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Christen Ravn Experimental biofilm and additional effects of sonication in diagnosing prosthetic joint infections 2018

Experimental biofilm and additional effects of sonication in diagnosing prosthetic joint infections

PhD thesis

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SDU

Bacteria are ancient and highly adaptive organisms. Biomaterials are new, but they imitate basic substrata for which bacteria, but not tissue cells, have already evolved colonization and survival strategies.

Anthony G. Gristina, 1987[*]

[*] Gristina A G. Biomaterial-centered infection: microbial adhesion versus tissue integration. Science 1987; 237 (4822): 1588-95.

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Preface

The idea of conducting a study on the diagnosis of prosthetic joint infection (PJI) originates from my earliest orthopedic experience at Vejle Hospital, where senior consultants Per Kjærsgaard-Andersen, Peter Revald, Per Wagner Kristensen and Dan Blohm discussed the shortcomings of available culture-based methods. It was increasingly accepted that low-virulent organisms residing in biofilm might be involved in chronic infections with symptoms mimicking aseptic prosthetic loosening. Hence, my interest was to study new microbiological methods for diagnosing PJI.

The clinical research field of PJI was dormant in our region at that time, but with great support, guidance and enthusiasm from Professor Søren Overgaard we defined research questions related to the clinical dilemmas of PJI. Further inspiration was found in the literature and scientific congresses, where bacterial detection by sonication and microcalorimetry were only recently introduced in orthopedic implant revision surgery [29, 224-226]. With scientific support from Prof. Søren Overgaard, Prof. Andrej Trampuz, Prof. Michael Kemp and Ass. Prof. Per Kjærsgaard-Andersen we defined two experimental studies and one clinical study involving the novel modalities, sonication and microcalorimetry. I will always be very grateful for the highly valuable inspiration and support from my four supervisors without which I would never had reached this far!

Research fellowship in Lausanne and Berlin

During almost 5 months fellowship at the research laboratory in Lausanne (February-June and September 2012), I did laboratory training and pilot studies, as well as completed **Study I** and half of **Study II**. I personally, despite orthopedic education, performed the vast majority of laboratory work with supervision and assistance from colleagues in the scientific environment of Andrej Trampuz' research group. This was also true in August 2013, when the laboratory had moved to Charité – University Medicine Berlin, Germany together with Andrej Trampuz entering a professorship at the Center for Musculoskeletal Surgery. I am very grateful for the scientific discussions and laboratory support my co-authors on **Study I** and **Study II**: Ulrika Furustrand Tafin, Inês Santos Ferreira, Elena Maiolo, and Bertrand Betriséy and other colleagues at both institutions.

Clinical study in Odense and Vejle

During one year (November 2012 – October 2013), we sampled prosthetic components after revision surgery in the orthopedic departments of Odense University Hospital and Vejle Regional Hospital. As project manager, I was blinded to the process of inclusion, surgery and microbiological interpretation. During these phases, the study conduct was directly dependent on active participation from the revision surgeons with committed support from project assistants Elisa Knudsen, Annie Gam-Pedersen, Jane Schwarz Leonhardt and Anette Møller Nielsen to whom I also owe great thanks. My project also received essential indirect support from the head of the participating departments, Niels Dieter Röck, Per Wagner Kristensen and Bente Gahrn-Hansen.

Among other collaborators to whom I would like to express my deepest thanks are Anna, Alice, Bjarke Frederik, Helle, Per and Torsten at the PhD-office as well as Jens Kjølseth Møller, Hanne Tange, Marie Ravn Nørgaard, Steen Larsen, Steen Schmidt.

Finally, I would like to thank the most important persons in my life, Kirstine, Oscar and Simon, for your support and patience!

Combining orthopedic surgery and clinical microbiology in one research field obviously calls for further education, which was achieved through active congress-participation in the European Bone and Joint Infection Society, international courses and fellowships – and a lot of literature reading, which I admit also characterize this thesis.

Financial support

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- The Danish Rheumatism Association
- Danish Orthopaedic Society Foundation

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Study I:

Reduced ability to detect surface-related biofilm bacteria after antibiotic exposure under in vitro conditions. Christen Ravn, Ulrika Furustrand Tafin, Bertrand Bétrisey, Søren Overgaard and Andrej Trampuz. Acta Orthopaedica [196]

See Appendix A

Study II:

Microcalorimetric detection of staphylococcal biofilm growth on various prosthetic biomaterials after exposure to daptomycin. Christen Ravn, Inês Santos Ferreira, Elena Maiolo, Søren Overgaard and Andrej Trampuz.

Accepted for publication at Journal of Orthopaedic Research 2018, See Appendix B

Study III:

The additive effect of sonication on bacterial diagnosis in a prospective cohort study of 211 revisions of total hip and knee arthroplasty. Christen Ravn, Michael Kemp, Per Kjærsgaard-Andersen and Søren Overgaard.

Submitted for publication May 2018, See Appendix C

Abbreviations and explanations

THA/TKA/TJA	Total hip/knee/joint arthroplasty	**ESM**	Electronic supplementary material
DHR/DKR	Danish hip/knee arthroplasty register	**ICM**	International consensus meeting
PJI	Prosthetic joint infection	**TSC**	Tissue sample culture
PIOC	Positive intraoperative culture	**SFC**	Sonication fluid culture
DCM	Department of Clinical Microbiology	**OUH**	Odense University Hospital
AsL	Aseptic loosening	**LHV**	Lillebælt Hospital Vejle
DInf	Deep infection	**1st**	One-stage revision
Disl	Dislocation	**2st1**	Two-stage, 1st step
PFx	Periprosthetic fracture	**DAIR**	Debridement and implant retention
LFU	Latest follow-up	**CRP**	*C*-reactive protein

Distinction between deep infection and prosthetic joint infection: These expressions are used to clarify the distinction between the preoperative revision indication (deep infection) and the postoperative diagnostic conclusion (prosthetic joint infection).

Introduction

Total joint arthroplasty

Total hip and knee arthroplasty (THA and TKA) improving the lives of thousands of patients in the active part of their third age are often referred to as successful and cost-effective procedures with few but serious complications [38, 55, 71, 95, 188]. The most common indication for these procedures is osteoarthritis (OA) as reported to the Danish Hip and Knee Arthroplasty Registers [165, 172]. OA is a complex and multifactorial joint condition related to cartilage degradation, and is diagnosed by an overall clinical impression based upon the patient's history, findings on physical examination, and radiographic findings. Treatment options for OA are multiple, but in the disease end-stage, a total joint arthroplasty is often the treatment of choice [88].

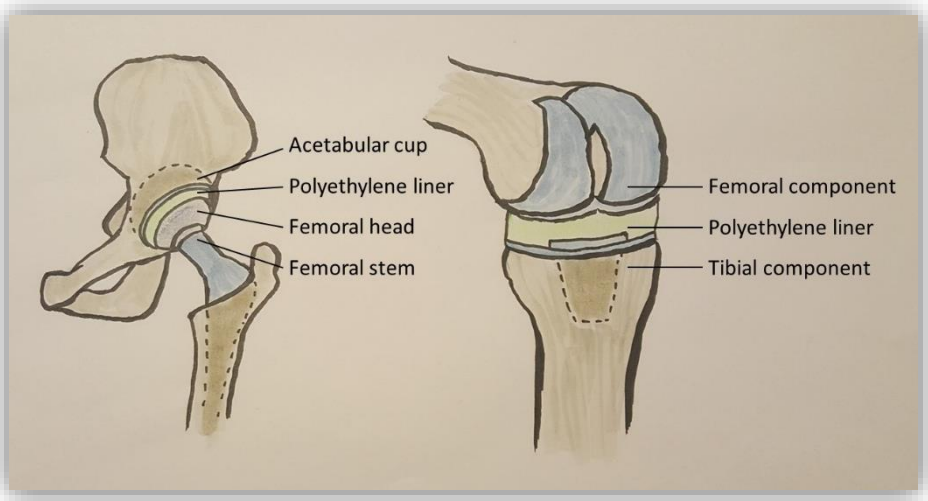


Figure 1: Schematics of typical joint implant devices.
Illustration by Kirstine Kjems 2017

THA and TKA are indicated in patients who have failed non-operative or previous surgical treatment options for a deteriorated hip or knee joint and who continue to have persistent, debilitating pain and a significant decrease in the activities of daily living. With primary total arthroplasty surgery, the diseased articular surfaces of a hip or knee joint are replaced with synthetic materials (Figure 1), thus relieving pain and improving joint kinematics and function. Almost 2/3 of all patients with primary THA or TKA are between 60-79 years of age at the time of operation [122, 209].

The Danish Hip arthroplasty Register (DHR) and Danish Knee arthroplasty Register (DKR) have been monitoring the epidemiology and outcome of hip and knee arthroplasty surgery in Denmark since 1995 and 1997 respectively. In 2014, the total number of patients provided with either primary THA or TKA in Denmark was 17,980, which corresponds to annual incidences of THA in 165/100,000 and TKA in 148/100,000 [165, 172]. The current popularity and availability of these large joint replacement procedures is illustrated by the marked increase in annual incidence over a decade from 114/100,000 THA and 90/100,000 TKA in 2004 [144, 145].

Revision surgery

The Danish arthroplasty registers' reported 10-year prosthesis survival rate is approximately 92%. In 2014, the number of revisions performed in Denmark were approximately 1400 THA and 1200 TKA [165, 172].

Whereas the long term revision indications mainly include aseptic loosening and component failure, revisions performed during the first couple of years after implantation are more often due to complicating reasons such as instability, dislocation and deep infection [18, 24, 140, 190].

With increasing numbers of primary procedures performed annually over the last decade, there are expectations of an increasing number of patients with a need for prosthetic revision in the coming years [99, 129, 162, 163, 181, 183]. The 5-year prosthesis survival rate after first revision due to any indication is approximately 87%, but the 5-year prosthesis survival rate is only about 55% for first time revisions indicated by deep infection [172].

As reported to DHR in 2014, deep infection was the third most common indication of index revisions of THA, whereas aseptic loosening was more frequent (Table 1). This proportion is turned upside down at subsequent revision numbers. Deep infection is the most common indication with 42% of second revisions and above, whereas aseptic loosening constitutes 22% [172].

Revision indication	THA	
	1st revision	≥2nd revision
Aseptic loosening	33%	22%
Dislocation	21%	18%
Deep infection	13%	42%
Periprosthetic fracture	12%	5%
Component failure	4%	2%
Pain	5%	3%
Other indications	12%	9%

Table 1: Revision indications at first and subsequent revisions of THA
Reported to the Danish Hip Register in 2014 [172].

The change in relative infection rate from primary to secondary arthroplasty surgery and subsequent revisions may reflect an increasing risk of perioperative contamination with longer and more complicated revision operations. In addition, soft tissues covering the prosthetic joint may be increasingly damaged with each surgery. Finally, the consequence of an unsuccessfully treated PJI is most often another round of revision surgery [109].

Interestingly, the reported indication rates have changed markedly over the last 18 years in DHR, as the cumulative reporting of re-revision indications from 1995-2012 was indicated by 38% of aseptic loosening and 31% of deep infection [172]. Similar findings have been reported from the Nordic Arthroplasty Register Association [56]. Whether these changes in reported infection incidence are reflecting a true and worryingly increase in the relative number of septic revisions or simply reflects behavioral changes among surgeons reporting to the national registers is still to be elucidated [94].

Prosthetic joint infection

Prosthetic joint infection (PJI) remains one of the most serious complications of TJA as it causes significant morbidity and account for a substantial proportion of health care expenditures [83, 100, 118, 130, 159, 236, 253, 255]. From a Danish register study, the 'true' incidence was estimated 1.03% of surgically treated deep infection with 5 years follow-up after primary THA [93], whereas register studies from Finland indicate a 1.3% rate of revision due to deep infection in primary TKA [107]. High risk of re-infection and re-revision after treatment for PJI was recently reported in a large cohort of TKA [141]. Furthermore, some reports have stated that suffering a PJI is a significant risk factor of one-year mortality [42, 255].

Risk factors related to deep infection

Many register-based studies have identified risk factors for revision due to deep infection (Figure 2). They may be related to male gender, diabetes, obesity and elevated comorbidity scores [56-58, 111, 184, 185, 231, 245].

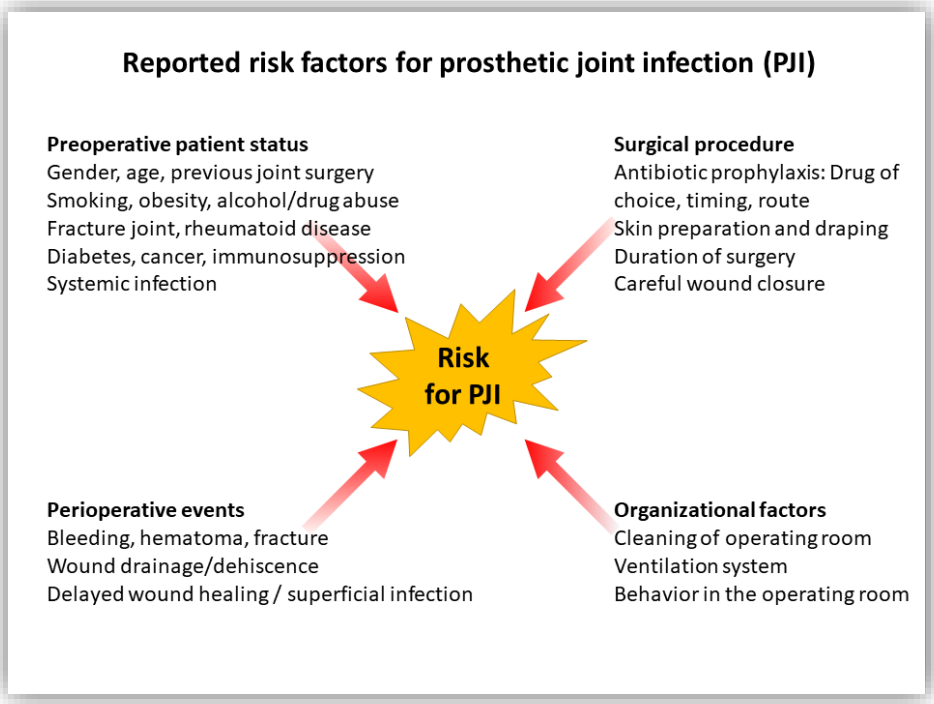


Figure 2: Reported risk factors for PJI and microorganisms

Illustration by C. Ravn 2017, based on data from various sources [17, 56, 58, 110, 117, 120, 185, 189, 214, 215, 234, 243, 245]

The underlying joint diagnosis leading to the indication of primary TJA is also influencing the risk of prosthetic joint infection (Figure 2). Compared to osteoarthritis there is a significant risk of deep infection after primary THA due to rheumatoid arthritis and hip fracture [56, 61, 185, 208]. In addition, previous surgery to the same joint, and especially TJA revision surgery, is related to increased risk of developing deep infection [109, 120, 139].

Factors in the perioperative setup may also influence the risk of subsequent PJI (Figure 2). This includes cleaning and ventilation of the operating room, behavior of the personnel present in the operating room, patient hygiene and use of antibiotic prophylaxis [41].

Diagnosing prosthetic joint infection

PJI is difficult to diagnose, still a correct and timely diagnosis is a crucial step for planning the treatment. Determination of a TJA as being infected traditionally depends on recovery of a microorganism from the joint [253]. Even though sampling and culture of periprosthetic tissue biopsies is cornerstone in the microbiological diagnostics, the sensitivity reportedly is as low as 50-80%, leaving a substantial proportion of cases without a bacterial diagnosis and antibiogram [75, 155, 186, 205, 225]. For more details on conventional sampling methods and microbiological diagnostics, please see 'Appendix H: Periprosthetic tissue samples and synovial fluid'.

Due to lack of a gold standard for PJI, the diagnosis is often defined by a combination of objective criteria [253], though no indisputable definition of PJI has yet been achieved [103]. A multi-criteria reference standard is recommended, since it is believed that the composite reference standard has better discriminatory properties than each of the reference standard components in isolation [169, 170, 198]. The International Consensus Meeting (ICM) on PJI in 2013 suggested the following definition of PJI (Table 2):

PJI is present when one of the major criteria exists or three out of five minor criteria exist	
Major criteria	Two positive periprosthetic cultures with phenotypically identical organisms, OR
	A sinus tract communicating with the joint, OR
Minor criteria	1) Elevated serum C-reactive protein (CRP) AND erythrocyte sedimentation rate (ESR)
	2) Elevated synovial fluid white blood cell (WBC) count OR ++change on leukocyte esterase test strip
	3) Elevated synovial fluid polymorphonuclear neutrophil percentage (PMN%)
	4) Positive histological analysis of periprosthetic tissue
	5) A single positive culture

Table 2: Definition of PJI according to ICM-2013
From 'Proceedings of the International Consensus Meeting on Periprosthetic Joint Infection' [85].

With 85% agreement in the delegate vote, the consensus of this definition was classified as 'strong'. The following consideration was also stated in the proceedings: 'Clinically, PJI may be present without meeting these criteria, specifically in the case of less virulent organisms (e.g. *P. acnes*)'. Another concern with this definition is that paraclinical investigation of periprosthetic histopathology, synovial fluid levels of white blood cells and polymorphonuclear neutrophil percentage may not be available at all institutions performing revision surgery [128].

The newly introduced synovial fluid leukocyte esterase test strip was also adopted as minor criterion. ICM-initiator, Javad Parvizi, took place in the Rothman Institute-based study group publishing the primary studies on this method [178, 221].

Clinical presentation

The starting point of the diagnostic strategy is clinical suspicion, which may include the symptomatology of an acute infection, namely elevated skin temperature, pain, redness, swelling and fever. It is well

documented that the majority of PJI are reported to the arthroplasty registers within the first couple of postoperative months [56, 93, 108, 131, 142]. These 'early' infections are acquired either during implantation or in the early postoperative period, and often accompanied by elevated acute phase reactants, wound healing problems, and even drainage [168]. Acute symptoms of PJI may also develop as a result of haematogenous seeding from a recent infection or bacteremia at any time after surgery. Haematogenous infections are characterized by a sudden onset of pain in the affected prosthetic joint after a relatively asymptomatic period [168, 233].

When low-grade infections may develop several months to years following the prosthetic implantation at which they were probably initiated, they can also be classified as 'delayed' or 'chronic'. The slow progression of pain and other vague clinical signs may be difficult to distinguish from aseptic loosening and thus lead to a delayed diagnosis of infection. Ultimately, these infections may give rise to sinus tract formation (Figure 3) and/or implant loosening [168, 249].

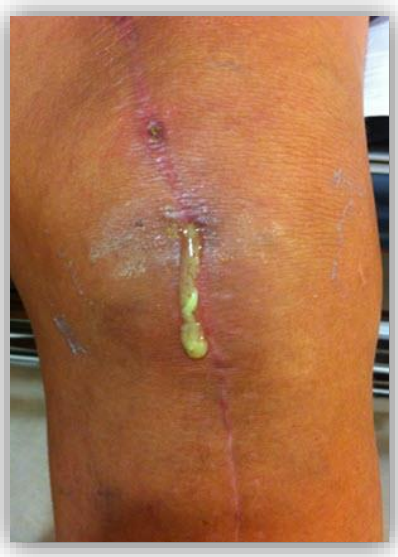


Figure 3: Sinus tract in a prosthetic knee.
A pathognomonic feature of chronic PJI [64].

Microbiological challenge with biofilm bacteria

Isolation of the causative organism is central in PJI diagnostics. To fully understand the diagnostic challenge of this approach, it is important to understand the different growth conditions of bacteria in the planktonic (free-floating cells) and biofilm (sessile aggregates) life forms [20]. Infection that is associated with TJA is typically caused by microorganisms that grow in biofilms [253]. The concept of bacterial biofilms is often referring to J. W. Costerton's definition from his Science-review:

> 'A structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface'[54]

Whereas the planktonic mode of growth is characterized by high metabolic activity and rapid replication requiring a higher nutritional supply, biofilm bacteria are less metabolically active and may switch to a

stationary (dormant) growth phase. Furthermore, the biofilm phenotype is characterized by decreased susceptibility to antimicrobial agents and higher tolerance against the immune response [21].

The biofilm phenotype can be considered as an evolutionary strategy for bacteria to survive in unfavorable conditions, but is also a response to local growth conditions. Because of this resilience of biofilm bacteria, implant infections may persist despite lack of clinical symptoms [97].
Culture-based diagnostics depend on recovery of the pathogen in the growth medium during the incubation period, but starvation and antimicrobial pressure has been demonstrated as inducers of a 'viable but non-culturable' (VBNC) state, which again complicates detection of implant-related infections [137, 180].

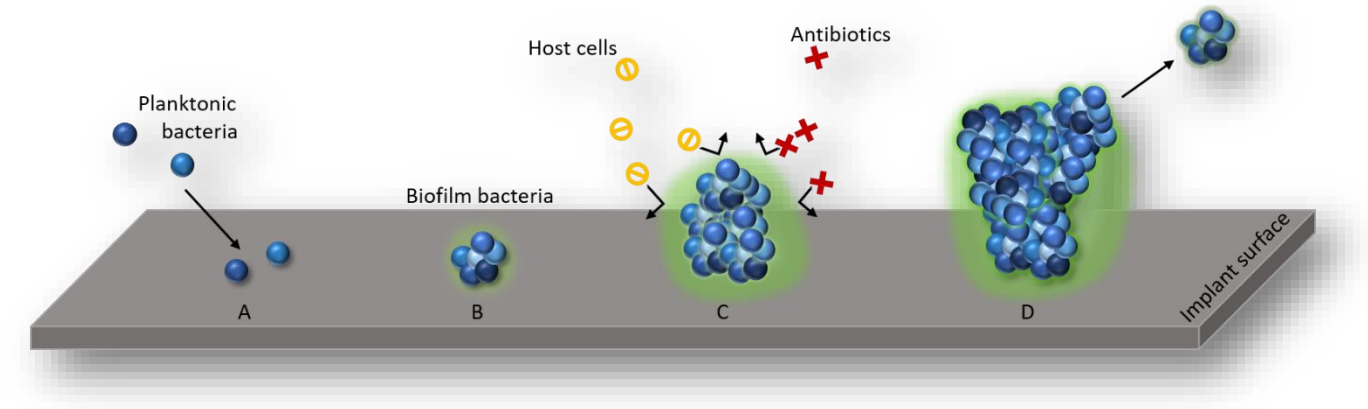


Figure 4: Biofilm formation
A: Attachment of free-floating bacteria to the implant surface. B: Bacterial replication and immature biofilm formation (green color). C: Biofilm maturation and increased tolerance against host immune cells and antibiotics. D: Biofilm dispersal. Illustration by C. Ravn 2017, inspired by Stoodley *et al* [219]

Biofilm growth on orthopedic biomaterials

Development of biofilm is a major challenge in implant-related infections [52]. Biofilm formation (Figure 4) is a multifaceted interaction between bacterial, biomaterial and host factors. Of these 3 factors, the biomaterial factors are most available for preventive measures [59]. Therefore, many experimental studies have investigated the effects of physico-chemical surface preparations on tissue cells and/or bacterial adhesion [8, 12, 14, 33, 46, 48, 63, 76, 121, 126, 171, 194, 206, 213, 240, 241, 244, 246].

Presence of a biomaterial surface in the body exponentially enhance bacterial virulence [252]. In a classic study by Elek and Conen it was demonstrated that the infecting bacterial inoculum could be as low as 100 staphylococci when a silk suture acted as foreign body in healthy human volunteers. This was >100.000-fold lower than in the absence of foreign material [68].

Implants used in orthopedic arthroplasty surgery include (Figure 5)

- Metals: titanium (and its alloys), stainless steel, and cobalt-chromium
- Polymeric biomaterials: ceramics, hydroxyapatite, and polyethylene
- Bone cement: polymethyl-methacrylate (PMMA)

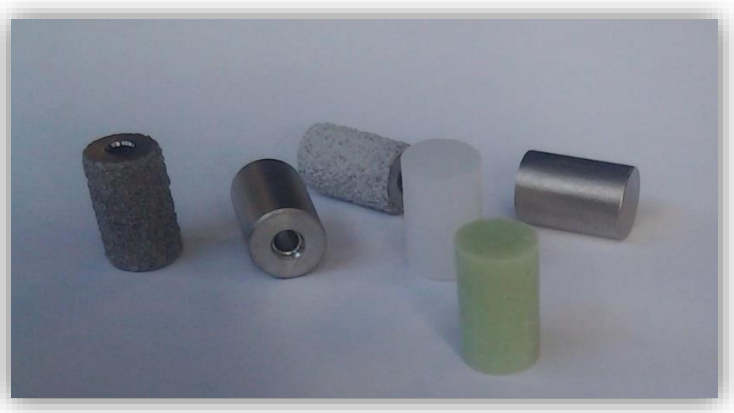


Figure 5: Cylindrical samples of biomaterials commonly used in orthopedic surgery. Left-right: Porous coated titanium, cobalt-chrome, hydroxyapatite covered titanium, polyethylene, polymethyl-metacrylate, and stainless steel (extract from **Study II**)

These biomaterials are aimed at maximizing mechanical properties, minimizing material deterioration and facilitating long-term incorporation of the implants into the musculoskeletal system [242]. In terms of biocompatibility cellular adhesion or physicochemical integration is required, weather bony anchorage of the TJA is provided by cementation or osteointegration [91]. These fundamental principles both apply to tissue integration and microbial adhesion [92].

It is suggested that the fate of an available surface may be conceptualized as a 'race for the surface', which is a contest between tissue cell integration and bacterial adhesion to that same surface. If the race is won by tissue, then the surface is occupied and defended and is thus less available for bacterial colonization. [92]. The articulating parts of the joint prosthesis are modular constructions often composed of a plastic liner and smooth metal surfaces. These surfaces will after implantation be covered by a thin layer of collagen tissue, which is less protective of biofilm formation [164].

The sonication-culture method

Sonication of explanted joint prostheses has been suggested to increase the culture yield and sensitivity in biofilm-associated infections [225]. The mechanism is based on ultrasonic fluctuations of waves that produce high- and low-pressure areas on the surface of a solid body submerged in liquid solution (Figure 6). During the low-pressure stage, millions of microscopic vapor bubbles are formed in the biofilm (cavitation), which collapse (implosion) during the high-pressure stage releasing an enormous amount of energy on the surface, e.g. of a prosthetic component [223].

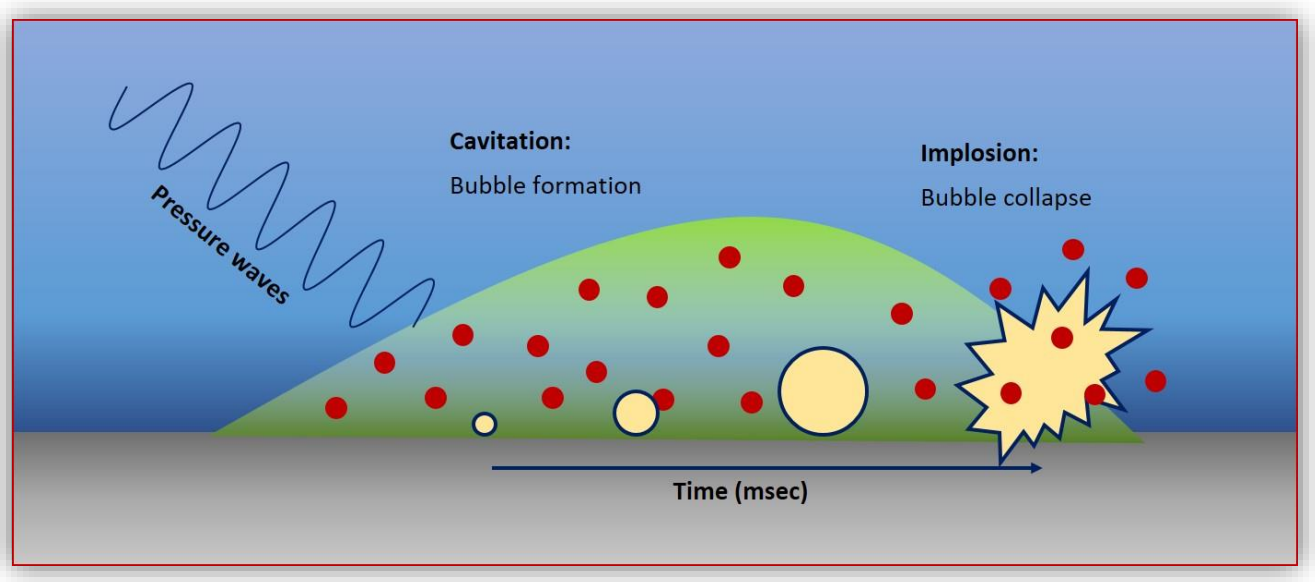


Figure 6: Mechanism of sonication on surface-related biofilm bacteria
Bacteria are red and biofilm is green.
Illustration by C. Ravn 2017 as described by Trampuz *et al* [223]

Recently, a meta-analysis evaluated twelve studies on sonication of prosthetic components for diagnosis of PJI [248], but no consecutive, large series have compared sonication fluid culture against conventional culture of five periprosthetic tissue samples from THA and TKA revision surgery since the groundbreaking study of Trampuz et al [225]. For more details on the sonication-culture method, please see 'Appendix G: Sonication procedure'.

Diagnostic modalities not routinely used

Other diagnostic modalities that are not routinely used, may also contribute to the investigation for PJI. Molecular techniques include direct microscopy and polymerase chain reaction (PCR) with detection of bacterial 16S DNA [98]. Diagnostic imaging includes a large spectrum of modalities including plain radiography, ultrasonography scan, computed tomography scan, magnetic resonance imaging, scintigraphy, single photon emission computed tomography and positron emission tomography [173, 230, 237]. Finally, a long list of serum and synovial biomarkers has been suggested [72, 87].

Principles in management of PJI

It is not possible to eradicate PJI by antibiotics alone

In implant-associated infections, spontaneous healing does not occur, and antibiotic treatment alone faces several challenges. Biofilms complicate infection treatment because surface properties of many implanted biomaterials are inviting for bacterial adhesion. The implant surface is devoid of microcirculation, which is crucial for delivery of systemically administered antibiotics, and dormant bacteria are less susceptible to bactericidal antibiotics that are dependent on bacterial cell division [52, 102]. Thus the antibiotic concentration necessary to kill biofilm bacteria is significantly higher (often unachievably) than for the planktonic counterparts [251]. For this reason PJI typically show recurring symptoms, after cycles of antibiotic therapy, until the biofilm embedded bacteria are surgically removed from the body, i.e. revision surgery [53].

PJI involves surgical treatment if eradication of the infection is the goal

Revision surgery involves thorough debridement of infected soft tissues as well as partial removal or complete removal of the TJA. The optimal strategy for removal and re-implantation is widely debated and may be individual to the situation [119, 132, 136, 138]. Current concepts include one-step exchange, two-step exchange and partial exchange of the prosthetic implants (debridement and implant retention).

Other treatment concepts include resection arthroplasty (no re-implantation), exarticulation (amputation) or chronic antibiotic suppression therapy. Due to the disabling consequences of these latter treatment options, they are mostly suggested when the patient already suffers from significant comorbidity or has undergone unsuccessful previous attempts of prosthetic re-implantation [253].

Study motivation

PJI is one of the most feared complications to TJA. This condition, indicating almost every third TJA revision, is not only potentially disabling to the patient, but also very expensive in health economic perspectives. A correct and timely diagnosis of PJI is crucial to the patient as well as to the health care professionals taking care of the patient. In the clinical situation with the patient, it is not only a question of presence or absence of deep infection. The bacterial diagnosis is also relevant in order to facilitate the antimicrobial treatment.

The motivation of this PhD study was to improve the diagnostic approach to PJI. The overall purpose was to diagnose microorganisms in experimental and clinical biofilm studies. We respectively address the influence of antibiotic exposure, implant material and clinical presentation on the ability to detect surface-related biofilm bacteria.

Purpose

Study I:

The primary aim of this experimental study was to determine the antimicrobial susceptibility of tested planktonic and biofilm bacteria in order to correlate biofilm detection with antibiotic exposure. The secondary aim was to investigate the effect of antibiotic exposure on detection of biofilm bacteria by sonication, culture, and microcalorimetry. We specifically investigated the effects of individual antibiotic agents inhibiting the growth of planktonic and biofilm bacteria. Microcalorimetric measurement of heat flow was compared with conventional viable counting of dislodged biofilm bacteria.

Study II:

The primary aim of this *in vitro* study was to test the efficacy of daptomycin to eradicate staphylococcal biofilms on various orthopedic implant surfaces and materials. The secondary aim was to quantitatively estimate the formation of staphylococcal biofilm on various implant materials with different surface properties. We hypothesized that bacterial growth and biofilm formation on prosthetic materials would vary with the different surface properties, and also be variably influenced by exposure to the potent antimicrobial effect of high-dose daptomycin.

Study III:

The primary aim of this clinical, cohort study was to investigate the additive effect of the sonication-culture method on diagnosis of bacteria in revision TKA and THA in relation to the categorization of PJI as either verified, possible or non-existing. The secondary aim was to describe the fate of prosthetic joint revisions, which had additional microbial findings in sonication fluid culture.

Methodological considerations

The relation between implant-related biofilm infection, microbiological diagnostics and antibiotic susceptibility is of interest. Seen from an orthopedic point of view this also relates to the challenge of prosthetic joint infection. Our primary intention was to conduct a study on the diagnosis of PJI by investigating novel diagnostic culture-based modalities in order to, hopefully, reduce the diagnostic uncertainty. At the time of protocol-writing, bacterial detection by sonication and microcalorimetry were only recently introduced in orthopedic implant revision surgery [29, 224-226].

Experimental studies I and II

We formulated two experimental study protocols to investigate two basic research questions in the light of diagnostic challenges in microbial PJI diagnostics:

1. How does various antibiotic exposure affect the ability to detect biofilm bacteria by sonication and microcalorimetry (**Study I**)?
2. Are sonication and microcalorimetry capable of demonstrating biofilm formation on various prosthetic materials (**Study II**)?

In relation to clinical research, an experimental study design is mainly suitable for direct manipulation of certain variables while controlling others to observe any direct reflections on the outcome. Compared with the use of laboratory animals or human subjects, repeated experiments are easily performed under in vitro conditions.

Study I, was designed as a susceptibility test of relevant combinations of bacteria and antibiotics commonly involved in PJI. Biofilms of four bacteria commonly causing PJI (*S. aureus*, *S. epidermidis*, *E. coli* and *P. acnes*) were formed on porous glass beads during 24-hour incubation [197]. We evaluated the effect of exposure to different antibiotics, typically used in the treatment of PJI, namely vancomycin, daptomycin, rifampin, flucloxacillin, or ciprofloxacin. This method of biofilm formation and antimicrobial exposure was adopted from previous studies [78, 250] as well as currently running or scheduled pharmacodynamic studies [10, 51, 147, 154, 166].
While controlling the combination of bacterial and antibiotic concentrations, triplicate experiments were performed. Growth medium was supplemented with $CaCl_2$. Thorough washing of the biofilm-covered beads after incubation was instituted in order to avoid carry-over of planktonic bacteria, and sonication fluid underwent centrifugation before plating of the dissolved pellet in order to avoid carry-over of excessive antibiotics. Outcome after direct incubation of sonication fluid was +/- bacterial growth, as well as quantification of the number of colony forming units (CFU) by serial dilution and viable counting (Figure 7).

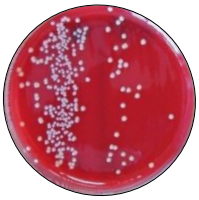


Figure 7: Dilution of sonication fluid with *staphylococcus epidermidis*
After exposure of to 8 mg/µL vancomycin, biofilm bacteria were dislodged by sonication and the sonication fluid was diluted $1{:}10^3$ (left) and $1{:}10^4$ (right) for viable counting (from **Study I**)

We also measured the microbial heat flow in sonication fluid by isothermal microcalorimetry, since initial pilot studies had confirmed the inverse relationship between microbial quantity and delayed regrowth of dislodged biofilm bacteria (Figure 22, see Appendix F: Isothermal microcalorimetry). Furthermore, the minimal heat inhibitory concentration (MHIC) was defined as the lowest antibiotic concentration killing bacteria on the beads, and equivalent to the minimal inhibitory concentration (MIC) of free-floating planktonic bacteria. Comparisons between MIC and MHIC was evaluated as an indication of the altered antimicrobial susceptibility in biofilm bacteria.

In **Study II**, staphylococcal biofilms were formed on various orthopedic implant surfaces and materials. As in **Study I**, sonication and microcalorimetry was used to estimate the biofilm formation and evaluate the efficacy of daptomycin to eradicate the biofilm bacteria on the surface of various biomaterials (Figure 8). In **Study II**, the biomaterial samples were custom made in a cylindrical shape that fitted into the calorimeter ampoules. Hence, real-time measurement of heat flow came directly from the remaining biofilm on the surface of each sample after antibiotic exposure. This protocol was inspired by a study of biofilm formation on bone grafts and substitutes published from the same institution one year before [48], and several times thereafter [46, 47, 76].

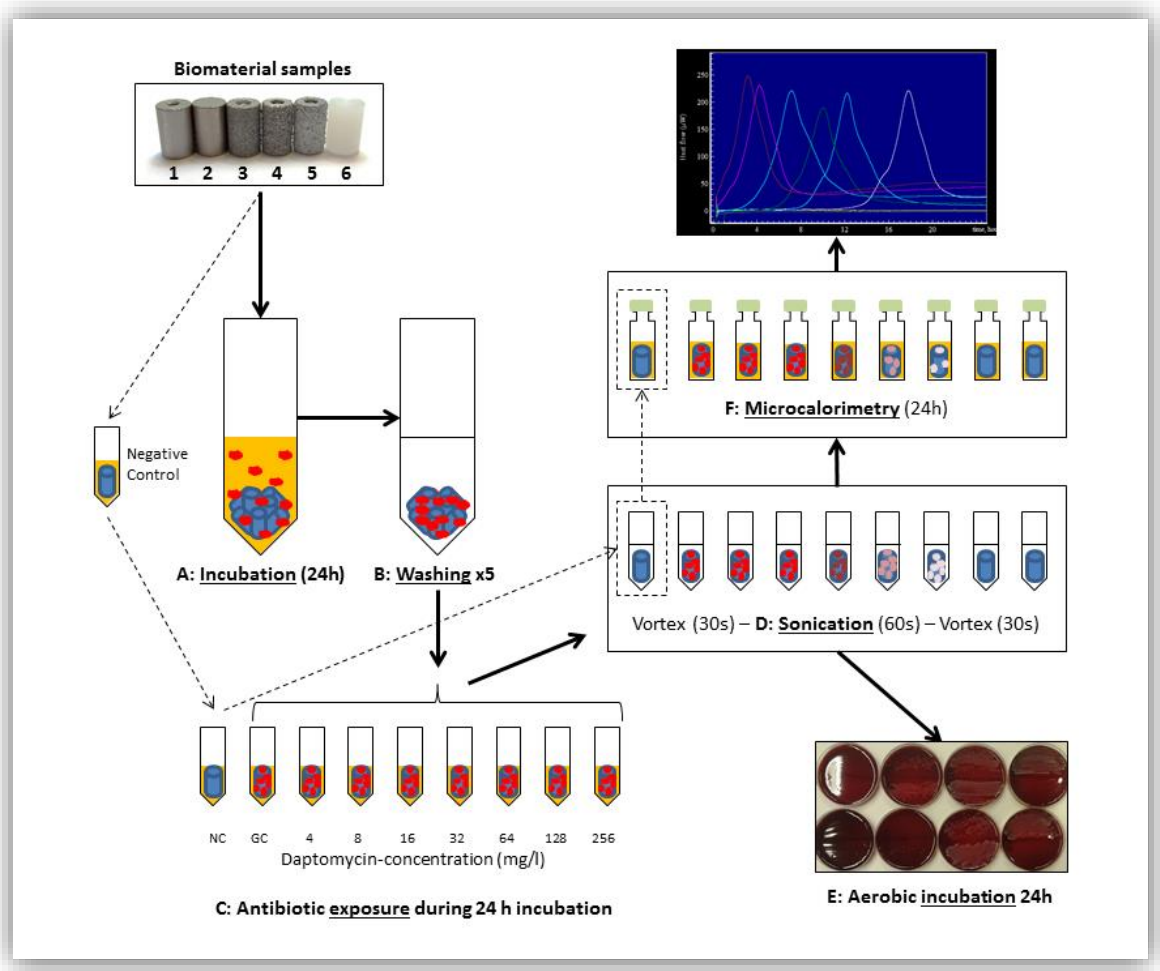


Figure 8: Schematic overview of the methods in Study II
Experiments with cylindrical samples of typical orthopedic implant materials covered in staphylococcal biofilm

Limitations of experimental study design

In order to answer the research question in a clinical context, our study design obviously lack important in vivo conditions such as antimicrobial pharmacokinetics (dose, tissue penetration, repeated administration, duration of treatment), host immune response and the artificial porosity of glass beads not necessarily reflecting commonly used prosthetic material. Furthermore, bacterial strains used in these studies are all laboratory strains (ATCC), whereas in the clinical context, bacteria isolated from a PJI might have behaved differently under antibiotic pressure. However, including clinical strains would most likely have introduced more experimental variability. In addition, they may not be well characterized. If using clinical isolates, a large number of strains would have needed to be included for each bacterium, in order to exclude strain-specific results. Therefore, we chose to use biofilm-forming ATCC strains of different bacterial species typically causing PJI, which also represent different virulence patterns, metabolism, and Gram-stainability.

For further description and discussion of the experimental methods in **Study I and II**, please see

- Appendix A: Manuscript in study I
- Appendix B: Manuscript in study II

Clinical Study III

This prospective cohort study was designed to improve internal validity through sampling of well-defined data in a limited time-period with no other modifications of the clinical microbiology approach. Regional scientific authorities and the Danish Data Protection Agency approved the study protocol (Ref: 2012-41-0826). The cohort study is reported according to the STROBE guidelines (www.strobe-statement.org).
We believe that involvement of both regional and university hospitals in this study may have improved the external validity. We evaluated a new diagnostic approach for PJI, but due to lack of an appropriate reference standard, we could not reasonably design a diagnostic accuracy study.

Inclusion criteria

We included all revisions of THA and TKA regardless of the indication. Hence, patient experience included both biomechanical discomfort due to instability, malposition, repeated dislocations or even periprosthetic fracture, and inflammatory conditions related to debris and septic necrosis [50]. One could ask, 'Is this comparison of apples and oranges?' and 'What is the significance of a microbial finding after revision for expected mechanical failure?'
With inclusion of the whole revision cohort, we were independent on preoperative interpretation of the possible pathogenesis leading to indication for revision surgery. We believe that the microbiological methods would detect bacteria where bacteria are present. If the pathogenesis is truly aseptic, these revisions may serve as negative control. On the other hand, a high number of aseptic revisions dilutes the prevalence of the target disease, PJI.

Data sampling and documentation

One dedicated project coordinator at each institution performed case identification and collection of checklists. Clinical data were prospectively sampled on standardized checklists (Appendix E) by the revision surgeons during the one-year study period (2012.11.01-2013.10.31), though no screening or inclusion was conducted during two weeks of summer holiday (2013.07.11-2013.07.26). Data were electronically entered in an online database (Topica, hosted by the Region of Southern Denmark) by independent data-managers.

Composite reference standard

Study III was conducted immediately before the International Consensus Meeting (ICM) suggested a new multi-criteria definition of PJI (Table 2). In a communication from the ICM chairpersons, the intention was later expressed as an 'aid to clinicians in their effort to diagnose and treat PJI' [177]. In addition, 'the availability of a standardized definition for PJI will allow a meaningful comparison of the medical literature reporting on issues related to PJI'.

We did not include analysis of synovial leukocyte count and periprosthetic histopathology that are listed under minor criteria in the ICM-definition. These tests were not routinely performed at our institutions and we found that lack of experience might impose interpretative uncertainty rather than clarity. Finally, diagnostic thresholds of these two culture-independent investigations are not clearly defined despite widespread use in the literature [62, 112, 127, 160, 207, 222, 232].

We have instead formulated a composite reference standard of *Verified PJI* based on multiple readily available tests that are also included in the ICM- and other definitions [177, 249, 253]. A diagnosis of PJI is

verified when one of four situations are identified (Table 3). A sinus tract communicating with the prosthetic joint (soft tissue criterion) has been considered pathognomic for PJI in research literature for decades [9, 37, 169, 253, 254]. Also, positive culture of ≥3/5 tissue samples with identical organisms (culture criterion) have been considered synonymous with PJI since Kamme and Lindbergs study was published in 1981 [116]. In our reference standard, another culture criterion for verified PJI was defined as positive culture of identical organisms in synovial fluid and 2/5 tissue samples. This criterion is motivated by the non-validated diagnostic threshold for positive TSC (≥2/5 tissue samples) in more recent literature and international guidelines [40, 142, 177, 179, 193]. Lastly, the diagnosis of PJI is 'verified' according to our composite reference standard, when a combination with three out of four minor criteria are present. These minor criteria are motivated by the PJI-definition presented by the American Musculoskeletal Infection Society in 2011 [179].

Verified prosthetic joint infection (either of the following four situations are identified)
1. Presence of a sinus tract communicating with the prosthetic joint, **OR** 2. ≥3 identical microbial findings in culture of 5 tissue samples, **OR** 3. Identical microbial findings in culture of 2/5 tissue samples AND 1 synovial fluid sample, **OR** 4. Combination of at least three of the following four minor criteria: - Microbial growth in ≥1 periprosthetic tissue samples, **AND/OR** - Culture of identical microorganism in synovial fluid, **AND/OR** - Purulence of synovial fluid or implant site, **AND/OR** - Elevated plasma C-reactive protein in blood test, threshold below i. 0-90 days postoperative: CRP > 100 mg/L ii. >90 days postoperative: CRP > 10 mg/L (not fracture, rheumatoid disease, cancer)

Possible prosthetic joint infection (at least two of the following eight observations are identified)
- Microbial growth in ≥1 periprosthetic tissue samples, **AND/OR** - Culture of a microorganism in synovial fluid, **AND/OR** - Purulence of synovial fluid or implant site, **AND/OR** - Elevated plasma C-reactive protein in blood test (threshold below), **AND/OR** i. 0-90 days postoperative: CRP > 100 mg/L ii. >90 days postoperative: CRP > 10 mg/L (not fracture, rheumatoid disease, cancer) - Painful AND warm/red prosthetic joint with acute onset (recent 0-3 weeks) , **AND/OR** - Suppurating wound defect 14 days after previous TJA surgery, **AND/OR** - Antibiotics administered for ≥2 days within 2 weeks prior to prosthetic revision surgery - Previous revision indicated by deep infection within last year

Non-infected prosthetic joint at revision surgery (all of the following conditions are fulfilled)
- No sinus tract communicating with the prosthetic joint, **AND** - No postoperative suppurating wound defect 14 days after previous TJA surgery, **AND** - Not warm, red and painful prosthetic joint, **AND** - No purulence of synovial fluid or implant site, **AND** - Negative OR single-positive culture of synovial fluid and tissue samples (5 biopsies)

Table 3: Composite reference standard for classification of revision THA and TKA.

In the first category, any of the above-mentioned three situations characterize verified PJI. In the second category, a prosthetic joint is suspicious of PJI when a combination with at least two observations are present. In the third category, a prosthetic joint is regarded as non-infected, when all conditions are fulfilled.

Based on clinical observations and the ICM-consideration, that PJI may be present without meeting the multi-criteria definition, we suggest second category of *Possible PJI*, categorized by further combination of observations (Table 3). Clinical suspicion of PJI arouses when more than one of eight observations are identified, including positive culture, macroscopic purulence, elevated p-CRP, prolonged wound defect with dehiscence, recent debut of articular inflammatory symptoms (painful AND warm/red prosthetic joint), recent antibiotic administration, or history of revision indicated by deep infection. This intermediate category corresponds to the clinical dilemma of difficult-to-diagnose situations where the treatment strategy may be difficult establish [98, 170]. Finally, the prosthetic joint is considered *Non-infected* at revision surgery, when five negative conditions are concurrently fulfilled (Table 3).

It is essential for evaluation of a diagnostic test, accurately to be able to identify patients with and without the target condition tested for [198, 203]. However, this is unaccomplished for PJI. Thus, with three infection-categories and an unclear definition of true and false positive/negative cases, it is not meaningful to estimate the diagnostic accuracy in **Study III**.

Though not directly communicable with other diagnostic studies of PJI, we find a composite reference standard based on best-practice modalities relevant for evaluation of the additive effect of the sonication-culture method, since this is the setting in which implementation would take place.

Routine microbial sampling and interpretation

Concerning the conventional culture methods, a detailed method description can be seen in 'Appendix H: Periprosthetic tissue samples and synovial fluid'. We applied a diagnostic threshold of ≥3 identical microbial findings in EITHER five periprosthetic tissue samples OR positive TSC 2/5 combined with identical finding in one synovial fluid sample after five days incubation. This threshold was adopted from the local guidelines, as it represents a compromise between the threshold of ≥3/5 culture-positive biopsies in the classical validation studies [9, 116] and the ICM-threshold of ≥2/5 [177, 253]. If the diagnostic threshold is too conservative, false negative results may occur, whereas lowering of the diagnostic threshold would obviously increase the risk of false positive culture results due to contamination.

In case of clinical suspicion of PJI, synovial fluid is often sampled preoperatively in order to confirm the diagnosis and plan the surgical strategy [62, 74, 133, 174, 207, 216, 251]. Furthermore, direct sampling of intraarticular culture material is sometimes desirable before initiation of preoperative antibiotic treatment in septic patients awaiting revision surgery. Despite sterile technique, preoperative synovial sampling may be a risk factor for intraarticular contamination, just like contamination of the synovial sample may impose interpretation flaw. Hence this is not a routine procedure, especially not when revision indication is regarded as aseptic. Finally, aspiration is not always achieved due to inadequate percutaneous technique or low intraarticular volume, this condition is often referred to as 'dry tap' [5, 125, 167, 176, 216, 221, 227]. Regardless of preoperative sampling status, we sampled synovial fluid intraoperatively before arthrotomy, and in accordance with the ICM-consensus, cases of dry tap was NOT substitute to saline injection.

Another concern related to the conventional microbial sampling is handling of the specimens after collection. Based on a systematic literature review by a Danish research group, the evidence base for optimizing culture methods for diagnosis of PJI have been investigated [133]. The most pertinent issue identified were lack of independent evaluation of results related to the culture process. At both hospitals of our study group, clinical guidelines ensured fast delivery from the operating room to the local department of clinical microbiology, as well as incubation procedure, microbial enumeration and species identification.

Prolonged incubation time (14 days) was discussed during protocol writing, since Schäfer, *et al* in 2008 had described relevant bacterial findings of low-virulent microorganisms (e.g. *P. acnes*, *Corynebacterium* sp.) after 5 days culture in non-quantitative thioglycollate [205]. From a clinical point-of-view, we found validation of this approach inadequate at the beginning of the study period, and therefore kept the culture-procedure unchanged. Occasionally though, prolonged culture was anyway performed due to clinical decision in the study period, but only data of the predefined 5-day culture period was analyzed in the study.

The sonication procedure

A detailed description of the sonication-culture method can be viewed in 'Appendix G: Sonication procedure'. This method, like other culture-based methods, entails a potential risk of contamination, but the prosthetic components were immediately transferred to a sterile, airtight plastic-box upon explantation. The box was transported to the department of clinical microbiology, where it was only opened twice (under laminar airflow): first, when saline was filled, and second, upon sonication fluid sampling. Hence, we do not consider this procedure more risky of contamination than the conventional method of tissue sample inoculation. We did on the other hand not perform additional centrifugation of the sonication fluid and cultivation of the sediment as previously suggested [70, 90, 104], since we found this extra step too labor-intensive and risky of contamination to include in the present study.

The distance between Lillebælt Hospital Vejle (LHV) and Odense University Hospital (OUH) is 80 km and sonication only took place at the DCM at OUH. From LHV a service car transported the prosthetic components in the morning after revision surgery, except in weekends when the transport-related delay was up to 3 days. Until sonication was undertaken in the DCM, the prostheses were stored in an airtight plastic box with no water or nutrients and kept in the fridge (5°C). We do not know how these conditions affected the potential bacteria on the prosthetic surface, but it has been hypothesized that depletion of nutrients in the interior of the biofilm can lead to starvation-induced dormancy, which could possibly induce false negative sonication-culture results [153, 182]. On the other hand, delayed sonication could lead to multiplication of contaminating microorganisms in the tissue remnants on the prosthetic surface, which would potentially induce false positive results.

We incubated sonication fluid in 0.2 mL aliquots on agar plates in aerobic and anaerobic conditions for 5 days, which corresponds to the incubation time for conventional samples in this study. Additionally, one 0.2 mL aliquot was incubated in thioglycollate broth and discontinued after 14 days. The latter was inspired by other reports of prolonged incubation of sonication fluid [39, 90]. In other studies, the diagnostic threshold of SFC varies between 5-100 CFU/mL [101, 133, 143, 248]. We used a pragmatic threshold at 20 CFU/ml,

which is equivalent with finding of minimum 4 CFU on the agar plate after incubation of 0.2 mL sonication fluid. Still to this date, validation of this cutoff remains.

Postoperative antibiotic treatment

Empirical antibiotic treatment with dicloxacillin (cefuroxime in case of penicillin allergy) was administered for maximum 5 days from the day of revision. As soon as the results of TSC were analyzed with antibiogram a targeted antibiotic treatment was initiated. In culture-negative cases with revision indicated by expected deep infection, dicloxacillin was typically administered intravenously for 2 weeks followed by 4 weeks of oral treatment.

Follow-up

Revision patients were routinely seen in the outpatient clinic after 3 and 12 months, where clinical examination and any antibiotic treatment were documented. Furthermore, the medical records were additionally checked for information related to infection treatment or subsequent reason to re-operation of the arthroplasty joint.

Statistical methods

In **Study I** and **Study II**, experiments were performed in triplicate and descriptive statistics were used to express median and range of MHIC and MBEC. Heat flow (μW) and time (hours) are continuous numeric data presented as mean ± standard deviation (SD)

In **Study I**, non-parametric comparisons of antimicrobial susceptibility in 3 Gram-positive microorganisms were based on the multiplication factors and they were performed by Wilcoxon signed-rank test using Prism 7.0 (GraphPad Software, San Diego, CA, USA). Direct comparison with the MHIC of ciprofl oxacin in Gram-negative bacteria (E. coli) was deemed unsuitable.

In **Study II**, continuous data were compared between groups by standard (non-parametric) ANOVA. Post-hoc mutual comparisons were performed by unpaired t-test and Wilcoxon test adjusting for multiple comparisons using the statistical software R (www.R-project.org) and Prism 7.0 for comparison of mean HFP and mean TTD-50 of S. aureus and S. epidermidis biofilms. P-value <0.05 was accepted as significant.

In **Study III**, data are presented with numerical values and percentage of total. Concordance rate is the percentage of similar microbial findings in conventional samples and SFC relative to the number of verified PJI-cases. Descriptive statistics were applied.

Ethical consideration

In our investigation of the additive effect of sonication on the bacterial diagnosis in revision of THA and TKA, our comparator was culture of conventional samples of periprosthetic tissue and synovial fluid. The sonication procedure only involved the prosthetic components that were otherwise discarded upon removal, and revision surgeons were blinded to the result of sonication fluid culture. Hence, the clinical course was not directly influenced by the study conduct. This was also the opinion of the local ethical committee, which replied that formal approval was not required.

Summary of results

Study I

In an experimental setup, we investigated the suppressive effect of five relevant antibiotics (vancomycin, daptomycin, rifampin, flucloxacillin and ciprofloxacin) on the growth of four different planktonic and biofilm bacteria commonly causing PJI (*Staphylococcus aureus*, *S. epidermidis*, *Escherichia coli* and *Propionibacterium acnes*). After antibiotic exposure, beads were washed and sonicated. Sonication fluid was subject to microcalorimetric analysis as well as culture with viable counting.

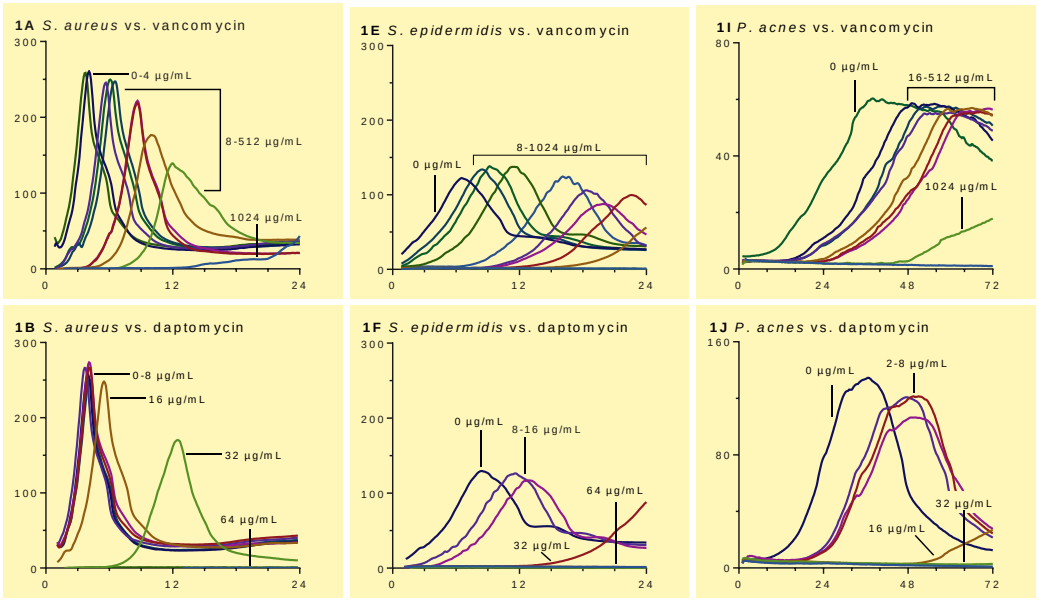


Figure 9: Heat flow over time (extract from Study I)

Heat flow (Y-axis, µW) development over time (X-axis, hours) of *S. aureus* (A-B), *S. epidermidis* (E-F) and *P. acnes* (I-J) exposed to vancomycin and daptomycin. The numbers above each curve indicate the respective antibiotic concentrations. X- and Y-axis range is different with *P. acnes* (72h, 160 µW). Biofilms on beads previously not exposed to antibiotics (0 µg/mL) represent the positive controls. Experiments were performed in triplicate and a representative experiment is shown.

In triplicate experiment, vancomycin did not inhibit the heat flow of staphylococci and *P. acnes* at concentrations ≤1024 µg/mL (Figure 9). Daptomycin inhibited heat flow of *S. aureus*, *S. epidermidis* and *P. acnes* at lower concentrations (32-64 µg/mL).

Organism	Vancomycin		Daptomycin		Rifampin		Flucloxacillin		Ciprofloxacin	
	MIC	MHIC	MIC	MHIC	MIC	MHIC	MIC	MHIC	MIC	MHIC
S. aureus	1	*>1024*	0.5	64	0.007	8	0.25	128	ND	ND
S. epidermidis	2	*>1024*	1	64	0.007	8-16[a]	IR	IR	ND	ND
E. coli	IR	IR	IR	IR	IR	IR	IR	IR	0.015	0.063-0.25[a]
P. acnes	1	*>1024*	1	32	0.007	8-16[a]	ND	ND	ND	ND

Table 4: Antimicrobial susceptibility of test organisms (Study I)

Results are expressed as minimal inhibitory concentration (MIC) for non-adherent, planktonic bacteria and as minimal heat inhibitory concentration (MHIC) for biofilm bacteria. Numbers are µg/mL. IR, intrinsic resistance. ND, not done.

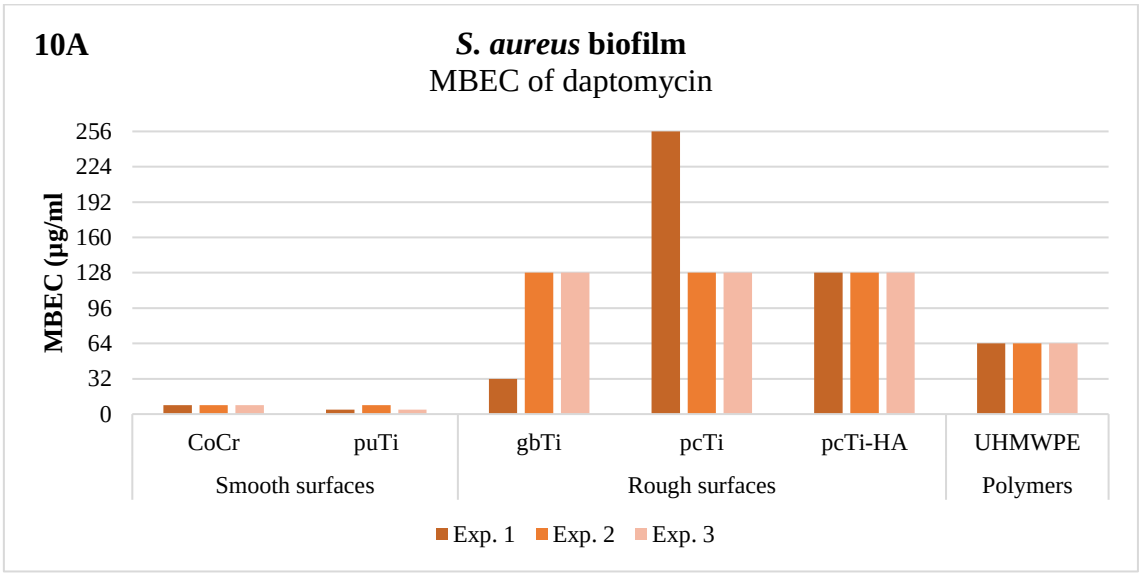
[a] In three occasions where small variations were observed in triplicate experiment the result is presented as range.

Rifampin demonstrated inconsistent results in staphylococci due to random emergence of resistance (Table 4), which was observed at concentrations ≤1024x MIC (8 µg/mL). Flucloxacillin inhibited *S. aureus* >512xMIC (128 µg/mL). Ciprofloxacin inhibited heat flow of *E. coli* at ≥4x MIC (≥0.06 µg/mL).

Study II

In vitro formation of staphylococcal biofilm on various implant materials with different surface properties was quantitatively estimated by time to detection (TTD) of growth related heat flow in isothermal microcalorimetry. Furthermore, the efficacy of daptomycin to eradicate staphylococcal biofilms on various orthopedic implant surfaces and materials was investigated, as we determined the lowest concentration of daptomycin required to eradicate a bacterial biofilm on the sample (minimal biofilm eradication concentration, MBEC). Two laboratory strains of bacteria commonly causing PJI were used, namely *Staphylococcus aureus* and *S. epidermidis*. Tested biomaterials include cobalt chrome alloy, pure titanium, grid-blasted titanium, porous plasma-coated titanium with/without hydroxyapatite, and polyethylene. We also tried to test biofilm formation on samples of bone cement (polymethyl methacrylate, PMMA) with and without gentamycin. Whereas no staphylococci grew from the antibiotic-impregnated cement samples, PMMA alone did not give reproducible results in repeated attempts. Hence, both materials were excluded from the final study.

Biofilm bacteria on orthopedic materials were variably influenced by exposure to the potent antimicrobial effect of high-dose daptomycin (Figure 10 A+B).
With smooth metallic surfaces of cobalt-chrome and pure titanium regrowth of *S. aureus* biofilm was absent at 4-8 µg/ml, equivalent to daptomycin concentrations 8-16 times higher than the MIC (0.5 µg/ml). With the remaining test samples having a macroscopic rougher surface structure the MBEC of S. aureus biofilms to daptomycin was higher (32-256 µg/ml). In experiments with *S. epidermidis* biofilms (Figure 10B), the MBEC variated from 8 to 256 µg/ml, but no pattern was seen in relation to the surface roughness.



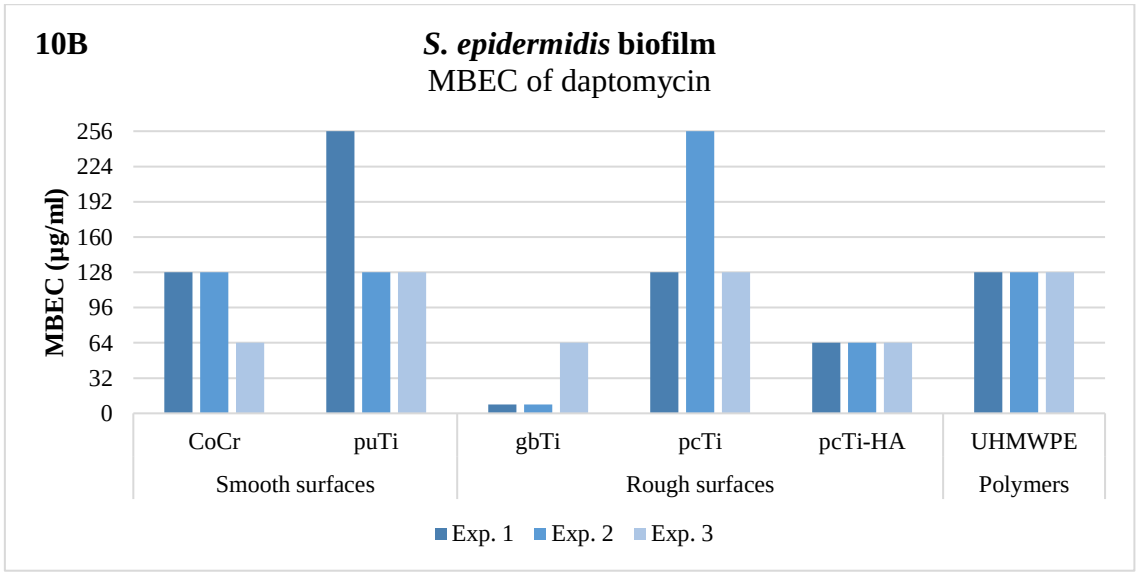


Figure 10 A+B: Minimal biofilm eradication concentration (Study II)
Minimal biofilm eradication concentration (MBEC, median and range) of daptomycin against staphylococcal biofilms in triplicate experiment (Exp. 1-3). Smooth metal surface: Cobalt-chrome and pure titanium. Rough metal surface: Grid blasted titanium and porous plasma coated titanium with/without hydroxyapatite. Polymer: Ultra-high molecular weight polyethylene

Figure 11A shows variations of TTD-50 in triplicate experiment with *S. aureus* biofilm on various materials. The heat flow developed differently between the surfaces (Friedman's test, $p<0.01$) and post hoc comparisons showed that the heat flow developed faster with the rough surfaces (gbTi, pcTi and pcTi-HA) in comparison to the smooth surfaces (CoCr, puTi) and polymer (UHMWPE) ($p<0.001$, Bonferroni corrected). This indicates a higher quantity of *S. aureus* biofilm on the macroscopically rough surfaces solely, as the smooth surfaces (CoCr, puTi) and polymer (UHMWPE) did not indicate any difference ($p>0.2$). Regarding *S. epidermidis* (Figure 4B), we also observed significantly faster heat flow development with rough gbTi and pcTi-HA in comparison to smooth CoCr and puTi.

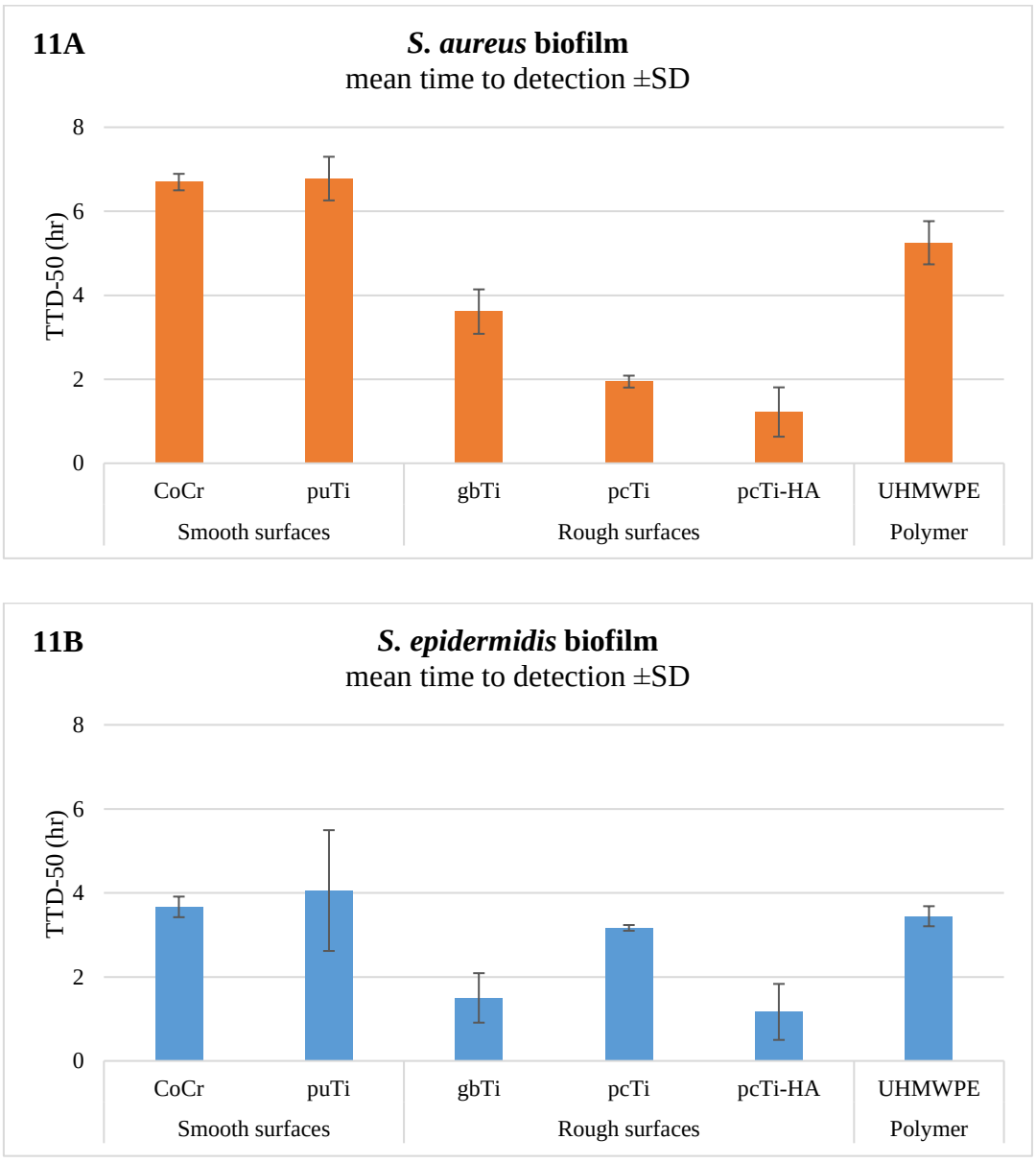


Figure 11 A+B: Time to detection of biofilm growth (Study II)

Microcalorimetric analysis of staphylococcal biofilm in triplicate experiment. Time to detection (TTD-50, mean ± SD) indicates the time needed to reach the heat flow of 50 µW. Smooth metal surface: Cobalt-chrome and pure titanium; Rough/porous metal surface: Grid blasted titanium, porous plasma coated titanium with/without hydroxyapatite; UHMWPE: Ultra-high molecular weight polyethylene.

Study III

In this prospective cohort study, we investigated the additive effect of sonication fluid culture (SFC) on diagnosis of bacteria in revision TKA and THA. We included 211 revisions indicated as aseptic loosening (n=71), deep infection (n=54) and 'other indications' (n=86). Based on the composite reference standard PJI was verified in 54 cases of which 40 cases (74%) fulfilled more than one criterion. In three verified PJI cases, only the first criterion (sinus tract) was fulfilled. Another six verified PJI cases fulfilled only the second criterion of ≥3 identical microbial findings in culture of 5 tissue samples. Three cases fulfilled the third criterion of identical microbial findings in culture of 2/5 tissue samples AND 1 synovial fluid sample. Finally, two verified PJI cases were identified as such due to a combination of minor criteria alone, namely elevated CRP and concordant culture in synovial fluid and 1/5 tissue sample.

The microbial diagnosis was achieved in 40 (74%) verified PJI cases by growth in ≥3/5 tissue samples (Figure 12). Correspondingly, SFC was positive in 48 (89%) of verified PJI-cases (Figure 13). Additionally, SFC was positive in 3/10 revision cases highly suspicious of PJI, though not fulfilling the composite reference standard.

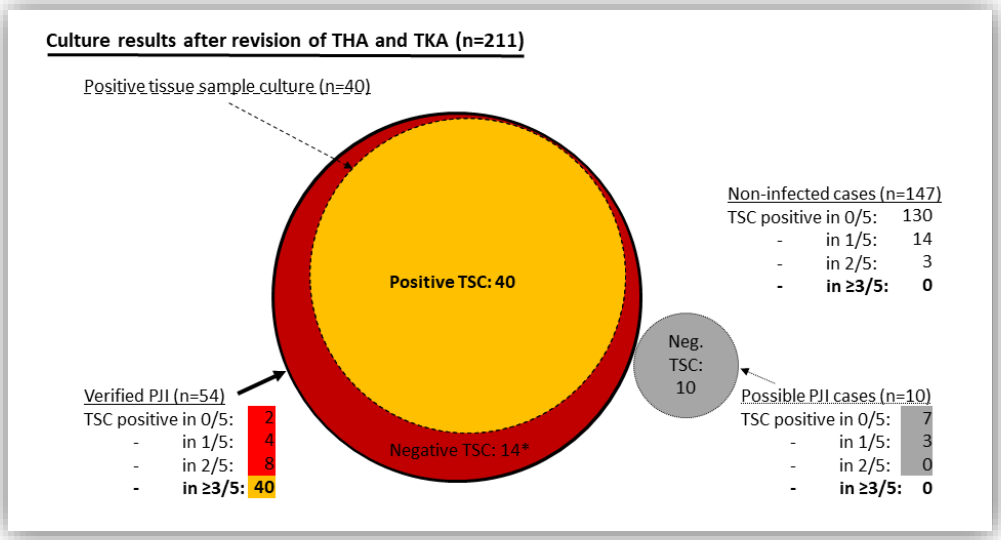


Figure 12: Culture results of conventional tissue samples (Study III)

Revision cases are classified as either Verified PJI, Possible PJI or non-infected. Yellow color represent positive tissue sample culture (TSC) with a threshold of ≥3/5. Red color represent the cases verified as PJI, though negative (*below threshold) in TSC.

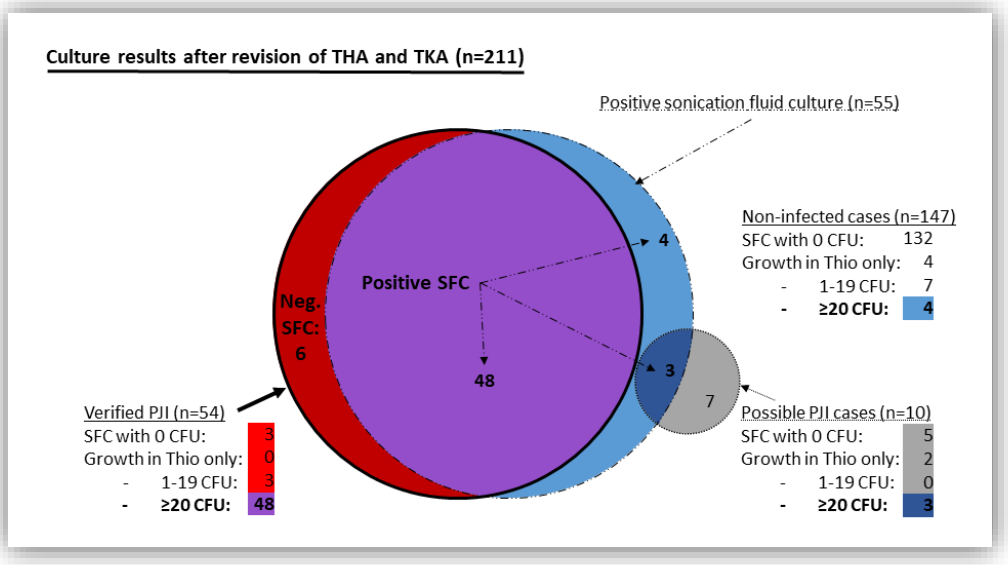


Figure 13: Culture results of sonication fluid (Study III)
Blue color represent positive sonication fluid culture (SFC) with a threshold of ≥20 CFU/ml. Red color represent the cases verified as PJI, though culture-negative in SFC.

Sonication fluid culture identified 11 verified PJI cases that in TSC were either negative or below the diagnostic threshold (≥3/5). 5 of them were culture-positive in a combination of synovial fluid and 2/5 tissue samples, and hence fulfilled the second diagnostic criterion anyway. In relation to TSC, additional bacterial growth was found in 11 verified PJI cases, 3 possible PJI cases and 4 cases categorized as non-infected (Figure 14).

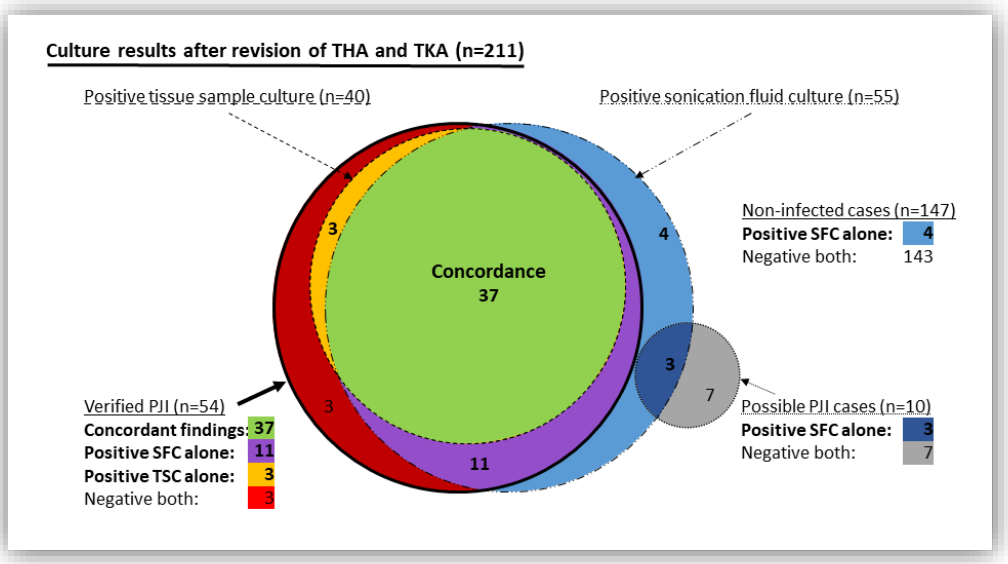


Figure 14: Concordant findings in tissue and sonication fluid culture (Study III)
Positive culture results from tissue samples (≥3/5) and sonication fluid (≥20 CFU/ml) compared to the classification of PJI. Green (concordant) cases are positive with both modalities, whereas the yellow cases are positive in tissue samples alone, and blue are positive in sonication fluid alone. Red represents the cases verified as PJI, though culture-negative in both tissue samples and sonication fluid.

Subgroup analysis of the additional findings of SFC in relation to prosthetic joint, revision indication, preoperative antibiotic administration, debut of symptoms, clinical manifestation and intraoperative evaluation of the prosthesis are listed in Table 5.

	Verified PJI (n=54)		Possible PJI (n=10)
	Positive in TSC / total number	Additional in SFC (%)	Positive in SFC / total
Joint prosthesis under revision			
THA	22/32	+8 (25%)	3/8*
TKA	18/22	+3 (14%)	0/2
Revision indication			
Deep infection, expected	36/46	+8 (17%)	1/7*
Aseptic loosening	3/6	+2 (-)	1/2
Mechanical failure	1/2	+1 (-)	1/1
Preoperative antibiotic administration within 14 days			
No antibiotics	25/36	+9 (25%)	1/4
Antibiotics (>1 day)	15/18	+2 (11%)	2/6*
Timing of symptoms according to Zimmerli [249]			
Early post-interventional	17/21	+3 (14%)	2/5*
Chronic PJI	11/20	+7 (35%)	1/3
Late acute PJI	12/13	+1 (8%)	0/2
Clinical joint symptoms			
Painful, warm and red	26/32	+6 (19%)	1/4*
Not warm and red	14/22	+5 (23%)	2/6
Intraoperative prosthetic status			
Fixed component	29/35	+4 (11%)	0/6*
Loosened component	11/18	+6 (33%)	2/3
Periprosthetic fracture	0/1	+1 (-)	1/1

Table 5: Subgroup analysis of additional effect of SFC

Numbers of cases with positive tissue sample culture (TSC) and additional findings by sonication fluid culture (SFC), respectively, when PJI is either Verified or Possible. Asterisk (*) indicates two further cases SFC-positive in thioglycollate medium alone.

Further analysis of the culture results in verified and possible PJI-cases with respect to the PJI-class and preoperative antibiotic treatment are listed in Table 6. Please note that the term 'conventional samples' includes culture results of both synovial fluid and tissue samples. The threshold of positive growth in conventional samples is finding of ≥3 identical microbes in culture of 5 tissue samples AND/OR 1 synovial fluid sample.

Characteristics of verified and possible PJI-cases *Culture-positive no (%)*	**Conventional samples** ≥3 samples	**Sonication fluid** ≥20 CFU/ml
Overall verified and possible PJI-cases, n=64	46 (72%)	51 (80%)
Early postoperative symptoms, n=26	19 (73%)	22 (85%)
- With preoperative antibiotics, n=15	10	12
- No preoperative antibiotics, n=11	9	10
Chronic symptoms, n=23	14 (61%)	16 (70%)
- With preoperative antibiotics, n=2	2	1
- No preoperative antibiotics, n=21	12	15
Late acute symptoms, n=15	13 (87%)	13 (87%)
- With preoperative antibiotics, n=7	5	5
- No preoperative antibiotics, n=8	8	8

Table 6: Distribution of positive culture results from verified and possible PJI-cases (Study III)
Influence of preoperative antibiotics (administered for ≥2 days within 2 weeks prior to prosthetic revision surgery) and PJI-class (Zimmerli 2014) is further specified.

Bacterial findings were similar in all concordant cases (Table 7). The additive effect of SFC resulted in a bacterial diagnosis in 6 (11%) verified PJI-cases that were unsolved by culture of conventional samples, and the aggregated culture results of conventional and sonication samples were positive in 53/54 (98%).

Microbiological findings All revisions (n=211)	**Synovial + tissue** ≥3 samples	**Sonication fluid** ≥20 CFU/ml	**Concordance**
Staphylococcus aureus	10	10	10
Staphylococcus epidermidis	12	16	10
Other coag. neg. staph.	5	4	3
Hemolytic streptococci	6	6	6
Enterococcus faecalis	2	5	2
Gram positive, other	2	4	2
Enterobactericae	3	3	3
Polymicrobial	6	7	5
Total	46	55	41

Table 7: Microbiological findings (Study III)
Results from five days incubation of conventional samples (synovial fluid and tissue samples, positive with growth of identical organisms in ≥3 samples) and sonication fluid (positive culture with ≥20 CFU/ml).

Postoperative fate of revision cases with additional findings in SFC

In total, we identified 11 cases of verified or possible PJI with additional microbial findings in SFC (Table 8). Re-revision was performed in 5/11 cases after additional findings in SFC. Hereof 2 ended up with permanent Girdlestone status. Among 5/11 cases that were not re-revised after minimum 1 year follow-up, only 1 case had an asymptomatic prosthesis, whereas 1 case had a strategy of antibiotic suppression, and in 3 cases a painful joint prosthesis was unsolved. Finally, 1/11 patients diseased in circulatory failure after 466 days.

In the 8/11 cases with preoperative suspicion of deep infection, the prosthetic joint revision was either debridement and implant retention (2 cases), 1-stage exchange (1) or 2-stage exchange (5). Empirical antibiotic treatment typically consisted of dicloxacillin (2 weeks iv. and 4-6 weeks oral), except for one case of re-infection when initial iv.-administration of vancomycin for 2 weeks was followed by 4 weeks of dicloxacillin.

In cases revised for deep infection, 4/8 were culture-negative in all conventional samples. Another 4/8 cases had microbial growth below the threshold (1 tissue sample and/or synovial fluid), but only 2/4 of microbial findings in conventional samples were similar to SFC. Finally, 2 cases revised for aseptic loosening had microbial growth below the threshold, both were concordant with SFC.

ID	**Classifi-cation**	**Indication**	**Revision type**	**Antibiotic** iv. / oral	**Microbial finding** Synov | TSC | SFC	**Postoperative fate** LFU: Latest follow-up
#34 Verified	Early PJI	Deep infection	2-stage	Dicloxa 2w / 4w	*E. faecalis* Pos. | 0/5 | >500	Spacer-exchange after 12 days (0/5) Prior revision with *E. faecalis*
#80 Verified	Chronic PJI	Deep infection	DAIR	Dicloxa 2w / 4w	*P. acnes* Neg. | 0/5 | >500	Permanent Girdlestone procedure after 13 months
#116 Verified	Chronic PJI	Aseptic loosening	1-stage, partial	Dicloxa 2w / 4w	*S. epidermidis* Pos. | 1/5 | >500	No more revisions LFU (30 months): possibly loose again
#137 Verified	Early PJI	Deep infection	1-stage, total	Dicloxa 2w / 6w	*S. epidermidis* Pos. | 1/5 | >500	2-stage revision after 12,5 months with *S. epidermidis*
#146 Verified	Chronic PJI	Deep infection	2-stage	Dicloxa 2w / 6w	*E. faecalis* Neg. | 1/5 | >500	Permanent Girdlestone procedure after 3 months
#206 Verified	Chronic PJI	Deep infection	2-stage	Dicloxa 2w / 4w	*F. magna* Pos. | 1/5 | >500	Diseased in circulatory failure 466 days later
#50 Possible	Chronic PJI	Aseptic loosening	1-stage, partial	Dicloxa 2w / 4w	*S. epidermidis* Pos. | 0/5 | 45	No more revisions LFU (12 months): lumbar stenosis
#71 Possible	Early PJI	Periprosth fracture	1-stage, partial	Dicloxa 5 days	*S. capitis* Neg. | 0/5 | 450	No more revisions LFU (24 months): Recurring troch bursit
#149 Possible	Early PJI	Deep infection	DAIR	Dicloxa 2w / 6w	*F. magna* Dry | 0/5 | Thio	No more revisions LFU (12 months): OK Prior revision w/ polymicrobial finding
#161 Possible	Early PJI	Deep infection	2-stage	Vancomyc 2w	*S. agalacticae* Neg. | 0/5 | Thio	No more revisions LFU (14 months): Permanent spacer Prior DAIR with *S. agalacticae*
#212 Possible	Early PJI	Deep infection	2-stage	Dicloxa 2w / 4w	*E. faecalis* Neg. | 0/5 | 30	Spacer-exchange after 30 days with *E. faecalis* in 5/5 tissue samples

Table 8: Fate of prosthetic joint revisions

Brief description of the clinical course after in 6 verified and 5 possible PJI cases with additional microbial findings in sonication fluid culture (SFC). Classification, revision indication, revision type, antibiotic treatment, and microbial findings are listed for index revisions. Microbial findings are specified for synovial fluid, tissue sample culture (TSC, no. positive in 5 samples) and SFC (no. CFU/mL). See appendix D for more detailed data on each case.

Abbreviations in table 8:

ID and Reference standard		**Antibiotic administration**		**Microbial finding**	
See Appendix D		iv.	Intravenously, 2 weeks	Synov	Synovial fluid culture, positive/negative
Revision type		oral	Tablets, 4-6 weeks	TSC	Tissue sample culture, positive no. / 5 samples
1-stage	One-stage revision	**Antimicrobial dosage**		SFC	Synovial fluid culture, no. culture forming units
2-stage	Two-stage, 1st step	Diclox	Dicloxacillin, 1g x 4	**Postoperative fate**	
DAIR	Debridement and implant retention	Vanco	Vancomycin, 1g x 2	LFU	Latest follow-up

Discussion

Differentiation between septic and aseptic etiology of a painful TJA is essential for successful treatment. There is clearly a challenge with inadequate sensitivity in conventional culture methods [16, 43, 106, 148, 175, 217]. On the other hand, confusion may occur when several intraoperative culture specimens unexpectedly grow positive [13, 204, 216]. Furthermore, it has been argued that presence of low-virulent bacteria in the TJA may be involved in the osteolytic process of what is expected to be 'aseptic loosening' [27, 124, 158, 161, 199].

Challenge of defining PJI reference standard

A comparison of different diagnostic modalities should ideally be done in a study of diagnostic accuracy, and reported according to the Standards for the Reporting of Diagnostic Accuracy Studies (STARD) [32]. I will in the following pages explain, how the lack of an indisputable reference standard of PJI makes analysis of diagnostic accuracy unreliable.

Whereas conventional culture methods has served as gold standard for detection of planktonic bacteria for more than 150 years, it is considered insufficient for detection of biofilm bacteria that to some extent may be characterized by viable but non-culturable bacteria [103, 137, 218].

In 2010, the American Academy of Orthopaedic Surgeons (AAOS) published a 286 pages guideline and evidence report on 'The diagnosis of periprosthetic joint infection of the hip and knee' [7]. In the review of 53 included diagnostic studies, they identified at least 18 different reference standards for PJI (Table 9).

Reference Standard (Infection defined as positive results on following tests)	No. of studies
Intraoperative cultures	18
Intraoperative cultures and histology	7
Histology	4
At least 2 of intraoperative cultures, purulence, and histology	4
Intraoperative cultures or histology	3
Open wound or sinus communicating with the joint OR systemic infection with pain in the hip and purulent fluid within the joint OR positive result on at least 3 tests (ESR, CRP, joint aspiration, intraoperative frozen section, and intraoperative culture)	3
Histology or purulence or sinus tract communicating with the prosthesis	2
Intraoperative cultures or purulence	2
Aspiration or intraoperative cultures	1
At least 2 of intraoperative cultures, purulence, and histology; or 2 positive cultures	1
Histology and gross operative findings	1
Intraoperative cultures or histology or purulence	1
Intraoperative cultures or histology or deep abscess	1
Cultures or purulence or histology or sinus tract communicating with the prosthesis	1
Correlation between intraoperative cultures and histology; the appearance of the tissue intraoperatively; and the clinical course	1
Intraoperative cultures and gross sepsis	1
At least 3 of CRP, ESR, aspiration culture, intraoperative purulence, intraoperative culture	1
Abscess or sinus tract communicating with the joint space OR aspiration culture OR ≥2 intraoperative cultures OR 1 culture and purulence or histology OR purulence and histology	1

Table 9: Variety of Reference standards for PJI

Diagnostic studies reviewed in the AAOS Guideline and Evidence Report 2010, reprint from page 6 [7].

Recent scientific literature on PJI often work with multi-criteria definitions [85] and the American Musculoskeletal Infection Society is currently advocating for the ICM-definition as reference standard in comparison of the medical literature reporting on issues related to PJI (Table 2). In our experience, some of the included standard criteria are not implemented in clinical practice and may not be sufficiently available for diagnostic purpose. Especially, histopathological analysis of periprosthetic tissue samples requires a dedicated and interested pathologist to gain experience in interpreting the specimens [62, 207]. Furthermore, the diagnostic threshold is still debated [127].

Risk of diagnostic misclassification

The major problem with an imperfect reference standard for a dichotomous condition, such as the PJI diagnosis, is the risk of misclassification [203]. Arthroplasty patients undergoing revision surgery may falsely be classified as either infected or non-infected in their prosthetic joint due to insufficiency of each available diagnostic modality as well as an imperfect reference standard.

In terms diagnostic accuracy, the effect of misclassification can be an upward or downward bias dependent on the correlation between the index test and an imperfect reference standard [198]. The error of a reference standard may disfavor certain conditions that is undetectable by the index test. For example, plasma C-reactive protein is often elevated in acute infections, but may be normal in chronic infections [152]. Hence, reliance on p-CRP to rule out PJI might disfavor infections with low-virulent organisms that

are difficult to diagnose by TSC. These errors are positively correlated and misclassification will erroneously increase agreement (non-differential misclassification).

In other situations, the error of a reference standard may differ from the error of an index test (differential misclassification). For example, the accuracy of some nuclear scanning methods are reportedly very high for chronic osteomyelitis, but also very sensitive for other reasons of local inflammation. Hence, in the first couple of months after implantation of an arthroplasty, the natural course of postoperative tissue reactions may be confused with PJI [235].

Due to the dilemma of misclassification in a dichotomous condition, we introduced the intermediate category of 'Possible PJI' (Table 3). Among the 10 possible PJI-cases, 7 were indicated as prosthetic revision due to deep infection by the operating surgeon, nevertheless none of them were culture-positive in conventional samples. Sonication fluid on the other hand was culture-positive with >20 CFU/mL in 3/10 cases cases, whereas another 2/10 cases grew an unquantifiable amount of bacteria in thioglycollate culture media only (see Appendix D for a descriptive overview of selected revision cases and culture results). Since exact categorization of these 10 possible PJI-cases as either 'PJI' or 'non-PJI' is unachievable, we find it impossible to estimate the diagnostic accuracy of the different culture samples in our cohort.

Challenges of a culture-dependent reference standard

The composite reference standard used in the clinical **Study III** (Table 3) may, except from the sinus-tract criterion, overall seem rather culture-dependent. The index test, culture of sonication fluid, is correspondingly dependent on bacterial growth (potential non-differential misclassification). Hence, concordant bacterial findings in conventional and sonication samples is less surprising (Figure 14).

The majority of verified PJI cases fulfilled more than one criterion of the composite reference standard, but 17% of verified PJI cases only fulfilled the second criterion of ≥3 identical microbial findings in culture of 5 tissue samples AND/OR 1 synovial fluid sample. The fact that conventional culture results plays such a dominant role in verification of PJI obviously makes this and other culture-dependent reference standards unsuitable for estimation of accuracy with TSC.

In **Study III,** additional bacterial growth in sonication fluid was found in 11 verified PJI cases, 3 possible PJI cases and 4 cases categorized as non-infected (Figure 14). This raises another concern related to the analysis of SFC, namely that sonication under some circumstances might be more sensitive than the composite reference standard. Whether this would be different with the ICM-definition cannot be determined. More interesting is the clinical implication of the additional bacterial findings (see below).

How to diagnose PJI

In PJI, where presence of microorganisms in the prosthetic joint will expectedly lead to deep infection, demonstration of bacteria in specimens from the joint space defines the disease. Hence, the principle of sampling periprosthetic tissue biopsies is still cornerstone to PJI diagnostics. However, specimen sampling, transport and incubation carries a risk of contamination, which may lead to false positive culture results that are difficult to interpret since sources of specimen contamination might as well be the source of perioperative inoculation [98]. Multiple specimen sampling enables estimation of the diagnostic probability, since identical bacterial findings in several independently sampled specimens are considered to represent the periprosthetic flora [9, 116]. Nevertheless, unexpected positive intraoperative culture occurs in revision surgery [13, 150, 204, 233].

In Study III, we identified 8/54 cases (15%) with positive intraoperative culture (PIOC) in ≥3 conventional samples despite a revision indication other than deep infection. The majority (6/8) were characterized by chronic symptoms of prosthetic loosening, and only two of them had elevated C-reactive protein levels (for more details, see Appendix D). If we had lowered the diagnostic threshold to ≥2 culture-positive periprosthetic tissue samples, another 3 revision cases indicated by aseptic loosening would have been considered as PIOC despite no other signs of PJI (Figure 15; purple color, third column).

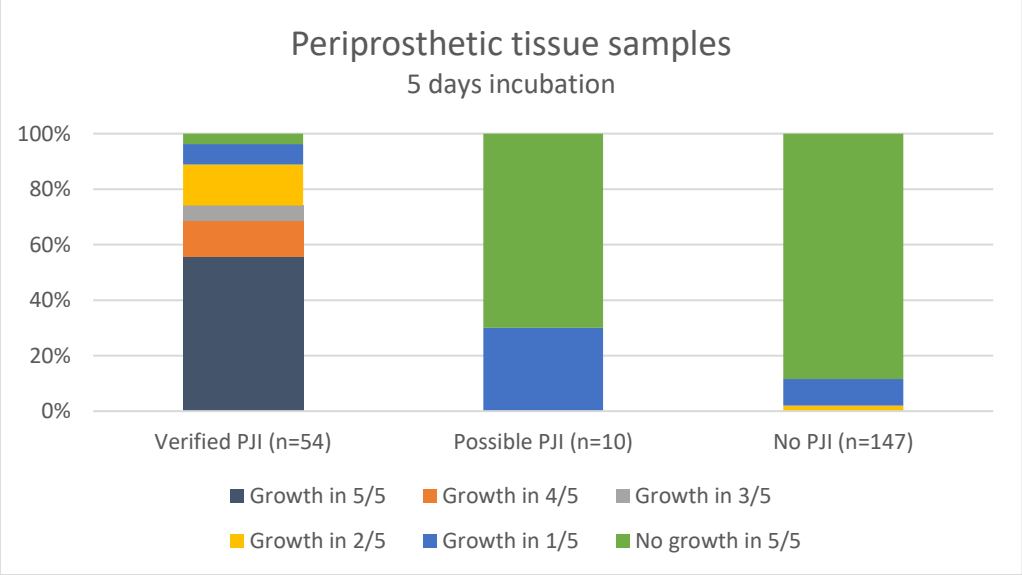


Figure 15: Number of culture-positive periprosthetic tissue samples (Study III)
Data are distributed in three groups according to the reference standard of prosthetic joint infection (Table 3). The diagnostic threshold was ≥3/5 positive TSC. If this threshold was lowered to 2/5, three revision cases (purple color, third column) with no other signs of PJI would have been included in the category of verified PJI.

Hence, the prevalence of unexpected PIOC in our cohort with 211 revisions of THA and TKA is 8/211 (4%) with a threshold at ≥3/5 or 11/211 (5%) with a threshold at ≥2/5. We are not able to determine whether these microbial findings actually represent contamination or causative microorganisms, though. Correspondingly, a retrospective study by Tsukayama et al reported a prevalence of 31/275 (11%) deep infections of THA diagnosed by PIOC with a threshold at ≥2/5 [233].

Standard incubation time at our institutions was 5-7 days during the study period, but this has also been investigated in recent publications. In a prospective study of patients undergoing revision for prosthetic hip or knee loosening, Schäfer *et al* suggested prolonged culture (14 days) of tissue samples in order to optimize PJI diagnostics [205]. After the first week of incubation culture plates tend to dry out, though and hereafter only non-quantitative culture in broth media is possible. The optimal incubation period was studied in a recent publication on PJI, but no clear conclusion was made on this issue [15].

The discussion of prolonged culture, diagnostic thresholds, false negative culture results, and risk of contamination in culture of periprosthetic tissue samples reveals two opposing approaches, since the quest for increased diagnostic sensitivity may sacrifice the specificity through contamination. Thus, cautious handling of microbiological material is imperative [133]. At the same time, diagnostic support from culture-independent modalities may further increase the likelihood of coupling the microbiological finding to the correct diagnosis.

Culture-independent diagnostic methods

1. Clinical assessment

Presence of a sinus tract communicating with the prosthetic joint has been considered pathognomic for chronic PJI in research literature for decades [9, 37, 169, 253, 254], but it is unclear how this condition is defined. We defined it as a permanent abnormal passageway between the prosthetic joint and the exterior of the body, but did not routinely perform fistulography. A sinus tract was registered in 8 cases (Table 10), whereof 5 also fulfilled other criteria of 'verified PJI'. The remainder 3 were accompanied by wound leakage as they occurred between 100-400 days after latest joint surgery.

Correspondingly, the term 'wound leakage' was defined as a suppurating wound defect persisting more than 14 days after surgery. This condition was preoperatively found in 19 cases (Table 10), whereof 1 case (#129 in appendix D) was categorized as non-infected due to lack of other signs of infection (sonication fluid was culture-positive though and the joint was 22 days later revised for deep infection; #149).

The visual impression of purulence in the prosthetic joint is a subjective condition that may be evaluated upon joint aspiration or most reliably at the prosthetic joint revision. This intraarticular finding cannot be defined as 'lack of normal joint fluid', since blood and metallosis blurs the interpretation. In a retrospective study, intraoperative purulence was recently evaluated against the ICM-definition of PJI and found unreliable for diagnosing PJI [6]. We have prospectively registered 24 cases with finding of intraarticular purulence (Table 10). Only 2 of them did not additionally fulfill a major criteria of verified PJI, though they showed local infection signs of infection in the early postoperative phase (both received continuous intravenous antibiotic treatment; #137 and #212).

Finally, classical signs of joint infection also includes tumor, rubor, dolor, calor et functio laesa. We prospectively registered all findings of a painful prosthetic joint AND acute onset of redness and elevated skin temperature, since we found that this condition was specific for joint inflammation and may increase the suspicion of PJI (Table 10). This condition was only included as 1/8 criteria of 'possible PJI' in the composite reference standard though.

Pre- and intraoperative findings	Verified PJI n=54	Possible PJI n=10	No PJI n=147
Preoperative clinical findings			
- Sinus tract communicating with the TJA	8	0	0
- Wound leakage (>14 days after surgery)	16	3	1
- Painful, warm, red joint (recent 3 weeks)	32	4	0
Intra-operative evaluation			
- Normal synovial fluid	3	4	110
- Purulent synovial fluid	23	1	0
- Uncertain synovial fluid*	28	5	37
Preoperative blood test			
- Elevated CRP/total number**	32/51	6/10	9/108

Table 10: Pre- and intraoperative data of 211 revisions.
* Synovial fluid quality was termed 'uncertain', when neither 'normal' nor 'purulent' were adequate
** C-reactive protein (CRP) was not analyzed in three verified PJI-cases and 39 non-infected revision cases; hence, the reduced total number is indicated as the denominator. The threshold of elevated CRP is 10 mg/l when the latest surgery was >3 months before, periprosthetic fracture was not present and no accompanying comorbidity of cancer or rheumatic disorder (otherwise 100 mg/l).

2. Biomarkers in blood and plasma

Acute phase reactants are proteins that exhibit marked increase in serum concentration as a response to inflammation and tissue injury [82]. The most widely used indicators of the acute phase response are the blood erythrocyte sedimentation rate (ESR) and plasma C-reactive protein (CRP) levels. These biomarkers may be considered as surrogates of the host immune response and may, when elevated, raise the suspicion of infection (e.g. PJI). However, in clinical use ESR and CRP are not specific to any particular disease, nor can they distinguish infection from other causes of acute and chronic inflammation [151].

3. Synovial fluid analysis

The level of acute phase reactants in synovial fluid aspirated from the prosthetic joint has been suggested to increase the specificity for PJI. This includes synovial CRP [167], synovial leukocyte esterase [221] and synovial alpha-defensin [60]. None of them was included in our investigation, as they have only recently been suggested; furthermore, validation studies are still awaited on the two latter biomarker tests. Elevated synovial fluid white blood-cell count (WBC) and the percentage of polymorphonuclear cells (PMN%) constitute two minor criteria in the ICM-definition of PJI, but diagnostic thresholds and precautions against dry aspiration remains controversial [40, 44, 45, 85, 86, 211, 222]. As previously explained we did not include these synovial fluid tests in our reference standard, as this was not routine investigations at our institutions during the conduct of **Study III**. In future research on the diagnosis of PJI, these analyses should be considered though.

4. Histopathology of tissue samples

This investigation is the most common culture-independent PJI-criterion reported in the literature (Table 9). However, it is our impression that the test is not routinely performed. Tissue sampling from the synovial membrane or the bone-implant interface was recently recommended [127]. The primary outcome parameter is numbers of neutrophil granulocytes per high-power field (x400 magnification) in sections of paraffin embedded tissue, but this analysis is subject to individual interpretation and warrants availability of a dedicated and experienced clinical pathologist. As previously mentioned, histopathological analysis of periprosthetic tissue samples was not routinely performed at our institutions, and we assumed that initiation of this praxis would impose high risk for interpretational bias.

Furthermore, the diagnostic thresholds for acute and chronic PJI still have not been established [85].

5. Molecular techniques

Culture-independent methods for microbial detection include polymerase chain reaction with molecular detection of bacterial 16S DNA and/or direct microscopic examination of infected materials, which have other disadvantages that make them unsuitable for inclusion in a composite reference standard for diagnosing PJI [53, 98, 103, 173].

6. Diagnostic imaging

Plain radiographs are important for identification of causes for joint pain and for preoperative planning before revision surgery, but not accurate markers of PJI [220]. Magnetic resonance imaging (MRI) and computed tomography (CT) may also be helpful for differential diagnostics, but the artifact caused by the metallic implant often disturbs the image [85].

Imaging modalities available in the area of nuclear medicine includes bone and leukocyte scintigraphy [66, 173, 230], as well as single-photon emission computed tomography and positron emission tomography [4, 235]. Due to cost, availability and radiation hygiene these modalities are not suitable screening tests for PJI, and were thus not included in the present study.

7. Clinical course

Another approach for validation of a PJI diagnosis would be to follow the clinical course after revisions surgery. In theory, an undiagnosed bacteriology may result in poor treatment outcome despite long-term administration of empirical antibiotics after thorough debridement and exchange of prosthetic components. In practice, culture-negative PJI might be caused by microbes that are susceptible to the empirical agent (most often Dicloxacillin in Denmark), hence the treatment course might end successful anyway. This also counts for cases of symptomatic superficial surgical site infection (SSI) that are treated with empirical antibiotics alone due to low suspicion of deep prosthetic joint involvement.

We noted 23 cases revised for expected deep infection in the early post-interventional period (<30 days) and two cases revised for early periprosthetic fracture with a concurrent leaking wound defect. Amongst these 25 cases, 15 had preoperatively received antibiotic treatment for SSI (median 9 days) within 14 days prior to revision surgery (Table 6). Hereof 10/15 (67%) were culture-positive in conventional samples whereas 12/15 (80%) were positive in sonication fluid culture (14/15 or 93% were positive if growth in thioglycollate alone was included). These findings both depicts the challenge with clinical assessment of SSI

as well as the risk of a culture-negative result with conventional samples after preoperative antibiotic treatment.

When positive intraoperative cultures (PIOC) are found after an expected aseptic revision, a strategy of antibiotic-free expectancy may be considered [204]. The rationale for this strategy is to wait and see whether a symptomatic infection develops or if the culture result was false positive (contamination). If another round of revision surgery is unnecessary, the accompanying risk of further prosthetic joint morbidity is avoided. In our cohort, we found 8 cases with PIOC in ≥3 conventional samples. 6/8 cases had concordant findings in sonication fluid culture. Another 5 cases were culture-positive in sonication fluid despite expected aseptic revision (See Appendix D for more details).

Introduction of new microbiological modalities

Inspiration for the investigation of bacterial detection by sonication and microcalorimetry in the present project was found in the literature and scientific congresses, where these modalities were only recently introduced in orthopedic implant revision surgery [29, 224-226].

Sonication

Bacteria growing as biofilm on an implant surface may be more difficult to recover from conventional samples and are in large inherently resistant to antibiotic treatment [103]. Advantages of implant sonication has been described as the ability to dislodge biofilm bacteria and make the available for further diagnostic procedures such as cultivation [223]. In clinical studies, the sonication-culture method reportedly improved the recovery of bacteria in low-grade PJI and in cases with previous antimicrobial therapy [2, 26, 29, 104, 187, 191, 200, 202, 210, 225].

In **Study I**, we investigate the ability of the sonication-culture method to detect microorganism after antibiotic exposure under in vitro conditions (Appendix A). Both highly virulent microorganisms (*S. aureus* and *E. coli*) and bacteria with slower growth rate (*S. epidermidis* and *P. acnes*) were included. Moreover, antibiotics tested include both time-dependent (flucloxacillin, vancomycin and rifampicin) and concentration-dependent (daptomycin and ciprofloxacin) drugs. With high culture-yields in sonication fluid at antibiotic concentrations well above the minimal inhibitory concentrations (MIC) of planktonic bacteria, we found the method suitable for detection of biofilm bacteria after antibiotic exposure under in vitro conditions. This was also accomplished in **Study II**, where staphylococcal biofilms were incubated on different prosthetic implant materials (Appendix B).

The Lausanne study group had already performed validation of the sonication protocol for terms of duration and intensity in unpublished studies. With a new and different sonication apparatus (BactoSonic™, Bandelin) than the one used in Andrej Trampuz' 2007 New England Journal of Medicine publication, the sonication time was lowered from five to one minute and saline was used equally with Ringer's solution [28]. In 2009, Monsen *et al* validated his experimental sonication protocol with a Transonic Digital S sonicator [157], and similar studies are warranted with any applied apparatus.
In **Study III**, we also used a BactoSonic™ sonication bath with a one-minute protocol (Appendix G), when a trained laboratory technician performed the sonication-culture procedure in the Department of Clinical Microbiology (DCM). We found that the procedure was very easy to perform and hands-on time of approximately 5 minutes per test was acceptable in comparison with handling of for example periprosthetic tissue samples.

Microcalorimetry

Isothermal microcalorimetry has been used in microbiological pharmacology studies where reduced, delayed and absent heat flow is related to the reduced inoculum after antimicrobial exposure [77]. The study group of Andrej Trampuz has conducted a long series of biofilm-experiments in the microcalorimeter with or without prior antimicrobial treatment [11, 34, 48, 73, 79-81, 147, 201, 239].

In **Study I** and **Study II**, we studied isothermal microcalorimetry for real-time evaluation of bacterial heat flow and growth after antibiotic exposure. We found obvious similarities and high reproducibility in the

graphic presentation of heat flow curves from the same bacterial strain indicated species specific heat flow behavior (Figure 9 and Figure 21). This observation is highly relevant to ensure purity or contamination of an experimental test sample, but not specific enough to allow for species identification when the microbial origin is unknown.

The effect of exposure to gradually increasing antibiotic concentrations was a stepwise reduction in bacterial quantity and corresponding delay of time to detection and heat flow peak. A flat heat flow curve was observed when bacterial regrowth was inhibited during the entire 24-hour measurement. This resembles susceptibility testing of planktonic bacteria according to the Clinical and Laboratory Standards Institute guidelines [49]. Instead of MIC, the lowest concentration of antibiotic exposure resulting in a flat heat flow curve was termed 'minimal heat inhibitory concentrations' (MHIC, Study I) and 'minimal biofilm eradication concentrations' (MBEC, Study II), respectively.

Our studies demonstrated a very fast detection (hours) of bacterial heat flow under in vitro conditions, which confirms the previous reports of microcalorimetry as a fast and sensitive method for microbial detection even in low inoculum [25, 36, 80, 228, 238, 247].

The use of sonication and microcalorimetry for microbial detection was recently tested in a clinical study of 39 consecutive patients who underwent complete removal of osteosynthesis material and prosthetic implants [30]. With a diagnostic threshold at 20 µW, bacterial detection was positive in all 12 (100 %) infection cases within mean 10.9 hours (range: 0.2-20.9 hours). This and other clinical studies might give inspiration for more diagnostic studies on implant-related infections, while the acquisition costs probably are too high for this method to become standard in clinical microbiology.

Considerations with clinical implementation of sonication fluid culture

The search for additional diagnostic tests in PJI is justified by the poor sensitivity of conventional culture methods when the free-floating planktonic bacteria are either considerably reduced in number or 'viable but non-culturable' due to antibiotic pressure and biofilm growth [103]. Furthermore, when a bacterial diagnosis and resistance pattern is desired, only microbiological tests may have the potential of giving that answer.

In the present and several other studies, we have seen that culture of sonication fluid has the potential of giving a microbial diagnosis after PJI revision [22, 69, 70, 90, 101, 104, 112-114, 187, 192, 193, 195, 212, 224, 225]. Still the method has not found its way to many departments of clinical microbiology [3]. What considerations need to be addressed during implementation of sonication fluid culture for diagnosis of PJI?

1. What is the indication for sonication?

In **Study III**, we included all revision cases in a consecutive series. We found positive culture results for other indications than expected deep infection in both tissue samples and sonication fluid (Table 11). The distinction between aseptic loosening and chronic PJI may be difficult, and it has previously been shown that bacteria may exist in specimens after revision surgery for expected aseptic loosening [124, 158, 161, 190, 195, 199]. In 4 THA-cases with early revision (14-25 days after latest surgery) for mechanical failure we found positive culture results in either both TSC and SFC (1) or SFC alone (3). Two of these cases (#35 and

#155, see Appendix D) fell under the category 'Verified PJI' according to our composite reference standard, whereas 1 case was 'possibly infected' (#71) due to ongoing antibiotic treatment only 14 days after revision of deep infection. The last case (#129) did not fulfill any other infection-criteria than persistent wound leakage 14 days after primary THA, though it was 22 days later re-revised due to suspicion of deep infection (#149). We thus find it plausible that the positive SFC in 6% of revisions indicated as aseptic loosening and 5% indicated as mechanical failure are truly positive.

Revision indication (expected pathology)	N	Positive TSC	Positive SFC
Deep infection	54	36 (67%)	47 (87%)
Aseptic loosening	71	3 (4%)	4 (6%)
Mechanical failure*	86	1 (1%)	4 (5%)
Total	211	40 (19%)	55 (26%)

Table 11: Positive culture results related to revision indication (Study III)
TSC: tissue sample culture; SFC: sonication fluid culture
*Mechanical failure: Periprosthetic fracture (3) and dislocation (1)

Two studies from the same Barcelonan study group found sonication most beneficial compared to tissue culture in chronic, low-virulent infections [190, 195]. This was also true in our study (Table 6), but even in the early postoperative phase (first month), we found more cases of positive SFC (22 cases) than positive TSC (17).
PJI may be unrecognized, at the time of revision surgery due to an acute mechanical failure. In other words, sonication and other microbiological tests may be indicated in cases where PJI cannot be excluded by other means, regardless of timing and concurrent mechanical failure. The present results indicate that SFC give additional microbiological information in TJA revision surgery which could be valuable in patient treatment.

2. Which implant materials are suitable for sonication?

This question was addressed in two clinical studies from the same Austrian study group, which concluded that the highest bacterial load was found on polyethylene liners. Going through the numbers and statistics reveals major interpretation flaws, though [105, 135]. One clinical study from Spain 'could not confirm a significantly higher adherence to a particular component or to a particular biomaterial' [89].
In **Study II**, we estimated numbers of biofilm bacteria on different experimental implant materials by heat flow measurement in isothermal microcalorimetry. With two different staphylococcal laboratory strains we observed that the quantity-equivalent measures, TTD-50 and HFP, were significantly different between the strains and among the different macroscopic surface structures. We saw no indication of a higher bacterial load on the hydrophobic polyethylene surface.

3. How to avoid contamination and destruction of the microbial content?

Neither our investigation, nor other available studies to the best of our knowledge have addressed this pertinent question on implant sonication. However, in 2006 Trampuz *et al* concluded that sonication of prosthetic components in double-layered polyethylene bags was associated with risk of contamination [224]. An interesting strategy has been suggested from the Aalborg-based 'Prosthetic-Related Infection and Pain' (PRIS) - Innovation project [134].

In **Study III**, we were not able to identify systematic bias related to handling of the prosthesis from explantation to sonication (see Appendix G), but especially the effect of logistic delay should be considered before implementation of the sonication-culture method.

4. Which sonication procedure will give the most reliable outcome?

Biofilm dislodgement by sonication is described by the bioacoustic effect of low-frequent ultrasound [67, 227]. The commonest type of liquid added to the prosthetic container is either saline or Ringer's solution [248]. Few studies have systematically addressed variable features of the sonication procedure for culture-based PJI-diagnostics [123, 157, 192], but none of them used the same ultrasound bath (BactoSonic™, Bandelin, Germany) as applied in the present and several other studies. Even with the BactoSonic ultrasound bath, different protocols has been described with sonication time from 1 to 5 minutes [30, 101, 114]. Portillo *et al* evaluated the effect of vortexing alone against the vortexing-sonication procedure (Branson Ultrasonic ultrasound bath, 5 minutes) and found lower biofilm removal efficiency with vortexing alone [192]. In all 3 studies, we used isotonic saline in a 30-60-30 second protocol of vortex-sonication-vortex as described in Appendix G.

5. How to manage sonication fluid?

After sonication and vortexing, the plastic container holds the prosthetic components and 2-300 mL of sonication fluid incl. dislodged bacteria and debris [133]. Hereafter, the main task is to ensure a sonication fluid sample representative of the potential microbial content. If direct inoculation is intended, aliquots of 0.1-0.5 mL are applicable, but if prior centrifugation is performed, the sample volume is usually larger (most often 50 mL Falcon tubes, dependent on the centrifuge). The rationale for centrifugation is obvious – increased bacterial concentration in the pellet after the supernatant has been discarded. However, handling of the container with prosthetic components and a large volume of sonication fluid imposes a risk of contamination. At the same time, it is unclear how to deal with large amounts of debris in the sediment after centrifugation, but a solution could be to redissolve the sediment before sampling.
Different culture media for sonication fluid has been suggested in the literature, but blood and chocolate agar plates are repeatedly used for aerobic and anaerobic incubation, respectively. Common for studies reporting sonication-culture of more than 5-7 days is that use of thioglycollate broth is reported [248]. Nutrient-rich broth agar as a culture medium for sonication fluid has the advantage of being more gracious to fastidious and slow growing bacteria [1, 133]. Unfortunately, growth conditions are also optimized for contaminants, and the inability of quantitative evaluation of the bacterial concentration in positive broth culture is a disadvantage. This is also true for inoculation in blood culture bottles as suggested in a few studies [193, 212, 225].
In **Study III**, solid blood and chocolate agar plates were used for aerobic and anaerobic incubation, respectively, whereas thioglycollate broth mainly was introduced to register growth after the first week of incubation. Positive culture in thioglycollate broth alone was seen in 6 cases during the first week of incubation and counted as 'uncertain culture outcome' (see Appendix C). We found no additional growth in thioglycollate after 7 days of sonication fluid incubation. Esteban *et al* reported similar findings [70].

6. How to interpret the culture result?

In a diagnostic study of 79 cases with PJI and 252 cases of aseptic loosening, Trampuz *et al* introduced the modern concept of sonication-culture [225]. After thorough analysis, a diagnostic cutoff in sonication fluid

culture at ≥10 CFU/mL was identified as the best trade-off between sensitivity and specificity. As previously mentioned, several changes have been suggested, including sonication bath manufacturer, sonication time, sonication fluid centrifugation and the definition of PJI. Partially as a consequence of this, the threshold of sonication fluid culture has varied between 1-100 CFU/mL [143].

In **Study III**, we defined our diagnostic threshold as ≥20 CFU/mL corresponding to 4 CFU in 0.2 mL sonication fluid on agar plates (see Methodological considerations, above). Though we did not clearly define presence and absence of PJI, we did analyze the bacterial concentration in sonication fluid culture towards the number of culture-positive tissue samples (Table 12). We did not take into account that low-virulent bacteria and PJI with preoperative antibiotic treatment may not grow as numerous as high-virulent bacteria.

Diagnostic concordance	Tissue culture, number of positive samples			
n=211	0	1	2	3-5
Sonication fluid culture				
Negative	121	13	4	2
Uncertain culture result*	6 (5)	1 (1)	2	1
20-100 CFU/ml	3	1	0	10
>100 CFU/ml	4	3	7	27

Table 12: Quantitative comparison of TSC and SFC (Study III)

Quantitative findings after culture of periprosthetic tissue samples (TSC) compared to sonication fluid culture (SFC). Green color indicates the concordant cases positive with both modalities. Discordant cases with positive tissue samples alone are colored yellow (≥3/5) and grey (2/5). Blue depicts the distribution of tissue culture results, when sonication fluid culture was positive, furthermore the darker blue represents cases with positive sonication fluid culture and concordant bacterial findings in 2/5 tissue samples.

*Uncertain culture results: bacterial concentrations <20 CFU/mL or microbial growth in thioglycollate alone (non-quantitative).

Clinical implication of additional bacterial findings

Introduction of the sonication-culture method as a new diagnostic tool for PJI may alter the base for clinical decision on treatment strategy. It is therefore natural to reflect on the clinical implications related to additional microbial findings.

Additional microbial findings in SFC may represent the causative organisms involved in the pathology of a present PJI. In **Study III**, positive bacterial findings in TSC and SFC were concordant in 41 cases and SFC was positive in additional 11/64 cases of verified and possible PJI. We described the postoperative fate in these 11 cases and found several links in the microbiological history of recurring infections.
Since sonication is not a routine procedure in our departments, the clinicians were blinded to the results of SFC and thus not influenced by the additional microbial findings. In cases with suspected deep infection, revision surgery included thorough debridement and postoperative administration of dicloxacillin for 6-8 weeks. Hence, it is not possible to correlate a poor treatment outcome after negative TSC directly with the additional microbial findings in experimental SFC though. To the best of our knowledge, no diagnostic studies has previously described the fate of prosthetic joint revisions, which had additional microbial findings in sonication fluid culture.

Despite reporting of higher diagnostic accuracy with SFC compared to TSC in the literature, there are no recommendations of excluding TSC in favor of SFC. Regardless whether the intention is to find the causative organism in obvious PJI or exclude microbial presence, when infection is less likely, SFC should generate valuable information enough to compensate the effort.

The direct and indirect costs of SFC also has to be taken into account whether the health care system, health insurance or the patient itself finances it. As an example, the billing fee to a patient for a SFC is $228 (approximately DKK 1500) at Geisinger Medical Center in Danville, Pennsylvania [149].
First, investment in a sonication bath would be necessary for a department of clinical microbiology in order to be able to offer sonication fluid culture. Several producers offer sonication baths, in our experience the investment cost is around DKK 30.000. Second, the sterile plastic boxes represent special consumables, which in our experience could be individually purchased at a price of DKK 30-60 (excl. sterilization). Third, consumables are also related to the process of sterilization of boxes and incubation of sonication fluid. Fourth and finally, the logistic setup affects existing jobs in the hospital system related to transport, reception, storage, operation, education, maintenance and repair.

As reported in the American literature, each case of PJI pose a substantial burden [100, 118, 130]. Seen from a health economic perspective, effective measures to improve treatment and prevention of reoccurrence therefore may be cost-effective. So far, no Scandinavian health economy studies have addressed this topic, though [31].

Conclusion

Study I:

Biofilm bacteria are less susceptible to antibiotics than their non-adherent, planktonic counterparts are. Weak growth inhibition was demonstrated with the time-dependent antibiotics (vancomycin and flucloxacillin), whereas the concentration dependent drugs (daptomycin and ciprofloxacin) considerably reduced the ability to detect biofilm bacteria. In perspective these findings support a recommendation of thorough surgical debridement in order to reduce the bacterial load rather attempts to cure an implant-related deep infection with antimicrobial therapy (Zimmerli et al. 2004).

Seen from a diagnostic point of view, these results might also indicate that preoperative administration of antibiotics has heterogeneous effects on the ability to detect biofilm bacteria. The quantitative and highly reproducible outcome of these procedures calls for further research in diagnosis and treatment of implant-related infections.

Study II:

Daptomycin eradicated *S. aureus* biofilm at lower concentrations on the smooth surfaces of CoCr and puTi compared to the rough surfaces of gbTi, pcTi and pcTi-HA, as well as polyethylene. In experiments with daptomycin against *S. epidermidis* biofilms, no pattern was seen in relation to the surface roughness. Furthermore, we demonstrated a significantly faster detection of staphylococcal heat flow due to higher biofilm quantity on the rough surfaces compared to smooth samples and polyethylene. This is an indication of a higher biofilm affinity and holding capacity of rough surfaces.

Findings in the present study indicate that orthopedic biofilm infections may not be sufficiently treated with parenteral administration of daptomycin in clinically achievable doses alone.

Study III:

This study demonstrated that the additive effect of the sonication-culture method was bacterial growth in 11/211 revisions of THA and TKA, which were not diagnosed by conventional methods. The fate of these 11 cases included re-revision in 5 cases. From a clinical perspective, patients with additional microbial findings by SFC had a discouraging prognosis and may represent true positive findings that have to be taken into consideration in the infection treatment.

Perspectives in diagnostic research on PJI

PJI is characterized by surface-related biofilm bacteria that may be termed 'difficult to diagnose' [98, 103, 251]. Inadequate PJI diagnostics are not only a challenge in the clinical practice between patient and surgeon, it is obviously also a problem in literature review and comparison of diagnostic studies [133, 248]. Furthermore, institutions designated to supervise trends in development of nosocomial infections may suffer from valid surveillance systems [58, 84, 107]. Thus, heterogeneous definition of PJI also affect the ability to monitor epidemiology and determine treatment success [65].

The ideal diagnostic test for PJI should be

- Sensitive enough to rule-in PJI with few cases of false-negative test results – i.e. detect any microorganism that is involved in the pathology of PJI.
- Specific enough to rule-out PJI with low numbers of false-positive results – i.e. exclude infectious etiology, when microorganisms are not the underlying reason for an inconclusive symptomology.

Culture of periprosthetic tissue samples diagnose the majority of PJI cases; also in the present clinical study. Hence, this diagnostic modality will continue to play a key role in microbiological investigation for PJI. Moreover, we found that sonication fluid culture is easy to perform and holds a potential of achieving additional bacterial diagnoses in septic TJA revision. Further validation of the SFC method with regard to clinical significance may determine to which extend positive SFC should be implemented in clinical practice and scientific definitions of PJI.

With potential improvements of the diagnostic approach, a patient suffering from PJI may have higher chance of being correctly diagnosed. Hereby, efficient revision surgery and targeted antimicrobial strategy may be initiated earlier. Furthermore, shortening of the pathophysiological process may reduce the extent of bone and soft tissue breakdown, which in turn reduce the need for extended surgery, repeated hospitalizations and handling of the adverse effects of antimicrobial treatment. Hence, improved microbiological diagnostics could expectedly improve the patients' quality of life and decrease PJI-related mortality. Finally, more effective PJI management is also desirable in the light of health economy and reimbursement [96].

In biofilm-associated infections, the antimicrobial susceptibility cannot sufficiently be predicted from conventional susceptibility tests due to a higher tolerance of biofilm bacteria against the antibiotic mechanisms [146, 156]. Experimental use of sonication fluid culture and real-time microcalorimetry are examples of measures to address this challenge [11, 73, 77, 79-81, 147].
In two experimental studies, we applied these new diagnostic tests, as we studied the effect of different antibiotic drugs in combination with various implant materials on the ability to detect biofilm bacteria in vitro. The results from these studies cannot be generalized to the clinical situation, but they may indicate that rough implant surfaces may hold more biofilm. And in such cases antibiotic treatment may be less effective in eradicating staphylococcal biofilm. These modalities may also be helpful in future studies of antimicrobial efficacy, biomaterial coating and other approaches against biofilm-associated infections.

Overall, a reliable diagnostic test for PJI is important from several perspectives. To fully understand the significance of these perspectives, a multidisciplinary team approach is important and should of course include informed consent by the patient.

Dansk resume

I Danmark udføres ledprotese-operationer i form af primær total hofte- og knæalloplastik (THA og TKA) årligt i ca. 18.000 tilfælde. En af de mest alvorlige komplikationer til denne procedure er dyb ledprotese-infektion (DLI), som rammer ca. 400 patienter årligt i Danmark. Tilstanden er stærkt invaliderende for patienterne, hvoraf de alvorligste forløb inkluderer mange re-operationer, livstruende infektioner med multiresistente bakterier, amputation og for tidlig død. Samtidig er de lange sygdoms- og behandlingsforløb ved DLI en stor belastning set fra et sundhedsøkonomisk perspektiv.

Ved infektion i relation til et fremmedlegeme (f.eks. ledprotese) har bakterier en tendens til hurtigt at danne en såkaldt biofilm, der beskytter dem i det næringsfattige miljø på overfladen af implantatet. Biofilmen beskytter også bakterierne mod kroppens immunforsvar og antibiotika, hvilket gør det svært at behandle fremmedlegeme-infektioner med medicin alene. Endelig er biofilm en af årsagerne til at DLI kan være svært at diagnosticere. Således viser flere studier at de diagnostiske metoder, vi anvender i dag, er utilstrækkelige i op imod hvert 5. tilfælde.

Det overordnede formål med dette ph.d.-studium var at undersøge to nye mikrobiologiske metoder, sonikering og microcalorimetri til diagnosticering af DLI. Sonikering anvendes til at løsne den bakterieholdige biofilm fra implantatoverfladen, mens microcalorimetri med stor nøjagtighed kan måle varmestrømme mens bakteriedeling foregår.

Vi udførte to eksperimentelle studier under fem måneders forskningsophold på professor Trampuz' laboratorium i Lausanne, Schweiz, efterfulgt af en måned i Berlin, Tyskland. Anvendelsen af sonikering og microcalorimetri blev anvendt til påvisning af overfladerelaterede biofilmbakterier i kombination med forskellige antibiotika og implantatmaterialer.
I **Studium I** viste vi, hvordan biofilmbakterier er mindre følsomme for antibiotika end ikke-klæbende, planktoniske bakterier. I **Studium II** observerede vi, hvordan biofilmbakterier på forskellige ortopædiske materialer (titanium, stål, polyætylen osv.) blev påvirket af høje doser af det potente antibiotikum, daptomycin.
I **Studium III** sammenlignede vi resultaterne af sonikeringsvæske-dyrkning mod vævsprøve-dyrkning i et prospektivt kohortestudium med 211 revisioner af THA og TKA. Ved sonikeringsvæske-dyrkning kunne vi finde bakterievækst i 11/211 revisionscases, som ikke var diagnosticeret i de konventionelle prøver. Ved at kombinere dyrkningsresultaterne fra konventionelle prøver og sonikeringsvæske faldt antallet af falsk negative resultater fra 15% til 2% blandt de verificerede DLI-tilfælde.

En god diagnostisk procedure er en grundlæggende betingelse for både behandling og yderligere forskning i biofilminfektioner. Vi har set, at sonikeringsvæske-dyrkning har potentiale til at påvise yderligere bakterielle diagnoser i forbindelse med DLI.

English summary

The overall purpose of this PhD study was to investigate two new microbiological modalities, sonication and microcalorimetry, for diagnosing prosthetic joint infection (PJI).

Total hip and knee arthroplasty (THA and TKA) are often referred to as successful and cost-effective procedures. In Denmark, primary THA and TKA are annually performed in approximately 18,000. PJI is one of the most feared complications of total joint arthroplasty (TJA). PJI is not only a potentially disabling disease, but also very expensive in health economic perspectives. The incidence of PJI within 2 years after implantation is around 1%, but markedly increasing in subsequent reoperations.

Bacteria living close to a foreign body (e.g. a joint prosthesis) will in a few hours typically attach to the implant surface and start developing a protective extracellular slime called biofilm. The biofilm growth mode is characterized by slow replication and few clinical symptoms such as pain developing over several months. With a diagnostic sensitivity of 50-80% in conventional culture samples, biofilm infections are called 'difficult-to-diagnose'. This has motivated the use of sonication, which was introduced to improve the culture yield of PJI by dislodgement of biofilm bacteria from implant surfaces. In order to further address the diagnostic challenges of slow-growing bacteria and reduced bacterial numbers after antibiotic pre-exposure, isothermal microcalorimetry was previously found a highly sensitive method for real-time detection of growth related heat flow within few hours.

We have performed two experimental studies during five months of research fellowship at Professor Trampuz ' laboratory in Lausanne, Switzerland followed by one month in Berlin, Germany. The use of sonication and microcalorimetry was analyzed for detection of surface-related biofilm bacteria in combination with various antibiotics and biomaterials.
In **Study I**, we demonstrated how biofilm bacteria are less susceptible to antibiotics than their non-adherent, planktonic counterparts are. Weak growth inhibition was demonstrated with the time-dependent antibiotics (vancomycin and flucloxacillin), whereas the concentration dependent drugs (daptomycin and ciprofloxacin) considerably reduced the ability to detect biofilm bacteria.
In **Study II**, we noted that biofilm bacteria on different orthopedic materials (titanium, steel, polyethylene, etc.) are variably influenced by exposure to the potent antimicrobial effect of high-dose daptomycin. Irregular surface topography was the main factor to decisively influence biofilm quantity and daptomycin susceptibility of *S. aureus* biofilms in this study.
High inter-experimental repeatability of the microcalorimetry assay in these and other studies suggests that the method is a valid test for investigation of biofilms without disturbing their sessile habitat on the biomaterial surface.

In **Study III**, we compared sonication fluid culture (SFC) against tissue sample culture (TSC) in a prospective cohort study of 211 revisions of THA and TKA. We handled the challenge of an inadequate definition of PJI by designing a composite reference standard. Based on multiple best-practice modalities that were readily available, we identified three diagnostic groups: Verified PJI, Possible PJI and Non-infected. We could not realistically estimate the accuracy of the diagnostic tests involved, since this would require a dichotomous definition such as infected/not infected.

The additive effect of SFC was bacterial growth in 11/211 revisions of THA and TKA, which were not diagnosed by conventional methods. By combining culture results from conventional samples and sonication fluid the rate of culture-negative results dropped from 8/54 (15%) to 1/54 (2%) of verified PJI-cases. Furthermore, bacterial growth in SFC of 5/10 possible PJI-cases and 1/157 revision cases categorized as non-infected might add to the diagnosis of PJI.
Subgroup analysis showed that the additional microbial findings in SFC were detected in both THA and TKA regardless of symptom duration, preoperative antibiotics, implant age and fixation. We are therefore not able to specify which patients have the highest diagnostic benefit of SFC.

A good diagnostic procedure is necessary in order to found the basic conditions for both treatment and further research in biofilm infections. We have seen that sonication fluid culture holds a potential of achieving additional bacterial diagnoses in prosthetic joint infection.

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Appendix A: Manuscript in study I

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Reduced ability to detect surface-related biofilm bacteria after antibiotic exposure under in vitro conditions

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Background and purpose — Antibiotic treatment of patients before specimen collection reduces the ability to detect organisms by culture. We investigated the suppressive effect of antibiotics on the growth of non-adherent, planktonic, and surface-related biofilm bacteria in vitro by using sonication and microcalorimetry methods.

Patients and methods — Biofilms of *Staphylococcus aureus, S. epidermidis, Escherichia coli,* and *Propionibacterium acnes* were formed on porous glass beads and exposed for 24 h to antibiotic concentrations from 1 to 1,024 times the minimal inhibitory concentration (MIC) of vancomycin, daptomycin, rifampin, flucloxacillin, or ciprofloxacin. The beads were then sonicated to dislodge biofilm, followed by culture and measurement of growth-related heat flow by microcalorimetry of the resulting sonication fluid.

Results — Vancomycin did not inhibit the heat flow of staphylococci and *P. acnes* at concentrations ≤ 1,024 µg/mL, whereas flucloxacillin at > 128 µg/mL inhibited *S. aureus*. Daptomycin inhibited heat flow of *S. aureus, S. epidermidis,* and *P. acnes* at lower concentrations (32–128 times MIC, p < 0.001). Rifampin showed inconsistent results in staphylococci due to random emergence of resistance, which was observed at concentrations ≤ 1,024 times MIC (i.e. 8 µg/mL). Ciprofloxacin inhibited heat flow of *E. coli* at ≥ 4 times MIC (i.e. ≥ 0.06 µg/mL).

Interpretation — Whereas time-dependent antibiotics (i.e. vancomycin and flucloxacillin) showed only weak growth suppression, concentration-dependent drugs (i.e. daptomycin and ciprofloxacin) had a strong suppressive effect on bacterial growth and reduced the ability to detect planktonic and biofilm bacteria. Exposure to rifampin rapidly caused emergence of resistance. Our findings indicate that preoperative administration of antibiotics may have heterogeneous effects on the ability to detect biofilm bacteria.

Prosthetic joint infection (PJI) is a serious complication with a reported incidence rate of 0.6–2.2% (Dale et al. 2012, Kurtz et al. 2012). The true incidence can be expected to be higher, though, due to under-reporting in the national databases (Huotari et al. 2010, Gundtoft et al. 2015) and imperfect diagnostic methods (Zimmerli et al. 2004).

For successful treatment, the diagnostic procedure involves isolation of the causative organism (Kamme and Lindberg 1981, Spangehl et al. 1999). Microorganisms living on the biologically inert surfaces of prosthetic components produce an organized community called biofilm (Gbejuade et al. 2014). These adherent biofilm bacteria can withstand host immune responses and are much less susceptible to antibiotics than their non-adherent, planktonic counterparts (Costerton et al. 1999).

Biofilm bacteria are often inaccessible with conventional sampling of synovial fluid and periprosthetic tissue samples, especially after eradication of the non-adherent bacteria by previous antibiotic treatment—e.g. empirical antibiotic therapy during postoperative wound healing complications or previous infections (Berbari et al. 2007, Malekzadeh et al. 2010). This has led to the use of sonication, which was introduced to improve the sensitivity of culture from biofilm infections by dislodgement of adherent bacteria from implant surfaces (Trampuz et al. 2007a).

To address the challenges of detecting slow-growing bacteria and reduced bacterial numbers after previous exposure to antibiotic, isothermal microcalorimetry has been found to be a highly sensitive and accurate method (Trampuz et al. 2007b, von Ah et al. 2008, Furustrand Tafin et al. 2012a, Maiolo et al. 2014). The principle of microcalorimetry relies on microbial heat production from bacterial growth and metabolism (Boling et al. 1973). Real-time measurement of exponential bacterial growth results in a pyramid-shaped heat-flow curve. The effect of antibiotics on recovery of bacterial growth can be followed by delayed detection of heat flow and reduction of the peak heat flow (Furustrand Tafin et al. 2011, Furustrand Tafin et al. 2012b, Mihailescu et al. 2014).

The main aim of this study was to determine the antimicrobial susceptibility of tested planktonic and biofilm bacteria in order to correlate biofilm detection with antibiotic exposure. A secondary aim was to investigate the effect of antibiotic exposure on detection of biofilm bacteria by sonication, culture, and microcalorimetry. We specifically investigated the effects of individual antibiotics in inhibiting the growth of planktonic and biofilm bacteria. Microcalorimetric measurement of heat flow was compared with conventional viable counting of dislodged biofilm bacteria.

Material and methods

This experimental study was designed to determine the effect of different antibiotics that are usually used in the treatment of PJI on the detection of biofilm bacteria by sonication and microcalorimetry, and to compare these results with those from conventional quantitative culture.

Test organisms and antimicrobial agents

Laboratory strains of bacteria that commonly cause PJI were studied, including methicillin-susceptible *Staphylococcus aureus* (ATCC 29213), methicillin-resistant *Staphylococcus epidermidis* (ATCC 35984), *Propionibacterium acnes* (ATCC 11827), and Gram-negative ciprofloxacin-susceptible *Escherichia coli* (ATCC 25922). Bacterial strains were stored at −80°C and cultured overnight on sheep blood agar plates before each experiment. Anaerobic culture conditions (Anaerogen system; Oxoid) and prolonged incubation (72 h) were used for all experiments with *P. acnes* in order to get visible colonies. The following antibiotics were used: vancomycin (Teva Pharma AG), daptomycin (Novartis Pharma AG), rifampin (Sandoz AG), flucloxacillin (Actavis SA), and ciprofloxacin (Bayer AG). For comparative reasons, the minimal inhibitory concentration (MIC) was initially determined for each combination of antibiotics and bacteria according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2012).

Biofilm formation on glass beads

Biofilms were investigated using porous glass beads (VitraPOR; Robu Glasfilter-geräte GmbH; diameter 4 mm, pore size 40–100 µm) (Corvec et al. 2013). For biofilm formation, beads were placed in 50-mL Falcon tubes containing tryptic soy broth (TSB) (1 mL per bead) and inoculated with 2 CFU of microorganisms as specified above. The beads were then incubated aerobically for 24 h at 37°C (brain heart infusion (BHI), 72 h, anaerobically for *P. acnes*). After the incubation, they were washed 5 times through rinsing with 10 mL saline, gentle shaking, and aspiration in order to minimize carryover of planktonic and loosely attached bacteria on the biomaterial surface.

Antibiotic exposure of biofilm

2-fold dilutions of antibiotic were prepared in TSB, or BHI for *P. acnes*, with concentrations ranging from 1 MIC up to 1,024 times MIC. Beads with biofilm bacteria were transferred with sterile forceps to the individual tubes (containing TSB and different antibiotic concentrations) and incubated for 24 h at 37°C (anaerobically, 72 h for *P. acnes*). Experiments were performed independently in triplicate and were accompanied with a growth control consisting of an inoculated glass bead without antibiotics and one negative control of a sterile glass bead in antibiotic-free medium.

Removal of biofilm by sonication

After exposure to antibiotic, the beads were transferred to individual Eppendorf tubes with 1 mL saline, vortexed for 30 s with maximum power (Vortex Genie 2; Scientific Industries), sonicated for 60 s (BactoSonic; Bandelin Electronic), and vortexed for 30 s again to dislodge biofilm-embedded bacteria. For conventional culture, 50-µL samples of the resulting sonication fluid were serially diluted and plated on blood agar plates. Bacteria in the sonication fluid were quantified by viable count of colony-forming units per mL (CFU/mL).

Detection of dislodged bacteria by microcalorimetry

In parallel to taking viable counts, 0.1 mL of sonication fluid from each experiment was added to 4-mL microcalorimetry glass ampoules with 1 mL TSB (3.9 mL BHI for *P. acnes*) (Clauss et al. 2010, Furustrand Tafin et al. 2012b, Corvec et al. 2013). After sealing the ampoules, they were lowered into a 48-channel batch microcalorimeter (thermal activity monitor model 3102 TAM III; TA Instruments). Heat flow (in µW) at 37.0000°C was measured continuously for 24 h (72 h for *P. acnes*) with an analytical sensitivity of ± 0.2 µW. The experimental detection limit was set at 10 µW to distinguish microbial heat production from the thermal background. Time to detection (TTD) was defined as the length of time (in hours) for a microcalorimeter experiment to reach the detection limit, which was therefore inversely proportional to the initial quantity of bacteria and the growth rate. The results were plotted as heat fl ow over time using the manufacturer's software (TAM Assistant; TA Instruments) and Prism 7.0 (GraphPad Software, San Diego, CA). The minimal heat inhibitory concentration (MHIC) was defined as the lowest antibiotic concentration (in µg/mL) to kill bacteria on the beads, or to show a post-antibiotic effect of inhibiting bacterial growth, leading to absence of heat production from biofilm-dislodged bacteria after 24 h of incubation in the microcalorimeter (72 h for *P. acnes*). In relation to the MIC of planktonic bacteria, the MHIC value is a multiplication factor on an ordinal scale from 1 MIC to 1,024 times MIC.

Statistics

Experiments were performed in triplicate, and descriptive statistics were used to express the median and range of all data. Non-parametric comparisons of antimicrobial susceptibility in 3 Gram-positive microorganisms were based on the multiplication factors and they were performed by

Susceptibility of test organisms to vancomycin, daptomycin, rifampin, flucloxacillin, and ciprofloxacin. The results are expressed as minimal inhibitory concentration (MIC) for non-adherent, planktonic bacteria and as minimal heat inhibitory concentration (MHIC) for biofilm bacteria. Numbers are in µg/mL

Organism	Vancomycin		Daptomycin		Rifampin		Flucloxacillin		Ciprofloxacin	
	MIC	MHIC	MIC	MHIC	MIC	MHIC	MIC	MHIC	MIC	MHIC
S. aureus	1	> 1,024	0.5	64	0.007	8	0.25	128	ND	ND
S. epidermidis	2	> 1,024	1	64	0.007	8–16 [a]	IR	IR	ND	ND
E. coli	IR	IR	IR	IR	IR	IR	IR	IR	0.015	0.063–0.25 [a]
P. acnes	1	> 1,024	1	32	0.007	8–16 [a]	ND	ND	ND	ND

IR, intrinsic resistance; ND, not done.
[a] In 3 cases where small variations were observed in experiments done in triplicate, the results are given as range.

by Wilcoxon signed-rank test using GraphPad Prism 7.0. Direct comparison with the MHIC of ciprofloxacin in Gram-negative bacteria (*E. coli*) was deemed unsuitable.

Results

Susceptibility of planktonic and dislodged biofilm bacteria to antibiotics

The Table summarizes the inhibition of microbial growth, expressed as the antimicrobial susceptibility of biofilm bacteria— as determined by sonication and microcalorimetry (MHIC)—relative to the MIC of planktonic bacteria. MHIC was considerably higher than MIC for all test strains and antibiotics (4 times to more than 1,024 times). According to CLSI breakpoints, the bacterial strains were susceptible to all antibiotics tested. The MHIC for vancomycin was more than 1,024 µg/mL for both staphylococci and *P. acnes*. The high MHIC values for rifampin reflect the emergence of resistance in staphylococci and *P. acnes*, as confirmed by susceptibility testing of the organisms after antibiotic exposure. The MHIC values were lowest for ciprofloxacin in *E. coli* (0.063 µg/mL), except in 1 of 5 experiments, where the MHIC was 0.25 µg/mL, reflecting emergence of ciprofloxacin resistance. In relation to the multiplication factor, the MHIC of daptomycin in Gram-positive bacteria was significantly lower than that of vancomycin ($p < 0.001$), rifampin ($p < 0.001$), and flucloxacillin ($p = 0.03$).

Effect of antibiotic exposure on detection of biofilm bacteria by microcalorimetry

Figure 1 shows the heat flow of bacteria after exposure to antibiotic, as a function of time. The heat-flow curves of organisms without previous antibiotic exposure had specific characteristics for each test bacterium, including the peak heat flow and shape of the curve. The time-shift of the curves to the right shows the delayed bacterial detection due to the lower quantity of bacteria in the sonication fluid from beads exposed to increasing concentrations of vancomycin, daptomycin, rifampin, flucloxacillin (only for *S. aureus*), and ciprofloxacin (only for *E. coli*). With rifampin, emergence of resistance was observed in *S. aureus* (Figure 1C) and *S. epidermidis* (Figure 1G) from heat production at higher antibiotic concentrations.

Figure 2 shows the time to heat detection. Fast-growing, high-virulence bacteria such as *S. aureus* (Figure 2A–D) and *E. coli* (Figure 2H) are detected earlier and produce a higher peak of heat flow compared to less virulent and more slowly growing bacteria (*S. epidermidis* (Figure 2E–G) and *P. acnes* (Figure 2I–K)). In addition, we observed the different modes of action of the antibiotics tested. Exposure to fast-acting antibiotics (such as daptomycin, rifampin, and ciprofloxacin) resulted in inhibition of heat flow at lower concentrations relative to the MIC and a more potent anti-biofilm activity compared to more slowly acting drugs (such as vancomycin and flucloxacillin). The high variability in heat flow after exposure to rifampin was due to spontaneous development of resistance, as confirmed by susceptibility testing.

In parallel to the microcalorimetric assay, the presence of bacteria in the sonication fluid was assessed by viable counts on conventional cultures (also shown in Figure 2). The culture results from repeated experiments with *S. aureus* (Figure 2C) and *S. epidermidis* (Figure 2G) exposed to rifampin showed a high degree of heterogeneity due to random emergence of resistance, and the data are therefore not shown.

Discussion

A correct and timely microbial diagnosis is a crucial step in determining the treatment of PJI, but antibiotic treatment before collection of periprosthetic tissue samples may lead to false-negative culture results (Kamme and Lindberg 1981, Spangehl et al. 1999, Trampuz et al. 2004, Achermann et al. 2010). In the present study, we investigated the effect of exposure of a young (24-h) biofilm on porous glass beads to antibiotic under in vitro conditions, using sonication and microcalorimetry.

We believe that this experimental study has clinical relevance regarding the challenge of establishing a bacterial diagnosis in cases with previous exposure to antibiotics (e.g. in recurrent infections or antibiotic prophylaxis).

Our materials included 4 bacteria that commonly cause PJI (Zappe et al. 2008, Stefansdottir et al. 2009), but which have differences in virulence pattern, metabolism, and Gram-stainability. Empirical antimicrobial treatment for PJI may include vancomycin and flucloxacillin for Gram-

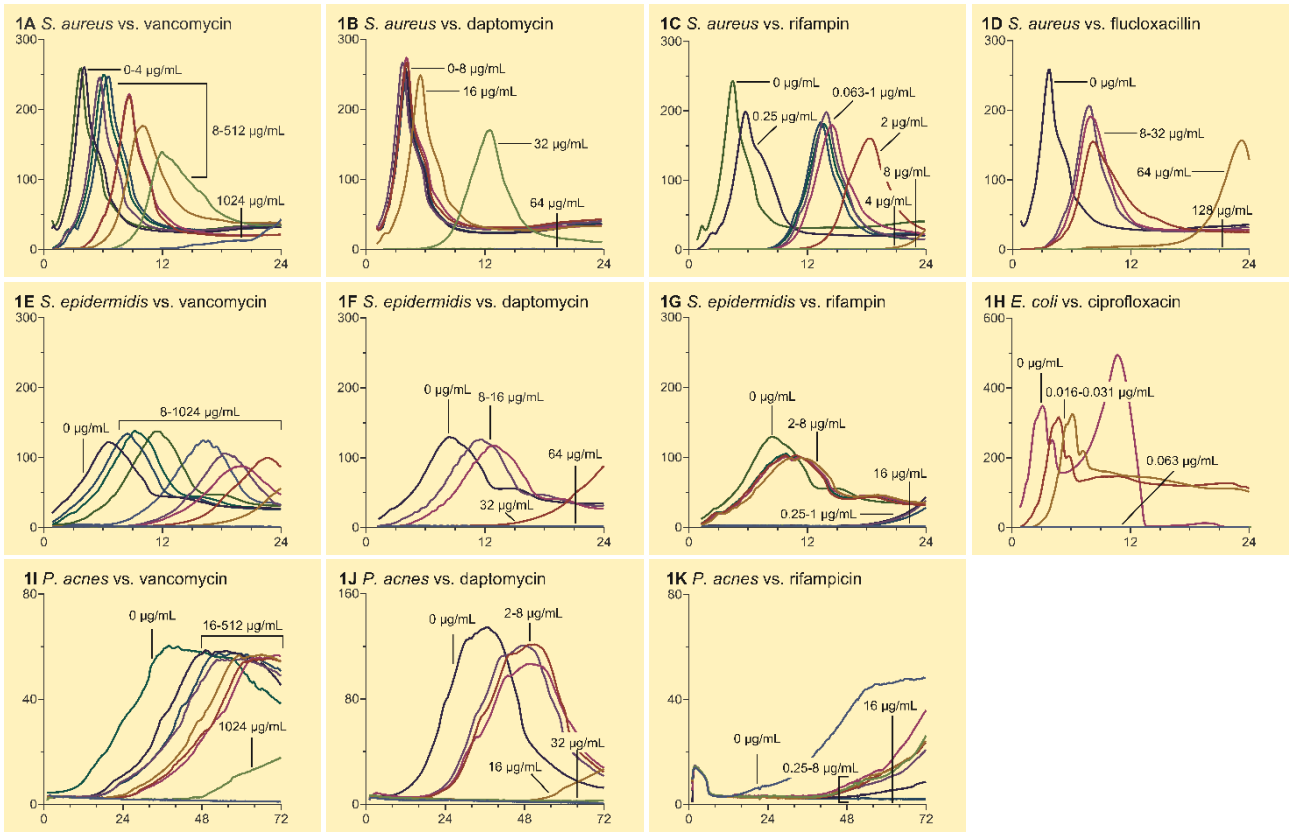

Figure 1. Heat flow (y-axis, µW) development over time (x-axis, hours) of *S. aureus* (panels A–D), *S. epidermidis* (E–G), *E. coli* (H), and *P. acnes* (I–K) exposed to different antibiotics. The numbers above each curve indicate the respective antibiotic concentrations. The range for x-axis is different in the case of *P. acnes* (72 h). Furthermore, y-axis range was changed for non-staphylococci that showed markedly different heat flow levels. The positive controls were biofilms on beads not previously exposed to antibiotics (0 µg/mL). The experiments were performed in triplicate, and a representative experiment is shown.

positive microorganisms or ciprofloxacin for Gram-negative microorganisms (Zimmerli and Moser 2012). Specific combinations with biofilm-penetrating rifampin are recommended under certain circumstances, and novel antibiotics such as daptomycin have been introduced in recent therapeutic studies (Zimmerli and Moser 2012). The rationale for antibiotic selection in this study was to test drugs with different properties regarding mode of action (concentration- or time-dependent), bactericidal activity (fast- or slow-killing), and existence of anti-biofilm activity (Asin et al. 2012).

The limitations of our study design included lack of important in vivo information about conditions such as antimicrobial pharmacokinetics (dose, tissue penetration, repeated administration, duration of treatment), the host immune response, and the fact that the artificial porosity of glass beads might not necessarily reflect that of commonly used prosthetic material. Also, we did not try to investigate the sonication fluid for the possible presence of viable—but non-cultivable—bacteria that produce less metabolism-related and growth-related heat flow.

Concerning antimicrobial susceptibility testing, for all test strains and antibiotics, the biofilm bacteria were 4 to > 1,024 times more resistant than non-adherent, planktonic bacteria, as determined by comparison of microcalorimetry (MHIC) and the MIC. The low doses achieved with systemic antibiotic therapy may alleviate symptoms caused by planktonic bacteria, but this barely affects the biofilm bacteria (Costerton 2005). According to CLSI breakpoints, the planktonic strains were susceptible to all the antibiotics tested. The MHIC for vancomycin was > 1024 µg/mL in the case of staphylococci and *P. acnes* biofilms. Vancomycin has previously been found to be less effective against staphylococcal biofilms (Monzon et al. 2002, Molina-Manso et al. 2013). The inconsistently high MHIC values for rifampin reflect the random emergence of resistance in staphylococci and *P. acnes*, as confirmed by susceptibility testing of the organisms after antibiotic exposure. The observation of spontaneous development of resistance to rifampin shows that a high bacterial concentration is a risk factor for emergence of resistance. These results confirm that rifampin should not be used as a single therapy or before sufficient surgical debridement, as has been recommended previously (Zimmerli et al. 2004, Furustrand Tafin et al. 2012b). The MHIC values were lowest for ciprofloxacin in *E. coli* (0.063 µg/mL), except in 1 of 5 experiments, where the MHIC was 0.25 µg/mL—reflecting the emergence of ciprofloxacin resistance.

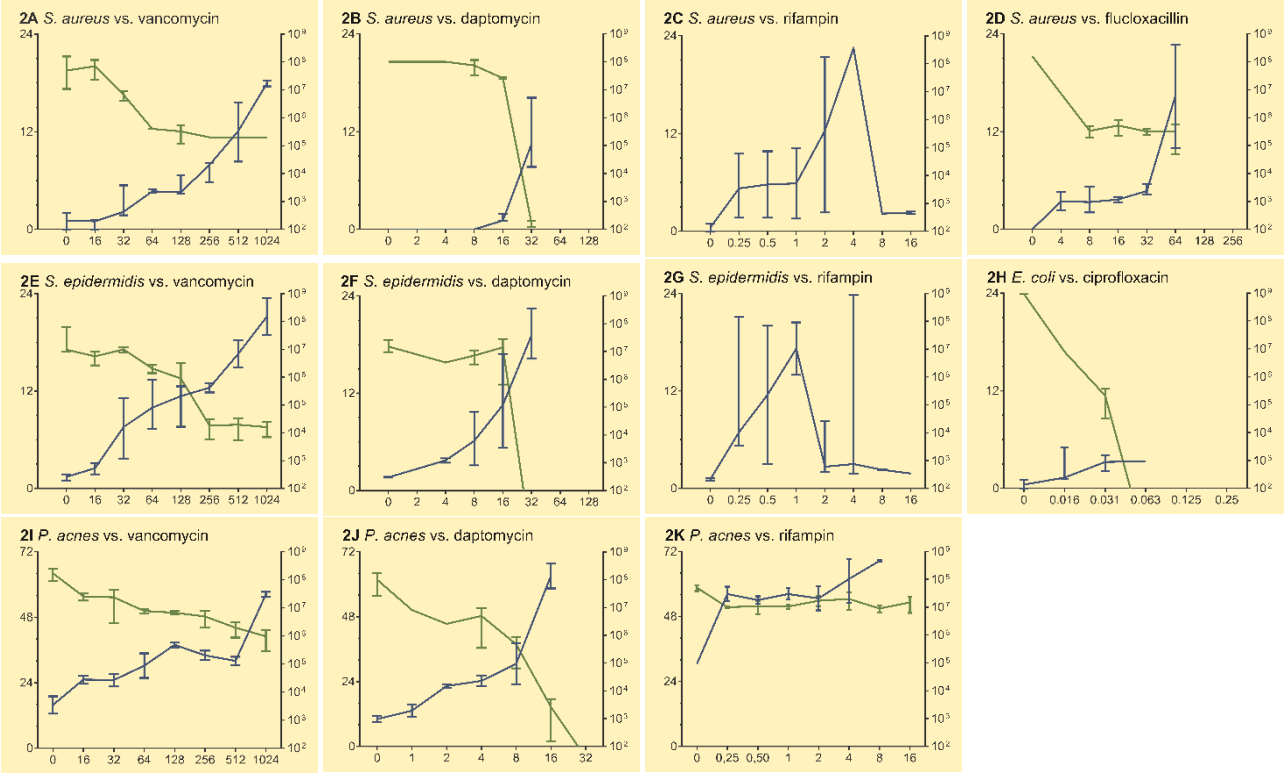

Figure 2. Growth inhibition of various antibiotics on *S. aureus* (panels A–D), *S. epidermidis* (E–G), *E. coli* (H), and *P. acnes* (I–K) in sonication fluid, determined by microcalorimetry and culture. The x-axis shows increasing antibiotic concentrations (µg/mL). The green line represents median bacterial concentration (corresponding to y-axis on right, in CFU/mL) determined by viable count in 3 independent experiments; bars indicate range. The blue line represents median time to detection (corresponding to y-axis on left, in hours) in the microcalorimeter experiment. It demonstrates how significant bacterial heat flow (>10 µW) is detectable within a few hours. Spontaneous emergence of staphylococcal resistance to rifampin was observed in replicates of experiments C and G.

Concerning the effect of antibiotic exposure on detection of biofilm bacteria, vancomycin did not reduce the ability to detect staphylococci and *P. acnes* in biofilms, even at very high (non-physiological) concentrations. Thus, our results call into question the short-term (24-hour) effect of vancomycin, which is often used in bone cement spacer to give high local concentrations, on eradication of biofilm bacteria. Daptomycin was the most active antibiotic, inhibiting staphylococci at 64 µg/mL and *P. acnes* at 32 µg/mL. Ciprofloxacin inhibited *E. coli* even at very low concentrations (MHIC 0.063 µg/mL). Flucloxacillin and rifampin inhibited Gram-positive bacteria, but only at higher concentrations than the MIC. The high culture yield from sonication fluid found in this study suggests that sonication is an efficient method to facilitate detection of biofilm bacteria despite antibiotic exposure (Trampuz et al. 2007a). However, our findings also suggest that using concentration-dependent antibiotics preoperatively may reduce the ability of a sonication-based method to detect the bacteria, whereas time-dependent antibiotics may be safer.

A recent randomized clinical trial did not find impaired intraoperative culture results in PJI as a consequence of using single-dose antibiotic prophylaxis (Tetreault et al. 2014). The authors suggested that perioperative antibiotic prophylaxis should be administered before surgery and before intraoperative sampling. We investigated a young (24-hour) biofilm, which might not be entirely relevant to the clinical situation where infection has usually been present for more than 24 hours. Under these conditions, it appears that a single dose of antibiotics does not reduce the sensitivity of a sonication-culture method. Whether this would also be true for mature biofilms remains to be elucidated.

The combined action of sonication and microcalorimetry might be useful in investigation of pre-exposure of biofilm-embedded bacteria to antibiotic. Furthermore, the high inter-experimental repeatability of the microcalorimetry assay in this and other studies suggests that the method is a valid test for investigation of dislodged biofilms (Clauss et al. 2010, Borens et al. 2013). Additional advantages of microcalorimetry are the fast detection of heat flow (within a few hours) from microbial metabolism in sonication fluid and real-time evaluation of the interaction between antibiotics and biofilm bacteria (Furustrand Tafin et al. 2011). The accurate measuring temperature controlled at ± 0.0001°C allows an analytical sensitivity of ± 0.2 µW, but in this study the experimental detection limit was determined at 10 µW to distinguish microbial heat production from the thermal background (e.g. non-specific heat flow generated by degradation of the growth medium). Time to detection was defined as the duration of a

microcalorimetry experiment to reach 10 μW, and was therefore not equivalent to the earliest sign of increase in heat flow. TTD is inversely proportional to the initial quantity and growth rate of bacteria, and we cannot exclude the possibility that discrete metabolism in viable but non-cultivable bacteria is neglected because of this detection limit.

In conclusion, biofilm bacteria are less susceptible to antibiotics than their non-adherent, planktonic counterparts. Weak growth inhibition was demonstrated with the time-dependent antibiotics (vancomycin and flucloxacillin), whereas the concentration-dependent drugs daptomycin and ciprofloxacin considerably reduced the ability to detect biofilm bacteria. These findings appear to support a recommendation of thorough surgical debridement in order to reduce the bacterial load rather attempts to cure an implant-related deep infection with antimicrobial therapy (Zimmerli et al. 2004).

Seen from a diagnostic point of view, these results might also indicate that preoperative administration of antibiotics has heterogeneous effects on the ability to detect biofilm bacteria. The quantitative and highly reproducible outcome of these procedures calls for further research in diagnosis and treatment of implant-related infections.

All the authors contributed to writing of the protocol. The laboratory work was mainly conducted by CR, with help from UFT and BB. CR and AT wrote the first draft of the manuscript, and all the authors revised it and approved the final version.

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No competing interests declared.

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Appendix B: Manuscript in study II

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Microcalorimetric detection of staphylococcal biofilm growth on various prosthetic biomaterials after exposure to daptomycin

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Running title: Daptomycin against prosthetic biofilm

Contribution of authors

All authors contributed in protocol writing. Laboratory work was mainly conducted by CR with help from ISF and EM. CR wrote the first draft of the manuscript and all authors have revised it and approved the final version.

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Conflict of interest

None of the authors have professional or financial affiliation to bias the presentation. Financial support was provided by the Regional Foundation of Southern Denmark, and the Danish Rheumatism Association.

ABSTRACT

Primary aim of this *in vitro* study was to test the efficacy of daptomycin to eradicate staphylococcal biofilms on various orthopedic implant materials. Secondary aim was to quantitatively estimate the formation of staphylococcal biofilm. We tested six clinically important biomaterials: cobalt chrome, pure titanium, grid-blasted titanium, porous plasma-coated titanium with/without hydroxyapatite, and polyethylene. Biofilms of *S. aureus* and *S. epidermidis* were formed on the samples and thereafter exposed to daptomycin. Samples were subsequently sonicated in order to detect dislodged biofilm bacteria and transferred to a microcalorimeter for real-time measurement of growth-related heat flow. Minimal biofilm eradication concentration (MBEC) was determined as the lowest concentration of daptomycin required to eradicate biofilm bacteria on the sample. Median MBEC of *S. aureus* biofilm on smooth metallic surfaces was lower than the rough metallic surfaces. In experiments with *S. epidermidis*, no pattern was seen in relation to the surface roughness. Regarding the quantitative estimation of staphylococcal biofilm formation on the sample, we found a significantly higher amount of biofilm growth on the rough surfaces than the smooth samples and polyethylene.

In conclusion, the presented study showed that daptomycin could eradicate *S. aureus* biofilm at lower concentrations on the smooth surfaces compared to the rough surfaces, as well as polyethylene. In experiments with daptomycin against *S. epidermidis* biofilms, no pattern was seen in relation to the surface roughness. Furthermore, we demonstrated a faster detection of staphylococcal heat flow due to higher biofilm quantity on the rough surfaces compared to smooth samples and polyethylene.

Keywords: Biofilm, prosthesis, sonication, microcalorimetry, daptomycin

INTRODUCTION

Prosthetic biomaterials are increasingly implanted to restore joint function in a growing elder population.[1,2] Developments in orthopedic arthroplasty surgery have not only increased the mechanical properties and biocompatibility of implant materials, but also improved the surface texture for tissue integration.[3,4]

Factors responsible for metallic implant fixation include physico-chemical bonding mechanisms such as surface charge, hydrophobicity, roughness and porosity.[3,5] These surface-related properties for implant-tissue integration are also believed to influence the ability for microbial adhesion in the 'race for the surface'.[6,7]

Bacteria that adhere to a prosthetic implant grow predominantly in slime-enclosed biofilms. These adherent biofilms are inherently resistant to host defenses and to conventional antibiotic therapy.[8] Comparison and quantification of surface-adherent bacteria on different prosthetic materials was previously performed in experimental and clinical studies.[9,10] In these studies sonication was used for biofilm dislodgement in order to make the surface-attached bacteria eligible for incubation and quantification.

Isothermal microcalorimetry and sonication were recently used to analyze the influence of bone graft material properties on the initial adhesion and formation of biofilm under *in vitro* and *in vivo* settings.[11-14] Microcalorimetry is a highly sensitive and accurate method for the detection of slow-growing microorganisms and reduced bacterial numbers after antibiotic pre-exposure.[15-17] The principle of microcalorimetry relies on microbial heat production related to bacterial growth and metabolism. Without disturbing the growth process, real-time heat flow measurement can estimate bacterial quantity in surface-related biofilms. Decreasing number of replicating bacteria, e.g. due to inhibition after antimicrobial exposure, is equivalent to decreased calorimetric heat production and/or delayed heat flow. Furthermore, our study group recently applied this analytical tool when a strong suppressive effect of daptomycin against staphylococcal biofilm growth on porous glass beads was demonstrated under *in vitro* conditions.[18]

Daptomycin is a fast acting, concentration dependent lipopeptide with potent activity against Gram-positive bacteria. Daptomycin targets the cell membrane rather than the metabolic active pathways, making it more active against metabolically stationary bacteria, such as biofilm bacteria.[19] Though previous *in vitro* studies have demonstrated superior activity of daptomycin against staphylococcal biofilms,[20-22] the influence of physicochemical properties of orthopedic implant materials on the efficacy of daptomycin to eradicate staphylococcal biofilms has not been systematically elucidated. In the present study we directly investigate the ability for daptomycin to inhibit (delayed heat production) and eradicate (no heat production) mature staphylococcal biofilms after 24 hours.

The primary aim of this *in vitro* study was to test the efficacy of daptomycin to eradicate staphylococcal biofilms on various orthopedic implant surfaces and materials. The secondary aim was to quantitatively estimate the formation of staphylococcal biofilm on various implant materials with different surface properties.

We hypothesized that bacterial growth and biofilm formation on prosthetic materials would vary with the different surface properties, and also be variably influenced by exposure to the potent antimicrobial effect of high-dose daptomycin.

MATERIALS AND METHODS

Biomaterials and test organisms.

We tested six clinically available orthopedic implant materials (Table 1) that were purchased at the manufacturers (Biomet Aps, Horsens, Denmark; Ortotech, Kolding, Denmark). Cylindrical test samples were custom-made to fit in a microcalorimeter ampoule (L 10 mm, Ø 6 mm, surface area 270 mm^2) and sterilized at 121°C for 20 minutes.

Table 1: Specifications of the 6 implant materials

Abbreviation	Material	Surface structure	Manufacturer
CoCr	Cobalt-chrome alloy	Smooth, metal*	Biomet
puTi	Pure titanium	Smooth, metal	Ortotech
gbTi	Grid blasted titanium	Rough, metal*	Biomet
pcTi	Porous plasma coated titanium	Porous, metal*	Biomet
pcTi-HA	Pc titanium w/ hydroxyapatite	Porous, metal*	Biomet
UHMWPE	Ultra-high molecular weight polyethylene	Rough, hydrophobic	Biomet and Ortotech

*Four test samples were due to the production method provided with a threaded canal.

Staphylococcal sp. are the most common findings in culture of periprosthetic tissue samples during PJI revision surgery.[23] We investigated two well-characterized reference strains of *S. aureus* (ATCC 29213, methicillin-susceptible) and *S. epidermidis* (ATCC 35984, methicillin-resistant).[13] These ATCC-strains, capable of biofilm production through adherence to and aggregation on biomaterial surfaces, have been used in numerous experimental studies,[12,13,24] and were considered representative of staphylococcal species commonly causing PJI. The susceptibility of these strains has been determined in a previous experimental study analyzing the inhibitory effect of daptomycin against a 24 hours biofilm compared to the minimal inhibitory concentration (MIC) of planktonic bacteria.[18] MIC for daptomycin against *S. aureus* and *S. epidermidis* was assessed as 0.5 µg/ml and 1.0 µg/ml respectively, whereas the minimal heat inhibitory concentration (MHIC) measured by microcalorimetry with sonication fluid was respectively 128 and 64 times higher than MIC.

The bacterial strains were stored at -80°C and cultured overnight on blood agar plates before each experiment. Hereafter a bacterial test-suspension was prepared by 1:100 dilution of a 0.5 McFarland solution. Hence bacterial concentrations in each test-suspension were approximately $1x10^6$ CFU/ml of *S. aureus* and $3x10^5$ CFU/ml of *S. epidermidis* respectively.

Biofilm formation on test samples.

An illustration of the experimental protocol is found in the Electronic supplementary material. For each experiment 6 samples were placed in a 50 ml Falcon tube containing 5 ml tryptic soy broth (TSB), inoculated with 0.25 ml of the bacterial test-suspension and incubated aerobically for 24 hours at 37°C. After the incubation, samples were washed 5 times through rinsing with 20 ml saline and gentle shaking in order to minimize carry-over of planktonic and loosely attached bacteria on the biomaterial surface. After the washing step, one of the samples was selected to act as growth control during the rest of the experiments.

Antibiotic exposure of biofilm.

Serial two-fold dilutions of daptomycin (Novartis Pharma AG, Bern, Switzerland) were prepared in TSB with concentrations ranging from 4x MIC up to 256x MIC. As daptomycin belongs to a calcium-dependent antibiotic group [25], the TSB that received daptomycin, was enriched with calcium chloride (0.3 µg/ml [equivalent to 0.1 µg/ml of Ca^{2+}]). Samples with biofilm embedded bacteria were transferred with sterile forceps to seven individual 4 ml tubes (containing 2 ml TSB and different antibiotic concentrations) and further incubated for 24 hours at 37°C. The eighth tube contained TSB without daptomycin, and the submerged sample acted as growth control when continued incubation resulted in formation of a 48-hour biofilm. Experiments were performed independently in triplicate and accompanied with a negative control of one sterile sample in antibiotic-free medium.

Biofilm dislodgement by sonication.

After antibiotic exposure test samples were transferred to individual 15 ml Falcon tubes with 3 ml saline, vortexed 30 s with maximum power (Vortex Genie 2, Scientific Industries, Bohemia NY, USA), sonicated for 60 s (BactoSonic™, Bandelin electronic, Berlin, Germany) and vortexed for 30 s again to dislodge biofilm embedded bacteria. The mild sonication process made loosely attached biofilm bacteria eligible for plate incubation, whereas more firmly attached biofilm bacteria remained on the material samples for subsequent microcalorimetric evaluation. Sonication fluid in 100 µl aliquots was plated on blood agar plates to detect growth of dislodged biofilm bacteria that survived the antibiotic exposure. Biofilm bactericidal concentration (BBC) was measured as +/- growth in sonication fluid after 24 hours incubation on blood-agar plates.

Detection of biofilm bacteria by microcalorimetry.

The sonicated test samples were transferred to individual 4 ml glass ampoules containing 1 ml TSB. After sealing the ampoules, they were lowered into a 48-channel batch microcalorimeter (thermal activity monitor, model 3102 TAM III; TA Instruments, New Castle, DE). The initial 15 minutes of equilibration ensured a temperature of 37.0000°C to minimize exogenous heat disturbance before the measurements started. Heat was measured continuously for 24 hours with an analytical sensitivity of ±0.2 µW. The results were plotted as heat flow (in µW) over time using the manufacturer's software (TAM Assistant; TA Instruments) and analyzed with Microsoft Excel 2013 (Microsoft Corporation, Richmond, USA).

The minimal biofilm eradication concentration (MBEC) was defined as the lowest antimicrobial concentration killing biofilm bacteria on the sample, leading to absence of regrowth after 24 hours of incubation in the microcalorimeter, indicated by the absence of growth-related heat flow [26].

To distinguish microbial heat production from the thermal background an experimental threshold was set at 10 µW and all measurements below this value were recorded as negative. The time to detection (TTD-50) was defined as the time from the insertion of the ampoule into the calorimeter until the exponentially rising heat flow signal exceeding 50 µW. This measure indirectly quantifies the amount of biofilm bacteria, with a shorter TTD-50 representing a larger amount of bacteria [11]. The maximum heat flow during the experiment, heat flow peak (HFP), is specific to the strain and reduced with decreasing biofilm fitness due to growth inhibition factors, such as antibiotic exposure.[27]

Statistics

MBEC-data on the ordinal scale from 2-256 µg/ml daptomycin are expressed as median and range. Heat flow (µW) and time (hours) are continuous numeric data presented as mean ± standard deviation (SD) and compared between groups by standard (non-parametric) ANOVA. Post-hoc mutual comparisons were performed by unpaired t-test and Wilcoxon test adjusting for multiple comparisons using the statistical software R (www.R-project.org) and Prism 7.0 (GraphPad Software, San Diego, CA, USA) for comparison of mean HFP and mean TTD-50 of *S. aureus* and *S. epidermidis* biofilms. P-value <0.05 was accepted as significant.

RESULTS

In the present study we directly investigate the efficacy of daptomycin to inhibit (delayed heat production) and eradicate (no heat production) mature staphylococcal biofilms after 24 hour incubation on various orthopedic implant surfaces and materials.

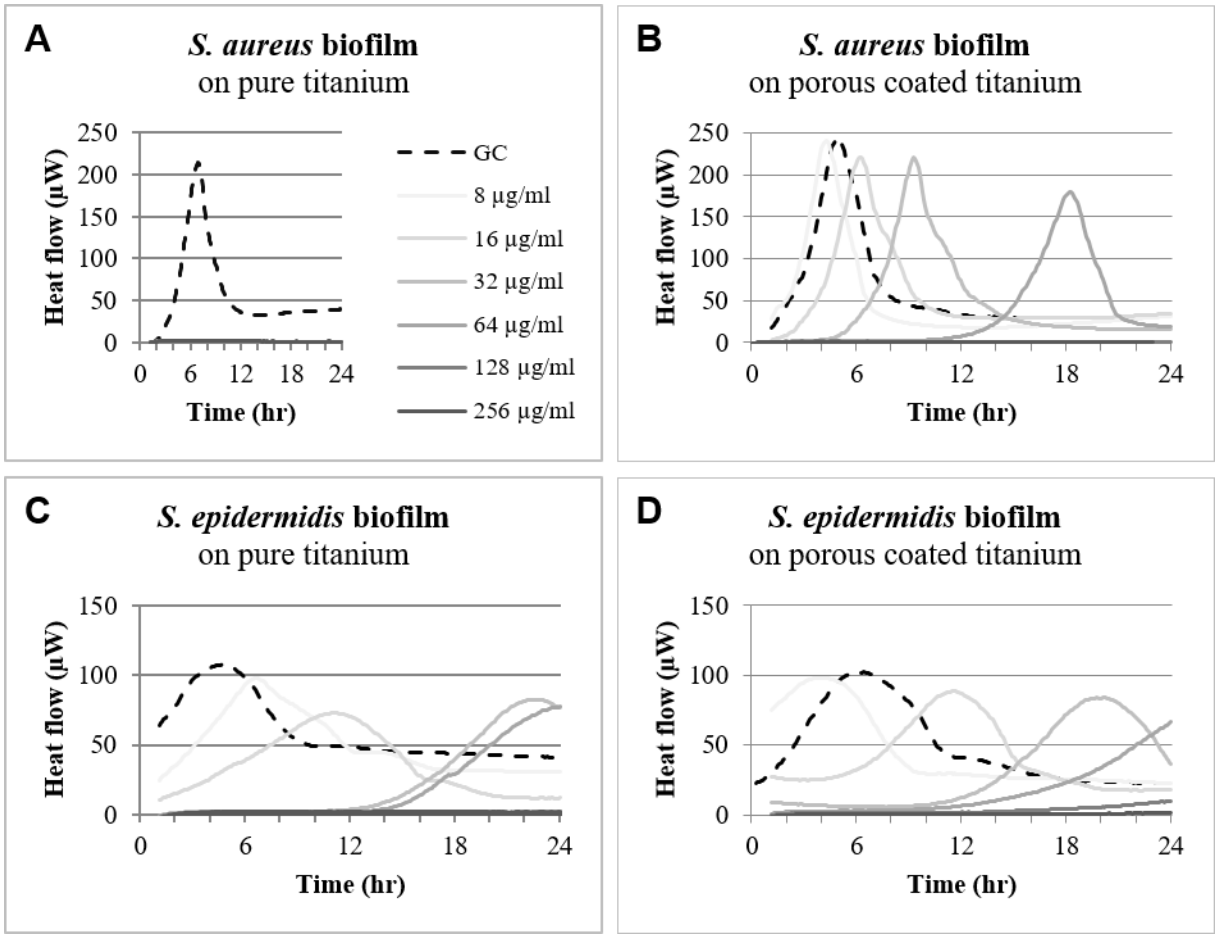

Figure 1 A-D: Heat flow-time curves generated by S. aureus (A+B) and S. epidermidis (C+D) biofilm on pure titanium and porous plasma-coated titanium after exposure to increasing daptomycin concentrations (8-256 µg/ml). Patterns indicate daptomycin concentrations (in µg/ml). GC denotes growth control (biofilm-covered sample submerged in TSB without daptomycin).

The efficacy of daptomycin to eradicate staphylococcal biofilms.

Figure 1 A-D show four representative heat flow curves of viable biofilm bacteria on the samples after antibiotic exposure. The time shift of curves to the right shows the delayed bacterial detection due to lower quantity of biofilm bacteria on the test samples after exposure to increasing concentrations of daptomycin. This picture shows that bacterial regrowth was successively inhibited until the lowest antimicrobial concentration killing biofilm bacteria on the sample, indicated by absence of growth-related heat flow, which is defined as the MBEC. Median MBEC of daptomycin against staphylococcal biofilms on various orthopedic implant surfaces and materials is displayed in Table 2.

Table 2: Minimal biofilm eradication concentration of daptomycin

MBEC (mg/l)	*S. aureus* (ATCC 29213)	*S. epidermidis* (ATCC 35984)
CoCr	8 (8;8)	128 (64;128)
puTi	4 (4;8)	128 (128;256)
gbTi	128 (32;128)	16 (8;64)
pcTi	128 (128;256)	128 (128;256)
pcTi-HA	128 (128;128)	64 (64;64)
UHMWPE	64 (64;64)	128 (128;128)

Results from sonication and microcalorimetry on biofilm embedded test samples after exposure to increasing daptomycin concentrations (µg/ml), displayed as median (range). Test samples consisted of various biomaterials: cobalt-chrome (CoCr), pure titanium (puTi), grid blasted titanium (gbTi), porous plasma coated titanium (pcTi), porous plasma coated titanium with hydroxyapatite (pcTI-HA), and ultra-high molecular weight polyethylene (UHMWPE).

Comparison of the MBEC of daptomycin against *S. aureus* biofilms on various test samples show that variations are related to the surface structure of the material used (Figure 2A). With smooth metallic surfaces of cobalt-chrome and pure titanium regrowth of *S. aureus* biofilm was absent at 4-8 µg/ml, equivalent to daptomycin concentrations 8-16 times higher than the MIC (0.5 µg/ml). With the remaining test samples having a macroscopic rougher surface structure the MBEC of *S. aureus* biofilms to daptomycin was higher (32-256 µg/ml). In experiments with *S. epidermidis* biofilms (Figure 2B) the MBEC variated from 8 to 256 µg/ml, but no pattern was seen in relation to the surface roughness.

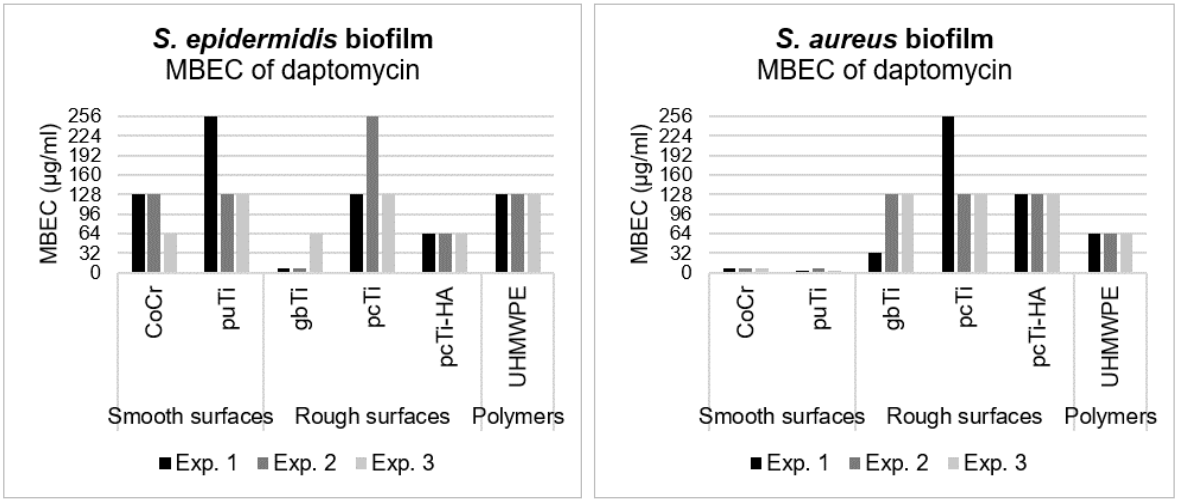

Figure 2 A-B: Minimal biofilm eradication concentration (MBEC, median and range) of daptomycin against staphylococcal biofilms in triplicate experiment (Exp. 1-3).

Quantitative estimation of staphylococcal biofilm.

The heat flow curves of growth controls without previous antibiotic exposure (broken lines in Figure 1 A-D) have a characteristic shape for each test bacterium, including the heat flow peak. Table 3 shows heat flow of staphylococcal biofilm on different materials without antibiotic exposure. HFP did not vary significantly in experiments with the same bacterial strain on different test materials. On the contrary when comparing growth-related heat flow of *S. aureus* and *S. epidermidis*, mean (±SD) HFP showed significant difference among the strains (202 ±22 µW and 105 ±9 µW respectively; $p<0.001$).

Table 3: Microcalorimetric analysis of biofilm formation on different biomaterials

		TTD-50	**HFP**
S. aureus (mean ±SD)		(hour)	(µW)
Smooth	CoCr	6.7 ±0.2	180 ±3
	puTi	6.8 ±0.5	180 ±22
Rough/porous	gbTi	3.6 ±0.5	200 ±7
	pcTi	1.9 ±0.1	228 ±13
	pcTi-HA	1.2 ±0.6	208 ±19
Polymer	UHMWPE	5.3 ±0.5	214 ±9
Overall, *S. aureus*		4.2 ±2.2	202 ±22
S. epidermidis (mean ±SD)			
Smooth	CoCr	3.7 ±0.2	103 ±6
	puTi	4.1 ±1.4	113 ±10
Rough/porous	gbTi	1.5 ±0.6	103 ±6
	pcTi	3.2 ±0.1	103 ±7

	pcTi-HA	1.3 ±0.8	111 ±3
Polymer	UHMWPE	3.4 ±0.2	98 ±8
Overall, *S. epidermidis*		2.9 ±1.3	105 ±9

Heat flow of bacterial growth on sonicated samples with no antibiotic exposure. Time to detection (TTD-50): Experimental duration (hours) when heat flow exceeds 50 µW. Heat flow peak (HFP): Maximum heat flow peak reached during the experiment. Cobalt-chrome (CoCr), pure titanium (puTi), grid blasted titanium (gbTi), porous plasma coated titanium (pcTi), porous plasma coated titanium with hydroxyapatite (pcTI-HA), ultra-high molecular weight poly-ethylene (UHMWPE).

The bacterial amount on the biofilm embedded test samples was indirectly quantified by microcalorimetric analysis of the time to reach 50 µW (TTD-50) after 24 hour incubation. A highly viable and quantitatively strong biofilm has faster initial heat flow development due to growth and metabolism than a weak and more dormant biofilm. Initial heat flow developed faster with S. epidermidis biofilms (mean TTD-50: 2.9 ±1.3 hours) compared to S. aureus (4.2 ±2.2 hours).

Figure 3A shows variations of TTD-50 in triplicate experiment with S. aureus biofilm on various materials. The heat flow developed differently between the surfaces (Friedman's test, $p<0.01$) and post hoc comparisons showed that the heat flow developed faster with the rough surfaces (gbTi, pcTi and pcTi-HA) in comparison to the smooth surfaces (CoCr, puTi) and polymer (UHMWPE) ($p<0.001$, Bonferroni corrected). This indicates a higher quantity of S. aureus biofilm on the macroscopically rough surfaces solely, as the smooth surfaces (CoCr, puTi) and polymer (UHMWPE) did not indicate any difference ($p>0.2$). Regarding S. epidermidis (Figure 3B), we also observed significantly different heat flow development between the surfaces (Friedman's test, $p=0.03$). Post hoc comparisons showed that the heat flow developed faster with the rough surfaces (gbTi and pcTi-HA) in comparison to 2/3 of the smooth surfaces (CoCr, puTi) and polymer (UHMWPE) ($p<0.01$, Bonferroni corrected). TTD-50 for CoCr, puTi, pcTi and UHMWPE did not indicate any difference ($p>0.40$).

The vortex-sonication method allowed visual growth-detection of dislodged bacteria on blood agar plates after 24 hour incubation. These results confirmed those obtained by microcalorimetry (data not shown).

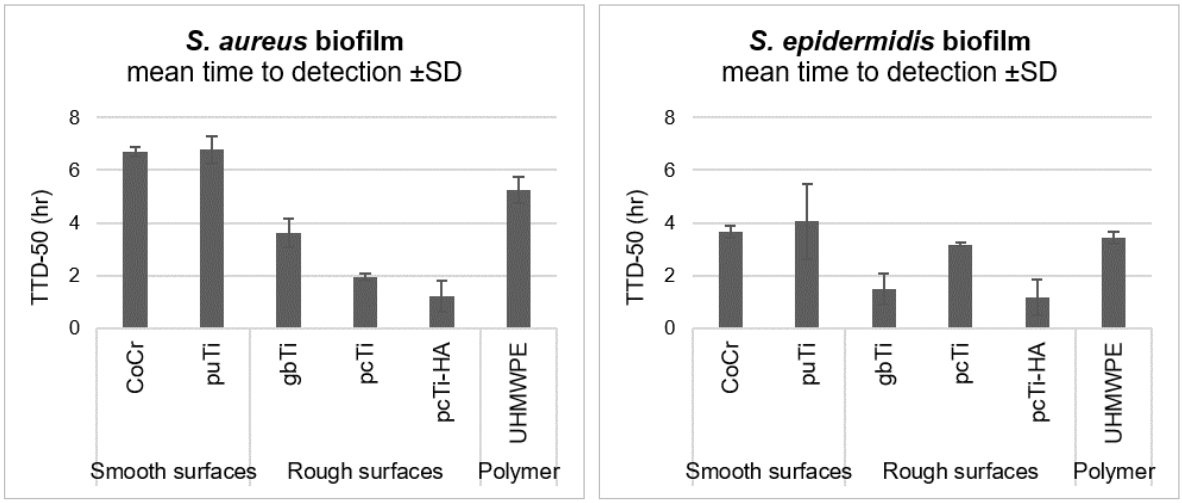

Figure 3 A-B: Microcalorimetric analysis of staphylococcal biofilm in triplicate experiment. Time to detection (TTD-50, mean ± SD) indicates the time needed to reach the heat flow of 50 µW.

DISCUSSION

In the present *in vitro* study we directly investigated the efficacy of daptomycin to eradicate mature staphylococcal biofilms after 24 hour incubation on various orthopedic implant surfaces and materials. With *S. aureus* biofilm on smooth metallic surfaces, the MBEC of daptomycin was 4-8 µg/ml, whereas the efficacy of daptomycin against *S. aureus* biofilm on test samples with rough surface structure was lower (MBEC 32-256 µg/ml). In experiments with *S. epidermidis* biofilms, MBEC variated from 8 to 256 µg/ml, but no pattern was seen in relation to the surface roughness.

We furthermore examined microbial heat production of the antibiotic-free growth controls in order to investigate quantitative variations of staphylococcal biofilm formation. With *S. aureus* biofilms we found a significantly higher heat flow rate on the rough surfaces than the smooth samples and polyethylene. In experiments with *S. epidermidis* biofilms, the heat flow rate showed less variation, but again we observed a significantly higher heat flow rate on 2/3 of the rough samples.

We investigated mature staphylococcal biofilms on six clinically important orthopedic implant materials, namely cobalt chrome alloy, pure titanium, grid-blasted titanium, porous plasma-coated titanium without/with hydroxyapatite, and ultra-high molecular weight polyethylene. We hypothesized that bacterial growth and biofilm formation on prosthetic materials would vary with the different surface properties, and would also be variably influenced by the exposure to the potent antimicrobial effect of daptomycin.

The biofilm evaluations were performed according to a well-established protocol including sonication, culture and microcalorimetric heat flow measurement previously used to analyze the influence of physicochemical biomaterial properties,[11-14] as well as studies of antimicrobial susceptibility of biofilm bacteria.[15-17,20,26,28]

We were able to demonstrate microbial heat production during growth and regrowth of biofilm bacteria on all biomaterials after 24 hour incubation. In order to minimize the influence of free-floating and loosely attached planktonic bacteria, samples underwent a 5-step washing procedure. After a 24-hour antibiotic exposure the more loosely attached bacteria were removed during a mild sonication process. Thus leaving the more firmly attached biofilm bacteria for subsequent microcalorimetric investigation. We benefited from the ability to insert the biofilm-covered samples into the microcalorimeter, as we measured the heat flow directly from bacterial growth on the samples rather than detached bacteria in sonication fluid.

Limitations of this study design include lack of important *in vivo* conditions, such as antimicrobial pharmacokinetics (dose, tissue penetration, repeated administration, duration of treatment) and host factors (tissue reaction, immune response). Furthermore different production methods were applied by the manufacturers, hence the test samples of four materials were provided with a threaded canal with resulting alteration in the macroscopic surface structure and surface area. Finally, during microcalorimetric measurement of real-time heat flow, direct demonstration of biofilm bacteria by traditional fluorescent staining and microscopy is not applicable.

The efficacy of daptomycin to eradicate staphylococcal biofilms.

The range of tested daptomycin concentrations in this study runs from 4-256 µg/ml in two-fold steps of categorical variables, and interpretation of MBEC results should be seen in this light. Thus median MBEC of 128 µg/ml (range 64-256) correlate with median plus/minus one concentration step. Seen from a clinical point of view, a local tissue concentrations of daptomycin above e.g. 20 µg/ml is hardly achievable, though.[29]

Median MBEC of daptomycin was found at 128 µg/ml in 7 out of 12 different combinations of material and staphylococcal spp. Similar levels of daptomycin susceptibility have been reported in other experimental studies of staphylococcal biofilms.[18,21,30] The experimental detection limit was determined at 10 µW to distinguish microbial heat production from the thermal background (e.g., nonspecific heat flow generated by degradation of the growth medium). We cannot exclude that discrete metabolism in viable but non-culturable bacteria is neglected due to this detection limit, and complete bacterial eradication hence was not achieved at the reported MBEC levels.

Biofilms of *S. aureus* on smooth metallic surfaces were eradicated at lower daptomycin concentrations (8-16 times MIC) compared to rough/porous metallic surfaces (64-512 times MIC). This finding is in line with theories of biofilm resistance and tolerance mechanisms including incomplete penetration and altered chemical microenvironment in the extracellular biofilm matrix.[27,31,32] With *S. epidermidis* biofilms, heterogeneity was observed in daptomycin susceptibility and variations could not be correlated to the surface structure in our study. Whether this difference between the bacterial strains is an effect of physicochemical properties which has not been taken into account or simply a matter of measuring variation is however unclear. Biofilm formation is a multifactorial process, and opposing physicochemical properties related to the sample materials (e.g. hydrophobicity, charge and pH) or unintendedly by the study protocol (e.g. chemical composition of culture medium) may blur the overall results in this experimental study.

Quantitative estimation of staphylococcal biofilm.

In order to investigate quantitative variations of staphylococcal biofilm formation on different biomaterials we examined microbial heat production of the antibiotic-free growth controls. When studying biofilm with microcalorimetry, duration of an experiment to reach a certain heat flow limit or heat flow peak are inversely proportional to the bacterial quantity and growth rate.[13,15,33] TTD-50 was defined as the time from insertion of an ampoule into a microcalorimeter until exponential growth produced a heat flow value of 50 µW. This initial lag phase exceeds the experimental threshold of 10 µW in order to take into account the heterogeneous starting point of those experiments that already produced high amounts of heat flow when measurements started after the equilibration period [11].

TTD-50 was used to compare biofilm performance on different biomaterials. We found significantly lower time variables when grid-blasted and porous titanium samples were used with *S. aureus* and *S. epidermidis* (except the combination of *S. epidermidis* and pcTi). This indicates a higher surface area, biofilm affinity and holding capacity of rough/porous metallic surfaces in comparison to smooth surfaces and polyethylene. Our findings confirmed previous reports showing a higher staphylococcal biofilm production on biomaterials with rough and porous topography due to more available biofilm binding-sites.[9,13,34]

Factors determining biofilm formation specifically on polyethylene includes altered hydrophobicity and surface charge, which in turn is differently influencing bacterial subspecies.[7,35] Clinical reports in the literature do not agree whether polyethylene components of infected joint prosthesis should be regarded

as more or equally prone to biofilm formation than other prosthetic materials.[10,36,37] Recent *in vitro* studies found reduced adhesive ability of *S. aureus* and *S. epidermidis* on vitamin E blended UHMWPE in comparison to standard UHMWPE samples.[38,39]

In conclusion, the presented study showed that daptomycin could eradicate *S. aureus* biofilm at lower concentrations on the smooth surfaces of CoCr and puTi compared to the rough surfaces of gbTi, pcTi and pcTi-HA, as well as polyethylene. In experiments with daptomycin against *S. epidermidis* biofilms, no pattern was seen in relation to the surface roughness. Furthermore, we demonstrated a significantly faster detection of staphylococcal heat flow due to higher biofilm quantity on the rough surfaces compared to smooth samples and polyethylene. This is an indication of a higher biofilm affinity and holding capacity of rough surfaces. Findings in the present study indicate that orthopedic biofilm infections may not be sufficiently treated with parenteral administration of daptomycin in clinically achievable doses alone.

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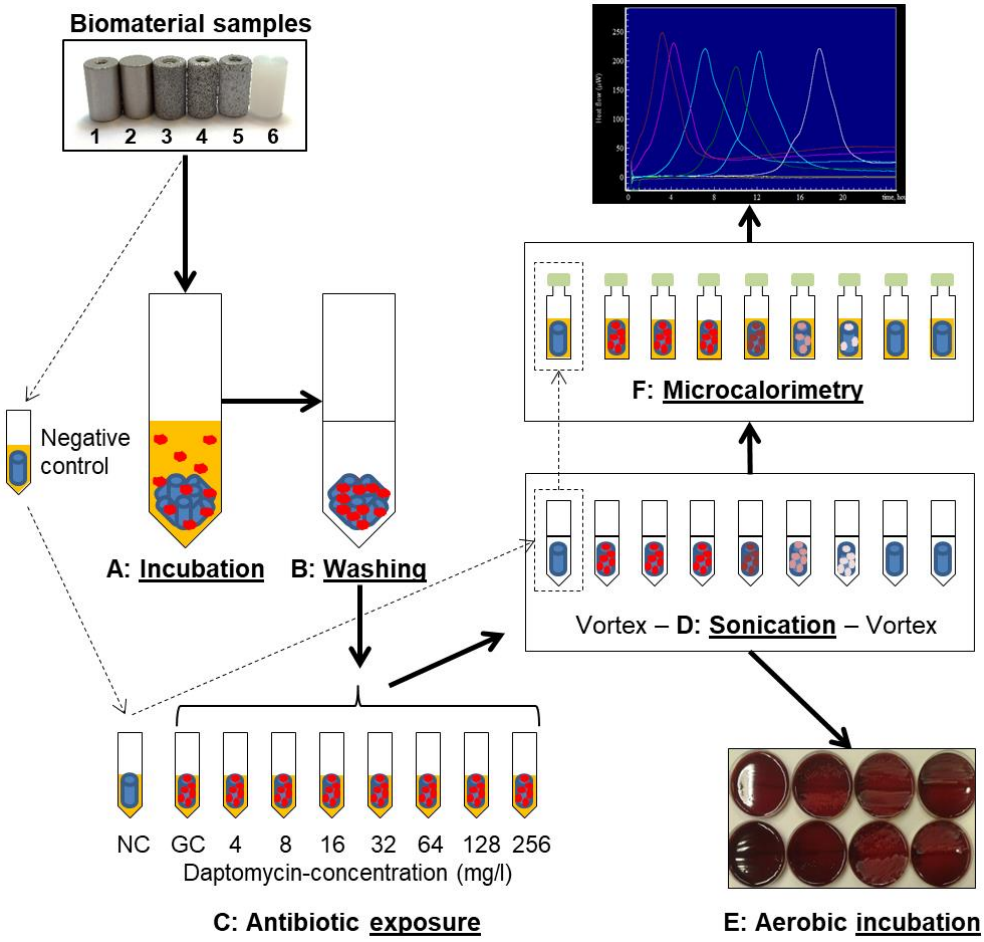
Electronic supplementary material:



Schematic overview of the study protocol. A: Biofilm formation (24 h). B: Gentle washing. C: Exposure to daptomycin (24 h). D: Sonication. E: Culture of sonication fluid. F: Microcalorimetry of samples (24 h).

Appendix C: Manuscript in study III

Submitted for Acta Orthopaedica

The additive effect of sonication on bacterial diagnosis in a prospective cohort study of 211 revisions of THA and TKA

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Conflict of interest statement (this information must be consistent with the information entered in the ICMJE Uniform Disclosure Form for Potential Conflicts of Interest):

This work was financially supported by the Research Councils of the Region of Southern Denmark, Odense University Hospital and Lillebaelt Hospital Vejle, as well as Danish Rheumatism Association.
Each author certifies that he has no commercial associations (e.g., consultancies, stock ownership, equity interest, patent/licensing arrangements, etc.) that might pose a conflict of interest in connection with the submitted article.

Running title: Prosthetic sonication I n revision TJA

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Ethical review committee statement

In this non-interventional study design, clinicians were blinded to the results from sonication-culture and the included patients were thus not exposed to any risk due to the study conduct. After presentation of the study protocol, the local ethical committee stated that this study did not require formal approval. Regional scientific authorities and the Danish Data Protection Agency approved the study protocol (Ref: 2012-41-0826).

Statement of the location where the work was performed

This work was performed at the Department of Orthopaedic Surgery and Traumatology, Odense University Hospital, Odense, Denmark; the Department of Orthopaedic Surgery, Lillebaelt Hospital Vejle, Vejle Denmark; and the Department of Clinical Microbiology, Odense University Hospital, Odense, Denmark.

ABSTRACT

Background: Addition of sonication fluid culture (SFC) to the diagnostic strategy for prosthetic joint infection (PJI) may increase the diagnostic yield in comparison to tissue sample culture alone. Until the present investigation, no large series of consecutive total hip (THA) and knee arthroplasty (TKA) revision surgery have compared the sonication-culture method against a PJI reference standard independent of SFC since the index study from 2007. Furthermore, no previous study has evaluated the clinical course of patients having additional microbiological findings after sonication fluid culture.

Purpose:

1. What is the additive effect of the sonication fluid culture (SFC) on diagnosis of bacteria in THA and TKA revisions in relation to PJI?
2. What is the fate of prosthetic joint revisions, which had additional microbial findings in sonication fluid culture?

Methods: We prospectively analyzed explanted prosthetic materials from consecutive THA and TKA revision surgery performed on any indication during one year. We used a multi-criteria reference standard to verify the diagnosis of PJI. Microbiological results from conventional periprosthetic tissue sample culture (TSC) and SFC was compared against the reference standard. In cases with suspected deep infection, thorough debridement was performed during revision surgery, and routine antibiotic treatment was dicloxacillin for 6-8 weeks. Minimum follow-up was 1 year.

Results: In 211 included revision cases, sonication showed 11 more case with positive culture, which were not found in conventional culture samples of synovial fluid and periprosthetic tissue. PJI was verified in 54 cases, and microbial diagnosis was achieved in 40 (74%) and 48 (89%) cases by either TSC or SFC. Bacterial findings were similar in all concordant cases. The combined culture result of conventional samples and sonication fluid gave a microbial diagnosis in 53/54 (98%) verified PJI-cases.
The fate of 11 revision cases, which had additional findings in SFC, included re-revision in 5 cases, antibiotic suppression in 1 case, and a painful and unsolved joint condition in 3 cases, whereas only 1 case had an asymptomatic prosthesis. Finally, 1 patient diseased in circulatory failure after 466 days.

Conclusion: The additive effect of the sonication-culture method was bacterial growth in 11/211 revisions of THA and TKA, which were not diagnosed by conventional methods. The fate of these cases included re-revision in 5/11. From a clinical perspective, patients with additional microbial findings by SFC had a discouraging prognosis and may represent true positive findings that have to be taken into consideration in the infection treatment.

INTRODUCTION

Background

Prosthetic joint infection (PJI) remains one of the most serious complications of prosthetic joint implantation with an estimated incidence of 1% [9, 10]. The diagnosis of PJI is often estimated by a combination of objective criteria [23]. A new multi-criteria definition of PJI have been suggested from The International Consensus Meeting (ICM) on PJI [15]. An accompanying consideration in the proceedings state that 'clinically, PJI may be present without meeting these criteria, specifically in the case of less virulent organisms'. This means that, these diagnostic criteria do not exhaustively divide a cohort of prosthetic joint revisions into definite PJI and non-infected, but may introduce a third category of 'possible PJI' [14].

Determining the treatment strategy in a clinical case of PJI is dependent on a correct microbial diagnosis, and conventional microbiological sampling based on periprosthetic tissue samples and synovial fluid is often insufficient [6, 16]. A suggested explanation to false-negative culture results is related to the biofilm-nature of implant-related infections, where the surface is colonized with aggregates of microorganisms encased in a self-produced matrix [4, 7]. Thus, culture-negative results in conventional synovial and tissue samples do not necessarily exclude infectious etiology.

Rationale

Sonication has been suggested to dislodge the surface attached biofilm bacteria and make them available for subsequent culture. Culture of sonication fluid from removed hip and knee implants after 331 prosthetic revisions (79 cases of PJI) was investigated in a prospective trial by prof. A. Trampuz and colleagues [20]. Sonication fluid culture (SFC) was found more sensitive than periprosthetic tissue culture for the microbial diagnosis of PJI. Recently a meta-analysis evaluated twelve studies on sonication of prosthetic components for diagnosis of PJI [21], but no large series of consecutive total hip (THA) and knee arthroplasty (TKA) revision surgery have compared the sonication-culture method against a PJI reference standard independent of SFC since the groundbreaking study of Trampuz et al [20]. Furthermore, no previous study has evaluated the fate of patients having additional microbiological findings after sonication fluid culture.

Objectives

1. What is the additive effect of the sonication fluid culture (SFC) on diagnosis of bacteria in total hip (THA) and knee arthroplasty (TKA) revisions in relation to PJI?
2. What is the fate of prosthetic joint revisions, which had additional microbial findings in sonication fluid culture?

METHODS

Study design and setting

This prospective cohort study was performed at our institutions (XXX University Hospital and regional YYY Hospital) during the one-year study period (2012.11.01-2013.10.31). This cohort study is reported according to the STROBE guidelines (www.strobe-statement.org).

Participants

Inclusion criterion was revision of THA or TKA, which was defined as removal of prosthetic components on any indication. The perioperative management of patients was conducted in a setup of care-as-usual. Routine antibiotic treatment with dicloxacillin was administered for maximum 5 days from the day of revision. As soon as the results of TSC were analyzed with antibiogram, a targeted antibiotic treatment was initiated. In culture-negative cases with revision indicated by expected deep infection, empirical antibiotic treatment was dicloxacillin administered intravenously for 2 weeks followed by 4 weeks of oral treatment. Revision patients were routinely seen in the outpatient clinic after 3 and 12 months, where clinical examination and any antibiotic treatment were documented. Furthermore, the medical records were additionally checked for information related to infection treatment or subsequent reason to re-operation of the arthroplasty joint.

Variables, Data Sources and Bias

Before the inclusion period, all surgeons were instructed in data collection with standardized checklists. One project coordinator at each institution performed case identification and collection of checklists. Data were electronically entered in an online database (Topica, hosted by the Region of ZZZ) by independent data-managers. A serial number linking to civil registration number, patient identity, gender and birthdate, as well as prosthetic joint location (hip/knee, right/left) identified each revision case.

Pre- and intraoperative investigation: Preoperative data included symptoms, signs, revision indication and comorbidity. Patients were asked for recent (within four weeks) events of either surgery, dental treatment, wound injuries, infections (e.g. urinary tract infection with *E. coli* 10 days prior to symptom debut) and antibiotic treatment (e.g. Mecillinam, 400 mg x3 from 14-8 preoperative days). Latest surgery of the same joint was registered with indication, type and date (or year, if the latest surgery was more than two years prior to the present revision). Based on the duration of symptoms and time interval from latest total joint surgery patients were categorized as proposed by Zimmerli in 2014 [22]: Early postoperative symptoms (debut within first month), chronic symptoms (debut after first month, manifest >3 weeks), or late acute symptoms (debut after first month, manifest <3 weeks). The level of C-reactive protein in plasma (p-CRP) was analyzed according to thresholds of the international consensus meeting [15]: 10 mg/l when the latest surgery was >3 months before (no accompanying fracture, cancer or rheumatic disorder), otherwise 100 mg/l. Intraoperatively, the surgeon described synovial fluid quality as either normal, purulent, shady or bloody. The latter two observations may be difficult to distinguish from purulence, and are hence merged under the term 'uncertain synovial fluid'.

Conventional culture tests: In every case, one sample of synovial fluid was aspirated during revision surgery immediately before arthrotomy in order to secure access to the joint space and reduce the risk of contamination from skin flora. Situations with fruitless joint aspiration are referred to as 'dry tap' [1, 19]. Sampling of five periprosthetic tissue biopsies was performed according to local guidelines with a sterile set

of five forceps and five surgical knives from a representative area adjacent to the prosthesis [13]. The first dose of perioperative antibiotic prophylaxis was administered immediately after biopsy sampling. Each biopsy was placed in a separate container, transported to the local department of clinical microbiology (DCM). The standard culture method implies plating on 5% horse blood agar (Statens Serum Institut (SSI), Copenhagen, Denmark), and incubation in an aerobic atmosphere with 5% CO2 for five days, K-vitamin enriched chocolate agar (SSI) cultured anaerobically for five days. Cultured organisms were identified by Matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF), using either MALDI Biotyper (Bruker, Bremen, Germany) or Vitek MS (bioMérieux, Marcy l'Etoile, France) instrument.

Culture-results of the tissue samples are registered with the proportion of culture-positive per five samples (e.g. 3/5). When more than five biopsies were sampled (i.e. 6-10 samples) and all are culture-positive, the result is registered as 5/5, whereas situations where all samples are culture-negative are registered as 0/5. Furthermore, one situation (#113) with 2/6 culture-positive samples is registered as 2/5 (this interpretation is indicated with † in Table 3). We will in the following distinguish between positive tissue sample culture (≥3/5) and positive culture of conventional samples (≥3 identical microbial findings in either 5 periprosthetic tissue samples and/or positive TSC 2/5 combined with identical finding in one synovial fluid sample).

The sonication-culture test: Handling of the explanted prosthetic components was conducted in parallel to the conventional diagnostic procedure. One dedicated laboratory technician blinded to the clinical situation performed all steps from registration and sonication to interpretation and documentation. All prosthetic components were transferred to a sterile plastic box with a suitable volume of 0.6-3.9 liters (HPL-series, Lock&Lock GMBH, Frankfurt am Main, Germany), which was sealed airtight and labeled. At XXX University Hospital, the boxes were sent directly after revision surgery to the DCM and processed either immediately or the following day. From YYY Hospital, boxes were transported to the DCM at XXX by car (one hour) the following morning. No transportation from YYY or sonication at XXX was undertaken during weekends and holidays. The sealed boxes were kept at 5 °C from ex-plantation until beginning of the diagnostic procedure. Approximately 300 ml saline (0.9% NaCl) was added to each box in order to cover the prosthesis. The following sonication-culture method was previously described [3]: initial shaking for 30 sec., followed by sonication for 60 sec. using a ultrasound bath (BactoSonic 14.2, Bandelin, Berlin, Germany) and final shaking for another 30 sec. Aliquots of 0.2 ml sonication fluid were sampled under laminar airflow and plated on 5% horse blood agar (incubated in an aerobic atmosphere with 5% CO2 for five days), K-vitamin enriched chocolate agar (cultured anaerobically for five days), and in thioglycollate enrichment broth for fourteen days. Growth media were checked daily to register time (days) from incubation to visible growth. The number of colony-forming units (CFU/ml) was enumerated. A diagnostic threshold at 20 CFU/ml was used to distinguish infected from possibly contaminated prostheses. The non-quantitative result of thioglycollate-culture was noted for comparison, but not in itself accountable for the diagnostic outcome. Cultured organisms in sonication fluid were identified by MALDI-TOF.

Reference standard: Where an indisputable gold standard does not exist (i.e. PJI), the best available practice may be referred to as 'reference standard' [17]. In the present study, we use a reference standard containing elements from leading European and American expert opinions [15, 23] (Table 1). A diagnosis of PJI is verified when one of four situations are identified.

In situations where a clinical suspicion of PJI cannot be clearly determined, we suggest a second category of 'possible PJI' with ≥2/8 observations identified. Finally, the prosthetic joint is considered non-infected at revision surgery, when five negative conditions are concurrently fulfilled.

Table 1: Reference standard for categorization of revision THA and TKA

Verified prosthetic joint infection (either of the following four situations are identified)
5. Presence of a sinus tract communicating with the prosthetic joint, OR 6. ≥3 identical microbial findings in culture of 5 tissue samples, OR 7. Identical microbial findings in culture of 2/5 tissue samples AND 1 synovial fluid sample, OR 8. Combination of at least three of the following four minor criteria: - Microbial growth in ≥1 periprosthetic tissue samples, AND/OR - Culture of identical microorganism in synovial fluid, AND/OR - Purulence of synovial fluid or implant site, AND/OR - Elevated plasma C-reactive protein in blood test, threshold below iii. 0-90 days postoperative: CRP > 100 mg/L iv. >90 days postoperative: CRP > 10 mg/L (not fracture, rheumatoid disease, cancer)

Possible prosthetic joint infection (at least two of the following eight observations are identified)
- Microbial growth in ≥1 periprosthetic tissue samples, AND/OR - Culture of a microorganism in synovial fluid, AND/OR - Purulence of synovial fluid or implant site, AND/OR - Elevated plasma C-reactive protein in blood test (threshold below), AND/OR iii. 0-90 days postoperative: CRP > 100 mg/L iv. >90 days postoperative: CRP > 10 mg/L (not fracture, rheumatoid disease, cancer) - Painful AND warm/red prosthetic joint with acute onset (recent 0-3 weeks) , AND/OR - Suppurating wound defect 14 days after previous joint surgery, AND/OR - Antibiotics administered for ≥2 days within 2 weeks prior to prosthetic revision surgery - Previous revision indicated by deep infection within last year

Non-infected prosthetic joint at revision surgery (all of the following conditions are fulfilled)
- No sinus tract communicating with the prosthetic joint, AND - No postoperative suppurating wound defect 14 days after previous joint surgery, AND - Not warm, red and painful prosthetic joint, AND - No purulence of synovial fluid or implant site, AND - Negative OR single-positive culture of synovial fluid and tissue samples (5 biopsies)

Statistical analysis

Data are presented with numerical values and percentage of total. Concordance rate is the percentage of similar microbial findings in conventional samples and SFC relative to the number of verified PJI-cases. Descriptive statistics were applied.

RESULTS

Participants

We assessed 221 consecutive cases for eligibility and included 211 revision cases (129 THA and 82 TKA) from XXX hospital (117 cases) and YYY hospital (94 cases). Mean age was 70 ±11 years (111 women). The flow diagram in Figure 1 summarizes the inclusion procedure. Revision indications are displayed in Table 2. The Electronic Supplementary Material (ESM, Appendix D) display original data of selected cases that are individually referred by serial numbers (e.g. #137) in the results section.

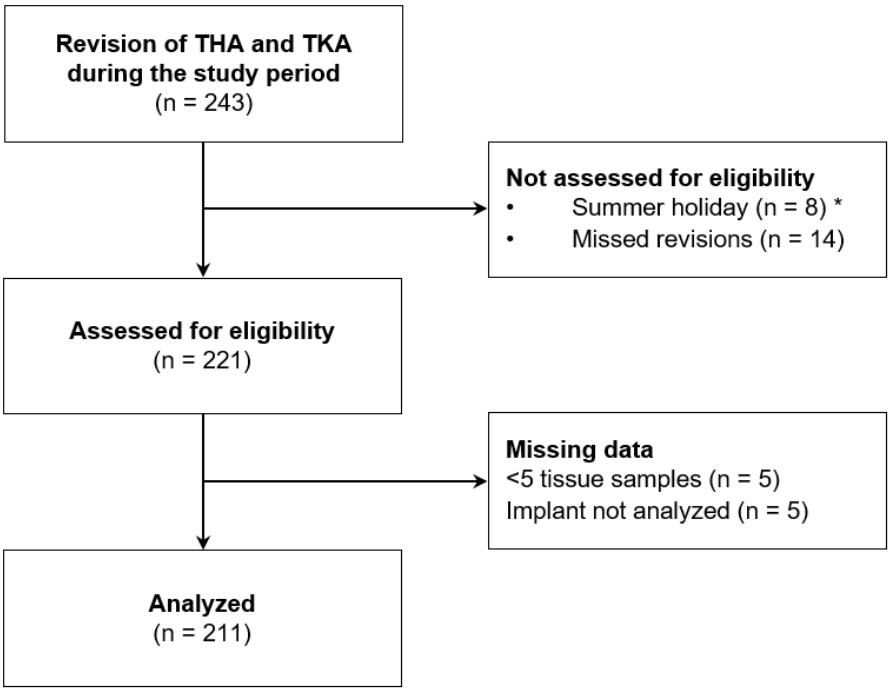

Fig 1: A flow diagram of the included revision THA and TKA cases during the one-year study period is shown.* No screening or inclusion was conducted during two weeks of summer holiday.

Descriptive and outcome data

Pre- and intraoperative findings: In 54 verified PJI-cases, 40 fulfilled more than one major diagnostic criterion, whereas 3 cases were categorized only due to presence of a draining sinus tract (Table 2). Another 9 cases were categorized alone due to identical findings in ≥3 culture-samples of periprosthetic tissue and synovial fluid. Finally, 2 PJI-cases were verified only by a combination of 3/4 minor criteria.

Table 2: Pre- and intraoperative data of 211 revisions categorized as verified PJI, possible PJI and non-infected

Pre- and intraoperative findings	Verified PJI n=54	Possible PJI n=10	No PJI n=147
Revision indication			
- Expected deep infection	46	7	1
- Expected aseptic loosening	6	2	63
- Other indications*	2	1	83
Preoperative clinical findings			
- Sinus tract communicating with the TJA	8	0	0
- Painful, warm, red joint (recent 3 weeks)	32	4	0
- Wound leakage (>14 days after surgery)	16	3	1
- Antibiotics (≥2 days within 2 weeks)	20	6	4
- Elevated p-CRP/total number**	32/51	6/10	9/108
- None of the above	6	0	133
Intra-operative evaluation			
- Normal synovial fluid	3	4	110
- Purulent synovial fluid	23	1	0
- Uncertain synovial fluid***	28	5	37

*Other indications include dislocation, instability, periprosthetic fracture, wear, malalignment, metal-on-metal, hardware failure, heterotopic ossification, tibia collapse, reduced range of motion and pain of unknown etiology.

** Plasma C-reactive protein (p-CRP) was not analyzed in three verified PJI-cases and 39 non-infected revision cases; hence, the reduced total number is indicated as the denominator. The threshold of elevated CRP is 10 mg/l when the latest surgery was >3 months before, periprosthetic fracture was not present and no accompanying comorbidity of cancer or rheumatic disorder (otherwise 100 mg/l).

*** Synovial fluid quality was termed 'uncertain', when neither 'normal' nor 'purulent' were adequate.

Forty-six of the verified PJI were indicated as presumed deep infection at the surgeon's discretion. The remainder of the verified PJI-cases were indicated as expected aseptic loosening (see #13, #113, #116, #195, #196 and #200 in the ESM, Appendix D), dislocation (#155) and periprosthetic fracture (#35).

Ten revision cases were categorized as possible PJI due to a combination of criteria. Seven of these revision cases were also indicated as presumed deep infection by the revision surgeon (#16, #30, #149, #161, #167, #172 and #212), whereas another two cases were expected aseptic loosening (#50 and #222) and one cases was revised for a periprosthetic fracture two weeks after the second stage operation in a long course of culture-negative PJI (#71).

All 5 criteria of non-infected prosthetic joint at revision surgery were fulfilled in 147 cases. This includes 1 case revised for presumed deep infection (#147). For further details, see the Appendix D.

Overall, preoperative antibiotics were administered in 30 revision cases due to either ongoing treatment of PJI (14 cases), new surgical site infection (6 cases) or recent infections with a distant focus (10 cases). Furthermore, presence or absence of pus in the periprosthetic joint fluid was not clearly determined in 52% of the verified PJI-cases and 26% of the non-infected revision cases. Finally, intraoperative aspiration

of synovial fluid from the prosthetic joint was fruitless (dry tap) and thus not available for incubation in 29% of the total number of revisions.

Culture of conventional samples: Phenotypically identical microorganisms in ≥3/5 periprosthetic tissue samples were found in 40/54 verified PJI-cases (74%). Another 6/54 (11%) cases were culture positive with identical organisms in synovial fluid AND 2/5 tissue samples (Table 3). Observation of microbial growth in 1 to 2 of 5 tissue samples without concordance in either synovial fluid or sonicate was found in 17/147 non-infected cases (12%); these observations may be regarded as possibly contaminated. Positive intraoperative culture (PIOC) of ≥3 tissue samples and synovial fluid was observed in 8/157 (5%) of expected aseptic revisions (#13, #35, #113, #116, #155, #195, #196, #200).

Table 3: Culture results of conventional samples and sonication fluid in revision of THA and TKA.

Microbiological results	**Verified PJI** n=54	**Possible PJI** n=10	**No PJI** n=147
Conventional culture samples			
- Growth in 1/5 tissue samples	4	3	14
- Growth in 2/5 tissue samples, alone	2	0	3
- Growth in synovial fluid AND 2/5 tissue samples†	6	0	0
- Growth in ≥3/5 tissue samples	40	0	0
- No growth in 5/5 tissue samples 5 days culture	2	7	130
Sonication fluid culture			
- Growth in thioglycollate only*	0	2	4
- Growth of 1-19 CFU/ml*	3	0	7
- Growth of ≥20 CFU/ml	48	3	4
- No growth in sonication fluid after 5 days culture	3	5	132

† One situation with 2/6 culture positive samples was registered as 'Growth in 2/5'

* Microbial growth in low concentrations (1-19 CFU/ml) or in thioglycollate alone was regarded as 'uncertain culture outcome'

Culture of sonication fluid: Bacterial growth above the diagnostic threshold of SFC was found in 48/54 verified PJI-cases (89%), as well as 7 'unverified PJI-cases'. All 55 SFC results above the diagnostic threshold were consistently detected on agar plates within five days incubation; 47 (85%) within 48 hours and 8 (15%) from day 3-5 (data not shown). Only 1 culture-positive result was registered after more than 5 days incubation, namely growth of *P. acnes* only in thioglycollate after 7 days incubation (#89). Microbial growth in thioglycollate alone was found in 6 cases recorded as 'uncertain culture outcome', and bacterial findings in concentrations below 20 CFU/mL was found in SFC of 7/147 non-infected cases (5%) without concordance in neither culture of tissue samples nor synovial fluid.

Main findings

What is the additive effect of SFC on diagnosis of bacteria in THA and TKA revisions in relation to PJI?

In 54 verified PJI-cases, 6 were culture-positive above the threshold in sonication fluid, but not in conventional sampling of synovial fluid and periprosthetic tissue (#34, #80, #116, #137, #146 and #206). Among 10 revision cases with possible PJI, SFC was positive in 3 cases (#50, #71 and #212), whereas

another 2 cases of presumed deep infection grew positive with sonicate in thioglycollate only (#149 and #161).

What is the fate of prosthetic joint revisions, which had additional microbial findings in sonication fluid culture?

In total, we identified 11 cases of verified or possible PJI with additional microbial findings in SFC (Table 4). Re-revision was performed in 5/11 cases after additional findings in SFC. Hereof 2 ended up with permanent Girdlestone status. Among 5/11 cases that were not re-revised after minimum 1 year follow-up, only 1 case had an asymptomatic prosthesis, whereas 1 case had a strategy of antibiotic suppression, and in 3 cases a painful joint prosthesis was unsolved. Finally, 1/11 patients diseased in circulatory failure 466 days after the revision operation.

Table 4: The fate of prosthetic joint revisions which had additional microbial findings in sonication fluid culture of 6 verified and 5 possible PJI cases.

Case no.	**Classifi-cation**	**Indication** of prior revision	**Revision type**	**Antibiotic** iv. / oral	**Microbial finding** Synov | TSC | SFC	**Fate after prosthetic joint revision** LFU: Latest follow-up
Verified PJI (6 cases)						
#34	Early PJI	Deep infection	2-stage	Diclox 2w / 4w	*E. faecalis* **Pos.** | 0/5 | >500	Spacer-exchange 12 days later Prior revision with *E. faecalis*
#137	Early PJI	Deep infection	1-stage, total	Diclox 2w / 6w	*S. epidermidis* **Pos. | 1/5** | >500	2-stage revision 12,5 months later with *S. epidermidis*
#80	Chronic PJI	Deep infection	DAIR	Diclox 2w / 4w	*P. acnes* Neg. | 0/5 | >500	Permanent Girdlestone procedure 13 months later
#146	Chronic PJI	Deep infection	2-stage	Diclox 2w / 6w	*E. faecalis* Neg. | **1/5** | >500	Permanent Girdlestone procedure 3 months later
#206	Chronic PJI	Deep infection	2-stage	Diclox 2w / 4w	*F. magna* **Pos. | 1/5** | >500	Diseased in circulatory failure 4 days after 2nd stage operation
#116	Chronic PJI	*Aseptic loosening*	1-stage, partial	Diclox 2w / 4w	*S. epidermidis* **Pos. | 1/5** | >500	No more revisions LFU (30 months): possibly loose again
Possible PJI (5 cases)						
#149	Early PJI	Deep infection	DAIR	Diclox 2w / 4w	*F. magna* Dry | 0/5 | *Thio*	No more revisions LFU (12 months): OK Prior revision w/ polymicrobial finding
#161	Early PJI	Deep infection	2-stage	Vanco 2w	*S. agalacticae* Neg. | 0/5 | *Thio*	No more revisions LFU (14 months): Permanent spacer Prior DAIR with *S. agalacticae*
#212	Early PJI	Deep infection	2-stage	Diclox 2w / 4w	*E. faecalis* Neg. | **1/5** | 30	Spacer-exchange 30 days later with *E. faecalis* in 5/5 tissue samples
#71	Early PJI	*Periprosth fracture*	1-stage, partial	Diclox 5 days	*S. capitis* Neg. | 0/5 | 450	No more revisions LFU (24 months): Recurring trochanteric bursitis
#50	Chronic PJI	*Aseptic loosening*	1-stage, partial	Diclox 2w / 4w	*S. epidermidis* **Pos.** | 0/5 | 45	No more revisions LFU (12 months): lumbar stenosis

Abbreviations:

ID and Reference standard		Antibiotic administration		Microbial finding	
See Electronic supplement material and Table 1		iv.	Intravenously, 2 weeks	Synov	Synovial fluid culture, positive/negative
Revision type		oral	Tablets, 4-6 weeks	TSC	Tissue sample culture, positive no. / 5 samples
1-stage	One-stage revision	Antimicrobial dosage		SFC	Synovial fluid culture, no. culture forming units
2-stage	Two-stage, 1st step	Diclox	Dicloxacillin, 1g x 4	Postoperative fate	
DAIR	Debridement and implant retention	Vanco	Vancomycin, 1g x 2	LFU	Latest follow-up

Regarding revision surgery in 8/11 cases with preoperative suspicion of deep infection, the prosthetic joint revision was either debridement and implant retention (2 cases), 1-stage exchange (1) or 2-stage exchange (5). Empirical antibiotic treatment typically consisted of dicloxacillin (2 weeks iv. and 4-6 weeks oral), except for one case of re-infection when initial iv.-administration of vancomycin for 2 weeks was followed by 4 weeks of dicloxacillin. Of these 8 cases suspected for deep infection, 4 were culture-negative in all conventional samples. Another 4 cases had microbial growth below the threshold (1 tissue sample and/or synovial fluid), but only 2/4 of microbial findings in conventional samples were similar to SFC.

Among 3 cases with revision indication other than deep infection, 2/11 cases were revised for aseptic loosening and had microbial growth below the threshold in conventional samples, both were concordant with SFC. Finally, in 1 case of periprosthetic fracture only SFC was positive, despite septic joint revision 14 days earlier.

Other analyses

Preoperative antibiotic administration for ≥2 days within 2 weeks prior to prosthetic revision surgery demonstrated little clinical difference in culture-positivity proportions of TSC and SFC (Table 5). In relation to the Zimmerli-classification based on duration of symptoms and time interval from latest total joint surgery, TSC and SFC overall showed similar frequencies of culture-positivity in 64 verified and possible PJI-cases.

Table 5: Distribution of positive culture results from verified and possible PJI-cases of revision THA and TKA

Characteristics of verified and possible PJI-cases *Culture-positive no (%)*	**Conventional samples** ≥3 pos. samples	**Sonication fluid** ≥20 CFU/ml
Overall verified and possible PJI-cases, n=64	46 (72%)	51 (80%)
Early postoperative symptoms, n=26	19 (73%)	22 (85%)
- With preoperative antibiotics, n=15	10	12
- No preoperative antibiotics, n=11	9	10
Chronic symptoms, n=23	14 (61%)	16 (70%)
- With preoperative antibiotics, n=2	2	1
- No preoperative antibiotics, n=21	12	15
Late acute symptoms, n=15	13 (87%)	13 (87%)
- With preoperative antibiotics, n=7	5	5
- No preoperative antibiotics, n=8	8	8

Microbial findings: Compared to the reference standard of verified PJI, the aggregated culture results of conventional and sonication samples were positive in 53/54 (98%). The concordance rate was 76% due to similar microbial findings in 41/54 verified PJI-cases (Table 6). Four cases were culture-positive in conventional samples, but negative by sonication-culture (#3, #108, #195 and #196). Only 1 verified PJI-case was culture-negative in all 3 materials (#6). With regard to the revision indication of presumed deep infection, a microbial diagnosis was achieved in 39/54 (72%) cases by conventional sampling and in 45/54 (83%) by SFC, whereas the aggregated culture result from both sampling methods was positive in 49/54 (91%). Data not shown.

Table 6: Results from five days incubation of conventional samples and sonication fluid

Microbiological findings All revisions (n=211)	**Synovial + tissue** ≥3 samples	**Sonication fluid** ≥20 CFU/ml	**Concordance**
Staphylococcus aureus	10	10	10
Staphylococcus epidermidis	12	16	10
Other coag. neg. staph.[1]	5	4	3
Hemolytic streptococci[2]	6	6	6
Enterococcus faecalis	2	5	2
Gram positive, other[3]	2	4	2
Enterobactericae[4]	3	3	3
Polymicrobial	6	7	5
Total	46	55	41

1. Other coagulase negative staphylococci: *Staphylococcus lugdunensis, s. capitis, s. caprae, s. warneri, s. hominis, s. schleiferi, s. saprophyticus*

2. Hemolytic streptococci*: Streptococcus pyogenes, s. agalacticae, s. dysgalacticae*

3. Gram-positive, other: *Micrococcus luteus, Gemella sp., Corynebacterium sp., Bacillus sp., Propionibacterium acnes, Finegoldia magna*

4. Enterobacteriaceae: *Escherichia coli, Enterobacter cloacae, Proteus mirabilis, Hafnia alvei, Serratia marcescens*

DISCUSSION

Key results

The present study evaluated the additive effect of the sonication-culture method on diagnosis of bacteria in revision THA and TKA. Our study showed that microbes were additionally found by SFC in 11 cases of verified and possible PJI, which were otherwise culture-negative or below the diagnostic threshold in synovial fluid and tissue samples. The fate of these 11 cases after minimum 1 year follow-up included re-revision in 5 cases, antibiotic suppression in 1 case, and a painful and unsolved joint condition in 3 cases, whereas only 1 case had an asymptomatic prosthesis. Finally, 1/11 patients diseased in circulatory failure 466 days later.

Limitations

This study had a number of limitations. **First**, we were not able to adapt the PJI definition as suggested by the International Consensus Meeting (ICM) in end 2013[15]. Two minor criteria of the ICM-definition, synovial leukocyte count and periprosthetic histopathology, were not included in the present study since they were not routinely performed at our institutions. However, we have formulated a multi-criteria reference standard of verified PJI based on readily available tests that are also included in the ICM and other definitions [15, 22, 23]. Though not directly communicable with other diagnostic accuracy-studies of PJI, we find a reference standard based on best-practice modalities relevant for evaluation of the additive effect of the sonication-culture method, since this is the setting in which implementation would take place.

Additionally, we introduced a category of possible PJI to identify culture-negative cases with strong suspicion of PJI though not meeting the criteria of verified PJI. This intermediate category corresponds to the clinical dilemma of difficult-to-diagnose situations where the treatment strategy may be difficult to establish [11, 14]. We identified 10 cases under this category, including 7 cases of presumed deep infection.

Second, we applied a diagnostic threshold of ≥3 identical microbial findings in either five periprosthetic tissue samples or positive TSC 2/5 combined with identical finding in one synovial fluid sample after five days incubation. This was adopted from the local guidelines and supported by the classical validation study of Bridget L. Atkins et al [2]. The threshold is somewhat more conservative than the latest recommendations of ≥2/5 culture-positive samples, though [15, 22]. Nevertheless, we found unexpected positive intraoperative culture (PIOC) in ≥3 conventional samples in 5% of expected aseptic revisions. A diagnostic threshold at ≥2/5 culture-positive tissue samples in the reference standard would categorize three more revision cases as PJI despite lack of preoperative suspicion. Hence, a lower diagnostic threshold might increase the risk of false positive culture results due to contamination.

Third, the risk of contamination also exists in relation to the sonication-culture method. We used a sterile, airtight plastic-box, which was only opened twice in the laboratory (under laminar airflow): when saline was filled, and upon sonication fluid sampling. Previous studies have suggested additional centrifugation of the sonication fluid and cultivation of the sediment [5, 8]. We found this extra step too labor-intensive and risky of contamination to include in the present study.

Fourth, the sonication-culture procedure was delayed during weekends and holidays, when the retrieved prosthetic components were stored in a sealed, sterile plastic box in a laboratory refrigerator. The direct effect of prolonged storage on bacterial viability after sonication has not been investigated systematically, but we believe that this was not a major confounder because surface-related bacteria residing in biofilm are capable of surviving in hostile environments [12].

Interpretation

What is the additive effect of SFC on diagnosis of bacteria in THA and TKA revisions in relation to PJI?

The additive effect of the sonication-culture method was bacterial growth in 11/211 revisions of THA and TKA, which were not diagnosed by conventional methods. The diagnostic interpretation of SFC was unbiased, since one dedicated laboratory technician blinded to the clinical situation performed the procedure of sonication, incubation and documentation. The aggregated culture result of conventional samples and sonication fluid was positive in 98% of verified PJI-cases. Corresponding findings has been reported in previous diagnostic sonication-studies [21]. Direct comparison is not feasible though, since individual studies cite different methods and reference standards.

What is the fate of prosthetic joint revisions, which had additional microbial findings in sonication fluid culture?

Description of the postoperative fate in 11 cases with additional microbial findings in SFC shows several examples of similar bacterial findings in present SFC and conventional samples of prior or subsequent revisions. To the best of our knowledge, no diagnostic studies has previously described the fate of prosthetic joint revisions, which had additional microbial findings in sonication fluid culture. In our study, the clinicians were blinded to the results of SFC and thus not influenced by the additional microbial findings. In cases with suspected deep infection, revision surgery included thorough debridement and postoperative administration of dicloxacillin for 6-8 weeks. Hence, it is not possible to correlate a poor treatment outcome after culture-negative TSC directly with the additional microbial findings in experimental SFC.

Other relevant findings: Preoperative antibiotic administration has previously been hold accountable for culture-negative results with conventional sampling, whilst the sonication-culture method demonstrated a higher culture yield [20]. In the present study, this correlation was not clinically significant. The microbial findings in the present study is in accordance with Scandinavian arthroplasty register studies [9, 18].

Generalizability

We included revisions of THA and TKA performed on any indication at both regional and university hospitals, and the conventional diagnostic measures were evaluated due to best practice. We believe that these measures improve the external validity of this study. Furthermore, the study protocol was designed to secure internal validity through prospective sampling of well-defined data in a limited time-period with no other modifications of the clinical microbiology approach.

Conclusion

In conclusion, the present study demonstrated that the additive effect of the sonication-culture method was bacterial growth in 11/211 revisions of THA and TKA, which were not diagnosed by conventional methods. The fate of these 11 cases included re-revision in 5 cases. From a clinical perspective, patients with additional microbial findings by SFC had a discouraging prognosis and may represent true positive findings that have to be taken into consideration in the infection treatment.

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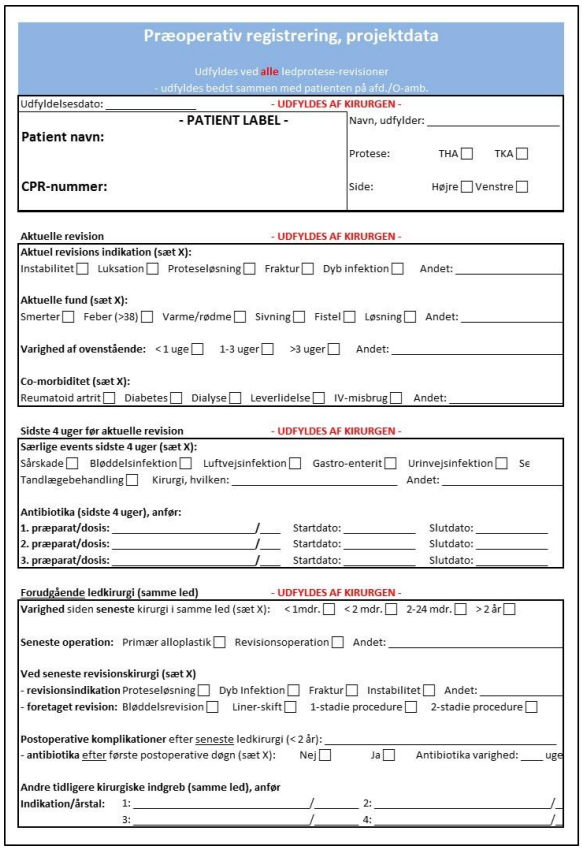
Appendix D: Descriptive overview of selected revision cases (Study III)

PJI status	Case ID-number	Total joint arthroplasty	Revision indication	Classification Zimmerli 2014	Latest TJA surgery	Purulence	CRP, mg/L	Painful, warm, red	Wound leak (>14d)	Preop. antibiotics	Tissue culture	Synovial fluid culture	Microbiology, conventional tests	Microbiology, sonication fluid	CFU/ml in sonicate	Remarks
Verified PJI	#3	TKA	DInf / DAIR	Early postop	rDInf, 14d		43		**1**	**1**	2/5	Concord	*S. epidermidis*		Neg	Recent rDInf w/ *S. aureus* + *S. epidermidis* (DAIR + Cefuroxim)
	#6	THA	DInf / 2st1	Chronic	rDInf, 168d		<10		**1**		0/5	Neg	Neg	*M. luteus*	1-19	PJI verified by sinus tract Recent rDInf w/ Moraxella sp.
	#13	THA	AsL / 1st	Chronic	pTHA, 2y		<10				4/5	Concord	S. epidermidis	S. epidermidis	>500	PIOC in early AsL
	#34	TKA	DInf / 2st1	Early postop	rDInf, 65d		21	**1**	**1**		(1)/5	Positive	*E. faecalis*	*E. faecalis*	>500	PJI verified by sinus tract Recent rDInf w/ *S. aur* + *E. faecalis*
	#35	THA	PFx / 1st	Early postop	rPFx, 25d		52				2+2/5	Concord	*S. aureus, S. epidermidis*	*S. aur., S. epid., S. capitis*	>500	PIOC in PFx Recent rPFx (1st, #26)
	#80	THA	DInf / DAIR	Chronic	pTHA, 104d		**20**	**1**			0/5	Neg	Neg	*P. acnes*	>500	PJI verified by sinus tract Recent pTHA
	#108	TKA	DInf / DAIR	Chronic	pTKA, 11y	**1**	**211**	**1**		**1**	5/5	Concord	*S. lugdunensis*	*M. luteus*	1-19	Preop. Cefuroxim liner soaked in dicloxacillin
	#111	THA	DInf / 2st1	Chronic	rDInf, 370d	**1**	**68**		**1**		2/5	Neg	*S. epidermidis*	*S. epidermidis*	>500	PJI verified by sinus tract
	#113	THA	AsL / 1st	Chronic	pTHA, 3y		**26**				2/6†	Concord	*Corynebacterium sp.*	*Corynebacterium sp.*	1-19	PIOC in early AsL Superficial SSI at pTHA
	#116	THA	AsL / 1st	Chronic	pTHA, 14y		**36**				1+1/5	Concord	*S. epidermidis, S. hominis*	*S. epidermidis*	500	PIOC in late AsL PJI verified by minor criteria
	#137	THA	DInf / 1st	Early postop	pTHA, 19d	**1**	**102**	**1**	**1**	**1**	1/5	Concord	*S. epidermidis*	*S. epidermidis*	>500	PJI verified by minor criteria Recent UTI w/ *E. coli*, Cefuroxim
	#146	THA	DInf / 2st1	Chronic	rDInf, 2y		ND		**1**	**1**	2+1/5	Neg	*S. aureus* *P. acnes*	*E. faecalis*	>500	PJI verified by sinus tract Latest rDInf w/ *S. aureus*, Cefuroxim
	#155	THA	Disl / 1st	Early postop	rPFx, 22d		16				5/5	Neg	*S. schleiferi*	*S. schleiferi*	20-50	PIOC in Disl Recent rPFx (1st, #135)
	#195	TKA	AsL / 1st	Chronic	rDInf, 154d		<10				3+3/5	Neg	*S. capitis, P. acnes*		Neg	PIOC in early AsL Recent PJI w/ *S. epidermidis*
	#196	THA	AsL / 1st	Chronic	pTHA, 24y		<10				4/5	Neg	*S. capitis*		Neg	PIOC in late AsL
	#200	THA	AsL / 1st	Chronic	rDInf, 648d		ND				2/5	Neg	*S. epidermidis*	*S. epidermidis*	100-500	PIOC in early AsL Recent PJI w/ *S. capitis*
	#206	THA	DInf / 2st1	Chronic	rDInf, 276d	**1**	**148**	**1**			1/5	Concord	*F. magna*	*F. magna*	>500	PJI verified by minor criteria Recent rDInf w/ *E. coli* (#31)
Possible PJI	#16	TKA	Dinf /2st1	Chronic	pTKA, 3y		**15**				0/5	Positive	*Bacillus sp.*		Neg	Expected deep infection due to intraoperative findings
	#30	THA	DInf / DAIR	Early postop	pTHA, 16d		19		**1**		1+1/5	Dry	*S. epidermidis, Corynebact.*		Neg	Expected deep infection due to wound leakage
	#50	THA	AsL / 1st	Chronic	pTHA, 2y		**38**				0/5	Positive	*S. epidermidis*	*S. epidermidis*	20-50	Early AsL Possible PJI w/ CRP + culture
	#71	THA	PFx / 1st	Early postop	rDInf, 14d		63			**1**	0/5	Neg	Neg	*S. capitis*	100-500	Recent rDInf (culture-neg) Ongoing Dicloxacillin
	#149	THA	DInf / DAIR	Early postop	rPFx, 22d		71	**1**	**1**	**1**	0/5	Dry	Neg	*F. magna*	Thio	Expected deep infection Recent rPFx (Dicloxacillin; #129)
	#161	THA	Dinf /2st1	Early postop	rDInf, 47d		**145**	**1**		**1**	0/5	Neg	Neg	*S. agalacticae*	Thio	Recent rDInf w/ *S. agalacti*cae (DAIR + Amoxacillin; #118)
	#167	THA	Dinf /2st1	Late acute	pTHA, 4y		**368**			**1**	0/5	Neg	Neg		Neg	Current sepsis w/ *S. aureus* Ceftriax+Piperac+Tazobact joint appears normal
	#172	TKA	DInf / DAIR	Late acute	pTKA, 114d		**98**	**1**		**1**	0/5	Neg	Neg		Neg	Recent pneumonia, unknown bact. Amox+Clavulan/Piperac+Tazobact
	#212	THA	Dinf /2st1	Early postop	rDInf, 14d	**1**	60	**1**	**1**	**1**	1/5	Neg	*S. hominis*	*E. faecalis*	20-50	Recent rDInf w/ *S. aureus* (DAIR + Cefuroxim; #197) - 30d later rDInf w/ *E. faecalis*
	#222	THA	AsL / 1st	Chronic	pTHA, 2y		**11**				1/5	Neg	*S. saprophyticus*		Neg	Early AsL Possible PJI w/ CRP + culture
Non-infected	#27	THA	Disl / 1st	Chronic	rDisl, 217d		<10				0/5	Neg	Neg	*P. acnes*	100-500	Second revision due to dislocation within 6 months
	#38	TKA	Wear / 1st	Chronic	pTKA, >2y		<10				0/5	Neg	Neg	*S. epidermidis*	20-50	20 year-old prosthesis
	#89	THA	AsL / 1st	Chronic	pTHA, 9y		ND				0/5	Neg	Neg	*P. acnes*	Thio	Difficult to close box Culture-pos in Thio after 7d
	#129	THA	PFx / 1st	Early postop	pTHA, 14d		34		**1**		0/5	Neg	Neg	*S. aureus, S. capitis*	20-50	Early re-revision hereafter (#149): 22d later rDInf W/ *S. aureus*
	#147	THA	Dinf /2st1	Chronic	pTHA, 8y		**26**				0/5	Neg	Neg	*S. epidermidis*	100-500	Expected deep infection, no PJI Preoperative PET-CT suspect of PJI

Abbreviations in the table:

THA	Total hip arthroplasty	1st	One-stage revision	CRP	C-reactive protein, plasma
TKA	Total knee arthroplasty	2st1	Two-stage, 1st step	ND	Not done
TJA	Total joint arthroplasty	DAIR	Debridement and implant retention	Wound leak	Suppurating wound defect >14 days
AsL	Aseptic loosening	pTHA / pTKA	Primary THA/TKA	0/5; 3/5; 5/5	No. of culture-positive samples
DInf	Deep infection	rAsL, rDInf	Revision of AsL, DInf, etc.	Concord	Concordant in synovial and tissue culture
Disl	Dislocation	14d / >2y	Days/Years; time since latest operation	SSI	Surgical site infection
PFx	Periprosthetic fracture	PJI	Prosthetic joint infection	PIOC	Positive intraoperative culture (unsuspected)

Appendix E: Data registration checklists (Study III)



Præoperativ registrering, projektdata

Udfyldes ved alle ledprotese-revisioner
- udfyldes bedst sammen med patienten på afd./O-amb.

Udfyldelsesdato: _____ - UDFYLDES AF KIRURGEN -

- PATIENT LABEL -

Patient navn:

CPR-nummer:

Navn, udfylder: _____

Protese: THA ☐ TKA ☐

Side: Højre ☐ Venstre ☐

Aktuelle revision - UDFYLDES AF KIRURGEN -

Aktuel revisions indikation (sæt X):
Instabilitet ☐ Luksation ☐ Proteseløsning ☐ Fraktur ☐ Dyb infektion ☐ Andet: _____

Aktuelle fund (sæt X):
Smerter ☐ Feber (>38) ☐ Varme/rødme ☐ Sivning ☐ Fistel ☐ Løsning ☐ Andet: _____

Varighed af ovenstående: < 1 uge ☐ 1-3 uger ☐ >3 uger ☐ Andet: _____

Co-morbiditet (sæt X):
Reumatoid artrit ☐ Diabetes ☐ Dialyse ☐ Leverlidelse ☐ IV-misbrug ☐ Andet: _____

Sidste 4 uger før aktuelle revision - UDFYLDES AF KIRURGEN -

Særlige events sidste 4 uger (sæt X):
Sårskade ☐ Bløddelsinfektion ☐ Luftvejsinfektion ☐ Gastro-enterit ☐ Urinvejsinfektion ☐ Se
Tandlægebehandling ☐ Kirurgi, hvilken: _____ Andet: _____

Antibiotika (sidste 4 uger), anfør:
1. præparat/dosis: _____/___ Startdato: _____ Slutdato: _____
2. præparat/dosis: _____/___ Startdato: _____ Slutdato: _____
3. præparat/dosis: _____/___ Startdato: _____ Slutdato: _____

Forudgående ledkirurgi (samme led) - UDFYLDES AF KIRURGEN -

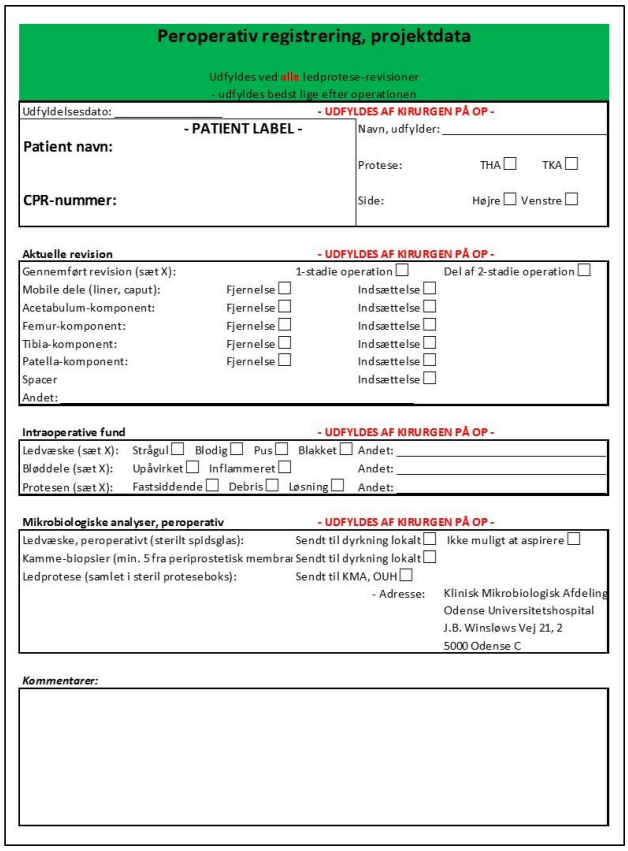
Varighed siden **seneste** kirurgi i samme led (sæt X): < 1mdr. ☐ < 2 mdr. ☐ 2-24 mdr. ☐ > 2 år ☐

Seneste operation: Primær alloplastik ☐ Revisionsoperation ☐ Andet: _____

Ved seneste revisionskirurgi (sæt X)
- **revisionsindikation** Proteseløsning ☐ Dyb Infektion ☐ Fraktur ☐ Instabilitet ☐ Andet: _____
- **foretaget revision:** Bløddelsrevision ☐ Liner-skift ☐ 1-stadie procedure ☐ 2-stadie procedure ☐

Postoperative komplikationer efter seneste ledkirurgi (< 2 år): _____
- **antibiotika** efter første postoperative døgn (sæt X): Nej ☐ Ja ☐ Antibiotika varighed: ___ uge

Andre tidligere kirurgiske indgreb (samme led), anfør
Indikation/årstal: 1: _____/_____ 2: _____/_____
3: _____/_____ 4: _____/_____



Peroperativ registrering, projektdata

Udfyldes ved alle ledprotese-revisioner
- udfyldes bedst lige efter operationen

Udfyldelsesdato: _____ - UDFYLDES AF KIRURGEN PÅ OP -

- PATIENT LABEL -

Patient navn:

CPR-nummer:

Navn, udfylder: _____

Protese: THA ☐ TKA ☐

Side: Højre ☐ Venstre ☐

Aktuelle revision - UDFYLDES AF KIRURGEN PÅ OP -

Gennemført revision (sæt X):	1-stadie operation ☐	Del af 2-stadie operation ☐
Mobile dele (liner, caput):	Fjernelse ☐	Indsættelse ☐
Acetabulum-komponent:	Fjernelse ☐	Indsættelse ☐
Femur-komponent:	Fjernelse ☐	Indsættelse ☐
Tibia-komponent:	Fjernelse ☐	Indsættelse ☐
Patella-komponent:	Fjernelse ☐	Indsættelse ☐
Spacer		Indsættelse ☐
Andet:		

Intraoperative fund - UDFYLDES AF KIRURGEN PÅ OP -

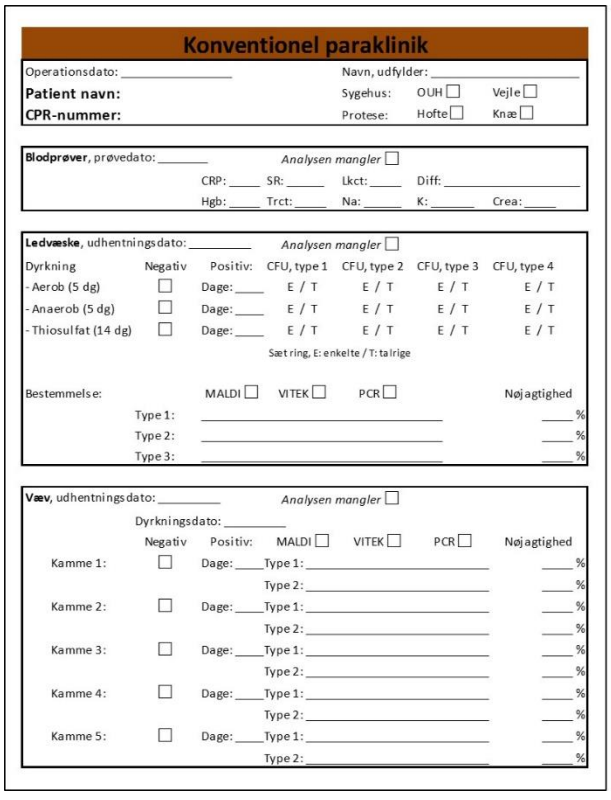
Ledvæske (sæt X): Stråguł ☐ Blodig ☐ Pus ☐ Blakket ☐ Andet: _____
Bløddele (sæt X): Upåvirket ☐ Inflammeret ☐ Andet: _____
Protesen (sæt X): Fastsiddende ☐ Debris ☐ Løsning ☐ Andet: _____

Mikrobiologiske analyser, peroperativ - UDFYLDES AF KIRURGEN PÅ OP -

Ledvæske, peroperativt (sterilt spidsglas): Sendt til dyrkning lokalt ☐ Ikke muligt at aspirere ☐
Kamme-biopsier (min. 5 fra periprostetisk membran Sendt til dyrkning lokalt ☐
Ledprotese (samlet i steril proteseboks): Sendt til KMA, OUH ☐
- Adresse: Klinisk Mikrobiologisk Afdeling
Odense Universitetshospital
J.B. Winsløws Vej 21, 2
5000 Odense C

Kommentarer:

Figure 16 and 17: Checklists for registration of pre- and intraoperative



Konventionel paraklinik

Operationsdato: _____ Navn, udfylder: _____
Patient navn: Sygehus: OUH ☐ Vejle ☐
CPR-nummer: Protese: Hofte ☐ Knæ ☐

Blodprøver, prøvedato: _____ *Analysen mangler* ☐
CRP: ____ SR: ____ Lkct: ____ Diff: _____
Hgb: ____ Trct: ____ Na: ____ K: ____ Crea: ____

Ledvæske, udhentningsdato: _____ *Analysen mangler* ☐

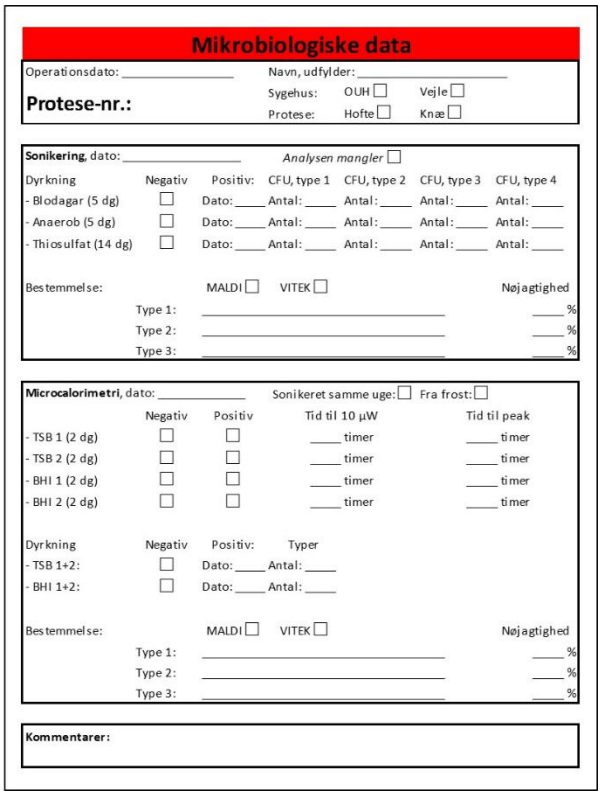
Dyrkning	Negativ	Positiv:	CFU, type 1	CFU, type 2	CFU, type 3	CFU, type 4
- Aerob (5 dg)	☐	Dage: ____	E / T	E / T	E / T	E / T
- Anaerob (5 dg)	☐	Dage: ____	E / T	E / T	E / T	E / T
- Thiosulfat (14 dg)	☐	Dage: ____	E / T	E / T	E / T	E / T

Sæt ring, E: enkelte / T: talrige

Bestemmelse: MALDI ☐ VITEK ☐ PCR ☐ Nøjagtighed
Type 1: _____ ____%
Type 2: _____ ____%
Type 3: _____ ____%

Væv, udhentningsdato: _____ *Analysen mangler* ☐
Dyrkningsdato: _____

	Negativ	Positiv:	MALDI ☐	VITEK ☐	PCR ☐	Nøjagtighed
Kamme 1:	☐	Dage: ____	Type 1: _____			____%
			Type 2: _____			____%
Kamme 2:	☐	Dage: ____	Type 1: _____			____%
			Type 2: _____			____%
Kamme 3:	☐	Dage: ____	Type 1: _____			____%
			Type 2: _____			____%
Kamme 4:	☐	Dage: ____	Type 1: _____			____%
			Type 2: _____			____%
Kamme 5:	☐	Dage: ____	Type 1: _____			____%
			Type 2: _____			____%



Mikrobiologiske data

Operationsdato: _____ Navn, udfylder: _____
Protese-nr.: Sygehus: OUH ☐ Vejle ☐
Protese: Hofte ☐ Knæ ☐

Sonikering, dato: _____ *Analysen mangler* ☐

Dyrkning	Negativ	Positiv:	CFU, type 1	CFU, type 2	CFU, type 3	CFU, type 4
- Blodagar (5 dg)	☐	Dato: ____	Antal: ____	Antal: ____	Antal: ____	Antal: ____
- Anaerob (5 dg)	☐	Dato: ____	Antal: ____	Antal: ____	Antal: ____	Antal: ____
- Thiosulfat (14 dg)	☐	Dato: ____	Antal: ____	Antal: ____	Antal: ____	Antal: ____

Bestemmelse: MALDI ☐ VITEK ☐ Nøjagtighed
Type 1: _____ ____%
Type 2: _____ ____%
Type 3: _____ ____%

Microcalorimetri, dato: _____ Sonikeret samme uge: ☐ Fra frost: ☐

	Negativ	Positiv	Tid til 10 µW	Tid til peak
- TSB 1 (2 dg)	☐	☐	____ timer	____ timer
- TSB 2 (2 dg)	☐	☐	____ timer	____ timer
- BHI 1 (2 dg)	☐	☐	____ timer	____ timer
- BHI 2 (2 dg)	☐	☐	____ timer	____ timer

Dyrkning	Negativ	Positiv:	Typer
- TSB 1+2:	☐	Dato: ____	Antal: ____
- BHI 1+2:	☐	Dato: ____	Antal: ____

Bestemmelse: MALDI ☐ VITEK ☐ Nøjagtighed
Type 1: _____ ____%
Type 2: _____ ____%
Type 3: _____ ____%

Kommentarer:

Figure 18 and 19: Checklists for registration of conventional and experimental microbiology

Appendix F: Isothermal microcalorimetry

Among the more opportunistic culture-based methods to determine bacterial presence in clinical samples is isothermal microcalorimetry that offers real time detection of heat flow produced by microbial metabolism and growth. Finding their way to Nature in 1973, Boling *et al*, described the use of microcalorimetry as a means of rapid and specific 'differentiation of one microbial species from another' by recognition of characteristic profiles for different Enterobacteria sp. [23].

We used one example of such, namely the Thermal Activity Monitor, model 3102 TAM III; TA Instruments, New Castle, DE, USA). Allowing very small variations of the sample temperature (±0.0001 °C) the TAM III equipment is virtually isothermal. With a sensitivity of 0.2 µW, TAM III can detect the heat produced by a small number of microorganisms. Assuming that a typical single bacterial cell produces approximately 2 pW when active, only 10^5 bacteria/ml are required to produce a detectable heat flow signal [35].

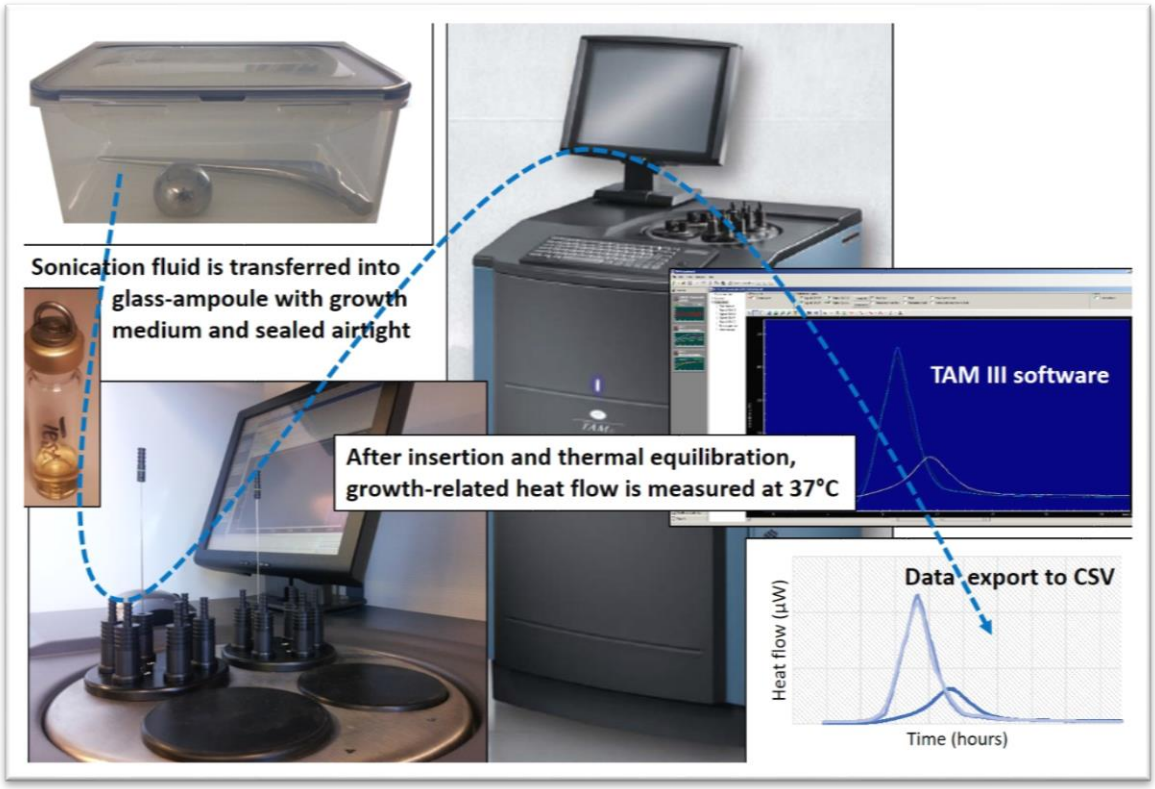


Figure 20: Microcalorimetry procedure (TAM III)

A sonication fluid sample is transferred to the glass ampoule containing growth medium, and lowered into the isothermal calorimeter. After thermal equilibration (37.000 °C), growth related heat-production is measured continuously and displayed live in the TAM-software as heat flow (µW) vs. time (hours). Data can be exported as comma-separated values (CSV)

In our protocol, the manual handling implied transfer of the test specimen or sample into a 4-ml glass ampoule containing liquid growth medium (tissue soy broth, TSB). Hereafter the ampoule cap was sealed airtight to avoid disturbance from outgassing and microbial contamination of the apparatus. The ampoule was inserted into the microcalorimeter (Figure 20) and remained 15 minutes in the thermal equilibration position to reach the target temperature before it was lowered into the measurement position [79].

After further stabilization of the heat signal, TAM III provided continuous real-time measurements (e.g. at 10 second intervals) proportional to the heat being produced in the test ampule, and output was expressed as heat-flow over time (in microwatts [µW]). In theory, the heat signal is a sum of all physicochemical processes taking place in the ampoule, and there may be simultaneous exothermic and endothermic processes taking place [35].

Examples of heat flow-time curves generated through 24-hour continuous measurement of heat flow in a TAM III microcalorimeter is depicted in Figure 21, below. In this experiment, four different bacterial laboratory-strains were individually incubated in TSB at preset concentrations. Bacteria in nutrient-rich growth medium at 37°C undergo rapid replication at exponential rate with resulting exponential heat flow development. The upper limit of heat flow forms a peak (peak heat flow, PHF) on the heat flow-time curve, when the replication rate reaches its maximum for the specific bacterium under the given physiological conditions. After this point, the amounts of nutrients and oxygen are approaching exhaustion and metabolic waste is building up in the growth medium, resulting in less favorable growth conditions and decreasing heat flow.

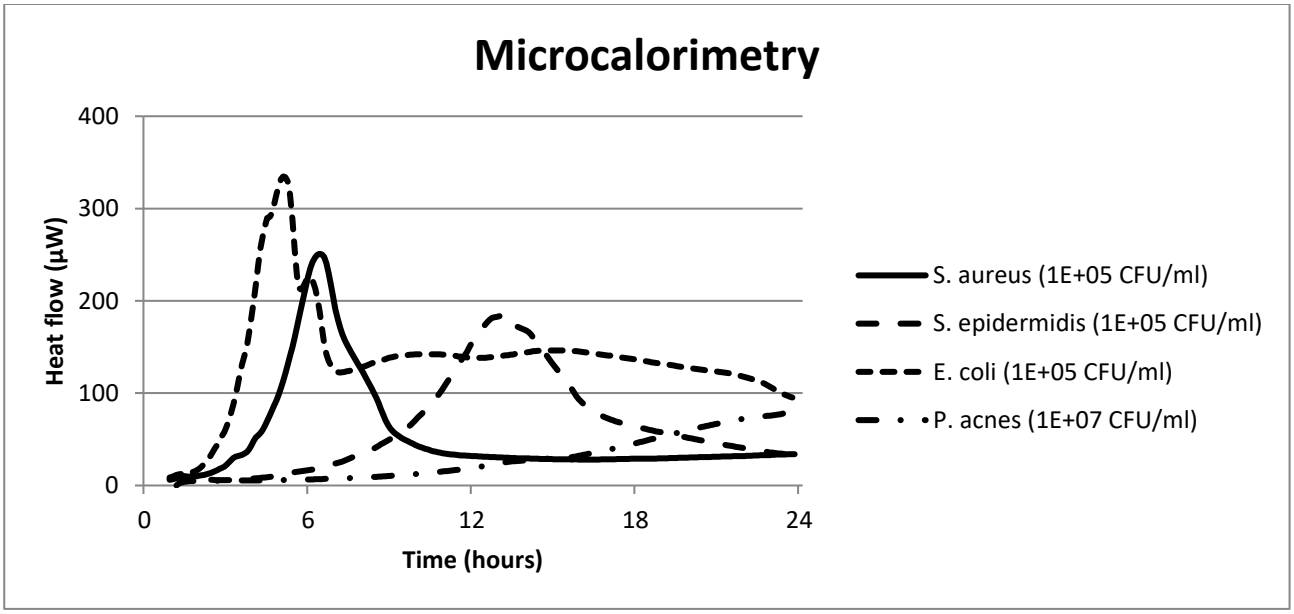


Figure 21: Heat flow-time curve of a 24-hour microcalorimetric measurement.
Four different bacteria in preset concentrations (10^5 CFU/ml of *S. aureus, S. epidermidis* and *E. coli*; 10^7 CFU/ml of *P. acnes*)

To distinguish microbial heat production from the thermal background a detection limit was set at 10 µW in the experimental studies. The calorimetric time to detection (TTD) is defined as the time from insertion of the ampoule into the calorimeter until the exponentially rising heat flow signal exceed that detection limit. Replication time and growth related heat flow is individual to the bacterial species and genera (Table 13).

Microcalorimetry	*S. aureus*	*S. epidermidis*	*E. coli*	*P. acnes*
ATCC-code	29213	35984	25922	11827
Conc. (CFU/ml)	10^5	10^5	10^5	10^7
TTD (hours)	2.83	6.42	2.00	12.17
PHF (µW)	251	185	335	>80
ttPHF (hours)	6.33	13.17	5.00	>24

Table 13: Analysis of microcalorimetric heat flow measurement

Time to detection (TTD) indicates the time to exceed the heat flow detection limit at 10 µW. Peak heat flow (PHF) indicates the maximum level of heat flow through the experiment, and time to reach PHF (ttPHF) measures the time to reach this maximum. All values indicate a typical course of heat flow individual to the four bacterial species and genera.

In experimental pilot studies, we tested the same bacteria at different concentrations (Figure 22). From the graphical appearance, some degree of inverse proportionality between bacterial concentration and delayed TTD and ttPHF was evident. Thus these two time-measures indirectly quantifies the amount of bacteria in a test sample. Note that experiments with *P. acnes* were conducted over 4 days, unlike 24 hours in the other.

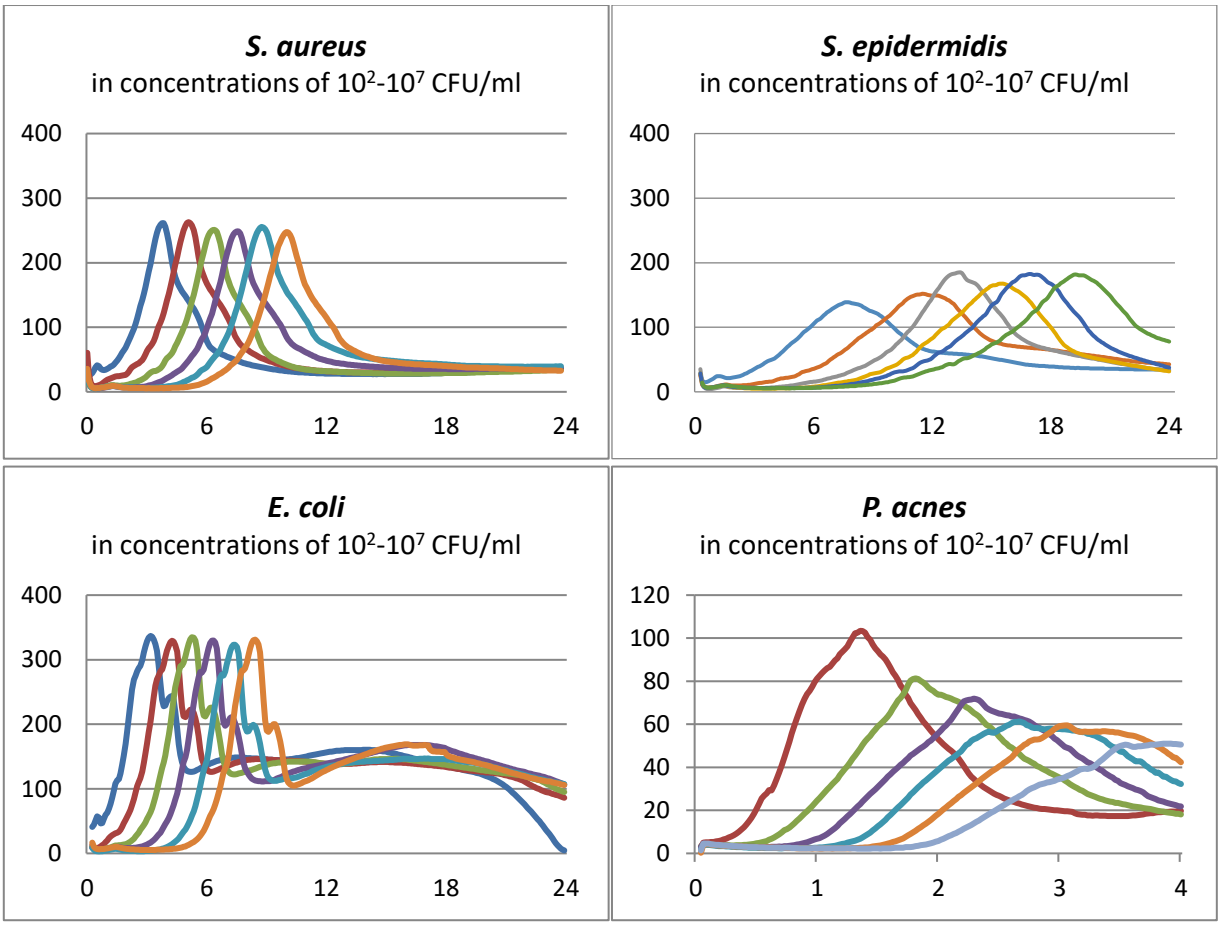


Figure 22: Microcalorimetric experiments with four bacteria at different concentrations.

Blue (10^7 CFU/ml), Red (10^6 CFU/ml), Green (10^5 CFU/ml), Purple (10^4 CFU/ml), Turquoise (10^3 CFU/ml), and Orange (10^2 CFU/ml). Note that experiments with *P. acnes* were conducted over 4 days, unlike 24 hours in the other.

Isothermal microcalorimetry has been useful in microbiological pharmacology studies where reduced, delayed and absent heat flow was related to the reduced inoculum after antimicrobial exposure [11, 34, 48,

73, 77, 79-81, 147, 201, 239]. The study group of Andrej Trampuz has conducted a long series of biofilm-experiments in the microcalorimeter with or without prior antimicrobial treatment, from which we found inspiration. In **Study I**, we studied the heat flow of dislodged biofilm bacteria after sonication, and in **Study II** we directly studied the surface-attached biofilm on a biomaterial sample fitting inside the calorimetric ampoule.

Appendix G: Sonication procedure

In all three studies, we used the same ultrasound bath model BactoSonic™ 14.2 from Bandelin (Berlin, Germany) with 100% power (frequency of 40 ±2 kHz and power density 0.22 ±0.04 W/cm2). We followed the sonication procedure (Figure 23), as recommended by Trampuz A., *et al.* [1, 30, 76, 229]:

1. Transfer the sample/implant to a solid and sterile container
2. Cover the sample/implant by isotonic saline or Ringers Lactate
3. Close the container airtight to avoid leakage and contamination
4. Shake (or vortex) the sample/implant inside the container for 30 seconds
5. Place the container in the ultrasound bath and sonicate for 60 seconds (100% power)
6. Shake (or vortex) the sample/implant inside the container for another 30 seconds
7. Open the container and take appropriate culture samples for further microbiological examination

For each sample in the experimental studies, we used one sterile container of suitable volume (2 ml Eppendorf tubes in **Study I** and 15 ml Falcon tubes in **Study II**) to enable vortexing (Vortex Genie, Scietific Industries) and sonication as described above.

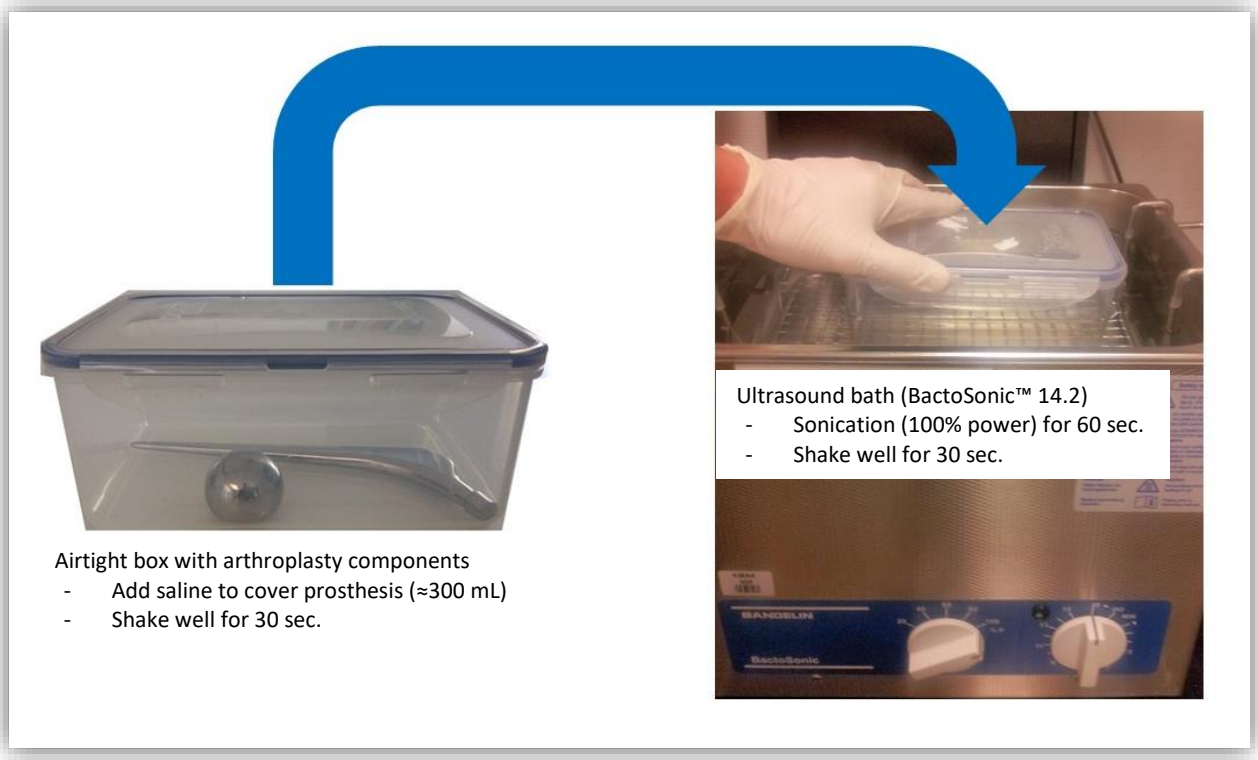


Figure 23: Sonication procedure

Handling of prosthetic components in Study III

For transport and sonication of explanted prosthetic components in the clinical **Study III**, we purchased plastic containers (LOCK&LOCK GmbH, HPL-series, Frankfurt am Main, Germany) of various sizes (0.6-3.9 liters) from www.BrixDesign.dk.

Treatment instructions for implant boxes as recommended by Bandelin follows EN ISO 17664:2004. In short, the local sterilization department assisted us in cleaning, packing and sterilization by either autoclaving at 121°C (Odense University Hospital) or plasma sterilization (Vejle Hospital).

In each orthopedic department, sterile boxes in different sizes were kept available for the surgeon to choose a box size containing the total amount of explanted components without too much excess space; no plastic box was reused after sonication. In the operating room, explanted prosthetic components were transferred to a plastic box, which was sealed airtight and labeled.

At Odense University Hospital (OUH), the boxes were sent directly after revision surgery to the Department of Clinical Microbiology (DCM) and processed either immediately or the following day. In Vejle, boxes were transported to the DCM at OUH by car (1 hour) the following morning. No transportation from Vejle or sonication at OUH was undertaken during weekends and holidays. The sealed boxes were kept at 5 °C from ex-plantation until beginning of the diagnostic procedure. Handling of the explanted prosthetic components was conducted in parallel to the conventional diagnostic procedure, and one dedicated laboratory technician blinded to the clinical situation performed all steps from registration to interpretation. Under laminar airflow, approximately 300 ml saline (0.9% NaCl) was added to each box in order to cover the prosthesis before shaking, sonication and repeated shaking (Figure 23).

After the vortex-sonication procedure, aliquots of 0.2 ml sonication fluid were sampled under laminar airflow and plated on 5% horse blood agar (incubated in an aerobic atmosphere with 5% CO2 for five days), K-vitamin enriched chocolate agar (cultured anaerobically for five days), and in thioglycollate enrichment broth for 14 days. Growth media were checked daily to register time (days) from incubation to visible growth. The number of colony-forming units (CFU/ml) was enumerated. A diagnostic threshold at 20 CFU/ml was used to distinguish infected from possibly contaminated prostheses. The non-quantitative result of thioglycollate-culture was noted for comparison, but not in itself accountable for the diagnostic outcome. Cultured organisms in sonication fluid were identified by MALDI-TOF.

Appendix H: Periprosthetic tissue samples and synovial fluid

In **Study III**, sampling of 5 tissue biopsies was performed according to local guidelines with a sterile set of five forceps and five surgical knives from a representative area adjacent to the prosthesis [116, 155]. Each biopsy was placed in a separate container (Figure 24). The first dose of perioperative antibiotic prophylaxis was administered immediately after biopsy sampling.

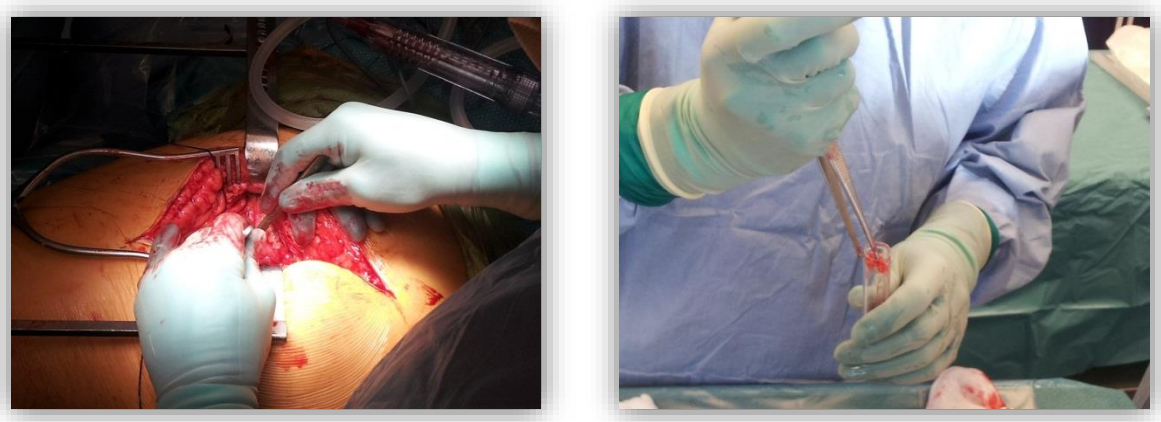


Figure 24: Intraoperative biopsy sampling from the periprosthetic tissue in THA revision surgery

Synovial fluid was aspirated during revision surgery immediately before arthrotomy in order to secure access to the joint space and reduce the risk of contamination from skin flora (Figure 25). Upon aspiration, the surgeon described synovial fluid quality as either normal, purulent, shady or bloody. Situations with fruitless joint aspiration were referred to as 'dry tap' [5].

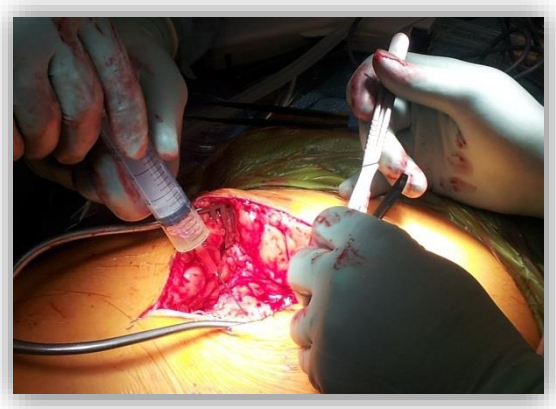


Figure 25: Intraoperative joint fluid aspiration before arthrotomy in THA revision surgery.

At the end of surgery, synovial fluid and periprosthetic tissue samples were transported to the local department of clinical microbiology. Synovial fluid and tissue samples were individually plated on 5% horse blood agar, eosin methylene blue agar and thioglycollate enrichment broth (incubated in aerobic atmosphere with 5% CO_2 for 5 days), as well as K-vitamin enriched chocolate agar (anaerobic incubation for 5 days). Experienced laboratory technicians perform quantification by viable counting of colony forming units (CFU) and susceptibility testing according to local standards. Cultured organisms were identified by Matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF), using either MALDI Biotyper (Bruker, Bremen, Germany) or Vitek MS (bioMérieux, Marcy l'Etoile, France) instrument [19, 115].