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# Increased Susceptibility of *Pseudomonas aeruginosa* to Macrolides and Ketolides in Eukaryotic Cell Culture Media by modulation of Outer Membrane Permeability


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## Abstract


**Background:** *P. aeruginosa* (PA) is reported as intrinsically resistant to macrolides and ketolides when tested by microdilution in broth according to CLSI guidelines. We observed that the MICs of PA to azithromycin drastically decrease when assayed in eukaryotic cell culture media. Our aim was to examine how general this effect is for macrolides/ketolides and whether it could be related to modulation of outer membrane (OM) permeability.

**Methods:** MICs of 36 clinical and laboratory strains of PA with known efflux phenotype were measured by microdilution in cation-adjusted Muller-Hinton broth (CA-MHB) or in RPMI medium (commonly used in eukaryotic cell culture). Phe-Arg-β-naphthylamide (PaβN, 50 mg/L) and EGTA 5mM used to inhibit efflux pumps and alter OM integrity, respectively.

**Results**: The Table shows the results obtained with PAO1 (wild-type), PA12 (overexpressing 4 efflux pumps), and PA403 (disrupted for genes coding for 5 efflux pumps). MICs of all molecules were high against PAO1 and PA12 in CA-MHB but considerably reduced if tested in either RPMI or in the presence of PaβN (reaching values close to those of PA403). EGTA reduced the MICs of ketolides in CA-MHB and had an additive effect in RPMI. For clinical strains, MICs were reduced from 2.8 (ERY) to 5.1-fold (TEL) (geometric means) in RPMI vs. CA-MHB.

**Conclusions:** The susceptibility of PA to macrolides and ketolides is highly dependent on the culture medium, probably through modulation of efflux pump activity and OM integrity. Because RPMI medium more closely mimics the composition of body fluids than CA-MHB, our data may have direct clinical significance.


## Background and aim

*Pseudomonas aeruginosa,* a major pathogen in pulmonary infections of cystic fibrosis or ICU patients, is considered as resistant to macrolides (1). However, macrolides, and azithromycin in particular, are widely used in such patients and contribute to reduce symptoms associated with infection and/or inflammation (2, 3). The mechanisms responsible for this activity remain unclear and may include anti-inflammatory effect (4), inhibition of the production of virulence factors by *P. aeruginosa* (5), and/or a direct effect on the outer membrane of the bacteria (6).

In this context, we have compared the activity of macrolides and ketolides against *Pseudomonas* strains differing in the expression of multidrug efflux systems and have examined the influence of the medium (broth versus media used for eukaryotic cell culture, which better mimics biological media), on this activity.

## Methods

**Bacterial strain and susceptibility testing.** *P. aeruginosa* strain ATCC PAO1 was used as reference. PA12 is a clinical strain overexpressing the 4 main efflux systems (MexAB, MexCD, MexEF, MexXY). PA403 is a laboratory strain deleted in the genes coding for the 4 efflux systems (7). A series of reference strains or of clinical isolates for witch the expression of genes coding for efflux pumps was known has been also used for MICs determinations (see Table 1 for genotype). MICs were measured by microdilution in MH broth or in RPMI medium (used for eukaryotic cells culture) supplemented with 10% of fetal calf serum, or in MH broth supplemented by increasing amounts of serum. EGTA (5mM) was used as a chelating agent (disrupting outer membrane integrity) and PaβN (50 mg/L) as an unspecific efflux inhibitor.

## Results

### MIC of macrolides and ketolides

| Strains | Efflux expression | ERY | | CLR | | AZM | | TEL | | CEM-101 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | MHB | RPMI | MHB | RPMI | MHB | RPMI | MHB | RPMI | MHB | RPMI |
| 12 | AB+CD+EF+XY+ | 512 | 32 | 512 | 16 | 256 | 2 | 128 | 4 | 128 | 4 |
| 434 | AB+CD+XY+ | 512 | 128 | 512 | 128 | 512 | 4 | 128 | 4 | 128 | 8 |
| 63 | AB+ EF+XY+ | 512 | 64 | 512 | 32 | 256 | 2 | 128 | 4 | 64 | 4 |
| 207 | AB+ EF+XY+ | 512 | 128 | 512 | 64 | 512 | 4 | 128 | 4 | 32 | 4 |
| 48 | CD+EF+XY+ | 512 | 64 | 512 | 64 | 256 | 2 | 128 | 8 | 128 | 4 |
| 49 | CD+EF+XY+ | 512 | 64 | 512 | 32 | 256 | 2 | 128 | 4 | 128 | 4 |
| 11 | AB+CD+ | 256 | 16 | 512 | 16 | 128 | 2 | 64 | 1 | 16 | 2 |
| 266B | AB+CD+ | 512 | 64 | 512 | 64 | 256 | 2 | 256 | 4 | 256 | 4 |
| 333A | AB+ EF+ | 512 | 64 | 512 | 64 | 256 | 2 | 64 | 2 | 128 | 2 |
| 335 | AB+ EF+ | 512 | 64 | 512 | 64 | 512 | 4 | 128 | 4 | 128 | 2 |
| 16 | AB+ XY+ | 512 | 32 | 512 | 64 | 256 | 4 | 128 | 4 | 32 | 4 |
| 68 | AB+ XY+ | 512 | 64 | 512 | 64 | 256 | 2 | 128 | 4 | 64 | 4 |
| 168B | CD+ XY+ | 512 | 256 | 512 | 256 | 512 | 4 | 256 | 4 | 128 | 4 |
| 133 | EF+XY+ | 512 | 64 | 512 | 64 | 256 | 4 | 128 | 4 | 64 | 4 |
| 156 | EF+XY+ | 512 | 16 | 512 | 32 | 512 | 4 | 128 | 2 | 64 | 2 |
| 1 | AB+ | 512 | 8 | 512 | 32 | 128 | 4 | 128 | 4 | 128 | 2 |
| 21 | AB+ | 512 | 64 | 512 | 64 | 256 | 2 | 64 | 2 | 128 | 4 |
| 2 | CD+ | 512 | 256 | 512 | 128 | 512 | 4 | 256 | 16 | 256 | 8 |
| 41 | CD+ | 512 | 64 | 512 | 64 | 256 | 2 | 256 | 4 | 256 | 4 |
| 3 | EF+ | 256 | 8 | 256 | 16 | 64 | 2 | 64 | 0.25 | 8 | 1 |
| 40 | EF+ | 256 | 32 | 256 | 16 | 256 | 2 | 64 | 2 | 32 | 4 |
| 4 | XY+ | 512 | 32 | 512 | 32 | 256 | 2 | 128 | 4 | 128 | 4 |
| 22 | XY+ | 512 | 32 | 512 | 32 | 256 | 2 | 128 | 2 | 64 | 4 |
| PAO1 | REFERENCE | 512 | 32 | 512 | 32 | 256 | 4 | 256 | 2 | 128 | 4 |
| 397 | AB- | 16 | 2 | 16 | 2 | 8 | 1 | 8 | 0.03 | 2 | 1 |
| 392 | CD- | 256 | 16 | 256 | 16 | 128 | 2 | 32 | 0.5 | 16 | 1 |
| 398 | CD- | 16 | 4 | 32 | 4 | 16 | 1 | 8 | 0.25 | 4 | 2 |
| 391 | EF- | 256 | 32 | 256 | 16 | 128 | 2 | 64 | 2 | 32 | 2 |
| 394 | XY- | 512 | 32 | 512 | 32 | 256 | 4 | 64 | 1 | 32 | 4 |
| 400 | XY- | 16 | 4 | 16 | 4 | 8 | 2 | 8 | 0.25 | 4 | 1 |
| 395 | HI- | 256 | 32 | 256 | 32 | 128 | 2 | 32 | 2 | 64 | 2 |
| 396 | ompH- | 128 | 16 | 64 | 16 | 64 | 2 | 16 | 0.25 | 8 | 2 |
| 401 | ompH- | 8 | 4 | 8 | 2 | 8 | 2 | 4 | 1 | 2 | 2 |
| 399 | AB – EF - | 16 | 4 | 16 | 4 | 16 | 2 | 4 | 0.25 | 8 | 1 |
| 403 | AB –CD – EF – XY - | 16 | 4 | 16 | 4 | 8 | 2 | 4 | 0.5 | 4 | 1 |
| 405 | AB –CD – EF – XY - | 8 | 4 | 8 | 4 | 8 | 2 | 8 | 0.25 | 4 | 1 |

**Table 1.** MIC (mg/L) of CLR, AZM, ERY, TEL and CEM-101 in MHB and RPMI for the 36 strains with known efflux phenotype

## Results

### MICs of macrolides against PA and *E. coli*

| | PAO1 | | *E. coli* | |
|---|---|---|---|---|
| | MHB | RPMI | MHB | RPMI |
| AZM | 128 | 2 | 4 | 0.01 |
| CLR | 512 | 16 | 64 | 2 |
| CLI | 512 | 512 | ND | ND |

**Table 2.** MICs (mg/L) of CLR, AZM, and CLI in MHB and RPMI against PAO1 strain and ATCC 27853 *E. coli* reference strain.

✓ Effect specific to macrolides
✓ Effect observed on another Gram(-)

### Influence of culture medium on MICs

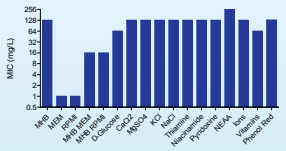


**Figure 1.** MICs (mg/L) of AZM against PAO1 in MHB supplemented by RPMI constituants.

✓ Effect not dependent on specific RPMI constituents; loss of susceptibility related to MH constituents

### MIC of macrolides in the presence of EDTA or of an efflux pump inhibitor

| Macrolide | PAO1 [a] | | | | | | PA12 [b] | | | | | | P403 [c] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CA-MHB | | | RPMI | | | CA-MHB | | | RPMI | | | CA-MHB | | | RPMI | | |
| | CT | PABN | EGTA | CT | PaBN | EGTA | CT | PaBN | EGTA | CT | PaBN | EGTA | CT | PaBN | EGTA | CT | PaBN | EGTA |
| ERY | 512 | 16 | 256 | 32 | 32 | 32 | 512 | 32 | 512 | 32 | 32 | 2 | 16 | ND | 16 | 4 | 4 | 0.5 |
| CLR | 512 | 8 | 256 | 32 | 4 | 32 | 512 | 16 | 256 | 16 | 16 | 2 | 16 | ND | 32 | 4 | 4 | 0.5 |
| AZI | 128 | 4 | 8 | 2 | 4 | 0.25 | 256 | 2 | 256 | 2 | 2 | 0.5 | 8 | ND | 2 | 2 | 2 | 0.125 |
| TEL | 128 | 4 | 32 | 2 | 4 | 1 | 128 | 4 | 32 | 4 | 4 | 1 | 4 | ND | 2 | 0.5 | 1 | 0.06 |
| CEM-101[d] | 32 | 8 | 8 | 2 | 2 | 1 | 32 | 4 | 16 | 4 | 4 | 1 | 4 | ND | 1 | 1 | 2 | 0.25 |

Table 3. **MIC (mg/L) in control conditions (CT), in the presence of PaβN 50 mg/L or EGTA 5 mM.** [a] wild type strain **b** clinical isolate overexpressing MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM [c] Δ(MexAB-OprM), Δ(MexCD-OprJ) , Δ(MexEF-OprN), Δ(MexJK) , Δ(MexXY) [7] [d] fluoroketolide (see structure in 8)

✓ Potential role of efflux/outer membrane permeability

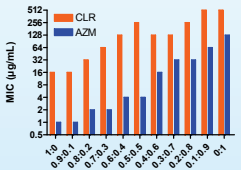


**Figure 2.** MICs (mg/L) of AZM and CLR against PAO1 in mixtures of RPMI and MH at different ratios

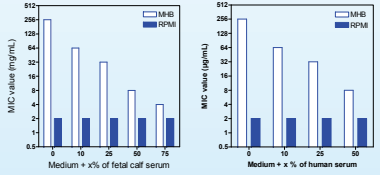


**Figure 3.** MICs (mg/L) of AZM in MHB supplemented by fetal calf or human serum with PAO1 strain.

✓ Lower MIC in « biologically relevant » milieu

## References


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*This poster will be made available for download after the meeting: http://www.facm.ucl.ac.be/posters.htm*

## Conclusions

The susceptibility of *P. aeruginosa* (and perhaps other Gram-negative bacteria) to macrolides and ketolides is highly dependent on the culture medium, probably through modulation of efflux pump activity and of OM integrity. Thus, MICs are considerably lower in RPMI medium and serum. Since these more closely mimic the composition of body fluids than CA-MHB, our data may have direct clinical significance.


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