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Evaluation of a Single Diagnostic Platform to Test Three *Vibrio* Species (*Vibrio parahaemolyticus, Vibrio vulnificus* and toxigenic *Vibrio cholerae*) in White Shrimp and Tiger Prawn for Field Monitoring and Laboratory Confirmation

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Abstract

Vibrio species, including *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and toxigenic *Vibrio cholerae*, are important bacterial pathogens associated with shrimp aquaculture and seafood safety. Routine monitoring is often limited by the lack of diagnostic platforms that can be applied consistently across field and laboratory settings. This study evaluated a unified diagnostic workflow based on a single TaqMan probe assay that can be used for both PCR screening and laboratory qPCR confirmation.

A total of 100 seafood samples comprising 50 white shrimp (*Litopenaeus vannamei*) and 50 tiger prawns (*Penaeus monodon*) were collected from five wet markets in Singapore. DNA was extracted using a rapid Chelex-based method and analyzed using a three-tube assay targeting the toxR, vvhA, and ctxA genes of *V. parahaemolyticus, V. vulnificus,* and toxigenic *V. cholerae,* respectively. Amplification was performed using four instruments comprising two PCR systems and two qPCR systems. Diagnostic agreement between the PCR and qPCR systems was evaluated using Cohen's kappa analysis.

The assays demonstrated amplification efficiencies of 90.1–96.7% with limits of detection ranging from 14.25 to 15.67 copies per reaction. Overall agreement between the PCR and qPCR systems ranged from 94.3% to 96.0%. Cohen's kappa values ranged from 0.88 to 0.92, indicating almost perfect agreement according to the Landis and Koch (1977) interpretation scale. The highest agreement was observed between the Mini8/Mini16 (MiniPCR bio) and CFX96 (Bio-Rad) systems ($\kappa = 0.92$), whereas the lowest was observed between the Turbo Cycler (Blue-Ray Biotech) and Maverick (Anitoa) systems ($\kappa = 0.88$).

These findings demonstrate that a single assay design can provide reliable field-based qualitative screening and laboratory-based quantitative confirmation without requiring separate diagnostic workflows. The proposed diagnostic platform offers a practical approach for rapid *Vibrio* surveillance in shrimp and prawn production systems and supports timely disease monitoring and food safety management.

Keywords: *Vibrio parahaemolyticus, Vibrio vulnificus, Vibrio cholerae, Litopenaeus vannamei, Penaeus monodon*, Portable PCR, Quantitative PCR (qPCR), diagnostic concordance, Cohen's kappa.

Introduction

Vibrio species such as *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Vibrio cholerae* are of concern in shrimp and prawn farming. They are naturally present in aquatic environments and can rapidly proliferate under favorable conditions. This can lead to disease outbreaks, resulting in significant economic losses and posing risks to food safety and public health **[1, 2, 3, 4].** To control disease outbreaks, routine surveillance is important for early intervention. Current monitoring approaches, including conventional microbiological culture, on-site screening methods, and testing by centralized laboratories, have several limitations.

Conventional microbiological culture methods remain widely used for the presumptive detection of *Vibrio* species. They are time-consuming, labor-intensive, and may underestimate bacterial presence when cells enter a viable but non-culturable (VBNC) state **[5, 6, 7].** Field-deployable tests provide rapid results but may have limitations related to sample preparation, analytical sensitivity, and validation under field conditions **[8].** While these tests offer the advantage of on-site detection, studies evaluating their agreement with laboratory-based qPCR remain limited. The common practice is to send samples to a centralized laboratory for qPCR testing, which is highly sensitive and reliable but requires sample transportation and longer turnaround times **[9].** These logistical requirements may affect sample quality and delay decision-making, particularly when testing facilities are located far from the sampling site.

To address these limitations, we developed a unified diagnostic workflow based on a single TaqMan probe assay that can be used for both on-site screening and confirmatory quantification. During amplification, probe cleavage by DNA polymerase generates a fluorescent signal **[10, 11].** This study used FAM-labelled probes because their fluorescence is compatible with the optical configuration of the P51 fluorescence viewer, enabling direct visualisation of amplified products without agarose gel electrophoresis while also supporting qPCR-based quantification **[12].** This allows the same assay chemistry to be applied for both field-based qualitative screening and laboratory-based quantitative confirmation. Agreement between the two approaches was evaluated through a concordance study involving two PCR instruments and two qPCR instruments using Cohen's kappa analysis **[13, 14].**

Materials and Methods

Sample collection and preparation

A total of 100 seafood samples, comprising 50 white shrimp (*Litopenaeus vannamei*) and 50 tiger prawns (Penaeus monodon), were collected from five wet markets across the North, Central, West, East, and North-East regions of Singapore between March and May 2026. Samples were purchased directly from retail vendors under normal consumer conditions to reflect typical market exposure. At each wet market, approximately 500 g of raw white shrimp and 500 g of raw tiger prawns were purchased based on product availability at the time of sampling. Species identity was determined based on vendor labeling. No additional ice or cooling was applied during transport. All samples were transported to the laboratory and processed on the same day of collection.

From each purchased batch, 10 individual shrimp or prawns were randomly selected for analysis, resulting in a total of 50 samples per species. Each shrimp or prawn was treated as an individual sample. The head was first removed using sterile scissors. The body tissue, including the gastrointestinal tract, was aseptically dissected, cut into small pieces, and transferred into sterile 50 mL Falcon tubes (Falcon, USA) containing 30 mL of phosphate-buffered saline (PBS; Ultra Pure Grade, 1st BASE). The tubes were gently inverted three to five times to mix the tissue with the buffer. The suspension was incubated at room temperature for 30–45 min to allow bacterial release into the buffer prior to DNA extraction for *Vibrio* detection.

DNA extraction

DNA was extracted using a rapid Chelex-based method. A 10% Chelex 100 Resin suspension (molecular biology grade, 200–400 mesh, sodium form; Bio-Rad, USA) was prepared. A 500 µL aliquot of the bacterial suspension was transferred to a 1.5 mL microcentrifuge tube (BIOFIL) and centrifuged at 8000 rpm for 10 min. The supernatant was discarded, and the pellet was resuspended in 100 µL of 10% Chelex solution by thorough pipetting. The mixture was transferred to a 0.2 mL PCR tube (Axygen) and heated at 95°C for 20 min using a Turbo Cycler TCST-9622 (Blue-Ray Biotech). After heating, the tubes were centrifuged at 2000 rpm for 1 min. The DNA-containing supernatant was carefully transferred to a clean 0.2 mL PCR tube while avoiding carryover of the Chelex resin. The extracted DNA was stored at −20°C until use. For all PCR and qPCR assays, 2 µL of extracted DNA was used as the template.

PCR and qPCR Assay

Information on the target genes for the three *Vibrio* species is summarized in Table 1. Primers were synthesized in Lab Ready format, and hydrolysis probes were synthesized as double-quenched probes labeled with ZEN/Iowa Black™ (Integrated DNA Technologies, IDT, USA). PCR and qPCR assays were performed in a 20 µL reaction volume using SensiFAST™ No-ROX Probe Master Mix (Bioline), with 2 µL of extracted DNA as the template. Thermal cycling conditions were standardized across all instruments, comprising an initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 10 s and combined annealing/extension at 60°C for 20 s.

Amplification was performed using four instruments: two PCR systems, the miniPCR mini8 and mini16 Thermal Cyclers (MiniPCR bio, USA) and the Turbo Cycler TCST-9622 (Blue-Ray Biotech Corp., Taiwan), and two qPCR systems, the Maverick MQ8 Portable qPCR System (Anitoa Systems, USA) and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, USA).

PCR amplicons were visualized using a P51 fluorescence viewer (miniPCR bio) under blue light excitation (450–480 nm) with an orange filter for visualisation. Synthetic DNA targets (toxR, vvhA, and ctxA; 4000 copies/µL; Integrated DNA Technologies, IDT, USA) served as positive controls, while Chelex extraction blanks were used as negative controls.

Target	Target Gene	Amplicon Length	Genbank Accession	Amplification Efficiency (%)	LOD Copies/ reaction	Cq value at LOD
Vibrio parahaemolyticus	toxR	147 bp	CP114186.1	90.1	14.69	35.95
Vibrio vulnificus	vvhA	179 bp	CP126699.1	92.2	14.25	37.79
Toxigenic *Vibrio cholerae*	ctxA	176 bp	CP189277.1	96.7	15.67	36.03

Table 1: Analytical performance of diagnostic assay for *Vibrio* spp. targeting toxR (*V. parahaemolyticus*), vvhA (*V. vulnificus*) and ctxA (toxigenic *V. cholerae*) (Pang HY, unpublished).

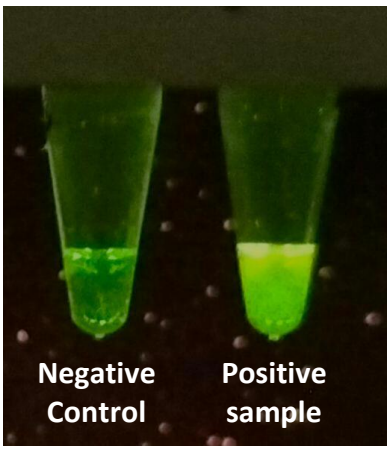
Detection and result interpretation

End-point PCR products generated using the Mini8/Mini16 (MiniPCR bio) and Turbo Cycler (Blue-Ray Biotech) were visualized using a P51 fluorescence viewer (Amplyus Ltd., USA). Samples exhibiting visible fluorescence were interpreted as positive. For qPCR, amplification data were analyzed using the instrument software of the Maverick (Anitoa) and CFX96 (Bio-Rad). A cycle quantification (Cq) cut-off value of 40 was applied. Samples with Cq ≤ 40 were considered positive, whereas samples with no amplification or Cq > 40 were considered negative.

Results

Detection of *Vibrio* targets

Representative positive results for *Vibrio* targets obtained by PCR and qPCR are shown in Figure 1. Representative negative samples are as shown in Figure 2.



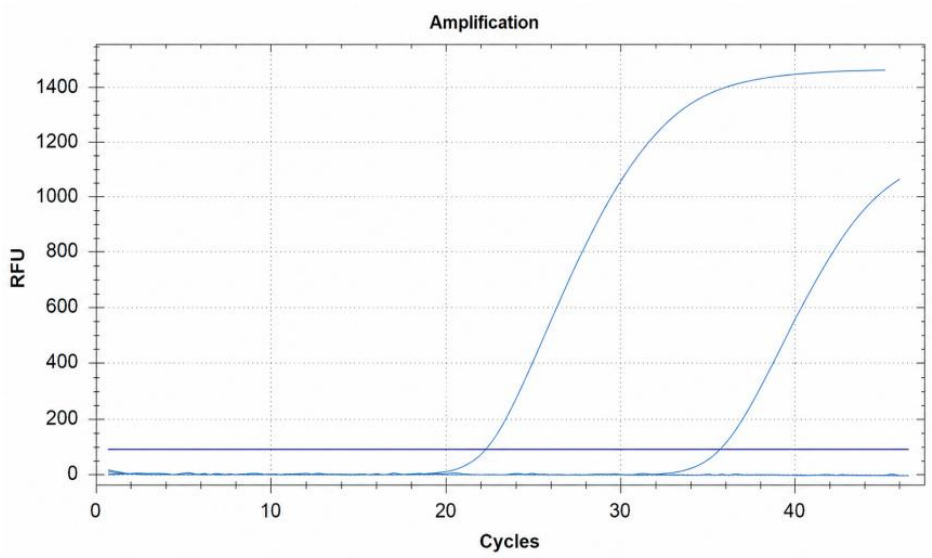


Figure 1: Positive detection by the PCR system was indicated by a stronger fluorescence signal in the sample compared with the negative control (left). Positive detection by the qPCR system was indicated by an amplification curve with a Cq value of ≤ 40 (right).

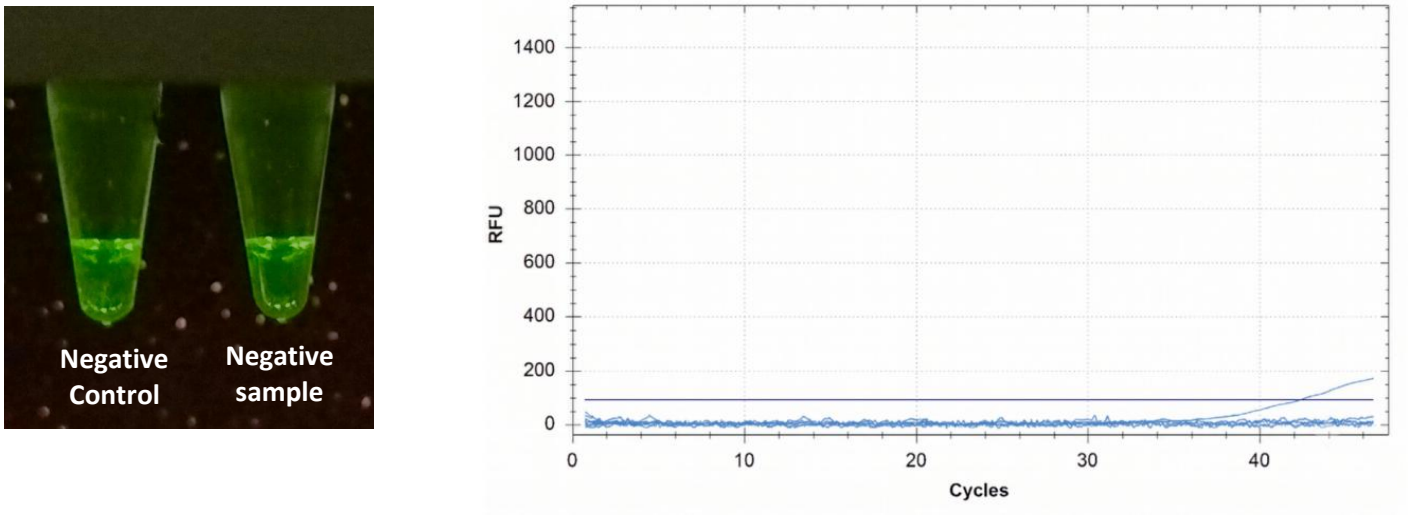


Figure 2: Negative samples in the PCR system exhibited fluorescence intensities similar to the negative control (left). Negative samples in the qPCR system showed no amplification curve or amplification at a Cq value > 40 (right).

Agreement between field PCR and laboratory qPCR systems

To evaluate agreement between PCR and qPCR systems, qualitative results from 300 reactions covering three *Vibrio* species were compared. Cohen's kappa was calculated to measure agreement **[14],** and the Landis and Koch interpretation scale was used to classify the level of concordance **[15].**

Cohen's kappa formula:

$$\kappa = \frac{p_0 - p_e}{1 - p_e},$$

where,

p_0 : Observed Agreement. The actual percentage of times the raters agree. Calculated as the total number of agreements divided by the total number of ratings.

p_e : Expected Chance Agreement. The hypothetical probability of chance agreement, calculated using the raters' individual probabilities of assigning each category.

The Landis & Koch Interpretation Scale 1977

< 0.00	: Poor agreement (less than chance)
0.01 - 0.20	: Slight agreement
0.21 - 0.40	: Fair agreement
0.41 - 0.60	: Moderate agreement
0.61 - 0.80	: Substantial agreement
0.81 - 1.00	: Almost perfect agreement

Table 2 shows the confusion matrix for four combinations of PCR and qPCR systems. Concordance analysis demonstrated almost perfect agreement across all evaluated platforms (Table 3). The highest level of concordance was observed between the Mini8/Mini16 (MiniPCR bio) vs CFX96 (Bio-Rad), yielding a Cohen's kappa (κ) coefficient of 0.92 (95% CI: 0.879–0.961). The lowest, yet still robust, level of agreement occurred between the Turbo Cycler (Blue-Ray Biotech) vs Maverick (Anitoa) with κ = 0.88 (95% CI: 0.831–0.929). Overall, all PCR/qPCR comparisons achieved κ values ≥ 0.88 with tight 95% confidence intervals, supporting the reliability and equivalence of both the PCR systems against standard qPCR frameworks.

1)

		qPCR System: Maverick (Anitoa)		Total
		-	+	
PCR System: Mini8/Mini16 (MiniPCR bio)	-	110	6	116
	+	8	176	184
Total		118	182	300

2)

		qPCR System: CFX96 (Bio-Rad)		Total
		-	+	
PCR System: Mini8/Mini16 (MiniPCR bio)	-	111	5	116
	+	7	177	184
Total		118	182	300

3)

		qPCR System: Maverick (Anitoa)		Total
		-	+	
PCR System: Turbo Cycler (Blue-Ray Biotech)	-	109	8	117
	+	9	174	183
Total		118	182	300

4)

		qPCR System: CFX96 (Bio-Rad)		Total
		-	+	
PCR System: Turbo Cycler (Blue-Ray Biotech)	-	110	7	117
	+	8	175	183
Total		118	182	300

Table 2: Confusion Matrix:
Mini8/Mini16 (MiniPCR bio) vs Maverick (Anitoa)
Mini8/Mini16 (MiniPCR bio) vs CFX96 (Bio-Rad)
Turbo Cycler (Blue-Ray Biotech) vs Maverick (Anitoa)
Turbo Cycler (Blue-Ray Biotech) vs CFX96 (Bio-Rad)

PCR/qPCR Comparison	Agreement (%)	Cohen's kappa, κ	Standard Error, SE	95% Confidence Interval, CI	Interpretation (Landis and Koch 1977)
1	95.3	0.90	0.026	0.852 to 0.952	Almost perfect agreement
2	96.0	0.92	0.024	0.869 to 0.963	
3	94.3	0.88	0.028	0.826 to 0.936	
4	95.0	0.90	0.026	0.843 to 0.947	

Table 3: Agreement analysis between PCR and qPCR system.
Mini8/Mini16 (MiniPCR bio) vs Maverick (Anitoa)
Mini8/Mini16 (MiniPCR bio) vs CFX96 (Bio-Rad)
Turbo Cycler (Blue-Ray Biotech) vs Maverick (Anitoa)
Turbo Cycler (Blue-Ray Biotech) vs CFX96 (Bio-Rad)

Disagreement between field PCR and laboratory qPCR systems

A total of 27 discordant sample-target combinations were identified across all PCR and qPCR platform comparisons. Of these, 25 were associated with *V. vulnificus* and 2 with *V. parahaemolyticus*, while no discordant results were observed for toxigenic *V. cholerae*.

Discussion

Degree of PCR and qPCR Agreement

Cohen's kappa incorporates the possibility of agreement occurring by chance and is commonly used for diagnostic concordance studies involving categorical positive or negative outcomes. The Cohen's kappa results indicate strong qualitative agreement beyond chance. Based on the interpretation criteria proposed by Landis and Koch, all platform comparisons were classified as demonstrating almost perfect agreement **[15].** These findings indicate high qualitative consistency between portable PCR systems used for field screening and laboratory-based qPCR systems used for confirmatory analysis.

Most discordant observations involving *V. vulnificus* were associated with low level target detection, with corresponding qPCR Cq values ranging from Cq 35.13 to Cq 39.98, with the mean Cq 38.1, which is close to the assay limit of detection (LOD Cq = 37.79). These findings suggest that a substantial proportion of the observed discrepancies were attributed to low target concentration and stochastic effects near the detection threshold, rather than to systematic differences between PCR and qPCR systems **[16].** Only two discordant observations were associated with *V. parahaemolyticus* (samples B07S and E10P). Notably, sample B07S yielded relatively low qPCR Cq values (20.03–20.94), indicating that factors other than target concentration may have contributed to this isolated discrepancy.

Despite these occasional discordant results, all platform comparisons demonstrated almost perfect agreement based on Cohen's kappa analysis (Table 3), supporting the suitability of the proposed workflow for both field screening and laboratory confirmation.

The use of Chelex Extraction Method

Chelex-based extraction provides a rapid and reliable method for nucleic acid preparation in aquatic pathogen detection. Compared with conventional commercial extraction kits, the Chelex extraction method requires fewer processing steps, shorter extraction time, lower cost, less technical expertise and less equipment, making it more suitable for portable or resource-limited testing conditions **[17, 18].** Although Chelex extraction may produce DNA of lower purity and retain higher levels of potential PCR inhibitors, previous studies have demonstrated that it provides acceptable amplification performance **[17, 19].**

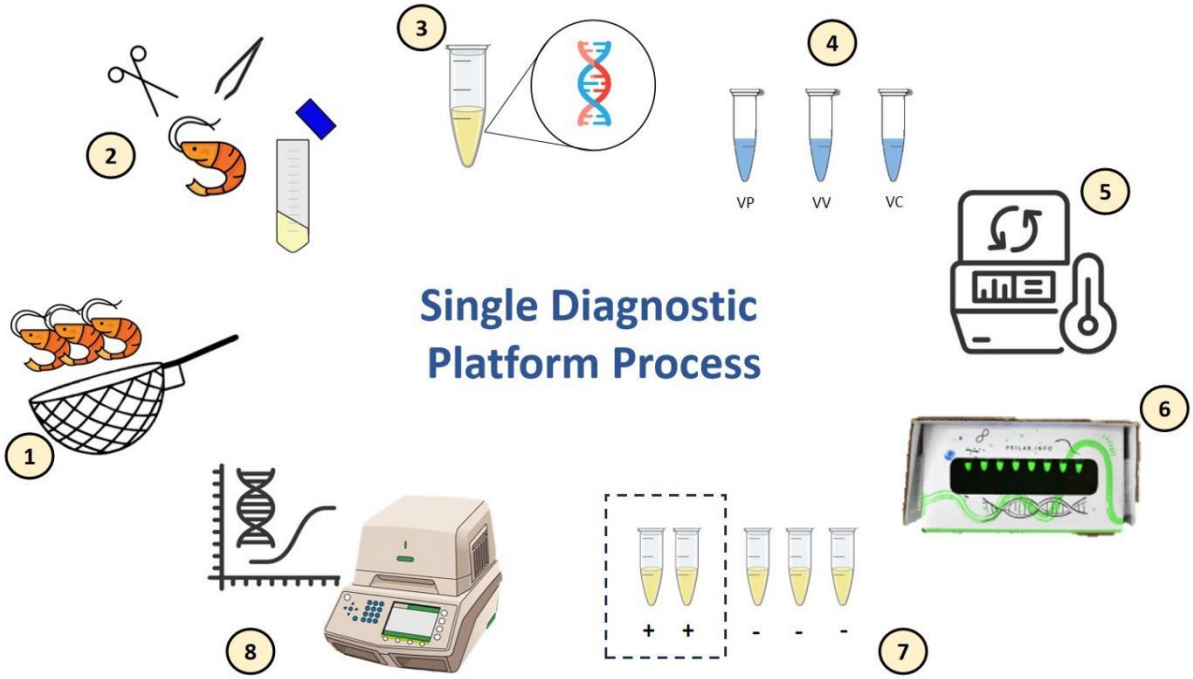


Diagram 1: Single diagnostic platform process and estimated processing time for 20 samples

Proposed Single Diagnostic Platform Process

1) **Sampling (approx. 30 min)**
   Fresh shrimp and prawn samples were collected and processed immediately on-site to preserve DNA integrity and minimize transport delays.
2) **Sample processing (approx. 1 hour)**
   Tissues were dissected and homogenized in PBS buffer to prepare sample suspensions for DNA extraction.
3) **DNA extraction (approx. 1 hour)**
   DNA was extracted from the prepared suspensions using a rapid Chelex-based method.
4) **Assay preparation (approx. 30 min)**
   Extracted DNA was directly added to PCR reaction mixtures targeting *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and toxigenic *Vibrio cholerae*.
5) **PCR amplification (approx. 1.5 hour)** Amplification was performed using portable thermocycler systems under standardized conditions.
6) **Fluorescence detection (approx. 5 min)** Amplification products were visualized using a portable blue-light viewer, where fluorescence indicated target presence.
7) **Transport of positive samples (distance-dependent)** Only positive DNA extracts were transported to the laboratory for confirmatory analysis, reducing transport load and contamination risk.
8) **qPCR confirmation (approx. 1.5 hour)**
   Positive extracts were directly subjected to qPCR for confirmation and quantification based on Cq values.

Using the proposed workflow, the estimated total turnaround time from sample collection to preliminary field-based detection was approximately 4.5 hours. The field-based detection demonstrated high concordance with qPCR, providing a reliable preliminary result to support timely farm management decisions and early intervention. Quantitative confirmation by qPCR required an additional 1.5 hours, excluding transportation time.

Advantages and Limitations in *Vibrio* Surveillance

Routine surveillance is a fundamental component of disease prevention in shrimp and prawn aquaculture because pathogenic *Vibrio* species can persist in the aquatic environment and colonize animals before clinical signs become evident **[20, 21].** Beyond supporting early outbreak detection and management, surveillance provides essential data for risk assessment and policy development while reducing the likelihood of underestimating the presence and distribution of pathogenic *Vibrio* **[20].** The proposed diagnostic platform provides a practical tool for routine farm-level monitoring and disease prevention throughout the aquaculture production chain, from broodstock and hatchery screening to nursery, grow-out ponds, harvest, and post-harvest seafood testing. Its portability enables more frequent on-site testing than conventional laboratory-based approaches, facilitating timely pathogen detection, early biosecurity interventions, and preventive disease management.

Multiplex PCR and qPCR assays enable the simultaneous detection of multiple *Vibrio* species in a single reaction, reducing assay time and overall reagent consumption. However, the inclusion of multiple primer sets in a single reaction may introduce challenges such as target competition, altered amplification efficiency, primer–dimer formation and non-specific amplification, often requiring extensive assay optimization **[22, 23, 24].** In contrast, the present workflow employs separate singleplex assays under a common thermal cycling protocol, avoiding these potential interactions while maintaining a simple and unified diagnostic workflow. Furthermore, because each target is amplified in an individual reaction, fluorescence signals can be interpreted independently during endpoint visual inspection, simplifying result interpretation.

Portable molecular methods such as loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) have been widely explored for rapid field detection of *Vibrio* species. However, studies directly comparing these methods with qPCR remain limited. Available studies have reported higher analytical sensitivity for qPCR than LAMP or real-time LAMP despite comparable diagnostic performance **[25-27].** In contrast, real-time RPA demonstrated complete concordance with qPCR for *Vibrio* detection, although this agreement was established using positive samples with qPCR Ct values ≤ 26.13, and its performance at higher Ct values remains unclear **[28].** Furthermore, because LAMP and RPA employ amplification mechanisms distinct from PCR and qPCR, they require independent assay development and validation. In contrast, the proposed workflow uses a single TaqMan probe assay compatible with both PCR and qPCR, enabling seamless transition from field screening to laboratory confirmation using the same molecular assay. A limitation of the endpoint fluorescence PCR assay is that it provides only qualitative results and cannot estimate target abundance. Therefore, it is intended as a rapid field screening tool, with qPCR using the same assay providing quantitative confirmation when required.

The endpoint fluorescence PCR assay demonstrated high qualitative agreement with the corresponding qPCR assay, supporting its application as a reliable field-screening tool. Although its analytical limit of detection was not formally evaluated, the present findings support diagnostic agreement rather than equivalent analytical sensitivity. Future studies should determine the analytical limit of detection through serial dilution experiments and directly compare its analytical performance with other portable amplification methods under identical experimental conditions.

Improvement and Recommendation

The initial field-based screening approach was designed to provide rapid qualitative detection of target *Vibrio* species but a positive result does not necessarily indicate an active disease outbreak. It is because they are natural microflora in shrimp and prawn aquaculture environments, and their detection may occur even in clinically healthy samples **[29].** Consequently, diagnostic results should be interpreted within a broader epidemiological context, including bacterial

load, animal health status, environmental parameters, and farm conditions, to more accurately assess disease risk. Based on these combined field and farm-level assessments, a Cq threshold for disease risk classification can be established and refined.

Establishing a Cq threshold for disease risk would enable the assay to be advanced beyond a simple presence–absence test into a practical and quantitative risk screening tool. This approach aligns with quantitative microbial risk assessment principles, where molecular detection outputs are translated into meaningful risk categories for decision making **[30].** Such refinement may be supported through optimisation of assay parameters to improve control of fluorescence signal intensity in PCR detection. The agreement between PCR-based results and the proposed Cq-based risk classification could be evaluated using Cohen's kappa analysis.

The present study was conducted using samples collected from a limited number of wet markets in Singapore and may not fully represent the broader diversity of *Vibrio* contamination patterns across different aquaculture systems or geographic regions. A larger sample size would be required to improve the robustness of the findings **[31].**

Conclusion

Overall, the proposed workflow demonstrates a practical approach for rapid detection of multiple *Vibrio* species by combining endpoint fluorescence PCR for field screening with laboratory confirmatory qPCR across the aquaculture production chain. Although each target is detected using a separate species-specific assay, the shared thermal cycling conditions allow multiple *Vibrio* species to be tested within the same PCR run without requiring separate amplification protocols. This simplifies routine surveillance, enabling earlier detection of pathogenic *Vibrio* species, timely diagnostic decision-making, and more effective biosecurity interventions. In addition, rapid screening of both aquaculture samples and seafood products supports food safety by enabling the early detection of foodborne *Vibrio* pathogens before contaminated products enter the consumer market. With further validation, this workflow may also provide a foundation for the development of risk-based surveillance strategies to support aquaculture health management.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationship that could have appeared to influence the work reported in this paper.

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