Validation of the Detection Kit for Pathogenic Bacteria *Salmonella typhi* Causes Food Poisoning with Real Time Polymerase Chain Reaction

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Abstract. *Salmonella typhi* is a gram-negative bacteria that causes food poisoning. *Salmonella typhi* bacteria can cause typhus, which can produce lipopolysaccharide complex endotoxin, which plays an important role in the pathogenesis oftyphoid fever. Cases of food poisoning are still common and are one of the causes of death and death in Indonesia. In 2019, there were 77 cases of food poisoning in Indonesia. A fast, accurate, and specific detection method is needed to detect poisoning. In previous studies, the optimum conditions and formulas for detecting these bacteria have been obtained using Real Time PCR. The results of previous studies are used as the basis for the development of a prototypedetection kit. In this study, validation was carried out, which aimed to confirm the results of previous studies so that a reproducible and accurate product for the detection of *Salmonella typhi* bacteria could be obtained. The results of this study showed that fim-C primers for *Salmonella typhi* were amplified at 95 base pairs (bp) with an annealing temperature of 60ºC and a standard DNA concentration of 50 ng/µL. The results of the Real Time PCR confirmation test of *Salmonellatyphi* bacteria at a pre-denaturation of 3 minutes with a concentration of 10 pmol obtained the Ct value according to thestandard with previous studies. The Ct value obtained was 13.96 for *S. typhi* bacteria. Based on the results obtained, it can be conclude that the condition validation stage for pure cultures was successfully carried out by producing consistentand reproducible data.

1 Introduction

The General Center for Food and Drug Administration reported 77 food poisoning cases in Indonesia in 2019, exposing 7,244 people [1]. Food poisoning cases can be caused by microorganisms,one of which is the *Salmonella typhi* bacteria. *Salmonella typhi* is a pathogenic bacteria that is classified as gram-negative bacteria that cancause typhus in humans and is transmitted through food or drink [2][3]. *S. typhi* bacteria can spread through contaminated foods or drinks. After entering the body, these bacteria will spread through the blood to the lymph glands, intestines, liver, and spleen, where they will multiply [4] Most cases of food poisoning caused by Salmonella bacteria affect children under 5 years of age, which equates to 93.8 million cases worldwide [5].

In Indonesia, many cases of food poisoning occur due to food made in households, services, factory processing, restaurants, households, and unlisted household industries [1]. In addition to bacteria, viruses and parasites, food poisoning can be caused by improper food storage, improper cooking and food contamination [6] . *Salmonella, E. coli, Campylobacter, Shigella, Listeria, Vibrio, Yersenia,* and *Staphylococcus* are common bacteria that can cause food poisoning [7].

Researchers have developed a variety of techniques to identify cases of food poisoning, but these incidents are still responsible for many deaths and painful illnesses in Indonesia. They are a very significant problem that can lead to unusual cases of food poisoning [3]. To find the source of food poisoning and lower the risk of death, this calls for swift, precise, and focused detection procedures. Real-time polymerase chain reaction techniques were used in the short-mixing methods based on nucleic acid research, that is,

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approaches that use template hybridization processes with primary or short nucleotide strands.

This study is part of the UNJ Salmonella research team. Previous research has successfully detected *Salmonella typhi* bacteria using a real-time polymerase chain reaction method. The principle of real-time PCR is that the amount of DNA can be measured through fluorescent signals, which can enhance the fluorescence signal and are directly proportional to the amplification of the DNA amount [8][9].

The results obtained in previous studies were successful in designing specific primers for *Salmonella typhi* and *Salmonella typhimurium* bacteria[10][11]. In this research, formula validation will be carried out to produce a prototype detection kitto detect *Salmonella typhi* bacteria, which cause food poisoning. Validation aims to confirm and improve the results of previous studies so that a reproducible product is obtained.

2 Material and Methods

2.1 Bacterial Culture

Culture of *Salmonella typhi* bacteria on Blood Agar media using a specific medium, namely Salmonella Shigella Agar. Bacterial culture was carried out by streaking a zig-zag (streak plate) with an ose needle and instilling an ose of bacteriaon glycerol stock into Blood Agar media. then incubated for 1 day at 37 ºC (overnight culture); after incubation, a colony of S. typhi bacteria was formed.

After that, the single colonies that formed on the agar medium were taken with a sterile loop anddipped into 10 mL of liquid medium, the liquid medium used is lactose broth. then incubated at 37ºC for 18 hours (overnight culture) with a shaker speed of 150 rpm until it produced turbidity.

2.2 Isolation of Salmonella typhi bacterial DNA

Isolation of DNA from pure cultures of bacteria was carried out using the commercial. The process of isolating gram- negative bacteria follows the protocol contained in the handbook kit. The DNAse membran combine micro spin technology with the binding characteristics of a silica gel based membrane. The buffer conditions used in the DNeasy extraction technique are created to enable the adsorption of DNA specific to the silica-gel membrane and to provide the best possible removal of carbohydrates, polyphenols, and other plant metabolites [12]. After that, the isolated results were characterized by agarose gel electrophoresis and quantified using a nanodrop spectrophotometer.

2.3 Validation of the annealing temperature of the fimC gene of*Salmonella typhi* bacteria by PCR gradient

Primer annealing temperature validation was carried out using a Gradient PCR instrument. This process was carried out at an annealing temperature of 60 ºC according to previous studies. At this stageit starts from making a recipe for the gradient PCR amplification process with a total volume of 25 µL of one reaction mixture containing a pair of primers (forward and reverse) of the fim-C gene, a template of bacterial DNA isolate *S. typhi* Colorless Master Mix and Nuclease Free water (NFW). The amplification results were characterized by 2% agarose gel electrophoresis and the DNA bands wereobserved with a UV transllluminator.

2.4 Validation of the confirmation test for the fimC gene of *Salmonella typhi* bacteria by real-time PCR

Confirmation tests were carried out using pure cultured DNA isolates of *S. typhi* bacteria as a positive control and NTC (No Template Control) asa negative control. A mixture of positive and negative control reactions containing 20 µL each was made, which contained the master mix, primers (forward and reverse), bacterial DNA isolates, and Nuclease Free Water (NFW). However, negativecontrols were made without DNA isolates.

The real-time PCR program used included initial denaturation at 95°C for 3 minutes, denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds, and repetition for 40 PCR cycles. However, this study varied the predenaturation or initial denaturation time from 3, 4, and 5 minutes and varied the primary concentrations from 5, 10, and 15pmol.

3 Result and Discussion

3.1 Bacterial Culture

Bacterial culture on agar media is the first step in this research. *Salmonella typhi* bacteria werecultured on Salmonella Shigella Agar media. Culture on SSA media resulted in the formation of black colonies. This happens because SSA media contain bile salts, brilliant green, and sodium citrate to inhibit the growth of gram-positive bacteria. In addition, Salmonella bacteria cannot ferment lactose, but can reduce sodium thiosulfate in SSA media to produce sulfite and H_2S gas [13]. The resultscan be seen in figure 1.

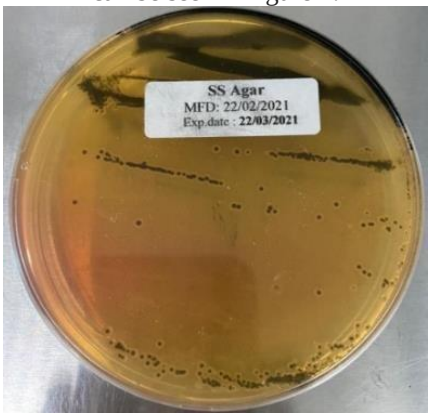

Fig. 1. Culturing bacteria on Salmonella- Shigella agar media

Lactose broth is the medium used after successful culture on agar and then culture on liquid media. Lactose is one carbohydrate that can differentiate to produce coliform. Gram-negative basil and optional aerobic and anaerobic spores are members of the coliform group. They digest lactose with gas production in 48 hours at a temperature of 35 ºC [14] .The result that is formed in liquid media is turbidity on the media, which indicates that the bacteria growing successfully [15]. The purpose of culturing in liquid media is to enrich the growth of bacteria so that it can easily isolate bacterial DNA.The results can be seen in figure 2.

Fig. 2. Culturing bacteria on Lactose Broth

3.2 DNA Isolation

DNA isolation aims to obtain pure DNA isolates without contamination of protein, RNA, or other components. The results of DNA isolation were subjected to quantitative analysis using a nanodrop spectrophotometer, to determine the concentration and purity of the isolated DNA. The concentration and purity of DNA isolates can be seen in Table 1.

Table 1. Results of nanodrop spectrophotometer measurements

Sample	Concentration (ng/µL)	Purity (A260/280)
Salmonella typhi	49	1,837

Based on the table above, the purity of the resulting DNA isolates was 1.837. The purity of DNA isolates is said to be good, when the purity value is between 1.8-2.0 if the purity is below 1.8, then the isolate is contaminated with protein, if the purity is above 2.0, then the isolate is contaminated with RNA then the concentration of DNA used for real-time PCR testing has a minimum concentrationof 0.1–500 ng [16]. The isolation results were then characterized by electrophoresis on 2% agarose, which was used to determine whether or not there was bacterial DNA present [17]. The results of agarose electrophoresis can be seen in Figure 3.

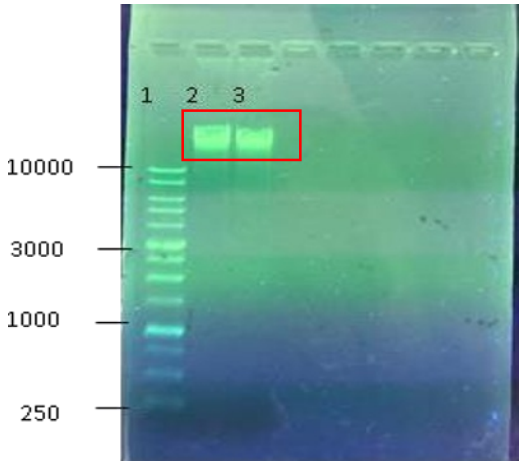

Fig. 3. Agarose Electrophoresis: (1) DNA ladder 1 kb; (2) Bacterial DNA isolates of *S. typhi*; (3) Bacterial DNA isolates of *S. typhi*.

Based on the results of the above agarose gel electrophoresis a band was formed above the 1 kb ladder. This is due to the larger genome size of *Salmonella typhi* 5 x 10^6 [18]. Based on these results,it can be stated that bacterial DNA isolation has been successful

3.3 Validation of the annealing temperature of the fimC gene of*Salmonella typhi* bacteria by PCR gradient

In previous research, annealing temperature optimization has been carried out using gradientPCR with a temperature range of 56–61ºC. The selection of this temperature is based on the melting temperature when the primer is synthesized, and usually the temperature range for this optimization is 5ºC below the Tm of the primer[8][19][20]. The optimum temperature for *Salmonella typhi* bacteria in thistemperature range is 60ºC [10]. The results of validation annealing temperature can be seen in Figure 4.

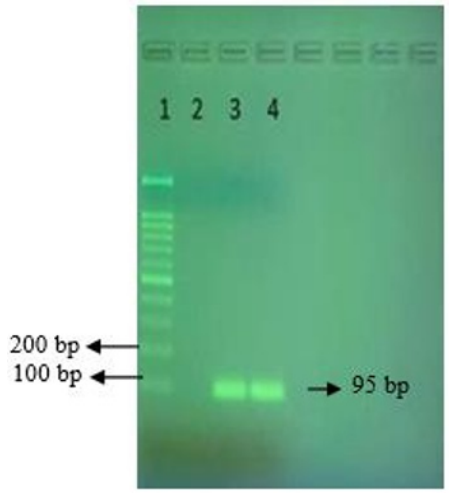

Fig. 4. Result of validation annealing temperature(1) DNA Ladder 100 bp; (2) NTC;(3)(4) Fragmen fim-C gene of bacterial *S. typhi*.

Based on the results obtained, it can be seen that there is a band on the fim-C gene fragment of *Salmonella typhi* bacteria with an amplicon size of 95 bp at 60 ºC. From these results it can be concluded that the annealing temperature validation is in accordance with the results of previous studies which showed that there was aband with an amplicon size of 95 bp [10].

Validation of the annealing temperature is necessary because it is a step that influences the success of the PCR amplification process. In addition, this step is also used for attachment of primers to the template DNA, so this attachment process requires optimum timing, the temperature is too high, it will cause amplification failure because the primer does not stick to the template DNA; if it is too low, it will make the primer stickto the other side of the genome, resulting in DNA that is formed having low specificity[19].

3.4 Validation of the confirmation test for the fimC gene of *Salmonella typhi* bacteria by real-time PCR

The confirmation test aims to determine whether or not the primer is capable of detecting and amplifying the target bacteria. Confirmation tests have been carried out in previous studies witha predenaturation time of 3 minutes and a primaryconcentration of 10 pmol. In this research, optimization of pre-denaturation time and primary concentration was carried out, optimizing the variations of pre-denaturation time used, namely 3 minutes, 4 minutes, and 5 minutes, while also optimizing the variations of primary concentrations used, namely 5 pmol, 10 pmol, and 15 pmol. The validation results of the confirmationtest with variations in predenaturation time and variations in primary concentration are shown in Figures 5-7.

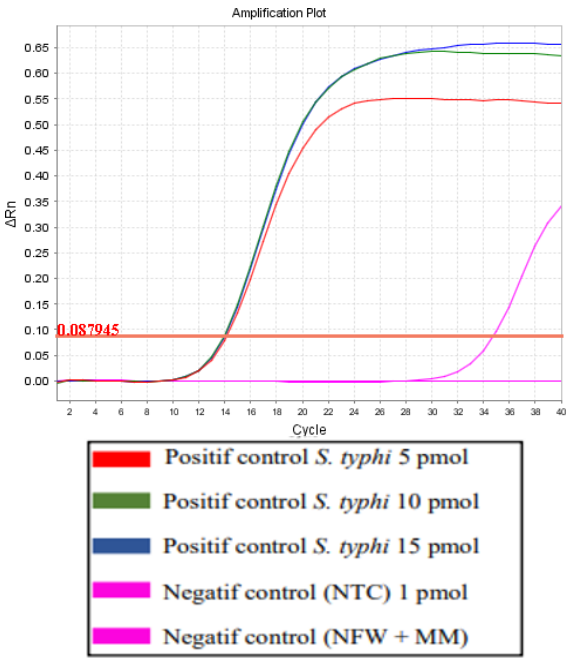

Fig. 5. Result of confirmation test Pradenaturation 3 minutes.

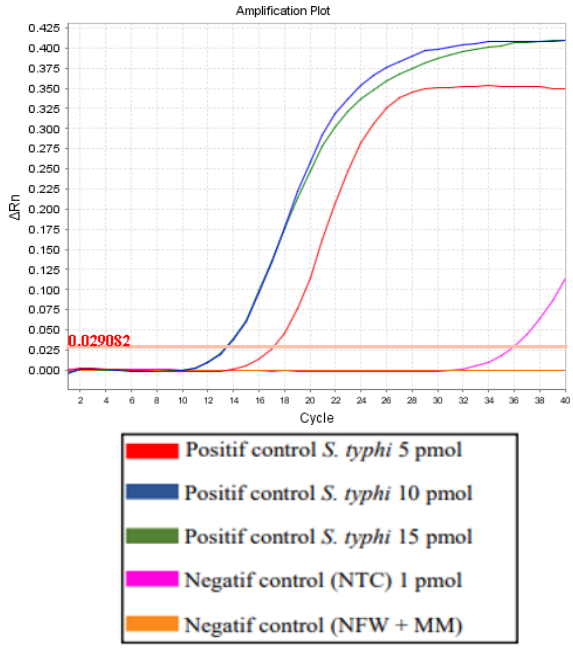

Fig. 6. Result of Confirmation testpradenaturation 4 minutes

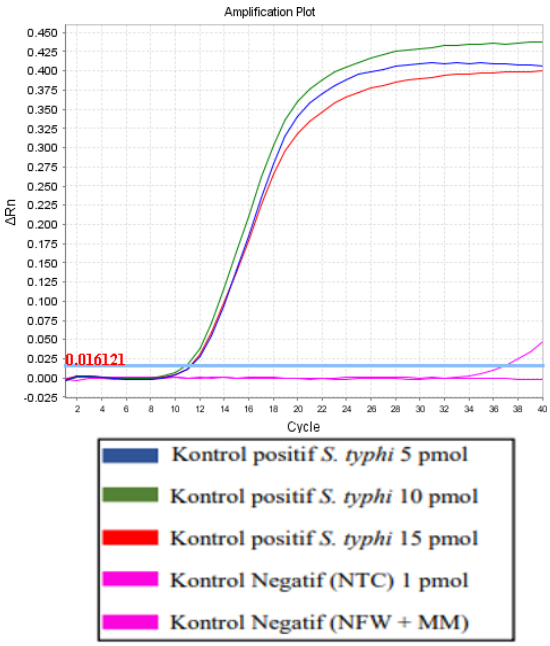

Fig. 7. Result of Confirmation test Pradenaturation 5 minutes.

Based on the results obtained by varying the primer concentration and predenaturation time, the amplification process in real-time PCR produces a stable curve and the optimum conditions for each. but to make a detection kit product, of course, it is necessary to streamline the time and materials to be used, so the results of 3 minutes of predenaturation with a concentration of 10 pmol will be used as a reference and compared with the results in previousstudies. The Ct value obtained in the previous studywas 14.35, with a negative control Ct value (NTC) of 32.63 [10]. The Ct value obtained from the results of 3 minutes of predenaturation validation and 10 pmol of concentration was 13.96, with a negative control Ct value (NTC) of 34.76. Shifts in CT valuescan indeed

occur; ideally, the dynamic range for real-time PCR is 7-8 fold for plasmid DNA, and a range of 3-4 for genomic DNA [8]. However, the Ct value was also found in the negative control (NTC),while the negative control NFW and master mix didnot amplify. but the curve can be ignored if the negative control CT is more than 10 positive controlcycles [9]. From the ct value data obtained, it can be said that the validation using real-time PCR was declared successful and reproducible.

4 Conclusion

Based on the results obtained by validating the annealing temperature with gradient PCR, the same results were obtained in the previous study, namely, that there was an amplification band at 95 bp at 60°C. A validation confirmation test showed that the primer was able to recognize the target DNA, resulting in amplification, which was indicated by the presence of Ct values and shifts in Ct values obtained in previous studies with validation results not too far away, so this validation can be said to be successful and reproducible.

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