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Performance characteristics of two real-time TaqMan polymerase chain reaction assays for the detection of WSSV in clinically diseased and apparently healthy prawns

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ABSTRACT: This study aimed to generate data on performance characteristics for 2 real-time TaqMan PCR assays (CSIRO and WOAH WSSV qPCRs) for the purposes of (1) detection of white spot syndrome virus (WSSV) in clinically diseased prawns and (2) detection of WSSV in apparently healthy prawns. Analytical sensitivity of both assays was 2 to 20 genome copies per reaction, and analytical specificity was 100% after testing nucleic acid from 9 heterologous prawn pathogens and 4 prawn species. Results obtained after testing more than 20 000 samples in up to 559 runs with the CSIRO WSSV qPCR and up to 293 runs with the WOAH WSSV qPCR demonstrated satisfactory repeatability for both assays. Both assays demonstrated median diagnostic sensitivity (DSe) 100% (95% CI: 94.9–100%) when testing clinically diseased prawns. When 1591 test results from apparently healthy prawns were analysed by Bayesian latent class analysis, median DSe and diagnostic specificity (DSp) were 82.9% (95% probability interval [PI]: 75.0–90.2%) and 99.7% (95% PI: 98.6–99.99%) for the CSIRO WSSV qPCR and 76.8% (95% PI: 68.9–84.9%) and 99.7% (95% PI: 98.7–99.99%) for the WOAH WSSV qPCR. When both assays were interpreted in parallel, median DSe increased to 98.3 (95% PI: 91.6–99.99%), and median DSp decreased slightly to 99.4% (95% PI: 97.9–99.99%). Routine testing of quantified positive controls by laboratories in the Australian laboratory network demonstrated satisfactory reproducibility of the CSIRO WSSV qPCR assay. Both assays demonstrated comparable performance characteristics, and the results contribute to the validation data required in the WOAH validation pathway for the purposes of detection of WSSV in clinically diseased and apparently healthy prawns.

KEY WORDS: Validation · qPCR · Sensitivity · Specificity · Repeatability · WSSV · Bayesian latent class model

1. INTRODUCTION

White spot syndrome virus (WSSV) is a World Organisation for Animal Health (WOAH, formerly OIE)-listed pathogen of a broad range of crustaceans, particularly decapods such as prawns and crabs (WOAH 2018). WSSV infection in farmed penaeid prawns often results in disease associated with mass mortality and significant economic loss. WSSV is a large double-stranded DNA virus with a circular

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genome of approximately 300 kb (van Hulten et al. 2001, Yang et al. 2001, Oakey & Smith 2018). While some genotypic variability is apparent in strains from different geographical locations (Lo et al. 2012), all isolates are classified as a single species by the International Committee on Taxonomy of Viruses (ICTV), which recognises WSSV as the sole member of the genus *Whispovirus* within the family *Nimaviridae* (Wang et al. 2019).

Since the early 1990s when WSSV was first reported in Fujian Province, China (Zhan et al. 1998), the virus has spread throughout the major shrimp farming regions of Asia, the Americas, Africa (Leu et al. 2009), and most recently, Australia (WOAH WAHIS Report IN_21737, https://wahis.woah.org/#/report-info?reportId=8409). Australia was considered free of WSSV until, in 2000, the virus was found in cultured mud crabs *Scylla serrata* and black tiger prawns *Penaeus monodon* from 2 experimental aquaculture facilities in Darwin (East et al. 2004). The source of infection was traced to imported commodity prawns used as feed. In response, a program of intensive destocking and disinfection was undertaken with the aim of eradication. Subsequently a national survey based on PCR screening was undertaken to determine if WSSV was more generally present in the Australian environment (East et al. 2004). With no reports of disease and no evidence of WSSV in the survey it was concluded that Australia's crustacean populations remained free of WSSV at that time.

In Australia, the CSIRO Australian Centre for Disease Preparedness (ACDP) has primary responsibility for the confirmation/exclusion of exotic pathogens in animals. For the WOAH-listed diseases of aquatic animals, diagnostic procedures are based on the WOAH Manual of Diagnostic Tests for Aquatic Animals (https://www.woah.org/en/what-we-do/standards/codes-and-manuals/aquatic-manual-online-access/). In addition, several alternative assays based on published literature or developed in-house are also used. Much of the testing, particularly for pathogens of crustacean and molluscan species, is based on molecular methods, with screening by real-time PCR and confirmation by conventional PCR and sequence analysis of amplicons. Initially, WSSV PCR testing at the CSIRO ACDP Fish Diseases Laboratory (AFDL) was based on the WOAH conventional nested PCR (WOAH WSSV nPCR) (Lo et al. 1996, WOAH 2018). During the response to the detection of WSSV in Darwin in 2000, a real-time TaqMan PCR (CSIRO WSSV qPCR) which targets the same region as the nested PCR was implemented as an additional screening assay (East et al. 2004, Sritunyalucksana et al. 2006). When a real-time TaqMan PCR, developed by Durand & Lightner (2002), was described in the WOAH Aquatic Manual (WOAH WSSV qPCR, WOAH 2018), this assay was added as a follow-up assay for detection of WSSV at AFDL.

Although the WOAH-recommended PCR tests have been in use for a considerable time, published information about performance characteristics is limited. For example, published information for the WOAH WSSV nPCR was limited to analytical characteristics (Lo et al. 1996). At the time of the Australian national survey for WSSV, the performance characteristics of the CSIRO WSSV qPCR had not been evaluated using field samples to determine the diagnostic characteristics of the assay (East et al. 2004). Sritunyalucksana et al. (2006) carried out comparative analytical sensitivity testing with CSIRO WSSV qPCR and commonly used WSSV PCR protocols in Thailand. The WOAH WSSV qPCR was developed to quantify WSSV in samples, with estimations of analytical sensitivity and analytical specificity with inclusivity and exclusivity provided (Durand & Lightner 2002). However, no estimates of diagnostic sensitivity (DSe) or diagnostic specificity (DSp) were determined, and these are key test parameters required for assay validation and are particularly important to determine appropriate sample sizes when undertaking surveillance activities, especially when used for prevalence studies in populations of apparently healthy animals

In December 2016, WSSV was confirmed in *P. monodon* at a prawn farm on the Logan River in Queensland, Australia, resulting in notification to the WOAH (WAHIS report IN_21737). Despite attempts to limit the spread of virus, WSSV was subsequently confirmed in a further 6 *P. monodon* farms in the Logan River area. In response, intensive destocking and disinfection was undertaken at infected farms, and an on-going program of active surveillance, largely based on real-time PCR screening, was put in place. Surveillance of wild prawn and crab populations resulted in further detections of WSSV in northern Moreton Bay in Queensland, which is in the WSSV Movement Regulated Area (Queensland Government 2017) where movement of potential carriers (e.g. polychaete worms and decapod crustaceans) is prohibited. A national surveillance testing program for WSSV to substantiate freedom in areas outside the WSSV Movement Regulated Area was also undertaken. This required the use of validated as-

says for the design of effective surveillance activities and correct interpretation of results.

Test validation data provide evidence that the performance characteristics of a test are appropriate for their proposed application. The WOAH defines validation as 'a process that determines the fitness of an assay, which has been properly developed, optimised and standardised, for an intended purpose' (WOAH 2021b, p. 11). WOAH further recommends that all diagnostic assays 'should be validated for the species in which they will be used' (WOAH 2021b, p. 11). The WOAH Validation Pathway describes a 4-stage approach (WOAH 2021a). Stage 1 includes analytical characteristics such as analytical sensitivity (ASe), analytical specificity (ASp) and repeatability. Stage 2 describes diagnostic characteristics, including DSe, DSp and cut-off values, and Stage 3 and Stage 4 include reproducibility and implementation.

The purpose of this study was to, using the stages in the WOAH Validation Pathway as a guide, evaluate the performance characteristics of 2 real-time TaqMan PCR assays, where the purposes of the tests are (1) detection of WSSV in clinically diseased prawns (confirmation/exclusion of disease) where confirmed positive detections in prawns from a pond would result in the farm being classified as positive, and (2) detection of WSSV in apparently healthy prawns (screening/exclusion to determine presence/absence during surveillance). For the testing of surveillance samples obtained from apparently healthy animals, data collection was planned before running the tests. Reporting of the design and statistical analysis of test accuracy data as described in Stage 2 of the WOAH validation pathway follow published standards (Gardner et al. 2016, Kostoulas et al. 2017), where possible.

2. MATERIALS AND METHODS

2.1. Processing and nucleic acid extraction for Logan River WSSV clinically diseased prawn samples, during an outbreak

On 30 November 2016, samples of *Penaeus monodon* with evidence of disease (e.g. mortalities, aggregation of prawns at pond edges, increased bird activity) from the index farm (Farm A) on the Logan River, were forwarded to AFDL for WSSV confirmatory testing. The samples had tested positive for WSSV by real-time PCR at the Biosecurity Sciences Laboratory (BSL) in Queensland. The submission consisted of 20 samples from 10 prawns (10 gill homogenates and 10 carapace homogenates), transported on ice and received within 9 h of dispatch. Given the significance of the detection of WSSV at BSL, which represented the first detection of an exotic pathogen associated with significant disease, all submitted samples from this index case submission were processed and tested immediately on arrival. Each sample was clarified by centrifugation at 10 000 × *g* for 10 min before 140 µl of each clarified supernatant was added to 560 µl AVL (Qiagen) containing carrier RNA. Nucleic acid was extracted using the QIAamp Viral RNA Mini Kit (Qiagen) and eluted in 60 µl of elution buffer.

Five subsequent submissions from index case ponds were received over a 10 wk period from additional farms (Farms B to F) in the Logan River area for confirmation of WSSV infection in *P. monodon* exhibiting clinical signs of disease. Each submission consisted of 10–11 samples of gill tissue fixed in 80% analytical grade ethanol (Table 1). These samples had been collected on the farm and transported to BSL for specimen receipt and processing, where tissues from

Table 1. Source of samples for white spot syndrome virus (WSSV) quantitative PCR (qPCR) assay validation. C_T: cycle threshold. NA: not applicable. C_T range is from results after testing both neat and 1/10 dilutions of nucleic acids

Submission	Population disease status	Sample type	No. of samples	CSIRO WSSV qPCR No. positive	CSIRO WSSV qPCR C_T range	WOAH WSSV qPCR No. positive	WOAH WSSV qPCR C_T range	Both assays No. positive
Farm A (index case)	Clinically diseased	Gill homogenate	10	10	13.31–23.34	10	14.39–24.43	10
		Carapace homogenate	10	10	12.96–19.18	10	14.00–20.42	10
Farm B	Clinically diseased	Ethanol-fixed gills	10	10	13.92–30.47	10	15.04–31.88	10
Farm C	Clinically diseased	Ethanol-fixed gills	11	11	12.36–23.41	11	13.90–24.64	11
Farm D	Clinically diseased	Ethanol-fixed gills	10	10	14.80–30.20	10	16.12–31.25	10
Farm E	Clinically diseased	Ethanol-fixed gills	10	10	15.38–23.82	10	16.22–24.60	10
Farm F	Clinically diseased	Ethanol-fixed gills	10	10	15.37–23.85	10	16.19–24.81	10
Wild surveillance prawns								
Moreton Bay	Apparently healthy	Ethanol-fixed gills	1196	133	22.30–44.40	122	22.81–42.16	94
Gulf of Carpentaria	Apparently healthy	Pleopods	395	0	NA	0	NA	0

each prawn were placed into individual 2 ml O-ring screw-cap tubes prior to transport to AFDL. Tissue from each sample was added to 600 µl phosphate-buffered saline A (PBSA; pH 7.4) in a 2 ml bead-beating tube (Lysing Matrix D, MP Biomedicals), and samples were homogenised for 2 cycles of 6 m s^{-1} for 1 min, with a 2 min cooling period on wet ice in between each cycle, using a FastPrep-24 5G homogeniser (MP Biomedicals). Following clarification by centrifugation, nucleic acid was extracted using the QIAamp Viral RNA Mini Kit. Dilution of nucleic acid extracted from prawns has previously been demonstrated to overcome PCR inhibition in real-time PCR (Cao et al. 2010). Thus, a 10-fold dilution of extracted nucleic acid was prepared for each sample in nuclease-free water. If not tested immediately, extracted nucleic acids were stored at −80°C. A negative extraction control, consisting of PBSA, was included with the samples from each submission.

2.2. Processing and nucleic acid extraction from apparently healthy wild prawn surveillance samples from Moreton Bay

Wild prawns of various species, including *P. merguiensis*, *P. esculentus*, *P. plebejus*, *Metapenaeus endeavouri* and *M. bennettae*, were caught by trawler in Moreton Bay, and transported to BSL for specimen receipt and processing. Samples of gill tissue fixed in 80% analytical grade ethanol were submitted to AFDL for testing. From mid-December 2016, wild prawn surveillance samples were submitted after processing of whole prawns at BSL and the Elizabeth Macarthur Agricultural Institute (EMAI), New South Wales, Australia, for WSSV screening, where they were tested as pools of 5 prawns. All individual prawns from any pools testing positive for WSSV at EMAI were submitted to AFDL and tested as individual samples (Table 1). From both BSL and EMAI, a total of 1196 wild prawns from Moreton Bay were submitted and tested in 81 submissions with an average of 15 prawns per submission. A gill or pleopod from each prawn was homogenized and 50 µl of clarified supernatant extracted using the MagMAX-96 Viral RNA Isolation Kit (Applied Biosystems) on the MagMAX Express-96 Magnetic Particle Processor (MME-96; Applied Biosystems) with nucleic acid eluted in 50 µl of elution buffer. A 10-fold dilution of extracted nucleic acid was prepared for each sample in nuclease-free water with extracted nucleic acids stored at −80°C if not tested immediately. A negative extraction control (NEC), consisting of a blank well run through the extraction procedure, was included with each extraction plate.

2.3. Processing and nucleic acid extraction from apparently healthy wild prawn surveillance samples from the Gulf of Carpentaria

As part of the national surveillance program to demonstrate freedom of WSSV outside the Movement Regulated Area in south-eastern Queensland, AFDL undertook testing of wild prawns caught in the Northern Prawn Fishery in the Gulf of Carpentaria in northern Australia. A total of 395 frozen prawns were submitted (Table 1) in 3 submissions, collected by trawl in July 2018 (n = 53 *P. merguiensis*, n = 30 *P. semisulcatus* and n = 24 *P. esculentis*), September 2018 (n = 104 *P. merguiensis*) and April 2019 (n = 184 *P. merguiensis*). To mitigate against false-negative results, due to PCR inhibitors present in extracted nucleic acids, the use of T4 bacteriophage as an internal extraction control was implemented for these samples. After partial thawing, a pleopod was removed from each prawn, bead-beaten in MagMAX-96 Viral RNA Isolation Kit (Applied Biosystems) lysis buffer. Nucleic acids were extracted and stored, after the addition of 5 µl per sample of quantified T4 bacteriophage stock (Attostar LLC) to the extraction kit lysis buffer. As the NEC was also spiked with T4, this was also used as the T4 positive extraction control, with the quantified stock used prepared to achieve an expected T4 qPCR threshold cycle (C_T) value of approximately 27 to 30.

2.4. WSSV real-time PCR protocols

Two real-time PCR assays, the WOAH WSSV qPCR (Durand & Lightner 2002; Table 2) and the CSIRO WSSV qPCR (East et al. 2004, Sritunyalucksana et al. 2006; Table 2), were utilised for the detection of WSSV nucleic acid. Both WSSV qPCR assays have been included in the AFDL ISO 17025 scope of accreditation since 2014, all staff undertaking testing have been deemed competent according to documented procedures in the laboratory quality system, and participation in external proficiency testing for WSSV has been satisfactory. For testing all prawns, both assays were performed, with duplicate reactions of both neat and 10-fold diluted nucleic acid templates. Testing both neat and 1/10 dilutions of extracted nucleic acid reduced the risk of false-negative results due to PCR inhibition (data not

Table 2. WSSV PCR primer and probe sequences, amplicon sizes and cycling conditions

PCR	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	50°C 2 min, 95°C 10 min, 94°C 15 s + 60°C 60 s (45 cycles)	Sritunyalucksana et al. (2006)
WOAH WSSV qPCR	WOAH WSSV 1011F WOAH WSSV 1079R WOAH 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	50°C 2 min, 95°C 10 min, 94°C 15 s + 60°C 60 s (45 cycles)	Durand & Lightner (2002)
WOAH WSSV nPCR	WSSV 146F1 WSSV 146R1 WSSV 146F2 WSSV 146R2	ACT ACT AAC TTC AGC CTA TCT AG TAA TGC GGG TGT AAT GTT CTT ACG A GTA ACT GCC CCT TCC ATC TCC A TAC GGC AGC TGC TGC ACC TTG T	95°C 15 min, 94°C 30 s + 55°C 30 s + 72°C 90 s (40 cycles) 72°C 7 min	Lo et al. (1996)
T4 qPCR	T4F T4R T4 probe	CCA TCC ATA GAG AAA ATA TCA GAA CGA CGC TGG GAA AAG AGG AAT TAT TTA VIC-AAC CAG TAA TTT CAT CTG CTT CTG ATG TGA GGC-QSY	50°C 2 min, 95°C 10 min, 94°C 15 s + 60°C 60 s (45 cycles)	Ninove et al. (2011)

shown), and it was not practical to run additional house-keeping gene assays given the number of samples being received. During the initial response phase, due to the numbers of samples submitted (>22 000 samples over a 5 mo period), screening of wild prawns from Moreton Bay was undertaken with only the CSIRO qPCR. Subsequently, to determine DSe and DSp in wild prawns, all prawns in a submission of wild prawn samples originating from Moreton Bay, where at least one prawn had tested positive using the CSIRO WSSV qPCR, were also tested using the WOAH WSSV qPCR. This created a subset of 1196 prawns. For both qPCR assays, each 25 µl qPCR reaction mixture contained 2 µl nucleic acid template, 12.5 µl TaqMan Universal PCR Master Mix (Life Technologies) and a final concentration of 900 nM for each primer and 250 nM for the probe. The qPCR assays were performed in a 7500 Fast Real-Time PCR System (Life Technologies) and analysed with the 7500 Fast software. Prawns were deemed to be WSSV test-positive if one replicate generated a typical amplification curve, for either assay at either nucleic acid dilution that crossed a threshold of 0.1 within 45 cycles. To increase sensitivity and avoid false-negative results, no C_T cut-offs were used. Two WSSV positive controls (20 000 and 200 plasmid copies per reaction), a negative extraction control and a no template control were included in duplicate on each assay plate, with control results to be as expected for each assay to be deemed valid. Based on in-house experiments, positive control results were deemed acceptable if the positive control mean C_T value was within ± 2 C_T values of the mean C_T value determined from the first 10 runs using that plasmid batch. If a sample generated a negative test result in both real-time PCRs, the prawn was deemed negative. The T4 qPCR (Ninove et al. 2011; Table 2), used as an internal extraction control for the wild prawn samples collected from the Gulf of Carpentaria, used the same 25 µl qPCR reaction mixture and cycling conditions as the CSIRO and WOAH WSSV qPCRs with samples tested in singlicate. For a sample to be deemed free of PCR inhibitors, the acceptance range for the T4 spiked samples was ±3 C_T values of the T4 spiked NEC well. Samples with T4 C_T values >3 of the T4 spiked NEC were diluted 1/10 in nuclease-free water and re-tested.

2.5. WSSV conventional PCR protocols

Confirmatory testing of index case samples from each farm using the WOAH WSSV nPCR (Lo et al. 1996; Table 2) was performed with cycling conditions as described in Table 2. Briefly, the primary PCR was performed in 25 µl reactions, with 2 µl extracted nucleic acid template added to a 23 µl reaction mix containing 12.5 µl HotStarTaq Master Mix (Qiagen) and 360 nM of each primer. The nested PCR had 2 µl primary PCR product added to a 23 µl reaction mix. A single WSSV genomic control, a negative extraction control and a no template control were included with each assay. Amplicons were visualised after electrophoresis on 1.5% agarose gels stained with SYBR

Safe DNA Gel Stain (Life Technologies). Amplicons of the expected size were excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen). Each amplicon was sequenced using forward and reverse primers by direct product sequencing using the BigDye Terminator v3.1 Cycle Sequencing chemistry and 3130xl Genetic Analyzer (Applied Biosystems). Chromatogram analysis, consensus sequence generation, BlastN searches of the NCBI database and multiple sequence alignments were undertaken using Geneious Pro (Biomatters).

Successful extraction of nucleic acid was confirmed by performing the WOAH Decapod PCR on each sample (Lo et al. 1996). The visual identification of amplicons of the expected size from either or both neat and 10-fold diluted nucleic acid templates confirmed that nucleic acid had been extracted and PCR inhibition had not occurred.

2.6. Analytical performance characteristics of WSSV real-time PCR assays

To determine the analytical performance characteristics of the CSIRO and WOAH WSSV qPCR assays, estimates of ASe, ASp and repeatability were determined. For determination of ASe, or limit of detection (LOD), of the assays, positive control plasmids were designed that contained the nucleotide sequence between the forward and reverse primer for each assay. Plasmids were produced commercially (Integrated DNA Technologies) and based on transcript length and concentration, determined by Qubit, stock plasmids were made to 10^8 copies μl^{-1} in nuclease-free water. Ten-fold dilutions of each plasmid were prepared in 2 diluents viz. nuclease free water and WSSV-negative prawn DNA (50 ng μl^{-1}). Five replicates for each 10-fold dilution in each diluent were tested, with at least 3 replicates required to test positive for a dilution to be considered positive. Two concentrations of each plasmid of 20 000 and 200 copies per reaction were prepared as laboratory network quality controls (NQC) for use in each test run. For these NQCs, NQC-1 and NQC-2 were specific for the CSIRO WSSV qPCR and NQC-3 and NQC-4 were specific for the WOAH WSSV qPCR. Depending on the purpose of the testing, controls were run in either singlicate (surveillance samples) or duplicate (confirmation samples). Analysis of results of testing the NQC positive controls over multiple runs by multiple operators were used to determine the repeatability of each assay.

For assessment of ASp, nucleic acid extracted from a selection of prawn pathogens, prawn species and plasmid controls were tested with each assay in duplicate (Table 3). The identity of each pathogen tested was confirmed by conventional PCR followed by amplicon sequence analysis. Additionally, *in silico* analysis of primers and probes for each qPCR was undertaken with WSSV sequences obtained from GenBank (https://www.ncbi.nlm.nih.gov/).

2.7. Intra-laboratory repeatability of WSSV real-time PCR assays and inter-laboratory reproducibility of the CSIRO WSSV qPCR

For assessment of repeatability within AFDL, qPCR results for testing undertaken for the 2 concentrations of plasmid control, for each WSSV qPCR assay were analysed. For the CSIRO WSSV qPCR, up to

Table 3. Analytical specificity of the CSIRO and WOAH WSSV qPCR assays. ND: not detected

Sample	Mean C_T value	
	CSIRO WSSV qPCR	WOAH WSSV qPCR
Prawn pathogens		
WSSV	22.93	23.86
Acute hepatopancreatic necrosis disease	ND	ND
Infectious hypodermal and haematopoietic necrosis virus	ND	ND
Infectious myonecrosis virus	ND	ND
Taura syndrome virus	ND	ND
Monodon baculovirus	ND	ND
Mourilyan virus	ND	ND
Yellow head virus genotype 1	ND	ND
Yellow head virus genotype 2	ND	ND
Yellow head virus genotype 7	ND	ND
Prawn species		
Penaeus monodon	ND	ND
P. merguiensis	ND	ND
Melicertus latisulcatus	ND	ND
Litopenaeus vannamei	ND	ND
Assay controls		
CSIRO qPCR plasmid NQC-1	24.02	ND
CSIRO qPCR plasmid NQC-2	30.71	ND
WOAH qPCR plasmid NQC-3	ND	27.65
WOAH qPCR plasmid NQC-4	ND	34.99
No template control	ND	ND

559 plates were run over an 11 mo period, with 6 batches of primer and probe used, and testing undertaken by 13 different operators. For the WOAH WSSV qPCR, up to 293 plates were run over a 15 mo period, with 7 batches of primer and probe used, and testing undertaken by 10 different operators. To determine between operator variability, data were analysed using repeated measures ANOVA with the Bonferroni post-hoc test correction in GraphPad Prism 9 (GraphPad Prism Software)

The positive control plasmids (NQC-1 and NQC-2) have been distributed to state government laboratories within Australia that are part of the Laboratories for Emergency Disease Diagnosis and Response (LEADDR) network with test results submitted for reporting and discussion. Laboratories test the plasmids with the specific qPCR assay but are free to use their own cycling conditions, PCR master mixes, thermocyclers, thresholds etc. in place within their laboratories. The NQC-2 results submitted between 2016 and 2019 from 5 laboratories were analysed to assess the inter-laboratory reproducibility of the CSIRO WSSV qPCR.

2.8. Bayesian latent class model (BLCM) to estimate DSe and DSp for apparently healthy wild prawn surveillance samples

2.8.1. Model overview

A 2-test in 2-population BLCM that allowed for DSe and DSp dependence between tests was used because both assays were based on detection of sequence in the same gene of the WSSV genome. The populations were comprised of wild prawns of unknown status from Morton Bay (n = 1196), and the second population (n = 395) was wild prawns from the Gulf of Carpentaria, which was believed to be free of WSSV. The model used sensitivity and specificity covariances (Dendukuri 6 Joseph 2001) rather than correlations (Georgiadis et al. 2003) as dependence parameters. In the model, there are 8 parameters (2 DSe, 2 DSp, 2 covariances, and 2 prevalence) to be estimated and only 6 degrees of freedom. Hence, the model is non-identifiable (i.e. there is no unique set of parameters that generated the counts of pairwise test results (T_1+T_2+, T_1+T_2−, T_1−T_2+, T_1−T_2−) without informative prior information on at least 2 parameters. Briefly, incorporation of scientifically justifiable priors for 2 parameters allows the researcher to distinguish a plausible solution from an infinite number of solutions when the model is non-identifiable.

2.8.2. Prior distributions

The beta (a,b) prior for the DSe of the WOAH WSSV qPCR was based on expert opinion of the lead author (N.J.G.M.), who had used the WOAH assay in his laboratory to test other samples not included in the present study. His best guess (mode) for DSe was 90% with 95% confidence that it was greater than 85%. The corresponding DSe prior as calculated in Betabuster 1.0 (https://betabuster.software.informer.com/) was beta (130.7, 15.4). After review of the prior, the DSe prior was downweighted 5-fold (the values of a and b were both divided by 5) to reduce influence of priors on the posterior distribution for DSe. The downweighting allowed the DSe prior (beta 26.14, 3.08) for the WOAH assay to be centred at approximately the same value but to be more spread out. The prior median for DSe was 90.4% with a 95% probability interval (PI) from 76.3 to 97.6%. The respective weights of the DSe prior (a + b) were approximately 146 for the original and 29 for the downweighted DSe prior with the latter selected for use. The same prior was used for DSe of the CSIRO WSSV qPCR because the assays had been used in parallel in the same laboratory with few discordant results and were expected to have similar DSe and DSp. Prevalence in population 1 was modelled as beta (1,1) and in population 2 was modelled as uniform (0, 0.0001) because prevalence was expected to be zero but also could have been very low.

2.8.3. Model inferences

OpenBUGS v3.2.3 (Lunn et al. 2000) was used for inferences about medians and 95% PI for parameters of interest. The STEP function in OpenBUGS was used to approximate the posterior probability of a difference in the DSe of the CSIRO and WOAH WSSV qPCRs (see code in Appendix) assuming conditional dependence of the 2 assays. Model convergence was assessed by evaluation of history plots, running quantiles and the Gelman-Rubin statistic (bgr) which compares the within and between-chain variability of 3 chains that were run from dispersed starting values. A sensitivity analysis of the influence of the selected DSe priors for the CSIRO and WOAH WSSV qPCR assays was done by lowering and increasing the respective median values by 5–10% as recommended by Johnson et al. (2019), while maintaining approximately the same PI width as in the default DSe priors described above.

3. RESULTS

3.1. Analytical performance characteristics of real-time PCR assays

The ASe of both qPCRs were calculated from testing 10-fold dilutions of plasmid DNA, diluted in either nuclease-free water or prawn DNA. The 10-fold dilutions were tested, and based on positive acceptance criteria being 60% of the replicates produce a positive result, both assays had an ASe of between 2 and 20 copies per reaction in both diluents (Table 4). The dynamic range of the PCRs was at least 7 logs, and the efficiencies of the qPCRs were within the expected range of 90–110%. To determine the ASp of the assays, extracted nucleic acids from other prawn pathogens were tested. None of the 9 heterologous pathogens tested generated a positive test result using either assay (Table 3). DNA extracted from 4 prawn species also generated negative test results. ASe and ASp testing indicated the assays demonstrated acceptable sensitivity, as the LOD was 2–20 genome copies per reaction, and specificity, as no positive WSSV detection were recorded when testing other prawn pathogens or host tissues (Table 3), according to fitness-for-purpose criteria for assay evaluation at AFDL. No primer and probe mismatches for either WSSV qPCR assay were observed after *in silico* analysis with representative sequences from Asia (China, India, Australia, Thailand, Taiwan, South Korea, Bangladesh and Vietnam), Iran and the Americas (United States, Mexico, Ecuador, Brazil and Peru).

3.2. Intra-laboratory repeatability of WSSV real-time PCR assays and inter-laboratory repeatability of the CSIRO WSSV qPCR

Two concentrations of plasmid control, specific for each WSSV real-time PCR, were tested on each PCR plate run. The AFDL acceptance criterion for positive control C_T is set at the mean ± 2 C_T of the first 10 plates tested with a new batch of NQC positive control. Bonferroni corrected repeated measures ANOVA indicated there was no significant statistical difference ($p > 0.05$) between operators for either the NQC1 or NQC2 positive controls for the CSIRO WSSV qPCR. For the WOAH WSSV qPCR, there was a significant statistical difference ($p < 0.05$) between several operators for both the NQC3 and NQC4 positive controls, respectively (Table 5). However, while the statistical analyses indicate the CSIRO WSSV qPCR is more repeatable than the WOAH WSSV qPCR, as coefficients of variation were <5% for all NQCs and all fell within the laboratory acceptance limits of ± 2 C_T values of the expected value, the statistical differences for the WOAH WSSV qPCR are considered minor. Based on these criteria, both assays demonstrated acceptable repeatability when tested by the same operator and acceptable precision when tested by different operators, based on 95% predictive intervals (Table 5).

The results using the C_T values obtained for NQC-2 (LEADDR positive control) in 5 laboratories around Australia, during 2016–2019 provided an indication of acceptable inter-laboratory reproducibility of the CSIRO WSSV qPCR. The mean C_T values (95% predictive interval) obtained for thQC-2 by the participating laboratories were 31.09 (30.23–31.95), 31.90 (29.60–32.52), 31.74 (29.22–34.26), 30.68 (27.96–33.40), and 30.75 (28.73–32.77), respectively. All coefficients of variation were <5%.

3.3. Confirmation of WSSV in clinically diseased prawns from the Logan River prawn farms

All 20 samples screening WSSV positive at BSL, from the index prawn farm on the Logan River, tested positive with both CSIRO and WOAH WSSV qPCR assays with C_T values ranging from 12.96 to 23.34

Table 4. Analytical sensitivity of the CSIRO and WOAH WSSV qPCR) assays. At least 3 of 5 replicates were required for a positive result and C_T values to be included. ND: not detected

Plasmid copies	C_T value (mean ± SD)			
	CSIRO WSSV qPCR		WOAH WSSV qPCR	
	Water	Prawn DNA	Water	Prawn DNA
2×10^8	5.68 ± 0.74	6.94 ± 1.34	10.48 ± 0.39	10.85 ± 0.14
2×10^7	7.55 ± 0.31	11.11 ± 1.90	13.49 ± .012	14.28 ± 0.13
2×10^6	12.21 ± 0.14	15.84 ± 0.23	17.11 ± 0.13	17.47 ± 0.64
2×10^5	15.75 ± 0.11	18.30 ± 1.94	20.66 ± 0.38	21.09 ± 0.65
2×10^4	19.49 ± 0.40	22.91 ± 0.18	24.31 ± 0.13	24.74 ± 0.12
2×10^3	23.15 ± 0.35	26.86 ± 0.59	27.45 ± 1.40	28.18 ± 0.33
2×10^2	27.30 ± 0.07	30.31 ± 0.28	31.97 ± 0.16	31.61 ± 0.74
2×10^1	30.82 ± 0.23	34.13 ± 0.46	35.60 ± 1.73	35.87 ± 0.43
2×10^0	34.51 ± 0.81	ND	39.02 ± 1.19	37.96 ± 0.37
2×10^{-1}	ND	ND	ND	ND
Efficiency	91.5%	91.6%	94.5%	97.0%

Table 5. Repeatability of the CSIRO and WOAH WSSV qPCR assays. NQC: network quality control. Identical superscript letters (a to e) indicate statistically different ($p < 0.05$) results between operators based on Bonferroni-corrected repeated measures ANOVA

Operator	CSIRO WSSV qPCR NQC-1			CSIRO WSSV qPCR NQC-2			WOAH WSSV qPCR NQC-3			WOAH WSSV qPCR NQC-4		
	No. of assays	C_T Mean±SD	95% PI	No. of assays	C_T Mean±SD	95% PI	No. of assays	C_T Mean±SD	95% PI	No. of assays	C_T Mean±SD	95% PI
1	14	23.90±0.33	23.24–24.56	14	31.05±0.66	29.72–32.37	9	24.83±0.22	24.38–25.28	9	31.50±0.21^{d}	31.08–31.91
2	8	23.96±0.32	23.32–24.59	8	31.03±0.40	30.22–31.84	8	25.02±0.28	24.46–25.58	9	31.73±0.45^{e}	30.84–32.63
3	102	24.04±0.32	23.40–24.68	102	31.10±0.48	30.14–32.05	64	25.12±0.32^{a}	24.49–25.76	61	32.14±0.43^{d}	31.28–32.99
4	9	24.10±0.36	23.37–24.82	9	31.20±0.34	30.51–31.88	9	25.13±0.27	24.58–25.67	9	32.04±0.39	31.26–32.81
5	22	24.03±0.16	23.71–24.36	22	31.37±0.27	30.84–31.90	43	25.19±0.31^{b}	24.56–25.82	44	32.09±0.40^{d}	31.28–32.89
6	106	24.12±0.37	23.39–24.85	102	31.21±0.40	30.41–32.00	25	24.87±0.29^{a,b,c}	24.29–25.45	23	31.91±0.39	31.13–32.70
7	86	24.04±0.27	23.49–24.58	91	31.06±0.42	31.20–31.90	12	25.00±0.46	24.07–25.93	12	31.95±0.51	30.93–32.97
8	2	24.30±0.16	23.97–24.62	2	31.76±0.54	30.67–32.84	113	25.12±0.33^{c}	24.46–25.78	114	32.14±0.38^{d,e}	31.38–32.90
9	37	23.91±0.46	22.99–24.84	37	31.11±0.51	30.09–32.13	3	25.20±0.61	23.98–26.42	3	32.07±0.46	31.15–32.98
10	110	23.95±0.33	23.30–24.61	110	31.08±0.44	30.20–31.96	7	25.01±0.23	24.54–25.47	6	31.99±0.38	31.22–32.75
11	13	23.96±0.32	23.31–24.61	13	31.04±0.38	30.29–31.79						
12	40	24.06±0.18	23.70–24.42	41	31.16±0.25	30.66–31.66						
13	10	24.03±0.18	23.93–24.64	4	31.56±0.19	31.19–31.93						
Overall	559	24.03±0.33	23.37–24.69	555	31.12±0.44	30.24–32.00	293	25.09±0.33	24.42–25.76	290	32.06±0.42	31.22–32.90

and 14.00 to 24.43, respectively (Table 1). As this represented the index case for a suspected exotic virus the samples were also tested by the WOAH WSSV nPCR for amplicon sequencing and comparison to confirm the presence of WSSV DNA. All 20 samples tested positive in the primary PCR and nPCR. Eight primary PCR amplicons were sequenced with primer-trimmed consensus sequences sharing 100% nucleotide identity with each other and reference WSSV nucleotide sequences, including WSSV strain CN01 (Ref Seq NC_003225.3). Subsequently, all 10 ethanol-fixed gill samples from the index pond from a second farm (Farm B) with *Penaeus monodon* mortalities tested positive with the CSIRO and WOAH WSSV qPCR assays, with C_T values ranging from 13.92 to 30.47 and 15.04 to 31.88, respectively. During the subsequent emergency response 100% of ethanol-fixed gills samples from clinically diseased *P. monodon* from index ponds from 4 additional farms (Farms C to F) tested positive by both assays (Table 1). All 71 clinically diseased prawns shared a similar frequency distribution of real-time PCR C_T values (Fig. 1) with a median C_T value of 17.77 using combined results from both assays. Based on positive test results for both real-time assays (CSIRO and WOAH WSSV qPCR) in 71 clinically diseased *P. monodon* from the index farm and 5 other farms (Table 1, Farms A to F), the median DSe was estimated to be 100% (95% exact binomial confidence interval of 94.9 to 100%). Samples from Farm C and Farm E were also tested by the WOAH WSSV nPCR with all samples testing positive and amplicons sharing 100% nucleotide identity with each other, samples from Farm A (index farm) and WSSV reference sequences. No gross clinical signs were visible in any of the prawns from Farms B to F. The positive test results for the 10 or 11 prawns sampled from each index pond from each of the 5 prawn farms were sufficient evidence for the pond to be classified as WSSV positive, which resulted in the prawn farm being classified as WSSV positive.

3.4. Surveillance testing of apparently healthy wild prawns from Moreton Bay for WSSV

Based on lack of evidence of mortality when samples were collected by trawl and absence of gross clinical signs observed during specimen receipt and processing in the receiving laboratory, these wild prawn surveillance samples were deemed to be apparently healthy. Of the 1196 wild prawn samples tested with both the CSIRO and WOAH WSSV qPCR

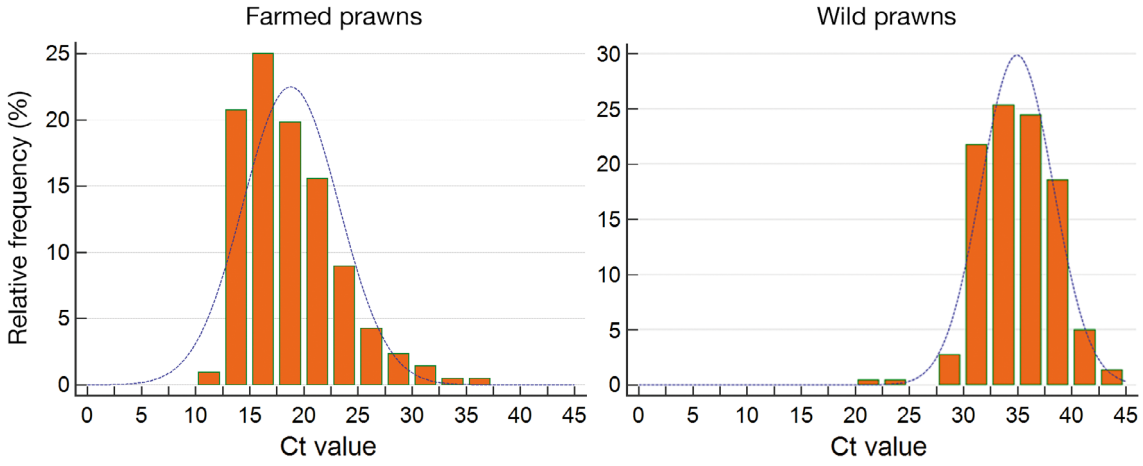

Fig. 1. Frequency distributions of C_T values, combined for both WSSV qPCR assays, for farmed prawns (clinically diseased) and wild prawns (apparently healthy). Median C_T values for the 2 populations of prawns were 17.77 and 34.03, respectively

assays, 1035 were test negative and 161 were test positive with either assay. Of these 161 test-positive samples, 133 (82.6%) were test-positive with the CSIRO WSSV qPCR, 122 (75.8%) were test-positive the WOAH WSSV qPCR, and 94 (58.4%) were test-positive by both assays. The C_T values for these samples ranged from 22.30 to 44.40 and 22.81 to 42.16 for the CSIRO WSSV qPCR and WOAH WSSV qPCR assays, respectively (Table 1), and the frequency distribution was markedly different to that seen for clinically diseased prawns (Fig. 1) with a median C_T value of 34.83.

3.5. Surveillance testing of apparently healthy wild prawns from the Gulf of Carpentaria for WSSV

All 395 samples of prawns sourced from the Gulf of Carpentaria were test-negative with both the CSIRO and WOAH WSSV qPCR assays. Of the 395 neat nucleic acid extracts tested, 390 (98.7%) had T4 qPCR C_T values within ±2 C_T values of the expected range for the T4 internal extraction positive control tested concurrently with the samples, indicating the absence of PCR inhibitors. When the 5 samples failing T4 quality control checks were retested after 1/10 dilution, all returned T4 C_T values within the expected range.

3.6. Bayesian latent class model

The CSIRO WSSV qPCR had a posterior median DSe of 82.9% (95% PI: 75.0–90.2%) in the conditional dependence model (code is in the Appendix) compared with a median DSe of the WOAH WSSV qPCR of 76.8% (95% PI: 68.9 –84.9%) but the posterior intervals overlapped (Table 6, Model A). Both the CSIRO and WOAH WSSV qPCR assays had a posterior median DSp of 99.7%. The Bayesian posterior probability value of 0.864 for the DSe difference can be interpreted as indicating that the DSe of the CSIRO WSSV qPCR was higher than that of the WOAH qPCR with 86.4% probability based on the specified model priors. The 95% PIs for the DSe and DSp covariances included zero, but they were left in the model to minimize any bias in DSe and DSp estimates. The median prevalence (95% PI) was 13.2% (11.0 to 15.5%). Inspection of history plots, brg plots, running quantiles, and small Monte Carlo error estimates indicated no evidence of lack of model convergence and there was minimal autocorrelation of the iterates.

A sensitivity analysis of a 10% decrease in the CSIRO and WOAH DSe prior medians (from 90.4 to 80.4%) resulted in a decrease of 3.0% in the posterior median DSe of the CSIRO DSe and a 2.6% decrease of the WOAH DSe median (Table 6, Model B). A 5% increase in the DSe prior medians for the CSIRO and WOAH WSSV qPCR assays from 90.4 to 95.3% (Table 6, Model C) resulted in the posterior median DSe for the CSIRO WSSV qPCR increasing by 1.1% (from 82.9% in Model A to 84.0% in Model C) and a 0.8% increase in the posterior median DSe for the WOAH WSSV qPCR. The DSp of both assays was unaffected by changes in the DSe priors of both assays and prevalence estimates were changed by <1%. When the joint tests results were interpreted in parallel (i.e. either or both tests were positive in infected prawns), the median DSe was 98.3% (95% PI: 91.6–99.9%). The corresponding

Table 6. Median diagnostic sensitivity (DSe) and specificity (DSp) and 95% probability intervals (PI) for the CSIRO and WOAH WSSV qPCR assays in 2 populations of apparently healthy prawns in Moreton Bay, Queensland (MB, Population 1), and the Gulf of Carpentaria (GOC, Population 2). Model A is the default model and Models B and C (sensitivity analysis) are for a 10% decrease and 5% increase, respectively, in the DSe of both assays

Parameters	Model A —DSe CSIRO, WOAH: beta (26.14, 3.08)—		Model B —DSe CSIRO WOAH: beta (26.14, 6.62)—		Model C — DSe CSIRO WOAH: beta (26.14, 1.6)—	
	Prior median (95% PI)	Posterior median (95% PI)	Prior median (95% PI)	Posterior median (95% PI)	Prior median (95% PI)	Posterior median (95% PI)
DSe: CSIRO	0.904 (0.763–0.976)	0.829 (0.750–0.902)	0.804 (0.647–0.915)	0.799 (0.706–0.869)	0.953 (0.832–0.995)	0.840 (0.764–0.929)
DSe: WOAH	0.904 (0.763–0.976)	0.768 (0.689–0.849)	0.804 (0.647–0.915)	0.742 (0.650–0.820)	0.953 (0.832–0.995)	0.776 (0.699–0.870)
DSp: CSIRO	0.5 (0–1)	0.997 (0.986–0.9999)	0.5 (0–1)	0.998 (0.989–0.9999)	0.5 (0–1)	0.997 (0.984–0.9999)
DSp: WOAH	0.5 (0–1)	0.997 (0.987–0.9999)	0.5 (0–1)	0.998 (0.989–0.9999)	0.5 (0–1)	0.997 (0.985–0.9999)
Prevalence MB	0.5 (0–1)	0.132 (0.110–0.155)	0.5 (0–1)	0.136 (0.114–0.163)	0.5 (0–1)	0.131 (0.107–0.154)
Prevalence GOC	0.5 (0–1)	0.0005 (0.00002–0.001)	0.5 (0–1)	0.0005 (0.00002–0.001)	0.5 (0–0.1)	0.005 (0.00002–0.001)

DSp (i.e. both tests negative in uninfected prawns) decreased slightly to 99.4% (95% PI: 97.9–99.9%).

The posterior medians for DSe in a conditional independence model (DSe and DSp covariances set to zero) with flat priors on all parameters except prevalence were about 5% lower than the corresponding DSe values in the dependence model. The median DSe for the CSIRO assay was 78.2 (95% PI = 69.8–87.6%), and the median DSe for the WOAH assay was 71.7% (95% PI = 63.3–80.1%). Because the conditional independence model did not include the scientific opinion of the lead author, we did not prefer it over the default Model A in Table 6. Inferences for specificities and prevalence changed minimally compared to Model A.

4. DISCUSSION

The data presented describe the evaluation of 2 real-time, TaqMan qPCR assays. Prior to the WSSV outbreak in Queensland in 2016, determination of the analytical characteristics of the WOAH and CSIRO WSSV qPCR assays had been undertaken following the standard WOAH pathway for the evaluation and implementation of new assays. The ASe of both the CSIRO and WOAH WSSV qPCR assays was ≤20 plasmid copies per reaction. An ASe value of ≤20 copies per reaction for a qPCR assay is deemed acceptable within AFDL to justify further evaluation of the performance characteristics of an assay. Within AFDL, no C_T cut-off values are used for real-time PCR assays as all real-time PCR tests generating a typical amplification curve are considered positive and further investigations are undertaken, which can include re-testing, testing by conventional PCR and amplicon sequence analysis and collection of additional samples for testing from the source population for increased monitoring of the population. This is considered a valid alternative to using C_T cut-off values (Caraguel et al. 2011). For this reason, ASe was not used to determined cut-offs for subsequent testing. Acceptable results were also obtained for ASp where the qPCR assays did not detect genomic material from 9 heterologous prawn pathogens. The failure of the assays to generate a positive result with DNA extracted from different prawn species was also documented to ensure there were no ASp concerns with host tissues. The use of T4 bacteriophage as an internal extraction control was implemented as a control for PCR inhibition and was also useful due to a lack of specificity of the WOAH Decapod PCR (Lo et al. 1996), for some prawns and crabs, and a Shrimp EF1 qPCR (Cowley et al. 2018), for some prawns, identified when testing submissions during subsequent WSSV surveillance activities (data not shown). While not as accurate as species-specific housekeeping gene assays, the T4 assay was considered a viable alternative as decapods of unknown, or uncertain, identity were submitted for testing. Testing at ACDP during and after the WSSV disease outbreak enabled generation of repeatability data for the CSIRO and

WOAH WSSV qPCRs which were generated by 13 and 10 different operators, respectively. The narrow range of the 95% PI values, coefficients of variation of <5% for each operator, and statistical analysis within the laboratory overall (Table 5), support the repeatability of the assays as acceptable. This also gives credibility to the acceptance range for positive control results of ±2 C_T values, as appropriate and not overly broad. Inter-laboratory testing of the plasmid positive control also demonstrated the repeatability of the CSIRO WSSV qPCR. Overall, the ASe, ASp and repeatability results indicated that the CSIRO and WOAH WSSV qPCR assays were performing to an acceptable level according to AFDL test evaluation criteria for fitness for purpose. This testing completed Stage 1 of the WOAH assay validation pathway and justified undertaking Stages 2 and 3, to estimate DSe and DSp, and reproducibility in different laboratories, respectively.

The WSSV outbreak provided the samples required to generate DSe data for the purpose of confirmation in clinically diseased prawns. Index pond specimens, consisting of 71 clinically diseased prawns, from 5 farms in the affected region all generated positive results with both the CSIRO and WOAH WSSV qPCRs. Therefore, the DSe when testing clinically diseased prawns for the purpose of designating a pond, and therefore a farm, as WSSV-positive was estimated to be 100%. Further confidence in the qPCR results was obtained after selected samples from each of the index ponds from each of the farms were confirmed WSSV-positive using the WOAH WSSV nPCR followed by sequencing of amplicons, as all sequences shared 100% nucleotide identity with WSSV reference sequences. For the index case, the approach of screening by qPCR, using 2 assays where followed by confirmation of the presence of WSSV by conventional PCR and amplicon sequence analysis, enabled notification of regulatory authorities within 14 h of sample receipt into AFDL and notification to the WOAH within 24 h of confirmation of the presence of WSSV.

To calculate the minimum sample size to detect WSSV with appropriate confidence in areas outside the WSSV control zone, accurate estimates of DSe and DSp for the purpose of WSSV detection in apparently healthy prawns was required. As no perfect reference standard test was available to classify with certainty the true infection status of individual prawns, a BLCM was used. This is a complicated, time-consuming and expensive exercise, particularly when the pathogen under study is exotic or has a very limited distribution. The number of test results generated from apparently healthy prawns during the emergency response to the WSSV incursion, provided an opportunity to estimate DSe and DSp in this target population. Individual test results from samples were available from surveillance testing of apparently healthy wild prawns in Moreton Bay, Queensland (within the WSSV Movement Regulated Area), as well as from prawns sourced from the Gulf of Carpentaria in Queensland (>3500 km from the WSSV Movement Regulated Area), where WSSV had never been detected. Due to the use of standard sampling and testing protocols, it was considered reasonable to expect that the assumption of constant DSe and DSp across tested populations would be met. The BLCM estimated that the WOAH WSSV qPCR and CSIRO WSSV qPCR had median DSe of 76.8 and 82.9%, respectively, and median DSp of 99.7% for both assays for surveillance in apparently healthy prawns when either replicate well testing positive classified a sample as positive. When both assays were interpreted in parallel, the median DSe increased to 98.3% and the DSp decreased slightly to 99.4%. Parallel testing of apparently healthy animals with increased DSe and only slightly reduced DSp reduces the risk of false-negative results while only slightly increasing the risk of false-positive results, which is particularly important when screening for exotic pathogens.

Estimates of the DSe and DSp enable calculation of appropriate sample sizes to ensure that surveillance programs, achieve the desired level of confidence (WOAH 2021b). Generation of 95% probability intervals for DSe and DSp allows a range to be considered when determining sample size, depending on how conservative the surveillance requirements are. The ability to accurately calculate appropriate sample sizes based on using a single assay or 2 assays in parallel will also contribute to efficiencies in testing where it may be more economical to reduce sample sizes by increasing the number of tests, particularly if lethal sampling of expensive animals (i.e. broodstock) is required, or samples may be difficult to obtain due to geography or density (i.e. wild samples). Determining DSe and DSp for detection in clinically diseased and apparently healthy animals completed Stage 2 of the WOAH assay validation pathway.

The generation of the performance characteristics described herein reduces the need for laboratories to undertake their own studies as equivalence (comparability) testing would provide confidence that the assays have been successfully implemented and are fit for purpose. Both the CSIRO and WOAH WSSV qPCR assays demonstrated comparable performance character-

istics, and the data generated contribute to the validation of the assays for the purpose of detection of WSSV in clinically diseased prawns and detection of WSSV in apparently healthy animals. As the CSIRO WSSV qPCR had slightly higher DSe and was statistically more repeatable, compared to the WOAH WSSV qPCR, the CSIRO WSSV qPCR is the primary screening assay, with the WOAH WSSV qPCR considered an acceptable alternate assay within AFDL. Inclusion of the results described in this study, in the WOAH Manual of Diagnostic Tests for Aquatic Animals Chapter for Infection with WSSV (WOAH 2018) will contribute to improved international standards.

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Appendix. OpenBUGS code for diagnostic sensitivity (Se) and specificity (Sp) of CSIRO WSSV qPCR (Test 1) and WOAH WSSV qPCR (Test 2) and prevalence in 2 wild prawn populations allowing for Se and Sp dependence between tests. The conditional independence (CID) model is a special case of the conditional dependence (CD) model with the CovSe12 and CovSp12 terms set to zero and the upper and lower limits removed

```
model {
y1 [1:Q, 1:Q] ~ dmulti(p1[1:Q, 1:Q], n1)
y2 [1:Q, 1:Q] ~ dmulti(p2[1:Q, 1:Q], n2)
p1[1,1] <- pi1*(Se1*Se2 + CovSe12) + (1-pi1)*((1-Sp1)*(1-Sp2) + CovSp12)
p1[1,2] <- pi1*(Se1*(1-Se2) - CovSe12) + (1-pi1)*((1-Sp1)*Sp2 - CovSp12)
p1[2,1] <- pi1*((1-Se1)*Se2 - CovSe12) + (1-pi1)*(Sp1*(1-Sp2) - CovSp12)
p1[2,2] <- pi1*((1-Se1)*(1-Se2) + CovSe12) + (1-pi1)*(Sp1*Sp2 + CovSp12)
p2[1,1] <- pi2*(Se1*Se2+CovSe12) + (1-pi2)*((1-Sp1)*(1-Sp2) + CovSp12)
p2[1,2] <- pi2*(Se1*(1-Se2)-CovSe12) + (1-pi2)*((1-Sp1)*Sp2 - CovSp12)
p2[2,1] <- pi2*((1-Se1)*Se2 -CovSe12) + (1-pi2)*(Sp1*(1-Sp2) - CovSp12)
p2[2,2] <- pi2*((1-Se1)*(1-Se2)+CovSe12) + (1-pi2)*(Sp1*Sp2 + CovSp12)
Se1 ~ dbeta (26.14, 3.08) #median is 0.904 (95% PI = 0.763 to 0.976)
Se2 ~ dbeta (26.14, 3.08)
Sp1 ~ dbeta(1,1)
Sp2 ~ dbeta(1,1)
pi1~dbeta(1,1)
pi2~dunif(0,0.0001)
Se12diff<-Se1-Se2
PrSe12diff<-step(Se12diff)
Sp12diff<-Sp1-Sp2
PrSp12diff<-step(Sp12diff)
CovSe12~dunif(LowerCovSe12,UpperCovSe12) # CovSe12<-0 changes this to a CID model
LowerCovSe12<- (Se1-1)*(1-Se2)
UpperCovSe12<- min(Se1,Se2) - Se1*Se2
CovSp12~dunif(LowerCovSp12,UpperCovSp12) # CovSp12<-0 changes this to a CID model
LowerCovSp12<- (Sp1-1)*(1-Sp2)
UpperCovSp12<- min(Sp1,Sp2) - Sp1*Sp2
ParallelSe12<-1-((1-Se1)*(1-Se2) + CovSe12) # One or both PCR results are positive in infected prawns
ParallelSp12<-Sp1*Sp2 + CovSp12 #Both PCR results are negative in uninfected prawns
}
list(n1=1196, n2=395, Q=2,
y1=structure(.Data = c(94,39,28,1035),. Dim = c(2,2)), # Morton Bay population
y2 =structure(.Data = c(0,0,0,395),. Dim = c(2,2))) # Gulf of Carpentaria population
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