564/91P

A RAPID AND VERSATILE METHOD TO QUANTIFY THE EXPRESSION LEVEL OF *mexAB-oprM* AND *mexXY-oprM* GENES IN *Pseudomonas aeruginosa*

N. Mesaros, N. Caceres, F. Van Bambeke, M.P. Mingeot-Leclercq and P.M. Tulkens

Unité de Pharmacologie cellulaire et moléculaire

Université catholique de Louvain - Brussels - Belgium

Mailing address:
P. Tulkens
Pharmacologie cellulaire et moléculaire
UCL 73.70 av. Mounier 73
1200 Brussels - Belgium
tulkens@facm.ucl.ac.be

ABSTRACT

OBJECTIFS: In order to optimize the empirical therapeutic choices and to influence prescription in a manner that will slow down the further development of resistance, it is not only desirable, but also essential, to survey individual isolates and to characterize them in terms of mechanisms of resistance. This, nowadays, must include the efflux systems which are rarely identified as such in the clinical microbiology setups. To date, six RND-type multi-drug efflux systems have been identified in *Pseudomonas aeruginosa*, three of them having a constitutional expression. The Western Blot technique can reveal the overexpression of these systems in a first instance. Alternatively, when antibodies against these proteins are not currently available, assaying mRNA would be essential. In this context, we developed, validated, and adapted a new method, a QC-RT-PCR (Quantitative Competitive RT-PCR) to quantify the levels of expression of antibiotic resistance related genes *mexAB-oprM* and *mexXY-oprM* in *P. aeruginosa*.

METHODS: *P. aeruginosa* PAO1 wild type strain, *P. aeruginosa* MexAB-OprM overproducer strain and *P.aeruginosa* MexXY-OprM overproducer strain were used in the study. Total RNA from all strains was isolated and used in RT-PCR reactions. The genes of interest were amplified by PCR from their respective cDNA in presence of primer -F and -R. For each gene an internal competitor DNA was generated by PCR [primer-40mer (primer-F+20 internal pb) and primer-R]. Triplicate PCR reactions were performed adding the internal competitor DNA at defined concentrations, identical aliquots of cDNA and primers -F and -R.

RESULTS: With this method we detected that in the case of the MexAB-OprM overproducer strain the expression level is at least 5 fold higher than the level observed for the same gene in the wild-type strain. In MexXY-OprM overproducer bacteria compared to the wild-type strain, we observed a *mexXY-oprM* gene expression level of at least 8 fold higher.

CONCLUSIONS: The QC-RT-PCR is an advantageous method and allows for both a sensitive and rapid diagnostic procedure to quantify the expression level of efflux genes of interest.

INTRODUCTION

Pseudomonas aeruginosa is primary 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 particular, polyspecific efflux pumps play a central role in the multiresistance of *P. aeruginosa* towards both antibiotics and antiseptics.
Overexpression of 2 constitutive efflux pumps, namely MexAB-OprM and MexXY-OprM, has indeed been shown to occur in clinical isolates and to contribute to multidrug resistance (Llanes *et al.*, 2004). In this context, the development of rapid and reliable methods for the early detection of this overexpression in clinical isolates may positively assist for the selection of appropriate therapeutic agents in a given patient and also be used for the screening of resistance in epidemiological surveys.

QC-RT-PCR uses the properties of a thermostable polymerase, Taq polymerase, to amplify small segments of cDNA through several rounds of synthesis (PCR). The segments that will be exponentially amplified depend upon the DNA oligonucleotide primers placed into the reaction mixture, which are the basis for specificity of the reaction. Because the amplification process is exponential and may vary from one experiment to the other due to changes in environmental factors, the addition of a competitor (internal standard - IS) in known concentration during the PCR will greatly improve the quality of the quantification (the amounts of target product (T) and IS being compared in order to obtain an accurate estimation of the target's starting concentration). QC-RT-PCR appears nowadays as the most sensitive method to measure mRNA levels, especially when the number of copies is low or when small amounts of sample are available for analysis.

OBJECTIVES

To quantify the expression level of *mexAB-oprM* and *mexXY-oprM* genes using quantitative-competitive-RT-PCR (QC-RT-PCR) as a novel detection method

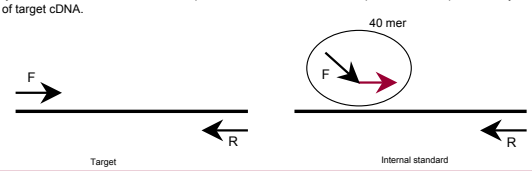
METHODS

Strains and growth conditions: we used *P. aeruginosa* wild type (PAO1), *P. aeruginosa* overexpressing MexAB-OprM (*nalB* mutant) (Kohler *et al.*, 1997) and *P. aeruginosa* overproducing MexXY-OprM (Hocquet *et al.*, 2003). The strains were grown on Muller-Hinton broth (MHB) at 37°C under gentle agitation at 100 rpm.

RNA extraction: 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. Five µg of tRNA was used in RT-PCR reactions.

QC-RT-PCR: The genes of interest 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).

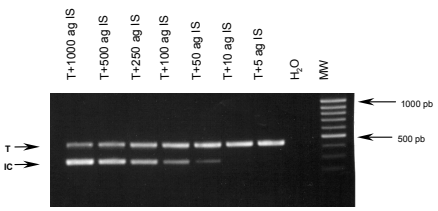
Quantification: PCR reactions were performed in triplicates by adding the internal competitor DNA at defined concentrations, identical aliquots of cDNA and promers - F and - R. The two amplified products were separated in 1.7% agarose, stained with ethidium bromide, and quantified by densitometry. The concentration of internal competitor DNA at which the two amplimers show equal intensity was assigned as the concentration of target cDNA.



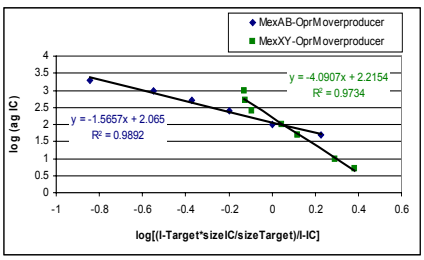
RESULTS

Validation of the method

QC-RT-PCR of *mexXY-oprM* in MexXY-OprM overproducer: amplification products of QC-RT-PCR are separated in agarose 1.7 % and stained with ethidium bromide. Varying amounts of the internal competitor (IC) (1000 ag to 5 ag) were co-amplified with 1 µl target cDNA (T) for 30 cycles. Ladder in the right side is 100 pb marker.

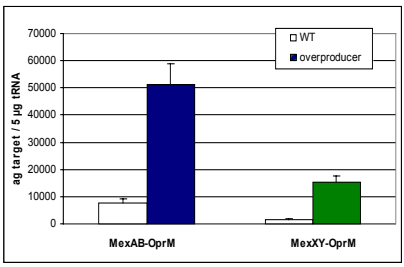


Quantification of *mexA* gene in MexAB-OprM overproducer and *mexX* gene in MexXY-OprM overproducer. The raport between the intensity of two bandes (T and IC) was ploted in function of known amount of IS in log scale. The amount of IS were the intensity of two bandes was equal, was taken as target cDNA concentration



Application of the method for the quantification of the expression level of the genes

Expression of mexAB-oprM and mexXY-oprM genes in wild type and overproducer strains. The expression of the *mexB* gene is more important than that of the *mexX* gene in the wild type strain. The expression of the *mexB* gene is increased about 6 fold and that of the *mexX* gene, about 8 fold in overproducer strains as compared to wild type strain.



CONCLUSIONS

- **we developed a fast and robust method for the quantification of *mexAB-oprM* and *mexXY-oprM* genes expression using a versatile PCR approach that could be setup for routine clinical assessment**
- **the basal level of expression in a wild type strain is higher for MexAB-OprM efflux pump than MexXY-OprM efflux pump**
- **the expression of the gene encoding MexB in the MexAB-OprM efflux pump is around 6 fold higher in an overproducer strain than in a wild type strain**
- **the expression of the gene encoding mexY in the MexXY-OprM efflux pump is around 8 fold more expressed in an overproducer strain than in a wild type strain**

REFERENCES

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**: pp 1371-1375.

Kohler T, Michea-Hamzehpour M, Plesiat P, Kahr A L and Pechere J C (1997) Differential Selection of Multidrug Efflux Systems by Quinolones in Pseudomonas Aeruginosa. *Antimicrob Agents Chemother* **41**: pp 2540-2543.

Llanes C, Hocquet D, Vogne C, Benali-Baitich D, Neuwirth C and Plesiat P (2004) Clinical Strains of Pseudomonas aeruginosa Overproducing MexAB-OprM and MexXY Efflux Pumps Simultaneously. *Antimicrob Agents Chemother* **48**: pp 1797-1802.

ACKNOWLEDGMENTS:

NM is recipient of a fellowship from the program Prospective Research in Brussels

NC is postodoctoral fellow, and FVB chercheur qualifié of the Belgian Fonds National pour la Recherche Scientifique