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Development of a Multiplex Quantitative PCR Assay for Simultaneous Detection of Multiple *Vibrio* Species in Aquaculture Samples

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ABSTRACT

Aquaculture associated *Vibrio* species are important bacterial pathogens causing significant economic losses in aquaculture and zoonotic infections in humans. Rapid and accurate detection of *Vibrio* species is therefore essential for diagnosis, surveillance and control. In this study, a multiplex quantitative PCR (qPCR) assay was developed for the simultaneous detection of *Vibrio* spp. together with *V. alginolyticus*, *V. anguillarum*, *V. harveyi*, *V. ordalii*, *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae*. Species-specific primers and probes targeting *ompA, glutamate synthase, mreB, vohB, dnaJ, gyrB* and *lolB* genes were designed, while the *rpoA* gene was selected for genus level *Vibrio* detection. The assay demonstrated 100% inclusivity for all target strains without cross reactivity against non-target bacterial species. PCR efficiency/inhibition analyses showed stable amplification dynamics across serial dilutions, with R^2 values above 0.99, amplification efficiencies between 96.8% and 98.2%, and ΔCq values ≤0.13 for all targets. Limit of detection (LOD) studies performed using artificially contaminated matrices demonstrated a detection limit of 10^1–10^0 CFU/mL for all targets, with detection performance comparable to the culture-based recovery method. Overall, the developed multiplex qPCR assay provides a rapid, sensitive and analytically reliable approach for simultaneous detection of multiple *Vibrio* species associated with aquaculture and zoonotic infections.

1 | Introduction

Aquaculture is a developing industrial sector that requires continuous research and innovation. Bacterial infections are a common and recurrent problem in aquaculture systems. Bacterial pathogens primarily affect fish and shellfish, causing diseases that can lead to reduced growth rates, increased mortality and deterioration in product quality. Various bacterial pathogens, particularly *Vibrio* species, are known to cause infections in aquaculture and can also cause water and food related infections (Austin and Austin 2007; Ajin et al. 2016; Kirchberger et al. 2016; Machado and Bordalo 2016; Shaheen et al. 2016). Diseases associated with *Vibrio* species are commonly referred to as vibriosis (Baker-Austin et al. 2018). Vibriosis, is a human disease caused by pathogenic strains of bacteria within the *Vibrio* genus (excluding *Vibrio cholerae* O1 and O139 strains responsible for cholera). Among more than 80 *Vibrio* species, over 20 are capable of causing vibriosis (Bell and Bott 2021; Ina Salwany et al. 2019; Ma et al. 2023). The *Vibrio* species isolated from vibriosis cases in fish are reported to include *Vibrio anguillarum*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Vibrio alginolyticus*, *Vibrio ordalii* and *Vibrio harveyi* (Öztürk and Altınok 2014). Human pathogenic species include *Vibrio carchariae*, *Vibrio mimicus*, *Vibrio cincinnatiensis*, *Vibrio fluvialis*, *Vibrio cholerae*, *V. parahaemolyticus, V. vulnificus, V. alginolyticus*,

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Vibrio furnissii, *Vibrio metschnikovii*, *Vibrio hollisae* and *Vibrio damsela* (Finkelstein 1996; Levinson and Jawetz 1996; Morris 2019). The principal species responsible for vibriosis are *V. parahaemolyticus* and *V. vulnificus* (Bell and Bott 2021; Ina Salwany et al. 2019; Ma et al. 2023).

Due to the increasing production and consumption of aquatic animals, the global risk of zoonotic diseases is also rising. Zoonotic bacteria can be transmitted to humans through various routes: 64% via the consumption of raw or undercooked fish products, 23% through skin contact and cutaneous ulcers, and 19% through exposure to water contaminated with these bacteria (Farzadnia and Naeemipour 2020). Among the most important zoonotic bacteria transmitted from fish to humans are *Vibrio* species, including *V. cholerae, V. alginolyticus, V. parahaemolyticus, V. vulnificus, V. anguillarum, V. ordalii* and *V. harveyi* (Farzadnia and Naeemipour 2020; Gauthier 2015). Within the *Vibrio* genus, *V. cholerae* is pathogenic only to humans and does not cause symptoms in fish (Farzadnia and Naeemipour 2020). Species causing vibriosis can enter the food chain through contaminated seafood, leading to vibriosis in humans, which is characterized by symptoms such as gastroenteritis, septicemia, vomiting, abdominal cramps and fever (Kobuszewska and Wysok 2023). Transmission is generally known to occur through the consumption of raw or undercooked contaminated seafood or through wound exposure (Bell and Bott 2021).

Zoonotic *Vibrio* species are classified into two groups based on the infections they cause: those responsible for cholera and those that are not. *V. cholerae* is the causative agent of cholera, a disease characterized by severe diarrheal symptoms. Cholera cases reported to date have occurred in the form of epidemics or pandemics (Martins et al. 2022). Globally, an estimated 4 million people contract cholera each year, with approximately 100,000 deaths reported. Outbreaks observed in many countries across Asia, Africa and the Americas pose a significant public health risk (Baker-Austin et al. 2018).

To date, numerous foodborne vibriosis outbreaks have been reported in many countries, and the number of such outbreaks is known to be increasing (CDC 2015, 2016). Furthermore, it is anticipated that global warming will contribute to an expansion in the geographic distribution, prevalence and impact of pathogenic *Vibrio* species (Baker-Austin et al. 2018).

Bacterial infections in fish farming are also of considerable economic importance in aquaculture. The increasing incidence of infectious diseases adversely affects the production, profitability and sustainability of the global aquaculture industry. Therefore, early and accurate diagnosis of disease agents, along with appropriate disease management, is critically important in aquaculture (Ina Salwany et al. 2019; Pinto et al. 2017).

Traditional diagnosis of *Vibrio* infections has primarily relied on culture-based methods, such as bacterial isolation on agar media (Ina Salwany et al. 2019). Although conventional culturing techniques can be used to detect and quantify pathogenic members of the *Vibrionaceae* family, they are time-consuming and labour-intensive. In addition, direct colony counting on thiosulfate citrate bile salts sucrose (TCBS) agar, a selective medium commonly used for *Vibrio* spp., may lead to underestimation of bacterial loads (Munro et al. 2020). The accurate identification of *Vibrio* species is further complicated by the high degree of similarity among species within the genus in terms of morphology, virulence factors, genomic characteristics and clinical manifestations. Current detection approaches include culture-based, immunological and molecular techniques; however, conventional symptom or culture-based methods often lack the specificity required for reliable differentiation at the species level (Rui et al. 2008; Zhou et al. 2013). PCR-based molecular diagnostics are widely applied due to their high specificity, and several conventional PCR assays have been developed for the detection of some *Vibrio* species (O'Hara et al. 2003; Osman et al. 2022; Pang et al. 2006; Xiao et al. 2009). Nevertheless, these methods are often labour intensive and may have limited sensitivity, particularly when applied to complex samples.

Most existing molecular assays target a limited number of *Vibrio* species, whereas comprehensive multiplex detection of multiple clinically and aquaculture relevant *Vibrio* species remains limited. Therefore, this study aimed to develop a multiplex quantitative real-time PCR (qPCR) assay for the rapid and simultaneous detection of *Vibrio* species (*V. alginolyticus*, *V. anguillarum*, *V. harveyi*, *V. ordalii, V. parahaemolyticus, V. vulnificus* and *V. cholerae*), including zoonotic species associated with foodborne infections in humans, as well as genus level detection of *Vibrio* spp.

2 | Materials and Methods

2.1 | Bacterial Strains

A total of 88 bacterial strains, including target *Vibrio* species and non-*Vibrio* strains, were included in this study. The *Vibrio* panel consisted of *V. alginolyticus*, *V. anguillarum*, *V. harveyi*, *V. ordalii*, *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae*, representing both reference strains and field isolates. The target panel included 10 field isolates and one reference strain for each *Vibrio* species. In addition, 11 closely related aquatic bacteria and non-target organisms were included to evaluate assay specificity. The field isolates were obtained from aquaculture-related samples and archived at the Department of Microbiology, Faculty of Veterinary Medicine, Ankara University, Türkiye. Field isolates and the other bacterial isolates used in the analytical panel were recovered by culture and their species-level identities were confirmed by Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) prior to inclusion in the study. Reference strains were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), including *V. alginolyticus* DSM 2171, *V. anguillarum* DSM 21597, *V. harveyi* DSM 19623, *V. ordalii* DSM 19621, *V. parahaemolyticus* DSM 11058, *V. vulnificus* DSM 10143 and *V. cholerae* DSM 100200. All bacterial strains were cultured on tryptic soy agar (TSA) supplemented with 1.5%–2% NaCl overnight at 37°C ± 1°C. Bacterial density was standardized to 0.5 McFarland (~1.5×10^8 CFU/mL) prior to DNA extraction, and glycerol stocks (20% v/v) were stored at −80°C in nutrient broth supplemented with NaCl.

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2.2 | Nucleic Acid Extraction

Genomic DNA was extracted from *Vibrio* isolates using the Bio-speedy Rapid Nucleic Acid Extraction kit (Bioeksen R&D Technologies Ltd., Türkiye) in combination with the Zybio EXM3000 robotic system following the manufacturer's instructions and the 9-min B-200 protocol. The extracted nucleic acids were stored at −80°C until qPCR analysis. An exogenous heterologous DNA template derived from a non-target organism was used as the internal amplification/inhibition control. The control template was added directly to each qPCR reaction mixture to verify successful amplification and to detect potential PCR inhibition caused by inhibitory substances carried over from the sample matrix or nucleic acid extraction process.

2.3 | Oligonucleotides

Target loci were selected based on previously published molecular studies and comparative bioinformatic analysis. The *rpoA* locus was selected for genus-level *Vibrio* detection (Dalmasso et al. 2009), while *ompA* for *V. alginolyticus* (Bunpa et al. 2020), *mreB* for *V. harveyi* (Mougin et al. 2020), *vohB* for *V. ordalii* (Avendaño-Herrera et al. 2014), *dnaJ* for *V. parahaemolyticus* (Nhung et al. 2007), *gyrB* for *V. vulnificus* (Kumar et al. 2006) and *lolB* for *V. cholerae* (Cho et al. 2013) were selected with reference to previous reports. For *V. anguillarum*, a discriminatory region within the glutamate synthase locus was identified through comparative bioinformatic sequence analysis performed in the present study. Species- and genus-specific primers and hydrolysis probes targeting these loci were newly designed in this study using Primer3 (v.4.1.0). Primer design parameters were optimized to obtain amplicon sizes ranging between 135 and 241 bp, with melting temperatures and GC content adjusted to ensure compatibility within the multiplex format.

Following primer and probe design, theoretical specificity was evaluated by *in silico* sequence comparison and NCBI Primer-BLAST analysis against publicly available nucleotide databases to assess potential cross-reactivity, including with closely related non-target *Vibrio* sequences. Hydrolysis probes were labelled with FAM, ROX and Cy5 fluorophores. The exogenous amplification/inhibition control was monitored in the HEX channel. The sequences and characteristics of the primers and probes evaluated in this study are presented in Table 1.

2.4 | Multiplex qPCR Assay

The assay was configured as a four-well multiplex qPCR panel consisting of four separately prepared reaction mixes that were run in parallel. Mix 1 targeted genus-level *Vibrio* spp. (FAM), *V. parahaemolyticus* (ROX) and *V. cholerae* (Cy5). Mix 2 targeted *V. alginolyticus* (FAM) and *V. vulnificus* (ROX). Mix 3 targeted *V. anguillarum* (FAM) and *V. harveyi* (ROX). Mix 4 targeted *V. ordalii* (FAM). The exogenous amplification/inhibition control was monitored in the HEX channel. Multiplex qPCR reactions were performed using a Magnetic Induction Cycler (Mic) platform under optimized touchdown cycling conditions. The optimized amplification conditions were 95°C for 10 s, followed by 12 touchdown cycles comprising denaturation at 95°C for 1 s and annealing/extension for 15 s with the annealing temperature decreasing by 1°C per cycle from 67°C to 56°C. This was followed by 30 additional amplification cycles consisting of denaturation at 95°C for 1 s and annealing/extension at 55°C for 15 s.

Each well reaction was carried out in a final volume of 22 μL, containing 1× PCR master mix, 500 nmol/L of each forward and reverse primer, 200 nmol/L of each probe, 2 μL of inhibition control template and 4 μL of extracted nucleic acid. The template DNA was adjusted to a working concentration of 10 ng/μL prior to analysis. Multiplex qPCR reaction setup details are given in Table 2.

Positive and negative controls were included in each qPCR assay run. Genomic DNA extracted from reference strains was used as a positive control, while nuclease-free water was used as a negative control. The validity of each run was assessed based on the performance of these controls. Samples were considered positive when a characteristic sigmoidal amplification curve was observed within the acceptable Cq range (positive if Cq < 28), and negative when no amplification signal was detected. Samples yielding borderline amplification signals were reanalysed to confirm the results.

2.5 | PCR Efficiency/Inhibition

PCR efficiency and inhibition analysis of the multiplex qPCR assay were performed using extracted nucleic acids from representative *Vibrio* strains. Bacterial cultures were grown under appropriate conditions, and genomic DNA was extracted as described above. DNA concentration and purity were determined prior to analysis using a Micro-Spectrophotometer, Nanodrop ND-1000 (Thermo Scientific, USA).

Extracted nucleic acids were adjusted to a working concentration of 10 ng/μL, and a six-point 4-fold serial dilution series (1/2, 1/4, 1/16, 1/64, 1/256 and 1/1024) was prepared. Each dilution was tested in duplicate within the same run using the multiplex qPCR assay. Calibration curves were constructed by plotting the mean Cq values against the logarithm of the dilution factors. Linear regression analysis was performed to determine the slope and correlation coefficient (R^2) for each target. Amplification efficiency (E) was calculated using the equation.

$$E = (10\text{^}(-1/\text{slope}) - 1) \times 100$$

PCR inhibition was assessed by calculating ΔCq values between the observed Cq at the working concentration and the extrapolated Cq derived from the regression equation. The following acceptance criteria were applied: ΔCq < 0.5, $R^2 \geq 0.98$, slope between −3.6 and −3.1, and amplification efficiency between 90% and 110%.

2.6 | Selectivity

In silico specificity of the newly designed primers and probes was evaluated using NCBI Primer-BLAST and sequence comparison against publicly available nucleotide databases, including non-target Vibrio sequences, to assess potential cross-reactivity.

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TABLE 1 | Primers and probes used for multiplex qPCR detection of *Vibrio* species.

Primer/Probe	Sequence (5′–3′)	Amplicon size (bp)	Target gene	Reference/ Selection basis
Vibrio spp.—Forward	TCACCTGACGGATGACAACG	196	*rpoA*	Dalmasso et al. 2009
Reverse	AGTACGCTGTTCNACACGAGC			
Probe (FAM-BHQ1)	GTTCCAGCTTCTGCGCGTATCC			
V. alginolyticus—Forward	TGGCGCTTTTGTTGGTTACG	176	*ompA*	Bunpa et al. 2020
Reverse	CGCCACCCACTTTACCGTAA			
Probe (FAM-BHQ1)	GCAGTAGAAGCGGGTTTCGACA			
V. anguillarum—Forward	TACCAGCCGCAAAGTTACGT	186	*glt synthase*	Present study: comparative bioinformatics
Reverse	ACGTCTGTTTCTATGGCGCA			
Probe (FAM-BHQ1)	ACGACCACTTTGGCTCCTGAGT			
V. harveyi—Forward	AAGCCCACGTGTACTGGTTT	135	*mreB*	Mougin et al. 2020
Reverse	CTCAGAGACACGCAGACCTG			
Probe (ROX-BHQ2)	GCGTCGTGCGATTCGTGAATCT			
V. ordalii—Forward	CGTGGTTGATTGCGCTTTCT	236	*vohB*	Avendaño-Herrera et al. 2014
Reverse	CAAGGTCGGTGTCTCAGCTT			
Probe (FAM-BHQ1)	TGCGACCACAATAAGCGATGCA			
V. parahaemolyticus—Forward	GATATCTTCGGTGGCGGTCG	166	*dnaJ*	Nhung et al. 2007
Reverse	CACCGCTACCGTCACAAGAG			
Probe (ROX-BHQ2)	CGCTAGAAGAAGCCGTTCGTGG			
V. vulnificus—Forward	CAACAACATCCCACAGCGTG	180	*gyrB*	Kumar et al. 2006
Reverse	GGATCCGGCACTTTGACTGA			
Probe (ROX-BHQ2)	CCGCGACTTCTGGTGATGATGC			
V. cholerae—Forward	CTTTGTGCATCTTGGTCGCG	241	*lolB*	Cho et al. 2013
Reverse	GGCAATCACACCAAGTCACTC			
Probe (Cy5-BHQ2)	TCATAAGTTTCGACCCGCGCAC			

TABLE 2 | Multiplex qPCR reaction setup details.

Reagent	Final concentration
2X PCR Master Mix	1X
Forward primer (each)	500 nmol/L
Reverse primer (each)	500 nmol/L
Probe (each)	200 nmol/L
Inhibition Control	2 μL
Template nucleic acid	4 μL
Final Volume	22 μL

Note: Template DNA was standardized to a working concentration of 10 ng/μL prior to qPCR analysis.

Experimental selectivity was evaluated using the target Vibrio strains and non-target bacterial strains. Target organisms included *V. alginolyticus*, *V. anguillarum*, *V. harveyi*, *V. ordalii*, *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae*. Each target Vibrio strain was assessed across the species-specific assays to evaluate cross-amplification; the expected genus-level Vibrio signal was also monitored. Non-target organisms included *Aeromonas hydrophila*, *Aeromonas veronii*, *Aeromonas salmonicida*, *Edwardsiella tarda*, *Yersinia ruckeri*, *Streptococcus iniae*, *Streptococcus agalactiae*, *Lactococcus garvieae*, *Lactococcus petauri*, *Escherichia coli* and *Salmonella enterica*. Additional non-target *Vibrio* species were not included as independent experimental strains; their potential cross-reactivity was assessed *in silico*.

All samples were tested under the same amplification conditions, and Cq values were monitored in the corresponding channels.

2.7 | Limit of Detection (LOD)

The limit of detection (LOD) was defined as the lowest concentration of target bacteria yielding positive detection in ≥95% of

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TABLE 3 | Data of PCR efficiency/inhibition tests.

Target	Dilution factor	Cq1	Cq2	Cq mean	Cq difference (prev)	Log10 of dilution factor
Vibrio spp.	1/2	16.82	16.88	16.85	—	0
	1/4	18.89	18.95	18.92	2.07	−0.602
	1/16	21.00	21.06	21.03	2.11	−1.204
	1/64	23.05	23.14	23.10	2.07	−1.806
	1/256	25.18	25.25	25.22	2.12	−2.408
	1/1024	27.24	27.33	27.29	2.07	−3.010
V. alginolyticus	1/2	17.40	17.47	17.44	—	0
	1/4	19.51	19.57	19.54	2.10	−0.602
	1/16	21.63	21.70	21.67	2.13	−1.204
	1/64	23.69	23.77	23.73	2.06	−1.806
	1/256	25.84	25.90	25.87	2.14	−2.408
	1/1024	27.91	27.99	27.95	2.08	−3.010
V. anguillarum	1/2	17.05	17.12	17.09	—	0
	1/4	19.12	19.19	19.16	2.07	−0.602
	1/16	21.25	21.33	21.29	2.13	−1.204
	1/64	23.31	23.39	23.35	2.06	−1.806
	1/256	25.46	25.55	25.51	2.16	−2.408
	1/1024	27.54	27.63	27.59	2.08	−3.010
V. harveyi	1/2	16.30	16.36	16.33	—	0
	1/4	18.35	18.42	18.39	2.06	−0.602
	1/16	20.49	20.56	20.53	2.14	−1.204
	1/64	22.52	22.60	22.56	2.03	−1.806
	1/256	24.65	24.74	24.70	2.14	−2.408
	1/1024	26.71	26.80	26.76	2.06	−3.010
V. ordalii	1/2	17.62	17.69	17.66	—	0
	1/4	19.70	19.78	19.74	2.08	−0.602
	1/16	21.83	21.92	21.88	2.14	−1.204
	1/64	23.88	23.97	23.93	2.05	−1.806
	1/256	26.06	26.14	26.10	2.17	−2.408
	1/1024	28.10	28.20	28.15	2.05	−3.010
V. parahaemolyticus	1/2	16.92	16.99	16.96	—	0
	1/4	18.99	19.06	19.03	2.07	−0.602
	1/16	21.12	21.19	21.16	2.13	−1.204
	1/64	23.16	23.25	23.21	2.05	−1.806
	1/256	25.33	25.41	25.37	2.16	−2.408
	1/1024	27.38	27.47	27.43	2.06	−3.010

(Continues)

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TABLE 3 | (Continued)

Target	Dilution factor	Cq1	Cq2	Cq mean	Cq difference (prev)	Log10 of dilution factor
V. vulnificus	1/2	17.22	17.29	17.26	—	0
	1/4	19.30	19.37	19.34	2.08	−0.602
	1/16	21.42	21.49	21.46	2.12	−1.204
	1/64	23.49	23.57	23.53	2.07	−1.806
	1/256	25.65	25.74	25.70	2.17	−2.408
	1/1024	27.70	27.80	27.75	2.05	−3.010
V. cholerae	1/2	16.66	16.73	16.70	—	0
	1/4	18.73	18.81	18.77	2.07	−0.602
	1/16	20.87	20.94	20.91	2.14	−1.204
	1/64	22.93	23.01	22.97	2.06	−1.806
	1/256	25.06	25.15	25.11	2.14	−2.408
	1/1024	27.13	27.22	27.18	2.07	−3.010

replicates (Hougs et al. 2017). LOD studies were conducted using artificially contaminated matrices, including fish tissue samples and chicken cloacal swabs. Prior to spiking, all matrices were confirmed to be free of the target *Vibrio* species using the culture-based method. Bacterial suspensions of each target species were prepared separately and adjusted to a defined concentration. Serial dilutions were performed to obtain target contamination levels of 10^2–10^1 and 10^1–10^0 CFU/mL. Each target *Vibrio* species was spiked into the matrices independently; target species were not combined within the same artificially contaminated sample, and therefore no interspecies mixing ratio was applied.

For each matrix and contamination level, 20 independent replicates were prepared and analysed across two separate experimental runs. All samples were processed under identical conditions and tested using the developed multiplex qPCR assay. Each sample was analysed in duplicate by two different analysts on different days by multiplex qPCR.

Positive and negative controls were included in each run to ensure assay validity. Results obtained by the multiplex qPCR assay were compared with culture-based recovery of the corresponding previously identified target strains. The identities of the field isolates used in the study had been confirmed by MALDI-TOF MS before the experiments. For culture-based recovery during the LOD study, artificially contaminated samples were cultured on TSA supplemented with 1.5%–2% NaCl and incubated overnight at 37°C ± 1°C. Growth of the known inoculated organism was used as the culture-based comparator during LOD determination; culture was not used as a stand-alone biochemical species-identification procedure in this experiment.

3 | Results

3.1 | PCR Efficiency/Inhibition

PCR efficiency and inhibition performance of the multiplex qPCR assay were evaluated using 4-fold serial dilutions (1/2 to 1/1024) of extracted nucleic acids for all eight targets. Amplification curves obtained from the dilution series exhibited typical sigmoidal profiles, with consistent Cq shifts of approximately 2.0 cycles between consecutive dilution steps, indicating stable amplification dynamics across the tested range. The data for the qPCR efficiency/inhibition tests are presented in Table 3, while the corresponding amplification curves are shown in Figure 1a–h.

Linear regression analysis of the calibration curves demonstrated excellent linearity for all targets, with coefficients of determination (R^2) ranging from 0.9985 to 0.9993. Slope values ranged between −3.365 and −3.401, falling within the predefined acceptance criteria ($-3.6 \leq \text{slope} \leq -3.1$) and corresponding to amplification efficiencies between 96.8% and 98.2%. All values satisfied the acceptance criteria (90%–110%; Bustin et al. 2009). The calibration curvesare shown in Figure 2a–h, and the corresponding results are presented in Table 4.

ΔCq values calculated between observed and extrapolated Cq values were ≤0.13 for all targets, remaining well below the threshold of 0.5, indicating the absence of significant PCR inhibition. Overall, these findings demonstrate stable amplification performance and high analytical robustness of the multiplex qPCR assay.

3.2 | Selectivity

The multiplex qPCR panel demonstrated 100% inclusivity by correctly identifying all tested target strains (10 field isolates per species). Genus-level detection targeting *rpoA* successfully amplified all *Vibrio* species included in the study, whereas each species-specific primer/probe set amplified only its corresponding target organism. No cross-amplification was observed between the species-specific target assays. No amplification was observed in the non-target organisms, including *A. hydrophila*, *A. veronii*, *A. salmonicida*, *E. tarda*, *Y. ruckeri*, *S. iniae*, *S. agalactiae*, *L. garvieae*, *L. petauri*, *E. coli* and *S. enterica*. All non-target samples remained negative in

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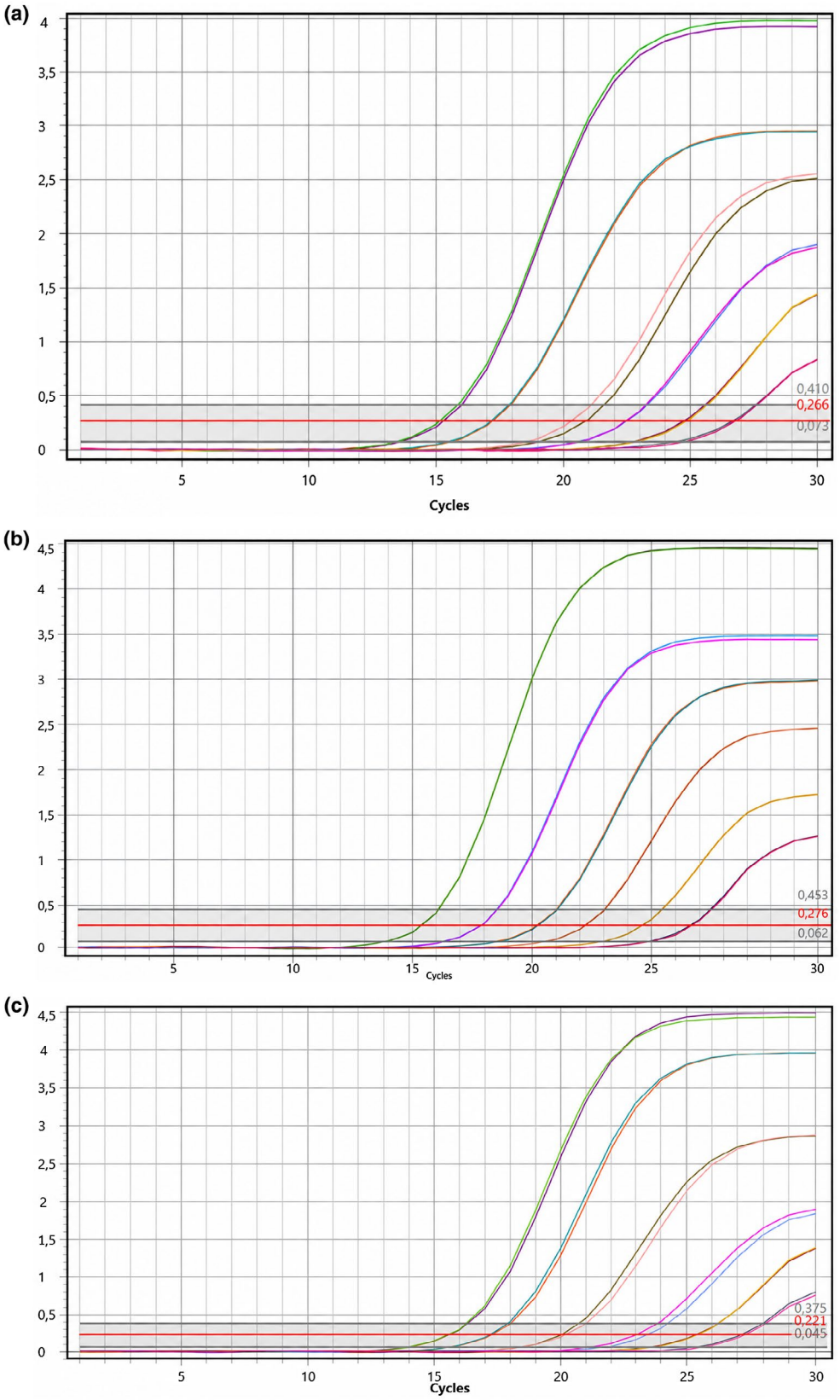


FIGURE 1 | The amplification curves of the PCR efficiency/inhibition tests: (a) *Vibrio* spp.; (b) *V. alginolyticus*; (c) *V. anguillarum*; (d) *V. harveyi*; (e) *V. ordalii*; (f) *V. parahaemolyticus*; (g) *V. vulnificus*; and (h) *V. cholerae*.

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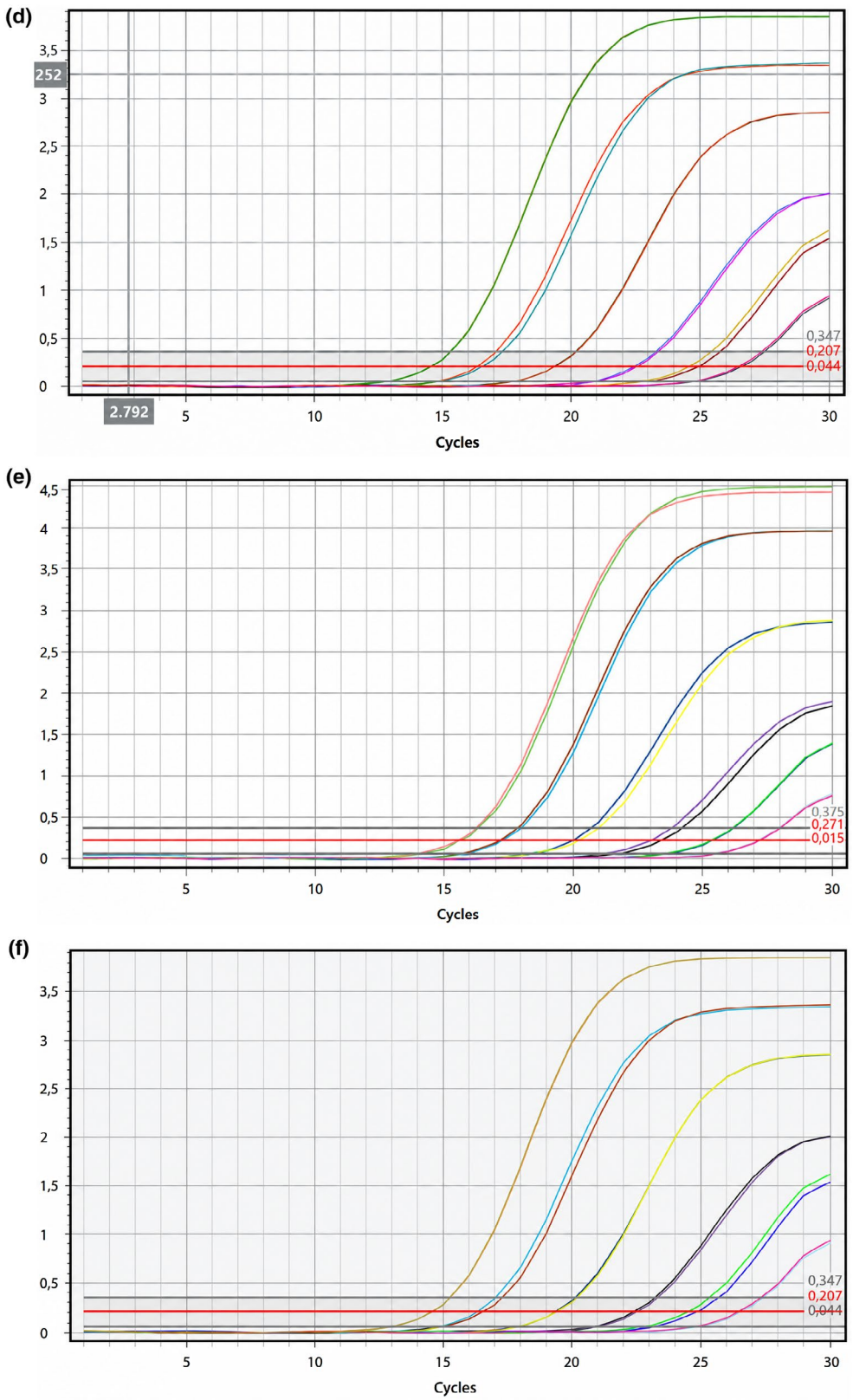


FIGURE 1 | (Continued)

the species-specific detection channels while yielding valid amplification of the internal amplification/inhibition control, confirming assay validity and the absence of experimentally observed cross-reactivity.

In addition, *in silico* specificity analysis using NCBI Primer-BLAST and sequence comparison showed no relevant off-target matching within the primer/probe binding regions for the selected targets, including the non-target *Vibrio* sequences

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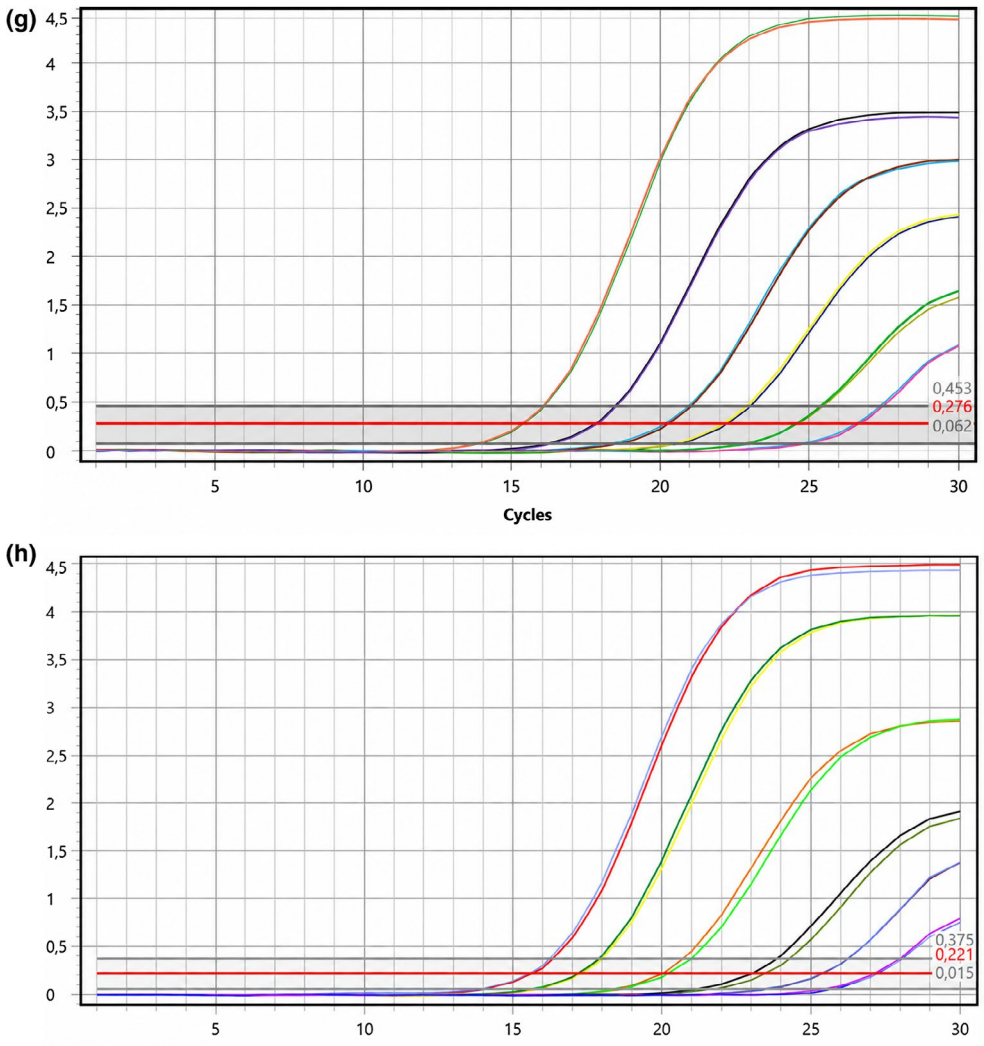


FIGURE 1 | (Continued)

evaluated *in silico*. Because the experimentally tested non-target reactions showed no amplification, individual negative amplification curves were not plotted; the corresponding negative results are reported in the specificity dataset. Together, the *in silico* and laboratory results supported the analytical specificity of the developed multiplex qPCR panel.

3.3 | Limit of Detection (LOD)

LOD studies were performed using artificially contaminated matrices and the results were compared with those obtained by the culture-based recovery method (Table 5). At contamination levels of 10^2–10^1 CFU/mL, all targets were detected in 100% of replicates (20/20), with mean Cq values ranging between 19.86 and 21.42. At contamination levels of 10^1–10^0 CFU/mL, ≥95% detection (19/20) was achieved for all targets, while mean Cq values ranged between 23.12 and 24.80. Under the same conditions, the culture method yielded positive results in all replicates (20/20). Based on the predefined acceptance criterion of ≥95% positive detection, the analytical limit of detection of the multiplex qPCR assay was determined as 10^1–10^0 CFU/mL for all targets under the tested conditions. All negative and positive controls included in the multiplex qPCR runs were found to be valid.

4 | Discussion

The multiplex qPCR assay developed in this study enables the simultaneous detection of multiple *Vibrio* species associated with aquaculture and zoonotic infections. Rapid and accurate identification of *Vibrio* spp. is critically important because conventional culture-based methods are labour intensive, time consuming and may lack sufficient specificity for reliable discrimination of closely related *Vibrio* species. For this reason, molecular diagnostic methods, particularly PCR based assays, have increasingly been used for the detection and differentiation of *Vibrio* pathogens (Chapela et al. 2017; Pinto et al. 2017). To date, numerous PCR based studies have been conducted in Türkiye and worldwide for the detection and typing of important pathogens associated with aquaculture (Ador et al. 2022; Bartkova et al. 2017; Chapela et al. 2017; Diyie et al. 2022; Fernández-Álvarez et al. 2016; Pinto et al. 2017). Since fish diseases are often associated not with a single pathogen but with synergistic interactions between two or more infectious agents, the application of

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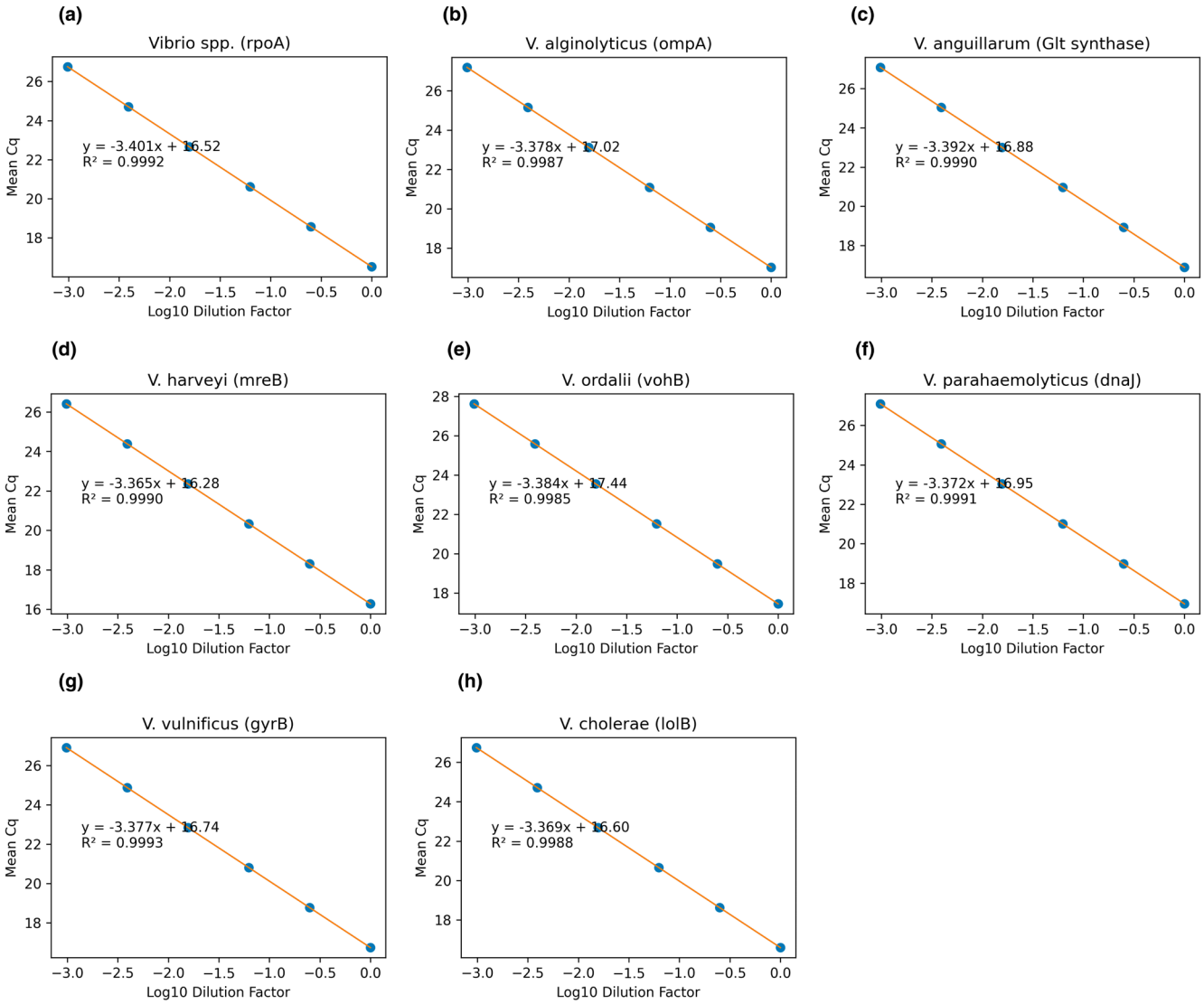


FIGURE 2 | The calibration curves of the PCR efficiency/inhibition tests (from left to right: *Vibrio* spp., *V. alginolyticus, V. anguillarum, V. harveyi, V. ordalii, V. parahaemolyticus, V. vulnificus*, *V. cholerae*). For each target, line/point colours correspond to the six dilution factors reported in Table 3 (1/2, 1/4, 1/16, 1/64, 1/256 and 1/1024), and each plotted dilution is associated with the corresponding mean Cq value for that target.

multiplex assays in disease diagnosis is particularly important (Kotob et al. 2017). Nonetheless, to the best of our knowledge, there is currently no multiplex qPCR assay capable of simultaneously detecting seven *Vibrio* species and genus level *Vibrio* targets included in the present study within a four-well panel. Pinto et al. (2017) previously developed a conventional multiplex PCR assay targeting four bacterial pathogens, including *V. alginolyticus*, *V. anguillarum*, *V. harveyi* and *E. tarda*. In contrast, the present study simultaneously detects additional *Vibrio* species of both aquaculture and zoonotic importance together with genus level *Vibrio* detection within a four-well panel using a probe based multiplex qPCR platform incorporating an internal inhibition control. In addition, the present study determined the analytical limit of detection using artificially contaminated matrices and replicate based detection analysis, and directly compared assay performance with a culture-based recovery method under conditions more closely reflecting practical aquaculture samples.

Similarly, Abioye et al. (2021) employed conventional simplex, duplex and triplex PCR assays for the detection of medically important *Vibrio* species in freshwater and brackish water environments. Several *Vibrio* species targeted in the present study, including *V. cholerae*, *V. vulnificus*, *V. parahaemolyticus* and *V. alginolyticus*, were also investigated by Abioye et al. (2021). However, Abioye et al. (2021) employed an enrichment-based workflow involving most probable number coupled with polymerase chain reaction (MPN-PCR) analysis using turbid enrichment tubes prior to PCR confirmation, whereas the present study focused on the development of a rapid multiplex qPCR platform for simultaneous detection of targeted *Vibrio* species. In addition, the developed assay

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TABLE 4 | Results of PCR efficiency/inhibition tests.

Parameter	Acceptance criteria	Results			
		***Vibrio* spp.** ($y = -3.401x + 16.52$)	***V. alginolyticus*** ($y = -3.378x + 17.02$)	***V. anguillarum*** ($y = -3.392x + 16.88$)	***V. harveyi*** ($y = -3.365x + 16.28$)
ΔCqa	< 0.5	0.12	0.09	0.11	0.11
R^2	≥ 0.98	0.9992	0.9987	0.9990	0.9990
Slope	−3.6 ≤ slope ≤ −3.1	−3.401	−3.378	−3.392	−3.365
PCR efficiency	90% ≤ E ≤ 110%	96.8%	97.6%	97.1%	98.1%

Parameter	Acceptance criteria	Results			
		V. ordalii ($y = -3.384x + 17.44$)	***V. parahaemolyticus*** ($y = -3.372x + 16.95$)	***V. vulnificus*** ($y = -3.377x + 16.74$)	***V. cholerae*** ($y = -3.369x + 16.60$)
ΔCqa	< 0.5	0.10	0.13	0.12	0.09
R^2	≥ 0.98	0.9985	0.9991	0.9993	0.9988
Slope	−3.6 ≤ slope ≤ −3.1	−3.384	−3.372	−3.377	−3.369
PCR efficiency	90% ≤ E ≤ 110%	97.4%	97.9%	97.7%	98.2%

was analytically validated using specificity, sensitivity and culture-based recovery comparison analyses. These methodological differences indicate that the present study provides a diagnostic oriented multiplex qPCR platform intended for rapid and simultaneous detection of *Vibrio* species relevant to aquaculture and zoonotic infections.

Zhang et al. (2025) developed a TaqMan probe-based multiplex qPCR assay for the simultaneous detection of multiple *Vibrio* pathogens associated with aquaculture including *V. anguillarum, V. alginolyticus, V. harveyi* and *V. scophthalmi* using species-specific virulence associated genes (*empA, toxR, vhhP2* and *luxR*). In agreement with the findings of Zhang et al. (2025), the multiplex qPCR assay developed in the present study demonstrated high analytical specificity without cross reactivity against non-target bacterial species. Zhang et al. (2025) reported R^2 above 0.99 and amplification efficiencies ranging between 96% and 108%, indicating highly reliable amplification performance. Similarly, the present study demonstrated stable amplification dynamics across serial dilutions, with R^2 values ranging between 0.9985 and 0.9993, slope values between −3.365 and −3.401, amplification efficiencies between 96.8% and 98.2%, and ΔCq values ≤ 0.13 for all included targets, indicating highly reproducible and analytically robust amplification performance.

In the present study, both *in silico* and laboratory-based specificity analyses demonstrated high analytical specificity of the developed multiplex qPCR assay. The genus specific *rpoA* target successfully amplified all included *Vibrio* species, whereas species specific primer and probe sets amplified only their corresponding target organisms without cross reactivity with non-target bacterial species. In addition, no significant off-target homology was identified in Primer BLAST analyses, further supporting the specificity of the selected oligonucleotide sets.

The developed multiplex qPCR assay also demonstrated high analytical sensitivity, with a detection limit of 10^1–10^0 CFU/mL for all targets under the tested conditions. The detection limits achieved in the present study were comparable to those previously reported for multiplex and single-target qPCR assays (Fukui and Sawabe 2008; Hickey et al. 2015; Osman et al. 2022; Zhao et al. 2011). Furthermore, the assay showed detection performance comparable to the culture-based recovery method while providing rapid and consistent amplification results across all tested species. Overall, the findings of the present study demonstrate that the developed multiplex qPCR assay provides a rapid, sensitive and analytically reliable approach for the simultaneous detection of multiple *Vibrio* species associated with aquaculture and zoonotic infections.

The present study primarily focused on analytical validation using DSMZ reference strains, aquaculture-derived field isolates, non-target bacterial strains and artificially contaminated matrices. Although the field isolates represented naturally occurring aquaculture-associated strains and were confirmed by MALDI-TOF MS, direct prospective validation using a large set of naturally infected field matrices was not performed. Further studies comparing the developed four-well multiplex qPCR

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TABLE 5 | Summary of LOD studies in artificially contaminated matrices for the multiplex qPCR method and culture-based recovery method.

Organism (Target gene)	Contamination level (CFU/mL)	Positive rate	Cq mean ± SD	Culture recovery positive rate
V. alginolyticus (*ompA*)	10^2–10^1	20/20	20.62 ± 0.34	20/20
	10^1–10^0	19/20	23.91 ± 0.58	20/20
V. anguillarum (*Glt synthase*)	10^2–10^1	20/20	20.44 ± 0.30	20/20
	10^1–10^0	19/20	23.70 ± 0.55	20/20
V. harveyi (*mreB*)	10^2–10^1	20/20	19.86 ± 0.28	20/20
	10^1–10^0	19/20	23.12 ± 0.52	20/20
V. ordalii (*vohB*)	10^2–10^1	20/20	21.05 ± 0.33	20/20
	10^1–10^0	19/20	24.39 ± 0.62	20/20
V. parahaemolyticus (*dnaJ*)	10^2–10^1	20/20	20.28 ± 0.29	20/20
	10^1–10^0	19/20	23.60 ± 0.56	20/20
V. vulnificus (*gyrB*)	10^2–10^1	20/20	20.76 ± 0.32	20/20
	10^1–10^0	19/20	24.10 ± 0.63	20/20
V. cholerae (*lolB*)	10^2–10^1	20/20	20.33 ± 0.31	20/20
	10^1–10^0	19/20	23.68 ± 0.54	20/20
Vibrio spp. (*rpoA*)	10^2–10^1	20/20	20.18 ± 0.27	20/20
	10^1–10^0	19/20	23.44 ± 0.50	20/20

panel with established reference diagnostic procedures using naturally infected aquaculture samples are warranted before routine diagnostic implementation.

5 | Conclusions

The multiplex qPCR assay developed in the present study demonstrated high analytical specificity and sensitivity together with stable amplification performance and reliable parallel multi-target detection across a four-well panel. In addition, the assay incorporates genus level *Vibrio* detection and an internal inhibition control, thereby increasing diagnostic reliability during routine analysis. The developed multiplex qPCR assay may provide a practical and efficient tool for routine monitoring, rapid pathogen detection and early surveillance of *Vibrio* species associated with aquaculture and zoonotic infections.

Author Contributions

İnci Başak Müştak: conceptualization, investigation, writing – original draft, methodology, writing – review and editing. **Hamit Kaan Müştak:** conceptualization, investigation, funding acquisition, writing – review and editing, project administration, supervision, methodology. **Seyyide Sarıçam İnce:** investigation, writing – review and editing. **Doğukan Uğun:** methodology, validation, visualization, formal analysis. **Görkem Cengiz:** investigation, methodology. **Gültekin Ünal:** formal analysis, validation, methodology. **Ebru Torun:** data curation, investigation. **Muammer Göncüoğlu:** supervision, conceptualization, investigation, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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