MEROPENEM-INDUCED REDUCTION IN COLISTIN SUSCEPTIBILITY IN *PSEUDOMONAS AERUGINOSA* STRAIN ATCC 27853

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Antibiotic-resistant strains of *Pseudomonas aeruginosa* are a global threat to public health. The knowledge of mechanisms underlying antibiotic resistance is essential to counter *P. aeruginosa* infections. This study describes the phenomenon of meropenem-induced cross-resistance to colistin in the ATCC 27853 strain of *P. aeruginosa*. The study was conducted in the specimens of *P. aeruginosa* grown from the reference ATCC 27853 strain in the medium containing meropenem gradients. Susceptibility of the isolates to carbapenems and colistin was assessed using the agar dilution method; susceptibly to colistin was assessed using the broth microdilution method. A total of 93 *P. Aeruginosa* isolates were analyzed; of them two demonstrated reduced susceptibility to carbapenems (meropenem, imipenem) and colistin. Whole-genome sequencing of the isolates was performed on a MGISEQ-2000 platform. Missense mutations in the *oprD* and *mexD* genes and a nonsense mutation in the *phoQ* gene were detected. We conclude that exposure of *P. aeruginosa* to meropenem can lead to cross-resistance to colistin, a last resort drug for *P. aeruginosa* infections.

Keywords: antibiotic resistance, *Pseudomonas aeruginosa*, meropenem, colistin

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МЕРОПЕНЕМ-ИНДУЦИРОВАННОЕ СНИЖЕНИЕ ЧУВСТВИТЕЛЬНОСТИ К КОЛИСТИНУ У *PSEUDOMONAS AERUGINOSA* ATCC 27853

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Нечувствительные к антибиотикам штаммы *Pseudomonas aeruginosa* представляют собой глобальную проблему в здравоохранении. Исследование механизмов возникновения резистентности лежит в основе разработки способов борьбы с *P. aeruginosa*. Целью работы было исследовать возникновение кросс-резистентности у *P. aeruginosa* в процессе адаптации к популярному антибиотику меропенему. Объектами исследования были образцы *P. aeruginosa*, полученные при росте референтного штамма *P. aeruginosa* ATCC 27853 на среде с возрастающей концентрацией меропенема. Чувствительность изолятов к карбапенемам и колистину определяли при помощи разведения в агаре, чувствительность к колистину оценивали методом серийных разведений. Было получено 93 изолята *P. aeruginosa*, два из которых имели сниженную чувствительность одновременно к карбапенемам (меропенем, имипенем) и колистину. Геномы изолятов секвенировали на полногеномном секвенаторе MGISEQ-2000; обнаружены миссенс-мутации в генах *oprD* и *mexD* и нонсенс-мутация в *phoQ*. Получнные результаты показывают, что при воздействии меропенема на штаммы *P. aeruginosa* может развиваться кросс-резистентность к колистину — препарату резерва для лечения синегнойной инфекции.

Ключевые слова: антибиотики, резистентность, *Pseudomonas aeruginosa*, меропенем, колистин

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Pseudomonas aeruginosa is a significant opportunistic pathogen and a serious burden to public health and economy [1]. Especially dangerous are carbapenem-resistant strains of *P. aeruginosa* regarded by WHO as critical priority pathogens [2]. This breeds the need for understanding mechanisms underlying bacterial resistance to carbapenems. Research into the molecular genetic underpinnings of carbapenem resistance focuses mostly on β-lactamase-associated mechanisms that are determined by plasmid genes and therefore can be acquired through horizontal gene transfer. However, there is another contributor whose role should not be overlooked: induced mutations in the core genome of *P. aeruginosa* resulting in high-level carbapenem resistance [3]. There are two approaches to the study of mutations conferring resistance to carbapenems. The first involves the analysis of drug-resistant isolates obtained from clinical, agricultural or environmental sources. In the second approach, the evolution of antibiotic resistance is modeled *in vitro*. For that, bacteria are grown in antibiotic concentration gradients. A smart method for studying mutational resistance has been proposed in [4]. Its authors

created a spatiotemporal model that enabled migration of *Escherichia coli* in trimethoprim and ciprofloxacin gradients and generated a variety of mutants for further analysis. Interestingly, some of the *E. coli* clones carried mutations that were not linked to trimethoprim or ciprofloxacin resistance [4]. So, we became curious to explore the direction and implications of such mutations. Specifically, we were interested in the clinically significant phenomenon of cross-resistance, in which a mutation induced by exposure to an antibiotic could confer resistance to other antibiotics [5, 6]. The aim of this study was to test the hypothesis that *P. aeruginosa* can develop cross-resistance to other antibiotics while adapting to meropenem.

METHODS

Bacteriological study

In our experiment, we used the spatiotemporal model of antibiotic resistance in motile bacteria [4]. The reference ATCC 27853 strain of *P. aeruginosa* was precultured in semi-solid LB agar (0.28% agarose) in a Petri dish at 37 °C for 24 h. After 24 h, the cells were harvested from the propagating colony front and seeded onto another Petri dish with semi-solid LB agar. The procedure was repeated 3 times. Then, 10 µl of the grown bacterial culture was picked up with an inoculation loop and introduced into the top layer (semi-solid agar) of the culture medium contained in a device shown in the Figure. The medium had a sandwich composition. The bottom layer was LB Miller broth (Becton Dickinson; USA) supplemented with 1.6% agarose, 30 µg/ml kanamycin sulfate, 100 µg/ml cycloheximide, and meropenem taken at one of the concentrations shown in the Figure. The optimum thickness of the bottom layer equaled three-fifths of the total medium thickness (~2.0 cm). The bottom layer was distributed into 5 isolated compartments of the dish containing different concentrations of meropenem. The middle layer (one-fifth of the total medium thickness) was LB Miller broth supplemented with 2.0% agarose, 30 µg/ml kanamycin sulfate, 100 µg/ml cycloheximide, and ink (4.0 ml per 1L culture medium) added as a contrasting background for photography purposes. The middle layer spread over the bottom layer was solid. The top layer (one-fifth of the total medium thickness) was semi-solid agar (Miller LB broth) with 0.3% agarose, 30 µg/ml kanamycin sulfate and 100 µg/ml cycloheximide.

The cells were incubated in air at 37 °C for 216 h. Every 12 h, *P. aeruginosa* samples were collected from the propagating colony front and reseeded on Mueller–Hinton agar (Becton Dickinson; USA) to obtain a sufficient amount of bacteria for the subsequent analysis of their phenotypic traits (antibiotic resistance profiles) and genomic changes.

Resistance to meropenem and imipenem was tested using the agar dilution method described in [7]. Resistance to colistin was assessed using the broth microdilution method following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [8].

Bacterial DNA was isolated from the 24-h culture of *P. aeruginosa* grown on Mueller–Hinton agar (Becton Dickinson; USA) using a QIAamp DNA Mini Kit (Qiagen; Germany) according to the manufacturer's protocol. The obtained DNA samples were stored at –20 °C.

To prepare genomic DNA libraries, 400 g of the isolated bacterial DNA was sheared in an ultrasonicator (Covaris; USA). The fragments were then end-repaired and ligated to MGI adapters (MGI; China). The libraries were purified on Agencourt AMPure XP beads (Beckman; USA). Concentrations of the bacterial DNA and DNA libraries were measured using a Qubit 4 fluorometer (Thermo Fisher Scientific; USA).

Whole-genome sequencing was performed using the MGISEQ-2000 platform (MGI; China). Read length was 250 bp.

The quality of the raw sequence data was tested in FASTQC; the reads were trimmed in Trimmomatic v.0.38. Bacterial genomes were assembled de novo using SPAdes 3.14 [9]. The assembled sequences were tested for contamination using Contest16S. The obtained genome assemblies were evaluated in QUAST 5.0 [10]. Genetic similarity between the assembled genomes was assessed in MUMmer [11]. The genomes were annotated using RAST [12] and Prokka software [13]. To detect the presence of single nucleotide polymorphisms (SNPs), the short reads were mapped to the reference genome in Snippy [14]. ATCC 27853 was used as a reference genome. The detected variants were annotated and their influence on the genes was predicted in SnpEff [15]. The search for antibiotic resistance genes in the genomes assembled de novo, their analysis and validation of the detected SNPs were all carried

Table. Characteristics of carbapenem-resistant strains of *P. aeruginosa* with reduced susceptibility to colistin

Methodology		Characteristic	*P. aeruginosa* ATCC 27853, reference	*P. aeruginosa*, isolate E62	*P. aeruginosa*, isolate E74
Phenotypic antibiotic susceptibility assessment	Agar dilution method, susceptibility to	meropenem	0.25 µg/ml, S	16 µg/ml, R	16 µg/ml, R
		imipenem	0.001 µg/ml, S	128 µg/ml, R	256 µg/ml, R
	Broth microdilution method, susceptibility to	colistin	0.5 µg/ml, I*	2 µg/ml, I*	4 µg/ml R
Changes in genome	Carbapenem resistance genes	*oprD*	wt	Mutation resulting in G307D	Mutation resulting in G307D
		mexD	wt	Mutation resulting in E89K	Mutation resulting in E89K
	Colistin resistance gene	*phoQ*	wt	Nonsense mutation resulting in Y290stop	Nonsense mutation resulting in Y290stop
	Polymorphism	SNP	—	134	177

Note: S — susceptible; I — intermediate; R — resistant; wt — wild type, matches the reference genome; SNP — single nucleotide polymorphism; * — according to the CLSI criteria, the term "susceptible" cannot be applied to describe the susceptibility of *P. aeruginosa* to colistin; all *P. aeruginosa* strains for which colistin MIC ≤ 2 µg/ml are classified as susceptible at increased exposure.

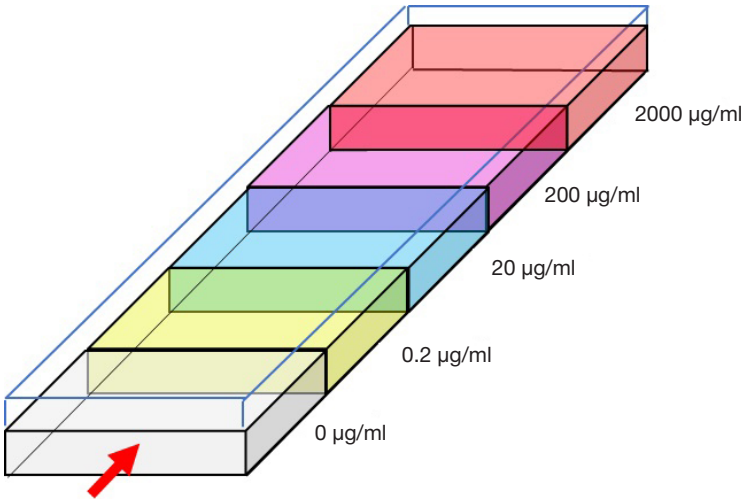

Fig. A device (MEGA-plate, 40 × 20 cm) for spatiotemporal modeling of meropenem resistance in *P. aeruginosa*. The bottom of the device is divided into 5 sections separated by 2.5-cm walls. The culture medium in the MEGA plate has a sandwich composition. The bottom layer contains solid LB agar (the description is provided in the article) distributed into the sections. Each of the sections contains different concentrations of meropenem (shown on the right). The middle layer is LB agar without meropenem. The top layer is semi-solid LB agar (Miller). The arrow indicates the inoculation site and the direction in which *P. aeruginosa* propagates along the surface of semi-solid agar.

out in BLASTn. The analysis of resistance determinants was aided by ResFinder and the AMRFinderPlus algorithm included in the NCBI Pathogen Detection pipeline [16, 17]

RESULTS

A total of 93 *P. aeruginosa* mutants with various phenotypic traits (colony color, antibiotic resistance profile, mucoid/non-mucoid phenotype) were harvested during 216 h of incubation. Among those isolates, two strains (E62 obtained at 192 h of incubation and E74 obtained at 216 h of incubation) demonstrated significantly reduced (four- to eightfold) susceptibility to colistin and high resistance to meropenem and imipenem. Phenotypic and genotypic characteristics of these 2 strains are shown in the Table.

For E62, meropenem and imipenem MICs were 16 μg/ml and 128 μg/ml, respectively; for E74, they were 16 μg/ml and 256 μg/ml, respectively, which satisfied the CLSI criteria for antibiotic resistance. According to CLSI criteria, the E62 isolate was characterized as susceptible to colistin at increased exposure; for this strain, colistin MIC was 4 times higher than for the baseline strain. According to the CLSI criteria, the E74 strain was characterized as resistant to colistin (MIC: 4 μg/ml).

Both strains carried a mutation in the porin gene (*oprD*) resulting in the substitution of glycine with aspartic acid at position 307 of the protein. Besides, both isolates had a missense mutation in the *mexD* gene (this gene encodes the subunit of the MexCD-OprJ efflux pump). Also, both E62 and E74 had a nonsense mutation in the *phoQ* gene resulting in the premature termination of protein synthesis (289 out of 448 amino acids).

DISCUSSION

P. aeruginosa strains with simultaneous resistance to carbapenems and polymyxins are not rare. For example, among multidrug resistant *P. aeruginosa* representatives, 22.2% of meropenem-resistant isolates were unsusceptible to colistin [18]. The evolution of such isolates is rarely described in the literature. It is possible that they acquire their resistance profiles through consecutive or simultaneous therapeutic exposure to carbapenems and colistin. The phenomenon observed in our study proves that *P. aeruginosa* can reduce their susceptibility to colistin following exposure to meropenem. The hypothetical mechanisms underlying induction of cross-resistance to colistin by meropenem fall into the “all roads lead to resistance” concept, meaning that in *P. aeruginosa* any stressor causes hypermutability and leads to the emergence of multiple clones with novel properties [3]. Such a mutational explosion can lead to the emergence of persisting mutations disrupting synthesis of lipopolysaccharides, the primary target of colistin.

Genomes of the E62 and E74 isolates carried mutations that can explain their resistance to meropenem/imipenem and reduced susceptibility to colistin. The missense mutation in the *oprD* gene reported in this study may have caused a structural change in the OprD porin, which transports meropenem and imipenem inside the bacterial cell [19]. The search of GeneBank (https://www.ncbi.nlm.nih.gov/genbank) identified only one clinical isolate with a similar amino acid sequence of the OprD porin (GCA_003194245.1). Similar to our mutant, this isolate obtained in 2013 was also resistant to meropenem and imipenem (MIC > 32 μg/ml). Another mutation that could have reduced susceptibility to carbapenems was the missense mutation in the *mexD* gene encoding the subunit of the MexCD-OprJ efflux pump. The MexCD-OprJ system is involved in the efflux of β-lactams; its hyperexpression is correlated with carbapenem resistance in *P. aeruginosa* [20]. The *phoQ* gene codes for the sensor histidine kinase, which is part of the two-component regulatory PhoPQ system. Mutations in *phoQ* were reported to cause resistance to polymyxins in *P. aeruginosa*, including specimens isolated from patients with cystic fibrosis [21, 22].

Thus, all phenotypic characteristics of carbapenem-resistant isolates of *P. aeruginosa* with reduced susceptibility to colistin observed in our study were associated with mutations.

CONCLUSIONS

The phenomenon of cross-resistance described in this paper may be due to the fact that the rate of point mutations in *P. aeruginosa*, specifically in the genes implicated in antimicrobials resistance, increases under stress conditions. Our findings prove that exposure to meropenem can lead to resistance not only to other β-lactams but also to colistin used as a last resort drug for *P. aeruginosa* infections, which seriously complicates the treatment strategy and limits its options.

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