MOLECULAR DETECTION OF VEB AND OXA-23 RESISTANCE GENES IN *PSEUDOMONAS AERUGINOSA* ISOLATES AT DR. WAHIDIN SUDIROHUSODO HOSPITAL MAKASSAR

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ABSTRACT

Pseudomonas aeruginosa is a common gram-negative pathogen in nosocomial infections in immune-compromised patients. It exhibits high rates of intrinsic resistance to many classes of antibiotics, especially β-lactam antibiotics. Production of extended-spectrum β-lactamase (ESBL) and genes belonging to the carbapenem-hydrolyzing class D subgroup β-lactamases (CHDL) are a problem for increasing antibiotic resistance worldwide. This study aimed to identify *P. aeruginosa* containing the VEB and OXA-23 genes, which are the genes that cause antibiotic resistance in gram-negative bacteria. There are eighty-five clinical isolates from various types of clinical samples identified as *P. aeruginosa* and tested for antimicrobial susceptibility using VITEK 2 compact. VEB and OXA-23 genes were detected using the Polymerase Chain Reaction (PCR) method. The PCR results revealed that 13 (15.3%) of *P. aeruginosa* isolates were positive OXA-23 gene, but no isolate was positive for the VEB gene in *P. aeruginosa* isolates. The study results demonstrated the spread of the OXA-23 gene that was proven to be present and distributed in *P. aeruginosa* isolates in Makassar, South Sulawesi.

Keywords: OXA-23; *Pseudomonas aeruginosa*; Resistance gene; VEB.

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative pathogen that causes life-threatening nosocomial infections in immunocompromised patients.[1] *Pseudomonas aeruginosa* is included in the critical priority pathogen resistance category to many classes of antibiotic agents which require urgent new antibiotic intervention.[1,2] The development of pathogen resistance is due to selective suppression of mutations in chromosomal genes leading to broad-spectrum beta-lactamase (ESBL) production, oprD inactivation or repression, AmpC hyperexpression, and overexpression of the efflux pump, which ultimately leads to treatment failure.[3,4]

β-lactamases are classified into four classes by Ambler: A, B, C, and D, and have been found in *P. aeruginosa*.[5] The Vietnamese extended-spectrum β-lactamase (VEB) gene, one of class A β-lactamase, encodes a rare enzyme responsible for broad-spectrum cephalosporin resistance and is linked to high levels of resistance to ceftazidime, cephems, and monobactams,.[6,7]

Oxacillinase (OXA) or OXA type enzyme belongs to class D β-lactamase. This enzyme can hydrolyze oxacillin much faster than penicillin and benzylpenicillin. 350 OXA-type enzymes are genetically distinct among gram-negative bacteria, widely distributed, and responsible for most antibiotic resistance.[8,9] Since one more

decade ago, OXA enzymes with carbapenemase activity have increased and become a global public health crisis. OXA-23 belongs to the class D β-lactamase with carbapenemase activity which was first reported in Scotland; this gene was detected in *Acinetobacter baumanii* isolates mediated by a plasmid.[9,10] OXA-23 could cause resistance to carbapenems like meropenem (MRP), doripenem (DRP), and imipenem (IMP).[11]

The distribution of VEB and OXA-23 genes has been widely reported in *P. aeruginosa*, but studies of these genes in *P. aeruginosa* isolates from Makassar have not been reported. Therefore, it is essential to detect this gene for infection control purposes and explain the epidemiology existence among isolates obtained in Dr. Wahidin Sudirohusodo Hospital, Makassar.

MATERIAL AND METHODS

Bacterial Isolates

Eighty-five stored isolates of *P. aeruginosa*, which originated from patients in the Clinical Laboratory of Dr. Wahidin Sudirohusodo Hospital, were isolates derived from various clinical samples, namely urine, sputum, blood, pus, gastric lavage, bronchial washings, ascitic fluid, pleural fluid, ear secretions, and tissue which was identified using VITEK 2 Compact.

All isolates were revived by inoculation into MacConkey agar and incubated at 37ºC overnight. The colonies that grew were confirmed as isolates of *P.aeruginosa*. Testing samples were carried out from March 2021 to August 2021 at the Universitas Hasanuddin Hospital Laboratory.

Antimicrobial Susceptibility Test

P. aeruginosa isolates were tested for antimicrobial susceptibility using VITEK 2 Compact (bioMérieux, France) and AST cards (GN93 and N317).

DNA Extraction

The DNA Extraction process followed the instructions on the gSync DNA Extraction Kit (Geneaid Biotech Ltd). DNA extraction was started by making bacterial suspension (Mac Farland Bacteria 0.5 – 1) in an Eppendorf tube, centrifuged for 5 minutes at a speed of 300 x g, discarding the supernatant then resuspend cells in 200 ul of PBS by pipette, added 20 µl of Proteinase K then mix by pipetting, and incubated for 5 minutes at 60ºC. Cells lysed by adding 200 µl of GSB buffer, incubated at 60°C for 20 minutes, where every 5 minutes vortexed. 200 µl of ethanol was added in the DNA binding step, then mixed for 10 seconds, put it into the GS Column in a 2 ml collection tube, centrifuged for 1 minute at 14,000 – 16,000 rpm, then discard the liquid in the collection tube. The sample was washed with 400 µl of W1 Buffer and centrifuged for 30 seconds at 14,000 – 16,000 rpm, discarded the liquid in the collection tube, 600µl Wash Buffer was added and centrifuged for 30 seconds at 14,000 – 16,000 rpm. Next, replace the collection tube with a new one and centrifuge for 3 minutes at 14,000 – 16,000 rpm. Then, transfer the GS Column to a sterile Eppendorf tube in the elution stage and add 100 µl of the previously heated elution buffer. After that, centrifuged for 30 seconds at 14,000 – 16,000 rpm, then discarded the GS Column. The liquid contained in the Eppendorf tube is a DNA product ready for PCR.

Molecular Detection of *bla*$_{VEB}$ and *bla*$_{OXA23}$

Amplification reactions for *bla*$_{VEB}$ were prepared in a total volume of 25 µl containing 0.5 µl of forward primer (5'-cgacttccatttcccgatgc-3'), 0.5 µl of reverse primer (5'-ggactctctgcaacaaatacgc-3'), 12.5 µl of Go Taq Master Mix, 5.0 µl of DNA sample, and 6.5 µl of nuclease-free water. The following temperatures were used to detect the VEB gene: 95ºC for 5 minutes (Pre-Denaturation); 30 cycles of 95ºC for 1 minute (denaturation), 54.6ºC for 1 minute (annealing), and 72ºC for 72 minutes(extension); 72ºC for 10 minutes

(final extension).[3] PCR amplification for blaOXA23 was carried out in a final reaction mixture of 25 µl containing 12.5 µl of Go Taq Master Mix, 0.5 µl for each primer (forward: 5'-gatcggattggagaaccaga-3') and (reverse: 5'-atttctgaccgcatttccat-3'), 5.0 µl of DNA Sample, and 6.5 µl of nuclease-free water. The optimal conditions for the Detection of the *bla*$_{OXA23}$ gene are with the following temperatures: 94ºC for 5 minutes (Pre-Denaturation); 30 cycles of 94ºC for 25 seconds(denaturation), 52ºC for 40 seconds(annealing), and 72ºC for 50 seconds(extension); 72ºC for 5 minutes (final extension).[12] Electrophoresis was processed with 2% agarose gel containing ethidium bromide (20 µl) at 50 V/cm for 180 minutes and detected by UV transillumination.

RESULT

Characterization of Specimens

In the present research, 85 *P. aeruginosa* isolates grouped by gender were dominated by men (n = 54/63.5%), with the group mostly over 45 years (n = 46/54%), and the most clinical specimens were found in pus (n = 33/38.8%) and sputum (n = 26/30.6%).

Table 1. Distribution of *P. aeruginosa* isolates according to gender, age group, and clinical specimens.

Isolates	Percentage (%)
Gender	
Man	54(63,5%)
Female	31(36,5%)
Age group (year)	
Up to 11	11(13%)
12 to 25	11(13%)
26 to 45	17(20%)
Over 45	46(54%)
Clinical specimens	
Blood	5(5,9%)
Pus	33(38,8%)
Urine	3(3,5%)
Sputum	26(30,6%)
Ear secretion	3(3,5%)
Sputum ETT	3(3,5%)
And others	6(7,6%)

*Others**:** gastric lavage, bronchial washings, ascitic fluid, and pleural fluid

Phenotypic Test

Antimicrobial susceptibility tests to several antibiotics revealed that doripenem and meropenem had the lowest resistance rates, with 15.3% and 16.5%, respectively, followed by imipenem and ceftazidime 18.8% and 27%, respectively (Table 2).

Table 2. *P. aeruginosa* sensitivity test results in several types of antibiotics

Antibiotic	S(%)	I(%)	R(%)	N(%)
Ceftazidime	68,2%	4,7%	27%	-
Meropenem	78,8%	4,7%	16,5%	-
Imipenem	61,2%	1,2%	18,8%	18,8%
Doripenem	64,7%	3,5%	15,3%	16,5%

*S: Susceptible, I: Intermediate, R: Resistant, None: no phenotypic test

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Genotypic Test

The results of the PCR examination of *P. aeruginosa* isolates showed no VEB gene was found, and 13 (15.3%) isolates found the OXA-23 gene (Table 3).

Table 3. Distribution of Genes Found in *P. aeruginosa* clinical isolates

Gene	Positive(%)
bla_{VEB}	0%
bla_{OXA23}	15,3%

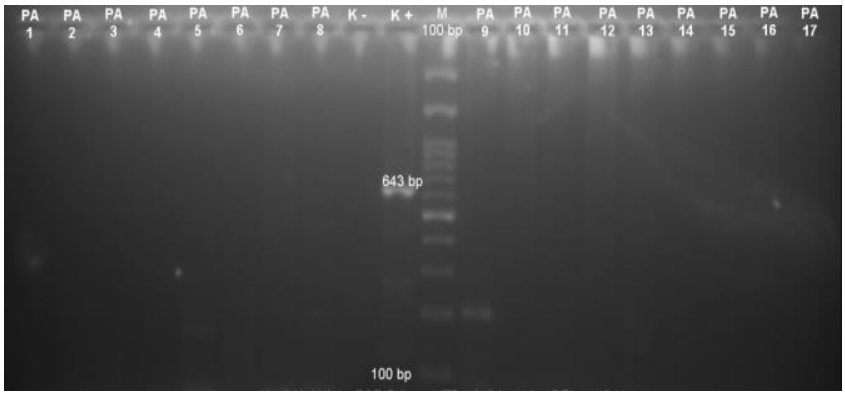

Figure 1. Electrophoresis results of the VEB gene PCR product with 643 bp marker., K-: negative control, K+: positive control (643 bp), and M: marker 100 bp: DNA ladder.

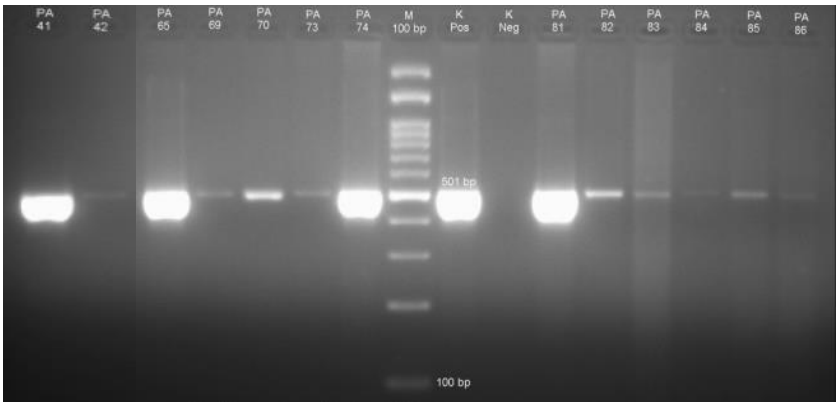

Figure 2. Electrophoresis results of the OXA-23 gene PCR product with 501 bp marker. From left to right: PA41, PA42, PA65, PA69, PA70, PA73, PA74, M 100 bp: DNA ladder, K Pos: positive control (501 bp), K Neg: negative control, PA81, PA82, PA83, PA84, PA85, and PA86.

Figure 1 shows the absence of a specific band, which means that there is no VEB gene in *P. aeruginosa* isolates. Figure 2 shows the presence of specific bands corresponding to positive controls in samples PA41, PA42, PA65, PA69, PA70, PA73, PA74, PA81, PA82, PA83, PA84, PA85, and PA86, which means these samples were positive for the OXA-23 gene.

DISCUSSION

Pseudomonas aeruginosa is one bacteria that causes a difficult-to-treat infection due to its resistance to several clinically significant antibiotics, including third-

generation cephalosporins, aztreonam, and imipenem.[13] The increasing resistance of *P. aeruginosa* to several antibiotics due to over-administration of antibiotics and its multifactorial resistance mechanisms, such as changes in target antibacterial agents, inactivation or inhibition of enzymes, overproduction by the efflux system, and loss of outer membrane porin.[14,15]

In this study, resistance to carbapenem antibiotics (imipenem, doripenem, and meropenem) showed 16 isolates (18.8%), 13 isolates (15.3%), and 14 isolates (16.5%), respectively. Meanwhile, resistance to cephalosporin antibiotics, namely ceftazidime, was in 23 isolates (27%). A study conducted in the Philippines in 2017 yielded similar results, which stated *P. aeruginosa* resistance to carbapenems, including meropenem (17.1%) and imipenem (16%).[15] According to research in China from 2007 to 2014, there was a significant increase in carbapenem antibiotic resistance; meropenem (38.5% to 87.3%) and imipenem (42.3% to 86.2%). Meanwhile, the antibiotic ceftazidime in the last year was 14.3% to 46.2%.[14] According to studies from various countries, *P. aeruginosa* resistance to ceftazidime ranges between 10% and 50%.[16] Intensive and widespread use of carbapenems and cephalosporins led to increased resistance in clinical isolates of P. aeruginosa.[16]

One of the global public health challenges and epidemiological scenarios is the spread of carbapenem antibiotic resistance among *P. aeruginosa* isolates. It is often associated with carbapenem-coding genes associated with endemic carbapenemase genes and the use of carbapenems in clinical medicine.[17] The increased resistance to cephalosporin antibiotics can be attributed to the development of cephalosporinases among *P. aeruginosa* isolates. The emergence of cephalosporinases can affect their usefulness in clinical medicine, considering that cephalosporin antibiotics have a broad spectrum of activity in controlling several infections.[18]

The results of the PCR test showed that there was no VEB gene (0%) which is one of the carriers of the ESBL resistance encoding gene. The spread of genes encoding extended-spectrum beta-lactamases (ESBL) is starting to worry because of the highly adaptive nature of the ESBL gene and the large number of variants that have emerged. The VEB gene has been reported in several countries in *P. aeruginosa* isolates, such as Thailand, Poland, Saudi Arabia, and Iran.[1,13,18,19] On the contrary, in this study, none of the VEB genes were detected in 85 isolates of *P. aeruginosa*. Similar results were also shown by a study conducted by Lee in 2015 in Korea, which did not find the presence of the VEB gene from 252 *P. aeruginosa* clinical isolates.[20]

The absence of the VEB gene can be caused by the VEB gene belonging to the minor type of class A β-lactamase gene, which is unrelated to any of the three major ESBL identified—reported CTX-M, TEM, and SHV. In addition, the protein homology level of the VEB gene with the well-known class A beta-lactamase is less than 20%, while the PER-1 and PER-2 genes have the highest percentage of amino acid identity (38%).[21,22]

This study detected 13 (15.3%) OXA 23 genes in *P. aeruginosa* isolates. A study in Iran found similar results, reporting that 15 (11.19%) of *P. aeruginosa* isolates contained the OXA-23 gene.[11] Another study conducted in India using 74 *P. aeruginosa* clinical isolates reported that the PCR results showed 5 (6.7%) positive isolates of the OXA-23 genes.[23]

The potential for decreased sensitivity of *P. aeruginosa* to carbapenem antibiotics is due to the OXA-23 resistance gene belonging to the carbapenem-hydrolyzing class D β-lactamases (CHDL) subgroup. It can be a clinical problem that needs attention that this gene can also be found in transferable elements. CHDL gene transmission between different bacterial strains may be caused by various gene transfer mechanisms such as horizontal gene transfer (including transposable elements).

This raises concerns about its spread which is likely to become a global problem threatening all countries and communities.[11,24,25] The limitation of this research is the small number of samples collected because sampling occurred only at one place; nevertheless, it is hoped that more samples can be used in future research.

CONCLUSION

The results of the above research proved that the distribution of the OXA-23 gene was present in *P. aeruginosa* isolates using the PCR method. Furthermore, it is necessary to conduct an extensive surveillance study of gram-negative organisms producing VEB and OXA species in Indonesia to determine the prevalence of various VEB and OXA variants spread in Indonesia.

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