In vitro models for the study of antibiotic PK/PD in biofilms

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Biofilms in human infections

Biofilms are associated to 65\textsuperscript{a}-80\textsuperscript{b} % of human infections and can colonize virtually all organs …

\textbf{Sites of Primary and Secondary Biofilm Infection}

\begin{itemize}
  \item ear
  \item nose
  \item throat
  \item mouth & teeth
  \item eye
  \item lung
  \item heart
  \item kidney
  \item gall bladder
  \item pancreas
  \item nervous system
  \item skin
  \item bone
  \item ***
  \item implanted medical devices
\end{itemize}

\textsuperscript{a}CDC 1999; \textsuperscript{b}Lewis et al, Nat Rev Microbiol. 2007; 5:48-56
## Main pathogens in biofilm-related diseases

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Biofilm infection</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>Acute and recurrent urinary tract infection, catheter-associated urinary tract infection, biliary tract infection</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Cystic fibrosis lung infection, chronic wound infection, catheter-associated urinary tract infection, chronic rhinosinusitis, chronic otitis media, contact lens-related keratitis</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Chronic osteomyelitis, chronic rhinosinusitis, endocarditis, chronic otitis media, orthopaedic implants</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>Central venous catheter, orthopaedic implants, chronic osteomyelitis</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>Colonization of nasopharynx, chronic rhinosinusitis, chronic otitis media, chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>Colonization of oral cavity and nasopharynx, recurrent tonsilitis</td>
</tr>
</tbody>
</table>

Antibiotics and biofilms in clinical practice

When and how should we treat biofilms in chronic sinusitis?
Jain R, Douglas R.

Reduced Vancomycin Susceptibility in an In Vitro Catheter-Related Biofilm Model Correlates with Poor Therapeutic Outcomes in Experimental Endocarditis Due to Methicillin-Resistant Staphylococcus aureus
Wesam Abdalla, c, Arnold S. Bayer, a,b Kati Sperl, c Cynthia C. Nix, a Margaret R. Kiellstrand, a Alexander R. Horvath,a Michael R. Yeaman a Van D. Kwon1

Biofilm formation or internalization into epithelial cells enable Streptococcus pyogenes to evade antibiotic eradication in patients with pharyngitis
Talji Ogasawara a,b, Yutaka Tanaka a,b, Hisashi Okumura a,b, Keiko Ninomiya b, Hiroshi Sakata a,b

The presence of antibiotic-resistant nosocomial pathogens in endotracheal tube biofilms and corresponding surveillance cultures.
Vandecandelaere L, Mathijs N, Nelis HJ, Depuydt P, Coenye T

→ Treatment failure is not rare...
Studying antibiotic PK/PD against biofilms

Very complicated?

Very simple?
Static models … for dynamic studies

pegs

multiwell plates
Static models: Calgary Biofilm Device

Determination of Minimal Biofilm Eradication Concentration (MBEC)

### PD parameters: planktonic vs. biofilm cultures

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimal inhibitory concentration</td>
<td>MIC</td>
<td>The lowest concentration of an antibiotic that inhibits the visible growth of a planktonic culture after overnight incubation</td>
</tr>
<tr>
<td>Minimal biofilm inhibitory concentration</td>
<td>MBIC</td>
<td>The lowest concentrations of an antibiotic that resulted in an OD650 difference at or below 10% (1 Log difference in growth after 6 h of incubation) of the mean of two positive control well readings.</td>
</tr>
<tr>
<td>Minimal bactericidal concentration</td>
<td>MBC</td>
<td>The lowest concentration of an antibiotic producing a 99.9% CFUs reduction of the initial inoculum of a planktonic culture.</td>
</tr>
<tr>
<td>Biofilm bactericidal concentration</td>
<td>BBC</td>
<td>The lowest concentration of an antibiotic producing a 99.9% reduction of the CFUs recovered from a biofilm culture compared to growth control.</td>
</tr>
<tr>
<td>Minimal biofilm eradication concentration</td>
<td>MBEC</td>
<td>The lowest concentration of an antibiotic that prevents visible growth in the recovery medium used to collect biofilm cells.</td>
</tr>
<tr>
<td>Biofilm prevention concentration</td>
<td>BPC</td>
<td>Same as MBIC but bacterial inoculation and antibiotic exposure occur simultaneously.</td>
</tr>
</tbody>
</table>

Macià et al, Clin Microbiol Infect. 2014 Apr 26
PD parameters: planktonic vs. biofilm cultures

Ampicillin and levofloxacin vs. *H. influenzae* from middle ear fluid

Slowly bactericidal antibiotic: MBEC >> MBC >> MIC

Rapidly bactericidal antibiotic: MBEC > MBC ~ MIC

*Takei et al, J Infect Chemother (2013) 19:504–9*
Static models: 96-well polystyrene plates

appropriate dyes to evaluate biomass or bacterial load
Quantifying biomass and metabolic activity in biofilms

**biofilm mass**

**crystal violet**

*Christensen et al, Infect. Immun. 1982; 37:318–26*
Quantifying biomass and metabolic activity in biofilms

biofilm mass

crystal violet

Gram(+) bacteria

metabolic activity

Gram(-) bacteria

resazurin

resorufin

fluorescein diacetate

fluorescein


CFU counting vs. RF fluorescence

An example for *S. aureus*

CFU & RF signal proportional

sensitivity depending on incubation time

Distinguishing between static and cidal effect

CFU counting

Hardrson et al, Nature Protocols 2010; 5:1236-54
Distinguishing between static and cidal effect

**CFU counting**

- **Biofilm cell density (log_{10} CFU mm^{-2})**

  - Day 1, Day 2, Day 3, Day 4, Day 5, Day 6
  - Technician 1, Technician 2

  - Overall mean = 5.03

**NADH release**

- **S. pneumoniae**

  - **NADH oxidase (NOX)**

- **NADH oxidase**

  - NADH → NADH oxidase → NAD^+

**References**

- Yu et al, Microbiology. 2001;147(Pt 2):431-8
RF fluorescence vs. NADH oxidase

An example for S. pneumoniae

Resorufin fluorescence (% control value)

NADH absorbance (% control value)

**Residual living bacteria**

**Dead bacteria only**
PK/PD studies: a few examples

Cooncentration effects

Time effects
S. aureus & S. pneumoniae models

Kinetics of biofilm formation

**S. aureus**

- Crystal violet absorbance
- Resorufin fluorescence

**S. pneumoniae**

- ATCC 49619
- R6

Young biofilm vs. mature biofilm
Pharmacodynamic model for antibiotic activity

An example with young biofilm of *S. aureus*

**S. aureus mature biofilms: comparison of drugs**

### ATCC33591 (MRSA)

- **vancomycin**
- **delafloxacin**
- **daptomycin**

- More active on viability than on matrix
- Huge difference among drugs

*Bauer, Siala et al, Antimicrob Ag Chemother. 2013;57:2726-37*
S. pneumoniae biofilms - influence of maturity

maximal efficacy \(\bowtie\) with maturity

Vandevelde et al, Antimicrob Ag Chemother. 2014; 58:1348-58
S. pneumoniae biofilms - influence of maturity

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**Moxifloxacin**

**Relative potency** with maturity

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**Vandevelde et al, Antimicrob Ag Chemother. 2014; 58:1348-58**

**antibiotic PK/PD in biofilms**
PK/PD parameters in biofilms

- **Pharmacokinetics**
  - diffusibility through the matrix
  - bioavailability within the biofilm
  - access to bacteria
  - efflux out of bacteria

- **Pharmacodynamics**
  - bacterial responsiveness (metabolic activity of bacteria)
  - antibiotic expression of activity (local environment [O₂, pH, ..])

Catheter, bone, skin, cardiac valve, ...

Nutrients & oxygen
Parameters affecting antibiotic activity in biofilms

2 clinical isolates of *S. aureus*

What makes the difference?

**viability**

**biomass**

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*Siala et al, in preparation*

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UCL

11/05/2014

antibiotic PK/PD in biofilms
PK parameter: antibiotic penetration

more potent if better penetration

more strains

Siala et al, in preparation
PD parameter: environmental pH

pH and fluoroquinolones

**Proteus mirabilis**
planktonic cultures vs. biofilms

<table>
<thead>
<tr>
<th>pH</th>
<th>Ciprofloxacin (mg l⁻¹)</th>
<th>MIC</th>
<th>MBC</th>
<th>MBEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td></td>
<td>0.66</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>0.08</td>
<td>0.31</td>
<td>125</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>0.04</td>
<td>0.04</td>
<td>25</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>0.04</td>
<td>0.08</td>
<td>25</td>
</tr>
</tbody>
</table>

→ less potent in acidic biofilms

PK/PD : on the way to biofilm bkpts ?

Ceftobiprole and comparators vs. S. aureus

Table 2
Dose-response of ceftobiprole (BPR) and rifampicin (RIF) with 11-day *Staphylococcus aureus* colony biofilms.

| Antibiotic concentration | CFU decrease (log_{10}) after 7 days of antibiotic exposure
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BPR</td>
</tr>
<tr>
<td></td>
<td>MSSA ATCC 0598</td>
</tr>
<tr>
<td>fC_{max}</td>
<td>1.0</td>
</tr>
<tr>
<td>1/2 fC_{max}</td>
<td>1.0</td>
</tr>
<tr>
<td>1/4 fC_{max}</td>
<td>1.0</td>
</tr>
<tr>
<td>1/8 fC_{max}</td>
<td>2.1</td>
</tr>
<tr>
<td>1/16 fC_{max}</td>
<td>2.7</td>
</tr>
<tr>
<td>1/32 fC_{max}</td>
<td>3.8</td>
</tr>
<tr>
<td>1/64 fC_{max}</td>
<td>2.7</td>
</tr>
</tbody>
</table>

fC_{max}: maximum free-drug plasma concentration attained during clinical use; MSSA, meticillin-susceptible *S. aureus*; MRSA, meticillin-resistant *S. aureus*; N/T, not tested.

CFU decreases were calculated relative to biofilm CFUs of Day 11 non-drug controls and were averaged from 24 individual biofilms.

In vitro dynamic models

- permanent fluid stirring
- unidirectional flow replacement
- constant conditions
Dynamic models: bioreactors

CDC reactor:
- constant mixing by stirring
  → kinetic experiments with change in medium composition over time
- high shear stress
Antibiotic activity - mimicking human exposure

**CDC reactor**

**S. aureus**

Simulated regimens:
DAP (10 mg/kg once daily) / LZD (600 mg twice daily)

Planktonic cultures

Biofilm (CDC reactor)

Combination more useful against biofilm than planktonic bacteria

Dynamic models: bioreactors

Drip flow reactor:
- progressive, unidirectional change in medium
- low shear stress

Expression of antibiotic resistance

**Flow cell reactor**

- **P. aeruginosa**

**96-well plates, static**

Fixed conc. of ceftazidime over time

**PAO1**

β-lactamase overproducer

Expression of antibiotic resistance

Flow cell reactor

96-well plates, static

P. aeruginosa

Fixed conc. of ceftazidime over time

PAO1 \( \rightarrow \) time-dependent

\( \beta \)-lactamase overproducer \( \rightarrow \) concentration-dependent ~ antibiotic access ??
Selection of resistant populations

P. aeruginosa

Fixed conc. of ciprofloxacin over time (MPC)

Amplification of mutator population during antibiotic treatment and accumulation of resistance mechanisms

Dynamic models: bioreactors

(non)-constant depth biofilm fermenter:
• constant conditions
• low shear stress
• ~ biofilms on implants

Selection of resistant populations

Constant depth fermenter

Fixed antibiotic conc. over time;
multispecies biofilm (strains isolated from human wounds)

Addition of flucloxacillin (15 mg/L)

Addition of ciprofloxacin (5 mg/L)

Low activity at clinically-relevant concentration

Take home messages …. 
Conclusions

- many methods to evaluate biomass / bacterial survival
  - no real consensus on the best options
- many models to grow biofilms *in vitro*
  - comparison between studies difficult
  - more relevant model?

*Smith et al, Eur J Clin Microbiol Infect Dis 2013; 32:1327–32*
Conclusions

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- many models to grow biofilms *in vitro*
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- antibiotic activity on biofilms <<< planktonic bacteria
  - no or limited effect on the matrix
  - determining PK parameters: diffusion / bioavailability
  - determining PD parameters: expression of activity / bacterial responsiveness
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  - determining PK parameters: diffusion / bioavailability
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combination with agents modifying matrix properties or bacterial metabolic state?
Still a lot of work ahead …
Acknowledgments

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Transparency declaration