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Evidence for interspecies transmission of oyster herpesvirus in marine bivalves

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Since 1991, numerous herpesvirus infections associated with high mortality have been reported around the world in various marine bivalve species. In order to determine whether these infections are due to ostreid herpesvirus-1 (OsHV1), a previously characterized pathogen of the Japanese oyster (*Crassostrea gigas*), PCR analysis was carried out on 30 samples of larvae collected from four bivalve species (*C. gigas*, *Ostrea edulis*, *Ruditapes decussatus* and *Ruditapes philippinarum*), most exhibiting mortality prior to collection. All samples were shown to be infected by OsHV1. Viral genomes in three samples of *C. gigas* and three of *R. philippinarum* that originated from the same hatchery were unusual in bearing a deletion of at least 2·8 kbp in an inverted repeat region. The results demonstrate that OsHV1 is capable of infecting several bivalve species, and this raises the possibility that interspecies transmission may be promoted by intensive rearing in modern hatcheries.

The family *Herpesviridae* comprises over 120 viruses that infect a wide range of vertebrates (Minson *et al.*, 2000). Particles with herpesvirus morphology have also been observed in invertebrates, specifically in marine bivalve species. The first such observation was made with adults of the Eastern oyster, *Crassostrea virginica* (Farley *et al.*, 1972). Subsequently, herpesviruses were detected in other farmed oyster species, such as the Japanese oyster, *Crassostrea gigas* (Hine *et al.*, 1992; Nicolas *et al.*, 1992; Renault *et al.*, 1994*a*, *b*), and the European flat oyster, *Ostrea edulis* (Comps & Cochennec, 1993; Renault *et al.*, 2000*b*). These infections were associated with high levels of mortality in larvae and juveniles. More recently, herpesvirus infections have also been described in *Ostrea angasi* adults (Hine & Thorne, 1997), in *Tiostrea chilensis* larvae (Hine *et al.*, 1998) and in the larvae of two clam species, the grooved carpet shell, *Ruditapes decussatus* (T. Renault, unpublished data), and the Manila clam, *Ruditapes philippinarum* (Renault, 1998).

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Sporadic virus infections of *C. gigas* larvae and juveniles have been observed in France each summer since 1991 (Renault, 1998). In the case of larvae, symptoms typically appear 4–5 days after fertilization. Larvae reduce their feeding and swimming activities and sediment from the water (Le Deuff *et al.*, 1994). Substantial mortality occurs by day 6, reaching 100% by days 8–12 (Renault *et al.*, 1994*a*, 1995). A causative role for the virus in larval mortality is supported by transmission experiments (Le Deuff *et al.*, 1994). In the case of juveniles, high mortality levels (80–90%) occur sporadically among farmed spat at months 3–12 (Renault *et al.*, 1994*b*). Prior to death, no gross physiological signs are detectable. The main histological changes consist of enlarged and abnormally shaped nuclei and abnormal chromatin patterns throughout the connective tissues (Renault *et al.*, 1994*b*, 1995). Mortality or morbidity has not been reported in adult oysters in France.

As histological lesions are not specific to herpesvirus infection and no bivalve cell line is available to facilitate virus culture, infections are routinely diagnosed by detection of virus particles by transmission electron microscopy (TEM). The size, structure and sequence of the genome support the hypothesis that the virus that infects *C. gigas* larvae is a member of the *Herpesviridae* (Le Deuff & Renault, 1999; A. J. Davison, unpublished data), now termed oyster herpesvirus or ostreid herpesvirus-1 (OsHV1; Minson *et al.*, 2000). Specific PCR tools have been developed to enable rapid diagnosis of OsHV1 in large numbers of samples (Renault & Lipart, 1998; Renault *et al.*, 2000*a*).

Herpesviruses have been detected in several bivalve species in different parts of the world, but it is not known whether these agents represent different viruses, as might be anticipated from the fact that vertebrate herpesviruses are invariably associated closely with individual host species. In order to answer this question, samples of larvae from different bivalve species obtained from different locations were analysed by PCR, restriction endonuclease digestion of PCR products and DNA sequencing.

Animals were obtained at 1–26 days after fertilization from three hatcheries on the Atlantic and Channel coasts of France; in Normandy, Vendée (370 km south of Normandy) and

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Charente-Maritime (530 km south of Normandy). Samples were collected during the period 1995–1999, in some cases from broods that presented abnormally high mortality, and were stored at −20 °C. Thirty infected samples were selected for analysis (Table 1). Twenty consisted of *C. gigas* larvae that had sedimented before sampling. Three of these samples (10–12) and the three samples of *R. philippinarum* larvae (13–15) originated from the same hatchery and were obtained during an episode of high mortality. Four samples consisted of *O. edulis* larvae that did not exhibit mortality (1–3 and 29). Lastly, three samples (4–6) consisted of *R. decussatus* larvae presenting high mortality.

Samples of larvae were prepared as described previously (Renault *et al*., 2000*a*). Briefly, 50 mg samples of frozen larvae were macerated by grinding in 50 µl double-distilled water with a disposable tissue homogenizer, boiled and centrifuged. Supernatants were diluted tenfold in double-distilled water and stored at −20 °C. PCR primers were designed for three regions of the genome, based on initial OsHV1 genome sequence data. The sequences of the primers are shown in Fig. 1(*c*). After the analysis was carried out, determination of the DNA sequence of OsHV1 was completed (A. J. Davison, unpublished data), enabling retrospective location of the three regions on the genome (Fig. 1*a*, *b*). Region A encodes an inhibitor of apoptosis belonging to the IAP family, members of which include proteins specified by cellular genomes and by other viruses of invertebrates. Region B encodes a protein of unknown function. Region C encodes two proteins of unknown function and, being located in an inverted repeat, is present as two copies in the genome.

Primary (not nested) PCR was employed for all analyses. Thermal cycling (35 cycles) was carried out as described previously (Renault *et al*., 2000*a*) and products were visualized by agarose gel electrophoresis alongside molecular mass markers. Reference OsHV1 DNA purified from infected *C. gigas* larvae (Le Deuff & Renault, 1999), which was also used for genome sequencing, constituted the positive control and the negative control was double-distilled water. Negative controls were included at every fifth sample during each PCR experiment. It should be noted that specimens were selected on the basis that they were likely to be positive, and that negative samples are detected frequently in routine PCR diagnosis carried out in the laboratory. In some experiments, PCR products were analysed by restriction endonuclease digestion and Southern blot hybridization or were cloned and sequenced.

The primers A3/A4 and B1/B2 amplified fragments of the sizes predicted for OsHV1 from all larval samples and from reference DNA (summarized in Table 1). In contrast, the primers C1/C6 amplified the predicted fragment from only 24 samples. The six negative samples originated from two bivalve species obtained from the same hatchery (10–12 and 13–15) and were shown to contain herpesvirus particles by TEM (Table 1). Further investigations were undertaken on these samples, with the primer pairs C2/C4, C2/C6 and C1/C4 (Fig. 2*a*). No product was obtained with samples amplified with C1/C4. Products were obtained with C2/C6 and C2/C4, but were aberrant in being about 180 bp smaller than those obtained with reference DNA, at 530 and 170 bp, respectively. The negative controls did not yield PCR products in any experiment, indicating that laboratory contamination had not occurred.

PCR products of the anticipated sizes from the A, B and C regions were subjected to restriction endonuclease digestion in order to assess their origins. Digestion profiles of the A3/A4 and B1/B2 products with two endonucleases, each cleaving at between one and three sites, were identical to each other and were exactly as predicted from the genome sequence (data not shown). Digestion of C2/C6 products with three restriction endonucleases (*Xho*I, *Sty*I and *Acs*I) yielded the expected restriction patterns for 24 samples (data not shown). Southern blot hybridization analysis of the smaller PCR products obtained with the primer pair C2/C6 from the six aberrant samples (samples 10–15) yielded fragments consistent with the loss of approximately 180 bp at or near the end of the fragment defined by C2 (Fig. 2*b*). Digestion of the reference PCR product of 709 bp with *Xho*I gave fragments of 335 and 374 bp, *Sty*I yielded fragments of 258 and 451 bp and *Acs*I produced fragments of 153, 263 and 293 bp, as predicted from the genome sequence. *Xho*I generated a fragment of about 160 bp instead of 335 bp from the two aberrant samples shown (samples 11 and 14) and *Sty*I and *Acs*I digests lacked the 258 and 263 bp fragments, respectively.

C2/C4 and C2/C6 products from one of the aberrant *C. gigas* samples (sample 11), one of the aberrant *R. philippinarum* samples (sample 14) and reference DNA were cloned into plasmids and sequenced. Three independent plasmids were sequenced for each product in order to rule out errors induced by PCR amplification. As expected, the reference sequence was identical to the appropriate part of the genome sequence. The sequence of the aberrant fragment from *C. gigas* was identical to that from *R. philippinarum*, but differed from that of the reference (Fig. 1*d*). The differences included several single nucleotide substitutions, insertions and deletions and, more notably, a deletion of 200 bp near the C2 sequence, accompanied by an insertion of 27 bp. These differences would be expected to disrupt the functions of the two proteins encoded by the C region. The provenance of the 27 bp insert is unknown; it is not present in the entire reference virus genome. It is possible that primer C2 functioned as a result of fortuitous hybridization to a non-specific sequence located nearer to the C4 and C6 primer recognition sites in the aberrant genome than the cognate sequence in the OsHV1 genome. Further assays with combinations of six additional primers up to 2·8 kbp upstream from the deletion end-point failed to yield products from the aberrant samples, indicating that a region of the inverted repeat of at least 2·8 kbp is absent from the virus genome. PCR products of the expected sizes were obtained when reference DNA was amplified with these primers. The

Table 1. PCR and TEM analysis of larval bivalve samples

Sample	Hatchery*	Bivalve species	Date of sampling	Mortality before sampling†	TEM analysis‡	Product with PCR primers§		
						A3/A4	B1/B2	C1/C6
1	N	*O. edulis*	28/04/1997	−	+	+	+	+
2	N	*O. edulis*	28/04/1997	−	+	+	+	+
3	N	*O. edulis*	28/04/1997	−	+	+	+	+
4	N	*R. decussatus*	30/09/1998	+	ND	+	+	+
5	N	*R. decussatus*	30/09/1998	+	ND	+	+	+
6	N	*R. decussatus*	30/09/1998	+	ND	+	+	+
7	N	*C. gigas*	07/06/1997	+	+	+	+	+
8	N	*C. gigas*	07/06/1997	+	+	+	+	+
9	N	*C. gigas*	07/06/1997	+	+	+	+	+
10	N	*C. gigas*	15/06/1997	+	+	+	+	−
11	N	*C. gigas*	15/06/1997	+	+	+	+	−
12	N	*C. gigas*	15/06/1997	+	+	+	+	−
13	N	*R. philippinarum*	15/06/1997	+	+	+	+	−
14	N	*R. philippinarum*	15/06/1997	+	+	+	+	−
15	N	*R. philippinarum*	15/06/1997	+	+	+	+	−
16	V	*C. gigas*	08/07/1995	+	+	+	+	+
17	V	*C. gigas*	09/07/1995	+	+	+	+	+
18	V	*C. gigas*	10/07/1995	+	+	+	+	+
19	V	*C. gigas*	10/07/1995	+	+	+	+	+
20	V	*C. gigas*	25/07/1995	+	+	+	+	+
21	V	*C. gigas*	25/07/1995	+	+	+	+	+
22	V	*C. gigas*	27/07/1995	+	+	+	+	+
23	V	*C. gigas*	04/08/1995	+	+	+	+	+
24	V	*C. gigas*	04/08/1995	+	+	+	+	+
25	V	*C. gigas*	24/08/1995	+	+	+	+	+
26	V	*C. gigas*	24/08/1995	+	+	+	+	+
27	V	*C. gigas*	24/08/1995	+	+	+	+	+
28	V	*C. gigas*	17/03/2000	+	ND	+	+	+
29	C	*O. edulis*	11/04/2000	−	ND	+	+	+
30	C	*C. gigas*	01/04/1997	+	+	+	+	+

* Abbreviations: N, Normandy; V, Vendée; C, Charente-Maritime.
† Scored as: +, mortality and sedimentation observed; −, mortality and sedimentation not observed.
‡ Scored as: +, virus particles detected; ND, analysis not done.
§ Scored as: +, product obtained; −, product not obtained.

upstream end-point of the deletion was not determined. Sequencing was also carried out for 380 bp at each end of the 1001 bp PCR product from the A region and for 332 bp in the B region. The reference sequence was identical to the appropriate part of the genome sequence in both regions. Samples 11 and 14 were identical to each other in regions A and B, region B being identical to the reference sequence and region A differing by a single synonymous nucleotide substitution.

The results of this study indicate that four bivalve species belonging to three genera were infected with OsHV1 and that infection of more than one species was not confined to a single hatchery. Infection with the variant of OsHV1 was detected in two bivalve species in a single hatchery during one episode of mortality. The variant did not persist to later episodes, however, as infected samples taken from the same hatchery at subsequent dates yielded PCR products characteristic of reference DNA (data not shown). We conclude that OsHV1 (and the variant) may be transmitted from one species of bivalve to another, and therefore that the natural host of OsHV1 must be considered a matter of uncertainty.

In the natural setting, vertebrate herpesviruses are invariably associated with a single host species. Moreover, the implication that the majority of these viruses have evolved with their hosts over long periods of time finds strong support from molecular phylogenetic studies (McGeoch & Cook, 1994; McGeoch *et al.*, 1995). Exceptionally, transmission can occur from one species to another in the context of farms or zoos. Examples include infection of humans with the simian herpesvirus B virus (Whitley, 1996) and of cattle, sheep, dogs and cats with the porcine herpesvirus pseudorabies virus (Gustafson, 1981). Interspecies transmission may also have

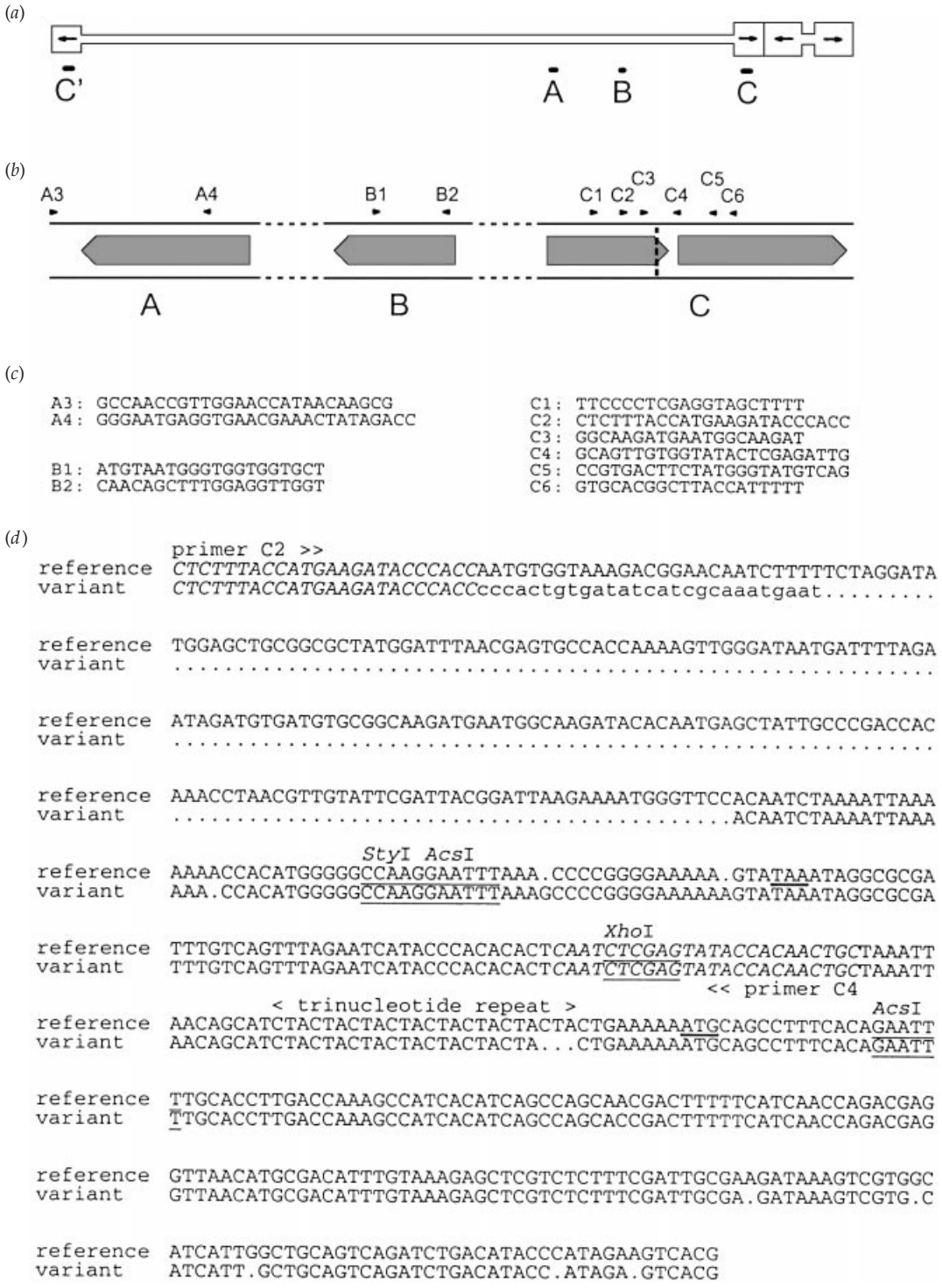

Fig. 1. (*a*) Scale diagram of the three regions (A, B and C) aligned with the 206 kbp OsHV1 genome. Two sets of inverted repeats are depicted as rectangles orientated by arrows. Region C is also present as a second copy (C′). (*b*) Scale diagrams of regions A, B and C, with PCR primers (arrowheads) aligned with predicted protein-encoding regions (shaded arrows). The horizontal dotted lines linking the three regions are of varied length. Sizes of PCR products are: A3/A4, 1001 bp; B1/B2, 464 bp; C1/C6, 896 bp. The sequence that is deleted in aberrant samples is located to the left of the vertical dashed line in region C. (*c*) Sequences of primers. (*d*) Aligned DNA sequences of part of the C region in the reference and variant genomes. Dots indicate missing nucleotides. The locations of primers C2 and C4 are shown, with their sequences in italics. The sequence of the 27 bp insert in the variant is shown in lower case. Relevant restriction endonuclease sites are underlined. The termination codon (TAA) for one coding region and the initiation codon (ATG) for the other are marked by thick underlining. The location of a trinucleotide repeat between the coding regions is indicated.

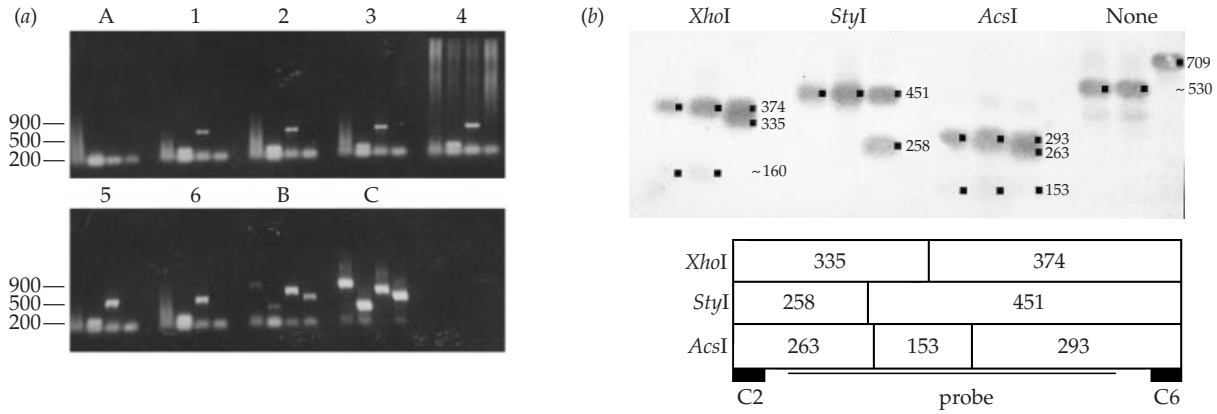

Fig. 2. (*a*) PCR products of aberrant larval samples electrophoresed on a 1% agarose gel. Each group of four lanes shows the results of amplification with C1/C6, C2/C4, C2/C6 and C1/C4, respectively. Groups: A, negative control (double-distilled water), 1–6, the samples listed in Table 1 (samples 10–12 from *C. gigas* and 13–15 from *R. philippinarum*); B, 0·5 pg reference OsHV1 DNA; C, 10 ng reference DNA. Fragment sizes are indicated in bp above the relevant bands for sample 15 and positive control C. Each lane contains a fast-migrating primer artefact. (*b*) Southern blot of restriction fragments of C2/C6 PCR products electrophoresed on a 2% agarose gel and hybridized to a digoxigenin-labelled probe made by PCR of reference DNA with primers C3/C5. Bound probe was detected by using a mouse IgG antibody against digoxigenin followed by a peroxidase-conjugated antibody against mouse IgG, and enzyme activity was visualized colorimetrically with diaminobenzidine in the presence of hydrogen peroxide. Fragments mentioned in the text and their sizes in bp are indicated by dots. Each group of three lanes shows the results for sample 11 in Table 1 (*C. gigas*), sample 14 in Table 1 (*R. philippinarum*) and reference DNA. Restriction maps of the reference PCR product deduced from the genome sequence are shown below, with fragment sizes in bp.

played a limited part in herpesvirus evolution (McGeoch *et al.*, 1995). It is possible that bivalve herpesviruses, like vertebrate herpesviruses, are confined to single host species in nature, but that intensive farming conditions, under which different bivalve species are kept in large numbers in unnaturally close proximity, promote transmission to new host species. It is also possible that OsHV1 is itself a mutant of a virus infecting a single bivalve species that has gained the ability to cross species boundaries.

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