26°C). Heart and embryonic cell in cultures may be obtained as readily with temperature varying from 15°C to 26°C, the optimum temperature for herpes viral infection. We have verified that oyster cell cultures may be sent by express mail to participant 1 (T. Renault - La Tremblade - France) without problems of surviving, following a procedure established by a french private company (Biopredic - Rennes)
- d) Cultivation of herpes-like virus in oyster primary cultures. We have made four assays of cell contamination by herpes-like virus. The first assay involved the incubation of cells with the virus in culture during 48 hours (Annex, Figure 6). We have study the cytopathogenesis of herpes-like virus (inverted microscope) and we have also fixed samples for electron microscopy (they have to be examined in 2000). We have observed a negative effect of virus on cell cultures at a dilution of 1/2 , 1/10 or 1/20 after 48 hours :
 - the contractile fibroblastic heart cells reverted to a former round shape and progressively detached from the plastic,
 - for the embryonic cell cultures, a decrease of ciliary beatings was observed.

Photographs of the Figure 7 (Annex) illustrate this negative effect : the first photograph is the control with many adherent fibroblastic heart cells. The second picture shows infected cells reverting their round shape. The question remains : is this observed effect cytotoxic or cytopathogenic ? It is expected that if this effect is cytopathogenic, exposure under conditions of decreased dilutions for longer periods would result in the observed negative effects. In order to answer this question, samples were fixed and prepared for electron microscope to verify if the virus was introduced into the cells. The results of this observation are pending. In parallel, different exposure regims (Annex, Figure 6) were designed to minimise a potential toxic effect of the larval homogenate and to optimize conditions favourable for the introduction of virus into the cells by cyto centrifugation for 1 hour of the cells with the virus and by treatment with DEAE dextran. Cells were fixed and treated for microscopic observations. For the heart cell cultures, the same

observations have been made regardless of the experimental setup as for the first assay. Samples were cytocentrifuged and coloured for microscopic study and a first assay has been made using *in situ* hybridisation (in La Tremblade - France - T. Renault). This result was negative. For the embryonic cell culture, a decrease of ciliary beatings was observed. These preliminary results have been yet to be verified by other experiments (Annex, Figure 8). It is hoped that the results of observations using electron microscopy will verify the introduction of the virus into the cells validating these exposure techniques for the *in vitro* diagnosis of herpes virus in the oyster.

Task 4 - General organisation of the programme and synthesis of results

Participation in annual meetings and in writing intermediate and final reports.

G. Dorange has participated to the first annual meeting in Paris. For the second annual meeting in december in Paris, due to health problems, she has only sent to T. Renault (the coordinator of the research program) overheads which summarised results obtained in Brest.

SIGNIFICANT DIFFICULTIES OR DELAYS

None.

Figure 1 : Dissociation procedure of embryonic and larval cells from *Crassostrea gigas*

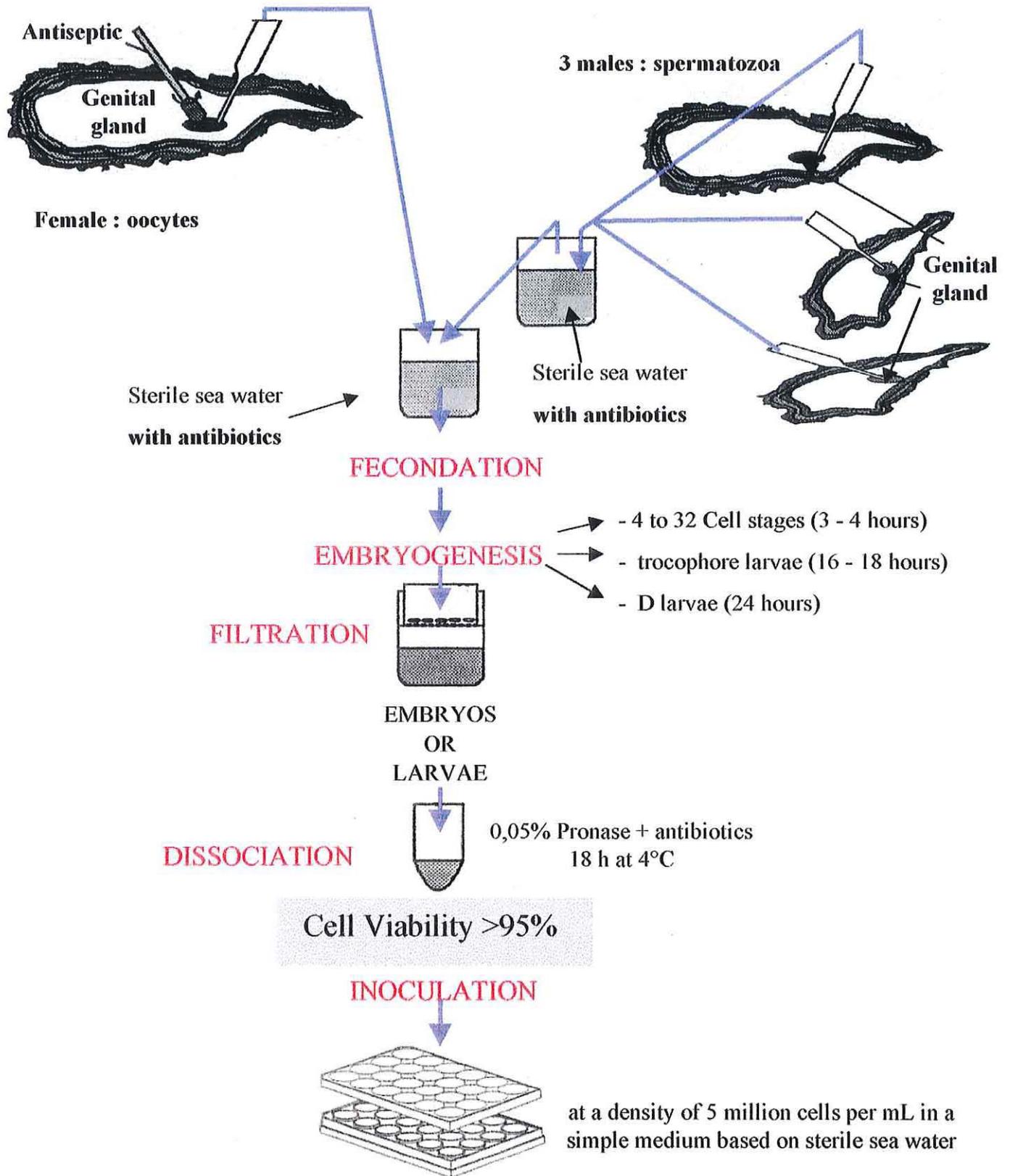


Figure 2
Embryos at 2-64 cell stages (1)



Embryoids (E) and Isolated cells (Ic) after pronase treatment (2)

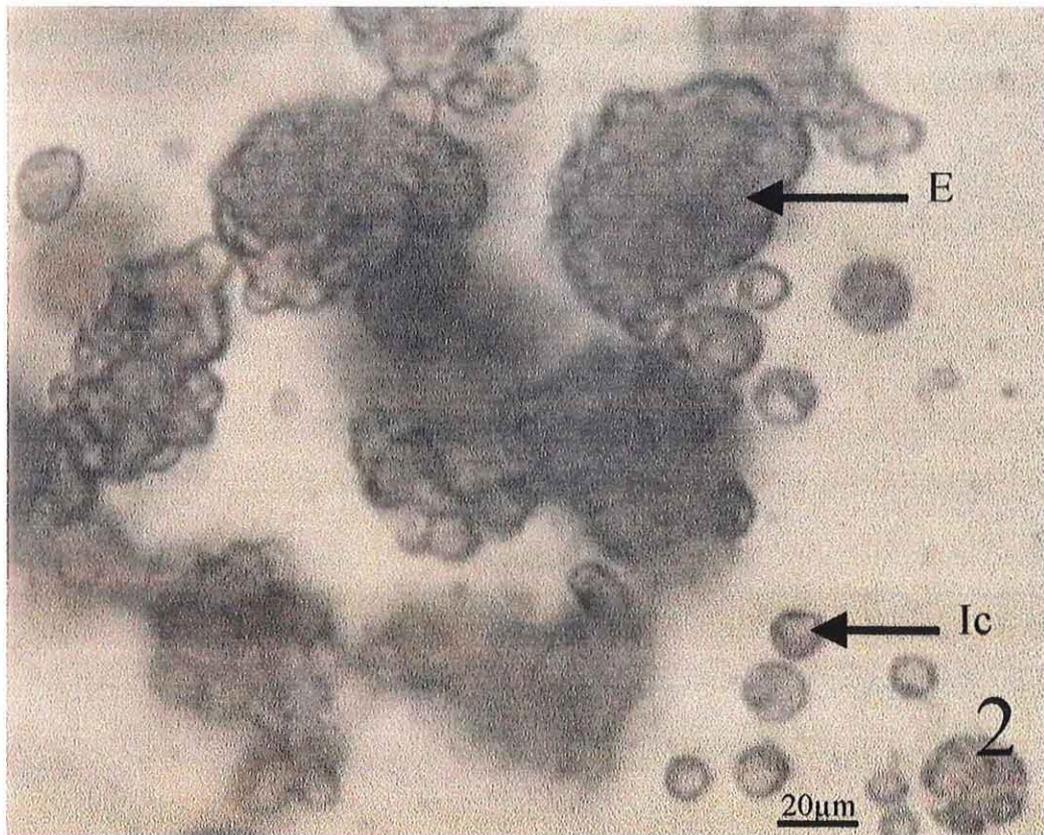


Figure 3 : Protocol for cell cryopreservation

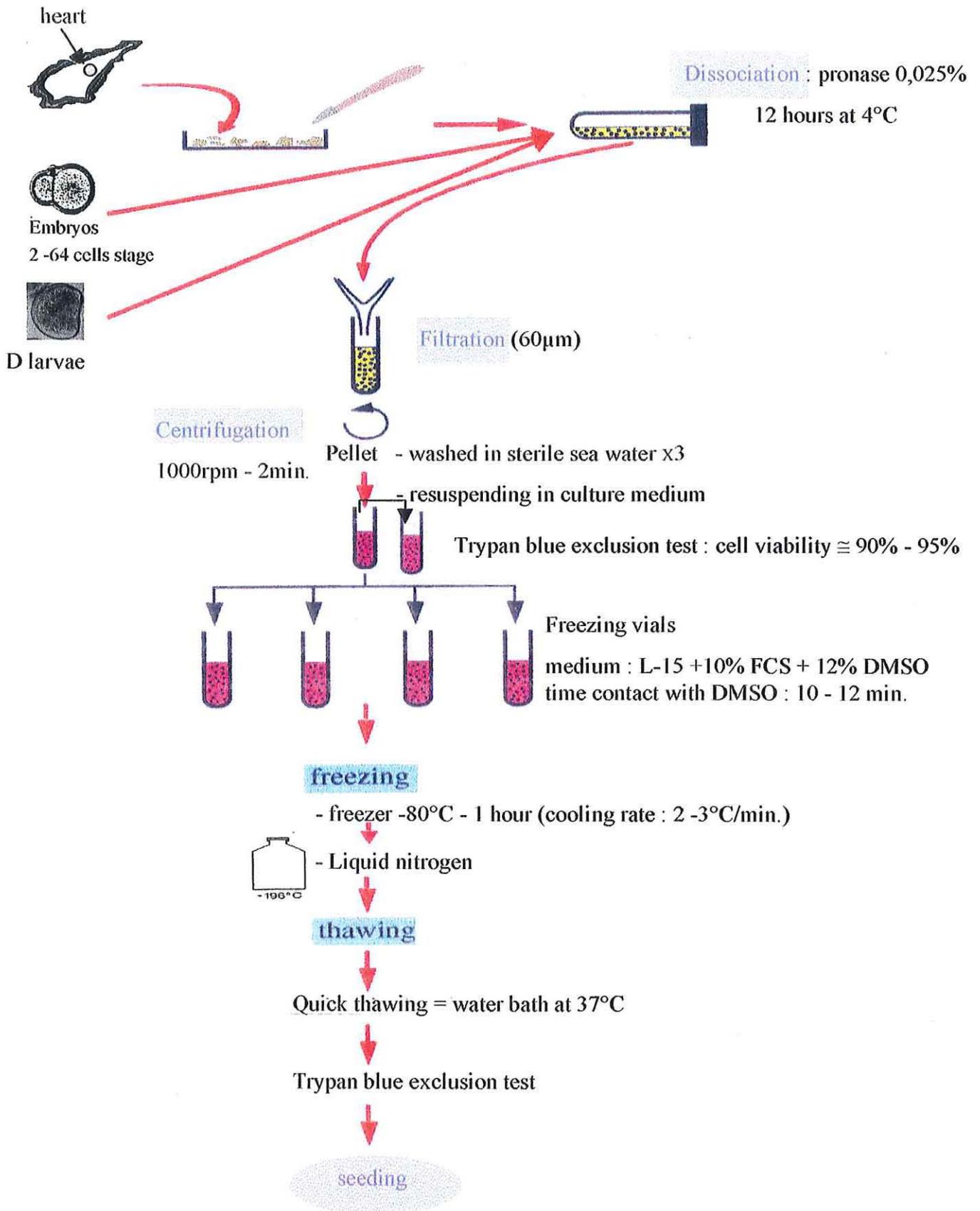
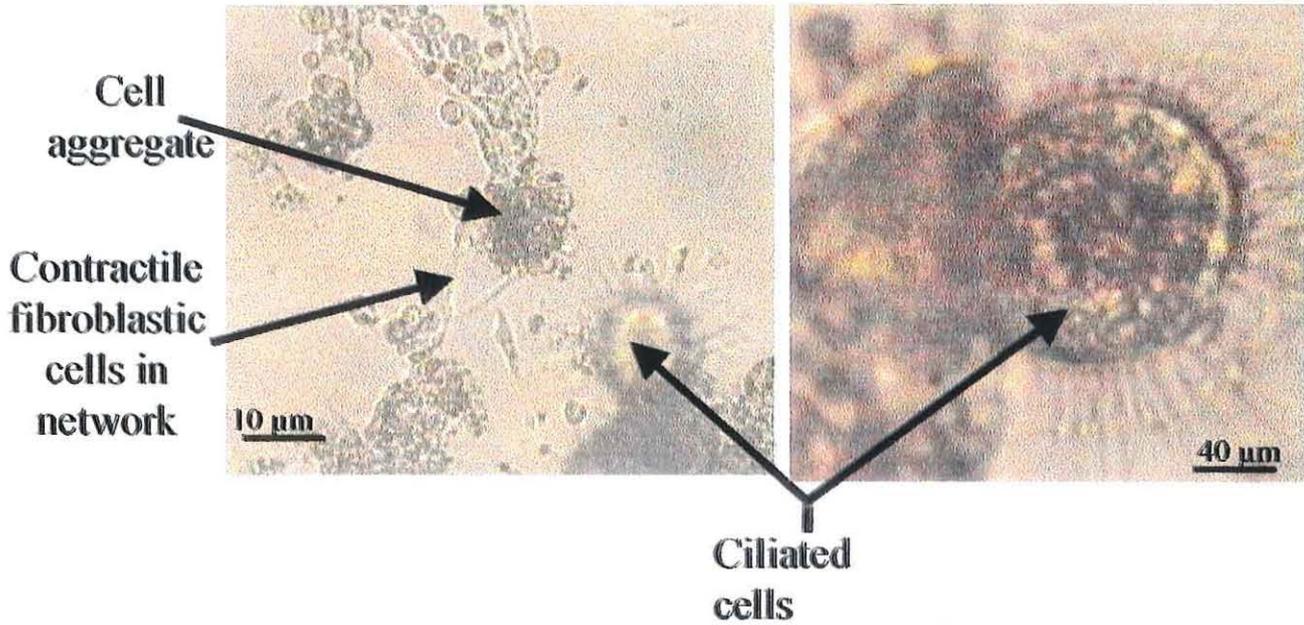


Figure 4

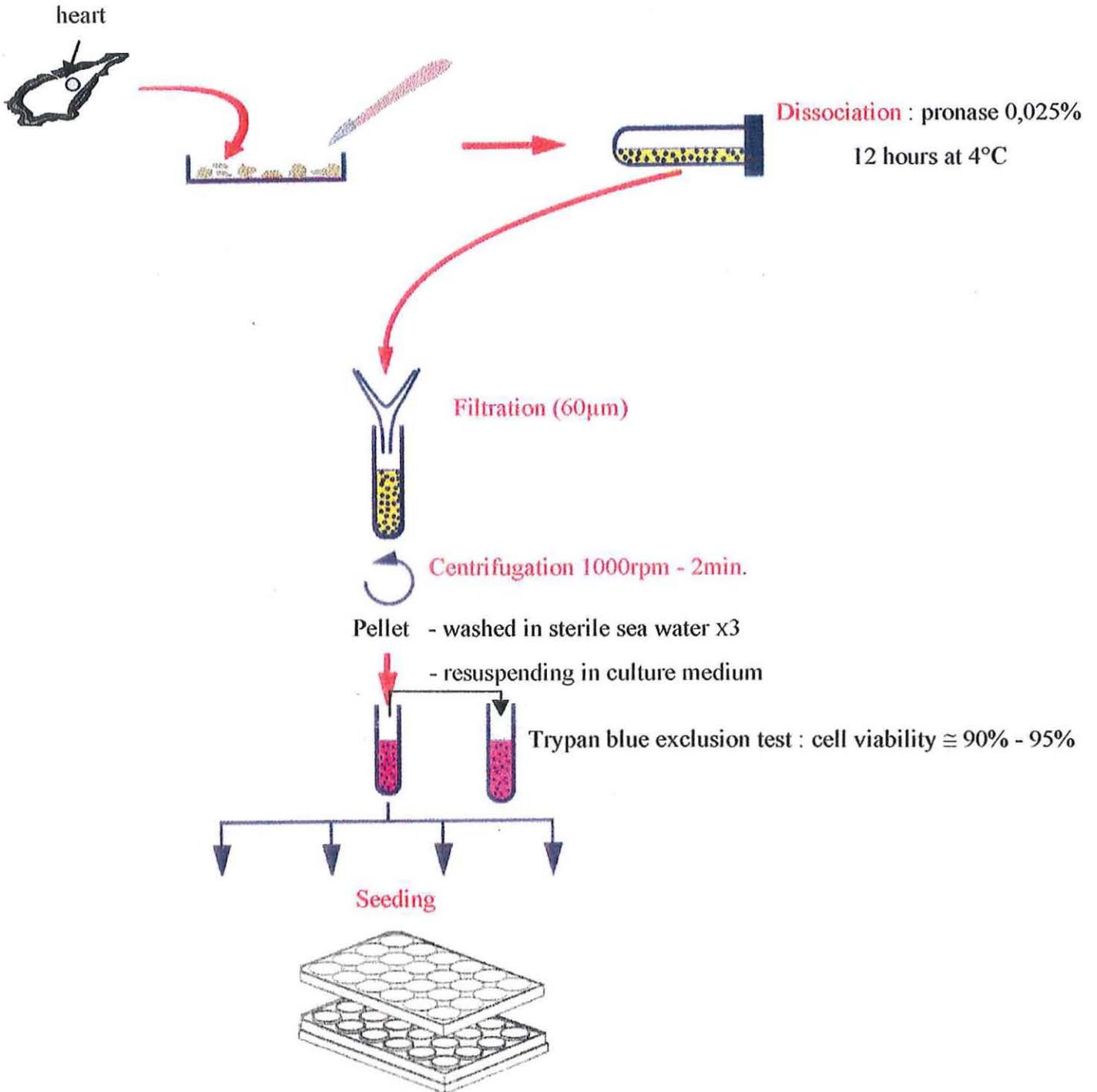
***Crassostrea gigas* embryonic cells after 4 days in culture**



***Crassostrea gigas* embryonic cells after 7 days in culture**



Figure 5 : Protocol for heart cell cultures



Culture at a cell density = 2,5 million cells per 500µl of medium / well

Figure 6 : Inoculation of Herpes virus in cell cultures

Collaboration : Participant 1 (IFREMER_v - Dr. T. RENAULT)



Homogenate from *C. gigas*
larvae infected by the *Herpes*
virus

First assay

C.gigas heart cell cultures (from fresh and thawed cells)

Incubation temperatures : 15°C and 26°C



1/2 Virus



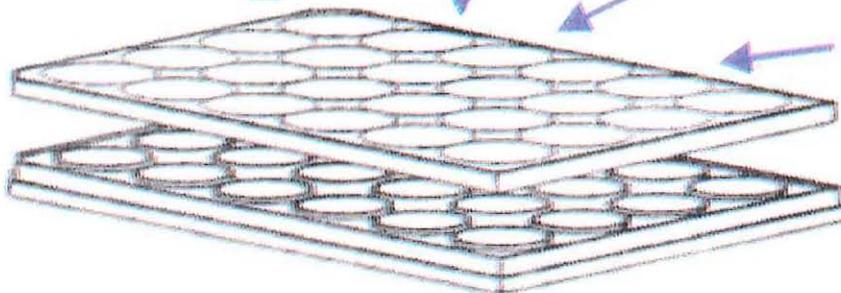
1/20 Virus



1/200 Virus

(final concentrations in the culture medium)

3 replicates
per dilution



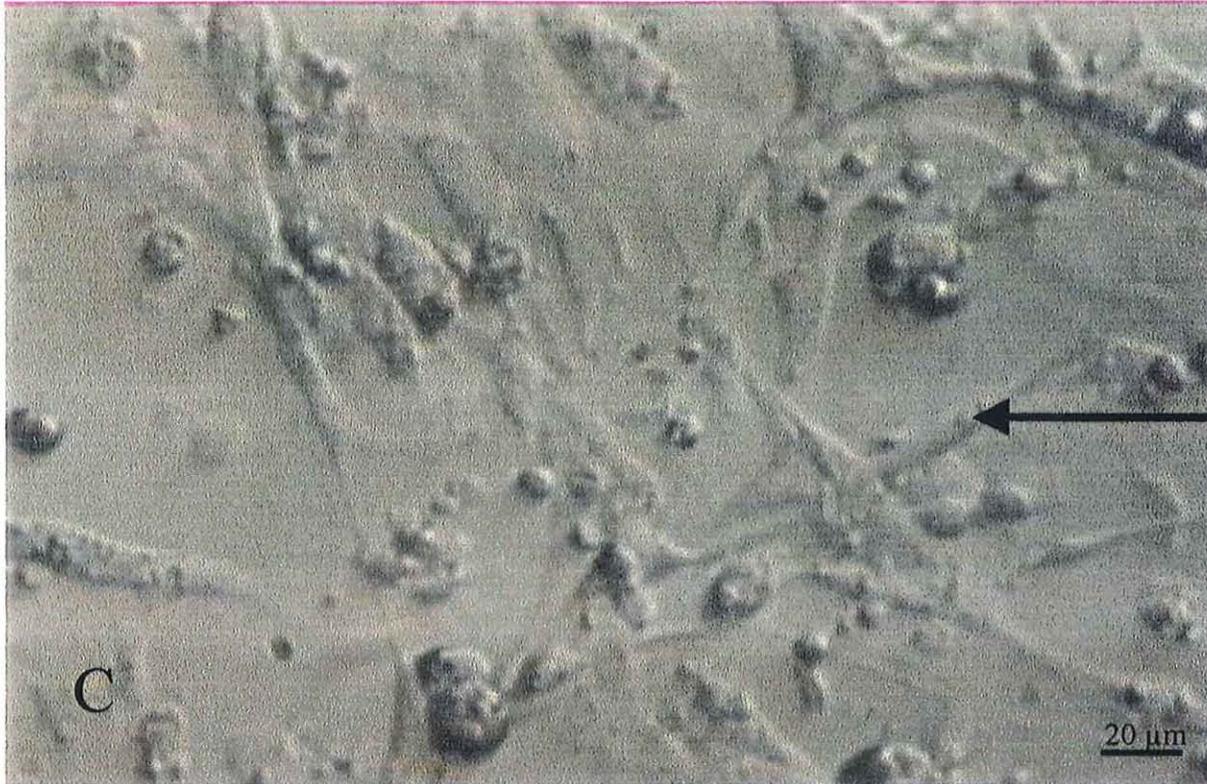
Control without virus

Incubation during 48 hours

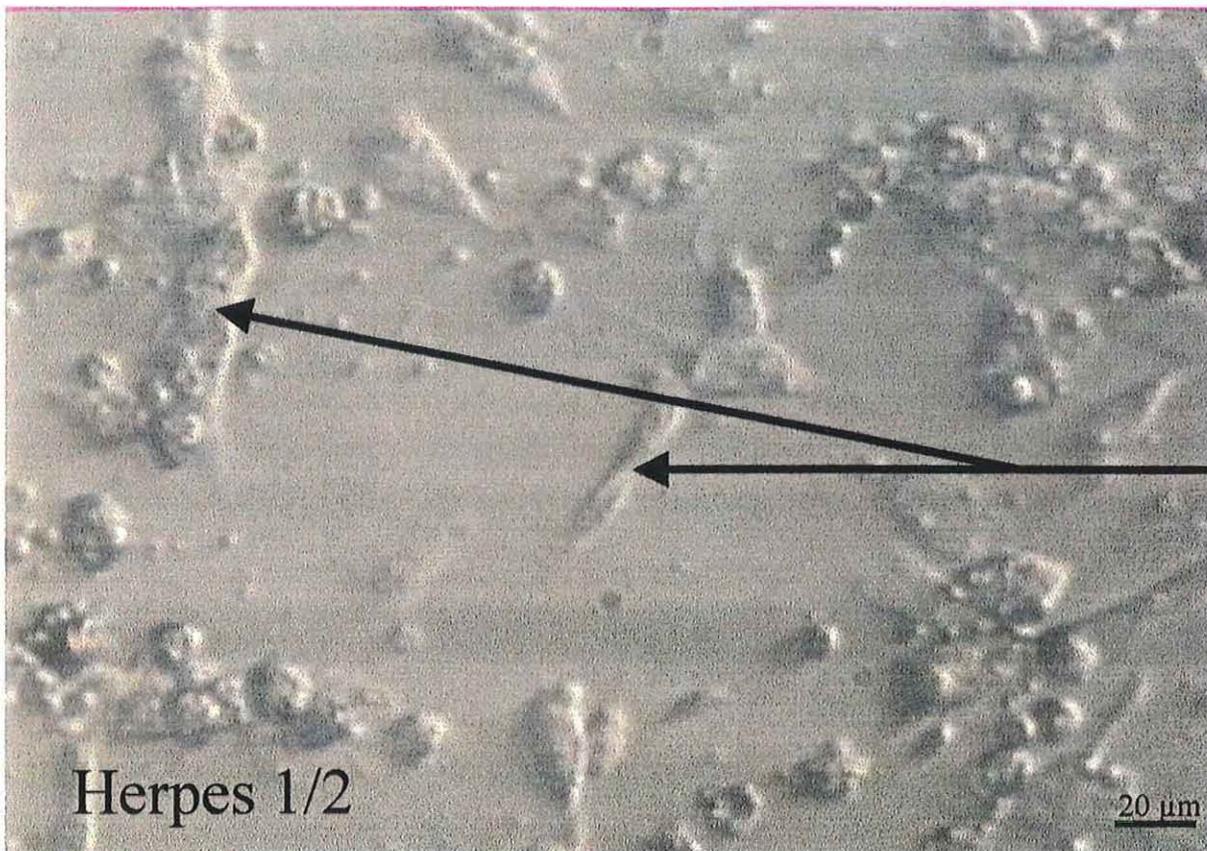
Cytopathogenesis of Herpes virus

Transmission electron microscopy study

Figure 7 : Control heart cell culture at D7 (C) and heart cell culture 48 hours after herpes virus infection



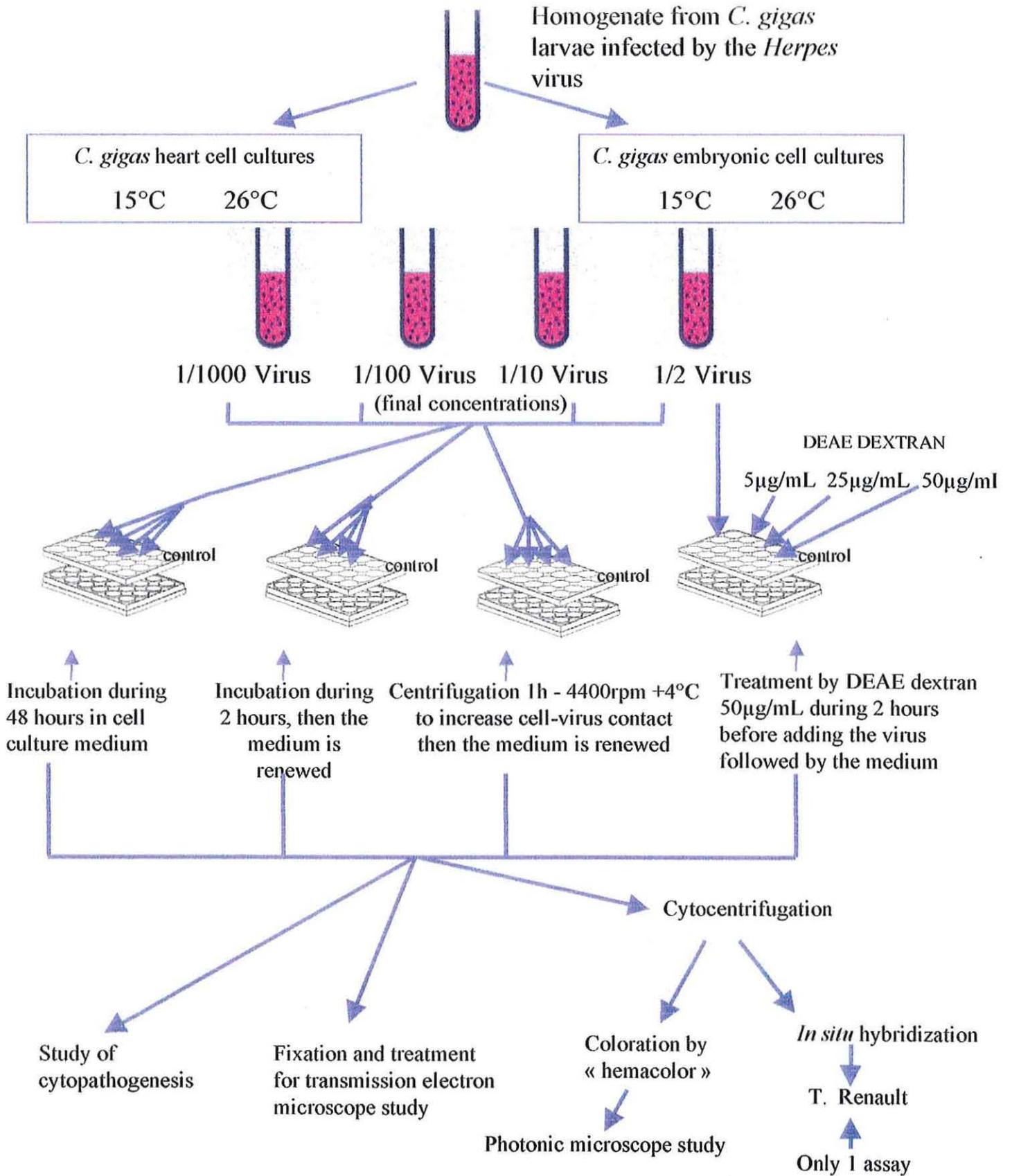
adherent
fibroblastic
cells



Retracted
fibroblastic
cells
detaching
from the
plastic

Figure 8

Following assays (X3)



European Commission

Contract FAIR-CT98-4334

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« VINO »

Individual Progress Report

**Reporting Period :
4th January 1999 to 3rd January 2000**

Participant no. 5

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European Commission

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« VINO »

Individual Progress Report

**Reporting Period :
4th January 1999 to 3rd January 2000**

Participant no. 5

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FAIR- CT98-4334

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

Individual Progress report for the period

from **04-01-99** to **03-01-00**

Type of contract: Shared-cost research project

Total cost: 1,283,805.5 ECU **EC contribution:** 649,738 ECU
(50.5%)

Participant no. 5 total cost: 88,066 ECU **EC contribution to partner no. 5:** 88,066 ECU (100%)

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

Completion date: 03-01-02

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INDIVIDUAL PROGRESS REPORT

Participant no. 5: University College Cork, Ireland

Scientific team: Dr. Sarah CULLOTY
Pr. Maire MULCAHY
Ms. Yvette KELLY

OBJECTIVES

- ♦ Use of diagnostic tools for herpes-like virus detection in oyster samples

ACTIONS IN THE PROJECT

Task 3 - Application of DNA probes, immunological reagents and cellular tools for virus detection (i.e. validation)

Sub-task 3.1 - Application of molecular methods for the diagnosis of oyster herpes-like virus, carrying out analysis of oyster samples originating from Ireland

Sub-task 3.2 - Application of immunological methods for the diagnosis of oyster herpes-like virus, carrying out analysis of oyster samples originating from Ireland

Sub-task 3.3 - Application of primary oyster cell cultures and vertebrate cell lines for the diagnosis of oyster herpes-like virus, carrying out analysis of oyster samples originating from Ireland

Task 4 - General organisation of the programme and synthesis of results

Participation in annual meetings, participation in writing intermediate and final reports

PLANNED RESEARCH ACTIVITIES

Task	Year 1	Year 2	Year 3
3. 1.	1 st sample collection/P. R. ^a	2 nd sample collection/Laboratory analysis/P. R.	3 rd sample collection/Laboratory analysis /F. R. ^b
3. 2	1 st sample collection/P. R.	2 nd sample collection/Laboratory analysis/P. R.	3 rd sample collection/Laboratory analysis /F. R.
3. 3	1 st sample collection/P. R.	2 nd sample collection/Laboratory analysis/P. R.	3 rd sample collection/Laboratory analysis /F. R.
4	2 meetings with all participants	1 meeting with all participants	1 meeting with all participants

^aP. R. : Periodic Progress Report; ^bF. R. : Final Report

RESEARCH ACTIVITIES DURING THE FIRST REPORTING PERIOD

Task 3 - Application of DNA probes, immunological reagents and cellular tools for virus detection (i.e. validation)

Collecting oyster samples

a) Sites. Some initial months were spent making contact with farmers and finding some that were prepared to provide samples. Two sites on the south coast of Ireland were sampled over July, August and November 1999. Cork harbour is the site of mixed production of *Ostrea edulis* and *Crassostrea gigas*. Dungarvan bay is the site of large-scale production of Pacific oysters only. Both sites have experienced significant mortalities in *C. gigas* stocks at different times since 1994. A small amount of spat is produced in Ireland but most spat are imported from Guernsey and Seasalter hatcheries. Half-grown oysters are imported from France.

Sampling began on 13 July 1999 at Cork harbour. Spat had been bought in from Sligo, Ireland. 30 spat were sampled. Sampling subsequently took place at this site on a weekly basis up to 30 August 1999. It had been intended to sample *Ostrea edulis* spat as well but this was not possible due to late settlement. Further samples were taken at this site in November but this years *O. edulis* spat were still too small to sample (Annex, Table 1). 80% mortalities occurred in spat in Dungarvan in early August 1999. However it was 25 August before samples were obtained from this site due to late notification of the mortalities and no access at the site due to poor tides. These spat had been imported from Guernsey and Seasalter hatcheries. Further samples were taken in November from this site (Annex, Table 1).

b) Oyster samples. On being returned to the laboratory all oysters were dissected aseptically. Each animal was dissected in two. One half was frozen at -20°C for future PCR analysis and the other half placed in individually numbered and dated histocassettes and placed in Davidsons fixative.

c) PCR analysis. Methodology for this technique has just recently been developed and provided by the Coordinator. Sample preparation and PCR analysis is now being practised in the laboratory.

The progress in this sub-task is as anticipated. The work was carried out by Dr. S. Culloty with laboratory support from Ms. Y. Kelly.

Task 4 - General organisation of the programme and synthesis of results

Participation in annual meetings and in writing intermediate and final reports.

Dr. S. Culloty and Pr. M. Mulcahy attended the first meeting in January 1999. Dr. S. Culloty attended and presented results at the second annual meeting, in December 1999, held at the IFREMER building in Paris. She also wrote this intermediate report.

SIGNIFICANT DIFFICULTIES OR DELAYS

- It is possible that samples were not obtained soon enough at the Dungarvan site after mortalities occurred to detect herpes-like virus.
- A major problem is that farmers do not monitor their stocks frequently enough during the year so mortalities are not observed quickly enough.
- Not all farmers are prepared to provide samples due to the fear of a herpes-like virus being found in their stocks.
- Samples of *Ostrea edulis* were not available due to late settlement and the small size of the spat in November - samples will be taken in early 2000.

ANNEX

Table 1 : 1999 samples for Ireland

Date Sampled	Site	Species	Farmer	n	Origin	Comments
13/07/99	Cork Harbour	<i>C. gigas</i>	Atlantic Shellfish	30	Sligo	
27/07/99	"	"	"	"	"	
03/08/99	"	"	"	"	"	
17/08/99	"	"	"	"	"	
30/08/99	"	"	"	"	"	
30/08/99	"	"	"	29		1+ age group
25/08/99	Dungarvan	"	P. Harty	30	Guernsey 15/06/99	Bags not turned 80% Mortality
25/08/99	"	"	P. Cullen	29	Seasalter 03/99	Bags turned 11/07/99 80% Mortality
12/11/99	"	"	"	30	Seasalter 03/99	
12/11/99	"	"	P. Cullen	30	French Hatchery	Bought in 11/99
12/11/99	"	"	P. Harty	30	Guernsey	
23/11/99	Cork Harbour	"	Atlantic Shellfish	30	Sligo	

European Commission

Contract FAIR-CT98-4334

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

« VINO »

Individual Progress Report

**Reporting Period :
4th January 1999 to 3rd January 2000**

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European Commission

Contract FAIR-CT98-4334

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

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Individual Progress Report

**Reporting Period :
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FAIR-CT98-4334

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

Individual Progress report for the period

from 04-01-99 to 03-01-00

Type of contract: Shared-cost research project

Total cost: 1,283,805.5 ECU *EC contribution:* 649,738 ECU (50.5%)

Participant no. 6 total cost: 222,605 ECU *EC contribution to partner no. 6 :* 81,780 ECU (36.7%)

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

Completion date: 03-01-02

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INDIVIDUAL PROGRESS REPORT

Participant 6 : Instituto de Investigaciones Marinas, Consejo Superior de Investigaciones Científicas (CSIC) Eduardo Cabello 6, 36208 Vigo, Spain

Scientific team : Dr. Antonio FIGUERAS
Dr. Beatriz NOVOA
Marías PERNAS
Jose Ramón CALDAS
Begoña VILLAVERDE

OBJECTIVES

- ♦ Development of cellular tools for oyster herpes-like virus detection
- ♦ Use of diagnostic tools for herpes-like virus detection in oyster samples

ACTIONS IN THE PROJECT

Task 2 - Developing tools for the diagnosis of herpes-like virus infections

Sub-task 2. 3. : Testing oyster primary cell cultures and vertebrate cell lines, carrying out infection tests on vertebrate cell lines (collaboration with Participant 1 in furnishing infected material)

Task 3 - Application of DNA probes, immunological reagents and cellular tools for virus detection (i. e. validation)

Sub-task 3. 1. : Application of molecular methods to the diagnosis of oyster herpes-like virus, carrying out analysis of oyster samples originating from Spain

Sub-task 3. 2. : Application of immunological methods to the diagnosis of oyster herpes-like virus, carrying out analysis of oyster samples originating from Spain

Sub-task 3. 3. : Application of primary oyster cell cultures and vertebrate cell lines to the diagnosis of oyster herpes-like virus, carrying out analysis of oyster samples originating from Spain

Task 4 - General organization of the programme and synthesis of results

Participation in annual meetings, participation in writing intermediate and final reports

PLANNED RESEARCH ACTIVITIES

Task	Year 1	Year 2	Year 3
2.3		Technique development and application/P. R.	F. R. ^b
3.1	1 st sample collection/P. R. ^a	2 nd sample collection/Laboratory analysis/P. R.	3 rd sample collection/Laboratory analysis /F. R.
3.2	1 st sample collection/P. R.	2 nd sample collection/Laboratory analysis/P. R.	3 rd sample collection/Laboratory analysis /F. R.
3.3	1 st sample collection/P. R.	2 nd sample collection/Laboratory analysis/P. R.	3 rd sample collection/Laboratory analysis /F. R.
4	2 meetings with all participants	1 meeting with all participants	1 meeting with all participants

^aP. R. : Periodic Progress Report; ^bF. R. : Final Report

RESEARCH ACTIVITIES DURING THE REPORTING PERIOD

Task 2 - Developing tools for the diagnosis of herpes-like virus infections

Sub-task 2.3. : Testing oyster primary cell cultures and vertebrate cell lines, carrying out infection tests on vertebrate cell lines (collaboration with Participant 1 in furnishing infected material)

Infected and uninfected (negative control) oyster larvae have been inoculated into two fish cell lines: one of marine origin TV-1 (derived from turbot (*Scophthalmus maximus*) and another from a freshwater fish: EPC (from carp epithelioma), in order to detect a cytopathic effect of the herpesvirus. Cells were grown with minimal essential medium with penicilin and streptomycin and 10% of fetal calf serum (MEM 10%). Infected and uninfected larvae were frozen and thawed and then, homogenized and filtered though 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 °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.

No cytopathic effect was detected in any of the passages conducted in these two fish cell lines. In the first passage alteration of the cell monolayer were observed in wells inoculated with infected larvae but in the second passage the morphology of the cells did not change in 30 days of incubation at 15°C.

The progress of this sub-task is as anticipated. The works was carried out by Dr. B. Novoa with scientific support from Dr. A. Figueras.

Task 3 - Application of DNA probes, immunological reagents and cellular tools for virus detection (i. e. validation)

Collecting oyster samples

- c) Sites and bivalve species. Although the aim of this task was to validate the technology to detect herpesvirus in oyster larvae, we had to widen the range of molluscan species sampled in Spain because of the lack of oyster hatcheries in our country. Moreover, since severe mortalities were detected in Spain in different bivalve species (mainly carpet shell clam), of different sizes (Annex), we decided to sample these clams to identify the possible cause of the mortalities trying to determine if the herpes-like virus was present in the diseased batches.
- d) Oyster samples. The individuals of each batch were removed from the shell, sagittally sectioned, then half of the animal was frozen at -80°C and the other half was fixed in Davidson and for electron microscopy studies (in osmium tetroxide). In some cases, when the size of the animal was too small (batches with asterisk), less than 5 mm total length, the batch was divided in three parts: one part was frozen for molecular studies, other part was fixed in Davidson for histology and another part was fixed directly in osmium tetroxide for electron microscopy.
- c) TEM analyses. Several samples have been already examined by transmission electron microscopy. Herpes-like viruses were not detected in oysters or clams. However, in diseased clams many icosahedric, unenveloped particles with 27-35 nm of diameter were observed in the cytoplasm of connective tissue cells. They appeared free in the cytoplasm or associated to endoplasmic reticulum membranes. Nuclei of infected cells were enlarged with dispersed chromatin which sometimes was condensed near the nuclear membrane. The cytoplasm content of these cells was reduced compared with uninfected cells. The size, morphology and the replication in the cytoplasm in association with endoplasmic reticulum, suggest that they belong to the *Picornaviridae* family.
- d) PCR analyses. Samples were taken and frozen for their PCR analysis. We are now conducting the PCR with the positive controls (infected larvae sent by the coordinator).

The progress of this sub-task is advance of that anticipated because some analyses have been done using the PCR technique developed by Participant 1. The works was carried out by Dr. B. Novoa with scientific support from Dr. A. Figueras.

Subtask 3.3. Application of primary oyster cell cultures and vertebrate cell lines to the diagnosis of oyster herpes-like virus, carrying out analysis of oyster samples originating from Spain.

Due to the lack of positive results when the infected material (positive control) was inoculated in cell lines, the inoculation on vertebrate cell lines of the sampled material is not being conducted. We only have inoculated samples of clams suffering high mortalities in Galicia (see sampling : Sub-task 3.1) with the same methodology explained in that section.

No clear cytopathic effect was detected in the inoculated monolayers (first and two subsequent blind passages). Inoculated cells were fixed and processed for electron microscopy.

The progress of this sub-task is as anticipated. The work was carried out by Dr. B. Novoa with scientific support from Dr. A. Figueras.

Task 4 - General organisation of the programme and synthesis of results

Participation in annual meetings and in writing intermediate and final reports.

Drs. B. Novoa and A. Figueras attended the first meeting in January 1999. Dr. B. Novoa also wrote this intermediate report.

SIGNIFICANT DIFFICULTIES OR DELAYS

None.

ANNEX

1999 samples for Spain

First sampling (9.3.99)

- Batch 1: *Crassostrea gigas*
- * Batch 2: *C. gigas*
- * Batch 3: *Ostrea edulis*
- * Batch 4: *Ruditapes philipinarum*
- * Batch 5: *R. philipinarum*
- Batch 6: *R. philipinarum*
- * Batch 7: *R. decussatus*
- Batch 8: *R. decussatus*

Second sampling (21.4.99)

- Batch 9: *Crassostrea gigas*
- Batch 10: *Ostrea edulis*
- Batch 11: *Ruditapes philipinarum*
- Batch 12: *R. decussatus*

Third sampling (25.10.99)

- Batch 13: *Ruditapes decussatus.*
- * Batch 14: *R. decussatus-a*
- * Batch 15: *R. decussatus-b*
- * Batch 16: *R. decussatus-c*
- * Batch 17: *R. decussatus-d*
- * Batch 18: *R. decussatus-e*
- * Batch 19: *R. decussatus-f*
- * Batch 20: *R. decussatus-g*
- * Batch 21: *R. decussatus-h*
- * Batch 22: *R. decussatus-i*

European Commission

Contract FAIR-CT98-4334

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

« VINO »

Individual Progress Report

**Reporting Period :
4th January 1999 to 3rd January 2000**

Participant no. 7

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European Commission

Contract FAIR-CT98-4334

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

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Individual Progress Report

Reporting Period :
4th January 1999 to 3rd January 2000

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FAIR-CT98-4334

**« Diagnosis of Oyster herpes-like virus: Development and validation
of molecular, Immunological and Cellular tools »**

Individual progress report for the period

from **04-01-99** to **03-01-00**

Type of contract: Shared cost research project

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

**Participant no. 7
total cost:** 116,000 ECU **EC contribution
to partner no. 7 :** 54,752 ECU (47.2%)

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

Completion date: 03-01-02

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PLANNED RESEARCH ACTIVITIES

Task	Year 1	Year 2	Year 3
3. 1	1 st sample collection/P. R. ^a	2 nd sample collection/Laboratory analysis/P. R.	3 rd sample collection/Laboratory analysis /F. R. ^b
3. 2	1 st sample collection/P. R.	2 nd sample collection/Laboratory analysis/P. R.	3 rd sample collection/Laboratory analysis /F. R.
3. 3	1 st sample collection/P. R.	2 nd sample collection/Laboratory analysis/P. R.	3 rd sample collection/Laboratory analysis /F. R.
4	2 meetings with all participants	1 meeting with all participants	1 meeting with all participants

^aP. R. : Periodic Progress Report; ^bF. R. : Final Report

RESEARCH ACTIVITIES DURING THE FIRST REPORTING PERIOD

Task 3 - Application of DNA probes, immunological reagents and cellular tools for virus detection (i.e. validation)

Collecting oyster samples

- a) 1999 summer sampling. Nine batches of 30 individuals (Annex, Table 1) were randomly sampled in August. No mortalities were associated with these samples. All samples originated from spawns produced in hatcheries. Individual animals were cut into three sections. These sections were treated as follows:
- Fixed in Davidson's fluid at 4°C and embedded in paraffin.
 - Fixed in Carson's fluid and stored at 4°C
 - Placed in individual bags and frozen at -70°C for PCR analysis.
- b) 1999 winter sampling. This comprised of four batches of spat molluscs (Annex, Table 1). All samples originated from spawns produced in hatcheries. One batch was from a sample associated with 50% mortalities. Mortalities were relatively recent compared to the date of sampling. These animals were too small to be sectioned into three.

Animals of 1cm:

- 30 individuals were fixed without shell, in Davidson's fluid at 4°C and will be embedded in paraffin.
- 30 other individuals were fixed without shell, in Carson's fluid and stored at 4°C
- More than 30 other individuals were placed all in the same bag with their shells and frozen at -70°C for PCR analysis.

Animals of less than 1cm:

- More than 30 individuals were fixed with their shells in Davidson's fluid at 4°C and will be embedded in paraffin after decalcification with EDTA.

- More than 30 individuals were fixed with their shells in Bouin's fluid at 4°C and will be embedded in paraffin.
- More than 30 individuals were fixed with their shells in Carson's fluid and stored at 4°C.
- More than 30 other individuals were placed all in the same bag with their shells and frozen at -70°C for PCR analysis.

The progress of this sub-task is as anticipated. The works was carried out by S. Bark with scientific support from Dr. R. M. Le Deuff.

Task 4 - General organisation of the programme and synthesis of results

Participation in annual meetings and in writing intermediate and final reports.

Dr. P. Dixon attended the first meeting in January 1999. Dr. R. M. Le Deuff on behalf of Dr. P. Dixon and Sam Bark attended and presented results at the second annual meeting, in December 1999, held at the IFREMER headquarters in Paris. Sam Bark also wrote this intermediate report.

SIGNIFICANT DIFFICULTIES OR DELAYS

None.

ANNEX

Table 1: 1999 samples for United Kingdom

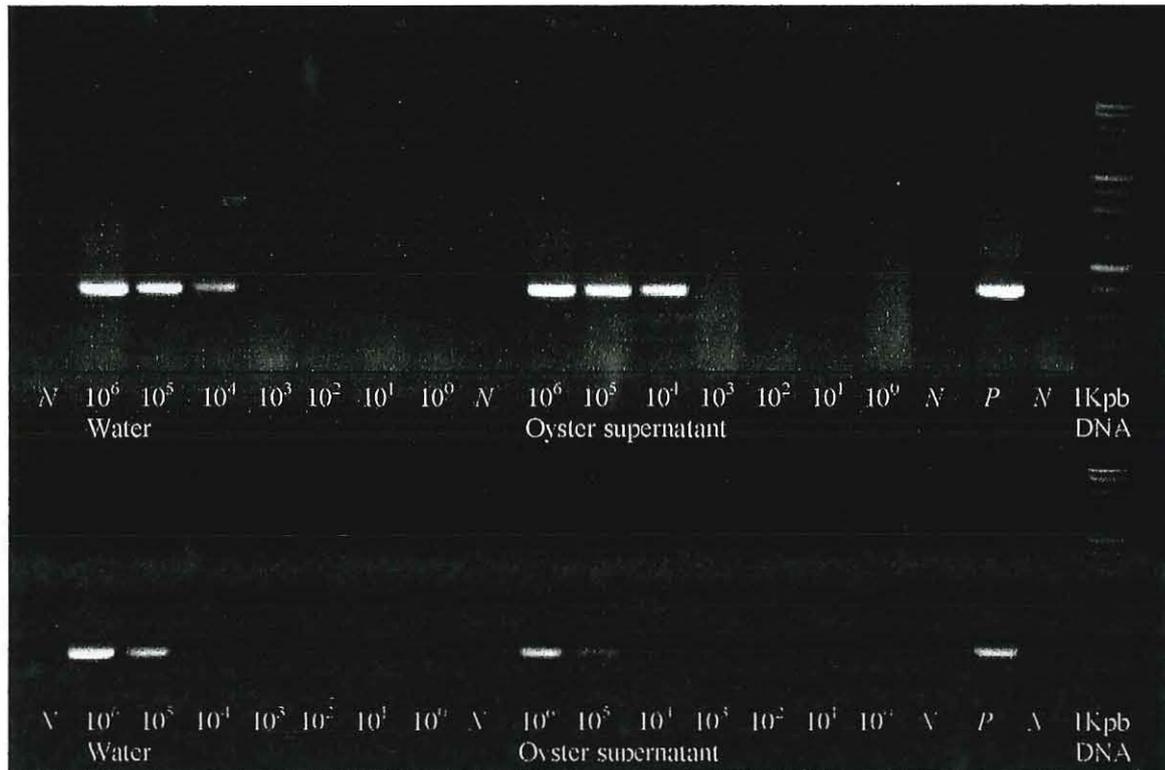
Sites	Batch code	Species	Age or size	Mortality rate	Date of sampling
Bangor, Gwynedd	002	<i>Crassostrea gigas</i>	5 month	0%	08/99
Newton Abbot, Devon	003	<i>Crassostrea gigas</i>	3 month	0%	08/99
	004	<i>Crassostrea gigas</i>	12 month	0%	08/99
	005	<i>Crassostrea gigas</i>	12 month	0%	08/99
Wadebridge, Cornwall	006	<i>Crassostrea gigas</i>	3 month	0%	08/99
	007	<i>Crassostrea gigas</i>	3 month	0%	08/99
Falmouth, Cornwall	008	<i>Ostrea edulis</i>	12 month	0%	08/99
	009	<i>Ostre.edulis</i>	12 month	0%	08/99
	010	<i>Ostrea edulis</i>	12 month	0%	08/99
Kent	011	<i>Ruditapes decussatus</i>	5mm	0%	12/99
	012	<i>Ruditapes decussatus</i>	5mm	0%	12/99
	013	<i>Crassostrea gigas</i>	1cm	0%	12/99
	014	<i>Oostrea edulis</i>	1-2cm	50%	12/99

PCR system trials and standardisation

First trial:

A dilution of OHV genomic DNA 10 ng/ μ l and sequences of primers OHV3 + OHV114 were provided by Dr. T. Renault (Participant 1). Ten fold serial dilutions of the above virus DNA were prepared in duplicate, in both molecular grade water and recovered oyster supernatant (previously identified as negative by PCR) (Figure 1).

Fig.1: Titrated OHV positive control (10 ng of viral DNA) within both water and recovered oyster supernatants. PCR products were ran in duplicate.



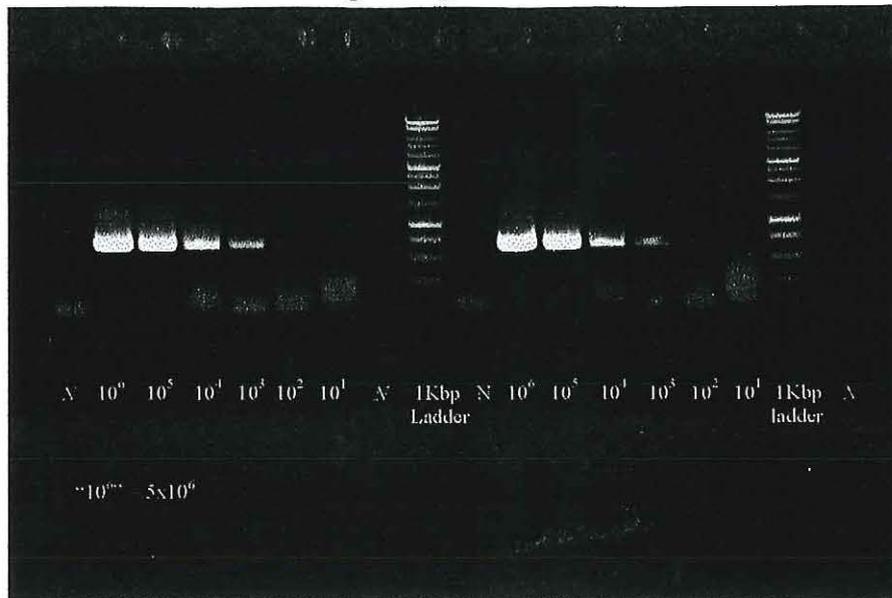
†The apparent differences are due to Ethidium Bromide staining and unidirectional migration.

The limit of detection of PCR products was corresponding to 10 pg of viral DNA present in a PCR tube. Detection sensitivity was not impaired by the positive control being titrated in oyster supernatant.

It can thus be concluded, that oyster supernatant holds no inhibitory agents/properties that directly affect PCR. However the sensitivity limit attained was significantly different compared to the sensitivity limit attained at IFREMER (Participant 1).

A second trial was conducted using a new batch of virus DNA (10 ng/ μ l) sent by Dr. T. Renault (Participant 1). This batch contained a freshly diluted virus DNA. PCR on serial dilutions was done as described above in duplicate. This trial was conducted in parallel in our laboratory and at IFREMER, by Dr. T. Renault. The limit of detection found in our laboratory was corresponding to 100 fg of viral DNA (Figure 2). This result may be compared to the 10 fg limit of detection found at IFREMER (Participant 1). This ten times difference in sensitivity could be explained by the use of different suppliers of chemicals and enzyme, and also the use of different models of thermocyclers.

Fig. 2: Titrated OHV positive control. PCR amplification products corresponding to 100 fg of viral DNA can be visualised on agarose gel.



Solutions to variations in the detection limit were proposed during the last meeting (December 1999). They include training of all partners in La Tremblade (February/March 2000) in order to ensure the standardisation of methods, use of common suppliers and the exchange of blind samples between associate laboratories to ensure quality control.