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Research Report

The molecular phenomena of the blaZ genes forming beta-lactamase enzymes structure in *Staphylococcus aureus* resistant to beta-lactam antibiotics (ampicillin)

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ABSTRACT

Background: *Nowadays, infectious disease still an important problem. One of the bacteria causing infectious diseases is Staphylococcus aureus (S. aureus). In the effort to deal with infections caused by S. aureus, beta-lactam antibiotics, such as ampicillin, are used. In fact, it is unfortunately known that many of S. aureus bacteria are resistant to this group of antibiotics. Because of nucleotide base changes in the structure of the genes blaZ which encode beta-lactamase enzymes in S. aureus.* ***Purpose:*** *The objective of this study was to analyze the nucleotide base changes in the structure of the genes blaZ forming beta-lactamase enzymes in S. aureus resistant to ampicillin based on molecular point of view.* ***Methods:*** *Molecular examinations was conducted by isolating the genes, forming beta-lactamase enzyme, which length was 845bp, from 7 isolates of S. aureus resistant to ampicillin by using PCR technique. The results of blaZ amplification were then subjected to homology by using Tn 552 of S. aureus obtained from bank of genes.* ***Results:*** *Based on the result of the homology, it was found that there was a change in purine base T→G, which was a pyrimidine base at the -37 position of the initial codon of blaZ. This change, however, did not affect the strength of the promoter since the number of A and T is still more than the number of G and C. In the structure of the blaZ gene there was even no mutation or deletion or nucleotide base substitution found, so it would not affect the effectiveness of beta-lactamase enzyme.* ***Conclusion:*** *It can be concluded that the resistance of S. aureus towards ampicillin was not caused by nucleotide base deletion/substation. It is suspected that there were other causes leading to the resistance, including the overproduction of beta-lactamase enzyme of the blaZ gene, causing the degradation of beta-lactam antibiotics.*

Key words: *Staphylococcus aureus, ampicillin, blaZ*

ABSTRAK

Latar belakang: *Penyakit infeksi sampai saat ini masih merupakan masalah. Salah satu bakteri penyebab infeksi yaitu Staphylococcus aureus (S. aureus). Upaya menangani infeksi yang disebabkan S. aureus dapat menggunakan antibiotik golongan betalaktam, salah satunya ampisilin. Pada kenyataannya banyak S. aureus resisten terhadap antibiotik ini. Salah satu penyebab timbulnya resistensi ampisilin terhadap S. aureus yaitu adanya dugaan perubahan basa nukleotida dari gen struktur (blaZ) yang mengkode enzim betalaktamase.* ***Tujuan:*** *Untuk menganalisis perubahan basa nukleotida gen struktur pembentuk enzim betalaktamase pada S. aureus yang resisten ampisilin ditinjau secara molekuler.* ***Metode:*** *Pemeriksaan enzim betalaktamase secara molekuler dilakukan dengan mengisolasi gen pembentuk ensim betalaktamase (blaZ) yang memiliki panjang 845 pb terhadap 7 isolat S. aureus hasil isolasi yang berasal dari abses yang resisten terhadap ampisilin dengan mengunakan PCR . Hasil amplifikasi blaZ kemudian dilakukan homologi dengan Tn 552 S. aureus yang diperoleh dari bank gen.* ***Hasil:*** *Hasil homologi ditemukan adanya perubahan basa purin T → G yang merupakan basa pirimidin pada posisi –37 dari kodon awal blaZ.* ***Perubahan ini tidak mempengaruhi kekuatan promoter karena jumlah*** *A dan T masih lebih banyak dari G dan C. Pada gen struktur blaZ ini tidak terdapat adanya mutasi ataupun delesi maupun subsitusi basa nukleotida hingga tidak akan mempengaruhi efektifitas kerja enzim betalaktamase.* ***Kesimpulan:*** *Terjadinya resisten S. aureus terhadap ampisilin bukan disebabkan adanya mutasi maupun delesi/ subsitusi basa nukleotida dari blaZ namun diduga adanya sebab lain yaitu produksi berlebih enzim betalaktamase hingga semua antibiotik betalaktam akan didegradasi oleh enzim betalaktamase.*

Kata kunci: *Stafilokokus. aureus, ampisilin, blaZ*

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INTRODUCTION

Nowadays, infectious disease still an important issue, especially in developing countries. One of the bacteria causing infectious diseases is *Staphylococcus aureus (S. aureus*), which is a major bacterium causing osteomyelitis with the average percentage about 83% of 589 clinical isolates.[1,2] Antibiotics are commonly used to treat the infectious diseases caused by *S. aureus*. One of them is beta-lactam antibiotics, namely ampicillin.

Unfortunately, the use of antibiotics often does not follow the right rules, such as unapproriate dosage and usage procedure, and misuse (not in accordance with the illness or indications), as a consequence, it can make the resistance of the bacterium towards ampicillin increased.[3] *S. aureus,* for instance, is often resistant to beta-lactam antibiotics, one of which is ampicillin, not only because of the activity of beta-lactamase enzymes in inactivating beta-lactam antibiotics, but also because of the characteristics changes of beta-lactamase enzymes encoded by blaZ gene, from inductive into constitutive.[4]

The use of ampicillin related with *beta-lactamase enzyme,* is to split the active site of serine until this enzyme is not able to break the *beta-lactam* ring. Then, ampicillin will bind to proteins binding to penicillin (transpeptidase). This activity occurs since it has a structure similar to the peptidoglycan precursor, which is D-alanin type-D-alanin penta-peptide of N-Acetyl muramics. Ampicillin then disrupts the formation of inter-peptide, so the final stage of the formation of peptidoglycan is disrupted since instead trans-peptidase cuts penta-peptide (D-ala), trans-peptidase bind to ampicillin, as a consequence, the spliting of D-ala is disrupted, and the formation of murrain sac becomes imperfect.[5]

The use of ampicillin currently still generates a lot of problems as *S. aureus* is known to be resistant. The mechanism of the resistance is not only due to the inability of the antibiotics to split serine, considered as the active site of beta-lactamase enzyme, but also due to the amount of beta-lactamase enzymes produced is so high that ampicillin cannot inactivate beta-lactamase enzymes produced. The reason is because of the disruption of genes that regulate the production of beta-lactamase enzymes which change to be constitutive.[6] Some researchers even say that the character of resistance is suspected to be encoded in plasmids that have genes resistant to ampicillin, as a result, these plasmids can conjugatively be transferred.

Actually, ampicillin is molecularly resistant to *S. aureus* allegedly either because of the mutation of the binding area or the active site of serine in the structure of blaZ genes forming beta-lactamase enzyme, or because of the deletion of the structure of the genes forming beta-lactamase enzymes or the signal interference from the regulator genes in order to suppress the production of beta-lactamase enzyme.[6–8]

Beta-lactamase enzyme, on the other side, is enzyme produced extracellularly. In Gram-positive bacteria, this enzyme will be produced if there is the induction of beta-lactam antibiotics. As a consequence, in Gram-positive bacteria, the formation of beta-lactamase enzymes is considered to be inductive. This formation even is 10 to 100 times higher than that in Gram-negative bateria constitutively forming beta-lactamase enzyme. It means that if there is overproduction, it is supposed to be caused by the disruption of the regulation in the formation of beta-lactamase enzyme, thus, the amount of beta-lactamase enzymes will get higher. In other words, the disruption of the regulation in the production of beta-lactamase enzymes is caused by the loss of protein function regulation.[9–11]

Another reason that causes the increasing of beta-lactamase enzymes is the changing of the nucleotide sequence in the structure of the genes encoding beta-lactamase enzyme, blaz, which is phenotypically increased 2–3 times. There was also some researchers stating that if there is a change of nucleotide base, blaZ, it will then cause the effectiveness of this enzyme decreased.[9,10]

Therefore, the purpose of this study is to analyze the nucleotide base changes of the structure of the blaz gene in forming beta-lactamase enzymes in *S. aureus* resistant to ampicillin.

MATERIALS AND METHODS

The selection of samples then was conducted with consecutive sampling method based on the following inclusion criteria: a) *S. aureus* produces beta-lactamase enzymes tested by savinase disc; b) *S. aureus* produces total DNA isolations, chromosomes and plasmids; and c) blaZ gene fragments are isolated well.

The study consists of two stages: conducting a laboratory examination for selecting *S. aureus* resistant to ampicillin by using 10 μg ampicillin disc, and determining minimal inhibitor concentration (MIC) of *S. aureus* resistant to ampicillin. Besides that, savinase disc is also used to determine whether *S. aureus* produces beta-lactamase enzyme. Next, the molecular examination of total DNA isolation in which 5 ml bacterial culture is washed with 2 ml of 50 mM Tris-HCl and 200 μg/ml of RNase.[11–12] Afterwards, the suspension is incubated while shaken at 50rpm at the temperature of 37° C for about 30 minutes, and then is added with 400 ul of 50 mM 0.5% Tris sodium dodecyl sulfate, 0.4 M EDTA, and 1 mg proteinase K, and after that it is incubated in 500 C water bath for about one hour. The result of DNA isolation, finally, is precipitated with ethanol, and then is resuspended in 50–100 μl of 10 mM Tris-HCL containing 1 mM EDTA.[13]

Moreover, blaZ amplification used primer pairs and PCR. Primer pairs used are as the following:[13]
Forward: 5' TACAACTGTAATATCGGAGGG 3'
Reverse: 5' CATTACACTCTTGGCGGTTTC 3'

The sequence of PCR condition at blaZ amplification is: initial denaturation process occured at the temperature of 94° C for about 4 minutes; the cycle of denaturation process

occured at the temperature of 94° C for about 30 seconds; the attachment of primer occured at the temperature of 50° C for about 30 seconds; polymerization process occured at the temperature of 72° C for 2 minutes; and PCR performs in the 35th cycle and the stabilization phase occured at the temperature of 72° C. For one cycle, it takes 7 minutes with the last storage phase occured at the temperature of 4° C. If blaz fragments are amplified, sequencing then must be conducted to obtain nucleotide sequences, and finally homology process with Tn552 derived from the Gen-Bank is conducted by using the DNA Star program.

RESULTS

From this laboratory examination, 102 samples of *S. aureus* isolates derived from various types of abscess were obtained. Based on the result of the sensitivity test, it is then known that 69 isolates of them were resistant to ampicillin. To prove that these bacteria produce beta-lactamase enzyme, savinase discs then were used by applying *S. aureus* colonies on the savinase discs. If the color alters into pink, it will indicate that the bacteria produce beta-lactamase enzyme.

Molecular examination was also conducted to analyze the nucleotide changes that occur in the structure of the genes (blaZ fragment) by isolating the total DNA of 8 isolates that were resistant to ampicillin first. The results of the isolation observed through the analysis of agarose gel electrophoresis 1% then can be seen in the following figure 1.

As shown with a thin band, it is also known that the result of the isolated total DNA in obtaining plasmid was less satisfied since it migrated faster than the chromosomes did. The reason is because plasmid carriying genes encoding beta-lactamase enzymes in *S. aureus* are relatively small, which is about 6,7 kb, and has 4–10 copies, as a consequence, it can be concluded that the plasmid is classified into a kind of plasmid with small number of copies. Besides that, unlike in *E. Coli,* in which beta-lactamase enzymes are more greatly encoded in the plasmid, in *S. aureus, beta*-lactamase enzymes are more greatly encoded in the chromosome than in the plasmid.[9,10]

The electrophoresis results of amplified blaZ gene fragments of some isolates of *S. aureus* in agarose gel 1% is considered to be PCR products with 845 bp length. In amplification process, there were only 7 isolates of 8 ones amplified by blaZ. The results of the amplification

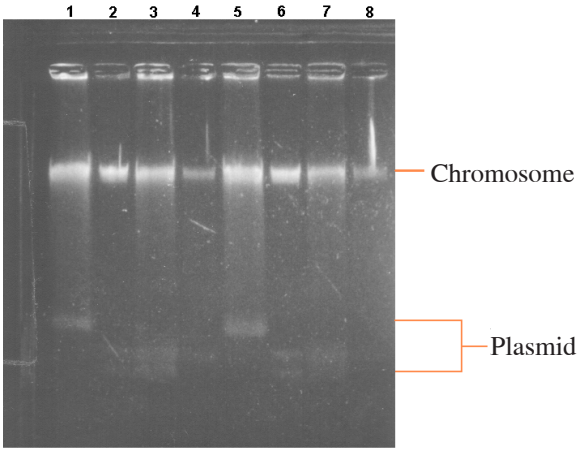

Figure 1. The isolated total DNA of 8 isolates of *S. aureus* resistant to ampicillin.

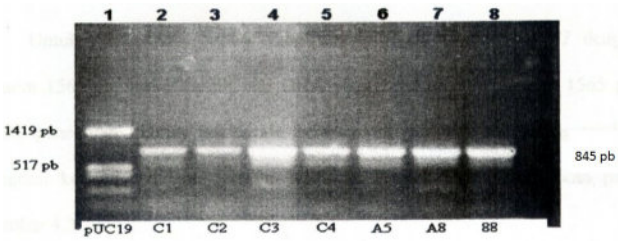

Figure 2. The results of blaZ isolation with 845 bp length.

process then were sequenced. Afterward, the nucleotide base sequence of the sequencing results were homologed, and the amino acid was deduced based on the sequence of *S. aureus,* Tn 552, obtained from the Gene Bank. This analysis used DNA star program in order to determine the homology of the nucleotide sequence and the deduction of amino acid sequence. The homology result of blaZ isolates of S. *S. aureus* and Tn 552 of *S. aureus* then can be seen in the following figure 3.

DISCUSSION

The amplification process of blaZ gene created DNA with 845 bp length, which was in line with the size of the base surrounded by a pair of primers located at nucleotide position 5372–5392 as primer 1 (forward) and at nucleotide positions 6192–6212 as primer 2 (reverse). It means that the amplification of blaZ was in line with the expected

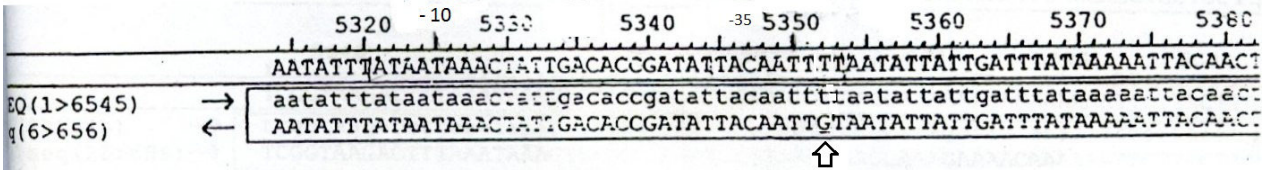

Figure 3. The homology result of *S. aureus* isolates resistant to ampicillin and *S. aureus* obtained from the Bank of Genes in promoter area showed that there were nucleotide changes at –37 (T→G) position.

length of base pairs since blaZ was relatively stable and even at the nucleotide sequence there would be not any secondary structure of DNA in blaZ fragment until blaZ could be amplified well. This condition actually has already been predicted that the blaZ does not form any secondary structures meanwhile genes that regulate the production of beta-lactamase enzymes either as repressor genes or as regulator genes are located in areas where secondary DNA was found, as a consequence, it is difficult to amplify those two genes.

The analysis results of sequencing blaZ isolates of *S. aureus* then was conducted by using the same pair of primers used during the blaZ amplification process. After that, the homology analysis of nucleotide sequence and *S. aureus* Tn 552 was conducted by using DNA star program. Next, the result was deduced into amino acids. Based on the result of the sequencing analysis of the nucleotide sequence of the promoter, ribosome binding side (RBS) of blaZ then was conducted to determine whether possesing strong promoter and RBS. The result of the homology of blaZ of *S. aureus* that is resistant to ampicillin has the same nucleotide composition with *S. aureus* Tn 552.

Based on the homology analysis of sequencing result of blaZ fragments, it is then known that there was mutation in the –37 position of the initial codon of T-G purine base of pyrimidine bases. However, this alteration did not affect the strength of the promoter since the number of A and T was still more than that of C and G. Similarly, another study also states that the alteration of the nucleotide sequence around the promoter consensus area with the number of A-T more than that of G-C still makes the promoter so strong that the transcription process can run well.[14]

Generally, the forming process of beta-lactamase enzymes in Gram-positive bacteria is actually considered to be inductive, as a consequence, strong promoter is needed. gram-positive bacteria has a strong promoter by generating *beta-lactamase* that is 8-18 times higher than gram-negative ones.[16] Beta-lactamase enzymes generated by gram-possitive bacteria is 30–60 times higher than that generated by gram-negative ones which production is considered to be constitutive.[14] The reason is because gram-positive bacteria have strong promoter, as a consequence, if the induction characteristics alters into the constitutive one with the strong promoter, the amount of beta-lactamase enzymes generated will be increasing. Besides that, the analysis of RBS homologed with th 552 of *S. aureus* indicates that there was no mutation, as a result, the number of A-T (6) (purine base) was still more than that of G-C (1) (pyrimidine bases). Therefore, it can be concluded that *S. aureus* that has strong RBS will have strong translation process which can increase the initiation complex on the 30S ribosomal subunit that still has the higher amount of purine base than the pyrimidines. The alteration of the nucleotide sequence about +1 in the consensus area caused by the addition of base A or T is very important for the strength of the promoter.[17] The mutation in this area then led to the increasing of the production of beta-lactamase enzyme. The observation conducted by Rowland and Dyke[19] even gives an illustration that the promoter of the blaz structure genes overlaps with the promoter of regulator gene.

In addition, in gram-positive bacteria, beta-lactamase enzymes will only be produced if there is the induction of beta-lactam antibiotics, and then the production beta-lactamase enzymes will be higher than that in gram-negative bacteria which beta-lactamase enzymes are produced as much as their base products.[9,11] This condition makes strong promoter needed for a strong transcription process in order to produce large amounts of beta-lactamase enzyme.

The results of nucleotide sequence analysis of the 7 isolates of *S. aureus* resistant to ampicillin at blaZ coding areas, called coding sequences. It indicates that there was no mutation, deletion, or substitution of nucleotide bases. Beta-lactamase will become ineffective if there is mutation in the blaZ structure genes, particularly in the active site, serine.[18] However, in this study, the resistance in S. aureus was not caused by the alteration of the active site of serine, but it was caused by another factor, which was the mutation of genes regulating the product of beta-lactamase enzyme.

S. aureus resistant to ampicillin was suspectedly caused by the excessive product of beta-lactamase, as a result, an ampicillin molecule could only be degraded by a molecule of beta-lactamase enzymes by breaking beta-lactam ring. Thus, ampicillin could bind to PBP 1–3 until the bacteria could stay alive or become resistant. To find out how many doses were needed to kill bacteria, a test of MIC then was also conducted in order to determine the inhibition dose of ampicillin. According to some researchers, the value of MIC is supposed to be 32u g/ml,[4,15] while the results of this study showed that the value of MIC was about 39–312 ug/ml. The high MIC volume then caused *S. aureus* become resistant because of the high MIC.

The treatment of infection still becomes problem until nowadays since there are still many bacteria, such as *S. aureus,* resistant to antibiotics, one of which is ampicillin. This resistance occurs because of the interference of beta-lactamase enzymes produced by *S. aureus*. Besides that, based on several studies, it is known that the resistance occurs because of mutations occured either in the binding areas or in serine active site in the structure genes of beta-lactamase enzyme, namely blaZ. However, in this study it is found that the resistance occured was not caused by the mutations in blaZ, but it was caused by another reason, which was supposed to be caused by interference in the regulator genes that caused the overproduction of beta-lactamase enzyme.[20] This condition is based on the test which result showed that the value of MIC obtained was high, about 39–312 ug/ml. It then can be concluded that to kill *S. aureus* a high amount of ampicillin is required since one molecule of ampicillin can be degraded by one molecule of beta-lactamase enzyme.

In conclusion, the resistance of *S. aureus* towards ampicillin is not caused by nucleotide base deletion/

substation. It is suspected that there are other causes leading to the resistance, including the overproduction of beta-lactamase enzyme of the blaZ gene, causing the degradation of beta-lactam antibiotics.

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