Detection of *Salmonella typhimurium* and *Escherichia coli* in artificially inoculated Milk sample using *Real Time PCR* method

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**Abstract.** *Salmonella typhimurium* and *Escherichia coli* are the bacteria the most common cause of food-borne illness. The aim of the study is to detection *S. typhimurium* and *E. coli* by *Real Time PCR* from milk samples. Gene targeted of *S. typhimurium* is *pef* and the *E. coli* is *fimC*. Confirmation test of all primers in contaminated milk with *S. typhimurium* and *E. coli* gave different cycle threshold (Ct) value in range 12-13. The detection limit for *S. typhimurium* with primer *pef* was in 7, 92 pg/μL and detection limit for *E. coli* with primer *fimC* in 7, 12 pg/μL. Each primer produces distinctive melting peak. The melting peak curve *S. typhimurium* with *pef* primer is 84,17°C and the melting peak curve *E. coli* with *fimC* primer is 79,97°C. Based on the result of this study; all the primers were successfully applied in detecting *S. typhimurium* and *E. coli* bacteria in the milk sample quickly, sensitive and specific as well as can determine the number of bacteria accurately.

1. Introduction

Food contains many nutrients that can support the growth of microorganisms often associated in poisoning cases. Types of foods that are susceptible to bacteria include meat and processed meat products, milk, seafood (fish, shellfish, and shrimp) and vegetables and fruits [1]. Microbes found in food have the potential to common damage to food components, causing changes in texture, taste and color [2]. Microorganisms contained in food come from the production process environment, the cleanliness of production equipment, storage processes, packaging materials and distribution processes [3].

Bacteria which are often associated with food poisoning are *Yersinia enterocolitica*, *Vibrio Cholera*, *Staphylococcus aureus*, *Shigella* species, *Salmonella* species, *Pseudomass*, *Escherichia coli*, and *Listeria monocytogenes*. *Salmonella* species 23% are involved in food poisoning, such as meat, fish, milk and eggs, while *E. coli* generally causes diarrhea disease. Worldwide, there are nearly 1.7 billion cases of diarrhea disease every year [4,5]. Symptoms occur 24-48 hours after infection, usually characterized by nausea, vomiting, abdominal cramps, and runny diarrhea or sometimes bleeding [6].
The faster method is an interesting to develop especially for food safety. The method developed at this time is no longer based on the cellular level, but has begun to be at the molecular level, one of which is Polymerase Chain Reaction (PCR). The PCR method is a chain reaction that involves an enzyme polymerase with the principle of amplifying (multiplying) the target DNA fragments [7]. Modification of the PCR technique which results in faster detection capabilities, high specificity and sensitivity, are namely the Real Time PCR method [8]. This is evidenced by the study of Mohammadiha et al., 2013 by comparing traditional PCR methods and Real Time PCR in detecting Leishmania infantum in human and dog blood samples, where the results obtained are the level of validation of the Real Time PCR method (95-99%) higher compared to use the conventional PCR method (60-80%) [9]. Research by Malorny also proves that detection time with Real Time PCR technique only takes 24 hours, while analysis of traditional culture methods takes 4 to 5 days [10]. The potential of Salmonella spp and Escherichia coli as a cause of disease is due to several virulence factors possessed by each bacterium. Virulence factors possessed by S. typhimurium include fimbria, flagellin, ion transporters, superoxide dismutase [11]. Gene that encodes virulence factors can be utilized at the molecular level to recognize the presence of a bacterium.

In previous studies, the primers E. coli fimC gene and pef S. typhimurium genes were shown to be perfectly to amplify the target bacteria in pure culture samples. The fimC gene primers are known to be able to detect E. coli bacteria up to a concentration of 7.12 pg/μL, while the pef primer could detect S. typhimurium to a concentration of 0.284 pg/μL [12]. Based on those results, this study aims to apply the Real Time PCR method in detecting the presence of S. typhimurium and E. coli bacteria in dairy products as the basis for developing the Real Time PCR application in food samples.

2. Research methods

The materials used for this research are: (1) QIAamp® DNA Mini Kit Cat No. 51304 (Qiagen); (2) SYBR® Green qPCR kit Master mix (3) Nuclease Free Water [Qiagen]; (4) pure culture bacterial samples of E. coli ATCC 25922 and S. typhimurium ATCC 14028 [Microbiologist supplier]; (5) Luria Bertani (LB) media [Deben Diagnostic, Ltd.], and selective media Salmonella Shigella Agar [Deben Diagnostic, Ltd.]; (6) Milk [Ultra Milk].

The research stage consists of: (1) culture of E. coli ATCC 25922 and S. typhimurium ATCC 14028 bacteria on Salmonella Shigella Agar (SSA) and LB media; (2) Inoculation of E. coli and S. typhimurium bacteria in milk samples (3); Isolation of DNA genomes of S. typhimurium and E. coli (4); Amplification using Real Time instrument PCR 7500 FAST [Applied Biosystems] includes (a) The primer confirmation test of the fimC gene and the pef gene in pure culture (b) The primer confirmation test of the fimC gene and the pef gene in milk sample (c) Negative Control (d) No Template Control. Preparation of the mixture reaction follows the standard protocol. Total volume in a single reaction of 20 μL, consisting of masters mix SYBR Green I, forward primer, reversed primer, bacterial DNA isolated or testing sample, and 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

Cultivation of S. typhimurium ATCC 14028 bacteria on selective media Salmonella Shigella Agar (SSA) showed the formation of black colonies on the surface of SSA media (Figure 1a), while culturing E. coli bacteria ATCC 25922 produced pink colonies (Figure 1b). The formation of dark and red colonies on SSA and media showed that the S. typhimurium and E. coli bacteria can grow [13]. These results are an indicator that the stage of bacterial growth has been successfully carried out.

One single colony of E. coli bacteria ATCC 25922 and S. typhimurium ATCC 14028 from SSA media was re-cultured in the liquid medium of Luria Bertani (LB) broth, which served to enrich the bacterial growth because it contained sources of peptides and peptones needed by bacteria to multiply...
Bacterial cultures that have been grown on LB media are sequential diluted starting from $10^{-1}$ until $10^{-6}$. The results of serial dilution were planted using the spread plate method on agar media and counted the number of bacteria growing in Colony Forming Unit (CFU)/mL used total plate count method [15,16]. In bacterial suspension $10^{-6}$ produce suitable colonies recommended by FDA BAM (Food and Drug Administration Bacteriological Analytical Manual) are producing 71 colonies of *E. coli* bacteria and 187 colonies of *S. typhimurium* bacteria. By calculation Total Plate Count, the concentration of *S. typhimurium* ATCC 14028 in uncontaminated (non-dilute) cultures was identical to $187 \times 10^5$ CFU/mL while the *E. coli* concentration of ATCC 25922 in pure (non-dilute) culture is equal to $71 \times 10^5$ CFU/mL. The number of colonies obtained is afterwards used as a standard for contaminating milk samples. The milk is later contaminated with a suspension of *S. typhimurium* ATCC 14028 with concentration $187 \times 10^5$ CFU/mL and *E. coli* ATCC 25922 with concentration $71 \times 10^5$ CFU/mL. Each milk contaminated with bacteria is used as a test sample.

![Figure 1. Cultivation of *E. coli* dan *S. typhimurium* bacteria on SSA media. (1a) Bacterial culture *S. typhimurium* in SSA media produces black colonies. (1b) Bacterial culture *E. coli* in SSA media produces pink colonies.](image)

One millilitre of prepared sample includes positive control (pure culture), test sample (Milk contaminated by bacteria), and negative controls were isolated using a commercial kit QIAamp® DNA Mini Kit [17]. Isolation of bacterial genomic DNA with this kit includes the stages of lysis, binding (precipitate), washing and elution [18]. The result from the DNA isolation process is 200 µL colourless DNA. After that the DNA storage at -20°C which is aims to keep the isolated DNA from being damaged and stored for a long time [19]. The purity of DNA samples was measured based on an absorbance ratio of 260/280 [20]. Quantitative test results through measurement of DNA concentration and purity using GE Nanovue Uv-vis Spectrophotometer shown in Table 1.

|                | *E. coli* ATCC 25922 | *S. typhimurium* ATCC 14028 |
|----------------|---------------------|----------------------------|
|                | Purity ($A_{260/280}$) | Concentration (ng/µL) | Purity ($A_{260/280}$) | Concentration (ng/µL) |
| Positive Control | 1,822               | 24,5                      | 1,93                     | 24,75                   |
| Test Sample     | 1,949               | 23,0                      | 1,89                     | 24,17                   |
| Negative Control| 1,980               | 19,5                      | 1,92                     | 20,5                    |

Based on the data shown in Table 1, it can be seen that DNA isolates from positive controls, negative controls and test samples of *E. coli* and *S. typhimurium* bacteria produce a good ratio of absorbance or purity because they have purity range of 1.8-2.0. Comparison of the absorbance shows pure DNA isolates this can indicate that the cell lysis process for the DNA isolation runs perfectly. If the ratio of
absorbance produced is less than 1.8, it means that the DNA isolated is still mixed with protein and if
the ratio of absorbance produced is more than 2.0 mean that there is an impurity in the form of RNA
[21]. The results of measurements of DNA concentration in the three samples had different DNA
concentration values (Table 1). The DNA concentration values obtained start from 19.5 ng/mL to 24.75
ng/mL. The difference in DNA concentration values from each sample of genomic DNA isolation is
caused by several factors, one of which is the homogenization factor with the solutions used for DNA
extraction [22]. The DNA isolated is one essential component into the Real Time PCR process.

The results of confirmation test of *E. coli* fimC gene and *pef* *S. typhimurium* gene primers in milk
samples are shown in Figures 2 and 3. The amplification curve showed that the fimC gene primer
successfully amplified the *E. coli* DNA isolates present in pure culture and milk contaminated. The same
results were also shown in the primer *pef* gene which could amplify *S. typhimurium* DNA isolates in
uncontaminated culture and contaminated milk samples.

The primer confirmation results of the fimC gene in the three samples showed an increase in the curve
past the threshold with a Ct value of 14, 45; 14, 98; 26, 53; 27, 43. From Figure 2, it can be seen that
the positive control of the *E. coli* bacteria ATCC 25922 appeared earlier (Ct 14, 45) from the
contaminated milk test sample (Ct 14.98). This is consistent with the theory that the lower the achieved
Ct value indicates the more DNA templates of the target genes that have been successfully amplified
[23]. The proximity of the green line (positive control) and red line (test sample) illustrates that *E. coli*
bacteria ATCC 25922 can live in milk samples so that the bacterial DNA that is extracted can be detected
in Real Time PCR.
Negative controls that are milk without bacteria, and No Template Control (NTC) are included into the process to help determine whether there are contamination factors that occurred during the study. The presence of contamination in the reaction mixture is characterized by the emergence of the NTC at the beginning of the cycle. In Figure 2 it appears that negative control and NTC, especially at the end of the cycle at Ct 26.53 and 27.43. The difference in Ct value between positive control and NTC is 12.98. These results are in accordance with the literature where the difference with the Ct value between positive control and NTC is very far. The appearance of amplification can be ignored if the difference in Ct is more than 10 cycles [24]. In a previous study, it was also known that the E. coli fimC primer had a Tm value of 78.65°C with sensitivity in detecting E. coli ATCC 25922 to DNA concentration 7.12 pg/µL [12]. It was concluded that this fimC primer could distinguish whether there was E. coli DNA ATCC 25922 in milk samples using the method.

The primer confirmation results of the pef gene in the three samples showed an increase in the curve past the threshold with successive Ct values of 13, 56; 13,933; 24, 83; 26, 03. It is the same as the fimC gene primer, which in Figure 3 shows that positive control of S. typhimurium ATCC 14028 bacterial culture appears earlier (Ct 11,56) from contaminated milk test samples (Ct 13,933). The proximity of the blue line (positive control of S. typhimurium ATCC 14028) and the red line (test sample) illustrates that the S. typhimurium bacteria are able to live in milk samples. Negative control and NTC on primary peers also appear in the last cycle.

![Amplification Plot](image)

**Figure 3.** Test confirmation of the *pef* primer gene in pure culture *S. typhimurium* ATCC 14028 (blue line), Milk contaminated with *S. typhimurium* (red line), Negative control (purple line), No template control (green line).

The appearance of amplification can be ignored if the difference in Ct is more than 10 cycles [24]. In a previous study, it was also known that the *pef* *S. typhimurium* primer had a Tm value of 82.68°C with sensitivity to detect *E. coli* ATCC 25922 to DNA concentration 0.284 pg/µL [12].
4. Conclusion
Based on this study it can be concluded that the fimC primer could detect *E. coli* ATCC 25922 and the pef primer could detect *S. typhimurium* ATCC 14028 in milk samples. From these results, both primers can be used as references to be used as detection of bacteria in food samples, especially in the case of food poisoning.

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