The detection of foodborne pathogens using multiplex-direct PCR

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ABSTRACT

Foodborne pathogens pose significant problems of public health and substantial economic problems. The conventional bacterial identification method relies on cultivation which is time-consuming and costly. Molecular technique thus can be alternative tool for rapid screening and identification. In this study, we aimed to develop and fully validate a rapid and simple heptaplex assay for simultaneous detection of seven common foodborne pathogens (*Shigella* spp., Shiga toxin-producing Escherichia coli (STEC), *Staphylococcus pyogenes*, *Campylobacter jejuni*, *Salmonella* Typhi, *Listeria. monocytogenes*, and *Staphylococcus aureus*) without the need for DNA extraction and bacterial enrichment. Previously reported and newly-designed primers were used to amplify the virulent genes of each target strain. The developed assay provided the expected size of PCR fragments of 583, 490, 415, 343, 224, 209 and 105 bp for *Shigella* spp., STEC, *S. pyogenes*, *C. jejuni*, *S.* Typhi, *L. monocytogenes*, and *S. aureus*, respectively. The assay was highly reproducible and a high degree of specificity was observed when tested with 9 other closely related species commonly found in food. The assay's limit of detection was found to be in the range of 106 - 100 CFU/ml. The assay proved to be applicable to detect the target pathogens in food samples by using 22 artificially contaminated food and 100 food samples and provide a statistically equivalent efficiency compared to the conventional culture method. The developed assay can be completely done two hours indicating it was rapid, inexpensive and reliable. Therefore, it is suitable and useful in bacterial identification, especially in food investigation and can be applied to other samples related to microbial forensic science and medical diagnoses.

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1.1 INTRODUCTION

Pathogenic bacteria and their toxins have the potential to be used as a biological weapon. Intentional or unintentional food poisoning using these agents have caused several incidences of illnesses and deaths (Budowle *et al.* 2005, Budowle 2004). These also become a major concern to public health and leads to economic problems in both developed and developing countries (Oh *et al.* 2009). Contamination of raw and processed food with these bacteria could occur inadvertently or deliberately (Budowle *et al.* 2003, Schutzer *et al.* 2005). Terrorists and criminals have used some of them to cause food poisoning in the United States (Bush *et al.* 2001) and worldwide (Budowle 2004, Budowle *et al.* 2005), affecting several hundred people. Microbial forensic thus plays an important role in bacterial species identification from relevant evidence to investigate these bio-crimes providing the cause of death, helping in drafting legislations to protect citizens and to improve food safety standards, and can be as a tool in law enforcement with any illegal market samples.

According to World Health Organization (WHO), the most common and virulent foodborne pathogens include *Salmonella* spp., *Shigella* spp., enterohaemorrhagic *Escherichia coli*, *Listeria monocytogenes*, *Campylobacter jejuni*, *Staphylococcus aureus*, and *Streptococcus pyogenes* (Chen *et al.* 2012, Lei *et al.* 2008). These category B bacteria belong to category B of pathogenicity class can cause diseases in humans and animals with moderate morbidity and mortality. There are adequate prophylaxis and therapy exist for these pathogens but require enhanced disease surveillance (Belkum and Erasmus 2010, Center for Occupational Safety Health and Environment Management 2012). *Shigella* spp. cause shigellosis with very low infectious dose (about 10-100 cell) (Shanmugakani and Ghosh 2012). 164.7 million people are infected by *Shigella* spp. annually, resulting in 1.1 million (Kotloff *et al.* 1999). Shiga toxin-producing *Escherichia coli* (STEC), especially serovar O157 H:7, is associated with severe gastrointestinal disease, hemorrhagic colitis (HC) and hemorrhagic-uremic syndrome (HUS) (Chen *et al.* 2012) through the fecal-oral route, causing 350 outbreaks

with 8,598 cases and 40 deaths in United States in 1998-2002 (Rangel *et al.* 2005). *L. monocytogenes* causes listeriosis, a disease with 24% mortality rate (Mook *et al.* 2011). This pathogen is often found in frozen food. The latest outbreak occurred in 2012, resulting in infection and four deaths. (CDC 2012). *S. aureus* infection can result in life-threatening food poisoning, pneumonia, endocarditis, and septic arthritis. It is major concern due to its ability to produce enterotoxin, invasiveness and antibiotic resistance. *S. aureus* is able to grow in a wide variety of foods especially in raw milk, cream and éclair (Le Loir *et al.* 2003). Multiple outbreaks occur every year (FDA 2013). *Salmonella* Typhi causes typhoid fever and through the fecal-oral route. S. Typhi causes about 22 million cases and 216,000 deaths annually, especially in developing countries (Kidgell *et al.* 2002, Crump *et al.* 2004). *C. jejuni* causes of gastroenteritis and is associated with Guillian-Barré syndrome and reactive arthritis (Ryan *et al.* 2002, Pragman and Schliever 2004, Persson and Olsen 2005, Tang *et al.* 2006, Tavakoli *et al.* 2010) through unpasteurized milk, unchlorinated water and poultry (Olsen *et al.* 2001). In the United States, *C. jejuni* infection occurs 2.5 million cases with 124 deaths annually and 80% of these causes by foodborne (Olofsson *et al.* 2013). Lastly, *Streptococus pyogenes* is a causative agent of streptococcal toxic shock syndrome and rheumatic fever (Levinson 2010). The outbreak of *S. pyogenes* in Japan in 2009, resulted in 461 infected students was caused by eating contaminated box lunches (Takayama *et al.* 2009). It can be transmitted by aerosol or droplets and fecal-oral route (Levinson 2010).

The standard for bacterial species identification depends on cultivation on selective media combined with biochemical tests. These methods include oxidase test, catalase test, carbohydrate fermentation, Methyl Red and Voges Proskauer test, and motility test (Levinson 2010). These methods are time-consuming, labor-intensive, expensive, and requires specialist knowledge (Samadi *et al.* 2007, Kawasaki *et al.* 2009, Tavakoli *et al.* 2010). It typically takes four to seven days to complete the analysis, needs at least four selective media to identify pathogen and particularly costs approximately $10 to analyze each pathogen. This cost will also increase when more pathogen species are suspected. Of these limitations, they are not suitable for pathogens

identification for those patients who are in the severe stage and need quick treatment or diagnostic (Oh *et al.* 2009, Tavakoli *et al.* 2010, Fukushima *et al.* 2010). A molecular approach is therefore used as an alternative tool as it can overcome these obstacles and can be more reliable and sensitive than the conventional one. In the last decades, a number of DNA-based techniques have been reported for foodborne pathogen identification (Feng 1997, Deng *et al.* 2008), including real-time PCR (Sharma and Dean-Nystrom 2003, Wang and Oian 2009, Chen *et al.* 2012, Fukushima *et al.* 2010, Huang *et al.* 2007), fluorescence in-situ hybridization (FISH) (Yanagita *et al.* 2000, Mackie *et al.* 2003, Deng *et al.* 2008), loop-mediated isothermal amplification (LAMP) (Wang *et al.* 2008, Ueda and Kawabara 2009), enzyme-linked immunosorbent assay (ELISA) (Lazcka *et al.* 2007), matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) (Hathout *et al.* 1999, Seng *et al.* 2009), and 16s rRNA sequencing (Drancourt *et al.* 2004). However, these techniques require complex equipments and special skill. They can also be expensive and time-consuming. Multiplex PCR offers a potentially simpler means as it is relatively cheaper and reduces the time of identification. Moreover, it also has been reported to be successfully developed for foodborne pathogens identification with high specificity, sensitivity and robustness (Chen *et al.* 2012, Lei *et al.* 2008, Tavakoli *et al.* 2010, Persson and Olsen 2005). However, they all require the time-consuming enrichment step before PCR.

Another way to reduce analysis time is “Direct PCR” – direct DNA amplification from sample without prior DNA extraction. It has been gathering attention lately in forensic community. Direct PCR speeds up the process due to the elimination of DNA extraction step and decreases contamination by minimizing sample handling (Fode-Vaughan *et al.* 2001, Fode-Vaughan *et al.* 2003). Omitting the DNA extraction step has the potential to increase the sensitivity of the assay, as no DNA is loss during purification; this enables trace samples obtained in forensic evidence to be analyzed. With novel polymerases and buffer additives to overcome PCR inhibitors, direct PCR has been demonstrated to be suitable for human and non-human forensic purposes. For human DNA analysis, direct PCR has been shown to be promising in recovering DNA from low-template samples and touch DNA; a higher peak height and full STR profiles

were retrieved from various surfaces (Swaran and Welch 2012, Kitpipit *et al.* 2014). For animal DNA analysis, direct PCR has also been proven to be applicable for several types of samples such as hair root, hair shaft, bloodstain, muscle, including trace, inhibitor-contaminated, or degraded samples (Hasap *et al.* 2013, Kitpipit *et al.* 2013). The study of Kitpipit *et al.* (2014) also reported the economical and efficient protocol for direct amplification from these sample types: pre-PCR treatment protocol was found to provide better result compared to the direct protocol. It could be due to the pre-PCR solution helping to dilute inhibitors and dilute DNA to be in the optimal range for amplification while the reagents used help to enhance cell lysis and release DNA (Kitpipit *et al.* 2014).

Even though direct PCR technique is widely applicable for various fields of forensic science and as mentioned above, the combination of multiplex and direct PCR has never been applied to bacterial species identification in microbial forensic science. In this study, we therefore aim to develop a DNA-based method called "heptaplex-direct PCR assay" to simultaneously detect seven common foodborne pathogens*: Shigella* spp.*,* Shigatoxigenic group of *Escherichia coli* (STEC), *S. pyogenes*, *C. jejuni*, *S.* Typhi, *L. monocytogenes* and *S. aureus*. The assay will also be fully validated for its reproducibility, sensitivity, specificity, and robustness with commercial foods. In addition, the assay's efficiency will be compared with the standard culture method.

1.2 OBJECTIVES

1. To develop a heptaplex-direct PCR assay to simultaneously detect seven foodborne pathogens; *Shigella* spp., STEC, *S. pyogenes*, *C. jejuni*, *S.* Typhi, *L. monocytogenes* and *S. aureus* which are commonly founded in foods.
2. To validate the developed assay for its reproducibility, specificity, and sensitivity as well as with commercial product.
3. To compare detection efficiency of the developed heptaplex-direct PCR with the standard cultivation method.

1.3 RESULTS AND DISCUSSION

1.3.1 Primer design

Seven species-specific primer pairs for seven target pathogens; *Shigella* spp., STEC, *S. pyogenes*, *C. jejuni*, *S.* Typhi, *L. monocytogenes* and *S. aureus*, were used in this study. They are modified from previous reports and newly designed to achieve the similar range of T_m. To do this, DNA sequences of the target gene sequences of the seven target and other bacterial species were firstly obtained from GenBank (www.ncbi.nih.gov). The accession number for these sequences are shown in Table 1. The sequences were then aligned by Mega 5 (Tamura *et al.* 2011).

Table 1 Accession number of the target gene sequences used in primer design

Bacterial species	Gene	Accession no.	Referenes
Shigella spp.	ipaH	M76445.1	Hartman *et al*., 1990
		M76444.1	Venkatesan *et al*., 1991
		M76443.1	Venkatesan *et al*., 1991
		M32063.1	Hartman *et al*., 1990
STEC O157:H7	Stx2	AB048837.1	Asakura *et al*., 2001
		AB048836.1	Asakura *et al*., 2001
S. pyogenes	Complete genome	AE004092.2	Ferretti *et al*.,2001
		CP000017.2	Sumby *et al*.,2005
		CP006366.1	Port *et al*., 2013
C. jejuni	hipO	FJ655194.1	Jiang *et al*., 2009
		FJ655193.1	Jiang *et al*., 2009
		FJ655192.1	Jiang *et al*., 2009
		AY168302.1	Kim and Lee, 2002
		AY168301.1	Kim and Lee, 2002
S. Typhi	Complete genome	CP002099.1	Xu *et al*., 2013
		CP003278.1	Ong *et al*., 2012
		AE014613.1	Deng *et al*., 2003
L. monocytogenes	hlyA	HM589601.1	Suba *et al*., 2010
		HM589596.1	Suba *et al*., 2010
		HM589602.1	Suba *et al*., 2010
		HM589597.1	Suba *et al*., 2010
		HM589598.1	Suba *et al*., 2010
S. aureus	Complete genome	HE579073.1	Vogel *et al*., 2012
		HE579069.1	Vogel *et al*., 2012
		HE579065.1	Vogel *et al*., 2012

Six of these seven pairs (stx, spy, hipo, styphi, lm, and s primer) were modified from previous reports to improve their suitability for multiplex amplification in one reaction (Table2). To do this, the target primer sequences were identified within the aligned DNA sequences and then edited at the 5' end to bind with the DNA template without compromise to its specificity. One specific primer pair for *Shigella* spp. was newly designed in this study. Likewise, virulent gene sequences of *Shigella* spp. were used as template to design species-specific primers. The physical parameters such as melting temperature (T_m), length, GC content, self-complementarity, and secondary structures, such as primer dimers and hairpin loop of the candidate primers, were evaluated by OligoCalc (http://www.basic.northwestern.edu/biotools/oligocalc.html). The expected candidate primers were 15-30 basepair long with the T_m in the range of 65-67˚C. The GC content was around 40-60%. Moreover, primer interaction was evaluated using Autodimer (http://www.cstl.nist.gov/biotech/strbase/AutoDimerHome page.htm) in order to check the complementary bases of primer sequences and avoid the chance of primer interaction during an amplification process. Details of species-specific primers for the seven target foodborne pathogens used in this study are shown in Table 2.

Table 2 Details of species-specific primers for the seven target foodborne pathogens used in this study, including: primer name, bacterial species, target gene, primer sequence, product size and their references.

Bacterial species	Target gene	Primer name	Primer sequence (5'-3')	Product size	Reference
Shigella spp.	*ipaH*	shi2F shi2R	CCTTTCCGATACCGTCTCTGC CTCATTCTCCAGCATCTCATACTTCTG	583	This study
STEC	*stx2*	stx2F stx2R	GGTTTTTCTTCGGTATCCTATTCCC TGGATGCATCTCTGGTCATTGTA	490	modified from Fode-Vaughan, 2003
S. pyogenes	*spy1258*	spyF spyR	TGTAAAGACCGCCTTAACCACCT AATACTGGCAAGGTAAACTTCTAAAGCA	415	modified from Liu *et al*., 2005
C. jejuni	*hipO*	hipoF hipoR	ACTTCGTGCAGATATGGATGCTT GCTATAACTATCCGAAGAAGCCATCA	343	modified from Persson, 2005
S. Typhi	*InvA*	sTyphiF sTyphiR	CATCACCCCGTAGCCCAATA CTTCAGGAGAGACGCATTCG	224	modified from Farrell *et al*., 2005
L. monocytogenes	*Hly*	lmF lmR	CGCAACAAACTGAAGCAAAGG TGGCGGCACATTTGTCAC	209	modified from Keith *et al*., 2000
S. aureus	16S rDNA	S1 S2	TGTCGGTACACGATATTCTTCACG CAACGTAATGAGATTTCAGTAGATAATACAACA	105	modified from Samadi *et al*., 2007

All target genes selected in this study are genes encoded for essential functions. Mutation in these genes could be fatal; therefore, low mutation rate is observed. (Chen and Shakhnovich 2009). For example, only 1-2% divergence is found in 16s rDNA per 50 million years (Ochman *et al.* 1999). This ensures that the developed assay would detect these pathogens in different samples.

1.3.2 Primer test and direct amplification

The target bacteria were prepared before amplification using the modified method of Kitpipit *et al* (2014). To do this, 1-2 bacterial colonies were picked from media plate and suspended into 1.5 ml microcentrifuge tube containing 20 µl of 1X PBS buffer (pH 7.4) and 0.5 µl of proteinase K. The solution was incubated at room temperature for 5 min then subjected to heat at 98˚C for 2 min and spun down for 15 s. The obtained solution was termed pre-PCR solution in this study and used instead of DNA template in a PCR reaction. The pre-PCR solution was stored at -20˚C until further analysis.

Preliminary tests were first conducted to compare the effectiveness of the direct and dilution protocol. One colony of target bacteria was either directly added to PCR reaction (direct protocol) or prepared as a pre-PCR solution (dilution protocol). The dilution protocol yielded PCR products of target species while no band was obtained from the direct protocol, indicating that the dilution protocol was more suitable for bacterial DNA amplification.

Uniplex-direct PCR was then performed to evaluate the primers according to dilution protocol. To do this, the seven species-specific primer pairs (shi, stx, spy, hipo, styphi, lm and s primer) were separately used to amplify their target DNA using gradient PCR. Annealing temperatures were varied at 65˚C, 68˚C, and 70˚C. PCRs were performed using the Q5 high fidelity DNA polymerase commercial PCR kit (New England Biolabs, UK). Each PCR reaction was conducted in a total volume of 20 µl, containing 1X PCR buffer (25 mM TAPS-HCl pH 9.3, 50 mM KCl, 2 µM $MgCl_2$, and 1 mM β-mercaptoethanol), 1.0 units DNA polymerase (Biolabs, England), 200 µM of each dNTPs, 0.5 µM of forward and reverse primer, sterile distilled water and 1 µl of pre-PCR solution. The initial PCR condition followed the manufacturer's recommended protocol: initial denaturation at 98˚C for 30 s, 36 cycles of denaturation at 98 ˚C for 5 s, annealing temperature at 65, 68, and 70˚C for 10 s, and extension at 72˚C for 20 s, followed by final extension at 72˚C for 2 min. The PCR products were held at 4˚C until subjected to agarose gel electrophoresis.

The result from gradient PCR showed that DNA from all target species (*Shigella* spp., STEC O157:H7, *S. pyogenes, C. jeuni, S.* Typhi, *L. monocytogenes, and S. aureus*) provided the expected PCR product sizes at at least one annealing temperature (Figure 1). DNA from *Shigella,* STEC O157:H7, *S. pyogenes*, *C. jejuni*, and *S.* Typhi were successfully amplified and provided the expected size of 583, 490, 415, 343, and 224 bp, respectively, at the three annealing temperature (Table 3). While DNA from *L. monocytogenes* and *S. aureus* provided the expected size of 209 and 115 bp, respectively, at 65˚C and 68˚C only. The absence of these two bands at 70˚C might be the result from too high annealing temperature used. At this annealing temperature,

these primer could not anneal effectively, resulting in no PCR product or the product concentration was lower than the detection limit of an agarose gel (Rychlik *et al.* 1990). The result from gradient PCR also illustrated that the lower annealing temperature can generate higher band intensity. However, too low annealing temperature leads to nonspecific binding of primer to the DNA template. The negative control of all experiments provided no PCR product fragment. This confirmed that the bands occurred were from the target bacteria and there was no DNA contamination in the PCR reactions (Butler 2005).

Table 3 The result of gradient PCR for seven primer pairs of each target species at three different annealing temperatures. (+) denotes the presence of PCR product and (-) denotes the absence of PCR product.

Bacterial species	Annealing temperature					
	65˚C		68˚C		70˚C	
	positive	negative	positive	negative	positive	negative
Shigella spp.	+	-	+	-	+	-
STEC	+	-	+	-	+	-
S. pyogenes	+	-	+	-	+	-
C. jejuni	+	-	+	-	+	-
S. Typhi	+	-	+	-	+	-
L. monocytogenes	+	-	+	-	-	-
S. aureus	+	-	+	-	-	-

After seven primer pairs successfully amplified their target species at the selected annealing temperature, primer specificity test was performed in order to cross-check if the primers can react among these seven target species. A uniplex PCR was conducted again with all seven target species at 68˚C annealing temperature. The result showed that at this temperature, styphi primer provided non-specific band with *Shigella*, *C. jejuni*, and *L. monocytogenes* (Figure 1)

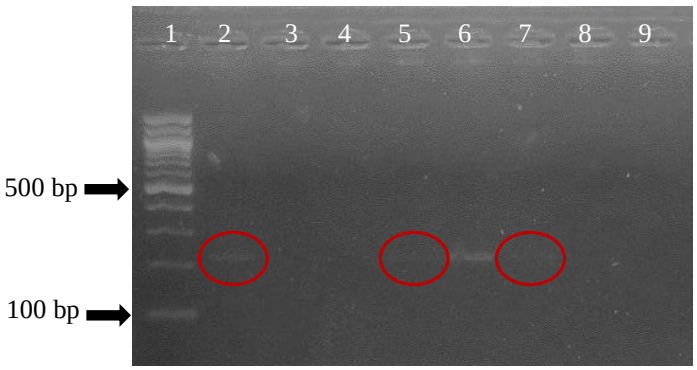

Figure 1 The primer specificity test of stphi primer at 68˚C annealing temperature. Lane 1 100 bp DNA ladder, lane 2 *Shigella* spp., lane 3 STEC, lane 4 *S .pyogenes*, lane 5 *C. jejuni*, lane 6 *S.* Typhi, lane 7 *L. monocytogenes*, lane 8 *S. aureus*, lane 9 negative control

The next experiment was performed by increasing annealing temperature from 68˚C to 70˚C and decreasing PCR cycles number from 36 to 30 cycles. The result demonstrated that at this condition, each primer pair was able to amplify DNA from its target species only and no cross-reaction was found the other target species (Figure 2a-f). However, primers for *L. monocytogenes* and *S. aureus* was not test at 70˚C as they could not amplify their target DNA at this temperature. Using 68˚C was high enough to provide good specificity for both primer pairs (Figure 2g-h).

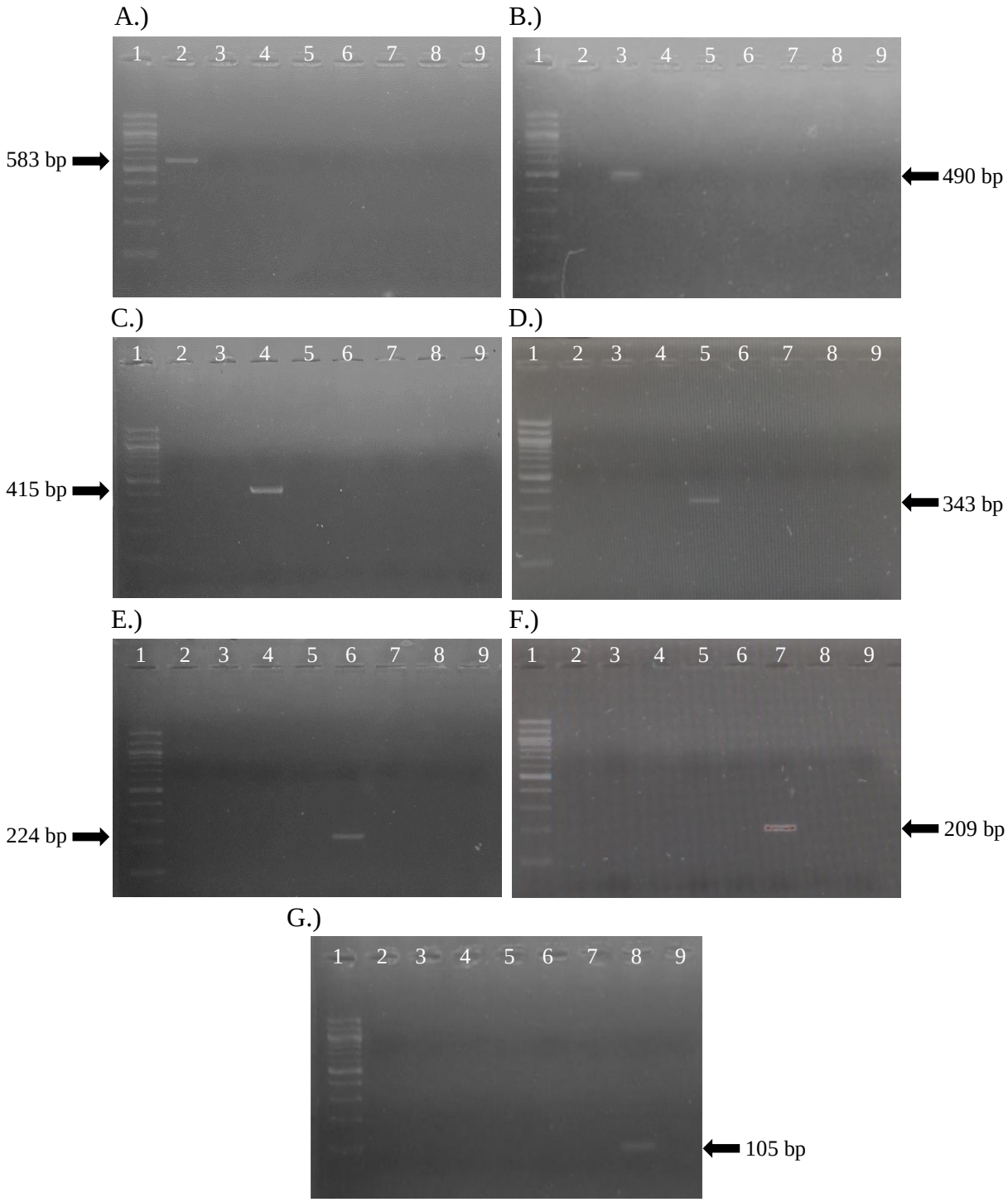

Figure 2 The primer specificity test of seven primer pairs at 70˚C annealing temperature and 30 PCR cycles. A.) shi primer for *Shigella* spp, B.) stx2 primer for STEC, C.) spy1258 primer for *S .pyogenes*, D.) hipo primer for *C. jejuni*, E.) styphi primer for *S.* Typhi, F.) lm primer for *L. monocytogenes*, G.) s primer for *S. aureus*. Lane 1 100 bp DNA ladder, lane 2 *Shigella* spp., lane 3 STEC, lane 4 *S .pyogenes*, lane 5 *C. jejuni*, lane 6 *S.* Typhi, lane 7 *L. monocytogenes*, lane 8 *S. aureus*, lane 9 negative control.

From the above result, it can assume that direct PCR was successfully used for amplifying pathogens DNA. One of the important keys for this success was the use of the Q5® High Fidelity DNA Polymerase commercial PCR, which contains a modified polymerase (a processivity-enhancing Sso7d DNA binding domain) and possibly other PCR additives. These chemicals are more tolerant to PCR inhibitions, such as peptidoglycan (Gram-positive bacteria's cell wall), lipopolysaccharide (Gram-negative bacteria's outer membrane), polysaccharide, and other organic and inorganic compounds composed in bacterial cell (Klančnik *et al.* 2012, Moreira 1998). These inhibitors could affect PCR performance by interactions or competition with polymerase, DNA template, or Mg^{2+} cofactor (Opel *et al.* 2010, Nguyen *et al.* 2012).

Dilution protocol used in this study are also the critical steps for the success. The chemicals for sample preparation in the dilution protocol, PBS buffer, helped to maintain the sample pH and prevent acid hydrolysis of DNA, while proteinase K helped break down some proteins in bacteria and DNase. The short period incubation of 2 min at 98˚C could also denature the cellular proteins, including DNase. This high temperature could also help improvement of cell lysis and DNA release process (Kitpipit *et al.* 2014). Diluting sample with PBS can also dilute the inhibitors contaminated in samples and dilute DNA template to be in the optimal range for amplification as well (Linacre and Tobe 2013, Kitpipit *et al.* 2014).

Bacterial amount used for sample preparation is also important for a successful direct amplification. In this study, the optimal amount of bacterial was found to be in a range of 1-2 colonies. Normally, the amount of DNA template recommended for the low complexity template like bacterial DNA or plasmid was 1 pg-10 ng (New England Biolabs, UK). Using too much of pre-PCR solution not only provided the high amount of PCR inhibitors, but also provided the excessive DNA template that can cause non-specific binding to primer and change the pH of reaction mixture, while using too low DNA template may result in low PCR product yield (National Forensic Science Technology Center 2007).

1.3.3 Multiplex PCR optimization

According to the result from uniplex PCR, the annealing temperature at 68˚C was selected for this experiment as this temperature was the highest temperature that provided the positive results for all primer pairs. Multiplex PCR were performed using the same reagents as described for the uniplex PCR. PCR were performed in a total volume of 20 µl containing 1X PCR buffer (containing 25 mM TAPS-HCl pH 9.3, 50 mM KCl, 2 µM $MgCl_2$, and 1 mM β-mercaptoethanol), 1.0 units DNA polymerase (Biolabs, England), 200 µM of each dNTPs and 0.5 µl of mixed target pathogen pre-PCR solution. The concentration for all primers were started at 0.5 µM as it is the optimal amount recommended for starting multiplex PCR (BioLabs 2011). The PCR condition was initial denaturation at 98˚C for 30 s, followed by 36 cycles of denaturation at 98˚C for 10 s, annealing at 68˚C for 10 s and extension at 72˚C for 20 s. The final extension was at 72˚C for 2 min. The PCR products were held at 4˚C until subjected to agarose gel electrophoresis.

With the primer concentration at 0.5 µM, the result showed that DNA from four of seven target foodborne pathogens; *C. jejuni*, *S.* Typhi, *L. monocytogenes*, and *S. aureus*, were successfully amplified and provided the expected PCR product size of 343, 224, 209 and 105 bp, respectively, in seven-template multiplex reaction (Table 4). Among four bands observed, fragments from *L. monocytogenes* and *S. aureus* showed low intensity. It should be due to too low primer concentrations used. DNA from *Shigella* spp. (583 bp), STEC O157:H7 (490 bp), and *S. pyogenes* (415 bp) failed to amplify in mixed-template multiplex reaction but still were observed in single-template multiplex reaction. Negative control was clean, indicating that there was no contamination in the reagents used for multiplex PCR. Thus, optimization of primer concentration of missing and faint bands were needed.

Table 4 The heptaplex PCR result using 68˚C annealing temperature and 0.5 µM of all primers. (+) denotes the intensity of the PCR product band and (-) denotes the absence of PCR product.

Bacterial species	Single-template	Mixed-template
Shigella spp.	++	-
STEC	+	-
S. pyogenes	++	-
C. jejuni	+++	++
S. Typhi	+++	++
L. monocytogenes	+++	+
S. aureus	+++	+

The next experiment was conducted by optimization of the primer shi (for *Shigella* spp.), stx (for STEC O157:H7) and spy (for *S. pyogenes*) that failed to amplify and the primer lm (for *L. monocytogenes*) and s (for *S. aureus*) that showed low intensity at the concentration of 0.5 µM. The concentration of these primers were increased from 0.5 to 1.0 µM. The remaining conditions were the same as previous experiment. The result showed that only four bands; 343 bp of *C. jejuni*, 224 bp of *S.* Typhi, 209 bp of *L. monocytogenes*, and 105 bp of *S. aureus*, were visible in the mixed-templated multiplex reaction (Table 5). However, an increase in band intensity of *Shigella* spp, STEC O157:H7 and *S. pyogenes* PCR products in their single-template reaction were observed. The three larger bands of *Shigella* spp. (583 bp), STEC O157:H7 (490 bp) and *S. pyogenes* (415 bp) still were not obtained using this condition.

Table 5 The heptaplex PCR result using 68˚C annealing temperature, 1.0 µM of shi, stx, spy, lm and s primer, and 0.5 uM of hipo and styphi primer. (+) denotes the intensity of the PCR product band and (-) denotes the absence of PCR product.

Bacterial species	Single-template	Mixed-template
Shigella spp.	+++	-
STEC	+++	-
S. pyogenes	+++	-
C. jejuni	+++	++
S. Typhi	+++	++
L. monocytogenes	+++	++
S. aureus	+++	++

The next experiment was performed by increasing concentration of the three primer pairs; shi (for *Shigella* spp.), stx (for STEC O157:H7) and spy (for *S. pyogenes*), from 1.0 to 1.25 µM. The same result was obtained as the three larger bands still was absent (data not shown).

Since *Shigella* spp., STEC O157:H7 and *S. pyogenes* were not detected in the previous condition and the concentration of all three primer pairs exceeded the maximal amount of optimal range (0.1-1.0 µM) recommended for multiplex PCR (Butler 2005). The next experiment thus was designed to reduce the annealing temperature from 68˚C to 66˚C to increase the chance of obtaining the absent bands (Roux 2009). The result of decreasing annealing temperature indicated that this condition provided six of seven target bands; 490 bp of STEC O157:H7, 415 bp of *S. pyogenes*, 343 bp of *C. jejuni*, 224 bp of *S.* Typhi, 209 bp of *L. monocytogenes*, and 105 bp *S. aureus* (Table 6). Only *Shigella* spp. DNA failed to amplify. This demonstrated that decreasing of annealing temperature allowed more primers to bind to their target template, resulting in more expected PCR fragments (Roux 2009). The absence of *Shigella* spp. fragment might be the consequence from its PCR fragment size which was the largest expected band, leading to insufficient PCR components to use when compete with other smaller bands. Among the six bands presented, two bands (343 bp of *C. jejuni* and 415 bp of *S. pyogenes*) were still faint (Table 6).

Table 6 The heptaplex PCR result using 66˚C annealing temperature; 1.25 µM of shi, stx, spy, 1.0 µM of lm and s primer, and 0.5 uM of hipo and styphi primer. (+) denotes the intensity of the PCR product band and (-) denotes the absence of PCR product.

Bacterial species	Single-template	Mixed-template
Shigella spp.	+++	-
STEC	+++	++
S. pyogenes	+++	++
C. jejuni	+++	+
S. Typhi	+++	++
L. monocytogenes	+++	++
S. aureus	+++	++

As the largest band of *Shigella* spp. was not present and the faint band of *C. jejuni* and *S.* Typhi were obtained in the previous experiment, the further experiment was thus performed as follow; 1) increasing amount of DNA polymerase from 0.5 unit to 1.0 unit, 2) elongating the extension time from 20 s to 30 s, 3) increasing primer concentration of the two faint bands from 0.5 µM to 0.75 µM. The result of multiplex PCR found that the largest band of *Shigella* spp. was observed but the band of *C. jejuni* was absent (Table 7). This resulted in the observation of six bands of target species; 583 bp of *Shigella* spp., 490 of STEC O157:H7, 415 bp of *S. pyogenes*, 224 bp of *S.* Typhi, 209 bp of *L moncytogenes*, and 105 bp of *S. aureus* (Table 7). However, the bands of *Shigella* spp. and *S. pyogenes* were still faint.

Table 7 The heptaplex PCR result using 66˚C annealing temperature and 1.25 µM of shi, stx, spy, 1.0 µM of lm and s primer, and 0.75 uM of hipo and styphi primer, (+) denotes the intensity of the PCR product band and (-) denotes the absence of PCR product.

Bacterial species	Single-template	Mixed-template
Shigella spp.	+++	+
STEC	+++	++
S. pyogenes	+++	++
C. jejuni	+++	-
S. Typhi	+++	++
L. monocytogenes	+++	++
S. aureus	+++	+

The next experiment was conducted by optimization of primer concentration of hipo primer (for *C. jejuni*) from 0.75 µM to 1 µM. Annealing temperature was also slightly decreased from 66˚C to 65˚C. The multiplex result found that the assay provided all expected PCR fragments of 583, 490, 415, 343, 224, 209, and 105 bp for *Shigella* spp., STEC O157:H7, *S. pyogenes*, *S.* Typhi, *L monocytogenes*, and *S. aureus*, respectively (Figure 3 lane 9). The high intensity bands were observed for all PCR fragments. Negative control was clean, indicating that there was no contamination in PCR reaction.

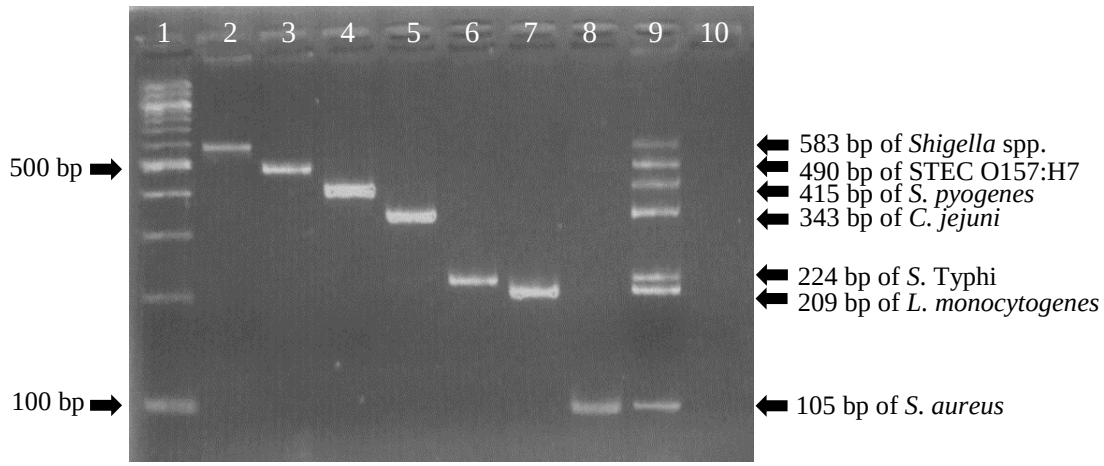

Figure 3 The heptaplex PCR result using the PCR condition and reagents as follows; 65˚C annealing temperature and 1.25 µM of shi, stx, spy, 1.0 µM of hipo, lm and s primer, and 0.75 uM of styphi primer; Lane 1 100 bp DNA ladder, lane 2 *Shigella* spp., lane 3 STEC O157: H7, lane 4 *S. pyogenes*, lane 5 *C. jejuni*, lane 6 *S.* Typhi, lane 7 *L. monocytogenes*, lane 8 *S. aureus*, lane 9 mixed seven target species, lane 10 negative control

1.3.3 Specificity test

The assay specificity test was conducted to evaluate if the developed assay could result in any cross reactivity with other commonly encountered bacterial species. To do this, seven target and nine non-target species were obtained from Songklanagarind Hospital, Songkhla, Thailand. The list of all bacterial species used in this study is shown in Table 8. All target and non-target species were used to prepare pre-PCR solution and amplified by the developed multiplex PCR with the previously optimized condition; 98˚C for 30 s, followed by 36 cycles of denaturation at 98˚C for 10 s, annealing at 65˚C for 10 s and extension at 72˚C for 30 s. The final extension was at 72˚C for 2 min, along with 1.25 µM of the primer shi, stx and spy, 1 µM of the primer hipo, lm, and s, and 0.75 µM of the primer styphi

Table 8 The bacterial species used in specificity test

Bacteria	Serovar/strain	Source
Target species		
Shigella flexneri		DMST 44237
Shigella spp.		DMST 1332
Shigella spp.		Clinical isolate
STEC	O157: H7	ATCC 43890
STEC	O157: H7	Clinical isolate
S. pyogenes		ATCC 19615
S. pyogenes		Clinical isolate
C. jejuni		ATCC 33291
C. jejuni		Clinical isolate
S. Typhi		DMST 22842
S. Typhi		Clinical isolate
L. monocytogenes		DMST 17303
L. monocytogenes		Clinical isolate
S. aureus		ATCC 6538
S. aureus		Clinical isolate
Other species		
Acinetobacter baumannii		ATCC 19609
A. baumannii		Clinical isolate
Aeromonas hydrophila		Clinical isolate
Enterobacter cloacae		Clinical isolate
Enterococcus faecalis		Clinical isolate
Klebsiella pneumonia		Clinical isolate
Pseudomonas aeruginosa		Clinical isolate
Proteus. Mirabilis		Clinical isolate
Streptococcus epidermidis		Clinical isolate
Serratia marcescens		Clinical isolate

The results of specificity test showed that DNA from all seven target species could be successfully amplified and provided the expected PCR product size (Figure 4 lane 2-8). However, one of nine non-target species, *P. aeruginosa*, was also amplified by the developed assay and gave the PCR product size of approximately 490 bp, which is close to the expected size of STEC O157:H7 (Figure 4 lane 14). This might be because these two species belong to the same family of *Enterobacteriaceae* (Levinson 2010), leading to the high similarity in their DNA sequences, including the binding regions of primer stx (STEC-specific primer).

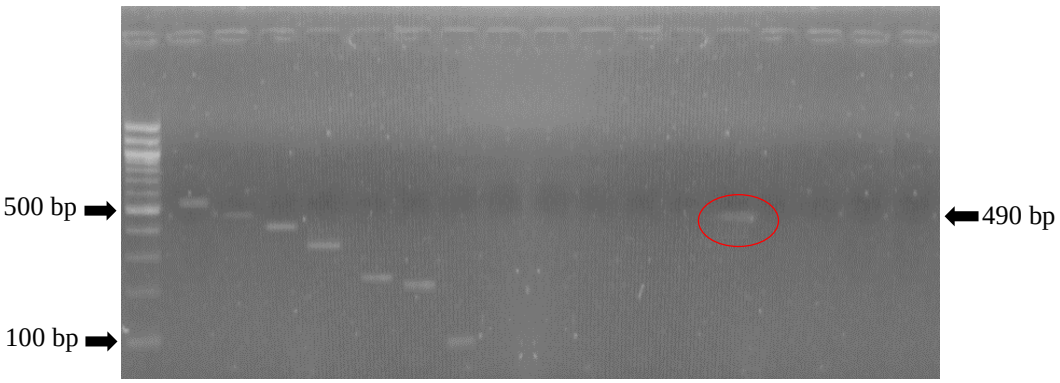

Figure 4 The specificity test with target and non-target foodborne pathogen species; Lane 1 100 bp DNA ladder, lane 2 *Shigella* spp, lane 3 STEC O157: H7, lane 4 *S. pyogenes*, lane 5 *C. jejuni*, lane 6 *S.* Typhi, lane 7 *L. monocytogenes*, lane 8 *S. aureus*, lane 9 *A. baumannii*, lane 10 *A. hydrophilus*, lane 11 *E. clocaeae*, lane 12 *E. feacalis*, lane 13 *K. pneumoniae*, lane 14 *P. aeruginosa*, lane 15 *P. mirabilis*, lane 16 *S. epidermidis*, lane 17 *S. marcescens*, lane 18 negative control

The next experiment thus was conducted by increasing of annealing temperature from 65 to 67˚C to solve the problem of non-specific band from *P. aeruginosa*. The seven target species were amplified along with *P. aeruginosa* and other three bacteria (*E. cloceae, K. pneumoniae,* and *S. epidermidis*) randomly chosen from non-target species list. The results showed that there still was a faint non-specific band of *P. aeruginosa* (Figure 5 lane 11) while all target species bands were appeared. The presence of non-specific product could be due several reasons, for example, too high primer concentration of stx, too many cycle number used in PCR process, or too low annealing temperature (Butler 2005, Roux 2009)

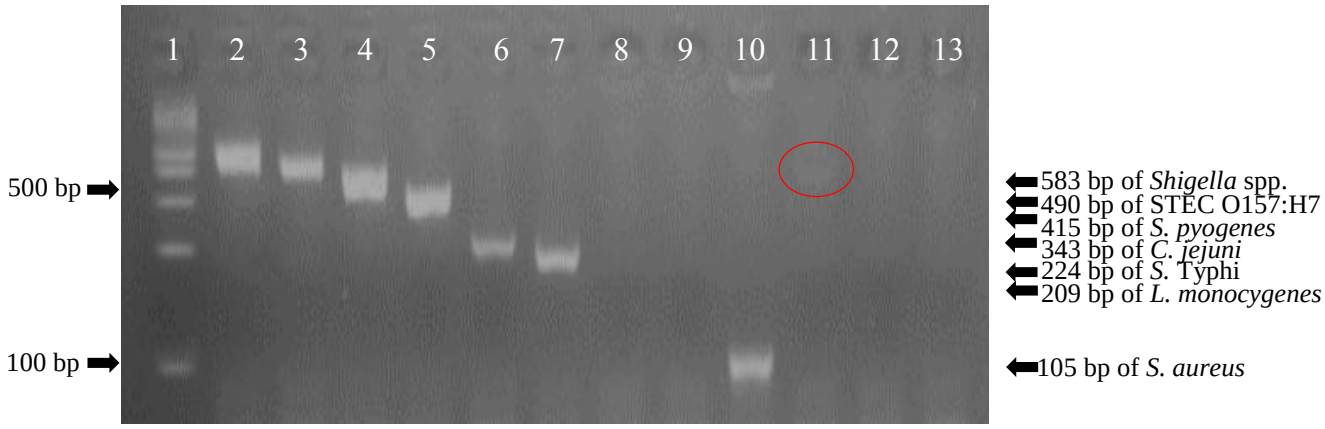

Figure 5 The specificity test with target and non-target foodborne pathogen species at 67˚C annealing temperature. Lane 1 100 bp DNA ladder, lane 2 *Shigella* spp, lane 3 STEC O157: H7, lane 4 *S. pyogenes*,

lane 5 *C. jejuni*, lane 6 *S.* Typhi, lane 7 *L. monocytogenes*, lane 8, *E. clocaeae*, lane 9 *K. pneumoniae*, lane 10 *S. aureus*, lane 11 *P. aeruginosa*, lane 12 *S. epidermidis*, lane 13 negative control

The next experiment was performed by decreasing PCR cycle number from 36 to 32 to eliminate non-target band of *P. aeruginosa*. The seven target species were amplified with *P. aeruginosa* and another non-target species of *S. epidermidis* that was randomly chosen. The result showed that non-specific band of *P. aeruginosa* was still seen but it was very faint (Figure 6 lane 3) while all seven target bands were observed in multiplex reaction (Figure 6 lane 2). Negative control was clean, indicating no contamination in PCR reaction.

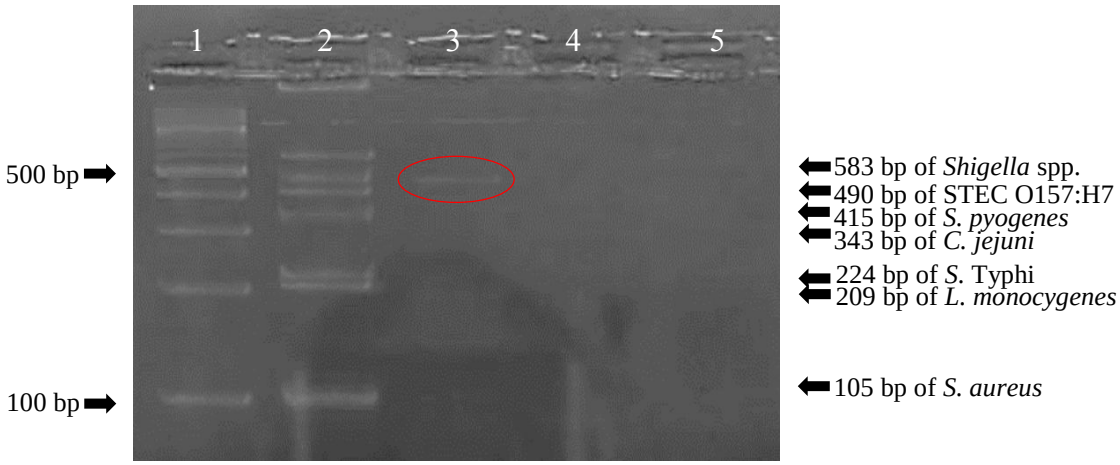

Figure 6 The specificity test with target and non-target foodborne pathogen species at 67˚C annealing temperature and 32 PCR cycles. Lane 1 100 bp DNA ladder, lane 2 multiplex PCR, lane 3 *P. aeruginosa*, lane 4 *S. epidermidis*, lane 5 negative control

The next experiment was conducted by further decreasing PCR cycle number from 32 to 30 to eliminate the faint band of non-target species, *P. aeruginosa*. All seven target species were amplified with nine non-target species (as listed in Table 8) at 67˚C annealing temperature. The result showed that seven target species were observed in the seven-template multiplex reaction (Figure 7 lane 2). No non-specific band was observed (Figure 7 lane 3-11, Table 4). The optimized condition for heptaplex-direct PCR can be summarized as follows; initial denaturation at 98˚C for 30 s, followed by 30 cycles of denaturation at 98˚C for 10 s, annealing at 67˚C for 10 s and extension at

72˚C for 20 s. The final extension was at 72˚C for 2 min. The optimal primer concentration were 1.25 µM for shi2, stx2, spy and hipo primers, 1.0 µM for lm and S primers, and 0.75 µM of sTyphi primer.

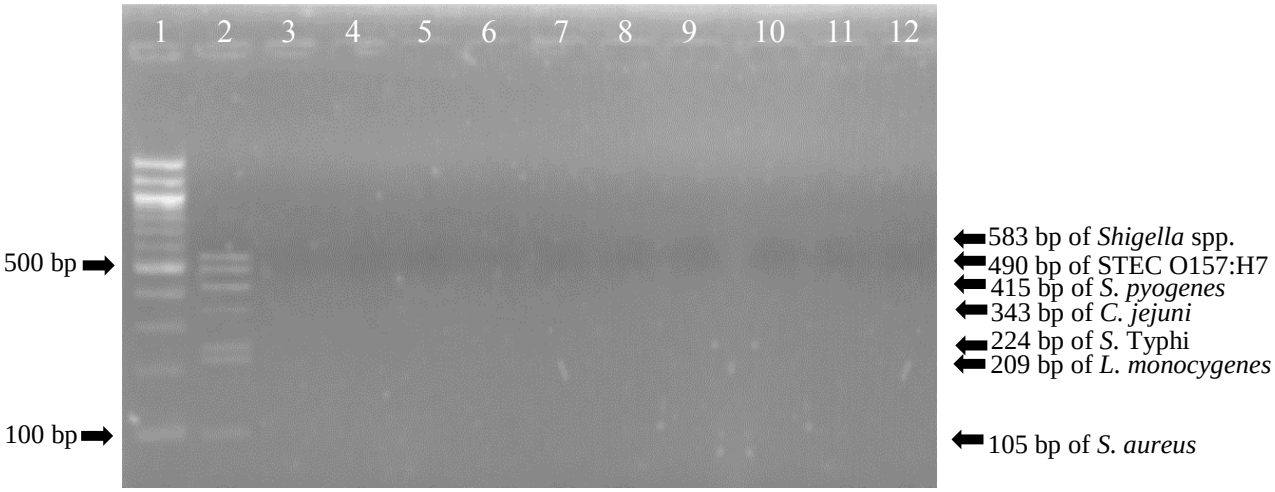

Figure 7 The specificity test with target and non-target foodborne pathogen species at 67˚C annealing temperature and 30 PCR cycles in agarose gel. Lane 1 100 bp DNA ladder, lane 2 multiplex PCR of seven target species, lane 3 *A. baumannii*, lane 4 *A. hydrophilus*, lane 5 *E. clocaeae*, lane 6 *E. feacalis*, lane 7 *K. pneumoniae*, lane 8 *P. aeruginosa*, lane 9 *P. mirabilis*, lane 10 *S. epidermidis*, lane 11 *S. marcescens*, lane 12 negative control

Table 9 The specificity test at the final optimized condition with seven target and nine non-target foodborne pathogen species.

	Shigella species-specific primer	STEC O157: H7 species-specific primer	*S. pyogenes* species-specific primer	*C. jejuni* species-specific primer	*S.* Typhi species-specific primer	*L. monocytogenes* species-specific primer	*S. aureus* species-specific primer
Shigella spp.	+	-	-	-	-	-	-
STEC O157: H7	-	+	-	-	-	-	-
S. pyogenes	-	-	+	-	-	-	-
C. jejuni	-	-	-	+	-	-	-
S. Typhi	-	-	-	-	+	-	-
L. monocytogenes	-	-	-	-	-	+	-
S. aureus	-	-	-	-	-	-	+
A. baumannii	-	-	-	-	-	-	-
A. hydrophilus	-	-	-	-	-	-	-
E. clocaceae	-	-	-	-	-	-	-
E. feacalis	-	-	-	-	-	-	-
K. pneumonia	-	-	-	-	-	-	-
P. aeruginosa	-	-	-	-	-	-	-
P. mirabilis	-	-	-	-	-	-	-
S. epidermidis	-	-	-	-	-	-	-
S. marcescens	-	-	-	-	-	-	-

To develop the heptaplex-direct PCR assay, the important keys for success were primer design, the optimization of annealing temperature, primer concentration, and extension time of PCR.

For primer design, the seven species-specific primer pairs were modified or designed to obtain a narrow T_m range of 65-67°C. They can therefore worked effectively at the optimized annealing temperature, resulting in a successful multiplex amplification. This step was also related to primer specificity. Species-specific primers used in this study were modified from previous reports or newly designed to bind to the target genes that are unique to each target species. For primer modification, target primer sequences were identified in the target species alignment and then edited at the 5' end without compromise its specificity. *Shigella* species-specific primers were designed from *ipaH* gene that encodes for invasion plasmid antigen H and has been proven to be carried by only carried by four *Shigella* species (Chen *et al.* 2012). In fact, *ipa* gene has been divided into five types (A, B, C D and F) but *ipaH* was widely used due to it is not temperature regulated (Hartman *et al.* 1990). The *stx2* gene coding for Shiga toxin (Verocytotoxin) was used for STEC primer design (Matise *et al.* 1998). The *spy1258* gene, encoding a putative transcriptional regulator, was used to target *S. pyogenes*. It is only found in a complete genome of *S. pyogenes* and absent in other bacterial genomes available in Genbank (Finegold and Bennion 2006). The hippuricase gene (*hipO*) was used to detect *C. jejuni*. It encodes for the hippuricase enzyme that can be used to characterize *C. jejuni* from other *Campylobacter* (Hani and Chan 1995, Persson and Olsen 2005). The *invA* gene encoding the invasive protein was used to detect *S.* Typhi. It is carried by Salmonella serovar Enteritidis species and has been verified to exit only in Salmonella used in this study (D'Souza *et al.* 2009, Suo *et al.* 2010). The *hlyA* gene, encoding listeriolysin O, was used for *L. monocytogenes* detection. This gene was used to differentiate *L. monocytogenes* from other *Listeria* (Chen *et al.* 2012). The 16S rDNA gene was used for *S. aureus*. It has been successfully validated for its specificity (Tang *et al.* 2006, Samadi *et al.* 2007, Chen *et al.* 2012).

Length of primers and their sequences also promote the specificity of assay. All primers were designed to be 18-28 bp in length, which were in the optimal length that provided least mismatch. A primer that is too long is more likely to cross-hybridize

with other primers and produces non-specific fragment in PCR reaction. Also, primer that is too short will decrease the assay specificity as it binds to a template nonspecifically (National Forensic Science Technology Center 2007). With the primer sequences, G and C are considered to promote the correct binding. G and C at 3' end of primer helps increasing of specificity due to their strong hydrogen bond (National Forensic Science Technology Center 2007).

Annealing temperature is one of factors affecting a successful amplification of the developed assay. In this study, the optimal annealing temperature that provided the best results was 67˚C. The range of 55-72˚C has been recommended for a PCR assay optimization to yield the best result (National Forensic Science Technology Center 2007). This temperature was higher than those of previous studies that reported the annealing temperatures in the range of 55-64˚C (Altinok *et al.* 2008, Chen *et al.* 2012, Persson and Olsen 2005, Tavakoli *et al.* 2010, Lei *et al.* 2008, Jofré *et al.* 2005). The optimal annealing temperature was experimentally determined, and specificity was a key issue for the selection of this temperature. Using high annealing temperature causes inefficient amplification, as the hydrogen bonds between primers and templates are disrupted. On the other hand, low annealing temperature causes non-specific binding of primer other sequences which are not the target due to partial complementarity. Consequently, non-specific bands are generated (National Forensic Science Technology Center 2007).

Primer concentrations, especially in a multiplex reaction, affect the amplification efficiency of all targets. The optimized primer concentrations were found to be 0.75 -1.25 µM. This is slightly higher than the optimal range recommended for PCR (0.1-1.0 µM) (Butler 2005). However, values that exceed the recommended range can be found in the literature, particularly for a large multiplex. Petri *et al*. (2013) used 2-3 uM in multiplex PCR to identify 13 lactic acid bacteria in wine. Another was the study of Jackson *et al*. (2004), in which 1.78 uM of primer concentration was used to identify five of 23 enterococci species in multiplex PCR. Nevertheless, too high primer concentration can promote mis-priming and primer dimers, which will compete with the true primer-target binding for other components in the PCR reaction like dNTPs or DNA polymerase, resulting in low yield of expected PCR products. On the other hand,

too low of primer concentration causes annealing to be inefficient and low yield of PCR products is obtained (Unit 2001).

The extension time generally depends on the length of PCR product. Usually, the rate of elongation is approximately 35 to 100 bases per second (National Forensic Science Technology Center 2007). In this study, the extension time that provided the complete PCR fragments for all primers was 30 s. This was sufficient for the largest size PCR product of 583 bp (*Shigella* spp.) to be amplified. The same extension time was found in the previous study of Jofre´ *et al*. (2005) using multiplex PCR to detect *Salmonella* (284 and 404 bp) and *L. monocytogenes* (215 and 406 bp) in cooked ham. When the PCR product size exceeds 500 bp, the extension time is increased accordingly. Several studies reported the extension time around 50-90 s, as their largest PCR products were 700-1500 bp (Chen *et al.* 2012, Lei *et al.* 2008, Tavakoli *et al.* 2010, Petri *et al.* 2013, Persson and Olsen 2005). Excessive extension time leads to non-specific amplification, while too short extension time results in insufficient time to complete the replication of target gene.

The optimal cycle number of PCR usually depends on the initial amount of DNA template. The optimized condition of 30 cycles yields the best result, as observed the complete bands for all seven targets. The similar cycle number of PCR was found in the identification of *Vibrio cholerae* using species-specific primer targeted to outer membrane protein *ompW* gene by Nandi *et al*. (2000), the detection and epidemiological typing of *Salmonella* in human clinical samples by Alvarez *et al*. (2004), the detection of pathogenic *Vibrio* spp. in shellfish by Panicker *et al*. (2004), and the detection of *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 in Iranian foods by Tavakoli *et al*. (2010). Forensic assays that aim to detect DNA from ancient and degraded samples may increase the cycle number to 40 at the expense of needing a very clean, DNA-free work environment. This is because minimal contamination of plasticware or circulated air could be amplified by the assay. Nonetheless, too low cycle number results in low yield of PCR product, while too many cycles lead to plateau effect and appearance of non-specific background product (National Forensic Science Technology Center 2007).

1.3.4 Reproducibility test

The reproducibility test was conducted to ensure that the result is reproducible when tested with other isolates of target species. In this step, a few isolates of each target species obtained from Department of Biomedical Science and Microbiological Laboratory of Songklanagarind hospital, Faculty of Medicine, Prince of Songkla University, were tested with the developed assay. The list of all species used in this study is shown in Table 10.

Table 10 List and details of target species used for reproducibility test

Bacteria	Strain	Total number (isolates)
Shigella flexneri	DMST 44237	3
Shigella spp.	DMST 1332	
Shigella spp. (isolate no.1)	Clinical isolate	
STEC O157:H7	ATCC 43890	2
STEC O157:H7 (isolate no.1)	Clinical isolate	
S. pyogenes	ATCC 19615	3
S. pyogenes (isolate no.1)	Clinical isolate	
S. pyogenes (isolate no.2)	Clinical isolate	
C. jejuni	ATCC 33291	2
C. jejuni (isolate no.1)	Clinical isolate	
S. Typhi	DMST 22842	3
S. Typhi (isolate no.1)	Clinical isolate	
S. Typhi (isolate no.2)	Clinical isolate	
L. monocytogenes	DMST 17303	3
L. monocytogenes (isolate no.1)	Clinical isolate	
L. monocytogenes (isolate no.2)	Clinical isolate	
S. aureus	ATCC 6538	3
S. aureus (isolate no.1)	Clinical isolate	
S. aureus (isolate no.2)	Clinical isolate	

The result of reproducibility test is shown in Figure 8. The results indicated that the assay was highly reproducible as the test provided the expected band of target strain for all corresponding isolates listed in Table 10

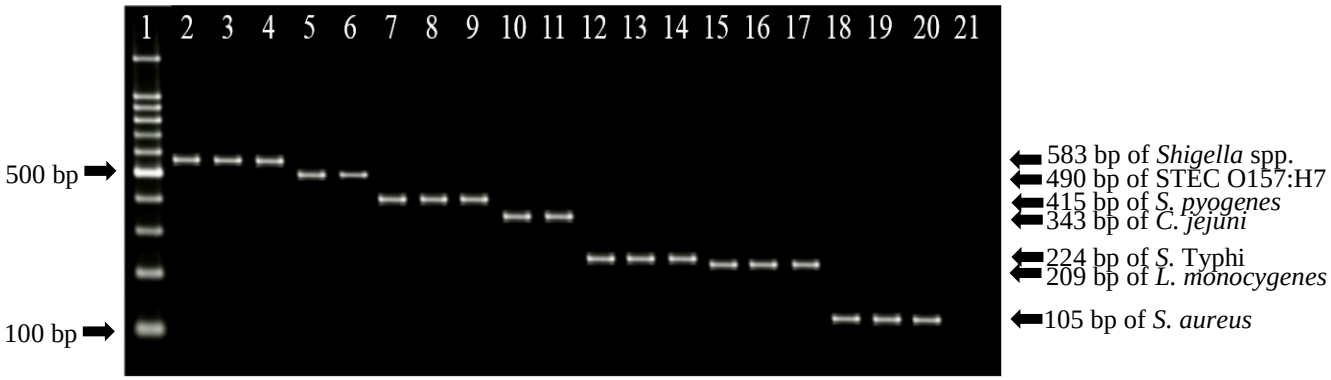

Figure 8 The diagram of agarose gel of reproducibility test for seven target pathogens (using other isolates of target pathogens) at 65˚C annealing temperature; Lane 1 100 bp DNA ladder, lane 2-4 *Shigella* spp., lane 5-6 STEC O157: H7, lane 7-9 *S. pyogenes*, lane 10-11 *C. jejuni*, lane 12-14 *S.* Typhi, lane 15-17 *L. monocytogenes*, lane 18-20 *S. aureus*, lane 21 negative control

1.3.5 Sensitivity test

The assay's sensitivity test was performed to determine the minimum amount of the target bacteria that can be detected by the assay. Bacterial colonies from each known species were suspended in 0.95% normal saline and the suspension's optical density (OD) at 260 nm was measured using UV-160A spectrophotometer (Shimadzu, Japan). The turbidity of each bacterial cell suspension was adjusted to 0.8 OD according to McFarland standard no.5. This approximated to 10^{10} CFU/ml. The number of bacteria in each suspension was confirmed using microbial drop plate method. Ten µl of the bacterial suspension was pipetted onto a nutrient media plate and incubated at 37˚C for 18 h, except for *C. jejuni* (42˚C for 48 h). The bacterial colonies were counted and reported as CFU/ml. The 10^{10} CFU/ml sample was serially diluted ten-fold to 10^{0} CFU/ml. These serial dilutions were then used as templates for sensitivity (limit of detection) analysis.

The limit of detection for each target species was determined (Table 11). The minimal amount of bacteria cell detected by the developed assay was 10^{0} CFU/ml for STEC and *L. monocytogenes*, 10^{1} CFU/ml for *S. pyogenes* and *S.* Typhi, 10^{5} CFU/ml for *C. jejuni*, and 10^{6} CFU/ml for *Shigella* spp. and *S. aureus*.

Table 11 The assay sensitivity test for each target at varying concentrations. (+) denotes the presence of PCR product and (-) denotes the absence of PCR product.

Species	Bacterial concentration (CFU/ml)										
	10^{10}	10^{9}	10^{8}	10^{7}	10^{6}	10^{5}	10^{4}	10^{3}	10^{2}	10^{1}	10^{0}
Shigella spp.	+	+	+	+	+	-	-	-	-	-	-
STEC	+	+	+	+	+	+	+	+	+	+	+
S. pyogenes	+	+	+	+	+	+	+	+	+	+	-
C. jejuni	+	+	+	+	+	+	-	-	-	-	-
S. Typhi	+	+	+	+	+	+	+	+	+	+	-
L. monocytogenes	+	+	+	+	+	+	+	+	+	+	+
S. aureus	+	+	+	+	+	-	-	-	-	-	-

For the sensitivity test, the pre-PCR solution preparation was a critical step that enhanced the effectiveness of cell lysis and DNA release process and increased the assay's sensitivity (Kitpipit *et al.* 2014). The results demonstrated that the developed assay provided a high sensitivity for detection of most pathogens, which are in a range of 10^{0}-10^{6} CFU/ml. The range obtained in this study is wider than those of the previous study of Kawasaki *et al*. (2009), Oh *et al*. (2009), and Chen *et al*. (2012) which were in the range of 10^{1}-10^{3} CFU/ml. Additionally, the developed assay was also sufficiently sensitive to detect the infectious dose of five foodborne pathogens; STEC (infectious dose of 10-100 CFU/ml), *S. pyogenes* (10^{7} CFU/ml), *S.* Typhi (10^{5} CFU/ml), *L. monocytogenes* (10^{2} CFU/ml), and *S. aureus* (10^{6} – 10^{8} CFU/ml) without the need of bacterial enrichment. For *Shigella* spp. (10^{2} CFU/ml) and *C. jejuni* (5×10^{2} CFU/ml) (FDA 2013), pooling of pre-PCR buffer with Micro-con or enrichment could increase the assay's sensitivity at the cost of speed and complexity. Interestingly, the sensitivity of *S. aureus* was considered lower when compared to the previous study of Kumar *et al*., 2009 which was around 10^{4} CFU/ml (Kuma *et al.* 2009). It should be noted that the previous study employed an overnight enrichment step.

The assay was also applicable for detection of some pathogens at the level specified by the Ministry of Public Health Standard of Thailand 2013 (Volume 364). All foodborne pathogens must not be found in any food, except for some pathogens specified in appendix 2 and 3. *Salmonella* must not be found in 25 g or ml of food, *S. aureus* must not be found in 0.1 g or ml of food, and *L. monocytogenes* must not be found in 25 g or ml of food. In the case of meat standards (established by Department

of Livestock Development, Ministry of Agriculture and Cooperatives), total bacterial count and *S. aureus* at 30˚C must not exceed 5×10^6 and 10^2 CFU/ml, respectively, without the presence of *Salmonella* spp. With an enrichment step, the assay should be able to detect *S. aureus* at these low levels, as enrichment should boost the number of bacteria to the limit of detection of the assay.

1.3.6 Mock case test

1.3.6.1 Artificially contaminated food investigation

The artificially contaminated food test was performed with 22 food samples, including four raw meats, one seafood, three frozen foods, and 14 processed foods, obtained from local supermarkets, restaurants, and food stalls in Songkhla, Thailand. The list of food samples is shown in Table 12. The samples were cut into small samples of approximately 5 mm × 5 mm. They were irradiated by UV radiation for 1 h to eliminate background bacteria and DNA and then spiked with 10^8 CFU/ml of mixed target template species. Spiked samples were divided into two portions: one was analyzed by the developed heptaplex-direct PCR assay and the other portion was suspended into 100 µl PBS and subjected to bacterial culture. The selective agar were as follows: MacConkey agar (MAC) for STEC, Salmonella-Shigella agar (SS) for *Shigella* spp. and *S.* Typhi., CAMPY agar for *C. jejuni*, blood agar for *S. pyogenes*, *L. monocytogenes*, and for *S. aureus*. The suspected colonies on selective agar were identified using biochemical tests at the Microbiology Unit, Department of Pathology, Faculty of Medicine, Prince of Songkla University. These tests were TSI slant, lysine decarboxylase test, citrate test, urease test, and motility test (Voravuthikunchai 2003).

Table 12 Lists of spiked food used in this study.

Type of food	Name	Source
Raw sample		
Meat	chicken	Tesco Lotus express, Hatyai, Songkhla
	pork	Local market, Hatyai, Songkhla
	beef 1	Local market, Hatyai, Songkhla
	beef 2	Sha-bu-ji Restaurant
Seafood	fish	Sha-bu-ji Restaurant
Frozen foods	frozen food 1	Big C extra supermarket, Hatyai, Songkhla
	frozen food 2	Tesco Lotus supermarket, Hatyai, Songkhla
	frozen meat ball	Tesco Lotus supermarket, Hatyai, Songkhla
Street samples		
Processed foods	fried fish	Food stall, Prince of Songkla University
	spicy fish salad	Food stall, Prince of Songkla University
	steamed egg	Food stall, Prince of Songkla University
	hot and sour fish soup1	Food stall, Prince of Songkla University
	sweet red sauce beef	Food stall, Prince of Songkla University
	sweet and sour chicken	Food stall, Prince of Songkla University
	pot-chicken stewed	Tesco Lotus supermarket, Hatyai, Songkhla
	mild pork soup	Tesco Lotus supermarket, Hatyai, Songkhla
	fried noodles	Big C extra supermarket, Hatyai, Songkhla
	fried noodles with soy	Food stall, Songklanagarind hospital, Songkhla
	chicken curry	Tesco Lotus supermarket, Hatyai, Songkhla
	chicken green curry	Big C extra supermarket, Hatyai, Songkhla
	fried pork	Food stall, Prince of Songkla University
	fried chicken	Food stall, Prince of Songkla University

The results of heptaplex-direct PCR and culture method of artificially contaminated foods are shown in Table 13. The multiplex PCR results showed 15 *Shigella*-positive samples, 15 STEC O157:H7-positive samples, 6 *S. pyogenes*-positive samples, 16 *C. jejuni*-positive samples, 21 *S.* Typhi-positive samples, 10 *L. monocytogenes*-positive samples, and 22 *S. aureus*-positive samples. While the culture method results showed 4 *Shigella*-positive samples, 12 *S. pyogenes*-positive samples, and 22 positive samples for STEC O157:H7, *C. jejuni*, *S.* Typhi, *L. monocytogenes* and *S. aureus*.

Table 13 The results of artificially contaminated food investigation with 22 food samples randomly obtained from local market, supermarket, restaurant and food stall in Songkhla, Thailand.

Food	*Shigella*-positive[b]		STEC O157: H7-positive[a]		*S. pyogenes* – positive[a]		*C. jejuni* – positive[a]		*S.* Typhi -positive[a]		*L. monocytogenes* -positive[b]		*S. aureuss*-positive[a]	
	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture
Raw sample														
chicken1	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Pork	-	+	-	+	-	-	-	+	-	+	-	+	+	+
beef1	+	-	+	+	+	+	+	+	+	+	+	+	+	+
beef2	+	-	+	+	-	+	+	+	+	+	+	+	+	+
fish2	+	-	+	+	-	-	+	+	+	+	-	+	+	+
frozen food1	+	-	+	+	+	+	+	+	+	+	-	+	+	+
frozen food2	+	-	+	+	-	+	+	+	+	+	-	+	+	+
meat ball	+	-	+	+	-	-	+	+	+	+	-	+	+	+
Street samples														
fried fish	+	-	+	+	-		+	+	+	+	-	+	+	+
spicy fish salad	-	-	-	+	-	+	-	+	+	+	-	+	+	+
steamed egg	-	-	-	+	+	+	+	+	+	+	-	+	+	+
hot and sour fish soup1	+	+	+	+	-	-	+	+	+	+	-	+	+	+
sweet red sauce beef	+	-	+	+	-	+	-	+	+	+	+	+	+	+
sweet and sour chicken	-	-	-	+	-	+	-	+	+	+	+	+	+	+
pot-chicken stewed	-	-	-	+	+	+	-	+	+	+	-	+	+	+
mild pork soup	+	-	+	+	-	-	+	+	+	+	+	+	+	+
fried noodles	+	-	+	+	-	+	+	+	+	+	+	+	+	+
fried noodles with soy sauce	+	-	+	+	-	-	+	+	+	+	+	+	+	+
Chicken curry	+	-	+	+	-	-	+	+	+	+	+	+	+	+
chicken green curry	+	-	+	+	+	-	+	+	+	+	-	+	+	+
fried pork	-	+	-	+	+	+	+	+	+	+	+	+	+	+
fired chicken	-	-	-	+	-	-	-	+	+	+	-	+	+	+
Total	15	4	15	22	6	12	16	22	21	22	10	22	22	22

Note: + is the presence of target PCR product - is not detected

[a] indicates no significant difference [b] indicates significant difference

To statistically compare the efficiency of both two methods; heptaplex-direct PCR and bacterial culture, the McNemar's test and Holm-Bonferroni correction were

used. The p-value lower than 0.05 indicates the significant differences between the two methods. The result showed that the heptaplex assay was significantly better than the culture method at detecting *Shigella* spp. Although the heptaplex detected pathogens in lower number of samples, no statistical differences were seen for five pathogens: STEC, *S. pyogenes*, *C. jejuni*, *S.* Typhi, and *S. aureus*. The culture method was significantly better at detecting *L. monocytogenes*

Comparison between the results obtained from multiplex PCR and culture method showed that the efficiency of culture method was not significantly different from multiplex PCR, except for *L. monocytogenes* and *Shigella* spp. All samples were positive by culture method for *L. monocytogenes* while only 10 samples were positive for multiplex PCR. For *Shigella* spp., the multiplex PCR was significantly better than culture method, as 15 samples was positive by PCR while only four samples were positive by culture. It should be due to the SS agar used for culture method inhibited some *Shigella* spp. (Leifson 1935, Taylor and Harris 1965) and the growth rate of *Shigella* spp. was relatively low when compared to other bacteria. They also cannot survive outside the host (Bryan 1978).

1.3.6.2 Naturally contaminated food investigation

To validate the robustness of the assay, 100 food samples (ten raw meats, five frozen pre-cooked meals, ten desserts and 75 processed food) were tested with the developed heptaplex-direct PCR. The list of food samples is shown in Table 14. Food samples were randomly chosen from local markets and supermarkets in Songkhla, Thailand. All samples were prepared as pre-PCR solution for multiplex PCR detection and some samples were also cross-checked by culture method as described in 1.3.7.1.

Table 14 List of food sample used in naturally contaminated food investigation

No.	Food		Source
1	Chicken with yellow rice	(ข้าวหมกไก่)	Restaurant in Hatyai, Songkhla, Thailand
2	Rice soup with chicken	(ข้าวต้มไก่)	Restaurant in Hatyai, Songkhla, Thailand
3	Steamed flour with coconut filling	(ขนมสอดไส้มะพร้าว)	Food stall, Songklanagarind hospital, Songkhla
4	Stir fried preserved mustard green with egg	(ผักกาดดองกับไข่)	Food stall, Prince of Songkla University
5	Stir fried sweet and sour pork	(ผัดเปรี้ยวหวาน)	Food stall, Prince of Songkla University
6	Spicy meatball salad	(ยำลูกชิ้น)	Food stall, Faculty of Science, PSU
7	Noodles with pork	(ก๋วยเตี๋ยวหมู)	Food stall, Faculty of Science, PSU
8	Gang-Kua with pork	(แกงคั่วหมู)	Food stall, Faculty of Science, PSU
9	Spicy minced pork	(ลาบหมู)	Food stall, Faculty of Science, PSU
10	Omelet	(ไข่เจียว)	Food stall, Faculty of Science, PSU
11	Caned mackerel in tomato sauce	(ปลากระป๋อง)	Food stall, Faculty of Science, PSU
12	Sa-lal-pao chinese mushroom filling	(ซาลาเปาเห็ดหอม)	7-11, Faculty of Medicine
13	Bolona sandwich	(แซนวิทโบโลน่า)	7-11, Faculty of Medicine
14	Glass noodle spicy salad	(ยำวุ้นเส้น)	Restaurant in Hatyai, Songkhla, Thailand
15	Stir fried rice with chilli dip	(ข้าวผัดน้ำพริก)	Restaurant in Hatyai, Songkhla, Thailand
16	Fried rice with seafood	(ข้าวผัดทะเล)	Restaurant in Hatyai, Songkhla, Thailand
17	Grilled chicken with rice	(ข้าวมันไก่ย่าง)	Food stall, Faculty of Science, PSU
18	Fried egg with tamarine sauce	(ไข่ลูกเขย)	Food stall, Faculty of Science, PSU
19	Hot and sour soup with snapper	(แกงส้มปลากระพง)	Food stall, Faculty of Science, PSU
20	Stuffed bitter gourd with clear soup	(ต้มมะระหมูสับ)	Food stall, Faculty of Science, PSU
21	Gold egg yolk drop	(ขนมทองหยอด)	Food stall, Prince of Songkla University
22	Stir fried bitter gourd with egg	(ผัดมะระไข่)	Food stall, Prince of Songkla University
23	Egg custard in pumpkin	(ขนมฟักทองสังขยา)	Food stall, Prince of Songkla University
24	Fish organs sour soup	(แกงไตปลา)	Food stall, Prince of Songkla University
25	Res curry with shrimp	(แกงคั่วกุ้ง)	Food stall, Prince of Songkla University
26	Mild soup with pork	(แกงจืดหมูสับ)	Food stall, Prince of Songkla University
27	Steamed flour with coconut filling	(ขนมสอดไส้)	Food stall, Prince of Songkla University
28	Stuffed glutinous rice with banana	(ข้าวต้มมัด)	Food stall, Prince of Songkla University
29	Fried chicken	(ไก่ทอด)	Food stall, Prince of Songkla University
30	Fried pork	(หมูทอด)	Food stall, Prince of Songkla University
31	Stir fried bitter gourd	(ผัดมะระ)	Food stall, Prince of Songkla University
32	Chilli in oil stir fried chicken	(ไก่ผัดน้ำพริกเผา)	Food stall, Prince of Songkla University
33	Omelet	(ไข่เจียว)	Food stall, Prince of Songkla University
34	Fried pork	(หมูทอด)	Food stall, Prince of Songkla University
35	Tom-Yam seafood noodles	(ก๋วยเตี๋ยวซีฟู้ดต้มยำ)	Food stall, Faculty of Science, PSU
36	Pink seafood noodles	(เย็นตาโฟทะเล)	Food stall, Prince of Songkla University
37	Roasted red pork	(หมูแดง)	Food stall, Songklanagarind hospital, Songkhla
38	Roasted pork	(หมูกรอบ)	Food stall, Songklanagarind hospital, Songkhla

No.	Food		Source
39	pork's leg with rice	(ข้าวขาหมู)	Food stall, Songklanagarind hospital, Songkhla
40	Chicken in sesame oil	(ไก่หมักน้ำมันงา)	Restaurant in Hatyai, Songkhla, Thailand
41	Shrimp	(กุ้งสด)	Tesco Lotus supermarket, Hatyai, Songkhla
42	Beef	(เนื้อวัวสด)	Tesco Lotus supermarket, Hatyai, Songkhla
43	Chicken curry soup	(แกงกะหรี่ไก่)	Restaurant in Hatyai, Songkhla, Thailand
44	Pork's liver	(ตับหมูสด)	Tesco Lotus supermarket, Hatyai, Songkhla
45	Fish's roe topped sushi	(ซูชิไข่ปลา)	Restaurant in Hatyai, Songkhla, Thailand
46	Pickled fish and noodles Thai papaya salad	(ตำซั่วปลาร้า)	Food stall, Prince of Songkla University
47	Shrimp topped sushi	(ซูชิหน้ากุ้ง)	Restaurant in Hatyai, Songkhla, Thailand
48	Bamboo shoots hot and sour soup	(แกงส้มหน่อไม้)	Restaurant in Hatyai, Songkhla, Thailand
49	Pak-Moh coconut filling	(ปากหม้อไส้หวาน)	Food stall, Prince of Songkla University
50	Salmon topped sushi	(ซูชิแซลมอน)	Food stall, Prince of Songkla University
51	Fried pork	(หมูทอด)	Restaurant in Hatyai, Songkhla, Thailand
52	Spicy stir fried pork	(ผัดเผ็ดหมู)	Restaurant in Hatyai, Songkhla, Thailand
53	Tapioca pork	(สาคูไส้หมู)	Restaurant in Hatyai, Songkhla, Thailand
54	Coconut milk stewed pumpkin	(ฟักทองบวด)	Restaurant in Hatyai, Songkhla, Thailand
55	Fried minced chicken	(ไก่สับทอด)	Food stall, Faculty of Nursing, PSU
56	Stir fried noodles with soy sauce	(ผัดซีอิ๊ว)	Food stall, Songklanagarind hospital, Songkhla
57	Boiled chicken with turmeric soup	(ไก่บ้านต้มขมิ้น)	Restaurant in Hatyai, Songkhla, Thailand
58	pickled fish	(ปลาส้ม)	Restaurant in Hatyai, Songkhla, Thailand
59	Snapper hot and sour soup	(แกงส้มปลากระพง)	Food stall, Faculty of Natural resources, PSU
60	Curry fried fish	(ฉู่ฉี่ปลา)	Food stall, Faculty of Natural resources, PSU
61	Spicy stir fried meatball	(ผัดเผ็ดลูกชิ้น)	Food stall, Faculty of Natural resources, PSU
62	Fish organs sour soup	(แกงไตปลา)	Food stall, Faculty of Nursing, PSU
63	Pork rib Tom-Yam	(ซี่โครงหมูต้มยำ)	Food stall, Faculty of Nursing, PSU
64	pickled fish and crab Thai papaya salad	(ตำปูปลาร้า)	Food stall, Prince of Songkla University
65	Chicken fried with ginger	(ไก่ผัดขิง)	Food stall, Songklanagarind hospital, Songkhla
66	Chilli in oil stir fried chicken	(ไก่กรอบผัดพริกเผา)	Food stall, Prince of Songkla University
67	Fried chicken	(ไก่ทอด)	Food stall, Faculty of Natural resources, PSU
68	Pork rib hot and sour soup	(แกงส้มซี่โครงหมู)	Food stall, Faculty of Nursing, PSU
69	Fried chicken	(ไก่ทอด)	Restaurant in Hatyai, Songkhla, Thailand
70	Spaghetti seafood with chilli and basil	(สปาเกตตี้ขี้เมาทะเล)	Restaurant in Hatyai, Songkhla, Thailand
71	Stired rice with garlic	(ข้าวผัดกระเทียม)	Restaurant in Hatyai, Songkhla, Thailand
72	Spicy stired fried beef	(ผัดเผ็ดเนื้อ)	Food stall, Prince of Songkla University
73	Stir fried Thai flat bean with shrimp	(ผัดสะตอกุ้ง)	Restaurant in Hatyai, Songkhla, Thailand
74	Minced beef steak	(สเต็กเนื้อบด)	Restaurant in Hatyai, Songkhla, Thailand
75	Grilled beef	(เสือร้องไห้)	Restaurant in Hatyai, Songkhla, Thailand
76	pork sausage	(ไส้กรอกหมูซีพี)	Tesco Lotus supermarket, Hatyai, Songkhla
77	Glutinous rice roasted in bamboo joint	(ข้าวหลาม)	Food stall, Songklanagarind hospital, Songkhla
78	Boiled chicken with turmeric soup	(ไก่ต้มขมิ้น)	Food stall, Faculty of Nursing, PSU

No.	Food		Source
79	Chilli in oil stir fried chicken	(ไก่ผัดน้ำพริกเผา)	Food stall, Faculty of Nursing, PSU
80	Stir fried mushroom	(ผัดเห็ด)	Food stall, Faculty of Nursing, PSU
81	Chicken bolona	(โบโลน่าไก่ BKP)	7-11, Faculty of Medicine
82	Pork soup	(น้ำซุปหมู)	Restaurant in Hatyai, Songkhla, Thailand
83	Spicy stir fried rice with chicken	(ข้าวผัดผัดเผ็ดไก่)	Restaurant in Hatyai, Songkhla, Thailand
84	Meatball	(ลูกชิ้นเทวดา)	Tesco Lotus supermarket, Hatyai, Songkhla
85	Honey roasted duck noodles	(หมี่เป็ดอบน้ำผึ้ง)	Restaurant in Hatyai, Songkhla, Thailand
86	Ka-Nom-Krok	(ขนมครกชาววัง)	Restaurant in Hatyai, Songkhla, Thailand
87	Stir fried rice with chilli dip	(ข้าวผัดน้ำพริก)	Restaurant in Hatyai, Songkhla, Thailand
88	Thai pork salad	(หมูน้ำตก)	Restaurant in Hatyai, Songkhla, Thailand
89	Stir fried chinese kale with chicken	(ผัดแขนงไก่)	Restaurant in Hatyai, Songkhla, Thailand
90	Salmon	(แซลมอนสด)	Tesco Lotus supermarket, Hatyai, Songkhla
91	Stir fried rice with chilli dip	(ข้าวผัดน้ำพริก)	Restaurant in Hatyai, Songkhla, Thailand
92	Curry fried fish	(ฉู่ฉี่ปลาดุก)	Food stall, Faculty of Natural resources, PSU
93	Bar-B-Q chicken	(ไก่บาร์บีคิว)	Tesco Lotus express, Hatyai, Songkhla
94	Beef	(เนื้อโคขุนสด)	Big C extra supermarket, Hatyai, Songkhla
95	Roasted red pork	(หมูแดง)	Restaurant in Hatyai, Songkhla, Thailand
96	Chicken sausage	(ไส้กรอกไก่)	Big C extra supermarket, Hatyai, Songkhla
97	Climbing wattle omelet	(ไข่เจียมชะอม)	Restaurant in Hatyai, Songkhla, Thailand
98	Stir fried pork with yellow curry paste	(คั่วกลิ้งหมู)	Food stall, Faculty of Natural resources, PSU
99	Steamed chicken with rice	(ข้าวมันไก่)	Restaurant in Hatyai, Songkhla, Thailand
100	Fried chicken	(ไก่ทอด)	Restaurant in Hatyai, Songkhla, Thailand

Only one sample (no. 2) provided the positive result of 490 bp of STEC by the developed heptaplex-direct PCR (Table 15), while no positive result was found by bacterial culture method. This low rate of contamination is similar to previous studies. Kawasaki *et al*. (2009), found one sample positive for STEC in 77 commercial foods using multiplex PCR. Lei *et al*. (2008) found that three of 77 meat samples were positive for STEC O157:H7 by multiplex PCR while only two samples were positive by culture. The presence of STEC was probably due to its tolerance for a range of condition. Although the optimal growth temperature is approximately 8-10˚C, this bacterium can survive for several weeks in refrigeration temperature (Buchanan and Bagi 1994, Rajkowski and Marmer 1995). Furthermore, STEC O157:H7 has high acid tolerance and can survive for many weeks to month in variety of acidic foods (IFT 1997). For the other bacteria, as most foodborne pathogens are heat sensitive, thus cooking with heat and pressure should have killed off the foodborne pathogens.

Adequate manufacturing standards of supermarket food, could also prevent bacterial contamination.

It was possible that the negative results could be due to bacterial count being lower than the limit of detection. However, this was deemed unlikely as the 25 food samples randomly selected for culture showed no contamination of the seven target pathogens. Adding an enrichment step should raise the sensitivity of the multiplex but comes at the cost of additional time and cost for analysis. The non-significant differences seen with both the artificially contaminated and naturally contaminated food samples showed that the assay could be used without enrichment.

Table 15 The results of naturally contaminated food investigation with 100 food samples randomly obtained from local market

Food	Total	*Shigella* spp.	STEC	*S. pyogenes*	*C. jejuni*	*S.* Typhi	*L. monocytogenes*	*S. aureuss*
Raw meat	10	0	0	0	0	0	0	0
Frozen pre-cook meal	5	0	0	0	0	0	0	0
Dessert	10	0	0	0	0	0	0	0
Processed food	7	0	1	0	0	0	0	0

1.4 CONCLUDING REMARKS

The standard for bacterial identification requires bacterial culture; this can be time-consuming, complicated, and expensive. A PCR-based method using bacterial DNA can overcome these problems. However, the process of DNA extraction is itself time-consuming, has low efficiency, and increases the possibility of contamination.

This study reported the developed heptaplex-direct PCR assay that omits the DNA extraction process. It is simple and can be achieved within 2 h. It can simultaneously detect seven pathogens; *Shigella* spp., STEC, *S. pyogenes*, *C. jejuni*, *S.* Typhi. *L. monocytogenens*, and *S. aureus*, in a single reaction without the step of bacterial enrichment. The important keys for success are the use of modified DNA polymerase and additives in the PCR buffer, optimization of sample amount during pre-PCR

solution preparation, and assay optimization in terms of primer concentration, annealing temperature and PCR cycle number.

To ensure applicability in everyday use, the developed assay was validated for its specificity, reproducibility, sensitivity, and robustness. High specificity was obtained from the developed assay mostly by the selection of virulent gene in the step of primer design. The assay is also sufficiently sensitive to detect the infectious dose of five foodborne pathogens without the need for bacterial enrichment. Application of the heptaplex-direct PCR to the artificially contaminated food, when compared to the standard culture method, showed no significant difference in the detection efficiency of most target foodborne pathogens. For *Shigella* spp., the assay provided significantly better efficiency than the culture method. Detection of 100 naturally contaminated food samples showed only one sample to be STEC-positive. Low contamination of food observed could be due to the cooking process and sanitation standards of food manufacturing.

In short, the heptaplex-direct PCR developed in this study offers a simply, rapid, and cost-effective choice for detection of seven foodborne pathogens. The assay is robust in terms of reproducibility, specificity and sensitivity. It is an alternative method that can be used for bacterial screening, especially to reduce use of broad-spectrum antibiotics. The assay is also useful for the field of epidemiological surveillance and is applicable to other related microbial forensic evidence. The combination of the developed technique and the standard culture method can be a powerful tool for bacterial identification and examination of food contaminated with the target pathogens to provide the cause of death and help in enforcing food safety legislations.

REFERENCES

Altinok, I., Capkin, E., and Kayis, S. 2008. Development of multiplex PCR assay for simultaneous detection of five bacterial fish pathogens. *Veterinary Microbiology*. 131(3–4): 332-338.

Asakura ,H., Makino, S., Kobori, H., Watarai, M., Shirahata, T., Ikeda, T., and Takeshi, K. 2001. Phylogenetic diversity and similarity of active sites of Shiga toxin (stx) in Shiga toxin-producing *Escherichia coli* (STEC) isolates from humans and animals. Epidemiology Infection. 127(1): 27-36.

Belkum, A. V., and Erasmus, M. C. 2010. Classification of bacterial pathogens. Netherlands: Department of Medical Microbiology and Infectious Diseases Unit Research and Development

BioLabs. 2011. Q5® Hot Start High-Fidelity DNA Polymerase. UK: New Enaland BioLabs.

Bryan, F. L. 1978. Factors that contribute to outbreaks of foodborne disease. *Journal of Food Protection*. 41: 816-827.

Buchanan, R. L., and Bagi, L. K. 1994. Expansion of response surface models for the growth of *Escherichia coli* O157:H7 to include sodium nitrite as a variable. *International Journal of Food Microbiology*. 23: 317-332.

Budowle, B. 2004. Genetics and attribution issues that confront the microbial forensics field. *Forensic Science International*. 146: 185-188.

Budowle, B., Murch, R., and Chakraborty, R. 2005. Microbial forensics: the next forensic challenge. *International Journal of Legal Medicine*. 119(6): 317-730.

Budowle, B., Schutzer, S. E., Einseln, A., Kelley, L. C., Walsh, A. C., Smith, J. A. L., Marrone, B. L., Robertson, J., and Campos, J. 2003. Building microbial forensics as a response to bioterrorism. *Science*. 301(5641): 1852-1853.

Bush, L. M., Abrams, B. H., Beall, A., and Johnson, C. C. 2001. Index case of fatal inhalational anthrax due to bioterrorism in the United States. *the New England Journal of Medicine*. 345(22): 1607-1610.

Butler, J. M. 2005. *Forensic DNA typing*. Edited by M. Listewnik, J. Soucy and P. Chester. second ed. UK: Elsevier.

CDC. 2013. *Multistate outbreak of listeriosis linked to imported Frescolina Marte Brand ricotta salata cheese (Final Update)*, 21 Nov 2012 2012 [cited 8 October 2013]. Available from http://www.cdc.gov/listeria/outbreaks/cheese-09-12/.

Center for Occupational Safety Health and Environment Management, C. 2012. *Mahidol University Biosafety guideline*. Vol. 2. Mahidol University: Thongsuk Print.

Chen, J., Tang, J., Liu, J., Cai, Z., and Bai, X. 2012. Development and evaluation of a multiplex PCR for simultaneous detection of five foodborne pathogens. *Journal of Applied Microbiology*. 112: 823-830.

Chen, P., and Shakhnovich, E. I. 2009. Lethal mutagenesis in viruses and bacteria. *Genetics*. 183: 639-650.

Crump, J. A., Luby, S. P., and Mintz, E. D. 2004. The global burden of typhoid fever. *Bull World Health Organ*. 82(5): 346-353.

D'Souza, D. H., Critzer, F. J., and Golden, D. A. 2009. Real-time reverse-transcriptase polymerase chain reaction for the rapid detection of *Salmonella* using *inv*A primers. *Foodborne pathogens and disease*. 6: 1097-1106.

Deng, W., Liou, S. R., Plunkett, G., Mayhew, G. F., Rose, D. J., Burland, V., Kodoyianni, V., Schwartz, D. C., and Blattner, F. R. 2003. Comparative genomics of *Salmonella enterica* serovar Typhi strains Ty2 and CT18. Journal Bacteriology. 185(7): 2330-2337.

Deng, W., Xi, D., Mao, H., and Wanapat, M. 2008. The use of molecular techniques based on ribosomal RNA and DNA for rumen microbial ecosystem studies: a review. *Molecular Biology Reports*. 35(2): 265-274.

Drancourt, M., Berger, P., and Raoult, D. 2004. Systematic 16S rRNA gene sequencing of atypical clinical isolates identified 27 new bacterial species associated with humans. *Journal of Clinical Microbiology*. 42(5): 2197-2202.

Farrell, J. J., Doyle, L. J., Addison, R. M., Reller, L. B., Hall, G. S., and Procop, G. W. 2005. Broad-range (pan) *Salmonella* and *Salmonella* serotype typhi-specific real-time PCR assays: potential tools for the clinical microbiologist. American Journal of Clinical Pathology. 123(3): 339-345.

FDA. 2013. *Foodborne pathogenic microorganisms and naatural toxins nandbook Staphylococcus aureus*, 05/08/2013 2013 [cited 13/10 2013]. Available from http://www.fda.gov/Food/FoodborneIllnessContaminants/CausesOfIllnessBadBugBook/ucm070015.htm.

FDA. 2014. *Foodborne pathogenic microorganisms and natural toxins handbook Campylobacter jejuni*, 15/3/2013 2013 [cited 5 June 2014]. Available from http://www.fda.gov/food/foodborneillnesscontaminants/causesofillnessbadbugbook/ucm070024.htm.

Feng, P. 1997. Impact of molecular biology on the detection of foodborne pathogens. *Molecular Biotechnology*. 7: 267-278.

Ferretti, J. J., McShan, W. M., Ajdic, D., Savic, D. J., Savic, G., Lyon, K., Primeaux, C., Sezate, S., Suvorov, A. N., Kenton, S., Lai, H. S., Lin, S. P., Qian, Y., Jia, H. G., Najar, F. Z., Ren, Q., Zhu, H., Song, L., White, J., Yuan, X., Clifton, S. W., Roe, B. A., and McLaughlin, R. Complete genome sequence of an M1 strain of *Streptococcus pyogenes*. Proceedings of the National Academy of Sciences. 98(8): 4658-4663.

Finegold, S., and Bennion, R. 2013. *Rapid identification of key pathogens in wound infection by molecular means* 2006 [cited 5 October 2013]. Available from http://oai.dtic.mil/oai/oai?verb=getRecord&metadataPrefix=html&identifier=ADA448241.

Fode-Vaughan, K. A., Maki, J. S., Benson, J. A., and Collins, M. L. 2003. Direct PCR detection of *Escherichia coli* O157:H7. *Letters in Applied Microbiology*. 37(3): 239-243.

Fode-Vaughan, K. A., Wimpee, C. F., Remsen, C. C., and Collins, M. L. 2001. Detection of bacteria in environmental samples by direct PCR without DNA extraction. *Biotechniques*. 31(3): 598-607.

Fukushima, H., Kawase, J., Etoh, Y., Sugama, K., Yashiro, S., Iida, N., and Yamaguchi, K. 2010. Simultaneous screening of 24 target genes of foodborne pathogens in 35 foodborne outbreaks using multiplex real-time SYBR green PCR analysis. *International Journal of Microbiology*. 2010: 1-18.

Hani, E. K., and Chan, V. L. 1995. Expression and characteriazation of *Campylobacter jejuni* benzoylglycine amidohydrolase (hippuricase) gene in *Escherichia coli*. *Journal of Bacteriology*. 177(9): 2396-2402.

Hartman, A. B., Venkatesan, M., Oaks, E. V., and Buysse, J. M. 1990. Sequence and molecular characterization of a multicopy invasion plasmid antigen gene, ipaH, of *Shigella flexneri*. *Journal of Bacteriology*. 172(4): 1905-1915.

Hasap, L., Thanakiatkrai, P., Singkhamanan, D., Linacre, A., and Kitpipit, T. 2013. Multiplex-direct PCR assay for foodborne pathogen identification: An application in forensic investigation. *Forensic Science International: Genetics Supplement Series*. 4(1): e103-e104.

Hathout, Y., Demirev, P. A., Ho, Y. P., Bundy, J. L., Ryzhov, V., Sapp, L., Stutler, J., Jackman, J., and Fenselau, C. 1999. Identification of *Bacillus* spores by matrix-assisted laser desorption ionization-mass spectrometry. *Applied and Environmental Microbiology*. 65(10): 4313-4319.

Huang, Q., Hu, Q., and Li, Q. 2007. Identification of 8 foodborne pathogens by multicolor combinational probe coding technology in a single real-time PCR. *Clinical Chemistry*. 53(10): 1741-1748.

IFT. 1997. Foodborne disease significance of *Escherichia coli O157:H7* and other enterohemorrhagic *E. coli*. *Food Technology*, 69-76.

Jiang, Y., Zhu, C., Luan, J., Xue, F., Chen, G., and Tang, T. Analysis of the hippurate hydrolase gene of *Campylobacter jejuni* isolated in China. Unpublished.

Jofré, A., Martin, B., Garriga, M., Hugas, M., Pla, M., Rodríguez-Lázaro, D., and Aymerich, T. 2005. Simultaneous detection of Listeria monocytogenes and Salmonella by multiplex PCR in cooked ham. *Food Microbiology*. 22(1): 109-115.

Kawasaki, S., Fratamico, P. M., Horikoshi, N., Okaka, Y., Takeshita, K., Sameshima, T., and Kawamoto, S. 2009. Evaluation of a multiplex PCR system for simultaneous detection of *Salmonella* spp., *Listeria monocytogenes*, and *Escherichia coli* O157:H7 in foods and in food subjected to freezing. *Foodborne pathogens and disease*. 6: 81-89.

Keith, A., Lampel L, Palmer, A., Orlandi, I., and Leroy, K. 2000. Template preparation for PCR-Bbased assays for detection of food-borne bacterial pathogens. Applied and Environmental Microbiology. 66(10): 4539-4545.

Kidgell, C., Reichard, U., Wain, J., Linz, B., Torpdahl, M., Dougan, G., and Achtman, M. 2002. *Salmonella typhi*, the causative agent of typhoid fever, is approximately 50,000 years old. *Infection Genetics Evolution*. 2(1): 39-45.

Kim, E. C. and Lee, J. K. Direct Submission. Submitted (24-OCT-2002) Laboratory Medicine, Seoul National University College of Medicine, 28 Yongondong Chongnogu, Seoul. 110-744, Korea

Kitpipit, T., Chotigeat, W., Linacre, A., and Thanakiatkrai, P. 2014. Forensic animal DNA analysis using economical two-step direct PCR. *Forensic Science Medicine and Pathology*. 10: 29-38.

Kitpipit, T., Sittichan, K., and Thanakiatkrai, P. 2013. Are these food products fraudulent? Rapid and novel triplex-direct PCR assay for meat identification. *Forensic Science International: Genetics Supplement Series*. 4(1): e33-e34.

Klančnik, A., Kovač, M., Toplak, N., Piskernik, S., and Jeršek, B. 2012. "PCR in food analysis." In, 195-220. Slovenia: Department of Food Science and Technology, Biotechnical Faculty, University of Ljubljana.

Kotloff, K. L., Winickoff, J. P., Ivanoff, B., Clemens, J. D., Swerdlow, D. L., Sansonetti, P. J., Adak, G. K., and Levine, M. M. 1999. Global burden of *Shigella* infections: implications for vaccine development and implementation of control strategies. *Bulletin of the World Health Organization*. 77(8): 651-666.

Kuma, T. D. K., Murali, H. S., and Batra, H. V. 2009. Simultaneous detection of pathogenic *B. cereus*, *S. aureus* and *L. monocytogenes* by multiplex PCR. *Indian Journal of Microbiology*. 49(3): 283-289.

Lazcka, O., Del Campo, F. J., and Munoz, F. X. 2007. Pathogen detection: a perspective of traditional methods and biosensors. *Biosens Bioelectron*. 22(7): 1205-17.

Le Loir, Y., Baron, F., and Gautier, M. 2003. *Staphylococcus aureus* and food poisoning. *Genetics and Molecular Research*. 2(1): 63-76.

Lei, I. F., Roffey, P., Blanchard, C., and Gu, K. 2008. Development of a multiplex PCR method for the detection of six common foodborne pathogens. *Journal of Food and Drug Analysis*. 16(4): 37-43.

Leifson, E. 1935. New culture media based on sodium desoxycholate for the isolation of intestinal pathogens and for the enumeration of colon bacilli in milk and water. *The Journal of Pathology and Bacteriology*. 40: 581.

Levinson, W. 2010. *Review of medical microbiology and immunology*. USA: McGraw-Hill Companies, Inc. .

Linacre, A., and Tobe, S. 2013. Wildlife DNA analysis: applications in forensic science. Oxford: Wiley.

Liu, D., Hollingshead, S., Swiatlo, E., Lawrence, M. L., and Austin, F. W. 2005. Rapid identification of *Streptococcus pyogenes* with PCR primers from a putative transcriptional regulator gene. Research in Microbiology. 156: 564-567.

Mackie, R. I., Aminov, R. I., Hu, W., Klieve, A. V., Ouwerkerk, D., Sundset, M. A., and Kamagata, Y. 2003. Ecology of uncultivated *Oscillospira* species in the rumen of cattle, sheep, and reindeer as assessed by microscopy and molecular approaches. *Applied and Environmental Microbiology*. 69(11): 6808-6815.

Matise, I., Shelton, M., Phillips, G., and Will, L. A. 1998. Sensitivity PCR method for detection of *Escherichia coli* O157:H7 and other Shiga toxin-producing bacteria in ground meat. *Iowa State University Swine Research Report*. 33: ASL-R1509.

Mook, P., Patel, B., and Gillespie, I. A. 2011. Risk factors for mortality in non-pregnancy-related listeriosis. *Epidemiology and Infection*. 21: 1-10.

Moreira, D. 1998. Efficient removal of PCR inhibitors using agarose-embedded DNA preparations. *Nucleic Acids Research*. 26(13): 3309-3310.

National Forensic Science Technology Center, N. 2014. *DNA Aanlyst Training* 2007 [cited 7 May 2014]. Available from http://www.nfstc.org/pdi/Subject04/pdi_s04_m01_02.htm.

National Forensic Science Technology Center, N. 2014. *DNA Analyst Training* 2007 [cited 7 May 2014]. Available from http://www.nfstc.org/pdi/Subject04/pdi_s04_m01_03_f.htm.

National Forensic Science Technology Center, N. 2014. *DNA Analyst Training* 2007 [cited 7 May 2014]. Available from http://www.nfstc.org/pdi/Subject04/pdi_s04_m01_02_c.htm.

National Forensic Science Technology Center, N. 2014. *DNA Analyst Training* 2007 [cited 7 May 2014]. Available from http://www.nfstc.org/pdi/Subject04/pdi_s04_m01_04.htm.

Nguyen, H., Bennett, H., Nhan, P., and Lee, S. B. 2012. New strategies to overcoming PCR inhibition using mutant *Taq* polymerase and PCR enhancers. USA: San Jose State University, .

Ochman, H., Elwyn, S., and Moran, N. A. 1999. Calibrating bacterial evolution. *Proceedings of the National Academy of Sciences*. 96(2): 12638–12643.

Oh, M. H., Paek, S. H., Shin, G. W., Kim, H. Y., Jung, G. Y., and Oh, S. 2009. Simultaneous identification of seven foodborne pathogens and *Escherichia coli* (pathogenic and nonpathogenic) using capillary electrophoresis-based single-strand conformation polymorphism coupled with multiplex PCR. *Journal of Food Protection*. 72(6): 1262-1266.

Olofsson, J., Axelsson-Olsson, D., Brudin, L., Olsen, B., and Ellström, P. 2013. *Campylobacter jejuni* Aactively invades the amoeba *Acanthamoeba polyphaga* and aurvives within non digestive vacuoles. *Plos one*. 8(11): 1-7.

Olsen, S. J., Hansen, G. R., Bartlett, L., Fitzgerald, C., Sonder, A., Manjrekar, R., Riggs, T., Kim, J., Flahart, R., Pezzino, G., and Swerdlow, D. L. 2001. An outbreak of *Campylobacter jejuni* infections associated with food handler contamination: the use of pulsed-field gel electrophoresis. *The Journal of Infectious Diseases*. 183(1): 164-167.

Ong, S. Y., Pratap, C. B., Wan, X., Hou, S., Abdul Rahman, A. Y., Saito, J. A., Nath, G., and Alam, M. 2012. Complete genome sequence of *Salmonella enterica* subsp. enterica Serovar Typhi P-stx-12. Journal of Bacteriology. 194 (8): 2115-2116.

Opel, K. L., Chung, D., and McCord, B. R. 2010. A study of PCR inhibition mechanisms using real time PCR. *Journal of Forensic Science*. 55(1): 25-33.

Persson, S., and Olsen, K. E. P. 2005. Multiplex PCR for identification of *Campylobacter coli* and *Campylobacter jejuni* frome pure cultures and directly on stool samples. *Journal of Medical Microbiology*. 54: 1043-1047.

Petri, A., Pfannebecker, J., Frohlich, J., and Konig, H. 2013. Fast identification of wine related lactic acid bacteria by multiplex PCR. *Food Microbiology*. 33(1): 48-54.

Port, G. C., Paluscio, E., and Caparon, M. G. 2013. Complete genome sequence of emm Type 14 *Streptococcus pyogenes* strain HSC5. Genome Announcements. 1 (4)

Pragman, A. A., and Schliever, P. M. 2004. Virulence regulation in *Staphylococcus aureus*: the need forin vivo analysis of virulence factor regulation. *FEMS Immunology and Medical Microbiology*. 2: 147-154.

Rajkowski, K. T., and Marmer, B. S. 1995. Growth of *Escherichia coli* O157:H7 at fluctuating incubation temperatures. *Journal of Food Protection*. 58: 1307–1313.

Rangel, J. M., Sparling, P. H., Crowe, C., Griffin, P. M., and Swerdlow, D. L. 2005. Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982–2002. *Emerging Infectious Disease*. 11(4): 603-609.

Roux, K. H. 2009. *Optimization and Troubleshooting in PCR*. 2 ed, *Cold Spring Harb Protocol*. USA: Cold Spring Habor Laboratory Press.

Ryan, C. A., Nickels, M. K., Hargrett-Bean, N. T., Potter, M. E., Endo, T., and Mayer, L. 2002. Massive outbreak of antimicrobial resistant salmonellosis traced to pasteurized milk. *The Journal of the American Medical Association* 278: 389-395.

Rychlik, W., Spencer, W. J., and Rhoads, R. E. 1990. Optimization of the annealing temperature for DNA amplification in vitro. *Necleic Acids Research*. 18(21): 6409-6412.

Samadi, N., Alvandi, M., Fazeli, M. R., Azizi, E., Mehrgan, H., and Naseri, M. 2007. PCR-based detection of low levels of *Staphylococcus aureus* contaminaton in pharmacertical preparations. *Journal of Biological Sciences*. 7(2): 359-363.

Schutzer, S. E., Budowle, B., and Atlas, R. M. 2005. Biocrimes, microbial forensics, and the physician. *Plos Medicine*. 2(12): 1242-1247.

Seng, P., Drancourt, M., Gouriet, F., La Scola, B., Fournier, P. E., Rolain, J. M., and Raoult, D. 2009. Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clinical Infectious Diseases*. 49(4): 543-551.

Shanmugakani, R. K., and Ghosh, A. R. 2012. Single primer designed for detection of *Shigella* species *African Journal of Microbiology Research*. 6(25): 5288-5293.

Sharma, V. K., and Dean-Nystrom, E. A. 2003. Detection of enterohemorrhagic *Escherichia coli* O157-H7 by using a multiplex real-time PCR assay for genes encoding intimin and shiga toxins. *Veterinary Microbiology*. 93: 247-260.

Suba, S., Rajeshwari, S., Prabhudas, K., and Shome, B. R. Molecular characterization of *Listeria monocytogenes* isolates by multi-locus sequence typing and multi-virulence locus sequence typing. Unpublished.

Sumby, P., Porcella, S. F., Madrigal, A. G., Barbian, K. D., Virtaneva, K., Ricklefs, S. M., Sturdevant, D. E., Graham, M. R., Vuopio-Varkila, J., Hoe, N. P., and Musser, J. M. Evolutionary origin and emergence of a highly successful clone of serotype M1 group a *Streptococcus* involved multiple horizontal gene transfer events. The Journal of Infectious Diseases. 192 (5): 771-782.

Suo, B., He, Y., Tu, S. I., and Shi, X. 2010. A multiplex real-time polymerase chain reaction for simultaneous detection of *Salmonella* spp., *Escherichia coli* O157, and *Listeria monocytogenes* in meat products. *Foodborne pathogens and disease*. 7: 619-628.

Swaran, Y. C., and Welch, L. 2012. A comparison between direct PCR and extraction to generate DNA profiles from samples retrieved from various substrates. *Forensic Science International: Genetics*. 6(3): 407-12.

Takayama, Y., Hikawa, S., Okada, J., Sunakawa, K., and Akahoshi, T. 2009. A foodborne outbreak of a group A streptococcal infection in a Japanese university hospital. *European Journal of Clinical Microbiology & Infectious Diseases* 28(3): 305-308.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, eeolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28: 2731-2739.

Tang, J. N., Shi, X. M., Shi, C. L., and Chen, H. C. 2006. Characterization of a duplex PCR assay for the detection of enterotoxigenic strains of *Staphylococcus aureus*. *Journal of Rapid Methods and Automation in Microbiology*. 14: 201-217.

Tavakoli, H. R., Najafi, A., and Ahmadi, A. 2010. Rapid, specific and concurrent detection of *Listeria*, *Salmonella* and *Escherichai coli* pathogens by multiplex

PCR in Irannian food. *African Journal of Microbiology Research*. 4(23): 2503-2507.

Taylor, W. I., and Harris, B. 1965. Isolation of shigellae. II. comparision of plating media and enrichment broths. *American Journal of Clinical Pathology*. 44: 476.

Ueda, S., and Kawabara, Y. 2009. The rapid detection of *Salmonella* from food samples by loop-mediated isothermal amplification (LAMP). *Biocontrol Science*. 14(2): 73-76.

Unit, T. R. S. C. a. R. G. D. 2001. Molecular Biology Techniques Manual 3 rd. In *Standard PCR protocol*. Thailand: Kasetsart University.

Venkatesan, M. M., Buysse, J. M., and Hartman, A. B. 1991. Sequence variation in two *ipaH* genes of *Shigella flexneri* 5 and homology to the LRG-like family of proteins. Molecular Microbiology. 5(10): 2435-2445.

Vogel, V., Falquet, L., Calderon-Copete, S. P., Basset, P., and Blanc, D. S. 2012. Short term evolution of a highly transmissible methicillin-resistant *Staphylococcus aureus* clone (ST228) in a tertiary care hospital. PLoS ONE. 7(6): E38969

Voravuthikunchai, S. P. 2003. *The identification of Gram-Positive and Gram-Negative bacteria*. Vol. 1. Bangkok: Chulalongkorn University Press.

Wang, L., Shi, L., Alam, M. J., Geng, Y., and Li, L. 2008. Specific and rapid detection of foodborne *Salmonella* by loop-mediated isothermal amplification method. *Food Research International*. 41(1): 69-74.

Wang, Y., and Oian, P. Y. 2009. Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. *Plos one*. 4(10): 1-9.

Yanagita, K., Kamagata, Y., Kawaharasaki, M., Suzuki, T., Nakamura, Y., and Minato, H. 2000. Phylogenetic analysis of methanogen in sheep rumen ecosystem and detection of *Methanomicrobium mobile* by fluorescence In Situ hybridization. *Bioscience, Biotechnology, and Biochemistry*. 64(8): 1737-1742.

Xu, D., Cisar, J. O., Poly, F., Yang, J., Albanese, J., Dharmasena, M., Wai, T., Guerry, P., and Kopecko, D. J. 2013. Genome sequence of *Salmonella enterica* serovar Typhi oral vaccine strain Ty21a. Genome Announcement. 1 (4): 00650-13.

APPENDICES

Appendix A

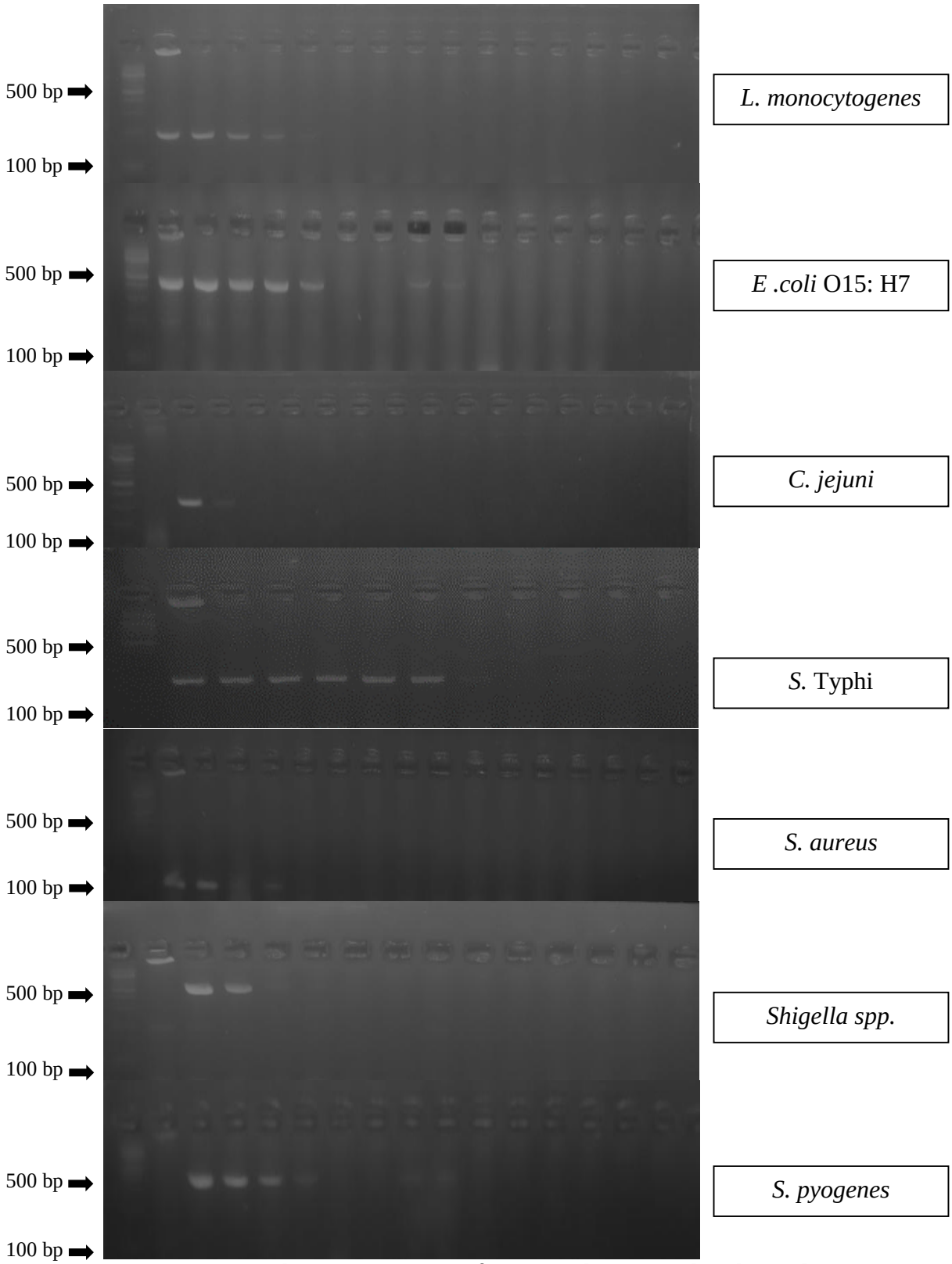

Figure 9 The sensitivity test of seven pathogen used in the study

Appendix B

McFarland Standard

Table B the preparation of McFarland standard

Volume in mL			
Standard	1% $BaCL_2$	1% H_2SO_4	Number of Bacteria/ mL/(10^8) represented
0.5	0.5	99.5	1.5
1	1.0	99.0	3
2	2.0	98.0	6
3	3.0	97.0	9
4	4.0	96.0	12
5	5.0	95.0	15
6	6.0	94.0	18
7	7.0	93.0	21
8	8.0	92.0	24
9	9.0	91.0	27
10	10.0	90.0	30

Reference: Pro-lab Diagnostics Inc, Canada

Purpose:

McFarland Standards are turbidity standards that are used to gauge approximately how many bacteria are present in a liquid suspension. The standards are used to visually compare the turbidity of a suspension of bacteria with the turbidity of the appropriate standard. Standards are prepared by adding barium chloride to sulfuric acid to obtain a barium precipitate. The volumes of the two reagents are adjusted to prepare standards of different turbidity that represent different concentrations of bacteria.

Reagents:

1. Sulfuric acid, 1%
2. Barium Chloride, 1.175%

Supplies:

1. Acid washed glass screw-cap tubes comparable to that used for test
2. Sterile serological pipettes and pipette bulb
3. Parafilm or paraffin

Equipment:

1. 100ml volumetric flasks
2. Vortex
3. Spectrophotometer
4. Magnetic stirrer and stirring rod

Procedure for the Preparation of a 0.5 McFarland Standard:

1. Add approximately 85 ml of 1% sulfuric acid (H_2SO_4) to a 100ml volumetric flask.
2. Using a volumetric pipette, add 0.5ml of 1.175% anhydrous barium chloride ($BaCl_2$) dropwise to the 1% sulfuric acid (H_2SO_4) while constantly swirling the flask.
3. Bring the volume to 100ml with 1% H_2SO_4.
4. Stir or mix for approximately 3 to 5 minutes while examining visually, until the solution appears homogeneous and free of clumps. A magnetic stirrer can be used for this step if available.
5. Check optical density, following the procedure described in the QC section below and record on QC sheet.
6. If QC is acceptable, dispense 2 to 7 ml volumes (depending on volumes routinely used in test) into each glass screw- cap tube.
7. Label the tubes appropriately including the expiration date and the initials of the person preparing the standards. Make sure that the labeling is positioned so that it does not interfere with spectrophotometer readings.
8. Cap the tubes tightly.
9. Draw a line to mark the meniscus on each tube. This mark can be used as a guide to check for evaporation at a later time.
10. Seal the tubes with paraffin or Parafilm.
11. Repeat the procedure to make additional standards using volumes indicated in Appendix A.
12. Store the prepared standards in the dark at room temperature for 3 months or longer as per QC acceptability.