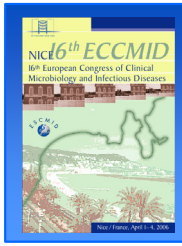


# Detection of *mexA* and *mexX* efflux genes in *P. aeruginosa*: correlation between QC-RT-PCR and Real-Time PCR.

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## Abstract

**Objectives:**

Efflux systems are rarely identified as such in clinical microbiology laboratories. Yet, overexpression of transporters such as MexAB-OprM and MexXY-OprM are likely to cause antibiotic multi- and cross-resistance in *Pseudomonas aeruginosa*, leading to potential clinical treatment failures because of their inducible character. We have previously developed and validated with reference strains a QC-RT-PCR method to quantify *mexA* and *mexX* expression levels (ECCMID 2005, P1731). In the present study, we have developed a Real-Time-PCR assay and present here the correlation between both methods using control strains and clinical isolates.

**Methods:**

Expression levels of *mex A* and *mexX* were measured by both techniques in (i) 4 reference strains expressing only one of these efflux mechanisms (mexA [2] or mexX [2]); and (ii) 8 clinical isolates, in comparison with the wild-type strain PAO1 (basal *mexA* and *mexX* expression levels).

**Results:**

Real-Time PCR showed an inter-day reproducibility of 95±5.3 % (triplicates of 10 strains). Among the clinical strains, 5 overexpressed *mexA* and 3 *mexX*. The Table shows (i) the mean level of overexpression of *mexA* and *mexX* in comparison with the wild type strain PAO1 (set at 1), as detected by Real-time PCR for all strains; (ii) the ratio of these values to those observed by QC-RT-PCR for the corresponding transporter.

| *mexA* (n=7) | | *mexX* (n=5) | |
|---|---|---|---|
| Real-Time-PCR | ratio to QC-RT-PCR | Real-Time-PCR | ratio to QC-RT-PCR |
| 5.99±0.29 | 1.03±0.22 | 6.83±0.31 | 0.98±0.24 |

**Conclusions:**

Both QC-RT-PCR and Real-Time-PCR are potentially useful in clinical laboratories as sensitive and rapid diagnostic tools to quantify the expression level of *mexA* and *mexX* in *P. aeruginosa*. Combined with phenotypic characterization, this approach may help in a better understanding of the resistance mechanisms and epidemiology of resistance in this difficult-to-treat nosocomial pathogen.

## Background

*Pseudomonas aeruginosa* is primarily a nosocomial pathogen, responsible for opportunistic infections. These are of great concern, because *P. aeruginosa* presents intrinsic as well as acquired resistance to a wide variety of antimicrobial agents. In this context, polyspecific efflux pumps play a central role in the multiresistance of *P. aeruginosa* towards both antibiotics and antiseptics.

Seven "Mex-type" efflux pumps able to transport various antimicrobial agents have been characterized in *P. aeruginosa,* but four of them (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM) have been shown to contribute to clinically significant resistance levels (Llanes *et al.*, 2004).

In this context, the development of rapid and reliable methods for the early detection of this efflux pumps in clinical isolates may positively assist for the selection of appropriate therapeutic agents in a given patient and also for the screening of resistance mechanisms in epidemiological surveys. We have previously developed and validated a Quantitative-Competitive RT-PCR method to quantify *mexA* and *mexX* genes expression levels in references strains and in clinical isolates (ICAAC 2005; P 1902).

## Aim of the study

**- to develop and validate a Real Time PCR method for the quantification of *mexA* and *mexX* genes expression levels in *Pseudomonas aeruginosa***

**- to correlate genes expression levels as measured by QC-RT-PCR and by Real Time PCR**

## Materials & Methods

***Strains and growth conditions:*** we used *P. aeruginosa* wild type (PAO1), two *P. aeruginosa* overexpressing MexAB-OprM (PT629 - Kohler *et al.*, 1997 and SLF30 - gift from José L. Martinez – Dept. Microbiologia, Centro Nacional de Biotecnologia, Madrid, Spain ), two *P. aeruginosa* overproducing MexXY-OprM (MutGR1 - Hocquet *et al.*, 2003 and SLF05 - gift from José L. Martinez – Dept. Microbiologia, Centro Nacional de Biotecnologia, Madrid, Spain ) and 6 clinical isolates. The strains were grown over-night on Muller-Hinton broth (MHB) at 37°C under aerobic conditions and gentle agitation (100 rpm).

***RNA extraction and cDNA synthesis:*** Total bacterial RNA (tRNA) was isolated for each strain from 1.5 ml of late-log-phase *P. aeruginosa* cultures and treated with Rnase-free DNase (1U of enzyme/µg RNA for 20 min at room temperature). All samples were checked for DNA contamination by PCR, using RNA like template. Five µg of tRNA was used in RT-PCR reactions.

***Quantification of mexA and mexX expression level***

1. ***Quantitative-Competitive-RT-PCR (QC-RT-PCR):*** The genes of interest (*mexA* and *mexX*) were amplified from their respective cDNA with primers -F and -R. Internal competitors were generated by PCR for each gene in the presence of primer 40mer (F+internal 20 pb) and -R primer (see below). During the PCR reactions, decreasing amounts of internal competitors (1000 ag to 5 ag) were co-amplified with the same amount of target cDNA (1 µl) (see below). PCR reactions were performed in triplicates. The two amplified products were separated in 1.7% agarose, stained with ethidium bromide, and quantified by densitometry. The concentration of internal competitor DNA at which the two amplimers show equal intensity was assigned as the concentration of target cDNA.
2. ***Real Time PCR (qPCR):*** Amplification reactions were performed in the presence of Sybr Green. We used 5 µl of a 5-fold dilution of cDNA samples as template for quantification. To correct for possible differences in the amount of starting material, the ribosomal *rpsL* gene (used as housekeeping gene) was amplified in parallel. For each gene measured, a sequence-specific standard curve was generated using 10-fold serial dilutions of purified PCR fragments, the concentration of which was determined by measuring their optical density in UV. Results were expressed as the ratio between the expression level of the target gene (*mexA* or *mexX*) and the expression level of the reference gene (*rpsL*), which were obtained according to the following equation:

$$\text{Ratio} = \frac{(E_{\text{Target gene}})^{\Delta Ct \text{ target gene}}}{(E_{rpsL})^{\Delta Ct \text{ house keeping gene}}}$$

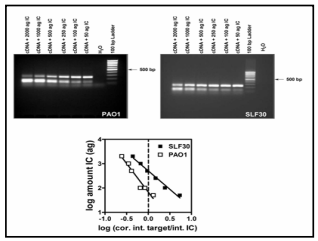
( = Ct target strain – Ct wild-type strain)

where E is the Real Time PCR efficiency for a given gene and $C_t$ is the crossing point of the amplification curve with the threshold.

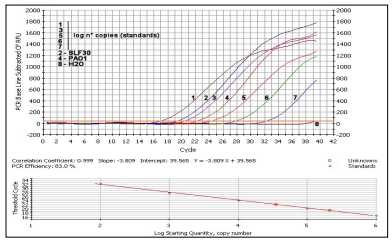
An effect on gene expression was considered significant when the corresponding ratios were > 2.0.

***Assessment of reproducibility of the quantitative methods:*** cDNA preparation was done in triplicates from 3 independent bacterial cultures. The PCR quantification reactions were routinely performed in triplicates for each of the cDNA preparations, generating a total of 9 data sets for each strain.

## Results




**QC-RT-PCR of *mexA*:** Quantification of mexA by Qc-RT-PCR in PAO1 strain (wild-type) and SLF30 strain (MexAB-OprM overproducer). Upper panels: ethidium bromide stained gels showing the amplification product of the target gene (T-252 bp) and 6 increasing amounts of internal competitor (IC – 127 bp; amounts given in ag). Ladder is 100 bp marker. Lower panel: the equation of the regression lines are y = -2.2393x + 1.9313 ($R^2$ = 0.9858) for PAO1 and y = 1.519 +/- 2.7082 ($R^2$ = 0.9825) for SLF30. The amount of target gene cDNA corresponds to the crossing between the regression line and the X axis (equal signal for target cDNA and competitor).




**Real Time PCR of *mexA*.** Upper panel: Amplification plot of real-time PCR of *mexA* for standards and cDNA of PAO1 (wild)-type strain) and of SLF30 (MexAB-OprM overproducer). The graph shows the PCR baseline subtracted relative fluorescence units (with the baseline set at 46.8 units) as a function of the number of cycles. The reaction was run in parallel for *rpsL* (housekeeping gene), the quantification of which was used for normalization of the results. Lower panel: standard curve of this assay, with the number of starting copies plotted against the threshold cycle. Circle shapes: standard samples; square shapes: unknown samples. The equation used to calculate the number of copies in unknowns is y = –3.809 x + 39.563 ($R^2$ 0.999).

| strain | *mexA* expression level | | | *mexX* expression level | | |
|---|---|---|---|---|---|---|
| | QC-RT-PCR | Real Time RT-PCR | QC-RT-PCR / Real Time-PCR | QC-RT-PCR | Real Time RT-PCR | QC-RT-PCR / Real Time-PCR |
| PAO1 | 1 | 1 | | 1 | 1 | |
| PT629 | 4.57 ± 0.11 | 4.18 ± 0.14 | 1.09 | ND | 0.18 ± 0.01 | |
| SLF30 | 6.45 ± 0.20 | 6.04 ± 0.07 | 1.07 | ND | 1.18 ± 0.02 | |
| MutGR1 | ND | 1.53 ± 0.17 | | 5.58 ± 0.19 | 6.38 ± 0.40 | 0.87 |
| SLF05 | ND | 1.27 ± 0.09 | | 7.09 ± 0.17 | 6.61 ± 0.07 | 1.07 |
| 324 | 5.73 ± 0.16 | 5.45 ± 0.45 | 1.05 | ND | 0.14 ± 0.03 | |
| 376 | ND | 2.50 ± 0.28 | | 4.77 ± 0.18 | 5.07 ± 0.87 | 0.94 |
| 411 | 7.71 ± 0.16 | 6.90 ± 0.37 | 1.12 | ND | 0.19 ± 0.02 | |
| 699 | 5.86 ± 0.07 | 5.84 ± 0.34 | 1.01 | ND | 0.67 ± 0.17 | |
| 785 | 7.18 ± 0.16 | 6.74 ± 0.29 | 1.07 | 8.12 ± 0.13 | 8.63 ± 0.21 | 0.94 |
| 905 | 5.96 ± 0.19 | 6.72 ± 0.43 | 0.87 | 7.84 ± 0.17 | 7.46 ± 0.34 | 1.05 |

**Quantification of the expression levels of *mexA* and *mexX* genes by QC-RT-PCR and Real Time PCR and correlation between the values determined by the two techniques.**

## Conclusions

**- we developed two tecniques for the quantification of *mexA*, and *mexX* genes in *Pseudomonas aeruginosa* clinical isolates.**

**- we observed a satisfactory correlation ( >88% ) between the genes expression levels determined by these 2 techniques.**

**- each of these techniques can therefore be used for the analysis of efflux pumps expression in clinical isolates.**

## References

- Llanes *et al.*, Clinical Strains of *Pseudomonas aeruginosa* Overproducing MexAB-OprM and MexXY-OprM Efflux Pumps Simultaneously. 2004; AAC,48:1797-1802.
- Mesaros *et al.*, Genotypic Method is More Reliable Method than Phenotypic Characterization to Detect Mex Efflux Pumps in Clinical Isolates of *Pseudomonas aeruginosa*. ICAAC Dec 16-21, 2005 - Washington DC, USA (P1902).
- Kohler T, Michea-Hamzehpour M, Plesiat P, Kahr A L and Pechere J C (1997b) Differential selection of multidrug efflux systems by quinolones in Pseudomonas aeruginosa. *Antimicrob Agents Chemother* **41**: 2540-2543.
- Hocquet D, Vogne C, El Garch F, Vejux A, Gotoh N, Lee A, Lomovskaya O and Plesiat P (2003) MexXY-OprM efflux pump is necessary for a adaptive resistance of Pseudomonas aeruginosa to aminoglycosides. *Antimicrob Agents Chemother* **47**: 1371-1375.

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