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Development of a polymerase chain reaction for the detection of abalone herpesvirus infection based on the DNA polymerase gene

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A 5781-base pair (bp) fragment of genomic DNA from the Taiwanese abalone herpesvirus was obtained and showed 99% (5767/5779) homology in the nucleotide sequence and 99% (1923/1926) in the amino acid sequence with the DNA polymerase gene of the abalone herpesvirus strain Victoria/AUS/2007. Homology of the amino acid sequence with the DNA polymerase of ostreid herpesvirus 1 was 30% (563/1856). In this study, a PCR-based procedure for detecting herpesvirus infection of abalone, *Haliotis diversicolor supertexta*, in Taiwan was developed. The method employed primer sets targeting the viral DNA polymerase gene, and was able to amplify DNA fragments of the expected size from infected samples. Primer sets of 40f and 146r were designed for amplification of an expected PCR product of 606 bp. Combining the new PCR protocol with histopathology, this assay can serve as a reliable diagnostic for herpesvirus infections in abalone.

1. Introduction

Herpesvirus infections have been reported in various marine bivalve molluscs worldwide since the early 1990s. The first description of a virus similar morphologically to members of the Herpesvirales (Davison et al., 2009) in a bivalve mollusk was in the eastern oyster, *Crassostrea virginica* in 1972 (Farley et al., 1972). A wide host range was then reported for herpesvirus infections in bivalves including various species of oysters, clams, and scallops (Renault and Novoa, 2004; Renault, 2008; Burge et al., 2011). Mortality of abalone (molluscan genus *Haliotis*) associated with herpes-like viral pathogens has been reported in several species and subspecies including *H. discus discus* (Otsu and Sasaki, 1997; Nakatsugawa et al., 1999), *H. discus hannai* (Wang and Li, 1997), *H. diversicolor* Reeve (Wang et al., 2000), *H. diversicolor aquatilis* (Song et al., 2000), and *H. diversicolor supertexta* (Zhang et al., 2001). Recent outbreaks of infection with a herpesvirus, *Haliotis* herpesvirus (AbHV) that induced acute and high mortality were described in cultured abalone, *H. diversicolor supertexta*, in Taiwan (Chang et al., 2005). A herpesvirus identified as *Haliotis* herpesvirus 1 (AbHV-1) also induced mortality in abalone including blacklip abalone *H. rubra*, greenlip abalone *H. laeviga*, and their hybrids in Australia. However, clinical signs of Australian AbHV infection differed from those of the Taiwanese abalone in that moribund Australian abalone had a swollen mouth and prolapsed odontophore (Hooper et al., 2007), while those in Taiwan diseased abalone exhibited mantle recession and muscle atrophy (Chang et al., 2005), but lacked the oral lesions observed in Australia.

AbHV is a neurotropic virus causing ganglioneuritis; abalone 1 year or older are typically affected (Huang et al., 1999; Chang et al., 2005). Lesions include necrosis of the cerebral ganglia and nerve bundles in the muscle of the foot as well as in the muscular layers beneath the visceral organs (Chang et al., 2005). Similar neurological involvement, also noted as ganglioneuritis was reported in the blacklip abalone and greenlip abalone by Hooper et al. (2007). A herpes-like virus was also reported from abalone causing amyotrophia characterized by the development of tumor-like lesions in the nerve trunk of Japanese black abalone, *H.* (=*Nordotis*) *discus discus* (Nakatsugawa et al., 1999).

Light microscopy has been used to diagnose both AbHV and AbHV-1 infections due to the prominent neurological lesions induced by these viruses in Taiwan and Australia, respectively (Chang et al., 2005; Hooper et al., 2007). Additional techniques, such as transmission electron microscopy, can be used to confirm the diagnosis. Both microscopic techniques are time consuming and inadequate for epidemiological studies because herpesviruses may

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persist in clinically healthy hosts (carrier or latent-state infection) and recur under stressful conditions (Whitley, 1996). Thus rapid, high-throughput molecular diagnostic methods are needed to aid in diagnosing AbHV.

The genome sequencing of one bivalve herpesvirus, ostreid herpesvirus (OsHV)-1, allowed the development of DNA-based diagnostic techniques. Polymerase chain reaction (PCR)-based methods for detecting OsHV-1 DNA isolated from fresh, frozen and paraffin-embedded samples have been published (Renault et al., 2000; Barbosa-Solomieu et al., 2004; Batista et al., 2007; Martenot et al., 2010; Segarra et al., 2010). Different OsHV-1-specific primer sets ($n = 20$) were used on abalone samples from Taiwan, but failed to amplify DNA from AbHV-infected abalone (Chang and Renault, unpubl. data). A TaqMan® PCR-based technique specific for gene containing motifs V and VI, characteristic of SF2 helicase was available for detection of herpesvirus infections in abalone (Corbeil et al., 2010).

Although the DNA polymerase gene seems to be highly conserved, some differences did exist between virus isolates from Taiwan and Australia. Thus, this virus gene appears of interest for generic diagnosis for detection of most of virus isolates in the field. In the present study a conventional PCR technique targeting the AbHV DNA polymerase gene was developed and tested on abalone samples from farms.

2. Materials and methods

2.1. Biological materials

AbHV-infected abalone, *H. diversicolor supertexta*, collected from a grow-out farm that suffered high mortalities in 2004 in northern Taiwan were used as the material source. Abalone collected from farms in southern Taiwan with no history of AbHV infection were used as negative controls. Nerve tissues of 300 moribund abalones collected from one batch from a AbHV-affected farm were excised and held at −80 °C until DNA extraction. Nerve tissues of 300 control abalones were also excised and similarly stored.

2.2. Viral purification, negative staining and DNA extraction

AbHV was purified using methods modified from Le Deuff and Renault (1999) and Tan et al. (2008). Briefly, 2 g of pleuropedal ganglia, pedal nerve cords, head and epipodial tissue from AbHV-infected abalone was used to purify virus particles. Cells were lysed using a combination of homogenization and sonication. Viral particles were separated from abalone nervous tissues by a discontinuous sucrose gradient composed of five fractions, of 60%, 50%, 40%, 30%, and 10% sucrose (w/v), prepared in seawater followed by ultracentrifugation at 112,398 × *g* (25,000 rpm using an SW28 rotor, Beckman, Brea, CA, USA) for 1 h at 4 °C. Fractions (1–2 ml) were collected at each interface, and each corresponding fractions were pooled from different gradient tubes. Fractions were diluted four times by adding seawater in a drop-wise manner, and the virus was pelleted at 300,000 × *g* for 90 min (Le Deuff and Renault, 1999). DNA from the virus pellets was extracted using a QIAamp Stool Mini Kit (QIAgen, Hilden, Germany) following the manufacturer's instructions. A portion of the purified virus pellets (100 μl) was centrifuged at 100,000 × *g* for 10 min, negatively stained with 2% phosphotungstic acid and examined with electron microscopy (Hitachi, HF-3300, Tokyo, Japan) (Bozzola and Russell, 1992).

2.3. DNA sequencing

Approximately 5 μg of genomic DNA from ganglia and nerve cords was sheared by nebulization, and DNA sequencing was performed following protocols for the Genome Sequencer GS FLX Titanium System (Roche, Branford, CT, USA). Reads generated by the GS FLX sequencer were trimmed of low-quality sequences and were assembled by a GS *de novo* Assembler. These sequences were compared using the NCBI blastx program (http://blast.ncbi.nlm.nih.gov). A gene homologous to the DNA polymerase of a herpesvirus was chosen in this study. Analyses were performed and sequences aligned using Phylogenetic Inference with the Automatic Likelihood Model (PALM) (Braithwaite and Ito, 1993; Chen et al., 2009).

2.4. PCR for the detection of Haliotis herpesvirus

PCR primers were designed from the above sequences using the program Primer3. Primers of 40f (5′-TCCATCGAGATTCCCAGTTC-3′) and 146r (5′-ACGCCACCCTGTATAACGAG-3′) were expected to yield a 606 bp PCR product. PCR amplification was performed as described by Renault et al. (2000). A 50-μl reaction mixture was prepared with 5 μl of 10× PCR buffer (10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% (w/v) gelatin), 400 μM dNTPs, 5 μM tetramethylammonium chloride, 40 pmol of each primer, 2 μl purified DNA, and 2 U of Taq polymerase, and was brought up to volume with sterile distilled water. PCR amplification was performed in a thermocycler (PTC-100, MJ Research, Alameda, CA, USA) with an initial denaturation step of 94 °C for 2 min, followed by 35 cycles of 1 min of denaturation at 94 °C, 1 min of annealing at 52 °C, and a 1-min extension at 72 °C, followed by incubation for 5 min at 72 °C. Control PCRs without a template were used to test for cross-contamination. All reactions were tested in triplicate. Amplified fragments were separated using a 1% agarose gel and visualized with UV illumination after staining with ethidium bromide. The presence of amplifiable DNA was confirmed in all negative samples using 18S ribosomal RNA gene primers kindly supplied by Dr. Mark Crane (CSIRO Livestock Industries, Victoria, Australia). Primer sets of 18S forward (5′-GGCTACCACATCCAAGGAA-3′) and 18S reverse (5′-GCTGGAATTACCGCGGCT-3′) were used in this study, and the procedure was carried out as described previously (Corbeil et al., 2010).

2.5. Histopathology and PCR concordance analysis

Batches were selected for the analysis based on mortality and low harvest rates of grow-out farms in an epizootic area. Archived samples collected between 2003 and 2009 from farms that showed persistent low rate mortality and high cumulative mortality associated with undertermined etiologies were included in this analysis. Samples of visceral organs were fixed in 10% seawater formalin, embedded in paraffin, sectioned at 5 μm, and stained with Mayer's haematoxylin and eosin (H&E) and examined by light microscopy. Results of histopathology and PCR were recorded.

3. Results

3.1. Nucleotide sequencing and sequence alignment

A 5781-bp fragment of AbHV showed 99% (5767/5779) homology with the polymerase region of the abalone herpesvirus strain Victoria/AUS/2007 (AbHV-1). When translated to its predicted amino acid sequence, the selected AbHV fragment also showed high sequence identity (99%; 1923/1926) with that of AbHV-1. AbHV showed moderate amino acid sequence identity (30%; 562/1856) with that of OsHV-1. This fragment of AbHV also showed moderate to low amino acid sequence identities (23–46%) with the DNA polymerase of other herpesviruses (Table 1). The nucleotide sequence of the AbHV DNA polymerase gene of the virus isolate from Taiwan has been deposited in GenBank under accession number HQ317456.