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Article

Improved Multiplex PCR Assay for Detection of *tlh*, *trh*, and *tdh* genes in *Vibrio parahaemolyticus*, Aligned with the U.S. FDA's Bacteriological Analytical Manual (BAM)

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Abstract: *Vibrio parahaemolyticus* is an important foodborne bacterium that causes severe gastroenteritis following the consumption of contaminated seafood. To identify *V. parahaemolyticus* and determine its pathogenicity, the U.S. Food and Drug Administration (FDA)'s Bacteriological Analytical Manual (BAM) recommends a multiplex polymerase chain reaction (PCR) protocol to simultaneously detect the species-specific thermolabile hemolysin (*tlh*) gene and the pathogenic thermostable-related hemolysin (*trh*) and thermostable-direct hemolysin (*tdh)* genes. However, this assay has shown two limitations: difficulty in separating the amplicons of the *trh* (486 bp) and *tlh* (450 bp) genes due to their similar sizes, and the weaker band exhibited by the *tdh* gene amplicon (270 bp). The present study aimed to improve the BAM's multiplex PCR assay by separating the three amplicons with similar intensity. A new primer set was applied for the *tlh* gene (369 bp) alongside the existing primers for the *trh* and *tdh* genes. The amplicons for the three genes were effectively separated by electrophoresis on a 2% tris-borate-EDTA (TBE) agarose gel within 45 minutes. Primer concentrations of 0.2 μM for *trh*, 0.2 μM for *tlh*, and 0.4 μM for *tdh* produced a significant amount of amplicons among various combinations of primer concentrations with 35 PCR cycles ($P < 0.05$). This assay exhibited a detection limit of 10 pg of bacterial DNA, demonstrating its high sensitivity. It did not display amplicons from nine *Vibrio* strains known to be human pathogens or from 18 well-documented foodborne pathogens. Therefore, the present multiplex PCR protocol could help overcome the limitations of existing assays and provide a more reliable method for detecting the three genes of *V. parahaemolyticus*.

Keywords: Improved multiplex PCR; U.S. FDA BAM; tlh; trh; tdh; Vibrio parahaemolyticus

1. Introduction

Vibrio parahaemolyticus poses a significant threat to public health through the consumption of contaminated seafood [1]. According to the U.S. Centers for Disease Control and Prevention (CDC), approximately 84,000 people suffer from Vibrio-related illnesses annually [2,3]. This Gram-negative halophilic bacterium inhabits estuarine and marine environments and can naturally infiltrate oysters [2–4]. However, the number of bacteria significantly increases in oysters during the warm-water season, and improper distribution or handling can further accelerate bacterial contamination, leading to public health concerns [5,6]. For instance, the Pacific *V. parahaemolyticus* strain (O4 serotype/sequence type 36) severely impacted Oyster Bay, NY, causing a significant increase in reported illnesses and extended closures in Long Island Sound, including major areas in Connecticut [7]. By 2013, the outbreak had spread from Virginia to Massachusetts, causing over 100 reported illnesses and resulting in unprecedented closures and recalls [8]. This crisis made the industry view the situation as an existential threat and become more open to implementing controls to restore their critical summer operations [9].

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A multiplex polymerase chain reaction (PCR) protocol is listed as one of the standard methods in the U.S. Food and Drug Administration (FDA)'s Bacteriological Analytical Manual (BAM) to identify *V. parahaemolyticus* and to determine its pathogenicity simultaneously, while information for the real-time PCR assay is still not available [10]. This assay is designed to yield three bands showing the thermolabile hemolysin (*tlh*) gene as a unique identification marker and the thermostable-related hemolysin (*trh*) and thermostable-direct hemolysin (*tdh)* genes as pathogenic markers of *V. parahaemolyticus*. Studies have demonstrated that all examined *V. parahaemolyticus* showed the amplification of *tlh* gene, with no positive results in closely related *Vibrio* spp. and other foodborne pathogens [11–13]. Moreover, clinical investigations have indicated that *V. parahaemolyticus* isolated from human patients carry the *trh* and *tdh* genes, which are potentially responsible for seafood-related illnesses and deaths [14–16]. Therefore, identifying *V. parahaemolyticus* and determining its pathogenicity in oysters are crucial steps to prevent foodborne illnesses and protect the domestic seafood industry.

Recently, we conducted the multiplex PCR recommended by BAM to identify *V. parahaemolyticus* and assessed its pathogenicity in oysters from the U.S. Gulf Coast. After 25 PCR cycles using a positive control strain (F11-3A), the amplicons were loaded onto a 1.5 % tris-borate-EDTA (TBE) agarose gel [2,11]. However, the three bands did not appear even after 90 minutes of electrophoresis. The bands for *trh* (486 bp) and *tlh* (450 bp) were not separated well, and the *tdh* band (270 bp) was weaker compared to others. This method was originally developed by Bej et al, and the major difference between the multiplex PCR method of BAM and Bej et al. was the number of PCR cycle (25 cycles of BAM and 30 cycles of Bej et al) [11]. We found that the original method produced thicker *tdh* band than BAM, but the bands for *trh* and *tdh* were not well-separated even after 90 minutes of electrophoresis.

In the present study, we aimed to improve the multiplex PCR method recommended by BAM by modifying the primers to achieve efficient separation of the three target genes and enhance the amplification of the *tdh* gene. Additionally, we examined the relative concentrations of primers, PCR cycling conditions, amounts of template DNA, the percentage of agarose, and electrophoresis time to produce three even amplicons, thereby confirming the specificity of the three sets of primers. The detection limit of the assay was evaluated using various concentrations of *V. parahaemolyticus* DNA, and the specificity of the assay was tested using nine *Vibrio* strains known to be human pathogens and 18 well-documented foodborne pathogens.

2. Materials and Methods

2.1. Bacteria, Genomic DNA, and Primers

Vibrio parahaemolyticus F11-3A was used as the reference strain to amply *tlh*, *trh* and *tdh* genes [2,11]. Bacterial DNA was extracted using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. The concentration of bacterial DNA was measured using the NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) by measuring the absorbance at 260 nm. Primers listed in table 1 were used for multiplex PCR. The *tlh* gene primers were designed using SnapGene software (version 5.2, San Diego, CA, USA) targeting a specific region within the gene (gene ID: GU971655.1, MH047289.1, OP270227.1, accessed on 15 January 2024). Primers for the amplification of *trh* and *tdh* genes were adopted from both Bej et al. and BAM protocols [2,11] (Table 1).

Table 1. Primers for the amplification of *tlh*, *trh* and *tdh* genes using the multiplex PCR.

Names	Sequences (5'-3')	size (bp)	References
VP_TLH_L	AAAGCGGATTATGCAGAAGCACTG	450	[11]
VP_TLH_R	GCTACTTTCTAGCATTTTCTCTGC		
VP_TRH_L	TTGGCTTCGATATTTTCAGTATCT	486	

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VP_TRH_R	CATAACAAACATATGCCCATTTCCG		
VP_TDH_L	GTAAAGGTCTCTGACTTTTGGAC	270	
VP_TDH_R	TGGAATAGAACCTTCATCTTCACC		
VP_TLH_F2	CTCAGTTTAAGTACTCAACACAAGAAGAGAT	369	This study
VP_TLH_R2	CTAAGTTGTTGCTACTTTCTAGCATTTTCT		

2.2. Multiplex PCR Condition

Three multiplex PCR methods were employed. The PCR mixture (50 µl) of both BAM and Bej et al consisted of the bacterial DNA (10 ng to 1 pg) from *V. parahaemolyticus* F11-3A, 1 µM of each of the primers (5 µl of each primer from 10 µM stock), 5 µl of a 10 X PCR buffer, 320 µM of each of the dNTPs (8 µl of a 8 mM stock dNTPs), 2.5 units of Dream Taq Green DNA polymerase (0.5 µl of 5 units/µL, Thermo Scientific, Vilnius, Lithuania), and 5.5 µl of water. The amplification condition of BAM was 1 cycle at 94 °C for 3 min, followed by 25 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min, with a final extension at 72 °C for 3 min, while the amplification condition of Bej et al (ref) was 1 cycle at 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 5 min.

Our PCR mixture was composed of the bacterial DNA (10 ng to 1 pg), 0.25 µM of each of the primers (1.25 µl of each primer from 10 µM stock), 5 µl of a 10 X PCR buffer, 320 µM of each of the dNTPs (8 µl of a 8 mM stock dNTPs), 1.5 units of Dream Taq Green DNA polymerase (0.3 µl of 5 units/µL, Thermo Scientific, Vilnius, Lithuania), and 28.2 µl of water. The amplification condition for this study was 1 cycle at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 5 min.

Electrophoresis was conducted using 1.5% TBE (TBE, Alfa Aesar, Ward Hill, MA, USA) agarose gels containing the SYBR Safe DNA gel stain (Invitrogen, Waltham, MA, USA) for various durations (30, 60, and 90 min) to optimize band separation. Similarly, electrophoresis through 2% TBE gel was run for 15 min, 30min, and 45min. The gel was visualized using the Gel Doc XR+ system (Bio-Rad, Hercules, CA, USA).

2.3. Optimization, Sensitivity, and Specificity of Multiplex PCR Assay

For the optimization of primer concentrations, 5 different PCR reaction mixtures were examined (Figure 2-1). Set A contained 0.25 µM of each of the primers. Set B contained 0.2 µM of primers for *tlh* and *trh*, and 0.3 µM of *tdh*. Set C contained 0.2 µM of primers for *tlh* and *trh*, and 0.4 µM of *tdh*. Set 4 contained 0.15 µM of primers for *tlh* and *trh*, and 0.3 µM of *tdh*. Finally, Set E contained 0.1 µM of primers for *tlh* and *trh*, and 0.5 µM of *tdh*. To determine the optimal PCR cycles for our multiplex PCR, the amplification condition was examined 1 cycle at 94 °C for 3 min, followed by 25, 30, or 35 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 5 min. Various concentrations (10 ng, 1 ng, 100 pg, 10 pg, 1 pg, and 100 fg) of *V. parahaemolyticus* F11-3A DNA were used to determine the sensitivity of the multiplex PCR. After genomic DNAs were extracted from other *Vibrio* strains and foodborne pathogenic bacteria listed in figure 4, one ng of each of bacterial DNA samples was employed to determine the specificity of the multiplex PCR.

2.4. Statistical Analysis

The band intensity of amplicons was quantified using the Image Lab Software (Bio-Rad, Hercules, CA, USA). The statistical analysis was conducted using the Prism software (Version 9, GraphPad, Boston, MA, USA) and significant differences were determined by Ordinary one-way ANOVA analysis (Dunnett's multiple comparisons test, $P < 0.05$). The data were presented as mean ± standard deviation (SD, n=3).

3. Results and Discussion

3.1. Multiplex PCR Assay of BAM and Bej et al.

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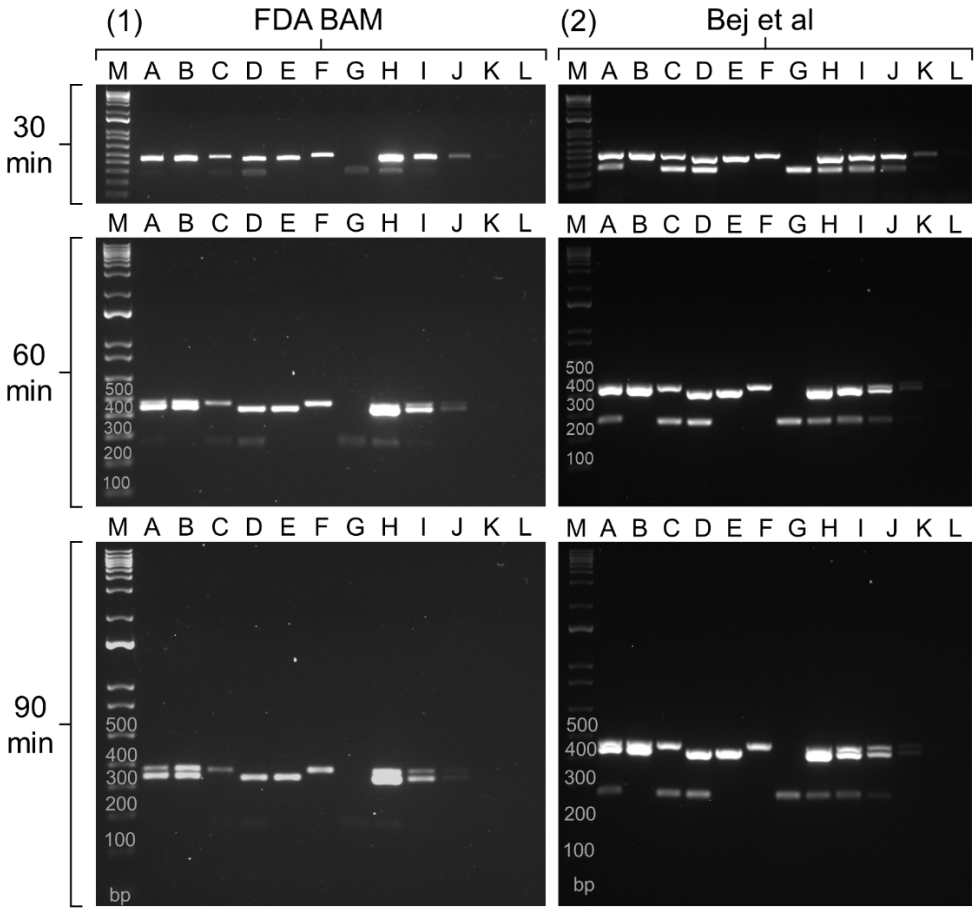
Multiplex PCR assays were conducted based on the method described by Bej et al and BAM to evaluate the simultaneous detection of *tlh*, *trh* and *tdh* genes of *V. parahaemolyticus* [10,11]. Since the BAM protocol originated from Bej et al., both protocols shared identical reaction components, including the PCR mixture and primer sequences except for modifications to the cycling conditions in the BAM protocol. To evaluate the performance of target gene amplifications, the assay was conducted with various primer combinations using 1 ng of F11-3A genomic DNA (Figure 1, panels A-G). Additionally, the sensitivity of the assay was assessed using different concentrations of F11-3A DNA with all three primer sets (Figure 1, panels H-L). Finally, the electrophoresis run time was optimized by running gels at different durations (30, 60, and 90 minutes) to achieve optimal band separation on the 1.5% TBE gel.



(3)

	A	B	C	D	E	F	G	H	I	J	K	L
trh primer (486 bp)	+	+	+	-	-	+	-	+	+	+	+	+
tlh primer (450 bp)	+	+	-	+	+	-	-	+	+	+	+	+
tdh primer (270 bp)	+	-	+	+	-	-	+	+	+	+	+	+
DNA concentration	1 ng	1 ng	1 ng	1 ng	1 ng	1 ng	1 ng	10 ng	1 ng	100 pg	10 pg	1 pg

Figure 1. Multiplex PCR analysis of *Vibrio parahaemolyticus tlh*, *trh* and *tdh* genes. The protocols were based on the Bacteriological Analytical Manual (BAM) of the U.S. FDA (1) and a study by Bej et al. (2). Panels A-G represent reactions with different combinations of *trh*, *tlh*, and *tdh* primers (3). Panels H-L depict the sensitivity of the PCR method tested with various bacterial DNA concentrations (3). M: Molecular weight marker. The electrophoreses were run for 30, 60, and 90 min to separate three bands through a 1.5% TBE agarose gel.

The multiplex PCR assay of BAM (Figure 1-1) exhibited insufficient separation of *trh* and *tlh* gene amplicons at 30 and 60 minutes of electrophoresis. While separation of both genes was achieved at

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90 minutes, bands remained unresolved with 10 ng of bacterial DNA (Figure 1-1H). The *tdh* gene displayed a faint band in the sample containing 1 ng of bacterial DNA (Figure 1-1A, C, D, G, and I) and became visible at 10 ng of DNA (Figure 1-1H). Therefore, the limit of detection (LOD) of the BAM multiplex PCR was determined to be 10 ng of genomic DNA (Figure 1-1H, I, J, K, and L).

The multiplex PCR assay of Bej et al. (Figure 1-2) showed stronger band intensity for all three genes compared to BAM. This difference was probably due to the increased number of PCR cycles employed by Bej et al. (30 cycles) compared to BAM (25 cycles). Although a band for the *tdh* gene appeared with 1 ng of bacterial DNA, its intensity diminished with longer electrophoresis times (Figure 1-2A, C, D, G, and I). Additionally, both *trh* and *tlh* genes remained inseparable even at 90 minutes (Figure 1-2A, B, D, G, and I). The LOD of this method was determined to be 100 pg of bacterial DNA (Figure 1-2H, I, J, K, and L).

Both multiplex PCR assays exhibited limitations in separating the amplicons of the *trh* (486 bp) and *tlh* (450 bp) genes due to their similar sizes. This small size difference makes it difficult to distinguish the two amplicons on a gel. Additionally, the *tdh* gene amplicon (270 bp) often displays weaker band intensity compared to *trh* and *tlh*, potentially hindering its detection. To address these limitations, our multiplex PCR was designed to effectively separate the amplicons of *trh* and *tlh* genes, allowing for clear identification of both targets. Furthermore, the assay would be optimized to generate amplicons for all three genes (*trh*, *tlh*, and *tdh*) with similar band intensities, facilitating easier detection and analysis. This improved design aimed to overcome the limitations of the existing assays and provide a more reliable method for detecting these genes.

3.2. Optimization of the Current Multiplex PCR

To optimize a multiplex PCR assay for efficient separation and detection of three genes (*tlh*, *trh*, and *tdh*) in *Vibrio parahaemolyticus*, three primer sets for *tlh* gene were examined for our enhanced multiplex PCR (Table 1). The middle band of lane A, B, and C in Figure 2 were amplified using the combination of VP_TLH_L and VP_TLH_R2 (403 bp), VP_TLH_F2 and VP_TLH_R (359 bp), and VP_TLH_F2 and VP_TLH_R2 (369 bp), respectively. All three candidates for *tlh* gene displayed specific amplicons along with the expected amplicons for *trh* (486 bp) and *tdh* (252 bp) genes on the gel, with no non-specific products observed. Notably, Panel C using the VP_TLH_F2/VP_TLH_R2 primers displayed the most consistent separation between all three target bands even after shorter electrophoresis times. Therefore, this primer set was selected for the optimized multiplex PCR.

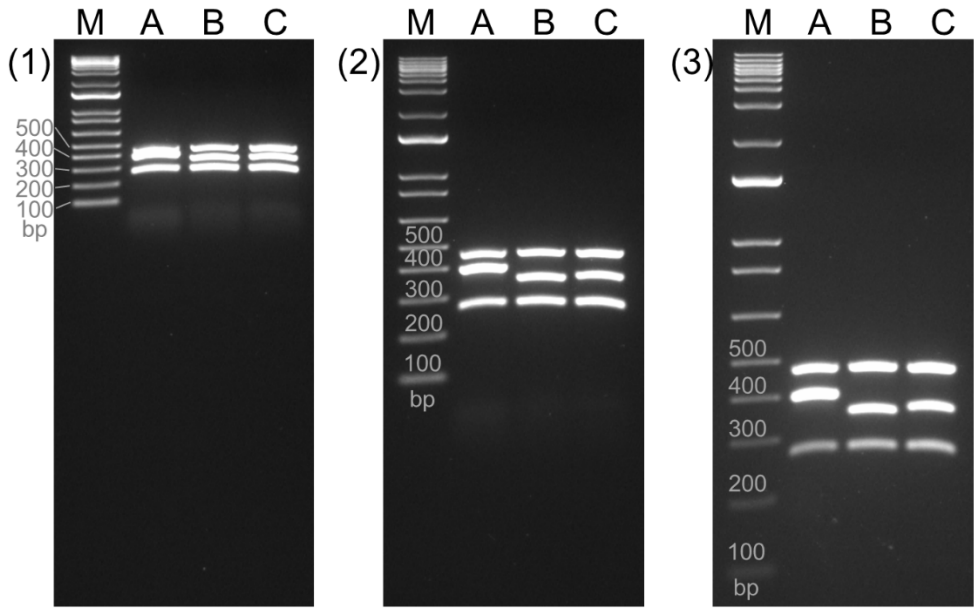

Figure 2. Multiplex PCR to enhance the discrimination of three genes of *Vibrio parahaemolyticus*. The primers for amplification of *trh* (486 bp) and *tdh* (270 bp) were adopted from the Bacteriological

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Analytical Manual (BAM) and a study by Bej et al [10,11]. The multiplex PCR included various combination of primers to amplify trh gene (A: 403 bp, B: 359 bp, and C: 369 bp). The amplicons were electrophorized onto 1.5% TBE agarose gel for 30 min (A), 60 min (B), and 90 min (C). M denotes the 100 bp molecular marker.

Our results, similar to those of previously reported methods (BAM and Bej et al.), showed a weaker band intensity for the *tdh* gene (Figure 2-3) compared to the *trh* and *tlh* genes at longer electrophoresis times (90 minutes). This was likely due to amplification bias, which was influenced by primer selection to a greater extent than by the template used [17]. To address this and improve band separation, five different primer concentrations (set A to E) were tested (Figure 3-1). The resulting amplicons were loaded onto 1.5% (Figure 3-2) and 2% (Figure 3-3) TBE agarose gels to evaluate the optimal electrophoresis conditions. While the Bej et al. multiplex PCR (Lane O in Figures 3-2 and 3-3) failed to separate *trh* and *tlh* genes, our optimized multiplex PCR (Lane A to E) achieved separation at shorter electrophoresis times: 60 min on a 1.5% gel and 30 min or 45 min on a 2% gel. Additionally, the bands displayed sharper and clearer intensity after 45 min on a 2% gel compared to 60 min on a 1.5% gel. Therefore, electrophoresis on 2% TBE gel for 45 min was used in further band intensity analysis for optimization of primer concentrations (Figure 3-4).

(1) Set	trh (μM)	tlh (μM)	tdh (μM)
A	0.25	0.25	0.25
B	0.2	0.2	0.3
C	0.2	0.2	0.4
D	0.15	0.15	0.3
E	0.1	0.1	0.5

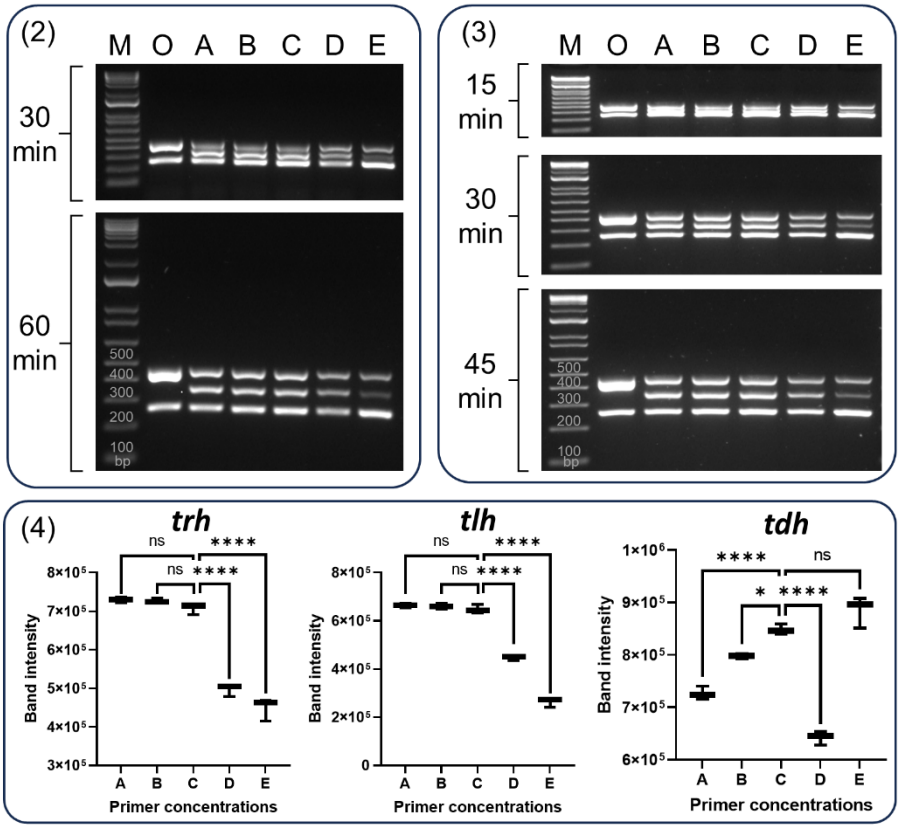

Figure 3. Optimization of primer concentrations of enhanced multiplex PCR. Various concentrations of the *trh*, *tlh*, and *tdh* primers were tested. Panel (1) shows the concentration of primers for set A, B, C, D, and E. The amplicons were separated using gel electrophoresis on either a 1.5% TBE agarose gel for 30 min or 60 min (2) or a 2% agarose TBE gel for 15

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min, 30 min or 45 min (3). Band intensities across the five tested concentration (lanes A-E) on the 2% TBE agarose gel for 45 min were compared to determine the optimal conditions for simultaneous amplification of all three target genes (4). Lanes M and O denote the 100 bp molecular marker and PCR conducted according to Bej et al (ref), respectively. Data represents the means of three independent replicates (One-way ANOVA, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001).

A previous study has indicated that the final concentration of the primers (0.04–0.5 μM) may vary considerably among the loci and was often established empirically [18]. To achieve even amplification in multiplex PCR, it was recommended to adjust primer concentrations. This involves increasing the concentration for "weak" loci and decreasing it for "strong" loci [19]. In this study, it was aimed to produce three amplicons evenly by decreasing the concentration of *trh* and *tdh* primers and by increasing the concentration of *tdh* primers. Both *trh* and *tlh* genes displayed the significant band intensity at a concentration of 0.2 μM within the set C combination (Figure 3-4). The *tdh* gene also displayed the significant band intensity in set C, which contained 0.4 μM concentration of the *tdh* primer. Interestingly, the same concentration of *tdh* primer (0.3 μM) resulted in different band intensities between set B and D. This observation highlights the importance of multiple attempts to optimize reaction conditions for efficient multiplex PCR, as noted by Markoulatos et al. [19].

3.3. Sensitivity and Specificity of our Multiplex PCR

A previous study reported a multiplex PCR for detection of *groEL*, *trh*, and *tdh* genes with a limit of detection (LOD) of 200 pg of *V. parahaemolyticus* DNA using 30 PCR cycles [20]. Another study demonstrated that the LOD of food-borne bacterial DNA in their multiplex PCR assay was 6.4 pg for *Staphylococcus aureus*, 32 pg for *Escherichia coli* O157:H7, 800 pg for *Listeria monocytogenes*, 160 pg for *Shigella flexneri*, and 32 pg for *Salmonella enterica* serovar Enteritidis using 35 cycles [21]. In this study, the sensitivity of our multiplex PCR assay was determined using various concentrations of F11-3A DNA (Figure 4-3). The LOD for the *trh*, *tlh*, and *tdh* genes were 10 pg, 10 pg, and 1 pg, respectively. The overall LOD for all three genes was 10 pg of bacterial DNA. This sensitivity was 1,000 times and 10 times higher than the results obtained using the BAM and Bej et al. methods, respectively (Figure 1).

The specificity of the multiplex PCR assay was evaluated using three *V. parahaemolyticus* (Lane 1-3 in Figure 5), nine *Vibrio* strains known to be human pathogens (Lane 4-12), and 18 well-documented food-borne pathogens (Lane 13-30) ([10,22–24]. All three target genes were successfully amplified using F11-3A (Lane 1). In contrast, *V. parahaemolyticus* ATCC 178802 showed only *tlh*-positive result, while *V. parahaemolyticus* ATCC 35118 displayed both *tlh* and *tdh*-positive, consistent with previous studies [11,13]. Notably, our multiplex PCR did not produce any amplified products from other *Vibrio* species and food-borne pathogenic bacteria. Therefore, this assay could be considered as an improved version of the multiplex PCR protocol recommended by BAM.

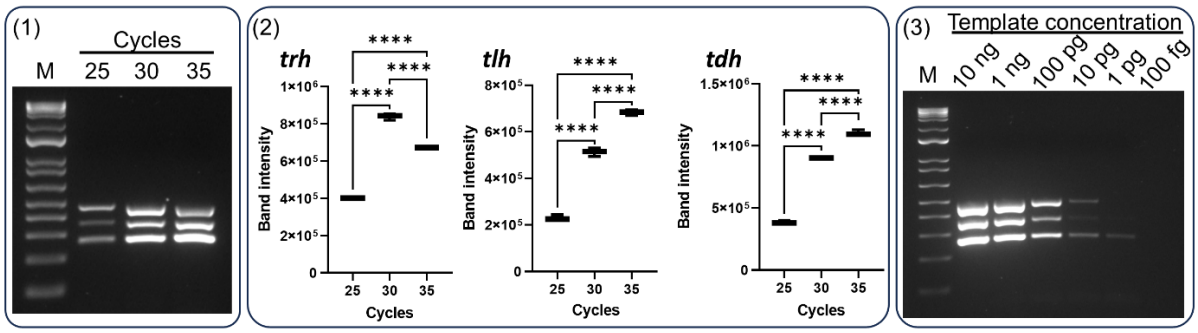

Figure 4. Optimization of multiplex PCR cycle number and detection limit. The PCR reactions were conducted with varying cycle numbers: 25, 30, and 35 (1). Panel (2) shows the corresponding band intensities for the amplified tlh, trh, and tdh genes at each cycles number. Panel (3) exhibits the

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detection limit (sensitivity) of the multiplex PCR with 35 cycles. Data represent the means of three independent replicates (One-way ANOVA, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$).

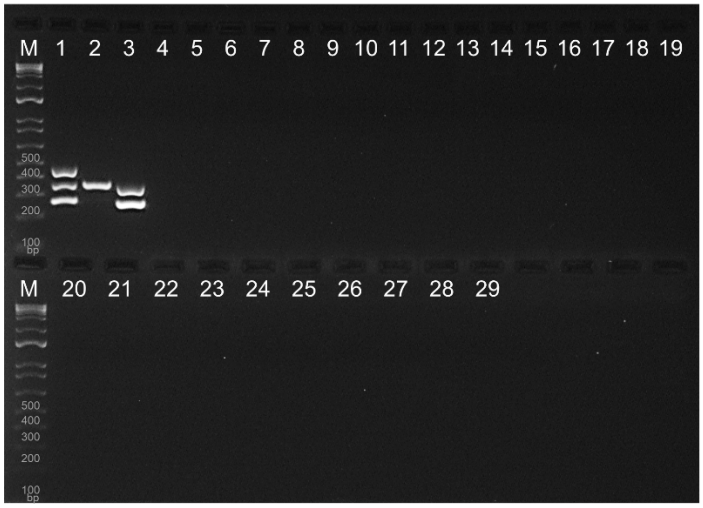

Figure 5. Specificity of the enhanced multiplex PCR. *Vibrio parahaemolyticus* F11-3A exhibited positive results for *trh*, *tlh* and *tdh* genes (1). *V. parahaemolyticus* ATCC 17802 was only positive for *tlh* (2). *V. parahaemolyticus* ATCC 35118 showed positivity for *tlh* and *tdh* genes (3). None of the other tested *Vibrio* strains and foodborne pathogenic bacteria displayed amplification of three genes. (4): *Vibrio vulnificus* ATCC 33147, (5): *Vibrio vulnificus* ATCC 27562, (6): *Vibrio vulnificus* ATCC 33815, (7): *Vibrio metschnikovii* ATCC 7708, (8): *Vibrio fluvialis* ATCC 33809, (9): *Vibrio mimicus* ATCC 33655, (10): *Vibrio furnissii* ATCC 35627, (11): *Vibrio cholerae* ATCC 39315, (12): *Vibrio alginolyticus* ATCC 33840, (13): *Escherichia coli* ATCC 51739, (14): *Escherichia coli* K-12, (15): *Escherichia coli* O157:H7 ATCC 43895, (16): *Listeria monocytogenes* F5069, (17): *Lactobacillus buchneri* ATCC 12936, (18): *Listeria innocua* ATCC 33090, (19): *Salmonella enterica* Serovar Typhimurium 14028, (20): *Salmonella enterica* Serovar Gaminara F2712, (21): *Salmonella enterica* Serovar Montevideo ATCC BAA-1735, (22): *Salmonella enterica* Serovar Senftenburg ATCC 43845, (23): *Salmonella enterica* Serovar Enteritidis E190-88, (24): *Salmonella enterica* Serovar Choleraesuis ATCC 10708, (25): *Bacillus subtilis* ATCC 9372, (26): *Clostridium perfringens* ATCC 13124, (27): *Enterococcus faecalis* ATCC 344, (28): *Lactobacillus acidophilus* NRRL B1910, (29): *Staphylococcus aureus* ATCC 25923, (30): *Shigella flexineri* ATCC 12022. M denotes the 100 bp molecular marker.

4. Conclusions

This study presents an efficient multiplex PCR assay for the detection of the species-specific *tlh* gene and two pathogenic *trh* and *tdh* genes. To enhance the multiplex PCR recommended by BAM, we redesigned primer set, optimized the concentration of primers, and adjusted the conditions of PCR cycles and gel electrophoresis. Our assay effectively separated the amplicons of three genes with similarly clear band intensities, facilitating their detection. Given its high sensitivity and specificity, this improved multiplex PCR method could significantly enhance the BAM recommended multiplex PCR protocol.

Author Contributions: Conceptualization, S.P. and Y.Z.; methodology, S.P; software, S.P.; formal analysis, S.P.; investigation, S.P.; resources, Y.Z.; data curation, S.P.; writing—original draft preparation, S.P.; writing—review and editing, Y.Z.; visualization, S.P.; supervision, Y.Z.; project administration, Y.Z.; funding acquisition, Y.Z. All authors have read and agreed to the published version of the manuscript.

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