Squalen Bull. of Mar. and Fish. Postharvest and Biotech. 15(2) 2020, 53-64

www.bbp4b.litbang.kkp.go.id/squalen-bulletin

Squalen Bulletin of Marine and Fisheries Postharvest and Biotechnology

ISSN: 2089-5690
e-ISSN: 2406-9272

Rapid and Simultaneous Detection of *Vibrio parahaemolyticus, Salmonella* spp. and *Escherichia coli* in Fish by Multiplex PCR

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Article history:
Received: 18 February 2020; Revised: 13 July 2020; Accepted: 17 July 2020

Abstract

Pathogenic bacteria are commonly found as natural contaminants in seafood and fish products. Globally, several countries have been imposing strict regulations on the maximum levels of pathogens and consequently require microbial testing of pathogens before the products can be marketed. A culture-based method with biochemical assay has been widely used to detect pathogenic bacteria in food, despite its long and extensive process. Meanwhile, the alternative molecular-based method to overcome this problem, cannot differentiate between viable and nonviable cells, which may lead to underestimation. This study aimed to develop a multiplex PCR (mPCR) method as a confirmatory assay for the culture-based method to detect pathogens in fish products simultaneously. This method applied a pre-enrichment step to ensure the growth of low-level pathogens and the injured cells in the sample. The target genes were *ToxR*, *InvA,* and *UidA* for *Vibrio parahaemolyticus*, *Salmonella* spp. and *Escherichia coli*, respectively. This assay also amplified the 16S rDNA gene of bacteria as an internal control for the PCR reaction. By implementing liquid-based DNA extraction during analysis, the developed-mPCR was comparable to detect the targeted bacteria in artificially-contaminated samples. The method was more sensitive in naturally-contaminated samples, where the number of *E. coli*, *Salmonella* spp. and *V. parahaemolyticus* detected were 28, 7, and 22, respectively. While the conventional method only detected 26, 5, and 19 of the respective pathogens. With a relatively shorter time and lower operation cost, the mPCR method is potential as an alternative for the culture-based method.

Keywords: multiplex PCR (mPCR), *Vibrio parahaemolyticus*, *Salmonella* spp., *Escherichia coli*, fish product

1. Introduction

Fish and fish products consumption provides many nutritional benefits for consumers (Hellberg, DeWitt, & Morrissey, 2012; Lund, 2013). However, these commodities often pose a health risk to consumers due to their exposure to contaminants, including heavy metals and microbial foodborne pathogens (EFSA, 2015; Marques, Maulvault, & Nunes, 2019; Rachmawati & Triwibowo, 2012; Yamaki & Yamazaki, 2018). In the last decade, microbial pathogens have been associated with some foodborne cases worldwide and have been responsible for disease burden in society. Amongst these pathogens, *Vibrio* spp., *Salmonella* spp., and *E. coli* are the most common bacteria present in fish and fish products associated with human enteric infection (CDC, 2018; Yamaki & Yamazaki, 2018). Hence, it is essential to perform routine microbiological testing for analyzing the presence of microbial pathogens in fishery products to ensure its safety before consumption. Microbial examination of pathogens (i.e., *V. parahaemolyticus*, *Salmonella* spp., and *E. coli*) is required for food quality and control prerequisite in fisheries products marketed in the US (FDA, 2011), EU (EC, 2007), Japan (JETRO, 2011) and China (Anonymous, 2013).

Due to its cost-effectiveness, reliability, and ease of use, the culture-based assay followed by biochemical test has been widely used to detect and to enumerate the pathogenic bacteria in food matrices

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DOI: http://dx.doi.org/10.15578/squalen.v15i2.444

(Dwivedi & Jaykus, 2011). One of the limitations of this method is time-consuming and it depends on the capability of bacteria to grow well in different culture media, i.e., pre-enrichment and selective media (Law, Ab Mutalib, Chan, & Lee, 2015). Moreover, the additional biochemical test as a confirmatory assay in this method needs a week or longer to complete the species identification (Zhao, Lin, Wang, & Oh, 2014).

Currently, different rapid methods for detection and enumeration of pathogenic bacteria have been developed to overcome the limitations of culture-based methods. These methods are required particularly by food industries as well as competent authorities (food inspectors) to take immediate responses if microbial contaminant present in food, environment, or production/manufacturing facilities (Law et al., 2015). Hence, these rapid methods should have comparable accuracy and sensitivity to the culture-based assay. A potential alternative method is the molecular-based assay that employs PCR to multiply the nucleic acid of bacteria. Unlike the culture-based method, this assay is still unable to distinguish between live and dead bacteria during the detection and quantification processes (Li et al., 2017); thus, the PCR result may not represent the actual number of live pathogens in the samples (food, water or environmental). A combination of the cultured-based method followed by PCR as a confirmatory assay may overcome this problem. Previous study has demonstrated the ability of PCR method as a confirmatory assay to quantify the number of Vibrios in naturally contaminated samples (Bonny, Hossain, Lin, & Ali, 2018).

Based on its sensitivity and accuracy, the PCR assay has been approved as foodborne pathogen detection assay by several international standards (Germini, Masola, Carnevali, & Marchelli, 2009), such as ISO/TS 13136:2012 on the detection of Shiga-toxin producing *E. coli* (STEC) in food and animal feed and Bacteriological Analytical Manual (BAM) on the detection of enterotoxigenic *Vibrio cholerae* in foods (ISO, 2017; Koch, Payne, & Cebula, 2001). PCR assay has also been applied to detect pathogenic bacteria in fisheries products such as *Salmonella* spp. and *Listeria monocytogenes* (Amagliani, Omiccioli, Brandi, Bruce, & Magnani, 2010; Zhang et al., 2015), *Vibrio* spp. (Espiñeira, Atanassova, Vieites, & Santaclara, 2010), *Staphylococcus aureus* (Zarei, Maktabi, & Ghorbanpour, 2012), *E. coli* (Surendraraj, Thampuran, & Joseph, 2010), *Campylobacter jejuni* and *C. coli* (Taminiau, Korsak, Lemaire, Delcenserie, & Daube, 2014).

There are two different types of PCR method based on the number of primer pair used i.e., single PCR (simplex PCR) and multiplex PCR (mPCR) (Zhao et al., 2014) which combines two or several pairs of primers for simultaneous gene amplification (Shen, 2019). The simultaneous detection of foodborne pathogen in fisheries products including seafood can be performed by conventional (Malcolm et al., 2015; Yasmin, Kawasaki, & Kawamoto, 2007) or real-time mPCR (Amagliani et al., 2010; Taminiau et al., 2014; Zhang et al., 2015). Compared to the conventional PCR assay, the real-time mPCR eliminated the post-amplification step of gel electrophoresis hence avoiding the possible use of carcinogenic staining-substances as well as reducing the analysis time (Zhao et al., 2014). However, this real-time mPCR assay is more expensive and more complicated than the conventional mPCR, for example, due to the additional probes on its primers and the use of sophisticated technology (Kralik & Ricchi, 2017).

This study aimed to develop a reliable mPCR assay, as an alternative confirmatory assay of the conventional method of pathogenic detection in fisheries products. The method should enable simultaneous, better sensitivity and more cost-effective detection of *V. parahaemolyticus*, *Salmonella* spp., and *E. coli*.

2. Material and Methods

2.1. Bacterial Strains

V. parahaemolyticus ATCC 17802, *S. typhimurium* ATCC 14028, and *E. coli* ATCC 25922 from Remel® (Thermoscientific, USA) were used as references of targeted species, while *S. aureus* ATCC 2593 from Oxoid® (Thermoscientific, USA) was used as a reference of non targeted species (negative control) during method development. Wild-type bacteria, i.e., *Vibrio* sp., *Salmonella* sp., and *E. coli* from the culture collections of Microbiology Laboratory, RCMFPPB were used for method verification.

2.2. Bacterial Culture Preparation

Bacterial cultures were prepared in Brain Heart Infusion (BHI) (Oxoid, UK) for *Salmonella* spp. and *E. coli*, and BHI+2% NaCl for *V. parahaemolyticus*. The cultures were incubated at 35±2 °C for 18-24 h (Ranjbar, Naghoni, Afshar, Nikkhahi, & Mohammadi, 2016). The bacterial cultures were diluted to obtain ± 10^1 CFU/ml and were confirmed by plating onto Tryptone Soya Agar (TSA) (Oxoid, UK) for *Salmonella* spp., *E. coli* and *S. aureus*; and TSA+2% NaCl for *V. parahaemolyticus.* Prior to confirmatory assay, all bacteria were grown in pre-enrichment media, i.e., Lactose Broth (LB) (Oxoid, UK) for *E. coli* and *Salmonella* spp., and Alkaline Peptone Water (APW)

for *V. parahaemolyticus*. The cultures were incubated overnight at 35-37 °C and plated onto selective agar media using CHROMagar™ *Vibrio* (CHROMagar, France) (Di Pinto, Terio, Novello, & Tantillo, 2011), Brilliance *Salmonella* Agar™ (Oxoid, UK) (Martiny et al., 2016), and Eosin Methylene Blue (EMB) Agar (Oxoid, UK) (Robison, 1984), for *V. parahaemolyticus, Salmonella* spp., and *E. coli*, respectively. To evaluate the sensitivity of mPCR as a rapid assay, the targeted species from the BHI media were diluted to obtain the stock culture with a concentration of 10^1 and 10^3 CFU/ml. The stocks were grown in pre-enriched media and confirmed by plating onto the respective selective media.

2.3. Bacterial Detection by Conventional Assay

The conventional assay was performed to detect the bacteria based on culture-method employing commercial selective agar media, followed by a biochemical test (Church, 2016). Briefly, 100 µl of bacteria culture from each pre-enrichment media was grown on the selective media, as previously described in section 2.2. The culture plates were incubated overnight at 37 °C. The colonies from each selective agar media were identified following the manufacturer's procedure. The suspected colonies were confirmed by a biochemical assay using API™ 20E (Biomereux, USA) for *V. parahaemolyticus* identification, and using MicroID™GnA+B (Microgen, UK) for *Salmonella* spp. and *E. coli* identification.

2.4. Bacterial Detection by PCR Assay

2.4.1. DNA extraction

The DNA extraction protocol was performed following the procedure modified from Kawasaki et al. (2005). One hundred µl of the pre-enriched culture (bacterial culture preparation (section 2.2) or fish samples (section 2.5)) were transferred into sterile microtubes (Eppendorf, Germany), and centrifuged at 7,500 rpm for 5 min at 4 °C. The suspension was disposed and the pellet was suspended in 100 µl of Tris/HCl – 1 mM EDTA (TE) buffer with 10 µl of *k*-proteinase (Thermo Fisher Scientific, USA) and incubated at 37 °C for 1 h. Three hundred µl of DNAzol (Thermo Fisher Scientific, USA) was added to the microtube and mixed by vortexing for 30 s, and then centrifuged at 12,500 rpm for 10 min. The supernatant was transferred to a new microtube containing 400 µl of cold isopropanol and centrifuged at 12,500 rpm for 15 min at 4 °C. The supernatant was discarded, and the remaining DNA pellet was washed with 300 µl of 75% isopropanol and centrifuged at 12,500 rpm for 15 min. The pellet was dried at room temperature by putting the tubes at a reverse position for 15–30 min. For the final step, 100 µl of TE buffer was added to the pellet, vortexed for 30 s and centrifuged at 9,000 rpm for 1 min. The DNA was stored at -20 °C for further analysis.

DNA extraction using the DNeasy™ Microbial kit (Qiagen, Germany) was performed as a comparison of the extraction method. The extraction was performed following the manufacturer's protocol. The quality and quantity of DNA obtained from both extraction methods were analyzed using gel electrophoresis and spectrophotometry assay with NanoDrop™ (Thermo Fisher Scientific, USA).

2.4.2. PCR optimization

The total of the PCR reaction was 25 µl, consisting of 2 µl DNA template and 23 µl PCR mixture (including primers, 12.5 µl of 2X master mix from Fermentas™ (Thermo Fisher Scientific, USA), $MgCl_2$ and nuclease-free water). The primers pairs for simplex and multiplex PCR assays were presented in Table 1. Gradient PCR (BioRad, USA) was used to optimize the mPCR amplification condition while the thermocycler (Applied Biosciences, USA) was used for simplex PCR. For the simplex PCR, the amplification condition was performed following the procedures from Kim et al. (1999), Rahn et al. (1992), and Jefferson, Burgess, and Hirsh (1986) for detection of *V. parahaemolyticus, Salmonella* spp., and *E. coli*, respectively. For the mPCR assay, another pair of primers was used to amplify 475 bp of 16S rDNA gene sequences of bacteria following procedure from Chiang et al. (2006). The gradient PCR assay for mPCR optimization was programmed at 94 °C for 5 to 10 min for denaturation; 40 cycles of the following: denaturation at 94 °C for 30 s, annealing at different temperatures (55-60 °C) for various times (40, 50, 60 s), elongation at 72 °C for 1 min; and a final extension at 72 °C for 5 min. The optimum cycling condition for the mPCR was as follows: (1) denaturation at 94 °C for 7 min, (2) 40 cycles of denaturation at 94 °C for 50 s, annealing at 59 °C for 40 s, elongation at 72 °C for 1 min, and (3) final extension at 72 °C for 5 min. The optimum primer concentration of *toxR, invA, uidA* and 16S rDNA were 0.5, 0.4, 0.2 and 0.1 mM, respectively, and the $MgCl_2$ concentration was 2.5 mM. The PCR products from both assays were analyzed by gel electrophoresis using 1.2% of agarose gel in 1X Tris–Boric acid–EDTA (TBE) solution. The gel electrophoresis condition was 100 volts with <100 mA for 60-90 min. The gels were stained by SYBR safe® (Invitrogen, USA), and documented using the BioDoc Analyzer system (Biometra, Germany).

Table 1. Primer pairs targeting the 16S rDNA, *ToxR, InvA,* and *UidA* genes

Targeted Genes (Species)	Size (bp)	Label	Sequence	Tm (ºC)	References
16S rDNA (All bacteria)	475	Forward	CCT ACG GGA GGC AGC AGT	60	Chiang et al. (2006)
		Reverse	CGT TTA CGG CGT GGA CTA C	58	
ToxR (*V. parahaemolyticus*)	368	Forward	GTC TTC TGA CGC AAT CGT TG	57	Kim et al. (1999)
		Reverse	ATA CGA GTG GTT GCT GTC ATG	58	
InvA (*Salmonella* spp.)	275	Forward	AAT TAT CGC CAC GTT CGG GCA A	68	Rahn et al. (1992)
		Reverse	TCG CAC CGT CAA AGG AAC C	63	
UidA (*E. coli*)	147	Forward	TGG TAA TTA CCG ACG AAA ACG GC	66	Jefferson et al. (1986)
		Reverse	ACG CGT GGT TAC AGT CTT	64	

2.5. Simultaneous Detection of Pathogenic Bacteria in Fish Samples by Conventional and Rapid mPCR Assays

To assess the performance of mPCR as a confirmatory assay and a rapid test, two types of samples (artificially-contaminated and naturally-contaminated) were used. Fresh white leg shrimp (*Litopenaus vannamei*) was used as a matrix for artificially-contaminated samples. To maintain the matrix freshness, shrimp was sterilized by irradiation at 20 kGy at the irradiation facility of the National Nuclear Energy Agency (BATAN) Jakarta, Indonesia. The sterile condition of shrimp was confirmed by plate count assay. Sterile shrimp were then subjected to artificial contamination by dipping in the bacterial culture at a concentration of 10^3 CFU/ml for 15 min at room temperature (25±3 °C).

For naturally-contaminated samples, 50 fisheries products, including shrimp, shellfish, and fish were randomly purchased from local fish markets at the Jakarta Bay area, i.e., Cilincing, Muara Baru, and Kamal in 2010. Samples were transported to the Laboratory of Microbiology, RCMFPPB with temperature maintained at <10 °C.

Both artificially- and naturally-contaminated samples were analyzed using conventional and rapid mPCR assays. The conventional assay consists of three steps, i.e., pre-enrichment in broth media followed by selective culture in agar media and biochemical test as a confirmatory assay. In comparison, the rapid mPCR assay consists of two steps, i.e., pre-enrichment in broth media followed by mPCR as a confirmatory assay. For the detection of *Salmonella* spp., *E. coli,* and *V. parahaemolyticus* by conventional assay, the assay procedures were performed following the method previously described in section 2.3.

Pre-enrichment of both conventional and rapid mPCR followed the method by Zhang et al. (2015), with modification. Briefly, 50 g of sample was minced and mixed with 450 ml of pre-enrichment media using a tissue homogenizer, then incubated at 37±1 °C. One ml of the pre-enriched sample was then centrifuged at 12,000 rpm for 10 min, and the supernatant was discarded. The pellet was dissolved with 1X TE-buffer and extracted using the best DNA extraction method obtained from the previous step (section 2.4.1). The extracted DNA was analyzed by mPCR to simultaneously detect pathogenic bacteria (*V. parahaemolyticus*, *Salmonella* spp., and *E. coli*), and the PCR products were subjected to gel electrophoresis.

3. Results and Discussion

3.1. Comparison of DNA Extraction Methods for Pre-enriched Bacterial Culture

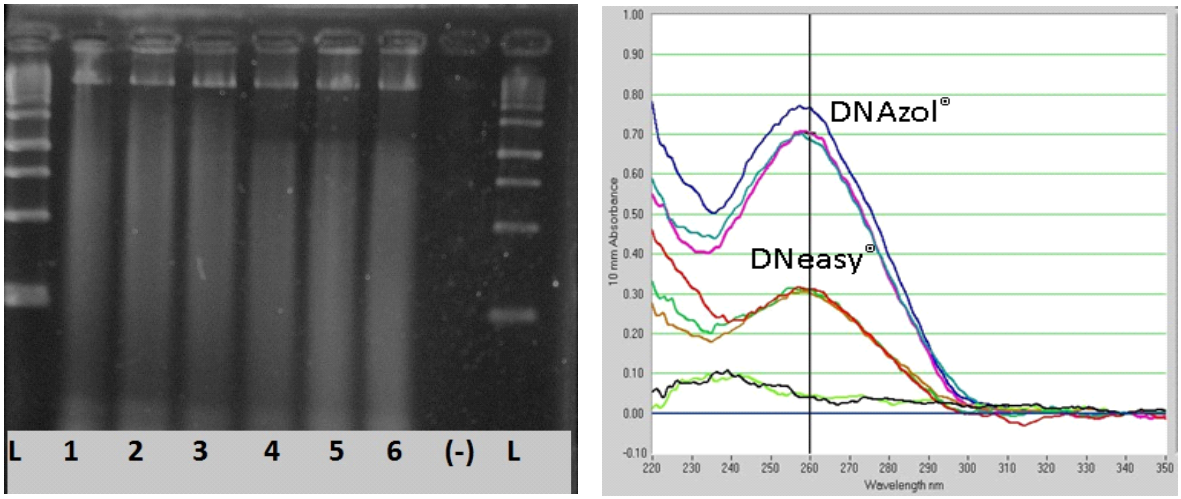
Extraction and purification of bacterial genomic DNA from culture media or directly from food matrices are generally performed using commercial kits, that are specifically designed for the rapid and cheap protocol. These extraction kits are mostly based on two different approaches to separate DNA from the suspensions, i.e., a solution-based protocol which

employs solutions (such as phenol, chloroform, isopropanol, and ethanol); and a column-based protocol (using silica matrices, glass particle, diatomaceous earth, magnetic bead or anion exchange resin) (Becker, Steglich, Fuchs, Werner, & Nübel, 2016; Tan & Yiap, 2009). Both protocols use lysis buffer containing Guanidinium Thiocyanate (GuSCN), Sodium Dodecyl Sulfate (SDS), Cetyltrimethylammonium Bromide (CTAB), Cesium Chloride (CsCl), or Proteinase Enzyme to extract the DNA from cells or tissues.

In this study, two different DNA extraction protocols to isolate bacterial genomic DNA from the pre-enrichment broth were compared, i.e., the solution-based method utilizing DNazol® (GuSCN-containing solution as a lysis buffer) (Thermo Fisher Scientific, USA) followed by organic phase separation, and the column-based method of DNeasy® Kit (Qiagen, Germany) applying enzymatic lysis buffer followed by silica column for the DNA separation. The DNA quality (Figure 1) showed that both extractions produced >10 ng/µl genomic DNA with absorbance ratio 260/280 nm ($A_{260/280}$) between 1.96 to 2.11 (data not shown), which indicated a good quality of purified DNA (Sambrook, Fritsch, & Maniatis, 1989). Although these protocols produced comparable results, the solution-based method was cheaper than the column-based method. The cost for DNA extraction, including the TE buffer, ethanol, and isopropanol for DNazol® and DNeasy® Kit, were estimated at USD 1-3 and USD 3-5 per sample, respectively. This cost excluded the electricity and labor cost, which are similar to both methods. Based on the manufacturer's protocols, both methods require less than 4 h of extraction, but the DNeasy® kit gives faster results than the DNazol® method. In our study, the solution-based method with DNazol® was selected as the preferred method for bacterial DNA extraction due to its cost-effectiveness. The solution-based method using GuSCN has been widely applied to extract bacterial DNA from cell culture, food, and environmental samples (Tan & Yiap, 2009). GuSCN is a chaotropic agent used to lyse cells as well as to inactivate indigenous nucleases during nucleic acids extraction (Boom et al., 1990; Chomczynski & Sacchi, 2006); hence this reagent is more popular than other reagents commonly used as the main ingredient of lysis buffer described previously. The GuSCN lysis buffer may contain phenol to separate nucleic acids from the suspension because of the non-polarity and the higher density of phenol than water (Sambrook & Russell, 2006). However, due to its toxicity to human health, the lysis buffer containing phenol must be used with precautions during nucleic acid extraction.

3.2. Pre-enrichment Step for Simultaneous Detection of Pathogenic Bacteria

Pre-enrichment is an essential step of pathogenic detection from food or environmental sample, to suppress the growth of competitive, non-targeted bacteria as well as to avoid false-negative results (Olsen, 2000; Villamizar-Rodríguez et al., 2015). For pathogenic bacteria where their presence at low levels already causes human health risk, a pre-enrichment step is compulsory in the most standardized microbial assay. To date, LB and Buffered Peptone Water (BPW)



(A) (B)

*Note: L: DNA ladder (1 kb); (-) in (A), light green and black curves in (B) are blank (1x TE buffer)

Figure 1. The extracted DNA by DNAzol® (Lane no. 1-3 (A); curves of dark blue, light blue, and pink colors (B) and DNeasy® (Lane no. 4-6 (A); curves of red, brown and dark green colors (B), analyzed using gel electrophoresis (A) and spectrophotometry (B) assay

are the most common liquid medium used as general pre-enrichment media for *E. coli*, *Salmonella* spp., and other *Enterobacteriaceae* (Olsen, 2000; Villamizar-Rodríguez et al., 2015). Meanwhile for *V. parahaemolyticus*, culture media containing 0.5 and 10% of NaCl is recommended, since this pathogen is more susceptible towards environmental change (temperature, pH and organic acids) when grown in low salinity medium (Beuchat, 1975; Huang & Wong, 2012; Whitaker et al., 2010), and becomes nonviable in agar medium (Wong & Wang, 2004).

In our study, we utilized APW (contains 2% of NaCl) instead of BPW (contains only 0.5% NaCl) as pre-enrichment broth for *V. parahaemolyticus*, while LB was used for *S. typhimurium* and *E. coli*. Results from pre-enrichment studies showed that all bacteria grew well in broth media and reached five times higher concentration compared to the initial level after 12 h incubation (Table 2), thus allowing the pathogen detection as low as 10^1 CFU/ml. These indicated that LB and APW were suitable as a pre-enrichment broth for *S. typhimurium* and *E. coli*; and for *V. parahaemolyticus*, respectively, for the subsequent assay (culture-based or molecular-based). Previous studies have also successfully applied LB as pre-enrichment broth for *Salmonella* spp. and *E. coli* assay (Daquigan, Grim, White, Hanes, & Jarvis, 2016; Hull-Jackson, Mota-Meira, & Adesiyun, 2019), and APW for subsequent PCR assay of *V. parahaemolyticus* (Taminiau et al., 2014; Zhang et al., 2015). By applying the pre-enrichment step, the aforementioned studies were able to detect as low as 10^2 CFU/g of *V. parahaemolyticus*, 10^1 CFU/25 g of *E. coli* and 10^0 CFU/25 g of *Salmonella* in contaminated samples.

3.3. Optimization of mPCR Assay

In our study, we utilized primers that have successfully amplified *UidA, InvA* and *ToxR* genes from *E. coli*, *Salmonella* spp., and *V. parahaemolyticus*, respectively (Jefferson et al., 1986; Kim et al., 1999; Rahn et al., 1992), instead of designing new primers for the mPCR. However, the previous studies performed amplification in a single and separate PCR reaction, and used different PCR reagents; therefore, a new thermal cycling condition for the mPCR needs to be optimized. In addition, amplification of the 16S rDNA gene as an internal control reaction was done to avoid false-negative results during PCR reaction due to ineffective nucleic acid extraction or the absence of genomic bacterial DNA in the samples.

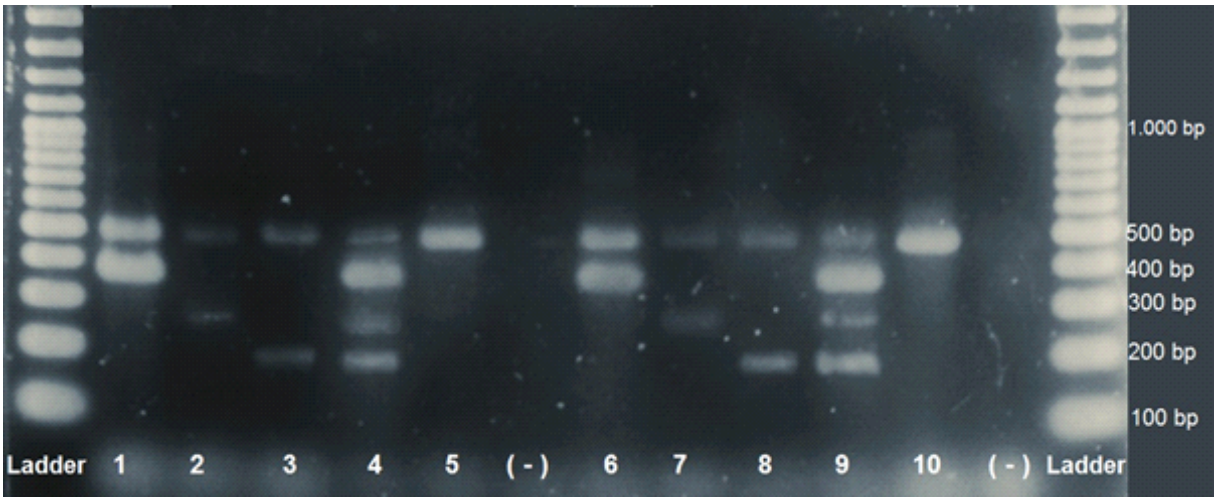
The mPCR assay with the pre-enrichment step amplified all targeted genes simultaneously (Figure 2) with good sensitivity (Limit of Detection = LOD) at 1 log CFU/ml from the diluted BHI stocks. The detection limit was lower than the findings from Germini et al. (2009) and Malcolm et al. (2015), which was > 2 logs CFU/ml. In Germini's study, the concentration of bacteria stocks was extracted from a non pre-enriched medium; hence it was likely to produce a higher LOD compared to the result of our study. As shown in Figure, 2, the genomic DNA extracted from the pre-enrichment medium was successfully amplified by this method. There were no nonspecific PCR products generated from this reaction, which indicated an excellent specificity of the assay. These observations were in accordance to the result of previous studies (Chiang et al., 2006; Germini et al., 2009; Malcolm et al., 2015) where the efficiency of PCR assay using these primers (i.e., *toxR, invA, uidA* or 16S rDNA) was not affected by the presence of multiple targeted genomic DNA in a single reaction.

3.4. The mPCR as a Confirmatory Assay for Culture-based Method and a Rapid Detection

The efficacy of PCR assay to detect the presence of foodborne pathogenic bacteria in food depends on the sample matrices, pre-enrichment methods, DNA extraction protocols, and PCR conditions (Law et al., 2015; Zhao et al., 2014). To ensure the reliability and specificity of the developed mPCR assay, the results from the mPCR assay were compared to those of the conventional assay using a commercial biochemical

Tabel 2. Total viable count of *S. typhimurium*, *E. coli,* and *V. parahaemolyticus* before and after pre-enrichment step

Bacteria	Viable Count (log CFU/ml)	
	Before	After
S. typhimurium ATCC 14028	0.91 ± 0.21	5.22 ± 0.24
E. coli ATCC 25922	1.09 ± 0.13	5.46 ± 0.39
V. parahaemolyticus ATCC 17802	0.87 ± 0.15	4.63 ± 0.28



Note: Lane 1 & 6 are *V. parahaemolyticus*; lane 2 & 7 are *S. typhimurium*; lane 3 & 8 are *E. coli;* lane 4 & 9 are a mixture of *V. parahaemolyticus, S. typhimurium*, and *E. coli*; lane 5 & 10 are *S. aureus* as bacterial DNA amplification control; and lane (-) were blank/negative control (1xTE buffer)

Figure 2. The mPCR products of pre-enriched bacterial culture at a concentration of 10^1 CFU/ml (lane 1-5) and 10^3 CFU/ml (lane 6-10)

kit (API® 20E and MicroID®). The targeted bacteria were inoculated to sterile shrimp (as sample matrix) to mimic the sample analysis procedure. Both methods were able to detect all of the targeted bacteria (Table 3), indicating that the mPCR was comparable to the conventional method to be used in the sample matrix. In addition, the mPCR assay could potentially reduce the time of analysis by at least 48 h compared to the conventional assay.

In terms of cost-effective and energy consumption issues, the mPCR offers a lower cost per sample compared to that of the conventional method due to the cheaper reagent and lower energy consumption for analysis. For example, in the present study, the PCR reagents (DNA extraction kit and consumable plasticware) cost approximately USD 7-10 per sample, while the total cost of the commercial biochemical test kit and selective agar was approximately USD 10-12 per sample. Meanwhile, the conventional method required additional energy and labor cost (for 48-72 h of incubation step at 35 °C).

Although PCR provides a rapid and reliable method, this assay may be insufficient to determine between viable and nonviable bacteria during detection or

Table 3. The number of artificially-contaminated shrimp showing positive results from confirmatory assay using commercial biochemical reagents and mPCR method

Spiked samples	Selective Agar Media	Number of samples tested	Biochemical Test			mPCR			
			API™ 20E	MicroID™GnA+B		16S rDNA	*ToxR*	*InvA*	*UidA*
			V. parahaemolyticus	*Salmonella* spp.	*E. coli*	Bacteria	*V. parahaemolyticus*	*Salmonella* spp.	*E. coli*
Shrimp+*V. parahaemolyticus* (ATCC 17802)	CHROMagar™ *Vibrio*	5	5	-	-	5	5	-	-
Shrimp+*S. typhimurium* (ATCC 14028)	Brilliance *Salmonella* Agar™	5	-	5	-	5	-	5	-
Shrimp+*E. coli* (ATCC 25922)	EMB Agar	5	-	-	5	5	-	-	5

quantification (Dwivedi & Jaykus, 2011). Thus, some additional steps are needed to confirm the existence of viable bacteria, such as by pre-enrichment or sample pre-treatment prior to DNA extraction. For that reason, PCR assay is likely more suitable for bacteria identification than enumeration, except for Most Probable Number PCR (MPN PCR) assay (Bonny et al., 2018).

3.5. Detection of Pathogenic Bacteria in Fish Samples by mPCR Method

The mPCR reliability as a rapid assay to detect the presence of foodborne pathogenic bacteria in naturally-contaminated seafood samples, was compared to the conventional assay. Detection of *E. coli*, *Salmonella* spp., and *V. parahaemolyticus* was done in fish, shrimp, and bivalve mollusks. As shown in Table 4, mPCR assay provided better performance than the conventional assay to detect the presence of targeted bacteria in the samples, except in bivalve mollusk samples where the conventional assay provides better detection of *E. coli*. It can be argued that the presence of a PCR inhibitor in bivalve mollusk tissue (as a filter feeder organism) could produce a false negative PCR reaction (Lees & Cen Wg, 2010). In general, the number of positive samples identified by mPCR assay was slightly higher than those detected by the conventional method. These findings were in agreement with the results from studies by Aabo et al. (1995), which concluded that the PCR method as a confirmatory assay produces a better sensitivity than the biochemical test to detect viable *Salmonella* spp. in naturally contaminated meat.

The biochemical test also has some limitations, especially its accuracy in detecting some bacterial strains (Donelli, Vuotto, & Mastromarino, 2013). For example, another study reported that this biochemical test using API 20E also failed to determine the difference between *V. parahaemolyticus* and *V. vulnificus* strains (Yi et al., 2014). Thus, molecular

Table 4. The detection of *E. coli*, *Salmonella* spp., and *V. parahaemolyticus* from fish samples analyzed by conventional and rapid mPCR method

Samples	Species	Number of samples	∑ Positive Samples					
			Conventional[1]			mPCR[2]		
			E. coli	*Salmonella* spp.	*V. parahaemolyticus*	*E. coli*	*Salmonella* spp.	*V. parahaemolyticus*
Fish	Indian Mackerel (*Rastrelliger kanagurta*)	5	2	0	1	3	1	1
	Golden Rabbitfish (*Siganus guttatus*)	5	3	1	0	3	1	1
	Scad Fish (*Decapterus* spp)	5	2	0	2	2	0	2
	Threadfin Bream (*Nemipterus hexodon*)	5	2	0	0	2	0	0
	Frigate Tuna (*Auxis thazard*)	5	3	1	0	4	1	1
Shrimp	White Leg Shrimp (*Penaeus vannamei*)	5	2	0	2	3	0	3
	Giant Tiger Prawn (*Penaeus monodon*)	5	3	0	2	3	0	3
Bivalve mollusks	Green Mussel (*Perna viridis*)	5	3	2	5	5	2	4
	Asian Hard Clam (*Meretrix lusoria*)	5	3	1	4	2	2	4
	Blood Cockle (*Anadara granosa*)	5	3	0	3	1	0	3
Total		50	26	5	19	28	7	22

Note: [1]Conventional method was conducted based on the culture-based method using selective agar media followed by the biochemical test as a confirmatory assay

[2]mPCR method was performed based on the culture-based method using pre-enrichment media (broth) followed by mPCR as a confirmatory assay

assay, including PCR, recently becomes more important as a rapid, sensitive, and easy tool of genotype-based speciation for identification and detection of microbes, including the non-culturable bacteria (Franco-Duarte et al., 2019).

Aquatic environments and its organisms, including fish, are known to be significant reservoirs for some foodborne pathogenic bacteria (i.e., *Salmonella* spp., *Vibrio* spp., and *E. coli*) (Amagliani, Brandi, & Schiavano, 2012; Elbashir et al., 2018). The presence of these bacteria in fisheries products, especially in Indonesia, has previously been reported by other studies. Kusmarwati, Hermana, Yennie, and Wibowo (2016) reported that the prevalence of *V. parahaemolyticus* in shrimp harvested from shrimp ponds in Java was 60.19% (62 out of 103 samples). Meanwhile, other studies investigating *Salmonella* spp. showed that the prevalence of this bacteria in fish products purchased from markets in Bogor, Jakarta, and Surabaya, as well as fish processing units in Ambon, ranged from 9.5 – 36% from the total sample collected (Kusumaningrum & Dewanti-Hariyadi, 2012; Narumi, Zuhriansyah, & Mustofa, 2009; Yennie, Aulia, & Handayani, 2017; Yennie et al., 2016). Furthermore, *E. coli* was found in fresh Indian Scad (*Decapterus russeli*) purchased from markets in Palu (Maruka, 2017) and salted-boiled fish from Pelabuhan Ratu (Mumpuni & Hasibuan, 2018). Following those findings, the results from our bacterial analysis in naturally-contaminated fish samples showed that *V. parahaemolyticus, Salmonella* spp., and *E. coli* were also present in the samples.

Based on its sensitivity and reliability, the mPCR assay developed in the present study, in combination with a pre-enrichment step, is potential to be proposed as a standardized assay to substitute the conventional method of pathogenic bacteria detection. The pre-enrichment step could avoid false-negative results due to the presence of low level or injured bacterial cells in the samples.

4. Conclusion

An mPCR assay with rapid and simultaneous detection of *V. parahaemolyticus*, *Salmonella* spp., *E. coli*, and 16S rDNA (as an internal control for the assay) in fisheries products has been developed. To the best of our knowledge, there are no previous studies that apply the method to detect the targeted genes in fisheries products simultaneously. By using a pre-enrichment step prior to DNA extraction, false-negative results due to the low level or injured cell of bacteria can be avoided, and the method was able to detect the targeted pathogens at a concentration as low as 1 log CFU/ml. The mPCR provides better performance to detect the targeted bacteria from both liquid medium and food matrix, compared to the conventional method. Although it requires advanced equipments, the analysis cost per sample is relatively cheaper than that of the conventional method. Thus, the developed-mPCR method has a potential to be used as a substitution or an alternative for the conventional method to detect pathogenic bacteria.

Acknowledgments

This research was funded by the Indonesian Ministry of Research and Technology (Grant number Kepmenristek No. 053/M/Kp/II/2010). The authors acknowledge our colleagues from the Laboratory of Microbiology and Laboratory of Biotechnology, Research Centre for Marine and Fisheries Product Processing and Biotechnology.

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