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ORIGINAL ARTICLE

Genetic and phenotypic of *Pseudomonas aeruginosa* sensitive to meropenem antibiotics after exposure to meropenem

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ABSTRACT

Background and Objectives: *Pseudomonas aeruginosa*, drug-resistant, causes health infections. Resistance to the preferred therapy meropenem is a serious threat. This study aimed to analyze changes in meropenem minimum inhibitory concentration (MIC), changes in *ampC*, *mexA*, and *oprD* gene expression, and the correlation between MIC and *ampC*, *mexA*, and *oprD* gene expression after meropenem exposure.

Materials and Methods: Ten isolates of *P. aeruginosa f*rom the Clinical Microbiology Department, Faculty of Medicine, Universitas Indonesia were used. After the bacteria were shown to be sensitive to meropenem phenotypically, intrinsic resistance genes were detected using PCR. After meropenem exposure on Days 5 and 12, sensitivity testing was carried out with the concentration gradient method and RNA was detected using real-time RT-PCR.

Results: All *P. aeruginosa* isolates that were phenotypically sensitive to meropenem had the *ampC*, *mexA*, and *oprD* genes. An increase in MIC, an increase in *ampC* and *mexA* gene expression, and a decrease in *oprD* gene expression were observed after meropenem exposure. There was a very strong and significant correlation ($p \leq 0.05$) between MIC and *oprD* gene expression after Day 12 of meropenem exposure.

Conclusion: Although there were no significant differences in MIC and *ampC*, *mexA*, and *oprD* gene expression between Day 5 and Day 12, there was a very strong and significant correlation between MIC and *oprD* gene expression on Day 12 ($p \leq 0.05$). This indicates that decreasing *oprD* gene expression has the potential to increase meropenem resistance in *Pseudomonas aeruginosa.*

Keywords: *Pseudomonas aeruginosa*; Meropenem; Antibiotic resistance; Minimum inhibitory concentration; Gene expression

INTRODUCTION

Pseudomonas aeruginosa is one of the main pathogens causing healthcare-associated infections, with a prevalence of 14.5%; moreover, 48.7% of cases are caused by multidrug-resistant variants (1-3). Generally, the preferred therapy for *P. aeruginosa* infections is meropenem (4); thus, resistance to meropen-

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em poses a serious threat. Resistance mechanisms to meropenem, both intrinsic and acquired, significantly challenge efforts to control infectious diseases (5, 6). Previous research by Pelegrin et al. (2019) showed the involvement of the acquired resistance genes bla_{GES-5}, bla_{IMP-1}, bla_{IMP-7}, bla_{IMP-43}, bla_{VIM-2}, and bla_{VIM-8} in the appearance of resistance to carbapenem in *P. aeruginosa* (7). However, the role of the intrinsic resistance genes *ampC, mexA,* and *oprD* in *P. aeruginosa,* which phenotypically shows sensitivity to meropenem, has not been studied extensively. *P. aeruginosa* was found to experience changes, such as gaining resistance, on Days 3 and 4 after antibiotic exposure (8). Previous research has also shown that meropenem often loses effectiveness due to resistance development in *P. aeruginosa*. Identifying the genes involved can provide important insights into how resistance develops and how it can be overcome (6, 9). This study focuses on meropenem because of its importance in first- and second-line therapy for Gram-negative bacterial infections, especially *P. aeruginosa* infections. Understanding the mechanisms of resistance to meropenem may help develop more effective treatment strategies and guide more judicious use of antibiotics (10-13). In this study, the use of 10 *P. aeruginosa* isolates was based not only on the need for adequate numbers but also on the urgency of obtaining comprehensive information about antibiotic resistance in this bacteria. This study aimed to analyze changes in minimum inhibitory concentration (MIC), changes in *ampC, mexA,* and *oprD* gene expression, and the correlation between MIC and *ampC, mexA,* and *oprD* gene expression after meropenem exposure.

MATERIALS AND METHODS

Study design and bacteria. This research used an *in vitro* pre-post experimental study design and was conducted at the Clinical Microbiology Department, Faculty of Medicine, Universitas Indonesia, from August 2020 to August 2023. The work was approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia with Approval Number KET-273/UN2.F1/ETIK/PPM.00.02/2020. The inclusion criterion was *P. aeruginosa* sensitive to meropenem, while the exclusion criterion was *P. aeruginosa* resistant to meropenem. Sample size was calculated using the Federer formula: $(n-1)(t-1) \geq 15$, with $t = 3$ (before, 5 days, and 12 days after meropenem exposure), so that $n \geq 8.5$. This study used 10 samples, including *P. aeruginosa* ATCC 27853.

Identification of *P. aeruginosa*. VITEK®2 (bioMerieux) an automated system used to identify microorganisms. All isolates were re-cultured in blood gel before testing. After 18-24 hours of incubation, the bacterial suspension matched McFarland 0.5. VITEK® 2 AST-GN93 (bioMerieux) cards were inoculated as per the manufacturer's recommendations (14). MALDI-TOF MS (Bruker) is a method used to identify microorganisms. In this process, some of the colonies to be tested are applied to a target slide. A matrix solution was added to the smear. The dry smear was loaded into the system. The sample spectra were compared to the system's microbial species-based spectra database. The spectra sample was analyzed to identify organisms based on trust level (15).

Antimicrobial susceptibility studies. Antimicrobial susceptibility was tested with VITEK®2 and the concentration gradient method. VITEK®2 is an antimicrobial susceptibility testing system. All isolates were regrown on blood agar before testing. Incubation for 18-24 hours yielded a 0.5 McFarland standard bacterial suspension. VITEK® 2 AST-GN93 (bioMerieux) cards were inoculated per the manufacturer's instructions. Using the Clinical and Laboratory Standards Institute (CLSI) breakpoint, the MIC results were clinically interpreted to determine whether the microorganism was susceptible, intermediate, or resistant. For meropenem is ≤ 2 mg/L, 4 mg/L, and ≥ 8 mg/L (16, 17). The concentration gradient method determines MIC using the strip Etest® (Liofilchem). Mueller–Hinton agar was inoculated with *P. aeruginosa* isolates. The strip Etest® with antibiotics of various concentrations was applied to the inoculated medium. The MIC value can be determined by observing the inhibition zone of bacterial growth around the strips after 24 hours of incubation at 35°C. MIC is calculated by comparing the inhibition zone to the strip Etest scale. The CLSI provision was used to interpret the MIC result and determine whether *P. aeruginosa* was sensitive, intermediate, or resistant to the tested antibiotics (18, 19).

Gene detection. Bacterial DNA was extracted from a single colony grown in blood agar using the QIAamp ® DNA Mini kit (QIAGEN). PCR was per-

formed to detect the presence of the *ampC, oxa-50, mexA, mexB, oprM,* and *oprD* genes. The master mix consisted of 8.5 µL diethyl pyrocarbonate, 1 µL 10 µM forward primer, 1 µM reverse primer, 12.5 µL 2× MyTaq Mix, and 2 µL DNA. Primers used to detect gene *ampC, oxa-50, mexA, mexB, oprM,* and *oprD* are presented in Table 1. The amplification was performed as follows: initial denaturation for 1 minute at 95°C, 40 denaturation cycles at 95°C for 15 minutes, extension at 72°C for 1 minute, annealing at standardized temperatures as shown in Table 1 for 15 minutes, extension at 72°C for 1 minute, and final extension at 72°C for 7 minutes. The PCR product was confirmed by electrophoresis in 2% agarose, and DNA was visualized using a gel documentation system (Bio-Rad). The DNA signal was 100 bp (Bioline).

Preparation of meropenem antibiotic solution. To prepare the stock solution, 4.45 mg of meropenem antibiotic powder (Merck) was weighed and dissolved in 25 mL of sterile water to create a 40 µg/mL stock solution. The MF-Millipore TH membrane filter (Merck) was used to sterilize the liquid. A working solution was prepared by adding 15 mL of 40 µg/mL meropenem antibiotic stock solution to 135 mL of sterile nutrition stock. The working solution had a volume of 150 mL and a concentration of 4 µg/mL.

Preparation of bacterial suspensions. Bacterial isolates of *P. aeruginosa* were inoculated on blood agar and incubated at 35°C for 24 hours. The turbidity of the bacterial suspension was measured until it reached 1 McFarland. *P. aeruginosa* ATCC 27853 was used as a quality control at all test stages.

Meropenem exposure. A total of 4 mL of bacterial suspension was added to 4 mL of meropenem antibiotic solution to achieve a final concentration of 2 µg/mL. Bacterial suspensions and antibiotics were prepared in 10 screw-capped tubes. The eleventh tube contained 0.5 McFarland bacterial suspension without antibiotics for use as a bacterial growth control, while the twelfth tube contained 2 µg/mL meropenem working solution without inoculum for use as an antibiotic control. Following exposure, observation was performed on Days 5 and 12, followed by antimicrobial sensitivity testing and gene expression analysis.

Antimicrobial susceptibility studies. Mueller–Hinton agar was inoculated with *P. aeruginosa* isolates. The strip Etest® (Liofilchem) with antibiotics of various concentrations was applied to the inoculated medium. The MIC value could be determined by observing the inhibition zone of bacterial growth around the strips after 24 hours of incubation at 35°C. The inhibition zone and strip Etest scale were checked for MIC. The CLSI provision was used to interpret the MIC result and determine whether *P. aeruginosa* was sensitive, intermediate, or resistant to the tested antibiotics (18).

Gene expression analysis. RNA was extracted from blood gel isolates using the SensiFASTTM SYBR® No-ROX One-Step Kit (Bioline). *ampC, mexA,* and *oprD* expression were measured by real-time RT-PCR. The master mix comprised 3.8 µL Diethylpyrocarbonate, 0.8 µL 10 µM forward and reverse primers, 10 µL SensiFASTTM SYBR® No-

Table 1. Primers used to detect gene

Primer	Sequence 5'-3'	Annealing (°C)	Size (bp)	Reference
ampC forward	GATGAAGGCCAATGACATTCCG	58	742	(20)
ampC reverse	CATGTCGCCGACCTTGTAGTAA			
oxa-50 forward	AATCCGGCGCTCATCCATC	55	869	
oxa-50 reverse	GGTCGGCGACTGAGGCGG			
mexA forward	CTACGAGGCCGACTACCAGA	59	772	(20)
mexA reverse	TGCAGGCCTTCGGTAATGAT			
mexB forward	CCGTGAATCCCGACCTGATG	59	355	
mexB reverse	TGACATGATGGCTTCCGCAT			
oprM forward	TACCAGAAGAGTTTCGACCTGAC	55	227	(20)
oprM reverse	ACTTCGAGCAGGGCCT			
oprD forward	CAACGAGAAGTCCTGGAAGC	55	201	
oprD reverse	ACGTACTGCAGGTCGAGCTC			

ROX One-Step Mix, 0.2 µL reverse transcriptase, 0.4 µL RNase inhibitor, and 4 µL RNA. Table 2 shows primary gene *ampC*, *mexA*, and *oprD* detection. The amplification process involved reverse transcription at 45°C for 10 minutes, polymerase activation at 95°C for 5 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 5 seconds, for 40 cycles. Melting was performed at 95°C for 5 seconds, 54°C for 30 seconds, and 97°C for 1 second. The reference gene was *rpoD*; test values were normalized to the gene expression of *rpoD* in the same strain. A significant increase or decrease in gene expression was defined as > 2 times and < 0.5 times, following previous criteria (21). The relative gene expression was calculated using the ΔΔCT method.

Statistical analysis. Statistical analysis was carried out using the Wilcoxon sign-rank test to measure differences in MIC and *ampC*, *mexA*, and *oprD* gene expression between Days 5 and 12 and Spearman rank correlation to measure the correlation between MIC and *ampC*, *mexA*, and *oprD* gene expression.

RESULTS

Ten isolates of *P. aeruginosa* were re-identified using VITEK®2 and MALDI-TOF. Bacterial sensitivity testing for meropenem was done using the VITEK®2 method and concentration gradients. All isolates were sensitive to the tested meropenem. All isolates had the intrinsic resistance genes *ampC*, *mexA*, and oprD. The MIC values ranged from 0.047 to 0.19 µg/mL. Six isolates had decreased MIC values from day 5 to Day 12, two isolates had increased MIC values during the same period, and two isolates showed no change between the two observation times (Table 3).

Two isolates showed increased *ampC* gene expression on Days 5 and 12. Three isolates showed decreased *ampC* gene expression on Day 12. One isolate showed increased *mexA* gene expression on Days 5 and 12. One isolate showed decreased *mexA* gene expression on Days 5 and 12. Two isolates showed decreased *mexA* gene expression on Day 5. One isolate showed decreased *mexA* gene expression on Day 12. Five isolates showed decreased *oprD* gene expression on Days 5 and 12. One isolate showed decreased *oprD* gene expression on Day 5. One isolate showed increased *oprD* gene expression on Day 5 (Table 3).

Statistical analysis showed no significant difference between MICs on Day 5 and Day 12. There was no significant difference in the expression of the *ampC*, *mexA*, and *oprD* genes on Day 5 and Day 12. The correlation between MIC and *ampC* gene expression was weak and not significant. The correlation between MIC and *mexA* gene expression was weak and not significant. The correlation between MIC and *oprD* gene expression was very strong and significant ($p < 0.05$) on Day 12.

DISCUSSION

This study highlights the urgency of addressing the threat of *P. aeruginosa* in healthcare-associated infections, especially considering the high rate of multidrug resistance. Although the mechanisms of resistance are understood, the role of intrinsic resistance genes in *P. aeruginosa*, which remains phenotypically sensitive to meropenem, has not been well-studied. The CLSI mentions changes in sensitivity on Days 3 and 4 after antibiotic exposure. However, the results of the present study showed that on Day 5, despite changes in gene expression, sensitivity did not change phenotypically *ampC* and *oxa-50* are

Table 2. Priners used to detect gene

Primer	Sequence 5'-3'	Size (bp)	Use	Reference
ampC F	CGGCTCGGTGAGCAAGACCTTC	218	Real-time RT-PCR	(22)
ampC R	AGTCGCGGATCTGTGCCTGGTC			
mexA F	CGACCAGGCCGTGAGCAAGCAGC	316		
mexA R	GGAGACCTTCGCCGCGTTGTCGC			
oprD F	ATCTACCGCACAAACGATGAAGG	156		
oprD R	GGCGAAGCCGATATAATCAAACG			
rpoD F	CGCAACAGCAATCTCGTCTGAAA	130	Real-time RT-PCR	(23, 24)
rpoD R	GCGGATGATGTCTTCCACCTGTT			

Table 3. Genetic and phenotypic analysis of *Pseudomonas aeruginosa* after meropenem exposure on days 5 and 12

Isolat	MIC (µg/mL)		Gene expression					
			ampC		*mexA*		*oprD*	
	Day 5	Day 12	Day 5	Day 12	Day 5	Day 12	Day 5	Day 12
AK25	0,125	0,094	1,0	0,8	1,3	1,1	2,3	1,3
AK36	0,125	0,125	0,9	0,9	3,2	3,6	0,4	0,4
AK40	0,19	0,125	0,8	0,8	0,5	0,7	0,7	0,6
AK59a	0,19	0,125	0,7	0,1	0,6	0,2	0,003	0,04
AK59b	0,094	0,125	0,7	0,1	0,1	0,7	0,03	0,006
AK204	0,047	0,064	1,8	0,8	1,1	1,2	0,8	0,9
AK237a	0,19	0,19	0,9	0,8	0,5	0,3	0,2	0,02
AK237b	0,19	0,125	2,3	2,5	1,2	1,2	0,3	0,09
AK203	0,125	0,094	1,7	0,5	1,5	1,0	1,8	1,8
ATCC 27853	0,19	0,125	2,1	2,6	1,5	1,2	0,2	0,7

Gene expression that increases or decreases is defined by expression values ≥ 2 and ≤ 0.5, and is indicated in bold (21).

genes that code the production of the beta-lactamase enzyme. *oxa-50* is a natural marker to identify the species *P. aeruginosa*. *mexA, mexB,* and *oprM* are responsible for the efflux system, and *oprD* is responsible for bacterial membrane permeability (25, 26).

This research discusses the impact of meropenem exposure on *P. aeruginosa* for a period of 12 days at a low concentration (2 µg/mL). The analysis of antimicrobial sensitivity shows that phenotypically, all isolates were initially considered sensitive to meropenem.

Meropenem-sensitive *P. aeruginosa* isolates showed a decrease in *mexA* gene expression, in accordance with previous research (21). This decrease in *mexA* gene expression causes the efflux system to work less optimally in pumping out meropenem so that the concentration of meropenem in cells remains high, thus reducing the MIC and increasing sensitivity. On the other hand, *mexA* gene expression increased in meropenem-sensitive *P. aeruginosa* isolates, which could cause an increase in the MIC, thereby potentially increasing resistance. This is in accordance with previous research, which showed an increase in *mexA* gene expression in meropenem-resistant *P. aeruginosa* isolates (21, 27). When expression of the *mexA* gene in *P. aeruginosa* is increased but meropenem sensitivity is retained, another mechanism is suspected to be at work; for example, the OprF porin may act as an alternative channel for the entry of meropenem into cells so that the concentration of meropenem remains sufficient to induce sensitivity.
Meropenem-sensitive *P. aeruginosa* isolates showed a decrease in *oprD* gene expression, in accordance with previous research (21, 28). Decreased expression of the *oprD* gene causes a decrease in membrane permeability so that antibiotics are prevented from entering the cell through the OprD porin, causing the concentration of meropenem in the cell to decrease. This decrease in meropenem concentration causes an increase in resistance; however, in this study, sensitivity was maintained, thought to be due to the existence of another mechanism that can increase meropenem concentration. The mechanism may involve the OprF porin as an alternative channel for the entry of meropenem (21). In contrast, there was an increase in *oprD* gene expression in meropenem-sensitive *P. aeruginosa* isolates. This increase in *oprD* gene expression causes an increase in membrane permeability so that antibiotics can enter cells through the OprD porin, increasing the concentration of meropenem so that sensitivity can be maintained.

Meropenem-sensitive *P. aeruginosa* isolates showed a decrease in *ampC* gene expression, in accordance with previous research (29). Decreased expression of the *ampC* gene causes a decrease in the production of the beta-lactamase enzyme, resulting in a decrease in meropenem degradation, thereby increasing sensitivity. In contrast, an increase in *ampC* gene expression in meropenem-sensitive *P. aeruginosa* isolates has been observed. This increase in *ampC* gene expression causes an increase in meropenem degradation, thus increasing resistance. However, in the present study, *P. aeruginosa* isolates remained sensitive. This finding can be attributed to other mechanisms,

such as the porin OprF serving as an alternative channel for meropenem entry so that sensitivity is maintained.

In this study, one meropenem-sensitive *P. aeruginosa* isolate had a combination of increased *ampC* gene expression and decreased *oprD* gene expression, and one meropenem-sensitive *P. aeruginosa* isolate had a combination of increased *mexA* gene expression and decreased *oprD* gene expression. This combination of gene expression changes has the potential for furthering antibiotic resistance. Previous research showed a combination of increased *ampC* and *mexA* expression and decreased *oprD* expression in meropenem-resistant *P. aeruginosa*. The potential for resistance is supported by statistical results showing a very strong and significant correlation ($p < 0.05$) between MIC and *oprD* gene expression after exposure on the twelfth day.

The novelty of this research is the use of exposure to meropenem at a dose of 2 µg/mL for 12 days of *P. aeruginosa* that was initially sensitive to meropenem. At a low concentration (2 µg/mL), therapy was still effective until the twelfth day. The findings show that at this stage, phenotypic and genetic changes occur, indicating the potential for further resistance.

Our study was limited to testing the effects of meropenem only, using a single concentration of 2 µg/mL. This limits the generalizability of our findings to the use of other antibiotics and the variability of doses commonly encountered in clinical settings. Therefore, we recommend further research that includes the use of other antibiotics such as ceftazidime, cefepime, and imipenem, which can provide additional insights into bacterial responses to various antimicrobial agents. Additionally, it is important to explore the effects of antibiotics at intermediate and resistant concentrations, as this can provide a deeper understanding of bacterial resistance mechanisms and guide the development of more effective therapies in the future. For specific implications in practice, it can be recommended that students or researchers new undertake advanced training in genetic and phenotypic analysis techniques, such as real-time RT-PCR.

CONCLUSION

Although there were no significant differences in MIC and *ampC*, *mexA*, and *oprD* gene expression between Day 5 and Day 12, there was a very strong and significant correlation between MIC and *oprD* gene expression on Day 12 ($p \leq 0.05$). This indicates that decreasing *oprD* gene expression has the potential to increase meropenem resistance in *Pseudomonas aeruginosa*.

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