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In silico and *in vitro* analysis primers for antibiotic resistance gene of bacteria

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ABSTRACT

The prevalence of Extended-Spectrum Beta-Lactamase (ESBL) occurs due to excessive use of antibiotics, resulting in antibiotic resistance. Research on ESBL has been conducted in several Indonesian cities, such as Surabaya, Malang, and Jakarta which identified the presence of ESBL bacterial isolates. Limited information about *bla*CTX-M and *bla*VEB primer data in *in silico* analysis such as primer length, GC base content, melting temperature, and organisms attached to the gene, is an important thing that needs further research, because *in silico* data will affect the annealing temperature in the *in vitro* process using Polymerase Chain Reaction (PCR). The purpose of this study was to determine that *bla*CTX-M and *bla*VEB primers can be used to detect the presence of ESBL genes from amplification results in PCR. *In silico* primer analysis was carried out using BLAST primers from the NCBI website which would then be continued through *in vitro* primer analysis using PCR to see the presence of DNA bands. The results of *in silico* research showed that there were 27 organisms detected in the *bla*CTX-M gene and 37 organisms in the *bla*VEB gene. The results of *in vitro* analysis on *bla*CTX-M primers can be amplified at ± 400 bp, while *bla*VEB cannot be amplified.

Keywords: *bla*CTX-M, *bla*VEB, ESBL, primer analysis

INTRODUCTION

Bacterial resistance to antibiotics is still a problem with a serious impact on the treatment of infectious diseases. Overuse of antimicrobials is one of the factors causing antibiotics to lose their effectiveness against bacteria. Various studies have found that around 40% - 60% of antibiotics are used inappropriately for diseases that do not require antibiotics, causing antibiotic resistance. The use of these antibiotics will increase cases of the spread of *Extended Spectrum Beta-Lactamase* (ESBL) producing bacteria. ESBL bacteria can increase morbidity and mortality higher than bacteria that do not produce ESBL (Lestari et al., 2022). An important factor that causes high rates of antibiotic resistance is irrational use where minimal public knowledge can affect health attitudes and behaviors (Gunawan et al., 2021).

The production of *extended-spectrum β-lactamase* (ESBL) bacteria is mostly bacteria from the Enterobacteriaceae family, especially *Klebsiella pneumoniae* and *Escherichia coli*. According to the *World Health Organization* (WHO), Indonesia shows a high level of antimicrobial resistance with 71% resistance cases of ESBL-producing *Escherchia coli* and 64% resistance of *Klebsiella pneumoniae* where antibiotic resistance rates are increasing every year (Bahi et al., 2023; Efrilia et al., 2023; Ramadhani et al., 2020). According to a WHO national survey in 2024 involving 80 hospitals

and 30 laboratories in 16 provinces revealed a high prevalence of resistant bacterial infections, where most of the high-prevalence resistant such as *Klebsiella pneumoniae* (21.6%), *Escherichia coli* (18.3%) and *Acinetobacter baumannnii* (70.7%) (Gach et al., 2024). Studies on ESBL have also been conducted in several cities in Indonesia, such as Surabaya (Sutandhio et al., 2017), Malang (Kuntaman et al., 2018) and Jakarta (Maharani et al., 2021). Based on the results of a previous study, researchers have successfully identified and found the presence of bacterial isolates that produce *Extended Spectrum Beta-Lactamase* (ESBL). The spread of ESBL-producing bacteria causes serious public-health problems. It leads to limited treatment options because many commonly used antibiotics become ineffective, increases treatment costs, prolongs hospital stays, and raises morbidity and mortality rates. This situation contributes to the global threat of antimicrobial resistance and makes infection control more difficult.

*bla*CTX-M and *bla*VEB are known to be at risk of causing antibiotic resistance with a resistance rate of *bla*CTX-M as much as 31.6% and *blaVEB* as much as 16.6% (Sales et al., 2021). *bla*CTX-M is a gene from the CTX-M enzyme which has a hydrophilic ability to cephalosporins, especially cefotaxime, so it is called *bla*CTX-M (Wibisono et al., 2020). *blaVEB* is a smaller group of class A beta-lactams and often appears in non-fermenting bacteria or species such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Enterobacteriaceae sp* (Al-Ouqaili et al., 2020). These two ESBL genes can encode the *Extended-Spectrum Beta-Lactamase* enzyme located on plasmids where its spread occurs through gene transfer.

This study is very important due to the limited information available on primary data for the *bla*CTX-M and *bla*VEB genes. These two primers are used to detect specific and common ESBL genes, with *bla*CTX-M being the dominant gene globally and *bla*VEB targeting the rarer VEB type. The combination of these two primers in PCR improves the coverage, speed, and accuracy of ESBL identification, including variants that are often missed by conventional primers such as *bla*TEM or *bla*SHV. The available information consists of in silico primer data, including primer length, melting temperature, GC content, and related bacterial organisms. These primer characteristics represent the initial step in the Polymerase Chain Reaction (PCR) process. This study aims to analyze, both in silico and in vitro, the *bla*CTX-M and *bla*VEB primers associated with antibiotic-resistant ESBL genes. These findings are expected to play an important role in identifying and controlling the spread of antibiotic-resistant bacteria, especially within communities, and contribute to overcoming the health challenges posed by antibiotic resistance.

METHOD

Tools and materials

The tools used for in vitro methods are laminar air flow, erlenmeyer, glass beaker, ose needle, petri dish, spatula, micropipette, centrifuge, vortex, balance, comb, tray, PCR machine, electrophoresis machine, UV Transilluminator, power supply, waterbath, microwave, thermal cycler, spindown centrifuge. Tools for in silico bioinformatics using BLAST primers from the NCBI website. The materials used in this study were resistant bacterial isolates randomly sampled, 20% SDS, 400 µL homogenizing buffer, 8 µL Proteinase-K, 1000 µL Isopropanol equal volume, 50 µL ddH2O, 300 µL, 6M NaCl, 300 µL Ethanol 70%, Gel green, TE buffer, agarose gel 0.3 gram, 1.5 µL loading dye or gel-loading buffer, 3 µL dH2O, 5 µL *blaVEB* primers, 5 µL *blaCTX-M*, 15 PCR master mix, 3.5 µL DNA template, distilled water, tip, microtube, Eppendorf tube, forward and reverse primers, PCR result DNA sample.

Preparation and analysis

The forward *bla*CTX-M primer with a product length of 499 bp, while the *bla*VEB primer with a product length of 643 bp. The

sequence results that have been obtained will then be copied on the BLAST primer website in the primer parameters forward and reverse columns, then change the MRNA refseq database to "nt" and the "*Homo sapiens*" organism column is deleted, then select "Get primers" and the primer data results will appear (primer length, GC%, melting temperature, and organism) for further analysis.

Dilution of *bla*CTX-M and *bla*VEB primers

Primer dilution is done by adding TE buffer to the primer according to the datasheet. The *forward bla*CTX primer added 254 µL of *TE buffer*, *reverse* 290 µL of *TE buffer*. Primer *bla*VEB *forward* 238 µL *TE buffer*, and primer *reverse* 260 µL *TE buffer*. The solution was *vortexed* until homogeneous and stored at -20˚C.

Bacterial DNA isolation

DNA isolation was performed by destroying the bacterial isolate by adding 400 µL of *homogenizing buffer*. The sample was transferred into an eppendorf tube and given 40 µL of 20% SDS and 8 µL of *proteinase-K* and then incubated for 2 hours at 65˚C in a waterbath. During the incubation process, the waterbath was opened to homogenize the Eppendorf tube by flipping, then 300 µL of 6M NaCl (salting-out) was added to the suspension and vortexed for 30 seconds. The result of the vortex process was then centrifuged at 10,000 rpm at 4˚C for 10 minutes. The resulting supernatant was transferred into a microtube, then given 1000 µL of cold equal isopropanol, then incubated at -20˚C for 3 minutes. After incubation, the sample was centrifuged again at 12,000 rpm at 4˚C for 5 minutes, and the supernatant was discarded. The pellet containing DNA was washed using 300 µL of 70% ethanol, then centrifuged at 10,000 rpm at 4˚C for 5 minutes, and the pellet was removed. The retrieved DNA pellet was dried at room temperature for 24 hours, after which 50 µL of sterile ddH2O was added and stored at -20˚C

PCR for bacterial DNA amplification

Table 1. PCR Reaction Composition

Reagent	Volume (µL)
Primer *bla*CTX-M & *bla*VEB	5 µL
dH_2O	3 µL
PCR mix	15 µL
DNA template	3,5 µL
Total	26,5 µL

The initial stage of PCR is pre-denaturation at 94˚C for 5 minutes to separate the DNA template and activate the polymerase enzyme. The second stage is denaturation, which separates double-stranded DNA into single strands at 94˚C for 1 minute, then the third stage is *annealing* so that the primer can attach to specific regions of template DNA. The *annealing* temperatures used in blaCTX-M primers are 58, 55 ˚C, 52, and 48 ˚C, while *blaVEB* primers with *annealing* temperatures of 55, 52 ˚C, 50.5 ˚C, and 48 ˚C, if *trial and error,* the annealing temperature can be reduced by 3-5 ˚C. The last stage is elongation or lengthening of the DNA strand at 72˚C for 5 minutes. All of these stages are repeated stages carried out for 30 cycles with a time of ± 3 hours.

Electrophoresis and visualization

The electrophoresis stage begins with the preparation of 20 ml agarose gel solution. Agarose gel solution is given 1 µL green gel dye, then poured into a mold (tray) that has been equipped with a comb (comb) and allowed to stand at room temperature until it hardens. The agarose gel that has been finished is inserted into the electrophoresis apparatus that has contained TBE 1x solution. DNA samples will be homogenized with loading buffer and inserted slowly in the agarose gel wells, then the power supply is turned on with a voltage of 50 volts within 50 minutes. Visualization of electrophoresis using BluPAD UV transilluminator which displays the results in the form of DNA bands.

RESULTS AND DISCUSSION

In silico study

The initial stage of in silico studies is to analyze the primary data obtained based on the research of Hassan et al. (2018) and Ranjabar et al. (2017) showing the presence of genes amplified by *bla*CTX-M and *bla*VEB primers. The results of BLAST parameter data listed in (Table 2.) show information about the length, Melting Temperature (TM), and percentage of GC%. Primer *blaCTX-M* has a primer length of 19-20 bp, while primer *blaVEB* has a length of 20 bp. This finding is in line with research conducted by Kuntaman et al. (2018), that generally primers have base lengths between 15-25 bp and do not have significant melting temperature differences. Primer length affects the annealing time in PCR reactions; primers that are too short (below 15 bases) can reduce specificity, while primers that are too long can inhibit the effectiveness of PCR reactions (Buchori et al., 2023).

Table 2. In silico result of *bla*CTX-M and *bla*VEB gene primers.

Primer *bla*CTX-M				
	5'-3' Sequence	Length	TM	GC%
Forward	GACGATGTCACTGGCTGAGC	20	61.08	60.00
Reverse	AGCCGCCGACGCTAATACA	19	61.78	57.89
Primer *bla*VEB				
	5'-3' Sequence	Length	TM	GC%
Forward	CGACTTCCATTTCCCGATGC	20	59.34	55.00
Reverse	GGACTCTGCAACAAATACGC	20	57.47	50.00

The in silico melting temperature results show that the *forward bla*CTX-M primer is 61.08˚C and the *reverse* primer is 61.78˚C, while the *forward bla*VEB primer is 59.34˚C and *the reverse* primer is 57.47˚C (Table 2). Both primers have corresponding TM values. Primer pairs should not have a melting temperature difference of more than 5 ˚C because it will cause a decrease in the amplification process or even no amplification process (Miranti et al., 2023; Sasmito et al., 2014). The value of GC will also affect the Melting temperature value Where the GC% content ranges from 45-60%, if it is lower it can reduce the primer's ability to annealing to the DNA template, but if it is too high it will encourage the primer to make dimers and reduce the concentration of DNA (Cahyadi et al., 2019; Siswanto et al., 2022).

Based on BLAST results (Table 3) showed that this primer is specific to the *blaCTX-M* gene in various bacterial organisms such as *Escherichia coli*, *Klebsiella pneumoniae*, *Shigella boydii*, *Aeromonas hydrophila*, *Edwardsiella tarda*, and *Salmonella enterica* with an amplification product length of 499 bp, while the blaVEB gene was found in various bacterial organisms such as *Aeromonas veronii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Serratia marcescens, Enterobacter cloacae, Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, and *Vibrio alginotycus* with an amplification product length of 643 bp. This is in line with the research of Lukey et al. (2021) that the *blaVEB* gene can be identified in *Proteus sp*, *Morganella morganii*, *Klebsiella oxytoca*, and *Acinetobacter baumannii*.

Table 3. BLAST results of *bla*CTX-M and *bla*VEB primers

Primer *bla*CTX-M			
No	**Gen**	**Organisms**	**Product Length**
1.	*bla*CTX-M	*Escherichia coli*	499
2.	*bla*CTX-M	*Klebsiella pneumoniae*	499
3.	*bla*CTX-M	*Shigella dysenteriae*	499
4.	*bla*CTX-M	*Shigella boydii*	499
5.	*bla*CTX-M	*Shigella sonnei*	499
6.	*bla*CTX-M	*Shigella flexneri*	499

Primer *bla*CTX-M			
No	**Gen**	**Organisms**	**Product Length**
7.	*bla*CTX-M	*Escherichia coli*	499
8.	*bla*CTX-M	*Klebsiella pneumoniae*	499
9.	*bla*CTX-M	*Klebsiella pneumoniae*	499
10.	*bla*CTX-M	*Escherichia coli*	499
11.	*bla*CTX-M	*Klebsiella pneumoniae*	499
12.	*bla*CTX-M	*Escherichia coli*	499
13.	*bla*CTX-M	*Aeromonas hydrophila*	499
14.	*bla*CTX-M	*Aeromonas hydrophila*	499
15.	*bla*CTX-M	*Aeromonas hydrophila*	499
16.	*bla*CTX-M	*Aeromonas hydrophila*	499
17.	*bla*CTX-M	*Edwardsiella tarda*	499
18.	*bla*CTX-M	*Edwardsiella tarda*	499
19.	*bla*CTX-M	*Edwardsiella tarda*	499
20.	*bla*CTX-M	*Edwardsiella tarda*	499
21.	*bla*CTX-M	*Escherichia coli*	499
22.	*bla*CTX-M	*Escherichia coli*	499
23.	*bla*CTX-M	*Escherichia coli*	499
24.	*bla*CTX-M	*Eschercihia coli*	499
25.	*bla*CTX-M	*Escherichia coli*	499
26.	*bla*CTX-M	*Escherichia coli*	499
27.	*bla*CTX-M	*Salmonella enterica*	499
28.	*bla*VEB	*Aeromonoas veronii*	643
29.	*bla*VEB	*Aeromonoas veronii*	643
30.	*bla*VEB	*Aeromonoas veronii*	643
31.	*bla*VEB	*Escherichia coli*	643
32.	*bla*VEB	*Klebsiella pneumoniae*	643
33.	*bla*VEB	*Klebsiella pneumoniae*	643
34.	*bla*VEB	*Klebsiella pneumoniae*	643
35.	*bla*VEB	*Aeromonas veronii*	643
36.	*bla*VEB	*Proteus mirabillis*	643
37.	*bla*VEB	*Aeromonas veronii*	643
38.	*bla*VEB	*Serratia marcescens*	643
39.	*bla*VEB	*Klebsiella pneumoniae*	643
40.	*bla*VEB	*Klebsiella pneumoniae*	643
41.	*bla*VEB	*Klebsiella pneumoniae*	643
42.	*bla*VEB	*Enterobacter cloacae*	643
43.	*bla*VEB	*Enterobacter cloacae*	643
44.	*bla*VEB	*Pseudomonas aeruginosa*	643
45.	*bla*VEB	*Enterobacter cloacae*	643
46.	*bla*VEB	*Acinetobacter baumannii*	643
47.	*bla*VEB	*Pseudomonas aeruginosa*	643
48.	*bla*VEB	*Acinetobacter baumannii*	643
49.	*bla*VEB	*Pseudomonas aeruginosa*	643
50.	*bla*VEB	*Pseudomonas aeruginosa*	643
51.	*bla*VEB	*Escherichia coli*	643
52.	*bla*VEB	*Vibrio parahaemolyticus*	643
53.	*bla*VEB	*Vibrio alginolyticus*	643
54.	*bla*VEB	*Vibrio parahaemolyticus*	643
55.	*bla*VEB	*Vibrio parahaemolyticus*	643
56.	*bla*VEB	*Proteus mirabillis*	643
57.	*bla*VEB	*Enterobacter cloacae*	643
58.	*bla*VEB	*Pseudomonas aeruginosa*	643
59.	*bla*VEB	*Escherichia coli*	643
60.	*bla*VEB	*Escherichia coli*	643
61.	*bla*VEB	*Pseudomonas aeruginosa*	643
62.	*bla*VEB	*Pseudomonas aeruginosa*	643
63.	*bla*VEB	*Pseudomonas aeruginosa*	643
64.	*bla*VEB	*Proteus mirabilis*	643

BLAST results (Table 3) bacterial organisms can be grouped into fecal and non-fecal coliform bacteria. The blaCTX-M primer contained 27 bacterial organisms, while blaVEB 37 bacterial organisms. The *blaCTX-M* gene organisms consisted of 19 fecal bacteria name ly *Escherichia coli*, *Shigella boydii*, and *Aeromonas hydrophila* and 8 non-fecal bacteria from *Klebsiella pneumoniae* and *Edwardsiella tarda* organisms (Oktaviani, 2018). The *blaVEB* gene was found in 20 fecal and 17 non-fecal bacterial organisms. The fecal bacteria are *Escherichia coli*, *Proteus mirabili*, *Enterobacter cloacae*, *Vibrio parahaemolyticus*, and *Vibrio alginotycus* while the non-fecal bacteria found in the *bla*VEB gene are *Klebsiella pneumoniae*, *Aeromonas veronii*, *Serratia marcescens*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* (Oktaviani, 2018; Sunarti, 2016; Wijaya, 2014).

Optimization of annealing temperature and PCR amplification

Optimization of annealing temperature was carried out with various temperatures. Table 4 shows the different temperatures used for each primer. The *bla*CTX-M primer used optimization temperatures of 58 ˚C, 55 ˚C, 52 ˚C, 50.5 ˚C, and 48 ˚C, while the *bla*VEB primer used temperatures of 55 ˚C, 52 ˚C, 50.5 ˚C, and 48 ˚C.

Table 4. Results of temperature optimization at annealing

Primer	Annealing Temperature	Results
***bla*CTX-M**	58 ˚C	-
	55 ˚C	-
	52 ˚C	-
	50,5 ˚C	-
	48 ˚C	+
***bla*VEB**	55 ˚C	-
	52 ˚C	-
	50,5 ˚C	-
	48 ˚C	-

These results show that *bla*CTX-M primers have an optimum annealing temperature of 48 ˚C, while *blaVEB* does not show any temperature options for annealing at 55 ˚C, 52 ˚C, 50.5 ˚C, and 48 ˚C. Annealing temperature settings are based on a range of -5˚C to +5˚C from the manufacturer's *melting temperature* (Tm) (Nuryady et al., 2020). Annealing temperatures that are below the optimum temperature can result in false priming, while if the annealing temperature is too high from the optimum temperature, the primers will not be able to bind to the target DNA, resulting in failure in the PCR process (Adikara et al., 2016).

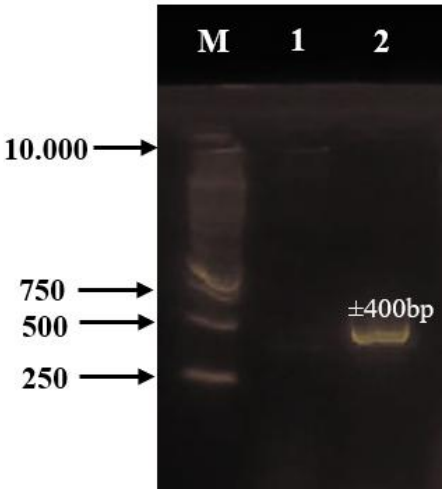

Figure 1. Results of DNA amplification visualization with *bla*CTX-M and *bla*VEB primers (M= Marker; 1= Primer *bla*VEB; 2= Primer *bla*CTX-M)

Visualization of electrophoresis with UV transilluminator showed positive results with the formation of DNA bands on *bla*CTX-M primers. The results (Figure 1.) Show the presence of genes amplified in 2 (primer *bla*CTX-M) with a product length of ± 400 bp with a fairly thick DNA band, this is in line with research of Korir et al. (2017) and Tabar et al. (2016) that the *blaCTX-M* gene has 499 bp. The difference may be due to the primer design and binding locations, which produce a smaller amplified fragment of the *bla*CTX-M gene. The amplicons show that the *blaCTX-M* primer can recognize or bind to the intended gene to ensure that only the DNA is to be reproduced or amplified, so it can be said to be a specific primer for the gene. A primer is said to be non-specific if in one well it produces amplification products of more than one fragment, or the length of the resulting DNA band does not match the prediction of the primer or base pair used (Nova et al., 2016).

The *bla*VEB primer (Figure 1) did not find any genes because the DNA band did not appear when visualized using a UV transilluminator. Non-detection of primer results. This may be due to the lack of specificity of these primers and the absence of the *bla*VEB gene in the DNA tested. In addition, the influence of GC content can also contribute to the non-appearance of DNA bands, because *bla*VEB has a lower GC percentage compared to *bla*CTX-M. In addition, the influence of GC content can also contribute to the non-appearance of DNA bands, because blaVEB has a lower GC percentage compared to blaCTX-M, which is 50-55% where low GC content can reduce the ability of primers to annealing on DNA templates, but if it is too high it will encourage primers to make dimers rather than bind to target genes, and reduce the concentration of DNA (Cahyadi et al., 2019; Siswanto et al., 2022). The presence of high Melting Temperature (Tm) content can also cause a decrease in the amplification process or even no amplification process occurs. Fragments that do not appear can also be caused by primer incompatibility with template DNA. A difference of one base pair can cause the primer to be incompatible, so amplification cannot occur (Safitri et al., 2024).

CONCLUSION

Based on the results of the study, the *bla*CTX-M primer can be said to be a specific primer because it successfully amplifies the *bla*CTX-M gene at ± 400 bp with a thick DNA band on PCR visualization, while the *bla*VEB primer fails to produce detectable amplification, characterized by the absence of DNA bands, which indicates a lack of efficiency or specificity. Therefore, redesign is required with further studies to validate the primers on more diverse bacterial isolates.

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