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Article

Dual Detection of Pathogenic *tdh* and *trh* Genes of *Vibrio parahaemolyticus* in Oysters Using Multienzyme Isothermal Rapid Amplification (MIRA) Combined with Lateral-Flow Dipstick (LFD) Assay

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Abstract: *Vibrio parahaemolyticus* is a foodborne pathogen commonly associated with the consumption of contaminated seafood, particularly oysters. While PCR and real-time PCR are widely used to detect its pathogenicity through *tdh* and *trh* gene detection, these methods may not be practical in resource-limited settings such as field environments. To address this limitation, a rapid, sensitive, and specific duplex detection method was developed using the multienzyme isothermal rapid amplification (MIRA) assay in combination with lateral flow dipstick (LFD) technology. The assay utilized specific primer sets and probes to simultaneously amplify *tdh* and *trh* fragments tagged with 3′-FAM and 5′-Digoxigenin or Biotin during MIRA amplification, enabling the detection via respective antibody capture on the LFD strip. This duplex MIRA-LFD assay demonstrated a detection limit of 100 fg of DNA, 300 CFU/reaction for bacterial culture, and 3,000 CFU/reaction for seeded oyster samples at 40 °C within 20 minutes. Notably, the assay exhibited no cross-reactivity with nine other *Vibrio* species or 18 foodborne pathogens, confirming its high specificity. Due to its simplicity, rapid turnaround time, and high sensitivity, this duplex MIRA-LFD assay offers a valuable tool for the surveillance of *V. parahaemolyticus* pathogenicity, aiding in public health protection and supporting the local seafood industry.

Keywords: *Vibrio parahaemolyticus*; *trh* gene; *tdh* gene; oysters; dual detection; MIRA-LFD

1. Introduction

Vibrio parahaemolyticus is a Gram-negative, facultative halophilic bacterium that inhabits estuaries and brackish water worldwide [1]. It thrives in warm water conditions above 15 °C and sodium chloride concentrations below 25 ppm, with a rapid doubling time of 8-9 minutes [2]. During the summertime, the bacterium can accumulate quickly in filter-feeding aquatic organisms, such as oysters [3]. Consequently, *V. parahaemolyticus* is a leading cause of gastroenteritis in humans, primarily associated with the consumption of contaminated seafood [4]. According to the U.S. Center for Disease Control and Prevention (CDC), an estimated 84,000 cases of foodborne illness are caused by *Vibrio* infection annually in the United States [3,4]. For instance, a specific strain of *V. parahaemolyticus* was identified as a causative agent for an outbreak across 13 Northeastern states in 2012 and 2013, which was associated with oyster consumption [5,6]. This outbreak had significant public health and economic repercussions, particularly for the local seafood industry [7].

Several virulence factors are known to play key roles in *V. parahaemolyticus* infection, including proteases, lipopolysaccharides, thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH), and type III secretion system [8–12]. Among these, TDH and TRH are regarded as critical

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markers for identifying pathogenic strains, as they are commonly found in clinical isolates from patients but are rare in environmental strains [8,13–16]. A genomic study has demonstrated that 90 % of clinical strains possess the *tdh* and/or *trh* gene, compared to less than 1 % of environmental strains [17]. Due to a 67 % similarity in the amino acid sequences, TDH and TRH share similar pathogenic mechanisms causing pore formation in gastrointestinal cell membranes [9,18]. This leads to massive ion and water efflux, resulting in symptoms such as diarrhea. Therefore, the simultaneous detection of *tdh* and *trh* genes is essential for predicting the pathogenicity of *V. parahaemolyticus* strains [19–21]. Given the global concern over *V. parahaemolyticus* contamination in seafood, there is an urgent need to develop rapid, sensitive, and specific detection tools for field applications.

Our previous study demonstrated that the multienzyme isothermal rapid amplification (MIRA), combined with lateral flow dipstick assay (LFD), could effectively detect the thermolabile hemolysin (*tlh*) gene, a marker for identifying *V. parahaemolyticus*, as well as two pathogenic marker *tdh* and *trh* genes separately, without requiring a laboratory setting [22]. The entire MILA-LFD process was completed within 20 minutes at temperatures ranging from 30 to 45 °C, exhibiting high specificity and sensitivity comparable to real-time PCR. MIRA is a recombinase-based nucleic acid technique which employs three key enzymes, including recombinase proteins to form nucleoprotein filaments with target-specific primers and facilitate strand invasion into double-stranded DNA, single-stranded DNA-binding proteins (SSBs) to stabilize the displaced strand and prevent re-annealing, and strand-displacing DNA polymerase to extend the primers once bound, synthesizing new DNA without high-temperature denaturation in a single-tube reaction [22–24]. MIRA amplifies and tags target DNA in one step using primers and probes labeled with haptens, such as FAM (fluorescein amidite), and biotin or digoxigenin. During LFD step, hapten-labeled amplicons bind to conjugated antibodies on a nitrocellulose membrane, producing visible bands at the test lines that indicate the presence of target genes, while a control line confirms proper flow and reagent function. Therefore, MIRA-LFD is well suited for on-site pathogen detection in resource-limited settings without specialized laboratory infrastructure.

In this study, we developed a duplex MIRA-LFD assay capable of simultaneously detecting two pathogenic markers, *tdh* and *trh*, using specifically modified primers and probes. The sensitivity of the assay was evaluated using extracted DNA, direct bacterial culture, and oyster samples artificially infected with bacteria. Furthermore, the specificity of the assay was assessed by testing various *Vibrio* strains and foodborne pathogens. This assay provides a rapid and practical tool for screening the pathogenicity of *V. parahaemolyticus* in oysters, complementing our previously developed MILA-LFD assay for the *tlh* gene, an identification marker.

2. Materials and Methods

2.1. Bacterial Strains and DNA Extraction

The reference strain *Vibrio parahaemolyticus* F11-3A, which contains both *tdh* and *trh* genes, was used in this study [19,21]. *V. parahaemolyticus* ATCC 35118 strain was used as a *tdh*-positive strain, while *V. parahaemolyticus* ATCC 17802 served as a negative control for both genes [25,26]. For the determination of specificity of MIRA-LFD, additional closely related *Vibrio* species and foodborne pathogens were tested. These included *V. vulnificus* ATCC 33147, *V. vulnificus* ATCC 27562, *V. vulnificus* ATCC 33815, *V. metschnikovii*, *V. fluvialis* ATCC 33809, *V. mimicus* ATCC 33655, *V. furnissii* ATCC 35627, *V. cholerae* ATCC 39315, *V. alginolyticus* ATCC 33840, *Escherichia coli* ATCC 51739, *E. coli* K-12, *E. coli* O157:H7 ATCC 43895, *Listeria monocytogenes* F5069, *Lactobacillus buchneri* ATCC 12936, *Listeria innocua* ATCC 33090, *Salmonella enterica* Serovar Typhimurium 14028, *S. enterica* Serovar Gaminara F2712, *S. enterica* Serovar Montevideo ATCC BAA-1735, *S. enterica* Serovar Senftenburg ATCC 43845, *S. enterica* Serovar Enteritidis E190-88, *S. enterica* Serovar Choleraesuis ATCC 10708, *Bacillus subtilis* ATCC 9372, *Clostridium perfringens* ATCC 13124, *Enterococcus faecalis* ATCC 344, *Lactobacillus acidophilus* NRRL B1910, *Staphylococcus aureus* ATCC 25923, and *Shigella flexineri* ATCC 12022. All bacterial strains were cultured in tryptic soy broth (TSB, Remel, San Diego, CA, USA) or

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on tryptic soy agar (TSA, Remel) at 37 °C. Genomic DNA was extracted using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, CA, USA), and DNA concentrations were measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The DNA samples were kept at -20 °C until further use.

2.2. Primers and Probes

The *tdh* (Gene ID: 1192010) and *trh* (GenBank: KP836460.1) were used to design the primers and probes using SnapGene software (version 5.2, San Diego, CA, USA). Table 1 provides the genetic information for the forward and reverse primer, as well as the probes used to amplify fragments of *tdh* and *trh* genes. The probes were labeled with a polymerase extension blocking group (C3 spacer) at the 5′ end, an internal abasic nucleotide analog (dSpacer tetrahydrofuran residue), and a carboxyfluorescein (FAM). The 5′ ends of reverse primers were labeled with either digoxigenin or biotin to enable attachment to the lateral flow dipsticks (LFD, HybriDetect 2T, Milenia Biotec, Giessen, Germany). The LFD features two test lines with anti-digoxigenin antibody and biotin ligand, which capture the amplicons labeled with digoxigenin or biotin.

Table 1. Primers and probes for amplification of *tdh* and *trh* genes of *Vibrio parahaemolyticus* using MIRA-LFD.

Gene	Name	Sequence (5' - 3')	amplicon size (bp)
TDH	VP_TDH_F1	CTGTGAACATTAATGATAAAGACTATACAA	145
	VP_TDH_Probe	/56-FAM/AGCTTCAACATTCCTATGATTCTGTAGCTA/idSp/CTTTGTTGGTGAAGA/3SpC3/	
	VP_TDH_R1_Dig	Digoxigenin-ATTACCAATATATTACCACTACCACTCTCATA	
	VP_TDH_R1_Bio	Biotin-ATTACCAATATATTACCACTACCACTCTCATA	
TRH	VP_TRH_F1	ACTCTACTTTGCCTTCAGTTTGCTATTGGCTTC	141
	VP_TRH_Probe	/56-FAM/TGAGCTACTATTTGTCGTTAGAAATACAAC/idSp/ATAAAAACTGAATCA/3SpC3/	
	VP_TRH_R1_Bio	Biotin-GAAGTCGTGAAAATAGATTGACCGTGAACGCT	
	VP_TRH_R1_Dig	Digoxigenin-GAAGTCGTGAAAATAGATTGACCGTGAACGCT	

2.3. Multienzyme Isothermal Rapid Amplification (MIRA) and Lateral-Flow Dipstick (LFD)

The MIRA-LFD assay was conducted using MIRA nfo kit (Amp-future, Changzhou, China) in a combination with lateral flow dipsticks (LFD, HybriDetect 2T, Milenia Biotec, Giessen, Germany). A 47.5 µL of MIRA mixture was prepared, consisting of 29.4 µL of Buffer A, 2 µL of each forward and reverse primer (10 µM) for both genes, 0.6 µL of each probe (10 µM), 1 µL of template and 7.9 µL of water (Table 1). This mixture was added to a test tube containing a lyophilized pellet. The MIRA reaction was carried out at 40 °C for 16 min by adding 2.5 µL of MgAc (280 mM). Then, 5 µL of the MIRA product was diluted in 195 µL of HybriDetect 2T assay buffer. The LFD sample pad was immersed in the diluted solution and incubated for 1.5 minutes. A clear control line, with or without test lines, was considered valid.

2.4. Optimization of MIRA-LFD Assay

Two different primer combinations were examined for simultaneous amplifications of *trh* and *tdh* genes of *V. parahaemolyticus* using F11-3A DNA in a MIRA reaction (Table 1). The amplified reactions, containing either *trh*-digoxigenin and *tdh*-biotin or *trh*-biotin and *tdh*-digoxigenin, were loaded onto LFD strips to confirm the presence of both test lines along with a control line. The incubation temperatures for MIRA reactions were set at 25, 30, 35, 40, 45, and 50 °C. Additionally, the optimal incubation time was evaluated at durations of 4, 8, 12, 16, 20, 24, and 28 minutes.

2.5. Sensitivity and Specificity of MIRA-LFD Assay

To evaluate the sensitivity of MIRA-LFD, both genomic DNA (ranging from 1 fg to 1 ng per reaction) and direct bacterial cultures (ranging from 3×10^1 to 3×10^5 CFU per reaction) of F11-3A were used. Fresh oysters were purchased from a local market, shucked, pooled, blended, and diluted with phosphate-buffered saline (PBS, pH 7.4) at a ratio of 1:4 (w/v). After confirming the absence of *V. parahaemolyticus* using a PCR assay [20], the oyster samples were seeded with F11-3A bacteria in

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concentrations ranging from 3×10^1 to 3×10^5 CFU for the MIRA-LFD assay. The specificity of the MIRA-LFD assay was evaluated by applying genomic DNA samples extracted from various *Vibrio* and foodborne bacteria mentioned above.

2.6. Multiplex PCR

A conventional multiplex PCR targeting the *tlh*, *tdh*, and *trh* genes was performed to compare its sensitivity with that of the MIRA-LFD assay [19,27]. PCR reactions (50 µL) contained bacterial genomic DNA (1 fg–1 ng), 0.25 µM of each primer (1.25 µL of a 10 µM stock), 320 µM of each dNTP (8 µL of an 8 mM stock), 5 µL of 10× DreamTaq Green PCR buffer, 1.5 U DreamTaq Green DNA polymerase (0.3 µL of 5 U/µL; Thermo Scientific, Vilnius, Lithuania), and nuclease-free water to volume. Thermal cycling was carried out on a standard PCR thermocycler as follows: initial denaturation at 94 °C for 3 min; 35 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 5 min. Amplified products were separated by electrophoresis on 2% agarose gels in TBE buffer (Alfa Aesar, Ward Hill, MA, USA) containing SYBR Safe DNA gel stain (Invitrogen, Waltham, MA, USA). The gel was electrophoresed for 45 min and visualized with a Gel Doc XR+ imaging system (Bio-Rad, Hercules, CA, USA).

2.7. Statistical Analysis

Test-band intensities were quantified using Image Lab™ v6.0.1 (Bio-Rad, Hercules, CA, USA) and normalized to the negative-control value. All statistical analyses were performed in GraphPad Prism™ v9 (GraphPad Software, Boston, MA, USA). Differences between groups were assessed by one-way ANOVA followed by Dunnett's multiple-comparisons test, with $p < 0.05$ considered significant. Data are presented as mean ± SD ($n = 3$).

3. Results and Discussion

3.1. Validation of Primers and Probes

Vibrio parahaemolyticus is a leading cause of foodborne illness in humans through the consumption of contaminated seafood, such as oysters [4]. Since most environmental *V. parahaemolyticus* strains are non-pathogenic, distinguishing them from pathogenic strains carrying the *trh* and/or *tdh* genes is crucial for seafood safety [19,28]. According to the U.S. FDA Bacteriological Analytical Manual (BAM) and the National Shellfish Sanitation Program (NSSP), DNA hybridization, PCR, and real-time PCR assays are the recommended methods for detecting *trh*- and *tdh*-positive strains in oyster samples [20,29]. However, these standard techniques are not well-suited for field applications due to their reliance on specialized laboratory infrastructure, equipment, and technical expertise.

Recombinase-based isothermal amplification assay, including multienzyme isothermal rapid amplification (MIRA) and recombinase polymerase amplification (RPA) offers significant advantages for on-site pathogen detection in field conditions without the need for specialized training or laboratory equipment [30]. In their lyophilized form, these assays are easy to transport and enable target gene amplification at relatively low incubation temperatures (30–45 °C) using a simple heat block or even body heat [31]. Recent studies have demonstrated the feasibility of simultaneous amplification of multiple target genes in a single RPA reaction, such as Genus *Clavibacter* and *C. nebraskensis*, *Campylobacter jejuni* and *C. coli*, *Listeria monocytogenes* and *Salmonella enteritidis*, as well as *Staphylococcus aureus*, *Vibrio parahaemolyticus*, and *Salmonella Enteritidis* [32–35]. This study is the first to report the simultaneous detection of two representative pathogenic genes from *V. parahaemolyticus* using the MIRA-LFD assay.

The primer sets and probes demonstrated 100% query coverage and 100% identity with both target genes (data not shown). Based on a previous study, the primer design criteria were established as follows: GC content ranging from 20 to 70%, annealing temperature between 50 and 100 °C, primer

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length between 30 and 36 bp, and a maximum allowable nucleotide repeat length of five [22]. The forward primers, reverse primers (tagged with biotin or digoxigenin), and probes (tagged with FAM) generated amplicons labeled with 5′ FAM and 3′ biotin or digoxigenin (Table 1 and Figure 1). Upon interaction with anti-FAM antibodies, the amplicons were captured at two distinct lines on the LFD pad: the upper line (anti-digoxigenin) and the lower line (anti-biotin) (Figure 1c). The control line on the LFD pad was visualized by capturing anti-FAM antibodies, and any results lacking a control line were deemed invalid. A valid MIRA-LFD assay can display possible outcomes: test negative, *tdh*-positive, *trh*-positive, and dual *trh*/*tdh*-positive.

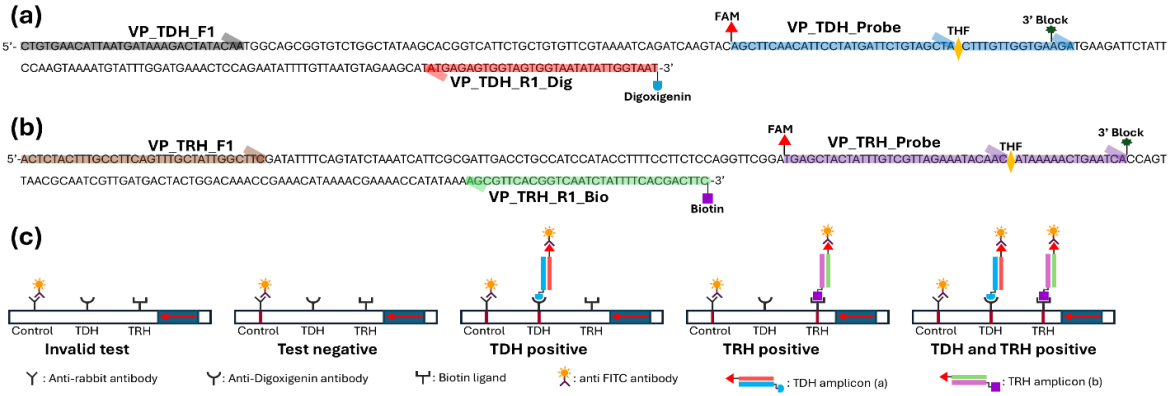

Figure 1. Arrangement of primers and probes for amplification of *tdh* (a) and *trh* (b) genes and attachment of amplified fragments to lateral flow dipsticks (c). The *tdh* and *trh* fragments were labeled with digoxigenin (a) and biotin (b), respectively. The *tdh* fragment was captured by an anti-digoxigenin antibody, while the *trh* fragment was captured by a biotin ligand of the lateral flow dipstick.

Three *V. parahaemolyticus strains*—F11-3A (*tdh*+/*trh*+), ATCC 17802 (*tdh*-/*trh*-), and ATCC 35118 (*tdh*+/*trh*-)—were used to evaluate primer sets and probes for the simultaneous amplification of *tdh* and *trh* fragments in a MIRA-LFD assay [19,21,25,26]. Prior to developing the dual MIRA-LFD assay, the primer sets and probes for each gene were evaluated using single-target MIRA-LFD assays. These assays yielded valid results without false positives or false negatives (data not shown). Dual MIRA-LFD assays were then tested using two different primer combinations: (1) *trh* primers tagged with digoxigenin (TRH(D)) and *tdh* primers tagged with biotin (TDH(B)), and (2) *tdh* primers tagged with digoxigenin (TDH(D)) and *trh* primers tagged with biotin (TRH(B)) (Figure 2a). Both combinations produced positive results on the LFD strip when using the DNA template of F11-3A (*tdh*+/*trh*+), with amplicons captured at the anti-digoxigenin (D) and anti-biotin (B) lines. However, combination (1) produced false-positive *tdh*+/*trh*+ results for ATCC 17802 (*tdh*-/*trh*-) and ATCC 35118 (*tdh*+/*trh*-). By contrast, combination (2) correctly identified ATCC 17802 as *tdh*-/*trh*- and ATCC 35118 as *tdh*+/*trh*-.

Previous studies have reported that recombinase-based isothermal amplification assays can produce false-positive results due to competition among primers and probes for recombinase proteins, as well as primer-dimer formation during multi-target amplification [32,33,35,36]. Although optimization of primer and probe concentrations has been suggested to mitigate false positives, adjusting the concentrations of primers, probes, and MgAc in combination (1) failed to eliminate false amplification in this study (data not shown). By contrast, combination (2), in which *tdh* and *trh* primers were tagged with digoxigenin and biotin, respectively, did not exhibit false-positive amplification. These findings suggest that modifying the tagging materials in primer sets can effectively prevent false amplification in duplex MIRA-LFD assays. This approach provides a potential solution for improving the specificity and reliability of multi-target isothermal amplification assays.

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3.2. Optimization of MIRA-LFD

Multiplex target gene amplification assays enable the simultaneous detection of multiple gene fragments in a single reaction, reducing operational steps, saving time, and minimizing reagent use [37]. Commonly used multiplex technologies include multiplex PCR, real-time PCR, and loop-mediated isothermal amplification (LAMP) [38–40]. Recently, the MIRA assay has emerged as a promising tool for rapid and reliable multiplex detection, offering advantages such as high tolerance to sample impurities, lower amplification temperatures compared to PCR (up to 95 °C) and LAMP (up to 65 °C), and significantly shortened incubation times [23,36]. However, due to the rapid amplification achieved by MIRA-LFD (lateral flow detection), optimizing incubation time and temperature is critical to maximize efficiency and minimize false-positive and false-negative results.

Figure 2b and 2d illustrate the results of duplex MIRA amplification across incubation temperatures ranging from 25 to 50 °C. The duplex MIRA assay was performed for 20 minutes using 1 ng of *V. parahaemolyticus* F11-3A genomic DNA as the template. While the control line remained consistent across all lateral flow detection (LFD) strips, *tdh* and *trh* band intensities varied with temperature. The *tdh* band first appeared at 25 °C, intensified up to 40 °C, and weakened at 45 °C. By contrast, the *trh* band was observed only at 35, 40, and 45 °C, while no amplification detected for both gene at 50 °C. Previous studies have also reported temperature-dependent variations in MIRA amplification efficiency. For example, MIRA targeting *Spiroplasma eriocheiris* failed to amplify at 30 °C and 40 °C [23], whereas MIRA for *Acinetobacter baumannii* was successful at 25 °C and 50 °C [41]. These findings indicate that 40 °C is the optimal temperature for simultaneous *tdh* and *trh* detection using MIRA-LFD ($p < 0.05$).

To determine the optimal amplification time, MIRA reactions were conducted at 40 °C using 1 ng of F11-3A genomic DNA, with incubation times ranging from 4 to 28 minutes at 4-minute intervals (Figure 2c and 2e). Both *tdh* and *trh* bands appeared after 8 minutes, with distinct, intense bands observed at 16 minutes. Band intensities remained consistent at 16, 20, 24, and 28 minutes, confirming that 16 minutes was the optimal amplification time ($p < 0.05$). For LFD detection, an incubation time of 1.5 minutes was selected, as no significant differences in band intensity were observed beyond this duration (data not shown), which was consistent with previous findings [22]. In summary, the optimized MIRA-LFD assay enabled the detection of *tdh* and *trh* genes within a total reaction time of 20 minutes.

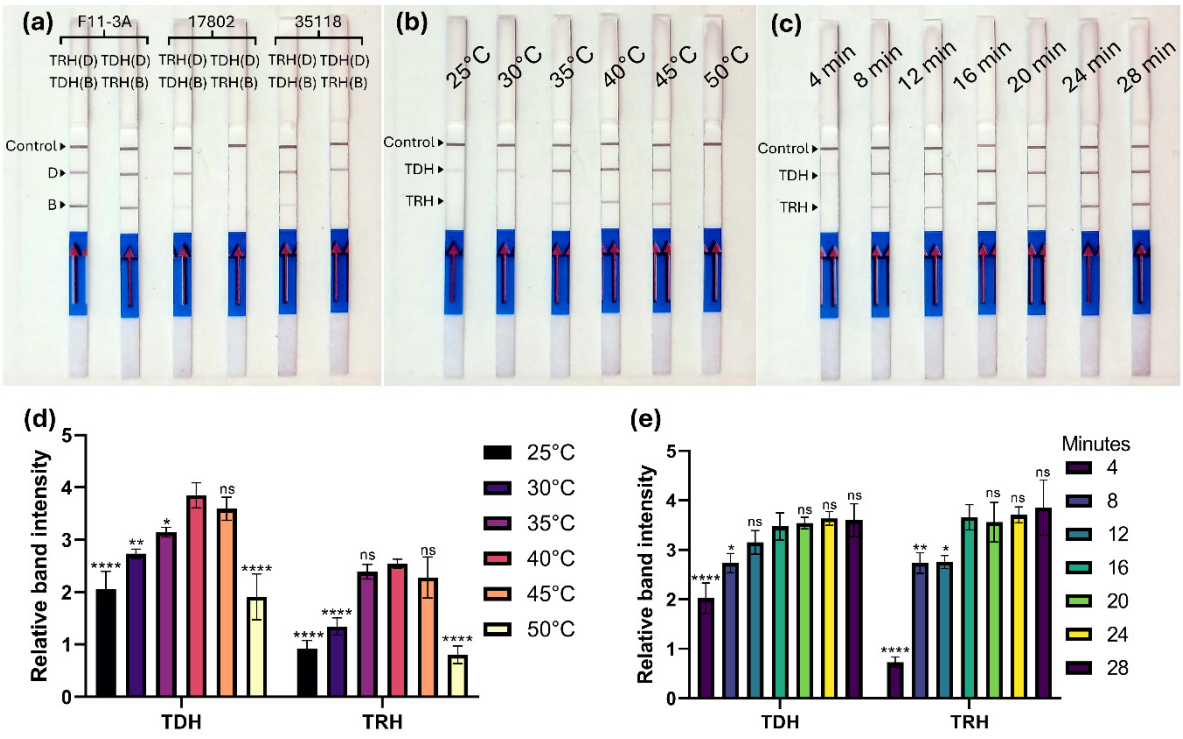

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Figure 2. Primer selection for simultaneous detection of *tdh* and *trh* genes of *Vibrio parahaemolyticus* (a) and optimization of incubation temperature (b, d) and time (c, e) for MIRA-LFD. The *trh* and *tdh* gene amplicons were labeled with digoxigenin (D) or biotin (B) for simultaneous detection on an LFD strip. The LFD strip displayed an upper control line, a middle *tdh* line, and a lower *trh* line on an LFD strip when both genes were successfully detected. Data represent the means of three independent replicates (one-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$, and ns (non-significant)).

3.3. Sensitivity and Specificity of MIRA-LFD Assay

Surveillance of pathogenic *Vibrio parahaemolyticus* in seafood is crucial for ensuring food safety and protecting the local seafood industry [3,4,7,42]. Recombinase-based isothermal amplification assays have been recognized as highly sensitive and specific tools for detecting foodborne pathogens under field conditions [30,36]. In this study, we evaluated the detection limits of our assay using various template concentrations and assessed its specificity against different pathogenic bacteria.

To determine the sensitivity of the MIRA-LFD assay, genomic DNA and bacterial cultures of *V. parahaemolyticus* strain F11-3A (*tdh*+ / *trh*+) were used as templates. Additionally, artificially contaminated oyster samples were prepared to assess detection limits in a food matrix. Templates were serially diluted (10-fold) and subjected to MIRA-LFD analysis. Clear visualization of control and two test lines on the LFD strip indicated a *tdh* and *trh* positive result, while only the control line signified a negative result. While the multiplex PCR detected both *tdh* and *trh* genes down to 100 fg (Figure 3a), the MIRA-LFD assay improved sensitivity by detecting *tdh* gene at 10 fg and *trh* gene at 100 fg, yielding an overall detection limit of 100 fg for both genes (Figure 3b). Notably, a recent multiplex PCR-LFD assay targeting *tlh* and *tdh* genes of *V. parahaemolyticus* demonstrated detection limits of 0.39 ng of bacterial DNA [43], highlighting that MIRA is more sensitive than conventional multiplex PCR-based assays for simultaneous detecting *V. parahaemolyticus* genes. In direct culture and seeded oyster samples, positive bands were observed at detection limits of 300 CFU/reaction and 3,000 CFU/reaction, respectively (Figure 3c and 3d). Previous MIRA-LFD studies have reported detection limits ranging from 97 pg to 64 fg of genomic DNA and 760 to 6 CFU for bacterial cultures of various pathogens [23,41,44–47]. Similarly, a multiplex RPA-LFD assay exhibited a 10-fold higher detection limit in direct culture (2.8 to 7.6×10^2 CFU/ml) than in artificially contaminated food samples (2.8 to 7.6×10^3 CFU/ml), suggesting that food matrix inhibitors, including polysaccharides, polyphenols, and elevated magnesium levels in fruits, vegetables, and seafood can impair MIRA reaction [48,49]. Therefore, after initial detection in complex food matrices, it is advisable to confirm target genes using purified bacterial DNA.

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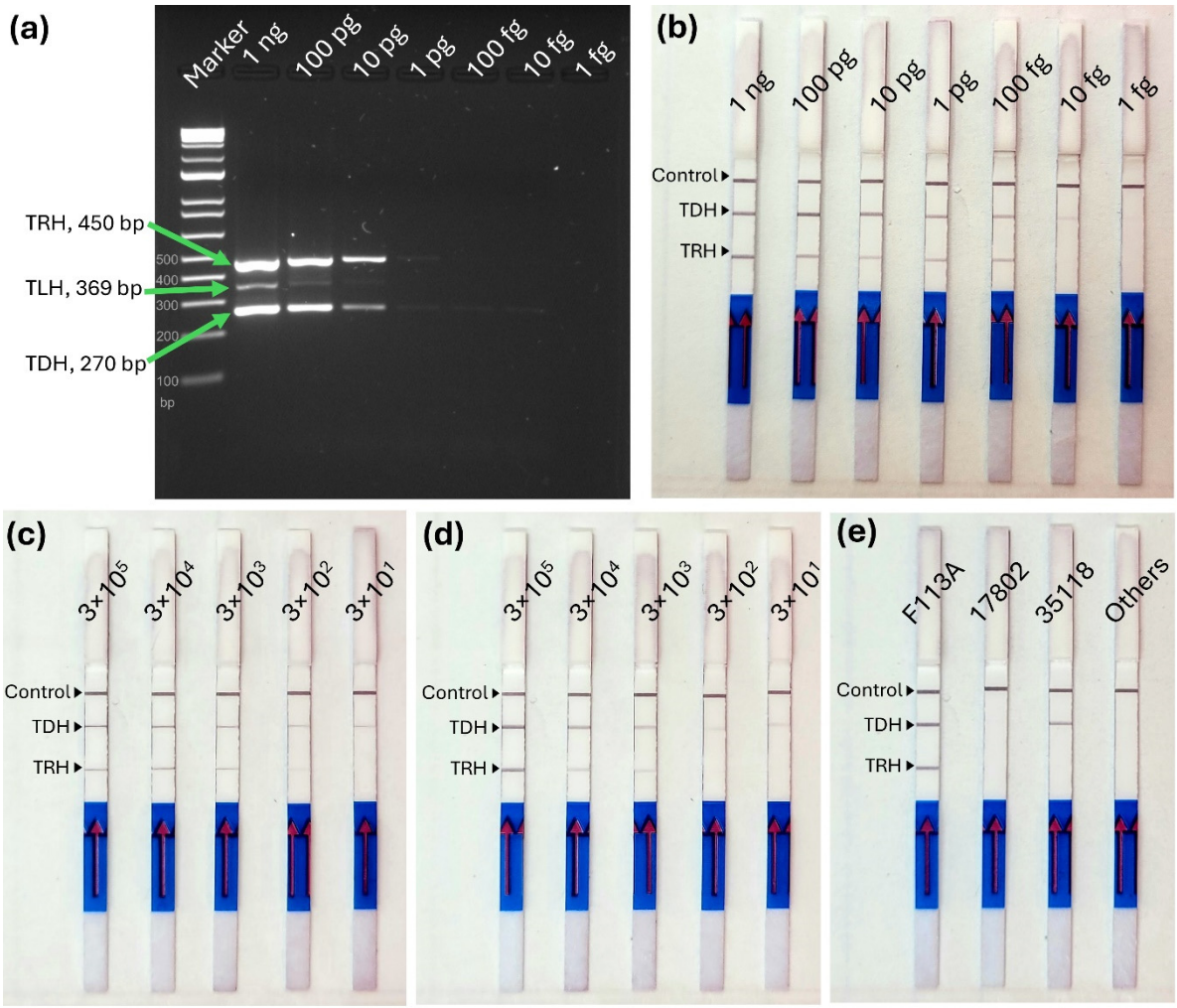

Figure 3. Sensitivity and specificity of MIRA-LFD for detecting *tdh* and *trh* genes of *Vibrio parahaemolyticus*. (a) A conventional multiplex PCR was conducted using the bacterial DNA ranging from 1ng to 1 pg. (b) Genomic DNA ranging from 1 ng to 1 fg, (c) bacterial cultures from 3 × 10^5 to 3 × 10^1 CFU/reaction, and (d) seeded oyster samples from 3 × 10^5 to 3 × 10^1 CFU/reaction were tested using the MIRA-LFD assay. (e) To assess specificity, the assay was performed on *V. parahaemolyticus* strains F11-3A (*tdh+/trh+*), ATCC 17802 (*tdh-/trh-*), and ATCC 35118 (*tdh+/trh-*), along with nine *Vibrio* species and 18 other foodborne bacteria (Others). The LFD strips displayed an upper control line, a middle *tdh* line, and a lower *trh* line when the assay successfully amplified *V. parahaemolyticus* strains containing both *tdh* and *trh* genes.

The specificity of the duplex MIRA-LFD assay was evaluated using nine *Vibrio* species and 18 foodborne pathogenic bacteria (Figure 3e and Supplemental Figure 1). The assay correctly identified *tdh+* / *trh+* for strain F11-3A, *tdh-* / *trh-* for ATCC 17802, and *tdh+* / *trh-* for ATCC 35118, demonstrating its ability to specifically amplify target genes in different *V. parahaemolyticus* strains. No cross-reactivity was observed with other *Vibrio* species or foodborne pathogens. While *Vibrio* species are known to exchange genetic elements, including pathogenic genes, and the *tdh* and *trh* genes of *V. parahaemolyticus* share high sequence similarity with those of *Vibrio alginolyticus*, *Vibrio cholerae*, *Vibrio mimicus*, and *Vibrio hollisae* [50–53], the MIRA-LFD assay exhibited no false positives or negatives. This specificity was likely due to the use of long primers (approximately 35 bp) and the unique recombinase-driven amplification system of MIRA, ensuring precise targeting of *tdh* and *trh* genes. However, further evaluation of this assay using clinical and environmental samples is necessary to verify its detection efficacy.

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4. Conclusions

A duplex MIRA-LFD assay was developed for the rapid detection of *Vibrio parahaemolyticus* by targeting the *tdh* and *trh* genes, key pathogenic markers associated with foodborne illness from oyster consumption. This assay provides visually interpretable results within 20 minutes at 40 °C using target-specific primer sets, making it a simple and efficient tool for field applications. With its high sensitivity and specificity, the MIRA-LFD assay may hold great potential for monitoring the pathogenicity of *V. parahaemolyticus*, not only to prevent foodborne outbreaks but also to support the local seafood industry by ensuring product safety.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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