Evaluation of Primers Detection Capabilities of the pef Salmonella typhimurium Gene and the fimC Eschericia coli Gene Using Real-Time PCR to Develop Rapid Detection of Food Poisoning Bacteria

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Abstract. The aim of the research is to obtain information about detection capabilities of the pef S. typhimurium and the fimC E. coli primer using Real time PCR method to develop kit detection of food poisoning bacteria. Evaluation of primer's capability was determined by accumulation of fluorescent signal in amplification curves that are connected by Cycle threshold (Ct) and intensity of amplicon signal. The results show the pef primer gene amplifies DNA target of the S. typhimurium fragments in Ct 17.85 and the fimC E. coli primer gene in Ct 12.25. Sensitivity evaluation of the pef S. typhimurium primer and the fimC E. coli primer showed a minimum detection level on 0,284 pg/μL and 7,12 pg/μL. Specificity evaluation of the pef gene of S. typhimurium primer on non-target E. coli bacteria showed Ct 18.05 almost similar to DNA target, but on primer fimC E. coli to non-target DNA bacteria S. typhimurium showed Ct 21.53. Therefore, it concluded the pef S. typhimurium primer gene and the fimC E. coli primer genes are suitable for rapid detection kit with good sensitivity. It needs further development in specificity of pef S. typhimurium primer, although the primer pef gene on non-target bacteria showed the different cycle threshold.

1. Introduction
The safety and hygiene of food quality from the contamination of pathogenic bacteria are very important to note [1, 2]. Based on data from the World Health Organization (WHO) federation, the most common bacterial pathogens are 31 species, some of which are identified to cause poisoning are E. coli 0:157 and Salmonella typhimurium bacteria [3, 4]. In Indonesia, the Food and Drug Supervisory Agency noted that in the year of 2016 there have been 1378 cases of food poisoning and 35 cases were caused by consumption of household food, catering food, market snacks and schools due to the contamination of harmful microorganisms [5]. One method to detect the presence of pathogenic bacteria is the method of Real-time Polymerase Chain Reaction (RT-PCR or q-PCR).

Real-time PCR or quantitative PCR is a method of detecting or quantifying a product of polymerase chain reaction amplification based on the corporation of a fluorescent-reporter dye or fluorescent signal along with the addition to a proportion as the number of products per cycle [6, 7].
This method began to be developed to overcome the limitations of conventional methods and be an option in identifying pathogenic microbes such as *Escherichia coli*, *Campylobacter*, *Shigella* and *Salmonella* species [8, 9,10]. The discovery of this molecular-based technique is based on various aspects of detection, such as sensitivity, rapidity, the ability to distinguish between pathogen species (selectivity) and ease of conducting suitability compared to traditional methods [9]. Another advantage of RT-PCR (qPCR) is that it can directly monitor the process of amplifying the target DNA during the reaction in each cycle by detecting the fluorescent signal emission from each DNA amplification product generated. So that the target DNA quantities can be identified and quantified accurately and fast without having to go through the characterization process using agarose gel electrophoresis [7, 9, 11].

Previous research reported by the UNJ *Salmonella* team, shows that it has successfully designed and synthesized a nucleotide sequence of specific areas of the *fimbral*-C gene (*fim-C*) of *Eschericia coli* bacteria and *Salmonella typhimurium*. The primer pairs of the *fim-C E. coli* and *pef S. typhimurium* genes have also been used for the amplification process by conventional PCR methods, which produce consecutive amplicon measuring 121 base pairs (bp) and 139 bp [12,13].

This research is part of UNJ *Salmonella* research umbrella study about development model of rapid kit detection method of foodborne pathogen bacteria. The purpose of this study was to test the primer genes of *pef* and *fim-C* gene primers in detecting *S. typhimurium* and *E. coli* bacteria using RT-PCR method. This method is expected to produce faster, sensitive and specific data in target bacterial DNA detection, so the primer genes *pef* and primer *fim-C* gene can be applied later for testing of target bacteria *S. typhimurium* and *E. coli* in the sample food.

2. Methods
The materials used for this research are: (1) GeneJET Genomic DNA purification isolation kit (Digestion Solution, Lysis Solution, RNase A Solution, Proteinase K Solution, Wash Buffer, and Elution Buffer [ThermoScientific]; (2) 2x Q-PCR Master Mix (3) Nuclease Free Water [Qiagen]; (4) pure culture of *E. coli* and *S. typhimurium* bacteria [Lab Microbiology UI]; (5) Luria Bertani (LB) media [Deben Diagnostic, Ltd.], and selective media *Salmonella Shigella* Agar [Deben Diagnostic, Ltd.].

The preparation stage consists of: (1) culture of *E. coli* and *S. typhimurium* bacteria on *Salmonella Shigella* Agar (SSA) and LB media; (2) Isolation of DNA genomes of *S. typhimurium* and *E. coli* (3) Amplification using real time instrument PCR 7500 FAST [Applied Biosystems] includes (a) The primer confirmation test of the *fimC* gene and the *pef* gene into the DNA target (b) sensitivity test by diluting the concentration of the DNA isolate, and (c) the specificity test by cross-examining the gene primers to the bacteria non target. Preparation of the mixture reaction follows the standard protocol [14]. Total volume in a single reaction of 20 μL, consisting of 10 μL masters mix SYBR Green, I [SMBIO], 1 μL forward primer (10 μM), 1 μL reversed Primer (10 μM), 2 μL bacterial DNA isolated, and 6 μL Nuclease Free Water. The amplification process was carried out for 40 cycles with pre-denaturation stage at 95 °C for 3 minutes, denaturation stage at 95 °C for 10 seconds, annealing stage at 60 °C for 30 seconds, and extension stage at 72 °C for 30 seconds.

3. Result and Discussion
The results from the amplification of the primer confirmation test of *S. typhimurium* *pef* gene and the *fimC E. coli* gene primer in pure culture bacterial samples are shown in Figure 1 (a) and 1 (b). Tests of each bacterial DNA isolate sample were performed in duplicate with No Template Control (NTC) as a negative control. The amplification curve data showed that the *fimC* gene primer successfully amplified the *E. coli* genomic DNA isolate sample and the *pef* gene primer successfully amplified samples of *S. typhimurium* genome DNA isolates. The success is shown by the formation of red and yellow sigmoid charts for *E. coli* DNA isolate samples. The sigmoid curve is formed because the fluorescent signal passes through the baseline threshold by forming the intersection point on the Cycle
threshold (Ct) 12,251 and 12,252. The intensity of the fluorescence signal achieved is 0, 0579 shown by the pink line (Figure 1a).

As for the genes pef positive test results are shown by the amplification curve that forms the red and yellow sigmoid line on the sample of S. typhimurium DNA isolate that is able to pass the baseline threshold by forming the intersection point at Ct 17,857 and 17,923 with the accumulation of fluorescence signal intensity reached 0,568 (Figure 1b). No Template Control (NTC) shown by the sigmoid graph on amplification curve 1(a) or curve 1(b) is a negative control without the presence of a bacterial target DNA template in the reaction component. Based on the NTC data on the amplification curve shows that in Figure 1(a) the amplification curve of E. coli occurs the amplification process in the threshold cycle of 31.909 while on the curve of 1 (b) S. typhimurium with Ct 35, 107. Based on good NTC threshold cycle (Ct) literature should be above the 35 cycle indicating that no non-targeted DNA is involved in amplification. However, if the NTC's cycle threshold has a range far exceeding 10 threshold cycles of the final threshold cycle achieved by target DNA, then it remains within the limit of tolerance for the amplification of non-targeted DNA contamination in the reaction [15, 17]. Therefore, the results of DNA amplification target on both E. coli and S. typhimurium bacteria are still categorized to give good results.

The primers confirmation test is also performed by Melting Curve Analysis (MCA). The yield of the E. coli melting peak curve shows one peak with a value of Tm 78, 65°C (Fig. 1a). The peak curve of S. typhimurium melting also produce one melting peak with a value of Tm 82, 68°C (Fig. 1b) The yield of the melting point curve indicates that there is no mispriming in the test meaning that the primer fim-C and pef used do not amplify the gene other than the target gene shown by only the formation of a peak [18].

(a)
Sensitivity testing with dilution of multilevel DNA samples up to a certain concentration gives good results. This is shown by the formation of the amplification curve and the efficiency value of the standard curve which is at the desired vulnerability (95-110%) (Fig. 2a and 2b). The resulting slope value for the E. coli sample is in the -3.137 range, and the resulting slope value for the S. typhimurium sample is -3.44. Based on the result of slope value, it can be known that the efficiency value obtained for the dilution concentration of E. coli sample is 108% and for S. typhimurium, the efficiency value can be 95.36%. The results of the amplification curve in Fig. 2 (a) for E. coli and Fig 2 (b) for S. typhimurium show that the real-time PCR process operates at good efficiency as described by the number of amplified DNA templates multiplying at each cycle according to dilution concentration, this is evident to the increasing threshold cycle (Ct) significantly on the sigmoid graph formed [16, 19, 20]. The result of the amplification curve at the lowest concentration of the template DNA is shown by the formation of the sigmoid line on the amplification curve of E. coli in Fig. 2 (a) that can reach the threshold with a Ct value of 23,923 at the concentration of 7.12 pg/μL. For S. typhimurium on the amplification curve in Fig 2 (b) shows the lowest amplification that can still be amplified at 0.284 pg/μL with a value of Ct 26,908.

**Figure 1.** The amplification curve and Melting curve of confirmatory test results. **1(a)** Primer gene fimC E. coli: DNA samples E. coli 1 (red line), and DNA samples E. coli 2 (green line), No template control (NTC, pink line), **1(b)** Primer gene pef Salmonella typhimurium: S. typhimurium 1(red line), S. typhimurium 2 (orange line), Negative control (NTC, green line). Melting curve of E. coli and S. typhimurium give the single curve on temperature 78, 65 °C and 82,68 °C.
Figure 2. Amplification curve and standard curve of sensitivity test result. **Fig 2 (a)** Primer of fimC gene with isolate concentration E. coli DNA: (A) 4450 pg/μL, (B) 890 pg μL, (C) 178 pg/μL, (D) 35.6 pg/μL, (E) 7.12 pg/μL. **Fig 2 (b)** primer gene pef with a concentration of S. typhimurium DNA isolates: (A) 1783 pg/μL, (B) 356 pg/μL, (C) 71.2 pg/μL, (D) 14.24 pg/μL, (E) 0.284 pg/μL.

Figure 3. Specificity test curve. **Fig 3 (a)** Primer gene fim C: (A) DNA samples E. coli (B) DNA sample of S. typhimurium. **Fig 3 (b)** Primer gene pef: (A) S. typhimurium DNA samples, (B) DNA samples of E. coli
Based on the amplification data in Fig. 3 (a) shows the Ct value obtained for the fimC gene primer with the S. typhimurium template of 21.53 whereas for the primer positive control of the fimC gene the E. coli template exhibits amplification by achieving a 14.04 threshold cycle lower than the value of Ct bacteria non target S. typhimurium. Achieving the threshold cycle of target bacteria E. coli with lower Ct values indicates that the fimC gene primers are more selective in recognizing DNA samples of E. coli target bacteria than with S. typhimurium bacteria as non-target bacteria.

Different results were shown by S. typhimurium primer gene in Fig. 3 (b), the sigmoid A graph shows gene primer pef with target S. typhimurium bacterial isolate with Ct value 17, 65 while the sigmoid B graph shows pef genes with the non-target DNA bacteria templates E. coli at Ct 18, 05. Based on the result of the amplification curve 3 (b) it shows that the non-targeted E. coli bacteria are amplified by the pef gene primer in the threshold cycle similar to the target bacteria. The occurrence of amplification in non-target bacteria may be due to the temperature at the annealing stage used in the primary specificity test of pef S. typhimurium gene is not optimal. According to Pestana (2010), the less optimum annealing temperature will cause the primer to attach to the other side of the non-target DNA genome resulting in the amplified DNA resulting in a non-targeted amplicon product by forming a low specificity [15, 18].

4. Conclusions

The results of the primers assay evaluation using the real time PCR method concluded that the primers of the fim-C E. coli gene, and the pef S.typhimurium primer genes can be used as sensitive and rapid detection devices, but still require improvement in the primers specificity of S.typhimurium genes, although the primer of pef genes against non-target bacteria gives different Ct values.

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