Development of a laboratory model for infectious challenge of Pacific Oysters (*Crassostrea gigas*) with ostreid herpesvirus type-1

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Executive Summary

What the report is about

Between 2010 and 2013 there were devastating outbreaks of Ostreid herpesvirus 1 (OsHV-1) that caused the almost entire loss of commercially farmed and wild populations of Pacific Oysters in the Georges River estuary in NSW and later in the Hawkesbury River. Scientists at the NSW Department of Primary Industries' Elizabeth Macarthur Agriculture Institute (EMAI) at Menangle have now developed a well characterised laboratory infection model to rapidly measure the level of resistance that new generations of Pacific Oysters might have against this virus. This will allow oyster geneticists and breeders to select families of oysters from which future generations can be derived and reduce the impact of this devastating disease. In collaboration with scientists at the NSW DPI Fisheries Institute at Port Stephens, CSIRO Marine and Atmospheric Research, Tasmania and Australian Seafood Industries, the research team has successfully applied the model infection system to screen several generations of Pacific Oysters to assess the level of inherited resistance to herpesvirus infection. This model will now be used as a key component in future breeding programs as one of the tools to reduce the impact of this disease.

Background

In November 2010, a herpesvirus was identified as the cause of a disease outbreak which resulted in almost 100% mortality of farmed and wild Pacific Oysters in the Georges River. Three years later the virus was detected in the Hawkesbury River in NSW. Commercial Pacific Oyster production is no longer viable in these estuaries. Since 2008 this variant herpesvirus (Ostreid herpesvirus type I µVar – OsHV-1) has devastated farmed Pacific Oysters (Crassostrea gigas) in many countries, particularly France and New Zealand. Early observations by scientists from the NSW Department of Primary Industries indicated that there may be some evidence of genetic resistance to this virus infection. However, to identify and breed from oyster lines that have resistance to OsHV-1 infection, selection requires exposure to infection under highly controlled conditions to ensure reliable data are obtained. While oysters could be exposed to natural infection under field conditions, there are many confounding variables. These include the presence and dose of virus, variations in virus strain, effects of temperature fluctuation and the influence of a number of other environmental factors. Because this virus will devastate Pacific Oyster populations within weeks of exposure, biosecurity and containment is also an important consideration to ensure that the virus is not spread to other regions in NSW or elsewhere in Australia.

Aims/objectives

The aim of this project was to develop a laboratory based system that simulates the conditions of natural infection as much as possible but overcomes the limitations of exposing oysters to herpesvirus infection in the field. Factors that had to be taken into account included the source of the virus, availability of the same virus over a long period of time, a method to ensure that the virus remained stable and the infectivity did not decline during storage in the laboratory, methods to infect oysters of different ages and sizes and a way to monitor virus infection in the oysters and confirm that any disease or death was due to the virus infection. These studies also had to be undertaken under conditions that would ensure that the virus is fully contained and could not be inadvertently released into the wild. The ultimate objectives were to produce a stable virus preparation that could be used in a biosecure system designed to screen large numbers of experimentally infected oysters of different genetic profiles.

Methodology

To achieve these objectives, research was undertaken in the NSW Department of Primary Industries' Elizabeth Macarthur Agriculture Institute at Menangle NSW. High level containment, controlled temperature facilities are available to undertake these studies and the research team led by Dr Peter Kirkland in the Virology Laboratory has extensive experience in research with herpesviruses as well as undertaking experimental infections of animals. Initially virus was amplified by injecting healthy Pacific Oysters from Port Stephens with an extract of tissue collected from oysters affected in the Georges River, immediately after the first outbreak of disease. Subsequently, large amounts of virus were produced, semi-purified and stored frozen in a cryo-preservative solution at approximately -80°C.

With a stabilised cryo-preserved virus preparation available, a series of experiments involving the immersion of oysters in a range of different virus concentrations were undertaken. Oysters were initially placed in a very small volume of water that contained virus and, after overnight exposure, the volume of artificial sea water was increased to a volume consistent with standard husbandry in the laboratory. Oysters were then held for up to 7 days, with food and complete changes of water every second day and were examined for signs of disease daily. Water samples were collected daily to test for the presence of virus. A real time PCR assay was used to determine the quantity of virus in oyster tissues and also in the water in which oysters were held. To provide a more reliable measure of resistance to herpesvirus infection, oysters from a wide range of different genotypes were exposed to virus at several different ages.

Results/key findings

Stability studies have shown that this virus preparation has remained infectious for more than 18 months with little recognisable decline in infectivity when frozen at ultra-low temperatures (-80°C or lower). Although the virus remains infectious for moderate periods at 4°C, there is a gradual decline in infectivity to an extent that this is not suitable for use in comparative studies.

In nature, newly introduced Pacific Oysters quickly become infected when placed in waters where OsHV-1 virus is endemic. During experimental studies conducted by French scientists it had only been possible to infect oysters when they were immersed in water that had held other oysters that had been recently infected by injection. Such an approach has significant limitations because it depends on infection of large oysters by injection and the need for a ready source of virus from naturally infected oysters. The resulting quantity of virus released from the injected oysters cannot be readily determined and varies from one experiment to another. These limitations have now been overcome and the research team has successfully and repeatedly infected oysters by immersion in water containing a known quantity of OsHV-1. The availability of a stabilised cryo-preserved virus preparation has also allowed oysters to be exposed to the same batch and dose of virus in a series of experiments. Consequently a reliable and reproducible infection model has been developed that essentially simulates natural exposure. Infection and disease have been induced in small spat as well as juvenile and sub-adult Pacific Oysters.

The infection model developed now allows experimental virus challenges to be undertaken to assess the genetic basis for any variability in the susceptibility or resistance to OsHV-1 infection. The determination of virus load in holding water at 4-5 days after challenge has proven to be a simple non-invasive method to confirm virus replication in susceptible Pacific Oysters. Scientists from the Cawthron Institute, New Zealand have also been trained in the establishment and use

of this infection model and have successfully applied it to oyster herpesvirus research in New Zealand.

As well as applying this infection model to assess the genetic basis of resistance in Pacific Oyster family lines, this system was later used to assess the susceptibility of Australian flat oysters (*Ostrea angasi*). Flat oysters were tested at ages ranging from a few months through to about 18 months of age. No disease was observed and no evidence of virus infection was detected, even after testing of tissues by a highly sensitive real time PCR assay.

Implications for relevant stakeholders

The infection model that has been developed now provides a key tool that will allow geneticists and oyster breeders to select genotypes that are resistant to infection with OsHV-1. When genetically resistant oysters become available to farmers this should form a firm foundation on which to develop strategies to minimise the impact of this highly contagious disease. This may allow farmers in affected areas of Australia to eventually resume Pacific Oyster production and reduce the risk of major outbreaks in areas that are currently free of infection. The confirmation of resistance of flat oysters provides an alternative product that can be farmed in OsHV-1 endemic waters.

Recommendations

While an infection model has been developed and provides reproducible results, one of the long term limitations to its use is the need for a highly susceptible oyster line or cell culture to use as a standard substrate to monitor the stability and dose of the challenge virus. As resistant oysters are developed, if they are used as a control to determine virus levels, it is likely that they will give results that would suggest less virus has been used or that the stored virus has deteriorated. If the challenge virus is then adjusted on the basis of these results, it is probable that an artificially high level would be used and may not accurately reflect the level of genetic gain that has been achieved. Consequently there is a need to continue to breed small populations of highly susceptible Pacific Oysters to use as a standard against which genetic gain can be measured and virus levels accurately determined.

Keywords

genetic resistance, genotype, infection, ostreid herpesvirus, Pacific Oyster, real time polymerase chain reaction assay, susceptibility.

Introduction

The microvariant of Ostreid herpesvirus type-I (OsHV-1 μVar) was first recognised in France in 2008 (Segara et al., 2010) as the cause of high mortality disease outbreaks in Pacific Oysters (*Crassostrea gigas*). This strain of OsHV-1 has emerged rapidly in many countries throughout the world, with a devastating impact on Pacific Oyster production, particularly in France and New Zealand. This variant oyster herpesvirus was first detected in Australia in November 2010, and was diagnosed as the cause of a disease outbreak which resulted in almost 100% mortality of farmed and wild Pacific Oysters in the Georges River south of Sydney (Jenkins et al., 2013). Due to potential adverse public reaction to the association of a herpesvirus infection with oysters, the disease outbreak was referred to as the Pacific Oyster mortality syndrome (POMS). Soon after, OsHV-1 μVar was also detected in the Parramatta River. The disease response resulted in successful containment of OsHV-1 μVar to the two affected waterways for a period. However, in January 2013 there was a major disease outbreak north of Sydney in the Hawkesbury River system. Almost all Pacific Oysters were lost during this outbreak. The virus has remained present in the Georges and Hawkesbury Rivers despite the cessation of commercial production of Pacific Oysters. OsHV-1 continues to pose a major threat to the valuable Pacific Oyster sector which operates in 3 states of Australia, and is worth in excess of $55 million per annum. The urgent need for continued applied research has been recognised by industry, government and scientists in Australia and internationally.

Early field observations by scientists from NSW Department of Primary Industries indicated that there may be some evidence of genetic resistance to this virus infection. When Pacific Oysters of a range of different genotypes were placed in the Georges River after the outbreak, differences in mortality rates were observed. Testing of oyster samples by real time polymerase chain reaction (qPCR) assay also showed that there were different levels of virus in the tissues. Some oysters did not even become infected during the exposure period. However, to identify and breed from oyster lines that have resistance to OsHV-1 infection, selection requires exposure to infection under highly controlled conditions to ensure reliable data are obtained. While oysters could continue to be exposed to natural infection under field conditions, there are many confounding variables. These include the virus dose, variations in virus strain, effects of temperature fluctuation and the influence of a number of other environmental factors. Further, it is impossible to know when an oyster is actually exposed to the virus in the field. Therefore a negative result could potentially be due to a lack of exposure. If undertaken in the field using natural exposure, such studies are obviously limited to areas where there have been outbreaks. Biosecurity and containment are also important considerations to ensure that the virus is not spread to other regions in NSW or elsewhere in Australia

A number of research activities have been directed towards managing the risks associated with OsHV-1 infection of Pacific Oysters. A critical component is the development of a laboratory model which enables oysters to be subject to a standardised infectious challenge with OsHV-1 under controlled conditions. The aim of this project was to identify the essential materials and operating protocols for a standardised, repeatable, reproducible and transferable infectious challenge system that could be applied to a range of research questions. The infection model will find an immediate application in the screening of selectively bred Pacific Oyster family pedigrees to discriminate genetic differences in susceptibility to infection and disease. Such a model could also provide a precise method of testing the role of factors suspected of influencing the biology of OsHV-1 infection and in turn devise additional strategies to reduce the impact of disease. An effective infection model system would also allow studies to investigate the susceptibility of other oyster species to be undertaken.

Objectives

The objectives of the original project to develop an infection model were as follows:

1 Undertake the production and long-term storage of large quantities of a standard OsHV-1 inoculum

2 Evaluate methods to provide a standard, measured OsHV-1 infection challenge

3 Define the outcomes of infectious challenge including mortality, development of histopathological lesions and quantities of OsHV-1 present in tissues

4 Determine the dose-response under standardised challenge conditions, including variation due to the age of the oysters

5 Determine the repeatability, reproducibility and transferability of the standard infection challenge

After good progress had been made towards the development of an infection model, a submission was submitted for a TRF proposal to investigate whether native flat oysters (*Ostrea angasi*) were susceptible to infection with OsHV-1 and, if they became infected, would this lead to disease. Flat oysters have been identified by producers as a possible alternative to Pacific Oysters. Knowledge about their susceptibility to infection with OsHV-1 would guide oyster farmers in their decisions to investigate commercial production of flat oysters. This funding proposal was supported and the original contract was varied to include 2 additional objectives:

1. Determine if OsHV-1 μVar causes disease in flat oyster spat in a standard laboratory infection challenge system.

2. Determine if OsHV-1 μVar is capable of causing disease, subclinical replication or persistence in the tissues of adult flat oysters

Methods

The methods (and where applicable, materials) described below were used during the course of this project to:

- ensure that there was a stock of OsHV-1 virus that would remain infectious and stable during low temperature storage over a prolonged period (ideally years);
- quantify the amount of virus that was being used to infect oysters;

- establish a system of infection of oysters that would simulate natural exposure;
- confirm that oysters had become infected with OsHV-1;
- compare the susceptibility of oysters of different genotypes;
- demonstrate that the infection model was reliable and reproducible and could be used in other facilities
- determine whether other species of oysters were susceptible to OsHV-1 infection.

Specific details are as follows:

1. Source of oysters

***a) Pacific Oysters*:**

During the initial stages of the project, wild diploid Pacific Oysters were sourced from Port Stephens by the NSW Department of Primary Industries aquaculture facilities at the Port Stephens Fisheries Institute (PSFI), Taylor's Beach NSW. However, in the later stages of the research the source was changed due to concern that unexplained mortalities in Pacific Oysters at Port Stephens might confound the results and progress of this project. From January 2014 onwards only limited stocks of Pacific oysters were sourced from PSFI. Most were sourced directly from Australian Seafood Industries (ASI), Hobart, Tasmania. Specific populations of oysters that have been used are as follows:

(i) ***Wild diploid Pacific Oysters*** were collected from Port Stephens and transported to EMAI by overnight courier. These oysters were used as controls and to amplify master and working stocks of OsHV-1. They were estimated to be between 4-7, 8-12 and 12-15 months of age at the time of exposure to virus.

(ii) ***Pacific Oysters (diploid) - spat and juvenile:*** produced by the Australian Seafood Industries (ASI) 2012 breeding program. These had been held in Port Stephens and transported to EMAI overnight immediately prior to use. The oysters that were used had been and continued to be retained as discrete family lines. They were about 3 and 12-14 months of age when used in this project.

(iii) ***Juvenile Pacific Oysters (diploid)*** produced by the ASI 2012 breeding program that were transported overnight directly to EMAI from Tasmania. These oysters were retained as discrete family lines. They were about 9-10 and 12-16 months of age when challenged.

(iv***) Pacific Oysters spat (diploid)*** produced by the ASI 2013 breeding program that were transported overnight to EMAI from Tasmania. The oysters that were used were retained as discrete family lines and ranged from 3-6 months of age when used.

b) Flat oysters:

These were included as a TRF enhancement to this project aimed at determining whether native flat oysters (Ostrea angasi) are susceptible to OsHV-1 virus. These oysters ranged in age from spat to juveniles and were aged approximately 6 and 18 weeks and 6 and 12 months of age at the time of exposure to virus. These flat oysters had been bred and supplied by PSFI, Taylor's Beach and transported to EMAI by overnight courier.

2. Oyster husbandry

All oysters included in these studies were held in a secure animal holding facility at Elizabeth Macarthur Agriculture Institute, Menangle NSW. The facility consisted of a series of separate fully enclosed rooms that have impermeable walls and floor and operate under negative pressure (relative to atmosphere) and controlled temperature. All waste leaving these facilities is treated to inactivate any infectious material to meet AQIS and Australian Standards for Microbiology Laboratories.

The facilities to hold the oysters were designed to use inexpensive equipment and a simple set-up that would be appropriate for adaptation for use in a laboratory without specialised aquaculture facilities but with appropriate biosecurity measures. The use of low cost equipment and materials would allow disposal of materials after exposure to virus if deemed necessary. Otherwise materials were selected to withstand exposure to chemical disinfectants.

Juvenile oysters were kept raised off the bottom of 10 L plastic buckets with airlift circulation of artificial seawater (ASW) prepared with Ocean Nature sea salt (Aquasonic) in dechlorinated tapwater. Each bucket contained 4L of ASW, in which 10-15 oysters were held, depending on size. Spat <5mm (15-30 depending on size) were maintained in 120 ml sterile specimen containers without circulation or aeration. A complete water exchange was performed every 2 or 3 days. The water temperature was held at 20 – 21˚C for all trials and the specific gravity was 1.030 – 1.035. In some trials the oysters were fed at each water exchange by addition of a combination of Chaetoceros sp., Isocrysis sp. and Pavlova sp. cultured microalgae supplied by PFSI staff.

3. Source of virus

OsHV-1 virus was obtained on two different occasions from oysters that had been naturally infected in the Georges River. The first had been stored at -80°C as tissue samples from sentinel oysters collected 2 weeks after translocation from the Hawkesbury River to the Georges River (CV11/0367) in January 2011 and represented virus associated with the first recognised outbreak in Australia, as described by Jenkins et al. (2013). The second was derived from fresh tissues collected in December 2012 (CV12/7056) from Pacific Oysters from an OsHV-1 affected Georges River population, thereby representing the virus that was being transmitted in the field at the start of this project. Both had been confirmed by qPCR as being infected with OsHV-1.

Confirmation that these samples contained infectious OsHV-1 virus was achieved by challenge of live oysters. A filtered (0.45 µm) extract of homogenised mantle and gill tissue was administered by intramuscular injection of naive Pacific Oysters from Port Stephens. Oysters were inoculated with 2 different concentrations of virus (2-3 oysters for each), representing 10^5 and 10^7 copies of viral DNA (see below). Samples of gill and mantle were collected from inoculated oysters that died; material for the 2 isolates (2011 and 2012) was kept separate. Replication of OsHV-1 was confirmed by qPCR. Pools of tissues from oysters infected with these 2 isolates were subsequently stored frozen at -80°C until used for the preparation of virus stocks. These preparations were identified as “Pre-master” stocks.

4. Virus amplification and stabilisation

To ensure that variability in an experimental infection system could be minimised, the aim was to prepare sufficient virus to last for a long period of time and to store the virus under conditions that minimised any decline in infectivity. A seed-lot system (involving master, seed and working stocks) was also established to ensure that, if there was a need to prepare additional batches of virus, these would be derived from a common source. The number of times that the virus was further amplified in the laboratory was also minimised to ensure that the virus in use was as close to the original 'wild' strain as was practical. Depending on the volume of material required, 4 to 12 wild diploid adult Pacific Oysters were inoculated intramuscularly with 100uL of either a 1/100 or 1/1000 dilution of the relevant inoculum (Pre-master, master, and seed stocks respectively). On each occasion, inoculated oysters were held until showing signs of disease or for up to 4 days. Mantle and gill tissue was then collected aseptically from each oyster and held at 4°C until the samples had been tested. Tissues from the individuals with the highest concentration of virus (as judged by the lowest Ct values) from each treatment were selected and pooled for further preparation. A 10% (w/v) homogenate of mantle and gill tissue was prepared in 0.2µm filtered artificial seawater (ASW) using a tissue blender. Homogenates were centrifuged at 1000 g for 10 min, the pellet was discarded and the supernatant was filtered successively using 1.2 µm, 0.8 µm, 0.45 µm syringe filters.

To investigate optimal conditions for long term storage, the clarified tissue extracts were held at either 4˚C or stored frozen in liquid nitrogen vapour or at -80°C after the addition of various cryoprotectants. These temperatures were selected because it is well known that many herpes viruses are relatively stable at 4°C for moderate time periods whereas freezing at -20°C immediately inactivates these viruses. To enhance stability, especially during ultra-low temperature storage, a range of cryoprotectants were investigated. These included the addition of 10% foetal bovine serum (FBS) and 5% or 10% glycerol or dimethyl sulfoxide (DMSO). For freezing at ultra-low temperatures (-80°C and approximately -150°C in liquid nitrogen vapour), the virus preparations were frozen slowly overnight (at a rate used for viable cell cultures) by lowering the temperature by about 1˚C per minute. This was achieved by placing the vials in a polystyrene box held overnight in a -80˚C freezer. The vials were then transferred directly for long term storage at either -80°C or in liquid nitrogen vapour. The retention of infectivity was investigated by the experimental infection of Pacific Oysters by various routes of infection. Although testing of samples by qPCR only provides an estimate of the amount of viral DNA present and is not a direct measure of virus infectivity, it still provides a guide to the relative amounts of virus that may be present. Consequently, on each occasion that a batch of virus was first prepared and subsequently diluted for use, the number of copies of viral genome in the preparation was estimated by qPCR.

5. Infection of oysters

Pacific Oysters and other molluscs have been successfully infected with herpes viruses by intramuscular injection. However, alternative methods of challenging oysters were investigated at an early stage because of the tedious nature of challenge by injection, its inapplicability to small spat, and because it bypasses many of the barriers and host responses involved in the natural pathogenesis of the disease. A range of options were investigated to expose oyster tissues to virus in water because these are more likely to resemble natural exposure in the field. These included virus adsorption directly onto tissues and immersion of oysters in water containing virus with injection used as a control.

For the intramuscular injection, oysters were "relaxed" by immersion in 50 g/L magnesium chloride for periods of 15 min to 5 h, until the oysters were open sufficiently to enable access

to the oyster muscle. The different virus preparations (derived from tissue homogenates) were diluted in 0.2µm filtered ASW to provide a dose of 10^7 or 10^6 genome copies in a total volume of either 50 µl or 100 µl. These virus concentrations were prepared by making approximately 1/100 and 1/1,000 dilutions of the tissue homogenates in ASW. After injection into the adductor muscle by 27 g needle, the oysters were placed directly into ASW and observed daily under the husbandry conditions described previously.

For challenge by adsorption, juvenile/sub-adult Pacific Oysters were relaxed in magnesium chloride, all fluid was tipped out of the shells and then a 1 – 2 ml volume of inoculum was applied to the surface of the mantle and gill whilst the oyster shells were held open. The oysters were then held out of water for 10-15 mins. The inoculum had been diluted in ASW to provide a total dose of approximately 10^5 or 10^7 genome copies.

Infection by immersion included "relaxation" of the oysters by immersion in 50 g/L magnesium chloride prior to holding oysters overnight in ASW containing the inoculum. The volume was reduced compared to standard maintenance volumes. For example, 10-12 juveniles were held in a volume of 1L or approximately 15-20 spat were held in 10 ml to enable the highest concentration of virus to be obtained. The virus exposure was performed at 4 or 5 different concentrations that were prepared as 10-fold dilutions from a highest maximum concentration available that contained 10^7 genome copies of viral DNA per ml. Thus, the concentrations of virus to which oysters were exposed by immersion were 400, 40, 4 and 0.4 times higher than the doses given by injection. The following day the volume of ASW in the buckets was then made up to 8 L with a total water exchange every 48 hours. Virus infection was monitored by collecting a sample of ASW each day and before the water change. Oysters showing signs of disease or death were removed and mantle and gill tissue collected and stored frozen at -80°C until prepared for testing by qPCR. The effect of magnesium chloride prior to infection by immersion was investigated and the procedure was attempted with and without the inclusion of physical damage (stress) by allowing the gills to dry for 5 min prior to exposure to the virus.

6. Virus detection by PCR

To extract the DNA prior to testing by qPCR, small (approximately 20mg) samples of tissue were enzymatically digested (Proteinase K, in ATL buffer total volume 20 µl, Qiagen). The DNA was then purified from the tissue extract using a commercially available magnetic bead based kit and run in accord with the manufacturer's instructions (MagMax-96, Life Technologies). The magnetic beads were handled using an automated magnetic particle handling system. Purified nucleic acids were eluted in a 50uL volume and then either immediately tested or held frozen at -20°C.

The qPCR was an in house assay (Jenkins et al., 2013) that used a commercial mastermix (Agpath, Life Technologies). The assay was run according to the manufacturer's specifications for 45 cycles on either a 7500 or a 7900 HT Fast Real-Time PCR System (Applied Biosystems), each in standard mode. The background fluorescence was adjusted automatically and the threshold was set manually at 0.05. Results were expressed as cycle-threshold (Ct) values, being the cycle at which the amplification curve crossed the 0.05 threshold. Ct values ≥ 37 were interpreted as inconclusive and ≥ 40 were considered to be negative. Full details of the tissue digestion, DNA purification and qPCR have been published by Jenkins et al. (2013). This qPCR assay was able to successfully detect purified nucleic acids from both the OsHV-1 µVar and OsHV-1 "reference strain" detected in France and kindly provided by Dr Tristan Renault (IFREMER, France).

7. Virus quantification

A critical aspect of this project was to ensure that the quantity of infectious virus to which oysters were being exposed was known. To achieve this, levels of infectious virus in different preparations were determined by experimental infection of susceptible Pacific Oysters. As a comparison, levels of viral DNA were also quantified by qPCR. Although qPCR only detects DNA and cannot be used to identify the titre of infectious virus, in most instances qPCR results could be used to confirm that oysters had been infected or to compare virus levels in water or different tissues. Specific details are as follows:

a) in vivo:

To determine the amount of infectious virus in a preparation, ten-fold dilutions were prepared in ASW. Initially juvenile Pacific Oysters were inoculated intramuscularly as this was the proven route of infection. Depending on the purpose of the titration and the level of accuracy required, groups of 4-12 oysters were infected with each dilution. Later, after a method for infection by immersion was established, groups of 8-12 oysters were infected with a limited number of dilutions close to the likely endpoint of the titration. qPCR results were also used to guide the number of dilutions required. The volumes of inoculum and handling of oysters was the same as described previously (see section 5 above). Oysters were not managed individually and those that had been exposed to the same concentration of virus were held together. An uninfected 'negative' control group was included in each assay. To establish whether oysters had become infected, they were usually held for 5 days, and their health monitored daily during this period. After 5 days, tissues were collected from all oysters and tested by qPCR to estimate the viral load or absence of infection. As there was no precise method of describing the infectious endpoint (other than ID_{50} which could vary depending on the age and genotype of the oysters), the virus concentration was expressed as a dilution of the preparation being tested. This provided a relative measure that could be used over time.

b) PCR:

Although qPCR is limited in that it only detects DNA concentrations, the results of qPCR do nevertheless provide a guide to the concentration of virus or extent to which there has been virus replication. qPCR does have other significant advantages which include that results can be available quickly (within a few hours if necessary), a large number of samples can be tested at one time, it is very sensitive and reproducible. When using qPCR, results were usually expressed as cycle-threshold (Ct) values but these could be readily converted to an estimate of virus load expressed as copies of viral DNA per unit mass of tissue.

The relationship between qPCR reactivity and the number of copies of viral genome present were established by the use of a dilution series of a partially purified virus preparation. This was tested on a number of different occasions and has extremely high repeatability across different qPCR runs. A Ct value of 12 was shown to be equivalent to approximately 10^7 copies of OsHV-1 DNA per microlitre. Since there are two copies of the qPCR target in each OsHV-1 genome, a Ct of 12 equates to approximately 5×10^6 OsHV-1 genomes per µl.

When assessing the relative extent of virus replication in tissues, the use of Ct values provided an adequate measure. It should be noted that the proportion of this nucleic acid which was derived from intact, infectious virions was potentially variable, depending on the stage of infection of the host oyster at which samples were collected. However, this measure provided a valuable comparison of the relative levels of OsHV-1 replication between different batches of oysters. When oysters were being infected, it was considered beneficial to quantify the number of genome copies of OsHV-1 in the inoculum. For this purpose, 2ml of an inoculum with a Ct of 12/ul would result in exposure to approximately 10^{10} OsHV-1 genome equivalents.

8. Evidence of virus replication in oysters

When undertaking these studies, as the ultimate goal was to have a system to infect oysters of different genotypes (presumptively linked to different levels of resistance to OsHV-1 infection), it was essential to establish whether any disease was associated with this herpes virus infection and, in the absence of disease, whether there still had been infection. Several measures were utilised to determine whether oysters had been infected. These were:

a) Clinical observations – oysters were inspected once or twice a day for signs of illness (eg partially open ('gaping'), when shells were open, sluggish responses to stimuli);

b) Pathology – dead oysters were examined for gross abnormalities and later for microscopic changes by histopathology;

c) qPCR – by testing of tissues from both sick and apparently normal oysters;

d) Detection of viral DNA in water – this was an approach that was developed during the course of the project. Water in which potentially infected oysters had been held was collected at various times from day 3 through to the end of an experiment, or in many cases, just at Day 5. These samples were then tested in the qPCR.

Interpretation of qPCR data:

While clinical observations and pathology examinations provide an indication of ill-health, the changes detected are not specific to OsHV-1 infection. Confirmation of herpes virus infection and quantification of the amounts of virus in different tissues or oysters can only be achieved by direct virus detection methods. qPCR is the most appropriate and reliable tool for this purpose. However, care must be exercised when interpreting results, especially when low levels of viral DNA are detected. The presence of residual viral DNA that may remain from the inoculum/holding water for short to moderate times after challenge has to be considered. Hence a semi quantitative assessment of DNA concentrations by qPCR was required. The detection of an increased level of viral DNA in the gill and mantle tissue above background levels arising from residual inoculum, or significant increases in the viral DNA concentrations in the water in which the oysters were held were used as an indication that amplification of the virus had occurred.

9. Infection model

After a series of studies had been completed to produce cryopreserved virus stocks and to identify ways in which oysters could be consistently infected following immersion in a diluted virus preparation, a prototype infection model was developed. After minor modifications (largely to refine the range of dilutions of virus that were used), this model system has been used on more than 20 occasions over a period of nearly 18 months. Standard operating conditions have been identified and are documented in a Standard Operating Procedure (SOP). A copy of this SOP is attached (Appendix 1). The key features of the model system include:

- Long term use of the same inoculum (working stock H050) throughout this entire period. This consisted of the filtered tissue extract to which 10% foetal bovine serum (FBS) and 10% glycerol had been added as a cryopreservative prior to storage at -80°C. Each time a challenge was conducted, a new vial of virus was taken from -80°C storage.

- Oysters were held in a biosecure facility in ASW at approximately 20-21°C
- After dilution for use, on each occasion, the number of copies of viral genome in each dilution of the inoculum was checked by qPCR. Although this did not give a direct measure of the quantity of infectious virus present, it did confirm that similar DNA concentrations were present on each occasion and that dilutions had been prepared correctly and consistently.
- Prior to exposure to virus, oysters were 'relaxed' by immersion in 50 g/L magnesium chloride for 1hr then returned to ASW for approximately 1hr.
- Oysters were held overnight in a reduced volume of ASW containing the inoculum;
- After approximately 18 hrs, without removing the inoculum, ASW was added to restore the maintenance water volume.
- After 24 hours, water was completely changed and normal volumes restored. Maintenance water volumes varied with the size/age of oysters.
- On each occasion that a challenge was conducted, both negative (uninfected) and positive (exposed proven susceptible oysters) were included. The positive controls were exposed to a range of dilutions of virus as described above (See section 7a).
- Oysters were monitored daily. Dead oysters were removed and tissues frozen for later testing by qPCR
- Water samples were collected on days 3-5 before the water was changed.
- Oysters under test were exposed to virus at several dilutions, usually ranging from 10^{-2} to 10^{-4} though dilutions of 10^{-1} and 10^{-5} were sometimes included.
- Samples of tissue and ASW were tested by qPCR to establish OsHV-1 concentrations.

10. Evaluation of model

The aim of these studies was to develop an experimental system to infect and cause disease in oysters after challenge that simulates natural exposure. This has been achieved using the model described above. Consequently, there was a requirement to:

- Determine the dose-response under standardised challenge conditions, including variation due to the age of the oysters;
- Determine the repeatability, reproducibility and transferability of the standard infection challenge;

These objectives were achieved as follows:

- The model challenge system was run on several occasions over successive weeks using Pacific Oysters of the same age group;
- The same virus preparation (H050) that had been held frozen at -80°C was used throughout all of the studies thereby minimising the impact of one potential variable and providing a much higher degree of standardisation;

- To investigate the variability due to age, Pacific Oysters of ages ranging from approximately 3-15 months and of different genotypes were tested over a period of 4 months. These oysters were also challenged at 2-3 different dilutions of the virus stock. These dilutions varied with age, with higher doses being administered to very young spat compared to juvenile spat.

- To investigate the suitability of the infection model, parallel field and laboratory challenges were undertaken for 2012 family lines (20 genotypes) on 2 occasions and a laboratory challenge was completed for 75 lines from the 2013 stock. Ten to fifteen oysters of each genotype were challenged at two standardised virus doses and infection was confirmed by testing virus concentrations in the water and from observed mortality rates. When high mortality rates were not observed by 7 days or equivocal virus levels were found in the water, tissues of individual oysters (both survivors and dead oysters from groups with a low mortality rate) were tested to establish whether infection had occurred.

11. Longitudinal study

One of the requirements for this project was define the outcomes of OsHV-1 challenge in terms of mortality, the development of histopathological lesions and quantities of OsHV-1 present in tissues. As virus levels and histopathological changes can be quite different at different stages of the disease, an intensive longitudinal study was undertaken to investigate the progression of the infection and document the associated changes. Two hundred and ten 13-14 month old Pacific Oysters from a single highly susceptible family line were obtained from ASI, Hobart, Tasmania. These oysters were simultaneously challenged with 2 different virus doses ($10^{-3.5}$ and 10^{-5} dilutions of H050) by immersion as separate groups (84 oysters each) in large containers with a volume of water and inoculum proportionately greater for the larger number of oysters being held. Thirty three oysters were held in normal ASW as uninfected controls. No ‘control’ inoculum (normal tissue extract and cryoprotectant) was administered as this had already been shown to have no adverse effect on oysters. Six challenged oysters were collected from each virus infected group along with 3 unexposed controls every 12 hours for up to 7 days. From each of the infected groups, at each time point, a minimum of 3 live oysters were collected and the balance (to a total of 6) made up with dead oysters. Once there were high mortalities, all dead oysters were collected at each time point. Mortality data was collected at each time point and a 1mL water sample was taken from both containers at each time point. From the 9 oysters (or more when there was a high mortality rate) collected at each time point, fresh mantle and gill tissue was taken to determine virus loads by qPCR. Fresh tissue and water samples were held frozen at approximately -80°C until tested. A tissue sample was collected by making a transverse section through the major organs of each oyster at the relevant time point and fixed overnight in 10% neutral buffered formalin prior to preparation of sections for histopathology.

12. Histopathology methods

Pacific Oysters were examined following fixation in 10% neutral buffered formalin, routine embedding and sectioning followed by staining with haematoxylin and eosin. Histopathologic scoring, based on primary lesions noted in the epithelium of the gills, labial palps and adjacent mantle, was used to obtain semiquantitative data from the tissues as described by Gibson-Corley et al (2013). Pathology features examined include presence of erosion (partial loss of epithelium with an intact basement membrane present), ulceration (full thickness loss of epithelium), intraepithelial focal accumulations of haemocytes (clusters of haemocytes within the epithelium), presence of underlying necrotic haemocytes (haemocytes with

pyknotic nuclei and scattered associated karyorrhectic debris) and subepithelial oedema (expansion of the subepithelium with clear space and separation of stromal fibres).

Each pathology criterion was scored based on severity, including 0 (non-existent), 1 (mild), 2 (moderate), 3 (severe). Severity was based on the focal size of the lesion and the multifocal distribution of the lesion. It was noted that at the time of sampling, both living and dead oysters were collected. The same scoring technique was applied to live and dead oysters, however an oyster with marked autolytic change (loss of nuclear detail and cellular structure) was excluded.

13. Assessment of OsHV-1 susceptibility of flat oysters

The objectives of the original project were augmented to include an assessment of the susceptibility of native flat oysters (*Ostrea angasi*) to OsHV-1 infection. This evaluation was completed by exposing flat oysters to virus infection in the experimental model system generally using the standard conditions unless specified. These flat oysters were bred and supplied by PSFI, Taylor's Beach NSW and were aged 6 and 18 weeks and 6 and 12 months old. Relaxation with magnesium chloride was not applied to the small spat used in the first 2 exposures of flat oyster spat (6 & 18 weeks) but was used with the large spat/juveniles (6 and 12 months).

The standard virus working stock (H050) was diluted in 0.2μm filtered ASW to provide a starting concentration of 10^7 genome copies per ml. This preparation was then used to challenge flat oysters by exposure to very high concentrations of virus using dilutions of working stock ranging from 10^{-1} to 10^{-3}. In short, a 10-100 fold higher concentration of virus was used to challenge the flat oysters compared to what had been used to infect Pacific Oysters. The inoculum volume was either 1L (for approximately 10-15 juveniles), 100mL (12-15 spat) or 10 mL (6 small spat) to enable the highest concentration of virus to be maintained. The following day the water volume was increased to 4L but was changed daily.

A proven susceptible line of Pacific Oysters was used as a 'control' for the challenge of the flat oysters and to confirm the reproducibility of the laboratory challenge. These oysters were exposed to 2 to 5 serial 10 fold dilutions of the standard virus preparation, commencing at a dilution of either 10^{-2} or 10^{-3}. After the exposure period, the volume of ASW in the buckets was made up to 4 L with a total water exchange every 48 hours.

Results

Preparation of a stable virus stock

A large quantity of infectious OsHV-1 virus was produced by amplifying the 'pre-master' virus stock derived from a strain collected in the Georges River in January 2011. This isolate (OsHV-1GR1-11) was selected for subsequent use because it represents the earliest known infectious source of the strain of OsHV-1 from the initial disease outbreak in Australia, before any selection pressure was applied to the virus. Virus stocks were prepared at Master, Seed and Working stock levels. Initially oysters were infected by intramuscular injection (Master stocks) but Seed and Working stocks were the product of infection by immersion. Oysters were held at 4^oC until digests of a 20 mg portion of mantle and gill tissue were tested by

qPCR. Tissues that were selected for processing contained very high virus concentrations as indicated by low Ct values in the qPCR (Ct range 7 -15). Master and Seed stocks (several batches) were stored frozen at -80°C both as tissue homogenates and clarified tissue extracts in a cryopreservative. After the successful production of small lots of apparently stable virus, one large working stock (H050), comprising more than 100 vials, was produced, stored frozen at -80°C as a clarified tissue extract in a cryopreservative and was subsequently used extensively during the course of the project.

While this inoculum is currently 'in use', a sufficient amount will be retained to allow ongoing assessment over a longer period of time.

Cryopreservation A number of different methods were investigated to allow long term storage of the virus. It was known that many herpesviruses are stable for moderate periods of time at approximately 4°C. A filtered tissue homogenate stored at 4°C was still infectious after 4 months, although a significant decrease in titre over this time was observed. Several different cryoprotectants were investigated to enhance the stability of the virus during freezing at ultra-low temperatures. These were all readily available substances and included foetal bovine serum, glycerol and DMSO. Although other combinations of solutions provided improved stability, it was shown that adding foetal bovine serum and glycerol (each at a rate of 10% v/v) provided good long term stability when preparations are frozen at approximately -80°C. There has been no apparent decline in infectivity of the main working stock (H050) after storage in small aliquots to support a "single use" strategy and avoid multiple freeze-thaw cycles that are likely to be deleterious. A small quantity of this working stock has also been stored in liquid nitrogen vapour and appears to be stable under these conditions. When used at the working dilution of 1/100 in ASW, these cryoprotectants did not have any adverse effect on oysters injected with uninfected (negative control) tissue homogenate. After freezing, the infectivity of the stabilised virus was checked by exposing groups of susceptible Pacific Oysters to a range of dilutions of the virus and using different routes of infection.

Comparison of infection methods

Initially, intra-muscular injection of juvenile oysters after relaxation in magnesium chloride was used successfully to confirm the presence of infectious virus in samples collected from the Georges River in 2011 and was later used to amplify OsHV-1 for the production of a Master stock. Compared to injection, a lower proportion of oysters became infected when infected by adsorption. In all experiments where oysters were challenged by injection or by adsorption, deaths were only observed from 2-5 days after inoculation or exposure to virus. OsHV-1 DNA was detected by qPCR in approximately 40% of the individuals that were still alive at the completion of the trial. While both injection and adsorption were shown to be effective methods to infect Pacific Oysters, these methods are tedious. In published studies, when oysters have been infected by intramuscular inoculation to expose other oysters to virus either by co-habitation or placement in water that has held the infected oysters, there is also a degree of variability each time the virus is amplified.

Infection by immersion was initially unreliable when attempting to infect juvenile oysters and required a very high dose. Encouraging feeding behaviour by the addition of algae did not increase the susceptibility of these oysters to infection. Deaths were only observed in juvenile oysters challenged by immersion when the oysters were relaxed in magnesium chloride before immersion. Using this approach, 1 - 2 cm spat that had been apparently refractory to the effects of the virus were repeatedly infected and deaths were observed.

Spat were susceptible to immersion in dilutions of both fresh and cryopreserved tissue homogenates diluted in sterile ASW as well as ASW taken directly from a bucket containing OsHV-1 infected oysters. During subsequent investigations it was shown that oysters of all ages could be infected by immersion, most consistently after 'relaxation' with magnesium chloride (Table 1). Consequently, relaxation prior to exposure to virus was introduced as a standard element of the model system.

It was shown that infection of Pacific Oysters was both age and dose dependent, with very young spat less susceptible than older animals when using the same dose of virus. There was also confirmation that oyster genotype was also a factor, as had been observed in the field. Initially the infection model had utilised wild Pacific Oysters, and later juvenile commercial spat of mixed genotype. When very young (2-3 month old) commercial spat were exposed to the same virus concentrations that induced high mortality rates in some defined genetic lines of juvenile (14-15 month old) oysters, no mortality was observed. Exposure of young spat from several different family lines to a range of higher virus concentrations did establish infection, although it was difficult to observe deaths due to the very small size of the oysters. Repeated exposures at higher virus concentrations confirmed that a 10-100 fold higher virus concentration was needed to successfully infect 3 month old spat. Overall, the data suggests very young spat are less susceptible but that by about 6 months of age, Pacific Oysters are as susceptible as juvenile stock aged about 12-14 months. However, susceptibility to viral infection also declined in mature adult stock compared to spat and juveniles. These trends have been observed consistently and with less variability than initial experiments where either wild Pacific Oysters or commercial spat of mixed genotype had been used.

Although a virus dose of approximately 10^7 OsHV-1 genome copies per ml (a 10^{-2} dilution of the standard virus working stock H050) was used in initial experiments, investigations were undertaken to establish the lowest virus dose that would infect oysters by immersion in an endeavour to identify a uniform concentration that could be used in the infection model. To achieve this, batches of oysters were exposed to 5 or 6 ten-fold virus dilutions. Although this range of dilutions could be reduced, it was not possible to use a single dose what would infect all oysters regardless of age or genotype. It was subsequently shown that some oysters could become infected using a virus concentration as low as 10^4 OsHV-1 genome copies per ml (10^{-5} dilution) while minimum infective concentrations for other oysters was 10^5 – 10^6 OsHV-1 genome copies per ml (10^{-4} &10^{-3} dilutions respectively). In order to achieve an outcome on each occasion that the model was used, 2 or 3 different virus doses were used for each challenge. The range of concentrations was adjusted depending on the age of oysters under study, with young spat (less than 4 months) usually exposed to 10^5 – 10^6 OsHV-1 genome copies per ml (sometimes 10^7 copies) and older spat and juvenile oysters 10^4 – 10^5 OsHV-1 genome copies per ml. In preliminary experiments with spat of different genotypes, 3 virus concentrations were often used, with a reduction to 2 concentrations when the susceptibility of a genotype had been established

Within these ranges of virus concentrations, deaths were consistently observed from 2-5 days after inoculation, with an occasional death occurring on days 6 or 7. However, if oysters were held beyond day 7, no further deaths were observed. Most trials were terminated at 7 days post challenge but in 2 trials, oysters were maintained for 2 weeks post-exposure. No deaths were noted after the first week.

Table 1: Comparison of infection and mortality in oyster spat challenged by immersion with and without relaxation in magnesium chloride prior to challenge.

Method	Dose*	Trial 1			Trial 2		
		Mortality	qPCR (Ct)		Mortality	qPCR (Ct)	
			Tissue	Water		Tissue	Water
Immersion + relaxation	1	6 / 6	12.75**	22.51	5 / 6	17.89	20.99
	2	4 / 6	11.54	24	5 / 6	13.21	19.74
	3	3 / 6	13.53	24	4 / 6	17.62	19.97
	4	0 / 6	12.81	22.58	3 / 6	13.81	18.56
	Negative control	0 / 6	Negative	Negative	0 / 6	Negative	Negative
Immersion	1	6 / 6	30.35	34.14	4 / 6	14.41	22.46
	2	3 / 6	10.53	22.87	5 / 6	14.80	21.09
	3	0 / 6	13.73	24.74	0 / 6	32.12	34.15
	4	0 / 6	13.45	23.65	1 / 6	36.12	Negative
	Negative control	0 / 6	Negative	Negative	0 / 8	Negative	Negative

* Doses 1 - 4 are 10-fold dilutions of a tissue homogenate in water starting at a concentration of approximately 10^7 copies per ml.

** Mean Ct values for tissues from all oysters in the group (dead and alive).

Although not frequently observed, at the end of the 7 day trial period, the mortality rate in some groups was sometimes lower than had previously been observed for the same dose of inoculum. However, when surviving oysters were tested, high virus loads, consistent with infection and active virus replication, were observed. As very high Ct values (usually >34) are detected after the inoculum had been removed and the water first changed, a Ct value of 30 or less was used as an indication that virus replication had occurred. This then allowed an assessment of an overall infection rate to be determined. This was based on a combination of the number of oysters that were dead at day 7 and those still alive but in which there was convincing evidence of virus replication. This not only provided quantitative measure of the response of the oysters to a standard virus dose but also provided a measure of the stability of the virus preparation while held frozen. Using a calculation that was based on a combination of mortalities and infections, it was found that the response to challenge was relatively similar on each occasion. No obvious decline in the titre of infectious virus has been detected but it is recognised that in the current system low levels of virus deterioration are unlikely to be detected. To monitor low levels of virus degradation in the long term, the ideal substrate for virus titrations would be an oyster population of uniform genotype and standard age. This is difficult to achieve in the absence of oyster cell cultures.

Confirmation of viral infection

Throughout the challenge studies, tissue samples were collected and tested by qPCR to confirm that deaths were due to OsHV-1 infection. The viral DNA profiles in water samples were also shown to be a useful means of assessing responses on a group basis without testing a large number of individual tissues. Water samples were initially collected daily before and after the first water change, which removes the residual inoculum. The viral DNA concentration of the inoculum at the start of challenge was used as a standard against which it was determined whether virus replication had occurred. Often no viral DNA was detected, even before the water change. When viral DNA was detected after the water change, the Ct values were very high (mid to high 30s). A Ct value of 30 or less has been used as an indication that virus replication had occurred. Detection of a high load of OsHV-1 DNA in water at days 3 - 5 post infection was a reliable indicator of widespread infection within a group and confirmed OsHV-1 replication. The virus level on day 5 post exposure alone has to date been found to be a reliable indicator of batches of oysters in which virus replication has occurred to a high level (Table 2). It should be noted however, that this is only used as a monitoring tool and cannot be used to detect limited virus replication in oyster tissues. Changing of the water and the number and size of oysters in a container all have an influence on the potential virus load that accumulates in a period of 48 hours. When high mortality rates were not observed by 7 days or equivocal virus levels were found in the water, tissues of individual oysters (both survivors and dead oysters) from groups with a low mortality rate were tested to establish whether infection has occurred and to estimate the incidence.

Table 2: Comparison of mortality rates, virus levels in tissues and virus concentrations in ASW holding oysters that had been experimentally infected by immersion in different concentrations OsHV-1

Oysters	Age	Dilution	Mortality	Water (day 5)	All Survivors*	
					Ct Range	Mean
Commercial spat	9m	-1	12/12	25.71	NT	NT
Commercial spat	9m	-2	12/12	27.18	NT	NT
Commercial spat	9m	-3	7/10	26.80	12.3 - 17.5**	14.23
Commercial spat	9m	-4	7/10	26.40	14.2 - 17.9**	15.82
Commercial spat	9m	-5	0/12	ND	33.6 - >40	35.16
Commercial spat	9m	-6	0/12	ND	32.8 - >40	34.78

* All spat surviving after 5 days were sampled and tested individually

** Virus was detected in all surviving oysters

Longitudinal study

While mortality rates and virus levels in tissues had been determined on numerous occasions, an intensive longitudinal study was undertaken to support a systematic prospective sampling regime. This investigation studied the development of infection, disease and pathology changes in 250 13-14 month old Pacific Oysters from a single highly susceptible family line. These oysters were divided into 2 equal sized groups (96 each plus 44 controls) which were simultaneously challenged with one of 2 different virus doses. Mortality data, fresh tissues to determine virus loads and samples for histopathology were collected every 12 hours. Whenever possible, both live and dead oysters were collected at each time point. As the experiment progressed, high mortality rates were observed in both the group exposed to a high and to a low concentration of virus. As a consequence, the study was terminated at 5 days post challenge. No deaths were observed until 48 hours after exposure to virus but then increased rapidly for the high dose group, with a peak point mortality of 50% observed at 60 hours (Fig 1). All oysters in this group were dead 96 hours after challenge (Fig 2). In the low dose group, there was a gradual progression of mortalities, peaking at 120 hours. With the exception of a single animal, no deaths were observed in the control group.

Extremely high virus loads were detected in all dead oysters (mean Ct = 10.1) and were similar regardless of the time of death. Viral loads in some dead individuals were as low as 5.8. Very high virus levels (mean Ct=10.8) were also detected in all live oysters sampled from 72 hours onwards (Fig 3). These would have inevitably died with the next 24-48 hours had they not been collected alive. No virus was detected in the tissue of the unexposed controls.

As the virus loads in the tissues of OsHV-1 infected oysters were very high within 48 hours of exposure, it is should not be surprising that moderately high quantities of viral DNA (Ct values of approximately 20-25) were detected in the water in which these oysters were maintained (Fig 4). No viral DNA was detected in the water holding the control oysters.

Figure 1: Point mortality of juvenile Pacific Oysters infected with 2 different doses of OsHV-1.

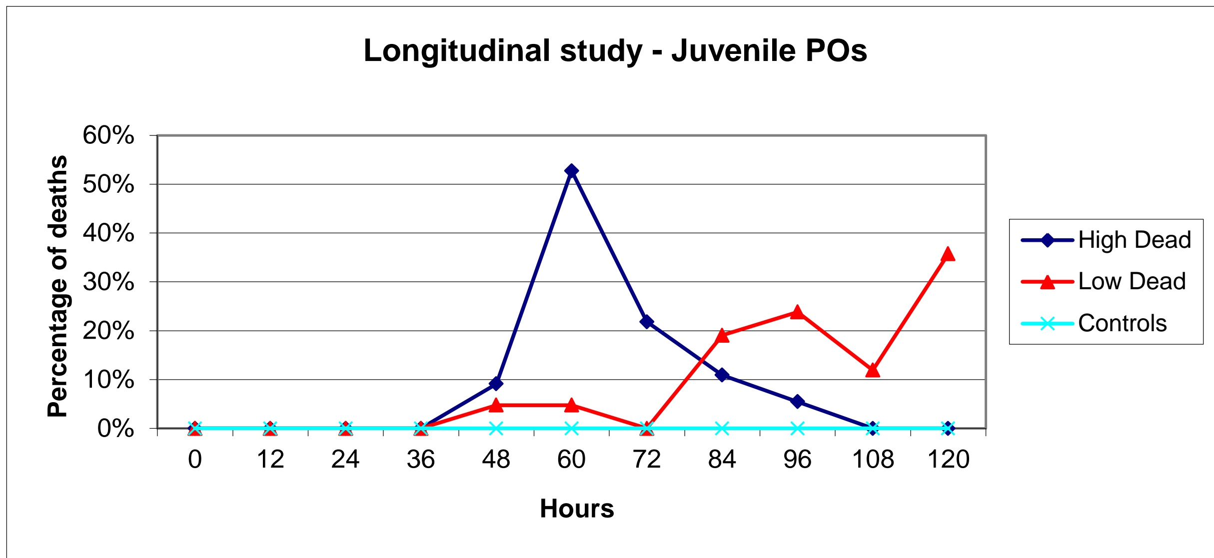


Figure 2: Cumulative mortality of juvenile Pacific Oysters infected with 2 different doses of OsHV-1.

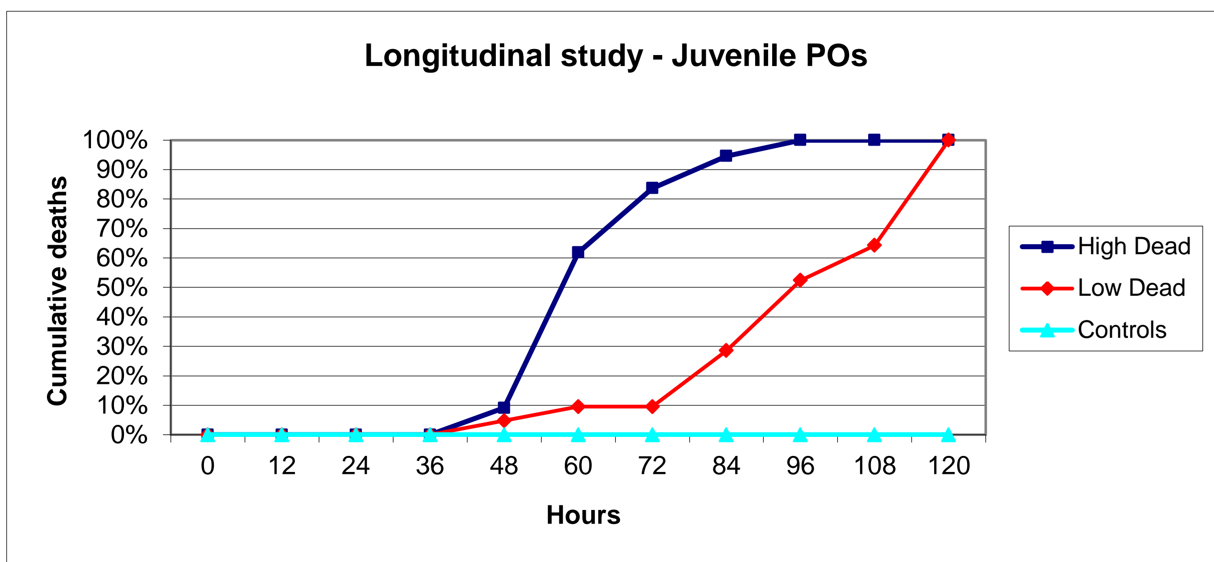


Figure 3: Virus concentrations in the tissues of both live and dead juvenile Pacific Oysters after infection with 2 different doses of OsHV-1.

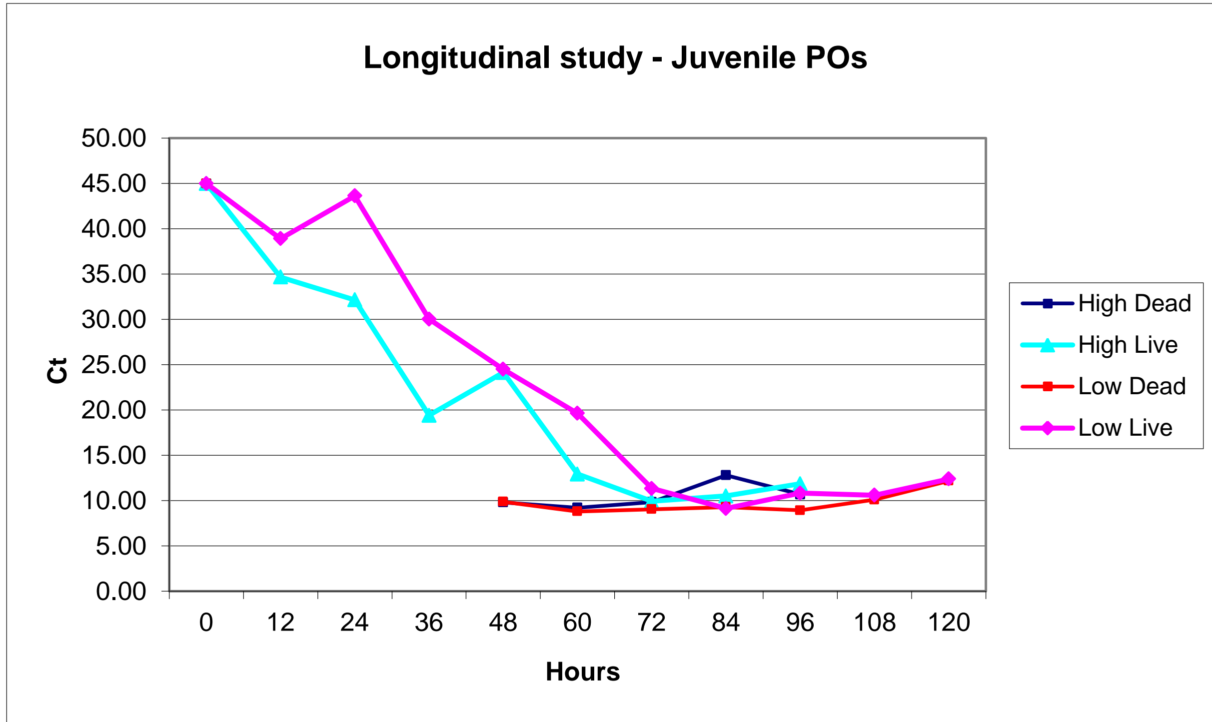
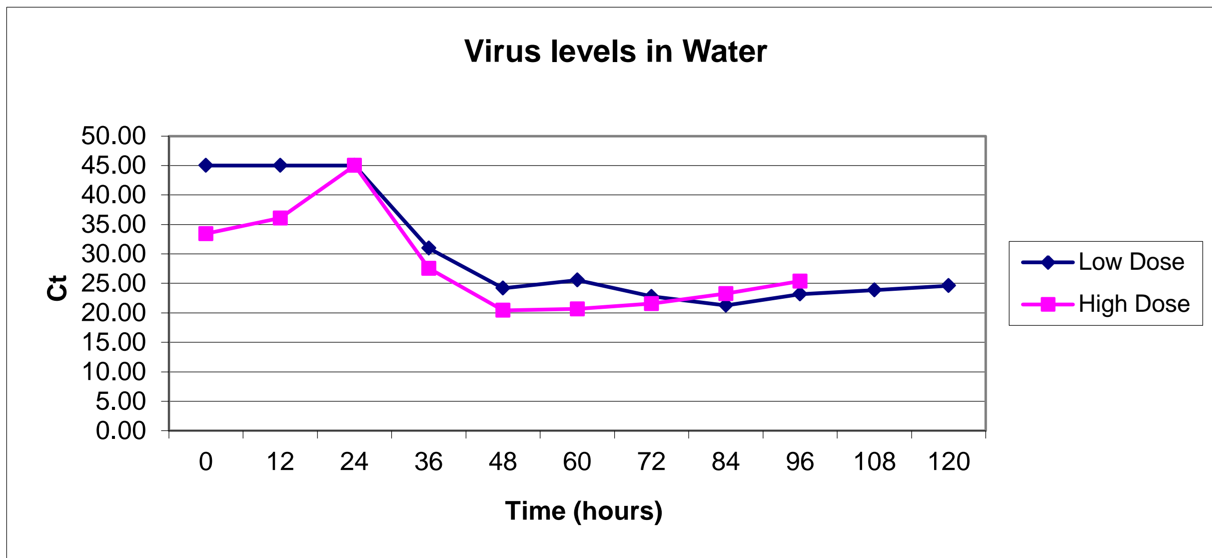


Figure 4: Virus concentrations in water holding juvenile Pacific Oysters infected with 2 different doses of OsHV-1. The holding water was changed at 24 hours and then every 48 hours afterwards.



Pathology features observed included the presence of erosion, ulceration, intraepithelial focal accumulations of haemocytes, presence of underlying necrotic haemocytes and subepithelial oedema. Both live and dead oysters were examined at most time points. All negative control oysters were alive at the time of sampling and examination of these oysters did not reveal significant histopathology findings. Mild subepithelial oedema of the palps, but

not the gills was noted, particularly in later time points (72, 84, 96 hours post infection). A few oysters had mild erosions on the palps. The average histopathology score was <2.0 at all time points examined. Details of the histopathology scoring for all groups are summarised in Table 3.

Unequivocal pathological changes were observed in tissues of infected oysters from 36 hours after exposure to virus, with lesions more severe in dead oysters. Whether oysters were exposed to high or low doses of virus, in general, the histopathology score increased at each time point examined and was greater in high dose oysters than low dose oysters at the same time point. The exception was a decrease in average histopathology score from 96 to 108 hours in low dose oysters. However, this reduction in total scores should not be misinterpreted and does not indicate a resolution of the pathology. Rather, at this time point, while specific changes appeared to be less severe, the tissue architecture was becoming disrupted. This was not taken into consideration in the scoring system. In both high and low dose oysters there was also a decrease in histopathology score from 24 to 36 hours and was associated with a decrease in severity of observed subepithelial oedema.

Epithelial erosion was one of the first histopathology changes seen and was noted from 12 hours post infection. In high dose oysters, the erosion was focally extensive and often multifocal. At earlier time points, (<60 hours) epithelial erosion was often associated with moderate to marked subepithelial oedema, prior to the development of epithelial ulceration. At later time points (>60 hours), focally extensive or multifocal loss of mantle, palp or gill epithelial cells was often adjacent to areas of ulceration.

Epithelial ulceration consisted of full thickness loss of palp, mantle and gill epithelium, often with necrotic epithelial cells at ulcerative margins. Ulceration was often associated with epithelial erosion, with increasing severity and distribution over time and was more severe in the high dose group of oysters. This change was not noted in any control oyster examined.

Intraepithelial focal accumulations of haemocytes were noted, in both low and high dose oysters appearing at low levels from 48 hours. This change was not noted in any control oyster examined.

Accumulation of necrotic haemocytes with pyknotic nuclei and associated karyorrhectic debris was specific to tissue underlying focal erosions and ulceration or areas expanded with subepithelial oedema. This change was of greater severity in high dose oysters and was not observed at time points less than 48 hours post infection. This change was not noted in any control oyster examined.

Subepithelial oedema was noted in high and low dose groups of oysters as both focally extensive expansion of the subepithelial tissue with clear space and separation of stromal fibres, or as focal expansile bullae of clear space elevating the overlying epithelium. It was noted in the gills causing expansion of the lamellae and in the palps. Accumulations of haemocytes were often associated with subepithelial oedema.

Eosinophilic intranuclear inclusion bodies were noted in few haemocytes in high dose oysters at 48 hours post infection. Although clear inclusions were rare, many haemocytes exhibited marginated chromatin and central clearing of chromatin at this time point. Affected haemocytes were prevalent in the subepithelial stroma underlying areas of ulceration, and within the spongiosum of the heart.

In summary, the histopathology changes generally increased in severity over time, were more marked in oysters exposed to high viral loads and sequentially demonstrated a progression of erosion, ulceration, subepithelial oedema and focal accumulation of necrotic haemocytes. In later time points, tissue architecture was compromised.

TABLE 3 – Summary of histopathology scoring at different time points for infection with 2 different doses of OsHv-1

SAMPLING TIME (hrs)	TREATMENT GROUP	TOTAL PATHOLOGY SCORE (mean)	TREATMENT GROUP	TOTAL PATHOLOGY SCORE (mean)	TREATMENT GROUP	TOTAL PATHOLOGY SCORE (mean)
0hr	negative control - live	0	N/A	N/A	N/A	N/A
12hr	negative control oyster - live	1.0	low dose 10^{-5} oyster - live	2.5	high dose $10^{-3.5}$ oyster - live	3.0
24hr	negative control oyster - live	0.3	low dose 10^{-5} oyster - live	3.0	high dose $10^{-3.5}$ oyster - live	4.2
36hr	negative control oyster - live	0.3	low dose 10^{-5} oyster - live	2.5	high dose $10^{-3.5}$ oyster - live	3.7
48hr	negative control oyster - live	2.0	low dose 10^{-5} oyster - live	6.2	high dose $10^{-3.5}$ oyster - dead	7.8
60hr	negative control oyster - live	0	low dose 10^{-5} oyster - live	5.3	high dose $10^{-3.5}$ oyster - dead	9.4
72hr	negative control oyster - live	1.3	low dose 10^{-5} oyster - live	7.3	high dose $10^{-3.5}$ oyster - dead	10.6
84hr	negative control oyster - live	1.0	low dose 10^{-5} oyster - live	8.1	high dose $10^{-3.5}$ oyster - live	12.1
96hr	negative control oyster - live	1.3	low dose 10^{-5} oyster - dead	10.8	N/A	N/A
108hr	negative control oyster - live	0.7	low dose 10^{-5} oyster - dead	8.6	N/A	N/A
120hr	negative control oyster - live	0.3	low dose 10^{-5} oyster - live	9.1	N/A	N/A

N/A= Not applicable – either oysters had not been infected at this time point or there were no oysters left for examination

Application of challenge model to assessment of genetic resistance

After challenge doses had been defined for oysters of different ages and the model system was working consistently, it was employed to assess the variation in resistance/susceptibility of different genotypes of oysters from the ASI oyster breeding program. To ensure that variability due to components of the model system and oysters (other than genotype) was minimised, standardised doses of the same virus stock were used through all experiments and a large numbers of families (up to 40 at a time) of oysters of the same age were tested concurrently.

Spat ranged in age from 3-6 months and juvenile stock from 14 to 16 months at the time of challenge. Infection was achieved by immersion after relaxation and used at least 2 virus doses on each occasion. Groups of 10-15 oysters were exposed to each virus dose. While encouraging feeding behaviour by the addition of algae did not affect the susceptibility to infection, oysters were fed on alternate days to maintain good condition during trials. The number of copies of viral genome in the inoculum was checked on each occasion by qPCR after dilution for use and showed little variation. The infectivity of the challenge virus was quantified on each occasion by exposing juveniles of the same highly susceptible genotype to selected $\log_{10}$ dilutions to provide doses virus near the end point of infectivity that had been established for this genotype and age. Single use vials of virus were also utilised to minimise the potentially deleterious effects of freezing and thawing. The challenges were also repeated over successive weeks to reduce variation as a result of increasing age of the oysters.

Based on these dose response studies, it has been possible to complete parallel field and laboratory challenges for progeny of the ASI 2012 breeding program (20 family lines) on 2 occasions and to run a laboratory challenge for 74 lines from the 2013 stock. These data demonstrate the variability in the susceptibility of different family lines (Figures 5 & 6). Parallel studies have also been undertaken in the field by collaborators from PSFC with comparable results.

On each occasion that oysters of a defined genotype were exposed to virus, similar mortality and infection rates were observed at the doses of virus that had been employed previously. In addition to the detection of high levels of virus in dead oysters, moderate levels of virus were again consistently detected by qPCR in the water in which the oysters had been held.

Figure 5: Survival rates for different 2013 Pacific Oyster family lines after exposure to a low dose of OsHV-1 in the experimental infection model

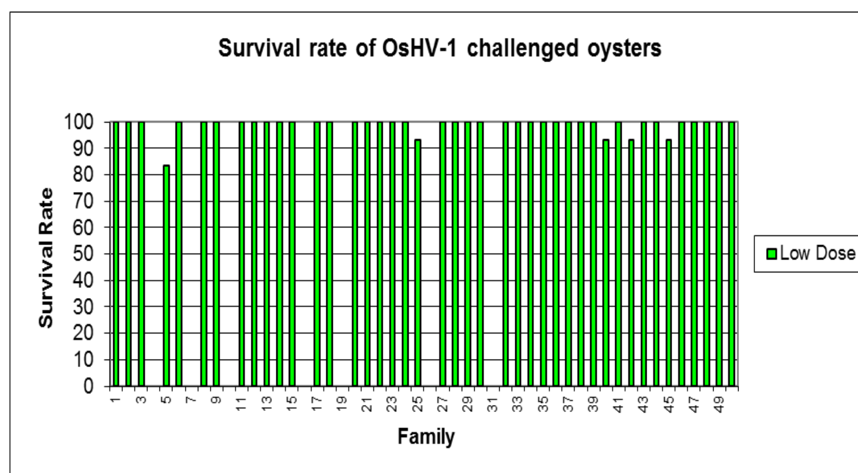
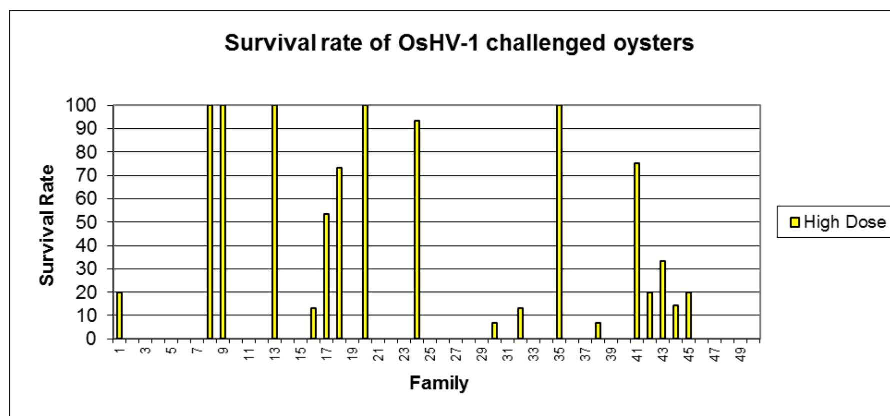


Figure 6: Survival rates for different 2013 Pacific Oyster family lines after exposure to a high dose of OsHV-1 in the experimental infection model



Infection of Flat Oysters

In order to assess the susceptibility of Flat oysters (Ostrea angasi) to OsHV-1 infection as quickly as possible, oysters were challenged with the standard OsHV-1 inoculum (H050) in the experimental model system on 4 different occasions. Use of the infection model allowed experiments to be undertaken during the winter months when natural infection of Pacific Oysters has not occurred.

Stock from an experimental production batch were first tested as spat at approximately 6 weeks of age (Table 4), then at 18 (Table 4) and 26 weeks and 12 months of age (Table 5). Very high virus doses were used in the experimental challenge. The 12 month old flat oysters were exposed to concentrations of virus resulting from a dilution of $10^{-1.3}$ (approximately $10^{7.7}$ copies of viral genome, the highest possible concentration with the current stock) to 10^{-3} (10^{6} copies) to confirm that there was a high level of resistance.

Some of the oysters that had been exposed to virus were killed for sample collection after 5, 7, or 11 days and the remainder at 14 days after challenge. After 2 weeks monitoring, there was no evidence of disease due to OsHV-1 infection and no virus was detected in the water in which the oysters were held. Testing of oyster tissues by qPCR did not reveal any evidence of infection or virus replication. Very low levels of viral DNA were detected in the tissues of some oysters when tested at both 7 (Ct range 35.6 to 36.6) and 14 days (Ct range 36.2 to 39.5) after exposure to OsHV-1. These DNA levels were at or near the limit of detection, and were most likely the result of surface contamination from the inoculum which contained DNA concentrations that were more than 10^{6} times higher.

In contrast, high mortality rates and high levels of virus replication were observed in Pacific Oyster spat that were challenged concurrently with the same levels of virus. Unlike the results for the Pacific Oysters, virus was not detected in the water in which the flat oysters were held. Collectively, these results suggest that flat oysters do not become infected with OsHV-1.

Table 4. Experimental challenge of Flat Oyster spat with OsHV-1 by immersion

Date	Oyster age (wks)	Inoculum		DNA in Water	DNA in tissues	
		Dilution	Ct	Day 5 (Ct)	No	Ct
20/06/2013	6	10^{-1}	18.6	32.8	10 (pool)	29.5
				36.3	10 (pool)	28.1
		10^{-2}	23.2	ND	10 (pool)	31.7
				34.7	10 (pool)	32.0
		10^{-3}	27.7	ND	10 (pool)	33.5
				ND	10 (pool)	35.1
		10^{-4}	31.9	ND	10 (pool)	36.4
11/09/2013	18	10^{-3}	NA	34.77	-	NA
		10^{-4}	NA	35.45	-	NA

Key: NA – not available; ND – not detected

* Oysters were exposed to OsHV-1 by immersion in artificial sea water containing a range of virus dilutions. Oysters were not treated with magnesium chloride prior to immersion..

Table 5. Experimental challenge of juvenile Flat Oysters with OsHV-1 by immersion*

Group	No oysters	Inoculum	PCR	PCR - ASW						
				Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
1	6	$10^{-1.3}$	18.22	24.67	34.66	Neg	Neg	Neg	Neg	Neg
2	12	10^{-2}	20.69	28.22	Neg	Neg	Neg	Neg	Neg	Neg
3	12	10^{-3}	24.52	28.59	Neg	Neg	Neg	Neg	Neg	Neg

Day 7 - PCR Tissues

Group	oyster 1	oyster 2	oyster 3	oyster 4	oyster 5	oyster 6
1	Neg	36.59	35.97			
2	35.95	Neg	36.44	35.57	36.59	Neg
3	Neg	Neg	Neg	Neg	Neg	Neg

Day 14 - PCR Tissues

Group	oyster 1	oyster 2	oyster 3	oyster 4	oyster 5	oyster 6
1				Neg	37.72	Neg
2	36.2	Neg	36.1	Neg	Neg	Neg
3	39.5	37.8	Neg	Neg	Neg	38.12

Technology Transfer

One of the special conditions for this project was to make the outcomes of this project available to researchers in New Zealand. As part of the transfer of the model system to other laboratories, a standard operating procedure was written (Appendix 1) and supplied to Dr C. Johnston, Aquaculture New Zealand. In late May 2014, two scientists from the Cawthron Institute, NZ, visited EMAI for training in running of the model, monitoring of infected oysters, collection of samples and running of the qPCR. They have since been able to infect oysters successfully in New Zealand.

Each of the critical aspects of the infection model has also been used to successfully infect oysters with OsHV-1 at the University of Sydney. These include the use of a cryopreserved stock of virus and infection of oysters by immersion challenge. A successful outcome was demonstrated as a differential mortality between challenged and control oysters and measurement of virus amplification using a qPCR assay

Project co-ordination and communication

One of the requirements for development of the infection model was that it be applicable to New Zealand conditions. Based on discussions with Dr C Johnston at the commencement of the project, the water temperature used during infection challenges has ranged between 19 – 21˚C. The equipment and facilities used have been simple with a view to establishment in a non-specialised biosecure laboratory or animal housing facilities.

To ensure that results from all Australian OsHV-1 research projects were effectively communicated in a timely manner, an OsHV-1 Research Coordination Reference Group was established and included the principal investigators of the three main OsHV-1 research projects in Australia (CSIRO, EMAI, and University of Sydney) and their research associates and collaborators. Meetings were held at EMAI on 10.12.2012, PSFI, Port Stephens on 2 August 2013 and on 10 April 2014 in Sydney. Some of these meetings were attended by Mark Camera (Cawthron Institute, New Zealand), Colin Johnston (NZ Oyster Industry) and Scott Parkinson (Shellfish Culture, Tasmania). Between these times there was informal email communication as required. At the inaugural meeting in December 2012 Professor R. Whittington provided an overview of virus stability studies undertaken in his laboratory for Project 2012/032. In general, these results were as expected for a herpesvirus (moderately good stability at 4°C and with complete loss of infectivity at -20°C) although the loss of infectivity after storage at -80°C and in liquid nitrogen was unusual and unexpected. These data confirmed that the approaches that were being adopted at EMAI for this project were appropriate. The use of a cryoprotectant and controlled freezing at ultra-low temperatures, as are used routinely for some herpesviruses, had been recognised as potentially important elements from the start of the George's River outbreak when tissues were being archived for future use.

Discussion

Overall, this project has met each of the stated objectives with a high degree of success. An infection model has been developed and applied to experimental infections of oysters on more than 20 occasions. A method has also been identified that allows long term storage of the virus at ultra-low temperatures without any apparent loss of infectivity and supports infection of oysters by immersion.

The capacity to store semi purified preparations of OsHV-1 virus successfully for a long period without obvious loss of infectivity and to infect by immersion has overcome many of the limitations encountered by other researchers. In most laboratories, spat or juvenile oysters have only been successfully infected after intramuscular injection (Schikorski et al., 2011b; Paul-Pont et al., 2015) or after co-habitation with virus amplified by other oysters that had been infected by intramuscular injection (Schikorski et al., 2011a). For abalone herpesvirus, stored virus has been first amplified by intramuscular injection to produce virus laden water to expose experimental animals by immersion (Corbeil et al., 2012). To overcome long term storage difficulties, repeated cycles of short term storage of

homogenates at 4°C followed by amplification by injection have been proposed to support maintenance of infectious virus through periods between outbreaks (Schikorski et al., 2011b), a practice thought to be still in use in France.

Apart from convenience, the use of a single stabilised virus preparation minimises genetic variability and allows a high level of standardisation from one experiment to the next, using a uniform challenge dose over very long time periods. To support this standardised approach, a seed lot system was developed for the systematic production of low passage virus obtained during the first Australian outbreak of ostreid herpesvirus infection. A single large working stock of virus has been in use for more than 18 months. No obvious decline in the titre of infectious virus has been detected but it is recognised that in the current system low levels of virus deterioration during storage are unlikely to be detected. To monitor low levels of virus degradation in the long term, the ideal substrate for virus titrations would be an oyster preparation of uniform genotype and standard age at each time point that a titration is performed. However, this is difficult to achieve in the absence of oyster cell cultures or life-stages of oysters that will remain viable after cryopreservation, thereby allowing ongoing use of the same standard preparation. Alternatives that should be explored include the potential to use embryonic stages or to develop cell cultures, both of which could be prepared in large quantities and stored frozen for long term use.

The inclusion of a basic cryoprotectant solution combined with a controlled slow freezing protocol are possibly factors that have overcome the loss of infectivity of semi-purified preparations that were observed by Paul-Pont et al. (2015) in Project 2012/032. The long term stability observed during storage at -80°C is in keeping with trends for mammalian herpesviruses and consistent with results for abalone herpes virus described by Corbeil et al. (2012). Indeed, the demonstration of infectivity of virus from unprocessed oyster tissues after storage for 6 months at -80°C (Paul-Pont et al., 2015) and after almost 2 years in the current project suggest that the loss of infectivity is probably related to damage to the virus during laboratory manipulations or freezing rather than the exposure to ultra-low temperature conditions. The fact that infectious virus is rescued after almost 2 years from whole oyster tissues that had been 'snap' frozen at about -80°C indicates that the loss of stability is not directly low-temperature related. A similar conclusion was made by Corbeil et al. (2012) for abalone herpes virus.

While it has been suggested that there have been different experiences with the storage of aquatic herpesviruses at 4°C, it is also possible that similar results have been obtained in the experiments involving both OsHV-1 and abalone herpes virus. Paul-Pont et al., (2015) indicate that there was a high level of infectivity after 3 months at 4°C and imply that there may not have been much loss of infectivity as observed by Corbeil et al, (2012) and in the current project. However, this would be difficult to assess using the methods described by Paul-Pont et al., (2015). These methods would at best provide a qualitative result and injections at a range of dilutions would be needed to determine the level of infectious virus present. Although ostreid herpesvirus, even when mixed with a cryostabiliser, does gradually lose infectivity over several months when held chilled at approximately 4°C, it is sufficiently stable to support short term holding (at least a few days to weeks) at normal refrigeration temperatures. This alleviates the need to freeze a virus preparation while laboratory manipulations are in progress, for example, testing by qPCR to confirm that a batch has sufficient virus to warrant distribution into ampoules and freezing at ultra-low temperatures.

The introduction of a method to produce muscle "relaxation" of oysters by immersion in magnesium chloride prior to exposure to the virus appears to be an important element that underpins the success of the infection model described in this report. After considerable success and less variable results when a one hour relaxation period was used (compared to the 3 hours first adopted), the investigation of other "relaxation" times was discontinued. However, there may be merit in investigating whether a longer period of treatment has any

effect on the susceptibility of mature oysters that appear less susceptible than younger stock. Nevertheless, this is possibly an indication of a greater resistance of older stock which are considered to be more resistant to disease and in which virus levels tend to be lower (Arzul et al., 2002). In contrast, pre-treatment of larval stages and perhaps very young spat is not necessary. Larvae have been successfully infected after immersion in virus extracted from tissues that had been stored frozen at -80°C for at least 1-2 years (Burge and Friedman, 2012) or from larvae that had been frozen at -20°C (Arzul et al., 2001)

Unlike methods in which oysters have been experimentally infected by intramuscular injection, challenge by immersion more closely reflects a natural situation and does not require extensive handing of individuals. It is clearly the preferred and most practical method for a laboratory infection model. The size and potentially the number of individuals that can be infected by injection is limited. In experiments involving co-habitation or water contaminated by virus from injected oysters, it is extremely difficult to control the dose to which the subjects are exposed. In contrast, the model developed in this project allows challenge of individuals or groups of oysters of a wide age/size range with a defined virus dose. The capacity to use small spat facilitates the inclusion of a large number of individuals to provide laboratory efficiency and provide a higher degree of statistical confidence

Throughout the course of these studies, the effects of age and genotype have been very apparent. On some occasions a virus concentration 10-1000 fold higher was needed to successfully infect very young (2-3 month old) spat compared to juvenile (14-15 month old) oysters. Some results suggest that by about 6 months of age Pacific Oysters are as susceptible as juvenile stock aged about 12-14 months. There has been speculation that some of the difference in the virus concentration needed to establish infection may be due to the higher filtration rate of larger oysters. Although they had been placed in an environment containing a lower virus concentration, they were also placed in a larger volume of ASW. When this larger volume of water is combined with a higher filtration rate, it is possible that the gills and mantle of larger oysters are eventually exposed to greater amounts of virus. This effect was also observed with oysters of varying sizes that were tested at the same age. In general, the larger individuals were the first to succumb to disease.

The genetically influenced variation in resistance to OsHV-1 infection that was first observed in the field soon after the Georges River outbreak has also been observed following exposure to the virus in the experimental model. Some family lines have a relatively high level of resistance to both infection and disease. In the future, in order to reliably measure the progress of genetic selection for resistance, it will be essential to have available a 'benchmark' of susceptible stock against with progress can be reliably measured.

One of the objectives of this project was to compare experimental infections with natural challenge. While this has been done and with a reasonably good outcome, the 2 comparisons involving spat and juvenile stock have shown the limitations of undertaking a field challenge study. Firstly, the logistics of placing and observing stock under field conditions is labour intensive and dependent on favourable environmental conditions (especially weather and tides). Secondly, there is no certainty of a consistent and uniform exposure to the virus. The April-May 2014 challenge of the 2013 spat confirmed this – there were sporadic mortalities in the spat but no evidence of OsHV-1 infection. Other factors such as other concurrent disease and biosecurity constraints on the interstate or inter-regional movement of oysters are additional considerations. With a model system, it is possible to ship oysters interstate overnight for a laboratory challenge. Even international movements are possible as there are fewer quarantine concerns in a contained, quarantine approved laboratory environment.

The capacity to test holding water for OsHV-1 DNA and to use these data as the main determinant of virus replication in a population has proven to be advantageous and

significantly reduces costs by minimising the need to collect and test by qPCR tissue samples from individual oysters. The observations and data generated in this project are consistent with those from other studies for either OsHV-1 or abalone herpes virus infections (Schikorski et al., 2011a; Corbeil et al., 2012; Paul-Pont et al., 2015).

The pathology findings of epithelial erosion and ulceration of gills and labial palps is consistent with lesions seen in oysters naturally infected with OsHV-1. However this study provided the opportunity to observe the linear progression of pathology lesions and revealed a more complex picture of the pathology changes. Additional pathology features of intraepithelial haemocytes, subepithelial oedema and lesion-associated necrotic haemocytes appear to be time specific findings that may be missed when samples are collected at a single time point. This may be particularly the case if the viral dose is high and progression to death is rapid. There are few pathognomonic signs of diseases of oysters in the scientific literature, in the absence of observation of an aetiologic agent. Thus the presence of a combination of pathology findings, while not pathognomonic, is ~~a strong diagnostic tool~~ highly suggestive of OsHV-1 infection. Gills and labial palps continue to be the target tissues for the changes observed. The use of pathology scoring provides an insight into disease progression over time. In this disease, progression appears to be viral dose and time dependent and is consistent with the known pathogenesis of herpesvirus infections. Overall, these findings indicate that the infection induced in this model closely resembles natural infection. There are no other studies where the histopathology has been described for the experimental infection of spat or juvenile oysters with the µVar genotype following infection by immersion. Most other studies involve infection by the intramuscular route and in the sole study (Schikorski et al., 2011a), histopathology was not described. However, during studies of abalone herpes virus infection induced by immersion, Corbeil et al., (2012) noted that the lesions observed microscopically also resembled natural infection whereas histological changes were not observed following intramuscular injection. Notwithstanding the fact that this experimental model can induce disease and histological changes consistent with natural infection, it is not considered that histopathology is a suitable tool for most applications for which this model would be used. Rather, direct agent detection and quantification by qPCR would be, and has already been, the most frequently used diagnostic tool.

An interesting observation was the occasional detection of surviving oysters that contained relatively high virus loads. Although all deaths ceased at 7 days after exposure, these survivors were still alive after 14 days. Similar observations have been made on several occasions for oysters surviving natural infection (Arzul et al., 2002; Dundon et al., 2011; Schikorski et al., 2011a) It is not known whether these survivors support high virus loads for lengthy periods or whether they would succumb under the stress of a field situation where they are also exposed to many other potentially pathogenic organisms. The possibility that they develop persistent or even latent infections, as commonly occurs for most mammalian herpesviruses, cannot be discounted at this stage. The implications for the persistence and spread of OsHV-1 are obvious and warrant further study.

Although the infection model that is currently in routine use is reliable and gives reproducible results, one of the limitations is that it is only a group based exposure system. At present groups of 10-15 oysters are exposed to the same treatment concurrently. Consequently, it is possible that only some oysters could become infected but that they do amplify the virus to a higher level and then infect other animals with which they are housed. This could artificially increase the proportions that are judged to have become infected after exposure to a particular virus dose. This potential for secondary infections could be overcome by segregating all individuals and holding them separately after the exposure period has been completed. Although this could complicate the logistics and operation of the system, such an approach may support a higher level of precision and provide new insights into the pathogenesis and dose response to OsHV-1 infections.

A major advantage of the model that has already been utilised is the capacity to undertake susceptibility evaluations at any time – even when outdoor environmental temperatures are below the threshold at which natural infection has been observed. The flexibility that the model offers will now allow a range of parameters such as the effect of temperature on infection thresholds to be determined in detail. An improved knowledge of the biology and pathogenesis of OsHV-1 infection should be possible.

While development of the infectivity model has been achieved, ongoing research to assess repeatability and within "run" variability and the reproducibility of a standard infection challenge in another laboratory is justified. In time, results from researchers in New Zealand may provide suitable evidence for the reproducibility of this system in a different geographical environment.

CONTACT WITH BENEFICIARIES:

Throughout the course of this project there has been frequent contact with oyster breeders, most notably Matt Cunningham (ASI, Tasmania) but also Scott Parkinson (Shellfish Culture, Tasmania). Scientists from CSIRO (Marine and Atmospheric Research, Hobart, Tasmania) and PSFI (Taylor's Beach NSW) have been regularly updated with progress of this research.

PROGRESS AGAINST COMMUNICATION & EXTENSION PLAN:

An overview of the development and operation of the infection model was presented at the Farmer's Day at the World Aquaculture Society conference in Adelaide on 8 June 2014. This presentation and the progress achieved was extremely well received by industry leaders and other producers from both Australia and New Zealand. Through NSW DPI newsletters, farmers, especially those affected by the OsHV-1 outbreaks in NSW, were advised the outcomes of the assessment of the resistance of native flat oysters to OsHV-1 infection.

Conclusion

The principal goal of this project was to develop a laboratory based model through which Pacific Oysters could be experimentally infected with ostreid herpesvirus 1. This goal has been achieved and it is now possible to experimentally infect oysters ranging from young spat through to sub-adult oysters. As well as establishing the viral infection, disease and death have ensued under controlled environmental conditions and in the absence of any other infectious agents. These infections have been induced with a standardised virus preparation that is stable and has been stored frozen for more than 18 months. A large number of different genetic lines of Pacific Oyster progeny of different ages have been exposed to the virus under controlled conditions, allowing estimates of resistance to this viral infection to be determined. This experimental model will be an important tool in the selection and breeding of Pacific Oysters that are resistant to OsHV-1 infection. The model had also been used to show that native flat oysters are not susceptible to infection with this virus, giving producers confidence to farm this species as an alternative, especially in areas where OsHV-1 infection has halted Pacific Oyster production.

Implications

The capacity to readily and consistently identify Pacific Oysters that are resistant to OsHV-1 infection will be a significant component in the battle against the devastating impact of this herpesvirus. When combined with an array of management strategies, the impact of the virus may be minimised, should its current distribution expand into areas that are currently virus free. In the long term, it may be possible to identify a range of options to resume Pacific Oyster production in areas where outbreaks have occurred.

Recommendations

While an infection model has been developed and provides reproducible results, one of the long term limitations to its use is the need for a highly susceptible oyster line or cell culture to use as a standard substrate to monitor the stability and dose of the challenge virus. As resistant oysters are developed, if they are used as a control to determine virus levels, it is likely that they will give results that would suggest less virus has been used or that the stored virus has deteriorated. If the challenge virus is then adjusted on the basis of these results, it is probable that an artificially high level would be used and may not accurately reflect the level of genetic gain that has been achieved. Consequently there is a need to continue to breed small populations of highly susceptible Pacific Oysters to use as a standard against which genetic gain can be measured and virus levels accurately determined.

Extension and Adoption

The use of this experimental infection model will of necessity be limited to laboratories in Australia and New Zealand that are conducting research involving OsHv-1 virus. Details of the experimental system have been supplied directly to researchers and their managers. Researchers from New Zealand have visited EMAI for training to develop batches of virus and in the establishment and use of the model. In Australia there are direct links between the research team and the breeders of Pacific Oysters. After the initial development phase, the model has already been applied, through the Seafood Co-operative Research Centre, to the assessment of the susceptibility of new generations of Pacific Oysters that are being bred in Australia. This partnership with the hatcheries is expected to continue.

Project materials developed

During the course of this project, standardised methods were developed for the production and long term storage of OsHv-1 virus. Subsequently, the key components of an experimental infection model were identified. The relevant equipment and materials and application of the model have been documented in a Standard Operating Procedure (see Appendix 1) which has been supplied to key laboratories in Australia and New Zealand.

Appendices

- **Appendix 1:** Development of a Laboratory Challenge System for Ostreid Herpesvirus 1 – Standard Operating Procedures.
- **Appendix 2:** Researchers and project staff.
- **Appendix 3:** Scientific References.

Appendix 1: Development of a Laboratory Challenge System for Ostreid Herpesvirus 1 – Standard Operating Procedures.

Virology Laboratory - EMAI

Development of a Laboratory Challenge System for Ostreid Herpesvirus 1

VLP180

Approved by: P.D Kirkland

Date: 10/03/2014

1. Introduction.

Herpes viruses are known to infect a range of different types of oysters and cause disease. In particular, a strain of ostreid herpesvirus-1 (OsHV-1) known as the OsHV-1 microvariant (μ-var) infects and causes severe disease in Pacific Oysters (Crassostrea gigas), usually with very high mortality rates. Most other species of oysters remain unaffected and usually do not even become infected with this virus. Within the Pacific Oyster (PO) populations, there are some genotypes that have varying degrees of genetic resistance to OsHv-1 infection and to the development of disease. A laboratory based challenge system is the most effective way of screening populations for various degrees of resistance because most of the variables found in the field can be controlled to a large extent in a laboratory model. Further, a reproducible infection model would support the investigation of the influence of other factors (age, temperature, environmental factors, infection with other agents) on the expression and severity of disease in POs. This protocol describes methods for the preparation of a stock of infectious OsHV-1, requirements to store the virus frozen and with long term stability and the conditions for the operation of a challenge system which will consistently and reliably infect Pacific oysters. It should be noted that some of the procedures described this document are still being evaluated and may be further refined. As a consequence, this document is subject to change and a check should be made to ensure that the current version is being followed.

2. Principles of Procedure.

The procedures listed in this document describe methods for:

- the isolation of OsHV-1 virus from the tissues of infected oysters by collecting, homogenising and filtering appropriate tissues,
- preparation of master and working stocks of virus by amplification of the virus in susceptible Pacific oysters. This involves infection of oysters by injection, monitoring for signs of disease, collection of tissues from diseased oysters and testing by real time polymerase chain reaction (qPCR) assay to confirm OsHV-1 infection and establish virus concentrations in the tissues;
- selection of tissues with high virus concentrations, extraction and partial purification of virus from the tissues, confirmation of virus loads, addition of a stabilising solution to these virus preparations prior to freezing and a freezing method to maximise infectivity of the virus;
- specifications and methods to assemble a laboratory based oyster culture capability with relevant biosecurity requirements;
- methods to determine appropriate concentrations of virus for oysters of different sizes/ages;
- exposure of oysters of different ages to OsHV-1 by immersion to simulate natural infection;
- maintenance and monitoring of oysters after exposure to virus to determine the occurrence of disease;
- collection and testing of samples to assess virus loads in different treatments/batches of oysters.

3. Safety and Health.

Oyster herpes viruses are not known to be infectious for humans and there are no known risks to human health from handling samples. However, some of the procedures involved in this protocol involve the amplification of OsHV-1 virus to very high titres and would present a risk to susceptible oysters whether

farmed or wild. Therefore, strict bisoecurity measures should be followed at all times and all material should be considered as potentially infectious and handled accordingly.

From the perspective of containment of infectious materials and to minimise cross contamination of materials and specimens, it is essential that as much work as possible is carried out in a Class II Biosafety Cabinet. It is extremely important to use strict aseptic techniques to avoid cross contamination of specimens or contamination of the environment during the collection and handling of specimens. This will require use of multiple sets of instruments and associated equipment. Suitable protective clothing should be worn at all times. Note that disposable vinyl or latex gloves are worn at all times during this procedure, and in some instances, “double” gloving may be specified.

Preparation of reagents, purification of nucleic acid from specimens and the conduct of the qPCR assay should all be conducted in specific, designated areas for PCR. The biological safety cabinet should be cleaned with disinfectant (usually Virkon) and the UV light should be used between individual batches of specimens and on completion of work to inactivate residual virus and nucleic acid.

All waste should be disposed of in a manner that will render all virus non-infectious. Autoclaving is preferred whenever possible. Any waste water from the culture facility should be treated to inactivate virus prior to discharge and all equipment and the holding rooms should be decontaminated with a suitable disinfectant.

4. Equipment, Reagents and Solutions.

4.1. Oyster culture/housing

a. Temperature controlled rooms;

b. Housing – various sizes depending on the age of oysters being handed (See Table 1 below) but 10L plastic buckets as a standard

c. Large water container (>200L) to mix and store artificial sea water.

d. Refractometer, thermometer,

e. Large container (Approx 200L) to disinfect used water.

f. Air Pump + aeration equipment (eg air stones, tubing etc).

g. Trays / bags to hold oysters (eg Mesh bags).

h. Oysters – for virus amplification and controls - juvenile or sub-adult diploid Pacific, no exposure to OsHV-1 or previous selective breeding.

i. Artificial sea-salt (Ocean Nature Sea Salt, Aquasonic 20 kg buckets).

j. Commerical algae concentrate (not available in Aust, use fresh cultured microalgae from PSFI)

k. Sodium hypochlorite (Bleach) to prepare 200 ppm disinfectant (4% available chlorine = 5 ml / L).

l. Boots, overalls and disposable gloves

m. Virkon disinfectant

n. Chlorhexidine (Microsheild handwash)

4.2 Preparation and administration of inoculum

a. Stored infectious material (Held frozen at or below approximately -80oC or for a few days at [4oC]). DO NOT use material that has been frozen at approximately -20oC

b. Magnesium chloride (APS Ajax Finechem Magnesium Chloride UNILAB #MAG297)

c. Glycerol, AR grade

d. Foetal bovine serum – sterile filtered, suitable for cell culture use

e. Syringes (1mL, 20mL), needles (25g) and syringe filters (5, 1, 0.8, 0.45 and 0.22 µm).

f. Penicillin and streptomycin solution (cell culture grade).

g. Artificial sea water prepared to 30 ppt (or manufacturer's directions) with purified (milliQ) water.

h. Balance

i. Laboratory bench centrifuge

j. Tissue blender.

4.3 Preparation / storage of master stock

a. Sterile scalpel handles, forceps and disposable sterile scalpel blades

b. 5ml screw topped vials; 1.5 ml screw topped cryovials

4.4 qPCR

a. Reagents, consumables and equipment for DNA extraction from tissue samples;

b. Mastermix, consumables and equipment for real time PCR.

c. qPCR primers and probe

All reagents and methods above should follow either Martenot et al (2010) or Jenkins et al (2013)

5. Procedure for Preparation of Virus Stocks

5.1 Precautions for containment of infectious material and aerosols of DNA fragments

The strict precautions described in Section 2 (above) and the use of specifically designated work areas MUST be followed at all times. Additionally, ensure that pipettors and similar equipment are used exclusively for PCR purposes, including initial sample handling and preparation.

5.2 Oysters and husbandry

At least 2 days prior to use, prepare a large volume (>150L) of ASW by filling a large vessel with good quality (dechlorinated and settled) tap water or laboratory grade purified water (preferred for master and seed stock amplification). Add the appropriate amount of sea salt. Allow to dissolve and mix thoroughly several times daily. Check the specific gravity prior to use. As water changes are usually made every second day, if storage capacity is limited a new batch of water should be prepared when each water change has been completed.

Although a wide range of ages of oysters may be used for the preparation of a virus stock, juvenile Pacific Oysters with a shell height of 5-8 cm (mass 39 +/- 9 grams when relaxed and emptied of water) will provide sufficient tissue to produce a master stock of virus. These oysters are estimated to be between 4 and 7 months of age. Pacific Oyster spat of 1- 2 cm shell height (approximately 3 months of age) are used for initial virus titrations by immersion.

Juvenile oysters (maximum of 6) are held, raised off the bottom of in 10 L plastic buckets (8L working volume) with airlift circulation (prepared using 25mm PVC pipe) of artificial seawater (ASW) with a specific gravity was 1.030 – 1.035. Spat (40-100 depending on size – see Table 1 below) are maintained in 120 ml sterile containers without circulation/aeration. The water is completely exchanged every 2nd day. The water temperature should be maintained at 20 – 22˚C for all trials. Ideally water temperature is maintained by housing the entire culture system in a temperature controlled room. If required, oysters are fed at each water exchange by the addition of a combination of Chaetoceros sp., Isocrysis sp. and Pavlova sp. cultured microalgae (supplied by PFSI, Port Stephens NSW).

5.3 Storage of virus preparations

OsHV-1, like most herpesviruses, is extremely sensitive to storage at inappropriate temperatures. Freezing samples or tissue extracts at about -20oC will rapidly and almost completely inactivate the virus. In contrast, the virus is relatively stable for 5-7 days (sometimes longer depending on the sample type and any prior preparation) at 4-6oC. Storage under chilled conditions for longer periods will be affected by degradation of tissue and release of enzymes etc. For long term storage, tissue (ideally in small pieces of about 1cm3 or less), homogenates and extracts can be stored frozen at approximately -80oC or lower without significant loss of infectivity of OsHV-1. Some but acceptable loss of infectivity will occur after a single freeze/thaw cycle but repeated freezing and thawing will accelerate loss of infectivity and should be avoided.

5.4 Seedlot system and summary of workflow.

Infectious virus preparations that are to be used in an experimental challenge system should follow the principles of a seedlot system so that passage levels of all material can be clearly identified, the sequential/serial passaging of infectious material is limited and there is low passage material available in a stabilised form to provide material for long term use. Each of these principles is designed to minimise virus variability and maximise reproducibility of results during use of these virus stocks. The sequence of steps that are followed for the preparation of master, seed and working stocks are generally similar and consist of the following:

- Acquire and acclimatise oysters prior to preparing tissue homogenate for the master stock to ensure that, ideally, it can be used as soon as it has been prepared.
- Prepare homogenates of both control and infected tissues. For amplification of seed and working stocks, extracts that have been frozen at -80oC will be available.
- Relax oysters and inject with homogenates.
- Monitor the health status of oysters twice daily.

- Collect dead or moribund oysters as soon as observed and hold at 4 oC. Collect all remaining live oysters 4 days after inoculation.

- Collect gill and mantle tissue from all oysters and hold at 4 oC. If testing cannot be completed within 24-48 hours, take an aliquot for testing and store remaining tissue frozen at -80oC.

- Determine virus load by qPCR using an enzymatic digest of tissue samples from individual oysters.

- Select tissues to be pooled for preparation of batch of virus (highest viral load estimated by qPCR).

- Prepare tissue homogenate or store as unprocessed tissue at -80oC.

- Add cryoprotectant to virus batch and freeze at -80oC.

- Titrate virus batch after being held frozen for at least 48 hours.

5.5 Preparation of OsHV-1 inoculum for injection

- Test affected oysters by qPCR and select at least 3-4 that have Ct values across a range of approximately 10-14 to ensure that there is a high probability that infectious virus is present.

- Prepare ASW in milliQ water (1 L milliQ water, + 33.3 g sea salt) containing double strength cell culture grade antibiotics (specific gravity = 1.022, 24˚C). Filter 0.22 µM

- Partially thaw the selected oysters and, using sterile instruments, endeavour to collect only gill and mantle. Pool all tissues in a sterile container.

- Prepare a 10% (w/v) homogenate of tissue in ASW using a mini tissue blender. Keep samples cool at all times by holding on ice.

- Prepare sufficient tissue to give at least 20 ml of homogenate (eg at least 2.0gm in 20 ml). Note that about 40% of the solution will be lost during processing and filtration.

- Centrifuge the homogenate at 3000 g for 10 min at 4˚C.

- Remove the supernatant and repeat centrifugation step.

- Filter the supernatant progressively through 5.0, 1.2, 0.8, 0.45 and 0.22 µm filters.

- Confirm the virus concentration by qPCR.

- Hold a small volume at 4˚C until use.

- Repeat the procedure with negative control oyster tissue (OsHV-1 negative mantle and gill)

- Add cryoprotectants (10% glycerol and 10% fcs) to the bulk of the suspension and mix well.

- Fill 1.0mL sterile cryovials with 3-500ul of suspension and freeze slowly overnight (at a rate used for viable cell cultures – do not lower the temperature by more than 1˚C per minute. This can be achieved by placing the vials in a polystyrene tube rack in a polystyrene box and holding in a -80˚C freezer overnight.

- Transfer frozen vials to ultra-low temperature storage (in a freezer at <-70˚C or in liquid nitrogen vapour). Assuming that this material has an acceptable virus level confirmed by qPCR, it should be designated as the pre-Master Stock and used on a strictly limited basis to amplify virus for new batches of Master Stock.

5.6 Production of Master Stock

This stage involves the inoculation of oysters, collection and preparation of tissue extracts and then the addition of cryoprotectants prior to freezing as the Master Stock.

- Prepare a solution of magnesium chloride (50-70 g/L) in distilled water containing artificial sea-salt up to salinity 30-35 ppt (Butt et al 2008)

- Immediately prior to use, dilute the premaster stock of OsHV-1 in ASW. The two target doses for injection are 105 and 107 OsHV-1 genome equivalents per oyster (i.e. a concentration of 103 and 105 OsHV-1 genome equivalents per µl. This can be estimated from qPCR data as described in Section 6.3.2). The tissue homogenate should be diluted by a factor of at least 100 to prevent toxicity in the injected oysters.

- Inject oysters with either a high and low dose (total 105 and 107 viral genome copies per injection). The genome copy number in acutely infected oysters should be measured by comparing the PCR results with those for quantitative DNA standards. In the absence of an accurate standard, the Ct of the filtered tissue homogenate should be in the range of 12-14.

The oyster homogenate must be diluted at least 100X before injection to avoid toxicity.

- Use the qPCR result on the virus stock prior to freezing as a guide for these calculations. As a safeguard, the first time a batch is used, also prepare a dilution of inoculum that contains 10 times more virus than the target range to ensure that oysters become infected and the virus stock is adequately amplified. A volume of 1.5-2.0 mL for each dilution will be more than sufficient.

- To induce relaxation of the adductor muscle, immerse oysters in the MgCl2 solution for 2-6 h when 100% of oysters are relaxed. A high proportion of oysters may be relaxed after 1-3 hours immersion. Allow 10 oysters for each dilution to be used.

- Using a 25g needle inject 100 µl of the appropriate dilution of tissue homogenate into adductor muscle and then return to ASW in the holding vessel.

- Check the oysters twice daily for signs of ill-health (‘gaping’, partly open shells; sluggish closure)

- Change the water and supply feed every second day.

- Place all used water into an appropriate holding vessel and decontaminate by adding chlorine (200 ppm) and holding for 2 hrs before discharge.

- Collect a 1mL sample of water daily (just prior to the time of any water change) to monitor the dynamics of virus excretion by qPCR.

- Remove any moribund/dead oysters (Should be shedding virus at about 48 h post inoculation and probably most will be dead at 4 dpi).

- Hold sick/dead oysters at 4˚C until day 4 (collect any surviving oysters)

- Collect samples of tissue from each oyster as described in 5.4 above. Collect sufficient tissue to provide approximately 30 ml of filtered supernatant.

- Homogenise, clarify and filter tissue extracts as described for the ‘premaster’ stock.

- Add cryoprotectant and freeze the virus stock in 500uL lots using a controlled freezing method as described previously. Check the concentration and infectivity of 1-2 vials after the stock has been frozen for at least 48 hrs.

- This material should be labelled as the Master Stock.

5.7 Production of Seed and Working Stocks

In practice, the methods for the preparation of a Seed and Working stock of virus are the same as for the preparation of the Master stock. However care should be taken to maintain this successive hierarchy for the derivation/amplification of virus so that the extent to which it has been passaged under laboratory conditions from the original ‘wild-type’ virus can be clearly established.

In order to minimise the potential for genetic variability the highest passage material should be used routinely for research purposes and virus at the lower passage levels (especially Master Stock) should be preserved for long term use. Infrequently and at long time intervals the viability of the Master Stock should be confirmed by passage. Assuming viability at that time, a small quantity may be frozen for ‘backup’ purposes in the event that the master stock unexpectedly deteriorates. However, under optimal conditions, viability of virus for decades would be expected.

Once alternative methods of infection have been established (e.g. immersion – see Section 6 below), they may possibly be used as an alternative to injection to amplify a virus stock, provided the seed lot generations are maintained.

6.0 Laboratory Infection Model

The procedures described in the preceding sections result in the production of a large quantity of high titred virus that should be relatively stable during ultra-low temperature storage. These virus preparations can then be employed for a range of research studies involving the infection of oysters. The information that is provided in this section is a summary of methods that have been successfully used to infect Pacific Oysters with OsHV-1. A range of methods can be used to infect oysters but this document concentrates on approaches that resemble natural infection as much as is possible. Only methods for infection of oysters by immersion are described.

6.1 Precautions for containment of infectious material and aerosols of DNA fragments

The procedures involved in the experimental infection of oysters can result in the amplification of OsHV-1 virus to very high titres. Both oyster tissues and the water in which they are held are likely to contain very high virus loads. There are also risks of transfer of infectious virus and DNA during specimen collection. Consequently, strict biosecurity practices and aseptic techniques (as described in Section 2 above) must be adhered to at times.

6.2 Oysters and husbandry

A wide range of ages of Pacific Oysters have been successfully infected. However, the virus dose needed to establish infection may vary. In general, the husbandry practices for the oysters follow those described above (see Section 5.2). However, in general vessel sizes and water volumes are kept to a minimum to limit

the quantities of virus required and to ensure that virus concentrations are kept at the target levels. Oysters are usually managed and tested as a group as it is not practical to handle them individually. As there are likely to be different treatments being applied to different groups of oysters, a culture system that provides a large number of self-contained vessels is essential. The water for each vessel must be contained and sharing or circulation of water between vessels is not appropriate. In most circumstances, a culture system must allow the maintenance of about 12-15 oysters for 1 week and permit complete water changes every second day. When very small oyster spat are being handled, a higher ratio of water to oysters will usually alleviate the need for aeration but for larger spat, juveniles and adults aeration will most likely be needed. A guide to vessel sizes and water volumes for oysters of different sizes/ages follows.

Table 1. Volumes of water and inoculum required for different age/size oysters.

Class	Size range* (mm)	Number	Container size (L)	Water volume	Inoculum volume (mL)
Spat	2 - 10	~100	0.12	0.1	20
Spat	10 - 20	12 - 40	0.12	0.1	20
Spat	20 – 30	> 15	10	4	100
Juvenile	30 - 60	12-15	10	4	100
Adult	60 - 80	10 - 12	10	4	100

* Shell height.

6.3 Infection of oysters

For optimal infection rates, it has been found necessary to undertake the virus exposure in several stages. These include:

- relaxation of adductor muscle so that shells are partially open;
- immersion of the oysters in a small volume of inoculum overnight;
- immersion of the oysters in a ‘normal’ volume of water for a further 24 hrs (without replacing the water containing the inoculum
- changing the water every 48 hours.

The specific details are as follows:

6.3.1. Facilities and oysters

Ensure that the facilities that will be utilised for virus transmission trials are ready for use - all decontamination from previous usage has been completed, sufficient ASW is available and air lines and pumps are fully operational. Ensure that the water temperature is being reliably maintained between 20-22 ^{o}C. Place oysters in the facility preferably at least 24 hours prior to use to allow them to acclimatise and depurate using a complete water exchange after 2 - 4 hours. The vessel size, stocking density and water volumes vary with size. Table 1 above provides a guide.

6.3.2 Preparation of inoculum

While the minimum virus concentration that will kill a high proportion of susceptible oysters can vary with age/size and genotype of oysters, when challenging spat aged from approximately 2-6 months, a starting virus concentration of approximately 107genome copies (per mL of ASW in which the oysters are held) should be selected. This concentration can be determined by the PCR results available for the working stock of virus. The virus should be diluted in the laboratory as close to the time of use as possible and held chilled at approximately 4°C until used. Until optimal virus concentrations have been determined for oysters of different ages, it is recommended that several ten fold lower dilutions are also prepared for use.

To ensure accurate measurement and dispersion in the oyster housing facility, these dilutions can be prepared in the laboratory and moved to the animal accommodation in a volume of 2 ml in ASW. A quantification standard can be used to estimate the number of genome copies of OsHV-1 in the inoculum by qPCR. It should be noted that the proportion of this nucleic acid sequence which is present in intact, infectious virions is variable, depending on the stage of infection of the host oyster at which samples were collected as well as other factors such as the dose of inoculum. As a guide, a Ct value of 12 is equivalent to approximately 107 copies/µl and a Ct value of 15 is equivalent to approximately 106 copies/µl. Since there are two copies of the qPCR target in each OsHV-1 genome, a Ct of 12 equates to approximately 5 x 106 OsHV-1 genomes per µl. For an inoculum with a Ct of 12, a total dose of 2 ml will provide 1010 OsHV-1 genome equivalents. When diluted in 1L, this will provide immersion of oysters in a dose of 107 genomes per ml of ASW. Alternatively, 200 µl of this inoculum can provide the highest dose when mixed in a vessel with 100 ml ASW.

An working stock with a Ct of 15 can be used with a starting dose of 2 ml of undiluted inoculum per litre of ASW to provide a maximum immersion dose of 106 OsHV-1 genome equivalents per ml of ASW. Such an inoculum can provide a dose of 107 OsHV-1 genome equivalents per ml of ASW when mixed with 100 ml in a vessel used to challenge small spat.

When preparing the inoculum, ensure that there is sufficient prepared to challenge the positive controls and that 3-4 dilutions are prepared below the highest concentation. For each dilution allow for at least 12-15 oysters. At least one (and preferably 2) similar sized group should be exposed to the extract of uninfected oysters. This may potentially be omitted after there has been sufficient use of the current working stock to prove that it does not show evidence of toxicity at the dilutions in use.

6.3.3 "Relaxation" of oysters

For challenge by immersion, oysters are "relaxed' by bathing in magnesium chloride solution (see section 5.6 above). For convenience, oysters from different batches or that are being prepared to receive different doses of virus may be placed in mesh bags to retain their group identity but can then be immersed in one large container of magnesium chloride. They should be immersed in magnesium chloride for 1-3 hours. Further investigation may be required to confirm an optimal time.

6.3.4 Exposure to inoculum

Towards the end of the time during which the oysters are being held in magnesium chloride solution, finalise preparation of the virus spiked ASW and place the appropriate volumes (see Table 1) in the holding

containers. At the end of the ‘relaxation’ period, the groups of oysters to be challenged are removed from the magnesium chloride solution, briefly drained then placed in the containers that hold the appropriate volume of virus suspension. Initially the volume of virus spiked water is kept as small as possible to enable the highest concentration of virus to be obtained. The airlift circulation is turned off during this period. The oysters are held in these volumes overnight and then, without removal of the virus, the containers are filled with additional ASW to the required final volume (Table 1).

6.3.5 Oyster management

Approximately 40-48 hours after first being exposed to virus, the water in each container is carefully removed and placed in a holding tank for inactivation of the virus. Once all virus contaminated water has been removed from the oyster containers, add chlorine (200 ppm) and holding for 2 hrs before discharge. Each oyster container is refilled with the appropriate volume of fresh ASW. It is usual practice to include groups of uninfected oysters that have only been negative control oyster homogenate. During any manipulation and especially changing of water, these uninfected (‘negative control’) groups should always be handled first to minimise risk of cross contamination. Similarly, oysters infected with the lowest levels of virus should be handled next and those exposed to the highest levels are managed last. Once all water has been changed, oysters are provided with algae as required.

6.4 Monitoring of oysters

All groups should be examined twice daily for signs of ill-health (‘gaping’, partly open shells; sluggish closure). Oysters are considered moribund if there is movement when the upper and lower shell are pushed together. Remove any moribund/dead oysters. Collect a 1mL sample of water daily (just prior to the time of any water change) to monitor the dynamics of virus excretion by qPCR. All oysters should be checked for at least 7 days from the time of first exposure to virus. Deaths are uncommon after 7 days and the trial is usually terminated at that stage. However, it should also be noted that deaths after 7 days could be caused by exposure to a higher dose of virus that has been excreted by a more susceptible cohabitant.

6.5 Sample collection and testing

The water samples and any sick or dead oysters can either be processed on the day of collection or held until the end of the trial. If samples will be prepared for testing within 48 hours of collection, they can be held at 4 ^{o}C, otherwise they should be held frozen. If there is no need to use tissue or water samples for collection of infectious virus, freezing at -20°C is suitable, otherwise hold at approximately -80 ^{o}C. Any oysters that are alive at the end of the trial can be frozen prior to tissue collection as this will facilitate opening of shells.

As well as observations on the occurrence of disease and death, some oysters may be infected but not die. Detection of these oysters will rely on testing of tissue samples by qPCR. Collect samples of tissue from each oyster as described in 5.4 above. Extract viral DNA and test all tissue and water samples by qPCR (see Section 4.4).

6.6 Decontamination of equipment and waste disposal

After oysters have been experimentally infected with OsHV-1, there are high levels of virus both in the oysters and any water in which they have been held. No material should be removed from the rooms in which virus transmission has been attempted until it has either been fully decontaminated or appropriately contained. All solid waste should be appropriately contained (e.g. held in sealed, leakproof containers or in at least 2 layers of very thick plastic bags that are each sealed) and transported securely for decontamination by autoclaving. Liquid waste should be decontaminated by the addition of chlorine (see 5.6 above) prior to discharge into a sewerage system. After all oysters and water have been removed, the vessels in which the oysters have been held can be safely decontaminated by wiping over with paper towel and immersing in a solution with 200 ppm chlorine for 2 hours. Air tubing and airstones should be discarded and replaced for subsequent trials. The floors and walls of any holding rooms should also be rinsed with a suitable disinfectant (e.g. iodine) and left to completely dry. Once equipment has been treated it should be left for a

minimum of 24 h hours then rinsed with tap water and allowed to dry before re-use. Ensure that all PPE is also decontaminated and footbaths are emptied and filled with new disinfectant just before the room is used again.

7. Quality Control Procedures

The laboratory challenge system for OsHV-1 that is described in this document is a living biological system and consequently involves many components that are subject to variation. While every effort is made to minimize variability, it is inevitable that there will be some differences in outcomes from one trial to another. Factors that have the greatest influence on variability are the genetic composition of the oysters, their age and the environment from which they have been sourced. While there can be variation in the quantity of virus in individual ampoules, measures are taken to stabilize the virus and minimize loss during freezing and thawing. It is important to note that qPCR does not measure infective virus. A stable PCR result does not automatically infer retention of infectivity. To monitor variations in virus titre, it is essential to include sufficient "positive" controls that are infected with different virus dilutions. These must be included in each trial. Whenever possible, the oysters used must be of a constant genotype and age variation should also be minimized. In the absence of a semi-continuous breeding program to provide stock of limited age range, this will present some challenges. Nevertheless these controls are critical to monitor the titre of virus that is used on each occasion. While temperature has a profound effect on OsHV-1 infection, it should be possible to limit temperature effects on virus infection and replication.

While all standard procedures should be followed closely to minimize the variability of PCR results from day to day, special care should be taken to prevent cross contamination. The nature of the infectivity model means that samples with very high viral load will be prepared for PCR. This should be noted, and routine laboratory workflow should be adjusted accordingly. The physical environment under which the assay is conducted, with segregation of different stages, and the preparation of reagents and solutions in different segregated rooms with dedicated equipment, should ensure that cross-contamination and false positives do not occur. The use of a blank and negative sample in each test 'run' provides some confirmation of freedom from contamination while the use of an internal control with each sample provides a measure of the quality of the sample and the performance of the assay in that specific well.

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Appendix 2: Researchers and project staff.

1. Elizabeth Macarthur Agriculture Institute

All laboratory aspects of this project were conducted by staff of the Virology Laboratory at the NSW Dept of Primary Industries' Elizabeth Macarthur Agriculture Institute, Menangle NSW. The key researchers were:

- Dr Xingnian Gu, and Dr Paul Hick, set up the model system and were responsible for the day to day operation of the model and sample collection and processing. Dr Hick moved to The University of Sydney, Camden NSW in mid 2013.

- Sarah Gestier and Robyn Hall conducted the longitudinal study and prepared samples for histopathology.

- Dr Peter Kirkland – planned and co-ordinated the research program

- Dr Mel Gabor examined the histopathology slides.

- Technical support for the conduct of laboratory assays was provided by Rodney Davis and staff of the Molecular Diagnostics Team

2. Port Stephens Fisheries Institute, Taylor's Beach, NSW

- Mike Dove, Wayne O'Connor and staff of the NSW Dept of Primary Industries' Port Stephens Fisheries Institute, Taylor's Beach, NSW provided advice during the set-up of the oyster holding facilities then supplied Pacific Oysters, native flat oysters and feed throughout the trial. They also conducted the parallel field studies for the challenge of the 2012 and 2013 ASI family lines in the Georges River, Sydney.

3. CSIRO Marine and Atmospheric Research, Tasmania

- Peter Kube and Nick Elliott were responsible for the analysis of genetic resistance data

4. Australian Seafoods Industries, Glenorchy, Tasmania

- Matt Cunningham and Gabby Bennett arranged the supply and delivery of Pacific Oysters from various ASI family lines for the last 9 months of the project.

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