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Mutations in 23S rRNA among *Pseudomonas aeruginosa* isolates from Cystic Fibrosis patients confer higher resistance to macrolides.

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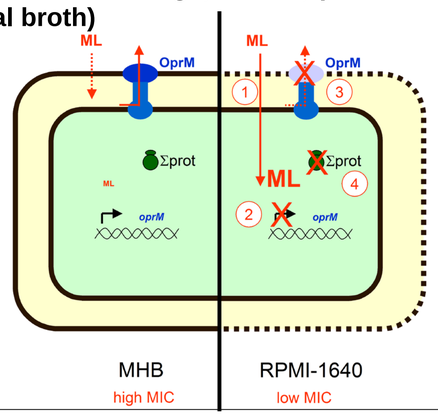
Background

Pseudomonas aeruginosa is the main microorganism causing chronic respiratory tract infections in cystic fibrosis (CF) patients older than 25 years (1). These patients, therefore, require repetitive and prolonged antibiotic treatments with anti-pseudomonal drugs.

The majority of these patients also receive long-term macrolide treatment for their anti-inflammatory properties.

Macrolides are deemed to be ineffective against *P. aeruginosa,* showing high MICs in conventional broth. However, our laboratory has shown that when cultured in biologically-relevant media (see Figure 1), macrolides could actually exert antimicrobial activity against *P.aeruginosa* (2). This is achieved by an increased accumulation of macrolides inside bacteria due to increased permeability of the outer membrane in these media and to repression of the expression of efflux systems.

Figure 1: Mechanism of action of macrolides in biological media (RPMI-1640; [culture medium for eukaryotic cells]; broncho-alveolar lavage, serum,) vs MHB (bacterial broth)



In CA-MHB macrolides show a high MIC because of a poor diffusibility through the outer membrane and active efflux by the constitutively-expressed efflux systems MexAB-OprM and MexXY-OprM. In RPMI-1640 medium, the outer membrane becomes more permeable, which favors macrolide penetration within bacteria (1), allows them to impair, through a still undefined mechanism, the expression of *oprM* (2), and therefore reduces the activity of the efflux systems MexAB-OprM and MexXY-OprM (3). In combination, steps 1 and 4 allows for an increased intracellular accumulation of macrolides, and allows them to impair protein synthesis at lower extrabacterial concentrations (4) and, thereby, to exert true antibacterial activity.

It is therefore not surprising that mutations in the macrolide target (domain V of 23S subunit of rRNA) could occur in *P.aeruginosa* isolated from CF patients (3).

Aims

Our aims were

- to characterize susceptibility to azithromycin (AZM) in *P.aeruginosa* isolates collected from CF patients (potentially pre-exposed to AZM) *vs.* intensive care patients with healthcare associated pneumonia (HAP; no AZM pre-exposure), using in parallel CA-MHB and RPMI-1640.
- to explore the presence of mechanism of resistance to macrolides (mutations in domain V of 23S ribosomal RNA, macrolide site of action) in CF isolates vs HAP isolates.

Materials & Methods

Bacterial isolates: we used
- 333 isolates from CF patients
- 48 isolates from HAP patients and hospitalized in intensive care units (4).
- ATCC 27853 as a quality control for susceptibility testing.
- PAO1 (fully sequenced genome) as reference strain and a derivative thereof containing a plasmid encoding the mutated (A2045G) rRNA operon (3) kindly provided by RL Marvig (Technical University of Denmark, Lyngby, Denmark). This plasmid was extracted and used as a backbone to introduce other mutations found in Table 1.

Susceptibility testing: MIC were determined by microdilution in cation-adjusted Mueller-Hinton broth (following CLSI recommendations), and Roswell Park Memorial Institute medium (RPMI-1640), as previously described (2). AZM was purchased from Teva, Plantex, Israel.

Sequencing of 23S ribosomal RNA gene: The gene corresponding to the domain V of 23S subunit of bacterial ribosome (625 bp; macrolide target) was amplified by PCR, purified and sequenced, focusing on regions were mutations were previously described (A2058G, A2059G and C2611T (5) in the 23S subunit of *E. coli*, corresponding to positions 2045, 2046 and 2598 in *Pseudomonas* species; see Figure 2).

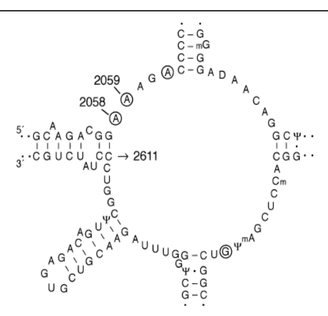


Figure 2: Secondary-structure model of the peptidyl transferase center in domain V of 23S rRNA of *E.coli* (6) (nucleotides at which the macrolide interact are encircled)

Results

1. CF isolates were more resistant to AZM in both RPMI-1640 and CA-MHB compared to HAP isolates (Figure 3). This suggests that some sort of resistance mechanism may have been selected among CF strains.

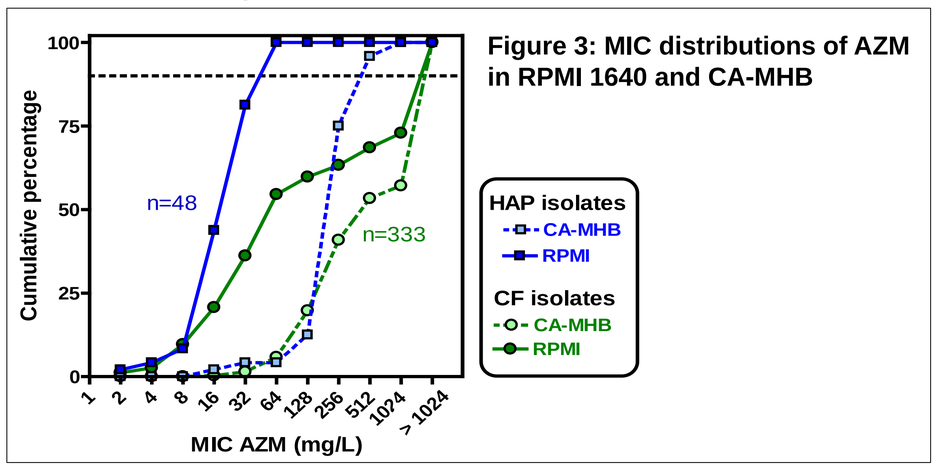


Figure 3: MIC distributions of AZM in RPMI 1640 and CA-MHB

2. Mutations were detected in 46% of CF isolates on 3 specific positions (A2045G, A2046G, and C2598T), which were previously described to cause resistance to AZM in *P.aeruginosa* (3). Three new mutations on these 3 positions but with different base change (A2045T, A2046T, and C2598G) were also detected.
3. No mutation was detected in all 48 HAP isolates.
4. Mutations in the 2 first sites (2045 and 2046) globally cause higher resistance as measured in RPMI-1640 (Table 1)

Table 1: MICs values of AZM in CA-MHB and RPMI-1640 for PAO1, PAO1-pMES-23S(A2045G) (or other mutations) (2) and CF isolates presenting mutations

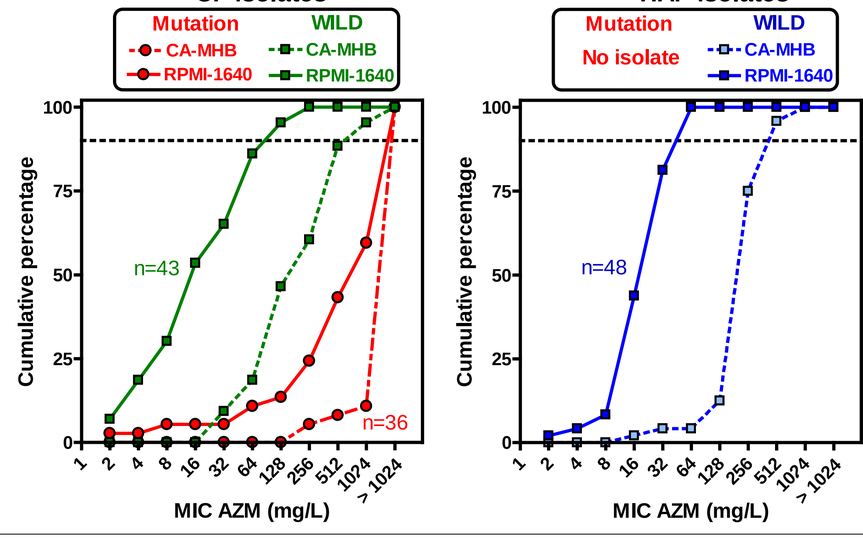
Strain	MIC of AZM in CA-MHB (mg/L)	MIC of AZM in RPMI-1640 (mg/L)
PAO1	**128**	**32**
Mutation : A2045G		
Control strain : PAO1-pMES-23S(A2045G)	**1024**	**512**
PA929	>1024	>1024
PA948	>1024	>1024
PA954	>1024	>1024
PA1024	>1024	>1024
PA1051	>1024	512
PA1062	>1024	>1024
PA1064	>1024	>1024
PA1065	>1024	>1024
Mutation : A2045T		
Control strain : PAO1-pMES-23S(A2045T)	**1024***	**512***
PA1111	>1024	>1024
Mutation : A2046G		
Control strain : PAO1-pMES-23S(A2046G)	**1024***	**512***
PA941	>1024	256
PA942	>1024	1024
PA1001	>1024	>1024
PA1007	>1024	>1024
PA1012	>1024	1024
PA1074	>1024	>1024
PA1151	>1024	>1024
PA1154	>1024	64
Mutation : A2046T		
Control strain : PAO1-pMES-23S(A2046T)	**1024***	**512***
PA1008	>1024	>1024
PA1009	>1024	1024
PA1036	>1024	>1024
PA1037	>1024	512

Strain	MIC of AZM in CA-MHB (mg/L)	MIC of AZM in RPMI-1640 (mg/L)
PAO1	**128**	**32**
Mutation : C2598T		
Control strain : PAO1-pMES-23S(C2598T)	**512**	**64**
PA928	>1024	>1024
PA999	>1024	64
PA1007	>1024	1024
PA1020	>1024	1024
PA1021	>1024	1024
PA1027	>1024	512
PA1028	>1024	512
PA1066	>1024	512
PA1071	>1024	1024
PA1102	>1024	512
PA1214	>1024	128
PA1243	>1024	256
PA1274	>1024	256
Mutation : C2598G		
Control strain : PAO1-pMES-23S(C2598G)	**512***	**64***
PA1069	>1024	512
PA1110	>1024	64

*predicted MIC (to be confirmed) based on the MICs of control strains PAO1-pMES-23S(A2045G) and PAO1-pMES-23S(C2598T)

5. CF strains presenting no mutation on either of these 3 sites were significantly more susceptible than those harboring mutations (Figure 4).

Figure 4: MIC distributions of AZM in CA-MHB and RPMI-1640 for CF and HAP isolates presenting mutations (Mutation) or not (WILD) on one of the three specific nucleotides on domain V of rRNA



Conclusions

- Mutations on 23S of bacterial rRNA could explain the high MICs of AZM recorded for strains coming from CF patients.
- Mutations in positions 2045 and 2046 confer higher resistance levels than those in 2598, probably because these two positions constitute the macrolide binding site while mutations in third site may rather alter the conformation of the center (7).
- Frequent administration of AZM to CF patients may help to select for ribosomal mutations conferring macrolide resistance in *P.aeruginosa* by exposing bacteria to low, sub-inhibitory concentrations.
- Even though these mutations should not compromise the anti-inflammatory or anti-biofilm properties of macrolides, they raise concern over the prolonged use of this class of antibiotics as they cause genetic modifications that may compromise the activity of future therapeutic options.
- These findings may support the evaluation of the beneficial effects of ketolides in CF patients, as these also bind to domain II of 23S rRNA and therefore keep activity on macrolide-resistant strains.

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