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THE FIRST INVESTIGATION OF AAC(6')-Ib ENZYME IN CARBAPENEM-RESISTANT *ENTEROBACTERIACEAE* ISOLATED FROM INDONESIAN PATIENTS

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ABSTRACT

Background: Over the past decade, numbers of Carbapenemase Producing-Carbapenem Resistant *Enterobacteriaceae* (CP-CRE) has been increasing worldwide and it has been becoming a threat because of its resistance against carbapenem which is considered as the “last resort” antibiotic. Therapy options for its infection are still limited. Aminoglycoside serves as one of the most commonly used antibiotics, but the resistance against it has already been presented for a long time. Aminoglycoside Modifying Enzyme (AME) is the most important resistance mechanism against aminoglycoside. AAC(6')-Ib enzyme is one of the most common AME produced by the gram-negative bacteria.

Objectives: This study wished to identify the gene of this enzyme among CRE isolated from infected Indonesian patients in Dr. Mohammad Hoesin Hospital Palembang.

Methods: Twenty-eight isolates collected from CRE-infected patients identified by Vitek 2 Compact (bioMerieux, USA) in dr. Mohammad Hoesin Hospital Palembang during September—November 2017. AAC(6')-Ib gene was identified using PCR method, then visualize by electrophoresis. The result is then analyzed by comparing it with a susceptibility test.

Results: Out of 28 samples, AAC(6')-Ib is identified in 22 (78.57%) samples. Samples with AAC(6')-Ib showed to be less resistant to various antibiotics, significantly to amikacin ($p=0.023$).

Conclusion: AAC(6')-Ib gene is found in most of samples implying its frequent occurrence in Indonesian patients.

Keywords : AAC(6')-Ib, Carbapenem Resistant *Enterobacteriaceae*, Carbapenemase

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INTRODUCTION

The usage of antibiotic has been increasing each year. Principally, antibiotic is used to treat infection, but in many cases, it is prescribed to treat patients without any indication of infection, or even if there is an infection, proper microbiological diagnosis is rarely done. Broad spectrum antibiotic is commonly used because of its wide range acting against bacteria. This leads to an increase in antibiotic resistance, which is one of the most concerning problems worldwide. Carbapenem is one of the beta-lactam antibiotics commonly used as the last resort because of its unique molecular structure that confers exceptional stability against most beta-lactamases. [1][2]

Enterobacteriaceae is the biggest group of gram-negative bacteria, which commonly confers high-level resistance against various antibiotics.[1] The carbapenemase genes are encoded inside the plasmids which explain its quick spreading over many countries.[2] The emergence of Carbapenem-resistant *Enterobacteriaceae* (CRE) is growing rapidly as it becomes a world's concern. Indonesia is reported to have a high prevalence of CRE, even the highest when compared to other countries in Asia.[3]

There are limited options for the treatment of infection caused by CRE.[4] Studies of the most effective regimens are still under investigation. Combination therapy is shown to be the most effective because of its fewer side effects and on treatment resistance. Aminoglycoside is one of the class of antibiotics used to treat CRE infection, but this antibiotic is different from others as it can be used as monotherapy, especially for urinary tract infection.[4] This antibiotic is also commonly available which makes it more likely to be used.

Aminoglycoside has been used since a long time ago to treat gram-negative bacterial infection, even before the emergence of carbapenem-resistant bacteria. So the resistance against it has been existing for a long time. The resistance against gentamicin (one of aminoglycoside) was shown to be higher than resistance against carbapenem in Dr. Mohammad Hoesin Hospital during 2017.[5] Additionally, aminoglycoside-modifying enzymes (AMEs) as the most clinically important resistance mechanism-against aminoglycoside are encoded in plasmids, which can also carry other resistance genes, including carbapenemase.[6]

This study analyzed the presence of AAC(6')-Ib gene, one of the most prevalent AME over the world in CRE isolates.[7,8] The results were then compared with the minimal inhibitory concentration (MIC) against a different class of antibiotics.

MATERIAL AND METHODS

Samples were obtained from specimens isolated from infected patients in Dr. Mohammad Hoesin Hospital Palembang from September through November 2017. CRE was identified using Vitek 2 Compact (bioMerieux, USA) for its susceptibility against ertapenem and meropenem. MICs against other antibiotics were also obtained by this method. From that period, 28 isolates were used for this study.

Antibiotics analyzed in this study were ampicillin, ertapenem, meropenem, amikacin, gentamicin, aztreonam, ceftazidime, ciprofloxacin, cefazolin, cefepime, nitrofurantoin, ampicillin-sulbactam, trimethoprim-sulfamethoxazole, tigecycline, and piperacillin-tazobactam.

The interpretation of MICs was made based on CLSI 2018 criteria.

Table 1. PCR optimization setting

Gene	Condition	T	Time	cycles
AAC(6')-Ib	Initiation of cell lysis	94°C	45 s	1x
	Denaturation	94°C	45 s	34x
	Annealing	51°C	45 s	1x
	Extension	72°C	45 s	1x
	Final Extension	72°C	5 min	1x

Identification of AAC(6')-Ib gene was done by Polymerase Chain Reaction (PCR) using specific primers (F: 5'-TTGCGATGCTCTATGAGTGGCTA-3'; R: 5'-CTCGAATGCCTGGCGTGTTT-3')[9]. The PCR mixtures used were 12 µL ddH₂O, 8 µL Green GoTaq®, 1 µL forward primer, 1 µL reverse primer and 3 µL of sample's DNA. The thermal setting condition for PCR was shown in table 1. The result was then visualized by agarose gel electrophoresis. The positive sample would display 482 bp band of the amplicon.

The presence of AAC(6')-Ib gene then analyzed statistically by comparing it with samples' median MIC values. The statistical analysis was made by Mann Whitney test. Significance was defined as $p \leq 0.05$ (two-tailed).

RESULTS

From twenty eight samples, 15 samples belong to *Klebsiella pneumoniae*, 7 samples belong to *Escherichia coli*, 4 samples belong to *Enterobacter cloacae* and 2 samples belong to *Serratia marscescens*. This study identified 22 samples (78.57%) carrying AAC(6')-Ib gene. *Klebsiella pneumoniae* is the most dominant (45.45%), followed by *Escherichia coli* (25%), then *Enterobacter*

cloacae (10.7%) and *Serratia marscescens* (7%). The visualization of the positive sample is shown in figure 1.

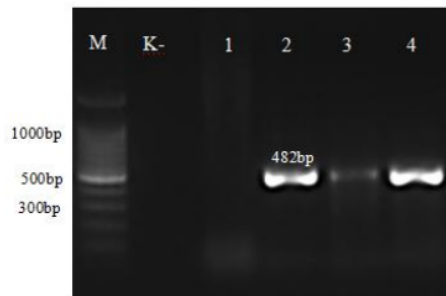


Figure 1. Electrophoresis result. The ladder used was 100 kb ladder. Positive result defined as displaying 482 bp band.

All isolates were resistant to ampicillin, ertapenem, and meropenem. Overall, samples with AAC(6')-Ib gene had a lower median MICs, except for tigecycline (Table 2). However, there was almost no significant difference between them. The only significant value was found in amikacin ($p=0.023$). The positive samples also had a lower median MICs than the negative samples in case of amikacin.

DISCUSSION

AAC(6')-Ib is the most prevalent detected AME.[7,8] AAC(6')-Ib belongs to AAC group which mediates the transfer of acetyl group from acetyl-CoA to 6' position of amine in aminoglycoside molecule.[10] This enzyme was first identified in *Klebsiella pneumoniae* in 1986, and now becomes widely spread among gram-negative bacteria. The reason behind of this spreading is the encoding site of this enzyme, which is located in a highly mobile genetic structure such as plasmids, integrons, and transposons.[11]

Table 2. Susceptibility test to various antibiotics of samples with and without AAC(6')-Ib gene

Antibiotics	AAC(6')-Ib Positive MIC (µg/mL) Median	AAC(6')-Ib Negative MIC (µg/mL) Median	p-value
Amikacin	2	64	0.023
Aztreonam	64	64	0.351
Ceftazidime	16	64	0.242
Ciprofloxacin	1	2	0.894
Ceftriaxone	64	64	0.492
Cefazolin	64	64	0.197
Cefepime	2	64	0.129
Nitrofurantoin	64	128	0.924
Gentamicin	12	16	0.253
Ampicillin-sulbactam	32	32	1
Trimetoprim-sulfamethoxazol	320	320	0.435
Tigecycline	2	1	0.897
Piperacillin-tazobactam	128	128	0.398

AAC(6')-Ib enzyme confers resistance mainly against amikacin[7,12], but the result of this study showed otherwise. The AAC(6')-Ib positive isolates are shown to be more resistant against gentamicin (50%) than against amikacin (22.7%). Overall, amikacin was the second most sensitive antibiotic among AAC(6')-Ib positive isolates (16 of 22 samples were shown to be sensitive). The presence of AAC(6')-Ib decreased median MICs significantly, from 64 to 2 µg/mL (p=0.026). Other studies showed that there are likely mutations (Leu119Ser, Leu120Ser, Glu167Ala, Phe171Ala, and Tyr166Ala in N-termini) occurring in *Enterobacter cloacae* isolates.[13–15] Similarly, all of the *Enterobacter cloacae* isolates in this study showed to be sensitive against amikacin. But there still needs further investigation for this. Furthermore, the mutation on different site was shown resulting in various degree of resistance level.[13]

Besides the probability of having carbapenemase genes, these isolates might also have ESBL genes. Other study states that the presence of at least three beta lactamases determines the presence of

AME because these genes are encoded on the same plasmid.[6]

Overall, the AAC(6')-Ib positive isolates were shown to be having lower MICs : they had lower median MICs against ceftazidime (p=0.242), however the values are still classified as resistant; they also had lower median MICs of cefepime, aztreonam, and nitrofurantoin, but the differences did not differ significantly.

Ciprofloxacin belongs to a class of antibiotics called fluoroquinolones. Theoretically this antibiotic is not included in AAC(6')-Ib's spectrum of action. However, 10 out of 22 AAC(6')-Ib positive samples are shown to be resistant against this antibiotic. It is likely because there are another variants of AAC(6')-Ib, which is known to have significant microheterogeneity at the N-termini[11]. One of the most prevalent variants present in gram negative bacteria is AAC(6')-Ib-cr [16] which also acetylates fluoroquinolones, including ciprofloxacin.[17]

There are mutations in AAC(6')-Ib-cr gene (102 codon Trp → Arg, 179

codon Asp → Tyr) which differentiate it from the original gene. These differences can not be detected by conventional PCR; it should be detected by the Restriction Fragment Length Polymorphism (RFLP) using BtsC1 enzyme. The original AAC(6')-Ib gene has a restriction site for that enzyme, as it shows 272 bp and 210 bp fragments, while AAC(6')-Ib-cr original gene stays as 482 bp.[18]

The presence of AAC(6')-Ib-cr variant could not be determined in this study. Despite probability having this gene, AAC(6')-Ib positive samples were shown to have a lower MIC of ciprofloxacin than the negative samples, however the difference was not significant. Other study states that even this gene is present, it would not significantly affect the MICs because this gene confers a low resistance level of ciprofloxacin.[19] There is should be at least 3-4 chromosomal mutations to increase the MIC value up to resistant category.[16]

Overall, fifty percent of CRE isolates were shown to be resistant to ciprofloxacin. It likely happens because ciprofloxacin is commonly used to treat bacterial infection since a long time so its resistance level is already high.[20] The most common mechanism is the presence of the other plasmid-mediated quinolone resistance (PMQR) like quinolone resistance proteins (qnrA, qnrB, qnrC, qnrD, and qnrS), which correlates with ESBL genes as they were often encoded on same plasmid.[21] Almost all of our isolates possessed ESBL, so the presence of other PMQR was also higher.

Seventy-five percent of samples were shown to be sensitive to tigecycline, making tigecycline as the most sensitive drug among all antibiotic tested. Samples with AAC(6')-Ib positive showed to have higher median MICs (2 µg/mL than 1.5

µg/mL in samples without this gene, p=0.897). It is not surprising as this antibiotic has already known as the option for CRE infection, but its use as monotherapy correlates with a high mortality rate.[22] Although this antibiotic exhibits high sensitivity in vitro, there is a problem during therapy since on treatment resistance often occurs.[23]

Administering a high dose of tigecycline only increases a small amount of its level in the plasma because it accumulates in intracellular and tissues, making it inappropriate for treating bacteremia.[24] High dose admission leads to an increase of its gastrointestinal side effects such as nausea, vomit, and diarrhea.[4] Tigecycline is also not suitable for treating urinary tract infections because of its low concentration in urine.[25] Furthermore, the Food and Drug Administration (FDA) in 2013 warned the usage of this antibiotic against nosocomial pneumonia because of its high mortality rate.[26]

Besides all of these limitations, tigecycline can be used effectively as combination therapy. One of the effective combinations is tigecycline and gentamicin or colistin which has 92% of effectiveness for treating multiple sites of CRE infections.[27]

CONCLUSION

The AAC(6')-Ib gene was detected in 78.57% CRE isolates. Overall, these isolates possessed lower median MIC values than isolates without this gene, making them more sensitive against various antibiotics, significantly was found against amikacin which is supposed to be affected by this gene. The most sensitive antibiotic found in this study was tigecycline.

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