Investigación original

Detection of carbapenem resistance genes in *Pseudomonas aeruginosa* isolates with several phenotypic susceptibility profiles

Detección de genes de resistencia a carbapenémicos en aislados de Pseudomonas aeruginosa con diferentes perfiles de susceptibilidad fenotípica

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Abstract

Introduction: *Pseudomonas aeruginosa* display several resistance mechanisms to carbapenems and such variety makes it difficult to infer from the antibiogram. The aim of this study was to determine the carbapenem resistance genes in *P. aeruginosa* isolates with different profiles of phenotypic susceptibility to these antibiotics. **Materials and methods:** From a microbial collection of *P. aeruginosa* isolates from infected patients, 40 isolates with different carbapenem resistance profiles were selected. The carbapenemases genes, and expression of the OprD porin, the MexAB-OprM efflux pump and the β-lactamase AmpC were determined. **Results:** From a total of 40 isolates evaluted, in 21 (52.5%) any mechanism of resistance evaluated were detected. In the meropenem-resistant group, overexpression of AmpC (n = 1) and decreased expression of MexAB-OprM (n = 2) and OprD (n = 1) were found. A decrease in the expression of MexAB-OprM was observed in imipenem-resistant group (n = 3) and mutations in the gene encoding the OprD porin (n = 1). Finally, the presence of carbapenemases (VIM, n= 3, KPC-2 / VIM, n = 1) was detected in imipenem-meropenem resistant isolates. **Conclusion:** The phenotypic susceptibility profiles in *P. aeruginosa* isolates were not explained by the molecular mechanisms explored, with the exception of carbapenemase-producing isolates. These results evidence the complexity of the antibiotic resistance mechanisms involved in this bacterium.

Keywords: *Pseudomonas aeruginosa*; Carbapenems; Carbapenemases; Resistance mechanisms.

Resumen

Introducción: *Pseudomonas aeruginosa* presenta diferentes mecanismos de resistencia a los carbapenémicos, dificultando su inferencia a partir del antibiograma. El objetivo fue determinar los genes de resistencia a carbapenémicos en aislados de *Pseudomonas aeruginosa* con diferentes perfiles de susceptibilidad a estos antibióticos. **Materiales y métodos:** a partir de una colección microbiana de aislados de *P. aeruginosa* provenientes de pacientes infectados se seleccionaron 40 aislados con diferentes perfiles

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de resistencia a carbapenémicos y en los cuales se determinaron los genes de carbapenemasas, la expresión de la porina OprD, la bomba de expulsión MexAB-OprM y la betalactamasa AmpC. **Resultados:** el 52,5 % de los aislados no presentó ninguno de los mecanismos de resistencia evaluados. En los resistentes a meropenem se encontró sobreexpresión de AmpC (n=1) y disminución de la expresión de MexAB-OprM (n=2) y OprD (n=1). En los resistentes a imipenem se observó disminución en la expresión de MexAB-OprM (n=3) y mutaciones en el gen que codifica la porina OprD (n=1). En aislados resistentes a imipenem y meropenem se detectó la presencia de carbapenemasas (VIM, n=3, KPC/VIM, n=1). **Conclusión:** los mecanismos moleculares hallados no explican el fenotipo de resistencia a carbapenémicos, excepto en los aislados productores de carbapenemasas. Estos resultados evidencian la complejidad de los mecanismos implicados en la resistencia antibiótica en esta bacteria.

Palabras clave: *Pseudomonas aeruginosa*; Carbapenémicos; Carbapenemasas; Mecanismos de resistencia.

Introduction

Pseudomonas aeruginosa is a gram-negative bacillus that causes a broad spectrum of infections, from urinary tract infections to bacteremia and pneumonia. This microorganism possesses intrinsic resistance mechanisms to different antibiotics including β-lactams, tetracyclines, trimethoprim/sulfamethoxazole and chloramphenicol (1,2). Besides, *P. aeruginosa* may acquire other resistance mechanism, through mobile genetic elements, which further complicate the treatment of the infections it causes (3).

Carbapenems are a group of broad-spectrum β-lactams of last resort for the treatment of infections caused by multiresistant bacteria due to their high capacity of entry, low toxicity, high affinity for penicillin-binding proteins (PBP´s) and stability against β-lactamases (2). However, in recent years the emergence of *P. aeruginosa* strains resistant to these antibiotics has emerged as a seroius threat causing higher hospital stay costs and mortality rates (4,5).

Resistance to carbapenems in *P. aeruginosa* can be mediated by several mechanisms (6). Non-carbapenemase resistance mechanisms include MexAB-OprM, AmpC, and OprD (7,8). MexAB-OprM is an efflux system belonging to the Resistance-Nodulation-Division (RND) family, which has the broadest substrate profile of β-lactams including meropenem; therefore, an expression level increase has been extensively related to carbapenem resistance (9,10). AmpC cephalosporinase is a chromosomally encoded enzyme in *P. aeruginosa*, whose basal expression confers inherent resistance to β-lactams, except ceftazidime, cefepime, and carbapenems (10).

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However, an overexpression of AmpC plus low permeability or overexpression of efflux pumps may confer additional resistance to carbapenems (9,10). Low membrane permeability is commonly acquired due to the decrease in the expression of membrane porins such as OprD or mutations in the genes encoding it; OprD porin is imipenem substrate-specific and facilitates the diffusion of basic amino acids and small peptides (3, 9). Non-carbapenemase resistance mechanisms are the most important in *P. aeruginosa*.

Within carbapenamase-mediated resistance mechanisms, Ambler class B enzymes such as VIM and IMP have been described more frequently; however, the emergence of KPC- producing *P. aeruginosa*, (Ambler class A carbapenemase), initially restricted to Enterobacteriacae, has acquired great importance (10,11).

In this regard, some authors have proposed the possibility of inferring the resistance mechanism from the antibiogram; thus, a meropenem-resistant but imipenem-susceptible profile could be due to an increase in the expression of MexAB-OprM efflux pump genes. On the other hand, an imipenem-resistant profile could be caused by diminution in the expression or mutations of the OprD porin, in addition to the overexpression of AmpC carbapenemase (11,12).

Furthermore, the presence of carbapenemase enzymes has shown multiple resistant profiles to different families of antibiotics and resistance to all β-lactams (6). Non-enzymatic mechanisms, unlike carbapenemase-mediated mechanisms, cannot be detected by routine laboratory phenotypic methods, hence the requirement for specific molecular techniques such as qRT-PCR (13,14).

Carbapenem resistance percentages reported in *P. aeruginosa* in Medellín exceed those reported in other main cities of Colombia, such as Bogotá, with 22% and 28% of isolates resistant to imipenem and meropenem respectively (15). Likewise, a local surveillance network for antibiotic resistance (GERMEN group) registers a percentage of resistance to carbapenems of around 30% in intensive care units; a context that highlights the need for decision-making and prevention measures to control infections caused by this microorganism (15).

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In this regard and given the difficulties to implement molecular methods in the daily laboratory routine like qRT-PCR, the aim of this study was to determine the carbapenem resistance genes in *P. aeruginosa* isolates with different profiles of susceptibility to these antibiotics.

Materials and methods

Bacterial isolates and study population

Bacterial isolates were selected from microbial collection of *P. aeruginosa* as part of a previous cross-sectional study conducted at several tertiary care hospitals located in Medellín, during 2012 to 2016.

Sample size

The selection of isolates was performed by a non-probabilistic sampling. A total of 40 isolates classified into four groups according to the carbapenem-resistance profile. *Group 1*: nine meropenem-resistant and imipenem-susceptible isolates; *Group 2*: eight imipenem-resistant and meropenem-susceptible isolates; *Group 3*: 16 isolates with resistance to both antibiotics, and *Group 4*: seven isolates with susceptibility to both antibiotics.

Bacterial identification and sensitivity testing

Identification and determination of antibiotic susceptibilities were carried out with the Vitek 2® automated system (bioMérieux Clinical Diagnostics), according to the criteria established by *Clinical and Laboratory Standards Institute* (CLSI 2016). The antibiotics evaluated were piperacillin/tazobactam, ceftazidime, cefepime, amikacin, gentamicin, ciprofloxacin, imipenem, meropenem, and colistin.

Detection of carbapenemases

The detection of these enzymes was performed using phenotypic and molecular methods. Phenotypic detection was performed using three dimensional test, whereas molecular identification was conducted by conventional and multiplex PCR amplifi-

cation of bla_{KPC}, $bla_{OXA-48,}$ $bla_{NDM,}$ bla_{VIM} y bla_{IMP} genes, using described protocols and previously identified negative and positive controls (16).

Detection of non-enzymatic resistance mechanisms

Analysis of outer membrane protein OprD, MexAB-OprM efflux pump and AmpC carbapenemase expression was performed by measuring the mRNA levels of *oprD, mexA* and *ampC* genes, according to the qRT-PCR protocol described by Tomas *et al*. (9).

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Total RNA was isolated and purified from late-log-phase cultures in BHI broth, using the RNeasy Mini Kit (Qiagen Inc., Crawley, United Kingdom). RT-PCR was performed in triplicate from different RNA extractions of the same sample using 50 ng of RNA per reaction mixture, QIAGEN® LongRange 2Step RT-PCR kit. The qPCR was then performed using Biorad C1000 Touch™ Thermal Cycler and SoFast™EvaGreen®Supermix. A negative control was used to verify that the reaction was not contaminated. Gene expression was normalized versus the *rpoD* gene in the same strain and then calibrated relative to *P. aeruginosa* PAO1, which was assigned a value of 1.0. Increases or decreases in gene expression of ≥2-fold and ≤0.5-fold were taken as significant, according to criteria previously reported (9). The ΔΔCT method for calculating relative gene expression was used.

OprD porin and MexA transmembrane protein sequencing: oprD gene sequencing was performed in three isolates from groups 1, 2 and 3. Meanwhile, MexA sequencing was performed in two isolates of group 1. Analyses of the sequences were carried out using FinchTV software v.1.4.0 and BioEdit Sequence Alignment Editor. Protein alignment was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). In each case the nucleotide sequence and the amino acid sequence were compared to the reference strain PAO1 (GenBank OprD Gene ID: 881970 MexA Gene ID: 877855). Images of OprD tridimensional (3D) structures were represented using Swiss-PdbViewer.

There is no obvious relationship between expression levels and resistance to carbapenems regarding non-enzymatic mechanisms when comparing the expression levels of the genes evaluated with the MIC to the carbapenems.

Statistical analysis: Categorical variables were described using absolute and relative frequencies and median or mean were used for continuous variables. Statistical analysis were performed using the sotfware package SPSS v20.0® (SPSS Inc., Chicago,USA).

Results

Detection of Carbapenem resistance mechanisms

Group 1, meropenem-resistant (n=9): In one isolate AmpC relative expression increased and in two isolates MexA relative expression decreased was observed. OprD porin expression was found to be altered in one isolate of this group (table 1).

Group 2, imipenem-resistant (n=8): In three isolates there was a decrease in the MexA relative expression gene (table 1).

Group 3, resistance to imipenem and meropenem (n=16): The presence of VIM carbapenemase was observed in four isolates and one of these carried VIM and KPC-2 simultaneously; all carbapenemase-producing isolates were positive for the 3-dimensional test. Additionally, three isolates showed overexpression of MexA, one isolate presented decreased MexA expression, and another isolate showed increased expression of OprD (table 1).

Group 4, susceptibility to imipenem and meropenem (n=7): A decrease in the expression of MexA in an isolate was found (table 1).

Table 1. Carbapenem resistance and molecular mechanisms found in each phenotypic susceptibility profiles

Profile	*Isolate*	*Carbapenems MIC µg/ml*		*Carbapenemases Detection*		*AmpC, MexA y OprD Expression level*		
		Imipenem	*Meropenem*	*3D-test*	*PCR*	*AmpC*	*MexA*	*OprD*
Group 1: Meropenem (R) Imipenem (S)	PL7	2	4	-	-	0,65	0,70	0,70
	PR3	<=1	>=16	-	-	***2,04***	1,96	1,91
	PL29	2	8	-	-	0,88	0,72	0,88
	PR55	<=1	4	-	-	0,69	***0,44***	***0,50***
	PL32	<=1	4	-	-	1,62	1,56	0,85
	PH39	2	>=16	-	-	1,23	1,54	0,24
	PL34	2	4	-	-	0,68	0,67	1,32
	PC2	2	8	-	-	1,03	***0,25***	1,05
	RP010	2	4	-	-	0,68	0,67	1,32
Group 2: Meropenem (S) S) Imipenem (R)	PR30	>=16	1	-	-	0,69	0,91	0,58
	PL38	>=16	2	-	-	0,90	***0,35***	0,76
	PH1	8	1	-	-	1,11	1,67	1,28
	PR7	8	2	-	-	1,18	1,68	1,20
	PC11	8	2	-	-	0,80	0,91	1,42
	PH91	>=16	2	-	-	0,68	***0,48***	1,18
	PH41	8	2	-	-	1,34	1,58	1,87
	PH89	>=16	<=0,25	-	-	1,28	***0,44***	1,25
Group 3: Meropenem (R) Imipenem (R)	PH23	4	>=16	-	-	0,94	0,92	1,15
	PH15	>=16	>=16	**+**	***VIM***	1,09	***2,07***	1,19
	PH36	>=16	>=16	-	-	0,66	***2,45***	1,36
	PC13	>=16	>=16	-	-	1,22	1,67	***2,41***
	PP9	>=16	>=16	**+**	***VIM,KPC-2***	1,17	1,66	1,04
	PR33	>=16	8	-	-	1,56	***2,76***	1,66
	PR28	>=16	>=16	-	-	1,21	1,33	1,23
	RP04	16	16	-	-	0,71	0,69	1,02
	RP06	16	8	-	-	1,37	0,75	1,12
	RP08	16	4	-	-	1,28	1,38	1,00
	RP13	16	16	-	-	1,15	0,51	0,84
	RP14	16	8	+	-	1,05	1,36	1,16
	RP009	>=16	>=16	-	-	1,56	1,12	1,32
	RP015	>=16	>=16	-	-	1,45	1,62	1,22
	PL28	>=16	>=16	**+**	***VIM***	1,14	1,33	1,33
	PH85	>=16	8	**+**	***VIM***	0,78	***0,48***	1,01
Group 4 Meropenem (S) Imipenem (S)	PS12	<=2	<=2	-	-	1,22	1,62	0,81
	PS15	<=2	<=2	-	-	1,10	0,98	1,20
	PS17	<=2	<=2	-	-	1,39	0,95	1,40
	PS9	<=2	<=2	-	-	1,28	1,70	1,13
	PS1	<=2	<=2	-	-	0,92	1,01	0,68
	RP03	2	2	-	-	1,75	1,47	1,17
	RP017	2	2	-	-	1,16	***0,45***	0,61

- Negative
+ Positive
- 'In bold significative changes in level expression and positive carbapenemases. R=Resistant S=Susceptible

Sequencing of OprD and MexA

Regarding OprD, an imipenem-resistant isolate showed two substitutions at positions 310 and 315, changing from arginine to glutamine and from alanine to glycine respectively (table 2), changing the conformational 3-dimensional structure of the protein (figure 1).

Table 2. Mutations present in isolates PH1, PC2, PL7, PR33 and PR55

Isolate	*Gene*	*Mutation*
PH1	OprD	310Arg→Glu 315Ala→Gly
PC2	MexA	Insertion at 259
PL7	MexA	None
PR33	OprD	None
PR55	OprD	None

It was interesting to observe that the isolates carrying carbapenemases presented a multiresistant-profile, to β-lactams, quinolones, and aminoglycosides.

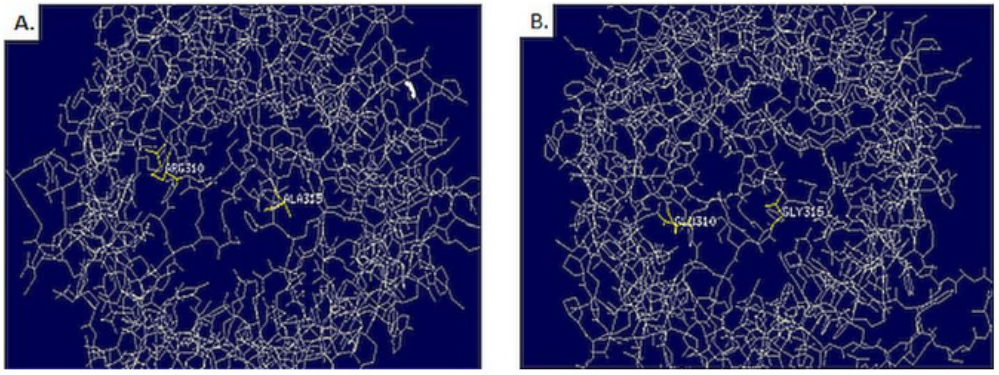

Figure 1. OprD Tridimensional protein structure upper view. **A**. Reference strain PAO1. **B.** PH1 isolate, mutation at 310 and 315

Regarding MexA, a meropenem-resistant isolate showed an insertion at position 259 of a single nucleotide (cytosine), changing the reading frame of the transmembrane protein MexA from the MexAB-OprM efflux pump.

Relationship between resistance mechanisms and antimicrobial profiles

It was noticed that there is no obvious relationship between expression levels and resistance to carbapenems regarding non-enzymatic mechanisms when comparing the expression levels of the genes evaluated with the minimal inhibitory concentration (MIC) to the carbapenems. Moreover, the isolate´s behavior was highly variable and did not depend on MIC values.

On the other hand, it was interesting to observe that the isolates carrying carbapenemases presented a multiresistant-profile, to β-lactams, quinolones, and aminoglycosides (figure 2).

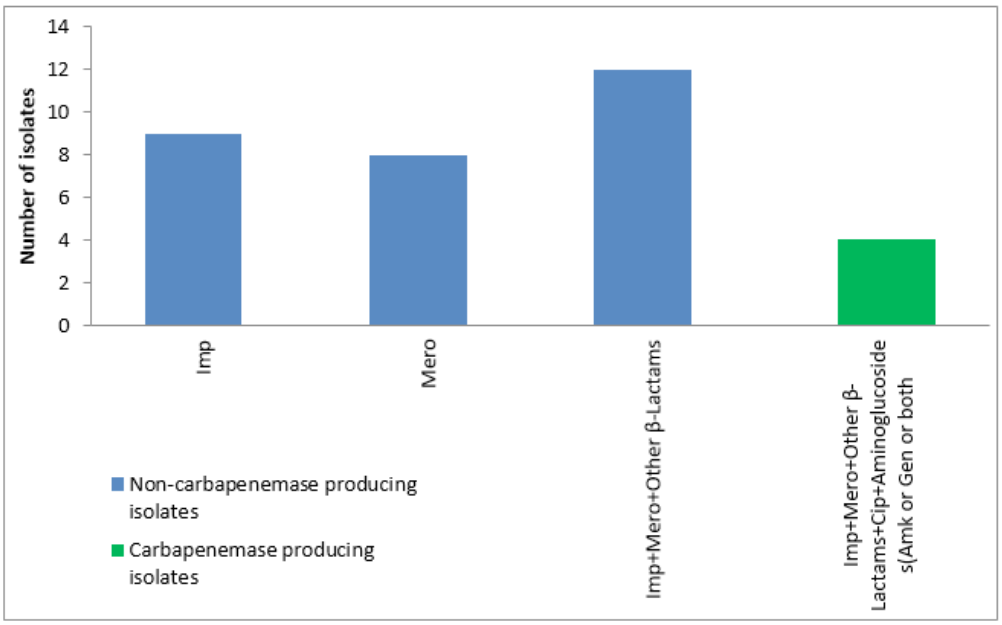

Figure 2. Comparison between non-enzymatic and enzymatic isolates and carbapenem resistance

Discussion

Alterations in the expression of resistance mechanisms in *P. aeruginosa* cannot explain the resistance profile, which may be due to the multifactorial condition of the resistance regulation process.

The results obtained in this study evidence that alterations in the expression of resistance mechanisms in *P. aeruginosa* cannot explain the resistance profile, which may be due to the multifactorial condition of the resistance regulation process (17). Addiotionallly, the fact that this microorganism can simultaneously express several mechanisms of resistance to the same type of antimicrobial such as carbapenems makes difficult the interpretation of the antibiogram (18).

In this study, the MexAB-OprM efflux pump was overexpressed only 3 of 15 meropenem resistant isolates. This result is in concordance with other studies that concluded that a synergistic effect for carbapenem resistance between efflux pumps and AmpC is not necessary (9, 11).

Paradoxically, three isolates resistant to meropenem showed decreased expression of the efflux pump. This finding suggests alterations in the genes that encode components of other non-studied efflux systems, such as MexCD-OprJ, MexEF-OprN, and MexXY, compensating the loss of MexAB-OprM expression in the membrane and provoking the resistance profile to carbapenems (19,20).

In one of these isolates with resistance to meropenem and susceptibility to imipenem, the *mexA* gene was sequenced, evidencing an insertion at the 259 position of a single nucleotide (cytosine) that had not previously been reported. This mutation causes a change in the reading frame of the protein that could lead to the formation of premature stop codons. This could explain the decreased expression of this gene in the isolate (21).

Some studies suggest that in many strains of *P. aeruginosa* the MexXY-OprM system is overexpressed to a greater extent than MexAB-OprM (19). Antibiotic pressure present in most hospitals may contribute to the selection of isolates that overexpress

MexXY-OprM because, unlike MexAB-OprM, the expression of the MexXY-OprM genes can be induced by the presence of Aminoglycosides, which are frequently used for the treatment of *P. aeruginosa* infections (17,19).

Remarkably, four isolates sensitive to meropenem showed decreased expression of the MexA gene, which may lead to an increase in the concentration of the antibiotic in the intracellular level and therefore an increase in the susceptibility. This result is congruent with other studies describing how defects in intrinsic mechanisms can lead to susceptibility (10).

Concerning the alteration of the OprD porin, in this study there was no relationship between imipenem resistance and decreased permeability by low expression of the genes encoding this porin, similar to that found in other investigations (10,17). The regulation of OprD porin genes can occur at both transcriptional and translational levels. It is important to emphasize that the data found do not necessarily highlight the true expression of the porin since the methodology used allows analyzing the transcripts without knowing how many are translated and what the functionality of the translated protien is (22).

Carbapenemases was observed in the isolates with simultaneous resistance to meropenem-imipenem.

On the other hand, the decrease in OprD expression was observed in a susceptible isolate, which could be evidence of the presence of other entry channels for these molecules like OprF, a porin involved in the entry of antibiotics into the bacterium (11, 17).

Some authors have described OprD as encoded in a chromosomal hypervariable region which can acquire mutations or deletions in the structural gene with greater ease, causing the emergence of strains resistant to imipenem by defective porins and without alterations in expression levels (10, 23). OprD gene sequencing shows the presence of intra and interspecies recombination events, resulting in a mosaic structure, thus a series of independent and rapidly mutations in the gene can occur (24).

According to the above, mutations in an isolate of the present study with MIC> = 16 µg/ml for imipenem were found. Conformational changes were observed after 3D modeling affecting the loops that bind β-sheets in the region of the outer surface of the protein that also possesses binding sites for imipenem, causing a possible porin closure and a subsequent decrease of antibiotic entry (24, 25).

OprD overexpression was observed in an imipenem-meropenem resistant isolate, which can be associated with the presence of basic amino acids in the culture medium. This can positively regulate transcription genes that encode the protein, giving rise to high levels of expression (25).

Regarding AmpC cephalosporinase, the results of the study are inconsistent with those described in the literature where the overexpression of this enzyme has been one of the most common mechanisms involved in the increase of the MIC to carbapenems in non-carbapenemases isolates (6,9). This finding may be explained by the lack of antibiotic pressure in the isolates (3,9).

The expression of the gene encoding this cephalosporinase is induced by the presence of peptidoglycan monomers produced by natural degradation of the cell wall (3). In the presence of β-lactam antibiotics (inhibits cell wall biosynthesis), these fragments increase their concentration within the bacterium, producing a positive regulation of the ampC gene transcription (3).

In this study, we found only one isolate with overexpression of AmpC, which had a MIC >=16 µg/ml for meropenem and susceptibility to the other β-lactams. Other studies have already found similar results and may be cohesive with the fact that an induction of this mechanism requires the participation of different molecules such as the regulatory protein AmpR, the permease AmpG, and homologs of the amidase. This process needs the presence of an inductive cofactor held by an amidase homologue, the anhydromuropeptide (3). A mutation or change in the expression of any of these molecules may prevent the activation of AmpR and suppress the induced expression of ampC (3,10).

The presence of carbapenemases was observed in the isolates with simultaneous resistance to meropenem-imipenem, in which a phenotype of multi-resistance to other antibiotics, such as aminoglycosides and flouroquinolones, was also noticed, thus explaining the presence of these enzymes encoded in mobile genetic elements containing resistance genes to other antibiotics (6,26).

It is possible that some of the isolates that showed resistance to carbapenems and did not reveal the presence of any of the mechanisms evaluated carried a carbapenemase not analyzed in the study like bla_{SPM} bla_{GIM}, bla_{SIM}, bla_{AIM}, bla_{DIM}, bla_{GES}, bla_{OXA-40}, bla_{OXA-50} (14).

All isolates with carbapenemases showed simultaneous phenotypic resistance to both carbapenems, a characteristic that would lead to their initial suspicion from the antibiogram.

The study limitations include: (I) samples were cryopreserved with glycerol for an extended of more than six months. Their recovery did not include culture media supplemented with antibiotics, which could influence the results of gene expression by not having the antibiotic pressure; however, antimicrobial MIC´s were confirmed before molecular testing, to confirm antibiotic resistance profiles. (II) The analysis of OprD porin expression was performed only at the transcriptional level further analysis of the translated membrane protein and sequencing the encoding genes to clarify the results it is necessary. (III) Lack of information about the regulatory genes of the different mechanisms, which could have mutations related to the observed resistance phenotype (17).

In summary, although alterations in the expression of these mechanisms do not explain the resistance profile in the isolates, results demonstrate the complexity in the regulation of the mechanisms involved in *P. aeruginosa* antibiotic resistance; therefore, explaining the resistance phenotype is challenging. It is necessary to carry out additional studies that include not only the detection and expression of carbapenem resistance genes, but also the analysis of regulatory genes and the changes in structure of proteins that code for this resistance.

Interestingly, all isolates with carbapenemases showed simultaneous phenotypic resistance to both carbapenems, a characteristic that would lead to their initial suspicion from the antibiogram. Antibiotic pressure is one of the situations that can contribute to this resistance phenomenon, which highlights the importance of establishing antibiotic use policies in hospitals to avoid the dissemination of resistant isolates.

Conflict of interest

The authors declare no conflict of interest.

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Ethical approval

The study protocol was approved by the Bioethics Committee for Human Research at Universidad de Antioquia (CBEIH-SIU) (approval no. 11-35-415).

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