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hpmA Gene as a Detection Method of Proteus mirabilis Bacteria using real-time Polymerase Chain Reaction

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Abstract

Proteus mirabilis is a pathogenic bacterium that can cause gastrointestinal infections, bacteremia, and Urinary Tract Infections (UTI). Therefore, it is necessary to have a fast, sensitive, specific, and accurate detection method to identify Proteus mirabilis. This study aims to determine the confirmation, specificity, and sensitivity of the hpmA gene primer to detect Proteus mirabilis swiftly and precisely using the real-time Polymerase Chain Reaction method. Gradient Polymerase Chain Reaction results showed the hpmA primer has an amplicon length of 195 bp and an optimum annealing temperature at 60°C. The primer pair produced a Ct value of 10.40±0.18 and showed one peak in the melting curve with a Tm value of 81.84°C±0.02 by real-time PCR. In addition, the hpmA primer can distinguish target and non-target bacteria based on the variation in Ct and Tm values formed. Based on these results, the concentration of bacterial DNA detected by primers reached 3.2 pg/µL, equivalent to the concentration of target bacteria that can be detected by primers, which is 10.24×102 CFU. In the next step, the hmpA primer will be developed to detect Proteus mirabilis in artificially contaminated samples using real-time PCR

Introduction

Food is the most fundamental necessity for human life. Every individual requires food as a source of energy and various essential nutrients for the body. Quality food is characterized by high nutritional value, cleanliness, and the absence of harmful substances. However, insufficient attention to proper food handling factors can lead to contamination with pathogenic microorganisms, potentially resulting in foodborne infections. The World Health Organization (WHO) estimates that more than 600 million people get sick and 420,000 people die annually, making foodborne pathogens a serious public health issue globally (Elbehiry *et al*., 2023). The first causative agent of foodborne pathogens is pathogenic bacteria, which are caused by bacteria in the digestive tract of food consumed by humans or the ingestion of large numbers of microbes, which then live and multiply in the body (Foddai & Grant, 2020). One of the pathogenic bacteria is *Proteus mirabilis*, a gram-negative, rod-shaped, and facultative anaerobic (I Nyoman & Ni luh,

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Proteus mirabilis is a pathogenic bacterium that can cause gastrointestinal infections, bacteremia, and Urinary Tract Infections (UTI) (Schaffer, J. N. & Pearson, M., 2015). This bacterium can be found in contaminated meats, vegetables, and seafood (Ronanki *et al*., 2022). *Proteus mirabilis* produces hemolysins, one of which is the *hpmA* hemolysin. *hpmA* hemolysin can cause cytotoxicity that allows bacteria to enter the kidney (Lazm *et al*., 2019). In recent years, cases of food poisoning caused by *Proteus mirabilis* bacteria have been reported in China. In a case in Zhejiang Province in 1998, 256 students at a middle school were diagnosed with a foodborne infection due to *Proteus mirabilis*. A similar case in Guangxi Province in 2006 resulted in 34 people getting sick, and in Beijing in 2013 resulted in 4 people being identified with a foodborne pathogen due to *Proteus mirabilis*. According to the Datong Food and Drug Inspection and Testing Center, foodborne pathogen incidents associated with *Proteus mirabilis* accounted for 3.61% of reported foodborne pathogen incidents in Datong (Shanxi Province, China) from 2016 to 2017 (Gong *et al*., 2019). An optimal approach to bacterial detection is needed to treat this instance of a foodborne pathogen.

Research and development have been carried out on the detection of pathogenic bacteria. One of the methods is Polymerase Chain Reaction (PCR). The ideal detection method needs to qualify the main terms, which are sensitivity, specificity, fast, simple operation, and cost-effective (Priyanka *et al*., 2015). Real-time PCR (rt-PCR) is an ideal method used for identifying and measuring various microbial agents in clinical diagnostics and food safety (Kralik & Ricchi, 2017). In previous research, the detection of *Proteus mirabilis* bacteria has been completed using Multiplex PCR. But the method used is not fast and sensitive (Priyanka *et al*., 2015; Wojno *et al*., 2020). Therefore, this study aims to determine the confirmation, specificity, and sensitivity test of the *hpmA* gene primer to detect *Proteus mirabilis* swiftly and precisely using the real-time Polymerase Chain Reaction method (rt-PCR).

Methods

Primers in *Proteus mirabilis* ATCC 7002 bacteria were identified using an in-silico primer utilizing the National Center for Biotechnology Information (NCBI) website and the Primer-BLAST (Primer-Basic Local Alignment Search Tool) program. Furthermore, the selected primers will be re-analyzed using the OligoAnalyzer and NetPrimer programs to identify a secondary structure. The Macrogen Synthesis, Inc.-Korea commercial facility synthesized the designed primers. The *Proteus mirabilis* culture, designed in a kwik-stick format, was resuspended using a hydration solution and inoculated into Luria Bertani Broth (LB) media (Merck). The inoculated culture was then incubated with aeration using an Orbital Shaker-Incubator (YIHDER LM-400) set at 150 rpm and 37°C for 18-24 hours. After the incubation period, the bacterial culture was examined; the formation of turbidity in the Luria Bertani Broth (LB) indicated successful bacterial growth. For further assessment, the Optical Density (OD) at a wavelength of 600 nm was measured using a UV-Vis spectrophotometer. Furthermore, the bacterial culture obtained was inoculated in *McConkey Agar* (MCA) (Merck) at a dilution of 10^{-6} and 10^{-7} for 24 hours at 37°C.

The DNA isolation process of a pure culture of *Proteus mirabilis* bacteria was performed using the Viogene-Geno Plus Genomic. A microcentrifuge tube containing 3 mL of a pure cultivated bacteria inoculum was centrifuged (Sorvall™ Legend™ Mikro 17R Microcentrifuge) at 12,000 x g for five minutes, or until a pellet was formed. Furthermore, the Viogene-Geno Plus Genomic instructions were followed to complete the isolation procedure. After the separation of *Proteus mirabilis* bacterial DNA, nanodrop testing (Nanovue™ Plus Spectofotometer) and electrophoresis were conducted. Gradient PCR (Heal Force X960) was used to optimize the annealing temperature within the 54°C - 62°C temperature range. The test involved a 25 µL reaction mixture containing NZYTaq II 2x Green Master Mix, *Proteus mirabilis* DNA isolate, forward and reverse *hpmA* primers, and Nuclease Free Water (NFW). The amplification process consisted of 35 cycles, beginning with

an initial denaturation process at 95°C for 100 seconds, followed by denaturation at 95°C for 30 seconds, annealing at temperatures ranging from 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. The results of the optimization of annealing temperature will be seen using electrophoresis.

Confirmation test of *hpmA* primer for the detection of *Proteus mirabilis* bacteria using the real-time PCR method, with a concentration of target bacteria of 50 ng/µL. A 20 µL reaction mixture was prepared, including ExcelTaq 2X qPCR Master Mix, forward and reverse *hpmA* primers, a pure DNA isolate template of *Proteus mirabilis*, and Nuclease Free Water (NFW). The amplification of samples was made twice (duplo). Additionally, two negative controls consisting of Nuclease-Free Water with Master Mix (NFW+MM) and a Non-Template Control (NTC) were included in the test. The specificity test using Real-Time PCR is designed to assess the primers' ability to differentiate between *P. mirabilis* target bacteria and non-target bacteria. This test used non-target bacteria, like *Escherichia coli, Enterococcus faecalis, Shigella flexneri, Campylobacter jejuni, Enterobacter sakazakii, Pseudomonas aeruginosa, Salmonella typhi, Listeria monocytogenes, Klebsiella pneumonia,* and *Yersinia enterocolitica.* The reaction mixture was prepared in a volume of 20 µL each. The results obtained from the specificity test are represented by the Ct value and Tm value based on the amplification curve and the melting curve. The Sensitivity testing aims to determine the Limit of Detection (LoD) value of the *hpmA* gene primer pair. This test went through a series of seven dilutions of pure *Proteus mirabilis* DNA isolates. A 1 µL aliquot of pure *Proteus mirabilis* bacterial DNA was mixed with 9 µL of Nuclease Free Water (NFW) for each dilution step. The results obtained from the specificity test are represented by the concentration and Ct value based on the amplification curve and the standard curve.

Results and Discussion

Primer design for *Proteus mirabilis* ATCC 7002 bacteria targeting the *hpmA* gene fragment. This gene was selected based on its role as one of the hemolysins produced by *Proteus mirabilis* bacteria (Fox-Moon *et al*., 2015). The *hpmA* gene encodes the hpm hemolysin. The *hpmA* hemolysin is a cell-independent, calcium-independent, pore-forming protein. *hpmA* hemolysin can cause cytotoxicity that allows *Proteus mirabilis* bacteria to enter the kidney (Lazm *et al*., 2019). Primer design in the *hpmA* gene requires several parameters, such as amplicon length, primer length, dimers, % GC, melting temperature, and specificity, which have a significant impact because they are interrelated with the stability and sensitivity of primers to detect target bacteria (Bustin, *et al*., 2020), in silico results obtained that the *hpmA* gene is 4,733 base pairs (bp) in the 1,075,493 - 1,070,760 region. The designed primers had an amplicon length of 195 bp and were identified as F5'CATTACTGGGCACGCCAAAG-F3', measuring 20 bp, and R5'GCATTCCCTGCGGTAGTCTT-R3', measuring 20 bp.

Table 1. Sequence and Design of hpmA Primer Pairs

Primer	Sequences	Primer Length	Molecular Wt	TM (°C)	% GC	Self-Dimer (kcal/mol)	Hairpin (kcal/mol)	Cross dimer	Amplicon Length
hpmA-f	CATTACTGGG CACGCCAAAG	20 bp	6111,07	61,47	55	-6,02	-1,62	-3.94	195 bp
hpmA-r	AAGACTACCG CAGGGAATGC	20 bp	6075,03	59,29	55	-	-0,69		

The cultivation of *Proteus mirabilis* bacteria aims to obtain a single colony of bacteria. Luria Bertani Broth (LB) was applied to enrich the bacterial suspensions for test cultures. The results of bacterial growth on liquid medium are shown by the presence of turbidity. Using UV-VIS spectrophotometry, the optical density at a wavelength of 600 nm (OD600) was measured to show the turbidity. In bacterial analysis, 600 nm is generally used, indicating that OD correlates directly with cell concentration (McBirney *et al.,* 2016). After 18 hours of bacterial growth, the optical density value was 2,270. After that, the bacterial culture obtained was inoculated in McConkey Agar (MCA). MCA is a selective medium that contains bile salts (to inhibit gram-positive bacteria), neutral red dye (as a pH indicator to determine the presence of lactose fermentation), lactose, and peptone (Artauli *et al*., 2023). The results of *P. mirabilis* produced colourless, round, single colonies after 18 hours of incubation.

In this study, DNA extraction uses *Viogene – Geno Plus Genomic DNA Extraction Miniprep System Kit*, which consists of *Lysis solution, Proteinase K, RNAse, Wash Buffer I, Wash Buffer II,* and *Elution Buffer*. The results of the bacterial DNA isolation test are pure bacterial DNA isolates, which will be tested qualitatively and quantitatively. A qualitative test using agarose gel electrophoresis, which aims to confirm that the isolation of bacterial genomic DNA is successful, is characterized by a band that matches the size of the *P. mirabilis* bacterial genome. Electrophoresis is a method of separating proteins and nucleic acids based on their size, as early detection of an infection (Wardoyo & Badri, 2020). Electrophoresis results show that the results of *P. mirabilis* bacterial DNA isolates are more than 10,000 bp, and there is a match with the size of the whole genome of *P. mirabilis* ATCC 7002 of 3,999,612 bp based on the results of in-silico analysis so that the results of this electrophoresis can be confirmed that there is a genomic DNA isolate from the isolation of *P. mirabilis* bacterial DNA.

Furthermore, quantitative tests using a nanodrop spectrophotometer aim to determine the purity and concentration of bacterial isolates. DNA purity can be measured by the A260/A280 ratio, where the wavelength of 260 nm is the maximum wavelength of DNA that can absorb UV light, as the wavelength of 280 nm is the maximum wavelength of contaminants such as proteins and phenols that will absorb UV light (Dewanta & Mushlih, 2021). The measurement results of *P. mirabilis* isolates obtained a concentration of 160 ng/µL and a purity of 1.80. Based on the measurement, good DNA purity were obtained in the range of 1.8 - 2.0. If the purity is above 1.80, it indicates RNA contamination, and a purity below 1.80 indicates contamination with protein (Lucena-Aguilar *et al*., 2016). Based on the concentration and purity, the DNA isolation was carried out successfully and purely.

The primer annealing temperature optimization aims to determine the optimal temperature of the primer from the annealing process in real-time PCR. Hydrogen bonds are formed between the primer and the template at this stage, which can only occur if the primer and template sequences are perfectly complementary (Barnes & Lewis., 2016). In this study, the temperature range used for annealing temperature optimization was 54-62°C using gradient PCR. When the annealing temperature used is too high, the annealing process becomes suboptimal and results in low amplification products. Conversely, if the temperature is too low, non-specific amplification will occur (Ehtisham *et al*., 2016). The temperature range used is ±5°C, the melting temperature of *hpmA* primer from the results of in silico analysis (Rodríguez *et al.,* 2019). The results of the *hpmA* primer annealing temperature optimization are shown in **Figure 1**. Based on the result, the primer was able to amplify the *hpmA* gene fragment at all temperatures. This is indicated by the presence of a band at a length of 195 bp at 54-62°C; thus, this size is consistent with the in-silico size of the *hpmA* primer. At a temperature of 60°C, it is considered the optimal annealing temperature because 60°C is the standard or default temperature of the real-time PCR machine. Choosing the highest temperature within the temperature range is also to avoid the formation of non-specific amplification and the formation of dimers. It is because the higher the annealing temperature, the greater the specificity of the primer. The positive

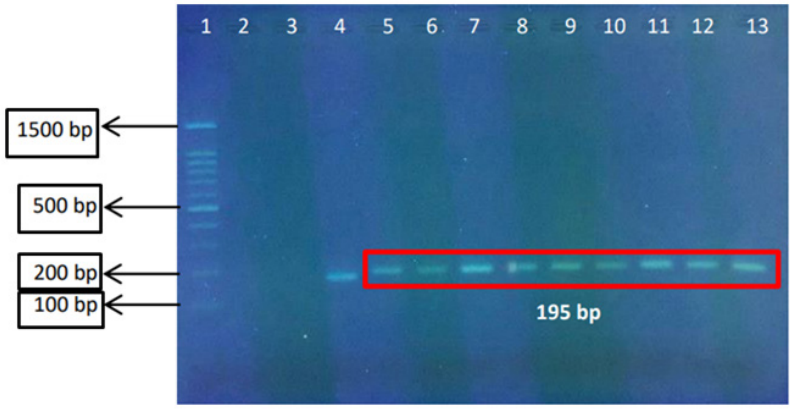

Figure 1. hpmA gene Annealing Temperature Optimization Results. (1) DNA Ladder 100 bp; (2) NTC; (3) NFW + MM (Negative control); (4) Positif control codY Bacillus subtilis 175 bp; (5) 54°C; (6) 55°C; (7) 56°C; (8) 57°C; (9) 58°C; (10) 59°C; (11) 60°C; (12) 61°C; (13) 62°C

control used is *codY Bacillus subtilis* with a size of 175 bp. There are two negative controls used, which are the NTC (*Non-Template Control*) and NFW (*Nuclease Free Water*) + MM (*Master Mix*). NTC aims to determine the presence of dimers in the primer, and NFW + MM aims to determine the absence of impurities in the PCR process.

A method for detecting and measuring different microbial agents for clinical diagnostics and food safety is real-time PCR (Kralik & Ricchi, 2017). Using the real-time PCR. The primer confirmation test using real-time PCR aims to determine the ability of primers designed to recognize and amplify target bacteria. The *hpmA* gene fragment of *P. mirabilis* was amplified using the BMS MicqPCR Cycler instrument as the target. In this study, the annealing temperature was 60°C based on the optimum results in the previous test. The confirmation test used 40 cycles, each cycle divided into three phases. They were denaturation, annealing, and extension. In the denaturation stage, the DNA is denatured at a high temperature between 92 and 98°C, causing the double-stranded DNA to separate into single-stranded DNA. Then, a temperature between 55 and 65°C allows the primers to bind to the complementary DNA template. Lastly, DNA polymerase enzymes bind to the annealed primers and synthesize a complementary DNA strand at a temperature range of 60°C to 78°C (polymerase extension) (Wu *et al.,* 2020). The results obtained in the confirmation test using RT-PCR are data in the form of an amplification curve and melting curve. The cycle threshold (Ct) value of the PCR process is examined to read the amplification curve in the test results. The cycle threshold (Ct) refers to the number of amplification cycles required for fluorescence to exceed the threshold. A lower Ct value indicates a higher amount of nucleic acid in the sample. A higher Ct value suggests a lower amount of nucleic acid, as the Ct value is inversely related to the number of nucleic acid targets present in the sample (Health Ontario, 2020). The results of the confirmation test in this study are shown in **Figure 2**.

According to the amplification curve results, the primer was able to detect the presence of *Proteus mirabilis* target bacteria with a concentration of 50 ng/µL. The primers used were *hpmA Forward* (*hpmA-f*) and *hpmA Reverse* (*hpmA-r*) primer pairs, with a concentration of 2 pmol. The appearance of Ct at cycles 10.23 and 10.58 (duplo) indicates the ability of the primer to detect target DNA in the sample. It shows that the primer successfully amplified the *hpmA* fragment of *Proteus mirabilis*. Based on the results of the positive control using *Listeria monocytogenes*, the *hly* Ct gene appeared at cycle 14.12, indicating that the reaction went well. In addition, the negative control is indicated by the appearance of NTC at cycle 28,15, which shows the formation of dimers and not contamination. It is confirmed

by the non-appearance of the NFW + MM sigmoid curve in the reaction results. The Ct value of a good NTC is 35-40 cycles. If the difference between the sample and the NTC is higher than 10 cycles, then the NTC can be tolerated (Nurjayadi *et al.* 2018). Based on the melting curve, the *P. mirabilis* sample showed one single peak, indicating the absence of non-specific amplification with TM values of 81.82°C and 81.86°C. However, if the sample produces more than one peak and several amplicons, it indicates that the designed primer is considered non-specific to the target DNA fragment or target gene (Nurjayadi *et al.*, 2018). The Tm value of the NTC was 81.16°C, indicating a difference in peaks compared to the sample attributed to GC composition factors present in both amplicons (Dorak, 2007). Furthermore, the Tm value of the positive control using *hly Listeria monocytogenes* shows a consistent value, in accordance with previous research, which is 81.67°C. Based on the temperature difference formed, it indicates that the product formed on the NTC is not the target product. So, based on the melting curve, a specific temperature is obtained to confirm the presence of *Proteus mirabilis*.

The specificity test with Real-Time

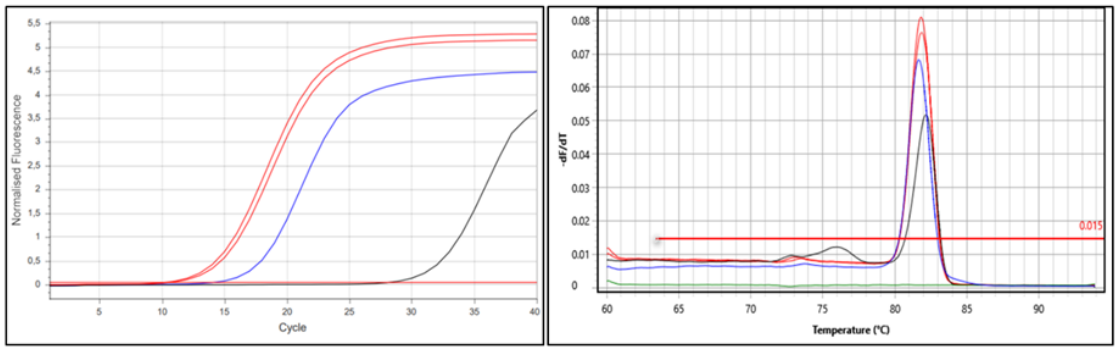

Figure 2. Amplification Curve and Melting Curve of Primer Confirmation Test. Amplification Curve (Left); Melting Curve (Right). Proteus mirabilis 2 pmol (duplo) (Red); Non-Template Control (NTC) (Black); Control Positif (L. monocytogenes) (Blue).

Table 2. Ct and Tm Value of Specificity Test

Sample	Ct	Tm (°C)
Proteus mirabilis 2 pmol (1)	11.49	82.05
Proteus mirabilis 2 pmol (2)	11.11	82.08
NTC	29.81	82.48
Control positive (*L. monocytogenes*)	17.34	81.30
NFW + MM	-	-
E. coli	27.53	82.59
C. jejuni	29.44	77.35; 83.36
C. sakazakii	28.58	82.53
E. faecalis	28.53	77.41; 83.35
K. pneumonia	28.09	83.81
L. monocytogenes	28.89	76.54; 82.49
P. aeruginosa	27.79	82.86
S. flexneri	29.04	76.54; 82.49
S. typhi	27.07	64.07; 73.80; 78.95
Y. enterolitica	27.76	82.50

PCR aims to determine the ability of primers to distinguish non-target bacteria from *P. mirabilis* target bacteria. This test is represented by an amplification curve, where the Ct value is obtained, and a melting curve, where the melting temperature (Tm) is obtained in **Table 2**. Based on the Ct values, the bacteria target *P. mirabilis* appeared at Ct at cycle 11.49 and 11.11 (duplo). Meanwhile, non-bacteria had Ct around cycle 27-29. Furthermore, the control positive was found at Ct cycle 17.34, and the control negative was found at Ct cycle 28.15. The difference in Ct between the target bacteria and the 10 non-target bacteria is 16 to 18 cycles. It can be ignored and taken as negative since the non-target bacteria and target bacteria had a Ct of more than 10 cycles (Dorak, 2007). Meanwhile, according to the Tm value, each non-target bacteria has a different Tm than the target bacteria. In non-target bacteria, the Tm value shifts, indicating the amplicon difference between the target and non-target bacteria. Furthermore, more than one Tm in non-target bacteria indicates the presence of non-specific amplification. According to the Ct value and Tm value, the *hpmA* primer can distinguish between target bacteria and 10 non-target bacteria.

The Sensitivity testing aims to determine the Limit of Detection (LoD) value of the *hpmA* gene primer pair. The initial concentration obtained from the isolation of pure DNA is 160 ng/µL. Based on the results, the smaller the concentration, the larger the resulting Ct. Because the smaller the concentration, the more cycles are needed, so the fluorescein formed passes the threshold, resulting in a larger Ct value. Therefore, there is an inverse proportional relationship between Ct value and concentration. Sensitivity test for *hpmA* primers is shown by a standard curve (**Table 3**) with equation y = -3.359 x + 16,89. The optimal slope for the standard curve is -3.32, which corresponds to 100% RT-qPCR efficiency. However, slopes ranging from -3.1 (indicating 110% efficiency) to -3.58 (indicating 90% efficiency) are also considered acceptable (Bivins, 2021). The efficiency result is 0.985, with amplification efficiencies ranging from 90% - 110% being accepted. The coefficient of determination (R^2) shows the linearity of the standard curve. The R^2 is 0.9957, showing a strong linear fit with R^2 values in the range of 0.980 to 1.00 (Bivins, 2021). PCR efficiency can be determined from the slope, with an efficiency of 0.985. Based on the amplification curve (**Table 3**), the concentration of bacterial DNA detected by primers reached 3.2 pg/µL at Ct 24.17, equivalent to the concentration of target bacteria detected by primers, which is 10.24×10^2 CFU. Based on the sensitivity test, the real-time PCR method can detect negligible sample DNA and amplify DNA specifically and accurately (Marbawati & Pramestuti, 2017).

Table 3. Concentration and Ct Value of Sensitivity Test

Sample	Concentration (ng/µL)	Ct
D0	50	11.13
D1	10	15.91
D2	2	13.27
D3	0.4	18.31
D4	0.08	21.02
D5	0.016	23.20
D6	0.0032	24.71
NTC	-	25.96
Equation y	-3.359 x + 16.89	
Efficiency	0.985	
R^2	0.9957	

Conclusion

The Rapid detection of *Proteus mirabilis* bacteria using the real-time PCR method has been successfully performed using the *hpmA* gene with an amplicon length of 195 bp at an annealing temperature of 60°C. The *hpmA* gene showed the ability to detect *Proteus mirabilis* with a Ct of 10.40±0.18 and Tm of 81.84°C±0.02. Based on the difference in Ct and Tm value, the hpmA primer was also successful in distinguishing between target and non-target bacteria. The Limit of detection for the *hpmA* primer was determined to be 3.2 pg/μL, which is equivalent to 10.24×10^2 CFU.

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