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Novel approaches to detecting and circumventing antibiotic resistance in Pseudomonas aeruginosa

Objectives *Pseudomonas aeruginosa* is an opportunistic pathogen in humans that is notorious for its resistance to antibiotics and dreaded in hospitals. This project is developing new methods for investigating its resistance mechanisms and for novel treatment approaches that weaken its virulence determinants.

Conclusions A quantitative real-time-PCR assay was developed for the analysis of the antimicrobial resistance gene expression in *P. aeruginosa* and was then applied for the detection of resistance mechanisms in clinical isolates. Using this method, the appearance of antibiotic resistance and of the underlying resistance mechanisms can be followed, thus helping to optimize antibiotic therapy.
It could be demonstrated that antimicrobial therapies can rapidly select (6 to 10 days) resistant isolates that may persist for several weeks in the lungs of patients once the selecting agent is withdrawn. Furthermore, it was shown that macrolides at subinhibitory concentrations have quorum-sensing inhibition properties by interacting with the ribosome. Quorum sensing was shown to be associated with the progression of colonization by *P. aeruginosa* of intubated patients towards pneumonia. These observations open the way for the clinical use of anti-virulence strategies as a novel approach to circumventing antibiotic resistance.

Main results and findings

Quantitative real-time PCR for the detection of resistance in clinical isolates A quantitative real-time PCR for the determination of antibiotic resistance gene expression of 13 resistance genes was set up, which allowed comparison of gene expression of the major drug efflux pumps (MexAB-oprM, MexCD-OprJ, MexEF-oprN, MexXY), the chromosomal AmpC ß-lactamase and the carbapenem specific porin OprD. The results can be summarised as follows:

– In a susceptible wild type strain and isogenic resistant mutants with defined mutations, expression levels of *ampC* and *oprD* genes paralleled beta-lactamase activity and OprD protein levels, respectively.
– This method was applied to study the emergence and persistence of resistance mechanisms in tracheal aspirate isolates obtained during a longitudinal observation from two intubated patients (who had received beta-lactam, quinolone and/or aminoglycoside treatments) and a total of 109 isolates collected during a 3-month observation period. The outcomes can be summarised as follows:
  - Within 6 to 10 days after initiation of therapy, all treatment regimens selected resistant isolates that remained detectable for at least 3 weeks after treatment termination.
  - Imipenem resistance was attributable to decreased OprD expression.
  - Fluoroquinolone (FQ) resistance resulted from overexpression of the MexCD-OprJ efflux pump.
  - Piperacillin, ceftazidime and aztreonam resistance resulted from derepression of the chromosomal AmpC ß-lactamase.
  - Combined ß-lactam-aminoglycoside resistance was associated with appearance of multidrug resistant small colony variants.
  - Resistance to beta-lactam and FQ was correlated with *ampC* and *mexC* gene expression levels, respectively.
  - Imipenem and FQ resistance persisted for a prolonged time, once the selecting antimicrobial treatment had been discontinued. In contrast, resistance to beta-lactams rapidly disappeared after removal of the selective pressure, to reappear promptly upon renewed exposure.

These results suggest that resistant *P. aeruginosa* are rapidly selected independently of the antimicrobial class. Even if resistant isolates are not cultured, these may persist in the lung and can rapidly be reselected.

Detection of targets responsible for mediated-attenuation of virulence It could be shown that azithromycin (AZM) reduced production of elastase, rhamnolipids and swarming motility, through transcriptional downregulation of the quorum-sensing (QS) system. This effect required interaction of AZM with the ribosome, since methylation of the 23S rRNA, the macrolide target, abrogated the effect on virulence-factor production. It was further demonstrated that the proposed AZM mediated cell killing of stationary phase cells also requires interaction with the ribosome. Moreover, uptake of AZM was facilitated by the production of rhamnolipids, a detergent like molecule previously shown to be involved in solubilisation of hydrophobic compounds.

Clinical use of quorum-sensing inhibitors Although not described in the initial project, our group participated in a clinical trial on the prevention of pneumonia in intubated patients. Using genotypic and phenotypic analysis it was shown that QS is correlated with the progression from colonisation to ventilator-associated pneumonia (VAP).
These results are encouraging for the use of QS-inhibitors as alternative strategies to classical antimicrobial treatments.

Impact of antibiotic resistance mechanisms on virulence gene expression To establish whether antimicrobial treatments and/or resistance mechanisms have an impact on the virulence of *P. aeruginosa,* elastase and rhamnolipid production were analysed in tracheal isolates from five patients (selected from 42 patients based on a prolonged follow-up) and correlated with the administration of antimicrobials as well as resistance phenotypes. The outcomes can be summarised as follows:

- While isolates collected during antimicrobial treatments (quinolones and b-lactam/aminoglycoside combination therapy) produced elevated elastase and rhamnolipid levels, an important fluctuation in the level of production of these two virulence factors was observed once the antimicrobial treatments were stopped.
- Interestingly, different genotypes predominated during (virulent phenotype) and after (avirulent phenotype) antimicrobial therapies.
- Sequencing of the *lasR* gene, which controls elastase and rhamnolipid expression, revealed point mutations and deletions in the avirulent isolates. Therefore, virulence fluctuations resulted in this case from antimicrobial-mediated enrichment of less virulent strains (*lasR* mutants).
- Surprisingly, antimicrobial resistance was not required for this phenomenon, as in one patient both the virulent and avirulent isolates remained susceptible to all antimicrobials.

These observations suggest that *lasR* wild type isolates have a selective advantage during antimicrobial treatments, whereas *lasR* mutants are favoured in the absence of treatments. These differences might be due to the stress induced by antimicrobials.

Publications of the NRP 49 project

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