Acta Biochimica Indonesiana
https://doi.org/10.32889/actabioina.159

RESEARCH ARTICLE
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Rapid detection of *Vibrio alginolyticus* in seafood using *flgL* gene and real-time polymerase chain reaction

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ABSTRACT

Background: Seafood is highly nutritious but poses health risks when contaminated with pathogenic bacteria like *Vibrio alginolyticus*, which causes food poisoning and can infect marine animals and humans.

Objective: This research aimed to determine the sensitivity and specificity of real-time polymerase chain reaction (rt-PCR) using the *flgL* primer pair to detect *V. alginolyticus* bacteria in seafood.

Methods: The rt-PCR method was used to detect *V. alginolyticus* quickly, specifically, and sensitively. The *flgL* primer pair was evaluated for amplicon length, Ct value, Tm value, and its ability to differentiate between target and non-target bacteria. In this research, the samples tested were red snapper and blood clams.

Results: The *flgL* primer produced an amplicon length of 224 bp. At 50 ng concentration, it yielded a Ct value of approximately 11.00 and a Tm of approximately 83°C. The *flgL* primer successfully differentiated between target and non-target bacteria. In sensitivity tests, it detected *V. alginolyticus* at concentrations as low as 1.86 x 10^{-3} ng/µL. Detection in seafood samples was also successful.

Conclusion: The rt-PCR assay using the *flgL* primer pair effectively detects *Vibrio alginolyticus* in seafood with high specificity, sensitivity, and rapidity. These findings support its use for rapid and accurate detection of pathogenic bacteria in seafood.

Keywords: Vibrio alginolyticus, seafood, flgL primer, real-time polymerase chain reaction, sensitivity, specificity

Introduction

As an archipelagic country, Indonesia has abundant marine resources that can serve as a vital food source for the community, including fish, shellfish, and shrimp. The nutritional content of marine biota includes protein, carbohydrates, fat, and vitamins, which are highly beneficial for human health [1]. However, contamination by pathogenic bacteria can pose serious health risks. According to the Indonesian Food and Drug Agency, there were 128 cases of extraordinary foodborne disease events in 2011, affecting 18,144 individuals [2].

One such pathogenic bacterium is *Vibrio alginolyticus*, which causes food poisoning and can infect both humans and marine animals. This Gram-negative bacterium is found in both fresh and saltwater [3]. Infections caused by *V. alginolyticus* can lead to bacteremia, septic shock, multiple organ failure, and even death [4]. Real-time polymerase chain reaction (rt-PCR) is a method that can detect the genetic material of pathogenic bacteria in food using specific primers. Detection using rt-PCR is faster, more effective, specific, and sensitive compared to other methods [5].

Previous study has successfully used rt-PCR to detect *Staphylococcus aureus* in milk and meat with the *nuc* gene, yielding good results [6]. Similarly, studies on *V. alginolyticus* have employed multiplex polymerase chain reaction with the *gyrB* gene, but these methods lack speed and accuracy [7]. This study aims to develop a rapid detection method for *V. alginolyticus* in seafood using the *flgL* primer and rt-PCR. The results of this study are expected to provide a reference for the quick, sensitive, and specific detection of pathogenic bacteria such as *V. alginolyticus* in food.

Method

Primers design

The primer design to detect *Vibrio alginolyticus* ATCC 17749 bacteria was carried out using the National Center for Biotechnology Information (NCBI) website and the Primer-BLAST program. The selected primer pairs were then analyzed using the NetPrimer and OligoAnalyzer programs to determine their secondary structures. The designed primer pairs were synthesized in the commercial laboratory of Macrogen Synthesis, Inc. Korea.

Optimization of annealing temperature

Optimization of the annealing temperature for the *flgL* primer pair was performed using Gradient Polymerase Chain Reaction over a temperature range of 53°C to 62°C. This test utilized a 25 µL reaction mixture containing *V. alginolyticus* DNA isolates, forward and reverse *flgL* primers, NZYTaq II 2x Green Master Mix, and nuclease-free water (NFW). Thirty-five amplification cycles were carried out, starting with initial denaturation at 95°C for 100 seconds, followed by denaturation at 95°C for 30 seconds, annealing at 53°C to 62°C for 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes.

Confirmation test of *Vibrio alginolyticus DNA with flgL* primers

The *flgL* primer confirmation test to detect *V. alginolyticus* bacteria was conducted using rt-PCR. This quantitative technique can specifically and sensitively detect DNA from samples at very low concentrations [8]. The confirmation test comprised a 20 µL reaction mixture containing ExcelTaq 2X qPCR Master Mix, *flgL* forward and reverse primers, *V. alginolyticus* DNA isolate template, and nuclease-free water. Two negative controls were used: a non-template control (NTC) and a mixture of nuclease-free water and master mix. The positive control used was *Salmonella typhi* with the *fimC* gene, as confirmed in previous research [9]. The rt-PCR process involved 40 cycles, starting with initial denaturation at 95°C for 3 minutes, denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 420 seconds.

Specificity and sensitivity test

The specificity test was carried out using the *flgL* primer pair and non-target bacteria such as *Staphylococcus aureus, Listeria monocytogenes, Salmonella typhi, Klebsiella pneumoniae,* and *Vibrio parahaemolyticus*. Each reaction mixture consisted of 20 µL. The results of the specificity test included an amplification curve and a melting curve.

The sensitivity test involved serial dilution of pure *V. alginolyticus* DNA isolates seven times. 1 µL of the pure isolate of *V. alginolyticus* bacteria was taken, and 9 µL of nuclease-free water was added. The sensitivity test results included an amplification curve and a standard curve.

Confirmation test on food samples

The food samples used in this test were red snapper and blood clams. This test involved DNA

Table 1. In silico data of *flgL* primer pair

Primer	Sequence	Tm (°C)	%GC	Self-dimer (kcal/mol)	Hairpin (kcal/mol)	Cross dimer (kcal/mol)	Amplicon length	Primer length
flgL_F	GAACGGCTCTCTTACTGAC	49.02	52.63	-	-	-	224 bp	19 bp
flgL_R	CCATCGTGACACCTTTAG	48.14	50.0	-	-			18 bp

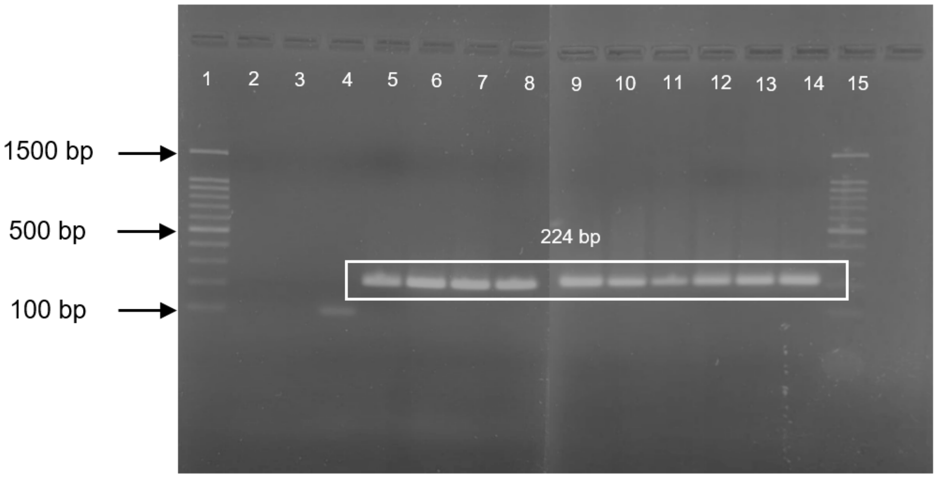

Figure 1. Annealing temperature optimization results for the *flgL* gene. (1) DNA ladder 100 bp, (2) NTC, (3) Negative control, (4) Positive control *fimC Salmonella typhi* 95 bp, (5) DNA fragment at 53°C, (6) DNA fragment at 54°C, (7) DNA fragment at 55°C, (8) DNA fragment at 56°C, (9) DNA fragment at 57°C, (10) DNA fragment at 58°C, (11) DNA fragment at 59°C, (12) DNA fragment at 60°C, (13) DNA fragment at 61°C, (14) DNA fragment at 62°C, (15) DNA ladder 100 bp.

isolates from test samples (red snapper and blood clams that were artificially contaminated), positive controls (pure DNA isolates of *V. alginolyticus*), and negative controls (uncontaminated red snapper and blood clams, non-template control, and master mix + nuclease-free water). Each reaction mixture consisted of 20 μL. The confirmation test results on food samples included an amplification curve and a melting curve.

Results

Primers design

The *flgL* gene of *Vibrio alginolyticus* ATCC 17749 is located in the region 669990-670889 on chromosome 2 and is 900 bp in size. This gene is involved in cell motility, enabling cells to move from one place to another through energy consumption. The designed *flgL* primer pairs were selected based on amplicon length, %GC content, melting temperature (Tm), primer length, and secondary structure. Table 1 presents the in silico data for the designed *flgL* primer pair.

Optimization of annealing temperature

Gradient PCR was used to determine the optimal annealing temperature, testing within the range of 53°C to 62°C. Electrophoresis was then performed to evaluate the results. All temperatures produced a single band of 224 bp, but the brightest band was observed at 60°C. Figure 1 shows the electrophoresis results for determining the optimal annealing temperature.

Confirmation test of *Vibrio alginolyticus* DNA with primers *flgL*

rt-PCR was used to confirm the *flgL* primer pair for detecting *V. alginolyticus* bacteria. This test produced an amplification curve and a melting curve. The amplification curve for the *flgL* primer (purple curve) resulted in Ct values of 11.12 and 11.24 (duplo), and the melting curve yielded Tm values of 83.32°C and 83.25°C (duplo) (Figure 2). The positive control, *Salmonella typhi fimC* (red curve), had Ct values of 13.52 and 13.72 (duplo) and Tm values of 85.74°C and 85.58°C (duplo). The

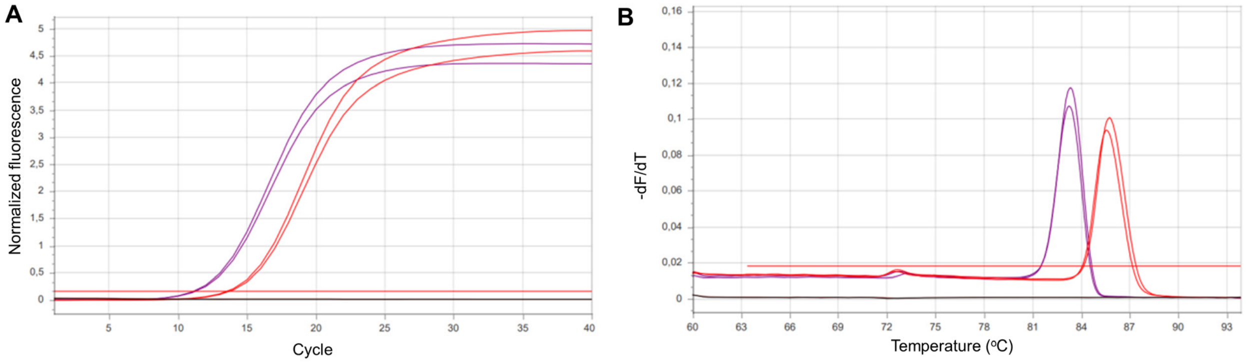

Figure 2. Confirmation test of *V. alginolyticus* DNA with *flgL* primers. (A) Amplification curve, (B) Melting curve

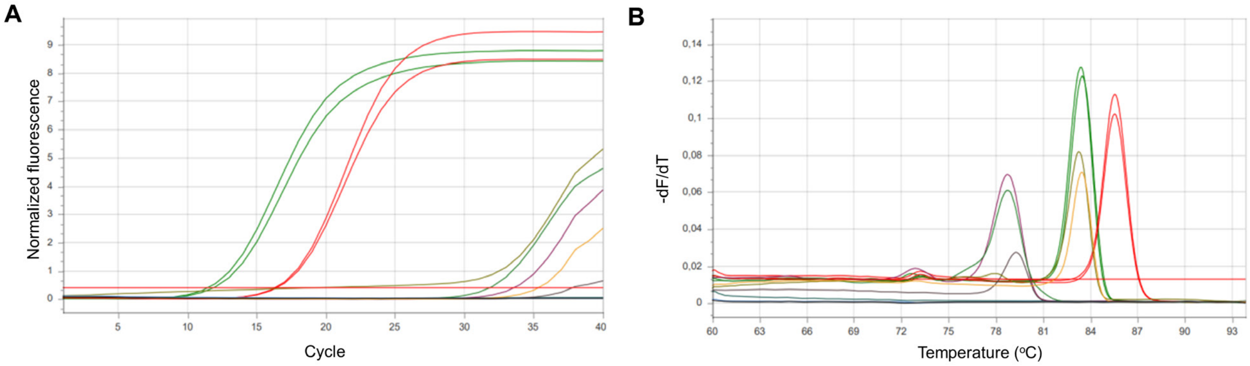

Figure 3. Specificity test of *flgL* primers. (A) Amplification curve, (B) Melting curve

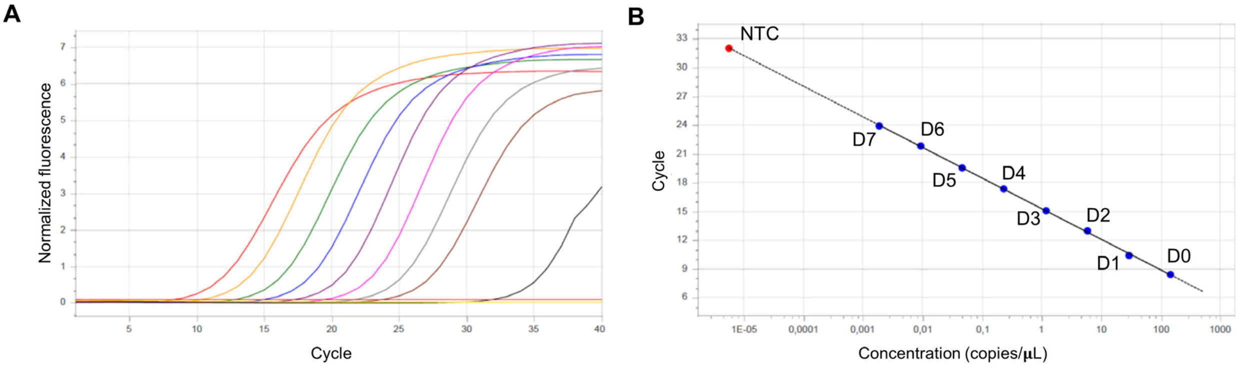

Figure 4. Sensitivity test of *flgL* primers. (A) Amplification curve, (B) Melting curve

negative controls, non-template control and master mix + nuclease-free water, did not produce any Ct or Tm values.

Specificity and sensitivity rest

The specificity test involved six non-target bacteria: *Vibrio parahaemolyticus, Cronobacter sakazakii, Listeria monocytogenes, Klebsiella pneumoniae, Salmonella typhi,* and *Staphylococcus aureus*. These bacteria produced Ct values of more than 31 cycles, and the melting curve showed more than two peaks (Figure 3).

The sensitivity test involved seven dilutions, resulting in a final concentration of 1.86 x 10^-3 ng/µL with a Ct value of 23.90 (Figure 4). The resulting line equation was $y = -3.20x + 15.27$, with an efficiency of 1.05 and an R^2 value of 0.9997.

Confirmation test on food samples

The final stage involved testing food samples, specifically red snapper and blood clams, which

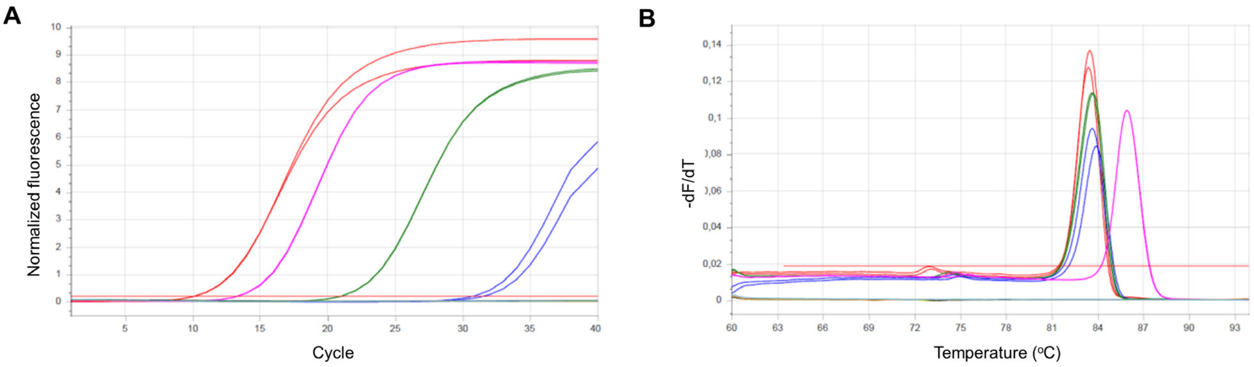

Figure 5. Confirmation test results on food samples. (A) Ct values, (B) Tm values

were artificially contaminated. The Ct values obtained were 21.00 and 21.03 (duplo) for red snapper, and 31.54 and 30.77 (duplo) for blood clams (Figure 5A). The Tm values were 83.61 and 83.70 (duplo) for red snapper, and 83.90 and 83.64 (duplo) for blood clams (Figure 5B). The negative controls, non-template control and master mix + nuclease-free water, did not produce any Ct or Tm values.

Discussion

The designed *flgL* primer pair meets the requirements for a good primer, exhibiting no secondary structures [10] that could prevent it from binding to the target bacterial DNA template. In the annealing temperature optimization test [11], 60°C was determined to be the optimal temperature, as it produced the brightest band and had the appropriate amplicon length without any non-specific amplification.

In the confirmation test for *V. alginolyticus* using the *flgL* primer, a concentration of 50 ng was used. The *flgL* primer successfully amplified *V. alginolyticus* DNA, evidenced by a small Ct value and a melting curve with a single peak. The presence of one peak on the melting curve indicates the formation of a single specific amplicon product [12].

The specificity test demonstrated that the *flgL* primer could distinguish between target and non-target bacteria, even those causing food poisoning similar to *V. alginolyticus*. Non-target bacteria produced small Ct values with a difference of more than ten cycles compared to pure *V. alginolyticus* DNA, which is considered negative [11]. The flgL primer also detected target bacteria at very low concentrations, namely 1.86 x 10^{-3} ng/µL at a Ct value of 23.90. The amplification curve showed that as dilutions increased, the Ct value also increased due to the decreasing bacterial DNA concentration [13]. The standard curve equation $y = -3.20x + 15.27$ indicates a detection limit of 119.3 CFU for *V. alginolyticus*.

The final stage involved testing the *flgL* primer on food samples, specifically red snapper and blood clams, which were intentionally contaminated. The *flgL* primer detected *V. alginolyticus* in both red snapper and blood clams, despite high Ct values due to inhibitors such as algae, glycogen, and polysaccharides commonly found in seafood [14]. The melting curve, however, produced the same peak as pure bacteria, confirming detectability. Red snapper contains approximately 0.23% polysaccharides, while blood clams have a glycogen content of around 1-7% [15].

Conclusion

This research successfully detected *Vibrio alginolyticus* bacteria quickly, specifically, and sensitively in seafood samples using the *flgL* gene and rt-PCR.

Acknowledgment

We would like to acknowledge PT Sinergi Indomitra Pratama for providing the instrumentation used in this research. We also extend our thanks to the Forensic Laboratory Centre of the Indonesian

National Police (PusLabFor), Sentul Bogor, Indonesia, for facilitating this research under the UNJ-Puslabfor MOU; Kimia Farma Tbk.; and the Salmonella UNJ team under the Center of Excellence for Pathogenic Bacteria Detection (PUI Pendeteksi Bakteri Patogen) LPPM UNJ, under contract agreement number 2/PPUI/LPPM/IV/2023, for their contributions to this research. Our gratitude is also extended to our international partner from Innovation Center in Agritechnology for Advanced Bioprocessing (ICA), Universiti Teknologi Malaysia (UTM), Pagoh, Johor, Malaysia.

Funding

This research was funded by Kemendikbud Ristek by the scheme of PDUPT under the contract agreement number 4/E5/DRTPM/IV/2023 and RIIM periode 2-BRIN under the contract agreement number 2/PG.02.00.PT/LPPM/IV/2024.

Declaration of interest

The authors declare no conflict of interest.

Author contributions

Conceptualization, M.N. and G.I.P.; Methodology, M.N., V.S., and I.M.W.; Software, J.L.D., T.F., I.K., and D.A.J.; Validation, M.N., G.I.P., and F.K.; Formal Analysis, J.L.D., T.F., S.R., D.S., and I.R.K.; Investigation, G.I.P., A.M.A.P., R.G.A., A.N.R., M.A., I.M., and A.B.; Resources, M.N., F.K., and D.S.; Data Curation, J.L.D., G.I.P., and D.A.J.; Writing – Original Draft, M.N., G.I.P., and J.L.D.; Writing – Review & Editing, B.A. and H.A.E.; Visualization, M.N., G.I.P., and J.L.D.; Supervision, M.N., B.A., and H.A.E.; Project Administration, M.N.; Funding Acquisition, M.N., F.K., and D.S.

Received: March 13, 2024
Revised: July 15, 2024
Accepted: July 29, 2024
Published online: August 4, 2024

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