Setting up a NADH oxidase (NOX)-based assay to quantify the bactericidal activity of an antibiotic in planktonic cultures and biofilms of *S. pneumoniae*.

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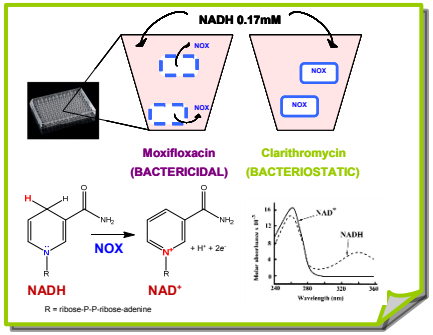
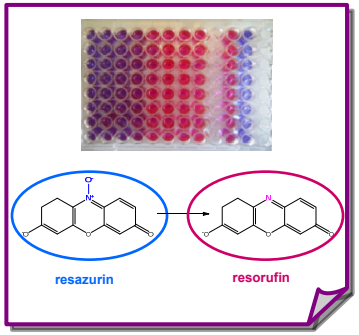
INTRODUCTION & AIM

- **Measuring the release of a soluble cytosolic enzyme is widely used for cytotoxicity assay in eukaryotic cells, but is rarely applied to bacteria. Yet, it could help for the rapid screening of bactericidal antibiotics towards both planktonic cultures (PK) and biofilms (BF).**
- **In *S. pneumoniae*, NADH oxidase (NOX), a soluble flavoprotein involved in NADH reoxidation, is essential for virulence.[1] As NADH does not permeate through membranes,[2] its oxidation when added to the medium will be proportional to the amount of NOX release from lysed bacteria[3].**
- **The aims of the study were (i) to set-up a NOX-based assay using moxifloxacin (MXF) and clarithromycin (CLR) as representatives of bactericidal and bacteriostatic antibiotics, respectively,[4,5] and (ii) to compare its performance *vs.* CFU counting (PK) and resazurin reduction assay [6,7] (PK and BF).**

MATERIALS & METHODS

- **Bacteria :** *S. pneumoniae* ATCC49619; suspension (0.5 McFarland) in caMHB+ 5% lysed horse blood.
- **Models and antibiotic exposure :**
  - **Planktonic model :** incubation of bacterial suspension with antibiotics (0.03 to 8000 x MIC_{broth}) during 24h at 37°C, following the CLSI MIC microdilution protocol.
  - **Biofilm model :** 25µL of bacterial suspension added to 175 µL of medium (caMHB + 5% lysed horse blood + 2% glucose[7]) in 96-well plates and incubated during 48h at 37°C in 5% CO_2 atmosphere; biofilm incubated with antibiotics (10^{-6} to 10^{3} x MIC_{broth}) during 24h in the same conditions.
- **CFU counting :** enumeration of CFUs after plating on agar plates.
- **NADH oxidase-based assay :** measure of the reduction in NADH OD_{340nm} after 30 min.of incubation at 37°C (initial conc.: 0.17mM). Daptomycin (2 mg/L) is used as positive control (100 % mortality)
- **Resazurin assay :** measure of the increase in fluorescence signal (λ_{ex} 560nm; λ_{em} 590nm) of resorufin (pink, fluorescent) formed by resazurin (0.001%; blue, non fluorescent) reduction by living bacteria after 30 min (planktonic cultures) or 72 h (biofilms).



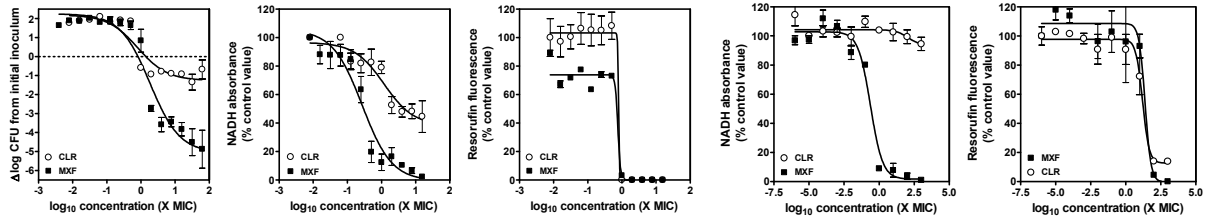


RESULTS

Characterization of moxifloxacin (MXF) and clarithromycin (CLR) activities (concentration-effect curves) on *S. pneumoniae* ATCC49619 planktonic cultures and biofilms, using 3 different assays

24h planktonic culture | **48h biofilm**

CFU counting | NADH oxidase (NOX) assay | Resazurin assay | NADH oxidase (NOX) assay | Resazurin assay



Antibiotic	Maximal changes in tested parameter				
	24h planktonic culture			48h biofilm	
	Δ log_{10} CFU (cell enumeration)	NOX assay (membrane integrity)	Resazurin assay (metabolism)	NOX assay (membrane integrity)	Resazurin assay (metabolism)
Moxifloxacin (bactericidal)	-3.8 ± 0.5 (A)	-100.4% ± 4.4 (A)	-100.0% ± 1.6 (A)	-98.3% ± 3.0 (a)	-100.4% ± 12.1 (a)
Clarithromycin (mainly bacteriostatic)	-0.7 ± 0.7 (B)	-61.7% ± 6.6 (B)	-100.0% ± 3.8 (A)	-6.5% ± 6.4 (b)	-87.4% ± 6.2 (a)

Statistical analysis : values with different letters are statistically different from each other (unpaired t-test, p<0.05).
uppercase letters: planktonic culture; lower case letters: biofilm

- **the resazurin-assay, which shows a very sharp slope, did not distinguish between bactericidal and bacteriostatic antibiotics (both caused a concentration-dependent reduction in signal that reached a minimum at low multiples of the MICs for planktonic cultures as well as in biofilms);**
- **conversely, in both planktonic cells and biofilm, the NADH oxidase assay allowed for a unambiguous differentiation between bactericidal (MXF) and bacteriostatic (CLR) antibiotics.**

SUMMING UP & CONCLUSIONS

- We set up a new sensitive method allowing for the rapid quantification of antibiotic bactericidal and bacteriostatic activities in pneumococcal planktonic cultures and biofilms.
- As this NADH oxidase-assay can be completed in 30 min and is amenable to performance in various media, it may offer advantages for the rapid screening of antibiotic activities in different culture conditions.
- Application to other bacterial species warrants further investigation.

REFERENCES

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