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Detection of *Salmonella* spp., *Escherichia coli*, and *Listeria monocytogenes* in Tuna by Multiplex PCR

Kristina Natalia[1,2], Mariana Wahjudi[1] and Tjie Kok[1*]
[1]Faculty of Biotechnology, University of Surabaya, Jalan Raya Kali Rungkut, Surabaya 60293, East Java, Indonesia
[2]Regional Fish Quarantine and Inspection Agency, Surabaya I 61254, East Java, Indonesia

*Correspondence :
tjie_kok@staff.ubaya.ac.id

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Abstract

Fishery products are types of commodity with various levels of development including the addition of food additives. They are very susceptible to pathogenic microbial contamination hence they can cause food poisoning and disease outbreaks in consumers. In this study, tuna fish spiked with *Salmonella* spp., *Escherichia coli*, and *Listeria monocytogenes* bacteria is examined for bacteria contamination. The examination of these pathogenic bacteria is carried out using multiplex PCR (mPCR), a modern technique developed to detect target DNAs of bacteria simultaneously. The results of mPCR are compared to those of biochemical tests for sensitivity and time-efficiency. Based on the results obtained, the mPCR method is $\pm$1250-fold more sensitive and $\pm$80-fold more time efficient than the biochemical test. Therefore, mPCR method could be recommended to be used as a tool for the detection of bacteria in fishery products.

INTRODUCTION

Fish is a source of protein that is generally the people's choice because it has a relatively cheap price. Fresh fish products are the main commodities of fishery products in Indonesia, one of which is tuna. Tuna is a kind of fish with a very high protein content (22.6-26.2 g/100 g) and fat (0.2-2.7 g/100 g). Tuna also contains minerals (calcium, phosphorus, iron, sodium), vitamin A (retinol), and vitamin Bs (thiamin, riboflavin, and niacin). In general, the edible portion of tuna (edible portion) ranges from 50-60% of the fish body (Alverson and Stansby, 1963).

Original fresh tuna products are usually sold in the local market and are usually purchased and then reprocessed into various processed fish, both raw and cooked. Post-catching fresh fish products have several drawbacks due to the lack of quality control processes starting from catching, distribution and marketing, hence the quality of fresh fish products produced is very diverse.

Fresh tuna has a very high risk of contamination because metabolic processes in dead fish are uncontrolled. The catabolism process results in the formation of substrates for bacteria, and hence fresh fish are growth substrates for bacteria so raw fish products damage faster than processed fish products (Palawe *et al.*, 2016). The presence of bacterial contamination in fresh tuna is one of the quality parameters for checking. Based on data on the FDA's RAS website, in 2020, for tuna fish products in

Indonesia, there were 13 cases of *Salmonella* spp. contamination. In June 2021, there were 7 cases of *Salmonella* spp. contamination in tuna fish products and 1 case of *L. monocytogenes* contamination in shrimp.

Test for pathogenic bacteria (*Salmonella* spp., *Escherichia coli,* and *L. monocytogenes*) on tuna fish products that are used by most state laboratories in Indonesia is using the biochemical SNI method. The test takes approximately 10-12 days to obtain results. In addition, the equipment and media needed for the test vary a lot, and they render higher operational costs.

Considering that there are shortcomings in the biochemical method, the development of a new diagnostic method providing solutions for lower cost and more time efficiency is essential. The singleplex PCR method for the test of several food-borne pathogenic bacteria has been used with sensitive results (Patil-Joshi *et al*., 2021). The quality of fishery products must fulfill export requirements before the delivery of products to destination countries such as Japan, the USA, the UK, Belgium, Canada, and some European countries. The ingestion of fish with high histamine levels is toxic, hence maximum permissible levels of < 200 or < 400 mg/kg of histamine are applied. The levels of pathogenic bacteria contamination are also regulated, for example, the level of *L. monocytogenes* contamination should not exceed 10^2 cfu/g (based on shelf life of fewer than 5 days) (Health Protection Agency Working Group, 2016).

In this study, a more time-efficient test is developed to detect the presence of the bacteria in fresh tuna fish products. Triwibowo *et al*. (2020) reported that mPCR offers a lower cost per sample than the biochemical method due to cheaper reagents and lower energy consumption for analysis. Hence mPCR is considered a promising new diagnostic method for the detection of food-borne pathogens. In this study, the sensitivity and time-efficiency of mPCR method are compared to those of biochemical test for the detection of *Salmonella* spp., *E. coli,* and *L. monocytogenes* in fresh tuna fish products.

METHODOLOGY

Place and Time

This research was carried out in the Laboratory of Regional Fish Quarantine and Inspection Agency (Laboratorium Balai Karantina Ikan, Pengendalian Mutu dan Keamanan Hasil Perikanan – KIPM) Surabaya I for three months, January to March 2022.

Research Materials

The materials used in this study are *Salmonella* spp. test media according to SNI ISO 6579:2015: Bismut sulfite agar, Buffered peptone water, MKTTN, Xylose lysine deoxycholate agar, Triple sugar iron agar, Tryptone broth, Rappaport-Vassiliadis medium, Reagen covacs, ONPG, nutrient agar; *E. coli* test media according to SNI 2332.1-2015: Lauryl Tryptose broth, EC broth, Levine's eosin methylene blue, Tryptone broth, MR-VP broth, Simmon citrate broth, Plate count agar, Lactose broth, Covacs reaction, Methyl red indicator; *L. monocytogenes* test media according to SNI ISO 11290-2:2015: Fraser Broth, Fraser listeria ammonium iron III supplement, Listeria agar acc Ottaviani and agosti, Listeria agar selective-supplement, PALCAM agar acc van et al base, PALCAM listeria selective supplement, Rhamnose monohydrate, Xylose, Yeast extract, Enzymatic digest animal tissue) and Polymerase Chain Reaction (PCR) materials: Silica kit extraction, Nuclease free water, TAE buffer, primer, DNA ladder 20, dNA staining, agarose.

Meanwhile, the equipment used in this study were stomacher, incubator, water bath, autoclave, oven, laminar air flow, microwave, microcentrifuge, vortex, thermal cycler, electrophoresis, spin down, micro tube, and micro pipette.

Research Design

This study used a descriptive research design. The results of biochemical and PCR tests on tuna spiked with *Salmonella* spp., *E. coli*, and *L. monocytogenes* are presented in the Table and Figures based on data from Laboratory tests.

Work Procedure

This research was performed in several stages starting with checking pure cultures of *Salmonella* spp., *E. coli*, and *L. monocytogenes* bacteria. Detection of the pathogenic bacteria was carried out using biochemical and mPCR tests. For PCR, a singleplex PCR was initially performed to find the optimum temperature for each bacterium. After obtaining the optimum temperature of the three bacteria, mPCR test was performed using the optimum temperature obtained during the singleplex PCR. Tuna free from pathogenic bacteria (unspiked tuna) was used as a negative control for tuna contaminated with each of the three pathogenic bacteria (spiked tuna).

Bacterial Culture Preparation

Salmonella spp. ATCC 14028, *E. coli* ATCC 25922, and *L. monocytogenes* ATCC 7644 were respectively on NaCl TSA growing media. After each bacterium was grown on selective media, XLD agar, LEMB agar, and ALOA agar, biochemical test. Meanwhile, to enrich *Salmonella* spp., *E. coli*, and *L. monocytogenes*, the corresponding isolate was cultured on non-selective Brain Heart Infusion Broth (BHIB) for 18-24 hours at 37 °C (*Salmonella* spp.), on Brain Heart Infusion Broth (BHIB) for 24 hours at 5 °C (*E. coli*), and on Listeria Enrichment Broth (LEB) for 42-48 hours at 27-30 °C (*L. monocytogenes*) in order to get stock (Sjahriani *et al*., 2021). Then each culture was divided into two parts: part 1 for the colony test (encoded A for *Salmonella* spp., B for *E. coli* , and C for *L. monocytogenes*) (Table 1) and part 2 for the tuna sample test (Zhang *et al*., 2015).

Tuna Sample Preparation

Tuna fish samples were crushed and divided into 4 parts. Part 1 was the unspiked tuna sample, encoded D. Part 2 was the tuna sample spiked with *Salmonella* spp. encoded E. Part 3 was tuna sample spiked with *E. coli* encoded F. Part 4 was a tuna sample spiked with *L. monocytogenes*, encoded G. In addition, 50 gr of each B, C, and D were taken and mixed to be sample encoded H (Table 1). Each sample weighing 25 g and 20 mg was taken for biochemical and PCR tests respectively. The sample was initially homogenized using a vortex and allowed to stand for 15 min-1 hour before the test.

Table 1. Sample coding.

Sample type	Code
Pure colony of *Salmonella* spp.	A
Pure colony of *Escherichia coli*	B
Pure colony of *Listeria monocytogenes*	C
Unspiked tuna sample	D
Tuna sample spiked with *Salmonella* spp.	E
Tuna sample spiked with *Escherichia coli*	F
Tuna sample spiked with *Listeria monocytogenes*	G
Mixture of samples E+F+G	H

Biochemical Test

Pure bacterial colonies and the tuna samples, both unspiked and spiked with target bacteria, were tested biochemically. Test for *Salmonella* spp. followed the SNI ISO 6579:2015, *E. coli* SNI 2332.1-2015, and *L. monocytogenes* follows SNI ISO 11290-2:2015 procedure.

Bacterial DNA Isolation

Each isolate of the bacterial colony was performed using the silica extraction

method, following the standard procedure at the KIPM Biomolecular Lab. The isolate of unspiked and spiked tuna samples were also extracted using the same procedure. In addition, the extracted DNA from each spiked tuna sample was taken, and mixed together to obtain a mixture containing DNAs of *Salmonella* spp., *E. coli,* and *L. monocytogenes* The primers used for PCR were as follows in Table 2.

Table 2. Primer for PCR.

Gen Target (species)	Dimension (bp)	Label	Sequence (5' → 3')	Tm* (°C)	Reference
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	
hlyA (*Listeria monocytogenes)*	730	Forward	CAT TAG TGG AAA GAT GGA ATG	N/A	(Bilung *et al*., 2018)
		Reverse	GTA TCC TCC AGA GTG ATC GA	N/A	
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	

*) Tm: primer melting temperature.

Optimization of PCR Condition

Optimization of PCR conditions was carried out by selecting a primer master mix according to the corresponding DNA templates for optimizing the primer annealing temperature. The compositions of the mixtures and primers used in PCR were as follows (Table 3).

Table 3. Composition of mixture in PCR processes.

Single PCR	(μl)	Multiplex PCR	(μl)
Master mix	12.5 μl	Master mix	12.5 μl
Forward primer	1 μl	Forward primer inv A	1 μl
Reverse primer	1 μl	Reverse primer inv A	1 μl
Nuclease free	8.5 μl	Forward primer uid A	1 μl
Water		Reverse primer uid A	1 μl
Template	2 μl	Forward primer hly A	1 μl
Total	25 μl	Reverse primer hly A	1 μl
		Nuclease free water	4.5 μl
		DNA mixture	2 μl
		Total	25 μl

Note: Final concentration for each primer is 10 μM.

Orientation processes of the singleplex PCR process were carried out using annealing temperatures ranging at 55.0-63.0 °C to find the optimum annealing temperature for each target DNA of bacteria. Next, mPCR was performed at the annealing optimum temperature, i.e., 58.1 °C. Using optimum annealing temperature can increase PCR efficiency for unknown DNA (Wei *et al*., 2007). The complete PCR stage and temperatures were as follows (Table 4):

Table 4. PCR stages and temperatures.

Stage	Temperature (°C)	Time (minutes)	
Pre-denaturation	95	3	
Denaturation	95	0.5	35 cycles
Annealing	58	0.5	
Elongation	72	0,5	
Final extension	72	5	

The PCR results were visualized by gel electrophoresis. A total of 2 g of agarose was dissolved in TAE buffer which has been two-fold diluted. The solution was heated using a microwave at 100°C for 4 minutes to ensure complete dissolution. Five µl of fluoro-safe DNA was then added and the mixture was allowed to stand for a while before being poured into molds and left to freeze. The frozen gel was placed in the placed in electrophoresis chamber and 1x TAE running buffer was poured into the chamber until the gel was submerged. The sample containing DNA and the marker were then put into the well of the gel. The electrophoresis process was run at 120 Volts for 35 min until completion. The gel was then put on a UV transilluminator to be visualized.

RESULTS AND DISCUSSION

Biochemical Test Results

In the biochemical test, there are three steps that must be carried out: pre-enrichment in broth media, followed by selective culture on agar media, and biochemical test. Pre-enrichment is an important step for the detection of pathogens from food or environmental product samples to suppress the growth of competitive, non-target bacteria, and also to avoid false negative results (Hoorfar and Baggesen, 1998). Based on the biochemical test results of *Salmonella* spp., *E. coli,* and *L. monocytogenes* it took approximately 5 days, 10 days, and 5 days, respectively. The biochemical test of unspiked tuna (sample D) for *Salmonella* spp., *E. coli,* and *L. monocytogenes* showed negative results (Figure 1).

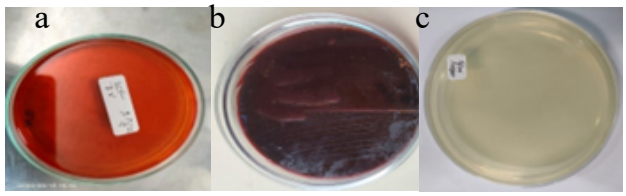

Figure 1. Biochemical test results of sample D on (a) XLD media, (b) LEMB Media, (c) ALOA media.

For the *Salmonella* spp. there was no colony growing on the XLD media (Figure 1a). For *E. coli*, the LEMB media was clear (Figure 1b), indicating there is no H2S produced. And for *L. monocytogenes,* there was no colony growing on ALOA media (Figure 1c).

The biochemical test for pure colonies (samples A, B, and C) gave positive results (Figure 2) and so did that for spiked tuna samples (samples E, F, G, and H) (Figures 3 and 4).

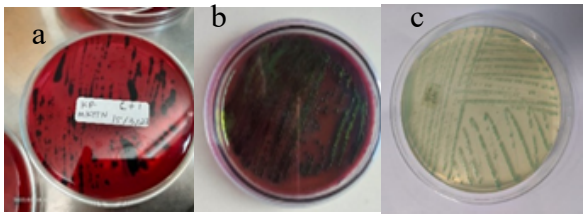

Figure 2. Biochemical test results of (a) sample A on XLD media, (b) sample B on LEMB media; (c) sample C on ALOA media.

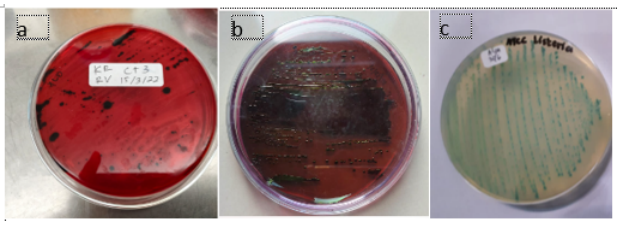

Figure 3. Biochemical test results of (a) sample E on XLD media, (b)sample F on LEMB media, (c) sample G on ALOA media.

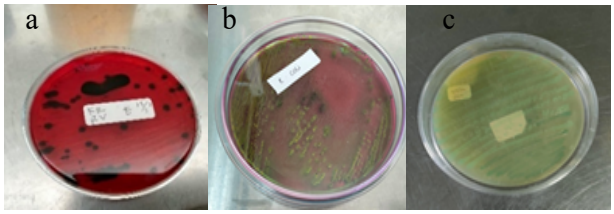

Figure 4. Biochemical test results of sample H on (a) XLD media, (b) LEMB media, (c) on ALOA media.

For pure colony of S*almonella* spp. (Figures 3a and 4a), there were black colonies growing on XLD media indicating that S*almonella* spp. is producing H_2S. Further biochemical test results confirmed the presence of the bacteria (Table 5).

Table 5. *Salmonella* spp. biochemical test results.

Media Test	Appearance	Results		
		C+1	C+3	E
TSI agar	Yellow	no	no	No
Urea Agar	Pink	yes	yes	Yes
L-Lysine decarboxylation medium	Purple	yes	yes	Yes
Galactosidase Reaction	Yellow	no	no	No
Voges-Proskauer reaction	Red/Pink	no	no	No
Indo Production	Red ring	no	no	No
	Positive for	***Salmonella typhi***	***Salmonella typhi***	***Salmonella typhi***

For a pure colony of *E. coli* and tuna samples spiked with *E. coli* initially, LTB test gave positive results, i.e., cloudy and gassy appearance. Then, some portions from the LTB tube were taken using an ose needle and inoculated into EC broth tubes containing Durham tubes, this also gave positive results. Hence, they were further grown on LEMB media. There were black and metallic green colonizing appearing (Figures 2b, 3a, and 4b). Further biochemical test results for samples B, F, and H confirmed the presence of the bacteria (Table 6).

Table 6. *Escherichia coli* biochemical test results.

Criteria	Sightings	Results		
		B+1	B+3	E
LTB	Gas	yes	yes	yes
Indo	Red ring	No	no	no
Methyl red	Red	yes	yes	yes
Voges Proskauer	red/pink	No	no	no
Citric	Blue	yes	yes	yes
	Positive for	***Escherichia coli***	***Escherichia coli***	***Escherichia coli***

For the pure colony of *L. monocytogenes test* (Figs. 2c) and tuna samples spiked with *L. monocytogenes* (Figure 3c and 4c), there were greenish blue colonies with or without a white halo growing on ALOA media. Further biochemical test results confirmed the presence of the bacteria (Table 7).

Table 7. *Listeria monocytogenes* test results.

Criteria	Appearance	Results		
		D+1	D+3	E
Microscopic test	Gram positive, thin, short rods or cocci-shaped bacilli	Gram positive, thin, short rods or cocci-shaped bacilli	Gram positive, thin, short rods or cocci-shaped bacilli	Gram positive, thin, short rods or cocci-shaped bacilli
Hemolysis	lines are narrowed, clean, and the hemolysis area is bright	Yes	yes	yes
L- Rhamnose	Yellow	Yes	yes	yes
D-Xylose	Yellow	No	no	no
	Positive for	***Listeria monocytogenes***	***Listeria monocytogenes***	***Listeria monocytogenes***

PCR Test

Prior to the mPCR, a singleplex PCR was carried out by initially performing the process at a temperature range of 55 to 63.0 °C to determine the optimum annealing temperature for each target DNA of bacteria.

The electrophoresis visualization of singleplex PCR result for *Salmonella* spp. DNA was shown in Figure 5.

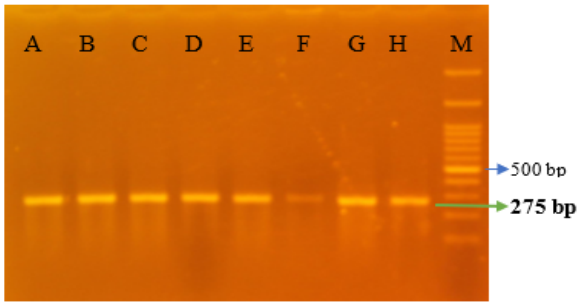

Figure 5. Visualization of singleplex PCR result for *Salmonella* spp. DNA at various temperatures: A. 63.0 °C; B. 62.4 °C; C. 61.4 °C; D. 59.9 °C; E. 58.1 °C; F. 56.5 °C; G. 55.6 °C; H. 55 °C; M is the marker.

The singleplex PCR for *Salmonella* spp. DNA at various temperatures gave a band at 275 bp on an electrophoresis gel.

The electrophoresis visualization of singleplex PCR results for *E. coli* DNA was shown in Figure 6.

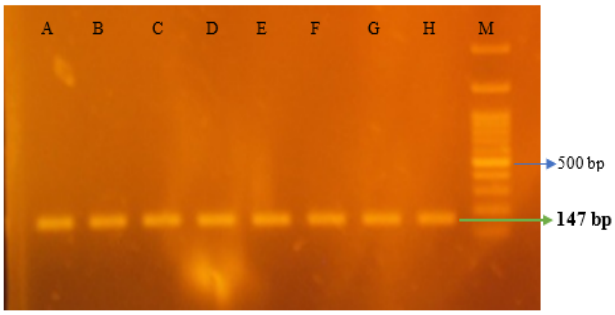

Figure 6. Visualization of singleplex PCR result for *E. coli* DNA at various temperatures: A. 63.0 °C; B. 62.4 °C; C. 61.4 °C; D. 59.9 °C; E. 58.1 °C; F. 56.5 °C; G. 55.6 °C; H. 55 °C; M is the maker.

The Singleplex PCR for *E. coli* DNA at various temperatures gave a band at 147 bp on the electrophoresis gel. The Electrophoresis visualization of singleplex PCR result for *L. monocytogenes* DNA was shown in Figure 7.

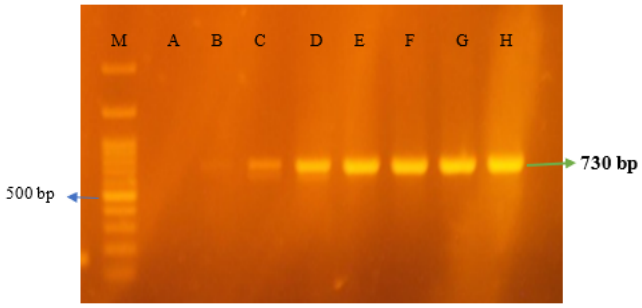

Figure 7. Electrophoresis visualization of singleplex PCR result DNA of *L. monocytogenes* at various temperatures: M is the marker, A. 63.0 °C; B. 62.4 °C; C. 61.4 °C; D. 59.9 °C; E. 58.1 °C; F. 56.5 °C; G. 55.6 °C; H. 55 °C.

The singleplex PCR for *L. monocytogenes* DNA at various temperatures gave a band at 730 bp on an electrophoresis gel.

Based on the results of the singleplex PCR and confirmed by mPCR, the optimum annealing temperature for the three target DNAs of bacteria was 58.1 °C. Hence, mPCR was carried out at the temperature to examine the three target DNAs of positive controls (mixture of samples A, B, and C) and spiked tuna samples (mixture of samples E, F, and G) (Figure 8).

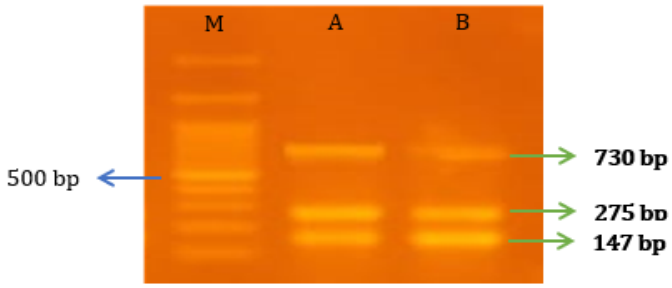

Figure 8. Visualization of mPCR results for the three target DNAs of positive controls and spiked tuna samples at 58.1 °C. M is the marker, A is target DNAs of positive controls, B is the target DNAs of spiked tuna samples.

The sensitivity of PCR to detect the presence of pathogenic bacteria in a food product, in this case, tuna product, depending on the sample matrix, pre-enrichment method, DNA extraction, and PCR condition (Law *et al*., 2014). The specificity of mPCR result on electrophoresis gel showed the bands at respective bp of all the three target DNAs of bacteria (Figure 8), indicating the specificity of this method.

Based on this study, mPCR is more sensitive than a biochemical test: mPCR requires much less sample than a biochemical test. In the biochemical test, for every 225 mL of media $\pm$ 20 mg is needed. Hence, mPCR is $\pm$(25x1000)/20 = 1250-fold more sensitive than biochemical test. In addition, mPCR provides faster analysis than the biochemical test. In the biochemical test, it takes at least 5 days for *Salmonella* spp. analysis, 10 days for *E. coli* analysis, and 5 days for *L. monocytogenes* analysis. Meanwhile, in mPCR, it takes $\pm$ 3 hours to finish the analysis. Hence for the examination of *Salmonella* spp., *E. coli*, and *L. monocytogenes*, mPCR is $\pm$(10x24)/3= 80-fold more time-efficient than the biochemical test.

Even though mPCR provides an advantage over the biochemical test, i.e., more sensitive and more efficient, this method has some weaknesses. First, if the extraction process is not good, then the isolate obtained is not pure and when the PCR is run, there is a possibility of obtaining false negative results. DNA is a material that is most likely covered by fat. Hence, DNA extraction is somewhat tricky and care is needed to obtain pure target DNAs from bacteria. Secondly, mPCR is unable to distinguish whether the DNAs are from living or not living bacteria (Dwivedi and Jaykus, 2011). Hence, an additional step is needed to confirm the presence of living bacterial DNAs rather than for quantification, excluding the Most Probable Number PCR (MPN PCR) test (Bonny *et al*., 2018). Moreover, PCR cannot be applied to samples with a low PH.

Further researches need to be conducted to provide a more comprehensive study on the use of mPCR for simultaneous detection of pathogenic bacteria in fish products, for standardization of this method.

CONCLUSION

The biochemical test and mPCR can be applied to detect the presence of pathogenic bacteria in tuna samples. In this case, mPCR provides several advantages: it can detect *Salmonella* spp., *E. coli,* and *L. monocytogenes* simultaneously, and it is more sensitive and more time-efficient than a biochemical test. Hence mPCR could be recommended as a standard test for replacement of biochemical tests.

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