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VALIDATION OF GENETIC MARKERS FOR AmpC, AAC, AND RNR3 GENES IN POTENTIALLY PRESENT PATHOGENS ORGANISMS IN FECAL SLUDGE

Ummu Syauqah Al Musyahadah[1]*, Radhiyah Mardhiyah Hamid[2], & Alricha Nasir[3]
[1]Bioinformatics Study Program, Health Technology Faculty, Megarezky University, Antang Raya Street, Makassar, South Sulawesi 90234, Indonesia
[2]Bioinformatic Research Center, Institut Bioinformatika Indonesia, Sarimadu Housing B3 Number 3A, Malang, East Java 65162, Indonesia
[3]Environmental Engineering, Civil Engineering Department, Tadulako University, Soekarno Hatta Street Km. 9, Palu, Central Sulawesi 94148, Indonesia
**Email: syauqah.almusyahadah@unimerz.ac.id*
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ABSTRACT: The issue of sanitation and water pollution due to fecal waste has become a serious concern in Indonesia, particularly after it was revealed that approximately 70% of the community's drinking water sources are contaminated with human feces. Determining safe waste management requirements necessitates indicators confirming the contamination-free nature of the waste management methods. Pathogen testing indicators for fecal waste are highly diverse. The current standard testing is the culture-based method for microorganisms and microscopy observation for helminths. However, molecular detection tools for these pathogens can give higher sensitivity and specificity in a relatively shorter time of technical processing. In this research, we designed primers for molecular-based diagnostics on three pathogens exhibiting extreme survival capabilities under various environmental conditions. The species-coupled genes are AmpC for *Escherichia coli*, AAC for *Mycobacterium tuberculosis*, and RNR3 for *Ascaris lumbricoides*. The design of molecular-based biomarkers and their specificity tests were conducted using web-based bioinformatics programs to prevent unintended dimers. Ultimately, primers were validated by conventional gradient PCR on available positive controls and a sample from processed fecal sludge. The PCR validation test showed that the designed primers were validated to amplify the intended target with the right size without unspecific bands on the positive controls. Meanwhile, the AAC gene for *E. coli* was detected in the sample from the fecal sludge.

Keywords: Pathogen, PCR, Web-Based Bioinformatics Programs, Oligonucleotide, Molecular.

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INTRODUCTION

Fecal sludge is waste that is continuously increasing along with population growth. The population growth rate supports the urgency of further waste management because, gradually, it will lead to excess capacity at the Waste Treatment Plant (IPLT). Excessive IPLT capacity can be indicated by fecal sludge overflowing from the holding pond and will impact pollution. The case is currently occurring and can potentially occur in several IPLTs in Indonesia (Sinthawaty,

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2021). Several parties have conducted various studies on the utilization of fecal sludge waste. Among these are the use of fecal sludge in compost for agriculture (Fadila, 2021; Fitriana et al., 2017) and biomass briquettes (Lubis, 2019). However, this processing has not been accompanied by verification that the product or the process is free from active pathogens. As it comes from a large area and is communal, fecal sludge in IPLTs and the results of their processing have the potential to be a source of disease-causing pathogens. Regardless of its intended use, wastewater should be adequately treated to be fit for purpose and will not harm human health or the environment (Chahal et al., 2016).

Fecal waste can contain microbes, organic material, worm eggs, and residual nutrients such as nitrogen and phosphorus. Among the microbes found, some were pathogenic or harmful to human health. The pathogens most frequently detected in fecal waste include *Salmonella* spp. and *Escherichia coli* (Krzyzanowski et al., 2014). Apart from microorganisms, there are also pathogens of the Helminth type or worms, including the Nematoda group. Therefore, in determining the type of processing and product, special waste processing is needed, as well as appropriate genetics markers as molecular elements to validate the resulting environmental safety and health aspects (Benito et al., 2020).

In Indonesia, procedures based on *Escherichia coli* bacterial culture are applied to monitor and analyze water quality to determine the presence of fecal contamination. *E. coli* can be cultivated on synthetic growth media, which qualifies it as a viable, cultivable form of bacteria common in the environment (Irianto et al., 2021). Although not limited to human origin, *E. coli* bacterial cultures cannot accurately represent all harmful organisms in waste samples or contaminated materials. Many genes provide livability and can indicate an organism's presence in various extreme conditions. Genes in the DNA represent different functions. AmpC, mostly detected in *E. coli*, mediates resistance to any combination of antibiotics targeting the beta-lactamase enzyme (Gupta et al., 2014). Meanwhile, the AAC gene is a gene that is commonly found in cases of *M. tuberculosis* multiple antibiotic resistance (Joshi et al., 2013). The last gene used in this research is the ITS1 of RNR3, which is a gene encoded the ribosome and encodes ribonucleoside diphosphate reductase subunit 3, which has the function in DNA damage response, and the sequence taken is specific from *A. lumbricoides* (Koh & Sarin, 2018; Ulaganeethi et al., 2023). All these genes were chosen based on the critical function of each organism's survival ability, either for antibiotic resistance or response to environmental stress.

Primer oligonucleotide is a short sequence used as the molecule that recognizes the specific region to amplify in PCR. Oligonucleotide sequence in research should be considered whether or not it can answer the research question; thus, the determination of it is important. Most open-access programs for primer design can be run via the website. These programs mostly adopt Primer3 source code, including NCBI Primer Blast. Moreover, NCBI Blast can be used for specificity checks as it has a comprehensive database availability (Ye et al., 2012). The newest version of Primer3 is Primer3Plus, which has a reduced chance of primer-dimer or hairpins by improving the thermodynamics model and melting temperature prediction (Untergasser et al., 2012). Additionally, we carried out a

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series of validation of primers using different web-based programs to be more sure of the primer specificity before synthesis. In-silico validation of PCR primers can be performed to prevent off-target or primer pairs dimers (Davi et al., 2021). Some critical parameters for primer design that should be considered are oligonucleotide length, melting temperature, specificity, secondary structure format, and primer dimers. Developing an efficient oligonucleotide design workflow demands the right balance between the precision of the applied computer models, the general expenditure of time and computational workload (Hendling & Barišić, 2019). Developing an efficient primer design requires tools to predict the unspecific bands that can be formed during the PCR process, thus having comprehensive parameters and employing the relevant thermodynamics formula (Miranda & Weber, 2021).

Therefore, this research tried to introduce PCR primer design workflow and the result in PCR primer sequences as the diagnostic tools for the presence of pathogens with an elevated survival ability potentially present in human waste. Previous studies have stated that Primer3Plus and Primer-BLAST performed the best prediction of Tm among 22 web-based programs (Bakhtiarizadeh et al., 2016). Thus, these programs have been widely used for various needs in molecular biology and genetics. Besides, OligoPerfect by ThermoFisher has proven its great use in different strategies for the molecular characterization of pathogens (Medeiros et al., 2018). In this research, we created genetic markers (PCR primer) using these open-access web-based programs to detect pathogens' survival genes in fecal waste processing products, provided information regarding potential web-based primer design processes, and performed in-vitro validation using Polymerase Chain Reaction (PCR) method.

METHODS

Study Overview

This research is intended to generate primers for pathogen survival gene detection and validate the designed primers in silico, which were carried out using web-based programs. The in-vitro validation for primers was performed using PCR against the pure DNA of *Mycobacterium tuberculosis* H37RV, *Escherichia coli* ATCC, and *Ascaris lumbricoides* originating from extraction available at Humrc Hasanuddin University. Another sample obtained from the SSC (Solid Separation Chamber) pond at ILT Nipa-nipa Makassar is included to test its validity to the environmental sample. IPLT Nipa-nipa Makassar. SSC pond is the first collection pond after collection from household septic tanks which further proceed in the laboratory of HUMRC.

Procedures

Pathogen Determination and Primer Design

The initial studies were carried out by determining the criteria and type of pathogens. Some types of pathogens can survive in extreme conditions. The survival ability of pathogen types is then used as material to search for the genes involved in this ability. Target pathogens are determined based on the population's level or number of organisms causing high prevalence and significant organisms for transmitting certain diseases. The literature search was performed on the NCBI (https://www.ncbi.nlm.nih.gov/) and WormBase (https://wormbase.org/)

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databases. Most of the search of genes was conducted using the NCBI database, while wormbase was used to cross-reference the gene from the parasite group. All candidate genes and sequences were selected based on the criterias: 1) the availability of data showing that the candidate gene has appropriate functionality, supported by naming the gene in the NCBI database (Babben et al., 2015; Ye et al., 2012); and 2) the suitability of the sequence, which is mainly indicated by the low number of nucleotide repeats in the gene sequence (Wang et al., 2018).

After determining the type of pathogenic organism, determining the candidate genes, and collecting sequence data representing the candidate genes, primers were then designed randomly for each sequence using the Primer3 coded programs; they are Oligo Perfect (https://apps.thermofisher.com/apps/oligoperfect/#!/design) and Primer3Plus (https://www.primer3plus.com/index.html). Primer design with OligoPerfect was performed by choosing the End-point PCR menu, entering all the reference sequences, and setting the parameters following the default PCR condition of the PCR kit manufacturer. The intended primer size was 18-25 bp with a 40-60% GC content. The maximum number of G or C allowed in the last five 3' bases was set at 2 to minimize hairpin. The primer parameters and selection process following the previous review article with certain settlements (Hendling & Barišić, 2019).

The results of these two programs were then listed in a group of candidate primers. The candidate primers were run through several tests nucleotide BLAST NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to check the specificity of the primer test the possibility of primer-dimer and hairpin formation using Primerstat (https://www.bioinformatics.org/sms2/pcr_primer_stats.html), Oligocalculator (http://biotools.nubic.northwestern.edu/OligoCalc.html), and iDT OligoAnalyzer (https://sg.idtdna.com/pages/tools/oligoanalyzer). The PrimerStat operated by entering the multi-FASTA text of the primers and submitting it. In this study, the OligoCalc program was run for a self-complementarity check by inputting single primer sequences individually into the section provided. This program resulted in many thermodynamics parameters, which, in this study, we only took the output of the self-complementarity check. With the iDT OligoAnalyzer, the hairpin, self-dimer, and heterodimer analysis was automatically performed after single or batch input with an Excel format text. The selected primers were purchased from iDT (Integrated DNA Technology, Singapore). The overall workflow is illustrated in Figure 1.

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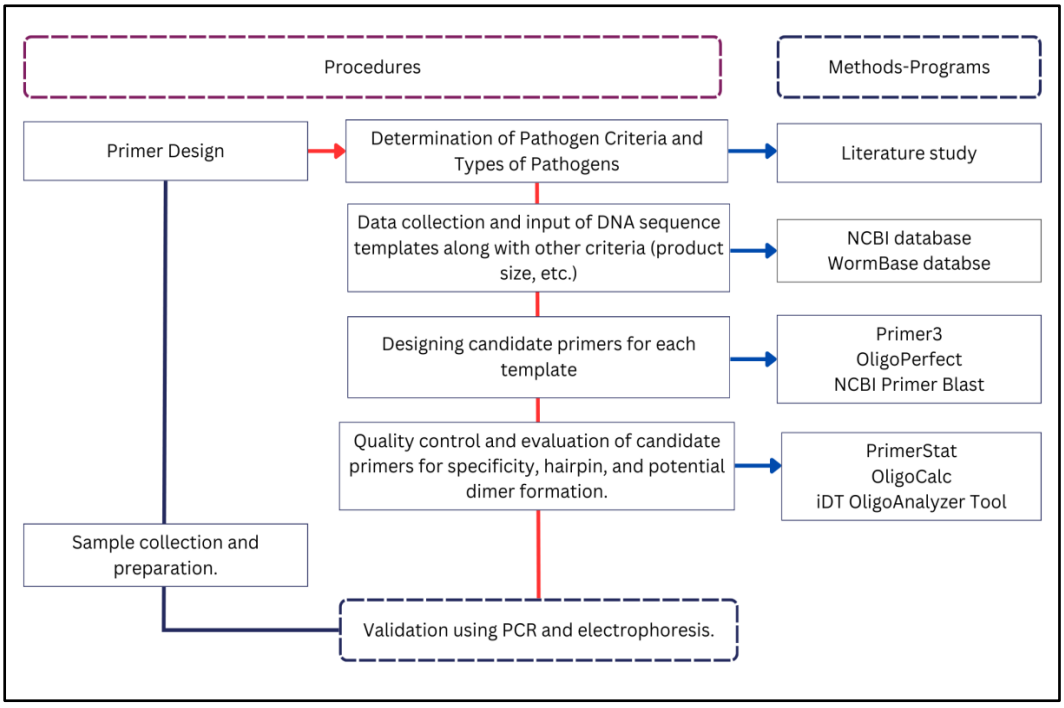

Figure 1. Overall Workflow of the Study.

Environmental Samples and DNA Extraction

The sample was randomly taken from three pond sides and mixed in a sample container. The sample was then stored at -4°C before further processing. Total DNA was isolated directly from fecal waste samples using the TIAnamp Stool DNA Kit. Before the extraction, the sample that had a muddy phase was homogenized and weighed 200 milligrams. The extraction was performed according to the manufacturer's protocol. Meanwhile, three positive controls for each gene-organism coupled testing, *Mycobacterium tuberculosis* H37RV, *Escherichia coli* ATCC, and *Ascaris lumbricoides*, are all available in the extracted DNA form, previously prepared by the laboratory analysts at HUMRC Hasanuddin University.

PCR and Electrophoresis

Gradient PCR was used for PCR primer validation by setting the melting temperature (Tm) range between 56,4°C and 62°C (based on the primers' lowest and highest Tm from the iDT instruction). PCR was done using Thermo Scientific Phusion U Green Multiplex PCR Master Mix (Thermo Scientific Inc., US). The master mix was prepared following the manufacturer's instructions, with a 5 µL DNA template and 0,5 µL of 10 µM primer each for a 20 µL reaction. It was done by preparing the PCR master mix and Nuclease-free water at the beginning, then aliquoting the master mix into four batches, mixing each batch with positive controls, and finally putting in the primers separately. PCR reactions were run on a Thermal Cycler (Bio-Rad iCycler) with the following thermocycling program: Pre-denaturation 30 sec 98°C Denaturation 5 Sec 98°C Annealing 10 Sec 50°C Elongation 10 Sec 72°C Post elongation 7 Min 72°C Cool down ∞ 12°C. The annealing temperatures were (56.4°C, 56.9°C, 57.6°C, 58.6°C, 60.1°C, 61.1°C,

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61.7°C, 62.0°C). The iCycler is a conventional PCR amplifier that results in band visualization after gel electrophoresis. The gel for electrophoresis was made at 1.7% concentration and 20 µL of EtBr. The electrophoresis was run at 800 volts for around one hour. The ladder used in this study was a 100 kb PCR ladder.

Data Analysis

The data generated by the web-based programs were in the form of primer pairs and information regarding the thermodynamics properties, Tm, and the potential secondary structures (hairpin, primer dimer, or hetero-dimer). The output of PrimerStat contains information about the single primer sequence's general properties and PCR suitability by stating Pass or Warning. The OligoCalc program resulted in many thermodynamics parameters, which, in this study, we only took the output of the self-complementarity check. The output of iDT OligoAnalyzer was analyzed on the Tm, the ΔG of the predicted secondary structure, and the Tm of the predicted secondary structure. All the primer candidates were classified into a 'Go' or 'No Go' for each program until one pair of primer sequence designs for each gene was chosen for oligonucleotide synthesis. Finally, the PCR-electrophoresis result was visualized and photographed using the Bio-rad Molecular Imager Gel-Doc XR System under UV excitation. The gel picture was analyzed based on the presence of the bands and the possible dimerization.

RESULTS AND DISCUSSION

Selected Pathogen and Genes

This initial research targets a few genes with significant potential for resistance to antimicrobials or genes significant for the organisms' survival ability. Some of the genes detected are antimicrobial resistance genes, which can move between microorganisms through conjugation, transformation, and transduction, resulting in the presence of these genes in the environment (Coleman & Smith, 2014). *E. coli* represents gram-negative bacteria, and *M. tuberculosis* represents gram-positive bacteria. Other than microbes, we tried to detect a gene from a parasitic worm, *Ascaris lumbricoides*, to represent a parasite. The selected genes and accordingly, functions are shown below (Table 1). Genes for bacteria detection were chosen from the anti-microbial resistance gene, especially the genes that cause multidrug resistance.

Table 1. Pathogen Species and Gene Target Functions for Primer Designs.

Species Pathogen	Target Gene	Gene Function	References
Escherichia coli	Cephalosporinase-hydrolysis of cephalosporin antibiotics	Antimicrobial resistance	(Gupta et al., 2014; Kon et al., 2023; Nurfadillah et al., 2023)
Mycobacterium tuberculosis	Aminoglikosida 2'-N-Acetyltransferase	Antimicrobial resistance	(Joshi et al., 2013; Sanz-García et al., 2019; Sari et al., 2019)
Ascaris lumbricoides	Ribonucleoside diphosphate reductase subunit 3; Internal Transcribed Spacer 1	DNA Damage response	(Das et al., 2015; Koh & Sarin, 2018; Ulaganeethi, et al., 2023)

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Primer Design

There are ten primers designed for each organism (Supplementary file 1). After the analysis and selection process, three primer pairs of organisms coupled with the Tm were stated based on the iDT program. One of the primers has the predisposition to forming a hairpin to have a wider view of the in-silico validation of newly generated primers and as the comparison for the in-silico and in-vitro validation. The primers and in-silico validation results can be seen in the table below (Table 2).

Table 2. Primer Designs Used and Validation Results from Web-Based Programs.

Target Genes	Species Target	Accession Number	Primer Pair	Tm (iDT/ °C)	Salt Adjusted Tm (Thermo Fisher/° C)	Product Size (bp)	Primer Blast	Primer Stat	OligoCalc	iDT
EC1_AmpC	*E. coli*	NC_000913.3	F: CGTCACACAGCAAACGTTGT R: CAGCAGTGTAGGTTGCGAGA	56.4 57.2	63.7 64.1	185	*E. coli*	Pass	None None	Supplementary file 2.
MT2_AAC	*M. tuberculosis*	NC_000962.3	F: ATCCAGCGGCGACTGATCTA R: TTGGTGCCAGTACCGATGTC	58.1 57.3	65.0 64.2	238	*M. tuberculosis*	Pass	None None	
AL7_RNR3	*A. lumbricoides*	OQ778747.1	F: AGGCATGCCGCTAGCGCTTA R: CCTCCAAGCTGAGGCTCATTGAGT	62.0 60.8	68.7 68.4	164	*A. lumbricoides*	Self-annealing and Haipin formation	None Hairpin at 5'CCTC_____GAGG_____3'	

In this study, we compare the analysis result of the web-based program on the Tm, sequence specificity, and the possibility of secondary structure formation. The Tm provided by iDT, based on the OlygoAnalyzer tool. The tool takes into account the monovalent salts concentration (NaCl) contained together in the primer. The Salt adjusted Tm (ThermoFisher) added the analysis of the divalent salt concentration (Mg^{2+}) with the value following the PCR reagent. Both Tm were calculated based on (Basílio Barbosa et al., 2019; SantaLucia & Hicks, 2004). The Tm (melting temperature) of the primer is needed for the targeted double-stranded DNA to be opened up so the primers can bind. The annealing temperature of the PCR is when the primers bind to the DNA, and the temperature should be lowered around 6-10°C below the Tm. Thus, these Tm were used to rule the PCR gradient temperature range: 56.4°C to 62°C. Further comparison of the stated annealing temperature and the result of in-vitro validation is described in the next part.

The result of in-silico validation also reviews the ΔG values of the possible secondary structure. The ΔG is the free energy of binding, which indicates the

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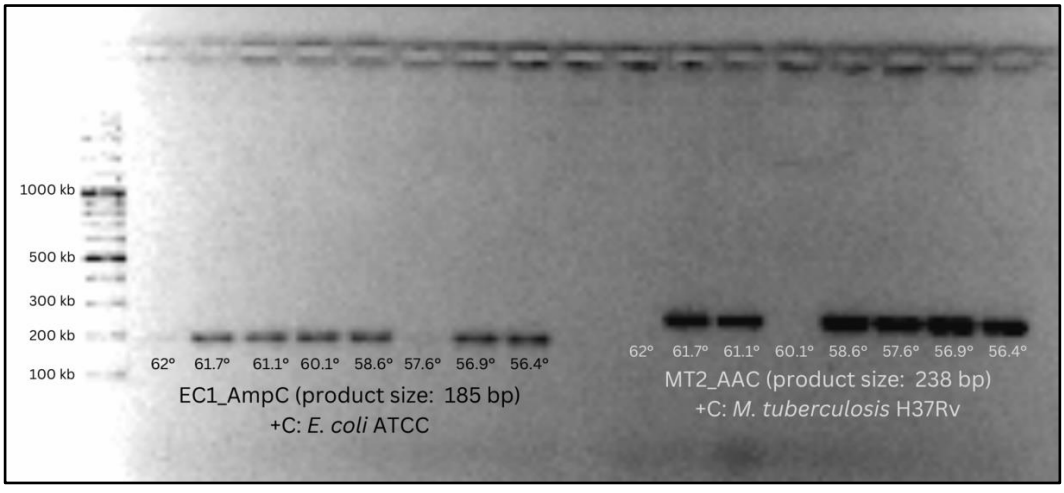
energy required to break a secondary DNA structure completely. The energy originated from a certain temperature needed to break the structure. This temperature should be far lower than the lowest annealing temperature settled. The closer it is to the melting temperature, the higher the possibility of a secondary structure (Yesudas et al., 2015). Based on PrimerStat and OligoCalc programs, the primers EC1_AmpC and MT2_AAC will be safe from secondary structure formation, as stated by the 'pass' and 'none' status (Table 2). The AL7_RNR3 primer pair based on both programs is prone to hairpin formation, as shown in Table 2. The detailed information can be seen in the Supplementary file 2. There is a difference in the result of PrimerStat and OligoCalc programs; PrimerStat shows that both the forward and reverse primers of AL7_RNR3 primer have the potential self-annealing (Supplementary file 2.), but OligoCalc reported only the reverse primer would potentially form a hairpin. However, both programs predict unfavorable results on the AL7_RNR3 primer pair. We can subsequently comprehend the results provided by the programs with the PCR electrophoresis result.

PCR and Electrophoresis

The possibility of AmpC *E. coli*, AAC *M. tuberculosis*, and RNR3;ITS1 of *A. lumbricoides* detections using newly generated PCR primers were tested using gradient PCR against the DNA extracted from *E. coli* strain ATCC, *M. tuberculosis* strain H37Rv, and *A. lumbricoides*. In the temperature range from 56.4°C to 62°C, the singleplex PCR clearly shows that all primer pairs could generate the expected bands indicated by the size (Figure 2).



(a)

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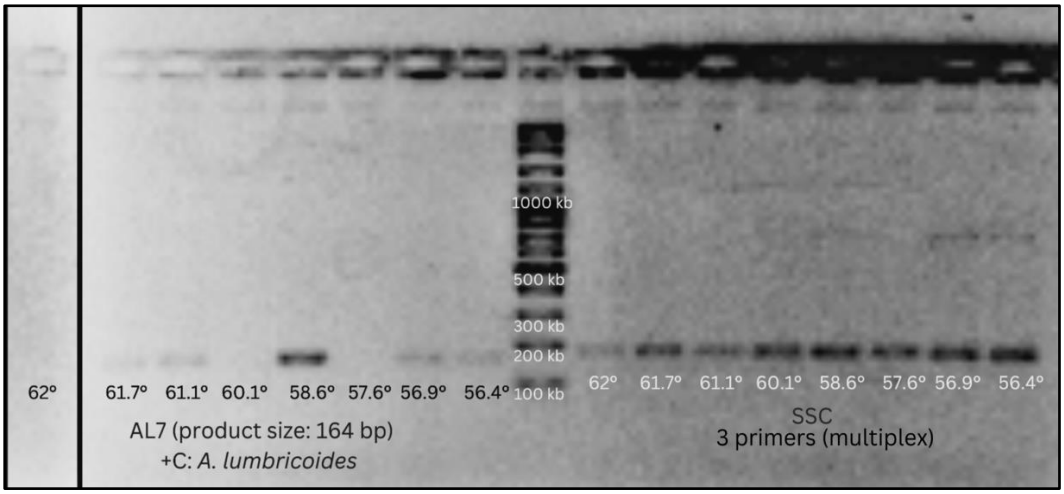
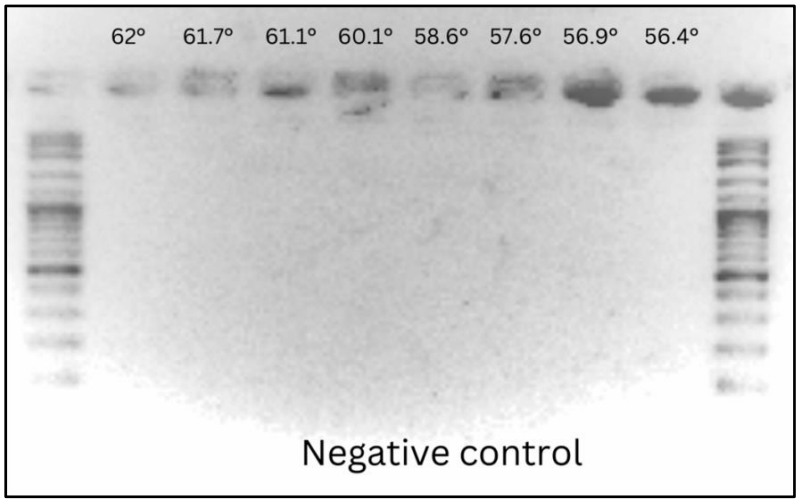


(b)



(c)

Figure 2. Images of Agarose Gel Displaying Singleplex Gradient PCR for Each Primer-Positive Control. a) The Result of the PCR Temperature Gradient Validates Primer AmpC_EC (Left) and Primer MT2_AAC (Right); b) Result of PCR Temperature Gradient for AL7_RNR3 Primer (Left) and Sample SSC (Right); and c) Negative Control Contains Primer without DNA Template.

The EC1_AmpC primer can perform amplification with the annealing temperatures used are 61.7°C, 61.1°C, 60.1°C, 58.6°C, 56.9°C, and 56.4°C (Figure 2a). There were unclear bands that could be visualized at 62°C and 57.6°C. The primer EC1_AmpC has a basic melting temperature of 56.4 and 57.2°C with salt adjusting Tm of 63.7 and 64.1°C. From the PCR results (Figure 2a. left), we can see that the EC1_AmpC primer pair could not amplify the gene target when the annealing temperature was set to 57.6°C even though this temperature should be reachable by the basic melting temperatures of the primer pairs (56.4-57.2°C). In contrast, the amplification can still occur with the annealing temperature of 61.7°C, which has more than a five °C temperature range from the basic Tm of one of the pairs. However, it is still in the range of the salt-adjusted Tm. The primer pair itself

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can pass the validation by PrimerStat and OligoCalc and is predicted to have dimerization (according to iDT OligoAnalyzer) occur under the temperature of 29.6-33.9°C with the ΔG values between -0.35 and -0.81 (Table 2 and Supplementary file 2). The negative value of ΔG is considered favorable and free from self-folding or unintended hybridization (Hata et al., 2018). Thus, the ΔG values of the EC1_AmpC primer pair show a relatively risk-free PCR reaction supported by the absence of secondary structure formation and the visualized bands with the expected size in almost every temperature of the gradient PCR. The Gibbs free energy (ΔG) compatibility is one of the most important thermodynamics parameters besides the GC content and primers' length when selecting primer pairs, which is also related to the Tm of a PCR reaction condition (Hendling & Barišić, 2019).

The MT2_AAC primer can successfully amplify the gene target when the annealing temperatures are set as 61.7°C, 61.1°C, 58.6°C, 57.6°C, 56.9°C, and 56.4°C (Figure 2a. right). The MT2_AAC primer pair failed the amplification with the melting temperatures of 62°C and 61°C (Figure 2a. right). The MT2_AAC primer has a basic Tm of 58.1 and 57.3°C with the salt adjusted Tm 65.0 and 64.2°C (Table 2). This primer pair passed the PrimerStat and OligoCalc programs for the secondary structure formation checks. Based on the batch analysis using iDT OligoAnalyzer, it is predicted to have ΔG values between -0.68 and -1.38 with the Tm between 33-42°C. The highest ΔG value of the potential secondary structure formed by MT2_AAC primer needs 42°C. It is considered safe from the secondary structure formation as there is more than a ten °C temperature distance to the lowest basic Tm of the primer pair. Additionally, the ΔG of the potential secondary structure itself is negative, indicating the prospective reliable amplification.

The generated primer for RNR3-ITS1 can perform amplification of the gene target at 61.7°C, 61.1°C, 56.9°C, and 56.4°C, even though the amplification result was not very clear. The AL7_RNR3 can start to amplify at its best when the annealing temperature is 58.6°C (Figure 2b. left). This primer pair has a high potential end to internal dimerization formed according to the PrimerStat, OligoCalc, and the iDT OligoAnalyzer programs (Table 2 and Supplementary file 2). Furthermore, the secondary structure would occupy the primer's availability, so even though no primer dimer is shown in the gel, the amplification would fail, and the expected band would be seen as unclear despite the presence of a target that should have been amplified (Park et al., 2020; Rychlik et al., 1990).

This study also used gradient PCR to test the primer in a sample from processed fecal waste (Figure 2b. right). From the figure, we can see that the sample contains only bands corresponding with the AmpC_EC1 primer product that detects the AmpC gene of *E. coli*. Two unspecific bands formed in the reactions at the annealing temperatures 56.9°C and 56.4°C, with the sizes between 700-800 bp. It means that a secondary structure formed around that temperature. The secondary structure usually forms when the annealing temperature exceeds the lower primer's melting temperature range. Moreover, the PCR performed on the sample was however, based on the result of the self-complementary check, in addition to the gradient PCR, the result has given the overview about the annealing temperature that should be placed at least at 58.6°C overall the primers to prevent secondary

structure. Besides, the undetected AAC and RNR3 genes in the fecal sludge sample from SSC pond sample is the indication of the absence of *M. tuberculosis* and *A. lumbricoides* traces in the processed fecal sludge. However, this result should be further tested especially in the multiplex PCR settings.

CONCLUSION

In-silico validation of primers after primer design using a series of web-based programs has results aligned with the PCR results in the case of primer design with minimum secondary structure. However, optimization using gradient PCR must be still conducted to find the fittest annealing temperature. The primer pairs can be used to detect the corresponding species initially for singleplex uses.

RECOMMENDATION

Checking the potential secondary structure of a primer pair can be useful in creating a primer pair with a wider range of annealing temperatures. However, there is different information regarding how to set the annealing temperature for a PCR reaction. The best consideration is to include the salt adjusting parameters, monovalent salt, divalent salt, and dNTPs concentration, for deciding the annealing temperature. Additionally, choosing the genes with a certain function based on the research question can be useful.

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Appendix A. Supplementary Data

Supplementary File 1. PCR Primer Candidates https://doi.org/10.6084/m9.figshare.24550915

Supplementary File 2. Additional Result of PCR Primer Validation https://doi.org/10.6084/m9.figshare.24550888