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Application of *gyrB* targeted SYBR green based qPCR assay for the specific and rapid detection of *Vibrio vulnificus* in seafood

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ABSTRACT

A SYBR green based qPCR assay targeting a unique region of *gyrB* was developed for the detection of *Vibrio vulnificus*. The specificity of the assay was studied using *V. vulnificus* and other bacterial strains belonging to Vibrio and non-Vibrio species. The assay unambiguously distinguished *V.vulnificus* with a sensitivity of 10^1 CFU/mL in pure culture while 10^2CFU/g was detected in clam meat homogenate with an efficiency of ≥98%.The utility of the qPCR assay was validated with naturally incurred seafood samples, where 24 out of 59(40.67%) seafood samples tested positive for *V. vulnificus* after 6–8 h enrichment in APW-P broth. In contrast, conventional PCR could detect only 11 samples (18.64%). Our results showed that qPCR assay developed in this study could be used as a rapid method for screening seafood samples for the presence of *V. vulnificus*, as the assay can be completed within 9–12 h including the enrichment of seafood in APW-P broth. The *gyrB* targeted qPCR developed in this study can provide excellent results on the presence and load of *V. vulnificus* in naturally contaminated samples quickly and efficiently; thus it could find application as a routine test in the seafood industry for the analysis *V. vulnificus*.

1. Introduction

Vibrio vulnificus is a Gram-negative halophilic bacterium found in association with biotic and abiotic components of the marine (DePaola et al., 1997; Strom and Paranjpye, 2000). This organism is capable of causing life-threatening septicemia, wound infections and acute gastroenteritis in certain high risk populations with certain underlying debilitations (Drake et al., 2007; Horseman and Surani, 2011; Mead et al., 1999; FAO/WHO, 2005). Human illness due to *V. vulnificus* generally occurs either by ingestion of raw or undercooked molluscan shellfish or wound exposure to seawater (Hlady and Klontz, 1996; Strom and Paranjpye, 2000). This pathogen has been an increasing concern and reported over wide geographical area, with cases from countries in United States, Europe and the Far East (Baker-Austin et al., 2010; Chuang et al., 1992; Dalsgaard et al., 1996; Hlady and Klontz, 1996). Infection due to *V. vulnificus* often progress rapidly into primary septicemia and may result in fatalities within 24 h, even with prompt diagnosis and aggressive antibiotic treatment (Baker-Austin and Oliver, 2018). In the United States, every year approximately 100 cases primary septicemia cases are reported from *V. vulnificus*, and almost all cases are associated with consumption of raw oysters harvested from the Gulf Coast (Jones and Oliver, 2009). Nearly 85% such infections were reported during the warmers as salinity and temperature of water strongly influence the density of *V. vulnificus* (Huehn et al., 2014; Rippey, 1994). Several studies have shown the relationship between the abundance of *V. vulnificus* in oysters to warmer temperatures and salinity of marine waters (Motes et al., 1998; Parvathi et al., 2004). Even though the prevalence and ecology of *V. vulnificus* in the Indian coastal waters have been extensively studied, infections due to this organism are rarely reported, probably due to lack of awareness among both clinicians and laboratory personnel. To-date only five clinical reports of *V. vulnificus* infections in India have been documented (Bhat et al., 2019; D'Souza et al., 2018; De and Mathur, 2011; Madiyal et al., 2016; Saraswathi et al., 1989). The gold standard is the conventional method for the study of *V. vulnificus* and requires enrichment with alkaline peptone water and isolation using selective agar [(cellobiose colistin (CC) or modified cellobiose polymyxin colistin (mCPC)] agar followed by phenotypic identification (Kaysner and Angelo DePaola, 2004). However, these assays require several days to complete. Furthermore, conventional methods may be limited by low sensitivity, and it also

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Table 1
Bacterial isolates used in this study.

Name of organism	Number isolates showing positive by qPCR
Vibrio vulnificus MTCC1145	01
Vibrio vulnificus (*n* = 15)	15
Vibrio parahaemolyticus (*trh*$^+$), (n = 15)	0
V. parahaemolyticus (*tdh*$^+$), (*n* = 04)	0
V. parahaemolyticus (*tdh*$^+$/*trh*$^+$), (*n* = 08)	0
V. parahaemolyticus (non-pathogenic), (n = 10)	0
V. cholerae, (*n* = 02)	0
V. harveyi, (*n* = 05)	0
V. alginolytiucs, (n = 02)	0
V. anguillarum, (*n* = 01)	0
Aeromonashydrophila, (n = 10)	0
Escherichia coli, (*n* = 10)	0
Salmonella species, (n = 10)	0

misses the viable but non-culturable (VBNC) state of the pathogen (Li et al., 2014). The failure to detect *V. vulnificus* may thus result in an underestimation of its true prevalence, thereby increasing the transmission risk. Currently available methods for the detection of *V. vulnificus* in food and/or clinical samples have limited application in disease surveillance or as a routine test in the food industry. The Joint FAO/WHO Expert meeting held in 2010 has underlined the need to harmonize and standardize molecular based methods to detect the organism. Therefore, there is a necessity to develop a method for rapid and accurate detection of *V. vulnificus* to lower the disease burden and provide safe food to the public and ensure the safety of export food. In this study, we report a simple and reproducible SYBR green-based qPCR assay for the direct detection of *V. vulnificus* that addresses the core issues of sensitivity and specificity.

2. Materials and methods

2.1. Bacterial strains

The bacterial isolates used in this study are listed in Table 1. They were revived from the stock preserved at −80 °C (Panasonic, Japan) in 30% glycerol broth. Each isolate was grown in 5 mL of Luria-Bertani broth (HiMedia Laboratories Pvt. Ltd., India) at 37 °C overnight with shaking.

2.2. Genomic DNA extraction

Genomic DNA was extracted from pure cultures of *V. vulnificus* and other bacterial isolates according to Ausubel et al., (1992) with minor modifications. The purified genomic DNA was precipitated with isopropanol and resuspended in 100 µl of 1× Tris-EDTA (TE, pH 8.0) buffer. The concentration and purity of the extracted DNA were determined using spectrophotometer at 260 and 280 nm (Bio Spectrophotometer, Eppendorf, Germany) and the purified DNA was stored at −20 °C until use.

2.3. Optimization of qPCR assay

The primers used in this study were gyr-vv1: 5′-GTCCGCAGTGGA ATCCTTCA-3'and gyr-vv2: 5′-TGGTTCTTACGGTTACGGCC-3′ (Kumar et al., 2006) and yielded a 285 bp amplicon (Table 2). Lyophilized primers were suspended in Tris-EDTA buffer (1 mM Tris–HCl, 0.01 mM EDTA, pH 8.0) to make a working solution of 2.5 pmol/µl. Different combinations of forward and reverse primer concentrations were tested across a range of template DNA concentrations (0.01 to 100 ng) to optimize the primer concentration as well as to verify that PCR efficiency is independent of the initial amount of target DNA. The qPCR mix contained 12.5 µl of 2XPower SYBR™ Green PCR Master Mix (Thermo Fischer Scientific, USA), the appropriate concentration of forward and reverse primers and 2 µl of template DNA. The volume of each reaction mixture was adjusted to 25 µl by adding sterile ultra-pure water. The qPCR was performed on real-time PCR system (Step One Plus™, Applied Biosystems, USA and CFX96 Touch™, BioRad, USA) with initial activation at 50 °C for 2 min, initial denaturation at 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 20 s, primer annealing at 55 °C for 20 s and elongation at 72 °C for 30 s. Data acquisition was performed by the respective software at the end of each elongation step. The optimized PCR conditions and primer concentration were used in subsequent experiments.

2.4. Specificity and sensitivity of detection

For the specificity validation of the qPCR method, a panel of isolates comprising *V. vulnificus* and other strains belonging to vibrio and non-vibrios confirmed by biochemical and molecular methods were used for the assay. For sensitivity analysis, pure culture of *V. vulnificus* MTCC1145 was grown in alkaline peptone water (APW, pH 8.5) at 37 °C to an absorbance of 0.6 at 600 nm and viable counts performed on mCPC agar plates. The *V. vulnificus* culture was 10-fold serially diluted in physiological saline from 10^5 CFU/ml to extinction. Next, 1 ml, aliquot from each dilution was centrifuged at 12,000*g* for 10 min. The resultant cell pellets were resuspended in 100 µl 1× TE and lysed at 95 °C for 10 min, followed by snap cooling on ice to release the DNA. The qPCR was then carried out twice in triplicate. Cycle threshold (Ct) values were plotted against log concentration of cells and correlation coefficient as well as the slope of the curve was determined. To verify the formation of any nonspecific products or primer dimers, the dissociation curves of final PCR products were analyzed from 60 to 95 °C at 1 °C interval.

2.5. Detection of V. vulnificus in artificially spiked seafood

For spiking experiment, the standard culture of *V. vulnificus* (MTCC1145) culture was grown in Luria-Bertani broth (HiMedia Laboratories Pvt. Ltd., India) at 37 °C with shaking (200 rpm) until the absorbance was 0.6 (10^8 CFU/ml). Clam meat confirmed to be negative for *V. vulnificus* was used in this study for preparing the homogenates. Ten grams of clam meat homogenate was added to 90 mL of enrichment broth, Alkaline Peptone Water with polymyxin (APW-P) in a conical flask and artificially spiked with dilutions of *V. vulnificus* to give a final count ranging from 10^0 to10^6 cells/g. Un-spiked homogenate served as a negative control. DNA lysate for qPCR was prepared by drawing aliquots of 1.5 ml of enrichment broth from each flask and centrifuged at low speed (800 ×*g* for 10 min) to sediment the meat particles. The resultant supernatant was subjected to centrifugation at 12,000 ×*g* for 10 min to pellet the bacteria. Pellets were suspended in 100 µl of 1× TE buffer (pH 8.0) and DNA was extracted from the pellet by heating at 95 °C for 10 min followed by cooling in ice. Aliquots of 2 µl of crude DNA lysate extracted was used as a template DNA for qPCR assay.

2.6. Detection of V. vulnificus in natural molluscan seafood samples

Fifty-nine samples of molluscan shellfish comprising clam, oyster and mussel were obtained from the fish market and retailers of Mangaluru and tested for the presence of *V. vulnificus* by qPCR assay targeting *gyr*B gene. The shellfish were washed well in clean water to remove adhering sand, shucked and homogenized using sterile mortar and pestle. Ten grams of homogenized meat was added to 90 ml of APW-P and incubated at 37 °C for 6–8 h and 16 h respectively. Following incubation, 1.5 ml of aliquots of enrichment broth was drawn from each flask at two-time points and DNA extracted as per the procedure described in Section 2.5. Two microliter DNA lysate was used as template DNA for qPCR assay for the detection of *V. vulnificus* in

Table 2
Primers used in this study.

Target	Sequence (5′-3′)	Amplicon size (bp)	Annealing temp (°C)	Reference
gyrB	F:GTCCGCAGTGGAATCCTTCA R: TGGTTCTTACGGTTACGGCC	285	55	Kumar et al. (2006)
vvhA (Ist step)	F: GACTATCGCATCAACAACCG R: AGGTAGCGAGTATTACTGCC	704	55	Lee et al. (1998)
vvhA (IInd step)	F: CTATCGTGCACGCTTTGGTA R: ACCGTTTTGTCACCGTTCTC	213	55	This study

Table 3
Sensitivity and linearity of detection of *V. vulnificus* purified genomic DNA.

DNA concentration	Ct Mean ± SD	Dissociation temperature (°C)
Non template control	ND	ND
0.6 pg	36.80 ± 0.040	83.52
6 pg	34.51 ± 0.48	83.52
60 pg	31.45 ± 0.06	83.52
600 pg	27.27 ± 0.25	83.52
6 ng	22.43 ± 0.07	83.52
60 ng	19.10 ± 0.11	83.52
Slope	−3.6843	
Intercept	41.493	
R^2	0.9876	

Note: "ND" means not detected.

conjunction with conventional PCR targeting *vvhA* genes and *gyrB* gene (Kumar et al., 2006) Simultaneously; conventional culture-based technique was also undertaken. A loopful of enrichment broth was streaked onto mCPC agar plates and incubated overnight at 37 °C. Typical colonies that grew on the mCPC plates were streaked onto Luria Bertani agar and a battery of biochemical test was performed for the phenotypic identification of the pathogen (Kaysner and Angelo DePaola, 2004). Samples which were negative by conventional PCR and detected by qPCR were additionally confirmed by nested PCR targeting *vvhA*. Details of the oligonucleotide primers used for the nested PCR are detailed in the Table 2.

3. Results and discussion

The role of *V. vulnificus*, in causing life-threatening illnesses in individuals with a mortality rate over 50% or even higher is being increasingly recognized (DePaola et al., 1997; Horseman and Surani, 2011; Strom and Paranjpye, 2000). In the last decade, the regions impacted by *V. vulnificus* infection have increased which is partly attributed to global warming, which affects ocean temperatures and currents (Böer et al., 2013; Jones et al., 2013). The establishment of an effective control programme for *V. vulnificus* necessitates reliable, accurate and sensitive methods to assess its presence in seafood or clinical specimens and could be extremely useful for an application in HACCP control plans in seafood industry. The Joint FAO/WHO Expert meeting held in 2010 has emphasized the role of molecular methods for the detection and enumeration of pathogenic vibrios that are evolving rapidly. Further recommendation of any single method for the purposes of monitoring these pathogens is difficult as well as of limited value as any method is likely to be superseded within a few years with the advent of superior methods (FAO/WHO, 2016). There exist several fluorescent-probe-based real-time PCR protocol to detect *V. vulnificus* using TaqMan probes but is limited by the need for expensive probes and rigid procedural outlines. Hence the objective of the present study was to establish a simple and specific method to detect *V. vulnificus* using qPCR based on SYBR Green DNA dye-binding fluorophore that detects by targeting a specific region of *gyrB* coding for B subunit of the gyrase (topoisomerase II). Use of the simple and less expensive double-stranded DNA-binding dye SYBR green I for detection of PCR amplicons overcomes the inherent limitations of traditional detection methods. The primer pair selected in this study is based on a previous study, where the genetic variations of *gyrB* sequences among *V. vulnificus* were studied its usefulness as a unique marker for the species specific identification was demonstrated (Kumar et al., 2006; Parvathi et al., 2005).

3.1. qPCR assay optimization

The optimization of primer concentration and assay conditions is one of the main challenges in developing a SYBR green based qPCR assay which is to ensure sensitivity and specificity of the target of interest. A set of qPCR experiments were performed on genomic DNA extracted from a pure culture of *V. vulnificus* MTCC-1145 set to a reported optimal annealing temperature and primer concentrations. The cycle threshold (Ct) values at 100, 200 and 300 nM primer concentrations for three different DNA concentrations 100 ng, 1 ng, and 0.01 ng were compared. Based on the results generated200 nM each of forward and reverse primer with a primer annealing temperature of 55 °C was found to be optimum for the best results, with the following thermal cycling conditions: 20 s for denaturation, 20 s for annealing and 30 s for elongation. In the melt curve, a single distinct peak was observed, indicating that all the amplicons had similar Tm values of approximately 83.52 °C. The qPCR assay on serially diluted genomic DNA of *V. vulnificus* showed consistent amplification of *gyr*B gene with the detection limit of 0.1 pg. An increase in Ct value was noticed as the concentration of DNA decreased (Table 3) Analysis of melt curve as well as agarose gel electrophoresis of PCR product did not show any primer dimer formation or nonspecific amplification at the condition described above (Figs. 1 & 2). Consistent results were observed in three consecutive runs with three replicates within each experiment.

3.1.1. Evaluation of the specificity of qPCR

The specificity of the qPCR was tested with the primers and protocol designed in this study on genomic DNA extracted from a panel of *V. vulnificus* species and other genera are listed in Table 1. It was observed that all *V. vulnificus* isolates were efficiently amplified by the qPCR, while all other vibrios (n = 47) and non vibrios (n = 30) were not amplified for *gyr*B gene (Table 1). Amplified products were resolved on 2% agarose gels pre-stained with ethidium bromide confirmed the

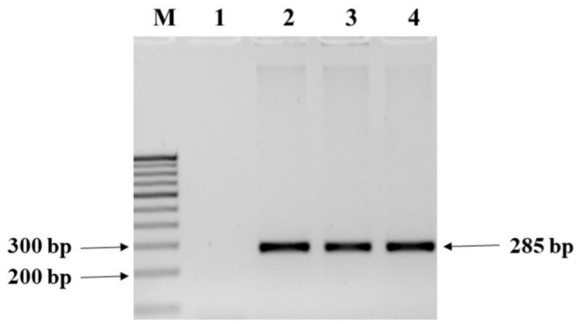

Fig. 1. Confirmation of amplification product of qPCR for the detection *Vibrio vulnificus* by targeting *gyrB* gene. Lane M: 100 bp DNA ladder, Lane 1: Non template control, Lane 2–4: Amplification of product of qPCR targeting *gyrB* gene of *Vibrio vulnificus* MTCC1145.

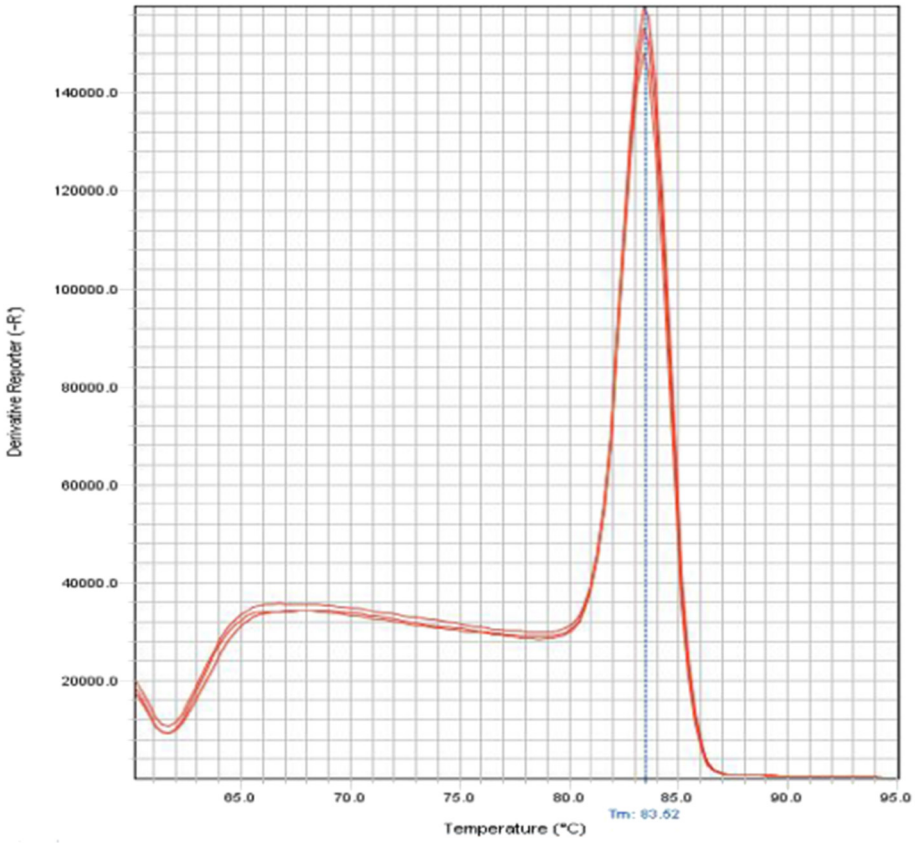

Fig. 2. Melting curve analysis of qPCR amplification products targeting *gyrB* gene of *V. vulnificus* MTCC1145.

absence of primer-dimer and non-specific amplification (data not shown). This demonstrated that qPCR targeting *gyrB* gene is highly specific for the detection of *V. vulnificus* and in conformity with other studies about specificity of qPCR for *V. vulnificus* (Campbell and Wright, 2003; Panicker and Bej, 2004; Panicker et al., 2004).

3.2. Sensitivity analysis

The sensitivity of the qPCR assay was performed with a serial 10-fold dilution of the standard isolate *V. vulnificus* MTCC1145. The standard curve was created by plotting Ct values against known serial dilutions of standard isolate, ranged from 10^1 to 10^7 copies/ml of pure culture (Fig. 3). The standard curve based on the dilutions of DNA showed a linear relationship between log CFU and threshold cycles (Ct) with a detection limit of approximately 10^1 CFU/ml of the standard pure culture of *V. vulnificus*. The standard curves showed a higher degree of similarity coupled with very low coefficient of variation (CV) among Ct values of three independent experiments thus indicating that the qPCR targeting *gyrB* gene of *V. vulnificus* is highly sensitive and reproducible with minimum variations. The slope of the curve was −3.9, and the R^2 after the linear regression was 0.987, which demonstrates the quantitative potential of the qPCR assay.

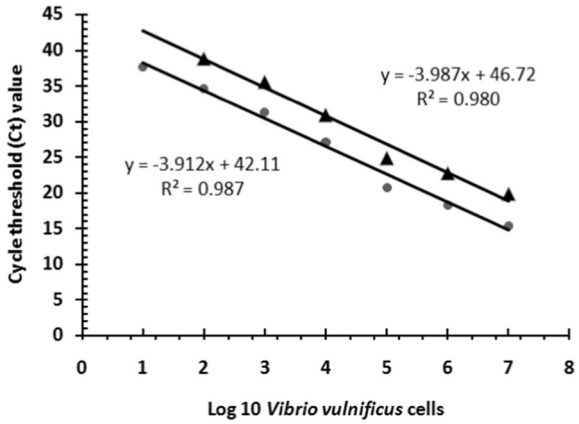

Fig. 3. Detection of *V. vulnificus* in pure cultures (●) and seafood homogenates (▲). Ct values were plotted against log CFU vales. Trend line equations shown separately for each experiment.

3.3. Detection of V. vulnificus in spiked clam meat homogenates

Clam meat homogenate confirmed negative for *V. vulnificus* was artificially contaminated with decimal dilutions of *V. vulniifcus* MTCC1145 culture with known CFU.DNA lysate from each decimal dilution was subjected to qPCR for the detection of *V. vulnificus*. The assay generated a linear correlation between Ct values and log bacterial concentration of *V. vulnificus* ranging from 10^2 to 10^7 CFU/g from clam meat homogenates with R values of 0.987 (Fig. 3). This experiment with clam meat homogenate showed 3–4 cycle difference in the Ct values than that from pure cultures for a given concentration. The difference in Ct values between pure culture and non-enriched clam meat homogenates could be due to PCR inhibitors in the tissue matrix (Richards, 2016; Schrader et al., 2012; Wang and Levin, 2006). Seafood contains many polysaccharides that are responsible for PCR inhibition (Atmar et al., 1993, 1995). Additionally, it has been surmised that the glycogen content in the tissues of bivalve molluscs influences PCR (Richards, 2016; Schrader et al., 2012). The clam meat homogenate control serving as control was negative for *V. vulniifcus*. The detection limit of 10^2 CFU *V. vulnificus*/g of clam meat homogenate achieved in this study is noteworthy, as this could be used for prescreening the seafood samples for *V. vulnificus* without any enrichment. Further, the

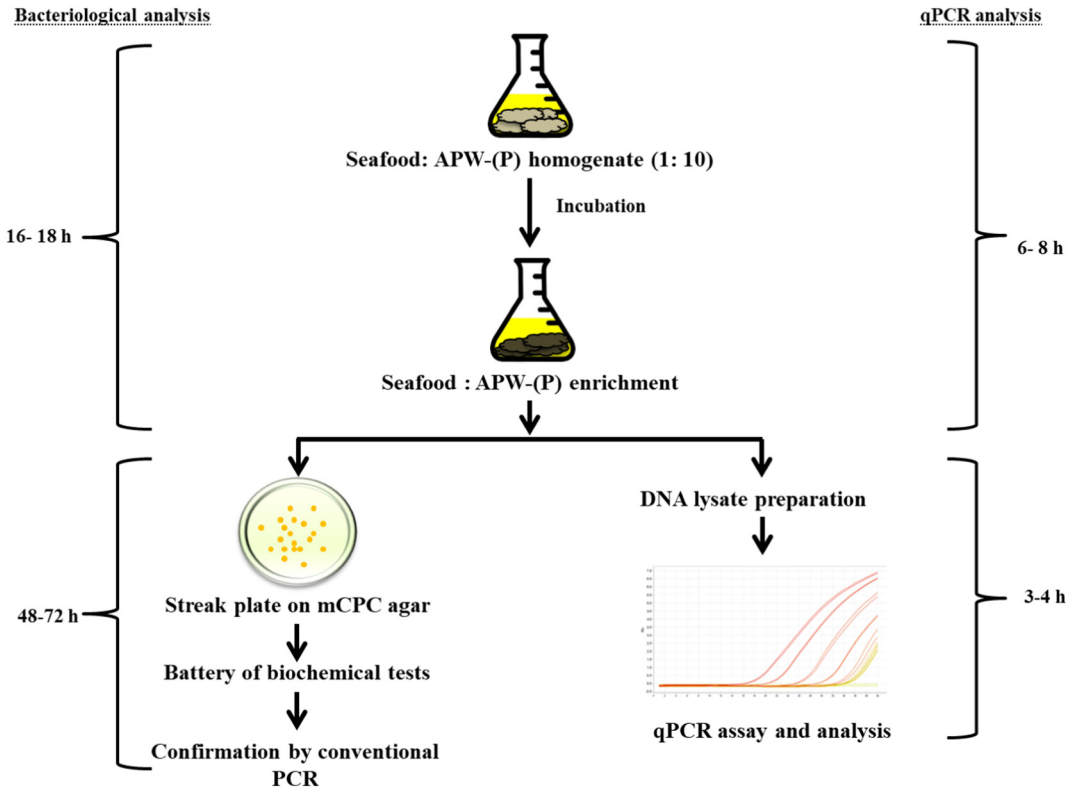

Fig. 4. Overall workflow for the detection of *V. vulnificus* by bacteriological analysis and qPCR assay.

samples negative by prescreening can be enriched; thus, labour saving and optimizing the time of reporting.

3.4. Performance of qPCR for the detection of V. vulnificus in seafood samples

To determine the applicability of the developed qPCR for the detection of *V. vulnificus* in samples by qPCR targeting *gyrB* gene, 59 seafood samples were analyzed. The overall workflow for the detection of *V. vulnificus* by qPCR assay developed in this study was depicted in Fig. 4. Twenty four (40.67%) samples were found to be positive for *V. vulnificus* by qPCR after 6–8 h enrichment in APW broth in contrast to conventional PCR which detected *V. vulnificus* in 11 samples (16.41%) after 16–18 h enrichment (Table 4). To validate the result obtained, the samples which were positive in qPCR but negative in conventional PCR were further subjected to nested PCR amplifying the *vvhA* gene and detected *V. vulnificus* in 13 such samples (Fig. 5). Additionally, melt temperature analysis was done to rule out nonrandom amplification. Overall observation suggests that *V. vulnificus* was actually present in all of the samples analyzed and it is clear that the detection rate of *V. vulnificus* in the seafood enrichments was significantly higher with qPCR than the conventional PCR. However, there was no difference in the detection rate of *V. vulnificus* between the two enrichment times used in the experiment. When a loopful of enrichment lysate was streaked on mCPC agar plates, only 9 of 59 samples yielded *V. vulnificus* after 18 h enrichment in APW-P. All these samples were confirmed positive by *gyrB* PCR. The results show that enrichment duration of 6–8 h is sufficient to detect *V. vulnificus* in seafood homogenates by this qPCR, which makes this method suitable for routine application in screening of seafood samples for the presence of *V. vulnificus* pathogen. Although the bacteriological methods are considered the gold standard for the detection of *V. vulnificus* in seafood samples, it is not widely used for routine analyses as it is very time consuming, involving multiple steps, limited sensitivity and inability to analyze large number samples. In contrast, the qPCR developed in this study can provide excellent results on the presence and load of *V. vulnificus* in naturally contaminated samples quickly and efficiently. This protocol could also find application also in monitoring the marine waters and seafood harvesting sites to assess the risk *V. vulnificus* and to permit the development of rational public health interventions for its control.

Declaration of Competing Interest

None.

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Table 4
Summary of detection of *V. vulnificus* in seafood samples by various methods.

Sample analyzed (n)	No. of samples positive for *V. vulnificus* by PCR		
	FDA bacteriological method	Conventional PCR targeting *vvhA* and *gyrB*	qPCR developed in this study
Molluscan shellfish (59)	9 (15.25%)	11 (18.64%)	24 (40.67%)

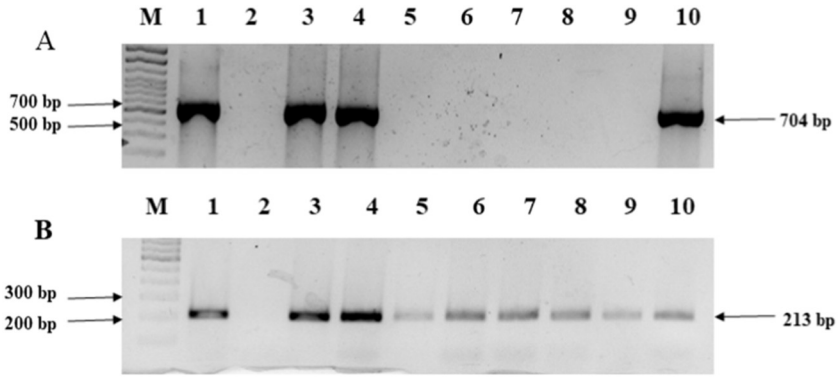

Fig. 5. Representative gel image showing the nested PCR amplifying the *vvhA* gene for the confirmation of *V. vulnificus* in seafood samples (Panel A: Amplicon of the first step PCR; Panel B: Amplicon of second step PCR). Lane M: 100 bp Marker, Lane 1: Positive control *V. vulnificus* MTCC1145, Lane 2: Negative control, Lane 3–4 and 10: Samples positive both by conventional PCR and qPCR, Lane 5–9: Samples negative by conventional PCR and positive by qPCR.

manuscript and sourcing few clinical isolates of *V. vulnificus* for the study.

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