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Detection of ostreid herpesvirus-1 (OsHV-1) by PCR using a rapid and simple method of DNA extraction from oyster larvae

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ABSTRACT: A DNA extraction procedure was developed for the detection of ostreid herpesvirus-1 (OsHV-1) using the polymerase chain reaction (PCR) in oyster larvae. The DNA extraction procedure developed was tested on 8 larval samples. Abnormal nuclei with characteristic features associated with OsHV-1 infections were only observed in samples in which the viral DNA was detected by PCR. A previously described competitive PCR method was applied to detect inhibition during PCR reactions. The results show that the method can be used on small amounts of oyster larvae (3 mg) for the detection of OsHV-1 DNA by PCR.

KEY WORDS: Herpesvirus · OsHV-1 · Oyster · Larvae · Detection · DNA extraction

INTRODUCTION

Herpes-like virus infections of bivalve molluscs were first reported in *Crassostrea virginica* by Farley et al. (1972). Viruses presumed to belong to the *Herpesviridae* family have subsequently been associated with mortalities in larvae and juveniles of various bivalve species around the world (Hine et al. 1992, Nicolas et al. 1992, Renault et al. 1994, Renault & Arzul 2001). The pathogenicity of the herpes-like virus infecting *Crassostrea gigas* larvae was demonstrated by experimental transmission of the disease to axenic healthy larvae (Le Deuff et al. 1994). The virus was isolated from moribund *Crassostrea gigas* larvae and its genome characterized (GenBank number AY509253), which allowed it to be included in the *Herpesviridae* family. The virus was named ostreid herpesvirus-1 (OsHV-1) (Minson et al. 2000, Arzul & Renault 2002, Davison 2002). The characterization of the virus genome has facilitated the development of molecular tools including detection of viral DNA using the polymerase chain reaction (PCR) (Renault et al. 2000, Arzul et al. 2001, 2002, Lipart & Renault 2002). PCR is a suitable tool for the diagnosis of OsHV-1 infections due to specificity, high sensitivity, time and cost efficiency. The method used to prepare samples for DNA detection using the PCR is known to be critical for the success of the amplification reaction. The procedure currently used for preparing samples for OsHV-1 detection using the PCR (Renault et al. 2000) requires large amounts of larvae (50 mg). The aim of the present study was to develop an alternative rapid and simple procedure of DNA extraction for small samples of oyster larvae (3 mg) that allows the detection of OsHV-1 DNA using PCR.

MATERIALS AND METHODS

Oyster samples. Larvae from a hybridization study between *Crassostrea angulata* and *C. gigas* were reared in 4 independent 150 l tanks. Larvae were collected in September 2003 after 10 and 15 d of development, and fresh-frozen at –80°C.

Sample preparation. Two procedures for total nucleic acid extraction were carried out: (1) a DNA

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sample preparation procedure previously described (Renault et al. 2000), (2) a variation of this procedure using a pre-treatment with Proteinase K/Tween-20. In the first extraction procedure (1), 50 mg of frozen larvae were measured in 1.5 ml microfuge tubes and ground in 50 µl of distilled water. Samples were vortexed and boiled in a water bath for 10 min, followed by quick chilling in ice (Renault et al. 2000). In the second extraction procedure (2), 3 mg of fresh-frozen larvae were measured in 0.2 ml microtube and washed in double distilled water to remove any traces of seawater. Larvae were then ground in 50 µl of extraction buffer solution (5 µl of Goldstar Taq DNA Polymerase buffer, 45 µl of double distilled water and 0.5% of Tween-20) using a single-use curved steel needle. Five microlitres of Proteinase K (10 mg ml^{-1}) were added to each tube. The samples were then incubated at 55°C for 60 min followed by a second incubation step at 100°C for 20 min in a thermal cycler (PTC-100™, MJ Research). Samples were then mixed and centrifuged at 10 000 rpm (1200 × *g*) for 5 min at 4°C. Supernatants obtained by both procedures were recovered in 0.5 ml microfuge tubes, diluted 10-fold in double distilled water and immediately frozen at –20°C.

Detection of OsHV-1 by nested PCR. Two primer-pair combinations were used for the detection of OsHV-1 DNA, the external C5 (5′ CCG TGA CTT CTA TGG GTA TGT CAG 3′)/C13 (5′ CCT CGA GGT AGC TTT TGT CAA G 3′) combination which generated a 765 bp product, followed by the internal C2 (5′ CTC TTT ACC ATG AAG ATA CCC ACC 3′)/C4 (5′ GCA GTT GTG GTA TAC TCG AGA TTG 3′) pair which yielded a 352 bp product (Arzul et al. 2001). All PCR reactions were performed on a Crocodile III thermal cycler (Appligene Oncor). Fifty microlitre PCR reactions were prepared using 2.5 U of Goldstar Taq DNA Polymerase (Eurogentec), 0.05 mM of each dNTP, 100 ng of each primer, 2.5 mM $MgCl_2$ and 1 µl of extracted DNA. After heating samples for 2 min at 94°C, 35 cycles consisting of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min were carried out, followed by a final elongation step of 5 min at 72°C. Nested PCR was carried out using 0.5 µl of the primary reaction as template following identical reaction conditions. Negative controls were incorporated in all PCR runs containing all the PCR reagents and double distilled water instead of template DNA. Genomic OsHV-1 DNA extracted from purified virions infecting *Crassostrea gigas* larvae (Le Deuff & Renault 1999) were used as positive control.

PCR inhibition controls. A competitive PCR method previously developed (Arzul et al. 2002, Renault et al. 2004) was used to detect inhibition during PCR reactions using the primer pair C2 (5′-CTC TTT ACC ATG AAG ATA CCC ACC-3′)/C6 (5′-GTG CAC GGC TTA CCA TTT TT-3′). These primers amplify an OsHV-1 DNA fragment of 710 bp as well as an internal standard competitor that differs from the target viral DNA by a 76 base pair deletion. One microliter of both extracted and competitor DNA was added to the reaction mixture, while the conditions for the PCR reaction were the same as described in the previous paragraph. For the internal standard control, 1 µl of double distilled water was added instead of extracted DNA.

Detection of oyster DNA. A 16S mitochondrial fragment (16SrDNA: the large subunit rRNA-coding gene) was amplified from the oyster larvae following the protocol of Boudry et al. (1998) using the primers described by Banks et al. (1993).

Electrophoresis of PCR products. All PCR products were visualized by electrophoresis in 1.5% agarose gels containing ethidium bromide (0.5 mg l^{-1}) in 1X Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Molecular mass markers (100 bp DNA ladder, Promega) were included in each gel to determine the approximate size of the PCR products, and DNA bands were photographed under UV transillumination.

Microscopical examination. Larvae were fixed in 2.5% glutaraldehyde in 0.2 M cacodylate buffer at pH 7.2 at 4°C. After 2 washes in 0.2 M cacodylate buffer, samples were then post-fixed in 1% osmium tetroxide in the same buffer at 4°C. Larval samples were dehydrated by serial ethanol baths (70 to 100%), cleared twice for 15 min in propylene oxide and infiltrated for 1 h in 50:50 propylene oxide:Epon resin. After 1 h infiltration in pure Epon resin, they were embedded in resin and cured for 48 h at 60°C. One micrometer sections were stained with 0.5% toluidine blue in 1% sodium borate solution for light microscopic examination.

RESULTS AND DISCUSSION

PCR products with the expected size were observed after the first (C5/C13) and second (C2/C4) reactions in one (15 d old *Crassostrea gigas* larvae) of the 8 analysed samples using both DNA extraction methods. Cells with enlarged nuclei of abnormal shape and chromatin pattern characteristic of herpes-like viral infections in mollusc bivalves (Hine et al. 1992, Renault et al. 1994) were observed in semi-thin sections in the 15 d old larvae in which the virus was detected by PCR. No nuclear abnormalities were observed in the 7 samples that did not yield PCR products. These results suggest that: (1) only 1 of the larval batches was infected with OsHV-1 and (2) that the DNA extraction method used was sensitive enough to allow the detection of OsHV-1 DNA from a small amount of larvae. This conclusion is further supported by the mortality

rate of 100% (3 d after the detection of OsHV-1 using PCR) in the infected larval batch, while no subsequent abnormal mortality was observed (data not shown) in any other batch in which the virus was not detected by PCR. The absence of OsHV-1 detection in other batches does not necessarily demonstrate the complete absence of the virus, as low levels of viral DNA in larval tissues may be below the threshold of our methods.

Factors that inhibit the PCR reaction can interfere by nucleic acid degradation or capture and may inhibit polymerase activity for amplification of the target DNA (Wilson 1997). No inhibitory effect was detected in the samples obtained by both DNA extraction methods (Fig. 1). Amplification of the internal standard (634 bp) was observed in all samples in which the nested PCR did not detect the viral DNA. In the positive sample a band of expected size (710 bp) corresponding to viral DNA was observed, as well as a lighter band corresponding to the internal standard (634 bp) (Fig. 1, Lane 7).

False-negative results can also result from inadequate cell lysis and subsequently low levels of DNA for amplification (Wilson 1997). Extraction by boiling alone has been noted to reduce PCR sensitivity due to poor lysis efficiency and insufficient separation of DNA from structural proteins (Todd et al. 1992). Moreover, the high concentration of salt in fresh frozen larvae can inhibit cell lysis and negatively affect PCR. The combined use of Proteinase K/Tween-20 and larval wash with distilled water may thus improve cell lysis and hence increase the efficiency of PCR (Estoup et al. 1996). On the other hand, alternative methods of DNA extraction from *Crassostrea gigas* larvae including Chelex-100 and phenol/chloroform extractions occasionally yield false-negative results (Renault et al. 2000). We successfully amplified the oyster 16S fragments in all our samples (data not shown), hence supporting our extraction methods for good quality DNA.

The DNA extraction procedure described is simple and rapid, and allows the detection of OsHV-1 DNA from small amounts of oyster larvae (3 mg). Moreover, the number of required steps is low when compared to other extraction protocols, hence reducing the risk of sample contamination. This method can be useful for disease diagnosis and studies concerning the mechanisms involved in infections by OsHV-1 when only small quantities of larvae are available. Furthermore, this method may also be used for detection of the virus by PCR in individual larva since it was successfully used in extracting DNA from single *Crassostrea gigas* larva for microsatellite multiplexing (Taris unpubl. data). Therefore, this method can open new perspectives in studies concerning OsHV-1 transmission and putative resistance in early life stages.

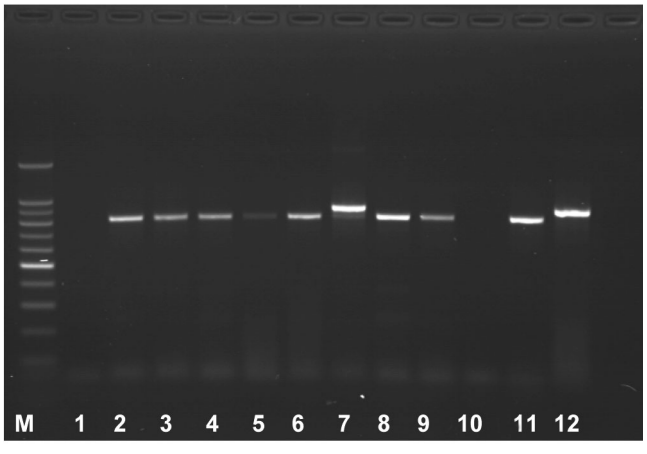

Fig. 1. Ostreid herpesvirus-1. Inhibition controls using a competitive PCR method. Lanes 1 and 10: negative controls; Lanes 2 and 6: 10 and 15 d old larvae (dol) from Tank 1, respectively; Lanes 3 and 7: 10 and 15 dol from Tank 2, respectively; Lanes 4 and 8: 10 and 15 dol from Tank 3, respectively; Lanes 4 and 8: 10 and 15 dol from Tank 4, respectively; Lane 11: internal standard; Lane 12: genomic viral DNA; Lane M: size markers (100 bp DNA ladder, Promega)

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