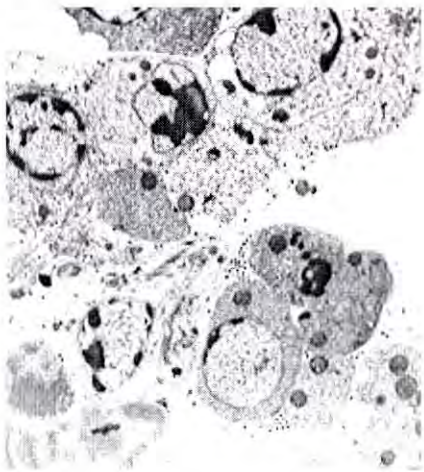
European Commission

Contract FAIR-CT98-4334

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

« VINO »

SECOND PERIODIC PROGRESS REPORT: 4th January 2000 to 3rd January 2001



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Abstract

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Abstract of the Progress Report for the period

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

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
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EC contact:	DG XIV.C.2 (F. Vander Elst: +32 2 299 54 08 and T. Tiainen: +32 2 299 52 56)		

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FAIR-PL98-4334

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

Abstract of the Progress Report for the period

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

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

II. DESCRIPTION OF WORK

Virus DNA cloning in cosmids and plasmids has been carried out in 1999 (Participant 2). The cloned viral DNA fragments have been used for characterising the virus genome and preparing specific diagnostic probes (Participants 1, 2 and 3). Identification of immunogenic viral proteins provided information in 1999 that facilitated production of viral recombinant proteins in 2000 (Participant 3). Mice and rabbits have been immunised in 2000 using recombinant proteins in order to produce specific antibodies (Participant 3).

A molecular biology workshop has been organised in May 2000 at the IFREMER laboratory (Participant 1) in La Tremblade (Charente Maritime, France) with Participants 4, 5, 6 and 7, in order to ensure that common protocols are used for PCR and *in situ* hybridisation methods developed in 1999 by Participant 1. Following this workshop, preliminary PCR experiments on serially diluted viral genomic DNA furnished by Participant 1 have been carried out. Participants 1, 4, 5, 6 and 7 have performed the PCR analysis in their own laboratory using the OHV3/OHV114 primer pair or the OHV1/OHV2 primer pair and the defined protocol. Positive and negative material (frozen larval samples and sections from fixed oysters) were

also supplied by Participant 1. Frozen material has been analysed by PCR. Histological sections have been used in order to test two *in situ* hybridisation protocols by Participants 1, 4, 5, 6 and 7.
A PCR procedure has also been developed in 2000 (Participant 1) and allowed to amplify short fragments of the OsHV-1 DNA after extraction of DNA from wax sections. Two primer pairs have been designed for this purpose: OH1/OH4 and IAP1/IAP2.

Tests of oyster primary cell cultures and vertebrate cell lines have been pursued in 2000 in order to study the ability of the virus to replicate *in vitro*.

The laboratories involved in mollusc epidemiological surveys (Participants 1, 5, 6 and 7) have taken bivalve samples during 1999 and 2000 in order to perform analyses to search herpes-like virus infections using the developed tools. In 2000, some samples have been analysed by PCR in order to diagnose herpes-like virus infections in bivalves.

III. STATE OF PROGRESS

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

Viral genomic DNA

At completion, 96.1% of the sequence was determined on both strands. The overall genome structure is: TR_L - U_L - IR_L - X - IR_S - U_S - TR_S with a 207439 bp total genome size. A detailed analysis of the coding potential of the genome sequence indicated the presence of 132 unique protein-coding open reading frames (ORFs). Amino acid sequence comparisons failed to identify a single protein which has homologues only in other herpesviruses. However, a genetic indication of a possible common origin between OsHV-1 and vertebrate herpesviruses resided with the ATPase subunit of the terminase.

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

PCR technique development

Two primer pairs (IAP1/IAP2 and OH1/OH4) were developed in order to analyse archived samples that were fixed for indeterminate periods. Both primer pairs yielded PCR products, which were the size expected for the OsHV DNA. DNA extracted from histological sections of infected oyster spat (viral particles detected by transmission electron microscopy) has served as template.

Preparation of recombinant proteins and antibodies for diagnosis use

Two clones identified by immunoscreening of the lambda library furnished by Participant 2 have been selected to produce recombinant antigens. Both resulting viral fusion proteins have been analysed by SDS-PAGE and immunological techniques. After purification by electrophoresis, they have been used for immunisation of laboratory animals (mice and rabbits) in order to produce specific monoclonal and polyclonal antibodies.

Testing culture cells for virus replication

Tests of oyster primary cell cultures and vertebrates cell lines have been pursued in 2000. Several assays of OsHV-1 cultivation have been performed in primary cultures of embryonic oyster cells. No obvious cytopathic effect was detected during experiments. However, PCR and *in situ* hybridisation assays showed the presence of viral DNA in examined cell samples. No cytopathic effect was detected in any passage conducted in the tested fish cell lines.

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

Collecting samples in 2000

The laboratories involved in epidemiological surveys among bivalves (Participants 1, 5, 6 and 7) have taken bivalve samples during 2000 in order to perform analyses to search for herpes-like virus infections using the developed tools.

PCR and in situ hybridisation analysis for virus diagnosis

A molecular biology workshop has been organised in May 2000 (15th to 19th May 2000) at the IFREMER laboratory (Participant 1) in La Tremblade (Charente Maritime, France) with Participants 1, 4, 5, 6 and 7, in order to ensure common protocols are used for PCR and *in situ* hybridisation. Following the molecular biology workshop, Participant 1 furnished reference material (viral DNA, 15 larval samples and histological slides as positive and negative reference material). Preliminary assays on serially diluted positive control material showed that detection of 1 fg of OsHV-1 genomic DNA may be routinely achieved in PCR experiments (Participants 1, 4, 5, 6 and 7). Positive and negative material (histological sections from fixed spat) were also used to test two different *in situ* hybridisation protocols.

Some bivalve samples taken in 1999 and 2000 have been analysed (Participants 1, 5, 6 and 7). No positive result was observed by Participants 5 and 7. Participant 6 reported a PCR positive result in a clam sample. However, in this case, the sample presented a lower band than the expected band. Three positive PCR batches have been observed by Participant 1 for animals collected in 2000.

IV. ACHIEVEMENTS

The task 1 - Obtaining a complete virus genomic library and DNA sequence - was completed at this time by Participant 2. All cloned materials are available to other participants.

Participant 3 has identified two immunogenic viral proteins and prepared two recombinant proteins as anticipated. These recombinant proteins have been used to immunise mice and rabbits in order to produce monoclonal and polyclonal antibodies.

V. FUTURE ACTIONS

Monoclonal and polyclonal antibodies will be produced using immunised mice and rabbits. The specificity of these antibodies will be characterised by ELISA and western blotting.

Tests of primary cultures of embryonic oyster cells will be pursued in 2001. PCR, *in situ* hybridisation and transmission electron microscopy will be used to confirm or not the presence of OsHV-1 in cells.

Molecular (PCR and *in situ* hybridisation) and immunological (monoclonal and polyclonal antibodies) tools will be used to diagnose herpes-like virus infections in bivalves by laboratories involved in epidemiological surveys. In order to validate the different techniques and reagents (PCR primers, labeled DNA probes and antibodies), Participant 1 will furnish to Participants 5, 6 and 7 reference material in 2001 : 20 frozensamples of infected oyster larvae and spat and histological sections of 30 infected oyster spat. This material will be used by

each participant involved in the task 3 (Application of DNA probes, immunological reagents and cellular tools for virus detection) in order to control if the different techniques used are reliable in the different laboratories.

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Consolided Progress Report

Reporting Period :
4th January 2000 to 3rd January 2001

FAIR-CT98-4334

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

Consolided Progress Report for the period

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

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EC contact:	DG XIV.C.2 (F. Vander Elst: +32 2 299 54 08 and T. Tiainen: +32 2 299 52 56)	

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

An initial step involved cloning of virus DNA in cosmids and plasmids. This work provided cloned viral DNA fragments suitable for characterising the virus genome and preparing specific diagnostic probes (PCR primers, labeled DNA probes and specific antibodies). Tests of oyster primary cell cultures and vertebrate cell lines are planned in order to study the ability of the virus to replicate *in vitro*. Successful cultures *in vitro* will provide a means of obtaining virus particles easily and tools to perform diagnostic assays. The development of molecular, immunological and cellular tools for OsHV-1 diagnosis will facilitate virus detection in infected material. 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

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

Viral genomic DNA

At the time of the last report, the sequence database took the form of three large fragments. Since then, the genome sequence has been completed and analysed. Specific regions in the database where information was absent or of poor quality were dealt with by PCR amplification of viral DNA and PCR products were sequenced. At completion, each nucleotide was determined an average of 10.8 times and 96.1% of the sequence was determined.

The overall genome structure is: TR_L - U_L - IR_L - X - IR_S - U_S - TR_S with a 207439 bp total genome size. TR_L and IR_L (7584 bp) are inverted repeats flanking a unique region (U_L, 167843 bp). TR_S and IR_S (9774 bp) are inverted repeats flanking a unique region (U_S, 3370 bp), and X (1510 bp) is located between IR_L and IR_S. A similar genome structure has evolved independently in certain vertebrate herpesviruses (e.g. herpes simplex virus and human cytomegalovirus).

Several experiments were undertaken to confirm the proposed genome structure.

- The sequences of the genome termini were determined. They are not located uniquely, but a predominant form is apparent for each.
- The nature of the sequence between IR_L and IR_S was determined. As with the termini, the IR_L - IR_S junction is not located uniquely, but the predominant form corresponds to a fusion of the two termini if each possesses two unpaired nucleotides at the 3' end. Unpaired nucleotides are characteristic of herpesvirus genome termini.
- Southern blot hybridisation experiments using PCR-generated probes from the ends of U_L and U_S showed that the two orientiations of U_L and U_S are present in approximately equimolar amounts in viral DNA, giving rise to four genome isomers. This is also a feature of the vertebrate herpesvirus genomes with similar structures.
- Both the database and restriction endonuclease digests indicated that a minor proportion (approximately 20-25%) of genomes contain a 4.8 kbp region in U_L in inverse orientiation.

These data indicate that the virus contains a mixture of genome forms. In light of the fact that the virion DNA that was sequenced originated from a virus that had not been clonally purified, this was not unexpected.

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

- Seven genes encode enzymes (DNA polymerase, deoxyuridine triphosphatase, two subunits of ribonucleotide reductase, helicase, a putative primase and the ATPase subunit of terminase).
- Seven proteins bear sequence similarities with viral or cellular inhibitors of apoptosis proteins. IAPs are also encoded by baculoviruses and entomopoxviruses (both of which have insect hosts) underscores the importance of the apoptotic responses of invertebrates against viral infections.
- Ten ORFs encode class I membrane proteins. An additional 17 proteins contain a hydrophobic domain indicating a possible assocation with membranes.
- A total of 39 proteins share sequence similarities with other proteins encoded by the virus, defining 13 multigene families in addition to the IAPs.
- An additional notable feature, located between ORFs 50 and 51, is a large palindrome. By analogy with certain vertebrate herpesviruses, this palindrome is a candidate origin of DNA replication.

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

Developing molecular tools and techniques

Two primer pairs have been developed in order to amplify small DNA fragments from OsHV-1 DNA. The first primer pair, called OH1/OH4, yielded 196 bp amplicons when genomic viral DNA was used as template. The size of PCR products obtained with the second primer pair (IAP1/IAP2) was 207 bp. Both primer pairs have been designed in order to obtain PCR amplification when DNA extracted from histological blocks was used as template. Several primer pairs (OHV3/OHV4, OHV3/OHV114, A3/A4 and A5/A6) have already been tested using this type of DNA. They failed producing amplicons. These results could be explained

by DNA fragmentation. Both primer pairs OH1/OH4 and IAP1/IAP2 allowed the production of amplicons when DNA extracted from wax blocks was used. A classical technique was choosen for DNA extraction from histological sections using dewaxing in xylene and treatment with proteinase K. Archive material has been used. Five histological blocks prepared in 1995 corresponding to *Crassostrea gigas* spat have been selected. Animals presenting high mortality rates have been fixed individually in Davidson's fluid during the summer of 1995. Transmission electron microscopy examination allowed to detetect viral particles. PCR analysis using DNA extracted from these blocks showed clear bands presenting expected sizes when both primer pairs were used. This suggested that both primer combinations were reliable tools to detect viral DNA in archive material. Moreover, the primer pairs have been designed in two different areas of the genomic viral DNA enhancing the specificity of the detection. The OH1/OH4 primer pair recognises a gene coding for a protein of unknow function and the IAP1/IAP2 primer combination amplifies a fragment of a gene corresponding to a putative inhibitor of apoptosis (IAP).

Identification of immunogenic proteins
The immunoscreening with specific anti-OsHV-1 antibodies and the results of sequencing of the virus genome allowed to identify two open reading frames (ORFs) encoding for putative immunogenic viral proteins. The first ORF (ORF94), codes for a protein of 748 amino acids. This protein contains a highly hydrophobic C-terminal domain, potential N-glycosylation sites (Asn-X-Ser/Thr) and a potential signal peptide at the N-terminal end. This ORF could code for a membrane glycoprotein, the typical profile of surface viral antigens. The second identified ORF (ORF43) codes for a protein of which different regions are recognized by anti-OsHV-1 ascitis. This protein of 364 amino acids doesn't present the characteristics of membrane proteins but seems to be a potential immunogenic protein. This ORF codes for a protein presenting homologies with baculovirus, insect and mammal IAPs (Inhibitor of Apoptosis). The two ORFs, ORF43 and ORF94, encoding for putative immunogenic viral proteins have been cloned in baculovirus expression system in order to prepare recombinant proteins and antibodies for diagnosis use. Others plaques have been analysed in order to isolate a third candidate. The other sequenced clones were not predicted anymore to express real OsHV-1 proteins. Thus, the search of a third candidate has not been successful.

The baculovirus system has been choosen rather than *Escherichia coli* as insect cells are more convenient for folding recombinant glycoproteins in a native conformation and with post-translationnal modifications. The recombinant baculoviruses have been constructed by co-transfection in Sf9 cells with BaculoGold (Pharmingen) and baculovirus transfer vectors. The vectors produced recombinant proteins with a His-tag and a Flag-tag. Recombinant baculoviruses obtained were called respectively ORF43s, ORF43c and ORF94 and were amplified in Sf9 cells. In futher steps, Tn5 cells have been used since these insect cells are recommended for the secretion of recombinant proteins. Both proteins were detected in the intracellular fraction, by Coomassie blue staining. The ORF43 had an apparent molecular weight of ~50 kDa and was in a doublet form. The ORF94 had an apparent molecular weight of 90 kDa and had a quite diffused coloration which is the characteristic of glycosylated proteins. The secreted forms were not detected by Coomassie blue staining, but they were weakly detected by western blotting. Since the level of secreted proteins was too weak, the purification of both proteins was performed from intracellular fractions.

For both proteins, the best conditions selected for solubilization of the insoluble cell fraction before purification was 10 mM NaOH. A 4 l production for both proteins has been performed in Tn5 cells. After 48h, infected cells were lysed with Triton X-100 and recombinant proteins

solubilized with NaOH 10mM. Solubilized proteins were submitted to an electrophoresis through a cylindrical acrylamid gel with SDS. Purified ORF43 and ORF94 proteins were identified by western blotting with an anti-Flag antibody. 2.8 mg of ORF43 and 2.3 mg of ORF94 have been purified and served for mouse and rabbit immunisation. Two rabbits and four mice were injected. ELISA for selection of antisera and monoclonal antibodies will be performed on insect cells rather than on purified antigen. Assays were done to determine the cell concentration for plate coating with infected Tn5 cells. Revelation was performed with an anti-Flag antibody. 5 10^4 cells/well will be used in further ELISA. Specific insect cell preparations were obtained for ELISA coating. Tn5 cells have been infected by the recombinant baculovirus ORF43s and the recombinant baculovirus ORF94. Uninfected cells were also prepared as control for antibodies against insect cell epitopes. As the baculovirus infected cells are sensitive to the proteases, they have been kept at –80°C until using in ELISA and blotting assays.

Testing culture cells for virus replication
Several assays of herpes-like virus cultivation in oyster primary cultures and fish cell lines have been carried out in 2000. OsHV-1 cultivation experiments have been performed in primary cultures of embryonic oyster cells. No obvious cytopathic effect was detected. However, PCR analysis using the OHV1/OHV2 primer pair showed amplicons presenting the expected size when DNA extracted from infected cells was used as template. *In situ* hybridisation assays have also been carried out on cell samples showing positive PCR results. Using a peroxydase/DAB indirect detection system, some cells presented a positive labeling at the cellular periphery and sometimes in the cytoplasm. No cytopathic effect was detected in any passage conducted in the tested fish cell lines.

Task 3. - Application of DNA probes, immunological reagents and cellular tools for virus detection
Collecting samples in 2000
The laboratories involved in epidemiological surveys among bivalves (Participants 1, 5, 6 and 7) have taken bivalve samples during 2000 in order to perform analyses to search for herpes-like virus infections using the developed tools.

PCR and in situ hybridisation analysis for virus diagnosis
A molecular biology workshop has been organised in May 2000 (15th to 19th May 2000) at the IFREMER laboratory (Participant 1) in La Tremblade (Charente Maritime, France) with Participants 1, 4, 5, 6 and 7, in order to ensure common protocols are used for PCR and *in situ* hybridisation.

Following the molecular biology workshop, Participant 1 furnished reference material (viral DNA, 15 larval samples and histological slides as positive and negative reference material). Preliminary assays on serially diluted positive control material showed that detection of 10 to 100 fg of OsHV-1 genomic DNA may be routinely achieved using the OHV3/OHV114 primer pair in the different laboratories involved in this test (Participants 1, 4, 5, 6 and 7). Morover, the OHV1/OHV2 primer pair allowed to amplify systematically 1 fg of viral DNA. 15 frozen larval samples have also been analysed by PCR. Five samples appeared systematically positive when they were tested in the different laboratories. However, other larval samples appeared positive or negative depending of the laboratories. Positive and negative material (histological sections from fixed spat) were also used to test two different *in situ* hybridisation protocols. The protocol based on a alkaline phosphatase/BCIP-NBT direct

detection system seemed to give better results than the protocol based on a peroxydase/DAB indirect detection system.

Some bivalve samples taken in 1999 and 2000 have been analysed (Participants 1, 5, 6 and 7). No positive result was observed by Participants 5 and 7. Participant 6 reported a PCR positive result in a clam sample. However in this case, the sample presented a lower band than the expected band. Three positive PCR batches have been observed by Participant 1 for animals collected in 2000.

Discussion-Conclusion

The sequence data demonstrate that the oyster herpes-like virus (OsHV-1) in not closely related to herpesviruses with vertebrate hosts (including fish). Amino acid sequence comparisons failed to identify a single protein which has homologues only in other herpesviruses. Several OsHV-1 proteins have homologues that are distributed widely in nature (e.g. DNA polymerase), but these are no more closely related to homologues in other herpesviruses that to homologues in other organisms. In this context, phylogenetic analyses are not of great utility in determining whether OsHV-1 and vertebrate herpesviruses have a common origin. However, a genetic indication of a common origin between OsHV-1 and vertebrate herpesviruses resides with the ATPase subunit of the terminase. Homologous genes are present in all herpesviruses, and the only non-herpesvirus counterparts are specified by T4 and related bacteriophages. The T4 and OsHV-1 genes are unspliced, whereas those in herpesviruses of mammals and birds contains one intron and those in herpesviruses of fish and amphibians contains two introns. Moreover, a similar genome structure was observed in certain vertebrate herpesviruses. The presence of several isomers described in the OsHV-1 genome is also a feature reported in vertebrate herpesvirus genomes.
The available data support the view that herpesviruses of mammals and birds, herpesviruses of fish and amphibians and herpesviruses of invertebrates form three major lineages of the herpesviruses. OsHV-1 would have established a separate lineage about a billion years ago, and the fish viruses about 400 million years ago. OsHV-1 is currently the single representative of what may be a large number of invertebrate herpesviruses. Morover, recent data shown that OsHV-1 can infect several bivalve species. This contrasts with vertebrate herpesviruses, which are generally confined to a single species in nature. Consequently, the true host of OsHV-1 is unknown. The apparent loss of several gene functions in OsHV-1 prompts the speculation that this may have promoted interspecies transmission in the context of introduction of non-native bivalve species and use of modern aquaculture techniques. It is possible that the parental virus still resides in its natural host.

Two putative immunogenic viral proteins have been identified by immunoscreening of a lambda library. Corresponding clones have been used to produce two recombinant proteins using the baculovirus system. The search of a third immunogenic viral protein failed using the immunoscreening of the lambda library. However, the prodcuction of polyclonal and monoclonal antibodies specific for the two selected viral proteins will furnish specific immunological reagents. These reagents will be used for immunological diagnosis of OsHV-1 infections. However, a delay in producing monoclonal antibodies will be observed. Analyses using these reagents will be carried out in the last 6 months of the programme. Participants 1, 5, 6 and 7 have been informed of this delay for the sub-task 3.2. - Application of immunological methods to the diagnosis of oyster herpes-like virus. However, polyclonal

antibodies production will be completed as anticipated at the beginning of 2001. They will permit first immunological analysis of oyster samples.

No cytopathic effect has been observed in 1999 and 2000 in tested fish cell lines. OsHV-1 may be not able to multiply in fish cell lines or under cultivation conditions used viral replication doesn't occur. Tested fish cell lines cannot be used for the herpes-like virus infection diagnosis. Preliminary assays performed in primary cultures of embryonic oyster cells showed the presence of viral DNA in infected cultures using PCR and *in situ* hybridisation. However, experiments must be reiterated and other techniques as transmission electron microscopy used in order to demonstrate the presence of the virus in embryonic cells. Although promising results have been observed, primary cultures of embryonic oyster cells are not at this time a reliable tool to detect OsHV-1. Cellular tools are not available and analysis of oyster samples using such tools as anticipated will not be performed in 2001.

Most of the PCR analyses of bivalve samples failed to show positive results. Validation of the developed tools should be difficult in these conditions. Validation will be carried out using reference materials. Participant 1 will furnish in 2001 some positive reference oyster samples and Participants 1, 5, 6 and 7 will be used this material to test molecular and immunological tools in their own laboratory.

Future actions

Monoclonal and polyclonal antibodies will be produced using immunised mice and rabbits. They will be characterised by ELISA and western blotting in 2001. ELISA for selection of antisera and monoclonal antibodies will be performed on insect cells rather than on purified antigen.

Tests of oyster primary cell cultures will be pursued in 2001 in order to investigate whether viral replication occurs *in vitro*. Some assays will be performed in order to try to cryopreserve « embryoïds » and to cultivate the herpes-like virus in heart cells. Some experiments will be also carried out to verify if the herpes-like virus introduces insides the embryonic cells or if it is only adsorbed on their plasmic membrane (by *in situ* hybridization and PCR). This information could be obtained after an enzymatic treatment of cells, which could detach viral particles from the plasmic membrane and by increasing the time of contact of the virus and the cells in culture.

Molecular (PCR and *in situ* hybridisation) and immunological (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 2000 and 2001 will be used.
Participant 1 will furnish again reference material (viral DNA, frozen positive larval and spat samples, and infected oyster histological sections) as positive reference material in order to validate the developed reagents and techniques. OH1/OH4 and IAP1/IAP2 primer pairs will be used to define the status of the selected wax blocks which will serve to prepare positive histological sections. For each block (30), *in situ* hybridisation and immunochemistry will be used to detect the presence of herpes-like viruses in the four laboratories involved in epidemiological surveys. Several techniques will be used on the same material and comparisons be done.

METHODOLOGY AND RESEARCH TASKS

Task 1 - Obtaining a complete virus genomic library and DNA sequence
Participant : 2
Duration : 24
Current status : completed
Total estimated man-month : 20
N° of man-month devoted already to the task : 25.5

Objectives :

- Obtaining the complete DNA sequence of the viral genome and determining the virus genome structure
- Preparating a lambda expression library in order to identify immunogenic proteins
- Studying the relationships of the OsVH-1 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. For the second, large quasi-random fragments generated from the viral genome were inserted into a cosmid vector. For the third, viral DNA fragments were inserted into a lambda expression vector. For DNA sequencing, random fragments of viral DNA were inserted into a standard M13-based sequencing vector. The sequences of several thousands of clones were derived by standard automated methods. The sequences were assembled into a database using Staden's software.

Deliverables during the second reporting period :

- Sequence and general structure of OsHV-1 genome
- Sequence comparisons with vertebrate herpesviruses

Links with other tasks :
Concomitant derivation of the complete DNA sequence provided information and materials for use in Sub-task 2. 2.

Progress in the second reporting period :
This part of the programme has been successfully completed.

Sub-task 1. 1. : Cloning of viral DNA in bacterial vectors
Participant : 2
Duration : 6
Current status : completed during the first reporting period
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. Ligated DNA was transfected into competent *Escherichia coli* DH5α and ampicillin-resistant colonies isolated. A library of clones representing the majority of the genome was established.
- Viral DNA was digested partially with *Bam*HI or *Sau*3AI. The fragments were ligated into a cosmid vector. Ligated DNA was packaged into phage lambda particles. Packaged particles were used to infect *E. coli* XL-1 Blue MR, and ampicillin-resistant clones isolated.
- Information about the genome structure has been produced from restriction enzyme cleavage data and Southern blot hybridization experiments.
- Viral DNA was digested partially with *Sau*3AI. The fragments was ligated into a lambda expression vector. Ligated DNA was packaged into phage lambda particles. Packaged particles were used to infect *E. coli* XL-1 Blue MRF', and an expression phage library was established.

Progress during the second reporting period :
(This sub-task has already been completed during the first reporting period).

Sub-task 1. 2. : Sequencing the virus genome
Participant : 2
Duration : 12
Current status : completed during the second reporting period
Total estimated man-month : 8
N° of man-month devoted already to the task : 10

Objective :
Obtaining the complete virus DNA sequence

Methodology :

- Random fragments 600-1000 bp in length were produced from viral DNA. The fragments were ligated to M13mp19. The ligation was transfected into competent *Escherichia coli* DH5αF' and plated onto a lawn of *E. coli* XL-1 Blue. Recombinant phages were produced in 96-well trays and template DNA prepared by treatment with 4 M sodium iodide followed by ethanol precipitation.
- The templates were sequenced using an ABI PRISM 377 DNA sequencer.
- The resulting random sequences were assembled into a database using Staden's Gap4 software. Residual problems have been resolved by specific experiments involving PCR of viral or cloned DNA and resequencing of problematic regions.

Progress during the second reporting period :
This sub-task has been successfully completed.

Sub-task 1. 3. : Phylogenetic analysis of the oyster virus
Participant : 2
Duration : 6
Current status : completed during the second reporting period
Total estimated man-month : 6
N° of man-month devoted already to the task : 9.5

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

Methodology :

- The completed DNA sequence has been analysed for genetic content using the standard suite of programs available in the Genetics Computer Group (GCG) package. Identification of open reading frames (ORFs) potentially encoding viral proteins has been carried out. Genes were identified from their sequence properties using appropriate programs (e.g. Pepplot).
- Global comparisons of predicted protein sequences with proteins encoded by other herpesviruses and other organisms has been 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 OsHV-1, phylogenetic comparisons have been carried out with vertebrate herpesviruses using appropriate software available from the GCG suite (e.g. Pileup) and other sources.

Progress during the second reporting period :
This sub-task has been successfully completed.

Task 2 - Developing tools for the diagnosis of herpes-like virus infections
Participants : 1, 3, 4 and 6
Duration : 36
Current status : 8 months to completion
Total estimated man-month : 63 (10, 34, 15 and 4)
N° of man-month devoted already to the task : 62.5 (7, 37, 11 and 7.5)

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 and specific primer pairs have been developed and applied for virus detection in bivalve samples. An *In situ* hybridization method was also developed using labelled DNA probes obtained by incorporation of digoxigenin-dUTP during PCR. It was applied for virus detection on histological sections of oyster tissues fixed in Carson's and Davidson's fluids. A first protocol based on an alkaline phosphatase/BCIP-NBT direct detection system has been tested. The second protocol was based on a peroxidase/DAB indirect detection system.

Immunoscreening of the expressed recombinant proteins was performed onto nitrocellulose filters. Proteins were screened with mouse anti-virus antibodies (supplied by Participant 1). Fixed antibodies were revealed with an appropriate peroxidase conjugated antisera. Specific antigens were revealed by colorimetric enzymatic reaction. Plasmids were extracted from immunologically reactive clones. The baculovirus system has been choosen in producing recombinant glycoproteins. The recombinant baculoviruses have been constructed by co-transfection in Sf9 cells with BaculoGold (Pharmingen) and baculovirus transfer vectors. In further steps, Tn5 cells have been used. Since the level of secreted proteins was too weak, the purification of proteins was performed from the intracellular fractions by electrophoresis. Purified recombinant proteins were identified by western blotting. Immunization of laboratory animals have been performed following conventional methods with purified recombinant proteins.

Oyster embryos have been obtained using conventional techniques of *in vitro* fecondation. Isolation of oyster embryonic cells has been carried out following a method described for scallop heart and gills and oyster embryos. Oyster cells were cultivated in a sea water-based medium. Infected and non infected larval tissues were inoculated in primary oyster cell cultures and several fish cell lines following routine procedures. DNA probes specific for herpes-like virus have been applied in order to detect the virus in centifuged oyster cells. PCR analysis has been also performed on the same biological material.

Deliverables during the second reporting period :
- Development of a PCR method and specific primer pairs to detect the viral genome in DNA samples extracted from histological blocks
- Obtaining and producing viral recombinant proteins
- Immunisation of mice and rabbits
- Detection of viral DNA in infected oyster embryonic cells by PCR and *in situ* hybridisation

Links with other tasks :
Deliverables obtained in task 2 provided information and techniques used in the task 3.

Progress during the second reporting period :
A PCR protocol has been developed in order to amplify the herpes-like viral genome in DNA samples extracted from archive histological blocks.
Immunisation of mice and rabbits has been carried out using two recombinant viral proteins.
Viral DNA has been detected in infected embryonic oyster cells using PCR and *in situ* hybridisation techniques.

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 during the first reporting period
Total estimated man-month : 6
N° of man-month devoted already to the task : 6

Objective :
Development of PCR and *in situ* hybridisation techniques for herpes-like virus detection in bivalves

Methodology :

- Using sequences of cloned viral DNA fragments (Task 1), primer pairs were designed. PCR parameters were optimised using virus DNA extracted from purified particles as template (1 fg to 100 ng). The specificity of the PCR was tested using DNA extracted from healthy oysters and different vertebrate herpesvirus DNAs as template. 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.
- Oyster samples were fixed in Carson's and in Davidson's fluids. Sections were cut 7µm thick. Digoxigenin-labeled DNA probes were obtained by incorporation of digoxigenin-dUTP during a PCR step using oyster herpes-like virus genomic DNA and the OHV4/OHV4 primer pair. After proteolysis with proteinase K treatment and denaturation of the target DNA and probes at 94°C, the tissue sections were hybridised in buffer containing 5ng/µl of digoxigenin labelled probes overnight at 42°C. Immunological detection of labeled probes have been carried out using two protocols. A first protocol based on an alkaline phosphatase/BCIP-NBT direct detection system has been developed. A second protocol was based on a peroxidase/DAB indirect detection system.

Progress during the second reporting period :
This sub-task has been succesfully completed during the first reporting period. However, additional information has been obtained about PCR amplification using DNA extracted from histological blocks during the second reporting period.

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 : 8 months to completion
Total estimated man-month : 34
N° of man-month devoted already to the task : 37

Objectives :
- Identification of immunogenic virus proteins
- Preparation of recombinant proteins
- Preparation of antibodies specfic for the recombinant proteins

Methodology :
The lambda library prepared in Task 1 was screened using mouse antibodies generated against virus particles in order to identify immunogenic viral proteins. Selected proteins have been expressed via a plasmid in *Escherichia coli*. Production of selected recombinant proteins provided material for positive controls included in immunological diagnostic assays. The viral recombinant proteins have been used to prepare specific antibodies.

- The recombinant proteins expressed by the lambda library have been immunologically screened using polyclonal antibodies. Lysed bacterial colonies were transfered on nitrocellulose membranes. Membranes were then incubated with specific antibodies followed by appropriate antibodies conjugated with horse radish peroxidase. Specific antigens were revealed by colorimetric enzymatic reaction.

- Plasmids were extracted from immunologically reactive clones and sequenced. The sequencing work was performed on an ABI PRISM 377-based fluorescent system. In a second step, specific oligonucleotides have been designed at both extremities of the coding sequences and used to amplify the DNA fragments to be cloned. Restriction sites have been included at the extremities of the chosen oligonucleotides. The baculovirus system has been choosen rather than *Escherichia coli* as insect cells are more convenient for folding recombinant glycoproteins in a native conformation and with post-translationnal modifications. The recombinant baculoviruses have been constructed by co-transfection in Sf9 cells with BaculoGold (Pharmingen) and baculovirus transfer vectors. The vectors produced recombinant proteins with a His-tag and a Flag-tag. Recombinant baculoviruses obtained were called ORF43s, ORF43c and ORF94 respectively and amplified in Sf9 cells. In further steps, Tn5 cells have been used since these insect cells are recommended for the secretion of recombinant proteins. Both proteins were detected in the intracellular fraction using Coomassie blue staining. Secreted forms were not detected in acrylamide gels, but they were weakly detected by western blotting. Since the level of secreted proteins was too weak, the purification of both proteins was performed from the intracellular fractions.
- For both viral recombinant proteins, the best conditions selected for solubilization of the insoluble cell fraction before purification was 10 mM NaOH. A 4 l production for both proteins in Tn5 cells has been performed. After 48h, infected cells were lysed with Triton X-100 and recombinant proteins solubilized with NaOH 10mM. Solubilized proteins were submitted to an electrophoresis through a cylindrical acrylamide gel with SDS. Purified ORF43 and ORF94 proteins were identified by western blotting with an anti-Flag antibody.
- Two rabbits and four mice were injected using conventioal techniques. ELISA for selection of antisera and monoclonal antibodies will be performed on insect cells rather than on purified antigen. Assays were done to determine the cell concentration for plate coating. 5 10^4 cells/well will be used in further ELISA. Specific insect cell preparations were done for ELISA coating. Cells were infected and harvested according to the previous results. Uninfected cells were also prepared as control for antibodies against insect cell epitopes. As the baculovirus infected cells are sensitive to the proteases, they were aliquoted and kept at –80°C until using in ELISA and blotting assays.

Progress during the second reporting period :
Two viral recombinant proteins have been produced and served for mouse and rabbit immunisation. Polyclonal and monoclonal antibodies will be obtain in 2001.

Sub-task 2. 3. : Testing oyster primary cell cultures and vertebrate cell lines
Participants : 1, 4 and 6
Duration : 21
Current status : 6 months to completion
Total estimated man-month : 23 (4, 15 and 4)
N° of man-month devoted already to the task : 19.5 (1, 11 and 7.5)

Objectives :
- 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 have been used in order to grow *in vitro* different cell types originating from *Crassostrea gigas* embryos, larvae and heart from adult Japanese oysters. The resulting primary cell cultures have been used to test the ability of the virus to multiply *in vitro*. Fish cell lines were also tested.

- Frozen oyster larvae are used to try to infect primary oyster cell cultures. Larvae were ground in sterile culture medium or in sterile sea water and filtered to 0.45 or 0.22µm. The virus suspensions were inoculated on cell cultutres and incubated 2 hours before adding new culture medium.
- Infected primary cultures of embryonic cells and infected fish cell lines were observed daily during several weeks in order to detect eventual cytopathic effects. For transmission electron microscopy, cultured cells were 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 were double stained with uranyl acetate and lead citrate. PCR and *in situ* hybridisation analyses have also been carried out in order to detect viral DNA in infected embryonic oyster cells. A step of DNA extraction has been added prior to the PCR reaction in order to avoid inhibition. *In situ* hybridisation assays have been carried out using the methodology developed by Participant 1.

Progress during the second reporting period :
No cytopathic effect has been reported in tested fish cell lines and in primary cultures of embryonic oyster cells. However, PCR and *in situ* hybridisation analysis demonstrated the presence of viral DNA in infected embryonic oyster cells at the cell surface or in the cytoplasm.

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 : 12 months to completion
Total estimated man-month : 62.6 (17, 19, 17 and 9.6)
N° of man-month devoted already to the task : 23.5 (7.5, 3.75, 6.5 and 5.75)

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

Overview of the methodology :
Different geographical sites have been sampled. 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 several bivalve species were cultivated, samples of different species have been taken. 30 animals have been taken on each occasion from each site. Time restrictions may prevent examination of all animals from all sites, but extensive sampling

ensured that, even if not looked at immediately that samples will be available for future reference. Samples were divided into two : one for histology and tansmssion electron microscopy sections for use in routine diagnosis, immunological labeling and *in situ* hybridisation; the other will be stored frozen for PCR.

Deliverables :
♦ Organisation of a molecular biology workshop
♦ First assays for intercalibartion of the PCR technique between the different laboratories involved in this task
♦ First analysis of bivalve samples using PCR

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

Progress during the second reporting period :
A molecular biology workshop took place in La Tremblade in May 2000 and allowed to ensure that the same protocols will be used in the different laboratories involved in sample analysis.
No viral detection was reported by PCR in samples from Ireland and United Kingdom (Participants 5 and 7). A positive sample was detected in Spain (Participant 6) and corresponded to a clam species. Three samples obtaining during summer mortalities in France (participant 1) in 2000 showed the presence of viral DNA.

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 : 12 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 : 22.25 (7.25, 3.75, 5.5 and 5.75)

Objective :
Detection of herpes-like virus in oysters using molecular tools

Methodology :
- Several batches have been taken in 1999 and 2000. For each batch, 30 individuals have been sampled. The oysters were removed from the shell, sagitally sectioned, then half was frozen at - 80°C or at - 20°C. The other half is again sagittaly sectioned and one part was fixed in Davidson's fluid and the other one in Carson's fluid. PCR analysis has been carried out on frozen oyster samples using the OHV3/OHV114 primer pair or the OHV1/OHV2 primer pair and the PCR protocol defined in Sub-task 2.1.
- Molecular tools in virus detection will be validated using reference material furnished by Particpant 1. Oyster samples fixed in Carson's or in Davidson's fluids will be used to prepare paraffin-embedded sections (7μm) and labeled DNA probes will be used to detect viral DNA.

Progress during the second reporting period :
No viral detection was reported by PCR in samples from Ireland and United Kingdom. A positive sample was detected in Spain and corresponded to a clam species. Three samples obtaining during summer mortalities in France in 2000 showed the presence of viral DNA.

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 : 12 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)
- Immunological methods in virus detection will be validated using reference material furnished by Particpant 1. 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 specfic for viral recombinant proteins will be used as immunological probes. The detection will be done using a peroxidase conjugate and diaminobenzidine.

Progress during the second reporting period :
No analysis has been performed using immunological tools in oyster samples.

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 : 6 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 :
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 0.45μm. Cell cultures will be inoculated with virus suspensions and incubated 2 hours before adding new culture medium. Primary cell cultures will be observed daily during several days in

order to detect eventual cytopathic effects. However, analysis can be made only if the cellular tools are available and reliable.

Progress during the second reporting period :
No analysis has been performed using cellular tools in oyster samples.

Task 4 - General organisation of the programme and synthesis of results
Participants : : 1, 2, 3, 4, 5, 6 and 7
Duration : 36
Current status : 12 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 (1, 0.6, 0.6, 0.6, 0.6, 0.6 and 0.6)

Objectives :

- General organisation of the programme
- Preparation and discussion of protocols
- Coordination of tasks between the differents 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 organised during the first month (January 1999) after the contract was signed in order to adjust the calendar and the content of the different tasks. 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. The first and the second annual meetings took place at the IFREMER headquarter in Issy-les-Moulineaux (France) in December 1999 and in December 2000 respectively.

Links with other tasks :
Link with tasks 1, 2 and 3 between all participants

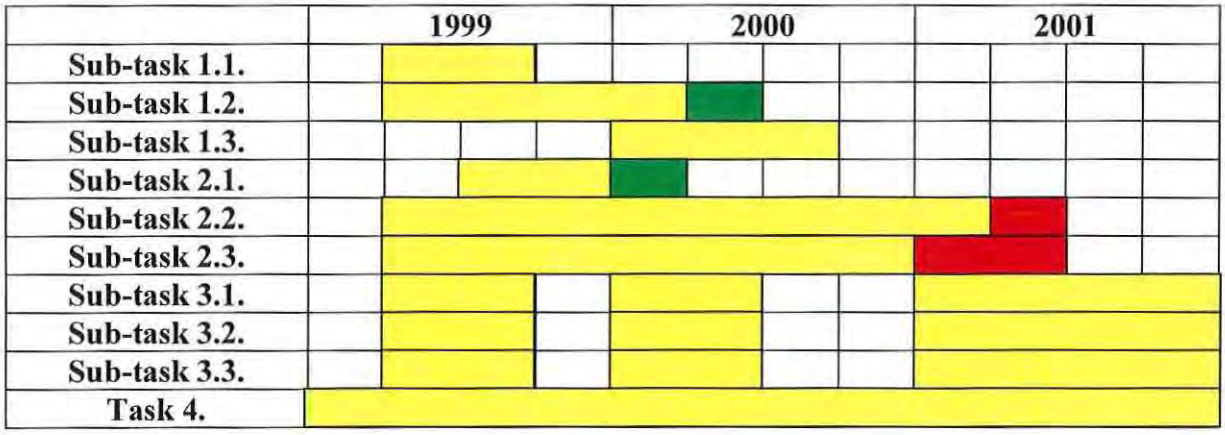
Progress during the second reporting period :
A molecular biology workshop was organised in La Tremblade (IFREMER, Charente Maritime, France) in May 2000 and the second annual meeting took place in December 2000 at the IFREMER headquarter in Issy-les-Moulineaux (France).

MILESTONES

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 the OsHV-1 sequence: 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 and *in situ* hybridisation) for OsHV-1 detection: the methodology was fully developed. The development of sensitive and specific techniques has ben 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 have been identified in the lambda library. Two viral recombinant proteins have been produced and served to immunise laboratory animals.
5. Obtaining oyster herpes-like virus multiplication in oyster primary cell cultures and/or on vertebrate cell lines: the results obtained concerning Sub-task 2. 3. determine the feasibility of Sub-task 3. 3. No vertebrate cell line allowed the detection of herpes-like viral replication. The results obtained in embryonic oyster cells were unclear and need to be reiterated. Molecular techniques showed the presence of viral DNA although no cytopathic effect was detected in infected embryonic oyster cells.
6. Collecting oyster samples: application of molecular probes and immunological reagents for OsHV-1 detection will be possible only if mortalities occur among bivalves during the course of the programme. If no mortality is observed, validation of the diagnostic reagents will be difficult.
   However, positive samples are available in the laboratory from La Tremblade (France, Participant 1) and will serve as reference material for comparative studies.

The following tables sum up the progress status of each sub-task with regard to the scheduled project.

Operation	In preparation	Started	Running	Finished
Sub-task 1.1.				Finished
Sub-task 1.2.				Finished (in advance)
Sub-task 1.3.				Finished (in advance)
Sub-task 2.1.				Finished (in advance)
Sub-task 2.2.		Started with 2 month delay	Prolonged until June 2001	
Sub-task 2.3.		As scheduled	Prolonged until June 2001	
Sub-task 3.1.		In advance		
Sub-task 3.2.	As scheduled			
Sub-task 3.3.	As scheduled			
Task 4.		As scheduled	As scheduled	



Initial planning:

Prolongation of task duration:

Reduction of task duration:

DELIVERABLES

First periodic progress report (February 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 (Participants 1, 3 and 4)

- ♦ Obtaining specific primers and probes for diagnosis by PCR and *in situ* hybridisation (Participant 1)
- ♦ Screening of the lambda expression library (Participant 3)
- ♦ Preparation of primary cultures of embryonic oyster cells (Participant 4)
- ♦ Tests of primary cultures of oyster cells and vertebrates cell lines for herpes-like virus cultivation (Participants 1 and 6)

Second annual progress report (February 2001)

Task 1 - Obtaining a complete virus genomic library and DNA sequences (Participant 2)
♦ Determination of the genetic contents of the oyster herpes-like virus genome
♦ Phylogenetic analysis of the herpes-like oyster virus

Task 2 - Developing tools for the diagnosis of herpes-like virus infection (Participants 1, 3 and 4)
♦ Development of a PCR procedure for viral DNA detection in samples extracted from wax sections
♦ Production of recombinant proteins and immunisation of mice and rabbits

Task 3 - Application of DNA probes, immunological reagents and cellular tools for virus detection
♦ Organisation of a molecular biology workshop (Participant 1)
♦ First assays for intercalibartion of the PCR technique between the different laboratories involved in this task (Participants 1, 4, 5, 6 and 7)
♦ First analysis of bivalve samples using PCR (Participants 1, 5, 6 and 7)

Third annual report (final report)

This report will describe results concerning :

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

DISSEMINATION OF RESEARCH RESULTS

Publishing in international scientific journals and participation in national and international meetings have been used to ensure proper dissemination of information.

- Contribution to articles related to OsHV :

I. Arzul, **T. Renault**, C. Lipart and **A.J. Davison** (in press). Evidence for interspecies transmission of oyster herpesvirus in marine bivalves. Journal of General Virology.

P.F. Dixon, **S.J. Bark**, **R.M. Le Deuff** and **P.D. Martin** (2000). Herpesviruses in marine molluscs. Shellfish News, 10: 18-20.

T. Renault (2000). Surveillance des virus de type herpès chez les larves et les juvéniles d'huître creuse. Lettre médias, IFREMER, 52:2 et 7.

T. Renault and **I. Arzul** (in press). Herpes-like virus infections in hatchery-reared bivalve larvae in Europe: specific viral detection by PCR. Journal of Fish Diseases.

T. Renault, C. Lipart and **I. Arzul** (in press). A herpes-like virus infects a non-ostreid bivalve species: virus replication in *Ruditapes philippinarum* larvae. Diseases of Aquatic Organisms

T. Renault, **R.M. Le Deuff**, C. Lipart and **C. Delsert** (2000). Development of a PCR procedure for the detection of a herpes-like virus infecting oysters in France. Journal of Virological Methods, 88:41-50.

T. Renault, **R.M. Le Deuff**, **B. Chollet**, **N. Cochennec** and **A. Gerard** (2000). Concomitant herpes-like virus infections among hatchery-reared larvae and nursery-cultured spat of both oyster species *Crassostrea gigas* and *Ostrea edulis*. Diseases of Aquatic Organisms, 42: 173-183.

R.M. Le Deuff and **T. Renault** (1999). Purification and partial genome characterization of a herpes-like virus infecting the Japanese oyster, *Crassostrea gigas*. Journal of General Virology, 80:1317-1322.

- *Posters and presentations*

Particpant 1 has published the results corresponding to the Sub-task 2.1 as 2 posters at the World Aquaculture Society Aqua 2000 in Nice (France) and 2 posters at Les Journées Francophones de Virologie in Paris (France).

The work concerning the OsHV-1 genome and sequence (Participant 2) was presented during the Herpesvirus Workshop at the Millennium Meeting of the Society for General Microbiology in Warwick, U.K. (10-14 April 2000). It was also publicised as a poster and as an oral presentation at the 25th International Herpesvirus Workshop in Portland, Oregon, U.S.A. (29 July - 4 August 2000).

- *E. C. Programme reports :*

Detection of herpes-like virus DNA by PCR in bivalve larval samples (2000). Final Report. Progamme E. C. no FA-S2 9052 (Research Objectives : Mortality in European Oysters).

Diagnosis of oyster herpes-like virus : development and validation of molecular, immunological and cellular tools (2000). First Periodic Progress Report. Programme FAIR-CT98-4334 (VINO).

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 2000 to 3rd January 2001

Participant no. 1 (coordinator)
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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 **04-01-00** to **03-01-01**

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	
EC contact:	DG XIV/C/2 (fax: +32 2 295 78 62)	

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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 medecine)

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 analsyis 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 analsyis 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	1st sample collection/P. R.	2nd sample collection/Laboratory analysis/P. R.	3rd sample collection/Laboratory analysis /F. R.
3. 2	1st sample collection/P. R.	2nd sample collection/Laboratory analysis/P. R.	3rd sample collection/Laboratory analysis /F. R.
3. 3	1st sample collection/P. R.	2nd sample collection/Laboratory analysis/P. R.	3rd 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

[a]P. R. : Periodic Progress Report; [b]F. 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 specific primer sequences and probes for diagnosis by PCR and in situ hybridisation

A PCR procedure and an *in situ* hybridisation technique have been developed during the first reporting period in 1999. The PCR method allowed the detection of viral DNA in frozen larval and spat samples. The *in situ* hybridisation technique demonstrated the presence of viral DNA in histologiacl sections of infected *Crassostrea gigas* and *Ostrea edulis* spat. PCR assays have been carried out in 2000 using serial ten-fold dilutions of genomic viral DNA and the OHV1/OHV2 primer pair. The PCR conducted with this primer pair showed a sensitivity of 1 fg of viral DNA (Fig. 1).

Figure 1. PCR assays on serial ten-fold dilutions of herpes-like virus DNA using the OHV1/OHV2 primer pair. M : molecular weight markers ; 1 and 1 ' : 1 fg of viral DNA ; 2 and 2 ' : 10 fg ; 3 and 3 ' : 100 fg ; 4 and 4 ' : 1 pg ; 5 and 5 ' : 10 pg ; 6 and 6 ' : 100 pg ; 7 and 7 ' : 1 ng ; 8 and 8 ' : 10 ng (5 10^7 copies of viral genome)

During the second reporting period (2000), two primer pairs have been developed in order to amplify small DNA fragments from OsHV-1 DNA. The first primer pair, called OH1/OH4, yielded 196 bp amplicons when genomic viral DNA was used as template. The size of PCR products obtained with the second primer pair (IAP1/IAP2) was 207 bp. Both primer pairs have been designed in order to obtain PCR amplification when DNA extracted from histological blocks was used as template. Several primer pairs (OHV3/OHV4, OHV3/OHV114, A3/A4 and A5/A6) have already been tested using this type of DNA. They failed producing amplicons. These results could be explained by DNA fragmentation. Both primer pairs, OH1/OH4 and IAP1/IAP2, allowed the production of amplicons when DNA extracted from wax blocks was used. A classical technique was choosen for DNA extraction from histological sections using dewaxing in xylene and treatment with proteinase K.

Archive material has been used. Five histological blocks prepared in 1995 corresponding to *Crassostrea gigas* spat have been selected. Animals presenting high mortality rates have been fixed individually in Davidson's fluid during the summer of 1995. Transmission electron microscopy examination allowed to detetect viral particles. PCR analysis using DNA extracted from these blocks showed clear bands presenting expected sizes when both primer pairs were used (Fig. 2). This suggested that both primer combinations were reliable tools to detect viral DNA in archive material. Moreover, the primer pairs have been designed in two different areas of the genomic viral DNA enhancing the specificity of the detection. The OH1/OH4 primer pair recognises a gene coding for a protein of unknow function and the IAP1/IAP2 primer combination amplifies a fragment of a gene corresponding to a putative inhibitor of apoptosis (IAP).

The OH1/OH4 and IAP1/IAP2 primer pairs will be used to select archive histological blocks which will be supplied to Participants 5, 6 and 7 by Participant 1. Participants 1, 5, 6 and 7 will use this material to validate molecular and immunological reagents.

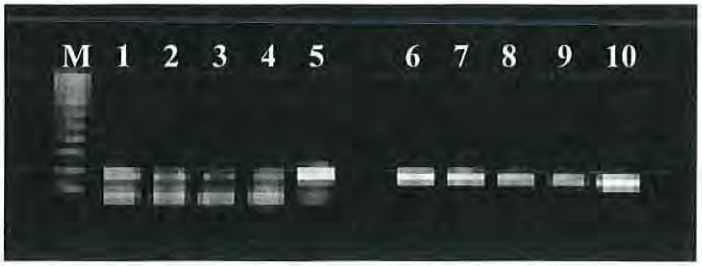

Figure 2. The OH1/OH4 and IAP1/IAP2 primer pair s have been tested using DNA extracted from wax blocks of infected *Crassostrea gigas* spat (M: molecular weight markers, 1 to 5:: PCR products obtained from five histological blocks using the IAP1/IAP2 primer pair, 6 to 10: PCR products obtained from the same histological blocks using the OH1/OH4 primer pair). In lanes 1 to 5, the lowest band corresponds to primer dimers.

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

1. Molecular biology workshop

A molecular biology workshop has been organised in May 2000 at the IFREMER station in La Tremblade (Charente Maritime, France) in order to ensure that each participant involved in epidemiological surveys will use the same protocols and procedures for molecular diagnosis of OsHV-1 infections. The time schedule and the items of the molecular biology workshop were given in the Annex. The workshop took place at the IFREMER station in La Tremblade during one week (15-19 May 2000). The list of participants is also presented in the Annex.

The first day of the meeting, PCR and *in situ* hybridisation protocols have been presented and discussed. Participant 1 showed how to carry out PCR diagnosis using 15 larval samples and the OHV1/OHV2 primer pair. Participants 4, 5, 6 and 7 have then analysed by theirself the same larval samples using the defined PCR protocol. The results obtained by each participant have been compared (Fig. 3 and Table 1). All negative controls appeared negative for all participants indicating the absence of contamination. All positive controls appeared positive. This suggests that the PCR procedure is reliable. Five larval samples showed a clear band presenting the expected size in agarose gels in all experiments (Fig. 3 and Table 1). Amplicons were detected in three other samples in three of the four PCR assays (Fig. 3 and Table 1). In other samples (7), faint bands were obserevd by one or two participants (Fig. 3 and Table 1). These results indicated that no contamination occured during PCR experiments and that the genomic viral DNA was systematically amplified (positive controls). Moreover, some larval samples (5) appeared systematically positive when PCR analysis was carried out.

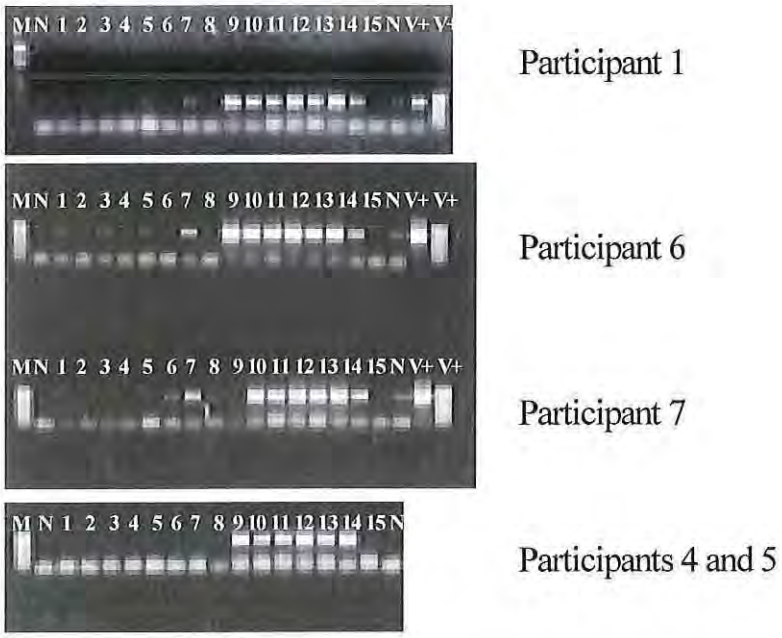

Figure 3. PCR results / May 2000 / OHV1-OHV2 primer pair (M: molecular weight markers, N: negative control, 1 to 15: larval samples, V+: positive control)

Participant 1 showed also how to carry out *in situ* hybridisation analysis. Two infected *Crassostrea gigas* juveniles and two non infected *C. gigas* spat have been tested using two different protocols. Positive and negative material were used to test two different *in situ* hybridisation protocols. The first protocol was based on a alkaline phosphatase/BCIP-NBT direct detection system and the second protocol on an peroxydase/DAB indirect detection system. Infected oysters showed the presence of labeled nuclei and cells in connective tissues of different organs. No staining was observed in non infected animals.
At the end of the molecular biology workshop, Participant 1 supplied genomic viral DNA and 15 larval samples in dry ice, and histological sections from two infected and from two non infected oyster spat to Participants 4, 5, 6 and 7 as reference material.

2. Sites and bivalve species

The aim of this task is to validate the technology to detect herpes-like virus in bivalve samples. Since mortalities were detected in France among *Crassostrea gigas* spat in Fouras (Charente Maritime, France) during the summer 2000, we decided to sample spat to identify the possible cause of the mortalities. 15 spat batches (30 individuals) have been taken in Fouras between March and August 2000.

3. PCR analysis

Samples were taken and frozen for PCR analysis. PCR analysis permitted the detection of viral DNA in three batches of oyster spat among the 15 analysed batches.

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

Table 1. - PCR assays using the OHV1/OHV2 primer pair and larval samples (molecular biology workshop, May 2000, La Tremblade IFREMER, Charente Maritime, France)

Samples or controls	*First negative control*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*Second negative control*	*First positive control*	*Second positive control*
Participant 1	-	(+)	-	(+)	-	-	-	+	-	+	+	+	+	+	+	+	-	+	+
Participant 6	-	(+)	(+)	(+)	(+)	(+)	-	+	-	+	+	+	+	+	+	+	-	+	+
Participant 7	-	-	-	-	-	-	(+)	+	(+)	-	+	+	+	+	+	+	-	+	+
Participants 4 and 5	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	**ND**	+

+: positive result ; (+): positive result with a faint band ; -: negative result ; ND: not done

All the larval samples tested corresponded to larvae presenting mortalities (they may be infected at different levels with different amounts of viral DNA).

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 a molecular biology workshop in La Tremblade (Charente Maritime, France) in May 2000 and the second annual meeting in December 2000 at the IFREMER headquarter in Issy les Moulineaux near Paris (France). Drs T. Renault and I. Arzul attended and presented results at second annual meeting. Dr T. Renault also wrote this intermediate report.

Dissemination of results (contribution to articles and posters related to OsHV)

- Contribution to articles related to OsHV :

I. Arzul, **T. Renault**, C. Lipart and **A.J. Davison** (in press). Evidence for interspecies transmission of oyster herpesvirus in marine bivalves. Journal of General Virology.

T. Renault (2000). Surveillance des virus de type herpès chez les larves et les juvéniles d'huître creuse. Lettre médias, IFREMER, 52:2 et 7.

T. Renault and **I. Arzul** (in press). Herpes-like virus infections in hatchery-reared bivalve larvae in Europe: specific viral detection by PCR. Journal of Fish Diseases.

T. Renault, C. Lipart and **I. Arzul** (in press). A herpes-like virus infects a non-ostreid bivalve species: virus replication in *Ruditapes philippinarum* larvae. Diseases of Aquatic Organisms

T. Renault, **R.M. Le Deuff**, C. Lipart and **C. Delsert** (2000). Development of a PCR procedure for the detection of a herpes-like virus infecting oysters in France. Journal of Virological Methods, 88:41-50.

T. Renault, **R.M. Le Deuff**, **B. Chollet**, **N. Cochennec** and **A. Gerard** (2000). Concomitant herpes-like virus infections among hatchery-reared larvae and nursery-cultured spat of both oyster species *Crassostrea gigas* and *Ostrea edulis*. Diseases of Aquatic Organisms, 42: 173-183.

R.M. Le Deuff and **T. Renault** (1999). Purification and partial genome characterization of a herpes-like virus infecting the Japanese oyster, *Crassostrea gigas*. Journal of General Virology, 80:1317-1322.

- Posters and presentations

I. Arzul and **T. Renault** (2000). Experimental herpes-like viral infection assays in *Crassostrea gigas* adult oysters. World Aquaculture Society, Aqua 2000, Nice, France.

I. Arzul, C. Lipart et **T. Renault** (2000). Herpèsvirus de l'huître : deux espèces différentes de bivalve marin infectées par un même "variant". Journées Francophones de Virologie, Paris, France.

T. Renault, C. Lipart et **I. Arzul** (2000). Construction et utilisation d'un standard interne dans le cadre de la détection par PCR de virus de type herpès chez les huîtres. Journées Francophones de Virologie, Paris, France.

T. Renault, C. Lipart, P. Trintignac, R.-M. Le Deuff, F. Hennequart, R. Pajot and **A. Gérard** (2000). Detection of herpes-like virus by PCR in oysters spat is correlated to genitor sources. World Aquaculture Society, Aqua 2000, Nice, France.

- *E. C. Programme reports :*

Detection of herpes-like virus DNA by PCR in bivalve larval samples (2000). Final Report. Progamme E. C. no FA-S2 9052 (Research Objectives : Mortality in European Oysters).

Diagnosis of oyster herpes-like virus : development and validation of molecular, immunological and cellular tools (2000). First Periodic Progress Report. Programme FAIR-CT98-4334 (VINO).

SIGNIFICANT DIFFICULTIES OR DELAYS

None have occurred so far.

ANNEX

(Molecular biology workshop)

PCR and *in situ* hybridisation for herpes-like virus detection in bivalves

Training at IFREMER's station in La Tremblade

15-19 May 2000

PCR and *in situ* hybridisation for herpes-like virus detection in bivalves

PCR and *in situ* hybridisation for herpes-like virus detection in bivalves

I - Time schedule

- *Monday 15th May 2000*

9H-12H : presentation of PCR and *in situ* hybridisation techniques

14H-17H : demonstration of PCR analysis of larval samples (15) : PCR prepration, PCR reaction and agarose gel preparation

- *Thuesday 16th May 2000*

9H-12H : agarose gel electrophoresis of PCR products and PCR analysis for each participant

14H-17H : preparation of histological slides for *in situ* hybridisation

PCR and *in situ* hybridisation for herpes-like virus detection in bivalves

I - Time schedule

- *Wenesday 17th May 2000*

9H-12H : preparation of the digoxigenin labeled probe by PCR

14H-18H : agarose gel electrophoresis of PCR products (16th May) and of the labeled probe and *in situ* hybridisation (digoxigenin labeled probe hybridisation overnight)

- *Thursday 18th May 2000*

9H-12H : *in situ* hybridisation (detection of the labeled probe)

14H-18H : *in situ* hybridisation (detection of the labeled probe)

- *Friday 19th May 2000*

9H-12H : observation of *in situ* hybridisation slides and discussion

PCR and *in situ* hybridisation for herpes-like virus detection in bivalves

II - Participants : 8

- N. Leon (UBO, Brest, France, Partner 4)

- S. Culloty (University College, Cork, Ireland, Partner 5)

- B. Novoa and A. Figueras (CSIC, Vigo, Spain, Partner 6)

- R.-M. Le Deuff and P. Martin (CEFAS, Weymouth, United Kingdom, Partner 7)

- I. Arzul and T. Renault (IFREMER, La Tremblade, France, Partner 1)

PCR and *in situ* hybridisation for herpes-like virus detection in bivalves

III - Protocols

III 1 - PCR

- Primer pair : OHV1/OHV2

OHV1: 5' CCG TGA CTT CTA TGG GTA TGT CAG 3'
OHV2 : 5' CCT CGA GGT AGC TTT TGT CAA G 3'

(OHV3/OHV114 not used)

- Samples

- ❖ **15 larval samples tested (larvae presenting anomalous mortalities)**
- ❖ **50 mg of frozen larvae ground in double distilled water**
- ❖ **10 minutes in boiling water bath**
- ❖ **Centrifugation 10 000 rpm / 5 minutes**
- ❖ **Recovery of supernatant and dilution ten-fold in double distilled water (-20°C)**

PCR and *in situ* hybridisation for herpes-like virus detection in bivalves

III - Protocols

III 1 - PCR

- PCR conditions

❖ Thermal cycling carried out in a Crocodile III thermal cycler (Appligene Oncor)

❖ 50 µl PCR reaction undertaken :

Bidistilled water	31.5 µl
Taq buffer 10X	5 µl
MgCl2 25 mM	5 µl
dNTP 20mM	5 µl
OHV1	1 µl
OHV2	1 µl
Taq DNA polymerase	0.5 µl
Template	1 µl

PCR and *in situ* hybridisation for herpes-like virus detection in bivalves

III - Protocols

III 1 - PCR

- PCR conditions

❖ **PCR cycle :**

2 min 94°C

1 min 94°C
1 min 50°C
1 min 72°C
35 cycles

5 min 72°C

❖ **PCR analysis :**

10 µl of each PCR product size selected on 1% agarose gel (0.5 µg / ml ethidium bromide)

PCR and *in situ* hybridisation for herpes-like virus detection in bivalves

III - Protocols

III 2 - *In situ* hybridisation

- Probe labeling by PCR and digoxigenin incorporation

The DNA probes synthesised by incorporation of digoxigenin-11-dUTP during PCR using OHV3/OHV4 primers (C1/C6)

❖ **50 µl PCR reaction undertaken :**

Bidistilled water	**35 µl**
Taq buffer 10X	**5 µl**
MgCl2 25 mM	**1.5 µl**
dNTP 20mM	**5 µl**
OHV3	**1 µl**
OHV4	**1 µl**
Taq DNA polymerase	**0.5 µl**
Viral DNA (100 pg / µl)	**1 µl**

PCR and *in situ* hybridisation for herpes-like virus detection in bivalves

III - Protocols

III 2 - *In situ* hybridisation

- Probe labeling control by electrophoresis

Incorporation of digoxigenin was indicated by increase in molecular mass

- Slide preparation

- **Tissues fixed in Davidson or Carson, embedded in paraffin**
- **Blocks cut 7 µm thick, placed on silanised slides (Sigma) and air dried à 37°C 12 hours**
- **Sections dewaxed in xylene (2 x 5 min), treated in absolute ethanol (2 x 5 min) and air dried**
- **Sections permeabilised with proteinase K (100 µg / ml) 30 min at 37°C. Proteolysis inactivated by 0.1M TrisHCl / 0. 4M NaCl buffer pH 7.5 (3 min)**

PCR and *in situ* hybridisation for herpes-like virus detection in bivalves

III - Protocols

III 2 - *In situ* hybridisation

- *Slide preparation*

❖ **Dehydration in ethanol (1 min in ethanol 95, 1min in ethanol 100) and air dried**

- Hybridisation and washing steps

❖ **Addition of prehybridisation buffer 30 min at 42°C**

❖ **Addition of hybridisation buffer and probe (5 ng / µl)**

❖ **Denaturation of the target and the probe (95°C - 5 min)**

❖ **Incubation at 42 °C overnight**

❖ **Wash in 1X SSC (O.2 BSA) at 42 °C, 10 min**

❖ **Detection of the digoxigenin labeled probe (direct or indirect detection)**

PCR and *in situ* hybridisation for herpes-like virus detection in bivalves

IV - Results

IV 1 - PCR

Strongly positive results appear positive in each assay

IV 2 - *In situ* hybridisation

Indirect versus direct detection of the digoxigenin labeled probe

PCR and *in situ* hybridisation for herpes-like virus detection in bivalves

V - Assays in the different laboratories involved in herpes-like virus diagnosis

V 1 - PCR on viral DNA (first results)

V 2 - PCR on larval samples : in progress

V 3 - *In situ* hybridisation : in progress

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 2000 to 3rd January 2001

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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 **04-01-00** to **03-01-01**

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	
EC contact:	DG XIV/C/2 (fax: +32 2 295 78 62)	

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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
M 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

This sub-task was completed during the previous reporting period.

Sub-task 1.2 - Sequencing the virus genome

This sub-task has been completed. The part of the work supported by the EC contract was carried out by Ms M. Watson and Dr A. Davison with laboratory support from Mr C. Cunningham.

1. Sequence database

At the time of the last report, the sequence database assembled using Staden's Gap4 program took the form of three large fragments. Since then, the genome sequence has been completed and analysed.

Specific regions in the database where information was absent or of poor quality were dealt with by PCR amplification of viral DNA using a set of 60 custom primers. PCR products were either purified from agarose gels and sequenced directly using the relevant primers, or cloned into pGEM-T and sequenced using universal primers. Ambiguities in the database were resolved using the standard editing functions of Gap4. In addition, every nucleotide was checked by reference to the best data available for that region. At completion, each nucleotide was determined an average of 10.8 times and 96.1% of the sequence was determined on both strands.

2. Genome structure

Comparison of the predicted sizes of restriction endonuclease fragments with those determined by digestion of viral DNA indicated that the overall genome structure is:

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

The total genome size is 207439 bp. TR_L and IR_L (7584 bp) are inverted repeats flanking a unique region (U_L, 167843 bp), TR_S and IR_S (9774 bp) are inverted repeats flanking a unique region (U_S, 3370 bp), and X (1510 bp) is located between IR_L and IR_S. A somewhat similar genome structure has evolved independently in certain vertebrate herpesviruses (e.g. herpes simplex virus and human cytomegalovirus).

Several experiments were undertaken to confirm the proposed genome structure.

- The sequences of the genome termini were determined. The initial approach was to digest viral DNA with *Pst*I, tail with dG residues using terminal transferase, and anneal to pUC18 which had been digested with *Sph*I and tailed with dC residues. This standard strategy reconstitutes the *Sph*I and *Pst*I sites, and allows both terminal and internal *Pst*I fragments to be cloned. Unfortunately, clones containing terminal fragments were not

identified among the resulting library. An alternative approach was successful. Intact viral DNA was treated with T4 DNA polymerase in the presence of the four dNTPs in order to produce flush ends. A partially double-stranded oligonucleotide (the cDNA adaptor from Clontech's Marathon kit) was ligated to the ends. Each terminal fragment was then amplified by PCR using one primer annealing to the adaptor and another primer annealing within a few hundred base pairs of the putative genome terminus. The PCR product generated for each terminus was cloned, and several clones were sequenced. The results are shown in Fig. 1. The genome termini are not located uniquely, but a predominant form is apparent for each.

- The nature of the sequences between IR_L and IR_S was determined. PCR products were generated from viral DNA using primers within these elements, close to their boundaries with X (i.e. equivalently close to the genome termini). Two products were obtained. The larger corresponded to the relevant portion of IR_L - X - IR_S, as expected. The smaller corresponded to the relevant portion of IR_L - IR_S (i.e. X was absent, equivalent to the genome termini joined together). The sequence is shown in Fig. 1. As with the termini, the IR_L - IR_S junction is not located uniquely, but the predominant form corresponds to a fusion of the two termini if each possesses two unpaired nucleotides at the 3' end. Unpaired nucleotides are characteristic of herpesvirus genome termini.

- Southern blot hybridisation experiments were carried out to determine the relative amounts of the two types of junctions (IR_L - X - IR_S and IR_L - IR_S) present in viral DNA. Only the former was detected, indicating that the latter is present in no more than a small proportion of virion DNA molecules. The hybridization experiments also indicated that a small proportion of molecules contain an additional X sequence at the left end of the genome.

- Further Southern blot hybridisation experiments using PCR-generated probes from the ends of U_L and U_S showed that the two orientiations of U_L and U_S are present in approximately equimolar amounts in viral DNA, giving rise to four genome isomers. This is also a feature of the vertebrate herpesvirus genomes with similar structures, and results from recombination between inverted repeats during DNA replication.

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

These data indicate that the virus contains a mixture of genome forms. In light of the fact that the virion DNA that was sequenced originated from a virus that had not been clonally purified, this was not unexpected. The major form has the basic structure:

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

By virtue of inversion of U_L and U_S (to give U_L' and U_S'), this comprises four isomers in approximately equimolar amounts:

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

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

Small proportions of molecules either lack the X sequence or contain an additional X sequence at the left terminus. Although the situation might be more complex, this would most simply represent:

X - TR_L - U_L - IR_L - IR_S - U_S - TR_S (as four isomers).

Since herpesvirus genomes are packaged into capsids from head-to-tail concatemers (conceptually a circularised form of the parental genome), this minor genome form is explained most readily as resulting from rare cleavage of concatemers at X - TR_S rather than at IR_L - IR_S.

A minority of genomes also contain a 4.8 kbp region within U_L that is inverted. Presumably, this form also exhibits the 8 permutations described above.

3. Genetic content

A detailed analysis of the coding potential of the genome sequence indicated the presence of 132 unique protein-coding open reading frames (ORFs). Their layout is shown in Fig. 2. Owing to the presence of inverted repeats, 13 ORFs are duplicated, resulting in a total of 145 ORFs in the genome. This is an approximation of the gene number, chiefly because of the presence of fragmented genes that might not encode functional proteins (see point 7 below).

Amino acid sequence comparisons of the proteins encoded by the 132 ORFs with public and private databases using Blast and FastA yielded the following information about their properties and relationships.

a) Seven genes encode enzymes. These include DNA polymerase (ORF106), deoxyuridine triphosphatase (ORF81), two subunits of ribonucleotide reductase (ORF21 and ORF53), helicase (ORF72), a putative primase (ORF25) and the ATPase subunit of terminase (ORF117).

b) Seven proteins bear sequence similarities with viral or cellular inhibitors of apoptosis proteins (IAPs; ORF43, ORF102, ORF103, ORF114, ORF125, ORF93, ORF105). Of these, the first five listed contain a zinc-binding domain known as a RING finger. One (ORF43) is under investigation by Partner 3 as a potentially immunogenic protein. The observation that IAPs are also encoded by baculoviruses and entomopoxviruses (both of which have insect hosts) underscores the importance of the apoptotic responses of invertebrates against viral infection. Vertebrate herpesvirus or poxvirus do not encode IAPs, and subvert the battery of host defences by other pathways.

c) Three additional proteins contain RING fingers (ORF10, ORF35, ORF126), and two others possess alternative zinc-binding domains (ORF129, ORF132).

d) One protein is related to a eukaryotic protein of unknown function which is brain-specific in vertebrates (ORF59).

Left terminus

2	TGGGGGGGCTGT
4	TGGGGGG

Right terminus

4	TCCCCA
1	CCCCA

IR_L - IR_S junction

1	TGGGGGGGCTGTTACTCCCCAA
1	TGGGGGGGCTGT..CTCCCCAA
1	TGGGGGGGCTGT...TCCCCAA
1	TGGGGGGGCTGC...TCCCCAA
1	TGGGGGGGCTG....TCCCCAA
5	TGGGGGGGC.....TCCCCAA

Fig. 1. - Sequence of the genome termini

The number of clones obtained for each sequence is shown to the left. The termini have been inverted and complemented so that they align with the junction.

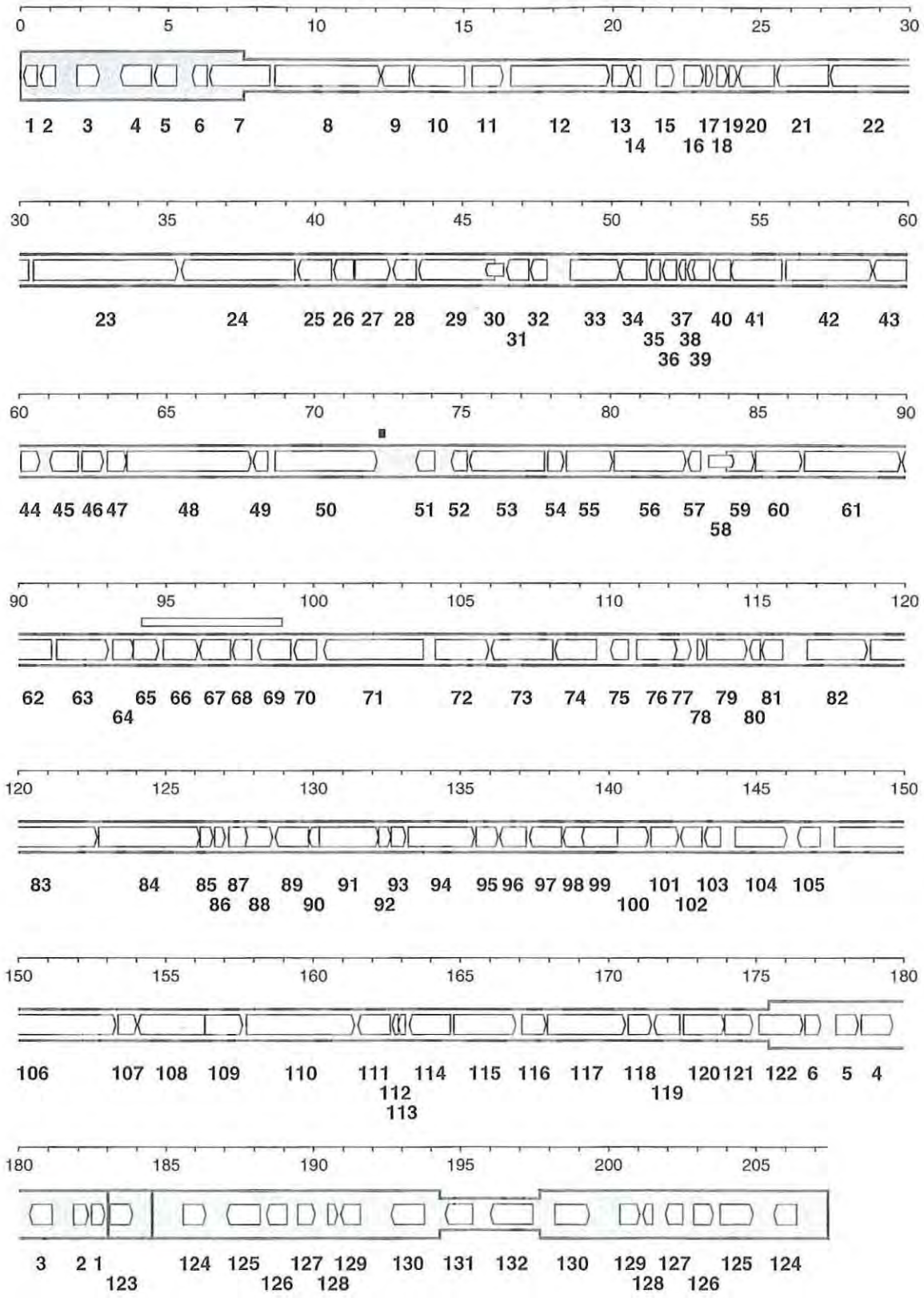


Fig. 2. - Arrangement of ORFs in the genome

Thicker genome regions indicate inverted repeats. ORF123 is in the X region. The filled rectangle indicates the palindrome, and the unfilled rectangle the region that is inverted in a minority of molecules. The scale is in kilobase pairs.

e) Seven ORFs encode class I membrane proteins that traverse the membrane once, and three encode proteins that traverse the membrane more than once. One member of the former class (encoded by ORF94) is under investigation by Partner 3 as a potentially immunogenic protein. An additional 17 proteins contain a hydrophobic domain indicating a possible assocation with membranes.

f) A total of 39 proteins share sequence similarities with other proteins encoded by the virus, defining 13 multigene families in addition to the IAPs. The generally ancient nature of the gene duplication events which have led to this situation is indicated by the fact that relationships are distant and that homologues are generally widely distributed in the genome.

g) Of those ORFs in multigene families, 16 appear to represent eight genes that have become fragmented relatively recently in evolution. It is questionable whether all of these ORFs encode functional proteins.

An additional notable feature, located between ORFs 50 and 51, is a large palindrome (Fig. 3). The sequence of this element was particularly difficult to solve. By analogy with certain vertebrate herpesviruses, this palindrome is a candidate origin of DNA replication.

Sub-task 1.3 - Phylogenetic analysis of the oyster virus

This sub-task has been completed. The work was carried out by Dr A. Davison.

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

In this context, detailed phylogenetic analyses are not of great utility in determining whether OsHV-1 and vertebrate herpesviruses have a common origin. The strongest genetic indication of a common origin resides with the ATPase subunit of the terminase (see above in point 1), which is involved in packaging DNA into the capsid. Homologous genes are present in all herpesviruses, and the only non-herpesvirus counterparts are specified by T4 and related bacteriophages. The T4 and OsHV-1 genes are unspliced, whereas those in herpesviruses of mammals and birds contains one intron and those in herpesviruses of fish and amphibians contains two introns.

Sequence determined from clones, from which most of the palindrome was deleted:

```
5'-AATATTTTAA
            AAAATGGTCTACTCGG
            TTTTACCAGATGAGC
3'-TCATACCGAA
```

Sequence of the complete palindrome from viral DNA, in monopartite form:

```
5'-AATATTTTAA
            CCATATA
            AAAATGGTCTACTCGACGAGTGGATCACTTTGGTATAAATTATAGGTTTATATCTTTTAAAAAATTGCCCGGTCGGGGGTCTTCACGCTCTTA
            TTTTACCAGATGAGCGGCTCATCTAGTGAAACCATATTTAATATCCAAATATAGAAAATTTTTTAACGGGCCAGCCCCCAGAAGTGCGAGAAT
3'-TCATACCGAA
            ATGTACC
```

Sequence of the palindrome from viral DNA, in tripartite form:

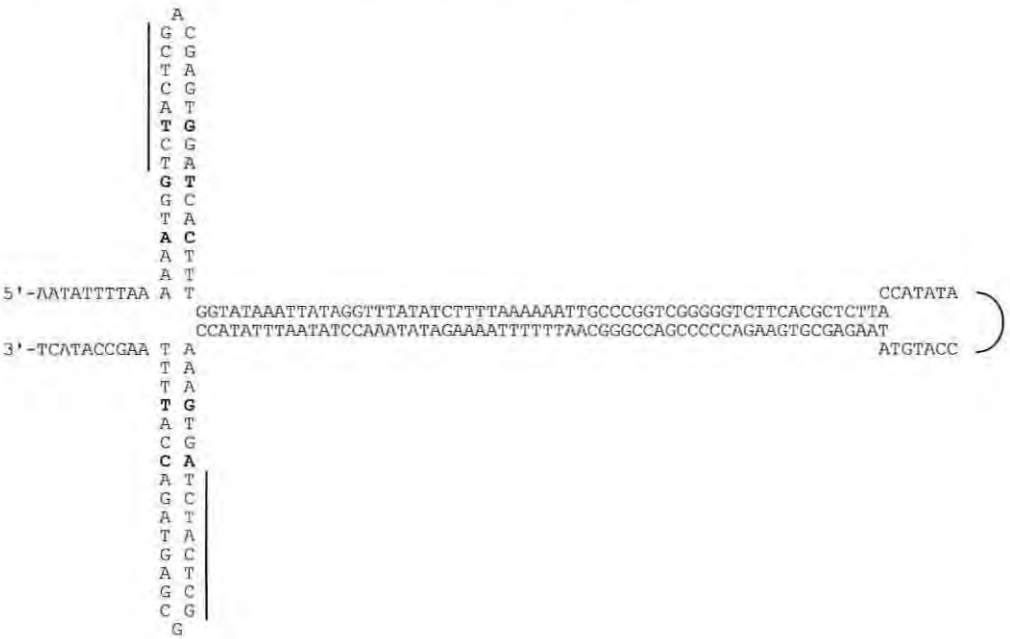


Fig. 3. - Structure of the palindrome

Base pairing within one of DNA two strands is shown. Non Watson-Crick base paired nucleotides within the paired regions are shown in bold. The regions between which the deletion occurred are indicated by horizontal or vertical lines.

The available data support the view that herpesviruses of mammals and birds, herpesviruses of fish and amphibians and herpesviruses of invertebrates form three major lineages of the herpesviruses (Fig. 4). This scheme is consistent with the generally accepted model of evolution of herpesviruses with their hosts. In the context of this model, OsHV-1 would have established a separate lineage about a billion years ago, and the fish viruses about 400 million years ago. OsHV-1 is currently the single representative of what may be a large number of invertebrate herpesviruses.

Recent data obtain by Partner 1 have shown that OsHV-1 can infect several bivalve species. This contrasts with vertebrate herpesviruses, which are generally confined to a single species in nature. Consequently, the true host of OsHV-1 is unknown. The apparent loss of several gene functions in OsHV-1 prompts the intriguing speculation that this may have promoted interspecies transmission in the context of introduction of non-native bivalve species and use of modern aquaculture techniques. It is possible that the parental virus still resides in its natural host.

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 the annual meeting held at the IFREMER building in Paris (14-15 December 2000). He also wrote this progress report.

Dissemination of results

The work conducted for task 1 is complete and is actively being prepared for publication. Some additional analyses of a minor nature may prove necessary during this processs, and will be carried out as required. A database entry has been prepared for the genome sequence, and will be submitted to Genbank when the paper is finalised.

The work was presented during the Herpesvirus Workshop at the Millennium Meeting of the Society for General Microbiology in Warwick, U.K. (10-14 April 2000). It was also publicised as a poster and as an oral presentation at the 25th International Herpesvirus Workshop in Portland, Oregon, U.S.A. (29 July - 4 August 2000).

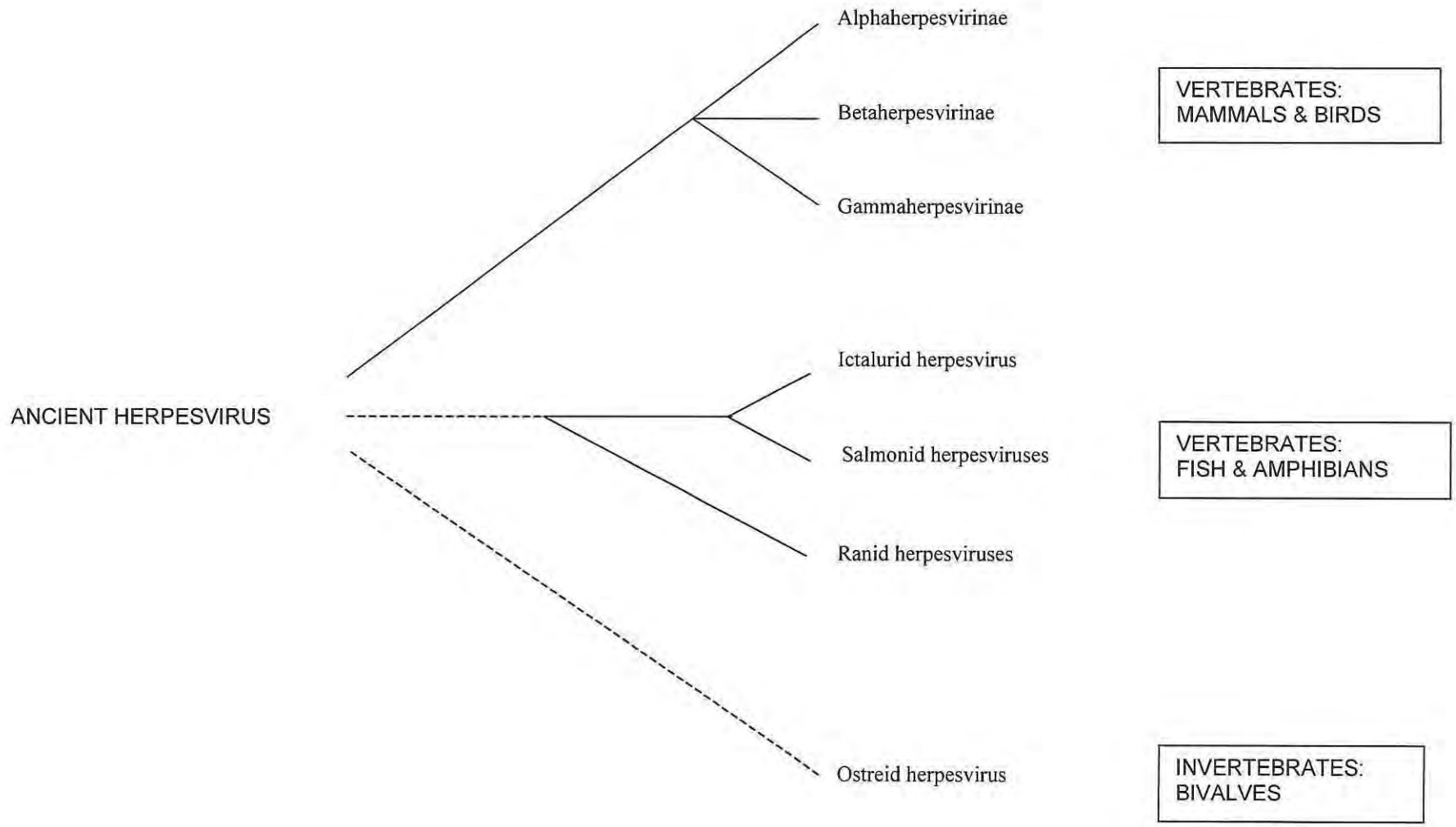
Sequence data were made available to other participants as required

SIGNIFICANT DIFFICULTIES OR DELAYS

None have occurred.

Fig. 4. - A scheme illustrating the evolution of herpesviruses

The limited nature of genetic data supporting evolution of the three major lineages from a common ancestor is indicated by the dashed lines. Relative orders of divergence cannot be imputed reliably for such ancient lineages.



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 2000 to 3rd January 2001

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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 **04-01-00** to **03-01-01**

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

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Yves LECOCQ

OBJECTIVES

- Identification of immunogenic virus proteins
- Preparation of recombinant proteins
- Preparation of antibodies specfic 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

[a] P. R. : Periodic Progress Report; [b] F. 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

1. Identification of immunogenic proteins

The immunoscreening with specific anti-OsHV-1 antibodies and the preliminary results of sequencing of the virus genome have allowed to identify two open reading frames (ORF) encoding for putative immunogenic viral proteins. The first ORF, called ORF94, codes for a protein of 748 amino acids. This protein contains a highly hydrophobic C-terminal domain, potential N-glycosylation sites (Asn-X-Ser/Thr) and a potential signal peptide at the N-terminal end (Fig. 1). Thus, this ORF could code for a surface glycoprotein with a transmembrane C-terminal end, the typical profile of surface viral antigen. The second identified ORF, called ORF43, codes for a protein of which different regions are recognized by anti-OHV ascite. This protein of 364 amino acids doesn't present the characteristics of membrane proteins but seems to be a potential immunogenic protein (Fig. 2).

In the beginning of the year, we have characterized others plaques in order to isolate a third candidate.

Secondly, the two ORFs, ORF43 and ORF94, encoding for putative immunogenic viral proteins have been cloned in Baculovirus expression system in order to prepare recombinant proteins and antibodies for diagnosis use.

2. Characterization of the selected plates

Some plaques previously selected (see progress report for 1999) were characterized. For some of them (plaques n° 2, 5-1 and 19-3), excision and characterization of the phagemids were necessary as described in previous report. For the others, the inserts were just sequenced from old plasmid preparations (issued from plates n° 2-3, 7-2, 7-4, 17-5-3 and 17-5-4). Fourteen clones in total, with different patterns and with the insert size above 400 bp, were sequenced with T3 primer. The obtained sequencing results were analyzed in looking for open reading frames and compared with the sequencing results of the Participant 2. The sequences of two clones (issued from plates n° 2-3 and 2-5) are identical and homologous to the C-terminal end of ORF19 sequenced by Participant 2. The insert of one other sequenced clone (issued from plates n° 7-2) correspond to the C-terminal end of one complete ORF, called ORF93. Unfortunately, these two ORFs, ORF19 and ORF93, don't seem to encode for a predicted membrane protein. Moreover these proteins aren't similar to any other protein in the available database. In conclusion, these two ORFs don't seem to code for potential immunogenic protein.
The other sequenced clones are not predicted anymore to express real OsHV proteins. So, we have decided to abandon the search of third candidate to put all our energies in expressing the two putative immunogenic viral proteins previously identified in an appropriate system.

ATGATCATTATGAAATCCATAATATTATTACTCGCTTGGTTTTTAACAAAAACACAGGCGAATATGTTGACCGAATCTTT
M I I M K S I I L L L A W F L T K T Q A N M L T E S
GTACTTGTCAGAATATGAGGGGAGTGTTGTGCTAAACATCATAGACAAAAATTTAAACGGGATATCCACCCTGTCAATAT
L Y L S E Y E G S V V L N I I D K N L N G I S T L S I
TTAACGATTCTACTAAATTACAAGAAGTAAGATATGTTGCCAGTGTATGCAGCCTCAGATCTGGATCGTTCAATATCACT
F **N D S** T K L Q E V R Y V A S V C S L R S G S F **N I T**
TGCAATGTAATAACCTACGGCACTTATCATGTCAGGATGTTTTTATCAGGTCTTAACATGTCCGCATTTGATTTATACAG
C N V I T Y G T Y H V R M F L S G L **N M S** A F D L Y
ACTTCGATATGTGTACGTTGGTCTTAGAGACGCCATAAATTACAACCCAAAATATGCAGAGGCGGTAATGGCACCGTTTG
R L R Y V Y V G L R D A I N Y N P K Y A E A V M A P F
CTTTAATTGGCAATAATAATATAGTTACAATTAAACTTATAAAAGACGGTGATAATATCACCGTCGGTTGTGGGTTTGGA
A L I G N N N I V T I K L I K D G D **N I T** V G C G F G
AATGTAGATTTGAGCACTGTAAACACCCATGCCAGCAAAATTGGCAGAAATATAAACCCTAGATTCATGGTCGGTGTTTA
N V D L S T V N T H A S K I G R N I N P R F M V G V
TACAAACGATAGCAATAAGTTAATCGAGGATGATATATACAGCCGTTACACAGATTCAGAATCTGCCGGTGTTATGAGAA
Y T **N D S** N K L I E D D I Y S R Y T D S E S A G V M R
AATGTAATTTAAACGAGGTTAAAACCACTCCCCAGGAAGATTGCATTCAGCCCTTTTGCACCAAAGGAACAGTGTATGGA
K C N L N E V K T T P Q E D C I Q P F C T K G T V Y G
AATAACCTCGTTTATGGAAGTAGATTACGATGTTTCAGTAGGACCAGGTGTTCACAGAGAAGTAGAACCGTTCCTCAATC
N N L V Y G S R L R C F S R T R C S Q R S R T V P Q
AGTCCCTTGGTATATACCATCTGGATTTACAGGAAAACAGTTTATGTACCTGGATAATAGACTGGGATATCTTTTGGGAT
S V P W Y I P S G F T G K Q F M Y L D N R L G Y L L G
TAGACCTTACCACGGCTATTTTTAAATATACCCCAATTGTTGTCGGACATATAGTAAGTGAATACCTGACGGGAATCATG
L D L T T A I F K Y T P I V V G H I V S E Y L T G I M
AACTATAAGCGTCTCAGTGTCAGGAAAGGACCGAATATAGACATGCGAGGTATTATAGGTGGAGAAATCAAAATGATATT
N Y K R L S V R K G P N I D M R G I I G G E I K M I
GATAAGAAACTACAGAAAGATGTTGGACATGAGTGGATTTACACCTTTGCCGGTGAATGGATGTTATGTCACAGTGATAA
L I R N Y R K M L D M S G F T P L P V N G C Y V T V I
AATTCATCGGGGATAAACGCGTTTTTAATCGAGTTTGGACGCCTAGTAATAACACCGATGACGGAGAAGAACATGTTTTC
K F I G D K R V F N R V W T P S **N N T** D D G E E H V F
GTCTTTCATCAAAAAAGGTCAAGCAATATAAGAGATTATACTTTAAGAATTATTCCCCGATAGCGGTATGGACACAGAGGG
V F H Q K R S S N I R D Y T L R I F P D S G M D T E
AAGTAAATATACAATGAACACCATAACAGATGTGGGTTGTAGTCGTGAAACCCACCACAAATCGGTTTATCCCGCAACAA
G S K Y T M N T I T D V G C S R E T H H K S V Y P A T
TCAAAAAGGCAATAGAAAGGTTCTGCGTCGACCAACCTAATATATCATGTGAATATGTTAAGGATATTGACAGGGTTGAT
I K K A I E R F C V D Q P **N I S** C E Y V K D I D R V D
ATTAACCCTTGCGGATGTAAACAAAGAGCCAACAGATGTGGTGAGCGGTATTCCAACAACACACTTAAAGCAACTATAGA
I N P C G C K Q R A N R C G E R Y S **N N T** L K A T I
ATTTGAAGTGCCAAAGATTTACGATACACCTTACACGTGTGAATTCTTGGGATATAAAAGTGTGAATTCATTAACATTCG
E F E V P K I Y D T P Y T C E F L G Y K S V N S L T F
ATTCACCACCACCGCCACCAACTACCACTCAGGCGCCTCCTCCACCACCCACCACCACTCAAGCTCCTCCACCCCCGCCA
D S P P P P P P T T T Q A P P P P P P T T T Q A P P P P P
ACCACCACACAAGCTCCTCCTCCACCTATCGTTATTAATACCACAGCAGCACCTTTGGCGCCCATTACCAATGCCACATT
T T T Q A P P P P P I V I **N T T** A A P L A P I T **N A T**
GCCACCAAGTGATGTGATCACACCGGAGGCTGTGAATTTAACAGATGATACCCCTGTTGTAAATGAACCGGTAAATTCTA
L P P S D V I T P E A V **N L T** D D T P V V N E P V **N S**
CATTTATCAATGATACGGATGTATTAGATGATTCT**C**CCACCACCTCTGCTCCACAAGCGCCTGGTATAGTTGGTATAATT
T F I **N D T** D V L D D S P T T S A P Q A P G I V G I I
GTAAATAAGATTACAACAACACCTGCACCATCCATCGGTAGGGCGCCTATCCCACCACCAGATGTACCAGTTGAACCACC
V N K I T T T P A P S I G R A P I P P P D V P V E P
CAGATCTATCCCTACAACCAACGCACCTTCACCCGAAGAGGATACAGTGGTTTTATCTAAATCTGACATTATGCGACGGT
P R S I P T T N A P S P E E D T V V L S K S D I M R R
TTTTGATAAGGTTAAAGACTAGAGATGGAGAAACCGTCGATATTTATACATGGCCAGAACTTAACCTTGCGCCTTTTAAA
F L I R L K T R D G E T V D I Y T **W P E L N L A P F K**
ACTTTAAGCTATGCCGGAATTGGTGTCGTGTCATTTGCTTTGTTATTCACTATTCTAGTTGTCTGCTTAATAAAATTCTC
T L S Y A G I G V V S F A L L F T I L V V C L I K F
AATATAG
S I .

Fig.1. Nucleotidic sequence and translation in amino acids of the ORF94 The protein contains a highly hydrophobic C-terminal domain (**in bold**), potential N-glycosylation sites [**A-X-S/T**] and a potential signal peptide at the N-terminal end (underlined). The most likely cleavage site of the putative signal peptide is located between position 22 and 23: ANM-LT.

```
ATGGGGATTTCAGAATTAGAATACCTGCCCCAGACAACTGTTAACGCATTACTGCAATATGATAATGTGAGATTGGCAAC
  M  G  I  S  E  L  E  Y  L  P  Q  T  T  V  N  A  L  L  Q  Y  D  N  V  R  L  A
GTTTAGAGGATATGAATATGCTACCGATCAATGGAAGAAATATCTTTCAGACACTAAGTTTTTCAAAGTAGGCGAGGTGG
T  F  R  G  Y  E  Y  A  T  D  Q  W  K  K  Y  L  S  D  T  K  F  F  K  V  G  E  V
ATCAGATACAATGTGTATTTTGTAGAATGAAAACATCGATACGCAACGAAGAAAGGATAAAGAAACACGTTGCCGATTGC
 D  Q  I  Q  C  V  F  C  R  M  K  T  S  I  R  N  E  E  R  I  K  K  H  V  A  D  C
AGAGAAGGAGTTGTGAGTGCACCACAACAACAACCACCACCCCCACCCTCGACCTCCATTGGTGCTGTTGGTGGAGATCC
  R  E  G  V  V  S  A  P  Q  Q  Q  P  P  P  P  P  S  T  S  I  G  A  V  G  G  D
AAGACCGGAGGATATGAATGTACCAGAAAGGGGGTGGGATCCACCAATGTCTAAAGATCCCAAATCTACTTTCTTGGGTA
P  R  P  E  D  M  N  V  P  E  R  G  W  D  P  P  M  S  K  D  P  K  S  T  F  L  G
AATGGCCACACAGTGAATATATTTCAATAGACAGCATGGTTGCAGAGGGATTTGAATTTATTGGCCCAGGTGATAGAGTT
 K  W  P  H  S  E  Y  I  S  I  D  S  M  V  A  E  G  F  E  F  I  G  P  G  D  R  V
CAATGTAGACATTGTAAAGTGATTTTACGAAACTGGGAGACCACAGACATTCCAAGTAGCGAACATGAAAGAAACGCACC
  Q  C  R  H  C  K  V  I  L  R  N  W  E  T  T  D  I  P  S  S  E  H  E  R  N  A
AAGGTGTCCTTTGGTGGTTCAGAGATACCTGACAAGGATGAGGGAAGATGATGAGAGGCGTGACAGGGAATTAAAAGAGG
P  R  C  P  L  V  V  Q  R  Y  L  T  R  M  R  E  D  D  E  R  R  D  R  E  L  K  E
TGCAACAGCGCAGGAAAATGGATATGAACAAAGCCTTTAGCCAGGACATGTCAAAATTGGAAAATAGAATAGCGAGTTTG
 V  Q  Q  R  R  K  M  D  M  N  K  A  F  S  Q  D  M  S  K  L  E  N  R  I  A  S  L
AAATTTTGGCCAGGTCCAATAAGAGACATTGAAAAGGTTGCCAGAACTGGATTTTTCTACACGGGAGAGAAAGATATGCT
  K  F  W  P  G  P  I  R  D  I  E  K  V  A  R  T  G  F  F  Y  T  G  E  K  D  M
CACCTGTTATGCCTGCGCTTGCAAGCTGATAAATTGGGAGAAGAATGACGACCCCATCAAAGAACATAAAATCAATTTCC
L  T  C  Y  A  C  A  C  K  L  I  N  W  E  K  N  D  D  P  I  K  E  H  K  I  N  F
CCCATTGCGCAAACATGGCAGATGTAAAATGGAGCGATGTGGGATTTAGCAATGACGAGGAATGTGTGATTTGTTTGGGA
 P  H  C  A  N  M  A  D  V  K  W  S  D  V  G  F  S  N  D  E  E  C  V  I  C  L  G
GCAAAAGCAGACACTATTTTGAAACCTTGTTTACATTATTCTTTGTGCTATGGATGTAGTACACAGGTGCAAAAATGTCC
  A  K  A  D  T  I  L  K  P  C  L  H  Y  S  L  C  Y  G  C  S  T  Q  V  Q  K  C
CTTGTGTAGAAAGAAAATAGAAAAAAGAGTACAGACAACAAATGTTTTACAATGA
P  L  C  R  K  K  I  E  K  R  V  Q  T  T  N  V  L  Q  .
```

Fig.2. Nucleotidic sequence and translation in amino acids of the ORF43.

3. Construction of baculovirus transfer vectors

We chose the baculovirus system rather than *Escherichia coli* as insect cells are more convenient for folding recombinant glycoproteins in a native conformation and with post-translationnal modifications.

3.1. Construction of baculovirus His-tag transfer vectors

We synthetized oligonucleotides with 6 successive His-codons and one "Stop" codon, flanked with appropriate restriction sites. We cloned them respectively at the 5' extremity of the MCS of pVL1392 and pAcGP67A Baculovirus transfer vectors (Pharmingen). This was done to have the possibility to clone a heterologuous gene in fusion with a C-terminal His-tag to facilitate the purification of the protein (affinity chromatography).
The pAcGP67A transfer vector harbor the gp67 signal sequence followed by multiple cloning sites. The gp67 signal peptide mediates the forced secretion of the recombinant protein, even if it is normally not secreted. This theoritically also facilitates the purification of the protein.

3.2. PCR-cloning of ORF43 into pVL1392-HisC and pAcGP67A-HisC vectors

The ORF43 was amplified by polymerase chain reaction (PCR) from the cosmid 7 of the Participant 2 and using the primers 43A and 43B for the cloning into pVL1392-HisC or the primers 43C and 43B for the cloning into pAcGP67A-HisC (figure 3). These primers were designed in order to introduce restriction sites at the extremities and a second sequence functioning as a tag, encoding DYKDDDDK (the FLAG Tag), at the C terminus of the protein. This flag was added to facilitate the detection of the protein in cell samples (good antibodies available). Once constructed, the nucleotide sequence of the entire ORF 43 was confirmed by single-strand sequencing. The sequences of the recombinant proteins will be:

MGI.....LQ**DYKDDDDK**SRG(**H**)$_6$G
Flag tag **His tag**

M-gp67 secretion signal sequence↓-ADPLGI.....LQ**DYKDDDDK**SR(**H**)$_6$
Flag tag **His tag**

3.3. PCR-cloning of ORF94 into pVL1392-HisC vector

The ORF94 was amplified by PCR from OsHV DNA using the primers 94A and 94B (figure 4). These primers were designed in order to introduce restriction sites at the extremities and a FLAG tag at the C-terminal end but to suppress the hydrophobic C-terminal region of the ORF94 (Y_{709} to I_{748}). We eliminated the hydrophobic C-terminal part of the protein to eliminate the risk of interference with the affinity purification process. This should have no influence on the antigenicity of the protein as this region is probably naturally anchored in the cell membrane and not directly accessible to the immune system. We kept the natural N-terminal potential peptide signal sequence. The amplified fragment was cloned into an intermediate vector, the pCR2.1 (Invitrogen) and the sequence of the ORF94 was checked by single-strand sequencing. Once checked, the insert was sub-cloned into pVL1392-HisC expression vector. The sequence of the recombinant protein will be:

MII.....VDI$_{708}$ **DYKDDDDK**SRG(**H**)$_6$G
Flag tag **His tag**

We also tried to replace the natural signal peptide by the baculovirus gp67 one but we had some cloning problems and decided to stop this strategy after some unsuccessful assays.

Primer 43A	5'- ACG TGC GGC CGC **ATG** GGG ATT TCA GAA TTA GAA TAC C -3' NotI
Primer 43B	5'- A CGT TCT AGA **CTT GTC ATC GTC GTC CTT GTA GTC** TTG XbaI **Flag tag** TAA AAC ATT TGT TGT CTG TAC TC –3'
Primer 43C	5'- AC GTA GAT CTC GGG ATT TCA GAA TTA GAA TAC CTG C –3' BglII

Fig. 3. Sequence of used primers for amplification of the ORF43 and its cloning into pVL1392-HisC (primers 43A and 43B) and pAcGP67A (primers 43B and C) Baculovirus transfer vectors.

Primer 94A	5'-ACG TGC GGC CGC **ATG** ATC ATT ATG AAA TCC ATA ATA TTA NotI TTA C-3'
Primer 94B	5'-ACGT TCT AGA **CTT GTC ATC GTC GTC CTT GTA GTC** AAT XbaI **Flag tag** ATC GAC GGT TTC TCC ATC TCT AG-3'

Fig. 4. Sequence of used primers for amplification of the ORF94 and its cloning into pVL1392-HisC Baculovirus transfer vector.

4. Construction and amplification of the recombinant baculoviruses

We constructed the recombinant baculoviruses by co-transfection in Sf9 cells with BaculoGold (Pharmingen) and the baculovirus transfer vectors pAcGP67-HisC-FlagC-ORF43, pVL1392-HisC-FlagC-ORF43 and pVL1392-HisC-FlagC-ORF94. We perform one single selection by end point dilution. Recombinant baculoviruses obtained were called respectively ORF43s, ORF43c and ORF94. We amplified them in Sf9 cells and produced 400 ml of high titer stock. Supernatant of high titer stock was harvested 3 days post-infection, clarified by centrifugation and stored at + 4°C. Titer was determined by TCID50. We obtained good titers: 1.610^8/ml, $2.6\ 10^8$/ml and $4.7\ 10^8$/ml for ORF43s, ORF43c and ORF94 respectively.

5. Expression study of the recombinant proteins in insect cells

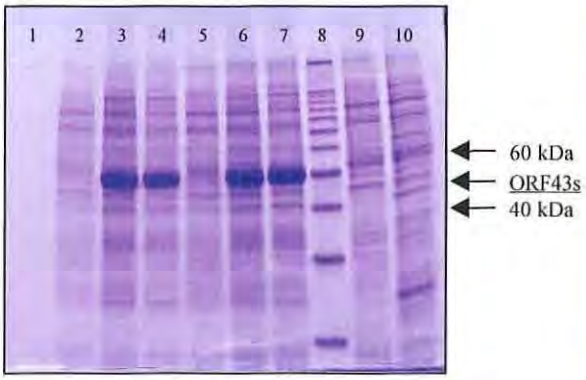
5.1. Preliminary assay in SF9 cells

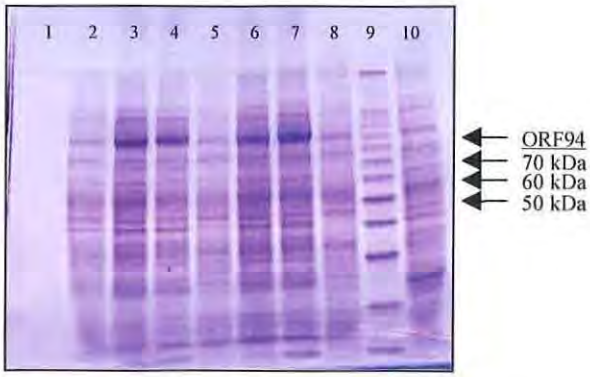
In order to prove the capacity of the recombinant viruses to produce the proteins expected in insect infected cells, we performed a slot blot analysis with an anti-Flag M2 antibody (Sigma) on the culture supernatant and on the cell pellets. Sf9 cells were separately infected with the three recombinant viruses at a MOI of ten and harvested two days post infection. We loaded from 500 µl to 0.5 µl equivalent cell culture of cell culture supernatant and of cells lysed by Triton X-100. We detected expression with the three recombinant tested. The majority of the three proteins are expressed in the intracellular fraction, in all tested dilutions. We observed also expression in the culture supernatant with the recombinant ORF43s and ORF94. In this case, the slot is positive until 5 µl and 0.5 µl for ORF43s and ORF94 respectively. By this way, we have demonstrated the secretion of the protein ORF94 without its hydrophobic C-terminal region and the capacity of the insect cells to recognize the natural signal peptide of this protein. We decided to carry out the kinetic of expression with the ORF43s and ORF94.

5.2. Kinetic study of the expression of ORF43s and ORF94 in Tn5 cells

We performed a kinetic study in order to assess the best production conditions and the harvest time in roller bottle. We use the Tn5 cells since these insect cells are recommended for the secretion of recombinant protein. When the cell density reached the density desired, we infected them with each virus at two multiplicity's of infection (0.1 and 5 virus/cells for ORF43s and 0.1 and 10 virus /cell for ORF94). Cell samples were harvested 24, 48, 72h post infection. In parallel, we infected cells with a virus coding for a known flag-tag protein as positive control. We took samples at each kinetic point and centrifuged them at 700 g to separate cell pellets and culture supernatants. We kept the cell pellets at –80°C. The supernatant were boiled immediately in loading buffer and kept at –20°C. We performed analysis by SDS-PAGE followed either by Commassie blue-staining or Western blot revelation with the anti-Flag M2 antibody (Sigma).

As shown on figures below, both proteins were well detected in the intracellular fraction, by Coomassie blue staining. The ORF43 has an apparent molecular weight of ~50 kDa and is in a doublet form. It is expressed at high yield. The ORF94 has an apparent molecular weight of 90 kDa and has a quite diffused coloration which is the characteristics of glycosylated proteins. The secreted forms were not detected by this way but we detected them weakly by immuno western blotting. We determined that the best production conditions are 48h of infection, to avoid important cells mortality. The two MOI tested were equivalent.





ORF 43s (10µl equivalent culture)
lane 1 : /
lane 2 : MOI of 5, 24h post-infection
lane 3 : MOI of 5, 48h post-infection
lane 4 : MOI of 5, 72h post-infection
lane 5 : MOI of 0.1, 24h post-infection
lane 6 : MOI of 0.1, 48h post-infection
lane 7 : MOI of 0.1, 72h post-infection
lane 8 : 10 µl of 10kDa ladder (Gibco BRL)
lane 9 : Uninfected Tn5 cells
lane 10 : Positive control (25kDa)

ORF 94s (10µl equivalent culture loaded)
lane 1 : /
lane 2 : MOI of 10, 24h post-infection
lane 3 : MOI of 10, 48h post-infection
lane 4 : MOI of 10, 72h post-infection
lane 5 : MOI of 0.1, 24h post-infection
lane 6 : MOI of 0.1, 48h post-infection
lane 7 : MOI of 0.1, 72h post-infection
lane 8 : uninfected Tn5 cells
lane 9 : 10 µl of 10kDa ladder (Gibco BRL)
lane 10 : Positive control (25kDa)

5.3. Optimization of the immuno Western-blotting analysis

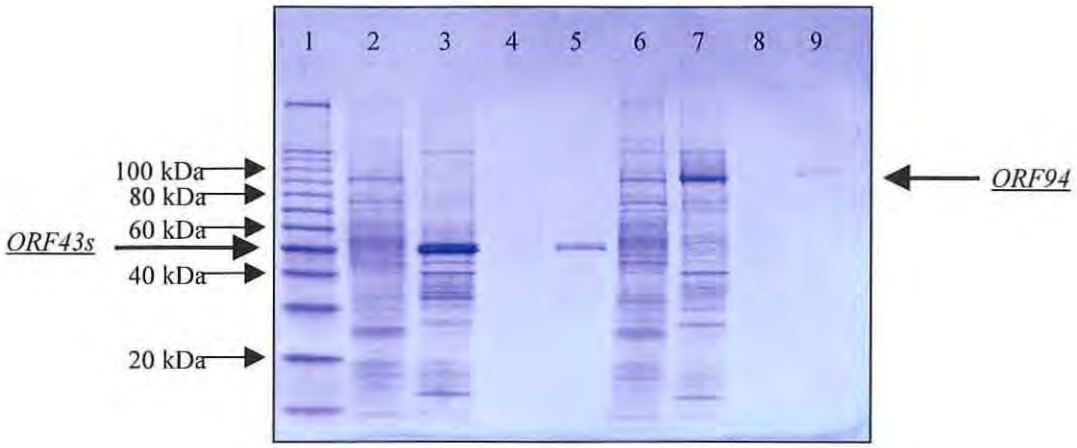
Even if we were capable to identify both proteins by Western-blot analysis when we use the protocol defined by the supplier, the background remained important against the uninfected insect cells and we observed a weak discrimination of bands. Concentration of primary antibody and incubation time were optimized to ensure a specific signal and to reduce the background. A dilution 1/20000 and an incubation of 1 hour at room temperature or overnight at 4°C were chosen for future analysis.

5.4. Analysis of the secreted ORF43s and ORF94

In order to estimate the concentration of the two proteins in culture supernatant, we precipitated them by TCA and analyzed concentrated protein pellets by SDS-PAGE and Coomassie blue staining. Despite the ten fold concentration of culture supernatants, both proteins could not be detected. Since the level of secreted proteins was too weak, we decided to perform the purification of both proteins from the intracellular fractions.

5.5. Solubility analysis of the intracellular ORF 43s and ORF 94

The proteins have to be soluble before being properly purified. So, we checked the solubility of the proteins expressed. We lysed infected cell pellets with 1% Triton X-100, centrifuged samples to separate cytoplasmic soluble and insoluble fractions and analyzed samples by SDS-PAGE and Coomassie blue staining. Western-blots are also performed and revealed with anti-Flag antibody. The major part (> 90%) of the two proteins remained associated to the insoluble pellet (see figure below).

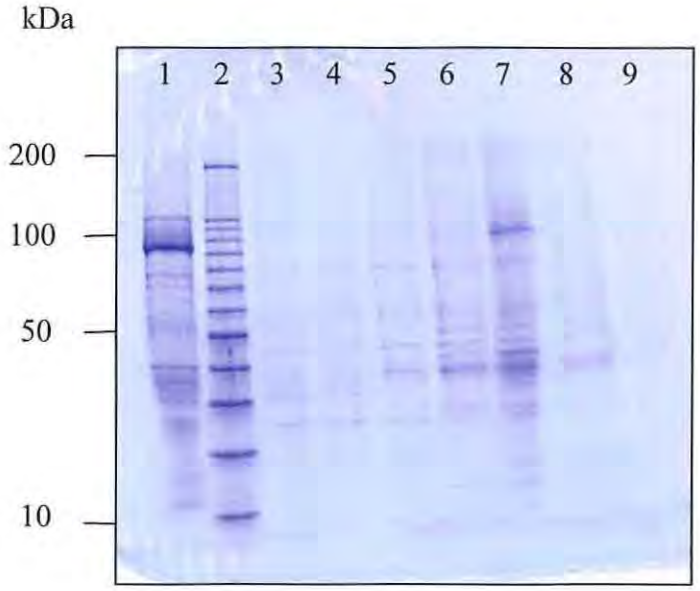


Triton lysis analysis (10µl equivalent culture loaded)

lane 1 : 10 µl of 10kDa ladder (Gibco BRL)
lane 2 : soluble fraction ORF43s
lane 3 : insoluble fraction ORF43s
lane 4 : soluble fraction ORF43s, 10x diluted
lane 5 : insoluble fraction ORF43s, 10x diluted
lane 6 : soluble fraction ORF94
lane 7: insoluble fraction ORF94
lane 8 : soluble fraction ORF94, 10x diluted
lane 9 : insoluble fraction ORF94, 10x diluted

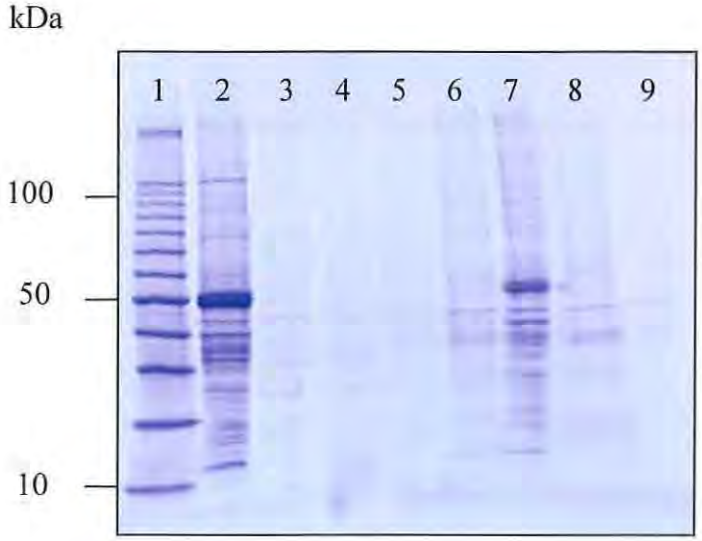
5.6. Solubilization assays

We lysed infected cells with Triton X-100, centrifuged lysates and performed different solubilization assays on insoluble pellets. We used Triton X-100 (1 and 4 %), extreme pH (10 mM NaOH, 1 mM HCl), an osmotic shock (2 M NaCl) and urea (1 and 4 M). Centrifuged supernatants and pellets of these assays were analysed by SDS-PAGE and Coomassie blue staining. For both proteins, the best conditions selected for solubilization of the insoluble cell fraction before purification is 10 mM NaOH (see figures below).



Solubilisation analysis of ORF94 protein

13.3 µl (= 10 µl culture)/lane
Lane 1 : insoluble fraction after cell lysis
Lane 2 : 10 kDa ladder (Gibco BRL)
Lane 3 : supernatant of sample treated with 1 % triton
Lane 4 : supernatant of sample treated with 4 % triton
Lane 5 : supernatant of sample treated with 1 M urea
Lane 6 : supernatant of sample treated with 4 M urea
Lane 7 : supernatant of sample treated with 10 mM NaOH
Lane 8 : supernatant of sample treated with 2 M NaCl
Lane 9 : supernatant of sample treated with 1 mM HCl



Solubilization analysis of 43S protein

13.3 µl (= 10 µl culture)/lane
Lane 1 : 10 kDa ladder (GibcoBRL)
Lane 2 : insoluble fraction after cell lysis
Lane 3 : supernatant of sample treated with 1 % triton
Lane 4 : supernatant of sample treated with 4 % triton
Lane 5 : supernatant of sample treated with 1 M urea
Lane 6 : supernatant of sample treated with 4 M urea)
Lane 7 : supernatant of sample treated with 10 mM NaOH
Lane 8 : supernatant of sample treated with 2 M NaCl
Lane 9 : supernatant of sample treated with 1 mM HCl

6. Production of ORF43s and ORF94

6.1. Expression in insect cells

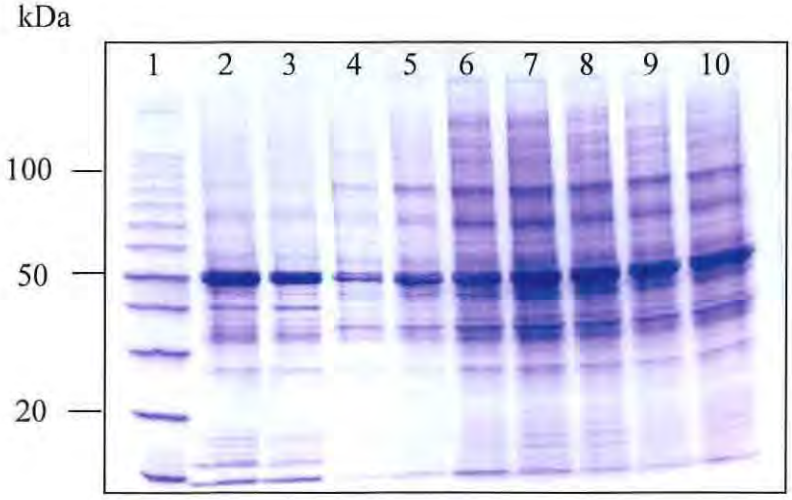
We performed a 4 l production for both proteins at the highest MOI in Tn5 cells. We harvested the cells after 48h, concentrated them 14x in PBS with anti-proteases cocktail and kept them frozen at –80°C.

6.2. Purification

6.2.1. Chelating Sepharose

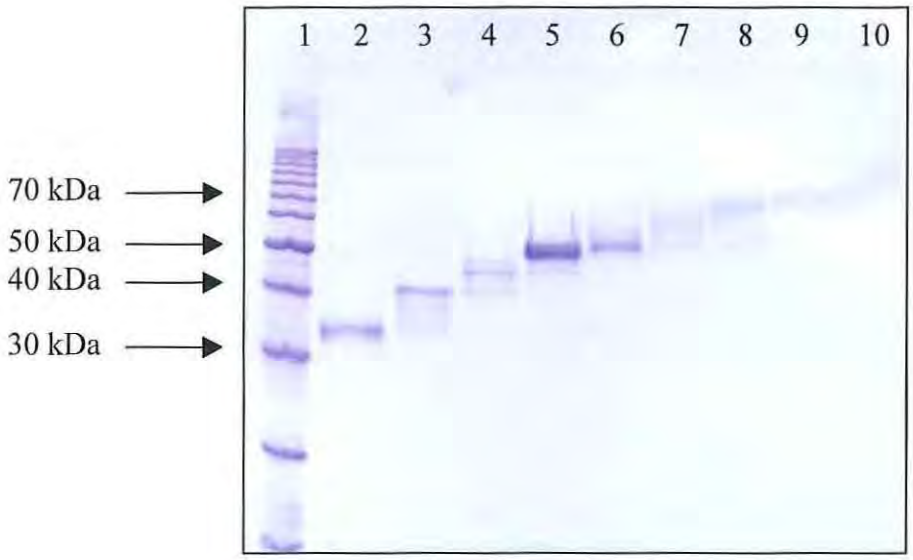
Cells infected with ORF43s baculovirus were first lysed with Triton X-100 and recombinant protein solubilized with NaOH 10mM according to the previously selected conditions. Purification of the protein was then performed on a Chelating Sepharose column (IMAC, Pharmacia) which has theoritically a high affinity for the His-tag added at the C-terminal part of the protein. We used our standard protocol and eluted with 200 mM imidazole. Unfortunately, the protein did not bind properly to the column and the eluted fractions are not

pure but contaminated by a lot of other cellular proteins (see figure below). It seems that the ORF43 was not completely solubilized leading to non efficient chromatography.
So, we changed our strategy for antigen purification and did not try this one for ORF94 anymore. We decided to purify proteins for animals immunization by preparative electrophoresis.



ORF43s purification on Chelate Sepharose column
20 µl/lane
Lane 1 : 10 kDa ladder (GibcoBRL)
Lane 2 : load
Lane 3 : flow through
Lane 4 : elution fraction 1
Lane 5 : elution fraction 2
Lane 6 : elution fraction 3
Lane 7 : elution fraction 4
Lane 8 : elution fraction 5
Lane 9 : elution fraction 6
Lane 10 : elution fraction 7

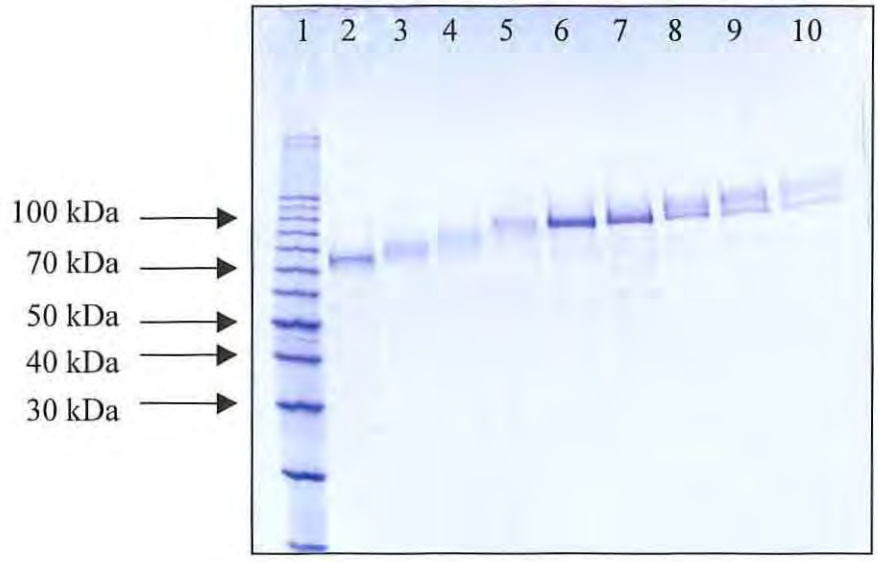
6.2.2 Preparative electrophoresis
Cells infected with ORF43s and ORF94 baculoviruses were lysed with Triton X-100 and recombinant protein solubilized with NaOH 10mM according to the selected conditions. Solubilized proteins were submitted to an electrophoresis through a cylindrical acrylamid gel (10 % for ORF43s and 6.5 % for ORF94) with SDS (Model 491 Prep Cell, Biorad). As molecules migrate through the gel matrix, they are separated into ring shaped bands. Individual bands migrate off the bottom of the gel where they pass directly into the elution chamber for collection. Electrophoresis conditions have had to be optimized. 110 fractions were finally collected and analyzed by SDS-PAGE and Coomassie blue staining. Fractions 53 to 65 and fractions 56 to 68 containing ORF43s and ORF94 proteins respectively (see figures below) were pooled and concentrated on Ultrafree-15 devices for immunizations.



SDS-PAGE analysis of ORF43s purification by preparative electrophoresis

25 µl/lane

Lane 1: molecular weight marker (10kDa, GibcoBRL)
Lane 2: elution fraction 30
Lane 3: elution fraction 40
Lane 4: elution fraction 50
Lane 5: elution fraction 60
Lane 6: elution fraction 70
Lane 7: elution fraction 80
Lane 8: elution fraction 90
Lane 9: elution fraction 100
Lane 10: elution fraction 110



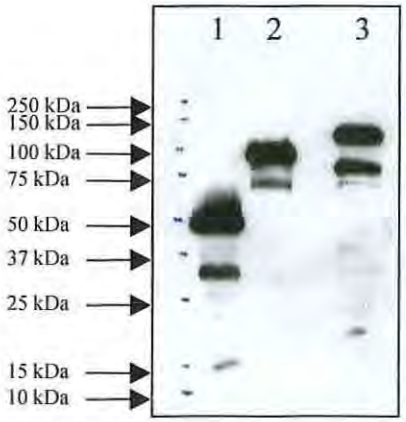
SDS-PAGE analysis of ORF94 purification by preparative electrophoresis

20 µl/lane

Lane 1: molecular weight marker (10kDa, GibcoBRL)
Lane 2: elution fraction 40
Lane 3: elution fraction 45
Lane 4: elution fraction 50
Lane 5: elution fraction 55
Lane 6: elution fraction 60
Lane 7: elution fraction 65
Lane 8: elution fraction 70
Lane 9: elution fraction 75
Lane 10: elution fraction 80

6.3. Quality Control

Purified ORF43 and ORF94 proteins were identified by ECL Western blotting with the anti-Flag antibody (see figure below).



Western blot analysis of purified ORF43 and ORF94 proteins

Lane 1 : 1 µg of purified ORF43 protein Lane 2 : 1 µg of purified ORF94 protein
Lane 3 : positive control (110 kDa)

The protein concentration of the preparations were dosed by microBCA (Pierce) and was ~1.4 mg/ml for ORF43 and ~1.2 mg/ml for ORF94. We purified by such a way 2.8 mg of ORF43 and 2.3 mg for ORF94, sufficient quantities for obtention of rabbit antiserum and monoclonal antibodies

Both preparations were also tested by immuno western-blotting with anti-OsHV cite and serum from Participant 1. The ORF43 could be detected by one of them (serum n°3) but curiously not the ORF94.

7. Immunization

7.1. Rabbit immunization

Immunization is in progress to obtain antisera. Two rabbits were injected at day 1, 14, 28 and 56 with 100µg of the recombinant proteins per injection. Bleedings will be tested by ELISA and immuno western blotting to determine their specific titer.

7.2. Mice immunization

Immunization is in progress to obtain monoclonal antibodies. Four mice were injected 4 times at three weeks interval with 50µg of the recombinant proteins per injection. Bleedings will be tested by ELISA and immuno western blotting to select the best responding mouse for fusion.

7.3. ELISA optimization

ELISA for selection of antisera and monoclonal antibodies will be performed on insect cells rather than on purified antigen. Assays were done to determine the best cell concentration for plates coating. We tested in parallel Tn5 cells infected by a recombinant baculovirus with a Flag-His Tag, Tn5 cells infected by a recombinant baculovirus without tag and Tn5 cells uninfected. Revelation was performed with anti-flag antibody. Experimental conditions for sensitive detection without background on negative controls were found. 5 10^4 cells/well will be used in further ELISA.

7.4. Cell preparations for ELISA

Specific insect cell preparations were done for ELISA coating. We infected Tn5 cells by the recombinant baculovirus ORF43s, the recombinant ORF94 and a well known Flag-His Tag recombinant as control for antibodies against Tag epitopes. Cells were infected and harvested according to the previous results. Uninfected cells were also prepared as control for antibodies against insect cell epitopes. As the baculovirus infected cells are very sensitive to the proteases, we aliquoted and kept them at –80°C until using in ELISA and blotting assays.

The progress in this sub-task is as anticipated.

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

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

Dr F. Xhonneux wrote this progress report.

Dissemination of results

The work conducts for sub-task 2.2 should permit to produce recombinant proteins and specific antibodies. Participants 1 and 3 want to try to deposit patents for these reagents.

SIGNIFICANT DIFFICULTIES OR DELAYS

A 2 month delay in monoclonal antibodiy production will be observed. They will be obtained in June 2001. This delay corresponds to a delay in the beginning of the sub-task 2.2.

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 2000 to 3rd January 2001

Participant no. 4
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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 **04-01-00** to **03-01-01**

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
Christine DELBARD
Jean-Paul GUIAVARC'H

OBJECTIVES

- Preparation of oyster primary cell cultures
- Herpes-like virus replication in 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

[a]P. R. : Periodic Progress Report; [b]F. 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)

1. Preparation of oyster primary cultures : embryonic and heart cell cultures

During this year, we continued to work on the optimization of the primary cultures of fresh and frozen-thawed *Crassostrea gigas* « embryoïds » obtained by enzymatic treatment of embryos at 2 to 64 stages of cell development, in particular to decrease the risk of microbial contamination of cultures by bacteria, and, more often, by yeasts.
We cultivated also oyster heart cells. Such cultures, which can be obtained routinely, are favorable to the observation of the cytopathogenic effect of virus, because the majority of adherent cells, which are cardiomyocytes, are fibroblastic-like cells. The effect of the incubation temperature on the embryonic and heart cell adherence and cell growth has been studied by measuring the rate of proteins and/or DNA and by using BrdU test, knowing that the optimal temperature for the herpes-like virus may be 26°C (Le Deuff *et al.*, 1996).
We observed, as illustrated in Figures 1 to 7, a similar evolution of embryonic and heart cell cultures at 26°C, 20°C and 15°C, up to 6 days. There is a slight increase of proteins and DNA between 2 and 4 days for embryoïds and between 2 and 8-10 days for heart cells. After BrdU incorporation, some positive dark nuclei were detected by immunocytochemistry, from 2 to 6 days (for embryoïds) or 8 days (for heart cells), showing that there is probably replicative DNA synthesis. However, we noted that at 26°C, culturing cells are contractile earlier and that cell networks are established after a shorter time than at 15°C : e.g., in cultures of embryoïds, contractile cells are observed after 1 day at 26°C and only after 3 days at 15°. Cell networks are established after 2 days at 26°C and 6 days at 15°C.

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

To control that the infectiosity of infected frozen samples sent by express mail by IFREMER - La Tremblade - T. Renault, was preserved after thawing, we cultivated also Crassostrea gigas D larvae, obtained in the laboratory by in vitro fecondation, in the same culture conditions as for cells.

Figs 1. and 2. Culture of embryonic cells at 26°C and 15°C up to 6 days

Figure 1

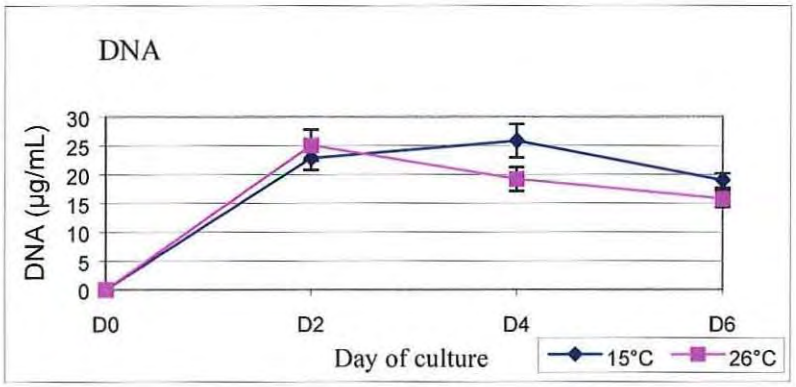


Figure 2

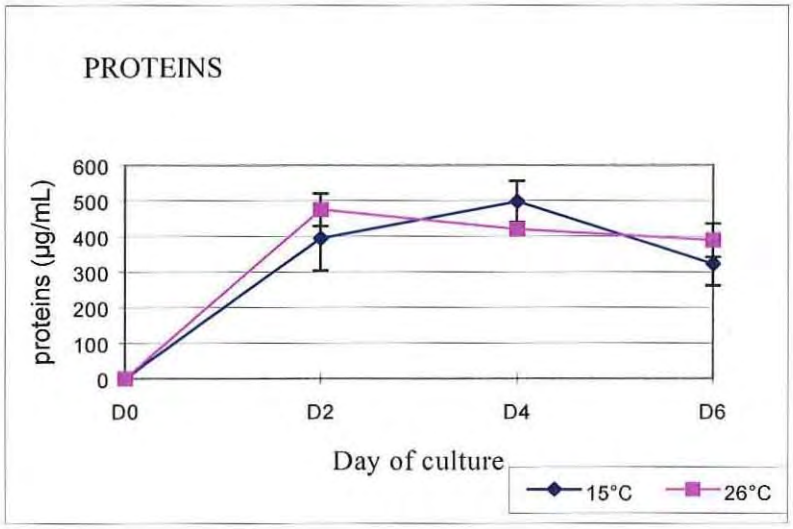


Fig. 3. BrdU test - Embryonic cells after 2 days in culture at 26°C

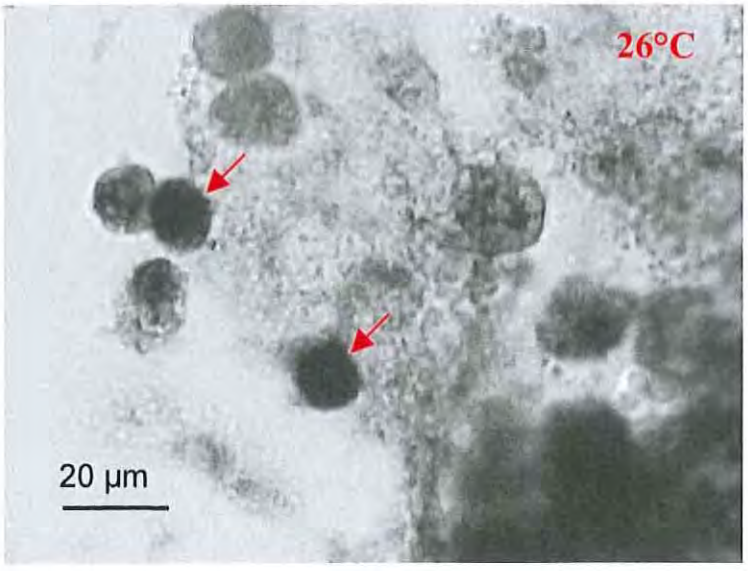


Fig. 4. BrdU test - Cardiac cells after 2 days in culture at 26°C

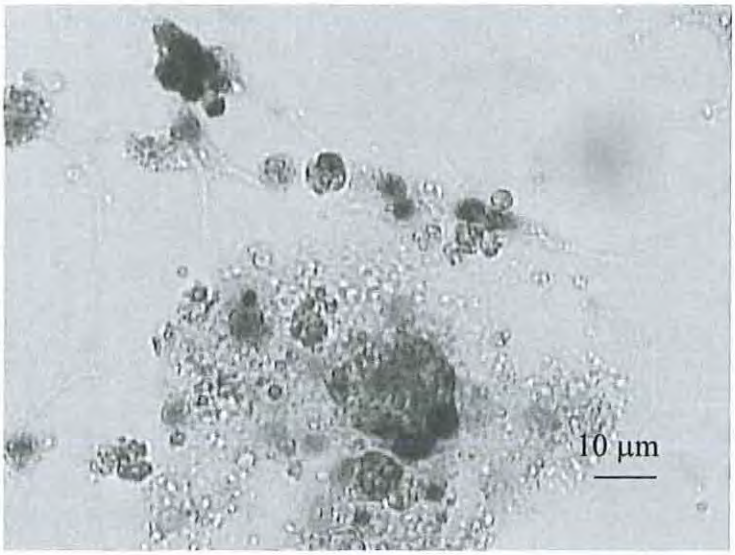


Fig. 5. Culture of heart cells at 15°C and 26°C up to 10 days

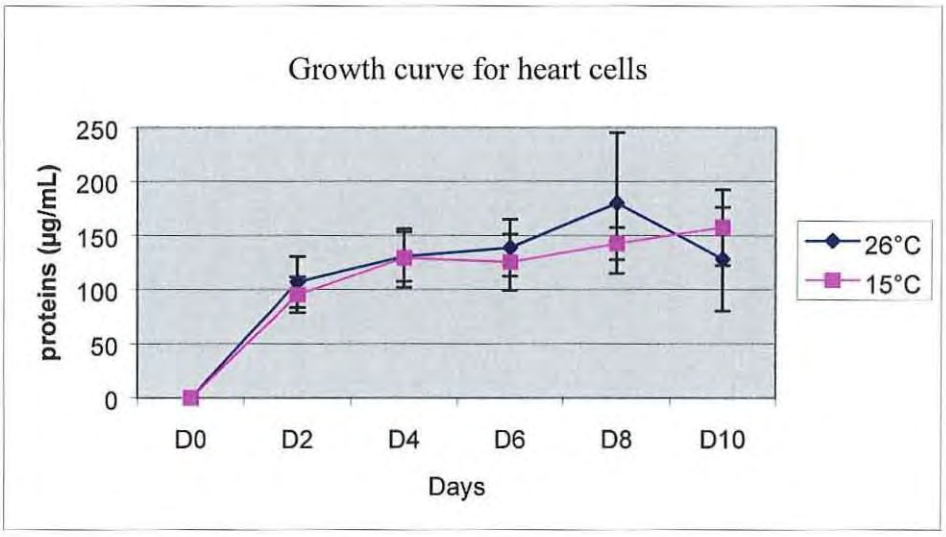


Fig. 6. BrdU incorporation by heart cells after 6 days at 15°C

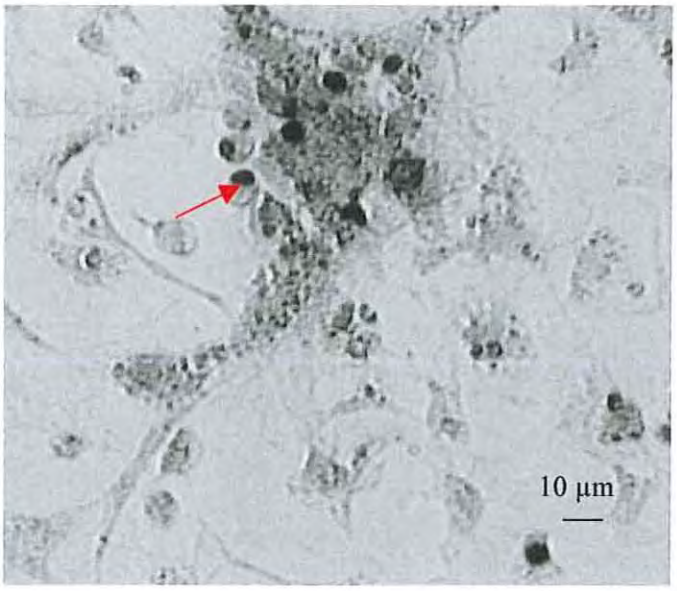
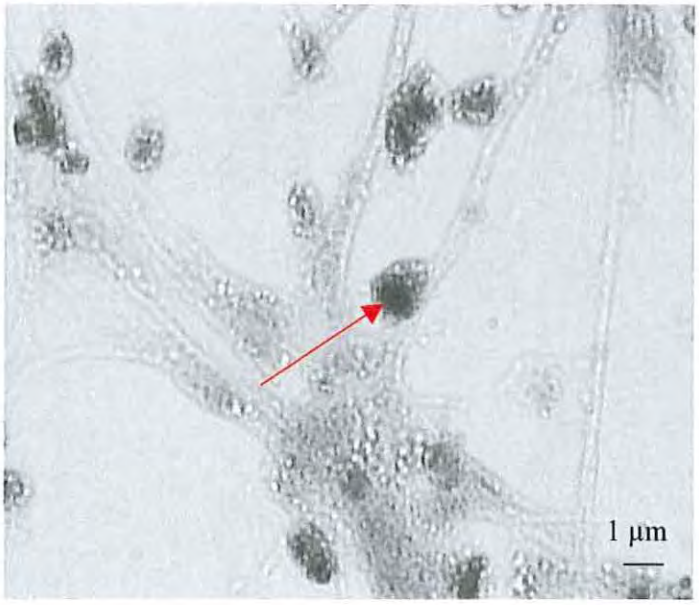


Fig. 7. BrdU incorporation by heart cells after 6 days at 26°C



2. Cryopreservation of dissociated heart cells and embryoïds

If heart cell cultures may be obtained from fresh cells and from cryopreserved cells using the protocol described by Le Marrec *et al.* (1998, 1999), we did not succeed to maintain frozen embryonic cells in aggregates : they separate when they are thawing. However, even if the viability percent of thawed cells is at least 80%, cultured cells did not organize in networks as described for embryoïds in the first VINO report. First attempts of cryopreservation of the embryoïds in biopolymers as alginate are in progress. This strategy will perhaps enable us to resolve this problem.

3. Cultivation of herpes-like virus in oyster primary cultures

3.1. Validation and optimization of the analytical methods used to control if cultured cells are infected by the herpes-like virus :

In a first step, we verified that tools and experimental procedures used to detect virus infections in cells were optimal. These controls concerned:

- The PCR amplification method,
- The digoxygenin labeling of DNA probes used for *in situ* hybridization,
- *The In situ* hybridization procedure, using histological sections of *Crassostrea gigas* spat (from IFREMER - La Tremblade : T. Renault) infected by the herpes-like virus, as positive control,
- The infectiosity of infected larval samples (from IFREMER - La Tremblade : T. Renault) using axenic *Crassostrea gigas* D larvae produced in our laboratory by *in vitro* fecundation.

❖ ***PCR procedure***

As illustrated in Figure 9, we validated the PCR procedure recommended by T. Renault, using different concentrations of purified herpes-like virus DNA (sent by T. Renault) : results of DNA amplification by PCR were dose-dependent from 10 ng to 100 fg of DNA, but not for lower concentrations (10 fg to 1 fg). According to T. Renault, such results were similar to those of the other participants in the VINO programme : the sensitivity level of this test could be 100 fg.

❖ ***Digoxigenin labeling of DNA probes used for in situ hybridization***

Firstly, we verified that UTP, a digoxigenin labeled analog of thymidin (digoxigenin-11-UTP) was incorporated in probes. As expected, the migration rate in electrophoresis of PCR amplification products labelled by digoxigenin was lowest than for unlabeled DNA (Fig. 10).

Secundly, digoxigenin labeled probes were applied to sections of *Crassostrea gigas* infected spat. As negative control, we used sections of larvae treated by heat (95°C) for DNA denaturation. As expected, we observed (Fig. 11) positive dark nuclei in sapt cells infected by the herpes-like virus (DAB as substrate of peroxidase).

Fig. 8. Validation of PCR procedure : purified herpes-like DNA at different concentrations from 10 ng to 1 fg

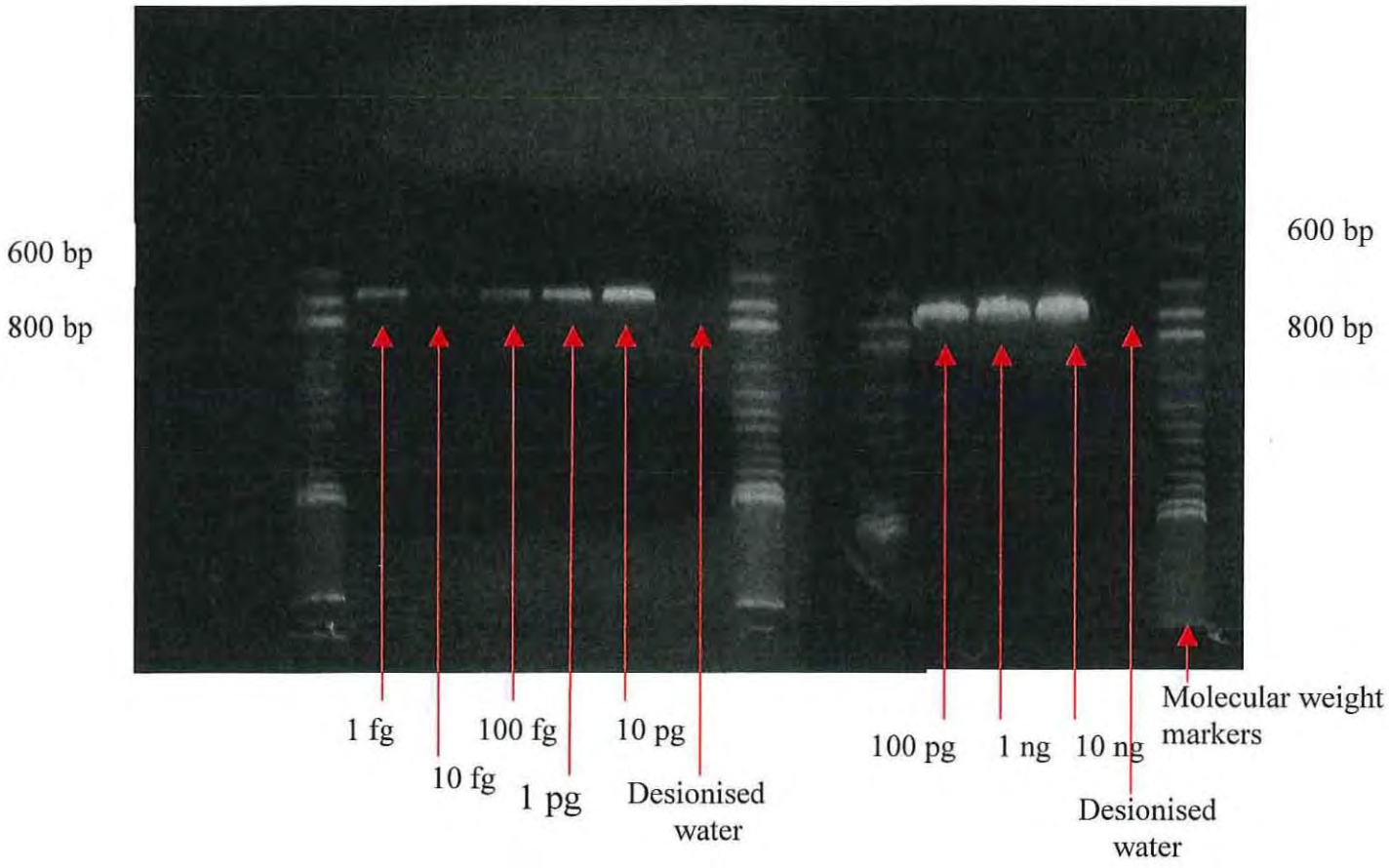
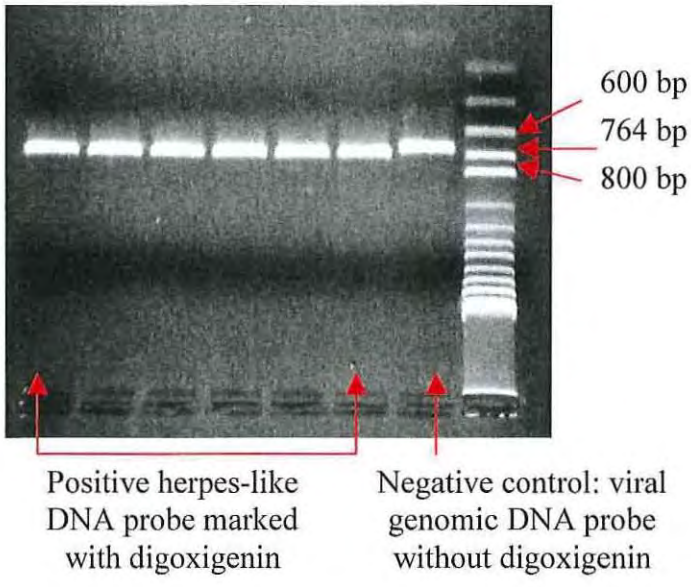


Fig. 9. DNA probes marked by digoxigenin for *in situ* hybridization



Figs. 10 and 11. Validation of *in situ* hybridization using infected spat by the herpes-like virus as positive controls

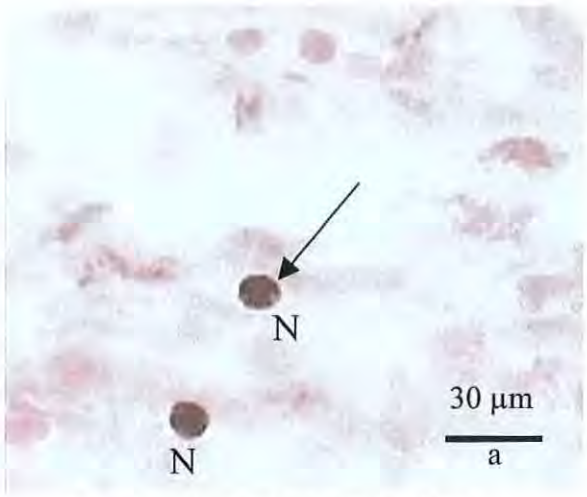


Figure 10: positive nuclei (N) : infected animal as positive control

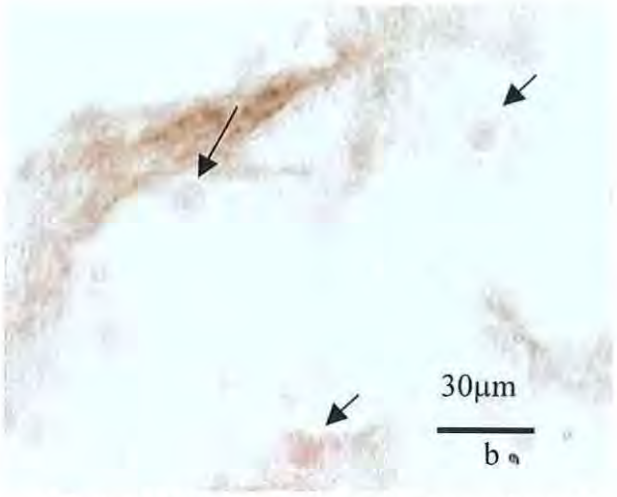


Figure 11: negative control: contaminated animal, without DNA denaturation by heat

❖ ***Infectiosity of infected larval samples***

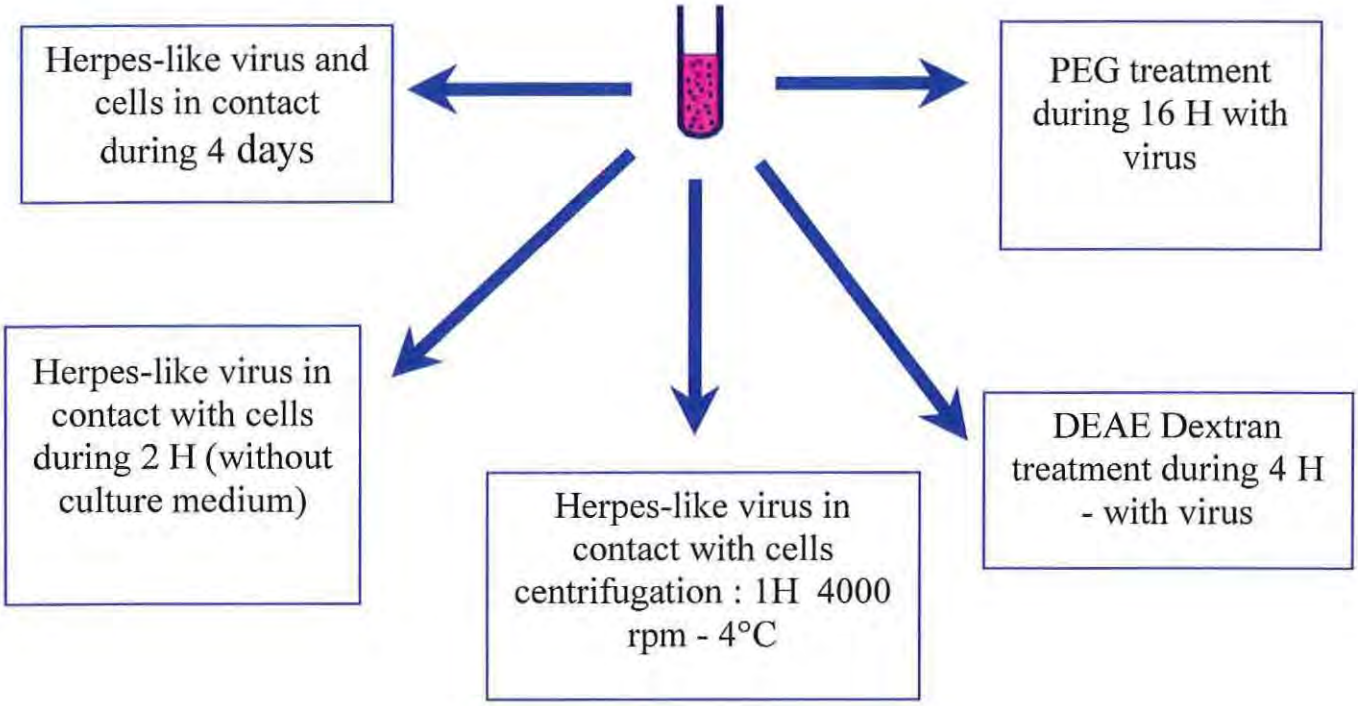
D larvae were used as experimental models to test the infectiosity of frozen-thawed infected larval samples, sent by express mail by T. Renault. These larvae are maintained *in vitro* in the same culture conditions as embryonic and heart cells (temperature of incubation : 26°C ; culture medium : sterile sea water complemented with Leibovitz (10%), fœtal calf serum (10%) and antibiotics ; 2000 larvae per well, in multiwell 24 ; 10 replicates per assay).

The infectiosity of the herpes-like virus was tested in the same experimental conditions (Fig. 12) as embryonic and heart cells. After 4 days, as related in litterature (Le Deuff *et al.*, 1994 ; Le Deuff, 1995), for all tested experimental conditions, infected larvae lost their motility and sedimented, suggesting a cytopathogenic effect of the herpes-like virus. However, unexpected results were obtained by PCR for these larval samples (after heat treatment): only a slight amplification was observed for the 764 pb DNA sequence. Viral DNA diluted in purified water (100 pg) was used as positive control and DNA from untreated larvae as negative control (Fig. 13).

To verify if the PCR could be inhibited by cellular extracts, two experiments were realised with purified viral DNA (1 ng to 100 fg) diluted in embryonic cellular extracts in the same way as viral DNA diluted in water. Figure 14 , which shows results of one of these assays, proved that PCR amplification was partially (10 ng DNA) or totally (lowest concentrations of DNA) inhibited by cellular extracts.

After these works, we could conclude that it was necessary to purify DNA extract from infected cells before PCR. In with this aim, we used the kit « SV total RNA Isolation System » commercialized by Promega, without Dnase extraction and after treatment of samples at 95°C (3 minutes) to eliminate RNA. The figure shows the results of one of two assays, obtained with the same samples as for the first experiments. They validated the interest of this procedure applied to cell cultures infected by the herpes-like virus.

Fig. 12. Experimental conditions to test the infectiosity of herpes-like virus samples



3.2. Cultivation of herpes-like virus in oyster cell cultures

The experimental conditions of these assays are resumed in Figure 14. Studies were realized 4, 7, 11 days after the inoculation of the herpes-like virus in heart cell cultures and 4, 5 and 6 days after the inoculation of the virus in embryonic cell cultures. It was difficult to conclude to a cytopathogenic effect of the herpes-like virus when we observed each day cell cultures under an inverted microscope. However, PCR, realised 4 and 6 days after the inoculation of the herpes-like virus, in the experimental conditions described in 3.1, showed positive results for all the conditions of viral infection of embryonic cells in culture, and, in particular, when the herpes-like virus was maintained in contact to the cells during 2 hours before adding cell culture medium and when PEG was added to the medium. Figure 15 shows results of 2 PCR obtained with purified DNA samples (5 µg) from embryonic cells. *In situ* hybridization (Fig. 16) applied to these « PCR positive » samples allowed to observed also positive reactions in some cells, in particular around the cells, but nuclei are not positively marked. We have to verify if the herpes-like virus penetrates or not into cells. For heart cells, the first results were negative but DNA samples were not purified before PCR. We have to control if such results are « false negative » or not.

Fig.13. PCR: DNA from infected larvae

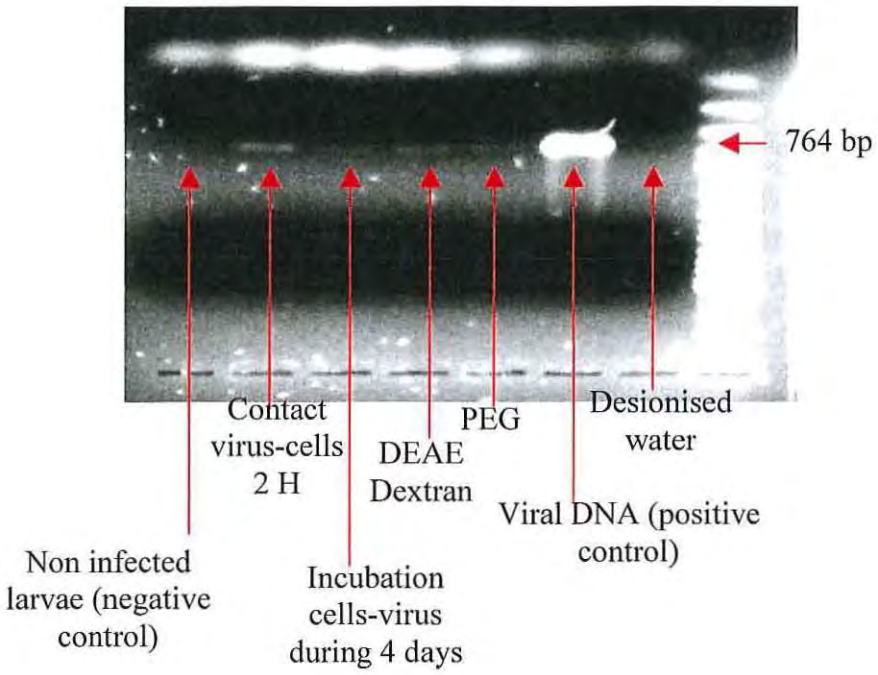
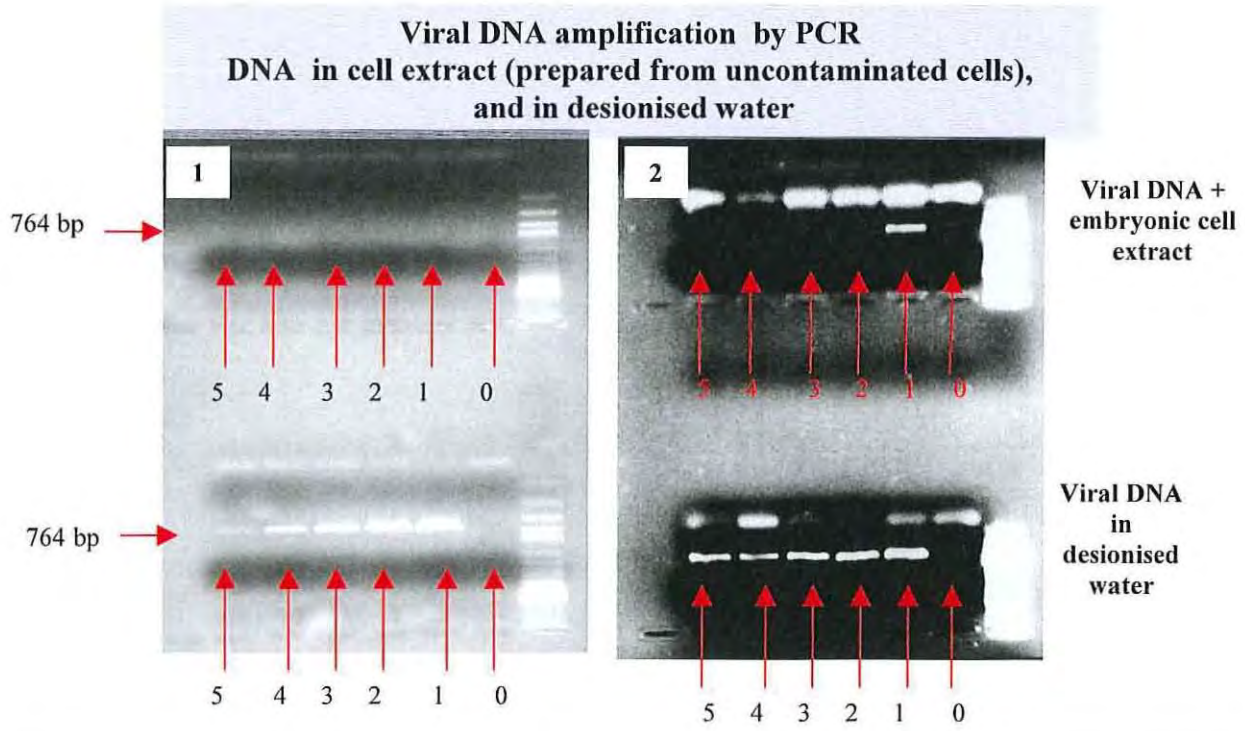
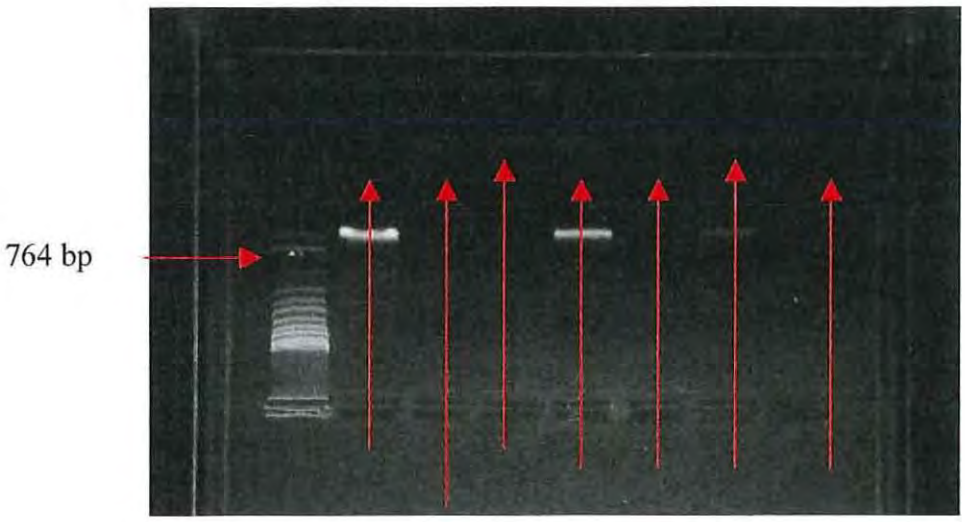


Fig. 14. PCR amplification of herpes-like DNA diluted in a cellular extract (from uncontaminated cells) and in water



Viral DNA: 1 = 1 ng ; 2 = 100 pg ; 3 = 10 pg ; 4 = 1 pg ; 5= 100 fg ;
0 = desionised water

Fig. 15. Purified DNA extracted from infected larvae : PCR



T: negative control (uninfected larvae) ; 1 : infected larvae with PEG ; 2 : with DEAE dextran ; 3: incubation with virus ; 4 : contact virus ; 5: larvae during 2 hours ; 100pg viral DNA: positive control

Fig. 16. Contamination of embryonic and heart cells in culture by the herpes-like virus

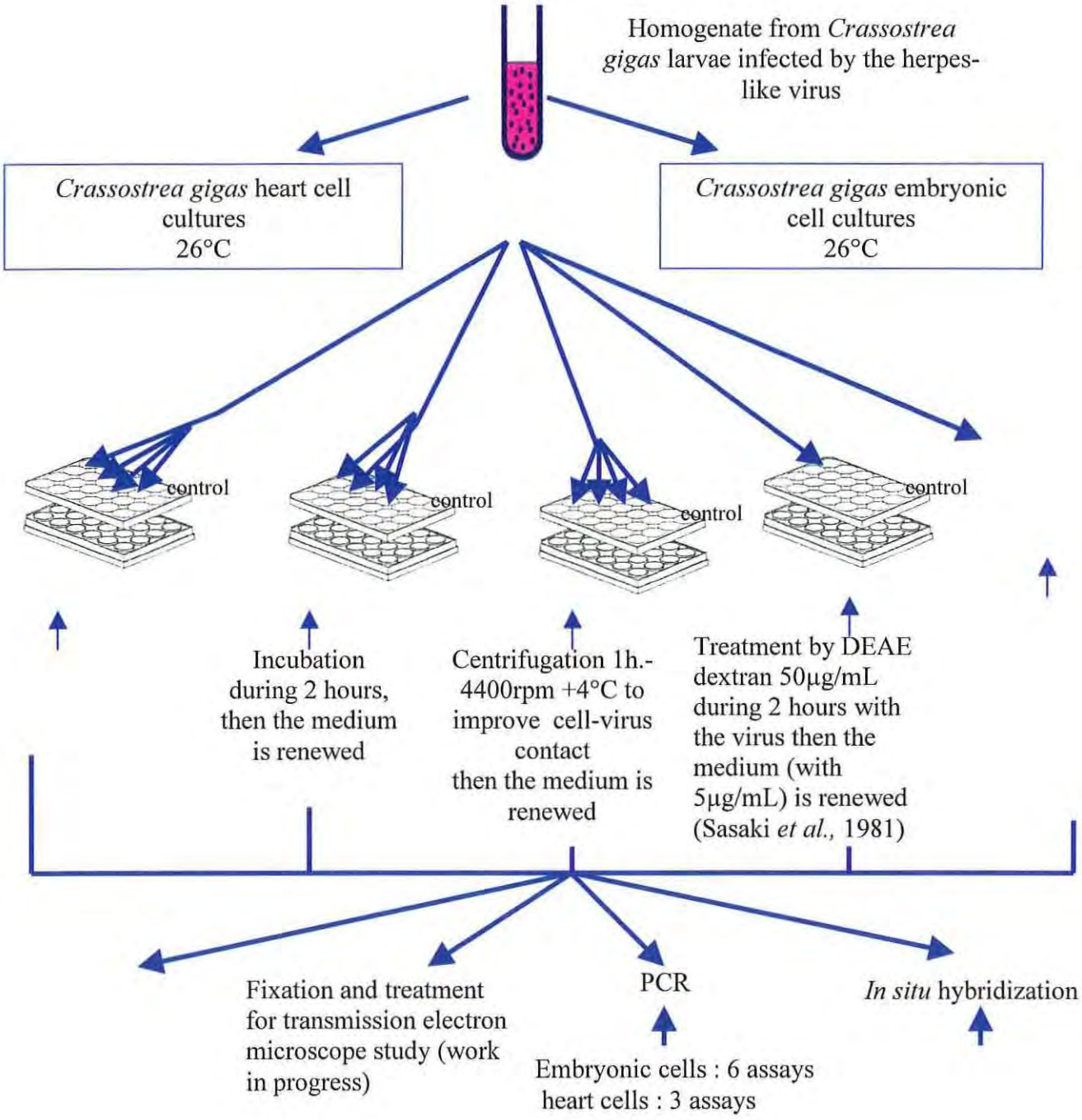


Fig. 17. PCR : embryonic cells infected by the herpes-like virus : purified DNA

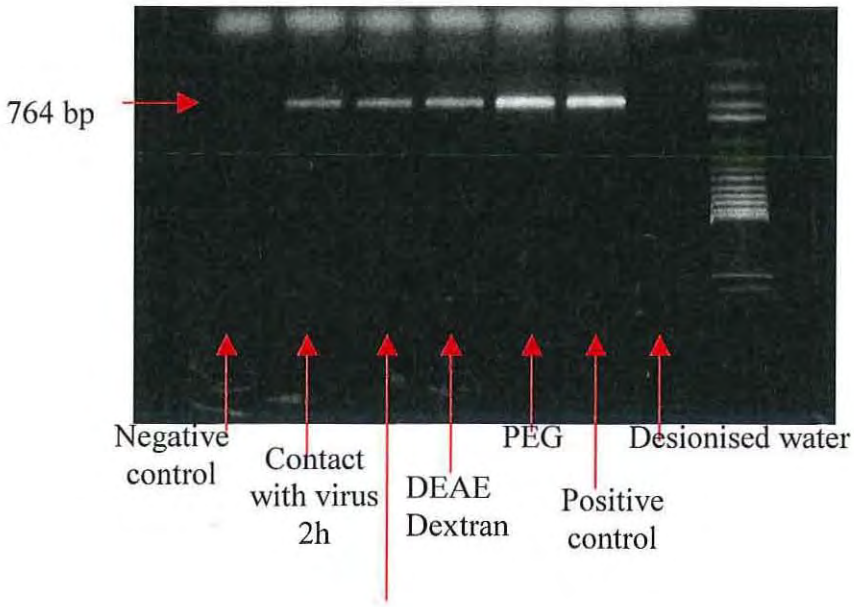
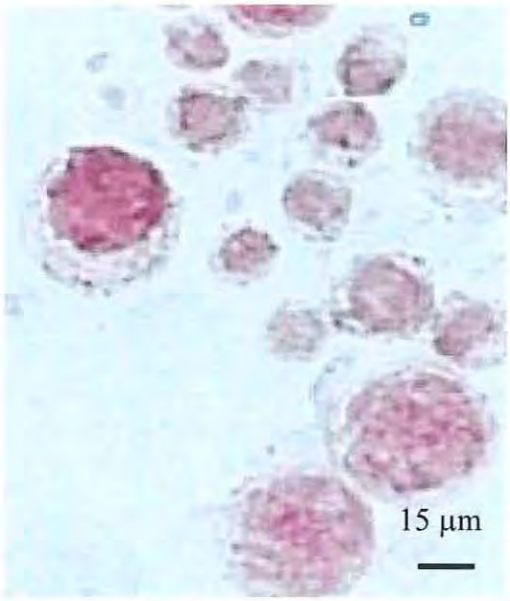
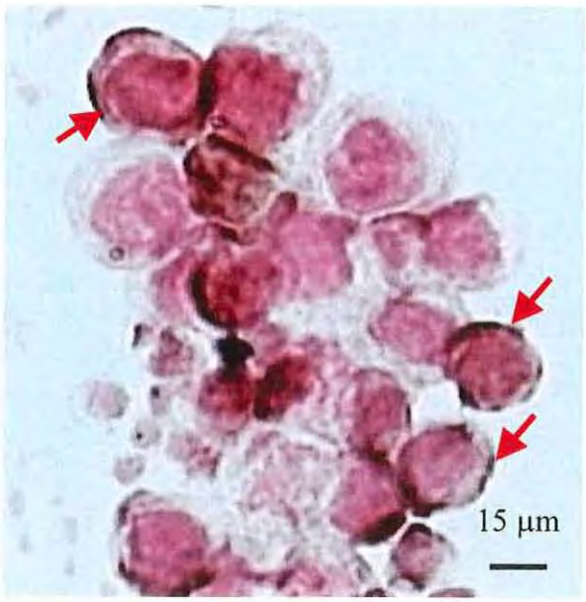


Fig. 18. *In situ* hybridization of cultured cells infected by the herpes-like virus



Negative control



Infected embryonic cells (arrows : labeled cells

4. Bibliography

LE DEUFF R.M. **1995.** Contribution à l'étude de virus de mollusques marins apparentés aux *Iridoviridae* et aux *Herpesviridae*. Thèse de Doctorat, univ-Bordeaux II, 234 p.

LE DEUFF R.M., RENAULT T. & GERARD A. **1996**. Effect of temperature on *Herpes*-like virus detection among hatchery-reard larval pacific oyster Crassostrea gigas . Dis. Aquat. Org., 24, 149-157.

LE MARREC-CROQ F., FRITAYRE P., CHESNE C., GUILLOUZO A. & DORANGE G. **1998**. Cryopreservation of *Pecten maximus* heart cells . Cryobiology, 37, 200-206

LE MARREC-CROQ F., GLAISE D., GUGUEN-GUILLOUZO C., CHESNE C., GUILLOUZO A., BOULO V. & DORANGE G. **1999**. Primary cultures of heart cells from the scallop *Pecten maximus* (Mollusca-Bivalvia). *In Vitro* Cell. Dev. Biol.- Animal, 35, 289-295.

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 second annual meeting in Paris (France) in December 2000. She also wrote the second Invividual Progress Report.

SIGNIFICANT DIFFICULTIES OR DELAYS

None.

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 2000 to 3rd January 2001

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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 **04-01-00** to **03-01-01**

Type of contract:	Shared-cost research project		
Total cost: (50.5%)	1,283,805.5 ECU	***EC contribution:***	649,738 ECU
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		

Coordinator: Dr Tristan Renault
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INDIVIDUAL PROGRESS REPORT

Participant no. 5: University College Cork, Ireland

Scientific team: Dr Sarah CULLOTY
Pr Maire MULCAHY
Ms Michelle CRONIN

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

[a]P. R. : Periodic Progress Report; [b]F. 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)

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

1. Collection of Samples /Sites.

Samples were again obtained in Year 2 from Cork harbour and Dungarvan as in Year 1 and this year oysters were also obtained from Galway Bay. Samples were taken in February, July-September and November 2000. *C. gigas* and *O. edulis* were obtained from Cork harbour (<1 year old), *C. gigas* from Dungarvan (< 1 year old) and *O. edulis* from Galway bay (< 1 year olds and adults) (Table 1). Some mortalities were observed in Cork harbour in the flat oyster spat while in holding tanks during the summer but otherwise no unusual mortalities were observed this year.

2. Oyster samples

All individuals were divided in half – one half frozen at -20°C for PCR analysis and one half fixed in Davidsons fixative.

3. Histology

All histological samples have now been processed from Year 1 and 2 and tissue sections of all blocks sectioned at 7µm and stained with Haematoxylin and Eosin to screen for morphological changes in the tissues. Some sections have been screened from Year 1 and a small number of sections appeared to show some abnormalities associated with nuclei of connective tissue cells (to be verified with the Coordinator) (Annex, Table 1).

4. PCR analysis

❖ *PCR - Validation*

Viral DNA – 5 x 10^7 copies of the viral genome (10 ng) was provided by the coordinator. A series of serial dilutions was carried out on this sample – down to 1 fg. This analysis has been carried out a number of times to continue to monitor sensitivity. All dilutions can be detected except for 1 fg on some occasions. This may be due to problems related to the dilution of samples at this level. A number of samples of supernatant were provided by the Coordinator following the training session at IFREMER in May 2000. PCR analysis was subsequently carried out on these samples in Cork and results compared with those obtained by different groups during the training session (Annex, Table 2).

❖ *Extraction of DNA*

All samples for Year 1 had been stored at -20°C. Following validation of the PCR technique, samples were removed and DNA extracted using the technique provided by the Coordinator. Animals were greater than 12 mm and so were ground within a plastic bag (individual samples) and a supernatant prepared.

❖ *PCR*

PCR on samples from Year 1 was initially carried out using Primers OHV3 and OHV14. All samples screened during the year were negative. Following the provision of new primers by the coordinator in early 2000 - OHV1 and OHV2, some

samples from Year 1 were rechecked with the new primers. Again all samples were negative (Annex, Table 1). PCR analysis has been carried out to date on samples taken in February 2000 from Cork harbour and Dungarvan using OHV1/OHV2 and all have to date been negative for herpes-like virus (Annex, Table 1).

5. *In situ* hybridisation

This work is just beginning in the laboratory. A number of sections have been screened but all have been negative (Annex, Table 1).

The progress in this sub-task is as anticipated. The work was carried out by Dr Sarah Culloty and Ms Michelle Cronin.

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 attended the training session at IFREMER, La Tremblade in May 2000. She also wrote this intermediate report. For the second annual meeting in December 2000 in Paris, due to health problems, Dr S. Culloty has sent to Dr T. Renault (the coordinator of the research programme) overheads which summarised results obtained in Cork.

SIGNIFICANT DIFFICULTIES OR DELAYS

None

ANNEX **Table 1.** 2000 samples for Ireland

Site	Date	Species	Origin	N	Histology	PCR		*In situ* hybridisation
					Samples processed	Screened	OHV3/OHV14	OHV1/OHV2	
Cork	13/07/99	*C. gigas*	Irish hatchery	30	Yes	30/30 (2)*	All negative		10/30 screened (all negative)
	27/07/99	*C. gigas*	Irish hatchery	30	"	" (1)	"		10/30 screened (all negative)
	03/08/99	*C. gigas*	Irish hatchery	30	"	" (1)	"		
	17/08/99	*C. gigas*	Irish hatchery	30	"	"	"		
	30/08/99	*C. gigas*	Irish hatchery	30	"	"	"		
	30/08/99	*C. gigas*	Irish hatchery	29	"	29/29	"		
	23/11/99	*C. gigas*	Irish hatchery	30	"	"	"		
Dungarvan	25/08/99	*C. gigas*	Guernsey	30	"		"		
	25/08/99	*C. gigas*	Seasalter	29	"		"		
	12/11/99	*C. gigas*	Seasalter 03/99	30	"		"		
	12/11/99	*C. gigas*	French hatchery	30	"		"	All negative	
	12/11/99	*C. gigas*	Guernsey	30	"		"		

Cork	13/02/00	*C. gigas*	Irish hatchery	30	"	"
	18/07/00	*C. gigas*	Irish hatchery	30	"	
		O. edulis	Cork harbour	30	"	
	27/07/00	*C. gigas*	Irish hatchery	30	"	
		O. edulis	Cork harbour	30	"	
	04/08/00	*C. gigas*	Irish hatchery	30	"	
		O. edulis	Cork harbour	20	"	
	08/08/00	*C. gigas*	Irish hatchery	30	"	
		O. edulis	Cork harbour	28	"	
	24/08/00	*C. gigas*	Irish hatchery	30	"	
		O. edulis	Cork harbour	30	"	
	26/11/00	*C. gigas*	Irish hatchery	30	"	
		O. edulis	Cork harbour	30	"	
Dungarvan	27/02/00	*C. gigas*	English hatchery	30	"	"
		"	French hatchery	30	"	"
		"	"	30	"	"
	02/08/00	*C. gigas*	English hatchery	29	"	
		"	French hatchery	30	"	
		"	"	30	"	

	24/08/00	*C. gigas*	English hatchery	29	"
		"	French hatchery	30	"
		"	"	30	"
	26/12/00	*C. gigas*	English hatchery	30	"
		"	French hatchery	30	"
		"	"	30	"
Galway Bay	11/08/00	*O. edulis*	Galway	30	"
		O. edulis	Galway	29	"
		O. edulis	Galway	25	"
	25/08/00	*O. edulis*	Galway	30	"
	27/09/00	*O. edulis*	Galway	30	"

Table 2. - Comparison of PCR results using the OHV1/OHV2 primer pair and reference larval samples (molecular biology workshop, May 2000, La Tremblade IFREMER, Charente Maritime, France)

Samples or controls	*First negative control*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*Second negative control*	*First positive control*	*Second positive control*
Participant 1 (La Tremblade)	-	(+)	-	(+)	-	-	-	+	-	+	+	+	+	+	+	+	-	+	+
Participant 6 (La Tremblade)	-	(+)	(+)	(+)	(+)	(+)	-	+	-	+	+	+	+	+	+	+	-	+	+
Participant 7 (La Tremblade)	-	-	-	-	-	-	(+)	+	(+)	-	+	+	+	+	+	+	-	+	+
Participants 4 and 5 (La Tremblade)	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	**ND**	+
Participant 5 (Cork)	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	**ND**	+

+: positive result ; (+): positive result with a faint band ; **-**: negative result ; ND: not done

All the larval samples tested corresponded to larvae presenting mortalities (they may be infected at different levels with different amounts of viral DNA).

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 2000 to 3rd January 2001

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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 **04-01-00** to **03-01-01**

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	

Coordinator: Dr Tristan Renault
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INDIVIDUAL PROGRESS REPORT

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

Scientific team: Dr Antonio FIGUERAS
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Marías PERNAS
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Begoña VILLAVERDE

OBJECTIVES

- Development of cellular tools (fish cell lines) 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	1st sample collection/P. R.[a]	2nd sample collection/Laboratory analysis/P. R.	3rd sample collection/Laboratory analysis /F. R.
3. 2	1st sample collection/P. R.	2nd sample collection/Laboratory analysis/P. R.	3rd sample collection/Laboratory analysis /F. R.
3. 3	1st sample collection/P. R.	2nd sample collection/Laboratory analysis/P. R.	3rd sample collection/Laboratory analysis /F. R.
4	2 meetings with all participants	1 meeting with all participants	1 meeting with all participants

[a]P. R. : Periodic Progress Report; [b]F. 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 different fish cell lines (Table 1) at two temperatures 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 or 20 °C. Plates were observed daily to detect a cytopathic effect (CPE). Blind passages were conducted although no CPE was detected in order to allow the virus to replicate in these cells.

The results of different experiments are summarized in Table 2. We only could detect an alteration of the cell monolayer in the first passage of the first experiment. When we conducted the second passage to new cells, no definitive cytopathic effect was observed. In the second experiment we repeated the same protocol but the incubations were done at 20 °C. No cytopathic effect was detected in any of the passages. The third experiment was conducted simultaneusly at 15 and 20 °C using 7 fish cell lines, and again no cytopathic effect was detected.

Table 1. Fish cell lines used in the experiments

Cell line	Fish	Morphology	Reference
TV-1	Turbot juvenile (*Scophthalmus maximus*)	Fibroblastic	Fernandez-*Puentes et al.*, 1993
RTG-2	Rainbow trout (*Oncorhynchus mykiss*)	Fibroblastic	Wolf and Quimby, 1962
SSN-1	*Channa striatus*	Fibroblastic	Frerich *et al.*, 1991
TFC	Turbot (*Scophthalmus maximus*)		
CHSE-214	Chinook salmon (*Oncorhynchus tshwytscha*)	Epithelial	Nims *et al.*, 1970
EPC	*Cyprinus carpio*	Epithelial	Tomasec and Fijan, 1971
BB	Catfish (*Ictalurus nebulosus*)	Epithelial	Wolf and Quimby, 1969

Table 2. Results of the inoculation of infected larvae homogenate into different cell lines

A. First experiment 15 °C

	TV-1	EPC	BB	RTG-2	SNN-1
1st passage	9 – 13	16	6 – 10	8	7
2nd passage	26 ?	29 ?	13 ?	20 ?	?
3rd passage	-	-	-	-	-

Data show the day postinoculation in which a cytopathic effect was observed.

B. Second experiment 20 °C

	TV-1	EPC	BB	RTG-2	SNN-1
1st passage	No CPE	No CPE	No CPE	No CPE	No CPE
2nd passage	No CPE	No CPE	No CPE	No CPE	No CPE
3rd passage	No CPE	No CPE	No CPE	No CPE	No CPE

C. Third experiment 15 and 20 °C

	TV-1	RTG-2	SNN-1	BB	CHSE	EPC	FTC
1st passage	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE
2nd passage	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No

The progress of this sub-task is as anticiped. 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

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, we decided to sample these clams to identify the possible cause of the mortalities trying to determine if the herpes virus was present in the diseased batches.
The individuals of each batch were removed from the shell, sagitally sectioned, then half of the animal was frozen at –80 °C and the other half was fixed in Davidson for histopatholgical studies 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 groups: one was frozen for molecular studies, another was fixed in Davidson for histology and the theird was fixed directly in osmium tetroxide for electron microscopy.

The progress of this sub-task is advance of that anticiped 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.

Table 3. Sampling of *Crassostrea gigas* and *Ostrea edulis* 2000

Date	Origin	Species	Stage
Mar 16	Hatchery	*Crassostrea gigas*	Adult
Jun 15	Santa Pola	*Ostrea edulis*	Adult
	Tabarca	*O. edulis*	Adult
	Guardamar	*O. edulis*	Adult
Sep 23	San Vicente de la Barquera	*C. gigas*	Juvenile
	San Vicente de la Barquera	*C. gigas*	Adult
Nov 13	Ría Pontevedra (Raxo)	*O. edulis*	Juvenile

Table 4. Sampling of *Ruditapes decussatus* and *R. philippinarum* 2000

Date	Origin	Species	Stage
Mar. 16	Hatchery	*Ruditapes philippinarum*	Adult
	Hatchery	*R. decussatus*	Adult
Mar. 23	B. Santander	*R. decussatus*	Adult
	Camariñas	*R. philippinarum*	Adult
Apr. 15	Camariñas	*R. philippinarum*	Adult
	B. Santander	*R. decussatus*	Adult
Jun. 8	Camariñas	*R. philippinarum*	Adult
	B. Santander	*R. decussatus*	Adult
Jul. 7	Hatchery	*R. philippinarum*	Adult
Sep. 15	Hatchery	*R. philippinarum* (S53)	Larvae
	Hatchery	*R. philippinarum* (S52SN)	Larvae
	Hatchery	*R. philippinarum*	Spat
	Hatchery	*R. decussatus* (GUERN)	Spat
	Hatchery	*R. decussatus* (SAT)	Spat
	Hatchery	*R. philippinarum* (SEAS T5)	Larvae
	Hatchery	*R. philippinarum* (S43)	Larvae
	Hatchery	*R. philippinarum* (LIS1)	Spat
Oct. 30	A Barqueiro	*R. philippinarum*	Larvae
	A Barqueiro	*R. philippinarum*	Spat

TEM: Several samples have been already examined by electron microscopy. Herpes-like virus were not detected in the cells of oysters or clams.

Subtask 3.1. Application of molecular methods to the diagnosis of oyster herpes-like virus, carrying out analysis of oyster samples originating from Spain

Three activities were conducted during the second year of the project related to this task:

a) Training meeting at La Tremblade, where all the participants worked together in techniques such as PCR, *in situ* hybridization, in order to use the same protocol in the different laboratories involved.

b) Determination of the sensibility and specificity of the PCR of herpesvirus with reference samples in our own laboratory in order to calibrate the results among labs. Setting up the *in situ* hybridization of herpes virus infected tissues.

c) Analysis of the samples from Spain taken in 1999 and in 2000 using molecular tools (PCR).

Results

a) Sensitivity and specificity of the PCR of herpesvirus with reference samples

Sensitivity:
We have compared the sensitivity of two primer pairs and two different Taq polymerases (Taq polymerase Roche and Supermix Gibco). The PCR conducted with the primers OHV1/OHV2 showed a sensitivity of 1 fg/µl (Annex, Fig. 1). However, the PCR of herpes-like virus with the OHV3/OHV114 primer pair only detected until 100 fg (–5 dilutions). This result was obtained with both Taq polymerases used (Annex, Fig. 2).

Specificity:
We have conducted a PCR reaction using the OHV1/OHV2 primer pair with 15 oyster larval samples furnished by the coordinator to all participants. Fig. 3 (Annex) showed the agarose gel with the results of this PCR.

b) Analysis of the samples from Spain taken in 1999 and in 2000 using molecular tools (PCR)

We have analysed by PCR all the bivalve samples taken in 1999 and 2000 using the OHV3/OHV114 primer pair. We also amplified the bivalve samples with universal primers in order to check the DNA isolation procedure. All the oyster batches analysed were negative in the specific PCR for herpes virus, although samples DNA was succesfully amplified with the conserved primers. However one clam batch from the sampling of the second year (2000) was positive with the OHV3/OHV114 primer pair in this study. The sample corresponded to a batch from adult *Ruditapes philippinarum* from hatchery taken in March 2000. Figure 4 (Annex) showed the agarose gels with the amplification products of samples. The positive PCR product (Annex, Fig. 4, sample 13) is higher than the product obtained after amplification of reference herpes-like virus DNA. At this moment we are sequencing the band for comparing it with the viral sequences available.

Subtask 3.2. Application of immunological methods to the diagnosis of oyster herpes-like virus, carrying out analysis of oyster samples originating from Spain

We have not received immunological reagents.

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.

The progress of this sub-task is as anticiped. The works 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 second meeting in December 2000 at the IFREMER headquarter in Paris (Issy les Moulineaux, France). Dr B. Novoa also wrote this intermediate report.

SIGNIFICANT DIFFICULTIES OR DELAYS

None.

ANNEX

Figure 1. Sensitivity of the PCR of oyster herpes-like virus using the primer pair OHV1/OHV2

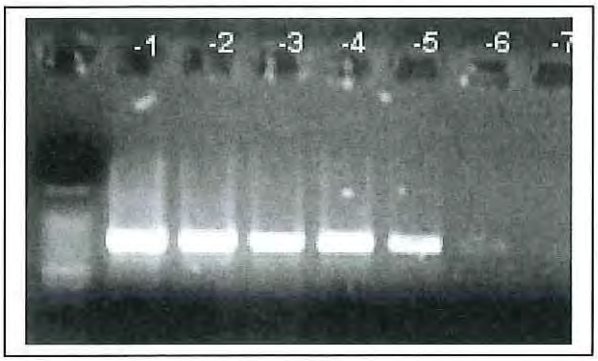
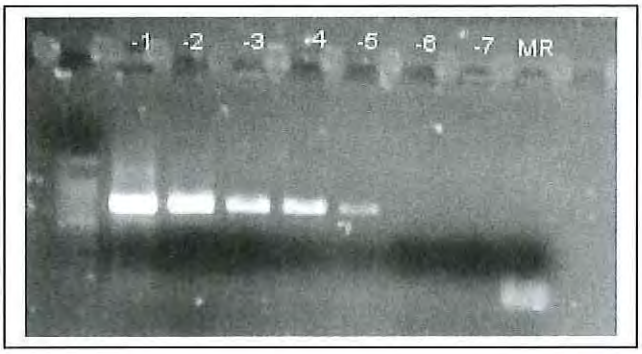


Figure 2. Sensitivity of the PCR of herpes-like virus using primer pair OHV3/OHV114

A. TAQ polymerase (Roche)



B. SUPERMIX (Life Technologies)

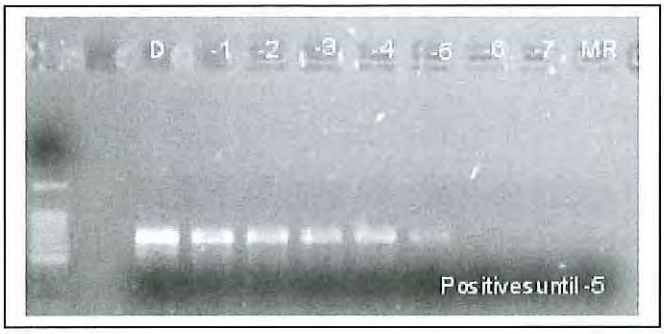
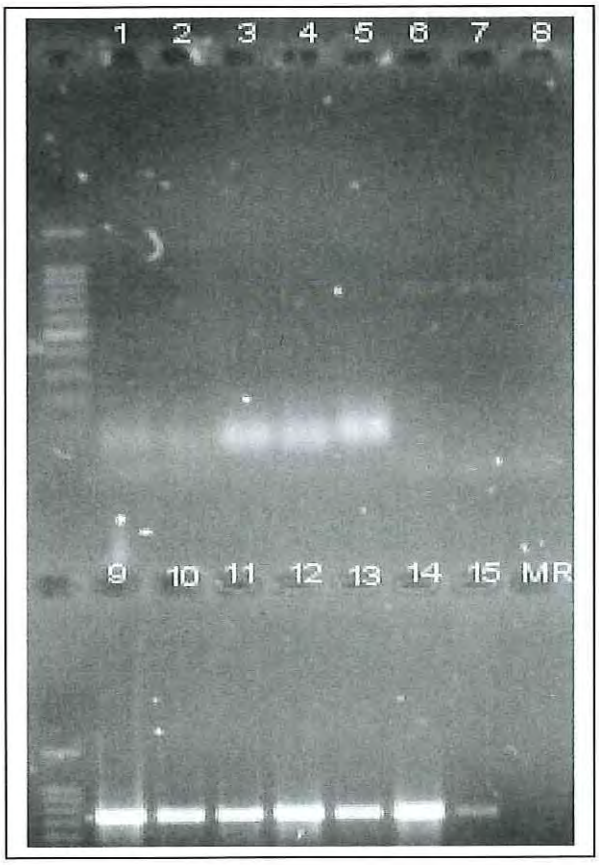
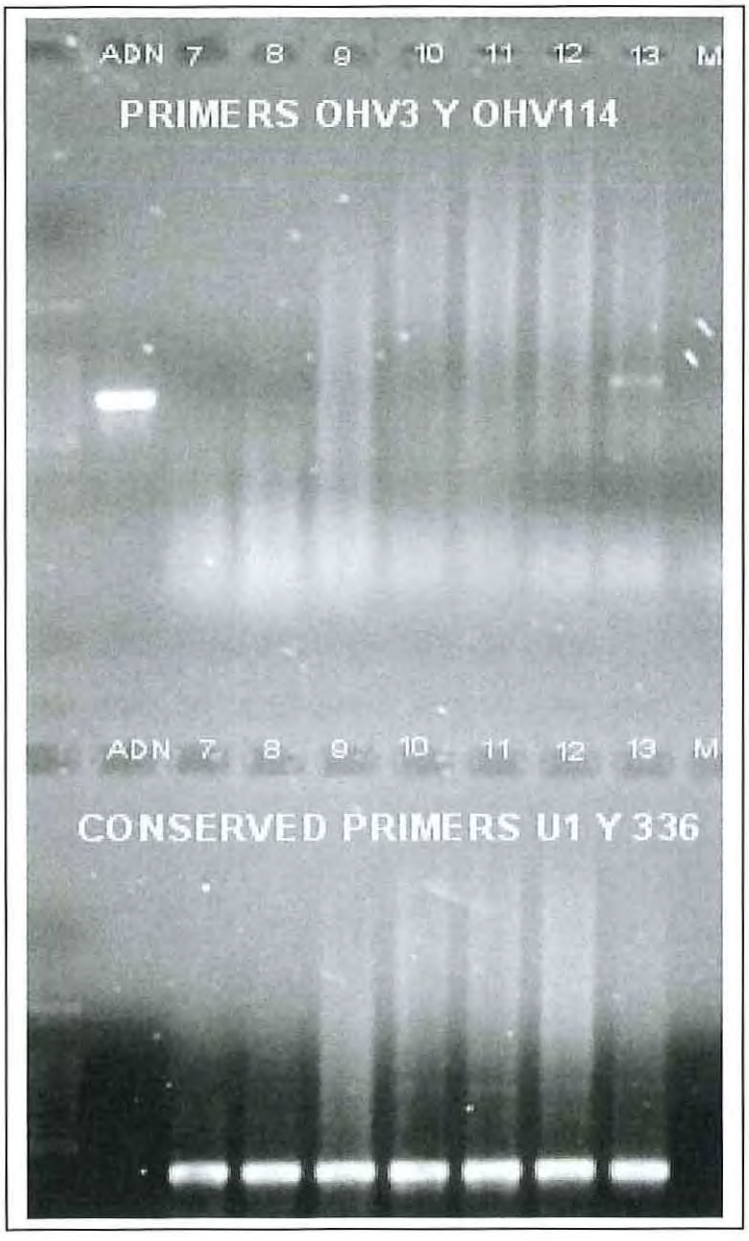


Figure 3. PCR of 15 Larval samples using the primer pair OVH1/OVH2 with TAQ polymerase (Roche)



Positive results 9,10,11,12,13,14,15 and positive result with a faint band 6 and 7

Figure 4. PCR amplification products corresponding to some Spanish samples visualised on agarose gel. The second line (ADN) shows the positive control The sample 13 corresponds to a positive of *Ruditapes philippinarum* (adult)



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 2000 to 3rd January 2001

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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 **04-01-00** to **03-01-01**

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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INDIVIDUAL PROGRESS REPORT

Participant No. 7: Centre for Environment, Fisheries and Aquaculture Science (CEFAS), United Kingdom.

Scientific Team: Dr Peter DIXON
Dr Rose-Marie LE DEUFF (replaces M K WAY)
M Paul MARTIN (replaces M S BARK)

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 to the diagnosis of oyster herpes-like virus, carrying out analysis of oyster samples originating from the United Kingdom.

Sub-task 3.2. : Application of immunological methods to the diagnosis of oyster herpes-like virus, carrying out analysis of oyster samples originating from the United Kingdom.

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 the United Kingdom.

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

[a] P. R.: Periodic Progress Report [b] F. R.: Final Report

RESEARCH ACTIVITIES DURING THE SECOND REPORTING PERIOD

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

1. Collecting oyster samples

a) 2000 winter/spring sampling. Twelve batches of 30 individuals (Annex, Table 2) were collected from a total of five randomly designated sites around the British Isles throughout January, February and March. No mortality was associated with any of these samples. All samples originated from spawns produced in hatcheries. Individual animals >2.5 cm diameter were cut into three sections which were treated as follows:

- Fixed in Davidson's fluid and embedded in paraffin.
- Fixed in Carson's fluid and stored at room temperature.
- Placed in individual bags and frozen at -70°C for PCR analysis.

Animals <1 cm were treated as follows:

- More than 30 individuals were fixed with their shells in Davidson's fluid at 4°C and embedded in paraffin after decalcification with 10% formic acid.
- More than 30 individuals were fixed with their shells in Carson's fluid and stored at room temperature.
- More than 30 individuals were placed in the same bag with their shells and frozen at -70°C for PCR analysis.

b) 2000 summer/autumn sampling. Twelve batches of 30 individuals (Annex 1 Table 3) were collected from a total of five randomly designated sites around the British Isles throughout in August, September and October. The summer/autumn samples were collected as late as October as the sea temperature was still high, and there was still the chance of the virus being expressed in the oysters. No mortality was associated with any of these samples. The samples were prepared as described for the winter/spring samples.

2. PCR analysis of oyster samples

PCR testing of all samples collected in both the 1999 and 2000 sampling periods was initiated using OHV3 and OHV114 sequence-specific primers. Preliminary experiments on serially diluted positive control material showed that detection of 50 copies of OsHV genomic DNA may be routinely achieved using this primer combination (Annex, Fig. 1).

The results are as follows:

a) PCR analysis of samples collected in summer 1999 has been completed. All samples were found to be negative for OsHV DNA (Annex, Table 1).

b) PCR analysis of samples collected in winter 1999/spring 2000 has yet to be completed. However, batch No 014 corresponding to *O. edulis* spat presenting 50% mortality, was analysed and was found to be negative for OsHV DNA (Annex, Table 2).

c) PCR analysis of samples collected in summer/autumn 2000 has been completed. All samples were found to be negative for OsHV DNA (Annex, Table 3).

3. *In situ* hybridisation (ISH)

Following the molecular biology workshop at La Tremblade in May 2000, positive and negative material (sections from fixed oysters) were supplied by Dr T Renault, in order to test two different ISH protocols.

A protocol based on an alkaline phosphatase/BCIP-NBT detection system gave good results. An intense blue labelling was observed, corresponding to nuclei of OsHV infected cells (Annex, Fig. 2). However, we have not been as successful using the second protocol based on a peroxidase/DAB detection system. Some background was observed and the intensity of the positive signal was low.

In 1976, *O. edulis* spat in north Wales experienced high mortality and samples were fixed for light and electron microscopy. The diagnosis at the time was of a possible herpesvirus infection. Stained slides were retrieved from the CEFAS archive and re-examined. Nuclei with marginated chromatin, typical of an OsHV infection, were observed in those samples, (Annex, Fig. 3). No further mortalities were recorded at that particular site but a follow-up sampling was done in 1976-77 and wax embedded tissues of several mollusc species from then are also available. We intend to apply the ISH protocol to a selection of this archive material. If it is successful, we will try to amplify (by PCR) and sequence fragments of OsHV

DNA after extraction of DNA from wax sections, then compare the sequences to OsHV DNA from other sources.

The progress of this sub-task is as anticipated. The work was carried out by P Martin with scientific support from Drs R-M Le Deuff and P Dixon.

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

Participation in annual meetings and in writing intermediate and final reports

Dr R-M Le Deuff and Mr P Martin participated in the molecular biology workshop held at the IFREMER Laboratory, La Tremblade in May 2000. Dr R-M Le Deuff and Mr P Martin attended and presented results at the annual meeting in December 2000 held at the IFREMER headquarters, Paris. Dr P Dixon wrote this intermediate report.

In addition to having discussions with shellfish farmers who are providing the samples, we have publicised this work by writing an article for *Shellfish News* (see below) which is distributed to all shellfish farmers and other interested parties in the UK.

Dissemination of results (contribution to articles related to OsHV)

P.F. Dixon, **S.J. Bark**, **R.M. Le Deuff** and **P.D. Martin** (2000). Herpesviruses in marine molluscs. Shellfish News, 10: 18-20. (Article reproduced in Annex).

T. Renault, **R.M. Le Deuff**, B. Chollet, N. Cochennec and A. Gerard (2000). Concomitant herpes-like virus infections among hatchery-reared larvae and nursery-cultured spat of both oyster species *Crassostrea gigas* and *Ostrea edulis*. Diseases of Aquatic Organisms, 42: 173-183.

T. Renault, **R.M. Le Deuff**, C. Lipart and C. Delsert (2000). Development of a PCR procedure for the detection of a herpes-like virus infecting oysters in France. Journal of Virological Methods, 88:41-50.

R.M. Le Deuff and T. Renault (1999). Purification and partial genome characterization of a herpes-like virus infecting the Japanese oyster, *Crassostrea gigas*. Journal of General Virology, 80:1317-1322.

SIGNIFICANT DIFFICULTIES OR DELAYS

None.

ANNEX

Table 1. Summer 1999 samples for the United Kingdom

Site[1]	Sample no	Species	Age/size	Mortality Rate (%)	Sample date	PCR result
Gwynedd	002	*Crassostrea gigas*	5 month	0	08/99	Negative
Devon	003	*C. gigas*	3 month	0	08/99	"
"	004	*C. gigas*	12 month	0	08/99	"
"	005	*C. gigas*	12 month	0	08/99	"
Cornwall	006	*C. gigas*	3 month	0	08/99	"
"	007	*C. gigas*	3 month	0	08/99	"
Cornwall	008	*Ostrea edulis*	12 month	0	08/99	"
"	009	*O. edulis*	12 month	0	08/99	"
"	010	*O. edulis*	12 month	0	08/99	"

[1] Only the county in which the sites are located are given in order to preserve their anonymity. Two sites were located in Cornwall.

Table 2. Winter 1999/Spring 2000 samples for the United Kingdom

Site[1]	Sample no[2]	Species	Age/size	Mortality Rate (%)	Sample date[2]	PCR result
Kent	011	*Tapes semidecussatus*	5mm	0	12/99	Pending
"	012	*Ruditapes decussatus*	5mm	0	12/99	"
"	013	*Crassostrea gigas*	1cm	0	12/99	"
"	014	*Ostrea edulis*	1-2cm	50	12/99	Negative
Gwynedd	015	*C. gigas*	spat	0	03/00	Pending
Cornwall	016	*C. gigas*	Spat	0	01/00	"
"	017	*C. gigas*	Spat	0	01/00	"
Kent	018	*T. semidecussatus*	Spat	0	02/00	"
"	019	*R. decussatus*	Spat	0	02/00	"
"	020	*C. gigas*	Spat	0	02/00	"
"	021	*R. decussatus*	Spat	0	02/00	"
"	022	*C. gigas*	Spat	0	03/00	"
"	023	*O. edulis*	Spat	0	03/00	"
Cornwall	024	*O. edulis*	Spat	0	02/00	"
"	025	*O. edulis*	Spat	0	02/00	"
"	026	*O. edulis*	Spat	0	02/00	"

[1] Only the county in which the sites are located are given in order to preserve their anonymity. Two sites were located in Cornwall.
[2] Sample numbers were allocated before the samples were collected, resulting in a disparity between some sample numbers and sampling dates.

Table 3. Summer 2000 samples for United Kingdom

Site[1]	Sample no	*Species*	Age/size	Mortality Rate (%)	Sample date	PCR result
Kent	027	*Crassostrea gigas*	Spat	0	8/00	Negative
Essex	028	*Ostrea edulis*	3-4cm	0	8/00	"
Gwynedd	029	*C. gigas*	1-3cm	0	9/00	"
Essex	030	*C. gigas*	3-4cm	0	9/00	"
"	031	*C. gigas*	3-4cm	0	9/00	"
Cornwall	032	*O. edulis*	3-4cm	0	10/00	"
"	033	*O. edulis*	3-4cm	0	10/00	"
"	034	*O. edulis*	3-4cm	0	10/00	"
"	035	*C. gigas*	3-4cm	0	10/00	"
"	036	*O. edulis*	3-4cm	0	10/00	"
"	037	*O. edulis*	3-4cm	0	10/00	"
"	038	*O. edulis*	3-4cm	0	10/00	"

[1] Only the county in which the sites are located are given in order to preserve their anonymity. Two sites were located in Cornwall.

Figure 1. PCR analysis using primers OHV3 and OHV114. Summer 1999 samples 007 (6 pools of five animals, lanes 2-7), 008 (6 pools of five animals, lanes 8-12 and 14) and 009 (6 pools of five animals, lanes 15-20). Negative controls (lanes 1 and 25). Serial 1/10 dilutions of positive control OsHV DNA, from 50 to 5 x 10^4 copies of the viral genome (lanes 21-24). 1kb DNA ladder (lanes 13 and 26).

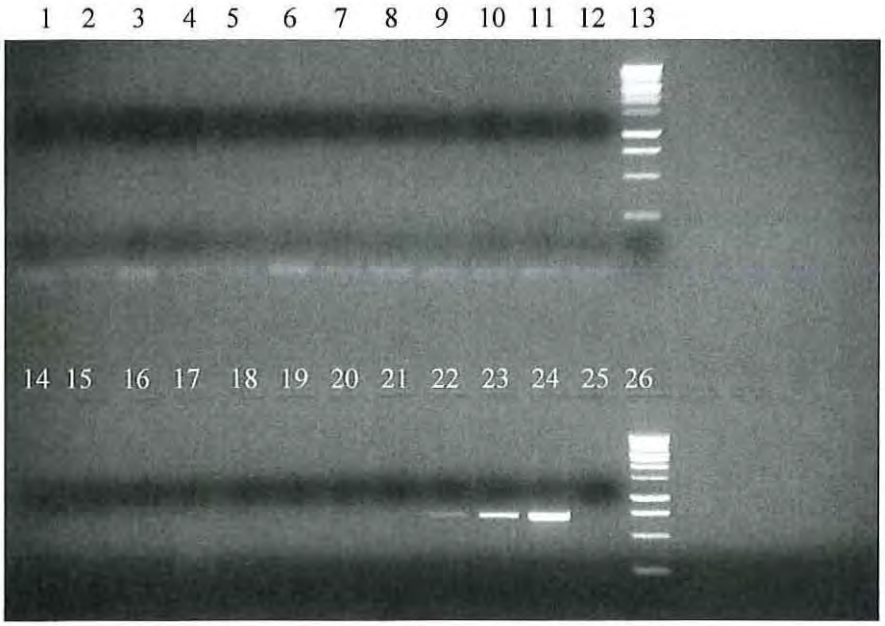


Figure 2. ISH using the alkaline phosphatase based protocol. a) Non infected negative control material, b) OsHV infected positive control material. Arrows indicate specific labelling.

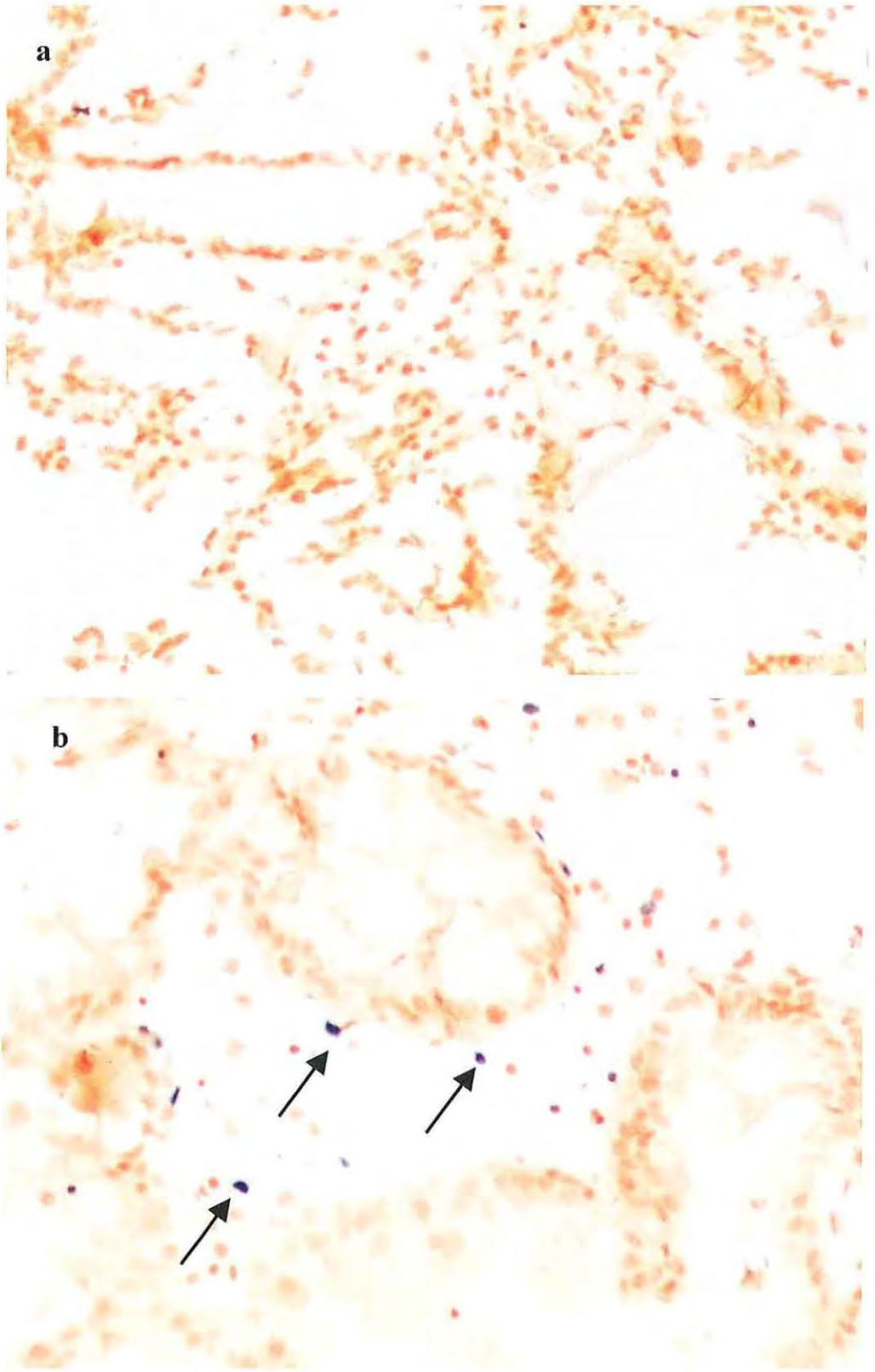
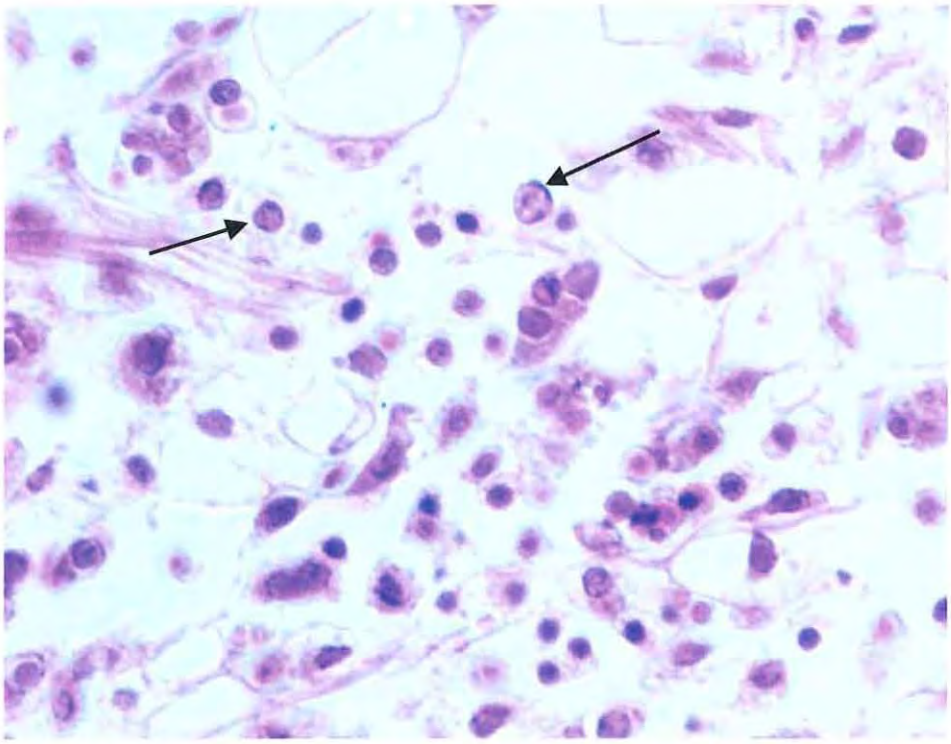


Figure 3. Oyster tissue archived since 1976. Nuclei with marginated chromatin were observed (arrows) suggesting an OsHV infection of these animals.



ANNEX

Herpesviruses in Marine Molluscs

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Background

The Pacific oyster, *Crassostrea gigas*, is the most important species of bivalve mollusc reared in the world and is of particular importance for European mariculture. Cultivation may be endangered by the occurrence of disease epizootics, especially of virus diseases. These affect many species of commercially valuable bivalve molluscs and mortalities associated with the viruses are considered to be one of the major risks to production.

In 1991 herpes-like viruses were associated with high mortality rates in hatchery reared *C. gigas* in France and New Zealand. In subsequent years, mortalities of French spat of *C. gigas* and the native flat oyster *Ostrea edulis* were reported during the summer months. Positive identification of herpes-like virus particles in the spat was made after analysis of a large number of samples at the IFREMER laboratory at La Tremblade in France. No mortality of adult *C. gigas* or *O. edulis* oysters was reported. As a direct result of these on-going mortalities, a project named VINO ('Viruses IN Ostreids') funded by the European Union was initiated in 1998.

Project VINO

World-wide, there is currently a lack of information concerning the occurrence of the oyster herpes virus (OsHV). This is probably due to the lack of suitable diagnostic tools. The basic method for identification and examination of suspect samples is predominantly histopathology. This enables the identification of any cellular changes (FIG.1), but is not conclusive identification of the OsHV. The use of electron microscopy is a necessity for visual confirmation (FIG.2). However, these techniques are time consuming and inadequate for epidemiological studies. The aim of the VINO project is therefore the development and validation of molecular, immunological and cellular tools for the diagnosis of, and studies on, the oyster herpesvirus.

The team consists of Dr. T. Renault (leader, IFREMER, La Tremblade) and various staff in Weymouth (Virology group, CEFAS), Scotland (MRC Virology, Glasgow), Ireland (University of Cork), Spain (Instituto de Investigationes Marinas, Vigo) Belgium (Eurogentech S.A., Seraing) and France (University of Brest).

The development of tools for the diagnosis of Oyster Herpesvirus.

Unlike virus research carried out on vertebrates, research into oyster viruses using cell cultures (a primary diagnostic technique used in virology) is impossible because of the lack of bivalve cell lines. Serological methods are also not available because of the absence of antibody production in molluscs, and until recently no molecular biology methods for diagnosing the OsHV have been available. The development of a protocol for purifying the OsHV particles from infected oyster larvae has subsequently served as an appropriate platform for generating molecular biology reagents to diagnose virus infections in oysters.

The major objective of the VINO project was to develop these 'state of the art' diagnostic techniques. They should be applicable for identification of the virus during a disease outbreak. In addition these techniques must also be suitable for the detection of subclinical infections and latent virus. To date, a

polymerase chain reaction (PCR) assay has been developed, which allows the rapid, specific and sensitive diagnosis of OsHV in oyster samples (FIG.3). Another technique that has also been developed is *in situ* hybridisation (ISH). ISH is very specific, but is relatively time consuming; however it appears to be most suited to the detection of OsHV in low level infections, or in possible latent stages such as occur with other herpesviruses.

VINO partners, including the CEFAS Weymouth laboratory, are currently conducting trials using PCR and ISH techniques in order to standardise and further develop the techniques in their respective laboratories. Applied to field samples, this calibration/standardisation step has provided an ideal opportunity to perform a preliminary epidemiological study. In the UK this is currently being achieved by the invaluable provision of oyster spat and larvae from private hatcheries and shellfish farms from around the country. None of the small number of UK samples that have been tested to date has been positive for the virus.

Experimental demonstration of the OsHV pathogenicity

The pathogenicity of the virus for larval stages of *C. gigas* was demonstrated in 1994 by experimental transmission to axenic larvae. Experimental studies on the OsHV also showed that it could be transmitted from *O. edulis* larvae to axenic larvae of *C. gigas*. To date, attempts to reproduce symptoms experimentally in spat and adult oysters have been inconclusive. The first experimental data indicated that it was possible to transmit the OsHV to spat of *C. gigas*, in cohabitation experiments using live infected larvae. A 40% mortality rate of challenged spat was only observed when the spat were kept in stressful conditions. In those experiments, control mock-challenged spat presented a mortality rate of 20%. However, when holding conditions were improved, so reducing the stress levels of the animals, no significant mortalities were observed. Dr. T. Renault and Dr. I. Arzul at IFREMER (La Tremblade) are currently undertaking further challenge studies on OsHV in adult and spat oysters. Furthermore, they have demonstrated that the herpesvirus can be transmitted from infected larvae of the manila clam *Ruditapes phiippinarum* to axenic larvae of *C. gigas*.

Relation of the OsHV to other herpesviruses

An additional part of the VINO project was to determine the relationship of the OsHV to other members of the *Herpesviridae* family. Dr. A. Davison (MRC Virology) has undertaken cloning and sequencing of the entire virus genome and analysis of sequences. The data demonstrated that the OsHV is not closely related to herpesviruses associated with vertebrate hosts. This is consistent with it being a herpesvirus that has evolved with an invertebrate, rather than being a contaminant from a vertebrate and acquired by filter feeding.

What is next?

In addition to continuing the calibration of PCR and ISH, the main targets for this forthcoming year are the development of oyster primary tissue cultures and the production of antibodies to the virus. The development of primary tissue cultures will facilitate fundamental research on the virus. For many other viruses (e.g. fish viruses), the identification of cytopathic effects as a result of virus replication in cell cultures is considered to be a primary diagnostic reference point. The production of antibodies against OsHV is a necessity for the development of any serological diagnostic/research technique. The development of IFAT (Immuno Fluorescent Antibody Test) and ELISA (Enzyme Linked Immunosorbent Assay) is now possible because of the availability of cloned sequences of OsHV which enables the synthesis of virus proteins for production of the required antibodies.

The realisation of these goals will permit a full epidemiological study of the virus and allow an assessment to be made on its impact to mariculture in the UK and the rest of Europe.

Acknowledgements

The authors wish to thank colleagues at IFREMER (La Tremblade) and the MRC Virology (Glasgow) for the information they kindly provided and for their critical advice on this article. Dr. T. Renault is particularly acknowledged for providing some of the illustrations shown in this article. The work on OsHV as a whole could not have been conducted without the invaluable contribution of private hatcheries and shellfish farmers.

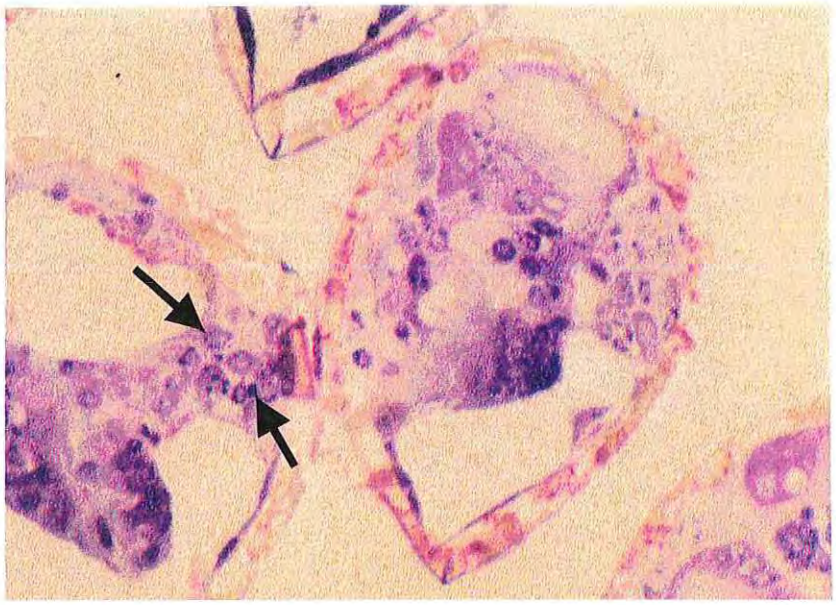

Figure 1: Histopathology showing characteristic nuclear lesions of an OsHV infection (arrows)

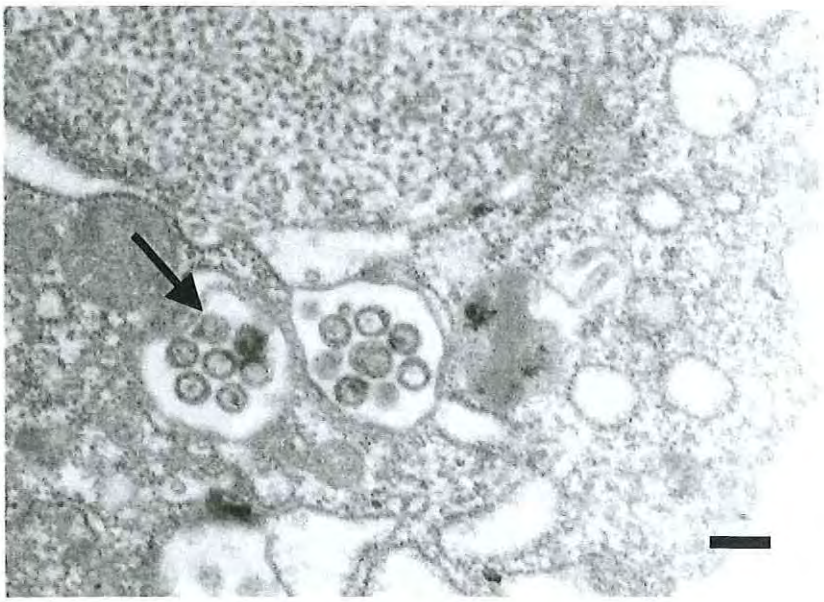

Figure 2: Electron micrograph showing OsHV particles (arrow), bar: 200nm.

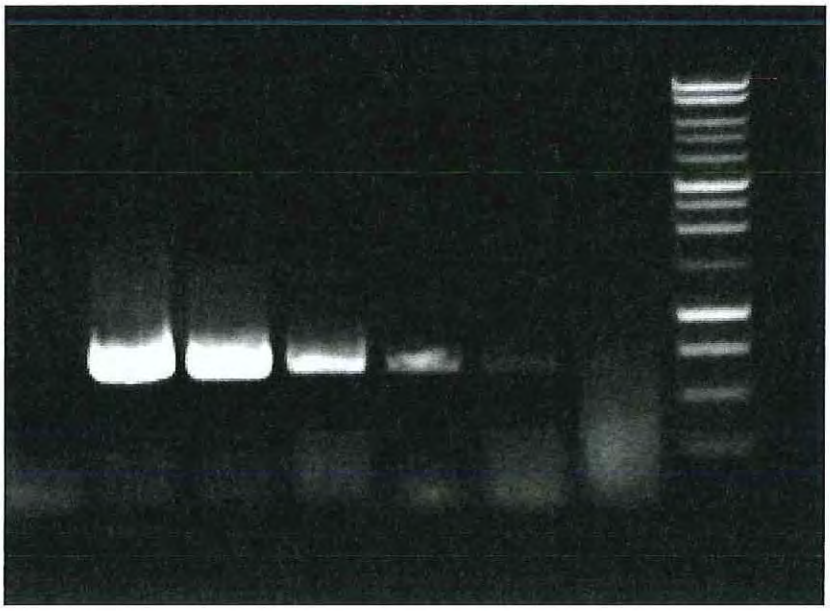

Figure 3: PCR agarose gel showing specific amplification of virus DNA fragments (white bands).