PROJECT ROMEO : E. C. no FA-S2 9052

Research Objectives : Mortality in European Oysters

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Detection of herpes-like virus DNA by PCR in bivalve larval samples

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Introduction

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 EU (FAO, 1989). Although no serious pathogen was detected previously in Japanese oysters in Europe, mortalities have been observed since the introduction of this bivalve species into the EU. Thus, oyster cultivation may be endangered by the occurrence of epizootics, especially virus 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 oyters, *Crassostrea virginica,* with the detection of particles indicating membership of the *Herpesviridae* (Farley *et al.*, 1972). Mass mortalities of adult Portuguese oysters, *C. angulata,* among French livestocks (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* (Elston, 1979; Farley, 1976; Farley, 1978; 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). The pathogenicity of the virus was demonstrated by experimental transmission of the infection to healthy *C. gigas* larvae (Le Deuff *et al.*, 1994). Since 1993, sporadic high mortalities occur also in some batches of Pacific oyster spat cultured in different French locations (Renault *et al.,* 1994b).

In addition, herpesvirus infections were also reported in spat and larvae of the European flat oyster (*Ostrea edulis*) in France (Comps and Cochennec, 199; Renault *et al.*, in press). Concomitant mortalities were observed among larvae and spat of *Crassostrea gigas* and *O. edulis*, in 1994 and 1995, with the detection of herpes-like virus particles by transmission electron microscopy (Renault *et al.*, in press). 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).

Unexplained mortalities were observed in recent years among larvae in the United Kingdom and Spain, although samples were not examined. High losses were reported among 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 belonging to the genera *Crassostrea*, *Ostrea* and *Ruditapes* seem to be ubiquitous and are associated with substantial mortalities. These observations highlight the importance of testing a range of efficient diagnostic methods in order to assess the causative role of the herpesviruses in bivalve mortalities.

To diagnose herpes-like virus infections, the basic method for examination of suspect samples is still light microscopy. This method appears poorly adapted to viral diseases and needs to be improved upon by other techniques such as transmission electron microscopy. Both techniques are time consuming and inadequate for epidemiological surveys. In addition, research into virus cytopathogenic effects in cell cultures is impossible because the lack of bivalve cell lines. Serological methods are also not available due to the absence of immunoglobulin production in molluscs. However, 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 C*rassostrea. 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.*, 2000). 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 developped (Renault *et al.*, 2000). 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.

Research objectives

The observed association between oyster mortality and herpes-like virus infection provides an imperative to determining the extent to which the virus is involved as a causative agent of massive mortalities in different European countries. It appears essential to survey infections epidemiologically in different European shellfish hatcheries.

The aim of the work proposed is to use molecular reagents (PCR) specific for oyster herpes-like viruses in order to analyse larval samples from different origins. Thus, two tasks have been defined :

- Analysis by PCR technique of bivalve larval samples originating from different European hatcheries in order to detect herpes-like virus DNA

- Validating new PCR primer pairs for herpes-like virus diagnosis

Material and methods

Clinical samples

81 larval bivalve samples were obtained from 5 different hatcheries located in France, Spain and United Kingdom : 21 samples for Hatchery 1, 7 samples for Hatchery 2, 34 samples for Hatchery 3, 10 for Hatchery 4 and 9 samples for Hatchery 5. They were investigated by PCR.

Samples corresponded to 4 different bivalve species : *Crassostrea gigas*, *Ostrea edulis*, *Ruditapes philippinarum* and *Ruditapes decussatus*. 63 larval *C. gigas* samples, 12 *R. decussatus* samples, 3 *O. edulis* samples and 3 *R. philippinarum* samples were obtained and analysed.

Samples were collected from broods, some of which presented abnormally high mortalities, from November 1997 to June 2000 and were frozen at -20°C as soon as possible after sampling. Frozen samples were sent to the IFREMER laboratory (La Tremblade, Charente Maritime, France) in dry ice.

Virus DNA

Herpes-like virus DNA was extracted from purified virus pellets. The protocol used was previously described (Le Deuff, 1995; Le Deuff and Renault, 1999). Briefly, purified virus particles were suspended in extraction buffer (100 mM NaCl; 10 mM Tris; 25 mM EDTA; 0.5% SDS; pH 8), then proteinase K (0.2 mg/ml) was added on two occasions. DNA was extracted using phenol/chloroform/isoamyl alcohol (24/23/1), and precipitated and dried using conventional methods. It was resuspended in TE buffer (10 mM Tris; 1 mM EDTA; pH 8). DNA concentration was determined using a Perkin-Elmer spectrophotometer at 260 nm (1 unit of optical density corresponding to 50 µg of DNA per ml). A virus DNA suspension was prepared at 100 ng/µl in TE buffer and used as positive control for PCR analysis.

Sample preparation for PCR

50 mg of frozen larvae were weighed out in an Eppendorf microtube using a fresh Pasteur pipette and ground in 50 µl of distilled water using a single-use tissue homogeneiser as previously described (Renault *et al.*, 2000). Larval samples were vortexed and denatured in a boiling water bath for ten minutes followed by quick chilling in ice. Samples were vortexed again and centrifuged at 10 000 rpm for five minutes. Supernatants were recovered, immediately ten-fold diluted in double distilled water and frozen at -20°C.

PCR primers

Seven PCR primers were derived from herpes-like viral DNA sequences (Renault *et al.*, 2000). Three forwards primers (OHV3, A3 and A5) and four reverse primers (OHV4, OHV114, A4 and A6) were designed which provided four primer-pair combinations (OHV3/OHV4, OHV3/OHV114, A3/A4 and A5/A6) with expected PCR products that ranged respectively in size 896 pb, 709 pb, 1001 pb and 917 bp.

Primers were synthesized commercially by Eurogentec (Parc Scientifique du Sart Tilman, 4102 Seraing, Belgium). Melting temperatures of primers were calculated using the nearest neighbor method (Richlik *et al.*, 1990). For the nested PCR protocol, A3/A4 were used as external primers and A5/A6 as internal primers. The primer pairs OHV3/OHV4 and OHV3/OHV114 were used in simple PCR protocols.

PCR conditions

The thermostable DNA polymerase used, Goldstar, was obtained from Eurogentec (Parc Scientifique du Sart Tilman, 4102 Seraing, Belgium). Thermal cycling was performed using a Crocodile III thermal cycler from Appligene Oncor (Illkirch, France). Fifty µl PCR reactions were performed, each containing the appropriate reaction buffer provided by the DNA polymerase manufacturer, 0.05 mM of each dNTP, 100 ng of each primer, 2.5 mM $MgCl_2$, 2.5 units of DNA polymerase and 1 µl of template DNA. Crocodile III apparatus possesses a heated lid, thus avoiding addition of mineral oil. After heating samples for 2 min at 94°C, 35 cycles were performed followed by a final elongation step of 5 min at 72°C. Each of the 35 cycles consisted of a DNA melting step at 94°C for 1 min, a primer annealing step for 1 min at 50°C for OHV3/OHV4, OHV5/OHV6, A3/A4 and A5/A6 pairs and a primer elongation step at 72°C for 1 min.

For the nested PCR using A3/A4 and A5/A6 primer pairs, reaction conditions were the same for the second reaction, excepted for template DNA volume. 0.5 µl of first PCR reaction products was used for the second PCR reaction.

PCR products were separated by size by electrophoresis on 1% agarose gels. Gels were stained with ethidium bromide (0.5 µg ml^{-1}). Ten µl of each PCR products were loaded per lane. The DNA was visualized using a 302 nm UV transilluminator and the gels photographied. Sizes of the DNA products were determinated relative to those of size markers (Eurogentec, Marker 1 or Marker 2). Relative amounts of amplification products within the same gel were made by comparing band intensities on photographs.

Avoiding contamination

Due to the high sensitivity of PCR, special precautions were adopted. All steps of this study, including sample preparation, preparation of amplification mixture components and PCR amplification were carried out in separate rooms. To limit contamination, all pipetting procedures were performed using filter tips.

Results

Sample nature

81 bivalve larval samples were obtained from 5 different hatcheries : 42 % from Hatchery 3 (France), 26 % from Hatchery 1 (United Kingdom), 12 % from Hatchery 4 (France), 11 % from Hatchery 5 (Spain) and 9 % from Hatchery 2 (United Kingdom) (Fig. 1).

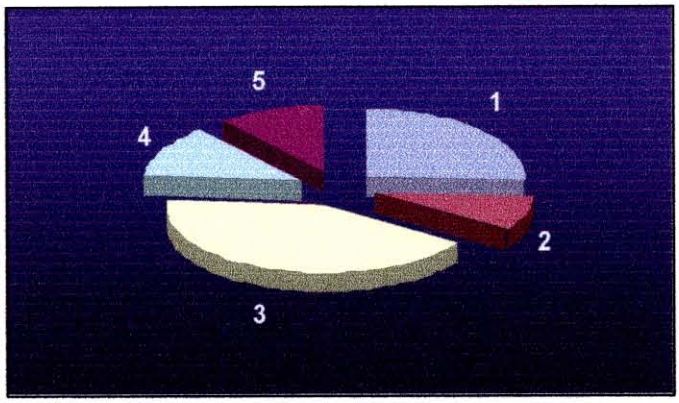

Figure 1 : distribution of bivalve larval samples depending on the hatcheries. 1 : Hatchery 1 (26 %), 2 : Hatchery 2 (9 %), 3 : Hatchery 3 (42 %), 4 : Hatchery 4 (12 %) and 5 : Hatchery 5 (11 %)

77 % of bivalve larval samples corresponded to *Crassostrea gigas*, 15 % to *Ruditapes decussatus*, 4 % to *Ostrea edulis* and 4 % to *Ruditapes philippinarum* (Fig. 2).

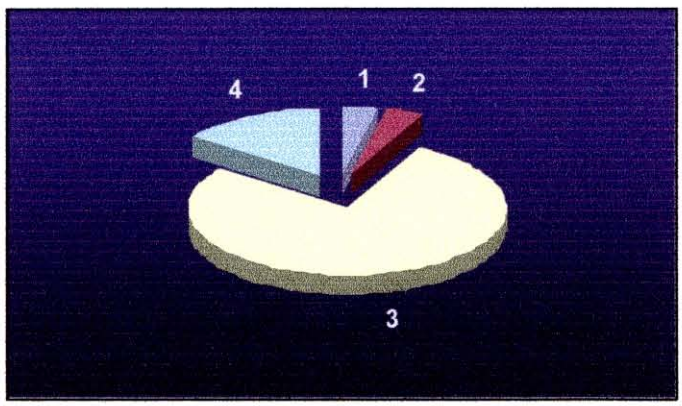

Figure 2 : distribution of larval samples depending on the bivalve species. 1 : *Ruditapes philippinarum* (4 %), 2 : *Ostrea edulis* (4 %), 3 : *Crassostrea gigas* (77 %) and 4 : *Ruditapes decussatus* (15 %).

However, the percentages of samples analysed for each bivalve species depended on the hatcheries (Fig. 3).

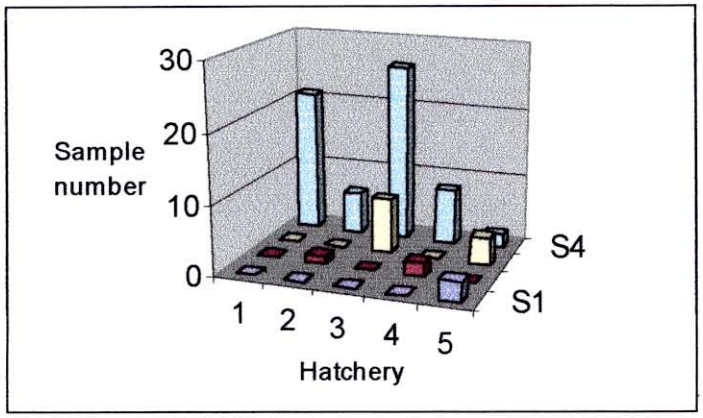

Figure 3 : distribution of laval samples depending on the hatcheries and bivalve species. 1 : Hatchery 1, 2 : Hatchery 2, 3 : Hatchery 3, 4 : Hatchery 4 and 5 : Hatchery 5. S1 : *Ruditapes philippinarum*, S2 : *Ostrea edulis*, S3 : *Ruditapes decussatus* and S4 : *Crassostrea gigas*.

Viral DNA detection by PCR

PCR analysis permitted to observe amplification products with expected sizes on agarose gels from different clinical samples presenting high mortality rates (Fig. 4, Tables 1 to 9). Several primer pairs were used and positive results were given in this part for any primer pair used.

Figure 4. Analysis of bivalve larval samples by nested PCR using A3/A4 and A5/A6 primer pairs. Agarose gel electrophoresis of the first PCR reaction products. Lane A shows the migration of molecular weight marker (Eurogentec M2). Lanes B, I and P : control reactions without template DNA; lane Q : positive control corresponding to 0.5 pg of virus DNA. Lanes B to H: *Crassostrea gigas* larval samples (Hatchery 1). Lanes J to O : *Ruditapes decussatus* larval samples (Hatchery 3). Positive larval samples : lanes J, L, M and N.

17 samples on the 81 analysed (21 %) appeared positive for the detection of herpes-like virus DNA by PCR (Table 10). However, for 2 samples (Hatchery 1), the band of expected size was very faint on agarose gels (Table 1).

Table 10 : Herpes-like virus DNA detection by PCR in bivalve larval samples

	C. gigas		*O. edulis*		*R. decussatus*		*R. Philippinarum*	
	+	-	+	-	+	-	+	-
Hatchery 1	**2**	19	**0**	0	**0**	0	**0**	0
Hatchery 2	**0**	6	**0**	1	**0**	0	**0**	0
Hatchery 3	**6**	20	**0**	0	**3**	5	**0**	0
Hatchery 4	**3**	5	**2**	0	**0**	0	**0**	0
Hatchery 5	**0**	2	**0**	0	**0**	4	**1**	2
Sub-Total	**11**	51	**2**	1	**3**	9	**1**	2
Total	63		3		12		3	

+ : detection of bands of expected sizes on agarose gels using primer pairs OHV3/OHV4, OHV3/OHV114 or A3/A4 and A5/A6. - : No band of expected size detected on agarose gels.

Positive samples were observed in the four bivalve species tested : 11 positive *Crassostrea gigas* samples, 2 *Ostrea edulis* samples, 3 *Ruditapes decussatus* samples and 1 *R. philippinarum* sample (Fig. 5, Table 10).

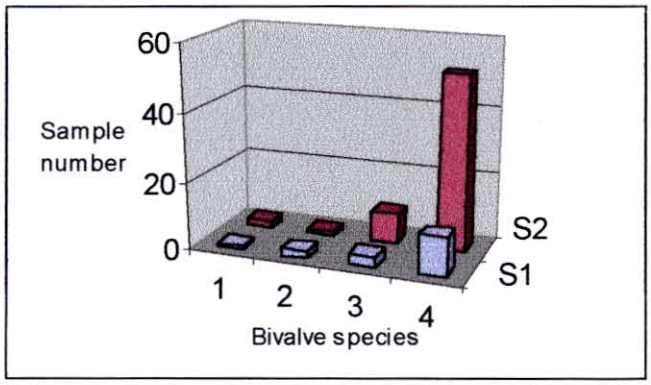

Figure 5 : detection of herpes-like virus DNA by PCR. 1 : *Ruditapes philippinarum*, 2 : *Ostrea edulis*, 3 : *Ruditapes decussatus* and 4 : *Crassostrea gigas*. S1 : PCR positive samples and S2 : PCR negative samples.

Positive samples were observed in 4 hatcheries : Hatchery 1; Hatchery 3, Hatchery 4 and Hatchery 5 (Fig. 6, Tables 1 to 9 and Table 10). The two positive samples originated from Hatchery 1 showed a very faint band on agarose gels (Table 1). All samples originating from Hatchery 2 were negative for the detection of herpes-like virus DNA by PCR.

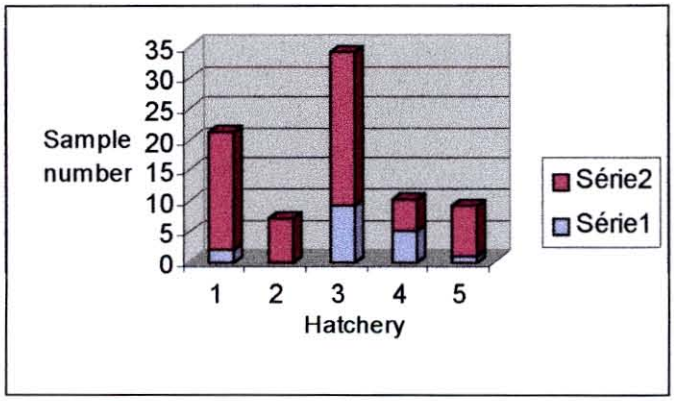

Figure 6 : detection of herpes-like virus DNA by PCR. 1 : Hatchery 1, 2 : Hatchery 2, 3 : Hatchery 3, 4 : Hatchery 4 and 5 : Hatchery 5. Série1 : PCR positive samples and Série2 : PCR negative samples.

PCR results depending on primer pairs used

170 PCR analyses were performed using different primer pairs : 65 (38 %) using the primer pair OHV3/OHV4, 66 (39 %) using the primer pair OHV3/OHV114 and 39 (23 %) using a nested PCR protocol with A3/A4 and A5/A6 (Fig. 7). They corresponded to 81 bivalve larval samples. Some samples have been analysed using the 3 sets of primers (Tables 1 to 9) and some samples have been analysed using only a primer pair or two primer pairs (Tables 1 to 9).

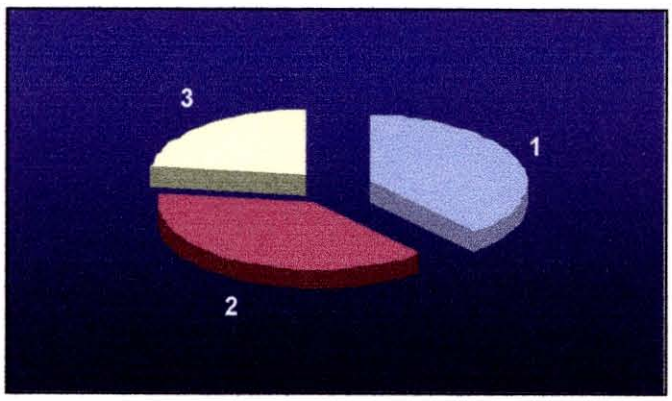

Figure 7 : distribution of PCR analyses depending on the primer pair used. 1 : OHV3/OHV4, 2 : OHV3/OHV114 and 3 : A3/A4 and A5/A6

The number of virus DNA positive samples depended on the primer pair used for PCR (Figure 8). Five PCR reactions appeared positive (5/65 : 7.7 %) when the primer pair OHV3/OHV4 was used. 23 PCR tubes (23/66 : 34.8 %) allowed the detection of a band of expected size on agarose gels when the PCR reactions were done with the primer pair OHV3/OHV114. A band of expected size was observed in 10 PCR reactions (10/39 : 25.6 %) when the nested PCR protocol (A3/A4 and A5/A6) was used.

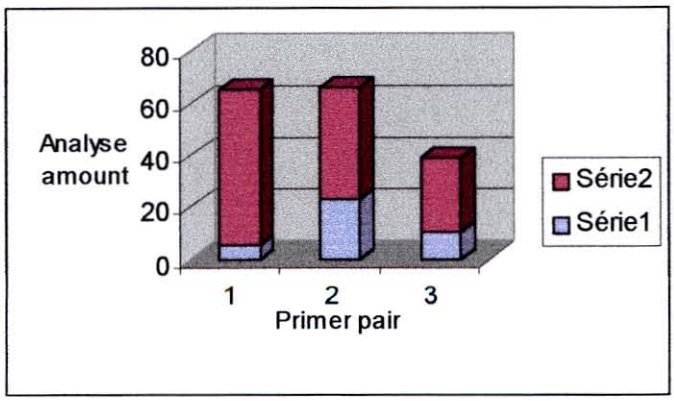

Figure 8 : detection of herpes-like virus DNA by PCR. 1 : OHV3/OHV4, 2 : OHV3/OHV114 and 3 : A3/A4 and A5/A6. Série1 : PCR positive analyses. Série2 : PCR negative analyses.

39 PCR reactions were performed using the 3 sets of primers (OHV3/OHV4, OHV3/OHV114 and A3/A4 - A5/A6) corresponding to 117 analyses (39 x 3) and using the same samples as templates. The percentages of PCR positive reactions were similar than the results given above (Fig. 9). Five PCR reactions appeared positive (5/39 : 5 %) when the primer pair OHV3/OHV4 was used. 15 analyses (15/39 : 38.4 %) allowed the detection of a band of expected size on agarose gels when the PCR reactions were done with the primer pair OHV3/OHV114. A band of expected size was observed in 10 PCR tubes (10/39 : 25.6 %) when the nested PCR protocol (A3/A4 and A5/A6) was used. The primer pair OHV3/OHV4 allowed less PCR amplification than OHV3/OHV114 (5 % versus 38.4 %) or A3/A4 - A5/A6 (5 % versus 25.6%).

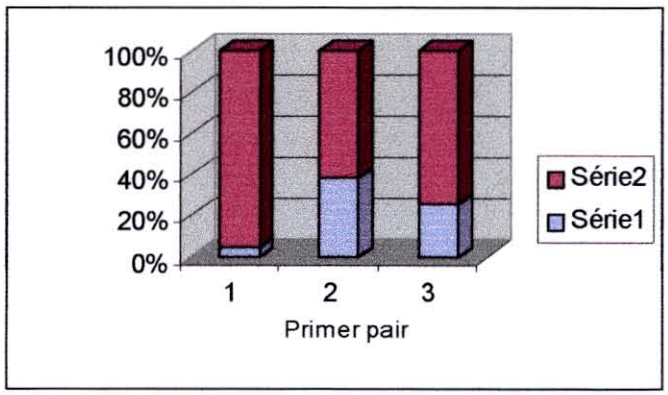

Figure 9 : detection of herpes-like virus DNA by PCR. 1 : OHV3/OHV4, 2 : OHV3/OHV114 and 3 : A3/A4 and A5/A6. Série1 : PCR positive tubes. Série2 : PCR negative tubes.

Mortality and positive PCR results

17 larval samples on 81 tested samples have been found positive by PCR. 12 PCR positive batches (70.6 %) corresponded to animals presenting mortalities. No information was available for 3 PCR positive samples. At least, 2 samples giving PCR positive results corresponded to *Ostrea edulis* larvae without mortality at the moment of sampling.

4. Discussion

The recent appearence of numerous herpes-like virus infections among different bivalve species highlights the usefulness of adapted methods for diagnosing these diseases. Currently available detection techniques, *i. e.* histology and transmission electron microscopy, are less than totally satisfactory with regards to sensitivity, time and cost efficiency. PCR based detection systems for vertebrate herpesvirus infections are well documented (Boyle and Blackwell, 1991; Casas *et al.*, 1996; Cassinotti *et al.*, 1996; de Gee *et al.*, 1996; Torigoae *et al.*, 1996). Progress has been recently made in developing methods for detection of human shellfish-transmitted viruses (Lees *et al.*, 1993; Le Guyader *et al.*, 1994; Atmar *et al.*, 1995). At least, molecular methods including PCR for detection of oyster herpes-like virus have been recently developed (Renault and Lipart, 1998; Renault *et al.*, 2000). Thus, a PCR-based detection procedure has been developed for a herpes-like virus infecting Pacific oyster, *Crassostrea gigas* larvae.

PCR analysis permitted to observe amplification products with expected sizes on agarose gels from different clinical samples. 17 samples on the 81 analysed (21 %) appeared positive for the detection of herpes-like virus DNA by PCR. However, for 2 samples (Hatchery 1), the band of expected size was very faint on agarose gels.

Positive samples were observed in 4 hatcheries : Hatchery 1 (United Kingdom), Hatchery 3 (France), Hatchery 4 (France) and Hatchery 5 (Spain). The two positive samples originated from Hatchery 1 showed a very faint band on agarose gels. All samples originating from Hatchery 2 (United Kingdom) were negative for the detection of herpes-like virus DNA by PCR. For Hatcheries 3 and 4, several samples (respectively 9 and 5) were positive for the detection of viral DNA by PCR. These results confirm previous data indicating that herpes-like virus infections may be observed in private French hatcheries (Nicolas *et al.*, 1992; Renault *et al.*, 1994b). Morover, some PCR positive results have been also obtained for bivalve larval samples originating from Spain and United Kingdom (respectively 1 and 2). Thus, herpes-like viruses exist in several European countries. The herpes-like virus infections seem ubiquitous and hatchery rearing conditions may favour viral disease expression. However, analysis must be pursued in order to confirm these results. Indeed, some samples originating from Spain and United Kingdom were thawed when they have been received in the laboratory (IFREMER La Tremblade, Charente Maritime, France) for PCR analysis. PCR negative results may correspond to damaged viral DNA. Number of positive PCR results may be underestimated.

Positive samples were observed in the four bivalve species tested : *Crassostrea gigas*, *Ostrea edulis*, *Ruditapes decussatus* and *R. philippinarum.* Thus, it is the first time that the detection of herpes-like virus DNA was observed in larval *Ruditapes decussatus*. In Europe, herpes-like viruses can be found in four different bivalve species : *Crassostrea gigas*, *Ostrea*

edulis, *Ruditapes philippinarum* and *Ruditapes decussatus*. This observation highlights the need of studying potential interspecific transmission of herpes-like virus infections among bivales.

17 larval samples on 81 tested have been found positive by PCR. 12 PCR positive batches have presented anomalous mortalities. The detection of viral DNA is associated in these cases with high mortality rates. No information was available for 3 PCR positive samples. Thus, numerous samples presenting viral DNA detection by PCR correspond to larval batches presenting mortalities. Herpes-like viruses may be interpreted as one of the causative agents of anomalous mortalities observed in bivalve hatcheries. Moreover, the pathogenicity of the herpes-like virus infecting *Crassostrae gigas* larvae has been previously demonstrated (Le Deuff *et al.*, 1994).

The number of virus DNA positive samples depended on the primer pair used for PCR. The differences observed for herpes-like virus DNA detection depending on the PCR primer pairs used indicate that some primer pairs are most adapted for epidemiologial surveys. Thus, this primer pair (OHV/OHV114) seems well adapted for herpes-like virus DNA detection because of processing ease (simple PCR reactions) and great sensibility. A rapid response can be obtained for herpes-like virus detection. This PCR protocol using the primer pair OHV3/OHV114 allows to limit reagent use and to reduce contamination.

The present results indicate that the PCR represents a fast, sensitive and reliable method for the detection of herpes-like virus infecting oysters. The procedure would be useful not only for disease diagnosis but also for studying the basic biology of the emerging bivalve herpes-like viruses. It is necessary to pursue the study in order to define the involvement of herpes-like viruses in larval bivalve mortalities in private hatcheries.

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Table 1 : PCR analysis results for larval oyster (*Crassostrea gigas*) samples originating from Hatchery 1

Batch	Species	Origin	Sampling date	Spawning date	Mortality	Comments	PCR results (OVH3/OHV4)	PCR results (OHV3/OHV114)	Nested PCR results (A3/A4 and A5/A6)
0	*C. gigas*	1	07/12/97	28/11/97	No mortality	Growth better than average	-	-	-
1	*C. gigas*	1	17/06/98	07/06/98	No mortality	160-285µm larvae after grading on 130µm mesh. Growth better than average	-	-	-
2 - Bin1	*C. gigas*	1	28/06/98	18/06/98	**Mortality**	No growth. Growth excellent on Day 9, but much mortality on Day 10 because salinity drop	-	-	-
2 - Bin2	*C. gigas*	1	28/06/98	18/06/98	**Mortality**	Small growth. Growth excellent on Day 9, but much mortality on Day 10 because salinity drop	-	-	-
G3	*C. gigas*	1	14/08/98	04/08/98	**But soon after metamorphosis 90% mortality, unlike batches before and after**	Started well but bad on day 2, then water source changed and they grew very well	-	-	-
G4	*C. gigas*	1	?	23/09/98	?	Bad start with only 7% of eggs becoming D-larvae. Water source suspect. From D stage G4 grew better than average	-	-	-

Table 1 (following): PCR analysis results for larval oyster (*Crassostrea gigas*) samples originating from Hatchery 1

Batch	Species	Origin	Sampling date	Spawning date	Mortality	Comments	PCR results (OVH3/OHV4)	PCR results (OHV3/OHV114)	Nested PCR results (A3/A4 and A5/A6)
5	*C. gigas*	1	10/10/98		No mortality		-	ND	ND
6	*C. gigas*	1	30/11/98		No mortality		+/-	ND	ND
7	*C. gigas*	1	22/12/98		No mortality		-	ND	ND
9	*C. gigas*	1	05/02/99		No mortality		-	ND	ND
10	*C. gigas*	1	26/04/99		No mortality		-	ND	ND
11	*C. gigas*	1	26/04/99		No mortality		-	ND	ND
12	*C. gigas*	1	21/06/99		No mortality		+/-	ND	ND
13	*C. gigas*	1			No mortality		-	ND	ND
13	*C. gigas*	1			No mortality		-	ND	ND
D larvae	*C. gigas*	1			No mortality		-	ND	ND
15	*C. gigas*	1	16/11/99		No mortality	J10	?	ND	ND
16	*C. gigas*	1	13/12/99	03/13/99	No mortality	J10	?	ND	ND
17	*C. gigas*	1	14/01/00	04/01/00	No mortality	J10	?	ND	ND
18	*C. gigas*	1		22/05/00	**A bit of mortality. Diet problem**	J10	?	ND	ND
21	*C. gigas*	1		05/04/00	No mortality	J10	?	ND	ND

N. B. : (?) - Detection of bands presenting unexpected sizes on agarose gels. N. D. not determined. +/- : a very faint band

Table 2 : PCR analysis results for larval oyster (*Crassostrea gigas*) samples originating from Hatchery 2

Batch	Species	Origin	Sampling date	Spawning date	Mortality	Comments	PCR results (OVH3/OHV4)	PCR results (OHV3/OHV114)	Nested PCR results (A3/A4 and A5/A6)
1	*C. gigas*	2	15/06/98	05/06/98	No mortality	Early eye stage from batch of 20 millions. Growth better than average	-	-	-
« Richard »	*C. gigas*	2	24/07/98	13/07/98	No mortality	Growth average or better	-	-	-
Bin 6	*C. gigas*	2	20/03/00		No mortality	Top	?	ND	ND
Bin 6	*C. gigas*	2	20/03/00		No mortality	Bottom	?	ND	ND
Bin 9	*C. gigas*	2	20/03/00		No mortality	Top	?	ND	ND
Bin 9	*C. gigas*	2	20/03/00		No mortality	Bottom	?	ND	ND

N. B. : (?) - Detection of bands presenting unexpected sizes on agarose gels. N. D. not determined

Table 3 : PCR analysis results for larval oyster (*Crassostrea gigas*) samples originating from Hatchery 3

Batch	Species	Origin	Sampling date	Spawning date	Mortality	Comments	PCR results (OVH3/OHV4)	PCR results (OHV3/OHV114)	Nested PCR results (A3/A4 and A5/A6)
G18/8>140<160	*C. gigas*	3	27/08/98	18/08/98	**High mortality**	Two water qualities for larval rearing	?	**+ (2+/2 sample tubes)**	- (2-/2 sample tubes)
G8/9 surface >100	*C. gigas*	3	15/09/98	08/09/98	**Low mortality**		?	**+ (2+/2 sample tubes)**	**+ (2+/2 sample tubes)**
G8/9 sand high >100	*C. gigas*	3	15/09/98	08/09/98	No mortality		?	- (2-/2 sample tubes)	- (2-/2 sample tubes)
G8/9 surface	*C. gigas*	3	16/09/98	08/09/98	**High mortality**		?	**+ (2+/2 sample tubes)**	**+ (2+/2 sample tubes)**
G8/9 sand high	*C. gigas*	3	16/09/98	08/09/98	No mortality on 16/09/98	**Total mortality on 17/09/98**	?	**+ (2+/2 sample tubes)**	**+ (2+/2 sample tubes)**
99- 4G	*C. gigas*	3	26/02/99	16/02/99	No mortality		ND	-	ND
99-5G	*C. gigas*	3	11/03/99	01/03/99	No mortality		ND	-	ND
99-6G	*C. gigas*	3	26/03/99	16/03/99	No mortality		ND	-	ND
99-7G	*C. gigas*	3	10/04/99	30/03/99	No mortality		ND	-	ND
99-8G	*C. gigas*	3	23/04/99	13/04/99	No mortality		ND	-	ND
99-10G	*C. gigas*	3	06/05/99	27/04/99	No mortality		ND	-	ND
99-11G	*C. gigas*	3	21/05/99	11/05/99	No mortality		ND	-	ND
99-12G	*C. gigas*	3	04/06/99	25/05/99	No mortality	> 180T	ND	-	ND
99-12G	*C. gigas*	3	04/06/99	25/05/99	No mortality	> 180 HT	ND	-	ND
99-12G	*C. gigas*	3	04/06/99	25/05/99	No mortality	> 180	ND	-	ND
99-12G	*C. gigas*	3	04/06/99	25/05/99	No mortality	> 180 H	ND	-	ND
99-13G	*C. gigas*	3	18/06/99	08/06/99	No mortality		ND	-	ND
99-1b	*C. gigas*	3	18/06/99	08/06/99	No mortality		ND	-	ND
99-15G	*C. gigas*	3	18/08/99	10/08/99	**High mortality**	**First mortality at J3**	ND	+	ND

N. B. : (?) - Detection of bands presenting unexpected sizes on agarose gels. N. D. not determined

Table 3 (following) : PCR analysis results for larval oyster (*Crassostrea gigas*) samples originating from Hatchery 3

Batch	Species	Origin	Sampling date	Spawning date	Mortality	Comments	PCR results (OVH3/OHV4)	PCR results (OHV3/OHV114)	Nested PCR results (A3/ and A5/A6)
99-21G	*C. gigas*	3			No mortality	< 275 µm	-	ND	ND
99-21G	*C. gigas*	3			No mortality	> 200 µm	-	ND	ND
00-3G	*C. gigas*	3		11/02/00	No mortality		-	ND	ND
00-6G	*C. gigas*	3		21/04/00	No mortality	> 180 µm	-	ND	ND
00-8G	*C. gigas*	3		26/05/00	No mortality		-	ND	ND
00-10G	*C. gigas*	3			No mortality	> 180 µm	-	ND	ND
00-11G	*C. gigas*	3			Mortality	> 100 µm	+	ND	ND

N. D. not determined

Table 4 : PCR analysis results for larval oyster (*Crassostrea gigas*) samples originating from Hatchery 4

Batch	Species	Origin	Sampling date	Spawning date	Mortality	Comments	PCR results (OVH3/OHV4)	PCR results (OHV3/OHV114)	Nested PCR results (A3/A4 and A5/A6)
P1	*C. gigas*	4			Mortality	Top - J10 – 60 µm	-	-	-
P1	*C. gigas*	4			Mortality	Bottom - J10	+	+	-
P2	*C. gigas*	4			Mortality	Top - J6 - 40 µm	-	-	-
P2	*C. gigas*	4			Mortality	Bottom - J6 - 40 µm	-	-	-
P2	*C. gigas*	4			Mortality	Top - J7 - 60 µm	-	-	-
P3	*C. gigas*	4			Mortality	Bottom	-	+	-
P13	*C. gigas*	4		27/06/99	Mortality	Top - J5	-	+	-
P13	*C. gigas*	4		27/06/99	Mortality (80 %)	Bottom - J5 –	-	-	-

Table 5 : PCR analysis results for larval oyster (*Crassostrea gigas*) samples originating from Hatchery 5

Batch	Species	Origin	Sampling date	Spawning date	Mortality	Comments	PCR results (OVH3/OHV4)	PCR results (OHV3/OHV114)	Nested PCR results (A3/A4 and A5/A6)
G2 (QG)	*C. gigas*	5	24/12/98	14/12/98	**All spat died within they were 30 day old in 2/3 days**	J10	ND	-	ND
G3	*C. gigas*	5	08/02/99	28/01/99	No mortality	J1I - 60 % survival from D-larvae	ND	-	ND

N. D. not determined

Table 6 : PCR analysis results for larval oyster (*Ostrea edulis*) samples originating from Hatchery 2

Batch	Species	Origin	Sampling date	Spawning date	Mortality	Comments	PCR results (OVH3/OHV4)	PCR results (OHV3/OHV114)	Nested PCR results (A3/A4 and A5/A6)
Bin 6	*O. edulis*	2	10/08/98?		No mortality		-	-	-

Table 7 : PCR analysis results for larval clam (*Ruditapes decussatus*) samples originating from Hatchery 3

Batch	*Species*	Origin	Sampling date	Spawning date	Mortality	Comments	PCR results (OVH3/OHV4)	PCR results (OHV3/OHV114)	Nested PCR results (A3/A4 and A5/A6)
	Ruditapes decussatus	3	25/09/98	22/09/98	No mortality	Pale digestive gland Two water qualities	- (for both water qualities)	- (for both water qualities)	- (for both water qualities)
	Ruditapes decussatus	3	26/09/98	22/09/98	No mortality	Two water qualities	- (for both water qualities)	- (for both water qualities)	- (for both water qualities)
	Ruditapes decussatus	3	27/09/98	22/09/98	No mortality	Two water qualities	- (for both water qualities)	- (for both water qualities)	- (for both water qualities)
	Ruditapes decussatus	3	28/09/98	22/09/98	**Mortality in both water qualities**	Two water qualities	- (for both water qualities)	- (for both water qualities)	**+ for one water quality**/- for the other one
	Ruditapes decussatus	3	29/09/98	22/09/98	**Mortality in both water qualities**	Two water qualities	**+ for one water quality**/- for the other one	**+ (for both water qualities)**	**+ (for both water qualities)**
	Ruditapes decussatus	3	30/09/98	22/09/98	**Mortality in both water qualities**	Two water qualities	- (for both water qualities)	**+ (for both water qualities)**	**+ for one water quality**/- for the other one
	Ruditapes decussatus	3	01/03/99 (J9)	22/02/99	**Mortality after metamorphosis**		ND	-	ND
	Ruditapes decussatus	3	15/06/99 (J8)	07/06/99	No mortality		ND	-	ND

Table 8 : PCR analysis results for larval oyster (*Ostrea edulis*) samples originating from Hatchery 4

Batch	Species	Origin	Sampling date	Spawning date	Mortality	Comments	PCR results (OVH3/OHV4)	PCR results (OHV3/OHV114)	Nested PCR results (A3/A4 and A5/A6)
	O. edulis	4		27/06/99	No mortality	Top - J11	ND	+	ND
	O. edulis	4		27/06/00	No mortality	Bottom - J13	ND	+	ND

N. D. not determined

Table 9 : PCR analysis results for larval clam (*Ruditapes philippinarum* and *R. decussatus*) samples originating from Hatchery 5

Batch	*Species*	Origin	Sampling date	Spawning date	Mortality	Comments	PCR results (OVH3/OHV4)	PCR results (OHV3/OHV114)	Nested PCR results (A3/A4 and A5/A6)
S12	*Ruditapes philippinarum*	5	16/10/98			J10	ND	+	ND
S15	*Ruditapes philippinarum*	5	24/11//98			J10	ND	-	ND
S3	*Ruditapes philippinarum*	5	08/02/99	28/01/99	Very good growth and survival	J11	ND	-	ND
D10	*Ruditapes decussatus*	5	24/12//98	11/12/98		J13	ND	-	ND
D11	*Ruditapes decussatus*	5	05/01/99	26/12/98	No problem at larval stages	J10	ND	-	ND
D11	*Ruditapes decussatus*	5	04/02/99	26/12/98	**Decreasing activity and mortality**	J40	ND	-	ND
D1	*Ruditapes decussatus*	5	29/01/99	19/01/99	**At 16th day thay went to the bottom and started to die**	J10	ND	-	ND