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Artículo científico

Analysis of Efflux Pump Genes in β-lactam Resistant Clinical Isolates of *Pseudomonas aeruginosa* from a Tertiary Level Hospital in Ecuador

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ABSTRACT.- *Pseudomonas aeruginosa* is a nosocomial microorganism that causes a wide spectrum of infections and is known as one of the primary multi-resistant microorganisms against β-lactam antibiotics. One of the main resistance mechanisms found in *P. aeruginosa* is the efflux pumps. This study is aimed at characterizing this mechanism by analyzing the expression of four genes (*mexA, mexX, oprJ and oprM*) involved in antibiotic efflux pumps in *Pseudomonas aeruginosa*. Forty clinical isolates (20 resistant, 20 susceptible) were collected from the Bacteriology Laboratory at the Carlos Andrade Marin Hospital, in Quito-Ecuador. Expression levels for the selected genes were assessed by RT-qPCR assays using *RpsL* as a housekeeping gene for *ΔΔCt* adjusted relative quantitation analysis. The importance of efflux pumps as a resistance mechanism was corroborated through analysis of efflux pumps genes that showed overexpression in all phenotypically resistant isolates. Furthermore, phenotype/genotype analysis was performed comparing Antibiotic Susceptibility Testing (AST) results with expression profiles. Results for the mexA genotype showed correlation with the TPZ resistance phenotype and the mexX genotype with the IPM, MEM and FEP resistance phenotypes. In conclusion, the expression pattern of the efflux pump genes suggests resistance mechanisms that are due to horizontal transmission or pathogens spreading into the hospital environment.

KEYWORDS: Bacterial Resistance, Efflux pumps, β-lactams, *Pseudomonas aeruginosa*, RT-qPCR.

RESUMEN.- *Pseudomonas aeruginosa* es un microorganismo responsable de una amplia variedad de infecciones y es uno de los principales patógenos multiresistentes ante antibióticos β-lactámicos. Uno de los principales mecanismos de resistencia en *P. aeruginosa* constituyen las bombas de eflujo. El objetivo del presente estudio fue caracterizar el mecanismo de bombas de eflujo mediante el estudio de expresión de 4 genes (*mexA, mexX, oprJ y oprM*) involucrados en este mecanismo en *Pseudomonas aeruginosa*. Cuarenta aislados clínicos (20 resistentes y 20 sensibles) fueron recolectados en el Laboratorio de Bacteriología del Hospital "Carlos Andrade Marin" en Quito-Ecuador. Los niveles de expression de los genes seleccionados fueron evaluados por RT-qPCR usando *RpsL* como gen constitutivo para el análisis de cuantificación relativa basado en el método *ΔΔCt* ajustado. La importancia de las bombas de eflujo como mecanismos de resistencia fue confirmada ya que el estudio de expresión de los genes relacionados con bombas de eflujo mostró sobreexpresión de ellos en todos los aislados fenotípicamente resistentes. Se realizó además un análisis fenotipo/genotipo comparando los resultados del antibiograma (AST) con los perfiles de expresión. La sobreexpresión de mexA (genotipo) mostró correlación con el fenotipo de resistencia a TPZ, mientras que el genotipo mexX se correlacionó con los fenotipos de resistencia a IPM, MEM y FEP. En conclusion, los patrones de expression de los genes relacionados con bombas de eflujo sugieren la presencia de mecanismos de resistencia basados en transmisión horizontal que posibilitan la diseminación de patógenos en el ambiente hospitalario.

PALABRAS CLAVES: β-lactamasa, bomba de flujo, *Pseudomonas aeruginosa,* Resistencia bacteriana, RT-qPCR.

INTRODUCTION

Pseudomonas aeruginosa is one of the main nosocomial microorganisms. It is responsible for 10% to 15% of nosocomial infections—such as pneumonia, urinary tract infections, wound infections and bloodstream infections—and it is mainly present in intensive care units and surgical wards (Lister et al. 2009). According to Guzmán-Blanco and Istúriz (2010), *Pseudomonas aeruginosa* infections have a prevalence of 16% in studies conducted in South American hospitals. In Ecuador, infectious episodes due to *Pseudomonas aeruginosa* rate at 23% of all β-lactam resistant episodes.

Pseudomonas aeruginosa shows a high level of resistance to the primary anti-pseudomonal antibiotics, such as β-lactams. Its genome is one of the largest of all microorganisms, allowing it to mutate and adapt to different stress conditions, such as exposure to antibiotics and through several complex resistance mechanisms (Meletis and Begkeri 2013). Resistance mechanisms are divided into three categories: acquired, adaptive and intrinsic. Adaptive resistance is due to environmental or nutritional stress, intrinsic resistance is caused by the low permeability of the outer membrane, while acquired mechanisms are represented by horizontal gene transfer and mutational events. The production of enzymes (β-lactamases), mutations in regulatory porines in the outer membrane and, most notably, overexpression of efflux pumps-encoding genes may result in a resistant phenotype (Breidenstein et al. 2011). Efflux pumps are part of the Nodular Resistance Division (RND), a tripartite system that includes a membrane fusion protein (MFP), an outer membrane factor (OMF) and a cytoplasmic membrane carrier. RND controls the inflow and expulsion of molecules from cytoplasm to periplasmic space. RND complex genes are arranged in operons in the *P. aeruginosa* chromosome. Twelve efflux pumps have been identified and are expressed in *P. aeruginosa* strains. MexAB-oprM, MexCD-oprJ, MexEF-OprN and MexXY have been widely studied, and all of them were identified in multiresistant isolates (Morita et al. 2012). Efflux pumps use antibiotics as substrate and expel them out of the cell by proton motive force using ions through the electrochemical gradient of the membrane, in a process known as chemiosmosis (Lister et al. 2009).

RT-qPCR assays allow RNA quantitation analysis. Their high sensitivity, reproducibility and efficiency have led qPCR to be the current main technique for gene expression analysis (Khan-Malek and Wang, 2011). Quantitative PCR (qPCR) uses specific primers and fluorescent probes. When the target sequence is detected, a fluorescent signal is emitted that has a higher intensity than the baseline-Threshold Cycle (Ct) (Nolan et al. 2006). Relative quantitation is the method used for measuring gene expression in quantitative PCR analysis. It relies on a housekeeping gene—whose expression is constant under different conditions—and utilizes the ratio of target gene expression over housekeeping gene expression for the quantitation of gene expression (Bolha et al. 2012).

The *ΔΔCt* adjusted method has been used for relative quantitation of gene expression in RT-qPCR. The method takes into consideration the assay´s Amplification Efficiency (AE) value. The Ct values of target and housekeeping genes are also considered in the equation and it allows for the calculation of gene expression ratios (Yuan et al. 2008).

The aim of this study was to implement RT-qPCR systems for quantitation of the expression of gene encoding efflux pumps in clinical isolates of *Pseudomonas aeruginosa* and to compare expression ratios in susceptible and resistant isolates in order to evaluate the role of efflux pumps in antibiotic resistance mechanisms.

MATERIALS AND METHODS

Collection and Storage of *Pseudomonas aeruginosa* Isolates.- Forty *P. aeruginosa* isolates were collected from the Microbiology Laboratory at the Carlos Andrade Marin Hospital in Quito-Ecuador. Demographics and collection site information for patients and isolates for this study are shown in Table S1 (Supplementary Information). Antimicrobial Susceptibility Testing (AST) was performed in the hospital´s Bacteriology Laboratory by qualified and experienced microbiology technicians in accordance with to current standards (CLSI). This study was conducted with 20 resistant and 20 susceptible isolates (AST profiles for all isolates are shown in Table S2 (Supplementary Information). Suspensions in 15% v/v glycerol/water were cryopreserved in our laboratory at the Universidad de las Fuerzas Armadas-ESPE.

RNA Extraction, Purification and Quantitation.- Prior to RNA extraction, 100µl of the cellular suspension in 15% glycerol was cultured in 4ml of Brain Heart Infusion and incubated at 37ºC for 18 hours. Afterwards, the recommended protocol for the *Purelink RNA Mini Kit (Ambion)*, DNa-

se treatment was performed using *TURBO DNase (Ambion),* and a phenol/chloroform RNA extraction protocol was then performed (Jacobs and White, 2013). Purified RNA was quantified using a *Qubit 1.0* fluorometer (*Invitrogen*) with *RNA BR kit* (*Invitrogen*). Finally, an aliquot of the extracted RNA was run in a 2% agarose electrophoresis gel in order to visually confirm RNA purification.

Design of Primers and Probes.- Five pairs of primers and four probes (Table S3 Supplementary Information) were designed with aid of the *Primer Express v3.0* software (Applied Biosystems). The additional bioinformatics tools *BLAST, Clustal Omega and Oligo Analyzer* were used for fine tuning of design parameters (melting temperature (Tm), primer size, GC content, homo dimers and hetero dimers formation).

RT-qPCR.- All 40 strains of *P. aeruginosa* were analyzed for each of the five genes included in the study. TaqMan RNA-to-Ct 1-Step Kit (Applied Biosystems) was used for *mexA, oprM, oprJ* and *rpsL* genes (housekeeping gene used as normalizer), and the Power SYBR Green RNA-to-Ct 1-Step kit (Applied Biosystems) was used for *mexX.* The assays were carried out in a 7300 Real Time PCR System (Applied Biosystems).

For the *mexA, oprM, oprJ* and *rpsL* amplification assays, a 10µl final reaction volume was used. The reaction mix contained 5µl of 2X Taqman RT-PCR Mix, 0.9µl of 10µM forward and reverse primers, 1µl of 2000nM MGB probe, 0.25µl of TaqMan RT enzyme, 1µl of 10ng/µl RNA, and 0.95µl of nuclease-free water. The thermal cycler program included a retro transcription step at 48ºC for 20 minutes, an initial denaturation step at 95ºC for 10 minutes, followed by 35 cycles consisting of a denaturation step at 95ºC for 15 seconds, an annealing step at 51ºC (for *mexA and oprJ*), 59ºC (for *oprM*) and 53ºC (for *rpsL*) for 30 seconds, and a final extension step at 60ºC for 30 seconds.

For the *mexX* gene, the 10µl final reaction mixture contained 5µl of 2X Power SYBR green mix, 0.2µl of 10µM forward and reverse primers, 0.08µl of RT enzyme mix, 1µl of 10ng/µl RNA and 3.52µl of nuclease-free water. The PCR thermal cycler program consisted of a reverse transcription step at 48ºC for 20 minutes, an initial denaturation step at 95ºC for 10 minutes, followed by 35 cycles of a denaturation step at 95ºC for 15 seconds, and an annealing step at 55ºC for 30 seconds. A final extension step at 60ºC for 30 seconds was included. For T*m* analysis, the thermal cycler was programmed as follows: denaturation at 95ºC for 15 seconds, renaturation at 60ºC for 1 minute, denaturation at 95ºC for 15 seconds and final renaturation at 60ºC for 15 seconds. A dissociation curve was used to determine if the predicted Tm (87ºC) for the *mexX* amplicon corresponded to the actual Tm of the cDNA PCR fragment obtained in the assay.

Amplification Efficiency and Statistical Analyses.- For calculation of the assay´s Amplification Efficiency (AE), two clinical strains were used as assay controls, corresponding to a resistant and a susceptible isolate (Wong and Medrano 2005). Four RNA dilutions were prepared from the original concentrated RNA (10ng/µl), by adding DEPC treated water to RNA final concentrations of 5ng/µl, 1ng/µl, 0.5ng/µl and 0.1ng/µl. A RT-qPCR reaction was run for all gene systems with the above RNA concentrations and Ct values were obtained for each PCR run. Ct values of the housekeeping gene *(rpsL*) were subtracted from the Ct values of the four target genes, and a ΔCt value for each system was obtained. A two-sample T-test was applied for comparison of Ct's for phenotypically resistant and susceptible strains in each targeted gene. A p-value of 0.05 was considered for statistical significance.

Relative Quantitation of Gene Expression.- Ct values were obtained and tabulated from the RT-qPCR for the five genes (n= 40 isolates). The mathematical model proposed by Yuan et al. (2008) was used for calculation of expression ratios.

$$\Delta\Delta CT^{\text{adjusted}} = \mu1 \times AE1 - \mu2 \times AE2 - \mu3 \times AE3 + \mu4 \times AE4 \quad (1)$$

Being:
µ1: Target gene Ct for sample X
µ2: Control gene Ct for sample X
µ3: Target gene Ct of control sample
µ4: Control gene Ct of control sample
AE1: Amplification Efficiency of target gene for sample.
AE2: Amplification Efficiency of control gene for sample.
AE3: Amplification Efficiency of target gene for control sample.
AE4: Amplification Efficiency of control gene for control sample.

$$\text{Ratio} = 2^{-}{}_{\Delta\Delta Ct} \quad (2)$$

Phenotype/Genotype Correlation.- A comparison between dominant (most common) phenotypes and genotypes was performed to esta-

blish the correlation and associations among the genotypes responsible for antibiotic resistance.

RESULTS

RT-qPCR.- The assay tested the purified RNA of all forty *P. aeruginosa* strains for each of the five target genes. Ct values, amplification and dissociation curves were obtained through the 7300-software system (Applied Biosystems) from all forty *P. aeruginosa* isolates. Amplification curves for *mexA* and *mexX* resistant isolates (Figures 1A and 1B) had a lower Ct than susceptible ones. A melting curve analysis for mexX (87 ºC) was used for confirmation of specific amplification of the PCR fragment (Figure 1C). Finally, the constant expression of the housekeeping gene (*rpsL*) is showed for both susceptible and resistant isolates (Figure 1D).

Amplification Efficiency and Statistical Analyses.- AE values were obtained from the resistant and susceptible isolates used as controls for each of the five gene expression systems. The Ct values obtained in all serial dilutions were analyzed by a two-sample T-test (Table 1 and Table 2).

Gene Expression.- Based on Yuan et al. (2008), Statistical methods for efficiency adjusted real-time PCR quantification, gene expression was quantified using Eq (1) and (2) (see Materials and Methods). Gene expression ratios for all strains and genes are showed in Table S4 (Supplementary information). A comparison of the expression ratios of resistant and susceptible phenotypes (average) was performed for each gene as shown in Figure 2. As expected, the expression ratios were higher in resistant isolates in comparison to susceptible ones. Over expression of efflux pump genes in clinical strains of *P. aeruginosa* was thus confirmed, stressing its importance as one of the main resistance mechanisms against β-lactam antibiotics. Furthermore, expression ratios for *oprM* and *oprJ* genes are lower than expression ratios for *mexA* and *mexX* genes, in spite of over-expression resistant isolates being greater than that in susceptible ones.

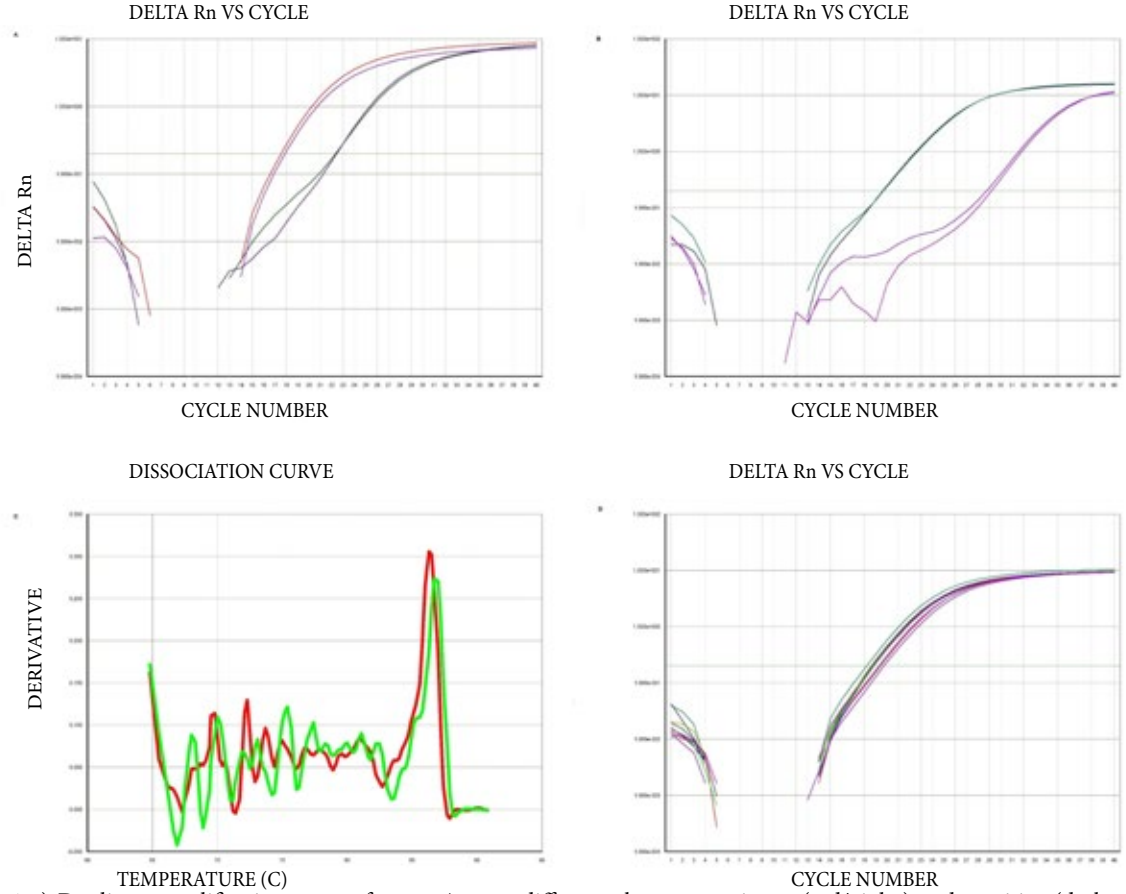

Figure 1 a) Duplicate amplification curves for *mexA* gene, difference between resistant (red/violet) and sensitive (dark purple/green) isolates. b) Duplicate amplification curves for *mexX* gene, difference between resistant (blue/green) and sensitive (violet/fuchsia) strains are showed. c) Duplicate dissociation curve for *mexX* gene (red/green), showing a melting temperature of 87 ºC. d) 10 amplification curves for *rpsL* gene with constant expression for resistant (purple spectrum) and sensitive isolates (green spectrum).

Table 1. Amplification Efficiency for each gene system

Gene	Phenotype	Slope	AE (%)
mexA	Resistant	-3.3058	100
	Susceptible	-3.3109	100
mexX	Resistant	-3.3239	99.92
	Susceptible	-2.045	100
oprM	Resistant	-3.5131	92.6
	Susceptible	-3.1858	100
oprJ	Resistant	-3.4104	96.44
	Susceptible	-3.4333	95.55
rpsL	Resistant	-3.4891	93.47
	Susceptible	-3.4549	94.73

Phenotype/Genotype Correlation.- Correlation between genotype and phenotype was performed using gene expression results as well as AST profiles. The results presented in Figure 3 showed *mexA* and *mexX* as dominant genotypes, data that was confirmed by the higher expression ratios shown in Table S4 Supplementary information. Out of the 20 resistant isolates tested in this study, in 95% of the isolates the *mexA* gene is overexpressed and in 100% the *mexX* gene is overexpressed, showing the presence of active genes encoding efflux pumps in clinical isolates of *P. aeruginosa.*

Furthermore, AST analysis showed that the main resistance phenotypes were to MEM (meropenem) and to IPM (imipenem), with a 100% resistance profile, and to TPZ (tazobactam) and to FEP (cefepime), with a 95% resistance profile (Figure 4).

AST profiles for susceptible isolates showed that more than 50% of the isolates express resistance to at least one β-lactam antibiotic. When a comparison performed of the antibiotic susceptibility profiles of the resistant strain with expression ratios for both susceptible and resistant isolates (Figure 5), we concluded that the *mexA* overexpression genotype is closely related to TPZ phenotype, while the *mexX* over expression genotype correlates to IMP, FEP and MEM resistance.

Table 2. Two-sample t-test results for resistant and sensitive isolates (p-value is < 0.05 in all cases)

Gene	Phenotype	Mean
mexA	Resistant	2.35
	Susceptible	6.2
mexX	Resistant	7.89
	Susceptible	12.86
oprM	Resistant	-2,02
	Susceptible	1.61
oprJ	Resistant	2.05
	Sensitive	4.39

DISCUSSION

In this study, we developed RT-qPCR assays for relative quantification of the expression of *P. aeruginosa* genes encoding efflux pumps (*mexA*, *mexX*, *oprJ* and *oprM)*. We then analyzed changes in gene expression in both resistant and susceptible clinical isolates. This technique allowed us to confirm the important role of efflux pumps as one of the main resistant mechanisms expressed in *P. aeruginosa* clinical isolates in the collection

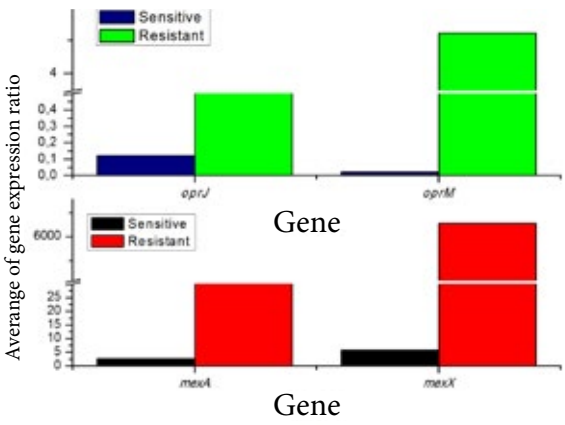

Figure 2. *oprJ, oprM, mexA and mexX* average expression ratios from sensitive and resistant strains.

analyzed. This finding is supported by the expression analysis of four genes performed in this study. Despite different expression ratios, over-expression in resistant isolates is much higher than in susceptible ones (Bolha et al. 2012). The RT-qPCR technique developed in this study is as a robust evidence for gene expression analysis in susceptible and resistant *P. aeruginosa* The model proposed by Yuan et al. (2008) considers amplification efficiency to be reliable method for relative quantification, since 100% amplification value is not possible to achieve because due to the different substrate conditions and the efficiency of the nucleic acid extraction kit used. Usually, amplification efficiencies for RT-qPCR assays are within 70–100%. We obtained an average efficiency of 90% in all PCR systems tested.

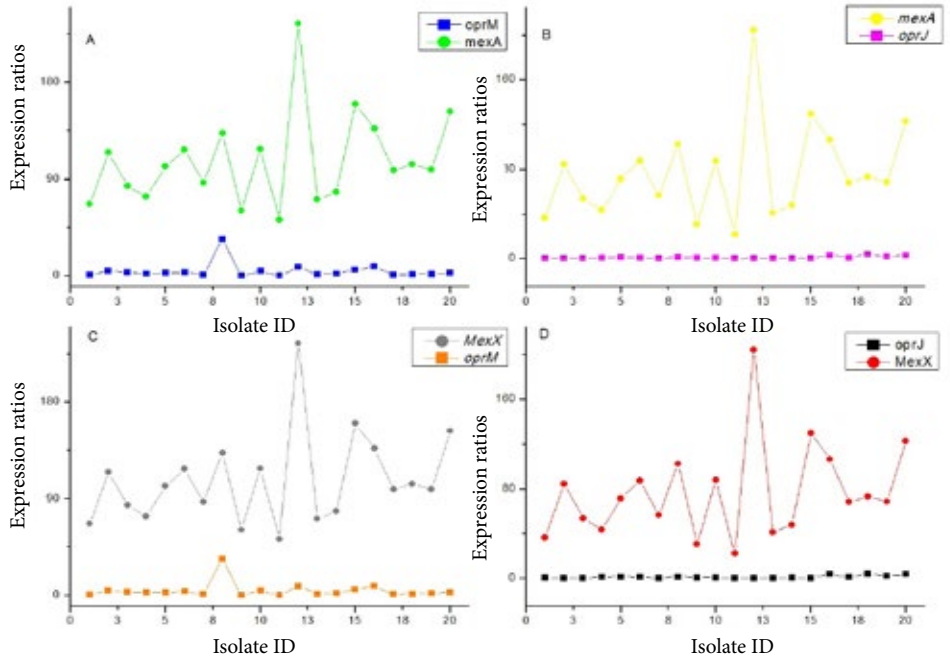

Figure 3. Efflux pumps encoding genes expression ratios differences in resistant strains of *P. aeruginosa.* A) *mexA* and *oprM*, B) *mexA* and *oprJ,* C) *mexX* and *oprJ* and D) *mexX* and *oprM.*

Expression ratios were considerably higher in all resistant isolates. *mexA* and *mexX* genes are the dominant genotypes, being over-expressed in almost all resistant isolates. Overexpression of efflux pump genes might suggest the presence of a nosocomial strain of *P. aeruginosa* in the hospital environment. However, it is important to note that horizontal transmission might be another mechanism for the spreading of infection among patients coming from another health facility.

The evident difference in the expression ratios of *mexA* and *mexX* in comparison with *oprM* and *oprJ* is related with their function in the RND complex. The *mexA* and *mexX* genes are important Membrane Fusion Proteins (MFP) in the pump structure. They act as assemble adaptors that ensure proper channel conformation in order to connect the cytoplasm and the membrane. Therefore, the overexpression of MFP's in all strains suggests a well-conformed and functional efflux pump. Low expression of the *oprJ* gene in comparison to the other three sys-

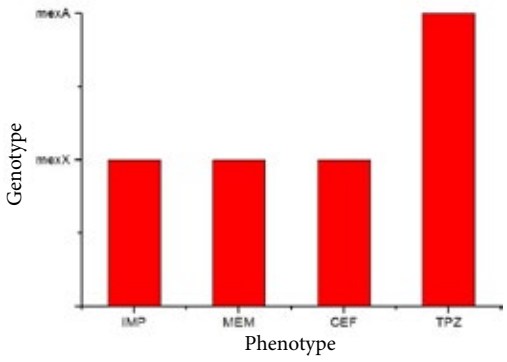

Figure 4. Resistance percentages for the β-lactams antibiotics: ceftazidime (CAZ), Azithromycin (ATM), tazobactam (TPZ), imipenem (IMP), cefepima (FEP) and meropenem (MEM).

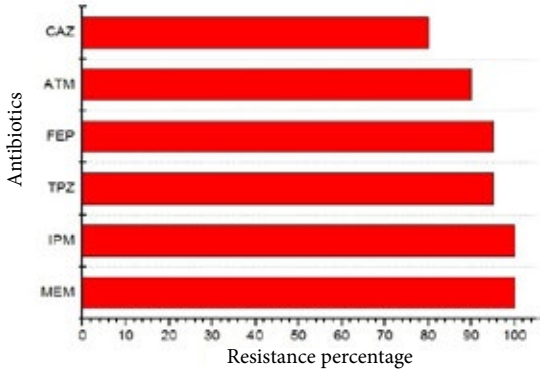

Figure 5. Correlation among dominant phenotype and genotype for both sensitive and resistant isolates. Genes mexA and mexX related with phenotypes resistant to tazobactam (TPZ), imipenem (IMP), cefepima (FEP) and meropenem (MEM).

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tems analyzed is due to the fact that it is acquired from plasmids, rather than chromosomally encoded in the *P. aeruginosa* genome (Lister et al. 2009).

The importance of analyzing the phenotype and genotype correlation in multiresistant *P. aeruginosa* was first stressed by Pommerenke and collaborators (2010). In the present study, we compared AST profiles for susceptible and resistant clinical isolates to the isolate's expression ratios. Our findings show that a number of susceptible isolates have measurable gene expression for the resistance-related genes *mexA* and *mexX* and show resistance to one or more of the phenotypically dominant antibiotics (tazobactam (TPZ), imipenem (IMP), cefepima (FEP) and meropenem (MEM)).

The most remarkable case was the resistant isolate R11, which showed *mexX* overexpression, providing resistance to ATM, MEM and IPM. Phenotypically susceptible isolates (5, 7, 8, 10, 13, 15, and 20), which presented resistance to TPZ, showed high *mexA* expression. Therefore, we were able to establish a correlation for the presence of the *mexA* genotype in the TPZ-resistant phenotype and a correlation of the *mexX* genotype the ATM, MEM and IPM-resistant phenotypes.

CONCLUSIONS

Gene expression (*mexA, oprM, mexX* and *oprJ*) pattern analysis in resistant isolates confirmed the predominate role of efflux pumps as one of the most important β-lactam resistance mechanisms.

Efflux pumps encoding genes are mainly acquired by mutations and horizontal gene transfer among strains present in the inner hospital environment. *P. aeruginosa* resistant isolates play an important role on this acquired resistance mechanism inside the hospital. This study found the same genetic pattern in more than 95% percent of all clinical isolates studied. However, larger studies are recommended in order to achieve a better comprehension of the mechanisms involved in β-lactam resistance in *Pseudomonas aeruginosa* clinical isolates.

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Suplementary information

Table S1. Isolates collection sites and patient demographics (susceptible isolates-S, resistant isolates-R)

Isolate	Age	Sex	Collection site	Ward
S1	67	F	Pharynx	Surgery
S2	44	M	Trachea	Neurosurgery
S3	74	F	Trachea	Neurosurgery
S4	24	M	Pharynx	Surgery
S5	25	M	Femoral	Surgery
S6	48	M	Blood culture	Gastroenterology
S7	74	F	Trachea	Neurosurgery
S8	74	M	Trachea	Neurosurgery
S9	71	F	Trachea	UCI
S10	25	M	Femoral	Surgery
S11	28	M	Catheter	Burns unit
S12	23	F	Catheter	Surgery
S13	27	F	Trachea	Burns unit
S14	27	M	Blood culture	Ortophedics
S15	74	M	Trachea	Neurosurgery
S16	72	M	Trachea	Surgery
S17	48	M	Peritoneal	Surgery
S18	74	M	Trachea	Neurosurgery
S19	48	M	Secretion	Surgery
S20	74	M	Trachea	Neurosurgery
R1	62	M	Femoral	Ortophedics
R2	67	M	Pancreatic tissue	Surgery
R3	43	M	Fracture	Ortophedics
R4	19	M	Femoral head	Surgery
R5	19	M	Left forearm	Surgery
R6	28	M	Secretion	Surgery
R7	69	M	Bile	Gastroenterology
R8	55	M	Wound	Surgery
R9	50	M	Limb	Surgery
R10	44	F	Skin (breast)	Surgery
R11	11	M	Secretion	Preventive medicine
R12	28	M	Sacral ulcer	Surgery
R13	22	F	Wound	Surgery
R14	8	M	Peritoneal	Pediatrics
R15	46	M	Trachea	Neurosurgery
R16	58	F	Peritoneal	Neurosurgery
R17	42	M	Catheter	Dialysis unit
R18	25	M	Trachea	Chest medicine
R19	43	F	Trachea	Neurosurgery
R20	54	F	Catheter	Surgery

Table S2. Primers and probes used in this study

	Probes 5' - 3'	Primers 5' - 3'		
Gene	MGB (Minor Groove Binding) with FAM dye	Forward	Reverse	Product length (bp)
mexA	CTGCTGCCCGGCAT	GTTCCCCAACCCGAACAAC	TTGACGCCTTCCTGCAACT	69
mexX	N/A (Syber Green)	CCATGCGTGCCCTGTTC	TCGCCTGCGGGTTCAC	93
oprJ	CTGCGTGCCAGCCT	GATCGGCAGCGTCAACGT	AATGTCCGGCCTGTTCGA	67
oprM	CTCCAGGAGCGCGAG	GGTTCGGGTTCCTGGTTGTT	CGTTGATGTCCTTCTGGATCTTC	106
rpsL	ACGCGGGTGCATAC	TTTTCGGCGTGGTGGTGTA	CAAAACTGCCCGCAACGT	58

Table S3. AST profiles for susceptible (S) and resistant (R) isolates. Antibiotics used for the analysis were: MEM (meropenem), IPM (imipenem), TPZ (tazobactam), FEP (cefepime) and CAZ ceftazidima.

Isolate	CAZ	FEP	ATM	MEM	IPM	TPZ
S1	S	S	S	S	S	S
S2	S	S	S	S	S	S
S3	R	S	S	R	S	S
S4	S	S	S	S	S	S
S5	S	S	S	S	S	R
S6	S	S	S	R	S	S
S7	S	S	S	S	R	R
S8	S	S	S	S	S	R
S9	S	R	S	S	S	S
S10	S	S	S	S	S	R
S11	S	S	S	S	R	S
S12	S	S	S	S	R	S
S13	S	S	S	S	S	R
S14	S	S	S	S	S	S
S15	S	S	S	S	S	R
S16	S	S	S	S	R	S
S17	S	R	S	S	S	S
S18	S	S	S	S	S	S
S19	S	S	S	S	S	S
S20	S	S	S	S	S	R
R1	R	R	R	R	R	R
R2	S	R	R	R	R	R
R3	R	R	R	R	R	R
R4	R	R	R	R	R	R
R5	S	R	R	R	R	R
R6	-	R	S	R	R	R
R7	R	R	R	R	R	R
R8	R	R	R	R	R	R
R9	R	R	R	R	R	R
R10	R	R	R	R	R	R
R11	S	S	R	R	R	S
R12	R	R	R	R	R	R
R13	R	R	R	R	R	R
R14	S	R	R	R	R	R
R15	R	R	R	R	R	R
R16	R	R	R	R	R	R
R17	R	R	R	R	R	R
R18	R	R	R	R	R	R
R19	R	R	R	R	R	R
R20	R	R	S	R	R	R

Table S4. Expression ratios obtained for all efflux pumps encoding genes in susceptible and resistant strains.

Strain	Susceptible				Strain	Resistant			
	mexA	mexX	oprJ	oprM		mexA	mexX	oprJ	oprM
S-1	1,726	1,773	0,229	0,002	**R-1**	14,530	224,300	0,503	1,030
S-2	1,457	0,042	0,175	0,004	**R-2**	62,400	41,690	0,387	4,717
S-3	0,212	0,682	0,018	0,003	**R-3**	31,380	19,790	0,329	3,159
S-4	3,213	0,027	0,015	0,003	**R-4**	21,400	60,580	1,080	2,327
S-5	5,059	0,822	0,090	0,027	**R-5**	49,510	1920,000	1,247	2,954
S-6	2,729	29,370	0,139	0,042	**R-6**	65,190	36,830	1,003	3,748
S-7	1,138	0,307	0,125	0,036	**R-7**	34,560	1046,000	0,394	1,158
S-8	6,012	0,816	0,311	0,080	**R-8**	80,150	110900,000	1,487	34,210
S-9	3,561	19,330	0,379	0,023	**R-9**	8,535	612,300	0,947	0,300
S-10	1,574	0,456	0,013	0,026	**R-10**	65,580	753,000	0,795	4,332
S-11	3,409	6,220	0,295	0,040	**R-11**	0,003	27,120	0,000	0,091
S-12	1,701	6,813	0,124	0,014	**R-12**	182,200	7490,000	0,292	8,514
S-13	4,390	0,591	0,096	0,051	**R-13**	18,940	9,946	0,305	1,591
S-14	3,037	1,089	0,094	0,021	**R-14**	25,760	18,510	0,697	2,097
S-15	4,040	1,299	0,048	0,049	**R-15**	107,400	136,600	0,437	5,720
S-16	1,677	0,133	0,038	0,008	**R-16**	84,420	211,400	3,469	8,700
S-17	0,823	42,500	0,086	0,009	**R-17**	46,050	89,970	1,139	1,151
S-18	1,093	2,881	0,052	0,006	**R-18**	51,170	442,400	3,945	1,427
S-19	1,971	1,350	0,074	0,008	**R-19**	46,510	423,400	2,080	2,023
S-20	2,247	0,766	0,070	0,005	**R-20**	100,700	997,700	3,656	3,030
Average	2,553	5,863	0,124	0,023		54,819	6273,077	1,210	4,614