RND efflux pumps in *P. aeruginosa*: an underestimated resistance mechanism

An adequate initial antibiotic therapy is a key determinant of therapeutic success in *Pseudomonas aeruginosa*-infected patients. Antibiotic efflux is an underestimated resistance mechanism because it may occur in strains categorized as susceptible. It is rarely or not at all diagnosed in routine laboratories and often masked by high-level resistance mechanisms.

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P. aeruginosa: state of the art of antibiotic susceptibility

P. aeruginosa is a Gram-negative bacterium recognized as a major cause of infections in hospitalized patients or in patients with impaired defences as observed in burn wounds or cystic fibrosis. In spite of improved hygiene measures, the risk of infection by *P. aeruginosa* in ICU remains high (infection incidence > 30/100 patients hospitalized in ICU). *P. aeruginosa* infections are associated with mortality rates as high as 30 % to 50 % in bacteremia [1] and up to 70% in patients with nosocomial pneumonia [2].

Yet, empirical selection of antibiotics is made difficult by the continuously evolving resistance of *P. aeruginosa* to antibiotics, notably due to the emergence of Multi Drug Resistance (MDR) phenotype (R ≥ 3 antibiotic classes). The MDR status of the strain as well as an initial inappropriate treatment negatively influence patient outcome [3].

Acquired high level resistance is due to the acquisition of genes coding for aminoglycoside-modifying enzymes or beta-lactamases, or to mutations in fluoroquinolone targets. Intrinsic antibiotic resistance is due to low outer membrane permeability mediated either by under production of the oprD porin, or by the expression of multidrug resistance efflux pumps. The genome of *P. aeruginosa* codes for numerous efflux pumps, among which MexAB-OprM and MexXY-oprM are of first clinical importance due to their large prevalence in clinical strains and their ability to expel several classes of chemically-unrelated antibiotics.

RND efflux pumps in *P. aeruginosa*

The main efflux pumps in *P. aeruginosa* belong to the Resistance-Nodulation-Division (RND) superfamily, which uses proton motive force as energy source. They constitute a tri-partite system, composed of an integral cytoplasmic membrane drug-proton transporter, an outer membrane channel and a periplasmic fusion protein linking the two other proteins. This assembly allows expelling the substrate from the inner membrane directly to the extracellular medium [Fig. 1, reproduced from [4]].

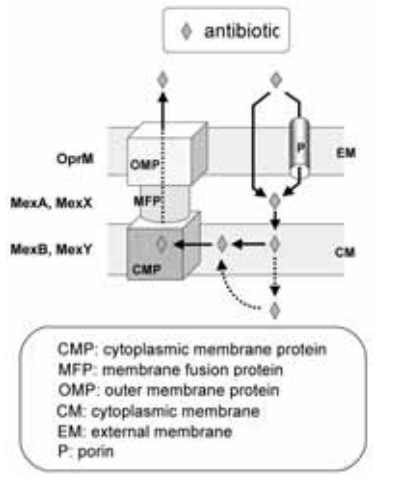

Figure 1. Resistance-Nodulation-Division (RND) transporter in P. aeruginosa.
In Gram (-), the antibiotic molecules must first cross the external membrane (EM), either by passive diffusion or by passage through a porin (P). The RND system is composed of three membrane proteins: the cytoplasmic membrane protein (CMP) recognizes the substrate and expels it by the proton motive force to the outer membrane protein (OMP), through a membrane fusion protein (MFP).

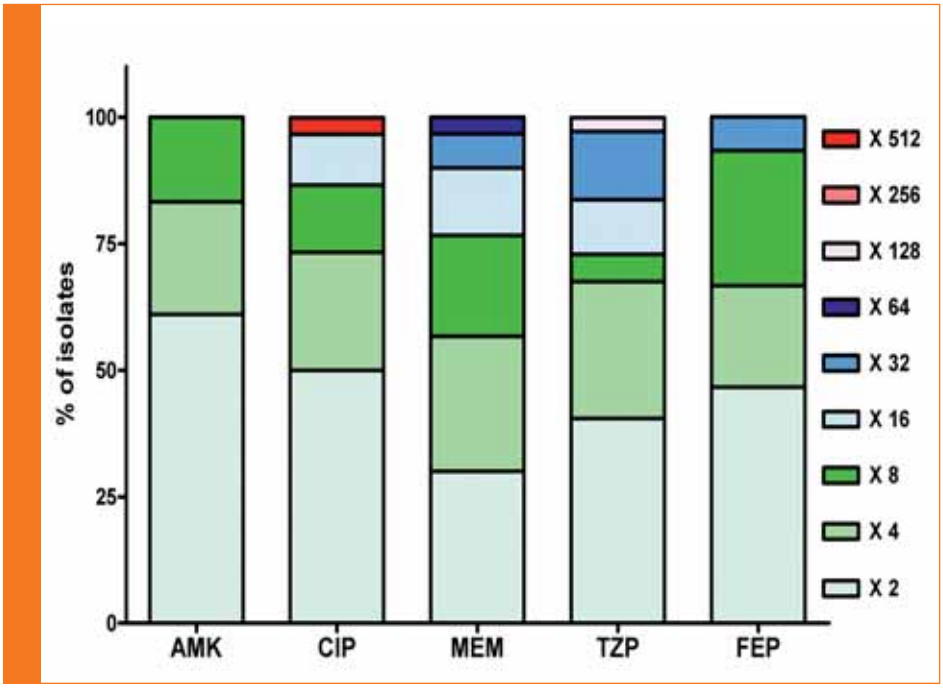

Figure 2. Increases in the minimum inhibitory concentration (MICs) of five antibiotics used in empirical antipseudomonal therapy between the first isolate and last isolate (clonal pairs) collected from the same individual patient in a population of 59 patients hospitalized in ICUs. (reproduced from [7])
The y-axis shows the percentage of clonal pairs with a given increased MIC [from 2x(1log2 dilution) to 512 x (9log2 dilution) the value of the initial isolate] out of all those showing an increase MIC (n=18 for AMK, n=30 for CIP, n=30 for MEM, n=37 for TZP and n=30 for FEP), AMK, amikacin; CIP, ciprofloxacin; MEM, meropenem; TZP, piperacillin/tazobactam; FEP, cefepime.

Ten efflux systems have been characterized in *P. aeruginosa*, among which MexAB-OprM and MeXY-OprM are constitutively expressed at a basal level in wild-type strains (expression of MeXY-OprM being however much lower than that of MexAB-OprM). Both systems are inducible when exposed to antibiotic substrates. The other systems (MexCD-OprJ, MexEF-OprN, MexJK, MexGHI-OpmD, MexVW, MexPQ-OpmE, MexMN, and TriABC are not expressed in wild type strains but may contribute to antibiotic or biocide resistance when expressed in resistant strains [5].

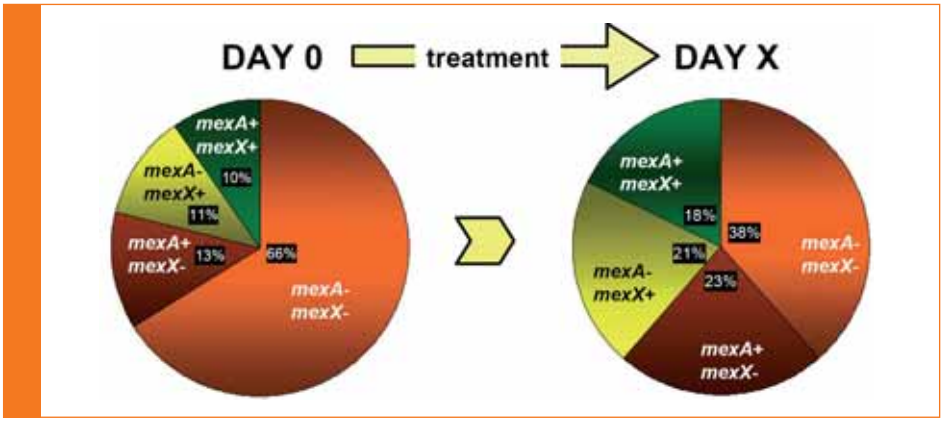

Figure 3. Prevalence of mexA+ (≥ 2 x basal level) and mexX+ (≥ 5 x basal level) or both, in 62 pairs of Pseudomonas aeruginosa collected from ICU patients at the initiation of treatment (day 0) or in the last isolate (day X) (adapted from [8]).

Antipseudomonal antibiotics released by *P. aeruginosa* multidrug efflux systems

RND efflux systems release multiple antimicrobials components including first-line antipseudomonal antibiotics such as β-lactams and β-lactamase inhibitors, fluoroquinolones, aminoglycosides [Table 1]. More specifically MexAB-OprM transports β-lactams fluoroquinolones, macrolides, tetracyclines, trimethoprim, sulfamides and chloramphenicol; MexXY-OprM, aminoglycosides, fluoroquinolones, macrolides, and tetracyclines; MexCD-oprJ, some β-lactams, fluoroquinolones, macrolides, tetracyclines, trimethoprim and chloramphenicol, and MexEF-OprN, fluoroquinolones, trimethoprim and chloramphenicol. The latter is also involved in resistance to meropenem and doripenem, but this may rather result from the fact that the OrpD porin is downregulated in strains expressing this efflux system.

Colistin, the last resort drug for MDR *P. aeruginosa*, is not substrate for these efflux pumps. Thus, efflux is responsible for multidrug resistance, a single pump being able to transport several classes of drugs while at the same time some redundancy exists among transporters, fluoroquinolones for example being universal substrates for the main efflux systems. Moreover, the subsequent reduction in antibiotic concentration inside the bacteria may help selecting high level resistance mechanisms, in particular target mutations [6].

Over-expression of efflux pumps: impact on antimicrobial susceptibility

A study published in 2010 examined the impact of antibiotic treatment on the susceptibility of *P. aeruginosa*, by collecting successive isolates from ICU patients at the time of diagnosis of infection and during treatment [7]. Globally, mean minimum inhibitory concentration (MIC) values increased after exposure to antibiotics, with statistically significant effects being observed for amikacin, ciprofloxacin, cefepime, meropenem and piperacillin/tazobactam, bringing mean MICs to values higher than the EUCAST susceptibility breakpoints. Three quarters of the isolates showed a moderate elevation of the MIC (≤16X initial MIC), suggesting the involvement of low to moderate levels resistance mechanisms as those affecting membrane permeability [Fig 2, reproduced from [7]].

The analysis of the expression of efflux pumps in this collection revealed that a high proportion of the strains (34 %) did overexpress MexAB-OprM and MexXY-OprM in the initial isolate, but that this proportion further increased during the antibiotic treatment, with about two third of the strain overexpressing at least one of these efflux systems [Fig.3, adapted from [8]].

Diagnosis of efflux in clinical laboratory

Because efflux in *P. aeruginosa* almost always co-operates with other mechanisms of resistance, differential diagnosis by phenotypic antimicrobial analysis is complex, high levels resistance mechanisms masking the effect of the expression of efflux systems on MICs. Moreover,

	Antibiotics recommended by EUCAST*																
RND SYSTEMS	PENICILLINS				CEPHALOSPORINS		CARBAPENEMS			MONOBACTAMS	FLUOROQUINOLONES		AMINOGLYCOSIDES				OTHERS
	PIPERACILLIN	PIPERACILLIN-TAZOBACTAM	TICARCILLIN	TICARCILLIN-CLAVULANATE	CEFEPIME	CEFTAZIDIME	DORIPENEM	IMIPENEM	MEROPENEM	AZTREONAM	CIPROFLOXACIN	LEVOFLOXACIN	AMIKACIN	GENTAMICIN	NETILMICIN	TOBRAMYCIN	COLISTIN
mexAB-oprM	X	X	X	X	X	X	X (LOW)		X	X	X	X					
mexCD-oprJ	X	X			X					X	X	X					
mexEF-oprN								X	X		X	X					
mexXY-oprM					X						X	X	X	X		X	

Table 1. Known antipseudomonal antibiotics supported by efflux systems.
** EUCAST: European Committee on Antimicrobial Susceptibility Testing*

efflux pumps can be overexpressed during treatment, which may explain therapeutic failures with antibiotics that are considered active based on the original determination of the susceptibility profile.

Resistance by efflux can be detected using Efflux Pumps Inhibitors (EPI), which revert MICs to those strains that do not express efflux systems. Among them MC-207,110 (phenylalanine arginyl beta-naphthylamide) is a broad spectrum inhibitor that has been widely used *in vitro* to investigate the impact of efflux on susceptibility to antibiotics of *P. aeruginosa*. Inhibitors specific of a given transporter are also under investigation. Yet, in MDR strains with additional resistance mechanisms, EPI do not allow restoring antibiotic activity, which may lead to false-negative results [9].

In this context, molecular methods appear as the only way to evidence the expression of efflux pumps in clinical isolates. Immunoblotting methods were developed first but were rapidly replaced by Reverse Transcriptase quantitative PCR (RT-qPCR) due to its higher specificity and rapidity. RT-qPCRs were thus developed to detect and quantify the expression of the genes coding for the different proteins of a given RND pump. This method remains applicable whatever the other resistance mechanisms present in the clinical strain and can thus be applied in clinical laboratories. Typically, a 2-fold increase in the expression of *mex*A and *mex*B genes causes a decrease in antibiotic susceptibility, while overexpression of *mex*X needs to be higher (≥ 5-fold) to increase MIC values. This low level of overexpression implies that all the steps for RT-qPCR should be carefully standardized [10]. The commercial mex Q-TesT kit includes two housekeeping genes to standardize the RT-qPCR and facilitates the interpretation of *mex*A and *mex*X genes expression of clinical *Pseudomonas aeruginosa* strains in comparison to wild type strain PAO1.

Conclusion

Resistance by efflux has now well been characterized in specialized laboratories but is still rarely or not at all diagnosed in routine laboratories. The complexity of resistance in *P. aeruginosa* with MDR phenotypes and the lack of diagnostic tools are probably the main reasons why this mechanism is neglected. Because this resistance mechanism can also contribute to therapeutic failures, accurate diagnosis is of prime importance for selecting adequate therapy.

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