Journal of General Virology (1999), **80**, 1317–1322. Printed in Great Britain

Purification and partial genome characterization of a herpes-like virus infecting the Japanese oyster, *Crassostrea gigas*

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First observed in 1972 in *Crassostrea virginica*, herpes-like viruses of bivalves were more recently found to be associated with high mortality rates in other cultured oyster species, such as *Crassostrea gigas* and *Ostrea edulis*. The diagnosis of herpes-like virus infections is performed currently by laborious histological and transmission electron microscope examinations. Preparation of specific reagents for use in more amenable diagnostic techniques prompted purification of virus particles and investigation of the viral genome. This paper is the first description of the purification of a virus pathogen from a bivalve mollusc. A procedure was developed which facilitated purification of large amounts of virus particles on the 40–50% interface of sucrose gradients. Transmission electron microscopy showed that a purified virus suspension contained capsids and enveloped virus particles. High molecular mass viral DNA was extracted, and the genome size was estimated by the summation of the sizes of restriction endonuclease fragments to be approximately 180 kbp. Partial cloning of the virus genome was achieved and the specificity of certain cloned fragments was established by dot blot hybridization.

Introduction

More than 100 different herpesviruses have been described in higher and lower vertebrates (Roizman & Baines, 1991). Concerning invertebrates, viruses morphologically related to the *Herpesviridae* have been identified in different oyster species (Renault *et al*., 1994 *a*). The first was observed in *Crassostrea virginica* (Farley *et al*., 1972). Subsequently, herpes-like viruses in other cultivated oyster species, such as *Crassostrea gigas* (Hine *et al*., 1992; Nicolas *et al*., 1992; Renault *et al*., 1994 *b*) and *Ostrea edulis* (Comps & Cochennec, 1993), have been found to be associated with high mortality rates. More recently, other herpes-like virus infections have been described in *Ostrea angasi* adults (Hine & Thorne, 1997) and in *Tiostrea chilensis* larvae (Hine, 1997).

Herpes-like virus infections in bivalves seem to be ubiquitous and are associated with substantial mortality levels. Since 1991, sporadic high mortality rates of larval *C. gigas* have been regularly observed in some French private hatcheries, occurring each year during the summer period in association with the detection of a herpes-like virus (Renault *et al*., 1994 *b*). Three to four days after the spond, reduction in larval feeding and swimming activities are observed. Significant mortality occurs by day 6, with 100% mortality by day 8–12 in most affected batches (Renault *et al*., 1994 *b*).

As seen by electron microscopy, infected cells in infected bivalves exhibit intranuclear and intracytoplasmic virus particles. The nuclei contain spherical or polygonal particles, 70–100 nm in diameter, depending on the infected oyster species (Farley *et al*., 1972; Hine *et al*., 1992; Nicolas *et al*., 1992; Comps & Cochennec, 1993; Renault *et al*., 1994 *a*; Hine, 1997; Hine & Thorne, 1997). Some particles appear empty and consist of structures assumed to be capsids; other contain an electron-dense core and are interpreted as being nucleocapsids. Enveloped particles are detected in cytoplasmic vesicles and extracellular spaces. These particles consist of a capsid with an electron-dense nucleoid that is in turn surrounded by a unit-membrane-like structure. The enveloped virions, 90–180 nm in size, exhibit spike-like protrusions on the surface, and sometimes a tail (Farley *et al*., 1972; Hine *et al*., 1992; Nicolas *et al*., 1992; Comps & Cochennec, 1993; Renault *et al*., 1994 *a*; Hine, 1997; Hine & Thorne, 1997).

To date, studies of viruses of bivalve molluscs have been

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The GenBank accession number of the sequence reported in this paper is HJ223507.

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limited to descriptive aspects. Moreover, diagnosis of herpes-like virus infections is currently performed by laborious histological and transmission electron microscope (TEM) examinations. Also, marine mollusc cell lines for virus cultivation are not available. Thus, specific probes are needed to aid the development of rapid diagnostic techniques capable of processing large sample numbers.

Most progress has been made with the herpes-like virus described in *C. gigas* (Nicolas *et al*., 1992). Experimental reproduction of the disease in larval *C. gigas* has been achieved (Le Deuff *et al*., 1994), thus establishing the pathogenicity of the virus for individuals at this stage of development and enabling further studies of the virus *in vivo*. The effect of temperature on the development of the virus in larvae has been demonstrated (Le Deuff *et al*., 1996). Attempts to propagate the virus in fish and insect cell lines and in primary cultured *C. gigas* cells were unsuccessful (Le Deuff, 1995). In this report, purification of *C. gigas* herpes-like virus has been achieved. This paper is the first describing the purification of a virus pathogen from a bivalve mollusc.

Methods

Biological material. Although both larval and juvenile *C. gigas* can be affected by herpes-like virus infections, observations by TEM revealed that larvae exhibit generalized infections, whereas only focal infections were generally detected in spat (Renault *et al*., 1994*a*). For this reason, several batches of *C. gigas* larvae (60–250 μm) were used as a source of infected material. Larvae were provided by private French hatcheries suffering mortalities associated with herpes-like viruses. They were stored at 4 °C for a maximum of 48 h until processed. Infection by herpes-like virus in these batches of larvae was confirmed by examination of specimens using TEM. Several batches of juvenile and larval *C. gigas*, healthy or infected with herpes-like virus, and several healthy adult *C. gigas* were also stored at −20 °C for DNA extraction.

Virus purification. Purification of virus particles was achieved using sea water as ‘buffer’. Sea water was filtered through 0·2 μm membranes to remove particulate contaminants and autoclaved. Just before use, it was filtered again through 0·2 μm membranes and PMSF was added to 5 ng/ml. Initial amounts of 10–20 g of *C. gigas* larvae were used for each purification, and subsequent additions were scaled appropriately. A 10 g batch of larvae was mixed with 50 ml sea water–PMSF and 20 g of 400 μm sand. The sand had been washed previously with 5% SDS, rinsed with tap water and then distilled water and baked at 450 °C for 1 h. The mixture was homogenized using an Ultra-Turrax tissue homogenizer. The suspension was diluted by adding 100 ml sea water–PMSF and homogenized again. The resulting suspension was sieved successively through 250 μm and 60 μm nylon meshes to remove sand and larger shell debris, and was then clarified in three steps at 250 ***g***, 1000 ***g*** and 4000 ***g*** for 30 min each. The resulting supernatant was centrifuged for 1 h at 200000 ***g***. The pellet was resuspended in 50 ml sea water–PMSF and mixed at 4 °C using a magnetic stirrer until it had completely resuspended (30–60 min). The suspension was layered on discontinuous sucrose gradients composed of five fractions, 60% (6 ml), 50% (5 ml), 40% (6 ml), 30% (6 ml) and 10% (5 ml) sucrose (w/w) prepared in sea water without PMSF. Volumes of 5–6 ml of virus suspension were layered onto each gradient tube. The gradients were centrifuged for 30 min at 80000 ***g***. Fractions (1–2 ml) were collected at each interface, and corresponding fractions were pooled from different gradient tubes. The fractions were diluted four times by adding sea water drop by drop, and the virus was pelleted for 90 min at 300000 ***g***.

Transmission electron microscopy. Virus pellets from sucrose gradients were treated in two ways. For negative staining, a small piece of pellet was resuspended in filtered (0·2 μm) sea water, particles were adsorbed on collodion-coated 200 mesh copper grids and negatively stained using filtered (0·2 μm) 2% phosphotungstic acid (PTA). A small piece of each pellet was also fixed in 3% glutaraldehyde in 0·2 M cacodylate buffer (pH 7·2), post-fixed with 1% osmium tetroxide in the same buffer and embedded in Epon resin using methods already described (Renault *et al*., 1994*b*). Ultrathin sections were contrasted with uranyl acetate and lead citrate, using conventional methods. *C. gigas* larvae were treated as above, but a decalcification step in a solution of 5% EDTA was performed prior to Epon embedding (Renault *et al*., 1994*b*). Observations were performed using a JEOL JEM 1200EX transmission electron microscope operating at 60 kV.

Nucleic acid extraction. Purified virus pellets were suspended in 0·5 ml extraction buffer (100 mM NaCl, 10 mM Tris–HCl, 25 mM EDTA, 0·5% SDS, pH 8) using a Potter homogenizer. Proteinase K was added to a concentration of 0·2 mg/ml and the suspension was incubated at 50–56 °C for 3 h. Proteinase K was added again to a final concentration of 0·4 mg/ml and the incubation proceeded at 50–56 °C for 15 h. Nucleic acid was extracted twice with phenol–chloroform–isoamyl alcohol (25 : 24 : 1) and twice with chloroform–isoamyl alcohol (24 : 1). A 10 μl aliquot of 10 mg/ml glycogen was then added to the aqueous phase in order to improve nucleic acid recovery, followed by half a volume of 7·5 M ammonium acetate and two volumes of absolute ethanol. The nucleic acid was allowed to precipitate for 1 h at −80 °C, pelleted, rinsed with 70% ethanol, dried and resuspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8). DNA was directly extracted from larval *C. gigas* as described above but an additional RNase treatment was carried out (Sambrook *et al*., 1982). DNA from adult and juvenile *C. gigas* was extracted by powdering them in liquid nitrogen, treating with an electric mincer, and extracting DNA as described above for larvae.

Agarose gel electrophoresis. DNA extracted from purified virus was digested with *Eco*RI or *Bgl*II. Electrophoretic separation of restriction fragments was performed in 0·7% agarose. Plasmid digests were run in 1% agarose gels. Ethidium bromide incorporated into the buffer (0·5 μg/ml) was used to stain DNA bands. Phage lambda *Eco*RI and *Hin*dIII DNA double digests were used as mass markers (Eurogentec). Patterns of DNA bands were analysed using Electro 2.00 (Alcatel) image analysis software.

Partial cloning and screening. Viral DNA was digested with *Eco*RI and ligated into the *Eco*RI site of pBluescript KS II−. Transformation was carried out using competent *Escherichia coli* XL-1 Blue cells. The boiling method was used to obtain minipreparations for screening transformed plasmids, and large-scale preparations made by alkaline lysis and CsCl purification were used for plasmids of interest (Sambrook *et al*., 1982).

DNA labelling, dot blot preparation and hybridization. After denaturation, DNA was spotted onto nylon Hybond-N+ membranes (Amersham) following established procedures (Costanzi & Gillespie, 1987). Dot blots were prepared with positive controls of DNA extracted from purified virus particles, and recombinant plasmids corresponding to the cloned fragments were used as probes. The negative control was

DNA extracted from healthy adult *C. gigas*. Other blots were performed using DNA extracted from virus-infected juveniles (one batch) and larval *C. gigas* (four different batches). For each sample and control DNA, quantification was determined by spectrophotometer at 260 nm. Then spots were added to the membrane in tenfold serial dilutions containing 1 µg to 0·1 pg DNA. Probes were prepared from cloned fragments purified after agarose gel electrophoresis of *Eco*RI-digested plasmids. Cloned DNA fragments were recovered from excised agarose bands (Sambrook *et al*., 1982) and the ECL kit (Amersham) was used according to the manufacturer's instructions.

■ **DNA sequencing.** The OmniBase DNA sequencing kit (Promega) was used for sequencing according to the manufacturer's instructions. Sequences were obtained after dried polyacrylamide gels were exposed to X-ray films.

■ **Sequence alignment.** Alignment of nucleotide sequences was performed using the BLAST algorithm described by Altschul *et al*. (1990) and obtained through web site http://www.ncbi.nlm.nih.gov/. Both Blastn and Blastx programs were used.

Results and Discussion

Purification

Before carrying out the purification steps, it was necessary to grind infected material efficiently in order to release virus particles, as oyster larval tissues are surrounded by a shell. As no reference procedure was available, several approaches were taken, including decalcification in 5% EDTA and different mechanical treatments. Efficient dissociation of tissues was achieved when decalcification in 5% EDTA for 2 h was carried out followed by treatment using an Ultra-Turrax tissue homogenizer. However, this method did not lead to purification of virus particles, perhaps because of disassembly of particles during the decalcification step. An alternative method was successful. This consisted of adding sand to the larval samples before treatment using a Ultra-Turrax tissue homogenizer. This mechanical treatment efficiently ground the shells and dissociated tissues and cells.

Different buffers were also assessed for virus purification, taking into account that the larvae were obtained from a marine environment of approximately 1000 mOsm. First attempts using a standard Tris–NaCl–EDTA (pH 8) buffer (Hayashi *et al*., 1987; Seal & St Jeor, 1988) adjusted to 1000 mOsm with NaCl failed to yield purified virus. The best results were obtained using sea water. This empirical 'buffer' was used for further purification attempts. Also, as material used for purification consisted of diseased and dead animal tissues, it was supplemented with a protease inhibitor in order to protect virus particles from degradative enzymes.

Sucrose gradients were used for purifying virus particles as it was likely that the ground oyster tissues contained virus particles in relatively small concentrations, perhaps partially degraded in comparison to other herpesviruses usually obtained from infected cell cultures. Preformed sucrose gradients are frequently used for purification of herpesvirus particles (Hayashi *et al*., 1987, 1989; Seal & St Jeor, 1988; Kanto *et al*., 1994), and enable particles to be separated according to size and density. Other methods of separation, such as isopycnic CsCl gradients, were considered less promising since they are more likely to result in damage to virus particles.

Analysis of sucrose gradient fractions was performed by TEM. TEM after negative staining with PTA did not lead to clear identification of virions (Fig. 1*a*). This was due in part to the presence of a virus envelope and to the presence of cellular debris in most of the purified fractions. Therefore, it was necessary to use an alternative method based on the observation of ultrathin sections of Epon-embedded pellets. This method was time consuming, but facilitated clear identification of the different types of virus particles (capsids and enveloped virions). These particles were found mainly at the 40–50% interface of sucrose gradients. A virus suspension composed of capsids and enveloped particles was recovered from this fraction (Fig. 1*b*). Spherical or polygonal particles, 70–80 nm in diameter, were observed. Some particles appear empty and others contain an electron-dense core. Enveloped particles larger in diameter (110–130 nm) and with a reduced tegument were also detected. A few particles were also found at the 30–40% interface, but were contaminated by abundant cellular debris. Only the 40–50% pure fractions were retained for further studies of the viral genome. The purification method used was reproducible and yielded sufficient quantities of herpes-like virus purified particles to permit subsequent studies of the genome.

Indeed, herpesviruses provide a more difficult challenge than most viruses with regard to the preparation of pure virus particles. Techniques which work well with one virus strain in a particular cell line do not work for other strains of virus in the same cells or for the same strain in different cells. Using 5–45% sucrose gradients, herpes simplex viruses can be clearly observed as a fluffy white band at the centre of the gradient. This band contains the single peak of infectious virus (Killington & Powell, 1985). For gallid herpesvirus 3 (Marek's disease herpesvirus type 2), enveloped particles are detected after centrifugation in sucrose gradients at 48–50% sucrose (Cauchy, 1985).

Nucleic acid extraction and characterization

For this study, a batch of 17·5 g virus-infected *C. gigas* larvae was used. DNA was extracted from approximately half of the final pellet of purified virions. A total of 500 µg of genomic viral DNA was obtained. Analysis on a 0·7% agarose gel revealed a single band of high molecular mass DNA (Fig. 2, lane c). The lack of degraded DNA showed that both the virus purification and DNA extraction methods adequately preserved the integrity of viral DNA.

Digestion with *Eco*RI or *Bgl*II yielded different patterns composed of more than 24 different bands each (Fig. 2, lanes a and b). The sizes of the *Eco*RI and *Bgl*II restriction fragments

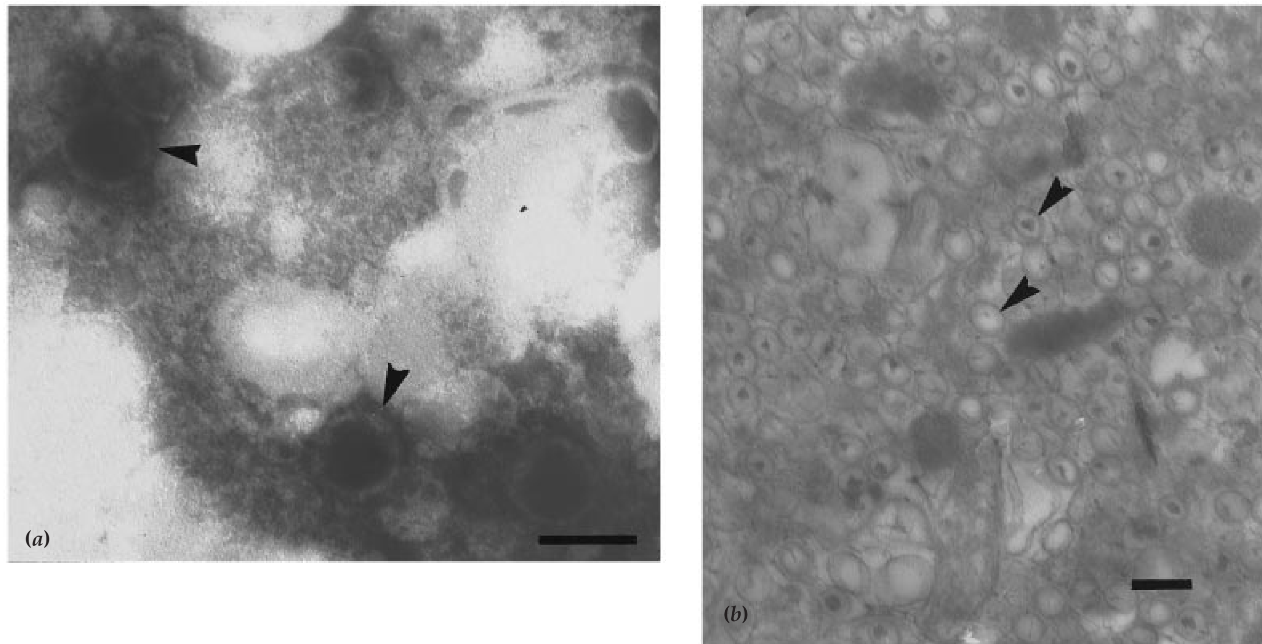

Fig. 1. TEM examination of purified virus particles from infected oyster larvae. (*a*) Negative staining of virus suspensions with PTA. Bar, 100 nm. (*b*) Virus pellets were fixed and embedded in Epon resin, and stained ultrathin sections were then contrasted with uranyl acetate and lead citrate. Bar, 200 nm. Arrowheads indicate virus particles.

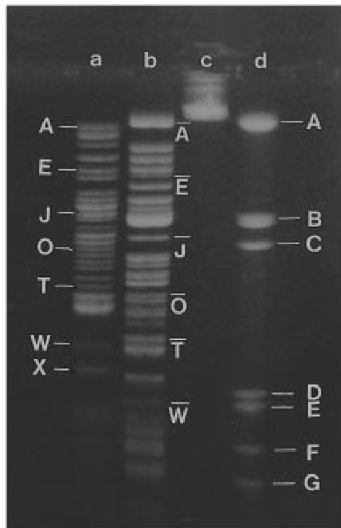

Fig. 2. Ethidium bromide-stained agarose gel showing restriction endonuclease patterns of oyster herpes-like virus DNA digested with *Bgl*II (lane a) or *Eco*RI (lane b). Fragment nomenclature is indicated. Undigested DNA is shown in lane c, and molecular mass markers in lane d (A, 21·23 kbp; B, 5·15–4·98–4·25 kbp; C, 3·52 kbp; D, 2·02 kbp; E, 1·91 kbp; F, 1·58 kbp; and G, 1·38 kbp).

were calculated using the Electro 2.00 software (Table 1). The size of the viral genome was estimated by addition of the 23 largest *Eco*RI restriction fragments (A to W) and, considering that the intense bands A and I each consist of two fragments, an estimated genome size of 177·63 kbp was obtained. The addition of the 24 largest *Bgl*II restriction fragments (A to X) gave a value of 177·19 kbp. Considering the presence of several smaller bands of undefined sizes, the genome size was estimated as approximately 180 kbp. Although this value remains to be defined more precisely using an appropriate method, such as pulsed field gel electrophoresis, it does lie within the range of genome sizes characteristic of the *Herpesviridae* (125–250 kbp).

Partial cloning of the virus genome and specificity of cloned fragments

Our objective was to clone several restriction fragments and check their specificity in order to prepare specific probes for further investigations. Preparation of a complete clone library for this virus remains to be accomplished. To date, 300 transformants have been screened by plasmid isolation. Sizes of cloned fragments ranged from approximately 0·5 to 3·5 kbp.

Four cloned *Eco*RI fragments were chosen arbitrarily for further investigations. From their sizes, these fragments were thought to be the *Eco*RI fragments K (3·4 kbp), T (2·57 kbp), V (2·18 kbp) and Z (1 kbp). The results for fragment V are shown in Fig. 3. Hybridization to dot blots specifically identified positive control DNA (Fig. 3*a*) and also DNA extracted from virus-infected spat and larvae (Fig. 3*b*). No hybridization was detected to DNA extracted from healthy oysters (1 µg to 0·1 ng) (Fig. 3). The other three fragments also showed specific hybridization (data not shown).

The best sensitivity was obtained with peroxidase-labelled probes K and V, which enabled detection of positive hybridization to as little as 1 ng (8×10^{-6} pmole) of genomic

Table 1. Restriction fragments and sizes

After digestion of virus genomic DNA by *Eco*RI or *Bgl*II, restriction patterns exhibit more than 24 fragments of different sizes, summarized below. From the restriction patterns, an approximate size for the virus genome was calculated.

*Eco*RI restriction pattern		*Bgl*II restriction pattern	
Fragment	Size (kbp)	Fragment	Size (kbp)
A	> 21·23	A	20·84
B	16·87	B	19·29
C	14·98	C	17·74
D	13·47	D	15·61
E	12·90	E	13·28
F	10·44	F	12·12
G	8·17	G	9·60
H	6·85	H	8·25
I	5·52	I	6·89
J	3·82	J	5·53
K	3·40	K	5·15
L	3·34	L	4·38
M	3·22	M	3·97
N	3·14	N	3·72
O	3·03	O	3·56
P	2·97	P	3·45
Q	2·85	Q	3·37
R	2·81	R	3·34
S	2·71	S	3·20
T	2·57	T	3·10
U	2·45	U	3·01
V	2·18	V	2·88
W	1·96	W	2·64
X and other smaller bands	< 1·95	X	2·27
		Other smaller bands	< 2·25
Total A to W (A and I repeated twice)	**177·63**	**Total A to X**	**177·19**

virus DNA, and also to 100 ng (38×10^{-6} pmole) of K plasmid or 10 ng ($2{\cdot}9 \times 10^{-6}$ pmole) of V plasmid. For DNA extracted from virus-infected animals, the sensitivity of hybridization depended on the sample. The detection limit was 100 ng for DNA extracted from infected spat and 1–10 ng for DNA extracted from different batches of infected larval *C. gigas*.

Partial sequencing and sequence alignment

Partial sequencing of one cloned fragment was performed. The accession number of the 266 bp sequence obtained is HJ223507. No significant similarities with any other available DNA or imputed protein sequence was obtained, even with sequences from other *Herpesviridae* or other viruses.

Conclusions

This study reports the first successful purification of a virus pathogen for *C. gigas*, a bivalve mollusc. It was prompted by reports of substantial mortalities in cultured larval and spat Japanese oysters since 1991, in France and in other countries (Hine *et al*., 1992; Nicolas *et al*., 1992; Renault *et al*., 1994*a*, *b*). Selected cloned fragments proved to be specific for hybridization to blotted virus DNA or DNA from infected animals. These cloned fragments may now form the basis for establishment of new methods, particularly those utilizing PCR, for diagnosis of this virus infection in oysters. The availability of a procedure enabling purification of virus particles from infected animals may also permit production of specific antibodies by immunization of laboratory animals, and establishment of diagnostic methods based on immunological detection. Development of methods for detecting this virus in oysters is likely to aid understanding of the infection at different stages of oyster development and epidemiological surveys of oyster stocks.

This paper also reports the first description of the genome of a herpes-like virus with an invertebrate host. The DNA sequence data obtained in this study are too limited to enable

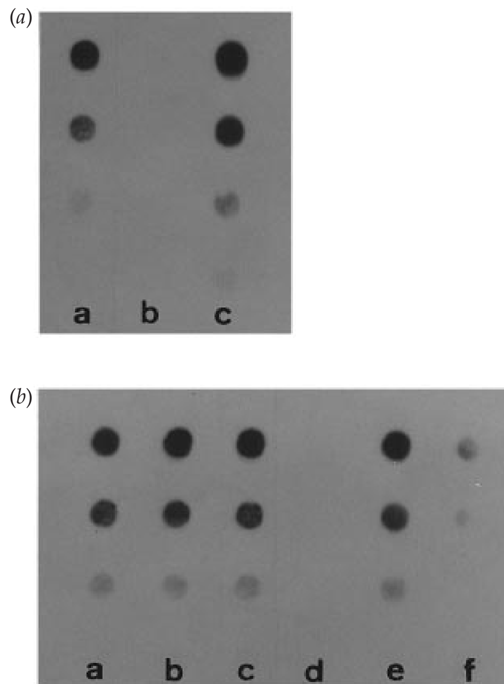

Fig. 3. (*a*) Dot blot showing hybridization of peroxidase-labelled *Eco*RI fragment V to DNA from V plasmid (a), healthy oysters (b) and purified virus (c). Samples of 100 ng to 0·1 ng of DNA were used. (*b*) Dot blot showing hybridization of peroxidase-labelled *Eco*RI fragment V to DNA from virus-infected spat (a–c), healthy oysters (d) and virus-infected larvae (e, f). The samples contained tenfold dilutions of DNA from 0 µg to 10 ng (a–c) or 1 µg to 1 ng (d–f).

conclusions to be drawn regarding relationships between the oyster virus and herpesviruses of vertebrates. However, derivation of more extensive DNA sequence information will facilitate detailed phylogenetic studies in future.

This study could not have been completed without the valuable help and confidence of some private French shellfish farmers. This research was supported in part by the Conseil Général de la Charente-Maritime and by a grant of the Ministère de l'Aménagement du Territoire, de l'Equipement et des Transports (Fonds d'Aide à la Recherche et à l'Innovation, no. 95.07.01). We also thank Dr A. Davison (MRC Virology Unit, Glasgow) for critical comments on the manuscript.

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Received 29 October 1998; Accepted 6 January 1999