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Detection of the *Salmonella typhi* bacteria in contaminated egg using real-time PCR to develop rapid detection of food poisoning bacteria

Muktiningsih Nurjayadi[a,*], Y.P. Pertiwi[a], N. Islami[a], N. Azizah[a], U.R. Efrianti[a], V. Saamia[b], I.M. Wiranatha[b], L. Nastassya[c], H. Ali El-Enshasye[e,f]

[a] Department of Chemistry, Faculty of Mathematics and Natural Science, Universitas Negeri Jakarta, Gedung KH. Hasjim Asj'ari, 6th Floor, Jl. Rawamangun Muka Jakarta Timur, 13220, Indonesia
[b] Pusat Laboratorium Forensik Badan Reserse Kriminal Kepolisian Republik Indonesia, Jl. Kavling Agraria, Duren Sawit, Jakarta Timur, Indonesia
[c] Balai Besar Uji Standar Karantina Pertanian, Jl. Pemuda Rawamangun, Jakarta Timur, 13220, Indonesia
[e] Institute of Bioproduct Development, Universiti Teknologi Malaysia (UTM), Skudai, Johor Bahru, Malaysia
[f] City of Scientific Research and Technology Applications, New Burg Al Arab, Alexandria, Egypt

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ABSTRACT

Salmonella typhi is one of the bacteria that cause food poisoning. Food poisoning and food safety become important studies along with increasing awareness and attention around the world. Various methods for detecting food poisoning bacteria are still being developed. The aim of the research is to develop detection kits of food poisoning bacteria with high sensitivity, specificity, accuracy and fast detection through RT-PCR method. The results showed that the primer pair could amplify fim-C of *S. typhi* gene DNA fragments as template as much 56 ng with Ct 14.783 and 14.923. The results showed that the primer pair could amplify fim-C of *S. typhi* gene DNA fragments at template concentration of 4.528 pg/μL with Ct 23.9. The specificity test showed that the primer can distinguish target and non-target bacteria, namely Shigella with Ct 27,949 and *E. coli* with Ct 26,036. Concentration of S.typhi as much of 10^{-6} CFU/mL in eggs can still be detected by producing a value of Ct 15,736 and 15,895. Based on the results it can be concluded that the development of the detection of *S. typhi* in contaminated food was successfully carried out, so that this detection device can be used for forensic laboratories or food testing laboratories with a small sample number, and provide specific, sensitive and fast results.

1. Introduction

The causes of food poisoning are microorganisms such as bacteria, viruses, fungi and several other parasites, which have an ability to infect humans through contaminated food or drink (Dwivedi and Jaykus, 2011; ICHRC, 2016; Liu, 2010). One type of bacteria that common food poisoning is Salmonella spp, and it can cause salmonellosis in the human body (Velusamy et al., 2010). *Salmonellosis* results in around 30% of all cases of food poisoning in developed countries such as the United States (Olsen et al., 2000; Taitt et al., 2004). Based on CDC (Centers for Disease Control and prevention) in 2006, it is estimated that every year, there are one million cases of food borne diseases in the US caused by *Salmonella* (Jackson, 2006).

Food that is often contaminated by *Salmonella* serotypes is an egg (Olsen et al., 2000). Egg can be a good medium for bacteria to grow and reproduce. Therefore, eggs are categorized as a source in a number of extraordinary case investigations (WHO, 1997). Some *Salmonella* serotypes can bind isthmus secretion and enter the inside of the egg shell, so that they are protected from antimicrobial factors in egg white (Buck et al., 2003). The Real Time PCR method is an alternative method for detecting food borne pathogenic bacteria; one of them is *Salmonella typhi* bacteria (Fach et al., 1999; Malorny et al., 2004; Priyanka et al., 2016). The Real Time method has high sensitivity and specificity compared to conventional PCR methods and the time needed is faster than traditional methods or culture methods (Dorak, 2006; Drahovská et al., 2001; Liu, 2010). The sensitivity test of Real Time PCR developed by Fusco (Fusco et al., 2011) using SYBR Green and Taqman master mix can detect bacteria up to 1 x 10^3 and 1 x 10^4 CFU/mL pathogens found in milk.

The use of Real Time PCR method requires functional genes for virulence and infectivity as markers in the PCR process. In this work, the pathogen determining gene for *Salmonella typhi* used is the *fim*C gene, which encodes a chaperone involved in the synthesis of type 1 fimbriae. These are proteinaceous filaments localized on the surface of

* Corresponding author.
E-mail address: muktiningsih@unj.ac.id (M. Nurjayadi).

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the bacterial cell and are responsible for binding to specific receptors on the epithelial cell during infection. (Drahovská et al., 2001). Previous study conducted by Nurjayadi et al. (2017) in collaboration with the forensic team of BARESKRIM POLRI has succeeded in designing a pair of primer fimC genes for *Salmonella typhi*. The primer pairs of *fim*C-F (forward primer) and fimC-R (reverse primer) successfully amplified the DNA of *Salmonella typhi* in 95 base pairs (bp) using conventional PCR method. (M Nurjayadi et al., 2017). Based on homology analysis through the Basic Local Alignment Search Tool (BLAST) method, the primer indicated that the fimC *S. typhi* primer can identify *Salmonella enterica serotype typhi* by 100% and "no significant similarity" with other bacteria such as Shigella flexneri and *Escherichia coli*. Previous research also provides information that the pairs of fimC-F primers (forward primer) and fimC-R (reverse primer) had high sensitivity and specificity in detecting *Salmonella typhi* stock cultures by RT PCR Method (Muktiningsih Nurjayadi et al., 2018). It refers to the previous research; this study continued to detect *Salmonella typhi* in food samples (eggs). Therefore, it is necessary to test the ability of the primer pairs of *fim*C-F (forward primer) and *fim*C-R (reverse primer) in egg samples artificially contaminated by *Salmonella typhi* bacteria.

2. Material and method

2.1. Preparation of culture sample

Reference culture of *Salmonella typhi* used in this study was clinical bacteria obtained from UI microbiology Laboratories Jakarta in solid medium and was then made culture as glycerol stock. *Salmonella typhi* cultures were grown for 24 h in SSA-specific media (Merck) at 37 °C with the streak plate method. The bacterial isolates formed (colonies with black center) were then grown overnight (18 h) in Luria Broth media (Deben Diagnostics.Ltd) at 37 °C using orbital shaking incubator-LM Series (Yihder Co., LTD) at a speed of 200 rpm. For artificially contaminated egg samples with *Salmonella typhi*, six dilution series were carried out from the *Salmonella typhi* overnight suspension in LB media (Deben Diagnostics.Ltd). To determine the number of colonies contaminated in egg samples, 1 mL of suspension at 10^{-4}, 10^{-5}, and 10^{-6} dilutions were grown in SSA media (Merck) using the spread plate method and incubated at 37 °C for 24 h. The suspension chosen for the calculation of bacterial concentration is a suspension that produces a number of colonies between 30 and 300 based on plate count standard of the FDA BAM (Food and Drug Administration Bacteriological Analytical Manual). The number of colonies formed was calculated as CFU/mL culture.

2.2. Artificial contaminated egg sample with Salmonella typhi

Egg samples were purchased from traditional markets then boiled until cooked.The cooked egg sample was crushed until smooth in sterile plastic and then transferred to the plate and irradiated with UV light for 30 min. Each egg sample was transferred into Erlenmeyer (Pyrex) and contaminated with bacterial suspension in a volume ratio of 1:1. The test samples used bacterial suspension at 10^{-6} dilutions, while positive controls used suspension of non-diluted bacteria to be inoculated on egg samples and negative controls, i.e. only samples of eggs without bacteria contamination. Each sample was incubated at 37 °C for 18 h using orbital shaking incubator-LM Series (Yihder Co., LTD) at a speed of 200 rpm.

2.3. DNA isolation

One milliliter of each culture stock, positive control, test sample and negative control were transferred into the Eppendorf tube and centrifuged (Sorval Legend Micro 17R) with a speed of $5000 \times g$ for 5 min to produce residue/pellets. Bacterial DNA were isolated from pellets/residues produced using a commercial kit from QIAamp DNA Mini Kit (ATL, AL, AW1, AW2, AE buffer) by following the stages of the Gram-negative bacterial DNA isolation protocol from the kit (Qiagen, 2016). The purity of the isolated DNA was measured at the A260/A280 ratio and DNA concentration using GE Nanovue Uv–Vis Spectrophotometer.

2.4. Design and synthesis primer

The sequences of the *Salmonella typhi* gene data were obtained from GenBank accession numbers NC_003198.1(NCBI, 2016). The primer pair of *fimC* gene in this study was the NCBI Primer BLAST design carried out in previous studies (M Nurjayadi et al., 2017) and has been adjusted to the primer design requirements (Dorak, 2006). The primer pair of *fimC* gene produces an amplicon of 95 base pairs (bp) (M Nurjayadi et al., 2017). The primers designed are synthesized at Macrogen, Inc-Korea commercial laboratories. Furthermore, the synthesized primer is optimized in the annealing temperature (56 °C, 57 °C, 58 °C, 59 °C, 60 °C, 61 °C) range based on ± 5 °C melting temperature of the primer (Innis, 1997).

2.5. Optimization of annealing temperature

The annealing temperature optimization process used a Gradient PCR (Applied Biosystem). The PCR reaction containing the primer pair of *fimC* gene (M Nurjayadi et al., 2017), The Dreamtaq Green PCR Master Mix (ThermoScientific), *Salmonella typhi* culture stock as DNA templates and Nuclease Free Water (NFW) with a total reaction component of 25 μL. The PCR amplification process used the appropriate method of the *Dreamtaq Green PCR Master Mix* procedure (ThermoScientific) that are denaturation temperature 95 °C for 2 min, denaturation 95 °C for 30 s, annealing (56 °C, 57 °C, 58 °C, 59 °C, 60 °C, 61 °C) for 30 s, extension at 72 °C for 1 min and final extension at 72 °C for 10 min, repeated in 40 PCR cycles (ThermoScientific, 2016).

The amplicon DNA was visualized by 2% agarose gel electrophoresis (Promega), in TAE 1X buffer (*Tris-acetate-EDTA*) (Thermo Scientific) and ethidium bromide dyes (Promega). Electrophoresis was carried out at 70 V for 60 min then DNA bands were observed with UV Trans illuminator (Fortune). DNA size was compared with a ladder (Thermo Scientific) to determine the size of the sample DNA (Magdeldin, 2012). The result of annealing temperature optimization was applied to the annealing temperature for the Real Time PCR method.

2.6. Confirmation test of Salmonella typhi stock culture

The confirmation test of the primer pair of *Salmonella typhi fimC* gene was conducted to determine the primer performance in detecting the presence of bacterial DNA by amplifying DNA in the Real Time PCR 7500 CAST detection system (Applied Biosystem) using SYBR Green I master mix. The testing was conducted by reacting SYBR Green master mix, forward primer, reverse primer, NFW (Nuclease Free Water) and DNA template. Overall volume of reaction is 20 μL. Then the same mixture is reacted without the bacterial DNA template as NTC (No Template Control). Then the 96 well reaction plate was closed by PCR *sealer ™ Microseal* and inserted into a real time PCR using PCR protocol at initial denaturation temperature 95 °C for 3 min, denaturation 95 °C for 15 s, annealing 60 °C for 30 s, extension at 72 °C for 30 s, repeated in 40 PCR cycles (SMOBIO, 2017).

2.7. The sensitivity and specificity testing of fim-C primer to Salmonella typhi from stock culture

The primer sensitivity test is obtained by dilution series from the template DNA stock of *Salmonella typhi* to find out the lowest detection limit that can be achieved by the primer on Real Time PCR method (Dorak, 2006). The sensitivity testing of the primer pairs of *fim*C-F (forward primer) and *fim*C-R (reverse primer) was conducted a fivefold dilution from DNA stock that can be achieved by the primer in detecting

template sample. The results of each dilution are used as DNA templates. The sensitivity results are indicated by a linear standard curve. While the specificity test was carried out by cross reaction tests on non-target bacterial DNA templates. In this study, the primer *fim*C gene *Salmonella typhi* was tested on the DNA template of *Shigella* and *Escherichia coli* and *Salmonella typhi* DNA from pure culture as positive controls. The total reaction of each sample was 20 μL with the same PCR profiles in previous testing.

2.8. Confirmation test of the firm-C primer to contaminated egg sample

The primer pairs of *Salmonella typhi fim*C genes were tested on artificially contaminated egg samples with DNA *Salmonella typhi* bacterial stock as positive controls, egg samples with *Salmonella typhi* bacterial suspension in 10^{-6} dilutions as test samples, and egg samples without bacterial contamination as negative controls. The total reaction of each sample was 20 μL. The Real Time PCR conditions were same as the previous confirmation test.

3. Result

3.1. Quantitative test of DNA sample

The purity and concentration of DNA were isolated from stock culture and egg samples contaminated by *Salmonella typhi* bacteria using GE Nanovue UV-VIS Spectrophotometer was shown in Table 1

3.2. Qualitative test

The visualization of the optimization results of the primer temperature annealing using of much as the DNA template was 56 ng in the amplification process. DNA bands produced at the temperature range of 56 °C–61 °C when compared to DNA ladder are in the size of 95 base pairs was shown in Fig. 1.

3.3. Amplification by real time PCR

The evaluation of a pair of *Salmonella typhi fimC-F* and *fimC-R* primers were carried out in a previous study (Muktiningsih Nurjayadi et al., 2018) through confirmation test using stock culture with as many DNA template of 56 ng and NTC as negative control. The amplification results formed a sigmoid curve that appeared at the threshold cycle (Ct) of 14.783 and 14.923 while the NTC appeared at Ct 32.631. *Salmonella typhi* peak curve was produced at temperature melting (Tm) of 83, 01 °C. The amplification and melting curve were shown in Figs. 2 and 3.

The study also showed the sensitivity of a primer pair of *Salmonella typhi fim*C-F (forward primer) and *fim*C-R (reverse primer) can detect DNA templates up to the lowest concentration of 4528 pg/μL and the amplification curve at the smallest concentration at a value of Ct 23.90 (Fig. 4).

The specificity evaluation of a pair of *fim*C-F (forward primer) and *fim*C-R (reverse primer) of *Salmonella typhi* showed that the primer could recognize *Salmonella typhi* target DNA that is indicated by Ct 14,770. It was considered unable to recognize *Shigella dysenteriae* DNA as non-target DNA despite amplification at Ct 28 and *Escherichia coli* DNA as the non-target at Ct 26,036 (Fig. 5). The concentration used is 56 ng for each DNA template.

Table 1
The purity and concentration of DNA isolated of stock culture and egg samples contaminated by *Salmonella typhi* bacteria.

	Sample	Purity $A_{260/280}$	Concentration (ng/μL)
A	Non diluted bacterial + egg sample	1953	28,33
B	10^{-6} suspension bacterial + egg sample	2003	7,43
C	Egg sample without bacterial suspension	2342	15,7
D	Stock culture	1850	27,83

Table 2
Results of evaluation the primer sensitivity of the *fimC gene Salmonella typhi*.

Primer	DNA Concentration (pg/μL)	Line sigmoid	*Thresholds Cycle* (Ct)
Salmonella typhi fim-C gene	2783		14.80
	556.6		17.09
	113.2		19.29
	22.64		21.93
	4.528		23.90
	NTC		31.36
Slope: 3. 295; R^2 : 0.999; Eff %: 101.132%; y-Int: 19.156			

The amplification results of *Salmonella typhi fim*C-F (Forward primer) and *fim*C-R (Reverse primer) primer pairs with three egg samples showed different results. That are eggs contaminated with non-diluted bacteria (sample A, green line) amplified with Ct 13,010 and 12,970, eggs contaminated with bacterial suspension at dilution 10^{-6} (sample B, blue line) amplified with a value of Ct 15,736 and 15,895, and egg samples that were not contaminated with bacteria (sample C, pink line) were amplified with a value of Ct 24,902 and 25,015. The amplification curve and the melting curve were shown in Fig. 6.

4. Discussion

In a case of food poisoning caused by pathogenic bacteria, a very fast, sensitive and specific detection method is needed in accurately the bacteria contained in foods so that victim handling becomes more effective and efficient (Laude et al., 2016; Priyanka et al., 2016; Zhao et al., 2014). The Real Time PCR is a method for rapid analysis, reduces the risk of contamination and sensitive in detecting targets for pathogenic bacteria *Salmonella typhi* (Hein et al., 2006) so that this study uses the method in detecting *Salmonella typhi* in egg samples, which often becomes the cause of food poisoning (WHO, 1997).

This study used the primer *Salmonella typhi fim*C gene with the design of *fim*C-F (forward primer) and *fim*C-R (reverse primer) from the previous study (M Nurjayadi et al., 2017) that has an amplicon length of 95 base pairs (bp). Based on amplification results by PCR Gradient the annealing temperature showed 56°C–61 °C produce good results. One of the condition that is 60 °C was chosen afterward, because that temperature is appropriate with the RT PCR machine in standard condition and information from some literature (Innis, 1997; Dorak, 2006). Incorrect annealing temperatures can result in mispriming or primer attachment errors (Kary, 1985). Therefore, before the Real Time PCR process, the primer pairs of *fimC-F* (*forward* primer) and *fimC-R* (*reverse* primer) were optimized in annealing temperature range 56 °C–61 °C, annealing temperature selection based on ± 5 °C from the temperature of the primer pair *fimC-F* (*forward* primer) and *fimC-R* (*reverse* primer) *Salmonella typhi,* melting temperature can be theoretically calculated by a formula Tm = 4 (G + C) + 2 (A + T) (Innis, 1997). Based on the data in Fig. 1, it shows the presence of specific DNA bands of *Salmonella typhi* bacteria with an amplicon size of 95 base pair at all temperature of 56°C–61 °C so that the product has been successfully amplified. This is because the amplicon size of the amplification results is in accordance with the size of the primer design results. Fig. 1 also provides information that the DNA band at a temperature of 60 °C and 61 °C produces the thickest and brightest band that indicates that at both temperatures the annealing produces optimum amplification so that it can be used in the Real Time PCR process.

The primer confirmation test of the *fimC* gene on *Salmonella typhi* stock culture in the previous study (Muktiningsih Nurjayadi et al., 2018) shown in Fig. 2 consistently amplified at ± 15 Ct and the melting point curve yielded one peak with a value of Tm 83,01 °C. The results of the melting point curve show that there is no mispriming in

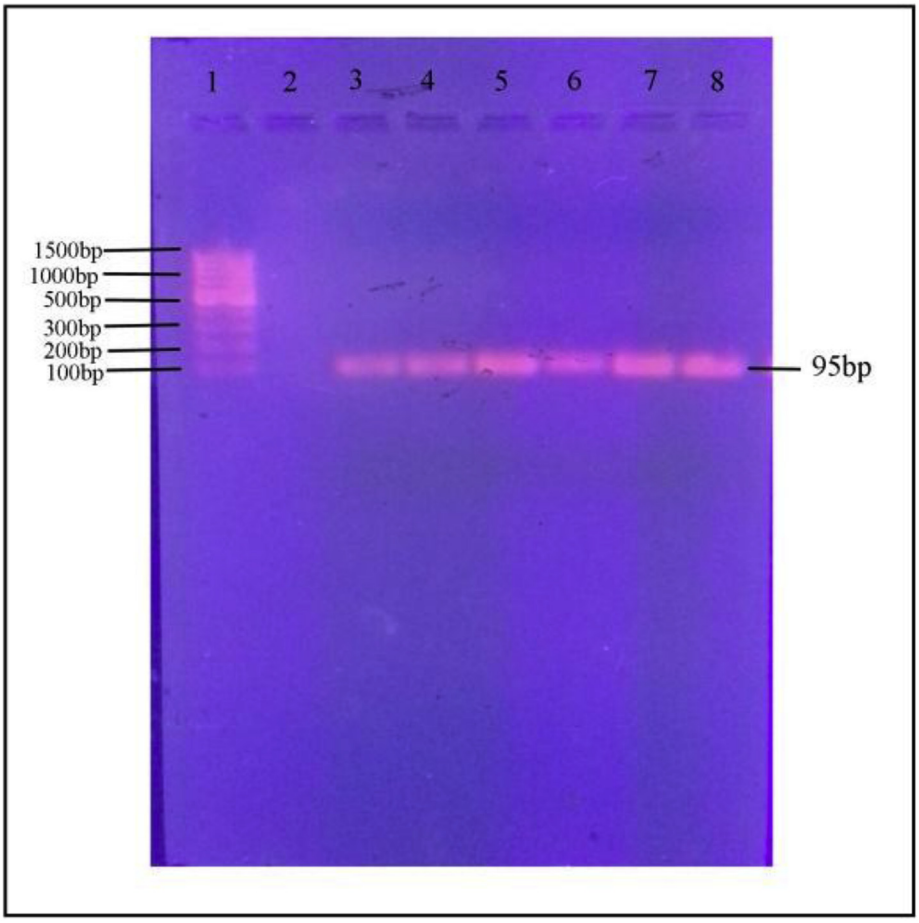

Fig. 1. Visualization of the results of optimization of primer annealing temperature of the *fimC* gene *Salmonella typhi*, (1) DNA Ladder 100 bp; (2) Negative controls; (3) DNA fragment at temperature of 56 °C; (4) DNA fragment at temperature of 57 °C; (5) DNA fragment at temperature of 58 °C; (6) DNA fragment at temperature of 59 °C; (7) DNA fragment at temperature of 60 °C; (8) DNA fragment temperature of 61 °C.

the test which means that the primer used only amplifies the target gene as indicated by the formation of one peak. In addition, sensitivity test data states that at the lowest concentration of the DNA template can still be detected by the primer *fimC* gene and reach the boundary with a value of Ct 23.90 at the lowest concentration of 4.528 pg/μL. The figure of standard curve data in Fig. 4 can be said to be ideal, having a regression value of $r^2 = 0.999$, which shows good linearity (Dorak, 2006) and the slope range from −3.1 to −3.6 (Pestana, 2010) is −3295 and efficiency 101,132%. Efficiency is an important factor for each quantitative PCR method that is reliable, reproducible, and forceful (Siebert, 1999).The ideal efficiency value is in the range of 90–110%, if the efficiency value is low (< 90%) can be caused by contamination of taq inhibitors, high annealing temperature, poor primer design results or amplicons with secondary structures while high-efficiency values (> 110%) due to non-specific primer dimer or amplicon results. However the most common cause of high and low efficiency is caused by poor pipetting techniques (Taylor et al., 2009). Specificity test states that the primer *Salmonella typhi fimC* gene can recognize *Salmonella typhi* target DNA as indicated by Ct 14,770 while in *Shigella dysenteriae* DNA amplified on Ct 27,949 and *Escherichia coli* on Ct 26,036 it is assumed that the primer *fimC* gene *Salmonella typhi* cannot recognize both samples (Fig. 5). According to Bustin SA, Nolan T

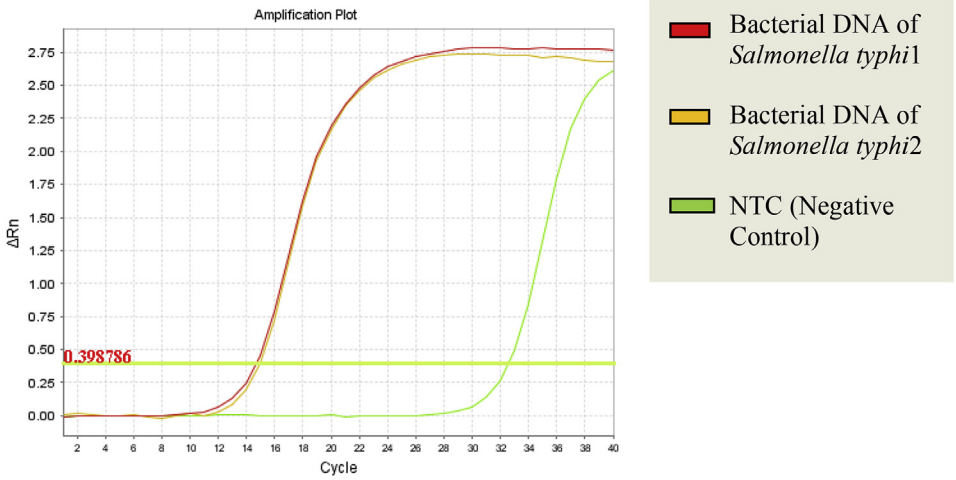

Fig. 2. Amplification curve of *Salmonella typhi* bacterial stock culture with concentration DNA template of 56 ng and negative control.

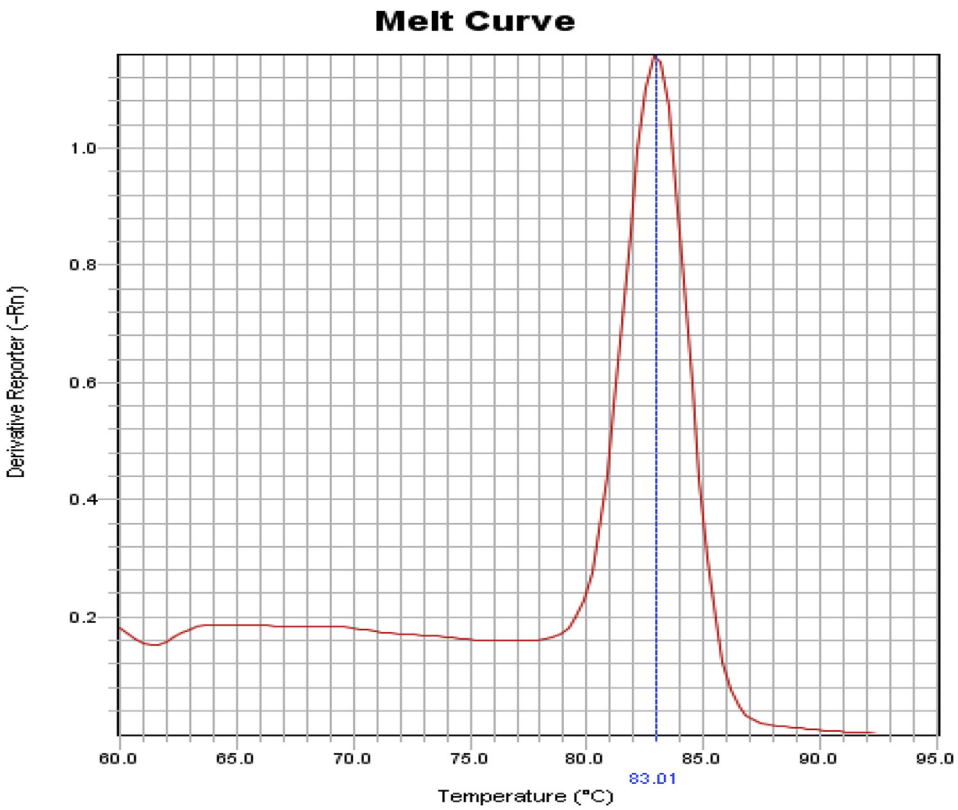

Fig. 3. Melting Curve of *Salmonella typhi* stock culture with DNA template of 56 ng.

(2004), the difference in amplification of 10 cycles with target DNA in one reaction is considered to be amplified bacterial DNA is the non-target (Bustin and Nolan, 2004).

In this study, the concentration of artificial contaminated *Salmonella typhi* in egg samples was determined in the plate count method in SSA media. The bacterial suspension at 10^{-6} dilutions was chosen to be artificially contaminated in egg samples grown on SSA media with spread plate techniques that produced 64 colonies, the number of colonies included in the range recommended by the Food and Drug Administration Bacterial Analytical Manual (FDA BAM), 25–250 colonies. The culture concentration of *Salmonella typhi* bacteria contaminated in egg samples was calculated to be 64 x 10^5 CFU/mL whereas in negative control samples which were not contaminated with *Salmonella typhi* black colonies were not produced so that it could be stated that negative control samples had no growing *Salmonella typhi*.

Through a confirmation test the primer pairs of *fimC-F* (*forward* primer) and *fimC-R* (*reverse* primer) in the three samples included non-diluted bacterial suspension contaminated with egg samples (sample A), bacterial suspension 10^{-6} dilutions contaminated with egg samples (sample B) and eggs without contaminated bacteria (sample C) produced the amplification curve shown in Fig. 6. Based on the results of the amplification curve it can be stated that the Ct value of the first detected sample is: Sample A < Sample B < Sample C. This shows that the concentration of 64 x 10^{-6} CFU/mL of *Salmonella typhi* bacteria in egg samples can still be detected, which is consistently amplified at Ct 15,736 and 15,895. The results of the melting curve analysis shown in Fig. 6, the peak curve of *Salmonella typhi* in samples A and B produced a melting curve with a value of Tm 85 °C. The results of the

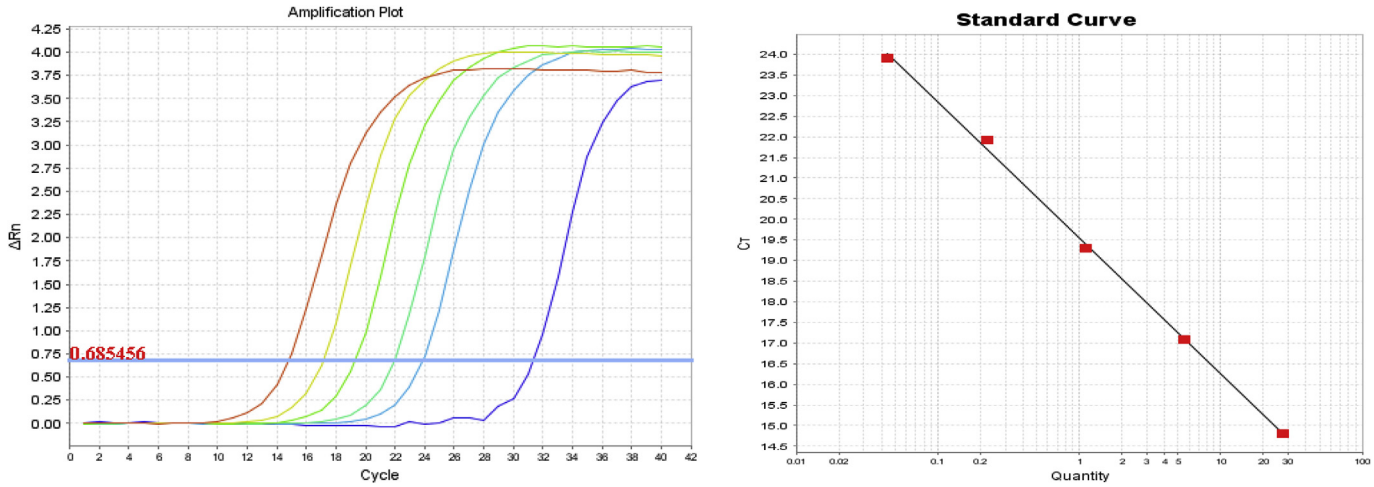

Fig. 4. The amplification curve and sensitivity test curve for *fimC Salmonella typhi* gene.

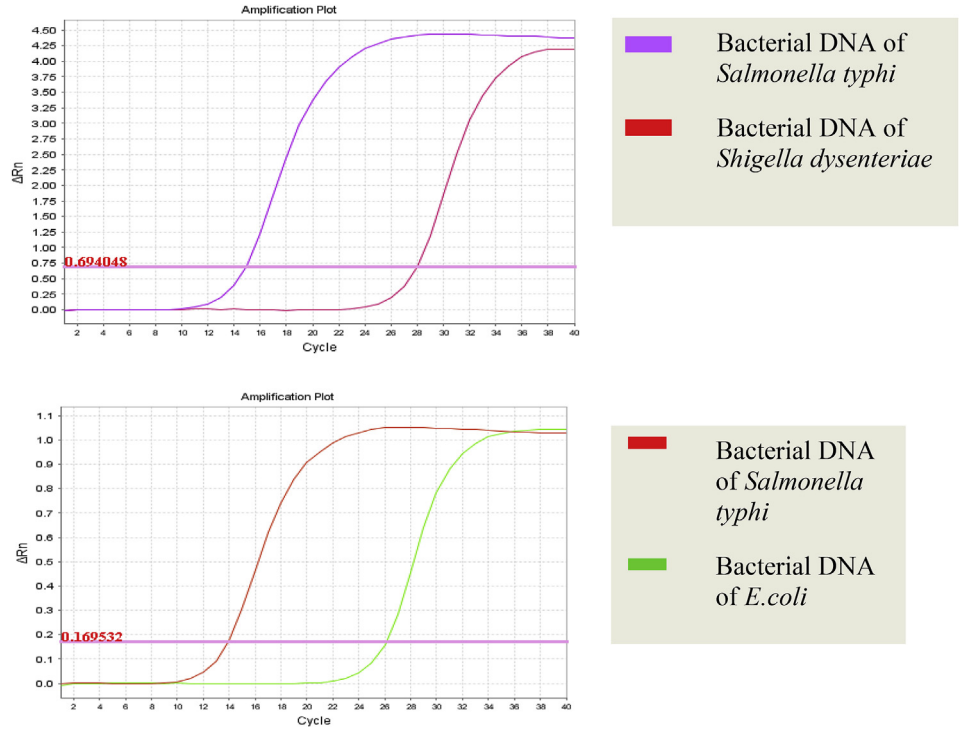

Fig. 5. Amplification curve of the primer specificity of the *Salmonella typhi fim-C* gene.

melting peak curve show that there is no mispriming indicated by the formation of one peak while in sample C (pink line) which is a negative control a melting curve is different from sample A (green line) and sample B (blue line) that is ± 84 °C. The results of the melting peak curve show that sample C is non-target DNA of *Salmonella typhi*.

5. Conclusion

The Real Time PCR method was successfully developed for the rapid detection of *Salmonella typhi* bacteria in egg samples up to 64 x 10^{-6} CFU/mL bacteria using primer *Salmonella typhi fim-C* gene. The Limit of detection of fim-C *S. typhi* primer 4528 pg/μL. The *fim*-C *S. typhi* primer specifically can differentiate *S. typhi* as target with *Shigella dysenteriae* and *E. coli* bacteria as non-target sample. These method potential to develop as detection model for other foodborne bacteria with quickly, sensitively and specifically.

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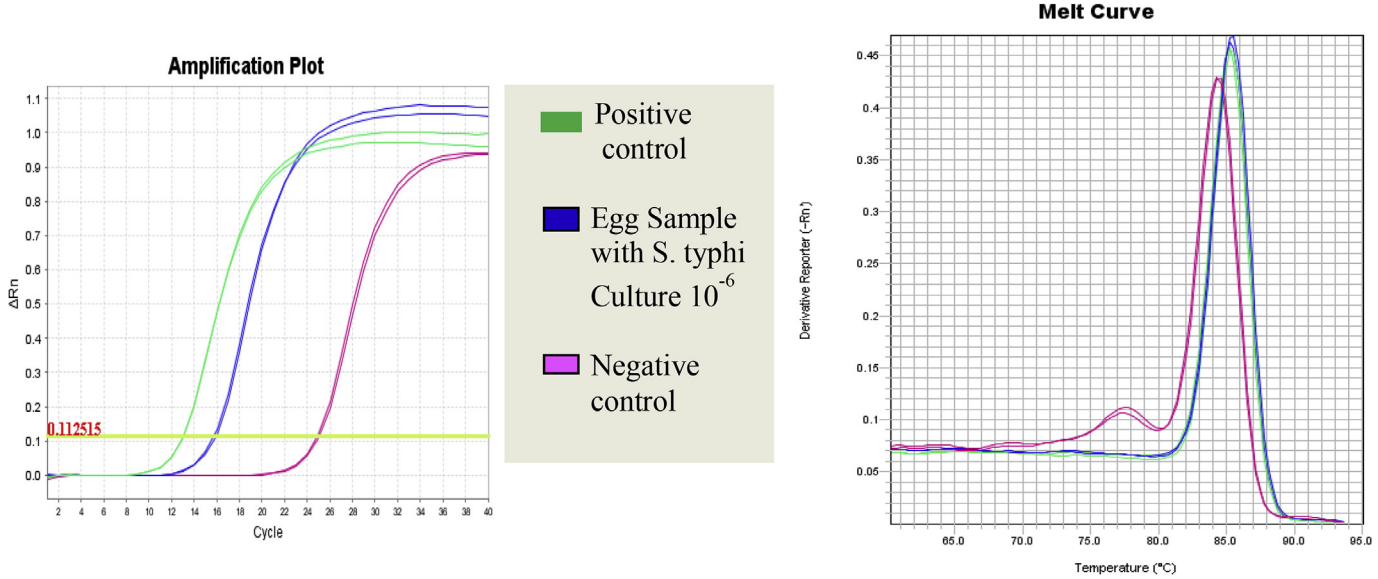

Fig. 6. The amplification curve and melting curve in the confirmation evaluation of *fimC-F* (*forward* primer) and *fimC-R* (*reverse* primer) *Salmonella typhi* with three egg samples.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101214.

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