FINAL REPORT

Aquatic Animal Health Subprogram: Development of molecular diagnostic procedures for the detection and identification of herpes-like virus of abalone (*Haliotis* spp.)

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Non-Technical Summary

OUTCOMES ACHIEVED

The real-time PCR, developed during the course of this project, has been validated and transferred to diagnostic laboratories in Australia and New Zealand. The real-time PCR has become the preferred test for the detection and identification of AbHV for surveillance programmes, and is also the test used for confirmatory diagnosis of presumptive cases of AVG (in lieu of examination by electron microscopy).

The real-time PCR was used extensively in the recent state-wide surveillance of abalone populations in Tasmania, following the discovery of suspect AVG (confirmed using the PCR test) in a processing plant on the east coast of Tasmania. Over 2000 samples of abalone, collected from around the Tasmanian coast, were examined using this test. Similarly, state diagnostic laboratories in Victoria, South Australia and Western Australia are using the real-time PCR as either the primary or the confirmatory diagnostic test for AVG.

While abalone herpes-like viruses appear to cause different disease manifestations in abalone species from different geographical locations, viz. Victoria and Tasmania Australia, and Taiwan, the real-time PCR test was shown to be pan-specific for the viruses from these locations.

The *in situ* hybridisation (ISH) test, also developed during the course of this project, provides a confirmatory test to be used to demonstrate the presence of AbHV within tissue lesions observed by light microscopy.

An Australian and New Zealand Standard Diagnostic Procedure (ANZSDP) has been drafted for submission to the Sub-committee on Aquatic Animal Health for peer-review.

In December 2005/January 2006, a disease outbreak caused high mortalities in abalone from two land-based and two marine-based farms in Victoria. The disease investigation identified lesions (ganglioneuritis involving infiltration of haemocytes) in multiple ganglia and nerves, and examination by electron microscopy detected a herpes-like virus in the pleuropedal ganglion. The disease was named abalone viral ganglioneuritis (AVG).

The emergence in Australia of AVG caused by abalone herpes-like virus (AbHV) is recognised as a major commercial threat to both the wild capture and the fledgling aquaculture industries, and an environmental threat to wild populations in general. Initial diagnosis of AVG relied on generic light and electron microscopy technologies which are labour intensive and are unlikely to detect low-level infections. In readily identifiable positive results, these methods contributed to knowledge of the geographical distribution of disease which could then inform disease control and management. However, the ability to detect AbHV infection in the absence of clinical disease is essential for investigating the biology of the virus and its interaction with abalone and other possible host species in more detail. The primary aims of this project

were to sequence the genome of AbHV and to develop and validate sensitive molecular diagnostic techniques, including a real-time PCR method that could be transferred to the state diagnostic laboratories.

To achieve these aims the herpes-like virus from infected abalone was concentrated and sequenced using state-of-the-art technology. This process provided the sequence of essentially the entire abalone viral genome which was subsequently able to be aligned to the genome of the only other known and sequenced herpesvirus of molluscs, the Ostreid herpesvirus-1. The sequence generated allowed the development of a sensitive and specific PCR assay for detection and identification of abalone herpes-like virus and of an ISH assay also specific for the abalone herpes-like virus – two tests the development of which was considered the highest research priority following the emergence of AVG. These tests would facilitate not only diagnosis of AVG but also further research on this disease.

The newly developed diagnostic tests have been documented in a draft Australian and New Zealand Standard Diagnostic Procedure (ANZSDP) that is ready to submit for external review. In addition, following validation, the TaqMan PCR has been transferred to state diagnostic laboratories in Australia and to MAF Biosecurity New Zealand.

The tests have been used to estimate the current geographical range of AbHV which includes regions of Victoria and Tasmania (even though disease has not been observed in Tasmanian open waters or farms).

These newly developed tests will facilitate the planned research identified in the National Abalone Health Work Plan drafted by the Federal Government's AAHC (Aquatic Animal Health Committee) and MACC (Marine and Coastal Committee) Working Groups which includes:

- A comparison of the Tasmanian and Victorian strains of AbHV
- Validation of the ISH diagnostic test including roll out to other States
- Development of a quantitative assay (qPCR) to determine AbHV infectious dose
- Determination of the sensitivity of AbHV to physico-chemical conditions including its stability in water/on fomites and its sensitivity to inactivation agents
- Determination of the role of mucus in AbHV transmission
- Determination of whether a latent stage exists in AVG
- Determination of the susceptibility of remnant populations of abalone previously exposed to AVG and known unexposed wild populations in South Australia.

Project results have been reported to government and industry on a regular basis through written communication (progress reports) as well as presentations at scientific and industry workshops/conferences.

KEYWORDS: Abalone herpes-like virus, AbHV, abalone viral ganglioneuritis, AVG, diagnosis, PCR, *in situ* hybridisation, ISH

Acknowledgments

The number of organisations and their staff that have had input to this project is large. There has been high interest in this project within governments and industry and we are grateful for their contributions which have enhanced the project outputs. Firstly, there are the members of the industry organisations - the Abalone Council of Australia and the Australian Abalone Growers Association and in particular the Abalone Divers' Associations and Great Southern Waters Inc. for obtaining and providing abalone that have been used in this project. In addition to the industry contributors there are the various departments within State governments that have made contributions and include:
DPI Fisheries, Department of Primary Industries, Victoria
Primary Industries and Resources, South Australia
Department of Primary Industries, Parks, Water and Environment, Tasmania
Department of Fisheries, Western Australia
Department of Primary Industries, New South Wales

Background

In December 2005/January 2006, a disease outbreak caused high mortality rates in abalone from two land-based farms in Victoria (Hooper *et al*., 2007). Two other, marine-based farms also experienced disease but to a lesser extent. The abalone species affected by the disease were *Haliotis laevigata*, *H. rubra*, and the *H. laevigata* x *H. rubra* hybrid. Histology performed on moribund animals identified ganglioneuritis involving infiltration of haemocytes in multiple ganglia and nerves (Hooper *et al*., 2007). Examination by electron microscopy detected a herpes-like virus in the pleuropedal ganglion (Hyatt *et al*., unpublished results). Preliminary transmission studies undertaken within AAHL's high biosecurity facility showed that this emerging virus is highly pathogenic and can be transmitted to healthy abalone through the water column (McColl *et al*., 2007). So far, the virus has not been isolated or grown in tissue culture.

Attempts by farm managers to eliminate the disease from affected abalone farms failed in part due to the lack of detection methods allowing early diagnosis of the aetiological agent before the onset of disease. Moreover, the disease has spread to wild abalone in the vicinity of one of the affected farms and is currently spreading, albeit at a slow rate, along the Victorian coast-line. If not managed appropriately, this emerging virus has the potential to spread further to other Victorian abalone farms, to wild abalone broodstock and to abalone and farms in other States and could jeopardise Australia's high market-value abalone industry.

It is generally accepted that rapid, reliable and sensitive diagnostic tests are essential for the effective control/management of viral disease outbreaks. Due to this need, DNA-based molecular diagnostic techniques such as quantitative real-time PCR are being developed for disease-monitoring programs for the most economically important mollusc pathogens (Corbeil *et al*., 2006) and to detect low-level viral infection in sub-clinical carriers (e.g. wild abalone broodstock and/or other aquatic animal species). Following extensive consultation with the FRDC Abalone Aquaculture Subprogram, DPI Victoria, Western Abalone Divers Association, other abalone industry sectors, international experts brought in by industry and DPI Victoria, as well as other abalone and disease experts nationally, it was agreed that this collaborative project to characterise and develop molecular diagnostics for the abalone virus was the highest priority for the industry. Presentations detailing the research plans were made at various forums including Australasian Aquaculture 2006 (30 August 2006), the FRDC Abalone Aquaculture Subprogram Workshop (31 August 2006), DPI Victoria/WADA Abalone herpesvirus Workshop (20 September 2006), DPI Victoria/WADA National Scientific Forum (21 September 2006), biannual WADA meeting (5 October 2006) and Abalone Council Australia R&D Workshop (17 October 2006) and the proposed plan received support from all stakeholder groups.

N.B. During the course of this project, AVG was discovered in a processing plant on the east coast of Tasmania (Ellard *et al*., 2009).

Need

Currently, diagnosis of abalone ganglioneuritis associated with infection by the recently discovered herpes-like virus is dependent on visualisation of gross clinical signs at the macroscopic level, of histological lesions evident by light microscopy and the observation of herpes-like virus particles by electron microscopy. Thus, while detection of diseased abalone is relatively straightforward, it is labour-intensive and time-consuming. The purpose of this project is to develop molecular diagnostic procedures for the rapid, sensitive and specific detection and identification of abalone herpes-like virus infections in the presence, or absence, of clinical signs. Thus, potential broodstock that are apparently healthy can be screened for the presence of sub-clinical herpes-like virus infections prior to on-farm use.

In addition to PCR-based surveillance tools to detect and identify sub-clinical infections, better procedures/reagents for overt disease diagnosis and characterisation are required. While the presence of histological lesions provides a presumptive diagnosis, the development DNA probe-based *in situ* hybridisation or diagnostic antiserum-based immuno-histochemical methods to detect abalone herpes-like virus infection within histological lesions will provide an alternative means for definitive diagnosis with ultimate confidence.

In addition to providing enhanced capability for disease diagnosis (detection and identification of herpes-like virus), molecular reagents and procedures developed will assist future research aimed at better understanding the pathogenesis (e.g. tissue distribution of the virus, effect of host factors such as age) and epidemiology (e.g. determination of host and geographic ranges, modes of transmission) of this disease. Such knowledge is crucial for efficient management of current and future disease outbreaks.

Objectives

1. To purify the herpes-like virus from infected abalone

2. To extract, clone and sequence the entire viral genome and align it to the Ostreid herpesvirus-1 genome

3. To develop a sensitive and specific PCR assay for detection and identification of abalone herpes-like virus

4. To develop an *in situ* hybridisation assay specific for the abalone herpes-like virus

5. To document a draft Australian and New Zealand Standard Diagnostic Procedure (ANZSDP) and submit for external review

6. To transfer of molecular techniques to State diagnostic laboratories and to MAF Biosecurity New Zealand

7. To validate the developed TaqMan PCR diagnostic test

8. To estimate the current geographical range of the causative agent of abalone viral ganglioneuritis

Methods

Purification of abalone herpes-like virus

A modification of the method described by Le Deuff and Renault (1999) was used to purify the abalone herpes-like virus for subsequent nucleic acid extraction and sequencing (Tan *et al*., 2008). Ganglioneuritis-affected abalone were collected from farms and from the wild along the south coast of Victoria and included blacklip abalone *Haliotis rubra*, greenlip abalone *H. laevigata* and hybrids of various sizes. Abalone were also collected from farms with no history of ganglioneuritis for use as negative controls. Ganglia and nervous tissue were dissected from infected abalone. A total of 40 g of pleuropedal ganglia, head, pedal nerve cords and epipodia tissue from abalone were homogenised with a 5-fold volume of 0.2 µm-filtered and autoclaved seawater. The homogenate was frozen/thawed (using three −80°C/37°C cycles), sonicated and centrifuged at 250 × *g* for 30 min to release virus from the tissues and to remove abalone tissue particles. The supernatant was sieved (74 µm pore size), and centrifuged (1000 × *g* and 4000 × *g* for 30 min each). Following ultracentrifugation at 4°C, the pellet, containing any virus, was resuspended in 50 mL seawater. Aliquots (6 mL) of the virus suspension were layered onto discontinuous gradients composed of five fractions, 60% (6 mL), 50% (5 mL), 40% (6 mL), 30% (6 mL) and 10% (5 mL) sucrose (w/v) prepared in seawater. The gradients were centrifuged at 25,000 rpm (SW28 rotor, Beckman) for 1 h at 4°C. Fractions were collected at each interface, and corresponding fractions from each tube were pooled. The harvested fractions were diluted 4-fold with seawater, and centrifuged at 25,000 rpm (SW28 rotor, Beckman) for 1.5 h at 4°C to pellet any virus present. The pellets from each fraction were resuspended in 200 µL phosphate buffered saline (PBS), and aliquots (5 µL) were adsorbed onto parlodion- and carbon-coated 400 mesh copper grids, negatively stained using 2% phosphotungstic acid (pH 6.8) and examined with 75 kV in a Hitachi transmission electron microscope (H7000). To determine the buoyant density of the purified virus and to increase its purity, isopycnic gradient ultracentrifugation (Brakke, 1960) was performed by layering purified virus from the 40-50% sucrose interface onto a discontinuous caesium chloride (CsCl) gradient (1.298 and 1.363 g/mL) and a continuous potassium tartrate gradient (20, 30, 35, 40 and 50%, w/v). After ultracentrifugation for 17 h at 25,000 rpm (SW28 rotor, Beckman) at 4°C, 3 mL fractions were collected from each gradient, diluted 4-fold, and pelleted by ultracentrifugation for 1.5 h at 25,000 rpm (SW28 rotor, Beckman) at 4°C. The pellet was resuspended in 200 µL of PBS for examination by TEM, and the buoyant density of the fraction containing viral particles was determined.

Abalone herpes-like virus genome sequence

The enriched virus preparation was suspended in 500 µL extraction buffer (100 mM NaCl, 10 mM Tris–HCl, 25 mM EDTA, 0.5% SDS, pH 8) and nucleic acid was extracted using the protocol of Le Deuff and Renault (1999). The primary DNA precipitate was resuspended and further purified using a QIAmp DNA Mini Kit spin column (QIAGEN) following the manufacturer's instructions.

The quality of high molecular weight DNA in the final elution in 100 µL TE buffer was assessed by electrophoresis of 5 µL DNA eluate in a 1% (w/v) agarose gel stained with 0.5 µg/mL ethidium bromide. Since the DNA yield from the viral fraction was low (at best ~5 µg/prep at a concentration of 100 ng/µL), it was amplified to generate sufficient template for pyrosequencing by isothermal multiple displacement amplification using the QIAGEN Repli-G Midi kit. Extracted high molecular DNA amplified from DNA isolated from the virus concentrate was sequenced using the 454 Roche Biosciences Genome Sequencer *GS-FLX* System. The gsAssembler software (version 1.1.02.15) supplied by Roche was used to assemble sequence reads. To identify viral DNA sequences potentially encoding peptides, the BLAST program (Altschul *et al*., 1997) was used to compare contigs generated by gsAssembler to the GenBank nucleotide and non-redundant peptide databases and to databases containing genomes and predicted peptides of all published viruses (obtained from NCBI). Assembled contigs were also examined, and potential open reading frames (ORFs) located, using the Artemis genome viewer (Rutherford *et al*., 2000). For use in alignments and phylogenetic analyses, peptide sequences from various dsDNA viruses were obtained from the NCBI clusters of related viral proteins (VOG at http://www.ncbi.nlm.nih.gov/genomes/VIRUSES/shagog.cgi?data=dsdna.defl &clust=VOG&) or from individual GenBank entries. DNA polymerase peptides from dsDNA viruses are from VOGd0007. Peptide sequences of the ATPase subunit of terminase were from individual GenBank sequence entries at NCBI as were Ostreid herpesvirus 1 sequences for each of the above enzymes. DNA polymerase sequences for Cyprinid herpesvirus 3 and Ranid herpesviruses 1 and 2 were obtained from their GenBank entries.

PCR assays for detection of abalone herpes-like virus

Genomic DNA sequences of the abalone herpes-like virus were used to design PCR primers (Primer Express Software, PE Applied Biosystems). Primers were synthesised by Geneworks Inc. and the specificity of the real-time PCR test was established by testing DNA extracted from diseased and healthy abalone as well as from herpesviruses that infect oyster, fish and frog. The detection limit of the PCR was determined using serial dilutions of plasmid DNA containing the AbHV target sequence.

Nucleic acid (including AbHV DNA) was extracted from tissue samples (abalone ganglia) using the QIAamp DNA® mini kit (QIAGEN Cat No. 51306) following the manufacturer's instructions). Whilst any extraction method could be used, the PCR was optimised using DNA isolated using this kit, and comparative testing should be undertaken if other methodologies are employed.

Conventional PCR

PCR primer sequences
The SE Central Software was used to design the PCR primers 007F and 007R specific to the contiguous DNA sequence labeled 007 (19,998 bp) were:
Forward primer (007F): 5'-GCCTTCGCTGGAAGCATAC-3'
Reverse primer (007R): 5'-GTGGTCGCGAGAAGAGAAC-3'

Single-step Conventional PCR
DNA extracted from pleuropedal ganglion and associated neural tissues dissected from control uninfected and AbHV-infected abalone was used as template for PCR. Each reaction contained 9.5 µL water; 12.5 µL HotStart Taq Master mix; 0.5 µL forward primer (18 µM); 0.5 µL reverse primer (18 µM); and 2 µL of DNA. For multiple samples, reaction volumes are multiplied appropriately. The thermal cycling conditions used were 94°C for 15 min, 35 cycles of 94°C for 15 sec, 52°C for 30 sec and 72°C for 30 sec followed by 72°C for 5 min. Amplified DNA was detected by agarose (2%) gel electrophoresis using standard procedures.

Real-time PCR

Real-time PCR has several advantages over conventional one-step PCR including higher throughput and sensitivity. A TaqMan probe-based test was employed to amplify AbHV DNA although alternative real-time PCR systems could be used if optimised and benchmarked against the TaqMan test. An Applied Biosystems 7500 Fast Real-Time PCR System is used for amplification and data acquisition.

TaqMan PCR primers and probe design
The TaqMan PCR incorporates two primers and a probe specific for abalone herpes-like virus (AbHV) designed using the default parameters of the Primer Express Software version 3.0 (PE Applied Biosystems) and targeted ORF49 in the AbHV sequence.

PCR primer and probe sequences:
Forward primer (AbHVORF49F): 5'-AACCCACACCCAATTTTTGA-3' Tm=48^{o}C
Reverse primer (AbHVORF49R): 5'-CCCAAGGCAAGTTTGTTGTT-3' Tm=50^{o}C
6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA), labeled probe (AbHV49Pr): 6FAM-CCGCTTTCAATCTGATCCGTGG-TAMRA Tm=57^{o}C

The PCR used primers at 300 nM final concentration, the FAM probe at a 100 nM final concentration and amplified a product 126 bp in length.

A PCR using TaqMan® Ribosomal RNA Control Reagents (Cat. No. 4308329; Applied Biosystems), 18S primers and probe was used to confirm the integrity of extracted DNA and the absence of PCR inhibitors. The endogenous 18S control primers and probe sequences are:

Forward primer (18S Forward) 5'-CGGCTACCACATCCAAGGAA-3'
Reverse Primer (18S Reverse) 5'-GCTGGAATTACCGCGGCT -3'
Probe (18S VIC probe) 5'-TGCTGGCACCAGACTTGCCCTC-3'

Both the 18S primers and the probe are used at a 100 nM final concentration.

PCRs were undertaken in wells of a 96-well plate containing 25 µL reaction mixture incorporating 12.5 µL TaqMan® Fast Universal PCR Master Mix (2X) (No AmpErase® UNG AB Applied Biosystems, part #4352042), 2 µL (~100 ng/µL) each DNA sample and deionised water to 25 µL. The thermal cycling conditions used were: 95^{o}C for 59 sec followed by 45 cycles of 95^{o}C for 3 sec and 62^{o}C for 30 sec. All samples are tested in duplicate (at a minimum) to ensure the reproducibility of the results.

A DNA sample is considered above background noise when the change in fluorescence (ΔR_N) of FAM or VIC, relative to that of ROX (internal reference signal), exceeds the cycle threshold (Ct) value arbitrarily set in the linear expansion range of the amplification plots. The Ct value is defined as the cycle at which a statistically significant increase in fluorescence output above background is detected.

The results of a TaqMan PCR are expressed in the form of software-generated characteristic amplification curves (Figure 4). Amplification curves from positive and negative (no template controls) should be compared to the test sample. Results of a TaqMan PCR can also be, and often are, expressed as cycle threshold (Ct) values representing the number of cycles necessary for a statistically significant rise in reporter dye emission. Threshold bars are used to determine these Ct values and can be set generally in two ways: (1) to a consistent ΔR_N value, specific to the assay or (2) half way along the linear portion of an amplification plot.

Preliminary assessment and optimisation of TaqMan PCR test
Following selection of the primers and probe for the AbHV TaqMan PCR, its specificity was assessed using samples of Victorian abalone infected either naturally (in the wild or on-farm) or experimentally (at AAHL) as well as uninfected abalone collected either from the wild (interstate – outside of Victoria) or from Great Southern Waters Pty Ltd, Victoria. In addition, a range of viruses (herpesviruses and iridoviruses) as well as other abalone pathogens (e.g. *Perkinsus olseni*), were examined as non-specific targets. Data acquired from these experiments were used to optimise the PCR prior to formal validation.

Preparation of a plasmid DNA standard
To establish the detection limit of the TaqMan PCR, the 126 bp AbHV sequence targeted by the PCR was amplified and cloned into pCR4 Blunt Topo vector (Invitrogen). Plasmids with AbHV inserts were confirmed by sequence analysis and one (designated pTopoORF49) was used to generate high quality DNA, purified using a QIAquick plasmid miniprep kit (QIAGEN). The A_{260nm} of three aliquots of the pTopoORF49 DNA was determined and averaged to obtain an accurate DNA concentration. The mass of a single

plasmid DNA molecule was calculated using the formula 1 bp=660 g/mol and the 4082 bp size of pTopoORF49 following the method described in the ABI manual of absolute real-time RT-PCR quantification (Applied Biosystems, 2009). A 10-fold dilution series of pTopoORF49 was prepared in sterile, DNAse-free, distilled water as well as in 10 ng/µL total DNA obtained from uninfected abalone tissue for testing, using the TaqMan PCR.

Validation of TaqMan PCR

Performance characteristics of the AbHV TaqMan PCR were established using a total of 1675 abalone samples and using histopathology as the 'gold standard', with Microsoft Excel 2003 (11.8237.8221) SP3, and Microsoft Cooperation MedCal Version 9.6.4.0 used for data collation and analysis.

A group of 32 abalone tissue samples from areas in Victoria where AVG outbreaks with high mortalities had been reported comprised the positive reference population with a high prevalence of AbHV. Specifically, these comprised moribund and dying abalone collected between January 2007 and June 2008, along Victorian coastal waters of which 23 were classified as positive and 9 as negative by histological examination. Another group of 18 abalone was sourced from a Tasmanian processing plant experiencing an AVG disease outbreak.

A negative reference population with low prevalence of AVG comprised tissue samples from a total of 1625 abalone collected from open waters around the entire coast of Tasmania. These abalone were considered to represent a reference population with an extremely low prevalence (i.e. <1%) of disease. All samples originated from abalone with no clinical signs of disease and were classified as negative by histological examination (i.e. absence of any ganglioneuritis).

TaqMan PCR precision
Establishment of intra- and inter-assay variability was performed using DNA samples obtained from 6 and 7 different abalone, respectively (OIE, 2008a), 6 confirmed AbHV-infected abalone (positive controls) from Victoria, and 7 negative controls from healthy uninfected abalone originating from NSW where AbHV or the associated abalone viral ganglioneuritis disease have never been reported.

In situ hybridisation test for abalone herpes-like virus

The *in situ* hybridisation test employed a digoxygenin (DIG)-labeled DNA probe to detect AbHV in formalin-fixed, paraffin-embedded (FFPE) tissue sections. If the pathogen is present in the tissue, hybridisation of viral DNA to the DIG-labeled probe will result in the formation of a stable hybrid which can be visualised using colourimetric detection procedures. The application of anti-DIG antibody conjugated to alkaline phosphatase and a substrate for the alkaline phosphatase leads to the formation of a precipitate at the site of the

hybrid. For AbHV-positive samples, blue-black staining is visible under light microscopy of the stained section. Positive and negative sections are necessary so that the specificity of the reaction can be confirmed and the absence of background staining due to the presence of endogenous alkaline phosphatase can discounted.

Reagents

20 x SSC pH7

175.32 g/L	NaCl
88.23 g/L	Sodium citrate

100 x Denhardt's Solution

2 g/100 mL	Bovine serum albumin (Fraction V)
2 g/100 mL	Ficoll 400
2 g/100 mL	Polyvinylpyrollidone

Hybridisation buffer

25 mL	Formamide
10 mL	20 x SSC
2.5 mL	100 x Denhardt's solution
10 mL	50% Dextran sulphate in distilled water
500 µL	10 mg/mL herring sperm DNA

Make up to 50 mL with MilliQ water

10 x Tris-buffered saline (TBS)

23.6 g/L	Tris base
127 g/L	Tris-HCl
87.66 g/L	NaCl

Preparation of DIG-labeled DNA Probe

A PCR was performed using AbHV DNA and a PCR DIG Probe Synthesis Kit (Roche Cat No. 11636090910) according to the manufacturer's instructions. The PCR used the primers AbHV_ORF66f1 (5'-TCCCGGACACCAGTAAGAAC-3') and AbHV_ORF66r2 (5'-CCCGGACACCAGTAAGAAC-3'), the thermal cycling conditions 95ºC for 5 min, 30 cycles of 95ºC for 30 s, 55ºC for 30 s, 72ºC for 60 s followed by 72ºC for 10 min, and amplified a 848 bp DNA product.

Preparation of Tissue Sections for Histology

Sections (3-µM thick) of paraffin-embedded AbHV-infected abalone tissues were placed onto Superfrost Plus slides (Menzel Cat No. SF41296SP) and allowed to dry. The slides were heated at 65ºC for 30 min and tissue was deparaffinised in 2 stages by immersion in xylene followed by rehydration by immersion of the slides in absolute ethanol for 2 min, 90% ethanol for 2 min, 70% ethanol for 2 min and then in distilled water. The slides were placed in 0.2 N HCl for 20 min and rinsed in distilled water for 10 min. A 50-100 µL aliquot of 100 µg/ml proteinase K in Tris-buffered saline (TBS) was applied to

the tissue section and incubated at 37ºC for 30 min. The slide was then rinsed in 0.2% glycine for 2 min and then gently washing under running water for 10 min. The tissue section was then dehydrated by immersing the slide in 70% ethanol for 2 min, 90% ethanol for 2 min and 100% ethanol for 2 min, and the slides allowed to air dry.

In situ Hybridisation Procedure

Hybridisation solution (4 x SSC, 5 x Denhardt's solution, 10 mg/mL Herring sperm DNA, 10% Dextran Sulphate, 50% Formamide, approximately 5 ng/µL DIG-labeled DNA probe) was heated at 95-100ºC for 5 min to denature the dsDNA probe and placed on ice until ready for use. Sufficient hybridisation solution (50-100 µL) to completely wet the section was applied and covered with a coverslip. The slides were heated to 95ºC for 5 min to denature cellular nucleic acid, placed into a humidified chamber that had been preheated to 37ºC and then incubated at 37ºC overnight (12-16 h).

Coverslips were removed by immersing slides in 2 x SSC and the slides were placed in a rack immersed in 2 x SSC and rocked gently for 15 min to ensure complete washing of the slides. The slides were placed in 0.5 x SSC (pre-warmed to 37ºC), rocked gently at 37ºC for 15 min and washed briefly in TBS buffer at room temperature. The slides were then transferred to blocking solution (0.5% (w/v) skim milk powder in TBS) for 30 min at room temperature. The slides were removed from blocking solution and tissue sections were covered with 100-200 µL sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche Cat. No. 1093274) diluted 1:100 in blocking solution and incubated at room temperature for 1 h. Excess conjugate was removed by washing (3 x 3 min) in TBS buffer and the slides were allowed to equilibrate in Solution II (0.1 M Tris-HCl pH 8, 0.5 M NaCl, 0.1 M $MgCl_2$, pH 9) for 3 min at room temperature.

Ready-to-Use staining solution was prepared by adding a NBT/BCIP Ready-to-Use tablet (Roche Cat. No. 11697471001) to 10 mL of a 10% solution of polyvinyl alcohol (high molecular weight, 40-100 kDa) in distilled water. Tissue sections were covered with the staining solution and covered with a coverslip before placing them in the dark for 3-4 h in a humidified container to ensure they did not dry out). Colour development was monitored periodically by light microscopy and when necessary, slides were incubated overnight to obtain adequate colour development. Coverslips were removed by immersing the slides in distilled water and the sections were gently washed under running water for 5 min. The slides were mounted with mounting medium (DAKO Cat. No. S3023) and a coverslip for examination using light microscopy

Optimisation of Disease Model

Prior to the commencement of this project, preliminary infectivity experiments had indicated that disease could be transmitted from diseased to healthy abalone using:

i. intramuscular (i.m.) injection of filtered homogenates of neural tissue from

diseased abalone
ii. cohabitation of diseased and healthy abalone.

One of the objectives of this project was to build on these preliminary experiments to develop a reliable infectivity model important for addressing research questions about the biology of the virus and its interaction with the host species, such as:
i. Infectious dose required to induce disease
ii. Storage conditions for the virus-infected material to preserve infectivity
iii. Mechanisms of transmission

Experimental animals. Healthy abalone (1-2 years old) were obtained from a local farm (Great Southern Waters Pty Ltd, Indented Head, Victoria) with no history of abalone viral ganglioneuritis (AVG), and from which abalone had consistently tested negative using the real-time TaqMan PCR described in this report. The abalone were placed in aquaria containing aerated, artificial seawater (using reconstituted Ocean Nature Sea Salt, Aquasonic, NSW) and maintained at 15-18°C. All tanks underwent a 50% water change every third day during each experiment. Approval for all experimental infections was obtained from the AAHL Animal Ethics Committee.

Viral inoculum, storage and titration. Viral inocula were prepared by dissecting out the pleuropedal ganglia and associated nerve cords (Figure 1) from several diseased abalone, homogenising the tissue (approximately 500 mg per abalone) on ice in Eagle's minimal essential medium supplemented with 10% (v/v) foetal bovine serum (FBS) using a Dounce homogeniser and clarifying the homogenate by centrifugation (1500 x *g*, 20 min, 4°C). The supernatant was decanted, filtered (0.45 µM) and 1-mL aliquots were stored at either -20°C, -80°C or in liquid nitrogen until use.

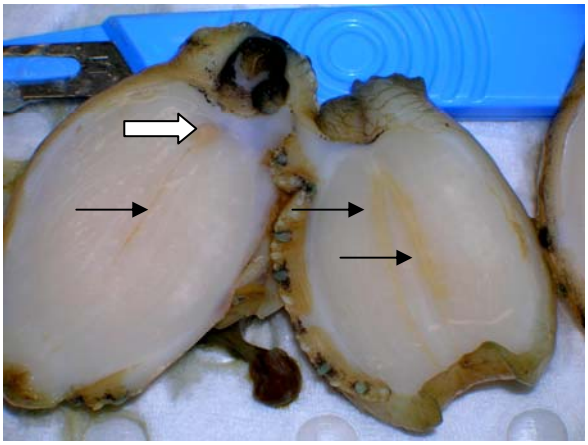


Figure 1. An abalone dissected to reveal the pleuropedal ganglion (white arrow) and nerve cords (black arrows)

To obtain base-line infectivity (LD_{50}) data on each inoculum, 10-fold dilutions were prepared to infect healthy abalone by using either intramuscular (i.m.) injection of the foot muscle or by immersion exposure of homogenate added to the aquarium sea water. For inoculation, 100 µL each stock virus inoculum

was added to 900 µL medium (1 mL of a 10^{-1} dilution) from which 10-fold dilution series to 10^{-8} was prepared. Healthy abalone (6-8 per dilution) were injected in the foot with 100 µL inoculum. Negative control abalone received 100 µL PBS-A alone. Following injection, each abalone was placed into a separate tank containing approximately 2 L of aerated artificial seawater (Figure 2).

Figure 2. Experimental tanks with air-lines housing individual abalone in approximately 1 L artificial seawater

Horizontal transmission trials. In order to determine whether direct contact between abalone was required for horizontal transmission, 4-6 infected abalone (either moribund abalone obtained from an infected premises, or experimental abalone injected with virus) were placed in porous baskets which were suspended in 50 L tanks containing 12-14 healthy uninfected abalone. Using this system, direct contact between the infected and uninfected abalone was impossible but virus could be shed from infected abalone into the water and thus expose uninfected abalone. The following experimental groups were established:

Group 1: Co-habitation of healthy abalone with 4 abalone injected with 0.1 mL virus inoculum (50 L tank; no direct contact between injected and healthy abalone).
Group 2: Co-habitation of healthy abalone with 4 moribund farmed abalone (no direct contact between sick and healthy abalone).

In addition, a semi-quantitative titration, using water taken from the Group 2 tank when the healthy abalone became moribund, was performed by using the following experimental groups:

Group 3: Healthy abalone held in undiluted water originating from the co-habitation tanks (Group 2).

Group 4: Healthy abalone held in water originating from the co-habitation tanks (Group 2) and diluted 10^{-1}.
Group 5: Healthy abalone held in water originating from the co-habitation tanks (Group 2) and diluted 10^{-2}.
Group 6: Healthy abalone held in water originating from the co-habitation tanks (Group 2) and diluted 10^{-4}.
Group 7: Healthy abalone held in water originating from the co-habitation tanks (Group 2) and diluted 10^{-5}.

Histopathology. Based on preliminary data, it was expected that infected abalone would die within 48 h of becoming moribund (e.g., demonstrating loose attachment to the substrate). Abalone were thus sampled for histopathology immediately they were identified as being moribund. Abalone were anaesthetised by placing them on a bed of ice (covered with a paper towel) for approximately 5 min and then dissected to expose the pleuropedal ganglion and nerve cords (Figure 1). The neural tissue was removed and placed in formalin. The following day, formalin-fixed tissue sections containing the pleuropedal ganglion and nerve cords were prepared by routine histological procedures including dehydration through an alcohol concentration series, paraffin embedding, sectioning (3-4 µm), and staining with haematoxylin and eosin.

Electron Microscopy. Small tissue pieces containing pleuropedal ganglion for examination by electron microscopy were fixed using 2.5% (v/v) glutaraldehyde, 2-4% (v/v) paraformaldehyde in 0.1 M cacodylate buffer and post-fixed in 1% (w/v) osmium tetroxide. The samples were washed in reverse osmosis water (3 x 5 min), dehydrated in a graded concentration series of analytical grade ethanol (70%, overnight at 4°C; 95%, 20 min; 100%, 3 x 20 min), infiltrated in 100% Spurr's resin (overnight) and then embedded in Spurr's resin.

PCR Analysis. Tissue containing the pleuropedal ganglion was dissected from dead abalone and processed for PCR analysis using both the single-step conventional PCR and real-time PCR tests described in this report.

Australian and New Zealand Standard Diagnostic Procedure (ANZSDP)

An ANZSDP was drafted for submission to SCAAH/SCAHLS for review and publication (Appendix 4). The draft conformed to the standard format and included sufficient detail to allow diagnostic laboratories to undertake presumptive and definitive diagnosis of abalone herpes-like virus infections.

Technology Transfer

Once validated by performing replicate testing at AAHL and DPI Victoria Attwood laboratories, the TaqMan PCR method was transferred to all interested diagnostic laboratories within Australia and New Zealand including:

- Mount Pleasant Laboratories, Department of Primary Industries and Water, Tasmania (contact: Dr Stephen Pyecroft)

- South Australian Research and Development Institute, Primary Industry and Resources - South Australia (contact: Dr Nathan Bott)

- Fish Health Section, Department of Fisheries – Western Australia (contact: Dr Melanie Crockford)

- Department of Virology, Elizabeth Macarthur Agricultural Institute, New South Wales (contact: Dr Peter Kirkland)

- Aquatic Animal Diseases, MAF Biosecurity New Zealand, Wallaceville, New Zealand (contact: Dr Colin Johnston)

In addition to transferring the methodology to these laboratories, a member of the project staff (Dr Serge Corbeil) visited laboratories in Tasmania, South Australia and Western Australia to assist diagnostic staff with establishing the tests in each laboratory including any trouble-shooting, if required.

Geographic Range

Prior to developing the PCR tests, samples of abalone from Tasmania, Western Australia, South Australia, Victoria and New South Wales had been collected by the respective jurisdictions and stored in readiness for when the test became available. Portions of these samples were used for test optimisation, validation and technology transfer. In addition, as part of the routine surveillance activities in Victoria and in response to the AVG outbreak in the Tasmanian processing plant, numerous additional abalone samples have been tested from these jurisdictions.

Results and Discussion

Virus Purification

The abalone herpes-like virus has not been isolated in cell culture. The lack of cell-culture-derived virus presented a number of challenges for virus purification:

- Limited source of naturally infected host material (sporadic reports of disease and associated sampling of fresh infected animals)
- Challenging source material/tissues for virus purification and nucleic acid preparation
- Low yields of “purified” virus

After ultracentrifugation, bands of opaque material appeared at each of the five sucrose interfaces (Figure 3). Examination by TEM revealed the presence of cellular debris in most of the gradient fractions. The faintest band, at the 40-50% sucrose interface, contained the highest number of viral particles. The virus particles were icosahedral with a clearly defined nucleocapsid composed of prominent capsomers. Many of the capsids were surrounded by a single envelope. The naked nucleocapsid diameter ranged from 92 to 109 nm, and the enveloped particle was approximately 150 nm in diameter. No virus particles were visualised by TEM in the negative control samples. After isopycnic centrifugation, one band appeared in the middle of each of the CsCl and potassium tartrate gradients. Virus particles were observed in the band from both gradients, with more observed in the potassium tartrate gradient than in the CsCl gradient. The buoyant density of the herpes-like virus was determined by refractometry to be 1.17 and 1.18 g/mL at 25°C in the potassium tartrate and CsCl gradients, respectively. The morphology, size and the buoyant density of the viral particles matched those described for the family *Herpesviridae* (Roizman and Pellett, 2001), and are consistent with the herpes-like virus from abalone described by Chang *et al*. (2005). Whilst this procedure did not totally purify AbHV away from all other abalone cellular components, it was enriched and concentrated sufficiently to allow nucleic acid sequencing (Tan *et al*., 2008).

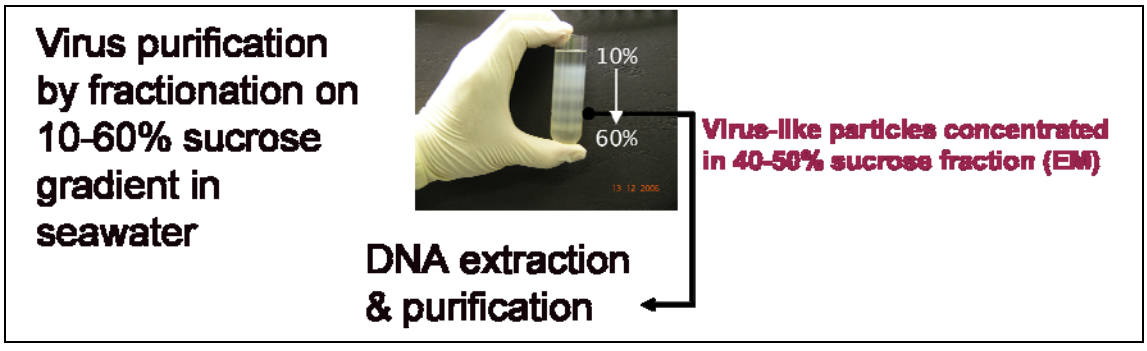


Figure 3. Fractionation of infected abalone tissue by sucrose gradient centrifugation

Abalone herpes-like virus genome sequence

Good quality sequence reads were generated by 454 sequence analysis. Sequencing the viral genomic DNA generated 83,554 sequence reads averaging 244 nucleotides in length. Upon assembly using the gsAssembler software provided by Roche, 3613 contiguous sequences (contigs) were generated, 236 of which were greater than 500 bases. The longest contig was 46 kb. The longest 20 contigs are described in Table 1. BLAST interrogation of the GenBank nucleotide database or a database of dsDNA viral genomes using sequences of the 20 contigs failed to identify any sequences with similarity greater than the E value cut-off of 0.02. However, BLAST analysis based on predicted amino acid, rather than nucleotide sequences, of the contigs identified matches with full-length open reading frames (ORFs) for several proteins, including 18 ORFs that matched gene homologues in the Ostreid herpesvirus-1 (Batista *et al*., 2005). Sequences encoding a DNA polymerase and the ATPase subunit of the terminase protein were chosen for phylogenetic analysis. DNA polymerase gene homologues occur in all dsDNA viruses while the terminase gene appears to be specific to herpesviruses and some bacteriophages (Davison, 1992).

Table 1. Summary of the twenty largest contigs obtained from 454 sequencing

Contig Id	Contig length (bp)	Number of reads
contig03033*	46316	5530
contig00007*	19998	2382
contig03304*	18596	2217
contig03197*	12673	2023
contig00039*	10862	1090
contig03200*	9793	1021
contig03026*	7138	964
contig03032*	6968	969
contig03230	6614	208
contig03209*	5326	1212
contig00246	5172	189
contig03225	4723	100
contig03233	4496	165
contig03083	4411	114
contig03170*	4132	381
contig03554	3955	1427
contig00188	3671	62
contig03229	3631	85
contig00129	3483	57
contig03333	3440	80

BLASTX searches of the GenBank nr peptide database identified some contigs* that contained ORFs with similarity to Ostreid herpesvirus-1.

Figure 4 illustrates the locations of several oyster virus (Ostreid herpesvirus-1)-related genes (ORFs) present in the three largest AbHV contigs described in Table 1.

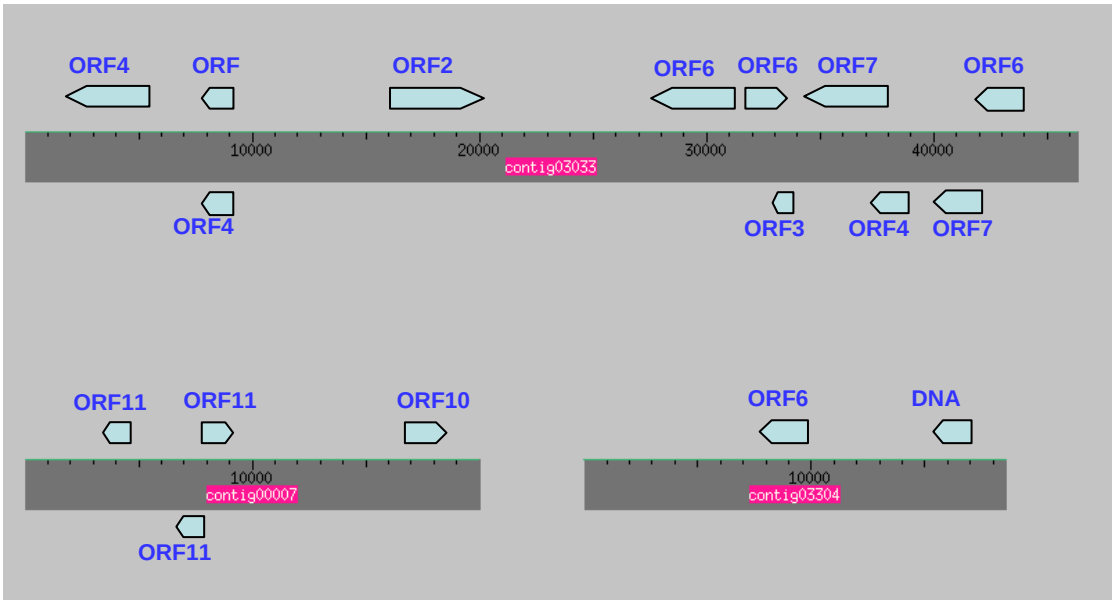


Figure 4. Location of Ostreid herpesvirus-1 ORFs along the three largest abalone virus contigs

To examine the evolutionary relationship of AbHV to other double-stranded DNA viruses, a molecular phylogenetic (phylogenomic) analysis was undertaken using the amino acid sequence encoded by the DNA polymerase gene. This gene was chosen because of its conservation across diverse dsDNA viruses. Some viral multimeric polymerase proteins encoded by multiple genes were omitted from the analysis. The coding sequences of AbHV polymerase gene contig03200, which encodes a monomeric enzyme, and cognate DNA polymerase genes of 55 other DNA viruses (the VOG0007 cluster, from the DNA polymerase B family, obtained from NCBI's clusters of related viral proteins) were analysed using SATCHMO (Simultaneous Alignment and Tree Construction using Hidden Markov Models**,** from Berkeley University, USA; Edgar and Sjölander, 2003). As expected, the alignment and phylogenetic analysis of the DNA polymerase protein sequences indicated that, like other aquatic herpes-like viruses, the abalone virus is not typical of the *Herpesviridae* family (Figure 5). A similarly distant relationship was evident in phylogenetic analyses using the sequence of the ribonucleotide reductase small subunit protein. Phylogenetic analyses (Savin *et al*., in preparation) indicate that the virus should be assigned to the Malacoherpesviridae family (Davison et al., 2009).

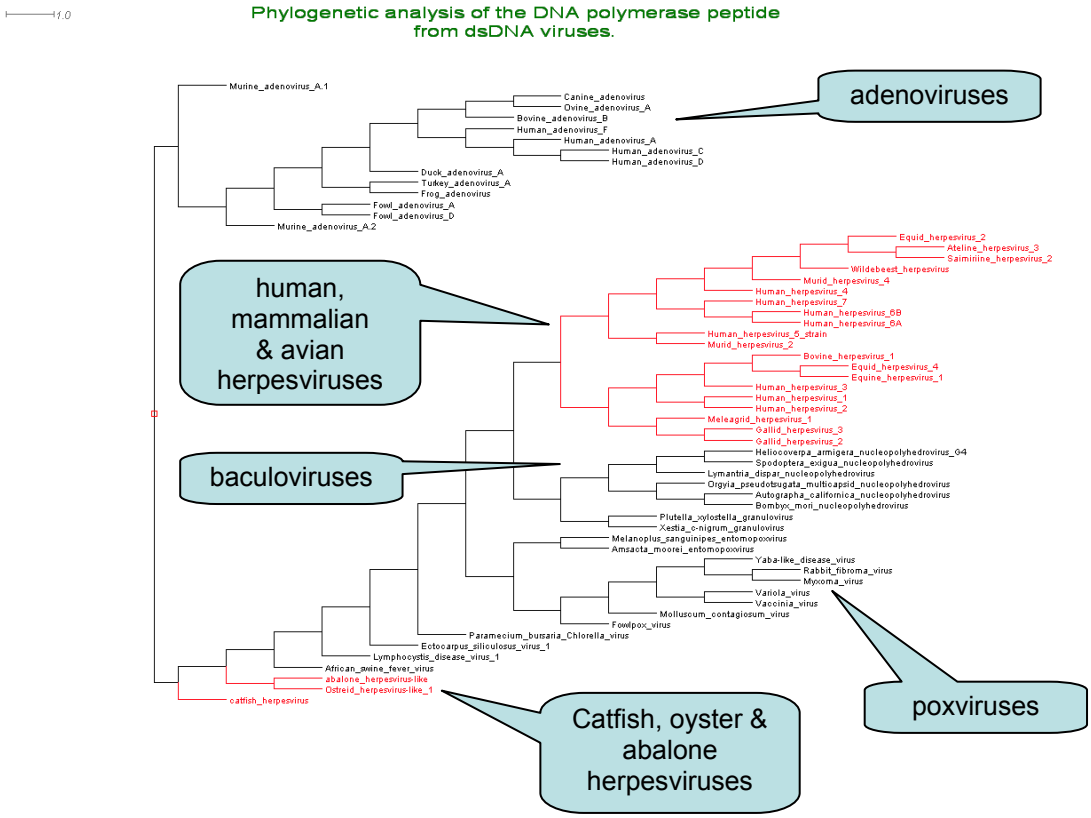


Figure 5. Phylogenetic analysis of the DNA polymerase proteins from dsDNA viruses including AbHV

PCR tests to detect abalone herpes-like virus

Conventional PCR

Based on the AbHV genome sequence obtained from the 454 sequencing, a number of conventional PCR primers were designed to amplify regions of various contigs and tested for their ability to amplify viral DNA in infected abalone. For example, PCR using primers targeting sequences of contig00007 (Table 1) amplified the expected 486 bp DNA product from AbHV-infected abalone tissues but not from healthy abalone tissues (Figure 6). Sequence analysis confirmed that the amplicon originated from the AbHV genome DNA region spanning contig00007. Based on data quality obtained using the various PCR tests, that used to amplify the contig00007 sequence was selected for further use.

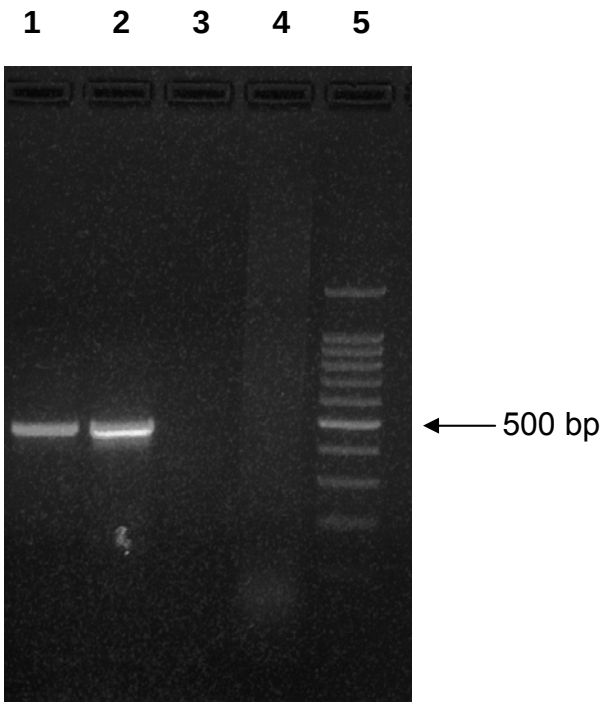


Figure 6. Photograph of an ethidium bromide-stained agarose gel. A PCR was performed on AbHV-infected abalone tissues (lanes 1 and 2) and healthy abalone tissues (lanes 3 and 4). The 486 bp amplicon seen in lanes 1 and 2 is virus-specific. Lane 5 contains a 100 bp DNA ladder (Promega).

Specificity of the conventional PCR

Preliminary analysis of 454 sequence data suggested the presence of some AbHV sequences with characteristics of iridoviruses in addition to herpes-like viruses (Figure 5). The 007 PCR was therefore assayed for specificity using DNA to a range of both herpesviruses and iridoviruses (obtained from reference stocks of frozen cell culture supernatants held at AAHL). Figure 7 shows PCR data obtained using a limited range of these viruses. While the expected 486 bp product was amplified with the AbHV-positive control sample, very low amounts of amplicon of similar size were also detected with Ostreid herpesvirus-1 and African swine fever virus. However, amplicon yields from these viruses were insufficient to allow sequence analysis to confirm their origin. Nevertheless, this result does indicate the importance of sequence analysis when using conventional PCR for diagnostic purposes.

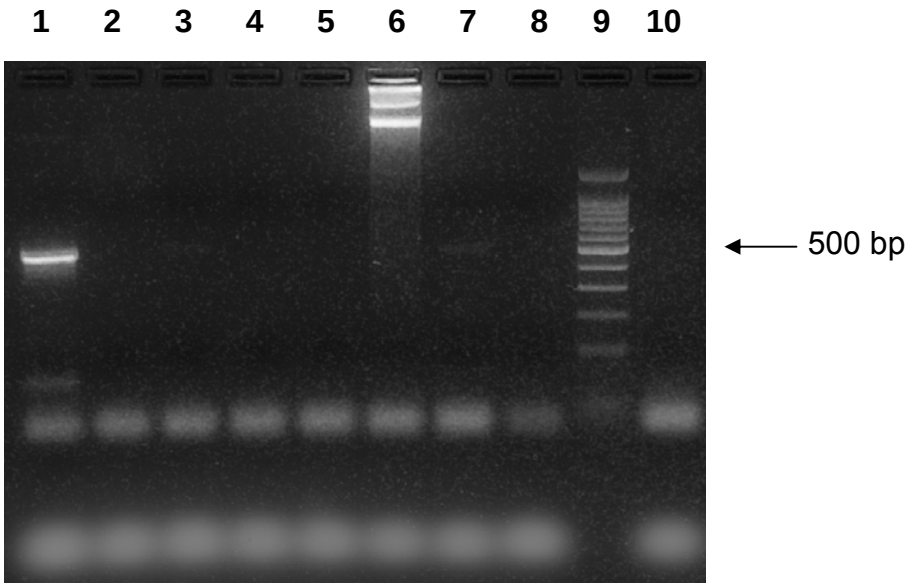


Figure 7. Electrophoresis of amplicons derived from PCRs using nucleic acid targets extracted from a range of herpesviruses and iridoviruses: Lane 1 contains positive control AbHV-infected abalone) from an experimental infection (strong band at 486 bp); lane 2 contains koi herpesvirus; lane 3 contains Ostreid herpesvirus-1 (faint band at 486 bp); lane 4 contains *Oncorhynchus masou* virus (Salmonid herpesvirus type 2); lane 5 contains Pilchard herpesvirus; lane 6 contains Bohle iridovirus; lane 7 contains African swine fever virus (iridovirus) (faint band at 486 bp); lane 8 contains Lymphocystis disease virus (a piscine iridovirus); lane 9 contains 100 bp ladder (Promega); lane 10 contains uninfected abalone tissue.

Conventional PCR sensitivity

The performance of the 007 conventional PCR as a diagnostic test was assessed further using ganglia tissues sampled from uninfected and AbHV-infected abalone sourced from infectivity trials undertaken in the biosecure facility at AAHL. Figure 8 shows conventional PCR data obtained with ganglia from 7 experimentally-infected abalone and 6 uninfected abalone. In this PCR, ganglia from 2 of the 7 experimentally-infected abalone did not yield amplicons. Moreover, amplicon yields obtained with the other 5 ganglia samples from infected abalone were variable, suggesting variability of viral loads in the abalone. Thus, it can be concluded that while the 007 conventional PCR is likely to be adequate to confirm AbHV infections in ***diseased*** abalone, it might not detect some lower-level sub-clinical infections.

With more extensive use of the 007 conventional PCR, overall, a small proportion (<10%) of known AbHV-infected abalone from a number of experiments did not yield an amplicon, confirming the diagnostic limitations of this test. Use of the more sensitive real-time PCR described below is thus recommended for detection and identification of AbHV in Australian abalone.

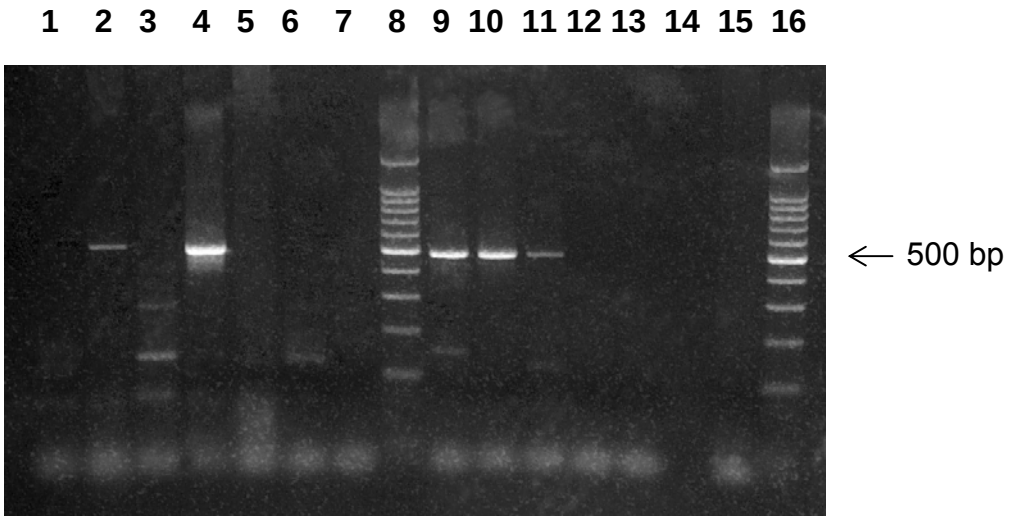


Figure 8. Electrophoresis of amplicons derived from PCRs using nucleic acid targets extracted from a range of infected and uninfected abalone. Lanes 1-4: Samples from AbHV-infected abalone; Lanes 5-7: Samples from uninfected abalone; Lane 8: 100 bp ladder Promega; Lanes 9-11: Samples from AbHV-infected abalone; Lanes 12, 13, 15: Samples from uninfected abalone; Lane 14: Empty well; Lane 16: 100 bp ladder (Promega).

Real-time PCR

Based on the 454 sequence data, a number of TaqMan PCR tests were designed to sequences of various contigs and tested for their ability to amplify AbHV DNA from infected abalone in a specific and sensitive manner. For example, a test based on primers and a probe designed to sequences of Contig03033 (Table 1), the real-time PCR amplified DNA isolated from AbHV-infected abalone tissues but not from healthy abalone tissues (Figure 9). A range of PCR primers and probes were compared based on this type of analysis and the primers and probes designed from contig03033 were selected for further study in TaqMan PCR.

A range of experiments designed to provide preliminary data on the specificity and sensitivity of the contig03033 real-time PCR were undertaken for optimisation prior to more formal validation of the test required prior to making it generally available for diagnostic use. A range of specimens from AbHV-infected abalone, uninfected abalone and DNA from a range of other viruses and pathogens were analysed under various conditions to optimise test performance.

For any diagnostic test to possess ultimate performance, it would need to be provide absolute specificity (i.e. yield zero false-positive results) and sensitivity (i.e. yield zero false-negative results). Unfortunately, few, if any diagnostic tests can accommodate such strict requirements. Thus, in considering the purpose of the test, decisions need to be made regarding which direction would be acceptable, that is more specific/less sensitive or less specific/more sensitive. Based on the potential devastating consequences of false-negative results, the probability of false-negatives

occurring needs to be reduced as much as possible (i.e. some false-positive results are more acceptable than a single missed positive sample).

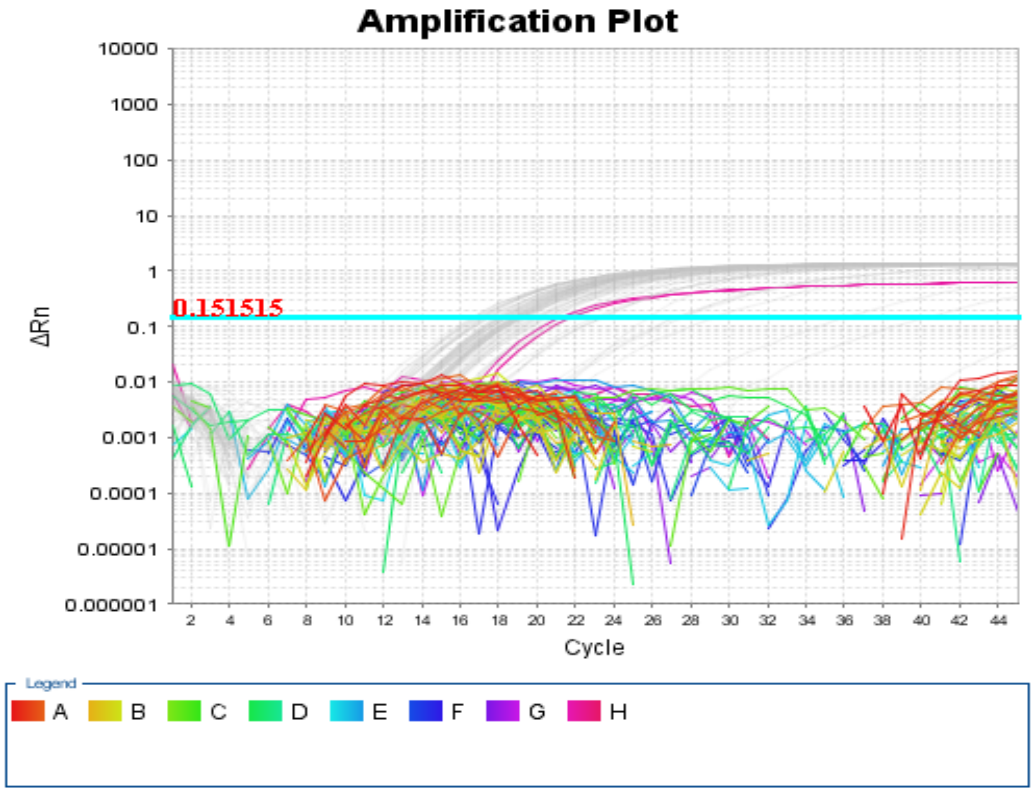


Figure 9. AbHV Real-time TaqMan PCR assay. Example of DNA amplification traces demonstrating positive samples with Ct values <35.00 and negative samples with Ct values >36.00. The Ct value is the cycle number where the curve for each sample crosses the horizontal green line (at 0.151515) which is related to DNA template copy number (the lower the Ct number, the higher the DNA template amount present in the sample).

Of interest, the sequence alignment and phylogenetic analysis of DNA polymerase proteins (Figure 5) indicate that the abalone and oyster viruses are not clear members of the *Herpesviridae* family. Their more distant phylogenetic relationship suggests that they might represent a lineage derived from a DNA virus ancestor that underwent a separate evolutionary course compared to lineages resulting in current herpesviruses or pox/irido viruses (c.f. Gao and Qi, 2007). A similar hypothesis can be derived from analyses undertaken using sequence comparisons of the ribonucleotide reductase small subunit protein. In an attempt to fine-tune the specificity of the TaqMan PCR, a range of herpesviruses and iridoviruses were tested at two primer annealing/extension temperatures (Table 2). Interestingly, the TaqMan PCR amplified a product from red sea bream iridovirus (RSIV) at 62°C and increasing the PCR stringency by increasing the primer annealing temperature to 63°C did not substantially alter amplification and make the assay more specific. As alluded to above, it is possible that some iridoviruses share sufficient sequence similarity with the abalone herpes-like virus for DNA

amplification to occur at reduced efficiency based on levels of primer/probe sequence similarity.

Moreover, performing the PCR at a 63°C primer/probe annealing temperature reduced its specificity for AbHV as evidenced by Ct values generated with other iridoviruses as well as uninfected abalone tissue being shifted to lower values than those generated at 62°C. Based on data shown in Table 2, at 62°C the cut-off point for a clearly negative sample was set at a Ct value of 33. Under these conditions, RSIV would still be detected. The TaqMan PCR could still be used as a screening test under these conditions, however, a more specific assay would need to be used for confirmatory diagnosis, if required.

Table 2. TaqMan PCR results using non-target viruses as specificity controls

Sample ID	C_T values [62.0°C]			Interpretation	C_T values [63.0°C]			Interpretation
	Rep 1	Rep 2	Rep 3		Rep 1	Rep 2	Rep 3	
Inf. abalone #1	27.62	27.56	27.57	Clearly +ve	16.19	16.15		Clearly +ve
Inf. abalone #14	ND			NA	23.60	23.54	23.50	Clearly +ve
Bohle iridovirus (BIV)	>40.00	>40.00	>40.00	Clearly -ve	>40.00	>40.00	>40.00	Clearly -ve
BIV repeat	36.36	35.57		indeterminate				
Frog virus 3 (FV3)	>40.00	>40.00	>40.00	Clearly -ve	>40.00	>40.00	>40.00	Clearly -ve
FV3 repeat	38.62	>40.00		Clearly -ve				
Red sea bream iridovirus (RSIV)	33.22	33.12	33.24	False positive	31.36	30.91	31.38	False positive
RSIV repeat	39.91	38.38		Clearly -ve				
African swine fever virus	35.40	35.36		indeterminate				
Epizootic haematopoietic necrosis virus (EHNV)	>40.00	>40.00	>40.00	Clearly -ve	38.90	>40.00	37.30	Clearly -ve
EHNV repeat	37.39	38.13		Clearly -ve				
European sheatfish virus	>40.00	>40.00	>40.00	Clearly -ve	37.51	38.29	36.61	Clearly -ve
Lymphocystis disease virus (LDV)	>40.00	>40.00	>40.00	Clearly -ve	>40.00	38.70	>40.00	Clearly -ve
LDV repeat	35.88	35.53		indeterminate				
Oncorhynchus masou virus	>40.00	>40.00	>40.00	Clearly -ve	>40.00	>40.00	>40.00	Clearly -ve
Pilchard herpesvirus	>40.00	>40.00	>40.00	Clearly -ve	>40.00	>40.00	>40.00	Clearly -ve
Koi herpesvirus (KHV)	>40.00	>40.00	39.58	Clearly -ve	>40.00	>40.00	>40.00	Clearly -ve
KHV repeat	35.21	35.42		indeterminate				
OsHV-1	>40.00	>40.00	>40.00	Clearly -ve	>40.00	>40.00	>40.00	Clearly -ve
OsHV-1 repeat	36.58	36.77		Clearly -ve				
Perkinsus	>40.00	>40.00		Clearly -ve				
Uninf. abalone #1	ND			NA	39.71	38.95	38.50	Clearly -ve
Uninf. abalone #6	ND			NA	38.49	37.43	39.75	Clearly -ve
Uninf. abalone #13	33.91	33.86		False positive				
NSW abalone	37.48	37.69		Clearly -ve				
Std +ve control	15.18	15.25	15.22	Clearly +ve	14.23	14.39	14.41	Clearly +ve

Std +ve control	16.60	16.66		Clearly +ve			
Std -ve control	>40.00	>40.00	>40.00	Clearly -ve		ND	NA
No template control	>40.00			Clearly -ve	>40.00		Clearly -ve

Ct values are either from single, duplicate or triplicate test wells

In an attempt to fine-tune the sensitivity of the TaqMan PCR, a range of tissues from infected and uninfected abalone were assayed at two annealing temperatures (Table 3).

Table 3. TaqMan PCR results using experimentally infected animals

Sample ID	Ct values [61.5°C]		Interpretation	Ct values [62.5°C]		Interpretation
	Rep 1	Rep 2		Rep 1	Rep 2	
Inf. Ab#13	22.35	22.03	Clearly +ve	16.19	16.15	Clearly +ve
Inf. Ab#17	>40.00	>40.00	False -ve	35.04	36.08	Indeterminate
Inf. Ab#31	29.62 (error)	17.59	Clearly +ve	17.14	16.93	Clearly +ve
Inf. Ab#34	21.44	20.14	Clearly +ve	20.16	22.08	Clearly +ve
Inf. Ab#38	39.14 (error)	21.02	Clearly +ve	20.76	20.83	Clearly +ve
Inf. Ab#41b	19.50	18.47	Clearly +ve	17.66	17.11	Clearly +ve
Inf. Ab#45	25.92	26.88	Clearly +ve	26.29	25.63	Clearly +ve
Inf. Ab#49	16.34	16.48	Clearly +ve	15.51	15.76	Clearly +ve
Inf. Ab #53	17.09	17.48	Clearly +ve	17.29	17.18	Clearly +ve
Inf. Ab#57	36.68	33.79	False -ve	36.92	36.23	False -ve
Inf. Ab#60	>40.00	>40.00	False -ve	37.73	37.18	False -ve
Inf. Ab#66	20.28	20.28	Clearly +ve	16.61	16.52	Clearly +ve
Inf. Ab#67	18.75	18.16	Clearly +ve	18.02	18.27	Clearly +ve
Inf. Ab#78	19.39	19.60	Clearly +ve	18.83	19.03	Clearly +ve
Inf. Ab#79	16.73	16.73	Clearly +ve	16.45	16.00	Clearly +ve
Uninf. Ab#64	34.93	35.17	Indeterminate	33.85	33.59	False positive
Uninf. Ab#65	38.34	37.83	Clearly -ve	32.69	32.51	False positive
Uninf. Ab#128	35.20	36.81	Clearly -ve	34.15	35.39	Indeterminate
Uninf. Ab#129	39.04	38.60	Clearly -ve	39.73	38.70	Clearly -ve
Uninf. Ab#130	>40.00	>40.00	Clearly -ve	>40.00	>40.00	Clearly -ve
Uninf. Ab#131	>40.00	>40.00	Clearly -ve	>40.00	>40.00	Clearly -ve
Uninf. Ab#132	>40.00	>40.00	Clearly -ve	>40.00	>40.00	Clearly -ve
Uninf. Ab#133	>40.00	39.33	Clearly -ve	>40.00	>40.00	Clearly -ve
Uninf. Ab#134	>40.00	>40.00	Clearly -ve	>40.00	>40.00	Clearly -ve
Uninf. Ab#135	>40.00	>40.00	Clearly -ve	>40.00	>40.00	Clearly -ve
Std +ve control	13.63	13.25	Clearly +ve	14.72	14.87	Clearly +ve
Std -ve control	ND		NA	33.74		
No template control	>40.00		Clearly -ve	>40.00		Clearly -ve

Samples tested in duplicate and both Ct values given.

At 61.5°C, the range in Ct values for known negative abalone was 34.90 to >40.00. Unfortunately 3 infected abalone samples yielded Ct values in this range (false-negative results) with one known positive yielding a Ct value >40.00, indicating that under these conditions the test would yield false-

negatives even if the cut-off point was set at a Ct value of 40.00. Interestingly, at 62.5°C, the range in Ct values for known negative abalone ranged from 32.60 to >40.00. Under these conditions, 3 infected abalone samples yielded Ct values in this range (false-negative results) but no known positive abalone yielded a Ct value >40.00, indicating that under these conditions, the PCR would yield less false-negatives if the cut-off point was set at a Ct value of 38.00. Using this cut-off value, the test would yield some false-positives.

A further option exists such that at 62.5°C, a cut-off point for clear negatives is set at >37.90, a cut-off point for clear positives is set at <32.50 and samples falling within the range 32.50-37.90 are reported as undetermined, with additional testing required for confirmation. Such additional testing would involve a repeat of the real-time PCR test with a different sample collected from the same abalone. If this second sample yields an undetermined result, then a more specific test would need to be employed.

The possibility exists that the two abalone samples assumed to be false-negatives are in fact true-negatives coming from uninfected abalone but misclassified due to either mislabeling or to them not becoming infected following injection in the experimental challenge experiment. In an attempt to clarify these results, the TaqMan PCR was used at 3 different temperatures to test samples of abalone sourced from Tasmania prior to the discovery of AVG in a Tasmanian processing plant as well as Western Australia, South Australia and New South Wales (Table 4).

Table 4. TaqMan PCR Ct values obtained using three different annealing temperatures and tissue samples from experimental infections and from abalone sourced from Tasmania

Sample ID	Ct values [61.5°C]	Interpretation	Ct values [62.0°C]	Interpretation	Ct values [62.5°C]	Interpretation
Tasmanian samples						
Tas F1	>40.00	Clearly -ve	>40.00	Clearly -ve	>40.00	Clearly -ve
	>40.00		>40.00		>40.00	
Tas F5	39.79	Clearly -ve	38.95	Clearly -ve	>40.00	Clearly -ve
	38.76		37.65		>40.00	
Tas F7	38.97	Clearly -ve	38.84	Clearly -ve	>40.00	Clearly -ve
	37.47		36.84		>40.00	
Tas F17	>40.00	Clearly -ve	>40.00	Clearly -ve	>40.00	Clearly -ve
	37.01		39.41		>40.00	
Tas F32	37.51	Clearly -ve	37.69	Clearly -ve	>40.00	Clearly -ve
	36.99		36.22		39.76	
Tas F34	>40.00	Clearly -ve	38.80	Clearly -ve	>40.00	Clearly -ve
	36.81		37.87		>40.00	
Tas F35	>40.00	Clearly -ve	38.98	Clearly -ve	>40.00	Clearly -ve
	37.25		38.26		>40.00	
Tas F36	>40.00	Clearly -ve	38.50	Clearly -ve	>40.00	Clearly -ve
	35.76		38.74		>40.00	
Tas F37	39.24	Clearly -ve	37.84	Clearly -ve	>40.00	Clearly -ve
	37.78		37.97		>40.00	
Tas F38	37.62	Clearly -ve	36.95	Clearly -ve	>40.00	Clearly -ve
	35.53		35.84		38.37	
Tas F74	37.07	Clearly -ve	37.10	Clearly -ve	>40.00	Clearly -ve

	36.67		37.11		38.83	
Tas F75	36.73	Clearly -ve	37.12	Clearly -ve	38.43	Clearly -ve
	34.96		36.66		37.19	
Experimentally infected abalone						
AbHV13	16.31	Clearly +ve	17.01	Clearly +ve	16.83	Clearly +ve
	16.81		16.86		16.01	
AbHV17	35.07	False -ve	35.06	False -ve	35.56	False -ve
	34.50		34.51		36.96	
AbHV31	16.35	Clearly +ve	16.75	Clearly +ve	17.39	Clearly +ve
	16.16		16.94		17.51	
AbHV34	21.86	Clearly +ve	22.46	Clearly +ve	22.10	Clearly +ve
	21.10		21.65		22.11	
AbHV38	20.55	Clearly +ve	20.18	Clearly +ve	19.53	Clearly +ve
	20.58		20.09		19.50	
AbHV41b	17.73	Clearly +ve	17.55	Clearly +ve	18.64	Clearly +ve
	17.07		17.41		17.30	
AbHV45	34.23	Clearly +ve	25.97	Clearly +ve	26.07	Clearly +ve
	33.95		26.00		26.01	
AbHV49	20.49	Clearly +ve	15.60	Clearly +ve	20.53	Clearly +ve
	20.52		15.64		20.77	
AbHV53	18.90	Clearly +ve	16.73	Clearly +ve	20.85	Clearly +ve
	16.70		16.77		16.32	
AbHV57	33.57	Clearly +ve	33.52	Clearly +ve	34.16	Clearly +ve
	34.30		35.32		34.30	
AbHV60	35.49	False -ve	35.02	False -ve	35.04	False -ve
	35.17		36.40		36.19	
AbHV66	26.03	Clearly +ve	15.94	Clearly +ve	16.31	Clearly +ve
	16.12		15.47		15.95	
AbHV67	31.50	Clearly +ve	18.30	Clearly +ve	18.25	Clearly +ve
	18.71		18.36		18.56	
AbHV78	27.37	Clearly +ve	18.70	Clearly +ve	27.16	Clearly +ve
	27.59		18.68		27.33	
AbHV79	21.53	Clearly +ve	21.54	Clearly +ve	16.16	Clearly +ve
	21.60		16.34		16.16	
Negative controls from experimental infectivity trials						
-ve 64	34.42	Clearly -ve	33.14	False +ve	35.44	Clearly -ve
	36.22		33.42		35.32	
-ve 65	33.66	Clearly -ve	33.16	False +ve	33.61	False +ve
	34.96		33.81		34.20	
-ve 128	36.23	Clearly -ve	34.43	Clearly -ve	35.83	Clearly -ve
	35.59		37.79		35.17	
-ve 130	39.11	Clearly -ve				
	>40.00					
-ve 131	38.41	Clearly -ve	36.26	Clearly -ve	36.03	Clearly -ve
	38.62		39.67		36.10	
-ve 132	36.15	Clearly -ve	37.10	Clearly -ve	36.45	Clearly -ve
	37.57		37.15		38.49	
-ve 133	38.71	Clearly -ve	38.32	Clearly -ve	37.39	Clearly -ve
	38.36		36.97		36.92	
-ve 134	38.73	Clearly -ve	37.20	Clearly -ve	36.20	Clearly -ve
	38.84		38.72		37.63	
-ve 135	>40.00	Clearly -ve	>40.00	Clearly -ve	>40.00	Clearly -ve
	>40.00		>40.00		nd	
Std +ve	14.68	Clearly +ve				
	14.48					
Std -ve	36.70	Clearly -ve	39.31	Clearly -ve		
	>40.00		39.41			
Water	35.17	Clearly -ve	34.51	Clearly -ve	34.79	Clearly -ve
	34.58		34.95		34.94	
Cut-off	34.50				34.50	

Based on these results, the TaqMan PCR annealing temperature was standardised at 62°C. Additional negative control abalone were submitted from NSW (Table 5), Western Australia (Table 6) and South Australia (Table 7) and these were tested using these standardised conditions.

Table 5. TaqMan PCR results using NSW abalone

Sample ID	C_T values [62.0°C]		Interpretation	Sample ID	C_T values [62.0°C]		Interpretation
	Rep 1	Rep 2			Rep 1	Rep 2	
F1	>40.00	>40.00	Clearly -ve	G1	>40.00	>40.00	Clearly -ve
F2	39.55	>40.00	Clearly -ve	G2	39.10	>40.00	Clearly -ve
F3	>40.00	>40.00	Clearly -ve	G3	>40.00	>40.00	Clearly -ve
F4	>40.00	>40.00	Clearly -ve	G4	39.17	>40.00	Clearly -ve
F5	>40.00	>40.00	Clearly -ve	G5	>40.00	>40.00	Clearly -ve
F6	>40.00	>40.00	Clearly -ve	G6	38.31	38.44	Clearly -ve
F7	38.26	>40.00	Clearly -ve	G7	39.47	>40.00	Clearly -ve
F8	39.54	>40.00	Clearly -ve	G8	37.99	>40.00	Clearly -ve
F9	>40.00	>40.00	Clearly -ve	G9	39.68	38.43	Clearly -ve
F10	38.26	>40.00	Clearly -ve	G10	>40.00	>40.00	Clearly -ve
F11	39.76	>40.00	Clearly -ve	G11	>40.00	>40.00	Clearly -ve
F12	>40.00	>40.00	Clearly -ve	G12	39.72	>40.00	Clearly -ve
F13	>40.00	>40.00	Clearly -ve	G13	39.26	37.65	Clearly -ve
F14	39.95	>40.00	Clearly -ve	G14	39.02	>40.00	Clearly -ve
F15	38.42	39.60	Clearly -ve	G15	39.75	>40.00	Clearly -ve
Std -ve	33.64	>40.00	Clearly -ve	Std +ve	16.95	17.20	Clearly +ve

Range for mean Ct values (F) (n=15): 39.01-undetected
Range for mean Ct values (G) (n=15): 38.38-undetected

Table 6. TaqMan PCR results using WA abalone

Sample ID	Ct values [62.0°C]		Interpretation
	Rep 1	Rep 2	
WA 76-1	>40.00	>40.00	Negative
WA 76-2	>40.00	38.18	Negative
WA 76-3	39.72	30.26	Negative
WA 76-4	>40.00	39.58	Negative
WA 76-5	39.73	39.03	Negative
WA 76-6	38.70	39.30	Negative
WA 77-1	>40.00	>40.00	Negative
WA 77-2	38.50	39.08	Negative
WA 77-3	>40.00	>40.00	Negative
WA 77-4	>40.00	>40.00	Negative
WA 77-5	>40.00	>40.00	Negative
WA 77-6	>40.00	>40.00	Negative
*WA 78-1	22.13	22.12	Positive
Std +ve	18.95	16.92	Positive
NSW -ve	39.94	>40.00	Negative

Range of mean Ct values for WA-sourced abalone (n=12): 34.99-undetected
*Note that these abalone were not sourced from WA waters but were seized from a restaurant aquarium

Apart from the abalone obtained from a Perth restaurant, all WA abalone were known to have been caught in WA waters. The exact origin of the abalone found dead in the Perth restaurant aquarium was unknown at the time of testing and demonstrated the usefulness of the TaqMan PCR test. Later investigations indicated that the restaurant abalone had been imported from a seafood supplier in Victoria.

Table 7. TaqMan PCR results using SA abalone

Sample ID	Ct values	Mean Ct value	Sample ID	Ct values	Mean Ct value	Sample ID	Ct values	Mean Ct value
3085-1	39.49	39.21	3106-1	>40.00	>40.00	3106-24	36.09	36.09
	38.92			>40.00			36.09	
3085-2	37.01	37.37	3106-2	>40.00	>40.00	3106-25	>40.00	>40.00
	37.73			>40.00			>40.00	
3085-3	38.17	39.09	3106-3	>40.00	>40.00	3106-26	38.90	>39.45
	>40.00			>40.00			>40.00	
3085-4	38.03	37.69	3106-4	>40.00	>40.00	3106-27	>40.00	>40.00
	37.34			>40.00			>40.00	
3085-5	37.41	36.74	3106-5	>40.00	>40.00	3106-28	38.71	38.63
	36.07			>40.00			38.54	
3085-6	>40.00	>40.00	3106-6	>40.00	>40.00	3106-29	>40.00	>40.00
	>40.00			>40.00			>40.00	
3085-7	38.49	37.85	3106-7	>40.00	>40.00	3106-30	>40.00	>40.00
	37.21			>40.00			>40.00	
3085-8	38.04	38.86	3106-8	38.47	38.32	3106-31	>40.00	>40.00
	39.67			38.16			>40.00	
3085-9	38.53	38.89	3106-9	>40.00	>39.73	3106-32	39.33	>39.67
	39.25			39.45			>40.00	
3085-10	39.09	38.23	3106-10	>40.00	>40.00	3106-33	38.78	38.96
	37.36			>40.00			39.13	
3085-11	38.61	38.72	3106-11	38.91	38.84	3106-34	38.49	38.33
	38.82			38.77			38.17	
3085-12	37.94	37.57	3106-12	>40.00	>39.56	3106-35	39.55	>39.78
	38.52			39.11			>40.00	
3085-13	>40.00	>40.00	3106-13	>40.00	>40.00	3106-36	37.77	>38.89
	>40.00			>40.00			>40.00	
3085-14	36.51	36.43	3106-14	>40.00	>40.00	3106-37	>40.00	>40.00
	36.34			>40.00			>40.00	
3085-15	37.15	38.23	3106-15	>40.00	>40.00	3106-38	37.26	37.29
	38.31			>40.00			37.32	
3085-16	>40.00	>39.15	3106-16	>40.00	>40.00	3106-39	38.99	38.54
	38.30			>40.00			38.08	
3085-17	>40.00	>40.00	3106-17	39.20	38.60			
	>40.00			37.99				
3085-18	38.31	38.87	3106-18	38.30	>39.15			
	39.43			>40.00				
3085-19	>40.00	>40.00	3106-19	>40.00	>40.00			
	>40.00			>40.00				
3085-20	>40.00	>40.00	3106-20	38.06	37.99			
	>40.00			37.92				
3085-21	>40.00	>40.00	3106-21	38.88	38.65			
	>40.00			38.42				
3085-22	>40.00	>40.00	3106-22	37.93	37.38			
	>40.00			36.82				
3085-23	>40.00	>40.00	3106-23	37.10	37.07			

	>40.00			37.03	
+ve #13	22.88	23.07	+ve #13	18.89	18.82
	23.25			18.75	
NSW F3	>40.00	>40.00	NSW F3	38.28	38.65
-ve cont	>40.00		-ve cont	39.01	

Range for mean Ct values for SA abalone (n=62): 36.09-undetected

At this stage of development of the TaqMan PCR, results indicated that under the standardised cycling conditions (95°C for 59 sec followed by 45 cycles of 95°C for 3 sec and 62°C for 30 sec), the following Ct value cut-off points should be used:
Ct value >36.00 = Clearly negative
Ct value <34.50 = Clearly positive
Ct values: 34.50-36.00 = indeterminate (confirmation required).

It was around this time in the development of the TaqMan PCR test that the Tasmanian Department of Primary Industries and Water (DPIW) reported on a suspect case of AVG in a processing plant on the Tasmanian east coast. Although the TaqMan PCR test had not been formally validated, it had been run with very good reproducibility between AAHL and DPI Victoria. Therefore this test was used in the subsequent emergency disease investigation led by the Department of Primary Industries and Water (DPIW), Tasmania. AAHL provided PCR testing of all samples, while DPIW undertook all histological examinations.

The data collected from the Tasmanian AVG investigation provided opportunities to examine a range of issues (see below) and also proved to be invaluable for validation of the TaqMan PCR.

Early in the development of the TaqMan PCR, false positive Ct values <34.50 were sometimes obtained with “no template control” wells that containing the reaction reagents minus abalone nucleic acid. These “false positive” occurrences subsequently “disappeared” upon further use of the test. There are several possible explanations for this phenomenon. However, it would take considerable effort to identify its cause, and this was deemed to be unwarranted as this problem no longer occurs. The then non-validated TaqMan PCR test was being used in Tasmania and South Australia for surveillance activities, with the negative and positive control samples provided yielding expected results. For example, in an experiment in which the performance of a new batch of the PCR primers and probe were checked, nucleic acid was extracted from a strongly positive abalone and then serially diluted 10-fold to a final dilution of 10^{-10}. TaqMan PCR data obtained in 2 independent tests using this abalone DNA dilution series are shown in Table 8. The Ct values obtained using the DNA dilution were found to approach the theoretical performance limits of TaqMan real-time PCR. Moreover, no template control reactions yielded Ct values >40.00 (data not shown). Although the AbHV TaqMan PCR performs well in most circumstances, further validation is required to identity the reasons behind occasional inconsistencies in specificity and sensitivity with clinical samples submitted for AbHV detection.

Table 8. Quality control experiments on TaqMan PCR primers and probe

Experiment 1				Experiment 2			
Sample Id	**Ct 1**	**Ct 2**	**Mean**	**Sample Id**	**Ct 1**	**Ct 2**	**Mean**
Controls							
Tas 2 (+)	22.94	23.06	23.00	NTC (-)	>40.00	>40.00	Negative
Neg F6 (-)	37.82	39.26	38.54	Neg F6 (-)	not done	not done	not done
Serial dilution							
Tas 3	**Ct 1**	**Ct 2**	**Mean**	**Tas 3**	**Ct 1**	**Ct 2**	**Mean**
Neat	19.05	19.45	19.25	Neat	18.90	19.18	19.04
10^{-1}	21.57	21.63	21.60	10^{-1}	21.72	21.50	21.61
10^{-2}	25.24	25.30	25.27	10^{-2}	24.94	24.83	24.885
10^{-3}	28.56	28.44	28.50	10^{-3}	28.32	28.63	28.475
10^{-4}	31.55	31.51	31.53	10^{-4}	32.45	32.30	32.375
10^{-5}	35.36	35.61	35.485	10^{-5}	36.35	35.91	36.13
10^{-6}	39.77	38.76	39.265	10^{-6}	39.60	39.52	39.56
10^{-7}	>40.00	>40.00	Negative	10^{-7}	>40.00	>40.00	Negative
10^{-8}	>40.00	>40.00	Negative	10^{-8}	>40.00	>40.00	Negative
10^{-9}	>40.00	>40.00	Negative	10^{-9}	>40.00	>40.00	Negative
10^{-10}	>40.00	>40.00	Negative	10^{-10}	>40.00	>40.00	Negative

One explanation for the improved performance of the TaqMan PCR (apart from technical familiarity obtained through extensive surveillance activities) is that latter batches of reagents were exhausted rapidly, thus providing little opportunity for degradation, compared to earlier batches that were used over more prolonged periods during test development.

The single-step conventional PCR and TaqMan real-time PCR appear to be specific for the abalone herpes-like virus. Interestingly, however, DNA products of the expected size are occasionally amplified in very low abundance with DNA isolated from Ostreid herpesvirus, OsHV-1 (Hine *et al*., 1992; Renault *et al*., 1994) and African swine fever virus (an iridovirus) (King *et al*., 2003). DNA product amounts amplified by the PCR with Ostreid herpes-like virus or African swine fever virus were, however, insufficient to allow direct sequence analysis.

It has been suggested (Gao and Qi, 2007), based on genome sequence relationships, that invertebrate herpes-like viruses, including the Ostreid herpes-like virus (Hine *et al*., 1992; Renault *et al*., 1994), should be classified in their own taxon. An interesting feature of the Ostreid herpes-like virus is that, unlike typical herpesviruses that demonstrate a high level of host-species specificity (Davison, 2002), it possesses a broader host range for several mollusc species (Arzul *et al*., 2001; 2001a; 2001b). Of possible significance to the recent detection of AbHV in local Abalone, it should be noted that herpesviruses have been detected in oyster species present in Australian waters (Hine and Thorne, 1997).

Validation of TaqMan PCR

In the analyses reported in the following sections, diagnostic samples have been considered positive when the TaqMan PCR Ct value is less than 35.8

and negative when higher than this value. This Ct value was chosen after testing large numbers of samples for assay validation (as described in the *Performance characteristics of the AbHV TaqMan PCR* section below).

Performance characteristics of the AbHV TaqMan PCR

Analytical specificity

In order to determine the specificity of the AbHV TaqMan PCR, DNA extracted from a range of other aquatic animal herpesviruses, as well as aquatic animal iridoviruses which also possess a double-stranded DNA genome, were tested. Unlike the AbHV single-step conventional PCR that showed cross-reactivity for some of these viruses, the TaqMan PCR was found to be specific for AbHV (Table 9). In addition, since the parasitic pathogen *Perkinsus olseni* is present in some abalone populations in Australia, the TaqMan PCR was also tested using *Perkinsus olseni* DNA but yielded negative results (Table 9). This absence of cross-reactivity is essential for AbHV surveillance in regions where both potential pathogens can be prevalent.

Viral DNA from the Taiwanese abalone herpes-like virus (Chang *et al*., 2005) was detected by the TaqMan PCR, indicating genome sequence similarity between the Taiwanese and Australian strains (Table 9).

Table 9. AbHV TaqMan PCR specificity

Herpesviruses	**Mean Ct value +/- std dev**	**Iridoviruses**	**Mean Ct value +/- std dev**
Oncorhynchus masou virus	>40[a]	Epizootic haematopoietic necrosis virus	>40[a]
Ostreid herpesvirus-1	>40[a]	Frog virus 3	>40[a]
Koi herpesvirus		Red sea bream iridovirus	38.96+/-0.04
Pilchard herpesvirus	>40[a]	Bohle iridovirus	39.98+/-0.03
Taiwanese herpes-like virus	26.04+/-0.4	*Lymphocystivirus*	39.37+/-0.19
Protozoan parasite *Perkinsus olseni* infected abalone tissues	>40[a]		
Positive control Abalone experimentally infected with AbHV	19.68+/-0.11	**Negative control** Uninfected abalone tissue	>40[a]

DNA samples with Ct values less than 35.8 are positive, those with Ct values equal to or greater than 35.8 are negative.

[a]All Ct values obtained were >40.0

Analytical sensitivity

Since no cell cultures are available to support the replication and quantification of AbHV, TaqMan PCR quantification of AbHV DNA copy numbers, using the recombinant plasmid pTopo-ORF49 containing an AbHV insert as a standard, was established to estimate viral infection loads. The TaqMan PCR was assessed using 10-fold serial dilutions of pTopo-ORF49 DNA and was able to detect down to a 10^{-8} dilution of the DNA prepared either in water (Table 10a) or in DNA extracted from uninfected abalone tissues (Table 10b). This finding suggests that the presence of excess non-specific DNA has little effect on the detection of AbHV DNA.

Assuming that the AbHV genome possesses a single copy of the ORF49 gene, it was calculated that the TaqMan PCR could theoretically detect ~30 genome copies. Infectivity trials in which abalone are sampled over time, however, remain to be undertaken to establish the earliest time point at which AbHV DNA can be detected during the infection process.

Table 10a. AbHV TaqMan PCR limit of detection

pTopo-ORF49 DNA diluted in de-ionised water	Plasmid copy number	Test 1 Mean Ct value +/- standard deviation	Test 2 Mean Ct value +/- standard deviation
10^{-2}	30 000 000	12.14 +/- 0.09	11.99 +/- 0.13
10^{-3}	3 000 000	13.88 +/- 0.36	14.39 +/- 0.00
10^{-4}	300 000	17.55 +/- 0.36	17.86 +/- 0.62
10^{-5}	30 000	22.27 +/- 0.09	20.82 +/- 0.54
10^{-6}	3000	24.77 +/- 0.12	25.21 +/- 0.23
10^{-7}	300	28.63 +/- 0.26	29.00 +/- 0.08
10^{-8}	30	30.44 +/- 0.01	32.67 +/- 0.60
10^{-9}	3	33.66 +/- 0.54	36.39 +/- 0.38
10^{-10}	0.3	36.88 +/- 0.82	38.89 +/- 1.23
10^{-11}	0.03	>40[a]	>40[a]
10^{-12}	0.003	>40[a]	>40[a]

Note that for diagnostic purposes, DNA samples with Ct values less than 35.8 are considered positive, whilst those with Ct values equal to or greater than 35.8 are considered negative.

[a]All Ct values obtained were >40.0.

Table 10b. AbHV TaqMan PCR limit of detection

pTopo-ORF49 DNA diluted in uninfected abalone DNA	Plasmid copy number	Test 1 Mean Ct value +/- standard deviation	Test 2 Mean Ct value +/- standard deviation
10^{-2}	30 000 000	13.02 +/- 0.03	13.28 +/- 0.13
10^{-3}	3 000 000	16.35 +/- 0.14	16.39 +/- 0.02
10^{-4}	300 000	18.94 +/- 0.14	19.18 +/- 0.18
10^{-5}	30 000	22.14 +/- 0.24	22.35 +/- 0.04
10^{-6}	3000	25.12 +/- 0.01	25.28 +/- 0.04
10^{-7}	300	28.61 +/- 0.09	28.62 +/- 0.27
10^{-8}	30	34.90 +/- 0.39	34.58 +/- 0.82
10^{-9}	3	37.43 +/- 1.06	38.79 +/- 1.6
10^{-10}	0.3	39.09 +/- 1.2	38.43 +/- 0.34
10^{-11}	0.03	>40[a]	39.03 +/- 1.37
10^{-12}	0.003	>40[a]	>40[a]

Note that for diagnostic purposes, DNA samples with Ct values less than 35.8 are considered positive, whilst those with Ct values equal to or greater than 35.8 are considered negative.
[a]All Ct values obtained were >40.0.

Samples from positive reference population with a high prevalence of abalone viral ganglioneuritis

A group of 32 abalone samples were obtained from a locality in Victoria where AVG outbreaks with high mortalities had been reported. Of these samples, 23 were classified as positive and 9 as negative by histological examination.

Of the 23 histology-positive samples, 22 were positive by TaqMan PCR (i.e. Ct values ≤ 35.8). The TaqMan PCR negative sample generated a Ct value of 39.0. Of the 9 histology-negative samples, 7 were negative by TaqMan PCR (i.e. Ct values ≥ 35.8). The 2 TaqMan PCR positive samples generated Ct values of 26.58 and 35.58. The possible reasons for the discrepancy between the two diagnostic tests are discussed later.

Another group of 18 abalone samples were obtained from a processing plant in Tasmania experiencing an AVG disease outbreak. Of these, 7 were positive by both histology and TaqMan PCR and 11 were negative by either test.

Samples from a negative reference population abalone with a low prevalence of abalone viral ganglioneuritis

A group of 1625 abalone samples were collected from open waters around the entire coast of Tasmania. These samples were considered to be from a reference population with an extremely low prevalence (i.e. <1%) of AbHV. All samples originated from abalone with no clinical signs of disease and were classified as negative by histological examination (i.e. absence of any ganglioneuritis). When tested by TaqMan PCR, the majority (1498 samples, 92.18%) returned Ct values ≥40, 126 samples (7.75%) returned Ct values between 35.8 and 40 and 1 sample (0.06%) returned a Ct value of 34.36 (i.e. below the Ct threshold of 35.8 for a negative result).

A summary of the positives and negatives scored by histology and the TaqMan PCR with these two reference populations is presented in Table 11.

Table 11. Assessment of AbHV infection of Victorian and Tasmanian abalone tissues by histology and by TaqMan PCR at a Ct threshold of 35.8

TaqMan	Histopathology		
PCR	(+)	(-)	
(+)	29	5	34
(-)	1	1640	1641
Total	30	1645	1675

Receiver operator analysis (ROC) curve
Receiver operator analysis (ROC) was used to evaluate the discriminatory power of the TaqMan PCR (Figure 10).

Positive reference samples used in the analysis:
A total of 30 positive abalone samples, based on the presence of histopathology (ganglioneuritis), were tested. Of these, 23 were obtained from Victoria and 7 from a processing plant in Tasmania (see above). The AbHV TaqMan PCR assigned 29 of the 30 samples as positive (i.e. Ct value ≤35.8). The other sample positive by histology that was not assigned as TaqMan PCR positive (i.e. Ct value of 39.3) originated from Victoria.

Negative reference samples used in the analysis:
A total of 1645 negative abalone samples based on the absence of any histopathological lesions (specifically ganglioneuritis), were tested. Of these, 9 originated from Victoria and 1636 from Tasmania (11 from a processing plant and 1625 from open waters). The AbHV TaqMan PCR assigned 1640 samples as negative (i.e. Ct value >35.8) and 5 samples as positive (i.e. Ct value ≤35.8). Two of these samples originated from Victoria (Ct values of 26.6 and 35.6) and 3 originated from Tasmania (2 from processing plant with Ct values of 31.6 and 35.4 and one from Tasmanian open waters with a Ct value of 34.4).

The AbHV TaqMan PCR had the highest diagnostic sensitivity (Se) and specificity (Sp) at a Ct value threshold of 35.8 [i.e. 96.7 (95% CI 82.7-99.4) and 99.7 (95% CI 99.3-99.9), respectively] (Figure 10).

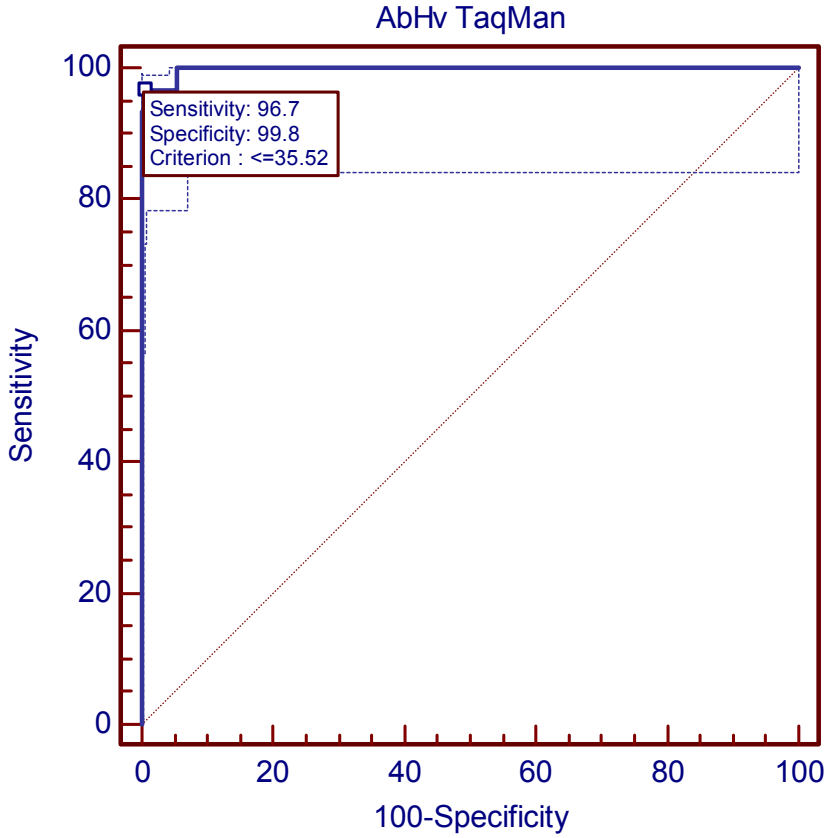


Figure 10. ROC curve (solid blue line). The blue dotted line indicates the 95% confidence interval. For reference, the diagonal dotted line represents a ROC curve for a test that cannot discriminate between positive and negative samples.

The area under the ROC curve (AUC) is a global summary statistic of diagnostic accuracy. Plots for diagnostic tests with perfect discrimination between negative and positive reference samples (i.e. no overlap of values of the two groups) pass through the co-ordinates 0,1, and represents 100% Se and Sp. In this case the AUC would be 1. In the analysis of the AbHV TaqMan PCR, the AUC was 0.998. According to an arbitrary guideline, one could distinguish between non-informative (AUC=0.5), less accurate (0.5<AUC≤0.7), moderately accurate (0.7<AUC≤0.9), highly accurate (0.9<AUC<1) and perfect tests (AUC=1) (Greiner *et al*., 2000).

Interactive dot diagram

The interactive dot diagram shown in Figure 11 illustrates the best separation (minimal false negative and false positive results) between the positive and negative samples at a Ct cut-off of 35.8 shown as a horizontal line. The AbHV TaqMan PCR results included one “false-negative” in 30 histology-positive samples and 5 “false-positives” in 1645 histology-negative samples, with histology used as the reference diagnostic method.

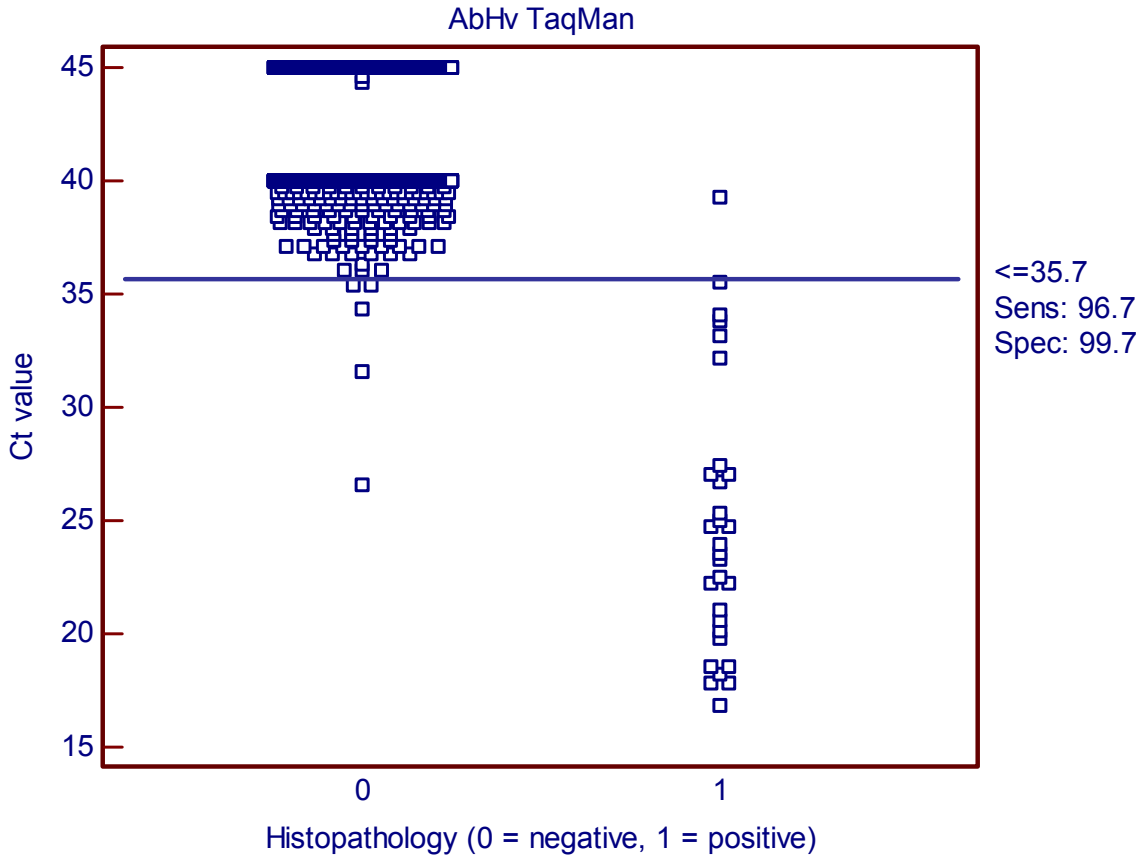


Figure 11. Interactive dot diagram. The interactive dot diagram illustrates the best separation (minimal false-negative and false-positive results) between the positive and negative groups at a Ct cut-off of 35.8, shown as a horizontal line. Values on the y-axis and x-axis are expressed as Ct values and diagnosis by histology (positive = 1 or negative = 0), respectively.

Plot versus criterion

For a diagnostic test, the higher the cut-off, or in this case the Ct value, the higher the diagnostic sensitivity, with a respective drop in the diagnostic specificity resulting in more “false-positive” results. Higher diagnostic sensitivity is normally required for screening tests used in surveillance programs. In contrast, a lower cut-off increases the diagnostic specificity with a respective drop in diagnostic sensitivity resulting in a higher percentage of “false-negative” results. A higher specificity is normally required for a confirmatory test. The graph (Figure 12) indicates that at a TaqMan PCR threshold Ct value ≤ 38.9, the diagnostic sensitivity (96.7) and specificity (95.7) are almost identical.

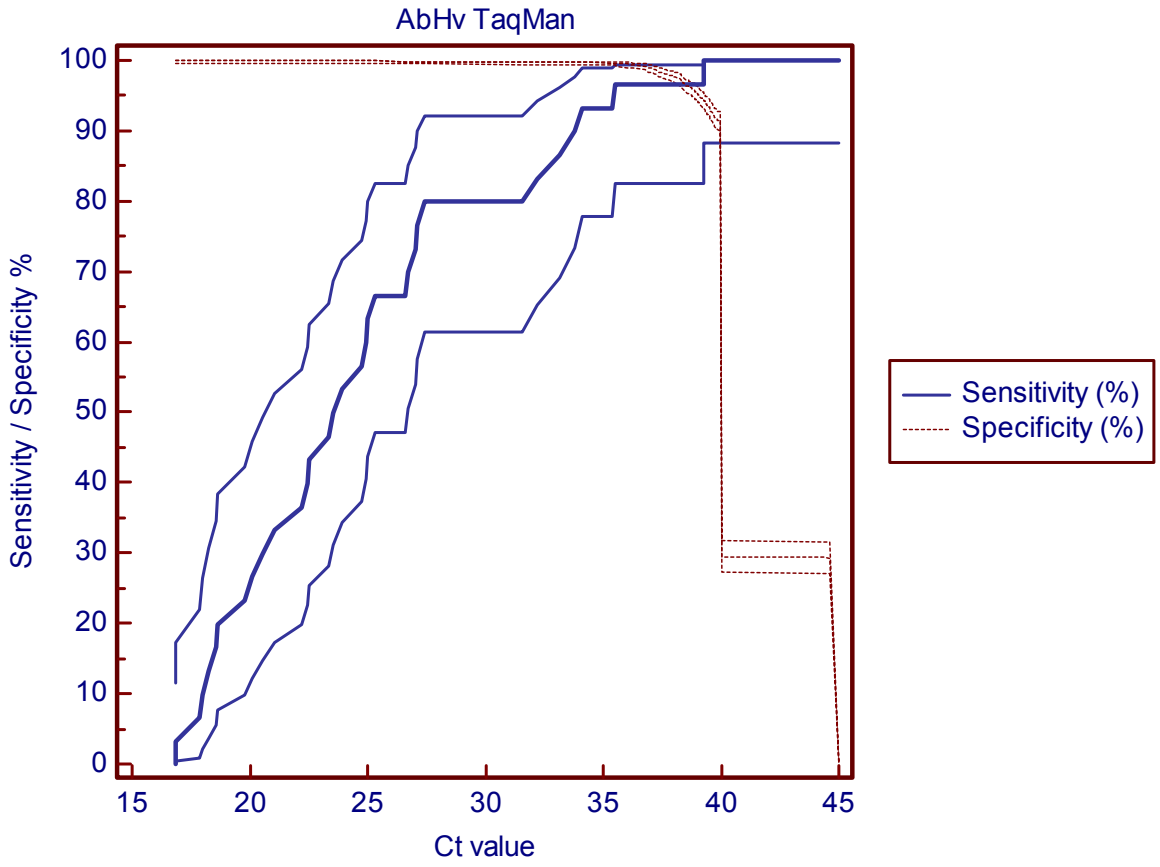


Figure 12. Plot versus criterion graph. The plot versus criterion graph illustrates the changes in diagnostic sensitivity and specificity, including their 95% confidence intervals, at different Ct value cut-off levels. Values on the x-axis and y-axis are expressed as Ct values and as percent sensitivity and specificity, respectively.

Inter- and intra-assay variation of the AbHV TaqMan PCR

With positive control samples (C+), the mean Ct value was 22, with a standard deviation of 2, a coefficient of variation of 10, and minimum and maximum Ct values of 18 and 27, respectively. For the negative control samples (C-), the mean Ct value was 40 with a standard deviation of 3, a coefficient of variation of 7 and minimum and maximum values of 35 and 45, respectively (Table 12a and Figure 13). Based on these data, preliminary upper and lower control limits using 1, 2 and 3 standard deviation intervals were established for C+ and C- as shown in Tables 12b and 12c.

Table 12a. Summary statistics for positive and negative controls in the AbHV TaqMan PCR

	C+	*C-*
Mean	22	40
Standard Error	0	0
Median	23	40
Mode	23	40
Standard Deviation	2	3
Sample Variance	5	7
Kurtosis	-1	0
Skewness	0	1
Range	9	10
Minimum	18	35
Maximum	27	45
Coefficient of variation	10	7
Count	106	98
Confidence Level (95.0%)	0	1

Table 12b. Upper and lower control limits for positive controls in the AbHV TaqMan PCR

	1 STD	2 STD	3 STD
UCL	24	27	29
LCL	20	18	16

Table 12c. Upper and lower control limits for negative controls in the AbHV TaqMan PCR

	1 STD	2 STD	3 STD
UCL	42	45	48
LCL	37	35	32

Reproducibility

For PCR-based detection tests it is important to obtain robustness estimates at the early stages of development. The TaqMan PCR methodology was transferred to the Victorian Department of Primary Industries (VicDPI) diagnostic laboratory and parallel testing of 47 Victorian abalone samples was performed at AAHL and VicDPI. Results indicate that AAHL consistently produced slightly lower Ct values (i.e. mean values for all samples were 29 for AAHL and 31 for VicDPI), indicating slightly increased sensitivity of the test methodology implemented at AAHL (Figure 14).

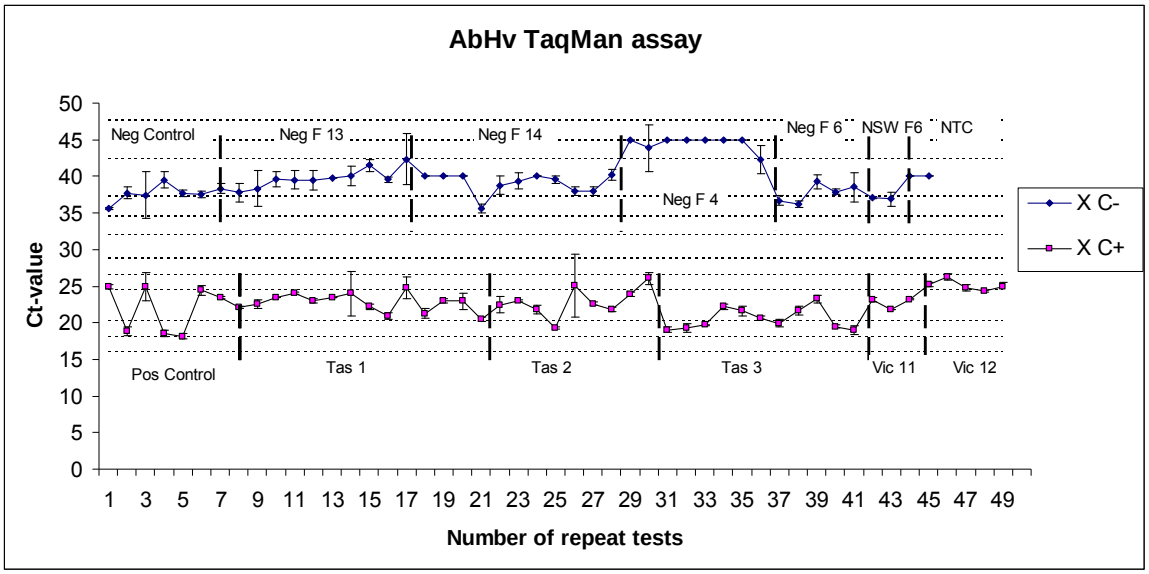


Figure 13. Graph demonstrating AbHV TaqMan PCR repeatability. Inter- and intra-assay variation for AbHV TaqMan PCR for different batches of positive and negative control samples (Sep-Nov 2008). The data points on each of the two graphs represent the mean values of the controls tested in either replicate or triplicate. The error bars represent 2 standard deviations and the dotted lines represent 1, 2 and 3 standard deviations after 49 runs of the positive samples (C+) and 45 runs of the negative samples (C-). Inter- and intra-assay variation within the same batch did not include variation between nucleic acid extractions.

In the absence of any other diagnostic test for comparative purposes, the existing standard test used for comparison was histological examination. It is likely that virus replication and thus AbHV DNA will be detectable by TaqMan PCR before pathological changes become evident (i.e. the TaqMan PCR can detect subclinical infection). This circumstance may result in positive TaqMan PCR detection of AbHV when abalone are still negative by gross disease signs or histology. Based on the preference for histology as the reference diagnostic test, such PCR data would be assigned as false-positive, thus underestimating the diagnostic specificity of the TaqMan PCR. It is possible, however, that histopathological lesions may remain long after the infection has cleared and viral DNA is no longer present in amounts detectable by TaqMan PCR. Such abalone would thus be positive by histology and be assigned as a (false) negative by TaqMan PCR, thus also underestimating the diagnostic sensitivity of the TaqMan PCR. To minimize the effects of an imperfect reference diagnostic test, additional information, such as epidemiological evidence of AbHV infection (e.g. prevalence and mortality data) were considered for selecting abalone sampling sites. Moreover, in histological examinations, it was noted that in some instances, lesions were quite focal in nature, and it is thus possible that abalone assigned as “false-negatives” by either histology or TaqMan PCR occurred simply as a result of neural tissue being selected for examination by either test that was clear of infection, whist other areas were not (i.e. sampling error).

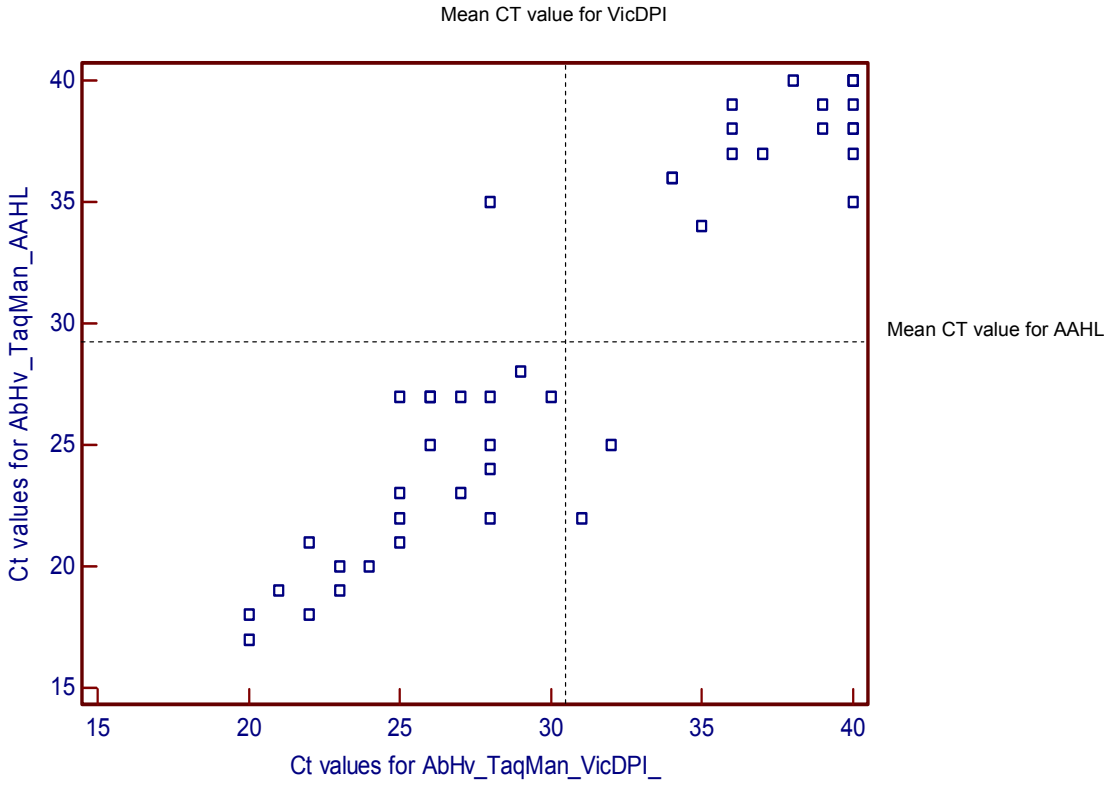


Figure 14. Graph demonstrating the AbHV TaqMan PCR reproducibility. Scatter diagram for results expressed as Ct values for 47 samples from Victoria using AbHV TaqMan PCR tests performed at AAHL (y-axis) and at VicDPI (x-axis). Note mean Ct values of samples tested at AAHL are slightly lower than samples tested at VicDPI.

For convenience, the preliminary diagnostic performance characteristics of the AbHV TaqMan PCR were estimated using a limited number of histology-positive samples from a high prevalence abalone population and a high number of samples from a population in which AbHV prevalence was expected to be low. In addition, the reference AbHV infection status of samples was determined by histological examination of all samples from the high prevalence population and some samples from the low prevalence population (Greiner & Gardner, 2000).

At a Ct threshold of 35.8, the AbHV TaqMan PCR had a diagnostic sensitivity and a diagnostic specificity of 96.7 (95% CI 82.7-99.4) and 99.7 (95% CI 99.3-99.9), respectively (Figures 10, 11 and 12). Because of the limited number of positive samples, the 95% confidence intervals for the diagnostic sensitivity are relatively broad and more samples from AbHV-infected abalone are needed for a more robust estimate of this parameter. The estimate of diagnostic specificity was based on 1625 samples from an abalone population with a low prevalence (<1%). When tested with the AbHV TaqMan PCR, the majority of these samples (1498 samples, 92.18%) returned Ct values ≥40, 126 samples (7.75%) returned Ct values between 35.8 and 40 and 1 sample

(0.06%) returned a Ct value of 34.4 (below the 35.8 Ct threshold). Thus if the purpose of the AbHV TaqMan PCR is as a screening test, such as surveillance studies to demonstrate freedom of infection in live abalone or abalone products (OIE, 2008b), the cut-off should be adjusted towards a high sensitivity which can be achieved by selecting a relatively high Ct value as the cut-off threshold. Currently, under the conditions detailed above, cut-off values have been adjusted as follows:

Clearly negative:	Ct value >36.00
Clearly positive:	Ct value <35.00
Indeterminate (requires retesting):	Ct value range 35.00-36.00.

Ultimate evidence of the usefulness of a new diagnostic test is its successful application in other laboratories and its inclusion in national, regional or international control programs. This requires acceptable robustness. Results for intra- and inter-assay variation of the AbHV TaqMan PCR performed at AAHL were satisfactory and preliminary upper and lower control limits were established. Coefficient of variation values of 10 and 7 for different batches of positive and negative controls respectively indicate good assay repeatability. When transferred and implemented at another laboratory (VicDPI), the PCR also showed good reproducibility of results with a range of positive and negative samples. The reliability and accurate performance of the AbHV TaqMan PCR thus make it a robust diagnostic test for detecting AbHV infection.

The development and validation of the AbHV TaqMan PCR has provided a powerful tool for detecting and identifying AbHV that can be applied to future epidemiological studies. When used to support routine histological analysis currently used to diagnose abalone viral ganglioneuritis, the TaqMan PCR provides sensitive, rapid data acquisition and is amenable to high throughput application. Its high sensitivity and specificity also provide a means of assessing AbHV prevalence within abalone populations. Moreover, unlike conventional PCR tests requiring gel detection of amplicons, the AbHV TaqMan PCR uses reagents and a methodology that minimises the risks of cross-contaminating diagnostic samples during processing.

Application of the TaqMan PCR should help address questions of an epidemiological nature, such as AbHV persistence in subclinical carriers, AbHV infection of other potential hosts (e.g. other mollusc species), possible routes of AbHV transmission as well as AbHV tissue distribution and infection loads in abalone tissues. In addition, preliminary data that the Taiwanese strain of AbHV is detected by the TaqMan PCR suggest significant genome sequence conservation with the Australian strain. Thus the use of the TaqMan PCR in Taiwan would also support disease management strategies implemented by Taiwanese fisheries authorities. Genome sequence analysis and phylogenetic analyses are needed to obtain more detailed knowledge on the relationship of the two viruses.

In situ hybridisation test for abalone herpes-like virus

Two DNA probes were generated and initially tested for their suitability in detecting AbHV in histological tissue sections using *in situ* hybridisation (ISH). The two probes employed targeted the sequences of two open reading frames, ORF49 and ORF66, identified in 454 sequence analysis of the AbHV genome. AbHV genomic DNA was used as template for PCR to amplify DNA probes labeled with digoxygenin (DIG) by use of the PCR DIG probe synthesis kit (Roche). Sequences of the PCR primers used to amplify the AbHV DIG DNA probes are shown in Table 13.

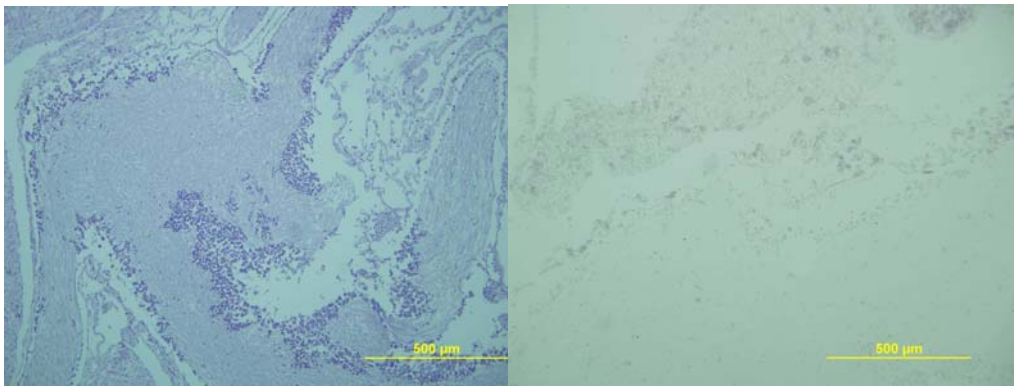
Table 13. Details of primers used to amplify probes for ISH

Primer Designation	Primer sequence (5'-3')	Amplicon (probe) size
AbHV_ORF49f1	AACCCACACCCAATTTTTGA	126 bp
AbHV_ORF49r1	CCCAAGGCAAGTTTGTTGTT	
AbHV_ORF66f1	TCCCGGACACCAGTAAGAAC	146 bp
AbHV_ORF66r1	CAAGGCTGCTATGCGTATGA	

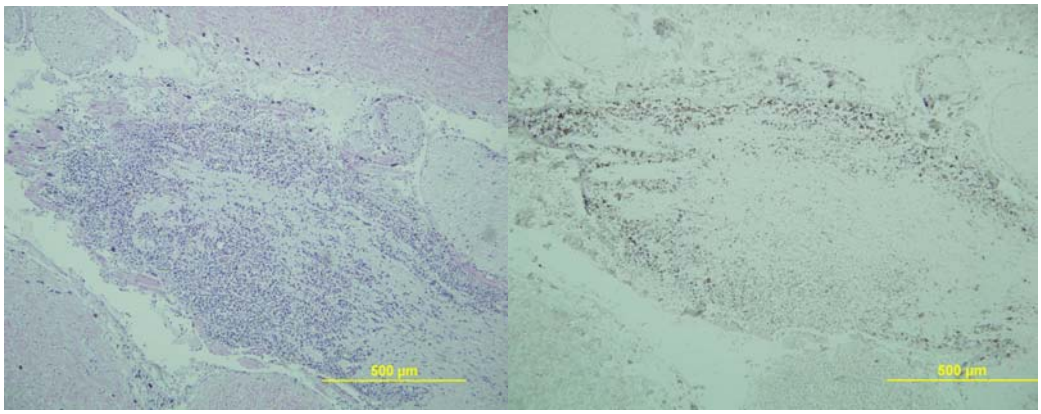
Preliminary ISH results obtained using the two DNA probes indicated that the 146 bp ORF66 sequence performed better and therefore it was used in subsequent analyses.

The ISH protocol was optimised using tissue sections from abalone either not infected or infected at high levels with AbHV. Tissue sections were dewaxed, rehydrated and permeabilized by treatment with proteinase K. Sections were then dehydrated in a graded series of ethanol concentrations and prehybridised in prehybridisation buffer (50% formamide, 4 x SSC, 10% dextran sulphate, herring sperm DNA and 5 x Denhardt's solution) for 15-30 min. The prehybridisation buffer was replaced with hybridisation buffer (prehybridisation buffer containing DIG-labeled probe). Target DNA in the tissue section and the DIG-labeled probe were denatured at 95°C for 5-15 min and hybridisation performed overnight at 37°C. Post-hybridisation, the tissue sections were washed in 2 x SSC for 5 min followed by a 15 min wash with 0.5 x SSC. DIG-labeled probe bound to the tissue section was detected using a DIG detection kit (Roche) and a standard colourimetric protocol.

Using the ORF66 DIG-DNA probe and the established ISH conditions, AbHV-positive tissue samples (e.g. sample no. 08-1666) identified by histology and PCR could be clearly distinguished from uninfected abalone samples (e.g. 07-1511-1) (Figure 15). However, in an attempt to improve the detection sensitivity (probe colour intensity) a longer DIG-DNA probe was amplified by PCR using the original forward primer AbHV_ORF66f1 (5'-TCCCGGACACCAGTAAGAAC-3') with an alternative reverse primer AbHV_ORF66r2 (5'-CCCGGACACCAGTAAGAAC-3'). This primer set similarly targeted the ORF66 sequence but amplified an 848 bp DNA product. Use of this longer DNA probe for ISH enhanced staining intensity (Figure 16) and was thus included in the ANZSDP and further developed and optimised.



A. Tissue from uninfected abalone B. ISH on section equivalent to A.



C. Tissue from infected abalone D. ISH on section equivalent to C.

Figure 15. *In situ* hybridisation test. Light microscopy images shown at low and high power magnifications. Note the pale-grey, washed-out appearance of uninfected tissues versus the intense, focal staining observed in infected tissues. A and C: H&E stained sections. B and D: Sections from the same blocks subjected to ISH using the 146 bp ORF66 DIG-DNA probe.

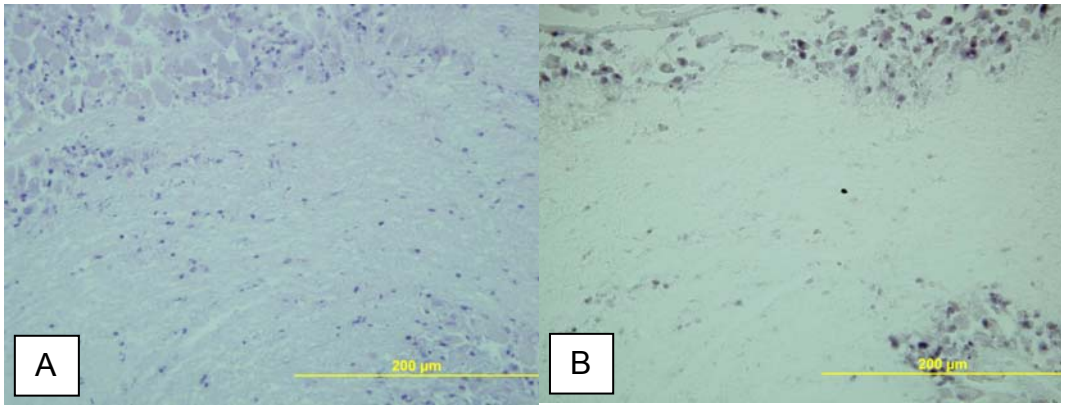


Figure 16. *In situ* hybridisation test. H&E stained tissue section of AbHV-infected abalone (A) and another section from the same block subjected to ISH using the 848 bp ORF66 DIG-DNA probe. Note the intense blue-black focal staining observed in infected cells.

In vivo infectivity model optimisation

Virus titration

The typical pattern of mortality following i.m. injection of healthy abalone with a 10-fold dilution series (0.1 mL per abalone) of a virus stock inoculum is shown in Figure 17. An LD_{50} value ($10^{-6.39}$) was calculated from the equation representing the dose-response curve derived from the cumulative mortality curves (Figure 18). While initial investigations of mortality events at abalone farms in Victoria suggested that ganglioneuritis had an infectious aetiology (Hooper *et al*., 2007), the exact cause of disease was not identified. This project confirmed that abalone viral ganglioneuritis (AVG) can be reproduced in healthy abalone by injection them with filtered (0.45 µM) homogenates of neural tissue from diseased abalone obtained from affected farms and that these homogenates contained virions with a herpes virus-like structure (Tan *et al*., 2008) but no other structures resembling an infectious agent. While not satisfying Henle-Koch postulates (Evans, 1976) in the strictest sense, mainly due to the inability to isolate virus in cell culture, the available data provide strong evidence that AbHV is the aetiological agent of abalone viral ganglioneuritis.

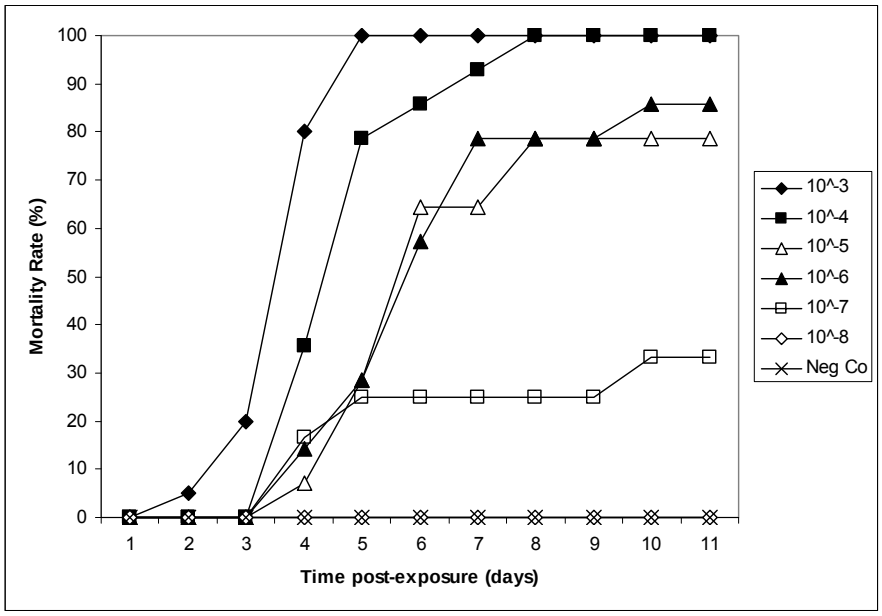


Figure 17. Cumulative mortality curves. Graphs showing the cumulative mortality of hybrid abalone injected intramuscularly with various doses of a stock AbHV homogenate and then maintained in separate tanks at 15-18°C for 11 days. The cumulative mortality curves represent the total number of dead and moribund abalone for each group over 14 days (there was no change in the cumulative mortality between day 11 and 14 post-infection).

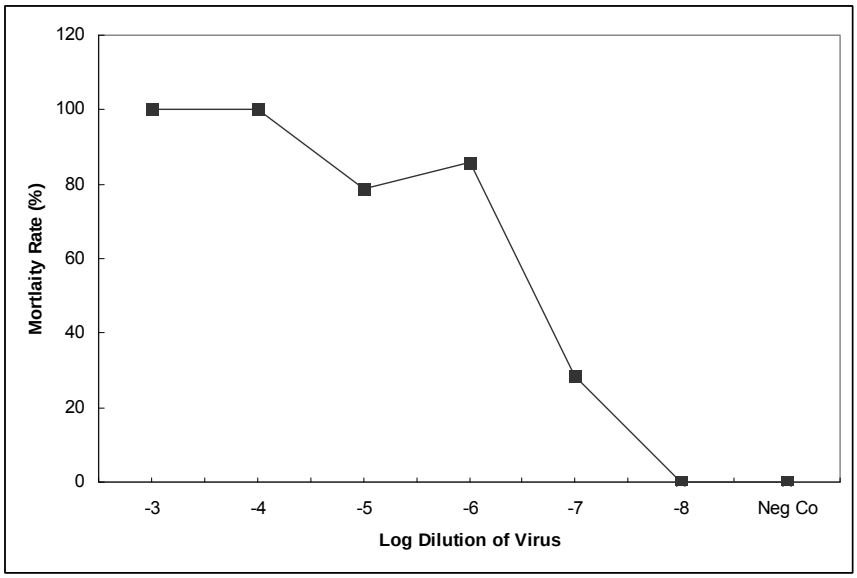


Figure 18. Dose-response curve. Graph of mortality rate vs homogenate dilution obtained from the data represented in Figure 17. LD_{50} for this particular virus stock was calculated to be $10^{-6.39}$.

Horizontal transmission trial

In addition to transmitting AVG by intramuscular injection, disease was also transmitted by co-habitation of healthy abalone with moribund abalone or by exposing healthy abalone to virus-contaminated water. In the co-habitation experiment, no direct contact of moribund abalone with healthy abalone demonstrating that AbHV can be transmitted via water. Deaths arising in this transmission trial are shown in Figure 19. The healthy abalone from group 1 (co-habitation with virus-injected abalone) began dying at day 3 post-exposure (p.e.) and were all dead by day 8 p.e. The healthy abalone from group 2 (co-habitation with diseased abalone) began dying at day 4 p.e. and were all dead by day 8 p.e.

AbHV appears to be highly pathogenic in these species of abalone as demonstrated by the dose-response curves. Stock virus homogenates diluted by six orders of magnitude remain infectious and pathogenic to abalone causing disease with associated mortality within 2-5 days of exposure. The amount of infectious virus in these diluted preparations is clearly dependent on how many diseased abalone were used to prepare the homogenate, the state of those diseased abalone, and what volume in which it was prepared.

A summary of mortalities obtained with abalone groups exposed to different dilutions of virus-contaminated water is shown in Table 14. The group 3 abalone (undiluted co-habitation water) began dying at day 3 p.e. and were all dead by day 10 p.e. The group 4 abalone (10% co-habitation water) began dying at day 3 p.e. and were all dead by day 19 p.e. The group 5 abalone from 1 tank (1% co-habitation water) began dying at day 8 p.e. and were all dead by day 14 p.e. However, in the duplicate tank of group 5 abalone, no mortality occurred over the duration of the experiment. Abalone from groups 6 and 7 (0.01 and 0.001% co-habitation water, respectively) showed no morbidity or mortality during the course of the experiment.

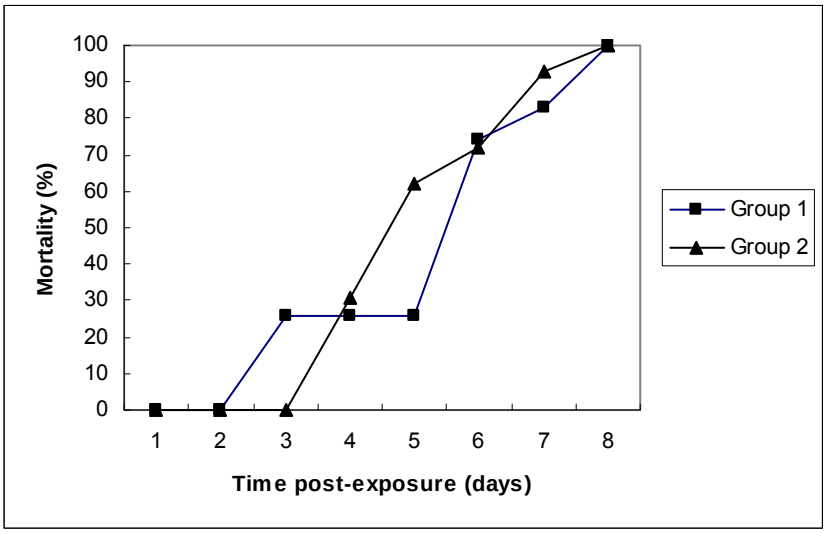


Figure 19. Horizontal transmission trial. Cumulative mortality of abalone in co-habitation with either abalone injected with a virus preparation (i.e. abalone from group 1) or moribund abalone obtained from an affected farm (i.e. abalone from group 2).

Table 14. Summary of abalone mortalities in water transmission trials

Group	Water dilution	Time of onset of mortality (days post-exposure)	Mortality (%)		Total mortality (%) for both tanks (# dead animals/total # animals)
			Tank 1	Tank 2	
3	None	3	100	100	100 (23/23)
4	10^{-1}	3	100	100	100 (27/27)
5	10^{-2}	8	100	0	46 (12/26)
6	10^{-4}	Not applicable	0	0	0 (0/28)
7	10^{-5}	Not applicable	0	0	0 (0/23)

Histopathology

Moribund abalone from groups 1 to 5 were sampled at various time-points during the course of the experiment and were processed for histological examination. For all abalone demonstrating abnormal clinical signs (weak attachment to the substrate and curling of the foot), there were lesions in the pleuropedal ganglion that were consistent with abalone viral ganglioneuritis (AVG, Figure 20).

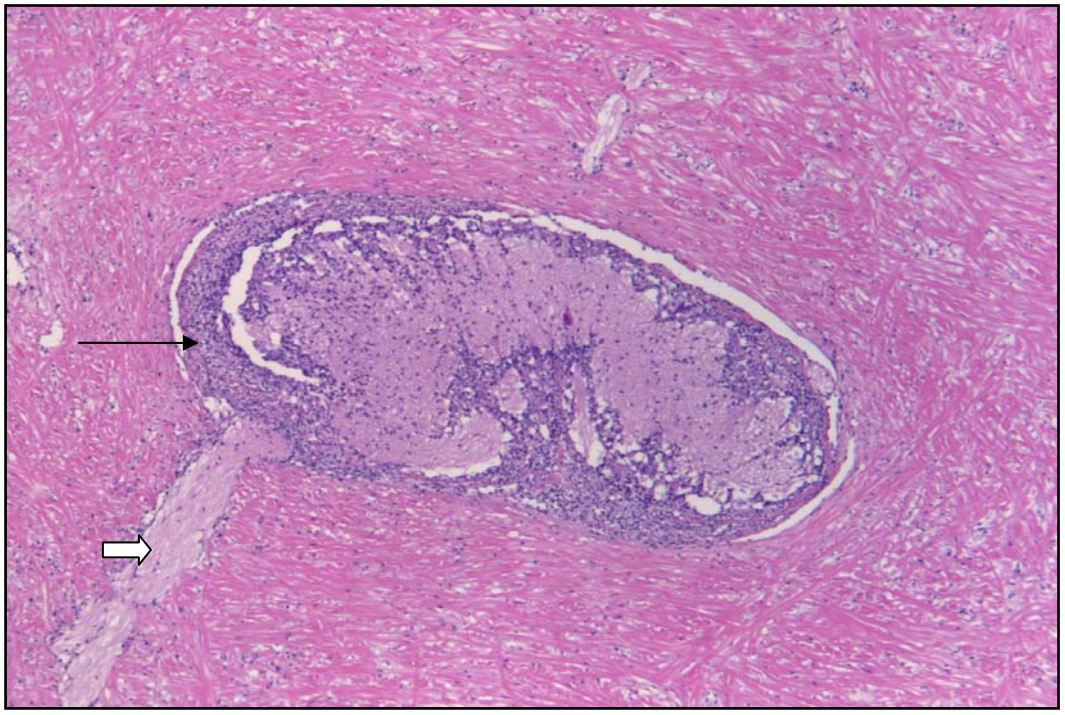


Figure 20. H&E-stained tissue section from an AbHV-infected abalone at 3 days post-infection. There is ganglioneuritis in the pleuropedal ganglion (black arrow). The white arrow indicates a lateral nerve cord.

PCR Analysis

Abalone that had died during the course of the experiment were examined for the presence of AbHV by PCR. Tissue samples from abalone that had died from AVG yielded the expected 486 bp amplicon when analysed by conventional PCR. However, as discussed previously, not all infected abalone yielded an amplicon using the conventional PCR, suggesting that there are limits to the diagnostic capability of this test. Using the TaqMan PCR, all abalone that were AbHV-positive by the conventional PCR and/or had histological lesions consistent with AVG yielded Ct values <35.00 and were thus assignable as positive for AbHV.

Electron microscopy. Virions with morphology that closely resembled viruses belonging to the *Herpesviridae* (Figure 21) were detected in experimentally infected abalone but not in uninfected abalone.

While this is the first viral disease associated with high mortality that has occurred in abalone in Australia, it is interesting to note that a herpes-like virus and a “spherical virus” with a diameter of 100-130 nm have been detected in diseased abalone (*Haliotis diversicolor*) in Taiwan (Chang *et al*., 2005) and in China (Wang *et al*., 2004), respectively. The relationship of the virus from Australian abalone to these other viruses is unknown. Other diseases of abalone with a suspect viral aetiology include abalone amyotrophia (Nakatsugawa *et al*., 1999; Otsu and Sasaki, 1997) reported in farmed abalone from Japan. It has markedly different characteristics from abalone viral ganglioneuritis. While disease, caused by AbHV, has not been discovered in other aquatic animals, it is important for future studies to determine whether or not other molluscs are susceptible to AbHV, and also to determine the

relationship of AbHV not only to other abalone herpes-like viruses but also to other molluscan herpesviruses including those that infect bivalves, such as OsHV-1 of oysters (Comps and Cochennec, 1993).

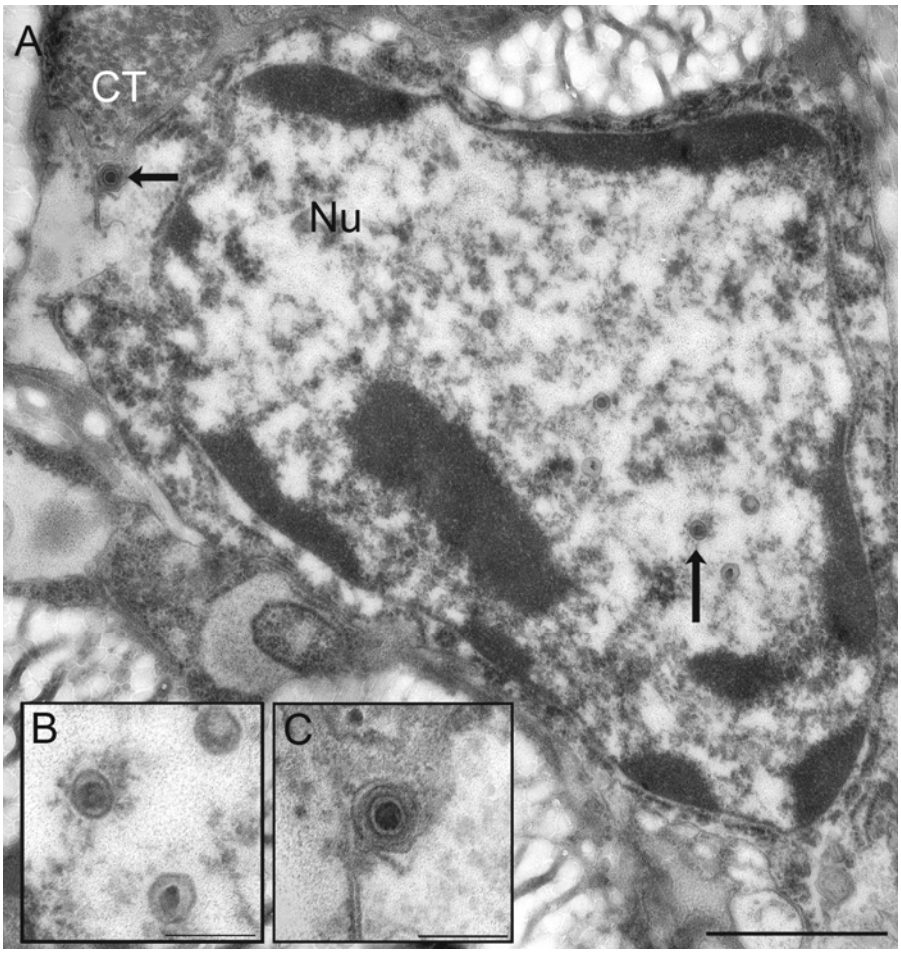


Figure 21. Transmission electron micrograph of an ultrathin section of a pleuropedal ganglion from an AbHV-infected abalone. A: Transmission electron micrograph of a putative glial cell and surrounding connective tissue (CT). Long arrow: Capsids within a nucleus (Nu); Short arrow: Enveloped herpes-like virus within non-defined, extracellular, granular material, bar represents 900 nm. B: Higher magnification of capsids within nucleus, bar represents 200 nm; C: Higher magnification of an enveloped herpes-like virus indicated in (A), bar represents 200 nm.

Viral stability at different storage temperatures

Early experiments demonstrated that virus stocks stored at -20°C lost infectivity (i.e. failed to induce disease and mortality) within 6 months of storage (results not shown). Therefore, a fresh stock of virus was prepared as described in the Materials and Methods and aliquots were stored at -20°C, -80°C and in liquid nitrogen. These stocks were subsequently titrated in abalone (6-14 abalone/virus dilution) at various times post-storage. These transmission experiments (Table 15) indicated a decrease in the mortality of abalone inoculated with virus stock stored at -20°C for 6 months and 21 months. Mortality of abalone also decreased with virus stored at -80°C. Storage of stock

virus in liquid nitrogen was associated with only a marginal decrease in abalone mortality, even after 21 months storage.

Table 15. Mortality in abalone i.m. injected with AbHV stored for various periods of time at -20°C, -80°C and in liquid nitrogen

Storage period	Storage temp. (°C)	Mortality (%) at 14 days post-inoculation with the indicated dilution of the virus stock				
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
<6 weeks	-80°C	100	100	79	86	33
6 months	Liquid N_2	ND	100	100	28.6	71.4
	-80°C	ND	100	87.5	62.5	62.5
	-20°C	ND	87.5	25	25	62.5
12 months	Liquid N_2	ND	100	62.5	12.5	12.5
	-80°C	ND	87.5	25	0	0
	-20°C	ND	0	25	0	0
21 months	Liquid N_2	100	100	75	25	ND
	-80°C	ND	ND	ND	ND	ND
	-20°C	75	50	12.5	0	ND

ND = not done

While the stability of the virus at ambient temperature has not yet been studied, the temperature for storage of viral stocks was investigated over a 21 month period. It appears that storage in liquid nitrogen prolongs viral infectivity and pathogenicity for longer periods of time than storage at either -80°C or -20°C. Since this virus cannot be cultured using standard fish cell lines and a mollusc cell line (results not shown) available at AAHL, it is important for sustaining future research that viral stocks are replenished on a regular basis.

Technology transfer

The TaqMan PCR methodology has been successfully transferred (all positive and negative controls yielded expected results) to State diagnostic laboratories in Tasmania (DPIW), South Australia (PIRSA), Western Australia (AHL) and NSW (EMAI) and to a laboratory in New Zealand (MAF Biosecurity). Samples of locally sourced abalone from open waters tested during technology transfer yielded negative results. Samples previously frozen and stored in readiness for analysis following technology transfer will be processed by State laboratories. AAHL will provide confirmatory diagnosis should the State laboratories obtain suspect AVG-positives.

Geographical range

In Victoria, abalone viral ganglioneuritis (AVG) continues to spread in both easterly and westerly directions, albeit at a relatively slow rate of progress. Due to this slow rate of spread, monitoring activity is regular but infrequent. The current confirmed geographical range for the disease, in Victoria, is from

the Discovery Bay Marine Park in the west of the state to reef areas west of Johanna Beach (approx 15 km North East of Cape Otway) (Figure 22). However, there is evidence that the whole Western Zone coastline is affected.

In Tasmania, AVG has been detected in a processing plant on the east coast. Interestingly, the disease has not been detected in the wild. While the abalone in the processing plant were sourced from Tasmanian waters, the causative agent for AVG, abalone herpes-like virus, has only been detected (by TaqMan PCR) in one out of over 1600 abalone samples obtained as part of a state-wide surveillance program, indicating a very low prevalence.

All abalone samples from Western Australian, South Australian and NSW waters have tested negative by the AbHV TaqMan PCR test.

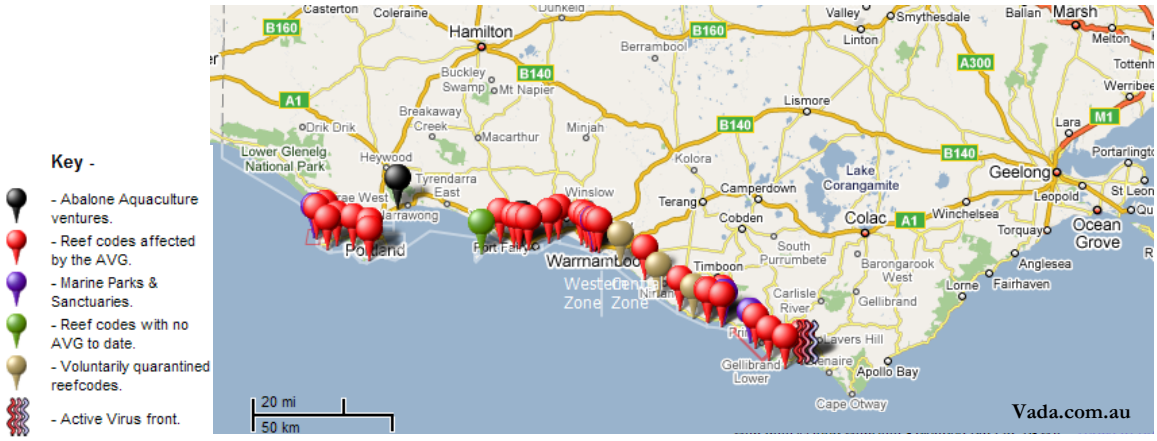


Figure 22. Regions of the Victorian coastline where AVG has been confirmed (red markers) (adapted from the Victorian Abalone Divers Association Inc, (VADA) website, www.vada.com.au)

Benefits

Industry sectors that will benefit from these outcomes include abalone wild-capture and abalone aquaculture in Tasmania, New South Wales, Victoria, Western Australia and South Australia. Identification of infected and uninfected stock will allow industry and State officers to implement a disease management plan based on accurate information. Isolation of infected and uninfected abalone populations will be possible thus enhancing our capability to control and/or eradicate AVG.

State diagnostic laboratories will benefit by being provided with sensitive and specific reagents and procedures that have been validated using both exotic and enzootic isolates. Based on accurate diagnoses, State officials responsible for conducting activities of the Local Disease Control Centre (LDCC) will be able to make informed decisions during implementation of disease management procedures.

Infections with abalone herpes-like virus are listed by the OIE and, as a Member State, Australia is obliged to report on their presence or absence in Australia. Such reporting can only be achieved if there are sensitive and specific reagents and procedures in place that are recognised by international agencies. Using internationally accepted procedures, Australian authorities can be confident of providing accurate information to the OIE, other international agencies and to our international trading partners.

Further Development

The absence of reliable, sensitive and specific diagnostic procedures for the detection and identification of AbHV infections has been the major hindrance to research on this emerging viral pathogen of wild and farmed abalone. Consequently, the outputs from this project, an optimised infectivity model, conventional and real-time PCR assays, and an *in situ* hybridisation test, will have a major impact not only on diagnosis of infections but also on planned research identified in the National Abalone Health Work Plan drafted by the Federal Government's AAHC (Aquatic Animal Health Committee) and MACC (Marine and Coastal Committee) Working Groups.

Planned research includes:

- A comparison of the Tasmanian and Victorian strains of AbHV
- Validation of the *in situ* hybridisation diagnostic test including roll out to other States
- Development of a quantitative assay (qPCR) to determine AbHV infectious dose
- Determination of the sensitivity of AbHV to physico-chemical conditions including its stability in water/on fomites and its sensitivity to inactivation agents
- Determination of the role of mucus in AbHV transmission
- Determination of whether a latent stage exists in AVG
- Determination of the susceptibility of remnant populations of abalone previously exposed to AVG and known unexposed wild populations in South Australia.

Planned Outcomes

Development of molecular tools for the sensitive and specific detection and identification of herpes-like virus infections that could lead to abalone ganglioneuritis will provide diagnostic laboratories with the necessary reagents and procedures to allow not only definitive diagnoses of overt disease, but also enable identification of sub-clinical infections. Thus State authorities will be able to provide all sectors of the abalone industry with an enhanced health service in relation to this specific disease. It is anticipated that these tools will provide the means to obtain accurate information on the host and geographic distribution of this virus. Armed with this information, State authorities and abalone industry sectors will be able to make informed decisions on the future management of this fishery.

Of utmost importance is restricting the movement of infected animals. It is possible that the current disease situation could be self-limiting due to the distribution and density of wild abalone. If this is the case, then translocation of infected animals to uninfected zones needs to be avoided. This can only be achieved if infected and uninfected animals can be identified with a high level of confidence. Testing of abalone broodstock using the TaqMan PCR as part of the translocation program from the wild to aquaculture facilities is in progress.

Currently, the TaqMan PCR is being used by state authorities in Tasmania, Victoria, South Australia and Western Australia as the primary diagnostic assay for the detection and identification of AbHV infections. The diagnostic tools developed as part of this project will form the basis for progressing future research on this emerging disease of farmed and wild abalone.

Conclusions

The aetiological agent of AVG has been confirmed to be a herpes-like virus (AbHV), not reported previously (prior to 2006) anywhere in Australia. Greater than 90% of its genome has been sequenced and compared to Ostreid herpesvirus-1. It is likely that AbHV will be the second species of virus assigned to the newly proposed family, *Malacoherpesviridae* (Minson *et al*., 2000; Davison *et al*., 2009). Prior to the work undertaken in this project, diagnosis of AVG was limited to the observation of clinical signs and histopathology and the detection of a herpes-like virus by electron microscopy. Detection of viral infections in sub-clinical abalone was not possible. The TaqMan real-time PCR has become the 'gold standard' for the detection and identification of AbHV. The technology has been transferred to other diagnostic laboratories that are now using it as the primary diagnostic tool for the detection and identification of AbHV infections.

Of further interest, the abalone herpes-like virus affecting *H. diversicolor* in Taiwan yields a positive reaction with the TaqMan real-time PCR indicating that the Taiwanese strain of AbHV is quite closely related to the Australian strain.

The use of 454 sequencing technology as part of this project to obtain the AbHV genome sequence was instrumental in successfully achieving all of project objectives. The importance of this technology and its applicability to 'sequencing projects' of any type cannot be overstated.

Apart from providing diagnostic laboratories, regulatory authorities and industry with effective diagnostic tools for AVG, the development of the conventional and real-time PCR tests and the *in situ* hybridisation test will facilitate further research to better understand the biology of AbHV and its interaction with its host species.

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Appendix 1: Intellectual Property

All information arising from this project has been used for the development and/or establishment of standard diagnostic procedures for use by diagnostic laboratories. No intellectual property has been identified.

Appendix 2: Project Staff

Name	Position	Organisation
Dr Ken McColl	Senior Research Scientist	AAHL Fish Diseases Laboratory
Dr Serge Corbeil	Experimental Scientist	
Dr Mark Crane	Project Leader, AFDL	
Ms Lynette Williams	Senior Technical Officer	
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Dr Mark Fegan	Senior Research Scientist	
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Dr Judith Handlinger	Senior Veterinary Pathologist	
Dr Kevin Ellard	Senior Veterinary Officer	
Dr Marianne Douglas	Research Scientist	
Dr Rod Andrewartha	Chief Veterinary Officer	
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Dr Hamish Aiken	Aquatic Animal Health Officer	Primary Industries and Resources - South Australia
Dr Jeffrey Go	Veterinary Officer, Aquatic Animal Health	Department of Primary Industries, New South Wales

Appendix 3. External communication

Progress reports to industry

During the course of this project, all milestone progess reports have been forwarded to the Abalone Council Australia (ACA) and the Australian Abalone Growers' Association (AAGA). In addition, project staff have attended the following industry meetings and provided presentations on project progress:

Crane M, Lancaster M, Corbeil S, Wong F, Savin K, Williams L, Tan J and Warner S. 2007. FRDC Project No. 07/006: Aquatic Animal Health Subprogram: Development of molecular diagnostic procedures for the detection and identification of herpes-like virus of abalone (*Haliotis* spp.). 2007 FRDC Abalone Aquaculture Subprogram Industry Workshop, SARDI, West Beach, SA, 27-28 August 2007.

Warner S, Wong F, Lancaster M, Cogan N, Savin K, Tan J, Corbeil S and Crane M. 2007. Development of molecular diagnostic procedures for the detection and identification of herpes-like virus of abalone (*Haliotis* spp). 2nd National Abalone Virus Scientific and Management Forum, Melbourne, 2 October 2007.

Crane M. Abalone herpesvirus: R&D Plans. Australian Abalone Growers' Association Annual General Meeting, Brisbane, 5 August 2008.

Crane MStJ, Corbeil S, Wong F, Williams LM, Kvalheim N, Savin K and Warner S. 2008. Development of molecular diagnostic procedures for the detection and identification of abalone herpes-like virus. 4th National Abalone Convention, Port Lincoln, 17-19 September 2008.

Crane MStJ, Corbeil S, Colling A, Williams LM, Wong F, Savin K, Cogan N, Fegan M, Mohammad I, Sunarto A, Handlinger J, Pyecroft S, Douglas M, Murdoch B and Warner S. 2009. Development and validation of a real-time (TaqMan) PCR assay for the detection and identification of abalone herpes-like virus (AbHV). Australian Abalone Growers' Association Annual General Meeting, Geelong, 7-8 September 2009.

Warner S, Fegan M, Kvalheim N, Wong F, Mohammad I, Savin K, Lancaster M and Mark Crane. 2009. Development of an *in situ* hybridisation assay for the detection and identification of the abalone herpes-like virus. Australian Abalone Growers' Association Annual General Meeting, Geelong, 7-8 September 2009.

Reports at Scientific Conferences

Wong F, Lancaster M, Crane M, Corbeil S, Hyatt A, Tan J, Savin K, Cogan A, Sawbridge T and Warner S. 2007. Characterisation of a herpes-like virus infecting Australian populations of abalone, *Haliotis* spp. by whole genome pyrosequencing. 13th Int. Symp. World Assoc. Vet. Lab. Diag., Melbourne, Victoria, Australia, 11-14 November 2007.

Warner S, Wong F, Lancaster MJ, Tan J, Cogan N, Savin K, Sawbridge T, Corbeil S and Crane M. 2007. Development of molecular diagnostic procedures for the detection and identification of a herpes-like virus infecting Australian populations of abalone, *Haliotis* spp. 4th Scientific Meeting of the Australian Virology Group, Fraser Island, Queensland, Australia, 9-13 December 2007.

Warner S, Savin K, Wong F, Kvalheim N, Fegan M, Lancaster M and Crane M. Development of Molecular Diagnostic Procedures for the Detection and Identification of Abalone Herpes-like Virus – Part 1 *In situ* hybridisation assay. Australian Association of Veterinary Laboratory Diagnosticians Annual Meeting, 6-7 November 2008, Brisbane, Queensland.

Crane M, Corbeil S, Savin K, Wong F, Williams L, Fegan M, Moody N and Warner S. 2008. Development of Molecular Diagnostic Procedures for the Detection and Identification of Abalone Herpes-like Virus – Part 2: PCR assays. Australian Association of Veterinary Laboratory Diagnosticians Annual Meeting, 6-7 November 2008, Brisbane, Queensland.

Corbeil S, Williams L, Hyatt A, McColl K, Colling A, Wong F, Savin K, Murdoch B, Warner S, Lancaster M, Tan J, Cogan N, Chang P and Crane M. 2009. Emergence of an abalone herpes-like virus in Australia. 7th International Abalone Symposium, Pattaya, Thailand, 19-24 July 2009.

Manuscripts in preparation

Corbeil S, Colling A, Williams LM, Wong FYK, Savin K, Warner S, Murdoch B, Cogan NOI, Sawbridge TI, Fegan M, Mohammad I, Sunarto A, Handlinger J, Pyecroft S, Douglas M, Chang PH and Crane MStJ. Development and validation of a TaqMan PCR for the Australian abalone herpes-like virus (AbHV).

Savin K, Wong FYK, Sawbridge TI, Savage D, Cogan NOI, Cocks B and Warner S. Phylogenetic analysis of a herpesvirus causing ganglioneuritis in abalone identifies a new member of the Malacoherpesviridae family.

McColl KA, Corbeil S, Williams LM, Hyatt AD Mackay E, Crameri SG and Crane MStJ. Abalone viral ganglioneuritis: Herpes-like viral infection causes acute disease associated with high mortality rates in Australian abalone.

Appendix 4: Draft Australian and New Zealand Standard Diagnostic Procedure

Abalone herpes-like virus

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SUMMARY

Abalone herpes-like virus (AbHV) is the aetiological agent for abalone viral ganglioneuritis (AVG) a contagious viral disease of abalone in Australia (Hooper et al., 2007; Ellard et al., 2009) and possible abalone species in other countries (Wang et al., 2004; Chang et al., 2005). However, the relationship between the Australian viral isolate(s) and the exotic isolates has not, as yet, been elucidated. In Australia, the disease was first reported in farmed abalone populations (greenlip abalone (*Haliotis laevigata*), blacklip abalone (*H. rubra*) and hybrids of these two species) in aquaculture facilities situated along the Victorian coast-line in 2005 (Hooper et al., 2007), and has since caused mortalities in wild abalone in Victorian open waters. The known host range is currently limited to greenlip abalone (*Haliotis laevigata*), blacklip abalone (*H. rubra*) and hybrids of these two species in Australia but this aspect has not been studied in detail. The exotic viral isolates have caused disease in the Taiwan abalone, *H. diversicolor supertexta* and there are reports of viral disease (as early as 1999) of *H. diversicolor* from China (Wang et al., 2004).

Diseased abalone may display irregular peripheral concave elevation of the lateral foot margins, swollen mouth parts, protrusion (eversion) of the radula, minimal movement of the pedal muscle, reduced pedal adhesion and absence of the righting reflex of healthy abalone when turned onto their backs. Diseased abalone exhibit ganglioneuritis in neural tissue (ganglia and peripheral nerves) - inflammation observed in tissues stained with haematoxylin and eosin (Hooper et al., 2007; Ellard et al., 2009).

Identification of the agent: Presumptive diagnosis of AVG is based on clinical signs and histopathology with confirmation using molecular tests. A conventional polymerase chain reaction is available for the identification of AbHV in clinically affected animals. In addition, a real-time (TaqMan) PCR has been developed which can detect AbHV in sub-clinically infected abalone. Moreover, an *in situ* hybridisation test has been developed for the localisation of virus associated with histopathology in tissue sections.

Status of Australia and New Zealand: AbHV first detected in regions of Victoria (2005 onwards) and Tasmania (2008), Australia and is exotic to New Zealand.

Introduction

Abalone viral ganglioneuritis (AVG) was first reported from Australia (Victoria) in 2005/6 from cultured abalone (Hooper et al., 2007). Since then, the disease has been found in an expanding geographical range along the Victorian coast. In 2008, AVG was reported from a processing plant in Tasmania (Ellard et al., 2009). In contrast to the situation in Victoria, AVG has not occurred in farmed abalone in that state nor in wild abalone in Tasmanian open waters. A similar disease has been reported in abalone from Taiwan (Chang et al., 2005) and China (Wang et al., 2004).

Aetiology

AVG is a viral disease that appears to be restricted to abalone species and has been associated with a herpes-like virus (abalone herpes-like virus - AbHV), based on the morphological characteristics of the virion (Roizman & Pellet, 2001). Recently, nucleic acid sequence analysis has demonstrated that AbHV is related to another invertebrate herpes-like virus – Ostreid herpesvirus type 1 (OsHV-1) which has been assigned to the newly created Malacoherpesviridae (Minson et al., 2000; Davison et al., 2009). However, based on sequence analyses, these invertebrate herpes-like viruses are not typical of the Herpesviridae and it has been suggested that they should form a separate group within the classification of dsDNA viruses (Gao & Qi, 2007).

Host and Geographical Ranges

Host Range

Abalone herpes-like virus appears to be restricted to species of abalone. To date, the occurrence of disease associated with herpes-like viruses in abalone has been reported from greenlip abalone (*Haliotis laevigata*), blacklip abalone (*H. rubra*) and hybrids of these two species in Australia, and *H. diversicolor supertexta* (Chang et al., 2005) in Taiwan and *H. diversicolor* in China (Wang et al., 2004).

Geographical range
The disease is found in abalone populations along the Victorian coast in Australia (Hooper et al., 2007). Abalone herpes-like virus has been found in the absence of disease in abalone populations from Tasmania (Ellard et al., 2009). Similar disease/agent has been reported from China (Wang et al., 2004) and Taiwan (Chang et al., 2005).

Epidemiology

Transmission is horizontal via water. Vertical transmission has not been proven. All life-cycle stages (juvenile to adult) of affected abalone species appear to be susceptible. High mortality rates (up to 90%) have been associated with disease outbreaks on farms in Victoria. In Tasmania, there have been no reports of disease in wild abalone in open waters while, in Victoria, mortality rates between 5% and 90% have been reported in wild populations.

Experimental infections have demonstrated that the incubation period between exposure of abalone to virus-contaminated water and the onset of clinical disease is 4 days (McColl et al., 2007).

The related Ostreid herpesvirus type 1 (OsHV-1) can persist in healthy adult oysters (Arzul et al., 2002). In Tasmania, the occurrence of AbHV in healthy abalone indicates that this virus can persist in subclinical hosts and explains how this virus can remain undetected in wild abalone populations.

Clinical Signs

AVG outbreaks in both farmed and wild populations can be associated with high mortality rates (up to 90%). Clinically, a variable proportion of affected abalone may demonstrate irregular peripheral concave elevation of the foot, swollen and protruding mouth parts and eversion of the radula. Affected abalone may demonstrate minimal movement of the pedal muscle, excessive mucus production, absence of the marked extension of the foot shown in the righting reflex when healthy abalone are turned onto their backs and reduced pedal adhesion to the substrate. In Tasmania, affected abalone observed in processing plants exhibit ‘hard foot’ or tetany; excessive mucus production; abnormal spawning; ‘bloating’ (Ellard et al., 2009).

Pathology

Abalone affected with AVG demonstrate inflammation (increased infiltration by haemocytes) and necrosis confined to neural tissue (cerebral, pleuropedal and buccal ganglia, branches of the pedal nerve and peripheral nerves) as observed in tissues stained with haematoxylin and eosin and examined by light microscopy (Hooper et al., 2007; Ellard et al., 2009).

Diagnostic Tests

A range of diagnostic tests, including conventional PCR, real-time PCR and *in situ* hybridisation (Crane et al., 2009), are available for confirmation of a presumptive

diagnosis of AVG based on clinical signs and presence of ganglioneuritis observed in tissue sections of affected abalone.

Case definition

Presence of AbHV should be suspected if affected abalone display clinical signs and pathology as described above. The presence of AbHV shall be considered confirmed if, in addition to clinical signs and consistent pathology, virus is positively identified in tissue sections by *in situ* hybridisation and/or by real-time PCR or conventional PCR (followed by sequence analysis of the amplicon to demonstrate virus identity).

Range of tests available and appropriate applications

Since the occurrence of a carrier state is, as yet, unknown for infection by this virus, there are no validated tests for surveillance. Nevertheless, real-time PCR would be considered the most sensitive test available to date, and is the test most likely to be appropriate for surveillance activities. For confirmatory diagnosis of disease, real-time PCR, *in situ* hybridisation and/or conventional PCR (followed by sequence analysis of the amplicon to demonstrate virus identity) is recommended.

Samples

Tissue samples suitable for examination are tissues dissected from affected abalone that include neural tissue - cerebral, pleuropedal and buccal ganglia, branches of the pedal nerve and peripheral nerves. Samples for histopathological (including *in situ* hybridisation) or electron microscopic examination should be placed in the appropriate fixative immediately after euthanasia of moribund or freshly dead abalone.

Samples for PCR analysis should ideally be placed in preservative prior to transportation:

80% reagent grade ethanol
19.75% glycerol
0.25% β-mercaptoethanol

or, alternatively, 95% ethanol

OIE Reference Laboratory
None

Other Reference Laboratories

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Appendix 1. Virus detection and identification by conventional PCR

Introduction

With many infectious diseases, especially where the aetiological agent cannot be easily propagated *in vitro*, development of polymerase chain reaction (PCR) for the specific and sensitive detection and identification of the agent has proved useful for diagnosis. Even where the causative agent can be propagated, if specific PCR primers are available, PCR provides a powerful method for the rapid identification of the agent. Furthermore, subsequent sequencing of any PCR product may enable a molecular epidemiological analysis of the agent, and, for exotic diseases in particular, this may be very important in control, prevention and eradication. However, there are some pitfalls that need to be noted. Firstly, the technique depends on the specific interaction of the nucleotide primers with a known, defined nucleic acid sequence within the genome of the infectious agent. If this sequence has been changed (for example, by mutation creating a related but distinct variant of the infectious organism) the PCR primers may not bind to the mutant sequence, subsequently yielding a false-negative reaction. Secondly, for nested PCRs especially, cross-contamination between reaction tubes/wells can occur, potentially yielding a false-positive diagnosis. Thus reliance on PCR results alone for a diagnosis is not recommended and it is important that other tests, for example, immunoassays, are used, if available.

The use of PCR as a diagnostic tool requires a large investment in human and physical resources. Specialised equipment for exclusive molecular diagnosis by qualified and experienced operators is essential. All equipment and procedures need to be strictly controlled to ensure validity of results. It is recommended that diagnostic PCR activities be carried out in a dedicated suite of laboratories with strict control on the transfer of materials and personnel in and out of the suite. Diagnostic laboratories are likely to have designed their PCR facilities and generic procedures according to their own specific requirements and therefore only general direction can be given here.

Reagents

100% ethanol AR grade
Primers (20μM)
HotStar Taq® PCR Mastermix (QIAGEN Cat# 201443)*
100bp DNA ladder & loading dye
QIAamp DNA Mini Kit (QIAGEN) that contains:
Agarose
SYBR® Safe (Invitrogen Cat# S33102)
50 x TAE Buffer

*Any Taq Mastermix could be used, however this method was validated only using HotStar Taq Mastermix and comparative testing should be undertaken if reagents are changed.

List of equipment

Apart from the normal range of equipment required in the standard diagnostic laboratory (for example, refrigerators, freezers, mixers, micropipettes, biological safety cabinets, centrifuges, balances, microwave oven, thermometers), specialised equipment required to undertake diagnostic PCR may include dry-heat blocks, thermocycler, gel electrophoresis equipment, UV transilluminator, camera system and sequencer.

Quality control

Molecular diagnosis should be operated under an ISO 17025 accredited and audited quality assurance program. Thus, such a program would include initial evaluation of kits and validation of performance; ongoing internal evaluation through mandatory use of appropriate quality control samples where available; and performance monitoring through quality assessment or proficiency programs.

External quality control samples over the appropriate range of testing must be obtained or manufactured wherever possible. Wherever possible, quality control samples should be included in every assay run and the data presented so that run-to-run performance can be monitored. Positive, negative and reagent controls should be conducted as specified in the protocol. As a norm, formalin-fixed controls would be conducted with formalin-fixed test samples and appropriate unfixed controls would be conducted with fresh tissue samples. Stocks of controls should be established. These controls should be evaluated prior to storage and used in a QA check-testing regimen, and as controls for the conduct of disease investigations.

Procedures

Sample preparation

Due to the sensitivity of PCR tests, care at every step of sample preparation must be taken to ensure that cross-contamination of diagnostic samples does not occur. Thus all instruments and sample containers must be clean and uncontaminated, that is, not pre-exposed to aquatic pathogens. Wherever possible it is recommended that disposable containers are used.

Tissue samples and other specimens containing cells are homogenised at approximately 10% (w/v) in phosphate-buffered saline or similar buffer, frozen and thawed, and gently centrifuged to precipitate cellular debris. The supernatant fluid is then mixed with the appropriate volume of commercially prepared buffers (for example, ATL buffer in QIAgen kits).

Nucleic acids from submitted samples should be extracted in a Class II Biological Safety Cabinet in the PCR suite.

Nucleic acid extraction

Nucleic acid is extracted from tissue samples using the QIAamp DNA® mini kit (QIAGEN cat no. 51306) following the manufacturer's instructions.

Conventional single-step PCR

Following DNA extraction, a PCR is then conducted. PCR amplification is performed on DNA samples extracted from tissues derived from positive (AbHV-infected) control, negative (uninfected), and test (submitted) abalone samples. Positive and negative controls are available from AAHL.

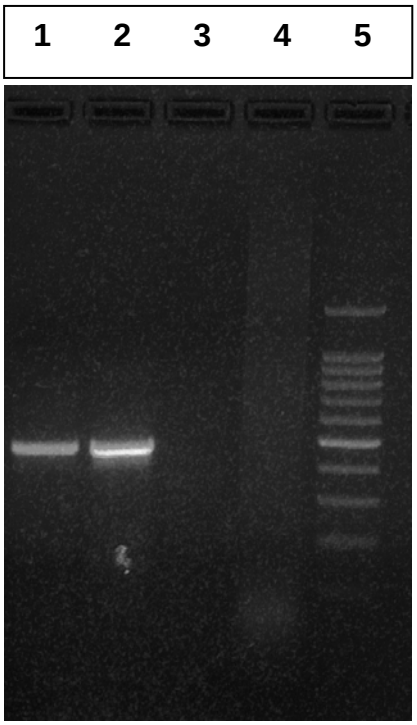
The PCR mixture for a single sample consists of the following reagents: 9.5 µL of water; 12.5 µL of HotStar Taq Master mix; 0.5 µL of the forward primer (20µM); 0.5 µL of the reverse primer (20 µM); and 2 µL of DNA. The mixture is incubated in an automatic thermal cycler that is programmed for: one cycle at 94^{O}C for 15 min; 35 cycles at 94^{O}C for 15 sec, 52^{O}C for 30 sec and 72^{O}C for 30 sec; and, finally, one cycle at 72^{O}C for 5 min. Amplified DNA is detected following resolution of the amplicons by agarose (2%) gel electrophoresis.

Primers used:
Forward primer (007F): 5'-GCCTTCGCTGGAAGCATAC-3'
Reverse primer (007R): 5'-GTGGTCGCGAGAAGAGAAC-3'

Interpretation

At the completion of the PCR, specific PCR fragments of the correct size (486 bp) are identified by agarose gel electrophoresis:

- The negative control sample must have no evidence of specific amplified products.
- A positive control sample must yield a specific AbHV fragment (486 bp).
- Amplified fragments of the correct size are then extracted from the gel, and the DNA sequence is determined (by using the PCR primers as sequencing primers).
- Sequence identity is determined by sequence alignment.



LEGEND

Lanes 1 and 2: AbHV-infected abalone

Lanes 3 and 4: Uninfected abalone

Lane 5: 100 bp ladder

Figure 1. Photomicrograph of SYBR safe stained gel demonstrating the AbHV-specific amplicon of the expected size (486 bp) in lanes 1 and 2.

An assay is valid only when all controls yield the expected results. Furthermore, a positive diagnosis is never made on the basis of the size of a PCR product alone. That product must be sequenced to confirm the diagnosis.

Appendix 2: Virus detection and identification by real-time (TaqMan) PCR

Introduction

An ABI Prism® 7500 Fast Real-Time Detection System and software Sequence Detector version 2.0 (PE Applied Biosystems) were used for the analysis and storage of data. Primers and probe for the TaqMan assay were designed using the Primer Express Software version 3.0 (PE Applied Biosystems). This TaqMan PCR assay incorporates specific primers and probes for abalone herpes-like virus (AbHV). The assay involves the use of the Applied Biosystems Sequence Detection System (SDS) model 7500 and TaqMan PCR assay technology. Other real-time systems (e.g. Corbett or Roche) could be used, however this method was validated using only TaqMan, and appropriate comparative testing should be undertaken if the real time system is changed.

Reagents

Reagents stored at room temperature:

QIAamp DNA Mini Kit (QIAGEN Cat. No. 51304 or equivalent)

Reagents stored at –20°C:

TaqMan® Fast Universal PCR Master Mix (2X) (No AmpErase® UNG, Applied Biosystems Cat. No. 4366073)

Reagents stored at -70^{o}C:

18S endogenous control primers and VIC probe (Applied Biosystems Cat. No. 4308329 or equivalent)
AbHV primers and probe

List of equipment

Applied Biosystems 96-well Optical reaction plates
Applied Biosystems Adhesive cover kit or equivalent
Applied Biosystems Sequence detection system Model 7500 or 7900
Applied Biosystems developed software SDS v1.4
Bench top centrifuge IEC model Centra and appropriate plate holders

DNA extraction from abalone tissue

Nucleic acid from abalone tissues (approximately 20 mg of muscle and neural tissue) is extracted using a QIAamp DNA® mini kit (QIAGEN) according to the manufacturer's instructions. Nucleic acid, bound to minicolumns, is eluted and resuspended in a final volume of 100 µL of AE buffer (~100 ng/µL).

TaqMan assay

Following DNA extraction, a real-time PCR is carried out in a 96-well plate in a 25 µL reaction volume containing 12.5 µL of TaqMan® Fast Universal PCR Master Mix (2X), 2 µL (~100ng per µL) of extracted DNA sample and the reaction mix is made up to 25 µL using deionised water after primers and probes are added at the

appropriate concentrations. The following thermal cycling conditions are used: 95^{o}C for 59 sec followed by 45 cycles of 95^{o}C for 3 sec and 62^{o}C for 30 sec.

The AbHV primers and probe sequences are as follows:
Forward primer (ORF49F): 5'-AACCCACACCCAATTTTTGA-3'
Reverse primer (ORF49R): 5'-CCCAAGGCAAGTTTGTTGTT-3'
6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA) labeled probe (ORF49Pr): 6FAM-CCGCTTTCAATCTGATCCGTGG-TAMRA.

The AbHV primers are used at a final concentration of 300 nM. The AbHV probe is used at a final concentration of 100 nM.

18S Ribosomal RNA gene primers and probe (Applied Biosystems) are used to validate the nucleic acid extraction procedure and the absence of PCR inhibitors. The 18S RNA gene endogenous control primers and probe sequences are as follows:
Forward primer (18S Forward) 5'-CGGCTACCACATCCAAGGAA-3'
Reverse Primer (18S Reverse) 5'-GCTGGAATTACCGCGGCT -3'
Probe (18S VIC – TAMRA probe) 5'-TGCTGGCACCAGACTTGCCCTC-3'
Both the 18S RNA gene primers and the probe are used at a final concentration of 100 nM.

All samples (including positive and negative controls – available from AAHL) are tested in duplicate or triplicate to ensure repeatability. The results of a TaqMan assay are expressed in the form of software-generated characteristic amplification curves. Amplification curves from positive and negative (no template controls) should be compared to the test sample. A sample is considered above the test background level when the change in fluorescence (ΔR_n) of FAM or VIC, relative to that of ROX (internal reference dye), exceeded the threshold value which was arbitrarily set at the upper end of the linear range of the amplification plots. Results of a TaqMan assay can also be, and often are, expressed as cycle threshold (C_T) values. The cycle threshold (C_T) is defined as the cycle number at which a statistically significant increase in fluorescence output above background was detected.

At the completion of the TaqMan PCR assay, the presence of AbHV DNA is demonstrated by the presence of specific amplicons, identified by software-generated characteristic amplification curves and cycle threshold values (C_T). No template controls (NTC) must have no evidence of specific amplicons (Figure 1).

If the test is deemed valid, the results for the test sample wells may be interpreted using the following criteria:

- Positive test results are defined as the presence of specific amplicons expressed as a characteristic amplification curve similar to the positive control(s) and having a cycle threshold (C_T) value less than 35.0.
- Negative test results are defined as the absence of specific amplicons expressed by a characteristic amplification curve similar to the no template control (NTC) and having a cycle threshold (C_T) value equal to or greater than 36.0.
- Indeterminate test results are defined as having a characteristic amplification curve similar to the positive control but a cycle threshold (C_T) value between 35.0-36.0. This necessitates repeating the assay with at least 3 test sample wells.

- Results for the test assay are kept in the form of a hard copy of the C_T values (experimental report) obtained from the instrument.

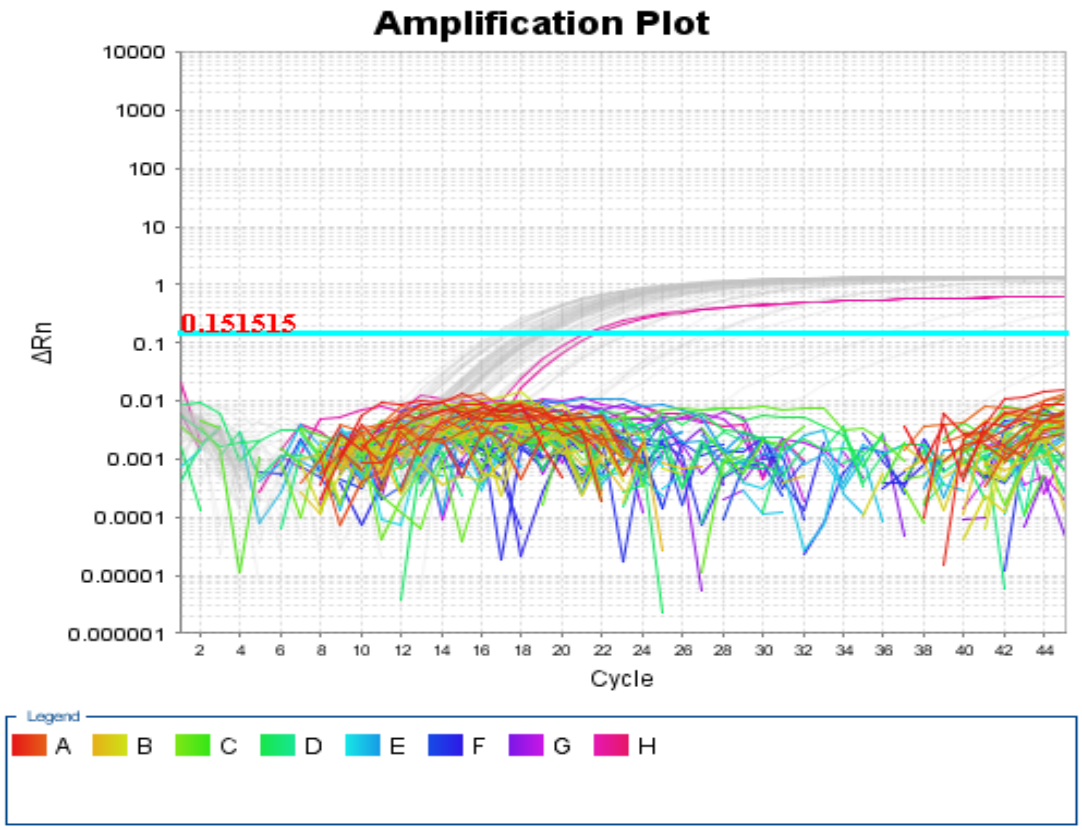


Figure 1. AbHV Real-time TaqMan PCR assay. Example of the read-out demonstrating clearly positive samples with C_T values <35.0 and clearly negative samples with C_T values >36.0. The C_T value is the cycle number where the curve for each sample crosses the horizontal green line.

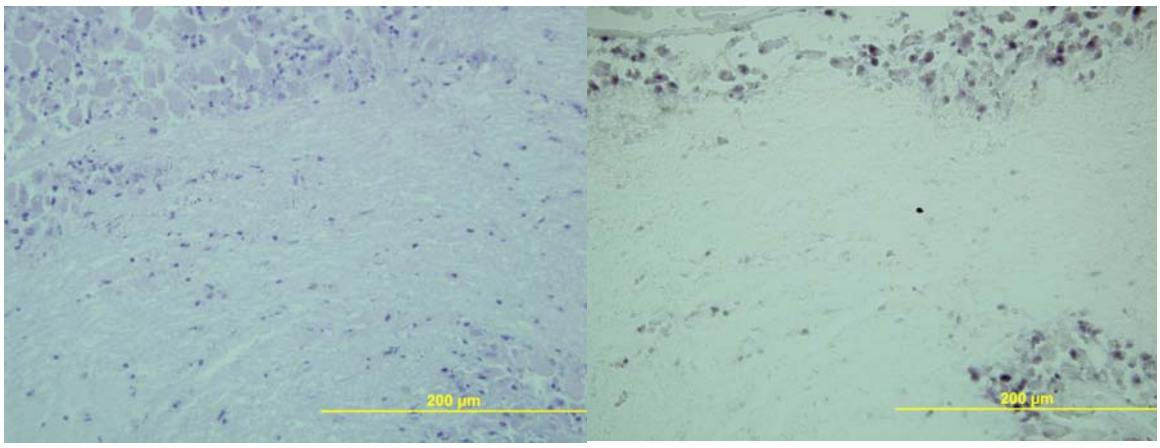
Appendix 3: *In situ* hybridisation

Introduction

The *in situ* hybridisation (ISH) procedure described here uses a digoxygenin (DIG)-labeled DNA probe to detect AbHV in formalin-fixed, paraffin-embedded (FFPE) tissue sections. If AbHV is present in the tissue, hybridisation of viral DNA to the DIG-labeled probe will result in the formation of a stable hybrid which can be visualised using colourimetric detection procedures. The application of anti-DIG antibody conjugated to alkaline phosphatase and a substrate for the alkaline phosphatase leads to the formation of a precipitate at the site of the hybrid. For AbHV-positive samples blue-black staining is visible under light microscopy of the stained section. Positive and negative sections are necessary so that the specificity of the reaction can be confirmed and the absence of background staining due to the presence of endogenous alkaline phosphatase can ruled out.

Interpretation of results

Specific dark blue-black intra-cellular staining (Figure 1b) is indicative of the presence of viral DNA. All controls must be included in the test and provided that the results of these controls are acceptable the result can be reported.



A. Infected abalone tissue (high power) B. ISH on section equivalent to A.

Figure 1. *In situ* hybridisation assay. (A) H&E stained section. (B) section from the same blocks subjected to ISH using the AbHV probe. Note the intense blue-black focal staining observed in infected tissues.

In situ hybridisation method

In each experiment a positive control slide, consisting of a section from an abalone exhibiting Abalone Viral Ganglioneuritis (AVG) should be included. A control slide of uninfected abalone tissue should also be included.

Preparation of DIG labeled Probes

1. Perform PCR on purified AbHV DNA or a sample known to contain AbHV using a PCR DIG Probe Synthesis Kit (Roche Cat. No. 11 636 090 910) as per the manufacturers' instructions.

a. Use the AbHV_ORF66f1 (5'-TCCCGGACACCAGTAAGAAC-3') AbHV_ORF66r2 (5'-CCCGGACACCAGTAAGAAC-3') primer pair which amplifies an 848bp product from AbHV DNA.
b. Use the following thermocycling profile: 95ºC for 5 min followed by 30 cycles of 95ºC for 30 s, 55ºC for 30 s, 72ºC for 60 s. Complete the PCR with a final elongation at 72ºC for 10 min.

Preparation of Sections

1. Section paraffin-embedded tissue at 3 µM thickness and place onto Superfrost plus slides (Menzel Catalogue No. SF41296SP) and allow to dry.
2. Heat sections at 65ºC for 30 min and deparaffinise in 2 stages of xylene.
3. Rehydrate by placing slides in absolute ethanol for 2 min followed by 90% ethanol for 2 min, 70% ethanol for 2 min and then into distilled water.
4. Place slides in 0.2N HCl for 20 min and rinse in distilled water for 5-10 min.
5. Apply 50-100 µL of 100 µg/mL proteinase K in Tris-buffered saline (TBS) and incubate at 37ºC for 30 min.
6. Rinse with 0.2% glycine for 2 min.
7. Wash in running water for 10 min.
8. Dehydrate sample in 70% ethanol for 2 min followed by 90% ethanol for 2 min and 100% ethanol for 2 min.
9. Allow slides to air-dry.

Hybridisation Procedure

1. Make 100 µL of hybridisation solution per section (4X SSC, 5X Denhardt's solution, 10 mg/mL herring sperm DNA, 10% dextran sulphate, 50% formamide, approximately 5 ng/µL probe).
2. Heat the hybridisation solution to 95-100ºC for 5 min to denature the probe and place on ice until ready for use.
3. Apply sufficient hybridisation solution to cover the section (approx 50 µL) and cover with a coverslip.
4. Heat the slides to 95ºC for 5 min to denature the nucleic acid in the specimen. To heat the slides to 95ºC a PCR heating block can be used or a purpose built hybridisation block such as the Invitrogen SPoT hybridiser.
5. Place the slides into a humidified chamber that has been preheated to 37ºC and incubate at 37ºC overnight (12-16 h).

Post-Hybridisation Procedure

1. Remove coverslips by immersing slides in 2X SSC at room temperature.
2. Place slides in a rack and immerse in 2X SSC at room temperature. Use a rocker or shaker at slow speed to ensure complete washing of the slides.
3. Wash, with gentle rocking/shaking, in 0.5X SSC (pre-warmed to 37ºC) at 37ºC for 15 min.
4. Wash slides briefly in room temperature TBS buffer (Solution I).
5. Incubate slides in blocking solution (0.5% skim milk powder in TBS) for 30 min at room temperature.
6. Cover sections with 100-200 µL of sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche Cat. No. 1093274) diluted 1 in 100 in blocking solution.

7. Incubate at room temperature for 1 h.
8. Wash in TBS buffer 3 x 3 min.
9. Equilibrate in solution II (0.1 M Tris pH 8, 0.5 M NaCl, 0.1 M $MgCl_2$, pH 9) for 3 min at room temperature.

Colour Development

1. Add 1 NBT/BCIP Ready-to-Use Tablet (Roche Cat. No. 11 697 471 001) to 10 mL of a 10% solution of polyvinyl alcohol (high molecular weight, 40-100 kD) in distilled water to prepare a ready-to-use staining solution.
2. Cover the sections with the staining solution and place a coverslip over them. Incubate in the dark for 3-4 h in a humidified container, making sure that the slides do not dry out.
3. Monitor the colour development by periodically checking the slides under a light microscope.
4. If required the slides can be incubated, in the dark at room temperature, overnight.
5. Stop the reaction and remove the coverslip by immersing the slides in distilled water
6. Wash the slides in running water for 5 min.
7. Mount the slides with mounting medium (DAKO Cat. No. S3023) and a coverslip.

Reagents

20X SSC pH7 (store at RT)
175.32 g/L NaCl
88.23 g/L Sodium citrate

100X Denhardt's solution (store at -20ºC)
2 g/100 mL Bovine serum albumin (Fraction V)
2 g/100 mL Ficoll 400
2 g/100 mL Polyvinylpyrollidone

Hybridisation buffer (store at -20ºC)
25 mL Formamide
10 mL 20X SSC
2.5 mL 100X Denhardt's solution
10 mL 50% dextran sulphate in distilled water
500 µL 10 mg/mL herring sperm DNA
Make up to 50mL with MilliQ water

10X Tris-buffered saline (TBS) (store at RT)
23.6 g/L Tris base
127 g/L Tris-HCl
87.66 g/L NaCl