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NEW PRIMER DESIGN OF MULTIPLEX POLYMERASE CHAIN REACTION (PCR) FOR DETECTION OF *Escherichia coli* AND *Salmonella enterica* IN FOOD SAMPLE

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Abstract

Background: Multiplex PCR techniques are used to detect multiple pathogens at the same time simultaneously. In order to achieve the specificity and sensitivity of detection, it is important to optimize the multiplex PCR method properly. Developing suitable primers and optimizing PCR temperature to boost particular genes from pathogens are key factors for this optimization.
Methods: For the simultaneous detection of Escherichia coli and Salmonella enterica in food samples, this research aims to develop a set of primers for multiplex polymerase chain reaction (PCR). Using Primer-BLAST software, the study utilizes specific primer designs for the phoA gene (Eschericia coli) and invA gene (Salmonella enterica). DNA isolation has confirmed successful extraction from the two bacterial samples. PCR was performed under different conditions, including Singleplex and Multiplex PCR, using two annealing temperatures of 53oC and 50oC.
Results: The results showed that this method can effectively amplify target genes and indicate their specificity and reliability at both temperature levels. Given these results, it has successfully conducted multiplex PCR using the built primer pairs. Both annealing temperatures of 50oC and 53oC can be used to perform multiplex PCR to detect E. coli and Salmonella enterica in one PCR reaction.
Conclusion: Through this research, we have created a new set of Multiplex PCR Primers and an optimized multiplex PCR technique for the simultaneous detection of E coli and Salmonella enterica in food samples. The rapid and simultaneous screening of E. coli and S. enterica, which contributes to improved food safety measures and pathogen detection in the food industry, is promising with this optimized PCR approach.

Keywords: *Escherichia coli; Multiplex PCR; Salmonella enterica; Specific primer design*

Abstrak

Latar Belakang: Teknik PCR multiplex digunakan untuk mendeteksi beberapa patogen secara bersamaan. Pengoptimalan metode PCR multiplex sangat penting untuk mencapai spesifisitas dan sensitivitas deteksi yang diinginkan. Pengembangan primer yang sesuai dan pengoptimalan suhu PCR untuk mengamplifikasi gen tertentu dari patogen merupakan faktor kunci dalam optimasi ini.
Metode: Penelitian ini bertujuan untuk mengembangkan sekumpulan primer untuk reaksi rantai polimerase (PCR) multiplex untuk deteksi simultan Escherichia coli dan Salmonella enterica dalam sampel makanan. Menggunakan perangkat lunak Primer-BLAST, penelitian ini merancang primer spesifik untuk gen phoA (Escherichia coli) dan gen invA (Salmonella enterica). Isolasi DNA telah mengkonfirmasi ekstraksi yang sukses dari kedua sampel bakteri. PCR dilakukan di bawah berbagai kondisi, termasuk PCR Singleplex dan Multiplex, dengan dua suhu annealing 53oC dan 50oC.
Hasil: Hasil menunjukkan bahwa metode ini dapat mengamplifikasi gen target secara efektif dan menunjukkan spesifisitas serta keandalan pada kedua tingkat suhu tersebut. Berdasarkan hasil ini, telah berhasil dilakukan PCR multiplex menggunakan pasangan primer yang dibuat. Kedua suhu annealing 50oC dan 53oC dapat digunakan untuk melakukan PCR multiplex guna mendeteksi E. coli dan Salmonella enterica dalam satu reaksi PCR.
Kesimpulan: Melalui penelitian ini, kami telah mengembangkan set primer PCR Multiplex baru dan teknik PCR multiplex yang dioptimalkan untuk deteksi simultan E. coli dan Salmonella enterica dalam sampel makanan. Pendekatan PCR yang dioptimalkan ini menjanjikan untuk skrining simultan E. coli dan S. enterica, yang berkontribusi pada peningkatan keamanan pangan dan deteksi patogen di industri makanan.

Kata Kunci: *Escherichia coli; PCR Multiplex;Salmonella enterica; Desain primer spesifik*

INTRODUCTION

Contamination in food samples due to pathogenic bacteria poses a significant threat to public health (Nguyen et al., 2016; C. Wei et al., 2018). Among the key pathogens responsible for foodborne infections are *Escherichia coli* and *Salmonella enterica* (Delbeke et al., 2015; Sahu et al., 2019). *Escherichia coli* can become pathogenic when certain strains produce toxins, such as *E. coli* O157:H7, while *Salmonella enterica* is known to cause food poisoning and gastrointestinal infections in humans and animals (Sahu et al., 2019). Traditional methods for detecting such infections in food samples are time-consuming and costly. Thus, Polymerase Chain Reaction (PCR) was adopted as a faster and more efficient alternative (Al-Jobori & Nader, 2016). However, conventional single PCR methods can only detect one type of bacteria at a time, necessitating the development of multiplex PCR methods to enable the simultaneous detection of multiple pathogens (Ludwig et al., 2020; Xu et al., 2016).

Despite adopting multiplex PCR for detecting pathogenic bacteria, a crucial research gap remains concerning optimizing this method for accurate and sensitive detection of Escherichia coli and Salmonella enterica simultaneously. To guarantee specificity and sensitivity in detection, it is necessary to optimize the PCR Multiplex Method (Tao et al., 2020). Selecting the correct primers and optimizing PCR temperature to add specific genes from both pathogens are key factors in this optimization (Molina et al., 2015; Tao et al., 2020). Poorly selected primers may result in incorrect or inconclusive results, while underestimation of the PCR temperature can affect sensitivity and specificity for detection (Nguyen et al., 2016; Sahu et al., 2019; Hernández et al., 2022; Lindsey et al., 2017; Molina et al., 2015).

This study aims to close the gap in research by increasing the robustness of multiplex PCR methods for pathogen detection in food samples. A primary focus has been optimizing the suitable primers and PCR temperature for the simultaneous detection of E. coli and Salmonella enterica. This optimization can provide a more accurate and precise detection rate for food samples containing both bacteria. This research aim to make a set of primers for Multiplex Polymerase Chain Reaction (PCR) that simultaneously identify Escherichia coli and Salmonella enterica in food samples, thereby ensuring the exact amplification of target genes from both pathogens. In order to fulfill these research objectives, in particular through multiplex PCR technology, this study is intended to contribute significantly towards developing methods for detecting contaminants in food samples. By providing more effective and accurate methods for detecting pathogenic bacteria in Food, these results are expected to enhance the safety of foods and human health while preventing possible disease outbreaks.

MATERIALS AND METHODS

An experiment designed for optimizing the multiplex PCR method to detect *E. coli* and *Salmonella enterica* in food samples has been adopted by this study. This study involves several key steps, including the cultivation of *E. coli* and *Salmonella enterica* bacterial strains, DNA isolation from cultured bacteria, and preparation of specific PCR primers for each bacterium that is to be used in either single or multiple simultaneous PCR reactions.

Appropriate equipment and facilities for bacterial cultivation, DNA isolation, and PCR reactions were used in a regulated laboratory environment. In order to carry out the necessary tests accurately and effectively, this laboratory is equipped with temperature-controlled incubators, PCR machines, gel electrophoresis equipment, or any of the needed instruments. *E. coli* and *Salmonella enterica* represent the majority of this research population.

These bacteria have been grown in nutrient agar media, and then five individual colonies of each bacterium will be selected for further experiments. These five colonies have been injected with separate food samples (in this case

using beef sausage), which are pre-processed and homogenized. In the initial procedure, *E. coli* and *Salmonella enterica* bacteria were grown in nutrients agar media at 37ºC for 24 hours.

After bacterial growth, DNA was isolated using the Chelex-100 method, extracting DNA from the cultured bacteria spiked into food samples. 100 mg sample was added to a 1.5 ml tube, 10 µl proteinase K, and 2 µl Merchaptoethanol. The mixture was vortexed for 20 seconds and then incubated at 56°C for 20 minutes, with homogenization by vortexing every 5 minutes.

The samples' isolated DNA was then processed in electrophoresis tools on a 1% agarose gel stained with ethidium bromide to ensure its quality and purity. Besides, the concentration and purity of DNA was assessed using Nanodrop. For Electrophoresis, 10 µl of each DNA sample was mixed with 2 µl of loading dye and loaded into the gel. The gel was run at 50 V for 1 hour to separate the DNA fragments, and the gel was visualized using a UV transilluminator.

Regarding the design of specific PCR primers for E. coli and Salmonella enterica, software primer design tools like Primer-BLAST or primer3plus have been utilized. These tools will assist in designing primers with suitable melting temperatures, appropriate lengths (typically 18-25 nucleotides), and minimal self-complementarity or hairpin formation to avoid non-specific amplification or primer-dimer formation. The primers used in this study are presented in Table 1.

Table 1. Primer used in this Study

Primer	Sequence
phoA F	5' AAAGATCACCCAACGATTCTG '3
phoA R	5’ ATATTGCCATGGTACGTTGCT ‘3
invA F	5' ATCTGGTTGATTTCCTGATCG '3
invA R	5' GACACGTTCTGAACCTTTGGT '3

Both single- and multiplex PCR reactions have been conducted for the PCR process. The single PCR will involve amplifying target genes specific to *E. coli* and *Salmonella enterica* separately using 2x Taq PCR-Plus from Tiangen. For each PCR reaction, 2 µl of the isolated DNA template have been used, along with 1 µl of each specific primer (10 pmol/µl), 12.5 µl of 2x Taq PCR-Plus Master Mix, and 8.5 µl of nuclease-free water. The PCR reactions have been carried out under defined conditions, including denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C or 53°C (as optimized) for 30 seconds, and elongation at 72°C for 50 seconds. The final elongation was performed at 72°C for 5 minutes.

Two pairs of specific *E. coli* and *Salmonella enterica* primers have been used in a single reaction for the multiplex PCR. Each multiplex PCR reaction will include 4 µl of the isolated DNA template, 1 µl of each specific primer (10 pmol/µl), 12.5 µl of 2x Taq PCR-Plus Master Mix, and 4.5 µl of nuclease-free water. The PCR conditions for the multiplex reaction have been the same as for the single PCR.

Data analysis will involve assessing the PCR products through agarose gel electrophoresis to visualize the amplified DNA bands. The presence or absence of bands corresponding to the target genes has been recorded and analyzed to evaluate the success of the PCR reactions. The optimization process has been conducted iteratively, adjusting the primers and PCR conditions until the desired sensitivity, specificity, and accuracy are achieved

RESULT AND DISCUSSION

This research begins with the primer design using tools from https://www.ncbi.nlm.nih.gov/tools/primer-blast/. Based on this, two sets of primers for the phoA gene (*Escherichia coli*) and invA gene (*Salmonella enterica*) were obtained with the following results that shown in Figure 1 and Figure 2

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AAAGATCACCCAACGATTCTG	21	56.24	42.86	4.00	1.00
Reverse primer	ATATTGCCATGGTACGTTGCT	21	58.07	42.86	6.00	0.00

Products on target templates

>NC_000913.3 Escherichia coli str. K-12 substr. MG1655, complete genome

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product length = 458
Forward primer  1       AAAGATCACCCAACGATTCTG  21
Template        402191  ......................  402211

Reverse primer  1       ATATTGCCATGGTACGTTGCT  21
Template        402648  ......................  402628
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Figure 1. Result of *E. coli* phoA gene PCR Primer Design

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	ATCTGGTTGATTTCCTGATCG	21	55.75	42.86	4.00	2.00
Reverse primer	GACACGTTCTGAACCTTTGGT	21	58.71	47.62	7.00	3.00

Products on target templates

>NC_003197.2 Salmonella enterica subsp. enterica serovar Typhimurium str. LT2, complete genome

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product length = 305
Forward primer  1        ATCTGGTTGATTTCCTGATCG  21
Template        3040361  ......................  3040341

Reverse primer  1        GACACGTTCTGAACCTTTGGT  21
Template        3040057  ......................  3040077
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Figure 2. Result of *S. enterica* invA gene PCR Primer Design

The results indicate the designed primers' specificity towards the target genes. For instance, the primer design for the phoA gene in *E. coli* species yielded an anticipated PCR result specific to *E. coli* str. K-12 substr. MG1655, with an estimated PCR product length of 458 bp. Similarly, the primer design for the invA gene in *S. enterica* species produced an expected PCR outcome specific to *S. enterica* subsp. Enterica serovar Typhimurium str. LT12, with an approximate PCR product length of 305 bp. The selection of primers for Multiplex PCR involves considering various factors. The PCR process occurs simultaneously at the same temperature, so the two primer pairs must have similar Tm temperatures (within a maximum difference of 5 degrees Celsius). Another consideration is that the PCR products resulting from these primer pairs should have different lengths. Based on these estimations, it can be observed that the two primer pairs meet the initial requirements for multiplex PCR. In a study by (Sint et al., 2012), a multiplex PCR technique was developed and optimized to detect multiple DNA fragments from different species or

genes in a single reaction. They utilized standardized DNA templates to ensure primer efficiency and sensitivity balance for each target, enabling result comparison across various systems and studies.

Subsequently, DNA was isolated for each bacterium, *E. coli* and *S. enterica*. Successful DNA isolation was confirmed through Electrophoresis, where the presence of DNA was evidenced by bands appearing on the agarose gel under UV light, as depicted in the Figure 3 A.

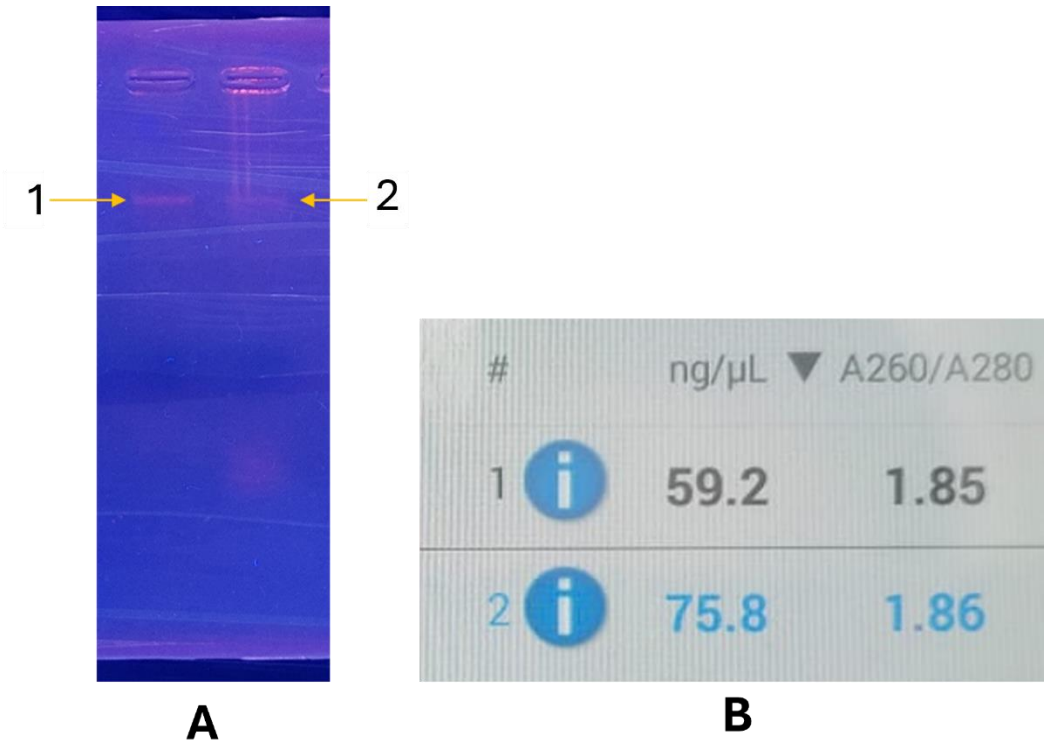

Figure 3. DNA Isolation Result (A.) The visible DNA band using electrophoresis; (B.) Concentration and Purity of DNA using Nanodrop (1: *E. coli* sample, 2: *S. enterica* sample)

Further, the Nanodrop analysis show the concentration and purity of DNA (Figure 3 B). This result show in the concentration of DNA for both sample is good because A260/A280 ratio close to 1.8. These findings illustrate the successful extraction of DNA from each *E. coli* and *S. enterica* bacteria sample, facilitating the subsequent step of PCR. In this study, DNA isolation was performed using a modified Chelex. The DNA isolation process commenced with sample treatment using proteinase K and mercaptoethanol to lyse the bacterial cells. Subsequently, DNA isolation was carried out utilizing Chelex 100. Chelex® 100 ion-exchange resins are composed of plastic with two iminodiacetate ions linked to it, enabling them to bind and retain metal ions with multiple positive charges effectively. Chelex extraction is a cost-effective and straightforward procedure (Ip et al., 2015).

PCR was executed under diverse conditions, encompassing singleplex PCR for each gene, namely phoA, and invA, and simultaneously multiplex PCR for both genes. Within this research, two annealing temperatures, 53°C and 50°C, were compared. The outcomes of both singleplex and multiplex PCR experiments are depicted in the Figure 4.

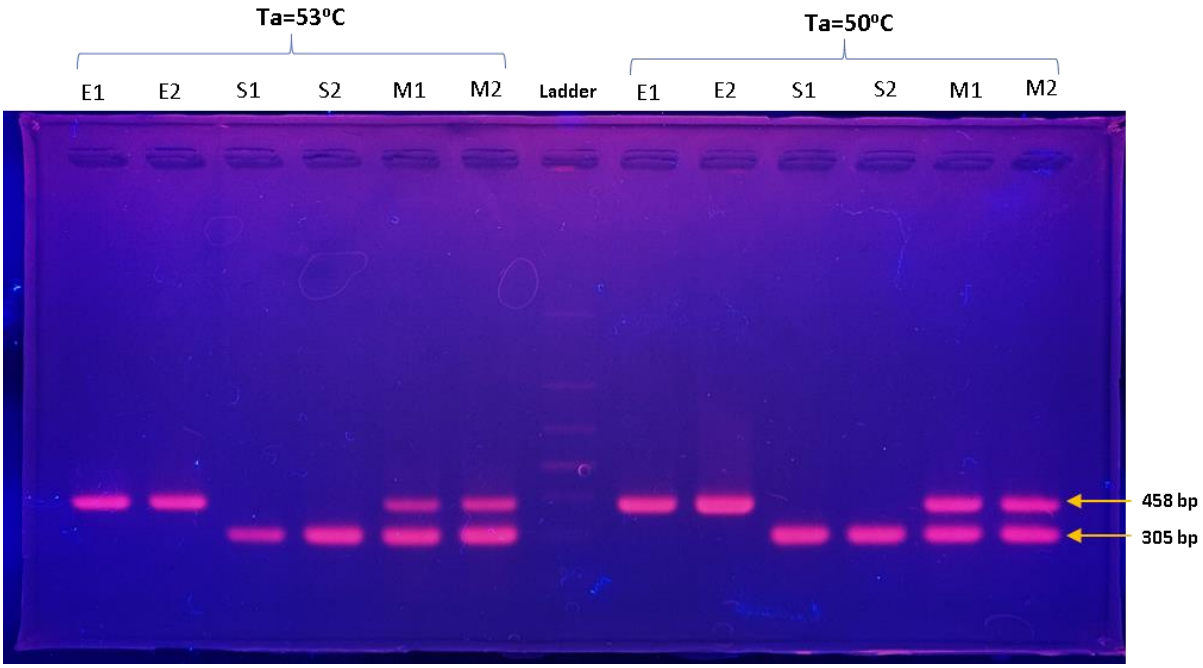

Figure 4. Result of Singleplex and Multiplex PCR (Sample E: *E. coli*; S: *S. enterica;* M: Multiplex *E. coli* and *S. enterica*)

Based on these outcomes, it is evident that successful singleplex and multiplex PCR was accomplished at both annealing temperatures, 53°C and 50°C. The success of the multiplex PCR process is indicated by the emergence of two bands at the anticipated sizes: 305 bp (PCR fragment of the phoA gene in *E. coli*) and 458 bp (PCR fragment of the invA gene in *S. enterica*). These findings imply that the designed primers and the specified annealing temperatures could serve as viable options for the simultaneous and rapid detection of *E. coli* and *S. enterica* bacteria through the PCR methodology. The success of the multiplex PCR process is evident from the bands observed under UV light. Based on the observations of the agarose gel under UV light, it is clear that the amplification results of the *E. coli* phoA gene and the *S. enterica* invA gene can be distinctly differentiated in the multiplex PCR reaction, highlighting the potential utility of this product for the simultaneous detection of these two bacteria.

In recent years, several studies have sought to address the critical issue of food safety by developing and applying multiplex PCR techniques. (Chen et al., 2021) demonstrated a method for detecting essential virulence genes in intestinal pathogens using optimized primer pairs for Staphylococcus aureus, Listeria monocytogenes, Shigella flexneri, Yersinia enterocolitica, and Clostridium difficile. Similarly, (S. Wei et al., 2019) employed multiplex real-time PCR to simultaneously identify Bacillus cereus, Listeria monocytogenes, and Staphylococcus aureus in various food types, showcasing the method's precision and sensitivity even at low bacterial levels.

(Delbeke et al., 2015) used a high-throughput multi-screening strategy, PCR-based techniques, and selective culture media to find *Salmonella* spp. and Shiga toxin-producing *Escherichia coli* (STEC) in strawberries, lettuce, and basil. This study supported the viability of PCR for finding small amounts of pathogens. Nguyen (2016) established a multiplex PCR assay to rapidly and concurrently detect *Escherichia coli* O157:H7, *Salmonella* spp., and Listeria monocytogenes, demonstrating the technique's efficacy in reliably identifying these foodborne pathogens

in artificially contaminated samples. Similarly, (Sahu et al., 2019) designed primers targeting the invA gene for the specific detection of *Salmonella* spp. in seafood processing, showcasing the potential of DNA-based molecular methods to address challenges in pathogen monitoring. Comparatively, our research adds to this body of work by introducing novel primers for a multiplex PCR targeting the phoA gene in *Escherichia coli* and the invA gene in *Salmonella enterica*, further enhancing the repertoire of techniques available for rapid and accurate detection of these bacterial species in food samples. This research aimed to optimize the multiplex PCR method for the simultaneous detection of *Escherichia coli* and *Salmonella enterica* in food samples. The primer design was successfully carried out using tools from https://www.ncbi.nlm.nih.gov/tools/primer-blast/, resulting in specific primers for the phoA gene (*E. coli*) and invA gene (*S. enterica*).

Overall, in this research, primers for Multiplex PCR (Table 1) have been successfully developed, and the PCR reactions have been carried out under defined conditions, including denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C or 53°C for 30 seconds, and elongation at 72°C for 50 seconds. For five minutes, the final elongation was carried out at 72 C. It promises to be a useful tool for simultaneously detecting these two bacterial species in food samples with this optimized Multiplex PCR technique, which is suitable for food safety and pathogen detection. It is necessary to carry out further studies and validation on a broader range of food samples to verify their suitability and reliability under real-world scenarios for food safety. Overall, this research contributes to the development of DNA diagnostics that will increase our capacity to monitor and control foodborne pathogens more effectively.

CONCLUSION

Finally, in this study, a new set of PCR primers for Multiplex was successfully developed, and the multiplex PCR conditions were optimized to detect E coli and *Salmonella enterica* in food samples simultaneously. The specially formulated primers have successfully isolated DNA and targeted gene amplification for both bacteria, validated through Electrophoresis. It is concluded that an optimal PCR condition to detect *E. coli* and *S. enterica* in the food sample can be achieved by using specific primers and annealing temperatures. The success of the multiplex PCR tests further highlights that this method can be used for rapid and simultaneous screening, providing valuable information on food safety applications.

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