International Journal of Engineering & Technology, 9 (2) (2020) 448-453

International Journal of Engineering & Technology

Website: www.sciencepubco.com/index.php/IJET

Research paper

Evaluation of multiplex SYBR green real-time PCR assay for detection of pathogenic *Escherichia coli*

Ema Komalasari [1], Winiati P. Rahayu [2, 3] *, Siti Nurjanah [2, 3]

[1] *Department of Food Technology, Faculty of Science and Technology, University Al Azhar Indonesia, Jakarta 12110, Indonesia*
[2] *Department of Food Science and Technology, Faculty of Agricultural Technology, IPB University (Bogor Agricultural University), Bogor. West Java 16680, Indonesia*
[3] *Southeast Asian Food and Agricultural Science and Technology (SEAFAST) Center, IPB University (Bogor Agricultural University), Bogor, West Java 16680, Indonesia*
**Corresponding author E-mail: wini_a@hotmail.com*

Abstract

Pathogenic *Escherichia coli* (*E. coli*) has been implicated in a wide range of disease causing infections. It is essential to generate a method for detecting and differentiating each pathotype of *E. coli* which is more quickly and efficiently by using less reagent. This study aimed to evaluate a SYBR Green multiplex real-time PCR method for detecting four types of pathogenic *E. coli*. Two of multiplex real-time PCR system, 6-plex and 3-plex, were set to detect six different virulence factors from ETEC, EPEC, EHEC, and EIEC and evaluate the melting curves and specificity compared to simplex method. The results showed that 3-plex rt-PCR method gave more reliable melting curves than 6-plex. The 3-plex rt-PCR also provided similar melting value (Tm) to simplex system. The results of this specificity assay supported the selection of 3-plex rt-PCR conditions for detection of pathogenic *E. coli*.

Keywords: *Melting Curve; Multiplex Rt-PCR; Pathogenic E. coli; Specificity; SYBR Green.*

1. Introduction

Pathogenic *Escherichia coli* (*E. coli*) has been implicated in a wide range of diseases causing infections, including urinary tract infections, diarrheal disease, and other clinical symptoms. Six types of this bacteria are known as diarrheagenic *E. coli* (DEC). Four of them are associated with food, i.e. enterotoxigenic *Escherichia coli* (ETEC), enteropathogenic *Escherichia coli* (EPEC), enterohemorrhagic *Escherichia coli* (EHEC), and enteroinvasive *Escherichia coli* (EIEC). In Indonesia, there have been several studies regarding the prevalence of *E. coli* in food. However, the prevalence of each type of bacteria is still unknown. Some food samples like fish meatball, melon, pineapple, watermelon, lettuce, cucumber, and beef are reportedly contaminated by *E. coli* [1,2]. *E. coli* isolates are also identified in 6.34% of iced beverage samples and 0.7% of them are confirmed as ETEC [3].

Identification of *E. coli* from DEC is crucial because information about this pathotype variation is a major barrier to control public health risks associated with foodborne pathogens. For example, EHEC has lower infective dose ($1 \times 10^2 – 2 \times 10^2$ cells) than EIEC ($2 \times 10^2 – 5 \times 10^3$ cells) or ETEC/EPEC (1×10^6 - 1×10^9 cells) [4], so the risk exposed by the existence of EHEC is higher if compared to ETEC or EPEC. It is essential to generate a method that detects and differentiates each pathotype of this bacteria more quickly and efficiently by less reagent use.

Multiplex real-time PCR (m-rtPCR) could separates and quantifies *E. coli* strains based on virulence genes. By this method, two or more sequence targets are amplified by using several pairs of primers in the same reaction. The multiplex method provides more advantages compared to simplex identification. It is more efficient and faster in time detection. Additionally, this assay is proved to be more sensitive for identification of *E. coli* isolates from a patient with diarrhea [5].

SYBR green is a fluorescent dye commonly used for labelling during analysis. SYBR is the simplest and cheapest if compared to other dyes for real-time PCR. SYBR green rt-PCR provides fluorescence while binding with the targeted double-stranded DNA formed during amplification. In a multiplex real-time method, several PCR products of targeted genes must have significantly different melting points (Tm) to distinguish each product of targeted genes. It is important that multiplex rt-PCR conditions using SYBR Green as a label do not generate nonspecific products that give an unexpected peak as background in the melting curve result. Moreover, primers also play a critical role and are influenced by internal characteristics; such as stability, melting temperature, secondary structure, concentration, or interference with each other [6]. Therefore, multiplex rt-PCR method often requires dedicated instrumentation and it is necessary to have default cycling parameters and protocols for optimum performance. This study aims to evaluate a fluorescence-based (SYBR Green I) multiplex rt-PCR for detecting four types of diarrheagenic *E. coli*, i.e. ETEC, EPEC, EHEC, and EIEC based on their virulence factors.

Copyright © Ema Komalasari *et al.* This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

2. Materials and methods

2.1. Bacterial strain and growth condition

Six pathogenic *E. coli* were used as positive control. All bacteria were obtained from National Culture Collection for Pathogens (NCCP) South Korea. A freeze dried culture stock of the isolates was revived in Tryptic Soy Broth (TSB) (Oxoid Ltd., UK) and incubated at 35˚C for 20 h. After incubation, one loop of the culture was streaked onto Tryptic Soy Agar (TSA) plate (Oxoid Ltd., UK). The single colony from TSA was transferred into 10 mL Brain Heart Infusion (BHI) broth (Oxoid Ltd., UK) and incubated at 35˚C for 20 h. For stock cultures, the bacteria were saved in TSA and stored at refrigerated temperature.

2.2. DNA extraction and quantification

Genomic DNA (gDNA) was extracted from each isolates using chelex100 microwave method based on Reyes-Escogido *et al.* [7] with some modification. A modification was done by changing the time of heating in the microwave (becoming three minutes at 100˚C). Each bacterial culture from the enrichment (TSB at 35˚C for 20 hours) was put into a 15 mL centrifuge tube then centrifuged for 25 minutes at the speed of 1 500 × g. The precipitated bacterial cells (around 1.5 mL) were then transferred to a 2.0 mL micro-centrifuge tube to be centrifuged again for five minutes at a speed of 8 000 × g at 4 ˚C. Bacterial pellets were washed with 1 000 µL TE buffer (10 mM TRIS-base pH 7.5; 1 mM EDTA pH 8.0) and homogenized by vortex machine. The mixture wasc centrifuged again for five minutes at a speed of 8 000 × g at 4˚C. The cells were re-suspended in 100 µL lytic buffer TES (10 mM TRIS-base pH 7.5; 1 mM EDTA pH 8.0; 0.5% SDS) then put in the microwave for three minutes. After that, 150 µg proteinase K and 20 µg RNase A were added immediately. The suspension mixture was then put back into the microwave for three minutes. After the lysis process, the suspension was incubated for two minutes at a room temperature then added by 150 µL of TE buffer contained 25 mg of chelex100. After this step, the mixture was microwaved again with the same condition then centrifuged at 12 000 × g for five minutes at 4˚C. The supernatant containing DNA was then precipitated with 10% 3M sodium acetate and 2.5 × volume of 95% ethanol. The supernatant mixture was then incubated at -20˚C for 20 hours. The DNA was then washed twice with 1 mL of 70% ethanol, dried at room temperature, then dissolved in 100 µL ddH_2O and quantified with NanoDrop 2000 Spectrophotometer (Thermo Scientific).

2.3. Optimization of primer concentration

Each primer pair was tested individually by simplex rt-PCR at four different concentrations, i.e. 0.125, 0.250, 0.375, and 0.500 µM. This step was conducted to verify that primers amplified a single target with a specific (unique) Tm value (actual Tm value). Real-time PCR was performed by using Applied Biosystem 7500 thermal cycler with final reaction volume of 20 uL containing 1X Dynamo SYBR Green PCR master mix (Thermofisher) and 100 ng DNA template. The amplification cycles consisted of initial denaturation (pre-denaturation) at 95˚C for three minutes, followed by 40 cycles consisting of denaturation at 95˚C for 60 seconds, annealing/extension step at 55˚C for 60 seconds. After that, the reactions were continued with melting analysis of the PCR products (melt curve) by increasing the temperature from 60 to 95˚C at 0.1°/s.
Optimum primer concentration was the primer concentration that showed the lowest Ct value without producing or generating a minimum primer-dimer and unspecific peak in the melting curve [8]. The optimum primer concentration was subsequently used as a parameter to the multiplex rt-PCR analysis. Isolate code and primer sequence are shown in Table 1.

Table 1: Isolates, Primer Sequences and Amplicon Size Used for This Study

E. coli Type	Isolate code	Gene Target*	Primer Sequence (5' – 3')	Size (bp)	GC content (%)
ETEC	NCCP 13717	lt	f- AGCGGCGCAACATTTCAG r- TTGGTCTCGGTCAGATATGTGATTC	113	45
ETEC	NCCP 13718	st	f- TTAATAGCACCCGGTACAAGCAGG r- CCTGACTCTTCAAAAGAGAAAATTAC	147	-
EPEC	NCCP 14038	eae	f- TGATAAGCTGCAGTCGAATCC r- CTGAACCAGATCGTAACGGC	229	45
EHEC	NCCP 13720	stx1	f- CAGTTAATGTGGTGGCGAAGG r- CACCAGACAATGTAACCGCTG	348	40
EHEC	NCCP 15958	stx2	f- ATCCTATTCCCGGGAGTTTACG r- GCGTCATCGTATACACAGGAGC	587	45
EIEC	NCCP 13719	inv	f- TTTCCCTCTTGCCTGCATATGCGC r- CTCACCATACCATCCAGAAAGAAG	466	35

*The specific virulence factors for pathogenic *E. coli* were enterolabile toxin (lt) and enterostable toxin (st) for ETEC, intimin (eae) for EPEC, shiga-like toxin 1 (stx1) and shiga-like toxin 2 (stx2) for EHEC, and invasion plasmid (inv) for EIEC [9].

2.4. Multiplex SYBR green rt-PCR condition trials

Trials of the multiplex assay were conducted by two different settings, i.e. 6-plex and 3-plex. The 6-plex reaction contained six primer pairs (with optimum primer concentration of the previous result) to detect six target genes and 3-plex reaction comprised two groups (A and B) containing three primer pairs to detect every three target genes on the pathogenic *E. coli* (Fig 1).
The group in the 3-plex system was chosen based on the difference in Tm values above 2˚C in the optimization of primer assay [5]. Reaction volume and cycling condition were performed the same as in the optimization of primers concentration with a simplex system. A good multiplex rt-PCR condition was chosen based on the consideration that the melting curve allowed separation of amplification product (amplicon) with specific Tm value, similar to Tm on the simplex assay. The results of rt-PCR were confirmed by electrophoresis on agarose 2%.

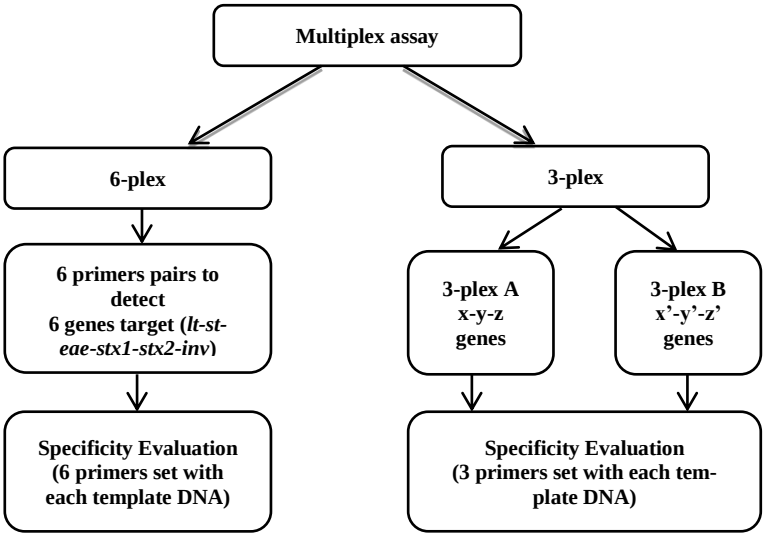


Fig. 1: Flow Chart for Multiplex Trial and Evaluation.

2.5. Specificity evaluation of multiplex assay

Specificity of the primer was evaluated during multiplex rt-PCR running. The assay was performed by testing the amplification of 6-plex primer set and 3-plex primer set against the simplex rt-PCR in each *E. coli* bacteria used in this study. Reaction and cycles were performed as previously described.

3. Results and discussion

3.1. Concentration and purities of extracted DNA

The concentration of *E. coli* DNA using chelex100-microwave method varied from 854.9 to 4301.6 ng/uL with purities of 1.80 – 1.94. This value was good as the extracted DNA was categorized as a pure DNA if the ratio between the absorbance of 260 and 280 was in the range of 1.8 – 2.0. The quality of the extracted DNA from pure culture or sample; such as food was important because this parameter was essential to achieve a reliable quantification by real-time PCR [10]. The presence of chelex during the heating step prevents DNA degradation with chelating metal ions that acted as catalysts in the breaking/destruction of DNA at high temperatures. Chelex 100 was known to be able to remove metals from culture media and reagents, purify dinucleotides, and remove metal ions in the blood [11].

3.2. Optimum primer concentration and melting temperature (Tm)

Optimum primer concentration was selected based on the lowest Ct value without producing a secondary peak in melting curves (non-specific products). Ct value was defined as the number of cycles required for the fluorescent signal to cross the threshold (exceeding background level). It was influenced by the concentration of the target (DNA template), mastermix, passive reference dyes, annealing temperature, primer concentration, and others.

The result of the primer concentration assay showed that the Ct value produced in the simplex rt-PCR ranged between 15.27 – 20.68 with Tm value 75.0 – 82.7˚C (Table 2). A melting curve with double peaks in some of concentration (Fig. 2) showed two products in one reaction that one of the products was nonspecific [12]. The curves were formed due to multiple phases in the melting process. This phase might be caused by additional sequence factors such as amplicon misalignment in A/T rich regions and designs that had a secondary structure in the amplicon region [13]. Based on Table 1, stx1, inv, and might st genes had a lower G/C content (abundant A/T content) than lt, eae, and stx2 genes. This approach was appropriate with the result in the simplex system (Fig 2 b,d,f).

Furthermore, excessive primer concentration was an important factor frequently triggered by false products in a PCR. The target genes of st, eae, stx1, stx2, and inv with primer concentration ≥ 0.25 uM indicated a tendency to form a secondary peak formation in melting curves. Too much primer reduced specificity and allowed primers to anneal in regions of the template that were not the target region and also to generate primer dimer [14,15]. This concentration should not choose for the primer in a multiplex assay. Therefore, the optimum primer concentrations for target gene lt were 0.25 uM and 0.125 uM for the primer pairs of st, eae, stx1, stx2, and inv.

Table 2: Ct and Tm Value for Optimization of Primer Concentration

Gene target	Concentration (µM)	Ct ± SD	Tm ± SD	Secondary peak* (unspecific product)
lt	0.125	15.65 ± 0.15	78.5 ± 0.35	None
	0.250	15.27 ± 0.74		None
	0.375	15.54 ± 0.70		None
	0.500	15.43 ± 1.24		None
st	0.125	20.68 ± 0.44	75.0 ± 0.07	None
	0.250	19.89 ± 0.84		Detected
	0.375	19.23 ± 0.59		Detected
	0.500	18.77 ± 0.35		Detected
eae	0.125	16.65 ± 0.25	81.6 ± 0.19	None
	0.250	16.38 ± 0.02		Detected
	0.375	16.27 ± 0.02		Detected
	0.500	16.24 ± 0.12		Detected
stx1	0.125	16.51 ± 0.06	79.8 ± 1.87	None
	0.250	16.80 ± 0.42		Detected

	0.375	16.94 ± 1.05		Detected
	0.500	16.97 ± 0.64		Detected
stx2	0.125	18.68 ± 1.08	82.7 ± 0.10	None
	0.250	17.76 ± 0.68		Detected
	0.375	17.62 ± 1.32		Detected
	0.500	17.37 ± 1.09		Detected
inv	0.125	17.93 ± 0.55	77.1 ± 0.14	None
	0.250	17.36 ± 1.05		Detected
	0.375	16.85 ± 0.19		Detected
	0.500	17.20 ± 0.04		Detected

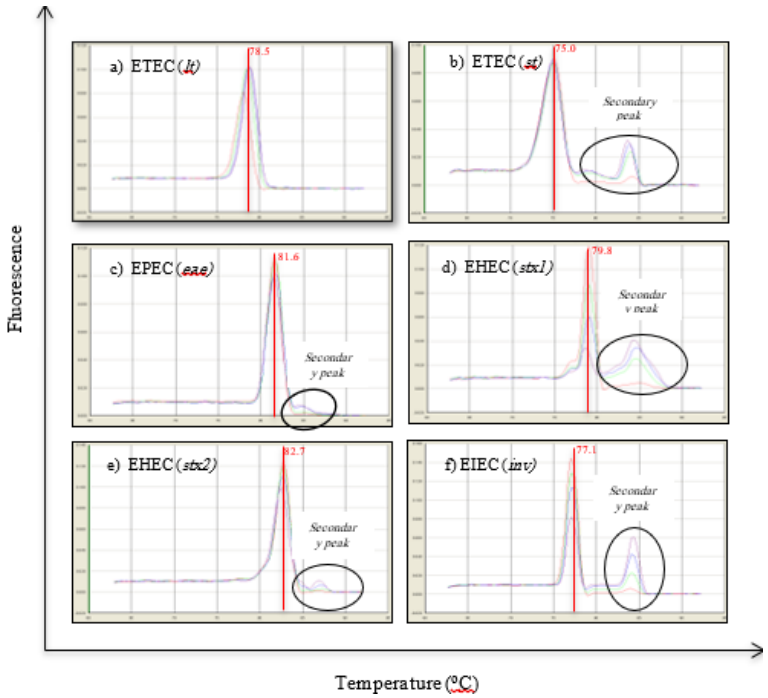


Fig. 2: Melting Curves of Each Gene Target. Red Line = 0.125 Um Concentration of Primer; Green Line = 0.25 Um Concentration of Primer; Blue Line = 0.375 Um Concentration of Primer; Purple Line = 0.5 Um Concentration of Primer.

3.3. Multiplex rt-PCR conditions trials for detecting pathogenic *E. coli*

Trials of multiplex rt-PCR method were carried out in two groups, namely 6-plex and 3-plex rt-PCR. The 3-plex rt-PCR was grouped based on different Tm values above 2°C in previous assay (Table 2). The first group (Group A) was st-lt-eae and Tm value ranged 75 – 78.5 – 81.6°C and the second group (Group B) was inv-stx1-stx2 with Tm value ranging 77.1 – 79.8 – 82.7°C respectively. Results showed that the melting curve from 6-plex rt-PCR only produced three peaks with Ct value 15.57 (± 0.14) and Tm 81.3 °C (± 0.00). Tm value was formed from the highest peak in the curve; while the other two peaks had Tm value between 75 – 80 °C and > 85°C. Electrophoresis results with 2% agarose also showed three bands that referred to three products that were DNA bands of lt (113 bp), eae (229 bp), and stx1 (348 bp); while the other three bands (the band for st, stx2, and inv) did not appear (Fig. 3a).

Meanwhile, trials of the 3-plex rt-PCR assay on three target genes with three primer pairs showed a melting curve with three peaks, although some peaks were not perfectly formed (Fig 3b, c). Previous study said that peak in multiplex system might be separated if Tm value had differences of about 2°C, However, a study conducted by Chassagne *et al.* [16] distinguished three target genes with SYBR green real-time PCR with the difference of Tm about 4°C. Zhang *et al.* [17] also succeeded in differentiating three target genes with Tm difference of about 3°C. It showed that parameter of the Tm value for each product (gene) became highly important in identification and detection of pathogenic bacteria using SYBR green multiplex rt-PCR, and each primer suggested had difference of about 4°C.

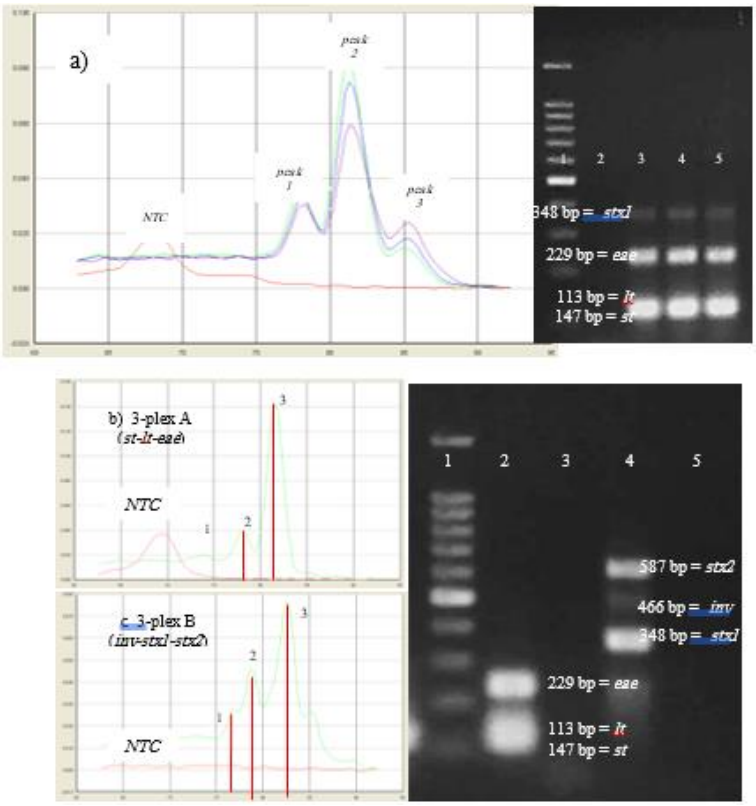


Fig. 3: (A) Melting Curve Profile and Electrophoresis Result from 6-Plex rt-PCR (1 = Ladder 100 Bp; 2 = No Template Control (NTC); 3, 4, 5 = 6-Plex Assay 3 Replication); (B) Melting Curve Profile from 3-Plex rt-PCR Assay Set A; (C) Melting Curve Profile From 3-Plex rt-PCR Assay Set B; (D) Electrophoresis Result from 3-Plex Assay (1 = Ladder 100 Bp; 2 = 3-Plex rt-PCR Assay Set A; 3 = No Template Control 3-Plex Assay Set A; 4 = 3-Plex rt-PCR Assay Set B; 5 = No Template Control 3-Plex Assay Set B).

According to Fig. 3, the area of the peaks was reduced when multiple targets were present in an isolate for multiplex reaction, especially for the smaller PCR product. One of the reasons was due to the degree of flexibility that was diminished or even lost with each additional primer set into reaction. Increased number of primers also increased the possibility of primer dimer formation and unspecific amplification. Thus, the 3-plex test showed better than the 6-plex test. Other than that, the application of rt-PCR method using SYBR green as a fluorescent label on four or more target genes (amplicon) had not been reported. In the previous studies, multiplex system was only aimed at two or three target genes. So far, multiplex PCR on more than three target genes had only been done in a standard or conventional multiplex by Kim *et al.* [18], Kagambega *et al.* [19], Mohammed [20], Tobias and Vutukuru [21], Sjoling *et al.* [22], and others.

3.4. The specificity of multiplex assay

Specificity assay was performed to ensure that the primers used in this study were specific with each target genes and could distinguish each product (amplicon). Specificity analysis was observed based on the Tm value. In multiplex systems, the resulted Tm value should be the same as that in the simplex system. Table 3 shows the comparison ratio of Tm values from multiplex assay (6-plex and 3-plex reaction) against with simplex assay. In a 6-plex assay, the specific product was formed with a lower fluorescent value than that in an unspecific product, so that the melting temperature showed on the instruments was unspecific product melting temperature value. This implied a false detection process.

In contrast, although a double peak was always generated on the melting curve in a 3-plex reaction, the specific product showed a higher fluorescent value than the unspecific product, so that the Tm value was equal to the desired Tm value. The use of SYBR green as the fluorescent label affected specificity because the dye and unspecific product fragments would bind to the primer and would be detected along with the desired product fragments. The use of a large number of primers increased the possibility of primer dimer and unspecific products exponentially [23]. The results of this specificity assay supported the selection of 3-plex rt-PCR conditions for detection of pathogenic *E. coli*. Further research is needed to obtain a more perfect melting curve in multiplex method that could separate each product (amplicon) more clearly without difference Tm value.

Table 3: Tm Value from Specificity Assay

E. coli type	Isolate code	Gene target	Tm (°C) simplex rt-PCR	3-plex rt-PCR	6-plex rt-PCR
ETEC	NCCP 13717	lt	78.5	78.4	82.4
ETEC	NCCP 13718	st	75.0	79.9	85.1
EPEC	NCCP 14038	eae	81.6	83.8	87.6
EHEC	NCCP 13720	stx1	79.8	79.3	84.9
EHEC	NCCP 15958	stx2	82.7	82.9	86.8
EIEC	NCCP 13719	inv	77.1	77.2	85.1

4. Conclusion

The use of 6-plex using SYBR green as a dye has not been able to separate each product perfectly due to increased the possibility of primer dimer formation and unspecific amplification as an effect of increased number of primer. Multiplex methods for the detection of

pathogenic *E. coli* designed in two sets of 3-plex rt-PCR based on the difference of Tm value 2°C could separate the product more reliable with primer concentration of LT gene was 0.25 uM and for the others gene was 0.125 uM.

Acknowledgment

The authors would like to thanks to Ministry of Research, Technology, and Higher Education, Indonesia for funding this research in the scheme of Competitive Grant year 2016-2017.

Conflict of interest

The authors declare no conflict of interest.

References

[1] N.A. Yunita, Identification and hazard characterization of pathogenic bacteria in food consumed by school children in Bogor, Master Thesis, IPB University Indonesia (2015)
[2] R. Anggreini, Contamination analysis of *Escherichia coli* (*E. coli*) O157:H7 in beef Makassar city, Undergraduate thesis, Hasanuddin University Indonesia (2015)
[3] N. Nababan, W.P. Rahayu, D.E. Waturangi, S. Suratmono, R. Puspitasari, N. Indrotristianto, E. Nikastri, S. Yulianingsih, N. Pusparini, Critical points and the presence of pathogenic bacteria in iced beverage processing line, Journal of Infection in Developing Countries 11 (2007) 2017 493-500. https://doi.org/10.3855/jidc.8934.
[4] FDA. Food and Drug Administration. Bad Bug Book, Foodborne Pathogenic Microorganisms and Natural Toxins, 2nd ed. Silver Spring: FDA (2012).
[5] T. B. Souza, M. L. Diego, M. S. K. Sonia, C. P. Liliana, P. S. Neusa, C. A. S. Isabel, Real time multiplex assay and melting curve analysis for identifying diarrheagenic *Escherichia coli*, Journal of Clinical Microbiology 51 (2013) 1031-1033. https://doi.org/10.1128/JCM.02478-12.
[6] A. Apte, S. Daniel, PCR Primer: A laboratory Manual. 2nd ed. Cold Spring Harbor. USA (2003).
[7] L. Reyes-Escogido, M. Balam-Chi, I. Rodriguez-Buenfil, J. Valdes, L. Kameyama, F. Martinez-Perez, Purification of bacterial genomic DNA in less than 20 min using *chelex*-100 *microwave*: examples from strains of lactic acid bacteria isolated from soil samples, Antonie van Leewenhoek 98 (2010) 456-474. https://doi.org/10.1007/s10482-010-9462-0.
[8] E. Pestana, S. Belak, A. Diallo, J.R. Crowther, G.J. Viljoen, Early, rapid and sensitive veterinary molecular daignostics real-time PCR application, Springer Netherlands, Netherland, 2010: pp. 252-253. https://doi.org/10.1007/978-90-481-3132-7.
[9] M.A. Croxen, B.B. Finlay, Molecular mechanism of *Escherichia coli* pathogenicity. http://www.natute.com/reviw/micro. Accessed January. 2015.
[10] M.R. Branquinho, R.T.B. Ferreira, P. Cardarelli-Leite, use of real-time PCR to evaluate two DNA extraction methods from food, Ciencia e Tecnologia de Alimentos Campinas 32 (2012) 112-118. https://doi.org/10.1590/S0101-20612012005000012.
[11] Bio-Rad Laboratories. *Chelex* 100 and *chelex* 20 chelating ion exchange resin instruction manual. http://www.biorad.com/webmaster/pdfs/9184_chelelx.PDF. Accessed Maret 2017.
[12] L.D. Moja, Artifacts in QPCR investigating unspecific product, master's degree Project, University of Skovde, Sweden (2014).
[13] IDT. Integrated DNA Technologies. Performing intercalating dye PCR/qPCR assays: Review examples of PCR melt curve data with our scientists to determine what it can/cannot tell us about resulting PCR amplicons. https://sg.idtdna.com. Accessed April 2, 2019.
[14] M.K. Gunnel, C.D. Lovelace, B.A. Satterfield, E.A. Moore, L.O. Kim, R.A.A. Robison, Multiplex real-time PCR assay for the detection and differentiation of *Francisella tularensis* subspecies, Journal of Medical Microbiology 61 (2012) 1525-1531. https://doi.org/10.1099/jmm.0.046631-0.
[15] Z. Chai, W. Ma, F. Fu, Y. Lang, W. Wang, G. Tong, Q. Liu, X. Cai, X. Li, A SYBR green-based real-time rt-PCR assay for simple and rapid detection and differentiation of highly pathogenic and classical type 2 porcine reproductive and respiratory syndrome virus circulating in China, Archives Virology 158 (2013) 407-415. https://doi.org/10.1007/s00705-012-1504-7.
[16] L. Chassagne, P. Nathalie, R. Frederic, L. Valerie, B. Richard, D. Julien, Detection of *stx1, stx2*, and *eae* genes of enterohemorrhagic *Escherichia coli* using SYBR green in a real-time polymerase chain reaction, Diagnostic Microbiology, and Infectious Disease 64 (2009) 98-101. https://doi.org/10.1016/j.diagmicrobio.2009.01.031.
[17] W. Zhang, M. Bielaszewska, A. Bauwens, A. Fruth, A. Mellmann, H. Karch, Real-time multiplex PCR for detecting shiga toxin 2-producing *Escherichia coli* O104:H4 in human stolls, Journal of Clinical Microbiology 50 (2012) 1752-1754. https://doi.org/10.1128/JCM.06817-11.
[18] K.H. Kim, J.I. Cho, C.Y. Cheung, J.M. Lim, S. Cho, D.H. Cho, C. S. Kang, D.H. Kim, Development of multipleks PCR assays to identify *Escherichia coli* pathogenic genes in food, Journal of Food Science and Biotechnology 19 (2010) 1205-1210. https://doi.org/10.1007/s10068-010-0172-1.
[19] A. Kagambega, O. Martikainen, T. Lienemann, A. Siitonen, A.S Traore, N. Barro, K. Haukka, Diarrheagenic *Escherichia coli* detected by 16-plex PCR in raw meat and beaf intestines sold at local markets in Ougadougou, Burkina Faso, International Journal of Food Microbiology 153 (2012) 154-158. https://doi.org/10.1016/j.ijfoodmicro.2011.10.032.
[20] M.A.M. Mohammed, Molecular characterization of diarrheagenic *Escherichia coli* isolated from meat products sold at Mansoura city, Egypt, Food Control 25 (2012) 159-164. https://doi.org/10.1016/j.foodcont.2011.10.026.
[21] J. Tobias, S.R. Vutukuru, Simple and rapid multilex PCR for identification of the main human diarrheagenic *Escherichia coli*, Microbiological Research 176 (2012) 564-570. https://doi.org/10.1016/j.micres.2011.11.006.
[22] A. Sjoling, L. Sadeghipoorjahromi, D. Novak, J. Tobias, Detection of major diarrheagenic bacterial pathogens by multiplex PCR panel. Microbiological Research 172 (2014) 34-40. https://doi.org/10.1016/j.micres.2014.12.003.
[23] B. C. Satterfield, Cooperative primers, 2.5 million-fold improvment in the reduction of nonspecific amplification, Journal of Molecular Diagnostic 16 (2014) 163-173. https://doi.org/10.1016/j.jmoldx.2013.10.004.