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Development and Evaluation of a Multiplex PCR for Simultaneous Detection of Five Foodborne Pathogens

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Abstract

Foodborne pathogens present serious concerns to human health and can even lead to fatalities. The gold standard for pathogen identification – bacterial culture – is costly and time consuming. A cheaper and quicker alternative will benefit in controlling food safety. In this study, we developed a multiplex-PCR protocol for simultaneous detection of five Foodborne pathogens including *Escherichia coli* O157:H7, *Staphylococcus aureus, Salmonella* spp., *Listeria monocytogenes,* and *Vibrio cholerae*, based on five genes *stx1*, *femA, invA, iap,* và *ctxA*, respectively. Specific primers for multiplex PCR amplification of the *stx* (Shiga-like toxin), *nuc* (thermo nuclease), *inv A* (invasion protein A), *iap* (invasive associative protein), and *ctx A* (cholera toxin A) genes that were established to amplify simultaneous detection of the target pathogens. The assay was also validated for its specificity, sensitivity, and applied to test some spiked food samples. The results showed the products expected multiplex PCR fragments of approximately 112, 244, 301, 453, 518 and 720bp for *S. aureus*, *Salmonella* spp. *V. cholera, L. monocytogenes*, *E. coli* O157:H7 and 16S rRNA, respectively. The assay was specific to the targeted pathogens and was sufficiently sensitive and robust to effectively analyze market samples. The whole process took less than 24 h to complete indicating that the assay is suitable for reliable and rapid identification of these five foodborne pathogens, which could be suitable in microbial epidemiology investigation.

Keywords: Foodborne pathogens; Multiplex-PCR; Five genes; Detection; Simultaneous

Introduction

The incidence of foodborne diseases has increased over the years and is a serious health hazard in both developing and developed countries. *Escherichia coli* O157:H7 (*E. coli* O157:H7), *Salmonella* spp., and *Vibrio cholera* (*V. cholera)* are likely the most common cause of foodborne disease [1, 2]. The well-known *E. coli* bacteria that produce Shiga toxin (STEC) is *E. coli* O157:H7 strains are foodborne infectious agents that cause a number of life-threatening diseases, including hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) [3]. According to recent reports by the Center for Disease Control and Prevention from 2011-2014, there were 11 multistate outbreaks of STEC in the United States of America with six of them attributed to *E. coli* O157:H7. With low infectious dose, an inoculation of fewer than 10–100 CFU of *E. coli* O157:H7, is sufficient to cause infection [4]. Shiga toxin (Stx) is one of the major virulence factors involved in *E. coli* O157:H7 pathogenesis [5]. The *stx* gene is well associated with a prophage and including a variety of subtypes shiga toxin that are described as stx1, stx1c, stxfc, stx2, stx2e, stx2d and stx2g [6]. With capable of producing enterotoxins and coagulase enzyme, *Staphylococcus aureus (S. aureus)* has already been involved in a number of food-poisoning outbreaks. Producing of an extracellular thermostable nuclease and coagulase with the same frequencies that were important phenotypic identifying markers of *S. aureus* [7-9]. The *nuc* gene encodes for the production of a thermostable endonuclease enzyme, which has been used for the correct identification of *S. aureus* in previous studies [10,11]. Approximately 20,000 hospitalizations and 378 deaths per year in the United States, *Salmonella* spp. are the leading bacterial cause of acute gastrointestinal illness. There are some serotypes as *S. enteritidis*, *S. typhi*, *S. paratyphi* A, *S. paratyphi*B, *S. paratyphi*C, and *S. choleraesuis* that can cause foodborne illnesses [12,13]. The *invA* gene is a good candidate gene to invade mammalian cells and subsequently cause disease [14,15], and it presents in all pathogenic serovars as a maker has been the most frequently used for *Salmonella* spp. Detection [16-18]. *Listeria monocytogenes* (*L. monocytogenes*) has been found from dairy, frozen aquatic and meat products [19,20] and is associated with listeriosisis, a servere disease with morbidity and of high mortality of 20–30% [21-23]. One of the main virulence genes, the *iap* gene encodes p60 protein, is associated with the presence of the invasion-associated as the mechanism of pathogenicity in *L. monocytogenes*. Similarity, cholera has been one of the most feared diseases for human. The vast majority of strains associated with epidemic cholera are attributed to toxigenic *Vibrio cholera* (*V. cholerae)* with the O1 serotype [24]. The ctxAB operon that encodes cholera toxin

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resides in filamentous bacteriophage CTXΦ genome confer the ctx operon to *V. cholerae* strains as a prophage that carries the *ctxA* and *ctxB* genes.

Illnesses resulting from the consumption of foods contaminated with pathogens and/ or their toxins have a wide range of economic and public health impact worldwide [25]. The current gold-standard method for detecting foodborne pathogen in food encompasses enrichment with subsequent plating on selective media, biochemical reactions, and serological tests, which are time-consuming and labor-intensive [26]. Currently,, the official procedure for detection of pathogenic bacteria also used to a cultural method, and this procedure could take from 3 to 5 days for confirmation, which is a disadvantage when the results are needed promptly [27,28]. Hence, faster technologies have been applied to develop rapid and enhance sensitive analytical protocol for the foodborne pathogens. The polymerase chain reaction (PCR) is still the most commonly used for detection of the targets bacterial, which based on the identification of the target gene of specific bacteria present after the exponential application with the high sensitivity and specificity. It has become an important tool for detecting and identifying pathogenic organisms in various foods [29-33]. Consequently, since multiplex PCR assay has been able to simultaneously amplify multiple gene targets by using several sets of target specific or degenerated primers in a single tube [34], it has greatly improved the sensitivity, specificity, and speed of detecting pathogenic organisms [35]. Furthermore, multiplex PCR assay, in comparison with uniplex PCR assays, could save considerable time and workload, and improve efficiency [26,30,31-36].

In this study, we developed a multiplex PCR assay for the rapid and simultaneous detection of five epidemic foodborne pathogens, namely *Escherichia coli O157:H7, Staphylococcus aureus, Salmonella* spp, *Listeria monocytogenes,* and *Vibrio cholera*. The performance of the multiplex assay, including its sensitivity, specificity, and precision in quantitative analyses, was comprehensively evaluated in comparison with the traditional methods. The capacity of the proposed assay to detect multiple target pathogens simultaneously was also tested, and the effect of non-target interference on the assay performance was evaluated. The results obtained with artificially contaminated food samples and real samples demonstrate that the multiplex PCR assay can simultaneously detect these five target foodborne pathogens in foods with high sensitivity and reliability.

Materials and Methods

Bacterial strains and their cultivation

The strains used for specificity testing are listed in Table 1. For the identification of five food-borne pathogens and the sensitivity of the multiplex PCR assay experiments, the following strains were used: three strains *E. coli* O157:H7, one produces both Stx1 and Stx2 (NLU), and one produces Stx1 only (NIHE), the last one has Stx2 only (HCMUS), all were obtained from previously worked [37,38], *S. aureus* ATCC6538, *S. enterica* ATCC 14028, *L. monocytogenes* A TCC15313, and *V. cholera* ATCC 17802. All strains were grown in tryptic soy broth (TSB) or brain heart infusion (Merck, Germany) at37°C for 24 h. The simultaneous enrichment broth (SEB), which used for simultaneous enrichment of five pathogenic bacteria in this study, was described previously by Kobayashi et al. [39].Then, the culture broth was used for DNA extraction and subjected to the multiplex PCR assay.

Pathogen detection by the conventional culture method

For detection of *E. coli* O157:H7, each 25 g of each sample was diluted in 225 mL of Modified Tryptone Soya broth (mTSB-Oxoid, UK) added with Novobiocin, homogenized for 2 min at 260 rpm using a Stomacher (Model 400 circulator, Seward, Norfolk, England) and incubated for 18–24 h at 41.5°C according to ISO 16654 (2001) method, as well as the remaining steps. After enrichment and immune magnetic concentration steps, the selective and differential isolation of enterohemorrhagic *E. coli* O157:H7 was carried out on MacConkey Agar with Sorbitol, Cefixime, and Tellurite (CT-SMAC—Oxoid, UK) and incubated overnight at 42°C. From each sample one well isolated suspected colony was transferred to tryptone soy agar (Oxoid) and incubated for 24 h at 37°C. Subsequently, one isolate from the subculture was further tested for agglutination with an *E. coli*

Table1. Bacterial strains and their sources were employed in this study

No	Bacteria	Serovar	Source
The target strains			
1	*E. coli*	O157:H7	NIHE
2	*E. coli*	O157:H7	HCMUS
3	*E. coli*	O157:H7	NLU
4	*S. aureus*		ATCC6538
5	*Salmonella enterica*		ATCC14028
6	*L. monocytogenes*		ATCC15313
7	*V. cholerae*		ATCC17802
The non-target strains			
1	*E. coli*		ATCC 11775
2	*E. coli*		ATCC 25922
3	*E. coli*(I1)		Clinical isolate
4	*E. coli*(I2)		Clinical isolate
5	*E. coli*(I3)		Chicken isolate
6	*E. coli*(I4)		Beef isolate
7	*E. coli*(I5)		Salad isolate
8	*Clostridium perfringens*		ATCC13124
9	*Bacillus cereus*		ATCC11778
10	*Shigella sonnei*		ATCC 9290

ATCC: American Type Culture Collection;
NIHE: National Institute Of Hygiene And Epidemiology;
HCMUS: HCM University of Science; NLU: Nong Lam University

O157:H7 latex test kit (Becton–Dickinson, USA) for sero group O157:H7 confirmation. For detection of *S. aureus*, each 10 g food sample was diluted with 90 mL of sterile Saline Petone Water (Merck, Germany) and pummeled in a Stomacher apparatus for 1 minute; One milliliter of the culture was added to 10 mL of Giolitti–Cantoni broth (Merck, Germany) and incubated at 37°C for 48 hours. One loop full of the culture with black color was then streaked onto Baird Paker Agar (Merck, Germany) and incubated at 37°C for 48 hours. The resulting presumptive *S. aureus* colonies were tested to biochemical screening using a coagulase test. For detection of *Salmonella* spp. Each 25g food sample was diluted with 225 mL of sterile Buffered Peptone Water (Merck, Germany) and pummeled in a Stomacher apparatus for 2 minutes; the mixture was then incubated for 18 hours at 37°C. One milliliter of the culture was added to 10 mL of Rappaport-Vassiliadis soy peptone broth (Merck, Germany) and incubated at 42, 5°C for 18 hours. One loop full of the culture was then streaked onto Xylose lysine desoxycholate agar (Merck, Germany) and incubated at 37°C for 24 hours. The resulting presumptive *Salmonella* colonies were tested to biochemical screening and serological confirmation using *Salmonella* polyvalent O, O1 antisera (Becton–Dickinson, USA). For detection of *L. monocytogenes*, 25 g of the food samples were mixed with 225 mL of sterile Fraser Broth Listeria enrichment broth (Merck, Germany) and pummeled in a Stomacher for 1 minute, followed by incubation for 48 hours at 30°C. One loopful of the culture broth was streaked onto Chromogenic *Listeria* agar with selective supplement (Oxoid, Hampshire, UK) and incubated at 37°C for 48 hours. Presumptive colonies were streaked onto horse blood agar and TSA plates and incubated at 35°C for 48 hours. The resulting presumptive *Listeria* colonies were submitted for biochemical screening (oxidase test, catalase test, and Gram staining). For detection of *V. cholera*, the composited sample of 25 g and to added 225 ml of enrichment medium alkaline saline peptone water (Merck, Germany)and pummeled in a Stomacher for 1 minute, followed by incubation for 24 hours at 41.5°C. One loopful of the culture was then streaked onto Thiosulfate Citrate Bile and Sucrose agar (Merck, Germany) and incubated at 37°C for 24 hours. The resulting presumptive *V. cholerae* colonies were submitted for biochemical screening and serological confirmation.

DNA isolation

DNA extraction was performed by boiling 1 ml of each overnight culture pre-enrichment samples or culture in all the strains was boiled for 10 min in an Isotemp heat block (Fisher Scientific, Pittsburgh, PA) and centrifuged at 12,000 × g for 2 min. The supernatants were saved for posterior use as DNA template for all PCR reactions at – 20°C.

Oligonucleotides primers and multiplex PCR assays

The oligonucleotide primers used in this study are shown in Table 2. For multiplex PCR analysis, two primers pairs were used: stx, which is specific primers to various *stx1* and *stx2* gene for *E. coli*O157:H7described by Yamasaki S, et al (1996) [28]; the primer inv A, specific for *Salmonella* spp. described by designed by Chiu et al [40]; the primer iap, specific for *L. monocytogenes* described by designed by Manzan et al [41]; and the primer ctxA, specific for *V. cholera* described by designed by Shirai et al [42]. The rest of target-specific primers in this work were designed to amplify for the *nuc*gene of *S.aureus* and 16S rRNA gene sequence of bacterial with a amplification product of 112 bp and 720 bp, respectively. The primers were selected from a complete sequence from *S. aureus* strain DQ399678 and 16S rRNA gene sequences for most bacteria and archaea are available on public databases from the Gen Bank database. Primers were designed using the software Fast PCR [43]. The specificity of pair of primers was evaluated by nucleotide similarity searched with the BLAST algorithm at the NCBI website (http: www.ncbi.nlm.nih.gov). Additionally, the PCR amplification was evaluated with DNA samples from the bacterial species listed in Table 2.

The 16S rRNA gene was also targeted as an internal control of the presence of amplifiable bacterial DNA. The single PCR was performed a total volume of 50 µl using the Veriti96-Well Thermal Cycler (Applied Biosystems, Foster City, CA)in reaction mixtures (Promega) containing 0.5 µM each primer, 200 µM each dNTP, 3 mM $MgCl_2$, 1.5 U Taq DNA polymerase, 1x PCR

Table2. Primer pairs and its characteristics employed for the multiplex PCR

Organisms	Forward primers (5' – 3')	Reverse primer (5'-3')	Target gene / primer	Amplicon size (bp)	Reference
Staphylococcus aureus	AATTACATAAAGAACCTGCGACT	GCACTTGCTTCAGGACCATATT	*nuc*	112	This study
Escherichia coli O157:H7	GAGCGAAATAATTTATATGTG	TGATGATGGCAATTCAGTAT	*stx*	518	[28]
Salmonella spp.	ACAGTGCTCGTTTACGACCTGAAT	AGACGACTGGTACTGATCGATAAT	*invA*	244	[40]
Listeria monocytogenes	GGGCTTTATCCATAAAATA	TTGGAAGAACCTTGATTA	*iap*	453	[41]
Vibrio cholerae	CTCAGACGGGATTTGTTAGGCACG	TCTATCTCTGTAGCCCCTATTACG	*ctxA*	301	[42]
Bacterial DNA	AGAGTTTGATCATGG CTCAGG	GGACTACCAGGGTATCTAATT	16S rRNA	720	This study

buffer and 2 l template. A negative control containing the same reaction mixture except the DNA template was included in every experiment. While the multiplex PCR conditions were the same as the single PCR assay except for the concentration of primers and incorporated Betaine into each reaction. The optimized concentrations of the six primer pairs in the multiplex were 0.135µM for *stx*F/ R; 1µM for *nuc*-F/ R; 1.2µM for *invA*-F/ R; 1.65µM *iap*-F/ R; 0.125 µM for *ctxA*-F/ R, and 0.8 µM for 16S -F/ R. Since PCR additives, such as dimethyl sulfoxide, glycerol, bovine serum albumin, or betaine, have been reported to be of benefit in multiplex PCR. The aim is to enhance amplification, adding with 2.5 M concentrations of Betaine were incorporated in all multiplex method.

The PCR program was carried out at 95°C for 5 min, followed denaturing by 35 cycles of 94°C for 1 min, 57. 5°C for 1 min, and 72°C for 1 min, and a final 5 min of 72°C for extension. PCR products were electrophoresed in 1% agarose at 100 V for 50 min followed by staining with ethidium bromide (0.5 g/ mL) then visualized under ultraviolet light, and the results were recorded by photography using an ultraviolet trans illuminator (Gel Doc XR system, Bio-Rad).

Specificity of the multiplex PCR assay

To assess the specificity of the multiplex PCR assay, cultures of 7 target pathogens and 10 non-target bacterial strains (Table 1) were prepared.The purity of the genomic DNA was assessed by determining the A260/ A280 ratio using a spectrophotometer(BioMate 3; Thermo spectronic, Rochester, NY). The multiplex PCR was performed individually for each of DNA samples of 17 strains in multiplex primer system using the experimental conditions described above. The inclusivity and exclusivity were calculated according to the MicroVal protocol [44]. Inclusivity is the ability of the PCR method to detect the target analyze from a wide range of strains. Inclusivity is defined as the percentage of target DNA samples that gave a correct positive signal. Exclusivity is defined as the percentage of non-target DNA samples that gave a correct negative signal.

Multiplex PCR evaluation in spiked food samples

In order to validate the multiplex PCR assay in food, the three Vietnam of food samples matrices including vegetables, seafood products and raw meat fork that were purchased from a local super market and were immediately transported in insulated coolers at 4°C to the laboratory for inoculation and analysis on the same day. Before inoculating experiments, the food samples were carefully tested for the presence of *E. coli* O157:H7, *S. aureus*, *Salmonella* spp. *L. monocytogenes*, and *V. cholera* using the conventional culture method as described above for each of once. Make sure none of these pathogens could be detected by culture in these food samples. The five food borne pathogens were cultured in TSB at 37°C for 18 h prior to decimal dilution in a sterile saline solution (0.85% NaCl) in order to obtain levels of inoculation (colony-forming units [CFU]/g). One mL in each dilution was spread on TSB agar to determine bacterial counts and the equal concentration also used to prepare bacterium representing to spiked 25g food samples. Each 25g food samples were inoculated with 1 mL of each level of inoculation, and then placed with 225 mL of sterile SEB medium. A nature sample with non-inoculated was employed as a negative control. The mixture was homogenized in a Stomacher apparatus for 1 minute. After each 12-h, 18-h and 24-hincubation times, a 1mL aliquot was collected from each sample in each period of time incubation and DNA was extracted as described.

Results and Discussion

Identification of food-borne pathogens

Rapid and simultaneous detection of multiple pathogenic bacteria in foods is of great importance to ensure food safety. In this study, we developed and evaluated a multiplex PCR for simultaneous detection of the five food borne pathogens including *E. coli* O157:H7*, S. aureus, Salmonella spp, L. monocytogenes* and *V. cholera* in a single reaction. In order to ensure specificity and sensitivity and toavoid cross-reactions, primer pair selection is critical in the multiplex PCR assay for the simultaneous detection of five foodborne pathogens. All six of primers (and one is 16S rRNA) that were erected, designed and analysed for the simultaneous detection of five food borne pathogens using the online available software as described by Kalendar et al. [43]. The table 2 shows all information of the primer 16S-F/ R for amplification of a 720-bp sequence from the 16S rRNA gene of bacterial DNA, stx-F/ R for amplification of a 518-bp sequence from the *stx* gene of *E. coli* O157:H7,iap-F/ R for amplification of a 453-bp sequence from the *iap* gene of *L. monocytogenes*, ctxA-F/ Rfor amplification of a 301-bp sequence from the *ctxA* geneof *V. cholerae*, inv-F/ R for amplification of a 244-bp sequence from the *invA* gene of *Salmonella* spp., and nuc-F/ R for amplification of a 112-bpsequence from the *nuc* gene of *S. aureus.* Furthermore, successful multiplexing of multiplex PCR assay requires careful experimental design and optimization of reaction conditions. To achieve accurate template quantification in a multiplex PCR assay, each reaction must efficiently amplify a single product, and amplification efficiency must be independent of template concentration and the amplification of other templates. The annealing temperature of a multiplex PCR assay is one of the most critical parameters for reaction specificity. We tested a range of temperatures above and below the calculated Tm of the primers. Fortunately, based on the yield of PCR products for the seven target genes, the results showed an optimal multiplex annealing temperature of 59, 5°C. As can be seen, the results showed that these six primer pairs in the multiplex PCR assay worked well independently, and could distinguish the five pathogens from each other with high specificity, and PCR amplification was obtained the size of the PCR product followed by identification in terms of the expected size. Five food-borne pathogens, including *E. coli* O157:H7 (NLU), *S. aureus* ATCC6538, *S. enterica* ATCC 14028, *L. monocytogenes* ATCC15313, and *V. Cholera* TCC 17802were detected simultaneously through the multiplex PCR assay using multiplex primer set and the conditions as described above. As the Table 3 and Fig. 1 show the multiplex PCR assay was successfully developed to simultaneously identify the five

foodborne pathogens based on the generation of the expected PCR fragments of 112 bp, 244 bp, 301 bp, 453 bp and 518 bp for *S. aureus* ATCC6538, *Salmonella enterica* ATCC 14028, *V. cholera* ATCC 17802, *L. monocytogenes* ATCC15313and *E. coli* O157: H7(NLU), respectively. In addition, no PCR product corresponding with target microorganism was detected in negative control using the multiplex primer. In addition, PCR products corresponding with the positive-control 16S rRNA gene (720 bp) were detected from pure cultures of seven pathogens.

Specificity of the multiplex PCR Assay

The specificity of the multiplex PCR conducted with the seven target strains and 10 non-target bacterial strains. All three *E. coli* O157:H7 strains *E. coli* O157:H7(NIHE), *E. coli* O157:H7(HCMUS), *E. coli* O157:H7(NLU), *S. aureus* ATCC6538, *S. enterica* ATCC 14028, *L. monocytogenes* ATCC15313, and *V. cholera* ATCC 17802strains were positive in the multiplex PCR assay and all non-target bacterial including *E. coli* ATCC 11775, *E. coli* ATCC 25922, *E. coli*(I1), *E. coli*(I2), *E. coli*(I3), *E. coli*(I4), *E. coli* (I5), *C. perfringens* ATCC13124, *B. cereus*ATCC11778 and *S. sonnei* ATCC 9290were negative in the assay, whereas 16S rRNA was amplified as expected. No mispriming or non-specific amplification was observed. Expectedly, the size of each pathogen amplicon was obtained only from the target foodborne pathogens, resulted in 100% inclusivity and 100% exclusivity. Even of target food borne pathogens and ten non-target pathogens were used to evaluate and verify the specificity of primers in this study, each primer pair by the multiplex PCR on DNA templates (Table 4). These results demonstrated that our multiplex PCR assay could be used to identify each of these five foodborne pathogens.

Evaluation of the multiplex PCR assay with spiked food samples

In order to assess the detection sensitivity of the multiplex PCR assay for its application to food samples, three of kind of food samples (vegetables, seafood products and raw meat fork) inoculated with *E. coli* O157:H7(NLU), *S. aureus* ATCC6538, *S. enterica* ATCC 14028, *L. monocytogenes* ATCC15313, and *V. cholera* ATCC 17802with seven level of the number of viable cells (0, 10^0, 10^1,10^2,10^3, 10^4 and 10^5 CFU/ ml) were employed and a nature sample of each categories was included as negative control after carefully tested using the conventional culture method as described above for the target pathogenic bacteria. Additionally, in order to archive the sensitivity and reproducibility of the multiplex PCR assay, the artificially inoculated and non-inoculated of these categories food samples that were incubated for 12, 18 and 24 h in SEB enrichment medium. As can be seen from Table 5 shows that the multiplex PCR assay was able to correctly identify the presence of the five foodborne pathogens at all different inoculated in the lowest concentration of 10 CFU/ mL in each category of the samples after enrichment for 12 hours in SEB medium (Figure 2). Comparatively, our multiplex PCR assay developed was similar or more sensitive with the same the lowest level of 10 CFU/ mL when compared with Kim et al. [26].

Table 3. Evaluation of the specificity of all PCR primers using various pathogenic bacterial

Strain	Source	Genes/ Primers					
		stx	*nuc*	*invA*	*iap*	*ctxA*	16S rRNA
E. coli O157:H7	NIHE	+	-	-	-	-	+
E. coli O157:H7	HCMUS	+	-	-	-	-	+
E. coli O157:H7	NLU	+	-	-	-	-	+
S. aureus	ATCC6538	-	+	-	-	-	+
Salmonella spp.	ATCC14028	-	-	+	-	-	+
L. monocytogenes	ATCC15313	-	-	-	+	-	+
V. cholerae	ATCC17802	-	-	-	-	+	+

Table 4. Specificity test for the multiplex PCR assay; a minus (-) indicates the absence of a band and a plus (+) indicates the presence of a band

Species	Genes					
	nuc	*invA.*	*ctxA*	*iap*	*stx*	16S rRNA
E. coli O157:H7 (NIHE)	-	-	-	-	+	+
E. coli O157:H7 (HCMUS)	-	-	-	-	+	+
E. coli O157:H7 (NLU)	-	-	-	-	+	+
*S. aureus*ATCC6538	+	-	-	-	-	+
*Salmonella entericaA*TCC14028	-	+	-	-	-	+
*L.monocytogenes*ATCC15313		-	-	+	-	+
*V. cholera*ATCC17802	-	-	+	-	-	+
E. coli ATCC 11775	-	-	-	-	-	+
E. coli ATCC 25922	-	-	-	-	-	+
E. coli(I1)	-	-	-	-	-	+
E. coli(I2)	-	-	-	-	-	+
E. coli(I3)	-	-	-	-	-	+
E. coli(I4)	-	-	-	-	-	+
E. coli(I5)	-	-	-	-	-	+
*C. perfringens*ATCC13124	-	-	-	-	-	+
*B. cereus*ATCC11778	-	-	-	-	-	+
S. sonnei ATCC 9290	-	-	-	-	-	+

Furthermore, in the more recently reported [45] multiplex PCR assays, Lee et al. (2014) reported a multiplex PCR for simultaneous detection of *E. coli* O157:H7, *B. cereus, V. parahaemolyticus, Salmonella* spp. *L. monocytogenes*, and *S. aureus* in various Korean ready-to-eat foods. The multiplex PCR assay developed by Lee et al. (2007) also allowed for simultaneous detection at concentrations of 10^0 CFU/ mL of the pathogenic bacteria, after only 24 h of incubation time. The multiplex PCR assay established in this study could similar the incubation time when compared with Lee et al. (2007). It could also detect the five foodborne pathogens with the lowest level of 10 CFU/ mL after 12 h of enrichment. Consequently, a 12-h enrichment period is

Table 5. Multiplex PCR results of five pathogens from artificially inoculated three food samples matrices

Pathogens	Incubation time (h)	CFU/ml	Multiplex PCR results detection in food samples		
			Vegetables	Seafood products	Raw meat fork
E. coli O157:H7 (*stx*)	12	0	-	-	-
		10^0	-	-	-
		10^1	+	+	+
		10^2	+	+	+
		10^3	+	+	+
		10^4	+	+	+
		10^5	+	+	+
	18	0	-	-	-
		10^0	-	-	-
		10^1	+	+	+
		10^2	+	+	+
		10^3	+	+	+
		10^4	+	+	+
		10^5	+	+	+
	24	0	-	-	-
		10^0	-	-	-
		10^1	+	+	+
		10^2	+	+	+
		10^3	+	+	+
		10^4	+	+	+
		10^5	+	+	+
S. aureus (nuc)	12	0	-	-	-
		10^0	-	-	-
		10^1	+	+	+
		10^2	+	+	+
		10^3	+	+	+
		10^4	+	+	+
		10^5	+	+	+
	18	0	-	-	-
		10^0	-	-	-
		10^1	+	+	+
		10^2	+	+	+
		10^3	+	+	+
		10^4	+	+	+
		10^5	+	+	+
	24	0	-	-	-
		10^0	-	-	-
		10^1	+	+	+
		10^2	+	+	+
		10^3	+	+	+
		10^4	+	+	+
		10^5	+	+	+
Salmonella spp. (*invA*)	12	0	-	-	-
		10^0	-	-	-
		10^1	+	+	+
		10^2	+	+	+
		10^3	+	+	+
		10^4	+	+	+
		10^5	+	+	+
	18	0	-	-	-
		10^0	-	-	-
		10^1	+	+	+
		10^2	+	+	+
		10^3	+	+	+
		10^4	+	+	+
		10^5	+	+	+
	24	0	-	-	-
		10^0	-	-	-
		10^1	+	+	+
		10^2	+	+	+
		10^3	+	+	+
		10^4	+	+	+
		10^5	+	+	+
L. monocytogenes (iap)	12	0	-	-	-
		10^0	-	-	-
		10^1	+	+	+
		10^2	+	+	+
		10^3	+	+	+
		10^4	+	+	+
		10^5	+	+	+
	18	0	-	-	-
		10^0	-	-	-
		10^1	+	+	+
		10^2	+	+	+
		10^3	+	+	+
		10^4	+	+	+
		10^5	+	+	+
	24	0	-	-	-
		10^0	-	-	-
		10^1	+	+	+
		10^2	+	+	+
		10^3	+	+	+
		10^4	+	+	+
		10^5	+	+	+
V. cholera (ctxA)	12	0	-	-	-
		10^0	-	-	-
		10^1	+	+	+
		10^2	+	+	+
		10^3	+	+	+
		10^4	+	+	+
		10^5	+	+	+
	18	0	-	-	-
		10^0	-	-	-
		10^1	+	+	+
		10^2	+	+	+
		10^3	+	+	+
		10^4	+	+	+
		10^5	+	+	+
	24	0	-	-	-
		10^0	-	-	-
		10^1	+	+	+
		10^2	+	+	+
		10^3	+	+	+
		10^4	+	+	+
		10^5	+	+	+

required in this multiplex PCR assay for detecting food samples contaminated with a low level of foodborne pathogens.

For the final analysis of multiplex PCR assay, we chose five of the representative bacteriaus ually associated with food-borne illnesses: *E. coli O157:H7, S. aureus, Salmonella spp, L. monocytogenes and V. cholera.* The multiplex PCR method is capable of detecting these five pathogens in approximately 16 hr (12 hr for enrichment, 1 hr for DNA extraction, 2 hr for PCR amplification, 45 min for capillary electrophoretic separation, and 15 min for interpretation). This is, by far, faster than 4 to 7 days to complete for each pathogen using a conventional detection method, which relies primarily on direct plating methods and biochemical tests.

Conclusion

In conclusion, the multiplex PCR assay for simultaneous detection of the five foodborne pathogens in food including *E. coli* O157:H7, *S. aureus, Salmonella spp, L. monocytogenes* and *V. cholera* was successfully developed and validated. It was able to

sufficient in specifically and simultaneously detecting as few as 10 CFU/ mL of the five pathogens in artificially inoculated food samples after enrichment for 12 h. Finally, each 25-g sample was mixed with 225mL of SEB medium and incubated at 37°C for 12-h. Then, each1mL of the culture broth was subjected to the multiplex PCR assay as the schematic representation of detection procedure is presented in Figure 3. The assay is reliable, rapid, specific, and robust. Therefore, it can be another tool for the investigation of microbial contamination in raw food and food products, and will also be useful for identifying the sources of food borne out break

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Authors' contributions

Vo Van Giau collected the samples, performed the experiments, analyzed and interpreted the data and wrote the manuscript. The other authors read, reviewed and provided feedback on the final manuscript.

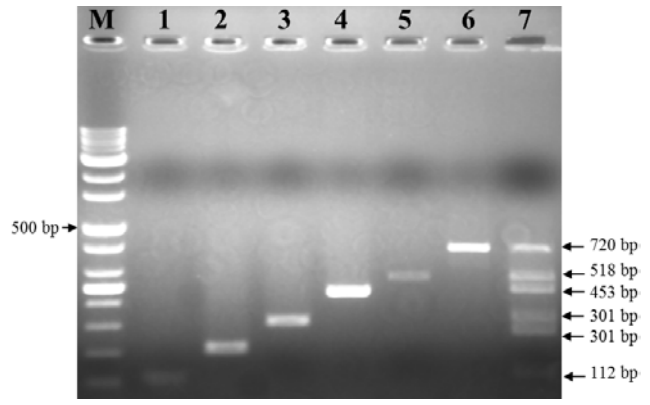

Figure 1: Multiplex PCR reaction applied tosingle and multiple pathogen detection.M, 100-bp DNAladder; lane 1, *S. aureus*ATCC6538; lane 2, *Salmonella enterica*ATCC14028; lane 3, *V. cholera*ATCC17802; lane 4, *L. monocytogenes*ATCC15313; lane 5, *E. coli* O157:H7NLU; lane 6, 16S rRNA; and lane 7,the five-pathogen mixture.

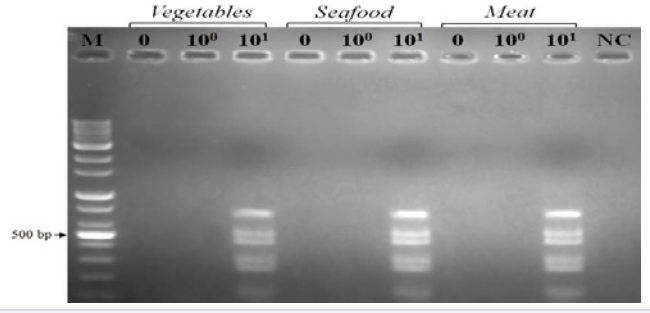

Figure 2: The results of the multiplex PCR assay in three categories of spiked food samples inoculated with different concentrations (showing 0, 10^0 and 10^1 colony-forming units mL^{-1}only) of five pathogens mixture after 12-h enrichment. M, 100 bp DNA ladder; N, negative control

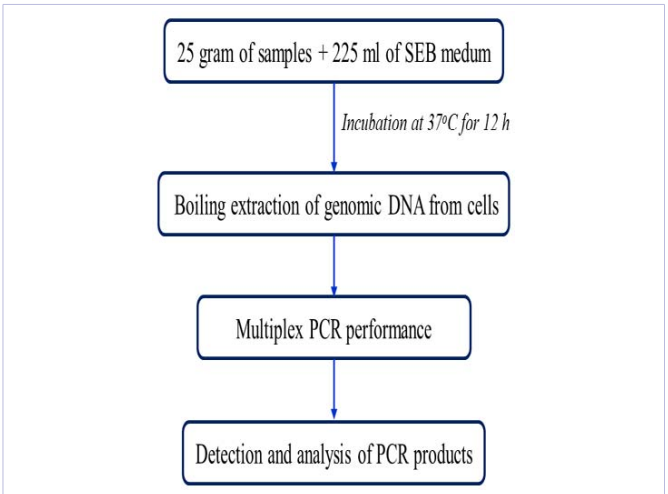

Figure 3: The scheme of multiplex PCR assay for simultaneous detection of *E. coli* O157:H7, *S. aureus*, *Salmonella* spp., *L. monocytogenes* and *V. cholera.*

Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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