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Rapid Detection of Foodborne Pathogen Bacteria *Vibrio parahaemolyticus* in Seafood using Gene *ToxR* with Real-Time Polymerase Chain Reaction Method

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Cases of food poisoning often occur due to food contamination caused by pathogenic bacteria. One of the pathogenic bacteria is *Vibrio parahaemolyticus* which is found in seafood. Thus, a fast, accurate and specific detection method is needed. The purpose of this study was to quickly detect *Vibrio parahaemolyticus* bacteria in seafood samples targeting the *ToxR* gene using Real Time PCR. In a previous study, gradient PCR was used to optimize ideal annealing temperature ranges from 53-62°C and revealed that 58°C produced the best outcomes for the *ToxR* primer with a size of 171 base pairs. Real-Time PCR was utilized to amplify, specify, and test for sensitivity under the ideal conditions from the PCR Gradient. The confirmation results show that the primer pairs could amplify *ToxR* of *Vibrio parahaemolyticus* with the amount of concentration as much as 50 ng μL^{-1} with Ct 10.69 and 10.32 and melting curve at temperature 82.18°C and 82.23°C. This primer pair can also distinguish non-target bacteria with different Ct and melting curve temperature. The sensitivity assay for this primer can amplify DNA templates at concentration 0.0032 ng μL^{-1}. Shrimp samples that are contaminated artificially can still be detected at Ct 13.02 and Ct 13.09. Based on these results, it can be concluded that Real Time PCR with *ToxR* primer can be applied to develop a detection kit for *Vibrio parahaemolyticus* in seafood.

Key words: Detection kit, Foodborne diseases, Real-Time PCR, *ToxR*, *Vibrio parahaemolyticus*

Kasus keracunan makanan sering terjadi karena kontaminasi makanan yang disebabkan oleh bakteri patogen. Salah satu bakteri patogen adalah *Vibrio parahaemolyticus* yang banyak ditemukan pada makanan laut. Oleh karena itu, diperlukan metode pendeteksian yang cepat, akurat dan spesifik. Tujuan dari penelitian ini adalah untuk mendeteksi secara cepat bakteri *Vibrio parahaemolyticus* pada sampel makanan laut yang menargetkan gen *ToxR* menggunakan Real Time PCR. Dalam penelitian sebelumnya, PCR gradien digunakan untuk mengoptimalkan suhu annealing ideal antara 53-62°C dan mengungkapkan bahwa 58°C menghasilkan hasil terbaik untuk primer *ToxR* dengan ukuran 171 pasangan basa. PCR Real-Time digunakan untuk memperkuat, menentukan, dan menguji sensitivitas di bawah kondisi ideal dari Gradien PCR. Hasil konfirmasi menunjukkan bahwa pasangan primer dapat mengamplifikasi *ToxR Vibrio parahaemolyticus* dengan jumlah konsentrasi sebanyak 50 ng μL^{-1} dengan Ct 10,69 dan 10,32 dan kurva leleh pada suhu 82,18°C dan 82,23° C. Pasangan primer ini juga dapat membedakan bakteri non-target dengan Ct dan suhu kurva leleh yang berbeda. Uji sensitivitas untuk primer ini dapat mengamplifikasi template DNA pada konsentrasi 0,0032 ng μL^{-1}. Sampel udang yang tercemar artifisial masih dapat dideteksi pada Ct 13,02 dan Ct 13,09. Berdasarkan hasil tersebut, dapat disimpulkan bahwa Real Time PCR dengan primer *ToxR* dapat diterapkan untuk mengembangkan kit deteksi Vibrio parahaemolyticus pada makanan laut.

Kata kunci: Keracunan Pangan, Kit Deteksi, *Real Time PCR, ToxR, Vibrio parahaemolyticus*

Foodborne disease is one of the public health challenges that causes death every year around the world. This disease comes from consuming contaminated food. Everyone is at risk for foodborne disease. However, some people may be at greater risk of serious symptoms or even death (Bari and Ukuku 2016). 52.4% of the 208 instances of food poisoning

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reported to DKI Jakarta in 2016 were attributable to seafood intake. (Mabruroh and Ciptaningtyas 2018).

Vibrio parahaemolyticus is one of the pathogenic bacteria that can cause foodborne diseases in seafood. In estuarine and marine environments around the world, *Vibrio parahaemolyticus* is a naturally occurring part of the microbial community and is a major cause of bacterial sickness linked to seafood consumption. (Newton *et al.* 2012). A culture method is one of the several approaches that have been proposed to identify *V. parahaemolyticus* (Duan and Su 2005). It is required to design a new quick, sensitive, and targeted way to identify the *Vibrio parahaemolyticus* because the culture method has a lengthy execution time and is difficult to define limits in samples at low concentrations (Dorak 2006). Bacterial identification at the lowest concentration is possible with Real-Time Polymerase Chain Reaction. Additionally, it only takes 2.5 hours, or, when combined with sample preparation, real-time PCR can be finished in about 5 hours (Nurjayadi *et al.* 2019).

A *Salmonella typhi* detection kit with the *fim- C* gene, whose product length is 95 base pairs, was successfully developed in a prior study. (Nurjayadi *et al.* 2017). The aim of this research is to create a precise, perceptive, and quick detection kit of *Vibrio parahaemolyticus* with *ToxR* gene using Real-Time PCR. Ideal annealing temperature *ToxR* gene has been optimized at 58°C with a size of 171 base pairs (Nurjayadi *et al.* 2022). Based on the previous experience and literature reviewed, the detection method of *Vibrio parahaemolyticu*s s as a rapid, sensitive, and specific detection kit is expected to be developed.

MATERIALS AND METHODS

Preparation of Culture Sample. *Vibrio parahaemolyticus* ATCC 17802 in KWIK-STIK™ (Microbiologist, Minnesota) was employed in this study. *V. parahaemolyticus* was further cultured in Thiosulfate Citrate Bile Sucrose (TCBS) Agar (Merck) at 37ºC for 24h. After incubation, one single colony was inoculated to Tryptic Soy Broth (TSB) + 2.5% NaCl and incubated in an incubation shaker (YIHDER LM-400D) at 37ºC for 24h and 150 rpm. For artificially contaminated food samples, *Vibrio parahaemolyticus* inoculum was then diluted at 10^{-1}-10^{-7} dilution with NaCl 0.85%. TCBS media (Merck) was used to grow a one mL solution of 10^{-5}-10^{-7}dilutions using spread plate method and incubated at 37ºC for 24h to determine the number of colonies contaminated food samples. Colonies result used for artificial contamination is a sample dilution that formed 30-300 colonies on a based on the FDA BAM's plate count standard (Food and Drug Administration Bacteriological Analytical Manual). CFU mL^{-1} was then determined based on the number of colonies.

Seafood Artificially Contaminated with *Vibrio parahaemolyticus*. First, shrimp samples were boiled. The boiled samples were then ground until smooth in a sterile plastic. Samples were put in a petri dish and exposed to UV light for a half-hour. After that, the shrimp sample was transferred to a sterile Erlenmeyer flask and infected with a suspension of *Vibrio parahaemolyticus* at a volume ratio of 8:2. The positive control used was in the form of non-dilute suspension of bacteria to be inoculated on shrimp samples, samples of uncontaminated food were used as a negative control. At a speed of 150 rpm, an orbital shaking incubator from the LM series (Yihder Co. Ltd.) was used to incubate each sample for 24 hours at 37ºC.

Isolation of DNA. Before DNA isolation, 1.5 ml of the *Vibrio parahaemolyticus* bacterial culture from the TSB + 2,5% NaCl was centrifuged at 5000 x g for 10 minutes. Thermo Scientific GeneJET Genomic DNA Purification Kit (ThermoFisher, 2012) and Geno Plus Genomic DNA Extraction Miniprep System (Viogene) were used to isolate DNA. The concentration of DNA isolation results was determined using Nanodrop (Nanovue Plus), then confirmed by electrophoresis on 0.7% agarose gel (NZYTech) and checked with an UV transilluminator (Vilber Lourmat).

Optimization Annealing Temperature of *ToxR* Primer Pairs: Synthesized primer pairs were then optimized using Gradient PCR with temperature length of 53-62°C. A PCR cocktail contained of Colorless Master Mix (NZYTaq), *V. parahaemolyticus* genome as a DNA template, Nuclease Free Water (Qiagen), *ToxR*-f (forward primer) and *ToxR*-r (reverse primer) with a total volume of single reaction is 25µL. Optimization results are followed by 40 cycles in reaction conditions of 95ºC for 3 minutes (initial denaturation), 95ºC for 10 second (denaturation), annealing temperature 53-62ºC for 30 second, 72 ºC for 30 seconds (extension), and 72 ºC for 7 minutes (final extension) (SMOBIO 2017).

***Vibrio parahaemolyticus ToxR* gene confirmation assay.** The confirmation assay of *Vibrio parahaemolyticus* was run in a Magnetic Induction Cycler qPCR (Bio molecular system). A PCR cocktail contained of qPCR Master Mix with SYBR green dye

Table 1 Design of cacao beans fermentation experiment

Sample	Concentration (ng μL^{-1})	Purity (A260/A280)
V. parahaemolyticus DNA isolated (Lane 1 & 2)	50	1,92
V. parahaemolyticus DNA isolated (Lane 3 & 4)	47	1,85

(Smobio), *Vibrio parahaemolyticus* genome as a DNA template, Nuclease Free Water (Qiagen), *ToxR*-f and *ToxR*-r with a total volume of single reaction is 20µL. To verify the fluorescence signals not contaminated by any contaminant; negative or non-template controls were also run. As a negative control, a PCR mixture does not contain any DNA template. Positive control is *Salmonella typhi* with *fimC* primers that have already study before (Nurjayadi *et al.* 2019). The PCR cycle conditions are followed by 40 cycles in reaction conditions of 95ºC for 3 minutes (initial denaturation), 95ºC for 10 second (denaturation), annealing temperature 58 ºC for 30 second, 72ºC for 30 seconds (extension), and 72ºC for 7 minutes (final extension) (Law *et al.* 2015)**.**

Specificity and sensitivity assay. The specificity assay of primer pairs *ToxR*-f and *ToxR*-r were tested with non-target bacteria, such as, *Vibrio alginolyticus, Cronobacter sakazakii, Listeria monocytogenes, Yersinia enterocolitica, Salmonella typhi, Klebsiella pneumoniae, Staphylococcus aureus*. Meanwhile, for the sensitivity assay, *Vibrio parahaemolyticus* culture stock was diluted to establish the Real-Time PCR primer pairs' limit of detection (LOD). By graphing the Ct (Cycle threshold) value against the plasmid DNA copy number, the standard curves for *Vibrio parahaemolyticus* copy number were created. (Mirasoli 2011). The PCR cycle conditions were described above.

Confirmation assay of seafood sample. Amount of 10 milligram shrimp contaminated artificially by *Vibrio parahaemolyticus* bacteria were used as DNA template samples in PCR mixture; meanwhile, a positive control of this assay is Genome DNA of culture stock of *Vibrio parahaemolyticus* bacteria non dilute results, and a negative control are DNA template from shrimp non-contaminated as samples. PCR mixture and cycle conditions were the same as described above.

RESULTS

Cultivation of *V. parahaemolyticus.* Bacteria on selective media Thiosulfate Citrate Bile Sucrose (TCBS) Agar showed the formation of green colonies on the surface of TCBS media (Fig 1). In bacterial suspension 10^{-6}achieve the FDA BAM (Food and Drug Administration Bacteriological Analytical Manual)-recommended colonies. There are reportedly 88 colonies of *V. parahaemolyticus* forming.

Qualitative and Quantitative DNA Analysis. DNA purity and concentration were measured using nanodrop (Nanovue plus) with a result as described in Table 1. *Vibrio parahaemolyticus* bacteria was successfully isolated, as shown in Fig 2**.** Based on the electrophoresis analysis of DNA isolates, *Vibrio parahaemolyticus*-specific DNA bands are visible in the upper section, higher than the 1 kb marker size.

Optimization of *ToxR* primer pairs Annealing Temperature. Before Real-Time PCR assay, primer pairs were optimized in annealing temperature range 53ºC-62ºC. The results of Gradient PCR were then visualized using 2% Agarose Gel Electrophoresis. This optimization showed in Fig 3, whereas the positive control is *Cronobacter sakazakii*, which produced 151 base pairs. Amplification band of *ToxR* primer pairs is at 171 bp, and the optimum annealing temperature result is at 58ºC.

Real-Time PCR amplification. Confirmation assay resulted from that *ToxR-f* and *ToxR-r* primer pairs can amplify *Vibrio parahaemolyticus* DNA at Ct 10.32 and 10.69, as shown in Fig 4. The melting curve (Fig 5) of *Vibrio parahaemolyticus* was at 82.18ºC and 82.23ºC. Non template control amplified ct 33.11 with the differences 23 cycles and different melt curve with low peak at 82.34 ºC.

Specificity and Sensitivity Assay. *Vibrio parahaemolyticus* isolate was diluted as much as five times of dilution. This assay gave the results that *Vibrio parahaemolyticus* DNA template can still detect at a lower concentration of 0,0032 ng μL^{-1} at 28.60 (Fig 6 and Table 2) Standard curve has a regression value of R^2= 0,9969 and equation y = -4.194 x + 18.68. *ToxR* primer pairs were tested with non-target bacteria as mentioned before and showed a good result as shown in Fig 7. Primer pairs can amplify to non-target bacteria

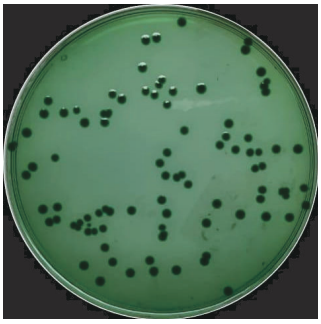

Fig 1 *Vibrio parahaemolyticus* culture results on TCBS Agar.

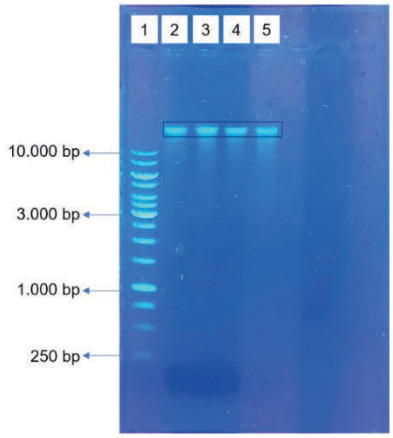

Fig 2 Gel Electrophoresis DNA isolates results of *V. parahaemolyticus*. Lane 1. DNA Marker 1 kb Ladder. Lane 2, 3, 4, 5. The Genome of *Vibrio parahaemolyticus* Bacteria.

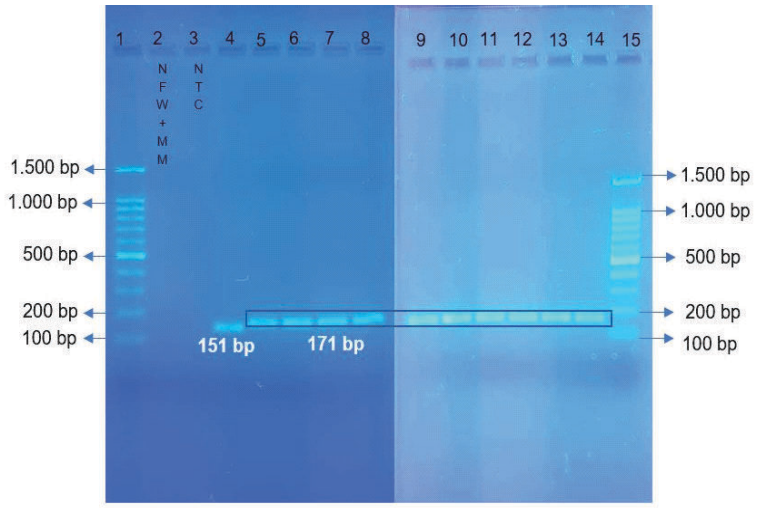

Fig 3 Optimization of *Vibrio parahaemolyticus ToxR* gene fragments with an annealing temperature range of 53-62°C (1) DNA Marker 100 basepair; (2) NFW+MM (Negative Control); (3) NTC; (4) Positive control *grxB Cronobacter sakazakii*; (5) DNA fragment at temperature 53ºC; (6) DNA fragment at temperature 54ºC; (7) DNA fragment at temperature 55ºC; (8) DNA fragment at temperature 56ºC; (9) DNA fragment at temperature 57ºC; (10) DNA fragment at temperature 58ºC; (11) DNA fragment at temperature 59ºC; (12) DNA fragment at temperature 60ºC; (13) DNA fragment at temperature 61ºC; (14) DNA fragment at temperature 62ºC; (15) DNA Ladder 100 bp.

with a difference range amount of 17-21 cycles. DNA and non-target, bacteria have a different melting curve (Fig 8 and Table 3).

Confirmation Assay of Seafood Sample. Artificial contaminated shrimp samples and negative control non contaminated shrimp samples checked

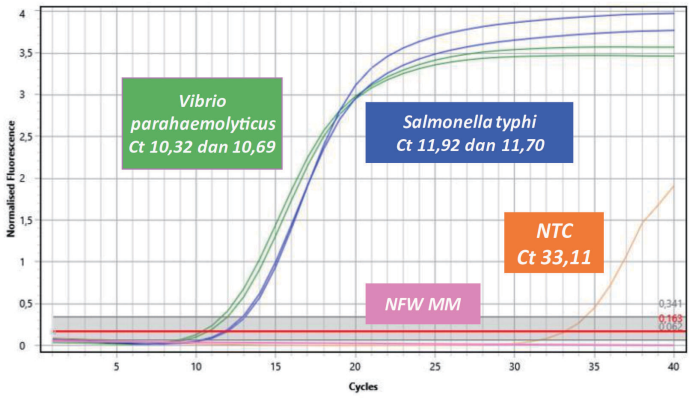

Fig 4 Amplification curve of a concentration 50 ng DNA templated *V. parahaemolyticus* bacterial stock culture, Positive control *Salmonella typhi*, and negative control NTC, NFW+MM.

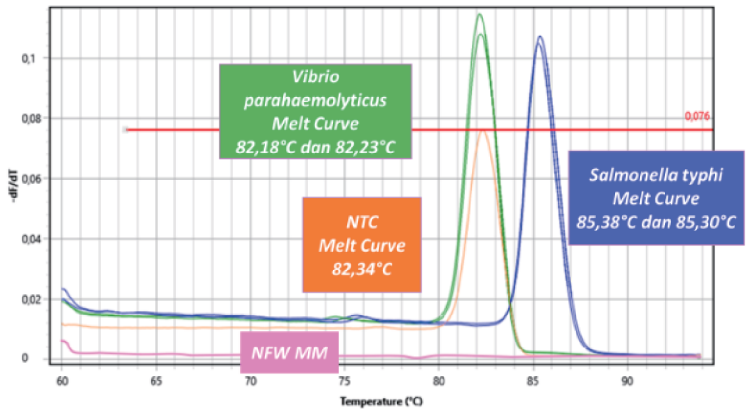

Fig 5 *V. parahaemolyticus* stock culture melting curve with 50 ng DNA template.

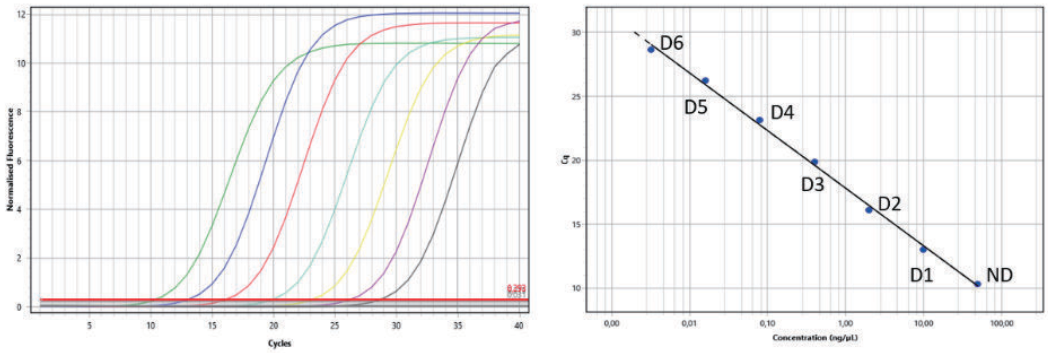

Fig 3 The sensitivity test and amplification curves for the *ToxR Vibrio parahaemolyticus* gene.

then compared with non-dilute positive controls (Fig 9). *ToxR* primer pairs can amplify the shrimp sample at the Ct 13,02 and Ct 13,09. Melt Curve in Fig 10, showed that both samples have the similar melting temperature at 82.28ºC and 82.22ºC.

DISCUSSION

Cultivation of *V. parahaemolyticus.* According to (Bisha *et al.* 2012) the resulting *Vibrio parahaemolyticus* colonies were in accordance with having a round size with a diameter of 2-3 mm and greenish colour on TCBS media. In the 10^{-6} dilution there were 88 bacterial colonies, so that the number of bacterial cultures in one milliliter were obtained: 88 x 10^{6} CFU mL^{-1}. The results of this calculation will be applied to determine the number of bacterial colonies formed in the confirmation test using seafood sample.

Qualitative and Quantitative DNA Analysis. The requirements for the purity of a good DNA isolate are 1.8-2.0. If it is less than 1.8, it indicates that there is still an impurity in the form of RNA and if it is more than 2.0, it indicates that there are still contaminants in the form

Table 2 The phytochemical component from ethanol extract of fermented cacao beans

Line	Concentration (ng/µL)	Ct
	50	10,30
	10	13.01
	2	16,08
	0,4	19,83
	0,08	23,11
	0,016	26,18
	0,0032	28,6

Table 3 Findings from an analysis of the *ToxR* gene *V. parahaemolyticus* primer specificity

Line	Sample	Ct	Tm
	V. parahaemolyticus	10,69	82,18
	S. typhi	29,28	82,18 & 86,65
	K. pneumoniae	31,06	82,16 & 87,95
	V. alginolyticus	30,57	81,83
	Y. enterocolitica	31,68	82,05 & 84,93
	S. aureus	28,26	82,06 & 87,02
	L. monocytogenes	31,09	81,77
	C. Sakazakii	30,88	82,12 & 88,67
	NTC	33,11	82,34

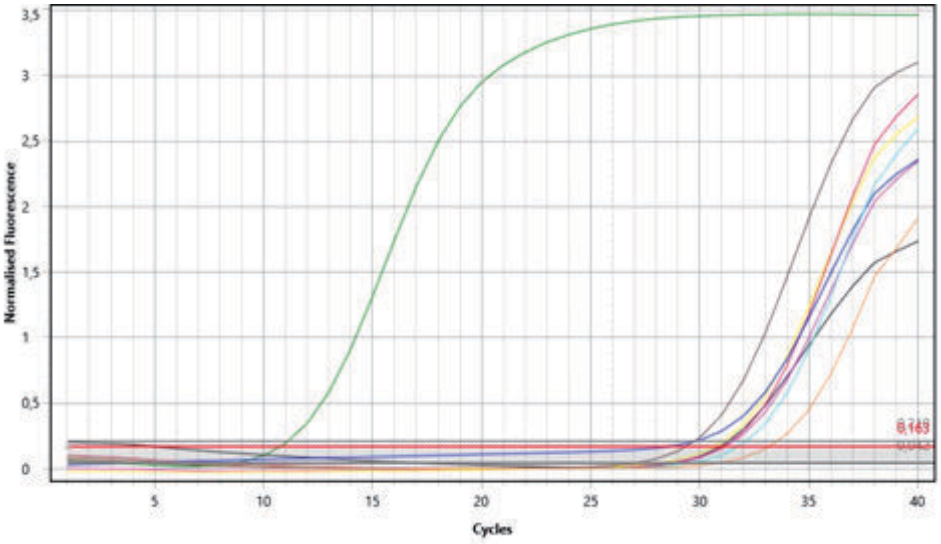

Fig 7 Amplification curve for the *Vibrio parahaemolyticus ToxR* gene's primer specificity.

of protein (Wasdili and Gartinah 2018). Measurement of concentration and purity of pure culture isolates of *Vibrio parahaemolyticus* DNA showed good results. *Vibrio parahaemolyticus*- specific DNA bands are visible in the upper section, higher than the 1 kb marker size, indicating that the results are consistent with data of the genome size that is 3.288.558 bp (Markino *et al.* 2003).

Optimization of *ToxR* primer pairs Annealing Temperature. An important factor in Real-Time PCR is optimization annealing temperature for primer pair design (Green and Sambrook 2019) Annealing temperatures were set based on ± 5 ºC from *ToxR-f* and *ToxR-r* primer pairs theoretical result which is ± 5ºC from 58 ºC and also this melting curve can be calculated by inserting the amount of nucleotides content in the formula Tm= 4(G+C) + 2(A+T) (Innis 1997). The results of Gradient PCR were then visualized using 2%

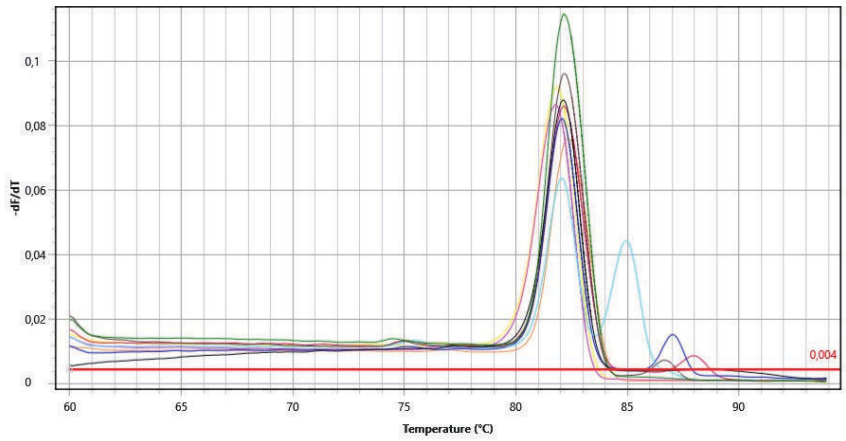

Fig 8 Melting curve of the primer specificity of the *Vibrio parahaemolyticus ToxR* gene.

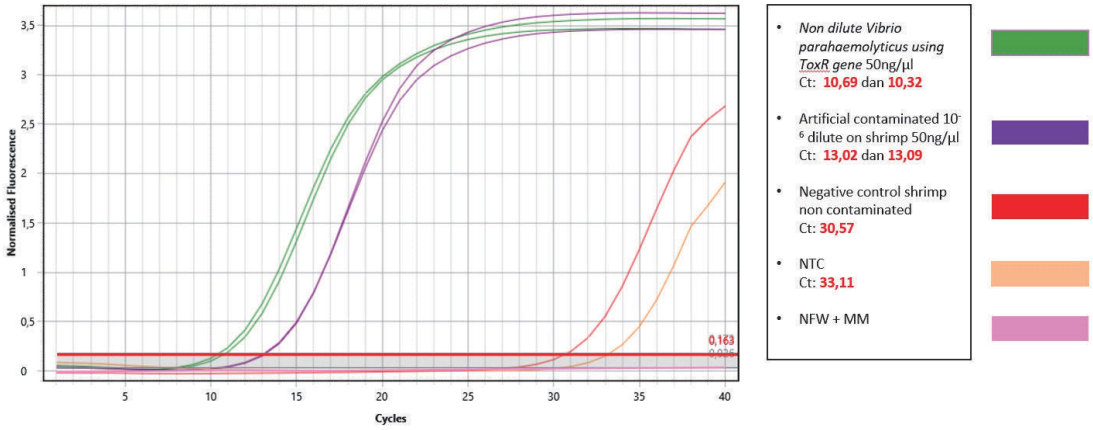

Fig 9 The amplification curve in the confirmation evaluation of *ToxR* Primer *Vibrio parahaemolyticus* with shrimp samples.

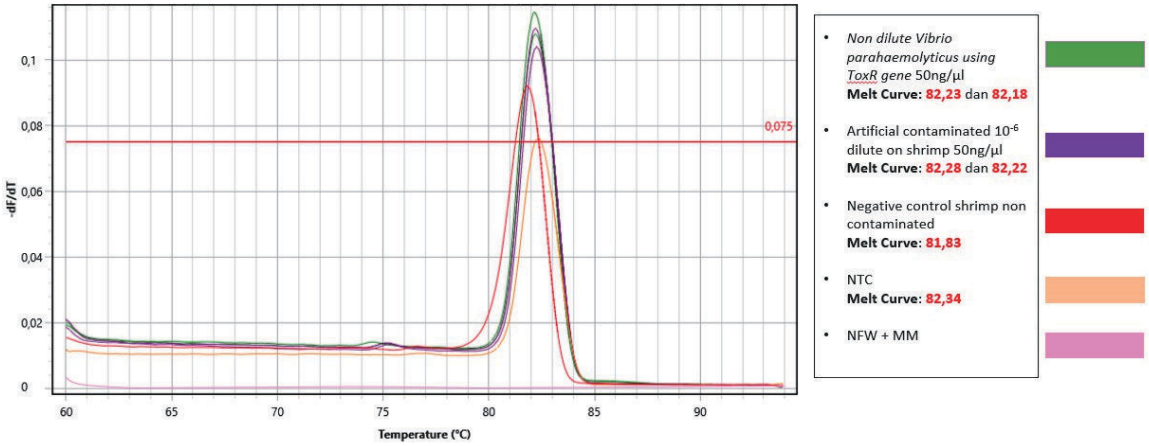

Fig 10 The Melting curve in the confirmation evaluation of *ToxR* Primer *Vibrio parahaemolyticus* with shrimp samples.

Agarose Gel Electrophoresis. This optimization showed a good result as revealed in Fig 2. Amplification band of ToxR primer pairs is at 171 bp, and the optimum annealing temperature result is at 58 ºC. The band has a generally constant band thickness when it is annealed at a temperature between 53-62 ºC. Based on the brightest, most stable, and single band that appears, the primer's ideal annealing temperature can amplify the template in the PCR process between 58-62 degrees celsius. Therefore, if used in conjunction with in silico analysis, 58 degrees Celsius is the most ideal annealing temperature for the PCR procedure (Nurjayadi *et al.* 2022).

Real-Time PCR Amplification. Confirmation

assay resulted from that *ToxR-f* and *ToxR-r* primer pairs can amplify *Vibrio parahaemolyticus*. the fewer number of cycles that required the fluorescent signal is first recorded as statistically significant above background and thus lower the Ct value. The melting curve result shows that there is no mis-priming, which means that the primer just amplifies the target DNA as indicated by the formation of one peak (Biorad 2006).

Specificity and Sensitivity Assay. The sensitivity assay gave the results that *Vibrio parahaemolyticus* DNA template can still detect at a lower concentration of 0.0032 ng μL^{-1} with LoD 0.00396 CFU mL^{-1} at Ct 28.60 (Fig 6 and Table 2). The standard curve can be determined by prolonging the amplification curve by the value of the x-axis, in which the value of the y- axis is the sample concentration. The curve's Ct connection and concentration were made. Standard curve has a regression value of R^2= 0,9969 and equation y = -4.194 x + 18.68 or value regression, which has superb linearity (Dorak 2006). *ToxR* primer pairs were tested with non-target bacteria as mentioned before and showed a good result as shown in Fig 7. Primer pairs can amplify to non-target bacteria with a difference range amount of 17-21 cycles. which can be considered as a negative control as stated by that if the cycles between target and non- target bacteria had a different range of 10 cycles, it considered that the primer pairs can amplify the non-target bacteria (Nurjayadi *et al.* 2017). DNA and non-target, bacteria have a different melting curve (Fig 8 and Table 3). so that this melting curve result can be a reference that non-target DNA can be considered as a negative control.

Confirmation Assay of Seafood Sample. Plate count results show that 10^{-6} dilution samples have 88 colonies. This result is chosen since it followed FDA BAM recommendation. So, the non-dilute bacteria that is contaminated artificially in food sample is $88x10^{6}$ CFU mL^{-1}. Artificial contaminated shrimp samples and negative control non contaminated shrimp samples checked then compared with non-dilute positive control (Fig 9). *ToxR* primer pairs can amplify the shrimp sample at the Ct 13.02 and Ct 13.09. Melt Curve in Fig 10, showed that both samples have the similar melting temperature compared to non-dilute at 82.28ºC and 82.22ºC. So, it can be stated that the concentration of *Vibrio parahaemolyticus* contaminated in the shrimp sample is $2,04x10^{1}$ CFU mL^{-1}. This result can be stated that the method could be developed as a detection kit model to determine pathogenic bacteria as rapid, sensitive, and specific and show an accurate result.

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