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Original Article

B-LACTAMASES INHIBITOR-PRODUCING SOIL BACTERIA FOR AMPICILLIN-RESISTANT UROPATHOGENIC *ESCHERICHIA COLI* ISOLATE

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ABSTRACT

Objective: The goals of this investigation were to identify the species of the producers and ascertain the dose-dependent effect of extracellular products of Indonesian bacteria that generate *β-lactamases* inhibitors.

Methods: An agar diffusion technique for the lactamase inhibitor activity assay was performed. Observation of bacteria using phenotypic analysis was performed by observing colony color and cell shape morphology, biochemical assays and a series of carbohydrate fermentation tests. Bacterial identification was performed by comparing the nucleotide sequence of the 16S rDNA gene of target bacteria with available nucleotide sequences in Gene Library (NCBI). Combining data from phenotypic and genotypic analyses allowed for the identification of the producers.

Results: According to our findings, none of the bacteria's extracellular products, which contain *β*-lactamase inhibitors in a range of concentrations, showed a discernible impact on the values of the inhibition zone. The producers are *Aeromonas popoffii*, *Alcaligenes faecalis*, *Streptomyces brasiliensis*, *Staphylococcus equorum*, *Pseudomonas putida*, *Pseudomonas fluorescens, Salmonella typhi, Enterobacter hormaechei, Serratia marcescens* and *Enterobacter sp.* The highest potency of *β*-lactamase inhibitor was provided by the extracellular product of VR3 isolate bacteria which was identified as *Serratia marcescens*.

Conclusion: In conclusion, this study clearly showed that our isolated bacteria have the potential to be further investigated in order to maximize the recovery of *β*-lactamase inhibitor compounds.

Keywords: ®-lactamase, Soil, Inhibitor, *Escherichia coli, Ampicillin, Serratia marcescens*

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INTRODUCTION

Limiting the use of beta-lactam antibiotics or their combination with beta-lactamase inhibitors for the initial treatment of cystitis is reported due to facts that these drugs are resistant to the uropathogenic bacteria, cystitis-causing bacteria [1]. As in Indonesia, antibiotic resistance is an obstacle in the treatment of cystitis. Based on medical record information from a hospital in Indonesia, urine culture examination results showed that the majority of bacteria found in the samples were Gram-negative bacteria with characteristics of multidrug resistance to several antibiotics, including cephalotin, cephazolin, ampicillin, sulphametoxazole, and trimethoprim. *Escherichia coli* was the most prevalent pathogen identified in urine culture among Gram-negative bacteria [2]. According to earlier research, *E. coli* is the main cause of cystitis, and ampicillin resistance was the highest case in isolated *E. coli* [3-5]. In order to combat bacterial resistance, serine ®-lactamases produced by *E. coli*, which inactivate the beta-lactam ring, are predominantly blocked by combinations of ®-lactamase inhibitors [6-8]. Since the inhibitor and beta-lactam antibiotics are competing for the same binding site on the beta-lactamase enzyme, evolutionarily, the beta-lactamase enzyme's gene may become mutated, making the enzyme more likely to be vulnerable to inhibitors [9]. Clavulanic acid, for example, is a ®-lactamase inhibitor that is beneficial in boosting the activity of ®-lactam antibiotics in the treatment of severe Enterobacteriaceae infections. For the treatment of severe Enterobacteriaceae infections, a number of ®-lactamase inhibitors are useful for repurposing the activity of ®-lactam antibiotics, including clavulanic acid, sulbactam and tazobactam. The development of resistance to the combination of ®-lactam-®-lactamase inhibitors is accelerated by the misuse of antibiotics. Additionally, there is a substantial rise in the occurrence of clinically significant inhibitor-resistant types of ®-lactamases. As a result, there is an urgent need for efficient inhibitors that can revive ®-lactam action [10].

Our earlier research demonstrated that a number of soil bacteria's extracellular products have ®-lactamase inhibitory action, which can improve the effectiveness of ampicillin from the ®-lactamases inactivation made by clinical isolates of ampicillin-resistant *E. coli*. Since ampicillin-resistant bacteria that produce ®-lactamases can be killed by the extracellular products of soil microorganisms, compounds of microbial products may contain new ®-lactamase inhibitors. The development of drugs will be significantly impacted by the discovery of novel ®-lactamase inhibitors. Because the potential sources of ®-lactamase inhibitors were found in Indonesian soil, this study has the potential to expand our understanding of microbial biodiversity. The objectives of this investigation were to determine the species of ®-lactamase and investigate the dose-dependent inhibition of bacterial extracellular products with possible ®-lactamase inhibitory activity and to determine the species of microbes that produce ®-lactamase inhibitors using phenotypic and genotypic methods.

MATERIALS AND METHODS

Materials

Co-amoxiclav (Indofarma), Sodium chloride (Merck), Potassium Chloride (Merck), Sodium Hydrophosphate (Merck), Potassium Hydrophosphate (Merck), Distilled water, Agarose (Boehringer Mannheim), forward primer (5'GGTTAC(G/C)TTGTACGACTT3') (Proligo), and reverse primer (5'AGAGTTTGATC(A/C)TGGCTCAG 3') (Proligo), deoxynucleotide triphosphate (dNTP) (MD Bio-Korea), Taq DNA polymerase (Stratagene), Marka 100 bp. Wizard Genomic DNA Purification Kit (Promega), absolute ethanol, 70% ethanol, distilled water, double distilled water, Tris-base 10 mmol, EDTA 1 mmol, pH 8.0, magnesium chloride (Merck), loading buffer (sucrose and 0.25% bromphenol blue (Merck)), ethidium bromide, Mueller Hinton Broth (MHB-Pronadisa), Mueller Hinton Agar (MHA-Pronadisa) and Luria Bertani (LB) medium.

Isolation of extracellular products

From our previous study, there are ten β-lactamase inhibitor containing bacteria isolated from soil in some areas in Indonesia, with the codes: JW2, DPA3, CNN2, DL3, VR3, HGM3.1, BS6, FN16, NLTA3 and SL1. Each of bacteria colony was cultured in 25 mlMHB and overnight incubated at room temperature. The bacterial suspension ($3x10^8$ CFU/ml) was centrifuged at 13 000× g for 10 min to isolate the extracellular product in the supernatant, then lyophilized. 0.5 g of each lyophilized extracellular product was then suspended in 500 µl of sterile PBS. The sample suspension was serially diluted to achieve various test concentrations, as follows: 200, 400, 600, 800 and 1000 mg/ml to perform dose-dependent activity against ampicillin-resistant *E. coli* using the agar diffusion method. For both *E. coli,* bacterial suspension it was prepared by taking 1 Ose of *E. coli* colonies and suspended in the sterile NaCl solution, then the bacterial solution is then adjusted to meet the 0.5 McFarland turbidity standard, which produces an optical density corresponding to that of a suspension containing 1.5 x 10^8cfu/ml. Ampicillin-resistant *E. coli* was grown on media containing ampicillin 25 µg/ml, while sensitive *E. coli* was inoculated on media without antibiotics.

Producer's identification

By combining phenotypic and genotypic analysis, the makers of β-lactamase inhibitor were identified. The observation of bacteria using phenotypic analysis was conducted by observing the colony color and cell shape morphology, biochemical assays and a serial of carbohydrate fermentation tests. The color of the colony was assessed visually, and Gram staining was used to view the cell morphologies under a microscope. Citrate acid, motile indole urea, red methyl-Voges Proskauer (MR-VP), and carbohydrate fermentation assays using glucose, maltose, sucrose, mannitol, and lactose as the substrates were used in biochemical studies.

Genotypic identification

By comparing the target bacteria's 16S rDNA gene's nucleotide sequence to other nucleotide sequences present in Gene Bank, bacteria were identified (NCBI). Utilizing the Wizard kit, the bacteria's chromosomes were separated and purified, after which they were subjected to agarose gel electrophoresis analysis. By measuring the absorbance at 240 and 280 nm, the purity of chromosomal DNAs was determined. The 16S rDNA genes were then amplified using two particular primers as follows: BacF1: 5'-AGAGTTTGATC (AC) TGGCTCAG-3' and Uni B1: 5'-GGTTAC (GC) TTGTTACGACTT3' (forward primer) (reverse primer). The conditions for the PCR were as follows: 94 °C for pre-denaturation for 2 min, 94 °C for amplification for 1 min, 48 °C for annealing for 1 min, 72 °C for elongation for 1 min, and post-elongation at 72 °C for 10 min. The expected size of 1400 bp for the PCR products was determined by agarose gel electrophoresis. The band was then isolated from the gel and purified using GFX column, then sequenced and analyzed using the Basic Local Alignment Search Tools (BLAST) program to find the sequences in Gene Bank that had the highest homology/identity values.

RESULTS

Dose-dependent activity

The effectiveness of ®-lactamase inhibitor activity for inactivating ®-lactamase produced by a clinical isolate of ampicillin-resistant *E. coli* was examined using a dose-dependent activity test. In table 1, the inhibition zone of microorganisms that produce inhibitors was displayed. The formation of an inhibitory zone surrounding the extracellular product against ampicillin-resistant *E. coli* can be used to detect β-lactamase inhibitor activity, but it did not inhibit the sensitive ones. The highest inhibitory diameter was present in the extracellular product of isolated bacteria from the VR3 region. Fig. 1 displays the relative inhibition of undiluted extracellular product to ampicillin-sensitive and-resistant *E. coli*. However, statistically (p = 5%), the value of F table (4.45) = 2575, which is greater than F count, indicates that the different concentrations of their extracellular product did not exhibit any discernible inhibitory diameter, as indicated in table 2.

Table 1: β-lactamase inhibitor activity

No	Sample	Diameter of inhibition (mm) of tested concentration (mg/ml)				
		200	400	600	800	1000
1.	VR3	11.7±0.10	11.7±0.00	11.8±0.02	11.9±0.00	12.1±0.10
2.	HGM 3.1	11.6±0.00	11.7±0.10	11.7±0.00	11.8±0.05	11.8±0.02
3.	DL3	11.5±0.04	11.5±0.10	11.6±0.00	11.6±0.00	11.7±0.00
4.	DPA 3	11.7±0.05	11.7±0.10	11.8±0.04	11.8±0.05	11.8±0.00
5.	SL 1	11.4±0.10	11.5±0.02	11.6±0.00	11.5±0.00	11.6±0.05
6.	NLTA 3	11.5±0.00	11.5±0.00	11.5±0.10	11.6±0.00	11.6±0.04
7.	CNN 2	11.6±0.02	11.6±0.00	11.6±0.05	11.6±0.02	11.6±0.10
8.	FN 16	11.7±0.10	11.7±0.05	11.7±0.05	11.8±0.04	11.8±0.05
10.	BS 6	11.6±0.00	11.6±0.02	11.6±0.02	11.6±0.00	11.6±0.00
11.	JW2	11.4±0.00	11.4±0.02	11.4±0.10	11.5±0.00	11.5±0.10

Values represent mean±SD, n=3.

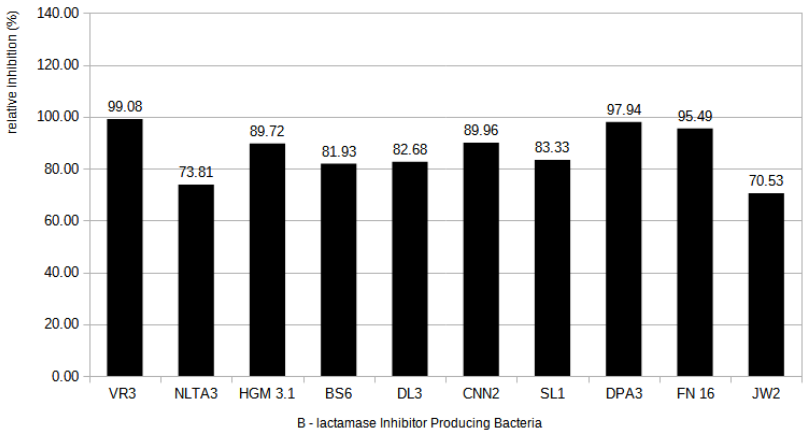

Fig. 1: Inhibition of undiluted extracellular product of resistant *E. coli*

Table 2: Statistical analysis

Source of variation	Df	Sum of squares	Middle square	F count
mean	1	6783.79	6783.79	
treatment (concentration)	4	0.008	0.002	0.1
error	45	0.902	0.020	
sum	50	6784		

From this study, we also found an interesting result; since DL3's extracellular product inhibited both ampicillin-resistant and-sensitive *E. coli*, it was suspected that it has potent to be both β-lactamase inhibitors and antibiotics, as shown in fig. 2. The difference of inhibitory diameter resulted by DL3's extracellular product can be clearly shown in fig. 3. This product provided greater inhibition to resistant *E. coli* than sensitive ones.

Phenotypic identification

Table 3-4 lists the results of biochemical tests and colony and cell morphology observations used to identify 10 tested bacteria using the phenotypic technique. Table 5 contains the details of the examined bacterium. Gram negative bacteria with rod forms and various colony morphologies made up the majority of the ®-lactamase inhibitor bacteria.

Genotypic identification

Fig. 4 shows the electrophoregram of the 16S rDNA PCR product. As expected, the PCR result is roughly 1410 bps in size. Table 6. displays the results of the forward and reverse specific nucleotide sequence analysis for the 16S rDNA gene.

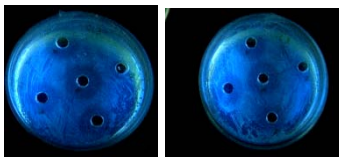

Fig. 2: Inhibition zones of DL3 Bacteria at various concentrations, Ampicillin resistant-*E. coli;* b. Ampicillin sensitive-*E. coli*

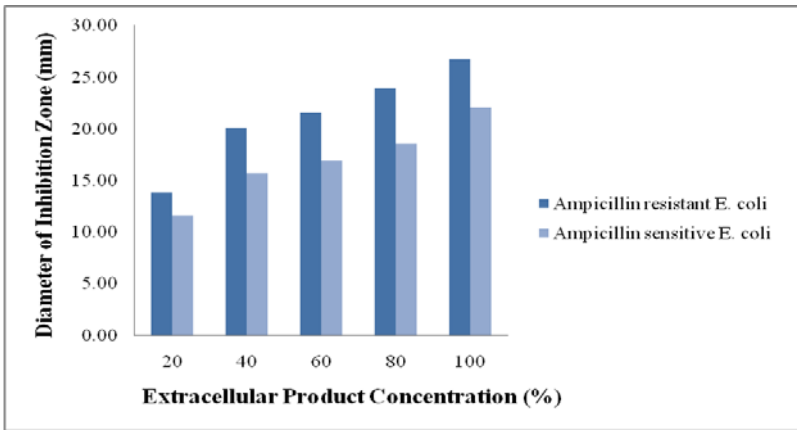

Fig. 3: The effect of DL3 extracellular product concentration to inhibition zone diameter

Table 3: The morphology of bacteria producing beta-lactamase inhibitory

Sample name	Shape	Gram grouping	Colony color
JW2	Rod	_	Yellowish white
CNN 2	Rod	_	Yellowish white, tranparant
DL3	Rod	_	Yellowish white, tranparant
DPA 3	Rod	_	Tranparant white
SL1	Coccus	+	Tranparant white
FN16	Coccus	+	White, opaque,
VR3	Rod	-	White, opaque
BS6	Rod	_	yellow
HGM 3.1	Coccus	_	Yellowish White
NLTA 3	Rod	_	White, transparant

Table 4: The result of biochemical assays

Sample	Citrate	Motil	Indole	Urea	MR	VP	Glucose	Maltose	Sucrose	Manitol	Lactose
JW2	+	+	-	+	+	-	+/gas	+/gas	+/gas	-	+/gas
DPA 3	+	+	-	+	+	-	+/gas	+/gas	+/gas	+/gas	-
CNN 2	+	+	-	-	+	-	+/gas	+/gas	+/gas	+/gas	+/gas
DL3	+	+	-	+	+	-	+/gas	+/gas	+/gas	+/gas	+/gas
VR3	+	+	+	+	+	-	+/gas	+/gas	+/gas	-	-
HGM 3.1	+	+	-	+	+	-	+/gas	+/gas	+/gas	+/gas	-
BS 6	+	+	-	+	-	-	-	+gas	+gas	+gas	-
FN 16	-	-	-	-	-	-	-	-	-	-	-
NLTA 3	+	+	+	-	+	-	-	-	-	-	-
SL 1	-	-	-	-	-	-	-	-	-	-	-

Table 5: Bacteria identity using phenotypic analysis

Sample	Genus
JW2	*Alcaligenes*
DPA 3	*Pseudomonas*
CNN 2	*Salmonella*
DL3	*Enterobacter*
VR3	*Serratia*
HGM 3.1	*Aeromonas*
BS 6	*Pseudomonas*
FN 16	*Stapylococus*
NLTA 3	*Enterobacter*
SL 1	*Staphylococcus*

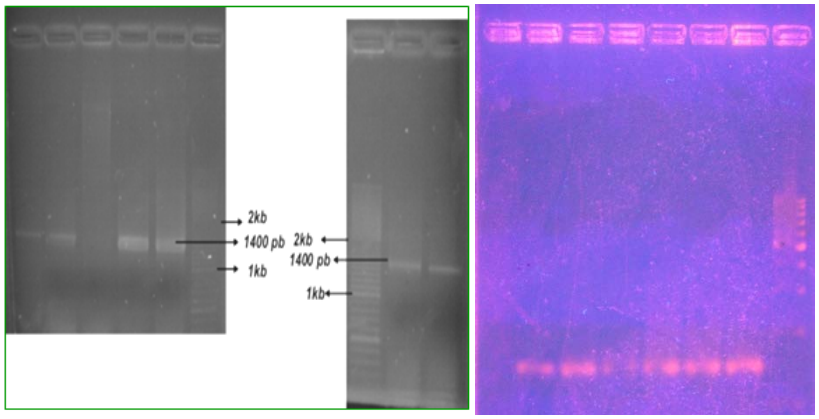

Fig. 4: PCR Product of 16S rDNA of marine and soil microorganisms under study, 1. CNN2; 2. VR 1.3; 3. FN16; 4. JW2; 5, 6, and 16. 100 bps DNA marker; 7. HGM 3.1; 8. SL1; 9. negative control; 10. Positive control; 11. BS6; 12. DL3; 13. DPA3; 14. HGM 3.1; 15. NLTA3

Table 6: Homology of 16s rDNA nucleotide sequence

No	Sample	Homology	Percentage of homology (%)
1.	JW2	*Alcaligenes sp.*	93
		Alcaligenes faecalis	93
2.	SL1	*Staphylococcus sp.*	98
		Streptomyces brasiliensis	98
		Staphylococcus equorum	98
3.	FN16	*Staphylococcus sp.*	99
		Staphylococcus equorum	99
4.	CNN2	*Salmonella typhi*	84
		Citrobacter sp.	84
5.	DPA3	*Pseudomonas fluorescens*	92
6.	BS6	*Pseudomonas sp.*	88
		Pseudomonas putida	88
		Pseudomonas syringae	88
		Pseudomonas alcaligenes	88
7.	NLTA3	*Enterobacter hormaechei*	94
8.	HGM 3.1	*Aeromonas sp.*	87
		Aeromonas popoffii	87
		Aeromonas veronii	87
		Aeromonas hydrophila	87
9.	DL3	*Enterobacter sp.*	80
10.	VR3	*Serratia sp.*	97
		Serratia marcescens	97

DISCUSSION

The prevalence of bacterial strains that are resistant to β-lactam antibiotics is effectively controlled by combining the antibiotics with β-lactamase inhibitors. The genus Streptomyces is known to be an outstanding natural source of antimicrobials and β-lactamase inhibitors such as clavulanic acid, most of which are applied in clinical practice [11]. But since years ago, resistance to the combinations of β-lactam and β-lactamase inhibitors in clinical isolates of *Escherichia coli* remains to be an important concern worldwide. This reason empowers the lead of new β-lactamase inhibitor discovery to improve the efficacy of ampicillin. Our data showed that the extracellular product of soil bacteria exhibit potency to contain β-lactamase inhibitor that inactivates β-lactamase produced by clinical isolate of ampicillin-resistant *E. coli*. The presence of inhibitory diameter around the extracellular products of all tested resistant bacteria indicated they contain β-lactamase inhibitor substances, considering there were no inhibition (0 mm in diameter inhibition) against the sensitive ones, except DL3 isolate. Based on morphology and biochemical assays, the tested bacteria can be identified at genus level only. Different isolates were characterized by observing colony characteristics and microscopic characteristics. Identification of bacteria using genotypic and phenotypic analysis gives a suitable result which is lead to the

correct identity of each bacterium. The species of ten tested bacteria were *Alcaligenes faecalis, Streptomyces brasiliensis, Staphylococcus equorum, Salmonella typhi, Pseudomonas fluorescens, Pseudomonas putida, Enterobacter hormaechei, Aeromonas popoffii, Enterobacter sp. and Serratia marcescens.* To our knowledge, the previously available β-lactamase inhibitors were not obtained from the β-lactamase-inhibiting microorganism investigated in our study. Among those spesies, extracellular product of VR3 isolate demonstrated the highest percentage of inhibition against resistant *E. coli.* The identity of VR3 isolate is *Serratia marcescens. S. marcescens* is a Gram-negative bacillus which can be found naturally in soil and water and at room temperature which produces a red color colony. It is linked to meningitis, endocarditis, osteomyelitis, septicemia, wound infections, eye infections, and respiratory and urinary infections [12]. In fact, *S. marcescens* has a significant trait due to their ability to produce a β-lactamase that gives resistance to β-lactam antibiotics, which frequently complicates therapy [13, 14]. This is interesting because as the β-lactamase producer, this bacterium resulted β-lactamase inhibitor against β-lactamase of other species as well. From these results, it can be assumed that the producer of this beta-lactamase inhibitor produces substances that can damage the catalytic site of the β-lactamase enzyme produced by ampicillin-resistant *E. coli*. At this time, approximately 1000 β-lactamases have been identified, representing a variety of structural classes, substrate promiscuities, and catalytic efficiencies [15, 16]. The type of β-lactamase enzyme produced by ampicillin-resistant *E. coli* used as the test bacteria in this study was the TEM β-lactamase encoded by the *bla* gene, as being probed by the used specific primers. The mechanism of unknown inhibitor substance from the extracellular products of those soil bacteria can be predicted from the mechanism of clavulanic acid and sulbactam as β-lactamase inhibitor in activating the TEM-1 β-lactamase [17]. They initially interact in ring scission with the beta-lactamase to produce an acyl enzyme with a conserved serine 70. This intermediate has the potential to hydrolyze, tautomerize to a more stable 3-aminoacrylate that promotes temporary inhibition, or result in considerable inactivation. It is hypothesized that the latter might be the outcome of a transamination process involving an unidentified active site residue [18, 19]. From this study, we also learned that the DL3 isolate's extracellular product inhibited both resistant and susceptible strains of *E. coli*. This sample has a larger inhibition zone for susceptible *E. coli* than for resistant *E. coli*. We hypothesized that the extracellular DL3 product would be more effective as an antibiotic at suppressing bacterial cell growth than its acting as a ®-lactamase inhibitor. The finding of antibiotics in extracellular products may have opened up new possibilities for treating *E. coli* infections that are resistant to treatment. This is related to the capability of soil bacteria to break down waste components and breakdown a wide range of organic compounds, including harmful bacteria and viruses [20].

CONCLUSION

In conclusion, this research strongly showed that the extracellular product of bacteria under study contain β-lactamase inhibitor activity and in less extend, contain activity to kill both resistant and sensitive *E. coli* strains. However, statistically, the different concentrations of their extracellular product did not exhibit any discernible inhibitory diameter.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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