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Evaluation of point of care (POC) tests for White Spot Syndrome Virus (WSSV)

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Abbreviations

ABI	Applied Biosystems™
ACDP	Australian Centre for Disease Preparedness
AEC	Animal Ethics Committee
AFDL	ACDP Fish Diseases Laboratory
AHC	Animal Health Committee
APFA	Australian Prawn Farmers Association
ASe	Analytical sensitivity
ASp	Analytical specificity
°C	degrees Celsius
C_q	quantity cycle
C_T	threshold cycle
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DSe	Diagnostic sensitivity
DSp	Diagnostic specificity
EAD	Emergency animal disease
EF1	Elongation factor 1
FRDC	Fisheries Research and Development Corporation
GAV	Gill associated virus
HPV	Hepatopancreatic parvovirus
IHHNV	Infectious hypodermal and haematopoietic necrosis virus
ISO	International Organization for Standardization
LOD	Limit of detection
MBV	monodon baculovirus
MRA	Movement restriction area
NPV	Negative predictive value
OIE	Office International des Epizooties
PCR	Polymerase chain reaction
p.i.	Post injection
POC	Point of care
PPV	Positive predictive value
QC	Quality control
qPCR	Real-time polymerase chain reaction
SCAAH	Sub-Committee for Aquatic Animal Health
TSV	Taura syndrome virus
Vp_{AHPND}	Vibrio parahaemolyticus acute hepatopancreatic necrosis disease
WOAH	World Organisation for Animal Health
WSD	White spot disease
WSSV	White spot syndrome virus
YHV	Yellow head virus

Executive Summary

The CSIRO Australian Centre for Disease Preparedness (ACDP) Fish Diseases Laboratory (AFDL), in collaboration with Biosecurity Queensland, conducted a laboratory-based evaluation of commercially available point-of-care (POC) tests designed to detect white spot syndrome virus (WSSV) in the field. The project was commissioned by the Fisheries Research and Development Corporation (FRDC) in response to requests from the Australian prawn farming industry for the potential use of WSSV POC tests on farms as an enhanced biosecurity measure, following a significant outbreak of this previously exotic pathogen in the Logan River region of Queensland in 2016/17. This study was the first side-by-side independent evaluation of commercial POC test devices for an aquatic animal pathogen in Australia.

Five commercially available WSSV POC tests were selected for evaluation, comprising 3 rapid antigen style tests and 2 PCR-based tests (Table I).

Table I. Commercial WSSV POC tests selected for evaluation

Test	Manufacturer	Type
Shrimple®	Fujikura Kasei Co Ltd, Japan	Immunochromatographic lateral flow rapid antigen test
ShrimpCheck WSSV Rapid Test Kit	Speedy Assay Sdn Bhd, Malaysia	Immunochromatographic lateral flow rapid antigen test
CDIA™ WSSV Rapid Test Kit	Creative Diagnostics, USA	Immunochromatographic lateral flow rapid antigen test
IQ Plus™ WSSV Kit with POCKIT™ System	GeneReach Biotechnology Corporation, Taiwan	Portable field-deployable PCR-based test
AgriGen POND on-site detection system for WSSV	AgriGen Biotech Pty Ltd, Australia.	Portable field-deployable PCR-based test

Key Outcomes

- A rapid antigen style WSSV POC test suitable for Australian circumstances and available for Australian procurement was not identified.
- The field-deployable PCR-based WSSV POC tests had lower sensitivity than validated laboratory reference WSSV qPCR tests.
- The cost, testing time and user training requirements were greater for the field-deployable PCR-based WSSV POC tests than for the rapid antigen style WSSV POC tests, due to their technical complexity and need for specialised equipment.

Background

Since the 2016/17 Logan River outbreak, WSSV has persisted as a significant threat to the 150+ million dollar Australian prawn aquaculture industry, with additional outbreaks occurring on farms in the QLD Logan River and NSW Clarence River areas in 2020 and 2023 respectively. With the potential to cause mass mortalities of up to 100% in farmed prawns within days, accurate early detection of the virus is essential so that rapid action can be taken to control spread and reduce losses. Clinical samples from WSSV outbreaks or suspected infection are tested at authorised State and Federal Government laboratories, with real-time qPCRs being the primary diagnostic tests. Turn-around time from sample collection at the farm to reporting of laboratory results can take more than 24 hours, particularly if the farm is in a remote location requiring samples to be transported long distances to receiving laboratories.

The ability to use POC tests on-farm for a preliminary WSSV diagnosis in a clinically affected population, in conjunction with confirmatory laboratory testing, could be advantageous for outbreak management. This is dependant, however, on the reliability of POC test results. Test performance data for commercially available WSSV POC tests was limited, so to improve knowledge of their suitability for Australian circumstances a side-by-side comparative assessment was undertaken whereby WSSV POC tests were compared to each other and to primary WSSV laboratory reference qPCR tests.

Scope and Methods

The approach used to evaluate the tests was based on the World Organisation for Animal Health (WOAH) assay validation pathway. The key performance characteristics assessed were analytical sensitivity (ASe), analytical specificity (ASp), diagnostic specificity (DSp), diagnostic sensitivity (DSe), repeatability and applicability (diagnostic window). The intended purpose of the POC tests was for the preliminary diagnosis of WSSV where suspect clinical signs or elevated mortalities are apparent in a farmed population, therefore the evaluation focused on the ability of the tests to detect WSSV in clinically affected *P. monodon*, Australia's primary farmed prawn species. The evaluation was laboratory-based and did not include a field trial.

Panels of test evaluation samples were prepared to assess each performance characteristic, using well characterised material with known WSSV status. Some material needed to be experimentally generated. For this *P. monodon* were experimentally infected at ACDP with WSSV/Australia/2016-Logan River to represent Australian circumstances. Experimental infection was via segregated cohabitation (i.e. shared water) with WSSV-injected *P. monodon,* to simulate a natural route of infection. Panel samples were tested with the POC tests and with two primary laboratory reference WSSV qPCRs, allowing a comprehensive side-by-side comparison of test performance. All testing and result interpretation was conducted in strict accordance with manufacturer's instructions. Operational characteristics were also assessed and validation data described in other studies was reviewed.

Results and Discussion

The foremost strength of this study is that the selected POC tests and laboratory reference tests were assessed using the same set of samples, allowing them to be directly compared. Due to procurement difficulties, discontinuation of one POC test by the manufacturer, and test specificity issues, all performance characteristics could not be assessed for all POC tests. A summary of the performance evaluation components completed for each test are provided in Table II.

Table II. Summary of performance evaluation components completed for each test

Test Evaluation Panel	CSIRO and OIE WSSV qPCR Reference Tests	Shrimple®[1]	ShrimpCheck WSSV Rapid Test Kit[2]	CDIA™ WSSV Rapid Test Kit[3]	IQ Plus™ WSSV Kit with POCKIT™ System	AgriGen POND System for WSSV
Analytical Specificity (ASp)	✓	✓ Partial (56%)	✓	✗	✓	✓
Analytical Sensitivity (ASe)	✓	✗	✓	✗	✓	✓
Relative Diagnostic Specificity (DSp)	✓	✓ Partial (76%)	✗	✗	✓	✓
Relative Diagnostic Sensitivity (DSe) for Clinically Affected Prawns	✓	✓ Partial (91%)	✗	✗	✓	✓
Repeatability	✓	✗	✗	✗	✓	✓
Applicability (Diagnostic Window)	✓	✗	✗	✗	✓	✓

[1] Shrimple® was discontinued by the manufacturer part-way through the study therefore exclusivity for heterologous prawn pathogens, ASe, repeatability and applicability could not be assessed and DSe and DSp assessments could only be partially completed.

[2] ShrimpCheck WSSV Rapid test was found to be unable to detect WSSV/Australia/2016-Logan River during the ASp assessment and therefore did not undergo assessment for diagnostic performance characteristics or repeatability due to these evaluation panels being prepared from WSSV/Australia/2016-Logan River infected prawns.

[3] CDIA™ WSSV Rapid Test Kit was unable to be procured and therefore could not undergo performance evaluation.

Analytical specificity was assessed for 7 heterologous prawn pathogens (yellow head virus genotypes 1, 2 and 7, *Vibrio parahaemolyticus*, taura syndrome virus, infectious hypodermal and haematopoietic necrosis virus and hepatopancreatic parvovirus), 5 prawn species (*P. monodon, P. esculentus, P. merguiensis, M. plebejus* and *M. latisulcatus*) and 4 WSSV strains (Australia/2016-Logan River, Vietnam/2017, China/2017 and Australia/2022-NSW broodstock). A critical finding was that the ShrimpCheck WSSV Rapid test was unable to detect the 2016 Logan River outbreak strain of WSSV. Importantly, no cross-reactivity was observed for any POC test to heterologous prawn pathogens or to prawn host genome. This study greatly increased existing knowledge of ASp performance for all evaluated tests with a focus on prawn species and pathogens relevant to Australian circumstances.

Determining the limit of detection of each POC test for 3 different WSSV strains (Australia/2016-Logan River, Vietnam/2017 and China/2017) revealed that ASe varied by several orders of magnitude between tests, with the PCR-based tests ranking higher for ASe than the rapid antigen style tests. For all 3 WSSV strains assessed, the laboratory reference qPCRs were 10-fold more sensitive than the most sensitive POC test; the AgriGen POND WSSV qPCR. The IQ Plus™ POCKIT™ test was 100-fold less sensitive than the AgriGen POND WSSV qPCR. The ShrimpCheck WSSV Rapid test was 10-fold less sensitive than the IQ Plus™ POCKIT™ test for the China and Vietnam strains, and was unable to detect WSSV/Australia/2016-Logan River. Our findings support the hierarchy of sensitivity expected following review of existing ASe data and consideration of the mode of action of the tests.

A panel of 240 *P. monodon* comprising 100 known WSSV-negative and 140 known WSSV-positive clinically affected experimentally infected specimens was used to determine the DSp and DSe of the POC tests relative to the WSSV laboratory reference qPCRs. All POC tests demonstrated 100% relative DSp with no false positive results observed. Differences in accuracy between tests were therefore dependent on relative DSe. For clinically affected prawns, the IQ Plus™ POCKIT™ test was the most sensitive (100%) relative to the

WSSV laboratory reference qPCRs, followed by Shrimple® (98.44%) and the AgriGen POND WSSV qPCR test (81.68%). DSe results for the AgriGen POND test were surprising given it was the highest ranked POC test for ASe. Further investigation indicated that issues related to operational characteristics and PCR inhibition were likely causing the AgriGen POND false negative test results.

The repeatability assessment demonstrated that for strongly positive or negative homogenised prawn tissue samples there was 100% concordance between test replicates for the assessed POC tests within test runs, between test runs and between operators. For weak positive samples, however, repeatability of the POC tests declined with only 56% - 78% of replicate weak positive samples testing positive, in contrast to the 100% concordance demonstrated by the laboratory reference qPCRs. Although the IQ Plus™ POCKIT™ test demonstrated the least overall concordance (85.19%), it is expected that discordance would increase for the AgriGen POND test when non-homogenised samples are used, as demonstrated in the DSe and applicability assessments where unhomogenised pleopod tissue strongly positive for WSSV (reference WSSV qPCR C_T values < 20) tested negative by AgriGen POND WSSV qPCR on numerous occasions.

The ability of the POC tests to detect WSSV in experimentally infected (cohabitated) *P. monodon* increased over time and with the progression of clinical signs. The first WSSV-positive detections occurred via qPCR reference test at 24 hours post exposure, a day earlier than the first positive detections by the assessed POC tests (AgriGen POND test and IQ Plus™ POCKIT™ test) at 48 hrs post exposure. The proportion of prawns testing WSSV-positive was reduced in POC tests compared to laboratory reference qPCRs in samples collected in the early stages of infection and in prawns with mild clinical manifestation or weak positive reference test results. The POC test with greater ASe (AgriGen POND test) detected a higher overall proportion of WSSV reference qPCR positive prawns (92.70%) than the IQ Plus™ POCKIT™ test (87.27%), despite returning negative results for a small number of strongly WSSV-positive samples.

In existing literature, all POC tests were reported to be able to detect WSSV in the early stages of infection. The live prawn experiments conducted in this study demonstrate that white spot disease (WSD) progresses much faster when prawns are injected with WSSV compared to when they are cohabitated with WSSV-infected cohorts. Thus, the predicted timeframe from WSSV exposure to WSSV detection by POC test in the field may be over-estimated in other studies where prawns were experimentally infected with WSSV via injection.

Importantly, this study is the first to compare WSSV POC test results with real-time qPCR reference test C_T values. Results indicate that detection of WSSV by rapid antigen test or IQ Plus™ POCKIT™ test may be greatly reduced in prawns with reference WSSV qPCR C_T values > 20. This is valuable information given the WSSV real-time qPCR reference test C_T value ranges of clinically affected and apparently healthy WSSV-infected prawns has been recently described (Moody et al., 2022).

A comprehensive assessment of operational characteristics clearly demonstrated that the rapid antigen style POC tests had numerous operational advantages compared to PCR-based POC tests. The rapid antigen style tests were easier to conduct and interpret, required less equipment and less sample processing steps, had a quicker turnaround time from sampling to result, were cheaper, and did not require any on-site training or additional equipment.

Conclusions, Implications and Recommendations

Overall, performance of the WSSV POC tests varied widely and was reduced compared to laboratory reference real-time qPCR tests, however, this is not unexpected given the trade-off between test performance and the simplified design and operational characteristics that make POC tests appropriate for field use.

The rapid antigen style POC tests, although potentially less sensitive than the PCR-based POC tests, offered several advantages. They were fast, simple and cheap, and did not require laboratory skills or specialised equipment to use or interpret them. This project, however, experienced obstacles procuring this style of test. Of the three rapid antigen style tests selected for this study (Shrimple®, ShrimpCheck WSSV Rapid Test and CDIA™ WSSV Rapid Test) the first was discontinued by the manufacturer during the study, the second was unable to detect the WSSV strain responsible for the 2016 Logan River outbreak and the third could not be procured at all. All attempts to obtain an additional rapid antigen style test to include in the evaluation were unsuccessful, meaning a rapid antigen style test suitable for Australian circumstances and available for Australian procurement was not identified.

The main advantage of the PCR-based POC tests (AgriGen POND test and IQ Plus™ POCKIT™ test) was improved ASe compared to the rapid antigen style tests. They were, however, more technically complex and required basic laboratory skills to conduct. They were also more expensive, took longer to obtain a result, and required specialised equipment. Implementing either of the two PCR-based POC tests on-farm in Australia may present challenges for operator training and maintaining on-going competency.

This report is intended to provide stakeholders with critical preliminary test performance information for the WSSV POC tests evaluated, so that governing bodies can make informed decisions regarding the fitness for purpose of the tests and management of their use in Australia. It is not the role of the authors to recommend or endorse a particular commercial product or products.

Keywords

White spot syndrome virus, WSSV, point of care test, POC, test evaluation, on-farm testing.

Introduction

White spot syndrome virus (WSSV) is a highly pathogenic virus of prawns that was exotic to Australia until December 2016, when it caused a large and devastating outbreak of clinical disease in farmed prawns in the Logan River area of Queensland. As well as response costs to State and Commonwealth governments, the outbreak caused direct losses of $43 million to the prawn farming industry and indirect losses of $383 million to Australian businesses. The virus was again detected in diseased farmed prawns and healthy wild crustaceans in Queensland's Moreton Bay area in 2020 and in diseased farmed prawns in the Clarence River area in 2023, and remains a significant threat to the 150+ million dollar Australian prawn aquaculture industry. White spot disease (WSD) can cause 100% mortality in farmed prawns within days, so rapid action is critical to identify the causative agent, control spread and reduce losses.

Testing of clinical samples from WSSV outbreaks or suspected infection is undertaken at approved State and Federal Government laboratories, with real-time qPCRs being the primary diagnostic tests. Turn-around time from sample collection at the farm to reporting of laboratory results can take more than 24 hours. Following the 2016/17 White Spot Disease (WSD) outbreak in Queensland, the Fisheries Research and Development Corporation (FRDC), in consultation with industry and government, identified rapid on-farm WSSV diagnosis as a potential key element to enhance biosecurity. Additionally, the WSD Response Plan (FRDC Project 2016/266; Stephens, 2017) recommended consideration of using point of care (POC) field tests on-farm as a first response tool for presumptive identification, allowing emergency control measures to be more rapidly implemented.

The Australian Prawn Farmers Association (APFA) approached Biosecurity Queensland (BQ) to explore options for on-farm WSSV testing, which BQ considered in accordance with the provisions of the Biosecurity Act 2015. While there were several commercial WSSV POC tests available in the global market, there was limited comparative test performance data to inform and support the use of the tests in Australia. It was agreed that a comparative evaluation of several commercial WSSV POC tests would be undertaken by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) at the Australian Centre for Disease Preparedness (ACDP) Fish Diseases Laboratory (AFDL), to determine the suitability of the tests for detection of WSSV in clinically affected prawns, based on key performance and operational characteristics.

While the tests were being evaluated, a commercial WSSV POC test (Shrimple®) was provided to Queensland prawn farms in 2019 as an interim measure until the evaluation could be completed, with use of the test subject to strict conditions. This test was successfully used in April 2020 when an outbreak of WSD occurred on two prawn farms in the Logan River. Initial identification of WSSV in clinically diseased prawns from index property 1 was via on-farm WSSV POC testing using the test kit supplied by BQ, followed by positive WSSV detections via qPCR at Biosecurity Sciences Laboratories QLD, and confirmatory testing via qPCR and conventional PCR and sequence analysis at ACDP. This case demonstrated how on-farm testing, supported by appropriate confirmatory testing, can be successfully deployed as a component of emergency animal disease (EAD) response.

Objectives

To evaluate the performance of 5 commercially available WSSV POC tests for their ability to detect WSSV in clinically affected prawns.

1. Determine the analytical and diagnostic performance characteristics (analytical specificity, analytical sensitivity, diagnostic specificity, diagnostic sensitivity, repeatability and applicability) of three commercially available immunochromatographic WSSV POC test kits for the detection of WSSV in clinically affected prawns.

2. Determine the analytical and diagnostic performance characteristics (analytical specificity, analytical sensitivity, diagnostic specificity, diagnostic sensitivity, repeatability and applicability) of two commercially available PCR-based WSSV POC testing platforms for the detection of WSSV in clinically affected prawns.

3. Using analytical and diagnostic performance data generated in objectives 1 and 2, conduct a comparative evaluation whereby the performance of the POC tests are a) compared to each other and b) compared to validated laboratory-based NATA accredited WSSV real-time qPCR reference assays.

1. Method

1.1 Scope of evaluation

The evaluation was designed and conducted based on the assay validation pathway described by the WOAH in their Manual of Diagnostic Tests for Aquatic Animals, Chapter 1.1.2 *Principles and methods of validation of diagnostic assays for infectious diseases*. The scope was for a laboratory-based evaluation to assess analytical and diagnostic performance characteristics of the selected commercialised POC tests, for the purpose of detecting WSSV in clinically affected prawns. POC test performance was compared to two NATA-accredited laboratory reference qPCR tests for WSSV. As WSSV was considered an exotic pathogen outside of the Movement Restriction Area (MRA) in south-east Queensland, the scope did not include a field-trial to assess the reproducibility of test results at different field sites.

1.2 POC test selection

To be considered for evaluation, WSSV POC tests were required to meet the following criteria:

- must be commercially available
- must be designed to specifically detect WSSV
- must be field deployable and designed for on-farm point-of-care disease diagnosis
- must produce rapid results (i.e. within ≤ 2 hours from sample collection to result)

It was also considered desirable if a test was well known or used internationally. Based on this criteria, five WSSV POC tests were selected for evaluation, comprising three immunochromatographic lateral flow rapid antigen tests and two field deployable PCR-based tests (Table 1).

Table 1. POC tests selected for evaluation

Test	Manufacturer	Type
Shrimple®	Fujikura Kasei Co Ltd, Japan	Immunochromatographic lateral flow rapid antigen test
ShrimpCheck WSSV Rapid Test Kit	Speedy Assay Sdn Bhd, Malaysia	Immunochromatographic lateral flow rapid antigen test
CDIA™ WSSV Rapid Test Kit	Creative Diagnostics, USA	Immunochromatographic lateral flow rapid antigen test
IQ Plus™ WSSV Kit with POCKIT™ System	GeneReach Biotechnology Corporation, Taiwan	Portable field-deployable PCR-based test
AgriGen POND on-site detection system for WSSV	AgriGen Biotech Pty Ltd, Australia	Portable field-deployable PCR-based test

The general mode of action of the rapid antigen style POC tests (Shrimple®, ShrimpCheck WSSV Rapid and CDIA™ WSSV Rapid) is presented in Figure 1. Briefly, a prawn sample is selected based on kit instructions (A), the sample is manually homogenised in sample buffer (B), buffer containing the sample is dripped into the sample window of the test cassette and the sample is drawn along the test strip membrane by capillary action, moving through the test (T) zone and control (C) zone. The membrane is pre-coated with specialised antibodies and is based on the principle of a sandwich immunoassay. If WSSV is present in the sample a visible coloured band will appear in the T zone, with test validity confirmed by a second coloured band in the C zone. Results are read visually by the operator without instrumentation. The time from sample collection to result is approximately 20 minutes.

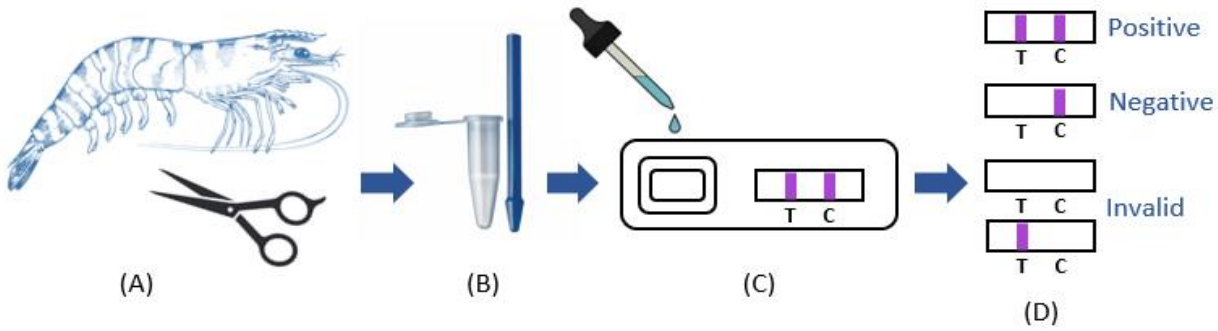

Figure 1. Mode of action of immunochromatographic lateral flow rapid antigen style tests

The IQ Plus™ WSSV POCKIT™ test is based on isothermal induction PCR (iiPCR) technology (Figure 2.) The prawn is sampled according to kit instructions (A). The sample is manually homogenised in a buffer solution (B) and the nucleic acid is extracted from the sample via a spin column purification system (C). The purified nucleic acid is added to reconstituted PCR reaction mix via transfer loop, then the mix is transferred by micropipette to an iiPCR reaction tube (D). Reaction tubes are loaded into a benchtop analyser, and qualitative (positive/negative) results are displayed on the instrument monitor at the end of the run.

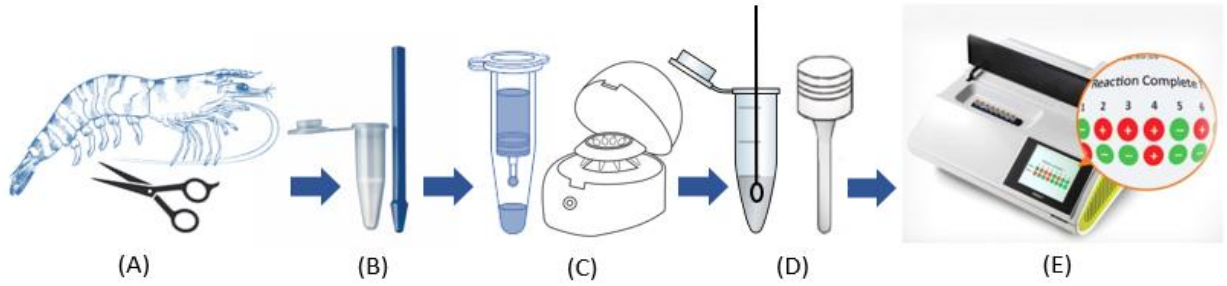

Figure 2. Mode of action of the IQ Plus™ WSSV POCKIT™ iiPCR test

The IQ Plus™ WSSV iiPCR uses thermal convection to drive fluid cycling in specialised capillary tubes. Fluorogenic probe hydrolysis chemistry generates a fluorescent signal when specific WSSV-targeted primers and probes amplify the WSSV DNA target sequence. To confirm test validity, internal control primers and probes are used to target a house-keeping gene of penaeid shrimp. Two optical channels (520 nm and 550 nm) are used for multiplex detection of WSSV and the internal control. The POCKIT analyser automatically interprets the iiPCR data, with no post run manipulation required from the operator. The time from sample collection to result is approximately 2 hours.

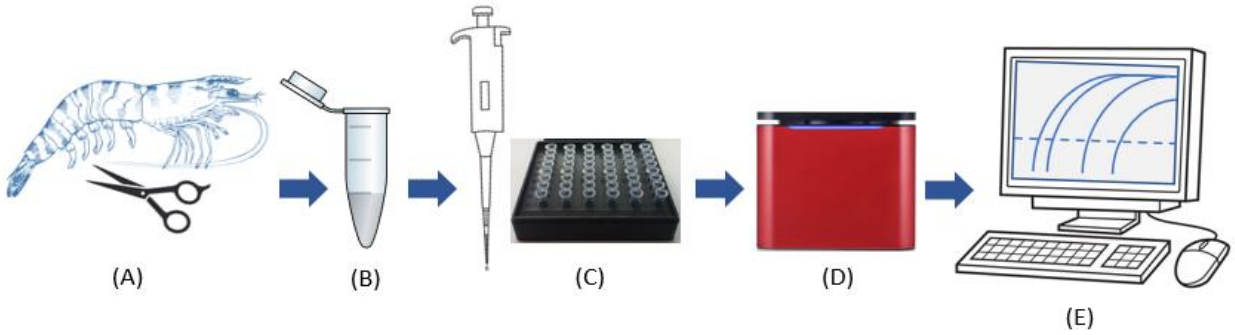

Figure 3. Mode of action of the AgriGen POND WSSV real-time qPCR test

The AgriGen POND WSSV test is based on real-time quantitative PCR (qPCR) technology (Figure 3.) The prawn is sampled according to kit instructions (A). The sample is added to a nucleic acid extraction buffer, with no homogenisation performed (B). A qPCR reaction mix is aliquoted into specialised reaction tubes, and a portion of the extraction buffer containing the sample is added directly into the reaction mix via micropipette (C). Reaction tubes are loaded into a benchtop rotary magnetic induction thermal cycler (D) and the qPCR run is set up and initiated using specialised software on a linked computer (E).

When WSSV-targeted primers and probe amplify WSSV DNA target sequence a fluorescent signal is emitted and measured in real-time, during each qPCR cycle. Data is visually recorded in a qPCR amplification plot as the reaction progresses. At the end of the run, the amplification data is analysed by the operator, using analysis parameters specific to the AgriGen POND WSSV qPCR assay. The time from sample collection to result is approximately 2 hours.

1.3 Laboratory reference tests

The primary tests used for laboratory diagnosis of WSSV in Australia are the CSIRO WSSV real-time qPCR and the OIE WSSV real-time qPCR (Table 2). POC test performance was compared to these two laboratory reference tests, both of which are within the ACDP ISO 17025 NATA scope of accreditation.

Table 2. Laboratory reference tests for WSSV

qPCR	Primers/probe	Sequence'(5'-3')	Cycling	Source
CSIRO WSSV qPCR	CSIRO WSSV-F CSIRO WSSV-R CSIRO WSSV probe	CCG ACG CCA AGG GAA CT TTC AGA TTC GTT ACC GTT TCC A 6FAM-CGC TTC AGC CAT GCC AGC CG-TAMRA	1 x 95°C for 10 min 45 x (95°C for 15 sec + 60°C for 60 sec)	Sritunyalucksana et al. (2006)
*OIE WSSV qPCR	OIE WSSV 1011F OIE WSSV 1079R OIE WSSV probe	TGG TCC CGT CCT CAT CTC AG GCT GCC TTG CCG GAA ATT A 6FAM-AGC CAT GAA GAA TGC CGT CTA TCA CAC A-TAMRA	1 x 95°C for 10 min 45 x (95°C for 15 sec + 60°C for 60 sec)	Durand and Lightner (2002)

*The nomenclature used in this document for the OIE WSSV qPCR is consistent with the test name listed in the Department of Agriculture Fisheries and Forestry (DAFF) guidance for WSSV testing 'Procedure for detection of white spot syndrome virus for biosecurity risk management'. It should be noted that this test may be referred to as the WOAH WSSV qPCR in recent publications such as Moody et al. (2022).

Samples were homogenised via bead-beating in MagMAX™ CORE lysis solution using a FastPrep-24™ 5G bead-beating instrument. The ABI MagMAX™ CORE nucleic acid purification kit and ABI MagMAX™ Express 96 automated magnetic particle processor were used for nucleic acid purification. The CSIRO and OIE WSSV

reference qPCRs were prepared using AgPath-ID™ one-step RT-PCR reagents and run on ABI 7500 Fast real-time PCR instruments in 25 µL reactions containing 2 µL nucleic acid template. The performance characteristics of both tests have been described in Moody et al. (2022).

1.4 Site and personnel

The laboratory-based POC test evaluation was conducted at the CSIRO ACDP Fish Diseases Laboratory which operates according to a certified Quality Management System (ISO 9001:2015) and conducts accredited testing according to ISO/IEC 17025:2017. Testing was performed by qualified and competent staff experienced in conducting, developing and assessing diagnostic methods for emergency animal diseases, including WSSV. The approach taken to evaluate the tests aligns with WOAH guidelines for test validation and was developed in consultation with ACDP's Principal Research Consultant and leader of the WOAH Collaborative Centre for Diagnostic Test Validation Science. Production of WSSV-infected material (AEC 2047) occurred at the CSIRO ACDP aquatic animal experiment facility, by staff experienced in conducting aquatic animal bioassays including infecting, maintaining and sampling prawns infected with WSSV.

1.5 Quality assurance

1.5.1 Storage

POC test kits were inspected on arrival at ACDP and components stored according to manufacturer's instructions. Components requiring cold storage were stored within temperature monitored fridges and freezers. Test components were not used after their specified expiry dates. Test evaluation panel samples (and the material used to prepare them), as well as any nucleic acid derived from these samples, were stored according to POC test manufacturer's instructions in temperature monitored fridges and freezers.

1.5.2 Testing

All POC testing was conducted in strict accordance with manufacturer's instructions. In-house SOPs and test worksheets were developed based on kit inserts, exactly describing manufacturer's instructions for sample preparation, testing and result interpretation including acceptance criteria for test controls. A pilot phase was conducted to familiarise staff with test platforms so that operators were confident and proficient with POC test procedures.

CSIRO and OIE WSSV qPCR testing was conducted in accordance with ACDP's ISO 17025 accredited test method for molecular detection of WSSV. Artificial probe was included in the CSIRO and OIE WSSV qPCR reaction mixes, and WSSV plasmids containing an artificial probe target sequence were used as positive controls. This artificial probe/plasmid strategy was an additional QC measure incorporated as a means of identifying false positives resulting from positive control contamination, should it occur. The CSIRO Shrimp EF1 qPCR internal control assay was run on all samples tested by CSIRO and OIE WSSV qPCR, to demonstrate successful nucleic acid purification.

Test results were interpreted as per manufacturer's instructions, with each individual test result classified as positive, negative, indeterminate or invalid based on the manufacturer's prescribed parameters. For PCR-based POC tests and reference tests, where sample nucleic acid extracts were tested in duplicate PCR reactions, the overall test result was interpreted as shown in Table 3.

Table 3. Final interpretation of duplicate PCR reactions for PCR-based POC and laboratory reference tests

Results from Duplicate PCR Reactions	Final Interpretation
Both PCR reactions positive	Positive
Both PCR reactions negative	Negative
One PCR reaction positive, one PCR reaction negative	Indeterminate
One or both PCR reactions indeterminate	Indeterminate
One or both PCR reactions invalid (e.g. positive control, housekeeping gene failure)	Invalid

1.5.3 Data management

In-house test worksheets were created for systematic capture of test results and quality control information such as sample ID, reagent batch numbers, operator ID, expiry dates etc. All raw data generated by PCR-based platforms was retained, and all rapid antigen tests were uniquely identified and photographed at the time of result interpretation, with the test image inserted into the test worksheet. All test results were reviewed by a second staff member prior to statistical analysis.

1.6 Preparation of test evaluation panels

1.6.1 Material required

Test evaluation panels to assess each performance characteristic were prepared from the material outlined in Table 4. This material was experimentally produced at ACDP or obtained from existing ACDP stocks or external sources.

Table 4. Material required to produce test evaluation panels

Evaluation Component	Material Required
Analytical Specificity (ASp) Degree to which the tests cross-react with other pathogens/antigens (exclusivity) and their ability to detect different WSSV strains (inclusivity)	- Exclusivity: 7 heterologous prawn pathogens - Inclusivity: 4 WSSV strains - Host genome: 5 prawn species
Analytical Sensitivity (ASe) Limit of detection of WSSV by the tests	10-fold dilutions of WSSV-positive prawn tissue homogenates; 3 WSSV strains
Diagnostic Specificity (DSp) Measure of how well the tests can identify true negatives, and the proportion of false positives generated by the tests	100 WSSV-negative *P. monodon*
Diagnostic Sensitivity (DSe) for Clinically Affected Prawns Measure of how well the tests can identify true positives, and the proportion of false negatives generated by the tests (for clinically affected prawns)	140 WSSV-positive, clinically affected *P. monodon*
Repeatability Ability of the tests to generate repeatable results for multiple preparations of the same sample, within run, run-to-run and operator-to-operator	Homogeneous aliquots of WSSV-positive (strong and weak) and WSSV-negative prawn tissue homogenates
Applicability Ability of the tests to detect WSSV throughout the course of infection (diagnostic window)	70 *P. monodon,* experimentally exposed to WSSV and sampled at pre-determined timepoints over the course of infection

1.6.2 Confirmation of source material

The WSSV status of all source material was confirmed by CSIRO and OIE WSSV qPCR testing.

- Confirmed WSSV-positive = testing positive (duplicate reactions) in one or both WSSV reference qPCRs (CSIRO and OIE qPCRs) with no artificial probe amplification, and testing positive in the CSIRO Shrimp EF1 internal control qPCR.
- Confirmed WSSV-negative = testing negative (duplicate reactions) in both WSSV reference qPCRs (CSIRO and OIE qPCRs) with no artificial probe amplification, and testing positive in the CSIRO Shrimp EF1 internal control qPCR.

Where different strains of WSSV were required, source material had undergone whole genome sequencing to confirm WSSV strain identity. Where non-WSSV pathogens were required, the disease agent status of the source material was confirmed by agent specific qPCR followed by conventional PCR and amplicon sequencing. Prawn species identities were confirmed by conventional PCR and amplicon sequencing.

1.6.3 Sample type

To comply with manufacturer's instructions for all POC tests, the prawn material used to evaluate the tests needed to be

- a tissue type suitable for all selected POC tests
- unfixed (ethanol fixed tissues were not suitable for some tests)
- fresh or stored frozen at ≤ -20°C

Pleopod tissue was selected as the preferred sample type as it was universally suitable for all POC tests. The POC tests are able to test prawns of any size, however, if a prawn is ≥ 20 g testing can be conducted using a single pleopod per test. This means that one ≥ 20 g prawn had enough pleopod material to allow comparative testing of that prawn across all platforms, including replicate testing where required. Therefore, prawns ≥ 20 g were used for panel preparation. An assessment of the distribution of WSSV in the pleopods and tail muscle segments of experimentally infected prawns indicated consistent distribution within these tissues in individual prawns (DAWE Pool-Level Sensitivity for Aquatic Animal Pathogens of national and Trade Significance Project [Ref ID 78260], 2018). For instances where pleopod tissue was not available, an equivalent amount of tail muscle tissue was used, following comparative determination of this tissue type as a suitable alternative.

1.6.4 Generation of WSSV-infected P. monodon

The samples held at ACDP from the 2016/2017 WSSV outbreak in Queensland's Logan River were ethanol fixed and therefore not suitable for use in this project. The project proposal indicated that WSSV-infected prawn material generated during experiments conducted as part of FRDC project 2017-190 *Assessment of gamma irradiation as a feasible method for treating prawns with White Spot Syndrome Virus* would be used for this project. Due to delays (e.g. COVID-19) and rescheduling affecting both FRDC projects, this was no longer viable. Therefore, WSSV-infected material for the DSe, applicability and repeatability panels was experimentally generated specifically for this project. So that test evaluation material would be representative of Australian circumstances, *P. monodon* (Australia's major farmed species) were infected with WSSV/Australia/2016-Logan River obtained from the index case of the 2016/17 WSSV outbreak in Queensland.

To simulate a natural route of WSSV infection in a laboratory-based setting, an experimental system of cohabitation was used whereby naïve *P. monodon* shared recirculating water with WSSV-injected *P. monodon*, facilitating environmental transmission of WSSV to the naïve cohort (Figure 4). This cohabitation

infection model had not previously been used for WSSV at ACDP, so a pilot trial (experiment 1) was conducted and successfully confirmed the model as fit for purpose. This was followed by a second experiment focused on the generation of material for the applicability panel. Experiment 2 was undertaken with approval from the ACDP Animal Ethics Committee (AEC 2047); experiment 1 was conducted prior to the requirement for AEC approval for experimental use of live decapod crustaceans.

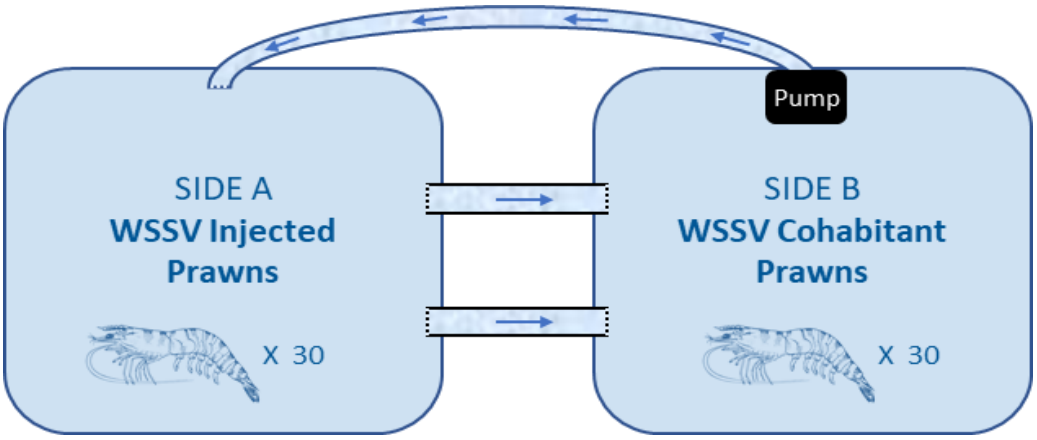

Figure 4. Conjoined tanks housing WSSV injected and cohabitated P. monodon – schematic diagram

1.6.4.1 Experiment 1 – Cohabitation pilot trial

P. monodon (≥ 20 g) were housed in conjoined tanks (30 prawns per tank) connected by pipes covered with mesh to segregate challenge groups while allowing unobstructed flow of pump circulated water through the conjoined tanks. Prawns in one tank were injected with WSSV, while cohabitant prawns in the adjoining tank received no treatment other than exposure to shared water which was pump-circulated through conjoined tanks. Dead/moribund prawns were removed for sampling twice daily until all had been collected. The trial was conducted in triplicate and included a parallel negative control cohort.

Information detailing specific experimental conditions including water temperature and flow rates, water quality monitoring and exchange, husbandry and feeding, inoculum composition and administration, specimen collection and storage are provided in Appendix I. The WSSV status of all samples was confirmed by WSSV reference qPCR and histopathological examination was conducted on representative prawns collected from each cohort at the end of the experiment. Results from experiment 1 are summarised in Appendix II. The trial successfully achieved the following important outcomes:

- Demonstrated the effectiveness of the cohabitation experimental transmission model in generating WSSV infection in prawns.
- Generated the mortality data that informed collection timepoints for the generation of applicability assessment panel samples (experiment 2).
- Generated clinically affected WSSV-positive *P. monodon* required for the DSe evaluation panel.

1.6.4.2 Experiment 2 – Generation of applicability panel material

P. monodon (≥ 20 g) were housed in conjoined tanks and experimentally exposed to WSSV via cohabitation with WSSV-injected *P. monodon*. Information detailing specific experimental conditions including water temperature and flow rates, water quality monitoring and exchange, husbandry and feeding, inoculum composition and administration, specimen collection and storage are provided in Appendix I. Prawns were monitored 3 times daily for 8 days and were systematically collected at pre-determined timepoints (0/baseline, 24, 48, 72, 96, 110, 120, 134, 144 and 168 hours) as the infection progressed through days 0 to

7 post exposure. Six cohabitated prawns were collected at each predetermined timepoint, apart from the final timepoint where only 2 prawns remained. A cohort of negative control cohabitated *P. monodon* were also sampled at parallel time-points. Any additional prawns presenting as moribund or dead during routing monitoring were also collected and the time of collection noted. At the time of collection, prawns were classified as follows:

- healthy or pre-clinical (no clinical signs)
- mildly clinically affected (slight decrease in activity compared to negative controls)
- moderately clinically affected (moderate decrease in activity compared to negative controls, decrease in feed intake or lack of feeding)
- severely clinically affected/moribund (lateral recumbency, minimal response to stimuli)
- dead

Note: At ACDP, gross anatomical signs such as the appearance of WSD-related white spots on the carapace, have not been observed during experimental infection of *P.* monodon with WSSV and are therefore not a reliable indicator of disease progression in experimental infection studies. Therefore, behavioural signs such as reduced activity or feed intake, atypical swimming and lateral recumbency were used to grade clinical disease.

The WSSV status of all samples was confirmed by WSSV reference qPCR and histopathological examination was conducted on representative prawns from each cohort, collected at the end of the experiment. Results from experiment 2 are summarised in Appendix III. The experiment successfully generated an applicability evaluation panel of 70 *P. monodon* experimentally exposed to WSSV via cohabitation, consisting of healthy and mildly, moderately and severely clinically diseased prawns collected from 0 to 168 hrs post exposure.

1.7 Generation of test evaluation data

1.7.1 Generation of analytical specificity data

Analytical Specificity was assessed using 3 panels (Table 5):

1. An exclusivity panel containing 7 heterologous prawn pathogens to assess cross-reactivity to non-target antigens/nucleic acid. All samples were strongly positive, with pathogen-specific qPCR C_T values < 20 for YHV genotypes 1, 2 and 7, Vp_{AHPND} and IHHNV, and C_T values between 20-25 for TSV and HPV.
2. An inclusivity panel containing 4 WSSV strains, to assess the ability of the tests to detect WSSV from different geographical regions. The panel included the 2016/2017 Australian Logan River outbreak strain, 2 strains of overseas origin (Vietnam/2017 and China/2017), and the strain of WSSV detected in *P. monodon* broodstock at a NSW commercial prawn hatchery in August 2022. All samples were strongly WSSV-positive, with CSIRO WSSV qPCR C_T values <19.
3. A host genome panel containing 5 prawn species of commercial importance in Australia to assess cross-reactivity to prawn host antigens/nucleic acid.

For the rapid antigen style tests each sample was tested in duplicate. For the PCR-based tests each sample was extracted in duplicate and each nucleic acid extract tested in duplicate PCR reactions.

Table 5. Analytical specificity panels

ASp Panel 1: Exclusivity Evaluation Panel		
No.	**Prawn Pathogen**	**Prawn Sample Type**
1	Yellow Head Virus 1 (YHV1)	Pleopod
2	Yellow Head Virus 7 (YHV7)	Tail muscle tissue homogenate spiked with YHV7-positive hemolymph
3	Gill Associated Virus (YHV2)	Pleopod
4	Vibrio parahaemolyticus (Vp_{AHPND})	Tail muscle tissue homogenate spiked with cultured Vp_{AHPND}
5	Taura Syndrome Virus (TSV)	Tail muscle tissue homogenate spiked with TSV-positive hemolymph
6	Infectious Hypodermal and Haematopoietic Necrosis Virus (IHHNV)	Pleopod
7	Hepatopancreatic Parvovirus (HPV)	Pleopod
ASp Panel 2: Inclusivity Evaluation Panel		
No.	**WSSV Strain**	**Prawn Sample Type**
1	WSSV/Australia/2016-Logan River	Pleopod
2	WSSV/Vietnam/2017	Tail muscle tissue
3	WSSV/China/2017	Tail muscle tissue
4	WSSV/Australia/2022-NSW Broodstock	Tail muscle tissue
ASp Panel 3: Host Genome Evaluation Panel		
No.	**Prawn Species**	**Prawn Sample Type**
1	*P. monodon* (Giant Tiger Prawn)	Pleopod
2	*P. esculentus* (Brown Tiger Prawn)	Pleopod
3	*P. merguiensis* (Banana Prawn)	Pleopod
4	*M. plebejus* (Eastern King Prawn)	Pleopod
5	*M. latisulcatus* (Western King Prawn)	Pleopod

1.7.2 Generation of analytical sensitivity data

Analytical sensitivity panels consisted of 10-fold dilutions of WSSV-positive prawn tissue homogenates with known viral load (genome copies/µL). Originally ASe was to be assessed using a single WSSV strain, however, after ASp testing highlighted variable WSSV specificity for one test, ASe was assessed using 3 WSSV strains:

- WSSV/Australia/2016-Logan River
- WSSV/Vietnam/2017
- WSSV/China/2017

Stock homogenates were prepared for each WSSV strain, whereby WSSV-positive prawn tail muscle tissue was mechanically homogenised in chilled Phosphate NaCl (PBSA) at a rate of 100 mg tissue : 1 mL PBSA and passed through a fine mesh (0.5 mm) sieve. A WSSV-negative prawn tail muscle tissue homogenate was prepared in the same way for use as a diluent. Prawn tail muscle tissue was used in place of pleopod tissue due to the large amount of shell-free tissue required. WSSV-positive tail muscle tissue was successfully trialled on all test platforms prior to use, and a buffer comparison trial ensured PBSA did not adversely impact test performance.

A recombinant plasmid of known copy number containing the CSIRO WSSV qPCR target sequence was serially diluted 10-fold in negative homogenate. Plasmid dilutions were extracted and tested by CSIRO WSSV qPCR alongside aliquots of WSSV-positive stock homogenates (triplicate PCR reactions) on 2 separate occasions, with plasmid standard curves used to quantify WSSV viral load (mean genome copies/µL) in the WSSV-positive stock homogenates (Tables 6 and 7).

Table 6. Plasmid-spiked prawn tissue homogenate standards used for quantification of WSSV stock homogenates

Plasmid copies/µL in prawn tissue homogenate	Extraction 1	Extraction 2
	C_T value (mean ± SD) from 3 replicates	C_T value (mean ± SD) from 3 replicates
1×10^6	13.70 ± 0.12	13.59 ± 0.13
1×10^5	16.87 ± 0.06	16.63 ± 0.05
1×10^4	19.70 ± 0.08	19.75 ± 0.24
1×10^3	23.35 ± 0.16	23.44 ± 0.11
1×10^2	26.07 ± 0.14	26.79 ± 0.14
1×10^1	29.50 ± 0.19	29.52 ± 0.19
Efficiency	103.84 %	101.52 %

Table 7. Quantification of WSSV stock homogenates

Stock Homogenate	Extraction 1		Extraction 2		Mean WSSV genome copies/µL from 6 replicates
	C_T value (mean ± SD) from 3 replicates	WSSV genome copies/µL calc. from std. curve	C_T value (mean ± SD) from 3 replicates	WSSV genome copies/µL calc. from std. curve	
WSSV/Australia/2016-Logan River	12.74 ± 0.11	1.96×10^6	12.41 ± 0.04	2.16×10^6	2.06×10^6
WSSV/China/2017	19.89 ± 0.11	1.05×10^4	19.73 ± 0.13	1.21×10^4	1.14×10^4
WSSV/Vietnam/2017	17.18 ± 0.08	7.60×10^4	16.59 ± 0.03	11.12×10^4	9.36×10^4

Stocks were then serially diluted 10-fold in negative homogenate to form panel samples ranging from 10^5 to 10^{-2} genome copies/µL and dispensed into single use aliquots. Two sets of panel samples from each strain were quantified as above, confirming the viral load in each homogenate dilution.

The input volume of ASe panel sample used for each test was standardised at 100 µL. For the rapid antigen style tests each sample was tested in triplicate, on 2 separate occasions. For the PCR-based tests, each sample was extracted in singlicate and the nucleic acid extract tested in triplicate PCR reactions, on 2 separate occasions. To determine a robust and conservative limit of detection (LOD) for each test, the LOD was defined as the final dilution where all test replicates (n=6) were positive.

1.7.3 Generation of diagnostic specificity data

Pleopods from a panel of 100 confirmed WSSV-negative *P. monodon*, comprising 76 wild-caught prawns from the Northern Prawn Fishery and 24 farmed prawns, were used to assess DSp. For the rapid antigen style tests each sample was tested in singlicate, and for the PCR-based tests each sample was extracted in singlicate and the nucleic acid extract tested in duplicate PCR reactions.

1.7.4 Generation of diagnostic sensitivity data for clinically affected prawns

Pleopods from 140 clinically affected (moribund or dead) WSSV-positive *P. monodon*, experimentally infected with WSSV/Australia/2016-Logan River, were used to assess DSe. For the rapid antigen style tests each sample was tested in singlicate, and for the PCR-based tests each sample was extracted in singlicate and the nucleic acid extract tested in duplicate PCR reactions.

1.7.5 Generation of repeatability data

Repeatability panels consisted of strong positive and weak positive dilutions of WSSV-positive (WSSV/Australia/2016-Logan River) *P. monodon* tissue homogenate appropriate for the ASe of each test, and a WSSV-negative *P. monodon* tissue homogenate. The weak positive sample for a given test contained a WSSV viral load ≥ 10 times the limit of detection determined for that test during the ASe assessment. Panel material was dispensed into single use aliquots, and a homogeneity assessment was conducted to ensure consistency between aliquots of the same dilution. As a quality control measure, coefficients of variation were determined for each preparation and assessed against the acceptance criteria for PCR of < 5%*. Coefficients of variation ≤ 1% were observed for each preparation, demonstrating that the repeatability panel samples were satisfactorily homogeneous and appropriate for repeatability assessment.

* In accordance with ISO 13528 (annex B).

For each POC test and laboratory reference test, two operators tested triplicate aliquots of each panel sample on 6 separate occasions per operator, with each test reaction performed in duplicate, generating 72 data points per panel sample, per test platform.

1.7.6 Generation of applicability (diagnostic window) data

The ability of the tests to detect WSSV throughout the course of infection was assessed using pleopods from a panel of 70 *P. monodon*, experimentally exposed to WSSV via cohabitation with WSSV-infected prawns, and systematically collected from 0 to 168 hours post exposure, representing disease progression through healthy and mildly, moderately and severely clinically affected animals. Six prawns did not have pleopods available for testing due to cannibalism, so in these cases an equivalent amount of tail muscle tissue was used as a proven suitable alternative. For the rapid antigen style tests each sample was tested in singlicate, and for the PCR-based tests each sample was extracted in singlicate and the nucleic acid extract tested in duplicate PCR reactions.

1.7.7 Review of other studies

Test performance information described in other studies, for the selected POC tests, was reviewed and compared to the performance data generated in this study.

1.7.8 Consideration of operational characteristics

The operational characteristics of the tests, such as clarity of kit instructions, ease of use, testing timeframe and technical complexity were evaluated, along with cost, shelf-life, equipment requirements and customer support.

1.8 Statistical methods

1.8.1 Statistical methods for diagnostic specificity and sensitivity

The relative DSe and DSp of the POC tests compared to the laboratory reference qPCR tests were calculated for a set of known positive and known negative samples according to Table 8.

Table 8. Calculating diagnostic sensitivity and specificity

		Reference Test Results	
		+ve	-ve
POC Test Results	+ve	A	B
	-ve	C	D
	Total	A + C	B + D

A = true positives B = false positives
C = false negatives D = true negatives

POC test relative diagnostic sensitivity = A / (A + C)
POC test relative diagnostic specificity = D / (B + D)

As the DSe and DSp panel results of the two reference qPCR tests were 100% concordant with each other, the POC tests were compared relative to the CSIRO WSSV qPCR reference test. Indeterminate POC test results were recorded as such and the number of indeterminate results generated by each POC test are presented in the results summary. Indeterminate results were eliminated from the DSe and DSp calculations.

1.8.2 Statistical methods for repeatability

For each repeatability sample (strong positive, weak positive and negative), the proportion of sample replicates producing the expected result (positive or negative) was calculated for each test and for each operator. MedCalc software was used to compare proportions, using the "N-1" Chi-squared test as recommended by Campbell (2007) and Richardson (2011).

2. Results

The CDIA™ WSSV Rapid test could not be procured and was removed from the project evaluation in accordance with the project risk analysis. Test evaluation data was generated for the remaining 4 POC tests, however, Shrimple® was discontinued by the manufacturer partway through the evaluation, resulting in an incomplete data set for this test. The ShrimpCheck WSSV Rapid Test was found to be unable to detect WSSV/Australia/2016-Logan River. Therefore, DSe, repeatability and applicability data could not be generated for this test as the strain used to produce the material for these panels was WSSV/Australia/2016-Logan River. A summary of the performance evaluation components completed for each test are provided in Table 9.

Table 9. Summary of performance evaluation components completed for each test

Test Evaluation Panel	CSIRO and OIE WSSV qPCR Reference Tests	Shrimple®[1]	ShrimpCheck WSSV Rapid Test[2]	CDIA™ WSSV Rapid Test[3]	IQ Plus™ POCKIT™ iiPCR	AgriGen POND WSSV qPCR
Analytical Specificity (ASp): Exclusivity for heterologous prawn pathogens	✓	✗	✓	✗	✓	✓
Analytical Specificity (ASp): Exclusivity for host genome	✓	✓	✓	✗	✓	✓
Analytical Specificity (ASp): Inclusivity for WSSV strains	✓	✓	✓	✗	✓	✓
Analytical Sensitivity (ASe)	✓	✗	✓	✗	✓	✓
Relative Diagnostic Specificity (DSp)	✓	✓ Partial (76%)	✗	✗	✓	✓
Relative Diagnostic Sensitivity (DSe) for Clinically Affected Prawns	✓	✓ Partial (91%)	✗	✗	✓	✓
Repeatability	✓	✗	✗	✗	✓	✓
Applicability (diagnostic window)	✓	✗	✗	✗	✓	✓

[1] Shrimple® was discontinued by the manufacturer part-way through the study therefore exclusivity for heterologous prawn pathogens, ASe, repeatability and applicability could not be assessed and DSe and DSp assessments could only be partially completed.

[2] ShrimpCheck WSSV Rapid test was found to be unable to detect WSSV/Australia/2016-Logan River during the ASp assessment and therefore did not undergo assessment for diagnostic performance characteristics or repeatability due to these test panels being prepared from WSSV/Australia/2016-Logan River infected prawns.

[3] CDIA™ WSSV Rapid Test Kit was unable to be procured and therefore could not undergo performance evaluation.

With Shrimple® discontinued, the ShrimpCheck WSSV Rapid test unsuitable for detection of the 2016/2017 Logan River WSSV outbreak strain, and the CDIA™ WSSV Rapid test unable to be procured, an additional rapid antigen style test was sought for inclusion in the evaluation. Numerous possibilities were pursued, including tests described in recently published journal articles or publicly promoted on commercial websites. All efforts to obtain an additional test were unsuccessful, due to the technology not yet being commercially available, the commercial product being discontinued or the supplier/manufacturer not responding to repeated product enquiries.

Note: The CSIRO and OIE laboratory reference qPCRs and the AgriGen POND WSSV qPCR express results quantitatively in the form of C_T (threshold cycle) or C_q (quantity cycle) values, whereas the IQ Plus™ POCKIT™ iiPCR, Shrimple® and the ShrimpCheck WSSV Rapid test express results qualitatively as positive/negative.

2.1 Analytical specificity results

Results for the 3 panels used to assess ASp are summarised in Table 10, and further presented in sections 2.1.1 to 2.1.3.

Table 10. Analytical specificity results summary

Prawn Pathogen	CSIRO WSSV qPCR	OIE WSSV qPCR	AgriGen POND WSSV qPCR	IQ Plus™ POCKIT™ iiPCR	Shrimple®	ShrimpCheck WSSV Rapid Test
Yellow Head Virus 1 (YHV1)	ND	ND	ND	ND	nt	ND
Yellow Head Virus 7 (YHV7)	ND	ND	ND	ND	nt	ND
Gill Associated Virus (YHV2)	ND	ND	ND	ND	nt	ND
Vibrio parahaemolyticus (Vp_{AHPND})	ND	ND	ND	ND	nt	ND
Taura Syndrome Virus (TSV)	ND	ND	ND	ND	nt	ND
Infectious Hypodermal and Haematopoietic Necrosis Virus (IHHNV)	ND	ND	ND	ND	nt	ND
Hepatopancreatic Parvovirus (HPV)	ND	ND	ND	ND	nt	ND
WSSV Strain	**Mean C_T**			**Qualitative result**		
WSSV/Australia/2016-Logan River	12.94	14.66	16.98	+ve	+ve	ND
WSSV/Vietnam/2017	18.93	20.38	23.01	+ve	+ve*	+ve
WSSV/China/2017	14.90	16.45	19.88	+ve	+ve*	+ve
WSSV/Australia/2022-NSW Broodstock	15.79	17.46	17.80	+ve	+ve*	+ve
Prawn Species						
P. monodon (Giant Tiger Prawn)	ND	ND	ND	ND	ND	ND
P. esculentus (Brown Tiger Prawn)	ND	ND	ND	ND	ND	ND
P. merguiensis (Banana Prawn)	ND	ND	ND	ND	ND	ND
M. plebejus (Eastern King Prawn)	ND	ND	ND	ND	ND	ND
M. latisulcatus (Western King Prawn)	ND	ND	ND	ND	ND	ND

ND: no positives detected *positive result generated outside QC scope due to expired test nt: not tested

2.1.1 Analytical specificity panel 1: exclusivity

Exclusivity panel testing was completed for the ShrimpCheck WSSV Rapid test, the IQ Plus™ POCKIT™ test, the AgriGen POND test and the CSIRO and OIE WSSV laboratory reference qPCRs. Shrimple® and the CDIA™ WSSV Rapid test did not undergo exclusivity assessment, due to discontinued manufacture and procurement issues, respectively.

All evaluated tests returned negative results for all exclusivity panel samples (Table 9). There was no cross-reactivity to YHV1, YHV2 (GAV), YHV7, Vp_{AHPND}, TSV, IHHNV and HPV for any test, with all tests demonstrating 100% concordance with CSIRO and OIE WSSV laboratory reference qPCR results.

2.1.2 Analytical specificity panel 2: inclusivity

Inclusivity panel testing was completed for the ShrimpCheck WSSV Rapid test, the IQ Plus™ POCKIT™ test, the AgriGen POND test and the CSIRO and OIE WSSV laboratory reference qPCRs. Shrimple® and the CDIA™ WSSV Rapid test did not undergo inclusivity assessment, due to discontinued manufacture and procurement issues, respectively.

The IQ Plus™ POCKIT™ test, the AgriGen POND test and the CSIRO and OIE WSSV laboratory reference qPCRs returned positive results for all four WSSV strains assessed (WSSV/Australia/2016-Logan River, WSSV/Vietnam/2017, WSSV/China/2017 and WSSV/Australia/2022-NSW Broodstock). The ShrimpCheck WSSV Rapid test returned negative results for WSSV/Australia/2016-Logan River, and positive results for the remaining three WSSV strains, indicating this test has limited ASp for WSSV (Table 9).

Although Shrimple® did not undergo inclusivity assessment due to discontinued manufacture, initial Shrimple® trials during the project's pilot phase along with the DSe evaluation data confirm that Shrimple® is able to detect WSSV/Australia/2016-Logan River. Although Shrimple® testing of WSSV/China, WSSV/Vietnam and WSSV/Australia/2022-NSW Broodstock samples occurred outside the QC scope of this evaluation using expired Shrimple® kits, positive results were produced indicating that this test is able to detect these strains.

2.1.3 Analytical specificity panel 3: host genome

Host genome panel testing was completed for Shrimple®, the ShrimpCheck WSSV Rapid test, the IQ Plus™ POCKIT™ test, the AgriGen POND test and the CSIRO and OIE WSSV laboratory reference qPCRs. The CDIA™ WSSV Rapid test was not assessed as it could not be procured.

All evaluated tests returned negative results for all host genome panel samples, indicating no cross-reactivity to *P. monodon* (Giant Tiger Prawn), *P. esculentus* (Brown Tiger Prawn), *P. merguiensis* (Banana Prawn), *M. plebejus* (Eastern King Prawn) and *M. latisulcatus* (Western King Prawn) host genomes (Table 9). The IQ Plus™ POCKIT™ test returned negative internal control results for *M. latisulcatus* and *M. plebejus* samples. Consultation with the test manufacturer confirmed that the test's internal control assay may not be able to detect the genome of all prawn species and is unlikely to be able to amplify the target sequence in *M. latisulcatus* and *M. plebejus*. This does not affect the ability of the test to detect WSSV in these species.

2.2 Analytical sensitivity results

ASe panel testing was completed for the ShrimpCheck WSSV Rapid test, the IQ Plus™ POCKIT™ test, the AgriGen POND test and the CSIRO and OIE WSSV laboratory reference qPCRs. Shrimple® and the CDIA™ WSSV Rapid test did not undergo ASe assessment, due to discontinued manufacture and procurement issues, respectively. A comparison of the limit of detection of each test for three WSSV strains is presented in Tables 11, 12 and 13.

Note: The CSIRO and OIE laboratory reference qPCRs and the AgriGen POND WSSV qPCR express results quantitatively in the form of C_T (threshold cycle) or C_q (quantity cycle) values, whereas the IQ Plus™ POCKIT™ iiPCR, Shrimple® and the ShrimpCheck WSSV Rapid test express results qualitatively as positive/negative.

A 100 µL standardised volume of quantified, serially diluted prawn tissue homogenate was used as the sample input volume at the beginning of each test process, so that ASe could be comparatively assessed across all tests, covering the different sample processing and target detection methods unique to each test.

Table 11. Comparative analytical sensitivity of WSSV POC tests and WSSV laboratory reference qPCRs for WSSV/Australia/2016-Logan River

WSSV/Australia/2016-Logan River					
WSSV genome copies/µL in prawn tissue homogenate	CSIRO WSSV qPCR	OIE WSSV qPCR	AgriGen POND WSSV qPCR	IQ Plus™ POCKIT™ iiPCR	ShrimpCheck WSSV Rapid Test
	C_T value (mean ± SD), No. +ve/6	C_T value (mean ± SD), No. +ve/6	C_q value (mean ± SD), No. +ve/6	No. +ve/6	No. +ve/6
1×10^5	16.83 ± 0.13, 6/6	18.17 ± 0.10, 6/6	20.11 ± 0.03, 6/6	6/6	test unable to detect this WSSV strain
1×10^4	20.31 ± 0.06, 6/6	21.77 ± 0.11, 6/6	23.50 ± 0.10, 6/6	6/6	
1×10^3	23.52 ± 0.15, 6/6	24.90 ± 0.21, 6/6	27.20 ± 0.12, 6/6	6/6	
1×10^2	26.57 ± 0.17, 6/6	27.99 ± 0.25, 6/6	30.47 ± 0.22, 6/6	4/6	
1×10^1	30.76 ± 0.19, 6/6	31.92 ± 0.10, 6/6	34.21 ± 0.40, 6/6	ND	
1×10^0	33.77 ± 0.51, 6/6	35.11 ± 0.83, 6/6	36.15 ± 0.07, 2/6	ND	
1×10^{-1}	35.81 ± 0.70, 3/6	37.21 ± 0.77, 2/6	ND	ND	
1×10^{-2}	ND	ND	ND	ND	
Rank	1	1	2	3	4

ND: no positives detected
Blue highlight denotes LOD. LOD defined as the final dilution where all 6 test replicates are positive.

Table 12. Comparative analytical sensitivity of WSSV POC tests and WSSV laboratory reference qPCRs for WSSV/China/2017

WSSV/China/2017					
WSSV genome copies/µL in prawn tissue homogenate	CSIRO WSSV qPCR	OIE WSSV qPCR	AgriGen POND WSSV qPCR	IQ Plus™ POCKIT™ iiPCR	ShrimpCheck WSSV Rapid Test
	C_T value (mean ± SD), No. +ve/6	C_T value (mean ± SD), No. +ve/6	C_q value (mean ± SD), No. +ve/6	No. +ve/6	No. +ve/6
1×10^4	20.02 ± 0.10, 6/6	21.34 ± 0.11, 6/6	22.87 ± 0.11, 6/6	6/6	6/6
1×10^3	23.26 ± 0.08, 6/6	24.57 ± 0.05, 6/6	26.73 ± 0.16, 6/6	6/6	2/6
1×10^2	26.84 ± 0.05, 6/6	28.12 ± 0.22, 6/6	30.25 ± 0.22, 6/6	4/6	ND
1×10^1	29.99 ± 0.33, 6/6	31.15 ± 0.38, 6/6	34.04 ± 0.72, 6/6	ND	ND
1×10^0	33.40 ± 0.57, 6/6	34.22 ± 0.52, 6/6	36.18 ± 1.05, 2/6	ND	ND
1×10^{-1}	36.48 ± 0.14, 3/6	37.27 ± 1.02, 4/6	ND	ND	ND
1×10^{-2}	ND	ND	ND	ND	ND
Rank	1	1	2	3	4

ND: no positives detected
Blue highlight denotes LOD. LOD defined as the final dilution where all 6 test replicates are positive.

Table 13. Comparative analytical sensitivity of WSSV POC tests and WSSV laboratory reference qPCRs for WSSV/Vietnam/2017

WSSV/Vietnam/2017					
WSSV genome copies/µL of prawn tissue homogenate	CSIRO WSSV qPCR	OIE WSSV qPCR	AgriGen POND WSSV qPCR	IQ Plus™ POCKIT™ iiPCR	ShrimpCheck WSSV Rapid Test
	C_T value (mean ± SD), No. +ve/6	C_T value (mean ± SD), No. +ve/6	C_q value (mean ± SD), No. +ve/6	No. +ve/6	No. +ve/6
1 x 10^4	20.20 ± 0.12, 6/6	21.58 ± 0.11 6/6	23.20 ± 0.37, 6/6	6/6	6/6
1 x 10^3	22.45 ± 0.19, 6/6	23.90 ± 0.21 6/6	27.12 ± 0.63, 6/6	6/6	ND
1 x 10^2	26.67 ± 0.21, 6/6	28.00 ± 0.11 6/6	30.75 ± 0.69, 6/6	4/6	ND
1 x 10^1	30.18 ± 0.21, 6/6	31.43 ± 0.19 6/6	34.94 ± 0.56, 6/6	1/6	ND
1 x 10^0	33.96 ± 1.00, 6/6	35.85 ± 0.80 6/6	36.95 n/a, 1/6	ND	ND
1 x 10^{-1}	36.34 ± 0.08, 2/6	37.79 ± 0.16 4/6	ND	ND	ND
1 x 10^{-2}	ND	ND	ND	ND	ND
Rank	**1**	**1**	**2**	**3**	**4**

ND: no positives detected
Blue highlight denotes LOD. LOD defined as the final dilution where all 6 test replicates are positive.

The CSIRO and OIE WSSV laboratory reference qPCRs were the most sensitive tests for all three WSSV strains (1 x 10^0 genome copies/µL homogenate) followed by the AgriGen POND WSSV qPCR (1 x 10^1 genome copies/µL homogenate), the IQ Plus™ POCKIT™ iiPCR (1 x 10^3 genome copies/µL homogenate) and the ShrimpCheck WSSV Rapid test (1 x 10^4 genome copies/µL homogenate). The ASe of each test was the same for each WSSV strain, except for the ShrimpCheck WSSV Rapid test which was unable to detect WSSV/Australia/2016-Logan River.

2.3 Diagnostic sensitivity (clinically affected prawns) and specificity results

Diagnostic sensitivity and specificity testing was completed for the IQ Plus™ POCKIT™ test, the AgriGen POND test and the CSIRO and OIE WSSV laboratory reference qPCRs. Testing was only partially completed for Shrimple® (91% and 76% completed for DSe and DSp respectively) due to discontinuation of test manufacture part way through panel testing. The ShrimpCheck WSSV Rapid test could not be assessed as it did not detect WSSV/Australia/2016-Logan River; the strain used to prepare the DSe panel material. The CDIA™ WSSV Rapid test was not assessed as it could not be procured.

Relative DSe and DSp results are presented in Tables 14 and 15. All assessed POC tests demonstrated 100% relative DSp with no false positive detections observed. The IQ Plus™ POCKIT™ test demonstrated 100% relative DSe, performing as well as the CSIRO and OIE WSSV laboratory reference qPCRs in detecting WSSV in clinically affected (moribund/dead) experimentally infected prawns. The AgriGen POND test had the lowest relative DSe at 81.68%, and was the only test to generate indeterminate results (n=9, 3.75%). For Shrimple® DSe (98.44%) and DSp (100%) had to be determined using a slightly reduced number of samples, as there were not enough tests available to test all 240 panel samples due to product discontinuation. The DSe and DSp results for this test should therefore be interpreted in this context.

Table 14. Relative diagnostic sensitivity (experimentally infected clinically affected prawns) and specificity results for WSSV POC tests and WSSV laboratory reference qPCRs

14A.

		CSIRO WSSV qPCR		
		+ve	-ve	Total
AgriGen POND WSSV qPCR	+ve	107	0	107
	-ve	24	100	124
	Total	131	100	231
	[1]Indet.	9	0	Grand Total 240

Relative DSe	81.68%
Relative DSp	100.00%
Proportion Indeterminate	3.75%

14B.

		CSIRO WSSV qPCR		
		+ve	-ve	Total
IQ Plus™ POCKIT™ iiPCR	+ve	140	0	140
	-ve	0	100	100
	Total	140	100	240
	[1]Indet.	0	0	Grand Total 240

Relative DSe	100.00%
Relative DSp	100.00%
Proportion Indeterminate	0.00%

14C.

		CSIRO WSSV qPCR		
		+ve	-ve	Total
Shrimple®	+ve	126	0	126
	-ve	2	76	78
	Total	128	76	204
	[1]Indet.	0	0	[2]Grand Total 204

Relative DSe	98.44%
Relative DSp	100.00%
Proportion Indeterminate	0.00%

14D.

		CSIRO WSSV qPCR		
		+ve	-ve	Total
OIE WSSV qPCR	+ve	140	0	140
	-ve	0	100	100
	Total	140	100	240
	[1]Indet.	0	0	Grand Total 240

Relative DSe	100.00%
Relative DSp	100.00%
Proportion Indeterminate	0.00%

[1] Test results classified as indeterminate

[2] Grand total of DSe and DSp panel samples tested by Shrimple® was reduced from 240 to 204 as there were not enough tests available to test all 240 panel samples due to product discontinuation

The CSIRO WSSV qPCR C_T values of the 2 positive samples not detected by Shrimple® were 17.67 and 16.21, at the upper end of the panel range (CSIRO WSSV qPCR mean C_T values of DSe panel samples ranged from 10.54 to 18.80). The CSIRO WSSV qPCR C_T values of the 33 samples testing negative or indeterminate by the AgriGen POND test ranged from 11.89 to 18.77.

Table 15. Comparison of relative diagnostic sensitivity (experimentally infected clinically affected prawns) and specificity of WSSV POC tests and WSSV laboratory reference qPCRs

Test	Relative DSe (95% CI)	Relative DSp (95% CI)	Rank
CSIRO WSSV qPCR	100.00% (97.40% - 100.00%)	100.00% (96.38% - 100.00%)	1
OIE WSSV qPCR	100.00% (97.40% - 100.00%)	100.00% (96.38% - 100.00%)	1
IQ Plus™ POCKIT™ iiPCR	100.00% (97.40% - 100.00%)	100.00% (96.38% - 100.00%)	1
Shrimple®	98.44% (94.47% - 99.81%)	100.00% (95.26% - 100.00%)	2
AgriGen POND WSSV qPCR	81.68% (73.98% - 87.89%)	100.00% (96.38% - 100.00%)	3

2.4 Repeatability results

Repeatability panel testing was completed for the IQ Plus™ POCKIT™ test, the AgriGen POND test and the CSIRO and OIE WSSV laboratory reference qPCRs. The Shrimple®, CDIA™ WSSV Rapid test and ShrimpCheck WSSV Rapid test did not undergo repeatability assessment, due to discontinued manufacture, procurement issues, and specificity issues respectively.

To account for the difference in ASe between the different tests, strong and weak positive samples were specifically prepared appropriate for each test, with the weak positive sample for a given test being ≥ 10 times the limit of detection. For each sample (strong positive, weak positive and negative) the proportion of test replicates producing expected test results is presented in Table 16.

Table 16. Proportion of repeatability sample replicates producing expected results

Test	Sample	Proportion of test replicates with expected results (%)			Rank
		Operator 1	Operator 2	Combined	
CSIRO WSSV qPCR	Strong Positive	100.00%	100.00%	100.00%	1
	Weak Positive	100.00%	100.00%	100.00%	
	Negative	100.00%	100.00%	100.00%	
	Combined	100.00%	100.00%	100.00%	
OIE WSSV qPCR	Strong Positive	100.00%	100.00%	100.00%	1
	Weak Positive	100.00%	100.00%	100.00%	
	Negative	100.00%	100.00%	100.00%	
	Combined	100.00%	100.00%	100.00%	
AgriGen POND WSSV qPCR	Strong Positive	100.00%	100.00%	100.00%	2
	Weak Positive	88.89%	66.67%	77.78%	
	Negative	100.00%	100.00%	100.00%	
	Combined	96.30%	88.88%	92.59%	
IQ Plus™ POCKIT™ iiPCR	Strong Positive	100.00%	100.00%	100.00%	3
	Weak Positive	66.67%	44.44%	55.56%	
	Negative	100.00%	100.00%	100.00%	
	Combined	88.89%	81.48%	85.19%	

For the strong positive and negative samples, 100% concordance was demonstrated for the IQ Plus™ POCKIT™ test, the AgriGen POND test and the CSIRO and OIE reference qPCRs, with all sample replicates (n=36 per test, per sample) returning the expected (positive or negative) test result in all duplicate test reactions (n=72 per test, per sample) in a total of 12 test runs per test, conducted by 2 different operators on different days.

Greater variability was observed for weak positive sample replicates for the AgriGen POND test and the IQ Plus™ POCKIT™ test for both operators, with only 77.78% and 55.56% of weak positive sample replicates testing positive for the respective tests. The difference in the proportion of positive detections between operators for weak positive samples was not significant for either test (p-values > 0.05). In contrast, 100% of weak positive sample replicates tested with the CSIRO and OIE qPCR reference tests produced expected results for both operators.

2.5 Applicability (diagnostic window) results

Applicability panel testing was completed for the IQ Plus™ POCKIT™ test, the AgriGen POND test and the CSIRO and OIE WSSV laboratory reference qPCRs. The Shrimple®, CDIA™ WSSV Rapid test and ShrimpCheck WSSV Rapid test did not undergo applicability assessment, due to discontinued manufacture, procurement issues, and specificity issues respectively. The number of cohabitated prawns collected each day post exposure, their clinical status, reference WSSV qPCR results and POC test results are summarised in Table 17.

Table 17. Applicability (diagnostic window) panel composition and results summary

Day post exposure	No. collected	Proportion of collected with no (n) mild (mi) moderate (mo) or severe (s) clinical signs or found dead (d)	CSIRO WSSV qPCR			OIE WSSV qPCR			AgriGen POND WSSV qPCR			IQ Plus™ POCKIT™ iiPCR % +ve
			% +ve	C_T range	Median C_T	% +ve	C_T range	Median C_T	% +ve	C_q range	Median C_q	
0 (0 hrs)	10 (baseline)	100.00% n	0.00%	n/a	n/a	0.00%	n/a	n/a	0.00%	n/a	n/a	0.00%
1 (24-47 hrs)	6	16.67% mi, 83.33% d	16.67%	35.89	35.89	16.67%	37.07	37.07	0.00%	n/a	n/a	0.00%
2 (48-71 hrs)	7	85.71% mi, 14.29% d	100.00%	16.99-30.28	20.94	100.00%	18.94-32.00	22.51	100.00%	19.23-35.73	31.30	42.86%
3 (72-95 hrs)	11	27.27% mi, 9.09% s 63.64% d	100.00%	11.81-15.68	12.69	100.00%	13.74-17.29	14.34	90.91%	14.98-20.81	15.94	100.00%
4 (96-119 hrs)	16	43.75% mo, 31.25% s, 25.00% d	100.00%	10.99-21.67	12.22	100.00%	12.12-23.48	13.88	100.00%	14.10-22.68	15.07	100.00%
5 (120-143hrs)	12	8.33% mo, 41.67% s, 50.00% d	100.00%	11.64-13.90	12.23	100.00%	13.11-15.68	13.86	91.67%	13.89-32.64	14.94	100.00%
6 (144-167 hrs)	6	83.33% mo, 16.67% s	100.00%	11.36-27.38	12.03	100.00%	12.92-28.97	13.53	100.00%	13.61-30.86	14.28	100.00%
7 (168 hrs)	2	100.00% mo	100.00%	30.61-31.98	31.16	100.00%	28.82-33.53	30.92	50.00%	30.99-37.19	34.02	0.00%
Total	70											

The first WSSV-positive detections occurred via CSIRO and OIE WSSV laboratory reference qPCRs in prawns collected 24 hrs post exposure, with 16.67% testing positive. In all subsequent collections, 100% of prawns tested positive by laboratory reference PCR, with median C_T values decreasing over time as the proportion of moderately and severely clinically affected specimens increased. For the AgriGen POND test, the first WSSV-positive detections occurred in prawns collected 48 hrs post exposure, with 90.91-100% of prawns collected on days 2 to 6 testing positive. The IQ Plus™ POCKIT™ test also first detected WSSV in prawns at 48 hrs post exposure although a lower proportion (42.86%) tested positive. One hundred percent of prawns collected between days 3 and 6 tested positive using the IQ Plus™ POCKIT™ test. Deaths occurring between 0 and 24 hrs were likely due to cannibalisation and natural attrition rather than WSD, as these prawns did not test WSSV-positive by reference qPCR or POC test.

The CSIRO WSSV qPCR C_T values of the WSSV-positive applicability panel samples ranged from 10.99 to 35.89. C_T value distributions are summarised in Table 18.

Table 18. CSIRO WSSV qPCR C_T value distributions of applicability (diagnostic window) panel samples testing positive for WSSV

CSIRO WSSV qPCR C_T range	No. collected	Time of collection (day post exposure)	Proportion of collected with no (n) mild (mi) moderate (mo) or severe (s) clinical signs or found dead (d)	OIE WSSV qPCR % +ve	AgriGen POND WSSV qPCR % +ve	IQ Plus™ POCKIT™ iiPCR % +ve
10-15	40	Days 3, 4, 5 & 6	7.50% mi, 22.50% mo, 27.50% s, 42.5% d	100.00%	95.00%	100.00%
15-20	4	Days 2, 3 & 4	50.00% mi, 25.00% mo, 25.00% d	100.00%	100.00%	100.00%
20-25	4	Days 2, 4 & 6	25.00% mi, 50.00% mo, 25.00% d	100.00%	100.00%	75.00%
25-30	3	Days 2 & 6	66.67% mi, 33.33% mo	100.00%	100.00%	33.33%
30-35	4	Days 1, 2 & 7	50.00% mi, 50.00% mo	100.00%	50.00%	0.00%
35-45	0	n/a	n/a	n/a	n/a	n/a
Total	55			Overall	92.70%	87.27%

Overall, the AgriGen POND test identified a higher proportion (92.70%) of reference WSSV qPCR positive prawns than the IQ Plus™ POCKIT™ test (87.27%). Of the 55 samples testing positive by laboratory reference qPCR, 3 tested negative and 1 tested indeterminate with the AgriGen POND test. The CSIRO WSSV qPCR C_T values of positive samples undetected by the AgriGen POND test ranged from 11.81 to 35.89. For the IQ Plus™ POCKIT™ test, 6 out of the 55 reference qPCR positive samples tested negative and 1 tested indeterminate, with all undetected positive samples having CSIRO WSSV qPCR C_T values > 20. Of the 11 applicability panel prawns with CSIRO WSSV qPCR C_T values > 20, only 5 (45%) tested positive in the IQ Plus™ POCKIT™ test. These 5 IQ Plus positive samples had CSIRO WSSV qPCR C_T values < 30. The IQ plus POCKIT™ test did not detect WSSV in any samples with CSIRO WSSV qPCR C_T values > 30.

2.6 Literature review of other studies

Test performance information described in other studies, as well as this study, is summarised for each of the WSSV POC tests in Table 19.

Table 19. Summary of POC test performance information described in other studies and this study

Test	Reference	Analytical specificity	Analytical sensitivity	Diagnostic specificity	Diagnostic sensitivity	Repeatability	Applicability (diagnostic window)
Shrimple®	Powell et al., 2006.	No cross-reactivity to *L. vannamei.* No exclusivity data presented for other prawn pathogens. Source/strain of WSSV used for inoculum not specified.	Results classified as faint positive were determined to contain 36 to 1784 viral copies/ng genomic DNA, or an average of 613.65 ± 551.42.	No false positives in negative control cohort.	Relative DSe in WSSV qPCR positive experimentally infected (injected) *L. vannamei* was 0% for 1-8 hrs p.i., 5.3% at 12 hrs p.i. increasing to 100% by 24 hrs p.i.	No information	Relative Se was 34.7% in WSSV qPCR positive experimentally infected (injected) *L. vannamei* sampled from 1 to 32 hrs p.i., prior to development of gross anatomical signs of disease.
	Takahashi et al., 2003.	Tests were reportedly used successfully on *M. japonicus, P. monodon* and *F. chinensis.* No exclusivity data presented for other prawn pathogens. Test detected WSSV circulating in farmed *P. monodon* in Thailand in 2003.	Sensitivity comparable to first-step WSSV conventional PCR and < sensitive (~50%) than second-step WSSV conventional PCR.	No information	Relative DSe in WSSV 2-step conventional PCR positive *M. japonicus* was 57.1% in live specimens and 100% in dead specimens. For *P. monodon* DSe was 96% in infected live specimens.	No information	Test displayed reduced sensitivity for WSSV-infected live shrimp (57.1% to 96%) compared to WSSV-infected dead shrimp (100%).
	Wangman et al., 2017.	No cross-reactivity to *L. vannamei.* No cross-reactivity to YHV, TSV or IHHNV. Source of WSSV was *P. vannamei* obtained from Khan province, Thailand (year not specified).	Test had 400 times lower sensitivity than an alternative rapid antigen test (ICP11 strip test since commercialised as ShrimpCheck WSSV Rapid Test). Limit of detection was not quantified.	No information	Article refers to the information provided in Powell et al, 2006.	No information	Article refers to the information provided in Powell et al, 2006.
	Shrimple® product brochure, date unspecified	No information	Reported to be able to detect 356 viral copies/ng genomic DNA, but no data presented.	96% accuracy reported but no data presented		No information	Reported to be able to detect WSSV at early infection stages, but no data provided.
	This study	No cross-reactivity to *P. monodon, P. esculentus, P. merguiensis, M. plebejus* and *M. latisulcatus.* Cross-reactivity to other prawn pathogens could not be assessed due to test manufacture discontinuation. Able to detect WSSV/Australia/2016-Logan River, WSSV/Vietnam/2017, WSSV/China/2017 and WSSV/Australia/2022-NSW Broodstock.	Could not be assessed due to test manufacture discontinuation.	100% relative DSp in WSSV qPCR negative wild caught and farmed *P. monodon.*	98.44% relative DSe in WSSV qPCR positive, clinically affected (moribund/dead), experimentally infected (cohabitated) *P. monodon.*	Could not be assessed due to test manufacture discontinuation.	Could not be assessed due to test manufacture discontinuation.

Table 19 continued. Summary of POC test performance information described in other studies and this study

Test	Reference	Analytical specificity	Analytical sensitivity	Diagnostic specificity	Diagnostic sensitivity	Repeatability	Applicability (diagnostic window)
ShrimpCheck WSSV Rapid Test	Speedy Assay validation report for ShrimpCheck Rapid series. Date unknown.	No cross-reactivity to shrimp host genome, however, the species identities of the shrimp used are not specified. No cross-reactivity to TSV, MBV, or YHV. Source/strain of WSSV used for evaluation not specified.	Limit of detection for WSSV was 2.0% (v/v).	100% specificity, 100% sensitivity, 100% PPV, 100% NPV, 100% test efficiency.		Reportedly all sample replicates produced the same results (100 % repeatability) and there was no difference in results generated by two different analysts (no data provided).	No information.
	Wangman et al., 2017	No cross-reactivity to *L. vannamei*. No cross-reactivity to YHV, TSV or IHHNV. Source/strain of WSSV used for inoculum not specified.	Test had 400 times higher sensitivity than an alternative rapid antigen test (Shrimple®) and 50 times lower sensitivity than one-step conventional PCR. Limit of detection approx. 5×10^4 copies of WSSV DNA.	No information	In experimentally infected (injected) *P. vannamei* test could detect one-step conventional PCR positive specimens from 12 hrs p.i. (37 %) and could detect WSSV in 100 % of specimens from 18 hrs p.i.	No information	In experimentally infected (injected) *P. vannamei* WSSV could be detected as early as 12 hrs p.i. and reliably detected from 18 hrs p.i. No information provided regarding clinical signs.
	This study	No cross-reactivity to *P. monodon, P. esculentus, P. merguiensis*, *M. plebejus* and *M. latisulcatus*. No cross-reactivity to YHV1, YHV7, YHV2, VpAHPND, TSV, IHHNV and HPV. Unable to detect WSSV/Australia/2016-Logan River. Able to detect WSSV/Vietnam/2017, WSSV/China/2017 and WSSV/Australia/2022-NSW Broodstock.	Conservative LOD (100% positive detection rate) is 1 x 10^4 WSSV genome copies/µL for a 100µL homogenised sample input. ASe was 10-fold < the IQ Plus™ POCKIT™ WSSV iiPCR, 1000-fold < the AgriGen POND WSSV qPCR and 10,000-fold < the CSIRO and OIE laboratory reference qPCRs, for the WSSV strains able to be detected.	100% relative DSp in WSSV qPCR negative wild caught and farmed *P. monodon*.	Could not be assessed due to the test being unable to detect the strain of WSSV used to prepare the DSe panel (WSSV/Australia/2016-Logan River).	Could not be assessed due to the test being unable to detect the strain of WSSV used to prepare the repeatability panel (WSSV/Australia/2016-Logan River).	Could not be assessed due to the test being unable to detect the strain of WSSV used to prepare the applicability panel (WSSV/Australia/2016-Logan River).
AgriGen POND WSSV qPCR	Other studies	No information.	No information.	No information.	No information.	No information.	No information.
	This study	No cross-reactivity to *P. monodon, P. esculentus, P. merguiensis*, *M. plebejus* and *M. latisulcatus*. No cross-reactivity to YHV1, YHV7, YHV2, VpAHPND, TSV, IHHNV and HPV. Able to detect WSSV/Australia/2016-Logan River, WSSV/Vietnam/2017, WSSV/China/2017 and WSSV/Australia/2022-NSW Broodstock.	Conservative LOD (100% positive detection rate) is 1 x 10^1 WSSV genome copies/µL for a 100µL homogenised sample input. ASe was 100-fold > the IQ Plus™ POCKIT™ WSSV iiPCR, 1000-fold > the ShrimpCheck Rapid test and 10-fold < the CSIRO and OIE laboratory reference qPCRs, for all WSSV strains assessed.	100% relative DSp in WSSV qPCR negative wild caught and farmed *P. monodon*.	81.68% relative DSe in WSSV qPCR positive, clinically affected (moribund/dead), experimentally infected (cohabitated) *P. monodon*.	100% concordance of test results for strong positive and negative sample replicates within runs, between runs, and between operators. Diminished repeatability was observed for weak positive sample replicates, with only 78% of replicates testing positive.	First WSSV-positive detections occurred at 48 hrs post exposure in experimentally infected (cohabitated) *P. monodon*. Overall proportion of true positives detected from 0 to 7 days post exposure was 92.70%.

Table 19 continued. Comparison of POC test performance information described in other studies and this study

Test	Reference	Analytical specificity	Analytical sensitivity	Diagnostic specificity	Diagnostic sensitivity	Repeatability	Applicability (diagnostic window)
IQ Plus™ POCKIT™ WSSV iiPCR	Tsai et al., 2014	No cross-reactivity to *L. vannamei*. No cross-reactivity to IHHNV, MBV, or HPV. Source of WSSV used for experimental infection was an isolate obtained in 1994 from naturally infected *P. monodon* in Taiwan.	Lowest dilution generating 100% positive signals contained 1 x 10^2 WSSV genome copies per reaction. LOD (95% positive detection rate) was 17 WSSV genome copies per reaction (95% CI: 13-24 copies).	Relative DSp in IQ2000™ WSSV DPS assay negative reference specimens (specimen details not provided) was 97% (95% CI: 94.31-98.50%).	Relative DSe in IQ2000™ WSSV DPS assay positive reference specimens (specimen details not provided) was 93.5% (95% CI: 90.61-95.56%).	100% intra and inter assay repeatability. 100% agreement for 2 operators. Inter-laboratory reproducibility demonstrated little heterogeneity (x^2 =0.54 and P= 0.76, Chi-square test; P =0.81, Fisher's exact test). No significant difference between lots or operators.	Reportedly able to detect WSSV in the early stages of infection based on sensitivity outcomes.
	IQ Plus™ WSSV Kit with POCKIT™ System User Manual, GeneReach Biotechnology Corporation 2014/11.	No cross-reactivity to *L. vannamei*. No cross-reactivity to IHHNV or HPV. Source/strain of WSSV used for specificity testing not specified.	Detection limit was up to 10 copies per reaction. Sensitivity was 20 ng genomic nucleic acid per reaction. 95% detection rate was 16.9 WSSV DNA copies per reaction. Serially diluted DNA from WSSV-infected *L. vannamei* showed that the detection endpoint (10^4 dilution) was similar to that of IQ2000™ WSSV DPS assay.	As per Tsai et al, 2014		100% agreement.	Certified by the WOAH as fit for: 1. to certify freedom from infection in individual animals or products for trade/movement purposes; 2. to confirm diagnosis of suspect or clinical cases (confirmation of a diagnosis by histopathology or clinical signs) and 3. to estimate prevalence of infection to facilitate risk analysis (surveys/herd health schemes/disease control).
	This study	No cross-reactivity to *P. monodon, P. esculentus, P. merguiensis*, *M. plebejus* and *M. latisulcatus*. No cross-reactivity to YHV1, YHV7, YHV2, VpAHPND, TSV, IHHNV and HPV. Able to detect WSSV/Australia/2016-Logan River, WSSV/Vietnam/2017, WSSV/China/2017 and WSSV/Australia/2022-NSW Broodstock.	Conservative LOD (100% positive detection rate) is 1 x 10^3 WSSV genome copies/µL for a 100µL homogenised sample input. ASe was 10-fold > the ShrimpCheck WSSV Rapid test, 100-fold < the AgriGen POND WSSV qPCR and 1000-fold < the CSIRO and OIE laboratory reference qPCRs, for all WSSV strains assessed.	100% relative DSp in WSSV qPCR negative wild caught and farmed *P. monodon*.	100% relative DSe in WSSV qPCR positive, clinically affected (moribund/dead), experimentally infected (cohabitated) *P. monodon*.	100% concordance of test results for strong positive and negative sample replicates within runs, between runs, and between operators. Diminished repeatability was observed for weak positive sample replicates, with only 56% of replicates testing positive.	First WSSV-positive detections occurred at 48 hrs post exposure in experimentally infected (cohabitated) *P. monodon*. Overall proportion of true positives detected from 0 to 7 days post exposure was 87.27%.
CDIA™ WSSV Rapid Test	Creative Diagnostics® validation report. Date unknown.	No cross-reactivity to shrimp host genome, however, the species identities of the shrimp used are not specified. No cross-reactivity to TSV, MBV, or YHV. Source/strain of WSSV used for evaluation not specified.	Limit of detection for WSSV was 2.0% (v/v).	100% specificity, 100% sensitivity, 100% PPV, 100% NPV, 100% test efficiency.		Reportedly all sample replicates produced the same results (100 % repeatability) and there was no difference in results generated by two different analysts (no data provided).	No information.
	This study	Could not be assessed as the test could not be procured.	Could not be assessed as the test could not be procured.	Could not be assessed as the test could not be procured.		Could not be assessed as the test could not be procured.	Could not be assessed as the test could not be procured.

2.7 Operational characteristics

Operational characteristics were assessed for Shrimple®, the ShrimpCheck WSSV Rapid test, the IQ PlusTM POCKIT™ test and the AgriGen POND test (Table 20). Operational characteristics were not assessed for the CDIA™ WSSV Rapid test as the test could not be procured.

Table 20. Operational characteristics of the evaluated POC tests

Test	Shrimple®	ShrimpCheck WSSV Rapid Test	IQ Plus™ WSSV Kit with POCKIT™ System	AgriGen POND on-site detection system for WSSV
Test type	Immunochromatographic lateral flow rapid antigen test	Immunochromatographic lateral flow rapid antigen test	Isothermal induction PCR (iiPCR)	Real-time PCR
Clarity of kit instructions	Printed instructions were clear and contained all necessary information	Printed instructions were clear and contained all necessary information	Printed instructions were clear and contained all necessary information	Printed instructions were clear but lacked some critically important information such as: required extraction buffer to tissue ratio, requirement to squeeze pleopod tissue out of the shell prior to adding the shell-free tissue to the extraction buffer, how to analyse and interpret test results including C_q cut-offs for positive results, expected values of positive controls etc. The information not included in written instructions was provided verbally during on-site training.
Ease of conducting the test	Low technical complexity	Low technical complexity	Moderate technical complexity	Moderate technical complexity
Ease of result interpretation	Low technical complexity	Low technical complexity	Low technical complexity	Moderate technical complexity
Approximate time from sampling to result	20 minutes	20 minutes	2 hours	2 hours
Maximum number of samples per run	1	1	8 including controls	48 including controls
Extra equipment / consumables required; not provided	No - kit contained everything required	No - kit contained everything required	Yes - extra equipment/consumables required but not provided included powder free gloves and 95% ethanol.	Yes - extra equipment/consumables required but not provided included a computer, pipettes and pipette tips, 2 mL tubes (RNAse/DNAse free) and tube racks, microfuge and powder free gloves.
Equipment maintenance / calibration required	No	No	The POCKIT analyser automatically performs a calibration run on start-up.	No

Table 20 continued. Operational characteristics of the evaluated POC tests

Test	Shrimple®	ShrimpCheck WSSV Rapid Test	IQ Plus™ WSSV Kit with POCKIT™ System	AgriGen POND on-site detection system for WSSV
Test contains quality controls	Yes - internal control embedded in test strip	Yes - internal control embedded in test strip	Positive control provided. Internal control assay (targeting prawn genome) included, however does not react to all prawn species (e.g. *M. latisulcatus and M. plebejus*).	Positive and negative control provided. Test does not include an internal control (e.g. housekeeping gene assay).
Cost* (AUD) *Costs are those paid at the time of purchase, from the chosen suppliers, and do not include shipping costs.	$11.33 per test	$14.60 per test	$14.10 total per test (nucleic acid extraction kit: $2.98 per extraction, WSSV test kit and R-tubes: $11.12 per reaction) plus equipment costs. Equipment: POCKIT COMBO $18,508.21.[1]	$19.20 total per test (Extraction reagent: $9.50 per extraction, WSSV reaction mix: $9.50 per reaction, reaction tubes: $0.20 per tube) plus equipment and training costs. Equipment: Mic 4-channel thermal cycler $17,500. Onsite training and installation: $1,000.00
Shelf life	2 years from date of manufacture	2 years from date of manufacture	3 years from date of manufacture	1 year from date of manufacture
Storage and operational temperature requirements	Room temperature storage and operation, away from direct sunlight, moisture and heat.	Room temperature storage (15-30°C) and operation, away from direct sunlight, moisture and heat.	Room temperature and 4°C storage. Extraction solution 1 may precipitate in cool temperatures and require warming prior to use. POCKIT analyser operational range is 15-35°C.	Room temperature and -20°C storage. Mic cycler operational range is 18-35°C.
Packaging	All components well packaged and clearly labelled. Test instructions provided with each set of test kits.	All components well packaged and clearly labelled. Test instructions provided with each set of test kits.	All components well packaged and clearly labelled. Test instructions provided with each set of test kits. Equipment packaged in a tough case for secure transport into the field. Equipment manuals provided.	All components well packaged and clearly labelled. Test instructions provided once, during initial training. Equipment manual provided.
Australian supplier available	No. Prior to discontinuation, tests were available via an Australian supplier.	Yes – however, purchaser needs to arrange their own import permit and customs paperwork.	No	Yes
Customer support	Satisfactory	Satisfactory	Satisfactory	Satisfactory
Additional comments	Nil	Nil	The POCKIT analyser used in this study was discontinued by the manufacturer at the time of writing this report. The manufacturer recommends the POCKIT™ Micro Duo as a suitable alternative for use with the IQ Plus WSSV kit.	AgriGen recommended the inclusion of a positive extraction control as their test does not include an internal control assay. Use of a positive extraction control on-farm is impractical and not viable due to the exotic nature of the pathogen.

[1] The version of the POCKIT™ COMBO purchased for this evaluation contained a mini automated extraction robot, which was not used due to a manual column-based extraction kit being supplied with the system. A cheaper version of the POCKIT™ COMBO that does not contain the automated extraction robot was available prior to discontinuation of the POCKIT™ analyser.

3. Discussion

3.1 Analytical specificity

No POC tests cross-reacted to heterologous prawn pathogens (n=7 pathogens) or prawn host genome (n=5 prawn species), showing 100% concordance with CSIRO and OIE laboratory reference qPCRs. Specificity to WSSV strains (n=4) from different geographical regions was 100% for all but one POC test. The ShrimpCheck WSSV Rapid test was unable to detect WSSV/Australia/2016-Logan River; the strain of WSSV responsible for the 2016/2017 WSSV outbreak in south-east Queensland. This test targets the highly expressed ICP11 non-structural protein of WSSV. To investigate the possible cause of the variable specificity observed for this test, the ICP11 coding regions of the 4 WSSV strains were compared (Figure 5).

The only difference was a single nucleotide polymorphism (SNP) observed in the WSSV/Australia/2016-Logan River ICP11 nucleotide sequence, which changes the corresponding amino acid (AA) to a stop codon, resulting in a truncated protein. A truncated ICP11 protein may be the reason that the ShrimpCheck WSSV Rapid test is unable to detect the 2016 Logan River strain of WSSV, as the truncation may affect antibody/antigen binding.

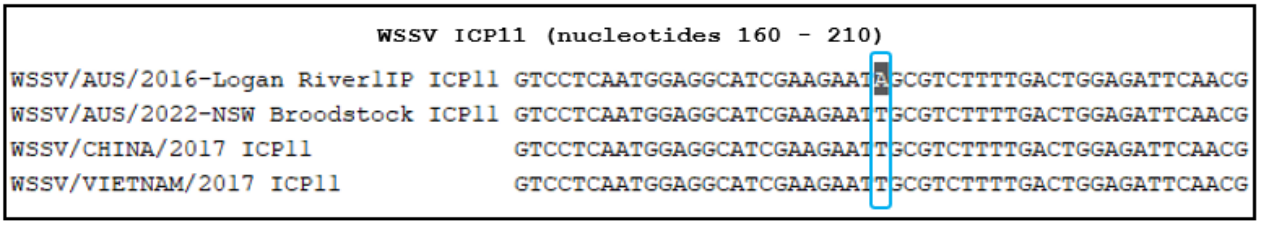

Figure 5. Nucleotide alignment of WSSV ICP11 coding region containing SNP

It was desirable to determine if this truncation was present in other global WSSV isolates, as this could infer specificity information for the ShrimpCheck Rapid test for WSSV from other regions. The SNP of interest was present in all Australian isolates from the 2016/2017 outbreak sequenced by ACDP (AFDL-ACDP, 2017, unpublished), as well as WSSV-AU MF768985 (Oakey and Smith, 2018) and WSSV/Australia/Logan River-2020 (AFDL-ACDP, 2020, unpublished). An examination of all available WSSV ICP11 sequences in NCBI determined that the SNP was not present in any international isolates within the database, indicating the test is likely to be able to detect these WSSV strains. This is predictive based on genome sequence information alone, and would require further testing with appropriate representative samples to accurately determine the specificity of this test for various WSSV isolates.

3.2 Analytical sensitivity

Quantifying WSSV viral load (genome copies/µL) in the starting material (WSSV-positive prawn tissue homogenate) and standardising the volume of starting material inputted into the beginning of each test process allowed for a direct comparison of LOD between all test types, regardless of the different target types (nucleic acid or protein) or preparation and detection processes (e.g. target purification or amplification processes) specific to each test.

ASe of the POC tests varied by several orders of magnitude, with the PCR-based tests ranking higher for ASe than the rapid antigen style tests. For the three WSSV strains assessed, the CSIRO and OIE WSSV laboratory reference qPCRs were 10-fold more sensitive than the most sensitive POC test; the AgriGen POND WSSV

qPCR. The IQ Plus™ POCKIT™ test was 100-fold less sensitive than the AgriGen POND WSSV qPCR for all WSSV strains. The ShrimpCheck WSSV Rapid test was 10-fold less sensitive than the IQ Plus™ POCKIT™ test for the China and Vietnam strains, and was unable to detect WSSV/Australia/2016-Logan River, making it the least sensitive test assessed.

3.3 Diagnostic sensitivity (clinically affected prawns) and specificity

All assessed POC tests demonstrated 100% relative DSp with no false positive results observed. Therefore, differences in accuracy between tests were dependent on relative DSe results. DSe was evaluated for clinically affected prawns to align with the intended purpose of WSSV POC test use on prawn farms; that is, for the preliminary diagnosis of WSD where it is suspected in a clinically affected population. Field samples held by ACDP from the 2016/2017 Logan River outbreak were ethanol fixed making them unsuitable for testing by several of the selected POC tests, therefore DSe was assessed using experimentally infected *P. monodon.* The clinical status of the experimentally produced DSe panel prawns was moribund or dead.

The IQ Plus™ POCKIT™ test was the most sensitive (100%) relative to the CSIRO and OIE WSSV laboratory reference qPCRs, followed by Shrimple® (98.44%*) and the AgriGen POND WSSV qPCR test (81.68%). The DSe results for the AgriGen POND test were surprising given it was the highest ranked POC test for ASe. Further investigation indicated that PCR inhibition was a factor contributing to the AgriGen POND false negative results. The test does not incorporate nucleic acid purification steps. Instead, shell free pleopod tissue is added to an extraction buffer for a specified period of time, after which a portion of this is added directly into the qPCR reaction mix. When a subset of the false negative DSe panel samples were re-tested neat and diluted 10-fold in AgriGen extraction buffer, numerous samples returned positive results only when diluted (data not shown).

*based on a reduced number of samples due to test manufacture being discontinued.

The CSIRO WSSV qPCR C_T value distribution of the experimentally generated DSe panel prawns ranged from 10.54 to 18.80. In comparison, C_T values from field infected animals collected from index ponds at five farms during the WSSV outbreak and tested by CSIRO WSSV qPCR at ACDP ranged from 12.36 to 30.47 (Moody et al, 2022), representing a wider C_T value range than the experimentally infected specimens used in this study. Based on the published C_T value range, it could be expected that there would be a greater number of false negative results for the IQ Plus™ POCKIT™ test in clinically affected populations containing specimens with reference qPCR C_T values > 20. A field-based assessment of DSe would build on the information determined from this assessment, however, it would only be possible to conduct this using samples collected during an active outbreak.

3.4 Repeatability

The laboratory reference qPCRs demonstrated excellent repeatability for the strong positive, weak positive and negative samples, with 100% of test replicates returning the expected (positive or negative) results. The assessed POC tests performed as well as the laboratory reference tests for the strong positive and negative samples, but their performance for weak positive sample replicates was reduced with only 77.78% (AgriGen POND qPCR) and 55.56% (IQ Plus™ POCKIT™ iiPCR) of replicates testing positive, despite the weak positive samples containing a viral load ≥ 10 times the POC test LODs. This variability for weak positive sample replicates was observed for both operators, on different days across multiple test runs, indicating the precision of the POC tests may be reduced as viral load in test specimens approaches the LOD. While this could be reasonably expected for any diagnostic assay, the operational characteristics of the POC tests may contribute to increased result variability in weak positive samples compared to the laboratory reference

qPCRs. For example, the IQ Plus™ POCKIT™ test uses an inoculation loop rather than a micropipette to transfer nucleic acid template into the PCR reaction mix, likely resulting in increased variability in the volume of nucleic acid added to test reactions, with the effect on test results becoming more apparent as viral load decreases.

Homogenised prawn tissue samples were used for the repeatability assessment to ensure adequate homogeneity between aliquots of the same sample. Although the AgriGen POND test correctly identified 100% of repeatability panel strong positive sample replicates as positive, this is likely to be reduced when unhomogenised samples are tested as it is the only POC test that doesn't incorporate a sample homogenisation step in it's methodology. In the DSe assessment, unhomogenised pleopod tissue strongly positive for WSSV (reference WSSV qPCR C_T values < 20) tested negative by AgriGen POND WSSV qPCR on numerous occasions. When prawn tissue homogenates were used to assess ASe and repeatability, consistency between the C_q values of AgriGen POND test replicates for a given sample was much greater (SD ≤ 3.60) than when unhomogenised tissue was tested in the DSe assessment (SD up to 10.15). In contrast, consistency between test replicates never exceeded SD 1.02 for the CSIRO and OIE laboratory reference qPCRs regardless of the sample starting material (data not shown).

3.5 Applicability (diagnostic window)

While the DSe assessment was focused on clinically affected prawns, the applicability assessment aimed to determine the diagnostic window of the tests to provide additional information on their ability to detect WSSV as infection progressed through a population, from the point of exposure through to severe clinical disease. The applicability assessment provides a useful comparison of how the WSSV reference qPCRs and POC tests performed over time post exposure, and how clinical manifestation and reference test C_T values correlate with WSSV-positive detections in the assessed POC tests. It should be noted, however, that the proportion of applicability panel specimens testing strongly positive (i.e. C_T values < 20) by WSSV reference qPCR was much higher than the proportion of moderate or weakly qPCR positive specimens, due to the rapid progression of WSD post exposure.

Overall, the ability of the POC tests to detect WSSV in experimentally infected *P. monodon* generally increased with the progression of time post exposure, the progression of clinical disease as observed by behavioural changes relative to negative controls, and as reference qPCR C_T values became stronger. The first WSSV-positive detections occurred via qPCR reference test 24 hours earlier than the first positive detections by the AgriGen POND and IQ Plus™ POCKIT™ tests at 48 hrs post exposure. Although the AgriGen POND test did not detect WSSV in a small number (n=2) of prawns with strongly positive reference qPCR C_T values (C_T < 15), it detected WSSV in a higher overall proportion (92.70%) of reference qPCR positive specimens than the IQ Plus™ POCKIT™ test (87.27%), primarily due to it's ability to detect WSSV in a greater proportion of samples with moderate or weak reference PCR C_T values (C_T > 20), and in prawns collected in the early stages of infection prior to 72 hrs post exposure.

The proportion of IQ Plus™ POCKIT™ positive detections was only 45% in samples with CSIRO WSSV qPCR C_T values between 20-30 and 0% for samples with C_T values > 30. Based on the published (Moody et al, 2022) WSSV reference qPCR C_T value range of 12.36-30.47 determined from clinically affected prawns tested by ACDP during the 2016/2017 Logan River outbreak, and the range of 22.30-44.40 determined for apparently healthy wild prawns collected during WSSV surveillance activities following the same outbreak, results indicate that the IQ Plus POCKIT™ test may fail to detect WSSV in a substantial proportion of apparently healthy WSSV-infected prawns or clinically affected prawns with C_T values > 20. This aligns with ASe results where the LOD dilutions for the IQ Plus™ POCKIT™ test had corresponding CSIRO WSSV qPCR C_T values of ~23.

3.6 Review of other studies

Test validation data for the selected POC tests was available in varying degrees from other sources outside of this study including scientific journal articles, manufacturer validation reports, test manuals and promotional material. The test performance information obtained from external sources, as well as the information derived from this study, has been summarised in Table 19.

The IQ Plus™ POCKIT™ test had the most comprehensive test validation data and was the only POC test with WOAH certification. Peer reviewed published data addressing several test performance components was available for the Shrimple® and ShrimpCheck Rapid tests, however, this information did not always align with claims made in promotional material. The test validation reports provided by the manufacturers of the ShrimpCheck Rapid test and the CDIA™ rapid test were identical, apart from company letterheads and signees, indicating these tests may in fact be the same, although this could not be confirmed. The AgriGen POND WSSV test had no test validation information of any source available. Performance characteristics were available for the MIC cycler (qPCR instrument used with the AgriGen POND test), however, this information does not specifically relate to the AgriGen POND test.

All sources that addressed ASp reported that the POC tests did not cross-react with prawn host genome or other prawn pathogens, although this was assessed with a limited number of pathogen and/or species representatives (n=1-3). ASp for WSSV was generally assessed using a single strain (of disclosed or undisclosed origin). The results from this study added to this existing knowledge by assessing ASp using 7 heterologous prawn pathogens, 5 prawn species of local commercial significance and 4 WSSV strains sourced from local and international regions, importantly revealing that one test (ShrimpCheck Rapid test) had variable specificity for different WSSV strains, being unable to detect WSSV/Australia/2016-Logan River.

Sensitivity assessments generally showed that the ASe of the rapid antigen style tests was equal to or less than that of one-step WSSV conventional PCR (Takashi et al, 2003 and Wangman et al, 2017) and less than that of two-step WSSV conventional PCR (Takahashi et al, 2003) or real-time WSSV qPCR (Powell et al, 2006). When the LOD of the Shrimple® and ShrimpCheck Rapid tests were directly compared (Wangman et al, 2017) the ShrimpCheck Rapid test was determined to be 400 times more sensitive than Shrimple®. The IQ Plus™ POCKIT™ test was reportedly as sensitive as two-step WSSV conventional PCR (GeneReach, 2014). This study was the first direct comparison of ASe for multiple WSSV POC tests and laboratory reference tests, assessed using multiple strains of WSSV. Our findings support the hierarchy of sensitivity expected from a) reviewing the existing information and b) considering the mode of action of the tests. The real-time qPCR-based tests demonstrated the greatest ASe, followed by the iiPCR-based test, and lastly the rapid antigen style tests which were the least sensitive.

Validation reports for the ShrimpCheck Rapid test and the CDIA™ rapid test reported 100% diagnostic sensitivity and specificity, with 100% PPV and NPV, based on a small sample size (n=28) containing 5 WSSV-positive and 23 WSSV-negative shrimp samples of unknown origin or clinical status. Although this current study could not assess the DSe of either of these tests due to specificity and supply issues respectively, it's likely that the overall accuracy of these tests would be less than the predicted 100%, when used under field conditions. For Shrimple®, comparative DSe ranged from 34.7% in pre-clinical experimentally infected prawns (Powell et al, 2006) to 100% in dead, field infected prawns (Takahashi et al, 2003). Shrimple® demonstrated 98.4% comparative DSe for the panel of severely clinically affected (moribund or dead) *P. monodon* used in this study.

The IQ Plus™ POCKIT™ test had the most comprehensive diagnostic performance assessment, using 400 positive (including at least 100 lightly infected) and 300 negative reference specimens, as well as 100 specimens of unknown WSSV status. Comparative DSe and DSp results were 93.5% and 97.0%, respectively. In this study, comparative DSe increased to 100% in severely clinically-affected prawns. This study found

the AgriGen POND test to have the lowest comparative DSe (81.68%) in severely clinically affected prawns, and reported PCR inhibition as the likely cause of this lower than expected DSe result. In all POC test studies, including this study, the rate of false negatives was higher than the rate of false positives.

There were no cases where POC test results were compared to WSSV qPCR C_T value ranges of diagnostic specimens. This study enabled POC test results to be compared to reference WSSV qPCR results, in specimens with clearly defined clinical status. This is important given the recent reporting of reference WSSV qPCR C_T value distributions in populations of WSSV-infected apparently healthy prawns from the wild versus clinically affected prawns sourced from farms during outbreaks (Moody et al, 2022).

The ShrimpCheck Rapid and CDIA™ rapid test validation documents reported 100% repeatability based on 2 operators, however, did not report the number of samples or replicates that were assessed. In this study, a repeatability assessment was not possible for these tests or for Shrimple®, due to supply and specificity issues. For the IQ Plus™ POCKIT™ test, Tsai et al (2004) reported 100% intra and inter-assay repeatability, 100% inter-operator repeatability and little heterogeneity between laboratories, with no significant difference found between lots or operators when inter-laboratory reproducibility was assessed. This study demonstrated that repeatability for this test is likely to be reduced for weak positive samples, with only 56% of replicate weak positive samples testing positive.

All POC tests were reported to be able to detect WSSV in the early stages of infection. Where test performance in early infection stages was assessed in experimental populations (Powell et al, 2006 and Wangman et al, 2017), it was inferred from experimentally infected prawns where the route of exposure to WSSV was via injection. The live prawn experiments conducted in this study demonstrate that WSD progresses much faster when prawns are injected with WSSV compared to when they are cohabitated with WSSV-infected cohorts. Thus, the timeframe from WSSV exposure to WSSV detection, and the ability of the tests to detect WSSV in the early stages of infection in field settings, may be over-estimated in these instances.

3.7 Operational characteristics

Operators from this study found that the timeframes required to conduct the tests (from sample collection to result) aligned with what was described by manufacturers in test manuals and promotional material. The rapid antigen style POC tests (Shrimple® and ShrimpCheck Rapid Test) were easier to conduct and interpret, required less equipment and less sample processing steps, had a quicker turnaround time from sampling to result, were cheaper, and did not require any on-site training or additional equipment not provided with the test kits. This is primarily due to the mode of action of this style of test, which requires minimal sample processing, does not require laboratory acquired skills such as low volume micro-pipetting, and allows all test components to be contained within a single-use, disposable test cassette. The Shrimple® and ShrimpCheck Rapid tests were equivalent for all operational characteristics assessed apart from cost, with the ShrimpCheck Rapid test costing slightly more at $14.60 per test than Shrimple at $11.33 per test.

Micro-pipetting, particularly of low volumes (i.e. < 10 µL), is an acquired skill. As is interpretation of real-time qPCR data. Both are required to conduct the AgriGen POND test. Although on-site training is provided for this platform during installation, the moderate technical complexity of conducting and interpreting this test may prove challenging for non-laboratory personnel in the field, particularly as printed instructions lacked some critical information. While the IQ Plus™ POCKIT™ test was also moderately technically complex to conduct, it did not involve low volume micro-pipetting ≤ 10 µL (lowest pipetting volume required was 50 µL), result interpretation was of low technical complexity, and printed instructions were clear and comprehensive, making this the more user-friendly option of the two PCR-based POC platforms evaluated.

3.8 Strengths and limitations of the evaluation

This study is the first side-by-side evaluation of commercial POC test devices for an aquatic animal pathogen in Australia. It is a comprehensive laboratory-based evaluation, using more than 350 well characterised reference specimens processed using standardised techniques and tested under strict QC conditions by experienced laboratory staff. Cohabitation was used as the route of exposure to generate experimentally infected specimens, rather than injection as used in other studies, to simulate a natural route of infection.

A comparative assessment of DSe using field-infected specimens was not possible as samples held by ACDP from the 2016/2017 Logan River outbreak were ethanol fixed, making them unsuitable for testing by several of the POC tests. Alternate prawn tissue types (e.g. gill, haemolymph), life cycle stages (e.g. post-larvae), or non-prawn host species (e.g. crabs) were not assessed as part of this evaluation. The effect of pooling samples on POC test performance was also not assessed.

As WSSV is an exotic and notifiable pathogen, a reproducibility assessment whereby POC tests are trialled with known WSSV-positive and negative specimens at multiple field sites, under varying environmental conditions and with testing performed by farm personnel, was not undertaken. During this project, while Shrimple® tests were deployed to Queensland prawn farms as an interim biosecurity measure, a Shrimple® test was successfully used on-farm to preliminarily diagnose WSSV in a clinically affected population of *P. monodon* in 2020. This instance is the only recorded and sanctioned field use of a WSSV POC test so far in Australia.

The evaluation of some tests was limited due to circumstances outside the author's control, including supply issues, product manufacturer discontinuation and test specificity issues, meaning some performance characteristics were unable to be assessed for some tests. The issues encountered, however, provide valuable insight into the challenges that may be encountered when trying to procure and validate POC tests, or when trying to facilitate a reliable supply to Australian end users.

4. Conclusion

Overall, performance of the WSSV POC tests varied widely and was generally reduced compared to laboratory-based reference tests, however, this is not unexpected given the trade-off between test performance and the simplified design and operational characteristics that make POC tests appropriate for field use.

All POC tests evaluated showed equivalent performance to the CSIRO and OIE WSSV qPCR laboratory reference tests for DSp and ASp/exclusivity, with no false positive detections observed for WSSV-negative reference samples, and no cross-reactivity observed for heterologous prawn pathogens or host genome. POC test ASe was reduced 10-fold to 10,000-fold compared to the laboratory reference qPCRs, with the PCR-based POC tests ranking higher for ASe than the rapid antigen style tests. The relative DSe of the POC tests ranged from 81.68% to 100% for clinically affected (moribund/dead) experimentally infected *P. monodon* with reference qPCR C_T values < 20, and may be further reduced for some tests in WSSV-positive apparently healthy or clinically-affected animals where reference qPCR tests return C_T values >20. The ability of the POC tests to detect WSSV in experimentally infected *P. monodon* increased over time and with the progression of clinical signs. The first WSSV-positive detections occurred via reference qPCR at 24 hours post exposure, a day earlier than the first positive POC test detections at 48 hrs post exposure.

Repeatability of the POC tests was excellent for strong WSSV-positive and negative prawn tissue homogenates, however declined for weak positive homogenates with only 56% - 78% of replicate weak positive samples testing positive, in contrast to the 100% concordance demonstrated by the laboratory reference qPCRs. The repeatability of the AgriGen POND test was also reduced in unhomogenised strong positive samples, as a result of manufacturer prescribed sample processing procedures and PCR inhibition.

Although potentially less sensitive than the PCR-based POC platforms, the rapid antigen style POC tests offered several advantages. They were fast, simple and cheap, and did not require laboratory skills or specialised equipment to use or interpret them. This project, however, experienced obstacles with procuring this style of test. Of the three rapid antigen tests selected for evaluation, one (CDIA™ WSSV Rapid test) could not be procured at all and another (Shrimple®) was discontinued by the manufacturer partway through the study. Efforts to source other commercial WSSV rapid antigen tests for inclusion in the evaluation were unsuccessful, despite repeated attempts with numerous lines of inquiry. With the ASp assessment revealing that the third rapid antigen test (ShrimpCheck Rapid test) was unable to detect the Australian 2016/2017 WSSV outbreak strain, a rapid antigen style test suitable for Australian circumstances and available for Australian procurement was not identified. This highlights that obtaining suitable rapid antigen style WSSV tests can be challenging.

The two PCR-based POC tests (AgriGen POND test and IQ Plus™ POCKIT™ test) were more technically complex and required basic laboratory skills to conduct. They were also more expensive, took longer to obtain a result, and required specialised equipment. Their main advantage was improved ASe compared to the rapid antigen style tests, however, this did not necessarily translate into improved DSe. Although the AgriGen POND test was ranked the most sensitive POC test in the ASe assessment, it was the lowest ranked test for relative DSe in clinically affected (moribund/dead) prawns due to issues with PCR inhibitors and inconsistent test replicates when un-homogenised samples were tested as per the test protocol, making it less accurate for this study's clinically affected DSe panel specimens than the IQ Plus™ POCKIT™ test. Result analysis interpretation was also more technically complex for the AgriGen POND test than for the IQ Plus™ POCKIT™ test.

The suitability of the evaluated POC tests for on-farm use in Australia needs to be considered in terms of the intended purpose, and intended end user. POC testing needs to be undertaken by end users who have the skills necessary to properly conduct the testing and interpret the results. Implementing either of the above two PCR-based POC tests on-farm may present challenges for operator training and maintaining on-going competency.

The intended use of WSSV POC tests in Australia would be to support existing laboratory-based systems for emergency diagnosis of WSD in farmed populations experiencing unusual mortalities or where suspect clinical signs are apparent. In this context, WSSV POC tests have the potential to improve EAD response by enabling capability for rapid detection of WSSV on-farm. However, as this study identified, not all commercially available WSSV POC tests may be suitable for this purpose on Australian prawn farms.

Implications

The results presented in this independent WSSV POC test assessment help to predict expected test performance outcomes, so that the advantages and risks associated with their use on-farm can be assessed, their purpose for use can be clearly defined, and strategies can be developed to minimise risks. The cost of implementing WSSV POC testing on prawn farms may be easily justified when considering the potential cost savings of early disease detection, however, if test performance is poor or operational characteristics are unsuitable for the intended user, unreliable results may impact potential benefits. This report will be used by governing bodies to decide if any of the POC tests evaluated in this study are suitable for use on Australian prawn farms, and to develop policies and procedures to manage their use.

This study will also support and influence POC test evaluation strategies at a broader level. The increased interest in POC testing technologies and their potential benefits and applications for veterinary testing has led to several recent initiatives to help formalise pathways for assessing, implementing and regulating point of care diagnostic tools. The Department of Agriculture, Fisheries and Forestry's Animal Health Committee (AHC) is currently conducting a consultancy study for POC testing policy development; the Australian Centre for International Research (ACIAR) recently conducted a project entitled *Assessing the potential of point of care diagnostic tools for developing countries* (LS/2018/203, 2021); and the WOAH is preparing a new chapter to present guidelines specific to evaluating POC test devices for infectious diseases.

Recommendations

This report is intended to provide stakeholders with critical preliminary test performance information for the WSSV POC tests evaluated, so that governing bodies can make informed decisions regarding the fitness for purpose of the tests and management of their use in Australia. It is not the role of the authors to recommend or endorse a particular commercial product or products.

Further development

If a WSSV POC test is selected for future implementation on Australian prawn farms, collation and assessment of field generated results will be important to increase our understanding of how the test performs at different sites, with different operators and under different environmental conditions. In the absence of an active WSSV outbreak, field specificity could be assessed, however, field sensitivity could only be assessed using samples collected during an active outbreak.

Additional WSSV POC tests not evaluated in this study may become commercially available to Australian consumers in the future, providing new opportunities for potential on-farm use. The development of national policies and procedures for POC test registration and use, as well as guidelines specific for POC test validation for infectious diseases, would greatly aid the identification and selection of appropriate WSSV POC tests for Australian prawn farms in the future.

Extension and Adoption

The target audiences are Australian prawn farmers and industry advisors, State and Federal government policy makers and regulators, and aquatic animal health scientists. Results of the project will be communicated to the target audiences via dissemination of milestone progress reports and the project final report to stakeholder groups, and by presentation of key findings at industry workshops and scientific conferences.

- Milestone progress report 1 was provided to the Australian Prawn Farmers Association (APFA), Biosecurity Queensland, Aquatic Animal Health and Biosecurity Subprogram and the Sub-Committee for Aquatic Animal Health (SCAAH) in May 2022.

- Milestone progress report 2 was provided to the APFA, Biosecurity Queensland, Aquatic Animal Health and Biosecurity Subprogram and SCAAH in November/December 2022.

- The project final report will be provided to the APFA, Biosecurity Queensland, Aquatic Animal Health and Biosecurity Subprogram and SCAAH following final approval from FRDC.

- Key findings are planned to be presented at the 6th Australasian Scientific Conference on Aquatic Animal Health in July 2023, and/or at the Australian Prawn Farmers Symposium 2023 or appropriate alternative workshop, conference or meeting.

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Appendices

Appendix I. Summary of experimental conditions for live prawn experiments 1 and 2

Appendix II. Experiment 1 results – WSSV cohabitation pilot trial

Appendix III. Experiment 2 results – generation of applicability panel material

Appendix III. List of project staff

Appendix I. Summary of experimental conditions for live prawn experiments 1 and 2

Table 21. Experimental conditions for live prawn experiments 1 and 2

Experimental Aspect	[1]Experiment 1 – Cohabitation Pilot Trial	[2]Experiment 2 – Generation of Applicability Panel Material
Prawns	*P. monodon* (~20g) sourced from Pacific Reef Fisheries (Ayr, QLD).	As per experiment 1.
Housing	Prawns were housed in conjoined tank sets (30 prawns per tank; stocking density 6g/L). Paired tanks were connected by 1-inch poly pipes covered with mesh to segregate injected and cohabitant challenge groups while allowing unobstructed water flow.	As per experiment 1
Water and temperature	Salt water (100L per Tank) was maintained at 28˚C – 30˚C via immersion heaters. Water was pump-circulated through conjoined tanks at an average flow rate of 8.3L/min.	As per experiment 1
Monitoring and maintenance	2 x daily monitoring (morning and afternoon). Morning monitoring included feeding, filter cleaning, water quality testing (GH, KH, pH, NO_2, NO_3, NH_3/NH_4, dissolved oxygen and temperature) and a 30% water exchange.	2 x daily monitoring (morning and afternoon) during non-disease periods and 3 x daily monitoring (morning, afternoon and evening) during disease periods. Morning monitoring included feeding, filter cleaning, water quality testing (GH, KH, pH, NO_2, NO_3, NH_3/NH_4, dissolved oxygen, salinity and temperature) and a 30% water exchange.
Feed	Commercial prawn feed pellets.	Diced, gamma-irradiated, wild caught (Australian northern prawn fishery), confirmed WSSV-negative prawns.
Inoculum	WSSV inoculum: WSSV/Australia/2016-Logan River infected haemolymph with a reference WSSV qPCR C_T of ~13. Negative control inoculum: PBSA.	As per experiment 1
Route of exposure	Cohabitated cohorts were exposed to WSSV via shared water with WSSV-injected prawns (intramuscular injection of inoculum [50 µL] between the carapace of the first and second abdominal segment).	As per experiment 1
Humane killing	Prawns were anaesthetised via immersion in chilled saltwater (<5°C) until there was no response to stimuli (~3 mins). Following anaesthesia, prawns were humanely killed by severing the supraoesophageal ganglia and nerve trunk.	As per experiment 1
Sample collection and storage	Following humane killing, whole prawns (or prawn tails in cases where heads were fixed for histology) were collected into individual containers and stored at -20°C.	As per experiment 1
Sample collection for histology	Following humane killing, the head of the prawn was completely removed from the tail and injected with (and collected into) Davidson's fixative.	As per experiment 1

Appendix II. Experiment 1 results – WSSV cohabitation pilot trial

By day 5 post exposure 80% of prawns in the injected cohort were dead/moribund and the cohabitant cohort was showing signs of reduced activity and food intake. Mortality/morbidity increased in the cohabitant prawns from 28% at day 5 to 90% by day 8 and all cohabitants were dead or moribund by day 10 (Figure 6). Mortalities were also observed in injected and cohabitated negative control cohorts due to cannibalisation (a behaviour often observed in healthy experimental prawns) and natural attrition.

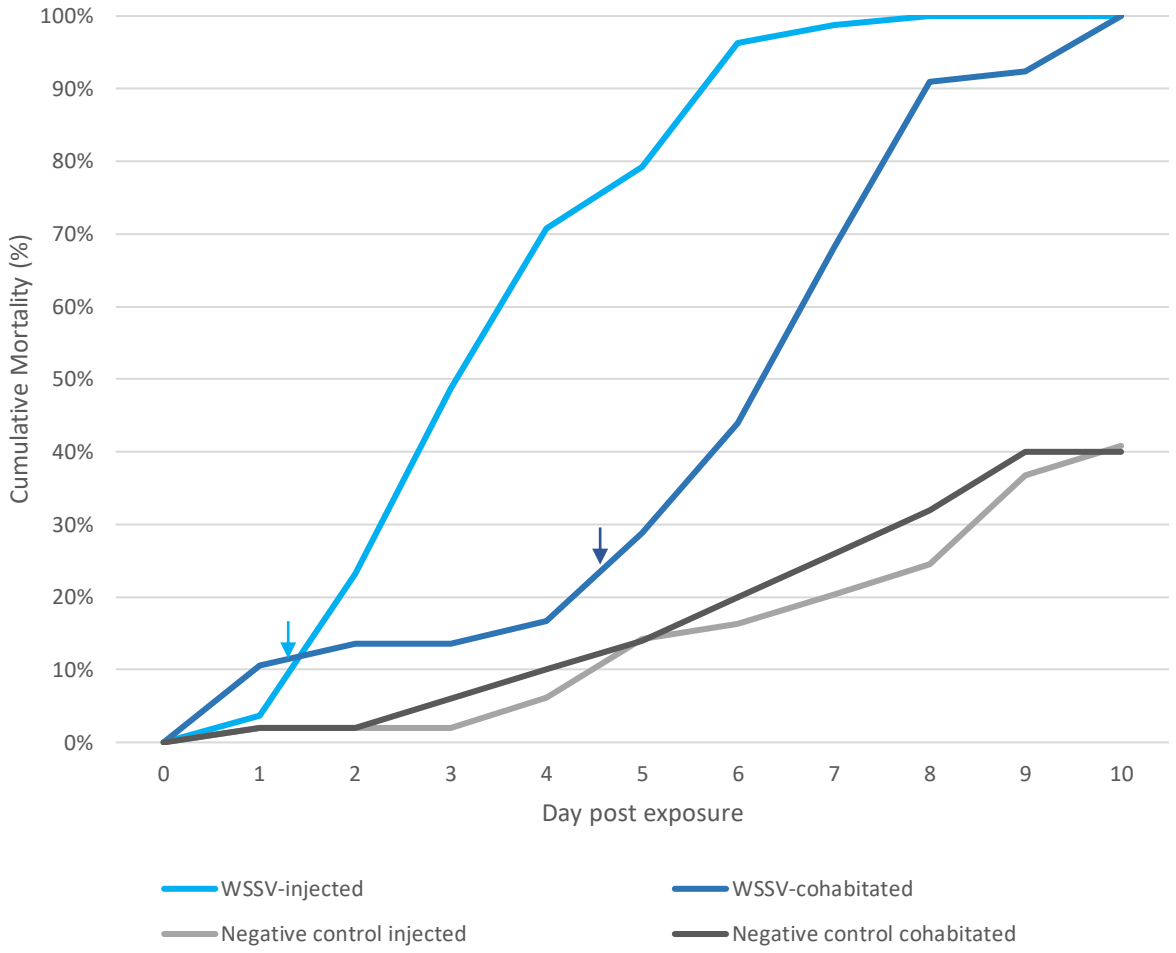

Figure 6. Cumulative mortality of WSSV-injected vs WSSV-cohabitated prawns

Arrows indicate first instance of observed behavioural changes associated with experimentally acquired WSD.

Note: mortalities occurring in negative control cohorts due to cannibalisation were included in cumulative mortality calculations

The WSSV status of all prawns was determined by WSSV reference qPCR testing. From 1 day post exposure, 100% of all dead/moribund injected prawns tested WSSV-positive by qPCR, with CSIRO WSSV qPCR C_T values ranging from 12 to 18. The first cohabitant prawn to test qPCR positive was collected on day 2. In the cohabitant cohort, Median C_T values consistently decreased over time, being > 32 at day 3 and then decreasing to 20.5 by day 4 and < 15.5 on days 5 to 10 (Figure 7). Pre-trial and negative control prawns did not test positive by CSIRO WSSV qPCR. Clinical observations, mortality/morbidity rates and WSSV qPCR results were consistent in all experimental replicates, supporting the repeatability of results.

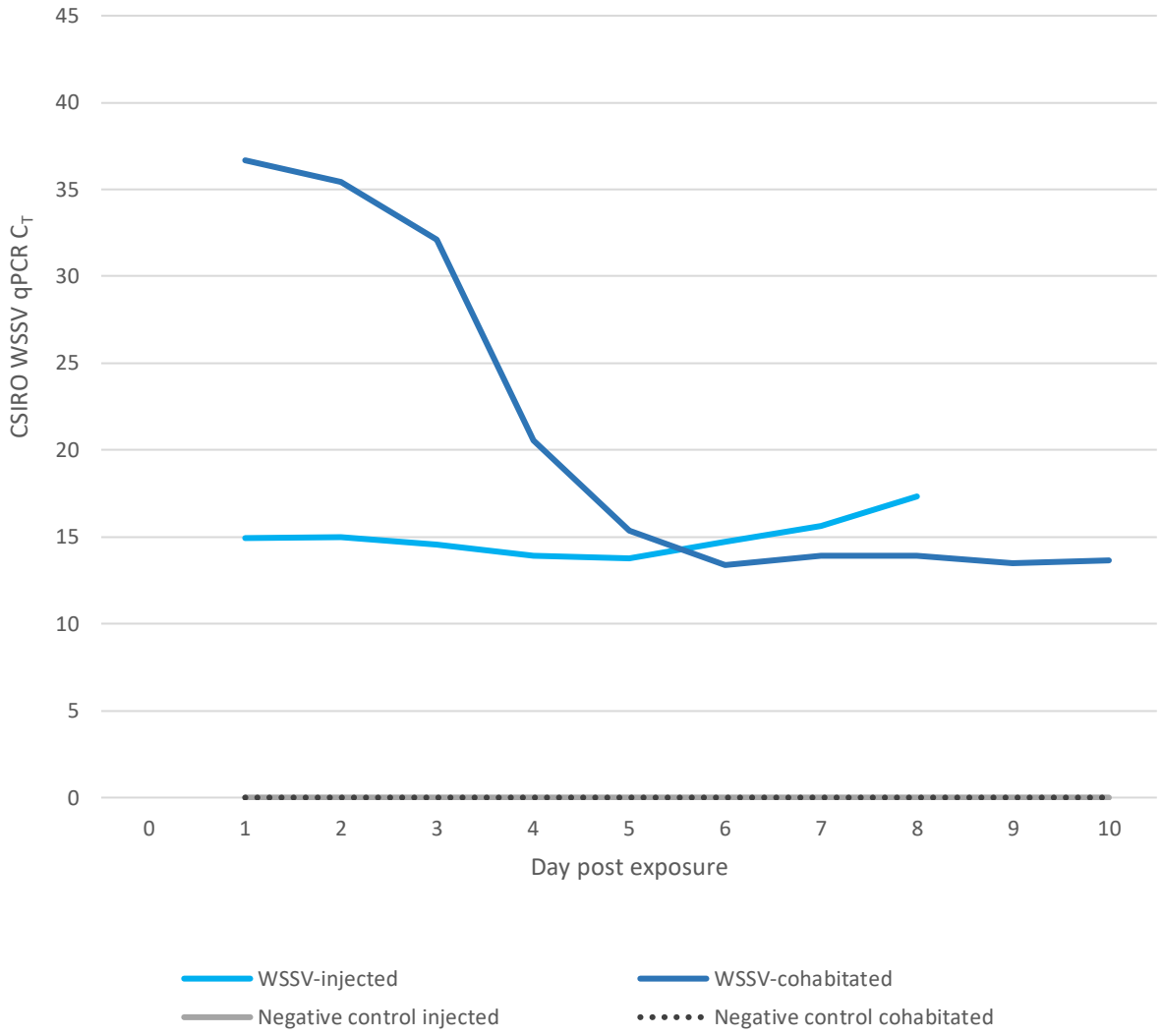

Figure 7. Median C_T values over time for WSSV-injected vs WSSV-cohabitated prawns

Histopathological examination was conducted on representative prawns from each cohort. All WSSV challenged prawns showed evidence of WSD, indicated by typical histopathological lesions consistent with WSSV infection. This included large basophilic intranuclear inclusion bodies in multiple tissues, observed most prominently in the subcuticular epithelium of the tegument and the gastrointestinal tract (Figure 8B). The lymphoid organ and the spongy connective tissue of the gastrointestinal tract also contained viral inclusions. Tissues in which inclusions were prominent often displayed varying degrees of degeneration and necrosis (Figure 8C). Pre-trial and negative control cohorts were negative for WSSV induced lesions.

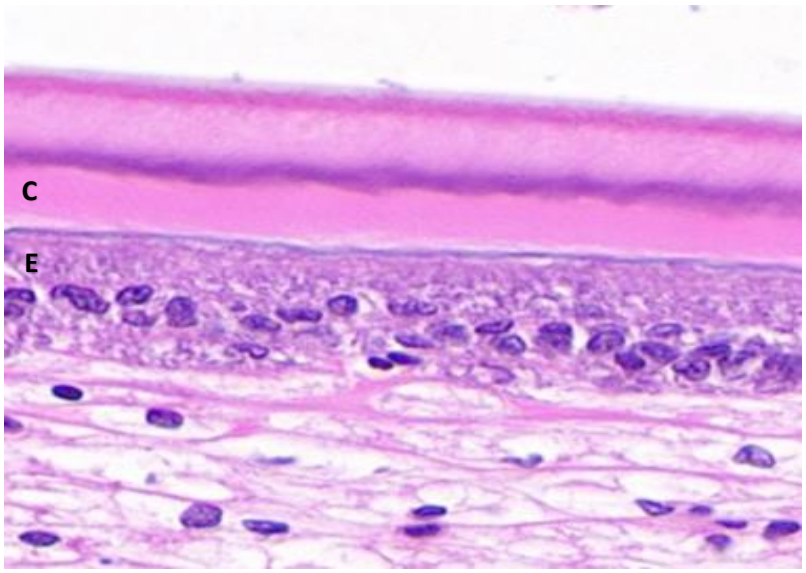

Figure 8A: Negative control prawn
Normal gastric epithelium. No WSSV inclusion bodies detected.

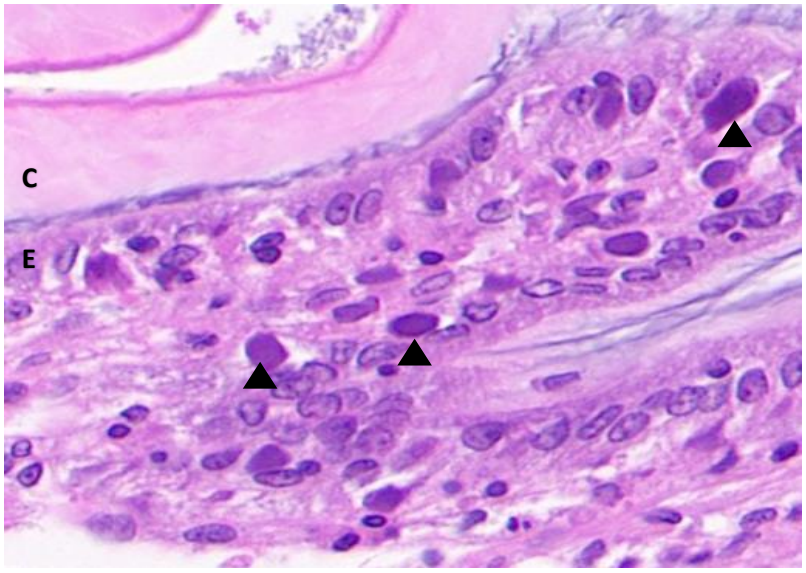

Figure 8B: WSSV-infected prawn
WSSV inclusion bodies in gastric epithelium

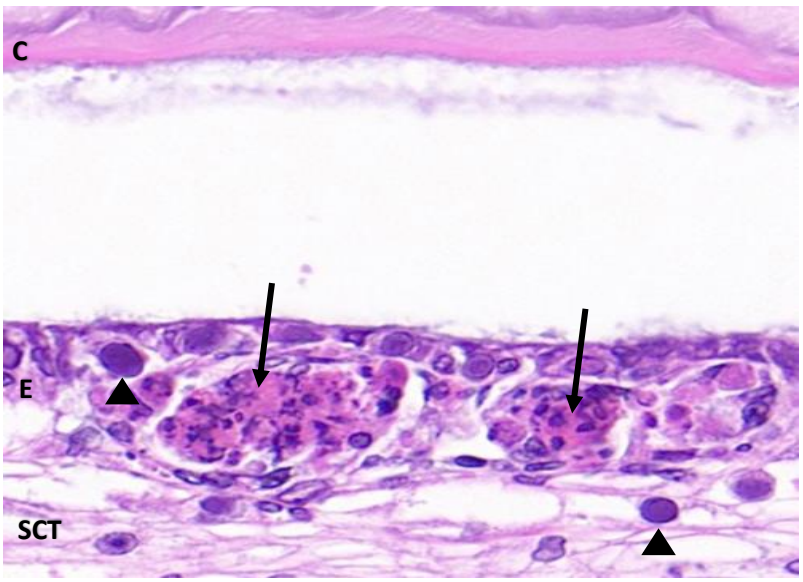

Figure 8C: WSSV-infected prawn
WSSV inclusion bodies and foci of necrosis in gastric epithelium

Figure 8. Comparative stomach histopathology of negative control (8A) and WSSV-infected (8B and 8C) *P. monodon*. Cuticle (C), epithelium (E), spongy connective tissue (SCT), WSSV inclusion bodies (arrowhead), foci of necrosis (arrow).

Appendix III. Experiment 2 results – generation of applicability panel material

The WSSV status of all prawns was determined by WSSV reference qPCR testing and histopathological examination was conducted on representative prawns from each cohort. Prawns derived from pre-trial and negative control cohorts were negative for WSSV induced lesions and tested negative for WSSV by WSSV reference qPCR, while typical histopathological lesions consistent with WSSV infection were observed in representative clinically-affected specimens from all exposed cohorts. Additionally, Chromogenic in situ hybridisation (ISH) was opportunistically conducted on representative cohabitated and negative control *P. monodon*, using DNA probes that were previously generated at ACDP (unpublished). Positive labelling was observed in the gastric epithelium of cohabitated prawns, while no positive labelling was identified in negative control prawns (Figure 9).

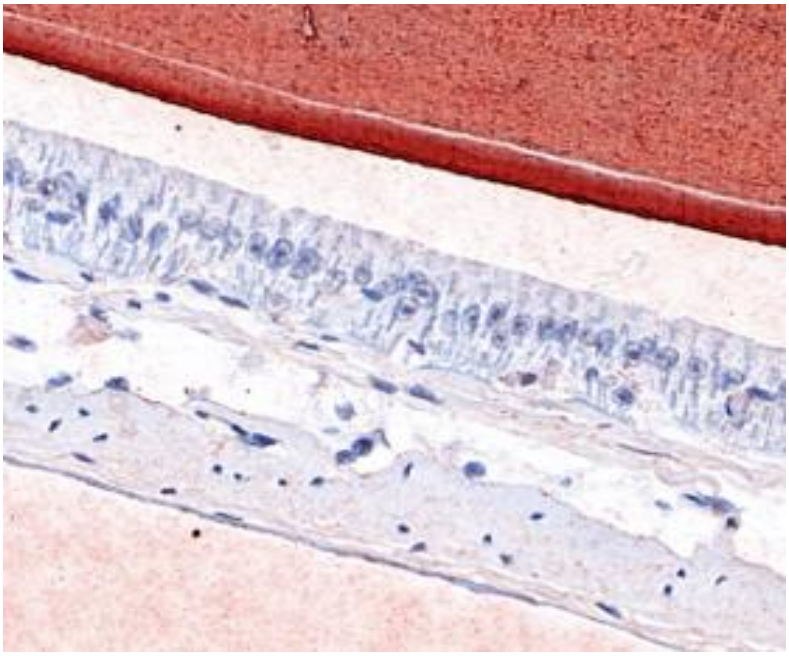

Figure 9A: Negative control prawn – normal gastric epithelium
No ISH-positive cells detected. Note: this test is not optimised and undesirable background and non-specific staining is visible in this view.

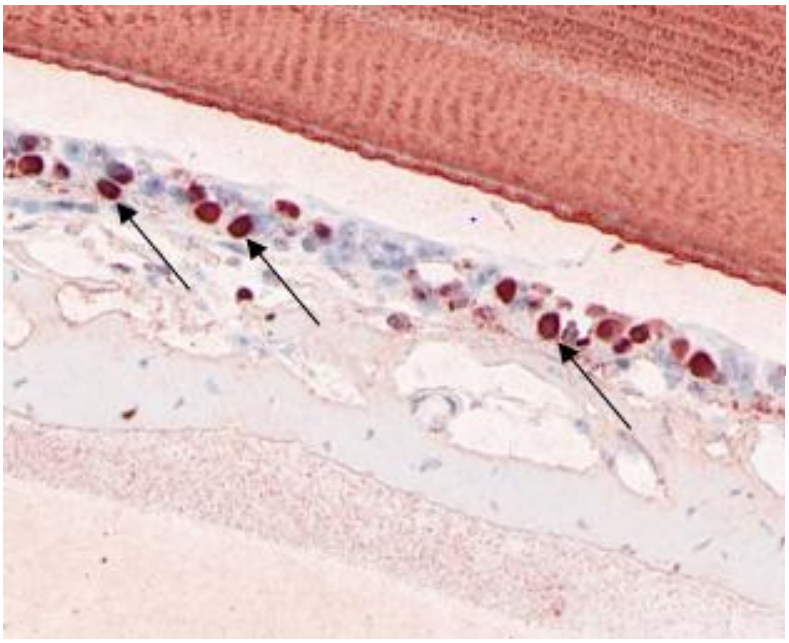

Figure 9B: WSSV-infected prawn – ISH positive cells detected
ISH-positive cells detected (arrows). Note: this test is not optimised and undesirable background and non-specific staining is visible in this view.

Figure 9. Comparative chromogenic in situ hybridisation (ISH) in negative control (9A) and WSSV-infected (9B) *P. monodon*. Foci of necrosis (arrow).

The experiment successfully generated an applicability evaluation panel of 70 *P. monodon* experimentally exposed to WSSV via cohabitation, collected from 0 to 168 hrs post exposure and consisting of healthy and mildly, moderately and severely clinically diseased prawns, with CSIRO WSSV qPCR mean C_T values ranging from 10.99 to 35.89. The composition of the applicability panel is summarised in Table 22.

Table 22. Composition of the applicability evaluation panel

Day post exposure	No. collected	Proportion of collected with no (n) mild (mi) moderate (mo) or severe (s) clinical signs or found dead (d)	CSIRO WSSV qPCR		
			% +ve	C_T range	Median C_T
0 (0 hrs)	10 (baseline)	100.00% n	0.00%	n/a	n/a
1 (24-47 hrs)	6	16.67% mi, 83.33% d	16.67%	35.89	35.89
2 (48-71 hrs)	7	85.71% mi, 14.29% d	100.00%	16.99-30.28	20.94
3 (72-95 hrs)	11	27.27% mi, 9.09% s 63.64% d	100.00%	11.81-15.68	12.69
4 (96-119 hrs)	16	43.75% mo, 31.25% s, 25.00% d	100.00%	10.99-21.67	12.22
5 (120-143hrs)	12	8.33% mo, 41.67% s, 50.00% d	100.00%	11.64-13.90	12.23
6 (144-167 hrs)	6	83.33% mo, 16.67% s	100.00%	11.36-27.38	12.03
7 (168 hrs)	2	100.00% mo	100.00%	30.61-31.98	31.16

Appendix IV. List of Project staff

Name	Position	Organisation
Dr Nagendra Singanallur Balasubramanian	Senior Research Scientist	CSIRO ACDP
Dr Jemma Bergfeld	Veterinary Pathologist	CSIRO ACDP
Dr David Cummins	Team Leader – Aquatic Research Capability	CSIRO ACDP Fish Diseases Laboratory
Dr Axel Colling	Principal Research Consultant	CSIRO ACDP
Mr John Hoad	Senior Research Technician	CSIRO ACDP Fish Diseases Laboratory
Mr Reuben Klein	Research Technician	CSIRO ACDP Fish Diseases Laboratory
Dr Peter Mohr	Team Leader – Aquatic Diagnostic Capability	CSIRO ACDP Fish Diseases Laboratory
Dr Nick Moody	Group Leader – AFDL, Co-investigator	CSIRO ACDP Fish Diseases Laboratory
Ms Joanne Slater	Research Technician	CSIRO ACDP Fish Diseases Laboratory
Ms Stacey Valdeter	Research Technician, Principal Investigator	CSIRO ACDP Fish Diseases Laboratory
Dr Stephen Wesche	Principal Scientist, Co-investigator	Biosecurity Queensland, Department of Agriculture and Fisheries
Ms Lynette Williams	Research Technician	CSIRO ACDP Fish Diseases Laboratory