The Potential of *htrA* Gene to Identify *Enterococcus faecalis* using real-time Polymerase Chain Reaction

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Abstract. *Enterococcus faecalis* is known as the opportunistic pathogens that can cause a variety of diseases. These bacteria are capable of producing enterotoxins that lead to gastrointestinal symptoms. This study aims to develop a real-time Polymerase Chain Reaction (PCR) method that is more efficient for identifying

T. A. Aziz et al. (eds.), *Proceedings of the 4th Science and Mathematics International Conference (SMIC 2024)*, Advances in Physics Research 11,
https://doi.org/10.2991/978-94-6463-624-6_12

Enterococcus faecalis by assessing the results of the confirmation and specificity test of the *htrA* primer. The concentration of isolated DNA is 107 nanograms per-microliter, and the purities of A260/280 are 2.02. *htrA* primer successfully amplified *Enterococcus faecalis* DNA fragments at an annealing temperature of 60 degrees Celsius with an amplicon length of 162 base pairs. These pairs of primers amplified target sequences at a Ct value of 17.62 ± 0.11 and a Tm value of 81.21 degrees Celsius ± 0.06. Specificity tests showed that the *htrA* primer could distinguish between target and several non-target bacteria. Based on these results, it can be concluded that the *Enterococcus faecalis* can be efficiently identified by these primers utilizing the real-time PCR. In order to create sensitive and specific detection methods, the next procedure will involve generating *htrA* primers to identify *Enterococcus faecalis* in sensitivity testing and artificially contaminated samples..

Keywords: *Enterococcus faecalis, htrA Gene, Pathogen,* Detection Method, Real-Time PCR, Bacteria.

1 Introduction

Enterococci are Gram-positive and facultatively anaerobic lactic acid bacteria that normally dwell in the gut flora of humans and numerous animals [1], [2]. Although most of these bacteria are thought to be innocuous to humans, two specific species—*Enterococcus faecalis* and *Enterococcus faecium*—have become important opportunistic pathogens in recent decades [3]. As opportunistic pathogens, enterococci can lead to infections such as bacteraemia, meningitis, and endocarditis [4], [5]. Additionally, enterococci are known to be involved in food contamination [6]. Enterococci are highly resilient organisms, capable of withstanding various unfavourable conditions [7]. The They can grow at pH values between 4.4 and 9.6 [8], temperatures between 10 °C and 45 °C, and in settings with high concentrations of NaCl [9], allowing them to flourish in a wide range of harsh and demanding conditions.

Enterococcus faecalis is a major cause of nosocomial infections, with infection rates 10 times higher compared to other Enterococcus species. [10], [11]. It is the third most frequently isolated pathogen in hospitals, accounting for up to 14% of nosocomial infections in US and Europe [12], [13], [14]. One of the virulence factors of *Enterococcus faecalis i*s serine protease [15]. Virulence factors are molecules that increase a microorganism's ability to cause disease [8]. *HtrA* gene is a serine protease induced by heat shock, has been identified in various bacteria [16], [17]. In *E. faecalis*, the *htrA* is essential for maintaining protein homeostasis during stressful conditions [18]. The ability of a system to control and preserve a stable internal environment is known as homeostasis [19]. This function is critical for the bacterium's survival and its ability to cause infection [18].

Enterococcus faecalis infections can be promptly, sensitively, and precisely detected by real-time Polymerase Chain Reaction (PCR) [20]. This method's capacity to track DNA amplification in real-time makes it more sensitive and accurate than traditional culture methods [21]. This method's capacity to identify harmful bacteria may help to

stop the spread of diseases. The purpose of this research is to evaluate the htrA gene primer's capacity for fast, accurate, and specific *Enterococcus faecalis* detection

2 Material and Methods

2.1 Primer Design

In order to determine specific primers for *Enterococcus faecalis* ATCC 19433, the National Centre for Biotechnology Information (NCBI) website and the Primer-BLAST (Primer-Basic Local Alignment Search Tool) application were utilized in the primer design process. In order to determine the secondary structure, the selected primers will be analysed again using the NetPrimer and OligoAnalyzer software. The designed primers were synthesized in Macrogen Synthesis, Inc.'s commercial facility in Korea.

2.2 Bacteria Preparation of *Enterococcus faecalis*

The process of bacterial rejuvenation was started by cultivating *Enterococcus faecalis* bacteria, which were supplied as kwik-sticks and then resuspended in a hydrating solution. After transferring *Enterococcus faecalis* ATCC 19433 from glycerol stock into 10 mL of Luria-Bertani (LB) Broth (Marck) using a sterile loop, the mixture was incubated in an orbital shaker incubator at 37°C for 18–24 hours at 150 rpm. Bacteria growth was monitored by observing turbidity formation using Optical Density (OD) at a wavelength of 600 nm. The bacterial suspension was subsequently analysed using UV/VIS Spectrophotometry (SHIMADZU UVMINI-1240). Next, *Enterococcus faecalis* shape was observed by growing bacteria on blood agar plates using the spread plate method. Next, the plates were placed in a Shaker-Incubator (YIHDER LM-400) and left for eighteen hours to watch the growth of bacteria.

2.3 DNA Isolation

To create a pellet, a 3 mL sample of pure *Enterococcus faecalis* culture was put in a microcentrifuge tube and centrifuged at 12,000 x g for five minutes. The Viogene kit was used to extract the DNA and Nanodrop spectrophotometer (a NanoVue Plus Spectrophotometer) was used to determine the DNA's concentration and purity. The DNA was characterized using 0.7% agarose gel electrophoresis, and then UV transilluminator was used to visualize the results. The isolated DNA was kept in storage at -20°C.

2.4 Optimization of Annealing Temperature of *htrA* Primer Pairs

The annealing temperature optimization was performed to determine the optimal temperature for amplifying the *htrA* gene using Gradient PCR across a range of 53°C to 62°C. The PCR reaction mixtures contained *Enterococcus faecalis* DNA template, htrA forward and reverse primers, and Nuclease-Free Water. Thirty-five cycles were used in the DNA amplification process: 100 seconds of initial denaturation at 95°C, 30 seconds

of further denaturation at 95°C, 30 seconds of annealing at the tested temperature range, 60 seconds of extension at 72°C, and 10 minutes of extension at 72°C. To analyze the PCR products, electrophoresis was performed on a 2% agarose gel, using a 1x TAE buffer and GreenSafe (Florovue) for visualization under UV light. Then, the Mupid-exU Submarine Electrophoresis was carried out for an hour at 70 V and 400 mA and the size of the product was then determined by comparing the amplified DNA bands to a DNA marker.

2.5 Specificity and Sensitivity Assay

The specificity of the *htrA*-f and *htrA*-r primer pairs was tested against non-target bacteria, including *Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Cronobacter sakazakii, Salmonella typhi, Yersinia enterocolitica, Shigella flexneri, Campylobacter jejuni, Listeria monocytogenes,* and *Escherichia coli*. The volume of the reaction mixture for the specificity test was set to 20 µL using real-time PCR (Mic qPCR Cycler from Bio Molecular Systems). The reaction mixture was prepared with 10 microliters of ExelTaq 2x qPCR Master Mix, 5 microliters of E. faecalis template DNA or non-target bacterial DNA, and 5 microliters of *htrA* primer. The specificity test results are visualized by melting and amplification curves.

3 Result and Discussion

3.1 Primer Design

The bacteria used in designing the primers was *Enterococcus faecalis* ATCC 19433. The *htrA* (High-temperature requirement A) gene encodes a serine protease that is activated in response to heat shock [16], [17] and plays a crucial role in maintaining protein homeostasis under stressful conditions [18]. The primer obtained resulted in an amplicon length of 162 bp shown in Fig. 1. Primer design is a crucial first step in determining the success of DNA amplification through the PCR method [22]. The length of the primer, the percentage of GC, the melting temperature (Tm), the difference in Tm between primer pairs, and the lack of dimers and hairpins are qualities for ideal primers [23]. The primer parameters for *htrA* are listed in Table 1, with this primer pair producing an amplicon of 162 bp. Since shorter amplicon lengths are essential for PCR amplification efficiency, particularly in cases when RNA quality is low, the primers that were chosen produced amplicons that fell within the usual range of 150–250 bp [24]. Furthermore, during gel electrophoresis, it may be challenging to discern particular PCR products from primer dimers when amplicon sizes are less than 100 bp.

ATGCAACGAAAAGATGTTACACCTAATTCAGATAAAAAAAGTTTATTGCAAAAATTTGGGATTGGTTTAGCTGGCGGCTTACTTGGTG
GCGCCCTTATTCTAGGAGGCGCTTATTCTGGGATTATTCCCACACCCAACGGCGGAAATAATGCGGCGACGACGACATCCACTAATCA
CGGCGACACAAAAGTCAGCAATGTAAGTTACAATGTGTCTAGCGATGTCACAAAAGCCGTGAAAAAAGTTCAAAATTCTGTGGTTTCT
GTCATTAATATGCAAAGTGCTAGCAACAATTCTTCGGCAGATGATCCTTTTGGGGGATTGTTCGGTGGGAATGAAGGTACTCAAGACT
CTTCCGGCAACAATGGTAACGATTTAGAAGCAGCCAGTGAAGGTAGTGGGGTTATTTATAAGAAAGATGGTAAAACGGCTTATGTCGT
GACAAATAACCACGTCGTAGATAAAGCGCAAGGATTGGAAGTTGTTTTATCTGATGGTACCAAAGTGAAAGGCGAATTAGTCGGAACC
GATGCTTACACGGATTTAGCCGTGATTAAAATTTCTTCCGACAAAGTTGATCAAGTCGCTGAGTTTGGAAATTCTAGTAAAATCACAG
TCGGTGAGCCTGCTATTGCAATTGGTTCCCCTCTAGGCTCTGATTATGCTAACTCTGTCACACAAGGAATCATCTCTTCTGTGAATAG
AAATATTACCAATAAAAACGAGTCTGGTGAAACCATCAATATTAATGCCATTCAAACCGATGCTGCCATCAATCCAGGAAACTCTGGT
GGTCCACTAATCAATATTCAAGGACAAGTCATTGGAATTAACTCAGTAAAAATTGTGCAATCAACTAGTCAAGTGAGCGTTGAAGGGA
TGGGCTTTGCCATTCCAAGTAATGACGTAGTCAACATTATCAACCAATTAGAAAAAGATGGTAAAGTGACGCGGCCAGCTCTCGGGAT
TACGATGTCTGATTTAACAGGTATCTCTTCACAACAACAAGAACAAATTTTAAAAATTCCAGCTTCTGTAAAAACTGGCGTAGTGGTT
CGTGGTGTTGAAGCAGCGACCCCTGCTGAAAAGGCTGGATTGGAAAAATACGATGTTATCACGAAAGTTGACGGCCAAGACGTAAGCT
CTACTACAGATTTACAAAGCGCGCTTTACAAGAAAAAAGTTGGCGACAAAATGGAAGTGACTTATTATCGTGGTTCTAAAGAAATGAA
AGCAACTATCGATTTAACCATTGATAAATCAGCTTTAACACAACAAAATAATCGCAGCAATCAATAA

Fig. 1. In Silico Genome Sequence of *htrA* gene *Enterococcus faecalis* ATCC 19433. Forward Primer (yellow); Reverse Primer (purple); Amplicon of *htrA*-f and *htrA*-r primer pair (blue).

Primer	Sequence (5'-3')	Primer Length	Tm (°C)	%GC	Self-Dimer (kcal/mol)	Hair-pin (kcal/mol)	Cross Dimer (kcal/mol)	Amplicon Length
htrA-f	CACGGCGACA CAAAAGTCAG	20 bp	60.04	55%	-3.92	-	-	162 bp
htrA-f	ATTCCCACCGA ACAATCCCC	20 bp	60.03	55%	-	-		

Table 1. Primer and Parameter of htrA Primer.

The primer length of *htrA* is 20 base pairs, which is still between the ideal primer range of 18–24 bp [25]. The %GC content, indicating the proportion of guanine and cytosine nucleotides, should fall between 40–60% [26]. Based on Table 1, the forward and reverse *htrA* primers have a GC content of 55%. Knowing the GC content is crucial because it indirectly influences the annealing process during PCR [27]. A high GC percentage can make primers increasing the likelihood of mis-priming at non-target sites. Furthermore, oligonucleotides with high %GC complicate denaturation during PCR, potentially reducing amplification efficiency [28]. The forward primer's Tm value for the *htrA* primer pair is 60.04°C, and the reverse primer's Tm value is 60.03°C. These values fall between the optimal 50-65°C range. It should not be more than 1-2°C for the Tm difference between the forward and reverse primers [26]. An ideal self-dimer formation has an internal ΔG of -6 kcal/mol and an external ΔG of around -5 kcal/mol, while a cross-dimer ΔG of no more than -5 kcal/mol is acceptable [29], [30]. The reverse primer does not form a self-dimer, and the forward primer has a self-dimer ΔG of -3.92 kcal/mol, which is still within an acceptable range. Furthermore, neither primer forms a cross-dimer. Based on the results, the *htrA* primer that was designed meets the criteria for a specific primer.

3.2 Bacterial Preparation and Cultivation of *Enterococcus faecalis*

Enterococcus faecalis was grown in Luria-Bertani (LB) broth for 18 hours in a shaker incubator with 150 rpm aeration at 37°C. Luria-Bertani broth is a commonly used medium for bacterial culture, containing tryptone, yeast extract, and sodium chloride (NaCl). After 18 hours of incubation, the culture became more turbid, indicating successful bacterial growth. The outcomes of the growth of the bacterial culture are indicated by turbidity in LB media, which was previously transparent yellow [31]. The formation of turbidity in the media indicates that the bacteria can flourish in the nutrient-rich Luria Bertani medium, which also features a shaking culture method that can aid increase the quantity of oxygen available for bacterial development [32]. Then, the cultured was verified through UV-VIS spectrophotometry at 600 nm, yielding an optical density (OD600). The OD600 value of bacterial growth for 18 hours is 1.542. Based on the Lambert-Beer law, OD is directly proportional to solution concentration, signifying light scattering as it passes through the bacterial culture.

Subsequently, when *Enterococcus faecalis* was cultured on sheep blood agar, it formed small, round, white colonies with a smooth texture, dispersed sparsely, indicating a lighter inoculation (Fig. 2). These colony characteristics are typical for *E. faecalis* [33]. There was no visible hemolytic activity around the colonies, consistent with *Enterococcus faecalis* typically displaying gamma hemolysis (γ-hemolysis) [34], where no red blood cells are lysed. This non-hemolytic behaviour was confirmed by the absence of a distinct zone surrounding the colonies [35]. Hemolytic strains (beta-hemolysis) were distinguished from non-hemolytic bacteria by the presence of clear zones resulting from blood cell lysis, while non-hemolytic strains either formed green zones surrounding the colonies (alpha-hemolysis) or had no effect on the blood plate (gamma-hemolysis) [36].

Fig. 2. *Enterococcus faecalis* ATCC 19433 Bacterial Cultured on Blood Agar

3.3 DNA Isolation

The cultivated bacteria are subsequently tested for DNA isolation. This procedure made use of the Viogene Geno Plus Genomic DNA Extraction Miniprep System kit, which contains reagents including TE Buffer, Proteinase K, RNAse, Lysozyme, and Wash Buffer I and II. Before using the DNA samples in analytical techniques, the isolated bacterial DNA was evaluated both qualitatively and quantitatively, their quality and usability must be confirmed by assessing DNA purity and integrity [37]. Qualitative analysis using 0.7% agarose gel electrophoresis with Green Safe as a fluorescent, and visualized with a UV-Transilluminator confirmed the successful isolation of *Enterococcus faecalis*. Whole genome sequence shows the size of the bacterial genome is 1,924,212 bp [38]. Fig. 3 shows that the Enterococcus faecalis genome has a size larger than the 10,000 bp marker, measuring 1,924,212 bp, as determined by in silico analysis. The electrophoresis results confirm the presence of genomic DNA from *Enterococcus faecalis.*

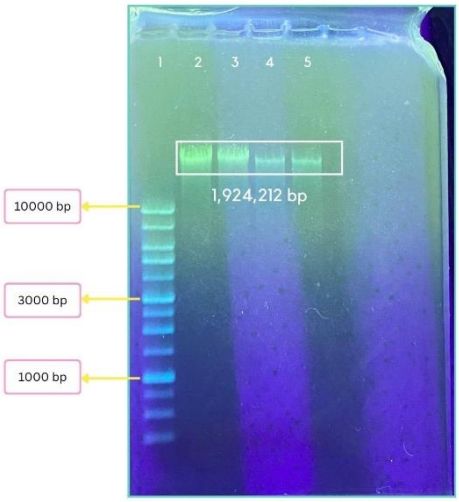


Fig. 3. DNA Isolates of *Enterococcus faecalis* on Agarose Gel Electrophoresis. (1) DNA Ladder 1 kb; (2-5) *Enterococcus faecalis* DNA

DNA purity was assessed using the absorbance ratio at 260 and 280 nm [39]. The results of the quantitative analysis conducted with a Nanodrop spectrophotometer are displayed in Table 2. The concentration of DNA was found to be 107 ng/µL, and the purity ratio (A260nm/A280nm) was 2.0, suggesting that the DNA was pure as it fell within the permissible range of 1.8-2.0 for pure DNA [31], [40]. For DNA, a ratio near 1.8 is usually regarded as "pure" [41]. A ratio less than 1.8 can point to the presence of proteins or other pollutants, whereas a ratio exceeding 2.0 indicates RNA contamination [37], [42]. The DNA concentration was adjusted to 10 ng for further use, as previous studies [43] demonstrated that PCR reactions yield optimal results at this concentration.

Sample	Concentration (ng/µL)	Purity (A260/A280)

Enterococcus faecalis isolat	107	2,0

Table 2. Purity and concentration DNA template for samples

3.4 Optimization Annealing Temperature

Through temperature optimization, the optimal annealing temperature for the *htrA* primer was discovered. The optimization was conducted within the 54°C–63°C temperature range. Different annealing temperatures were used in this gradient PCR technique [26], [43]. The results of the gradient PCR are shown in Fig. 4. Based on these results, a single bright DNA band was observed for each annealing temperature tested with the primer. This single band indicates the absence of nonspecific bands, suggesting that the selected primer is free from genomic DNA contamination [26]. Negative controls, including NTC and NFW+MM, confirmed that no contamination occurred during the *htrA* gene reaction. The band size matches the results of primer design and in-silico analysis, where the amplicon from the *htrA* gene with 162 bp, located above the 100 bp marker and below the 200 bp marker. With having been observed that DNA bands were seen at every temperature examined, an annealing temperature of 60°C was chosen for this investigation because, according to published research, it produced the best amplification [31], [44], [45].

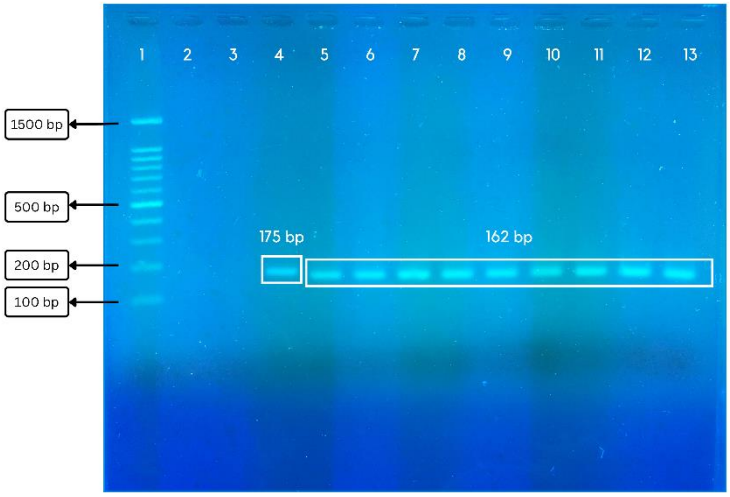
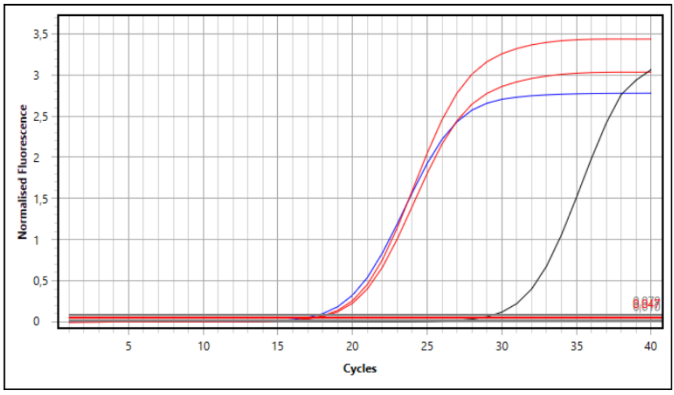


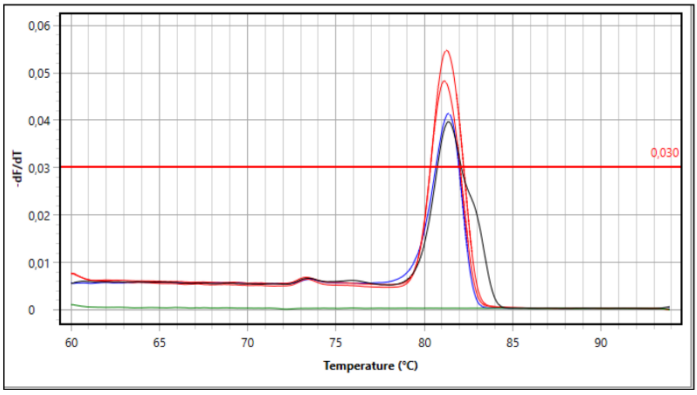
Fig. 4. Optimization of Annealing Temperature *htrA* Primer Pairs. (1) DNA Ladder 100 bp; (2) NTC; (3) NFW+MM (Negative control); (4) Positive control *codY Bacillus subtilis* 175 bp; (5) – (13) DNA fragment at temperature 54oC - 62oC

3.5 Confirmation Test with real-time PCR

In the confirmation test, the data were analyzed by interpreting the cycle threshold (Ct) value and melting temperature during the PCR process. The aim of this test was to verify that the primers effectively amplified and identified *Enterococcus faecalis*. Fluorescence dyes are used in real-time PCR to monitor the amount of DNA in each cycle; the amount of amplicon produced is directly proportional to the increase in fluorescence signal [46]. The PCR process generally includes three stages: denaturation, annealing, and extension [47]. For this test, the annealing temperature was optimized to 60°C based on previous experiments with the *htrA* primer. The test was conducted over 40 cycles, and the resulting Ct values were plotted as an amplification curve.



(A) Amplification curve



(B) Melting Curve

Fig. 5. Melting and Amplification Curves of the *htrA* Primer Confirmation Test.

Line Color	Sample	*htrA*	
		Ct	Tm
	Enterococcus faecalis (1)	17,29	81,27
	Enterococcus faecalis (2)	17,48	81,16

	Listeria monocytogenes (Positive Control)	16,82	81,36
	NTC	28,54	81,38
	NFW+MM	-	-

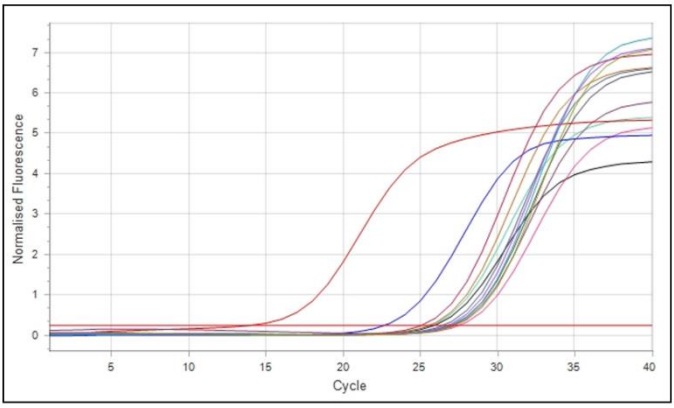
Table 3. The Results of Confirmation Assay *htrA* Primer

The Ct value, or the number of cycles required for the fluorescence signal to exceed the baseline, indicates the amount of DNA present. Higher DNA concentrations are correlated with lower Ct values [46], [48]. Table 3 provides confirmation of the primer's capacity to detect the *htrA* gene, with Ct values of 17.29 and 17.48 (Duplo). The positive control, on the other hand, demonstrated appropriate reaction function with amplification at cycle 16.82. The NTC showed amplification at cycle 28.54, indicating non-target amplification, as the difference from the target bacterium was more than 10 cycles [29], [31], [49].

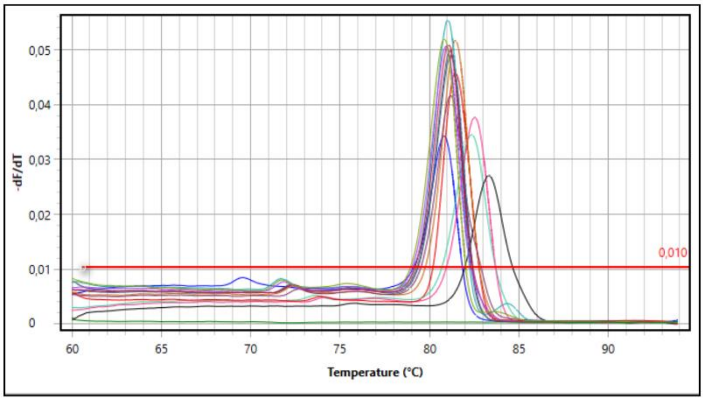
In the melting curve analysis, the melting temperatures (Tm) of the samples were 81.27°C and 81.16°C, while the NTC had a different Tm due to differences in GC content between the amplicons. The melting curve in Fig. 5 showed a single peak for *E. faecalis* at 81.27°C and 81.16°C, confirming that no primer dimers were formed and that the amplification was specific to the target DNA [49]. These results demonstrate that the *htrA* gene primers effectively confirmed the presence of *E. faecalis* without non-specific amplification.

3.6 Specificity Test with real-time PCR

The purpose of the specificity test was to determine if the *htrA* primer was made specially to identify *Enterococcus faecalis*. Real-time PCR amplification results were compared between *Enterococcus faecalis* DNA and several non-target bacteria, including *L. monocytogenes, C. sakazakii, Y. enterocolitica, S. flexneri, E. coli, C. jejuni, P. aeruginosa, P. mirabilis, K. pneumoniae*, and *S. typhi*. The amplification curve showed in Fig. 6 and Table 4, a cycle threshold (Ct) value of 13.97 for *Enterococcus faecalis*, while the non-target bacteria had Ct values between 25 and 27. The Ct value of the NTC was around 25.88, with no amplification curve detected, ruling out the presence of any unwanted products. confirming the absence of contamination or non-specific amplification in the PCR reaction. The cycle differences between the non-target and target bacteria provided more evidence for the *htrA* primer's specificity. An important difference was observed between Enterococcus faecalis and non-target bacteria, which was roughly 11 cycles. A difference of more than 10 cycles, according to the literature [29], [31], [49] is regarded as sufficient to rule out significant non-specific amplification, indicating that the *htrA* primer successfully distinguishes *Enterococcus faecalis* from other bacteria.



(A) Amplification curve



(B) Melting Curve

Fig. 6. Melting and Amplification Curves of the *htrA* Primer Specificity Test Results.

Line	Sample	*htrA*	
		Ct	Tm
	NFW + MM	-	-
	Positive Control (*L. monocytogenes*)	22.63	80.81
	E. faecalis	13.97	81.47
	L. monocytogenes	26.98	81.15
	C. sakazakii	27.46	82.53
	Y. enterocolitica	25.63	82.36
	S. flexneri	26.41	81.19
	E. coli	26.96	81.02

	C. jejuni	27.13	81.19
	P. aeruginosa	25.51	81.45
	P. mirabilis	25.07	81.06
	K. pneumoniae	27.21	83.82
	S. typhi	26.71	83.93
	NTC	25.88	83.32

Table 4. Specificity Test Results of *htrA* Primer with Bacteria Target and Non-Target Bacteria.

The melting curve reveals the distinct melting points of the amplicons, providing further confirmation of primer specificity. Through melting curve analysis, it is observed that each bacterium has a characteristic melting temperature (Tm), influenced by the sequence of the amplicon formed. *Enterococcus faecalis* (red line) has a pronounced peak with a Tm value of 81.47°C, according to the melting curve study in Fig. 6. A single peak suggests that no primer dimers are generated and that the primer specifically detects the target at the Tm temperature. Moreover, each non-target bacterium exhibited a shift in Tm compared to the *Enterococcus faecalis* sample, this demonstrates that the primer is successfully differentiated the specific bacteria [49]. These results confirm the *htrA* primer for *Enterococcus faecalis*, making it a reliable tool for detecting this particular bacterial species.

4 Conclusion

This study concludes that the *htrA* primer can amplify *Enterococcus faecalis*, generating a 162 bp amplicon at an annealing temperature of 60°C. The primer demonstrated its effectiveness in detecting *Enterococcus faecalis* with a Ct value of 17.62 ± 0.11 and a melting temperature (Tm) of 81.21 ± 0.06°C. Furthermore, based on differing Ct and Tm values, the *htrA* primer was able to distinguish *Enterococcus faecalis* from non-target bacteria. This emphasizes its capacity to differentiate between bacteria that are the target and those that are not. With the use of the *htrA* primer, the real-time PCR method exhibits significant promise for creating a sensitive and specific detection method for the rapid and specific identification of *Enterococcus faecalis*.

Acknowledgments. This research was funded by Lembaga Penelitian dan Pengabdian Masyarakat Universitas Negeri Jakarta (LPPM UNJ), Kemendikbudristek through the PDUPT scheme and Badan Riset Inovasi Nasional (BRIN) with the Riset Inovasi untuk Indonesia Maju (RIIM-LPDP) scheme with contract number: 2/PG.02.00.PT/LPPM/IV/2024. We would also like to thank PT Sinergi Indomitra Pratama for providing the research instruments. In addition, we would like to thank our international partners from Arnold Palmer Hospital Orlando Florida, USA and Universiti Teknologi Malaysia (UTM), Malaysia. Our appreciation also goes to the Pusat Laboratorium Forensik Polisi Republik Indonesia (Puslabfor Polri), Sentul Bogor, Pusat Kedokteran dan Kesehatan Polri, Kimia Farma, and the UNJ Salmonella team from the Pusat Unggulan Ipteks Pendeteksi Bakteri Patogen (PUI PBP) at LPPM UNJ for their contributions and capabilities in this research.

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