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Development of a novel multiplex-PCR technology for simultaneous detection of five major aquaculture pathogens

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Early and precise pathogen identification and corresponding disease management are primary concerns in aquaculture. Here, we attempted at diagnostic methods that can simultaneously identify multiple pathogens, where many samples, several pathogens, and concurrent infections are to be handled. Hence, a multiplex PCR assay targeting five major aquaculture pathogens, *viz. Vibrio parahaemolyticus*, *Vibrio anguillarum*, *Vibrio alginolyticus*, *Vibrio vulnificus* and *Vibrio harveyi* was developed for the first time. The primers targeting *toxR* of *V. parahaemolyticus*, *amiB* of *V. anguillarum*, *col* of *V. alginolyticus*, *vvhA* of *V. vulnificus*, and *topA* of *V. harveyi* were applied. Furthermore, the reaction included an internal amplification control against prokaryotic 16S rRNA to perceive false-negative results. The assay showed 100% specificity against 56 unique bacteria. The sensitivity was 0.25 ng for *V. harveyi*, 0.5 ng for *V. vulnificus*, 1 ng for *V. parahaemolyticus* and *V. anguillarum*, and 2 ng for *V. alginolyticus* DNA per µL assay. Sensitivity regarding CFU was 1.2, 5.2, 10, 5.6×10^1 and 3.8×10^2 per µL, for *V. harveyi*, *V. vulnificus*, *V. anguillarum*, *V. parahaemolyticus* and *V. alginolyticus*, respectively. The results suggest that the optimized method can be applied for sensitive and specific identification of five aquaculture pathogens through a single PCR.

Keywords: Internal amplification control, *Vibrio* spp.

Aquaculture is the most rapidly growing global food production sector and plays a significant role in meeting the increased need for high quality animal protein in human nutrition[1]. The total world aquaculture production in 2020 comprised 122.6 million tonnes in live weight, representing an increase of 6.7 million tonnes from 2018[1]. Nevertheless, increased incidences of infectious diseases adversely affect the production, profitability, and sustainability of the global aquaculture industry[2]. Therefore, the early and precise identification of the pathogen and corresponding disease management form a primary concern in commercial aquaculture practices[3].

Vibriosis is one of the most common diseases leading to massive mortality and substantial economic losses in the world's aquaculture industry[4]. The traditional way of bacterial disease diagnosis by *in vitro* cultivation has several drawbacks, such as insufficient sensitivity and specificity, laboriousness, and extreme slowness in the precise identification[5]. Accordingly, molecular techniques have been established for the diagnosis of aquaculture diseases to overcome the time, sensitivity, and specificity limitations[6]. Polymerase chain reaction (PCR)-based identification is a suitable option since it is a simple, rapid, and reliable form of detection with much sensitivity and specificity[7]. However, conventional PCR targeting a single pathogen takes a long time and is expensive to deal with several samples[8] and multiple pathogens in aquaculture, where concurrent infections from different pathogens are widespread[9]. As a result, the developing multiplex PCR (mPCR) tools for detecting fish pathogens will help in fish disease assessment and management through a cost, effort, and time-effective manner[10].

The *Vibrio* genus comprises >30 species with several primary food-borne human and aquaculture pathogens[11]. *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V. salmonicida* and *V. vulnificus* are the primary bacterial finfish pathogens[5]. The higher plasticity of the vibrio genomes makes precise species identification of vibrios a challenging topic[12]. The mPCR methods for detecting primary human pathogenic *Vibrio* spp. have

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been developed[13-17]. However, the mPCR approach has not been developed and evaluated for detecting fish pathogenic vibrios[18], except the three previous reports each target three *Vibrio* spp. either, *V. alginolyticus*, *V. anguillarum*, and *V. harveyi*[3], *V. anguillarum*, *V. harveyi* and *V. alginolyticus*[19] or *V. harveyi*, *V. campbellii* and *V. parahaemolyticus*[20]. In this context, here, we have made an attempt to develop an mPCR assay for simultaneous identification of five major vibrio pathogens of aquaculture. An internal amplification control (IAC) is included in PCR to avoid possible false-negative results.

Materials and Methods

Bacterial strains

Details of the bacterial strains used in this study are given in Table 1. In brief, a total of 56 bacterial strains belonging to 47 different species and 30 different genera were used to optimize and validate multiplex PCR. All the isolates used in the study were sourced from Marine microbial culture collection, ICAR-CMFRI (Indian Council of Agriculture Research-Central Marine Fisheries Research Institute), Kochi, Kerala, India. The

Table 1 — Details of bacteria used in the study and the optimized multiplex PCR assay results

Species	Isolate ID	Results					
		toxR of VP	*amiB* of VAn	*col* of VAl	*vvhA* of VV	*topA* of VHa	IAC
Vibrio parahaemolyticus	CMFRI/VP-08	+	-	-	-	-	+
	CMFRI/VP-07	+	-	-	-	-	+
	CMFRI/VP-05	+	-	-	-	-	+
V. anguillarum	CMFRI/VAn-2	-	+	-	-	-	+
V. alginolyticus	CMFRI/VAl-42	-	-	+	-	-	+
	CMFRI/VAl-43	-	-	+	-	-	+
	CMFRI/VAl-41	-	-	+	-	-	+
V. vulnificus	CMFRI/VV-02	-	-	-	+	-	+
	CMFRI/VV-03	-	-	-	+	-	+
	CMFRI/VV-04	-	-	-	+	-	+
V. harveyi	CMFRI/VHa-03	-	-	-	-	+	+
	CMFRI/VHa-06	-	-	-	-	+	+
	CMFRI/VHa-07	-	-	-	-	+	+
V. furnissii	CMFRI/Vfur-01	-	-	-	-	-	+
V. campbellii	CMFRI/VCa-1	-	-	-	-	-	+
V. owensii	CMFRI/VOw-01	-	-	-	-	-	+
V. cholera	MTCC 15025	-	-	-	-	-	+
V. japonicas	CMFRI/VJa-01	-	-	-	-	-	+
V. aestuarianus	CMFRI/VAe-01	-	-	-	-	-	+
Acinetobacter baumannii	CMFRI/ABa-01	-	-	-	-	-	+
Aeromonas caviae	CMFRI/ACa-02	-	-	-	-	-	+
A. hydrophila	CMFRI/AH-02	-	-	-	-	-	+
A. jandaei	CMFRI/AJ-01	-	-	-	-	-	+
Aeromonas schubertii	CMFRI/AS-02	-	-	-	-	-	+
Aeromonas veronii	CMFRI/AV-02	-	-	-	-	-	+
Aeromonas dhakensis	CMFRI/ADh-01	-	-	-	-	-	+
Bacillus subtilis	CMFRI/BS-21	-	-	-	-	-	+
Brevibacillus sp.	CMFR1/UnBr-01	-	-	-	-	-	+
Cronobacter sakazakii	CMFR1/Csa-01	-	-	-	-	-	+
Citrobacter amalonaticus	CMFRI/CAm-01	-	-	-	-	-	+
Enterobacter sp.	CMFRI/EAs-01	-	-	-	-	-	+
Enterococcus faecium	CMFRI/EF-02	-	-	-	-	-	+
Escherichia coli	ATCC25922	-	-	-	-	-	+
Halotalea sp.	CMFRI/UnH-01	-	-	-	-	-	+
Klebsiella pneumonia	CMFRI/KlP-01	-	-	-	-	-	+
	CMFRI/KlP-02	-	-	-	-	-	+
Kluyvera ascorbata	CMFRI/KlA-01	-	-	-	-	-	+
Lysinibacillus sp.	CMFRI/UnLy-01	-	-	-	-	-	+
Mesorhizobium sp.	CMFRI/UnMes01	-	-	-	-	-	+
Micrococcus luteus	CMFRI/Ml-01	-	-	-	-	-	+
Morganella morganii	CMFRI/MM-04	-	-	-	-	-	+
Paenibacillus alvei	CMFRI/PaA-01	-	-	-	-	-	+
Pantoea dispersa	CMFRI/PaD-01	-	-	-	-	-	+
Photobacterium damselae	CMFRI/PhD-03	-	-	-	-	-	+
Providencia rettgeri	CMFRI/PRe-01	-	-	-	-	-	+
Pseudomonas aeruginosa	CMFRI/PA-04	-	-	-	-	-	+
Pseudomonas oleovorans	CMFRI/PO-01	-	-	-	-	-	+
Salmonella typhimurium	MTCC3231	-	-	-	-	-	+
Serratia marcescens	CMFRI/SeMa-01	-	-	-	-	-	+
	CMFRI/SHa-13	-	-	-	-	-	+
Shewanella haliotis	CMFRI/Sha-03	-	-	-	-	-	+
Shigella flexneri	MTCC1457	-	-	-	-	-	+
Staphylococcus aureus	MTCC 1144	-	-	-	-	-	+
Staphylococcus epidermidis	CMFRI/Ste-02	-	-	-	-	-	+
Streptococcus agalactiae	CMFRI/SA-01	-	-	-	-	-	+
Virgibacillus halodenitrificans	CMFRI/VbHd-01	-	-	-	-	-	+

[VP, *Vibrio parahaemolyticus*; Van, *Vibrio anguillarum;* Val, *Vibrio alginolyticus*; VV, *Vibrio vulnificus*; VHa, *Vibrio harveyi*; and IAC, Internal amplification control]

purity of each strain was confirmed, and purified cultures were stored as glycerol stocks at −80°C.

Genomic DNA extraction

Genomic DNA was isolated from each strain using the recommended standard protocol[21]. Purified DNA was dissolved in 30 µL of Tris-EDTA buffer (pH 8.0) and stored at -20°C for future use. The integrity of each isolated DNA was checked by 0.7% agarose gel electrophoresis. Further, the purity and concentration of each isolated DNA were determined using a Biophotometer (Eppendorf, Germany). The DNA concentration of each strain was adjusted to 100 ng/µL before using in PCR. The concentration and $OD_{260/280}$ of each template used for PCR are given in Table 2.

Primers

The specific primers for each targeted species were selected following the general principles of multiplex PCR[22]. In brief, the genes which are reported to be specific and ubiquitous in each species were chosen. Several primers are used in single-target PCR for the species identification of the target pathogens of the present study. From these large data set, the primers were selected based on three criteria, their Tm (melting temperature) values were within a few degrees (°C) of each other, they could produce well definite amplicon (size range between 121 to 773 bp) of the target genes, and the amplicon can be well differentiated from the amplicons of the other targets through agarose gel electrophoresis. The details of the used primers are given in Table 3[14,23-27]. All the primers were commercially synthesized (Sigma, India) and used for the PCR reactions.

Optimization of PCR

Uniplex PCR

As the initial step, each primer set was individually validated for amplification, robustness, and specificity through the uniplex PCR reaction using the template DNA of each targeted species (Table 4). The PCR mixture and conditions were optimized to amplify the corresponding target gene from the respective template DNA at an annealing temperature of

Table 2 — Concentration and purity of DNA of bacterial isolates used for mPCR optimization

Species	Isolate ID	DNA concentration and purity	
		DNA Conc. (ng/uL)	$OD_{260/280}$ ratio
Vibrio parahaemolyticus	CMFRI/VP-08	100	1.83
	CMFRI/VP-07	100	1.71
	CMFRI/VP-05	100	1.68
V. anguillarum	CMFRI/VAn-2	100	1.85
V. alginolyticus	CMFRI/VAl-42	100	1.80
	CMFRI/VAl-43	100	1.77
	CMFRI/VAl-41	100	1.68
V. vulnificus	CMFRI/VV-02	100	1.85
	CMFRI/VV-03	100	1.80
	CMFRI/VV-04	100	1.88
V. harveyi	CMFRI/VHa-03	100	1.86
	CMFRI/VHa-06	100	1.79
	CMFRI/VHa-07	100	1.73
V. furnissii	CMFRI/Vfur-01	100	1.78
V. campbellii	CMFRI/VCa-1	100	1.68
V. owensii	CMFRI/VOw-01	100	1.87
V. cholera	MTCC 15025	100	1.63
V. japonicas	CMFRI/VJa-01	100	1.75
V. aestuarianus	CMFRI/VAe-01	100	1.84
Acinetobacter baumannii	CMFRI/ABa-01	100	1.71
Aeromonas caviae	CMFRI/ACa-02	100	1.68
Aeromonas hydrophila	CMFRI/AH-02	100	1.81
Aeromonas jandaei	CMFRI/AJ-01	100	1.84
Aeromonas schubertii	CMFRI/AS-02	100	1.67
Aeromonas veronii	CMFRI/AV-02	100	1.78
Aeromonas dhakensis	CMFRI/ADh-01	100	1.83
Bacillus subtilis	CMFRI/BS-21	100	1.83
Brevibacillus sp.	CMFR1/UnBr-01	100	1.68
Cronobacter sakazakii	CMFR1/Csa-01	100	1.76
Citrobacter amalonaticus	CMFRI/CAm-01	100	1.84
Enterobacter sp.	CMFRI/EAs-01	100	1.83
Enterococcus faecium	CMFRI/EF-02	100	1.87
Escherichia coli	ATCC25922	100	1.71
Halotalea sp.	CMFRI/UnH-01	100	1.88
Klebsiella pneumoniae	CMFRI/KlP-01	100	1.69
	CMFRI/KlP-02	100	1.82
Kluyvera ascorbata	CMFRI/KlA-01	100	1.67
Lysinibacillus sp.	CMFRI/UnLy-01	100	1.88
Mesorhizobium sp.	CMFRI/UnMes01	100	1.75
Micrococcus luteus	CMFRI/Ml-01	100	1.82
Morganella morganii	CMFRI/MM-04	100	1.81
Paenibacillus alvei	CMFRI/PaA-01	100	1.76
Pantoea dispersa	CMFRI/PaD-01	100	1.69
Photobacterium damselae	CMFRI/PhD-03	100	1.73
Providencia rettgeri	CMFRI/PRe-01	100	1.88
Pseudomonas aeruginosa	CMFRI/PA-04	100	1.70
Pseudomonas oleovorans	CMFRI/PO-01	100	1.77
Salmonella typhimurium	MTCC3231	100	1.84
Serratia marcescens	CMFRI/SeMa-01	100	1.77
Shewanella haliotis	CMFRI/SHa-13	100	1.85
	CMFRI/Sha-03	100	1.81
Shigella flexneri	MTCC1457	100	1.77
Staphylococcus aureus	MTCC 1144	100	1.74
Staphylococcus epidermidis	CMFRI/Ste-02	100	1.80
Streptococcus agalactiae	CMFRI/SA-01	100	1.81
Virgibacillus halodenitrificans	CMFRI/VbHd-01	100	1.73

Table 3 — Details of primers used in the study

Name of primers	Sequence (5'-3')	Product size (bp)	Gene targeted	NCBI Accession no. of gene sequence submitted/Ref.
Valg2-F	CTCTCCCAATTCAGCCCTCTA	737	*col* of *V. alginolyticus*	OP187079[23]
Valg2-R	GACTCTTCACAACAGAACTC			
VP1-F	TGTACTGTTGAACGCCTAA	503	*toxR* of *V. parahemolyticus*	MW168989[14]
VP1-R	CACGTTCTCATACGAGTG			
Vang-F	ACATCATCCATTTGTTAC	429	*amiB* of *V. anguillarum*	OP231632[24]
Vang-R	CCTTATCACTATCCAAATTG			
VV3-F	TTCCAACTTCAAACCGAACTATGA	205	*vvhA* of *V. vulnificus*	OP231633[25]
VV3-R	ATTCCAGTCGATGCGAATACGTTG			
VH-F	TATTTGTCACCGAACTCAGAACC	121	*topA* of *V. harveyi*	OP231634[26]
VH-R	TGGCGCAGCGTCTATACG			
NP1-F	GAGTTTGATCCTGGCTCA	1499	*16SrRNA* of prokaryotes	MN240447[27]
NP1-R	ACGGCTACCTTGTTACGACTT			

Table 4 — Bacterial strains used for PCR optimization

Species	Isolate ID	NCBI accession no. obtained for *16SrRNA* gene	Bacterial density in OD_{600} adjusted to one culture susp. (CFU/mL)
V. alginolyticus	CMFRI/VAl-45	MZ227006	9.5×10^8
V. parahaemolyticus	CMFRI/VP-07	MK156400	1.4×10^{10}
V. anguillarum	CMFRI/VAn-2	OP363866	2.5×10^9
V. vulnificus	CMFRI/VV-04	MK156402	1.3×10^{10}
V. harveyi	CMFRI/VH-07	MW142502	3×10^9

50-60°C. The amplification was carried out in a Veriti thermal cycler (Applied Biosystems, UK) with an initial denaturation at 95°C for 5 min, followed by 40 cycles of 94°C for 1 min, 50-60°C for various time (0.5-2 min) and 72°C for varied time (0.5-2 min), and a final extension of 72°C for 10 min. The reaction mixture was optimized by changing different reaction components, such as concentrations of primers (1-10 pmol), $MgCl_2$ (0-2 mM), and template DNA (1-150 ng/µL), to obtain the specific bands with good intensity of each targeted amplicon. Negative control without any template DNA was maintained in each PCR reaction. Finally, five µL of each PCR product was run on 1.5% w/v agarose in Tris Borate EDTA (TBE) buffer containing 0.5 µG/mL ethidium bromide. The gel was visualized under ultraviolet illumination using Molecular Imager®Gel Doc™ XR System (Bio-Rad, Hercules, CA, USA). The standard 100 bp molecular weight DNA marker (Himedia, India) was simultaneously run in each gel to determine the approximate size of each amplicon. Further, the amplicon from each reaction was sent for sequencing at Agrigenome Labs, India, for confirmation. The obtained sequences were then subjected to NCBI-BLAST search and were subsequently submitted to GenBank, NCBI (Table 3).

Multiplex PCR (*mPCR*)

The mPCR was optimized following the general principles by changing different PCR conditions and components[28]. In brief, annealing temperature (50-60°C), annealing time (30 s, 45 s, 1, 1.5 and 2 min), concentrations of $MgCl_2$ (0-2 mM), primers (1-10 pmol), and template DNA of each targeted species (1-150 ng/µL) were changed to obtain the specific bands with good intensity of each targeted amplicon. There were five pairs of species-specific primers, one IAC primer (*16SrRNA* gene), and template DNA of all the five targeted species in each reaction. Two negative controls, one without any template DNA and another with genomic DNA from *A. hydrophila* as the only template, were maintained in each PCR reaction. Finally, five µL of each PCR product was analyzed on 1.5% w/v agarose in TBE containing 0.5 µg/mL ethidium bromide. The standard 100 bp molecular weight DNA marker (Himedia, India) was simultaneously run in each gel.

Sensitivity evaluation of the optimized mPCR

The optimized assay was performed using two-fold dilutions of the genomic DNA of each targeted species (from 100 nG/µL) to evaluate the sensitivity. The lowest concentration of each template that can result in the formation of six distinct bands through the optimized assay was found. DNA quantification of the template was performed using Biophotometer (Eppendorf, Germany), and sensitivity was expressed as nanograms of genomic DNA of each targeted bacteria per µL PCR reaction volume.

The sensitivity of the optimized mPCR in terms of CFU of each pathogen was also evaluated[9]. Each pathogen was grown in Luria Bertani broth for 18-24 h, and bacterial suspensions were prepared in sterile phosphate-buffered saline (PBS). The OD (optical density) at 600 nM of each bacterial suspension was adjusted to one and heat-inactivated at 100°C for 15 min. Serial ten-fold dilutions of the heat inactivated cultures were prepared in PBS, and one µL from each dilution was used as a template in

the optimized mPCR. The highest dilution of each pathogen that can produce six distinct bands in the mPCR was determined. The number of bacteria corresponding to this dilution was then estimated in CFU/mL by the spread plate method[29]. The sensitivity was then expressed as the CFU of each targeted bacteria per µL PCR reaction volume.

Specificity evaluation of the optimized multiplex PCR

Specificity was verified by employing the genomic DNA of the bacteria from 30 different genera and 47 unique species (Table 1) as the template in the optimized reaction.

Results

Uniplex PCR conditions

The uniplex PCR reaction mixture to amplify the corresponding target gene from the respective template DNA (Fig. 1) at an annealing temperature of 50-60°C included 1X PCR buffer (Takara) containing 1.5 mM $MgCl_2$, 5 pmol of each primer, 2.5 mM of each dNTPs (Takara) and 1.5 U of *Taq* polymerase (Takara) in a final 25 µL reaction volume. The PCR conditions were; initial denaturation for 5 min at 95°C, followed by 40 cycles of 94°C for 1 min, 55°C for 30 s, and 72°C for 1.5 min, and a final extension of 72°C for 10 min. There was specific amplification of all six targeted gene fragments *viz.*, *toxR* of *V. parahaemolyticus* (503 bp), *amiB* of *V. anguillarum* (429 bp), *col* of *V. alginolyticus* (773 bp), *vvhA* of *V. vulnificus* (205 bp) and *topA of V. harveyi* (121 bp) and *16SrRNA* gene of prokaryotes (1499 bp) (Fig. 2) through the optimized uniplex PCR conditions, while all the control samples remained as negative. All the tested strains showed a positive reaction with *16SrRNA* primers. The specific amplification of each primer set was confirmed by the amplicon sequencing, and the sequences were submitted to GenBank, NCBI, under accession numbers (Table 3).

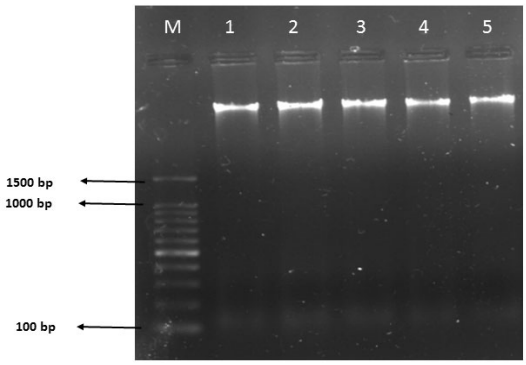

Fig. 1 — Agarose gel profiles of the template DNA of the pathogens used for multiplex PCR. [Lane M: Molecular size marker (100 bp ladder); Lane 1: *V. parahaemolyticus*; Lane 2: *V. alginolyticus V. parahaemolyticus*; Lane 3: *V. anguillarum*; Lane 4: *V. harveyi*; and Lane 5: *V. vulnificus*]

Multiplex PCR

The optimized mPCR reaction mixture contained 2.5 µL 10X PCR buffer (Takara) containing 1.5 mM $MgCl_2$, 2.5 mM of each dNTPs (Takara), 1.5 U *Taq* polymerase (Takara), 5 pmol of forward and reverse primers of six primer sets and 1 µL of template DNA prepared from all the five pathogens (concentration: 100 ng/µL) in a final 25 µL reaction volume. The addition of extra $MgCl_2$ was found to have no beneficial effect on the mPCR. The optimized multiplex PCR conditions were; Initial denaturation for 5 min at 95°C, followed by 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min, and a final extension of 72°C for 10 min. The optimized multiplex PCR successfully amplified all six gene fragments of the expected sizes (Fig. 3). The control sample with genomic DNA of *A. hydrophila* amplified only 16SrRNA gene, and the other control sample without any template DNA produced no bands in the optimized assay. The results of the optimized mPCR can be interpreted as shown in Table 5.

Sensitivity of the optimized mPCR assay

During sensitivity assay, the intensity of amplified products gradually decreased along with the decrease in template DNA concentration. The minimum detection limit was 0.25 ng for *V. harveyi*, 0.5 ng for *V. vulnificus*, 1 ng for *V. parahaemolyticus* and *V. anguillarum*, and 2 ng for *V. alginolyticus* DNA

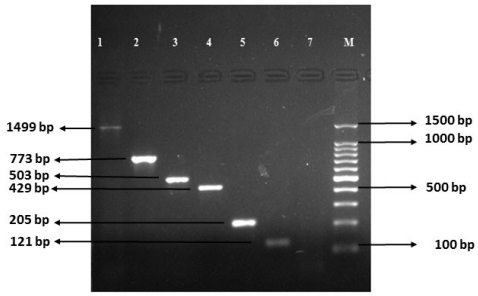

Fig. 2 — Amplification profiles in the uniplex PCR. [Lane M: Molecular size marker (100 bp ladder); Lane 1: ~1500 bp sized fragment from *16SrRNA* gene of prokaryotes (Internal amplification control); Lane 2: ~737 bp sized fragment from *col* of *V. alginolyticus*; Lane 3: ~503 bp sized fragment from *toxR* of *V. parahaemolyticus*; Lane 4: ~ 429 bp sized fragment from *amiB* of *V. anguillarum*; Lane 5: ~205 bp sized fragment from *vvhA* of *V. vulnificus*; Lane 6: ~121 bp sized fragment from *topA* of *V. harveyi*; and Lane 7: Negative control (without DNA template)]

Table 5 — Interpretation of optimized multiplex PCR

Results	Interpretation
Amplification of all the six fragments	Presence of all the five targeted pathogens and well-functioning of all the systems (True positive)
No amplification of any fragments	False negative reaction
Amplification of only one fragment (~1500 bp)	True negative reaction
Amplification of any of the targeted fragments along with ~ 1500 bp sized-amplicon	True positive reaction and presence of the pathogen corresponding to the amplified gene
Amplification without a fragment of ~1500 bp	False positive reaction

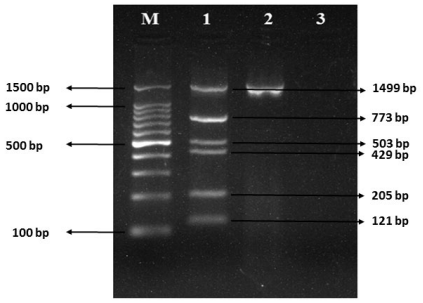

Fig. 3 — Amplification profiles in the optimized multiplex PCR. [Lane M: Molecular size marker (100 bp ladder); Lane 1: Amplification of all the targeted six fragments in the optimized Multiplex PCR showing ~1500 bp sized fragment from *16SrRNA* gene of prokaryotes, ~737 bp sized fragment from *col* of *V.alginolyticus*, ~503 bp sized fragment from *toxR* of *V. parahaemolyticus*, ~429 bp sized fragment from *amiB* of *V. anguillarum*, ~205 bp sized fragment from *vvhA* of *V. vulnificus*, ~121 bp sized fragment from *topA* of *V. harveyi*; Lane 2: Negative control with template DNA of *A. hydrophila* showing only ~1500 bp sized fragment amplification; and Lane 3: Negative control (without any template DNA)]

per µL of the optimized mPCR assay (Fig. 4). The detection sensitivity in terms of CFU of the five-target species was found to be ranging from 1.2 CFU to 380 CFU per µL mPCR. In detail, the minimum detection limit was 1.2 CFU for *V. harveyi*, 5.2 CFU for *V. vulnificus*, 1×10^1 CFU for *V. anguillarum*, 3.8×10^2 CFU for *V. alginolyticus*, and 5.6×10^1 CFU for *V. parahaemolyticus* per µL of the optimized mPCR assay, in a mixed template containing the heat-inactivated cultures of each pathogen (Fig. 5)

Specificity of the optimized mPCR assay

The specificity assay showed that none of the non-targeted bacterial genera and non-targeted species of the genus *Vibrio* produced any cross-reactivity or non-specific amplification in the optimized mPCR (Table 1). Nevertheless, all the strains tested showed a ~1500 bp-sized amplicon in the optimized assay (Fig. 6).

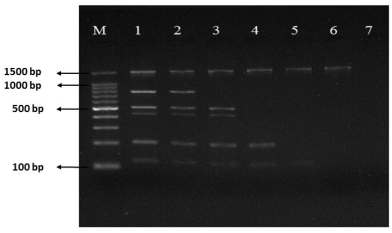

Fig. 4 — Sensitivity evaluation of the optimized multiplex PCR in terms of DNA. [The optimized assay was done using mixed template DNA of each targeted pathogen. Two-fold dilutions of genomic DNA from each targeted species were used. Lane M: Molecular size marker (100 bp ladder); Lanes 1-6: Multiplex PCR using 100, 50, 25, 12.5, 6.25 and 3.125 ng of template DNA from each targeted pathogen, corresponding to 4, 2, 1, 0.5, 0.25 and 0.12 ng/µL DNA, respectively from each pathogen; and Lane 7: Negative control (without DNA template)]

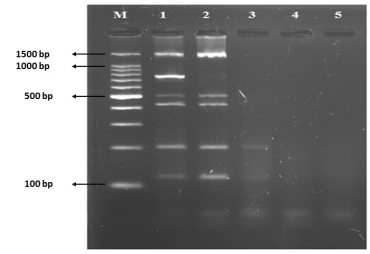

Fig. 5 — Sensitivity evaluation of the optimized Multiplex PCR in terms of colony-forming units. [The optimized assay was done using mixed heat-inactivated cultures of each targeted pathogen. Lane M: Molecular size marker (100 bp ladder); Lanes 1-4: Multiplex PCR using 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} diluted OD_{600}=1 adjusted heat inactivated cultures as templates, corresponding to 3.8×10^2, 5.6×10^2, 1.0×10^2, 5.2×10^2 and 1.2×10^2 CFU/µL assay, respectively for *V. alginolyticus*, *V. parahaemolyticus*, *V. anguillarum*, *V. vulnificus* and *V. harveyi*; and Lane 5: Negative control (without template)]

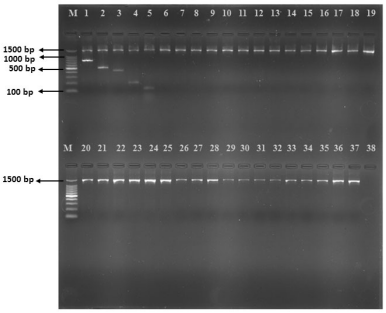

Fig. 6 — Specificity evaluation of optimized Multiplex PCR. [Lane M: Molecular size marker (100 bp ladder); Lanes 1-11: *V. alginolyticus*, *V. parahaemolyticus*, *V. anguillarum*, *V. vulnificus*, *V. harveyi*, *V. furnissii*, *V. campbellii*, *V. owensii*, *V. cholerae*, *V. japonicus* and *V. aestuarianus*, respectively; Lanes 12-37: *Acinetobacter baumannii*, *Aeromonas hydrophila*, *Bacillus subtilis*, *Brevibacillus* sp., *Cronobacter sakazakii*, *Citrobacter amalonaticus*, *Enterobacter* sp., *Enterococcus faecium*, *Escherichia coli*, *Halotalea* sp., *Klebsiella pneumoniae*, *Kluyvera ascorbata*, *Lysinibacillus* sp., *Mesorhizobium* sp., *Micrococcus luteus*, *Morganella morganii*, *Paenibacillus alvei*, *Pantoea dispersa*, *Photobacterium damselae*, *Providencia rettgeri*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Serratia marcescens*, *Shewanella haliotis*, *Shigella flexneri* and *Staphylococcus aureus*, respectively; and Lane 38: Negative control (without template)]

Discussion

The availability of rapid, sensitive, and specific detection methods for fish pathogens is essential for early and efficient disease management in aquaculture practices. The conventional microbiological methods lack many of these attributes; therefore, several individual PCR-based assays have established a place in diagnosing aquaculture diseases[30]. The major challenges in fish disease diagnosis, like the frequent occurrence of concurrent infections with multiple pathogens, the requirement to screen a large number of samples to reach a definite diagnosis, *etc.* make mPCR technology specifically essential for fish pathogens to have a cost, effort, and time-effective disease assessment and management[3]. Although simultaneous detection of several pathogens with the mPCR has been widely used in clinical and food specimens, this approach has not been applied in the detection of fish pathogens[18]. The present paper develops a multiplex PCR technology for the simultaneous detection of 5 major pathogens of aquaculture significance, *viz. V. parahaemolyticus*, *V. anguillarum*, *V. alginolyticus*, *V. vulnificus*, and *V. harveyi*. The factors, namely, DNA polymerase inactivation, presence of PCR inhibitors, instrument failure, *etc*., can affect the PCR efficiency and lead to false-negative results in many circumstances. Accordingly, an internal amplification control (IAC) targeting the highly conserved gene of bacteria namely, the 16S rRNA gene, was kept in the optimized mPCR platform to ensure the precision of the detection method[31].

Vibriosis is the most common infectious disease leading to massive mortality and substantial economic losses in the world's aquaculture industry[2]. Among the several species of *Vibrionaceae* associated with health problems of aquatic animals, *V. parahaemolyticus*, *V. salmoncida*, *V. anguillarum*, *V. alginolyticus*, *V. harveyi*, *V. vulnificus*, *V. owensii* and *V. campbelli* are the major finfish pathogens[32]. A new multiplex PCR targeting human and animal-specific virulence marker genes (pilF and fpcrp) was developed recently to detect seafood containing zoonotic *V. vulnificus*[33]. However, the challenges inherent in size discrimination among different PCR products by electrophoresis, and the complexity of optimizing PCR conditions for different PCR products of various lengths, make it difficult to incorporate more than six primer sets in a single mPCR platform[33,34]. Accordingly, we have targeted the five most common *Vibrio* spp., pathogens of tropical fishes, and the sixth primer set was fixed as IAC for the mPCR.

The species-level identification of *Vibrio* spp. has been challenging in the diagnostic field[35]. The ample phenotypic variability within the *Vibrionaceae* family makes the application of classical phenotypic identification impractical in many circumstances for species-level discrimination, pointing toward the need for the scheme based on the genomic data[36]. Even though the accurate identification of vibrios at the family and genus levels is possible by 16S rRNA gene sequencing, several vibrios have nearly identical 16S rRNA gene sequences making identification at the species and strain levels possible only through targeting the specific genes[37]. The recent research in *V. alginolyticus* strains by Praveena *et al*.[38] showed that there was a mixing and dispersal of multiple genotypes of same species within water samples. Accordingly, the present study targeted *toxR* for *V. parahaemolyticus*, *col* for *V. alginolyticus*, *vvhA* for *V. vulnificus*, *topA* for *V. harveyi* and *amiB* for *V. anguillarum*, all of which are reported to be specific for each targeted pathogens. Previously, these genes were utilized for individual identification of each of these species[23-25,39,40] During the validation, uniplex PCR produced products of the designed size, indicating the specific amplification, further confirmed by the amplicon sequencing. Following the confirmation, mPCR was optimized to get all six amplicons simultaneously, and the best results were obtained at 55°C with 1.5 mM $MgCl_2$ concentration and 5 pmol of each primer. The optimized mPCR with mixed template DNA of all five species produced precise amplification without interference in band intensity. Similar mPCR platforms that can identify up to 3 or 4 major human pathogenic *Vibrio* spp., such as *V. cholerae*, *V. parahaemolyticus*, *V. campbellii* and *V. vulnificus* were already reported[9,15,20]. Further, mPCR technology that can identify three *Vibrio* spp. like, *V. alginolyticus*, *V. anguillarum*, and *V. harveyi* [3], *V. anguillarum*, *V. harveyi*, and *V. alginolyticus*[19] and *V. harveyi*, *V. campbellii*, *and V. parahaemolyticus*[20] were reported. However, the simultaneous detection of five fish pathogens along with one IAC is not reported to date.

Concerning the sensitivity of the assay, the intensity of amplified products gradually decreased along with the decrease in template DNA concentration, as reported in other mPCR platforms[19]. The minimum detection limit of the optimized

mPCR was found as 0.2 ng for *V. harveyi* and 1 ng for *V. parahaemolyticus*, *V. vulnificus* and *V. anguillarum*, and 10 ng for *V. alginolyticus* DNA per µL in mixed templates. The detection sensitivity in terms of CFU of the five-target species was 1.2 CFU for *V. harveyi*, 5.2 CFU for *V. vulnificus*, 1×10^1 CFU for *V. anguillarum*, 3.8×10^2 CFU for *V. alginolyticus*, and 5.6×10^1 CFU for *V. parahaemolyticus* per µL of the optimized mPCR assay, in a mixed template containing the heat inactivated cultures of each pathogen. In general, mPCR is reported to be less sensitive than uniplex PCR due to the competition for reaction reagents[41]. Nevertheless, the detection limit of the present mPCR assay was similar to the one reported for the uniplex PCR technology[25,33]. More importantly, the three target mPCR for fish pathogens reported earlier demonstrated almost equal sensitivity to the present mPCR technology[3,19]. In detail, the mPCR technology demonstrated a sensitivity of 1.5, 0.4 and 5.6 ng per µL, respectively, for *V. harveyi*, *V. anguillarum* and *V. alginolyticus*[3], whereas the sensitivity of the present mPCR was 0.25, 1 and 2 ng per µL, respectively for these pathogens. A three-target mPCR was developed for *V. anguillarum*, *V. harveyi* and *V. alginolyticus* with 1 ng per µL sensitivity for all the pathogens[19]. The almost similar sensitivity of the present mPCR assay with five pathogens would account for the rapid generation of the test results. Another important observation was the bias to amplify small fragments in the current mPCR technology resulting in the lower detection limit of *V. harveyi* compared to the other four targeted pathogens. The observation was in parallel to the earlier reports on other mPCR platforms[8,28]. The difference in detection limits might further be contributed by the difference in primer amplification efficiency in the PCR reaction due to variations in primer length, nucleotide content, and secondary structure[16].

When the optimized five-species mPCR assay was evaluated for specificity against a collection of *Vibrio* and non-Vibrio species, none of the non-targeted bacterial species produced cross-reactivity, indicating the discrimination power without ambiguity of false-positive results from non-target species. Nevertheless, all the bacteria tested showed a ~1500 bp sized amplicon in the optimized assay, so the ambiguity of false-negative results can be avoided.

In brief, the paper narrates a molecular methodology for the simultaneous and accurate detection and identification of five major aquaculture pathogens in a convenient platform, which is essential for developing appropriate prophylactic measures in aquaculture settings. While applying the optimized mPCR, the researcher should know the possible challenges in getting precise PCR results. For example, the quality of template DNA has a marked effect on the results. A quality DNA with OD 260/280 ratio between 1.6-1.9 is recommended since the impure DNA with an increased or decreased OD260/280 ratio can produce false positive or negative results[28]. The same can also happen with excess primers or dNTPs in the solution, so the researchers should use the optimized PCR reaction mixture mentioned in the paper to ensure precision. The researchers should also follow the optimized PCR condition, especially initial denaturation and annealing. The initial denaturation step separates the double-stranded template DNA into single strands so that the primers can efficiently bind to the target region and cause amplification[28]. Furthermore, the high temperature at this step helps to inactivate heat-labile proteases or nucleases that may be present in the sample, with minimal impact on *Taq* DNA polymerases. Accordingly, if the denaturation temperature is not ensured, the DNA will not be completely denatured, resulting in low amplification efficiency. Similarly, if the denaturation time is too long, DNA might be degraded, resulting in low amplification efficiency[28]. Further, the researchers are recommended to use 1.5-3% agarose gel for visualizing the results to ensure the proper separation of multiple bands. Prolonged electrophoresis at lower voltage gradients can notably lower the sharpness of individual PCR bands[28], especially the bands >400 bp belonging to *V. anguillarum*, *V. vulnificus*, and *V. harveyi*.

Conclusion

The above study demonstrated a molecular methodology for simultaneous and accurate detection and identification of five major aquaculture pathogens in a convenient platform that forms the essential step for developing appropriate prophylactic measures in aquaculture settings. The optimized multiplex PCR conditions were; Initial denaturation for 5 min at 95°C, followed by 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min, and a final extension of 72°C for 10 min. The minimum detection limit was 0.25 ng for *V. harveyi*, 0.5 ng for *V. vulnificus*, 1 ng for *V. parahaemolyticus* and *V. anguillarum*, and 2 ng

for *V. alginolyticus* DNA per µL of the optimized mPCR assay. In terms of CFU, the detection limit was 1.2, 5.2, 10, 5.6×10^1 and 3.8×10^2 CFU/µL for *V. harveyi*, *V. vulnificus*, *V. anguillarum*, *V. parahaemolyticus* and *V. alginolyticus*, respectively, per µL assay. There was 100% specificity. The multiplex PCR assay developed can be used as a sensitive and specific method for the simultaneous detection of five fish pathogens causing significant concern in the aquaculture industry. Furthermore, four of these species (*V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus* and *V. harveyi*) are emerging human pathogens with a possible increase in virulence with the predicted climate change/global warming. In this context, the newly developed multiplex PCR can also be used for human clinical diagnostics, food industry, ecological and epidemiological studies. Nevertheless, future investigation of the suitability of this assay for direct detection in fish tissues, other marine organisms, and seafood is warranted.

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Conflict of interest

Authors declare no competing interests.

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