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## Ciprofloxacin/amikacin combination therapy: evaluation in biofilms of *Pseudomonas aeruginosa* overproducing efflux pumps

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## Introduction

- Eradicating biofilm-related infections without mechanical biofilm dispersal remains a challenge
- The contribution to tolerance of low-level resistance related to the over-expression of efflux systems has not been evaluated in biofilms
- **Combination therapies are still considered as an approach to enhance killing of biofilm-embedded cells and minimize the emergence of resistance**

### ❖ Objectives:

Efficacy of ciprofloxacin and amikacin used separately and in combination, against *P. aeruginosa* planktonic and biofilm cultures

Impact of efflux systems on antibiotic (ATB) efficacy in planktonic and biofilm cultures

## Methods

- ❖ PAO1, a wild type clinical strain (WT), and 3 clinical strains overexpressing the efflux MexAB-OprM (AB), MexXY-OprM (XY) and MexCD-OprJ (CD)
- ❖ Planktonic and 2-day-old biofilm (6-wells plate) cultures of *P. aeruginosa*
- challenged with **ciprofloxacin (4 mg/L)** and **amikacin (40 mg/L)** separately, in combination, and successively
- ❖ Number of viable and resistant cells determined on MH2 without ATB and supplemented with 4-fold MIC of ciprofloxacin or amikacin
- ❖ **Characterization of resistant mutants** = ATB susceptibility, QRDR sequencing, and efflux pumps gene expression
- ❖ Determination of ciprofloxacin and amikacin **group's MICs** (by recovering surviving cells from biofilms on MH2 plates after 72 h of ATB exposure)
- ❖ Measurement of ciprofloxacin and amikacin concentrations in the biofilm at the end of the ciprofloxacin and amikacin exposure

## Results

### ❖ Strains

Table 1. Main characteristics of the strains used.

	MIC (mg/L)				Mutation frequency	Mean relative gene expression		
	TIM (S≤16 -R>16)	CIP (S≤0.5 -R>0.5)	LVX (S≤1 -R>1)	AMK (S≤8 -R>16)		<i>mexB</i>	<i>mexY</i>	<i>mexC</i>
PAO1	16	0.125	0.25	2	$1.8 \times 10^{-8}$	1.0	1.0	1.0
WT	16	0.06	0.25	2	$3.4 \times 10^{-9}$	1.7	1.1	0.3
AB	>256	0.06	1	2	$8.1 \times 10^{-9}$	3.6	5.3	0.7
CD	4	0.5	4	0.5	$3.5 \times 10^{-8}$	1.3	1.7	14.6
XY	32	0.125	1	16	$2.9 \times 10^{-9}$	1.3	10.1	4.1

S, susceptible; R, resistant; TIM, ticarcillin/clavulanate; CIP, ciprofloxacin; LVX, levofloxacin; AMK, amikacin

- PAO1, WT, AB, and CD were susceptible to ciprofloxacin and amikacin
- **XY strain was intermediate to amikacin (CMI = 16 mg/L)**
- No mutation in the QRDR
- No hypermutable strains
- AB, CD and XY respectively overexpressed MexAB-OprM, MexCD-OprJ and MexXY-OprM

### ❖ Planktonic time-kill assay

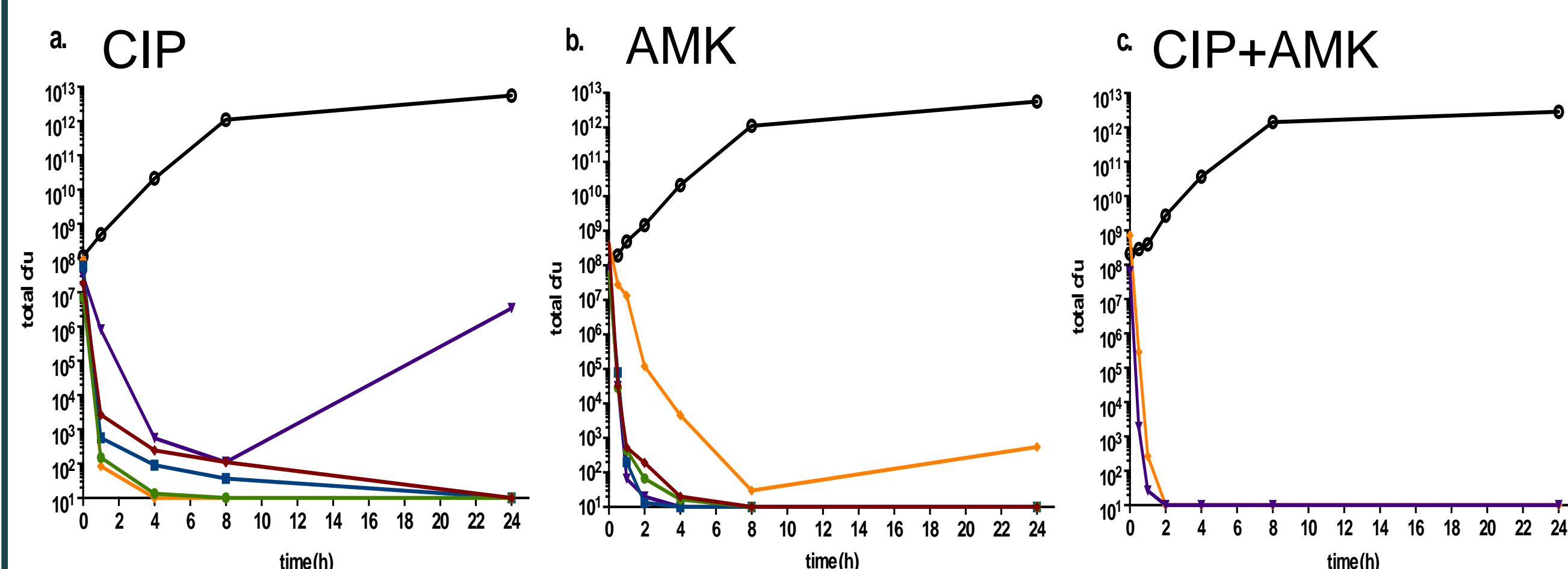


Figure 1. Planktonic time-kill assays with ciprofloxacin (a), amikacin (b) and the combination of ciprofloxacin and amikacin (c). The total cfu numbers are the mean value for three different experiments. GC: growth control

- $\geq 3 \log_{10}$  cfu/mL reduction after 4 h of ciprofloxacin or amikacin in all strains
- Regrowth of high-level resistant mutants:
  - when CD was exposed to ciprofloxacin (mutations in the QRDR of GyrA [Thr-83->Ile] and ParC [Ser-80->Leu])
  - when XY was exposed to amikacin
- **Eradication with a ciprofloxacin + amikacin combination in XY and CD strains**
- Eradication with ciprofloxacin or amikacin in all the other strains

### ❖ Biofilm time-kill assay

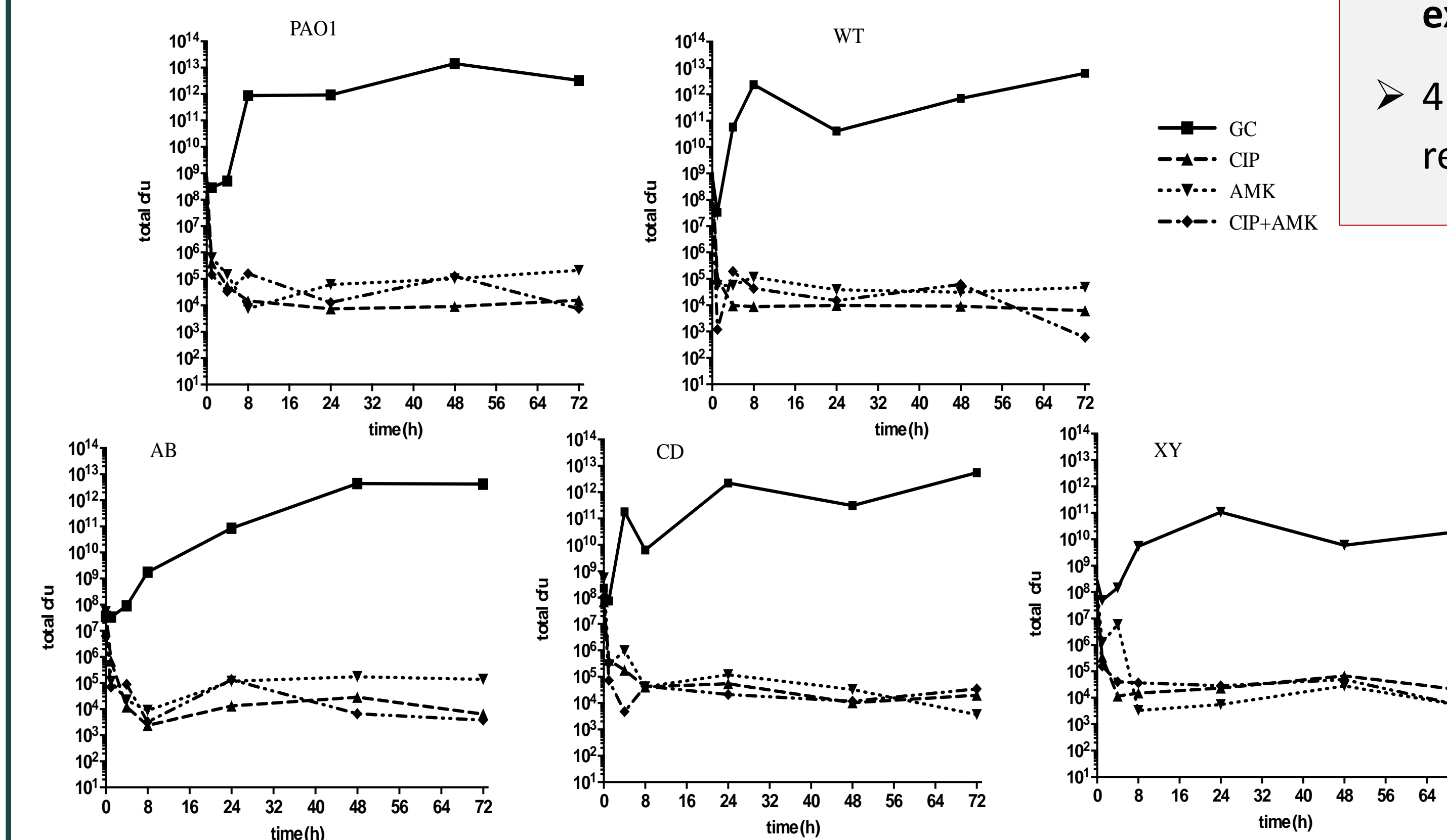


Figure 2. Biofilm time-kill assays with ciprofloxacin, amikacin and the combination of ciprofloxacin and amikacin. The total cfu numbers are the mean value for three different experiments. GC, growth control; CIP, ciprofloxacin; AMK, amikacin

- $\geq 3 \log_{10}$  cfu/mL reduction in all strains after 8 h of ATB exposure (similar killing rates for all regimens [p>0.05])
- 4 [3.62 – 4.17]  $\log_{10}$  cfu/mL plateau in all strains and for all regimens after 8 h and until 72 h of ATB exposure

- ❑ **No MIC creep** : group's MICs for the surviving cells after 72 h of ATB exposure **unchanged compared to pre-exposure MIC**
- ❑ Erratically, for all regimens including combination therapy, **4-fold MIC ciprofloxacin or amikacin resistant mutants** isolated from detached biofilm: low density, no mutation in the QRDR, and **no bacterial regrowth**
- ❑ **Sequential ATB exposure**: bacterial reduction after the first ATB exposure significantly higher than after the second ATB exposure (p<0.05)
- ❑ **Stable ATB concentrations along time**: no ATB degradation according ciprofloxacin and amikacin concentration measurements

## Conclusion

- ❖ **The ciprofloxacin-amikacin combination enhances killing and prevents emergence of resistance for low-level resistant strains in planktonic cultures, but not in biofilm.**
- ❖ The low-level resistance conferred by MexCD-OprJ and MexXY-OprM efflux pumps may reduce intracellular antibiotic concentrations and hence facilitate the selection of high-level resistant mutants by target site mutations ,
  - In planktonic conditions, we suggest that the **MIC value of ciprofloxacin** should be determined, and **levofloxacin evaluated**, in order to detect MexCD-OprJ overexpression.
- ❖ **In biofilm, the lack of bacterial eradication was related to an antibiotic-recalcitrant population composed of persister cells, refractory to antibiotics used alone or in combination.** No regrowth due to high-level resistant mutant was detected whatever the antibiotic/strain pair studied.