Preliminary Detection of *Salmonella enterica* on Chicken Eggshell by Multiplex Polymerase Chain Reaction (Multiplex PCR)

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Abstract

Salmonella enterica is a type of Gram-negative bacteria that can cause food poisoning from contaminated food, including eggs. Effective and rapid detection methods are necessary for food safety. In this research, Multiplex Polymerase Chain Reaction (Multiplex PCR) was used to track *S. enterica* on eggshells. Primers specific to *inv*A and *ttr* genes of *S. enterica* were added to the reactions. When using these two pairs of primers for both conventional and Multiplex PCR, the expected DNA bands were observed for *S. enterica* genome template without cross-reactivity to that of Escherichia coli. Preliminary results from four eggshell samples collected from grocery stores near Ramkhamhaeng University, Huamark Campus, revealed the expected conventional PCR products for *S. enterica*. However, only one DNA band for Multiplex PCR products was observed in some eggshell samples. Specifically, while both *inv*A and *ttr* gene products were detected in two samples, only the *ttr* gene was amplified in the remaining two samples. These results suggest that optimization of Multiplex PCR conditions is necessary for consistent detection. Due to the small number of samples, this method needs to be further improved for detecting *S. enterica* contamination on eggshells and other food products.

Keywords: *Salmonella enterica, Multiplex PCR, eggshell, invA gene, ttr gene*

1. Introduction

Salmonella enterica is a rod-shaped, flagellate, and Gram-negative bacterium responsible for foodborne illnesses, collectively known as salmonellosis. This pathogen is a major cause of food poisoning worldwide, with symptoms including diarrhea, fever, abdominal cramps, and vomiting. In severe cases, *S. enterica* infections can lead to systemic complications, particularly in children, the elderly, and immunocompromised individuals (Jajere, 2019). The most common *S. enterica* serovars responsible for human infections include Salmonella Typhi, Salmonella Typhimurium, and Salmonella Enteritidis, which are transmitted through contaminated food and water (Majowicz et al., 2010).

Given the significant public health risk posed by *S. enterica*, rapid and accurate detection methods are essential for preventing outbreaks. Traditional culture-based methods for *S. enterica* detection, including bacterial culturing and biochemical characterization, are time-consuming and labor-intensive. Molecular techniques, such as Polymerase Chain Reaction (PCR), have been widely employed to enhance the sensitivity and specificity of detection (Shanmugasamy et al., 2011).

Among the genetic targets for *S. enterica* identification, the *invA* gene is commonly used due to its role in encoding an *invA*sion protein essential for bacterial pathogenicity. The *invA* gene is a well-conserved genetic marker for *S. enterica* and has been widely utilized for PCR-based detection (Abdel-Aziz, 2016). However, despite its specificity, false-positive results have been reported in some studies due to variations in the *invA* gene across different *S. enterica* subspecies (Resendiz-Nava et al., 2019).

To improve detection reliability, additional genetic markers are often used in combination with *invA*. The *ttr* gene, encoding tetrathionate reductase, is another crucial target for *S. enterica* identification. This enzyme allows *S. enterica* to utilize tetrathionate as an alternative electron acceptor during anaerobic respiration, providing a selective advantage in contaminated environments (Mohammed, 2024). The *ttr* gene is highly specific to *S. enterica* and has been used to enhance the accuracy of PCR-based assays (Nair et al., 2019).

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Although single-gene PCR assays are effective, they are limited in their ability to confirm the presence of *S. enterica* with high specificity. Multiplex PCR, which allows simultaneous amplification of multiple target genes in a single reaction, offers a more efficient approach for bacterial detection. This technique reduces reaction time, reagent consumption, and the risk of false negatives while improving detection accuracy (Kawasaki et al., 2005). In this study, Multiplex PCR was employed to target both *invA* and *ttr* genes of *S. enterica*, ensuring a more reliable detection method for contaminated eggshells.

Thus, this research aims to optimize Multiplex PCR conditions for *S. enterica* detection and assess its effectiveness in identifying bacterial contamination on eggshells collected from grocery stores. By combining two genetic markers, *invA* and *ttr*, this study seeks to improve diagnostic accuracy and contribute to the development of rapid and effective food safety monitoring techniques.

2. Objectives

1) To optimize primers and reaction conditions of Multiplex PCR for *S. enterica* detection.
2) To apply Multiplex PCR for detecting *S. enterica* on eggshell samples.

3. Materials and Methods

3.1 Bacterial Reference Strains Used

Genomic DNA from *Salmonella enterica* subsp. *enterica* serovar Typhi (S. Typhi) was used as the positive control for PCR assays. Additionally, *Escherichia coli* (ATCC 25922) was included as a negative control to assess primer specificity and rule out cross-reactivity. Both bacterial strains were cultured under standard laboratory conditions before DNA extraction.

3.2 Preparation of DNA Samples

Genomic DNA was extracted using the NucleoSpin® Food Genomic DNA from food kit (Macherey-Nagel, Germany). DNA extraction was performed according to the manufacturer's protocol. DNA concentration and purity were measured using a spectrophotometer at 260 nm and 280 nm (Table 2). Extracted DNA samples were stored at -20°C until use.

3.3 Eggshell Sample Collection and DNA Preparation

Eggshell samples were collected from four different grocery stores near Ramkhamhaeng University, Huamark Campus. The samples were labeled as Sample 1, Sample 2, Sample 3, and Sample 4. Each eggshell was washed with sterile distilled water, dried, and ground into a fine powder. DNA extraction was performed using the NucleoSpin® Food Genomic DNA from food kit (Macherey-Nagel, Germany), following the same protocol as for bacterial DNA extraction. The DNA concentration and purity of each sample were determined using spectrophotometry (Table 4).

3.4 Primer Pair Selection

Two pairs of primers were selected to amplify the *invA* and *ttr* genes of *S. enterica*. The *invA* gene is associated with bacterial invasion, while the *ttr* gene encodes tetrathionate reductase, which is important for anaerobic respiration in *S. enterica*. These genes were chosen to improve detection accuracy and minimize false-positive results.

Table 1 Detail of primers for *S. enterica* detection

Primer name	Sequence (5'-3')	% GC	Tm (°C)	Size of PCR product (bp)	Target gene	Reference
Inv F	TTTCAATGGGAACTCTGC	44.4	45.8	172	*invA*	Csordas et al. (2004)
Inv R	AACGACGACCCTTCTTTT	44.4	45.8		*invA*	
*ttr*6 F	CTCACCAGGAGATTACAACATGG	47.8	55.3	94	*ttrA*	Malorny et al. (2004)
*ttr*4 R	AGCTCAGACCAAAAGTGACCATC	47.8	55.3		*ttrC*	

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It was noted that the *ttr6* forward primer targets *ttrA*, while the *ttr4* reverse primer targets *ttrC*. The specificity of these primers was verified by performing PCR assays with *S. enterica* and *E. coli* genomic DNA.

3.5 Detection of invA and ttr Genes Using Conventional PCR

Conventional PCR was performed to confirm the specificity of the selected primers before applying them in Multiplex PCR. The reaction components and cycling conditions are detailed below:

Table 2 PCR Reaction Components that each 50 µL reaction mixture contained

Component	Final Concentration	Volume per Reaction (µL)
10× PCR buffer	1×	5
dNTP mix (10 mM)	0.2 mM each	1
$MgCl_2$ (if not included in buffer)	1.5 mM	*As required*
Forward primer (10 pmol/µL)	0.5 µM	2.5
Reverse primer (10 pmol/µL)	0.5 µM	2.5
DNA template	~50 ng	2
Taq DNA polymerase	1.25 U	1
Nuclease-free water	-	Adjusted to 50

PCR Cycling Conditions

The PCR program was set as follows:

1. Initial denaturation: 95°C for 5 min
2. Denaturation: 95°C for 30 sec
3. Annealing: 55°C for 1 min
4. Extension: 72°C for 1 min
5. Final extension: 72°C for 10 min
6. Hold at 4°C

The PCR products were analyzed using 1.5% agarose gel electrophoresis.

3.6 Detection of invA and ttr Genes Using Multiplex PCR

Multiplex PCR was performed using both primer sets simultaneously in a single reaction. The reaction components and cycling conditions were optimized to ensure efficient amplification of both targets.

Table 3 Multiplex PCR Reaction Components that each 50 µL reaction mixture contained.

Component	Final Concentration	Volume per Reaction (µL)
10× PCR buffer	1×	5
dNTP mix (10 mM)	0.2 mM each	1
$MgCl_2$ (if not included in buffer)	1.5 mM	*As required*
Inv F primer (10 pmol/µL)	0.5 µM	2.5
Inv R primer (10 pmol/µL)	0.5 µM	2.5
ttr6 F primer (10 pmol/µL)	0.5 µM	2.5
ttr4 R primer (10 pmol/µL)	0.5 µM	2.5
DNA template	~50 ng	2
Taq DNA polymerase	1.25 U	1
Nuclease-free water	-	Adjusted to 50

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Multiplex PCR Cycling Conditions

The thermal cycling conditions were as follows:

1. Initial denaturation: 95°C for 5 min
2. Denaturation: 95°C for 30 sec
3. Annealing: 55°C for 1 min
4. Extension: 72°C for 1 min
5. Final extension: 72°C for 10 min
6. Hold at 4°C

3.7 Gel Electrophoresis and Visualization

PCR products were analyzed using 1.5% agarose gel electrophoresis in 1× TAE buffer, stained with RedSafe™ Nucleic Acid Staining Solution (Intron Biotechnology). A 100 bp DNA ladder was used as a size reference. The gels were visualized under UV illumination.

4. Results and Discussion

With the use of primer pairs targeting *invA* and *ttr* genes, several conditions for conventional PCR reactions were varied, and the condition mentioned above was selected. The *invA* gene, encoding an *invA*sion protein responsible for pathogenicity of *Salmonella* while the *ttr* gene, encoding tetrathionate reductase responsible for *S. enterica* metabolism are suitable target for *Salmonella* detection (Abdel-Aziz, 2016; Mohammed, 2024; Nair, et al., 2019). When the genome of *S. enterica* was used as the DNA template, the expected DNA bands were observed (Figure 1). Cross-reactivity to *E. coli* genome was not exhibited. The results of three replicates were similar. Multiplex PCR was conducted under similar conditions to conventional PCR. The results (see Figure 2) revealed two expected DNA bands when *S. enterica* genomic DNA was used as the template. The results suggested that our Multiplex PCR assay was relatively specific to *S. enterica*. Therefore, this method was chosen for further experiments.

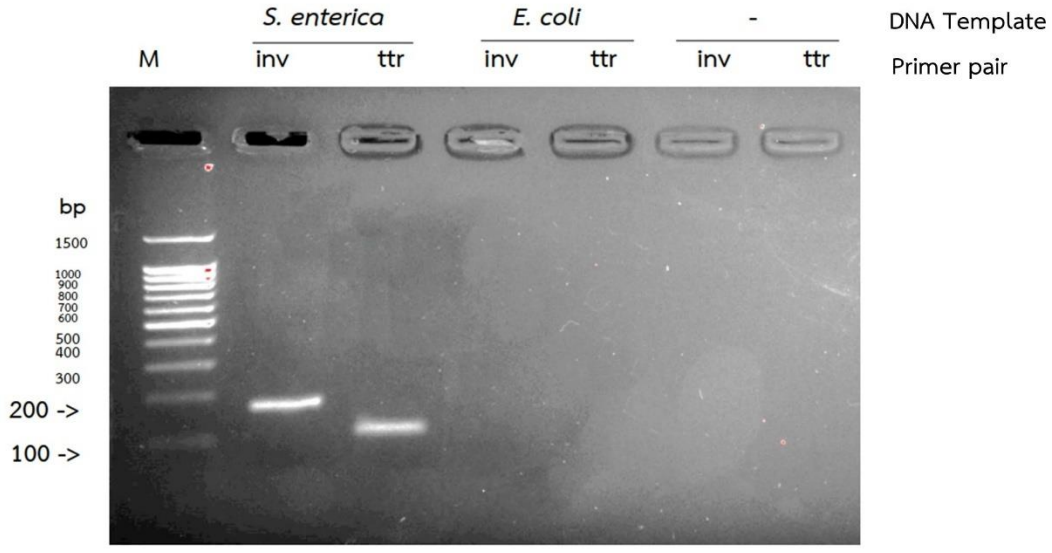

Figure 1 Visualization of Convention PCR products on 1.5% agarose gel electrophoresis. Primers specific to *invA* and *ttr* gene of *S. enterica* were used and expected bands of 172 and 94 bp, respectively, were observed. Cross reaction with *E. coli* DNA was not detected. Three replicates of experiments gave similar results (data not shown). (M = 100 bp DNA ladder, - = no template control)

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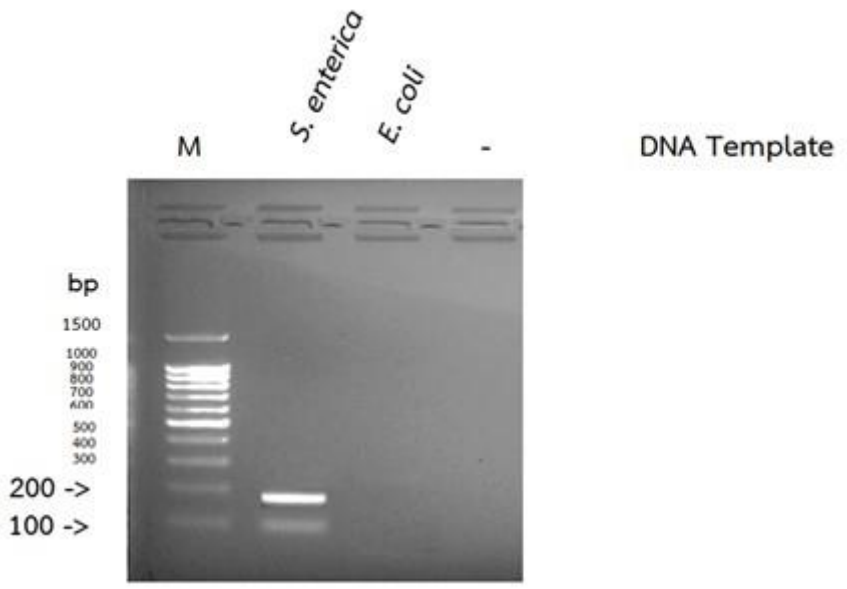

Figure 2 Visualization of Multiplex PCR products on 1.5% agarose gel electrophoresis. Primers specific to *invA* and *ttr* genes of *S. enterica* were added in the single reaction and expected bands of 172 and 94 bp were observed. Cross reaction with *E. coli* DNA was not detected. Three replicates of experiments gave similar results (data not shown). (M = 100 bp DNA ladder, - = no template control)

Four egg samples were bought from grocery stores near Ramkhamhaeng University, Huamark Campus. Only eggshells were collected and ground. One gram of eggshell powder was subjected to DNA extraction. The concentration and purity of harvested DNA from each eggshell sample were calculated from absorbance at 260 nm (A260) and A260/280, respectively (Table 4).

Table 4 Concentration and purity of DNA harvested from eggshell samples

Sample	A260	A280	DNA concentration (µg/ml)	DNA purity (A260/A280)
1	0.053	0.048	2.65	1.09
2	0.060	0.070	3.00	0.85
3	0.039	0.034	1.95	1.15
4	0.292	0.246	14.60	1.19

DNA from these eggshell samples was used as the DNA template for conventional and Multiplex PCR under the conditions described above. Only an expected band from the primer pair targeting the *ttr* gene of *S. enterica* was observed from Samples 2 and 3 (Figures 3 and 4).

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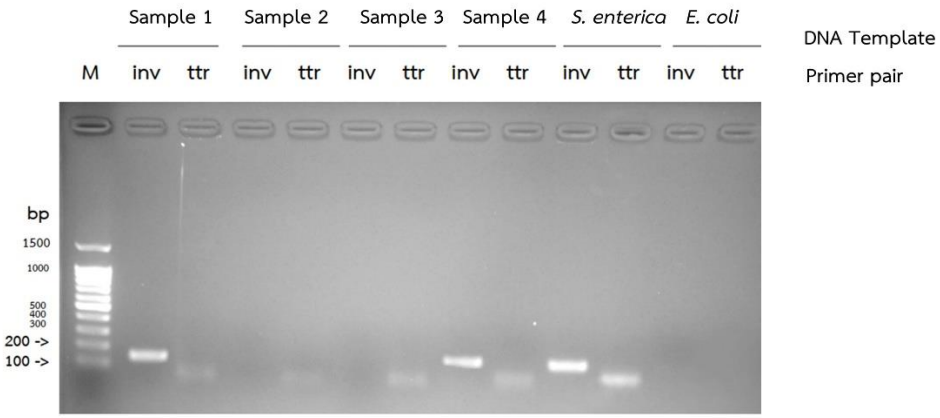

Figure 3 Visualization of Convention PCR products on 1.5% agarose gel electrophoresis. Primers specific to *invA* and *ttr* gene of *S. enterica* were used to detect *S. enterica* from eggshell samples. Both expected bands of 172 and 94 bp were observed in Sample 1 and 4. For Sample 2 and 3, only 94 bp band from *ttr* primer pair was observed. Three replicates of experiments gave similar results (data not shown).
(M = 100 bp DNA ladder)

Figure 3 Visualization of Multiplex PCR products on 1.5% agarose gel electrophoresis. Primers specific to *invA* and *ttr* gene of *S. enterica* were added in the single reaction. Two expected bands of 172 and 94 bp were observed from Sample 1 and 4. For Sample 2 and 3, only 94 bp band from *ttr* primer pair was observed. Three replicates of experiments gave similar results (data not shown).
(M = 100 bp DNA ladder)

In this research, although Multiplex PCR gave expected results when *S. enterica* was used as the DNA template, only one expected band was observed in some eggshell samples. As mentioned above, one of the major challenges of Multiplex PCR is to find the optimal condition that all primers added in the reaction appropriately work. Two selected pairs of primers have relatively different Tm (Table 1). They gave desirable results with genomic DNA; however, with the samples containing mixture of DNA, the effectiveness was possibly lower. Therefore, primer selection, optimal conditions, and more experiments with additional samples are necessary for our further study. Moreover, false-positive results were reported when targeting *inv* genes for PCR (Resendiz-Nava et al., 2019). Although this research gave contrast results to that report, the difference of *inv* genes among subspecies of *S. enterica* is a topic that needs to be studied.

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The detection of *S. enterica* using Multiplex PCR provided valuable insights into the presence of contamination on eggshells; however, discrepancies in gene amplification were observed. While both *invA* and *ttr* genes were successfully detected in Samples 1 and 4, and only the *ttr* gene was amplified in Samples 2 and 3. This selective gene detection raises important questions regarding the reliability of single-gene PCR assays. The absence of *invA* amplification in some samples could be attributed to biological factors such as genetic variability in *S. enterica* strains or differences in gene expression under specific environmental conditions. Prior studies have reported sequence variations or mutations in the *invA* gene that could hinder primer binding, leading to false-negative results (Resendiz-Nava et al., 2019). Alternatively, the technical efficiency of PCR may have played a role in this discrepancy. The *ttr* gene, which encodes tetrathionate reductase, is highly conserved and may have higher amplification efficiency than *invA* when using mixed DNA templates (Mohammed, 2024). The presence of PCR inhibitors in the extracted DNA or variations in DNA quality among samples could also contribute to differential amplification. These findings underscore the importance of using multiple genetic markers to enhance the reliability of *S. enterica* detection and reduce the risk of false negatives.

The limitations of Multiplex PCR were also evident when comparing the band clarity between Multiplex and conventional PCR assays. While conventional PCR produced distinct bands for both *invA* and *ttr* genes, Multiplex PCR bands appeared less defined. Several factors may contribute to this observation, including primer competition, reaction efficiency, and suboptimal PCR conditions. Primer competition is a common issue in Multiplex PCR, where multiple primers within the same reaction may compete for polymerase activity and template binding, leading to reduced amplification efficiency (Kawasaki et al., 2005). The difference in melting temperatures (Tm) between the *invA* and *ttr* primers (Table 1) may have also affected their simultaneous amplification, potentially resulting in preferential amplification of one gene over the other. Additionally, Multiplex PCR requires precise optimization of reaction conditions, including primer concentrations, annealing temperatures, and Mg^{2+} concentrations, to ensure equal amplification efficiency of all targets. The presence of PCR inhibitors in eggshell DNA extracts may have further impacted amplification efficiency, particularly in Multiplex PCR reactions. Despite these challenges, Multiplex PCR remains a valuable tool for rapid bacterial detection, as it allows for simultaneous identification of multiple target genes in a single reaction, reducing processing time and reagent consumption. Future studies should focus on optimizing reaction conditions to improve the clarity and consistency of Multiplex PCR results.

Epidemiological considerations are also crucial in interpreting the findings of this study. The detection of *S. enterica* genetic markers in all four eggshell samples suggests potential contamination in grocery stores near Ramkhamhaeng University, Huamark Campus. However, the study does not specify whether the samples originated from a single supplier or multiple sources. If all samples were collected from a single location, this could indicate localized contamination, whereas multiple sources would suggest a more widespread food safety issue. Previous studies have reported varying rates of *S. enterica* contamination in eggshells. Benevides et al. (2020) found that 15% of eggshell samples in Brazil tested positive for *S. enterica*, while Shanmugasamy et al. (2011) reported an 8.3% contamination rate in broiler carcasses in India. Compared to these findings, the presence of *S. enterica* in all four tested samples in this study suggests a potentially higher contamination rate, although the small sample size limits definitive conclusions. Further epidemiological studies with a larger dataset are needed to assess contamination risks in grocery stores and potential sources of bacterial transmission.

Future studies should focus on improving Multiplex PCR conditions to enhance detection reliability. Adjusting primer concentrations, optimizing annealing temperatures, and testing additional target genes could help improve assay sensitivity. Increasing the sample size and expanding the geographical scope of sample collection would provide more comprehensive data on *S. enterica* contamination trends. Additionally, whole-genome sequencing of detected strains could help identify specific serovars present in contaminated eggshells, contributing to a more detailed epidemiological analysis. Comparing Multiplex PCR results with traditional culture-based methods would also validate the accuracy of this molecular detection approach. By addressing these limitations, future research can further refine *S. enterica* detection techniques, ultimately improving food safety monitoring and reducing the risk of foodborne illness outbreaks.

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5. Conclusion

This research aims to develop Multiplex PCR techniques to detect *S. enterica* contamination on eggshells. Although the desirable results were obtained when using genomic DNA of *S. enterica* as the template, only one expected DNA product was observed in some samples. Therefore, to detect *S. enterica*, more than one gene region should be targeted. Multiplex PCR is a tool to amplify several specific target DNA regions in one reaction that is less time-consuming compared to conventional PCR; however, there are many challenges that need to be overcome. This research is a fundamental step to set up an effective technique to detect bacterial contamination in food.

6. Acknowledgements

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