Biofilms and their role in culture-negative infections

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Denmark
Background

- 60-85% of all infections are biofilm related
- Biofilm infections cost billions of € each year
- Formation of biofilm constitutes a challenge to current sampling-, diagnosis and treatment procedures
Biofilm

Biofilm is the natural way of living of bacteria.

A coherent cluster of bacterial cells imbedded in a biopolymer matrix—which are more tolerant to: most antimicrobials, mechanical stress and the host defense, than planktonic bacterial cells.
For the purpose of diagnosis: We have to differentiate between acute and biofilm infections.
Acute versus biofilm infections

- Planktonic phenotype
- Wide arsenal of evasion and virulence mechanisms
- Generally aggressive infections
- Quickly resolved by clearance or death of host

- Biofilm formation
- Down regulated virulence
- Microorganisms grow slowly and are heterogeneously distributed - complicates sampling
- Less susceptible to antibiotics, even if highly susceptible as individual cells
- Cannot be cleared by the immune system
- Physical removal is necessary
- Standard culture is not sufficient to diagnose biofilm infections
Biofilm related infections challenges

1. Sampling/logistics
2. Microbiological testing
3. Interpretation of results
4. Treatment
Toolbox for studying biofilm infections

Patient data
NMR spectroscopy
Culture
qPCR
Next generation sequencing
MS
Phage display
ELISA
FISH

Clinic
Sampling
Culture

In vitro
Static
MBEC™
Flow cells
Reactos

Molecular analysis

Biomarkers and protein analysis

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Culture-negative infections

Sinus
Known: *S. aureus, P. aeruginosa,*
Poorly described: *Cyanobacterium* sp.,
uncultured *Alphaproteobacterium,*
uncultured *Betaproteobacterium,*
uncultured *Deltaproteobacteria, uncultured Bacteroidetes,* uncultured *Acidobacterium* sp.

Central venous catheter
Known: *S. epidermidis* and other CoNS, *S. aureus, P. aeruginosa, K. pneumoniae, Enterococcus* sp.
Poorly described: *Acidovorax* sp.,
uncultured *Deltaproteobacteria, uncultured Bacillales* bacterium

Prosthetic implants
Known: *S. aureus, CoNS*
Poorly described: uncultured *Rhodoferax* sp., uncultured *Curvibacter* sp., uncultured *Betaproteobacteria, uncultured Burkholderia* sp., uncultured *Bacteroidetes,*

Chronic venous leg ulcer
Known: *S. aureus, P. aeruginosa, E. faecalis, CoNS, K. oxytoca*
Poorly described: uncultured *Porphyromonas, uncultured Clostridia* bacterium, uncultured bacterium

Cystic fibrosis
Known: *S. aureus, P. aeruginosa, Haemophilus influenzae*
Poorly described: uncultured *Bacteroidetes* bacterium, uncultured *Flavobacterium, uncultured Betaproteobacterium, Polaromonas* sp., uncultured *Saprospiraceae bacterium*

Infective endocarditis
Known: *S. aureus, CoNS, Streptococcus* sp., Enterococci
Poorly described: *Legionella* sp.,
*Stenotrophomonas* sp., *Clostridium* sp.,
*Propionibacterium* sp., *Prevotella* sp.,
*Finegoldia* sp., uncultured bacterium

Urinary catheters
Known: *E. coli, P. aeruginosa, enterococci, Klebsiella* species, *Citrobacter* species.
Poorly described: uncultured *Corynebacterium* sp.

Necrotizing fasciitis
Known: streptococci, *E. coli, Bacteroides fragilis*
Poorly described: *Mycoplasma* sp., uncultured bacterium
## Frequency in prosthetic implant procedures

<table>
<thead>
<tr>
<th>Implants</th>
<th>Reporting year</th>
<th>No of surgery</th>
<th>Cost (billion $)</th>
<th>Reporting year</th>
<th>Primary surgery (Revisions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Knee prostheses</td>
<td>NS</td>
<td>543,000</td>
<td>12</td>
<td>2012</td>
<td>8535 (1291)</td>
</tr>
<tr>
<td>Hip prostheses</td>
<td>2007</td>
<td>230,000</td>
<td>10.5</td>
<td>2014</td>
<td>9410 (1366)</td>
</tr>
<tr>
<td>Spinal fusion hardware</td>
<td>2008</td>
<td>413,000</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Implantable eye lenses (pseudophakos)</td>
<td>2006</td>
<td>2,600,000</td>
<td>8-102</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coronary stents</td>
<td>2007</td>
<td>560,000</td>
<td>7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Implantable cardioverter defibrillators (ICD)</td>
<td>2009</td>
<td>133,000</td>
<td>5.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pacemakers</td>
<td>2009</td>
<td>235,000</td>
<td>4.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trauma fracture repair</td>
<td>2007</td>
<td>453,000</td>
<td>4.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tympanostomy tubes</td>
<td>2006</td>
<td>715,000</td>
<td>1-22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast implants, purely cosmetic</td>
<td>2010</td>
<td>366,000</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intrauterine devices</td>
<td>NS</td>
<td>425,000</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Denmark

Infection rate 0.6-2%

McIntyre, 2011, July 18. The eleven most implanted medical devices in America. 247 Wall st. (www.247wallst.dk)

Danish hip alloplastic register 2015, Danish knee alloplastic register 2013, Dale et al. 2012, Gundtoft et al. 2015
Prosthetic joint infection (PJI)

Infection can present itself

- Acute
- Chronic

Infection routes

- Exogenous
- Haematogenous spread
PRIS project

Focus: to study infections, aseptic loosening and pain related to implanted joint prosthesis

Clinical study
1. Sampling
Pilot study: quantification of ex. *Propionibacteria* using a qPCR assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average ± STD (copies/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient 1</strong></td>
<td></td>
</tr>
<tr>
<td>Bone</td>
<td>43 ± 3</td>
</tr>
<tr>
<td>Tissue</td>
<td>101 ± 95</td>
</tr>
<tr>
<td>Prosthesis scraping</td>
<td>42 ± 14</td>
</tr>
<tr>
<td><strong>Patient 2</strong></td>
<td></td>
</tr>
<tr>
<td>Joint fluid</td>
<td>-</td>
</tr>
<tr>
<td>Bone</td>
<td>-</td>
</tr>
<tr>
<td>Tissue</td>
<td>39 ± 9</td>
</tr>
<tr>
<td>Prosthesis scraping</td>
<td>-</td>
</tr>
<tr>
<td><strong>Patient 3</strong></td>
<td></td>
</tr>
<tr>
<td>Joint fluid</td>
<td>-</td>
</tr>
<tr>
<td>Bone</td>
<td>-</td>
</tr>
<tr>
<td>Tissue</td>
<td>-</td>
</tr>
<tr>
<td>Prosthesis scraping</td>
<td>83 ± 7</td>
</tr>
</tbody>
</table>

Thomsen, T., Xu, Y., Lorenzen, J., Nielsen, PH, & Schønheyder, H. 2012
Improved diagnosis of biofilm infections using various molecular methods.
*Culture Negative Biofilm Infections*: Springer. Edited by J. W. Costerton.
Pilot study, uneven distribution of bacteria

Heterogenous distribution is a challenge to sampling

Important where the samples are taken!

Multiple specimens required

Sampling, “All in a box”-concept

Specimens types

- Soft tissue biopsy
- Joint fluid
- Bone biopsy
- Prosthesis scraping
- Prosthesis

E.g. Sample box for revision surgery


2. Microbiology testing

Current PJI diagnostics

Patient history

Imaging (X-ray, radionuclide imaging o.a.)

Biochemical parameters (CRP, leukocyte count a.o.)

Joint fluid (optional)

5 periprosthetic soft tissue biopsies

Culturing aerobic and anaerobic for 6 days

False-negative culture results in up to 50% percent of apparent infections (antibiotics, not culturable, biofilm, multispecies)
**Analysis flow diagram – clinical study**

- **Samples**
  - Synovial fluid, bone, tissue biopsy, prosthesis swab, prosthesis sonication fluid
- **Selective bacterial DNA extraction, Molysis**
  - Negative samples
  - Positive samples
- **Broad range 16S rRNA gene PCR**
  - Extended culture, 14 days
- **Illumina amplicon library construction**
  - Illumina sequencing
  - Bioinformatic analysis of sequences
- **Visualization**
  - PNA filtration FISH
  - Confocal laser scanning microscopy
- **Detection**
  - Human and dead bacterial DNA is removed, we only focus on intact cells
- **Identity**

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**ESCMID eLibrary**

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NGS in clinical microbiology

- Know the background
- Include appropriate positive and negative controls at all levels
Diagnostic algorithm


Patients included in aseptic, pain and infection group

Lone Heimann Larsen, Vesal Khalid, Yijuan Xu, Trine Rolighed Thomsen and Henrik C. Schønheyder, PRIS study group. In prep. Differential contribution of specimen types, culturing, and 16S rRNA sequencing in diagnosis of prosthetic joint infections.
During the 2-year clinical project period:

- 164 boxes were used by the surgeons,
- 1508 (89%) of 1685 scheduled samples were received
Multiple periprosthetic tissue biopsies, prosthetic component(s) and joint fluid the optimal specimen set for diagnosis.

Diagnostic contribution of different specimen types for PJI

Contribution of different specimen types to the diagnosis of prosthetic joint infections by culture and 16S rDNA sequencing. In prep. Lone Heimann Larsen, Vesal Khalid, Yijuan Xu, Trine Rolighed Thomsen, Henrik C. Schønheyder, PRIS Study Group.

- Benefit of a broad specimen collection more pronounced in difficult low grade/atypical/biofilm infections
- For each type of device, the best specimen set for infection diagnosis should be identified
3. Interpretation of results in the clinical study

- Heterogeneous distribution of bacteria
- Polymicrobial infections in ~30% of cases
- Extended culture performed well
- NGS particularly helpful to ‘difficult to diagnose, culture negative’ cases

Tendency:
- *S. aureus, S. epidermidis* and *E. faecalis* are not detected by culture in some cases. Biofilm mode of growth
- Co-existence of some bacteria

Lone Heimann Larsen, Vesal Khalid, Yijuan Xu, Trine Rolighed Thomsen and Henrik C. Schønheyder, PRIS study group. In prep. Differential contribution of specimen types, culturing, and 16S rRNA sequencing in diagnosis of prosthetic joint infections
Monomicrobial versus polymicrobial biofilms

Environmental isolates

PJI isolate collection

The composition matters

Ren; Burmølle et al. 2015

Larsen et al. In prep
Case: A 67-year old male with diabetes and rheumatoid arthritis

The primary knee arthroplasty was performed in 1994

2005 revision surgery for presumed mechanical problems
Infection with *S. epidermidis*

Next 7 years: Pain, culture negative results

In 2012 the patient was included in the clinical project

Advanced diagnostic hybrid imaging (bone scan, dual leukocyte/bone marrow SPECT-CT and PET-CT) showed a ‘hotspot’

Extensive microbiological diagnostics was performed. *S. epidermidis* identified

The susceptibility pattern of *S. epidermidis* similar to that reported in 2005, a **chronic biofilm prosthesis infection had persisted for the last 7 years**

The patient was treated with antibiotics and the infection parameters was normalized for the first time since 1994
Understanding microbial pathogenesis

Who is there?

What are they doing?

How to stop them?
Studying microbial pathogenesis

PHYSIOLOGY AND VIRULENCE

1. **Genome**
   - What can happen

2. **Transcriptome**
   - What appears to be happening

3. **Proteome**
   - What makes it happen

4. **Metabolome**
   - What has happened and is happening
AIM
To gain insight into the *in vivo* expression of virulence and metabolic genes of *Staphylococcus aureus* in a prosthetic joint infection in a human subject.
Case:

- The patient had a diagnosis of psoriatic arthritis.
- He had joint implants in one hip, both knees, one elbow and one shoulder.
- Admitted after a fall with subsequent swelling of the right knee.
- Fever (38.8°C) and highly elevated C-reactive protein.
- A joint puncture revealed $10^4$-$10^5$ colony forming units of \textit{S. aureus}, susceptible to penicillin, methicillin and 5 antibiotic classes other than β-lactam.
- On the 4\textsuperscript{th} day of admission revision surgery with removal of the implant, \textit{S. aureus} with the same antibiogram was obtained from blood culture and biopsies.
- The blood culture isolate was referred to Statens Serum Institut for \textit{spa-typing} (t908, annotated to Clonal Complex 45).
- Several months later the patient underwent surgical revision and removal of implants from the left elbow and the left hip. \textit{S. aureus} infection with the same antibiogram was confirmed.
Pathogen detection and identification

- Cultivation
- Fluorescence in situ hybridization (DNA and PNA)
- S. aureus monoinfection
  Genome sequencing and annotation
- 16S amplicon sequencing
Methods: gene expression and metabolite profiling

- RNA-seq
- NMR
- Centrifugation
- Supernatant
- Pellet

**Muscle**

**Bursa**

**Tendon**

**Joint capsule**

**Synovial tissue**

**Joint fluid**

**Bone**

**Cartilage**

**Culture**

*In vitro culture (OD$_{600}$~0.5)*

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Toolbox for understanding the function of pathogens
Diagnosis of biofilm related infections

- Remember fundamental approaches like optimal specimen types and standardised sampling

- Combination of several diagnostic tools on specified specimens may be needed when confronted with culture-negative results in the face of a strong clinical suspicion of infection

- Algorithm, resulting in a personalized diagnosis and treatment

- Translate studies on bacterial function to clinical practise
Understanding microbial pathogenesis

Who is there?

What are they doing?

How to stop them?
4. Antibiofilm approaches

Purpose: Attach and test new particles with remarkable multispecies antibiofilm effect

Antibiofilm particles at 1g/L can decrease biofilm by 100 fold


Elos MedTech Pino A/S, Neurodan A/S, Arla Foods Ingredients Group Aarhus University, Malmö Högskola, Danish Technological Institute

“ASTI” -project
FP7 project. Diagnosis of PJI, new biomarkers and development and test of new implant materials

Participants:
Otto-von-Guericke Universität Magdeburg
University of Tartu
Instytut Obróbki Plastycznej Metal forming unit, Poland
Hungarian Academy of Sciences
Danish Technological Institute
Progenika, Spain
Mathys Ltd, Switzerland
GABO:mi, Germany

Static test

Benchmarking of various materials

CDC reactor test
Our work was supported by

- Danish PWT Foundation - Investment in Public Welfare Technology (ABT-fonden)
- The Danish Arthritis Society
- EU
- The Ministry of Science Technology and Innovation
Thanks to

Technological Institute
- Jan Lorenzen
- Yijuan Xu
- Majbritt Hauge Kyneb
- Peter Jensen

Aalborg University
- Jane Ildal
- Susanne Bielidt
- Kåre Lehmann Nielsen
- Jeppe Lund Nielsen
- Vibeke Rudkjøbing
- Per Halkjær Nielsen

Copenhagen University
- Thomas Bjarnsholt

Rigshospitalet
- Claus Moser, Niels Høiby

Aalborg and Århus hospital
- Ole Simonsen, Sten Rasmussen, Mogens Brouw Jørgensen, Kjeld Søballe, Lone Larsen, Henrik Schønheyder

The PRIS study group

The Hyporth study group

The ASTI study group
Ekstra slides
Interest areas

Chronic wounds
Orthopaedic infections
Sepsis
Urinary catheters
Necrotizing fasciitis
Central venous catheters
Endocarditis
Cystic fibrosis lungs
Cystic fibrosis sinus
Pleura empyema
MRSA
Fungal infections
...

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Treatment

- Chemical cleaning
- Mechanical cleaning
- Physical methods – UV, temperature, radiation

Thomsen U & Lorenzen J 2005

Dirschl, 2105

© by author
What is a biofilm?

- Biofilm is the **natural way of living** of bacteria
  - Exponential, planktonic growth only occurs in test tubes in the laboratory

- Biofilms are sessile communities of
  - prokaryotic and/or eucaryotic **cells**, attached to a substratum or interface or to each other,
  - embedded in a **matrix** composed, at least partially, of selfproduced extracellular material,
  - exhibiting an **altered phenotype** compared to planktonic cells.

(Donlan and Costerton, 2002)
The process of biofilm formation

We have to differentiate between acute and biofilm infections

IA. Attachment to uncoated surface by physicochemical forces
IB. Attachment to protein coated surface by specific adhesins
Attached cell monolayer
IV. Detachment
The *in vitro* biofilm

Janus Haagensen
The *in vivo* biofilm

- Wound
- CF lung
- Implant mouse model

Bjarnsholt et al. Trends in Microbiology. 2013 Sep;21(9):466-74
Bacterial biofilms

Natural form

Extracellular matrix
  Polysaccharides, nucleic acid, protein and lipids
  + host component

Higher tolerance towards extremes
  pH
  Temperature
  Mechanical stress
  Avoidance of the immune defence
Sampling


Position

<table>
<thead>
<tr>
<th>Position</th>
<th><em>Staphylococcus aureus</em></th>
<th><em>Pseudomonas aeruginosa</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>200±2%</td>
<td>920±9%</td>
</tr>
<tr>
<td>3</td>
<td>86±8%</td>
<td>300±13%</td>
</tr>
<tr>
<td>6</td>
<td>290±8%</td>
<td>8200±8%</td>
</tr>
<tr>
<td>9</td>
<td>80±5%</td>
<td>800±10%</td>
</tr>
<tr>
<td>12</td>
<td>93±12%</td>
<td>15±5%</td>
</tr>
</tbody>
</table>
Antibiotic tolerance

Antibiotics have different targets in active dividing bacteria

Architecture of the biofilm
RNA-seq results

No of total reads
No of *S. aureus* reads
No of *S. aureus* mRNA reads
In vivo gene expression in a *Staphylococcus aureus* prosthetic joint infection characterized by RNA sequencing and metabolomics: a pilot study

Yijuan Xu¹, Raluca Georgiana Maltesen¹, Lone Heimann Larsen¹,², Henrik Carl Schønheyder²,³, Vang Quy Le⁵, Jeppe Lund Nielsen¹, Per Halkjær Nielsen¹, Trine Rolighed Thomsen¹,⁴ and Kåre Lehmann Nielsen¹*
S. aureus metabolism during human PJI
Free amino acids were abundant in the infected joint fluid; food for the bacterial growth.
Fermentation

The infected joint was severely hypoxic
Interest areas

Chronic wounds
**Orthopaedic infections**
Sepsis
Urinary catheters
Necrotizing fasciitis
Central venous catheters
Endocarditis
Cystic fibrosis lungs
Cystic fibrosis sinus
Pleura empyema
MRSA
Fungal infections
Prosthetic joint infection (PJI) or not?

Difficult to separate chronic infections from more aseptic failures

Often negative results when biochemical parameters suggest infection
## Advantages and disadvantages of culture

<table>
<thead>
<tr>
<th><strong>Advantages</strong></th>
<th><strong>Disadvantages</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>• Gold standard in clinical microbiology</td>
<td>• Slow, time consuming, and labour intensive</td>
</tr>
<tr>
<td>• Relatively inexpensive and widely available</td>
<td>• Samples require appropriate transport conditions and immediate processing</td>
</tr>
<tr>
<td>• Requires only few specialized instruments</td>
<td>• Restricted to culturable organisms</td>
</tr>
<tr>
<td>• Allows quantification of bacterial population</td>
<td>• Selection of growth media and condition can greatly affect results</td>
</tr>
<tr>
<td>• Allows for antimicrobial susceptibility testing</td>
<td>• Not all viable bacteria can be recovered</td>
</tr>
<tr>
<td>• Physiological and biochemical studies are possible</td>
<td>• Biofilm bacteria may not grow</td>
</tr>
<tr>
<td></td>
<td>• Antimicrobial treatment inhibits growth</td>
</tr>
</tbody>
</table>