

Experimental herpes-like viral infections in marine bivalves: demonstration of interspecies transmission

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ABSTRACT: Since 1972, herpes-like virus infections have been reported in several marine bivalve species around the world. Viral detection was often associated with high mortality rates in larvae and spat. To determine whether a single virus is able to infect different bivalve host species, we carried out experimental transmission assays. As a first step, 8 assays were performed to infect axenic *Crassostrea gigas* larvae with virus from infected *C. gigas* larvae using a previously described protocol. The protocol appeared reliable and PCR was confirmed as a powerful technique for detecting viral DNA in experimentally infected oysters. The defined protocol was then applied to infect different bivalve species. Interspecies viral transmission was demonstrated under laboratory conditions. The same phenomenon may occur in private hatcheries and may be promoted by intensive rearing conditions. This hypothesis is reinforced by reports of concomitant mortalities in the larvae of several bivalve species and by the first molecular analysis of infected larval samples.

KEY WORDS: Herpes-like virus · Marine bivalves · Interspecies transmission · Host specificity · Experimental infection

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INTRODUCTION

Oyster mortalities associated with the detection of a herpes-like virus were first reported in adult eastern oysters *Crassostrea virginica* in 1972 in the United States of America (Farley et al. 1972). Since 1991, further herpes-like virus infections have been documented in different parts of the world in different marine mollusc species. Viral detection was often associated with high mortality rates in spat and larvae of farmed bivalves, including Japanese oyster *C. gigas* (Hine et al. 1992, Nicolas et al. 1992, Renault et al. 1994a,b) and European flat oyster *Ostrea edulis* (Comps & Cochenne 1993, Renault et al. 2000b). More recently, herpes-like viruses were observed in Australia in adult *O. angasi* (Hine & Thorne 1997) and in *Tiostrea chilensis* larvae in New Zealand (Hine et al. 1998). Lastly, herpesviral infections have been observed in European clam *Ruditapes decussatus* (Renault &

Arzul 2001) and Manila clam *R. philippinarum* larvae (Renault 1998). Herpesviruses infecting bivalves are ubiquitous, but it is not known if these pathogenic agents represent different viruses specifically infecting a host species or a single virus infecting several host species. In natural settings, vertebrate herpesviruses are associated with a single host species, and the question arises as to whether bivalve herpesviruses also present high host specificity. There are only a few reports of transmission of viruses between different marine bivalve species. However, some results are considered preliminary, or the evidence for a viral aetiology for some of the diseases has been questioned (Elston 1997). No data are available on the specificity of herpes-like viruses for marine bivalves.

Considering mortality reports and economic importance of shellfish culture, specific and sensitive diagnosis methods such as the polymerase chain reaction (PCR) (Renault et al. 2000a) were developed to enable rapid detection of herpes-like viruses from many samples. As there were no bivalve cell lines in which to grow the virus, a protocol was perfected to experimen-

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tally transmit herpes-like viruses to axenic *Crassostrea gigas* larvae (Le Deuff et al. 1994). This method allows the study of viral infection, diagnosis and virion production. In the present study, those first trials were repeated and improved to confirm assay reliability. Moreover, infection results were monitored by PCR. We report herein protocol improvements, notably for recovery of infected larvae and in PCR analysis, and the application of a method to infect larvae of different bivalve species. The method developed must demonstrate that viral particles in challenged larvae are not simply those remaining from the inoculum. Trials were conducted in an attempt to transmit a herpes-like virus infecting *C. gigas* larvae to *C. angulata*, *C. rivularis* and *Ostrea edulis* larvae, and a herpes-like virus infecting *Ruditapes philippinarum* larvae, to *C. gigas* larvae.

MATERIALS AND METHODS

Larval production. Axenic larvae were obtained according to Langdon's method (1983) as previously described (Le Deuff et al. 1994). In brief, *Crassostrea gigas* adults were sterilized externally before opening them in a sterile area. Oysters were washed for 5 min with commercial Betadine solution. Gonads were incised and gametes were recovered using a pipette. Fertilization was carried out by mixing spermatozoa and ova at a ratio of 10:1. Axenic larvae were reared in sterile sea water with antibiotics (oxolinic acid, kanamycin, erythromycin and streptomycin sulphate, 0.1 mg l⁻¹; flumequin, 30 µg l⁻¹ and penicillin, 100 units l⁻¹) at a density of 300 larvae ml⁻¹ in 1 l containers. Larvae were maintained at 25°C. No algae were added during breeding periods. Non-axenic larvae were produced using the same protocol without decontaminating the broodstock and sea water. *Ostrea edulis* and *C. rivularis* larvae were reared in 0.22 µm filtered sea water without antibiotics.

Experimental design. The viral source consisted of 400 mg of thawed (from -20°C) *Crassostrea gigas* or *Ruditapes philippinarum* larvae infected with a herpes-like virus, ground using a Dounce tube in 20 ml of sea water and filtered through a 0.22 µm filter.

In the first step, 8 experiments were conducted to standardize the infection protocol, larval recovery and PCR analysis. For each experiment, one 1 l container of 3 d old axenic larvae was

inoculated with a viral suspension (8 ml) and one 1 l container of 3 d old axenic larvae was inoculated with sterile sea water (8 ml). When infected larvae ceased to swim, settlement was observed. Larvae were then recovered with the procedure shown in Fig. 1. Before filtering the water using a 20 µm mesh, 1 ml of water was sampled (Sample C). Larvae were concentrated and washed in filtered sea water 3 times (200 × g, 15 min). One ml of sea water was collected for PCR analysis after each rinse (Samples S1, S2 and S3). After rinsing, a portion of the larvae was treated as previously described for PCR analysis (Renault et al. 2000a). Another portion was fixed in 3% glutaraldehyde for transmission electron microscopy (TEM) examination.

In a second step, the protocol was applied to infect larvae belonging to different bivalve species. To infect *Crassostrea gigas* and *C. angulata* larvae, axenic larval cultures were directly inoculated with viral suspensions obtained from *Ruditapes philippinarum* infected larvae and *C. gigas* infected larvae, respectively.

Infection assays of *Ostrea edulis* and *Crassostrea rivularis* larvae required an additional step. That consisted of experimentally infecting *C. gigas* axenic larvae, which were then inoculated into separate cultures of *O. edulis* and *C. rivularis* larvae. Larval recovery was performed as described above. The bivalve species tested depended on the availability of broodstock ready to reproduce at the laboratory. For each assay, a bacteriological control was carried out and consisted of inoculating 0.2 ml of rearing water into 10 ml of pancreatic peptone casein (1.5% w/v in artificial sea water, pH 7.4).

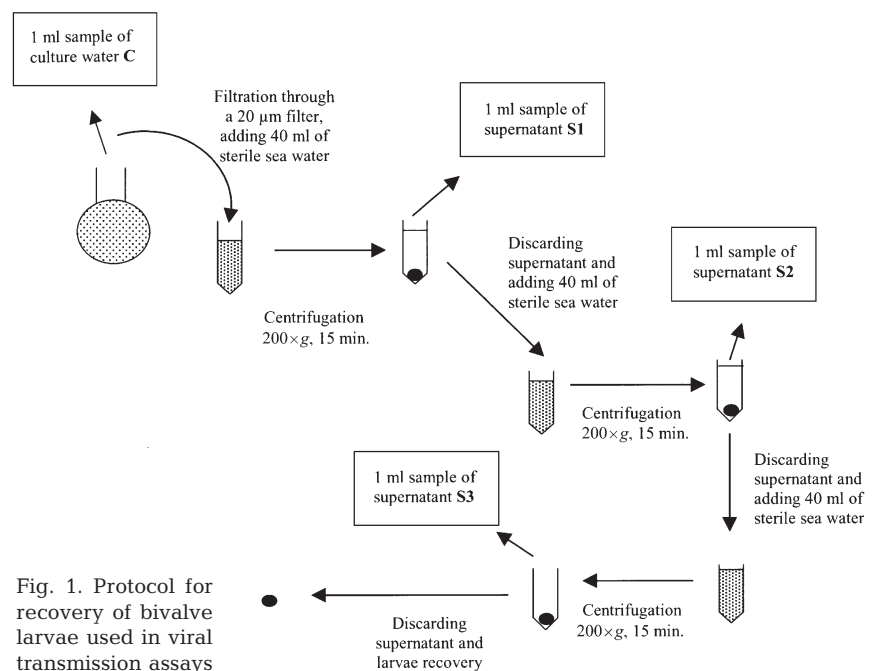


Fig. 1. Protocol for recovery of bivalve larvae used in viral transmission assays

TEM examination. After fixation in 3% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2), larval samples were treated as previously described (Renault et al. 1994a). Ultrathin sections stained with uranyl acetate and lead citrate were examined with a JEOL JEM 1200EX transmission electron microscope at 80 kV.

PCR analysis. PCR analyses were performed using the C2 (5' CTCTTTACCATGAAGATACCCACC 3') - C6 (5' GTGCACGGCTTACCATTTTT 3') primer pair designed from a viral sequence named C (Arzul et al. 2001). This viral sequence encodes 2 proteins of unknown functions, and the expected PCR product had a size of 710 bp. Thermal cycling was carried out as previously described (Renault et al. 2000a). Fifty μ l PCR reactions were performed using Goldstar Polymerase (Eurogentec, Seraing, Belgium), each containing the appropriate buffer, 0.5 mM of each dNTP, 100 ng of each primer, 2.5 mM $MgCl_2$, and 2.5 units of DNA polymerase. One μ l of water containing various amounts of template DNA was added. After 35 amplification cycles, 10 μ l of each PCR product were subjected to electrophoresis on 1% agarose gels, stained with ethidium bromide (0.5 μ g ml^{-1}) and visualized using a 302 nm UV transilluminator. Sizes of DNA products were determined relative to the 1 kb DNA ladder (Eurogentec). The positive control consisted of 10 ng/ μ l OsHV DNA extracted from purified virus particles (Le Deuff & Renault 1999). Larval samples were prepared as previously described (Renault et al. 2000a). In brief, 50 mg of frozen larvae were ground in 50 μ l double-distilled water. Ground larval samples were denatured in a boiling water bath for 10 min and centrifuged at $1000 \times g$ for 5 min. Supernatants were recovered and immediately diluted 10-fold in double-distilled water and frozen at $-20^\circ C$.

RESULTS

Intraspecies transmission assays

Six to 10 d after inoculation of the viral suspension into the containers of axenic larvae of *Crassostrea gigas*, larvae ceased to swim and settled to the bottom of the containers in all 8 transmission trials (Table 1). Larvae exhibited velar lesions and swollen cells as previously described (Le Deuff et al. 1994, 1996) (Table 1). The C, S1 and S2 samples were positive by PCR (Table 1, Fig. 2). The S3 samples showed negative results by PCR (Table 1, Fig. 2). After rinsing, viral DNA was still detected in larval samples (Fig. 2). TEM examination of larvae revealed intranuclear and intracytoplasmic herpes-like viral particles (Table 1, Fig. 3) as previously described (Le Deuff et al. 1994, 1996). No gross sign was observed in negative controls (Table 1).

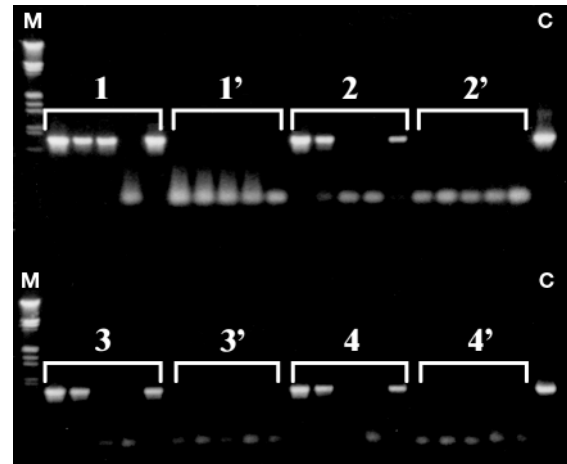


Fig. 2. PCR results of 1 intraspecies and 3 interspecies transmission assays using the primer pair C2-C6. 1: Infection of *Crassostrea gigas* axenic larvae by a viral suspension obtained from infected *C. gigas* larvae. 1': Negative control. 2: Infection of *C. angulata* axenic larvae by a viral suspension obtained from infected *C. gigas* larvae. 2': Negative control. 3: Infection of *Ostrea edulis* non axenic larvae by contact with infected *C. gigas* axenic larvae. 3': Negative control. 4: Infection of *C. rivularis* non axenic larvae by contact with infected *C. gigas* axenic larvae. 4': Negative control. Each group of 5 lanes shows the results of amplification of C, S1, S2, S3 and larval samples, respectively. M: 1 kb DNA ladder (Eurogentec) and C: positive control (10 ng of OsHV DNA)

PCR analysis was negative and no viral particles were detected by TEM (Table 1, Fig. 2). The bacteriological control was negative for each batch of larvae tested.

Interspecies transmission assays

Exposure of *Crassostrea gigas* axenic larvae to the viral suspension obtained from infected *Ruditapes philippinarum* larvae caused settling of larvae 8 d after challenge (Table 2). Gross signs were associated with

Table 1. PCR and TEM for the 8 axenic *Crassostrea gigas* larvae viral transmission assays. Challenge containers received the viral suspension while non-challenge containers received sterile water. The 8 trials showed the same results

	Gross signs ^a	C	S1	S2	S3	Larvae	TEM results ^c
Challenge containers	+	+	+	+	-	+	+
Non-challenge containers	-	-	-	-	-	-	-

^a+: mortality and settling observed, -: mortality and settling not observed
^b+: product obtained, -: product not obtained
^c+: viral particles observed, -: viral particles not observed

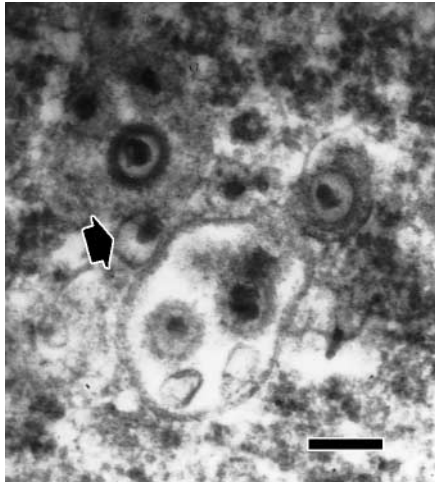


Fig. 3. Infected *Crassostrea gigas* axenic larva showing herpes-like viral particles (arrow). Scale bar = 150 nm

positive PCR results. Samples C, S1 and S2 showed positive PCR results whereas S3 samples were negative (Table 2). Lastly, larvae were positive using PCR (Table 2). Herpes-like viral particles were detected by TEM in inoculated larvae (data not shown). Non-exposed larvae did not settle, and PCR analysis was negative for Samples C, S1, S2 and S3 and larval samples (Table 2).

Six days after exposure of *Crassostrea angulata* larvae to *C. gigas* viral suspension, settling occurred (Table 2). PCR analysis was positive for Samples C, S1 and larval samples (Table 2, Fig. 2). S2 and S3 samples were neg-

ative as all negative control samples (Table 2, Fig. 2). TEM examination revealed herpes-like viral particles in challenged *C. angulata* larvae but none in non-challenged larvae (data not shown).

Two trials consisted of co-habiting 10 d old *Ostrea edulis* larvae and 2 d old *Crassostrea rivularis* larvae with infected *C. gigas* axenic larvae. *O. edulis* larvae settled 6 d after co-habitation. The C, S1 and larval samples showed positive PCR results; S2 and S3 were negative (Table 2, Fig. 2). Herpes-like viral particles were detected by TEM. Non-challenged larval samples were negative by PCR (Table 2, Fig. 2) and no viral particles were observed by TEM (Table 2).

Crassostrea rivularis larvae were recovered 3 d after cohabitation with infected *C. gigas* axenic larvae. Massive larval settlement was observed in the challenge container, although no gross sign was observed in the non-challenged container (Table 2). PCR analysis of the C, S1 and larval samples from the challenge container was positive whereas all the samples from the non-challenged container were negative (Table 2, Fig. 2). No TEM examination was carried out for *C. rivularis* larval samples.

DISCUSSION

Numerous authors have reported experimental herpesviral transmission experiments in higher and lower vertebrates, including fish (Kimura et al. 1981a,b, Wise et al. 1988, McAllister & Herman 1989). Conversely, there have been very few attempts to transmit viral

Table 2. PCR and TEM results for the 4 interspecies transmission assays

Infected species	Viral source	Gross signs ^a	PCR results ^b					TEM results ^c
			C	S1	S2	S3	Larvae	
Axenic larvae <i>Crassostrea gigas</i>	<i>Ruditapes philippinarum</i> viral suspension	+	+	+	+	-	+	+
	Sterile water	-	-	-	-	-	-	-
Axenic larvae <i>Crassostrea angulata</i>	<i>Crassostrea gigas</i> viral suspension	+	+	-	-	+	+	
	Sterile water	-	-	-	-	-	-	
Non axenic larvae <i>Ostrea edulis</i>	<i>Crassostrea gigas</i> infected axenic larvae	+	+	-	-	+	+	
	Healthy axenic larvae	-	-	-	-	-	-	
Non axenic larvae <i>Crassostrea rivularis</i>	<i>Crassostrea gigas</i> infected axenic larvae	+	+	-	-	+	ND	
	Healthy axenic larvae	-	-	-	-	-	ND	

^a+: Mortality and settling observed, -: mortality and settling not observed
^b+: Product obtained, -: product not obtained
^c+: Viral particles observed, -: viral particles not observed, ND = not done

infections to marine molluscs, particularly to bivalves. Some viruses infecting molluscs can be cultured in fish cell lines, including a birnavirus from cultured hard clam *Meretrix lusoria* (Chou et al. 1994) and the unclassified Akoya virus from Japanese pearl oysters *Pinctada fucata martensii* (Miyazaki et al. 1999). Both viruses are non-enveloped and may have a wider host range than enveloped ones. They can be cultivated *in vitro* on heterologous cell lines. Since the herpes-like virus isolated from infected *Crassostrea gigas* larvae was not able to multiply in fish and insect cell lines (Le Deuff 1995), a protocol was developed to experimentally transmit the virus to *C. gigas* axenic larvae (Le Deuff et al. 1994, 1996). This technique constitutes an alternative method to cell culture for diagnosis and production of *C. gigas* herpes-like virus and to study viral pathogenesis. However, the amounts of virus inoculated into containers of axenic larvae remain unknown. A PCR internal standard that allows viral DNA quantitation is available (T.R. unpubl. data), but the amount of DNA in larval samples is not representative of viral infectivity because only enveloped particles are infectious.

Concerning the 8 transmission trials with axenic *Crassostrea gigas* larvae, PCR and TEM analyses confirmed the first assays previously reported (Le Deuff et al. 1994, 1996). However, the protocol was improved, particularly for larval recovery (Fig. 1) and analysis. The real intent was to recover infected larvae with no remaining contamination from inoculated virus. Successive washes may eliminate residual viral DNA from the virus inoculum. Viral DNA detected after the rinsing steps probably corresponds to progeny virus, and hence indicates effective larval experimental infections. Specific molecular tools such as PCR (Renault et al. 2000a) have been developed since the first experimental transmission trials (Le Deuff et al. 1994, 1996). PCR gave results quickly, which were confirmed by TEM analysis (Fig. 3). Detection of intracellular particles by TEM demonstrated that positive PCR results correspond to real infections and not particles tightly adhered to larval surface.

This reliable experimental protocol was used to infect other bivalve species. PCR was used as the most suitable technique for further analysis of larvae. A herpesvirus infecting one bivalve species can infect other bivalve species, specifically, a herpesvirus infecting *Ruditapes philippinarum* was transmitted to *Crassostrea gigas*, and a herpesvirus infecting *C. gigas* was transmitted to *C. angulata*, *C. rivularis* and *Ostrea edulis* larvae. But does this phenomenon occur in the field or in hatcheries? Concomitant herpes-like viral infections were confirmed by TEM examination of cultured *C. gigas* and *O. edulis* (Renault et al. 2000b) and of cultured *C. gigas* and *R. philippinarum* (T.R. unpubl.

data). Interspecies transmission appears possible under hatchery rearing conditions. This hypothesis was reinforced by molecular analysis of infected larvae belonging to different bivalve species, *C. gigas*, *O. edulis*, *R. philippinarum* and *R. decussatus*. Viral DNA sequences were found to be similar for viruses infecting these different bivalve species (I.A. unpubl. data). The detection of viral DNA by PCR in some samples presenting anomalous mortalities may lead to some precautionary measures being taken in order to prevent the spread of viral infections among all larval broods in a hatchery. Moreover, the detection of herpes-like viruses in different bivalve species highlights the risk of rearing different mollusc species together in the same hatchery.

Herpesviruses could have evolved with their bivalve host for a very long period and they would then constitute good molecular markers to understand bivalve evolution and 'migration'. Evolutionary studies of vertebrate herpesviruses suggest that a common viral ancestor existed and that herpesviruses have evolved by substitution, deletion, insertion of nucleotides and recombination processes resulting in gene duplication and divergence, capture of host genes and gene rearrangement (McGeoch 1992, Davison & McGeoch 1995). Persistent viruses, such as herpesviruses, are bound to their hosts by the constraints of specificity which would certainly favor their coevolution (Khanna et al. 1997). Many studies consist of tracing relationships between genotypes and human ethnic groups by analysing DNA polymorphism of HSV-1 strains present in populations of various countries. HSV-1 genotyping appears to be a reliable molecular marker to understand population migrations (Umene & Sakaoka 1997, 1999). Molecular evolutionary genetic analysis shows that Epstein Barr Virus (EBV) isolates have evolved differently according to the regions of the world considered (Khanna et al. 1997).

Coevolution over a long period between a bivalve ancestor and a herpesvirus ancestor is suspected, possibly explaining herpesvirus detection in different host species belonging to different genera and presenting different ways of life and biotopes. In the field, oysters settle on hard substratum whereas clams, which are burrower bivalves, prefer soft substratum. However, both species now being developed together in hatcheries and the proximity it creates may facilitate pathogen transmission and infection of new host species. Bivalve herpesviruses, like vertebrate herpesviruses, may be confined to a single host species in the field, but intensive rearing conditions promote transmission to new host species. Since bivalves have differentiated and specialized, herpesviruses may use conserved systems among bivalves to enter and settle in their host.

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