

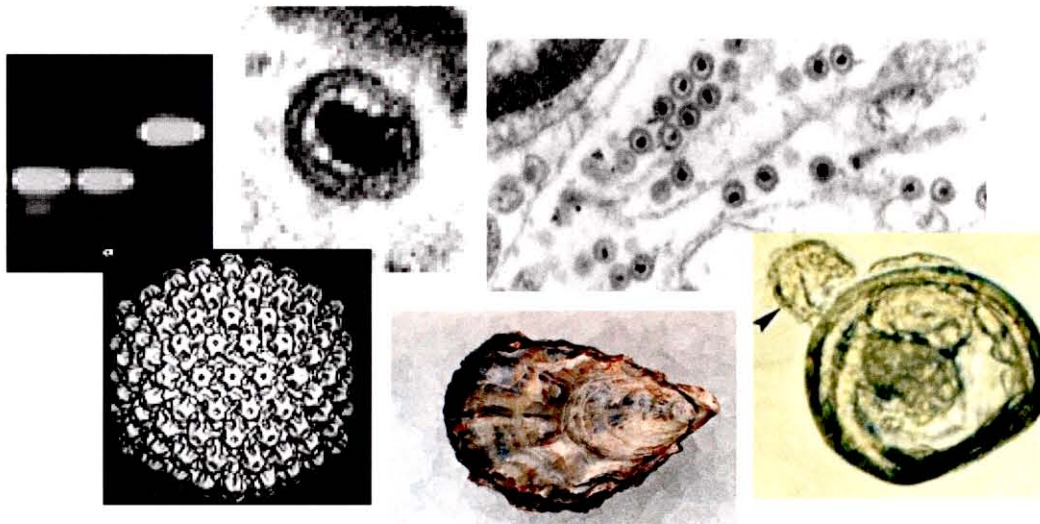
European Commission

Contract FAIR-CT98-4334

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

« VINO »

**THIRD PERIODIC PROGRESS REPORT:
4th January 2001 to 3rd January 2002**



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

European Commission

Contract FAIR-CT98-4334

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

« VINO »

**Reporting Period :
4th January 2001 to 3rd January 2002**

European Commission

Contract FAIR-CT98-4334

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

« VINO »

Abstract

**Reporting Period :
4th January 2001 to 3rd January 2002**

FAIR-CT98-4334

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

Abstract of the Progress Report for the period

from **04-01-01** to **03-01-02**

Type of contact: Shared-cost research project

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

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

Completion date: 03-01-02

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

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

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

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

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

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

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

Participant no. 7: Dr Peter Dixon (contractor)
CEFAS Weymouth Laboratory
Virology Group
Weymouth, Dorset, DT4 8UB
United Kingdom
Tel : +44 1305 206642
Fax : +44 1305 206638
Email : P.F.DIXON@cefas.co.uk

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 **04-01-01** to **03-01-02**

I. OBJECTIVES

Herpesvirus infections have been reported in different bivalves species around the world. Specific diagnostic tools are needed in order to assess the causative role of these agents in mortalities observed among bivalves. There is currently a lack of information concerning the occurrence of bivalve herpesviruses. This is probably due to the lack of suitable diagnostic tools. The basic method for examination of bivalve samples is predominantly histology. This enables the identification of any cellular changes, but is not conclusive for virus identification. Transmission electron microscopy is a necessity for visual confirmation of viral infections. However, histology and electron microscopy are time consuming and inadequate for epidemiological studies. The aim of the VINO project is therefore the development and the validation of molecular, immunological and cellular tools for the diagnosis of, and studies on, bivalve herpesviruses. They should be applicable for identification of viruses during disease outbreaks. Moreover, these techniques must also be suitable for the detection of subclinical or latent viral infections.

The specific aim of the programme is to develop specific tools for diagnosing herpesvirus infections in bivalves and to validate tools and reagents by using them in European laboratories involved with epidemiological surveys. The specific 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

Molecular techniques

A PCR procedure was developed in 2000 (Participant 1) and allowed to amplify short fragments of the OsHV-1 genome after extraction of DNA from wax sections. Two primer pairs have been designed for this purpose (OH1/OH4 and IAP1/IAP2). In 2001, the procedure was validated using DNA extracted from 31 archived histological blocks.

Obtaining antibodies for diagnosis use

Cloned viral DNA fragments have been used for the development of specific diagnostic probes (Participant 1) and identification of immunogenic viral proteins (Participant 3). Mice and rabbits have been immunised in 2000 using recombinant viral proteins in order to produce specific antibodies (Participant 3). The specificity of these antibodies was characterized in 2001 (Participant 3 and Participant 1). An immunochimistry protocol was specially developed (Participant 1) in order to analyze the specificity of monoclonal antibodies using histological material. Indeed, histology is the main technique used in laboratories involved with shellfish disease surveys. The availability of an immunological technique of diagnosis allowing virus detection on histological sections may be useful.

Validation of PCR and in situ hybridization for virus diagnosis

Some assays were carried out in 2001 by Participants 1, 5, 6 and 7 on reference material in order to ensure that common protocols are used for PCR and *in situ* hybridization. Positive and negative material (frozen larval and spat samples and sections from fixed oysters) were supplied by Participant 1 to Participants 5, 6 and 7. Frozen material has been analysed by PCR. Participants 1, 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 a previously defined protocol. Histological sections have been used in order to test direct and indirect *in situ* hybridization protocols. Immunochimistry labelling using a selected monoclonal antibody specific for a putative viral glycoprotein was carried out only by Participant 1 on the same reference material.

Sample analysis

The laboratories involved in mollusc epidemiological surveys (Participants 1, 5, 6 and 7) have collected bivalve samples during 2001 to search viral infections using the developed tools. In 2001, both PCR and *in situ* hybridization were used to diagnose herpes-like virus infections in bivalves.

III. STATE OF PROGRESS

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

This task has been completed in 2000.

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

Molecular techniques

Two primer pairs (IAP1/IAP2 and OH1/OH4) were developed in 2000 in order to analyse archived samples that were fixed for indeterminate periods. In 2001, a validation assay was carried out using DNA extracted from 30 histological blocks of infected and non infected oysters. Results confirmed that extracted material from histological blocks might be used for PCR diagnosis purpose. Indeed, the primer pairs OH1/OH2 and IAP1/IAP2 allowed to detect expected bands on agarose gels when PCR reactions were carried out using DNA extracted from histological sections. Thus, suspicion of viral infection (presence of abnormal cells on histological sections) may be confirmed.

Obtaining antibodies for diagnosis use

Two viral fusion proteins were selected and used to produce specific monoclonal and polyclonal antibodies. The specificity of these antibodies was characterised by ELISA and western blotting (Participant 3). Several hybridoma supernatants allowed to detect the recombinant antigens using both techniques. Hybridoma supernatants have been sent to Participant 1 in order to select some. An immunochemistry technique has been developed and used to analyze the specificity of hybridoma supernatants. Five hybridoma supernatants gave positive results using this technique.

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

Collecting samples in 2001

The laboratories involved in epidemiological surveys among bivalves (Participants 1, 5, 6 and 7) have collected bivalve samples during 2001 in order to carry out analyses to search for herpes-like virus infections using the developed tools.

PCR and in situ hybridization for virus diagnosis

In order to validate the different techniques and reagents (PCR primers and labeled DNA probes), Participant 1 furnished reference material in 2001: viral DNA, 15 larval samples, 15 spat samples and slides from 31 histological blocks as positive and negative reference material. This material was 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.

Some bivalve samples collected in 2001 were analysed (Participants 1, 5, 6 and 7). Molecular tools (PCR and *in situ* hybridization) were used to diagnose herpes-like virus infections in bivalves by laboratories involved in epidemiological surveys. No positive result was observed by Participants 5 and 6. Participant 7 reported PCR positive results (3 batches) in oyster samples. Positive PCR batches were observed by Participant 1 for animals collected in 2001 confirming results obtained in 1999 and 2000.

IV. ACHIEVEMENTS

Using specific primer pairs, archived fixed material may be used to detect herpesviruses. Small viral DNA fragments are targeted. To enhance viral detection, several primer pairs designed in different viral genome zones are needed. For routine diagnosis, it would be recommendable in a first step to carry out PCR using the primer pair OH1/OH4 and in a second step to confirm negative results with IAP1/IAP2.

Participant 3 produced monoclonal and polyclonal antibodies. Participant 3 and Participant 1 have selected some monoclonal antibodies specific for herpesviruses.

Participants 1, 5, 6 and 7 have carried out analysis on bivalve samples using developed techniques (PCR and *in situ* hybridization). Herpesviral infections were confirmed in France during 2001 and some positive samples (3) were also reported in The United Kingdom. In 2001, no positive sample was detected in Ireland and Spain.

European Commission

Contract FAIR-CT98-4334

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

« VINO »

Consolidated Progress Report

**Reporting Period :
4th January 2001 to 3rd January 2002**

FAIR-CT98-4334

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

Consolidated 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

Completion date: 03-01-02

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

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

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

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

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

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

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

Participant no. 7: Dr Peter Dixon (contractor)
CEFAS Weymouth Laboratory
Virology Group
Weymouth, Dorset, DT4 8UB
United Kingdom
Tel : +44 1305 206642
Fax : +44 1305 206638
Email : P.F.DIXON@cefas.co.uk

SCIENTIFIC SYNTHESIS

Introduction

Little information is available on viral infections that affect bivalve molluscs. Such a lack of data is due to a certain inadequacy of the diagnosis methods that are employed when massive mortality events occur. Most laboratories involved in mollusc pathology still analyse samples through light microscopy. This technique doesn't allow, by itself, to detect viruses unless it is completed by other methods, such as transmission electron microscopy, the study of cytopathogenic effects in cell cultures or the detection through specific reagents. At present, no bivalve cell-line is available: the detection of cytopathogenic effects in a homologous system is thus impossible. Since invertebrates lack antibody-producing cells, the direct detection of viral agents remains the only possible tool.

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.

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

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 were planned in order to study the ability of the virus to replicate *in vitro*. The development of molecular, immunological and cellular tools for OsHV-1 diagnosis may facilitate virus detection in infected material. Developed reagents have been used by four European laboratories to analyse a wide range of bivalve samples and to confirm the usefulness of the diagnostic tests.

Results

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

Developing molecular tools and techniques

Two primer pairs have been developed in 2000 in order to amplify small DNA fragments from OsHV-1 DNA. Both primer pairs have been designed in order to obtain PCR amplification when DNA extracted from histological blocks was used as template. 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 OH1/OH4 and IAP1/IAP2 allowed to produce amplicons when DNA extracted from five wax blocks was used. Histological blocks (5) prepared in 1995 corresponding to infected *Crassostrea gigas* spat have been tested in 2000. Results suggested that both primer combinations were reliable tools to detect viral DNA in archived material. Moreover, the primer pairs have been designed in two different areas of the genomic viral DNA enhancing the specificity of the detection. The OH1/OH4 primer pair recognises a gene encoding for a protein of unknown function and the IAP1/IAP2 primer combination amplifies a fragment of a gene corresponding to a putative inhibitor of apoptosis (IAP).

In 2001, a first validation assay of both primer pairs was carried out using 31 archived histological blocks. Histological blocks corresponding to the material used for preparing reference slides sent by Participant 1 to Participants 5, 6 and 7 for testing *in situ* hybridization protocols were used for the validation. Each block has already been analysed by histology. Histology examination revealed cellular and nuclear abnormalities suggestive of infection with oyster herpesvirus type 1 (OsHV-1) in 20 individuals. Eleven oysters presented no sign of infection and were interpreted as healthy individuals. Both primer pairs OH1/OH2 and IAP1/IAP2 allowed to detect viral DNA from infected oysters. However, some difference may be observed in amplification efficiency. Thus, to enhance viral DNA detection, several primer pairs designed in different genome areas are needed. For routine use, it would be recommendable to run PCR with OH1/OH4 and to confirm negative results with IAP1/IAP2.

Production of polyclonal and monoclonal antibodies

Anti-OsHV-1 specific rabbit antisera were obtained in this study and may be used for OsHV-1 diagnosis development. The best experimental conditions have been defined for their use in ELISA and Western blot analysis. A good immunization of mice was obtained with both viral recombinant proteins and clones producing anti-OsHV-1 specific monoclonal antibodies have been isolated and characterized by ELISA and Western blot analysis. All clones produce immunoglobulins IgG2A, kappa.

Twenty seven hybridoma supernatants were tested by immunochesmistry. The incubation with pure hybridoma supernatants was carried out one hour at room temperature. No signal was detected. Hybridoma supernatants were tested again. The immunochesmistry protocol was modified. A proteinase K treatment was added and a longer incubation period was used. Three hybridoma supernatants specific for a protein presenting homologies with a inhibitory apoptosis protein and three hybridoma supernatants specific for a putative glycoprotein yielded positive results on histological sections. Corresponding hybridoma were selected and subcloned. The subcloned hybridomas were tested using the immunochesmistry protocol including a proteinase K treatment. No signal was detected. Assays have been conducted three times.

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

Collecting samples in 2001

The laboratories involved in epidemiological surveys among bivalves (Participants 1, 5, 6 and 7) have collected bivalve samples during 2001 in order to carry out analyses to search for herpes-like virus infections using the developed tools.

Ring trials (PCR and in situ hybridization)

A molecular biology workshop took place 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 were used for PCR and *in situ* hybridisation.

Following the workshop in 2000, 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). Moreover, the OHV1/OHV2 primer pair allowed to amplify systematically 1 fg of viral DNA. Fifteen larval samples have also been analysed by PCR. Five samples appeared systematically positive when they were tested in the different laboratories. However, other larval samples appeared positive or negative depending of the laboratories. Positive and negative material (histological sections from fixed spat) were also used to test two different *in situ* 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 peroxidase/DAB indirect detection system.

In 2001, more reference material has been sent to Participants 5, 6 and 7 by Participant 1 in order to carry out PCR and *in situ* hybridization Ring trials.

- a) Fifteen *Crassostrea gigas* spat samples collected in several locations in Charente Maritime during the year 2001 were selected by Participant 1 and sent as reference material for PCR to other participants. Fifteen bivalve larval samples collected in 1997, 1998 and 1999 were also selected by Participant 1 and sent to Participants 5, 6 and 7 as reference material for PCR trials.
- b) Three histological blocks have been selected by Participant 1 in order to send them to Participants 5, 6 and 7. Selected blocks correspond to *Crassostrea gigas* oyster spat collected in 1994 and 1995 infected by OsHV-1. Moreover, *in situ* hybridization Ring trials were carried on a series of samples collected in the years 1994-1995. Thirty one histological blocks were selected by Participant 1 and sent to Participants 5, 6 and 7. Each block has already been analysed by histology. Histology examination revealed cellular and nuclear abnormalities suggestive of infection with OsHV-1 in 20 individuals. Eleven oysters presented no sign of infection and were interpreted as healthy individuals. Four slides of each histological block have been sent to Participants 5, 6 and 7 in June 2001. Slides were again sent to Participants 5, 6 and 7 in December 2001 in order to repeat *in situ* hybridization ring trial.

1. Interlaboratory comparison of PCR on frozen samples

Frozen larvae and seed samples analysed in the laboratory of IFREMER in La Tremblade (labelled A) were sent to other European laboratories (labelled B, C and D). B is Participant 5, D corresponds to Participant 6 and D to Participant 7.

Table I. PCR ring trial. Four laboratories were involved and two primer pairs were tested.

Reference	N°	PCR							
		OHV3/OHV114				OHV1/OHV2			
		A	B	C	D	A	B	C	D
317/1	1	-	-	+	-	-	-	+	-
317/2	2	-	-	-	-	-	-	+	-
317/3	3	-	-	+	-	-	-	-	-
317/4	4	-	-	-	-	-	-	+	-
317/5	5	-	-	-	-	-	-	-	-
T1	6	-	-	-	-	-	-	+	-
T2	7	-	-	+?	-	-	-	+	-
T3	8	-	-	+	-	-	-	+	-
T4	9	-	-	+	-	-	-	+	-
T5	10	-	-	-	-	-	-	+	-
PAL2	11	-	-	-?	-	-	-	+	-
74/1	12	+	+	+	+	+	+	-	-
93/5	13	+	+	+	+	+	+	+	+
98/6	14	+	+	+	+	+	+	+	+
99/4	15	+	+	+	+	+	+	+	+
99/5	16	+	+	+	+	+	+	+	+
105/4	17	+	+	+	+	+	+	-	+
106/1	218	+	+	+	+	+	+	+	+
115/4	19	+	+	+	+	+	+	+	+
116/3	20	+	+	+	+	+	+	+	+
117/3	21	+	+	+	+	+	+	+	+
PAL6	22	+	+	+	+	+	+	+	+
PAL8	23	+	+	+	+	+	+	+	+
PAL10	24	+	+	+	+	+	+	+	+
PAL12	25	+	+	+	+	+	+	+	+
SAT9	26	+	+	+	+	+	+	+	+
SAT10	27	+	-	+	+	+	+	+	+
SAT1	28	+	-	-	-	+	-	-	-
SAT6	29	+	+	+	+	+	+	+	+
SAT7	30	-	+	+	+	+	+	+	+

N. B. : reference information given on page 52

PCR with OHV3/OHV114 (C2/C6) gave consistent results considering laboratories A, B and D. As far as the primer pair OHV1/OHV2 (C5/C13) is concerned, false positives were obtained in laboratories B, C and D. Moreover, several false positive results were observed in the laboratory C with both primer pair. However, results obtained by Participants 1, 5 and 7 were similar using both primer pairs.

2. Interlaboratory methodology comparison: ISH on fixed samples

In situ hybridization analysis was carried on in the laboratory of IFREMER in La Tremblade (labelled A) on a series of samples collected in the years 1994-1995. Additional slides were prepared and sent for analysis to three other European laboratories, labelled B, C and D (see paragraph above).

Table II. Second hybridization ring trial. Four laboratories were involved. Positive results correspond to the detection of a staining on histological sections using the direct technique.

N°	ISH				
	A	B	C1/C6	C	D
VB1	-	?	-	-	-
VB2	-	+	-	-	-
VB3	-	+	-	-	-
VB4	-	+	-	-	-
VB5	-	+	-	-	-
VB6	-	+	+	+	-
VB7	-	ND	ND	ND	-
VB8	-	-	+	+	-
VB9	-	+	+	+	-
VB10	-	+	?	?	-
VB11	+	+	+	+	-
VB12	+	+	+	+	-
VB13	+	+	+	+	+
VB14	+	+	+	+	+
VB15	+	+	?	?	+
VB16	+	+	-	-	+
VB17	+	+	+	+	+
VB18	+	+	+	+	+
VB19	+	+	+	+	+
VB20	+	+	-	-	+
VB21	+	+	-	-	+
VB22	+	+	+	+	ND
VB23	+	+	?	?	ND
VB24	+	+	+	+	ND
VB25	+	+	-	-	ND
VB26	+	+	+	+	ND
VB27	+	-	+	+	ND
VB28	+	-	-	-	ND
VB29	+	+	+	+	ND
VB30	+	-	+	+	ND

N. B. : reference information given on page 44. ND : not done

The laboratories B, C and D found 3 false negative results, but also false positive results. On the other hand, results from laboratory B indicate that seven false positives were obtained among ten negative samples. Therefore, consistent results were obtained as far as laboratories A and D are considered. However, two false negative results were observed in the laboratory D.

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

PCR and in situ hybridization analysis for virus diagnosis

Some bivalve samples collected in 2001 were analysed (Participants 1, 5, 6 and 7). No positive result was reported by Participants 5 and 6. Participant 7 reported PCR positive results (3) in oysters. Two samples correspond to *Crassostrea gigas* oysters and the last one to *Ostrea edulis* oysters. Positive PCR batches have been observed by Participant 1 for animals collected in 2001 confirming results reported in 1999 and 2000.

Discussion

Two putative immunogenic viral proteins have been identified by immunoscreening of a lambda library and served to produce two recombinant proteins using the baculovirus system. Polyclonal and monoclonal antibodies specific for the two selected viral proteins have been produced. However, a two month delay in producing monoclonal antibodies was observed. Analyses using these reagents were not possible during the third period 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. Moreover, a supplementary delay (six months) was also observed in producing subcloned hybridoma supernatants. At the end, it was not possible to reproduce the results using these subcloned hybridoma supernatants. Indeed, several assays have been carried out, but all failed using the subcloned hybridomas. Polyclonal antibodies production has been completed as anticipated at the beginning of 2001. However, because of the limited amounts of the considered reagents, it has been decided to work only with monoclonal antibodies for diagnosis purpose.

Cellular tools were not available and analysis of oyster samples using such tools as anticipated was not carried out in 2001. Indeed, no cytopathic effect has been observed in 1999 and 2000 in tested fish cell lines. Tested fish cell lines cannot be used for the herpes-like virus infection diagnosis. Assays carried out in 2000 in primary cultures of embryonic oyster cells showed the presence of viral DNA in infected cultures using PCR and *in situ* hybridization. However, these results must be confirmed.

Molecular methods (PCR and *in situ* hybridization) were used for herpes-like virus infections in laboratories involved in epidemiological surveys among bivalves (Participants 1, 5, 6 and 7) during 2001. However, most of the PCR analyses of bivalve samples failed to show positive results. Thus, validation of molecular reagents and tools was carried out using reference material. Participant 1 furnished in 2001 some positive and negative reference oyster samples and Participants 1, 5, 6 and 7 used this material to test molecular tools in their own laboratory.

Conclusion

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

Applied to field samples, new developed tools of diagnosis provided an ideal opportunity to perform a preliminary epidemiological study. This was currently being achieved by the invaluable provision of oyster spat and larvae from private hatcheries and shellfish farms in France, Spain, the United Kingdom and Ireland.

The production of antibodies against bivalve herpesviruses 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 specific monoclonal antibodies. Delays in producing antibodies avoided the development of such methods during the third reporting period. However, because of the interest in developing such diagnosis techniques the work will be pursued after the end of the VINO contract.

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
- ◆ Preparing 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 third reporting period :

This part of the programme has been successfully completed during the second reporting period.

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 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.

Two OsHV-1 selected ORFs (ORF43 and ORF94) were expressed via the baculovirus system. The purified recombinant proteins were used for immunization of rabbits and mice in order to produce anti-OsHV-1 specific antibodies for diagnosis use. Immunization of laboratory animals have been carried out following conventional methods with purified recombinant proteins. Rabbit antisera and mouse monoclonal antibodies were characterized by ELISA and western-blotting against the OsHV-1 antigens and two control proteins. Characterization of antibody specificity was also carried out using an immunocytochemistry technique on histological sections.

Deliverables during the third 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 testing specific antibodies

Links with other tasks :

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

Progress during the third reporting period :

A PCR protocol previously developed to amplify herpes-like viral genome in DNA samples extracted from archived histological blocks was tested on 31 reference histological blocks. Results indicated that viral DNA might be detected in DNA samples extracted from archived material (histological blocks). *A posteriori* analyses may be possible using this material.

Monoclonal and polyclonal antibodies have been produced and selected using ELISA and western blotting. Some hybridoma supernatants (monoclonal antibodies) have also been checked using an immunocytochemistry protocol. Six hybridomas were selected and subcloned. Seven subcloned hybridoma supernatants were obtained and tested. They failed to produce expected positive results using immunoperoxidase technique on histological sections.

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 collected 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 transmission electron microscopy sections for use in routine diagnosis, immunological labeling and *in situ* hybridisation; the other will be stored frozen for PCR.

During the programme, most of PCR analyses of bivalve samples failed to show positive results excepted for Participant 1. Thus, validation of developed diagnosis tools was carried out using reference material. Participant 1 furnished some positive and negative reference material. Participants 1, 5, 6 and 7 used this material to test molecular tools (PCR and *in situ* hybridization) in their own laboratory.

Deliverables :

- ◆ Assays for intercalibration of the PCR technique between the different laboratories involved in this task
- ◆ Assays for intercalibration of the *in situ hybridization* technique between the different laboratories involved in this task
- ◆ Analysis of bivalve samples using PCR and *in situ hybridization*

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

Progress during the second reporting period :

No viral detection was reported by PCR in samples from Ireland and Spain (Participants 5 and 6) in 2001. Three positive samples were detected in the United Kingdom (Participant 7). Three Samples obtained during summer mortalities in France (participant 1) in 2001 showed the presence of viral DNA confirming the results obtained in 1999 and 2000.

PCR ring trials indicated that all positive samples were found as positive by the different participants. Some negative samples used as reference gave positive results in one laboratory.

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 different participants
- ◆ Presentation and analysis of results of each participant
- ◆ Production of intermediate and final reports
- ◆ Publishing in international scientific journals and participation in national and international meetings for proper dissemination of information
- ◆ Financial management of the project

Overview of the methodology :

A meeting of all participants was 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, the second and the third annual meetings took place in December 1999, in December 2000 and December 2001 respectively.

Links with other tasks :

Link with tasks 1, 2 and 3 between all participants

Progress during the second reporting period :

The third annual meeting took place in December 2001 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 been obtained and is necessary to perform Sub-task 3. 1.
4. Obtaining recombinant proteins and specific antibodies: these materials are necessary to perform Sub-task 3. 2. Immunologically reactive clones 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 herpesvirus detection is possible only if herpesviral infections occur among bivalves. Positive samples were available in the laboratory from La Tremblade (France, Participant 1) and served 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 November 2001	Finished (with delay)
Sub-task 2.3.		As scheduled		Finished
Sub-task 3.1.		In advance		Finished
Sub-task 3.2.	As scheduled			Not completed
Sub-task 3.3.	As scheduled			Not done
Task 4.		As scheduled	As scheduled	Finished (with delay)

	1999	2000	2001
Sub-task 1.1.	■		
Sub-task 1.2.	■	■	
Sub-task 1.3.		■	
Sub-task 2.1.	■	■	
Sub-task 2.2.	■	■	■
Sub-task 2.3.	■	■	
Sub-task 3.1.	■	■	■
Sub-task 3.2.	■	■	■
Sub-task 3.3.	■	■	■
Task 4.	■	■	■

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 intercalibration 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 progress report

This report describes results concerning :

- ◆ Obtaining specific antibodies
- ◆ Use of molecular (PCR and *in situ* hybridization) and immunological techniques in diagnosing herpes-like virus infections
- ◆ Comparison of results obtained by histology, PCR, *in situ* hybridization, 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 :

- Arzul I. & Renault T.**, 2002. Herpèsvirus et bivalves marins. *Virologie* **6** : 169-174
- Arzul I., Renault T. & C. Lipart**, 2001. Experimental herpes-like viral infections in marine bivalves : demonstration of interspecies transmission. *Diseases of Aquatic Organisms* **46**(1) : 1-6
- Arzul I., Nicolas J. L., Davison A. J. & T. Renault**, 2001. French scallops : a new host for ostreid herpesvirus-1. *Virology* **290** : 342-349
- Arzul I., Renault T., Lipart C. & A. J. Davison**, 2001. Evidence for interspecies transmission of oyster herpesvirus in marine bivalves. *Journal of General Virology* **82** : 865-870
- Arzul I., Renault T., Thébault A. & A. Gérard**, 2002. Detection of oyster herpesvirus DNA and proteins in asymptomatic *Crassostrea gigas* adults. *Virus Research* **84** : 151-160
- Lipart C. & T. Renault**, 2002. Herpes-like virus detection in *Crassostrea gigas* spat using DIG-labelled probes. *Journal of Virological Methods* **101** : 1-10
- Renault T. & I. Arzul**, 2001. Herpes-like virus infections in hatchery-reared bivalve larvae in Europe : specific viral DNA detection by PCR. *Journal of Fish Diseases* **24** : 161-167
- Renault T., Lipart C. & I. Arzul**, 2001. A herpes-like virus infects a non-ostreid bivalve species : virus replication in *Ruditapes philippinarum* larvae. *Diseases of Aquatic Organisms* **45** : 1-7
- Renault T., Lipart C. & I. Arzul**, 2001. A herpes-like virus infecting *Crassostrea gigas* and *Ruditapes philippinarum* larvae in France. *Journal of Fish Diseases* **24** : 369-376

- Posters and presentations

- Arzul I. & T. Renault**. Infections à virus de type herpès chez les bivalves : large spectre d'hôte et détection au stade adulte. IFREMER - Journées conchylicoles, Nantes, 3 – 4 avril 2001
- Arzul I., Nicolas J. L. & T. Renault**. Infection à virus de type herpès chez les larves de coquille Saint-Jacques. IFREMER - Journées conchylicoles, Nantes, 3 – 4 avril 2001
- Arzul I., Renault T. & J. L. Nicolas**, 2001. Study of a herpes-like virus infection in scallops, *Pecten maximus*. . 10th International Conference of the EAFP «Disease of fish and shellfish » 9th - 14th september 2001, Trinity College Dublin, Ireland.
- Arzul I. & T. Renault**, 2001. Detection of oyster herpes virus DNA and proteins in *Crassostrea gigas* adult oysters. 10th International Conference of the EAFP «Diseases of Fish and Shellfish », 9th - 14th September 2001, Trinity College, Dublin, Ireland.
- Renault T., Deniau S., Bourgougnon N. & A. Gérard**, 2001. Apoptosis of pacific oyster, *Crassostrea gigas* haemocytes maintend *in vitro*. . 10th International Conference of the EAFP «Disease of fish and shellfish » 9th - 14th September 2001, Trinity College Dublin, Ireland.
- Renault T. & I. Arzul**, 2001. Herpes-like virus infections in hatchery-reared bivalve larvae in Europe : specific viral DNA detection by PCR. . 10th International Conference of the EAFP «Disease of fish and shellfish » 9th - 14th September 2001, Trinity College Dublin, Ireland

- *E. C. Programme reports :*

Diagnosis of oyster herpes-like virus : development and validation of molecular, immunological and cellular tools (2001). Second Periodic Progress Report. Programme FAIR-CT98-4334 (VINO).

Diagnosis of oyster herpes-like virus : development and validation of molecular, immunological and cellular tools (2002). Third Periodic Progress Report. Programme FAIR-CT98-4334 (VINO).

- *Web sites :*

Detection of herpes-like viral DNA by PCR in European bivalve larval samples (Programme E. C. no FA-S2 9052, ordis Technology Marketplace, <http://www.cordis.lu/marketplace>)

Herpesviruses in Marine Bivalves (<http://www.ifremer.fr/latremblade/>, IFREMER laboratory located in La Tremblade, Charente Maritime, France). The document is presented in the annex (page 29).

ANNEX

Herpesviruses in Marine Bivalves

Contact : Dr T. Renault

IFREMER, Laboratoire de Génétique et Pathologie

17390 La Tremblade, France

Tel : 33 5 46 36 98 36, Fax : 33 5 46 36 37 51, E-mail : trenault@ifremer.fr

Background

Crassostrea gigas is increasingly being cultured in a great number of countries from animals originally introduced from Japan, and is one of the most exploited bivalve species in the European Union (EU). Although no serious pathogen was detected previously in Pacific oysters in Europe, mortalities have been observed since the introduction of this bivalve species into the EU. Oyster cultivation may be endangered by the occurrence of epizootics, especially viral diseases, which are considered one of the major risks to production. Indeed, mortalities have been described among different species of ostreids and are associated with the presence of viruses belonging to various families. The first description of a virus was reported in adult Eastern oysters, *C. virginica*, with the detection of particles indicating membership of the *Herpesviridae* (Farley *et al.*, 1972). Mass mortalities of adult Portuguese oysters, *C. angulata*, among French livestock (between 1967 and 1973) were associated with iridovirus infections (Comps *et al.*, 1976; Comps and Bonami, 1977; Comps and Duthoit, 1979). Other viruses described in ostreids are members of the *Iridoviridae*, *Papovaviridae*, *Togaviridae*, *Retroviridae* and *Reoviridae* (Farley, 1976; Farley, 1978; Elston, 1979; Meyers, 1979; Elston and Wilkinson, 1985).

Recently, in 1991, viruses interpreted as belonging to the *Herpesviridae* were associated with high mortality rates of hatchery-reared larval *Crassostrea gigas* in France (Nicolas *et al.*, 1992) and in New Zealand (Hine *et al.*, 1992). Since 1992 sporadic high mortalities of larval *C. gigas* are regularly observed in some private French hatcheries, occurring each year during summer period in association with a herpes-like virus (Renault *et al.*, 1994b). Since 1993, sporadic high mortalities occur also in some batches of Pacific oyster spat cultured in different French locations (Renault *et al.*, 1994a and b). In addition, herpesvirus infections were reported in spat and larvae of the European flat oyster, *Ostrea edulis*, in France (Comps and Cochenec, 1993; Renault *et al.*, 2001). Concomitant mortalities were observed among larvae and spat of *C. gigas* and *O. edulis*, in 1994 and 1995, with the detection of herpes-like virus particles by transmission electron microscopy (Renault *et al.*, 2000b). Replication of herpes-like viruses was also described in *O. angasi* adults in Australia (Hine and Thorne, 1997), in larval *Tiostrea chilensis* in New Zealand (Hine, 1997; Hine *et al.*, 1998) and in larval *Ruditapes philippinarum* in France (Renault, 1998; Renault *et al.*, 2001a and b). Unexplained mortalities were observed in recent years among *C. gigas* larvae in the United Kingdom and Spain, although samples were not examined. High losses were reported among Pacific oyster spat in Ireland in 1994 and 1995. No obvious cause of mortalities was determined (Culloty and Mulcahy, 1995). However, screening using conventional light microscopy yielded little apart from some cell damage most noticeably enlarged cell nuclei and marginated chromatin. Results would now indicate that herpes-like virus

is present in at least one site on the south coast of Ireland (Culloty and Mulcahy, unpublished data). Herpes-like virus infections in bivalves seem to be ubiquitous and are associated with substantial mortalities. The observed association between oyster mortality and herpes-like virus infections provides an imperative to determining the extent to which the virus is involved as a causative agent of massive mortalities. It appeared essential to survey epidemiologically infections in different European countries.

Experimental demonstration of the OsHV pathogenicity

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

Project VINO (contract FAIR-CT98-4334)

World-wide, there is currently a lack of information concerning the occurrence of the oyster herpesvirus (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, but is not conclusive identification of the OsHV. The use of electron microscopy is a necessity for visual confirmation. 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 Investigaciones Marinas, Vigo), Belgium (Eurogentec S.A., Seraing) and France (University of Brest). An 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. Another technique that has also been developed is *in situ* hybridisation (ISH) (Renault and Lipart, 1998). ISH is specific, but is relatively time consuming; however it appears to be most suited to the detection of OsHV in low level infections, or in possible latent stages such as occur with other herpesviruses. VINO partners 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. This is currently being achieved by the invaluable provision of oyster spat and larvae from private hatcheries and shellfish farms in France, Spain, the United Kingdom and Ireland. In addition to continuing the calibration of PCR and ISH, the main targets are development of oyster primary tissue cultures and 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. 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.

Obtaining a complete virus genomic library and DNA sequence

The genome sequence has been completed and analysed. Virus particles have been purified from fresh infected *Crassostrea gigas* larvae and viral DNA extracted from purified virions (Le Deuff and Renault, 1999). 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 orientations of U_L and U_S are present in approximately equimolar amounts in viral DNA, giving rise to four genome isomers. This is also a feature of the vertebrate herpesvirus genomes with similar structures. Both the database and restriction endonuclease digests indicated that a minor proportion (approximately 20-25%) of genomes contain a 4.8 kbp region in U_L in inverse orientation. 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 association with membranes. A total of 39 proteins share sequence similarities with other proteins encoded by the virus, defining 13 multigene families in addition to the IAPs. An additional notable feature, located between ORFs 50 and 51, is a large palindrome. By analogy with certain vertebrate herpesviruses, this palindrome is a candidate origin of DNA replication.

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

Development of diagnosis tools

To diagnose herpes-like virus infections, the basic method for examination of suspect samples is still light microscopy. This method appears poorly adapted to viral diseases and needs to be improved upon by other techniques such as transmission electron microscopy. Both techniques are time consuming and inadequate for epidemiological surveys. In addition, research into virus cytopathogenic effects in cell cultures is impossible because of the lack of bivalve cell lines. A breakthrough was achieved recently in the development of a protocol, based on sucrose gradient centrifugation, for purifying oyster herpes-like virus particles from fresh infected larval *Crassostrea gigas* (Le Deuff and Renault, 1999). This advance has served as an appropriate platform for generating molecular biological reagents to diagnose virus infections (Renault and Lipart, 1998; Renault *et al.*, 2000a). A procedure to detect herpes-like virus in French oysters using the polymerase chain reaction (PCR) (Saiki *et al.*, 1985; Mullis *et al.*, 1986) was developed (Renault *et al.*, 2000a). PCR offers many advantages for disease diagnosis (Henson and French, 1993; Jones and Bej, 1994; Martin, 1994). With regard to herpes-like viruses from oysters, important advantages include its extreme sensitivity, pathogen specificity, ease of sample processing, and availability of reagents. Another technique that has also been developed is *in situ* hybridisation (ISH) (Renault and Lipart, 1998). In addition to continuing the calibration of PCR and ISH, the main target is the production of antibodies to the virus. The development of immunochemistry and ELISA tests will be possible because of the availability of cloned sequences of an oyster herpes-like virus which enables the synthesis of recombinant virus proteins.

Developing molecular tools and techniques

A PCR-based procedure for detecting a herpes-like virus that infects the Pacific oyster, *Crassostrea gigas*, in France was developed (Renault *et al.*, 2000a). Two primer primers (A3/A4 and A5/A6) were designed to provide specific amplification products ranging in size 917 and 1001 bp when performed on oyster herpes-like virus DNA (Renault *et al.*, 2000a). No amplification was observed on oyster genomic DNA nor on the DNA from vertebrate herpesviruses. Crude samples were prepared and submitted to nested PCR, allowing the amplification of DNA fragments of the expected size when performed on infected larval and spat samples. The procedure used to prepare the sample for PCR was found to be critical because of the presence of unidentified substances in oyster tissues that inhibit the PCR reaction. A quick and convenient sample preparation using ground tissues allowed a sensitive detection of the herpes-like virus infected oysters. The ability of the defined PCR protocol to diagnose herpes-like virus infections in oysters was compared to the transmission electron microscopy technique from 15 *C. gigas* larval batches presenting or not mortalities. PCR amplification is as sensitive diagnosis assay for herpes-like virus as the transmission electron microscopy. However, the nested PCR protocol is more convenient and less time consuming. The relationship between reported mortalities among *C. gigas* oyster spat and herpes-like virus DNA detection by PCR was also investigated. Stastitcal analysis showed that virus detection and mortalities are correlated.

A competitive PCR method has also been developed using previously designed primers (Arzul *et al.*, 2001b) in order to detect and quantitate herpes-like virus DNA. The method is based on the use of oyster herpesvirus specific primer pairs and an internal standard competitor that differs from the target DNA by a deletion of 76 base pairs. The internal standard DNA molecule was generated by PCR and then co-amplified with the target DNA. The resulting PCR products which were different in size were separated on agarose gels. The assay was found to be specific and sensitive, allowing the detection of 1 fg of viral DNA among 0.5 mg of oyster tissues. The method was used to demonstrate the absence of PCR inhibitors in oyster spat ground tissues. PCR inhibition was observed in adult oyster samples when the same tissue preparation procedure was used. On the contrary, classical phenol/chloroform DNA extraction from adult oyster tissues allowed amplification of the internal standard competitor and the viral DNA. The method was successfully used to demonstrate the presence of viral DNA in asymptomatic adult oysters indicating that oyster herpes-like virus infects animals presenting no anomalous mortality. Quantitations of herpes-like virus DNA in infected spat and asymptomatic adult oysters were also carried out. Although between 1.5 pg and 325 pg of viral DNA per 0.5 mg of oyster tissues were detected in adults, amounts of viral DNA in infected oyster spat varied from 750 pg to 35 ng per 0.5 mg of ground tissues.

Two primer pairs 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 previously designed (Renault *et al.*, 2000 , Arzul *et al.*, 2001b) 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 detect viral particles. PCR analysis using DNA extracted from these blocks showed clear bands presenting expected sizes when both primer pairs were used. This suggested that both primer combinations were reliable tools to detect viral DNA in 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 unknown 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 codes for a protein of 748 amino acids. This protein contains a highly hydrophobic C-terminal domain, potential N-glycosylation sites (Asn-X-Ser/Thr) and a potential signal peptide at the N-terminal end. This ORF could code for a membrane glycoprotein, the typical profile of surface viral antigens. The second identified ORF codes for a protein of which different regions are recognized by anti-OsHV-1 ascitis. This protein of 364 amino acids doesn't present the characteristics of membrane proteins but seems to be a potential immunogenic protein. This ORF codes for a protein presenting homologies with baculovirus, insect and mammal IAPs (Inhibitor of Apoptosis). The two ORFs encoding for putative immunogenic viral proteins have been cloned in baculovirus expression system in order to prepare recombinant proteins and antibodies for diagnosis use. Corresponding clones have been used to produce two recombinant proteins using the baculovirus system. The production 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.

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. No cytopathic effect has been observed in tested fish cell lines. OsHV-1 may be not able to multiply in fish cell lines or under cultivation conditions used viral replication doesn't occur. Tested fish cell lines cannot be used for the herpes-like virus infection diagnosis. Preliminary assays performed in primary cultures of embryonic oyster cells showed the presence of viral DNA in infected cultures using PCR and *in situ* 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.

Herpes-like infection surveys

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

the 81 analysed appeared positive for the detection of herpes-like virus DNA by PCR. These results confirm previous data indicating that herpes-like virus infections may be observed in private French hatcheries. Moreover, some PCR positive results were also obtained for bivalve larval samples originating from Spain and United Kingdom. The number of virus DNA positive samples depended on the primer pair used. The primer pair C2/C6 appears well adapted for herpes-like virus DNA detection because of processing ease and great sensibility. Positive samples were observed in four bivalve species: *Crassostrea gigas*, *Ostrea edulis*, *Ruditapes decussatus* and *R. philippinarum*. Herpes-like virus DNA detection was reported herein in larval *R. decussatus* for the first time. Numerous samples presenting viral DNA detection by PCR correspond to larval batches presenting mortalities. Thus, herpes-like viruses may be interpreted as one of the causative agents of anomalous mortalities observed in bivalve hatcheries.

Study of bivalve antiviral immunity

An other major objective of the IFREMER laboratory in La Tremblade is to develop new approaches in order to control disease impact. The earliest antiviral response in vertebrate hosts is non specific. Upon viral infection, host cells are stimulated to change their transcription pattern, begin to secrete immune mediators or die. Thus, non specific antiviral mechanisms and molecules are investigated in oysters.

Acknowledgements

The authors wish to thank colleagues at the MRC Virology in Glasgow (Dr A. J. Davison), Eurogentec in Seraing (Dr F. Xhonneux and Dr D Vandenberg), Université de Bretagne in Brest (Dr G. Dorange), University College in Cork (Dr S. Culloty), CSIC in Vigo (Dr B. Novoa and Dr A. Figueras) and CEFAS in Weymouth (Dr P. F. Dixon and Dr R. M. Le Deuff) for the information they kindly provided. Dr. A. J. Davison is particularly acknowledged for providing OsHV-1 genome information shown in this document. The work on OsHV as a whole could not have been conducted without the invaluable contribution of private hatcheries and shellfish farmers. The work carried out on herpesvirus infections in bivalves at the IFREMER station in La Tremblade (Charente Maritime, France) was supported in part by European Union (FAIR-CT98-4334 and FAIR979052).

References

- Arzul, I., Renault, T. and Lipart, C. (2001a). Experimental herpes-like viral infections in marine bivalves: demonstration of interspecies transmission. *Dis aqua Org*, 46: 1-6.
- Arzul, I., Renault, T., Lipart, C. and Davison, A. J. (2001b). Evidence for inter species transmission of oyster herpesvirus in marines bivalves. *J Gen Virol*, 82: 865-870.
- Comps, M., and Bonami, J.R. (1977) Infection virale associée à des mortalités chez l'huître *Crassostrea angulata* Th. *C. R. Acad. Sc., D*, 285 : 1139-1140.
- Comps, M. and Cochenec, N., (1993) A herpes-like virus from the European oyster *Ostrea edulis* L. *J. Inv. Pathol*, 62, 201-203.
- Comps, M. and Duthoit, J.L. (1979) Infections virales chez les huîtres *Crassostrea angulata* (Lmk) et *C. gigas* (Th.). *Haliotis* 8 (1977), 301-308.
- Comps, M., Bonami, J.R., Vago, C. and Campillo, A. (1976) Une virose de l'huître portugaise (*Crassostrea angulata* Lmk). *C. R. Hebd. Séanc. Acad. Sc. D* 282, 1991-1993.
- Culloty, S.C. and Mulcahy, M.F. (1995). Mortalities in Pacific oyster *Crassostrea gigas* with particular reference to Ireland. *Dept. of Zoology, U.C.C.* 27pp.

- Elston, R.A. (1979) Virus-like particles associated with lesions in larval Pacific oysters (*C. gigas*). *J. Invert. Pathol.* 33, 71-74.
- Elston, R. and Wilkinson, M.T. (1985) Pathology, management and diagnosis of oyster velar virus disease (OVVD). *Aquaculture* 48, 189-210.
- Farley, C.A. (1976) Ultrastructural observations on epizootic neoplasia and lytic virus infection in bivalve mollusks. *Prog. Exp. Tumor Res.* 20, 283-294.
- Farley, C.A. (1978) Viruses and virus-like lesions in marine molluscs. *Mar. Fish. Rev.* 40, 18-20.
- Farley, C.A., Banfield, W.G., Kasnic, J.R.G., Foster, W.S. (1972) Oyster herpes-type virus. *Science, Wash.D.C.* 178, 759-760.
- Henson, J.M. and French, R. (1993) The polymerase chain reaction and plant diseases diagnosis. *A Rev. Phytopathol.* 31, 81-109.
- Hine, P.M. (1997) Trends in reserch on diseases of bivalve molluscs. *Bull. Eur. Ass. Fish Pathol.* 17(6), 181-183.
- Hine, P.M., Wesney, B. and Hay B. E. (1992) Herpesvirus associated with mortalities among hatchery-reared larval Pacific oysters, *C. gigas*. *Dis. aquat. Org.* 12(2), 135-142.
- Hine, P.M. and Thorne, T. (1997) Replication of herpes-like viruses in haemocytes of adult flat oysters *Ostrea angasi* (Sowerby, 1871): an ultrastructural study. *Dis. aquat. Org.* 29(3), 197-204.
- Hine, P.M., Wesney, B. and Besant, P. (1998) Replication of herpes-like viruses in larvae of the flat oyster *Tiostrea chilensis* at ambient temperatures. *Dis. aquat. Org.* 32(3), 161-171.
- Jones, D.D. and Bej, A.K. (1994) Detection of foodborne microbial pathogens using polymerase chain reaction methods. In : H. G. Griffin and A.M. Griffin (Eds), *PCR technology: current innovations*. CRC Press, Boca Raton, pp. 341-365.
- Le Deuff, R.-M. and Renault, T. (1999) Purification and partial genome characterization of a herpes-like virus infecting the Japanese oyster, *Crassostrea gigas*. *J. Gen. Virol.* 80, 1317-1322
- Le Deuff, R.-M., Renault, T. and Gérard A. (1996). Effects of temperature on herpes-like virus detection among hatchery-reared larval Pacific oyster *Crassostrea gigas*. *Dis. aqua. Org.*, 24: 149-157.
- Le Deuff, R.-M., Nicolas, J.L., Renault, T. and Cochenec, N. (1994) Experimental transmission of herpes-like virus to axenic larvae of Pacific oyster, *Crassostrea gigas*. *Bull. Eur. Ass. Fish Pathol.* 14(2), 69-72.
- Martin, W.J. (1994) Infectious diseases. In: K.B. Mullis, F. Ferre and R.A. Gibbs (Eds), *the polymerase chain reaction*. Birkhauser, Boston, pp. 406-417.
- Meyers, T. R. (1979) A reo-like virus isolated from juvenile American oysters (*Crassostrea virginica*). *J. Gen. Virol.* 46, 203-212.
- Mullis, K., Falcoma, F., Scharf, S., Snikl, R., Horn, G. and Erlich, H. (1986) Specific amplification of DNA *in vitro*: the polymerase chain reaction. *Cold Spring Harbor Symp. Quant. Biol.* 51, 260
- Nicolas, J. L., Comps, M. and Cochenec, N. (1992) Herpes-like virus infecting Pacific oyster larvae, *C. gigas*. *Bull. Eur. Ass. Fish Pathol.* 12(1), 11-13.
- Renault, T. (1998). Infections herpétiques chez les invertébrés : détection de virus de type herpès chez les mollusques bivalves marins. *Virologie* 2:401-403.
- Renault, T. and Arzul, I. (2001). Herpes-like virus infections in hatchery-reared bivalve larvae in Europe: specific viral DNA detection by PCR. *J. Fish Dis.*, 24: 161-167.
- Renault, T. and Lipart, C. (1998) Diagnosis of herpes-like virus infections in oysters using molecular techniques. *EAS Special Publication* 26, 235-236.
- Renault, T., Lipart, C. and Arzul, I. (2001a). A herpes-like virus infects a non-ostreid bivalve species: virus replication in *Ruditapes philippinarum* larvae. *Dis. aqua. Org.*, 45: 1-7.

- Renault, T., Lipart, C. and Arzul, I. (2001b). A herpes-like virusd infecting *Crassostrea gigas* and *Ruditapes philippinarum* larvae in France. *J. Fish Dis.*, 24: 369-376.
- Renault, T., Cochenec, N., Le Deuff, R.-M. and Chollet, B. (1994a) Herpes-like virus infecting Japanese oyster (*C. gigas*) spat. *Bull. Eur. Ass. Fish Pathol.* 14(2), 64-66.
- Renault, T., Le Deuff, R.-M., Cochenec, N. and Maffart, P. (1994b) Herpesviruses associated with mortalities among Pacific oyster, *C. gigas*, in France - Comparative study. *Rev. Med. Vet.* 145(10), 735-742.
- Renault, T., Le Deuff, R.-M., Lipart C. and Delsert C. (2000a) Development of a PCR procedure for the detection of a herpes-like virus infecting oysters in France. *J. Virol. Meth.* 88, 41-50.
- Renault, T., Le Deuff, R.-M., Chollet B., Cochenec N. and Gérard A (2000b). Concomitant herpes-like virus infections in hatchery-reared larvae and nursery-cultured spat *Crassostrea gigas* and *Ostrea edulis*. *Dis. aqua. Org.* 42(3), 173-183.
- Saiki, R.K., Scharf, S., Falcoma, F., Mullis, K.B., Horn, G.T., Erlich, H.A. and Arnheim, N. (1985) Enzymatic amplification of β -globulin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230, 1350-1354.

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 2001 to 3rd January 2002

Participant no. 1 (coordinator)

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

Laboratoire Génétique et Pathologie

17390 La Tremblade

France

Phone: +33 5 46 36 98 36

Fax: +33 5 46 36 37 51

Email: trenault@ifremer.fr

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 **04-01-01** to **03-01-02**

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)

Coordinator: Dr Tristan Renault
IFREMER - DRV/RA
Laboratoire Génétique et Pathologie
17390 La Tremblade
France
Phone: +33 5 46 36 98 36
Fax: +33 5 46 36 37 51
Email: trenault@ifremer.fr

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

INDIVIDUAL PROGRESS REPORT

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

Scientific team : Dr Tristan RENAULT
Dr Isabelle ARZUL (PhD student, Doctor in veterinary medicine)
Maeva ROBERT
Bruno CHOLLET

OBJECTIVES

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

ACTIONS IN THE PROJECT

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

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

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

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

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

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

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

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

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

PLANNED RESEARCH ACTIVITIES

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

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

RESEARCH ACTIVITIES DURING THE REPORTING PERIOD

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

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

1. First validation of a PCR procedure for viral DNA detection using fixed material

During the second reporting period (2000), two primer pairs (OH1/OH4 and IAP1/IAP2) have been designed in order to obtain PCR amplification when OsHV-1 DNA extracted from histological blocks was used as template. Several primer pairs (OHV3/OHV4, OHV3/OHV114, A3/A4 and A5/A6) failed producing amplicons because of DNA fragmentation in fixed material. Five histological blocks prepared in 1995 corresponding to *Crassostrea gigas* spat have been selected in 2000 to test the primer pairs OH1/OH2 and IAP1/IAP2. Animals presenting high mortality rates have been fixed individually in Davidson's fluid during the summer of 1995. Transmission electron microscopy examination allowed to detect viral particles. PCR analysis using DNA extracted from these blocks showed clear bands presenting expected sizes when both primer pairs were used. This suggested that both primer combinations were reliable tools to detect viral DNA in archived material.

In 2001, a first validation assay of both primer pairs has been carried out using 31 archived histological blocks.

1. 1. Selection of fixed material

Thirty one histological blocks corresponding to the material used for preparing reference slides sent by Participant 1 to Participants 5, 6 and 7 for testing *in situ* hybridization protocols (*in situ* hybridization ring trials) were selected. Each block has already been analysed by histology. Histology examination revealed cellular and nuclear abnormalities suggestive of infection with oyster herpesvirus type 1 (OsHV-1) in 20 individuals (infected animals, VB12 to VB31, Table I). Eleven oysters (VB1 to VB11) presented no sign of infection and were interpreted as healthy individuals (Table I).

Table I. Histological blocks selected to carry out viral DNA detection by PCR

N°	Histology	Reference	N°	Histology	Reference	N°	Histology	Reference
VB1	-	95 54-30	VB12	+	94 278-13	VB22	+	94 250-4
VB2	-	95 54-28	VB13	+	94 278-11	VB23	+	94 248-15
VB3	-	95 54-26	VB14	+	94 278-10	VB24	+	94 248-13
VB4	-	95 54-20	VB15	+	94 278-8	VB25	+	94 248-7
VB5	-	95 54 18	VB16	+	94 278-7	VB26	+	94 248-5
VB6	-	95 54 14	VB17	+	94 250-23	VB27	+	94 248 1
VB8	-	95 54 12	VB18	+	94-250-19	VB28	+	94 215-11
VB9	-	95 54 11	VB19	+	94-250-18	VB29	+	94 215-10
VB10	-	95 54-8	VB20	+	94-250-17	VB30	+	94 215-9
VB11	-	95 54-7	VB21	+	94-250-13	VB31	+	94 215-6

The reference number (N°) indicates the year of collection (1994 or 1995), the lot number and the particular organism of one lot. The « Histology » column indicates whether tissue lesions (herpes-like virus infection suspected) have been observed in histology slides at the time of sample collection.

1. 2. DNA extraction

DNA was extracted from histological sections using conventional techniques. The general protocol is summarized below. Ten µm thick tissue sections were cut and three sections for each block were put in a 1.5 ml sterile Eppendorf tube. Histological sections were dewaxed with xylene for 30 minutes and centrifuged 3 minutes at 8000 g (room temperature). Pellets were rinsed twice in pure ethanol (3 min, 8000 g at room temperature) and dried in hot-air steam cabinet at 55°C. Pellets were resuspended in 100 µl extraction buffer (50 mM Tris, 1 mM EDTA, 0.5% Tween 20) containing Proteinase K (200 µg/ml). The incubation was proceeded at 55°C overnight. Then, tubes were heated at 95°C (5 min) and centrifuged (5 min, 8000g). Supernatants were recovered and frozen at -20°C before PCR analysis.

1. 3. PCR reactions

Two primer pairs were chosen : IAP1/IAP2 and OH1/OH4. They allow the amplification of short viral DNA fragments (Figure 1). The primer pairs IAP1/IAP2 and OH1/OH4 were tested separately but cycling conditions were similar. Thirty five cycles of denaturing at 94 °C (1 min), annealing (1 min, 50°C) and elongation (1 min, 72°C) were preceded by a denaturation step (2 min, 94°C) and ended by a final elongation period (5 min, 72°C). Each reaction included negative controls introduced every five samples (sample DNA was replaced by double distilled sterile water) and a positive control (viral DNA).

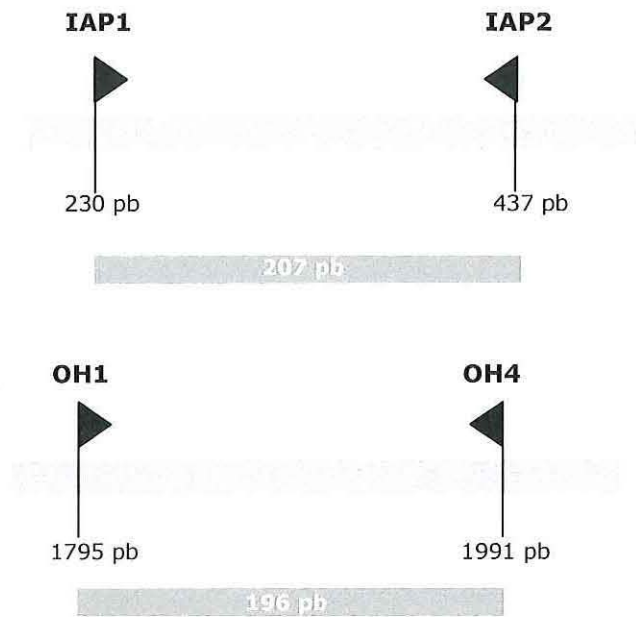


Figure 1. Position of the PCR primers IAP1/IAP2 and OH1/OH4 and expected amplification products

1. 4. Results

Both primer pairs gave products of the expected sizes. PCR results using the primer pair OH1/OH2 confirmed histological examination. DNA extracted from infected animals yielded amplicons presenting the expected size (Figure 2 and Table II). However, results obtained with the other primer pair (IAP1/IAP2) are less convincing. Indeed, only six individuals among the 20 infected oysters (suspicion of infection by histology) produced the expected band in agarose gels when PCR assays were carried out using DNA extracted from histological sections. Fifteen samples gave a positive result with OH1/OH4. Thirteen samples gave a positive result with OH1/OH4 whereas no amplification product was obtained using IAP1/IAP2. Four samples gave a positive result with IAP1/IAP2 and a negative one with OH1/OH4. All healthy oysters (11 individuals, VB1 to VB11) failed to produce amplicons with both primer pairs (Table II).

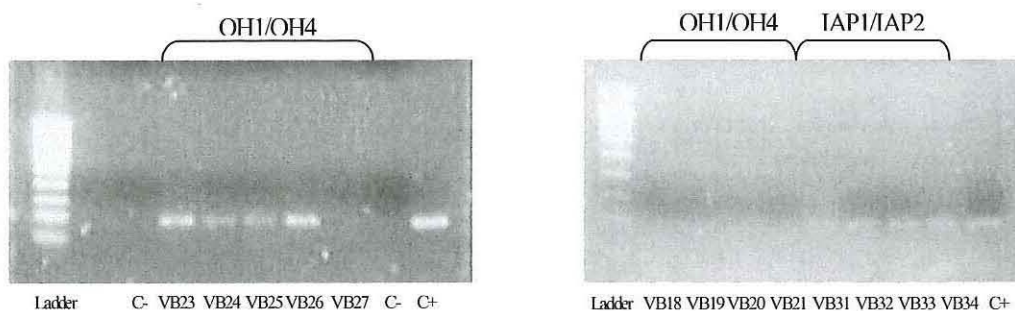


Figure 2. The OH1/OH4 and IAP1/IAP2 primer pairs have been tested using DNA extracted from wax blocks of infected and non-infected *Crassostrea gigas* spat

Table II. Detection of viral DNA from histological blocks by PCR using both primer pairs OH1/OH4 and IAP1/IAP2. The ø symbol indicates that no exploitable result could be obtained.

N°	OH1/OH4	IAP1/IAP2	N°	OH1/OH4	IAP1/IAP2
VB1 to VB11	-	-	VB 22	+	-
VB 12	ø	-	VB 23	+	-
VB 13	+	-	VB 24	+	-
VB 14	+	-	VB 25	+	-
VB 15	+	-	VB 26	+	-
VB 16	+	-	VB 27	+	-
VB 17	+	+	VB 28	-	+
VB 18	+	+	VB 29	-	+
VB 19	+	-	VB 30	-	+
VB 20	+	-	VB 31	-	+
VB 21	+	-			

We searched for differences in fixation procedures but the whole lot of samples appeared to have been fixed with Davidson's fixative. The primer pair OH1/OH4 targets a variable region. The samples that give a « false » negative result with this primer pair may be infected by a virus variant. The differences observed between the detection with OH primers and IAP primers may correspond to a higher efficiency of the OH primer pair.

Using specific primers, archived fixed material may be used in order to detect OsHV-1 genome. However, small viral DNA fragments must be targeted because of DNA breaks. Both primer pairs OH1/OH2 and IAP1/IAP2 appear adapted. However, some difference may be observed in amplification efficiency. Thus, to enhance viral DNA detection, several primer pairs designed in different genome areas are needed. For routine use it would be recommendable to run PCR with OH1/OH4 and to confirm negative results with IAP1/IAP2.

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

Considering the selection of monoclonal antibodies specific for OsHV-1, Participant 1 has developed an immunohistochemistry protocol. Indeed, it appears necessary to complete results obtained using ELISA and Western-blotting by Participant 3. Histological blocks remain the major material available in laboratories involved with shellfish disease diagnosis.

1. Histological material

In a first step, an OsHV-1 infected *Crassostrea gigas* individual previously characterized by transmission electron microscopy, PCR and *in situ* hybridization (histological block 94/215/11) was selected to develop the immunohistochemistry protocol.

2. Hybridoma supernatants

Twenty two hybridoma supernatants specific for ORF43 (a putative inhibitor of apoptosis, IAP) and five hybridoma supernatants specific for ORF94 (a putative viral glycoprotein) have been selected by Participant 3 (Table III) and sent to Participant 1 in June 2001 (5 ml for each hybridoma supernatant). A two month delay in monoclonal antibody production was observed. They were obtained in June 2001. This delay corresponds to a delay in the beginning of the sub-task 2.2.

Seven subcloned hybridoma supernatants (LG 5E5, LG 6C8, LG 6H8, LF 1A10, LF 3A10 G6A9, LF 3A10 H7D7 and LF 4H2) have been sent to Participant 1 in October 2001 (with a six month delay). The hybridomas were selected on the basis of ELISA and immunochemistry results (Participants 3 and 1). LF 3A10 G6A9 and LF 3A10 H7D7 correspond to subcloned hybridomas originating from the LF 3A10 hybridoma.

Table III. Hybridoma supernatants selected for immunochemistry assays

Reference	Specificity	ELISA (OD)
LG 2F6	Anti-glycoprotein (ORF 94)	0.6
LG 4A3	Anti-glycoprotein (ORF 94)	1.1
LG 5E5	Anti-glycoprotein (ORF 94)	0.9
LG 6C8	Anti-glycoprotein (ORF 94)	1.2
LG 6H8	Anti-glycoprotein (ORF 94)	1.0
LF 1A10	Anti-IAP (ORF 43)	1.3
LF 2A11	Anti-IAP (ORF 43)	0.4
LF 2C10	Anti-IAP (ORF 43)	0.4
LF 2C11	Anti-IAP (ORF 43)	1.2
LF 2D6	Anti-IAP (ORF 43)	0.4
LF 3A10	Anti-IAP (ORF 43)	0.5
LF 3B2	Anti-IAP (ORF 43)	0.6
LF 3D3	Anti-IAP (ORF 43)	0.6
LF 3D8	Anti-IAP (ORF 43)	0.9
LF 3E1	Anti-IAP (ORF 43)	0.6
LF 3E6	Anti-IAP (ORF 43)	0.6
LF 3E7	Anti-IAP (ORF 43)	0.5
LF 3G6	Anti-IAP (ORF 43)	0.6
LF 4G4	Anti-IAP (ORF 43)	0.4
LF 4H2	Anti-IAP (ORF 43)	0.6
LF 5G7	Anti-IAP (ORF 43)	0.6
LF 6A2	Anti-IAP (ORF 43)	0.5
LF 6C1	Anti-IAP (ORF 43)	0.7
LF 6C3	Anti-IAP (ORF 43)	0.4
LF 6D2	Anti-IAP (ORF 43)	0.4
LF 6D3	Anti-IAP (ORF 43)	0.6
LF 6F2	Anti-IAP (ORF 43)	0.5

N. B.: A bold font indicates strong responses by ELISA

3. Immunocytochemistry method

The immunocytochemistry method was developed and adapted from a previously developed protocol (T. Renault, unpublished data). Seven μm tissue sections on silane-prep slides (Sigma, France) were dewaxed in xylene and rinsed in alcohol. Endogenous peroxidases were inactivated 1 hour in pure methanol added with H_2O_2 (4% vol/vol). Tissue sections were washed twice in 1X PBS (150 mM NaCl, 12.5 mM Na_2HPO_4 , 3 mM K_2PO_4 , pH 7.5) and treated then with proteinase K (50 $\mu\text{g}/\text{ml}$ in distilled water) at 37°C for 15 minutes. Slides were washed again twice in 1X PBS. Sections were blocked for 90 minutes with skimmed dried milk (Régilait, France, 6% weight/vol) in 1X PBS. They were then incubated overnight at 4°C with monoclonal antibodies (pure hybridoma supernatants). Unbound antibodies were removed by six washes in 1X PBS (5 min for each wash). Specifically bound antibodies were detected using a peroxidase-conjugated antibody against mouse IgG (Sanofi Diagnostic Pasteur, France) diluted 1:400 in 1X PBS for 1 hour at room temperature in the dark. Unbound conjugate was removed by five washes in 1X PBS (5 min for each wash). A total of 200 μl of diaminobenzidine tetrahydrochloride (0.7 mg/ml of 1X PBS supplemented with 0.3% vol/vol hydrogen peroxide) were added on each tissue section and incubated for 10 minutes in the dark. Slides were washed twice with 1X PBS, counterstained for 20 seconds in Unna blue (RAL, France), dehydrated with ethanol and mounted in Eukitt resin after passing through a xylene bath. Negative controls included histological sections without primary antibodies (monoclonal antibodies), secondary antibody and revelation step.

4. Results

In a first step, 27 hybridoma supernatants received in June 2001 have been tested using the protocol described above. However, the incubation with pure supernatants was carried out one hour at room temperature. No signal was detected with the hybridoma supernatants tested.

Then, the hybridoma supernatants (received in June 2001) have been tested with the protocol described above without proteinase K treatment. Three hybridoma supernatants specific for ORF43 (LF 1A10, LF 2C11 and LF 3D8) and two hybridoma supernatants specific for ORF 94 (LG 5E5 and LG 6H8) yielded positive results on histological sections (Table IV). The staining appeared stronger with hybridoma supernatants specific for ORF94 than for hybridoma supernatants specific for ORF43. Incubation of hybridoma supernatants overnight at 4°C gave better results than a shorter incubation period (1 hour) at room temperature. Such results have already been observed in the laboratory (T. Renault, personal communication) when immunocytochemistry techniques were used on fixed material from bivalves.

The proteinase K treatment was included in some assays and results obtained with and without this type of treatment have been compared using the hybridoma supernatants received in June 2001. The hybridoma supernatants were tested using tissue sections obtained from the histological block 94/215/11. Five hybridoma supernatants (LF 1A10, LF 2C11, LF 3D8, LG 5E5 and LG 6H8) yielded positive results on histological sections in absence of proteinase K treatment confirming the first results (Table IV). Positive results were also observed with the hybridoma supernatant LG 6C8 when a proteinase K treatment was added in the immunocytochemistry protocol (Table IV). Moreover, the staining appeared more intense with hybridoma supernatants LG 5E5 and LG 6H8 when proteinase K was used. Staining was detected in muscle and some connective tissues (Figures 3a, 3b, 3c and 3d).

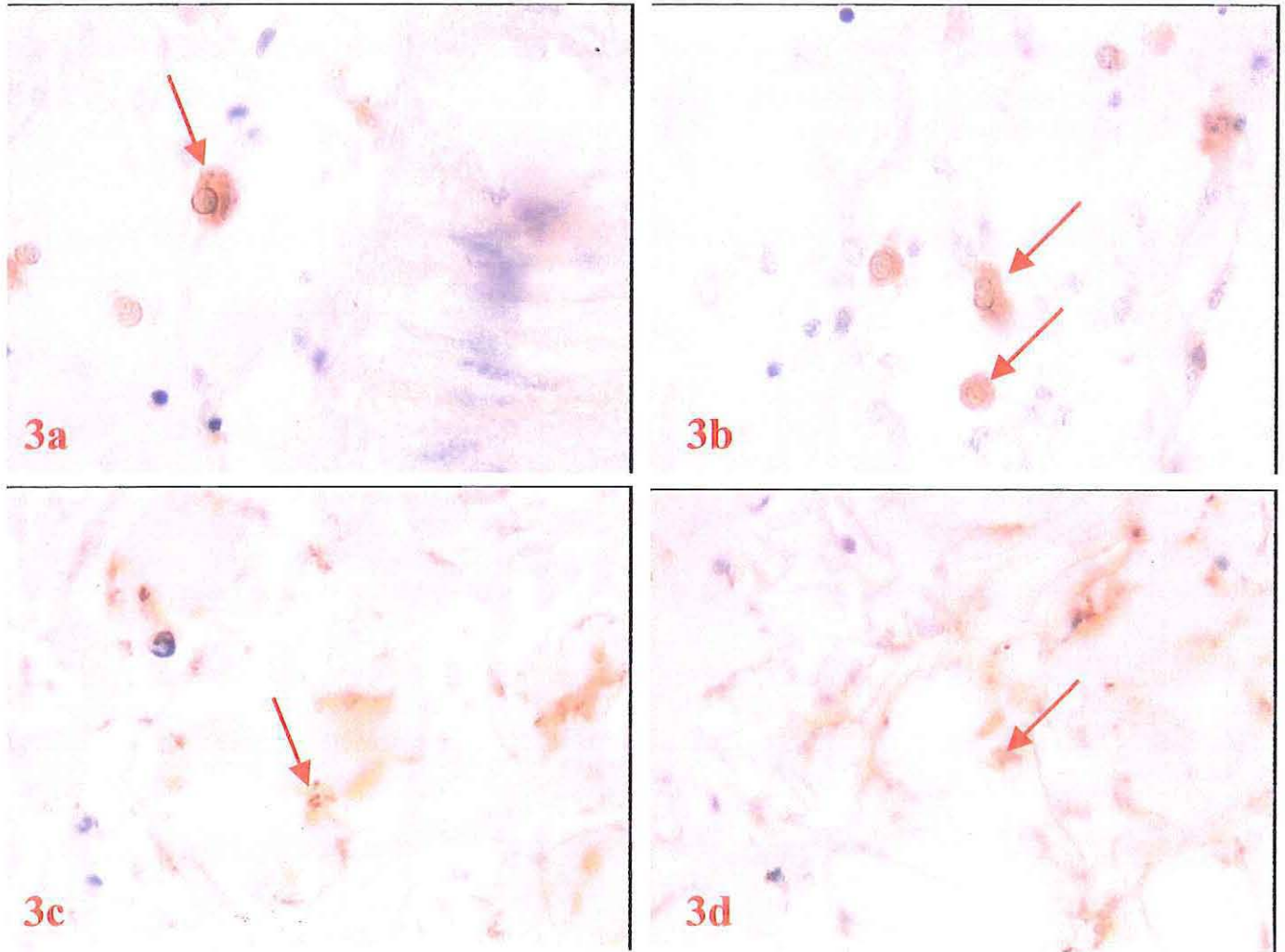


Figure 3. Immunostaining (arrows) observed with the hybridoma LG 6H8 supernatant unig proteinase K treatment. a and b : labelled cells (cytoplasm and nucleus) detected in connective tissue of the digestive gland. c and d : staining observed in the adductor muscle.

Table IV. Immunochemistry assays using pure hybridoma supernatants
(incubation overnight at 4°C)

Reference	Specificity	Immunochemistry	
		Without proteinase K treatment	With proteinase K treatment
LG 2F6	Anti-glycoprotein (ORF 94)	-	-
LG 4A3	Anti-glycoprotein (ORF 94)	-	-
LG 5E5	Anti-glycoprotein (ORF 94)	+	++
LG 6C8	Anti-glycoprotein (ORF 94)	-	+
LG 6H8	Anti-glycoprotein (ORF 94)	+	++
LF 1A10	Anti-IAP (ORF 43)	-	-
LF 2A11	Anti-IAP (ORF 43)	-	-
LF 2C10	Anti-IAP (ORF 43)	-	-
LF 2C11	Anti-IAP (ORF 43)	-	-
LF 2D6	Anti-IAP (ORF 43)	-	-
LF 3A10	Anti-IAP (ORF 43)	(+)	(+)
LF 3B2	Anti-IAP (ORF 43)	-	-
LF 3D3	Anti-IAP (ORF 43)	-	-
LF 3D8	Anti-IAP (ORF 43)	-	-
LF 3E1	Anti-IAP (ORF 43)	-	-
LF 3E6	Anti-IAP (ORF 43)	-	-
LF 3E7	Anti-IAP (ORF 43)	-	-
LF 3G6	Anti-IAP (ORF 43)	-	-
LF 4G4	Anti-IAP (ORF 43)	-	-
LF 4H2	Anti-IAP (ORF 43)	(+)	(+)
LF 5G7	Anti-IAP (ORF 43)	(+)	(+)
LF 6A2	Anti-IAP (ORF 43)	-	-
LF 6C1	Anti-IAP (ORF 43)	-	-
LF 6C3	Anti-IAP (ORF 43)	-	-
LF 6D2	Anti-IAP (ORF 43)	-	-
LF 6D3.	Anti-IAP (ORF 43)	-	-
LF 6F2	Anti-IAP (ORF 43)	-	-

N. B. : (+) : faint staining, + : clear staining, ++ : strong staining

Subcloned hybridoma supernatants received in October 2001 have been tested using the protocol described above including a proteinase K treatment. No signal was detected with the hybridoma supernatants tested (7). Assays have been carried out three times.

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

1. Collecting oyster samples in 2001

Bivalve samples were again obtained in Year 3 (2001). Since mortalities were yearly detected in France among *Crassostrea gigas* spat in France during the summer period, spat samples were collected to identify the possible cause of the mortalities. One hundred twenty two oyster batches have been collected in 2001 in different locations along the French Atlantic coast at the IFREMER laboratory in La Tremblade for epidemiological survey (Annex). Some of them consisted of Pacific oyster spat originating from Fouras (natural beds) and placed in May 2001 in different sites (6) of the Charente estuary (Fouras, Charente Maritime, France).

All individuals were divided in half – one half frozen at -20°C for PCR analysis and one half fixed in Davidsons fixative. The half fixed in Davidson might be subsequently processed for histology and for *in situ* hybridization. Frozen material was used to carry out PCR analysis. PCR analysis was carried out using pooled animals (5)

2. Preparation of reference material for Ring trials (2001)

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 (Participants 1, 5, 6 and 7) involved in epidemiological surveys used the same protocols and procedures for molecular diagnosis of OsHV-1 infections. The workshop took place at the IFREMER station in La Tremblade during one week (15-19 May 2000). The first day of the meeting, PCR and *in situ* hybridization protocols have been presented and discussed. Participant 1 showed how to carry out PCR diagnosis and *in situ* hybridization analysis. At the end of the 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.

In 2001, more reference material has been sent to Participants 5, 6 and 7 by Participant 1 in order to carry out PCR and *in situ* hybridization Ring trials.

2. 1. Reference material for PCR

Selection of frozen spat and larvae

Twenty two *Crassostrea gigas* spat samples collected in several locations in Charente Maritime in the year 2001 and analysed by PCR the same year using the primer pair OHV3/OHV114 during an epidemiological survey were tested by Participant 1 in order to select reference material for PCR. Indeed, spat samples were analysed again by Participant 1 in 2001 (Table V) using both primer pairs OHV3/OHV114 (C2/C6) and OHV1/OHV2 (C5/C13) in order to select negative and positive samples for PCR Ring trials.

Table V. Detection of viral DNA by PCR in *Crassostrea gigas* spat samples collected in 2001 using both primer pairs OHV3/OHV114 and OHV1/OHV2

Reference	PCR	Reference	PCR
01/74/1	+	01/113/5	+
01/78/1	+	01/115/4	+
01/78/5	+	01/116/3	+
01/93/5	+	01/117/3	+
01/98/6	+	01/117/4	+
01/99/4	+	01/175/1	+
01/99/5	+	01/317/1	-
01/105/3	+	01/317/2	-
01/105/4	+	01/317/3	-
01/106/1	+	01/317/4	-
01/113/3	+	01/317/5	-

The reference indicates the year of collection (01: 2001), the lot number and the particular pool of one lot.

Twenty one bivalve larval samples collected in 1997, 1998 and 1999 have also been tested in order to select negative and positive samples for PCR Ring trials. These samples were kept at the IFREMER laboratory in La Tremblade (Charente Maritime, France) at -20°C. They have already been analysed by PCR using the OHV3/OHV114 primer pair at the time of collection (Table VI). These larval samples were analysed again by Participant 1 in 2001 using both primer pairs OHV3/OHV114 (C2/C6) and OHV1/OHV2 (C5/C13).

Table VI. Detection of viral DNA by PCR in bivalve larval samples collected between 1997 and 1999 at the time of collection

Reference	Origin	Collection date	Status	Age (Days)	Reference	Origin	Collection date	Age (Days)	Status
SAT1	Normandy	16/09/98	+	7-8	PAL1	Normandy	25/09/98	3	-
SAT6	(hatchery)	15/09/98	+	7-8	PAL2	(hatchery)	25/09/98	3	-
SAT7		15/09/98	+	7-8	PAL3		26/09/98	4	-
SAT8		15/09/98	+	7-8	PAL4		26/09/98	4	-
SAT9		27/08/98	++	7-8	PAL6		27/09/98	5	-
SAT10		27/08/98	++	7-8	PAL8		28/09/98	6	-
P10	Vendée	05/99	+	10	PAL10		29/09/98	7	+
P11	(hatchery)	05/99	+	10	PAL12		30/09/98	8	+
T1 to T5	England	12/97-	-	10					
	(hatchery)	06/98		10					

The « Status » column indicates the information furnished by PCR analysis of the samples at the time of their collection (1998-1999). All samples are *Crassostrea gigas* except PAL larvae which are *Ruditapes decussatus*.

PCR with both primer pairs OHV3/OHV114 and OHV1/OHV2 was tested for frozen archived larvae and spat (Table VII, Fig. 4). Ten samples that had previously been controlled as negative (by TEM and PCR analysis for larvae and by PCR for spat) gave indeed negative results by PCR with both primer pairs (Table VII). Twenty samples among thirty-six that had been controlled positive by the same means gave positive results with both primer pairs (Table VII). Eleven positive samples gave negative results with both primer pairs (Table VII). Four positive samples gave a negative result with OHV3/OHV114 whereas a positive result was obtained with OHV1/OHV2 (Table VII).

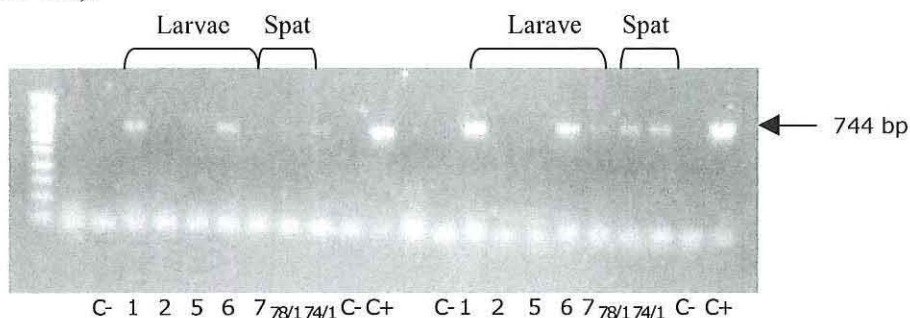


Figure 4. PCR analysis of frozen samples (selection of material for PCR Ring trials)

Table VII. PCR results (2001) for frozen archived samples

Samples	Spat			Samples	Larvae		
	PCR*	PCR C2/C6	PCR C5/C13		TEM* PCR*	PCR C2/C6	PCR C5/C13
01/74/1	+	+	+	SAT1	+	+	+
01/78/1	+	+	+	SAT2	+	-	-
01/78/5	+	-	+	SAT5	+	-	-
01/93/5	+	+	+	SAT6	+	+	+
01/98/6	+	+	+	SAT7	+	-	+
01/99/4	+	+	+	SAT 8	+	-	-
01/99/5	+	+	+	SAT 9	+	+	+
01/105/3	+	-	+	SAT 10	+	+	+
01/105/4	+	+	+				
01/106/1	+	+	+	P10	+	-	-
01/113/3	+	+	+	P11	+	-	-
01/113/5	+	-	-				
01/115/4	+	+	+	PAL 1	+	-	-
01/116/3	+	+	+	PAL 2	+	-	-
01/117/3	+	+	+	PAL 3	+	-	-
01/117/4	+	-	+	PAL 4	+	-	-
01/175/2	+	-	-	PAL 6	+	+	+
01/317/1	-	-	-	PAL 8	+	+	+
01/317/2	-	-	-	PAL 10	+	+	+
01/317/3	-	-	-	PAL 12	+	+	+
01/317/4	-	-	-				
01/317/5	-	-	-	T1 to T5	-	-	-

* : Analysis carried out at the time of sample collection. Samples in bold fonts correspond to the selected samples for PCR Ring trials

The primer pair OHV1/OHV2 has a better sensitivity than OHV3/OHV114 for the detection of OsHV-1 from archived frozen samples. However, on such samples, false negatives are observed. This appearance of false negatives may be explained by the conservation of the samples at -20°C . The reproducibility of PCR results could be improved by storage at -80°C of the biological material.

Fifteen spat samples were selected by Participant 1 in order to carry out PCR Ring trials (Table VII). Ten samples correspond to PCR positive animals (01/74/1, 01/93/5, 01/98/6, 01/99/4, 01/99/5, 01/105/4, 01/106/1, 01/115/4, 01/116/3 and 01/117/3) and five to PCR negative animals (01/317/1, 01/317/2, 01/317/3, 01/317/4 and 01/317/5). Fifteen larval samples were also selected by Participant 1 in order to carry out PCR Ring trials (Table VII). Nine samples correspond to PCR positive animals (SAT1, SAT6, SAT7, SAT9, SAT10, PAL6, PAL8, PAL10 and PAL12) at the time of collection and in 2001. One sample (PAL2) consisted of a PCR positive sample at the time of collection, but was negative in 2001 using both primer pairs (OHV3/OHV114 and OHV1/OHV2). Five PCR negative samples (T1, T2, T3, T4 and T5) were also selected for PCR Ring trials.

Sending selected samples

Selected larval and spat samples (30, Table VIII) analysed in the laboratory of IFREMER in La Tremblade (France, Participant 1) were sent to other European laboratories (Participants 5, 6 and 7). Samples consisted of 20 μl of supernatant from ground larvae or spat. The protocol of sample preparation was previously detailed in the First Annual Progress Report. Samples were sent in dry ice by express mail in order to avoid DNA degradation. Sample references were concealed before PCR Ring trials took place and replaced by a code. Indeed, no information was furnished to Participants 5, 6 and 7 excepted a number for each sample (Table VIII).

2. 2. Reference material for *in situ* hybridization

Histological blocks

Three histological blocks have been selected by Participant 1 in order to send them to Participants 5, 6 and 7 (reference material for *in situ* hybridization). Selected blocks correspond to *Crassostrea gigas* oyster spat collected in 1994 and 1995. The samples have already been analysed by histology and transmission electron microscopy at the time of collection. Histology examination revealed cellular and nuclear abnormalities suggestive of infection with OsHV-1 (infected animals, Table IX). Viral particles have been detected by electron microscopy (Table IX). Histological blocks have also been analysed in 2001 by *in situ* hybridization (indirect technique) and PCR using DNA extracted from histological sections as template. Both techniques gave positive results (Table IX). Blocks 94/250 (2), 95/240 (7) and 95/240 (9) have respectively been sent in November 2001 to Participant 5, Participant 6 and Participant 7.

Table VIII. Selected samples for PCR Ring trials

Reference	Status	N° (*)
317/1	-	1
317/2	-	2
317/3	-	3
317/4	-	4
317/5	-	5
T1	-	6
T2	-	7
T3	-	8
T4	-	9
T5	-	10
PAL2	+?	11
74/1	+	12
93/5	+	13
98/6	+	14
99/4	+	15
99/5	+	16
105/4	+	17
106/1	+	18
115/4	+	19
116/3	+	20
117/3	+	21
PAL6	+	22
PAL8	+	23
PAL10	+	24
PAL12	+	25
SAT9	+	26
SAT10	+	27
SAT1	+	28
SAT6	+	29
SAT7	+	30

(*) : Reference was concealed before PCR Ring trials took place and replaced by a number

Table IX. Histological blocks selected as reference blocks for Participants 5, 6 and 7

Reference	Histology	MET	PCR (OH1/OH4)	<i>In situ</i> hybridization	Participant
94/250/2	+	+	+	+	5
95/240/7	+	+	+	+	6
95/240/9	+	+	+	+	7

The reference indicates the year of collection (94: 1994 or 95: 1995), the lot number and the particular organism of one lot.

Histological sections

In situ hybridization Ring trials were carried on a series of samples collected in the years 1994-1995. Thirty one histological blocks corresponded to the material used by Participant 1 for testing PCR detection from fixed material (Table I). Each block has already been analyzed by histology. Histology examination revealed cellular and nuclear abnormalities suggestive of infection with OsHV-1 in 20 individuals (infected animals, Table I). Eleven oysters (VB1 to VB11) presented no sign of infection and were interpreted as healthy individuals (Table I).

Four slides of each histological block have been sent to Participants 5, 6 and 7 in June 2001. References were not concealed on histological slides. However, no information has been sent to Participants 5, 6 and 7 concerning the infection status of the selected oyster samples. 372 histological sections (4 x 31 x 3) have been prepared by Participant 1 for sending them. Moreover, some histological sections have also been prepared for local processing.

Slides (VB1 to VB30) were again sent to Participants 5, 6 and 7 in December 2001 in order to repeat *in situ* hybridization ring trial. In this case, no reference and no information have been sent to the involved participants. Participants have received histological slides identified by a code (VB1 to VB30). One hundred twenty histological sections have again been prepared in order to carry out this second *in situ* hybridization Ring trial.

Subtask 3.1. Application of molecular methods for the diagnosis of oyster herpes like virus carrying out analysis of oyster samples originating from Ireland.

1. Screening by PCR

1. 1. Screening of bivalves samples from France during 2001 by PCR

PCR analysis permitted the detection of viral DNA in batches of oyster spat among the analysed batches (Annex). Screening of all samples from February 2001 on was carried out by PCR using the primer pair OHV1/OHV2.

2. Screening using *In situ* hybridisation

2. 1. Validation of direct and indirect methods of *in situ* hybridisation

The labelled probe produced by PCR using the OHV3/OHV114 primer pair was controlled through electrophoresis in an agarose gel (Figure 5). The positive control (C+) corresponded to viral DNA amplified with the same primer pair but using non labelled dNTPs.



Figure 5. Labelled probes for *in situ* hybridization

Histological sections from the 31 selected histological blocks (VB1 to VB31) were screened using the direct and indirect revelation methods (first *in situ* hybridization Ring trial). Results were reported in Table XI.

All the samples (VB1 to VB11) that had been analysed as negative by histology gave negative results using both techniques (direct and indirect revelation). Among the nineteen samples that had been analysed as positive, seventeen gave positive results when the direct protocol is used. All these samples gave positive results when the indirect revelation procedure was used.

Consistent results were obtained in twenty-eight out of thirty analysed samples, with only three false negatives (VB12, VB19 and VB21) with the direct revelation technique. The indirect revelation protocol allows to detect viral DNA by *in situ* hybridization in all samples selected as positive by histology. Therefore, *in situ* hybridization appears to be a sensitive method for the detection of OsHV-1 in samples that have been stored for several years as histological blocks.

Table XI. *In situ* hybridization results using both revelation protocols and selected histological blocks (first *in situ* hybridization Ring trial)

N°	ISH		N°	ISH		N°	ISH		N°	ISH	
	1*	2*		1*	2*		1*	2*		1*	2*
VB1	-	-	VB10	-	-	VB17	+	+	VB24	+	+
VB2	-	-	VB11	-	-	VB18	+	+	VB25	+	+
VB3	-	-	VB12	-	+	VB19	-	+	VB26	+	+
VB4	-	-	VB13	+	+	VB20	+	+	VB27	+	+
VB6	-	-	VB14	+	+	VB21	-	+	VB28	+	+
VB8	-	-	VB15	+	+	VB22	+	+	VB30	+	+
VB9	-	-	VB16	+	+	VB23	+	+	VB31	+	+

* : 1 corresponds to results obtained using the direct revelation protocol and 2 to results obtained with the indirect revelation protocol

It was decided in December 2001 that Participant 1 would rescreen 30 blocks (coded VB1 to VB30) using the direct method (second *in situ* hybridization ring trial). The results of this analysis are similar to those observed for the first *in situ* hybridization Ring trial (Table XI).

2. 2 . Technique comparison (histology, PCR and *in situ* hybridization) in La Tremblade

Considering global PCR results (a positive result with either of the primer pairs meaning a positive global result, Table II), PCR and ISH with the direct revelation protocol (Table XII) give similar results including one false negative (VB 12) in twenty-eight out of thirty-one samples. In two cases (VB19 and VB21), PCR with OH1/OH4 gave a positive result whereas ISH gave a negative one.

Table XII. Technique comparison for OsHV-1 detection in histological blocks

Samples	Histology	Global PCR	ISH		Samples	Histology	Global PCR	ISH	
			1*	2*				1*	2*
VB1 to VB11	-	-	-	-	VB 22	+	+	+	+
VB 12	+	-	-	+	VB 23	+	+	+	+
VB 13	+	+	+	+	VB 24	+	+	+	+
VB 14	+	+	+	+	VB 25	+	+	+	+
VB 15	+	+	+	+	VB 26	+	+	+	+
VB 16	+	+	+	+	VB 27	+	+	+	+
VB 17	+	+	+	+	VB 28	+	+	+	+
VB 18	+	+	+	+	VB 29	+	+	+	+
VB 19	+	+	-	+	VB 30	+	+	+	+
VB 20	+	+	+	+	VB 31	+	+	+	+
VB 21	+	+	-	+					

* : 1 corresponds to results obtained using the direct revelation protocol and 2 to results obtained with the indirect revelation protocol

Both techniques (PCR and *in situ* hybridization) have a similar sensitivity when global PCR results are considered. The primer pair OH1/OH4 and ISH may be used for the detection of OsHV-1 from archived material with similar results. A good correlation is also observed between histology, PCR and *in situ* hybridization. Such result may be explain because of the selection of oyster samples. Indeed, positive samples correspond to highly infected individuals on the basis of abnormality detection at photonic microscope level.

2. 3. Screening of French bivalves for herpes like virus using ISH

Five *Crassostrea gigas* spat batches collected in 2001 were subjected to *in situ* hybridization analysis. PCR analysis was carried out using frozen material and fixed material (Davidson fixative) was used for *in situ* hybridization. Results of this screening are presented in Table XIII.

Table XIII. Technique comparison (PCR and *in situ* hybridization) for OsHV-1 detection

Reference	PCR (OHV3/OHV114)	HIS*	% of positive PCR **	% of positive HIS
01/36	0+/2	3+/20	0	15
01/74	1+/7	1+/20	2.8 to 14.3	5
01/78	2+/6	4+/20	6.6 to 33.3	20
01/93	1+/10	1+/20	2 to 10	5
01/94	0+/5	0+/20	0	0

The reference indicates the year of collection (01: 2001) and the lot number. * : *in situ* hybridization was carried out using a direct revelation protocol. ** : PCR analysis was carried out on pools of five oysters ; a PCR positive result may correspond to 1 to 5 infected individuals.

For four batches, the percentage of positive results is quite similar between both techniques. *In situ* hybridization doesn't appear less sensitive than PCR. However, comparison between PCR and *in situ* hybridization efficiency is difficult. Indeed, *in situ* hybridization was carried out using individuals. Pools of five oysters were used for PCR. Moreover, sample treatment for both analysis techniques was different. Samples were fixed in Davidson fluid for *in situ* hybridization analysis although oysters were frozen in the case of PCR.

In situ hybridization seems adapted to search herpes-like viral infection in oysters. This technique allows to detect and localize the viral DNA in infected animals.

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

Sub-task 3.2 Application of immunological methods

Monoclonal antibodies were not available until June 2001. Indeed, a two month delay in monoclonal antibody production was observed. They were obtained by Participant 1 in June 2001. These antibodies have been tested in order to define their potential interest using histochemistry techniques. Six hybridomas have been selected using indirect immunoperoxidase immunolabelling on reference histological material. They were then subcloned in order to validate their antigen specificity.

The subcloned hybridoma supernatants have been received by Participant 1 in October 2001 (with a 6 month delay). These subcloned hybridoma supernatants failed to give positive results on selected histological slides in three assays (November 2001 to January 2002).

Because of these results, the subcloned hybridoma supernatants were not available to screen bivalve samples. Thus, aliquots (1 ml) of a non subcloned hybridoma supernatant (LG 6H8, received by Participant 1 in June 2001) were sent to Participants 5, 6 and 7 in February 2002. However, the supernatant volume (1ml) is too low to analyze more than 10 histological slides). Moreover, Participants 5, 6 and 7 had no funding available to carry out the immunochemistry analysis. Thus, the application of immunological methods for OsHV-1 diagnosis was not developed.

Polyclonal antibodies have also been produced by Participant 3 and sent to Participant 1. However, these antibodies have not been characterized using immunochemistry by Participant 1. Because of busy time, it was decided to carry out the characterization on monoclonal antibodies. Thus, no polyclonal antibodies have been sent to other Participants in order to screen bivalves samples.

Sub-task 3.3 Application of primary oyster cell cultures

No cell cultures were available for diagnosis during the project.

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 the third annual meeting in December 2001 at the IFREMER headquarter in Issy les Moulineaux near Paris (France). Dr T. Renault attended and presented results at third annual meeting. Dr T. Renault also wrote this intermediate report.

Dissemination of results (contribution to articles and posters related to OsHV-1)

- Contribution to articles related to OsHV-1:

Arzul I. & Renault T., 2002. Herpèsvirus et bivalves marins. *Virologie* **6** : 169-174

Arzul I., Renault T. & C. Lipart, 2001. Experimental herpes-like viral infections in marine bivalves : demonstration of interspecies transmission. *Diseases of Aquatic Organisms* **46**(1) : 1-6

Arzul I., Nicolas J. L., Davison A. J. & T. Renault, 2001. French scallops : a new host for ostreid herpesvirus-1. *Virology* **290** : 342-349

Arzul I., Renault T., Lipart C. & A. J. Davison, 2001. Evidence for interspecies transmission of oyster herpesvirus in marine bivalves. *Journal of General Virology* **82** : 865-870

Arzul I., Renault T., Thébault A. & A. Gérard, 2002. Detection of oyster herpesvirus DNA and proteins in asymptomatic *Crassostrea gigas* adults. *Virus Research* **84** : 151-160

Lipart C. & T. Renault, 2002. Herpes-like virus detection in *Crassostrea gigas* spat using DIG-labelled probes. *Journal of Virological Methods* **101** : 1-10

- Renault T. & I. Arzul**, 2001. Herpes-like virus infections in hatchery-reared bivalve larvae in Europe : specific viral DNA detection by PCR. *Journal of Fish Diseases* **24** : 161-167
- Renault T., Lipart C. & I. Arzul**, 2001. A herpes-like virus infects a non-ostreid bivalve species : virus replication in *Ruditapes philippinarum* larvae. *Diseases of Aquatic Organisms* **45** : 1-7
- Renault T., Lipart C. & I. Arzul**, 2001. A herpes-like virus infecting *Crassostrea gigas* and *Ruditapes philippinarum* larvae in France. *Journal of Fish Diseases* **24** : 369-376

- *Posters and presentations*

- Arzul I. & T. Renault**. Infections à virus de type herpès chez les bivalves : large spectre d'hôte et détection au stade adulte. IFREMER - Journées conchylicoles, Nantes, 3 – 4 avril 2001
- Arzul I., Nicolas J. L. & T. Renault**. Infection à virus de type herpès chez les larves de coquille Saint-Jacques. IFREMER - Journées conchylicoles, Nantes, 3 – 4 avril 2001
- Arzul I., Renault T. & J. L. Nicolas**, 2001. Study of a herpes-like virus infection in scallops, *Pecten maximus*. . 10th International Conference of the EAFP «Disease of fish and shellfish» 9th - 14th september 2001, Trinity College Dublin, Ireland.
- Arzul I. & T. Renault**, 2001. Detection of oyster herpes virus DNA and proteins in *Crassostrea gigas* adult oysters. 10th International Conference of the EAFP «Diseases of Fish and Shellfish», 9th - 14th September 2001, Trinity College, Dublin, Ireland.
- Renault T., Deniau S., Bourgougnon N. & A. Gérard**, 2001. Apoptosis of pacific oyster, *Crassostrea gigas* haemocytes maintend *in vitro*. . 10th International Conference of the EAFP «Disease of fish and shellfish» 9th - 14th September 2001, Trinity College Dublin, Ireland.
- Renault T. & I. Arzul**, 2001. Herpes-like virus infections in hatchery-reared bivalve larvae in Europe : specific viral DNA detection by PCR. . 10th International Conference of the EAFP «Disease of fish and shellfish» 9th - 14th September 2001, Trinity College Dublin, Ireland

- *E. C. Programme reports :*

Diagnosis of oyster herpes-like virus : development and validation of molecular, immunological and cellular tools (2001). Second Periodic Progress Report. Programme FAIR-CT98-4334 (VINO).

Diagnosis of oyster herpes-like virus : development and validation of molecular, immunological and cellular tools (2002). Third Periodic Progress Report. Programme FAIR-CT98-4334 (VINO).

- *Web sites :*

Detection of herpes-like viral DNA by PCR in European bivalve larval samples (Programme E. C. no FA-S2 9052, ordis Technology Marketplace, <http://www.cordis.lu/marketplace>)

Herpesviruses in Marine Bivalves (<http://www.ifremer.fr/latremblade/>, IFREMER laboratory located in La Tremblade, Charente Maritime, France). In addition to having published this work by writing articles in different journals, we have prepared a document (Annex of the Consolidated Progress Report) available on the web site (<http://www.ifremer.fr/latremblade/>) of IFREMER laboratory located in La Tremblade (Charente Maritime, France).

SIGNIFICANT DIFFICULTIES OR DELAYS

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

Moreover, subcloned hybridoma supernatants from selected hybridomas have been received by Participant 1 in October 2001 (with a 6 month delay). These subcloned hybridoma supernatants failed to give positive results on selected histological slides in three assays. Thus, it has been decided to send a non subcloned hybridoma supernatant (LG 6H8) to Participants 5, 6 and 7 in February 2002. However, the available volume of this supernatant is low (1ml for each Participant) and Participants 5, 6 and 7 had no funding available to carry out the immunochemistry analysis.

ANNEX - French oyster samples collected in 2001 and analysed by PCR (OHV3/OHV114). PCR analysis was carried out using oyster pools (5 individuals per pool)

Code	Species	Date of sampling	Location	Number of animals	Age (months)	Positive PCR analysis
01/03	<i>C. gigas</i>	05/02/01	Arcachon	30	68	0/6
01/12	<i>C. gigas</i>	11/05/01	Arcachon	30	11	0/6
01/17	<i>C. gigas</i>	29/05/01	Fouras	10	10	0/2
01/18	<i>C. gigas</i>	29/05/01	Fouras	10	10	0/2
01/19	<i>C. gigas</i>	29/05/01	Fouras	10	10	0/2
01/20	<i>C. gigas</i>	29/05/01	Fouras	10	10	0/2
01/21	<i>C. gigas</i>	29/05/01	Fouras	10	10	0/2
01/22	<i>C. gigas</i>	29/05/01	Fouras	10	10	0/2
01/26	<i>C. gigas</i>	05/06/01	Fouras	10	11	0/2
01/27	<i>C. gigas</i>	05/06/01	Fouras	10	11	0/2
01/28	<i>C. gigas</i>	05/06/01	Fouras	10	11	0/2
01/29	<i>C. gigas</i>	05/06/01	Fouras	10	11	0/2
01/30	<i>C. gigas</i>	05/06/01	Fouras	10	11	0/2
01/31	<i>C. gigas</i>	05/06/01	Fouras	10	11	0/2
01/32	<i>C. gigas</i>	05/06/01	Fouras	10	11	0/2
01/33	<i>C. gigas</i>	05/06/01	Fouras	10	11	0/2
01/50	<i>C. gigas</i>	12/06/01	Fouras	10	11	0/2
01/51	<i>C. gigas</i>	12/06/01	Fouras	10	11	0/2
01/52	<i>C. gigas</i>	12/06/01	Fouras	10	11	0/2

Code	Species	Date of sampling	Location	Number of animals	Age (months)	Positive PCR analysis
01/53	<i>C. gigas</i>	12/06/01	Ronce	30	11	0/6
01/59	<i>C. gigas</i>	11/06/01	Ronce (hatchery)	30	5	5/6
01/60	<i>C. gigas</i>	11/06/01	Ronce (hatchery)	30	5	6/6
01/61	<i>C. gigas</i>	12/06/01	Fouras	10	11	0/2
01/62	<i>C. gigas</i>	12/06/01	Fouras	10	11	0/2
01/63	<i>C. gigas</i>	12/06/01	Fouras	10	11	0/2
01/66	<i>C. gigas</i>	15/06/01	Arcachon	30	10	0/2
01/67	<i>C. gigas</i>	15/06/01	Arcachon	30	11	0/6
01/68	<i>C. gigas</i>	15/06/01	Arcachon	30	11	0/6
01/70	<i>C. gigas</i>	29/05/01	Fouras	10	11	0/2
01/71	<i>C. gigas</i>	19/06/01	Fouras	10	11	0/2
01/72	<i>C. gigas</i>	19/06/01	Fouras	10	11	0/2
01/73	<i>C. gigas</i>	19/06/01	Fouras	10	11	0/2
01/74	<i>C. gigas</i>	19/06/01	Fouras	10	11	0/2
01/75	<i>C. gigas</i>	19/06/01	Fouras	10	11	0/2
01/76	<i>C. gigas</i>	19/06/01	Fouras	10	11	0/2
01/77	<i>C. gigas</i>	19/06/01	Fouras	10	11	0/2
01/78	<i>C. gigas</i>	19/06/01	Fouras	10	11	½
01/79	<i>C. gigas</i>	19/06/01	Fouras	10	11	0/2
01/80	<i>C. gigas</i>	19/06/01	Fouras	10	11	0/2

Code	Species	Date of sampling	Location	Number of animals	Age (months)	Positive PCR analysis
01/81	<i>C. gigas</i>	19/06/01	Fouras	10	11	0/2
01/84	<i>C. gigas</i>	22/06/01	Arcachon	30	11	0/6
01/88	<i>C. gigas</i>	22/06/01	Arcachon	30	11	0/6
01/89	<i>C. gigas</i>	22/06/01	Arcachon	30	11	0/6
01/92	<i>C. gigas</i>	26/05/01	Fouras	20	11	0/4
01/93	<i>C. gigas</i>	26/05/01	Fouras	50	11	1/10
01/94	<i>C. gigas</i>	26/05/01	Fouras	25	11	0/5
01/95	<i>C. gigas</i>	26/05/01	Fouras	5	11	1/1
01/96	<i>C. gigas</i>	26/05/01	Fouras	5	11	1/1
01/97	<i>C. gigas</i>	26/06/01	Fouras	5	11	0/1
01/98	<i>C. gigas</i>	26/06/01	Fouras	30	11	1/6
01/99	<i>C. gigas</i>	26/06/01	Fouras	25	11	2/5
01/100	<i>C. gigas</i>	26/06/01	Fouras	40	11	2/8
01/105	<i>C. gigas</i>	03/07/01	Fouras	35	12	2/7
01/106	<i>C. gigas</i>	03/07/01	Fouras	25	12	1/5
01/107	<i>C. gigas</i>	03/07/01	Fouras	20	12	0/4
01/110	<i>C. gigas</i>	03/07/01	Fouras	10	12	0/2
01/113	<i>C. gigas</i>	03/07/01	Fouras	25	12	2/5
01/114	<i>C. gigas</i>	03/07/01	Fouras	15	12	0/3
01/115	<i>C. gigas</i>	03/07/01	Fouras	20	12	¼

Code	Species	Date of sampling	Location	Number of animals	Age (months)	Positive PCR analysis
01/116	<i>C. gigas</i>	03/07/01	Fouras	20	12	¼
01/117	<i>C. gigas</i>	03/07/01	Fouras	20	12	2/4
01/118	<i>C. gigas</i>	03/07/01	Fouras	20	12	0/4
01/119	<i>C. gigas</i>	03/07/01	Fouras	15	12	0/3
01/120	<i>C. gigas</i>	03/07/01	Fouras	15	12	0/3
01/121	<i>C. gigas</i>	03/07/01	Fouras	15	12	0/3
01/124	<i>C. gigas</i>	04/07/01	Ronce	14	6	2/3
01/126	<i>C. gigas</i>	09/07/01	Fouras	15	11	0/3
01/127	<i>C. gigas</i>	09/07/01	Fouras	15	11	0/3
01/128	<i>C. gigas</i>	09/07/01	Fouras	15	11	0/3
01/129	<i>C. gigas</i>	09/07/01	Fouras	25	11	0/5
01/130	<i>C. gigas</i>	09/07/01	Fouras	10	11	0/2
01/131	<i>C. gigas</i>	09/07/01	Fouras	25	12	1/5
01/132	<i>C. gigas</i>	09/07/01	Fouras	15	12	0/3
01/133	<i>C. gigas</i>	09/07/01	Fouras	15	12	0/3
01/134	<i>C. gigas</i>	09/07/01	Fouras	15	12	0/3
01/113	<i>C. gigas</i>	03/07/01	Fouras	25	12	2/5
01/114	<i>C. gigas</i>	03/07/01	Fouras	15	12	0/3
01/115	<i>C. gigas</i>	03/07/01	Fouras	20	12	¼

Code	Species	Date of sampling	Location	Number of animals	Age (months)	Positive PCR analysis
01/142	<i>C. gigas</i>	17/07/01	Fouras	15	12	0/3
01/143	<i>C. gigas</i>	17/07/01	Fouras	15	12	0/3
01/144	<i>C. gigas</i>	17/07/01	Fouras	10	12	0/2
01/145	<i>C. gigas</i>	17/07/01	Fouras	5	12	0/1
01/146	<i>C. gigas</i>	17/07/01	Fouras	5	12	0/1
01/147	<i>C. gigas</i>	17/07/01	Fouras	5	12	0/1
01/148	<i>C. gigas</i>	17/07/01	Ronce	15	12	0/3
01/149	<i>C. gigas</i>	17/07/01	Fouras	15	12	0/3
01/150	<i>C. gigas</i>	17/07/01	Fouras	10	12	0/2
01/166	<i>C. gigas</i>	24/07/01	Fouras	10	12	0/2
01/167	<i>C. gigas</i>	24/07/01	Fouras	15	12	0/3
01/168	<i>C. gigas</i>	24/07/01	Fouras	10	12	0/2
01/169	<i>C. gigas</i>	24/07/01	Fouras	5	12	0/1
01/172	<i>C. gigas</i>	24/07/01	Fouras	10	12	0/2
01/173	<i>C. gigas</i>	24/07/01	Fouras	10	12	0/2
01/174	<i>C. gigas</i>	24/07/01	Fouras	10	12	0/2
01/175	<i>C. gigas</i>	24/07/01	Fouras	10	12	0/2
01/176	<i>C. gigas</i>	24/07/01	Fouras	10	12	0/2
01/177	<i>C. gigas</i>	24/07/01	Fouras	10	12	0/2

Code	Species	Date of sampling	Location	Number of animals	Age (months)	Positive PCR analysis
01/178	<i>C. gigas</i>	17/07/01	Fouras	10	12	0/2
01/1179	<i>C. gigas</i>	17/07/01	Fouras	10	12	0/2
01/180	<i>C. gigas</i>	17/07/01	Fouras	10	12	0/2
01/184	<i>C. gigas</i>	31/07/01	Fouras	5	12	0/1
01/190	<i>C. gigas</i>	31/07/01	Fouras	10	12	0/2
01/191	<i>C. gigas</i>	31/07/01	Fouras	10	12	0/2
01/192	<i>C. gigas</i>	31/07/01	Ronce	10	12	0/2
01/196	<i>C. gigas</i>	31/07/01	Fouras	10	12	0/2
01/197	<i>C. gigas</i>	31/07/01	Fouras	10	12	0/2
01/198	<i>C. gigas</i>	31/07/01	Fouras	10	12	0/2
01/223	<i>C. gigas</i>	07/08/01	Fouras	10	12	0/2
01/224	<i>C. gigas</i>	07/08/01	Fouras	10	12	0/2
01/225	<i>C. gigas</i>	07/08/01	Fouras	10	12	0/2
01/226	<i>C. gigas</i>	07/08/01	Fouras	5	12	0/1
01/227	<i>C. gigas</i>	07/08/01	Fouras	5	12	1/1
01/229	<i>C. gigas</i>	07/08/01	Fouras	10	12	½
01/230	<i>C. gigas</i>	07/08/01	Fouras	10	12	0/2
01/231	<i>C. gigas</i>	07/07/01	Fouras	10	12	0/2
01/1246	<i>C. gigas</i>	14/08/01	Fouras	10	13	0/2

Code	Species	Date of sampling	Location	Number of animals	Age (months)	Positive PCR analysis
01/247	<i>C. gigas</i>	14/08/01	Fouras	10	13	0/2
01/248	<i>C. gigas</i>	14/08/01	Fouras	10	13	0/2
01/252	<i>C. gigas</i>	14/08/01	Fouras	10	13	0/2
01/253	<i>C. gigas</i>	14/08/01	Fouras	10	13	0/2
01/254	<i>C. gigas</i>	14/08/01	Fouras	10	13	0/2

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 2001 to 3rd January 2002

Participant no. 2

Medical Research Council (MRC) Virology Unit

Institute of Virology

Church Street

Glasgow G11 5JR

United Kingdom

Phone: +44 141 330 6263

Fax: +44 141 337 2236

Email: a.davison@vir.gla.ac.uk

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 **04-01-01** to **03-01-02**

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)

Coordinator: Dr Tristan Renault
IFREMER - DRV/RA
Laboratoire Génétique et Pathologie
17390 La Tremblade
France
Phone: +33 5 46 36 98 36
Fax: +33 5 46 36 37 51
Email:

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

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 was completed during the previous reporting period. 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.

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 in December 2001. He also wrote this progress report.

Dissemination of results

The work conducted for task 1 is complete and is actively being prepared for publication. A database entry has been prepared for the genome sequence, and will be submitted to Genbank when the paper is finalised.

Sequence data were made available to other participants as required.

SIGNIFICANT DIFFICULTIES OR DELAYS

None have occurred.

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 2001 to 3rd January 2002

Participant no. 3

Eurogentec S.A.

Parc Scientifique du Sart-Tilman

B-4102 Seraing

Belgium

Phone: +32 4 366 61 58

Fax: +32 4 366 51 03

Email: xhonneux@eurogentec.be

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 **04-01-01** to **03-01-02**

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

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

Coordinator: Dr Tristan Renault
IFREMER - DRV/RA
Laboratoire Génétique et Pathologie
17390 La Tremblade
France
Phone: +33 5 46 36 98 36
Fax: +33 5 46 36 37 51
Email: trenault@ifremer.fr

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

INDIVIDUAL PROGRESS REPORT

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

Scientific team : Dr Florence XHONNEUX
Danièle VANDENBERGH
Doriano CINGOLANI
Alain PONCIN
Corinne BOULLE
Nathalie ROPPE
Yves LECOCQ

OBJECTIVES

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

ACTIONS IN THE PROJECT

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

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

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

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

TIMETABLE OF PLANNED RESEARCH ACTIVITIES

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

^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

The progress in this sub-task was delayed.

Introduction

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

Results

1. Production of proteins used for antibody characterization

The antigens used for antibody characterization were non purified antigens. They corresponded to Tn5 insect cells infected by different recombinant baculoviruses: one coding for the ORF43, one coding for the ORF94 and two coding for control proteins (Fig.1).

The first control protein named “T+” is a 25 kDa protein that has the same tags (His₆ and Flag) as the injected OsHV-1 antigens (ORF43 and ORF94), the second one named “T-” is the luciferase (60 kDa) and has no tag. These control recombinant baculoviruses were constructed as described for the ORF43 and ORF94 (see previous report).

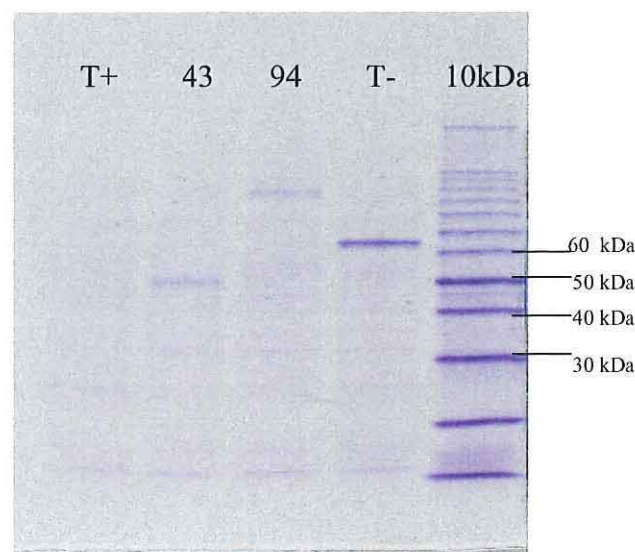


Figure 1. Characterization of the antigens. SDS-PAGE Novex 4-20% stained by Coomassie blue. T+: control protein with tags; 43 and 94: OHV antigens; T-: control protein without Tag.

2. Production and characterization of the polyclonal antibodies

Two rabbits were immunized with each purified OsHV-1 recombinant antigens (ORF94 and ORF43) (see previous report). 100 µg of recombinant antigen were injected per animal and 5 injections were carried out (Days 0, 22, 52, 71 and 113). Bleedings were carried out before all injections (except the second one) and a final bleeding was also performed after the last injection. All the sera were tested and characterized by ELISA and Western blotting.

2.1. ELISA characterization of ORF94 specific antisera

Experimental conditions for ELISA were optimized (see previous report). Each well was coated with 5×10^4 insect cells infected by one of the recombinant baculoviruses. After saturation, several dilutions of the sera collected from rabbits n°539 and n°540 (injected with ORF94) were prepared and plates were incubated overnight at 4°C. Peroxidase linked anti-rabbit antibodies (Amersham NA 934) were used as secondary antibody. Peroxidase revelation was carried out with o-phenylenediamine (OPD) according to standard protocols and the colorimetric response read at 490 nm. ELISA were performed with all the bleedings and against the 4 antigens described in §1 (ORF94 as positive antigen in this case and ORF43, T+ and T- as negative ones).

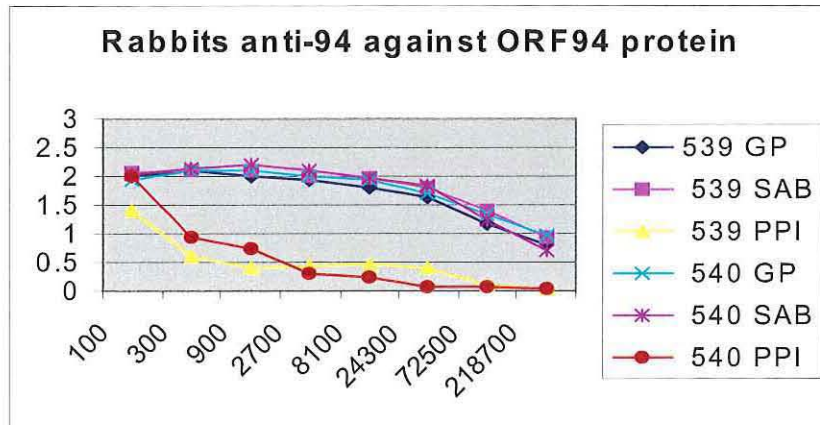


Figure 2. ELISA results with anti-94 polyclonal antibodies against the ORF94 preparation. The rabbits n°539 and 540 were immunized with ORF94 protein. Results are showed for sera taken before injections (PPI), taken after 4 antigen injections (GP) and after 5 injections (SAB).

A standard low background was observed with sera collected before injections (PPI). Serum titers raised after each injection but reached a maximum level after the fourth one. A high background was observed against control proteins. The specificity of the response (Δ OD) was calculated by subtracting the optical densities obtained with the control antigens from the optical densities obtained with the ORF94 antigen (see figure 3). The optimal serum dilutions could be thus determined and were found to be 1000x for bleedings after 2 and 3 antigen injections and 25000x for the sera collected after 4 and 5 antigen injections. The best titers and the best specificity were obtained with the sera taken from the rabbit n°540.

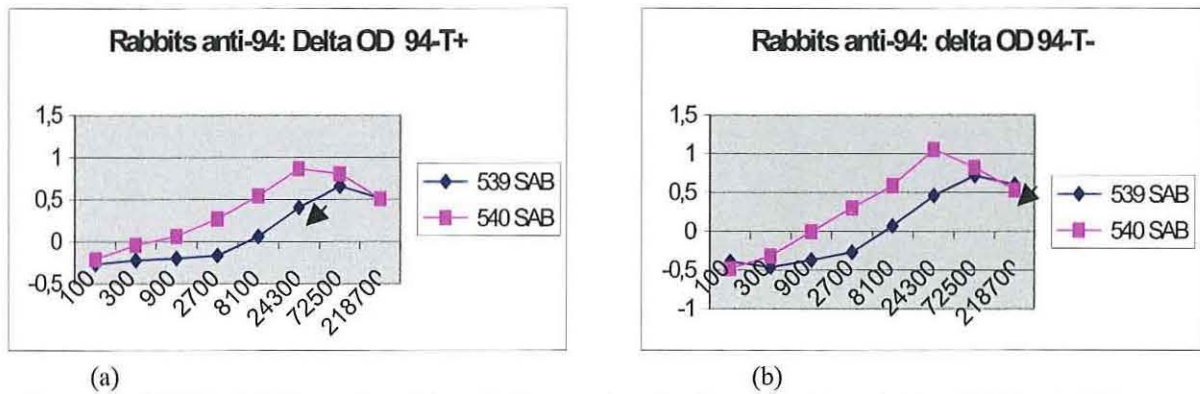


Figure 3. ELISA Δ OD results with anti-94 polyclonal antibodies: The rabbits n°539 and 540 were immunized with ORF94 protein. Are plotted on the graph the Δ OD obtained between ORF94 preparation and T+ (a) or T- (b) control preparations for each dilution of the final bleedings (SAB).

2.2. Western blot characterization of ORF94 specific antisera

The sera collected from rabbits n°539 and n°540 were also tested on western blots prepared with the 4 antigens described in §1 (ORF94 as positive antigen in this case, and ORF43, T+ and T- as negative controls). A commercial anti-Flag antibody was also tested in parallel as a control of the antigens. The equivalent of 2.5×10^3 insect cells ($1 \mu\text{l}$ of culture) infected by each recombinant baculovirus were loaded on Novex 4-20% gels and transferred on Immobilon membranes. After saturation, the antibodies diluted 1000x were kept in contact with the membranes overnight at 4°C . The enzyme conjugated secondary antibodies used were the same as for ELISA (see § 2.1) and were detected by a standard 4-chloro-naphtol method. More sensitive detection by chemiluminescence was also tested but too high background was observed. A good signal at the expected molecular weight for ORF94 was obtained with sera taken from both rabbits (see fig. 4). A background raised with sera taken after the fifth antigen injection. The best experimental conditions were determined as to be an incubation of the western blots with the rabbit sera taken after 4 antigen injections and minimum 1000x diluted.

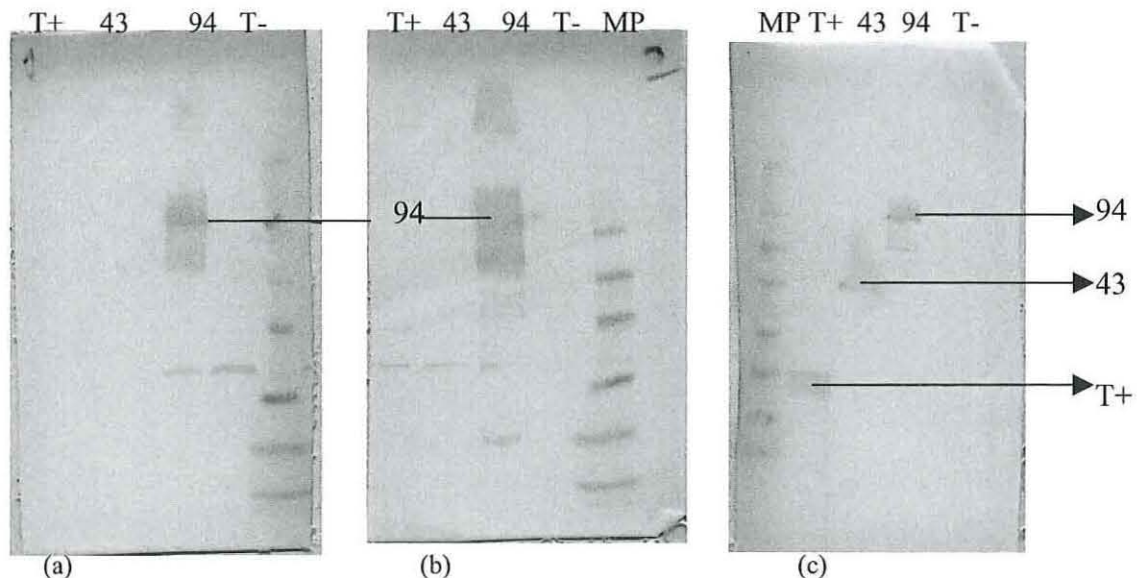


Figure 4. Western-blot analysis of the anti-94 polyclonal antibodies. Antigens blotted: T+ as control protein with tags, 43 and 94 as the OHV antigens and T- as control protein without tag. Sera collected after 4 injections from the rabbits n°539 (a) and 540 (b) immunized with ORF94 protein. Secondary antibodies: anti-rabbit peroxydase linked antibodies (a and b) or anti-Flag monoclonal antibodies (Eurogentec, 1/5000) followed by an anti-mouse peroxydase linked antibody (Amersham NA931) (c).

2.3. ELISA characterization of ORF43 specific antisera

ELISA were performed with all the bleedings of the rabbits n° 541 and 542 (injected with ORF43) and against the 4 antigens described in §1 (ORF43 as positive antigen in this case and ORF94, T+ and T- as negative ones) (see fig.5). The experimental conditions used were the same as described above (see §2.1).

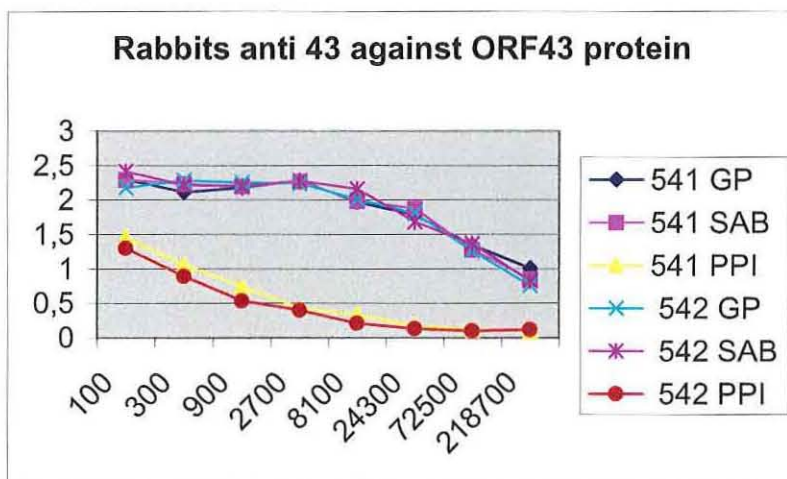


Figure 5. ELISA results with anti-43 polyclonal antibodies against the ORF43 preparation. The rabbits n°541 and 542 were immunized with ORF43 protein. Results are showed for sera taken before injections (PPI), taken after 4 antigen injections (GP) and after 5 injections (SAB).

As previously observed, a standard low background was seen with the bleedings before injections (PPI) and the sera titers raised after each antigen injection until reaching a maximum level after the fourth one. A high background was observed against the control proteins, but more especially against the T+ protein with tags.

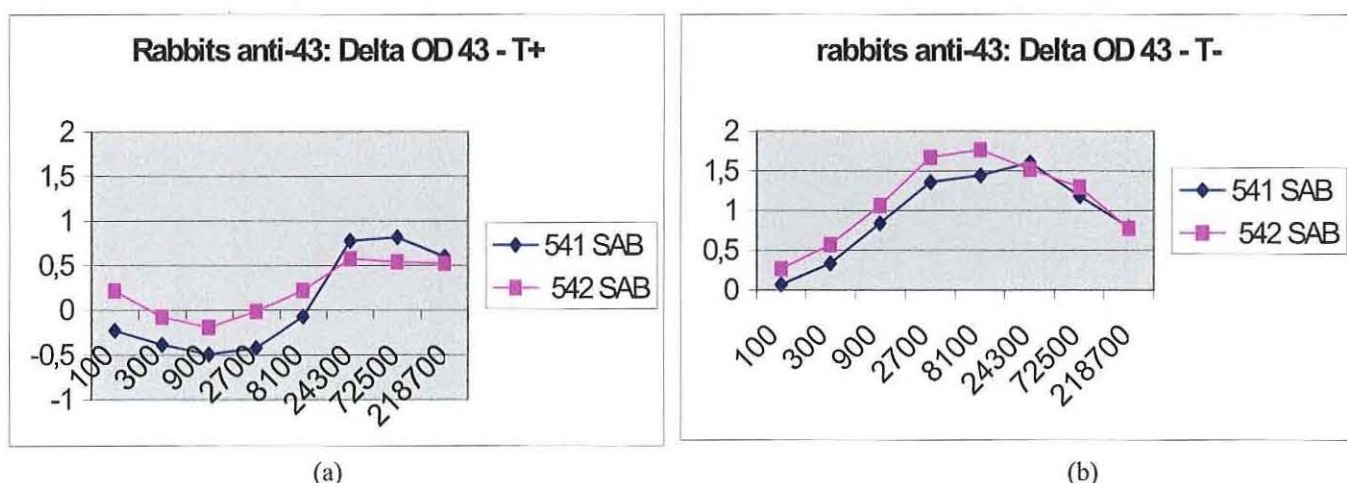


Figure 6. ELISA Δ OD results with anti-43 polyclonal antibodies: The rabbits n°541 and 542 were immunized with ORF43 protein. Are plotted on the graph the Δ OD obtained between ORF43 preparation and T+ (a) or T- (b) control preparations for each dilution of the final bleedings (SAB).

The optical densities obtained against T+ were equivalent to those obtained against the specific ORF43 antigen and were higher than those against ORF94 and T-. This unexpected result suggests the presence of common epitope(s) on T+ and ORF43 but different from His₆ and Flag tags (also present on ORF94). This is accidental and should have no influence on the quality of these antisera for OHV diagnosis use.

The specificity of the response was calculated by subtracting the optical densities obtained with the control antigens from the optical densities obtained with the ORF43 antigen (see figure.6). The optimal sera dilutions could be thus determined and were found to be 500x for bleedings after 2 and 3 antigen injections and 50000x for the sera taken after 4 and 5 antigen injections. The best titers and the best specificity were obtained with the sera taken from the rabbit n°542.

2.4. Western blot characterization of ORF43 specific antisera

The sera taken from rabbits 541 and 542 were also tested on western blots containing the 4 antigens described in §1 (ORF43 as positive antigen in this case, and ORF94, T+ and T- as negative controls). Experimental conditions used were the same as described above (see §2.2). A positive signal at the expected molecular weight for ORF43 was obtained with sera taken from both rabbits (see fig. 7). A cross-reactivity against the T+ protein was also observed as previously done in ELISA (see §2.3.). A raising general background was seen as the number of injections increased.

The best experimental conditions were thus determined as to be an incubation of the western blots with the rabbit sera taken after 4 antigen injections and minimum 1000x diluted.

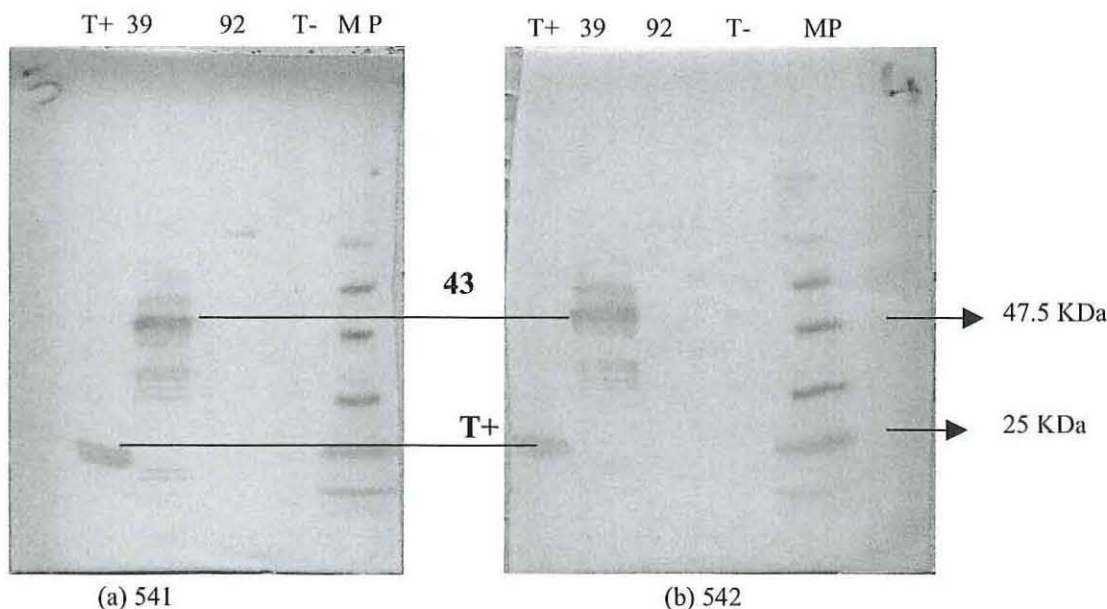


Figure 7. Western-blot analysis of the anti-43 polyclonal antibodies. Antigens blotted: T+ as control protein with tags, 43 and 94 as the OHV antigens and T- as control protein without tag. Sera taken after 4 injections from the rabbits n°541 (a) and 542 (b) immunized with ORF43 protein.

3. Production and characterization of the monoclonal antibodies

3.1. Phase 1: Immunisation and mouse selection for fusion

Four mice were immunized with each purified OsHV-1 recombinant antigen (ORF94 and ORF43) (see previous report). 50 µg of recombinant antigen were injected per animal and 3 injections were carried out at three weeks interval (Days 0, 21 and 42). Bleedings were carried out before all the injections and after the last one. All the sera were tested by ELISA and Western blotting assays in order to determine the mouse to be sacrificed for fusion procedure. Assays were carried out with all the bleedings and against the 4 antigens described in §1 (ORF 94, ORF43, T+ and T-).

ELISA was carried out as described above for the characterization of rabbit antisera. Dilutions of mouse sera were tested from 1000x to 128000x and an anti-mouse peroxidase linked antibody was carried out with the mouse antisera diluted 1000x and an incubation with antigens during 1h at room temperature. The revelation of the enzyme linked secondary antibody (Amersham NA931, 1/5000) was done by chemiluminescence.

The western blots analysis showed a rather high background with all the samples but a specific signal with the expected molecular weight could be detected in each case. The specificity of the responses obtained in ELISA was calculated as described above, by subtracting the optical densities obtained with the control antigens from the optical densities obtained with the immunization antigen. The mouse selection for hybridoma production was done on the basis of the best ratio. A high cross-reaction of the sera anti-ORF43 was obtained against the T+ protein as it was previously showed with the polyclonal antibodies. According to these results, mouse DJ was selected among mice immunized with ORF94 antigen and mouse BL among those immunized with ORF43 antigen.

3.2. Phase 2: Fusion

Selected mice were injected with a fourth dose of antigen (ORF94 and ORF43 respectively) before doing the fusion according to standard protocols. Lymphocytes were isolated from both spleens and fused with mouse myeloma cells (B-lymphocyte tumor, SP2/O-Ag 14, ATCC CRL 8287) by PEG method. Fused cells (hybridoma) were recovered in the classical selective HAT medium. This method of selection is based on the ability of the hybridoma to survive in the HAT medium contrary to the myeloma cells (HGPRT negative) and their division ability contrary to the unfused lymphocytes. This method usually allows the obtention of ~500 hybridoma per spleen. 5-20% of these hybridoma usually secrete antigen-specific antibodies. These clones had to be then selected. Six 96-wells plates were processed.

3.3. Phase 3: Screening of the hybridoma and amplification

All the obtained hybridoma were subcultivated in order to maintain them and to produce enough material for their characterization. The hybridoma that secreted anti-ORF94 and anti-ORF43 antibodies were screened by ELISA. Each culture well was incubated with the non purified ORF94 or ORF43 antigen (see §1) and the T+ protein as control. The specificity of the response (Δ O.D.) was calculated as described above for ELISA by subtracting control optical densities values from the specific ones. A screening was also done in parallel on the basis of the growth of the clones in the appropriate medium.

All the supernatants selected (from clones with good growth) showed a positive Δ O.D. and thus a specific response against injected proteins. After 2 runs, 47 clones still remained among the initial anti-ORF43 hybridoma and only 12 clones among the anti-ORF94 ones. An additional test was performed and 22 anti-ORF43 hybridoma were selected as they showed significant Δ O.D. Only 5 anti-ORF94 hybridoma remained and were selected. Six clones were finally selected on the basis of ELISA and immunochelistry results: 1A10, 2C11 and 3D8 for anti-ORF43 hybridoma and the clones numbers 4A3, 6C8 and 6H8 for anti-ORF 94 hybridoma.

Participant 1 has also tested all these clones (22 anti-ORF43 hybridoma and 5 anti-ORF94 hybridoma) using a immunohistochemistry method as this technique is also intended to be used for OsHV-1 diagnosis. A low specific signal was observed with all the supernatants anti-ORF43 but higher response was observed with the anti-ORF94 ones. The clone numbers 3A10, 4H2 and 5G7 were selected by this method among the anti-ORF43 hybridoma and the clone numbers 5E5, 6C8 and 6H8 were selected among the anti-ORF94 ones.

From all these results, three clones were selected for each antigen to be next subcloned and fully characterized. The clones 1A10, 3A10 and 4H2 were chosen among the anti-ORF43 hybridoma and the clones 5E5, 6C8 and 6H8 among the anti-ORF94 ones. These were the clones that gave the best response in ELISA and/or immunohistochemistry.

3.4. Phase 4: Subcloning of the selected hybridoma and characterization

The six selected hybridoma at this stage were not pure, some of them contained several clones yet as the technique used for the screening does not allow a real cloning of the hybridoma. The next step was to isolate monoclonal antibody-producing hybridoma cell lines by "limiting dilution cloning". The culture supernatant of each single colony obtained was checked by ELISA as described above against the ORF94 or ORF43 antigen and the T+ protein as control. Positive clones were subcloned again until all the supernatants of the individual next subclones showed an equally positive antibody response.

One subclone was then selected for each initial hybridoma except for the clone 3A10 (anti-ORF43) for which two subclones (3A10G6 and 3A10H7) were selected. All the subclones were then characterized by ELISA and Western blotting as described below. The isotype of the secreted antibodies was also determined for each of the subclones and all the selected antibodies were immunoglobulins G2A kappa.

3.4.1. Characterisation of the anti-ORF43 monoclonal antibodies

3.4.1.1. ELISA results

ELISA were performed as described above (see §3.1) (MnAb 1h30 RT) with the 4 selected subclones and against the 4 antigens described in §1. Background observed against the control proteins was low ($OD_{490} < 0.3$) and the specific response against the ORF43 antigen was very high even at the highest dilutions of the antibodies (see figure 8). No significant difference could be seen between the different antibodies.

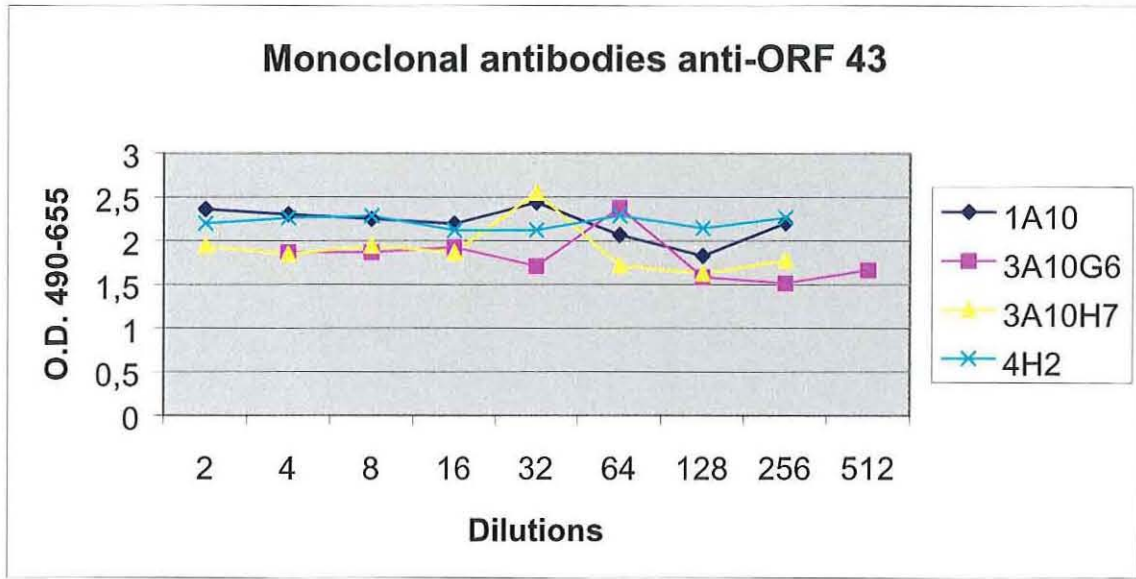


Figure 8. ELISA results with the anti-43 monoclonal antibodies against the ORF43 preparation. Mabs 1A10, 3A10G6, 3A10H7 and 4H2

3.4.1.2. Western-blotting results

Western-blotting were performed as described above (MAb 1/5 ovn 4°C) and against the 4 proteins described in §1.

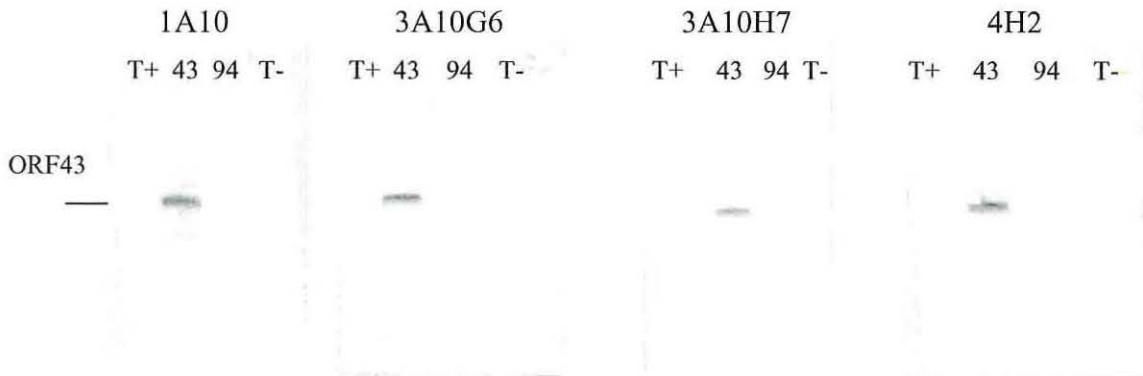


Figure 9. Western-blot analysis of the anti-ORF43 monoclonal antibodies. Antigens blotted: T+ as control protein with tags, 43 and 94 as the OHV antigens and T- as control protein without tag. Mabs 1A10, 3A10G6, 3A10H7 and 4H2. 4-chloro-naphtol detection.

A specific response against the ORF43 could be seen with the 4 monoclonal antibodies and no background was observed against the control proteins (see Figure 9).

3.4.2. Characterisation of monoclonal antibodies anti-ORF94

3.4.2.1. ELISA results

ELISA assays were carried out as described above (§3.4.1.1.) with the 3 selected subclones.

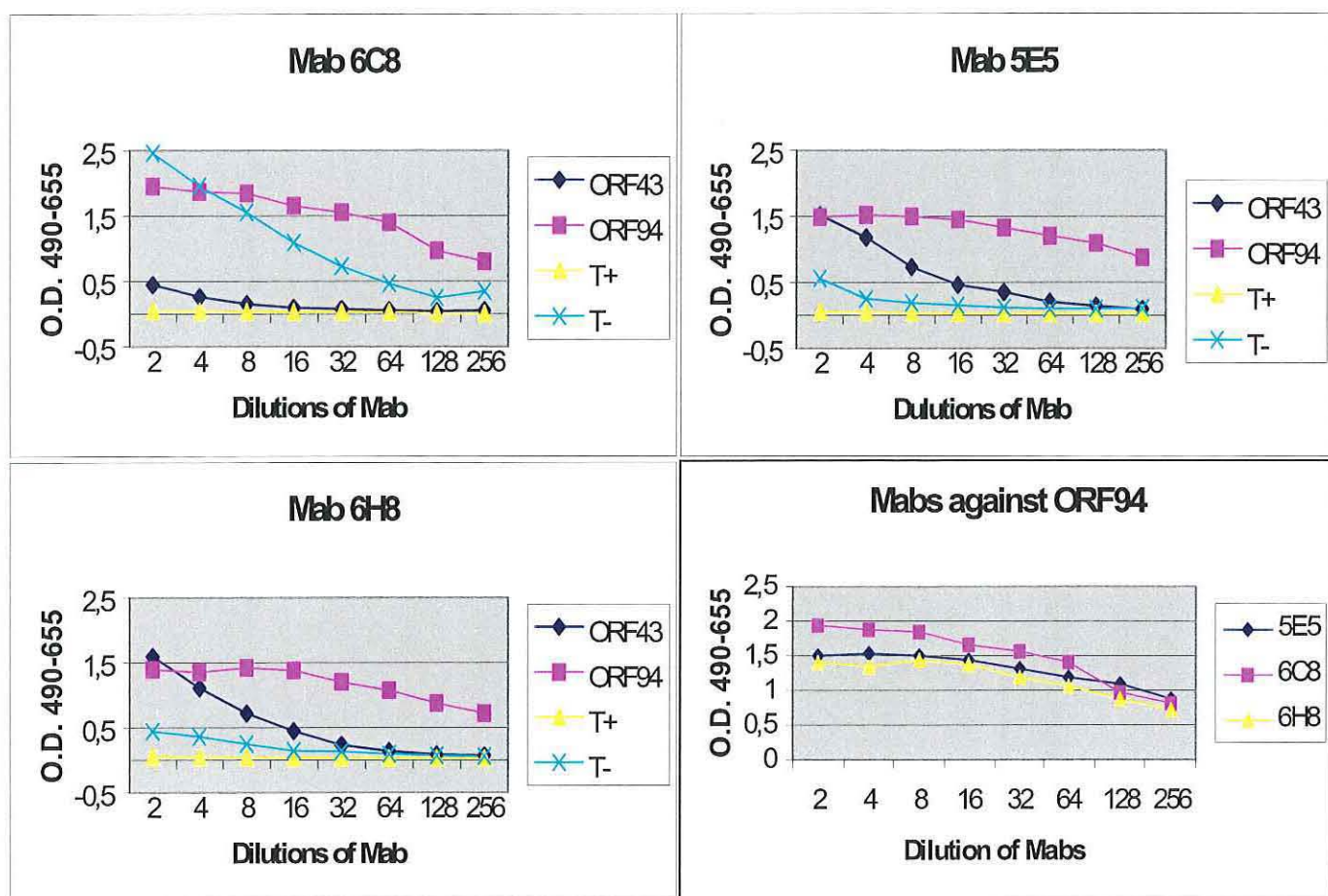


Figure 10. ELISA results with the anti-94 monoclonal antibodies against the ORF94 and controls preparations

Background was observed against the ORF43 and T- control proteins but at the highest concentrations of the 3 monoclonal antibodies. No background could be seen against the T+ control protein certainly because this protein was chosen as discrimination control in the screening of hybridoma. All Mabs recognized ORF94 and the response of Mabs 5E5 and 6H8 was significant from the 30x dilution. The Mab 6C8 showed higher background against T- antigen. The best conditions for a specific anti-OHV response are to use Mabs 5E5 and 6H8 30x diluted.

3.4.2.2. Western-blotting results

Western-blot assays were performed as described above (§ 3.4.1.2.).



Figure 11. Western-blot analysis of the anti-94 monoclonal antibodies. Antigens blotted: T+ as control protein with tags, 43 and 94 as the OHV antigens and T- as control protein without tag. Mabs 5E5, 6C8 and 6H8. Chemiluminescence detection.

Background was observed with the 3 monoclonal antibodies (except against the T+ protein as in ELISA) and no specific response could be obtained in these experimental conditions.

Conclusions

1. Polyclonal antibodies

Anti-OsHV-1 specific rabbit antisera could be obtained in this study and could be used for OsHV-1 diagnosis development. The best experimental conditions were defined for their use in ELISA and Western blot analysis (see frame below).

Rabbit polyclonal antibodies anti-ORF94:

In ELISA: Sera from rabbit n°540 more specific than those from n°539
Sera after 2 and 3 antigen injections, to be used 1000x diluted
Sera after 4 and 5 antigen injections, to be used 25 000x diluted

In Western-blotting:

Sera from rabbit n°539 equivalent to those from n°540
Sera after 4 antigen injections selected and to be used 1000x diluted or more
4-Chloro-naphtol detection selected (no chemiluminescence)

Rabbit polyclonal antibodies anti-ORF43:

In ELISA Sera from rabbit n°542 more specific than those from n°541
Sera after 2 and 3 antigen injections, to be used 500x diluted
Sera after 4 and 5 injections, to be used 10 000x diluted

In Western-blotting:

Sera from rabbit n°541 equivalent to those from n°542
Sera after 4 antigen injections selected and to be used 1000x diluted or more
4-Chloro-naphtol detection selected (no chemiluminescence)

2. Monoclonal antibodies

A good immunization of the mice was obtained with both OsHV-1 antigens and 7 clones producing anti-OsHV-1 specific monoclonal antibodies could be isolated and characterized by ELISA and Western blot analysis (see frame below). All clones produce immunoglobulins IgG2A, kappa.

Mab anti-ORF43:

- Clones 1A10, 3A10G6, 3A10H7 and 4H2
- High specific response in ELISA
- High specific response in Western blot analysis

Mab anti-ORF94:

- Clones 5E5, 6C8 and 6H8
- Less specific response in ELISA than with the anti-ORF43 Mabs.
- 5E5 and 6H8 supernatants to be used in ELISA 30x diluted
- No experimental conditions defined for Western blot analysis

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

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

Dr D. Vandendenbergh attended and presented results at the annual meeting in December 2001 held at the IFREMER headquarters, Paris. Dr D. Vandenberg and Dr F Xhonneux wrote this final 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 two month delay in monoclonal antibody production will be observed. They were obtained in June 2001. This delay corresponds to a delay in the beginning of the sub-task 2.2. However, subcloned hybridoma supernatants have been resent to Participant 1 in October 2001 (with a 6 month delay).

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 2001 to 3rd January 2002

Participant no. 4

Université de Bretagne Occidentale

Unité de Culture Cellulaire, ISSS, Hôpital Morvan

5, avenue Foch, 29609 Brest

France

Phone : +33 2 98 01 81 16

Fax. : +33 2 98 01 81 23

Email: germaine.dorange@univ-brest.fr

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 **04-01-01** to **03-01-02**

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

Coordinator: Dr Tristan Renault
IFREMER - DRV/RA
Laboratoire de Génétique et Pathologie
17390 La Tremblade
France.
Phone : +33 5 46 36 98 36
Fax : +33 5 46 36 37 51
email: trenault@ifremer.fr

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

INDIVIDUAL PROGRESS REPORT

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

Scientific team : Dr Germaine DORANGE
Dr Hélène TALARMIN
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

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

RESEARCH ACTIVITIES DURING THE REPORTING PERIOD

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

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

Several assays of herpes-like virus cultivation in oyster primary cultures have been carried out in 1999 and 2000. Virus cultivation experiments were carried out in primary cultures of embryonic oyster cells. No obvious cytopathic effect was detected. However, PCR analysis allowed to detect amplicons presenting the expected sizes. Moreover, *in situ* hybridization assays demonstrated positive labeling at the cellular level. Experiments must be reiterated and other techniques such as transmission electron microscopy used in order to demonstrate the presence of viral particles in primary cell cultures.

Although promising results were observed, primary cultures of oyster cells are not at this time a reliable tool to detect herpesviruses in bivalves.

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

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

Dr H. Talarmin has participated to the third annual meeting in Paris (France) in December 2001.

SIGNIFICANT DIFFICULTIES OR DELAYS

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. Obtaining herpes-like virus multiplication in oyster primary cell cultures determine the feasibility of Sub-task 3. 3.

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. 5

University College Cork

Department of Zoology and Animal Ecology

National University of Ireland

Lee Maltings, Prospect Row, Cork

Ireland

Phone : +353 21 904187

Fax. : +353 21 270562

Email: s.culloty@ucc.ie

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
(50.5%)

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
IFREMER - DRV/RA
Laboratoire de Génétique et Pathologie
17390 La Tremblade
France.
Phone : +33 5 46 36 98 36
Fax : +33 5 46 36 37 51
email: trenault@ifremer.fr

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

INDIVIDUAL PROGRESS REPORT

Participant no. 5: University College Cork, Ireland

Scientific team: Dr Sarah Culloty
Prof. Maire Mulcahy
Ms Eileen Dillane
Ms Elizabeth Cotter
Mr Brian Farrell

OBJECTIVES

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

ACTIONS IN THE PROJECT

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

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

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

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

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

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

PLANNED RESEARCH ACTIVITIES

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

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

RESEARCH ACTIVITIES DURING THE FIRST REPORTING PERIOD

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

1. Collection of Samples /Sites

Bivalve samples were again obtained in Year 3 (Jan-Dec 2001) from Cork harbour, Galway bay, Bannow bay and a hatchery in Co. Sligo (Figure 1). Samples were taken between March and September 2001. Native (*O. edulis*) and Pacific oysters (*C. gigas*) up to one year old were obtained from Cork harbour along with adult mussels (*M. edulis*). Adult *O. edulis* were obtained from Galway bay. Pacific oyster (*C. gigas*) spat and Manila clam (*R. philippinarum*) spat were obtained from a hatchery in Sligo. Pacific oysters of all sizes were obtained from Bannow bay in September 2001 (from 30 g up to market size). These collections took place following heavy mortalities (over 90%) in these stocks. The oysters from Bannow bay were taken from two farms that were experiencing mortalities of up to 90% and from one farm that did not experience mortalities. No abnormal mortalities were observed in any of the other sites throughout 2001.

2. Bivalve samples

All individuals were divided in half – one half frozen at -20°C for PCR analysis and one half fixed in Davidsons fixative. The half fixed in Davidsons were subsequently processed for histology and four sections taken from each block – one for routine histology, one for *in-situ* hybridisation and two for immunological staining.

3. Histology

During 2001 the screening of all slides of tissue sections taken from 1999-2001 was completed. All tissue sections from Years 1-3 were screened to monitor any morphological abnormalities (a total of 1983 slides). Of these, 71 bivalves appeared to show some abnormalities associated with nuclei of connective tissue cells (Figures 2a and 2b, Table I). The largest percentage of abnormal sections were observed during Year 1 (7.0% of the total sample).

Subtask 3.1 Application of molecular methods for the diagnosis of oyster herpes like virus carrying out analysis of oyster samples originating from Ireland.

1. Screening by PCR

1.1. PCR - Validation

Viral DNA – 10 ng of the viral genome 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 and all dilutions can be detected (Figures 3a and 3b).

1.2. PCR Ring Trial

Thirty frozen extracted samples were sent by the Co-ordinator in February 2002. These samples were screened for PCR using both the primer pair OHV1/OHV2 (C5/C13) and OHV3/OHV114 (C2/C6). Results of this screening are presented in Table II. 18/30 positive results were obtained following screening using the primer pair OHV1/OHV2 and 17/30 following screening using OHV3/OHV114.

1. 3. Screening of bivalves samples from Ireland during 2001 by PCR

All samples from 2001 were negative when screened using PCR. Screening of all samples from February 2000 on was carried out by PCR using the primer pair OHV1/OHV2 (1625 samples in total). All subsequent Irish samples taken from then until the end of the project were found to be negative when screened with this method (Table I).

2. Screening using In situ hybridisation

2. 1. Validation of direct and indirect methods of in situ hybridisation

Thirty slides were subsequently obtained from the Co-ordinator in Autumn 2001. These were screened using the indirect method, however problems were observed in the integrity of the slides possibly due to the amount of time spent in proteinase K. The time for treatment with proteinase K was reduced to 15 minutes and the remaining slides screened in this group (Table IIIa, Figure 4b).

A reference positive block was obtained in December 2001. As results of some slides were unclear it was decided that all partners would rescreen 30 blocks (coded VB1-30) using the direct method furnished by the Coordinator in December 2001. The results of this analysis is presented in Table IIIb.

2. 2. Screening of Irish bivalves for herpes like virus using ISH

Sections of all oysters were cut for *in situ* hybridisation (1983 in total). However due to the absence of any positive samples among the Irish samples following screening by PCR and it was decided at a yearly meeting of the partners that subsequent screening by ISH was of little use when samples were negative for PCR. Only samples showing abnormalities following screening of tissue sections or positive for PCR were to be screened. No bivalves from the Irish sites showed positive results by PCR. However a sample of slides were screened using ISH – these were slides that showed some morphological abnormalities when tissue sections were screened. Results of this screening are presented in Table I – of the small number screened all were negative following the indirect method for ISH.

Sub-task 3.2 Application of immunological methods

MAB not available until February 2002 - no funding available to carry out this analysis.

Sub-task 3.3 Application of primary oyster cell cultures

No cell cultures were available for diagnosis during the project.

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 last partner meeting at IFREMER headquarters in December 2001. She also wrote this third yearly report.

SIGNIFICANT DIFFICULTIES OR DELAYS

Monoclonal antibody not available until February 2002.

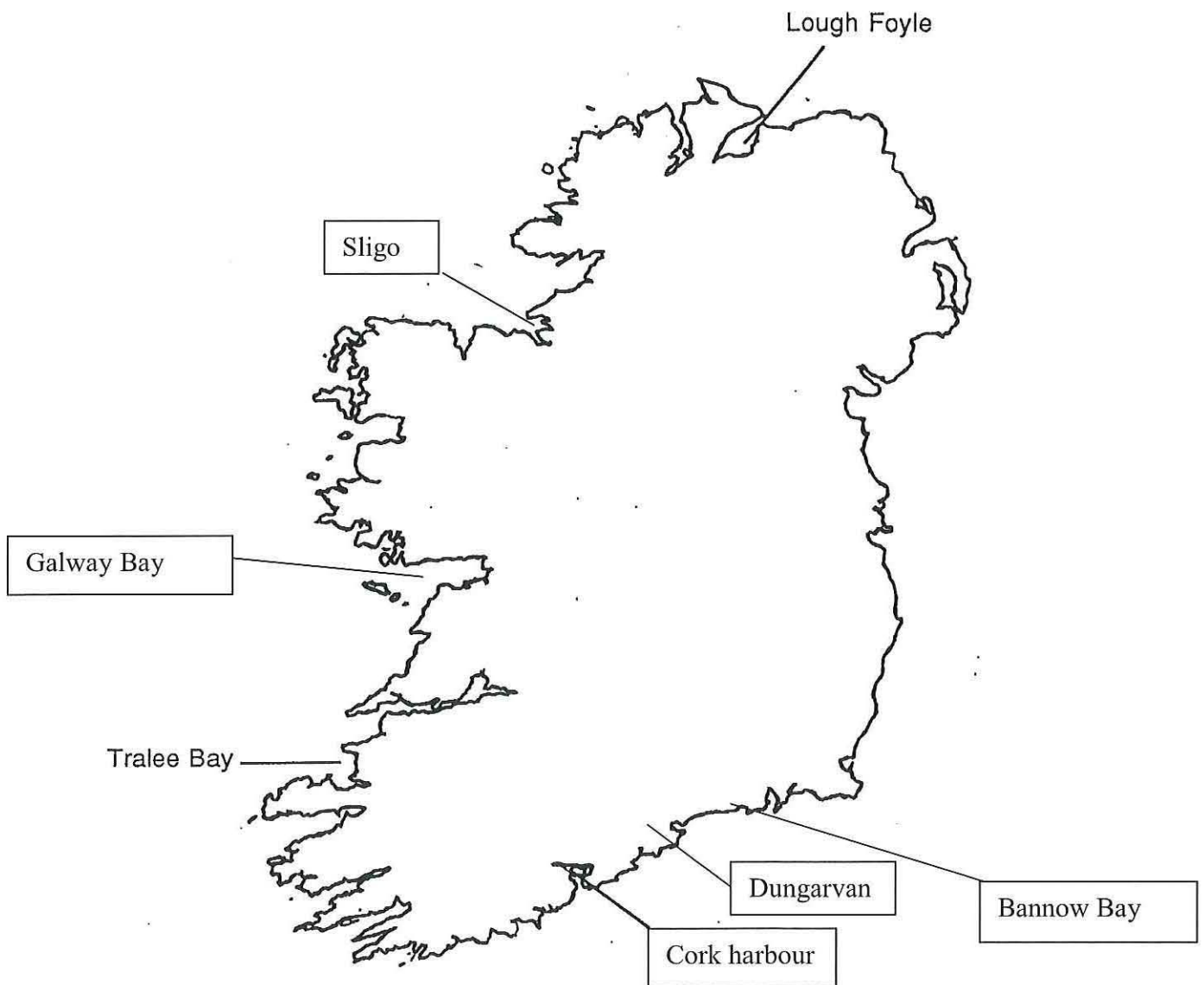
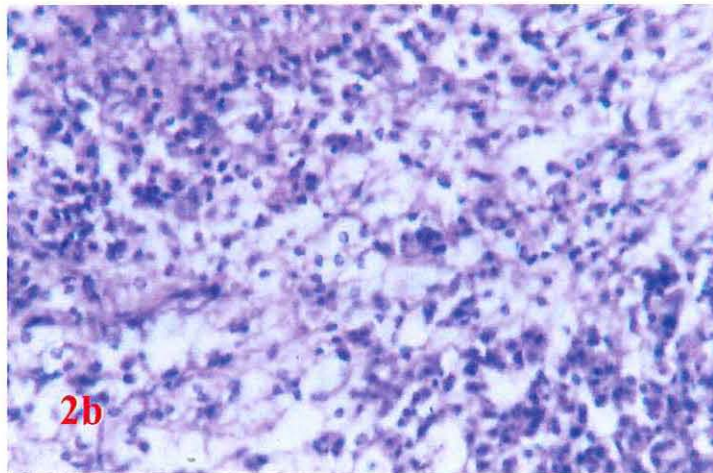
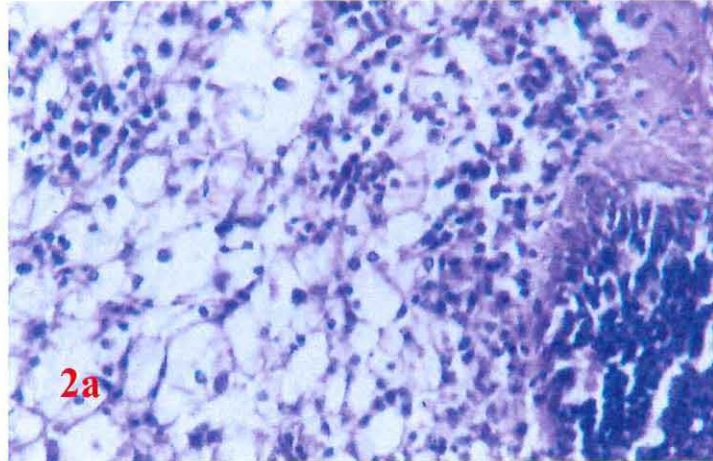


Figure 1. Some major oyster growing areas in Ireland along with study sites (boxed)



Figures 2a and 2b. Abnormalities observed in connective tissue cells in *C. gigas* from Ireland

Table I. Summary of all populations screened during the project (January 1999 - January 2002) and results of analysis

Year	Site	Species	Age	Origin	Date	Morts at site	N	Histology *	PCR **	ISH ***
1999	Cork harbour	<i>C. gigas</i>	< 12 months	Sligo, IE	13-juil	No	30	2	Neg.	Neg.
	"	"	"	"	27-juil	"	30	0	"	
	"	"	"	"	03-août	"	30	0	"	
	"	"	"	"	17-août	"	30	4	"	Neg.
	"	"	"	"	30-août	"	30	0	"	
	"	"	"	"	30-août	"	29	0	"	
	"	"	"	"	23-nov	"	30	0	"	
	Dungarvan (PH)	<i>C. gigas</i>	< 12 months	Guernsey (15/06/99)	25-août	80% morts	30	8	"	Neg (3)
	Dungarvan (PC)	"	"	Seasalter (03/99)	25-août	"	29	7	"	Neg (2)
	"	"	"	"	12-nov	No	30	0	"	
	"	"	"	French pop. (11/99)	12-nov	"	30	0	"	
	"	"	"	Guernsey	12-nov	"	30	4	"	

Year	Site	Species	Age	Origin	Date	Morts at site	N	Histology *	PCR	ISH
2000	Cork harbour	<i>C. gigas</i>	<12 months	Sligo	13-févr	No	30	0	"	"
	"	"	"	"	17-juil	"	30	0	"	"
	"	"	"	"	27-juil	"	30	0	"	"
	"	"	"	"	04-août	"	30	0	"	"
	"	"	"	"	08-août	"	30	0	"	"
	"	<i>C. gigas</i>	"	"	24-août	"	30	1	"	"
	"	"	"	"	26-nov	"	30	0	"	"
	"	<i>O. edulis</i>	<18 months	Cork harbour	13-févr	"	30	0	"	"
	"	"	<12 months	"	18-juil	"	30	0	"	"
	"	"	"	"	27-juil	"	30	2	"	"
	"	"	"	"	04-août	"	20	0	"	"
	"	"	"	"	08-août	"	28	0	"	"
	"	"	"	"	24-août	"	30	0	"	"
	"	"	"	"	26-nov	"	30	0	"	"
	Dungarvan	<i>C. gigas</i>	<12 months	English hatchery	27-févr	"	30	0	"	"
	"	"	"	French hatchery	"	"	30	0	"	"
	"	"	"	"	"	"	30	0	"	"

"	"	"	English hatchery	02-août	"	30	0	"
"	"	"	French hatchery	"	"	30	10	"
"	"	"	"	"	"	30	4	"
"	"	"	English hatchery	24-août	"	30	10	"
"	"	"	French hatchery	"	"	30	4	"
"	"	"	"	"	"	30	0	"
"	"	"	English hatchery	26-nov	"	30	0	"
"	"	"	French hatchery	"	"	30	0	"
"	"	"	French hatchery	"	"	30	0	"
Galway Bay	<i>O. edulis</i>	Adults	Clarenbridge outer	11-août	"	30	4	"
"	"	"	Clarenbridge inner	"	"	30	0	"
"	"	"	"	"	"	30	0	"
"	"	"	"	25-août	"	30	0	"
"	"	"	"	27-sept	"	30	0	"

Year	Site	Species	Age	Origin	Date	Morts at site	N	Histology *	PCR ISH	
2001	Cork harbour	<i>C. gigas</i>	<12 months	Sligo	24-avr	No	30	6	Neg	
		"	"	"	24-mai	"	30	0	"	
		"	"	"	25-juin	"	30	0	"	
		"	"	"	10-juil	"	30	0	"	
		"	"	"	24-juil	"	30	1	"	
	"	"	"	"	03-sept	"	30		"	
	Cork harbour	<i>O. edulis</i>	<24 months	Cork harbour	24-avr	"	"	30	2	"
		"	"	"	24-mai	"	"	30	0	"
		"	"	"	25-juin	"	"	30	1	"
		"	"	"	10-juil	"	"	30	1	"
		"	"	"	24-juil	"	"	"	0	"
	Cork harbour	<i>M. edulis</i>	Adults	"	"	24-mai	"	30	0	"
		"	"	"	"	25-juin	"	30	0	"
	Galway Bay	<i>O. edulis</i>	"	Clarenbridge outer	01-mars	"	"	0	"	
	Sligo	<i>T. semid.</i>	Spat	Sligo	10-juil	"	"	30	0	"
"		"	"	13-août	"	"	30	0	"	
<i>C. gigas</i>		"	"	10-juil	"	"	30	0	"	

Bannow bay	<i>C. gigas</i>	Adults	? Farmer 1 30-50g	04-sept	Yes (>90%)	15	0	"
"	"	"	F. 1 50-70g	"	"	15	0	"
"	"	"	F. 1 80-100g	"	"	30	0	"
"	"	"	F. 1 Triploids	"	"	15	0	"
"	"	"	F. 2 small	"	No	12	0	"
"	"	"	F. 2 mid sized	"	"	10	0	"
"	"	"	F. 2 market sized	"	"	11	0	"
"	"	"	F. 3 30-40g	"	Yes (>90%)	26	0	"
"	"	"	Mid-market sized	"	"	29	0	"
"	"	"	Market sized	"	"	34	0	"

* Tissue sections were screened for each bivalve sampled. The numbers in this column relate to the number of tissue sections observed with abnormalities within that sample.

** PCR was carried out on every individual animal that was sampled.

*** ISH was carried out on a small number of tissue sections only as all PCR screening was negative.



Figure 3a. Serial dilutions of viral DNA to determine specificity of PCR. (1- 2500 copies, viral DNA, 2 - 10 ng, 3 - 1 ng, 4 - 100 pg, 5 - 10 pg, 6 - H₂O, 7 - Molecular marker, 8 - 500 fg, 9 - 10 ng, 10 - 1 ng, 11 - 100 pg, 12 - 10 pg, 13 - H₂O. Samples 1-6 screened using Taq in freezer for 2 months, 8-13 screened with fresh Taq – both Eurogentec)



Figure 3b. Screening for efficacy of different Taq and primer pairs - 10 ng viral DNA used in all tubes (1 - Molecular marker, 2 - H₂O/Eurogentec Taq 3 - Viral DNA/Eurogentec Taq/OHV1-OHV2, 4 - Viral DNA/Promega pfu DNA polymerase 3u/μl/OHV1-OHV2, 5 - Stratgene native pfu polymerase 2.5 u/μl/viral DNA/OHV1-OHV2, 6 - Viral DNA/Promega Taq polymerase 5u/μl/OHV1-OHV2, 7 - Viral DNA/Promega Taq polymerase 5u/μl/OHV1-OHV2, 8 - Viral DNA/Eurogentec Taq/OHV3-OHV114, 9 - H₂O/Eurogentec Taq/OHV1-OHV2)

Table II. Comparison of screening of 30 samples (15 larval and 15 spat) by PCR using two primer pairs - supplied by co-ordinator

Sample Number	OHV1/OHV2	OHV3/OHV114
H ₂ O	-	-
1	-	-
2	-	-
3	-	-
4	-	-
5	-	-
6	-	-
7	-	-
8	-	-
9	-	-
10	-	-
11	-	-
12	+	+
13	+	+
14	+	+
15	+	+
16	+	+
17	+	+
18	+	+
19	+	+
20	+	+
21	+	+
22	+	+
23	+	+
24	+	+
25	+	+
26	+	+
27	+	-
28	-	-
29	+	+
30	+	+
H ₂ O	-	-
10 pg	+	+
10 ng	+	+

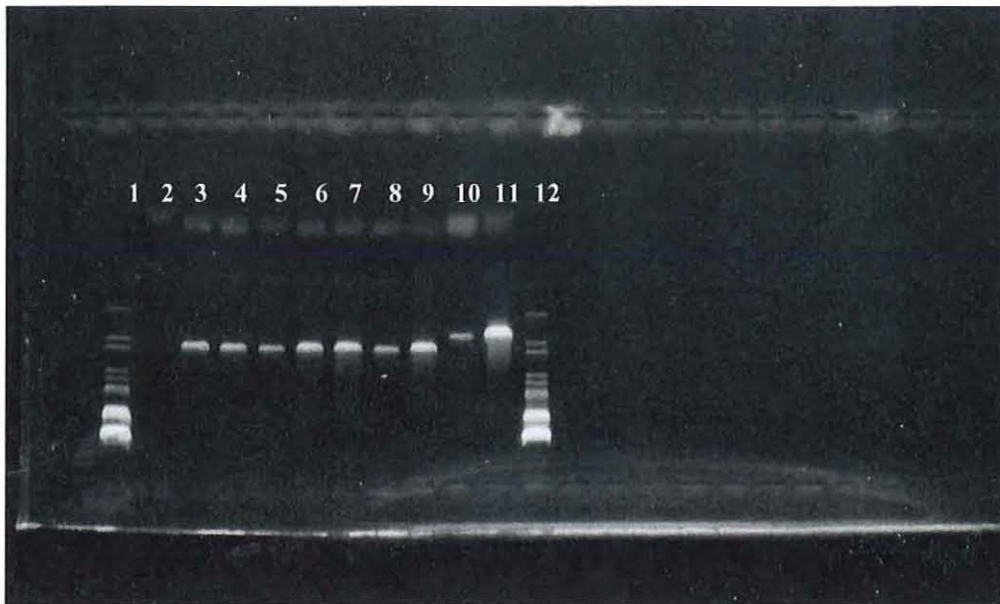


Figure 4a. Preparation of DIG labelled probe. (1 - Molecular marker, 2 - H₂O, 3 to 9 - DIG labelled probe, 10 - 1 pg, 11 - 10 ng, 12 - Molecular marker)

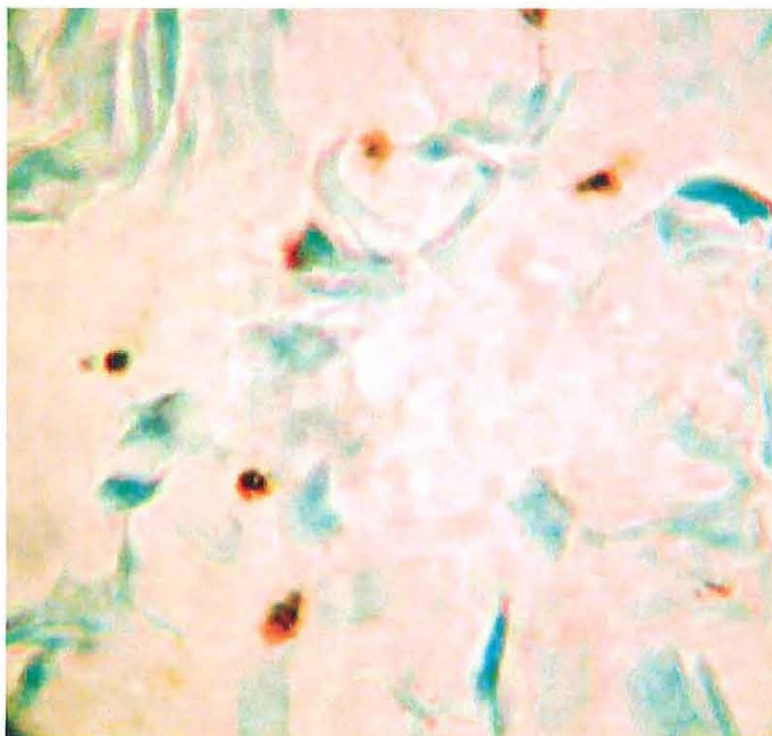


Fig. 4b. ISH - indirect method

Table IIIa. Screening of 30 reference slides by direct and indirect method for ISH

Sample	Indirect method	Direct method*
94/215 6	-	-
94/215 9	?	-
94/215 10	?	+
94/215 11	+	+
94/248 1	+	+
94/248 5	-	+
94/248 7	+	+
94/248 13	+	+
94/248 15	+	-
94/250 4	+	+
94/250 13	-	-
94/250 17	-	-
94/250 18	+	-
94/250 19	+	+
94/250 23	+	-
94/278 7	-	+
94/278 8	-	-
94/278 10	+	+
94/278 11	+	+
94/278 13	-	+
95/54 7	-	-
95/54 8	-	-
95/54 11	-	-
95/54 12	-	-
95/54 13	-	+
95/54 14	-	-
95/54 18	-	-
95/54 20	-	-
95/54 26	-	-
95/54 28	?	-
95/54 30	-	-

* Reference number was concealed before staining took place

Table IIIb. Staining of reference slides (VB1-30) by direct method

Sample	Results
VB1	?
VB2	+
VB3	+
VB4	+
VB5	+
VB6	+
VB7	
VB8	-
VB9	+
VB10	+
VB11	+
VB12	+
VB13	+
VB14	+
VB15	+
VB16	+
VB17	+
VB18	+
VB19	+
VB20	+
VB21	+
VB22	+
VB23	+
VB24	+
VB25	+
VB26	+
VB27	-
VB28	-
VB29	+
VB30	-

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. 6

Instituto de Investigaciones Marinas (CSIC)

Eduardo Cabello, 6

36208 Vigo

Spain

Phone : +34 986 231930

Fax. : +34 986 292762

Email: virus@iim.csic.es

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 **04-01-01** to **03-01-02**

Type of contract: Shared-cost research project

Total cost: 1,283,805.5 ECU
(50.5%)

EC contribution: 649,738 ECU

**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
IFREMER - DRV/RA
Laboratoire de Génétique et Pathologie
17390 La Tremblade
France.
Phone : +33 5 46 36 98 36
Fax : +33 5 46 36 37 51
email: trenault@ifremer.fr

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

INDIVIDUAL PROGRESS REPORT

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

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

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

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

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

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

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

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

PLANNED RESEARCH ACTIVITIES

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

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

RESEARCH ACTIVITIES DURING THE REPORTING PERIOD

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

1. Collecting bivalve 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.

2. Bivalve samples

The individuals of each batch were removed from the shell, sagittally sectioned, then half of the animal was frozen at -80 °C and the other half was fixed in Davidson for histopathological 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 third was fixed directly in osmium tetroxide for electron microscopy.

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

1. Screening by PCR

1. 1. PCR - Validation

Viral DNA (10 ng of the viral genome) 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 and all dilutions can be detected.

1. 2. PCR Ring Trial

We have conducted a PCR reaction using the primers OHV1/OHV2 (C5/C13) and OVH3/OHV114 (C2/C6) with thirty DNA samples distributed by the coordinator to all participants. Table 3 shows the the results of these PCRs. Figure 1 also shows the agarose gels with the analyzed samples

1. 3. PCR analysis of the samples from Spain collected in 2001

We have analyzed by PCR all the bivalves samples taken in 2001 using two primer pairs (OVH3- OHV114 and OHV1-OHV2). We also amplified the bivalves samples with universal primers in order to check the DNA isolation procedure. All the oysters batches analyzed were negative in the specific PCR for herpes virus, although samples DNA was successfully amplified with the conserved primers.

One clam batch from the sampling of the second year (2000), which was positive with the primers OHV3/OHV114 was also analyzed by PCR using the other primer pair. Moreover, the positive product was sequenced. The results of this study showed that the clam sample was negative using the second primer pair. The sequence of the product OHV3/OHV114 did not correspond to herpesvirus.

2. Screening by In situ hybridization

2. 1. In situ hybridisation Ring Trial

Three slides each of 31 coded OsHV positive and negative samples were supplied by Participant 1, to be tested by ISH. The protocol based on a peroxidase/DAB detection system (indirect detection) was used. Nineteen slides were considered to be definitely positive for OsHV, with an additional three considered possible positive (Table 4).

Sub-task 3.2 Application of immunological methods

MAB not available until February 2002 - no funding available to carry out this analysis.

Sub-task 3.3 Application of primary oyster cell cultures

No cell cultures were available for diagnosis during the project.

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

Participation in annual meetings and in writing intermediate and final reports

Dr B. Novoa and Dr A. Figueras attended and presented results at the annual meeting in December 2001 held at the IFREMER headquarters, Paris. Dr B. Novoa wrote this final report.

SIGNIFICANT DIFFICULTIES OR DELAYS

MAB not available until February 2002 and no cell cultures were available for diagnosis during the project.

Third year sampling

Table I. Sampling of *Crassostrea gigas* and *Ostrea edulis* in 2001

Number	Date	Origin	Species	Stage	PCR Primers (OVH3/OHV114 and OHV1/OHV2)
1-01	Feb. 7/01	Ría Vigo	<i>O. edulis</i>	Juvenile	-
2-01		(America Beach)			-
3-01	Mar. 10/01	Ría Vigo	<i>O. edulis</i>	Adult	-
4-01		(America Beach)			-
5-01	Apr. 19/01	Ría Pontevedr (Raxo)	<i>C. gigas</i>	Adult	-
6-01					-
7-01	Apr. 25/01	Hatchery	<i>C. gigas</i>	Larvae	-
8-01					-
11-01	May. 9/01	San Vicente Barquera	<i>C. gigas</i>	Juvenile	-
12-01					-
13-01	Jun. 15/01	Ría Arousa	<i>C. gigas</i>	Adult	-
14-01		(Cambados)			-
15-01	Aug. 9/01	Hatchery	<i>C. gigas</i>	Larvae	-
16-01					-
17-01	Sep. 3/01	Ría Vigo	<i>O. edulis</i>	Adult	-
18-01		(Domaio)			-
19-01	Oct 7/01	Hatchery	<i>C. gigas</i>	Juvenile	-
20-01					-

Table II. Sampling of *Ruditapes decussatus*, *R. philippinarum* and *V. pullastra* in 2001

Number	Date	Origin	Species	Stage	PCR Primers (OVH3/OHV114 and OHV1/OHV2)
21-01	Apr. 23/01	Hatchery	<i>R. decussatus</i>	Larvae	-
22-01					-
23-01		Hatchery	<i>R. philippinarum</i>	Juvenile	-
24-01					-
25-01	May. 5/01	Ría Pontevedra	<i>R. decussatus</i>	Adult	-
26-01		(A meixal)			-
27-01		Hatchery	<i>R. philippinarum</i>	Juvenile	-
28-01					-
29-01	May. 20/01	Ortigueira	<i>R. decussatus</i>	Adult	-
30-01					-
31-01	Jul. 10/01	Hatchery	<i>R. decussatus</i>	Larvae	-
32-01					-
33-01	Aug. 7/01	Ria Arousa	<i>R. decussatus</i>	Adult	-
34-01		(Carril)			-
35-01	Sep. 3/01	Hatchery	<i>V. pullastra</i>	Spat	-
36-01					-
37-01	Oct. 4/01	Hatchery	<i>R. decussatus</i>	Spat	-
38-01					-
39-01	Nov. 12/01	Hatchery	<i>R. philippinarum</i>	Spat	-
40-01					-
41-01		Hatchery	<i>V. pullastra</i>	Larvae	-
42-01					-

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

Table III. PCR assays/ primer pair OHV1/OHV2 and primer pair OHV3/OHV114

Sample	Primer pair OHV1/OHV2	Primer pair OHV3/OHV114
1	+	+
2	+	-
3	-	+
4	+	-
5	-	-
6	+	-
7	+	+?
8	+	+
9	+	+
10	+	-
11	+	-?
12	-	+
13	+	+
14	+	+
15	+	+
16	+	+
17	-	+
18	+	+
19	+	+
20	+	+
21	+	+
22	+	+
23	+	+
24	+	+
25	+	+
26	+	+
27	+	+
28	-	-
29	+	+
30	+	+

N. B. : Negative samples primer pair OVH1-OVH2: 5. Negative samples primer pair OVH3-OVH114: 7

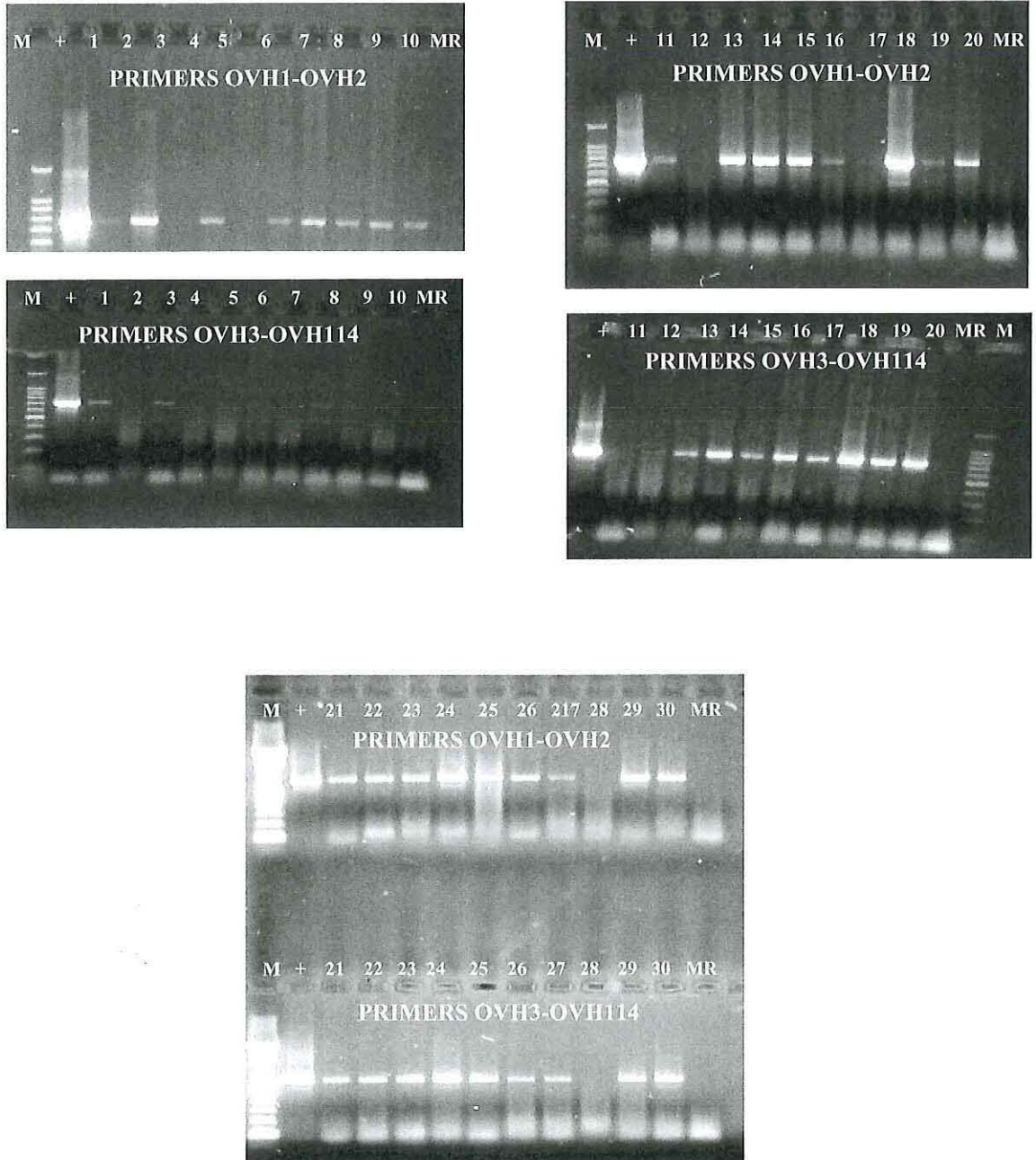


Figure 1. PCR amplification products corresponding to the 30 samples

Table IV. Samples for *in situ* hybridisation analysis

Number	Results
VB1	-
VB2	-
VB3	-
VB4	-
VB5	-
VB6	+
VB8	+
VB9	+
VB10	?
VB11	+++
VB12	+
VB13	+++
VB14	++
VB15	?
VB16	-
951240 7 *	+
VB17	+
VB18	+
VB19	+
VB20	-
VB21	-
VB22	+
VB23	?
VB24	++
VB25	-
VB26	++
VB27	+++
VB28	-
VB29	+++
VB30	+++
VB31	++
951240 7 *	+

* Sample +, +: Low, ++: Half, +++: high

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. 7

CEFAS Weymouth Laboratory

Virology Group

Weymouth, Dorset, DT4 8UB

United Kingdom

Tel : +44 1305 206642

Fax : +44 1305 206638

Email : P.F.DIXON@cefas.co.uk

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 **04-01-01** to **03-01-02**

Type of contract: *Shared cost research project.*

Total cost: *EC contribution:*

Participant no. 7 *EC contribution*
Total cost: *to partner no. 7:*

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

Completion date: 03-01-02

EC contact: DG XIV/C.2 0033-546363751

Coordinator: Dr Tristan Renault
IFREMER - DRV/RA
17390 La Tremblade
France
Tel: 00335-46369836
Fax: 0033-546363751
Email: trenault@ifremer.fr

Participant no. 7: Dr Peter Dixon (contractor)
CEFAS Weymouth Laboratory
Virology Group
Weymouth, Dorset, DT4 8UB
United Kingdom
Tel: +44 1305 206642
Fax: +44 1305 206601
Email: P.F.Dixon@cefas.co.uk

PLANNED RESEARCH ACTIVITIES

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

^a P. R.: Periodic Progress Report ^b F. R.: Final Report

RESEARCH ACTIVITIES DURING THE THIRD REPORTING PERIOD

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

1. Collecting bivalve samples

1. 1. Winter 2000-Spring 2001 sampling

16 batches of 30 individuals (Annex 1, Table 2) were collected from a total of 5 randomly designated sites around the British Isles throughout December, January, February and March. No mortality was associated with these samples. All samples originated from spawns produced in hatcheries. Individual animals > 2.5 cm in diameter were cut into three sections.

1. 2. Summer 2001 sampling

8 batches of 30 individuals (Annex 1, Table 2) were collected from 2 designated sites located in the South West of the British Isles throughout August and September. No mortality was associated with these samples. All samples originated from spawns produced in hatcheries.

2. Bivalve samples

Individual animals > 2.5 cm in diameter were cut into three sections.

- 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.

Subtask 3.1 Application of molecular methods for the diagnosis of oyster herpes like virus carrying out analysis of oyster samples originating from Ireland.

1. Screening by PCR

1.1. PCR - Validation

Viral DNA – 10 ng of the viral genome 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 and all dilutions can be detected.

1.2. PCR Ring Trial

Fifteen larval samples and 15 spat samples were supplied by Participant 1, to be tested by PCR, using primer pairs OHV1/OHV2 (C5/C13) and OHV3/OHV114 (C2/C6). The results are shown in Annex 1 (Table 6). The numbers of samples found to be positive were practically identical with both primer pairs except that sample 12 was positive with primer pair OHV3/OHV114 (C2/C6) but negative with OHV1/OHV2 (C5/C13). Overall, primer pair OHV3/114 (C2/C4) gave the best results as it produced very distinct, bright bands.

1.3. Screening of bivalves samples from the United Kingdom during 2001 by PCR

In addition to testing the winter 2000-spring 2001 sample and the summer 2001 sample, testing of the winter 1999-spring 2000 sample that was started in 2000 was completed. Only sample 014 (an oyster mortality) from the latter sample had been tested during the period of the second annual report. PCR testing of samples collected during all these sampling periods was initiated using OHV3 and OHV114 sequence-specific primers.

The results are as follows:

- a) PCR analysis of samples collected in winter 1999-2000 has been completed. Samples 016, 017 (from *C. gigas* spat from one site) and sample 023 (from *O. edulis* larvae from a second site) were positive in the PCR (Annex 1 Table 1). The nucleotide sequence (Annex 2) of the PCR product of samples 016 and 023 showed 94.4 % and 98.3 % homology, respectively, to reference OsHV DNA. There was not enough DNA from sample 017 to allow nucleotide sequencing, even after repeating the PCR on that sample. All other samples were found to be negative for OsHV DNA.

Both those sites were re-sampled in the following sampling periods and tested by the PCR, but were negative for OsHV DNA (Interim Report 2000, and this report).

- b) PCR analysis of samples collected in winter 2000-2001 has been completed. At one of the sampling sites there were significant mortalities in larval clams, *T. philippinarum* and *T. decussatus* and lesser mortalities in *C. gigas* spat. However, all samples were found to be negative for OsHV DNA (Annex 1, Table 2).

Further investigations were made into the clam and oyster mortalities. Samples of *C. gigas* larvae taken during a previous mortality episode had been fixed in glutaraldehyde, and those samples were processed further for examination under the electron microscope. Such examination revealed OsHV-like particles (Annex 1, Figures 1 and 2). Samples of oysters and clams from the winter 2000-2001 mortality were examined by light microscopy and by electron microscopy. Histological examination revealed occasional nuclei suggestive of a light infection with OsHV, but the virus was not observed by electron microscopy. Taken with the negative PCR results, it was concluded that OsHV was not present in the animals, and not the cause of the mortalities. It appears that the site has been infected with OsHV in the past, but there was no evidence of a current infection. However, organisms resembling a *Dermocystidium*-like organisms as described by Leibovitz *et al.* (1978)¹ and later re-designated as a possible colourless algae, *Hyachlorella* (Elston 1980)² were seen in the stomachs of several specimens by light microscopy (Annex 1, Figure 3). Examination by electron microscopy showed that the organism contained chloroplasts (Annex 1, Figure 4), and hence differed from the *Hyachlorella* -like organism, and more closely resembled the algae *Chlorella*. Further investigations on the relationship of the organism to the disease in the oysters and clams are not within the remit of this project, and will be continued if further funding is obtained.

- c) PCR analysis of samples collected in summer 2001 has been completed. All samples were found to be negative for OsHV DNA. (Annex 1, Table 3).

2. Screening by *In situ* hybridization

2.1. *In situ* hybridisation Ring Trial

Three slides each of 31 coded OsHV positive and negative samples were supplied by Participant 1, to be tested by ISH. The protocol based on a peroxidase/DAB detection system was used. Ten slides were considered to be definitely positive for OsHV, with an additional two considered possible positive (Annex 1, Table 4).

Following discussions with Participant 1 and other partners, a limited number of samples were tested using the C1/C6 probe. The samples were a new set provided by Dr. Renault. Twenty one of 31 samples were tested (the remainder were retained for serology) and nine were positive (Annex 1, Table 5).

¹ Leibovitz, L., Elston, R. A., Lipovski, V. P. and Donaldson, J. (1978). A new disease of larval Pacific oysters. Proc. World Maricult. Soc. 9: 603-615.

² Elston, R. A. (1980). Ultrastructural aspects of a serious disease of hatchery-reared larval oysters, *Crassostrea gigas* Thunberg. J. Fish Dis. 3: 1-10.

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

Sub-task 3.2 Application of immunological methods

MAB not available until February 2002 - no funding available to carry out this analysis.

Sub-task 3.3 Application of primary oyster cell cultures

No cell cultures were available for diagnosis during the project.

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

Participation in annual meetings and in writing intermediate and final reports

Dr P. Dixon and Mr P. Martin attended and presented results at the annual meeting in December 2001 held at the IFREMER headquarters, Paris. Dr P. Dixon wrote this final report.

Contribution to articles related to OsHV

The investigations into the clam and oyster mortalities in winter 2000-spring 2001 formed part of a poster presentation at the 10th Annual Conference of the European association of Fish Pathologists, Dublin, September 2001:

L.F. Richens, **R. M. Le Deuff**, A.E. Bayley, **P.D. Martin** and K.V. Thomas. "Chronic mortality in a bivalve mollusc hatchery: a case study."

SIGNIFICANT DIFFICULTIES OR DELAYS

MAB not available until February 2002 and no cell cultures were available for diagnosis during the project.

ANNEX 1

Table I. Winter1999/Spring2000 samples for the United Kingdom

Sites ¹	Sample no	Species	Age/size	Mortality Rate%	Sample date	PCR result
Kent	011	<i>T. semidecussatus</i>	5mm	0%	12/99	Negative
“	012	<i>T. decussatus</i>	5mm	0%	12/99	“
“	013	<i>C. gigas</i>	1cm	0%	12/99	“
“	014	<i>O. edulis</i>	1-2cm	50%	12/99	“
Gwynedd	015	<i>C. gigas</i>	spat	0%	03/00	“
Cornwall	016	<i>C. gigas</i>	Spat	0%	01/00	Positive; sequenced
“	017	<i>C. gigas</i>	Spat	0%	01/00	Positive; not sequenced
Kent	018	<i>T. semidecussatus</i>	Spat	0%	02/00	Negative
“	019	<i>T. decussatus</i>	Spat	0%	02/00	“
“	020	<i>C. gigas</i>	Spat	0%	02/00	“
“	021	<i>T. decussatus</i>	Spat	0%	02/00	“
“	022	<i>C. gigas</i>	Spat	0%	03/00	“
“	023	<i>O. edulis</i>	Larvae	0%	03/00	Positive; sequenced
Cornwall	024	<i>O. edulis</i>	Spat	0%	02/00	Negative
“	025	<i>O. edulis</i>	Spat	0%	02/00	“
“	026	<i>O. edulis</i>	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.

Table II. Winter 2000/Spring 2001 samples for the United Kingdom

Sites ¹	Sample no	Species	Age/size	Mortality Rate%	Sample date	PCR result
Cornwall	039	<i>C. gigas</i>	2-8cm	0%	29/01/01	Negative
“	040	<i>C. gigas</i>	2-8cm	0%	29/01/01	“
“	041	<i>O. edulis</i>	6cm	0%	13/02/01	“
“	042	<i>O. edulis</i>	6cm	0%	13/02/01	“
“	043	<i>O. edulis</i>	6cm	0%	13/02/01	“
“	044	<i>O. edulis</i>	6cm	0%	13/02/01	“
“	045	<i>O. edulis</i>	6cm	0%	13/02/01	“
“	046	<i>C. gigas</i>	3-5cm	0%	13/02/01	“
“	047	<i>O. edulis</i>	6cm	0%	13/02/01	“
Gwynedd	048	<i>C. gigas</i>	3-7cm	0%	15/02/01	“
Guernsey	049	<i>T. philippinarum</i>	larvae	signs	16/02/01	“
“	050	<i>C. gigas</i>	600 micro	signs	23/02/01	“
“	051a	<i>T. philippinarum</i>	larvae	diseased	23/02/01	“
“	052	<i>T. philippinarum</i>	larvae	50% ⁺	23/02/01	“
“	053	<i>T. philippinarum</i>	5mm spat	0%	23/02/01	“
“	054	<i>C. gigas</i>	2mm spat	0%	23/02/01	“
“	055	<i>C. gigas</i>	10mm spat	22%	23/02/01	“
Kent	056	<i>C. gigas</i>	2cm spat	6.7%	01/03/01	“
“	057	<i>C. gigas</i>	2mm spat	0%	01/03/01	“
“	058	<i>O. edulis</i>	5mm spat	0%	01/03/01	“
Guernsey	059	<i>T. decussatus</i>	Larvae	90%	03/04/01	“
“	060(a)	<i>T. decussatus</i>	Day4 Larvae	0%	07/04/01	“
“	060(b)	<i>T. decussatus</i>	Day8 Larvae	90%	11/04/01	“

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 III. Summer 2001 samples for the United Kingdom

Sites ¹	Sample no	Species	Age/size	Mortality Rate%	Sample date	PCR result
Cornwall	061	<i>C. gigas</i>	Spat 3-4cm	0%	08/01	Negative
“	062	<i>C. gigas</i>	“	0%	08/01	“
“	063	<i>C. gigas</i>	“	0%	08/01	“
“	064	<i>O. edulis</i>	Adult 5-7cm	0%	08/01	“
“	065	<i>O. edulis</i>	“	0%	08/01	“
“	066	<i>O. edulis</i>	“	0%	08/01	“
“	067	<i>C. gigas</i>	Spat 5cm	0%	09/01	“
“	068	<i>C. gigas</i>	“	0%	09/01	“

¹ Only the county in which the sites are located are given in order to preserve their anonymity. Two different sites were sampled.

Table IV. *In situ* hybridisation ring test results for the United Kingdom (first ISH Ring Trial using the indirect technique)

Sample Reference	ISH result	Comments
94/215 - 6	Positive	Moderate labelling
94/215 - 9	Positive	Strong labelling
94/215 - 10	Positive	Moderate labelling
94/215 - 11	Positive	Strong labelling
94/248 - 1	Negative	-
94/248 - 5	Negative	-
94/248 - 7	?Positive	Very few Red inclusions
94/248 - 13	Negative	-
94/248 - 15	Negative	-
94/250 - 4	Positive	Strong labelling
94/250 - 13	Negative	-
94/250 - 17	Negative	-
94/250 - 18	Negative	-
94/250 - 19	Negative	-
94/250 - 23	Negative	-
94/278 - 7	Negative	-
94/278 - 8	Negative	-
94/278 - 10	Negative	-
94/278 - 11	Negative	-
94/278 - 13	Negative	-
95/54 - 7	Negative	Red/purple inclusions
95/54 - 8	Negative	-
95/54 - 11	Negative	-
95/54 - 12	Positive	Very few labelled cells with inclusions
95/54 - 13	Positive	Very few labelled cells with red/purple inclusions
95/54 - 14	Positive	Very few labelled cells with red/purple inclusions
95/54 - 18	Negative	Red/purple inclusions
95/54 - 20	Positive	Very few labelled cells + red/purple inclusions
95/54 - 26	Negative	-
95/54 - 28	Positive	Very few labelled cells + red/purple inclusions
95/54 - 30	?Positive	Red/purple inclusions

Table V. *In situ* hybridisation ring test results for the United Kingdom (second ring test using the direct technique)

Sample reference	ISH result	Comments
1	Negative	
2	Negative	
3	Negative	
4	Negative	
5	Negative	
6	Negative	
8	Negative	
9	Negative	
10	Negative	
11	Negative	
12	Negative	
13	Positive	Heavy infection
14	Positive	"
15	Positive	Moderate infection
16	Positive	"
17	Positive	"
18	Positive	"
19	Positive	"
20	Positive	"
21	Positive	Light infection
22	Not done	
23	Not done	
24	Not done	
25	Not done	
26	Not done	
27	Not done	
28	Not done	
29	Not done	
30	Not done	
31	Not done	

Table VI. PCR ring test results for the United Kingdom

Tube Number	Primer pair		Comments
	OHV1/OHV2	OHV3/OHV114	
1	Negative	Negative	
2	Negative	Negative	
3	Negative	Negative	
4	Negative	Negative	
5	Negative	Negative	
6	Negative	Negative	
7	Negative	Negative	
8	Negative	Negative	
9	Negative	Negative	
10	Negative	Negative	
11	Negative	Negative	
12	Negative	Positive	
13	Positive	Positive	
14	Positive	Positive	
15	Positive	Positive	
16	Positive	Positive	Faint band with OHV1/OHV2)
17	Positive	Positive	Faint band with OHV1/OHV2)
18	Positive	Positive	
19	Positive	Positive	
20	Positive	Positive	
21	Positive	Positive	
22	Positive	Positive	
23	Positive	Positive	
24	Positive	Positive	
25	Positive	Positive	
26	Positive	Positive	
27	Positive	Positive	
28	Negative	Negative	
29	Positive	Positive	
30	Positive	Positive	

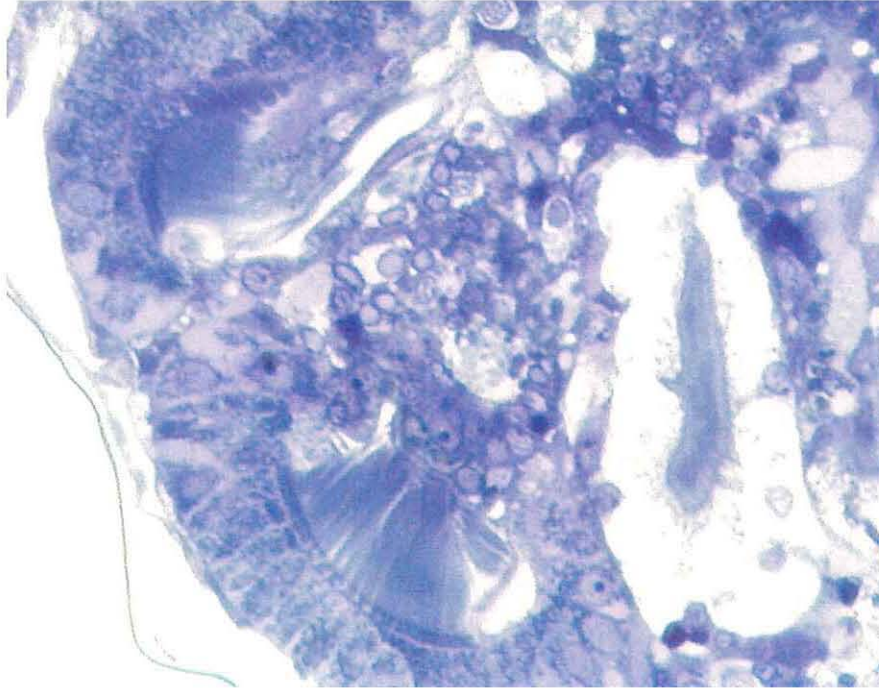


Figure 1. Semi-thin section of *C. gigas* larvae showing cells indicative of OsHV-1 infection

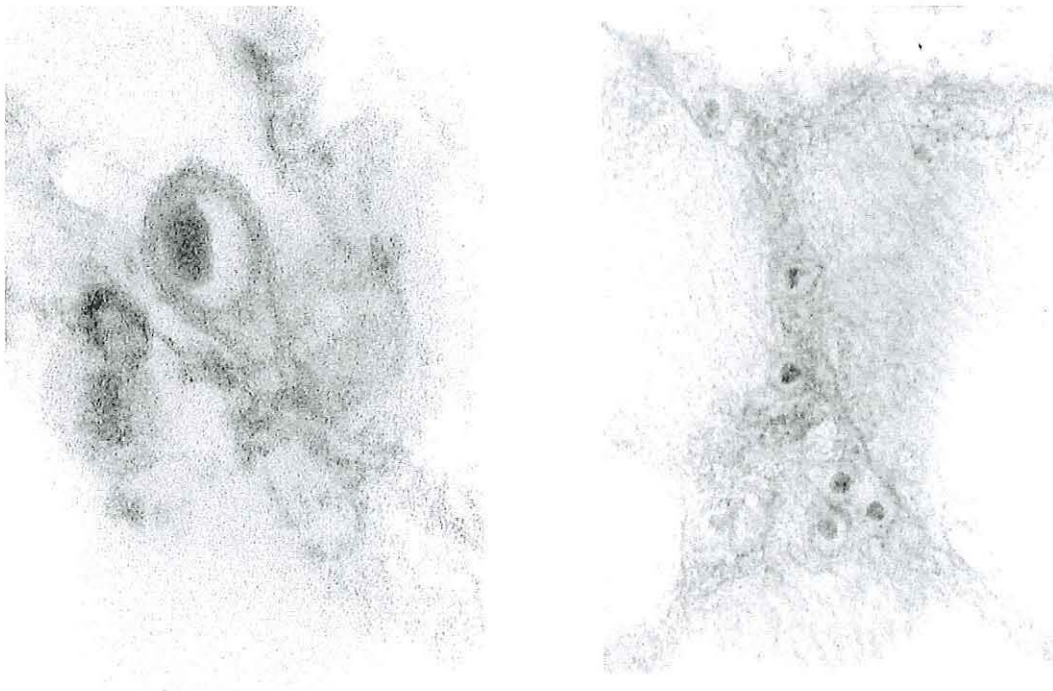


Figure 2. Transmission electron microscopy of OsHV-1 in *C. gigas*



Figure 3. *Dermocystidium*-like organisms (arrowed) in the gut of *C. gigas*.

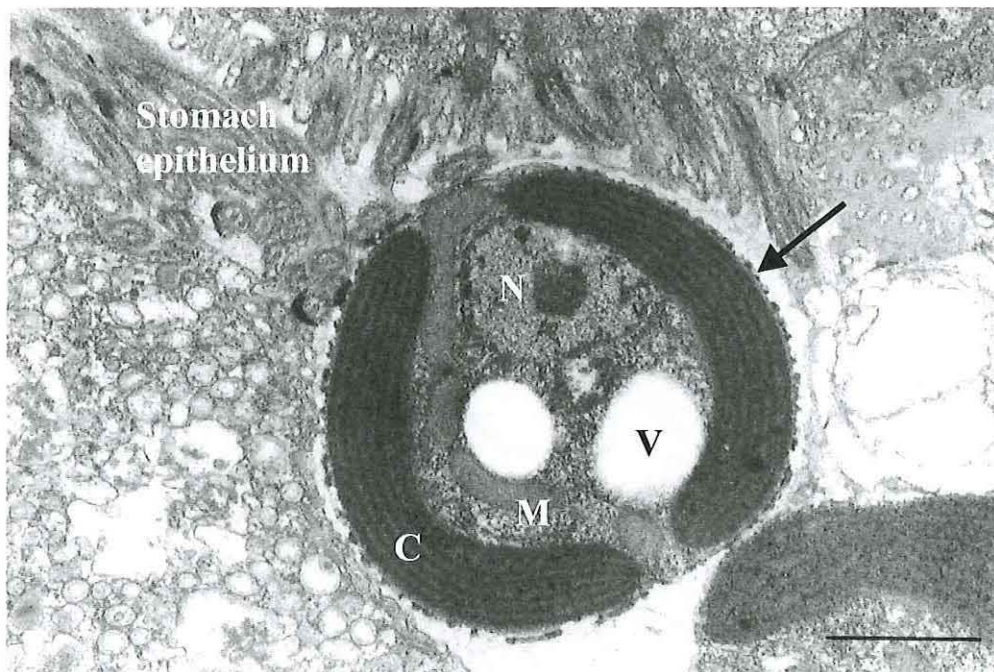


Figure 4. Ultrastructure of the organism by electron microscopy. C, chloroplast, M, mitochondrion, N, nucleus, V, vacuole. Arrow indicates the cell wall.

ANNEX 2

Nucleotide sequencing. PCR products were purified using the GLEANCLEAN® (Anachem, Luton, UK) and inserted into the pGEM-T vector (Promega, Southampton, UK) using the standard protocol. Both DNA strands were sequenced using the M13 universal sequencing primers and the ABI PRISM™ dye terminator cycle sequencing system (Applied Biosystems, Warrington, UK). Sequencing reactions were analysed on an ABI 310 genetic analyser and multiple alignments performed using Clustal V (Higgins & Sharp 1989).

Higgins, D. G. and Sharp, P. M. (1989). Fast and sensitive multiple sequence alignments on a microcomputer. *CABIOS* 2: 151-153.