

Generation of diagnostic reagents for pilchard herpesvirus

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FRDC Project Number: 1999/226

ISBN 0-643-06923-2



FISHERIES
RESEARCH &
DEVELOPMENT
CORPORATION

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Published by CSIRO Livestock Industries, Australian Animal Health Laboratory,
Geelong

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2002

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ISBN 0-643-06923-2

Front cover designed by Frank Filippi, CSIRO Livestock Industries using an electron micrograph of pilchard herpesvirus kindly provided by Alex Hyatt, CSIRO Livestock Industries, CSIRO and Richard Whittington, University of Sydney.

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Project title: Generation of diagnostic reagents for pilchard herpes virus

Project number: 1999/226

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NON TECHNICAL SUMMARY PRO- FORMA**1999/226 Generation of diagnostic reagents for pilchard herpes virus****PRINCIPAL INVESTIGATOR:** Dr B. Eaton**ADDRESS:** Australian Animal Health Laboratory
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Telephone: 03 5227 5000 Fax: 03 5227 5555**OBJECTIVES**

1. To develop a polymerase chain reaction assay and a set of protocols to detect pilchard herpesvirus nucleic acid in affected Australian pilchards.
2. To develop cultured pilchard cell lines and use susceptible cells to grow pilchard herpesvirus.

NON-TECHNICAL SUMMARY:**OUTCOMES ACHIEVED**

1. A polymerase chain reaction (PCR) assay has been developed to detect pilchard herpes virus nucleic acid in the tissues of affected fish. The assay is significantly faster than electron microscopic methods used extensively during the disease outbreaks. However, absence of methods to grow and quantify the virus means it is not possible to determine the sensitivity of the assay that is the number of virus particles needed to generate a positive PCR result.
2. Pilchard heart and liver cell lines were established. The failure to get pilchard herpesvirus to grow in the cells may reflect their innate resistance or absence of "live" virus in samples taken from affected pilchards that had been stored at -20°C . The cell lines may be an important resource if fish are processed quickly for virus isolation or stored at -80°C .

In the mid-to-late nineteen nineties in the waters around Southern Australia there were two massive disease outbreaks in pilchards. The first started in March 1995 off the coast of South Australia and in the six-month period to September 1995, the outbreak spread to affect the entire geographic range of the Australian pilchard fishery, more than 5000 km of coastline from Western Australia to Southern Queensland. A second outbreak occurred in late 1998. Although it too started in South Australian waters and spread in both directions, the 1998 event occurred later in the year, starting in November and continuing to mid 1999. The rate of spread of the diseases was remarkable, approximately 30 km per day in 1995. In 1998 it was slower and the numbers of dead, dying and diseased fish detected were fewer than in 1995. Although events that triggered the outbreaks are not understood, examination of affected pilchards revealed a common finding. By electron microscopy the gill tissues of diseased fish were seen to contain a virus whose size and structure indicated that it was a herpesvirus. In the laboratory small amounts of virus were isolated from diseased gill tissue, but the virus did not grow in fish cell lines that normally support replication of a range of

viruses infecting aquatic animals. Failure to grow the virus in cells was significant because it meant that scientists could not implement routine procedures to study the virus and develop diagnostic reagents. The major objectives of work described here were to (1) develop pilchard cell lines that could be used to grow large amounts of the herpesvirus in the laboratory and (2) develop tests that could be used to detect the virus in affected fish, by targeting the virus DNA, without the need to grow the virus in the laboratory.

Cultured pilchard cell lines

As the herpesvirus was seen only in gills of affected pilchards, initial attempts to develop cell lines focussed on gill tissue from putatively unaffected fish. These attempts were not successful. No cells grew continuously in culture and bacteria and fungi in gills contaminated cultures in spite of the presence of antibiotics to control the growth of microorganisms. Attempts to develop cell lines from other pilchard organs were more successful and lines derived from pilchard heart and liver tissue were established and continue to grow well in flasks in culture. The pilchard liver cell line, which has been cultured continuously for over three years, supports the replication of a number of piscine viruses. Unfortunately, partially purified pilchard herpesvirus and virus in ground-up gill tissues from diseased fish that had been frozen at -20°C did not grow in either pilchard liver or heart cells. This may not reflect the susceptibility of the cells to infection by the virus, but rather the absence of infectious virus in the samples added to cultured cells. Viruses that are purified in the laboratory may lose infectivity because of treatments required to isolate them. In addition, diseased tissues are best stored at -80°C because storage at -20°C does not effectively preserve virus infectivity. Continuous lines of pilchard cells may be useful in if there is another epizootic provided affected pilchards can quickly be processed for virus isolation or stored at -80°C .

Test to detect pilchard herpesvirus DNA

To develop a test to detect pilchard herpesvirus DNA requires knowledge of the virus DNA sequence. Herpesviruses have been isolated from many animal species including fish. Scientists believe that herpesviruses have evolved in close association with the species from which they are derived and generally infect only that species. So herpesviruses, even those from related species, tend to be quite different genetically. Herpesviruses also have the capacity to "hide" in very small numbers in specific cells in their natural host – a state known as latency. The presence of a latent herpesvirus infection frequently becomes obvious only after virus replication is stimulated by some other event. The appearance of herpesvirus-induced cold sores following stress in humans is a well-known example of latency.

The DNA sequence of some aquatic herpesviruses has been determined and the first step of the project was to compare the DNA of known aquatic herpesviruses and look for related sequences in the hope that similar regions might be present in pilchard herpesvirus. Comparison of the DNA of channel catfish herpesvirus and salmonid herpesvirus type 1 led to the identification of a partially conserved genetic region. From the DNA sequence of that region, it was possible to make reagents that were used to determine if a similar sequence was present in pilchard herpesvirus. DNA was extracted from pilchard herpesvirus that had been partially purified from gills of affected fish during the 1995 outbreak. Using reagents that reacted with other aquatic herpesviruses, a portion of pilchard herpesvirus DNA was amplified in a test called the polymerase chain reaction (PCR). The sequence of the DNA segment was found to differ slightly from that of other aquatic herpesviruses. Determination of the DNA sequence for that portion of pilchard herpesvirus made it possible to generate reagents that reacted only with pilchard herpesvirus and not with other herpesviruses. The PCR test did not react with DNA taken from pilchard cell lines established in the laboratory.

The ability of the PCR test to detect pilchard herpes DNA in clinical material has been confirmed using gills from affected pilchards in 1995 and 1999 and stored at -20° C. Although virus infectivity, a function of both the proteins and nucleic acid of the virus, may have been lost under these storage conditions, the DNA of the virus would be expected to remain essentially intact. The DNA from both sets of gills yielded a positive PCR result and the pilchard herpesvirus DNA amplified from gills had the same sequence as that amplified from partially purified virus. These results indicate that the PCR test is specific and capable of detecting pilchard herpesvirus in the gills of affected fish in disease outbreaks.

We don't know if the PCR test will detect herpesvirus in latently infected pilchards that show no sign of overt disease. To determine if the test is capable of doing this, we need to know the sensitivity of the assay. This remains unknown because we have no method of growing the virus and determining the number of virus particles needed to generate a positive PCR response. This is an important issue because the PCR test, like all other diagnostic assays, may yield positive results only with samples that contain more than a threshold number of virus particles. While results show that the test provides a very rapid means of virus detection in disease situations, it cannot be used with confidence to look for pilchard herpesvirus in Australian pilchards in the absence of overt disease or in imported fish.

KEYWORDS: Pilchards, pilchard herpesvirus, diagnostic assays, PCR, pilchard cell lines.

SECTION 1: INTRODUCTION

Background

More new viruses have emerged in Australia during the last decade of the twentieth century than at any other time. Hendra virus, Menangle virus and Australian bat lyssavirus "jumped" from flying foxes to cause fatal disease in animals and man. Such terrestrial diseases caused enormous concern, but their size, in terms of the number of animals and humans affected, pales into insignificance compared with the huge epizootics in pilchards (*Sardinops sagax neopilchardus*) in the waters of southern Australia in 1995 and 1998.

The first mass mortality occurred from March to September 1995 and covered the entire range of the pilchard fishery (more than 5000 km of the Australian coastline from Western Australia to Queensland and 500 km of the New Zealand coastline (Whittington et al. 1997). In 1998, a similar event occurred but with significant differences (Gaughan et al., 2000). While both epizootics appeared to start in South Australian waters and spread in both directions towards Western Australia and Victoria, the 1998 event occurred later in the year (starting in November and continuing to mid 1999) and appeared to involve juvenile (<10cm length) as well as older, larger fish. The speed at which the 1995 epizootic moved was spectacular, approximately 30 km per day, a speed not much slower than the maximum swimming speed of pilchards (Murray et al., 2001). The rate of spread in 1998 was slower than in 1995 and the numbers of dying/diseased fish detected were less than in 1995.

During and subsequent to the 1995 epizootic, nationwide investigations by several laboratories failed to link the mortalities with physical or biological oceanographic events (Fletcher et al., 1997; Griffin et al., 1997) or to unequivocally identify the cause of the mass mortality. However there was a positive correlation between the presence of a herpesvirus in gill tissue and diseased fish (Whittington et al., 1997; Hyatt et al. 1997). Histological lesions in gill epithelium were consistent with viral infection and the likely cause of death, but it was not proven that herpesvirus infection caused the mortality event. The circumstances triggering the mortalities are not fully understood

The following observations provide evidence of a causal relationship between the pilchard mortalities and the presence of a herpesvirus (called here pilchard herpesvirus, PHV) in the gills of dead and moribund pilchards. Herpesviruses have been isolated from fresh water salmonids (Wolf and Taylor, 1975), turbot *Scophthalmus maximus* L (Buchanan et al., 1978), channel catfish *Ictalurus punctatus* (Wolf and Darlington, 1971), carp *Cyprinus carpio*

(Schubert, 1966) and white sturgeon *Acipenser transmontanus* (Watson et al., 1995). The genome of CCV has recently been sequenced (Davison, 1992).

1. A virus with the morphological characteristics of herpesvirus has been observed in ultrathin sections of pilchard gill epithelium in which significant hyperplasia has been detected histopathologically (Hyatt et al., 1997).
2. Electron microscopic observations confirmed the presence of small numbers of herpesviruses in a partially purified preparation derived from affected pilchard gills.
3. Electron microscopic examination of gills from normal pilchards failed to reveal the presence of herpesviruses.
4. A fragment of DNA from partially purified PHV from the 1995 epizootic was amplified by polymerase chain reaction (PCR) technology using primers based on sequences derived from the thymidine kinase gene of channel catfish herpesvirus (Tham and Moon, 1996). The significance of this result remains uncertain because the amplified fragment was not sequenced. For the result to be significant, the sequence of the PCR product should not be identical to but manifest some similarity to that of the positive channel catfish DNA control.

In spite of the correlation between the presence of virus, observed pathology and death of adult pilchards in both the 1995 and 1998 epizootics, it remains to be shown that the herpesvirus is the sole causative agent of the pilchard mortalities. Classically, the correlation would be confirmed by satisfying Koch's postulates (eg producing disease in normal pilchards that are inoculated with this herpesvirus). However, because of the dual difficulties of culturing the virus, and maintaining pilchards in captivity, it has not been possible to satisfy Koch's postulates in this case. Attempts to isolate PHV by growth in poikilothermic cell lines such as CHSE-214, RTG-2, BF-2, FHM and EPC were not successful (Hyatt et al., 1997).

1.2 Need

The inability of the PHV to replicate in cultured cells is a major impediment to the development of diagnostic methods and tools for virus detection and analysis. It is suggested that the generation of a continuous cell line derived from pilchards offers the most likely mechanism to produce PHV in the laboratory.

During the 1995 and 1998 epizootics the only techniques available to examine diagnostic samples from pilchards were histopathology and electron microscopy. While the latter

provided definitive information that a herpesvirus was present, electron microscopy is not suitable for widespread, rapid screening of samples from a large number of pilchards from disparate parts of the Australian coastline. In addition electron microscopy does not provide the means to determine if the same PHV was involved in both outbreaks or if the virus evolved during each outbreak. Diagnostic laboratories traditionally rely on enzyme-linked immunosorbent assays (ELISA) for the detection of antibody to specific pathogens and ELISA or polymerase chain reaction (PCR)-based assays for the detection of pathogen antigens or nucleic acid respectively. There is a substantial need for rapid systems to detect either the proteins and/or the nucleic acid of PHV.

Fletcher et al. (1997) suggested that the size and nature of the epizootic in 1995 were consistent with infection of a naïve Australian pilchard population by an exotic herpesvirus to which they had never previously been exposed. He postulated that the use of imported pilchards to feed caged southern bluefin tuna in South Australian waters provided a potential source of such a virus. Consequently, diagnostic tests to detect PHV are needed, not only to confirm infection of affected pilchards, but also to investigate the possible presence of herpesvirus in imported pilchards.

Herpesviruses are highly disseminated in nature with most animal species yielding at least one herpesvirus (Roizman, 1996). Herpesviruses have the capacity to remain latent in their natural host and the existence of latent herpesviruses frequently remains unknown until a predisposing environmental factor leads to their recrudescence (Rock, 1993). The factor most commonly recognised in herpesvirus reactivation is stress that can itself be induced by a variety of stimuli including infection by another, unrelated microorganism. Fletcher et al. (1997) suggest that the severity of the disease observed in Australian pilchards and its emanation from a single geographic locality, are not consistent with reactivation of a latent infection. However, we can not rule out the possibility that the herpesvirus is endemic and present in a latent state in Australian pilchards and is activated following infection by an, as yet, unidentified microorganism that spreads from a point source in South Australia. Imported pilchards may or may not be the source of such a microorganism. Thus a panel of diagnostic reagents and tools is needed to determine if Australian pilchards are latently infected by a herpesvirus.

Objectives

1.3.1 Original objectives

1. Prepare a panel of diagnostic reagents and a set of protocols to detect herpes virus in affected Australian pilchards
2. Provide reagents and protocols to State laboratories and offer training in their use to screen Australian and imported pilchards for the presence of the virus
3. Develop cultured pilchard cell lines and use susceptible cells to grow pilchard herpes virus.
4. Investigate the cause of juvenile pilchard deaths by pathological and ultrastructural examination of juvenile fish

In February 2001, Dr Helen Byers resigned after 16 months on the project, precipitating a reassessment of project objectives. At that time objectives 1 and 3 were partially completed and work had not commenced on objectives 2 and 4. The thirteenth meeting of the Joint Pilchard Scientific Working Group (JPSWG) on March 1st 2001 considered a number of modified objectives generated in response to Dr Byers' resignation and agreed that staff with experience in PCR should be transferred from other areas within AAHL, rather than undertake a formal recruitment process to replace Dr Byers. The primary objective should be validation of the pilchard herpes virus PCR test already developed. The revised objectives are listed in section 1.3.2. The JPSWG also requested that AAHL should liaise with Dr Brian Jones, Fisheries Western Australia, to maximise output of FRDC 99/226. Dr Brian Jones and Dr Brad Chadwick were informed by email on May 8 and 25, 2001 respectively on the nature of the primers used in the PCR at that time.

1.3.2 Revised objectives

1. To develop a PCR assay and a set of protocols to detect pilchard herpesvirus in affected Australian pilchards.
2. To develop cultured pilchard cell lines and use susceptible cells to grow pilchard herpesvirus.

SECTION 2: METHODS

2.1 Preparation, characterisation and virus susceptibility of pilchard cell lines

2.1.1 Capture of pilchards and isolation of pilchard organs

Juvenile pilchards approximately 10 months of age were netted from a fishing trawler off the coast of Lakes Entrance in Victoria, Australia in November 1998. Pilchards were alive when netted and died, or became moribund, shortly thereafter and were immediately placed on ice. The operculum was removed and gills excised from each fish by cutting through the cartilage at the base of the gill arches and the gills were placed in ice cold transport medium (Eagle's minimum essential medium with Earle's salts (EMEM, Gibco-BRL Cat. # 41500-034) supplemented with 10mM hepes (BDH Cat. # 44285 6A), 2mM glutamine (ICN Biomedicals Inc. Cat. # 1580115), 2% (v/v) foetal bovine serum (CSL), 50µg/ml gentamycin sulphate (Sigma Cat. # G-3623) and 5µg/ml amphotericin B (Bristol-Myers Squibb Co "Apothecon" Fungizone-Amphotercin B). After removal of the gills, the fish were immersed in 80% (v/v) ethanol for ten minutes to sterilise the surface and air dried on a paper towel. The body cavity and internal organs were exposed taking care not to cut the digestive tract. The liver, heart, spleen and kidney were removed aseptically and each placed in 20-50ml volumes of transport medium. To reduce the possibility of microbial contamination, instruments were rinsed in 80% (v/v) ethanol, followed by sterile water after each dissection. Tissues arrived at AAHL within approximately 6 hours of collection.

In a laminar flow cabinet excess blood was removed from each tissue by washing 4 times with phosphate buffered saline (PBSA pH 7.4), without Ca^{2+} and Mg^{2+} ions and supplemented with 50µg/ml gentamycin sulphate and 5µg/ml amphotericin B. Half the volume of each tissue was placed in a 50ml centrifuge tube containing PBSA and placed at 4°C for subsequent treatment with trypsin. The other half was used to prepare tissue explants.

2.1.2 Tissue explant preparation

Tissues, with the exception of gill tissue, were individually minced into small fragments using two scalpel blades in approximately 10ml PBSA prior to centrifugation at 100 x g for 5 minutes at 4°C. The supernatants were removed and the pellets resuspended in 6ml PBSA. Six 1ml aliquots were mixed with 5 ml of six different media (see below), placed in sealed 25cm² (Corning) tissue culture flasks and incubated at 22°C.

Due to the volume of blood contained in gills, these tissues were processed differently. Gill arches were removed with a scalpel blade and gill filaments minced to small fragments. The minced tissue was aspirated into a 10ml pipette, whose tip was covered with gauze to exclude large pieces of arch and filament. The filtered supernatant was dispensed into a sterile tube and 2 ml aliquots mixed with 5ml of six different media and incubated at 22°C in sealed 25cm² (Corning) tissue culture flasks. The remaining gill arch and filament tissue was resuspended in 10ml washing medium and centrifuged at 100 x g for 5 minutes at 4°C. The pellet was resuspended in washing medium and 1ml aliquots added to each 25cm² tissue culture flasks containing 5ml of one of the six media types. Cultures were incubated at 22°C in sealed flasks.

2.1.3 Trypsinisation of tissues

PBSA was decanted from tubes containing kidney, liver, spleen, and heart tissues and replaced with 5ml trypsin (0.25% (w/v) trypsin (Difco Trypsin 1:250 Cat # 0152-15) in hepes buffered saline, adjusted to pH 7.2 with 1N sodium hydroxide and filtered through a 22µm filter). Tissues were slowly agitated using a magnetic stirrer at ambient temperature (22-24°C). Trypsinisation times varied according to the tissue (Table I). After the first trypsinisation period, suspensions were allowed to sit for a few minutes prior to removal of the supernatants. Foetal bovine serum was added to the removed cell suspensions to a concentration of 5% (v/v) to prevent the deleterious effect of trypsin digestion on cells. Remaining material from the first trypsinisation was reincubated at ambient temperature for times given in Table I and cell suspensions removed and treated as described above. The two trypsinised aliquots of each tissue were combined, divided into 6 portions, each of which was mixed with a different tissue culture medium and incubated at 22°C in sealed, 25cm² (Corning) tissue culture flasks.

Table 1. Summary of trypsin treatments applied to individual pilchard tissue

Tissue	1 st trypsin treatment	2 nd trypsin treatment
Heart	4 min	7 min
Liver	4 min	7 min
Kidney	12 min	5 min
Spleen	19 min	13 min
Gill	13 min	15 min

2.1.4 Tissue culture media

Six media were used. (1) Eagle's minimum essential medium with Earle's salts (EMEM) (Gibco-BRL) containing 10mM hepes, (2) double strength EMEM with 10mM hepes, (3) medium 199 (M199) (ICN Biomedicals Inc.) containing 10mM hepes, (4) double strength M199 containing 10mM hepes, (5) Leibovitz (L-15) (Gibco-BRL) containing 1mM hepes, and (6) double strength L-15 containing 1mM hepes. Media also contained 2mM glutamine, 20% (v/v) foetal bovine serum (FBS), 50µg/ml gentamycin sulphate and 5µg/ml amphotericin B.

2.1.5 Culture maintenance

Attached cells were removed from plastic substrates by decanting the culture medium, rinsing with PBSA and incubating with trypsin-versene at room temperature. Trypsin-versene contains 5g trypsin, 2g ethylenediaminetetraacetic acid tetrasodium salt and 8.5 g NaCl per litre and is diluted 1 in 10 with PBSA. Trypsinisation times varied depending on the cell type, age of the cell monolayer and the cell density, but never exceeded 30 min. After cell detachment, 10ml of growth medium was added and the cells were gently aspirated using a 10 ml pipette to obtain a single cell suspension. Cells were subcultured at a 1:3 ratio and when established, new cultures grew to confluency in 5 to 7 days at 22^oC. Only liver and heart cells survived the first few months of attempted subculturing. Within the first few months cells established from other tissues did not thrive and were eventually terminated. Cells were initially cultured in media containing 20% FBS but a subsequent reduction to 10% enhanced the rate of cell division. Cell cultures have been grown in 10% FBS and maintained for longer periods in 5% FBS. Others have also observed that 20% FBS may be supra optimal for establishment of cell lines from marine finfish (Fernandez et al., 1993; Nicholson, 1985; Watanabe et al., 1981).

2.1.6 Storage of cells in liquid nitrogen

Cultured liver and heart cells were preserved in liquid nitrogen using dimethyl sulphoxide (DMSO; Sigma D-5879) as cryoprotectant and successfully recovered at passage numbers 8, 13, 15, 16, 29, 48, 53 and 64 (liver cells) and 9, 12 and 14 (heart cells). For storage trypsinised cells were pelleted and resuspended in growth medium containing 10% (v/v) FBS and 10% (v/v) DMSO to a density of 3 million cells/ml. Aliquots of 1.5ml were placed in cryotubes, kept at -80^oC for 24 h and then placed in liquid nitrogen storage.

2.1.7 Cell cloning

Individual cells were derived from a monolayer of pilchard liver cells by treatment with trypsin-versene followed by cloning using the limiting dilution technique (McFarland, 2000). Increasing dilutions of the cell suspension were placed in wells of 96-well culture plates and incubated at 22°C. At the highest dilution, cultures derived from single cells were grown to confluency. Such cells sub-cultured and expanded until sufficient numbers were obtained to permit cryopreservation. Three clones were sub-cultured 12 times prior to cryopreservation and have been successfully preserved and recovered.

2.1.8 Cellular Proliferation Assay

Actively replicating cells were identified using a commercial kit that relied on an immunoperoxidase-based, immunocytochemical detection of 5-bromo-2'-deoxy-uridine (BrdU) incorporated into cellular DNA (Roche, catalogue number #1 299 964). Cells were grown, labelled with BrdU and the incorporated nucleotide analog detected according to the manufacturer's instructions. The fixative and temperature of fixation were modified for use with piscine cells. Cells were fixed with acetic acid:ethanol (1:3) at ambient temperature (approximately 22°C) for 45 minutes. BrdU-labelled cell cultures were stained with either Mayer's haematoxylin (Dako, Lillie's modification) and Diff-Quik II counterstain (Lab Aids Pty. Ltd, modification of Wright's stain) to determine the most appropriate counterstain for each cell type. Cell cultures were treated with Mayer's haematoxylin for 1 minute, washed with tap water, and a blueing agent, Scott's tap water, was added for 1 minute before being washed off with tap water. Diff Quik was added directly to cell cultures, drained and washed off in tap water. After drying, coverslips were applied using an aqueous mounting medium (Gurr* Aquamount improved). Mayer's haematoxylin was the better counterstain for the control RTG-2 cell line and "Diff-Quik" was the preferred counterstain for pilchard cells.

2.1.9 Confirmation of pilchard origin of cell lines

The pilchard origin of cell lines was established using a polymerase chain reaction (PCR) specific for *Sardinops spp.* and confirmed by sequence analysis of the amplified PCR product. PCR primers were designed from the consensus sequence of four DNA mitochondrial control regions from *Sardinops neopilchardus* previously lodged with GenBank (Accession Numbers U95929 to U95932). DNA was extracted from actively growing pilchard cell cultures using the Puregene® DNA Isolation Kit (Gentra Systems). DNA, for use as a positive control, was also isolated from the spleen of a frozen pilchard. DNA was quantified using a GeneQuant II® DNA calculator (Invitrogen™).

PCRs were performed in 0.2ml thin walled PCR tubes (Quantum Scientific Pty Ltd) in a GeneAmp® 9600 thermal cycler (Perkin Elmer-Cetus). Each 25µl reaction contained 1-10ng template DNA, 0.25U of Platinum™ Taq DNA Polymerase, 6mM MgCl₂, 0.2mM of each of the four deoxynucleotide triphosphates, PCR buffer (Life Technologies™) and 8pmoles each of the amplification primers, 5'-ATCTTATCATTCATGATATGCACGC-3' and 5'-TTATACTACACCGCGCGTTCG-3'. Reaction mixes were held for 2 min at 94°C and amplified for 25 cycles, with denaturation for 30 sec at 94°C, annealing for 30 sec at 56°C, and elongation for 1 min at 72°C. A final extension was performed at 72°C for 3 min. The expected PCR product was 472bp in length.

2.1.10 Virus susceptibility

Liver cell cultures (passage 56) were seeded in 25cm² tissue culture flasks and incubated at 22°C overnight prior to infection with viruses listed in Table 2. Virus was adsorbed for 1 h at either 15 or 19°C and 5 ml growth medium added to each culture. Cultures were examined by light microscopy daily for signs of virus-induced cytopathic effect (CPE). The presence of virus particles in cultures displaying CPE was confirmed by ultrathin electron microscopy.

Table 2. The source of piscine viruses

<u>Virus</u>	<u>Source</u>
Infectious pancreatic necrosis virus (IPNV) [Sp serotype]	CEFAS, Weymouth laboratory, Weymouth, United Kingdom
Infectious haematopoietic necrosis virus (IHNV) [WRAC strain]	Western Fisheries Research Centre, Seattle, USA
Viral haemorrhagic septicaemia virus (VHSV) [serotype 23.75]	INRA, France
<i>Oncorhynchus masou</i> virus (Salmonid herpes virus 2 (SHV-2)) [serotype P-17]	Faculty of Fisheries, Hokkaido University, Japan
Epizootic haematopoietic necrosis virus (EHNV)	Regional Veterinary Laboratory, Benalla, Australia
Pilchard orthomyxo-like virus	AFDL, AAHL, Geelong, Australia
Spring viraemia of carp virus (SVCV) [S-30 reference strain]	CEFAS, Weymouth laboratory, Weymouth, United Kingdom
Infectious salmon anaemia virus (ISAV) [Canadian reference strain]	Department of Fisheries and Oceans, New Brunswick, Canada
Atlantic salmon reovirus	AFDL, AAHL, Geelong, Australia

To determine if cloned cell lines differed from the parental population in susceptibility to virus infection, the uncloned parental cell line and the three cloned cell lines were inoculated with the viruses in Table 2 and incubated at 15°C. Virus-inoculated cultures were examined by light microscopy for the development of viral CPE.

2.2 Partial purification of pilchard herpes virus

Approximately 100gm gills from affected pilchards were added to TNE buffer (10mM Tris, pH 7.2, 10mM NaCl, 1.5mM EDTA) to a final volume of 500ml and homogenised in a Sorvall Omni Mixer for approximately 1 min. The homogenate was spun at 60 x g for 1 min in 50ml bluemax tubes in the bench-top centrifuge and the supernatant removed and 5ml volumes given 20 strokes in a Dounce homogeniser. The pellets in blue-max tubes were combined, homogenised again in the blender and spun up to 60 x g in the bench top centrifuge. The supernatant following the second spin was also Dounce homogenised in 5ml volumes. The Dounce homogenates were pooled and spun at 1000 x g in the bench-top for 3 min to generate pellet and supernatant (fractions 1 and 2 respectively).

2.2.1 Virus isolation

Fraction 2 (the supernatant from section 2.2) was clarified by centrifugation at 15,000 x g in a JA14 rotor for 30min and the resulting supernatant centrifuged at 140,000 x g in a SW28 rotor for 2hr to pellet virus. The pellet was resuspended overnight in TNE, layered over a 20% sucrose column and centrifuged at 150,000 x g in a SW41 rotor for 1hr. The pellet was resuspended overnight in 1ml TNE, layered over 20-40% sucrose in TNE and centrifuged at 150,000 x g in a SW41 rotor for 90min. Gradient fractions of 1ml were collected, diluted in 11ml TNE and centrifuged at 150,000 x g in a SW41 rotor for 1hr. Pellets were resuspended in 200ul TNE. Herpes virus was observed amongst extensive cellular debris in fraction number 8. Lesser amounts of virus were found in fractions 7 and 9. Complete enveloped virus was not observed and many nucleocapsids were fragmented.

2.2.2 Nucleocapsid isolation

Non-ionic detergents Brij-58 and Triton-X were added to fraction 1 (the pellet from section 2.2) to concentrations of approximately 0.5 and 1.0 respectively and the mixture was clarified by centrifugation at 1000 x g for 30 min. The supernatant was centrifuged at 140,000 x g in a SW28 rotor for 2hr. The pellet was resuspended in TNE, layered over a 20% sucrose column and centrifuged at 150,000 x g in a SW41 rotor for 90min. The pellet (putative nucleocapsids) were resuspended and mixed with 40% CsCl to a density equivalent to 34%

and a volume of 12ml. The nucleocapsid-CsCl solution was spun at 150,000 x g overnight. Two opalescent bands were observed near the top of the gradient and a brown band below. Both upper bands were removed diluted in TNE, repelleted and resuspended overnight in TNE. Electron microscopic observation indicated that the top band contained intact and fragmented nucleocapsids. CsCl fractions adjacent to the virus band contained virtually no virus.

2.3 PCR

2.3.1 Methodology and reagents

DNA was extracted from partially purified virus preparations, pilchard cell lines and affected tissues using the Puregene[®] DNA Isolation Kit (Gentra Systems¹⁰) as per the manufacturer's instructions. Other reagents such as Platinum[™] Taq DNA Polymerase and PCR buffer were obtained from Life Technologies.

Each 25 μ L PCR reaction contained 1 μ L of template DNA preparation, 2.5 μ L of 10x reaction buffer (Gibco BRL), 2.0mM MgCl₂, 50ng of each amplification primer (Table 3), all four deoxynucleotide triphosphates at 0.2mM each, and 1.0U of Platinum[™] Taq DNA Polymerase (Gibco BRL). Reaction mixes were held for 2min at 95°C and amplified for 30 cycles using a GeneAmp 9600 thermal cycler (Perkin Elmer Cetus), with denaturation for 30 sec at 94°C, annealing for 30sec at 55°C, and elongation for 1min at 72°C. Templates for nested or semi-nested PCRs were prepared by transferring 1 μ L of primary PCR into the nested PCR mix. Cycling conditions for the nested PCR are as for the primary PCR. PCR products were detected by electrophoresis (10 V cm⁻¹) in a 1 x TBE agarose gel (1.5%) supplemented with 0.5 μ g mL⁻¹ ethidium bromide.

Table 3 PHV Primers

Primer	Sequence 5'-3'	Length
1f	GATTTTGGATAAACTGGGGTTGGC	24
4r	CTTGGTAGTTCCTTTGTAATCTGG	24
2f	GATATGGACCTGGTGGTCCAAGC	23
3r	CATTCTTGTGTAGCGGTGTTCTTCTC	27

2.3.4 Analysis of PCR products

PCR products were purified using a Qiagen™ PCR product purification kit and both DNA strands were sequenced using BigDye® Chemistry (Applied Biosystems™). Sequencing was performed with an Applied Biosystems™ 7700 DNA sequencer. The resulting sequence data were analysed by blastn analysis (Altschul *et al.* 1997) and bioinformatics analyses were conducted on BioNavigator.com as provided by Entigen Corporation (<http://www.entigen.com>).

SECTION 3: RESULTS

3.1 Cultured pilchard cells

3.1.1 Development of continuous cell lines

The worldwide growth of the marine aquaculture industry in recent years has led to an increased interest in the development of marine fish cell lines especially for use in virus isolation and growth (Chang *et al.*, 2001; Ganassin *et al.*, 1999; Perez-Prieto *et al.*, 1999; Qin *et al.*, 2001). Establishment of marine fish cell lines representing important aquaculture species has almost become routine and the methods used in this study closely follow those developed for other vertebrate species with some modifications (Freshney, 1994; Nicholson, 1985). Trypsinised cells and explants from pilchard heart, liver, spleen and kidney were incubated at 22°C in a number of single and double strength media. Double-strength media were used in an attempt to mimic the higher salt content of marine environments, but under these conditions cells did not attach to the substrate within a 50-day observation period. Gill cell cultures did not survive in any medium, a problem compounded by the fact that gill cultures frequently suffered extensive bacterial and fungal contamination. Despite extensive washing of the excised gill tissue with antibiotic-supplemented media, cultures were almost always overcome by contamination. It was noted however that in spite of major contamination, gill fibroblastic cells became attached and appeared to grow in single strength L-15 medium.

Cultures derived from trypsinised and explanted kidney and spleen tissues attached and spread to varying extents. Some cultures were sub-cultured once but subsequently failed to show evidence of cell division and were eventually discarded. Cells derived from explanted heart and liver tissue did not attach to the substrate and all cultures were discarded after 13 days incubation.

Trypsinised liver and heart were the only tissues to produce long-term cultures, although sustained growth was not obtained in all culture media tested. Cells derived from trypsinised heart tissues grew only in single strength EMEM. After the first 10 passages the cell morphology changed from fibroblastic to epithelial. The reason for this transformation is unknown but the epithelial appearance of the heart cells appears to be a stable characteristic. Cells derived from trypsinised liver grew best in single strength EMEM but cultures maintained in L-15 or M199 media could also be sustained.

Confluent monolayers of heart and liver cells in EMEM were obtained after approximately 5 weeks incubation. After a period of sub-culturing and incubation for various time periods, monolayer cultures were sub-cultured on a weekly basis. Pilchard liver cells were trypsinised at ambient temperature (22-24°C) for approximately 5 min. Initially pilchard heart cells were trypsinised for 15-25 min, but this was reduced to less than 10 min on subsequent passage. The pilchard liver and heart cell lines have been sub-cultured over 70 times and 40 times respectively during a 3-year period. Stock cultures are grown in EMEM supplemented with 10% FBS, 2mM glutamine and 10mM hepes. Although M-199 and L-15 media were used on initial cultures, further trials using these and other medium types were not pursued.

3.1.2 Incubation temperature

Cultures were initiated and maintained at 22°C. To determine the optimal growth temperature replicate pilchard liver cell cultures (passage 63) were incubated at 15, 22, 25 and 30°C and examined daily by light microscopy for a period of 4 weeks. Incubation temperature had no effect on cellular morphology but cultures at 15°C reached confluency at a slower rate than at other incubation temperatures. Cultures incubated at higher temperatures (25-30°C) showed some deterioration towards the end of the 4-week incubation period. Consequently, cell cultures were grown at 22°C, the temperature at which the primary cultures were established and which is well within the normal temperature range for pilchards. It is likely that during the initial culture period, cells best suited to this temperature were selected and it is not surprising that this temperature appeared to be optimum for cell growth.

3.1.3 Characterisation of pilchard cell lines by mitochondrial DNA sequencing

A PCR specific for *Sardinops spp.* mitochondrial DNA was used to confirm that the established cell lines were derived from pilchard tissue and were not derived as a result of contamination by non-pilchard cells already established in the laboratory that may have been

co-cultivated in primary cultures and selected during repeated passage. The PCR-based amplification of DNA from liver and heart cell cultures and pilchard spleen yielded the expected 471bp amplicon. Sequencing and alignment of both DNA strands from all amplicons yielded unequivocal sequence data for a 295bp region whose sequence was identical in both pilchard spleen DNA and that of the cultured cells (data not shown). This sequence was used for analysis. A blastn search of GenBank revealed that the 295bp region had 97 to 99% homology with the mitochondrial DNA sequences of 8 *Sardinops neopilchardus* and *Sardinops ocellatus* sequences previously lodged with Genbank. A further 13 *Sardinops* sequences including *Sardinops melanostictus* and *Sardinops sagax* were found to be between 92 to 98% homologous if the region used for analysis was reduced to 271bp. The high degree of homology between the DNA of the cultured cells, pilchard spleen and *Sardinops spp.* sequences in Genbank confirms the pilchard origin of both the liver and heart cells. The data also establish the likely origin of the cultured cells to be either *Sardinops neopilchardus* or *Sardinops ocellatus*. As *Sardinops ocellatus* is not known in the waters of South-Eastern Australia, where the pilchards from which the cell cultures were derived were obtained, it is reasonable to assume that the cells are derived from *Sardinops neopilchardus*.

3.1.4 Susceptibility of pilchard cell lines to infection with piscine viruses

Pilchard liver cell cultures were susceptible to infection by a range of piscine viruses in the families *Rhabdoviridae* (IHNV, VHSV, SVCV), *Iridoviridae* (EHNV), *Birnaviridae* (IPNV) and *Reoviridae* (Atlantic salmon reovirus). The cells were also susceptible to a putative member of the family *Orthomyxoviridae* (pilchard orthomyxo-like virus). Cell susceptibility was determined by electron microscopic detection of virus particles in infected cells manifesting a CPE. The pilchard liver cell line was refractory to infection with the herpesvirus SHV-2 (OMV), and ISAV, a member of the family *Orthomyxoviridae*. These results were confirmed by examination of cell culture material by electron microscopy.

Purified PHV and PHV in ground-up gill tissues from diseased fish that had been frozen at -20°C did not grow in either the pilchard liver or heart cells. The fact that no virus grew in the cells may not however reflect the susceptibility of the cells to infection by PHV, but rather the absence of infectious virus in the material added to the cell monolayers. Viruses that have been purified in the laboratory and which may appear intact in the electron microscope are frequently not infectious because of the treatments and conditions they have endured during the purification procedure. In addition diseased tissues are best stored at -80°C because storage at -20°C does not effectively preserve virus infectivity (ref).

3.2 Development of a PCR detection system for pilchard herpes virus

3.2.1 A search for regions of homology between herpesviruses of aquatic species

DNA sequences of two piscine herpes viruses, CCV and SHV-2 and an amphibian herpesvirus (Ranid herpesvirus I) were compared and a region of limited homology was detected in open reading frame (ORF) 62 of CCV (unpublished data, Brad Chadwick). The limited homology observed in this region is consistent with the fact that CCV ORF 62 encodes an enzyme known as terminase whose function is conserved among herpesviruses

Table 4. Universal primers derived from aquatic herpesviruses alignments

Primer	Primer Sequence 5' → 3'	Number of degeneracies	Primer size	Primer location from CCV sequence (nt)
U-1F	GAMGAWCTSTTYAARTAY AARCAYAT	8	26-mer	82280-82305
U-2F	GACATYGAGATYGTGGTC CARGC	3	23-mer	82505-82524
U-3R	CTGGATSGTASMKAAACT CYCGRGTYTC	7	28-mer	82643-82670
U-4R	AARTGRACCYKGGTMGTT CCTTTRTA	6	26-mer	82673-82698

Sequence Codes: R=AG, Y=CT, M=AC, K=GT, S=GC, W=AT, H=ACT, B=GCT, V=AGC, D=AGT, N=AGCT. U: universal; F: forward; R: reverse.

(Davidson, 1992). Terminases cleave newly synthesized viral DNA as it is inserted into nucleocapsids during viral morphogenesis. On the assumption that PHV contained a similar, related gene, a number of degenerate PCR primers were made to target this region of the PHV DNA genome. The primers that were synthesized and their location within the CCV genome are shown in Table 4.

3.2.2 Amplification of PHV DNA using degenerate primers

PHV DNA for PCR amplification was extracted from partially purified virus isolated from the gills of affected pilchards frozen after the 1998 mass mortality. The primary PCR, using the primers U-1F and U-4R, yielded no visible product after the first round of amplification. A 1:10 dilution of the first amplification round was used as template in a second round of amplification with nested and semi-nested primer sets. The nested primer pair U-2F and U-

3R, failed to yield a product, but the semi nested primer set, U-2F and U-4R, generated a PCR product of the expected size, approximately 200 base pairs (bp). This product was designated U2F-U4R DNA and the sequence is shown in Fig. 1.

The sequence of PHV U2F-U4R DNA was used to construct 2 new putative PHV-specific primers P-3R and P-3BR, the locations of which are indicated in Fig. 1. Significant differences were observed between the universal U-3R primer and the sequence of PHV DNA in that region and these differences are the most likely explanation for the failure to amplify DNA using the original U-3R primer (Table 2).

U-2F	<u>GATATGGACCTGGTGGTCCAAGC</u> TCAAACATAAACATGTGTGAGCAAACCTTCAAGGGC
	P-3R
	GTTGTCGCGCTGATGGACGACATCATGGACGAGCCGACCTT <u>CGAGAAGGAACACCGCTA</u>
	P-3BR
	<u>CACAAGAATGGTTGGTACCATGGAGGGGGCGCGGTT</u> CATCTTCAACCCAGATT <u>TACAAAG</u>
U-4R	<u>GAACTACCAAGGTCCACTT</u>

Fig 1. The sequence of PHV U2F-U4R DNA with the sequence at the U-2F and U-4R primer sites underlined and in bold and the primers indicated above the sequence in bold. The positions of putative PHV-specific primers P-3F and P-3BR are also indicated.

Table 5. Alignment of universal and PHV-specific primers

Primer	Primer Sequence 5' → 3' ¹
U-3R	CTGGATSGTASMKAAACTCYCGRGTYTC
P-3R	CGAGAAGGAACACCGCTACACAAGAATG

¹ Regions of identity between the sequences are in bold

3.2.3 Amplification of PHV DNA using universal and putative PHV-specific primers

With a view to generating a nested PCR, the efficacy of the new, putative PHV-specific 3R and 3BR primers was tested using a 1:10 dilution of PHV U1F-U4R DNA amplified as described in section 3.2.2. Initial attempts to amplify PHV DNA from the primary (U1F-U4R)

reaction using the U-2F and either P-3R or P-3BR primers failed. This failure using primers believed to be PHV-specific, suggested that PCR conditions were not optimal. In an attempt to increase the efficiency of the procedure, individual test parameters were modified in a system using degenerate U-2F and U-4R primers and SHV-2 DNA as template. Increased efficiency of amplification was achieved by raising the annealing temperature from 50°C to 55°C (data not shown). Using the new annealing conditions and a 1:10 dilution of the U1F-U4R primary amplification product as template, a further attempt was made to amplify PHV DNA using the universal primer U-1F and the new, putative PHV-specific P-3BR primer. The reaction generated a strong band of expected size (approximately 370), a weak band at approximately 350 bp and a very faint band greater than 1000 bp. The dominant band was gel purified and sequenced (Fig 2).

<p>U-1F</p> <p><u>TGACGGATCTGTTTAAGTATAAGCAYATGATTTTGGATAAACTGGGGTTGGCCACACCGGAGATTG</u> <u>CCGCACACTAATCCCAAATGTGCATCACCCAGTACTGTGTTACAGATTGATAAAATTTTCACAAGATA</u> <u>CAGTAAATGCTACTCGTTGGTTTTAGCCCCTCGACAATGTGGGAAGACCACCATCATGGTCTTATTG</u> <u>GTCGCCGCGATGATTCTTTACACGGATATGGACCTGGTGGTCCAAGCTCAAACATAAACATGTGT</u> <u>GAGCAAACCTTCAAGGGCGTTGTCGCGCTGATGGACGACATCATGGACGAGCCGACCTTCGAGAA</u></p> <p style="text-align: center;">P-3BR</p> <p><u>GGAACACCGCTACACAAGAATGGTTGGTACCATGGAGGGGGCGCGGTTTCATCTTCAACCCAG</u></p>
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Fig 2. The sequence of U1F-P3BR DNA with primer sites underlined and in bold and the primer designation indicated above the sequence in bold.

3.2.4 Analysis of the compiled PHV DNA sequences

The U2F-U4R and U1F-P3BR PHV sequences (Figs. 1 and 2 respectively) were combined, generating a total of 422 bp from U-1F to U-4R (Fig. 3). Analysis of the DNA revealed two, out-of-phase and overlapping ORFs, potentially encoding two peptides 24 and 130 amino acids in length (Fig. 4). The short peptide (peptide 1) is partially encoded within the U-1F primer region. The first encoding nucleotide of the short peptide is the second nucleotide of the primer and the sequence terminates 72 nucleotides down stream at a TAA termination codon (Fig. 3). The sequence of peptide 2 is in a different reading frame from the sequence encoding peptide 1 (Fig. 3).

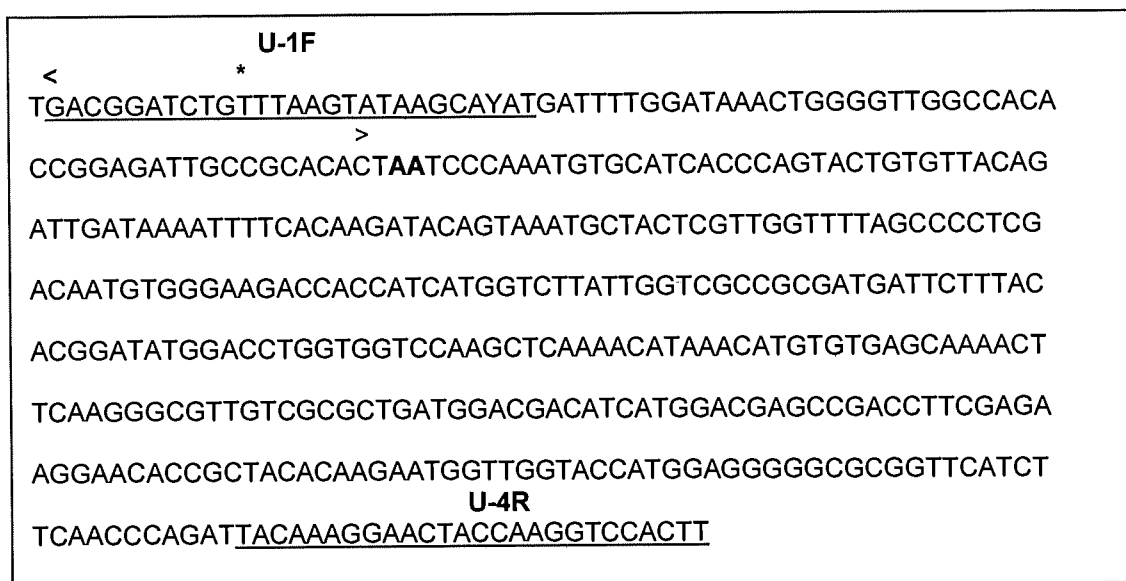


Fig 3. The sequence of PHV U1F-U4R DNA with U-1F and U-4R primer sites underlined and the designated primers indicated above the sequence in bold. The start and finish sites of the sequence encoding peptide 1 are designated by < and > respectively with the TAA termination codon in bold. The sequence encoding peptide 2 starts at the asterisk.

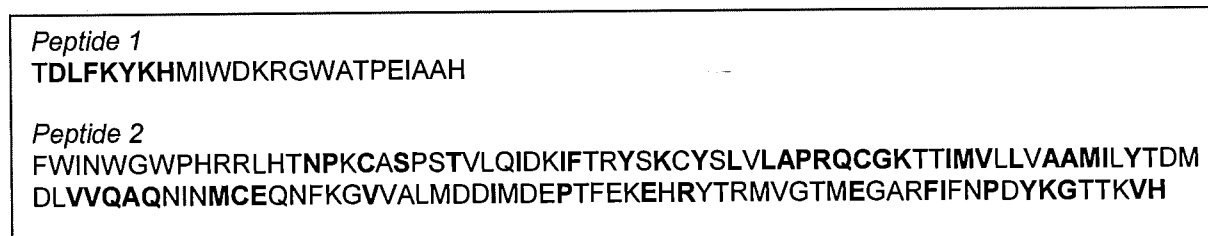


Fig 4. Peptides 1 and 2 encoded by U1F-U4R DNA. Amino acids in bold are identical to those found in channel catfish herpesvirus terminase protein.

Both the 422 nucleotide U1F-U4R DNA segment and the 130 amino acid peptide were subjected to blast and fasta searches on ANGIS to identify related nucleotide and protein sequences. The closest matches were aquatic herpesviruses SHV-2 and CCV, followed by a lower match with Ranid herpesvirus (Table 6).

Table 6. Blast and Fasta analyses of putative PHV DNA and amino acid sequences

Search program	Basis for search	Match	Smallest sum probability
Blastn	Nucleotide vs nucleotide	CCV ORF 62	3.3×10^{-8}
Blastp	Protein vs protein	CCV ORF62 SHV-2 Ranid HV	1.6×10^{-35} 2.4×10^{-35} 2.8×10^{-10}
Fasta	Nucleotide vs nucleotide	CCV	4.1×10^{-29}
Fasta	Protein vs protein	CCV ORF62 SHV-2 Ranid HV	7.4×10^{-21} 1.3×10^{-20} 2.3×10^{-6}
Tfasta	Protein vs nucleotide	SHV-2 CCV Ranid HV	1.1×10^{-22} 4.9×10^{-21} 5.0×10^{-5}

These results strongly support the view that the DNA fragment amplified by PCR using primers U-1F and U-4R was derived from the DNA of PHV, a virus related to but different from several aquatic herpesviruses. PHV peptides 1 and 2 respectively display 77.8% and 44% amino acid identity with the terminase protein of CCV. The presence of two out-of-phase open reading frames encoding CCV-related peptides suggests that the nucleotide sequence immediately after primer U-1F may not be correct. While the failure to detect a single ORF limits precise determination of the amino acid sequence of PHV terminase protein in this region, it has little impact on studies to determine the effectiveness of primers derived from sequences upstream and down-stream of the region in question.

3.2.5 Synthesis of PHV-specific DNA sequence

The U1F-U4R PHV DNA sequence (Fig. 3) was used to generate new PHV-specific primers (Table 7), and their location is seen in Fig. 5.

Table 7. PHV-specific Primers

Primer	Sequence 5'-3'	Length	%GC	Tm
P-1F	GATTTTGGATAAACTGGGGTTGGC	24	37	69
P-4R	CTTGGTAGTTCCTTTGTAATCTGG	24	41	70
P-2F	GATATGGACCTGGTGGTCCAAGC	23	56	77
P-3R	CATTCTTGTGTAGCGGTGTTCTTCTC	27	48	79

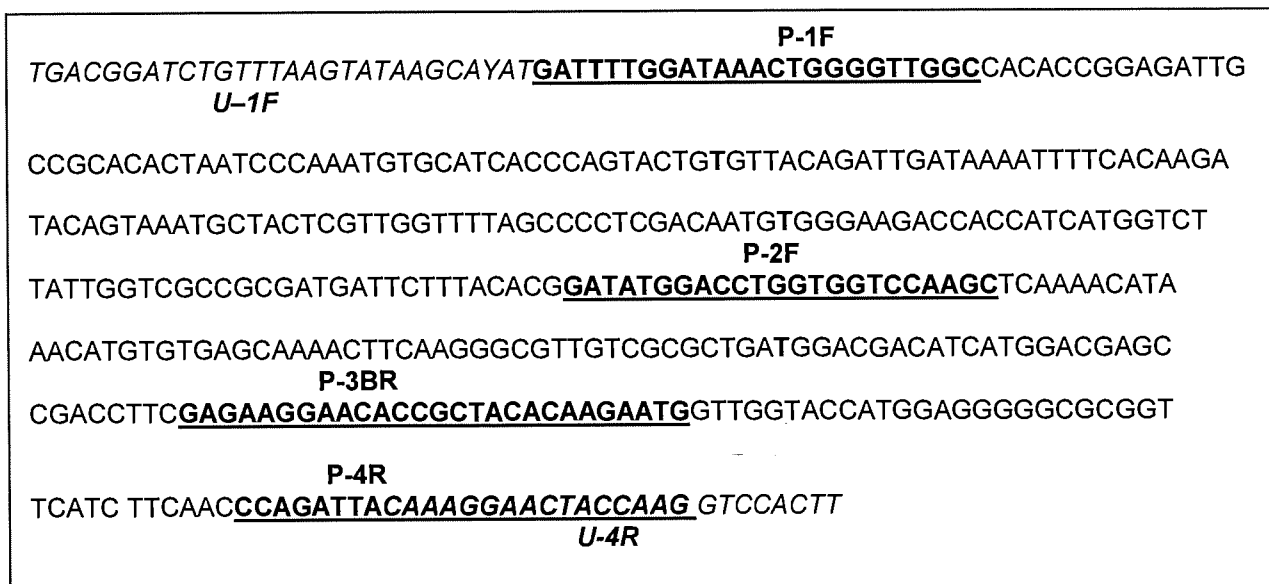


Fig 5. The sequence of U1F-U4R DNA with PHV-specific primer sites underlined and the primer designation indicated above the sequence in bold. The universal primer sites used in Fig. 1 are in italics and the primer designation below the sequence in bold.

The expected product sizes of various combinations of PHV primers are outlined in Table 8.

Table 8: Product sizes of various PHV primer sets

PHV Primer Set	Product Size
P-1F to P-4R	414 bp
P-2F to P-3R	128 bp
P-1F to P-3R	354 bp
P-2F to P-4R	188 bp

3.2.6 Specificity of primers derived from PHV sequence data

To examine the specificity of the PHV primers an attempt was made to amplify DNA from CCV, SHV-1, SHV-2, white sturgeon herpesvirus type 1 (WSHV-1) and white sturgeon herpes virus type 2 (WSHV-2). In a nested reaction with the PHV primers shown in Fig. 5 and Table 7 there was no amplification of the DNA of any of these aquatic herpesviruses (Fig 6B). Under identical conditions the universal aquatic herpesvirus U-2F and U-4R primers generated a product of the expected size (approximately 200 bp) from CCV, SHV-1 and SHV-2 DNA but not from WSHV-1 or WSHV-2 DNA (Fig 6A).

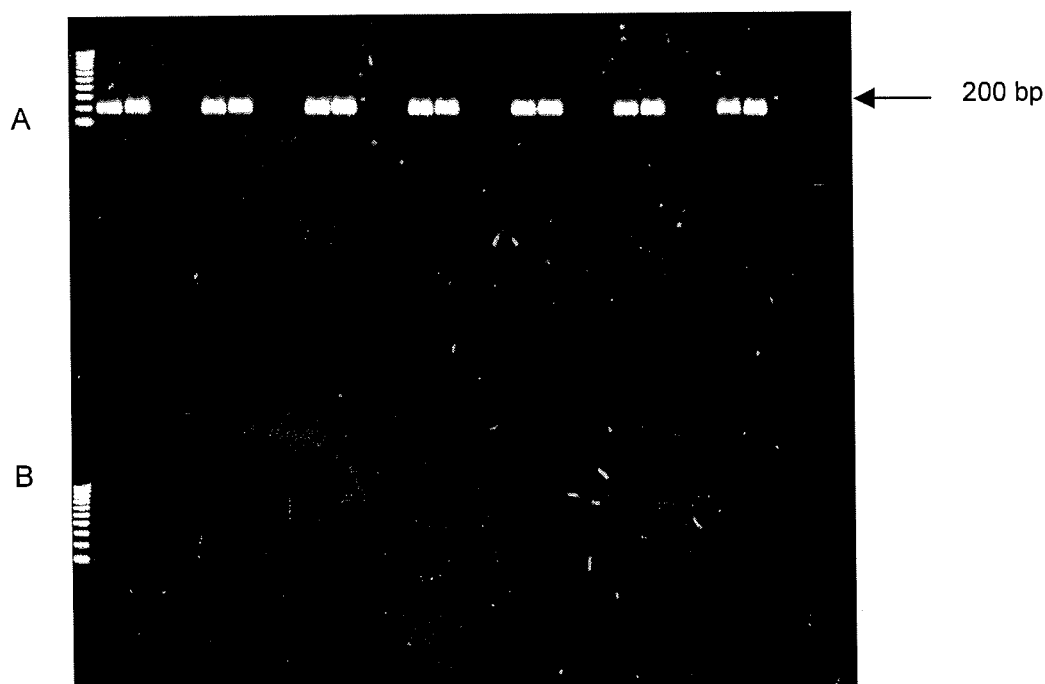


Fig 6. PCR of aquatic herpesviruses DNA using (A) universal primers U-2F and U-4R and (B) PHV-specific primers P-1F and P-4R followed by nested primers P-2F and P-3R. Both A and B contain viral DNA templates from the following sources: CCV, SHV-1, WSHV-1, WSHV-2 (repeated 6 times) followed by SHV-2 (repeated 2 times). The molecular weight marker is a 100-bp ladder. The location of the expected amplified product is indicated on the right.

primers were also tested on nucleic acid extracted from normal, uninfected pilchard liver cells. No products, specific or non-specific, were detected following amplification, demonstrating that the primers do not cross-react with sequences in normal pilchard DNA.

3.2.7 A search for a positive control for the PHV PCR

DNA was extracted from partially purified PHV isolated from affected pilchards during the 1995 epizootic, including two preparations generated at AAHL (designated G5 & G6) and one prepared in Western Australia and kindly provided by Dr Brian Jones, WA Fisheries. The latter was divided into 2 and designated WA1 and WA2. WA and AAHL preparations were generated by a series of differential centrifugation steps following homogenisation of affected pilchard gills. Preparations contained low levels of partially disrupted PHV visible by electron microscopy among extensive debris. Primary PCR using primers P-1F and P-4R did not yield a visible product (expected size 414 bp) with any of the templates (Fig 7A).

Attempts were made to amplify a fragment from the primary PCR using the nested primers. An aliquot of each primary PCR mix was used as template in a nested PCR using primers P-2F and P-3R. The nested PCR resulted in amplification products of the expected size (128

bp) from templates G5, G6 and WA2 (Fig 7B). These products were sequenced and the sequence shown to be identical to that in Fig. 5.

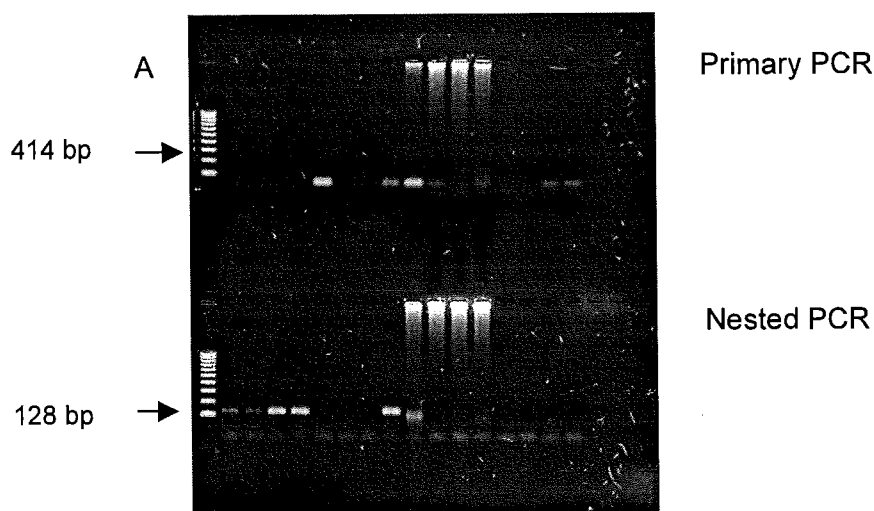


Fig 7. Amplification of PHV DNA. A. Primary PCR using primers P-1F and P-4R. B. Nested PCR using primers P-2F and P-3R. Viral DNA templates are in the following order in both A and B: G5, G5, G6, G6, WA1, WA1, WA2, WA2 (diluted 1:10 prior to addition to the PCR); and WA1, WA1, WA2, WA2 (all undiluted), and 4 negative controls (water only). The molecular weight marker is a 100-bp ladder (Advanced Biotechnologies) and locations of expected amplified products are indicated on the left.

primer set was derived from P1F-P4R DNA, the putative primary amplification product by secondary amplification or was generated using primers P-2F and P-3R in a primary amplification. Nucleic acid was extracted from the gills of affected pilchards and amplified using either primary and nested primer sets or the nested set alone. A similar amount of specific 128 bp product was obtained for each gill sample following amplification with primary and nested primers, or with nested primers alone. This suggested that the primary primers (either one, or both of them) were not working, and that the nested primers were in fact acting as primary primers.

SECTION 4: DISCUSSION

During the 1995 and 1998 epizootics the only techniques available to examine diagnostic samples from pilchards were histopathology and electron microscopy. While the latter provided evidence that a herpesvirus was present in the gill tissues of affected pilchards the technique was not appropriate for screening large numbers of fish, nor did it provide the means to compare the herpesviruses involved in both epizootics and herpesviruses from other geographical locations and other species. Such comparisons require gene sequence information.

The greatest impediment to the generation of diagnostic tests during the outbreaks was the inability of partially purified PHV, laboriously isolated from pilchard gills, or herpesvirus in homogenized gill tissue to replicate in a range of non-pilchard, piscine cell lines. The later finding was consistent with the frequently observed species specificity of herpesviruses, a fact that suggested that derivation of continuous cell lines from pilchards may offer the best option for in vitro cultivation of PHV.

Thus the two major thrusts of this project after revision of the objectives in March 2001 were the development and validation of a PCR assay to detect pilchard herpesvirus in clinical material from affected fish and the establishment of continuous pilchard cell lines that support the replication of pilchard herpes virus.

The development of a pilchard herpesvirus-specific PCR requires information on the DNA sequence of pilchard herpesvirus. At the start of this project no DNA sequence data were available for the pilchard herpesvirus, but the sequence of some aquatic herpesviruses had been determined in previous studies. Consequently the first step was to identify related sequences among aquatic herpesviruses in the hope that a similar DNA sequence might be present in pilchard herpesvirus. Comparison of the DNA of several aquatic herpes viruses such as channel catfish herpesvirus (Davison, 1992) and salmonid herpesvirus type 1 (Davison, 1998) led Dr Brad Chadwick during his tenure at AAHL to identify a region of DNA partially conserved between these two aquatic herpesviruses. Using universal, redundant primers with specificity towards all known aquatic herpesviruses, a portion of pilchard herpesvirus DNA was amplified, the sequence of which differed slightly from that of the other aquatic herpesviruses. From this sequence a set of primers was constructed that amplified only pilchard herpesvirus and did not react with other aquatic herpesviruses or with the DNA from established pilchard cell lines. The ability of the PCR test to detect pilchard herpes DNA in clinical material was been confirmed using gills removed from affected pilchards in 1995 and stored at -20°C and gills removed from affected pilchards that had been stored at

-20°C since 1999. The DNA from both sets of gills yielded a positive PCR result. The signal from the second sample was particularly strong and PHV DNA amplified from the gills had the same sequence as that found in partially purified virus.

These results indicate that the PCR test is specific and capable of detecting pilchard herpesvirus in the gills of affected fish. However, the absence of test sensitivity data means that we cannot be sure that the PCR assay will detect herpesvirus in all affected fish. Acquisition of test sensitivity data requires methods to grow the virus and either assay it or isolate it in pure form to determine the number of virus particles needed to generate a positive PCR response. This is a critical issue because the PCR test, like all other diagnostic assays, may yield positive results only in samples that contain more than a threshold number of virus particles. While our results indicate that the PCR test can be used for rapid diagnosis in outbreak situations, the lack of sensitivity data means that the test cannot be used with confidence to look for pilchard herpesvirus in latently infected Australian pilchards or in imported fish.

As the herpesvirus was seen only in the gills of affected pilchards, initial attempts to develop cell lines focussed on gill tissue from unaffected fish. These attempts were not successful for a number of reasons not the least of which was the fact that bacteria and fungi present in gill tissue contaminated the cell cultures in spite of the presence of a range of antibiotics. However, attempts to develop cell lines from pilchard heart and liver tissue were successful and the cells have grown well in flasks in culture over a period of several years. Unfortunately, purified pilchard herpesvirus and herpesvirus in ground-up gill tissues from diseased fish that had been frozen at -20°C did not grow in either the pilchard liver or heart cells. The fact that no virus grew in the cells may not however reflect the susceptibility of the cells to infection by pilchard herpesvirus, but rather the absence of infectious virus in the material added. Viruses that have been purified in the laboratory and appear intact in the electron microscope are frequently not infectious. In addition diseased tissues are best stored at -80°C because storage at -20°C for significant lengths of time does not effectively preserve virus infectivity.

The pilchard liver cells support the replication of a number of piscine viruses and a new, uncharacterised pilchard virus (an unclassified member of the myxovirus family) not associated with the disease outbreaks. This suggests that such cells may be useful in the future if there is a further occurrence of pilchard mortality associated with PHV provided affected pilchards can quickly be processed for virus isolation without freezing at -20°C or stored at -80°C .

SECTION 5: BENEFITS

This report describes the development of a PCR test to detect the herpesvirus that was implicated in the pilchard epizootics in 1995 and 1998. The assay can be used to either confirm the presence of this, or a closely related virus in the gills of pilchards affected in any future epizootic. Conversely, the assay may be used to rule out the involvement of the 1995 and 1998 PHV in further outbreaks of disease in pilchards, or indeed in any aquatic species. Thus this test is a very valuable addition to the ever increasing number of molecular diagnostic assays that may be used at AAHL for either confirmatory or exclusion purposes.

Although the susceptibility of the developed cell lines for PHV virus remains uncertain, in the event of another outbreak of disease in pilchards these cells would play a vital role in our attempts to isolate disease-causing agent. Given the difficulty faced in developing diagnostic tests, especially the more user-friendly immunologically-based ones, in situations where virus can not be grown in vitro the availability of pilchard cell lines represents a substantial resource that did not exist prior to undertaking this project.

SECTION 6: INTELLECTUAL PROPERTY

The procedures and cell lines developed during the course of this project are extensions of previously published methodologies and it is unlikely that any intellectual property has been generated.

SECTION 7: FURTHER DEVELOPMENT

While the availability of a PCR for PHV is a significant diagnostic achievement, the inability to readily grow the virus still presents significant difficulties. These include the inability to determine the sensitivity of the PCR assay because of the absence of other methods to quantify virus. Sensitivity is an issue if the PCR were to be used to seek evidence of low levels of virus such as might be present in persistently infected fish. The lack of antibody-based reagents to detect virus also represents a significant gap in our diagnostic repertoire. There is currently not enough PHV available, nor is the small amount of sucrose gradient purified virus pure enough, to generate anti-virus antiserum. New methods, which permit isolation of virus nucleic acid from infected cells without the need for viral nucleic acid sequence data upon which to base PCR primers, offer the possibility to rapidly generate complete genome sequence information. Such a method has recently been used to

determine the genome sequence of a novel, uncharacterised paramyxovirus without any reliance on the availability of genome sequence data from any other member of the Paramyxoviridae family (Bowden et al., 2001). Knowledge of the complete sequence of PHV and its comparison with that of other herpesviruses (Davison, 2002) and their genetic organisation would permit expression of genes identified as major capsid genes and the raising of antibody to virus proteins synthesized "in vitro". Such antisera would be highly specific and could be used for ELISA and immuno-histological examination of pilchard tissues. Such a molecular approach to the generation of anti-virus antisera would avoid the requirement to grow PHV in cultured cells and should be considered.

SECTION 8: STAFF

Staff in the following institutions contributed to this project.

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Lynette Williams, Senior Technical Officer, AAHL Fish Diseases Laboratory

Ken McColl, Senior Research Scientist, AAHL Fish Diseases Laboratory

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SECTION 9: DISTRIBUTION

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Product Integrity, Animal and Plant Health

Dr Gardner Murray, Executive Manager and CVO

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