Biofilms and their role in culture-negative infections

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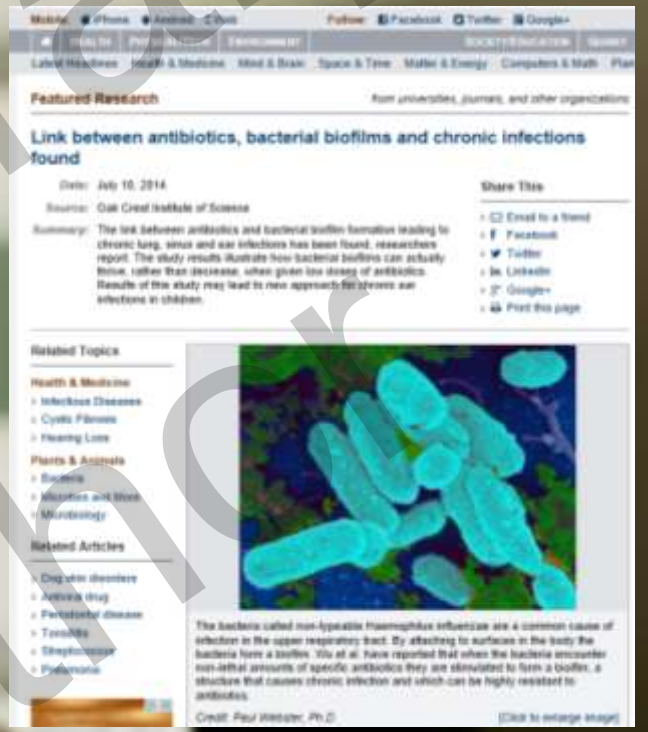
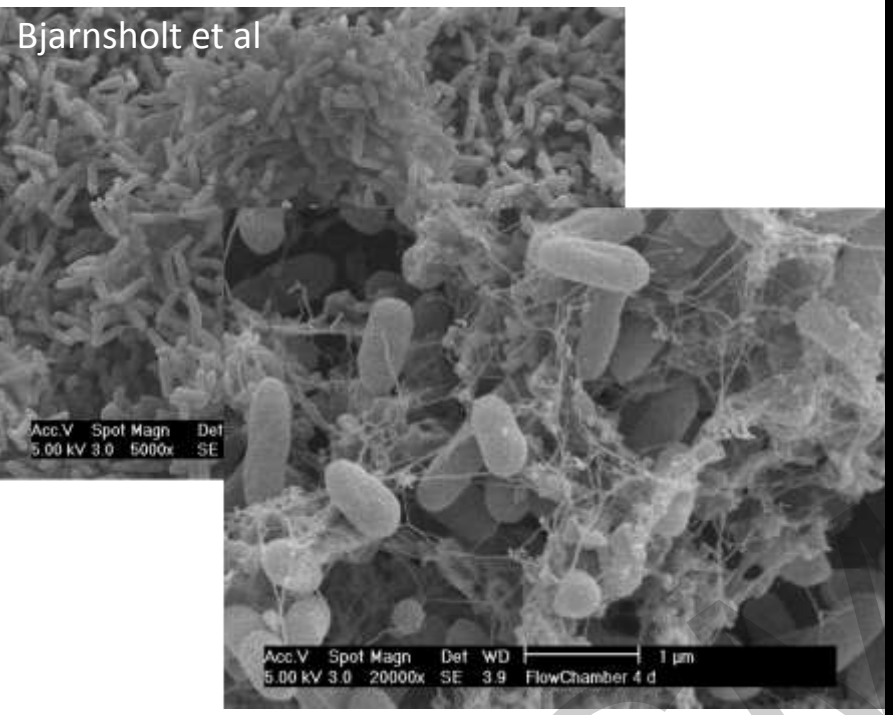
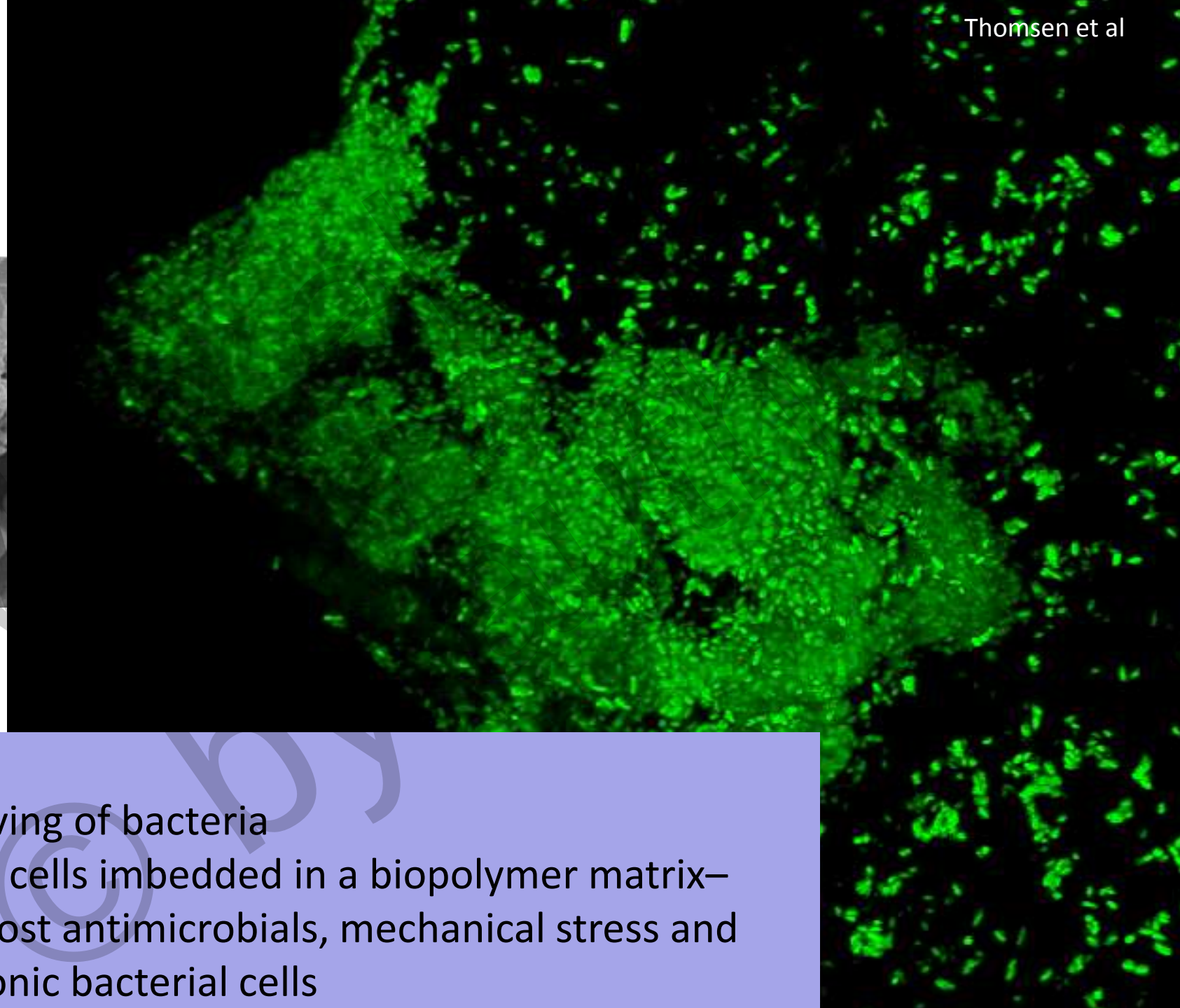
Background

- 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

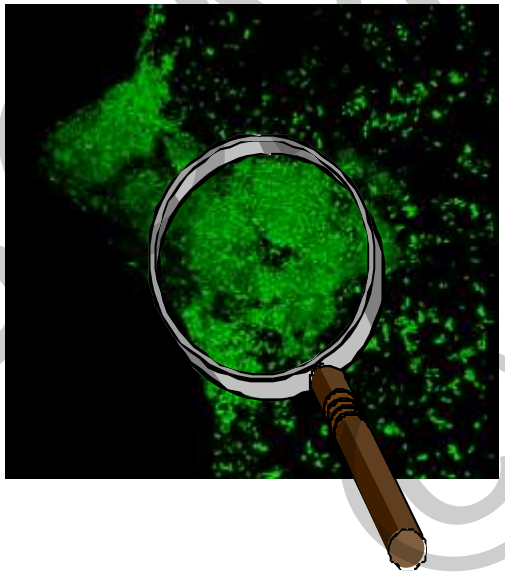


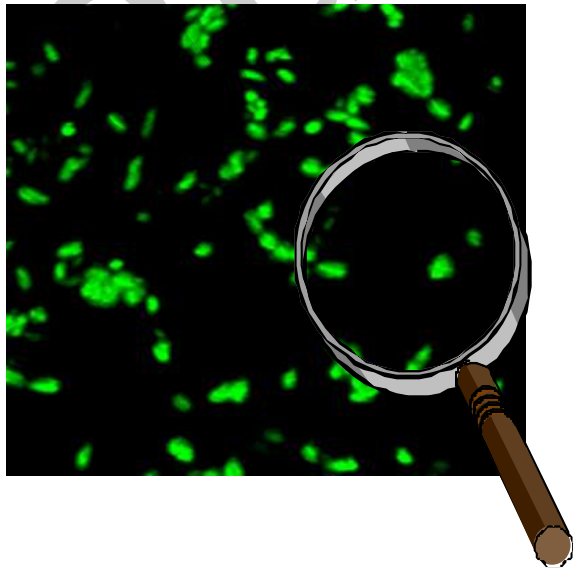


BIOFILM:

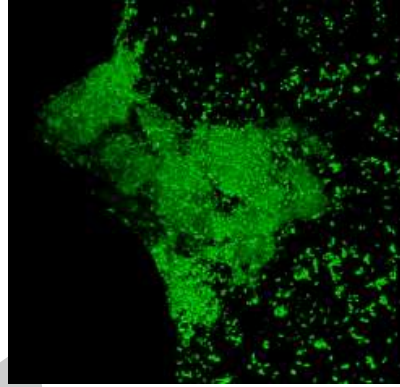
- Biofilm is the natural way of living of bacteria
- A coherent cluster of bacterial cells imbedded in a biopolymer matrix– which are more tolerant to: most antimicrobials, mechanical stress and the host defense, than planktonic bacterial cells

For the purpose of diagnosis:
We have to differentiate between acute and biofilm infections



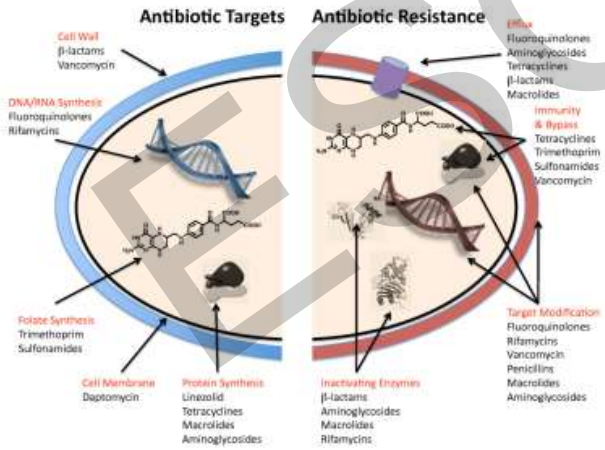


Acute versus biofilm infections



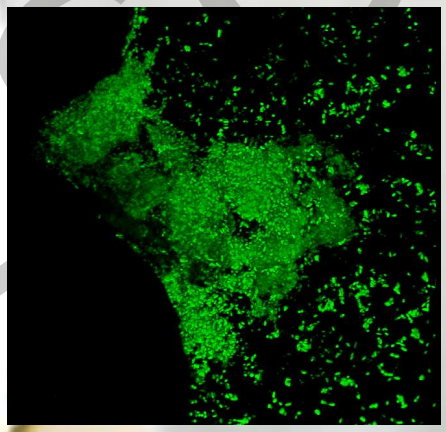
- Planktonic phenotype
- Wide arsenal of evasion and virulence mechanisms
- Generally aggressive infections
- Quickly resolved by clearance or death of host

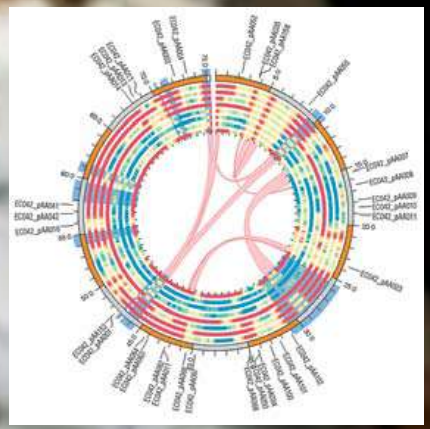
- Biofilm formation
- Down regulated virulence
- Microorganisms grow slowly and are heterogeneously distributed- complicates sampling
- Less susceptible to antibiotics, even if highly susceptible as individual cells
- Cannot be cleared by the immune system
- Physical removal is necessary
- Standard culture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

Clinic

Patient data

Sampling

Culture

In vitro

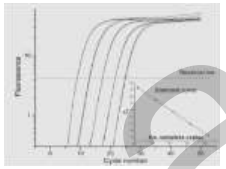
Static

MBEC™

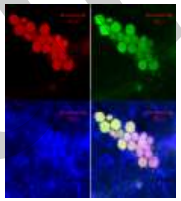
Flow cells

Reactos

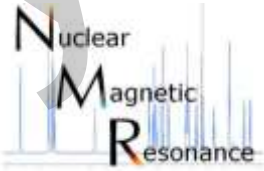
Molecular analysis



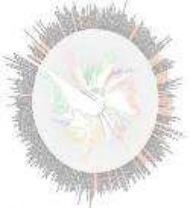
qPCR



FISH



NMR spectroscopy

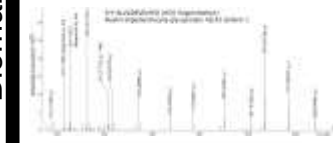


Next generation sequencing

Biomarkers and protein analysis

ELISA

Phage display



MS

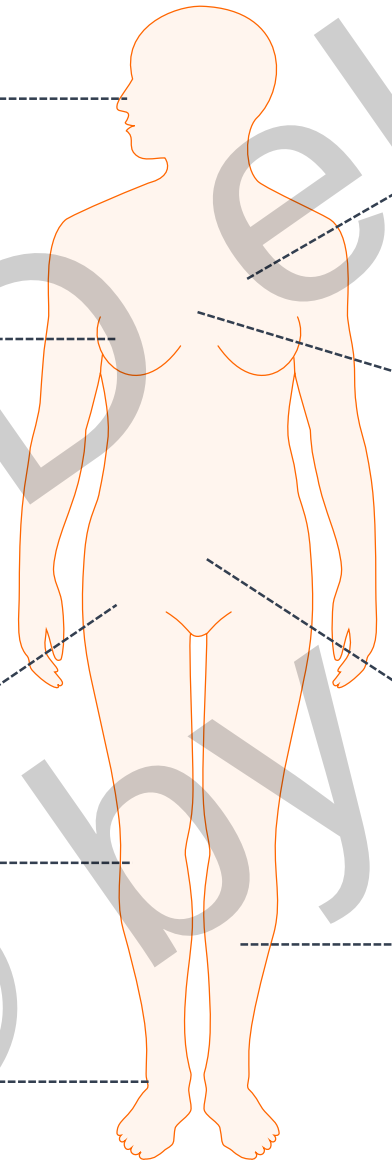
Culture-negative infections

Sinus
Known: *S. aureus*, *P. aeruginosa*,
Poorly described: *Cyanobacterium* sp., uncultured *Alphaproteobacterium*, uncultured *Betaproteobacterium*, uncultured *Deltaproteobaceria*, 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 *Deltaproteobaceria*, 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 influenza*
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

Implants	Reporting year	No of surgery	Cost (billion $)
Knee prostheses	NS	543,000	12
Hip prostheses	2007	230,000	10.5
Spinal fusion hardware	2008	413,000	10
Implantable eye lenses (pseudophakos)	2006	2,600,000	8-102
Coronary stents	2007	560,000	7.5
Implantable cardioverter defibrillators (ICD)	2009	133,000	5.5
Pacemakers	2009	235,000	4.5
Trauma fracture repair	2007	453,000	4.5
Tympanostomy tubes	2006	715,000	1-22
Breast implants, purely cosmetic1	2010	366,000	1.0
Intrauterine devices	NS	425,000	0.3

Denmark

Reporting year	Primary surgery	(Revisions)
2012	8535	(1291)
2014	9410	(1366)

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

AdvanDx

AALBORG UNIVERSITET

STORZ
KARL STORZ — ENDOSKOPE

nordicbioscience

AALBORG SYGEHUS
ÅRHUS UNIVERSITETSHOSPITAL

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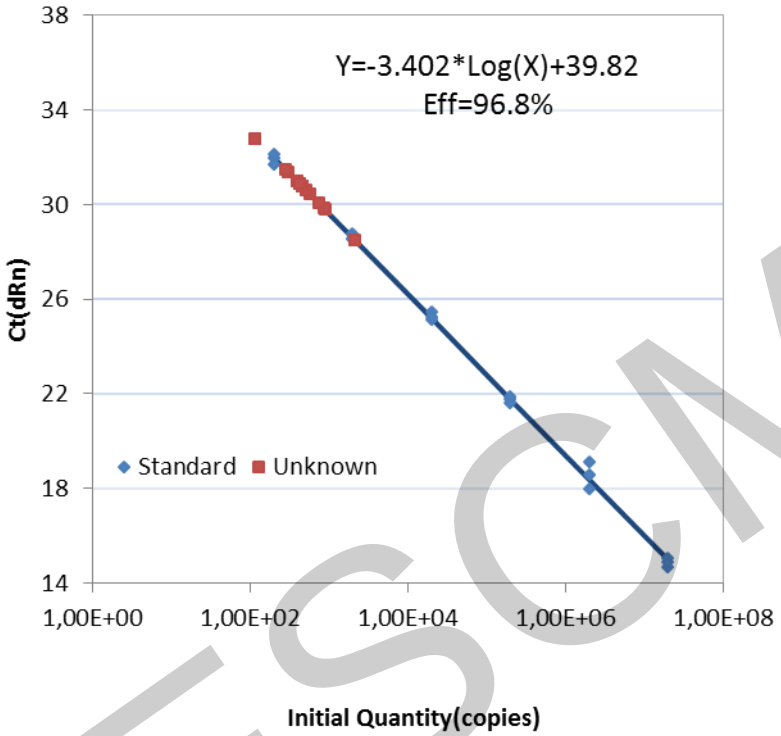
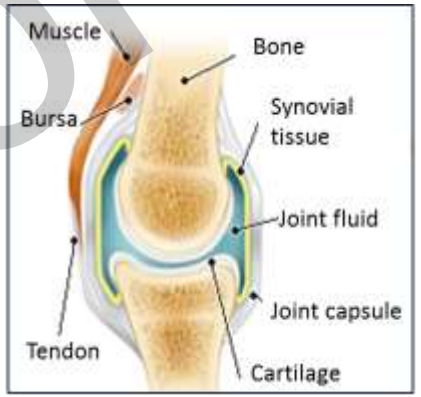
Danish Agency for Science Technology and Innovation
Ministry of Science Technology and Innovation

1. Sampling

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



	Sample	Average ±STD (copies/µL)
Patient 1	Joint fluid	-
	Bone	43±3
	Tissue	101±95
	Prosthesis scraping	42±14
Patient 2	Joint fluid	-
	Bone	-
	Tissue	39±9
	Prosthesis scraping	-
Patient 3	Joint fluid	-
	Bone	-
	Tissue	-
	Prosthesis scraping	83±7



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.

Sampling

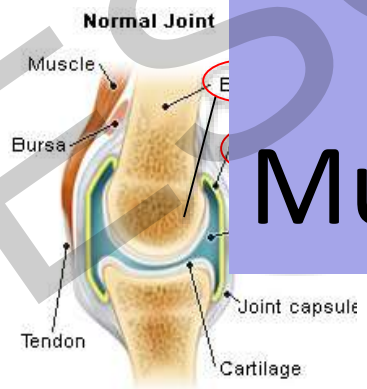
Pilot study, uneven distribution of bacteria

Heterogenous distribution is a challenge to sampling

Important where the samples are taken!

Multiple specimens required

Prosthesis sample
+
+
+
+



Xu, Y., V. Rudkjøbing, O. Simonsen, C. Pedersen, J. Lorenzen, H. Schønheyder, P. Nielsen, and TR. Thomsen. (2012). 'Bacterial Diversity in Suspected Prosthetic Joint Infections: An Exploratory Study Using 16S rRNA Gene Analysis', *FEMS Immunol Med Microbiol* DOI: 10.1111/j.1574-695X.2012.00949.x

Sampling, ”All in a box”-concept

Specimens types

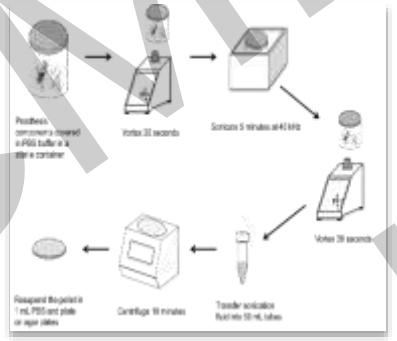
Soft tissue biopsy

Joint fluid

Bone biopsy

Prosthesis scraping

Prosthesis



Lone Heimann Larsen, Yijuan Xu, Ole Simonsen, Christian Pedersen. Henrik C. Schønheyder, Trine Rolighed Thomsen, PRIS Study Group.
All in a box! A novel concept for optimising microbiological diagnostic sampling in prosthetic joint infections. 2014. BMC Research Notes, 7:418

Kahn, 2006. Stryker. Copan, Italia.

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

Human and dead bacterial DNA is removed, we only focus on intact cells

Samples

Synovial fluid, bone, tissue biopsy, prosthesis swab, prosthesis sonication fluid

Bacterial DNA extraction & human DNA removal

Selective bacterial DNA extraction, Molysis

Detection

Broad range 16S rRNA gene PCR

Negative samples Positive samples

Identity

Illumina amplicon library construction

↓

Illumina sequencing

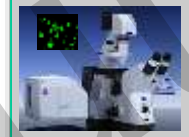
↓

Bioinformatic analysis of sequences

Visualization

PNA filtration FISH

↓



Confocal laser scanning microscopy

Extended culture, 14 days

NGS in clinical microbiology

- Know the background
- Include appropriate postive and negative controls at all levels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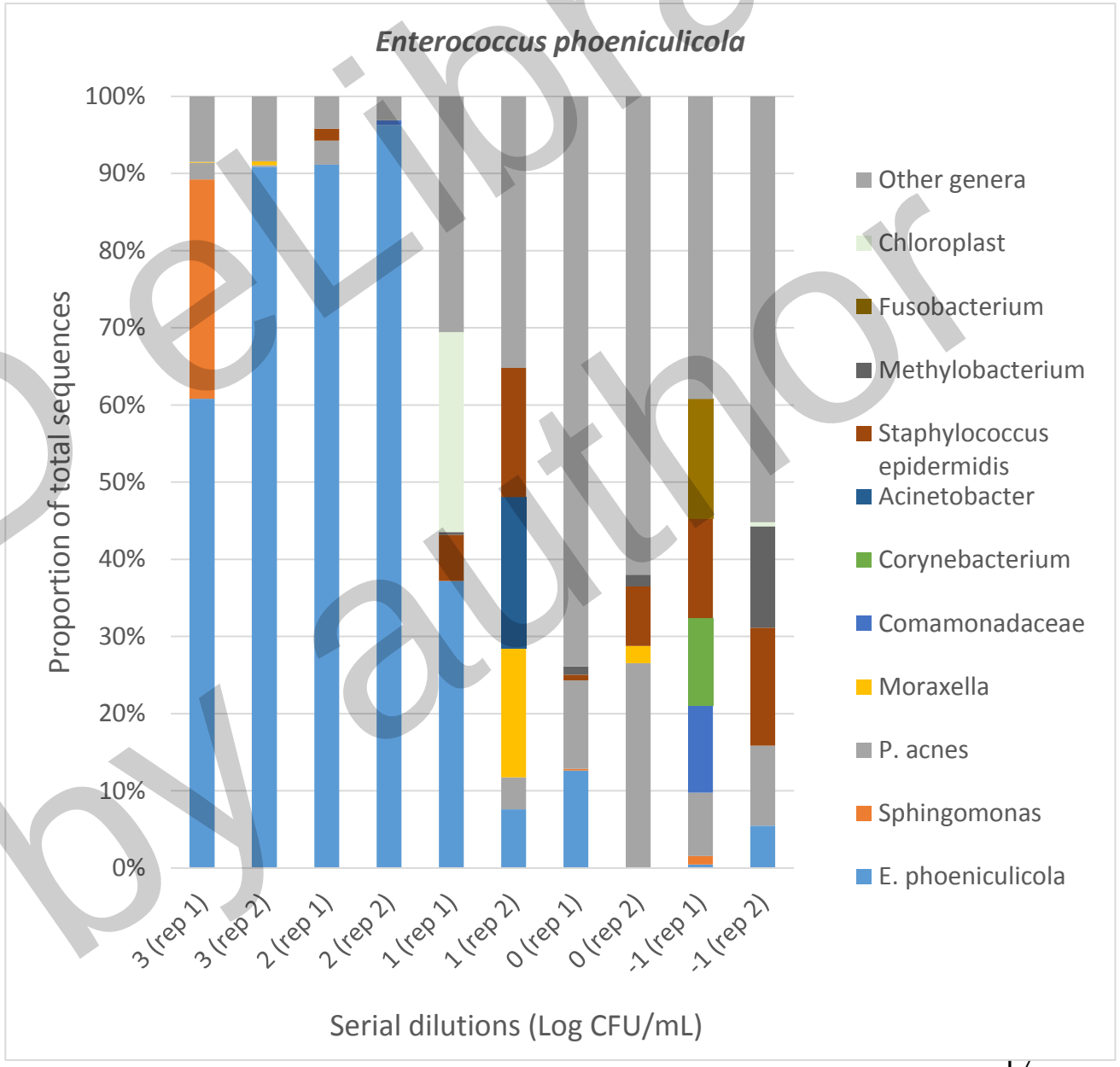
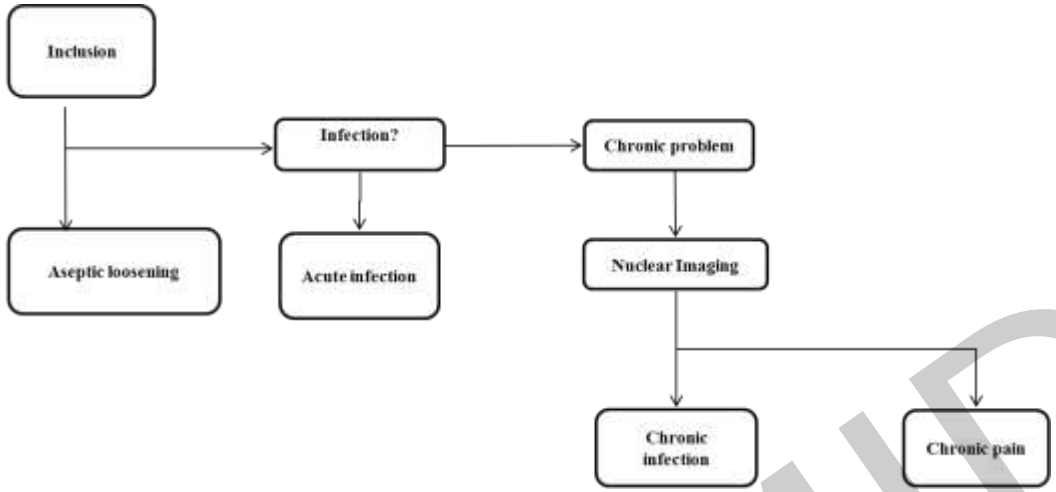
REVIEW ARTICLE

Microbiological diagnosis of device-related biofilm infections

YIJUAN XU,[1] LONE HEIMANN LARSEN,[2] JAN LORENZEN,[1] LUANNE HALL-STOODLEY,[3] JUDITH KIKHNEY,[4] ANNETTE MOTER[4] and TRINE ROLIGHED THOMSEN[1,5]

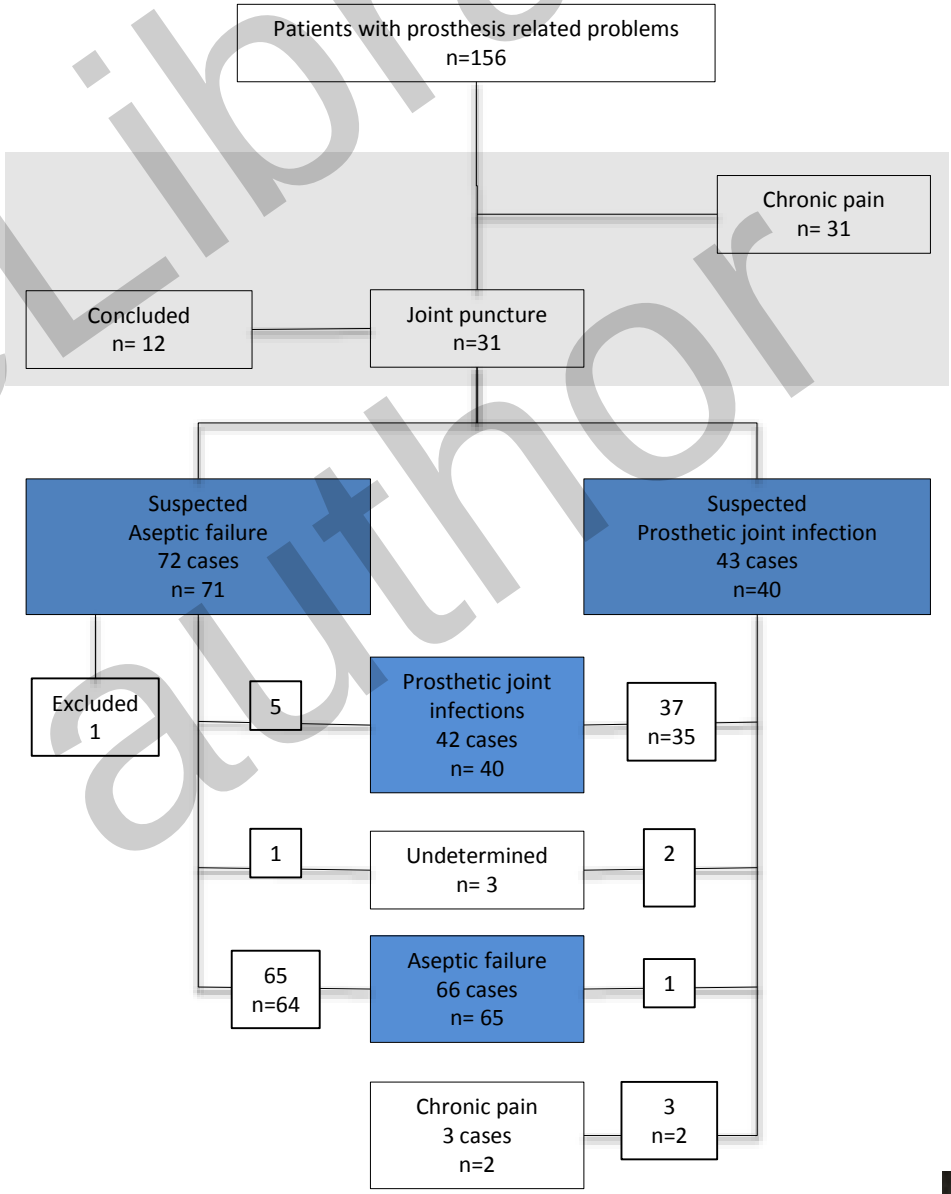


Diagnostic algorithm



Vesal Khalid, Trine Thomsen, Jan Lorenzen, Henrik Schønheyder, Sten Rasmussen, PRIS study group. In prep. Multidisciplinary diagnostic algorithm for evaluation of patients presenting with a prosthetic problem in the hip or knee: A prospective study

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

During the 2-year clinical project period:

- 164 boxes were used by the surgeons,
- 1508 (89%) of 1685 scheduled samples were received

- 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

Multi and joint fluid t

Peripros

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.

3. Interpretation

of results in the clinicial study

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

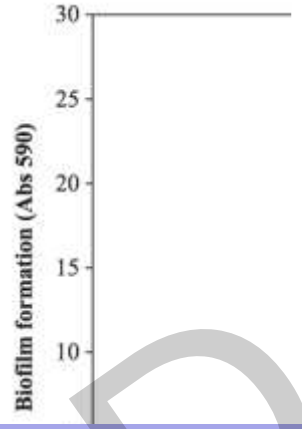
Tendency:

- *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

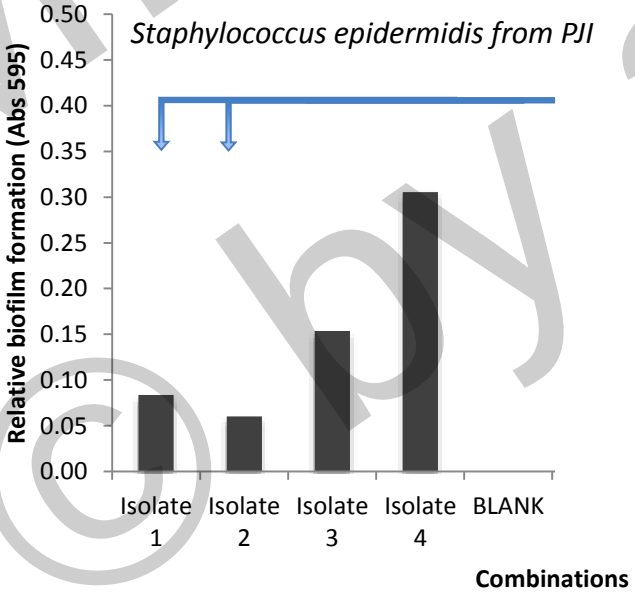
Environmental isolates



The composition matters

Ren; Burmølle *et al.* 2015

PJI isolate collection



Larsen e*t 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'

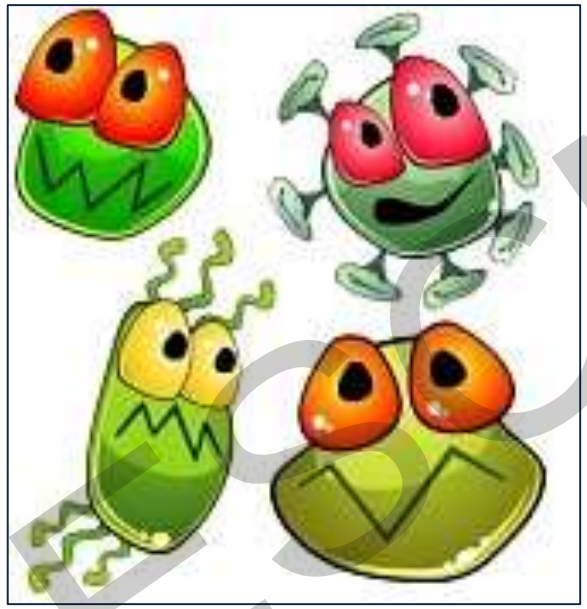
Extensive microbiological diagnostics was performed. *S. epidermidis* identified

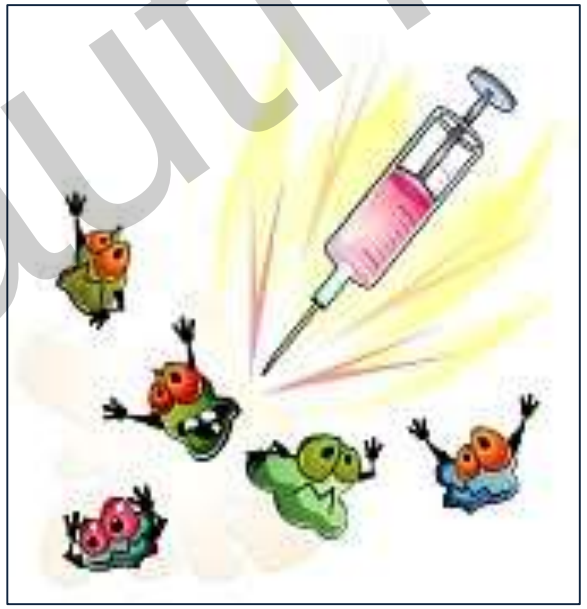
The susceptibility pattern of *S. epidermidis* similar to that reported in 2005, a **chronic biofilm prosthesis infection had persisted for the last 7 years**

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

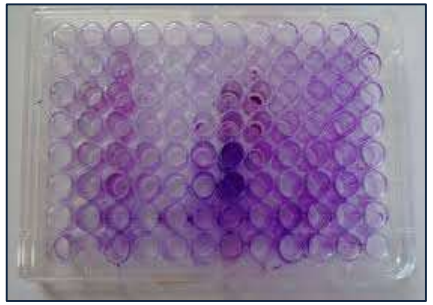
Understanding microbial pathogenesis

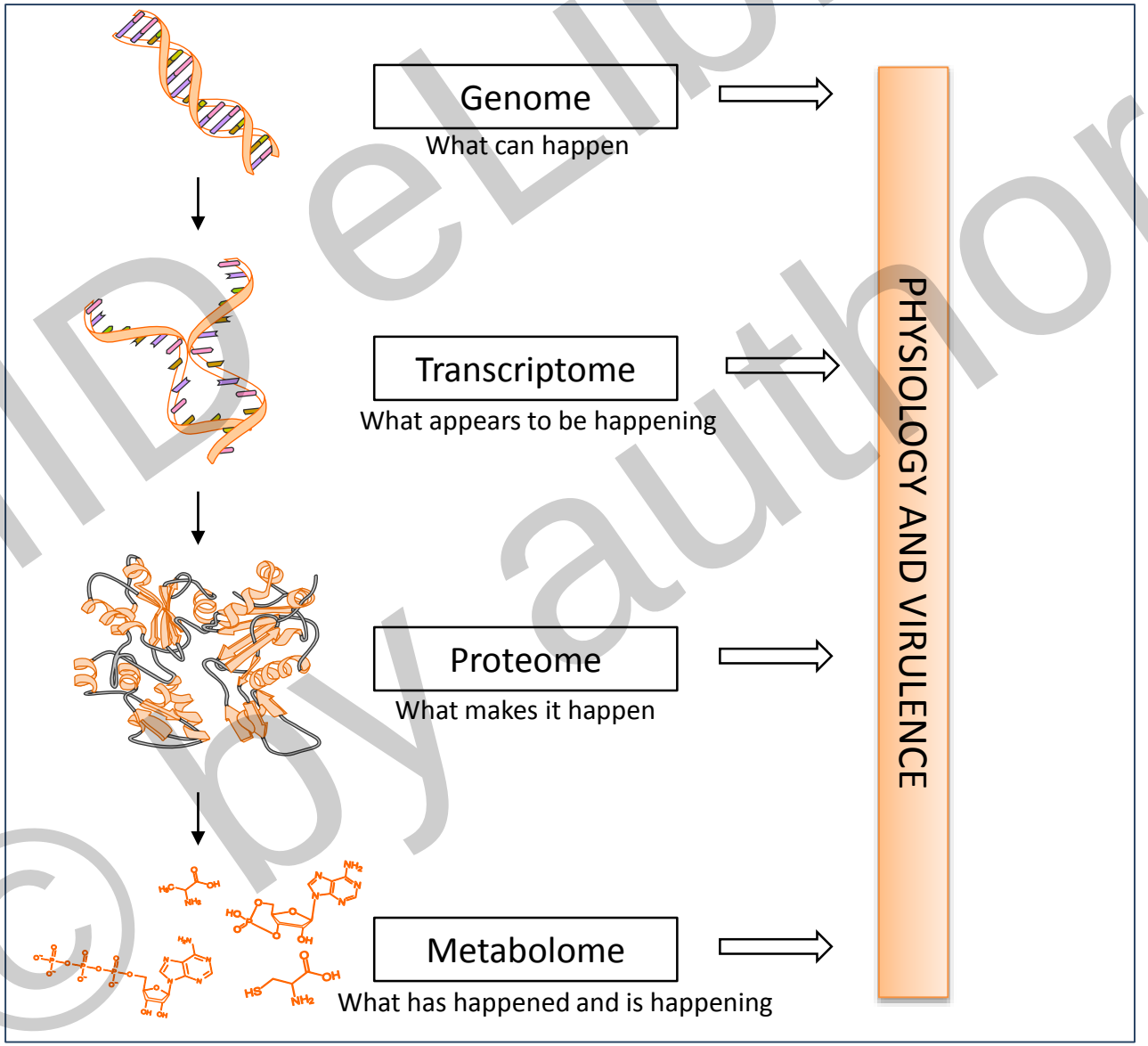
Who is there?	What are they doing?	How to stop them?





Studying microbial pathogenesis





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

Danish Agency for Science Technology and Innovation
Ministry of Science Technology and Innovation

nordicbioscience

AALBORG UNIVERSITY DENMARK

AdvanDx

COPAN

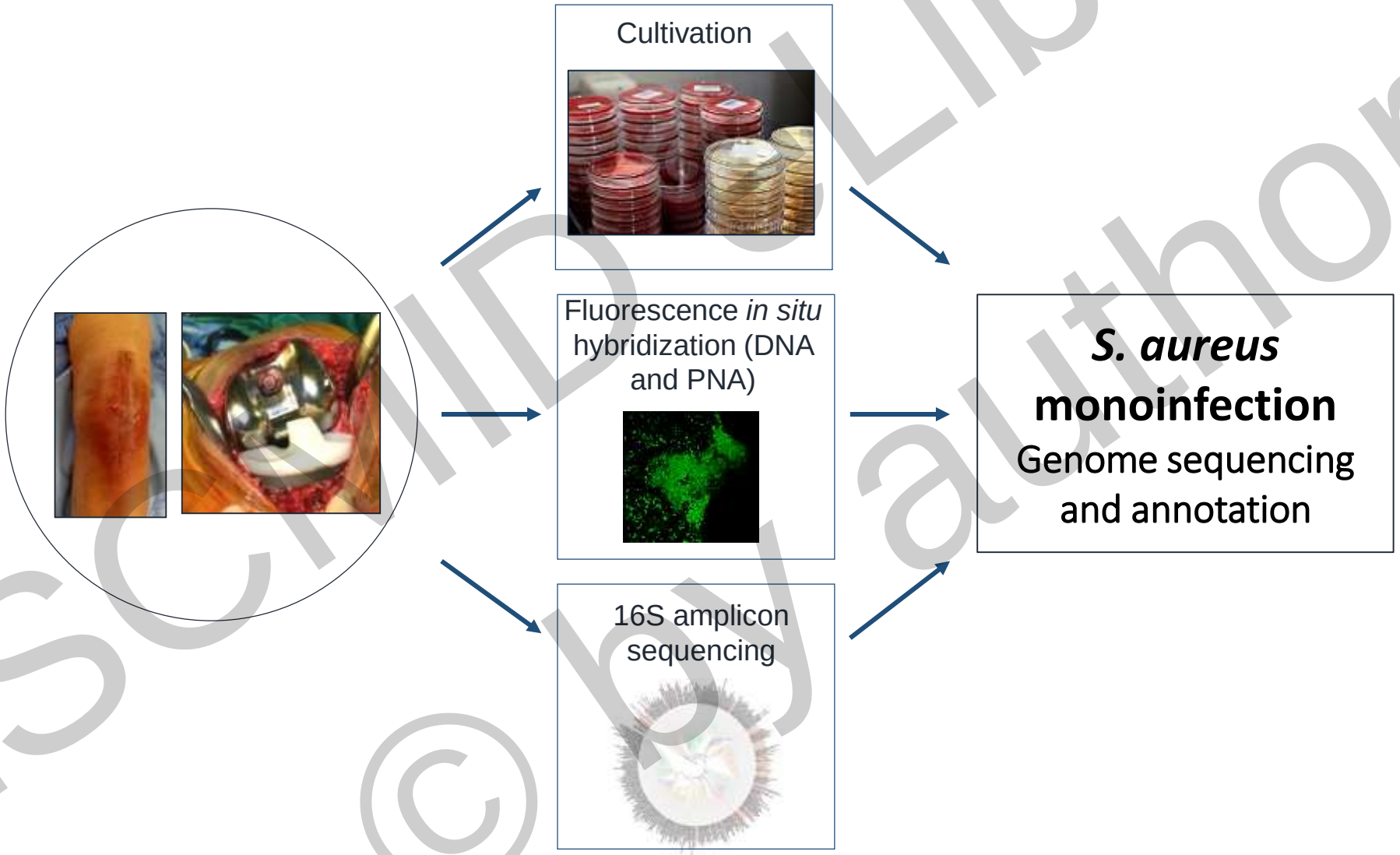
I gode hænder hos AALBORG UNIVERSITETSHOSPITAL

DANISH TECHNOLOGICAL INSTITUTE

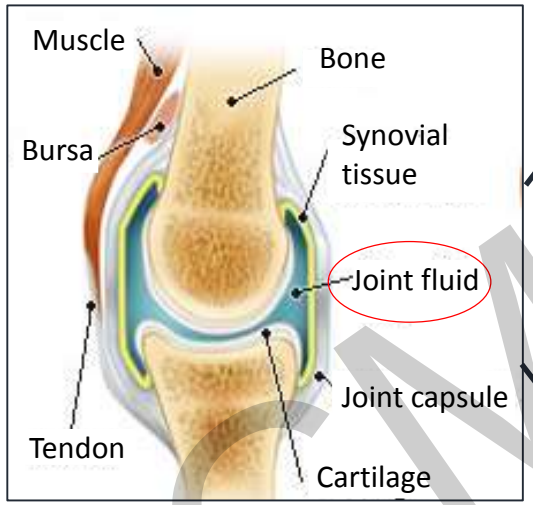
Case:

- 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 *S. aureus*, susceptible to penicillin, methicillin and 5 antibiotic classes other than β-lactam.**
- On the 4th day of admission revision surgery with removal of the implant, *S. aureus* with the same antibiogram was obtained from blood culture and biopsies.
- The blood culture isolate was referred to Statens Serum Institut for ***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. *S. aureus* infection with the same antibiogram was confirmed.

Pathogen detection and identification



Methods: gene expression and metabolite profiling



Centrifugation

NMR

Supernatant

RNA-seq

Pellet

Culture

In vitro culture (OD_{600}~0.5)

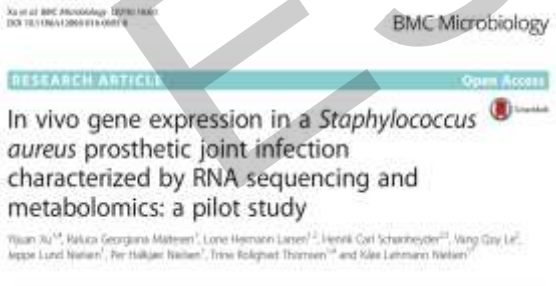
Centrifugation

NMR

Supernatant

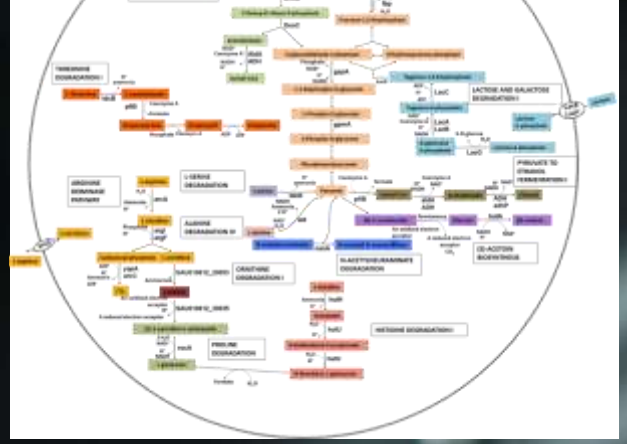
RNA-seq

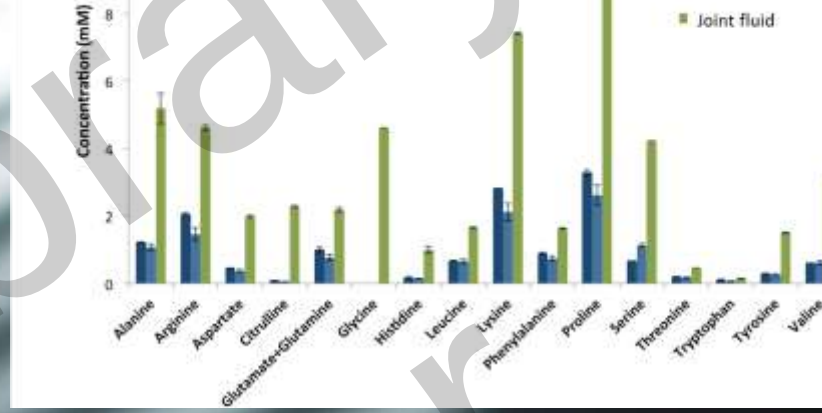
Pellet


BMC Microbiology

RESEARCH ARTICLE Open Access

In vivo gene expression in a *Staphylococcus aureus* prosthetic joint infection characterized by RNA sequencing and metabolomics: a pilot study

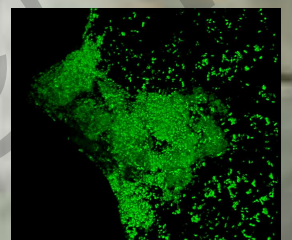
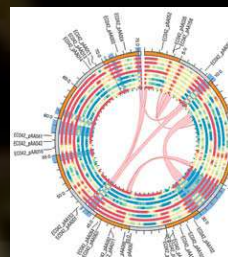




- ✓ Toolbox for understanding the function of pathogens

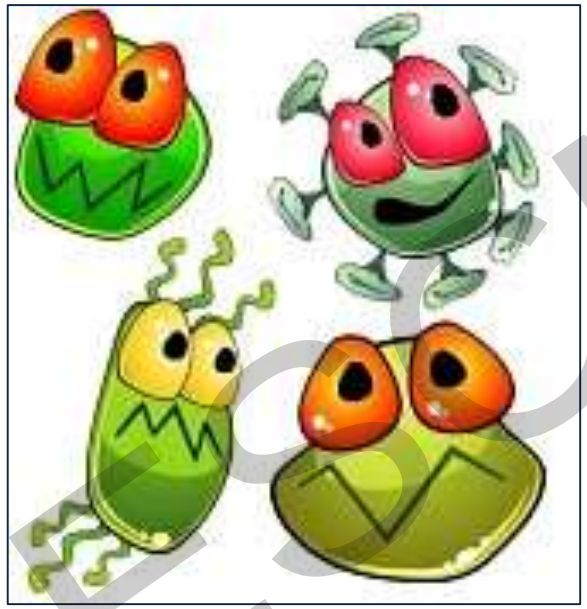
Diagnosis of biofilm related infections

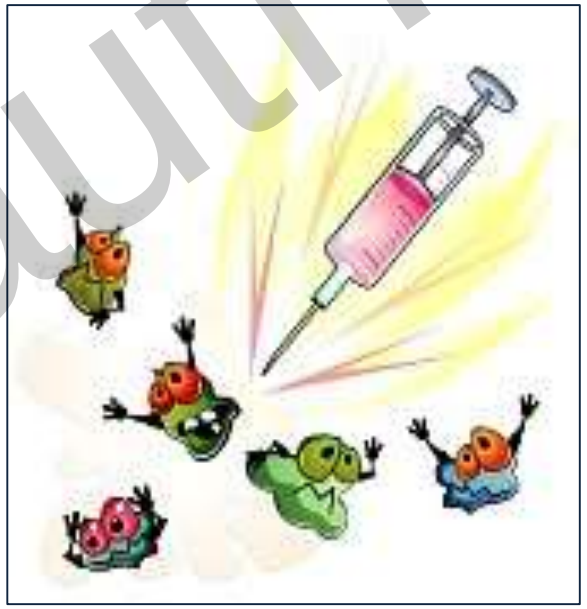
- Remember fundamental approaches like optimal specimen types and standardised sampling
- Combination of several diagnostic tools on specified specimens may be needed when confronted with culture-negative results in the face of a strong clinical suspicion of infection
- Algorithm, resulting in a personalized 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?

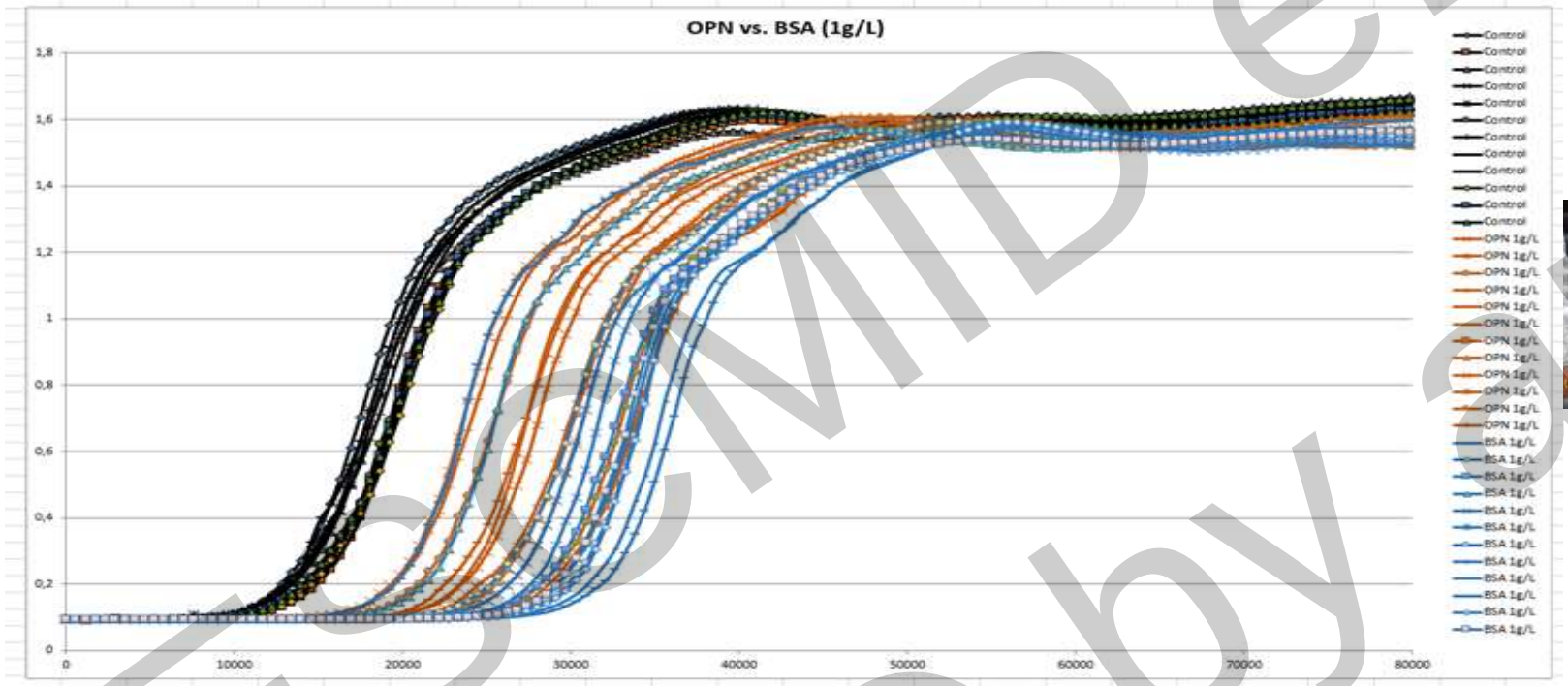




4. Antibiofilm approaches

http://www.dti.dk/projects/project-new-surface-coatings-ensure-enhanced-soft-tissue-integration-of-implants/35492

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

HYP ORTH

Participants:
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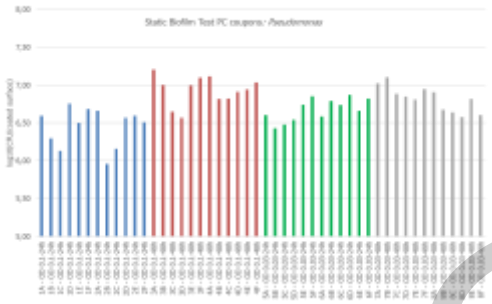
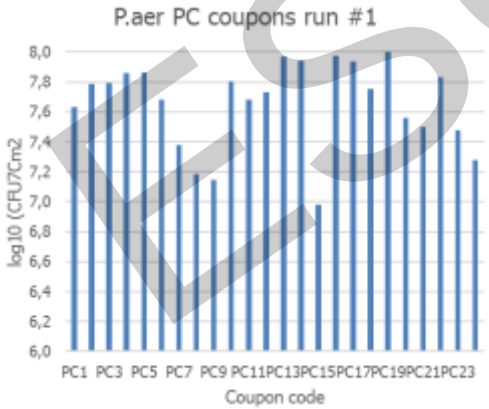
University of Tartu
Instytut Obròbki Plastycznej Metal forming unit, Poland
Hungarian Academy of Sciences
Danish Technological Institute
Progenika, Spain
Mathys Ltd, Schwitzerland
GABO:mi, Germany



Static test

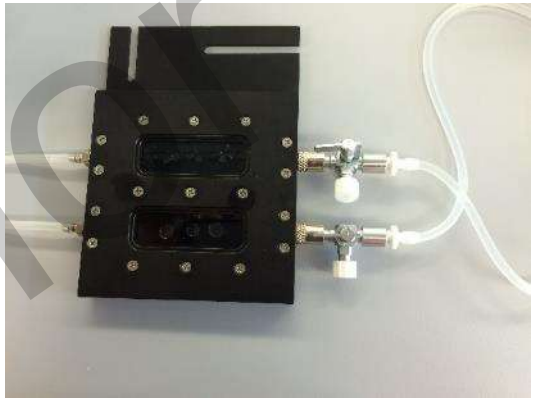
Benchmarking of various materials

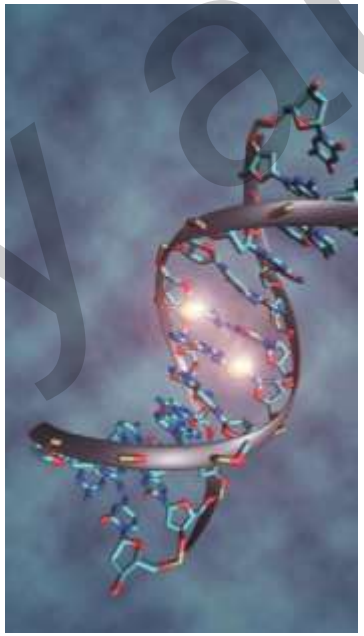


CDC reactor test

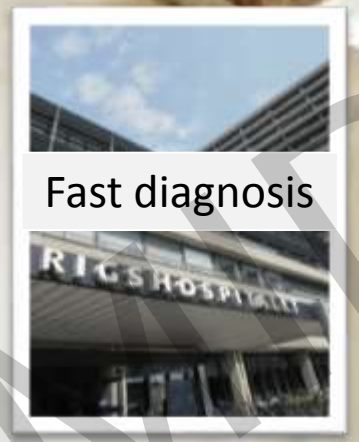
FP7 project.

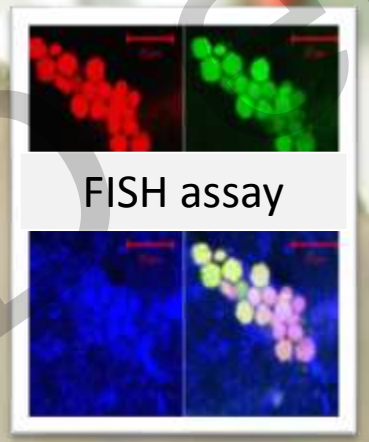
Diagnosis of PJI, new biomarkers and development and test of new implant materials

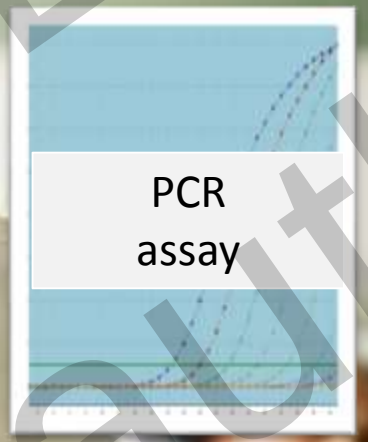


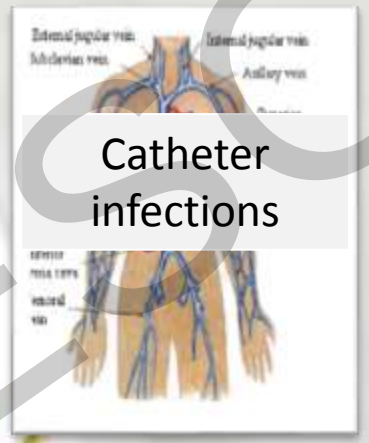


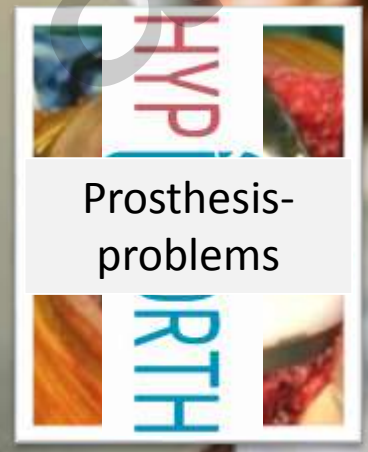
Our work was supported by











Danish PWT Foundation- Investment in Public Welfare Technology (ABT-fonden)

The Danish Arthritis Society

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The Ministry of Science Technology and Innovation

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The PRIS study group

The Hyporth study group

The ASTI study group

Ekstra slides

Interest areas

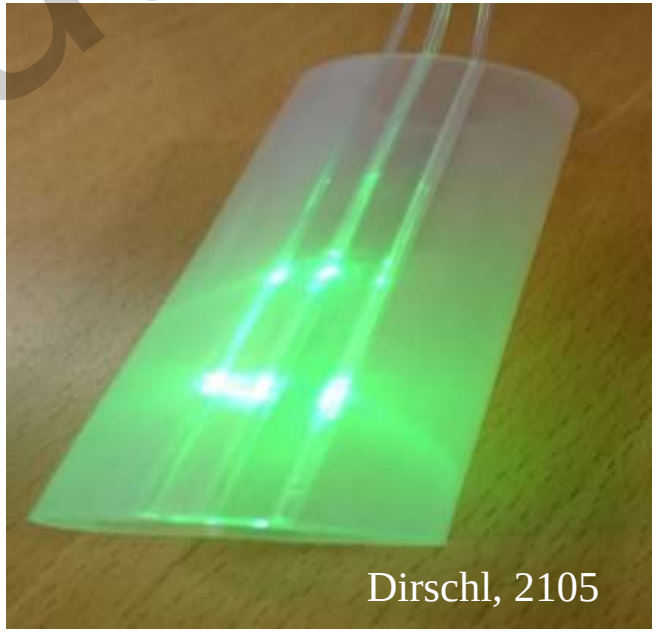
Chronic wounds
Orthopaedic infections
Sepsis
Urinary catheters
Necrotizing fasciitis
Central venous catheters
Endocarditis
Cystic fibrosis lungs
Cystic fibrosis sinus
Pleura empyema
MRSA
Fungal infections
…

Treatment

DANISH TECHNOLOGICAL INSTITUTE

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


Thomsen U & Lorenzen J 2005


Dirschl, 2105

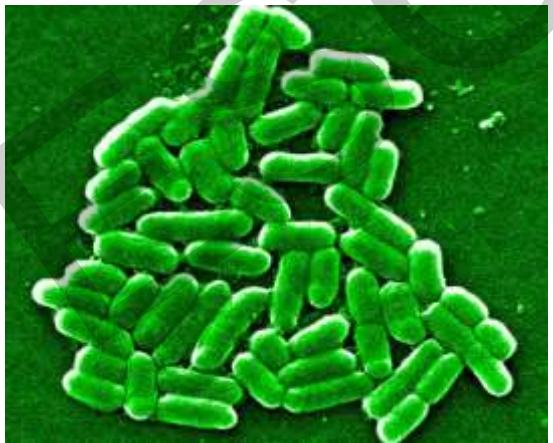
What is a biofilm?

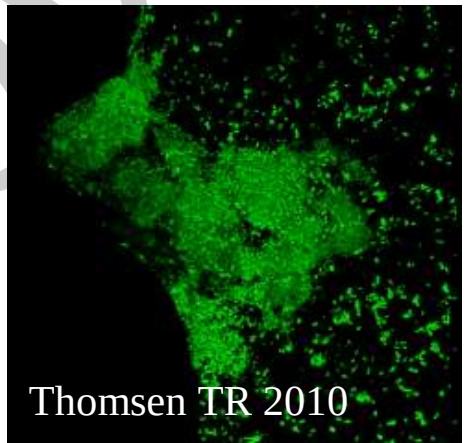
DANISH TECHNOLOGICAL INSTITUTE

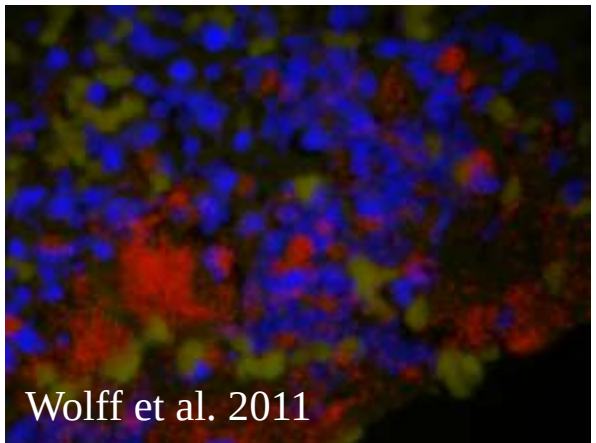
- Biofilm is the **natural way of living** of bacteria
  - Exponential, planktonic growth only occurs in test tubes in the laboratory

- 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.

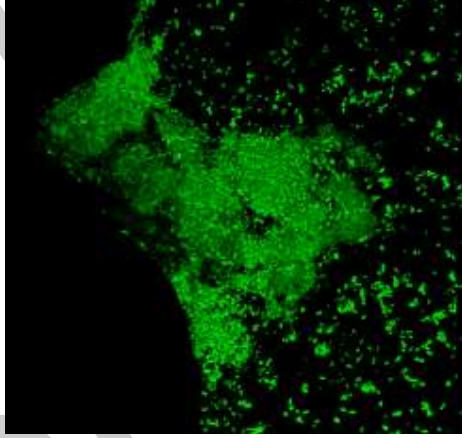
(Donlan and Costerton, 2002)

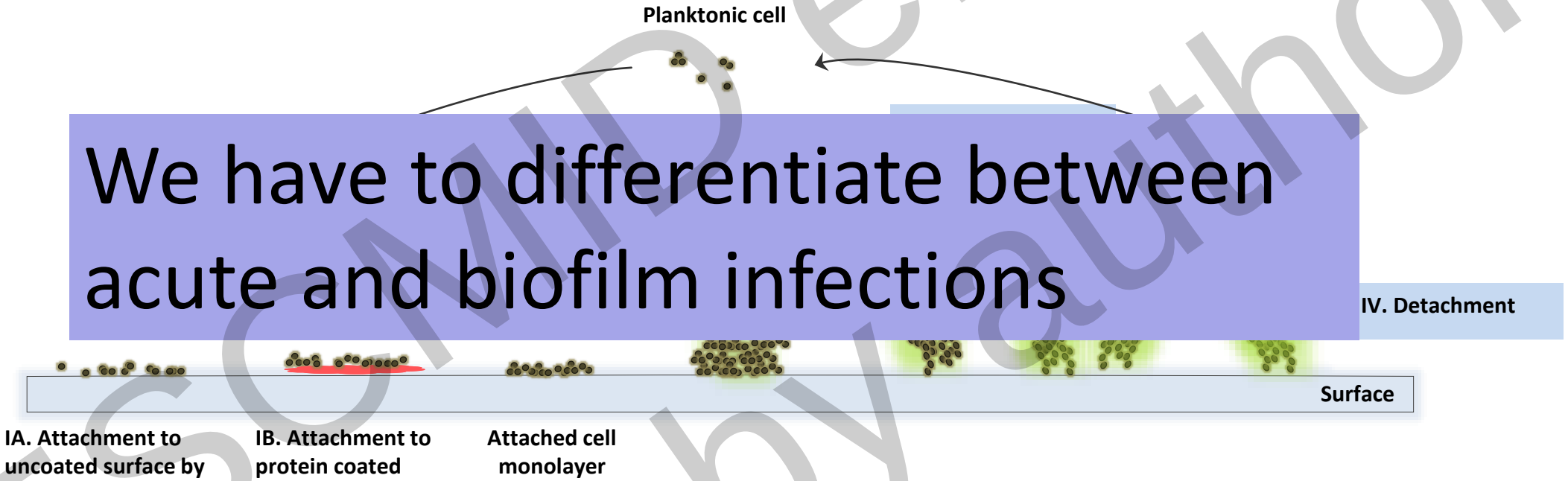






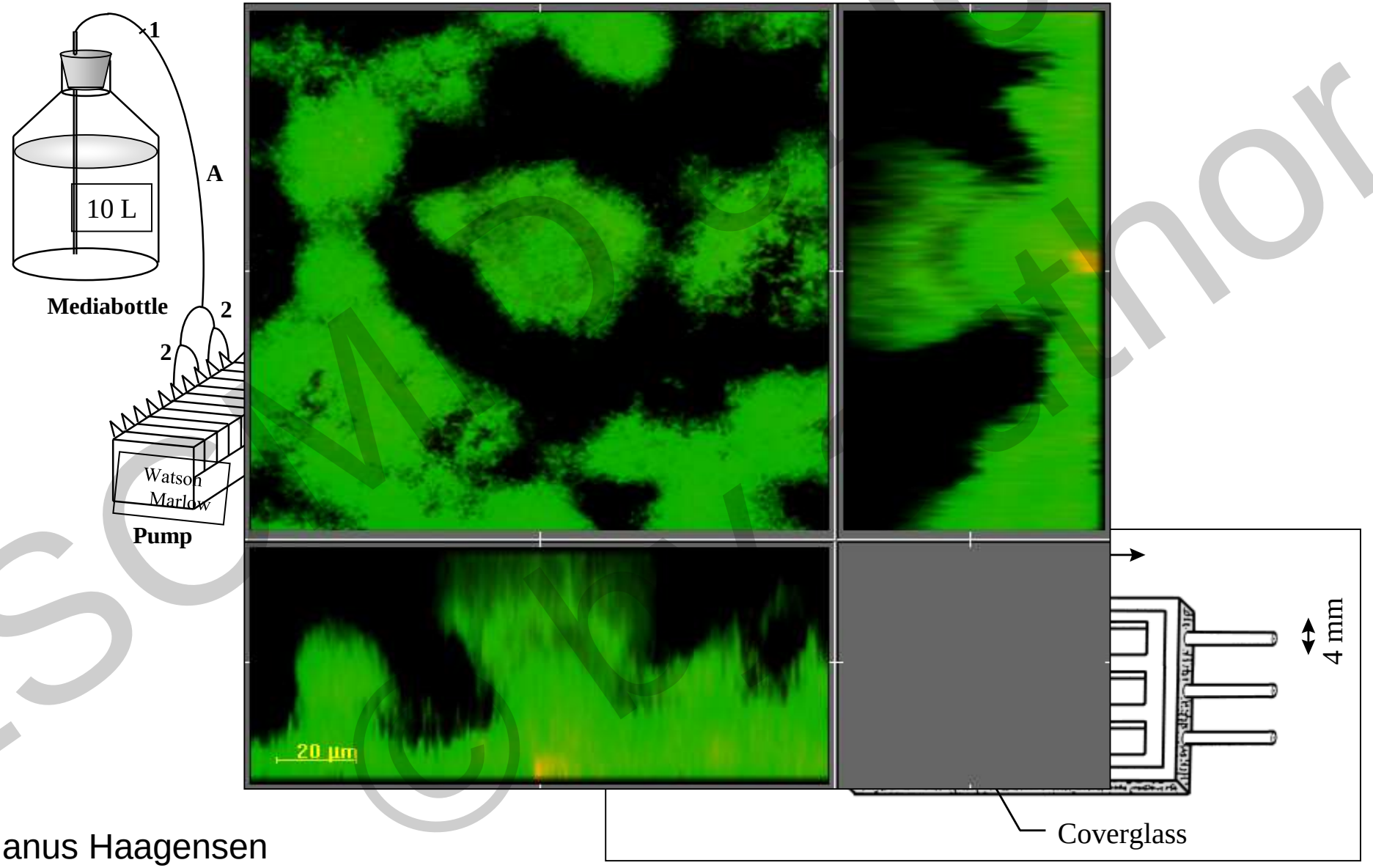
The proces of biofilm formation





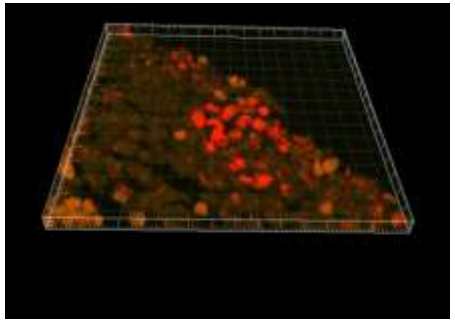
We have to differentiate between acute and biofilm infections

The *in vitro* biofilm

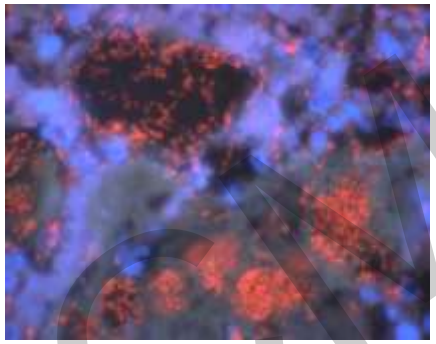


Janus Haagensen

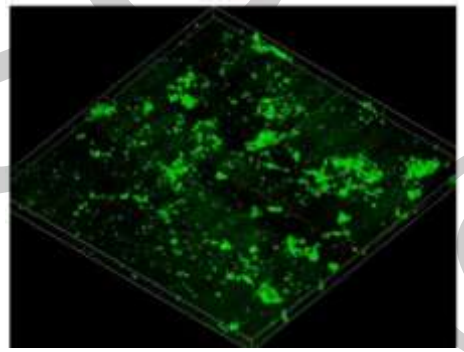
The *in vivo* biofilm



Wound



CF lung



Implant mouse model

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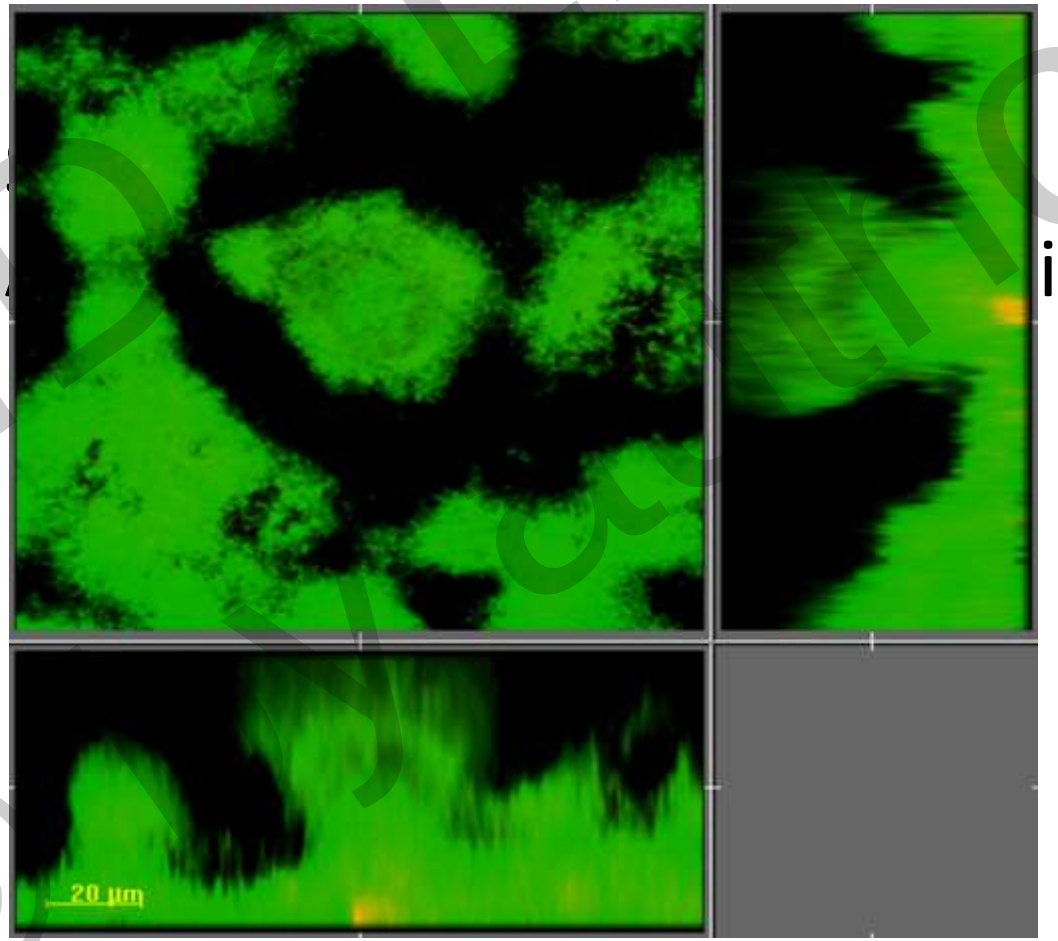


Bjarnsholt et al Trends in Microbiology Trends Microbiol. 2013 Sep;21(9):466-74

Bacterial biofilms

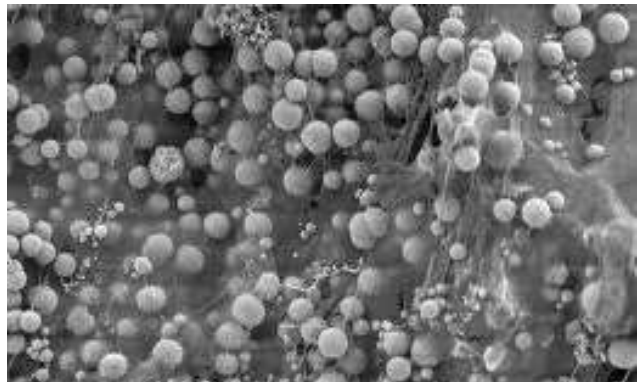
Natural form

Exstracellular matrix

- Polysaccharides, nucleic acid, protein and lipids
- + host component

Higher tolerance towards extremes

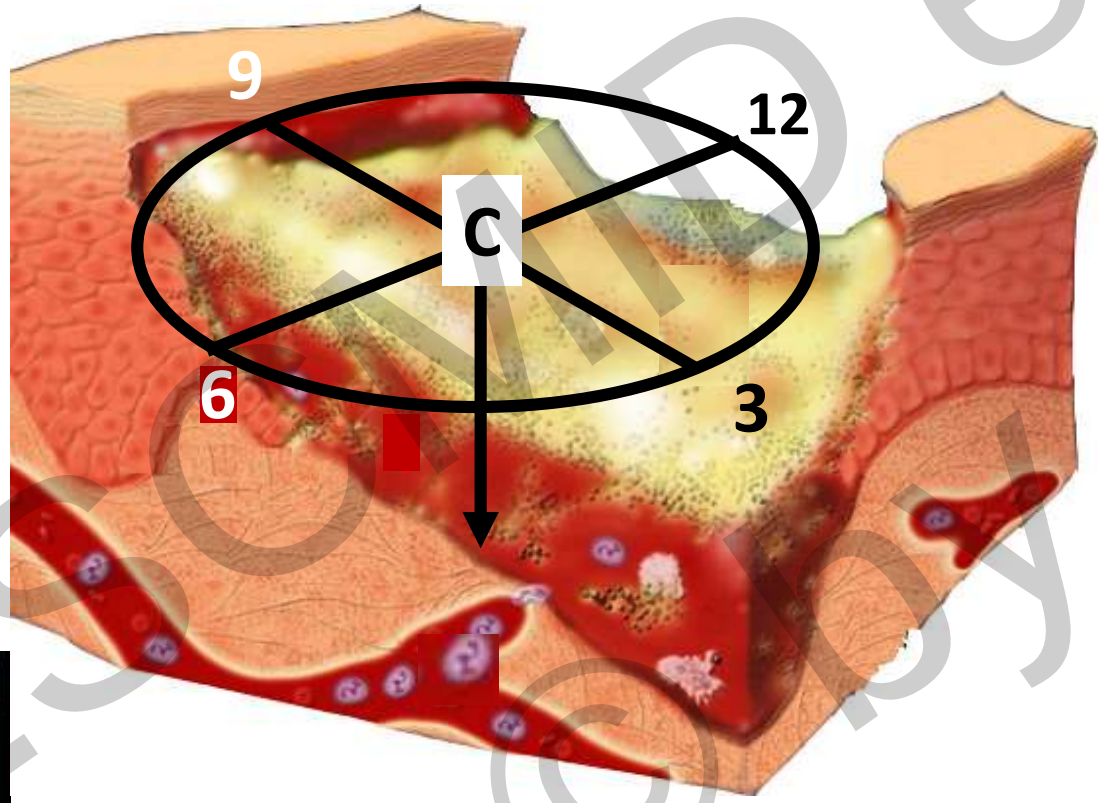
- pH
- Temperature
- Mechanical stress
- Avoidance of the immune defence



MSU center for Biofilm Engieering, Montanna, USA
www.djus.dartmouth.edu

Sampling

Thomsen TR, Rudkjøbing V, et al *WRR*, 2010, **18**(1):38-49.




The fight against biofilm infections:
Do we have the knowledge and means?

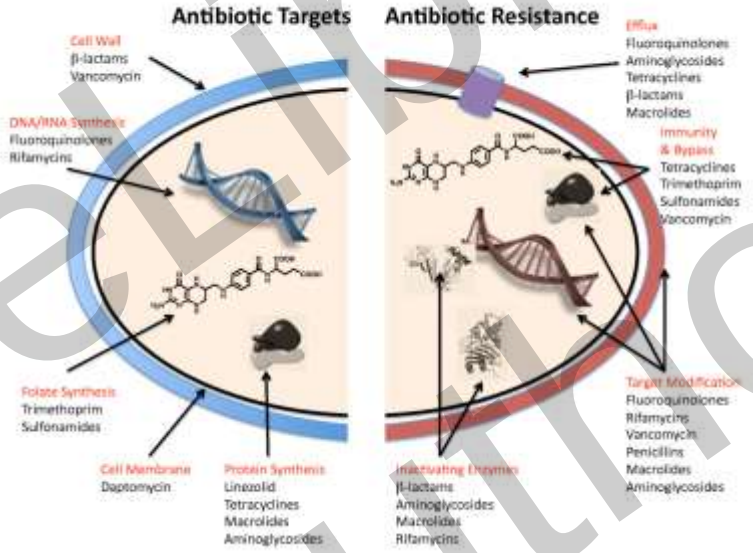
Picture from homepage of Montana State University

qPCR

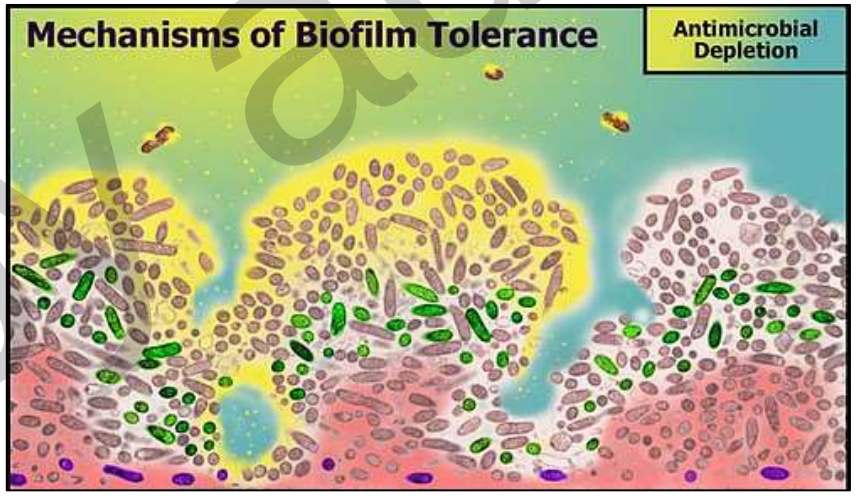
Position	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
C	200±2%	920±9%
3	86±8%	300±13%
6	290±8%	8200±8%
9	80±5%	800±10%
12	93±12%	15±5%

Antibiotic tolerance

Antibiotics have different targets in active dividing bacteria

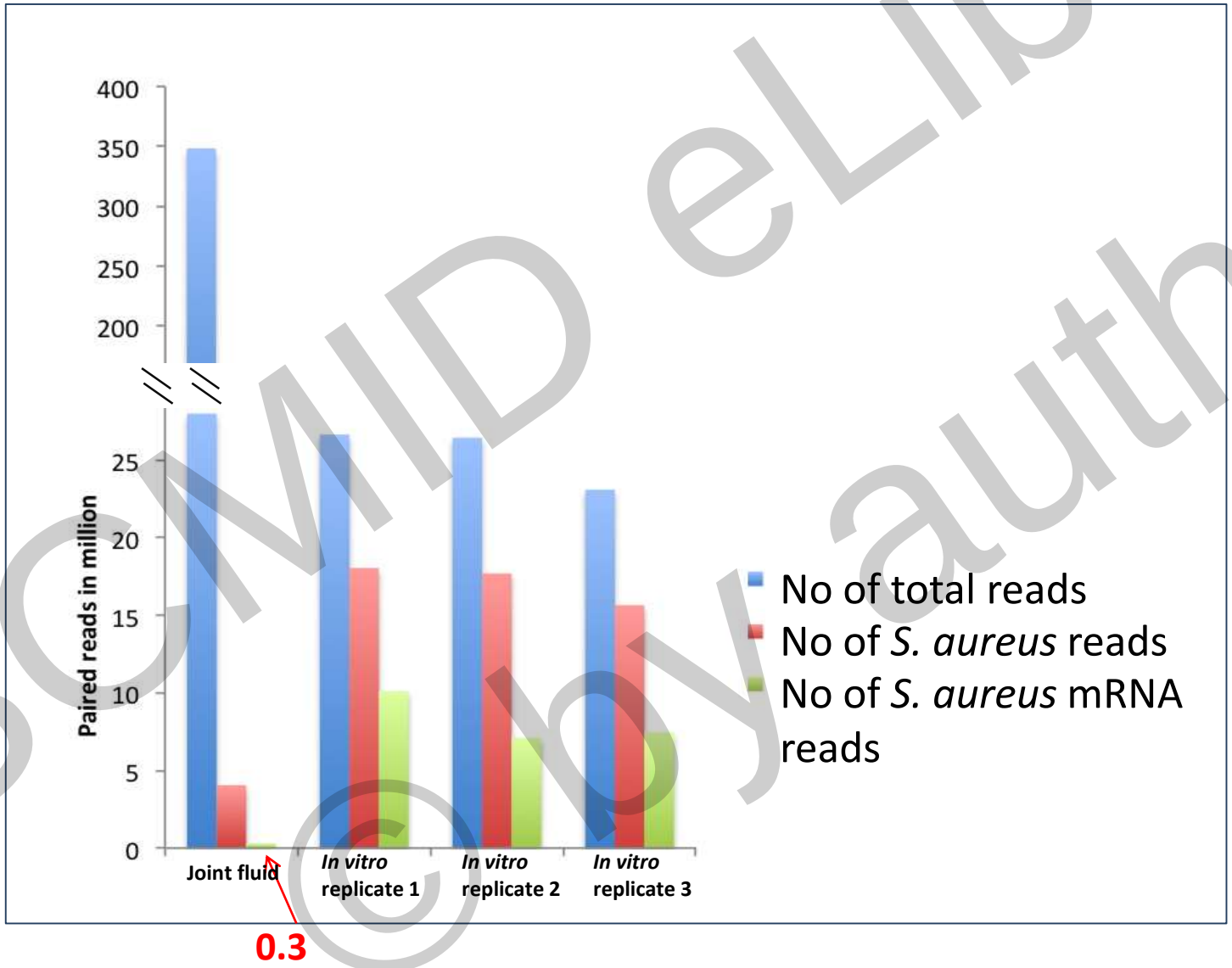


Architecture of the biofilm



MSU center for Biofilm Engieering, Montanna, USA

RNA-seq results



RNA-seq results

Xu et al. BMC Microbiology (2016) 16:80
DOI 10.1186/s12866-016-0695-6

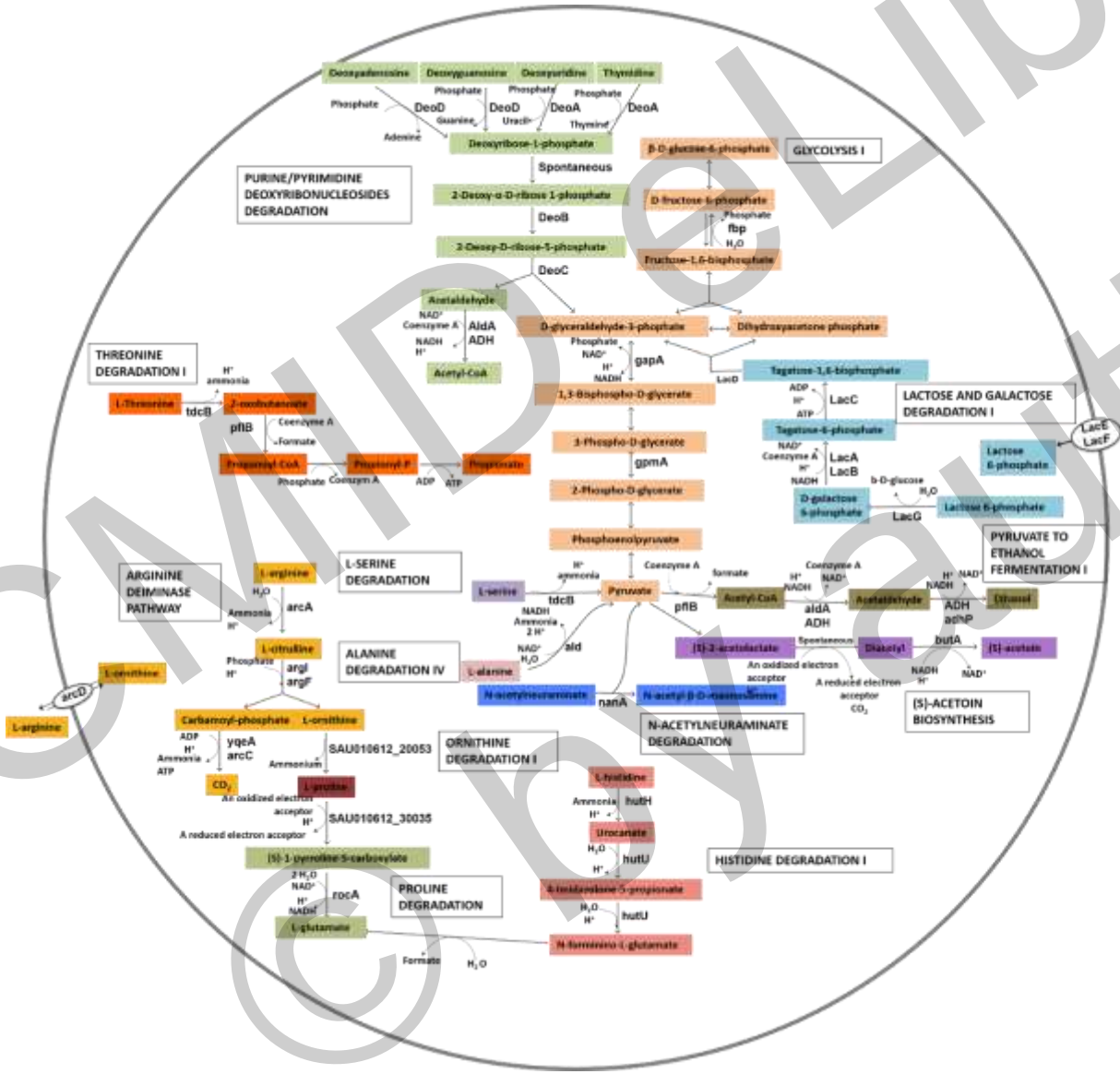
BMC Microbiology

RESEARCH ARTICLE Open Access

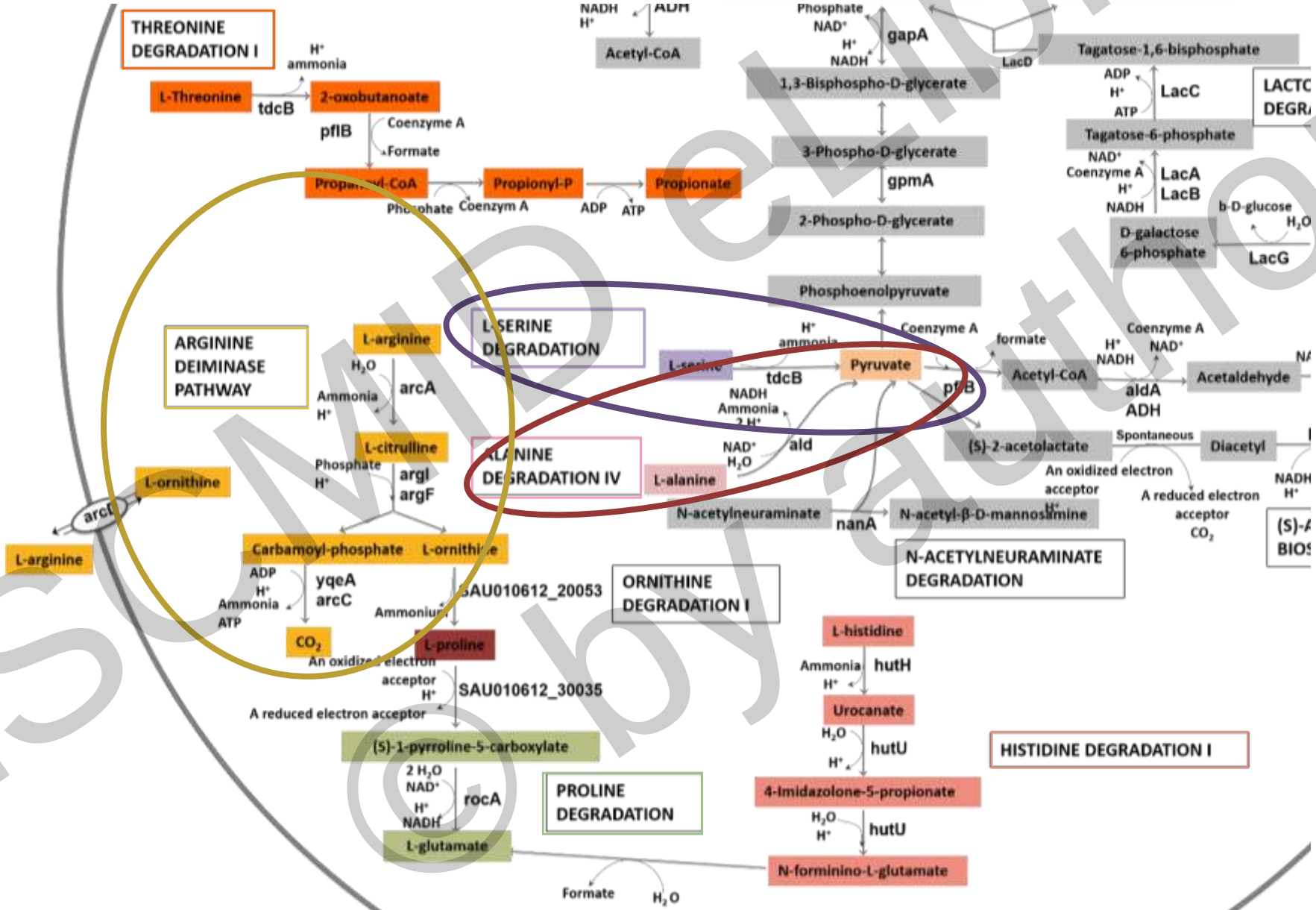
In vivo gene expression in a *Staphylococcus aureus* prosthetic joint infection characterized by RNA sequencing and metabolomics: a pilot study

Yijuan Xu[1,4], Raluca Georgiana Maltesen[1], Lone Heimann Larsen[1,2], Henrik Carl Schønheyder[2,3], Vang Quy Le[5], Jeppe Lund Nielsen[1], Per Halkjær Nielsen[1], Trine Rolighed Thomsen[1,4] and Kåre Lehmann Nielsen[1*]

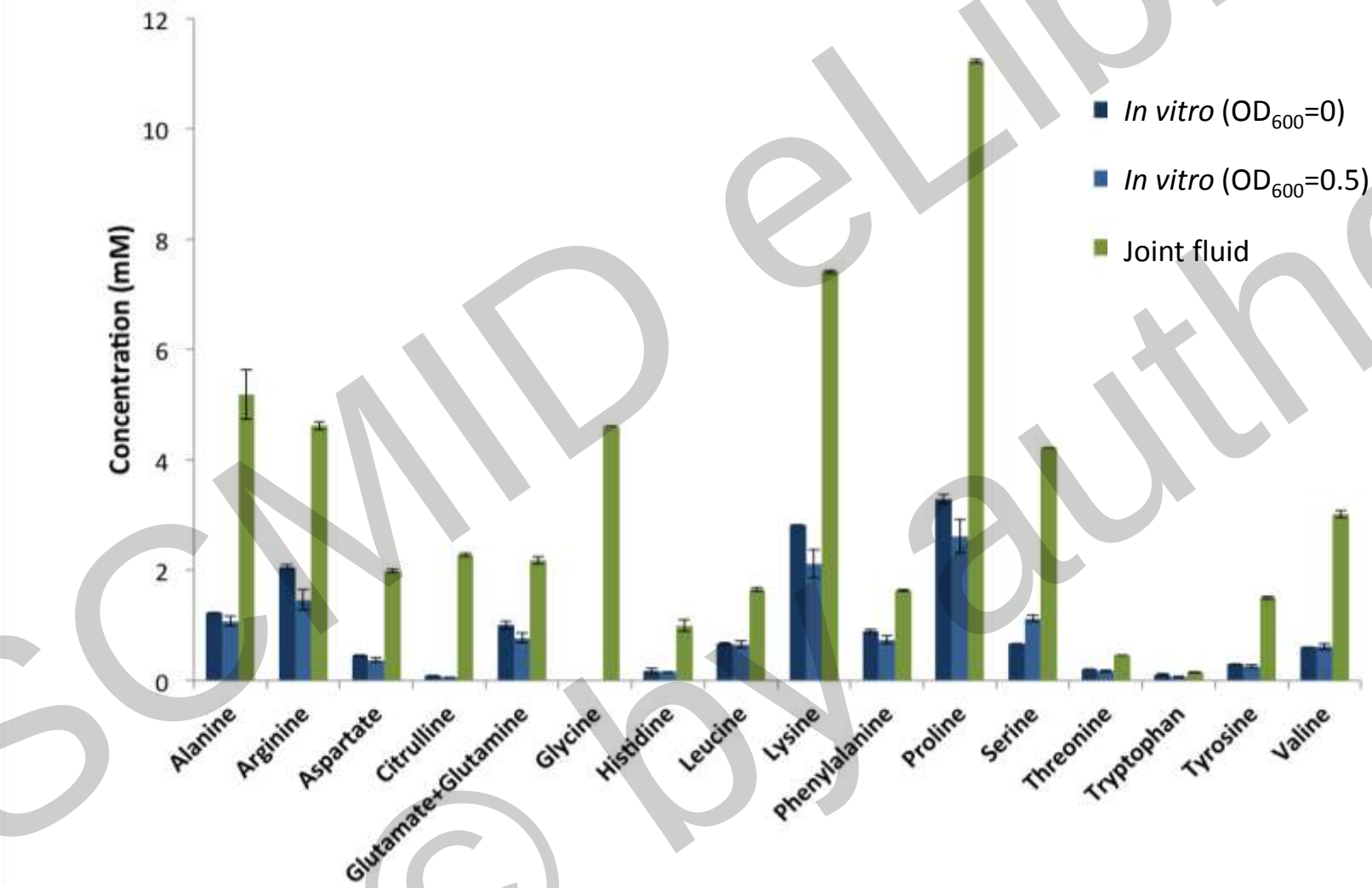
S. aureus metabolism during human PJI



Amino acid degradation

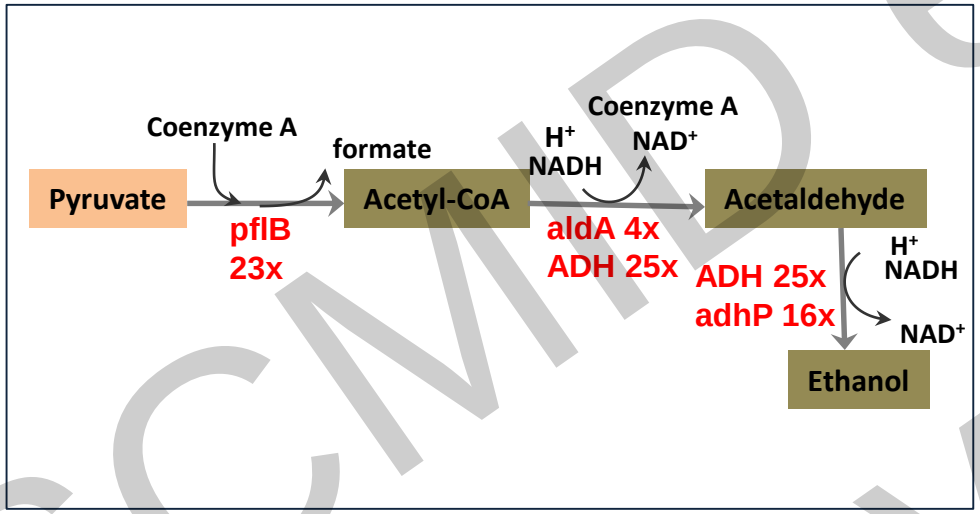


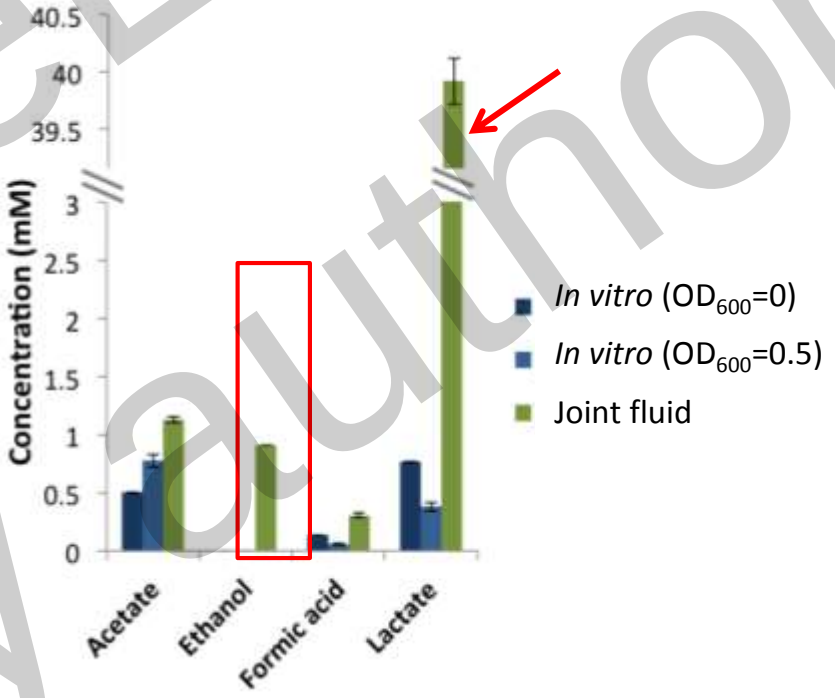
Amino acid concentration



Free amino acids were abundant in the infected joint fluid; food for the bacterial growth

Fermentation



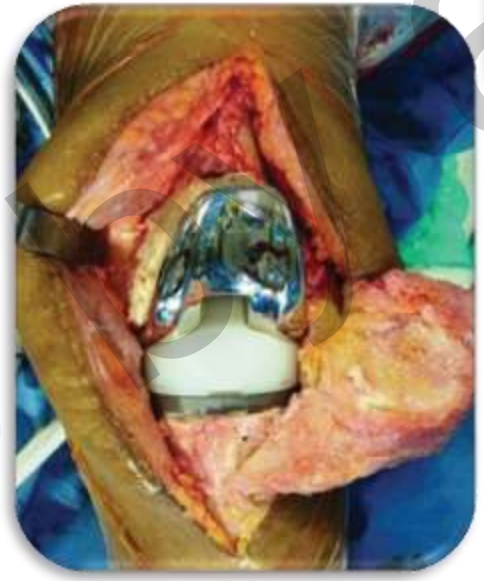
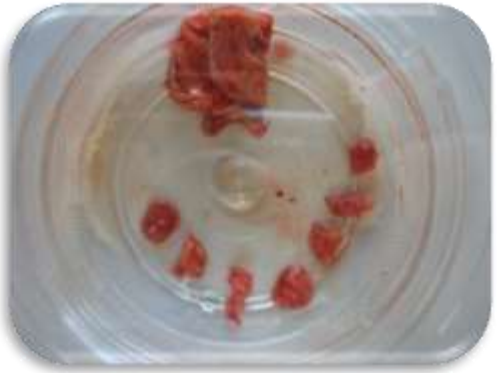


The infected joint was severely hypoxic

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

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