West Science Nature and Technology
Vol. 3, No. 02, June 2025, pp. 77~85

In Silico Design of Multiplex PCR Primers for the Detection of Foodborne Pathogens in Fermented Shrimp Paste (Terasi) from Lombok Island: In Silico PCR and Primer Verification

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Article Info

Article history:

Received March, 2025
Revised June, 2025
Accepted June, 2025

Keywords:

In Silico PCR
Multiplex PCR
Foodborne Pathogen Detection
Shrimp Paste
Primer Design

ABSTRACT

Fermented shrimp paste (terasi) is a traditional food product produced by micro, small, and medium enterprises (MSMEs) in Lombok Island, Indonesia. Due to its fermentation and handling processes, terasi is susceptible to contamination by foodborne pathogens. The Indonesian Food and Drug Authority has identified specific bacterial pathogens that pose a risk of contamination in shrimp paste, necessitating accurate and efficient detection methods. Molecular detection using polymerase chain reaction (PCR) is known for its high specificity and sensitivity, as well as its rapid processing time and cost-effectiveness. However, conventional PCR can only detect a single target pathogen in one reaction, limiting its efficiency in screening multiple contaminants simultaneously. This study aims to design and optimize Multiplex PCR primers for detecting multiple foodborne pathogens in terasi through an in-silico approach, followed by In Silico PCR verification to assess primer specificity and efficiency. Target pathogens include Salmonella spp., Staphylococcus aureus and Vibrio cholerae. DNA target sequences were retrieved from the National Center for Biotechnology Information (NCBI) database, and primer design was conducted using Primer3Plus software. The designed primer sets were validated using Primer-BLAST (NCBI), and in-silico PCR was performed to verify amplification efficiency and specificity. The designed primer set has been successfully obtained and evaluated using In Silico PCR and Primer-BLAST (NCBI), confirming its specificity to the target genes. These results demonstrate that the primers can be effectively used as a molecular detection tool for foodborne pathogens. The optimized conditions and validated primer set are expected to contribute to molecular-based food safety detection methods, particularly for Indonesian fermented food products.

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1. INTRODUCTION

Foodborne diseases caused by pathogenic microorganisms pose a serious global health threat [1], [2], [3]. Food contamination by such pathogens can trigger symptoms ranging from mild digestive disturbances to severe or even fatal conditions [4]. Various preventive measures have been adopted, including proper cooking, separation of cooked and raw food, and enforcement of food safety regulations [5], [6], [7]. One food product at particular risk is terasi (fermented shrimp paste), which may be contaminated by eight major pathogenicbacteria, namely *Salmonella spp*., *Clostridium perfringens*, *Clostridium botulinum*, *Listeria monocytogenes*, *Campylobacter jejuni*, *Staphylococcus aureus*, *Vibrio cholerae*, and *Bacillus cereus* [2].

To date, the detection of pathogenic bacteria in terasi typically involves three main approaches: culture methods, immunological assays, and molecular techniques. Culture methods are accurate but can take several days, whereas immunological methods such as Immunomagnetic Separation (IMS) and Enzyme-Linked Immunosorbent Assay (ELISA) tend to be costly [6], [8]. In contrast, molecular methods based on Polymerase Chain Reaction (PCR) are faster, more specific, and use fewer reagents [9], [10], [11], [12], although conventional PCR detects only a single pathogen per reaction, thus becoming less efficient when multiple pathogens must be targeted.

To overcome these limitations, Multiplex PCR was developed to simultaneously detect multiple pathogens in a single reaction by employing multiple primer pairs [13], [14], [15], [16], [17]. This method has proven effective in reducing both the time and cost of detection, especially in routine food contamination assessments [6]. In addition, In Silico PCR has gained popularity for predicting amplification outcomes computationally, allowing researchers to design and optimize primers before conducting laboratory experiments [9], [10], [13], [18].

2. METHODS

2.1 Primer Design Using Primer3Plus

2.1.1 Target Pathogens

The study focused on three major bacterial pathogens commonly associated with contaminated terasi or other high-risk foods:

- Staphylococcus aureus (±251 bp)
- Salmonella typhi (±155 bp)
- Vibrio cholerae (±637 bp)

2.1.2 Sequence Collection

Representative gene sequences for each pathogen were retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/). Each target region was selected based on published literature or known diagnostic gene targets relevant to foodborne pathogen detection.

2.1.3 Primer3Plus Configuration

Primer pairs for each pathogen were designed using Primer3Plus (https://primer3plus.com/). Default parameters were used, except for the following adjustments to optimize detection in a potential Multiplex PCR:

- Primer length: 18–25 nucleotides (nt)
- GC content: 40–60%
- Melting temperature (Tm): 55–60 °C
- (with a maximum Tm difference of 2 °C between forward and reverse primers)
- Amplicon size: Predefined target lengths for each pathogen as indicated above Each primer set was inspected to confirm the absence of hairpins, self-dimers, and cross-dimers that might affect multiplex reactions.

2.1.4 Primer Output

Primer3Plus generated a forward and reverse primer for each bacterium's target sequence, ensuring that the final predicted amplicon sizes matched the desired ranges. The recommended primer sets and their respective amplicon sizes were recorded for subsequent validation.

2.2 Primer Specificity Check Using Primer-BLAST (NCBI)

2.2.1 Specificity Assessment

The designed primer pairs were individually entered into Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to confirm specificity. For each pathogen:

- The target organism was specified under "Organism."
- The "Database" field was set to default NCBI databases to screen for any non-specific binding.
- Mismatch allowances and product size ranges were adjusted in accordance with the initial design from Primer3Plus.

2.2.2 Evaluation Criteria

- Specific Binding: Primers should ideally match only the intended pathogen's target region.
- Product Length: The predicted product size in Primer-BLAST output was checked against the intended amplicon size.
- Number of Hits: Off-target amplification, if present, was noted and minimized by adjusting primer parameters if necessary.

2.3 In Silico PCR Validation

The validated primers were further tested for predicted PCR band formation using three in silico PCR tools. Each website simulates PCR conditions, enabling visualization of expected amplicon sizes or virtual gel bands.

Insilico.ehu.es PCR

- URL: PCR - University of the Basque Country
- Enter the primer sequences along with the target DNA sequence (in FASTA format) or an equivalent reference.
- Adjust parameters such as mismatch allowance and maximum product size if needed.
- Run the simulation to verify the resulting virtual bands and check alignment details.

2.4 Data Analysis and Interpretation

2.4.1 Size Confirmation

All virtual PCR simulations were inspected to confirm that the expected amplicon sizes corresponded to the initial design.

2.4.2 Specificity Verification

- Primer-BLAST results were cross-checked with each in silico PCR tool to ensure there were no unintended amplification products.
- Any mismatched or additional bands detected in silico would prompt re-examination of the primers.

3. RESULTS AND DISCUSSION

Designing primers using Primer3Plus successfully yielded four primers pairs targeted to *Staphylococcus aureus*, *Salmonella typhi*, and *Vibrio cholerae*, each corresponding to the desired amplicon sizes of approximately 251 bp, 155 bp, and 637 bp, respectively (Table 1). Throughout the design process, default Primer3Plus parameters were adjusted to maintain optimal melting temperatures, GC content, and minimal self-complementarity, ensuring that the primer sets were both specific and suitable for potential multiplex applications. Careful inspection of the primer sequences confirmed the absence of significant hairpins, self-dimers, and cross-dimers, thereby minimizing the risk of undesired secondary structures or non-specific amplification in subsequent assays.

Table 1. Primer sets designed using Primer3Plus for the detection of four bacterial pathogens in terasi.

Pathogen	Forward Primer (5'–3')	Reverse Primer (5'–3')	Expected Amplicon Size (bp)
Staphylococcus aureus	TTGACATACATCAGCGAAAACA	TCAATTTCGTTGTTTGCTTCA	251
Salmonella typhi	CCAGCATGTTACGAATGTGG	CAGCCGCACTACGTGATAGA	155
Vibrio cholerae	TCGTGATGACAGCGAAAAAG	TCTTGGAAAAATCGCAATCC	637

Table 2. Primer-BLAST validation results

Staphylococcus aureus primer		Salmonella typhi primer		Vibrio cholerae primer	
CP181164.1	*Staphylococcus aureus* strain AG21-0620	CP141194.1	*S. enterica* strain 26	CP157384.1	*Vibrio cholerae* C6706 chromosome 1
CP181145.1	*Staphylococcus aureus* strain AG21-0629	CP173296.1	*S. enterica* subsp. enterica serovar Typhi strain 2CTRGujarat	CP173750.1	*Vibrio cholerae* strain 1901530 chromosome 1
CP181140.1	*Staphylococcus aureus* strain AG21-0599	CP173281.1	*S. enterica* subsp. enterica serovar Kentucky strain s202011-11	CP173752.1	*Vibrio cholerae* strain 01113756 chromosome 1
CP181134.1	*Staphylococcus aureus* strain AG21-0594	CP171351.1	*S. enterica* subsp. enterica serovar Bareilly strain UTK W1-0003	CP173754.1	*Vibrio cholerae* strain 01492768 chromosome 1

CP181131.1	*Staphylococcus aureus* strain AG21-0621	CP169503.1	*S. enterica* subsp. enterica serovar Infantis strain FSIS9916	CP175806.1	*Vibrio cholerae* strain N19-2759 chromosome 1
CP181128.1	*Staphylococcus aureus* strain AG21-0622	CP169513.1	*S. enterica* subsp. enterica serovar Infantis strain FSIS7823	CP173745.1	*Vibrio cholerae* strain 831317 chromosome 1
CP181138.1	*Staphylococcus aureus* strain AG21-0601	CP169511.1	*S. enterica* subsp. enterica serovar Infantis strain FSIS5221	CP171688.1	*Vibrio cholerae* strain M647 chromosome 1
CP181142.1	*Staphylococcus aureus* strain AG21-0630	CP169507.1	*S. enterica* subsp. enterica serovar Infantis strain FSIS9851	CP171686.1	*Vibrio cholerae* strain M686 chromosome 1
CP181136.1	*Staphylococcus aureus* strain AG21-0619	CP169517.1	*S. enterica* subsp. enterica serovar Infantis strain FSIS4897	CP171680.1	*Vibrio cholerae* strain M804 chromosome 1
CP181120.1	*Staphylococcus aureus* strain AG21-0606			CP178387.1	*Vibrio cholerae* isolate CTMA_1842 chromosome 1

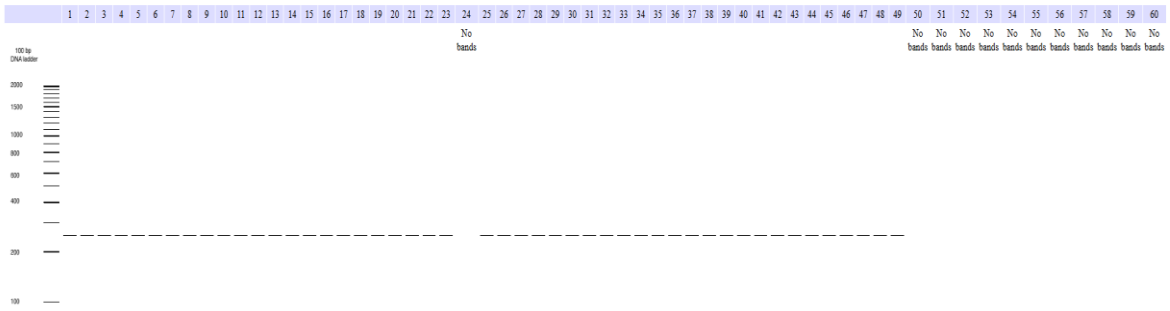

Figure 1. Evaluation results using in Silico PCR for primers designed for *Staphylococcus aureus*.

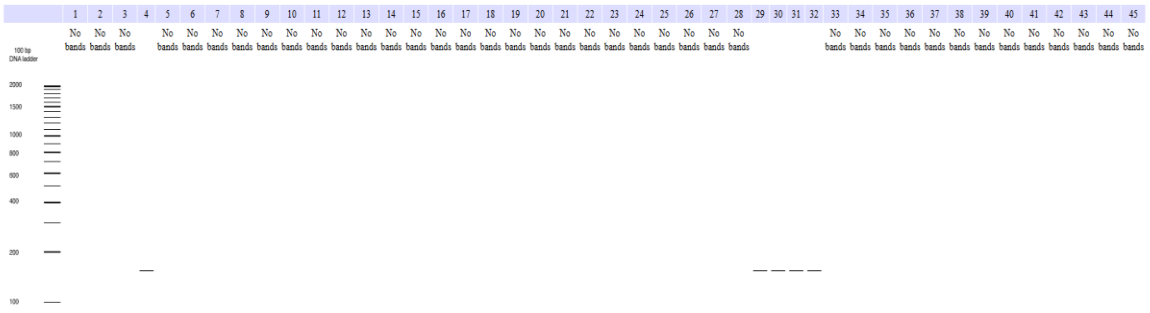

Figure 2. Evaluation results using in Silico PCR for primers designed for *Salmonella typhi*.

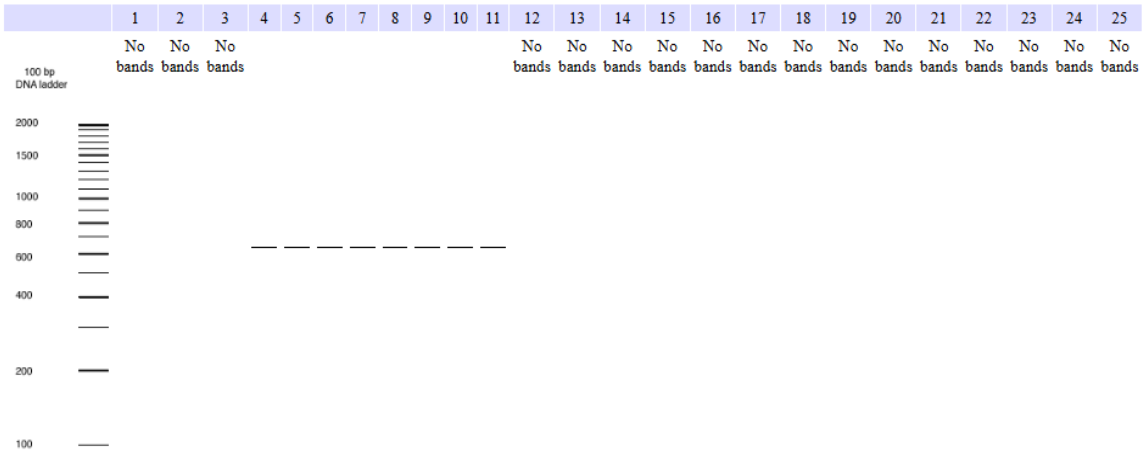

Figure 3. Evaluation results using in Silico PCR for primers designed for *Vibrio cholerae*.

The in Silico primer design process using Primer3Plus yielded four highly specific primer pairs targeting key foodborne pathogens commonly associated with fermented shrimp paste. The designed primer sets, validated by Primer-BLAST in NCBI (Table 2).

Figure 1 (gel image) shows the results of electrophoresis on 60 strains of Staphylococcus aureus via in Silico PCR. A band of approximately 200 bp was observed in 48 lanes using primers designed for *Staphylococcus aureus*.

Figure 2 (gel image) shows the results of electrophoresis on 45 strains of Salmonella spp via in Silico PCR. A band of approximately 150 bp was observed in 5 lanes using primers designed for Salmonella typhi. Based on these 5 bands, it can be seen that the primer is very specific for *Salmonella subspecies enterica* only.

Figure 3 (gel image) shows the results of electrophoresis on 25 strains of Vibrio spp via in Silico PCR. A band of approximately 600 bp was observed in 8 lanes using primers designed for Vibrio cholerae. Based on these 8 bands, the primer is very specific for *Vibrio cholerae*.

Based on Primer-BLAST evaluations and in silico PCR simulations, the three primer sets designed for detecting pathogenic bacteria in shrimp paste exhibited high specificity and produced distinct amplicon sizes. These findings confirm that the primers are well-suited for multiplex PCR applications, allowing simultaneous detection of multiple foodborne pathogens in a single assay.

The present study successfully designed and validated highly specific primers targeting *Staphylococcus aureus*, *Salmonella typhi*, and *Vibrio cholerae* for application in both in silico and laboratory-based PCR assays. Notably, 48 out of 60 tested isolates were identified as S. aureus via a distinct 200 bp amplicon, highlighting the

relatively high prevalence of this pathogen in the examined samples. These findings underscore the critical need for robust and rapid diagnostic methods, particularly for S. aureus, which is known to produce enterotoxins that can lead to foodborne illnesses [4], [19], [20], [21], [22], [23], [24].

The use of Primer3Plus for primer design, coupled with specificity checks via Primer-BLAST, ensured a high degree of target selectivity with minimal off-target amplification. This high specificity is essential in molecular diagnostics, as false positives or negatives could have significant public health ramifications and economic consequences [17], [25]. The efficiency and consistency of the designed primers were further confirmed by in silico PCR analysis, which mirrored experimental gel electrophoresis outcomes. This congruence between computational predictions and laboratory data underscores the practicality of integrating in silico methods into the workflow for developing PCR-based detection assays [9], [10], [13], [18].

Moreover, the successful detection of *Salmonella typhi* and *Vibrio cholerae* using the newly designed primers highlights the versatility of this molecular approach for multiple pathogens. Prior studies have also reported that PCR-based detection systems can effectively identify various bacterial contaminants in fermented seafood and other high-risk products [13], [16]. The inclusion of in silico PCR validation in this research provided a cost-effective and resource-efficient means of primer optimization, reducing the need for extensive trial-and-error in the laboratory [26], [27].

In terms of food safety implications, the high prevalence of *S. aureus* observed in this study raises concerns regarding potential outbreaks, given the pathogen's capacity to produce toxins under relatively mild conditions [4], [12]. Similar warnings apply to *Salmonella* and *V. cholerae*, both of which have been implicated in severe gastroenteritis and cholera outbreaks linked to contaminated seafood [15], [17]. Since traditional production methods for fermented foods often rely on artisanal practices with limited regulatory oversight, a targeted molecular surveillance program becomes crucial to mitigate contamination risks and safeguard public health [14], [16].

Based on Primer-BLAST evaluations and in silico PCR simulations, the three primer sets designed for detecting pathogenic bacteria in shrimp paste exhibited high specificity and produced distinct amplicon sizes. These findings confirm that the primers are well-suited for multiplex PCR applications, enabling the simultaneous detection of multiple foodborne pathogens in a single assay. Overall, the study's integrated strategy, combining Primer3Plus, Primer-BLAST, and in silico PCR simulations substantially reduces experimental costs and time, thus facilitating large-scale pathogen surveillance in food products, particularly in traditional and artisanal production settings [13], [17]. By enhancing detection capabilities, these methods contribute significantly to improving public health outcomes and reinforcing global food safety standards.

4. CONCLUSION

This study demonstrates that in silico primer design, combined with rigorous verification through Primer-BLAST and multiple PCR simulation platforms, provides a powerful framework for developing multiplex PCR assays targeting foodborne pathogens in fermented shrimp paste. By designing and validating primers specific to *Staphylococcus aureus*, *Salmonella typhi*, and *Vibrio cholerae*, we established an efficient, cost-effective, and reliable method for simultaneous pathogen detection in a single reaction. The high specificity and reproducibility of the primers underscore their applicability for large-scale food safety surveillance, particularly in artisanal or traditional processing settings where contamination risks may be elevated. This integrated molecular approach not only enhances the rapidity and accuracy of pathogen identification but also offers a valuable diagnostic tool to help safeguard public health and reinforce global standards in the monitoring of fermented food products.

ACKNOWLEDGEMENTS

The authors would like to thank the Food Technology Study Program, Faculty of Food Technology and Agroindustry, University of Mataram, for their support and facilities throughout this research. This study was funded through PNBP (Internally Generated Funds) of the University of Mataram. The authors are also grateful to the technical staff who assisted with laboratory work and data analysis, as well as to all colleagues who provided insightful feedback during the research process.

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