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# Aquatic Animal Health Subprogram: Characterisation of abalone herpes-like virus infections in abalone

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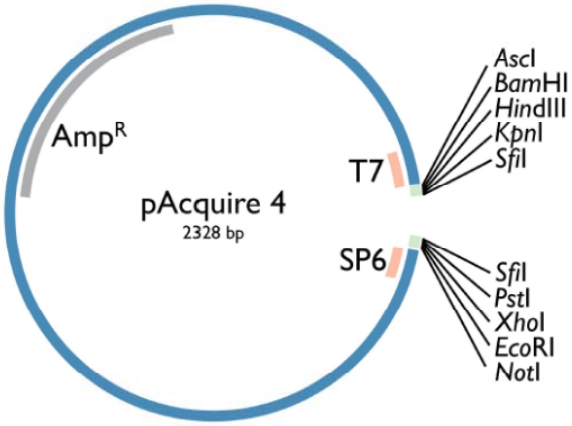


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**Aquatic Animal Health Subprogram: Characterisation of abalone herpes-like virus infections in abalone**

**FRDC Project No 2009/032**

**2014**

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In submitting this report, the researcher has agreed to FRDC publishing this material in its edited form.

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# Abbreviations

| | |
|---|---|
| AAGA | Australian Abalone Growers Association |
| AAHC | Aquatic Animal Health Committee |
| AAHL | Australian Animal Health Laboratory |
| AbHV | Abalone herpesvirus |
| ACA | Abalone Council Australia |
| AVG | Abalone viral ganglioneuritis |
| BF-2 | Bluegill fry cell line |
| CHSE-214 | Chinook salmon embryo cell line |
| CSIRO | Commonwealth Scientific and Industrial Research Organisation |
| DAFF | Department of Agriculture, Fisheries and Forestry |
| DEPI Vic | Department of Environment and Primary Industry, Victoria |
| DIG | digoxigenin |
| DNA | deoxyribonucleic acid |
| DPI | Department of Primary Industries |
| DPIW | Department of Primary Industries and Water |
| EDTA | Ethylenediaminetetraacetic acid |
| EZAIA | Eastern Zone Abalone Industry Association Inc. |
| FRAB | Fisheries Research Advisory Board |
| FRDC | Fisheries Research and Development Corporation |
| GF | Grunt fin cell line |
| GSW | Great Southern Waters Pty Ltd |
| h | hour(s) |
| ISH | *in situ* hybridisation |
| kD | kilodaltons |
| KF-1 | Koi fin cell line |
| $LD_{100}$ | 100% lethal dose |
| $LD_{50}$ | 50% lethal dose |
| min | minute(s) |
| NAHMACB | National Abalone Health Management Advisory and Coordinating Body |
| NaOH | sodium hydroxide |
| NBT/BCIP | nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate |
| ORF | open reading frame |
| PCR | polymerase chain reaction |
| PIRSA | Primary Industries and Regions, South Australia |
| qPCR | quantitative polymerase chain reaction |
| s | second(s) |
| SARDI | South Australian Research and Development Institute |
| SSC | saline-sodium citrate buffer |
| TAC | Tasmanian Abalone Council |
| TBS | Tris-buffered saline |
| $TCID_{50}$ | 50% tissue culture infectious dose |
| VADA | Victorian Abalone Divers Association |
| vgc | viral genome copies |

# Executive Summary

**What the report is about**

Scientists at CSIRO Australian Animal Health Laboratory, the Department of Environment and Primary Industries Victoria, South Australian Research and Development Institute, the Department of Primary Industries, Parks, Water and Environment Tasmania, and the University of Adelaide, collaborated on a major project investigating various aspects of the biology, detection and identification of abalone herpesvirus, the causative agent of abalone viral ganglioneuritis. The various aspects under investigation included determining the stability of the virus under various physico-chemical conditions, providing new information on the current diagnostic tests for the detection and identification of the virus including sub-clinical infections, and determining the susceptibility of various abalone and other molluscan species to infection and disease.

Much of the work built on research undertaken on previous FRDC-funded projects and involved using diagnostic tests and the *in vivo* infectivity model developed previously. All *in vivo* research was undertaken at the CSIRO Australian Animal Health Laboratory, a high-level bio-security laboratory located in Geelong.

**Background**

The project was undertaken in response to a scientific forum that was convened in 2006 involving national and international experts to discuss R&D requirements in the face of this newly emerging viral disease of abalone. A second scientific forum was convened the following year to develop a national work plan based on the outputs of the previous forum. The prioritised work plan was developed by the National Abalone Health Management Advisory and Coordinating Body (NAHMACB) for the then Federal Government Department of Agriculture, Fisheries and Forestry's Aquatic Animal Health Committee (AAHC). This project was developed to address many of the priorities identified in the consultative process coordinated by Fisheries Victoria.

**Aims/objectives**

1. Validate the developed *in situ* hybridisation diagnostic test including roll-out to other States.
2. Develop a quantitative assay (qPCR) for determining the infectious dose for this virus.
3. Determine the sensitivity of the virus to physico-chemical conditions including its stability in water/on fomites and its sensitivity to inactivation agents.
4. Determine the role of mucus in viral transmission.
5. Determine whether or not a latent stage exists in AVG.
6. Determine the susceptibility of remnant populations of abalone previously exposed to AVG and known unexposed wild populations in South Australia.
7. Using all three available qPCR tests, determine their relative sensitivities and specificities by re-testing previously collected samples from the abalone populations in Tasmania (additional objective added during the project).

**Methodology**

In the absence of *in vitro* culture systems such as molluscan cell lines for the replication of infectious virus, the research undertaken in this project made use of molecular technology (*in situ* hybridisation, polymerase chain reaction, dot-blot hybridisation), an archive collection of samples obtained from naturally infected abalone and experimentally infected abalone using the previously developed infectivity model for this virus (see FRDC Project 2007/006: Aquatic Animal Health Subprogram: Development of molecular diagnostic procedures for the detection and identification of herpes-like virus of abalone (*Haliotis* spp.)).

**Results/key findings**

The project was successful in achieving all its objectives. Firstly, the developed *in situ* hybridisation diagnostic test was validated and made available to diagnostic laboratories in Australia and overseas.

Secondly, it has been shown that a quantitative polymerase chain reaction (qPCR) can be used to determine the infectious dose for this virus. An analysis of the qPCR tests has determined that the ORF49 and ORF66 qPCR tests, when used in parallel, have a diagnostic sensitivity (DSe) of 86% and a diagnostic specificity (DSp) of >98% for the detection of sub-clinical infections.

Moreover, the sensitivity of the virus to various physico-chemical conditions including its stability in water/on fomites and its sensitivity to inactivation agents has been determined. While the virus can be present in mucus secreted by infected abalone, mucus does not appear to play a significant role in prolonging viral infectivity in seawater.

Available data indicate that abalone herpesvirus (AbHV) can exist as a sub-clinical infection, and this is supported by investigations into disease events.

In addition, it was shown that remnant populations of wild abalone (in Victoria) previously exposed to AbHV (studied here and in FRDC Project 2009/075: Determining the susceptibility of remnant populations of abalone previously exposed to AVG) remain susceptible to infection and disease. Furthermore, it has been shown that known unexposed wild populations in South Australia are susceptible to infection and disease.

**Implications for relevant stakeholders**

New information on technical aspects of the diagnostic tests will be of interest to diagnostic laboratories. In addition, results on the performance of the diagnostic tests provide industry and regulators with important information on the detection of sub-clinical infections that needs to be considered when designing surveillance programs e.g. sampling regime.

Moreover, results on stability of the virus under various conditions provide information of importance when developing control and management strategies.

To date, the known host range includes greenlip abalone (*Haliotis laevigata*), blacklip abalone (*H. rubra*), hybrids of these, and Roe's abalone (*H. roei*) which occur in Australia. Other mollusc species tested, *Turbo undulatus* (marine snails) and *Mimichlamys bifrons* (scallops), do not appear to be susceptible to infection with AbHV.

**Recommendations**

Information from this project should be used by industry and regulators when considering various aspects of this disease threat, e.g., during development of biosecurity plans, surveillance programs, and management and control strategies.

**Keywords**

Abalone viral ganglioneuritis (AVG); abalone herpesvirus (AbHV); diagnostic methods; *in situ* hybridisation (ISH); polymerase chain reaction (PCR); diagnostic test validation; virus transmission; virus inactivation; virus stability

# Introduction

In December 2005/January 2006, following an outbreak of abalone viral ganglioneuritis in both farmed and wild abalone, the aetiological agent was identified as a herpes-like virus that infected neuronal tissues of affected abalone (Hooper et al., 2007; Tan et al., 2008; Savin et al., 2010). Previous research was successful in developing nucleic acid-based diagnostic tests for the detection and identification of this virus (Corbeil et al., 2010; 2012). Thus it was recognised that we were in a position to undertake further research on various aspects of the biology of this virus which would lead to a better understanding of the disease associated with infections which, in turn, would lead to improved strategies for better management/control of the disease both on-farm and in the wild. If not managed appropriately, this emerging virus has the potential to continue to spread to all States involved in abalone fisheries and aquaculture and could jeopardise Australia's abalone industry.

In September 2006, as part of the response to the original outbreak, a scientific forum was convened with national and international experts to discuss R&D requirements in the face of this newly emerging disease. A second scientific forum was convened the following year to develop a national work plan. The prioritised work plan was developed by the National Abalone Health Management Advisory and Coordinating Body (NAHMACB) for the then Federal Government Department of Agriculture, Fisheries and Forestry's Aquatic Animal Health Committee (AAHC) and this project was developed to address many of the priorities identified in the consultative process coordinated by Fisheries Victoria.

At the National Abalone Health Work-plan Priority Setting Workshop held on 18 June 2008 in Melbourne, attended by representatives from DEPI Victoria, SARDI, FRDC, VADA, TAC, EZAIA, AAGA, ACA, CSIRO, DPIPWE Tasmania, RecFish, DAFF and NSW DPI, it was agreed that, to address the main epidemiological questions, research should be focussed on 4 areas:

1) Development of diagnostic techniques with the highest priorities being:
- A technique to isolate and concentrate the virus from abalone tissues (addressed in FRDC Project 2007/006 Aquatic Animal Health Subprogram: Development of molecular diagnostic procedures for the detection and identification of herpes-like virus of abalone (*Haliotis* spp.))
- Development of a rapid and specific diagnostic test development of a validated PCR test for the AVG virus (addressed in FRDC Project 2007/006 Aquatic Animal Health Subprogram: Development of molecular diagnostic procedures for the detection and identification of herpes-like virus of abalone (*Haliotis* spp.))
- Development of a secondary diagnostic test (ISH development addressed in FRDC Project 2007/006 Aquatic Animal Health Subprogram: Development of molecular diagnostic procedures for the detection and identification of herpes-like virus of abalone (*Haliotis* spp.))
- Validation of diagnostic tests (partly addressed in the proposed extension of FRDC Project 2007/006 (PCR validation) and this project (ISH validation)
- Roll-out of diagnostic tests across the States (addressed in proposed extension of FRDC Project 2007/006 (transfer of PCR tests) and this project (transfer of ISH test)

2) Disease aetiology and transmission with the highest priorities being:
- Other bio-vectors and abiotic factors (partly addressed by this project)
- Determination of the susceptibility of remnant populations following exposure to AVG (to be addressed by this project and FRDC Project 2009/075: Determining the susceptibility of remnant populations of abalone previously exposed to AVG.)

3) Virus inactivation with the highest priorities being:
- Determine the viability of the AVG virus, including disinfection efficacy; efficacy of treatments (to be addressed by this project)
- Survival of the virus in seawater (to be addressed by this project)

- Survival of the virus on fomites (to be addressed by this project)

4) Disease surveillance and modelling with the highest priorities being:

- National survey of stocks to determine current distribution of the virus, including latency, and resistance status of stocks (partly addressed by this project)
- Determine the mode(s) of spread of the AVG (not addressed)

Thus, following development of rapid, reliable and sensitive PCR-based diagnostic tests, the work plan detailed issues of fundamental importance for effective control/management of disease outbreaks and included determination of:

- the origin of virus
- virus/disease distribution (geographical range)
- differential mortality across year classes – larvae to adult stage
- the host range
- mechanisms of viral transmission (role of mucus?)
- latency/sub-clinical infection in animals and conditions triggering virus release
- stability of the virus to disinfectants/detergents
- antiviral immunity

Following on from these various forums, this project was developed as a collaboration between DEPI Victoria, SARDI and AAHL to address some of these needs. As the project developed and following the discovery of abalone herpes-like virus in Tasmania, DPIPWE Tasmania accepted an invitation to join the collaborative project.

Those state agencies (e.g. State Departments of Primary Industry) with responsibilities regarding the health of wild and farmed abalone stocks (PIRSA, DPIPWE Tasmania, Fisheries WA) were informed of the project and provided letters of support indicating that this research is rated as a high priority. The proposal was also forwarded to the Australian Abalone Grower's Association (AAGA) (Contact: Ann Fleming) and the Abalone Council of Australia (ACA) for review/comment. AAGA indicated that this project is a high priority and committed to contributing $5000.00 towards the research. In addition, the proposal was discussed with AAGA at their Annual General Meeting held in Brisbane, 5 August 2008. It was generally agreed that the proposal addresses many of the documented priorities (from the national forums convened by Fisheries Victoria) for research on this virus. Progress on current research and an outline of the planned research (i.e. the proposed extension to FRDC 2006/007 and this proposal) was also presented at the 4th National Abalone Convention held in Port Lincoln, 17-19 September 2008. Discussion at that meeting indicated that the planned research (extension to FRDC 2006/007 and the research covered by this proposal) was viewed as a high priority by ACA. Other letters of support were received from Fisheries WA, TasFRAB and VicFRAB.

# Objectives

1. Validate the developed *in situ* hybridisation diagnostic test including roll-out to other States.
2. Develop a quantitative assay (qPCR) for determining the infectious dose for this virus.
3. Determine the sensitivity of the virus to physico-chemical conditions including its stability in water/on fomites and its sensitivity to inactivation agents.
4. Determine the role of mucus in viral transmission.
5. Determine whether or not a latent stage exists in AVG.
6. Determine the susceptibility of remnant populations of abalone previously exposed to AVG and known unexposed wild populations in South Australia.
7. Using all three available qPCR tests, determine their relative sensitivities and specificities by re-testing previously collected samples from the abalone populations in Tasmania (additional objective added during the project).

# Methods

**Objective 1: Validate the developed *in situ* hybridisation diagnostic test including roll-out to other States**

The previously developed *in situ* hybridisation (ISH) diagnostic test (FRDC Project No. 2006/007 Aquatic Animal Health Subprogram: Development of molecular diagnostic procedures for the detection and identification of herpes-like virus of abalone (*Haliotis* spp.)) was validated using standard procedures. Following establishment of the test at DEPI Victoria, the test method was transferred and established at CSIRO-AAHL. Subsequently, the test was evaluated with respect to its analytical sensitivity and specificity, and offered to all other interested State diagnostic laboratories via workshop training at DEPI Victoria. Representatives from CSIRO-AAHL, DPIPWE Tasmania and Fisheries WA attended.

***Clinical samples and tissue preparation***

Abalone (*Haliotis* spp.) were collected from fisheries including commercial farms, or were experimentally infected with the abalone herpesvirus strain AbHV Vic1 at AAHL. A total of seven positive samples and 56 negative samples were tested.

All positive samples were confirmed as having histopathology typical of abalone viral ganglioneuritis (AVG) and all were confirmed as positive for the presence of the AbHV by real-time PCR (qPCR). Similarly, all negative samples were confirmed as not having histopathological lesions typical of AVG and were confirmed as being negative for the presence of the AbHV by qPCR.

The tissues were embedded in paraffin, sections (3µm thickness) were cut, placed onto Superfrost plus slides (Menzel) and allowed to dry. The sections were heated at 65$^{o}$C for 30 min, deparaffinised in 2 stages of xylene, rehydrated by placing the slides in absolute ethanol for 2 min followed by 90% ethanol for 2 min, 70% ethanol for 2 min and then into distilled water. Slides were placed in 0.2N HCl for 20 min, rinsed in distilled water for 5-10 min and permeabilised by treatment with 100µg/mL proteinase K in Tris-buffered saline (TBS) for 30 min at 37$^{o}$C. Slides were rinsed in 0.2% glycine for 2 min and washed in running water for 10 min. Sections were than dehydrated in 70% ethanol for 2 min followed by 90% ethanol for 2 min and 100% ethanol for 2 min and air-dried prior to ISH.

***Preparation of DIG labelled probes***

The probe was synthesised by incorporating digoxigenin 11-dUTP during PCR employing the AbHV-specific primer pair AbHV/ORF66f1 (5'-TCCCGGACACCAGTAAGAAC-3') and AbHV/ORF66r2 (5'-GCCGGTCTTTGAAGGATCTA-3') and using a PCR DIG probe synthesis kit (Roche) as per the manufacturer's instructions. The 848bp probe was amplified using the following thermocycling profile: 95$^{o}$C for 5 min followed by 30 cycles of 95$^{o}$C for 30s, 55$^{o}$C for 30s, 72$^{o}$C for 60s with a final elongation at 72$^{o}$C for 10 min.

***In situ hybridisation (ISH)***

In each experiment a positive control slide, consisting of a section from an abalone exhibiting AVG and a control slide of uninfected abalone tissue were included. Negative controls also included samples without digoxigenin-labeled probe in the hybridization mixture or without antibodies during the revelation step.

Hybridisation solution (4X SSC, 5X Denhardt's solution, 10mg/mL herring sperm DNA, 10% dextran sulphate, 50% formamide, approximately 5 ng/µL probe) was heated to 95-100$^{o}$C for 5 min to denature the probe and then placed on ice until ready for use. For each tissue section, sufficient hybridisation solution (approx. 50 µL) was applied to cover the section which was then covered with a coverslip. The section was heated to 95$^{o}$C for 5 min to denature the nucleic acid in the specimen and all slides placed in a humidified chamber preheated to 37$^{o}$C and incubated at 37$^{o}$C overnight (12-16h).

The coverslips were removed by immersing the slides in 2X SSC at room temperature after which the slides were washed in 0.5X SSC at 37°C for 15 min. Slides were washed briefly at room temperature with TBS buffer (Solution I) and then incubated in blocking solution (0.5% skim milk powder in TBS) for 30 min at room temperature. The presence of hybridised probe was detected by covering the sections with 100-200 µL sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche) diluted 1/100 in blocking solution and incubating at room temperature for 1 h. Sections were washed in three changes of TBS buffer for 3 min each. The sections were then equilibrated in solution II (1M Tris pH 8.0, 5M NaCl, 1M $MgCl_2$, pH 9.0) for 3 min at room temperature followed by colour development using NBT/BCIP. A NBT/BCIP ready-to-use tablet (Roche) was added to 10 mL 10% solution of polyvinyl alcohol (high molecular weight, 40-100 kD) in distilled water to prepare the ready-to-use staining solution. Sections were covered with the staining solution and a coverslip placed over the section. Slides were incubated in the dark for 3-4 h in a humidified container. The colour development was stopped and the coverslips removed by immersing the slides in distilled water for 5 min. Each section was counterstained for 1 min with Bismarck brown yellow and mounted using a commercial mounting medium (DAKO) and coverslip.

***Specificity testing using dot blot hybridisation***

As tissues from aquatic animals infected with other herpesviruses were not readily available, herpesvirus-infected cell cultures were used as a source of other herpesviruses for specificity testing of the ISH test. Thus genomic DNA was extracted from AbHV-infected and uninfected abalone and from fixed cell culture supernatants from representative herpesviruses and iridoviruses using QIAamp DNA Mini kit according to the manufacturer's instructions (Qiagen).

Fixed cell culture supernatants from representative herpesviruses and iridoviruses were produced at AAHL. Briefly, viruses were grown using appropriate cell lines (Table 1) and then harvested when 100% viral cytopathic effect had developed. Culture supernatants were clarified by centrifugation (1500 x *g* for 10 min) then fixed in ethanol by adding 8 mL 100% ethanol to 2 mL tissue culture supernatant.

**Table 1. Viruses used for determination of specificity of the AbHV probe**

| Virus | Cell Line |
|---|---|
| Bohle iridovirus (BIV) | BF-2 |
| Epizootic haematopoietic necrosis virus (EHNV) | BF-2 |
| Frog virus 3 (FV3) | BF-2 |
| Red sea bream iridovirus (RSIV) | GF |
| Koi herpesvirus (KHV) | KF-1 |
| *Oncorhynchus masou* virus (OMV) | CHSE-214 |

***PCR analysis***

Virus-specific PCR tests were used to confirm the presence of respective viral nucleic acids in the ethanol-fixed tissue culture supernatants.

***Dot Blot Hybridisation***

DNA solutions were denatured at 100°C for 10 min and transferred to ice for 5 min. The solution of denatured DNA was loaded onto a positively charged membrane (Roche) using a Bio-Rad Microfiltration Apparatus (Bio-Rad Laboratories), and rinsed with 100 µL 0.4M NaOH. DNA was immobilized by UV-cross-linking for 3 min using an UV-transilluminator. The membrane was placed in a hybridisation bottle containing pre-hybridisation buffer (Dig-Easy Hyb; Roche) pre-warmed to 37°C and incubated for 30 min at 37°C in a hybridisation oven (Hybaid). DIG-labelled probe was denatured by heating at 100°C for 10 min and kept on ice prior to addition to the hybridisation buffer (Dig-Easy

Hyb; Roche). A 4 µL volume of DIG-labelled probe was added to 4 mL hybridization buffer. The membrane was incubated overnight at 37°C in a hybridisation oven (Hybaid). The hybridised membrane was washed in 2X SSC (0.3 M NaCl, 30 mM sodium citrate; pH 7.0)/0.1% SDS for 10 min at room temperature followed by two washes in 0.5X SSC/0.1% SDS for 15 min each at 65$^{o}$C. The presence of hybridised probe was visualised by enzyme immunoassay and enzyme-catalysed colour reaction with NBT/BCIP using a DIG Nucleic Acid Detection Kit as per the manufacturer's instructions (Roche). Colour development was stopped with a 5 min TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) wash. The wet membrane was scanned after placing in a clear plastic bag and results captured by a document scanner.

**Objective 2: Develop a quantitative assay (qPCR) for determining the infectious dose for this virus**

Currently, the absolute infectious dose of AbHV that causes AVG in susceptible species is unknown. Since the virus cannot be grown in cell culture (that would allow determination of a $TCID_{50}$) qPCR assays were developed to allow an estimation of the absolute (rather than relative) infectious dose of viral stocks. This capability will, for example, allow an estimation of the absolute infectious dose required to cause disease in different abalone species of different ages. Cloning of the viral sequence targeted by the qPCR test into a DNA plasmid and its use as a standard control in the TaqMan assay is an alternative to approximating the number of viral particles in the tissue. The following steps are involved:

- Amplification of the target sequence (amplicon) by conventional PCR using the same set of primers used for the TaqMan assay
- Purification and cloning of the amplicon into a plasmid
- Transformation and growth of *Escherichia coli* for multiplication of the plasmid
- Purification of the plasmid
- Confirmation of the amplicon sequence
- Determination of plasmid copy number by optical density according to the exact molar mass derived from the plasmid and amplicon sequences
- Serial dilution of the plasmid and analysis by the TaqMan test to produce a standard curve
- Establishment of the minimal number of gene copies detectable (analytical sensitivity)

This process has been undertaken for each of the qPCR tests (ORF49, ORF66 and ORF77) and the standard curves can be used to determine the number of AbHV genome copies in virus preparations.

**Objective 3: Determine the sensitivity of the virus to physico-chemical conditions including its stability in water/on fomites and its sensitivity to inactivation agents**

In order to determine viral stability (1) in seawater, (2) on fomites and (3) following treatment with inactivating agents, viral suspensions (with a known infectious dose) were treated with and without putative inactivation agents at various temperatures, for various time periods after which the $LD_{100}$ of the treated suspensions were re-assessed using the previously developed infectivity model. Samples to be tested for infectivity included:

- Virus stored in seawater at 4$^{o}$C, at 15$^{o}$C and at 25$^{o}$C for 1, 5 and 12 days
- In order to investigate the effect of dehydration on AbHV, a stock of AbHV was generated by injecting 6 GSW abalone with 100 uL of AbHV Vic1. At 5 days p.i. (when abalone were demonstrating clinical signs) a sample (10 mL) of the infectious water was taken and aliquots (2 mL) were placed into 5 Petri dishes. The Petri dishes were placed on ice in a bio-safety cabinet and then transferred to a refrigerator (4$^{o}$C) o/n to allow the virus preparation to dehydrate. The following day it was confirmed that all aliquots were dehydrated. Distilled water (10 ml total) was used to rehydrate the virus samples (on ice). The rehydrated AbHV sample was added to 4L seawater into which seven experimental abalone were placed.

- State jurisdictions were consulted concerning inactivation agents of interest. Victoria, Tasmania and South Australia recommended evaluation of three agents (calcium hypochlorite, Buffodine®, *Impress*). Thus during the course of this project disinfection trials were undertaken using these products. AbHV was either pretreated with the products, the disinfectant removed and then the virus inoculated into experimental abalone or AbHV was pretreated with the products and the preparations were added to abalone in seawater:

***Injection (i.m.) challenges***

Aliquots (1 mL) of AbHV Vic1 isolate (~200 x $10^6$ viral gene copies per mL) were placed into 6 2-mL-tubes. Calcium hypochlorite (Sigma-Aldrich), Buffodine® (Malaguna Pty Ltd) or the non-ionic surfactant *Impress* (Ultimate Cleaning Products NQ) were added to the tubes to obtain the final concentrations specified in Table 2. A positive control virus preparation did not receive any chemical treatment. After 10 min incubation at 16$^o$C the contents of the 6 tubes were transferred to 6 Vivaspin columns - 30 kDa cut-off (Sartorius Stedim Australia Pty, Ltd). The columns were then centrifuged for 10 min at 1200 x *g* at 4$^o$C. The filtrate was discarded and 2 mL Hank's balanced salt solution (HBSS) was added to each column. The process was repeated twice more. After the third centrifugation the virus was resuspended in 1 mL HBSS and kept on ice. Groups of 6 abalone were injected (i.m.) with 100 µL of treated virus (ca. 20 x $10^6$ virus gene copies) using a 1-mL syringe fitted with a 26G needle. This virus titre was approximately 100,000 times greater than the $LD_{100}$. Abalone in the positive control group received 100 µL virus suspension that had not been exposed to any chemical. Six abalone receiving 100 µL of HBSS only (no virus) was the negative, uninfected control group. Treatment control abalone received 100 µL HBSS previously treated with calcium hypochlorite to evaluate its potential residual toxicity after filtration through the Vivaspin columns. Abalone were placed in individual aquaria containing 1.5 L seawater and monitored daily for 10 days. Animals that showed clinical signs (e.g. lethargy and difficulty to attach to the aquarium surface) were euthanased and tissue samples were fixed in 10% buffered formalin in seawater for histological examination.

**Table 2. Summary of the experimental groups to determine efficacy of chemical disinfectants**

***Injection trial 1***

| Chemical treatment | Low concentration (loading dose) | Medium concentration (loading dose) |
|---|---|---|
| AbHV + non-ionic surfactant *Impress* | 1% (6 abalone) | 5% (6 abalone) |
| AbHV + Buffodine® | 50ppm (6 abalone) | nd |
| AbHV + calcium hypochlorite | 5ppm (6 abalone) | 10ppm (6 abalone) |
| calcium hypochlorite alone | 5ppm (2 abalone) | 10ppm (2 abalone) |

The positive control group (6 abalone) received AbHV in HBSS.
The negative control group (6 abalone) received HBSS only.

***Injection trial 2***

| Chemical treatment | Low concentration (loading dose) | Medium concentration (loading dose) | High concentration (loading dose) |
|---|---|---|---|
| AbHV + calcium hypochlorite | 5ppm (6 abalone) | 15ppm (6 abalone) | 20ppm (6 abalone) |
| calcium hypochlorite alone | 5ppm (2 abalone) | 15ppm (2 abalone) | 20ppm (2 abalone) |

The positive control group (6 abalone) received AbHV in HBSS.
The negative control group (6 abalone) received HBSS only.

***Immersion challenge***

Infectious water containing 1.67 x $10^6$ viral gene copies/mL (a viral titre more than 100 times greater than the $LD_{100}$) were distributed into three different aquaria (1 L per tank) which were either left as is (positive control water) or treated with calcium hypochlorite to a final concentration of 10 and 15 ppm. Two more aquaria containing normal seawater (virus-free) were also treated with calcium hypochlorite to a final concentration of 10 and 15 ppm to evaluate any toxicity effect on abalone

during and after immersion challenge. Water was held at 16$^{O}$C for 15 minutes and then water samples from each treatment were taken to measure the level of residual free chlorine (using an ExStik CL200 Chlorine meter, Envco – Environmental Equipment) for each treatment. Following treatments, 100 mL of each treatment were added to 900 mL seawater in five new aquaria. Healthy abalone (6 per aquarium containing the virus, and 2 abalone per aquarium containing control water now containing diluted calcium hypochlorite) were added. Six abalone were also added to an aquarium containing seawater only (negative, uninfected, untreated control group):

Treatment 1: AbHV treated with 15 ppm calcium hypochlorite
Treatment 2: AbHV treated with 20 ppm calcium hypochlorite
Treatment 3: 15 ppm calcium hypochlorite
Treatment 4: 20 ppm calcium hypochlorite
Treatment 5: Seawater only.

Abalone were challenged with the viral preparations for only 40 mins to limit their contact with any residual calcium hypochlorite, before transfer to individual aquaria containing 1.5 L fresh seawater. Water was changed daily.

**Objective 4: Determine the role of mucus in viral transmission**

It would be useful to know whether mucus secreted by affected abalone contains infectious virus and, if so, whether or not the mucus has protective qualities that may facilitate virus transmission. Mucus shed from infected abalone was analysed by PCR and used as a source of inoculum in the bioassay to determine whether it contained infectious virus.

To generate the immersion inoculum five hybrid abalone (70 mm) were injected (i.m.) with 100 uL of a 1:100 dilution (4.9 x $10^5$ vgc per 100 uL) of AbHV Vic1 stock virus. Disease signs (excess froth) were observed on day 3 post-injection (p.i.) and on day 4 p.i. 4 of the 5 injected abalone were moribund. A scalpel was used to scrape some mucus from the abalone and was placed in a tube with ~5 mL infectious water. In addition, 1.0 mL infectious water was used to quantify the presence of virus by qPCR. The infectious water titre was estimated at 9.1 x $10^5$ vgc/mL.

The infectious water was stored at 4$^{O}$C (positive control) and at 15$^{O}$C (temperature control) and the mucus sample were stored at 15$^{O}$C for three days (to mimic natural conditions).

[To ensure that the water sample contained infectious virus a titration experiment was undertaken and demonstrated that 1:10 dilution of this water produced 100% mortality in 6 days].

Following the 3-day storage period, 4 groups consisting of 8 abalone each were placed in 4 treatment tanks:

(1) Infectious mucus stored at 15$^{O}$C for 3 days
(2) Infectious water stored at 15$^{O}$C for 3 days
(3) Infectious water stored at 4$^{O}$C for 3 days
(4) Negative control (seawater only)

After 24 hours exposure to the treatments all abalone were placed in individual tanks with fresh seawater and monitored daily for clinical signs.

**Objective 5: Determine whether a latent stage exists in AVG**

Abalone herpesvirus (AbHV) has been determined to belong to the herpesvirus group known as the *Malacoherpesviridae* (Savin et al., 2010). Confirmation of the taxonomic status of the causative organism of abalone viral ganglioneuritis as a member of the herpesvirus group has implications for the possibility that the virus may form latent infections as is typically found for other herpesviruses. The ability to establish a subclinical/latent infection in host cells preserves a reservoir of the virus, which may be subsequently reactivated to cause disease during periods of stress for the animal. Commonly herpesviruses maintain their genomes within host cells as circular episomes within the

nuclei of host cells although some herpesviruses have been found to integrate into the genome of host cells (Morissette & Flamand, 2010).

The major tenet of viral latency is the presence of the pathogen viral genome in the absence of disease in the animal. This has been shown for the *Herpesviridae* (Morissette & Flamand, 2010) and has been suggested to occur in infections caused by members of the *Alloherpesviridae* where low viral loads have been detected in subclinical or healthy animals (Eide et al., 2011). In the *Malacoherpesviridae*, Ostreid herpesvirus (OsHV) which causes disease in oysters, has also been shown to be present at low viral loads in the absence of disease (Arzul et al., 2002; Barbosa-Solomieu et al., 2004; Dundon et al., 2011). Therefore latent or subclinical infection of oysters with OsHV has also been speculated to occur.

Three lines of research were undertaken to determine whether or not a subclinical/latent infection of abalone with AbHV occurs:

- Relationship to herpesvirus integrated in the genome of *Branchiostoma floridae* (amphioxus)
- Detection of viral genomic sequence in abalone in the absence of disease
- Identification of genes associated with latency

### 5.1 Relationship to herpesvirus integrated in the genome of *Branchiostoma floridae* (amphioxus)

During the AbHV Vic1 genome sequence analysis, Savin et al. (2010) identified the presence of homologues of AbHV open-reading frames in the genome sequence of *Branchiostoma floridae* (amphioxus), thus indicating that there is a herpesvirus associated with this invertebrate chordate (Figure 1). Further analysis was undertaken.

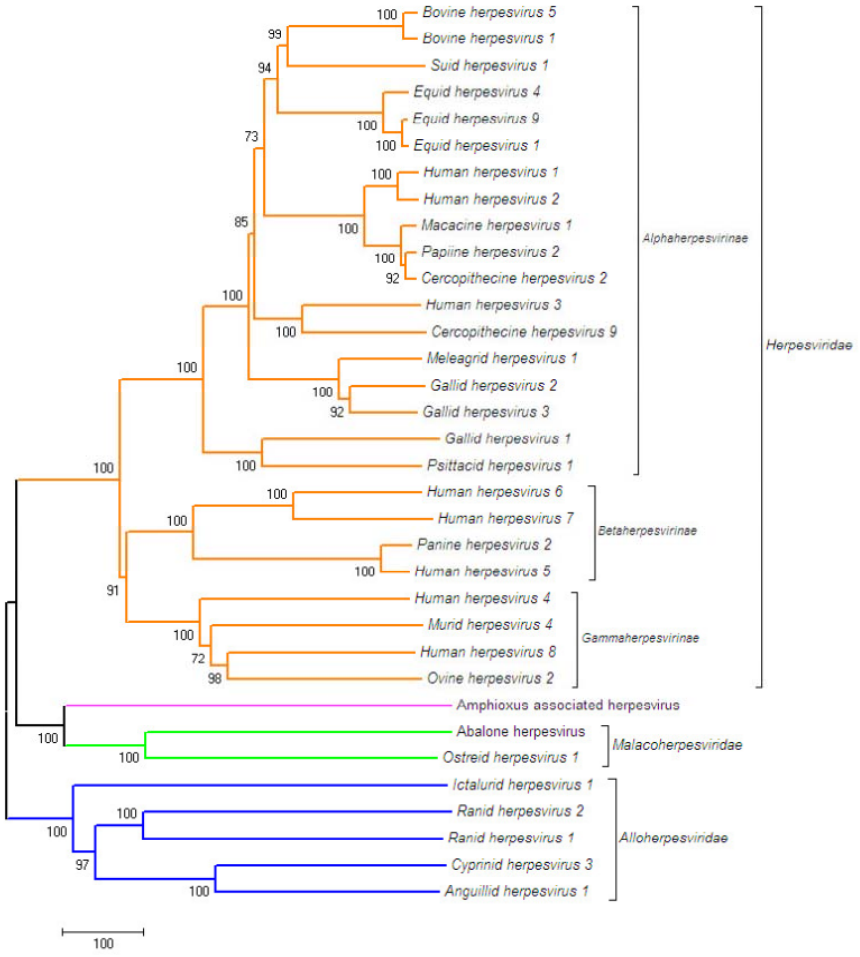


**Figure 1. Dendrogram of concatenated DNA polymerase and terminase protein sequences from 34 herpesviruses (from Savin et al., 2010).**

### 5.2 Presence of viral genomic sequence in the absence of disease

In a related project, coordinated by Dr Harry Gorfine (Fisheries Victoria), which aimed to determine the extent of spread of the AbHV in Victoria, a total of 767 live abalone exhibiting no apparent disease were collected from 81 sites along the coastline (Figure 2). The sites from where abalone were collected included areas where the disease was known to be active (MW1–4, MC1 and MC2) and others where disease had not been reported (MC4–10 and ME1–4) (Figure 2).

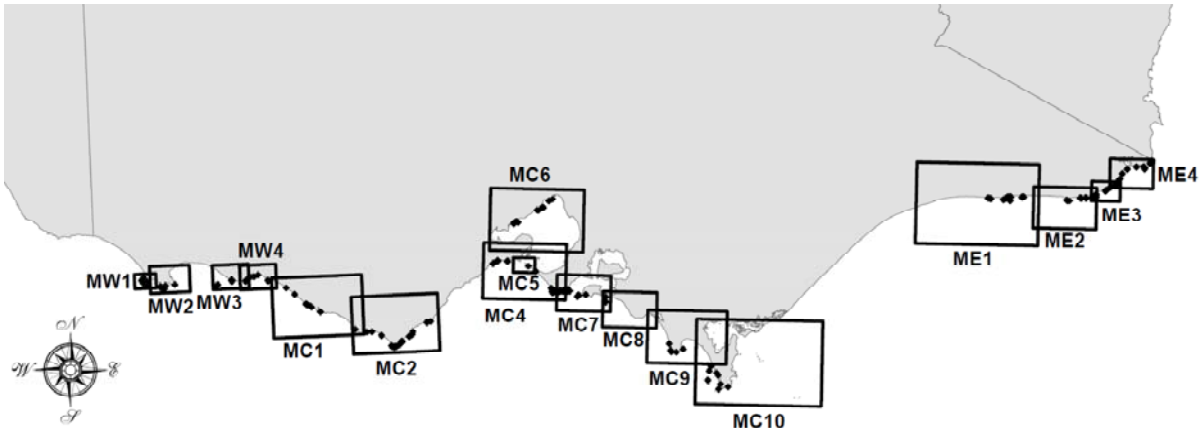


**Figure 2. Map indicating areas from where abalone were caught (supplied by Dr Harry Gorfine)**

***qPCR detection of AbHV***

All samples were tested at DEPI Victoria using qPCRs based upon ORF49 (Corbeil et al., 2010), ORF66, ORF77 and ORF95 (Table 3). ORF95 qPCR was developed at DEPI Victoria as part of a strategy to increase test sensitivity.

Reactions contained 12.5 µL TaqMan Fast Universal PCR Master Mix (2x), 2 µl (~100 ng/µl) of extracted DNA sample with the reaction mixture made up to 25µl using deionised water after addition of the appropriate primer and probe combinations (Table 3) at final concentrations of 300 nM for the primers and 100 nM for the probe. The following thermal cycling conditions were used for all qPCR assays: 95°C for 59s followed by 45 cycles of 95°C for 3s and 60°C for 30s.

**Table 3. Nucleic acid sequences of primers and probes for AbHV qPCR tests**

| Primer/Probe Name | Sequence |
|---|---|
| AbHV ORF66F1 | 5'-TCC-CGG-ACA-CCA-GTA-AGA-AC-3' |
| AbHV ORF66R1 | 5'-CAA-GGC-TGC-TAT-GCG-TAT-GA-3' |
| AbHV 66Prb1 | 5'-6FAM-TGG-CCG-TCG-AGA-TGT-CCA-TG-TAMRA-3' |
| AbHV ORF77F1 | 5'-CAA-CCA-CTT-GTT-CGG-GTT-CT-3' |
| AbHV ORF77R1 | 5'-CAG-GGT-GAT-TAA-TGC-GGA-GT-3' |
| AbHV 77Prb1 | 5'-6FAM-TCC-GTA-CGC-GGG-ATC-TTC-GT-TAMRA-3' |
| AbHV ORF95F1 | 5'-TGA-GCG-TTT-GGT-AGC-CTT-CT-3' |
| AbHV ORF95R1 | 5'-ACC-AGG-ACG-GGA-GTG-ACA-AG-3' |
| AbHV 95Prb1 | 5'-6FAM-TAT-CCC-CTC-TTC-CTC-GCT-TGC-ATG-T-TAMRA-3' |
| 18SF1 | 5'-CGG-CTA-CCA-CAT-CCA-AGG-AA-3' |
| 18SR1 | 5'- GCT-GGA-ATT-ACC-GCG-GCT-3' |
| 18S rRNA gene Probe | 5'-6VIC-TGC-TGG-CAC-CAG-ACT-TGC-CCT-C-TAMRA-3' |

***Development of nested qPCR protocols***

To improve the sensitivity of qPCR tests nested qPCR protocols were develop for the AbHV qPCR assays. Nested primer pairs for use in first round PCR were designed using aligned sequence data from all known variants of the AbHV to avoid areas of sequence variation (see Figure 3).

For the nested PCR assays all first round PCR reactions were completed using 200µM dNTPs, 1X PCR buffer, 1.5 mM $MgCl_2$, 400 nM of the appropriate forward and reverse primer (Table 4) and 1U Taq polymerase. The following thermal cycling conditions were used: 95°C for 5 min followed by 30 cycles of 95°C for 30s, 57°C for 30s and 72°C for 1 min. The PCR was completed by holding the reaction at 72°C for 10 min. A volume of 2 µL of the first round PCR reaction was used as template for the qPCR assays which were conducted as described above.

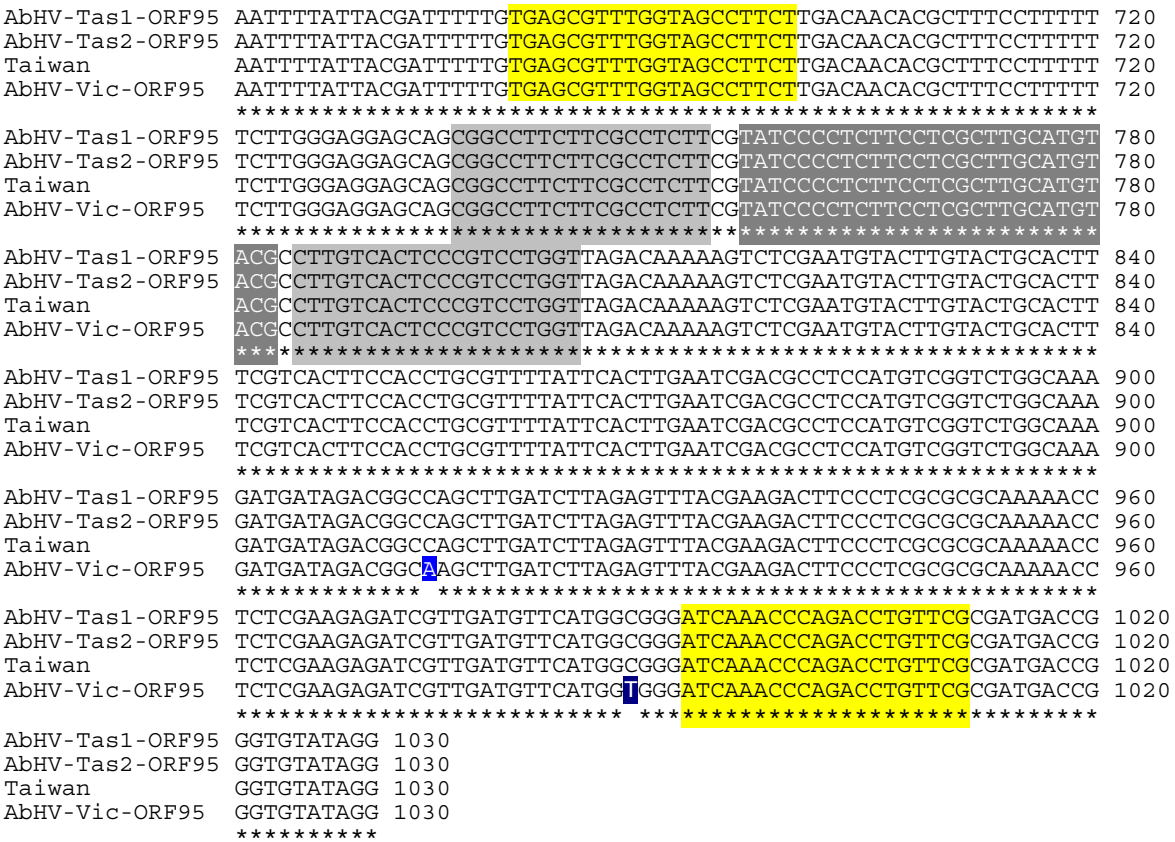


**Figure 3. Example alignment from ORF95 used to design AbHV nested qPCR.** Areas highlighted in yellow are the primer regions for the first round PCR; areas highlighted in light grey are primers for the qPCR and the area highlighted in dark grey with white text is the qPCR probe.

***Sequence analysis of amplicons***

Amplification products produced from PCR employing the primers described in Tables 3 and 4 were sequenced to confirm the sequence identity of the amplification products.

**Table 4: Nucleic acid sequences of primers for nested PCR protocols**

| Primer/Probe Name | Sequence |
|---|---|
| ORF66F Nested | 5'-GAC-TCC-CTT-TTC-CGT-GTG-AG-3' |
| ORF66R Nested | 5'-TAA-ACA-CCC-TGT-GGG-AGG-AC-3' |
| ORF77F Nested | 5'-TGT-TAA-GGA-CGG-CTG-TGT-3' |
| ORF77R Nested | 5'-TCA-ACC-GAT-AGA-CAG-GCA-GA-3' |
| ORF95F Nested | 5'-TGA-GCG-TTT-GGT-AGC-CTT-CT-3' |
| ORF95R Nested | 5'-CGA-ACA-GGT-CTG-GGT-TTG-AT-3' |

***Dot blot hybridisation***

Dot blot hybridisation was employed to identify whether or not AbHV DNA could be detected in samples using the ORF66 probe used in ISH, as described previously.

***In situ hybridisation***

*In situ* hybridisation, as described previously, was conducted on samples to identify whether or not the presence of AbHV could be detected in samples that tested positive for the pathogen using PCR.

**5.3 Identification of genes associated with latency**

During viral infections of multicellular organisms, induction of apoptosis is often observed and can be regarded as a primitive anti-viral mechanism (Kent et al., 2003). When infected with a virus cells that die by apoptosis limit the ability of the virus to replicate and spread. Therefore viruses that can subvert the apoptotic processes have a selective advantage. Several viruses have been found to carry genes that interfere with the host cell apoptotic machinery. Such genes have been found in mammalian herpesviruses (Kent et al., 2003) and baculoviruses (Huang et al., 2000), and genes encoding proteins significantly related to inhibitor of apoptosis proteins (IAPs) in mammalian and insect cells have been identified in the OsHV-1 genome (Davison et al., 2005).

Gradient-purified AbHV virions were obtained from dissected moribund abalone neuronal tissue collected during 2009 and genomic DNA was extracted from enriched virion preparations as described by Savin et al. (2010). The Roche-454 and Illumina HiSeq2000 technologies were used to generate DNA sequence data from genome fragments, as described by the manufacturers. The 454 sequence reads were assembled into contigs using Roche newbler software version 2.5.3 and the Illumina reads assembled using SOAPdenovo-127mer version 1.05. PCR fragments derived from the genome were sequenced using Sanger-based technology and were assembled in combination with the 454 reads using cap3. Several of the newbler, cap3 and SOAPdenovo-127mer contigs were then used as seed sequences and further assembled with the Illumina paired-end reads to establish the complete genome using PriceTI software and alignment to scaffolds generated from 454/newbler contigs using the ContigViewer in-house software. ContigViewer uses contig linking information from the newbler ace file. The genome sequence was annotated using Artemis and Blast.

**Objective 6: Determine the susceptibility of remnant populations of abalone previously exposed to AVG**

Not all abalone in virus-exposed areas died during the mortality event of 2005/6 and survivors continue to populate reefs in these areas. It would be useful to determine whether survival of these abalone was due to either some inherent resistance to the virus demonstrated by these abalone or that, for some reason, they were not exposed to a lethal dose. Samples of abalone (*Haliotis rubra*) from areas previously exposed to the virus were taken to determine whether or not they were resistant to experimental challenge with standard viral doses (previously determined in unexposed abalone of the same species and age).

Susceptibility of remnant populations of abalone previously exposed to AVG was determined for five populations in total (as part of this project – 2009/032 – and FRDC Project 2009/075 Tactical Research Fund: Determining the susceptibility of remnant populations of abalone previously exposed to AVG). FRDC Project 2009/075 reported on four of the populations. The results from the fifth population, obtained from Warrnambool Breakwater, 38 24 209S 142 28 627E, at 5 metres depth, with a water temperature of 13.3°C, are reported here.

In addition to these abalone, farmed and wild blacklip and greenlip abalone from South Australian waters were tested for susceptibility to infection and disease, using the developed infectivity model, as a service to the South Australian Government. The results are reported here for the sake of completion. It was agreed that, since the susceptibility to AVG of greenlip and blacklip abalone sourced from South Australia had already been studied as part of an independent investigation it would be more appropriate to extend this study to other species of abalone and molluscs from South Australia. Thus SARDI arranged for samples of Roe's abalone (*Haliotis roei*), the marine warrener snail (*Turbo undulatus*) and a scallop species (*Mimichlamys bifrons*) to be collected and transferred to AAHL. Experimental groups were exposed to either a low, medium or high dose of AbHV Vic1 and monitored for signs of disease.

Challenge dose: Three challenge doses were used that were estimated to be equivalent to a 10% lethal dose ($LD_{10}$), 50% lethal dose ($LD_{50}$) and 90% lethal dose ($LD_{90}$). Using three such challenge doses increased the probability of demonstrating whether or not any resistance to infection/disease existed. The challenge doses were prepared from infectious water generated as follows: Six 2-year-old farmed abalone (Jade Tiger from Great Southern Waters, Indented Head, Victoria) were inoculated (i.m.) with a standard dose (determined previously: 100 µL each of an AbHV stock containing ~$10^4$ gene copies) of stock virus and placed in 8 L of water which was changed daily. On day 4 post-inoculation (p.i.) the water was collected and analysed by qPCR. Previous experiments have shown that at day 4 post-inoculation there should be ~$10^6$ vgc $mL^{-1}$. The three challenge doses were prepared based on the $C_T$ value obtained using a plasmid standard control to estimate the viral titre (Corbeil et al., 2010). This infectious water was then used to make serial dilutions.

Experimental animals: Wild *H. rubra* (mature and juveniles) were collected and transported to AAHL where they were placed in the bio-secure aquarium and observed for 2-5 days to ensure that they had recovered from any handling/transportation stress. While these abalone were being "rested", the farmed hybrid (*Haliotis laevigata* X *H. rubra*) abalone (supplied by Great Southern Waters, Indented Head, Victoria) were used to prepare the challenge virus. For each exposure trial, 10 juvenile and 10 mature abalone from the wild, and 10 juveniles and 10 mature farmed abalone, were exposed to each dose (low, medium and high) of virus and then placed in individual tanks, each containing approximately 1.5 to 2.0 L aerated seawater (Figure 4). Uninfected control animals, both wild and farmed, were included in each trial.

Sampling: Following exposure to virus, abalone were monitored on a daily basis for clinical signs. A full change of water was performed daily. When abalone started to demonstrate clinical signs they were euthanased for laboratory examination (histology and PCR analysis). The experimental period was 10 days (from previous experience, it is known that infected abalone will die within 10 days of exposure to a lethal dose of Abalone herpesvirus).

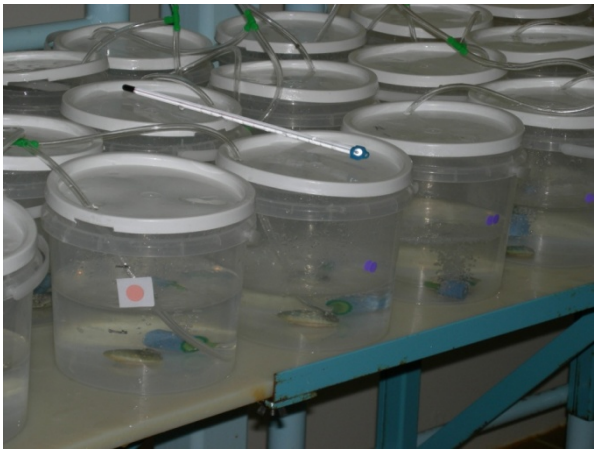

**Figure 4. Experimental tanks with air-lines housing individual abalone in approximately 1.5 L seawater**

Histopathology: Based on reports from natural disease outbreaks and some preliminary experimental infections, it was known that all moribund animals would die within 24 hours of showing clinical signs (e.g., loose attachment to the substrate). Therefore, moribund abalone were immediately sampled for histopathology and PCR analysis. For sampling, abalone were placed on a bed of ice (covered with a paper towel) for approximately 5 minutes. The abalone were dissected to expose the pleuropedal ganglion and nerve cords (Figure 5). The neural tissue with some surrounding muscle was removed and placed in formalin for a minimum of 24 h. Then the formalin-fixed tissues containing the pleuropedal ganglion and nerve cords were prepared by routine histological procedures (dehydration through an alcohol series, paraffin embedding, sectioning (3-6 µm), and staining with haematoxylin and eosin).

qPCR: Tissue containing the pleuropedal ganglion and/or nerve cords was dissected from moribund or dead animals and processed for PCR analysis using a real-time PCR (Corbeil et al., 2010). Nucleic

acid was extracted from tissue samples using the QIAamp DNA Mini Kit (Cat # 51306) following the manufacturer's instructions. Each sample was tested in duplicate.

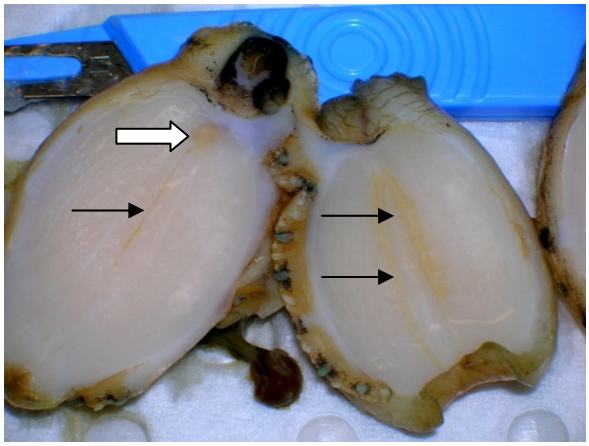

**Figure 5. An abalone dissected to reveal the pleuropedal ganglion (white arrow) and nerve cords (black arrows)**

**Objective 7: Using all three available qPCR tests, determine their relative sensitivities and specificities by re-testing previously collected samples from the abalone populations in Tasmania**

Currently, there are three qPCR tests (ORF49, ORF66 and ORF77) in use for detection and identification of AbHV, the causative agent of AVG. Previous studies demonstrated the performance of the original qPCR test (ORF49) which, at the time, detected all known isolates (AbHV Vic1, AbHV Tas1 and the Taiwanese strain) of AbHV (Corbeil et al., 2010). Since the development of the ORF49 qPCR, other genetic variants of AbHV have been discovered in Tasmania which were not recognised by this test (Ellard et al., 2009; Cowley *et al*., 2011). Thus two other tests, ORF66 and ORF77, were brought on-line in an attempt to identify a pan-specific test. Both of these tests were shown to detect all known genetic variants of AbHV (Table 5).

**Table 5. AbHV genetic variants: Information summary**

| | Vic1 | Tas1 | Tas2 | TAIWAN | Tas3 | Tas4 | Tas5 |
|---|---|---|---|---|---|---|---|
| **SAN*** | 06-00438 | 08-03285<br>09-02112 | 09-03016<br>10-01249<br>11-05745 | | 10-04643<br>11-00189<br>11-00221<br>11-00259 | 11-00590<br>11-00591 | 11-05369<br>11-05485 |
| **DATES**** | 2005/6 | Aug 2008 | Aug 2009<br>Nov 2011 | 2003 | Dec 2010<br>Jan 2011 | Feb 2011 | Nov 2011 |
| **Range** | Victoria | Tasmania | Tasmania<br>NSW | Taiwan | Tasmania | Tasmania | Tasmania<br>NSW |
| **ORF49** | + | + | - | + | - | + | + |
| **ORF66** | + | + | + | + | + | + | + |
| **ORF77** | + | + | + | + | + | + | + |

*AAHL Specimen Accession Number; **Date(s) detected

While these tests have been used to confirm presence of AbHV in suspect cases of AVG, their diagnostic sensitivity (DSe) and specificity (DSp) for detection of sub-clinical infections were unknown. This information is essential for development and result interpretation of surveillance programs designed to detect (sub-clinical) infections (rather than disease).

In the absence of a gold standard test (i.e. a perfect test with 100% DSe and DSp), the method of choice to evaluate the accuracy of a diagnostic test is the Latent Class Model analysis (LCM). There are three criteria for the use of this analytical approach:

(i) Test results from at least two assays on the same samples collected from at least two representative populations with distinct prevalence of infection must be used,
(ii) The DSe and DSp of the tests should not vary across populations,
(iii) The tests should be conditional independent on the disease status.

Testing material was sourced from the DPIPWE Tasmania diagnostic laboratory archives. Only specimens originating from abalone populations showing no evidence of clinical disease at the time of collection were targeted. The specimens were grouped according to their origin into 5 source populations with expected varying prevalences (Table 6). A total of 1,459 abalone tissue specimens were obtained from the 5 source populations. They included greenlip, blacklip, and hybrid abalone samples collected in Tasmania between 2008 and 2012 for various reasons including surveillance, routine investigation, and health certification. All the selected specimens were previously tested using histopathology as part of routine laboratory activity at DPIPWE, Tasmania.

**Table 6. Summary of source population characteristics used for the Latent Class Model analysis**

| Source Population | Description | Total number | % Blacklip (number) | % Greenlip (number) | % Hybrid (number) | Sampling period |
|---|---|---|---|---|---|---|
| 1 | Wild abalone direct from marine environment | 703 | 92.7% (652) | 7.3% (51) | 0% (0) | 2008 & 2011 |
| 2 | Wild abalone via processor | 440 | 81.1% (357) | 17.5% (77) | 1.4% (6) | 2008 to 2011 |
| 3 | Cultured abalone direct from farm | 138 | 14.5% (20) | 50% (69) | 35.5% (49) | 2008 to 2012 |
| 4 | Cultured abalone via processor | 7 | 100% (7) | 0% (0) | 0% (0) | 2011 |
| 5 | Wild abalone direct from motherboat | 171 | 60.8% (104) | 8.2% (14) | 41.0% (53) | 2008-9 & 2011 |
| **Total** | | 1,459 | 78.1% (1,140) | 14.6% (211) | 7.4% (108) | 2008 to 2012 |

# Results

**Objective 1: Validate the developed *in situ* hybridisation diagnostic test including roll-out to other States**

***DNA probe specificity***

Hybridisation specificity of the DNA probe was assessed by dot blot hybridisation to DNA extracted from infected and non-infected abalone and to genomic DNA from other herpesviruses and iridoviruses. The related herpes and iridovirus samples were confirmed as containing nucleic acid from the viruses by PCR analysis of extracted DNA (Table 7). The AbHV-DNA probe hybridised only to the DNA extracted from AbHV-infected abalone (Samples 3 and 4; Figure 6). No cross-hybridisation of the probe with any of the other viral genomic DNA was observed (Samples 5–12; Figure 6). The AbHV probe did not hybridise to DNA extracted from abalone which were not infected with AbHV (Samples 1 and 2; Figure 6).

**Table 7. Confirmation of nucleic acid content by specific PCR**

| Virus | Conventional PCR | | Taqman PCR | |
|---|---|---|---|---|
| | Ranavirus | OMV | RSIV | KHV |
| BIV | + | - | - | - |
| EHNV | + | - | - | - |
| FV3 | + | - | - | - |
| RSIV | - | - | + | - |
| KHV | - | - | - | + |
| OMV | - | + | - | - |

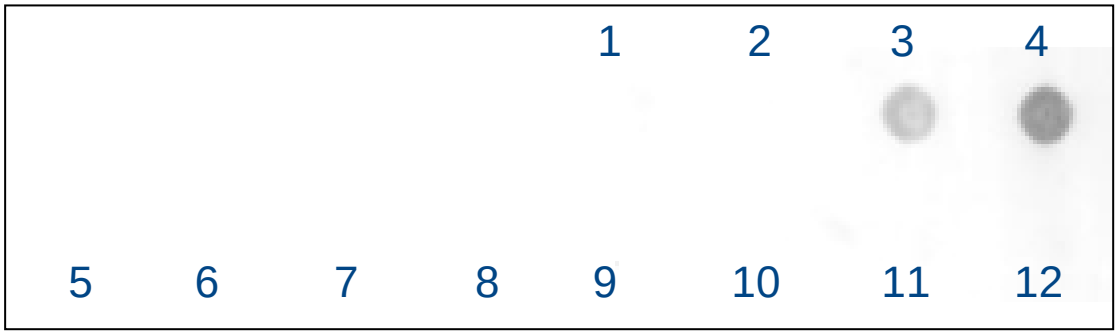


**Figure 6. Dot-blot hybridisation of extracted genomic DNA from AbHV-infected and non-infected abalone and from other herpes and iridoviruses.** Sample 1: Non-infected abalone 09/4333-2; Sample 2: Non-infected abalone 09/4333-1; Sample 3: AbHV-infected abalone 09/4479-2; Sample 4: AbHV-infected abalone 09/4479-1; Samples 5 & 6: Buffer negative controls; Sample 7: *Oncorhynchus masou* virus; Sample 8: Koi herpes virus; Sample 9: Red sea bream iridovirus; Sample 10: Frog virus 3; Sample 11: Epizootic haematopoietic necrosis virus; Sample 12: Bohle iridovirus.

***In situ hybridisation***

Positive reactions, characterised by the presence of a blue-black precipitate, were detected in AbHV-infected abalone tissues (Figures 7A, 8A and 8B). All confirmed AbHV-positive samples tested produced the characteristic blue-black precipitate in cells associated with the neural tissue. No signal (Figure 7B) was detected from abalone tissue taken from any of the non-infected abalone tested. No signal was detected in the tissue from fish infected with epizootic haematopoietic necrosis virus (Figure 9A) or Koi herpesvirus (Figure 9B). Similarly, no signal was produced for the controls without the addition of probe (Figure 10A) or the anti-DIG antibody (Figure 10B).

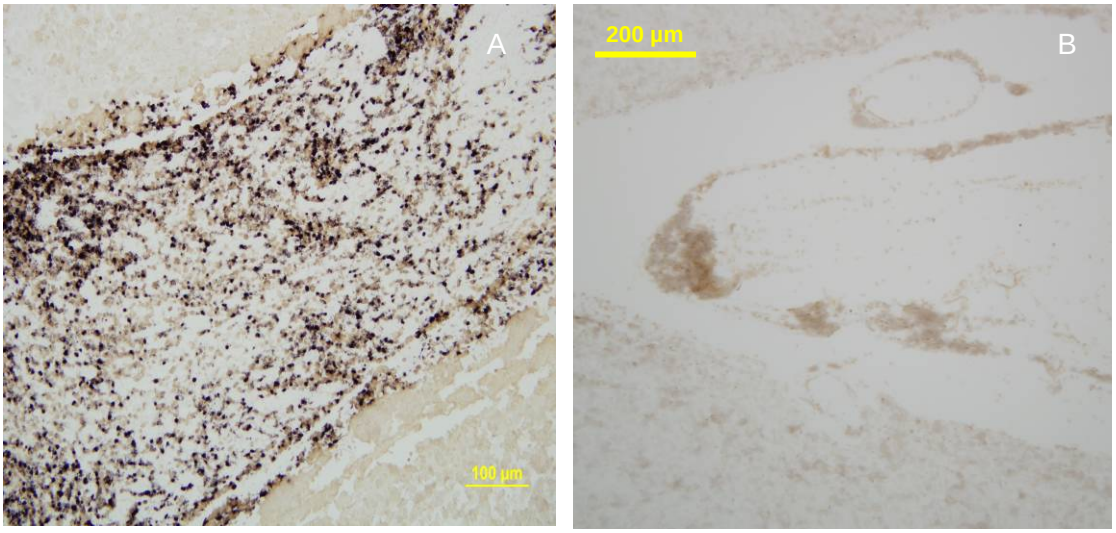


**Figure 7. *In situ* hybridisation of AbHV-infected (A) and non-infected (B) abalone.** Blue-black staining of cells indicates the presence of the target nucleic acid sequence. Brown colour is produced from counter-staining with Bismarck brown yellow. ISH-positive cells are only observed in the AbHV-infected sample (A).

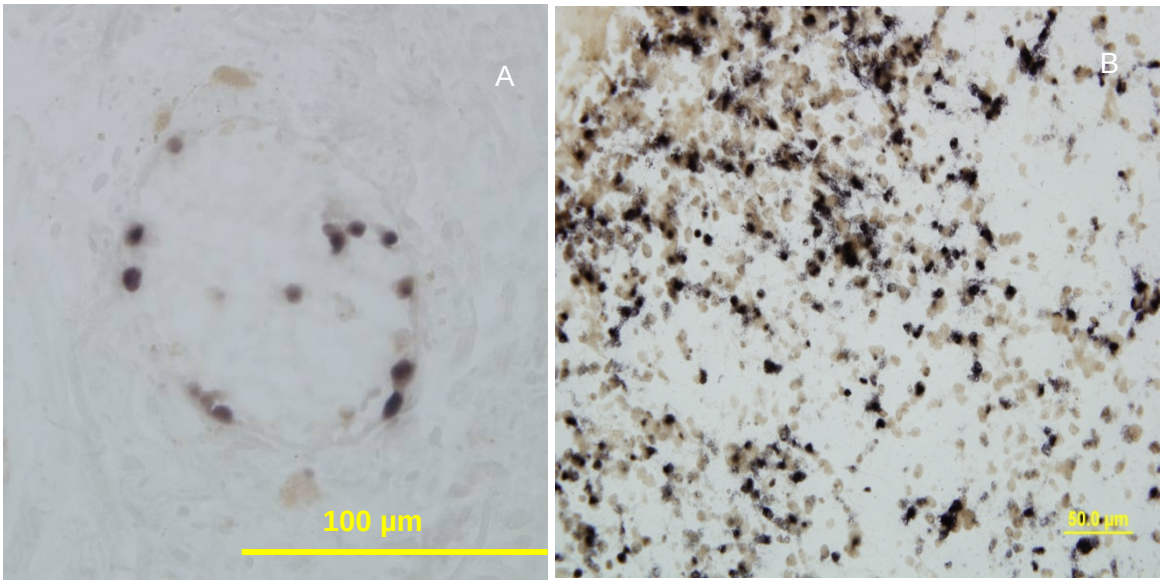


**Figure 8. *In situ* hybridisation of AbHV-infected abalone.** Note the intense blue-black staining of cells which indicates the presence of the target nucleic acid sequence. (A) A nerve showing cells containing the AbHV target sequence. (B) A close up section of the pleuro-pedal ganglion showing cells containing the AbHV target sequence.

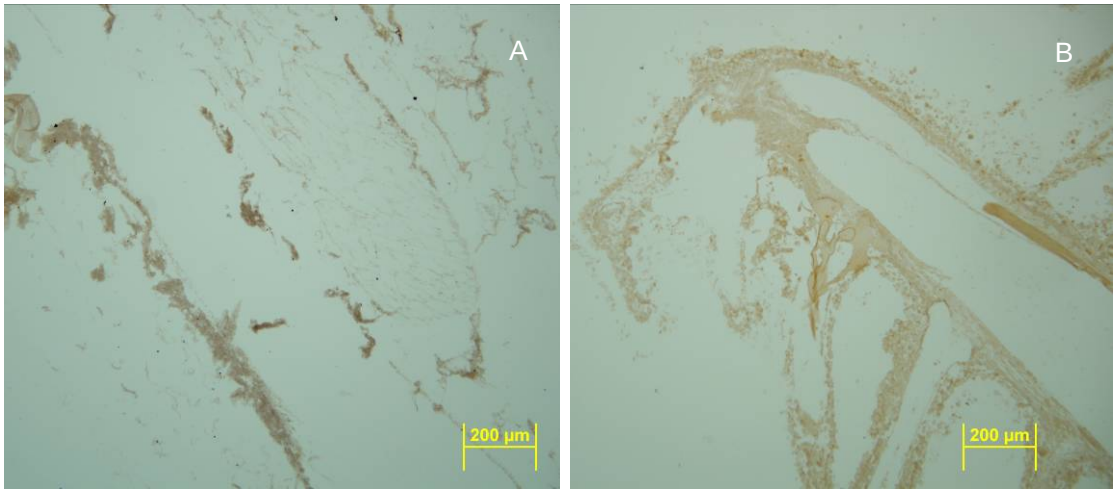


**Figure 9. *In situ* hybridisation of tissue from a fish infected with epizootic haematopoietic necrosis virus (A) and koi herpesvirus (B) with the DIG-labelled AbHV probe.** Note the absence of intense blue-black staining seen in positive samples.

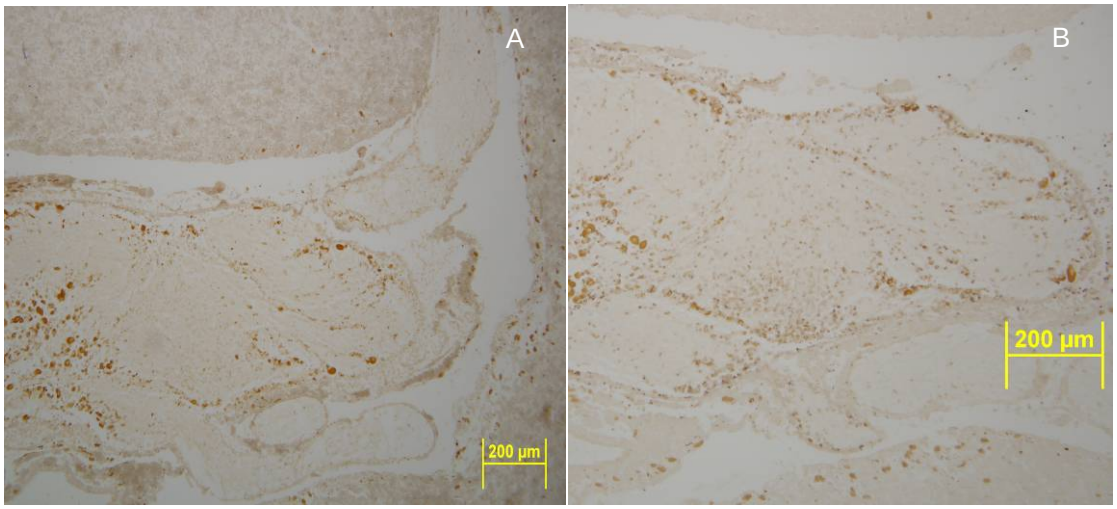


**Figure 10. Negative controls of *in situ* hybridisation of abalone tissue infected with the AbHV.** (A) Control slide minus DIG-labelled AbHV probe and (B) Control slide minus the anti-DIG antibody. Note the absence of intense blue-black staining seen in positive samples.

***ISH test transferred to diagnostic laboratories***

*In situ* hybridization (ISH) is a method that can detect specific viral nucleic acid sequences in cells and tissues by hybridization of a labelled gene probe to a specific target nucleic acid sequence of the viral pathogen. ISH has been applied to diagnosis of several marine animal viruses, and this project team has developed an ISH test specific for AbHV. The ISH test for the detection of AbHV was validated on a set of abalone sections identified as being either positive or negative for AVG. ISH is a specialised test and is not able to be conducted by all laboratories. Therefore this project sought to only transfer this test on request. The full testing methodology has been included in the recent revision of *the OIE Manual of Diagnostic Tests for Aquatic Animals* Chapter – Infection with abalone herpesvirus (OIE, 2014). Consequently, any laboratory with *in situ* hybridisation capabilities around the world, with access to the *OIE Manual*, has the full details of all aspects of the test and would be able to run the AbHV ISH test.

During this project, scientists from Japan visited the DEPI Attwood laboratories in order to be trained in the ISH method. On completion of the training, the Japanese scientists were competent to implement the test at their laboratories.

Given the specialised nature of this test, technology transfer of the ISH methodology within Australia was limited. In May 2012, a training workshop was conducted for Australian diagnostic laboratories with participants from AAHL, Tasmania and Western Australia. South Australia is not currently in a position to request the transfer of the ISH test. If, in the future, South Australia requires samples to be tested with the ISH test, their first response would be to send the samples to DEPI Victoria for testing.

**Objective 2: Develop a quantitative assay (qPCR) for determining infectious dose for this virus**

Events in Tasmania have indicated that infection with AbHV can occur in the absence of clinical disease (Ellard et al., 2009). Healthy abalone transferred from the wild to processing plants, where less-than-optimal conditions occur, demonstrated AVG signs. Of particular interest was the second outbreak that occurred in September 2009 (Table 7) which was associated with clinical disease, tissue lesions and mortality but samples were ORF49 qPCR negative, when tested both at DPIPWE Animal Health Laboratory, Tasmania and at CSIRO-AFDL. Fresh tissues from diseased abalone were therefore transferred to AFDL where they were used as inoculum in an infectivity trial. Experimentally inoculated abalone developed AVG which was confirmed by presence of clinical signs, histopathology and positive ISH (carried out by DEPI Victoria), and electron microscopy at AAHL confirmed presence of virus in the tissues. Samples prepared for PCR analysis using the ORF49 qPCR produced negative results. Therefore it was decided to test these samples using other qPCR primers and probes (ORF66 and ORF77) that were produced and evaluated in FRDC Project 2007/006 Aquatic Animal Health Subprogram: Development of molecular diagnostic procedures for the detection and identification of herpes-like virus of abalone (*Haliotis* spp.). The samples produced positive results using either of these primers/probe sets.

Subsequently, all suspect AVG cases submitted to CSIRO-AAHL have been, and continue to be, tested using all three qPCR tests. The September 2009 isolate from Tasmania (Tas2) has been sequenced and shown to demonstrate significant variation from Vic1 and Tas1 at the genome level. Subsequent isolates from Tasmania, in December 2010 and January 2011, were also ORF49-negative and ORF66- and ORF77-positive. A further isolate from Tasmania, in February 2011, was positive by all three qPCR tests. Taken together, these results indicate that there are several genetic variants of AbHV present in wild abalone stocks in Tasmania and that disease may occur when sub-clinically infected abalone are transferred to processing plants. There are insufficient data to determine whether or not sub-clinically infected abalone develop clinical disease, or shed virus into tanks resulting in disease outbreaks in co-habiting stock. Conventional PCR tests (AB1213; AB1617; AB2930) have also been developed to facilitate partial sequence analysis and differentiation of genomic variants (see Appendix 1 for PCR details). Tables 8a and 8b summarise the analysis of available variants using currently developed qPCR and conventional PCR tests.

**Table 8a. AbHV TaqMan PCR test detection summary**

| | Vic1 | Tas1 (Sep-08) | Tas2 (Sep-09) | TAIWAN | Tas3 (Dec-10) | Tas4 (Jan-11) | Tas5 (Feb-11) |
|---|---|---|---|---|---|---|---|
| **ORF49** | + | + | - | + | - | + | + |
| **ORF66** | + | + | + | + | + | + | + |
| **ORF77** | + | + | + | + | + | + | + |

**Table 8b. AbHV conventional PCR test detection summary**

| | Vic1 | Tas1 | Tas2 | TAIWAN | Tas3 (Dec-10) | Tas4 (Jan-11) | Tas5 (Feb-11) |
|---|---|---|---|---|---|---|---|
| **AB1213** | + | + | + | + | + | + | + |
| **AB1617** | + | + | + | + | + | + | + |
| **AB2930** | + | + | - | - | - | - | - |

In addition, an analysis was undertaken to determine the reason for non-reactivity of Tas2 with the ORF49 qPCR. There appeared to be mismatches in both the forward and reverse primers (Figure 11).

```
AbHV-Vic    TTCATCAAAAGCGTTTTTATCCTCGAGAGAGAAACCCACACCCAATTTTTGAGTGTAGGC
AbHV-Tas1   ..........................................................
AbHV-Tas2   .........C.......CG.....C..G.....C.....................A...T
                                                  AbHV_ORF49f1 ->
AbHV-Vic    GAATACATTTGCTTTCTTACCGCTTTCAATCTGATCCGTGGTTTCTTTAGTCGTTTTGAG
AbHV-Tas1   ..........................................................
AbHV-Tas2   A..C..G.C..AC.....GG..A................C..........T........
                                    AbHV_49Prb1
AbHV-Vic    AATCTGTTTGCATAAAGGAACAACAAACTTGCCTTGGGTGTTTGCTTGGATGTTTTCAAT
AbHV-Tas1   ..........................................................
AbHV-Tas2   ..A.........C.CG.....C........T.....T.....C.............G..
                                  <- AbHV ORF49r1
```

**Figure 11. Sequence analysis of AbHV Vic1, Tas1 and Tas2 demonstrating nucleotide mismatches in both the forward and reverse primers (highlighted in yellow) and the probe (highlighted in grey).**

### 2.1. Plasmid standards for ORF66 and ORF77 qPCR tests generated

Amplicons of AbHVORF66 and AbHVORF77 qPCRs were generated by a conventional PCR, utilizing HotStar Taq polymerase (Figure 12). Each amplicon was gel-purified (QIAquick Gel Extraction Kit), ligated into a pAcquire vector (Figure 13) according to the Acquire PCR Cloning Kit fast protocol and chemically transformed into *E. coli* (Alchemy Biosciences). Positive colonies were identified by colony PCR (Figure 14), cultured overnight (AbHVORF66 colonies 1 and 4, AbHVORF77 colonies 1 and 6) in selective LB broth before plasmids were purified using a Qiagen Spin Miniprep Kit.


**Figure 12. Electrophoresis gel of PCR amplicons.** Row 1: Lanes 1-4 CSIRO AbHVORF66 (146 bp) and Row 2: Lanes 1-4 CSIRO AbHVORF77 (190 bp) conventional PCR amplicons. N = no template control, M = 100 bp molecular weight marker

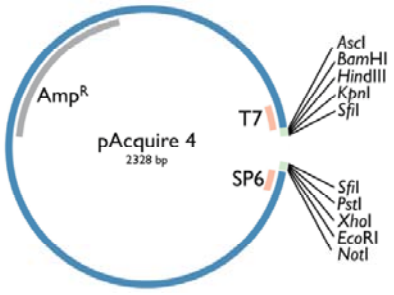


**Figure 13. pAcquire high copy number cloning vector**

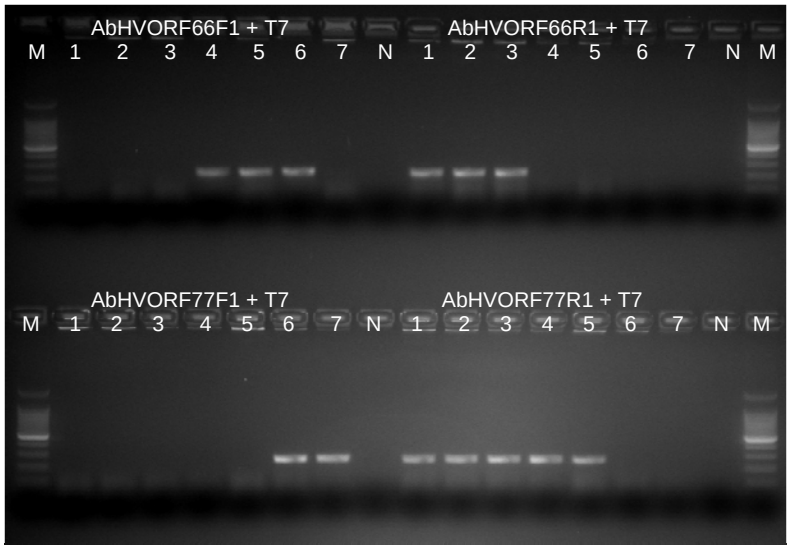


**Figure 14. PCR to detect colonies positive for AbHVORF66 (Row 1 colonies 1-7) or AbHVORF77 (Row 2 colonies 1-7) inserts, using insert-specific primers and a vector-specific primer (T7).** N = no template control, M = 100 bp molecular weight marker.

The presence of AbHVORF66 or AbHVORF77 within pAcquire plasmid minipreps was confirmed by TaqMan Fast reactions with $C_T$ values in the range 8-9. Both plasmid preparations for each target were combined and diluted to a final concentration of 80% ethanol, before being dunked out of the AAHL high-level bio-secure area. After dunk-out, the DNA was vacuum-dried and then re-constituted in nuclease-free water. Ten-fold dilutions of each plasmid were prepared and tested by TaqMan Fast reactions to determine the $C_T$ value for each dilution (Table 9).

**Table 9. TaqMan qPCR results for AbHVORF66/pAcquire and AbHVORF77/pAcquire plasmid ten-fold dilutions.**

| Plasmid Dilution | AbHVORF66/pAcquire (Mean $C_T$) | AbHVORF77/pAcquire (Mean $C_T$) |
|---|---|---|
| $10^{-1}$ | 9.90 | 12.01 |
| $10^{-2}$ | 13.04 | 15.12 |
| $10^{-3}$ | 15.62 | 19.19 |
| $10^{-4}$ | 20.43 | 22.01 |
| $10^{-5}$ | 23.58 | 25.57 |
| $10^{-6}$ | 26.81 | 28.95 |
| $10^{-7}$ | 30.25 | 32.45 |
| $10^{-8}$ | 33.76 | 36.39 |
| $10^{-9}$ | 37.53 | >40.00/>40.00 |
| $10^{-10}$ | 38.60/>40.00 | >40.00/>40.00 |
| $10^{-11}$ | >40.00/>40.00 | >40.00/>40.00 |
| $C_T$ linearity ($R^2$) | 0.9983 | 0.9991 |
| qPCR % efficiency | 97.05% | 97.38% |

Mean $C_T$ values from 25 µl duplicate Taqman Fast reactions containing 2 µl of plasmid dilutions

Each plasmid was quantified by both NanoDrop (Thermo, UV absorbance) and Qubit Fluorometer (Invitrogen, dsDNA HS Assay Kit) (Table 10).

**Table 10. Quantification of plasmid DNA by Nanodrop and *Qubit* fluorometer**

| Plasmid | Nanodrop (ng/µl) | Qubit (ng/µl) |
|---|---|---|
| AbHVORF66/pAcquire | 86.1 | 21.1 |
| AbHVORF77/pAcquire | 60.0 | 7.7 |

Based on Qubit DNA calculations, the copy number of each plasmid was determined. Dilutions were prepared for a high (10 000 copies $\mu l^{-1}$) and low (100 copies $\mu l^{-1}$) positive in 10mM Tris, pH 8.8 in molecular biology grade $H_2O$ with 50 ng/µl calf thymus DNA (Table 11). Aliquots of high and low positive plasmid dilutions were placed in 2 ml Sarstedt tubes (25 x 200 µl and 5 x 1000 µl). The homogeneity of the high and low positives for each plasmid was validated using 5 x 200 µl aliquots in duplicate by Taqman Fast assay (Table 11).

**Table 11. Homogeneity of high and low AbHVORF66 and AbHVORF77 plasmid positive controls**

| Plasmid | Mean $C_T$ | Std Dev |
|---|---|---|
| **AbHVORF66/pAcquire** | | |
| 20 000 copies | 26.56 | 0.13 |
| 200 copies | 33.34 | 0.28 |
| **AbHVORF77/pAcquire** | | |
| 20 000 copies | 27.45 | 0.08 |
| 200 copies | 34.32 | 0.31 |

$C_T$ values (mean and std dev) from Taqman Fast 25 µl reactions containing 2 µl plasmid control

## 2.2 Distribution of plasmids

Plasmids (4 x 200 µl) were distributed to relevant diagnostic laboratories both nationally and internationally, and on request as part of the responsibilities of an OIE Reference Laboratory (Table 12).

**Table 12. Laboratories to which AbHV qPCR positive controls were distributed.**

| International Laboratories | Australian Laboratories |
|---|---|
| Dr Pen H. Chang<br>Dept Veterinary Medicine<br>National Taiwan University<br>Taipei 10617, TAIWAN | Fish Health Unit<br>Department of Primary Industries, Parks, Water and Environment<br>Prospect TAS 7250 |
| Dr Cho Jae Bum<br>Experiment and Analysis Division<br>YeongNam Regional Headquarter Office<br>Animal, Plant, Fisheries Quarantine and Inspection Agency, SOUTH KOREA | Elizabeth MacArthur Agricultural Institute<br>Department of Primary Industries<br>Menagle NSW 2568 |
| Dr Jun Kurita<br>Fish Diseases Diagnostic Group<br>Diagnosis and Training Center for Fish Diseases<br>Mie 516-0193, JAPAN | WA Department of Fisheries<br>South Perth WA 6151 |
| Dr Tristan Renault<br>IFREMER<br>La Tremblade, FRANCE | SARDI Aquatic Sciences<br>Henley Beach SA 5022 |
| Sr. Marcos Godoy<br>Centro de Investigaciones Biologicas Aplicadas<br>Puerto Montt, CHILE | DEPI Victoria<br>AgriBio Centre<br>Bundoora VIC 3839 |
| Dr. J.K. Jena<br>National Bureau of Fish Genetic Resources<br>INDIA | |
| Dr Gary Meyer<br>Pacific Biological Station<br>Nanaimo, CANADA | |

## 2.3. Quantitative assay (qPCR) for determining infectious dose

Using the AbHV Vic1 strain and the ORF49 qPCR with a plasmid positive control standard, it is possible to quantify the infectious dose for each batch of challenge virus (Corbeil et al., 2010) used for immersion challenge (Corbeil et al., 2012).

It should be noted that $C_T$ values obtained from the standard curve relate to viral gene copies (vgc) which may overestimate infectious dose (i.e. a viral gene copy may not always represent an infectious virus particle because, while the viral nucleic acid target for the PCR may be present, the virus may not be fully mature and infectious). This is one source of variation that can contribute to variation observed in mortality rates between experiments.

Other sources of variation include (i) the conditions used for preparing the infectious water (size of inoculated abalone, age of the virus stock used for i.m. inoculation, water temperature at which the inoculated abalone are maintained, timing of harvesting the resultant infectious water); (ii) the conditions of the immersion challenge (water temperature, size of exposed abalone, exposure time period, proportion of infectious virus (in relation to total viral DNA) in the infectious water.

Thus, following the preparation of a virus working stock, the stock should undergo a ten-fold titration by i.m. inoculation in abalone to obtain estimates of $LD_{90}$ and $LD_{50}$ which are related to the $C_T$ values obtained from the standard curve using the plasmid positive control. These data are then used to determine the viral dose to inoculate abalone used to generate the infectious water for immersion challenges. The infectious water is also analysed using the ORF49 qPCR to determine the viral gene copies per ml.

Thus a ten-fold dilution series ($2.5 \times 10^6$ vgc/ml; $2.5 \times 10^5$ vgc/ml; $2.5 \times 10^4$ vgc/ml; $2.5 \times 10^4$ vgc/ml) was prepared from the infectious seawater. Each of four experimental groups with 8 abalone each was exposed to one of the four viral dilutions for one hour and then the abalone were placed individually in 2-litre aquaria. The abalone were monitored daily for the appearance of clinical signs/mortality. Moribund and dead abalone were sampled for laboratory analysis (histology and qPCR).

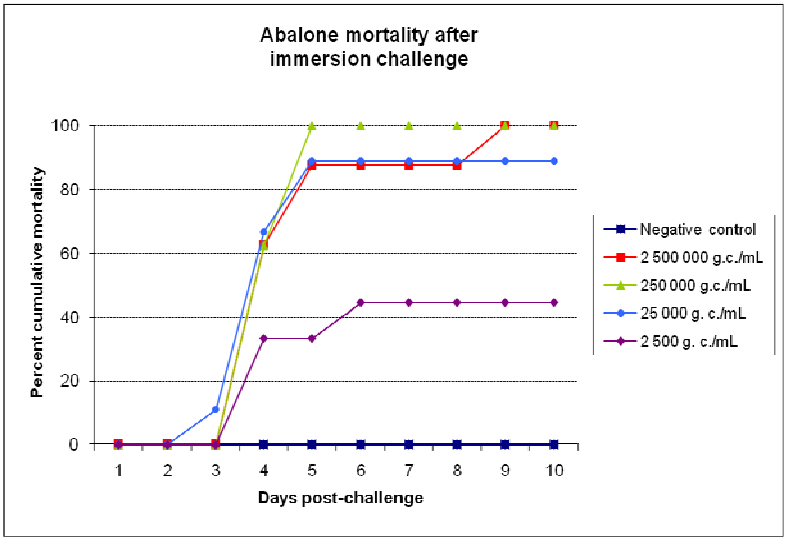


**Figure 15. Mortality curves for abalone infected with AbHV by bath immersion**

Mortality curves (Figure 15) demonstrated a clear correlation with viral challenge dose. In addition, histopathology typical of abalone viral ganglioneuritis (AVG) was observed in tissue samples taken. Thus depending on the aim of the experiment we can now choose from two routes of infection (intramuscular inoculation or bath immersion) using a quantifiable dose of virus based on viral genome equivalents (vgc).

**Objective 3: Determine the sensitivity of the virus to physico-chemical conditions including its stability in water/on fomites and its sensitivity to inactivation agents**

### 3.1. Virus Stability

#### 3.1.1 Stability in seawater

The standard bioassay was used to evaluate stability of AbHV stored in seawater at each of three temperatures. Three aliquots of stock AbHV (Vic1) of known titre were placed in seawater at each of three temperatures (4$^{o}$C, 15$^{o}$C and 25$^{o}$C). Viral infectivity was assayed by exposing abalone to each of the treated viral stocks following 1, 5 and 12 days incubation at their respective temperatures. A summary of the results is shown in Tables 13 and 14. Mortalities reached a plateau value at 7-8 days post-exposure (the experiment was allowed to run for 10 days to demonstrate that no further mortality occurred). Samples were taken for laboratory analysis (histology and ORF49 qPCR).

**Table 13. Abalone mortality at 10 days after exposure to AbHV incubated at three different temperatures in seawater**

| Incubation period at specified temperature (days) | Total mortality at 10 days post-exposure | | |
|---|---|---|---|
| | 4$^{o}$C | 15$^{o}$C | 25$^{o}$C |
| 1 | 87.5% | 75% | 12.5% |
| 5 | 12.5% | 0% | 0% |
| 12 | 0% | 0% | 0% |

Viral infectivity, as demonstrated by abalone mortality, decreased with increasing temperature and with increasing time period at each of the three temperatures. All abalone in the negative control group remained alive for the duration of the experiment.

The Victorian isolate of AbHV was used for all bioassays and so PCR analysis was undertaken using the ORF49 qPCR. The results are summarised in Table 14 and demonstrate that presence of virus correlates with the mortality rates.

**Table 14. Summary of ORF49 qPCR analysis of tissues sampled after exposure to AbHV incubated at three different temperatures in seawater**

| Incubation period at specified temperature (days) | ORF49 qPCR (mean $C_T$ value ± SD) | | |
|---|---|---|---|
| | 4$^{o}$C | 15$^{o}$C | 25$^{o}$C |
| 1 | 19.9±7.8 | 21.23±10.8 | 36.5±8.4 |
| 5 | 31.8±11.9 | 38.8±1.8 | 37.5±2.3 |
| 12 | 38.5±1.17 | 36.9±2.4 | 37.6±3.2 |

Negative control group mean $C_T$ value: 37.1±1.6

#### 3.1.2 Effect of dehydration on AbHV infectivity

The dehydrated virus preparation was rehydrated and added to 4L seawater into which seven experimental abalone were placed. Mortality curves (Figure 16) indicated that exposed abalone were infected and demonstrated clinical signs of AVG. AVG was confirmed based on histopathology. Presence of AbHV was confirmed by PCR analysis.

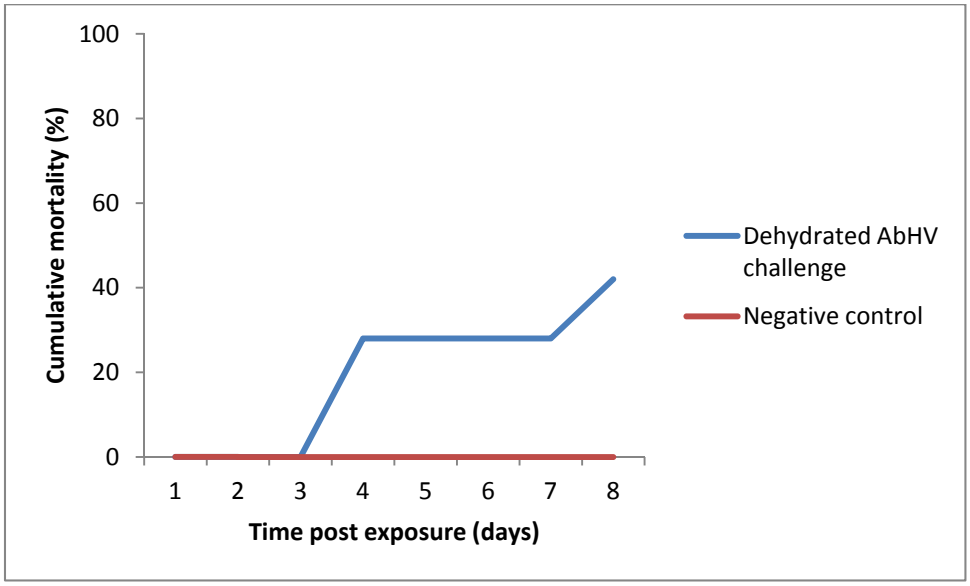


**Figure 16. Mortalities following exposure of abalone to AbHV that had been dehydrated overnight.**

These results indicate that dehydration for 24 hours is insufficient to totally inactivate AbHV. It is noted that dehydration here was undertaken at 4°C, the rationale being that it is known from previous studies that temperature also has an effect on virus infectivity. To reduce any temperature effects on virus infectivity, in an attempt to investigate dehydration alone, dehydration was undertaken at 4°C (AbHV infectivity is reduced to 87.5% of positive control virus – section 3.1.1).

### 3.1.3 Virus inactivation

***Injection trial 1***

Abalone injected with AbHV alone (positive control group) showed a typical mortality curve reaching 100% (6 out of 6 abalone) within 7 days post-challenge (Figure 17). The groups of abalone injected with the virus treated with 5 and 10 ppm of calcium hypochlorite showed mortality rates of 100% (6 out of 6 abalone) and 66% (4 out of 6 abalone), respectively. In addition, the 66% mortality rate (4 out of 6 abalone) of the 10 ppm group was delayed by two days in comparison to the 5 ppm group. Statistical analysis showed a significant difference between these mortality curves and the negative control group (uninfected abalone) curve ($P = 0.05$). Histological examination carried out on 12 moribund animals (including 5 positive controls) revealed typical AVG lesions in their neural tissues. The HBSS-injected animals, as well as all other groups of abalone challenged with treated virus (Buffodine® and *Impress*), remained healthy for the duration of the experiment. Histological examination of the target tissues for these animals revealed normal appearance with no AVG lesions. In contrast, calcium hypochlorite was less effective – at 5 ppm added dose (65% active chlorine) there was no effect on abalone mortality.

***Injection trial 2***

Abalone injected (i.m.) with AbHV treated with 5 and 15 ppm of calcium hypochlorite showed mortality rates of 100% ($P = 0.03$). The virus treated with 20 ppm of calcium hypochlorite induced 66% cumulative mortality (Figure 18), however, there was no difference with the accumulated 100% mortality of the positive control (untreated virus) group ($P = 0.44$). Histological examination carried out on 15 moribund animals (including 5 positive controls) revealed typical AVG lesions in their neural tissues. Abalone injected with filtered HBSS solutions (without virus) containing calcium hypochlorite (to determine effect of chemical residue on abalone) all survived and did not show any lesions in their tissues.

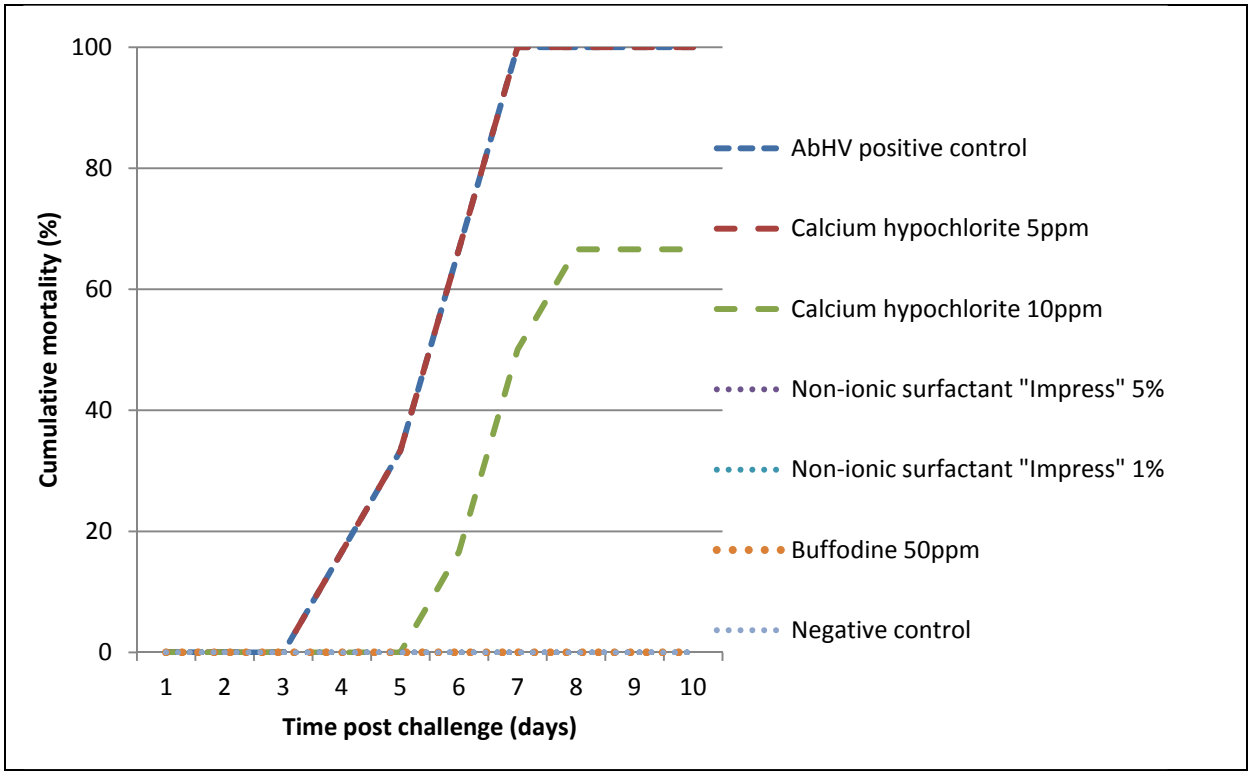


**Figure 17. Abalone herpesvirus inactivation by chemical agents.**

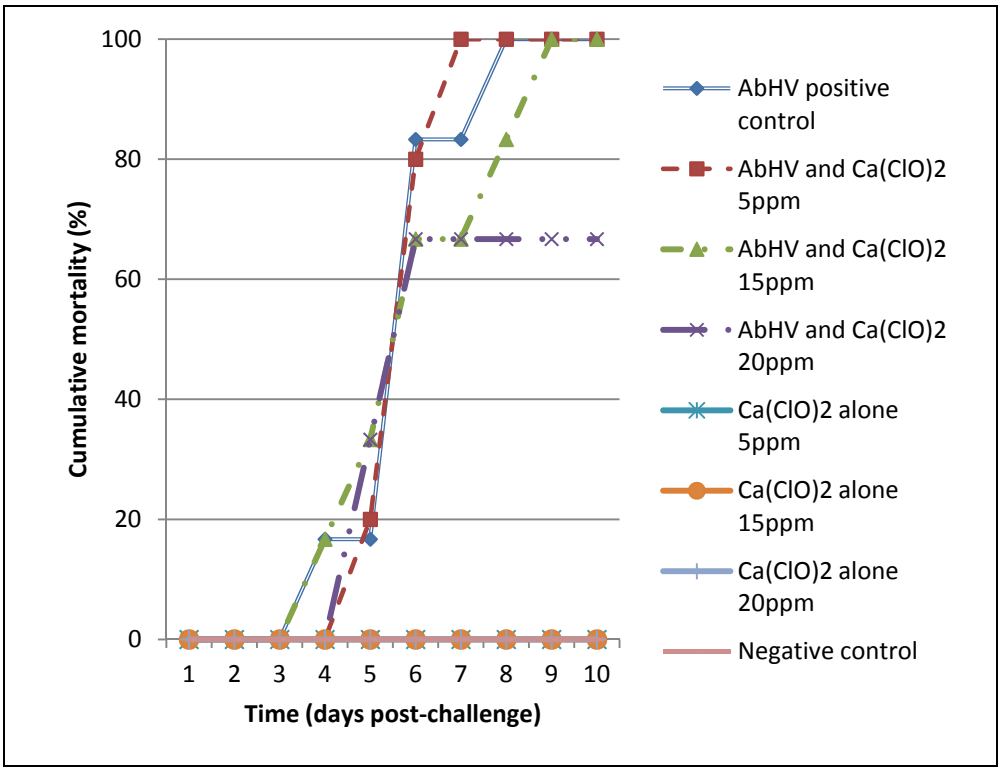


**Figure 18. Abalone herpesvirus inactivation by calcium hypochlorite.**

PCR analysis (Tables 15a and b) demonstrated a correlation between mortality and $C_T$ values.

**Table 15a. Chemical treatments (injection trial 1): TaqMan PCR results summary**

| Chemical treatments | Mean $C_T$ value ± sd in abalone tissues |
|---|---|
| AbHV positive control | 18.4±4.7 |
| Calcium hypochlorite (5ppm) | 19.5±3.0 |
| Calcium hypochlorite (10ppm) | 19.9±10.1 |
| Impress (1%) | 39.0±1.6 |
| Impress (5%) | 34.0±2.8 |
| Buffodine® (50ppm) | 33.0±1.7 |
| Negative control | 38.5±3.2 |

**Table 15b. Chemical treatments (injection trial 2): TaqMan PCR results summary**

| Chemical treatments | Mean $C_T$ value ± sd in abalone tissues |
|---|---|
| AbHV positive control | 18.03 ± 2.2 |
| AbHV + calcium hypochlorite (5ppm) | 15.6 ± 1.9 |
| AbHV + calcium hypochlorite (15ppm) | 14.86 ± 1.5 |
| AbHV + calcium hypochlorite (20ppm) | 22.67 ± 13.0 |

***Immersion trial***

Abalone exposed to untreated infectious water showed a 50% cumulative mortality (Figure 19). In this group, 3 abalone out of 6 showed histopathology typical of AVG. Groups of abalone exposed to infectious water treated with 10 and 15 ppm calcium hypochlorite (1.5 and 2 ppm of residual chlorine, respectively after 15 min exposure) all survived the challenge and did not show histopathological legions in their tissues. Abalone exposed to the calcium hypochlorite treated water only (no virus) all survived and did not show any lesions in their tissues.

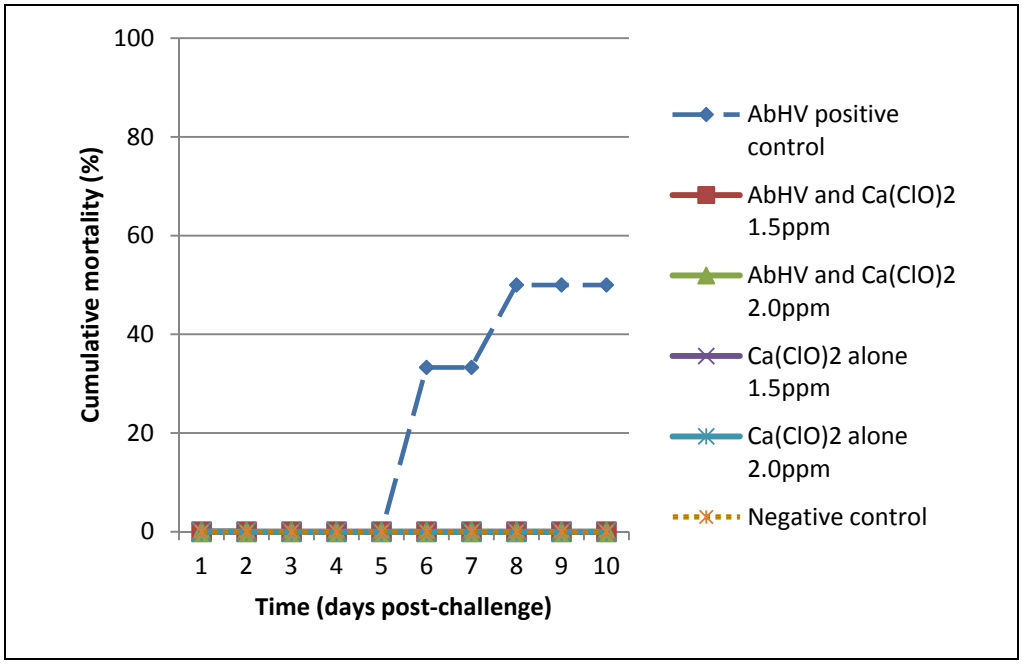


**Figure 19. AbHV pathogenicity in abalone after calcium hypochlorite (residual chlorine) treatment of infectious water.** Groups of abalone were exposed to infectious water treated with 10 and 15 ppm calcium hypochlorite (1.5 and 2 ppm of residual chlorine, respectively after 15 min exposure).

**Objective 4: Determine the role of mucus in viral transmission**

Previous results (section 3.1) demonstrated that virus infectivity is affected by length of time in seawater and by water temperature. The effect of mucus on viral infectivity is studied here to establish whether or not abalone mucus plays a role in the epidemiology of the disease by protecting the virus after being shed in seawater.

Mortality curves (Figure 20) of the abalone exposed by immersion to AbHV, maintained at 4$^{O}$C (positive control group) and 15$^{O}$C (in water alone or in mucus) for three days before challenge, indicated that all treatments led to some mortality. As expected from previous results, virus stored at 4$^{O}$C for 3 days had reduced infectivity (50% mortality) compared with virus that has been used immediately (100% mortality). The infectivity of virus stored in water at 15$^{O}$C was reduced further (37.5% mortality) and virus stored at 15$^{O}$C with mucus produced lower mortality (25%). Unchallenged control abalone remained alive for the duration of the experiment (0% mortality). The results suggest that infectivity of viral particles was not protected by the presence of mucus.

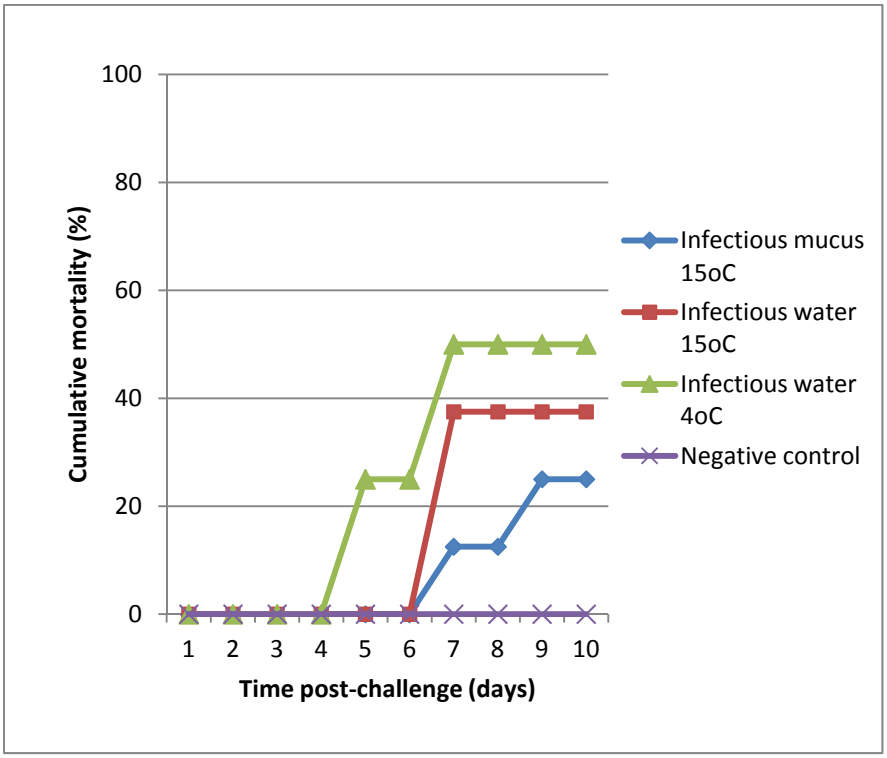


**Figure 20. Mortality curves for abalone challenged with AbHV stored in mucus or water for 3 days at either 4$^{o}$C or 15$^{o}$C.**

**Objective 5: Determine whether a latent stage exists in AVG**

**5.1 Relationship to herpesvirus integrated in the genome of *Branchiostoma floridae* (amphioxus)**

During AbHV Vic1 genome sequencing work conducted as part of this project, Savin et al. (2010) identified the presence of homologues of AbHV open reading frames in the genome sequence of *Branchiostoma floridae* (amphioxus), thus indicating that there is a herpesvirus associated with this invertebrate chordate. The virus discovered in the amphioxus genome is most closely related to AbHV and the OsHV-1, members of the family *Malacoherpesviridae* (Savin et al., 2010) (Figure 1). The virus identified in amphioxus appears to be integrated into the genome as 18 genes were found to be clustered within a 150 kb region of a single amphioxus scaffold. The virus-coding sequences therefore appear to be legitimately assembled within published genome sequence scaffolds and are therefore probably integrated within the amphioxus genome. Discovery of clustered intact herpesvirus genes in

amphioxus suggests an opportunistic integration of the virus has occurred in the amphioxus genome. Herpesviruses within the Alpha- Beta- and Gamma-herpesviridae (Figure 1) have been found to integrate in the genome of their hosts at low frequencies (Morissette & Flamand, 2010) and although integration into host genomes may not be a normal feature of infection and latency, integrated herpesviruses have been shown to play a role in latent stage infections (Morissette & Flamand, 2010).

### 5.2 Presence of viral genomic sequence in the absence of disease

Samples from sites MW1, MW3, MW4 and MC2 (areas where AVG is known to be active), and MC7, MC9, MC10 and ME1 (sites where AVG has not been recorded) tested positive for AbHV in a qPCR assay (Table 16). It should be noted that the results from those sites where AVG has not been recorded previously may be false positives. Samples from one site (201) within the ME1 area (Figure 2) were chosen for further study. In some cases not all tests could be carried out on all samples due to the limited amount of the remaining sample.

**Table 16. Results of qPCR assays targeting ORF49 for the sites along the Victorian coast**

| Map Reference[a] | Number of Sites | No. qPCR-positive Sites | No. qPCR-negative Sites |
|---|---|---|---|
| MW1 | 10 | 3 | 7 |
| MW2 | 9 | 0 | 9 |
| MW3 | 7 | 1 | 6 |
| MW4 | 7 | 2 | 4 |
| MC1 | 5 | 0 | 5 |
| MC2 | 8 | 5 | 3 |
| MC3 | 0 | 0 | 0 |
| MC4 | 6 | 0 | 6 |
| MC5 | 1 | 0 | 1 |
| MC6 | 4 | 0 | 4 |
| MC7 | 6 | 1[b] | 5 |
| MC8 | 0 | 0 | 0 |
| MC9 | 2 | 2[b] | 0 |
| MC10 | 4 | 4[b] | 0 |
| ME1 | 4 | 4[b] | 0 |
| ME2 | 2 | 0 | 2 |
| ME3 | 3 | 0 | 3 |
| ME4 | 3 | 0 | 3 |
| **TOTALS** | **81** | **22** | **58** |

[a]Map references as per Figure 2; [b]Faint positive results ($C_T$ >37.5)

***qPCR detection of AbHV***

Testing of the samples from site 201 using ORF49, ORF66, ORF77 and ORF95 qPCRs found that sample 201-1 tested positive using all qPCRs assays while samples 201-4, 201-6 and 201-8 tested positive in the ORF49 and ORF66 based qPCR assays (Table 17). Samples 201-5 and 201-9 tested positive only by the ORF49 based qPCR assay and 201-8 with the ORF66 based qPCR assay only (Table 17). The $C_T$ values for all samples which tested positive with the qPCR assays were relatively high (>37.5) indicating that the viral load in the samples, if present, was very low (Table 17). The low level of AbHV template DNA in the samples is potentially at the limit of detection of the assays which may have led to the failure of some assays for some samples from site 201 (Table 17).

**Table 17. Extended PCR testing on samples from site 201 within the ME1 map reference (Figure 2)**

| Sample Name | ORF 49 $C_T$ values | ORF66 $C_T$ values | ORF77 $C_T$ values | ORF95 $C_T$ values |
|---|---|---|---|---|
| 201-1 | **38.7** | **37.6** | **40.4** | **34.9** |
| 201-2 | Undetermined[a] | Undetermined | Undetermined | Undetermined |
| 201-3 | Undetermined | Undetermined | Undetermined | Undetermined |
| 201-4 | **40.7** | **39.6** | Undetermined | Undetermined |
| 201-5 | **41.8** | Undetermined | Undetermined | ND |
| 201-6 | **42.1** | **40.5** | Undetermined | ND |
| 201-7 | Undetermined | ND[b] | ND | Undetermined |
| 201-8 | **41.3** | **38.7** | Undetermined | ND |
| 201-9 | **40.3** | ND | ND | ND |
| 201-10 | Undetermined | Undetermined | ND | ND |

[a] Undetermined: $C_T$ value >45.00; [b] ND: Not done – sample not tested

***Nested PCR analysis of samples from site 201***

Use of all nested PCR assays to test samples from site 201 found that only sample 201-1 reproducibly tested positive (Table 18). The $C_T$ values for the nested PCR assays for ORF66 and ORF77 are significantly lower than the standard qPCR results for the same sample (Table 17). The $C_T$ value for ORF95 using the nested PCR protocol is not significantly lower (Table 18) than the standard qPCR result (Table 17). This indicates that the first round PCR for this sample did not amplify AbHV DNA from this sample. Template DNA from the other samples tested negative for the presence of AbHV DNA using the nested PCR. However, of the samples tested only sample (201-4) had previously tested positive for AbHV using the standard qPCR protocol. It is possible that the very low levels of AbHV template DNA in sample 201-4, as indicated by $C_T$ values >39.5 in standard qPCR, is below the level that can be amplified with the first round primers.

**Table 18. Nested PCR testing on samples from site 201 within the ME1 map reference (Figure 2)**

| Sample Name | ORF66 $C_T$ values | ORF77 $C_T$ values | ORF95 $C_T$ values |
|---|---|---|---|
| 201-1 | **12.3** | **16.1** | **33.4** |
| 201-2 | Undetermined | Undetermined | Undetermined |
| 201-3 | Undetermined | Undetermined | Undetermined |
| 201-4 | Undetermined | Undetermined | Undetermined |
| 201-5 | ND[b] | ND | ND |
| 201-6 | ND | ND | ND |
| 201-7 | Undetermined | Undetermined | Undetermined |
| 201-8 | ND | ND | ND |
| 201-9 | ND | ND | ND |
| 201-10 | Undetermined | Undetermined | Undetermined |

[a] Undetermined: $C_T$ value >45.00; [b] ND: Not done – sample not tested

***Sequence analysis of amplicons***

Sequence analysis of amplification products produced confirmed that amplicons from sample 201-1 had 100% sequence identity to the ORF on which the PCR assays were developed confirming that AbHV was present in the sample. This result was obtained for only one out of 10 samples obtained from this area. Further sampling would be required to confirm presence of AbHV at this site.

***Dot blot hybridisation***

The ORF66 probe, also used for ISH (see below), hybridised to DNA from samples 201-1 and 201-4 as well as DNA extracted from two AbHV positive control infected abalone. No hybridisation was detected for DNA extracted from uninfected control abalone or the no template controls (Figure 21).

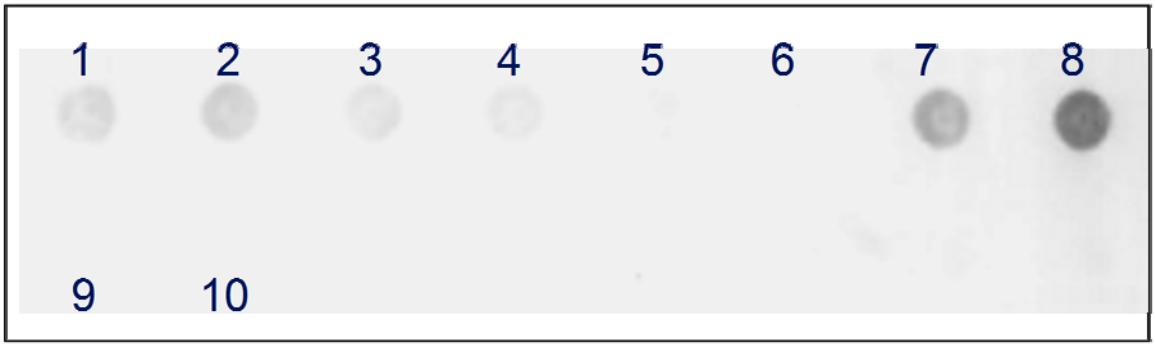


**Figure 21. Dot-blot hybridisation of extracted genomic DNA from abalone with a DIG-labelled ORF66 probe.** 1: Sample 201-1, 2: sample 201-4, 3: Sample 201-1, 4: sample 201-4, 5: Non-AbHV infected abalone 09/4333-2, 6: Non-AbHV infected abalone 09/4333-1, 7: AbHV infected abalone 09/4479-2, 8: AbHV infected abalone 09/4479-1, 9: Buffer negative control, 10: No sample control.

***In situ hybridisation***

*In situ* hybridisation (ISH) was conducted on samples 201-1, 201-3, 201-4, 201-6, 201-8 and 201-9 to identify if the presence of the AbHV could be detected in samples that tested positive for the pathogen using PCR. Testing of other samples from site 201 was not possible as the remaining material for these samples was not sufficient for preparation of blocks for ISH.

Of the samples tested samples 201-1 (Figure 22), 201-8 (Figure 23) and 201-9 tested positive for AbHV and samples 201-3, 201-4 (Figure 24) and 201-6 tested negative. The presence of blue/black staining cells (Figures 20 and 21) indicates the presence of AbHV target DNA in the abalone tissue.

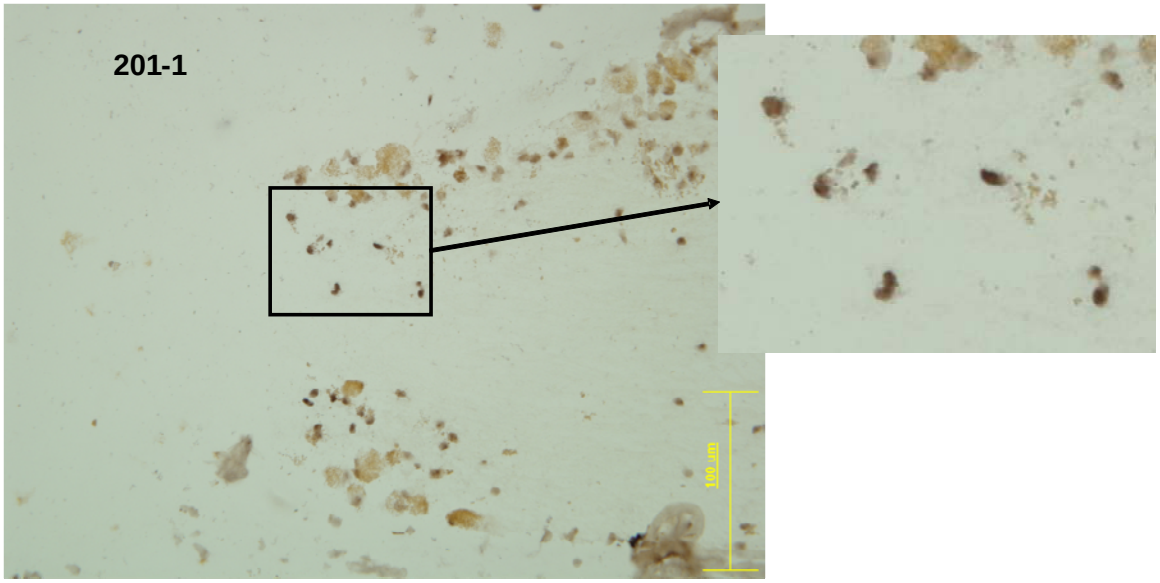


**Figure 22. *In situ* hybridisation of sample 201-1.** Blue-black stained areas indicate the presence of AbHV.

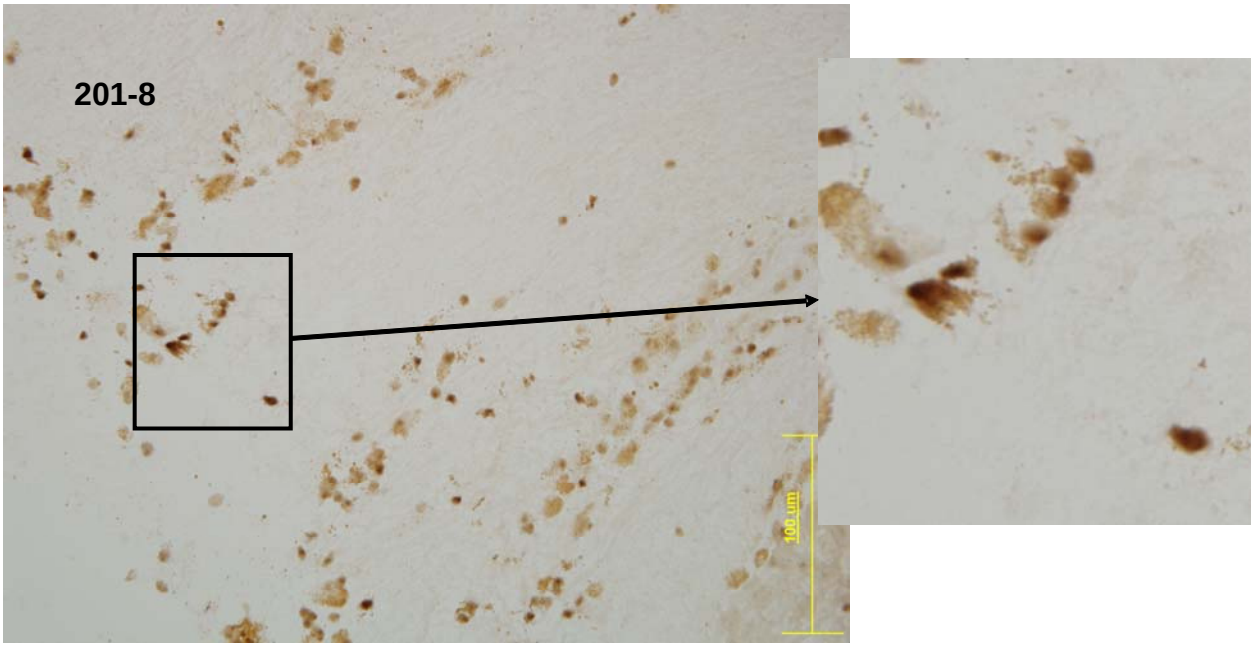


**Figure 23. *In situ* hybridisation of sample 201-8.** Blue-black stained areas indicate the presence of AbHV.

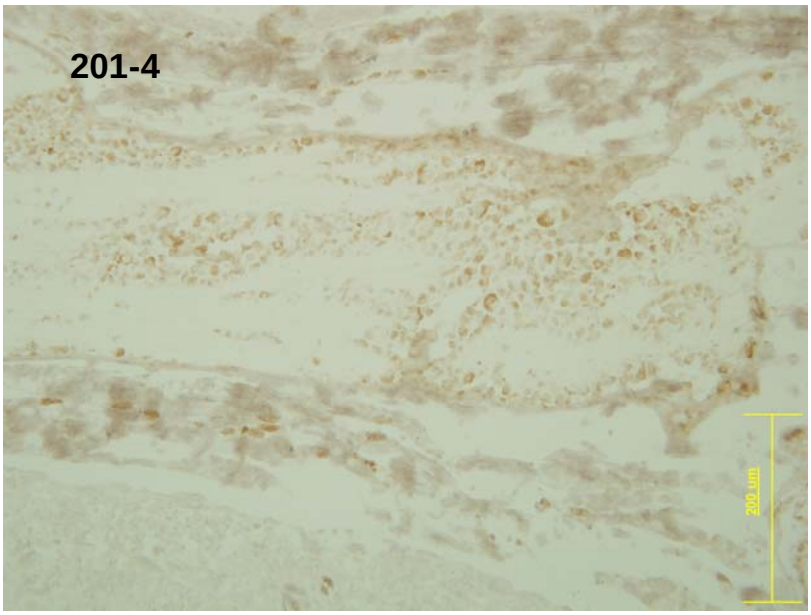


**Figure 24. *In situ* hybridisation of sample 201-4.** Absence of blue-black stained areas indicate the absence of AbHV.

Results of testing samples from site 201 from outside of the area where AVG has been reported suggests that low levels of virus may be present in abalone caught in the area in the absence of detectable disease. However, only one sample, 201-1, from the site tested positive for the presence of AbHV DNA using all methods (Table 19). This site (201-1) was positive for all qPCR assays, including nested qPCR assays (Table 19), as well as the two hybridisation assays used.

The low levels of AbHV DNA template within the DNA extracted from the abalone tissue is indicated by the very high $C_T$ values (Table 19). It is possible that the inability of all tests to detect the presence of AbHV within the extracted DNA is due to the amount of AbHV template within the samples being at the limit of detection of the qPCR assays. In an attempt to improve the sensitivity of the assays nested qPCR assays were developed. However, only sample 201-1 tested positive using these assays, again this is potentially due to the amount of AbHV template in the samples being below the level that can be amplified by the first round PCR.

Unexpectedly sample 201-4 which tested positive using two (ORF49 and ORF66) of the four qPCRs (Tables 17 and 19) was found to be positive on dot blot hybridisation but not on ISH. This failure to detect AbHV DNA using ISH is probably due to the section used not containing any infected cells. Although it is possible that another area of the sample 201-4 may have contained levels of AbHV DNA detectable by ISH the limited amount of sample available for testing makes this impossible to determine. ISH also detected the presence of AbHV in samples 201-8 and 201-9 which were not tested by dot blot analysis and only tested positive by ORF49 qPCR (both samples) and ORF66 qPCR (201-8 only). The use of the ORF95 test did not appear to offer any advantage over the other qPCR tests and is not used for diagnostic purposes (Table 19).

**Table 19. Compilation of results for samples from site 201**

| Sample | qPCR $C_T$ values | | | | Dot Blot | ISH |
|---|---|---|---|---|---|---|
| | ORF49 | ORF66 | ORF77 | ORF95 | | |
| 201-1 | 38.71 | 37.57 | 40.35 | 34.9 | + | + |
| 201-2 | ND[b] | ND | ND | Undetermined[a] | ND | ND |
| 201-3 | ND | ND | ND | Undetermined | ND | - |
| 201-4 | 40.65 | 39.55 | Undetermined | Undetermined | + | - |
| 201-5 | 41.81 | Undetermined | Undetermined | ND | ND | ND |
| 201-6 | 42.12 | 40.53 | Undetermined | ND | ND | - |
| 201-7 | ND | ND | ND | Undetermined | ND | ND |
| 201-8 | 41.28 | 38.68 | Undetermined | ND | ND | + |
| 201-9 | 40.28 | ND | ND | ND | ND | + |
| 201-10 | ND | ND | ND | ND | ND | ND |

[a] Undetermined: $C_T$ value >45.00; [b] ND: Not done – sample not tested

### 5.3 Identification of genes associated with latency

Within the assembled 211518 bp AbHV Vic1 genome sequence there are 112 different predicted ATG-initiated open reading frames (ORFs). Within these ORFs, two different homologues of baculovirus Inhibitor of Apoptosis Proteins (IAPs) have been recognised. Coding sequences ORF1 and ORF79 are evolutionarily most closely related to baculovirus and eukaryote genes encoding the IAP. Both ORF1 and ORF79 contain protein domains corresponding to pfam00653 and similar motifs and so are highly likely to represent the IAP protein (Figures 25 and 26). These ORFs are similar to genes encoded by the OsHV-1 (Davison et al., 2005) and a recently sequenced, but unpublished, Scallop herpesvirus genome (Ren *et al*., 2013). Genes inhibiting apoptosis in herpesviruses have been linked to an ability to become latent and to subsequently recover from latency to generate a productive infection (Kent et al., 2003). Therefore the presence of a family of IAP genes suggests AbHV has the capacity to exist in a latent form.

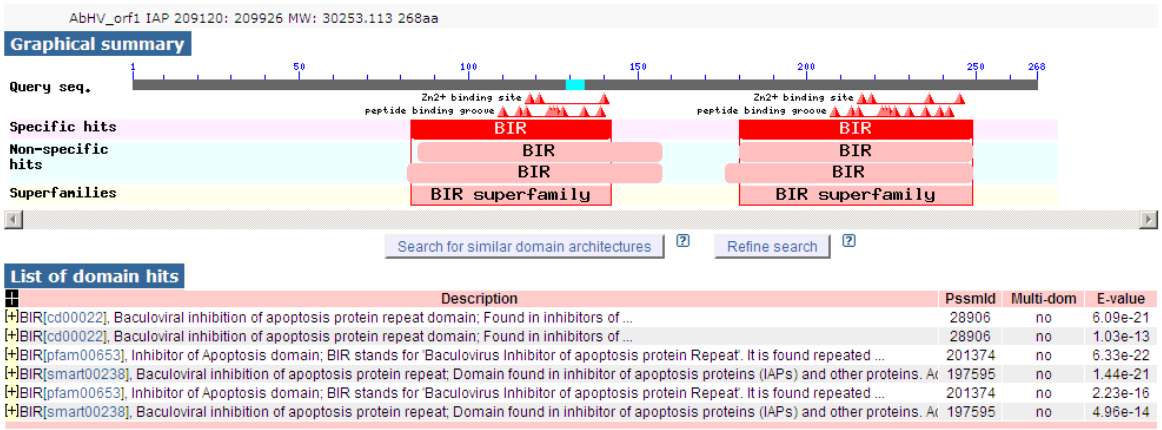


**Figure 25. Conserved domain database matches for ORF1.**

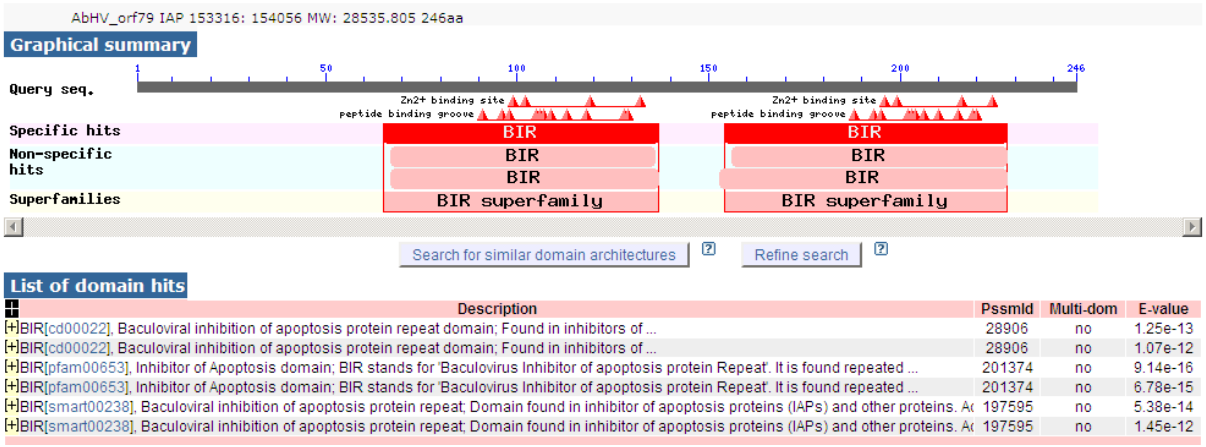


**Figure 26. Conserved domain database matches for ORF79.**

## Objective 6: Determine the susceptibility of remnant populations of abalone previously exposed to AVG

For infectivity trials the preferred method used for infecting abalone is by bath immersion rather than intramuscular injection. This more natural exposure ensures that the abalone's natural defences would not be circumvented by direct injection. This methodology was used for determining the susceptibility of abalone sourced from previously exposed reefs in Victorian waters, as well as samples of abalone and other mollusc populations sourced from South Australia.

### 6.1 Susceptibility of remnant populations of abalone previously exposed to AVG

The results presented here are for one of five populations tested in total. FRDC Project 2009/075 (Crane et al., 2012; 2013) reported on the other four populations. Samples of the abalone population tested here were obtained from Warrnambool Breakwater (coordinates 38 24 209S 142 28 627E), at a depth of 5 metres and a water temperature of 13.3°C.

Mortality curves (Figure 27) demonstrate that abalone (juvenile and mature) from this site were susceptible to infection with AbHV Vic1 and AVG. Moreover, the mortalities correlated with PCR analysis (Table 20). The $C_T$ values reflect the mortality rates for each group. In those groups with 100% mortality (e.g. high dose experimental groups) the $C_T$ values were consistently low indicating that all abalone had a high-level infection. In groups that did not demonstrate 100% mortality the surviving abalone had relatively high $C_T$ values indicating low-level or no infection. The abalone that died in these groups had low $C_T$ values indicating high-level infection rates. As expected, abalone in the uninfected control groups had high $C_T$ values (with two exceptions which could be due to laboratory contamination).

**Table 20. Summary of Results of Abalone exposed to Abalone Herpesvirus**

| Experimental group | Range in mean $C_T$ values and mortalities | | | |
|---|---|---|---|---|
| | High dose | Medium dose | Low dose | Uninf. Co. |
| GSW controls<br>Mortality at day 10 | 12.5-14.2<br>100% | 13.8-18.9<br>100% | 11.5-36.6<br>80% | 35.7->45.0<br>0% |
| Wild mature<br>Mortality at day 10 | 13.7-18.2<br>100% | 13.6-20.0<br>100% | 12.1-45.0<br>40% | 36.5->45.0<br>0% |
| Wild juvenile<br>Mortality at day 10 | 10.9-15.9<br>100% | 11.0-14.9<br>90% | 12.8-38.4<br>40% | 29.2-45.0*<br>0% |

*Two abalone in this group had $C_T$ values <30.0 and were therefore suspect positives. The samples were retested using ORF66 qPCR and resulted in similar $C_T$ values. Whether these results indicate low level infection or false positives (e.g. laboratory contamination) is unknown.

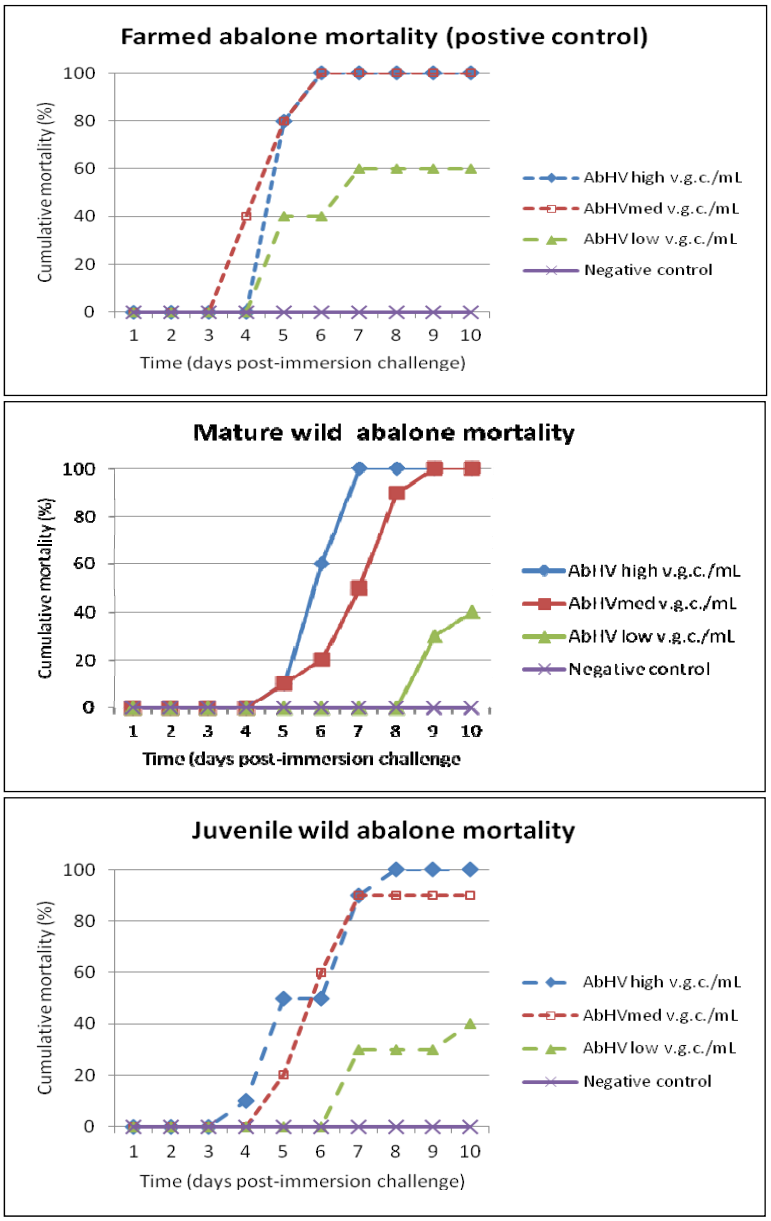


**Figure 27. Mortality curves for GSW abalone (positive controls), mature wild abalone and juvenile wild abalone (from Warrnambool Breakwater).**

Where possible (in some groups abalone died before suitable samples could be taken for histology) moribund abalone were processed for histology. Representative abalone from each of the groups were examined for lesions. Uninfected control abalone showed no histopathology. Abalone obtained from virus challenged groups showed lesions typical of AVG (results not shown).

Taken together with the results from Project No. 2009/075, these results indicate that the sampled abalone do not demonstrate resistance to infection/disease (Figure 28). The occasional relatively low $C_T$ value (<30.00) obtained from the uninfected wild abalone (Table 20) may suggest a low level (sub-clinical?) of infection in abalone in the wild but this would need to be investigated further for confirmation. Alternatively, these results could represent false positives due to cross-contamination.

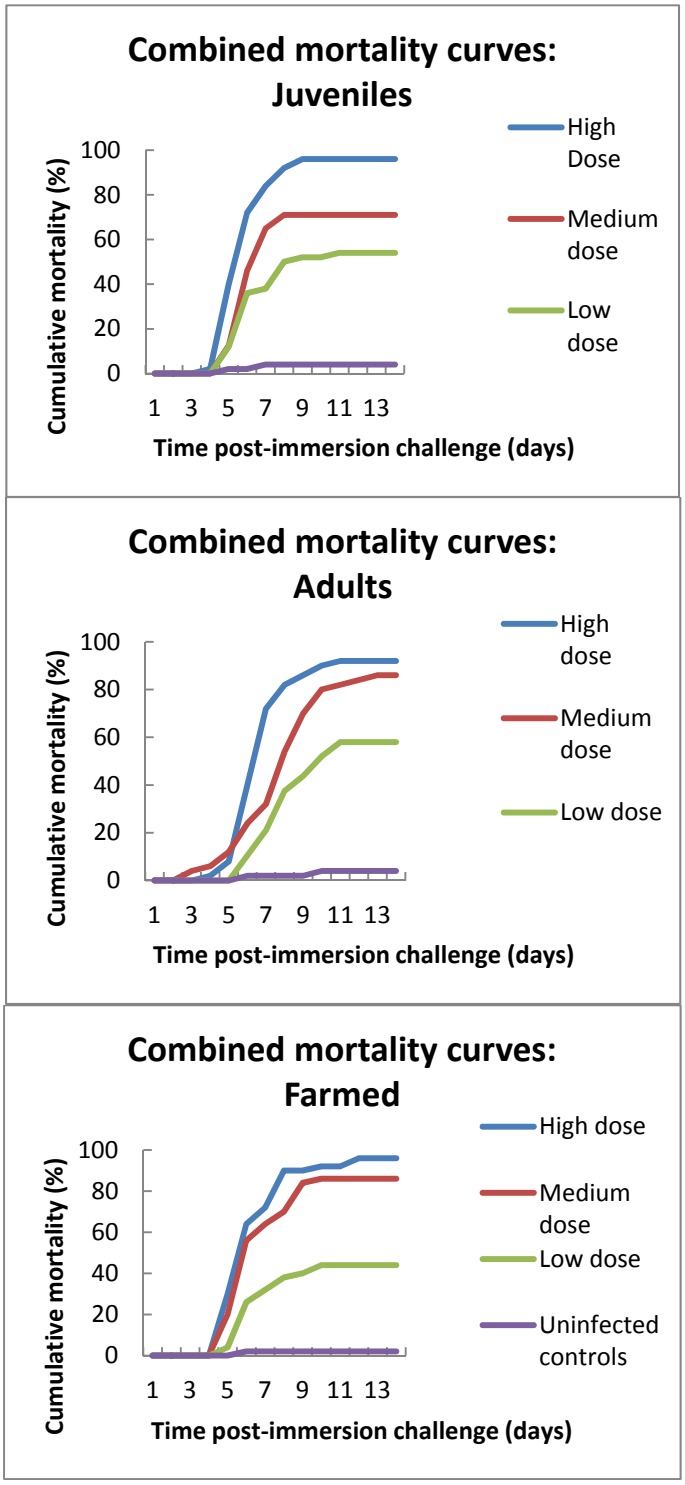


**Figure 28. Combined mortality curves for wild-caught juvenile, wild-caught adult (from all 5 locations – see Project 2009/075) and farmed abalone**

### 6.2. Susceptibility of known unexposed populations in South Australia

Abalone sourced from different locations within South Australia were exposed to ABHV Vic1 to determine their susceptibility to infection and AVG. Since abalone sourced from South Australia had not been used previously, experimental groups were exposed to a dilution series of AbHV to ensure that a range of doses was evaluated.

### 6.2.1 SA farmed greenlip abalone

Greenlip abalone sourced from South Australian aquaculture facilities were shown to be susceptible to infection and disease (Figure 29).

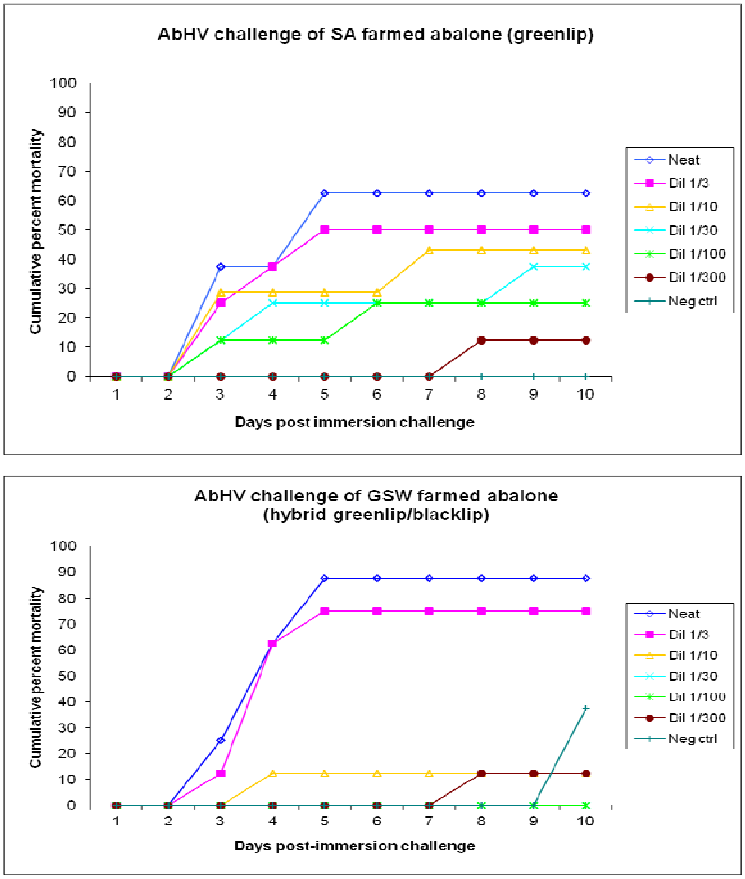


**Figure 29. Susceptibility of South Australian farmed greenlip abalone to infection with AbHV and AVG.**

It is interesting to note that the greenlip appeared to be more resistant than GSW farmed hybrids to the higher doses of virus. Further studies would need to be undertaken to confirm this preliminary observation.

### 6.2.2 SA wild blacklip abalone

Samples of wild blacklip abalone were sourced from two areas (DB and GB) within South Australian waters and exposed to AbHV Vic1. This species of abalone from South Australia were also shown to be susceptible to infection and disease (Figure 30). It is noted that the uninfected control groups demonstrated relatively high levels of mortality. These mortality rates were most probably due to the presence of excessive biofouling of the abalone shell causing water quality problems in the experimental aquaria. In subsequent experiments using wild-caught abalone (such as those from previously exposed reefs in Victorian waters) we have ensured that the abalone are “cleaned” of the biofouling and this has eliminated the problem.

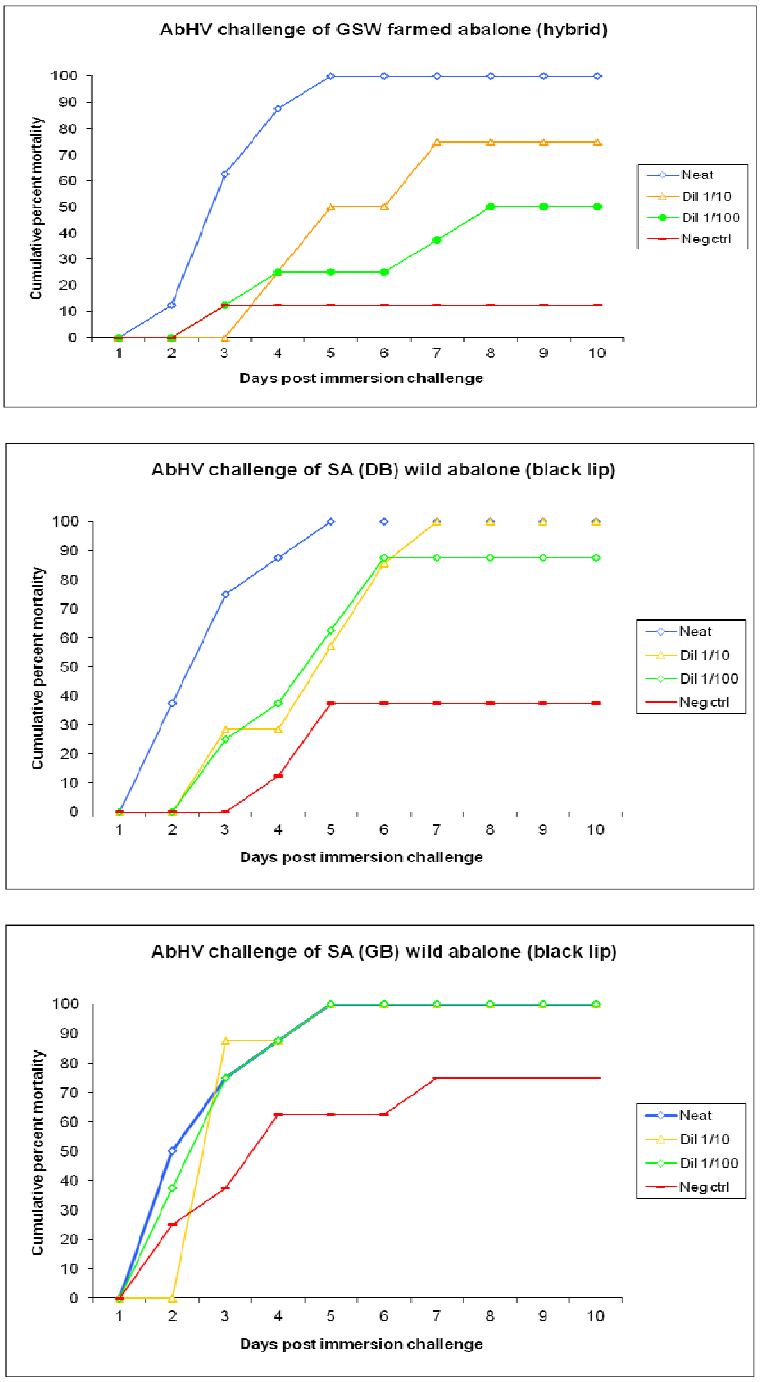


**Figure 30. Susceptibility of South Australian wild blacklip abalone to infection with AbHV and AVG.**

### 6.2.3 Susceptibility of Mollusc species to AbHV infection

It was agreed that, since the susceptibility to AVG of greenlip and blacklip abalone sourced from South Australia had already been studied as part of an independent investigation (results provided above for completeness), it would be more appropriate to extend this study to other species of abalone and molluscs from South Australia.

GSW hybrid abalone were used as positive controls and exposed to the high dose of virus to ensure that the viral batch and dose of AbHV used for these experiments was infectious and pathogenic. Exposure of GSW hybrid abalone resulted in 100% mortality by 5 days post-exposure, typical of the AbHV stocks used for other *in vivo* infectivity trials.

#### 6.2.3.1 *Haliotis roei*

Infectivity trials indicated that *H. roei* (using both juvenile and adult specimens) is susceptible to AVG. The mortality curves (results not shown) reached a "plateau" at 6 to 9 days post-exposure which is similar to that for hybrid abalone when exposed to similar viral doses. Similarly, PCR analysis correlated with mortalities; moribund/dead abalone had in general low $C_T$ values demonstrating high levels of viral DNA in their tissues. In addition, many unchallenged negative control *H. roei* that survived the trial and were sacrificed at the end of the experiment (day 10) had $C_T$ values in the low 30s (and one at 28.86) which would qualify them as positive (infected). However, these negative control abalone (those tested) were negative by ISH and histopathology. The positive PCR results for the negative control abalone (if real) could either be due to (1) cross-contamination, (2) these *H. roei* samples were from sub-clinical carriers of AbHV when collected from the wild. This second option would seem unlikely since AbHV (or AVG) has not been detected in any other susceptible abalone species in South Australia. Further investigation (beyond the scope of this project) is required for clarification.

#### 6.2.3.2 *Turbo undulatus*

Immersion challenge of *T. undulatus* with AbHV did not lead to any mortality within the 10-days duration of the experiment. Surviving snails were negative by the AbHV ORF49 qPCR assay and no histopathology was observed in tissue sections.

#### 6.2.3.3 *Mimichlamys bifrons*

Scallops (*Mimichlamys bifrons*) showed mortality during the acclimation period, beginning 24 hours after arriving at AAHL. Of the 14 remaining scallops that were challenged only 4 survived the duration (10-days) of the experiment. Animals were negative by the TaqMan qPCR test. No histopathology was seen in tissue sections of the exposed scallops. Thus it is likely that the scallops died for reasons other than AVG.

From these preliminary results it is concluded that the marine snail *Turbo undulatus* and the scallop *Mimichlamys bifrons* are not susceptible to infection/disease caused by AbHV. However, further studies are required to confirm this. In contrast, it was clearly demonstrated that *Haliotis roei* was as susceptible to AbHV infection/AVG as *Haliotis rubra, H. laevigata* and the *H. rubra X H. laevigata* hybrid.

**Objective 7: Using all three available qPCR tests, determine their relative sensitivities and specificities by re-testing previously collected samples from the abalone populations in Tasmania**

All 1,459 specimens collected previously during surveillance activities in Tasmania were re-tested with the three qPCR assays (ORF49, ORF66, and ORF77). The outputs of the different combination of test results used for the analysis are summarized in Table 21.

Using the open-source software OpenBUGS v.3.2.2 (www.openbugs.net), LCM analyses were conducted with a Bayesian framework to allow modelling flexibility. An initial burn-in of 10,000 iterations was used to allow convergence, and the subsequent 50,000 iterations were used for estimations and inferences. Markov chain conversion was assessed visually. Conditional dependence among the qPCR assays was investigated since these tests target a similar analyte (Gardner et al., 2000). Separate LCMs were run by including conditional covariance factors between two PCRs at the time (models 2-4, Table 22). A model without conditional dependence (conditional independence) was run for comparison (models 1, Table 22). Model comparison and improvement was conducted using the deviance information criterion (DIC) (the smaller the DIC, the better is the model).

**Table 21. Summary count for the 16 possible combinations of positive/negative (+/-) test results collected from histopathology and the three qPCR assays, for each of the 5 source populations**

| Histology | ORF49 | ORF66 | ORF77 | Source 1 | Source 2 | Source 3 | Source 4 | Source 5 | Total |
|---|---|---|---|---|---|---|---|---|---|
| + | + | + | + | 0 | 1 | 0 | 0 | 0 | 1 |
| + | + | + | - | 0 | 0 | 0 | 0 | 0 | 0 |
| + | + | - | + | 0 | 0 | 0 | 0 | 0 | 0 |
| + | + | - | - | 0 | 4 | 0 | 0 | 0 | 4 |
| + | - | + | + | 0 | 0 | 0 | 0 | 0 | 0 |
| + | - | + | - | 0 | 0 | 0 | 0 | 0 | 0 |
| + | - | - | + | 0 | 0 | 0 | 0 | 0 | 0 |
| + | - | - | - | 0 | 0 | 0 | 0 | 0 | 0 |
| - | + | + | + | 11 | 8 | 0 | 0 | 1 | 20 |
| - | + | + | - | 18 | 7 | 0 | 0 | 0 | 25 |
| - | + | - | + | 6 | 0 | 0 | 0 | 0 | 6 |
| - | + | - | - | 16 | 8 | 0 | 0 | 0 | 24 |
| - | - | + | + | 1 | 5 | 7 | 0 | 0 | 13 |
| - | - | + | - | 4 | 15 | 1 | 0 | 0 | 20 |
| - | - | - | + | 3 | 2 | 3 | 0 | 0 | 8 |
| - | - | - | - | 644 | 390 | 127 | 7 | 170 | 1338 |
| | | | **Totals** | **703** | **440** | **138** | **7** | **171** | **1459** |

| | | Histology | | ORF49 | | ORF66 | | ORF77 | | Covariance 49/66 | | Covariance 49/77 | | Covariance 66/77 | | Covariance 49-66/77 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Model** | **CD** | DSe | DSp | DSe | DSp | DSe | DSp | DSe | DSp | DSe | DSp | DSe | DSp | DSe | DSp | DSe | DSp |
| 1 | No | 4.83% | 99.95% | 61.39% | 99.31% | 65.55% | 99.77% | 39.22% | 99.75% | | | | | | | | |
| 2 | Yes | 5.47% | 99.92% | 63.07% | 99.07% | 68.59% | 99.56% | 40.35% | 99.57% | -0.033 | 0.002* | - | - | - | - | - | - |
| 3 | Yes | 5.30% | 99.92% | 64.12% | 99.24% | 65.36% | 99.47% | 40.96% | 99.71% | - | - | -0.026 | 0.001* | - | - | - | - |
| 4 | Yes | 6.57% | 99.93% | 85.56% | 99.71% | 59.18% | 98.10% | 33.88% | 98.65% | - | - | - | - | 0.054* | 0.008* | - | - |
| 5 | Yes | 5.24% | 99.92% | 55.17% | 98.49% | 59.14% | 99.19% | 19.24% | 97.88% | - | - | 0.021 | 0.041* | 0.010 | 0.012* | - | - |
| | | | | *ORF49 and ORF66 combined in parallel* | | | | | | | | | | | | | |
| 6 | Yes | 5.48% | 99.92% | Dse = 80.63% | | DSp = 98.14% | | 22.14% | 98.15% | - | - | - | - | - | - | 0.013 | 0.518* |
| 7 | Yes | 5.83% | 99.92% | Dse = 85.96% | | DSp = 98.16% | | 23.34% | 98.18% | - | - | - | - | - | - | - | 0.013 |

* Significantly different from 0 at the 5% level.

**Prevalence**

| **Model** | **CD** | Source Pop. 1 | Source Pop. 2 | Source Pop. 3 | Source Pop. 4 | Source Pop. 5 | **Bayes-P** | **DIC** |
|---|---|---|---|---|---|---|---|---|
| 1 | No | 7.93% (5.60-10.70%) | 11.14% (7.80-15.00%) | 8.05% (3.90-14.00%) | 9.0% (0.40-40.70%) | 1.04% (0.20-3.40%) | 0.059 | 180.1 |
| 2 | Yes | 7.37% (4.72-10.19%) | 10.81% (7.47-14.54%) | 7.57% (3.38-13.25%) | 11.66% (0.32-8.74%) | 1.22% (0.14-3.37%) | 0.066 | 181.0 |
| 3 | Yes | 7.78% (5.28-10.56%) | 10.60% (6.61-14.77%) | 8.30% (3.94-13.99%) | 11.98% (0.35-0.30%) | 1.23% (0.14-3.43%) | na | 182.8 |
| 4 | Yes | 7.74% (5.54-10.44%) | 8.18% (4.75-13.58%) | 1.71% (0.03-8.87%) | 11.61% (0.33-8.87%) | 1.20% (0.14-3.35%) | 0.105 | 167.8 |
| 5 | Yes | 8.02% (5.05-11.39%) | 12.34% (6.24-17.96%) | 5.20% (0.19-12.43%) | 13.76% (0.38-6.20%) | 0.84% (0.02-2.99%) | 0.080 | 169.0 |
| 6 | Yes | 8.75% (4.20-22.2%) | 12.97% (6.90-32.70%) | 5.03% (0.20-15.5%) | 14.44% (0.40-53.5%) | 0.93% (0.00-3.70%) | 0.242 | 83.77 |
| 7 | Yes | 7.38% (4.21-10.84%) | 10.97% (7.03-15.83%) | 4.39% (0.22-11.75%) | 12.73% (0.36-3.24%) | 0.77% (0.02-2.79%) | 0.263 | 85.26 |

**Table 22. Summary table of the Latent Class Models estimates of test diagnostic sensitivity (DSe) and specificity (DSp) and source population prevalence with and without conditional dependence (CD) among tests**

Substantial and significant conditional dependence was found between the ORF77 qPCR and the two other qPCRs (Table 22). Thus, the final LCM included conditional dependence between these two pairs of tests (model 5, Table 22). The estimates of DSe and DSp and their posterior 95% credibility intervals are presented in Table 23.

Given that the intended purpose of the developed assays is to screen apparently healthy abalone populations to demonstrate freedom from infection, the method of choice should be the assay with the highest DSe or the lowest likelihood ratio of a negative test result (LR-) (i.e. lowest chance that a negative test result is a false negative). The qPCR ORF66 should therefore be the single test of choice for surveillance. It was noted that the DSe and DSp of qPCR ORF49 is only slightly less than those of ORF66 (Table 23). However, the ORF49 test has specificity limits i.e. it does not detect all known genetic variants of AbHV (see Table 8a).

**Table 23. Diagnostic sensitivity, specificity, and respective likelihood ratios of the 4 tests and respective covariance in the infected and non-infected abalone.** LR+ = DSe/(1-DSp); LR- = (1-DSe)/DSp.

| Assays | Diagnostic Sensitivity (DSe) | Diagnostic Specificity (DSp) | Likelihood ratio of a positive test result (LR+) | Likelihood ratio of a negative test result (LR-) |
|---|---|---|---|---|
| Histology | 5.24% (1.80-10.89%) | 99.92% (99.71-100.0%) | 65.50 | 0.948 |
| RT-PCR ORF49 | 55.17% (34.30-88.04%) | 98.49% (97.03-99.68%) | 36.54 | 0.455 |
| RT-PCR ORF66 | 59.14% (43.57-75.67%) | 99.19% (97.45-99.98%) | 73.01 | 0.412 |
| RT-PCR ORF77 | 19.24% (4.67-39.33%) | 97.88% (96.31-99.44%) | 9.08 | 0.825 |
| | | | | |
| Covariance 49-77 | 0.021 (-0.048-0.074) | 0.041* (-0.014-0.093) | | |
| Covariance 66-77 | 0.010 (0.001-0.022) | 0.012* (0.001-0.024) | | |

* Significantly different from 0 at the 5% level.

Given that the ORF49 qPCR appeared to have a better analytical sensitivity and subsequent DSe with Tas1 virus type (i.e. the test can detect lower Tas1 virus loads) and that ORF66 qPCR detects all other virus types, it was considered possible that a combination of these two assays would improve the overall detection capacity. Any specimen testing positive to either or both assays would be deemed positive (i.e. interpretation **in parallel**). It is possible to predict the DSe and DSp of the two assays interpreted in parallel using the following formulae:

$$DSe_{49/66} = 1 - [(1-DSe_{49}) * (1-DSe_{66})] = 81.7\% \qquad DSp_{49/66} = DSp_{49} * DSp_{66} = 97.7\%$$

Using the two tests in parallel, where a positive result in either test is considered positive, would provide a substantial increase in DSe and a slight loss in DSp except for those areas where Tas2 and Tas3 are prevalent. The corresponding LR- (0.187) is greatly reduced which supports using the assay combination for screening apparently healthy abalone populations. To confirm this expectation, additional LCMs were run where the test result for ORF49 and ORF66 qPCRs were interpreted in parallel (models 6-7, Table 22). Conditional dependence between ORF77 and the ORF49/66 combination was included in the model for the infected and the non-infected abalone (model 6) and for the non-infected abalone only (model 7). For parsimony reasons (only minor increase in DIC), it was decided to use model 7 with less parameters as the final model (conditional dependence in non-infected abalones only). The estimates of DSe and DSp and their posterior 95% credibility intervals are presented in Table 24.

**Table 24. Diagnostic sensitivity, specificity, and respective likelihood ratios of the 3 tests and respective covariance in the infected and non-infected abalone.** LR+ = DSe/(1-DSp); LR- = (1-DSe)/DSp.

| Assays | Diagnostic Sensitivity (DSe) | Diagnostic Specificity (DSp) | Likelihood ratio of a positive test result (LR+) | Likelihood ratio of a negative test result (LR-) |
|---|---|---|---|---|
| Histology | 5.83% (1.99%-11.92%) | 99.92% (99.72%-100.0%) | 72.9 | 0.942 |
| qPCR ORF49/66 | 85.96% (57.91%-99.45%) | 98.16% (96.03%-99.87%) | 46.7 | 0.143 |
| qPCR ORF77 | 23.34% (4.10%-42.22%) | 98.18% (96.34%-99.82%) | 12.8 | 0.781 |
| | | | | |
| Covariance 49/66-77 | na | 0.013 (0.000-0.029) | | |

The parallel combination of ORF49/66 qPCRs provides a much better fit for screening when compared to any of the tests used individually. The confidence on a negative test result (negative predictive value) will be substantially higher with the qPCR ORF49/66 combination regardless of the suspected prevalence (Figure 31).

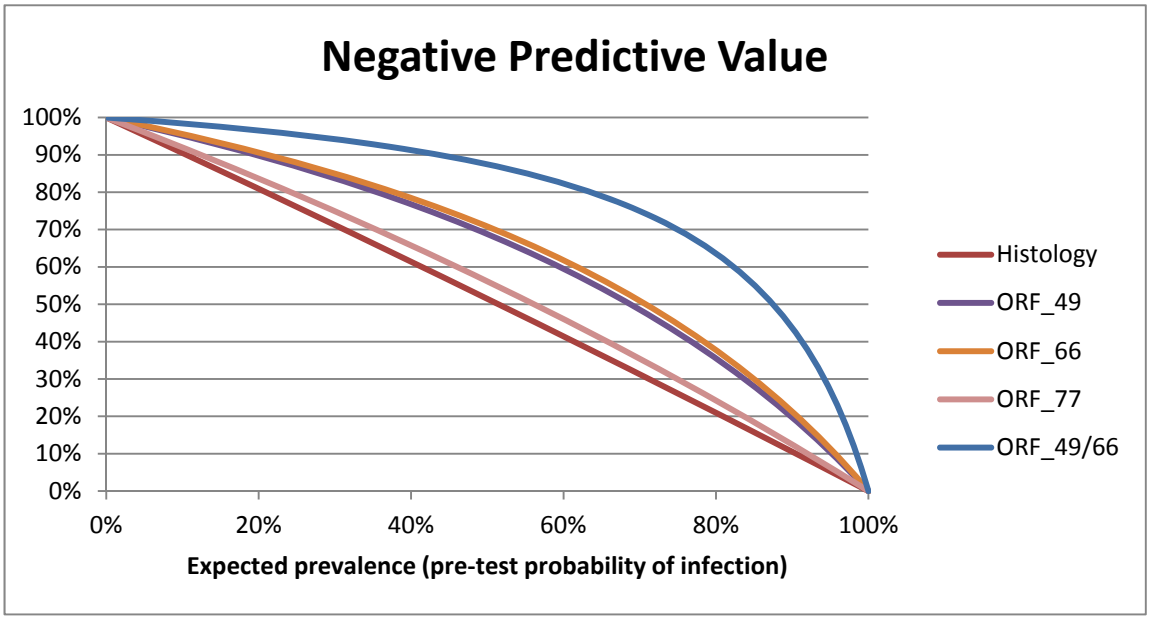


**Figure 31. Negative Predictive Value (probability of an abalone to be truly non-infected if tested negative) for the 5 studied tests according to the expected prevalence of infection before running the test.**

# Discussion

**Objective 1: Validate the developed *in situ* hybridisation diagnostic test including roll out to other States**

The *in situ* hybridisation (ISH) protocol was optimised using tissue sections from abalone previously diagnosed to have no infection (negative population) or a high level of infection (positive population). The ISH test has been shown to only produce a positive signal in confirmed AVG positive samples and all confirmed negative samples have tested negative using the ISH test.

The AbHV-probe was also confirmed to specifically hybridise to DNA extracted from abalone infected with AbHV by using dot blot hybridisation. The AbHV-probe hybridised to the two AbHV samples which were confirmed as being positive for the presence of the pathogen by real-time PCR analysis. No hybridisation signal was detected with DNA from other related herpesviruses or iridoviruses or DNA from abalone which were negative for the presence of the pathogen by qPCR, indicating that the probe sequence is highly specific for AbHV.

The procedure has been included in the chapter, "Infection with abalone herpesvirus", of the *OIE Manual of Diagnostic Tests for Aquatic Animals*, 2014.

**Objective 2: Develop a quantitative assay (qPCR) for determining infectious dose for this virus**

Using any of the three qPCR tests (ORF49, ORF66, ORF77) in conjunction with a standard curve generated with the relevant plasmid positive control, it is possible to quantify the amount of target DNA in abalone samples. The calculated figure represents genome equivalents (assuming one copy of the target sequence per genome) rather than the number of infectious virus particles. Nevertheless, in the absence of abalone cell lines susceptible to infection by AbHV, this metric provides a useful quantitative measure for a variety of applications.

**Objective 3: Determine the sensitivity of the virus to physico-chemical conditions including its stability in water/on fomites and its sensitivity to inactivation agents**

Based on the results of the current study, the stability of AbHV appears to be typical of other herpes viruses. For example, based on disease expression, AbHV infectivity was reduced to 75% after 1 day in seawater at 15$^{O}$C, to 37.5% after 3 days and to 0% at 5 days. At 25$^{O}$C the reduction in infectivity was more pronounced (e.g., 12.5% after 1 day). In addition, AbHV was inactivated by the various chemical agents (calcium hypochlorite, Buffodine®, *Impress*) used in this study.

**Objective 4: Determine the role of mucus in viral transmission**

While it has been shown that virus is contained within the mucus shed from infected abalone, the results obtained here indicate that mucus does not provide any additional protection for the virus i.e. infectivity is not prolonged for virus in mucus maintained at ambient temperature.

**Objective 5: Determine whether a latent stage exists in AVG**

Three lines of research have suggested that AbHV may latently and/or sub-clinically infect abalone: Firstly, the relationship of the virus to other herpesviruses, secondly the detection of AbHV genomic sequences (qPCR positive results from one abalone sample (201-1) obtained in Victoria – high $C_T$ values that could not be confirmed by other methods and could be a false positive) in abalone in the absence of disease, and finally the identification of anti-apoptotic genes within the AbHV genome. The one abalone that returned positive qPCR results originated from an area that has not experienced any disease. Whether or not this represents a latent or sub-clinical infection, or false positive results, is not known.

The situation in Tasmania is different; in several of the disease incidences in Tasmania, wild-caught abalone did not show any disease signs until they were transferred to live holding facilities in processing plants. Following transfer and presumably due to less-than-optimal conditions (involving stress factors such as poor water quality), the abalone showed clinical signs including mortality. Subsequent laboratory investigation confirmed the presence of histopathology typical of AVG, and the presence of virus by PCR analysis. Thus the absence of disease in wild populations in Tasmania followed by disease outbreaks in harvested abalone from these populations when transferred to live holding facilities provides good

evidence for the presence of latent/sub-clinical infection in the wild.

Thus an important objective (to determine the diagnostic specificity and diagnostic sensitivity of the qPCR tests for detection of sub-clinical infections) was added during the course of this project (Objective 7) which required additional resources and time to complete (see below).

**Objective 6: Determine the susceptibility of remnant populations of abalone previously exposed to AVG and known unexposed wild populations in South Australia**

The results from this study and FRDC Project 2009/075 demonstrated clearly that wild abalone sourced from areas previously exposed to the virus were susceptible to infection/AVG and do not demonstrate detectable levels of resistance (Crane et al., 2013).

**Objective 7: Using all three available qPCR tests, determine their relative sensitivities and specificities by re-testing previously collected samples from the abalone populations in Tasmania (additional objective added during the project)**

The diagnostic target for this study was an abalone (any species) from an apparently healthy population (no disease previously reported or present) infected with/carrying AbHV regardless of the virus type/strain (i.e. all previously identified Tasmanian variants).

The analysis demonstrated that the combination of the ORF49 and ORF66 qPCR tests provided the highest diagnostic sensitivity. The combination of the two tests (ORF49 and ORF66) should be considered as one unique test, the diagnostic sensitivity of which was determined to be approximately 86% using abalone from Tasmania (representing the mixture of Tasmanian “genotypes” in the study population). Therefore, so as long as the study population is a representation of what will be tested in the future, the combined sensitivity applies.

In conclusion, if the two tests are interpreted in parallel the combined sensitivity should be used regardless of the assumed Tasmanian genotype as this estimate is a weighted average of the sensitivity across the mix of Tasmanian genotypes in the study population. The caveat is that the study population represents the true target population of infected abalone in terms of Tasmanian genotypes.

Based on the results of this analysis, for surveillance purposes, it is recommended that the ORF49 and ORF66 qPCR tests are used in parallel to enhance DSe (ca. 86%). Diagnostic specificity is high for all tests (>98%).

# Conclusion

This project has made significant contributions to our knowledge about important aspects of abalone herpesvirus and the associated disease, abalone viral ganglioneuritis. This new knowledge will be of relevance to industry, government regulators, diagnostic laboratories and researchers interested in abalone diseases.

Firstly, the validated ISH method is available to all diagnostic laboratories both within Australia and internationally (Objective 1). The method is included in the *OIE Manual of Diagnostic Tests for Aquatic Animals* (OIE, 2014). The use of this test provides confirmation of the presence of AbHV associated with histopathology in abalone tissues suspected to be due to AVG. A positive result with this test provides the examining pathologist with additional confidence in diagnosis of AVG. Moreover, this test has proved useful in research on the pathogenesis of AVG (Corbeil et al., 2012).

Secondly, the development of a molecular assay for quantifying viral load in various preparations (Objective 2), in the absence of an *in vitro* assay (virus isolation and replication in cell lines) for quantifying infectious virus, provides an important tool for both diagnosis (detection and identification) and research. Validation of the qPCR tests with respect to diagnostic specificity (DSp) and sensitivity (DSe) (Objective 7) allows authorities to interpret test results with greater confidence as well as providing a sound basis for the development of surveillance programs designed to detect infection as opposed to disease.

Determining the stability of the virus in the environment (including in mucus shed from infected abalone) and sensitivity to inactivation agents (Objectives 3 and 4) provides important information for industry and government regulators, for example, when considering biosecurity plans for the abalone sector. Of further interest in this respect is the issue of latency/sub-clinical infection (Objective 5).

The ability of herpes viruses to establish a latent infection in host cells preserves a reservoir of the virus, which may be subsequently reactivated to cause disease during periods of stress for the animal. Commonly, herpes viruses maintain their genomes within host cells as circular episomes within the nuclei of host cells although some herpes viruses have been found to integrate into the host genome (Morissette & Flamand, 2010).

The major tenet of viral latency is the presence of the pathogen viral genome in the absence of disease in the animal. This has been shown for the *Herpesviridae* (Morissette & Flamand, 2010) and has been suggested to occur in infections caused by members of the *Alloherpesviridae* where low viral loads have been detected in sub-clinical or healthy animals (Eide *et al.*, 2011). In the *Malacoherpesviridae*, the ostreid herpes virus (OsHV) which causes disease in oysters, has also been shown to be present at low viral loads in the absence of disease (Arzul *et al.*, 2002; Barbosa-Solomieu *et al.*, 2004; Dundon *et al.*, 2011). Therefore latent or sub-clinical infection of oysters with OsHV-1 has also been speculated to occur.

Three lines of research have suggested that the AbHV may latently infect abalone: (1) the relationship of the virus to other herpesviruses, (2) the identification of AbHV DNA in abalone in the absence of disease and (3) the identification of anti-apoptotic genes within the AbHV genome. It is interesting to note the situation in Tasmania with respect to AVG. While disease outbreaks have not been detected in wild populations of abalone in that state, transfer of wild-caught abalone to live holding facilities within processing plants has precipitated the onset of AVG in a number of cases (Ellard et al., 2009), indicating the presence of sub-clinical/latent infection in wild populations. Indeed the presence of sub-clinical/latent infection in wild populations of abalone in Victoria, similar to that in Tasmania has been speculated (Crane et al., 2013) but not proven. Moreover, it is interesting to note that in recent times further outbreaks of AVG in wild abalone in Victoria have not been reported.

In the absence of further AVG outbreaks along the Victorian coast-line it could be speculated that, while it is unlikely that the virus has disappeared, the resident populations have acquired resistance to infection and/or disease or have not been exposed to a pathogenic dose of the virus. To test this hypothesis, samples of abalone from various locations along the Victorian coast-line were collected and exposed to pathogenic AbHV Vic1 strain (Objective 6). Results generated by this project and by FRDC Project 2009/075 demonstrated that abalone from reefs previously exposed to AbHV remain susceptible to infection and disease.

# Implications

Development of further understanding of the biology of abalone herpes virus will provide State authorities/industry with an enhanced capability for future management of the wild fishery and aquaculture enterprises. With increased knowledge and national diagnostic testing capability, the impacts of AbHV on abalone resources will be reduced.

An output from FRDC 2007/006 was a preliminary analysis of the abalone virus genome which indicated that the virus has both *Herpesviridae* and *Iridoviridae* characteristics. Precise classification of this virus may be important for determining strategies for managing disease outbreaks (Crane et al., 2009). For example, iridoviruses tend to be more stable in the environment than herpes viruses. On the other hand, typical herpes viruses have been shown to have a latent stage which has implications with respect to virus detection as well as the role of stress on disease expression.

Providing information on the most efficacious treatment (including chemicals and/or heat) for virus inactivation will have a direct impact on reducing virus spread. Determining the role of mucus in protecting the virus from the environment/inactivation agents and/or facilitating or inhibiting transmission is also important for understanding mechanisms of virus spread.

Development of the ISH test provides diagnosticians with enhanced capability in determining whether or not the virus is associated with observed tissue lesions. Thus the number of false-positive diagnoses based on histopathology alone will be reduced.

Development of qPCR will allow quantification of infectious dose and determination of viral distribution within abalone tissues – providing better information on the most appropriate tissues to sample for diagnosis – again reducing the number of false-positive and false-negative results.

Determining the status of wild abalone that have survived prior exposure to the virus may provide important information with regard to future management strategies. Results have demonstrated that the abalone remain susceptible to infection and disease. It is likely that the virus remains present in the wild abalone populations along the Victorian coast-line even though there have not been any recent disease outbreaks. This situation is reminiscent of that in populations of abalone in Tasmanian waters. The abalone may be sub-clinically infected. Exposure to stress factors may precipitate disease outbreaks.

# Recommendations

The outputs from this project are:

- a validated ISH diagnostic method
- a quantitative assay (qPCR) for determining infectious dose for AbHV
- information on the sensitivity of the virus to physico-chemical conditions including its stability in water/on fomites/in mucus and its sensitivity to inactivation agents
- information on the existence of a latent/sub-clinical infection
- information on the susceptibility of remnant populations of abalone previously exposed to AVG and known unexposed wild populations in South Australia
- information on the diagnostic sensitivities and specificities of the qPCR tests

This new knowledge contributes to better control and management of AbHV and the associated disease (AVG) and should be of interest to the abalone industry sector and government regulators. Thus the Australian Abalone Growers Association (AAGA), the Abalone Council Australia (ACA) and state governments (Department of Environment and Primary Industries, Victoria; and Department of Primary Industries, Parks, Water and Environment, Tasmania, Fisheries WA, Primary Industries and Regions South Australia, NSW Department of Primary Industries) will be provided with findings of this research as described in the Extension and Adoption section below.

The new information will also be published in peer-reviewed scientific journals for broader distribution. Some of the research described in this report has already been published (see section on *Project materials developed*).

## Further development

While this project has made significant contributions to our knowledge on AbHV and AVG directly applicable to disease management, there remain some pressing issues, particularly relating to factors affecting disease onset. It is clear that a sub-clinical infection can and does occur in wild populations of abalone and there are diagnostic tests available to detect sub-clinical infections. However, it is likely that there is a range of factors (viral, host and environmental) which are involved in determining whether or not a disease outbreak will occur.

At least 6 genetic variants of AbHV (Vic1, Tas1-5) have been detected which led to the requirement for additional qPCR tests to be developed. With respect to viral and host factors, it is not known whether or not there are differences in virulence between these variants. It is not known whether or not pathogenicity in different hosts differs between these variants. Research is continuing (FRDC Project 2013/001 Aquatic Animal Health Subprogram: Determination of susceptibility of various abalone species and populations to the various known AbHV genotypes) to investigate some, but not all, of these questions. Clearly, maintaining a virus-free state would ensure freedom from disease but this is not always achievable. We know commercial stocks of abalone are susceptible to infection and disease and there is little, if any, information on molluscan mechanisms of resistance to viral infections. With respect to other host factors it is not known whether there are mollusc species other than abalone that are susceptible to infection/disease.

Information on environmental factors that influence the onset of disease would be useful with respect to disease control with the understanding that (1) it will not always be possible to maintain a virus-free environment and (2) all abalone stocks tested to date are susceptible to infection. Research on environmental factors that influence onset of disease outbreaks should be aimed at identifying specific stressors that can be managed e.g. water quality factors. The issue of temperature is also basic to the understanding of the pathogen/host interaction. It is likely that there is a temperature range that is optimal for viral replication and for host physiological processes. It would be interesting to determine the influence that temperature has on infection and pathogenesis.

# Extension and Adoption

The purpose of this project is to increase our knowledge about abalone herpesvirus which is responsible for abalone viral ganglioneuritis (AVG) so that industry sectors and regulators are able to make better-informed decisions concerning management of this fishery and aquaculture enterprises in the presence of this virus. The non-technical summary will be provided in the next available issue of the *Health Highlights* Newsletter and the final report will be available on the FRDC website.

Results will be reported by face-to-face communication at scientific and industry conferences and through peer-reviewed scientific publications.

In addition, the developed diagnostic tests for the detection and identification of AVG in abalone and/or other mollusc species are available to national and international diagnostic laboratories. The methods are included in the *OIE Manual of Diagnostic Tests for Aquatic Animals* (OIE, 2014).

Copies of the final report will be forwarded to state authorities, AAGA and ACA.

**Presentations made by project staff at scientific and industry meetings/conferences**

Corbeil S, McColl KA, Williams LM, Mohammad I, Bergfeld J, Hyatt AD, Mackay E, Crameri SG, Fegan M, Crane MStJ. 2011. Early detection of herpesvirus infection in Australian abalone and susceptibility of the virus to chemical treatments. First FRDC Australasian Scientific Conference on Aquatic Animal Health, Cairns, 5-8 July 2011.

Corbeil S, Williams LM, Gannon V, Crane MStJ. 2012. Evaluation of abalone viral ganglioneuritis resistance amongst wild abalone populations along the Victorian coast. Australasian Aquaculture 2012, Melbourne, Victoria, Australia, 1-4 May 2012.

Corbeil S, Williams LM, Gannon V, Crane MStJ. 2012. Evaluation of abalone viral ganglioneuritis resistance amongst wild abalone populations along the Victorian coast. International Abalone Symp., Hobart, Tasmania, Australia, 6-11 May, 2012.

Corbeil S, Williams N, Moody N, Cowley J, McColl K, Bergfeld J, Hyatt A, Crameri S, Mackay E, Wong F, Colling A, Mohammad I, Fegan M, Warner S, Savin K, Murdoch B, Cogan N, Sawbridge T, Crane, M. 2011. Abalone viral ganglioneuritis: Current status of research. 5$^{th}$ National Abalone Convention, Hamilton Island, Queensland, 21-23 July 2011.

Corbeil S, Williams L, Moody N, McColl K, Crane M. 2010. Is there more than one abalone herpes-like virus emerging in Australia? Australasian Aquaculture 2010, Hobart, Tasmania, Australia, 23-26 May 2010.

Cowley JA, Corbeil S, Bulach D, Moody NJ, Ellard K, Fegan M, Savin K, Warner S, Crane MStJ. 2012. Complete genome sequences of abalone herpesvirus (AbHV) strains from Victoria and Tasmania provide insights into its origin and identify variations useful for epidemiology. International Abalone Symp., Hobart, Tasmania, Australia, 6-11 May, 2012.

Cowley JA, Corbeil S, Chen H, Bulach D, Wong F, Moody NJ, Ellard K, Fegan M, Savin K, Warner S, Crane MStJ. 2011. Sequence variations amongst abalone herpes-like virus (AbHV) strains provide insights into its origins in Victoria and Tasmania. First FRDC Australasian Scientific Conference on Aquatic Animal Health, Cairns, 5-8 July 2011.

Cowley JA, Corbeil S, Chen H, Wong F, Moody NJG, Ellard K, Fegan M, Savin K, Warner S, Crane MStJ. 2010. Preliminary investigations into the molecular epidemiology of abalone herpes-like virus (AbHV) and its origins in abalone in Victoria and Tasmania. Australian Association of Veterinary Laboratory Diagnosticians Annual Meeting, Brisbane, Queensland, 25-26 November 2010.

Mohammad I, Warner S, Kvalheim N, Crane MStJ, Corbeil S, Williams LM, Fegan M. 2011. Development of an *in situ* hybridisation assay for the detection and identification of the abalone herpes-like virus. First FRDC Australasian Scientific Conference on Aquatic Animal Health, Cairns, 5-8 July 2011.

Warner S, Savin K, Wong F, Fegan M, Sawbridge T, Lancaster M, Kvalheim N, Mohammad I, Corbeil S, Crane M. 2010. Full genome sequencing and its application in the identification of new biosecurity threats. Global

Biosecurity 2010: safeguarding agriculture and the environment, 28 Feb-3 Mar 2010, Brisbane Convention and Exhibition Centre, Brisbane, Qld.

# Project materials developed

Corbeil S, Williams LM, Bergfeld J, Crane MStJ. 2012. Abalone herpes virus stability in sea water and susceptibility to chemical disinfectants, *Aquaculture* **326**: 20-26.

Corbeil S, McColl KA, Williams LM, Mohammad I, Hyatt AD, Crameri SG, Fegan M and Crane MStJ. 2012. Abalone viral ganglioneuritis: Establishment and use of an experimental immersion challenge system for the study of abalone herpes virus infections in Australian abalone. *Virus Res* **165:** 207-213.

Crane MStJ, Corbeil S, Williams LM, McColl KA and Gannon V. 2013. Evaluation of abalone viral ganglioneuritis resistance amongst wild abalone populations along the Victorian coast of Australia. *J Shellfish Res* **32:** 67-72.

Infection with Abalone herpesvirus. 2014. OIE Manual of Diagnostic Tests for Aquatic Animals. World Organisation for Animal Health, Paris, France. Pp. 441-451.
http://www.oie.int/fileadmin/Home/eng/Health_standards/aahm/current/2.4.01_INF_ABALONE.pdf

# Appendices

## 1. Information on conventional PCR tests used for AbHV genotype identification

The range in amplicon size for the following conventional PCR tests is due to differences in sequence for the various genetic variants of AbHV.

### 1.1 CONVENTIONAL PCR FOR AbHV AB1213 REGION (AbHV AB1213 PCR)

| **AbHV AB1213 PCR – 469 to 485 bp amplicon** | | |
|---|---|---|
| **Reagent** | **Volume for 1 rxn** | **Volume for ____ rxns** |
| Water<br>Batch: _____ | 9.5 µl | |
| HotStarTaq Master Mix<br>Batch: _____ | 12.5 µl | |
| Primer name: **AbHV-12 (18µM)**<br>Batch: _____ | 0.5 µl | |
| Primer Name: **AbHV-13 (18µM)**<br>Batch: _____ | 0.5 µl | |
| **Total volume** | 23 µl | |

**Thermal Cycler Program**

| Cycles | Conditions |
|---|---|
| 1 | 95°C for 15 minutes |
| 40 | 94°C for 30 seconds<br>52°C for 30 seconds<br>74°C for 45 seconds |
| 1 | 72°C for 7 minutes |
| Hold | 4°C |

**Run Details**

Date run: _____

Thermal cycler: _____

Operator: _____

Primer sequences:

| Primer | Sequence |
|---|---|
| AbHV-12 | 5'- CCG TTT GAA AGT CTA GCG TGA AAG -3' |
| AbHV-13 | 5'- CCA TGG AGA AAG AAG AAG TGT CTC A -3' |

### 1.2 CONVENTIONAL PCR FOR AbHV AB1617 REGION (AbHV AB1617 PCR)

| **AbHV AB1617 PCR – 522 to 588 bp amplicon** | | |
|---|---|---|
| **Reagent** | **Volume for 1 rxn** | **Volume for ____ rxns** |
| Water<br>Batch: _____ | 9.5 µl | |
| HotStarTaq Master Mix<br>Batch: _____ | 12.5 µl | |
| Primer name: **AbHV-16 (18µM)**<br>Batch: _____ | 0.5 µl | |
| Primer Name: **AbHV-17 (18µM)**<br>Batch: _____ | 0.5 µl | |
| **Total volume** | 23 µl | |

**Thermal Cycler Program**

| **Cycles** | **Conditions** |
|---|---|
| 1 | 95°C for 15 minutes |
| 40 | 94°C for 30 seconds<br>52°C for 30 seconds<br>74°C for 45 seconds |
| 1 | 72°C for 7 minutes |
| Hold | 4°C |

**Run Details**

Date run: _____

Thermal cycler: _____

Operator: _____

Primer sequences:

| **Primer** | **Sequence** |
|---|---|
| AbHV-16 | 5'- GGC TCG TTC GGT CGT AGA ATG -3' |
| AbHV-17 | 5'- TCA GCG TGT ACA GAT CCA TGT CA -3' |

### 1.3 CONVENTIONAL PCR FOR AbHV AB2930 REGION (AbHV AB2930 PCR)

| AbHV AB2930 PCR – 418 to 481 bp amplicon | | |
|---|---|---|
| **Reagent** | **Volume for 1 rxn** | **Volume for ____ rxns** |
| Water<br>Batch: _____ | 9.5 µl | |
| HotStarTaq Master Mix<br>Batch: _____ | 12.5 µl | |
| Primer name: **AbHV-29**<br>Batch: _____ | 0.5 µl | |
| Primer Name: **AbHV-30**<br>Batch: _____ | 0.5 µl | |
| **Total volume** | 23 µl | |

**Thermal Cycler Program**

| Cycles | Conditions |
|---|---|
| 1 | 95°C for 15 minutes |
| 40 | 94°C for 30 seconds<br>52°C for 30 seconds<br>74°C for 45 seconds |
| 1 | 72°C for 7 minutes |
| Hold | 4°C |

**Run Details**

Date run: _____

Thermal cycler: _____

Operator: _____

Primer sequences:

| Primer | Sequence |
|---|---|
| AbHV-29 | 5'- AGA GGC TTC AAC ACG CTC AAG -3' |
| AbHV-30 | 5'- AGG CGA TGT AAG ATT CTT CCA ATC -3' |

# 2. References


Arzul I, Renault T, Thébault A, Gérard A. 2002. Detection of oyster herpesvirus DNA and proteins in asymptomatic *Crassostrea gigas* adults. *Virus Res* **84:** 151-160.

Barbosa-Solomieu V, Miossec L, Vázquez-Juárez R, Ascencio-Valle F, Renault T. 2004. Diagnosis of Ostreid herpesvirus 1 in fixed paraffin-embedded archival samples using PCR and in situ hybridisation. *J Virol Methods* **119:** 65-72.

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, Crane MStJ. 2010. Development and validation of a TaqMan PCR assay for the Australian abalone herpes-like virus. *Dis Aquat Org* **92:** 1-10.

Corbeil S, McColl KA, Williams LM, Mohammad I, Hyatt AD, Crameri SG, Fegan M, Crane MStJ. 2012. Abalone viral ganglioneuritis: Establishment and use of an experimental immersion challenge system for the study of abalone Herpes virus infections in Australian abalone. *Virus Res* **165:** 207-213.

Cowley JA, Corbeil S, Chen H, Bulach D, Wong F, Moody NJ, Ellard K, Fegan M, Savin K, Warner S, Crane MStJ. 2011. Sequence variations amongst abalone herpes-like virus (AbHV) strains provide insights into its origins in Victoria and Tasmania. First FRDC Australasian Scientific Conference on Aquatic Animal Health, Cairns, 5-8 July 2011.

Crane MStJ, Corbeil S, Fegan M and Warner S. 2009. Aquatic Animal Health Subprogram: Development of molecular diagnostic procedures for the detection and identification of herpes-like virus of abalone (*Haliotis* spp.). Fisheries Research and Development Corporation Project No. 2007/006.

Crane MStJ, Corbeil S, Williams LM, Gannon V. 2012. Determining the susceptibility of remnant populations of abalone previously exposed to AVG. FRDC Project 2009/075. CSIRO Livestock Industries, Geelong, Victoria 3220.

Crane MStJ, Corbeil S, Williams LM, McColl KA, Gannon V. 2013. Evaluation of abalone viral ganglioneuritis resistance among wild abalone populations along the Victorian coast of Australia. *J Shellfish Res* **32:** 67-72.

Davison AJ, Trus BL, Cheng N, Steven AC, Watson MS, Cunningham C, Deuff R-ML, Renault T. 2005. A novel class of herpesvirus with bivalve hosts. *J Gen Virol* **86:** 41-53.

Dundon WG, Arzul I, Omnes E, Robert M, Magnabosco C, Zambon M, Gennari L, Toffan A, Terregino C, Capua I, Arcangeli G. 2011. Detection of Type 1 Ostreid Herpes variant (OsHV-1 µvar) with no associated mortality in French-origin Pacific cupped *oyster Crassostrea gigas* farmed in Italy. *Aquaculture* **314:** 49-52.

Eide K, Miller-Morgan T, Heidel J, Bildfell R, Jin L. 2011. Results of total DNA measurement in koi tissue by Koi Herpes Virus real-time PCR. *J Virol Methods* **172:** 81-84.

Ellard K, Pyecroft S, Handlinger J, Andrewartha R. 2009. Findings of disease investigations following the recent detection of AVG in Tasmania. 4th FRDC National Aquatic Animal Health Scientific Conference, Cairns, 22-24 July 2009.

Gardner IA, Stryhn H, Lind P, Collins MT. 2000. Conditional dependence between tests affects the diagnosis and surveillance of animal diseases. *Prev Vet Med* **45:** 107-122.

Hooper C, Hardy-Smith P, Handlinger J. 2007. Ganglioneuritis causing high mortalities in farmed Australian abalone (*Haliotis laevigata* and *Haliotis rubra*). *Aus Vet J* **85:** 188-193.

Huang Q, Deveraux QL, Maeda S, Salvesen GS, Stennicke HR, Hammock BD, Reed JC. 2000. Evolutionary conservation of apoptosis mechanisms: Lepidopteran and baculoviral inhibitor of apoptosis proteins are inhibitors of mammalian caspase-9. *Proc Natl Acad Sci USA* **97:** 1427-1432.

Kent JR, Kang W, Miller CG, Fraser NW. 2003. Herpes simplex virus latency-associated transcript gene function. *J Neurovirol* 9: 285-290.

Morissette G, Flamand L. 2010. Herpesviruses and chromosomal integration. *J Virol* **84:** 12100-12109.

OIE, 2014. Infection with Abalone herpesvirus. OIE Manual of Diagnostic Tests for Aquatic Animals. World Organisation for Animal Health, Paris, France.
http://www.oie.int/fileadmin/Home/eng/Health_standards/aahm/current/2.4.01_INF_ABALONE.pdf

Ren W, Chen H, Renault T, Cai Y, Bai C, Wang C, Huang J. 2013. Complete genome sequence of acute viral necrosis virus associated with massive mortality outbreaks in the Chinese scallop, *Chlamys farreri*. *Virol J* **10:** 110.

Savin KW, Cocks BG, Wong F, Sawbridge T, Cogan N, Savage D, Warner S. 2010. A neurotropic herpesvirus infecting the gastropod, abalone, shares ancestry with oyster herpesvirus and a herpesvirus associated with the amphioxus genome. *Virol J*. 308.

Tan J, Lancaster M, Hyatt A, van Driel R, Wong F, Warner S. 2008. Purification of a herpes-like virus from abalone (*Haliotis* spp.) with ganglioneuritis and detection by transmission electron microscopy. *J Virol Methods* **149:** 338-341.

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