Primers Design and PCR Optimization for Developing *Salmonella* Sp. Detection Method on Refillable Drinking Water Sample

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Abstract. One of the parameters of drinking water quality is not contaminated by coliform bacteria such as *Salmonella* sp. This bacteria can infect humans through oral routes causing infection of the gastrointestinal tract (*Salmonellosis*). Generally, analysis testing of coliform bacteria in water can be done using microbiological methods. However, microorganisms contained in refillable drinking water are usually in very small quantities and cause errors in sampling or in the calculation of the number of microorganisms. Testing with PCR technique can be used because it offers different advantages. PCR is specific, sensitive, fast and accurate to detect small amounts of target nucleic acids in drinking water samples. The study aims to obtain a Salmonella-specific PCR primer sequence for developing detection methods of refillable drinking water samples contaminated with *Salmonella* sp. And optimize the PCR condition using genomic DNA of *Salmonella* sp. as a template. Primer was designed using Geneious Prime and the specificity was checked using Primer BLAST on NCBI (National Center for Biotechnology Information). Meanwhile the PCR optimization was conducted using gradient PCR. The results of this study were Salmonella-specific PCR primer forward 5'-CCGTCTTATCTTGATTGAAGCCG-3' and reverse 5'-CGTCATGATATTCCGCCCCA-3' that amplified *InvA* gene of *Salmonella* sp. With amplicon size of 559 bp. The optimum PCR condition was the PCR program using annealing temperature at 59,5 °C.

Keywords: Refillable drinking water · Salmonella sp. · Primer Design · Gradient PCR

1 Introduction

Water is a very important chemical compound in the human body. For humans, the most important water requirement is drinking water [1]. Human needs for drinking water consumption are increasing as the population increases, while the quantity and quality of drinking water is getting less [2]. The fulfillment of urban drinking water needs that continues to increase makes the Refillable Drinking Water Depot (DAMIU) an alternative source of drinking water by the community [3].

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Drinking water at a safe and healthy Refillable Drinking Water Depot (DAMIU) must meet physical, chemical and bacteriological qualities [4]. Based on the Decree of Surat Keputusan Menteri Kesehatan No.907/Menkes/SK/VII/ 2002, one of the parameters of drinking water quality that is worth consuming is not polluted by coliform bacteria such as *Salmonella* sp. Coliform bacteria such as *Salmonella* sp. Can infect humans through the oral route thus causing infection of the digestive tract (*Salmonellosis*) [5].

Salmonella sp. is one of the coliform bacteria that cause systemic infections in the long term. The genus *Salmonella* sp., included in the family Enterobacteriaceae, is a Gram-negative bacterium, has no spores, anaerobic basil, motile, facultative intracellular nature. The genus consists of two species, *Salmonella enterica* and *Salmonella bongori* [6]. In general, organisms belonging to the genus Salmonella are the source of a wide variety of infections, ranging from mild to severe gastroenteritis such as typhoid fever and bacterimia [7].

Salmonella sp. Has the *InvA* gene that causes pathogenicity [8]. *InvA* gene in *Salmonella* sp. is located on chromosomes that are able to produce proteins that can provide invasive properties to epithelial cells found in the intestine. The presence of this *InvA* gene causes *Salmonella* sp. Become pathogenic in the human body [9]. *Salmonella* genomic DNA consists of 4.86 Mb chromosomal DNA (NC_003197.2) and 0.09 Mb plasmid DNA (NC_003277.2). *Salmonella* sp chromosome DNA contains 4,605 genes, while the plasmid DNA contains 109 genes [10].

Testing of pathogenic microbes in water generally can be done using microbiological methods. In microbiological methods microorganisms contained in refillable drinking water are usually in very small amounts, causing errors in the detection testing of microorganisms contamination. Culture techniques used for the identification of pathogenic microorganisms in refillable drinking water take a long time and it is difficult to grow microorganisms in differential selective media. False negative results are often obtained at the beginning of contamination and take several days to give a positive culture result [11, 12]. In addition to microbiological tests, detection of pathogenic microbes in refillable drinking water can also be done molecularly using the PCR (Polymerase Chain Reaction) technique [13].

Testing to detect pathogenic microbes performed molecularly using the PCR technique offers a distinct advantage because it is specific, sensitive, fast and accurate enough to detect small amounts of target nucleic acids in drinking water samples [11]. Kary Mullis, the inventor of the technique, published and patented a gene replication method called "PCR" (Polymerase Chain Reaction) in 1985. There are three stages to PCR techniques: denaturation, annealing and extension [15]. The successful amplification of DNA fragments is influenced by several factors namely *DNA template, primer,* $MgCl_2$*, DNA Polymerase Enzyme*, temperature, time and number of cycles [16].

For PCR techniques to work specifically, effectively and accurately in detecting coliform bacteria such as *Salmonella* sp., one of the most important things to do is design a primer. A primer is a single-strand oligonucleotide molecule consisting of 18–30 bases. Primer design is done specifically because the primer is a determining factor in the success of amplification and DNA sequencing. The primer design should pay attention to the primer Tm in the range of 52-58^{0}C, with the tolerance of Tm difference between primers being 3-5^{0}C. Primers also have GC bases in the range of 30–60%, and

avoid primers that form self-dimers and hairpins. Self-dimer and hairpin are secondary structures resulting from primer intermolecular and intramolecular interactions that can interfere with PCR results [17, 18].

Primer design is done because many publications of less than optimal commercial primer designs such as primers lack specificity and can form secondary structures such as dimers and can only be hybridized within a limited temperature range. Commercial primer use has likely not been validated and has not been optimally tested. Commercial primers also cannot be ascertained that a set of such primers will produce the same results under different experimental conditions because testing performance can vary depending on what extraction methods are used to purify DNA, the reagents used for PCR reactions and the type of thermal cycler used for testing. Therefore, primer design is performed to ensure testing performance by conducting optimal validation experiments. This can save time, cost, and help avoid inconsistent process failures or experimental data [19].

In some studies, testing using commercial kits on Salmonella with the target *InvA* gene using 3 pairs of primers failed to amplify all strains of Salmonella. Both commercial rapid detection kits analyzed failed to detect *Salmonella enterica* on 1 log unit above the predicted LOD, and required concentrations 1,000 to 100,000 times higher to be able to amplify. Another study that detected DNA *Salmonella* sp. Using a BoxAir primer obtained the result that the primer can amplify *Salmonella* sp. However, the BoxAir primer is not enough to optimize the DNA band formed because the use of BoxAir primer requires the purity of the sample in order for the DNA tape formed to have a good resolution [20, 21].

The primer to be designed for PCR is a pair of primers known as forward primer and reverse primer. Primer design is done in silico and BLASTING Primer using BLAST tools. BLAST is one of the features on the NCBI (National Centre for Biotechnology Information) website that serves to analyze whether there are primer similarities (specificity) that we will use with other genomes in the GenBank DNA database. BLAST in NCBI can be used to perform calculations of the length of the resulting product based on the forward primer and reverse primer positions on gene the *Salmonella* sp. [8].

This study was conducted to obtain forward primer and reverse primer for PCR that is specific to *Salmonella* sp. to develop pathogen detection in the sample of refillable drinking water. The designed primers were simulated in silico and optimized the PCR condition using genomic DNA of *Salmonella* sp.. This in silico study was conducted to obtain the accuracy of results and efficiency of work time allocation during in vitro studies. PCR optimization was done so that the primer can work well on in vitro tests.

2 Material and Methods

2.1 Material

The material used in this study was a complete genome sequence of *Salmonella* sp. (NC_003197.2) downloaded from GenBank NCBI (http://www.ncbi.nlm.nih.gov) as template sequence and genomic DNA of *Salmonella* sp. Which was extracted from culture of *Salmonella* sp.. The tools used was Geneious Prime Bioinformatics Software 2021.1.1. And thermal cycle engine for PCR processes.

2.2 Methods

Salmonella sp. Primers specific *InvA* target gene were designed to meet the ideal primer criteria of having nucleotide lengths ranging from 18–30 bases based on consideration of random combinations that may be found in a single genome sequence, having a base composition of G and C of 40–60%, Tm between primer forward and reverse not having a difference of more than 5^0C, and not forming a secondary structure (hairpin or self dimer). Primer candidates were checked using Geneious Prime software to ensure they have met the ideal primer criteria above.

Primer specificity that has been designed is checked using primer BLAST tools on the NCBI website. The purpose of this check was to ensure that the primer that has been designed will only amplify the specific target genes of *Salmonella* sp. The primer pair obtained was simulated with in silico PCR using Geneious Prime. Based on these results, the designed primers which already met the ideal primer criteria were then ordered and synthesized in IDT, Singapore.

PCR optimization was conducted using gradient PCR to determine the optimum annealing temperature. The gradient annealing temperature was calculated based on the Tm of forward and reverse primer. The PCR reaction with total volume of 10 μL consists of 1x MyTaq HS Red Mix (Bioline), 0.4 μM forward primer, 0.4 μM reverse primer, 50 ng template DNA and nuclease-free water up to 10 μL. The gradient PCR program was set as follow: initial denaturation at 95 °C for 1 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at gradient temperature (Tm ± 5 °C) for 15 s, and elongation at 72 °C for 10 s. PCR reaction was terminated by final elongation at 72 °C for 5 min. The PCR products were analyzed using 1,5% agarose gel electrophoresis.

3 Result and Discussion

3.1 Primer Design

The primer design of the study was conducted in silico using Bioinformatics software and Primer BLAST tools at NCBI (National Centre for Biotechnology Information). On the complete genome sequence of *Salmonella* sp. (NC_003197.2) region 3038407 – 3040471 and length of 2065 bp with target *InvA* genes downloaded from NCBI pages was carried out for forward and reverse primer design using Geneious Prime software. Geneious Prime is a user-friendly bioinformatics application that integrates genomic data analysis. This software can also be used to ensure the primer candidate has met the ideal criteria for amplification [22].

The primer obtained was a series of nucleotide bases that are attempted to have a short size to minimize costs. Primer length ranges from 18 to 30 bases, based on consideration of random combinations that can be found on a single genome sequence [18]. Primers with a length of more than 30 bases are not recommended, as they do not show higher specificity. In addition, a long primer can result in hybridization with other primers so as not to form the polymerization of the target DNA. Table 1 shows the forward primer length (Salmonella 258 F) measuring 23 bases and the reverse primer length (Salmonella 816 R) 20 bases which means it already has an ideal primer length so as to reduce the risk of mispriming.

The optimal melting temperature (Tm) for primer is in the range of 52–58 °C [11] and between 50–65 °C [18] generally gives better results than primers with lower TMs. Primers with Tm above 65 °C should also be avoided as they have the potential for secondary annealing. The Tm difference between the primer forward and the best reverse primer is no more than 50 bases. A difference of Tm of more than 50 will cause a decrease in the efficiency of the amplification process. Primer criteria results in Table 1. Showing a forward primer Tm of 59.8 °C and a reverse primer of 60.0 °C with a difference of Tm of no more than 50 which is only 0.2° so it is good for the amplification process.

%GC is an important characteristic of DNA and provides information about annealing temperature. Primers must have GC content between 45 and 60 percent [23]. For primers with a G/C content of less than 50%, it may be necessary to extend the primer sequence beyond 18 bases to keep the melting temperature above the recommended lower limit of 50 °C [11]. For repeated Guanine (G) bases should be avoided, as they may prevent complete strand dissociation and also reduce amplification efficiency [19]. Primer criteria results in Table 1. It has met GC percentages of 47.8% and 55.0% for forward primers and reverse primers.

A good primer design should take into account the amplicon structure and ensure that the primer target location is free of secondary structures, such as not to form hairpins. If there is a hairpin, the primer will fold back by itself and produce a priming event that can decrease the overall signal obtained. Both primers should not have a T nucleotide base at the 3' end because it can cause mismatches. The number of mismatches or mismatches at the 3'-primer end can also cause hairpins [18, 24]. The primer should not contain a nucleotide sequence that would allow one primer molecule to form bonds on two similar primers, to itself or to another primer used in PCR reactions (primer dimer formation). This can be a problem because the primer will tend to stick to each other, not with the target gene and this can reduce DNA concentration [25]. Table 1 shows that the primer has been designed according to the requirements to meet the ideal criteria because there are no hairpins or self dimers.

DNA fold analysis also showed that amplicons have no secondary structure in the primer attachment area (Table 1). DNA fold analysis is important to find out if there is a secondary structure at the primer binding site. The presence of secondary structures in this area can affect primer attachment to the target gene. This indicates that there are no interruptions in the primer attachment process. DNA fold is a feature in Geneious Prime software used to predict nucleic acid folds (DNA or RNA) and their hybridization [26].

Primer forward and primer reverse specificity were re-examined with Primer BLAST on the NCBI page. This was so that the primer design meets the ideal criteria and only amplifies *Salmonella* sp. Table 2 shows the results of the primer specificity that has been designed.

BLAST is a feature in the NCBI that analyzes primer specificity used with other genomes in the GenBank DNA database. BLAST in NCBI can be used to perform calculations of the length of the resulting product based on the forward primer position and reverse primer on the *Salmonella* sp gene sequence. From Table 2 it appears that the primer will only amplify *Salmonella* sp. And *Salmonella enterica*. The designed primers cannot amplify other types of bacterial DNA templates. Based on the results of testing

Table 1. Geneious Prime Output of Designed Primer Characteristic

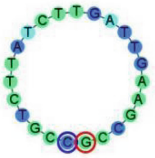
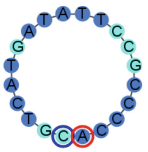
No	***Characteristics Primer***	***DNA Fold***	***Amplicon Size***
1	**Salmonella 258 F** **Sequence (5' to 3')**: CCGTCTTATCTTGATTGAAGCCG Type:Primer Length: 23 created by: primer3 %GC: 47,8 Tm: 59,8 Hairpin Tm: None Self Dimer Tm: None		559 bp
2	**Salmonella 816 R** **Sequence (5' to 3'):** CGTCATGATATTCCGCCCCA Type: Primer Length: 20 created by: primer3 %GC: 55,0 Tm: 60,0 Hairpin Tm: None Self Dimer Tm: None		

Table 2. Result of Primer Specificity Check Using Primer BLAST

Template Target	*Primer Salmonella F and Salmonella R*
Salmonella sp. Lignieres (1900)	Yes
Salmonella enterica Le Minor and Popoff (1987)	Yes
Shigella flexneri Castellani and Chalmers (1919)	No
Shigella boydii Ewing (1949)	No
Shigella sonnei Weldin (1927)	No
Shigella dysenteriae Castellani and Chalmers, (1919)	No
Escherichia coli Castellani and Chalmers (1919)	No
Legionella pneumophila Brenner dkk., (1979)	No
Vibrio cholerae Pacini (1854)	No
Klebsiella sp Trevisan (1885) emend. Carter dkk., (1999) emend. Drancourt dkk., (2001)	No
Enterobacter sp. Hormaeche and Edwards (1960)	No

at NCBI, the designed primers were specific to detect DNA sequence of *Salmonella* sp and *Salmonella enterica.*

Fig. 1. In silico PCR of Primer Salmonella 258 and Salmonella 816 of *InvA* sequences. Dark green arrow = forward primer; light green arrow = reverse primer.

3.2 In Silico PCR

To simulate the virtual amplification of genes with a designed primer pair, in silico PCR was performed using Geneious Prime. In silico PCR is a technique of using computer-based programs that rely on one or more theoretical heuristics or computational algorithms to predict or calculate the results of PCR amplification products, thus minimizing errors when PCR is performed in vitro [27].

PCR was performed for the primer pair with a sequence of *Salmonella* sp. Genomes. (NC_003197.2) using Geneious Prime software (Fig. 1). In silico PCR results showed the primer pair could attach to the *InvA Salmonella* sp gene. With region 3038407–3040471 and length of 2065 bp. The primer attaches to the 258–280 nucleotide for the Salmonella F primer and to the 797–816 nucleotide for the Salmonella R primer. This primer pair will amplify the *InvA* gene region that is on the chromosomal DNA, so that it will be obtained with an amplicon in size of 559 bp. These results are consistent with PCR product size predictions with Primer BLAST. The use of PCR in silico is to predict and simulate the attachment of the primer sequence on the DNA sequence template, so as to minimize errors when performing PCR in vitro.

3.3 PCR Optimization

At the PCR optimization stage, the optimal conditions for the PCR process need to be determined, including the optimal temperature at the time of the reaction [17]. In this study, gradient temperature annealing was carried out to optimize the stages of the PCR process. The right annealing temperature can produce optimal DNA bands for use in bacterial testing on drinking water samples. Annealing temperature is the temperature at which the primer will attach to the DNA template, the magnitude of the temperature can be calculated based on the melting temperature (Tm) value of each primer. The search for optimal conditions of annealing temperature is very important, as it relates to the specificity and sensitivity of PCR products [28]. High annealing temperatures can lead to primer bonds (self dimer and hairpin) resulting in less efficient PCR products. If the annealing temperature is too low it will keep the primer from sticking to the DNA in an nonspecific place [18].

PCR products from DNA *Salmonella* sp. (Fig. 2) for gradient annealing temperature showing positive amplification results. In general, DNA bands are consistently thick

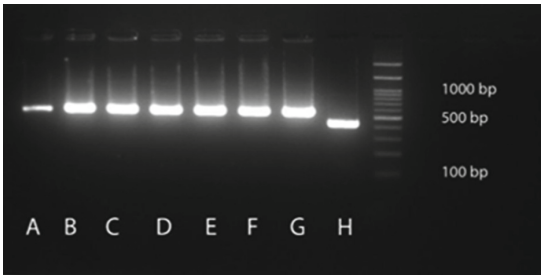


Fig. 2. Electropherogram resulting from *Salmonella* sp. DNA amplification with variations in annealing temperature. A: 59,5 °C; B: 58,7 °C; C: 56,7 °C; D: 55,6 °C; E: 53,3 °C; F: 52,5 °C; G: 50,5 °C; H:50,0 °C.

ranging from 50.0 °C–59.5 °C and the observed amplicon size is between 500–600 bp, corresponding to an amplicon length of 559 bp. This suggests that the primer can stick well to the target template using all the annealing temperatures tested. While in silico tests known Tm primer forward and reverse is at 59.8 °C and 60.0 °C. If the amplification product produced is equally good, in this case there is no secondary hybridization and the resulting DNA band is thick enough, then for further use the annealing temperature of 59.5 °C because it has a band size according to the target amplicon that is ±559 bp compared to others and classified as a high annealing temperature so that it will amplify the specific target, although the thickness of the band is thinner than other temperatures.

4 Conclusions

The designed primers were qualified as an ideal primer for the PCR amplification process. The primers sequence were 5'-CCGTCTTATCTTGATTGAAGCCG-3' as forward primer and 5'-CGTCATGATATTCCGCCCCA-3' as reverse primer. The primer pair has successfully detected the *InvA* gene region with a size of 559 bp according to primer BLAST results in NCBI and in silico analysis produced on Geneious Prime. PCR optimization results obtained the optimum temperature of 59.5 °C.

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