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Detection of *Listeria monocytogenes* in indigenous Indonesian snack using real-time polymerase chain reaction

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ABSTRACT

Aims: *Listeria monocytogenes* is an important foodborne pathogen that can cause serious human listeriosis. The aim of this study was to optimize real-time PCR method for *L. monocytogenes* detection in indigenous Indonesian snack (*pempek*).

Methodology and results: DNA isolation of *L. monocytogenes* was conducted by comparing three extraction methods: phenol:chloroform, heating, and commercial kit (QIAamp DNA Blood Mini Kit). DNA isolate was amplified by PCR using two variables, which were LIM 2 and LIMRE primers for *iap* gene detection, and DG69 and DG74 primers for *hlyA* gene detection. Phenol:chloroform method showed the best extraction result, while DG69 and DG74 primers showed more specific result on PCR compared with LIM 2 and LIMRE primers. The running conditions gave specific amplification curve until 27th cycle. DNA detection in *L. monocytogenes* culture generated standard curve equation of Ct = 37.9 – 3.11 C with limit of detection (LOD) at 3.2 × 10^3 CFU/mL, while DNA detection in *pempek* generated standard curve equation of Ct = 41.03 – 3.69 C with limit of detection (LOD) at 6.3 × 10^3 CFU/g.

Conclusion, significance and impact study: Real-time PCR with DG69 and DG74 primers could be considered as a reliable method for specific and sensitive detection of *L. monocytogenes*.

Keywords: Detection, Indigenous snack, *Listeria monocytogenes,* Real-time PCR

INTRODUCTION

Fish-based snacks have the risk of microbial contamination due to its nutritional content required by microbes and less hygienic handling of the seller. *Listeria monocytogenes* is often found in frozen and refrigerated ready-to-eat food (Moreno *et al.*, 2012). *Listeria monocytogenes* can cause illness in humans through listeriosis. Listeriosis impact will be more dangerous in young children, pregnant women, and people who have lost immunity due to a disease (Todd and Notermans, 2011). This bacteria can cause illness in healthy people such as gastroenteritis and fever that will heal by itself (Schuppler and Loessner, 2010).

Conventional culture method for *L. monocytogenes* detection has some drawbacks of time consuming and under estimated data due to existency of viable but not culturable colony. An alternative to conventional detection methods for *L. monocytogenes* is molecular techniques such as real-time PCR (Polymerase Chain Reaction).

This study aimed to determine the best extraction method for *L. monocytogenes* in *pempek* (a fish-based indigenous Indonesian snack), determine the specificity and sensitivity of the primer for *L. monocytogenes* detection test, and evaluate the limit of detection of real-time PCR ESCO Swift 48 for *L. monocytogenes* culture and spiked food samples.

MATERIALS AND METHODS

The standard microbe used in this study was *L. monocytogenes* ATCC 7644. Confirmation test was conducted using Agar Listeria Ottaviani Agosti (ALOA) (Merck, Germany) as a selective medium and GLISA Singlepath® L'mono immuno-assay kits (Merck, Germany). Microbes used as controls for determining primer specificity were *L. monocytogenes* ATCC 13932, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Lactobacillus plantarum* ATCC 8014, and *Cronobacter sakazakii* ATCC 29544. Spiked food sample

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analyzed was indigenous snack from South Sumatera-Indonesia named *pempek.*

Selection of DNA extraction method

This step was started with inoculation of *L. monocytogenes* in Tryptic Soy Broth (Oxoid, Hamsphire UK) medium for 18 h at 37 °C. The overnight culture was extracted with three methods.

Phenol:chloroform method

This method was modified from Sambrook and Russel (2001). The first modification was conducted by replacing lysis buffer with TE buffer in 1 mL of bacterial culture. A mixture of lysis buffer and 0.2 mg/mL of proteinase K was replaced with a mixture of 500 mL of 1× TE buffer and 100 mL of lysozyme. Furthermore, phenol:chloroform solvents (1:1 v/v) were used instead of phenol:chloroform:isoamyl alcohol solvents (25:24:1 v/v/v) and 10 M ammonium acetate at pH 7.4 was used instead of 0.3 M sodium acetate at pH 4.8. In addition, absolute ethanol was replaced with isopropanol. Vacuum-dried pellets were replaced with air-dried pellets.

Heating method

This method was modified from Rahayu *et al.* (2009) and Hein *et al.* (2001). Modification was conducted by lowering the heating temperature from 100 to 95 °C.

Commercial kit method (QIAamp® DNA Blood Mini Kit)

Modifications were conducted to several steps in the manufacturer's method, in which 1 mL of CTAB was used during initial sample preparation, followed by sample vortexing until homogeneous. The suspension was centrifuged for 1 min at 13000 rpm, then the supernatant was discarded leaving pellets in the microcentrifuge tube. The next steps were in accordance with the manufacturer's instructions.

DNA extracted from these three methods assessed by determining the quality and purity of DNA using electrophoresis with 15 g/L agarose gel at 100 V. Extraction method was selected based on the highest concentration number of genomic DNA.

Primer selection for *L. monocytogenes* detection

This study compared two primers: LIM 2/LIMRE (Choi and Hong, 2003) and DG69/DG74 (Dadkhah *et al.*, 2012). DNA used was DNA of *L. monocytogenes* as the target DNA, compared with DNA of *S. aureus* and *C. sakazakii* as other bacterial DNA. The best primer was selected using standard PCR based on its specificity. Selected primer would be used for the further steps.

Determination of primer specificity with Real-time PCR

Parameters measured for determining primer specificity were the patterns of amplification curve and melting curve. Primer specificity was indicated by the absence of amplification from other bacterial DNA. Primer specificity on melting curve was indicated by different peak value of the melting curve (Tm) between the target DNA, negative control and control of other bacterial DNA. DNA used was DNA of *L. monocytogenes* as the target DNA and DNA of *E. coli*, *S. aureus, L. plantarum,* and *C. sakazakii* as DNA from other bacteria.

Determination of detection method sensitivity of *L. monocytogenes* DNA

DNA detection test was conducted on pure cultures of *L. monocytogenes* and spiked food samples. The sensitivity of real-time PCR to detect *L. monocytogenes* was determined by making a standard curve to relate the cycle threshold value (Ct) and the log concentration of pure cultures. This step was initiated by conducting a serial dilution from highest to lowest dilution that still could be detected, with the concentration of bacteria in culture between 10^0-10^9 CFU/mL. The number of microbes was calculated using the standard plating method (Maturin and Peeler, 2001). DNA extracted from each sample was then amplified by real-time PCR.

The intersection of amplification curves generated with basement-threshold could be analyzed to produce cycle threshold (Ct) value. Determination of detection method sensitivity of *L. monocytogenes* DNA in spiked food sample was conducted through similar steps as *L. monocytogenes* pure cultures. Samples used for generating standard curve were determined based on these criteria:

1. Sample still showed a detectable amount of colonies during plating
2. Sample that has Ct value below the highest cycle value and still showed a primer specificity (according to primer specificity test)

RESULTS AND DISCUSSION

Relevant DNA extraction method of *L. monocytogenes*

Electrophoresis results of *L. monocytogenes* DNA (Figure 1) showed that phenol:chloroform extraction method modified from Sambrook and Russel (2001) produced the clearest bands when compared with the two other methods. This is possible because the DNA concentration resulting from phenol:chloroform method, which was 1857 ng/µL, was higher than those resulting from heating and commercial kit method, which were 1027 and 915 ng/µL, respectively. DNA concentration from the heating method was smaller than the phenol:chloroform method because of DNA damage due to high temperature (95 °C) effect.

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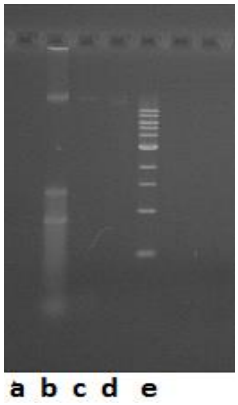
According to Sambrook and Russel (2001), heating might cause irreversible DNA denaturation. Modification using CTAB in commercial kit method even lowered concentration of obtained DNA.



Descriptions:
a. Control
b. DNA extracted from phenol:chloroform method
c. DNA extracted from heating method
d. DNA extracted from commercial kit method
e. 1 kb DNA ladder

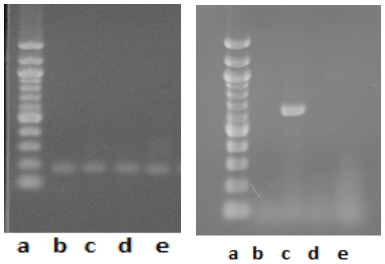
Figure 1: Electrophoresis results of three extraction methods.

DNA is considered as pure if the ratio between the two absorbance values of 260 and 280 nm is between 1.8 to 2.0 (Nolan *et al.*, 2007). Purity measurement of DNA extracted with phenol:chloroform method resulted 1.24 for A260/A280 ratio, which showed that the DNA produced still contained other impurities. The impurities were suspected due to phenol used. Brescia (2012) stated that 10 g/L phenol could cause a decrease in purity by 0.6.

Relevant primer for *L. monocytogenes* analysis

LIM 2/LIMRE primers were used to amplify the *iap* gene (invasion associated secreted endopeptidase). This gene plays an important role in cell viability of *L. monocytogenes*. This gene produces p60 protein that has a bacteriolitic activity (Kohler *et al.*, 1991). DG69/DG74 primers amplified *hlyA* genes that play a role in the production of 58 kDa listeriolysin O associated with virulence and widely used to detect *L. monocytogenes* (Choi and Hong, 2003).

Standard PCR result with LIM 2/LIMRE primers produced 175 bp amplicons in all types of microbes tested, while standard PCR result with DG69/DG74 primers showed 635 bp band only in *L. monocytogenes* DNA (Figure 2). Comparison of both primers showed that DG69/DG74 primers were more suitable for the detection of *L. monocytogenes*.



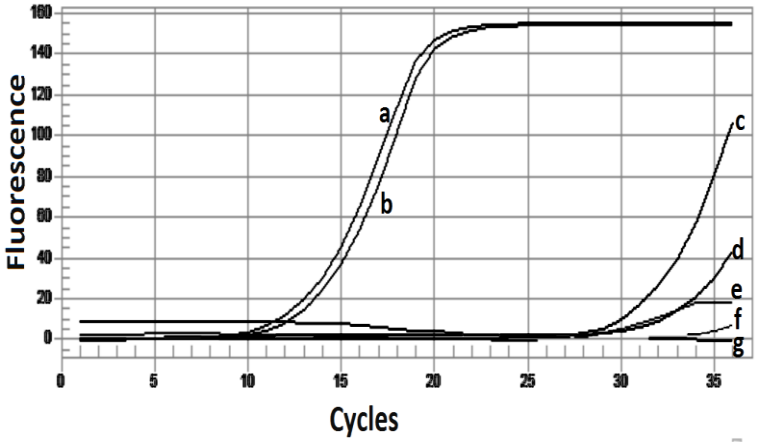
(1) (2)

Descriptions:
a. 100bp DNA Ladder
b. Control without DNA
c. *L. monocytogenes* DNA
d. *S. aureus* DNA
e. *C. sakazakii* DNA

Figure 2: Electrophoresis results of PCR products with (1) LIM 2/LIMRE primers and (2) DG69/DG74 primers.

Primer specificity

Specificity test of DG69/DG74 primers with real-time PCR can be seen in Figure 3. The running conditions were able to detect *L. monocytogenes* DNA with good amplification curve until 27th cycle. The running cycle was no longer specific after 27th cycle because it could amplify *L. plantarum*, *S. aureus*, *C. sakazakii*, *E. coli*, and negative control.



Descriptions:
a. *L. monocytogenes* ATCC 7644
b. *L. monocytogenes* ATCC 13932
c. *L. plantarum*
d. *S. aureus*
e. *C. sakazakii*
f. Control without DNA
g. *E. coli*

Figure 3: Real-time PCR amplification curve of specificity test with DG69/DG74 primer.

Melting temperature value (Tm) is a temperature at which 50% of double-stranded DNA has been denatured into a single-stranded DNA (Esco, 2009). The results of the melting temperature (Tm) with DG69/DG74 primers showed that *L. monocytogenes* (Tm = 79 °C) could be distinguished from *C. sakazakii* (Tm = 75.5 °C) and *E. coli*

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(Tm = 73.5 °C); whereas *L. monocytogenes, L. plantarum* and *S. aureus* could not be distinguished because they have the same Tm, which is 79 °C. An increase at amplification curve from non *L. monocytogenes* DNA after 27th cycle indicates formation of non-specific products or primer dimers.

Sensitivity of *L. monocytogenes* DNA detection with Real-time PCR

Detection sensitivity is the value of the highest dilution that still can be amplified by real-time PCR. This value can be considered as the limit of detection. Cycle threshold values (Ct) are shown in Table 1 for pure culture and spiked *pempek* sample.

Standard curve of relationship between Ct values and concentration of *L. monocytogenes* on pure culture can be generated based on Table 1 and produced a standard curve equation below:

Ct = 37.9 – 3.11 C

Ct: Ct value
C: Concentration Log value of bacteria (CFU/mL)

According to the specificity test this method was specific until 27th cycle, thus the limit of detection in pure culture samples was 3.50 log or 3.2 × 10^3 CFU/mL. Result of the detection limit was slightly larger than the result obtained by Dadkhah *et al.* (2012) that showed a detection limit in pure culture samples at 1.2 × 10^3 CFU/mL.

Table 1: Analysis result of *L. monocytogenes* pure culture and spiked sample with real-time PCR.

Bacteria concentration of pure culture (CFU/mL)	Ct	Bacteria concentration of spiked sample (CFU/g)	Ct
4.3 × 10^9	8.06	4.1 × 10^8	9.32
5.7 × 10^8	11.59	4.1 × 10^7	12.60
5.6 × 10^7	12.67	4.4 × 10^6	16.04
5.8 × 10^6	16.17	4.4 × 10^5	20.94
4.9 × 10^5	21.07	4.8 × 10^4	24.28
5.3 × 10^4	23.23	4.4 × 10^3	26.93
4.2 × 10^3	27.13	< 1.0 × 10^3	27.27
5.8 × 10^2	33.86	ND	ND

Sensitivity of DNA detection method in pure culture was different from *pempek* samples. This is caused by the presence of food components that may reduce the ability of the extraction method. Standard curves generated in spiked food samples produced a standard curve equation below:

Ct = 41.03 – 3.69 C

Ct: Ct value
C: Concentration Log value of bacteria (CFU/g)

According to the specificity test this method was specific until 27th cycle, thus the limit of detection in in spiked food samples was 3.80 log or 6.3 × 10^3 CFU/g. Result of the detection limit was slightly larger than the result acquired by Dadkhah *et al.* (2012) that showed a detection limit in milk samples at 2.1 × 10^3 CFU/mL. Food samples that did not contain *L. monocytogenes* were determined when the value of concentration log of the samples were < 0, thus the obtained Ct value = 41.03.

Constant value of the standard curve equation from pure culture (37.9) was much smaller than the spiked food samples (41.03). While the coefficients value of the standard curve equation from pure culture (3.11) was slightly lower than the spiked food samples (3.69). This indicates that the food matrix from snacks affecting the amplification results in real-time PCR. According to Dadkhah *et al.* (2012) and Camma *et al.* (2012) other food components can be the cause of differences in the amplification results, either due to the influence of extraction process or the influence of amplification process by real-time PCR.

CONCLUSION

Recommended extraction method for *L. monocytogenes* detection in *pempek* (Indonesian indigenous snack) was phenol:chloroform method. Recommended primer for real-time PCR analysis of *L. monocytogenes* was DG69/DG74. Running conditions produced specific amplification curve up to 27th cycle.

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