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Detection of ostreid herpesvirus 1 DNA by PCR in bivalve molluscs: A critical review

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Abstract:

Herpes-like viral infections have been reported in different bivalve mollusc species throughout the world. High mortalities among hatchery-reared larvae and juveniles of different bivalve species have been associated often with such infections. The diagnosis of herpes-like viruses in bivalve molluscs has been performed traditionally by light and transmission electron microscopy. The genome sequencing of one of these viruses, oyster herpesvirus 1 (OsHV-1), allowed the development of DNA-based diagnostic techniques. The polymerase chain reaction (PCR) has been used for the detection of OsHV-1 DNA in bivalve molluscs at different development stages. In addition, the PCR used for detection of OsHV-1 has also allowed the amplification of DNA from an OsHV-1 variant. The literature on DNA extraction methods, primers, PCR strategies, and confirmatory procedures used for the detection and identification of herpesviruses that infect bivalve molluscs are reviewed.

Keywords: Herpesvirus; OsHV-1; Detection; PCR; Bivalve molluscs; Oyster

1. Introduction

Herpes-like viral infections have been identified in various marine bivalve mollusc species throughout the world, including the USA (Farley et al., 1972; Friedman et al., 2005), New Zealand (Hine et al., 1992), France (Nicolas et al., 1992, Arzul et al., 2001b; Renault and Arzul, 2001; Renault et al., 2000b; Renault et al., 2001b), Australia (Hine and Thorne, 1997), and Mexico (Vásquez-Yeomans et al., 2004). The first description of a virus morphologically similar to members of the *Herpesviridae* family in a bivalve mollusc was made by Farley et al. (1972) in the eastern oyster, *Crassostrea virginica*. Since then, a wide host range has been reported for herpes and herpes-like viruses infecting bivalve species, including the Pacific oyster *C. gigas* (Hine et al., 1992), the European oyster *Ostrea edulis* (Nicolas et al., 1992), the Antipodean flat oyster *O. angasi* (Hine and Thorne, 1997), the Chilean oyster *Tiostrea chilensis* (Hine et al., 1998), the Manila clam *Ruditapes philippinarum* (Renault et al., 2001a), the carpet shell clam *R. decussatus* (Renault and Arzul, 2001), the Portuguese oyster *C. angulata* (Arzul et al., 2001a), the Suminoe oyster *C. rivularis* (Arzul et al., 2001a) and the French scallop *Pecten maximus* (Arzul et al., 2001b). High mortalities among hatchery-reared larvae (Hine et al., 1992; Nicolas et al., 1992; Renault et al., 1994; Renault and Arzul, 2001) and juveniles (Comps and Cochennec, 1993; Renault et al., 1994; Renault et al., 2000b; Friedman et al., 2005) of different bivalve species have often been associated with herpes and herpes-like virus infections. Observations by transmission electron microscopy (TEM) revealed that larvae exhibit generalized infections, whereas focal infections were generally observed in spat (Renault et al., 1994). Viral infections were also observed in adult bivalves (Hine and Thorne, 1997; Arzul et al., 2001b, 2002; Vásquez-Yeomans et al., 2004), but adults are apparently less sensitive to such infections as compared to younger stages (Arzul et al., 2002; Renault and Novoa, 2004). The pathogenicity of herpes-like viruses was demonstrated by experimental transmission assays either for larval stages of *C. gigas* (Le Deuff et al., 1994) and other bivalve species (Arzul et al., 2001a, 2001b). Similar experiments were conducted with juveniles and adults of *C. gigas*, but the results were inconclusive (Renault and Novoa, 2004). It is noteworthy that a highly pathogenic herpes-like virus was observed recently by TEM in the gastropod mollusc *Haliotis diversicolor supertexta* in Taiwan associated with high mortality rates (Chang et al., 2005).

The development of a method for purifying herpes-like virus particles from infected *C. gigas* larvae facilitated the extraction of viral DNA and a partial genome characterization (Le Deuff and Renault, 1999). The genome was subsequently completely sequenced (GenBank accession no. AY509253) revealing a tenuous relationship with other herpesviruses. This oyster virus was classified as a member of the *Herpesviridae* family under the name ostreid herpesvirus 1 (OsHV-1) (Minson et al., 2000) and was considered the only member of a new major class of herpesvirus (Davison, 2002; Davison et al., 2005). A variant of OsHV-1 (OsHV-1var) was also described in larvae of different bivalve species (Arzul et al., 2001b, 2001c) and OsHV-1 and OsHV-1var are considered representatives of a single viral species. In contrast with vertebrate herpesviruses, which are generally confined to a single host, OsHV-1 has been identified in several bivalve species and interspecies viral transmission was demonstrated (Arzul et al., 2001a; Friedman et al. unpublished data).

Detection of viruses in mollusc bivalves cannot be done by classic serological methods because molluscs do not produce antibodies. In addition, viral replication in cell culture to facilitate the diagnosis is not possible due to the absence of mollusc cell lines. The diagnosis of herpes-like virus infections has traditionally been performed by light microscopy, as a first approach in order to detect cytological abnormalities, followed by transmission electron microscopy to complete the diagnosis (Hine and Thorne, 1997; Renault et al., 2000b). These procedures are time consuming, impractical for epidemiological surveys, and some viruses can be difficult to detect and identify when present in low amounts. As a result of these limitations, other diagnostic methods have been developed such as immunochemistry and nucleic acid-based techniques. A protocol using polyclonal antibodies produced in BalbC mice immunized with viral particles purified from infected *C. gigas* larvae has been used to detect OsHV-1 proteins (Le Deuff, 1995; Arzul et al., 2002). Another technique that has also been developed is *in situ* hybridization, which allows the detection of viral DNA (Renault and Lipart, 1998; Lipart and Renault, 2002; Barbosa-Solomieu et al., 2004). Both techniques have high sensitivity and specificity, and allow the visualization of viral proteins or DNA, respectively. Polymerase chain reaction (PCR) diagnostic methods have also been developed for detection of OsHV-1 DNA (Renault et al., 2000a; Renault and Arzul, 2001; Arzul et al., 2001a, 2001b, 2001c). PCR is considered a suitable tool for the diagnosis of OsHV-1 infections owing its specificity, high sensitivity relative to other methods (e.g., TEM), ease of sample processing, availability of reagents, and time and cost efficiency. However, as this test only detects viral nucleic acid, other tests such as histology, ISH or antigen-based tests must confirm PCR tests, particularly in a new species or new geographic location. In areas where the pathogen is endemic, PCR alone may be sufficient for diagnosis of its presence. A competitive PCR method was developed that can be used to demonstrate the presence of PCR inhibitors or to quantify OsHV-1 DNA (Renault et al., 2004). Different methods of DNA extraction (Renault et al., 2000a; Arzul et al., 2002; Batista et al., 2005; Friedman et al., 2005) as well as various primer pairs (Renault et al., 2000a; Arzul et al., 2001 a, 2001b, 2001c; Renault and Arzul, 2001; Barbosa-Solomieu et al., 2004, 2005) have been designed and used to detect viral DNA using one-round or nested PCR. In addition, the PCR conditions used for detection of OsHV-1 also allow amplification of DNA from other closely related herpesviruses in different bivalve species, as was the case of OsHV-1var (Arzul et al., 2001b, 2001c). In order to confirm the authenticity of PCR products, different techniques have been used, including digestion of PCR products with restriction enzymes (PCR-RFLP) (Arzul et al., 2001c; Renault et al., 2004; Barbosa-Solomieu et al., 2004, 2005) and DNA sequencing. The objectives of the present work are: (1) to provide a review of the different DNA extraction methods, PCR protocols, primers and confirmatory procedures that have been used in the detection of OsHV-1 DNA by PCR; (2) to discuss the adequacy of the different methods based on published and unpublished data; (3) to address different aspects that should be considered in order to avoid false PCR results.

2. Nucleic acid extraction techniques

The exquisite sensitivity of the PCR reaction can allow the amplification of DNA even when a very small number of target molecules are present. Nevertheless, the type of samples analysed as well as the procedure used for nucleic acid extraction can greatly

affect the success of the amplification reaction. If samples cannot be processed immediately after harvesting they should be conserved and stored under conditions that preserve the integrity of nucleic acids. Extraction of nucleic acids from fresh frozen samples (e.g., larval or adult tissues), stored at -20 or -80 ºC, generally yields good quality DNA for OsHV-1 detection by PCR (Renault et al., 2000a; Arzul et al., 2002; Batista et al., 2005). Samples fixed and stored using non-denatured ethanol have also been used for amplification of OsHV-1 DNA (Friedman et al., 2005; Burge et al., in press; Renault et al., unpublished data). Other trials using formalin fixed samples (e.g., Davidson's and Carson's solutions) have also allowed amplification of herpesvirus DNA from bivalve mollusc samples (Barbosa-Solomieu et al., 2005). However, nucleic acid extraction from formalin-fixed tissues usually yields DNA that is degraded and can affect subsequent nucleic acid-based techniques (Jackson et al., 1990; An and Fleming, 1991), especially those involving larger amplified fragments such as that produced by the nested A primer test of OsHV-1 at 917 bp (Renault et al., 2000a).

Several techniques have been used for the extraction of nucleic acids from different types of bivalve mollusc samples in order to detect herpesvirus DNA by PCR (Table 1). Renault et al. (2000a) used a simple method of nucleic acid extraction from frozen oyster specimens by grinding and boiling the tissues. This method allowed the extraction of nucleic acids and subsequently amplification of viral DNA from larvae and spat of *C. gigas* infected with a herpes-like virus as confirmed by TEM. However, this technique does not appear suitable to amplify OsHV-1 DNA from adults. Other techniques of DNA extraction were also examined by Renault et al. (2000a) namely, Chelex-100 and a combination of proteinase K digestion with phenol/chloroform purification, but both techniques yielded false-negative PCR results. It was also noticed that extraction by boiling ground oyster tissues in some cases produced compounds that inhibited the amplification reaction (Renault et al., 2000a). A method of DNA extraction from oyster larvae was reported by Batista et al. (2005) that combines the use of proteinase K, Tween-20 and boiling. This method allowed the amplification of OsHV-1 DNA by PCR from a small amount of larvae (3 mg). Commercial DNA extraction kits (e.g. Qiagen's QIAamp DNA mini kit using the tissue protocol) have also been used to extract amplifiable herpesvirus DNA from *C. gigas* spat (Friedman et al., 2005, Burge et al. in press). These authors obtained good quality DNA but variable PCR results depending on the amount of template DNA used. Herpesvirus DNA has also been extracted from *C. gigas* adults by grinding, digestion with proteinase K and phenol/chloroform purification from fresh frozen tissues (Le Deuff, 1995).

For decades, specimens of bivalve molluscs have been routinely fixed in formalin and embedded in paraffin for histological analyses. Although extraction of sufficient quantity of amplifiable DNA from these tissues can be difficult (Jackson et al., 1990; An and Fleming, 1991), these samples constitute invaluable resources for pathological analysis using DNA-based techniques. Barbosa-Solomieu et al. (2004) could extract nucleic acids from fixed paraffin-embedded archival samples by deparaffination, digestion with proteinase K and heating. The method allowed the amplification of small fragments (196 and 207 bp) of OsHV-1 DNA, and the absence of PCR inhibitors was demonstrated by amplification of an internal standard. A similar protocol was successfully used by Barbosa-Solomieu et al. (2005), who extracted nucleic acids from oyster tissues preserved in Davidson's solution, which allowed the detection of OsHV-1

by PCR. A study in which the previous technique and a variation of this procedure, using phenol/chloroform purification and ethanol precipitation instead of the heating step, revealed PCR inhibition in 29 and 5 % of the cases, respectively (Batista, personal communication). The samples used in that study had been fixed in Davidson's solution and later transferred to 70 % ethanol. The greater inhibition observed with the boiling protocol could be due to the presence of cell components and DNA degrading enzymes that may have been removed with the phenol/chloroform purification step. Indeed, the removal of inhibitory effects by phenol/chloroform purification has been reported (An and Fleming, 1991). The effect of inhibitors may also be reduced during the PCR reaction with the use of bovine serum albumin (BSA) as outlined below (Friedman et al. 2005). However, methods of nucleic acid extraction with fewer steps should be used preferentially in order to reduce the risk of sample contamination.

3. PCR analysis

Different PCR strategies can be used to detect viral DNA. Some methods utilize one-round PCR (or single PCR) in which the DNA sequence is amplified using one primer pair. Other methods use nested PCR in which first round amplification products are subjected to a second round of amplification and hence two primer pairs are used.

3.1. PCR primers

Based on OsHV-1 sequence data several oligonucleotide primers have been designed for the detection of herpesvirus DNA in bivalve molluscs (Table 2). Different primer pair combinations (Table 3) have been used to amplify DNA by PCR from regions A, B, C and Gp of the virus genome (Fig. 1). The A region encodes a protein of unknown function; the B region encodes a putative inhibitor of apoptosis belonging to the IAP family; the C region encodes parts of two proteins of unknown functions and is present twice in the genome (being located in the inverted repeats TR_L and IR_L,) (Davison et al., 2005); and the Gp region encodes a putative glycoprotein (Arzul et al., 2001b, 2001c).

3.2. Diagnostic specificity and sensitivity

Specificity is defined as the ability of an assay to amplify DNA only from the target agent. Sensitivity (or limit of detection) is defined as the smallest quantity of viral DNA that can be systematically detected.

Two pairs of primers (A3/A4 and A5/A6) corresponding to the A region of the OsHV-1 genome have been used to detect herpesvirus DNA in larvae and spat via nested PCR (Renault et al., 2000a). The specificity of these primer pairs was evaluated using DNA from *C. gigas* as well as DNA from vertebrate herpesviruses. The detection procedure was found to be specific, only amplifying OsHV-1 DNA and not leading to false-positives. The sensitivity of nested PCR using on A3/A4 and A5/A6 primer pairs was also tested. As little as 500 fg of virus DNA extracted from purified particles were routinely detected. The amplification sensitivity decreased with increasing amounts of non-infected oyster spat tissues (Renault et al., 2000a). The one-round PCR assay with the A3/A4 primer pair not only allowed amplification of OsHV-1 DNA but also the detection of a variant of this virus (OsHV-1var) in *C. gigas* and *R. philippinarum* larvae (Arzul et al., 2001c).

The combination of primer pairs A3/A4 and A5/A6 allowed less PCR amplification than C2/C6 (21.4 % *vs.* 32.4 %) when the same larval samples were analysed (Renault and Arzul, 2001). The differences observed in herpesvirus DNA detection suggested that the one-round PCR with C2/C6 was more useful for epidemiological surveys than the nested PCR using A3/A4 and A5/A6 (Renault and Arzul, 2001). Moreover, Renault et al. (2004) observed that the C2/C6 primer pair systematically allowed the detection of 1 fg of purified viral DNA, and was more sensitive than the nested PCR with primer pairs A3/A4 and A5/A6 (detection limit of ca. 500 fg of purified viral DNA). The higher sensitivity of the one-round PCR with the primer pair C2/C6 can be partially explained by the fact that the targeted fragment is present twice in the OsHV-1 genome (Arzul et al., 2001b, 2001c). However, other primer pairs designed in the C region (C13/C5 and C15/C14) failed to produce detectable amplicons with amounts of purified viral DNA detected with the C2/C6 primer pair (Renault et al., 2004). Vigneron et al., (2004) observed a detection limit of 10 fg of purified viral DNA for both primer pairs C13/C5 and Gp3/Gp4 (Table 4), the later target region being only present in one copy in the OsHV-1 genome. On the other hand, Friedman et al. (2005) detected herpesvirus DNA in *C. gigas* juveniles with A3/A4 and A5/A6 whereas no PCR products were obtained with primer pair C2/C6. In addition, partial sequencing of the amplified A region revealed a single nucleotide difference compared to the reference sequence of OsHV-1 (Arzul et al., 2001c). The herpesvirus described by Friedman et al. (2005) is very closely related to OsHV-1 based on the sequence of the A region. Non-amplification with C2/C6 primer pair could be due to nucleotide substitutions and/or insertions/deletions in the C region. The variant of OsHV-1 described by Arzul et al. (2001b, 2001c) presented a major deletion of 2.8 kbp in the C region as well as other nucleotide deletions, substitutions and insertions. No amplification of OsHV-1var DNA was observed when primer pairs C1/C4 and C1/C6 were used. Whereas amplicons were produced with primer pairs C2/C4 and C2/C6; however, these amplicons were smaller than those obtained with reference viral DNA (Arzul et al., 2001c). In contrast, the PCR products obtained from samples infected with OsHV-1 and OsHV-1var using pair primers A3/A4, B1/B2, B3/B4 and Gp3/Gp4 were the same size (Arzul et al., 2001b, 2001c). Nevertheless, some nucleotide differences (substitutions) in the sequence of the Gp3/Gp4 fragment were observed between the two types of OsHV-1 (Arzul et al., 2001c). It is noteworthy that primers used to detect bivalve herpesviruses where designed based on specific OsHV-1 sequences. Hence, putative polymorphisms in primer-binding sites can result in non-amplification or lower sensitivity of the PCR analysis to detect other related herpesviruses such as OsHV-1var.

OsHV-1 was also detected by PCR in asymptomatic *C. gigas* adults using the primer pairs B3/B2 and C2/C6 (Arzul et al., 2002). Both primer pairs gave concordant PCR results. Although other techniques, *in situ* hybridization and immunohistochemistry, were also used by Arzul et al. (2002) to detect herpesvirus DNA and proteins, respectively PCR was the most sensitive method. Mantle and gills appear as organs of choice for OsHV-1 detection in adult oysters (Arzul et al., 2002).

Different PCR strategies and primer pairs were used to analyse hatchery reared oyster larvae (Fig. 2) (Batista, personal communication). The larvae were collected at different stages of development and immediately frozen at -80 ºC. Samples for PCR analysis were prepared according to the method used by Renault et al. (2000a). No

differences were observed in the number of virus DNA positive samples with the different primer pairs. Amplification artifacts were observed with the C9/C10 primer pair yielding PCR products of various sizes. However, no positive PCR results were obtained for the samples showing extraneous bands when other primer pairs were used. Amplicons approximately 200 bp smaller than the expected size were obtained with the C13/C5 primer pair. Based on their size, these PCR products could be interpreted as the amplification of OsHV-1var DNA. However, when PCR analyses were performed with others primer pairs (i.e., A3/A4, B3/B4 and Gp3/Gp4), that have been used to amplify DNA of OsHV-1var in previous studies (Arzul et al., 2001b, 2001c), no PCR products were obtained (data not shown). These results suggest that the PCR conditions used with the primer pairs C9/C10 and C13/C5 should be optimised in order to eliminate the non-specific amplification observed.

3.3. Amplification from formalin fixed samples

Samples for histopathological examination are usually formalin fixed using different types of fixatives (e.g., 10 % formalin, Davidson's solution, Carson's solution). The quality and quantity of the DNA extracted from these types of samples is generally low due to degradation and leaching of nucleic acids during the fixation procedure, particularly under prolonged fixation durations (more than 24-48 h). Primer pairs that amplify small fragments can be used to increase the efficiency of the amplification reaction, in particular, when the template DNA is highly fragmented (Coates et al., 1991). Barbosa-Solomieu et al. (2004) used primers pairs C9/C10 and B3/B4 successfully to amplify OsHV-1 fragments of 196 and 207 bp, respectively, from formalin-fixed paraffin-embedded archival samples. Despite the similar size of the amplified fragments, a higher number of PCR positive results were obtained with primer pair C9/C10 compared to B3/B4 (Barbosa-Solomieu et al., 2004). In contrast, no detectable PCR products were obtained in previous assays using primers that generate larger fragments (600-1000 bp) when the same type of samples were analysed (Renault, personal communication).

3.4. Competitive PCR

A competitive PCR method was developed in order to detect and quantify herpesvirus DNA (Renault et al., 2004). The method was based on co-amplification of OsHV-1 DNA and a mimic molecule, which differs from the target DNA by a deletion of 76 bp, using the same set of primers (C1/C6 or C2/C6). Production of the mimic molecule was done by PCR using the C1 primer and a modified primer (C116). The size differences of amplicons obtained from viral DNA and the mimic molecule can easily be observed by electrophoresis on agarose gel. This method has been successfully used to quantify herpesvirus DNA (Arzul et al., 2002) and to detect the presence or absence of PCR inhibitory factors (Barbosa-Solomieu et al., 2004; Renault et al., 2004; Batista et al., 2005).

4. Analysis of amplification products

Assessing the specificity of a PCR only on the basis of amplicon size determined by agarose gel electrophoresis can result in false-positive results, especially in PCR assays, that have not been properly validated. Different techniques have been used to verify the

specificity of PCR products from bivalve herpesviruses, including Southern blot analysis, restriction digestion and sequencing. Arzul et al. (2001c) used a digoxigenin-labelled probe in order to verify the specificity of amplification products by Southern blot hybridization. Digestion of PCR products with restriction enzymes has been the technique most frequently used to confirm the specificity of the amplification of herpesvirus DNA (Table 3). The method is simple and the results can be obtained in a few hours. Nevertheless, insufficient or absent digestion caused by the presence of certain inhibitory compounds or low enzyme activity due to suboptimal storage conditions can lead to misinterpretations of the results. Consequently, restriction digestion of PCR products obtained from reference OsHV-1 DNA (e.g., the positive control) should be used in every assay as an external control of the digestion reaction. Sequencing of PCR products is the most accurate method for determining the authenticity of the amplicons. Despite the accuracy of sequencing, this technique it is not very practical for amplicon confirmation and the cost is high specially when analysing a large number of samples. Nevertheless, it can provide unique information about the virus detected and hence allow the identification of new herpesviruses or variants of OsHV-1. If the previous confirmatory methods are not available, different primer pairs targeting different areas of the viral genome can also be used to confirm the presence of the target viral DNA.

During an outbreak of OsHV-1 in hatchery-reared larvae, PCR products obtained with the primer pairs C2/C4 and Gp17/Gp18 were cloned and sequenced (Batista, personal communication). Although no differences were observed in the sequence of the majority of clones when compared with the reference virus (OsHV-1) some clones presented single nucleotide polymorphism (Fig. 3). The polymorphism observed could be due to polymerase errors and not because of the presence of more than one type of viruses. In order to eliminate these uncertainties, high-fidelity polymerases (e.g., Goldstar Polymerase, Eurogentec, Belgium) should be used. Sequencing can also be performed directly from the PCR products since putative polymerase errors will be underrepresented.

5. PCR results and infection levels

Amplification of nucleic acid by PCR has revolutionized the diagnosis of viral infections, in particular when it is difficult or impossible to cultivate the infectious agent due to a lack of continuous cell lines. However, caution should be applied when assigning a virus to a disease based only on the detection of its genomic material. The presence of virus nucleic acid could be due to latent, low-grade persistent or active infections (Kleinschmidt-DeMasters et al., 2001), or to the presence of virions in the gastrointestinal tract and not due to actual infection. In the case of latent infections, even if highly sensitive PCR methods are used in association with well-established nucleic acid extraction procedures, viral DNA may not be detected (e.g., Khadijah et al., 2003). Consequently, negative PCR results always have to be interpreted with some caution.

Detection of herpesvirus DNA by PCR has been reported in larvae of bivalve species in which intracellular viral particles were observed by TEM (Arzul et al., 2001a; Renault et al., 2001b). Moreover, positive PCR results have been associated with high mortality rates during larval rearing (Renault et al., 2000a; Arzul et al., 2001a, 2001b, 2001c; Renault and Arzul, 2001). The failure to detect viral DNA by PCR does not necessarily mean that the animals are not infected, as low levels of viral DNA in tissues

may be below the threshold of the detection method. It has been observed that the number of positive PCR results in moribund *C. gigas* juveniles is higher than in apparently healthy individuals (C. Garcia, IFREMER LGP LaTremblade, personal communication, Burge et al. in press). OsHV-1 has been detected by PCR in apparently healthy *C. gigas* adults, which suggest that the virus is able to persist in adult oysters without inducing disease and mortality (Arzul et al., 2002).

6. Avoiding false PCR results

6.1. False-positive results

The high sensitivity that makes PCR a very powerful technique for the amplification of DNA can also be seen as a negative aspect because of inadvertent contamination by nucleic acids. Even very low levels of contamination can result in false-positive results. There are basically three major sources of contamination: (1) target DNA previously extracted (e.g., in reagents used for the PCR reaction); (2) PCR products obtained from previous assays; and (3) cloned target molecules in plasmids (Rys and Persing, 1993). Contamination of PCR reaction by previously amplified sequences is the most likely form of contamination and occurs especially in environments where the same amplification is run repeatedly (e.g., diagnostic laboratories).

In order to avoid contamination, separate work areas should be used for: (1) preparation and storage of reagents; (2) sample preparation; (3) preparation of amplification mixture components; (4) PCR amplification; and (5) analysis of amplicons. Equipment (e.g., pipetting devices) and reagents should not be exchanged between the separate work areas. Preparation of amplification mixture components is best carried out in a hood equipped with UV lights (Fox et al., 1991). These lights should be turned on before and after preparation of the PCR mixture. To limit contamination, all pipetting procedures should be performed using filter tips, and disposable gloves should always be worn and changed frequently. Cross-contamination can also be minimized throughout the elimination of contaminants from surfaces and equipments with sodium hypochlorite (e.g., decontamination of laboratory bench with 10% bleach). For each PCR analysis, at least one negative control (containing distilled water instead of template DNA) per five diagnostic samples should be used. Due to the high sensitivity of PCR (i.e., less than 1 fg of purified viral DNA can be detected), the nature of samples, the environment (e.g., seawater, hatchery) and the time of sampling (e.g., mortality outbreaks), particular care should be taken during sample collection and preparation. Although, this type of contamination is most frequently encountered, plasmid contamination may also be problematic due to the ease of plasmid aerosol formation. To avoid plasmid contamination, in addition to the precautions listed above, a dedicated room where plasmids are handled is recommended.

6.2. False-negative results

There are several factors that can cause false-negative PCR results such as: (1) pipetting errors; (2) inadequate sample preparation; (3) nucleic acid degradation and capture; and (4) polymerase inhibition. Inadequate sample preparation can yield insufficient amounts of target molecules as well as poor quality DNA (e.g., poor lysis efficiency). The method of nucleic acid extraction may also yield compounds that can inhibit the PCR reaction by degradation and sequestration of target DNA and/or inhibiting polymerase (Wilson,

1997). The presence of inhibitor factors can also depend on the development stage of the animal. For example, severe PCR inhibition has been observed in oyster larvae one day after fertilization, but not in older larvae from the same larval batch (Batista, personal communication). PCR inhibition was also observed in adult oyster samples using ground tissues, but not in spat samples (Renault and Novoa, 2004). Therefore, it is advisable to employ standard molecules as indicators of the efficacy of the reaction (Ballagi-Pordány and Belák, 1996), such as the internal standard developed by Renault et al. (2004). To assure that the extraction method used yields good quality DNA and also to evaluate the presence of inhibitory factors, universal primers that amplify DNA of the hosts can be used (Le Roux et al., 1999; Batista et al., 2005). The universal primers developed by Le Roux et al. (1999) have been successfully used to amplify DNA from several species including the oysters *C. gigas* and *O. edulis* (Audemard et al., 2002; Friedman et al., 2005). The dilution of inhibited samples provides a rapid and simple way to overcome this problem (Renault et al., 2000a). However, sample dilution can reduce the number of target molecules below the limit of the detection method. Commercial DNA purification kits procedures can also be used to remove inhibitors and at the same time concentrate the total DNA extracted (An and Fleming, 1991; Wiedbrauk et al., 1995). The addition of BSA can effectively reduce PCR inhibition (Friedman et al., 2005).

7. Final considerations

PCR has been used successfully to detect the DNA of herpesvirus infecting several species of bivalve molluscs. Although PCR specificity has been assessed for some of the primer pairs used to detect herpesvirus DNA (i.e., A3/A4, A5/A6, C1/C6, C2/C6, C13/C5 and C14/C15), this has not done been done for all primer pairs used routinely. Moreover, the amplification conditions that have been used in PCR assays using different primer pairs were based on the conditions optimised for A3/A4 and A5/A6 (Renault et al., 2000a). The sensitivity (between 1 and 10 fg of purified viral DNA) observed for the majority of the primer pairs (e.g., C2/C6, Gp3/Gp4) suggests that the PCR conditions did not have a significant negative influence on the detection limit. However, it should be noted that non-specific amplification has been reported, which may be due to sub-optimal PCR conditions (e.g., annealing temperature and Mg^{2+} concentration). For example, detection of bands with unexpected sizes was observed in PCR assays with the C2/C6 primer pair. The template DNA used for these assays was extracted from the gills (preserved in 95 % ethanol) of adult oysters by digestion with proteinase K followed by phenol/chloroform purification and ethanol precipitation. However, when using an annealing temperature of 60 ºC (an increase of 10 ºC) these extraneous bands were not observed (unpublished data). Under these conditions, the minimum template quantity needed systematically to detect amplicons was 10 fg of purified viral DNA extracted from purified particles. The same sensitivity was also observed when 500 ng of DNA from non-infected *C. gigas* was added (Fig. 4). Consequently, validation and standardization of the PCR conditions for the primer pairs that are apparently more useful for epidemiological surveys should be performed. The adequacy of some of the primer pairs often used is presented in Table 4, and practical recommendations for the detection of herpesvirus DNA by PCR in bivalve mollusc samples are given in Table 5. Development of a quantitative real-time PCR test is needed for simultaneous identification and quantification of virus copies (viral load) in hosts or environmental

samples as the conventional end-point PCR tested developed to date provide qualitative data (presence or absence).

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Table 1
Techniques used to extract nucleic acids from bivalve molluscs samples for detection of herpesvirus DNA by PCR

Development stage	Sampling conditions	Method	Reference
Larvae and spat	Frozen at -20 ºC	Grinding, boiling	Renault et al. (2000a)
Larvae	Frozen at -80 ºC	Washing, grinding, digestion with proteinase K, boiling	Batista et al. (2005)
Spat	Fresh material and samples fixed in 95 % ethanol	Commercial kit (lysis, digestion with proteinase K, DNA binding to silica gel)	Friedman et al. (2005) Burge et al. (2006)
Adults	Frozen at -80 ºC	Grinding, digestion with proteinase K, phenol-chloroform purification	Le Deuff (1995)
Adults and spat	Fixed in Davidson's and Carson's solutions, paraffin-embedded	Deparaffination, digestion with proteinase K, heating	Barbosa-Solomieu et al. (2004)
Adults	Fixed in Davidson's solution	Digestion with proteinase K, heating	Barbosa-Solomieu et al. (2005)
Adults	Fixed in Davidson's solution	Digestion with proteinase K, phenol-chloroform purification	Unpublished data
Adults	95 % ethanol (gill)	Digestion with proteinase K, phenol-chloroform purification	Unpublished data

Table 2
Oligonucleotide primers used for OsHV-1 detection by PCR

Name	Primer sequence (5' – 3')	Forward/Reverse	Reference
Region A			
A3	GCCAACCGTTGGAACCATAACAAGCG	Forward	Renault et al. (2000a)
A3	GCCAACCGTTGGAACCATAACA**G**GCG	Forward	*
A4	GGGAATGAGGTGAACGAAACTATAGACC	Reverse	Renault et al. (2000a)
A5	CGCCCCAACCACGATTTTTCACTGACCC	Forward	Renault et al. (2000a)
A6	CCCGCTAGATATAGGATGAGATTTG	Reverse	Renault et al. (2000a)
A6	CCCG**T**CTAGATATAGGATGAGATTTG	Reverse	*
Region B			
B1	ATGTAATGGGTGGTGGTGCT	Forward	Arzul et al. (2001c)
B2	CAACAGCTTTGGAGGTTGGT	Reverse	Arzul et al. (2001c)
B3	GTGGAGGTGGCTGTTGAAAT	Forward	Arzul et al. (2001b)
B4	ACTGGGATCCGACTGACAAC	Reverse	Arzul et al. (2001b)
Region C			
C1	TTCCCCTCGAGGTAGCTTTT	Forward /Reverse	Arzul et al. (2001c)
C2	CTCTTTACCATGAAGATACCCACC	Forward /Reverse	Arzul et al. (2001c)
C4	GCAGTTGTGGTATACTCGAGATTG	Forward /Reverse	Arzul et al. (2001c)
C5	CCGTGACTTCTATGGGTATGTCAG	Forward /Reverse	Arzul et al. (2001c)
C6	GTGCACGGCTTACCATTTTT	Forward /Reverse	Arzul et al. (2001c)
C9	GAGGGAAATTTGCGAGAGAA	Forward /Reverse	Barbosa-Solomieu et al. (2004)
C10	ATCACCGGCAGACGTAGG	Forward /Reverse	Barbosa-Solomieu et al. (2004)
C11	GAGGGAAATTTGCGAGAGAG	Forward /Reverse	Barbosa-Solomieu et al. (2005)
C13	CCTCGAGGTAGCTTTTGTCAAG	Forward /Reverse	Renault et al. (2004)
C14	CCGTGACTTCTATGGGTATG	Forward /Reverse	Renault et al. (2004)
C15	GATTACCCAGATTCCCCTC	Forward /Reverse	Renault et al. (2004)
Region Gp			
Gp3	GGTTGTGGGTTTGGAAATGT	Forward	Arzul et al. (2001b)
Gp4	GGCGTCCAAACTCGATTAAA	Reverse	Arzul et al. (2001b)
Gp7	TTACACCTTTGCCGGTGAAT	Forward	Unpublished data
Gp8	TCACATCACTTGGTGGCAAT	Reverse	Unpublished data
Gp10	AAGCAAATGACACGACACCA	Reverse	Unpublished data
Gp17	AACCACCACACAAGCTCCTC	Forward	Unpublished data
Gp18	ACATCTGGTGGTGGGATAGG	Reverse	Unpublished data

* The sequence of primer A3 and A6 based on OsHV-1 genome is presented in the table with a G instead of A for primer A3 in the underline position and with the insertion of a T in the underline position for primer A6

Table 3
Primer pairs, product size and restriction enzymes analysis of PCR products used for the detection of OsHV-1 (OsHV-1var) DNA

Primer pair	Product size, bp	Restriction enzyme analysis		
		Enzyme	Fragments size, bp	Reference
Region A				
A3/A4	1001 (ca. 1001)	BxtI	765/236	Renault et al. (2001b)
A5/A6	917 (UPS)			
Region B				
B1/B2	464 (ca. 464)			
B3/B2	332 (ca. 332)			
B3/B4	207(ca. 207)	Tru9I	121/85	Barbosa-Solomieu et al. (2004)
Region C				
C1/C4	538 (NA)			
C1/C6	896 (NA)	MboI	747/94/55	Renault et al. (2004)
C2/C4	352 (180)			
C2/C6	709 (ca. 529)	XhoI	375/335 (375/155)	Arzul et al. (2001c)
C13/C5	765 (UPS)			
C9/C10	197 (UPS)	Sau3AI	118/78	Barbosa-Solomieu et al. (2004)
C11/C10	197 (UPS)			
C15/C14	780 (UPS)			
Region Gp				
Gp3/Gp4	698 (698)			
Gp3/Gp10	1735 (UPS)			
Gp7/Gp8	699 (UPS)			
Gp17/Gp18	306 (UPS)			

UPS: unknown product size; NA: no amplification.

Table 4
Sensitivity and adequacy of some of the primer pairs that have been mostly used to detect herpesvirus DNA from bivalve mollusc samples by PCR

Primer Pair	Sensitivity	Positive points	Limitations
One-round PCR			
A3/A4	ND	Also allows the amplification of OsHV-1var and the OsHV detected in USA [e]	
C1/C6	1 fg [b]	Target fragment present twice	Failed to amplify OsHV-1var [f]
C2/C6	1 fg [b]	Target fragment present twice; Allows the differentiation of OsHV-1 from OsHV-1var [f]	Failed to amplify OsHV detected in USA [e]
C13/C5	10 fg [c]	Target fragment present twice	Extraneous bands observed
Gp3/Gp4	10 fg [c]	Target region apparently more conserved; Also allows the amplification of OsHV-1var [f]	
Nested PCR			
A3/A4 – A5/A6	500 fg [a]	PCR conditions optimized; Also allows the amplification of OsHV detected in USA [e].	Low sensitivity
C13/C5 – C2/C4	1 fg [d]	Target fragment present twice; Allows the differentiation of OsHV-1 from OsHV-1var [f]	Extraneous bands observed

ND: not determined. [a] Renault et al. (2000a). [b] Renault et al. (2004). [c] Vigneron et al. (2004). [d] unpublished data. [e] Friedman et al. (2005). [f] Arzul et al. (2001b, 2001c)

Table 5
Recommendations for the detection of herpesvirus DNA by PCR in bivalve mollusc samples

Steps	Recommendations
Sampling	- Samples should be preferably fresh frozen (-20 ºC, -80 ºC or liquid nitrogen), if not possible they can be fixed in 70 % ethanol and stored at 4 ºC
	- If samples are formalin-fixed (e.g., Davidson's and Carson's solutions), over-fixation should be avoided (over 24-48 h) and subsequently transferred to 70 % ethanol and stored at 4 ºC
	- Among the different formalin-based fixatives Davison's solution should be preferentially used in order to better preserve nucleic acids integrity
DNA extraction	- Sample preparation should be done in a specific area and protocols used should be adequate to the type of material
	- Extraction by boiling ground tissues can be used for larval samples or as below
	- DNA extraction from spat and adult animals can be performed by proteinase K digestion followed by phenol/chloroform purification and ethanol precipitation or Qiagen extraction kits
	- The presence of PCR inhibitors should always be checked (e.g., internal control)
	- The quality and quantity of the nucleic acids extracted as well as the presence of inhibitory factors can be assessed throughout the use of universal primers
	- To eliminate PCR inhibitors, dilution of the DNA preparation can be done. However, this procedure will also reduce the putative number of target molecules.
PCR and primers	- Positive (i.e., viral DNA) and negative (e.g., distilled water) controls should be always used. At least one negative control per five diagnostic samples should be used
	-BSA should be added to the PCR master mix to reduce inhibition
	- The selected primers should detect both OsVH-1 and OsHV-1var DNA
	- The use of more than one primer pair is advantageous, such as C2/C6 and Gp3/Gp4 (several primer pairs targeting different areas of the viral genome)
	- Since no gain in sensitivity has been documented by nested PCR procedures, its use should be avoided in order to reduce the risk of contamination
Analysis of amplicons	- Confirmation of positive PCR results can be done by restriction enzyme analysis or Southern blot hybridization
	- In each restriction enzyme analysis, amplicons obtained from reference OsHV-1 DNA should also be digested (positive control)
	- Sequencing of PCR products can also be used to confirm the authenticity of amplicons and will provide additional information about the identity of the fragments amplified

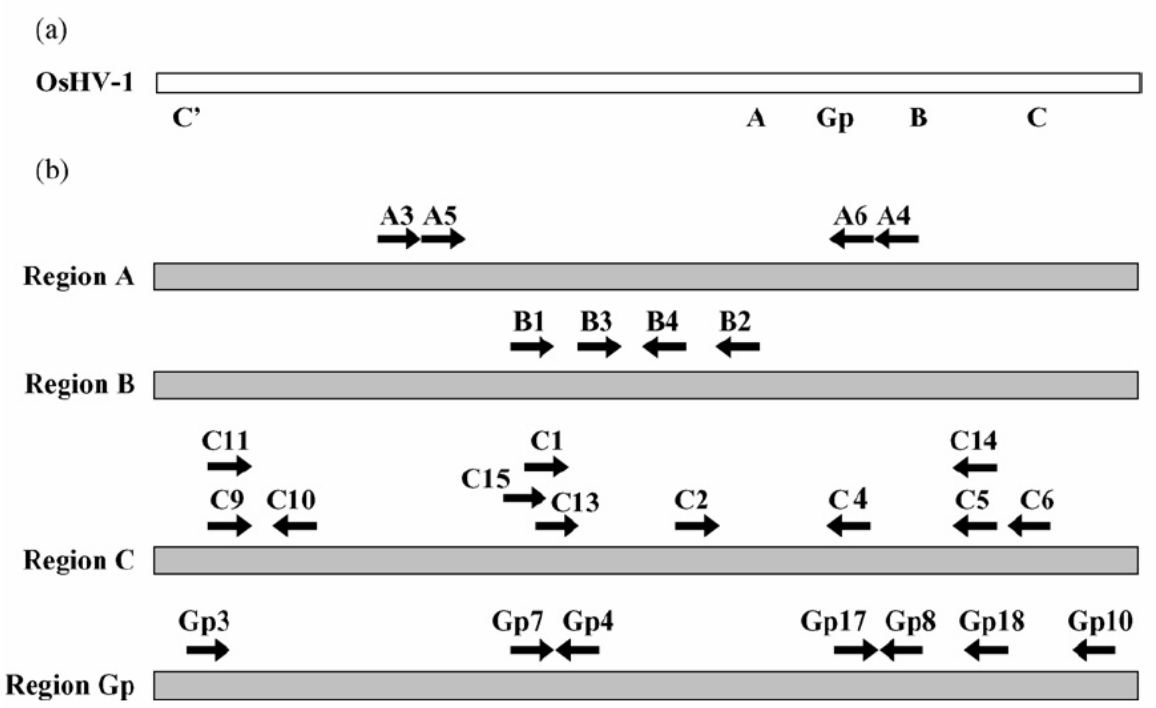

Fig. 1. General scheme of OsHV-1 genome. (a) Position of the regions A, B, C and Gp. C' represent the position of the inverted repeat of the C region. (b) Diagrams with primers (arrows) annealing sites on the four regions

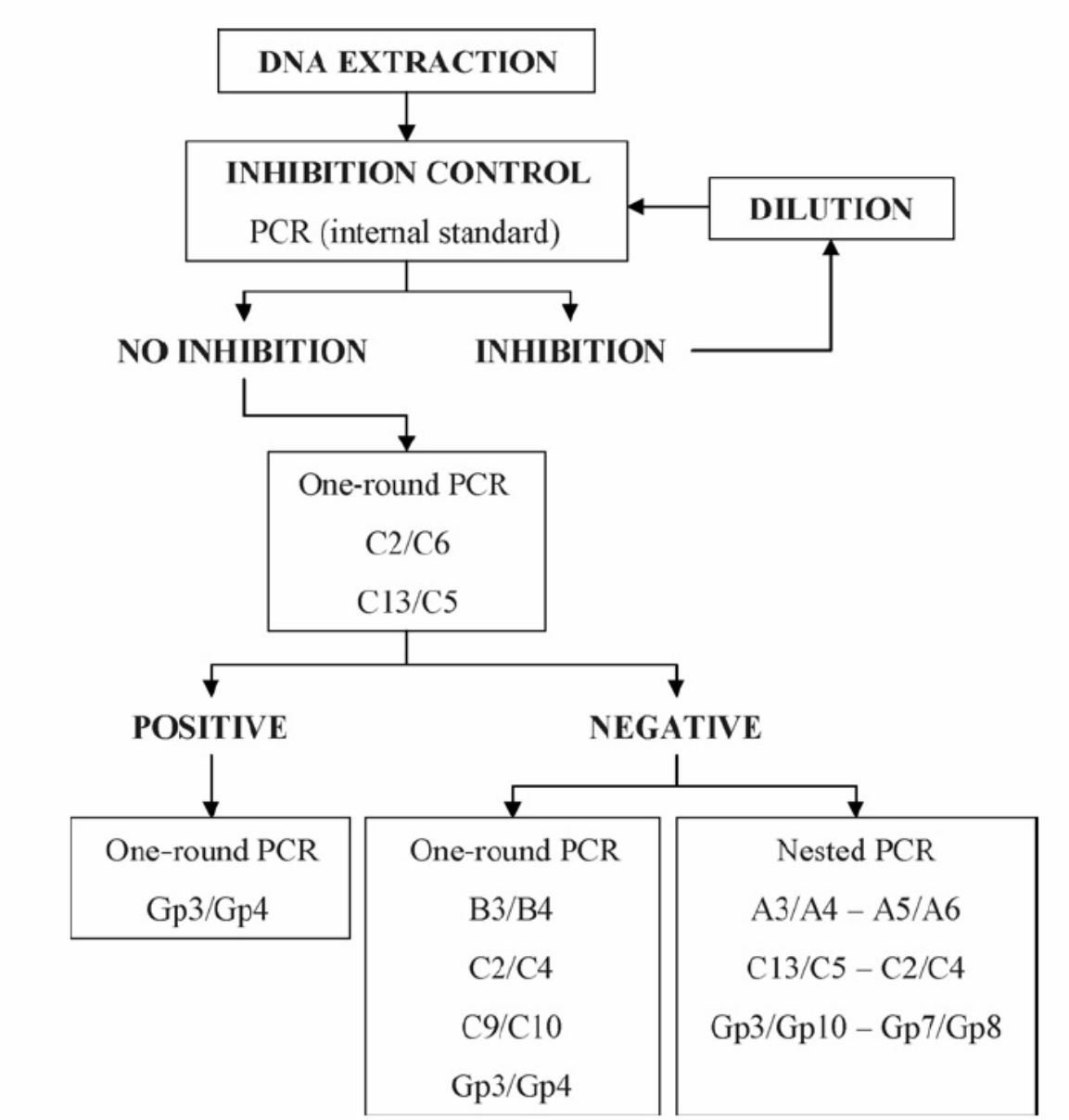

Fig. 2. Experimental procedure scheme used for the detection of OsHV-1 DNA

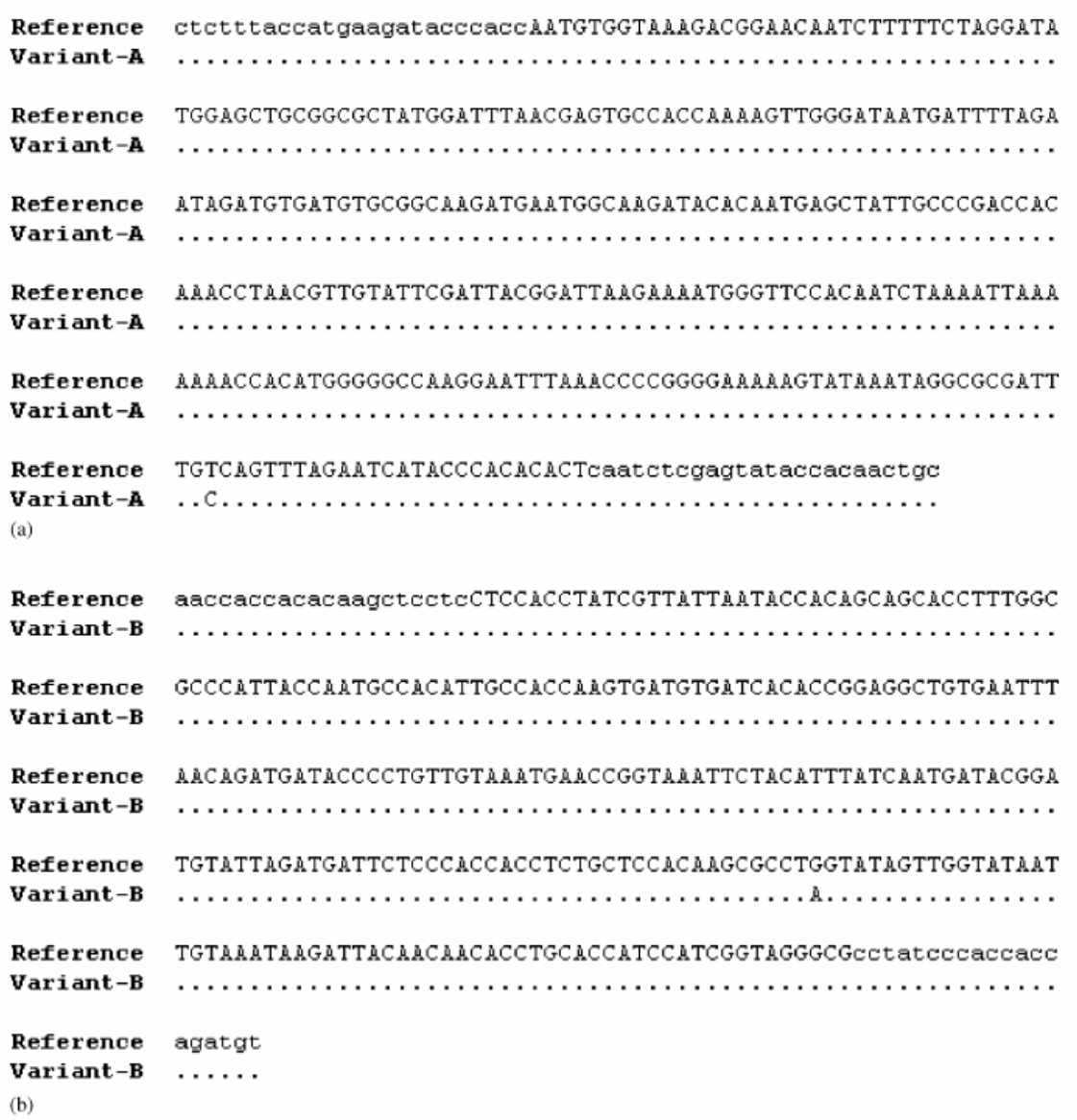

Fig. 3. Comparison of the nucleotide sequence of (a) the C2-C4 fragment (variant-A) and (b) the Gp17-Gp18 fragment (variant-B) from oyster larvae with reference OsHV-1 DNA. Primer sequences are in lower cases

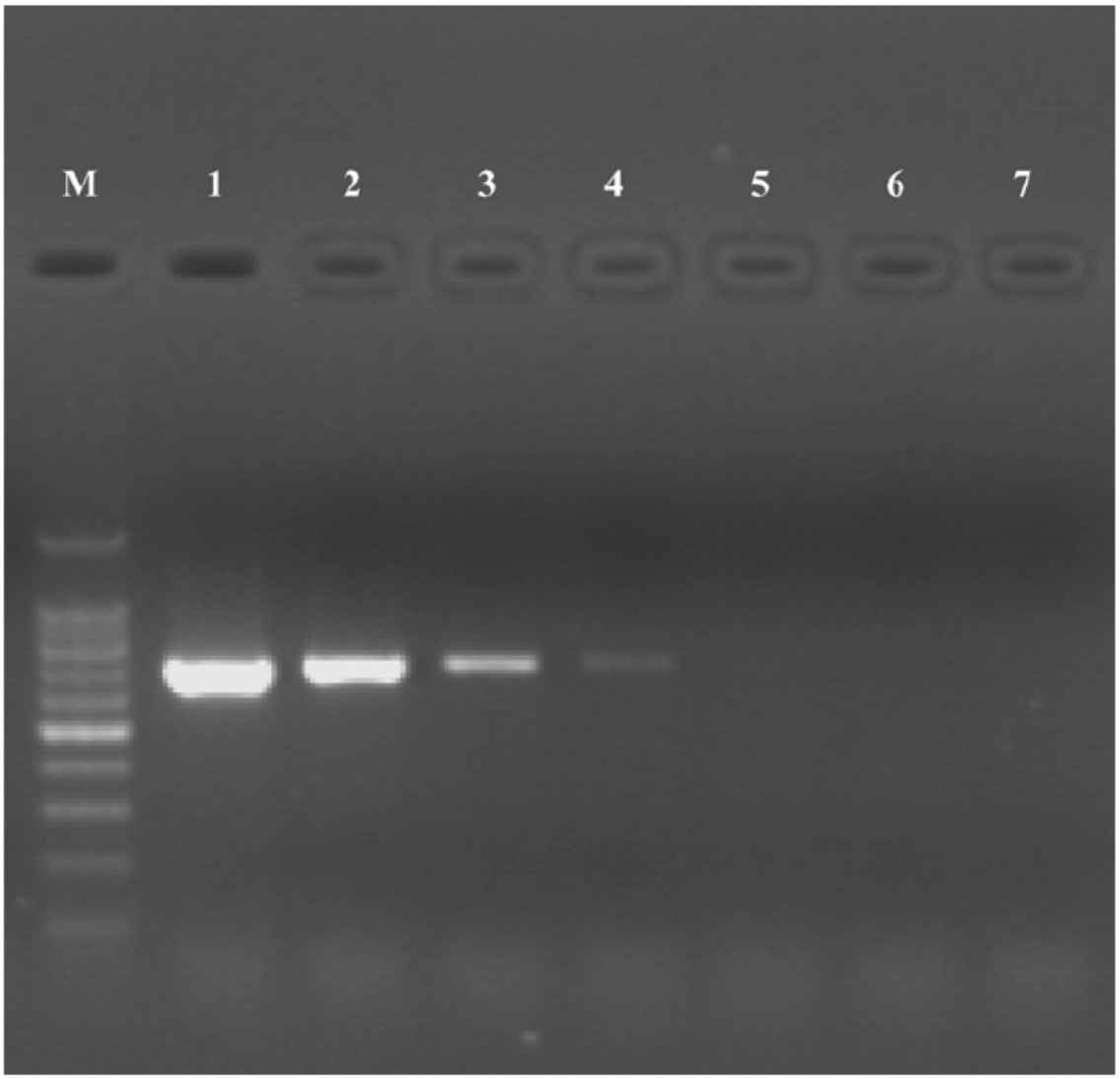

Fig. 4. Sensitivity of OsHV-1 DNA detection by PCR using the C2/C6 primer pair when 500 ng of non-infected *C. gigas* DNA was added. Results were obtained on a serial dilution (1:10) of purified viral DNA. Lane 1: 10 pg of purified viral DNA; lane 2: 1 pg of purified viral DNA; lane 3: 100 fg of purified viral DNA; lane 4: 10 fg of purified viral DNA; lane 5: 1 fg of purified viral DNA; lane 6: 100 ag of purified viral DNA; lane 7: negative control; lane M: size markers (100 bp DNA ladder, Promega)