Understanding ostreid herpesvirus-1 risk: Alternative hosts and in situ hybridization: evidence for infection of mussels.

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

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Executive Summary

South Australia (SA) has a large edible oyster industry primarily growing Pacific oysters (*Crassostrea gigas*). The industry is regionally-based, an important employer and a substantial contributor to regional economies. Pacific oyster mortality syndrome (POMS) is a serious infectious disease of *C. gigas* caused by ostreid herpesvirus-1 microvariant (OsHV-1 microvariant). The first outbreak of OsHV-1 in Australia occurred in 2010, in the Georges River-Botany Bay and Port Hacking-Sydney Harbor estuaries in NSW. OsHV-1 was subsequently detected in the Hawkesbury River system (2013) and eastern Tasmania (2016). The production and economic impacts of these outbreaks have been substantial.

OsHV-1 infection was confirmed in the Port River, SA, in February 2018 in association with high mortality (50-90+%) of feral Pacific oysters. POMS was not identified in SA outside the control area, and surveillance has shown commercial growing areas in SA to be free of OsHV-1. Surveillance has shown that *C. gigas* in the Port River system has high prevalence of OsHV-1 infection.

Protection of OsHV-1 free status for oyster growing regions is a priority for South Australia. Pursuant to the Fisheries Management Act 2007, PIRSA implemented a ban on the take and movement of bivalves from the Port River system to reduce the risk of POMS spreading. Bivalve organisms, including oysters, mussels, cockles and razorfish, are banned from being taken from the area for any purpose (including bait or berley). Controlled risk assessment, taking into account the controls in place, indicates that spread of OsHV-1 from the Port River is most likely to occur with biofouling on vessels. Understanding which biofouling types are infected is therefore important for management of the disease.

Mussels (*Mytilus* spp.) share similar habitats to Pacific oysters and are common in the Port River estuary (1). Mussels are demonstrated as hosts for OsHV-1 in Ireland (2), and show some histopathological signs of disease but outbreaks in mussels are not described. We aimed to implement an OsHV-1 *in situ* hybridization (ISH) assay and assess OsHV-1 infection in PCR positive non-*C. gigas* hosts using ISH.

An *in situ* hybridization (ISH) test for OsHV-1 was implemented at Flinders University based on published primers for detection of the virus. ISH showed a strong signal in sections from infected *C. gigas* and none in uninfected *C. gigas*. Bivalves were collected in the Port River by SARDI, sampled and tested for OsHV-1 using PCR. Three PCR positive samples of *Mytilus* spp. were also positive by ISH, showing infection with OsHV-1.

Confirmation that mussels are a host of OsHV-1 has a range of important management implications. Movement of mussels likely poses a risk for transmission of OsHV-1, so control activities designed to decrease host populations that target only Pacific oysters are unlikely to be successful; biofouling management should be general rather than targeting only Pacific oysters. Containment measures should target all bivalve species. Mussels for translocation to OsHV-1 free areas should be sourced from biosecure hatcheries and tested to provide evidence of OsHV-1 freedom.

Keywords

Ostreid herpesvirus-1 microvariant; Pacific oyster mortality syndrome (POMS); Pacific oysters (*Crassostrea gigas*); Mussels (*Mytilus* spp.); in situ hybridization.

Introduction

Infection with ostreid herpesvirus 1 microvariant (OsHV-1 microvariant), a specific genotypic group of ostreid herpesvirus 1 (OsHV-1), causes Pacific oyster mortality syndrome (POMS) and serious acute mortality in juvenile and adult Pacific oysters (*Crassostrea gigas*). OsHV-1 microvariant was first detected in Australia in 2010 (3). By June 2014, it was known to occur in Australia in three estuaries: the Georges River–Botany Bay, Port Jackson–Sydney Harbour and Hawkesbury River–Brisbane Water estuaries. In January 2016 it was detected in south-eastern Tasmania (4), and in February 2018 an outbreak occurred in feral Pacific oysters in the Port River, an urban waterway in metropolitan Adelaide, South Australia.

Pacific oysters (and *C. angulata*, the Portuguese cupped oyster) were the only species known to be susceptible to infection with OsHV-1 microvariant (5). Reference strains of OsHV-1, however, have a broad host range (6). *Mytilus* spp. in Ireland were shown (2) to be infected, at up to 100% prevalence, and OsHV-1 DNA was detected at low levels in *Mytilus* spp. In New South Wales (7). Knowledge of alternative hosts is important to understand transmission and spread, and for development of meaningful containment controls. This project, therefore, aimed to understand if species other than *C. gigas* were hosts of OsHV-1 in the Port River. This was investigated by broad scale sampling of bivalves and testing them using the Martenot (8)/OIE (5) and Jenkins (3) quantitative polymerase chain reaction (qPCR) assays for OsHV-1 and showing infection in qPCR positive animals using *in situ* hybridization (ISH).

Objectives

1. To implement and validate OsHV-1 *in situ* hybridization assay.

2. Assess OsHV-1 infection in PCR positive non-*C. gigas* hosts using ISH.

Methods

Sampling

Bivalves were collected manually from the Port River, South Australia (9). They were identified by a taxonomist and sampled at the South Australian Research and Development Institute (SARDI) facility at North Arm, South Australia. Preserved samples were transported to SARDI Aquatic Sciences at West Beach.

A 5 x 5 mm sample of mantle and gill and the tissue remaining from the heart smear were preserved in 70% ethanol. A diagonal 3-5 mm tissue section was taken from each oyster ensuring each sample included mantle, gills, digestive gland and gonad. This section was placed in a histology cassette and fixed in 10% formalin with filtered seawater for 48 h and transferred to 70% ethanol for storage. Equipment and surfaces were decontaminated in PCR Clean™ (Cat. No. 15-2025, Minerva Biolabs, Berlin, Germany) or DNA Away (Thermo Fisher Scientific, Thebarton, South Australia) between samples. The laboratory had not been used for sampling oysters for 3 months and was cleaned to remove DNA with steam and hypochlorite several times before this study commenced.

DNA was extracted with the QIAamp Mini kit (Cat. No. 51306, Qiagen, Hilden, Germany), following the manufacturer's protocol from a pooled heart, gill and mantle tissue sample (total mass of approximately 25 mg) from each oyster. A negative extraction control (no tissue) was also included. Extracted DNA quality and concentration was assessed using a Nanodrop® ND-2000 spectrophotometer (Thermo Fisher Scientific). Extracted DNA was stored at -20°C until qPCR testing.

Formalin fixed sections were embedded in paraffin wax, sectioned at 5 μm and mounted on silane-coated slides (Cat. No. S4651-72EA, Sigma-Aldrich, Missouri, United States).

PCR

To ensure samples contained amplifiable DNA, extracted samples were assayed by T4 PCR (Table 1) (10) using the Taqman® Ribosomal RNA Control Reagents kit (Cat. No. 4308329, Thermo Fisher Scientific). Thermal cycling was performed according to manufacturer's guidelines up to 45 cycles using a StepOnePlus (Thermo Fisher Scientific). Samples were considered suitable for further testing if they produced $C_T \leq 25$.

Two subsamples of 2 μl of nucleic acid extract were tested in 25 μl reactions using the OIE Martenot OsHV-1 quantitative PCR (qPCR) (8) and EMAI Jenkins OsHV-1 qPCR (3) assays (Table 1). Each PCR plate included a positive plasmid control (Australian Animal Health Laboratory, East Geelong, Victoria), a no-template control and a negative extraction control. A qPCR assay was described as positive when there was statistically significant increase in fluorescence output above the background, meaning the cycle threshold (C_T) was reached and a typical amplification curve was displayed. C_T values were calculated automatically using StepOne™ Software v. 2.3. When a test did not have a C_T value, or a typical amplification curve the test was described as negative.

Mussels were considered positive if all subsamples returned a positive qPCR result. Positive samples were tested with the Arzul OsHV-1 classic PCR (cPCR)(11) with 2 μl of nucleic acid in 25 μl reactions and the AAHL AFDL nested PCR (nPCR) using 5 μl of nucleic acid in 50 μl reactions (Table 1). Both of these reactions are specific for OsHV-1 microvariant (11).

ISH probe and detection system

DNA was extracted from OsHV-1 microvariant-infected *C. gigas* tissue using QIAamp DNA mini-kit (Cat. No. 51304) and used for PCR. Primers were OHVC/OHVD (AGGCGCGATTTGTCAGTTTAGAATCAT & AGGTTCAGGTCTTTGCGTTCCGT; (12)). Primers to generate the ISH probe were chosen because these were successfully used previously (13). Enzyme used was Phusion (Cat No. E0553S, New England Biolabs, Massachusetts, United States), with annealing temperature of 72°C, extension time of 10 seconds and 40 cycles.

Probe was labeled as described in PCR DIG Probe Synthesis Kit (Cat. No. 1 636 090, Roche, Basel, Switzerland). ISH primers were OHVC/OHVD (AGGCGCGATTTGTCAGTTTAGAATCAT & AGGTTCAGGTCTTTGCGTTCCGT; (12)). Template was previous PCR product diluted 1:100.

Dot Blot

Labelled probe (2µl) was dotted on nylon membrane at concentrations from 15ng/µl down to 15pg/µl (final quantities of 30ng down to 30pg). Membrane was microwaved for 2 minutes (14). Probe was detected using a peroxidase-conjugated antibody against digoxigenin (Anti-Digoxygenin-POD, Fab fragments, Cat. No. 11207733910, Roche; AD-POD) and stable DAB (Cat No. 750118, Invitrogen, Thermo Fisher Scientific).

In situ hybridization

ISH was performed by a modification of Arthur et al (15). Hybridization (65°C for 16-20 hours) stringency was Tm -25°C and washes were at Tm -15°C. Oyster tissue sections were dewaxed in xylene for 5 minutes, followed by 100% ethanol 2 × 5 minutes, then dried. Protein was digested with 100 µl of proteinase K (100 µg/ml in 20mM Tris pH 7.4, 2mM EDTA, 1.2% Triton X-100) in a humidified box at 37°C for 30 minutes. Proteolysis was stopped by rinsing with absolute ethanol for 1 minute, after which slides were briefly air-dried.

Tissue was saturated with 70 µl of hybridization buffer (2 × SSC, 6% skim milk powder), including 0.25 ng/µl of DIG-labelled DNA probe. Coverslip was added and sealed. DNA on the slide was denatured at 95°C for 2 minutes then slides were moved to a humidified box and incubated overnight at 65°C.

Post-hybridization stringency washes were of 2 × SSC, 6% skim milk powder at 65°C (2 × 15 minutes), followed by 0.5 × SSC, 6% skim milk powder (4 × 10 minutes) at 65°C. Probe was detected using AD-POD and Pierce DAB substrate kit (Cat No. 34002, Thermo Fisher Scientific), incubated for 5 minutes. Slides were counterstained for 20 seconds in 0.01% toluidine blue.

Table 1. OsHV-1 and T4 PCR assays.

Primer and probe sequences, cycling conditions and amplicon sizes

PCR	Primers/Probe	Sequence (5'-3')	Cycling	Reaction	Amplicon	Reference
OIE Martenot OsHV-1 qPCR[1]	BF B4 Probe B	GTC GCA TCT TTG GAT TTA ACA A ACT GGG ATC CGA CTG ACA AC **6FAM** TGC CCC TGT CAT CTT GAG GTA TAG ACA ATC **TAMRA**	95°C 20s, 95°C 3s + 60°C 30s (45 cycles)	2 µl NA 23 µl MM[3]	102bp	Martenot et al. 2010 (8)
EMAI Jenkins OsHV-1 qPCR[1]	CRF CRR CR probe	CGT TTT ATC CAC CAC GAT TTT TAT T TAC ATC AAA CCC ACT TTT CCT ATG AT **6FAM** CAC TCA TGA AAA CAC CGC TAA GAT CAC TGC **TAMRA**	95°C 20s, 95°C 3s + 60°C 30s (45 cycles)	2 µl NA 23 µl MM[3]	88bp	Jenkins et al. 2013 (3)
T4 FAST qPCR[1]	Phage F Phage R Phage Probe	CCA TCC ATA GAG AAA ATA TCA GAA CGA CGC TGG GAA AAG AGG AAT TAT TTA **VIC** AAC CAG TAA TTT CAT CTG CTT CTG ATG TGA GGC **QSY**	95°C 20s, 95°C 3s + 60°C 30s (45 cycles)	2 µl NA 23 µl MM[3]	101bp	Ninove et al. 2011 (10)
Arzul OsHV-1 cPCR[2]	C2 C6	CTC TTT ACC ATG AAG ATA CCC ACC GTG CAC GGC TTA CCA TTT TT	95°C 15 min, 94°C 30s + 50°C 60s + 72°C 60s (40 cycles) 72°C 7 min	2 µl NA 23 µl MM[4]	709bp	Renault et al. 2000 (16) Arzul et al. 2001 (11)
AAHL AFDL In-house OsHV-1 nPCR[2]	*Primary* C2 C6 *Nested* C3 C6	 As above As above GGCAAGATGAATGGCAAGAT As above	95°C 15 min, 94°C 30s + 55°C 30s + 72°C 60s (40 cycles) 72°C 7 min As per primary reaction	5 µl NA 45 µl MM[4] 5 µl NA 45 µl MM[4]	709bp 574bp	AFDL In-house

qPCR[1] = Performed with StepOne real-time PCR system, cPCR[2] or nPCR[2] = Performed with Eppendorf thermal cycler, NA = nucleic acid, MM[3] = TaqMan Fast Universal PCR master mix, MM[4] = HotStarTaq PCR master mix.

Results

qPCR survey

Three *Mytilus* samples were qPCR positive for both the OIE Martenot OsHV-1 quantitative PCR (qPCR) (8) and EMAI Jenkins OsHV-1 qPCR (3) (Table 2). One positive mussel was collected adhering to a Pacific oyster, which was also qPCR positive (Figure 1). None of the other bivalves tested yielded positive qPCR results (Table 2). We note this was a small study designed to inform an understanding of risk. It is beyond the scope of our work to identify the *Mytilus* hosts at the species level as the *Mytilus edulis* species complex is taxonomically unresolved (17, 18).

Table 2. qPCR survey results for non-*C. gigas* bivalves from Port Adelaide.

(Details of *Mytilus* PCR tests in Table 3, Appendix). All positive *Mytilus* samples were also positive for cPCR and nPCR, which are specific for OsHV-1 microvariant (11).

Species	n collected	n positive	Apparent prevalence	Comments
Anadara sp.	45	0	0%	Subtidal
Mytilus spp.	300	3	1%	Intertidal and subtidal
Pinna bicolor	120	0	0%	Subtidal
Tapes sp.	300	0	0%	Subtidal

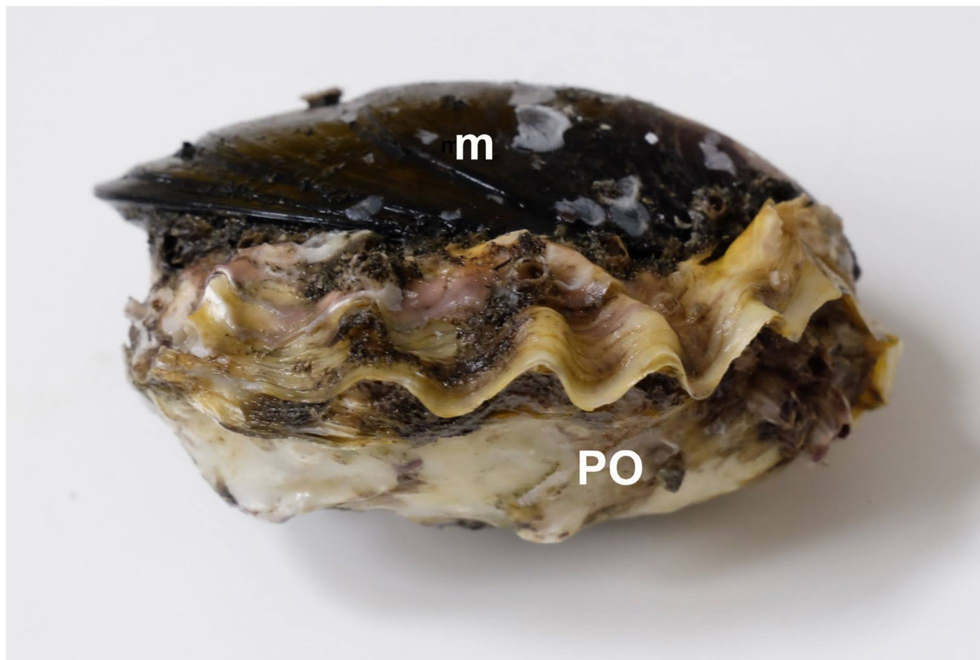


Figure 1. Mussel (m) shown adhering to Pacific oyster (PO).

Both animals were OsHV-1 positive when tested by qPCR.

Assessment of probe quality

Testing the probe indicated that 30pg of labelled probe could be detected on the nylon membrane (Figure 2), and that batches made on separate occasions were consistent.

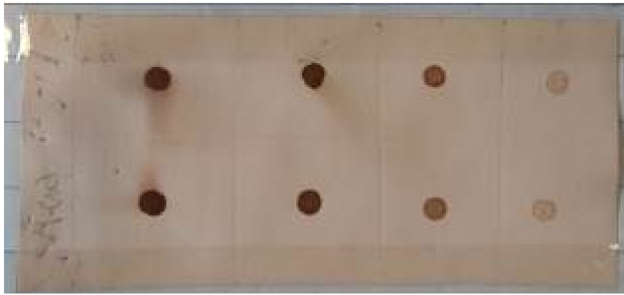


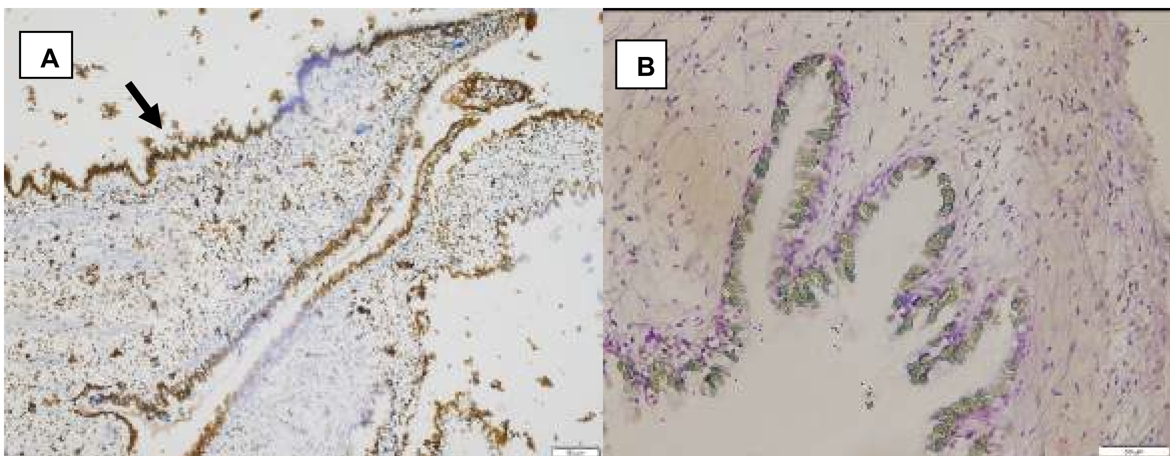
Figure 2. Dot Blot.

Amounts of labelled probe applied to membrane were titrated by serial 10^{-1} dilutions, from 15ng/µl down to 15pg/µl (final quantities of 30ng down to 30pg). Top row is first batch of probe, bottom row is second batch. Clearly, 30pg probe is equally detectable from both batches.

In situ hybridization

OsHV-1 viral DNA was detected as brown (diaminobenzidine) labelling in the nucleus and cytoplasm of cells (Figure 3). Heavy staining was evident in epithelium of infected *C. gigas* (Figure 3A, 3B), indicating substantial viral load. Arrow indicates typical area of high signal. No staining was observed in negative control *C. gigas* from farms in tested OsHV-1-free areas (Figure 3C, D).

Mussels that were qPCR-negative showed no staining (Figure 3G, H), while qPCR positive mussels showed evidence of abundant OsHV-1 DNA in epithelial tissues (Figure 3E, F). This high level of signal is highly unlikely to represent input viral genomes, such as would occur in mechanical carriage of the virus. The alternative and more attractive explanation is that there has been a degree of viral replication occurring in these tissues.



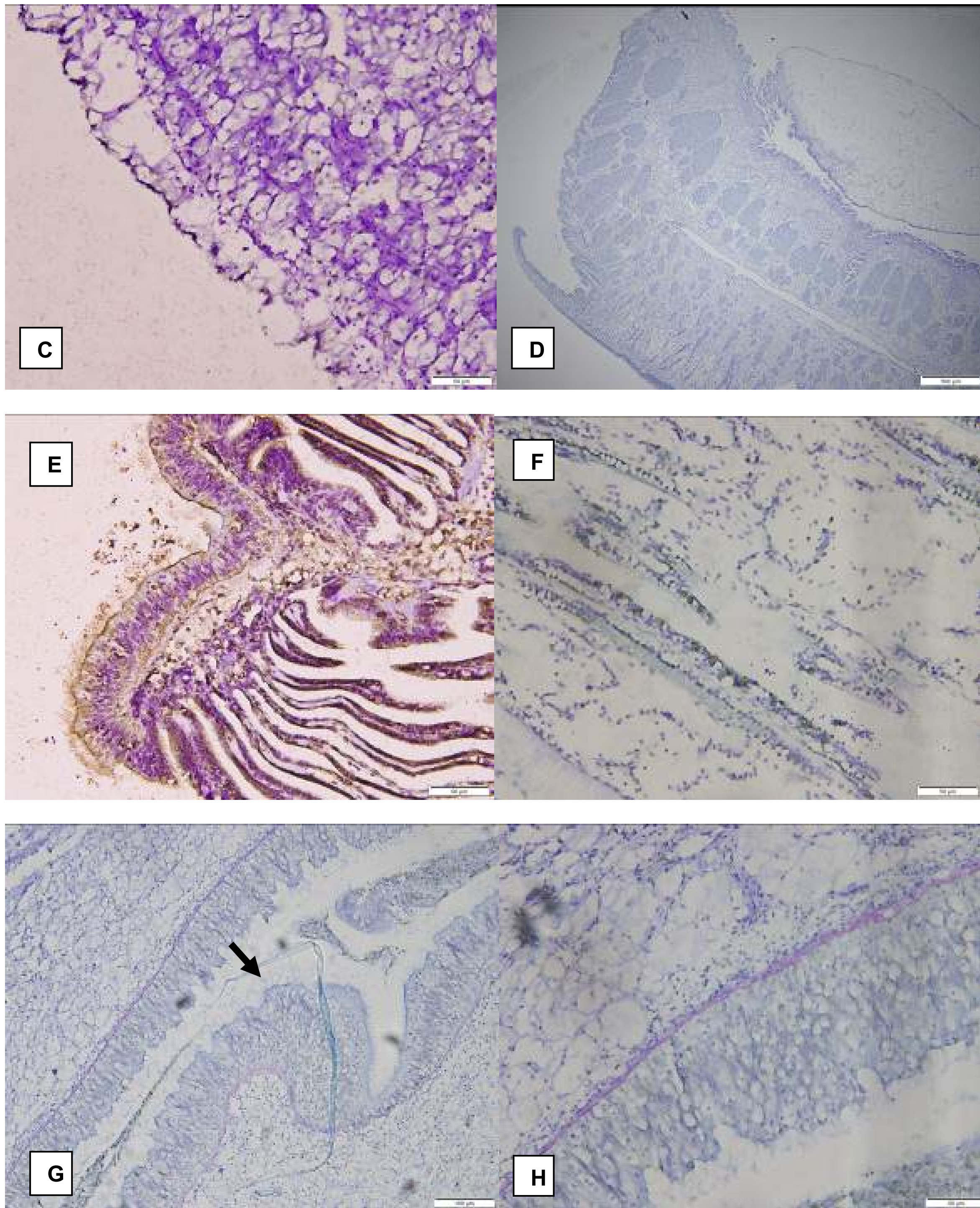


Figure 3. Results of ISH on *C. gigas* and *Mytilus* samples

A, B: OsHV-1 infected *C. gigas* from the Port River, South Australia. Arrow indicates typical area of high signal C, D: Uninfected *C. gigas* from the Port River. E, F: qPCR positive, infected mussel from the Port River showing OsHV-1 DNA staining in basal membrane of digestive tract, connective tissue of the gills and mantle, and viral DNA in the lumen of the gut. G, H: qPCR-negative mussel. Arrow indicates typical clear (no signal) area of epithelial tissue.

The counterstaining hue and density vary between preparations, although this appears largely a feature of the imaging system used to obtain the photomicrographs.

Discussion

OsHV-1 was detected, using PCR and ISH, in *Mytilus* spp. from the Port River. OsHV-1 microvariants had previously been detected in *C. gigas* and *C. angulata* (see (5)) but O'Reilly et al. (2) found that *Mytilus* spp. in Ireland were hosts of OsHV-1 and show histopathological signs of disease associated with OsHV-1 infection, although they did not determine if OsHV-1 causes mortality in *Mytilus* spp. It is apparent from our study that *Mytilus* spp. in Australia are also hosts of OsHV-1. Reference strains of OsHV-1 (those without the 12 base pair deletion upstream from ORF 4 which characterises the microvariant (5) have a broad host range (6), and further investigation of potential hosts of OsHV-1 in Australia may be relevant for risk management in some cases.

Infection in *Mytilus* spp. influences our understanding of the risks associated with OsHV-1 of bivalves. That the host range may encompass species beyond *C. gigas* has implications for containment of the virus to the Port River, which is PIRSA's long term aim for the disease. Preventing movement of bivalves from the Port River to other parts of South Australia and in particular to areas where there are commercial oyster farms or feral populations of *C. gigas* should remain a priority. To facilitate this control, the bivalve take closure over the Port River should be maintained and should continue to include all bivalves. The PCR-positive mussels had evidence of viral DNA in the gut and adhering to the mantle, indicating that the surfaces, gut and pallial cavity of bivalves other than *C. gigas* can contain OsHV-1. Our study did not survey all species of bivalves present in the Port River, and the sample size was limited by resources and season. It should not be discounted that other bivalves may be infected at low prevalence.

South Australia has a commercial mussel industry that grows *Mytilus* spp. The industry uses locally-caught spat and some translocated hatchery-reared stock. Translocations of mussels to South Australia must be sourced from OsHV-1 free areas and batch tested to show freedom from OsHV-1.

Activities such as dredging, energy industry exploration, and other marine commerce that can carry biofouling, should continue to be managed to minimise movement of bivalves out of the Port River and to limit movement to South Australia from other regions and systems that are, or are likely to be, infected by OsHV-1. Vessel traffic and biofouling remains the greatest, least-managed risk for OsHV-1 to establish itself in an oyster farming zone. The Department of Agriculture and Water Resources (DAWR) has been undertaking consultation for the upcoming *Australian Biofouling Management Requirements* but there are currently no biofouling management requirements in South Australia's State controls. Development of a domestic system that provides risk management equivalent to the national controls should be regarded as a priority.

In 2018 and 2019 PIRSA undertook, and later contracted, control activities for *C. gigas* in Port Adelaide. The aim of this program was to decrease Pacific oyster populations and to reduce the viral load. Although prevalence in mussels is not high, presence of the infection in other species makes the Pacific oyster control program less likely to be effective. Combined with the very heavy settlement of juvenile Pacific oysters observed following the summer 2018-2019 (unpublished observations) the management program should be reviewed, and its cost effectiveness considered.

Renault and Lipart (19), Corbeil et al. (20) and Bueno et al. (21) described methods for OsHV-1 ISH, finding the method useful and provided reliable results. We found that negative control animals showed negligible staining, indicating that the probe is specific, and that non-specific binding was insignificant, indicating that negative results were reliable. We observed variability in the intensity of both DAB staining of the target and the background staining, but this was also noted by Renault and Lipart (19). ISH stain is proportional to the density of target DNA in the sections, but conclusions about intensity of infection are difficult to draw from this method. Its main benefit relative to other diagnostic methods is to differentiate adhering DNA from infection (21). The positive control animals were heavily infected and although the qPCR positive mussels did not stain as intensely as the positive control oyster material the signal in the connective tissues shows clear viral replication. The relative

efficiency of *Mytilus* spp. as a host is less important than its capacity to facilitate transmission of the virus. ISH is useful for this type of study where confirmation of infection is required but is too slow, expensive and complicated for routine diagnostic use.

Conclusion, Implications and Recommendations

In situ hybridization (ISH) functions effectively as a test for OsHV-1. ISH shows a strong signal in sections from OsHV-1 infected *C. gigas* and none in uninfected *C. gigas*. qPCR positive samples of *Mytilus* spp. were also shown to be positive by ISH, demonstrating infection with OsHV-1.

Confirmation that mussels are a host of OsHV-1 in Australia as previously found in Ireland (2) has a range of important management implications. We are unable to quantify the risk of transmission of OsHV-1 from mussels to *C. gigas*, suggesting further work could include tank studies to measure this. However, movement of mussels likely poses a risk for transmission of OsHV-1, so control activities designed to decrease host populations that target only Pacific oysters are unlikely to be successful. Management of biofouling should include mussels specifically and, where specific controls are infeasible, all bivalve species should be controlled. Inclusion of mussels in surveillance programs however may not be efficient as the prevalence (and presumably viral load) is much lower than in Pacific oysters.

Further development

Not applicable.

Extension and Adoption

The project was intended to support PIRSA policy on management of Port River estuary. Results of the project have been communicated to PIRSA.

Project coverage

Not applicable.

Abbreviations

OsHV-1	Ostreid herpesvirus 1
PIRSA	Primary Industries and Regions South Australia
SARDI	South Australian Research and Development Institute
ISH	in situ hybridization
PCR	Polymerase chain reaction
cPCR	Classic polymerase chain reaction
qPCR	Quantitative PCR
nPCR	Nested PCR
POMS	Pacific oyster mortality syndrome
OEI	World Organisation for Animal Health.

Project materials developed

If the project creates any products such as books, scientific papers, factsheets, images these should be outlined in this section outline and attach them where possible.

Not applicable.

Intellectual Property

None

Appendix 1: Field and laboratory staff

SARDI

Kathryn Wiltshire

Ian Moody

Mandee Theil

Y Nhu Lieu

Flinders University

Jessica Buss

The University of Adelaide

Cheryl Day (histologist)

Tracey Murnane (histologist)

Appendix 2: Summary of results of tests used on *Mytilus* spp. samples for OsHV-1.

ID	18S control	OIE Martenot OsHV-1 qPCR C_T mean	EMAI Jenkins OsHV-1 qPCR C_T mean	In-house OsHV-1 nPCR	Arzul OsHV-1 cPCR
M2	+	Negative	Negative	-	-
M3	+	Negative	Negative	-	-
M4	+	Negative	Negative	-	-
M5	+	Negative	Negative	-	-
M6	+	Negative	Negative	-	-
M7	+	Negative	Negative	-	-
M8	+	Negative	Negative	-	-
M9	+	Negative	Negative	-	-
M10	+	Negative	Negative	-	-
M11	+	Negative	Negative	-	-
M12	+	Negative	40.20	-	-
M13	+	Negative	Negative	-	-
M14	+	Negative	Negative	-	-
M15	+	Negative	Negative	-	-
M16	+	Negative	Negative	-	-
M17	+	Negative	Negative	-	-
M18	+	Negative	Negative	-	-
M19	+	**33.50**	**31.69**	POSITIVE	POSITIVE
M20	+	Negative	Negative	-	-
M21	+	Negative	Negative	-	-
M22	+	Negative	Negative	-	-
M23	+	Negative	Negative	-	-
M24	+	Negative	Negative	-	-
M25	+	Negative	Negative	-	-

M26	+	Negative	Negative	-	-
M27	+	Negative	Negative	-	-
M28	+	Negative	Negative	-	-
M29	+	**31.83**	**30.62**	POSITIVE	POSITIVE
M30	+	Negative	Negative	-	-
M31	+	Negative	Negative	-	-
M32	+	Negative	Negative	-	-
M33	+	Negative	Negative	-	-
M34	+	Negative	Negative	-	-
M35	+	Negative	Negative	-	-
M36	+	Negative	Negative	-	-
M37	+	Negative	Negative	-	-
M38	+	Negative	Negative	-	-
M39	+	Negative	Negative	-	-
M40	+	Negative	Negative	-	-
M41	+	Negative	Negative	-	-
M42	+	Negative	Negative	-	-
M43	+	Negative	Negative	-	-
M44	+	Negative	Negative	-	-
M45	+	Negative	Negative	-	-
M46	+	Negative	Negative	-	-
M47	+	Negative	Negative	-	-
M48	+	Negative	Negative	-	-
M49	+	Negative	Negative	-	-
M50	+	Negative	Negative	-	-
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M82	+	Negative	Negative	-	-
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M138	+	Negative	Negative	-	-
M139	+	und, 38.69	Negative	-	-
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M166	+	Negative	Negative	-	-
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M173	+	39.87, und	Negative	-	-
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M221	+	Negative	Negative	-	-

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M227	+	Negative	Negative	-	-
M228	+	**31.30**	**30.11**	POSITIVE	POSITIVE
M229	+	Negative	Negative	-	-
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M231	+	Negative	Negative	-	-
M232	+	Negative	Negative	-	-
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M234	+	Negative	38.77, und	-	-
M235	+	Negative	Negative	-	-
M236	+	und,39.80	Negative	-	-
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M295	+	Negative	Negative	-	-
M296	+	Negative	Negative	-	-
M297	+	Negative	Negative	-	-
M298	+	Negative	Negative	-	-
M299	+	Negative	Negative	-	-
M300	+	Negative	Negative	-	-

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