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FULL PAPER

Simultaneous multiplex Polymerase Chain Reaction detection of *Salmonella* spp., *Escherichia coli* O157, *Vibrio parahaemolyticus*, *Vibrio cholerae*, *Listeria monocytogenes* and *Campylobacter* spp.

[1]Ling, S., [1]Noramirah, R., [1]Abidatul, A.A., [1]Nurfarhanah, N.M.J., [1,2]Noor-Azira, A.M., [1,2]Jambari, N.N., [3]Ungku Fatimah, U.Z.A., [1]New, C.Y. and [1,2,*]Son, R.

[1]*Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia*
[2]*Food Safety and Food Integrity, Institute of Tropical Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia*
[3]*Department of Food Service and Management, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia*

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Abstract

Foodborne illness is a global burden that impacts a country politically, economically and socio-economically. The severity of the burden can be unmeasurable as foodborne illness is often an underestimated problem. In order to enlighten the burden, appropriate food safety control measures should be taken. This study aimed to optimize a multiplex Polymerase Chain Reaction (mPCR) detection method to identify foodborne pathogens simultaneously. Six foodborne pathogens namely, *Salmonella* spp., *Escherichia coli* O157, *Vibrio parahaemolyticus*, *Vibrio cholerae*, *Listeria monocytogenes* and *Campylobacter* spp., were targeted in the mPCR detection method. Each mPCR parameter was tested and the outcome was analysed to obtain a successful mPCR protocol to detect the targeted foodborne pathogens. The amplified PCR products showed that the optimized mPCR protocol will be a potential rapid diagnostic tool in foodborne pathogen detection.

1. Introduction

Foodborne disease is caused by consumption of food that is contaminated by pathogens. Most of the disease-causing germs are infectious. In addition, harmful toxins and chemicals can also cause foodborne illness. The symptoms of foodborne disease can range from mild, self-limiting to severe with permanent damage. Children, the elderly, pregnant women, and the immunocompromised persons are greatly at risk when they contract foodborne pathogens. The impact of foodborne disease is greatly displayed in developing countries with wide economic inequality, particularly the African regions and South-East Asia regions. Furthermore, the tropical climate of these regions favours the growth of foodborne pathogens. The burden of foodborne disease impacts the economy of a country, particularly challenging the tourist, agricultural and food (export) industries (World Health Organization, (WHO) 2015).

Foodborne disease in Malaysia was reported to be fluctuating year by year (New, Ubong, Premarathne *et al.,* 2017). The total incident rate of food and waterborne diseases in Malaysia in 2015 was 50.32 per 100,000 population while the mortality rate of was 0.05 per 100,000 population according to Ministry of Health (MOH) Malaysia (2016). Among the diseases listed, food poisoning had the highest incident rate (47.34%), followed by typhoid (1.42%), cholera (0.80%) and dysentery (0.41%). However, this might not be the exact number of cases reported due to the rigid and strenuous protocols of reporting an outbreak. Additionally, not many who contracted foodborne disease will seek medical treatment due to the self-limiting symptoms.

Salmonella spp. (Najwa *et al*., 2015), *Escherichia coli* O157 (Radu *et al*., 1998), *Vibrio parahaemolyticus*, *Vibrio cholerae* (Letchumanan, 2013), *Listeria monocytogenes* (Goh, 2013) and *Campylobacter* spp. (New, Wong, Usha *et al*., 2017) are among the common foodborne pathogens in Malaysia. In the recent food poisoning cases (2013 - 2016), *Salmonella* was identified as the causative agent (Food Safety News, 2014; Astro Awani, 2014; Koris, 2016). Salmonellosis, a disease caused by non-typhoidal *Salmonella* is commonly associated with poultry, eggs, as well as meat, unpasteurized milk and juices, cheese, raw fruits and

*Corresponding author.
Email: *son@upm.edu.my*

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vegetables, and spices and nuts (Nguyen *et al*., 2016). Centers for Disease Control and Prevention (CDC) reported about half of the outbreaks of STEC in the United States from 2011 to 2014 were caused by *E. coli* O157: H7 also known as STEC O157, and contaminated ground beef and raw vegetables as the common food vehicles for infection. The low infectious dose of *E. coli* O157: H7 (10 - 100 CFU) is the reason for high number of cases in outbreaks (Settanni and Corsetti, 2007; Lim *et al*., 2010; Nguyen *et al*., 2016). In addition, *E. coli* infection is more common in lower-income countries (WHO, 2017). *L. monocytogenes* is associated with milk, dairy products, eggs, poultry and meat, and causes listeriosis. Listeriosis has a high mortality rate in immunocompromised populations, such as HIV/AIDS and chronic disease patients, elderly, pregnant women and unborn children. *L. monocytogenes* is a concern in the food industry as it often contaminates ready-to-eat food (Nguyen *et al*., 2016). According to WHO Regional Office for South-East Asia (SEARO) (2012), the incidence of listeriosis varied between 0.1 and 11.3 cases per 1, 000, 000 population per year in different countries. *V. cholerae* and *V. parahaemolyticus* are found in brackish and marine water, and prevalent in seafood, such as shrimp, shellfish, and finfish (Wei *et al*., 2014). These two *Vibrio* species have caused several outbreaks of diarrhoea especially *V. cholerae* for the seven cholera pandemics, and sporadic infections worldwide (Vinothkumar *et al*., 2013). *Campylobacter* infection or Campylobacteriosis is caused by consumption of drinking water, unpasteurised milk, and poultry which are contaminated by *Campylobacter* spp. (Silva *et al*., 2011). It is a significant public health hazard due to low infective dose, the serious sequelae, virulence genes and the clinical infection pattern (Al Amri *et al*., 2007).

The traditional method for detecting foodborne pathogens is a combination of selective cultivation techniques with standard biochemical tests. It is time-consuming, laborious and produces sampling and enumerating errors as well as subjective interpretation. This method also does not allow rapid detection of more than one pathogens in a large quantity of food samples. As a result, over the past few decades, researchers have developed various types of rapid methods based on molecular knowledge to detect pathogens in food samples. By mixing several sets of specific primers into a single PCR assay. Multiplex PCR (mPCR) is one of the well-known molecular methods that are able to detect multiple pathogens simultaneously in a single reaction. The advantages of using mPCR are that the method is sensitive, saves time and effort as well as laboratory cost (Settanni and Corsetti, 2007; Chen *et al*., 2012). There have been many studies done in the last decade in successfully developing mPCR procedures for simultaneous detection of three different foodborne pathogens (Park *et al*., 2006; Germini *et al*., 2009; Al-Jobori *et al*., 2016; Nguyen *et al*., 2016; Trimoulinard *et al*., 2017). mPCR is a very useful tool which has been used by other researchers to detect five different foodborne pathogens (Chen *et al*., 2012), distinguish between five closely-related *Salmonella* spp. (Tan, 2015) and also detect five virulence genes in *E. coli* O157: H7 (Jeshveen *et al*., 2012).

The development of rapid methods becomes a resource tool in the control of food safety and at the same time strengthening the capacity of countries to conduct assessment (WHO, 2017). Often the method development should be economical and in line with the national food safety systems. As such, there is a strive to develop more rapid methods. This study focused on the optimisation of simultaneous detection of six foodborne pathogens, which were *Salmonella* spp., *E. coli* O157, *V. parahaemolyticus*, *V. cholerae*, *L. monocytogenes* and *Campylobacter* spp. using mPCR.

2. Materials and methods

2.1 Preparation of bacterial strains

The six bacterial strains (*Salmonella* spp., *L. monocytogenes*, *E. coli* O157, *V. parahaemolyticus* and *V. cholerae* and *Campylobacter* spp.) were acquired from Bacteriology Food Safety Lab, UPM in glycerol stocks. The bacterial strains were revived by sub-culturing them in 10 ml Tryptic Soy Broth (TSB) and incubated at 37°C for 18 – 24 hours. At the same time, each strain was sub-cultured onto nutrient agar plates. For *V. parahaemolyticus* and *V. cholerae*, the strains are sub-cultured in 10 ml TSB with 1% sodium chloride, NaCl and on nutrient agar plates with 1% NaCl. The revived bacterial strains were then sub-cultured onto their respective selective agars for confirmation. *Salmonella* spp. on Xylose Lysine Deoxycholate (XLD) agar; *L. monocytogenes* on PALCAM agar; *E. coli* O157 on Eosin Methylene Blue (EMB) agar; *V. parahaemolyticus* and *V. cholerae* on Thiosulphate Bile Citrate Sucrose (TCBS) agar; *Campylobacter* spp. on *Campylobacter* Blood-Free Selective Medium. All media and chemicals used were purchased from Merck, Germany.

2.2 Preparation of DNA template

Confirmed bacterial strains were then subjected to DNA template extraction. Prior to that, the bacterial strains were sub-cultured into TSB and incubated at 37° C for 18 – 24 hours to obtain bacterial suspensions. Genomic DNA was extracted from the bacterial strain using modified boiling cell method following the method

described by Tang *et al.* (2009) with modification. One ml of bacterial suspension was transferred into 1.5 ml microcentrifuge tube. The tube was centrifuged at 10000 rpm for 3 mins. The supernatant was discarded, and the pellet was resuspended in 500 µl of sterile distilled water and then vortexed. The suspension was boiled for 10 mins, followed by immediate cooling at -20°C for 10 mins, then centrifuged at 10000 rpm for 1 min.

2.3 PCR amplification

Optimisation of PCR was carried out by performing singleplex first, then multiplex. Singleplex was performed by mixing 1X PCR buffer, 2.0 mM of $MgCl_2$, 0.2 mM of deoxyribonucleotide phosphate mix, 0.2 µM of primers, 1 U Taq DNA Polymerase and 2 µl of DNA template. The mixture was topped up with sterile distilled water to have the final volume of 25 µl. For the amplification, the samples underwent pre-denaturation at 95°C for 5 minutes and then cycled for 35 times of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s before incubated at 72°C for 7 min for the final extension and hold at 4°C. Primers used in this study were listed in Table 1.

There were several parameters adjusted in the optimisation of mPCR. The parameters include: 1. annealing temperature (55-60°C); 2. $MgCl_2$ concentration, (2.5-4.5 mM); 3. Primers concentration [Vc hlyA primer concentration (0.3-0.6 µM), C-23SrRNA primer concentration (0.15-0.30 µM), Styinva-JHO-2 primer concentration (0.2-0.4 µM), Vp *toxR* primer concentration (0.1-0.2 µM), *rfbE* primer concentration (0.2-0.3 µM)]; 4. deoxyribonucleotide phosphate (dNTP) mix concentration (0.3-0.5 mM); 5. Taq DNA polymerase concentration (1.5-2.0 U) and; 6. PCR buffer concentration (1 – 1.5X).

The final optimised protocol of the mPCR was performed in a 25 µl mixture containing 1.4X PCR Buffer, 3.0 mM MgCl2, 0.3 mM dnTP mix, 0.1 µM of Vp *toxR* primer, 0.5 µM of Vc *hlyA* primer, 0.2 µM of-of *rfbE*, LM-*hlyA* and Styinva-JHO-2 primer pairs and, 0.15 µM of C-23SrRNA primers, 1.5 U of Taq DNA Polymerase and 2.0 µL of DNA template. The PCR amplification was performed following pre-denaturation 95°C for 5 mins, 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 45 s and extension at 72°C for 30 s, then final extension at 72°C for 7 mins and lastly hold at 4°C.

All primers used in this study were purchased from Sigma Aldrich, Malaysia while the PCR reagents were purchased from Promega, USA.

2.4 Gel electrophoresis

Both singleplex and multiplex PCR products were separated on a 1.25% (w/v) agarose gel in 0.5X Tris-Borate-EDTA (TBE) buffer. The gel was pre-stained with Florosafe DNA Stain (1st base, Malaysia) and electrophoresed at 90V for 30 minutes and 60V for 1 hour for singlplex and multiplex system respectively. The gel was then viewed under UV light using Gel Documentation System (Syngene, USA). The lengths of amplified products were estimated by comparison against a 100 bp DNA Ladder (Promega, USA).

3. Results and discussion

Figure 1 shows the PCR products that were successfully amplified. In Figure 1, *invA* amplicon (119 bp) for *Salmonella* spp., *hlyA* amplicon (210 bp) for *L. monocytogenes*, *rfbE* amplicon (296 bp) for *E. coli* O157, *toxR* amplicon (368 bp) for *V. parahaemolyticus*, *hlyA* amplicon (427 bp) for *V. cholerae*, and 23S rRNA amplicon (650 bp) for *Campylobacter* spp. were identified easily via different amplicon sizes. When

Table 1. List of primers used in mPCR

Target Pathogens	Primers	Primer sequence 5' to 3'	Product length (bp)	Target Gene	References
Salmonella spp.	Styinva-JHO- 2 - F	TCGTCATTCCATTACCTACC	119	*invA*	Hoorfar *et al.* (2000)
	Styinva-JHO- 2 - R	AAACGTTGAAAAACTGAGGA			
Escherichia coli O157	*rfbE*-F	CAGGTGAAGGTGGAATGGTTGTC	296	*rfbE*	Bertrand and Roig (2007)
	rfbE-R	TTAGAATTGAGACCATCCAATAAG			
Vibrio parahaemolyticus	Vp *toxR*-F	ATACGAGTGGTTGCTGTCATG	368	*toxR*	Kim *et al.* (1999)
	Vp *toxR*-R	GTCTTCTGACGCAATCGTTG			
Vibrio cholerae	Vc *hlyA*-F	CAATCGTTGCGCAATCGCG	427	*hlyA*	Vinothkumar *et al.* (2013)
	Vc *hlyA*-R	TAATAAGCGAGCGGTACG			
Listeria monocytogenes	LM-*hlyA*-F	CGCAACAAACTGAAGCAAAGG	210	*hlyA*	Park *et al.* (2006)
	LM-*hlyA*-R	TTGGCGGCACATTTGTCAC			
Campylobacter spp.	C-23SrRNA-F	TATACCGGTAAGGAGTGCTGGAG	650	23S rRNA	Wang *et al.* (2002)
	C-23SrRNA-R	ATCAATTAACCTTCGAGCACCG			

developing a mPCR detection method, the amplicon sizes of the PCR product must be taken note based on the selected primers. Preferably, the difference between each amplicon should be not less than 100 bp and not more than 900 bp difference. This allows quicker separation during electrophoresis and maintaining the rapidness of the method. The primer design or selection if the primers are adopted must be ensured that they are not complementary to each other to ensure successful multiplexing and avoid cross-reactivity between primers and amplification of unwanted DNA fragments (Sint *et al.,* 2012). During the optimisation, it was noted that the parameters that affected the mPCR the most were the annealing temperature and the primer's concentration.

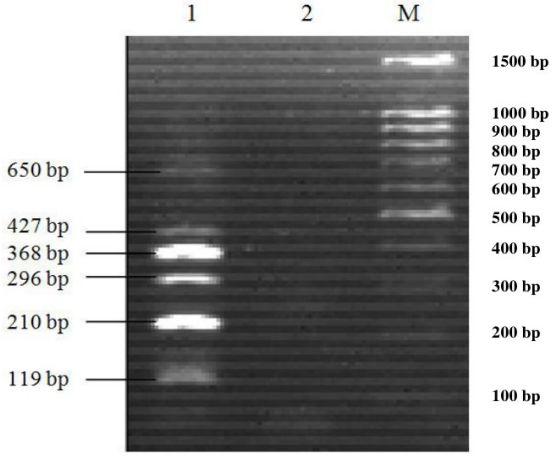

Figure 1. PCR amplification product of mPCR for simultaneous detection of six foodborne pathogens. M - 100 bp DNA ladder (Promega, USA). Lane 1- *invA* amplicon (119 bp) for *Salmonella* spp., *hlyA* amplicon (210 bp) for *L. monocytogenes*, *rfbE* amplicon (296 bp) for *E. coli* O157, *toxR* amplicon (368 bp) for *V. parahaemolyticus*, *hlyA* amplicon (427 bp) for *V. cholerae*, and 23S rRNA amplicon (650 bp) for *Campylobacter* spp. Lane 2- negative control.

In mPCR, it is essential to have all primers pairs work within the same range of melting temperature. Hence, prior to the mPCR optimisation, singleplex was performed to estimate the optimal range of the annealing temperature for each primer to produce the desired product (Sint *et al.,* 2012). The optimal range of the annealing temperature was then tested during multiplexing containing single extracts and a mixture of the bacterial templates to ensure that the right PCR products are produced. In this study, it was observed the *invA* amplicon for *Salmonella* spp. disappeared when the annealing temperature was increased to 56°C. Subsequent raise of the annealing temperature observed low signal strength (unclear bands) for the amplification of *invA* for *Salmonella* spp. Hence, it was deduced that the optimal annealing temperature for the multiplex was at 55°C whereby all targets produced the best signal strength.

Multiplexing single extracts will allow the developer to test the primers' specificity in which the exact primer pair should work in amplifying the target gene. In addition, it is also to check if there is any potential formation of primer cross-dimers (Sint *et al.,* 2012). This occurred when there is a primer interaction, usually weak interactions that produce non-specific products (Brownie *et al.,* 1997). Primer cross-dimers can be observed below 100 bp which often indicates a high concentration of primers being used or complementary primer pairs with no target amplification produced. It was observed that no primer cross-dimers were formed during the amplification (Figure 1), signifying the complete use of primers at the right concentration.

However, the amplicons showed unequal signal strength (Figure 1). *hlyA* amplicon for *V. cholerae*, 23S rRNA amplicon for *Campylobacter* spp. and *invA* amplicon for *Salmonella* spp. showed the same equal signal strength while *toxR* amplicon for *V. parahaemolyticus*, *rfbE* amplicon for *E. coli* O157 and *hlyA* amplicon for *L. monocytogenes* showed strong signal strength. This was one problem and the second problem noted in this study was the existence of non-specific product at the range of 120 – 150 bp. Throughout the optimisation, the primer concentrations were adjusted stepwise. This was done by starting off at the equal concentration of the primers (0.2 µM), observing the results and subsequently reducing the concentration of primers that produced stronger bands and increasing the primers that produced weaker bands. By balancing the primers' concentration, this yield a higher primer efficiency according to their varying amplification efficiency (Sint *et al.,* 2012). The final result as shown in Figure 1 was the best yield as the DNA template was not quantified.

C-23S rRNA primer pair was noted to be the cause of the non-specific product within the multiplex system which can be due to several other factors such as PCR inhibitors in the reaction. Hence, the reduction of the C-23S rRNA primer had successfully eliminated the non-specific product but produced weaker signal strength. Primer concentration that is slightly under will cause the PCR product unable to accumulate exponentially to exhibit strong signal (Markoulatos *et al.,* 2002). On the other hand, Vc – *hlyA* primers did not produce any targeted bands at low primer concentration in this study due to reason aforementioned. The increase of the primer concentration up to 0.5 µM produced a yield and further increase only produced higher primer dimer intensity.

To further suppress the production of non-specific bindings, hot-start PCR was employed (D'Aquila *et al.,* 1991; Chou *et al.,* 1992) coupled with higher PCR buffer concentration. The PCR buffer concentration consists of

KCl salt and Tri-HCl. The potassium salt reduces repulsive force between negatively-charged phosphate backbones of DNA double strands by neutralizing the charges. It facilitates annealing of primer to DNA template and stabilizes the primer-template structure (Rajendran, 2014). As the non-specific product was captured between 120 – 150 bp, the specificity was improved by increasing the potassium salt concentration according to Lorenz (2012).

The $MgCl_2$ concentration was also manipulated to further enhance the signal strength and reduce the non-specific bindings. It should be noted too that multiplexing more than 4 targets required a higher concentration of $MgCl_2$. Insufficient $MgCl_2$ concentration will lower the yield of a product or results in no amplicon production (Markoulatos *et al.,* 2002; Lorenz, 2012) while excessive $MgCl_2$ beyond 3.0 mM in the presence of the enzyme will cause spurious priming to non-specific template site, lowering the specificity and producing undesired products (Markoulatos *et al.,* 2002; Jeshveen *et al.,* 2012). Furthermore, the free Mg^{2+} ion will react with unbound dNTPs to form soluble complexes to produce the PCR amplicons. A balanced ratio of dNTPs and unbound Mg^{2+} will form amplicons with high specificity. Increasing concentration of dNTPs can immediately stop the PCR amplification as the increased amount of dNTPs will reduce the amount of Mg^{2+} ions that are available for the reaction (Markoulatos *et al.,* 2002; Roux, 2009; Jeshveen *et al.,* 2012). The final concentration of $MgCl_2$ and dNTP for the multiplex system to work best was at 3.0 mM and 0.3 mM respectively.

To ensure the best optimisation of a mPCR, primer/ assay sensitivity should be measured to validate the method. Theoretically, PCR is known as a specific and sensitive method as minute amounts of DNA templates is sufficient to achieve stable detection. This should be maintained within the multiplex system as multiple targets can be detected at low copies of DNA templates and avoiding biased detection rates (Sint *et al.,* 2012). The sensitivity can be gradually affected when there are large amounts of other taxa as well as PCR inhibitors. This study is in agreement with Sint *et al.* (2012) to use standard bacterial pathogens and quantify the DNA template before the start of optimising. It will improve the precision of the method by reducing the biasness, improves the sensitivity and also reduce workload.

In food safety, this specific, sensitive and rapid detection method will be able to finely discriminate the pathogens (Institute Food Technologist (IFT), 2000) and to provide leverage in early detection. Further, this helps in ensuring the safe food supply through identifying the routes of contamination and preventing foodborne diseases (Law *et al.,* 2014). Additionally, this approach allows one to study on the epidemiological of the foodborne pathogens and its evolution via downstream molecular analysis. Most importantly, it helps in building the capacity of assessment commitment in food industries. It is hoped that this successfully optimised method could be used in the pathogen surveillance system in Malaysia and in other countries burdened with foodborne illness.

Conflicts of Interest

The authors declare no conflicts of interest.

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