Animal models of biofilm infections.

Claus Moser, MD, Ph.d.
Department of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark
All models are wrong
But they can be useful

Testing of hypothesis generated from observations in the patients

Pre-clinical testing of agents showing promising *in vitro* effect
The Law (Denmark)

- Animal experiments on vertebrates allowed if the aim is significant, specified and scientific.
- Permission given to individual persons with sufficient education/background, after having passed a special animal experimental course and test.
- Permission from a council with a professional chairman (Judge) and ten members appointed by the Ministry of Justice.
- Only allowed if other methods cannot replace the experiments.
- Files approved for all individual experiments.
- Subject to unannounced visits from authorities.
- Annual report from the council in an anonymized form.
- Approximately 300,000 animals used/year in Denmark.
- Mortality not allowed as an intended end point.
- Animal wellfare increasing attention.
- Fanatic anti-animal experiment groups not a problem in Denmark, so far.
- Some rules not invented by animal facilities!!
Agenda

- Chronic lung infection model
- Chronic wound model
- Peritoneal implant model
- Tissue filler model
Acute lung infection

Ware LB, Matthay MA. NEJM 2000

Resolution

Moser Hamburg 2011
Chronic biofilm infection in CF – no resolution.

- Adaptive immune response accelerates inflammation and contributes to pathogenesis:
  - Skewing of Th1/Th2 balance
  - Immunecomplex disease
  - Paradox persistent acute type inflammation (PMNs)
  - High number of necrotic PMNs
  - Progressive loss of lung function
  - Fibrosis

- The type of adaptive immune response influenced by the inflammatory response.

- Provides possible treatment targets.

Ware LB, Matthay MA. NEJM 2000
Cystic fibrosis. Multiorgan disease

- Malabsorption, pancreatic insufficiency
- Male infertility
- Hepatic insufficiency
- Diabetes mellitus
- Allergy
- Kidney insufficiency
- Chronic endobronchial infection

Mucoid biofilm of P. aeruginosa in an alveolar surrounded by severely inflamed tissue (PMNs, pneumonia). Autopsy (BS242/74) of a CF girl (MLM) who died due to chronic P. aeruginosa lung infection and 21 precipitating antibodies against P. aeruginosa. HE stain x 40
Pro’s

• Numerous inbred strains
• Prolonged infection possible
• Inflammation CF-like
• Vast immunological tools available
• CF mice available (and ENaC mice)

Con’s

• Important histological differences
• Natural resistant to *P. aeruginosa*
• (No spontaneous lung infection in CF mice)
• Short lifespan
Dominated by rat and mouse models

The lung infection models

- Embedded models ("Classical")
  - Agar (Cash 1979)
  - Agarose (Starke 1987)
  - Seaweed alginate (Pedersen 1990)
  - Mucoid model in native alginate (Hoffmann 2005)
  - Adaptation model (Moser 2009)

- Planktonic models
  - Nebulizing chamber (Yu 1997)
  - Nasal application/inflation (Tang 1995)
  - Drinking model (Coleman 2003)

- Tube model ("Japanese") (Yanagihara 1997)
- Xenograft model (Tirouvanziam 2000)
Alginate
Agar- or alginate beads is necessary for chronic infections - the Cash model

Seaweed alginate beads 60 µm (30-110 µm)
Alginate beads with *Pseudomonas aeruginosa*
Characteristics.
The seaweed alginate model (Pedersen SS, et al. APMIS 1990)

- A clinical alginate producing isolate PAO 579 (provided by Govan, Edinburgh). $10^{7-8}$ CFU/ml, 0.1 ml/rat.
- Intratracheal infection with bead-tipped needle
- Sacrificed rats 4 weeks after challenge.
- Compared to the agar model.
- Macroscopic pathology:
  - Grey nodules (abscesses) and pleural adhesions.
- Histological pathology:
  - Pronounced PMN dominated inflammatory response. Beads with bacteria.
- Quantitative bacteriology:
  - Positive bacteriology in 10/12 rats.
- Antibody production:
  - Significantly higher number of precipitating antibodies in the alginate group.
Characteristics mice.
(Using PAO 579)

- Course of infection highly dependent on mouse strain.
- Shorter duration of the infection (1-2 weeks).
- Macroscopic abscesses are seldom.
- Similar histopathology.
- Antibody production, and activation of cellular immunity.
Histopathology

- Severe inflammation
- Both central and in the periphery
- Alginate area with biofilm like structures
Activation of CD4+ cells

Use of the model

- Vaccination studies (HK Johansen, O Ciofu)
- Immune modulation (C Moser, HK Johansen)
- Antibiotic resistance (O Ciofu)
- Immune responses (C Moser, PØ Jensen)
- Treatment studies (C Moser, Z Song, T Bjarnsholt, N Hoffman)
- Host-Pathogen interactions (C Moser, H Wu, T Bjarnsholt, PØ Jensen, S Prakhabar)
Improvements in care.

• Liquid post challenge (1 ml isotonic NaCl 37 C s.c.).
• Maintenance of body temperature.
• Analgetic treatment of surgical wound (bupivakain).
• Euthanasia strategy.
Modifications of the model

• Embedment in native alginate
• Adaptation model
• Niche model
Mucoid *P. aeruginosa*
(Hoffmann N, et al. IAI 2005)

- Stabile mucoid phenotype.
- Cultured for 28h, 37°C.
- Centrifuged, and resuspended in 2 ml ox broth.
- Adjusted to $1 \times 10^8$ CFU/ml ($1 \times 10^9$ for BALB/c mice) in crude or purified native alginate.
  - Crude: culture supernatant
  - Purified: supernatant heated to 80°C for 30 min. Precipitated with 99% ice-cold ethanol. Resuspended in sterile 0.9% saline.
- Mice challenged intra-tracheally with a bead-tipped needle.
  - CF-mouse: $cftr^{tm1Unc-TgN^{(FABPCFTR)}}$ (Jackson Lab.)
  - BALB/c mouse (M&B Lab.)
Mucoid *P. aeruginosa* embedded in crude native alginate induced higher mortality of lung infected BALB/c mice as compared to the non-mucoid isolate (p<0.05).

### Macroscopic pathology

**TABLE 3.** Macroscopic lung pathology after intratracheal *P. aeruginosa* challenge of CF mice and BALB/c mice with the mucoid strain +QS (NH57388A), the nonmucoid strain –QS (NH57388B), and the nonmucoid strain +QS (NH57388C)

<table>
<thead>
<tr>
<th>Score</th>
<th>Expt 1</th>
<th>Expt 2</th>
<th>Expt 3</th>
<th>Expt 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CF mice (mucoid +QS) NH57388A</td>
<td>BALB/c mice (mucoid +QS) NH57388A</td>
<td>BALB/c mice (nonmucoid –QS) NH57388B</td>
<td>BALB/c mice (mucoid +QS) NH57388A</td>
</tr>
<tr>
<td>1</td>
<td>1/15 (7)</td>
<td>5/12 (42)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10/15 (67)</td>
<td>7/12 (58)</td>
<td>2/6 (33)</td>
<td>1/7 (14)</td>
</tr>
<tr>
<td>3</td>
<td>2/9 (22)</td>
<td>3/15 (20)</td>
<td>4/10 (40)</td>
<td>8/10 (80)</td>
</tr>
<tr>
<td>4</td>
<td>7/9 (78)</td>
<td>1/15 (7)</td>
<td>6/10 (60)</td>
<td>2/10 (20)</td>
</tr>
<tr>
<td></td>
<td>CF mice (mucoid +QS) NH57388A</td>
<td>CF mice (nonmucoid +QS) NH57388C</td>
<td>CF mice (nonmucoid –QS) NH57388B</td>
<td>CF mice (mucoid +QS) NH57388A</td>
</tr>
</tbody>
</table>

*See Table 2, footnote a. Asterisks indicate probabilities as follows: *, P < 0.01 compared to day 13; **, P < 0.01 compared to the mucoid strain NH57388A +QS; ***, P < 0.001 compared to BALB/c mice; ****, P < 0.001 compared to the nonmucoid strain NH57388B –QS; *****., P < 0.001 compared to the mucoid strain NH57388A +QS.*

*b Day 13, in this case.

*c Score 1, normal; 2, swollen lungs, hyperemia, small atelectasis; 3, pleural adhesion, atelectasis, multiple small abscesses; 4, large abscesses, large atelectasis, and hemorrhages.*

Adaptation model.

- Mouse models of chronic *P. aeruginosa* lung infections are limited to 2 (-3) weeks!
- 20-30 years of chronic infection.
- ≥ 10,000 days (~120,000 bacterial generations) of mutual exposure to the inflammatory responses and bacterial virulence factors.
- ≥ 100 antibiotic i.v. courses (≥ 1,400 days) of antibiotic exposure.

Moser, van Gennip, et al APMIS 2009
van Gennip, Moser et al JAC 2009
Partial conclusion

For non-mucoid isolates:
1) Initial stages: Ability for **biofilm formation** correlates to pathogenecity.
2) Late stages: Virulence dominated by other factors - hyperproduction of exopolysaccharides?

<table>
<thead>
<tr>
<th></th>
<th>1988</th>
<th>1997</th>
<th>2003</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-mucoid</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mucoid</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Moser, van Gennip, et al APMIS 2009
van Gennip, Moser et al JAC 2009
Inflammatory response
Non-mucoid versus mucoid.

G-CSF: PMN mobilizer from the bone marrow

van Gennip and Moser 2010
Conclusion adaptation model

- **For non-mucoid isolates:**
  - Initial stages: Ability for biofilm formation correlates to pathogenicity.
  - Late stages: Virulence dominated by other factors - hyperproduction of exopolysaccharide.

- **For mucoid isolates:**
  - More CF-relevant pathology.
  - Virulence increases with time.

- **Combined:**
  - New infection strategy providing a CF-relevant time-perspective (years).
  - Possible to investigate the impact of bacterial adaptation on host responses.
Pulmonary niches or zones

The conductive and respiratory zone of the lungs.
Recognition and recruitment.

Five distinct lung DC subsets based on surface markers and location.

Type of generated response dependent on:
- Cytokine environment
- Danger signals
- Pathogen recognizing receptors (PRRs)
- Pathogen
- Antigen processing

If harmless antigen with minor additional signals induce Treg.


In the respiratory zone:
- 100% blood supply
- short distance from blood to lumen
- Pathogen recognizing receptors (PRR)

In the conductive zone:
- 1% blood supply
- long distance from blood to airway lumen
- limited PRR

Craig A et al IAI 2009

Lars Christophersen et al. CEI 2012
Quantitative bacteriology

Lars Christoffersen et al. CEI 2012
Day 1

Airways area SB<LB: n.s
Area beads SB<LB: p<0.0001

Day 2/3

Airways area SB<LB: p=0.002
Area beads SB<LB: p<0.0001

Day 5/6

Not analysed

L Christophersen et al. CEI 2012
Presence of *P. aeruginosa* in the biofilm-like structures
Inflammation: PMN mobilization by serum G-CSF

Lars Christophersen et al. CEI 2012
Inflammation: PMN chemoattraction by pulmonary MIP-2

Lars Christophersen et al. CEI 2012
Conclusion (bead size direction):
Biofilms in smaller airways, increases inflammation. Increases lung tissue damage. Direct antibiotic treatment at smaller airways!
Worldwide deleterious condition

- 2% of the population in developed countries\(^1\)
- Venous ulcers: 2.5 million\(^2\)
- Pressure ulcers: 3 million\(^2\)
- Diabetic foot ulceration: 15%\(^3\) - amputation 14% - 24%\(^4\)
- Annual health care expenses: $25 billion\(^5\)

---


Venous ulcers account for 70-90% of chronic wounds

Chronic wounds are thought to persist in the inflammatory state of wound healing.

A theory of exaggerated proteolysis in wound fluids from patients with chronic venous ulcers is dominating.

Lack of growth factors or angiogenic factors?

Larger chronic wounds are more likely to be colonized by *P. aeruginosa*

Once colonized by *P. aeruginosa*, the success rate of surgical skin grafting deteriorates.

Surgical experience: overall healing rate of chronic venous ulcers is probably reduced when infection by *Pseudomonas aeruginosa*
Demonstration of Biofilm in chronic wounds


Chronic *Pseudomonas aeruginosa* wound model


Inoculation of *Pseudomonas aeruginosa* biofilm after 2-4 days

Pseudomonas aeruginosa in chronic wounds
(H Trøstrup, K Thomsen et al. WRR 2013).

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1</th>
<th>Day 4</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate – PAO1</td>
<td>0/6</td>
<td>0/6</td>
<td>0/5</td>
</tr>
<tr>
<td>Alginate + PAO1</td>
<td>6/6</td>
<td>5/5</td>
<td>6/6</td>
</tr>
</tbody>
</table>
Histopathology

- Four spatial and temporal integrated phases occur in a well orchestrated dynamical process: hemostasis (though not perceived a phase among all authors), inflammation, proliferation and tissue remodeling.

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1. Type</th>
<th>Day 1. Degree</th>
<th>Day 4. Type</th>
<th>Day 4. Degree</th>
<th>Day 7. Type</th>
<th>Day 7. Degree</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate</td>
<td>5 PMN</td>
<td>1 +++</td>
<td>0 PMN</td>
<td>0 +++</td>
<td>0 PMN</td>
<td>0 +++</td>
</tr>
<tr>
<td>-PAO1</td>
<td>0 MN/PMN</td>
<td>3 ++</td>
<td>5 MN/PMN</td>
<td>3 ++</td>
<td>2 MN/PMN</td>
<td>1 ++</td>
</tr>
<tr>
<td>Alginate</td>
<td>1 MN</td>
<td>1 +</td>
<td>1 MN</td>
<td>2 +</td>
<td>2 MN</td>
<td>1 +</td>
</tr>
<tr>
<td>+PAO1</td>
<td>1 NI</td>
<td>1 -</td>
<td>0 NI</td>
<td>1 -</td>
<td>1 NI</td>
<td>3 -</td>
</tr>
<tr>
<td>(n=6)</td>
<td></td>
<td></td>
<td>(n=6)</td>
<td></td>
<td>(n=5)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1. Type</th>
<th>Day 1. Degree</th>
<th>Day 4. Type</th>
<th>Day 4. Degree</th>
<th>Day 7. Type</th>
<th>Day 7. Degree</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate</td>
<td>6 PMN</td>
<td>3 +++</td>
<td>4 PMN*</td>
<td>2 +++</td>
<td>4 PMN*</td>
<td>1 +++**</td>
</tr>
<tr>
<td>+PAO1</td>
<td>0 MN/PMN</td>
<td>2 ++</td>
<td>1 MN/PMN</td>
<td>2 ++</td>
<td>1 MN/PMN</td>
<td>3 ++</td>
</tr>
<tr>
<td>Alginate</td>
<td>0 MN</td>
<td>1 +</td>
<td>0 MN</td>
<td>1 +</td>
<td>1 MN</td>
<td>2 +</td>
</tr>
<tr>
<td>+PAO1</td>
<td>0 NI</td>
<td>0 -</td>
<td>0 NI</td>
<td>0 -</td>
<td>0 NI</td>
<td>0 -</td>
</tr>
<tr>
<td>(n=6)</td>
<td></td>
<td></td>
<td>(n=5)</td>
<td></td>
<td>(n=6)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2. Quantitative bacteriology in chronic wounds.

*Pseudomonas aeruginosa* in chronic wounds (H Trøstrup, K Thomsen et al. WRR 2013).
Figure 3. IL-1β in chronic wounds.

*Pseudomonas aeruginosa* in chronic wounds (H Trøstrup, K Thomsen et al. WRR 2013).
Phases and degree of healing

<table>
<thead>
<tr>
<th>Phases</th>
<th>Degree of Healing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Necrotic tissue area (pixels)</td>
<td>133,084</td>
</tr>
<tr>
<td>⇒ Inflamed/healing area</td>
<td>233,357 - 133,084 = 100,273 pixels</td>
</tr>
<tr>
<td>⇒ Relative inflamed/healing area</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Moser Berlin 2013
The peritoneal implant model
Implant related infections

- Microbial infections on medical implants occur in more than 2 million surgical cases each year, in the United States alone (Emerson & Camesano, 2004, Gristina et al., 1988)

- Infections are the main cause of biomedical implant failure in modern medicine (Verkerte et al., 1997)

- In general infections on foreign-bodies will not be cleared until the implants have been removed from the body (Neut et al., 2005)
Model for studying biofilms on implants

- **Aim**
  - To introduce an *in vivo* foreign-body infection model, with the aim of studying treatment of foreign body related biofilm infections.
  - The model is calibrated when the mice do not die upon insertion of a *P. aeruginosa* colonized implant and bacteria can be found on the implants 1 week post-surgical.
Model for studying biofilms on implants

- Square silicone implants are inserted in the peritoneal cavity of mice

- Implants pre-colonized with bacteria

- O/N culture Dilute to OD$_{600}$ 0.5 in 0.9% NaCl. Allow colonization for 20 hours with moderate shaking. Wash the implants before usage.

- Implants removed after different periods of time and studied using CLSM and CFU counting
Application

• The impact of *Pseudomonas aeruginosa* Quorum Sensing (bacterial cell to cell communication) on biofilm persistence on implants *in vivo*
Clearing of *P. aeruginosa* wild-type vs. the Δ*lasR* *rhlR* mutant

Balb/c mice

![Graph showing bacterial counts in Balb/c mice over time post-surgical implant.](image)

Red: Δ*lasR* *rhlR*

Blue: Wild type

NMRI mice

![Graph showing bacterial counts in NMRI mice over time post-surgical implant.](image)

Red: Δ*lasR* *rhlR*

Blue: Wild type

Louise Dahl Christensen et al. Microbiology 2007
The implant mouse model vers. 2

**wt**

**QS mutant**

Day 0, P.a. coated silicone tube prior to insertion
**Staphylococcus epidermidis (SE1457)**

**Treatment groups**

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Number of mice/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre colonised implants, treated with vancomycin</td>
<td>12</td>
</tr>
<tr>
<td>Pre colonised implants, treated with SAMP Ltx21</td>
<td>12</td>
</tr>
<tr>
<td>Pre colonised implants, placebo</td>
<td>12</td>
</tr>
<tr>
<td>Sterile implants, SAMP ltx 21</td>
<td>12</td>
</tr>
<tr>
<td>Sterile implants, saline</td>
<td>12</td>
</tr>
<tr>
<td>Control animals, without surgery or treatment</td>
<td>5</td>
</tr>
</tbody>
</table>

Pauline Cavanagh et al. JAC 2013
Pre colonisation of implants

Pauline Cavanagh et al. JAC 2013
CFU implants

Pauline Cavanagh et al. JAC 2013
Small colony variants

Pauline Cavanagh et al. JAC 2013
Conclusion I

• We have successfully established a model to investigate implant related infections (both *P. aeruginosa* and CoNS)

• The model confirmed that the wild-type *P. aeruginosa* is more virulent than the ΔlasR rhlR mutant

• QS plays a role in the ability of mice to clear a *P. aeruginosa* foreign-body infection
Aknowledgements

Louise Dahl Christensen

Maria Alhede
All models are wrong
But they can be useful

Testing of hypothesis generated from observations in the patients

Pre-clinical testing of agents showing promising *in vitro* effect
Acknowledgements

• Rigshospitalet, Department for Clinical Microbiology.
  – Niels Høiby
  – Peter Østrup Jensen
  – Henrik Calum
  – Lars Christoffersen
  – Dina Silke Malling
  – Kim Thomsen
  – Hannah Trøstrup
  – Zhijun Song
  – Hong Wu
  – Competent technicians

• Rigshospitalet, Copenhagen CF Centre.
  – Christian Koch†
  – Tanja Pressler
  – Marianne Skov
  – Christine Rønne Hansen
  – Kim G Nielsen
  – Frederik Buchwald
  –excelent nurses

• Rigshospitalet, Department of Infection Control.
  – Leif Percival Andersen

• Copenhagen University, ISIM.
  – Michael Givskov
  – Thomas Bjarnsholt
  – Maria van Gennip
  – Louise Dahl Christensen
  – Morten Alhede
  – Oana Ciofu

• Copenhagen University, Forensic medicine
  – Hans Petter Hougen

• Technical University of Denmark.
  – Søren Molin

• Technological Institute and Aalborg University
  – Trine Rolighed Thomsen
  – Per Halkjær
  – Jan Lorenzen
  – Tine Yding Wolff

• Tromsø University Hospital
  – Hildegunn Granslo
  – Pauline Cavanagh
  – Elizabeth Fredheim
  – Klaus Klingenberg
  – Trond Flægstad