

*bla*_{SHV-12} gene detection from *Klebsiella pneumoniae* producing Extended-Spectrum β -Lactamase using amplification-refractory mutation system method

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ABSTRACT

Extended-Spectrum β -lactamase (ESBL)-producing bacteria makes the bacterial disease more difficult to treat resulting in increased cost, longer duration of patient treatment along with increased morbidity and mortality. *Klebsiella pneumoniae* is one such bacteria known to produce ESBL. The objective of this study was to determine the presence of the *bla*_{SHV-12} gene in ESBL-producing *K. pneumoniae* from pneumonia patients at Dr. Hasan Sadikin Teaching Hospital, Bandung, and to determine the relationship of the *bla*_{SHV-12} gene with the incidence of antibiotic resistance. The *bla*_{SHV-12} gene was detected using Amplification-Refractory Mutation System (ARMS) method and visualized by UV spectroscopy following SYBR[®] safe DNA gel stain embedded agarose gel electrophoresis. The presence of the *bla*_{SHV-12} gene was indicated by the appearance of three bands at 756, 397, and 142 bp. To our knowledge, this is the first study to use the ARMS method with specific tetra primers in search of the *bla*_{SHV-12} gene. We collected antibiotic susceptibility data retrospectively from the Department of Clinical Pathology, testing with VITEK 2. Correlation test using phi coefficient tests. From 45 samples of ESBL-producing *K. pneumoniae* tested in this study, a total of 39 (87%) samples were *bla*_{SHV-12} gene-positive while 6 samples (13%) were *bla*_{SHV-12} gene negative. Our results indicate that the presence of the *bla*_{SHV-12} gene correlates with cefmethazole (*value* -0,544) but does not correlate with sensitivity to ampicillin/sulbactam, piperacillin/tazobactam, ceftazidime, meropenem, and meropenem.

Keywords: Amplification-Refractory Mutation System (ARMS), *bla*_{SHV-12} gene, Extended-Spectrum β -lactamase (ESBL), *Klebsiella pneumoniae*

Introduction

The inappropriate use of antibiotics leads to resistance. Based on a global analysis in 2019, antimicrobial resistance was responsible for 4.95 million deaths. Studies conducted at two teaching

hospitals in Indonesia showed that 30-80% of antibiotics usage was not based on any clear indication [1-3].

One of the commonly found antibiotic-resistant bacteria was *Klebsiella pneumoniae* (*K. pneumoniae*) which produces Extended-Spectrum β -Lactamase (ESBL) [4, 5]. Extended-Spectrum β -Lactamase is an enzyme with a wider resistance spectrum than the β -lactamase enzyme, due to mutations [6]. The ESBL enzyme triggers resistance to the β -lactam class of antibiotics, including penicillin as well as third-generation cephalosporin and aztreonam (**Figure 1**) [4, 7].

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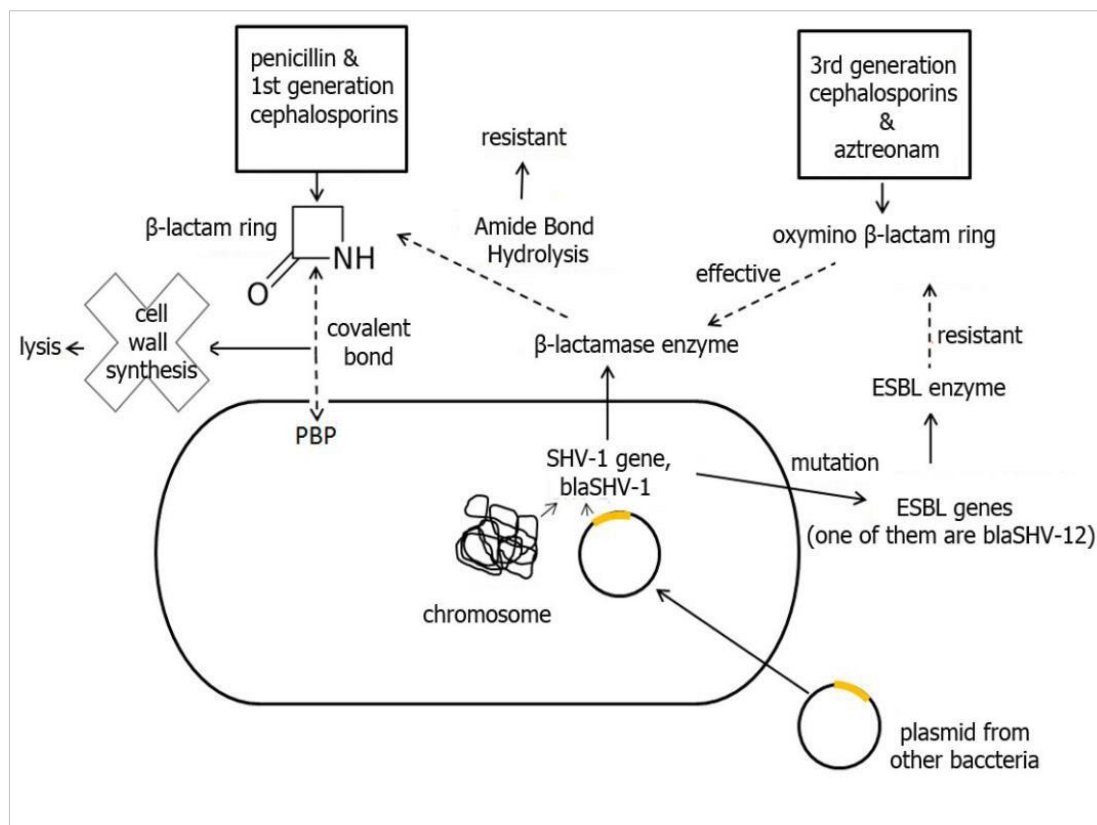


Figure 1. ESBL enzyme make bacteria more resistant to wider spectrum antibiotic than β -Lactamase enzyme

Infections caused by ESBL-producing bacteria increase the incidence of mortality in children and adults [8]. Moreover, because the resistance pattern to antibiotics is becoming wider, the existence of ESBL may increase the cost by prolonging treatment duration [9].

Three groups of most commonly found ESBL in bacteria including SHV, TEM, and CTX-M are expressed by *bla*_{SHV}, *bla*_{TEM}, and *bla*_{CTX-M} respectively [5, 10]. A previous study showed that SHV-12 is an ESBL enzyme that was most commonly found in *K. pneumoniae* [11]. A Korean study reported that the *bla*_{SHV-12} gene is more dominant in *K. pneumoniae* than other types of *bla*_{SHV} [12]. Data regarding the identification and analysis of these genes in Indonesia are still emerging.

The *bla*_{SHV-12} gene is a gene mutation from the *bla*_{SHV-1} gene. Mutations occur at codon L35Q (Leucine to Glutamine at codon 35) at the 92 bases, G238S (Glycine was changed to Serine at codon 238) at the 700 bases, and E240K (Glutamic Acid was changed into Lysine at codon 240) at the 703 bases [13, 14].

The objective of this study was to determine the prevalence of the *bla*_{SHV-12} gene in ESBL-producing *K. pneumoniae* from patients at the Dr. Hasan Sadikin Teaching Hospital, Bandung, and to determine the relationship of the *bla*_{SHV-12} gene with the incidence of antibiotic resistance.

We hypothesized that a high proportion of pneumonia patients at the Dr. Hasan Sadikin Teaching Hospital, Bandung are infected with ESBL-producing *K. pneumoniae* which has the *bla*_{SHV-12} gene and there is a correlation with antibiotic resistance.

Materials and Methods

Sample collection and preparation

The sample was collected from biological materials (blood) stored in the form of bacterial isolates of ESBL-producing *K. pneumoniae* obtained from the Department of Clinical Pathology, Dr. Hasan Sadikin Teaching Hospital, Bandung. The bacteria were derived from clinical specimens from the whole blood of patients infected with *K. pneumoniae* producing ESBL.

Bacterial isolates that were stored were recultured, then diluted with ultrapure DNase/ RNase free water, and boiled before DNA extraction. The DNA of bacteria was extracted using Purelink™ Genomics DNA Kit Invitrogen. The DNA was stored at 4°C until assay.

This research was conducted at the Laboratory of Molecular Biology, Dr. Hasan Sadikin Hospital, and Laboratory of Pharmaceutical Biotechnology, Faculty of Pharmacy, the University of Padjadjaran July to November 2015

Detection of *bla*_{SHV-12} gene using Amplification-Refractory Mutation System (ARMS)

Mutation analysis of the *bla*_{SHV-12} gene was performed using Amplification Refractory Mutation System (ARMS). The concentration of templates was analyzed using Lightwave II Biochrom. *bla*_{SHV-12} gene-positive in this study was indicated by

the appearance of three bands at 756 bp, 397 bp, and 142 bp, which showed there are mutations in three codons (L35Q, G238S, E240K).

Specific tetra primers of *bla*_{SHV-12} gene were used, forward primer 1 (F1) 5'-CAGCCGCTTGAGCAAATTAACAA-3', reverse primer 1 (R1) 5'-TGACGTTGTGCGCCGATCTG-3', forward primer 2 (F2) 5'-CGATAAGACCGGAGCTAGCA-3' and reverse primer 2 (R2) 5'-CCCGGCGATTTGCTGATTTTC-3'. The polymerase chain reaction was done using Maxima Hot Start Green PCR Master Mix (Thermo Scientific).

Stages of PCR of one cycle of an initial denaturation at 95° C for 4 minutes, 35 cycles of denaturation at 95° C for 30 seconds, 35 cycles of annealing at 55° C for 30 seconds, 35 cycles of extension at 72° C for 1 minute and 1 cycle of final extension at 72° C for 10 minutes.

Visualization of PCR products was done by electrophoresis from DNA fragment which has been amplified using 2% agarose gel which has been added with SYBR® safe DNA gel stain to aid visualize spotting. Furthermore, an hour of constant voltage of 100 Volts was given in 1x TAE buffer. Electrophoresis results were seen under UV light λ 312 nm using a fluorescence scanner (Gel Logic 2200 PRO®).

The susceptibility of samples data

The susceptibility of a sample obtained from computer data in the Clinical Pathology, Hasan Sadikin Hospital. Testing using VITEK 2 tools in the hospital.

Statistical analysis

The Chi-square test was used to analyze the categorical variables, with the assumed level of statistical significance at a $P < 0.05$. In

addition, the relationship between the *bla*_{SHV-12} gene and the occurrence of antibiotic resistance was measured using the phi coefficient tests.

Results and Discussion

Bacteria samples were derived from clinical specimens (whole blood) of 45 patients with *K. pneumoniae*. The characteristics of the study sample subjects are expressed in **Table 1**. Of the 45 samples, the majority from infants (44.44%) and males (51.11%).

Table 1. Characteristics of the study sample subjects (n = 45)

Characteristic	Quantity	Percentage
1. Gender :		
Male	23	51.11%
Female	22	48.89%
2. Age :		
0 days-27 days	20	44.44%
28 days-23 months	2	4.44%
2 years – 11 years	3	6.67%
12-18 years	1	2.22%
19-60 years	16	35.56%
>60 years	3	6.67%

Antibiotic susceptibility testing with VITEK 2 was used to detect bacterial resistance to antibiotics. Whether isolates were resistant, intermediately resistant, or sensitive. We collected data retrospectively from the Department of Clinical Pathology, Dr. Hasan Sadikin Teaching Hospital, Bandung. The susceptibility of samples in our study to specific antibiotics is shown in **Table 2**.

Table 2. The susceptibility of samples to a specific antibiotics

Antibiotic	Number samples tested	Resistant (%)	Intermediate (%)	Sensitive (%)
ampicillin	45	45 (100%)	-	-
ampicillin/sulbactam	41	39 (95.12%)	2 (4.88%)	-
piperacillin/tazobactam	44	11 (25%)	6 (13.64%)	27 (61.36%)
amoxicillin/ clavulanic acid	3	1 (33.3%)	1 (33.3%)	1 (33.3%)
cefoperazone/sulbaktam	3	-	-	3 (100%)
Aztreonam	31	31 (100%)	-	-
Cefazolin	31	31 (100%)	-	-
Cefalotin	9	9 (100%)	-	-
Cefoxitin	11	1 (9.09%)	-	10 (90.91%)
Cefmetazole	19	3 (15.79%)	-	16 (84.21%)
Ceftriaxone	34	34 (100%)	-	-
Cefotaxime	12	12 (100%)	-	-
Ceftazidime	45	45 (100%)	-	-
Cefepime	42	42 (100%)	-	-
Ertapenem	31	3 (9.68%)	-	28 (90.32%)
Meropenem	45	3 (6.67%)	-	42 (93.33%)
Doripenem	3	-	-	3 (100%)
Imipenem	3	-	-	3 (100%)

A positive result of the *bla*_{SHV-12} gene was shown with visualization of 3 bands due to amplification of DNA from a

mixture between the F1 with an R2 with a length of 756 bp, F2 with an R2 with a length of 397 bp, and F1 with an R1 with a

length of 142 bp (**Figure 1**). Visualization of the *bla*_{SHV-12} gene using electrophoresis in this study can be seen in **Figure 2** below:

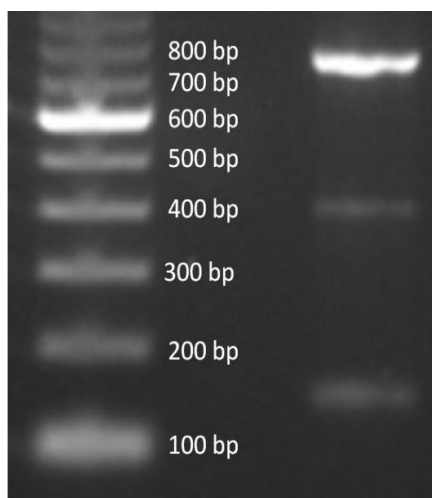


Figure 2. Visualization by electrophoresis, positive *bla*_{SHV-12} gene is indicated by visualization of 3 bands

Detection of the *bla*_{SHV-12} gene from ESBL-producing *K. pneumoniae* with the ARMS method showed that 86.67% (39 of 45) of the isolates were positive *bla*_{SHV-12} gene, and 13.33% (6 of 45) were negative for the *bla*_{SHV-12} (**Table 3**).

Table 3. The result of *bla*_{SHV-12} gene detection using ARMS method.

The result of <i>bla</i> _{SHV-12} gene detection	Quantity	Percentage
Positive	39	86.67 %
Negative	6	13.33 %

The incidence of antibiotic resistance combined with *bla*_{SHV-12} genes detection results can be seen in **Table 4**. Because of research data limitations, we cannot use the correlation test for ampicillin, cefotaxime, ceftazidime, ceftriaxone, and aztreonam. Ampicillin/sulbactam, piperacillin/tazobaktam, sefoksitin, ertapenem, meropenem has a *P* value > 0.05. Cefmetazole has a *P* value <0.05 (-0.544).

Table 4. Incidence of antibiotic resistance combined with *bla*_{SHV-12} genes detection results

Antibiotic	+ and R (%)	+ and S (%)	+ and I (%)	- and R (%)	- and S (%)	- and I (%)	R value*	P value**
ampicillin	39 (100)	-	-	6 (100)	-	-	-	-
Ampicillin /sulbactam	35 (94,6)	-	2 (5,4)	4 (100)	-	0 (0)	-0,074	0,634
piperacillin/ttazobactam	10 (26,3)	22 (57,9)	6 (15,8)	1 (16,7)	5 (83,3)	0 (0)	0,196	0,43
amoxicillin/clavulanic acid	-	-	1 (100)	1 (50)	1 (50)	-	1,000	0,223
cefoperazone/sulbactam	-	1 (100)	-	-	2 (100)	-	-	-
Aztreonam	27 (100)	-	-	4 (100)	-	-	-	-
Cefazolin	27 (100)	-	-	4 (100)	-	-	-	-
Cefalotin	9 (100)	-	-	-	-	-	-	-
Cefoxitin	1 (11,1)	8 (88,9)	-	0 (0)	2 (100)	-	0,149	0,621
cefmetazole	2 (11,1)	16 (88,9)	-	1 (100)	-	0 (0)	-0,544	0,018
ceftriaxone	28 (100)	-	-	6 (100)	-	-	-	-
cefotaxime	10 (100)	-	-	2 (100)	-	-	-	-
ceftazidime	39 (100)	-	-	6 (100)	-	-	-	-
Cefepime	38 (100)	-	-	4 (100)	-	-	-	-
Ertapenem	2 (7,4)	25 (92,6)	-	1 (25)	3 (75)	-	-0,199	0,267
meropenem	2 (5,1)	37 (94,9)	-	1 (16,7)	5 (83,3)	-	-0,157	0,292
Doripenem	-	1 (100)	-	-	2 (100)	-	-	-
Imipenem	-	1 (100)	-	-	2 (100)	-	-	-

+ = *bla*_{SHV-12} gene positive, - = *bla*_{SHV-12} gene negative, R= resistant to antibiotic, S = sensitive to antibiotic, I= intermediate to antibiotic

* measure of association for two variables with *phi coefficient tests*

** *Chi square*, significance at *P value* < 0,05

The research conducted in this study was performing a culture of bacteria isolates that met the inclusion criteria, which was ESBL-producing *K.pneumoniae* derived from patient's blood clinical specimens from the Department of Clinical Pathology, Dr. Hasan Sadikin Teaching Hospital, Bandung. Furthermore, bacteria isolates were extracted to obtain isolates of bacterial DNA. The DNA isolates were then amplified and the results were visualized by electrophoresis.

To our knowledge, this is the first study to use the ARMS method with specific tetra primers in search of the *bla*_{SHV-12} gene (**Figure 3**). DNA base sequence of the *bla*_{SHV-12} gene *K. pneumoniae* obtained from GenBank at the National Center for Biotechnology Information (NCBI) is one of the largest database sequences [15]. We then match it with the amino acid sequence of SHV-12 and SHV-1 at Lahey.org/studies that displays the β -lactamase classification and amino acid sequences for TEM, SHV, and OXA [14].



Figure 3. Specific Tetra Primers in Search of *bla_{SHV-12}* Gene

ARMS method used in this study is also known as allele-specific PCR (ASP). The ARMS is a simple and commonly method to detect single base mutations change or base deletions. In principle, ARMS use PCR primers with specific sequences which make DNA amplification test occurs only when the target allele is present in the sample. When the primer matches the template, amplification will take place [16]. The presence or absence of ARMS product results could then simply be analyzed by electrophoresis agarose gel [17], and visualized by UV spectroscopy.

Bla_{SHV-12} gene has a mutation at codons 35, 238, and 240. To determine the presence of mutations in codon 35 we were using specific primer F1 and R1 with a DNA amplification length was 142 bp. Meanwhile, to confirm the presence of mutations at codons 238 and 240, we were using specific primer F2 and R2 with a DNA amplification length was 397 bp. While one spot in 756 bp occurs unexpectedly, which we know later as amplification of DNA from a mixture between F1 and R2, that we can also use to confirm the mutation in codon 35.

In our study, most of the subject samples were in the age range of 0-27 days, as many as 20 (44.44%) subjects and followed by an age range of 19-60 years in 16 subjects (35.56%). According to UNICEF/WHO, Pneumonia: The Forgotten Killer of Children, it has been estimated that 26 percent of neonatal deaths, or 10 percent of all under-five deaths, are caused by severe infection. And a significant proportion of these infections is caused by pneumonia/sepsis [18]. Annually, an estimated three million newborn deaths occur, and 36% of these are caused by Neonatal septicemia (NNS). NNS is the main cause of neonatal morbidity and mortality worldwide [19]. The morbidity and mortality high in neonates due to their low immunity. *K.pneumoniae* is found a lot in stool specimens, so it is easy to contaminate neonate's environment and can cause nosocomial infection, even has potential to last for more than three years [7]. *K. pneumoniae* became an important cause of hospital-acquired infections, notably among patients in the neonatal intensive-care unit and mortality rates are very high (70%) [20]. Recently published reports said that males were more vulnerable to *Klebsiella* infection than females [21]. In the current study,

from the 45 subjects of bacterial samples tested, there were 23 (51.11%) male subjects and 12 (48.89%) women subjects. Similarly in one study in Canada, From 640 *K. pneumoniae* bacteremia incidence cases, 370 (58%) occurred in men [22]. Recent studies from Saudi Arabia accounted for 55% of 400 isolates are ESBL positive, whereas 97,3% are *bla*_{SHV} gene positive. Another study in Iran also showed a high frequency of the *bla*_{SHV} gene [23]. Other studies in Mexico, of 92 *K. pneumoniae* bacteria, SHV-12 are the enzymes predominantly found (51.5%) [11]. A study in Korea also found the *bla*_{SHV-12} gene more dominant than the other types of *bla*_{SHV} gene [12]. This suggests that the gene *bla*_{SHV-12} that passes through several stages of mutations is already emerging.

In line with this, the detection results of the *bla*_{SHV-12} gene with the ARMS method in this study showed that 39 samples (87%) of the 45 samples tested were positive *bla*_{SHV-12} gene, and 6 samples (13%) were the negative *bla*_{SHV-12} gene. This study shows that the gene *bla*_{SHV-12} has become the dominant gene in ESBL-producing *K. pneumoniae* in Dr. Hasan Sadikin Teaching Hospital. It also showed that the *bla*_{SHV-12} gene ESBL-producing *K. pneumoniae* plays a role in the incidence of resistance to an antibiotic in Dr. Hasan Sadikin Teaching Hospital. The high number of positive samples that had *bla*_{SHV-12} genes may show a third-generation cephalosporin antibiotic use and aztreonam were quite high.

We cannot use the correlation test for ampicillin, cefotaxime, ceftazidime, ceftriaxone, and aztreonam because of research data limitations. While ampicillin/sulbactam, piperacillin/tazobactam, cefoxitin, ertapenem, and meropenem have a *P*-value > 0.05, which means there is no significant correlation between the two variables tested, the incidence of antibiotic resistance, and the positive or negative *bla*_{SHV-12} gene. In contrast, cefmetazole has a *P*-value <0.05 (approx. sig 0,018), which means there is a significant correlation between the two variables tested. The correlation value of cefmetazole is 0.544, which indicates a moderate correlation level. Directions correlation is negative (-) indicates that the more samples showed positive *bla*_{SHV-12} gene, the less it resistant to these antibiotics.

Based on one research, all the ESBL phenotype *K. pneumoniae* were susceptible to some antibiotics including cefmetazole [24]. Also in other research, all ESBL producers were susceptible to imipenem [25]. In other journals, it proved that cefmetazole exhibited high activity against *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis* isolates [26]. Cefmetazole is still sensitive even though there is *bla*_{SHV-12} because it has a 7-methoxy structure that effective enough to deal with the ESBL-producing bacteria.

ESBL enzymes can inactivate β -lactam antibiotics, and oxyimino groups, such as oxyimino-cephalosporins (ceftazidime, ceftriaxone, or cefotaxime) or oxyimino-monobactam (aztreonam). ESBL can inactivate the antibiotic in the presence of serine in the active site that can attack the amide bond in the β -lactam ring so that the β -lactam ring can be hydrolyzed (Figure 1) [27].

In this study, *K. pneumoniae* that tested proved 100% resistant to penicillin, first-generation cephalosporins (cefazolin, cephalothin), and third-generation cephalosporins (ceftazidime, cefotaxime, ceftriaxone), and aztreonam. So that this antibiotic can no longer be used to treat bacterial infections caused by ESBL-producing *K. pneumoniae*.

Among antibiotics, the carbapenem class has a higher sensitivity against ESBL-producing *K. pneumoniae*. Where ertapenem, meropenem, doripenem, and imipenem have a sensitivity level of 90.32%, 93.33%, 100%, and 100% respectively. So imipenem or doripenem may be the primary choice for overcoming a bacterial infection of ESBL-producing *K. pneumoniae*.

This is in corroboration with an earlier report published that says carbapenem is the most effective drug of choice for infections caused by ESBL-producing *K. pneumoniae*. Carbapenem-resistant to hydrolysis activity of the enzyme ESBL because it has a trans-6-hydroxyethyl group [4]. Also imipenem was found to be the most effective antibiotic in Iran and Saudi Arabia for cases of infection due to *Klebsiella* [23].

Beside cefmetazole, cefoxitin also has a 7-methoxy structure that effective enough to deal with the ESBL-producing bacteria [4]. Cefoxitin and cefmetazole have a sensitivity level of 90.91%, and 84.21% respectively. So it can be used to treat infections caused by ESBL-producing *K. pneumoniae* but not as first-choice antibiotics.

Although it said that ESBL activity can be inhibited by clavulanic acid [4], in this study showed a combination of amoxicillin and clavulanic acid cannot be used as the drug of choice for the treatment of ESBL-producing *K. pneumoniae*. Of the three samples tested, 1 bacteria sample shows the sensitive result (33.3%), 1 shows a resistant result (33.3%), and one shows an intermediate result (33.3%).

The combination of other penicillin/beta-lactamase inhibitors such as ampicillin/sulbactam and piperacillin/tazobactam shows 0% and 61.36% sensitivity respectively, so cannot be used as the drug of choice for the treatment of ESBL-producing *K. pneumoniae*. While the combination of a third-generation cephalosporin with a beta-lactamase inhibitor that is cefoperazone/sulbactam can still be used, from 3 samples that were tested, all samples are still showing 100% sensitivity with antibiotics cefoperazone/sulbactam.

Cefepime is mentioned to be more resistant to ESBL hydrolysis than the third-generation cephalosporins [28], but in this study, the level of resistance of this fourth-generation cephalosporins is 100% resistant. This may be because these antibiotics have been widely used to treat a variety of infections. So cefepime cannot be used for therapy against ESBL-producing organisms in Hospital Dr. Hasan Sadikin.

The inappropriate use of antibiotics is responsible for the development of resistance to antibiotics. Hence, there is a need to emphasize the rational use of antibiotics.

The limitation of this study was this method can only identify one ESBL encoding gene, which was *bla*_{SHV-12}. The other ESBL encoding genes such as *bla*_{SHV-5}, *bla*_{SHV-2a}, *bla*_{CTX-M}, or *bla*_{TEM} in

K. pneumoniae isolates were not detected in this study. Another limitation is for ampicillin, aztreonam, cefotaxime, ceftriaxone, and ceftazidime correlation tests cannot be performed because of limited research data (all samples are positive *bla*_{SHV-12} gene, and we use no control groups to compare outcomes).

Conclusion

From 45 samples of ESBL-producing *K. pneumoniae* that were tested, there were a total of 39 (87%) samples that *bla*_{SHV-12} gene positive, and 6 samples (13%) that *bla*_{SHV-12} gene negative. presence of blast-12 gene correlates with cefmetazole (*value* - 0,544) but does not correlate with sensitivity to ampicillin/sulbactam, piperacillin/tazobactam, ceftaxime, ertapenem, and meropenem.

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Conflict of interest: None

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Ethics statement: This study was approved by the Ethics Committee for Health Research, Faculty of Medicine, the University of Padjadjaran Bandung with the letter number 329/UN6.C1.3.2/KEPK/PN/2015.

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