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Sensitivity and Specificity of Molecular-Based Approcach for Detecting Drinking Water Pathogen

Afifatul Achyar[1*], Irma Leilani Eka Putri[2], Dwi Hilda Putri[2]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences Universitas, Negeri Padang, Jl. Prof. Dr. Hamka, Padang, West Sumatra 25171, Indonesia

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Abstract

he existence of refillable drinking water depots helps the community to get affordable and practical drinking water. However, poor quality drinking water will, however, have an effect on health. One of the quality parameters of drinking water that is suitable for consumption is not contaminated by the bacteria *Escherichia coli*, *Salmonella* sp. and *Escherichia* sp. Measurement of the quality of drinking water, in addition to microbiological tests, can be carried out molecularly using PCR (Polymerase Chain Reaction) method. Therefore, the aim of this study was to examine the sensitivity and specificity of PCR for detection of drinking water pathogens. DNA was extracted from cultures of *E. coli*, *Salmonella* sp., *Escherichia* sp. and some non-coliform bacteria. PCR was performed separately using primer pairs of E. coli-AA-Forward and E.coli-AA-Reverse, Salmonella-OY-Forward and Salmonella-OY-Reverse, E. coli-DB-Forward and E. coli-DB-Reverse. The results of the PCR sensitivity showed that the minimum amount of DNA that can be detected by this method were 0.0025 ng/µL, 0,0005 ng/µL, 0,04 ng/µL for *E. coli*, *Salmonella* sp., *Escherichia* sp., respectively. The results of the PCR specificity of each primer pairs indicated that these methods were able to detect each coliform bacterium specifically according to PCR product size of ± 417 bp, ± 559 bp and ± 815 bp for *E. coli*, *Salmonella* sp., *Escherichia* sp., respectively.

INTRODUCTION

Drinking water is a primary human need in order to function properly. Adult humans need at least eight glasses of water a day. As the human population increases, the demand for drinking water also increases. This condition is an economic opportunity for home industries to provide refillable drinking water through the refill drinking water depot. The lifestyle of urban people who want to be practical causes refill drinking water to be in great demand (Agustia *et al*. 2019; Bambang *et al*. 2014; Wandrivel *et al*. 2012).

Although refilled water is thought to be more practical and affordable than bottled water, the physical, chemical, and microbiological quality of the water must still be assured. Based on the Decree of the Minister of Health No.492/MENKES/PER/IV/2010, one of the parameters of the quality of drinking water that is suitable for consumption is that it is not contaminated by *Escherichia coli* and total coliform bacteria, including *Salmonella* sp. and *Escherichia* sp. that are pathogenic to humans (Ministry of Health of the Republic of Indonesia, 2010).

*Coresponding Author: afifatul.achyar@fmipa.unp.ac.id
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Standards for testing the quality of drinking water for microbiological parameters are generally carried out through culturing the bacteria using Most Probable Number (MPN) testing (Cochran, 1950; Ahmed *et al*. 2013). However, when the number of bacteria present in the water sample is too small, it is often difficult to grow them on the culture medium and time-consuming (Nemati *et al*. 2016). In addition to microbiological tests, water analysis tests can be carried out molecularly, with PCR (Polymerase Chain Reaction) method. PCR is a molecular biology technique that has become the preferred method for rapid detection of bacterial DNA and requires a small number of samples (Sibley *et al*. 2012; Kralik and Ricchi, 2017). Therefore, this study was aimed to determine the sensitivity and specificity of PCR for pathogen detection of refill drinking water in order to obtain accurate results to determine pathogenic microbes contained in refill drinking water samples.

MATERIALS AND METHODS

From *E. coli*, *Salmonella* sp., and *Escherichia* sp. bacterial cultures, the genomic DNA of coliform bacteria was isolated. The genomic DNA of a number of non-coliform bacteria was also isolated from cultures of *Staphylococcus aureus*, *Bacillus substilis*, *Pseudomonas* sp., and *Pseudomonas mosselii*, which are held by the Microbiology Laboratory, Biology Department, Universitas Negeri Padang. PCR was carried out using three types of primers that would amplify the target gene in *E. coli*, *Salmonella* sp. and *Escherichia* sp. (Table 1).

Table 1. Primer List of Pathogen Detection PCR

Primer	Descriptions		
	Sequences	*Amplicon Size*	*Reference*
E.coli-AA-Fwd	5'-ATG CAG TGG TTC CTT ATC TCA CA-3'	417 bp	Putri *et al*., 2021
E.coli-AA-Rev	5'- ATC CTT AAT GGC ACT GCG CT-3'		
Salmonella-OY-Fwd	5'-CCG TCT TAT CTT GAT TGA AGC CG-3'	559 bp	Yuselman *et al*., in press
Salmonella-OY-Rev	5'-CGT CAT GAT ATT CCG CCC CA-3'		
E. coli-DB-Fwd	5'-GCT AAT GAA AAT GGC GCT GT-3'	815 bp	Purwakasih and Achyar, 2021
E. coli-DB-Rev	5'-AGC CGA CGG TTT GAA GTT AC-3'		

Genomic DNA from each bacterial culture was extracted using a boiling method. A colony was picked using sterile micropipette tip and mixed with sterile TE buffer pH 8.0. The mixture was incubated at 95 °C for 10 minutes using a heat block and centrifuged at 1000 rpm for 5 minutes. Supernatant which contained DNA was transferred to a new microtube. The DNA concentration and purity were measured using a nanophotometer.

Sensitivity of PCR was measured using serial dilution of DNA samples. Meanwhile, specificity of PCR was measured using various type of bacterial DNA such as coliform and non-coliform bacterial DNA. PCR was performed separately using primer pairs of E.coli-AA-Forward and E.coli-AA-Reverse, *Salmonella*-OY-Forward and *Salmonella*-OY-Reverse, *E. coli*-DB-Forward and *E. coli*-DB-Reverse.

The PCR reaction consist of 1x MyTaq HS Red mix Bioline, 0.4 µM forward primer, 0.4 µM reverse primer, 1 µL of template DNA and nuclease-free water was added to get a final volume of 10 µL. PCR condition was set as touchdownPCR as follows: initial denaturation at 95 °C for 1 minute, followed by 5 cycles consist of denaturation at 95°C for 15 seconds, annealing at 70°C for 15 seconds and elongation at 72°C for 10 seconds, followed by 10 cycles consist of denaturation at 95°C for 15 seconds, annealing start from 69°C to 60°C (decrement 1°C per cycle) for 15 seconds and elongation at 72°C for 10 seconds, then followed by 20 cycles consist of denaturation at 95°C for 15 seconds, annealing at 59.5°C for 15 seconds and elongation at 72°C for 10 seconds, and followed by final elongation at 72°C for 5 minutes. PCR products were analyzed using 1.5% agarose gel electrophoresis (Putri *et al*., 2021; Purwakasih and Achyar, 2021; Yuselman *et al*., in press).

RESULTS AND DISCUSSION

The PCR sensitivity test was carried out to determine the minimum limit of DNA concentration that could be detected. For the sensitivity test, the target DNA band would be at the same amplicon size but the thickness and clarity could be distinguished from each other. The sensitivity results (Figure 1-3)

show that the lower DNA concentration, the thinner and less clear the DNA band will be. *E.coli* sensitivity test electropherogram (Figure 1) show that template DNA at a concentration of 0.0025 ng/µL (M) yield a faint DNA band at ± 417 bp (Putri *et al*., 2021), meanwhile at a concentration 0.0013 ng/µL (N) the DNA band no longer visible. This indicated that the lowest concentration of *E. coli* DNA that could still be amplified was at a concentration of 0.0025 ng/µL (M). For *Salmonella* sensitivity test electropherogram (Figure 2), at a concentration of 0.0002 ng/µL (Q) the DNA band was no longer visible, this indicated that the lowest concentration of DNA that could still be amplified was at a concentration of 0.0005 ng/µL (P). Furthermore, the *E. coli* sensitivity test electropherogram (Figure 3) shows that the minimum concentration of template DNA was 0.04 ng/µL (J). These results indicated that the pathogen detection PCR method being developed in this study had high sensitivity. This is very important because generally the number of pathogens found in refill drinking water samples is very small.

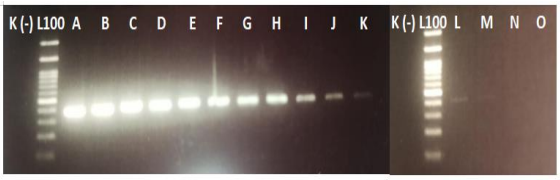

Figure 1. Electropherogram of *E. coli* PCR sensitivity test using serial dilution of *E.coli* DNA. The *E.coli* amplicon size is ± 417 bp. K(-): no template control; L100: DNA ladder 100 bp (Geneaid); A: DNA 10.15 ng/µL; B: DNA 5.08 ng/µL; C: DNA 2.54 ng/µL; D: DNA 1.27 ng/µL; E: DNA 0.63 ng/µL; F: DNA 0.32 ng/µL; G: DNA 0.16 ng/µL;H: DNA 0.08 ng/µL; I: DNA 0.04 ng/µL; J: DNA 0.02 ng/µL; K: DNA 0.01 ng/µL; L: DNA 0.005 ng/µL; M: DNA 0.0025 ng/µL; N: DNA 0.0013 ng/µL; O: DNA 0.0006 ng/µL.

The primer specificity was tested in vitro using the DNA of various types of bacteria classified as coliform and non-coliform bacteria. The coliform bacteria tested were *E.coli*, *Salmonella* sp., and *Escherichia* sp. Meanwhile, the non-coliform bacteria were *S. aureus*, *P. moselii*, *Pseudomonas* sp., and *B. subtilis*. Based on the electropherogram of the *E. coli* PCR specificity test (Figure 4), the target DNA was amplified only in the positive control (*E.coli* DNA), while the DNA of other bacteria was not detected. Thus, the PCR method used has worked specifically to only amplify *E. coli* DNA.

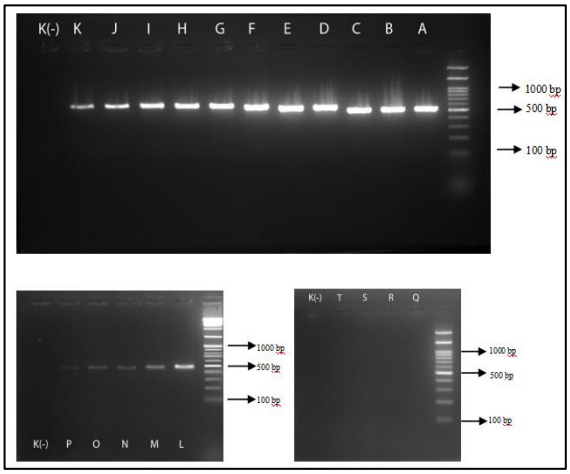

Figure 2. Electropherogram of *E. coli* sp. PCR sensitivity test using serial dilution of *Escherichia* sp. DNA. The *Escherichia* sp. amplicon size is ± 815 bp. K(-): no template control; L100: DNA ladder 100 bp (Geneaid); A: DNA 22.25 ng/µL; B: DNA 11.13 ng/µL; C: DNA 5.56 ng/µL; D: DNA 2.78 ng/µL; E: DNA 1.39 ng/µL; F: DNA 0.69 ng/µL; G: DNA 0.35 ng/µL;H: DNA 0.17 ng/µL; I: DNA 0.09 ng/µL; **J: DNA 0.04 ng/µL**; K: DNA 0.02 ng/µL; L: DNA 0.01 ng/µL

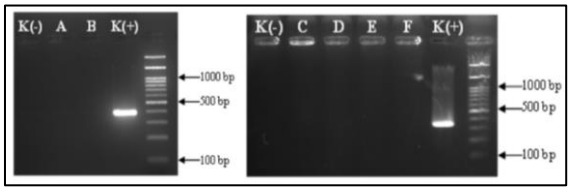

Figure 3. Electropherogram of *E. coli* PCR specificity test using various bacterial DNA. The *E.coli* amplicon size is ± 417 bp. K(-): no template control; A: *E. coli* sp. DNA; B: *Salmonella* sp. DNA; C: *S. aureus* DNA; D: *P. moselii* DNA; E: *Pseudomonas* sp. DNA; F: *B. subtilis* DNA; K (+): *E. coli* DNA; L100: DNA ladder 100 bp (Geneaid).

Based on the electropherogram in Figure 5, the results of electrophoresis visualization showed that the band was only found in the sample wells of the *Salmonella* sp. DNA PCR product. A suitable primer is an unique series of nucleotide bases in the DNA template, meanwhile it was not found in other sequences or locations in other DNA templates and the primer will amplify DNA and produce bands of a certain length (Abd-El-Haleem *et al*., 2003). Meanwhile, DNA from other Enterobacteria such as *E. coli* and *Escherichia* sp. and non-Enterobacteria such as *S. aureus*, *B. subtilis*, *P. moselii*, and *Pseudomonas* sp. There were no bands observed on the electropherogram. The electropherogram showed no detectable bands. This demonstrated that the *Salmonella* primer pair and PCR software were particularly designed to identify and amplify *Salmonella* sp. DNA. As shown in Figure 5, the

inconsistency of positive control (K+) amplicon size between the right and left gel electrophoresis occured even if the DNA templates were from the same source, this result was confirmed by sequencing (data not shown here) that both DNA bands were *Salmonella* sp. This fact can occur due to DNA binding dyes that are mixed directly with PCR products before loading the samples to the wells causing non-uniform migration of DNA.

Visualization of *E. coli* PCR product specificity test (Figure 6) showed that the DNA bands were only found in the wells of the *Escherichia* sp. DNA PCR product, while the DNA from other Enterobacteria and non-Enterobacteria such as *S. aureus, B. substilis, P. moselii,* and *Pseudomonas* sp. no bands were observed on the electropherogram. This indicates that the *E. coli* primer pair detects and amplifies the DNA of *Escherichia* sp. by applying the touchdown PCR program. Touchdown PCR was used as a method to determine the optimal annealing temperature of oligonucleotide primers and was able to reduce off-target priming and increase the specificity of PCR (Korbie and Mattick, 2008). Figure 6 shows the appearance of the light band on the *Salmonella* (lane B) and negative control (lane K (-)), this was caused by cross-contamination as a result of not using filtered-tips when performing PCR.

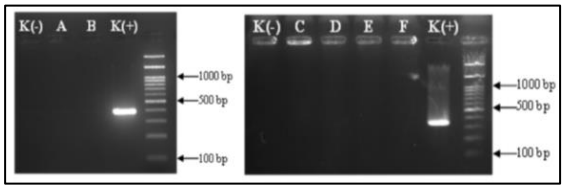

Figure 4. Electropherogram of *E. coli* PCR specificity test using various bacterial DNA. The *E.coli* amplicon size is ± 417 bp. K(-): no template control; A: *Escherichia* sp. DNA; B: *Salmonella* sp. DNA; C: *S. aureus* DNA; D: *P. moselii* DNA; E: *Pseudomonas* sp. DNA; F: *B. subtilis* DNA; K (+): *E.coli* DNA; L100: DNA ladder 100 bp (Geneaid).

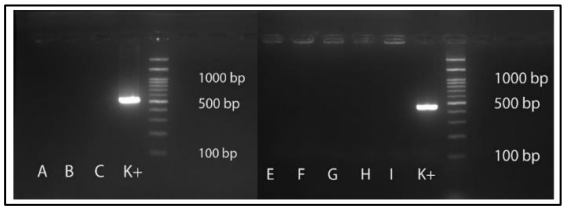

Figure 5. Electropherogram of *Salmonella* sp. PCR specificity test using various bacterial DNA. The *Salmonella* amplicon size is ± 559 bp. A & E: no template control; B: *E.coli* DNA; C: *Escherichia* sp. DNA; F: *S. aureus* DNA; G: *B. subtilis* DNA; H: *Pseudomonas* sp. DNA; I: *P. moselii* DNA; K (+): *Salmonella* sp. DNA; L100: DNA ladder 100 bp (Geneaid).

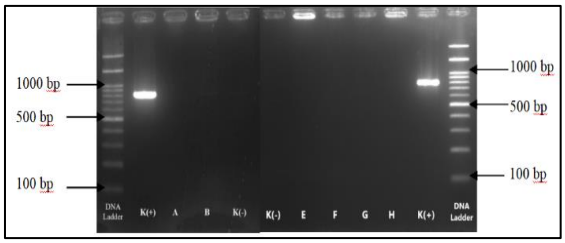

Figure 6. Electropherogram of *Escherichia* sp. PCR specificity test using various bacterial DNA. The *E. coli* amplicon size is ± 815 bp. L100: DNA ladder 100 bp (Geneaid); K (+): *Escherichia* sp. DNA; A: *E.coli* DNA; B: *Salmonella* sp. DNA; E: *S. aureus* DNA; F: *B. subtilis* DNA; G: *Pseudomonas* sp. DNA; H: *P. moselii* DNA; K(-): no template control.

PCR has been widely used as a tool for pathogen detection. However, the protocols used today must be further optimized to obtain truly specific results. To ensure a high specificity of detection, the specificity of the primer sequence to the target sequence should be thoroughly examined to rule out any major homology with other organisms (Sachse, 2004; Ruiz-Villalba *et al*.,2017; Achyar *et al*., 2021). In this case, *Escherichia* sp. and *E.coli* shared 99% similarity sequence, meaning that the nucleotide arrangement is very similar, generally preexisting *E. coli* PCR primer sequences can also amplify *Escherichia* sp. and vice versa (Putri *et al*. 2021; Purwakasih and Achyar, 2021). Nevertheless, the results of the PCR specificity test for all primer pairs in this study are in accordance with the results of the in silico primer specificity test conducted in previous studies (Putri *et al*. 2021; Yuselman *et al*., in press; Purwakasih and Achyar, 2021).

CONCLUSION

The PCR sensitivity test showed that the minimum amount of DNA that can be detected by this method were 0.0025 ng/µL, 0,0005 ng/µL, 0,04 ng/µL for *E. coli*, *Salmonella* sp., *Escherichia* sp., respectively. The PCR specificity test of each primer pairs indicated that these method were able to detect each coliform bacteria specifically according to PCR product size of ± 417 bp, ± 559 bp and ± 815 bp for *E. coli*, *Salmonella* sp., *Escherichia* sp., respectively.

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