VOLUME 10 NOMOR 2 DESEMBER 2023

ISSN 2548 – 611X

JURNAL

BIOTEKNOLOGI & BIOSAINS INDONESIA

Homepage Jurnal: http://ejournal.brin.go.id/JBBI/index

DETERMINATION OF OPTIMAL ANNEALING TEMPERATURE *Vibrio alginolyticus* PRIMERS USING POLYMERASE CHAIN REACTION METHOD

Penentuan Suhu Annealing Optimal Primer *Vibrio alginolyticus* Menggunakan Metode *Polymerase Chain Reaction*

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ABSTRACT

Food poisoning is a global issue of grave concern. If food is not properly cooked, it can be a medium for the spread of pathogenic bacteria. Vibrio alginolyticus is one of the pathogenic bacteria that can cause food poisoning. real-time Polymerase Chain Reaction (rt-PCR) can detect pathogenic bacteria in food, so it is necessary to determine the optimal annealing temperature. This research aims to obtain the optimal annealing temperature of the Va_Chr1_FR primer using Gradient PCR. The DNA concentration used was 174.5 with an A_{260}/A_{280} purity of 1.94. The temperature range tested, 53°C-62°C, corresponds to the melting temperature of the Va_Chr1_FR primers. The primers designed were F5'-TTCTTCTGTTGTAGGTTCCG-F3' and R5'-CCAGCCCTCACATCTAATAC-R3'. Based on these results, a temperature of 60°C is deemed as the most optimal annealing temperature because it produces one of the brightest bands on electrophoresis with an amplicon length of 146 bp. The findings of this study will be beneficial to the development of Va_Chr1_FR Vibrio alginolyticus primers testing on food samples using the real-time PCR method.

Keywords: *Annealing temperature, Foodborne diseases, Polymerase Chain Reaction, Va_Chr1_FR primers, Vibrio alginolyticus*

***Received**: 05 June 2023* *__Accepted__: 26 December 2023* *__Published__: 30 December 2023*

ABSTRAK

Keracunan makanan merupakan masalah global yang sangat memprihatinkan. Makanan yang tidak dimasak dengan benar dapat menjadi media penyebaran bakteri patogen. *Vibrio alginolyticus* merupakan salah satu bakteri patogen yang dapat menyebabkan keracunan makanan. Deteksi bakteri patogen pada makanan dapat menggunakan real-time PCR, sehingga harus dilakukan penentuan suhu annealing yang optimal. Penelitian ini bertujuan mendapatkan informasi suhu annealing optimal primer Va_Chr1_FR menggunakan PCR Gradien. Konsentrasi DNA yang digunakan yaitu 174,5 dengan kemurnian A_{260}/A_{280} sebesar 1,94. Rentang suhu yang diuji pada 53°C-62°C sesuai dengan melting temperature primer Va_Chr1_FR. Primer yang dirancang adalah F5'-TTCTTCTGTTGTAGGTTCCG-F3' dan R5'-CCAGCCCTCACATCTAATAC-R3'. Berdasarkan hasil tersebut, suhu 60°C dianggap sebagai suhu annealing yang paling optimal karena menghasilkan satu pita paling terang pada elektroforesis dengan panjang amplikon 146 bp. Hasil dari penelitian ini akan bermanfaat untuk pengembangan uji primer Va_Chr1_FR *Vibrio alginolyticus* pada sampel makanan dengan menggunakan metode real-time PCR.

Kata Kunci: Penyakit Keracunan Pangan, Polymerase Chain Reaction, Primer Va_Chr1_FR, Suhu annealing, *Vibrio alginolyticus*

INTRODUCTION

Food has a very important role in the spread of various diseases. Food that is processed improperly can be a good medium for the spread of pathogenic bacteria. Diseases caused by the contamination of food are called foodborne diseases (Nurmawati et al. 2019). Foodborne diseases occur because the food we eat contains bacteria, viruses, or fungi and they have the ability to quickly multiply in the human body.

Based on reports issued by the World Health Organization, 600 million cases of food transmission caused 420,000 deaths in 2010. Children under the age of five accounted for roughly 40% of those affected (Todd 2020). According to the Badan Pengawas Obat dan Makanan (BPOM), cases of foodborne diseases in Indonesia reached 128 cases of extraordinary events in 2011, with 18,144 people exposed to foodborne diseases (Siyam & Cahyati 2018). In Morocco, 7,118 cases of food poisoning were reported, and more than 86% of the bacteria in some cases are associated with the consumption of fishery products (Cohen et al. 2006; Sabir et al. 2013). Foodborne diseases are an iceberg phenomenon because not all incident cases are properly reported (Yulianto et al. 2019).

Vibrio alginolyticus is one of the microbes responsible for food poisoning. *Vibrio alginolyticus* is a halophilic gram-negative bacteria found naturally in seawater and freshwater and it can cause gastroenteritis (Reilly et al. 2011). *Vibrio alginolyticus* is commonly found in seafood. *Vibrio alginolyticus* bacteria is also present in approximately 80% of the waters (Jones 2014). This bacteria grows at a temperature of 42°C. *Vibrio* prefers alkaline conditions between 6.8-10.2 and its optimum pH is 7.4-9.6 (Chart 2012). Vibrio *alginolyticus* has two flagella positions, in the middle of the cell body and polar (at the ends of the cell body) (Aizawa 2019).

When *Vibrio alginolyticus* enters the human body, the immune system, such as macrophages, releases cytokines and activates other macrophages, causing inflammation. Inflammation is an immune response intended to eliminate pathogens; however, excessive inflammation can be harmful to the body. What is worrisome is when there is excessive inflammation that can result in disease, including gastroenteritis. (Wang et al. 2021).

To detect pathogenic bacteria in the food we can use real-time Polymerase Chain Reaction using specific primers. real-time PCR detection yields faster, more effective, more specific, and more sensitive results (Ayu & Nurdyansyah 2017). The

real-time PCR process consists of three stages that are repeated every 30-40 cycles: denaturation, annealing, and extension. (Dorado et al. 2019). To determine the optimal annealing temperature, research must be conducted to determine at which temperature the primer can attach to the DNA template optimally.

real-time Polymerase Chain Reaction (rt-PCR) method was previously used to detect *Salmonella typhi* bacteria in contaminated eggs in a fast, accurate, sensitive, and specific manner (Nurjayadi et al. 2019). In this research, the *fimC* gene with an amplicon length of 95 bp was used with an annealing temperature of 60°C. Research on the detection of *Vibrio alginolyticus* bacteria has also been carried out using Multiplex PCR with the *gyrB* gene (Wei et al. 2014). Therefore, this research aims to determine the optimal annealing temperature for the *Vibrio alginolyticus Va_Chr1_FR* primers, so that primers designed can appear to amplify *Vibrio alginolyticus* which can be continued by detecting *Va_Chr1_FR* primers using real-time PCR method on food samples.

MATERIALS AND METHODS

Place and time of the research

This study was carried out at the Biochemistry and Biotechnology Laboratory of Universitas Negeri Jakarta, Biology Laboratory of Universitas Negeri Jakarta, Integrated Laboratory of the Faculty of Medicine of Universitas Indonesia, Pusat Laboratorium Forensik MABES POLRI, Sentul, and PT. Indomitra Pratama Synergy. This research lasted from January to December 2022.

Materials

The materials used in this research were *Vibrio alginolyticus* ATCC 17749, Thiosulphate Citrate Bile Sucrose (TCBS) Agar, Tryptic Soy Broth (TSB) + 2.5% NaCl, GRS Genomic DNA Kit - Bacteria, *Va_Chr1_FR* primer synthesized (Macrogen), Nuclease Free Water (Qiagen), NZYTaq II 2× Green Master Mix (NZYTech), agarose (Promega), Loading Dye 6x (Promega), Buffer TAE 50x (Promega), greensafe (NZYTech), DNA ladder 1 kb dan 100 bp (Smobio), 96% ethanol, disinfectant (Wipol), and distilled water.

METHODS

Primers Design

Vibrio alginolyticus ATCC 17749 *Va_Chr1_FR* primers were designed using the National Center for Biotechnology Information or NCBI website and the Primer-BLAST (Primer-Basic Local Alignment Search Tool) program to identify gene specific to *Vibrio alginolyticus*. The selected primer pairs were then analyzed using NetPrimer and OligoAnalyzer programs to determine dimers, because if there is a dimer, it will result in the designed primer not attaching to the target bacterial DNA template. The designed primers were synthesized in the commercial laboratory of Macrogen Synthesis, Inc.-Korea.

Preparation of Culture Sample

Vibrio alginolyticus ATCC 17749 in the form of KWIK-STIK^{TM} (Microbiologist, Minnesota) was resuspended with hydrating liquid and planted in Thiosulphate Citrate Bile Sucrose Agar (TCBS Agar) media with the SWAB method, then streaked zigzag (streak plate method) on agar medium in a petri dish that has been sterilized using a sterile ose. After 24 hours (overnight culture) at 37°C in an incubator (MEMMERT Type INB 400), a large yellow *Vibrio alginolyticus* colony was formed. Using a sterile ose, colonies were harvested and immersed in 10 mL of Tryptic Soy Broth (TSB) + 2.5% NaCl medium. Then they were incubated at 37°C for 18 hours with an aeration shaker at 150 rpm using a shaker incubator (YIHODER LM-400D). Furthermore, the results of bacterial cultures were checked for turbidity using a UV/Vis Spectrophotometer (SHIMADZU UVMINI-1240).

DNA Isolation

1.5 mL of pure cultured bacteria that had been incubated for 18 hours at 37°C was put into a microtube and then centrifuged at 14.000 × g for 1 minute to produce pellets. Furthermore, isolation was carried out using the GRISP kit. The obtained bacterial genomic DNA isolates were analyzed qualitatively and quantitatively using

agarose gel electrophoresis and nanodrop spectrophotometer (Nanovue Plus).

Optimization of Annealing Temperature

Optimization was carried out using Gradient PCR (TaKaRa Model TP600) in a temperature range of 53°C - 62°C based on ± 5°C melting temperature (Tm) value of the primer pair *Vibrio alginolyticus Va_Chr1_FR*. The annealing process is related to the primer melting temperature value, the %GC value, and also the primer length. When the annealing temperature used is too low, the primer cannot stick to the DNA template, and if the temperature used is too high, it will damage the primer. In this research, 25 µL of the reaction mixture was used. The mixture contains 5 µL pure DNA isolates of *Vibrio alginolyticus*, 1 µL forward and 1 µL reverse primers *Va_Chr1_FR*, 12.5 µL NZYTaq II 2x Colorless Master Mix, and 5.5 µL Nuclease Free Water (NFW). Amplification was then performed, beginning with an initial denaturation process at 95°C for 1:40 minutes, followed by 30 seconds of denaturation at 95°C, 30 seconds of annealing at 53°C - 62°C, 1 minute of extension at 72°C, and 10 minutes of final extension at 72°C. This process was repeated 35 times in total.

Agarose Gel Electrophoresis

DNA amplification results from Gradient PCR were analyzed using 2% agarose gel electrophoresis with 1x TAE buffer and florovue in the form of Green Safe as fluorescence. Agarose gel electrophoresis serves to see whether the DNA obtained can be amplified or not, because agarose gel is cheaper and easier to apply (Kristianto et al. 2019). 18 µL of the mixture was used that contains 10 µL of DNA amplification results were used, 5 µL sterile distilled water, and 3 µL of Loading Dye 6x. The reaction was carried out on parafilm paper and homogenized using the pipetting method. Then the mixture was put into an agarose well and then soaked with 1x TAE buffer in the mini-sub DNA Electrophoresis Cell chamber. The electrophoresis process was carried out using an electric current of 400 A and 70 V for 70 minutes.

RESULTS AND DISCUSSION

Va_Chr1_FR Primers Design

The *Vibrio alginolyticus Va_Chr1_FR* primers were 509 bp in the region 168211-168720. *Va_Chr1_FR* primer pairs are a DNA fragment in the 168211-168720 region of *Vibrio alginolyticus* ATCC 17749 chromosome I. Based on homology analysis of the nucleotide sequence, 99% homology match is the same as *Vibrio alginolyticus* strain K09K1 chromosome II as a membrane transport protein. The primer pairs were selected based on the length of the amplicon, %GC, melting temperature (Tm), and primer length (Rahayu et al. 2018). The selected primer pairs were then analyzed using NetPrimer and OligoAnalyzer programs to determine dimers.

The primers designed were F5'-TTCTTCTGTTGTAGGTTCCG-F3' and R5'-CCAGCCCTCACATCTAATAC-R3' with an amplicon length of 146 bp. The nucleotide sequence of the *Vibrio alginolyticus Va_Chr1_FR* primers based on the NCBI website database was shown in Figure 1. Primers are sequences of DNA molecules that are specifically designed to detect target bacteria. In this research, the primer that was designed still had some tolerable dimers.

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GCTTCCATGTAAGTATCCGCAGCAAAAGAAAAAAGTGTTCCTGTTGCCTATTAAGCCACAAACCCGGAGG
GTAGGACAGTAACGTGATGAAATTTTCTTCTGTTGTAGGTTCCGGAGTCATTTACACTCCAAGTTATGGA
CGAGGTCCCAATTTAACAAAGCGGTACTGGTCCTACGTGCGATATGTTGATTAACTCGTTGTCGTGATGC
ATCCACATGCGTATTAGATGTGAGGGCTGGTCATCGTAGTTATGGATAGGAGTTCATCAATGAAAAGGAA
ACACAACAAAACTGTACTGTGTGCGTTTGTCTCTGGTTCGGTATTTGGTGCGGTTTTGGTCATTGCGATG
TCTTTACCAATGTAATCCTCTTAATTGGCTGTTGGAACACTCACTCTAATTCAGTACAAAACAGATTGAG
TTCTGCGGTATGTGCTGTTAGTTTAAAAAACATGAAAATACACGTTCGCCCTTCATTAACAACCTTCTCT
TGGTGGCGCTCTAAATAGAG
```

Figure 1. The nucleotide sequence of the 509 bp *Va_Chr1_FR* primers in 168211-168720. Forward primer (red); reverse primer (blue); amplicon of *Va_Chr1_FR* primers (yellow).

According to Jia (2012), good primer requirements such as a primer length of 18-24 bp, a GC value of 35-65%, an amplicon length of around 100-250 bp, and a maximum Tm difference between forward and reverse primers is 3°C. The *Vibrio alginolyticus Va_Chr1_FR* primer pairs in silico analysis were shown in Table 1. Based on the results in silico analysis, the *Va_Chr1_FR* primers have the requirements of a good primer, but there are still cross dimers in the primers which are considered tolerable. The delta G value determines the stability of the secondary structure, which can prevent the primer from attaching to the DNA template (Sasmito et al. 2014). For self-dimers, the maximum delta G value is -6 kcal/mol, cross dimers value is -5 kcal/mol, and hairpin is -3 kcal/mol.

Table 1. Data in silico *Va_Chr1_FR* primers

Primer	Sequence	Tm (°C)	%GC	Self-Dimer (kcal/mol)	Hairpin (kcal/mol)	Cross Dimer (kcal/mol)	Amplicon Length	Primer Length
Va_Chr1_F	TTCTTCTGTT-GTAGGTTCCG	52.77	45%	-	-	-3.3	146 bp	20 bp
Va_Chr_R	CCAGCCCTCA CATCTAATAC	52.08	50%	-	-			

Bacterial Culture of *Vibrio alginolyticus* ATCC 17749

In Figure 2, *Vibrio alginolyticus* bacterial colonies formed on TCBS agar medium were large yellow colonies after being incubated at 37°C for 24 hours. The bacteria culture process used the streak plate method. TCBS media contains sucrose, dipeptone, sodium citrate, sodium thiosulfate, NaCl, oxgall, sodium cholate, ferric citrate, bromothymol blue, agar, and yeast extract. The colonies formed were yellow due to acidification of the media resulting from the fermentation of sucrose by *Vibrio alginolyticus* which made bromotimol blue turn yellow (Agars et al. 1982).

Figure 2. *Vibrio alginolyticus* on Thiosulphate Citrate Bile Sucrose Agar (TCBS Agar)

One colony of *Vibrio alginolyticus* that grew on TCBS agar medium was then taken using a sterile ose and immersed in TSB+2.5% NaCl media. The turbidity indicates that the bacteria are flourishing. This turbidity is measured from the resulting OD600 value, which is 1.517 after being incubated at 37°C for 18 hours. One colony of *Vibrio alginolyticus* grown on TCBS agar media was then transferred to TSB+2.5% NaCl media using a sterile ose. The turbidity indicates that the bacteria are flourishing. This turbidity is determined by the resulting OD600 value, which is 1.517 after an 18-hour incubation at 37°C. The value of OD600 or Optical Density 600 is a way to measure the concentration of pure cultured bacteria in liquid media using a UV/Vis Spectrophotometer with a wavelength of 600 nm. The acceptable range of OD600 values in a spectrophotometer is 0.1-1.8 (Rahayu et al. 2018).

Analysis DNA Sample

The process of isolating *Vibrio alginolyticus* bacterial DNA used the GRS Genomic DNA Kit – Bacteria. The process of DNA isolation involves three main principles: destruction (lysis), extraction or DNA separation, and DNA purification (Dolphin 2014). The results of the *Vibrio alginolyticus* DNA isolates that had been obtained were then tested qualitatively and quantitatively.

In quantitative testing, the concentration of the resulting DNA isolates was 174.5 ng/µL with a purity of A_{260}/A_{280} was 1.94. The purity of DNA samples is measured by a comparison of two wavelengths between A_{260} and A_{280} because DNA can absorb UV light at a wavelength of 260 nm while the amino acid sequence of a protein absorbs UV light at a wavelength of 280 nm (Fatchiyah 2011). The resulting DNA purity has good purity. This is in accordance with the literature which explains that a good range of DNA purity is ≥1.8 to 2.0 (Boesenberg-smith et al. 2012). A DNA purity value above 2.0 indicates that the DNA sample still contains contaminants in the form of RNA, while a DNA purity value below 1.8 indicates that there are still contaminants in the form of protein compounds (Kusumaningrum et al. 2023).

In qualitative testing, 0.7% agarose gel electrophoresis was used to determine the length of the isolate. The appearance of DNA bands from electrophoresis is determined by the percentage of agarose gel used in electrophoresis (Luh et al. 2014). In Figure 3, a band appears above the ladder, this shows that the results of the DNA isolate of *Vibrio alginolyticus* are more than 10,000 bp and have been declared successful, because it corresponds to the size of the whole genome of *Vibrio alginolyticus* ATCC 17749, which is 3,334,467 bp. In lanes 2 and 3 the resulting band is brighter than in lanes 4 and 5. This is because the isolate used in lanes 2 and 3 is the first elution so more DNA is eluted than that in lanes 4 and 5 which are the second elution.

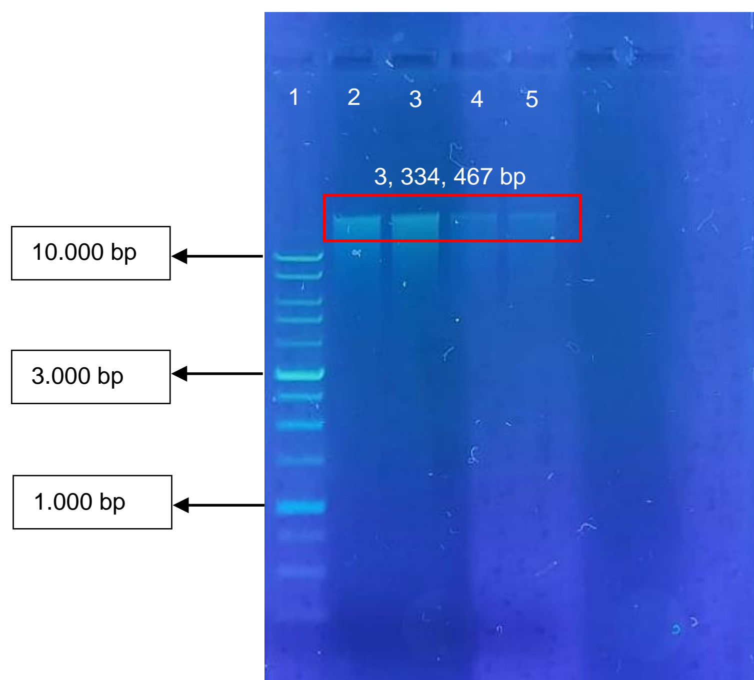

Figure 3. Characterization of *Vibrio alginolyticus* DNA isolates with agarose gel electrophoresis. (1) DNA Ladder 1 Kb; (2-3) first elution of *Vibrio alginolyticus* DNA; (4-5) second elution of *Vibrio alginolyticus* DNA.

Optimization Annealing Temperature of *Va_Chr1_FR* Primers

The temperature range used in the optimization of the annealing temperature is 53°C - 62°C using Gradient PCR. This temperature range is used because it is derived from ± 5°C melting temperature (Tm) of primers *Vibrio alginolyticus* (Balacs 1997).

Based on Figure 4, all temperatures produced a specific band at 146 bp, indicating that the *Va_Chr1_FR* primers were successfully amplified. At a temperature of 60°C a bright band is produced; therefore, this temperature is considered the optimal annealing temperature. There is still non-specific amplification at temperatures ranging from 53°C to 59°C, so this is not considered the optimal annealing temperature. The positive control used was *fimC Salmonella typhi* with a size of 95 bp and the annealing temperature is 60°C (Nurjayadi et al. 2019).

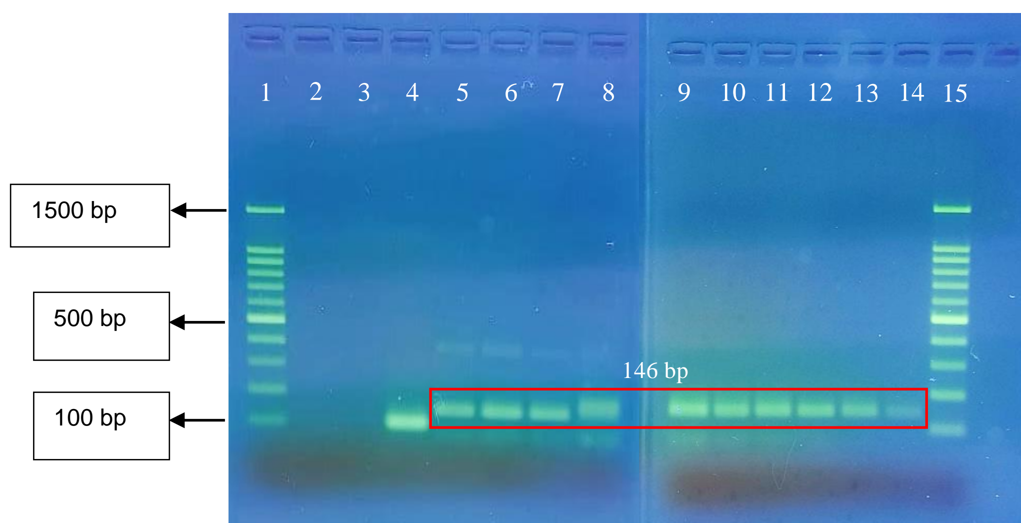

Figure 4. *Va_Chr1_FR* primers annealing temperature optimization results. (1) DNA Ladder 100 bp; (2) NTC; (3) Negative control; (4) Positive control *fimC Salmonella typhi* 95 bp; (5) DNA fragment at 53ºC; (6) DNA fragment at 54ºC; (7) DNA fragment at 55ºC; (8) DNA fragment at 56ºC; (9) DNA fragment at 57ºC; (10) DNA fragment at 58ºC; (11) DNA fragment at 59ºC; (12) DNA fragment at 60ºC; (13) DNA fragment at 61ºC; (14) DNA fragment at 62ºC; (15) DNA Ladder 100bp.

CONCLUSION

Based on this research, it can be concluded that the Polymerase Chain Reaction (PCR) method has been successful in optimizing the annealing temperature of *Vibrio alginolyticus Va_Chr1_FR* primers at 60°C. The results of the primers design of the *Va_Chr1_FR* primers that have been designed are also confirmed to be able to amplify the DNA fragment of *Vibrio alginolyticus* ATCC 17749 with an amplicon length of 146 bp.

ACKNOWLEDGEMENT

This research is funded by KEMENDIKBUD RISTEK by the scheme of PDUPT under the contract agreement number 1/PG.02.00.PT/LPPM/V/2022. We would like to acknowledge PT Sinergi Indomitra Pratama which has facilitated the instrument on this research, Forensic Laboratory Centre of Indonesian National Police (PusLabFor), Sentul Bogor Indonesia, which has facilitated this research under the UNJ-Puslabfor MOU; Kimia Farma Tbk. and Salmonella UNJ team under Center of Excellence Pathogenic Bacteria Detection (PUI Pendeteksi Bakteri Patogen) LPPM UNJ under the contract agreement number 446.2/UN39/KH.02/2022 for contributing to this research. Our gratitude is also delivered to our international partner from IBD-UTM who has helped all collaboration.

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