ESTABLISHMENT OF MULTIPLEX PCR ASSAY FOR SIMULTANEOUS DETECTION OF SALMONELLA SPP., SHIGELLA SPP. AND ENTAMOEBA HISTOLYTICA

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ABSTRACT

Objective: To establish a novel multiplex PCR assay for simultaneous detection of foodborne pathogens, including *Salmonella* spp., *Shigella* spp., and *Entamoeba histolytica*, with high sensitivity, specificity, cost-effectiveness and time-saving.

Method: Multiplex PCR, Semi-Nested PCR and melting analysis for specifically amplifying and distinguishing three highly conserved target gene regions (*invA, ipaH, 18S rRNA*), in order to simultaneously detect *Salmonella* spp., *Shigella* spp., *Entamoeba histolytica* in a single reaction tube. Specific pathogens are characterized based on the melting profiles of the PCR products collected on a realtime PCR instrument following the second round of amplification.

Results: The established multiplex PCR assay is capable of simultaneously detecting *Salmonella* spp., *Shigella* spp., and *Entamoeba histolytica* at a limit of detection of 5.86 copies/µl, 24.4 copies/µl và 53.6 copies/µl, respectively (confidence interval of 95%), the specificity reached 100% when evaluated with multiple microbial species provided by the Department of Microbiology, 103 Military Hospital.

Conclusion: The novel multiplex PCR assay for simultaneous detection of *Salmonella* spp., *Shigella* spp., *Entamoeba histolytica* has been successfully established.

Keywords: Multiplex PCR, Semi-Nested PCR, *Salmonella spp., Shigella spp.,* Entamoeba histolytica.

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1. INTRODUCTION

Along with the development of food technology and agricultural production, foodborne diseases, which tend to be a burden on economic and human health, have gradually become a major concern for community [9]. According to the World Health Organization (WHO), the most common foodborne pathogens in the West Pacific region including Norovirus, Salmonella, Campylobacter, *Shigella* spp., Giardia, *Entamoeba histolytica*, and Hepatitis A virus [2].

Shigella spp. is the causative agent of an infection called shigellosis. In 2002, many studies showed that *Shigella* spp. was the third most pathogenic bacteria present in food in the United States [6]. There are about 400 000 cases of shigellosis each year, however only about 20 000 cases are reported [4]. Therefore, determining the presence of *Shigella* spp. in food samples plays a vital role in accurate and effective monitoring and preventing the disease.

Salmonella is the most common cause of food poisoning caused by bacteria. Salmonella infection remains a serious global health problem and affects gravely in industrialized and undeveloped countries. There are plenty of serious complications that could be found such as bacteremia, osteomyelitis, and meningitis which have to be treated with antibiotics [3]. Besides, the continuous heredity and genomic evolution in Salmonella lead to increased virulence and drug resistance, which are of major public health concern [7].

Amoebiasis caused by *Entamoeba histolytica* tends to occur in areas with poor socioeconomic conditions and poor sanitation. WHO estimates that amoebiasis is one of the three most common causes of death due to parasitic diseases causing up to 100,000 deaths annually [1]. Meanwhile, molecular methods, including PCR and Realtime PCR are potential methods for determining the presence of *E. histolytica* in a variety of clinical specimens, including stool, tissue, and fluid aspirate liver abscesses [8]. The sensitivity and specificity of the PCR test have shown outstanding efficiency and overcome the limitations of the others .

Therefore, in this study, we aim to establish a novel multiplex PCR assay to simultaneously detect *Shigella* spp., *Salmonella* spp. and *E. histolytica,* with high sensitivity and specificity. The multiplex PCR assay is a method which combines traditional multiplex PCR, semi-nested PCR, and the melting analysis of the PCR products. As a result, the novel method can simultaneously detect multiple pathogens in one reaction tube while ensuring sensitivity and specificity for each amplified target gene. The limit of detection (analytical sensitivity) of the assay is evaluated using the PODLOD application. In addition, different microbial strains are also collected to evaluate the analytical specificity of the established multiplex.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Positive control

The target gene sequences *invA, ipaH, 18S rRNA* (*Salmonella* spp., *Shigella* spp., *Entamoeba histolytica*) were synthesized into double-stranded DNA fragments with G-block structure (Phu Sa Biochem, Can Tho). Lyophilized G-block DNA was recovered in

30 µl H2O according to the manufacturer's instructions and directly diluted to serve as a standard for evaluating the amplification efficiency of simplex PCR assay and multiplex PCR assay.

2.1.2. Other specimens and strains of other microorganisms:

Samples positive for Hepatitis B virus (02 samples), strains of *Enterobacter aerogenes*, *Citrobacterfreundii, Pseudomonas aeruginosa, Acinetobacter baumannii, Proteus mirabiles, Klebsiella pneumoniae, Staphylococcus homitis, Stenotrophomonas maltophilia, Neisseria gonorrhoae* were collected at Department of Microbiology, 103 Military Hospital. Sample types provided included blood samples, or cultures of bacterial strains and were extracted using the QIAamp DNA Mini Kit (50) according to the manufacturer's instructions.

2.2. Methods

2.2.1. Primer design

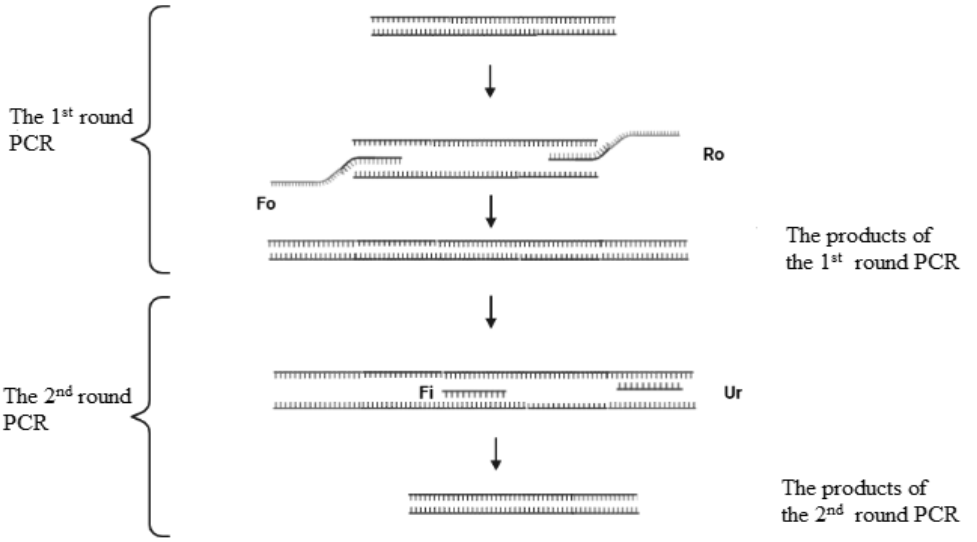
The primer sequences for PCR round 1, PCR round 2 were designed to amplify target gene regions of *invA, ipaH, 18S rRNA* specifically to detect *Salmonella* spp., *Shigella* spp., and *Entamoeba histolytica.* Through polymorphism assessment, the nucleotide sequence region 1919220-1919310 on the *ipaH* gene (access number NC_004337.2), the nucleotide sequence region at positions 3039930 to 3040040 on the *invA* gene (sequence access code NC_003197. 2), and the nucleotide sequence region 1266-1472 on the *18S rRNA* gene (sequence access number AB197936.1) is a highly conserved sequence region with low polymorphism in most strains of *Shigella* spp., *Salmonella* spp., and *Entamoeba histolytica.*

The BLASTN tool (NCBI) was used to compare primer sequences with the BLAST Nucleotide database to check the specificity of each primer. Other software and tools such as OligoAnalyzer (IDT), and DINAMelt are used in combination to evaluate the technical parameters affecting the PCR process of the designed primer pairs, including the length of an amplified gene fragment, annealing temperature, %GC, %AT, the possibility of mispairing (hetero-dimer), intramolecular pairing (self-dimer), and ability to form an intramolecular secondary structure (hairpin).

2.2.2. Principle of multiplex PCR

The multiplex RT-PCR method used in the study consisted of two rounds: the 1st round PCR uses outer primer pairs (Fo, Ro) which are specific for different target genes to enrich target sequences; then the products of the 1st round PCR are used as a template for the 2nd round PCR. More specifically, there are many different forward primers (Fi) which are specific for each target, and only the sole universal reverse primer (Ur) are applied in the 2nd round of PCR (Figure 1). The pathogens are distinguished from each other by the specific melting temperature of the product after amplification of the 2nd round PCR (amplicon) analyzed on a Real-time PCR instrument.

Figure 1: Principle of multiplex PCR



Note: The 1st round PCR uses forward outer primers (Fo) and reverse outer primers (Ro) for pre-amplification; the 2nd round PCR uses inner forward primers (Fi) which are specific to each target gene, and a sole universal reverse primer for all targets (Ur).

2.2.3. Multiplex PCR reaction components

Components of the 1st PCR reaction: The total volume of the 1st PCR reaction used is 20 µl including 4 µl of PCR buffer with reverse transcriptase enzyme addition, and 5 µl of amplification template volume; primer volume ensures final concentration of *Salmonella* spp., *Shigella* spp., *Entamoeba histolytica* were 40 nM, 80 nM, and 40 nM, respectively, with the remaining volume being nuclease-depleted water.

Components of the second round PCR reaction: The second round PCR reaction has a total volume of 20 µl with 4 l of PCR buffer, 2 µl of the amplification template volume, and the concentration of reverse primer for the second round PCR is 0.2 µM, the primer concentration optimal downstream for *Salmonella* spp., *Shigella* spp., and *Entamoeba histolytica* were 0.2µM, 0.8µM, and 0.2µM, respectively; de-nuclease water was added to ensure a total reaction volume of 20 µl.

2.2.4. Multiplex PCR thermal cycling program

Table 1: Thermal cycling program of 1st round PCR

Steps		Temperature	Time	Unit
Denaturation		95	15	min
Amplification 1	Denaturation	94	15	sec
Cycle: 6	Annealing	63	3	min
	Extension	72	30	sec
Amplification 2	Denaturation	94	15	sec
Cycle: 22	Annealing	76	3	min
	Extension	72	30	sec
Extension		72	10	min
Incubate		4	∞	

Table 2: Thermal cycling program of the 2nd round PCR

Steps		Temperature	Time	Unit
Denaturation		95	15	min
Amplification N° Cycle: 35	Denaturation	94	15	sec
	Annealing	63	30	sec
	Extension (Green)	72	30	sec
HRM		95	1	min
		70-95	0.2°C – 2s	

2.2.5. Limit of detection

First, we prepared the concentration range of *invA, ipaH, 18S rRNA* target genes by diluting 10 times from 10^{10} copies/µl to 10^{9} copies/µl, 10^{8} copies/µl, etc., 10^{1} copies/µl. The LOD was then investigated by performing 5 replicates with concentrations in the range of 10^{4} copies/µl, 10^{3} copies/µl, 10^{2} copies/µl, and 10^{1} copies/µl. Then, higher number of replicates (25 - 28) for dilutions with low concentrations was applied if the screening results show that the LOD of the assay is within the tested range; otherwise continue screening process with multiplex PCR assays using lower concentrations of DNA templates to determine the detection threshold.

The data collected from the laboratory results were used as input for the determination of the assay detection threshold using the PODLOD application (version 9).

2.2.6. Evaluation of the specificity

The specificity of the multiplex PCR assay are evaluated by cross-reactivity/no-misdetection test with DNA/RNA samples extracted from patient samples positive for *Enterobacter aerogenes, Citrobacter freundii,* Hepatitis B virus (02) samples), *Pseudomonas aeruginosa, Acinetobacter baumannii, Proteus mirabilis, Klebsiella pneumonia, Staphylococcus hominis, Stenotrophomonas maltophilia, Neisseria gonorrhoea*.

3. RESULTS AND DISCUSSION

3.1. Primer sequence

Target gene-specific primer sequences *invA, ipaH, 18S rRNA* for the 1st round PCR, the 2nd round PCR detect *Salmonella* spp., *Shigella* spp., and *Entamoeba histolytica* in multiplex PCR is showed in Table 3. The Blast results indicated all the primer sequences are specific to most *Salmonella* spp., *Shigella* spp., *Entamoeba histolytica* and did not mismatch with sequences of other species (Appendix).

Table 3: Primer sequences of multiplex PCR

Target gene	Primer	Sequences (5'-3')
ipaH	Fo	***CGACAACGTGCGACAGGAAGTTA***TTGTGGATGAGATAGAAGT
	Ro	***CTGGCATATCATGACATACGACCTGA***ACACGCCATAGAAACGCAT
	Fi	TCGCAGAGAAACTTCAGCT
18S rRNA	Fo	***CGACAACGTGCGACAGGAAGTTAATGA***TTTATTGGGTAGTGGTG
	Ro	***CTGGCATATCATGACATACGACCTGATAA***GAAGTGGTACTTACGCA
	Fi	GGTGGAGTGATTTGTCAGGTT
invA	Fo	***CGACAACGTGCGACAGGAAGTTAG***CGATTTTCTCTGGATGGTAT
	Ro	***CTGGCATATCATGACATACGACCTGA***TGGCTTTCCCTTTCCAGTA
	Fi	CGATTTTCTCTGGATGGTATGC
	Ur	CTGGCATATCATGACATACGACCTGA

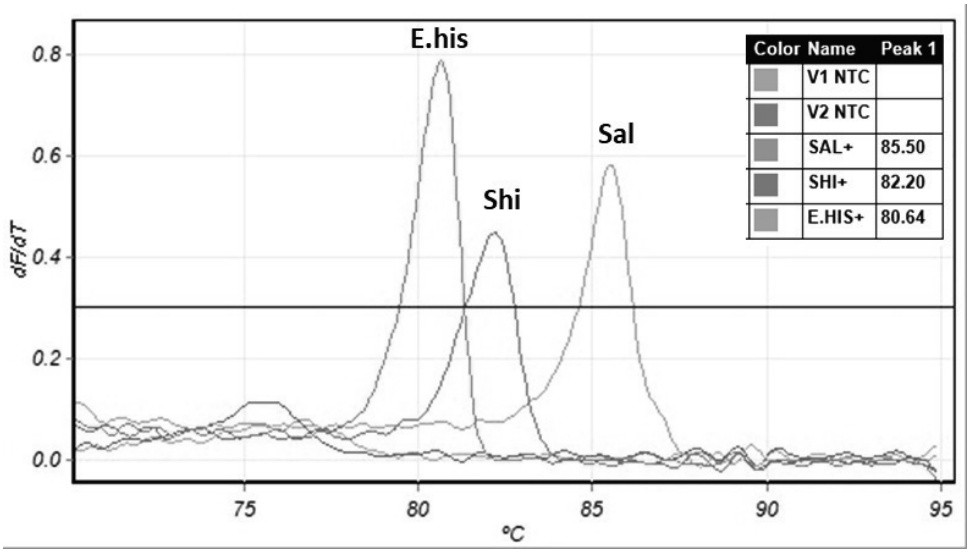
(Note: The italicized part corresponds to the added synthesis sequence)

3.2. Multiplex PCR for simultaneous detection of *Salmonella* spp., *Shigella* spp., *Entamoeba histolytica*

We performed multiplex PCR to test the ability to specifically detect amplification targets in the same reaction tube. The results of the analysis of the melting temperature presented in Figure 2 show that when performing multiplex PCR, the assay is capable of detecting specific pathogens with specific product melting peaks, consistent with the results obtained when analyzing the melting temperature spectrum

of simplex PCR products to detect *Salmonella* spp., *Shigella* spp., *Entamoeba histolytica* (Supplementary 5). Besides, PCR negative control round 1 (V1-) and negative control round 2 (NTC) have insignificant by-product peaks and are different from the main product melting peaks, thus, the multiplex PCR assay is capable of ability to simultaneously detect *Salmonella* spp., *Shigella* spp., and *Entamoeba histolytica.*

Figure 2: Multiplex PCR for simultaneous detection of Salmonella spp., Shigella spp., Entamoeba histolytica



In order to easily analyze the results by melting peak analysis, the analysis data of the melting temperature spectrum through different experiments were also collected, synthesized and analyzed by us to give a range of values specific melting peak values for *Salmonella* spp., *Shigella* spp., and *Entamoeba histolytica* were 84-86.34 °C, 81.5-82.9 , and 79.8-81.16 °C, respectively (Table 4). The corresponding standard deviations for relatively low ranges of values indicate that the melting analysis data collected over different runs do not differ or fluctuate significantly from the mean.

Table 4: Summarize the range of melting peak values specific to each amplification target

Pathogen	Number of experiments (N)	Melting peak average values	Range of melting peak	Standard deviation
Salmonella spp.	171	85.056	84-86.34	0.362
Shigella spp.	146	82.169	81.5-82.9	0.275
Entamoeba histolytica	47	80.306	79.8-81.16	0.368

3.3. Limit of detection

The results of the LOD study of the multiplex PCR are presented in Table 5. The LOD values determined at 95% confidence interval (PODLOD calculator program) show that the multiplex PCR assay is capable of simultaneously detecting *Salmonella* spp., *Shigella* spp., and *Entamoeba histolytica* with detection limit of 5.86 copies/µl, 24.4 copies/µl and 53.6 copies/µl, respectively (confidence intervals of 95%). Compared with the results of the LOD assessment with the simplex PCR assay (Supplementary 4)), it was found that the multiplex PCR assay has the same detection limit as the simplex PCR assay for detecting each pathogen. Thus, the established multiplex PCR assay is capable of simultaneously detecting *Salmonella* spp., *Shigella* spp., and *Entamoeba histolytica* while ensuring analytic sensitivity for each pathogen.

Table 5: Limit of detection of multiplex PCR assay

Concentration (copies/ µl)	**Replications**	**Multiplex PCR for detecting *Salmonella* spp.**	**Multiplex PCR for detecting *Shigella* spp.**	**Multiplex PCR for detecting *Entamoeba histolytica***
10^2	6	6	6	6
5 x 10^1	25	/	25	25
2 x 10^1	25	/	25	13
10^1	25	25	23	12
5 x 10^0	25	25	0	_
2 x 10^0	6	0	_	_
LOD (copies/ µl)		**5,86**	**24,4**	**53,6**
Confidence interval (95%)		**3,9-9,04**	**18,0-33,12**	**38,90-73,80**

Note: (_) did not perform the test to determine the limit of detection at this concentration because the percentage of positive results at the previous concentration was low. (/) for Salmonella spp. when conducting LOD detection with concentrations of 10^2, 10^1 copies/µl, the percentage for positive results was 100%, so we carried out LOD detection and repeated at lower positive control concentrations and do not repeat the experiment at concentrations between 5 x 10^1 and 2 x 10^1 copies/µl.

3.4. Analytical specificity

The specificity of **t**he established multiplex PCR assay was evaluated on different microbial strains as shown in Table 6. In a study in South Africa, when conducting microbiological analysis of different ready-to-eat food samples, researchers have shown that up to 18% of *Enterobacter* spp. present in the collected samples [5], cross-reactivity testing of established multiplex PCR on

enteric bacterial strains is necessary. The results (Table 6) indicate that the multiplex PCR assay does not have cross-reaction/ mismatch with *Enterobacter aerogenes, Citrobacter freundii, Proteus mirabilis strains* and ensure the specificity when evaluated on other microorganisms including Hepatitis B virus (02 samples); certain bacteria that cause hospital-acquired infections - *Pseudomonas aeruginosa, Stenotrophomonas maltophilia* and *Acinetobacter baumannii*; bacteria causing pneumonia *Klebsiella pneumoniae*; *Staphylococcus hominis;* and *Neisseria gonorrhea* bacteria. Thus, the established multiplex PCR assay has good specificity when evaluating in other strains of microorganisms.

Table 6: Analytical specificity of multiplex PCR assay

STT	Microorganisms	Result
1	*Enterobacter aerogenes*	Negative
2	*Citrobacter freundii*	Negative
3	Hepatitis B virus 1	Negative
4	Hepatitis B virus 2	Negative
5	*Pseudomonas aeruginosa*	Negative
6	*Acinetobacter baumannii*	Negative
7	*Proteus mirabilis*	Negative
8	*Klebsiella pneumoniae*	Negative
9	*Staphylococcus hominis*	Negative
10	*Stenotrophomonas maltophilia*	Negative
11	*Neisseria gonorrhoae*	Negative

4. CONCLUSION

The multiplex PCR assay for simultaneous detection of *Salmonella* spp., *Shigella* spp., and *Entamoeba histolytica* was established successfully with the limit of detection of 5.86 copies/µl, 24.4 copies/µl and 53.6 copies/µl, respectively (confidence intervals of 95%). The specificity of the assay reached 100% (no cross-reactivity) when tested with the other strains of microorganisms.

Thus, the multiplex PCR assay which can detect early and accurately multiple foodborne, will be the optimal solution for food safety control and contribute to eliminating the economic damage and protecting community health during an outbreak.

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