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Development and Evaluation of a Multiplex Fluorescence PCR for *Salmonella* Virulence Genes Analysis

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Abstract

Background: Salmonellosis, a disease caused by *Salmonella*, is a significant public health concern and economic burden worldwide. The ability of various *Salmonella* serovars to cause disease is closely linked to the virulence genes they possess.

Objectives: The aim of this study was to develop a multiplex fluorescence PCR for detecting ten major virulence genes (*ssaR*, *spvC*, *pefA*, *sipA*, *fimA*, *sifA*, *sopE2*, *sopB*, *prgH*, and *stn*) in *Salmonella*.

Methods: Primer pairs specific to ten target virulence genes were designed using Primer Premier 5.0 and distributed across two reaction tubes. The multiplex fluorescence PCR was optimized by adjusting one factor at a time.

Results: A total of sixty *Salmonella* strains were analyzed using the newly developed multiplex fluorescence PCR. All strains contained seven or more of the tested virulence genes. The positive rates of virulence genes *ssaR*, *sipA*, *sopE2*, *sopB*, *prgH*, and *stn* were high, each at 100%. The positive rate of *sifA* was also relatively high at 81.67%. However, the positive rates of *spvC* at 5% and *pefA* at 3.33% were relatively low.

Conclusions: The newly developed multiplex fluorescence PCR provides a straightforward, cost-effective, and high-throughput solution for detecting virulence genes in *Salmonella*. It has the potential to become a routine method for analyzing *Salmonella* virulence genes.

Keywords: *Salmonella*,Virulence, Gene, Fluorescence, PCR

1. Background

Salmonellosis, a disease caused by *Salmonella*, represents a significant public health problem and economic burden worldwide (1-3). The transmission routes of *Salmonella* to humans are diverse, including consumption of contaminated food or water and contact with infected animals and humans. According to estimates from the World Health Organization (WHO), more than 90 million cases of human salmonellosis occur globally each year (4). Therefore, the control and prevention of salmonellosis are crucial.

The pathogenicity of *Salmonella* serovars depends on the presence of a variety of virulence genes, such as *ssaR*, *spvC*, *pefA*, *sipA*, *fimA*, *sifA*, *sopE2*, *sopB*, *prgH*, and *stn*. The *ssaR* and *sifA* genes are essential for the intracellular survival and subsequent replication of *Salmonella* (5-7), while the *pefA*, *sipA*, *fimA*, *sopE2*, *sopB*, and *prgH* genes are involved in adhesion and invasion of host cells (7-10). The *spvC* gene can suppress the innate immunity of infected hosts (11), and the stn gene is implicated in the complex mechanisms of diarrhea (12). Additionally, virulence genes can be transferred from one strain to another, enhancing the pathogenicity of *Salmonella* strains and potentially leading to disease outbreaks. For example, during the mid to late 1980s, there was an outbreak of typhoid fever in China characterized by severe symptoms and high mortality rates.

Subsequent studies identified the main causative agent of the epidemic as *Salmonella enterica serovar typhi* (*S. typhi*) strains carrying the *Salmonella* plasmid virulence (spv) gene, which is typically absent in *S. typhi* (13). Investigating the presence of virulence genes in *Salmonella* strains is crucial for identifying pathogenicity, understanding potential transfer mechanisms, and assessing the risks posed by these

strains. Thus, developing an efficient method for detecting *Salmonella* virulence genes is necessary for disease prevention and control.

PCR, including conventional and fluorescence PCR, is one of the most commonly used techniques for gene analysis. Fluorescence PCR introduces a fluorescent reporter (dsDNA binding dye or fluorophore-labeled probe) that binds to the amplification product and indicates its presence by fluorescence, allowing for real-time detection of the amplification product during the PCR reaction. The entire fluorescence PCR process can be completed in a closed-tube setting without any manual operation. Compared to conventional PCR, fluorescence PCR is simpler, faster, and easily automated for high-throughput testing (14). However, reports on the application of fluorescence PCR for analyzing *Salmonella* virulence genes are limited.

2. Objectives

The aim of this study is to develop a multiplex fluorescence PCR for detecting ten major virulence genes (*ssaR*, *spvC*, *pefA*, *sipA*, *fimA*, *sifA*, *sopE2*, *sopB*, *prgH* and *stn*) in *Salmonella*.

3. Methods

3.1. Strains

A total of 60 *Salmonella* strains, 2 *Vibrio parahaemolyticus* strains, and 3 *Escherichia coli* strains were utilized in this study. One *S. typhimurium* strain and two *Salmonella* London strains were isolated from meat, while other strains were isolated from patients with diarrhea. All strains were isolated and confirmed by biochemical characterization according to GB 4789 (national standards for microbiological examination of food in the People's Republic of China) and WS 271 (health standards for the diagnosis of infectious diarrhea in the People's Republic of China). Five *Salmonella* strains, comprising 3 strains of *S. enteritidis* and 2 strains of *S. typhimurium* (Table 1), were chosen as positive controls. Additionally, two *V. parahaemolyticus* and three *E. coli* strains served as negative controls. The virulence gene profiles of these control strains were detected by previously reported conventional PCR (15-20). Genomic DNA from all strains was extracted using the KAPA Express extract kit, following the manufacturer's instructions.

3.2. Oligonucleotide Primers

The DNA sequences of ten major virulence genes in *Salmonella* were downloaded from GenBank and aligned using Clustal X 1.8. Based on this sequence alignment, relatively conserved regions were selected for primer design using Primer Premier 5.0. The details of the ten primer pairs targeting these genes are shown in Table 2.

3.3. Optimization of Multiplex Fluorescence PCR

Primer pairs for ten virulence genes were distributed across two reaction tubes, with the distribution of these pairs detailed in Table 2. The multiplex fluorescence PCR was optimized by adjusting one parameter at a time. The parameters evaluated included the target primer concentration, which ranged from 0.05 μM to 1 μM, and the annealing temperature, which varied from 55 °C to 65 °C.

The multiplex fluorescence PCR was conducted using a 20 μL reaction mixture for each tube, consisting of 1 μL of DNA template, 1 μL of EvaGreen dye, 10 μL of KAPA 2G Fast Multiplex PCR Mix, appropriate amounts of target primers, and the remaining volume filled with distilled water. The PCR amplification took place in a Bio-Rad CFX 96TM real-time PCR system with the following program: An initial denaturation at 95 °C for 3 minutes, followed by 30 cycles of denaturation at 95 °C for 15 seconds, annealing for 30 seconds at the specified annealing temperature, and extension at 72 °C for 10 seconds.

3.4. Melting Temperature Curve Analysis

Fluorescence melting temperature curve analysis was conducted following PCR amplification. The PCR products were initially cooled to 78 °C and subsequently heated to 91 °C at a rate of 0.1 °C per second. Fluorescence signals were continuously monitored throughout the melting temperature curve analysis process.

4. Results

4.1. Optimization of Multiplex Fluorescence PCR

To develop a multiplex fluorescence PCR capable of detecting ten primary virulence genes in *Salmonella*, a systematic study was conducted to optimize the conditions. The optimal primer concentrations were

Table 1. Virulence Genes Profiles of Control Strains

Isolates	Isolates No.	*ssaR*	*spvC*	*pefA*	*sipA*	*fimA*	*sifA*	*sopE2*	*sopB*	*prgH*	*stn*
Salmonella enteritidis	1	+	-	-	+	+	+	+	+	+	+
	1	+	-	-	+	+	-	+	+	+	+
	1	+	+	-	+	+	-	+	+	+	+
Salmonella typhimurium	1	+	+	+	+	+	+	+	+	+	+
	1	+	-	-	+	+	+	+	+	+	+
Vibrio parahaemolyticus	2	-	-	-	-	-	-	-	-	-	-
Escherichia coli	3	-	-	-	-	-	-	-	-	-	-

Table 2. Primer Sequences Used in This Study

Variables	Target Gene	Primer	Sequence
Tube1	*ssaR*	ssaR-F	5'- CAAAAAAACTCTGAAGAGAAGGAAGCC-3'
		ssaR-R	5'- GCCTGCGTTAACTGACTCACCGT-3'
	spvC	*spvC*-F	5'- TGGGGCGGAAATACCATCTACAAAT-3'
		spvC-R	5'- CTGAATAGTCAGGCACATCCAGCG-3'
	pefA	*pefA*-F	5'- ACCAGCCGGGTAATTTTGTTGACT-3'
		pefA-R	5'- CAGCAGAAGCCCAGGAAACAGTG-3'
	sipA	*sipA*-F	5'- CACGTCAGAAAAGGGCACTACGG-3'
		sipA-R	5'- CTCGACGCCAGCCAGTTGACTG-3'
	fimA	*fimA*-F	5'-TAATGACCTCTACTATTGCGAGTCTGATGTT-3'
		fimA-R	5'- AGAAAGCCACGGCAGCGTTG-3'
Tube2	*sifA*	*sifA*-F	5'- TTAAAAAGTGAAATCCTTACCAACTCCCC-3'
		sifA-R	5'- TCCGCTTTTGCTTTGCCAGTAGAA-3'
	sopE2	*sopE2*-F	5'- TAACATAACACTATCCACCCAGCACTACAG-3'
		sopE2-R	5'- TAAAACGATCTGACAGACTCGTCGACA-3'
	sopB	*sopB*-F	5'- ATAAAGATGGCGATCTACAGACGGTAAA-3'
		sopB-R	5'- CCCACATTAAATGCGGCGACG-3'
	prgH	*prgH*-F	5'- CGGCTGTGAGTTTCCATTGCTG-3'
		prgH-R	5'- TTCTAACTTCTCAGGCTGCTCGGG-3'
	stn	*stn*-F	5'- CGCCAGCCCTTTGATGGACG-3'
		stn-R	5'- GGCATCAGCGTTATCAGCGCTC-3'

determined as follows: In tube 1, 0.4 μM for *ssaR*, 0.4 μM for *spvC*, 0.3 μM for *pefA*, 0.1 μM for *sipA*, and 0.1 μM for *fimA*; in tube 2, 0.4 μM for sifA, 0.2 μM for sopE2, 0.2 μM for *sopB*, 0.05 μM for *prgH*, and 0.1 μM for *stn*. The optimal annealing temperature was established at 60°C.

4.2. Melting Temperature Curve Analysis of PCR Products

After PCR amplification and melting temperature curve analysis, ten distinct melting peaks were observed. The melting peak temperatures for the PCR products were as follows: *ssaR* primer pairs at 80.7 ± 0.3°C, *spvC* primer pairs at 82.8 ± 0.2°C, *pefA* primer pairs at 85.5 ± 0.1°C, *sipA* primer pairs at 87.4 ± 0.2°C, and *fimA* primer pairs at 89.0 ± 0.2°C (Figure 1). Additionally, the melting peak temperatures for *sifA* primer pairs were at 79.6 ± 0.2°C, *sopE2* primer pairs at 81.8 ± 0.3°C, *sopB* primer pairs at 84.3 ± 0.2°C, *prgH* primer pairs at 86.3 ± 0.1°C, and *stn* primer pairs at 88.1 ± 0.3 °C (Figure 2).

4.3. Evaluation of Specificity of Multiplex Fluorescence PCR

Analysis of five positive controls and five negative controls using the newly developed multiplex fluorescence PCR showed that the five positive controls produced specific melting peaks, whereas no obvious melting peaks were observed in the five negative controls. The virulence genes profiles in the five positive

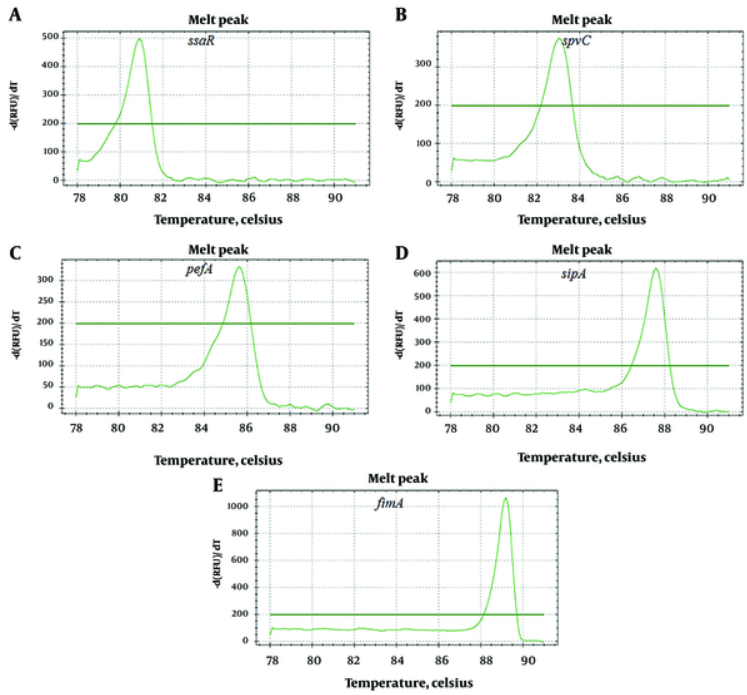

Figure 1. Melting peaks of virulence genes (*ssaR*, *spvC*, *pefA*, *sipA* and *fimA*) in tube 1

controls detected by the newly developed multiplex fluorescence PCR were consistent with our previous results achieved by conventional PCR.

4.4. Analysis of Salmonella Strains

The distribution of 10 virulence genes in 60 *Salmonella* strains is summarized in Table 3. All strains contained seven or more of the tested virulence genes. The positive rates for virulence genes *ssaR*, *sipA*, *sopE2*, *sopB*, *prgH*, and *stn* were 100%, while *sifA* was 81.67%. However, the positive rates for *spvC* and *pefA* were relatively low, at 5% and 3.33% respectively. These results were confirmed by previously reported conventional PCR (15-20).

5. Discussion

Infection with *Salmonella* is still one of the most important public health problems in the world. It can cause diseases ranging from gastroenteritis to typhoid fever (21). The pathogenicity of *Salmonella* strains is determined by their abilities to adhere, invade and replicate inside host cells, as well as to escape from or neutralize the host's defenses (7, 22). These abilities of *Salmonella* strains depend on the virulence genes that they possess. In many cases, when a *Salmonella* serotype acquires new virulence genes that were previously absent, it can enhance pathogenicity of *Salmonella* strains and cause disease outbreak (13). Therefore, analysis of virulence genes profiles of *Salmonella* strains is necessary for preventing outbreak of disease caused by *Salmonella* infection. As one of the most widely used techniques in genes analysis, several PCR methods have been developed for *Salmonella* virulence genes analysis (15-20). However, these reported PCR methods for Salmonella virulence genes analysis are conventional PCR.

The drawbacks of conventional PCR are obvious. First, post-PCR analysis depend on gel electrophoresis is necessary for conventional PCR. The procedure of post-PCR analysis is complex and labor-intensive, which results in low throughput of the conventional PCR. Second, conventional PCR has the relative high risk of cross contamination as the essential step of opening PCR tube for post-PCR analysis. Therefore, other method

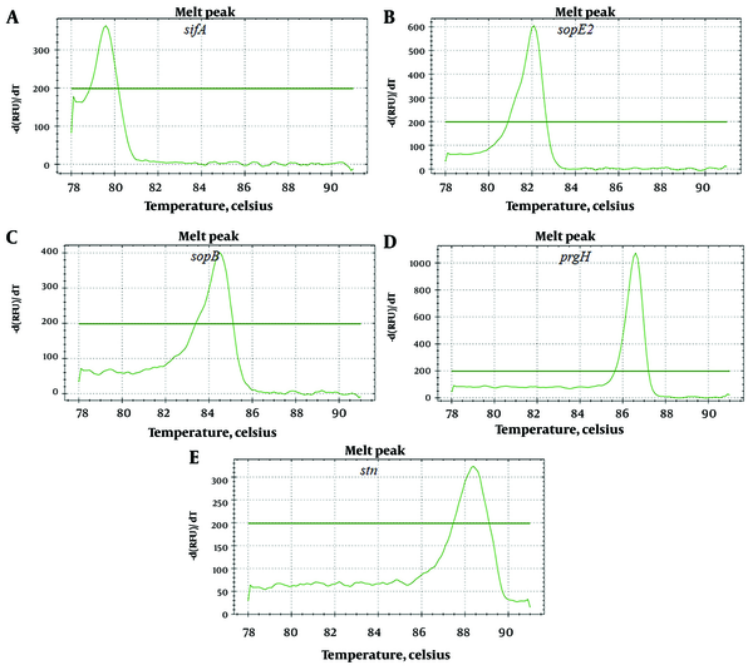

Figure 2. Melting peaks of virulence genes (*sifA*, *sopE2*, *sopB*, *prgH* and *stn*) in tube 2

Table 3. Virulence Genes Profiles of Tested *Salmonella* Strains

Isolates	Isolates No.	*ssaR*	*spvC*	*pefA*	*sipA*	*fimA*	*sifA*	*sopE2*	*sopB*	*prgH*	*stn*
Salmonella enteritidis	11	+	-	-	+	+	+	+	+	+	+
Salmonella enteritidis	5	+	-	-	+	+	-	+	+	+	+
Salmonella enteritidis	1	+	+	-	+	+	-	+	+	+	+
Salmonella typhimurium	1	+	+	+	+	+	+	+	+	+	+
Salmonella typhimurium	8	+	-	_	+	+	+	+	+	+	+
Salmonella stanley	7	+	-	_	+	+	+	+	+	+	+
Salmonella agona	3	+	-	_	+	+	-	+	+	+	+
Salmonella london	5	+	-	_	+	+	+	+	+	+	+
Salmonella london	1	+	-		+	+	-	+	+	+	+
Salmonella essen	3	+	-	-	+	+	+	+	+	+	+
Salmonella give	1	+	+	+	+	+	+	+	+	+	+
Salmonella tshiongwe	1	+	-	-	+	+	-	+	+	+	+
Salmonella montevideo	2	+	-	-	+	+	+	+	+	+	+
Salmonella abony	3	+	-	-	+	+	+	+	+	+	+
Salmonella weltevreden	5	+	-	-	+	+	+	+	+	+	+
Salmonella dublin	3	+	-	-	+	+	+	+	+	+	+

have been developed for *Salmonella* virulence genes analysis. Though these methods have their advantages, they can't completely replace PCR in the field of *Salmonella* virulence genes analysis. For example, microarray has a higher throughput than conventional PCR in *Salmonella* virulence genes analysis, PCR is still needed to validate the microarray data demonstrating weaker signals.

In this study, we developed a multiplex fluorescence PCR for *Salmonella* virulence genes analysis. Fluorescence PCR differs from the traditional PCR. The whole detection process of fluorescence PCR could be completed in a closed-tube setting and the post-PCR analysis depend on gel electrophoresis is not required.Without post-PCR analysis, fluorescence PCR increases assay throughput and reduces the risk of cross contamination (23). Thus, our newly developed multiplex fluorescence PCR for Salmonella virulence genes analysis overcomes the drawbacks of conventional PCR. Our multiplex fluorescence PCR encompasses ten main virulence genes (*ssaR*, *spvC*, *pefA*, *sipA*, *fimA*, *sifA*, *sopE2*, *sopB*, *prgH*, and *stn*), which play crucial roles in the diseases caused by *Salmonella*. These genes are significant indicators of pathogenicity, facilitating the ability of *Salmonella* to adhere to, invade, and replicate inside host cells, and to escape from or neutralize the host's defenses (5, 12).

In this study, all analyzed strains contained seven or more of the tested virulence genes. The positive rates for virulence genes such as *ssaR*, *sipA*, *sifA*, *sopE2*, *sopB*, *prgH*, and *stn* were relatively high, whereas those for *spvC* and *pefA* were relatively low. The distribution of most virulence genes in our study aligns with results from previous reports. For instance, Yue et al. (15) analyzed 61 *Salmonella* isolates from the feces of children with acute diarrhea and found that all isolates (100%) tested positive for *prgH*, 51 isolates (83.61%) for *sopB*, and 11 isolates (18.03%) for *pefA*. Another study by Qiao et al. (24) reported prevalence rates for the virulence genes *sipA*, *pefA*, and *spvC* at 77.6%, 10.3%, and 1.9%, respectively.

5.1. Conclusions

In summary, the newly developed multiplex fluorescence PCR provides a straightforward, cost-effective, and high-throughput method for detecting virulence genes in *Salmonella*. Thus, it has the potential to become a routine method for analyzing *Salmonella* virulence genes.

Footnotes

Authors' Contribution: PH and ZC designed research, GZ and YY identified strains, PH and ZZ analyzed data and wrote the paper with the input from other authors.

Conflict of Interests Statement: The authors declare no competing interests.

Data Availability: The data presented in this study are openly available in one of the repositories or will be available on request from the corresponding author by this journal representative at any time during submission or after publication.

Ethical Approval: Institute Ethics Committee, Jiaxing Center for Disease Control and Prevention has approved the work with the reference number: 2022-05/October 11, 2022.

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Informed Consent: Informed consent was obtained from all participants.

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