FINAL REPORT

Aquatic Animal Health Subprogram: pilchard herpesvirus infection in wild pilchards

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Fish for the future

Australian Government
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Authors: Brian Jones, Melanie Crockford, Richard Whittington, Mark Crane, Graham Wilcox

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LIST OF ABBREVIATIONS

AAHL	Australian Animal Health Laboratory
CCV	Channel catfish virus
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DNA	Deoxyribose nucleic acid
FFPE	Formalin fixed and paraffin embedded
FHL	Fish Health Laboratory
FRDC	Fisheries Research and Development Corporation
GFHNV	Goldfish haematopoietic necrosis virus
IQF	Individually quick frozen
ISH	*in-situ* hybridization
JPSWG	Joint pilchard scientific working group
KHV	Koi herpesvirus
MW	Molecular weight
NSW	New South Wales
NT	Northern Territory
OIE	Office International des Épizooties (World Organisation for Animal Health)
OMV	*Oncorhynchus masou* virus
ORF	Open reading frame (a DNA sequence that codes for a protein)
PCR	Polymerase chain reaction (a patented method of copying DNA)
PHV	Pilchard herpesvirus
QLD	Queensland
RC	Reverse complement
SA	South Australia
SARDI	South Australian Research Development Institute
SSC	Sodium chloride and Sodium citrate buffer
TAS	Tasmania
TEM	Transmission electron microscopy
VHS	Viral haemorrhagic septicaemia
VIC	Victoria
WA	Western Australia
WSHV1	White sturgeon herpesvirus 1
WSHV2	White sturgeon herpesvirus 2
YHV	Yellowhead virus

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2002/044 Aquatic Animal Health Subprogram: pilchard herpesvirus infection in wild pilchards

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OBJECTIVES:

- To improve the sensitive and specific diagnostic assays [polymerase chain reactions (PCRs) and *in situ* hybridization (ISH)] that are based on current limited sequence data. This will include the generation of further sequence data from the available stocks of virus.

- To independently establish the sensitivity and specificity of the PCRs and ISH at other laboratories, including AAHL.

- To then investigate basic aspects of the virus and the disease e.g. the tissue distribution of virus in infected fish, and the correlation between disease in fish and the presence of virus.

- To survey wild pilchard populations to determine whether the virus is still currently detectable and causing disease.

- To compare the herpesvirus strains from 1995 and 1998, and to compare, at the molecular level, this herpesvirus and this disease with two other similar herpesvirus fish diseases that have been reported elsewhere in the world.

NON-TECHNICAL SUMMARY

OUTCOMES ACHIEVED

1. Additional sequence data that is strongly suggested to be new sequence data for the pilchard herpesvirus (PHV).
2. A PCR with documented sensitivity and specificity that can be used to test fresh and frozen pilchard tissues for PHV.
3. An *in-situ* hybridization test with documented specificity that can be used to detect virus-containing tissues in sectioned histological material.
4. A paper describing the PCR and sequencing methods to obtain sequence data for PHV that places the information in public domain.
5. An insight into the presence of PHV at various times before, during and after the 1995 mortality event.
6. Confirmation that the transmission trial undertaken in 1999 was successful and that sub-clinical infection of previously unaffected pilchards was achieved.
7. A paper describing the correlation of histopathology, ISH, PCR and real-time PCR of archived transmission trial samples.
8. A survey of wild pilchard stocks giving evidence of a significant presence of PHV in the wild population.
9. Comparison of the 1995 and 1998 strains and comparison of these strains with 2 similar herpesviruses.

In 1995 and again in 1998/9, millions of pilchards (*Sardinops sagax neopilchardus*) were found dead or dying off the coast of Australia. Some pilchards in New Zealand were also affected during the 1995 epizootic. The epizootics moved progressively in a “bushfire-like” manner against the prevailing currents at a rapid speed. An uncharacterised herpesvirus, pilchard herpesvirus (PHV), was associated with both mortality events and, based on its occurrence in affected fish and the disease pathogenesis, generally accepted as the causative agent, although experimental evidence of its role was not established. Until recently, rapid and sensitive methods to detect PHV were not available and so research into the characteristics of this virus were limited. New methods developed and optimised for the detection of PHV include Polymerase Chain Reaction (PCR), *in situ* hybridization (ISH) and real-time PCR. These methods were then applied to a number of pilchard samples that were obtained at various times during the two outbreaks.

Some sequence data for PHV was obtained. These data are the first step in obtaining information at the molecular level as to how this virus fits within the *Herpesviridae*. Since acquiring this initial sequence data, techniques have been applied to try to further characterise PHV. At the time of starting this project, opportunities to increase the known sequence were limited due to the inability to grow the virus in culture, the limited virus stocks and the best virus material only being part purified and of unknown viability. The additional sequence data obtained is still preliminary at this stage and requires further analysis.

Three molecular methods have recently become available for detecting PHV (ISH, PCR and Real-time PCR) and were used to detect PHV in samples from fish collected pre-, during and post-outbreak. Samples collected before 1995 were not available to test. The real-time PCR

detected virus in fish collected 4 days before the epizootic front, during the front, and 8 days after the front had passed. The samples that were available for this study were archival and limited the information that could be obtained on the length of the virus incubation period and persistence of the virus after the epizootic front had passed. However, the information obtained was consistent with modelling by Murray *et al*. (2003) that predicted an incubation period of up to 12 days, depending on the wave speed of the epidemic. The detection of PHV in samples after mortalities had ceased, that appeared to be healthy by histology and were not positive by ISH or conventional PCR, indicates that virus persisted in the population of survivors at low levels.

In 1999 an experiment was carried out to transmit PHV to apparently healthy pilchards in an attempt to (1) show that the virus could be transmitted to uninfected pilchards, (2) demonstrate that the infected pilchards develop gill lesions characteristic of the disease and (3) show that the virus can be re-isolated from experimentally infected fish. At the time of the experiment the only available methods for detection of PHV were histology and electron microscopy. As attempts to grow the virus *in vitro* were unsuccessful the lack of virus detected in experimentally exposed pilchards suggested that virus transmission was unsuccessful. Because the technology is now available to apply molecular techniques to detect PHV, the transmission trial samples have been re-analysed.

Re-examination of archived transmission trial samples produced some interesting results. All samples were negative by ISH and PCR, yet 3 of 6 samples were positive by real-time PCR. Real-time PCR analysis indicated that these positives had low levels of virus present. The results suggest that PHV was transmitted to healthy pilchards during the trial, and results were also consistent with samples that showed some histological changes suggestive of early stages of disease, however disease was not produced within the time frame of the trial. It may be that only low levels of virus were transmitted and were not enough to cause disease. The positives detected by real-time PCR still confirm that sub-clinical infection was achieved.

Wild pilchard populations were surveyed in 2004 as part of the project, to determine whether the virus is currently detectable and causing disease. Results of this survey show that PHV is significantly present within the current pilchard population: PHV is now endemic. There does appear to be variation between sub-populations of Australian pilchards, with the possibility that NSW pilchards are not infected at all. Further studies are required to prove this, using more randomised samples. The suggestion that PHV is now endemic makes sense with the lack of further epizootics seen after the 1998/9 epizootic. Mortalities of the scale seen in 1995 and 1998 are not likely to be witnessed again due to the continued presence of PHV within the population and the likelihood of a degree of immunity in pilchards in the current population. It is expected, however, that small-scale mortality events will occur, but will either not be seen or not be reported. It is feasible that another PHV epizootic will never occur in Australian waters and further studies should include the testing of pilchards from overseas waters for the presence of PHV. If PHV is present elsewhere overseas, especially with no large-scale mortalities having been reported, then this may give an insight into how PHV was introduced into Australian waters.

PHV from the 1995 outbreak was compared over 373 bp of sequence data to PHV from the 1998 outbreak. The viruses are considered to be the same over this region. However, this is only a small amount of sequence data considering that the expected size of the genome is approximately 200 kbp. This should be interpreted as a preliminary investigation of sequence similarities between the 1995 and 1998 virus(es).

Based on the current limited data, PHV appears to be related to other fish herpesviruses but bears no resemblance to mammalian or avian herpesviruses. This sequenced region is known to be highly conservative in herpesviruses, suggesting that PHV is indeed unique, although still classed morphologically as a herpesvirus.

KEYWORDS: Pilchard herpesvirus; PCR; *in situ* hybridization; transmission trial.

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SECTION 1: INTRODUCTION

1.1 Background

In 1995 and again in 1998 there was major mortality of pilchards that spread from South Australia around the southern coastline of Australia until, eventually, the entire geographical range of pilchards in Australian waters was affected. The mortalities received widespread media coverage in 1995 and 1998. The disease resulted in estimates of mortality of about 70% and devastated large sectors of the WA pilchard fishery that by 2001 had still not recovered to pre-1998 levels of biomass.

Of the papers co-authored by the PI, three (Whittington *et al*., 1997; Hyatt *et al*., 1997; Fletcher *et al*., 1997) describe the 1995 outbreak of disease, identify a herpesvirus as the cause and demonstrate that environmental impacts were not the cause of the mortalities. Jones *et al*. (1997) reviewed knowledge about the disease in 1996.

Following public and media speculation about the cause of the 1998 fish kills and their similarity to 1995 mortalities, a Joint Pilchard Scientific Working Group (JPSWG) was established under the Consultative Committee on Exotic Animal Diseases. The working group set priorities and coordinated research on the virus. A transmission trial was attempted in Western Australia but the results were inconclusive. A project was also initiated to use mathematical modelling techniques to explore the observed behaviour of the epizootic and see what could be deduced about the virus. This project was very successful and resulted in three papers (Murray *et al*., 1999; Murray *et al*., 2001a,b).

The modelling suggested that on both occasions the virus originated from a point source, travelled without the need for vectors in a host-density independent manner, and has an incubation time of several days with a very short window of infectivity. These inferences from mathematical studies still need to be verified with biological data.

Development of molecular diagnostic techniques was given highest priority by the JPSWG. The Australian Animal Health Laboratory (AAHL) at Geelong developed a number of cell lines and a polymerase chain reaction (PCR) but progress has been difficult due in part to shortage of viable material, staff transfers, and the difficulty in working with a virus which has a probable latent phase and which cannot be cultured. The Department of Fisheries WA has also developed a PCR and an *in situ* probe for the pilchard virus.

At the time of beginning this project it was unknown whether the virus was dormant in the pilchard population or if the virus was being introduced in to the country through imported pilchard bait. With the development of molecular tools for the detection of PHV, it may now be possible to address these issues.

1.2 Need

This study continues the work begun at AAHL and Department of Fisheries WA. There is a need to independently validate the available tools from Western Australia and AAHL and to put them to use in elucidating the biology of the virus, including a survey of wild pilchards for the virus. This is especially important as a number of modelling papers have made inferences about the latency and infectivity of the virus that need to be verified. There is also a need to continue the sequencing of the viral genome which has been carried out both at AAHL and in WA, in order to design more specific tools and also to compare the virus

obtained in 1995 with that collected in 1998. This work was identified as a national priority in 1998 and the need has not diminished. It was also recognised by the JPSWG, from the outset, that progress would be slow.

1.3 Objectives

- To improve the sensitive and specific diagnostic assays (polymerase chain reactions (PCRs) and *in situ* hybridization (ISH)) that are based on current limited sequence data. This will include the generation of further sequence data from the available stocks of virus.
- To independently establish the sensitivity and specificity of the PCRs and ISH at other laboratories, including AAHL.
- To then investigate basic aspects of the virus and the disease eg. tissue distribution of virus in infected fish, and the correlation between disease in fish and the presence of virus
- To survey wild pilchard populations to determine whether the virus is still currently detectable and causing disease
- To compare the herpesvirus strains from 1995 and 1998, and to compare, at the molecular level, this herpesvirus and this disease with two other similar herpesvirus fish diseases that have been reported elsewhere in the world.

SECTION 2: METHODS

2.1 Objective 1: *"To improve the sensitive and specific diagnostic assays (polymerase chain reactions (PCRs) and in situ hybridization (ISH)) which are based on current limited sequence data. This will include the generation of further sequence data from the available stocks of virus."*

The first sequence data of the PHV genome was obtained using universal fish herpesvirus primers, designed to amplify approximately 400 bp of fish herpesvirus DNA from the ORF 62 gene, in a PCR to amplify PHV DNA (Eaton *et al*., 2002). From this initial sequence, primers PHV373F, designed at the Department of Fisheries WA, and PHV367R, sequence supplied by CSIRO Livestock Industries, Australian Animal Health Laboratory in Geelong, Victoria, were developed to target only PHV (Crockford *et al*., 2005).

Due to the difficulties in obtaining additional sequence data, validated tests were based on the 373 bases of known PHV sequence and not additional sequence data.

2.1.1 Samples used for PCR

The main sample used for obtaining original sequence data was partially purified PHV from the 1995 outbreak. A sample from frozen fish, labelled 95-4420, was also used for the optimisation of the PCR.

2.1.2 Preparation of partially purified virus for PCR

For partial purification of virus, PHV from the 1995 outbreak was detected by electron microscopy and partially purified by sucrose gradient centrifugation before being stored at -80ºC as a stock preparation of virus. When a working preparation was required, 10 µL of the stock preparation was resuspended in 100 µL of digestion solution (10 µL of 2 mg/mL Proteinase K in 100 µL of sterile Milli-Q [MQ] water), allowed to digest overnight at 37°C before being heated to 80°C in a heating block (Ratek Dry Block Heater, Scot Scientific, Australia) for 20 minutes, and then stored at –20°C. A 1:100 dilution of this was used in each PCR.

2.1.3 Extraction of DNA from frozen tissue for PCR

A modified DNAzol extraction procedure (Life Technologies, USA) was used to extract viral DNA suitable for PCR. DNA was also extracted by this method from frozen gill tissue of pilchards collected during the 1995 outbreak.

A tissue sample (while still thawing) of 25–50 mg was added to a 1.5 mL microcentrifuge tube containing 0.7 mL of DNAzol reagent (Life Technologies, USA), homogenised using a hand-held disposable pestle (Scientific Specialties Incorporated, USA) and centrifuged at 10,000 x *g* for 10 minutes at ambient temperature. The supernatant was transferred to a fresh microcentrifuge tube, and 0.5 mL of 100% ethanol (Sigma, USA) was added. The tubes were inverted several times to allow a precipitate to form, before being centrifuged at 10,000 x *g* for 5 minutes at ambient temperature. The supernatant was decanted, leaving a visible pellet at the bottom of the tube. The pellet was washed twice, by resuspending the pellet in 0.7 mL of 75% ethanol, centrifuging at 10,000 x *g* for 5 minutes at ambient temperature and decanting the supernatant after each wash. Any remaining ethanol was removed using a

pipette, and the pellet was allowed to dry on a heating block at 50ºC. Sterile water (Astral Scientific, Australia) was added to the DNA and the tube was placed on a heating block at 55ºC for up to 1 hour to resuspend the DNA. The concentration of the DNA sample was determined by spectrophotometry, and then more sterile water added, if necessary, to give a concentration of approximately 150 ng/µL DNA. Aliquots were stored at –20ºC.

2.1.4 PCR for the detection of Pilchard herpesvirus

The PHV373 PCR has been designed to amplify a region of PHV from ORF 62 of the genome, which is suggested to code for a putative terminase involved in DNA packaging and amplifies a product of 373 bp. Standard reaction volumes were 25 µL, with the following components included: 400 µM each dNTP, 2.5 mM $MgCl_2$, 0.5 µM primer PHV373F 5'-ACACATGATTTTGGATAAACTGGGG-3', 0.5 µM primer PHV367R 5'-ATCTGGGTTGAAGATGAACCGCGCC-3', 0.1 U Tth Plus DNA Polymerase (Fisher Biotec), and 10X Buffer at 1/10 the volume of the PCR. Approximately 150 ng of suitable template were added per 25 µL reaction.

PCRs were performed on a MJ Research Minicycler (Geneworks, Australia), and cycle conditions were as follows: initial denaturation step of 96°C for 1 minute, then 30 cycles of 96°C for 30 seconds, 60°C for 45 seconds, and 72°C for 1 minute, followed by a final extension at 72°C for 4 minutes, then held at 14°C.

PCR products were run with a 100 bp DNA ladder, Hyperladder IV (Bioline, Australia) on a 1.5% agarose gel (Progen, Australia) containing 0.5 $mgmL^{-1}$ ethidium bromide at 90 volts for 50 minutes, and visualised by a UV transilluminator (Pathtech Pty. Ltd., Australia).

2.1.5 Sequencing

PCR products or plasmids were cleaned up using DNAce Quick-Clean (Bioline, USA) and manufacturer's instructions, or bands extracted using a freeze-squeeze gel extraction method (described below) and were sequenced at the State Agricultural and Biotechnology Centre, Murdoch University, WA, using their standard method.

2.1.6 Freeze-squeeze method for extracting DNA from agarose gel

The PCR product was run on an agarose gel, the desired band cut out and placed in a 1.5 mL microcentrifuge tube, and frozen at –20ºC immediately for at least 30 minutes. The gel slice was squeezed between a parafilm sheet that had been folded in half, and all of the liquid that appeared as the gel slice thawed was extracted and placed in a sterile microcentrifuge tube. One tenth of the extract volume of 3 M sodium acetate pH 5.2 and double the extract volume of 70% ice-cold ethanol were added, and gently agitated using a vortex mixer. The preparation was centrifuged at 10,000 x *g* for 5 minutes, the supernatant discarded, and the pellet vacuum-dried. Twelve µL of sterile water was added to dissolve the pellet, and 5.75 µL of this was used in a sequencing reaction.

2.1.7 Samples used for *in situ* Hybridization

Sample 95-4420 that showed histology suggestive of PHV and was strongly positive by PCR was used for the optimisation of the ISH. PCR product was used to make a PHV-specific riboprobe. Gill tissue from this fish was formalin fixed and paraffin embedded (FFPE) and used as the positive control for the ISH process.

2.1.8 Riboprobe production

Four µL of PCR product was made up to 9.5 µL with sterile water (Astral Scientific, Australia) and used in a riboprobe reaction mix, consisting of 4 µL of 5X transcription buffer (Promega, USA), 2 µL of 100 mM DTT (Dithiothreitol) (Promega, USA), 3 µL RNA labelling mix (Boehringer-Mannheim, Australia), 1 µL of SP6 RNA polymerase (Promega, USA) and 0.5 µL of RNAsin (Promega, USA). The reaction mix was incubated at 37ºC for 2 hours, followed by the addition of 2 µL of 0.2 M EDTA (pH 8.0) (Sigma, USA) to stop the reaction. Labelled RNA was incubated at -80ºC for 30 minutes with 2.5 µL of 4 M lithium chloride (ICN, USA) and 75 µL of 100% ethanol to precipitate the labelled RNA. Following centrifugation at 10,000 x *g* for 20 minutes, the resulting pellet was washed in 70% ethanol (Sigma, USA), centrifuged at 10,000 x *g* for 15 minutes, and dried following decantation of the supernatant. The pellet was then resuspended in 100 µL of sterile water (Astral Scientific, Australia). Ten µL of the preparation was run on a 1.5% agarose gel (Progen, Australia) at 90 volts for 50 minutes, to check the quality of the riboprobe. One µL of RNAsin (Promega, USA) was added to the resuspended labelled RNA and the preparation stored in 5 µL aliquots at -80ºC.

2.1.9 *in situ* Hybridization for Pilchard herpesvirus

Paraffin-embedded tissues were sectioned at 5 µM thickness onto silanated slides and allowed to dry. Before enzyme treatment, sections were heated in a hot oven to 65ºC for at least 10 minutes before being deparaffinized in 2 stages of xylene (Rowe Scientific, Australia) for 5 minutes at each stage, then rehydrated in 95% ethanol (Scot Scientific, Australia) for 5 minutes, then 70% ethanol for 5 minutes, and then rinsed in MQ water for 5 minutes.

Three hundred µL of Protease VIII (Sigma, USA) diluted in Buffer 1 (100 mM Tris-HCl pH 7.5, 150 mM NaCl) to a concentration of 375 ng/µL was applied to each section and incubated at 37ºC for 20 minutes in a lunchbox moistened with MQ water. Following incubation, the sections were washed in MQ water, soaked in 95% ethanol (Scot Scientific, Australia) and left to air dry.

Prior to hybridization, salmon sperm DNA (Sigma, USA) was dissolved in MQ water at 10 mg/mL, stirred with a magnetic stirrer for 5 hours before being sheared several times through a 21-gauge hypodermic needle. The viscous solution was then boiled for 10 minutes in a beaker of MQ water on a hot plate and stored in 1 mL aliquots at -20ºC. For hybridization, an aliquot of the sheared salmon sperm was placed on a heating block at 100ºC for 5 minutes just prior to use to freshly denature the DNA, then placed on ice.

A stock solution of hybridization mixture was prepared and stored at room temperature, consisting of 5 mL formamide (Sigma, USA), 1 mL dextran sulphate in sterile water (Astral Scientific, Australia) (stock solution of 2 g dextran sulphate [ICN, USA] in 4 mL sterile water (Astral Scientific, Australia), filtered with 0.45 µM filter into a 5 mL vial and stored at 4ºC), and 1 mL of 20X SSC (175.3 g NaCl [ICN, USA], 88.2 g sodium citrate [ICN, USA], 800 mL sterile water (Astral Scientific, Australia), with the final solution adjusted to pH 7.0 with HCl [ICN, USA] and made up to 1 litre). Hybridization solution at 100 µL per section, was freshly prepared using 70 µL hybridization mixture (previously prepared), 4 µL freshly denatured salmon sperm DNA at 10 mg/mL, 25.5 µL of TE8 buffer (10 mM Tris-HCl pH 8.0 [ICN, USA], 1 mM EDTA pH 8.0 [Sigma, USA]) and 0.5 µL of riboprobe previously prepared by *in vitro* transcription of PCR product. Before application to the section, the

hybridization solution was vortexed, then placed on a heating block at 100ºC for 10 minutes to denature the probe, before being placed on ice ready to use. One hundred µL of this solution was applied per section and the section was coverslipped. Rubber bands doubled over were placed on each end of the slide to prevent the coverslip from moving off the section during boiling. The section was then placed in a vegetable steamer over boiling water with the lid on, for 10 minutes to denature the nucleic acid in the tissue. Sections were then quickly placed in a pre-warmed, humidified lunchbox moistened with 4X SSC (200 mL of 20X SSC in 1 L Milli-Q [MQ] water) and incubated in a pre-heated oven at 50ºC for 2.5 hours to allow hybridization of the probe to viral DNA in the section.

Following hybridization, coverslips were carefully washed off with 4X SSC, then gently washed on a rocker in a square petri dish containing 4X SSC for 2 x 5 minutes, then washed in 1X SSC (50 mL of 20X SSC in 1 L MQ water) for 2 x 5 minutes, before being stringently washed in 0.1X SSC (5 mL of 20X SSC in 1 L MQ water), previously heated to approximately 80ºC, for 15 minutes at ambient temperature.

Blocking solution (3% Skim Milk Powder [Coles Supermarket, Australia] and 0.05% Triton-X-100 [ICN, Australia] in 5 mL Buffer 1) was applied to the sections at room temperature for 30 minutes in a humidified lunchbox, after removing an amount for dilution of the antibody. Sheep anti-DIG (digoxigenin) antibody conjugated to alkaline phosphatase (Boehringer-Mannheim, Australia) was diluted 1:500 in the previously prepared blocking solution and applied at 300 µL per section, and left overnight at 4ºC in a humidified lunchbox.

The sections were washed in Buffer 1 for 2 x 5 minutes, then immersed in Buffer 3 (100 mM Tris-HCl pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$) for at least 2 minutes at ambient temperature. NBT/BCIP colour development solution (Boehringer-Mannheim, Germany), diluted 1:50 in Buffer 3, was applied at 300 µL per section and left in darkness for at least 2 hours, with the reaction monitored after 2 hours for determination of the end point of the probe staining. Immersing in 2 changes of MQ water stopped the reaction.

The section was counterstained by applying 0.1% neutral red (Sigma, USA) for 30 seconds, followed by 0.1% light green (Sigma, USA) for 2 minutes, and then rinsed in running tap water. An alternative counterstain was often used, consisting of 0.5% Bismark Brown (Sigma, USA) applied for 60 seconds. Non-specific staining was removed by soaking in acetone for 10 seconds. The section was further dehydrated by dipping 4-5 times in each of 2 changes of propanol, followed by 2 changes of xylene, before being mounted in DePeX (BDH Laboratory Supplies, Australia). Each section was examined by light microscopy for the presence of hybridized blue-purple probe against a light green background, or light brown background if Bismark Brown was the counterstain used.

2.1.10 Preparation of pZErO DNA library for use in obtaining new sequence data

Following the 1995 epizootic, a PHV DNA library was prepared at AAHL. Part-purified PHV was cut into fragments and the fragments were ligated into a plasmid vector, pZErO (Invitrogen, USA). PHV had not been confirmed to be present in this library, and so finding new PHV sequence data from this library was expected to be difficult, due to the fact that, at the time of construction of the pZErO library, the best material available was limited stocks of only part purified virus (Jones, J.B. and Hillier, P.W., pers.comm.).

The pZErO library was transformed using manufacturer's instructions (Invitrogen, USA) and pooled colonies were screened by the PHV373 PCR for clones containing the known

fragment of PHV. The first and second attempts failed and so a 3rd attempt was made. Four plates of transformations were grown, labelled A to D. Selected colonies were picked into pools, so that there were 3-5 pools per plate and 3-5 colonies per pool. Each pool was screened by the PHV373 PCR for an insert containing the 373 bp fragment. Three pools amplified a band of interest at 373 bp, and so individual colonies from these 3 pools were tested.

Three positive colonies, labelled A3-3, C3-2 and D4-3 were picked into SOB-Zeocin, as recommended by pZErO manufacturers, glycerol stocks were made. Plasmid minipreps were made using GenElute Plasmid Miniprep Kit (Sigma-Aldrich Co., USA) and manufacturer's instructions. The PHV373 PCR was performed to confirm the presence of the PHV insert in the plasmid minipreps.

The size and orientation of the inserts in clones A3-3, C3-2 and D4-3 were determined by PCR using combinations of M13F, M13R, T7 and PHV367R primers. Reaction conditions were changed to suit amplification of larger products, including longer extension times and increased magnesium in some tubes.

Sequencing of the plasmid DNA minipreps as well as PCR products was attempted using the protocol described earlier. Clone A3-3 was also tested at AAHL to verify the results being obtained from WA were reproducible and not due to technical error.

Standard restriction enzyme digests were set up to see if various enzymes cut at the same places for all 3 samples, indicating that the clones are the same. Enzymes used include Nsi1, Hind111, Kpn1, EcoR1 and Pst1.

Due to unsuccessful sequencing attempts a control run was performed. A 373 bp PHV PCR product was purified using DNAce Quick-Clean (Bioline, USA) according to manufacturer's instructions, and used with the PHV373 PCR to freshly amplify a 373 bp product of PHV. This product was processed as for the pZErO clones and submitted for sequencing.

The individual pZErO colonies that made up the pooled tubes A3, C3 and D4, but failed to amplify the specific 373 bp product, were used for further sequence analysis. A PCR was performed on these individual colonies using M13 primers to amplify the entire insert from each clone. Resulting bands were of sizes 450, 650, 750 and >1000 bp. Selected products were cleaned, quantified and sequenced.

It was also considered that the pZErO vector may have been causing the erroneous results, so the 1000 bp insert in pZErO was amplified, gel extracted and cloned into vector pCR2.1 (Invitrogen, USA), using manufacturer's instructions. A PCR was performed to test whether the insert was successfully cloned into the new vector and could be amplified with various primer combinations.

2.1.11 PCR for the detection of insert in pZErO plasmids

The PCR used for all amplification from pZErO plasmids consisted of the same PCR conditions as detailed above for the detection of PHV, with the exception of various primers used instead of the specific PHV primers, an annealing temperature of 55°C and extension time of 90 seconds. The various primers used are listed in Table 1.

2.2 Objective 2: *"To independently establish the sensitivity and specificity of the PCRs and ISH at other laboratories, including AAHL".*

Laboratory work has consisted of further improvement and validation of the PHV373 PCR and ISH developed at the Department of Fisheries WA. The methodology for the PHV373 PCR was taken to AAHL and University of Sydney by Ms Crockford and passed on to staff at those institutes. The methodology for the ISH developed in WA has been sent to AAHL for evaluation and the University of Sydney for use on histological material.

2.2.1 PCR optimisation

The specificity of the PHV373 PCR was tested at AAHL and the FHL in WA using a total of 27 known PHV negative samples, as listed in Table 2.

The sensitivity of the PHV373 PCR was tested at the FHL in WA using a PHV fragment cloned into pCR2.1 (Qiagen, USA) using manufacturer's instructions. The plasmid clone was purified using GenElute Plasmid Miniprep Kit (Catalogue #PLN-70, Sigma, USA) and their recommended protocol. Template added ranged from neat to $1:10^{18}$ dilutions.

2.2.2 *in situ* Hybridization optimisation

A research technician at the University of Sydney performed the PHV ISH technique to test reproducibility and robustness. The specificity of the PHV ISH was tested at the FHL in WA using known PHV-positive pilchard gill, PHV riboprobe, known PHV-negative pilchard gill and YHV-positive prawn section as positive and negative controls.

2.3 Objective 3: *"To then investigate basic aspects of the virus and the disease e.g. the tissue distribution of virus in infected fish, and the correlation between disease in fish and the presence of virus."*

2.3.1 Detection of PHV in different tissues

Samples were tested for the presence of PHV in different tissues of fish collected during the 1995 and 1998 outbreaks. Gill, heart, liver, spleen, intestine and kidney tissues were collected, the DNA extracted using the DNAzol method described previously, and tested for PHV using the PHV373 PCR. PCR products were sequenced using the method described previously.

2.3.2 Samples used for pathogenesis study

The samples used in this study have been examined previously by histology at the University of Sydney and are listed in Table 3. Fish were chosen based on time of collection and histological examination, with the aim of testing a range of fish from pre-epizootic healthy, mildly affected, heavily affected and post-epizootic healthy. Fish collected pre-epizootic in 1995 were healthy and labelled as 95-4667. Fish collected during the 1995 epizootic were unhealthy and morbid, and labelled as 95-4420. Fish collected post-epizootic in 1995 were healthy, and labelled as 95-5135. Fish showing mild histological changes and collected as the mortality front was approaching, designated 95-5000, has also been included in this study to determine if any of the methods used can detect the assumed low level of infection in these samples. Samples were tested by ISH, PCR and real-time PCR.

All samples available were tested by the PHV373 PCR, however some samples were from FFPE blocks and others were freshly extracted DNA samples from frozen fish. DNA extractions for PCR from frozen gill tissue of samples 95-4667, 95-4420 and 95-5135 were performed using the method previously described. DNA extractions from FFPE gill tissue of samples 95-4667 (fish # 11, 13 and 14), 95-4420 (fish # 1 and 2), 95-5000 (fish # 1, A2, 3, B3 and B4) and 95-5135 (fish # 2, 4, 5 and 6) were performed using a freeze/boil extraction method. A total of 3 PCRs were carried out across the range of samples available.

Samples that were prepared and used for PCR were also used for the PHV real-time PCR. A total of 8 real-time PCRs were carried out across the range of samples available.

2.3.3 Real-time PCR

Reaction conditions: Real-time PCR was carried out on an iCycler real-time thermalcycler (Bio-Rad, Australia) using 0.2 mL thin-walled PCR tubes (Bio-Rad, Australia). A master mix was prepared as follows: 1x IQ Sybr Green Supermix (Bio-Rad, Australia), 250 nmol primer PHVRltF 5'- AGATACAGTAAATGCTACTCGTTGGTTT-3', 250 nmol primer PHVRltR 5'- CGCGGCGACCAATAAGAC –3', sterile water (to make volume up to 19 µL). Reaction volume was 20 µL per tube, consisting of 19 µL of master mix and 1 µL of template. A known positive control and negative control, consisting of 1 µL water and master mix, was included with each real-time PCR.

Cycle conditions: 95ºC x 7 minutes, followed by 36 cycles of 95ºC x 15 seconds, 59ºC x 20 seconds, and 72ºC x 25 seconds, then 72ºC x 3 minutes, 40 cycles of 60ºC x 10 seconds with an increment of 0.5ºC per cycle, and finally held at 4ºC. Data collection and real-time analysis was enabled at the annealing step of each cycle (59ºC), and melt curve data collection and analysis was enabled at each increment step in the latter 40 cycles. Samples were analysed by agarose gel electrophoresis, using a 4% gel run at 78 volts for 1 hour 10 minutes. PCR products were visualised under UV for a band of 80 bp.

2.3.4 Confirmation of PHV positives for pathogenesis study

Positive real-time PCR products from samples 95-4667, 95-4420 and 95-5135 (frozen) were cleaned using DNAce Quick-Clean (Bioline, USA) according to manufacturer's instructions, and sequenced at the State Agricultural and Biotechnology Centre, WA, using their standard method.

2.3.5 Examination of archived transmission trial blocks

Archived transmission trial blocks (FFPE tissues) were analysed to determine whether sub-clinical infection was achieved. Blocks were examined previously by TEM and histology, but these methods lacked the sensitivity of the newly developed molecular methods for detection of PHV. Samples tested can be seen in Table 4. Sections were prepared for ISH, or DNA was extracted for PCR and real-time PCR using the methods described previously.

2.3.6 *in situ* Hybridization of transmission trial samples

ISH was used for all FFPE samples using the method previously described.

2.3.7 PCR of transmission trial samples

The PHV373 PCR was used for the detection of PHV in these samples. The amount of each DNA sample added was 1 µL of neat, 1:10 or 1:100 dilutions. DNA could not be accurately quantified due to the DNA extraction method used.

2.3.8 Real-time PCR of transmission trial samples

Samples were tested using the PHV real-time PCR method previously described. Positive samples were cleaned using DNAce Quick-Clean (Bioline, Australia) and sequenced at the State Agricultural and Biotechnology Centre, WA, using their standard protocol. A total of 56 DNA samples from negative control tanks 1, 2 and 3 were analysed in pools of 3-5 by real-time PCR in an attempt to rule out contamination as a factor contributing to positives.

2.4 Objective 4: *"To survey wild pilchard populations to determine whether the virus is still currently detectable and causing disease."*

2.4.1 Survey design

Professor Whittington of University of Sydney compiled the PHV survey design. Since the prevalence of positives, if any, needed to be determined, individual pilchards were tested as one unit. No pilchards were pooled.

In order to obtain an unbiased view of possible PHV occurrence in adult Australian pilchard stocks it was desirable to obtain a representative sample of fish from each sub-population, and therefore included fish from 4 areas:

1. South-eastern Queensland/northern New South Wales
2. Victoria/South Australia
3. South coast Western Australia
4. West coast Western Australia

Randomised samples were collected from the catch by professional fishers with the assumption that schools of fish targeted at any time by these fishers would be representative of the sub-population from which they were caught. Samples were forwarded to the laboratory by fishers.

Methods available at the time for the detection of PHV include histopathology, electron microscopy, PCR, ISH and a recently developed real-time PCR. The real-time PCR was the test chosen for the purpose of this survey due to its higher sensitivity and specificity, and applicability for testing a large number of samples.

2.4.2 Samples used for wild stock survey

For the wild stock testing, a total of 200 fish were collected from each of 4 sites from different pilchard fisheries within Australian waters – northern NSW, SA, Fremantle and the south west region of WA (Bremer Bay and Esperance), as listed in Table 5. All samples were

IQF (individually quick frozen) as this was the most practical method of collection and the real-time PCR has previously proven to be effective on frozen samples.

2.4.3 DNA extraction of wild stock samples

Gill tissues from each fish were taken into DNAzol (Life Technologies, USA) and the DNA extracted using the method previously described. Samples were tested as individuals and not pooled.

2.4.4 Real-time PCR for wild stock survey

Samples were tested using the PHV real-time PCR described previously. PCR product from two wild stock positives were cleaned using DNAce Quick-Clean (Bioline, USA) and their recommended method, and sequenced at the State Agricultural and Biotechnology Centre, WA, using their standard method.

2.5 Objective 5: *"To compare the herpesvirus strains from 1995 & 1998, and to compare, at the molecular level, this herpesvirus and this disease with two other similar herpesvirus fish diseases which have been reported elsewhere in the world."*

2.5.1 Samples used for the comparison of strains from 1995 and 1998

Samples used for sequence data comparison between the 1995 and 1998 outbreaks are listed in Table 6. Each of these samples were gill from the various fish tested, extracted using DNAzol (Life Technologies, USA) as described previously. Due to the difficulties experienced with obtaining additional sequence data, this study has been limited. Unfortunately, the only comparison that could be done in the time frame was a preliminary comparison of the 1995 virus to the 1998 virus. The 373 bases of known PHV sequence were compared with Channel catfish virus and *Oncorhynchus masou* virus previously.

SECTION 3: RESULTS

3.1 Objective 1: *To improve the sensitive and specific diagnostic assays (polymerase chain reactions (PCRs) and in situ hybridization (ISH)) which are based on current limited sequence data. This will include the generation of further sequence data from the available stocks of virus.*

3.1.1 Attempt to obtain additional sequence data from a pZErO DNA library

Pooled colonies from transformed pZErO plasmids were screened by the PHV373 PCR for clones containing the insert. The first and second attempts amplified a product ~450 bp that was too large for the target product, so a 3rd attempt was made. Three pools amplified a band of interest and can be seen in Figure 1. The positive pools were A3, C3 and D4.

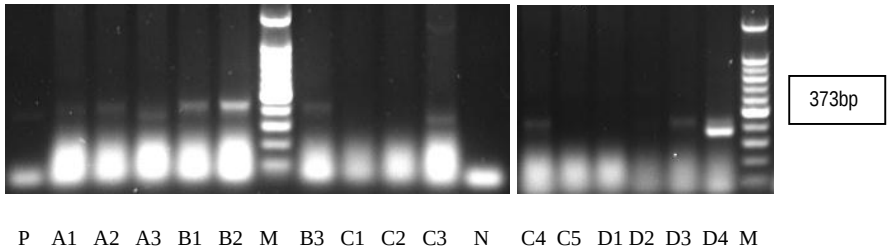


Figure 1. Pooled colonies of pZErO transformants were screened by PCR for the PHV 373 bp fragment and analysed by agarose gel electrophoresis. Pooled samples A3, C3 and D4 showed a band of interest. Other pooled samples showed a larger sized product or primer-dimer only. Lanes are labelled according to the pooled sample labelling. M = 100 bp DNA Ladder (Fisher Biotec, Australia).

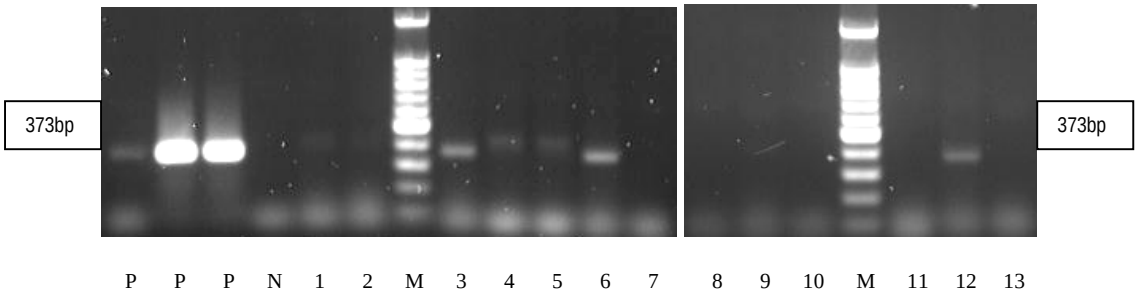


Figure 2. Agarose gel electrophoresis of individual colonies that made up the 3 pooled samples that produced a positive band with the PHV 373 PCR. Three single colonies for each of the pooled samples seen in Figure 1 have shown a band of ~370 bp, in lanes 3, 6 and 12. This confirms that colonies A3-3, C3-2 and D4-3 contain inserts that consist of at least the 373 bp PHV DNA, and possibly unknown sequence data also. The bands seen in lanes 4 and 5 are greater than 400 bp and therefore non-specific. Lanes 1-4 = Samples A3-1 to A3-4; Lanes 5-9 = Samples C3-1 to C3-5; Lanes 10-13 = Samples D4-1 to D4-4; P = Positive control; N = Negative control with water substituted for template.

Individual colonies from the three positive pools were screened to isolate the ones that contained the insert. Results can be seen in Figure 2, with positive clones identified as A3-3, C3-2 and D4-3. The PHV373 PCR confirmed the presence of the PHV insert in the minipreps made. All minipreps amplified a specific band of ~370 bp and no non-specific bands.

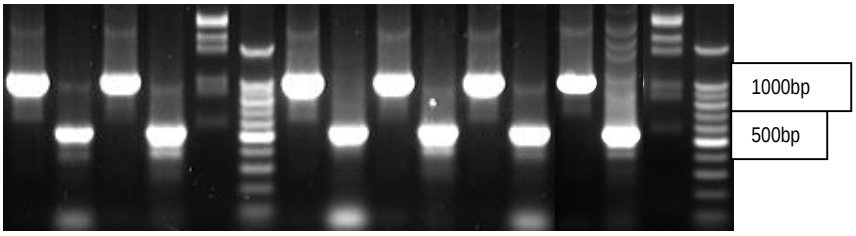


Figure 3. Agarose gel electrophoresis of PCR performed to determine the size of inserts in clones A3-3, C3-2 and D4-3 using various combinations of primers. All PCR products seen when M13F and M13R primers were used are the same size of ~1000 bp, and all PCR products seen when T7 and PHV367R primers are used have produced the same size band of ~500-550 bp. This suggest that the inserts for A3-3, C3-2 and D4-3 appear to be the same inserts or very similar. Lane 1 = A3-3 with M13F & M13R; Lane 2 = A3-3 with T7 & PHV367R; Lane 3 = A3-3 with M13F & M13R and increased magnesium; Lane 4 = A3-3 with T7 & PHV367R and increased magnesium; Lane 5 = C3-2 with M13F & M13R; Lane 6 = C3-2 with T7 & PHV367R; Lane 7 = C3-2 with M13F & M13R and increased magnesium; Lane 8 = C3-2 with T7 & PHV367R and increased magnesium; Lane 9 = D4-3 with M13F & M13R; Lane 10 = D4-3 with T7 & PHV367R; Lane 11 = D4-3 with M13F & M13R and increased magnesium; Lane 12 = D4-3 with T7 & PHV367R and increased magnesium; M1 = Lambda/*Hin*dIII MW Markers (Fisher Biotec, Australia); M2 = 100 bp DNA Ladder (Fisher Biotec, Australia).

The size of the insert in the 3 positive clones was determined, with results shown in Figure 3. All PCR products seen when M13F and M13R primers were used are the same size of ~1000 bp, and all PCR products seen when T7 and PHV367R primers are used have produced the same size band of ~500-550 bp. This suggest that the inserts for A3-3, C3-2 and D4-4 appear to be the same inserts or very similar. Sequencing of the plasmid DNA minipreps as well as PCR products was attempted. The sequence data obtained was not good quality, but matched with *E.coli* and vectors, and was inconclusive.

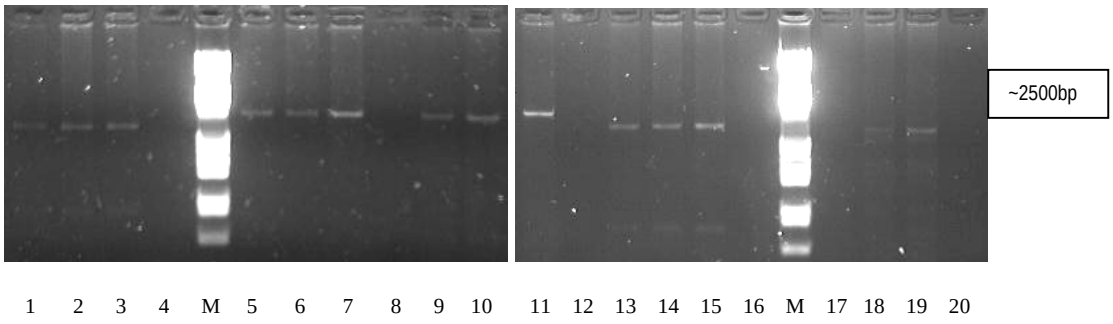


Figure 4. Restriction enzyme digests of the 3 clones thought to contain the known PHV insert. Bands are seen with each clone and each enzyme; negative controls have no visible band. All clones appeared to be cut the same with each enzyme, suggesting that all 3 clones are identical. Lane 1 = A3-3 cut with *Nsi*1; Lane 2 = C3-2 cut with *Nsi*1; Lane 3 = D4-3 cut with *Nsi*1; Lane 4 = Negative control consisting of *Nsi*1 digest mix; Lane 5 = A3-3 cut with *Hin*d111; Lane 6 = C3-2 cut with *Hin*d111; Lane 7 = D4-3 cut with *Hin*d111; Lane 8 = Negative control consisting of *Hin*d111 digest mix; Lane 9 = A3-3 cut with *Kpn*1; Lane 10 = C3-2 cut with *Kpn*1; Lane 11 = D4-3 cut with *Kpn*1; Lane 12 = Negative control consisting of *Kpn*1 digest mix; Lane 13 = A3-3 cut with *Eco*R1; Lane 14 = C3-2 cut with *Eco*R1; Lane 15 = D4-3 cut with *Eco*R1; Lane 16 = Negative control consisting of *Eco*R1 digest mix; Lane 17 = A3-3 cut with *Pst*1; Lane 18 = C3-2 cut with *Pst*1; Lane 19 = D4-3 cut with *Pst*1; Lane 20 = Negative control consisting of *Pst*1 digest mix; M = Lambda/*Hin*d111-*Eco*R1 MW Markers (Fisher Biotec, Australia).

Restriction endonuclease digests of the three positive clones are shown in Figure 4. All clones appeared to be cut the same with each enzyme, suggesting that clones A3-3, C3-2 and D4-3 are identical. A number of sequencing attempts were made but were inconclusive, mostly matching with *E.coli*. It was thought that the sequencing technique may not be

satisfactory, so a control PHV373 PCR was performed to amplify a 373 bp product of PHV. This product was processed as for the pZErO clones, and sequencing of this product was successful.

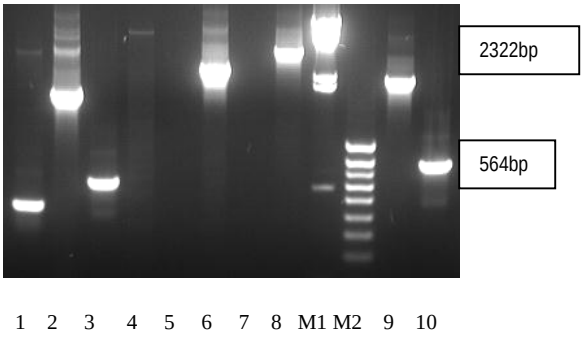


Figure 5. Agarose gel electrophoresis of PCR products from individual pZErO colonies that made up the pooled samples A3, C3 and D4, excluding those that had previously amplified a 373 bp product. All colonies were screened by PCR using M13 primers to amplify the entire insert within each plasmid clone. Controls worked as expected but have not been shown here. Lane 1 = colony A3-1; Lane 2 = colony A3-2; Lane 3 = colony A3-4; Lane 4 = colony C3-1; Lane 5 = colony C3-3; Lane 6 = colony C3-4; Lane 7 = colony C3-5; Lane 8 = colony D4-1; Lane 9 = colony D4-2; Lane 10 = colony D4-4; M1 = Lambda/*Hin*d111 MW Markers (Fisher Biotec, Australia); M2 = Hyperladder IV 100 bp DNA Ladder (Bioline, Australia).

The individual pZErO colonies that made up the pooled tubes A3, C3 and D4, but failed to amplify the specific 373 bp product, were used for further sequence analysis. Resulting PCR products were of sizes 450, 650, 750 and >1000 bp (see Figure 5). All sequences matched *E.coli*.

The PCR products seen by the testing at AAHL were consistent with those seen by the testing in WA. Sequencing results from AAHL were consistent with those from WA, except for 1 sequence, and are listed in Table 1 (date 20.10.03 on table). Sequence data from clone A3-3 mainly matched *E.coli*.

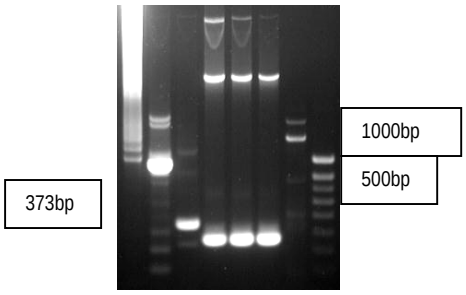


Figure 6. Agarose gel electrophoresis of the 1000 bp product from pZErO A3-3 clone after being cut out and cloned into a new plasmid, pCR2.1. Products of ~900 bp and ~450 bp when M13F or M13R is used with primer PHV373F. This suggests that the PHV373 known sequence is located close to the M13R site on the plasmid. Lanes 4-6 show that the PHV373 product can be amplified from this new clone in pCR2.1. The negative control is showing some large bands, which are certainly non-specific and not interfering with the 1000 bp or 373 bp products. Lane 1 = 1000 bp insert cloned into pCR2.1 with M13F and M13R; Lane 2 = 1000 bp insert cloned into pCR2.1 with M13F and PHV373F; Lane 3 = 1000 bp insert cloned into pCR2.1 with M13R and PHV373F; Lane 4 = 1000 bp insert cloned into pCR2.1 (1 uL) with PHV373F and PHV367R; Lane 5 = 1000 bp insert cloned into pCR2.1 (0.5 uL) with PHV373F and PHV367R; Lane 6 = 1000 bp insert cloned into pCR2.1 (1 uL of 1:10 dilution) with PHV373F and PHV367R; Lane 7 = Negative control (water substituted for template) with PHV373F and PHV367R; M = Hyperladder IV 100 bp Ladder (Bioline, Australia).

It was thought that the pZErO vector may have been causing the erroneous results, so the 1000 bp insert in pZErO was cloned into pCR2.1 and verified by PCR.

The sequencing attempt of the PHV plasmid clone in pCR2.1 was mildly successful with a small amount of data obtained, as shown in Table 7. This data, which was difficult to interpret, did not significantly match any sequence on Genbank and the known PHV 373 base sequence was not found within this new sequence. It is possible that this new sequence is PHV, but if so, then we would expect to find part of the known 373 base sequence within it. Further work was required to solve this problem.

It needed to be confirmed again that the PHV 373 bp fragment could be amplified from this clone in pCR2.1. The PHV373 PCR was repeated, and strong bands were seen at the 373 bp mark. Sequencing returned various results, as listed in Table 7.

Table 1 provides a summary of the sequencing attempts with the pZErO and pCR2.1 clones. As seen from Table 1, many reactions failed, many matched *E.coli*, and a few reactions either matched PHV or were inconclusive.

The forward and reverse primers proven to be specific for PHV, PHV373F and PHV367R, were analysed for other sequence matches using Blastn. Matches for the forward primer included Zebrafish, human and mouse sequences for only 20 of the 25 bases at best. Matches for the reverse primer included *Borrelia sp.*, *Mus musculus* and other insignificant sequences for only 19 of the 25 bases at best. No match of any sequence with PHV373F or PHV367R was considered significant.

Although the sequence data obtained for sample MC6 from the testing on 20.10.03 needed to be further interpreted manually to get clearer data, there was no doubt that the new data matched PHV over the known 373 base region. There are still unknown bases in this

sequence that need to be determined by further testing. This new sequence data, including the sequence data matching the known PHV data, is as follows:

CGGTNTCGGACTGNGNGAAGACAGCGAGNCGTGAGAGANGGTCACATAATGGTCGTAGCGAGAGGTGA
GCACGTTGAANTACGATCANGTGNACAGAGTGGAATGTTGTTAGCACAACCGATATCCGGAGCCAGTGC
NTAGTGATACTGTGCANTATAGATTAGTGATGTTCGAGGTGGTCAGNAGCGTGANATGATNGNTGACGA
ACGGAACNNGTANATNGGTAGTGGTACGATTTGCNGGCGTCCAATATTATNAGCGNATGGCGAGCGCN
ATAAACNCTACTGCANATAGATGNTTAGNCCAGAANACGCGTTGGGTGNAGTGCAGAGGCGCGAGAAG
CCACGGCAGACAAGATGAGTGAGGGCGTAATGNTNAGANACNGGTGTGCAGAGTATGGTGCAGTAGTN
AAAGTTNAGNACTTGAGGCGTGNCGATGATAGNAGANGCAANTGACGGTATCTGCTATAGACAAGAGT
GCNTGAGGTTTAGGCGATCAAACNATGGCAGTATAGCTATTCAGTGCAGCNGAACGATCGTGTAACTAT
AGAAGACATATGAANAGTGCGCATCTAGCAGTGGAGATGATTAAGCAATAANACTGAGCAGTACTCTAT
AATGCAGGATATCACGTANCTCCGTAAGAACTCGNGTCGCAGCTAGTAATAGTACGCGATGATCACACG
TAAGCAGGTATTCGTGTGAACCGTCACGTTACCGGCATCCATGTGCGATATGACGCTGGTATGANAGCG
TCATGTATAGCGAGAGGATAGACCGANATANGTATANCCGGTACGTTAGTGTAGCAATGAGCGTACGGA
TAGGTGGTTCGAGTGGATGGACGCGATCGAANATGTACTGTGCGGTCAAAGCGCGGGACCTGTGTGGC
CNGGGGCATCCGCTGTCATCGAGAGGGAAGTGAGGGCGGACCANGTGTCGANNAGGCTTGACGTNNNA
GCGCGGGTGGNATTTGTGGGATAATGTAGGCGGTAACAACGTACTTTGTATGGATTAGACTGCGGGTTA
GGCCCACACCCGGAGAGGTTCCCGGACCATATTCCCAAATGGCCATCTACCCCAGGAATGGTGTTCCAG
ATTGNTAAAATTTTCACAAGATACAGTAAATGCTACTCGGTGTGTTTNTAGCCCCTTCAGACAATGTGGG
AAGACCACCATCATGCGTCTTTATTGGTCGCCGCGATGGTTCTTCACACGGGATATGGACGTGTGTGGT
CCAAGCTCAAAACATAAACATGTGNAAGCAAAACTTCAAGGGCTGTTGTCGCGATTGATGGCGGACTTC
ATGGACCGAGCCGAACGTTAGAGAAGGGAACACCGCTACACAAGAATGGTTGGACCCATGGGGGAGGA
GTGANANAGNCCA**ANTTNNNCCCCCCCCCCTNCNTNNNNNNGNGAGAACATAGATNACGAGGCTGTNGT**
GANGTACGAANATGNGGGAGATGTATGTGTCGCTAGCANCGATGCGAAT

The red text denotes sequence that matches with the known PHV 373 base sequence. The black text represents unknown, and possibly new PHV-, sequence data.

This sequence data was analysed by performing a Blastn search. A total of 1491 letters were entered in the search and only 1 sequence in Genbank matched. This matching sequence produced a high alignment score and was determined to be Pilchard herpesvirus putative terminase.

3.1.2 PCR for the detection of Pilchard herpesvirus

Due to the difficulties in obtaining additional sequence data and time constraints, a PCR to detect PHV over a new region of sequence was not developed. The PHV373 PCR is currently the PCR of choice for the detection of PHV.

The 373 bp fragment, specific for PHV, is shown in Figure 7. It can be seen from Figure 7 that the PHV373 PCR did not amplify PHV from DNA samples from fish collected pre- or post-outbreak, but consistently produced a specific band of the correct size (373 bp) for samples from fish collected in the midst of the epizootic.

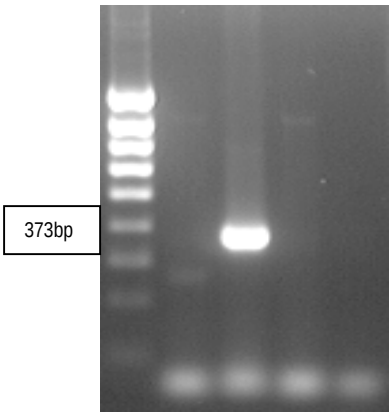


Figure 7. Agarose gel electrophoresis of PCR product amplified by the PHV373 PCR. Only one band of the expected size was amplified, and this was from the sample from a fish collected at the height of the mortality front. Samples from fish collected both several days before and after the mortality front failed to amplify a band of 373 bp. M = 100 bp DNA Ladder Hyperladder IV (Bioline, Australia); Lane 1 = DNA sample from fish collected pre-outbreak,1995 epizootic; Lane 2 = DNA sample from fish collected during 1995 epizootic; Lane 3 = DNA sample from fish collected post-outbreak, 1995 epizootic; N = Negative control (PCR master mix and sterile water).

The sequence that was consistently obtained using PCR product from the partially purified virus derived from fish in WA in 1998 was used as the reference sequence. The sequence, 373 bp in length including primers shown in bold lowercase, is consistent with initial sequence data previously described (Eaton *et al*. 2002), and is as follows:

acacatgattttggataaactggggTTGGCCACACCGGAGATTGCCGCACATAATCCCAAATGTGCATCACCCAGTACTGTG
TTACAGATTGATAAAATTTTCACAAGATACAGTAAATGCTACTCGTTGGTTTTAGCCCCTCGACAATGTGGGA
AGACCACCATCATGGTCTTATTGGTCGCCGCGATGATTCTTTACACGGATATGGACCTGGTGGTCCAAGCTCA
AAACATAAACATGTGTGAGCAAAACTTCAAGGGCGTTGTCGCGCTGATGGACGACATCATGGACGAGCCGAC
CTTCGAGAAGGAACACCGCTACACAAGAATGGTTGGTACCATGGAGGG**ggcgcggttcatcttcaacccagat**

This reference sequence has been listed as Genbank Accession Number AY995177. A paper describing the PHV373 PCR and PHV sequence was published in December, 2005 (Crockford *et al*. 2005).

3.1.3 *in situ* Hybridization for Pilchard herpesvirus

The PHV ISH was optimised using PCR product from the PHV373 PCR. An ISH technique to detect PHV over a new region of sequence was not attempted due to the problems encountered with obtaining additional sequence data.

Gill tissue appeared to contain the highest concentration of PHV compared to the other tissues, and therefore this was the tissue used for testing. Controls included gills that were known to be negative for PHV and PHV probe, Salmon gill showing an inflammatory reaction and PHV probe, prawn tissue and PHV probe, and Yellowhead Virus probe and PHV positive gill tissue (see Figures 8 and 9). No non-specific staining of probe is seen with these negative controls (see Figure 9). A large amount of probe staining can be seen with the PHV-positive sections shown in Figure 8.

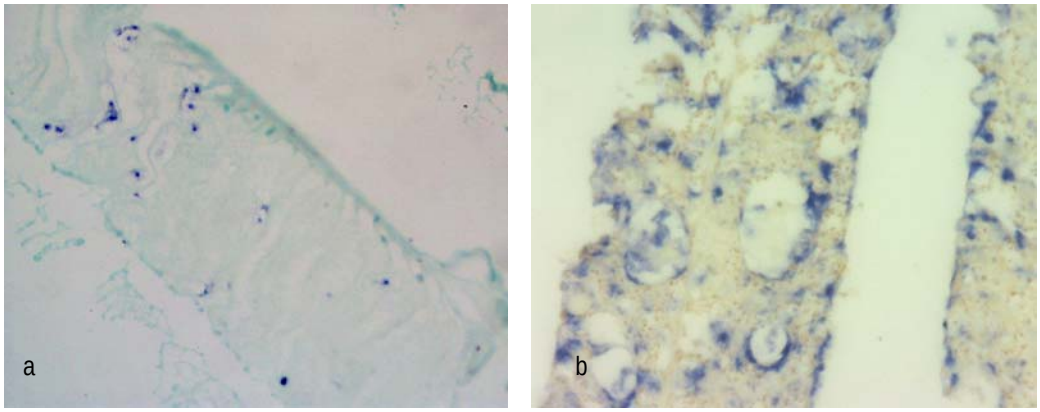


Figure 8. The distribution of PHV within the gills of a PHV-positive section. (a) PHV is represented by the dark blue staining against a Light Green counterstain, at x200 magnification. (b) PHV-positive gill tissue, against Bismark Brown counterstain at x400 magnification.

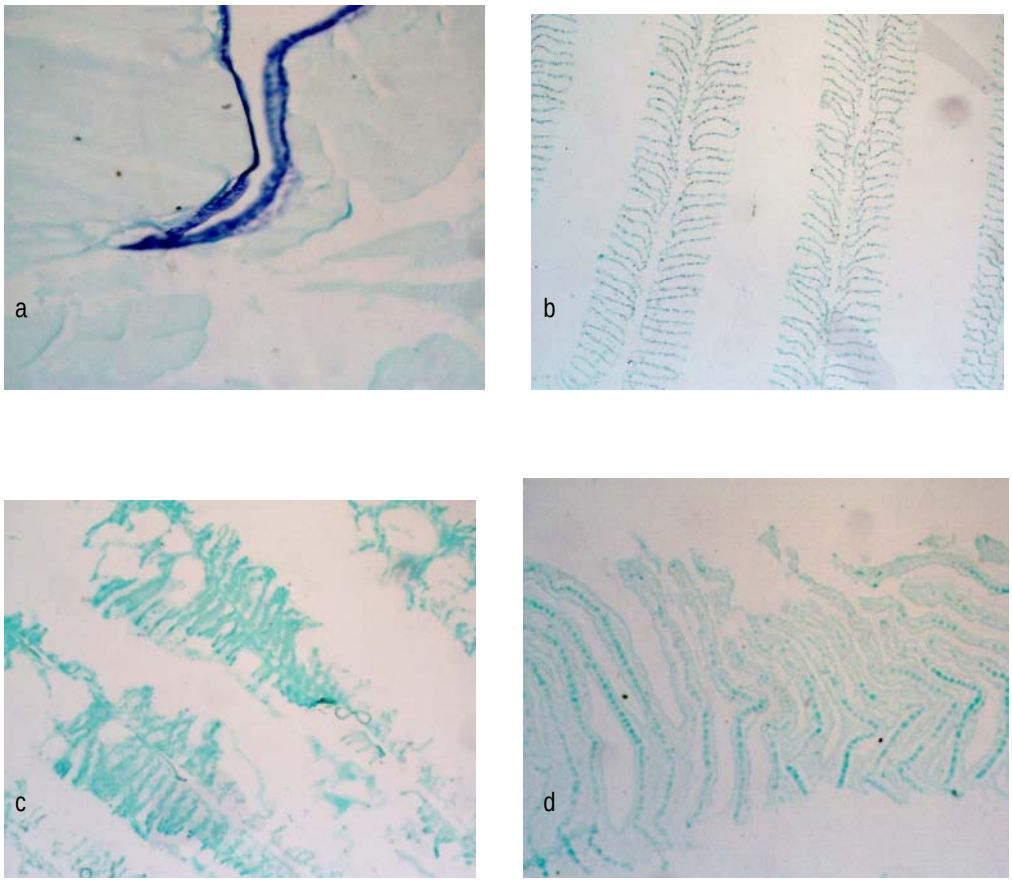


Figure 9. Various controls used for optimisation of the PHV ISH technique. (a) PHV riboprobe and YHV-positive prawn section showing some non-specific staining, however, this non-specific staining can clearly be differentiated from specific staining. There is no specific staining seen in this section (x200 magnification; Light Green counterstain); (b) PHV riboprobe and known PHV-negative pilchard gill, demonstrating the absence of PHV in this section (x100 magnification; Bismark Brown counterstain); (c) YHV riboprobe and known PHV-positive pilchard gill, illustrating no cross-reactivity (x100 magnification; Light Green counterstain); (d) PHV riboprobe and salmon gill showing signs of an inflammatory response. The PHV riboprobe is not cross-reacting with inflammatory cells (x100 magnification; Light Green counterstain). YHV = Yellowhead Virus, which causes disease in prawns.

A paper describing the PHV ISH protocol has been written for publication in Diseases of Aquatic Organisms (Crockford *et al*., under review).

3.2 Objective 2: *To independently establish the sensitivity and specificity of the PCRs and ISH at other laboratories, including AAHL.*

Laboratory work consisted of further improvement and validation of the PHV373 PCR and ISH developed at the Department of Fisheries WA. The methodology for the PHV373 PCR was sent to AAHL for evaluation. The methodology for the ISH developed in WA was sent to the University of Sydney for evaluation and use on histological material. The methodology for the PCR developed at AAHL was sent to WA.

3.2.1 Sensitivity of the PHV373 PCR

The PHV373 fragment cloned into pCR2.1, as described previously, was used to determine the sensitivity of the PHV373 PCR. PHV could be amplified by this PCR when a $1{:}10^7$ dilution of template or higher, were added to the PCR mix, which equates to the PHV373 PCR able to detect as few as 7.76×10^4 virus particles.

3.2.2 Specificity of the PHV373 PCR

Twenty-seven known PHV-negative samples (see Table 2) were tested using the PHV373 PCR to check that cross-reactivity was not an issue.

3.2.3 Sensitivity of the ISH

The sensitivity of the PHV ISH was not determined due to the unavailability of suitable material.

3.2.4 Specificity of the ISH

The specificity of the PHV ISH was previously determined and is shown in the results for Objective 1.

3.3 Objective 3: *To then investigate basic aspects of the virus and the disease e.g. the tissue distribution of virus in infected fish, and the correlation between disease in fish and the presence of virus.*

3.3.1 Detection of PHV in different tissues

PHV was most prevalent in heart tissue (73.3%), then gill tissue (72.7%) (see Table 3 and Figure 10), although the intensity of the PCR products from gill tissue was higher than that from heart tissue. Other tissues were infected with PHV, but not at such a high prevalence and some PCR products from these only produced a weak result.

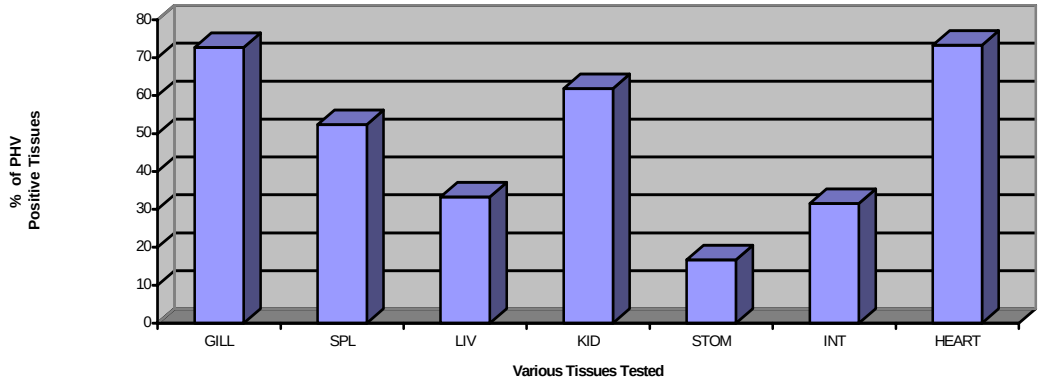


Figure 10. Prevalence of PHV in various tissues. Samples included tissues of pilchards from 1995 and 1998. Heart and gill tissue show a high prevalence, followed by kidney, spleen, liver, intestine and stomach.

Most gill samples tested had an intense band at the 373 bp mark. Some of the heart and kidney tissues tested showed a strong band at the 373 bp mark also, however these bands were not as intense as those shown in most of the gill tissues tested (data not shown).

3.3.2 Histology of samples for pathogenesis study

Histological examination of suitable samples was performed at the University of Sydney, with results shown in Table 8.

3.3.3 *in situ* Hybridization of samples for pathogenesis study

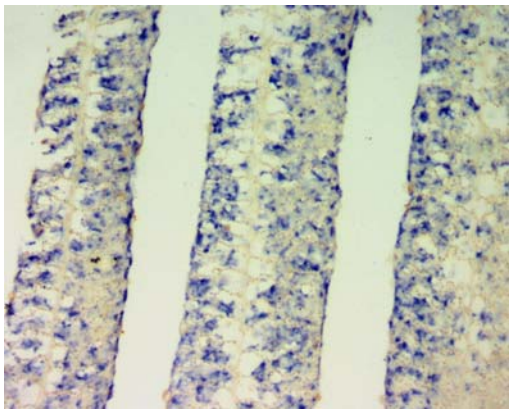
ISH results are shown in Figure 11.

Samples 95-4667 and 95-5135 were consistently negative by ISH.

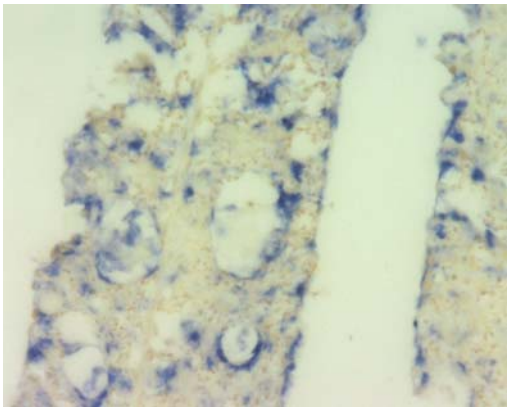
Sample 95-4420 was consistently positive, as seen in Figure 11a and 11b. Note that positive staining is both nuclear and cytoplasmic, as expected for this virus, and is consistent with previous electron microscopy studies performed at the FHL. Figure 11a and 11b demonstrate that this sample is heavily infected with PHV.

Sample 95-5000 was ISH positive but only a few cells were positive, as shown in Figure 11c. All positive cells were in close proximity and surrounded by negative cells and tissue, indicating that the positive staining seen is due to specific staining of riboprobe and not background staining.

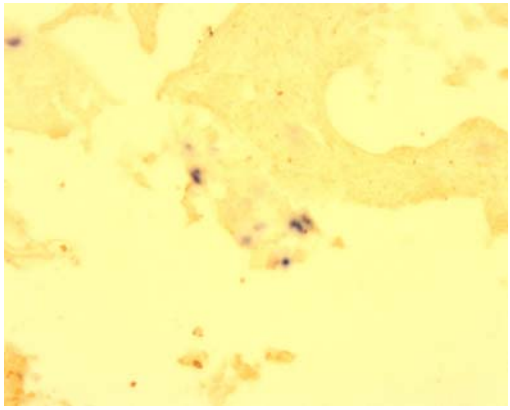
Figure 11d is a negative control slide for the ISH, and illustrates that non-specific staining is not evident, and any positive staining seen in other samples is likely to be due to hybridization of riboprobe to PHV.



(a) ISH of sample 95-4420.This picture is clearly illustrating the distribution of PHV DNA within the tissue, as seen by the dark blue staining against the brown counterstain. This tissue is markedly infected with virus. (x200 magnification)



(b) ISH of sample 95-4420 at higher magnification (x400). This photograph shows specific staining, both nuclear and cytoplasmic, of the PHV riboprobe to tissue heavily infected with PHV.



(c) ISH of sample 95-5000 B4. Here we can see a few cells with dark blue staining, indicating the presence of virus. There is clearly no background or non-specific staining seen on the section. This Tissue was collected just as the outbreak was beginning, so results are consistent with the low virus levels that may be expected at the beginning of a virus outbreak. (x400 magnification)

(d) ISH of a gill from an unaffected pilchard. Note that the lamellae are separate from each other, unlike the fused lamellae seen in (a) and (b), indicating normal gill tissue. No staining of PHV probe is seen. (x400 magnification)

Figure 11. ISH of gill tissue from various infected and uninfected fish. All section have been counterstained with Bismark Brown to illuminate the background tissue. Slides (a), (b) and (c) clearly demonstrate some positive staining of the PHV probe. Slide (d) is a negative control slide. Slides (a) and (b) show gill tissue from a heavily affected pilchard collected during the outbreak, demonstrating the marked presence of virus within this tissue. Slide (c) was tissue from fish collected at the time the outbreak was beginning, and therefore we would expect low levels of virus to be present as a minimum. In this slide, a small number of cells have stained positive with the PHV probe, indicating that virus is present within these cells.

3.3.4 PCR of samples for pathogenesis study

Samples 95-4667 and 95-5135 from frozen fish were consistently negative. Sample 95-4420 from frozen fish was consistently strongly positive. These results are as expected, since samples 95-4667 and 95-5135 appeared to be healthy and were not collected at the time of the peak of the outbreak. Sample 95-4420 was clearly infected at the time of collection and the positive PCR result is consistent with the history of this sample. A typical gel electrophoresis photo of these samples can be seen in Figure 12.

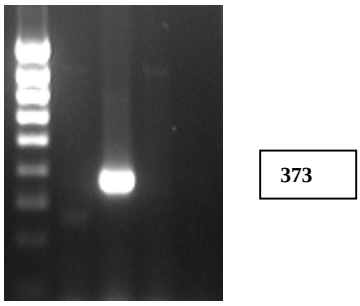


Figure 12. Agarose gel electrophoresis for PCR of samples from frozen fish collected pre-, during and post-outbreak. The only PCR product of the expected size is seen in Lane 2 (373 bp), the sample being from a fish collected during the outbreak. PCR product was not amplified from samples collected pre- and post-epizootic. M = Hyperladder IV 100 bp MW Marker; Lane 1 = Sample 95-4667 collected from frozen fish pre-epizootic; Lane 2 = Sample 95-4420 collected from frozen fish during the epizootic; Lane 3 = Sample 95-5135 collected from frozen fish after the mortality front had passed; Lane 4 = PCR negative control consisting of water and PCR master mix.

A summary of PCR results is shown in Table 8. PCR product from sample 95-4420 was sequenced for confirmation that it is PHV.

3.3.5 Real-time PCR of samples for pathogenesis study

Samples tested by the PHV real-time PCR are listed as a summary of results in Table 4. For DNA samples extracted from frozen fish, results were consistent upon repeated testing, with 95-4667 and 95-5135 positive, and 95-4420 strongly positive. For FFPE samples, 95-4667 and 95-5135 were negative. Gel electrophoresis and sequencing confirmed PHV positives.

3.3.6 *in situ* Hybridization of archived transmission trial samples

No positive stained cells were seen in any samples tested by ISH (see Table 9).

3.3.7 PCR of archived transmission trial samples

All samples tested by PCR, both at AAHL using the method developed by AAHL in the 1990's and at the FHL in WA using the PHV373 PCR, were negative (see Table 9).

3.3.8 Real-time PCR of archived transmission trial samples

Results of real-time PCR testing for transmission trial samples are shown in Table 10. Figure 13 shows the amplification graph for these samples. Figure 14 shows the melt curve graph for these samples.

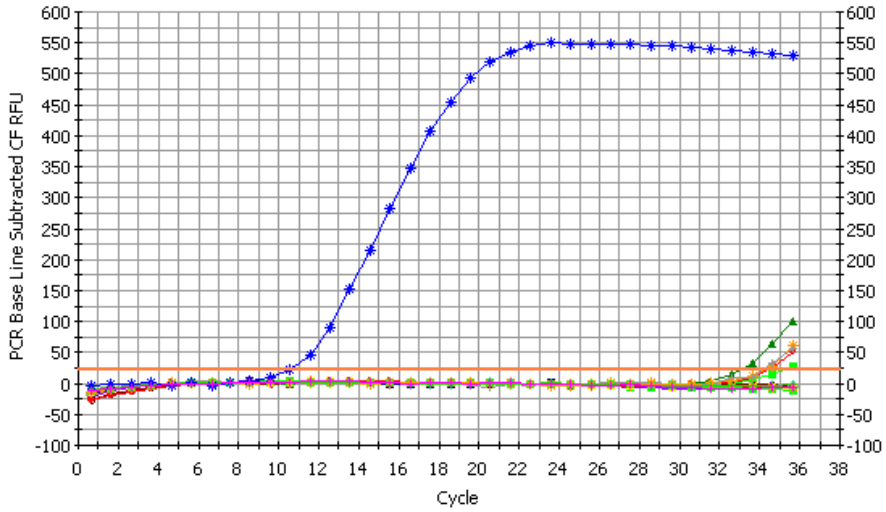


Figure 13. Amplification graph for transmission trial samples tested by the PHV real-time PCR. Positive samples are T1D3 neat, T1D1 neat, T2A6 neat and 1:10. All other samples are clearly negative, with no amplification. T1D3 1:10 (light green) is considered to be negative as this sample did not register a Ct value, even though the amplification graph suggests this sample is borderline positive. Red = T1D3 neat, Ct 34.5; Light green = T1D3 1:10, no Ct value; Dark green = T1D1 neat, Ct 33.0; Orange = T2A6 neat, Ct 34.2; Grey = T2A6 1:10, Ct 34.2; Blue = positive control (PCR product cloned into vector pCR2.1), Ct 10.5; Pink = negative control (water substituted for template), no Ct value.

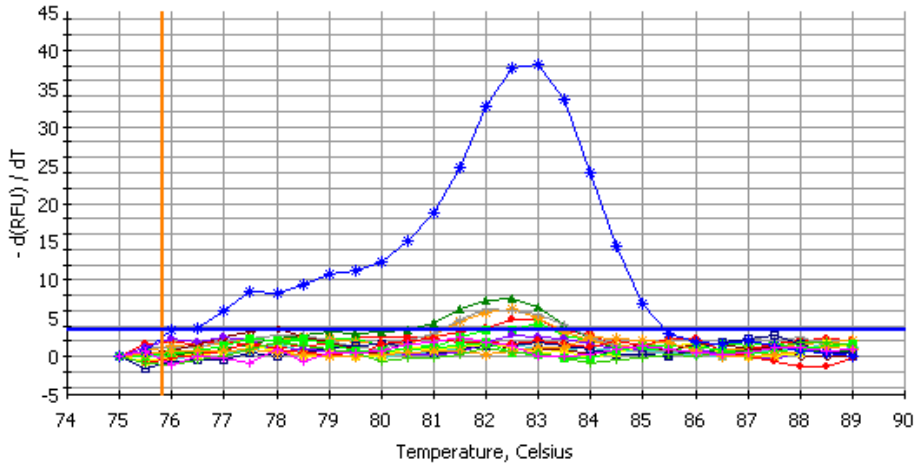


Figure 14. Melt curve graph for transmission trial samples tested by the PHV real-time PCR. As seen from the melt curve, no non-specific amplification is evident, and only a few samples showing small, but specific, melt peaks consistent with the PHV positive control. Positive samples are T1D3 neat, T1D1 neat, T2A6 neat and 1:10. All other samples are clearly negative, with no melt peak above the threshold. T1D3 1:10 (light green) is considered to be negative as this sample did not register a Ct value, even though the melt curve graph suggests that this sample is borderline positive. Red = T1D3 neat; Light green = T1D3 1:10; Dark green = T1D1 neat; Orange = T2A6 neat; Grey = T2A6 1:10; Blue = positive control (PCR product cloned into vector pCR2.1); Pink = negative control (water substituted for template).

All PCR products from the 3rd real-time PCR were analysed by agarose gel electrophoresis to confirm results. Samples T1D3, T1D1 and T2A6 were sequenced at the State Agricultural and Biotechnology Centre, WA, using their standard method. Data from all samples sequenced was consistent with PHV.

Due to the unexpected number of positive samples from the real-time PCR testing, all available negatives were tested to further validate that the positives being seen were not false positives. A total of 56 samples from negative control tanks 1, 2 and 3 were analysed in pools of 3-5 by real-time PCR and were concluded to be negative by this testing (see Table 11).

3.4 Objective 4: *To survey wild pilchard populations to determine whether the virus is still currently detectable and causing disease.*

3.4.1 Real-time PCR for wild stock survey

A real-time PCR was performed for 32 PHV-negative samples and 2 PHV-positive samples to check the specificity of the real-time PCR (see Table 12). All PHV-negative samples were negative and the 2 PHV-positive samples were positive. The amplification graph and melt curve graph for some of these samples are shown in Figures 15 and 16 respectively.

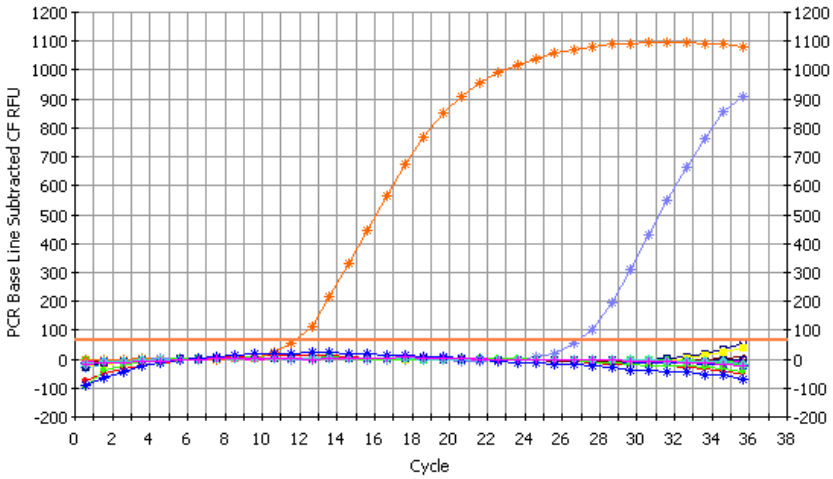


Figure 15. Amplification graph for some samples, listed in Table 12, tested using the PHV real-time PCR. Samples included in this real-time PCR are #1-12, 15, 17, 18, 21, and 23, plus positive and negative controls. The only amplification seen was from the 2 known PHV positive samples. Note that none of the known PHV negative samples have shown amplification above the threshold. Orange = positive control (PCR product cloned into vector pCR2.1), Ct 11.5; Mauve = sample #5 (AAHL PHV positive), Ct 26.5; Red = sample #10 (AAHL PHV negative), no Ct value; Light blue = negative control (water substituted for template), no Ct value. Other colours = other samples listed in Table 12, no Ct value.

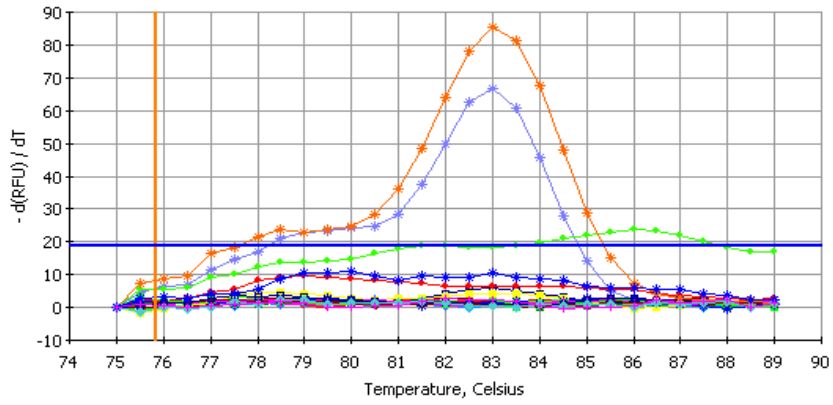


Figure 16. Melt curve graph for some samples, listed in Table 12, tested using the PHV real-time PCR. Samples included in this real-time PCR correlate with those seen in Figure 15. The only melt peaks seen are from the 2 known PHV positive samples, and these are specific at the melt temperature of 83.0ºC. None of the known PHV negative samples have shown a melt peak. All samples were chosen for further analysis by agarose gel electrophoresis. Orange = positive control (PCR product cloned into vector pCR2.1); Mauve = sample #5 (AAHL PHV positive); Red = sample #10 (AAHL PHV negative); Light blue = negative control (water substituted for template); Other colours = other samples listed in Table 12.

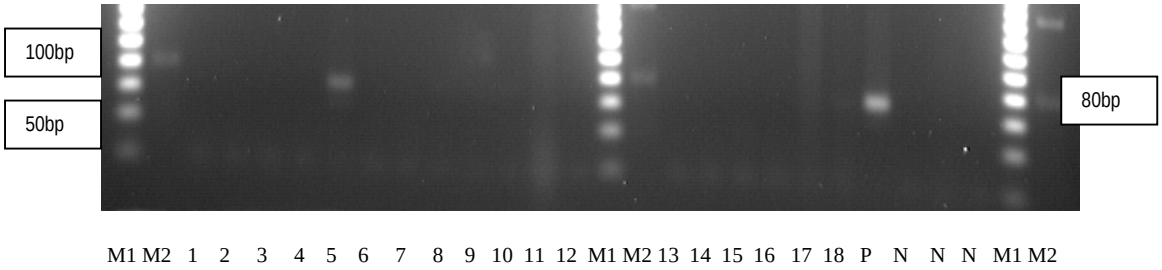


Figure 17. Agarose gel electrophoresis of samples seen in Figures 15 and 16. Specific bands of 80 bp can be seen in lane 5 (sample 5 from Table 12) and for the positive control, and is consistent with results illustrated by Figures 15 and 16. All other samples do not show any specific band, confirming that these samples are negative for PHV by this testing. Lanes 1-12 = samples #1-12 from Table 12; Lanes 13-18 = samples 15-18, 21 and 23 from Table 12; P = positive control (PCR product cloned into vector pCR2.1); N = negative control (water substituted for template); M1 = 25 bp DNA Step Ladder (Promega, USA); M2 = Hyperladder IV 100 bp MW Marker (Bioline, Australia).

A total of 800 samples, as shown in Table 5, were tested by real-time PCR for the presence of PHV. Results are shown in Table 13. The total prevalence calculated from all 800 fish tested using the PHV real-time PCR is 13.75% (see Table 13). The southwest region of WA, over three batches of fish collected, have an average prevalence of 21.7%. Fish collected in two batches from Fremantle, representing the west coast region, have an average prevalence of 12.5%. Fish collected from waters off the SA coast have a prevalence of 17%. Fish collected from the eastern coast have a prevalence of only 0.5%.

Table 6 shows samples that were selected for testing using the conventional PHV373 PCR. Positives were detected with the conventional PCR, thus reinforcing that PHV can be detected in wild pilchards. The sensitivity of the PHV373 PCR is limited compared to the PHV real-time PCR, as evident in Table 6. The PHV373 PCR has detected PHV in 2/25 samples. The real-time PCR has confirmed PHV in these two samples plus detected PHV in another 5/25 samples. Positives from the PHV373 PCR were sequenced and matched the known PHV 373 base sequence.

3.5 Objective 5: *To compare the herpesvirus strains from 1995 & 1998, and to compare, at the molecular level, this herpesvirus and this disease with two other similar herpesvirus fish diseases that have been reported elsewhere in the world.*

3.5.1 Comparison of 1995 and 1998 strains

PHV373 PCR products from pilchard gill samples (see Table 14) were used for obtaining sequence. Three samples were virus from the 1995 outbreak and 3 were from the 1998 outbreak. From these 6 samples, only one showed any sequence variation, which was from a 1998 sample. This could be a true inconsistency or it could be a sequencing inaccuracy. In either instance, the variation consisted of a silent base change with the codon still coding for histidine.

SECTION 4: DISCUSSION

4.1 Objective: *To improve the sensitive and specific diagnostic assays (polymerase chain reactions (PCRs) and in situ hybridization (ISH)) which are based on current limited sequence data. This will include the generation of further sequence data from the available stocks of virus.*

This was achieved.

Results of sequencing from Table 7 prove that the 373 bp fragment being amplified matches the PHV known sequence. Other results were not so easy to interpret. It appears that when the 1000 bp plasmid clone is used in direct sequencing with PHV367R, the sequence data does not match with anything significant in Genbank. When the 373 bp product amplified from the 1000 bp clone in pCR2.1 is sequenced with PHV373F primer, the sequence matched with the known PHV 373 bp product. When the 373 bp product amplified from the 1000 bp clone in pCR2.1 is sequenced with PHV367R primer, the sequence matched with *E.coli* and the sequences previously obtained from the pZErO 1000 bp clones.

Samples from Table 1 sequenced on 20.10.03 produced some interesting results. Sequence data was edited to obtain better data and analysed by Blastn for sequence similarity with organisms on the database. Samples MC7 and MC8 were included as positive controls and matched PHV, confirming that the sequencing run was successful. Samples MC1 and MC2 were from a fragment of approximately 1000 bp and matched *E.coli*. Samples MC3 and MC4 were from a fragment of approximately 600 bp and matched *E.coli*. Samples MC5 and MC6 were from a fragment of approximately 900 bp and produced varying results. MC5 was inconclusive. MC6 matched with PHV but also had some extra sequence that did not significantly match any other organism on the database. This new sequence data was analysed by several programs, including Blastn, Blastx, and Blastp, with no significant matches returned. This implies that the new sequence data is in fact PHV that has not been previously characterised. The match of MC6 sequence over the 373 bp of known PHV confirms that the fragment is derived from a sample containing PHV, and suggests that the uncharacterised sequence data is PHV.

With the number of unsuccessful attempts to obtain PHV sequence from these pZErO clones, it can be said that PHV sequence is not easily amplified using these clones and these combinations of primers. Unfortunately, due to time constraints, this investigation has not been explored further, although this work has shown very significant and promising results for obtaining uncharacterised PHV sequence.

It appears that additional sequence data for PHV was identified. However, this sequence data was of low quality, with some bases undetermined, and so further investigation is required. Specific primers should be designed from this new sequence data in areas where good sequence has been obtained and all bases determined, and sequenced from PCR product and by direct sequencing. The entire base composition needs to be determined by repetitive sequencing before it can be entered in Genbank as new PHV sequence. Upon determination of the complete base composition, further analysis can be carried out with regard to protein prediction studies, relation to other herpesviruses, and sequence similarity over this region for the 1995 PHV and the 1998 PHV.

The sequences in Table 1 listed as MC3 from 4.3.04 and MC5 and MC6 from 19.3.04 were aligned significantly enough to be considered the same sequence, with only a few bases different or not determined due to compromised sequencing reaction efficiency. No significant matches were found by Blast analysis. This suggests one of two things - this sequence data is new sequence and has not been characterised previously or this data is invalid. No part of the PHV 373 base sequence was found within the consensus sequence and it is therefore considered to be invalid until proven otherwise. It was noted that the sequence data obtained from all plasmid sequencing was low quality and this may have contributed to the consensus sequence not matching with the known PHV 373 base sequence.

This task of finding new sequence data with limited virus stocks, and part purified stocks at best of unknown viability, proved difficult. If live virus were available, different approaches would have been attempted and more resources would have been available. The pZErO library preparation was not documented and certain assumptions had to be made, which made analysis trying from the start. It is concluded that new PHV sequence data has been obtained from this library, after numerous attempts, and, although this data is preliminary and needs further testing, it is certainly a promising step in the right direction. Further studies should include an attempt to re-make the pZErO library, using virus in its purest form.

The PHV373 PCR and PHV ISH were optimised. With the difficulties of discovering additional sequence data for PHV due to the limited virus stocks available, these 2 methods were optimised based on the known 373 bases of PHV sequence. For the ISH, variable parameters were optimised, such as using skim milk powder instead of bovine serum albumin, and the optimised method was checked against controls for specificity (see Objective 2). For the PCR, reagent variables were optimised, such as primer and magnesium concentrations. The optimised method was checked for specificity and the sensitivity determined.

4.2 Objective 2: *To independently establish the sensitivity and specificity of the PCRs and ISH at other laboratories, including AAHL.*

Objective 2 was achieved.

The sensitivity and specificity of the PHV373 PCR were determined. The PHV373 PCR could detect as little as 7.76×10^4 copies of virus and was specific for PHV against the samples tested.

The specificity of the ISH was tested against all available controls. The positive control consisted of PHV riboprobe applied to a known PHV-positive pilchard gill section (see Figure 8), as determined by PCR. Negative controls are shown in Figure 9 and included PHV riboprobe applied to Yellowhead virus-positive prawn section, PHV riboprobe applied to PHV-negative pilchard gill section, PHV riboprobe applied to a salmon gill section showing an inflammatory response, and Yellowhead virus riboprobe applied to a PHV-positive gill section. The ISH was specific for PHV and did not react with any of the negative controls.

The PHV373 PCR was also performed at AAHL and the University of Sydney to confirm the reproducibility of results. The ISH was also performed at the University of Sydney to check consistency in the technique.

It should be noted that the reporting of samples that are negative for PHV in this project could be negative for virus or virus may be present but below the determined limit of sensitivity of the test. The reporting of a negative result could mean that virus is present but in such low numbers that PHV is not detectable.

4.3 Objective 3: *To then investigate basic aspects of the virus and the disease eg. the tissue distribution of virus in infected fish, and the correlation between disease in fish and the presence of virus.*

Objective 3 was achieved.

Table 3 and Figure 10 show the prevalence of PHV detected in different tissues. Heart showed the highest prevalence, although gill also showed a high prevalence. The intensity of the band after PCR from gill tissue was the often much more intense than the other tissues tested (data not shown), suggesting that this would be the best tissue to test for PHV, due to the likelihood of PHV being present in this tissue, if the fish is infected, as well as giving a strong PCR result. This was as expected, due to the fact that the primary lesions were observed in the gill of all affected fish, and therefore this is where we would expect the highest concentration of virus in the fish.

As listed in Table 8, sample 95-5000 appears to be mildly affected, with only slight histological changes noted and fish still live but showing signs of distress. Sample 95-4420 appeared to be heavily affected on collection (see Table 8), with fish dead or near death and showing classic symptoms of PHV disease. Histological examination of this sample showed marked changes in the gill tissue, including severe hyperplasia in the gill epithelium, a sign that is characteristic of PHV infection. Samples 95-4667 and 95-5135 appear to be non-affected either by not being exposed to the virus (pre-outbreak) or recovering from exposure to the virus (post-outbreak) (see Table 8). Histological examination of these samples could not reveal any significant changes seen that may be suggestive of viral infection.

For the PCR, all FFPE samples were negative as a neat and 1:10 template by PCR, as shown in Table 4. It was assumed from these results that samples prepared for histology were not suitable for PHV373 PCR testing. It is likely that the proteins were cross-linked and the DNA fragmented through processing, as no positive amplification was seen, even with tissue that was strongly positive by other methods. A positive control, consisting of sample 95-4420 DNA extracted from frozen fish plus PCR master mix, was included with the PCR to ensure that the PCR itself had worked, and a strong specific PCR product was amplified. The only positive by PHV373 PCR was sample 95-4420 from frozen fish.

Samples 94-4420 and 95-5000 showed positive cells when tested by the PHV ISH. Samples 95-4667 and 95-5135 were negative. Results are consistent with histology.

DNA samples that were extracted from FFPE blocks and tested by real-time PCR showed a pattern. All 95-4420 samples, both neat and 1:10 dilutions, were strong positives. This was also the case with 95-5000 samples. This is interesting because these samples were not positive by PCR and only one of these 95-5000 samples (#B4) was positive by ISH, yet all of these samples are positive by real-time PCR. All 95-5135 samples, both neat and 1:10 dilutions, were negative by this testing. All 95-4667 samples, both neat and 1:10 dilutions, were negative by this testing.

Re-examination of archived transmission trial samples produced some interesting results. All samples were negative by ISH, AAHL's PCR and the PCR developed in WA, yet 3 of 6 samples were positive by real-time PCR.

Samples that were consistently positive by real-time PCR were T1D3, T1D1 and T2A6. Samples that were concluded as negative by this test were T3B1, T1C3, T1D5, C1-2 (transmission trial negative control), AS-03-4361 (negative control, goldfish) and AS-03-1989 (negative control, koi carp). Positive transmission trial samples had a Ct value of >30 (positive control had a Ct of ~9), indicating low levels of virus present, and melt temperatures were within ± 1.0 of the positive control (melt temperature 83.0ºC). These results suggest that PHV was transmitted to healthy pilchards during the trial, although disease was not produced. It may be that only low levels of virus were transmitted, which is suggested by the real-time PCR results, and were not enough to cause disease.

4.4 Objective 4: *To survey wild pilchard populations to determine whether the virus is still currently detectable and causing disease.*

Objective 4 was achieved.

There are 2 life history scenarios that would contribute to the possible distribution of PHV:

1. All the fish that were infected in 1995 and 1998/9 died due to PHV, and those remaining in 1995 have since died. It is also possible that not all pilchards were infected. This would mean that PHV is now absent and exotic.

2. PHV is maintained by latency or a constant low level spread and only a small number of fish suffer. At times of low environmental viral load, high host immunity or other unfavourable circumstances, efficient propagation of infection does not arise. PHV is present either throughout the population or restricted to sub-populations. In this scenario, PHV is now present and endemic.

It was determined in the survey design that a prevalence of >10% of PHV in wild pilchards is significant. Results of this survey show that all samples tested have, on average, a prevalence of 13.75% of detectable PHV. This suggests that a significant number of wild pilchards are infected with PHV and that PHV is now endemic.

The results suggest variation in the prevalence of infection depending on the location of the pilchard subpopulation. However, the sample numbers were small relative to the total population of pilchards, and the sampling method was not ideal. Samples were collected following a randomised collection protocol but given that the professional fishers were relied upon to obtain samples, it can only be assumed that the samples collected were representative of the sub-population from which they were caught. Non-randomness in collection may contribute to some of the variation seen between sub-populations.

As seen from Table 13, 22% on average of wild stock from the south west coast sub-population were prevalent with PHV. This is a significant number of wild pilchards infected with virus. Samples from batch FH04-078 showed the greatest prevalence with 28% positive for PHV.

The prevalence of pilchards representing the SA sub-population of Australian pilchards (see Table 13) was consistent with the prevalence of PHV in those from the south coast and west coast regions of WA.

As shown by Table 13, pilchards representing the NSW sub-population of Australian pilchards have a low prevalence of PHV, with only 1 fish positive of the 200 tested. This is noticeably different to the other sub-populations tested. This leads to the question – is the eastern pilchard population actually infected? Further investigations are required to clarify this. More random sampling and more batches need to be tested.

Juveniles were omitted from this survey. Since virus was detected in adults in the current experiments, it would now be valuable to test juveniles. In 1998, juvenile pilchards died locally from PHV, indicating that they are not completely resistant (Murray *et al*., 2003), but juveniles also only erratically mix with adult pilchards and mostly live in isolated populations in individual bays (Neira *et al*., 1999), and so less infections would be expected.

This current study only addressed the presence or absence of PHV in wild pilchards, and further studies should be undertaken to quantify the titre of virus within positive fish, which could be done using real-time PCR. The model proposed by Murray *et al*. (2003) predicts levels of infection of PHV that are much higher than the estimated levels of mortality.

Mortalities of the scale seen in 1995 and 1998 are not likely to be witnessed again due to the continued presence of PHV within the population and the likelihood of a degree of immunity in pilchards in the current population. It is expected, however, that small-scale mortality events will occur, but will either not be seen or not be reported. It is feasible that another PHV epizootic will never occur in Australian waters and further studies should include the testing of pilchards from overseas waters for the presence of PHV. If PHV is present elsewhere overseas, especially with no large-scale mortalities having been reported, then this may give an insight into how PHV was introduced into Australian waters.

4.5 Objective 5: *To compare the herpesvirus strains from 1995 & 1998, and to compare, at the molecular level, this herpesvirus and this disease with two other similar herpesvirus fish diseases that have been reported elsewhere in the world.*

Objective 5 was achieved.

As seen in Table 14, it appears that the viruses from the 1995 and 1998 outbreaks are the same over the 373 bases compared. However, this is a small number of bases compared to the expected size of the genome (~200 kbp). This data should only be taken as a preliminary investigation of sequence similarities between the 1995 and 1998 virus(es).

At the level of the deduced amino acid sequence it appears that the herpesvirus sequence from fish affected during the 1995 outbreak was identical to the PHV sequence from fish affected during the 1998 outbreak. However, this research involved analysis of only 373 bp of the PHV and should be considered as preliminary only. The ORF 62 gene, which encodes a putative terminase and is thought to be involved in DNA packaging, appears to be highly conserved throughout the family *Herpesviridae* (Davison, 1998), and was used to generate generic primers and obtain the first sequence for PHV. There may be significant differences between the 1995 and 1998 PHV isolates in other parts of the genome.

The 373 base sequence of the ORF 62 of PHV appears to be sufficiently different from other fish herpesviruses that its detection would provide evidence of the presence of PHV. The fact that this region analysed is known to be highly conservative in herpesviruses suggests that PHV is indeed unique. Morphologically, PHV appears to be a herpesvirus. Based on the current limited data, PHV appears to be related to other fish herpesviruses but bears no resemblance to mammalian or avian herpesviruses. Indeed, CCV and OMV share only 61% similarity at the amino acid level over the ORF 62 region, indicating that, while the fish herpesviruses appear to be phylogenetically similar, they are still very different from one another.

SECTION 5: BENEFITS

This report describes 3 molecular methods that can be used to detect PHV in fresh or frozen fish, or in samples prepared for histology. The real-time PCR appears to be highly sensitive and will be useful in detecting low levels of virus. This test is recommended, should further outbreaks occur or for the testing of imported baitfish.

It appears that virus from 1995 and 1998 are the same from the limited sequence data available. Additional sequence data believed to be new PHV sequence has been documented and will be invaluable for further work with this virus, such as a more extensive comparison of 1995 and 1998 strains, and comparison of these strains with other herpesviruses.

There is new evidence linking histology of samples to the amount of virus present within the fish gills. This will be useful in future to know that a fish with mild histological changes may house low levels of virus and certain methods of testing may not be suitable for detection of such low levels of virus.

PHV was proven to now be endemic in the population, although the NSW population is questionable. This is beneficial to know with regard to fisheries management. If NSW pilchards are not infected, perhaps there should be more research conducted as to why NSW have remained uninfected when the rest of the pilchard population is infected.

Several publications are in preparation or published as a result of this work:

Crockford M., Jones J.B., Crane M.S.J. and Wilcox G.E. (2005) Molecular detection of a virus, Pilchard herpesvirus, associated with epizootics in Australasian pilchards *Sardinops sagax neopilchardus*. Diseases of Aquatic Organisms 68(1): 1-5

Crockford M., Jones J.B. and McColl, K. Comparison of 3 molecular methods for the detection of Pilchard herpesvirus in archived paraffin-embedded tissue and frozen tissue. (submitted for peer review)

Whittington R.J., Crockford M., Jordan D. and Jones J.B. Survey for herpesvirus in pilchard, a marine pelagic finfish, in Australia. (in preparation)

Crockford M., Jones J.B., Hillier P. and Wilcox G.E. Molecular detection of Pilchard herpesvirus in archived pilchard (*Sardinops sagax neopilchardus*) paraffin-embedded tissues from a transmission trial conducted in 1999. (in preparation)

SECTION 6: INTELLECTUAL PROPERTY

Nil

SECTION 7: FURTHER DEVELOPMENT

Work with the additional sequence data should continue. There is little effort needed to confirm that this new data is PHV and determine all bases within that region.

The fact that PHV is now endemic is a significant discovery. If the virus were grown in cell culture, many doors would open for further PHV research. AAHL have recently developed a pilchard cell line that could be used to grow PHV (Williams *et al*., 2003). Supplies of PHV are no longer a problem, with virus available from wild pilchards. There is now a possibility that PHV could be sourced from wild pilchards, purified and grown in cell culture. New virus stocks could also be used to make a better DNA library, using purified virus rather than the part-purified virus that was available for this and previous studies. Sequencing of the whole genome would be possible. It could then be determined exactly how this virus fits into the Herpesviridae. The ability to culture live virus also opens doors to protein work, such as protein expression studies or serological test development.

The results from the wild stock survey are also interesting in terms of sub-population comparison. It appears that the NSW sub-population is unlikely to be infected, however, this is based on samples that were not as random as other sub-populations. A more in-depth survey is required, for all sub-populations, before more conclusions can be drawn regarding the prevalence of PHV within these sub-populations.

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SECTION 9: REFERENCES CITED

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TABLES

Table 1. Summary of all sequencing results for pZErO and pCR2.1 plasmid clones. All attempts with M13 primers resulted in a failed reaction or matched *E. coli*. Some smaller PCR products were sequenced and also resulted in failed reactions or matched *E. coli*. Yet, the PHV 373 bp product could be amplified and the sequence matched PHV. MC5 and MC6 from 19.3.04 and MC3 from 4.3.04 matched with each other but did not match with any sequence on Genbank. MC6 from 20.10.03 matched with PHV plus had extra sequence data spanning the known PHV region, suggesting that this is new PHV sequence data.

Date samples sequenced	Tube Label	Template	Primer	Result	Interpretation
28.3.03	MC1	A3-3 1000 bp product	M13F	Failed reactions	N/A
	MC2	A3-3 1000 bp product	M13R		
	MC3	C3-2 1000 bp product	M13F		
	MC4	C3-2 1000 bp product	M13R		
	MC5	D4-3 1000 bp product	M13F		
	MC6	D4-3 1000 bp product	M13R		
1.5.03	MC1	A3-3 1000 bp product	M13F	*E. coli* match	*E. coli*
	MC2	A3-3 1000 bp product	M13R		
	MC3	C3-2 1000 bp product	M13F		
	MC4	C3-2 1000 bp product	M13R		
	MC5	D4-3 1000 bp product	M13F		
	MC6	D4-3 1000 bp product	M13R		
20.5.03	MC1	A3-3 plasmid pZ	PHV373F	Failed reactions	N/A
	MC2	A3-3 plasmid pZ	PHV367R		
	MC3	C3-2 plasmid pZ	PHV373F		
	MC4	C3-2plasmid pZ	PHV367R		
	MC5	D4-3 plasmid pZ	PHV373F		
	MC6	D4-3 plasmid pZ	PHV367R		
30.5.03	MC1	A3-3 plasmid pZ	PHV373F	Failed reactions	N/A
	MC2	A3-3 plasmid pZ	PHV367R		
	MC3	A3-3 plasmid pZ	PHV373F		
	MC4	A3-3 plasmid pZ	PHV367R		
25.6.03	MC1	A3-3 1000 bp product	M13F	MC1 and MC2 failed reactions; MC3 and 4 *E. coli* match	*E. coli*
	MC2	A3-3 1000 bp product	M13R		
	MC3	A3-3 600 bp product	T7		
	MC4	A3-3 600 bp product	PHV367R		
4.7.03	MC1	A3-3 600 bp product	T7	Failed reactions	N/A
	MC2	A3-3 600 bp product	T7		
	MC3	A3-3 600 bp product	T7		
	MC4	A3-3 600 bp product	T7		
12.7.03	MC1	A3-3 1000 bp product	M13F	Sequence ok to ~400 bases; *E. coli* match	*E. coli*
	MC2	A3-3 1000 bp product	M13R		
	MC3	A3-3 1000 bp product	M13F		
	MC4	A3-3 600 bp product	T7		
	MC5	A3-3 1000 bp product	M13F		
28.7.03	MC1	A3-3 1000 bp product	PHV373F	Failed reactions	N/A
	MC2	A3-3 1000 bp product	PHV367R		
13.8.03	MCA	A3-3 1000 bp product	PHV367R	Failed reactions	N/A
	MCB	A3-3 1000 bp product	PHV373F		
	MCC	A3-3 1000 bp product	PHV367R		
26.8.03	MC1	PHV 373 bp product	PHV367R	PHV 373 bp match	PHV 373, sequencing worked
	MC2	PHV 373 bp product	PHV373F		
3.9.03	MC1	A3-2 1800 bp product	M13F	*E. coli* match but different from	*E. coli*

Date samples sequenced	Tube Label	Template	Primer	Result	Interpretation
	MC2	A3-2 1800 bp product	M13R	pZ 1000 clones	
	MC3	D4-4 900 bp product	M13F		
	MC4	D4-4 900 bp product	M13R		
16.9.03	MC1	C3-2 plasmid pZ	PHV373F	MC1, 3, 6, 7 and 8 failed reactions; MC2, 4, 5 and 9 matched with *E. coli*	*E.coli*
	MC2	C3-2 plasmid pZ	PHV367R		
	MC3	C3-2 plasmid pZ	PHV373F		
	MC4	C3-2 plasmid pZ	PHV367R		
	MC5	A3-3 plasmid pZ	T7		
	MC6	A3-3 plasmid pZ	SP6		
	MC7	A3-3 plasmid pZ	PHV373F		
	MC8	A3-3 plasmid pZ	PHV367R		
	MC9	A3-3 plasmid pZ	M13R		
20.10.03	MC1	A3-3 1000 bp product	M13F	MC7 and MC8 matched the known PHV 373 sequence; MC1-4 matched with *E. coli*; MC5 was inconclusive; MC6 matched with PHV but had extra sequence that did not match with any organism in the database	*E.coli* for some, PHV match for others including fragment with extra uncharacterised data that is presumed to be new PHV sequence data
	MC2	A3-3 1000 bp product	M13R		
	MC3	A3-3 600 bp product	M13F		
	MC4	A3-3 600 bp product	PHV367R		
	MC5	A3-3 900 bp product	M13R		
	MC6	A3-3 900 bp product	PHV367R		
	MC7	PHV 373 bp product	PHV367R		
	MC8	PHV 373 bp product	PHV373F		
4.3.04	MC1	A3-3 plasmid pCR2.1	SP6	MC1 and 2 failed reactions; MC3 ok but matching with MC5 + 6 from 19.3.04	Unclassified data
	MC2	A3-3 plasmid pCR2.1	PHV373F		
	MC3	A3-3 plasmid pCR2.1	PHV367R		
19.3.04	MC1	A3-3 plasmid pCR2.1	SP6	MC1-4 failed reactions; MC8 + MC10 reverse complement match with *E. coli*; MC7 + 9 match with PHV373; MC5 + 6 match with MC3 from 4.3.04 (See Table 1)	Some PHV match, some *E. coli* match, some unclassified data (See Table 1)
	MC2	A3-3 plasmid pCR2.1	SP6		
	MC3	A3-3 plasmid pCR2.1	PHV373F		
	MC4	A3-3 plasmid pCR2.1	PHV373F		
	MC5	A3-3 plasmid pCR2.1	PHV367R		
	MC6	A3-3 plasmid pCR2.1	PHV367R		
	MC7	A3-3 373 bp product	PHV373F		
	MC8	A3-3 373 bp product	PHV367R		
	MC9	A3-3 373 bp product	PHV373F		
	MC10	A3-3 373 bp product	PHV367R		
6.4.04	MCA	A3-3 900 bp product	SP6	MCA and MCC failed reactions; MCB and MCD sequence ok to ~300 bases with no hits on database	Unclassified data, insignificant
	MCB	A3-3 900 bp product	PHV373F		
	MCC	A3-3 450 bp product	SP6		
	MCD	A3-3 450 bp product	PHV367R		

Table 2. PHV373 PCR specificity results, including a range of known negative samples and 2 PHV positive samples. Only the 2 known PHV-positive samples are positive and all known PHV-negative samples are negative by this testing. VHSV = *Viral haemorrhagic septicaemia virus*; OMV = *Oncorhynchus masou virus*; WSHV1 = *White sturgeon herpesvirus 1*; WSHV2 = *White sturgeon herpesvirus 2*; KHV = *Koi herpesvirus*; GFHNV = *Goldfish haematopoietic necrosis virus*; AAHL = samples were obtained from the CSIRO Australian Animal Health Laboratories; WA = samples were obtained from Western Australia.

Tube #	Sample description	Result from PHV373 PCR
1	VHSV known positive (AAHL)	Negative
2	OMV known positive (AAHL)	Negative
3	WSHV1 known positive (AAHL)	Negative
4	WSHV2 known positive (AAHL)	Negative
5	PHV known positive (AAHL)	**Positive**
6	KHV #1 (Indonesia)	Negative
7	KHV #2 (Indonesia)	Negative
8	Suspect KHV positive (WA)	Negative
9	Suspect KHV positive (WA)	Negative
10	GFHNV positive (WA) (kidney)	Negative
11	GFHNV positive (WA) (spleen)	Negative
12	GFHNV positive (WA) (kidney, spleen)	Negative
13	GFHNV positive (WA) (kidney, spleen, liver)	Negative
14	FH05-080 #1 Whiting #	Negative
15	FH05-080 #2 Whiting #	Negative
16	FH05-080 #3 Whiting #	Negative
17	FH05-080 #4 Whiting #	Negative
18	FH05-081 #1 Herring *	Negative
19	FH05-081 #2 Herring *	Negative
20	FH05-081 #3 Herring *	Negative
21	FH05-081 #4 Herring *	Negative
22	FH05-082 Baitfish (*Spratelloides robustus*)	Negative
23	FH05-083 Tin Sardines (*Sardinops sagax*)	Negative
24	FH05-085 #1 Indonesian pilchards **	Negative
25	FH05-085 #2 Indonesian pilchards **	Negative
26	FH05-085 #3 Indonesian pilchards **	Negative
27	FH05-085 #4 Indonesian pilchards **	Negative
28	FH05-085 #5 Indonesian pilchards **	Negative
29	WA Positive control (cloned PHV373 product)	**Positive**

*# = Sillago sp.; * = Arripis georgianus; ** = Tenualosa macrura*

Table 3. Prevalence of PHV in different tissues of fish from the 1995 and 1998 outbreaks. Heart and gill tissues showed a high prevalence of PHV. From the total samples tested, PHV was detected in all types of tissue.

Tissue	No. positives/ No. tissues types tested	% Prevalence
Gill	16/22	72.7
Spleen	11/21	52.4
Liver	5/15	33.3
Kidney	13/21	61.9
Stomach	1/6	16.7
Intestine	6/19	31.6
Heart	11/15	73.3

Table 4. Summary of results for all samples tested by ISH, PCR and real-time PCR. Frozen indicates that samples were from fish that were frozen at –20ºC. Other samples were formalin fixed, paraffin-embedded tissue that was initially processed for histological examination. These samples have been adapted to suit molecular testing as well, however it can be seen from the results that this method of preparation was less successful than using frozen samples. Sample 95-4420, collected during the epizootic, was consistently strongly positive by all 3 methods.

Sample	Preservation	ISH	PCR	Real-time PCR
Pre-outbreak 95-4667	Frozen	Negative	Negative	Positive
During outbreak 95-4420	Frozen	Strong positive	Strong positive	Strong positive
Post-outbreak 95-5135	Frozen	Negative	Negative	Positive
Pre-outbreak 95-4667 (#11, 13, 14)	FFPE	Negative	Negative	Negative
Start of outbreak 95-5000 (#1, A2, 3, B3, B4)	FFPE	Positive	Negative	Strong positive
During outbreak 95-4420 (#1,2)	FFPE	Strong positive	Negative	Strong positive
Post-outbreak 95-5135 (#2, 4, 5, 6)	FFPE	Negative	Negative	Negative

FFPE = Formalin fixed, paraffin-embedded tissue

Table 5. Wild stock samples collected for real-time PCR testing. Each designated area had a total of 200 samples collected. Each batch of samples was assigned a laboratory number as they entered the laboratory. Some areas, such as SA and NSW, proved to be difficult for obtaining samples and, as a result, 200 fish from the same supplier was used.

Area of collection	**Assigned laboratory no.**	**No. samples**
Fremantle (west coast WA)	FH04-078	50
	FH04-081	50
	FH04-191	100
Bremer Bay and Esperance (south coast WA)	FH04-085	50
	FH04-199	150
SA	FH04-221	200
Northern NSW	FH05-016	200
Total no. of samples for testing	**---**	**800**

Table 6. Results of a selection of samples tested by PHV373 PCR and the PHV real-time PCR. Two samples are positive by PHV373 PCR and by PHV real-time PCR, however some samples were positive by the real-time PCR but negative by PHV373 PCR, indicating that the real-time PCR is the more sensitive of the two tests. The 2 positives detected by both PCRs confirm that PHV is present in wild pilchards.

Tube #	**Sample #**	**PHV373 PCR Result**	**Result from real-time PCR**
1	FH04-078 #4	Negative	Negative
2	FH04-078 #12	Negative	Negative
3	FH04-191 #17	Negative	**Positive**
4	FH04-191 #22	Negative	Negative
5	FH04-191 #81	Negative	**Positive**
6	FH04-191 #96	Negative	Negative
7	FH04-081 #31	**Positive**	**Positive**
8	FH04-081 #32	Negative	Negative
9	FH04-199 #18	Negative	Negative
10	FH04-199 #66	Negative	Negative
11	FH04-199 #76	Negative	Negative
12	FH04-199 #96	Negative	**Positive**
13	FH04-199 #121	Negative	Negative
14	FH04-199 #141	Negative	Negative
15	FH04-085 #41	Negative	Negative
16	FH05-016 #13	Negative	Negative
17	FH05-016 #22	Negative	Negative
18	FH05-016 #110	Negative	Negative
19	FH05-016 #146	Negative	**Positive**
20	FH05-016 #160	Negative	Negative
21	FH04-221 #1	Negative	Negative
22	FH04-221 #33	Negative	Negative
23	FH04-221 #66	Negative	**Positive**
24	FH04-221 #100	**Positive**	**Positive**
25	FH04-221 #180	Negative	Negative
No. of Positives	---	**2/25**	**7/25**

Table 7. Results for sequencing performed on the 1000 bp clone in plasmid pCR2.1 and on the PCR product after PCR of this clone using specific PHV primers. Note that some of the sequencing matched the known PHV sequence, some matched *E. coli* and some did not match with anything significant in the database. MC5 and MC6 showed promising results, with new, unclassified sequence data obtained. However, any new sequence data was inconclusive as it could not be linked to PHV, or it matched with *E. coli*. Sequence data from MC7 and MC9 matched PHV, indicating that PHV is present within this clone in pCR2.1 and can be successfully amplified with primer PHV373F.

Sample Label	Template	Primer	Result	Interpretation
MC1	1000 bp clone in pCR2.1	SP6	Unsuccessful	N/A
MC2	1000 bp clone in pCR2.1 (higher concentration)	SP6	Unsuccessful	N/A
MC3	1000 bp clone in pCR2.1	PHV373F	Unsuccessful	N/A
MC4	1000 bp clone in pCR2.1 (higher concentration)	PHV373F	Unsuccessful	N/A
MC5	1000 bp clone in pCR2.1	PHV367R	RC match with MC6 RC and previous sequence data from MC3 4.3.04 (see Table 1)	Unknown sequence data, not matching with *E. coli*, human, mouse or PHV. Possibly new PHV sequence or invalid data.
MC6	1000 bp clone in pCR2.1 (higher concentration)	PHV367R	RC match with MC5 RC and previous sequence data from MC3 4.3.04 (see Table 1)	See MC5
MC7	373 bp product after PCR using PHV primers	PHV373F	Match with known PHV 373 sequence and MC9	PHV is present within the 1000 bp insert in pCR2.1
MC8	373 bp product after PCR using PHV primers	PHV367R	RC match with *E. coli* and pZErO 1000 base sequence; match also with MC10 RC	Amplified fragment containing *E. coli*
MC9	373 bp product after PCR using PHV primers (higher concentration)	PHV373F	Match with known PHV 373 sequence and MC7	See MC7
MC10	373 bp product after PCR using PHV primers (higher concentration)	PHV367R	RC match with *E. coli* and pZErO 1000 base sequence; match also with MC8 RC	See MC8

RC = Reverse complement

Table 8. Histological results for the samples used in this study. Information has been provided courtesy of University of Sydney. Note that samples collected pre- and post-outbreak appeared to be healthy fish, both on collection and histologically. Sample 95-4420 appeared to be heavily affected at the time of collection and showed the most severe changes histologically.

Sample	Location of sample collection	Collection in relation to 1995 epizootic	Status at time of collection	Histological examination
95-4667	Iluka	Pre outbreak (4 days before)	Healthy, live	Healthy, no significant changes seen
95-5000	Iluka	Just before mortality front (2 days before)	Affected, live	Slight changes seen, which may be consistent with viral infection
95-4420	Port Stephens	During outbreak	Affected, dead	Marked changes seen. Severe hyperplasia in the gill epithelium
95-5135	Iluka	Post outbreak (8 days after)	Healthy, live	Healthy, no significant changes seen.

Table 9. Histology, ISH and PCR results for selected suspect positive transmission trial samples. No virus was detected in any of the samples by any of the methods listed here. This suggests that virus transmission was unsuccessful or that these methods lack the sensitivity required to detect virus in these samples. Testing was performed at AAHL or the FHL in WA.

Sample	Original Histology Report (AAHL)	ISH (FHL)	Original PCR Report (AAHL)	PCR (FHL)
T1D3	Mild hyperplasia	Negative	Negative	Negative
T1D1	No significant changes noted	Negative	Negative	Negative
T3B1	Mild hyperplasia	Negative	Negative	Negative
T2A6	Some changes noted. Moderate focal acute hyperplasia*	Negative	Negative	Negative
T1C3	No significant changes noted	Negative	Negative	Negative
T1D5	N/A	Negative	N/A	Negative
C1-2	N/A	Negative	N/A	Negative
AS-03-4361	N/A	Negative	N/A	Negative
AS-03-1989	N/A	Negative	N/A	Negative

*Moderate focal acute hyperplasia consistent with PHV disease, although the lesion was not extensive.

N/A = Not applicable

Table 10. Real-time PCR results for samples tested. All samples were analysed at the FHL in WA. Templates consisted of neat (no dilution), 1:10 or 1:100 dilutions of sample. Positive samples are T1D3, T1D1 and T2A6. Negative samples are T3B1, T1C3, T1D5, C1-2, AS-03-4361 and AS-03-1898. Some samples, for example, T1D3 1:10 and T2A6 1:10, were on the limit of detection, being positive when neat sample were used but positive or negative when less template was added.

Sample	**1st real-time PCR**	**2nd real-time PCR**	**3rd real-time PCR**	**Conclusive result (Pos/Neg)**
T1D3	Pos neat + 1:10	Pos neat + 1:10; Neg 1:100	Pos 1:10; Neg neat; Gel pos 1:10	Pos
T1D1	Neg neat +1:10	Pos neat; Neg 1:10 + 1:100	Pos neat; Neg 1:10; Gel pos neat	Pos
T3B1	Neg neat + 1:10	Neg neat, 1:10 + 1:100	Neg neat + 1:10	Neg
T2A6	Pos neat + 1:10	Pos neat + 1:10; Neg 1:100	Pos neat + 1:10; Gel pos neat + 1:10	Pos
T1C3	Neg neat + 1:10	Neg neat, 1:10 + 1:100	Neg neat + 1:10	Neg
T1D5	Neg neat + 1:10	Neg neat, 1:10 + 1:100	Neg neat + 1:10	Neg
C1-2 (TT neg control)	Neg neat + 1:10	Neg neat, 1:10 + 1:100	Neg neat + 1:10	Neg
AS-03-4361 (Neg control, goldfish)	Neg neat + 1:10	Neg neat, 1:10 + 1:100	Neg neat + 1:10	Neg
AS-03-1898 (Neg control, koi carp)	Neg neat + 1:10	Neg neat, 1:10 + 1:100	Neg neat + 1:10	Neg

KEY: Pos = positive; Neg = negative; Gel = agarose gel electrophoresis result

Table 11. Negative control samples tested to rule out contamination for the PHV real-time PCR. Samples from fish from the 3 control tanks were taken into pools of 5 (with the exception of 2 pools of 3) and tested by real-time PCR. All samples were negative.

Tube #	**Pooled Samples**	**Result of real-time PCR**
1	Control tank 1 # 1-5	Negative
2	Control tank 1 # 6-10	Negative
3	Control tank 1 # 11-15	Negative
4	Control tank 1 # 16-18	Negative
5	Control tank 2 # 1-5	Negative
6	Control tank 2 # 6-10	Negative
7	Control tank 2 # 11, 12, 15-17	Negative
8	Control tank 2 # 18, 19, 21, 23, 24	Negative
9	Control tank 3 # 1-5	Negative
10	Control tank 3 # 6-8, 10,11	Negative
11	Control tank 3 # 12-16	Negative
12	Control tank 3 # 19, 21, 9	Negative

Table 12. Real-time PCR results for a range of known negative samples and 2 PHV positive samples. Only the 2 known PHV-positive samples are positive and all known PHV-negative samples are negative by this testing. VHS = Viral haemorrhagic septicaemia virus; OMV = *Oncorhynchus masou* virus; WSHV1 = White sturgeon herpesvirus 1; WSHV2 = White sturgeon herpesvirus 2; KHV = Koi herpesvirus; GFHNV = Goldfish haematopoietic necrosis virus; AAHL = samples were obtained from the CSIRO Australian Animal Health Laboratories; WA = samples were obtained from Western Australia.

Tube #	Sample description	Result from real-time PCR
1	VHS known positive (AAHL)	Negative
2	OMV known positive (AAHL)	Negative
3	WSHV1 known positive (AAHL)	Negative
4	WSHV2 known positive (AAHL)	Negative
5	PHV known positive (AAHL)	**Positive**
6	VHS negative control (AAHL)	Negative
7	OMV negative control (AAHL)	Negative
8	WSHV1 negative control (AAHL)	Negative
9	WSHV2 negative control (AAHL)	Negative
10	PHV negative control (AAHL)	Negative
11	KHV #1 (Indonesia)	Negative
12	KHV #2 (Indonesia)	Negative
13	Suspect KHV positive (WA)	Negative
14	Suspect KHV positive (WA)	Negative
15	GFHNV positive (WA) (kidney)	Negative
16	GFHNV positive (WA) (spleen)	Negative
17	GFHNV positive (WA) (kidney, spleen)	Negative
18	GFHNV positive (WA) (kidney, spleen, liver)	Negative
19	FH05-080 #1 Whiting #	Negative
20	FH05-080 #2 Whiting #	Negative
21	FH05-080 #3 Whiting #	Negative
22	FH05-080 #4 Whiting #	Negative
23	FH05-081 #1 Herring *	Negative
24	FH05-081 #2 Herring *	Negative
25	FH05-081 #3 Herring *	Negative
26	FH05-081 #4 Herring *	Negative
27	FH05-082 Baitfish (*Spratelloides robustus*)	Negative
28	FH05-083 Tin Sardines (*Sardinops sagax*)	Negative
29	FH05-085 #1 Indonesian pilchards **	Negative
30	FH05-085 #2 Indonesian pilchards **	Negative
31	FH05-085 #3 Indonesian pilchards **	Negative
32	FH05-085 #4 Indonesian pilchards **	Negative
33	FH05-085 #5 Indonesian pilchards **	Negative
34	WA Positive control (cloned PHV373 product)	**Positive**

*# = Sillago sp.; * = Arripis georgianus; ** = Tenualosa macrura*

Table 13. Prevalence of PHV found in wild stock samples using the PHV real-time PCR. This wild stock testing shows that significant numbers of PHV positive fish can be detected. It should be noted that with the rigorous cut-off values to eliminate any false positives, some false negatives might have been reported. The total prevalence of all fish tested is calculated at 13.75%.

	South west WA			***West coast WA***		***SA***	***NSW***
Sample no.	**FH04-078**	**FH04-081**	**FH04-191**	**FH04-085**	**FH04-199**	**FH04-221**	**FH05-016**
Prevalence	14/50 (28%)	7/50 (14%)	23/100 (23%)	3/50 (6%)	28/150 (19%)	34/200 (17%)	1/200 (0.5%)
Total Prevalence (all 800 samples) = 110/800 (13.75%)							

Table 14. Sequence comparison of Pilchard herpesvirus isolates from 1995 and 1998. Sequences of samples from various parts of WA, NSW and NZ were compared, with sequences from all but one sample identical over the 373 bases. The sequence from the sample from Garden Island in WA, 1998, produced a silent base change, which could be due to variance in sequencing accuracy between samples or it could be a true base change. The protein coded for by this codon was the same, and so the protein sequence for all samples sequenced were identical over the 373 bases.

Samples from PHV-affected fish	**Sequence Similarity with Reference Strain**
95-4420 (NSW) (1995)	100 %
Nelson Beach (WA) (1995)	100 %
Bay of Islands (NZ) (1995)	100 %
Smith's Beach (NSW) (1998)	100 %
FH/218 F Garden Island (WA) (1998)	99.7 %
FH/218 A Esperance (WA) (1998)	100 %