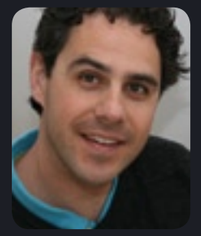
Curriculum Vitae

The author of this thesis was born on December 14th, 1976 in Utrecht, The Netherlands. After spending his childhood in Houten, he graduated from high school (Stedelijk Gymnasium, Utrecht) in 1995 and started to study medicine in the same year at the University of Utrecht. In 1999 he made his first steps in the field of orthopaedic research when he cooperated in a research project on cartilage tissue engineering (supervisor: Prof.dr. D.B.F. Saris). After obtaining his medical degree in August 2002, he started working as a non-training resident at the department of Orthopaedics of the Medical Center Alkmaar (head: Dr. A.J. de Gruijter/ Dr. B.J. Burger). In March 2003 he started working on a PhD-project on the diagnosis, prevention and treatment of implant-related infections at the department of Orthopaedics at the University Medical Center Utrecht (supervisors: Prof.dr. W.J.A. Dhert/ Prof.dr. R.M. Castelein; co-supervisors: Dr. H.Ch. Vogely/ Dr. L.M. Schouls). This work resulted in several publications, presentations at national and international conferences and this thesis. In 2006 he received the EBJIS Innovation Award at the annual meeting of the European Bone and Joint Infection Society. He started his surgical training in July 2006 at Tergooiziekenhuizen, Hilversum (head: Dr. J.W. Juttmann/ Dr. J.P. Eerenberg) and proceeded with his orthopaedic training 2 years later at the University Medical Center Utrecht (head: Prof.dr. D.B.F. Saris). Since July 2009 he is working at the Onze Lieve Vrouwe Gasthuis, Amsterdam (head: Dr. W.J. Willems). In January 2011 he will return to the University Medical Center Utrecht to complete his residency period. He lives in Utrecht with his wife Gemma and daughter Emeline. A second daughter or son is expected in early May 2010.



Implant-related Infections:
Application of PCR-based Diagnostics and New Antimicrobial Strategies in Prevention and Treatment
D.J.F. Moojen

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Implant-related Infections:
Application of PCR-based Diagnostics and New Antimicrobial Strategies in Prevention and Treatment

Implantaat-gerelateerde Infecties:
Toepassing van PCR Diagnostiek en Nieuwe Antimicrobiële Strategieën voor Preventie en Behandeling

(met een samenvatting in het Nederlands)

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Journal of Orthopaedic Research, 2008 Mar;26(3):404-10

In vitro release of antibiotics from commercial PMMA beads and articulating hip spacers.
D.J.F. Moojen, B. Hentenaar, H.Ch. Vogely, A.J. Verbout, R.M. Castelein, W.J.A. Dhert
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Prophylaxis of infection and effects on osseointegration using a tobramycin-peri-apatite coating on titanium implants - an experimental study in the rabbit.
D.J.F. Moojen, H.Ch. Vogely, A. Fleer, P.G.J. Nikkels, P.A. Higham, A.J. Verbout, R.M. Castelein, W.J.A. Dhert
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No efficacy of silver bone cement in the prevention of methicillin sensitive staphylococcal infections in a rabbit contaminated implant bed model.
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D.J.F. Moojen, G. van Hellemondt, H.Ch. Vogely, B.J. Burger, G.H.I.M. Walenkamp, N.J.A. Tulp, B.W. Schreurs, F.R.A.J. de Meulemeester, C.S. Schot, I. van de Pol, T. Fujishiro, L.M. Schouls, T.W. Bauer, W.J.A. Dhert
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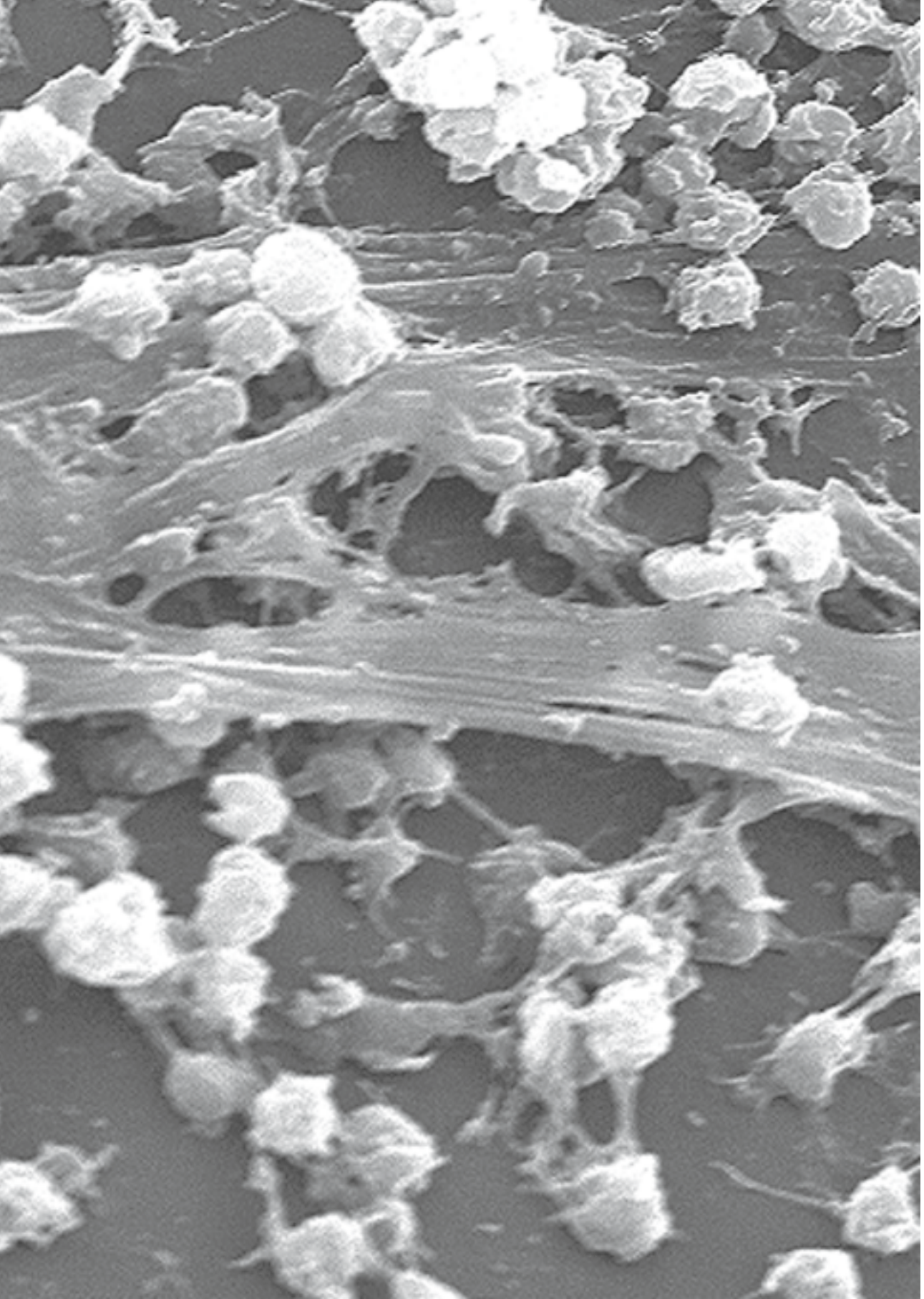

Chapter 1

Introduction and aims

Introduction

Since Sir John Charnley reported on the use of his low friction arthroplasty of the hip in the early 60's of the previous century, the use of implants in orthopaedic surgery has seen many innovations[1, 2]. Because of the clinical successes, indications were broadened (e.g. younger, more active patients) and numbers of prostheses implanted increased[3, 4]. In The Netherlands, currently more than 20.000 total hip arthroplasties are implanted annually and numbers are even expected to increase in the next few decades[5, 6]. For total knee arthroplasty these numbers are comparable.

Despite all long-term successes, total joint replacements may be accompanied by complications as well. Although the most common complication is thought to be aseptic loosening, the most devastating complication is infection[7-10]. This can be either early, late or haematogenous[11-14]. An infection is considered early if it becomes apparent during the early postoperative period, which, according to different authors, may range between 6 and 12 weeks[11, 12]. This type of infection usually presents itself with clear symptoms and is caused by virulent bacteria. Late infections appear after this early period and sometimes even after years. They present themselves as low-grade infections with more subtle signs and symptoms and are usually caused by low virulent bacteria. For both types of infection the contamination with bacteria is thought to have happened either during surgery or during the early postoperative period[11]. Haematogenous infections can be early, but most often appear years after implantation with acute signs and symptoms and are caused by a bacteraemia from infections elsewhere, like the urinary tract, skin or after dental procedures[13, 15, 16].

Again Charnley was the first to publish his results with respect to infection rates[2]. From his first series of patients he reported an infection rate of 9.5%, a number that would be considered unacceptable nowadays. He already identified the bacterial content in the air of the operating theatre as an important risk factor. Meanwhile many improvements have been made in the prevention of implant-related infection, and infection percentages of less than 1% are nowadays reported in literature[13, 17-20]. Of course one should keep in mind that these numbers are often biased, as centers of excellence with low infection rates are probably more likely to publish their results. In The Netherlands, postoperative surgical infection rates are documented in a registry called 'PREZIES'[a]. This register shows that infection rates of about 2.3 and 1.8% are obtained for total hip and knee arthroplasty, respectively[21]. For revision cases these numbers increase rapidly, with 4.5% for total hip arthroplasty and 5.0% for total knee arthroplasty.

[a] PREZIES (PREventie ZIEkenhuisinfecties door Surveillance) is a collaboration between the participating health care facilities, the Quality Institute for Health Care CBO and the National Institute for Public Health and the Environment (RIVM)

The bacteria cultured in patients with an implant-related infection, can be pathogens from regions elsewhere on the body, like the oropharyngeal cavity or urinary tract, but are most often skin commensals[22-25]. *Staphylococci* are found in more than 50% of the patients. Traditionally, *Staphylococcus aureus* was seen most often, however nowadays coagulase negative staphylococci are predominant[23, 26]. Other microorganisms often encountered are *Streptococci*, *Enterococi* and Gram-negatives, like *Enterobacter* and *Pseudomonas* species. Other bacteria found, like *Proprionibacterium acnes* and *Corynebacterium* species, were often regarded as contamination, but are now increasingly linked to low-grade infections[27-31]. However, these types of bacteria have a fastidious growth and are often missed[28, 29].

Although infection rates have decreased, the consequences and morbidity for the individual patient still remain considerable. In addition, the financial costs for the health care system because of long hospitalization, multiple operations and long-term antibiotics are considerable as well[32-36]. Therefore every effort should be made to make sure prevention and treatment are as successful as possible.

Diagnosis of infection

Obtaining a correct preoperative diagnosis in a patient with complaints after total joint arthroplasty is of high importance, as treatment will significantly differ between a septic and aseptic case. Unfortunately, this is not always easy. Although the surgeon has many diagnostic modalities available, there is not one test available, which can serve as a true gold standard[11, 12, 14, 37-41].

One will always start with a thorough anamnesis and physical examination. Although fever and pain in a joint with an arthroplasty is suggestive for infection, it does not provide a definite diagnosis. For physical examination, swelling and redness are suggestive, whereas a draining fistula can be considered diagnostic. Additional X-ray analysis should always be performed, but rarely shows specific signs of infection and mainly excludes other causes[42, 43].

Blood analysis determining leukocyte count, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) does have diagnostic value[39, 44-49]. Although useful in diagnosing non-arthroplasty arthritis, leukocyte count has been shown to have little value in implant-related infection, as it has a very low sensitivity[39]. ESR and CRP on the other hand do have good discriminatory value. Although exact cut-off values differ between studies, an ESR of more than 30 and CRP of more than 10 above baseline values for a patient are suggestive of infection with high sensitivity and specificity, with CRP and ESR combined the numbers are even higher[39]. In patients with an acute fulminant infection these parameters will rapidly increase; however, in low-grade infections both parameters may often be normal[45].

In addition to these tests, bone scintigraphy can be performed to evaluate implant loosening and infection. The three-phase technetium-99m bone scan is most often used, but has a low sensitivity for infection and it is often difficult to differentiate between aseptic loosening and infection[50-52]. Several different nuclear imaging scans are available to further differentiate between these diagnoses, like the indium-11 leukocyte scan, gallium-67 scintigraphy, technetium-99m polyclonal IgG scan, FDG-PET scanning and more recently PET-CT scanning[53-59]. Although some reports say these scans have higher sensitivity, results are variable and added clinical value for regular use is questioned.

Another preoperative test to evaluate infection is aspiration of joint fluid for culture[39, 60-64]. For the knee this can be even done at the outpatient clinic, for the hip it is usually performed in a more formal setting under guidance of fluoroscopy. With this test it is possible to identify the pathogenic microorganism preoperatively and it gives an opportunity to guide further treatment. To minimize the risk of a false negative result it is recommended the patient should not use antibiotics at least 2 weeks prior to aspiration[11, 38]. However, no hard evidence exists on how long this antibiotic free period should actually be. Likewise, any positive result should always be considered in its clinical context, as false positive results, due to contamination, can occur as well.

Intraoperative tests are microbiological and pathological analysis. Microbiological analysis can be differentiated into Gram stain and culture. Although often performed, several studies showed that a negative Gram stain has no diagnostic value in excluding infection, as it has a very low sensitivity[39, 65-67]. A positive result might have value in the early guidance of antibiotic treatment. Intraoperative culture on the other hand is often considered as gold standard or at least the best reference standard available[24, 38, 60, 68-72]. Culture can identify the microorganism involved and determine the antibiotic susceptibility to guide antibiotic treatment. Tissue cultures have superior sensitivity compared to swab cultures[73, 74]. In order to minimize the risk of a false negative result also here it is recommended that antibiotic use should be prohibited at least 2 weeks prior to surgery and prophylactic perioperative antibiotics should only be given after culture samples are obtained[11, 38]. As contamination and thereby false positive results can happen, there is debate on how many cultures have to be positive to reliably detect infection. Although different studies recommended different standards, only one study prospectively investigated this issue and recommended that at least 3 samples have to be positive for the same microorganism and that at least 5 tissue samples should be sent for culture[67].

Frozen sections on intraoperative pathology samples can be used as well[39, 68, 71, 75-84]. Samples for analysis should be taken from tissue that displays highest degree of inflammation. Although the pathogenic microorganism cannot be identified in frozen samples, they can be used to assess whether an infection is ongoing or not. This diagnosis is mainly based on the number of polymorphonuclear leukocytes (PMN) detected in the tissue. Unfortunately, multiple cut-off values, ranging for 1 to 10 PMN per high power field, are used in literature; all with variable sensitivity and specificity[77, 79, 81]. Another disadvantage of this test is that it is often difficult to efficiently arrange the necessary logistics and that one must have a skilled pathologist available, who can give a reliable histopathological diagnosis. If this is the case, frozen section analysis is as reliable as definite paraffin-embedded pathology analysis[39].

Although the routine tests and techniques described above are currently used in clinical practice, new diagnostic modalities are constantly being developed. Because of the emerging incidence of fastidious growing bacteria, like small colony variants and planktonic biofilm growth modes, these microorganisms are much more difficult

to detect[85-88]. These new tests use for example sonication to dislodge the bacteria from the biofilm[30, 89-93]. Many other studies use molecular biological techniques, like polymerase chain reaction of bacterial DNA[24, 30, 70, 91, 94-109]. For this, the 16S rRNA gene, which is exclusive to and present in all bacteria, is often used[24, 30, 70, 91, 95, 98, 102, 108]. Using molecular biological techniques it is possible to detect low numbers of low-virulent or fastidious growing bacteria, irrespective of prior antibiotic use. This provides the possibility to detect low-grade infections missed with standard techniques. Disadvantage of this high sensitivity is that these tests are more susceptible to contamination[24, 94, 110, 111]. Although not available for routine clinical practice yet, these developments do posses great potential for the future. This is especially true since there is growing evidence in literature that currently many low-grade infections are misdiagnosed as aseptic[7, 9, 18, 30, 73, 89, 101, 107, 108]. Both from the large arthroplasty registers, where the addition of antibiotics to the bone cement led to lower revision rates for aseptic loosening, and from several clinical studies, it is determined that substantial numbers of patients, who are regarded as aseptic and treated accordingly, are in fact low-grade infections missed with routine diagnostics. As this may increase the risk of early failure and thereby decrease long-term survival of these implants, these observations need further research.

Antimicrobial strategies in prevention and treatment

Prevention

Although many pre-, per- and postoperative factors, like for example comorbidities, airflow in the operating room and wound care, have been identified and improved, the use of systemic antibiotics during the perioperative period had the largest effect on the prevention of implant-related infections[17, 18, 112-117]. These prophylactic antibiotics should have good efficacy against the most common bacteria causing arthroplasty infections and should have good bone penetration. Therefore, most commonly a first or second-generation cephalosporin is chosen. It should be administered within 60 minutes before the start of surgery to be maximally effective[115, 118]. In addition, prophylaxis for 24 hours is sufficient, as longer prophylaxis was shown to have no beneficial effect[18].

Buchholz introduced the possibility of adding antibiotics to the PMMA bone cement[119, 120]. He reported reduced infection rates after the addition of gentamicin to Palacos cement[120]. Since then the use of antibiotics in bone cement has become widespread and nowadays has become standard of care for most surgeons[121, 122]. Different brands of PMMA cement, containing different types of antibiotic, are commercially available in many countries. Alternatively, some surgeons hand-mix the desired type and amount of antibiotic with the cement themselves. Its efficacy in preventing infection has been later confirmed in many clinical and experimental studies, as well as by the large Swedish and Norwegian arthroplasty registries[9, 17, 18, 120, 121, 123-131]. Despite its popularity, there are also critics that claim, that by routinely using antibiotic-loaded bone cement the emergence of antibiotic resistant bacterial strains is increased[122, 132-135]. This would increase the difficulty of effectively treating infected revision cases. No scientific studies, which showed this increase of antibiotic resistance due to the prophylactic use of antibiotics for bone surgery, were published. However, it is clear that continued selective pressure due to use of antibiotics will lead to resistance and the effects have to be carefully monitored.

For uncemented implants, no strategies for local antibiotic delivery are currently available. As the uncemented implant has to interact with the surrounding bone to obtain a solid fixation, surface characteristics are very important[136-141]. A potential antimicrobial coating should not interfere with this osseointegration. Therefore bulky coatings with large amounts of antimicrobial agents, comparable to the antibiotic-loaded cement, are not feasible. During the last decade, many studies on this topic have been published[142-163]. These studies investigated the possibility of coating different implants with antibiotics, cross-linked albumin or antiseptics. This was done by either direct attachment, additional stabilization with lipids or proteins or even

binding it covalently to the implant surface. The amount of antibacterial agent incorporated, release patterns and effectiveness varied considerably between the different coatings. Despite different studies with positive results, until now no coating has become available for clinical practice.

Because of the increasing problem of antibiotic resistance, some antimicrobial agents outside the field of regular antibiotics have been investigated for their efficacy in the prevention of implant-related infection as well. For example, the use of antimicrobial peptides, like Dhvar-5 and human lactoferrin 1-11, was investigated, but showed only moderate efficacy in preventing infection *in vivo*[164-167]. In addition silver, which is known to have antimicrobial activity, was coated on external fixator pins and prostheses and added to PMMA bone cement[168-174]. Already 30 years ago, silver was added to the cement as a silver salt[173, 174]. It showed reasonable efficacy against different bacteria, but disappeared after reports of potential neurological complications because of the release of silver ions[175, 176]. More recently, results of a new type of low-dose silver bone cement were reported[168, 169]. This cement contained NanoSilver, which is metallic silver with very small particle sizes (nanoparticulate), thereby increasing the active antimicrobial surface and preventing the systemic release of silver. This silver cement showed good efficacy against different sensitive and resistant bacteria and no toxicity against osteoblasts *in vitro* and might hold promise for future clinical application. Although the number of publications on new antimicrobial agents for orthopaedic use is currently limited, this will probably increase in the near future, as resistant bacteria will force us to explore other options than the antibiotics currently available.

Treatment

When the surgeon has to choose how to treat a patient with an infected joint arthroplasty, there are different strategies available[11, 13, 177, 178]. Patients with an early infection or an acute-onset late haematogenous infection can be treated with debridement with or without the use of local antibiotic carriers in an effort to retain the implant. Patients with a late infection should be treated with a 1 or 2-stage revision, resection arthroplasty or only life-long suppressive antibiotics, when they are not able to undergo surgery. In addition to the surgical treatment of the infection, the patient should receive systemic antibiotics, adjusted to the susceptibility pattern of the cultured pathogen, for a prolonged period as well. For hip arthroplasties a period of 3 months is recommended, for knee arthroplasties even 6 months[11, 179].

Although some centers have reported excellent results for 1-stage exchange arthroplasties in infected patients, the gold standard still is the 2-stage procedure[180-186]. During the first stage of this procedure, the old prosthesis is removed, soft tissues are debrided and in many cases a local antibiotic carrier is left in situ. During the interval the systemic antibiotics should further cure the infection and at the second stage, when the infection is cured, a new prosthesis is implanted.

Even though new treatment algorithms and their efficacy have been published, most attention during the last decade on the aspect of treatment has been on the different local antibiotic carriers[11, 177, 187-189]. These carriers should release antibiotics in very high concentrations locally, without toxic systemic side effects. Although not all surgeons use such a carrier between the first and second stage, traditionally the most commonly used are the antibiotic-loaded PMMA cement beads[190-195]. These have been used since the late 70's and are commercially available in most European countries loaded with gentamicin. As these beads have no FDA (Food and Drug Administration) approval, in the United Stated the surgeon fabricates them himself during surgery by mixing antibiotic powder with the PMMA cement and molding the cement to beads. Good efficacy and safety has been reported, but this strategy has disadvantages as well[190, 191, 196-200]. These beads release antibiotics for only a limited period of time and it is recommended they are removed or exchanged after approximately 2 weeks. In addition the beads provide no mechanical stability to the joint and soft tissues surrounding it. As a consequence the patient is bedridden and the reimplantation is often difficult because of shortening of muscles and other structures[201, 202]. Therefore, the use of cement spacers has become increasingly popular[202-215]. These spacers are made of antibiotic-loaded bone cement and frequently contain a central metal core. In addition to releasing antibiotics, these spacers fill the cavity of the removed prosthesis and, although they are generally not strong enough for full weight bearing, provide some stability and maintain congruence of the joint[206]. Positive clinical results have been reported for spacers loaded with large amounts of antibiotics[184, 202, 206, 212, 215]. However critics say, that a disadvantage of spacers is that they might not release enough antibiotics to effectively treat the infection[196, 207, 216, 217]. As antibiotic release is a surface phenomenon, the much larger total surface of the beads, compared to the spacers, would favor the use of beads in this respect[196, 216].

Aims of the thesis

This thesis will focus on different aspects of diagnosis, prevention and treatment of implant-related infections, according to the aims outlined in the following paragraphs. This was done, by performing *in vitro* laboratory experiments, animal infection studies and retro- and prospective clinical studies. The general aims and specific research questions we wanted to address were:

1. To determine the value of the combined use of broad range 16S rRNA PCR and reverse line blot hybridization (PCR-RLB) in the detection of orthopaedic implant-related infections
   a. Can the combined PCR-RLB protocol be optimized and validated for use in reliably detecting orthopaedic infections?
   b. Can the real incidence of aseptic loosening and low-grade infection of total hip arthroplasties be determined using the PCR-RLB protocol in combination with routine diagnostics?
2. To investigate new prophylactic antimicrobial strategies, both for uncemented and cemented implants and new applications
   a. Can an antibiotic coating on hydroxyapatite-coated titanium foam decrease infection susceptibility and increase osseointegration?
   b. Does silver containing bone cement have a good efficacy in preventing implant-related infection in comparison to antibiotic bone cement?
   c. Does platelet-leukocyte gel have an antimicrobial effect?
3. To evaluate the availability of antibiotics from different therapeutic carriers
   a. Do spacers release less antibiotics compared to cement beads?

Content of the thesis

A proper diagnosis and evaluation guides the surgeon's decision on what the most optimal treatment will be. With the aim to improve the sensitivity and reliability of detecting implant-related infections, we investigated the feasibility of new molecular biological techniques, using a combined polymerase chain reaction targeted at the 16S rRNA gene and a reverse line blot hybridization protocol. Chapter 2 describes the study in which these techniques were developed, refined and validated using a large collection of clinical samples of patients suspect or not suspect for infection.

As mentioned previously, there is a high suspicion that many patients with aseptic loosening of their arthroplasty have in fact low-grade infections, which are not detected, because routine diagnostics are not sensitive enough. Therefore our next aim was to more reliably determine the True Incidence of Aseptic and septic loosening in Revision Arthroplasty (TIARA) and investigate whether the combined 16S rRNA polymerase chain reaction (PCR) and reverse line blot hybridization (RLB) protocol described in chapter 2 would be a valuable tool in identifying these low-grade infections. Chapter 3 describes the results of this TIARA-study. In this multicenter study, patients receiving a revision of their total hip arthroplasty, because of the preoperative diagnosis aseptic loosening, were investigated using PCR-RLB and routine diagnostics, like culture and pathology.

Although accurately diagnosing implant-related infections is essential for correctly treating the patient, perhaps even more important is the prevention of infection. Therefore in the second part of this thesis we investigated several new antimicrobial strategies for their potential use in orthopaedic practice.
As uncemented implants experience an increase in popularity because of good long-term survival, there is also an increasing interest in local antimicrobial prophylaxis for these types of implants. Currently, no such option is available. Therefore, our aim was to investigate the usefulness for infection prophylaxis of a simple method to apply an antibiotic coating to a porous, hydroxyapatite-coated titanium biomaterial, which can be used for uncemented implants. Chapters 4 and 5 describe two studies in which the efficacy of applying a tobramycin coating to a periapatite-coated titanium foam implant is investigated in preventing staphylococcal implant-related infections. In chapter 4 the *in vitro* release characteristics of both tobramycin and gentamicin are determined, when added as a dip-coating to the implant. In addition, the *in vitro* inhibition of *Staphylococcus aureus* was determined. Chapter 5 describes a study where we used an animal implant-related infection model, to investigate the *in vivo*

efficacy of a tobramycin coating on periapatite-coated titanium foam in preventing *Staphylococcus aureus* infection after contamination with different amounts of bacteria. This was compared to uncoated implants and implants coated with periapatite only. In addition the effect on osseointegration of these different implants was studied as well.

Although for cemented implants the use of antibiotic containing bone cement has become standard for most surgeons both in primary and revision cases, the increasing problem of antibiotic resistance forces us to look for other antimicrobial agents to overcome this impeding problem. Silver is known to have antimicrobial activity against multiple different bacteria and is said to be even active against methicillin resistant *Staphylococcus aureus* (MRSA). Positive results of a new type of nanoparticulate silver added to PMMA cement were reported from *in vitro* tests and suggested good potential for clinical application[168, 169]. Our aim was to establish the *in vivo* efficacy of PMMA bone cement loaded with this type of nanoparticulate silver in preventing implant-related infection. In chapter 6 we investigated this using an animal implant-related infection model. In this study the efficacy of bone cement loaded with 2 different concentrations of silver in preventing methicillin sensitive *Staphylococcus aureus* infection was compared to tobramycin-containing bone cement and bone cement not containing an antimicrobial agent.

A totally novel antimicrobial strategy is the use of platelet-leukocyte gel, which is made from autologous blood and contains high concentrations of platelets and leukocytes. Although platelet-leukocyte gel is mainly investigated because of the potential effect on bone formation and wound healing, it could have an antimicrobial effect as well[218-227]. The leukocytes in the gel contain myeloperoxidase, which plays a key role in the oxidative killing of bacteria[228]. In addition the platelets contain many different antimicrobial peptides[229, 230]. As no research had been published on this topic, our aim was to investigate whether platelet-leukocyte gel indeed has an antimicrobial activity, how large this activity would be and try to identify to role of different mediators in this process. Chapter 7 describes a study in which the antimicrobial activity of platelet-leukocyte gel against *Staphylococcus aureus* was determined *in vitro*. In addition, the contribution of myeloperoxidase to this process was determined.

Although many improvements have been made with respect to prevention, implant-related infections do still occur. In treating these infections, surgical debridement of all infected tissue is the most important factor for success. Additionally, temporary local antibiotic carriers can be used. Traditionally small gentamicin-containing bone cement beads are used. However, the use of spacers made of antibiotic bone cement has gained popularity. As antibiotic release is mainly a surface phenomenon, it is questioned whether these spacers release enough antibiotics to adequately treat the infection. Although widely discussed, experimental data comparing these two modalities is lacking. In order to give some evidence to the discussion, the aim of the study of chapter 8 was to compare the *in vitro* antibiotic release of a clinically relevant amount of gentamicin cement beads to the release from hip spacers made of 2 types of commercially available antibiotic bone cement.

Finally in chapter 9 we give a summary and conclusions from the research performed for this thesis. Results of the studies will be discussed, not only with respect to clinical applicability, but also with respect to new possibilities for future research.

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Diagnosis of infection

Chapter 2

Identification of orthopaedic infections using broad range PCR and reverse line blot hybridization

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Abstract

Background

Culture remains the gold standard in the diagnosis of bacterial infection, but molecular biological techniques have yielded promising results. In this study we validated a combined PCR and reverse line blot (RLB) hybridization protocol for identifying orthopaedic infections.

Methods

Samples from 76 orthopaedic patients undergoing surgery for various aseptic and septic indications were obtained. The diagnosis of infection was based on a review of all available clinical and culture data. Next to routine culture for aerobic and anaerobic growth, analysis of the samples was performed with a broad range 16S rRNA PCR and subsequent RLB hybridization, using 28 group, genus and species-specific oligonucleotide probes.

Results

Thirty-one patients were diagnosed as infected. All patients with a bacterial infection were positive by PCR-RLB. Five of 45 patients not suspect for infection had at least 1 positive PCR result as well. Culture detected microorganisms in 25 infected patients and in 2 patients not suspect for infection. With the exception of a single patient, PCR-RLB species identification was in full accordance with culture. *Staphylococcus aureus* was found in more than 13% of all patients enrolled in this study. Coagulase negative staphylococci were found in 5% and streptococcal species in approximately 11% of the patients.

Conclusions

PCR-RLB performed well in detecting and identifying the various bacterial species and was more sensitive than routine culture. It identified *S. aureus* as the most frequently found microorganism. Five patients, clinically not suspect for infection, were positive by PCR, which may have been caused by contamination of the samples. However, 3 of these patients had aseptic loosening of their total hip arthroplasty, suggesting a low-grade bacterial infection that remained undetected by culture, but was detected by PCR-RLB.

Introduction

Infection after orthopaedic surgery is a serious complication, requiring early intervention and aggressive treatment. Especially in the case of an infected arthroplasty, the consequences, both for patient and healthcare system, can be considerable. Early treatment requires an early detection and identification of the infectious agent. Unfortunately, diagnosis of infection is in many cases difficult and inconclusive. For most surgeons, bacterial culture combined with the clinical presentation, blood analysis and pathology, remains the gold standard[1-3]. However, there is a growing number of publications, that show culture may not be accurate in a substantial number of patients, due to contamination or false negative results[4-6]. Particularly false negatives pose an important problem, as this may delay adequate therapy. False negatives may be caused by small colony variants and biofilm-related bacteria, often not detected by routine culture, as detection of these types of bacteria often requires special growth media and extended culture periods[7, 8]. Therefore new molecular biological detection techniques are being developed, which show promising results. Especially the use of the polymerase chain reaction (PCR), which multiplies minute amounts of bacterial DNA, is a valuable technique. During the last decade, several studies using PCR targeted at the 16S rRNA gene, present in all bacteria, have been published[9-17]. These studies showed that with broad range 16S PCR it was possible to detect the bacterial DNA in infected cases. In addition, they also showed the presence of bacterial DNA in a substantial number of patients not suspect for infection, possibly indicating low-grade infections, missed by routine diagnostics[10-12]. Despite these promising results, broad range 16S PCR also has some disadvantages. Because of the high sensitivity and the fact that each bacterial species is target for this PCR, it is very susceptible for contamination; theoretically even a single bacterium can be detected. High contamination rates have already been reported in patients receiving a primary total hip arthroplasty (THA)[18]. Another disadvantage of the 16S PCR protocols published until now is, that with most techniques used, bacterial DNA could be detected, but the bacterial species could not be further identified. Species identification would help to distinguish the presence bacterial species in the sample from contamination introduced during the PCR procedure. This would aid a more reliable diagnosis of infection and could serve as a global guide for treatment. The aim of this study was to develop a 16S rRNA PCR method in which sample contamination was kept at a minimum. Furthermore, we assessed the clinical applicability by combining PCR with reverse line blot (RLB) hybridization using oligonucleotide probes to detect and identify bacterial species using intraoperative tissue samples from a group of orthopaedic patients undergoing surgery for various aseptic and septic indications.

Materials and Methods

Sample collection

We received approval from the institutional board review committee of the University Medical Center Utrecht. Between September 1995 and January 2001, 180 intraoperative tissue samples for PCR were collected from 76 patients undergoing surgery for different aseptic and septic indications (Table 1) at the department of Orthopaedics. Specimens for PCR and culture (n=167) were obtained during the same operation. Culture samples were processed using standard protocols for aerobic and anaerobic culture.

Diagnosis of infection

As the reference for comparison we used the clinical diagnosis of infection. For each patient this final diagnosis of infection or no infection was based on a retrospective review of all available patient data, such as clinical data, x-rays, erythrocyte sedimentation rate, c-reactive protein, microbiology and pathology results, but not on the PCR data. This review was performed on average 6.6 years (range 4.2-9.5 years) postoperative. Both written medical files and the electronic hospital reference database were used for retrieving data. As a retrospective analysis was performed, not all tests were available for all patients. To make the diagnosis of infection or no infection as consistently as possible we used the criteria as previously used by Spangehl *et al.*[1].

Table 1

Indications for surgery of cases enrolled in the study

Indication for surgery	Number of patients
Aseptic revision arthroplasty	29
Removal infected arthroplasty	16
Debridement infected arthroplasty	12
Arthritis	6
Primary arthroplasty	2
Other, aseptic	6
Other, septic	5
Total	**76**

DNA extraction

After surgery, tissue samples for PCR were immediately transferred in digestion buffer (500 mM Tris.HCl pH 9, 20 mM EDTA, 10 mM NaCl, 1% sodium dodecyl sulphate (SDS), 0.5 mg/ml proteinase K) and stored at -20°C until further processing. For extraction, samples in digestion buffer were incubated overnight at 60°C to release total DNA. The DNA was further purified using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according the protocol provided by the manufacturer. Extracts were stored at -20°C until further use. To monitor the possible occurrence of false-positive results due to contamination during DNA extraction, an extraction control, consisting of digestion buffer without patient material, was added for every 4 clinical samples processed.

PCR of the 16S rRNA gene

Broad range primers based on the highly conserved regions on the 16S rRNA gene were used to set up the 16S PCR. The 5' end of the reverse primer was biotin-labeled. All primers and probes are listed in table 2. Probe selection was based on what was described in literature as the most frequently isolated pathogens in orthopaedic infections[19-21].

PCR was performed in 25 µl reaction volumes. Each reaction mixture contained 30 pmol of primer 16S8-27F and 45 pmol of primer B-16S509-525R(22) and 12.5 µl of HotStarTaq Master Kit (Qiagen). In addition 1U of 4 different restriction enzymes, *Alu*I, *Cfo*I, *Hae*III, *Sau3A*I, was added to the reaction mixture to digest contaminating DNA in any of the components. Mixes were prepared in an ultraclean laboratory with positive pressure and an airlock. Prepared mixes were transferred to another clean laboratory with positive pressure and an airlock, which was also used for DNA extraction from the clinical samples. The reaction mixture was incubated for 1 hour at 37°C for restriction. Then the mixture was filtered using a QIAquick column (Qiagen) to remove any residual whole bacteria containing DNA, which is not accessible for the restriction enzymes. The mix was applied directly to the QIAquick column and centrifuged at 10.000 rpm for 1 minute in a microfuge. Mixes were dispensed in 0.2 ml PCR tubes (Applied Biosystems, Foster City, CA, USA) and an additional hour of restriction at 37°C was performed to ensure all interfering DNA was cut. Subsequently, the restriction enzymes were inactivated at 65°C for 20 minutes, after which 2 µl of the DNA extraction sample was added to the mixture. To minimize non-specific amplification, a touchdown PCR program was used: 15 min at 95°C for activation of the HotStarTaq polymerase: two cycles of 30 sec at 95°C, 30 sec at 66°C, 1 min at 72°C. During subsequent two-cycle sets, the annealing temperature was lowered by 2°C until it reached 56°C and an additional 33 cycles were performed.

This program was followed by an extra incubation for 7 min at 72°C. PCR products were stored at 4°C until further processing. In each PCR 10 reagent controls were added, consisting solely of PCR mixture, to monitor the occurrence of contamination introduced during preparation of the PCR reagents. In addition, each PCR sample was spiked with 100 copies of an oligonucleotide, which contains the two 16S priming sites flanking a randomly selected DNA sequence (Table 2). The spike was added to serve as an internal positive PCR control to detect possible inhibition due to components in the samples. If no or a very weak spike signal was detected this indicated inhibition of the PCR and such samples were reanalyzed.

Reverse line blot hybridization

The use of the reverse line blot hybridization (RLB) technique has been described in detail previously[23]. Briefly, 28 oligonucleotide probes designed for detection of bacteria associated with joint infections (Table 2) were covalently coupled to Biodyne™ Transfer membranes (Pall Biosupport Division, Port California, NY, USA) in parallel lines using a miniblotter (Immunetics, Cambridge, MA, USA). All probes were designed to have a melting temperature of 55°C +/- 1°C, enabling simultaneous hybridization with the PCR fragments. Ten μl biotin labeled PCR product was diluted in 140 μl 2 x SSPE (360 mM NaCl, 20 mM NaH_2PO_4, 2 mM EDTA) + 0.1% SDS. Samples were denatured at 99°C for 10 minutes and applied on the membrane perpendicular to the probes using the miniblotter. Furthermore, to assess the quality of the hybridization, each assay included a biotin labeled oligonucleotide that hybridizes with 3 different probes on the RLB membrane. Hybridization was performed at 42°C for 1 hour. The membrane was washed twice in 2 x SSPE + 0.5% SDS at 56°C, then incubated with streptavidin-POD conjugate (Boehringer Mannheim Gmbh, Mannheim, Germany) for 45 minutes at 42°C, followed by 2 washes at 42°C. The membrane was rinsed with 2 x SSPE before it was colored with SuperSignal West Femto (Pierce, Rockfort, IL, USA). Chemoluminescence was measured at the LAS 3000 system (Fujifilm Lifescience, Düsseldorf, Germany). If a positive result was obtained, this was shown by a dark dot at the intersection of the sample line and the corresponding probe line. RLB data were stored in a BioNumerics database (Applied Maths, Sint-Martens-Latem, Belgium). After the result was obtained, PCR-products were stripped from the membrane by 2 washes of 30 minutes at 80°C with 1% SDS and rinsed with 2 x SSPE. The membrane was sealed and stored in 2 x SSPE at 4°C and could be reused at least 20 times. Positive and inhibited samples were amplified and hybridized again, to confirm or replace the initial results.

Table 2
Characteristics of oligonucleotide primers and probes used in the PCR-RLB

Oligonucleotide name	Sequence	Target	pmol oligo per lane on membrane	Nucleotide position in the 16S rRNA gene
Primers				
16S8-27F	AGAGTTTGATCMTGGCTCAG	Eubacteria		8-27
B-16S509-525R*	GCTGCTGGCACGDAGTT	Eubacteria		509-525
Probes**				
A-16S A339	TCCTACGGGAGGC(AT)GCA	Eubacteria	25 & 50	339-355
A-16S CAMPY113	GTGAGTAAGGTATAGTTAATC	*Campylobacter* genus	200	113-133
A-16S CORYN451	CGAAGCTTTTGTGACGG	*Corynebacterium* genus	20	451-467
A-16S Eaero474	GGCGATTGACGTTACTC	*Enterobacter aerogenes*	100	474-491
A-16S Eaggl471	CCTGTCGATTGACGTTACC	*Enterobacter agglomerans*	200	471-489
A-16S ECEAE136	CTGATGGAGGGGGATAA	*Enterobacteriaceae* group	1500	136-153
A-16S Ecoli451	AAGGGAGTAAAGTTAATACCT	*Escherichia coli*	50	451-471
A-16S Efaec220	TTTCGGGTGTCGCTGAT	*Enterococcus faecalis/faecium*	100	220-236
A-16S ENTER448	AGGAAGGTGTTGTGGTTAA	*Enterobacter* genus	50	448-467
A-16S Fmagn210	CAAAGATTTATCGGTCATAGA	*Finegoldia* magna *(Peptostreptococcus magnus)*	1500	210-230
A-16S Hpylo82	CTTGCTAGAGTGCTGATTA	*Helicobacter pylori*	1500	82-100
A-16S Kpneu73	CACAGAGAGCTTGCTCT	*Klebsiella pneumoniae*	1500	73-90
A-16S Mpneu75	TAGTAATACTTTAGAGGCGAA	*Mycoplasma pneumoniae*	1000	75-95
A-16S Mtube211	CTTTAGCGGTGTGGGAT	*Mycobacterium tuberculosis*	800	211-227
A-16S MYCOB72	AAAGGTCTCTTCGGAGATA	*Mycobacterium* genus	800	72-90
A-16S NEISS120	AACATATCGGAACGTACC	*Neisseria* genus	400	120-137
A-16S Ngono129	GAACGTACCGGGTAGC	*Neisseria gonorrhoeae*	200	129-144
A-16S Nmeni185	TCTTGAGAGAGAAAGCAGG	*Neisseria meningitidis*	50	185-201
A-16S Pacne69	GAAAGGCCCTGCTTTTG	*Proprionibacterium* acnes	1000	69-85
A-16S Pmira461	GGTTAATACCCTTATCAATTGA	*Proteus mirabilis*	200	461-482
A-16S PSEUD141	GGATAACGTCCGGAAAC	*Pseudomonas* genus	800	141-157
A-16S Pvulg463	TTAATACTCTTAGCAATTGACG	*Proteus vulgaris*	2	463-484
A-16S SALMO452	GGTGTTGTGGTTAATAACC	*Salmonella* genus	400	452-470
A-16S Saure195	CATGGTTCAAAAGTGAAAGAC	*Staphylococcus aureus*	400	195-215
A-16S Spyog85	TAACGCATGTTAGTAATTTAAAAG	*Streptococcus pyogenes*	50	85-108
A-16S STAPH129	AACCTACCTATAAGACTGG	*Staphylococcus* genus	400	129-147
A-16S STREP142	GCGGGGGATAACTATTG	*Streptococcus* genus	750	142-158
A-16S TpD	CCGCTTCTATGTGACAG	Spike DNA	25 & 100	
Hybridization control				
B-A339/Salg/Ngon101*	GCTACCCGGTACGTTCCAATAGTTATCCCCCGCTGC			
Spike oligonucleotide				
TpD spike	AGAGTTTGATCATGGCTCAGCCGCTTCTATGTGACAGAACTTCGTGCCAGCAGC			

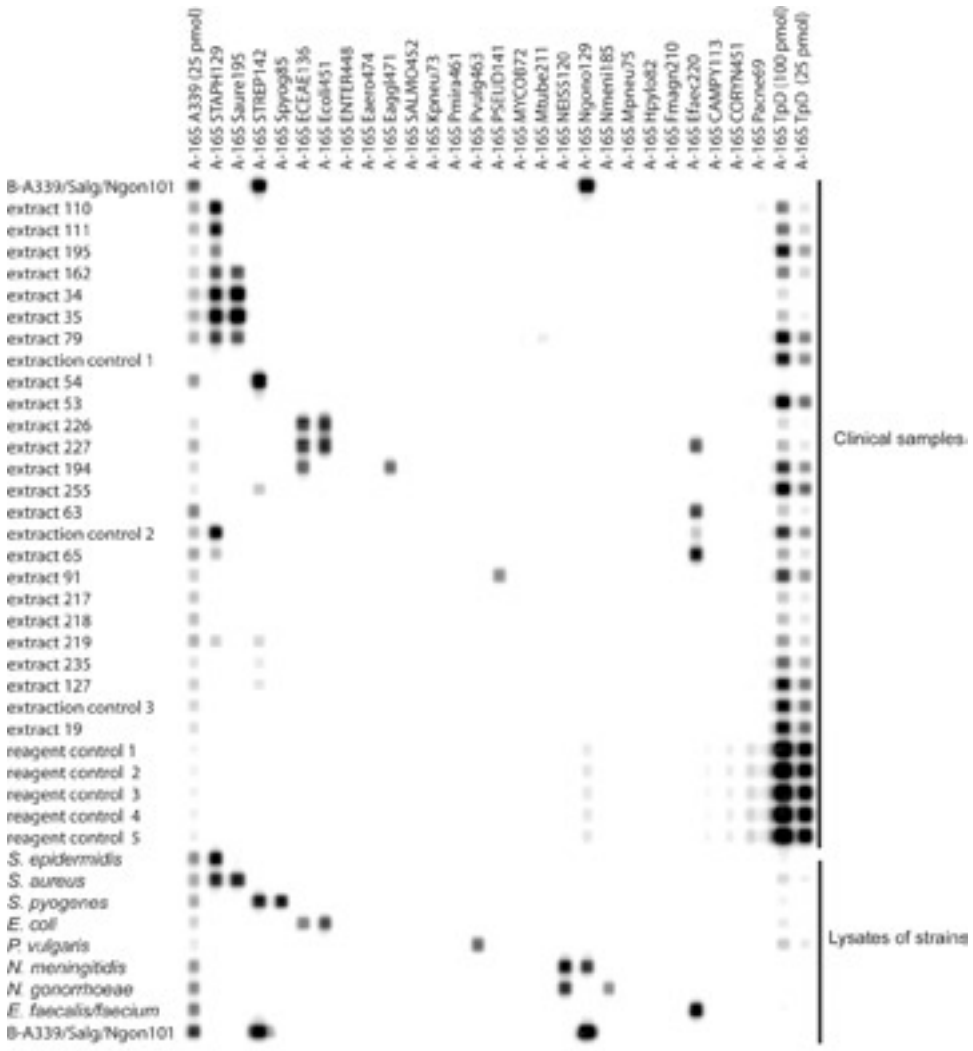

Figure 1
Reverse line blot analysis of 16S gene amplicons from patients suspect for orthopaedic infection. PCR products obtained from patients and controls were applied in the rows and oligonucleotide probes in the columns. The specificity of the oligonucleotide probes can be found in table 2. In the first and last row a biotin labeled oligonucleotide reacting with 3 different oligonucleotide probes was included as a positive hybridization control. The 8 lanes at the bottom of the blot display results obtained with lysates of a number of bacterial species. Extraction control 2 exemplifies a false positive extraction control. Extraction control 3 shows weak reactivity with the eubacterial probe, illustrating the normal background reactivity with this probe in the assay. Extract, PCR on extracts from clinical samples; extraction control, PCR on negative control obtained during DNA extraction from clinical samples; reagent control, negative control obtained by PCR on reagents only.

Real-time PCR

For real-time PCR, primers and pre-treatment of the reagents were identical to those described for the PCR-RLB. After inactivation of the restriction enzymes 2 μl of LightCycler Master SYBR Green I (Roche Applied Science, Almere, the Netherlands) and 2 μl template DNA was added. Samples were amplified in a LightCycler 2.0 System (Roche Applied Science) using the following protocol: an initial 30 sec 95ºC denaturation, followed by amplification in 45 cycles of 10 sec 95ºC, 5 sec 56ºC, 22 sec 72ºC, a melting curve analysis after a 15 sec at 65ºC ramping to 95ºC at a rate of 0.1ºC/sec and finally a hold temperature of 40ºC.

DNA sequence analysis

DNA sequencing was performed to verify the identification of the bacterial species. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and sequencing with the PCR primers 16S8-27F and B-16S509-525R) was performed using fluorescent Big Dye Terminator technology on a 3700 automated DNA analyzer (Applied Biosystems, Foster City, CA, USA).

Results

Optimizing the PCR-RLB

PCR targeting the 16S rRNA gene is extremely prone to contamination with traces of bacterial DNA present in the reagents used for PCR. To remove remnants of bacterial DNA we pre-treated the reagent mixture, including the primers, with a mixture of restriction enzymes[22]. The pre-treatment proved to be extremely helpful to reduce false positivity due to contamination of the reagents used in the PCR. Despite all precautions only if no more than 33 cycles were used in the PCR reagent, controls reproducibly remained negative and a detection limit of approximately 10 copies of the 16S rRNA gene was retained. Frequently extraction controls became weakly positive. In a quantitative real-time PCR we determined that extraction controls became positive if they contained an equivalent of 3 or more 16S rRNA gene copies. This showed that some contamination is introduced during processing of the samples. Figure 1 displays a RLB obtained with a selection of clinical samples.

Table 3
Comparison of PCR-RLB with clinical diagnosis and culture of samples from 76 patients

			PCR-RLB		Culture	
		total	**pos**	**neg**	**pos**	**neg**
Diagnosis infection	pos	31	30	1	25	6
	neg	45	5	40	2	43
Culture	pos	27	24	3		
	neg	49	11	38		

Detection of infection by PCR-RLB

After the retrospective review of all available patient data, 31 of the 76 patients were diagnosed as being infected at the time of surgery and all were treated accordingly. In 30 of the 31 infected patients PCR-RLB showed a positive result (Table 3). There was only a single patient diagnosed as infected for whom the PCR-RLB was negative. This patient had an infection of her THA with *Candida albicans*, a microorganism that does not carry a 16S rRNA gene and therefore is undetectable by 16S PCR. Of the 45 non-infected patients, 5 had a positive PCR-RLB in at least one of the samples collected. Three of these patients had undergone a revision of their THA, because of aseptic loosening, one patient received a primary THA and one patient underwent surgery for removal of osteosynthesis hardware. In comparison, with routine culture an infecting agent was detected in 25 patients of the 31 diagnosed as infection, but culture was also positive in 2 patients not suspect for infection. Of the 6 clinically infected cases missed by routine culture, 3 patients had undergone surgery for removal of their THA because of infection and despite culture positive preoperative aspiration of the joint, all intraoperative samples remained culture negative. Although because of the retrospective analysis of this study it can not be totally excluded, no evidence of obvious explanations for this discrepancy, like the accidental use of antibiotics during the interval between joint aspiration and intraoperative sampling, was found in the medical files of the patients concerned. In 24 infected patients the intraoperative culture and PCR-RLB results were both positive (Table 3). There were no infected patients in whom culture and PCR-RLB were both negative, neither had any of the patients not suspect for infection a positive result of both tests.

Bacterial species detected in clinical samples

Including the three patients with the culture positive preoperative aspiration, there were 27 patients with positive bacterial species identification with both culture and PCR-RLB. The most frequently detected bacterial species both by culture and PCR-RLB was *Staphylococcus aureus*, which was found in more than 10% of the patients (Table 4). To exclude a possible *S. aureus* contamination obtained during processing of the clinical samples, we determined the DNA sequence of the *aroA* gene of *S. aureus* in 6 of the positive samples. The aroA gene is one of the house keeping genes of *S. aureus* and is used as one of the targets in the multi-locus sequence typing technique[24]. This revealed 4 different *aroA* sequences in the 6 samples, indicating the *S. aureus* were different from the *S. aureus* strain used in the laboratory and that the *S. aureus* found originated from the clinical samples. The second most frequently found bacterial species belonged to the genus *Streptococcus*, which culture differentiated into α-hemolytic and β-hemolytic *Streptococci* and *Streptococcus oralis*. By RLB we could not speciate the members of the *Streptococcus* genus, as there was only a probe for *Streptococcus pyogenes* on the membrane in addition to the *Streptococcus* genus-probe. However, DNA sequencing of the PCR-RLB samples identified *Streptococcus dysgalactiae* in most cases. This species can be both α-hemolytic and β-hemolytic. Additional sequencing of the 16S PCR fragments from the positive PCR-RLB samples confirmed the species identifications of the RLB in the majority of the cases (Table 4). However, not all species identification could be confirmed by sequencing because some samples contained mixtures of bacterial species or yielded insufficient PCR product for DNA sequencing. There was one discrepancy in identification, where sequencing detected a *Haemophilus influenzae*, whereas culture and PCR-RLB both detected an *Enterococcus*. The fact that there

was no probe for *Haemophilus* on the RLB membrane may explain why only *E. faecalis* species was detected. Furthermore, *H. influenzae* is more fastidious in growth than *E. faecalis*, which may be responsible for the failure to culture this organism. In all but one patient, species identification by culture and PCR-RLB was in complete accordance. In the aberrant case, culture identified coagulase negative staphylococci, whereas RLB identified a *Corynebacterium* species, which was confirmed by DNA sequencing and further specified as *Corynebacterium xerosis*. In 2 cases RLB showed a signal for the eubacterial probe, but there was no signal with any of specific probes on the membrane. However, DNA sequencing revealed the presence of *P. canis* and *H. influenzae* corroborating the species identification performed by culture. In 1 patient PCR-RLB detected both *E. faecalis* and coagulase negative staphylococci, whereas culture only identified an *Enterococcus* species.

Table 4
Species identification by culture, PCR-RLB and DNA sequencing of PCR products in 76 patients

Culture	no. of patients (%)	RLB	no. of patients (%)	Sequencing of RLB samples	no. of patients (%)
S. aureus	8 (11)	*S. aureus*	10 (13)	*S. aureus*	8 (11)
				Not determinable	2 (3)
Coagulase negative staphylococci	5 (7)	*Staphylococcus* genus	4 (5)	*S. epidermidis*	2 (3)
S. lugdunensis	1 (1)			*S. lugdunensis*	1 (1)
No specification	4 (5)			Not determinable	1 (1)
Streptococcus sp.	5 (7)	*Streptococcus* genus	8 (11)	*S. dysgalactiae*	3 (4)
α-haemolytic streptococci	2 (3)			*S. mitis*	2 (3)
β-haemolytic streptococci	2 (3)			Not determinable	3 (4)
S. oralis	1 (1)				
E. faecalis	1 (1)	*E. faecalis/faecium*	1 (1)	*Haemophilus* sp.	1 (1)
E. cloacae	1 (1)	*E. agglomerans*	2 (3)	*E. cloacae*	1 (1)
				Not determinable	1 (1)
P. canis	1 (1)	Eubacteria	2 (3)	*P. canis*	1 (1)
H. influenzae	1 (1)			*H. influenzae*	1 (1)
		Enterobacteriaceae	1 (1)	Not determinable	1 (1)
		Corynebacterium genus	1 (1)	*C. xerosis*	1 (1)
		P. acnes	1 (1)	*P. acnes*	1 (1)
Mixed culture	4 (5)	Mixed culture	5 (7)		
M. morganii & Enterococcus sp. & *P. aeruginosa*	1 (1)	*E. agglomerans & E. faecalis & P. aeruginosa*	1 (1)	*M. morganii*	1 (1)
E. coli & Enterococcus sp.	1 (1)	*E. coli & E. faecalis*	1 (1)	*E. coli*	1 (1)
M. tuberculosis & S. aureus	1 (1)	*M. tuberculosis & S. aureus*	1 (1)	Not determinable	1 (1)
α-*haemolytic streptococci & S. aureus*	1 (1)	*Streptococcus* genus & *Staphylococcus* genus	1 (1)	*S. dysgalactiae*	1 (1)
		E. faecalis/faecium & Staphylococcus genus	1 (1)	*S. epidermidis*	1 (1)
C. albicans	1 (1)				
Negative	49 (65)	Negative	41 (54)		

Discussion

In the present study we showed that the combined broad range 16S rRNA PCR and RLB hybridization protocol performed well in detecting and identifying bacterial species in orthopaedic samples. The assay enabled the detection of as few as 10 16S rRNA gene copies in the DNA extract added to the PCR vial. As only 2 μl of the 1 ml DNA extract is used for PCR, this corresponds to 5000 gene copies in the clinical sample. By combining PCR with a hybridization of the amplicon also high specificity was obtained. We detected and identified bacterial species in samples from all patients suspect for bacterial infection. The 16S PCR-RLB assay was more sensitive compared to intraoperative cultures, identifying bacterial species in 6 clinically infected patients of whom culture failed to detect bacteria. In all but one case, species identification by RLB was in complete accordance with the identification obtained by culture. Only in a single patient we found a discrepancy between culture and RLB determination: culture identified coagulase negative staphylococci and RLB *Corynebacterium*. These species are easily distinguished and therefore a mistake in determination by culture is unlikely. Possibly, both pathogens were present in the tissue samples collected from the patient and only coagulase negative staphylococci were cultured. In a single patient culture identified the yeast *Candida albicans*. Yeasts do not possess 16S rRNA genes and therefore PCR-RLB did not detect infection with this pathogen.

Using PCR-RLB, *S. aureus* was found in more than 13% of the patients enrolled in this study and therefore was the most frequently detected bacterial species. Coagulase negative staphylococci were found less frequently (5%). In total 14 (18%) of the patients were infected with a member of the genus *Staphylococcus*. Streptococcal species were found in approximately 11% of the patients making it the second most frequently found bacterial genus of which *S. dysgalactiae* was found most often. Together, species belonging to the *Staphylococcus* and *Streptococcus* genera were found in 25 of the 76 (33%) patients and this amounts to 71% (25/35) of the PCR-positive patients. These findings are comparable to what has been previously reported as most common microorganisms in orthopaedic infections[15, 19, 20, 25, 26]. In 5 patients PCR-RLB detected mixtures of 2 or 3 different species. Remarkably, culture revealed identical mixtures in 4 of these 5 patients. Latter result indicated that the mixtures found were not the result of contamination during the PCR procedure. These samples may have been contaminated during surgery, but they may also represent a true mixed infection.

To detect the presence of bacterial species for which no specific probe was present on the RLB membrane we used a eubacterial probe. Often, PCR samples obtained by amplification of extracts of clinical samples yielded a weak hybridization signal of variable intensity, indicating the possible presence of bacterial DNA. However, sequencing of such samples almost never identified an unambiguous DNA sequence, but rather yielded sequencing trace files characteristic for mixtures of sequences. We therefore considered these signals as identifiable contamination, probably originating from contaminating DNA, present in the digestion buffer used for DNA extraction. However, in two patients, reactivity with the eubacterial probe appeared to be useful for detection of the infecting agent. One of these patients was shown to be infected with *Pasteurella canis*, a pathogen normally found in dogs, for which there was no specific probe on the membrane. Because of the intense signal of the eubacterial probe, the PCR product was sequenced and the *Pasteurella canis* was identified. *Haemophilus influenzae* was found by a similar way. This illustrates the fact that an intense hybridization signal with the eubacterial probe can be used to identify species for which no specific probe is available. Because the RLB only has a limited number of lines available for different probes, we based the selection of the probes on what is known from literature on bacterial species associated with orthopaedic infections. This could create the risk of missing rare species or species that have not been associated with orthopaedic infections before, but the use of the eubacterial probe in combination with DNA sequencing can circumvent this problem. Although DNA sequencing definitely has added value for the more specific species identification, the big advantage of the RLB hybridization is that it is capable of simultaneous detection of multiple bacterial species present in the same sample.

To our knowledge this is one of the first studies in orthopaedic patients in which broad range PCR combined with specific species identification is used. Most previously published PCR studies only reported the presence or absence of bacterial DNA, using the 16S gene, without any further species identification. This makes these methods more susceptible for false-positivity, as they do not offer a possibility for discriminating a true positive result from contamination. A recent publication did use specific bacterial identification by combining 16S and species specific PCR with DNA sequencing and cloning[15]. In addition to the microorganisms often found in orthopaedic infections, multiple anaerobes and other rarely described human pathogens were found. Using cloning, up to 8 different microorganisms were detected in patients with mixed infections, many of them not described in bone and joint infections. Another group studied the possibility of performing a molecular gram stain by combining real time-PCR aimed at the 16S rRNA gene with pyrosequencing[16, 17]. By using only the first three to five nucleotides sequenced they could differentiate between gram positive, gram negative and acid-fast bacilli with good sensitivity and specificity.

In addition to the positive samples from patients that were diagnosed as infected, 5 patients of the 45 patients (6 out of 85 samples) not suspect for infection had at least one of their samples positive by PCR-RLB. A possible explanation is that these are false-positive results caused by contamination. However, extraction and PCR controls never reacted with the specific probes, making it unlikely that contamination was introduced during the PCR-RLB procedure. Contamination of the samples may have occurred during surgery, but this will always be very difficult to control. Three of these 5 patients had undergone a revision of their THA, because of the diagnosis aseptic loosening. Therefore, these cases may have been patients with infections with small numbers of bacteria, or with bacteria, which were difficult to culture due to their fastidious nature. Alternatively, these patients may have been treated with antibiotics and therefore remained undetected with routine diagnostics. All 3 patients have undergone at least one subsequent revision of the concerning THA since then. Underdiagnosis of implant-related infections has been reported before in many different studies. Studies using 16S PCR by Mariani *et al.*, Tunney *et al.* and Clarke *et al.* even reported the presence of bacterial DNA in 63%, 72% and 52% of patients, respectively[10-12]. These high numbers probably are caused by contamination, as was already acknowledged by Clarke *et al.*, but there will definitely be a proportion of true positive patients with low-grade infections that were missed with the routine cultures[27]. This is also illustrated by the observations of Neut *et al.* that many more infections were detected using extensive culture protocols and confocal laser scanning microscopy of the implants compared to routine culture[28]. Due to the relative small number of patients with aseptic loosening and the lack of real negative controls, we cannot give a reliable answer to this question with our study, but it will remain a topic for further research.

During the last decades many diagnostic tests using new technologies have been studied. Unfortunately not many are currently used in the clinical setting, despite the fact that several techniques have shown promising results. Possibly, these techniques require particular expertise or equipment or do not offer any additional benefits to the already existing tests. Therefore, further development of new diagnostic tests should meet particular criteria. In order to become a real counterpart of the routine diagnostics, the new tests should be simple to perform, yield results within a limited amount of time, be sensitive and specific for infection and determine which microorganism has to be dealt with. Of course these criteria will be difficult to meet, but they will increase the chances of the technique in becoming a standard test that is available to the general orthopaedic surgeon. Although there are no cost-effectiveness studies available, as with most new technologies the costs of the new test will probably be higher than of the widely used, routine tests. Nevertheless, the costs of the materials used for PCR-RLB assay are relatively low, amounting approximately 5 US dollars per sample. The PCR-RLB probably will not replace culture or pathology in the near future and it should therefore be considered as an additional test. However, for the patients with suspected low-grade infections, the PCR-RLB may be helpful and the additional costs of the new diagnostic test may soon outweigh the costs involved in treating a patient in which the diagnosis of infection initially was missed.

In conclusion, we can say that the combined 16S rRNA PCR and RLB hybridization has been validated and was shown to be a sensitive and useful diagnostic instrument to detect and identify bacterial species associated with orthopaedic infections. Although it currently will not be a test for general use, because of the strict protocols and facilities needed to prevent contamination, it will definitely be of great scientific value to investigate the true incidence of aseptic and septic loosening in revision arthroplasty.

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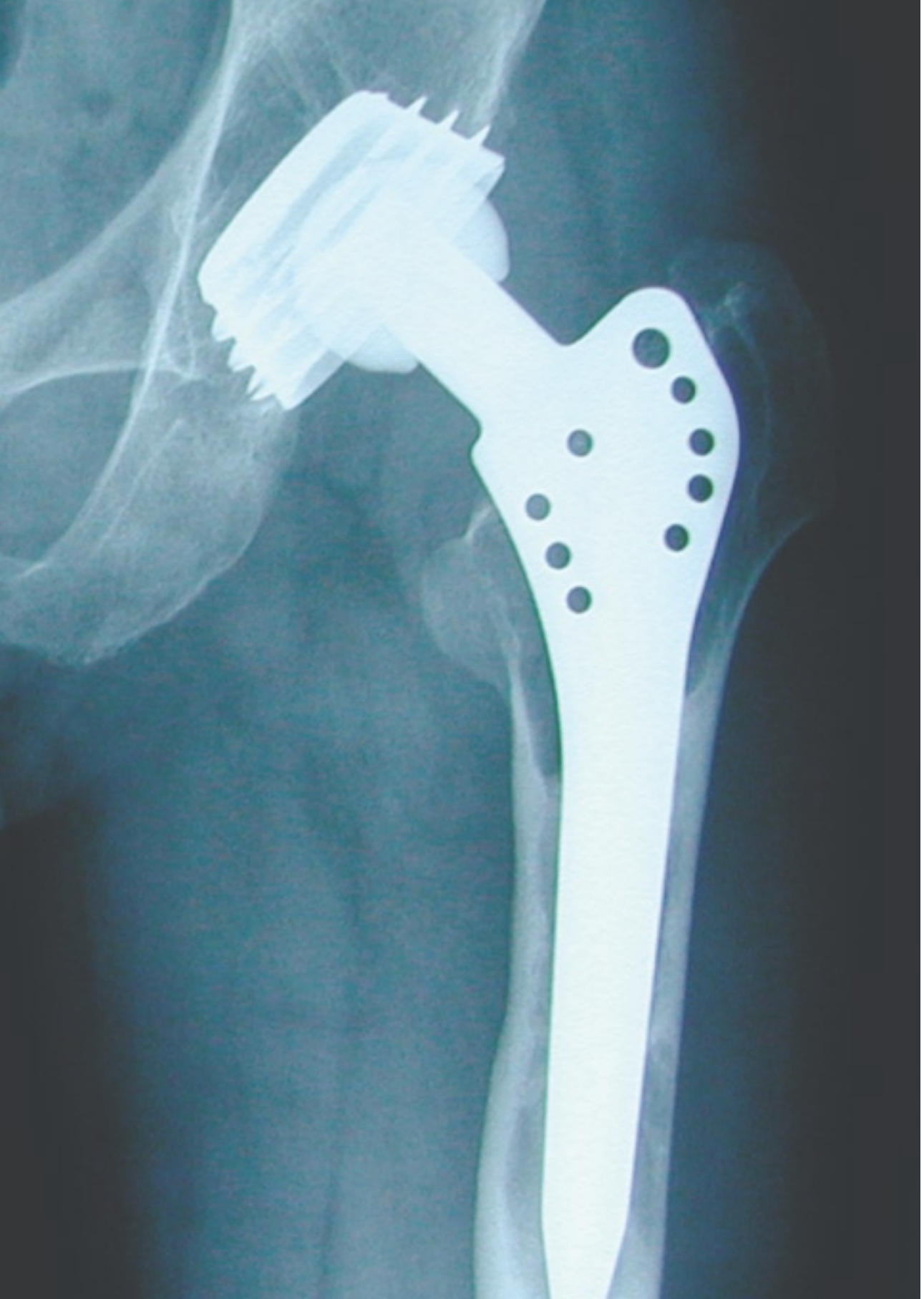

Chapter 3

A prospective multicenter study investigating the incidence of aseptic loosening in total hip revision arthroplasty using extensive routine and broad range 16S PCR with reverse line blot diagnostics

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Abstract

Background

This study investigated the hypothesis that many total hip arthroplasty revisions, classified as aseptic, are in fact low-grade infections missed with routine diagnostics.

Methods

In 7 Dutch hospitals, 176 patients with the preoperative diagnosis of aseptic loosening of their total hip arthroplasty were enrolled. From each patient, the preoperative history was obtained. During surgery, between 14 and 20 tissue samples were obtained for culture, pathology analysis and broad range 16S rRNA PCR with reverse line blot hybridization. The diagnosis of infection was set using strict, predefined criteria. Each patient had a follow-up visit after 1 year.

Results

Seven patients were classified as infected, of whom 4 were not identified by routine culture. An additional 15 patients were suspect for infection as well. Twenty of these 22 patients received a cemented prosthesis, fixated with antibiotic-loaded bone cement. All patients received prophylactic systemic antibiotics. Seven of the 22 patients reported complaints one year post-surgery, only one showing signs of early loosening. However, in none of the patients additional surgery was performed.

Conclusion

Although percentages were not as high as previously reported in literature, between 4 and 13 percent of patients with the preoperative diagnosis of aseptic loosening were infected. However, as thorough debridement was performed during surgery and prophylactic antibiotics were used, it did not have many clinical consequences, as most patients performed well at the 1-year follow-up. Whether it has implications for long-term implant survival remains to be seen.

Introduction

Total hip arthroplasty has become an increasingly popular orthopaedic intervention as it largely improves quality of life. Despite improvements in multiple factors influencing the outcome of total hip arthroplasty, approximately 8% of the prostheses need to be revised within 10 years[1]. More than 75% of these revisions are due to aseptic loosening, whereas primary or secondary infection accounts for approximately 8%[1]. Data gathered by the Scandinavian hip registries clearly suggest that antibiotic prophylaxis either systemically, locally or combined, increases implant survival[1, 2]. In fact, the widespread use of antibiotic containing bone cement in primary total hip arthroplasties in these countries was associated with a decrease in the number revisions due to aseptic loosening as well[1, 2]. As aseptic loosening is thought to be a non-infectious event, which should not be influenced by the activity of antibiotics, this observation suggests that there is an underdiagnosis of infection. The aseptic loosening group might contain a substantial number of patients with low-grade infections, which are missed with the routine diagnostic tests. As a result these patients might not get an optimal treatment, which might ultimately lead to early complications. Other studies have presented evidence of bacteria or bacterial DNA in patients thought to have developed aseptic loosening[3-9]. However, the prevalence of bacterial involvement in revision surgery has ranged from just a few percent up to 70 percent of all patients[3, 6].

A difficulty with confirming the diagnosis of infection in total hip arthroplasty is that there is no true gold standard[10]. Traditionally, microbiological cultures are seen as the reference standard, but also these are often false positive due to contamination or false negative due to changed growth characteristics of bacteria. Histopathological analysis of tissue samples is frequently used as well[10, 11]. However, there is no consensus on which number of polymorphonuclear leukocytes is the best cut-off value[12-14]. Although a valid study on the use of culture as a single diagnostic test was published, the best reference will probably be the combined results of multiple diagnostic tests[10, 11, 15].

In recent years, different studies on the use of new, more sensitive diagnostic techniques were published. Particularly the use of molecular biological techniques, like polymerase chain reaction (PCR), was investigated[5-8, 16, 17]. PCR techniques aim at the 16S rRNA gene, which is universally present in bacteria, and appear to have promising potential for the detection of orthopaedic infections. However, because this 16S gene is present in all bacteria and remnants of bacterial DNA, this technique is very susceptible to contamination. The key publications by Mariani *et al.* and Tunney *et al.* therefore probably also contained a substantial percentage of false

positive results, as was acknowledged by the authors in a later publication[5, 6, 18]. Moojen *et al.* previously published a study on the optimization and validation of a combined broad range 16S rRNA PCR and reverse line blot hybridization protocol[7]. Results showed that with this technique multiple microorganisms, often encountered in orthopaedic infections, could be reliably detected and identified at the genus- and species level and that the combined PCR-RLB technique was more sensitive than routine cultures.

The hypothesis of this study was that there is an underdiagnosis of infection in the group of patients undergoing revision total hip arthroplasty. We investigated this by extensively utilizing routine diagnostics, like microbiological cultures and pathology analysis, together with the previously validated broad range 16S rRNA PCR and reverse line blot hybridization technique in a group of patients, preoperatively suspect for aseptic loosening of their total hip arthroplasty.

Materials and Methods

Study design

The institutional board review committee of the University Medical Center Utrecht approved this study (13-09-2003, 03/133). Between November 2002 and July 2006, 176 patients with the preoperative diagnosis aseptic loosening of their total hip arthroplasty were included in 7 Dutch hospitals. All were scheduled for a 1-stage revision of cup, stem or both. During surgery, multiple tissue biopsies were obtained and sent for microbiological culture, pathology analysis and broad range 16S rRNA PCR with reverse line blot hybridization. Each patient had a 1-year follow-up visit.

Eligibility for inclusion

Patients were eligible for inclusion if they were at least 18 years old and were scheduled for a revision of either one or both components of their total hip arthroplasty, because of the preoperative diagnosis aseptic loosening. This diagnosis had to be based on at least anamnesis, physical examination, AP and lateral X-rays of the involved hip and the determination of the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). Both first and re-revision cases were included. All patients enrolled had provided written informed consent. Exclusion criteria were indication for revision surgery because of technical error, recurrent dislocation, periprosthetic fracture or solely polyethylene wear (e.g. only exchanging of a liner); history of septic arthritis, osteomyelitis or deep prosthesis infection on the symptomatic hip or a cli-nically apparent infection at another site. Finally, antibiotic use less than 2 weeks prior to revision surgery was also a reason for exclusion.

Sample acquisition and analysis

Preoperatively, for each patient medical history was obtained, plain X-rays were made and ESR and CRP levels were determined. In case of doubt, the treating orthopaedic surgeon was free to perform additional diagnostics, like aspiration and arthrography or scintigraphy, but this was not obligatory for the study. During surgery prophylactic antibiotics were withheld until sampling was done. Depending on whether a partial or total revision was performed, between 14 and 20 samples were obtained from the joint fluid, (neo)capsule, acetabular and femoral interface, resulting in 4 to 7 samples for each diagnostic test. The sampling was done according to a strict sampling protocol and for each location clean instruments and gloves were used. To minimize tissue handling, a sterile sample kit was available for each patient. Samples for culture were analyzed at the local Microbiology laboratory. In each hospital the samples were cultured according to comparable protocols, which meant culturing under aerobic and anaerobic conditions for at least 7 days. Samples for pathological analysis were processed at the local Pathology laboratory and centrally analyzed by

1 study pathologist (TB), specialized in histopathology of failed implants and blinded with respect to patient identification and results of all other tests. Criteria for infection were modified from those of Spangehl(11) as follows: When a maximum tissue concentration of 0 to 5 polymorphonuclear neutrophils (PMN) per 10 high power fields (HPF) were seen, this was regarded as definitely not infected. When a maximum tissue concentration of 6 to 10 PMN's per 10 HPF's were seen this was regarded as borderline, but probably not infected. When >10 PMN's per 10 HPF's, but not >5 PMS's in any one HPF were seen this was regarded as borderline, but most likely infected. When >5 PMN's per 1 HPF were seen this was regarded as definitely infected. PMN's entrapped in superficial fibrin or in capillaries were excluded[10]. Samples for the broad range 16S rRNA PCR with reverse line blot hybridization (PCR-RLB) were stored locally at -70°C and analyzed centrally, according to the techniques previously validated and published by our group[7]. In short, the technique consist of a broad range 16S rRNA PCR using universal primers targeted at the 16S rRNA gene, which amplifies the bacterial DNA. PCR products are detected and further specified at the genus or species level, using reverse line blot hybridization, which uses 28 oligonucleotide probes, specifically designed for the detection of bacteria associated with joint infections. Only a positive result on 2 separate PCR-RLB runs was regarded as positive. One year postoperative, each patient had a follow-up visit where postoperative clinical course, additional surgery or antibiotic use was recorded, an X-ray was made and ESR and CRP were determined.

Diagnosis of infection

To determine whether a patient should be regarded as infected or not we used both the criteria as described by Spangehl *et al.* and the criteria as described by Atkins *et al*.[11, 15]. According to Spangehl, a patient is infected when there is either an open wound or sinus to the joint, a systemic infection with pain in the hip and purulent fluid in the joint or a positive result on at least 3 diagnostic tests for infection: a CRP >10, an ESR >30, a positive preoperative aspiration, ≥5 PMN's per HPF on pathology analysis or at least one third of the culture samples positive with the same microorganism. The PCR-RLB was added to this list of diagnostic tests, using the same criteria as for culture. Atkins investigated the value of microbiology results as a single diagnostic. According to these criteria, at least 3 samples have to be positive with the same microorganism to be regarded as truly infected. For the PCR-RLB results this definition was used in the same manner. If the diagnostic results of a patient met the criteria by either Spangehl or Atkins, he or she was regarded infected in this study.

Results

General

176 patients were included, 49 men and 127 women (Table 1). The median age at the time of revision surgery was 72 years (range 28-92). The indication for primary total hip arthroplasty was osteoarthritis in 115 patients, resultant of childhood disease in 21, fracture in 10, inflammatory arthritis in 10, osteonecrosis in 9 and other reasons or unknown in 11. For the majority of patients it was a first revision. The median time the prosthesis had been in place was 11 years (range 0.5 to 35). Fixation of the implant was cemented in 83 patients, hybrid in 19 and uncemented in 74. In most cases where bone cement was used it contained either tobramycin or gentamicin. However, because of many referrals, the antibiotic content of the cement was unknown in about half of the cases. Prophylactic systemic antibiotics were used during the previous surgery in 71 of 79 patients; it was unknown in 97 patients. A first or second-generation cephalosporin was used in 75%; other antibiotics included clindamycin and amoxicillin/clavulanic acid.

Ninety percent of patients complained of pain and half of them reported functional impairment. X-rays showed signs of loosening of one or both components in 88% of patients; none showed signs suggestive for infection. Mean ESR was 17 (SD 14) and mean CRP was 5 (SD 2). In 72% of the patients additional diagnostic tests were performed, including aspiration and arthrography in 30% and scintigraphy in 55%.

Even though all patients were preoperatively planned for one-stage revision surgery, in 3 patients there was too much intraoperative doubt and a two-stage revision was performed. In 63 patients both components were revised, in 76 only the acetabular cup and in 37 only the femoral stem. All patients received intravenous antibiotics perioperatively.

Table 1
Patient demographics, surgical history and diagnostics

	Average/ Number of pts (+%)	Range
Age	70.0	(28 - 92)
Male/Female	49/127 (28/72%)	
Indication for THA		
Osteoarthritis	115 (65%)	
Childhood disease	21 (12%)	
Fracture	10 (6%)	
Inflammatory arthritis	10 (6%)	
Osteonecrosis	9 (5%)	
Unknown	9 (5%)	
Other	2 (1%)	
Revision number		
1st	130 (74%)	
2nd	34 (19%)	
3rd	8 (5%)	
4th	3 (2%)	
5th	1 (1%)	
Time in situ of arthroplasty	10.8	(0.5 - 35)
Method of fixation		
Cemented	83 (47%)	
Hybrid	19 (11%)	
Uncemented	74 (42%)	
Systemic antibiotics at previous surgery		
Yes	71 (40%)	
No	8 (5%)	
Unknown	97 (55%)	
Anamnesis		
Pain	161 (91%)	
Functional impairment	90 (51%)	
X-ray		
Normal	21 (12%)	
Loosening cup	74 (42%)	
Loosening stem	47 (27%)	
Loosening both components	34 (19%)	
Blood analysis		
ESR	17.1	(1 - 90)
CRP	5.1	(<1 - 45)
Additional diagnostics		
No	49 (28%)	
Aspiration & arthrography	53 (30%)	
Scintigraphy	96 (55%)	

Table 2
Results of diagnostic tests obtained in samples from 176 patients

	All samples negative	%	One or more samples positive	%	True positives	%
Microbiology	127	72	49	28	3	2
PCR-RLB	141	80	35	20	7	4
Pathology	157	89	19	11	5	3
Combined	98	56	78	44	7	4

Analyses and infection

In 127 patients (72%), every sample obtained during surgery was negative by culture (Table 2). Similarly, none of the samples was positive by PCR-RLB in 141 patients (80%). Pathology analysis suggested no infection in 157 patients (89%). If the 3 tests were combined they were all negative in 98 patients (56%). Although many patients had a positive result on one of the 3 tests (Table 2), not all of the remaining 78 patients were considered infected. In many patients only 1 or 2 samples were positive by culture or PCR-RLB or different microorganisms were cultured from different samples. Therefore, these results were regarded as contamination and interpreted as false positive. With respect to cultures, 46 patients (26%) were regarded as false positive, for the PCR-RLB 28 patients (16%) and for pathology analysis 14 patients (8%). If the results of the three tests were combined, 71 patients (40%) had a false positive result on one or more diagnostic tests. Culture was regarded as true positive in 3 patients (2%), PCR-RLB in 7 patients (4%) and pathology analysis in 5 patients (3%). Table 3 shows the concordance between the true positive results of the diagnostic tests. According to the strict criteria used for infection, 7 patients (4%) were classified as infected (Table 4). Six of those 7 patients had positive results on multiple diagnostic analyses and were classified as infection according to the criteria by Spangehl. Only one additional patient was classified infected according to the criteria by Atkins. This patient had 3 of his 5 PCR-RLB samples positive with *Enterobacter agglomerans*, while other tests were negative. Coagulase negative staphylococci were found in 3 patients and *Proprionibacterium acnes*, *Streptococcus* species, *Salmonella* species and *E.agglomerans* in one patient each. With culture a microorganism was found in only 3 of the 7 infected patients, while all were detected with PCR-RLB. The positive PCR-RLB results were supported in all but one case with pathology results suspect for infection. As the type of infections searched for in the study group will most likely be low-grade infections it is possible that the criteria for infection described above are too strict. Therefore, the results were re-analyzed with less strict criteria as well. If the criterion

of at least 2 culture or PCR-RLB samples positive with the same microorganism or >5 PMN per HPF on pathology analysis was used, 15 additional patients were identified as suspect for infection (Table 5); 8 patients were based on culture, 2 on PCR-RLB, 1 on both culture and PCR-RLB and 4 on pathology analysis. Microorganisms found in these patients were *P.acnes* in 4, coagulase negative staphylococci in 2, *Streptococcus* species in 2, and *Staphylococcus aureus*, *Micrococcus* species or a diphteroid rod in the other 3 patients.

Postoperative clinical course and 1-year follow-up

Of the initial group of 176 patients, 1-year follow-up was available for 170 patients (97%). As all patients were preoperatively thought not to be infected, all were scheduled for a 1-stage revision and only a short prophylactic course of systemic antibiotics. As PCR-RLB and pathology results were not available to the treating orthopaedic surgeon, these did not influence decisions for postoperative treatment. Because of intraoperative doubt, a 2-stage revision was performed in 3 patients, of whom only one indeed was infected.

In the 148 patients not suspect for infection 146 had a 1-stage revision. Five patients received prolonged antibiotics, because of intraoperative positive cultures or persisting postoperative wound drainage. Nine patients had additional surgery after the 1-stage revision. In 4 patients a debridement was performed because of suspected infection. Four patients had a 1-stage revision because of technical problems caused by the previous operation and one had a 2-stage re-revision because the clinical course was complicated by infection. At the 1-year follow-up, 121 patients had no complaints. Twenty-seven patients did have complaints, of which 21 showed no signs of infection or loosening, 5 showed signs of implant loosening and one had a resection arthroplasty.

In the 7 infected patients, all but one had a 1-stage revision. Six patients received an implant fixated with antibiotic bone cement and one patient received an uncemented implant. Only the patient with the 2-stage revision received antibiotics for a prolonged period. After one year, only 2 patients reported complaints, but there were no signs of loosening or infection. No debridement or re-revision was performed.

The 15 additional patients suspect for infection using less strict criteria all had a 1-stage revision. Fourteen patients received an implant fixated with antibiotic bone cement and one patient received an uncemented implant. One patient received prolonged antibiotics because of positive intraoperative cultures and did well at follow-up. After one year, 4 patients reported complaints without signs of infection or loosening. One patient showed loosening of the cup with an elevated ESR and CRP and IgG scintigraphy suggestive of low-grade infection. However, no additional surgery was performed in this group either.

Table 3
Concordance of true positive results

	Microbiology	PCR- RLB	Pathology
Microbiology	3		
PCR-RLB	3	7	
Pathology	2	5	5

Table 4
Results of diagnostic tests of patients considered infected

Patient	ESR	CRP	Aspiration	Culture	Pathology	PCR-RLB
1	55	18	not done	Gram+ *cocci* (2 of 5)	negative	*Staphylococcus* sp./CNS (1 of 6)
2	35	10	not done	negative	positive	*Salmonella* sp. (3 of 4)
3	17	<5	not done	negative	negative	*E.agglomerans* (3 of 5)
4	34	<5	not done	negative	positive	*P.acnes* (4 of 4)
5	20	4	not done	*S.epidermidis* (3 of 5)	positive	*Staphylococcus* sp./CNS (4 of 4)
6	12	11	negative	Skin flora (5 of 7)	positive	*Staphylococcus* sp./CNS (5 of 6)
7	31	21	not done	negative	positive	*Streptococcus* sp. (3 of 7)

Table 5
Results of diagnostic tests of patients suspect for infection

Patient	ESR	CRP	Aspiration	Culture	Pathology	PCR-RLB
8	11	1	not done	negative	negative	*Staphylococcus* sp./ CNS (2 of 7)
9	11	5	not done	*Micrococcus* sp. (2 of 6)	negative	negative
10	5	2	not done	*S.aureus* (2 of 7)	negative	negative
11	10	6	not done	*S.kominis* (2 of 5)	negative	negative
12	13	2	not done	*P.acnes* (2 of 4)	negative	negative
13	25	2	not done	*P.acnes* (2 of 5)	negative	negative
14	9	9	negative	Diphteroid rod (2 of 5)	negative	negative
15	14	<5	not done	*P.acnes* (2 of 5)	negative	negative
16	3	<5	not done	CNS (2 of 7)	negative	*Staphylococcus* sp./ CNS (2 of 7)
17	13	13	negative	negative	negative	*P.acnes* (2 of 6)
18	8	<7	negative	negative	positive	negative
19	10	<7	negative	*Streptococcus* sp. (2 of 4) & *Corynebacterium* sp. (2 of 4)	negative	negative
20	7	6	not done	negative	positive	negative
21	7	10	not done	negative	positive	negative
22	23	4	not done	negative	positive	negative

Discussion

This prospective study of 176 patients who underwent revision surgery of their total hip arthroplasty because of the preoperative diagnosis of aseptic loosening, showed that in 7 to 22 patients (4-13%) there was infection or suspicion of infection, based on the combined results of pre- and intraoperative diagnostic tests for infection. All patients but one had a 1-stage revision and were treated accordingly; only 2 patients received antibiotics over a prolonged period. At the 1-year follow-up most patients did well. None of these 22 patients had received additional surgery after one year.

Compared to several previous studies that investigated a similar hypothesis, our study found a relatively low rate of infection. For example, Tunney *et al.* showed in a comparable group of patients the presence of bacteria in 22% using sonication and culture, in 63% using immunofluorescence microscopy and in even 72% using 16S PCR[6]. A study by Clarke *et al*. using 16S PCR reported evidence of bacterial DNA in 46% of the patients with suspected aseptic loosening[8]. However, they found bacterial DNA in 21% of the tissue samples from primary total hip arthroplasties as well, indicating a high contamination rate. Mariani *et al*. found comparable results in a group of 50 patients with aseptic and septic revision of total knee implants[5]. In addition to the 15 patients identified by culture, 16S PCR revealed the presence of bacterial DNA in additional 17 patients. As their negative controls were negative, they considered the 17 patients to be true positives. The problem with these studies is that neither of them used a valid reference standard for infection, but only reported percentages of patients that had positive samples. In contrast, the 2 large studies previously mentioned on defining a reference standard for infection, did not focus on the identification of low-grade infections[11, 15].

Although we tried to use the best, published reference standards for infection, this will always be a topic of debate. Most studies have been designed to detect normal infections with good sensitivity and specificity, and used internal references for comparison. For example, Atkins *et al.* used the pathology diagnosis as gold standard to which they compared the culture results[15]. In the current study we anticipated low-grade infections, which would likely be difficult to detect. Using published criteria, we identified only 7 infected patients (4%), suggesting that those criteria have good specificity. Lowering the stringency for diagnosis of infection in an attempt to identify more low-grade infections would be expected to increase sensitivity, but decrease specificity. In our study it increased the number of patients suspect to be infected to 22 (13%). Perhaps the identification of the same microorganism in 2 separate tissue samples or only a high concentration of polymorphonuclear neutrophils in peri-implant tissues would increase sensitivity. On the other hand it is possible that 4% might just be the true incidence.

The types of microorganisms found in the infected patients consisted mainly of low virulent bacteria, like coagulase negative staphylococci and *Proprionibacterium acnes*. These bacteria are skin flora, but are also often associated with orthopaedic implant-related infections[17, 19, 20]. In one infected patient, a *Salmonella* species was found in 3 PCR-RLB samples. *Salmonella* is a microorganism not often related to infections of total hip arthroplasties, but it has been reported in literature before[21]. In some patients conventional cultures were negative, yet the PCR-RLB yielded positive results. The validity of the PCR-RLB results was supported by the pathology analysis, which indicated infection as well. Regarding the detection of microorganisms, there was a distinct difference between culture and PCR-RLB. PCR-RLB detected the presence of bacterial DNA in all 7 infected patients, even though in 1 patient only 1 of 6 samples was positive for coagulase negative staphylococci. In contrast routine cultures identified a microorganism in only 3 of the 7 patients and could only specify the type of microorganism found to Gram-positive cocci and skin flora in 2 patients. PCR-RLB showed coagulase negative staphylococci in both. This low sensitivity of routine cultures may be related to a more fastidious growth of these bacteria.

A point of criticism could be that in only a limited number of patients a preoperative aspiration was performed. As it is an invasive procedure, this was not obligatory for the study and was only performed in case of doubt by the treating orthopaedic surgeon. An aspiration could have added valuable information, which could have changed treatment strategies. On the other hand, in the patients where aspirations were performed results were negative and it does represent normal clinical practice for many hospitals.

Even though we found a substantial percentage of patients to be infected or suspect for infection in a group preoperatively not thought to be infected, the consequences of this finding remain to be seen. Almost all patients were treated with a 1-stage revision and only a short period of prophylactic systemic antibiotics, but at the 1-year follow-up none of those 22 patients had received any additional surgery on the revised hip. Compared to the group of patients not suspect for infection they performed comparable. One patient suspect for infection did have complaints and showed signs suggestive of low-grade infection at one year. Nevertheless, a 1-stage revision with thorough intraoperative debridement and the prophylactic use of systemic and perhaps additional local antibiotics in the bone cement might be sufficient treatment for most low-grade infections with low virulent bacteria. Although most of our patients received a cemented revision prosthesis fixated with antibiotic-loaded bone cement, the patients with an uncemented prosthesis did not seem to do worse, so no hard statements can be made regarding the potential additional value of local antibiotics. However, even though using antibiotic-loaded bone cement has been

proven valuable for infection prophylaxis and implant survival[2], it should always be remembered, that by the routine use of this type of cement, bacterial resistance against antibiotics like gentamicin and tobramycin can be induced.

Finally, we can conclude that in the group of 176 patients, requiring revision surgery of their total hip arthroplasty and preoperatively not suspect for infection, between 4 and 13% should be regarded as infected, based on the combined results of laboratory, culture, histology and broad range 16S rRNA PCR with reverse line blot hybridization diagnostics. These infections are mainly caused by low virulent bacteria, for which detection with PCR-RLB was more sensitive than routine cultures. For implant survival after 1 year, the presence of infection seemed to have little consequences. However, one year is still a too short follow-up interval to be fully confident on the absence of low-grade infection, so the consequences on long-term survival remain to be seen.

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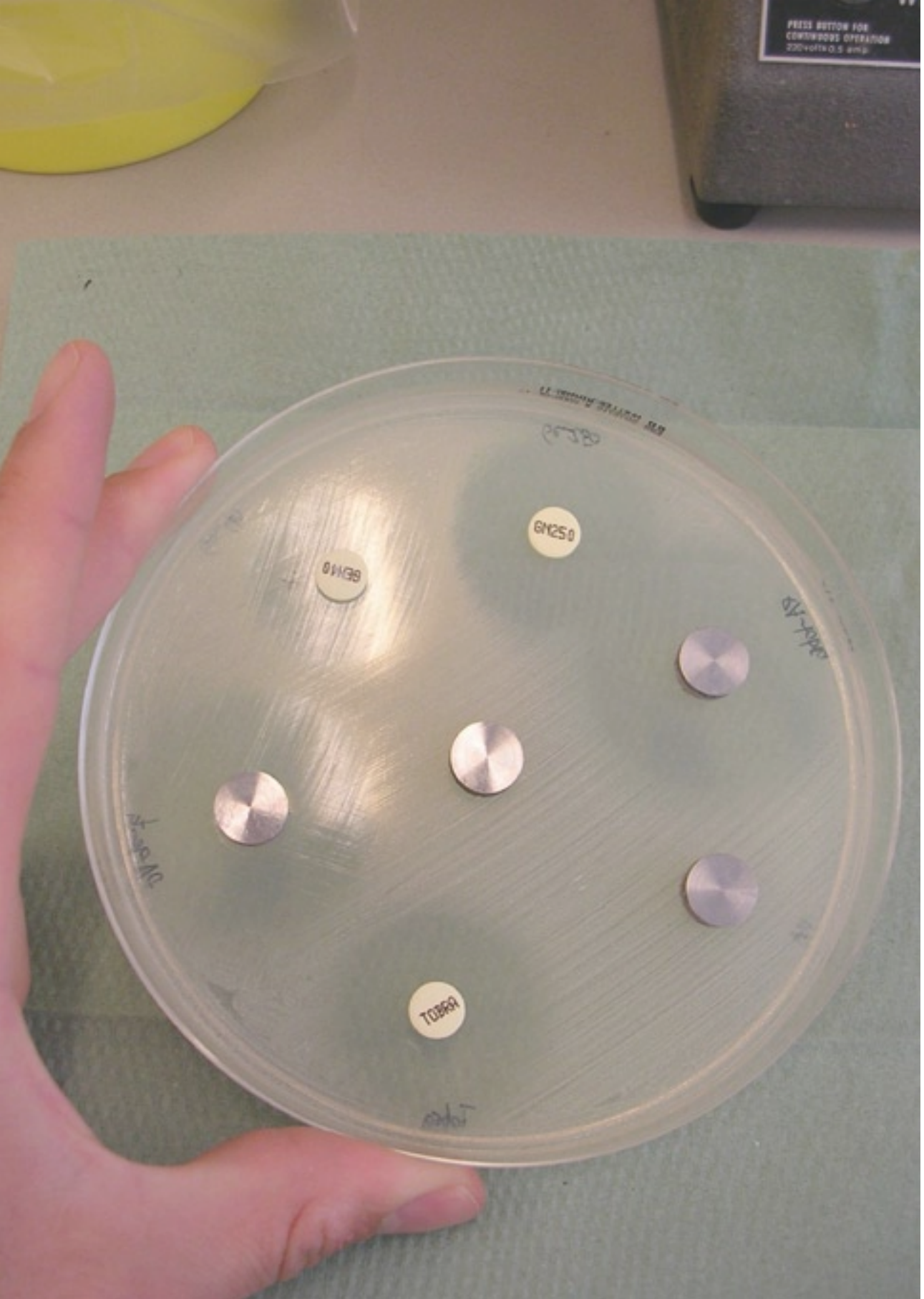
Antimicrobial strategies in prevention and treatment



Chapter 4

In vitro release of gentamicin and tobramycin from periapatite coated titanium foam implants

Abstract

Background

Hydroxyapatite (HA) coatings improve the fixation of uncemented implants, but previous reports suggested that the implants become more susceptible for infection compared to uncoated implants. By loading a porous titanium implant with a biomimetic HA-coating (Peri-Apatite, PA) with antibiotics, we could obtain adequate local antibiotic concentrations, which could reduce the infection susceptibility. This study determined the *in vitro* release and bacterial inhibition characteristics.

Methods

PA-coated titanium foam disks were loaded with 2.4 mg of gentamicin or tobramycin. For release testing, disks were placed in 5 ml sterile physiological saline at 37°C and sampled at nine time points. Antibiotic concentrations were determined by fluorescence polarization immunoassay. For inhibition testing, a disk diffusion assay using *Staphylococcus aureus* was performed.

Results

Both antibiotics showed a burst release, which rapidly decreased. Concentrations remained above the MIC of many pathogens for 12 hours. Release after 168 hours was complete and both antibiotics showed large inhibition zones. No significant differences between gentamicin and tobramycin were observed.

Conclusion

Our results demonstrate that both antibiotics are rapidly and completely released and can inhibit growth of *S. aureus*. Therefore, loading PA-coated titanium foam with antibiotics appears to be an easy procedure to achieve local antibiotic delivery for uncemented prostheses. However, further experiments have to prove its value in preventing infection *in vivo*.

Introduction

Total hip arthroplasty is one of the most successful operations in orthopaedic surgery, especially since infection rates have been reduced from 10% in the early 60's to between 0.5 and 2% currently[1-3]. During the last decade, the proportion of uncemented implants has rapidly increased with respect to the total number of prostheses implanted. Since the introduction of hydroxyapatite (HA) coatings, fixation of these implants has improved and their survival has significantly increased. For instance several recent reports with mid to long-term follow-up demonstrated survival rates for uncemented femoral stem implants similar to or even better than cemented implants[4-6].

In spite of these positive clinical results, Vogely *et al.* showed in an experimental infection model a significant difference in infection susceptibility between uncoated and HA-coated titanium implants[7, 8]. In this study, both types of implants were inserted in the medullary canal of the proximal tibia of rabbits after contamination with *Staphylococcus aureus*. After 4 weeks bacteriological analysis showed significantly more infected cases and higher numbers of bacteria in the group of HA-coated implants. Histopathological analysis showed more signs of infection next to the HA-coated implants as well. A possible explanation for this higher infection susceptibility could be the higher surface roughness of the HA-coated implants, making it easier for the bacteria to attach to the implant.

Furthermore, for cemented implants, local antibiotic delivery from the bone cement has proven its prophylactic effect against infection both in experimental and clinical studies[9, 10]. However, until now there were no strategies available for local antibiotic delivery from uncemented implants. This is partially related to the very high temperatures used in the process of applying HA by plasma spraying, but also due to the low porosity of many implants, thereby reducing the potential antibiotic load onto an implant surface.

Although no strategy is clinically available yet, different *in vitro* and experimental *in vivo* studies have been published, investigating the possibility of coating implants or external fixator pins with antibiotics, cross-linked albumin or antiseptics[11-25]. The amount of antibacterial agent incorporated in the coating varies largely between the different materials. Also the release patterns show a large variation with effective release ranging from hours to weeks. Nevertheless *in vivo* studies show, that these coatings do have a substantial effect on the prevention of infection[11, 14, 16, 20]. A disadvantage of these coatings is the fact that they need to be prefabricated and they are only available with a limited number of different antibacterial agents.

In this study we present an easy method for local antibiotic delivery from uncemented

implants. The theory is, that by loading antibiotics on a highly porous titanium foam implant with a biomimetic HA-coating (Peri-Apatite, PA), the antibiotic load on the implant can be improved in such a way that adequate local concentrations are reached postoperative, in order to prevent implant-related infections. The loading can be done by direct application of the antibiotic to the implant, for example by dipping the implant in an antibiotic solution. This can be done during surgery with any type of water-soluble antibiotic. It is hypothesized that by applying antibiotics to the PA-coated titanium foam implant, the infection susceptibility of this implant can be reduced.

In this study, we determined the *in vitro* release and bacterial inhibition characteristics of gentamicin and tobramycin loaded on a PA-coated titanium foam implant.

Materials and Methods

Disks

For both the release and bacterial inhibition testing, Ti6Al4V disks with a diameter of 12.7 mm were used, coated on one side with approximately 1.5 mm of titanium foam (Tritanium Dimensionalized Metal™, Stryker Orthopaedics, Mahwah, NJ, USA) (Figure 1). The titanium foam has an overall porosity of 65%, with pore sizes of 250 to 400 μm. The titanium foam was coated with a biomimetic HA-coating, which is applied using a solution deposition method (Peri-Apatite® (PA), Stryker Orthopaedics, Mahwah, NJ, USA). The coating is highly crystalline, contains 100% HA and has a uniform thickness of 20 μm[26]. Figure 2 shows a scanning electron microscopy (SEM) image of the PA-coating. The solution deposition method has the ability to penetrate macro-porous (>100 μm) structures and therefore the coating not only covers the outside of the implant, but also the inner surface of the titanium foam. As a result of the high porosity of the titanium foam and the hydrophilicity of the PA-coating a maximum volume of 100 μl of antibiotic solution could be applied to each disk.

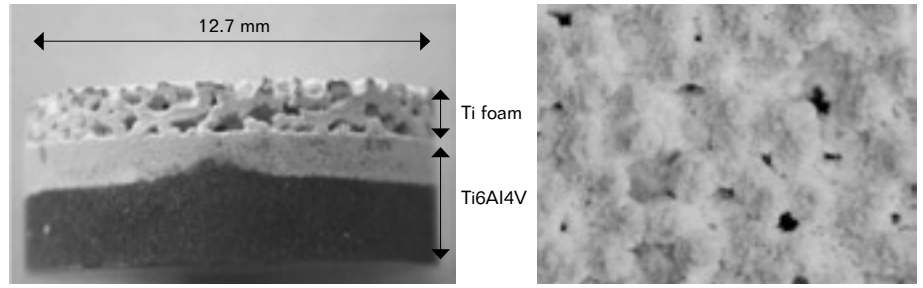


Figure 1
Left photo shows the porous, PA-coated titanium foam on top of the dense Ti6Al4V. Right photo is a stereomicroscopic enlargement of the PA-coated titanium foam surface.

Loading of antibiotics

The disks were loaded with commercial solutions of either gentamicin or tobramycin (Centrafarm, Etten-Leur, The Netherlands). Each solution contained 40 mg/ml of the corresponding antibiotic. Disks were loaded with a total of 2.4 mg antibiotic by applying a volume of 60 μl directly to the porous PA-coated titanium foam, using a micropipette.

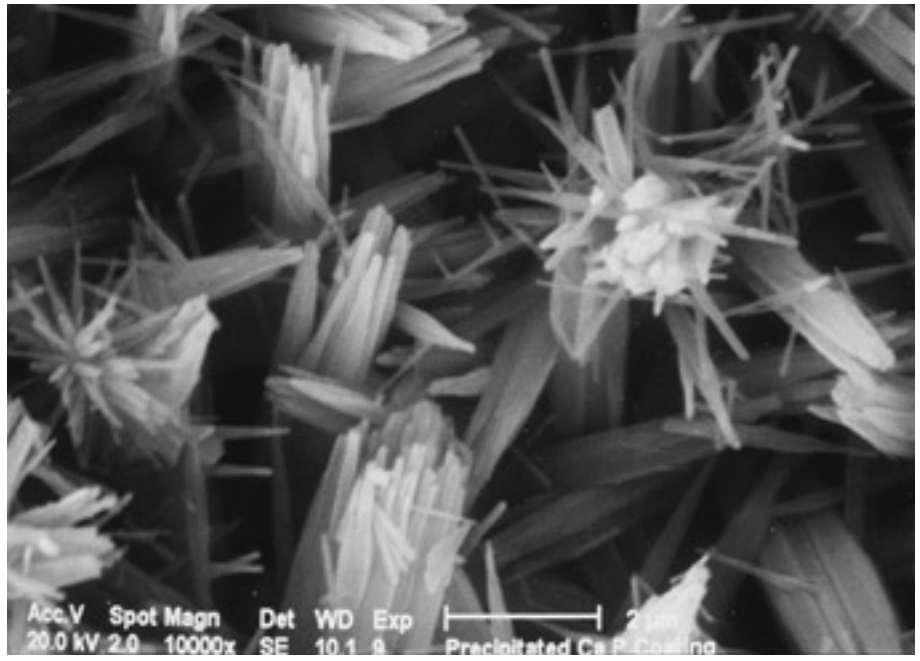


Figure 2
SEM image of the PA-coating (magnification 10.000x)

Release of antibiotics

After the application of the antibiotic solution, the disks were immediately put into a standard sterile tube containing 5 ml of sterile physiological saline (SPS). The tubes were placed in a water bath, which was shaking at 30 rounds per minute, at 37°C. The antibiotic concentration was determined at nine different time points: 30 minutes, 1, 3, 6, 12, 24, 48, 96 and 168 hours. At each time point, the disk was removed from the tube and was put in a new tube containing 5 ml of fresh SPS. Elution samples were stored in the freezer at -21°C until analysis was done. Antibiotic concentrations were determined using a fluorescence polarization immunoassay (AxSym system, Abbott Diagnostics, Hoofddorp, The Netherlands). The AxSym system has a sensitivity (lowest concentration distinguished from zero with 95% confidence) of 0.30 µg/ml for gentamicin and 0.18 µg/ml for tobramycin. Gentamicin and tobramycin controls in SPS (1, 2, 4, 6 and 8 µg/ml) were included in each sample run. Measured gentamicin and tobramycin release concentrations were adjusted to the trend lines, obtained from the SPS-controls.

Bacterial inhibition

For inhibition testing a disk diffusion assay was performed. Disks were sterilized in an autoclave at 120°C for 30 minutes. As bacterial agent *Staphylococcus aureus* Wood 46 (ATCC 10832) was used. For preparation of the stock 5 colonies were grown overnight in 200 ml Müller-Hinton broth (Oxoid Ltd, Basingstoke, England) at 37°C. Overnight cultures were centrifuged (15 minutes at 3500 rpm and 21°C) and resuspended in fresh broth. Standardized suspensions were frozen and held at -70°C until used. To determine the concentration of bacteria in the stock, a sample was thawed and plated in serial tenfold dilution on blood agar plates. After an overnight culture the number of viable bacteria were counted. For the inhibition testing, a sample from the stock was diluted with sterile phosphate buffered saline (pH 7.4) to a concentration of $5x10^7$ cfu/ml. Using a sterile cotton swab a Müller-Hinton plate (∅ 14 cm, Becton Dickinson, Heidelberg, Germany) was evenly inoculated with bacteria. After inoculation, disks loaded with gentamicin or tobramycin were placed on each plate with the titanium foam side down. A PA-coated and an uncoated titanium foam disk without antibiotics were placed on each plate as negative controls. In addition commercial tobramycin and gentamicin inhibition disks (Neo-sensitabs™, Rosco, Taastrup, Denmark), containing 40 µg of the antibiotic, were placed on each plate as positive controls. The topside of each disk was gently pressed to ensure full contact with the plate. Subsequently, plates were placed at 37°C. After an incubation of 24 hours inhibition zones were measured in millimeters. We defined the inhibition zone as the total diameter of the zone of inhibition, including the disk.

Statistics

Results are reported as mean ± standard deviation. For all testing, group sizes of $n=3$ were used. To determine statistical differences between the release of gentamicin and tobramycin a repeated measures analysis was performed. For statistical analysis of the inhibition zones one-way ANOVA and Tukey-HSD post-hoc testing was used. A p-value <0.05 was considered significant.

Results

Antibiotic release

Both gentamicin and tobramycin showed a burst release with concentrations of 399.5±28.2 and 353.9±24.9 μg/ml respectively after 30 minutes (Fig. 3). After this initial burst release, antibiotic concentrations rapidly decreased in time. However, concentrations of both antibiotics remained above the MIC of many pathogens, common in orthopaedic infections, for at least 12 hours, when levels of 0.52±0.09 and 0.47±0.11 μg/ml were measured for gentamicin and tobramycin respectively. At later time points, the measured antibioticconcentrations were below the sensitivity concentrations indicated for the AxSym-system and were therefore regarded as zero. Considering the cumulative release after 168 hours, we observed a complete release for each antibiotic (Fig. 4). Of course, the major amount was released within the first few hours. Although the initial release of gentamicin was somewhat higher compared to that of tobramycin, no significant difference was observed (p=0.487).

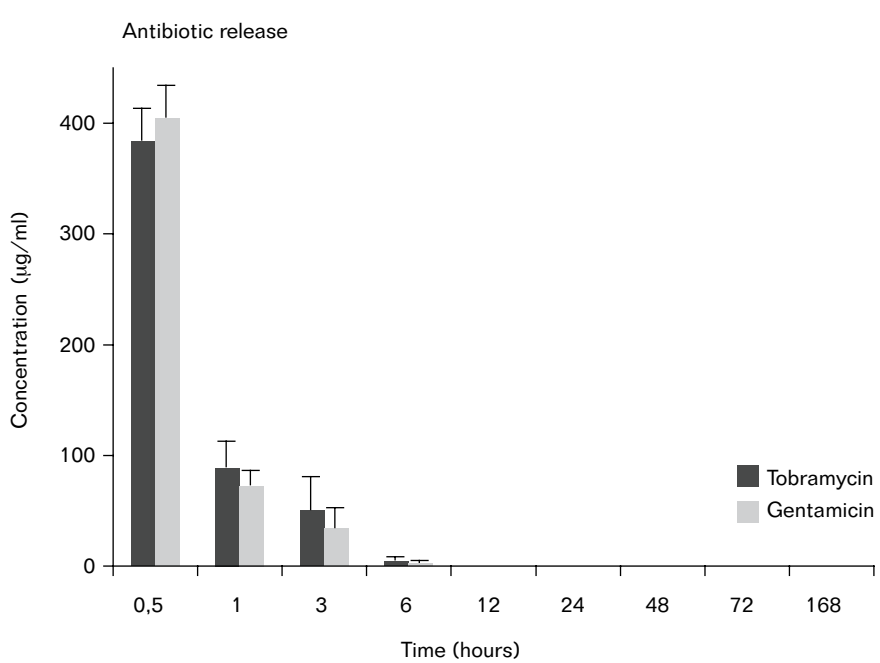


Figure 3
Gentamicin and tobramycin release at the different sampling time points

Table 1
Inhibition zones measured after 24 hours incubation of S.aureus Wood 46

Disk	Inhibition Zone (mm)
PA-gentamicin	41.7 ± 0.6
PA-tobramycin	42.7 ± 0.6
PA	0.0 ± 0.0
Uncoated	0.0 ± 0.0
Gentamicin 40 μg	32.3 ± 0.6
Tobramycin 40 μg	32.7 ± 1.2

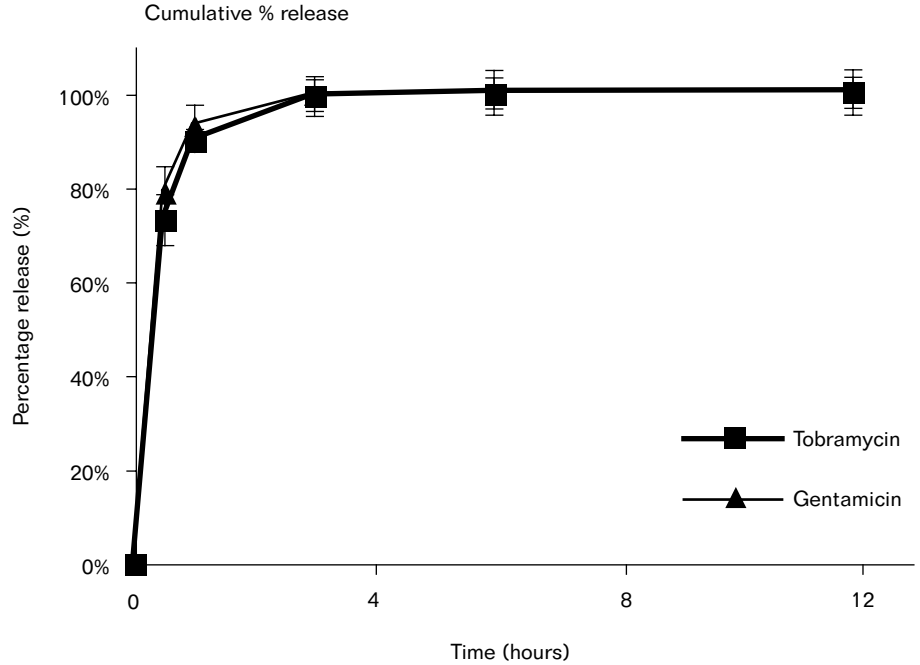


Figure 4
Cumulative release patterns of gentamicin and tobramycin (time scale until t=12)

Bacterial inhibition

Incubation for 24 hours showed that neither the titanium foam, nor the PA-coating themselves have any antibacterial effect, as the inhibition zones for both uncoated and PA-coated disks were zero millimeters in all cases (Table 1). In contrast, the commercial inhibition controls of gentamicin and tobramycin showed inhibition zones of 32.3±0.6 and 32.7±1.2 millimeter respectively ($p=0.948$), indicating comparable susceptibility of the *S.aureus* Wood 46 for both antibiotics. The inhibition zones of the PA-coated titanium foam disks, loaded with gentamicin or tobramycin, were even significantly larger ($p<0.001$) compared to the commercial controls. The difference between disks loaded with gentamicin or tobramycin was not significant ($p=0.428$).

Discussion

The results of the current study demonstrate that both gentamicin and tobramycin are rapidly released from the PA-coated titanium foam and that *in vitro* concentrations above the MIC of many pathogens are maintained for at least 12 hours. Microbiological testing showed that both antibiotics retain the activity to inhibit growth of *S. aureus* after being released from the implant. Both antibiotics also showed a complete release of the antibiotic load that was initially applied, which suggests that there is no permanent bonding of the tested antibiotics to the PA-coating. As the initial burst release with high gentamicin and tobramycin concentrations, is followed by a rather steep decrease in release rates, one might say that a longer and more controlled release pattern is desirable. However, this is not likely with the dip-coating technique. Besides, as it is believed that most bacteria are introduced in the implant bed during surgery, the intra- and direct postoperative period can be considered as the most important period for maintaining adequate antibiotic levels when being used as a prophylactic measure. Because of the smaller amounts of free fluid relative to the implant surface and because of gradually decreasing tissue concentrations compared to sudden fluid exchanges in this *in vitro* experiment, it can be hypothesized that *in vivo* antibiotic concentrations could remain higher as measured in this *in vitro* experiment, for a longer period of time.

The results from previous studies by others demonstrate that for other antibiotic-carrier combinations, the antibiotic release can last up to 8 weeks[15]. However, the coatings with a prolonged release regularly use additional measures to stabilize the coating, such as combining the agent with PLLA, PLGA or lipids[13, 14, 19-22]. Because of the gradual breakdown of the stabilizing agents, the antimicrobial release is also more gradual. A disadvantage is that these combined coatings will most likely have an influence on the surface characteristics of an implant and therefore seem less useful for application on HA-coatings. There are only two groups that studied antimicrobial agents in combination with HA-coatings. Stigter *et al.* studied a biomimetic HA-coating on titanium implants, which was loaded with different antibiotics by co-precipitation during the HA-coating process, and found a release period ranging between one and 16 hours depending on the type of antibiotic incorporated[23, 24]. Campbell *et al.* used external fixator pins coated with HA and chlorhexidine and found a release, which maintained for only three hours[13]. By adding a lipid layer they could prolong this release to approximately 24 hours. A subsequent *in vivo* study showed that the chlorhexidine-lipid-HA-coating could reduce infection rates from 100 to 17 percent, but unfortunately did not include any control animals to study the effect of the different coating components on the fixation of the pins[14].

A point of consideration in our study is the initial high antibiotic level of approximately 400 μg/ml after 30 minutes. Miclau *et al*. previously showed that 24-hour exposure to a tobramycin concentration of 400 μg/ml significantly inhibited osteoblast replication *in vitro*[27]. Although in our study the exposure to high concentrations lasted only for a short period of time and an *in vitro* set-up is only a simplification of the true *in vivo* situation, this might have a negative effect on bone growth onto the implant. For example, a recent study investigating a chlorhexidine coating showed good results in reducing bacterial colonization of the implant, but also was very cytotoxic to fibroblasts, thereby making it not useful for clinical application[28]. In the *in vivo* follow-up of our study, this potential problem is being assessed by performing a histopathological analysis of the surrounding bone and doing histomorphometry on bone ingrowth and bone-implant contact area.

Although we only tested gentamicin and tobramycin in this study, it can be hypothesized, that other types of antibiotics can be used as well. Theoretically any type of water-soluble antibiotic can be applied using the dip-coating technique. However, release patterns for other antibiotics may be different, as was shown by Stigter *et al.* using their technique of co-precipitation into the biomimetic HA-coating[23]. Although they found a total release for gentamicin, other types of antibiotics only released 65 to 90 percent of the total amount incorporated. An advantage of the dip-coating method presented in the current study is that the antibiotics are not incorporated in the coating, but are only added to the implant during surgery. This will potentially reduce the possibility of permanent bonding and incomplete release.

Based on the results of the study presented here, we conclude that the direct application of antibiotics to PA-coated titanium foam suggests an easy procedure to achieve local antibiotic delivery for a limited period. *In vitro,* it has a rapid release with high antibiotic concentrations in the first few hours, no measurable additional release after 12 hours and inhibited growth of *S. aureus*. Therefore, it can potentially be useful as a prophylactic measure for uncemented prostheses against peroperative bacterial contamination. However, further *in vivo* animal experiments are needed to investigate the feasibility in terms of reducing infection susceptibility of antibiotic-loaded PA-coated titanium foam implants.

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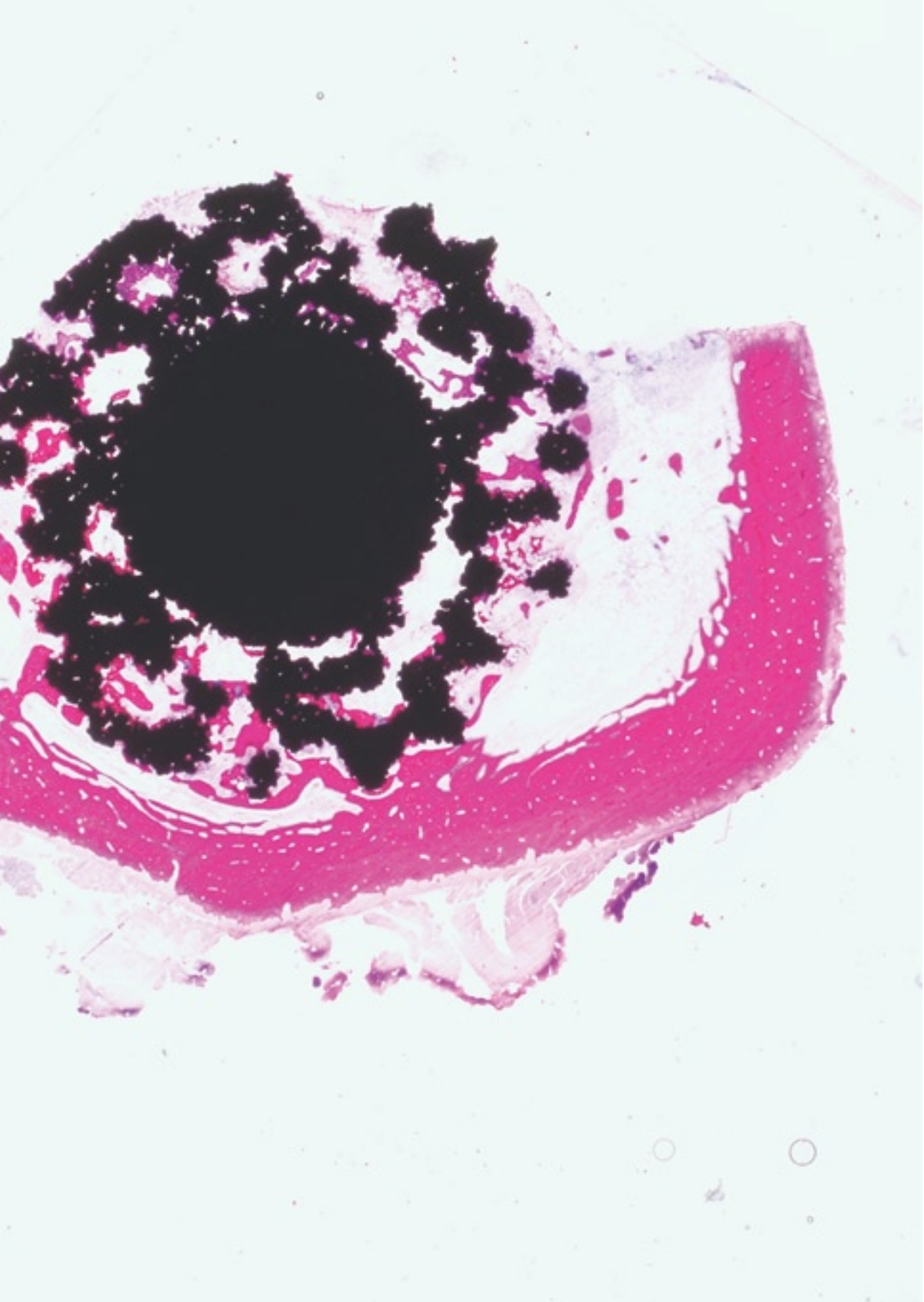

Chapter 5

Prophylaxis of infection and effects on osseointegration using a tobramycin - periapatite coating on titanium implants - an experimental study in the rabbit

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Journal of Orthopaedic Research, 2009 Jun;27(6):710-6

Abstract

Background

No options are available for local antibiotic delivery from uncemented implants. By loading a porous titanium implant with a biomimetic HA-coating (PeriApatite, PA) with antibiotics, we could obtain adequate local antibiotic concentrations and reduce infection susceptibility. This study investigated the efficacy of a tobramycin-loaded PA-coated titanium foam implant in preventing infection, as well as the effects on osseointegration.

Methods

In 72 New Zealand White rabbits, an uncoated (Ti), PA-coated (PA) or Tobramycin-PA-coated (PA-tobra) titanium foam rod was implanted intramedullary in the left tibiae after contamination of the implant bed with none (control), 10^3, 10^4 or 10^5 CFU *Staphylococcus aureus*. PA-tobra implants were loaded with 2.4 mg tobramycin. After 28 days analysis was done by bacteriology, histopathology and histomorphometry.

Results

Six percent of the contaminated PA-tobra rabbits were infected, whereas this was 53% and 67% for PA and Ti respectively ($p<0.001$). Quantitative cultures were also significantly lower in the PA-tobra group ($p=0.003$). None of the control rabbits were infected. Histopathological and histomorphometrical scores were both better for the PA-tobra group, although only significant compared to Ti. No significant differences were observed between PA and Ti rabbits.

Conclusion

We conclude that the application of tobramycin to PA-coated titanium foam implants appears to be an effective local antibiotic strategy for uncemented implants for infection prophylaxis and has a beneficial effect on implant fixation, which will result in improved long-term implant survival.

Introduction

During the last decade, the proportion of uncemented implants has rapidly increased with respect to the total number of prostheses implanted. Since the introduction of hydroxyapatite (HA) coatings, fixation of these implants has improved and their survival has significantly increased. Several recent reports with mid to long-term follow-up demonstrated survival rates for uncemented femoral stem implants similar to or even better than cemented implants[1, 2]. Therefore surprisingly, Vogely *et al.* showed in an infection model in rabbits that plasma-sprayed HA-coated implants had a higher infection susceptibility compared to uncoated titanium implants[3]. As a consequence, the advantage of better bone integration and thereby implant fixation was reduced[4]. A possible explanation for this higher infection susceptibility could be the higher surface roughness of the HA-coated implants, making it easier for the bacteria to attach.

For cemented implants, local antibiotic delivery from the bone cement has proven its prophylactic effect against infection both in experimental and clinical studies[5, 6]. However, until now no strategies were available for local antibiotic delivery from uncemented implants. This is partially related to the very high temperatures used in the process of applying HA by plasma spraying, but also due to the low porosity of many implants, thereby reducing the potential antibiotic load onto an implant surface.

Although no strategy is clinically available yet, different studies have been published, investigating the possibility of coating implants or external fixator pins with antibiotics, cross-linked albumin or antiseptics[7-18]. The amount of antibacterial agent incorporated in the coating and release patterns varied considerably between the different materials. Nevertheless, *in vivo* studies show that these coatings do have a substantial effect on the prevention of infection[7, 9, 10, 13, 18]. Disadvantages of these coatings are the fact that they need to be prefabricated and are only available with a limited number of different antibacterial agents.

In this study we investigated a simple method for local antibiotic delivery from uncemented implants. The theory is, that by loading antibiotics on a highly porous titanium foam implant with a biomimetic HA-coating (Peri-Apatite, PA), the antibiotic load on the implant can be improved in such a way that adequate local concentrations are reached postoperative and implant-related infections can be prevented. Previous *in vitro* experiments showed that it readily and completely released both gentamicin and tobramycin and that antibiotic release lasted for at least 12 hours, which could be sufficient for prophylaxis[19]. The loading of antibiotics is done by direct application of the antibiotic to the implant, for example by dipping the implant in an antibiotic

solution. This can be done during surgery with any type of water-soluble antibiotic. It is hypothesized that by combining the antibiotic and PA-coating on the titanium foam implant, the infection susceptibility can be reduced and better osseointegration can be obtained.

The aim of the current study was to investigate in an *in vivo* contaminated implant bed model, the efficacy of adding tobramycin to a PA-coated titanium foam implant in preventing implant-related staphylococcal infection and study the effects on osseointegration; this in comparison to both PA-coated and uncoated implants.

Animals, Materials and Methods

Design

The Animal Care and Use Committee of the Faculty of Medicine of Utrecht University approved the protocol for this study. A total of 72 female New Zealand White (NZW) rabbits received an uncoated (Ti, n=24), PeriApatite-coated (PA, n=24) or tobramycin-PeriApatite-coated (PA-tobra, n=24) titanium foam implant in the medullary canal of their proximal left tibia. Prior to insertion the implant bed was contaminated with none (control), 10^3, 10^4 or 10^5 colony-forming units (CFU) of *Staphylococcus aureus*. All rabbits had a follow-up of 28 days. Post-mortem, analysis was performed by bacteriology, histopathology and histomorphometry.

Animals, housing and feeding

Female New Zealand White rabbits (Harlan, Horst, The Netherlands (NL)) weighing 2.6 to 3.5 kg (mean weight 2.9 kg) were obtained from a professional stockbreeder. They arrived 1 week prior to surgery to acclimatize to the housing in the animal care facility. The rabbits were housed individually in cages and were daily fed with 80-100 gram antibiotic free rabbit diet STANRAB P (Special Diet Services, Witham, UK).

Implants: Cylindrical implants with a solid Ti6Al4V core, coated with titanium foam (Tritanium Dimensionalized Metal™, Stryker Orthopaedics, Mahwah, NJ, USA) with a thickness of approximately 1.5 mm were used. The implant had a total diameter of 4.5 mm and a length of 12 mm. The titanium foam has an overall porosity of 65%, with pore sizes of 250 to 400 μm. The implants were either uncoated or Peri-Apatite-coated. Peri-Apatite (PA) is a biomimetic HA-coating, which is applied using a solution deposition method (Peri-Apatite®, Stryker Orthopaedics, Mahwah, NJ, USA). The coating is highly crystalline, contains 100% HA and has a uniform thickness of 20 μm. The solution deposition method has the ability to penetrate macro-porous (>100 μm) structures and therefore not only coats the outside of the implant, but also the inner surface of the titanium foam. All implants were sterilized by gamma irradiation. During surgery, the rods in the PA-tobra group were loaded with 2.4 mg of tobramycin (Centrafarm, Etten-Leur, NL) just prior to implantation by directly applying 60 μl of antibiotic solution (40 mg/ml) using a micropipette.

Bacteria

Staphylococcus aureus Wood 46 (ATCC 10832) was used to contaminate the implant bed. This strain has been previously used in several other infection studies[3, 20, 21]. The *S.aureus* Wood 46 has a minimal inhibitory concentration for tobramycin of 0.125 μg/ml. For preparation of the stock, a colony was grown overnight in Müller Hinton broth at 37°C. Standardized suspensions were frozen at -70°C until a sample was used. On the day of use the suspension was diluted with sterile phosphate buffered saline (PBS, pH 7.4) to a concentration of 10^4, 10^5 or 10^6 CFU per ml. During surgery the rabbits were inoculated with 100 μl of bacterial suspension.

Surgery and anaesthesia

Surgery was performed under general inhalation anaesthesia and aseptic conditions. Premedication consisted of an intramuscular (im) injection with 4 mg acepromazinemaleate (Sanofi Sante, Maassluis, NL) and 4 mg methadone (Eurovet Animal Health, Bladel, NL). Anaesthesia was initiated by an intravenous (iv) injection of 8-10 mg etomidate (Janssen Pharmaceuticals, Tilburg, NL). After intubation, anaesthesia was maintained using a mixture of O_2/N_2O (ratio 1:1) and halothane 1% (Albic, Maassluis, NL).
Skin of left leg was shaven, disinfected with 10% povidone-iodine and isolated by sterile drapes. The knee joint was opened using a medial parapatellar incision. The patella was luxated laterally and anterior to the insertion of the anterior cruciate ligament, the tibial medullary canal was opened, reamed by hand until a depth of about 25 mm and a diameter of 4.5 mm were obtained and 100 μl of bacterial suspension was injected into the cavity using a micropipette. Subsequently, an uncoated, PA-coated or PA-tobra-coated titanium foam rod was implanted press-fit in the medullary canal. Finally, the patella was reduced and the joint was closed in layers. Pain relief was provided by 3 mg nalbufine (Lamepro, Raamsdonkveer, NL) iv and 0.1 mg buprenorphine (Reckitt and Colman Products, Kingston-upon-Hull, UK) im.

Postoperative follow up

Fluoroscopy of the left tibia was performed to check the position of the implant. Animals were examined daily for activity, eating and wound healing. Weight and temperature were measured as well. Preoperative and 1, 2, 3 and 4 weeks postoperative, an erythrocyte sedimentation rate (ESR), white blood count (WBC) and systemic tobramycin concentration (PA-tobra group only) were determined. For the PA-tobra rabbits, an additional sample was obtained on day 1 to measure systemic tobramycin concentrations. The rabbits were killed 4 weeks postoperative, using an overdose of pentobarbital (Apharmo, Arnhem, NL) iv.

Post-mortem sample acquisition and analysis

Tissue samples were obtained under aseptic conditions. A bone sample of the anterior right cortex was obtained as a negative control for contamination during sampling and culture. The left tibia was divided into an anterior and posterior part as well. The anterior part was divided in two different samples: a proximal sample, which was fully adjacent to the implant, and a distal sample, which was distal from the implant.
For bacteriology, a proximal and distal sample of approximately 1 gram of bone of the anterior half of the tibia were weighed, ground in a sterile metal mortar and homogenized in 10 ml PBS at 2500 rounds per minute (rpm) for 3 minutes and at 6000 rpm for 5 minutes using a tissue grinder (Polytron PT3100; Kinetica Benelux, Best, NL). Finally, 10 μl samples of serial 10-fold dilutions were plated on blood agar plates (Tryptic Soy Agar; Beckton Dickinson, Darmstadt, Germany) using a multichannel pipette. In addition a blood agar plate was incubated with 1 ml of the undiluted homogenate. After an overnight incubation at 37°C the number of colony forming units (CFU) were counted and calculated per gram of bone (detection limit 10 CFU/g of bone).
For histopathology, the posterior half of the tibia, containing the implant, was fixed in 4% buffered formaldehyde and dehydrated by graded series of ethanol. After that the samples were embedded in a mixture of methyl methacrylate (Merck Schuchardt, Hohenbrunn, Germany) and Plastoid N (Sigma-Aldrich, Steinheim, Germany) in a ratio of 4 to 1. Benzoylperoxide (Merck Schuchardt, Hohenbrunn, Germany) was added as a catalyst. After polymerization, non-decalcified sections (thickness 20 μm) on the transverse axis of the tibia were made, using a sawing microtome. The sections were stained with basic fuchsine and methylene blue. For analysis, a proximal and distal section of each tibia including the implant were blindly examined by 2 of the authors (DM and PN) using light microscopy and semi-quantitative scoring of infection was performed according to the scoring system previously used by Vogely *et al.*[3]. This system scores 11 different items for infection (cortical destruction, enlargement of Haversian canals, periosteal thickening and presence of leukocytes, microabscesses, granulation tissue and fibrosis in the cortex and medullary canal) and results in a score between 0 (no infection) and 56 (gross, acute infection). The scores of both sections were averaged.
For histomorphometry, digital photos (resolution: 2040x1536 pixels) of the slides were taken. Image analysis was carried out with a PC-based system equipped with the KS400 version 3.0 software (Carl Zeiss Vision, Oberkochen, Germany). A program was developed in KS400 to quantify different parameters concerning bone integration. For each section, both the bone-implant contact and bone area inside the

titanium foam were measured (Figure 1). Scores were reported as a percentage of the total possible contact length or total area inside the foam. Because bone was removed from the anterior part, only the posterior part of the foam was used for analysis. A proximal and a distal section were analyzed and the scores of both were averaged.
The diagnosis of infection was defined as a positive culture of either the proximal or distal bone sample, in combination with elevated infection parameters in the blood and histopathological signs of infection.

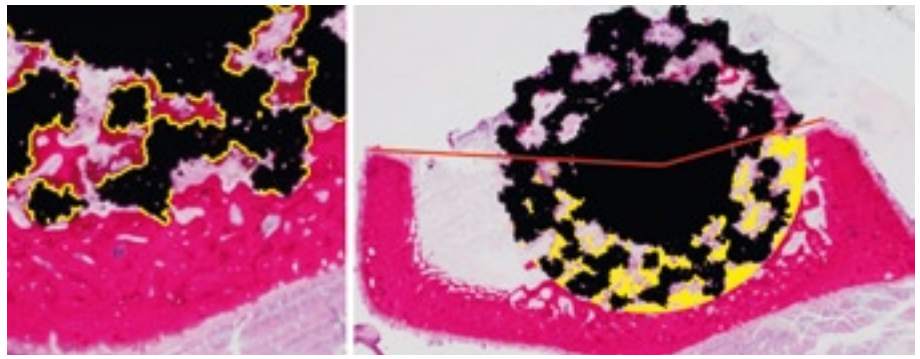


Figure 1
Both photos show examples of the histomorphometrical measurements; the yellow line in the photo on the left indicates the bone-implant contact, whereas the yellow area in the right photo indicates the area of new bone inside the titanium foam.

Statistical analysis

All results are reported as mean ± standard error of the mean (SEM). For the outcome infection a logistic regression analysis was performed, with implant type and inoculum dose as independent factors. For all other analyses we performed repeated measures analysis or two-way ANOVA (uni- and multivariate) and Bonferroni post-hoc testing. A p-value <0.05 was considered significant. SPSS 11 for Mac OS X was used for all analyses.

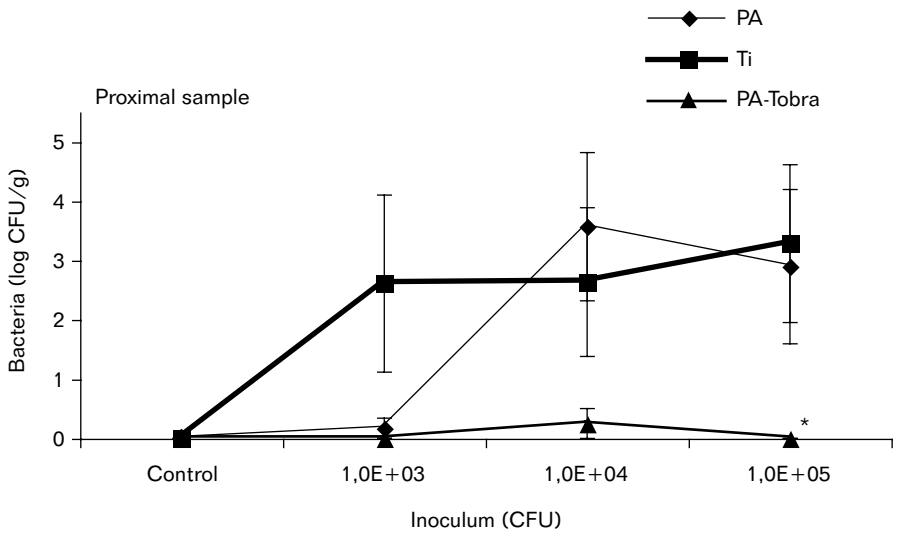
Results

General

One rabbit (PA implant, 10^4 CFU) died after 6 days because of septicemia. Because of the short follow-up, this rabbit was excluded from all analyses. All other rabbits recovered well from surgery. The ESR significantly increased to 19.6±5.0 and 20.6±5.2 in both the PA and Ti rabbits compared to the PA-Tobra rabbits ($p=0.004$ and $p=0.002$), in which it remained normal at 1.4±0.2. A less distinct change was observed in the WBC, but values of the PA-tobra rabbits also showed a significantly lower increase compared to both other groups ($p=0.003$ and $p=0.002$). For the PA and Ti rabbits the WBC increased from 7.0±0.5 to 9.2±0.7 and from 6.9±0.3 to 9.6±0.9 respectively, whereas for the PA-tobra rabbits it increased from 6.0±0.3 to 6.2±0.4. In the subsequent weeks, the ESR and WBC values of the PA and Ti rabbits returned to normal as well. Only for the ESR a significant effect of inoculum dose was observed ($p=0.004$). Average weight loss after 28 days for the PA-Tobra rabbits was 6±16 grams, whereas this was 188±66 grams for the PA rabbits ($p=0.013$) and 161±51 grams for the Ti rabbits ($p=0.037$). In comparison the non-contaminated control rabbits had an average weight loss of 13±26 grams. Although there was a positive trend between a higher inoculum dose and weight loss, this difference was not significant. Systemic tobramycin concentrations in the PA-tobra group were negligible, as all results remained below the detection level (0.18 μg/ml) of the assay.

Infection and bacteriology

Based on the criteria used for infection, 9 of 17 contaminated rabbits (53%) in the PA group and 12 of 18 contaminated rabbits (67%) in the Ti group were considered infected after 28 days. In the PA-tobra group 1 of 18 contaminated rabbits (6%) was infected, which was a significant reduction compared to both other groups ($p=0.009$ and $p=0.002$). Cultures were negative in all of the non-contaminated control rabbits and all right control tibiae. The actual number of CFU cultured from the proximal bone samples was also significantly less in the PA-tobra group ($p=0.004$ vs. Ti and $p=0.045$ vs. PA) (Figure 2). For the distal samples there only was a significant difference between the PA-tobra and Ti groups ($p=0.035$). The effect of increasing the inoculum dose on the number of infections or number of CFU was not significant ($p=0.455$ and $p=0.246$). No significant differences in infection rate or number of CFU were observed between the PA and Ti-group ($p=0.632$ and $p=1.00$).



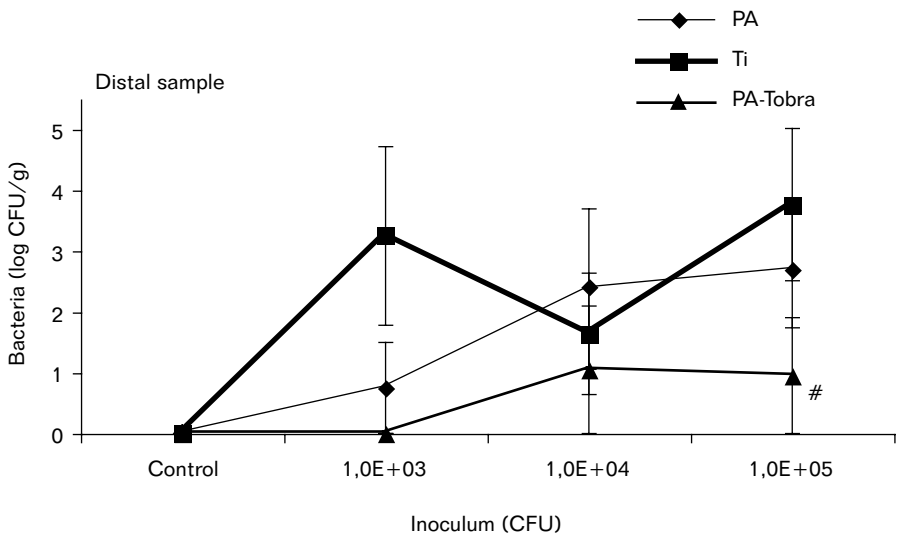


Figure 2
Quantitative culture results of the proximal and distal bone samples 28 days after contamination with different concentrations of S.aureus Wood 46 (* $p<0.05$ vs. PA and Ti, # $p<0.05$ vs. Ti).

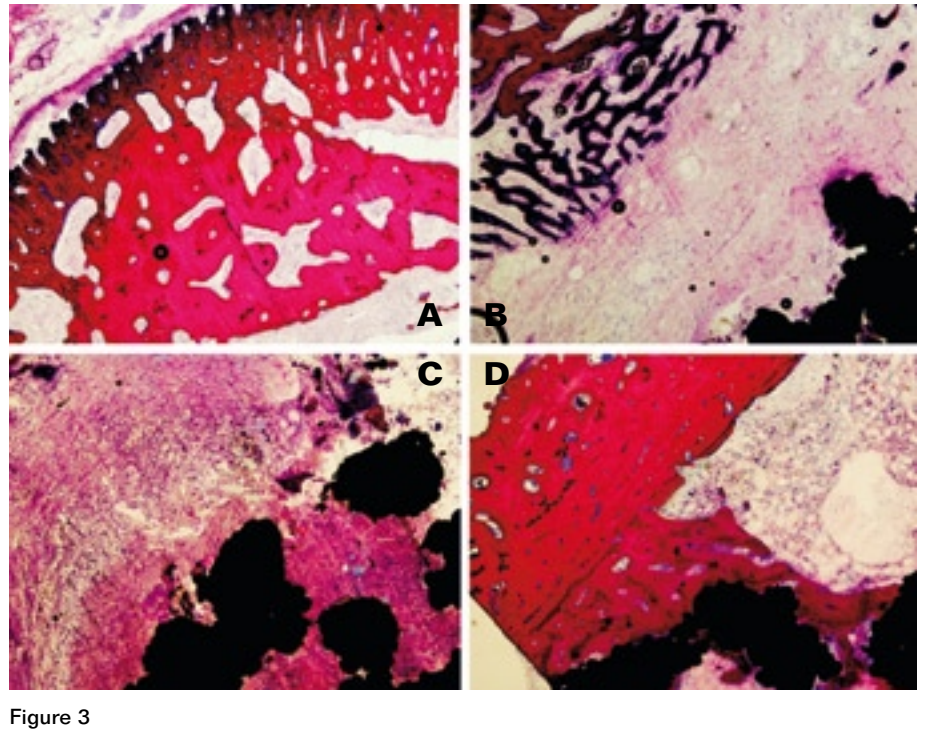


Figure 3
All photographs show signs of the presence or absence of infection seen during the histopathological analysis. Figure A shows periosteal thickening, enlargement of Haversian canals and destruction of the cortex; figure B shows extensive fibrosis and the total absence of cortical bone; figure C shows a large microabscess with debris. Figure D shows normal bone architecture, ingrowth of new bone into the titanium foam, and the presence of haematopoiesis.

Histopathology

Microscopic analysis of the slides mainly showed signs of chronic infection in all contaminated PA and Ti subgroups. Periosteal thickening, cortical destruction, enlargement of Haversian canals, microabscesses and fibrosis in the medullary canal were abundantly present (Figure 3). In only a few cases leukocytes and granulation tissue were seen. Signs of infection were absent in all the non-contaminated control rabbits. In the PA-tobra group, there was only 1 rabbit that showed histological signs of infection, all other rabbits appeared infection free. In both the PA and Ti group there were multiple rabbits, in which the implant bed appeared to be infected. As a result, the average histopathology scores of the PA-tobra rabbits were lower (Figure 4), however this was only significant compared to the rabbits in the uncoated titanium group

(p=0.011) and not to the PA-group (p=0.177). Even though the rabbits in the Ti group showed significantly more signs of infection compared to the rabbits in the PA group in the subgroup contaminated with 10^3 CFU, overall their scores were comparable (p=0.825). Excluding the non-contaminated control rabbits from the analysis did not lead to statistical significant differences (p=0.161 vs. PA and p=0.011 vs. Ti). There was no significant effect of increasing the inoculum dose on the pathology score. Histopathological signs of infection were present in the majority of rabbits with a positive culture and there was a high correlation between the number of bacteria cultured and pathology score (R^2=0.861, p<0.001).

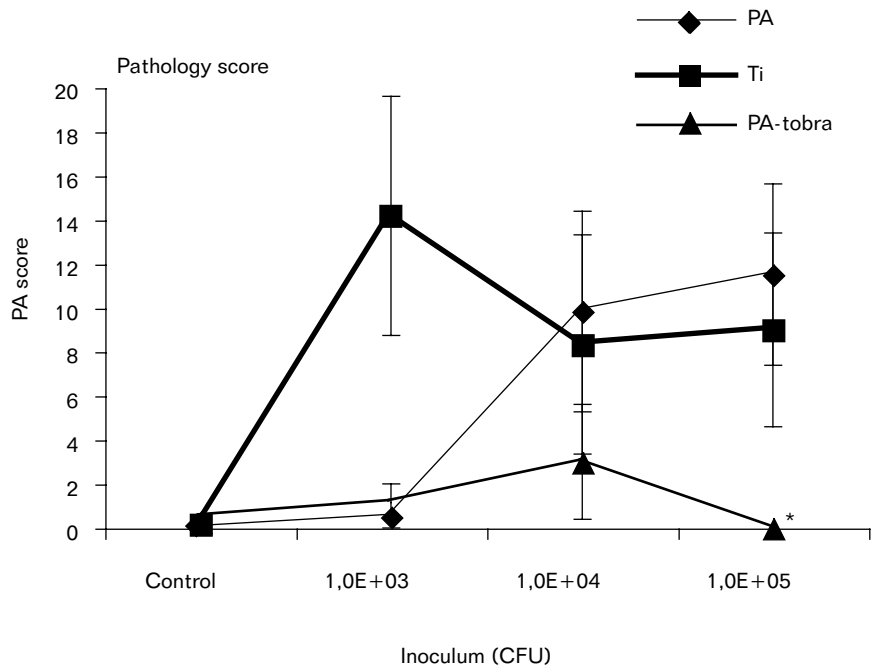


Figure 4
Pathology scores for infection according the scoring system by Vogely *et al.*[3], after contamination with different concentrations of *S.aureus* Wood 46. No signs of infection resulted in a score of 0, whereas acute, gross infection could results in a maximum score of 56 (* p<0.05 vs. Ti).

Histomorphometry

Image analysis showed that mean overall percentages of bone-implant contact were 16±3% for PA, 9±2% for Ti and 21±3% for PA-tobra rabbits, respectively (Figure 5). For bone area, mean overall percentages were 17±2%, 11±3% and 21±2%. On both parameters PA-tobra rabbits scored better than both other groups. The scores were significantly higher compared to Ti (both p=0.004), whereas differences between PA-tobra and PA rabbits (p=0.612 and p=0.463) and between PA and Ti rabbits were not significant (p=0.126 and p=0.182). Excluding the non-contaminated rabbits favored the results of the PA-tobra rabbits compared to the PA rabbits somewhat more, but did not lead to a significant difference (p=0.271 for bone-implant contact and p=0.418 for bone area). There was a significant effect of increasing inoculum sizes toward less bone-implant contact (p=0.007), whereas for the bone area it was just not significant (p=0.054).Looking at the effect of the actual presence of infection or no infection on bone integration, it was observed that bone-implant contact significantly decreased from 24±4% to 7±3% for the PA rabbits (p=0.003) (Figure 6). For the Ti rabbits the decrease from 12±3% to 6±2% and for PA-tobra rabbits from 22±3% to 5±4% was not significant (p=0.096 and p=0.062). A comparable effect for the different implants was observed for the bone area (PA p=0.012, Ti p=0.451 and PA-tobra p=0.019). It should be mentioned that for the PA-tobra group the decreased percentages of the infected group are based on only 2 infected rabbits in that group. To obtain further insight on the severity of infection and the effect on bone integration, both parameters were correlated with the ^{10}logCFU. This resulted in correlation coefficients of -0.50 and -0.532 (p<0.0001). This indicated a negative, however moderate, effect of the ^{10}logCFU on bone integration. The correlation between bone-implant contact and bone area was very good with a correlation coefficient of 0.893 (p<0.0001).

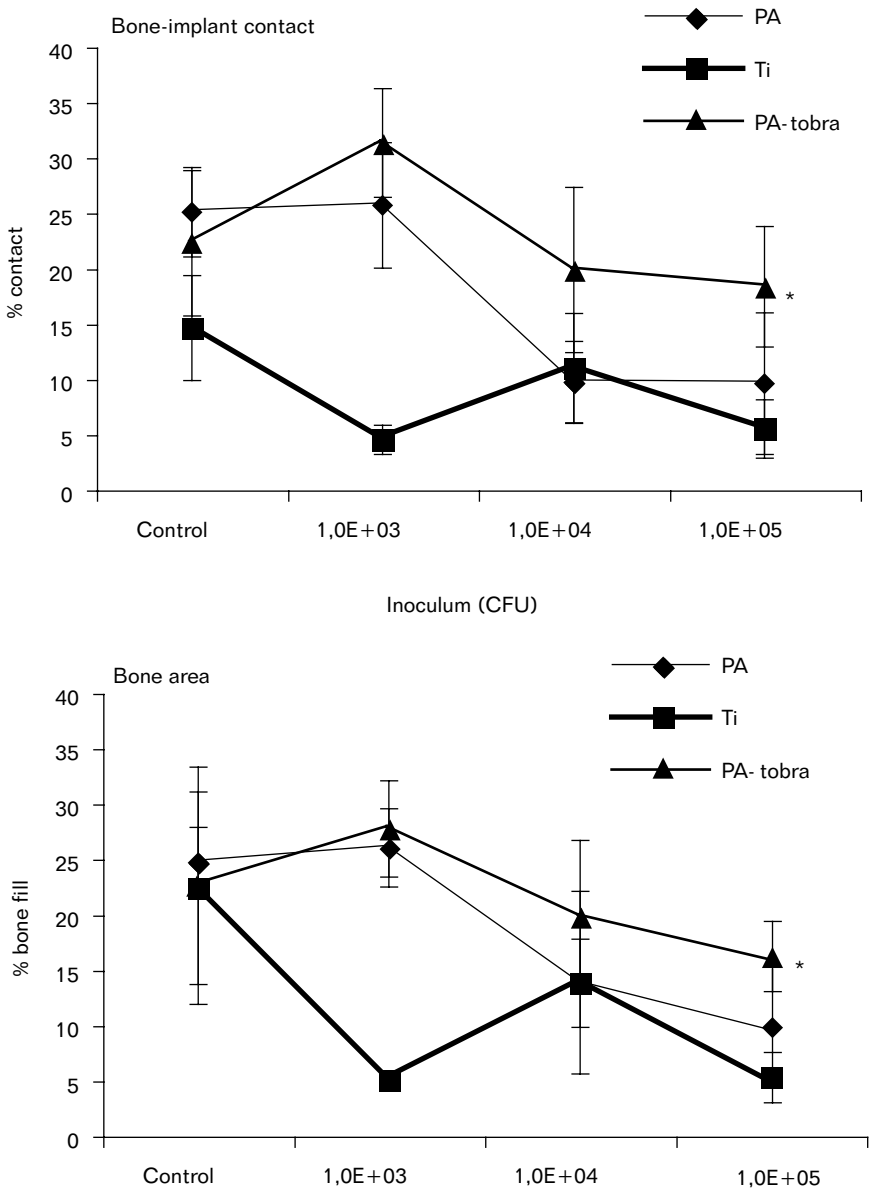


Figure 5
Osseointegration, expressed by amount of bone-implant contact and bone area with the titanium foam, after contamination with different concentrations of *S.aureus* Wood 46. Results are reported as a percentage of total contact with or fill of the titanium foam (* $p<0.05$ vs. Ti).

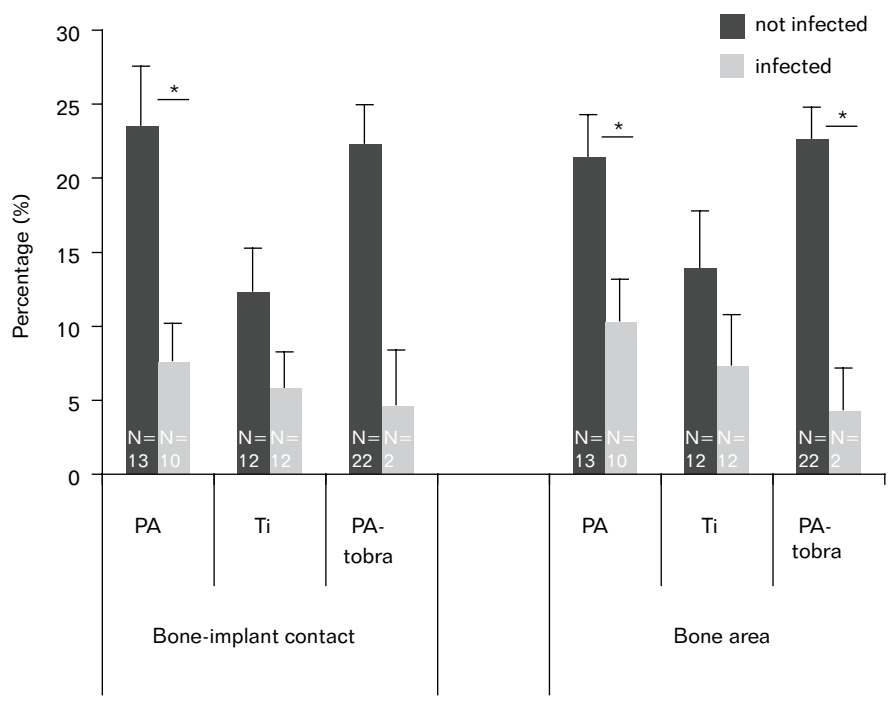


Figure 6
Osseointegration, expressed by amount of bone-implant contact and bone area with the titanium foam, in the absence or presence of infection. Results are reported as a percentage of total contact with or fill.

Discussion

Although it did not prevent infection in all rabbits, the addition of tobramycin to the PA-coated implants significantly reduced the infection susceptibility, compared to both PA-coated and uncoated implants. Apart from reducing the number of infections, it also significantly reduced the number of bacteria found in infected cases and the extent of histopathological signs of infection. In addition, it resulted in increased bone integration, although this was only significant compared to uncoated titanium.

In our model the rabbits were contaminated with different concentrations of bacteria, ranging from low to relatively high, thereby mimicking the array of possible peroperative contamination in the clinical situation. As expected, the control rabbits, which were not contaminated, showed no signs of infection in any of the analyses. For the others, unfortunately no real dose-response effect could be observed between the inoculum of contaminating bacteria and the infectious response. Although parameters like weight loss, number of CFU on quantitative cultures and histopathology all showed a trend towards higher outcomes in animals with higher inoculum doses, this was never significant. This can be explained by the variability in clinical responses between the rabbits, as some rabbits with a low inoculum developed severe infections and others with a high inoculum showed no signs of infection. The only outcome in which there was a significant effect of increasing inoculum doses was osseointegration. For this outcome higher doses of bacteria led to a marked reduction of the bone-implant contact and the amount of bone present in the titanium foam.

A point of discussion is what type of antibiotic to use for the dip-coating of the implant. For this study tobramycin was chosen, as it known to have good efficacy against the different microorganisms common in orthopaedic infections, like *staphylococci* and *Pseudomonas aeruginosa*[22]. In *in vitro* experiments it showed a good release from the implant and with the amounts used inhibited growth of *Staphylococcus aureus*[19]. Previously, the addition of tobramycin to bone cement was also shown to be effective in preventing implant-related infections[20]. Although we did not investigate this in our current experiment, theoretically one could choose any type of water-soluble antibiotic to load the implant, depending on the susceptibility of the anticipated microorganism. Because the dip coating is not prefabricated, an individual surgeon can even choose what type of antibiotic to apply during the actual surgical procedure. Conditions that have to be met, to be able to apply the antibiotic solution, are that one uses an implant with a high porosity on the outside and an external surface, which is hydrophilic. It should be mentioned that different antibiotics could have different release characteristics and that a different amount of the agent could be necessary to obtain the same effect.

Criticism on this study could be that by loading an uncoated titanium foam implant with antibiotics or even by directly applying antibiotics to the medullary canal, comparable results in infection prevention could be obtained. Most likely, the uncoated implant with antibiotic coating would have resulted in less infected cases as well and thereby better osseointegration compared to the uncoated group without antibiotics. As our main interest was in the value of combining the antibiotic coating for infection prevention and the PA-coating for better osseointegration, we decided not to add this extra group to the current study. With respect to just applying antibiotics to the implant bed before implantation, it could be hypothesized that this would have beneficial effects as well. However, it should be realized that this would be a totally uncontrolled situation without any consistent antibiotic concentrations at the direct implant surface. Reason to use both PA-coated and uncoated implants as controls, were the surprising results of the previous study with plasma-sprayed HA by Vogely *et al.*[3, 4], where the HA-coated implants were more susceptible for infection and because of that lost their advantage of better bone integration. In our study the PA-coated titanium foam implants showed similar infection rates and better bone integration compared to uncoated implants. An explanation for this could be the differences in surface characteristics of the PA-coating and the titanium foam itself. With a smooth, solid titanium implant, as used by Vogely *et al.*, the effect on surface roughness and porosity of the plasma-sprayed HA-coating will be significant compared to the uncoated implant. This increased roughness and porosity could make the coated implant more susceptible for bacterial attachment. In implants with a titanium foam external surface, the titanium foam itself will mainly determine the surface characteristics and the potential additional effect of the thin PA-coating will be much smaller, which will diminish the differences between the uncoated and coated implants.

The histopathological analysis showed that after 4 weeks mainly signs of chronic infection remained, as almost no indicators of acute infection, like polymorphonuclear leukocytes, were detected. As a result none of the rabbits even approached the maximum score of 56 point for acute, gross infection. However, pathology results were in line with the bacteriology results with respect to the observed differences between the different groups. Perhaps even more important, the rabbits of all 3 groups that were not contaminated with *Staphylococcus aureus* also did not show any signs of infection or inflammation. In many non-infected rabbits there were even signs of normal medullary haematopoiesis, a factor not included in the scoring system. This indicates a good biocompatibility for both the uncoated and PA-coated implants and that the addition of a tobramycin coating has no adverse effects, like cytotoxicity because of too high concentrations of released antibiotic.

Although several studies were published, which investigated the *in vivo* activity of

antibacterial coatings on the prevention of infection, there are only 2 studies that also used hydroxyapatite coatings. De Jong *et al.* reported a study in goats in which chlorhexidine-lipid-HA-coated external fixator pins reduced infection rates from 100% to 17% compared to pins only coated with HA[9]. More recently Alt *et al.* reported on the efficacy of gentamicin-HA and gentamicin-RGD-HA coatings on steel K-wires in the prevention of infection[18]. They showed good results, as infection rates decreased from 88% for HA-coated K-wires without antibiotic to 0% for both types of gentamicin-coated K-wires. Although this second study did look at histopathological signs of infection, neither study investigated the effects of either the presence of infection or the additional antimicrobial coating on osseointegration. This current study is the first to report this aspect of antimicrobial coatings in combination with HA on uncemented implants.

We found that the PA-tobra implants showed better osseointegration compared to both other types of implant. Even though differences in the control rabbits were not that large, the differences compared to uncoated titanium foam were apparent in all subgroups of contaminated rabbits. In comparison to the PA-coated implants, the amount of bone-implant contact and bone present in the titanium foam of the PA-tobra implants was higher as well. Although this was not significant, differences did increase with higher inoculum doses. Analysis showed that there was a large difference in osseointegration between rabbits that did or did not develop an infection; especially the PA and PA-tobra rabbits showed a large reduction. As PA-tobra and PA implants showed comparable bone integration in non-infected rabbits, the observed differences between these groups in the contaminated rabbits could probably be explained by the prevention of infection in the PA-tobra group.

From the current experimental study, we can conclude that the combined tobramycin and PA-coating on titanium foam implants appears to be an easy and effective local antibiotic strategy for uncemented implants for infection prophylaxis and thereby also improved implant fixation, which will most likely result in improved long-term implant survival.

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Chapter 6

No efficacy of silver bone cement in the prevention of methicillin-sensitive staphylococcal infections in a rabbit contaminated implant bed model

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Abstract

Background

Data from literature showed that a new type of metallic silver PMMA cement had good results in infection prophylaxis. This study investigated the *in vivo* efficacy of silver cement in the prevention of methicillin sensitive staphylococcal infections, compared to plain and tobramycin-containing cement.

Methods

In 48 rabbits 0.6%-silver, 1%-silver, plain or tobramycin PMMA cement was injected into the femoral medullary canal after contamination with 10^5, 10^6 or 10^7 CFU *Staphylococcus aureus*. After 14 days bone was collected for bacteriology and histopathology.

Results

All plain and silver cement rabbits were infected, whereas only 2 tobra rabbits were ($p<0.001$). The number of bacteria cultured (^{10}logCFU) from bone adjacent to the cement, was 6.4±0.3 and 6.1±0.3 for the 0.6% and 1%-silver rabbits. For the rabbits with plain and tobra cement this was 6.2±0.2 ($p>0.95$) and 0.0±0.0 ($p<0.001$) respectively. Two tobra rabbits had a positive culture of a distal bone sample. Histological sections of plain, 0.6% and 1%-silver rabbits all showed signs of infection; these signs were absent in the tobra rabbits.

Conclusion

Silver and plain cement were not effective in preventing infection, whereas tobra cement was. As silver cement predominantly exhibits an antimicrobial effect at the direct cement surface, this cement seems less useful in situations where there are bacteria present in surrounding tissues, like revision surgery. Whether silver cement has relevance in the prevention of bacterial colonization of cement remains to be determined.

Introduction

Although infection rates in orthopaedics have been significantly reduced by prophylactic measures, like stringent antiseptic surgical measures and intravenous antibiotics preoperatively, infection of a joint arthroplasty remains a major problem. The use of antibiotic-containing bone cement has proven its value in the prevention of infection in both experimental and clinical research[1-3]. Using cement as the carrier for antibiotics provides a way to deliver high levels of antibiotics locally, without increased risk of renal toxicity or ototoxicity due to high serum levels[4-8]. Therefore many orthopaedic surgeons use either commercially available or hand-mixed antibiotic-containing bone cement in primary and revision hip and knee arthroplasties nowadays.

Prosthesis related infections are mainly caused by Gram-positive bacteria such as *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococci*, followed by Gram-negative organisms such as *Enterobacter* and *Pseudomonas* species[9, 10]. Unfortunately an increasing number of these bacteria have become resistant to antibiotics. Especially *Methicillin-resistant Staphylococcus aureus* (MRSA) and *Methicillin-resistant Staphylococcus epidermidis* (MRSE) are increasingly found in infected arthroplasties. In these cases, the antibiotics in the bone cement have lost their protective effect and therefore constantly new antimicrobial agents have to be investigated.

Silver is well known for its antimicrobial effects and is already used in different fields in medicine, such as cardiovascular implants, contact lenses, urine catheters and wound care[11-14]. In the field of orthopaedics, there are multiple publications about silver-coatings of implants or pins for external fixation[15-19]. Even the use of silver in bone cement has already been reported in literature[20-23]. Although there were good antimicrobial effects, toxic side effects were reported as well and as a consequence its use was not continued.

More recently, a new type of low-dose metallic silver-containing bone cement showed good antimicrobial effects. The cement contained a type of nanoparticulate metallic silver, called NanoSilver[24, 25]. This silver has a much higher active surface and the nanoparticulate silver particles form small aggregates. Therefore the nanoparticulate silver has a much higher porosity compared to commercial silver powder. Because it is a metallic type of silver and not a silver salt, there is almost no local release and systemic spread of silver from the cement. This silver, which was added to a not commercially available PMMA bone cement, showed good *in vitro* results against *Staphylococcus epidermidis*, MRSA and MRSE and showed no *in vitro* cytotoxicity against osteoblasts[24]. In addition, it was also effective in preventing infection with

MRSA in an experimental rabbit infection model[26]. For this silver cement, the main antimicrobial activity is claimed to be a surface phenomenon, which prevents the presence of viable bacteria at the implant surface.
To determine the extent of antimicrobial activity of the new silver when added to Simplex bone cement, we compared in the present study the *in vivo* efficacy of silver-containing PMMA bone cement in preventing methicillin sensitive staphylococcal infections to plain and tobramycin-containing PMMA bone cement, in a rabbit model of a contaminated local implant bed.

Animals, Materials and Methods

Design

The Animal Care and Use Committee of the Faculty of Medicine of Utrecht University approved the protocol for this study. In 48 female New Zealand White rabbits 0.6% w/w silver, 1% w/w silver, plain or tobramycin-loaded (tobra) PMMA bone cement (n=12 per cement type) was injected into the medullary canal of the right femur. Prior to cement injection the implant bed was contaminated with 10^5, 10^6 or 10^7 colony-forming units (CFU) of *Staphylococcus aureus* (n=4 per subgroup). All rabbits had a follow-up of 14 days. Post-mortem, analysis was performed by bacteriology and histopathology.

Animals, housing and feeding

Female New Zealand White rabbits (Harlan, Horst, The Netherlands (NL)) weighing 2.8 to 3.9 kg (mean weight 3.0 kg) were obtained from a professional stockbreeder. They arrived 1 week prior to surgery to acclimatize to the housing in the animal care facility. The rabbits were housed individually in cages and were daily fed with 80-100 gram antibiotic free rabbit diet STANRAB P (Special Diet Services, Witham, UK).

Cement

For all 4 experimental cement types Simplex P bone cement (Howmedica International, Limerick, Ireland) was used. This PMMA bone cement is a mixture of 40 g of powder component (polymer) and 20 ml of liquid component (monomer). For the plain and tobramycin-containing cement the commercially available Simplex P and Simplex P with Tobramycin (1 g of tobramycin per package) bone cements were used. For both types of silver-containing cement, Simplex P bone cement was pre-blended with 0.6% w/w or 1% w/w silver. The silver used was the same as used in the previous studies, which is a nanoparticulate type of silver with particle sizes between 5 and 50 nm. The silver has an active surface of 4 m^2/g and particles form aggregates of 2 to 5 μm, which have a porosity between 85 and 95%. All cement types were sterilized, mixed under vacuum and used according to the manufacturer's guidelines.

Bacteria

Staphylococcus aureus Wood 46 (ATCC 10832) was used to contaminate the implant bed. This strain has been previously used in several other infection studies[1, 27]. For preparation of the stock, a colony was grown overnight in Müller Hinton broth at 37°C. Standardized suspensions were frozen at -70°C until a sample was used. On the day of use the suspension was thawed and diluted with sterile phosphate buffered saline (PBS, pH 7.4) to a concentration of 10^6, 10^7 or 10^8 CFU per ml. During surgery the rabbits were inoculated with 100 μl of bacterial suspension, resulting in inoculum sizes of 10^5, 10^6 or 10^7 CFU.

Surgery and anaesthesia

Surgery was performed under general inhalation anaesthesia and aseptic conditions. Premedication consisted of an intramuscular (im) injection with 4 mg acepromazinemaleate (Sanofi Sante, Maassluis, NL) and 4 mg methadone (Eurovet Animal Health, Bladel, NL). Anaesthesia was initiated by an intravenous (iv) injection of 8-10 mg etomidate (Janssen Pharmaceuticals, Tilburg, NL). After intubation, anaesthesia was maintained using a mixture of O_2/N_2O (ratio 1:1) and halothane 1% (Albic, Maassluis, NL).

The skin of right leg was shaven, disinfected with 10% povidone-iodine and isolated by sterile drapes. After skin incision, the trochanter tertius was exposed. Using a small drill, the cortex was penetrated and the femoral canal was reamed up to a diameter of 4 mm. Then cooled PMMA bone cement (4°C) was vacuum mixed according to the manufacturer's guidelines. After suctioning, 100 µl of bacterial suspension was injected in the medullary canal and subsequently ±1.2 gram of the cement was injected. Expansion of the cement sealed of the entrance of the canal, thereby preventing spill of bacteria. The cement was allowed to polymerize in situ. Finally, the wound was closed in layers. Pain relief was provided by 3 mg nalbufine (Lamepro, Raamsdonkveer, NL) iv and 0.1 mg buprenorphine (Reckitt and Colman Products, Kingston-upon-Hull, UK) im.

Postoperative follow up

Postoperatively, fluoroscopy of the right femur was performed to check the position of the cement (Figure 1). Animals were examined daily for activity, eating and wound healing. Weight and temperature were measured as well. Preoperative and 1 and 2 weeks postoperative blood samples were taken for erythrocyte sedimentation rate (ESR) and white blood count (WBC). Two weeks postoperative, the rabbits were killed using an overdose of pentobarbital (Apharmo, Arnhem, NL) iv.

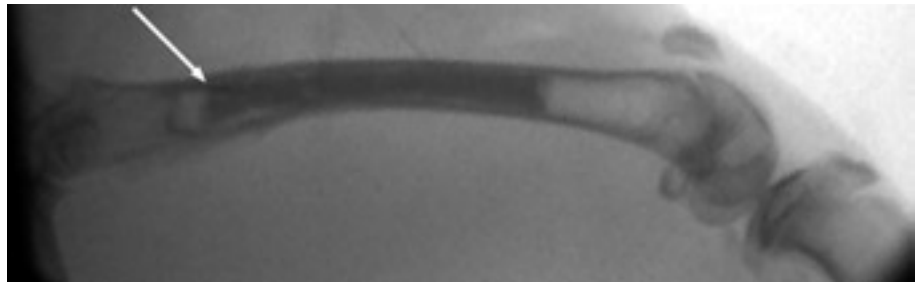

Figure 1
Fluoroscopic image of the right femur, showing cement in situ in the proximal medullary canal. The arrow indicates the entry point for cement injection.

Post-mortem sample acquisition and analysis

Post-mortem, tissue samples were obtained under aseptic conditions. A bone sample of the lateral left cortex was obtained as a negative control for contamination during sampling and culture. The right femur was excised and divided into a lateral and medial part as well. The lateral part was divided in 2 different samples: a proximal sample, which was fully adjacent to the cement, and a distal sample, which was distal from the cement.

For bacteriology, a proximal and distal sample of approximately 1 gram of bone of the lateral half of the femur were weighed, ground in a sterile metal mortar and homogenized in 10 ml PBS at 2500 rounds per minute (rpm) for 3 minutes and at 6000 rpm for 5 minutes using a tissue grinder (Polytron PT3100; Kinetica Benelux, Best, NL). Finally, 10 µl samples of serial 10-fold dilutions were plated on blood agar plates (Tryptic Soy Agar; Beckton Dickinson, Darmstadt, Germany) using a multichannel pipette. In addition a blood agar plate was incubated with 1 ml of the undiluted homogenate. After an overnight incubation at 37°C the number of colony forming units (CFU) were counted and calculated per gram of bone. The lowest detection limit was 10 CFU/g of bone.

For histopathology, the medial half of the femur was fixed in 4% buffered formaldehyde, decalcified and dehydrated by graded series of ethanol. The decalcified bone samples were embedded in paraffin and 5 μm sections were cut using a microtome. Slides were stained with haematoxylin and eosin and blindly analyzed using light microscopy for signs of infection, like periosteal thickening, enlargement of Haversian canals, destruction of the cortex and presence of leukocytes, microabscesses, fibrosis and granulation tissue.

The diagnosis of infection was defined as a positive culture of either the proximal or distal bone sample, in combination with elevated infection parameters in the blood and histopathological signs of infection.

Statistical analysis

All results are reported as mean ± standard error of the mean (SEM). For the outcome infection a logistic regression analysis was performed, with cement type and inoculum dose as independent factors. For all other analyses we performed repeated measures analysis or two-way ANOVA and Tukey-HSD post-hoc testing. A p-value <0.05 was considered significant. SPSS 11 for Mac OS X was used for all analyses.

Results

General

All rabbits recovered well from surgery. All rabbits showed a rise in temperature to about 40°C on the first postoperative day, which returned to baseline values the next day for the tobra rabbits and remained high until day 7 for all other groups. The overall ESR significantly increased to 25.1±7.3, 21.8±4.4 and 15.0±3.3 in the rabbits with plain, 0.6% and 1% silver cement compared to the rabbits with tobra cement ($p<0.01$ vs. plain and 0.6% silver, $p=0.111$ vs. 1% silver), in which it remained normal at 1.2±0.1. After the peak value after 7 days, the ESR of both silver groups returned to almost normal in the second week. A less distinct change was observed in the WBC, which only showed a significant difference between plain and tobra cement ($p=0.004$). Only for the WBC a significant effect of inoculum dose was observed ($p=0.029$). Average weight loss after 14 days for plain and tobra cement rabbits was 246±45 and 50±83 gram. Rabbits with 0.6% and 1% silver cement had a weight loss of 213±36 and 202±29 gram (0.6% silver $p=0.895$ and 1% silver $p=0.786$ vs. plain and both silver concentrations $p<0.001$ vs. tobra). Although there was a positive trend between higher inoculum dose and weight loss, this difference was not significant ($p=0.077$).

Infection rates and bacteriology

Based on the criteria defined for infection, in the plain and both silver cement groups all rabbits (100%) were infected, whereas with tobra cement only 2 of 12 rabbits (17%) were infected ($p<0.001$) after 14 days (Table 1). These infected rabbits all showed clinical signs of osteomyelitis to some extent. The average number of bacteria cultured from the proximal samples of bone adjacent to the cement (^{10}logCFU/g) was 6.4±0.3 and 6.1±0.3 for the 0.6% and 1% silver rabbits (Figure 2). For the rabbits with plain and tobra cement this was 6.2±0.2 ($p=0.953$ vs. 0.6% silver and $p=0.991$ vs. 1% silver) and 0.0±0.0 ($p<0.001$ vs. both silver concentrations). The quantitative culture results of the distal samples, not adjacent to the cement, were comparable to the results of the proximal samples (Figure 3). However, 2 tobra rabbits did have a positive culture of their distal bone sample, compared to no positive cultures for the proximal samples. The effect of increasing the inoculum dose on the number of infections was not significant; for the number of CFU cultured it was borderline not significant either ($p=0.051$). In 45 of the 48 non-contaminated left control femurs cultures were negative. The 3 control cultures that were positive all showed growth of *Staphylococcus aureus*.

Table 1

Incidence of *Staphylococcus aureus* infection after 14 days

Inoculum (^{10}log CFU)	Plain	0.6% Silver	1% Silver	Tobramycin
5	4/4	4/4	4/4	1/4
6	4/4	4/4	4/4	0/4
7	4/4	4/4	4/4	1/4
Total	**12/12 (100%)**	**12/12 (100%)**	**12/12 (100%)**	**2/12 (17%)**

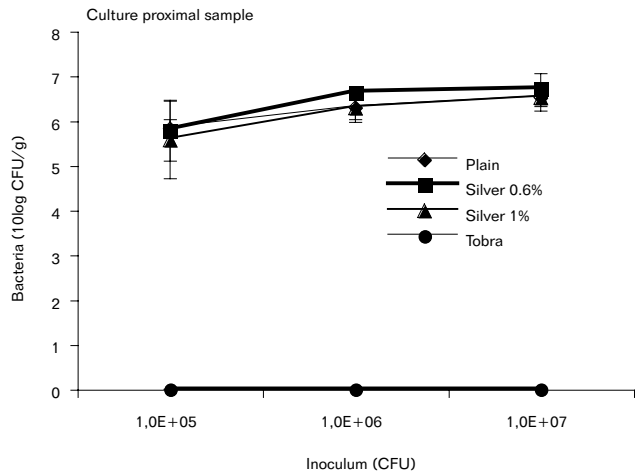

Figure 2

Quantitative culture results of the proximal bone samples of the right femur 14 days after contamination with different concentrations of *S. aureus* Wood 46.

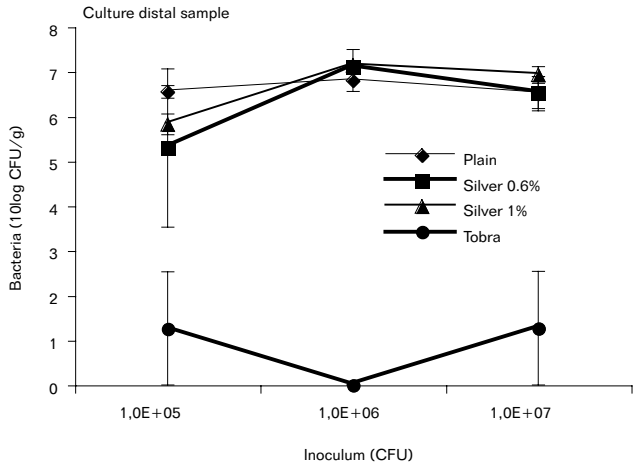

Figure 3

Quantitative culture results of the distal bone samples of the right femur 14 days after contamination with different concentrations of *S. aureus* Wood 46.

Histopathology

Microscopic analysis of the sections showed different signs of infection. As the medullary canal had been totally occluded by bone cement during the surgical procedure, these signs were limited to the cortical bone and periost. Signs most often seen were periosteal thickening, enlargement of Haversian canals and cortical destruction (Figure 4). In only a few rabbits, leukocytes, fibrosis and granulation tissue were seen. No microabscesses were found. In the rabbits with plain, 0.6% and 1% silver bone cement signs of infection were present in almost all rabbits. The extent, to which these signs were present, was comparable in all 3 groups and was not substantially influenced by the bacterial inoculum dose. In the group of tobra rabbits, there were only 2 rabbits that showed some enlargement of Haversian canals. All other signs of infection were absent in this group.

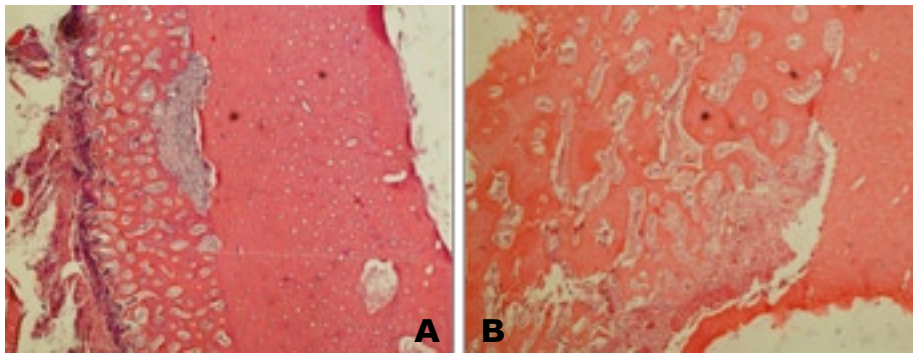

Figure 4
Photographs of haematoxylin/eosin colored sections of rabbits with plain (A) and 0.6% silver-containing (B) bone cement contaminated with *Staphylococcus aureus*. Both photos show examples of histopathological signs of infection. Photo A shows thickening of periost, the presence of leukocytes and granulation tissue and some enlargement of Haversian canals. Photo B shows extensive enlargement of Haversian canals and cortical destruction.

Discussion

Results showed that in this experimental, contaminated implant bed model, both concentrations of silver cement were not effective with respect to the prevention of methicillin sensitive staphylococcal infection in comparison to the tobramycin-containing bone cement, as all outcome measures of the silver groups were comparable to the plain cement.

As expected, infections developed in all plain PMMA cement animals, while the tobramycin cement was effective in the prevention of infection.

As it is known that in the presence of biomaterials much less bacteria are necessary to cause an infection and as plain Simplex P cement contained no antimicrobial agent to prevent this, it was not surprisingly that infection developed in all rabbits of this group. About the use and characteristics of the Simplex P with tobramycin cement much research has already been performed and published and its use is widely accepted in clinical practice[1, 5, 8, 28]. The results of this type of cement in this study were comparable to the results previously reported by Nijhof *et al.*, using the same experimental infection model[1]. Although 2 tobra rabbits did have a positive culture of a bone sample distal from the cement, it showed to be effective in the prevention of infection in the majority of the cases. The 2 infections found, can be explained by the presence of bacteria further distally from the cement, where antibiotic concentrations were probably not high enough to kill these bacteria.

The silver was expected to have a good antimicrobial activity as well, as it is know to be bactericidal and acts through different mechanisms of action[29]. It reacts with different membrane or enzyme proteins, leading to protein denaturation and interference of energy production. Other modes of action are interference of DNA replication and disruption of bacterial cell wall integrity, all ultimately leading to cell death. However, in this study neither the 0.6%, nor the 1% silver cement had any effect in the prevention of infection. In spite of the previous promising reports[24, 26], which showed good efficacy against antibiotic sensitive and resistant bacteria both *in vitro* and *in vivo*, the silver cement scored on no outcome parameter significantly different from the plain cement. On the primary outcome of 'infection/no infection' it even showed a 100% infection rate. The histopathological analysis supported the bacteriology results, as in almost all silver rabbits multiple microscopic signs of infection were present.

There are several possibilities to explain the lack of efficacy of the silver cement in this study. First, it should be realized that the current model is that of a contaminated implant bed, with bacteria present at the implant surface, but also at a distance from the implant. PMMA cement releasing an antimicrobial agent, such as tobramycin or

gentamicin, will be active at the cement surface, but will also have antibiotic concentrations exceeding minimal inhibitory concentrations of bacteria in the surrounding bone and therefore will likely be effective against bacteria distant to the implant surface as well. In contrast, the silver cement contains metallic nanoparticulate silver particles, which are not released from the cement and need to be ionized before they exhibit their antimicrobial activity. Therefore it predominantly exhibits an antimicrobial effect at the direct cement surface and not in the surrounding bone tissue as well. Because of the need to be ionized before the silver displays its antimicrobial activity, this is a delayed response as well. This provides the bacteria the possibility to replicate several times before the silver starts to interfere with its proliferation.

Another explanation could be the difference in contaminating bacteria used in the 2 studies. Although the *Staphylococcus aureus* Wood 46, used in this study, has often been used in infection research[1, 27], it could have had a higher virulence compared to the antibiotic sensitive and resistant coagulase negative staphylococci and *Staphylococcus aureus* used by Alt *et al.*[24]. Especially, because the contaminating concentrations of 10^5 to 10^7 CFU of the *Staphylococcus aureus* Wood 46 were lower than the 10^7 and 10^8 CFU Alt *et al.* used in their *in vivo* study.

Finally, small differences in how the surgical procedure was exactly performed in the 2 studies could be an explanation. Unfortunately this is difficult to determine, as the procedure performed by Alt *et al.* in their *in vivo* study is not described in detail. However, we think that the model as currently used by us is a good representation of what happens in clinical practice. During the operation and the immediate postoperative period, bacterial contamination can also be present both at the implant surface and in the surrounding tissues.

The publications on silver-containing PMMA cement from the late 70's and 80's shows variable results as well. The article by Spadaro *et al.* reported on an *in vitro* study using PMMA cement loaded with 4 different types and different concentrations of silver salts[20]. Dependent on the type and concentration, the silver cement showed good efficacy in inhibiting growth of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *E.coli*. In a later article by Dueland *et al.* this was further investigated in a study in rabbits, using cement loaded with 1% silver sulfate, resulting in significantly reduced mortality rates and bacterial counts compared to plain controls[23]. Two other publications represent a clinical case in which the use of 3% metallic silver-containing PMMA cement was complicated by neuropathy of the corresponding leg[21, 22]. Microbiological testing, using the bone cement removed during revision surgery, did not show any inhibitory effect on bacterial growth using both plate and liquid medium cultures. In comparison, control disks loaded with silver nitrate inhibited growth in concentrations down to 0.1% for *Staphylococcus aureus* and down to 0.01% for *E.coli*. The difference in antimicrobial activity between metallic silver and a silver salt in PMMA cement has never been investigated, but it is imaginable that silver as a salt is more easily released from the cement and therefore present in higher concentrations and more distant from the cement. However, this larger release does have the drawback of increasing the potential risk of silver toxicity.

Although it was not effective preventing infection, the nanoparticulate metallic silver-containing bone cement might have effective antimicrobial activity for preventing bacterial colonization of the cement at the direct surface. By using the current infection model this question cannot be investigated and a new model should probably be developed first. This model should mimic the clinical situation of late haematogenous infection, where the implant or cement gets colonized and later infected because of a transient bacteraemia. However, the results from the present study demonstrate that the nanoparticulate metallic silver-containing cement is at least less useful compared to antibiotic containing cement in situations where there are also bacteria present in the surrounding tissue, like revision surgery. Its use in primary arthroplasties is debatable as well, as the majority of arthroplasty infections are primary infections, established during the per- and early postoperative period. In most cases bacterial contamination will not be limited to the implant surface itself, but will spread to the surrounding tissues and wound haematoma. There the non-eluting silver cement will have no antimicrobial effect. This might limit the use of metallic silver-containing cement to surgeons, who still use plain instead of antibiotic containing cement as their standard fixation method, because of the risk of inducing antibiotic resistance. Although, because of the increasing incidence of antibiotic resistance, new antimicrobial strategies for prophylaxis against implant-related infections will become increasingly necessary during the upcoming decades, the role of metallic silver bone cement in its current form seems limited.

Finally, we can conclude, that in contrast to tobramycin-containing cement, metallic silver-containing PMMA cement was not effective in preventing infection. As the non-eluting silver cement predominantly exhibits an antimicrobial effect at the direct cement surface, this type of cement seems less useful in situations where there are also bacteria present in surrounding tissues, like revision surgery. Whether silver cement has relevance in preventing bacterial colonization of cement, for instance in late haematogenous infections, remains to be investigated.

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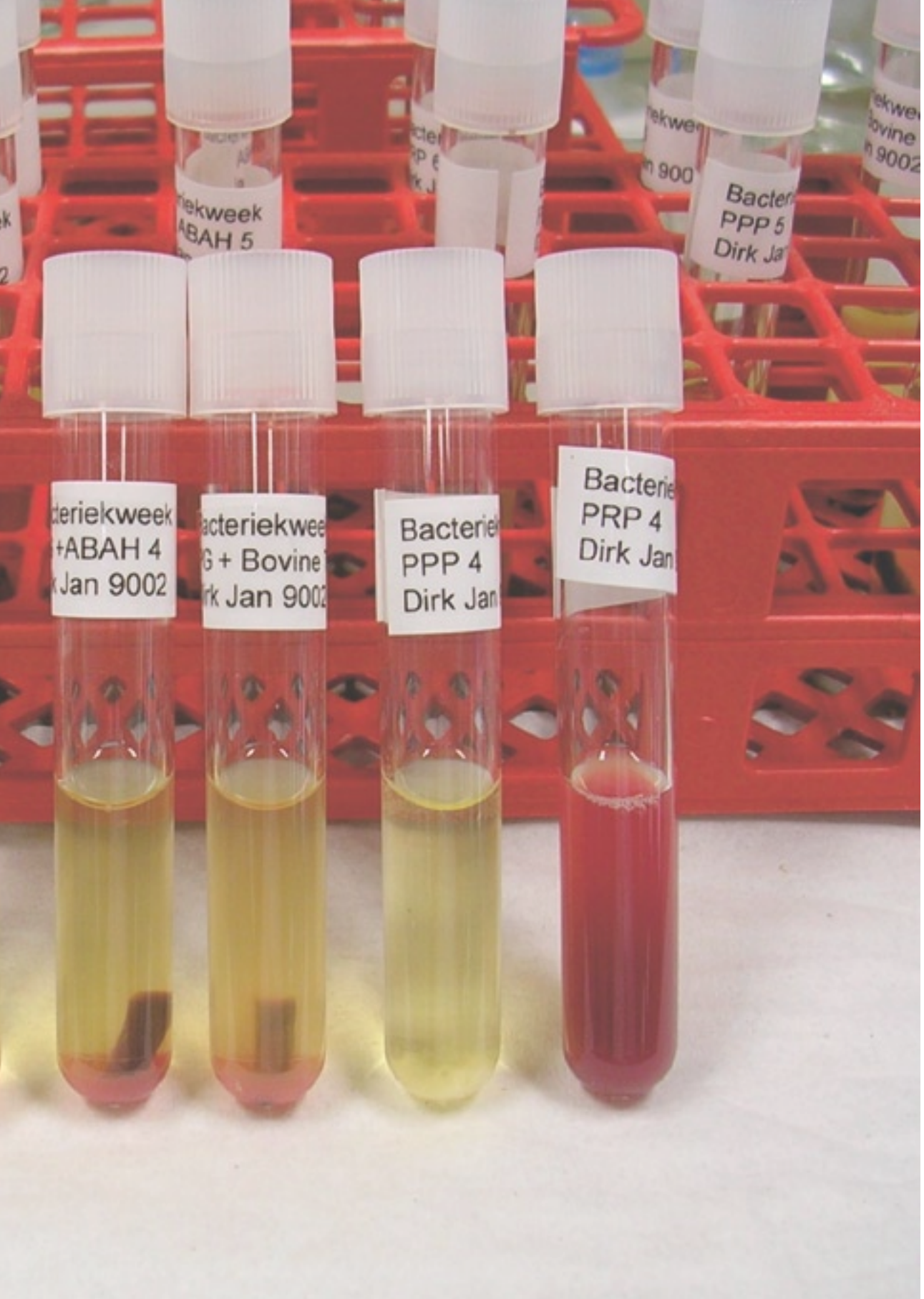

Chapter 7

Antimicrobial activity of platelet-leukocyte gel against *Staphylococcus aureus*

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Abstract

Background

Platelet-leukocyte gel (PLG) contains high concentrations of platelets and leukocytes. As leukocytes play an important role in the innate host-defense, we hypothesized that PLG might have antimicrobial properties. This study investigated the antimicrobial activity of PLG against *Staphylococcus aureus* and the contribution of myeloperoxidase (MPO), present in leukocytes, in this process.

Methods

Platelet rich plasma (PRP) and platelet poor plasma (PPP) were obtained from whole blood of 6 donors. PLG was prepared by mixing PRP with autologous (PLG-AT) or bovine thrombin (PLG-BT). Antimicrobial activity of PLG-AT, PLG-BT, PRP and PPP was determined in a bacterial kill assay. MPO release was measured by ELISA and activity was measured using a MPO activity assay.

Results

Cultures showed a rapid decrease in the number of bacteria for both PLG-AT and PLG-BT, which was maximal between 4 and 8 hours, to approximately 1% of the bacteria in controls. The effect of PLG-AT was largest and significantly different compared to PRP ($p=0.004$) and PPP ($p<0.001$), however not compared to PLG-BT ($p=0.093$). PLG-AT, PLG-BT and PRP showed a comparable, gradually increasing MPO release. MPO activity was comparable for all groups and remained stable. No correlation between MPO release, activity and bacterial kill could be found.

Conclusion

PLG appears to have potent antimicrobial capacity, but the role of MPO in this activity is questionable. PLG might represent a useful strategy against postoperative infections. However, additional research should elucidate its exact antimicrobial activity.

Introduction

The use of autologous platelet-leukocyte gel (PLG) is a relatively new perioperative technology, which is currently being used by a variety of surgical specialties[1-3]. The primary clinical applications are the support of soft tissue wound healing, the stimulation of bone formation and the control of postoperative bleeding[4-8]. PLG, prepared from a unit of whole blood, is a mixture of platelet rich plasma (PRP), leukocytes, thrombin and plasma proteins[9]. Thrombin is added and activates the platelets to form a viscous platelet clot, which results in degranulation and release of platelet growth factors, present in the αgranules of platelets. These autologous platelet growth factors initiate migration to and proliferation of cells at the wound sites. The effect of the released autologous platelet growth factors on tissue growth and repair has been investigated extensively[1, 10-14].

PRP is a buffy coat product, which apart from being enriched in platelets, also contains a high concentration of viable neutrophilic polymorphonuclear leukocytes or neutrophils. These cells play an important role in the innate immune defense against infections[9]. Activation of neutrophils results in the so-called respiratory burst, during which the highly bactericidal hypochlorous acid is formed through the action of myeloperoxidase (MPO), an enzyme produced mainly by neutrophils and monocytes[15]. Previous research suggested that this oxidative killing, compared to the non-oxidative killing, which is also present, contributes for the largest part to the antibacterial effect of neutrophils and that MPO plays an essential role in this process[16]. Furthermore, it has been demonstrated that platelets also have an antimicrobial efficacy, as they contain multiple antimicrobial peptides[17, 18].

For these reasons, the hypothesis is that PLG, as an engineered, biological blood product, also has antimicrobial properties, in addition to the effects of the platelet-derived growth factors that support healing. Despite the amount of publications on PRP and PLG during recent years, hardly any studies were published that investigated this potential antimicrobial activity. Only one recent publication reported this activity against different bacterial species[19]. The purpose of the current study was to investigate the *in vitro* antimicrobial activity of PLG against *Staphylococcus aureus*. As MPO released by neutrophils is an important mediator of the innate immune defense, the release and activity of MPO during incubation of the bacteria with different blood products was studied in parallel.

Materials and Methods

Donors

The Medical Ethical Review Committee of the University Medical Center Utrecht agreed to the study protocol. From 6 healthy, unmedicated volunteers (3 male, 3 female) blood was obtained by inserting a 17 G intravenous line into the median cubital vein.

PRP preparation method

Two 60 ml syringes were pre-filled with 7 ml of anticoagulant citrate dextrose-A solution and 53 ml of whole blood was slowly drawn via the infusion line. The syringes were inverted five times to ensure proper mixing with the anti-coagulant to avoid blood clotting in the syringes. The total volume of 120 ml was injected in the whole blood collection reservoir of the Angel Whole Blood Processing System™ (AWBPS; Sorin Group, Mirandola, Italy). The AWBPS is a semi-automated tabletop centrifuge system using a flat-disc. It uses a variable blood volume ranging from 60 to 180 ml to sequester the whole blood in components. Following centrifugation at 3,200 rounds per minute (rpm) for 19 minutes, the platelet-poor plasma (PPP), the platelet-rich plasma (PRP) and the erythrocyte concentrate were collected separately. An aliquot of 12 ml of PPP was used as the source to create autologous thrombin (AT). The remaining PPP volume and erythrocyte concentrate were discarded and not retransfused to the volunteers.

Autologous thrombin preparation

AT was produced using the activAT™ system (Sorin Group, Mirandola, Italy). The isolated PPP aliquot was mixed with the commercial ethanol reagent solution. Subsequently, this mixture was injected in an AT processing syringe containing glass beads to initiate clotting of the PPP mixture. After 25 minutes, a solid clot was formed in the processing syringe, which was then squeezed out through a microporous filter into another syringe. The liquid part in this syringe contains AT with a concentration of at least 50 IU/ml[20].

Platelet-leukocyte gel production

PLG was produced shortly before application to the bacterial cultures. PRP was mixed with AT in a 10:1 ratio to create PLG. As an alternative to AT, PRP was also activated with bovine thrombin (BT, 500 IU/ml; Jones Pharma Inc, St Louis, MO, USA) in a ratio 10:1, to determine if there were any differences in the source of platelet activator with regard to the kill of bacteria. As the amount of BT available was not dependent on an individual production process, a relatively high concentration of BT was used, to obtain maximal activation of the PRP in the PLG-BT samples.

Determination of platelet and leukocyte counts

Platelet and leukocyte counts were measured in samples of whole blood, erythrocyte concentrate, PRP and PPP as an estimate of the efficiency of the PRP separation process using the AWBPS sequestration equipment. Platelet count, white blood cell and differential counts were assessed with a fully automated analyzer (Cell-Dyn™ 4000, Abbott Diagnostics, Santa Clara, CA, USA), using a combination of optical and impedance methods for all blood samples. Platelets were counted in the impedance channel only, since this, in contrast to the optical method, is not affected by platelet activation.

Bacterial kill assay

One ml of phosphate-buffered saline (PBS, control), PLG activated with AT (PLG-AT), PLG activated with BT (PLG-BT), PRP or PPP was added to sterile tubes containing 4 ml of Müller-Hinton broth (Oxoid Ltd, Basingstoke, UK), which was inoculated with *Staphylococcus aureus* Wood 46 (ATCC 10832). This strain of *S. aureus* has been used previously in several other infection studies[21-24]. The final bacterial concentration after adding the sample was $1x10^6$ colony-forming units (CFU)/ml. After 1, 4, 8, 12 and 24 hours, a 50 μl sample was taken from each tube and the antibacterial activity of MPO was inactivated by adding excess catalase (Bovine catalase; Sigma-Aldrich, St Louis, MO, USA). Serial 10-fold dilutions of each sample were made and 10 μl samples were plated on blood agar plates (Tryptic Soy Agar + 5% sheep blood; Beckton Dickinson, Darmstadt, Germany) using a multi-channel pipette. After an overnight incubation at 37°C the number of viable bacteria was determined by counting the colonies (^{10}logCFU/ml).

Release and activity of MPO

The release and activity of MPO from the leukocytes in the different blood products into the culture medium was measured in separate series of vials containing the same concentrations of bacteria and platelet- derived products. MPO release and activity were measured after 1, 4, 8, 12 and 24 hours. Vials were centrifuged at 10,000 rpm for 3 minutes to pellet the bacteria and blood cells and the supernatant was stored at -20 and -80°C until analysis was done. To measure the presence of MPO, a commercial MPO-ELISA was performed (MPO ELISA Kit; Immundiagnostik AG, Bensheim, Germany) according to the manufacturer's guideline. To measure the activity of MPO, a commercial activity assay was performed (Myeloperoxidase Assay

Kit; CytoStore, Calgary, Canada) according to the manufacturer's guideline. This assay allows for a semi-quantitative and colorimetric determination of MPO. For both assays a sample of bacterium-inoculated Müller Hinton broth with 20% v/v PBS was used as a negative control to correct for background activity.

Statistical analysis

Results are reported as mean ± standard deviation. Analysis was performed using repeated measures analysis for bacterial kill, MPO release and activity and one-way ANOVA for platelet and leukocyte count. Tukey-HSD and Bonferroni post hoc-testing was performed. To determine the potential correlation between MPO release, activity and bacterial kill Spearman's rank correlation coefficients were measured. For each time point and group, MPO release and activity were correlated to both the absolute bacterial count at that time point and decrease or increase in bacterial count before and after that certain time point. Correlation between MPO release and activity was measured as well. P values <0.05 were considered to be significant.

Results

PRP collection

Six healthy donors with an age range of 22 to 44 years volunteered to participate in the study. The donor platelet count of whole blood was 262 ± 26 (10^6/ml) (Table 1). An average of 4.5 ml (range 4 to 5.5 ml) of concentrated PRP was obtained after the whole blood sequestration with the AWBPS, representing a high platelet yield of 64 ± 11% of the total circulating platelet concentration. In order to have the same amount of PRP from each donor available for testing, this concentrate was diluted with PPP to obtain a total PRP volume of 10 ml. The platelet count of the diluted PRP was $1{,}688\pm318$ (10^6/ml), a significant enrichment compared to the whole blood platelet count ($p<0.001$). The leukocyte count increased 3.1 fold from the baseline value of 5.7 ± 0.4 (10^6/ml) in whole blood to 18.0 ± 1.2 (10^6/ml) in PRP ($p<0.001$). The enrichment of platelets and leukocytes was comparable to those reported previously[7, 19]. There was no enrichment of platelets or leukocytes in the PPP or erythrocyte concentrate (Table 1).

Table 1

Platelets and leukocytes in different whole blood derived products

	WB	PRP	PPP	E-C
Platelets (10^6/ml)	262 ± 26	1688 ± 318*	18 ± 11	63 ± 22
Leukocytes (10^6/ml)	5.73 ± 0.95	17.9 ± 2.62*	0.0	8.28 ± 4.43

Legend: WB: whole blood; PRP: platelet rich plasma; PPP: platelet poor plasma; E-C: erythrocyte concentrate; * $p<0.001$

Bacterial kill

Cultures showed a rapid decrease in the initial number of bacteria for both PLG-AT and PLG-BT (Figure 1). Although less rapid and less pronounced, there also was a decrease in the bacterial counts for the PRP and PPP incubations. The maximum decrease for PLG-AT and PLG-BT was seen after 4 hours when only 0.35 ± 0.18% and 1.03 ± 1.07% of the number of bacteria present in the controls incubated without PRP-related products remained (Figure 2). For the PRP samples, this maximum decrease was seen after 8 hours, when the bacterial counts were 0.66 ± 0.49% of the controls. PLG-AT and PLG-BT could maintain a plateau in the proportional decrease compared to the control up to 8 hours. However, because the bacterial kill was not complete in any of the groups, the absolute number of bacteria increased again after the 4 hours

time point, when growth rates started to exceed bacterial kill. After 24 hours, bacterial growth had reached the stationary phase in all groups, although at lower levels in all experimental groups.

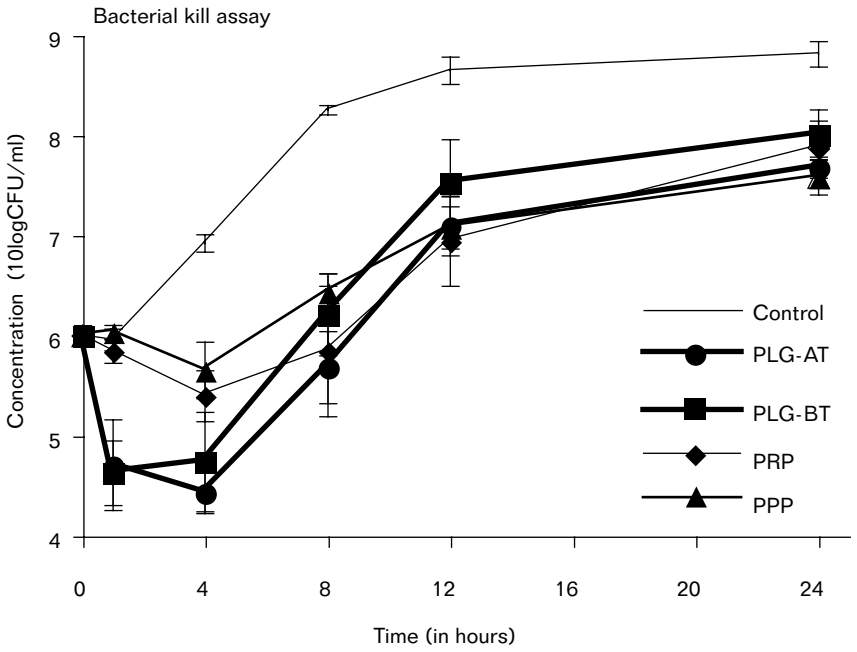

Figure 1
Bacterial growth of *Staphylococcus aureus* during 24-hour period (PLG-AT: platelet-leukocyte gel activated with autologous thrombin; PLG-BT: platelet-leukocyte gel activated with bovine thrombin; PRP: platelet rich plasma; PPP: platelet poor plasma)

Although PLG-AT, PLG-BT, PRP and PPP all induced a significant decrease in the number of bacteria compared to the control ($p<0.001$), the effect of PLG-AT appeared to be the largest for the entire 24-hour period ($p=0.093$ vs. PLG-BT; $p=0.004$ vs. PRP and $p<0.001$ vs. PPP). Although the initial bactericidal activity of PLG-BT was comparable to PLG-AT, no statistically significant differences were found compared to the effects of PRP and PPP for the entire 24-hour period.

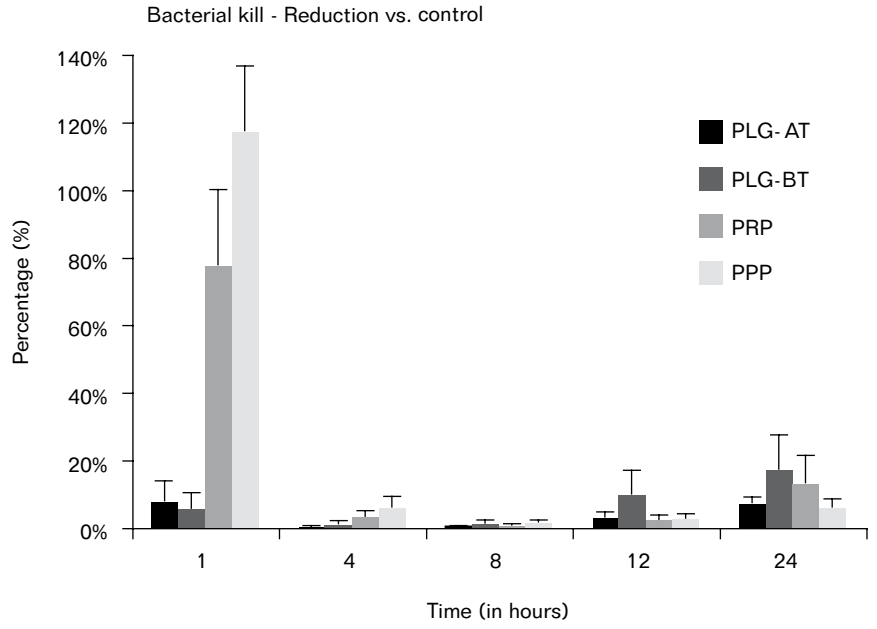

Figure 2
Percentage reduction of the absolute number of bacteria compared to the control (PLG-AT: platelet-leukocyte gel activated with autologous thrombin; PLG-BT: platelet-leukocyte gel activated with bovine thrombin; PRP: platelet rich plasma; PPP: platelet poor plasma)

MPO release and activity

The bacterial suspensions containing PLG-AT, PLG-BT and PRP all showed a gradual release of MPO during the first hours (Figure 3). PLG-AT and PRP reached a maximum MPO release after 8 hours, when concentrations of 474 ± 280 and 396 ± 198 ng/ml were measured. PLG-BT reached its maximum after 12 hours when the MPO concentration was 929 ± 989 ng/ml. Some MPO was also measured in the PPP samples, with concentrations ranging between 1.2 ± 0.6 and 2.1 ± 1.0 ng/ml during the 24-hour follow-up. No changes in MPO levels, indicating a release process, were observed. A large variation in the quantity of MPO released was observed between different donors at all time points. This resulted in large standard deviations for all groups, except PPP. As a consequence only the difference between PLG-BT and PPP was statistically significant ($p=0.038$).

For activity testing only samples of PLG-AT, PRP and PPP were available. Results showed that MPO activity was comparable for all 3 PRP-related products, with activity ranging between approximately 300 and 500 μUnits, and no real increase or decrease in activity was observed during the 24-hour period (Figure 4). No significant differences were found between the groups (PLG-AT vs. PRP p=1.0; PLG-AT vs. PPP p=0.63; PRP vs. PPP 0.99).
At no time point and for no experimental group a significant correlation between MPO concentration, activity and bacterial kill could be found (data not shown).

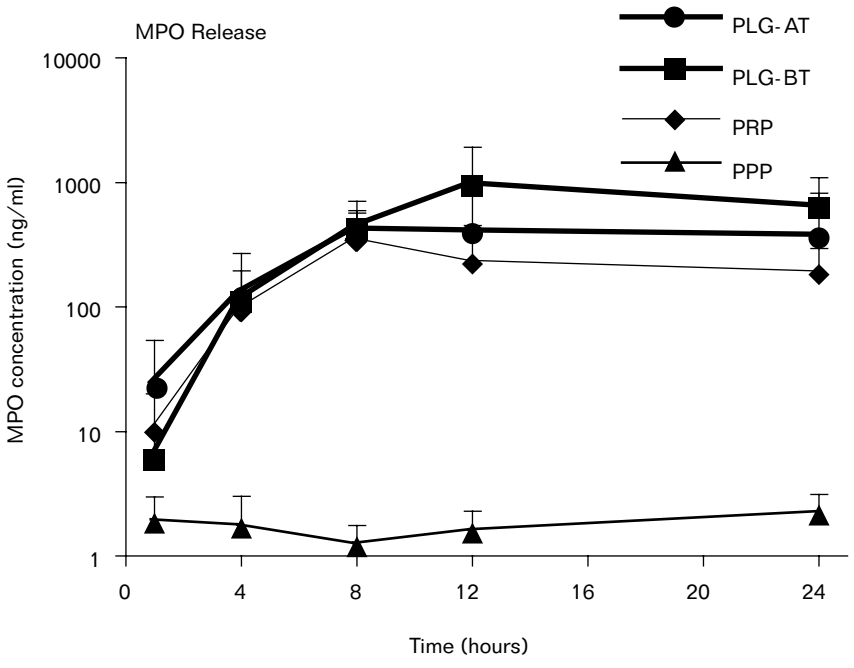

Figure 3
Presence of MPO released in the Müller Hinton broth culture medium during the assay (in ng/ml) (PLG-AT: platelet-leukocyte gel activated with autologous thrombin; PLG-BT: platelet-leukocyte gel activated with bovine thrombin; PRP: platelet rich plasma; PPP: platelet poor plasma)

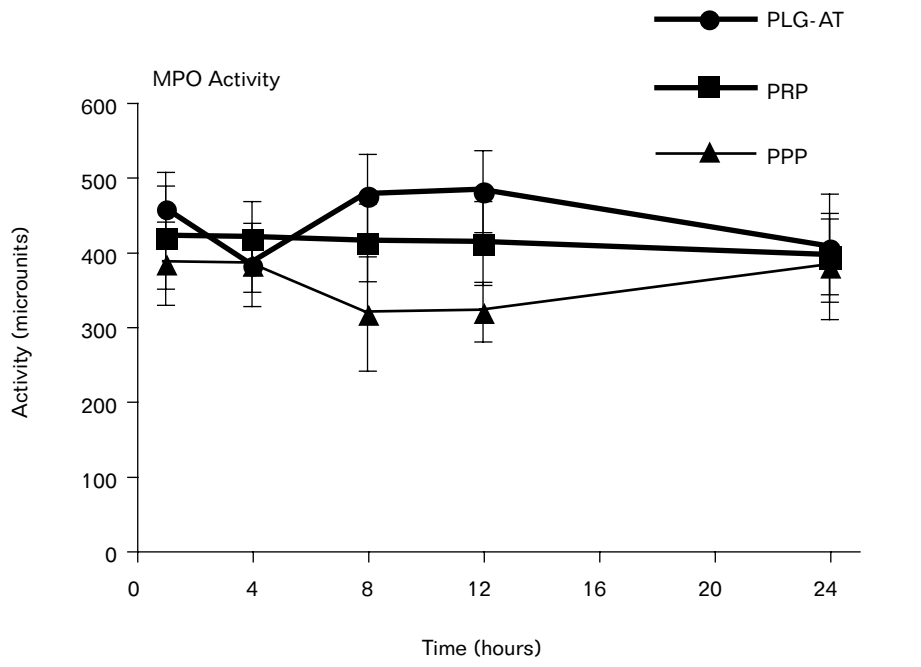

Figure 4
Activity of MPO released in the Müller Hinton broth culture medium during the assay (in μUnits) (PLG-AT: platelet-leukocyte gel activated with autologous thrombin; PRP: platelet rich plasma; PPP: platelet poor plasma)

Discussion

This study is one of the first to demonstrate that platelet-leukocyte gel (PLG), activated with either autologous or bovine thrombin, has a significant antimicrobial activity against *Staphylococcus aureus*. In an *in vitro* setting, the bacterial load was reduced to less than 1% of the controls by incubation in the presence of activated platelet-leukocyte gel. Also non-activated PRP and even PPP inhibited bacterial growth, although the decrease in number of bacteria was slower and less pronounced as compared to the effect of activated PLG. MPO release was comparable in both incubations with PLG and PRP, whereas in the cultures where PPP was added only low MPO levels were detected. Surprisingly, MPO activity was almost equal for all groups and remained stable during the entire follow-up period. MPO release and activity showed to have no significant correlation with the observed bacterial kill for any of the PRP-derived products.

The PRP produced was a buffy coat product, consisting not only of a high concentration of platelets, but also of concentrated leukocytes, composed of a mixture of neutrophils, monocytes and lymphocytes. *In vivo*, the neutrophils and macrophages are responsible for the phagocytosis of foreign pathogens. This system appears to represent one of the most important host defenses against infection[25]. Neutrophils are rich in granules containing MPO, which is released into the tissues after activation and degranulation. There MPO catalyzes the oxidation of chloride to generate hypochlorous acid and other reactive oxygen derivates. These substances act as potent bactericidal oxidants and are toxic to microorganisms and fungi[26]. Neutrophils and monocytes seemed not to be damaged or activated during the PRP production, as demonstrated by the low initial and rapidly increasing MPO concentrations measured in the Müller Hinton broth of the PLG and PRP-containing bacterial cultures during the 24-hour period. In comparison, with PPP only a low and stable concentration of MPO was measured. As determination of leukocyte counts of PPP had shown that it contained no leukocytes, the measured traces of MPO probably reflected basal MPO levels in plasma released upon phagocytosis[27, 28], or originated from a limited number of leukocytes that did get damaged or activated during the blood processing.

The bacterial culture assay used in the current study showed that PLG and PRP indeed have a strong antibacterial effect. This effect was not only growth inhibitory, but also bactericidal as was shown by an almost 100-fold reduction of the initial bacterial inoculum for PLG. For this early antimicrobial activity, it did not matter whether the PLG was activated with autologous or bovine thrombin. In PRP a slower and less strong decrease in the absolute number of bacteria was observed as well.

Activation of PLG by thrombin in the first hour of culture is therefore likely to have accounted for the strong decrease of bacterial load found, as the effect of non-activated PRP, containing exactly the same components, was minimal in this time period. In PRP these components will probably get partially activated by the presence of bacteria or in the course of spontaneous degeneration of the platelets/cells. Although this was not investigated in the current experiment, an explanation for the bactericidal effect of PPP might be the presence of antimicrobial peptides and other factors to some extent present in plasma, either released from the platelets during normal functioning or by breakdown of some platelets during the blood processing. Likewise, the plasma PPP may have contained some MPO released by leukocytes. Having demonstrated that PRP and PPP also exhibit antimicrobial activity to some extent, another interesting control would be whole blood, as this is always present in the natural situation of a wound bed. As the concentration of non-activated platelets and leukocytes in whole blood is much lower than in PRP, its antimicrobial activity would probably lie somewhere between the activity of PPP and PRP.

The strong antimicrobial effect of PLG seems to be limited to the first hours after application. After 4 hours less than 3% of the high initial bacterial inoculum was left in the presence of PLG activated with autologous thrombin. Although a proportional reduction of bacteria of approximately 99% compared to the control could be maintained up to 8 hours, the number of bacteria started to increase again after 4 hours. After this time point, the logarithmic growth of the bacteria exceeded the kill and growth continued until the stationary phase was reached. Adding a 48-hour time point to the experiment in pilot studies (data not included) showed that this stationary phase was already reached after 24 hours for all groups.

To determine the role of MPO in the antimicrobial activity of PLG and PRP the MPO release and activity was determined. As neither presence nor activity MPO could be correlated to bacterial kill in any way, it seems unlikely that this mechanism was an important mediator of the bacterial kill found in these settings. The discrepancy between MPO release and activity is remarkable. However, peroxidases are unstable enzymes and are easily inactivated by the substrate hydrogen peroxide[29]. Also other mediators can inhibit the activity of MPO[30, 31]. Therefore, part of the MPO released may not have been detected by the activity assay because of the subsequent inactivation by hydrogen peroxide in the course of the respiratory burst caused by the presence of the bacteria or other unknown factors present in the blood products.

Interestingly, recent evidence suggests that platelets are also involved in antimicrobial activity, since it has been demonstrated that they play a role in the innate immune defense by releasing a variety of platelet antimicrobial proteins (pAMP)[18, 32]. In both animal and human studies, pAMPs were released after platelet activation by thrombin

and displayed potent activities against blood borne pathogens[15]. MPO release was comparable for the not activated PRP and activated PLG samples and MPO activity was even comparable for all groups. This suggests that the addition of thrombin to PRP activates the platelets and not the leukocytes. As a consequence the difference in bacterial kill during the first hours might mainly be attributed to the effect of released antimicrobial peptides from the platelets and not so much to an effect of MPO from the leukocytes. As there will have been a combined activity of antimicrobial peptides and MPO in PRP and PPP as well, it is not possible to say from this experiment how large the contribution of either agent exactly is. Experiments where the activity of MPO is blocked by a specific inhibitor, like 4-aminobenzoic acid hydrazide (ABAH) could provide more insight into this matter[31]. Thus, although MPO has been reported to play a central role in the innate host-defense, it only seems to play a minor antimicrobial role in PRP and PLG-mediated bactericidal activity. However, whether this also goes for the *in vivo* contribution to the innate defense cannot be stated with certainty. In PRP the ratio of platelets and leukocytes is doubled from 50:1 to 100:1 in comparison to whole blood. This abundant presence of activated platelets could have overruled the effect of MPO in the current experimental setup. Although the use of PRP and PLG is relatively new, there have been many studies during the last decade investigating their applicability for many indications, either within or outside the orthopaedic field. Depending on the indications for use, the added value of PRP or PLG was found to be variable. As for the possible anti-infectious properties of PLG, this was investigated in 1 experimental and hinted at by circumstantial evidence in 2 clinical studies. The experimental study tested it using a disk-diffusion assay against 7 different bacterial species and found it to be active against methicillin sensitive and resistant *Staphylococcus aureus* and *E. Coli*[19]. In a study evaluating the effect of PLG on the post-operative wound healing process in patients receiving a total knee prosthesis 5% of patients not treated with PLG developed a superficial wound infection compared to no patients in the PLG group[7]. Finally, PLG was demonstrated to avoid superficial and deep wound infections in a study on the use of PLG in cardiac surgery[33].

As in the current study it was shown that especially PLG has a strong bactericidal activity, we hypothesize that it can also be used as a prophylactic antimicrobial agent against postoperative infection in orthopaedic surgery. Although many indications are possible, using PLG as a coating on uncemented implants seems like a beneficial option, especially since, in contrast to cemented implants, no options for local antimicrobial prophylaxis are available for these types of implants. As the antimicrobial activity of PLG seems to be at its maximum during the first hours after application, its use might be limited to infection prophylaxis and not treatment. However, as surgical debridement is of course the first and most important measure in infection treatment, the additional use of PLG might reduce the number of remaining bacteria enough to prevent reinfection as well.

Finally, we conclude that platelet-leukocyte gel, as an autologous blood product, which is safe to use for the patient and can be easily prepared during surgery, has a strong bactericidal activity and thus might be a potentially useful substance in the fight against postoperative infections. Further research should investigate its antimicrobial capacity compared to antibiotics, its exact antibacterial spectrum and, more importantly, prove its efficacy in the *in vivo* situation.

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Chapter 8

In vitro release of antibiotics from commercial PMMA beads and articulating hip spacers

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Abstract

Background

In vivo studies have shown the efficacy and benefits of antibiotic-loaded spacers compared to cement beads in the treatment of an infected prosthesis. These studies used hand-mixed, high-dose antibiotic bone cement. However, in clinical practice, commercial, low-dose antibiotic bone cement is often used for the preparation of spacers, which may lead to sub-therapeutic drug release. This study investigated the *in vitro* antibiotic release of hip spacers made from commercial, low-dose cement compared to commercial beads.

Methods

Three different antibiotic carriers were used: spacers made from Refobacin-Palacos-R or Antibiotic-Simplex-P and Septopal beads. Samples were placed in 1000 ml phosphate buffered saline at 37°C. Antibiotic concentrations were measured during 6 weeks.

Results

All carriers showed a burst release. Spacers showed little additional release after the first week, whereas for the beads release continued for at least 2 weeks. Cumulative release after 6 weeks was 27.5±2.3 mg for Palacos, 23.8±0.2 mg for Simplex and 188.3±9.3 mg for Septopal ($p<0.001$).

Conclusion

Despite the efficacy of spacers made from high-dose antibiotic bone cement and although other factors, such as providing stability to inflamed tissues, may have a beneficial effect *in vivo*, we think one should be cautious toward using low-dose antibiotic bone cement for spacers, as the lower and shorter drug release could result in an unsuccessful eradication of the infection.

Introduction

Nowadays less than 1% of all primary total hip arthroplasties (THA) need to be revised because of primary or secondary infection[1, 2]. However, the treatment of an infected THA is often a major challenge. Although some specialized centers have excellent results with one-stage procedures, the two-stage procedure still is the method of first choice for most surgeons and remains the gold standard. The traditional use of antibiotic-loaded PMMA beads for local antibiotic therapy during the interval between the first and second stage has proven to be effective in curing the infection, as success rates of more than 85% have been reported[3-6]. Although the beads work well to provide a high local concentration of antibiotics, morbidity for the patient is considerable and the implantation of the new prosthesis during the second stage is often complicated because of disuse osteopenia and peri-articular soft-tissue contractures.

Meanwhile, many clinical studies have shown the benefits of spacers made from antibiotic-loaded bone cement regarding these complications and show similar results regarding the infection-free rates[5, 7-12]. However, most of these studies are relatively small, uncontrolled case-series. There is only one study that directly compared spacers and beads[5]. This study did show a similar percentage of eradication of the infection for both strategies.

In almost all the clinical studies, hand-mixed, high-dose antibiotic bone cements are used for spacer preparation[10, 12]. Moreover, it was recommended that one should use an antibiotic dose of at least 3.6 gram of tobramycin combined with 1 gram of vancomycin to obtain effective elution levels for at least 6 weeks[13].

Based on the clinical successes presented in literature, an increasing number of orthopaedic surgeons are using spacers as their preferential therapeutic agent for local antibiotic delivery between the first and second stage. However, in clinical practice often commercially available, low-dose antibiotic bone cement is used for the preparation of the spacer. These types of bone cement regularly contain 1 gram or less of antibiotic per package, in order to retain their mechanical strength. Although this is sufficient when being used as a prophylactic measure, this creates the risk of having a sub-therapeutic antibiotic release from the spacers when used for a therapeutic indication[12]. Especially since antibiotic release is mainly a surface phenomenon and it has been shown that spacers, because of their lower surface area, have a lower antibiotic release compared to beads[14, 15]. As a consequence the local drug concentration may be too low or is not maintained long enough at a high concentration to successfully treat the infection. It may even induce antibiotic resistance of the bacteria[16, 17].

To investigate whether hip spacers made from commercially available, low-dose antibiotic bone cement exhibit effective release of antibiotics, we measured the *in vitro* antibiotic release characteristics and compared these to the antibiotic release of commercially available gentamicin-loaded PMMA beads.

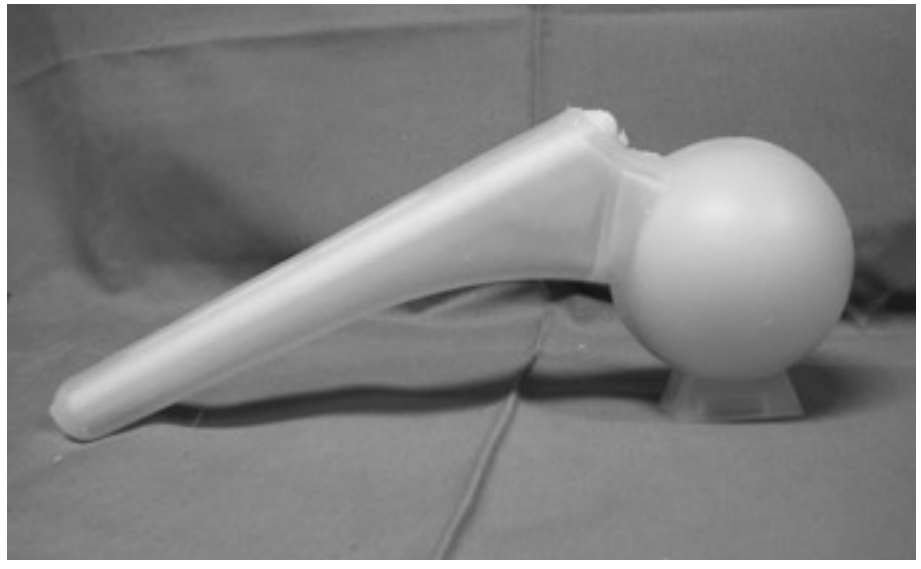

Figure 1
Hip spacer mold filled with bone cement

Materials and methods

Groups

Three groups of PMMA antibiotic-carriers were compared. Group one consisted of hip spacers made from Refobacin®-Palacos®-R bone cement, containing 0.5 gram gentamicin per 40 gram of dry PMMA powder (Biomet Orthopaedics, Kerzer, Switzerland). Group two consisted of spacers made from Antibiotic-Simplex®-P bone cement containing 1 gram tobramycin per 40 gram of dry PMMA powder (Howmedica Int., Limerick, Ireland). Group three consisted of commercially available Septopal®-beads (Biomet Merck, Darmstadt, Germany), containing 4.5 mg gentamicin per bead. Each Septopal study sample consisted of 120 beads.

Preparation of the spacers/beads

Palacos-R and Simplex-P bone cement were provided in a sterile pack containing 40 gram of dry PMMA and 20 ml of liquid monomer. Both cements were mixed according to the manufacturer's guidelines, using either the Optivac® (Biomet Cementing Technologies AB, Sjöbo, Sweden) or ACM® (Stryker Instruments, Kalamazoo, MI, USA) mixing and delivery system. In order not to reduce cement porosity and thereby antibiotic release, we did not use a vacuum (18). After mixing, the cement was injected in a silicone hip spacer mold (Figure 1). The spacer mold used, was a collarless bi-metric style stem with an endo-style head, a stem size of 9x125 mm and head diameter of 51 mm (Biomet Orthopedics Inc., Warsaw, IN, USA). To adequately fill the mold, approximately 150 gram of bone cement was needed (Palacos spacers 153.8±0.9 gram; Simplex spacers 153.3±1.7 gram). To ensure full polymerization, all spacers were allowed to polymerize for 30 minutes prior to the start of testing. The Septopal beads were preformed and consisted of 2 chains of 60 beads. The spherical beads had a diameter of 7 mm.

Release testing

The spacers and beads were placed in covered glass beakers, containing 1000 ml phosphate buffered saline (PBS, pH 7.4) at 37°C. A cumulative release was measured and 250 μl elution samples of the PBS were collected at 16 time points with a gradually expanding time interval during a period of 6 weeks (Day 1-7, 9, 11, 13, 16, 19, 23, 28, 35 and 42), which were stored at -21°C until analysis. Antibiotic concentrations were determined using a fluorescent polarizing immunoassay (AxSym system, Abbott Diagnostics, Hoofddorp, The Netherlands) The AxSym system has a sensitivity (lowest concentration distinguished from zero with 95% confidence) of 0.30 μg/ml for gentamicin and 0.18 μg/ml for tobramycin. Gentamicin and tobramycin

controls in PBS (1, 2, 4, 6 and 8 μg/ml) were included in each sample run. Measured gentamicin and tobramycin release concentrations were adjusted to the trend lines, obtained from the PBS-controls.

Statistical analysis

A sample size of 3 was used for each experimental group. Results are reported as mean ± standard deviation. To determine statistical differences between the release profiles of the different carriers we performed a repeated measures analysis and Tukey-HSD post-hoc testing. A p-value <0.05 was considered significant.

Results

All 3 groups showed a burst release during the first day, which was more than 3 times as high for the beads compared to both spacers (Figure 2). The release rates rapidly decreased during the next few days. For both the Palacos and Simplex spacers there was hardly any additional release after the first week, whereas for the Septopal beads there was a persistently increasing cumulative release for at least 2 weeks. From week 3 to 6 the beads still had an average additional release of 2491±383 μg per day (Figure 3), whereas this was only 55±5 and 32±9 μg per day for the Palacos and Simplex spacers respectively ($p<0.001$)

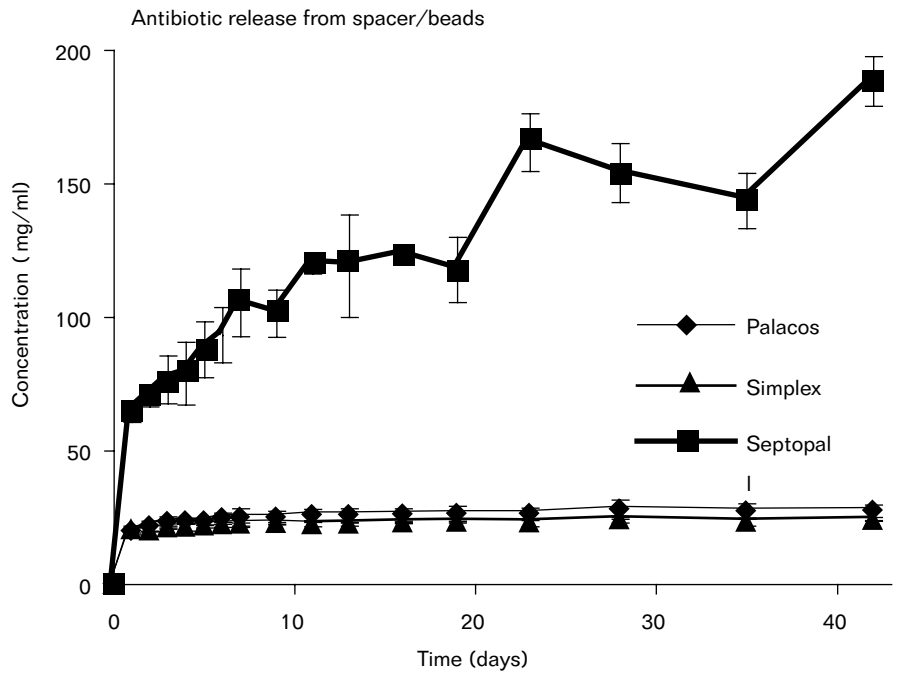

Figure 2
Cumulative antibiotic release during six weeks (concentrations in mg/ml)

The total cumulative release after 6 weeks was 27.5±2.3 mg for the Palacos spacers, 23.8±0.2 mg for the Simplex spacers and 188.3±9.3 mg for the Septopal beads. This represented 2.1±0.2%, 0.9±0.0% and 34.9±1.7% respectively of the total

amount of antibiotics present in each carrier. At all time points the cumulative release from the Septopal beads was significantly higher compared to both other groups ($p<0.001$). The difference between the Palacos and Simplex spacers was not significant ($p=0.782$).

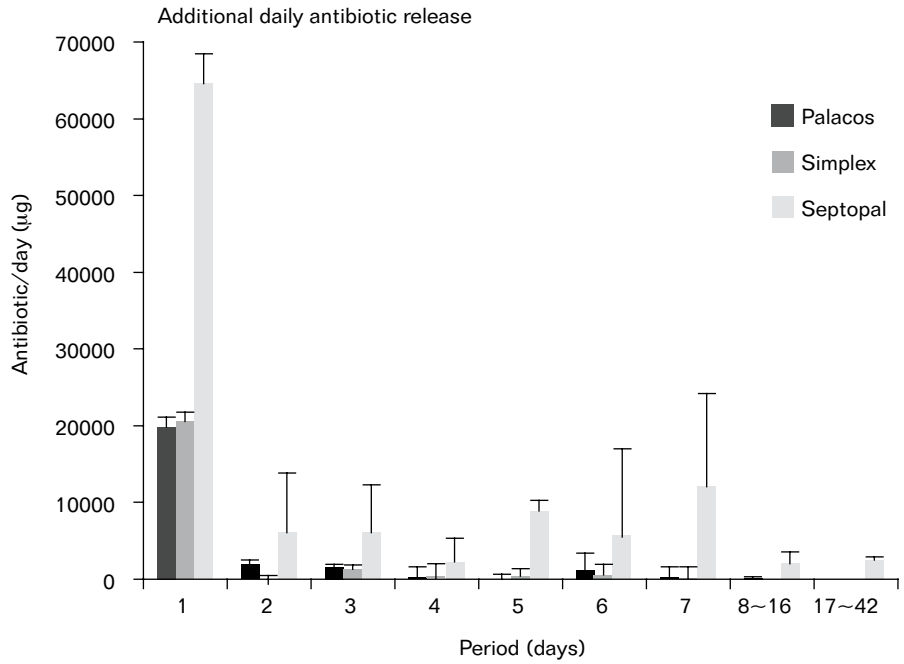

Figure 3
Additional absolute release per day (in µg)

Discussion

The Septopal beads revealed a much higher antibiotic release for a longer period of time compared to the hip spacers made of Refobacin-Palacos-R or Antibiotic Simplex-P bone cement. Both spacers had a comparable release, even though the Palacos spacers contained only half the amount of antibiotics. The antibiotic release of the beads was comparable to what was previously reported in literature[19, 20]. No studies on antibiotic release from spacers made of low-dose antibiotic bone cement are available, although it has been shown that antibiotic release from Palacos is higher than from Simplex[14, 21].

The use of Septopal beads has been proven effective against infections in both experimental and clinical studies. Pharmacological studies also showed that they are safe to use with respect to systemic concentrations and oto- or renotoxic complications[22, 23]. Even though we found a substantial additional antibiotic release after 2 weeks, it is recommended by the manufacturer to exchange the beads every 10 to 14 days to prevent the risk of sub-therapeutic drug release. In comparison, the idea of the spacer is that it should remain in situ for at least 6 weeks. Masri *et al.* previously showed that by using high-dose antibiotic bone cement adequate antibiotic concentrations could be maintained for such a period of time[13]. However, in our study the release of the spacers made from low-dose bone cement lasted for only 1 week. This may be sufficient in some cases, and it should be realized that the use of a spacer in infected hip revision has other surgical and clinical advantages such as providing stability to the joint, but it also creates the risk of having a foreign body in situ, which may prolong the infection instead of curing it. Unfortunately, it is not exactly known how high antibiotic concentrations should be and for how long these concentrations should be maintained when antibiotic bone cement is used for a therapeutic indication. As the antibiotic susceptibility of bacteria will have an important influence on these variables, there will also be a large variation for the different combinations of antibiotic types and bacterial strains.

Although it was not an objective of this study to explain the higher antibiotic release from beads, some possible explanations can be given: It probably is a combined effect of the surface area and the porosity of the bone cement. Reason to assume this, is the fact that the more than 7 times higher release can not only be attributed to the macroscopic surface area, as this was only 1.5 times higher for the beads compared to the spacers. Even though no vacuum was used during the mixing of the cement, it was clearly visible that the cement of the spacers had a smooth and dense appearance, whereas the cement of the beads appeared porous and had a rough texture. This will most likely implicate that the beads have a fairly higher microscopic

surface area available for antibiotic release and that fluids will penetrate into deeper cement layers, which will release their antibiotic content as well. This is illustrated by the fact that the beads released almost 35% of their total antibiotic content.

A point of criticism on this study is the fact that a large elution volume was used. To have the spacers and beads completely submerged, 1000 ml of PBS was used, whereas the volume *in vivo* will be less. Nevertheless, a smaller volume would probably not positively change the release patterns and would still result in a very small additional release from the spacers after the first week. However, in the *in vivo* situation spacers, because of their tighter fit in the bone and joint cavity compared to beads, will probably have less surrounding fluid, which will have a positive effect on antibiotic concentrations and to some extent reduce the observed differences.

Another point for discussion is the fact that a relatively small number of antibiotic beads was used, as some surgeons use up to 300 beads to fill the hip cavity after removal of the prosthesis. However, using a higher number of beads would make the difference between the beads and the spacers even larger and would only strengthen the current results.

Finally we can conclude, that literature shows that hip spacers made from high-dose antibiotic bone cement have proven their value in the eradication of infection and should therefore be seen as a step forward in the treatment of an infected arthroplasty compared to the traditional use of cement beads. However, although other factors, such as providing stability to inflamed tissues, may have a beneficial effect *in vivo*, we think one should be cautious toward using low-dose antibiotic bone cement for spacers in the treatment of an infected hip prosthesis, as the much lower antibiotic concentrations and short period of drug release could result in an unsuccessful eradication of the infection.

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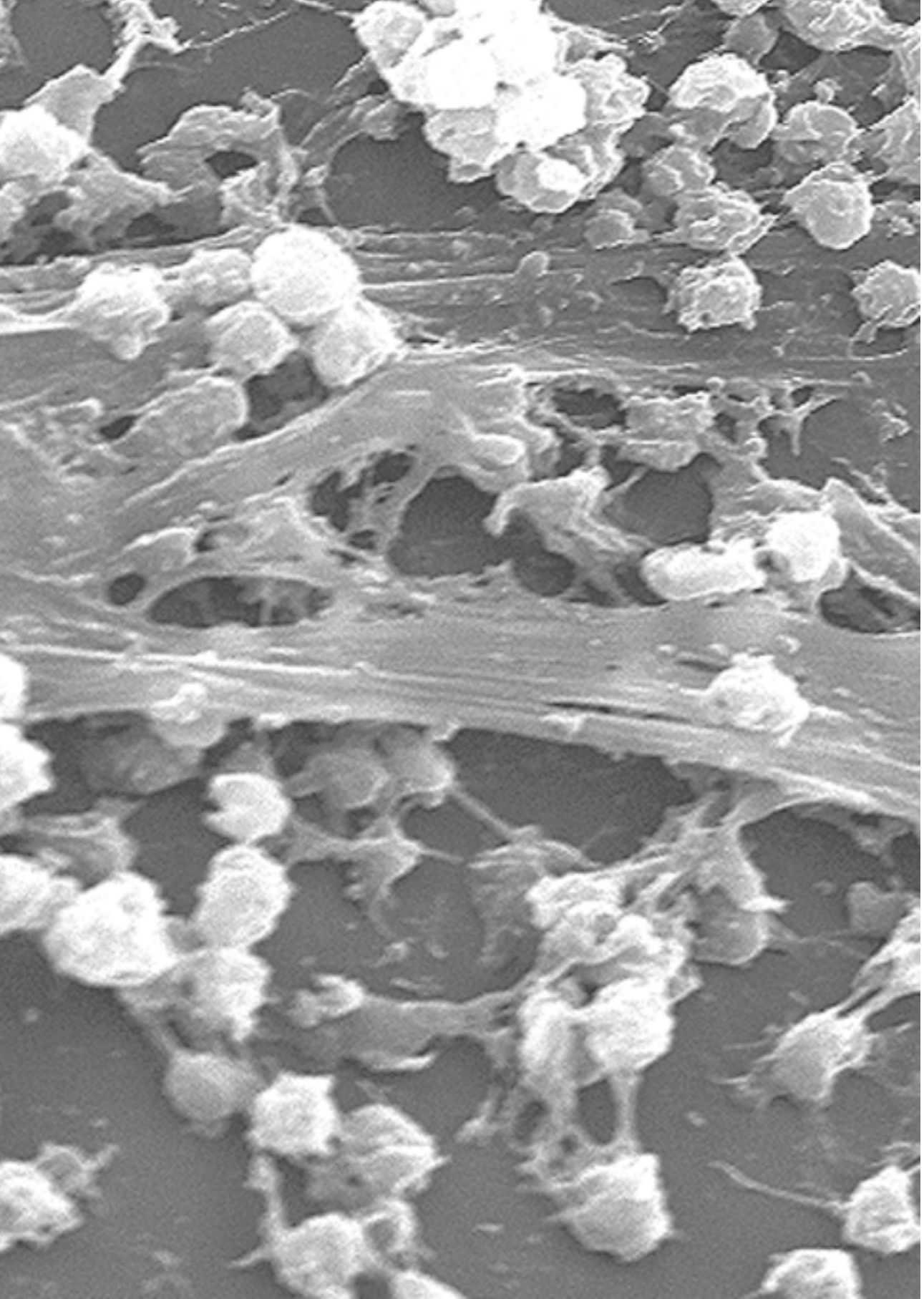

Chapter 9

Summary, discussion and conclusions

Summary, discussion and conclusions

The main aim of this thesis was to investigate new developments in the field of diagnosis, prevention and treatment of orthopaedic implant-related infections. We did this by performing *in vitro* laboratory experiments, animal infection-model studies and retro- and prospective clinical studies. This chapter summarizes and discusses the results of the different studies that were performed, by addressing the aims that were formulated in Chapter 1 (Introduction and aims).

Aim 1
To determine the value of the combined use of broad range 16S rRNA PCR and reverse line blot hybridization (PCR-RLB) in the detection of orthopaedic implant-related infections

New molecular biological techniques have yielded promising results in the detection of orthopaedic implant-related infections. Different studies were reported on the use of 16S rRNA PCR. Although promising, there was the problem of high contamination rates, because the universal presence of the 16S gene in all bacterial remnants, and the fact that these studies provided no further differentiation of the bacterial DNA found. In the study presented in **Chapter 2** we investigated a combined broad-range 16S rRNA PCR and reverse line blot hybridization protocol (PCR-RLB). The first part of the study was aimed at optimizing the technique by eliminating the contamination and designing group, genus and species-specific oligonucleotide probes. In the second part the protocol was validated using samples from 76 orthopaedic patients with various aseptic and septic indications for surgery. PCR-RLB detected all patients with a bacterial infection; the only microorganism not detected was a *Candida*, which has no 16S gene. In comparison, culture results were negative in 5 infected patients. However, 5 patients not suspect for infection had positive PCR result as well. PCR-RLB showed to be reliable in respect to species identification as it was in accordance with culture results. *Staphylococcus aureus* and coagulase negative staphylococci were found in most cases. From these results we concluded that the PCR-RLB protocol performed well in detecting and identifying the various bacterial species and was more sensitive than routine culture. What remained were the 5 patients, clinically not suspect for infection, with positive PCR-RLB results. This may have been caused by contamination. However, 3 of these patients had aseptic loosening of a total hip arthroplasty, suggesting low-grade infection detected by PCR-RLB.

These final results and suggestions from the PCR-RLB validation study were in line with results previously reported by others. From the results of the large arthroplasty registries and experimental studies, it was suspected that a percentage of all total hip arthroplasty revisions, classified as aseptic, are in fact low-grade infections missed with routine diagnostics. These percentages ranged from only a few to more than 70%. To investigate the real incidence of aseptic loosening and low-grade infection in revisions of total hip arthroplasty the study in **Chapter 3** was performed. In this prospective, multicenter study 176 patients with the preoperative diagnosis aseptic loosening of their total hip arthroplasty were investigated. Extensive diagnostic testing was performed, by using routine culture, pathology examination and the PCR-RLB protocol validated in Chapter 2. Results showed, that by using strict criteria for infection, 7 patients were classified as infected, of whom 4 were not identified by routine culture. An additional 15 patients were identified as suspect for infection as well. The microorganisms found were mainly low virulent bacteria, like coagulase negative staphylococci and *Proprionibacterium acnes*. Almost all of these patients were treated as not infected with a one-stage revision and only a short period of prophylactic systemic antibiotics. Most received a cemented implant fixated with antibiotic-loaded bone cement. One year post-surgery, 7 of the 22 patients reported complaints, only one showing early loosening and signs suggestive of infection. However, in none of the patients a debridement was performed or prosthesis was revised. From this we concluded, that although percentages were not as high as previously reported in literature, between 4 and 13 percent of patients with the preoperative diagnosis of aseptic loosening were infected. This supported the hypothesis that there is an underdiagnosis of low-grade infection in patients with loosening of their total hip arthroplasty. However, as thorough debridement was performed during surgery, systemic antibiotics were administered and antibiotic containing bone cement was used, this did not seem to have many clinical consequences, as most patients performed well at the 1-year follow-up. As one year is only a short period for arthroplasty survival, implications for long-term implant survival remain to be seen.

Aim 2
To investigate new prophylactic antimicrobial strategies, both for uncemented and cemented implants and new applications

In contrast to bone cement, no options for local antibiotic delivery are currently available for uncemented implants. This is related to the fact that most uncemented implants have low porosity and are often coated with plasma-sprayed hydroxyapatite with specific surface characteristics, which limits the options for antimicrobial coatings. However, new implant materials also provide new options for coatings. In the studies in **Chapter 4 and 5** the option of loading a porous titanium foam implant

with a biomimetic hydroxyapatite coating (Peri-Apatite, PA) with antibiotics to decrease infection susceptibility was investigated. In **Chapter 4,** PA-coated titanium foam disks were loaded with either tobramycin or gentamicin and *in vitro* antibiotic release and inhibition testing were performed. Results showed that both antibiotics had a burst release, which rapidly decreased. Concentrations remained above the MIC of many pathogens for 12 hours and release after 7 days was complete. Both antibiotics showed large inhibition zones of *Staphylococcus aureus*. No significant differences between gentamicin and tobramycin were observed.
With the knowledge that the antibiotic coating on PA-coated titanium foam is rapidly and completely released and can inhibit growth of *Staphylococcus aureus*, the study of **Chapter 5** was performed. In this study the *in vivo* efficacy of a tobramycin-loaded PA-coated titanium foam implant in preventing infection, as well as the effects on osseointegration, were investigated in a rabbit infection model. Tobramycin-PA-coated (PA-tobra) titanium foam rods were compared to uncoated (Ti) and only PA-coated (PA) rods. Compared to the PA-tobra rabbits, blood analysis showed significantly increased parameters for ERS and leukocytes for the PA and Ti rabbits. Both groups had significantly more weight loss as well. Cultures showed that 6% of the contaminated PA-tobra rabbits were infected, whereas with respectively 53% and 67% this was significantly higher for PA and Ti rabbits. Quantitative cultures were significantly lower in the PA-tobra group as well. None of the control rabbits showed signs of infection. Histopathological scores for infection were better for the PA-tobra group, although only significant compared to Ti. The histomorphometry parameters of bone-implant contact and new bone formation within the titanium foam were also both better for the PA-tobra group, but again only significantly higher compared to Ti. On none of the outcome parameters significant differences were observed between the PA and Ti rabbits.

Based on the results of both studies, we concluded that the application of antibiotics to PA-coated titanium foam implants appears to be an effective prophylactic local antibiotic strategy for uncemented implants and has a beneficial effect on implant fixation. This will hopefully result in improved long-term implant survival. In this study we investigated the efficacy of tobramycin, but as the antibiotic coating is applied by just dipping the implant in antibiotic solution other agents can be loaded as well, depending on the microorganism expected. Disadvantage of this type of coating is the fact that the antibiotics are very rapidly released and therefore only have a short effect.

Although the use of antibiotic bone cement has proven to be very effective for cemented implants in preventing infection, the increasing emergence of antibiotic resistant bacteria necessitates the search for other antimicrobial agents, which can be added to the bone cement. Previous *in vitro* research on a new type of low-dose, nanoparticulate silver PMMA bone cement showed promising results on infection prophylaxis, not only against antibiotic sensitive, both also resistant bacteria. To study the *in vivo* efficacy of this silver bone cement in preventing implant-related infections compared to plain and antibiotic bone cement the study in **Chapter 6** was performed. In a rabbit infection model the effects of 0.6%-silver, 1%-silver, plain and tobramycin PMMA bone cement were compared. Compared to the tobra rabbits, blood analysis showed significantly increased parameters for ESR and leukocytes for the plain and silver rabbits. These groups had significantly more weight loss as well. Results showed that all plain and silver cement rabbits were infected, whereas only 2 tobra rabbits were. The number of bacteria cultured were significantly lower in the tobra rabbits, whereas there was no difference between the silver and plain rabbits. In addition, histological sections of plain and silver rabbits all showed signs of infection like periosteal thickening and enlargement of Haversian canals. These signs were absent in the rabbits with tobramycin cement. As expected, the plain cement was not effective in preventing infection, whereas the tobramycin cement was. In contrast to the positive *in vitro* results previously gathered by others, both concentrations of silver cement showed no efficacy in preventing the staphylococcal infections. An explanation for this might be the fact that silver cement acts through ionized metal particles, which are not released and predominantly exhibit their antimicrobial effect at the direct cement surface. Therefore, this cement seems not useful in situations where there are bacteria present in surrounding tissues, like revision surgery. Based on the results of this study the indications for the use of silver cement seem very limited. Perhaps it might have an effect on the prevention of late bacterial colonization of cement, for example in the case of a haematogenous infection. However, also this remains to be determined.

A totally new approach is the use of platelet leukocyte gel (PLG) as an antimicrobial agent. Because it contains high concentrations of platelets and leukocytes, it was hypothesized that PLG might have antimicrobial properties. Myeloperoxidase, which is released by leukocytes, might play a key role in this process. The study in **Chapter 7** investigated this hypothesis. In an *in vitro* bacterial kill assay the antimicrobial activity of PLG-activated with autologous thrombin (AT), PLG-activated with bovine thrombin (BT), platelet rich plasma (PRP) and platelet poor plasma (PPP) against *Staphylococcus aureus* was determined. MPO release and activity was measured as

well. Results showed a rapid and large decrease in the number of bacteria for both PLG-AT and PLG-BT, which was maximal between 4 and 8 hours. Also PRP and PPP showed a substantial, however smaller effect. MPO release and activity was comparable for all groups and no correlation between MPO release, activity and bacterial kill could be found. Based on these results PLG appears to have a potent antimicrobial activity, but the role of MPO in this activity is questionable. PLG might represent a useful strategy against postoperative infections. However, additional research should elucidate its exact antimicrobial activity, *in vivo* efficacy and interaction with implants.

Aim 3

To evaluate the availability of antibiotics from different therapeutic carriers

The efficacy and benefits of high-dose antibiotic cement spacers compared to beads in the treatment of an infected prosthesis have been shown. However, in clinical practice, commercial, low-dose antibiotic bone cement is often used, which might lead to insufficient antibiotic release. To provide some objective knowledge to this discussion we determined the antibiotic release characteristics of different antibiotic carriers used in clinical practice. The study in **Chapter 8** investigated the *in vitro* antibiotic release of hip spacers made from Refobacin-Palacos-R or Antibiotic-Simplex-P cement compared to Septopal-beads. Results showed that all carriers had a burst release, but spacers showed little additional release after the first week. The cumulative release was 7 to 8 times as high for the beads compared to the spacers. We concluded that despite the efficacy of high-dose antibiotic bone cement spacers, we think one should be cautious toward using low-dose antibiotic bone cement for spacers, as this could result in insufficient antibiotic release and an unsuccessful eradication of infection.

General conclusions and future directions

All studies in this thesis investigated different new developments in the field of diagnosis, prevention and treatment of orthopaedic implant-related infections. As is normal with new developments, some of them turned out to be successful; others unfortunately were not. With respect to the improvement of diagnosing implant-related infections, the combined broad-range 16S rRNA PCR and reverse line blot hybridization protocol could be optimized to a level that it could reliably detect the infection and specify the bacterial species involved. It could be validated using a set of clinical tissue samples. Hereby, it provided us a powerful tool to answer our next question: Are many low-grade infections misdiagnosed as aseptic loosening in revisions of total hip arthroplasties?

This question was answered in prospective, multicenter study. Although percentages of infected patients were not as high as previously reported, a substantial number of patients were indeed diagnosed with low-grade infection. For this, PCR-RLB proved its value, as cultures were negative in many patients. For the survival of these patients with low-grade infections, there appeared to be little consequences on the short term. However, for arthroplasty survival 1-year follow-up is very short and longer follow-up might bring up increased failure rates compared to the patients not suspect for infection.

Regarding future directions on the aspect of diagnosing infection, the molecular biological PCR techniques are expected to become of increasing value for use in clinical practice. However, the techniques currently used still put a large demand on facilities and time, and the problem of contamination with 16S PCR will probably always remain to some extent. Therefore, future research should focus on simplifying the test conditions and decreasing the time needed to obtain a result. Perhaps the use of real time-PCR techniques might be of value for this.

For the individual orthopaedic surgeon, clinical practice will not immediately change because of the results of this thesis. However, the high index of suspicion for infection in patients with loosening of their arthroplasty should only get higher, as we showed that a substantial number of low-grade infection are easily missed. Although this finding seemed to have little effect on short-term, every effort should be made to identify these patients, as long-term effects are still unknown. With the already expected revision burden in the near future, surgical and medical treatment of arthroplasty loosening should be as optimal as possible in order not to increase the demands on health care even more.

With respect to the different strategies investigated for the prevention of infection, the use of an antibiotic coating on periapatite-coated titanium foam and the use of platelet leukocyte gel proved to be successful in their current experimental settings. Unfortunately, the use of nanoparticulate silver in bone cement was not successful.

Although the simple application of antibiotics to a porous uncemented implant, as presented in this thesis, can be immediately used in clinical practice for effective short-term prophylaxis, future research will most likely focus on strategies that provide longer antimicrobial protection. Other groups already investigated the possibilities of adding stabilizing coatings for more gradual release or covalently binding the antibiotic to the implant. Problems most likely to be encountered are changed surface characteristics with an influence on implant fixation and the fact these types of implants are no longer regarded as medical devices, but as therapeutic agents, which will need FDA approval. The search for antimicrobial agents other than antibiotics will continue as well. Different agents were already investigated with

variable results and presumably many others will follow. Our study with the platelet leukocyte gel was only a first step, but the gel did show a promising antimicrobial potential. Combined with the positive effects it is claimed to have on bone formation and wound healing, this autologous blood product might be very suitable for uncemented implants. However, additional research is needed to establish its exact position in the prevention of implant-related infections.
Finally, we showed there is a large difference in antibiotic release between cement beads and hip spacers made of commercial antibiotic cement. Although innovations, like spacers, should always be welcomed, results presented should be interpreted critically as there can be large differences with common clinical practice. As orthopaedic surgeons use spacers more and more often, better clinical reports on their efficacy are expected in the near future.

In this thesis we investigated a few aspects of the field of orthopaedic implant-related infections and the results obtained answered some questions. However, many more questions remain and hopefully much more research from many investigators will follow.

Nederlandse samenvatting

Samenvatting, discussie en conclusies

Het hoofddoel van dit proefschrift was het onderzoeken van nieuwe ontwikkelingen op het gebied van de diagnostiek, preventie en behandeling van orthopaedische implantaat-gerelateerde infecties. We hebben dit gedaan door het verrichten van *in vitro* laboratorium experimenten, dierenstudies in infectiemodellen en retro- en prospectieve klinische studies. Dit hoofdstuk vat de resultaten van deze studies samen en bediscussieert deze aan de hand van de doelen, zoals geformuleerd in Hoofdstuk 1 (Introduction and aims).

Doel 1
Het bepalen van de waarde van het gecombineerde gebruik van broad range 16S rRNA PCR en reverse line blot hybridisatie in het detecteren van orthopaedische implantaat-gerelateerde infecties

Nieuwe moleculair biologische technieken laten veelbelovende resultaten zien in het aantonen van orthopaedische implantaat-gerelateerde infecties. Verschillende studies zijn gepubliceerd over het gebruik van 16S rRNA PCR. Alhoewel veelbelovend, zijn er ook de problemen van hoge contaminatie percentages, vanwege het feit dat het 16S gen aanwezig blijft in alle bacteriële DNA resten, en het feit dat deze studies het gevonden bacteriële DNA niet nader konden specificeren. In de studie uit **Hoofdstuk 2** onderzochten we een gecombineerd broad-range 16S rRNA PCR en reverse line blot hybridisatie protocol (PCR-RLB). Het eerste gedeelte van de studie was gericht op het optimaliseren van de techniek door het elimineren van de contaminatie en het ontwerpen van groep-, genus- en soortspecifieke oligonucleotide probes. In het tweede gedeelte werd het protocol gevalideerd door het analyseren van weefselmonsters van 76 orthopaedische patiënten met verscheidene infectieuze en niet-infectieuze indicaties voor operatie. PCR-RLB detecteerde alle patiënten met een bacteriële infectie; het enige micro-organisme dat niet gevonden werd, was een *Candida*, welke geen 16S gen heeft. Ter vergelijking waren kweekresultaten negatief in 5 geïnfecteerde patiënten. Vijf patiënten niet verdacht voor een infectie hadden echter ook een positieve PCR uitslag. PCR-RLB toonde aan betrouwbaar de verschillende bacteriële species te kunnen differentiëren, aangezien PCR-RLB identificatie in overeenstemming was met de kweekresultaten. *Staphylococcus aureus* en coagulase negatieve staphylococcen werden in de meeste gevallen gevonden. Op basis van deze resultaten kunnen we concluderen dat het PCR-RLB protocol betrouwbaar infecties detecteert, de verschillende bacteriële species identificeert en zelfs sensitiever is dan routine kweken. Wat blijft, zijn de 5 patiënten met positieve PCR-RLB resultaten, klinisch niet verdacht voor infectie. Dit zou enerzijds kunnen duiden op contaminatie. Anderzijds zaten hierbij 3 patiënten met een aseptische loslating van hun totale heupprothese, hetgeen op de aanwezigheid van low-grade infectie zou kunnen duiden.

Deze laatste resultaten en suggesties uit de PCR-RLB validatie studie zijn in lijn met resultaten, zoals eerder gepubliceerd door anderen. Op basis van de resultaten van de grote protheseregistratie databases en experimentele studies, bestond reeds de verdenking, dat een gedeelte van alle revisies van totale heupprotheses voor aseptische loslating in feite low-grade infecties zijn, welke worden gemist met routine diagnostiek. Deze getallen varieerden van slechts enkele tot meer dan 70%. Om het daadwerkelijke voorkomen van aseptische loslating en low-grade infectie te onderzoeken werd de studie in **Hoofdstuk 3** verricht. In deze prospectieve, multicenter studie werden 176 patiënten met de preoperatieve diagnose aseptische loslating van hun totale heupprothese onderzocht. Bij alle patiënten werd uitgebreide diagnostiek verricht, te weten routine kweken, pathologie analyse en de PCR-RLB techniek uit Hoofdstuk 2. Resultaten toonden dat, volgens de gebruikte strikte criteria voor infectie, 7 patiënten geïnfecteerd bleken te zijn, waarvan er 4 niet werden aangetoond met de routine kweken. Nog 15 patiënten werden geclassificeerd als verdacht voor infectie. De gevonden micro-organismen waren voornamelijk laag virulente bacteriën, zoals coagulase negatieve staphylococcen en *Proprionibacterium acnes*. Vrijwel al deze patiënten zijn behandeld als niet geïnfecteerd met een 1-stage revisie en slechts een korte periode van prophylactische systemische antibiotica. Bij de meesten van hen werd een gecementeerde prothese gefixeerd met antibioticahoudend botcement geïmplanteerd. Bij follow-up na 1 jaar, rapporteerden 7 van de 22 patiënten resterende klachten. Bij slechts 1 patiënt was er inderdaad ook sprake van vroege loslating en tekenen suggestief voor infectie. In geen van de patiënten was de prothese gespoeld of gereviseerd. Op basis hiervan concluderen we dat, hoewel percentages niet zo hoog zijn als eerder gerapporteerd, tussen de 4 en 13% van de patiënten met de preoperatieve diagnose aseptische loslating toch geïnfecteerd blijken te zijn. Dit ondersteunt de hypothese dat er momenteel een onderdiagnose is van low-grade infecties bij patiënten met loslating van hun totale heupprothese. Door het verrichten van een uitgebreide nettoyage peroperatief, het gebruik van systemische antibiotica en antibioticahoudend botcement, lijkt dit weinig klinische consequenties te hebben. Met de meeste patiënten ging het goed bij de 1-jaars follow-up. Eén jaar is echter een korte follow-up voor prothese survival, dus mogelijke implicaties voor de langere termijn zullen afgewacht moeten worden.

Doel 2
Het onderzoeken van nieuwe prophylactische antimicrobiële strategieën voor zowel ongecementeerde, als gecementeerde implantaten en nieuwe toepassingen

In tegenstelling tot botcement, zijn er momenteel geen mogelijkheden beschikbaar voor lokale antibiotica afgifte voor ongecementeerde implantaten. Dit komt mede door het feit dat vele ongecementeerde implantaten weinig poreus zijn en frequent gecoat zijn met plasma-sprayed hydroxyapatiet met specifieke oppervlakte kenmerken. Dit beperkt de mogelijkheden voor antimicrobiële coatings. Nieuwe prothesematerialen geven echter ook nieuwe mogelijkheden voor coatings. In de studies uit **Hoofdstuk 4 en 5** werd de mogelijkheid om de infectiegevoeligheid van een poreus titanium foam implantaat met een biomimetische hydroxyapatiet coating (PeriApatite, PA) te verminderen onderzocht door er antibiotica op aan te brengen. In **Hoofdstuk 4** werden PA-gecoat titanium foam schijfjes geladen met tobramycine of gentamicine en werden *in vitro* antibiotica afgifte en inhibitie testen verricht. Resultaten toonden dat beide soorten antibiotica een burst afgifte hadden, welke snel afnam. Concentraties bleven boven de minimale inhibitoire concentratie van veel pathogenen gedurende 12 uur en afgifte na 7 dagen was volledig. Beide soorten antibiotica toonden een grote inhibitiezone tegen *Staphylococcus aureus*. Verschillen tussen gentamicine en tobramycine waren niet significant.

In de wetenschap dat de antibiotica coating op PA-gecoat titanium foam snel en volledig afgegeven wordt en dat het de groei van *Staphylococcus aureus* kan voorkomen, werd de studie in **Hoofdstuk 5** verricht. In deze studie werd in een konijnen infectiemodel de *in vivo* effectiviteit van een tobramycine-geladen PA-gecoat titanium foam implantaat onderzocht in het voorkomen van infectie en werd tevens gekeken naar het effect op de botingroei. Tobramycine-PA-gecoate implantaten (PA-tobra) werden hierbij vergeleken met ongecoate (Ti) en alleen PA-gecoate (PA) implantaten. In vergelijking met de PA-tobra konijnen, liet bloed analyse van de PA en Ti konijnen significant verhoogde waardes voor bezinking en leukocyten zien. Beide groepen hadden tevens significant meer gewichtsverlies. Kweken toonden dat 6% van de gecontamineerde PA-tobra konijnen geïnfecteerd was, in vergelijking met 53% en 67% voor respectievelijk de PA en Ti konijnen, wat wederom significant verschillend was. Kwantitatieve kweken van de PA-tobra konijnen waren ook significant lager. Geen van de controle konijnen was geïnfecteerd. Histopathologische scores voor infectie waren beter voor de PA-tobra groep, alhoewel alleen significant ten opzichte van Ti. De histomorphometrie parameters van bot-implantaat contact en hoeveelheid nieuwe botformatie in het titanium foam waren ook hoger voor de PA-tobra groep; echter wederom alleen significant ten opzichte van Ti. Op geen van de uitkomstmaten vonden we significante verschillen tussen de PA en Ti konijnen.

Op basis van de resultaten van deze beide studies, concluderen we dat de toevoeging van antibiotica aan PA-gecoat titanium foam implantaten een effectieve strategie lijkt te zijn voor prophylactische lokale antibiotica afgifte voor ongecementeerde implantaten en dat het een positief effect heeft op implantaat fixatie. Dit zal hopelijk leiden tot verbeterde lange termijn overleving van deze implantaten. In de huidige studie onderzochten we de effectiviteit van tobramycine. Afhankelijk van het verwachte micro-organisme, zouden andere soorten antibiotica ook gebruikt kunnen worden. De coating wordt namelijk op eenvoudige wijze toegevoegd aan het implantaat, door het in antibiotica oplossing te dopen. Nadeel van dit type coating is dat de toegevoegde antibiotica zeer snel worden afgegeven en derhalve slechts kort effectief is.

Ondanks dat antibioticahoudend botcement erg effectief is gebleken in het voorkomen van infecties bij gecementeerde implantaten, dwingt de toenemende incidentie van antibiotica resistente bacteriën ons naar andere antimicrobiële middelen, welke toegevoegd kunnen worden aan botcement, te zoeken. Eerder *in vitro* onderzoek met een nieuw type, lage dosis nanopartikel zilverhoudend PMMA botcement liet veelbelovende resultaten zien tegen zowel antibiotica gevoelige, als resistente bacteriën. Om de *in vivo* effectiviteit van dit zilverhoudend botcement te vergelijken met antibioticahoudend en geen antibioticahoudend botcement werd de studie in **Hoofdstuk 6** verricht. In een konijnen infectiemodel werden 0,6% zilver, 1% zilver, antibiotica vrij en tobramycine-houdend PMMA botcement vergeleken. In vergelijking met de tobra konijnen, toonde bloedanalyse van de antibiotica vrije en zilver konijnen significant verhoogde waardes voor de bezinking en leukocyten. Konijnen in deze groepen verloren tevens significant meer lichaamsgewicht. Kweekresultaten toonden dat alle antibiotica vrije en zilver konijnen geïnfecteerd waren, terwijl slechts 2 tobra konijnen dit waren. De aantallen gekweekte bacteriën waren ook significant lager in de tobra konijnen, terwijl er tussen antibiotica vrij en zilver cement geen verschil was. Daarnaast lieten histologische coupes van antibiotica vrije en zilver konijnen allen tekenen van infectie zien, zoals periostverdikking en vergrote Haverse kanalen. Deze tekenen waren afwezig bij de tobra konijnen. Zoals verwacht, was het antibiotica vrije cement niet effectief in het voorkomen van infectie, terwijl tobramycine-houdend cement dat wel was. In tegenstelling tot de eerdere *in vitro* resultaten verkregen door anderen, bleken beide concentraties zilverhoudend cement niet effectief in het voorkomen van staphylococcen infecties. Een mogelijke verklaring hiervoor is, dat zilverhoudend cement werkt via geïoniseerde metaal partikels, welke niet uitgescheiden worden en voornamelijk actief zijn op het directe cement oppervlak. Hierdoor lijkt dit cement

weinig bruikbaar in situaties waar er ook bacteriën aanwezig zijn in omringende weefsels, zoals revisie chirurgie. Op basis van de huidige resultaten lijkt de indicatie voor het gebruik van zilverhoudend cement erg beperkt. Het zou mogelijk een effect kunnen hebben op de late bacteriële kolonisatie van cement, bijvoorbeeld in het geval van een haematogene infectie, maar ook dit zal nog moeten blijken.

Een volledig nieuwe strategie is het gebruik van plaatjes-leukocyten gel (PLG) als een antimicrobieel middel. Omdat het hoge concentraties bloedplaatjes en leukocyten bevat, ontstond er de hypothese dat PLG antimicrobiële eigenschappen heeft. Myeloperoxidase (MPO), wat wordt uitgescheiden door leukocyten, zou hierin mogelijk een centrale rol spelen. De studie uit **Hoofdstuk 7** onderzocht deze hypothese. In een *in vitro* bacteriële groei assay werd de antimicrobiële activiteit van PLG geactiveerd met autoloog thrombine (AT), PLG geactiveerd met runder thrombine (BT), plaatjes rijk plasma (PRP) en plaatjes arm plasma (PPP) tegen *Staphylococcus aureus* bepaald. MPO afgifte en activiteit werden ook gemeten. Resultaten toonden een snelle en grote afname van het aantal bacteriën voor zowel PLG-AT, als PLG-BT, welke maximaal was tussen 4 en 8 uur. Ook PRP en PPP lieten een substantieel, echter kleiner effect zien. MPO afgifte en activiteit waren vergelijkbaar voor alle groepen. Er werd geen correlatie gevonden tussen MPO afgifte, activiteit en aantallen bacteriën. Op basis van deze resultaten, lijkt PLG een krachtige antimicrobiële activiteit te hebben, echter de rol van MPO hierin lijkt beperkt. PLG zou een bruikbare strategie kunnen zijn tegen postoperatieve infecties. Verder onderzoek zal de exacte antimicrobiële activiteit, *in vivo* effectiviteit en de interactie met implantaten nader in kaart moeten brengen.

Doel 3
Het evalueren van de beschikbaarheid van antibiotica uit verschillende therapeutische dragers

De effectiviteit en voordelen van hoge dosis antibioticahoudende cement spacers in vergelijking met kralen voor de behandeling van een geïnfecteerde prothese zijn aangetoond. In de klinische praktijk wordt echter vaak commercieel, lage dosis antibioticahoudend botcement gebruikt, wat kan leiden tot insufficiënte antibiotica afgifte. Om enige objectieve kennis toe te voegen aan deze discussie, bepaalden we de antibiotica afgifte karakteristieken van verschillende antibiotica dragers, zoals gebruikt in de klinische praktijk. De studie in **Hoofdstuk 8** onderzocht de *in vitro* antibiotica afgifte van heupspacers gemaakt van Refobacin-Palacos-R of Antibiotic-Simplex-P cement in vergelijking met Septopal kralen. Resultaten toonden dat allen een burst afgifte hadden, maar dat spacers na de eerste week nog maar weinig extra antibiotica afgaven. De cumulatieve afgifte was 7 tot 8 maal zo hoog voor de kralen in vergelijking met de spacers. We concluderen dat, ondanks de effectiviteit van hoge dosis antibiotica cement spacers, men voorzichtig moet zijn met het gebruik van lage dosis antibioticahoudend botcement voor spacers. Dit zou kunnen leiden tot onvoldoende antibiotica afgifte en daardoor niet succesvolle behandeling van infecties.

Algemene conclusies en toekomstperspectieven

Alle studies in dit proefschrift onderzochten nieuwe ontwikkelingen op het gebied van diagnose, preventie en behandeling van orthopaedische implantaat-gerelateerde infecties. Zoals gebruikelijk met nieuwe ontwikkelingen, bleken enkele hiervan succesvol, andere helaas niet.

Ten aanzien van de verbetering in het diagnosticeren van implantaat-gerelateerde infecties, kon het gecombineerde broad-range 16S rRNA PCR en reverse line blot hybridisatie protocol op zo'n niveau gebracht worden, dat hiermee betrouwbaar infecties konden worden aangetoond en de betreffende bacteriën konden worden gespecificeerd. De techniek werd vervolgens gevalideerd met behulp van een set klinische weefselmonsters. Hiermee hadden wij een krachtig middel in handen om onze volgende vraag te beantwoorden: Worden veel low-grade infecties foutief gediagnosticeerd als aseptische loslating tijdens revisies van totale heupprotheses? Deze vraag werd beantwoord in een prospectieve, multicenter studie. Ondanks dat percentages geïnfecteerde patiënten niet zo hoog waren als eerder gerapporteerd, werd er toch een substantieel aantal patiënten gediagnosticeerd als low-grade infectie. Hierbij toonde de PCR-RLB zijn waarde, aangezien kweken in meerdere patiënten vals negatief waren. Voor de korte termijn overleving van de protheses van deze patiënten met low-grade infectie leek het weinig consequenties te hebben. Voor protheseoverleving is 1 jaar echter zeer kort en de kans bestaat dat langere follow-up toch toegenomen faalpercentages laat zien in vergelijking met patiënten niet verdacht voor infectie.

Ten aanzien van de toekomstperspectieven van infectiediagnostiek, zullen de moleculair biologische technieken naar alle waarschijnlijkheid steeds belangrijker worden voor het gebruik in de kliniek. De huidige technieken vragen nog wel een flinke investering in zowel faciliteiten, als tijd en het probleem van de contaminatie met de 16S PCR zal altijd in enige mate aanwezig blijven. Derhalve zal toekomstig onderzoek zich moeten focussen op het vereenvoudigen van testvoorwaarden en het verkorten van de tijd die nodig is om een uitslag te krijgen. Mogelijk dat real time PCR technieken hierin een rol kunnen spelen.

Voor de individuele orthopaedisch chirurg zal de dagelijkse praktijk niet direct veranderen door de resultaten, zoals gepresenteerd in dit proefschrift. De reeds bestaande verdenking op infectie bij patiënten met loslating van hun prothese, dient echter nog groter te worden, aangezien we aantoonden dat een substantieel aantal low-grade

infecties makkelijk gemist wordt. Ondanks het feit dat dit op de korte termijn weinig consequenties had, dient alles in het werk gesteld te worden om deze patiënten toch te identificeren, omdat lange termijn effecten nog onbekend zijn. Met de reeds verwachte toename van het aantal revisies in de nabije toekomst, dient de chirurgische en medicamenteuze behandeling van prothese loslating zo optimaal mogelijk te zijn, om de druk op de gezondheidszorg niet nog meer toe te laten nemen.

Ten aanzien van de verschillende onderzochte strategieën voor infectiepreventie, bleken de toevoeging van een antibiotica coating aan periapatiet-gecoat titanium foam en het gebruik van plaatjes-leukocyten gel succesvol in hun huidige experimentele setting. Daarentegen was het gebruik van nanopartikel zilver-houdend botcement helaas niet effectief.

Ondanks dat de eenvoudige toevoeging van antibiotica aan poreuze, ongecementeerde implantaten, zoals gepresenteerd in dit proefschrift, direct kan worden gebruikt in de klinische praktijk voor effectieve korte termijn prophylaxe, zal verder onderzoek zich moeten gaan toeleggen op strategieën die langere antimicrobiële bescherming zullen bieden. Andere onderzoeksgroepen onderzochten reeds de mogelijkheden om stabiliserende coatings voor meer geleidelijke afgifte te gebruiken of het covalent binden van antibiotica aan het implantaat. Problemen die daarbij naar alle waarschijnlijkheid tegen gekomen zullen worden, zijn veranderde oppervlaktekenmerken met een invloed op implantaatfixatie en het feit dat dit soort implantaten niet meer worden gezien als medische apparaten, maar als therapeutische middelen, welke veel strengere goedkeuringen nodig zullen hebben.

De zoektocht naar antimicrobiële middelen anders dan antibiotica zal ook doorgaan. Verscheidene stoffen met wisselende resultaten werden reeds onderzocht en naar verwachting zullen nog vele volgen. Onze studie met de plaatjes-leukocyten gel was slechts een voorzichtige eerste stap, maar deze gel toonde wel een veelbelovend antimicrobieel potentieel. In combinatie met de positieve effecten, welke het zou hebben op botformatie en wondgenezing, zou dit autologe bloedproduct erg geschikt kunnen zijn voor ongecementeerde implantaten. Verder onderzoek is nodig om de exacte waarde ten aanzien van het voorkomen van implantaat-gerelateerde infecties te bepalen.

Tenslotte lieten we zien dat er een groot verschil bestaat in antibiotica afgifte tussen cement kralen en heupspacers, gemaakt van commercieel botcement. Alhoewel vernieuwingen, zoals spacers, verwelkomd moeten worden, dienen gepresenteerde resultaten altijd kritisch bekeken te worden. Er kunnen immers grote verschillen zijn met de dagelijkse praktijk. Aangezien de spacers steeds vaker gebruikt worden door orthopaedisch chirurgen, worden betere klinische publicaties ten aanzien van hun effectiviteit verwacht in de nabije toekomst.

In dit proefschrift hebben we enkele aspecten op het gebied van de orthopaedische implantaat-gerelateerde infecties onderzocht en de verkregen resultaten hebben enige vragen kunnen beantwoorden. Nog vele vragen resteren, welke hopelijk zullen leiden tot meer onderzoek van vele onderzoekers.

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