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Enterocytozoon hepatopenaei (EHP)

Molecular Assay Development and Performance assessment

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

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Abbreviations

AHPND	Acute Hepatopancreatic Necrosis Disease
ASe	Analytical Sensitivity
ASp	Analytical Specificity
BLAST	Basic Local Alignment Search Tool
BLCM	Bayesian Latent Class Model
Cas	CRISPR-associated protein
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Ct	Cycle Threshold
DNA	Deoxyribonucleic Acid
DSe	Diagnostic Sensitivity
DSp	Diagnostic Specificity
DTE	Dilution to Extinction
EHP	*Enterocytozoon hepatopenaei*
GAV	Gill-Associated Virus
IHHNV	Infectious Hypodermal and Hematopoietic Necrosis Virus
LOD	Limit of Detection
PCR	Polymerase Chain Reaction
PTP	Polar Tube Protein
PTP2G	PTP2 qPCR assay developed by Genics
PTP2W	PTP2 qPCR assay developed by Wang *et al.*, 2020
RPA	Recombinase Polymerase Assay
rRNA	ribosomal Ribonucleic Acid
RSD	Relative Standard Deviation
SD	Standard Deviation
SMP	Shrimp MultiPath
SMP EHP	Shrimp MultiPath *Enterocytozoon hepatopenaei* assay
SSU	Small Subunit
STD	Standard
SWP	Spore Wall Protein
SWP26G	SWP qPCR assay developed by Genics
TNA	Total Nucleic Acid
WSSV	White Spot Syndrome Virus

Executive Summary

What the report is about

This report summarises the development and performance assessment of novel PCR based assays to monitor and detect the microsporidian *Enterocytozoon hepatopenaei* (EHP) in Penaeid shrimp samples using a cost-effective and high-throughput approach.

The original Project objective was to collect White Spot Syndrome (WSSV) positive samples from infected shrimp ponds (international location) and to use the sample material to complete an assay validation pathway for Shrimp Multipath™ WSSV assays (SMP WSSV) in line with the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2021, Chapter 1.1.6. Principles and methods of validation of diagnostic assays for infectious diseases (https://www.oie.int/en/what-we-do/standards/codes-and-manuals/terrestrial-manual-online-access/). A large sampling effort was undertaken by colleagues in Soc Trang province, Vietnam, from one shrimp farm that observed "weak" shrimp on feeding tray and water surface of two independent ponds, suspected WSSV outbreak. Live animals (n = 576) were collected from two ponds and sent to Genics for testing / screening for a potential infectious agent. Instead of detecting WSSV in the shrimp specimens received a relatively high prevalence of EHP positive samples were established with the multiplexed SMP assay. As further sampling efforts would have shifted the timeline of the commencement of the project too much and other means to validate WSSV assays in SMP were explored it was decided to use the sample set for SMP EHP validation purposes and pivot the project to develop new assay targets and real-time PCR assays for EHP in penaeid shrimp.

Background

The occurrence and distribution of EHP around the world, since the formal description of this pathogen in 2009, is ever increasing. EHP is a major threat to the global shrimp aquaculture industry due to its potential for growth retardation, as well as increasing the susceptibility of farmed shrimp to co-infections with other pathogens.

In response to the rising threat of EHP several diagnostic molecular tools have been developed, ranging from standard PCR or nested PCR approaches to quantitative real-time PCR (qPCR) as well as isothermal amplification methods. Assay development efforts so far have focussed only on a handful of molecular targets of EHP including Spore Wall Protein 1 (SWP1) and the 18S rRNA gene (SSU rRNA).More recently the Polar Tube Protein 2 (PTP2) has been identified as a diagnostic target which has led to the development of a real-time PCR assay and a CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas12a coupled recombinase polymerase assay (RPA).

An additional concern is the fact that all conventional PCR methods developed for detection of EHP are relatively costly and lack sufficient throughput to offer a cost-effective solution for farmers to undertake regular health-checks on their farm let alone deploy entire farm pathogen screening/monitoring schemes for early detection and thereby enable risk mitigation to be implemented.

Objectives

1) Develop new qPCR assays for novel target genes to screen and detect EHP in farmed penaeid shrimp.
2) Assess and validate the relatively new, high throughput, and cost-effective diagnostic test Shrimp Multipath™ (SMP) targeting SSU rRNA of EHP (SMP EHP) in parallel with 12 additional shrimp pathogen targets.

Methodology

The assays using the two new targets, PTP2 and SWP26, were assessed for basic performance metrics such as amplification efficiency and Analytical Sensitivity (ASe) using a synthetic template of the two markers *in lieu* of the EHP virus itself. Analytical Specificity (ASp) was assessed on small selection of EHP positive and negative shrimp samples and successful amplification confirmed by bi-directional direct amplicon Sanger sequencing. The qPCR assays for PTP2 and SWP26, along with using the SMP EHP, were analysed for inter-run and intra-run repeatability using a pools of clinical shrimp samples determined to carry EHP. Final assessment of the new assays was aimed at determining the Diagnostic Sensitivity (DSe) and Diagnostic Specificity (DSp) using samples of shrimp with and without clinical signs of disease compared to two published reference assays. All positive results were confirmed by direct amplicon sequencing of PCR products; where appropriate, additional sequence data was submitted to GenBank, the public, worldwide repository of nucleic acid sequence data.

Results/key findings

The two new qPCR assays targeting PTP2 and SWP26, as well as SMP EHP targeting SSU rRNA, were shown to perform to a high standard. The findings highlight the importance of developing additional EHP gene targets for diagnostic test development to counter poorly performing reference PCR assays as well as to generate global sequence data to better capture genetic variation in EHP.

Implications for relevant stakeholders

This study provides novel gene target PCR assays for detection of EHP in Penaeid shrimp tissues, in addition to validation data on SMP EHP, which can be adopted for use by industry to monitor the potential spread of EHP through brood stock and post larvae but also live feed (e.g. live polychaetes).

Recommendations

An exhaustive sequencing study of EHP positive shrimp samples from different regions of the world is recommended to identify genetic variants of EHP. From the findings of such a study, recommendations could be made to identify and assess new molecular targets for incorporation into PCR diagnostic platforms. From this, further assessment of the new targets would be made regarding diagnostic specificity and sensitivity that would encompass a wider geographic range of EHP. The ability to detect a large majority of EHP variants would allow a more systematic screening for the EHP pathogen across the globe and would enable the monitoring of artificial spread through live animal trade (broodstock, post larvae, and feed). .

Keywords

Penaeid Shrimp, Microsporidian, *Enterocytozoon hepatopenaei,* EHP, Hepatopancreatic microsporidiosis, HPM, Spore Wall Protein, Polar Tube Protein, Shrimp MultiPath™, PCR, diagnostics, detection

1.0 Introduction

Enterocytozoon hepatopenaei (EHP) is a microsporidian and the causative agent of Hepatopancreatic microsporidiosis which is reported to infect different Penaeid shrimp species including *Penaeus (Litopenaeus) vannamei, Penaeus monodon* and *Penaeus stylirostris*, mainly in the Asia-Pacific region (Anderson *et al.*, 1989; Hudson *et al.*, 2001; Chayaburakul *et al.*, 2004; Tourtip *et al*., 2009). Early reports indicated the absence of clinical signs in animals infected with EHP and highlighted that the key issue of EHP infections was growth retardation of affected shrimp leading to a significant economic impact for affected aquaculture enterprises with the potential loss of tens of thousands of tons of production valued at hundreds of millions of USD (Patil and Geetha, 2021). EHP infection has been more recently shown to increase the susceptibility of shrimp to acute hepatopancreatic necrosis disease (AHPND) (Aranguren *et al.,* 2017).

Histopathological methods, combined with *in situ* hybridisation, have been used successfully to analyse and describe EHP infections and help identify the location of the parasite and respective spores within the tissue of infected shrimp (Tourtip *et al*., 2009; Tangprasittipap *et al*., 2013; Tang *et al*., 2015; Sanguanrut *et al*., 2018). Distribution of EHP in the hepatopancreas tubules is known to be uneven making detection of the microsporidian unreliable. Although histology has proven to be useful for EHP detection, it is not practical in terms of rapid test turnaround time or suitable as a surveillance or monitoring tool. As a diagnostic test neither specificity nor sensitivity has been established, factors which further limit the utility of histology as a tool of detection.

In preference to histology, a number of methods, based on the molecular detection of the parasite, have been developed for the detection of EHP. Of the molecular methods available, polymerase chain reaction (PCR)approaches have shown the greatest utility. An effective assay is a nested PCR method, which targets the EHP spore wall protein gene and is used as the main assay for EHP detection (Jaroenlak et al., 2016). Efforts are still ongoing to expand the molecular tools available to detect EHP and these are summarised by Chaijarashphong *et al.* (2020).

Interestingly, almost all efforts to develop molecular assays for the detection and surveillance of EHP are focussed on only a handful of genomic targets. Ordered by most to less frequently used targets these are: small subunit ribosomal RNA gene (SSU-rRNA), EHP spore wall protein 1 (SWP1), polar tube protein 2 (PTP2), beta-tubulin gene. qPCR and nested PCR assays show in general good sensitivity. Yet, assays targeting SSU-rRNA (Liu *et al.*, 2018; Hou *et al.*, 2021), a highly conserved gene, lack some degree of specificity compared to assays targeting specific gene targets like spore wall protein or polar tube protein genes (Jaroenlak *et al.*, 2016; Wang *et al.*, 2020) and are therefore at risk of reporting false positive results. In addition, sensitivity and complete assay failure issues have been observed (internal results) with the SWP1 nested assay as well as SSU-rRNA assay (Liu et al., 2018) when run against samples of Latin American origin that were later confirmed by Sanger sequencing to contain EHP.

The frequency of use of a very restricted number of assay targets and the limited amount of EHP related sequence information is reflected in the number of sequences that have been deposited in GenBank which are: 62 ribosomal RNA subunit entries, followed by 16 spore wall protein, four beta-

tubulin and one polar tube protein 2 entries.[1] In addition, only two Whole Genome Shotgun assemblies (GenBank Accession Nos. ASM208167v1 and ASM370911v1) are publicly available for mining of sequence data. This resource is vital to identify novel gene targets for diagnostic assay development and to subsequent understanding of EHP genetic diversity of highly conserved housekeeping genes and more importantly to elucidate genetic variation of species-specific gene targets like SWP1 and PTP2.

As indicated above, specificity and sensitivity issues exist with assays designed previously to both EHP gene targets 18sRNA and SWP1, which are the main diagnostic targets to monitor EHP. Therefore, new molecular assays, better access to cost-effective screening tools and a better understanding of genetic variation of the EHP pathogen are needed by the shrimp industry and regulators worldwide to help better manage and mitigate the risks posed by EHP. This study addresses the requirement for novel gene targets and assays by describing two new real-time PCR assays targeting Polar Tube Protein 2 (PTP2) and novel Spore Wall Protein 26 (SWP26) gene targets. In addition, this study takes a close look at the commercially available Shrimp MultiPath™ EHP assay which is a cost effective high-throughput PCR platform available globally, that can detect 13 shrimp pathogens simultaneously. Furthermore, this report offers a good insight into the molecular diversity of the two novel target genes and offers a plausible explanation on the failure of some gene specific assays that work in Asia-Pacific EHP strains to perform at all in Latin American strains of EHP.

[1] https://www.ncbi.nlm.nih.gov/search/all/?term=Enterocytozoon%20hepatopenaei

2.0 Objectives

1) Develop new qPCR assays for novel target genes to screen and detect EHP in farmed penaeid shrimp.
2) Assess and validate the relatively new, high throughput, and cost-effective diagnostic test Shrimp Multipath™ (SMP) targeting SSU rRNA of EHP (SMP EHP) in parallel with 12 additional shrimp pathogen targets.

3.0 Methods

3.1 Sample collection and nucleic acid extraction

A pool of tissue types (gill, lymphoid organ, hepatopancreas, stomach, epithelial cells, muscle) was taken from random live *Litopenaeus vannamei* shrimps collected from shrimp ponds where weak animals were observed in Soc Trang Province, Vietnam and shrimp ponds in Latin America (two locations). As a BICON Exemption Permit exists for shrimp samples preserved in 70% ethanol, an international import permit was not required as the pools of tissue types were submerged in 70% ethanol for preservation[2]. Total nucleic acid (TNA = RNA and DNA) was extracted using a MagMAX™ Core nucleic acid purification kit with the KingFisher FLEX robot (Thermo Fisher Scientific, CA, USA) from a pool of tissue types. Briefly, extraction buffer was added to up to 30 mg dissected sample and combined with 2 ceramic and 5 glass beads followed by a homogenisation step on a TissueLyser II (QIAGEN, Hilden Germany) twice for 1.5 minutes at maximum speed. After visual confirmation that tissue homogenisation had been achieved, sample homogenates were mixed for 10 min at 10,000 rpm on a plate shaker at 4°C. 200µL of the sample homogenate was then further processed for total nucleic acid extraction using a MagMAX™ Core nucleic acid purification kit with the KingFisher FLEX robot (Thermo Fisher Scientific, CA USA). Extracted sample TNA was eluted in 50 µl PCR grade water and directly used for the different PCR based assays or stored at -80°C under further use.

3.2. PCR based assay design and run parameters

With the exception of SMP EHP, which is available commercially as a service at Genics Pty Ltd (Genics, Brisbane, Australia), all PCR assays developed in this study were designed using the PRIMER 3 software (Untergasser *et al.*, 2012) as implemented in the NCBI interface[3]. In summary, two SYBR green based real-time PCR assays targeting spore wall protein 26 (SWP26) and polar tube protein 2 (PTP2) were designed for development as diagnostic assays for monitoring EHP infection. Both target genes were identified via various sequence comparison approaches and sequence entries in GenBank, and selected based on species-specific characteristics (e.g. sequence conservation) as they relate to EHP specific morphologies. In addition, and as means to monitor sequence variation that might potentially be encountered, two long amplicon standard PCR assays each targeting SWP26 and PTP2, respectively, were developed and deployed for direct amplicon sequencing. The PCR based SMP EHP assay, which targets the EHP 18S rRNA gene as well as nested PCR and real-time qPCR

[2] Bicon case: Preserved and fixed animal and human specimens Effective: 14 Jan 2022 (https://bicon.agriculture.gov.au/BiconWeb4.0/)

[3] https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome

assays targeting the EHP SWP1 and PTP2 gene (Jaroenlak *et al.*, 2016; Wang *et al.*, 2020), respectively, were assessed in parallel to compare performance with the two new target assays SWP26G and PTP2G. Assays used in this study are listed in Table 1.

qPCR assays SWP26G, PTP2G, and PTP2W were setup in 5µl aliquots of quadruplicate reactions in 384-well PCR plates using the PowerUp SYBR Green Master Mix with 0.4nM forward and reverse PCR primers and run on the QuantStudio 12K Flex Real-Time PCR system (Thermo Fisher Scientific, CA USA). Cycle conditions were an initial 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 30 sec. and a final extension step of 95 °C for 15 sec and 60 °C for 1 min and a terminal heating step to 95 °C at a ramp rate of 0.05 °C/sec. Synthetic Templates as positive controls for all three assays were purchased from Integrated DNA Technologies (IDT, Iowa, USA). Data were reviewed and analysed using QuantStudio 12K Flex Software v1.4 (Applied Biosystems, ThermoFisher Scientific, MA, USA).

Standard long amplicon PCR assays SWP26L2, SWP26L4, PTP2L1, and PTP2L10 were setup using the Platinum SuperFi II PCR Master Mix chemistry according to manufacturer's instructions and run on Veriti PCR thermocyclers (Thermo Fisher Scientific, CA, USA) in 96-well format with the following cycling conditions resembling a touchdown approach. Initial denaturation at 98 °C for 30 sec, 10 cycles of 98 °C for 10 sec, 65 °C for 10 sec and 72 °C for 30 sec (decrease of Tm by 0.5 °C every cycle), followed by 30 cycles of 98 °C for 10 sec, 65 °C for 10 sec and 72 °C for 30 sec, with a final extension step at 72 °C for 5 min. An aliquot of 5 µl was analysed on a 1% Agarose gel to confirm absence/presence of PCR amplicon bands.

Standard PCR (nested) was setup using the One*Taq*® Hot Start Quick-Load® 2X Master Mix with Standard Buffer (New England Biolabs, MA, USA) run as per manufacturer's instructions on Veriti PCR thermocyclers 96-well format (Thermo Fisher Scientific, CA, USA) as described by Jaroenlak *et al.* (2016). An aliquot of 5 µl was analysed on a 1% Agarose gel to confirm absence/presence of PCR amplicon bands.

Table 1. Assays and corresponding primer sequences used in this study.

Target Gene	Assay	Primer Sequences	Annealing Temp [°C]	Amplicon Size [bp]	Sequence Accession #	Note
Spore Wall Protein 26 (SWP26)	SYBR qPCR – SWP26G	SWP26G F 5'-AAGAGGGTGGTTACTGAAGTCAT-3' SWP26G R 5'-CTGGAAAGTCCGGCATCACA-3'	60	86	MN604022	This study[1]
Polar Tube Protein 2 (PTP2)	SYBR qPCR – PTP2G	PTP2G F 5'-ATGGTAAGGTGGTTGGCCTG-3' PTP2G R 5'-GAGTGCAAGAACACATGCGA-3'	60	88	MT249228	This study[2]
Polar Tube Protein 2 (PTP2)	SYBR qPCR – PTP2W	PTP2W F 5'-GCAGCACTCAAGGAATGGC-3' PTP2W R 5'-TTTCGTTAGGCTTACCCTGTGA-3'	60	238	MT249228	Wang *et al.* (2020)
Spore Wall Protein 1 (SWP1)	Nested PCR step1 – NestedPCR SWP1 1	SWP_1F 5'-TTGCAGAGTGTTGTTAAGGGTTT-3' SWP_1R 5'-CACGATGTGTCTTTGCAATTTTC-3'	58	514	KX258197	Jaroenlak *et al.* (2016)
	Nested PCR step2 – NestedPCR SWP1 2	SWP_2F 5'-TTGGCGGCACAATTCTCAAACA-3' SWP_2R 5'-GCTGTTTGTCTCCAACTGTATTTGA-3'	64	148		
Spore Wall Protein 26 (SWP26)	Standard long amplicon PCR - SWP26L2	SWP26L_2F 5'-ATTGTTAAATGACTTTCAATACCCT-3' SWP26L_2R 5'-GCATGCTCAAACAAGAACAAC-3'	56	600	MN604022	This study
	SWP26L4	SWP26L_4F 5'-AATGACTTTCAATACCCTCATCAC-3' SWP26L_4R 5'-ATTTTATGCATGCTCAAACAAGA-3'	56	600		
Polar Tube Protein 2 (PTP2)	Standard long amplicon PCR – PTP2L1	PTP2L_1F 5'-ACCAGATGGTAAGGTGGTTG-3' PTP2L_1R 5'-ACAGTAAACATGCCTTTGCC-3'	56	735	MT249228.1	This study
	PTP2L10	PTP2L_10F 5'-TTCACTTTCAGATGGAGTTGG-3' PTP2L_10R 5'-GGACATGAAGGTGGAAACAG-3'	56	703		
18S rRNA	Shrimp MultiPath™ SMP EHP	Proprietary	56	NA	KP759285.1	This study

[1] MNPJ01000024.1 (scaffold of MNPJ00000000.1 EHP TH1 whole genome shotgun sequencing)

[2] MNPJ01000011.1 (scaffold of MNPJ00000000.1 EHP TH1 whole genome shotgun sequencing) and QTJQ01000014.1 (scaffold of QTJQ00000000.1 EHP-ID6 whole genome shotgun sequencing

3.3 Direct Amplicon Sequencing

Standard PCR and real-time qPCR amplicons were sequenced directly to confirm sequence authenticity. PCR amplicons in original amplification reaction mix/volume and corresponding forward and reverse primers were submitted to the Australian Genome Research Facility (AGRF, Brisbane, Australia) for direct Sanger sequencing using Big Dye Terminator chemistry 3.1 and ABI Capillary Sequencer 3730xl (Thermo Fisher Scientific, CA USA).

The quality of sequence traces was checked and curated using the Sequencher Software (GeneCodes, MI, USA) and verified sequences confirmed using the BLASTn tool interface of NCBI[4].

3.4 Assay verification and validation

Real-time qPCR and SMP EHP assays were assessed against a list of performance criteria such as serial dilution and amplification efficiency assessment (qPCR), analytical sensitivity and specificity, repeatability and diagnostic sensitivity and specificity.

3.4.1 qPCR Standard Curve and Amplification Efficiency

Synthetic double stranded DNA templates (GBlocks; Integrated DNA Technologies, IA, USA) for each quantitative real-time qPCR assay were sourced from Integrated DNA Technologies and diluted in IDTE ((10 mM Tris, 0.1 mM EDTA) – Salmon Sperm DNA (10ng/µl) buffer in a 10-fold dilution series spanning 10,000 copies per reaction down to 10 copies per reaction. Four replicates per dilution step were run and amplification efficiencies calculated.

3.4.2 Analytical Sensitivity (ASe) and Specificity (ASp)

ASe or the limit of detection (LOD) to classify a sample positive or negative for EHP was determined via a dilution to extinction experiment and subsequent Logistic Regression analysis. Synthetic templates for each corresponding qPCR assay were diluted in non-symmetrical steps to reach extinction of the template in the highest dilution. The following dilution steps were assessed (in copies per reaction): 100, 10, 5, 2.5, 1.25, 0.625, 0.0625 and 0.00625. In parallel to determining LOD as a function of copy number concentration, the LOD experiment was used to also determine Cycle threshold cut-off range for each qPCR assay.

LOD was calculated via a Logistic regression analysis using MedCalc® Statistical Software version 20.008 (MedCalc Software Ltd, Ostend, Belgium) and Equation 1 below:

Equation 1:

$$X\,(Limit\ of\ Detection) = \frac{\ln\left(\frac{1-p}{p}\right) + b_0}{-b}$$

p = confidence interval 0.95
b_0 = Intercept or Constant
b = variable or Ct or value provided (e.g. copy #)

ASp was assessed initially on synthetic template in a shrimp TNA sample matrix background known to be EHP free. Further ASp assessment was done with 12 EHP positive shrimp samples (pool of tissue

[4] https://www.ncbi.nlm.nih.gov/home/about/; National Centre for Biotechnology Information, MD, USA

types) determined to be infected with EHP by qPCR, nested PCR, and SMP EHP, and confirmed with Sanger Sequencing. All assays were run on additional shrimp samples negative for EHP but with a background of different pathogens including Infectious hypodermal and hematopoietic necrosis virus (IHHNV), White Spot Syndrome virus (WSSV), Gill-Associated virus (GAV), and AHNPD PirA and PirB toxin genes determined via SMP screening.

3.4.3 Assay Repeatability

Assay repeatability was determined by pooling 30 infected EHP clinical *L. vannamei* samples (TNA) from a Latin American country, creating three two-fold dilutions (1:2 dilution; 1:4 dilution; 1:8 dilution) of the neat TNA pool and analysing 4 replicates per sample pool by three operators on three different days. Inter- and intra- run repeatability was assessed for primers PTP2G (Genics construct, this study), SWP26G (Genics construct, this study), and SMP EHP by calculating the Relative Standard Deviation (RSD) of Ct values (qPCR) or log transformed SMP EHP copy number results within and across runs. Transformation from linear copy number scale to log scale is necessary to allow for an even comparison to Ct values.

3.4.4 Diagnostic Specificity (DSe) and Sensitivity (DSp)

To estimate the diagnostic parameters of the qPCR assays SWP26G, PTP2G, and the SMP EHP assay, samples from two different populations of *L. vannamei* that had clinical signs of EHP (white faeces, slow growth) at time of sampling were analysed. In the absence of a perfect reference assay, DSe and DSp were estimated with a Bayesian latent class model (BLCM) constructed for five conditionally dependant tests (PTP2G, SWP26G, PTP2_Wang, SMP EHP assay and nested PCR) for 190 field samples from Penaeid shrimp populations in South-East Asia and for 95 samples from Penaeid shrimp population in Latin America. These models make no assumption of the true disease status of each individual sample and assume the tests under evaluation are imperfect. The diagnostic specifications (sensitivity and specificity), the prevalence amongst the samples for each population, and any conditional correlation terms, are all treated as unknown variables and jointly inferred. As detailed elsewhere (Cheung et al. 2021), such a model formulation was expected to be identifiable even with flat priors, as a five-tests-in-two-populations conditional dependence model is anticipated to have 62 degrees of freedom, for inferring 32 unknown parameters. A common assumption in such models is that the sensitivity and specificity of each test are constant across the populations sampled. As the nested PCR was considered to have potential for different performance in shrimp sampled from Southeast Asia and South America, the model was configured to test if this was the case by relaxing this assumption through the incorporation of ten additional unknown parameters (DSe and DSp in the 2nd population for the nested PCR, and conditional dependence terms with the other tests used). As little published information was available as a basis for priors for the prevalence or the test specifications, flat priors were used for all unknown parameters, i.e., Beta(1, 1) priors for prevalence in each population, diagnostic sensitivity and specificity of each test. The conditional dependence between all tests in the BLCM was modelled using covariance terms (Dendukuri and Joseph, 2001), with uniform hyper-priors constraining correlation terms for each test to the range (-1, 1). A 'saturated model' was constructed first, including all two-way covariance terms between tests in each BLCM and compared to models with covariance terms progressively excluded based on an assessment of deviance information criterion (DIC) in the fitted model (Spiegelhalter et al. 2002) and the inferred magnitude of each particular covariance term and its 95% highest probability density (Mathevon et al. 2017; Salgadu et al. 2021). The joint posterior distribution was modelled as two Markov Chain Monte Carlo (MCMC) chains of 20,000 iterations, with this chain length and discarding of the first 5000 as burn-in based on visual assessment of convergence and the chains, the Gelman-Rubin statistic (Gelman & Rubin, 1992) and estimates of effective sample size (ESS>200 for all

inferred parameters) and autocorrelation by lag using JAGS (Plummer, 2003), R2jags (Su & Yajima, 2015), mcmcplots (McKay Curtis et al. 2018) and the epiR (Stevenson et al. 2021) libraries in the R statistical software package (R Core Team, 2019). Final inferences are presented as the posterior median and 95% highest credibility interval based on quantiles of the joint posterior distribution.

4.0 Results

4.1 Novel EHP assay selection details

Out of a total of 4 potential EHP specific gene targets (SWP12; SWP1; SWP26; PTP2) and one generic gene target (SSU-rRNA) two candidate assays targeting PTP2 (assay PTP2G) and SWP26 (assay SWP26G) were selected in an initial screening approach. The criteria to select those assays in further investigations included (A) primer dimer formation in melt curve analysis and (B) concordance of real-time PCR results on a small subset of EHP positive *L. vannamei* samples that were pre-screened by SMP (data not shown) (Table 1).

4.2 Reference EHP real time qPCR assay

Three published PCR methods were tested to assess their suitability as a reference assay.

- Jaroenlak *et al.* (2016): Nested PCR targeting Spore Wall Protein 1 (nestedPCR SWP1)
- Wang *et al.* (2020): SYBR Green real-time PCR assay targeting Polar Tube Protein 2 (PTP2W)
- Liu *et al.* (2018): TaqMan real-time PCR assay targeting 18S rRNA (SSU rDNA)

The Jaroenlak *et al.* (2016) nested PCR assay and Wang *et al.* (2020) SYBR Green real-time PCR assay showed positive amplification results in the Vietnam *L. vannamei* samples (EHP positive by SMP). However, we were unable to get any positive amplification result using the Liu *et al.* (2018) TaqMan real-time PCR assay in the same samples. In the interest of time and resources this assay was dropped from further investigations.

4.3 Real time qPCR assay standard curve assessment

The two newly designed qPCR assays SWP26G and PTP2G, as well as the selected reference qPCR assay for EHP classification PTPW (Wang *et al.* 2020) were assessed against a standard curve titration using synthetic template DNA.

The new EHP qPCR assays showed a tight curve fit ($R^2 > 0.99$) along a dynamic range from 10,000 copies per reaction down to 10 copies per reaction run in quadruplicate replicates per titration point. Amplification efficiencies were 92.5% (SWP26G) (Figure 1) and 97.3% (PTP2G) (Figure 2, Table 2). However, reference assay PTP2W showed a reduced amplification efficiency of 88.6% using the dilution to extinction experimental dilutions (Figure 3, Table 2).

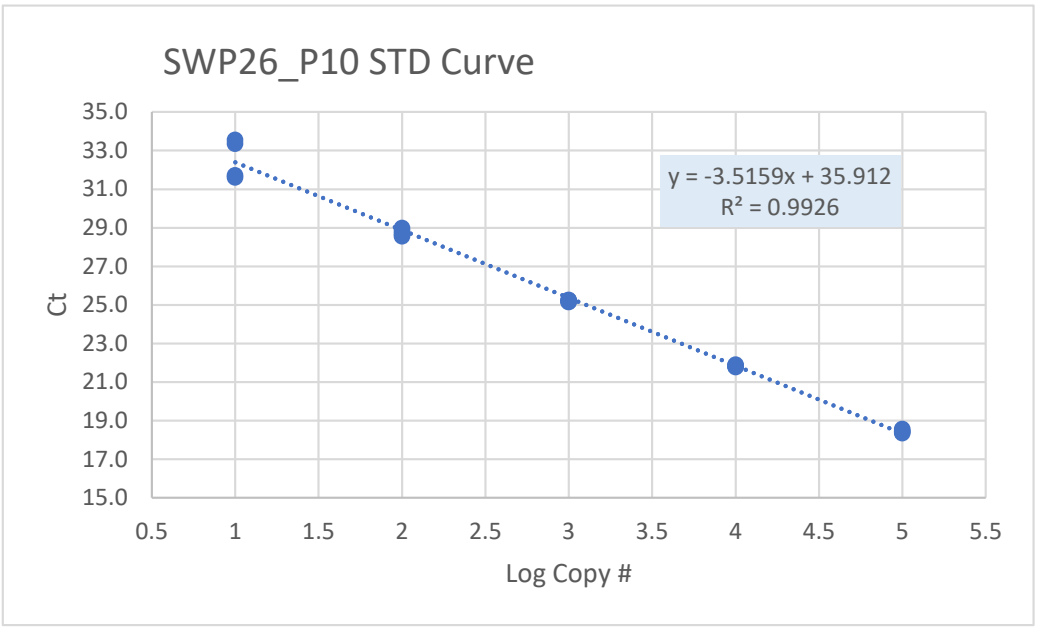


Figure 1. Standard curve for SWP26_ P10. R^2, coefficient of determination. Four replicates per data point using synthetic target DNA as template.

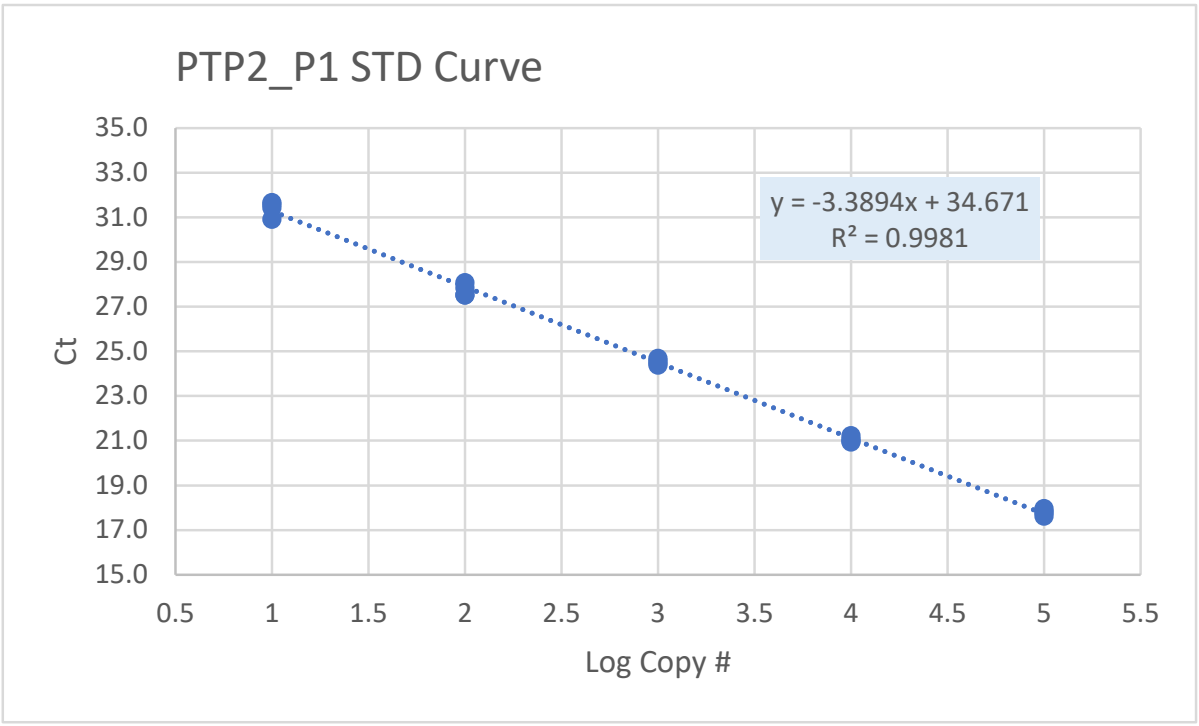


Figure 2. Standard curve for PTP2_P1. R^2, coefficient of determination. Four replicates per data point using synthetic target DNA as template.

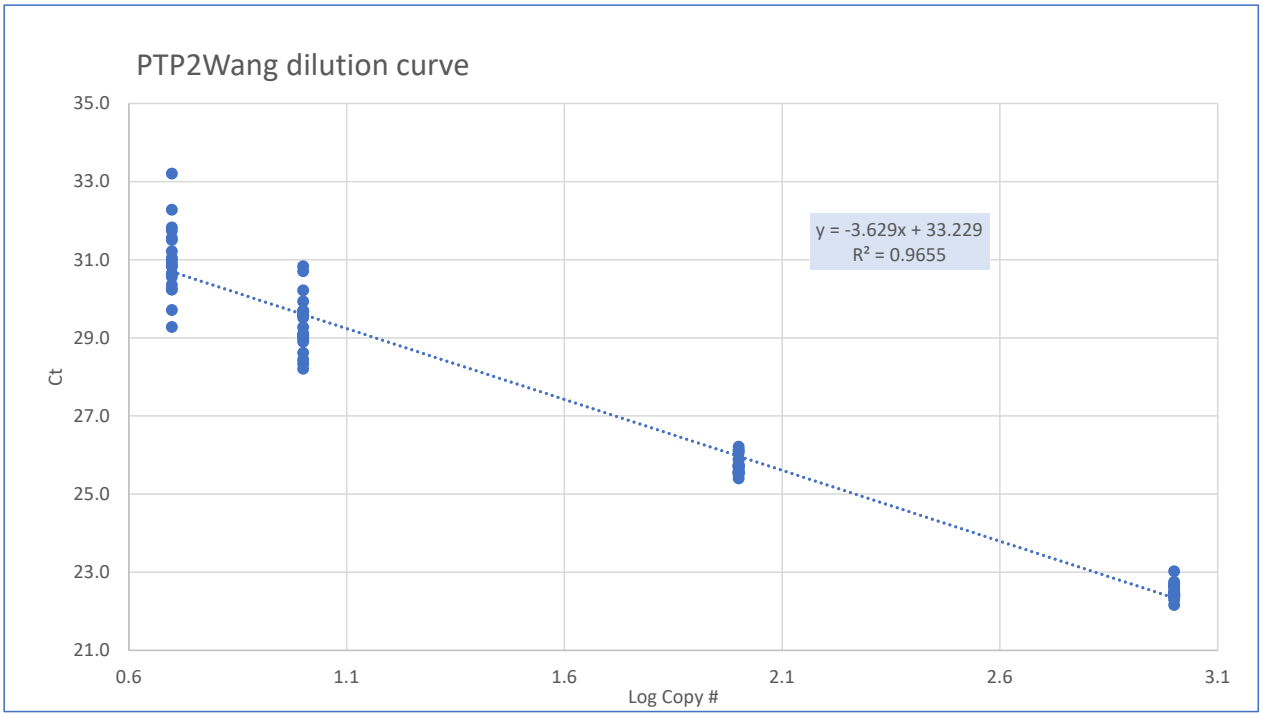


Figure 3. Standard curve for PTP2 Wang. R^2, coefficient of determination. Twenty replicates per data point using synthetic target DNA as template.

Table 2. ***EHP real-time PCR assay amplification efficiencies.***

Assay	Reference	Slope	Efficiency#	Amplification Efficiency [%]
SWP26G	This study	-3.5159	1.92	92.5
PTP2G	This study	-3.3894	1.97	97.3
PTP2W	Wang *et al*. 2020	-3.629	1.89	88.6
# Efficiency of 2 indicates perfect amplification dynamics with every cycle				

4.4 Real time qPCR assay direct amplicon sequencing, BLASTn confirmation, and analytical specificity

After confirming primer specificity *in-silico* using the Primer-BLAST[5] interface, all new qPCR assays were run on 12 clinical EHP positive samples (as identified with SMP EHP, 18S rRNA gene target and Sanger direct amplicon sequencing). Primers SWP26G and PTP2G showed 100% concordant identification results (12 of 12 samples positive).

To confirm target identity, real-time qPCR quadruplicate reactions (4 x 5 µl) were pooled and subjected to bi-directional amplicon Sanger sequencing, including the PTP2W reference real-time qPCR results on clinical samples.

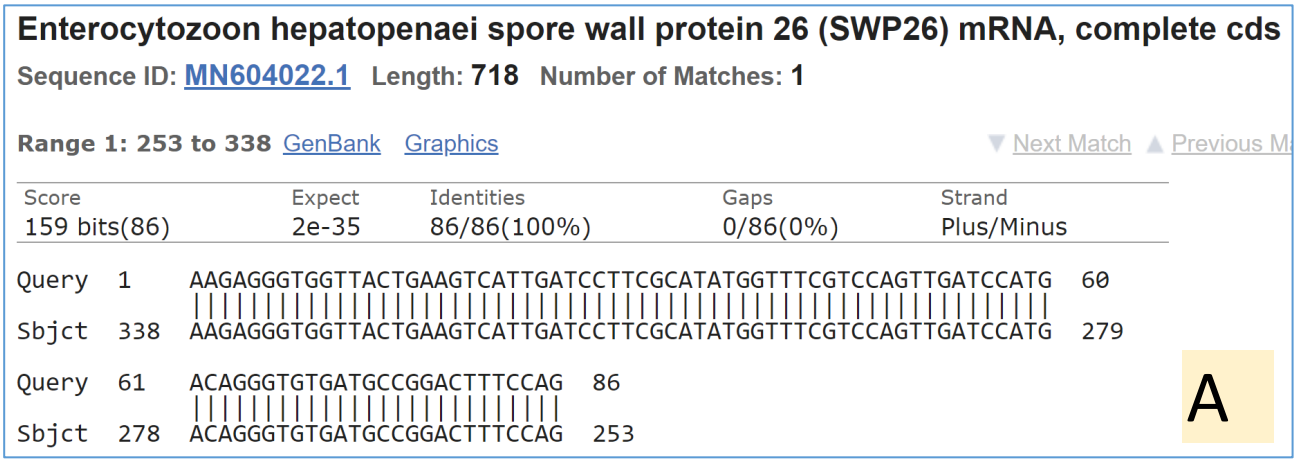
[5] https://www.ncbi.nlm.nih.gov/tools/primer-blast/

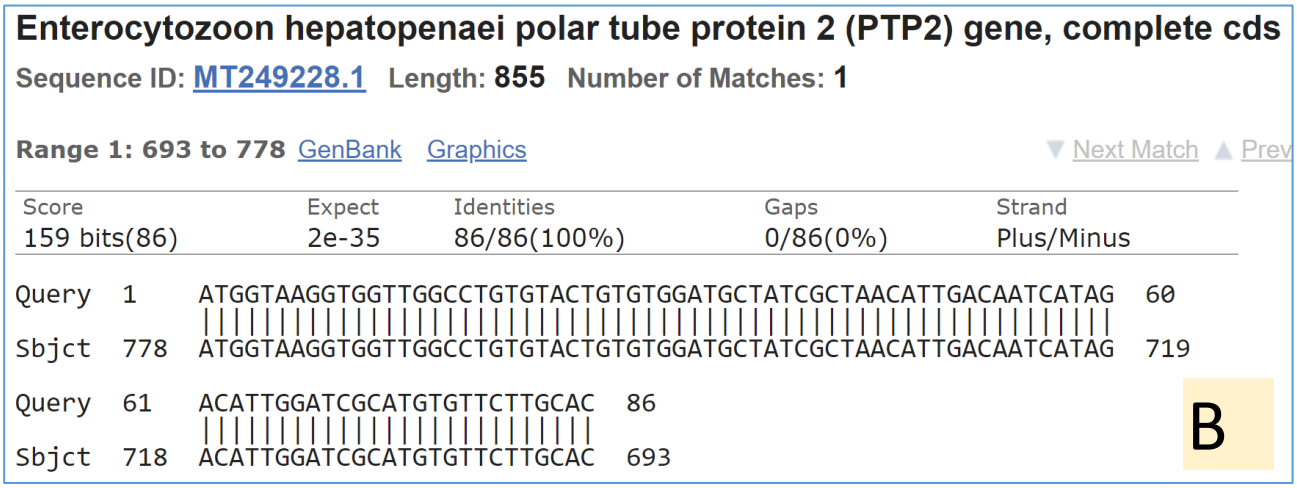
Direct amplicon sequencing was conclusive for all targets assessed. Top BLASTn hits confirmed a match with the expected EHP target gene/sequence (Table 3, Figure 4). All BLASTn parameters especially percent identity and query cover indicate a perfect match with the respective GenBank entry.

Table 3. Top BLASTn hits for PTP2G, SWP26G, and PTP2W sequenced PCR products.

Assay	Top Hit Description	Scientific Name	Max Score	Total Score	Query Cover	E value	%. ident	Acc. Len[$]	GenBank Accession
PTP2W	*Enterocytozoon hepatopenaei* polar tube protein 2 (PTP2) gene	*Enterocytozoon hepatopenaei*	435	435	100%	9.00E-118	100	855	MT249228.1
PTP2G	*Enterocytozoon hepatopenaei* polar tube protein 2 (PTP2) gene	*Enterocytozoon hepatopenaei*	159	159	100%	2.00E-35	100	855	MT249228.1
SWP26G	*Enterocytozoon hepatopenaei* spore wall protein 26 (SWP26) mRNA	*Enterocytozoon hepatopenaei*	159	159	100%	2.00E-35	100	718	MN604022.1

[$] Sequence length of accession entry





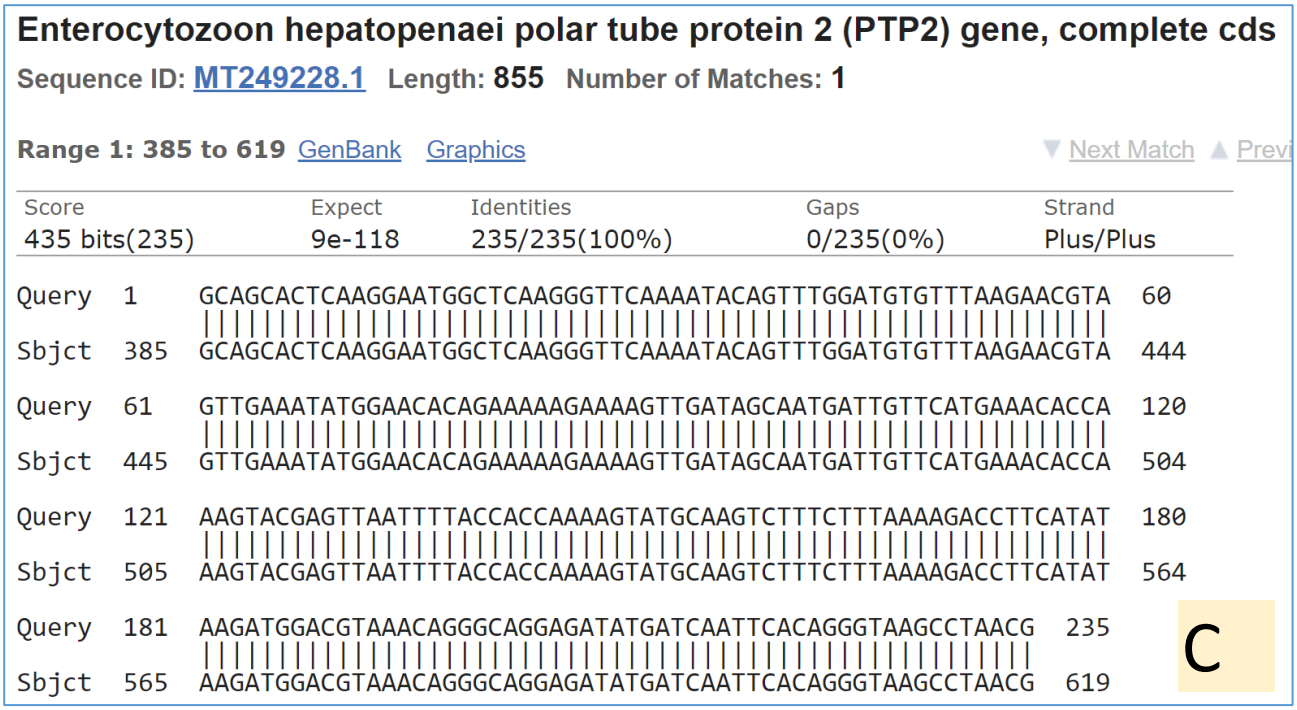


Figure 4. Top BLASTn hits and pairwise alignment of direct amplicon sequencing consensus of (A) SWP26G, (B) PTP2G, and (C) PTP2W.

4.5 Analytical sensitivity, limit of detection (LOD) via dilution to extinction (DTE) experiment

Analytical sensitivity calculated from the DTE real-time PCR data and expressed as limit of detection shows PTP2G and SWP26G, reaching a range of 2.0 – 3.6 copies per reaction in line with qPCR assay PTP2_Wang (Wang *et al.* 2020) (Table 4, Figures 5a,b,c). These results are comparable to other real-time PCR assays (Liu *et al.* 2016; Liu *et al*. 2018; Chaijarasphong *et al*. 2021). The SMP EHP assay limit of detection has been determined to be between 8-12 copies per reaction in a different project as part of the NATA accreditation process.

Table 4. Logistic regression analysis and limit of detection results.

Variable	PTP2G		SWP26G		PTP2_Wang	
	Conc.*	Const.	Conc.	Const.	Conc.	Const.
Coefficient	2.5155	-2.1491	1.7112	-3.1780	4.7494	-3.1331
Std. Error	0.5004	0.4393	0.3164	0.5377	0.9732	0.6606
LOD copy # [95% CI]	2.0 [0.7, 3.4]		3.6 [2.5, 4.6]		1.3 [0.5, 3.2]	
Ct cut-off	33.9 - 31.9		33.2 – 32.5		34.2 - 31.3	

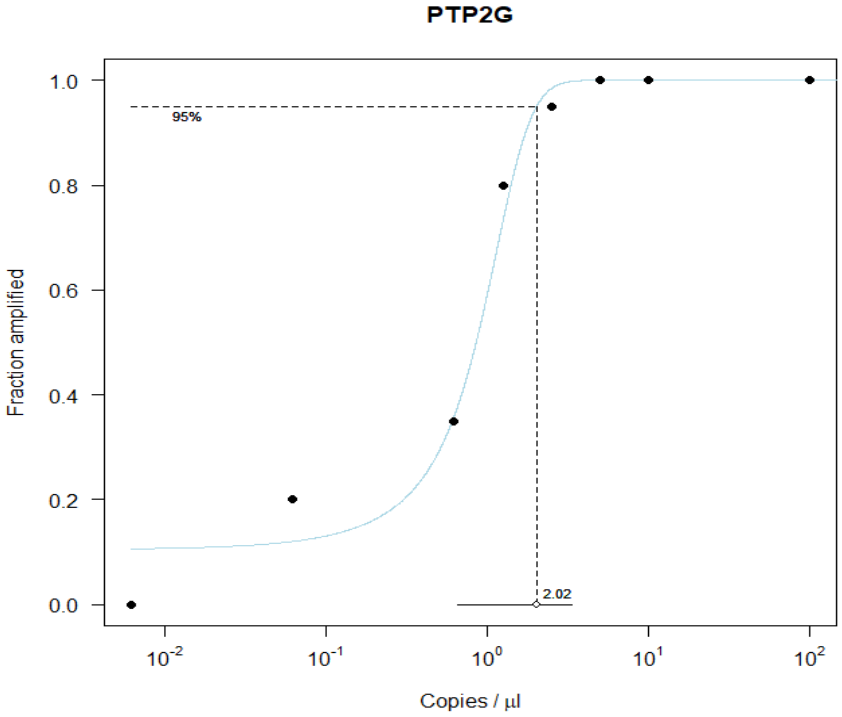


Figure 5a. Logistic regression limit of detection (LOD) analysis results shown as sigmoidal curve fit for qPCR assay PTP2G. LOD with 95% confidence interval at bottom of plot. Twenty replicates tested at each concentration.

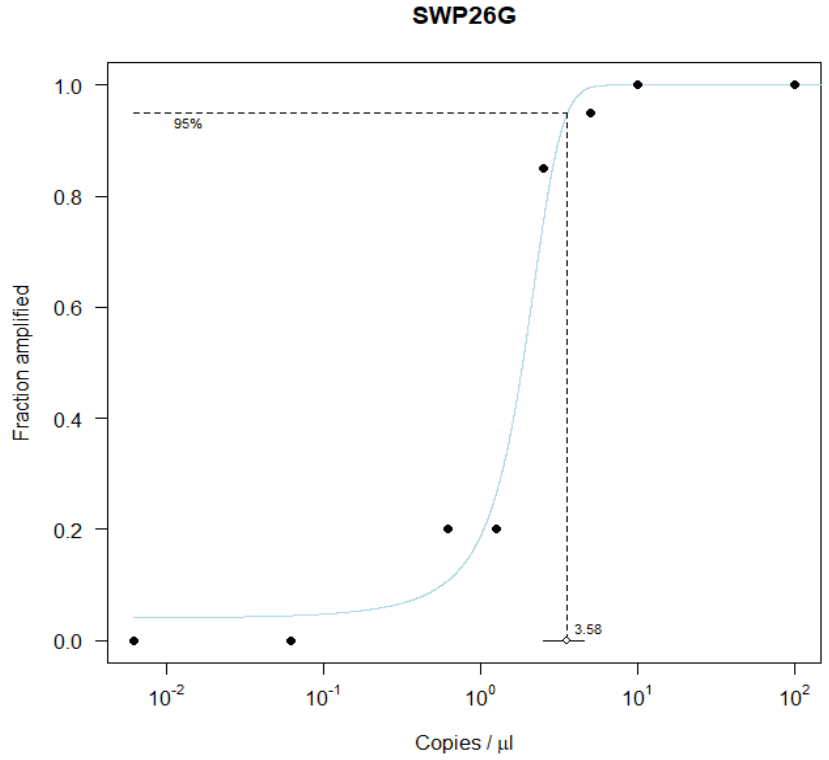


Figure 5b. Logistic regression limit of detection (LOD) analysis results shown as sigmoidal curve fit for qPCR assay SWP26G. LOD with 95% confidence interval at bottom of plot. Twenty replicates tested at each concentration.

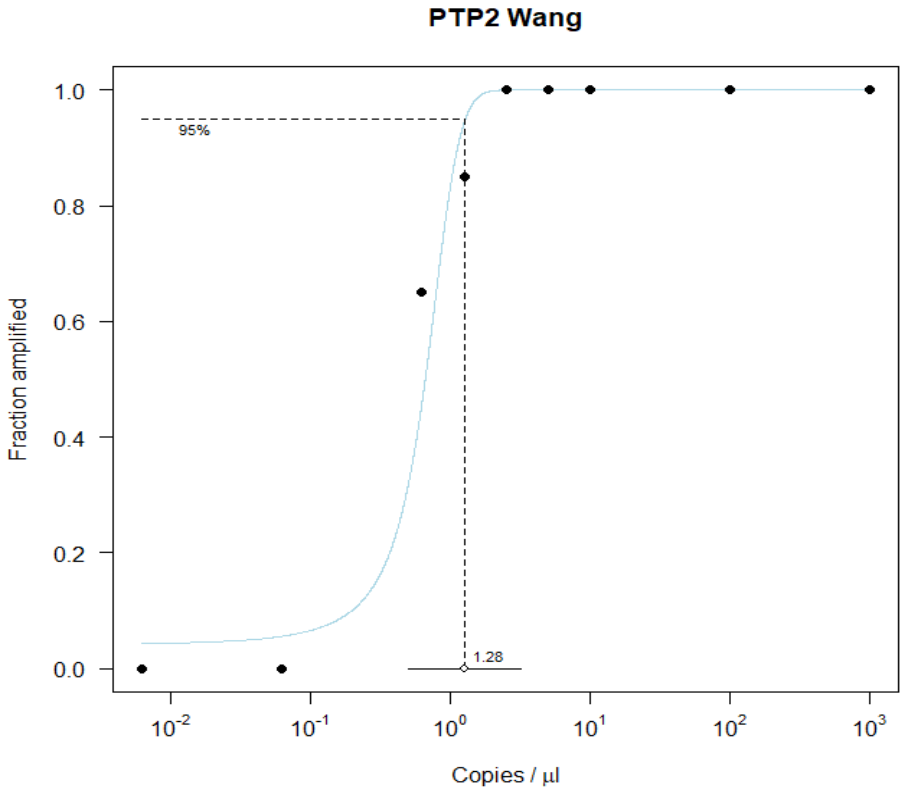


Figure 5c. Logistic regression limit of detection (LOD) analysis results shown as sigmoidal curve fit for qPCR assay PTP2_Wang. LOD with 95% confidence interval at bottom of plot. Twenty replicates tested at each concentration.

4.6 Repeatability assessment

Repeatability was assessed on *L. vannamei* samples from shrimp ponds from one of the two Latin American populations that were identified to be EHP positive using SMP EHP and Sanger sequencing. Since only a limited number of samples were available, a pooling approach was necessary as described in the Methods section.

The relative standard deviations of qPCR assay SWP26G within run (Intra-run repeatability) and between run (Inter-run repeatability) was extremely low and the results indicate SWP26G to be a highly repeatable assay. In comparison, PTP2G is showing slightly higher variation between 0.09 – 2.4% RSD within the boundaries of a good repeatable assay (Table 5). Of note is that the Ct values differ significantly between PTP2G (high Ct equals low copy number) and SWP26G (lower Ct equals higher copy number) by a magnitude of 2.6 – 4.8 Ct units. This curiosity would explain the higher variation of PTP2G among replicate reactions as the assay is approaching or even exceeding its limit of detection with Ct values being greater than 32. Further investigations conducted after submission of this report with results not shown here but summarised in scientific paper "Molecular assay development for *Enterocytozoon hepatopenaei* in globally diverse Penaeid shrimp populations" (Moser *et al*., 2022) confirmed the suspicion that the PTP2G assay might have been impacted by sequence variation or genetic variation in the gene region of the polar tube protein 2 that is targeted by the assay.

The repeatability of SMP EHP is showing a slightly higher variability in the intra run comparison in the range of 0.17 – 4.47% RSD while the inter-run comparison ranges from 0.91 – 2.49% RSD. The key difference that impacts those RSD values is the unit output of both analytical platforms. The real-time PCR assays RSD values were calculated from Ct values in line with common practice, whereas SMP EHP output is measured in log scale converted copy number (in attempt to scale and compare to Ct values).

4.7 Diagnostic Sensitivity (DSe) and Specificity (DSp)

Diagnostic sensitivity and specificity have been assessed on two independent *L. vannamei* populations from Vietnam (N = 190) and Latin American location (N = 95) (Table 6). The true prevalence of EHP in these populations was estimated to be ~5% and ~51%, respectively. Detailed outputs from the Bayesian latent class model are presented in Table 7. This model, with the best fit to the data, included the nested PCR with different diagnostic sensitivity and comparable diagnostic specificity in each population, and 26 correlation terms (see Tables 9 and 10).

All tests studied were highly specific. PTP2G has a DSe of 84.5% and DSp of 99.9%; and SMP EHP a DSe of 92.0% and DSp of 98.8%. SWP26G with a DSe of 70.2% lacks some degree of sensitivity but exhibits a high DSp of 99.8%. Interestingly, the nested SWP1 PCR assay had comparable diagnostic sensitivity and specificity to the SWP26G assay in *L. vannamei* shrimp from Vietnam but failed to detect shrimp samples from Latin America that were inferred to be truly EHP positive (DSe = 8.2%). Unfortunately, this observation completely abolishes the usefulness of the nested SWP1 PCR as universal EHP reference assay.

Emergence of caveats around the concept of universal EHP reference assays become even more pronounced when taking a closer look at several shrimp samples from Vietnam classified as EHP positive or negative by the various methods (Table 8). While classification results were overall consistent and concordant between the different methods in this shrimp population, some inconsistencies were identified using Sanger direct amplicon sequencing as the ultimate confirmatory application. For example, nested SWP1 PCR classified sample G-EHP_V015 as EHP negative but Sanger direct long amplicon sequencing (SWP26L) confirmed the unambiguous detection of SWP26 with a clear sequence trace and 100% BLAST hit. Another two samples G-EHP_V016 and G-EHP_V009 showed negative classification for EHP and only the long direct amplicon sequencing approach showed clear sequence trace for SWP26 with 100% BLAST hit of corresponding GenBank identification.

Table 5. Intra- and inter- run repeatability results of qPCR assays PTP2G and SWP26G, and Shrimp MultiPath EHP assay.

Sample ID	qPCR Assay	Repeatability Run 1			Repeatability Run 2			Repeatability Run 3			Inter-run Repeatability		
		Mean Ct	SD	RSD [%]	Mean Ct	SD	RSD [%]	Mean Ct	SD	RSD [%]	Mean Ct	SD	RSD [%]
Pool 1	PTP2G	33.44	0.27	0.80	34.03	0.71	2.10	33.89	0.37	1.09	33.79	0.31	0.91
Pool 2		32.21	0.52	1.62	31.57	0.10	0.33	32.58	0.40	1.21	32.12	0.51	1.60
Pool 3		35.27	0.69	1.94	35.28	0.12	0.35	35.33	0.85	2.40	35.30	0.03	0.09
Pool 4		35.70	0.32	0.90	34.96	0.47	1.35	35.48	0.47	1.32	35.38	0.38	1.06
Pool 1	SWP26G	29.72	0.05	0.19	29.12	0.03	0.12	28.96	0.11	0.37	29.27	0.40	1.38
Pool 2		29.59	0.05	0.16	29.27	0.13	0.44	28.97	0.07	0.23	29.28	0.31	1.06
Pool 3		30.51	0.02	0.06	30.13	0.22	0.74	29.71	0.10	0.33	30.12	0.40	1.33
Pool 4		30.86	0.09	0.29	30.61	0.09	0.28	30.19	0.28	0.92	30.55	0.34	1.11
Sample ID	**SMP**	**Mean log copy no.**	**SD**	**RSD [%]**	**Mean log copy no.**	**SD**	**RSD [%]**	**Mean log copy no.**	**SD**	**RSD [%]**	**Mean log copy no.**	**SD**	**RSD [%]**
Pool 1	EHP	9.57	0.19	1.94	9.12	0.02	0.17	9.82	0.12	1.27	9.50	0.09	0.91
Pool 2		8.28	0.15	1.87	7.95	0.10	1.30	8.57	0.31	3.56	8.27	0.10	1.27
Pool 3		7.41	0.33	4.47	7.15	0.11	1.53	7.54	0.11	1.52	7.37	0.13	1.72
Pool 4		6.57	0.03	0.41	6.52	0.31	4.77	7.01	0.02	0.24	6.70	0.17	2.49

Enterocytozoon hepatopenaei (EHP); Shrimp MultiPath (SMP); Relative Standard Deviation (RSD); Standard Deviation (SD); qPCR Cycle Threshold (Ct).

Table 6. Binary input data presented as frequency profile counts (each assay as Positive or Negative) for diagnostic sensitivity (DSe) and specificity (DSp) estimation using a Bayesian latent class model for five assays in one population (field samples from shrimp in Vietnam = Pop VTN) combined with four assays in a 2nd population (field samples from shrimp in Latin America = Pop LA). The table is a list of all possible Positive / Negative combinations and tallies the count for each combination observed. For example, combination of all assays calling a sample POSITIVE is five.

PTP2G	SWP26G	PTP2_Wang	SMP EHP	Nested SWP1 PCR	Freq. Obs. (Pop VTN)	Freq. Obs. (Pop LA)
+	+	+	+	+	5	0
+	+	+	+	-	1	31
+	+	+	-	+	0	0
+	+	+	-	-	0	0
+	+	-	+	+	0	0
+	+	-	+	-	1	0
+	+	-	-	+	0	0
+	+	-	-	-	0	0
+	-	+	+	+	0	0
+	-	+	+	-	0	8
+	-	+	-	+	0	0
+	-	+	-	-	0	0
+	-	-	+	+	0	0
+	-	-	+	-	0	1
+	-	-	-	+	0	0
+	-	-	-	-	0	1
-	+	+	+	+	0	0
-	+	+	+	-	0	1
-	+	+	-	+	0	0
-	+	+	-	-	0	0
-	+	-	+	+	0	0
-	+	-	+	-	0	0
-	+	-	-	+	0	0
-	+	-	-	-	0	0
-	-	+	+	+	0	0
-	-	+	+	-	0	5
-	-	+	-	+	0	0
-	-	+	-	-	0	0
-	-	-	+	+	0	0
-	-	-	+	-	3	1
-	-	-	-	+	0	0
-	-	-	-	-	180	47
			Total Number of Samples		190	95

Table 7. Diagnostic sensitivity and specificity estimates from a Bayesian latent class model for five assays (PTP2G, SWP26G, PTP2_Wang, SMP EHP and nested SWP1) in two populations.

Population/Test	Parameter	Posterior median (95% CrI)	Prior distribution
VTN	Prevalence	0.053 (0.025, 0.093)	Beta(1, 1)
LA	Prevalence	0.507 (0.404, 0.607)	Beta(1, 1)
PTP2G	Sensitivity	0.845 (0.743, 0.919)	Beta(1, 1)
	Specificity	0.999 (0.978, 1.000)	Beta(1, 1)
SWP26G	Sensitivity	0.702 (0.578, 0.810)	Beta(1, 1)
	Specificity	0.998 (0.984, 1.000)	Beta(1, 1)
PTP2_Wang	Sensitivity	0.876 (0.784, 0.950)	Beta(1, 1)
	Specificity	0.997 (0.983, 1.000)	Beta(1, 1)
SMP EHP	Sensitivity	0.920 (0.845, 0.981)	Beta(1, 1)
	Specificity	0.988 (0.965, 0.998)	Beta(1, 1)
Nested PCR	Sensitivity (VTN)	0.702 (0.380, 0.904)	Beta(1, 1)
	Specificity (VTN)	0.999 (0.981, 1.000)	Beta(1, 1)
	Sensitivity (ECU)	0.082 (0.027, 0.178)	Beta(1, 1)
	Specificity (ECU)	0.994 (0.952, 1.000)	Beta(1, 1)

Estimates of correlation terms provided in Supplementary Table S2. Model deviance information criterion (DIC) = 104.0.
Model configured to allow nested PCR to have different sensitivity and specificity in each population: VTN = Vietnam, LA = Latin America

Table 8. Long amplicon direct sequencing results comparison of clinical L. vannamei samples from Vietnam positive for EHP determined via nested PCR and compared to qPCR results and sequence trace recovery.

Sample Barcode	Seq_#	Nested SWP1 PCR[#]	qPCR[$]	SWP26L Seq trace [out of 4]	SWP26L BLAST hit	Coverage Amplicon vs [Acc-#] [bp]	Coverage Identity	PTP2L Seq trace [out of 4]	PTP2L BLAST hit	Coverage Amplicon vs [Acc-#] [bp]	Coverage Identity
G-EHP_V001	1	NEG	NEG	0	NA	NA	NA	0	NA	NA	NA
G-EHP_V002	2	WEAK	SWP26 POS	3	MN604022.1	583 [718]	100%	4	MT249228.1	761 [855]	100%
G-EHP_V003	3	WEAK	POS	4	MN604022.1	599 [718]	100%	4	MT249228.1	760 [855]	100%
G-EHP_V004	4	WEAK	SWP26 POS	4	MN604022.1	599 [718]	100%	2	MT249228.1	702 [855]	100%
G-EHP_V005	5	MEDIUM	POS	4	MN604022.1	584 [718]	100%	4	MT249228.1	761 [855]	100%
G-EHP_V006	6	HIGH	POS	4	MN604022.1	599 [718]	100%	4	MT249228.1	760 [855]	100%
G-EHP_V007	7	HIGH	POS	4	MN604022.1	598 [718]	100%	4	MT249228.1	761 [855]	100%
G-EHP_V008	8	HIGH	POS	4	MN604022.1	583 [718]	100%	4	MT249228.1	762 [855]	100%
G-EHP_V009	9	NEG	NEG	2	MN604022.1	578 [718]	100%	0	NA	NA	NA
G-EHP_V010	10	NEG	NEG	0	NA	NA	NA	0	NA	NA	NA
G-EHP_V011	11	NEG	NEG	0	NA	NA	NA	0	NA	NA	NA
G-EHP_V012	12	NEG	NEG	0	NA	NA	NA	0	NA	NA	NA
G-EHP_V013	13	NEG	NEG	0	NA	NA	NA	0	NA	NA	NA
G-EHP_V014	14	NEG	NEG	0	NA	NA	NA	0	NA	NA	NA
G-EHP_V015	15	NEG	SWP26 POS	2	MN604022.1	599 [718]	100%	0	NA	NA	NA
G-EHP_V016	16	NEG	NEG	4	MN604022.1	599 [718]	100%	0	NA	NA	NA

[#] Jaroenlak et al. (SWP1) - negative (NEG); weak, medium, high (amplicon band intensity directly stained by SYBR green)

[$] qPCR PTP2G and SWP26G summary result. Individual amplification indicated where appropriate.

Table 9. Bayesian latent class models with different covariance terms for estimating the cut-off value and diagnostic performances for five tests for *Enterocytozoon hepatopenaei* (EHP) nucleic acid in samples from Penaeid shrimp populations in Vietnam and Latin America.

Model	Covariance terms included	DIC	PD
$M_{0\ (Saturated)}$	$\rho D^{+,12}\ \rho D^{-,12}\ \rho D^{+,13}\ \rho D^{-,13}\ \rho D^{+,14}\ \rho D^{-,14}$ $\rho D^{+,15}\ \rho D^{-,15}\ \rho D^{+,16}\ \rho D^{-,16}\ \rho D^{+,23}\ \rho D^{-,23}$ $\rho D^{+,24}\ \rho D^{-,24}\ \rho D^{+,25}\ \rho D^{-,25}\ \rho D^{+,26}\ \rho D^{-,26}$ $\rho D^{+,34}\ \rho D^{-,34}\ \rho D^{+,35}\ \rho D^{-,35}\ \rho D^{+,36}\ \rho D^{-,36}$ $\rho D^{+,45}\ \rho D^{-,45}\ \rho D^{+,46}\ \rho D^{-,46}$	108.0	32.1
M_1	M_0 minus $\rho D^{+,25}\ \rho D^{-,34}$	104.0	29.4
M_2	M_1 minus $\rho D^{-,14}\ \rho D^{-,25}$	104.8	30.6
M_3	M_2 minus $\rho D^{+,26}\ \rho D^{+,45}$	104.5	30.8
M_4	M_0 reformulated with sp5 = sp6	109.4	34.5

DIC = deviance information criterion (lower implies better model fit); PD = effective number of parameters. Correlation terms represented as $\rho D^{+,12}$ for the correlation in results between tests 1 and 2 in samples that were truly infected with *Enterocytozoon hepatopenaei* (EHP). Tests: 1 = PTP2G PCR, 2 = SWP26G PCR, 3 = PTP2 Wang PCR, 4 = Shrimp MultiPath EHP, 5 = Nested PCR in Vietnam, 6 = Nested PCR in Latin America.

Table 10. Estimated correlation terms between tests from a Bayesian latent class model for five assays in one population (PTP2G, SWP26G, PTP2_Wang, SMP EHP and nested SWP1)).

Correlation	Posterior median (95% CrI)	Prior distribution
$\rho D^{+,12}$	0.255 (0.059, 0.460)	Uniform(-1, 1)
$\rho D^{+,13}$	0.127 (-0.005, 0.313)	Uniform(-1, 1)
$\rho D^{+,14}$	0.097 (-0.040, 0.259)	Uniform(-1, 1)
$\rho D^{+,15}$	0.062 (-0.186, 0.360)	Uniform(-1, 1)
$\rho D^{+,16}$	-0.099 (-0.306, 0.017)	Uniform(-1, 1)
$\rho D^{+,23}$	0.135 (-0.027, 0.347)	Uniform(-1, 1)
$\rho D^{+,24}$	0.072 (-0.070, 0.254)	Uniform(-1, 1)
$\rho D^{+,26}$	-0.047 (-0.246, 0.119)	Uniform(-1, 1)
$\rho D^{+,34}$	0.178 (0.003, 0.455)	Uniform(-1, 1)
$\rho D^{+,35}$	0.157 (-0.097, 0.446)	Uniform(-1, 1)
$\rho D^{+,36}$	-0.219 (-0.515, -0.016)	Uniform(-1, 1)
$\rho D^{+,45}$	0.072 (-0.170, 0.347)	Uniform(-1, 1)
$\rho D^{+,46}$	-0.204 (-0.482, -0.018)	Uniform(-1, 1)
$\rho D^{-,12}$	0.055 (-0.891, 0.846)	Uniform(-1, 1)
$\rho D^{-,13}$	0.115 (-0.818, 0.875)	Uniform(-1, 1)
$\rho D^{-,14}$	-0.017 (-0.874, 0.58)	Uniform(-1, 1)
$\rho D^{-,15}$	0.173 (-0.839, 0.927)	Uniform(-1, 1)
$\rho D^{-,16}$	0.110 (-0.915, 0.935)	Uniform(-1, 1)
$\rho D^{-,23}$	0.166 (-0.783, 0.835)	Uniform(-1, 1)
$\rho D^{-,24}$	0.063 (-0.682, 0.566)	Uniform(-1, 1)
$\rho D^{-,25}$	-0.041 (-0.934, 0.907)	Uniform(-1, 1)
$\rho D^{-,26}$	-0.161 (-0.957, 0.902)	Uniform(-1, 1)
$\rho D^{-,35}$	0.265 (-0.837, 0.945)	Uniform(-1, 1)
$\rho D^{-,36}$	0.210 (-0.879, 0.951)	Uniform(-1, 1)
$\rho D^{-,45}$	-0.333 (-0.958, 0.240)	Uniform(-1, 1)
$\rho D^{-,46}$	-0.180 (-0.945, 0.683)	Uniform(-1, 1)

Correlation terms represented as $\rho D^{+,12}$ for the correlation in results between tests 1 and 2 in samples that were truly infected with *Enterocytozoon hepatopenaei* (EHP). Tests: 1 = PTP2G PCR, 2 = SWP26G PCR, 3 = PTP2 Wang PCR, 4 = Shrimp MultiPath EHP5 = Nested PCR in Vietnam, 6 = Nested PCR in Latin America. Model deviance information criterion (DIC) = 104.0.

Conclusion

EHP is devastating global shrimp crops resulting in economic losses totalling hundreds of millions of US dollars. Current molecular detection methods focus mainly on a handful of target genes that originate from EHP pathogens in South-East Asia without much insight into the genetic diversity of global EHP species. This micro-focus is a serious short-sighted risk for the control of EHP infections and its infectious agent. Thus, the development of new EHP target genes for molecular assay development and the understanding of its underlying genetic variation in different geographical locations is needed to expand exploration and monitoring of the global presence of EHP. Importantly, new geographically calibrated assays will play a vital role to counter false assumptions on the status of universal reference assays that are based only on very limited amount of genetic information and understanding of genetic variation of the pathogen. For this purpose, new qPCR assays PTP2G and SWP26G were developed and validated in the presented sample set as great performing assays based on Analytical and Diagnostic metrics established. In addition, the cost effective and high-throughput Shrimp MultiPath^{TM} EHP assay has been validated with comparable good assay performance results. All three assays will form the basis of a toolbox to screen for EHP infection in shrimp populations. Moreover, this study opened a view to global gene specific variation in respective target genes and has led to scripting of a scientific research study that expands on the finding in this report.

Implications

This study provides novel gene target PCR assays for detection of EHP in Penaeid shrimp tissues which can be adopted for use by industry and regulators. Utility of this test expands to the improvement of border biosecurity and could potentially deployed as screening tool kit for EHP monitoring in fresh-frozen commodities.

Recommendations

An exhaustive sequencing effort of EHP positive shrimp samples from different regions of the world to identify genetic variants of EHP would be recommended followed by further assessment of these new PCR assays for their sensitivity and specificity across the current known geographical range of EHP. This will enable the development of more holistic EHP detection assays to capture geographically diverse EHP populations. This will allow the move away from generic phylogenetic target genes such as 18S rRNA.

Extension and Adoption

The key assay primer sequences, accession numbers of novel sequence data and methods for novel EHP assays are summarised both in this report and in the submitted scientific publication “Moser RJ, Franz L, Firestone SM, Sellars MJ (submitted). Molecular assay development for *Enterocytozoon hepatopenaei* in globally diverse Penaeid shrimp populations. DAO” These publications will be provided to the Australian Prawn Farmers Association, State and Commonwealth Government Authorities, and CSIRO (ACDP).

Data will be communicated with the Australian Prawn Farmers Association and Australian prawn farmers through way of face to face meetings, presentation of materials at meetings and conferences.

Data and outcomes are to be communicated with State and Commonwealth Government Authorities, and CSIRO (ACDP) through presentation and discussion at relevant meetings.

Project materials developed

In addition to the content presented in this report and accessible in the Materials and Methods section, a scientific paper has been published from this project in the Journal Diseases of Aquatic Organisms.

Moser RJ, Franz L, Firestone SM, Sellars MJ (submitted). Molecular assay development for *Enterocytozoon hepatopenaei* in globally diverse Penaeid shrimp populations. DAO DOI: https://doi.org/10.3354/dao03655

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